CLINICAL UTILITY OF THE p53 TUMOR SUPPRESSOR PROTEIN IN VARIOUS MALIGNANCIES

by

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ABSTRACT

Mutational inactivation of the p53 gene occurs more frequently than any other genetic alteration in human malignancies and typically results in accumulation of mutant p53 protein as well as a proliferative advantage and/or escape from apoptotic deletion. Because of its effects at the cellular level, p53 abnormalities may also be associated with the presence of p53 protein in serum, increased tumor aggressiveness, unfavorable prognosis, and reduced efficacy of apoptosis-inducing antineoplastic agents at the level of the cancer patient. To study these possibilities, a sensitive immunofluorometric assay for p53 protein was developed, subjected to analytical evaluation in patient sera, lysates of cultured cells, and extracts of normal and malignant tissues, and found to be comparable to immunohistochemical staining of 91 lung carcinoma tissues and to DNA sequencing of p53 exons 5 to 9 in 55 ovarian carcinomas with respect to the ascertainment of p53 alteration status. The assay demonstrated resistance to serum interferants and showed that p53 protein was undetectable in serum specimens collected pre- and post-operatively from 114 patients with lung cancer, 75 of whom also provided matched tumor tissue. In extracts of tumor tissues, however, p53 protein overexpression was shown to indicate, in

a dose-response manner, increased risks for cancer relapse and death in 86 patients with non-small cell lung cancer, 998 patients with breast cancer, and 120 patients with epithelial ovarian cancer treated with platinum-based chemotherapy. In the latter cohort of patients, p53 accumulation was associated with treatment failure, but not with immunoassay-determined expression levels of the p53-inducible WAF1 protein, itself not associated with patient prognosis. Evidence was also presented suggesting that the impact of p53 on survival may be greatest in the subgroups of patients with squamous cell carcinoma of the lung and estrogen receptor-positive, lymph-node positive breast carcinoma patients treated with adjuvant chemotherapy. Together, these findings demonstrate the prognostic value of p53 protein concentrations measured by immunoassay in extracts of lung, breast, and ovarian carcinomas, as well as the relationship between p53 expression levels and response to platinum-based chemotherapy in ovarian carcinoma, but provide no support for a role for p53 as a serum tumor marker.

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A	Alanine or adenine
ALP	Alkaline phosphatase
ARF	Alternative reading frame
ATCC	American Type Culture Collection
BCA	Bicinchoninic acid
BCTR	Breast Cancer Tissue Resource
BSA	Bovine serum albumin
C	Cysteine, cytosine, or carboxy (-terminal)
CDGE	Constant denaturant gel electrophoresis
CDK	Cyclin-dependent kinase
CI	Confidence interval
CK	Casein kinase
DFS	Disease-free survival
DNA-PK	DNA-activated protein kinase
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
F	Phenylalanine
FIGO	International Federation of Gynecologists and Obstetricians
G	Guanine
GADD45	Growth arrest and DNA damage inducible 45 gene
GaMIg	Goat anti-mouse immunoglobulin
GaRIg	Goat anti-rabbit immunoglobulin
HPV	Human papilloma virus
HAMA	Human anti-mouse antibodies
Ig	Immunoglobulin
IGFBP	Insulin-like growth factor binding protein
IHC	Immunohistochemistry
K	Lysine
kDa	KiloDalton

L	Leucine
LH	Luteinizing hormone
MAPK	Mitogen activated protein kinase
MDM2	Murine double minute 2 gene
N	Amino (-terminal)
NIRL	Nichols Institute Research Laboratories
NSCLC	Non-small cell lung cancer
OS	Overall survival
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PKC	Protein kinase C
PMSF	Phenylmethylsulfonyl fluoride
PR	Progesterone receptor
Py	Pyrimidine
R	Arginine
ROC	Receiver Operator Characteristic
RR	Relative risk
S	Serine
SAGE	Serial analysis of gene expression
SCLC	Small cell lung cancer
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SH3	Src homology 3
SSCP	Single strand conformation polymorphism
T	Thymine
TAF	TATA-associated factor
TNM	Tumor-Node-Metastasis
TSH	Thyroid stimulating hormone
UV	Ultraviolet
W	Tryptophan

WAF1	Wild-type p53-activated factor-1
WHO	World Health Organization
X	Any amino acid

LIST OF SUPPLIERS

Investigators	Generously Provided Gifts
Dr. Barry W. Brown Department of Biomathematics MD Anderson Cancer Center University of Texas Houston, TX 77030	STPLAN software
Dr. David P. Lane Department of Biochemistry University of Dundee Dundee, UK DD1 5EH	DO-1, PAb240 and PAb421 hybridoma cell lines
Dr. Donna Peehl Department of Urology Stanford Medical Center Stanford, CA 94305-5118	pRNS-1 cell line
Dr. Thierry Soussi Institut Curie Section de Reserche Paris, France 75248	Baculoviral p53 expression vector

Commercial Suppliers	Reagents or Materials
ACGT Toronto, ON M5G 1Z6	PCR primers for amplifying p53 exons 5 to 9
Abbott Laboratories Abbott Park, IL 60064-3500	Estrogen receptor and progesterone receptor enzyme immunoassays
American Type Tissue Collection Rockville, MD 20852-1776	COLO 320HSR, DU-145, MCF-7 and T-47D cell lines
Amersham (Pharmacia Biotech) Piscataway, NJ 08855-1327	Enhanced chemiluminescence Western blot detection kit, Exonuclease I, shrimp alkaline phosphatase, Thermo Sequenase kit, and CNBr-activated Sepharose 4B
Bio-Rad Laboratories Hercules, CA 94547	Econo-Pac Protein A columns
MDS Nordion Kanata, ON K2K 1X8 (formerly Cyberfluor)	Diflunisal phosphate

DAKO USA Carpinteria, CA 93013	Biotinylated goat anti-mouse antibody, horseradish peroxidase-conjugated streptavidin
Dynex Technologies Middlesex, UK TW15 1XB (formerly Dynatech Laboratories)	96-well polystyrene mictrotitre plates
Life Technologies (Gibco BRL) Rockville, MD 20849-6482	DMEM, Eagles MEM, keratinocyte serum-free medium, and RPMI-1640 cell culture media, fetal bovine serum, epidermal growth factor, bovine pituitary extract, sodium pyruvate, gentamycin, and phenol-chloroform
Perkin-Elmer Norwalk, CT 06859 (for Hoffmann-La Roche)	Amplitaq DNA polymerase
Invitrogen Carlsbad, CA 92008	Grace's insect medium and supplements
Jackson ImmunoResearch West Grove, PA 19390	Goat anti-mouse antibody and alkaline phosphatase-conjugated goat anti-rabbit antibody
Medix Biochemica Kauniainen, Finland 02700	Monoclonal antibodies against thyroid-stimulating hormone (clone 5404) and luteinizing hormone (clone 5301)
Molecular Biology Insights Cascade, CO 80809-1333 (for National Biosciences)	Oligo version 5.0 software and Cy5-labeled primers for sequencing p53 exons 5 to 9
Novex San Diego, CA 92121	Western blot reagents, minigels, and apparatus
Novocastra Laboratories Newcastle upon Tyne, UK NE12 8EW	Polyclonal CM-1 antiserum and monoclonal DO-7 antibody
Oncogene Research Cambridge, MA 02142	Lyophilized recombinant p53 protein calibrants, and WAF1 Quantitative ELISA Assay
Pierce Chemical Rockford, IL 61105	Bicinchoninic acid protein detection kit
SAS Institute Cary, NC 27513-2414	SAS versions 6.03 and 6.12 base software and STAT and SAS-GRAPH modules
Sigma-Aldrich St. Louis, MO 63103	Tween-20 and all other reagents and chemicals

CHAPTER I

GENERAL INTRODUCTION

I. 1. Overview

A complex array of homeostatic mechanisms have evolved that allow cellular adaptation to a diverse range of physiological and pathological stresses. Such responses must be carefully coordinated, but the mechanisms by which eukaryotes react to harmful exogenous insults by the cessation of cell cycle progression or by the regulated loss of damaged or unwanted cells by apoptosis remain poorly understood. Originally described 20 years ago as a cellular protein that interacted with a critical transforming antigen in the SV40 tumor virus (Lane and Crawford, 1979), p53 has emerged as an important, and perhaps pivotal, player in the coordination of the cellular responses to stress. It's perceived importance is reflected in almost 10,000 p53-related papers published since 1992. These papers have shown that abnormalities of the p53 gene are the most common genetic change in human neoplasia; missense point mutations in the sequence-specific DNA-binding domain and concomitant allelic loss at the p53 locus on chromosome 17 occur in slightly more than 50% of all human cancers (Hollstein *et al.*, 1994). A large body of data has also indicated that p53 functions as a transcription factor, activated by a variety of stresses such as DNA damage, hypoxia, and nucleotide deprivation, that can differentially up-regulate or down-regulate the expression of a constellation of genes which initiate either cell cycle arrest or programmed cell death, depending on the cellular environment or a number of other modifying factors inside the cell (Levine, 1997). In light of these findings, p53 is viewed as an integration point of a network of signaling pathways (Agarwal *et al.*, 1998; Giaccia and Kastan, 1998), inactivation of which, by direct mutation of p53 or obstruction of pathways signaling upstream or downstream of p53, may be essential for tumor development. Indeed, loss of p53 function during tumorigenesis has been shown to lead to inappropriate cell growth, increased cell survival, and genetic instability (Levine, 1997). Studies of the ability of p53 to serve as a diagnostic tool, prognostic factor, or therapeutic target have been inconclusive, however, and despite *in vitro* evidence that p53 can modulate cellular responses to DNA-damaging agents, whether p53 mutations may also influence treatment efficacy is also uncertain (Kirsch and Kastan, 1998). These areas of uncertainty have been the subject of intense research interest and were also addressed by the work summarized in this thesis.

I. 2. Molecular and Cellular Biology of p53

I. 2. 1. Structure-Function Relationships

Human p53 protein (Figure I. 2. 1. 1.) consists of 393 amino acids and can be divided structurally and functionally into five domains which are well-conserved in vertebrates (Soussi and May, 1996). The N-terminus contains a transactivation domain which is able to interact with the basal transcription machinery to positively regulate the expression of p53 target genes as a consequence of the binding of the central DNA-binding domain to DNA in a sequence-specific manner. The ability of p53 to interact with the basal transcriptional apparatus can itself be modified by binding of proteins, such as the product of the murine double minute 2 (MDM2) gene or the adenovirus E1B-55 kDa protein, to specific residues at the N-terminus of p53, particularly residues F19, L22, W23, and L26 (Kussie *et al.*, 1996). These same amino acids are required for the transcriptional activation of p53 *in vivo* and make contact with and bind to the TATA-associated factors $TAF_{II}70$ and $TAF_{II}31$, both of which are subunits of the TFIID general transcription factor (Lu and Levine, 1995).

The region between the N-terminal activation domain and the central DNA-binding domain contains five repeats of the motif proline-X-X-proline and is capable of binding to Src homology 3 (SH3) domains. Deletion of this domain reduces p53-mediated cell arrest or apoptosis but permits p53 transcriptional activation, as has been shown by Walker and Levine (1996), who speculated that interaction between p53 and SH3-containing proteins may be one way in which p53 can "tune into" signal transduction pathways, including those involving proteins such as c-abl, sensing

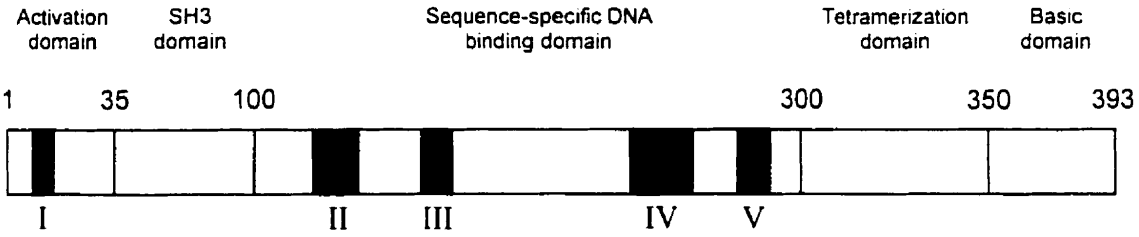


Figure I. 2. 1. 1. Schematic Representation of p53 Protein Domains
Human p53 is a 393 amino acid protein consisting of five evolutionarily conserved domains, shown as black boxes, and five regions (approximately demarcated) subserving different functions. These functions are interdependent, and regulation of one domain can influence other domains.

alterations in cellular homeostasis. Recent work has also suggested that this domain cooperates with the C-terminal domain in negatively regulating p53 function and maintaining p53 in a latent, low-affinity DNA binding conformation (Muller-Tiemann *et al.*, 1998).

The central DNA-binding domain of p53 (residues 102-292) confers the ability of p53 to act as a transcription factor, and it is within this region where the majority of point mutations are found in tumors (Hollstein *et al.*, 1994). Protease-resistant, independently folded, and containing a Zn^{2+} ion that is required for sequence-specific DNA binding, this domain has a number of critical residues that contact DNA and others essential for the stabilization of the amino acid backbone that orientate those contact residues. Specifically, structural studies have revealed that residues K120, S241, R273, A276, and R283 make contacts with the phosphate backbone in the major groove of the DNA duplex, while K120, C277, and R280 interact via hydrogen bonds to the DNA bases (Cho *et al.*, 1994). R248 then makes multiple hydrogen bond contacts in the minor groove of the DNA helix (Cho *et al.*, 1994). These contact residues are the mutational hotspots in the p53 gene, and more than 90% of missense mutations in p53 are found in this domain.

p53 functions as a tetramer as a consequence of homotypic binding through the oligomerization domain between residues 324 and 355 (Jeffrey *et al.*, 1995). Occasional mutations in this domain can inactivate p53 function (Lomax *et al.*, 1997). This tetramerization domain is linked to the DNA binding domain by a flexible linker of 37 amino acids (Jeffrey *et al.*, 1995). The tetrameric p53 protein (which is a dimer of a dimer) binds specifically to four repeats of a consensus DNA sequence 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3', and this motif is repeated in two pairs, each arranged as inverted repeats such as →←→←, where → is the sequence given above (El-Deiry *et al.*, 1992).

The C-terminus of p53 includes nine basic amino acid residues that bind to DNA or RNA with some sequence or structural preferences (Lee *et al.*, 1995b), may help to catalyze the reassociation of single-stranded DNA or RNA to double strands, and binds preferentially to DNA ends and to internal deletion loops in DNA as generated by replication errors that are then detected and fixed by mismatch repair processes. This domain also appears to have important regulatory functions. It was established that the

carboxy terminus of p53 functions to allosterically regulate the conversion of p53 between forms that are active and inactive for DNA binding (Hupp *et al.*, 1992). Small peptides and short single strands of DNA interacting with the C-terminal domain, phosphorylation of S378 by protein kinase C (PKC) or S392 by casein kinase II (CK II), binding by monoclonal antibody PAb421 (to residues 370-378), as well as deletion of the domain altogether, are each capable of converting the latent, inactive form of p53 into the active molecule (Hupp *et al.*, 1995).

I. 2. 2. Induction of the p53 Response

The induction of p53 accumulation following exposure to ultraviolet (UV) light was first reported by Maltzman and Czyzyk (1984), but the pivotal significance of this observation could not be appreciated at the time because p53 was considered a transforming oncogene rather than a tumor suppressor (Parada *et al.*, 1984). In the early 1990s, however, a series of observations led to a model of p53 function in which genotoxic damage induced p53 protein accumulation/activation, which in turn was able to trigger specific cell cycle arrest or apoptosis (Kastan *et al.*, 1991, 1992; Hall *et al.*, 1993; Lu and Lane, 1993). The development of p53 knockout mice allowed this model to be critically evaluated and established the absolute dependence on p53 function for some (but not all) of the growth arrest and apoptotic sequelae following genotoxic insult *in vivo* and *in vitro* (Clarke *et al.*, 1993; Lowe *et al.*, 1993; Clarke *et al.*, 1994; Merritt *et al.*, 1994). A critical issue that emerged from these studies was the role of p53 in inducing apoptosis in the protection of organisms from neoplastic transformation. Furthermore, the contribution of mutational inactivation of p53 to tumor suppression *in vivo* was strikingly highlighted by studies of transgenic mice in which controlled loss of p53 function was associated with loss of apoptosis and progression from benign to malignant tumors (Symonds *et al.*, 1994). In addition to a role in control of apoptosis, loss of p53 function also appears to contribute to neoplasia by allowing the development of genetic instability (Griffiths *et al.*, 1997).

p53 is well-known as a "mediator" or "sensor" of DNA damage and has been christened the "guardian of the genome" (Lane, 1992). Diverse types of genotoxic stress such as gamma-irradiation, UV light, and drugs such as etoposide and methyl methane

sulfate invoke a p53 response, causing the protein to stabilize and increase in level (Kastan *et al.*, 1991, 1992; Hall *et al.*, 1993). This is achieved by increasing the molecule's short half-life from approximately 20 minutes to several hours and presumably by switching the latent form to the active DNA-binding form. Transcriptional induction of the p53 gene does not contribute significantly to the acute up-regulation of p53 following genotoxic stress. It has been postulated that proteins that recognize DNA damage relay this information to p53 by an as yet poorly defined, but emerging, series of signal transduction pathways. For example, patients with the radiosensitive, cancer-prone disease ataxia telangiectasia lack the ionizing radiation-induced p53 response present in healthy individuals, indicating an abnormality upstream of the p53 signaling pathway (Kastan *et al.*, 1991, 1992; Zakian, 1995). However, as mentioned above, it is also possible that p53 itself senses DNA damage, as the molecule binds to single-stranded DNA, possibly at excision-repair damage sites (Bakalin *et al.*, 1994), and can localize to sites of damage (Coates *et al.*, 1995). Within the last three years, tremendous progress has been made toward understanding the biochemical mechanisms that regulate basal levels of p53 protein and the signal transduction pathways that are activated by incoming signals and finally culminate in the activation of p53.

In fact, whether DNA damage is the only insult that can cause p53 induction had

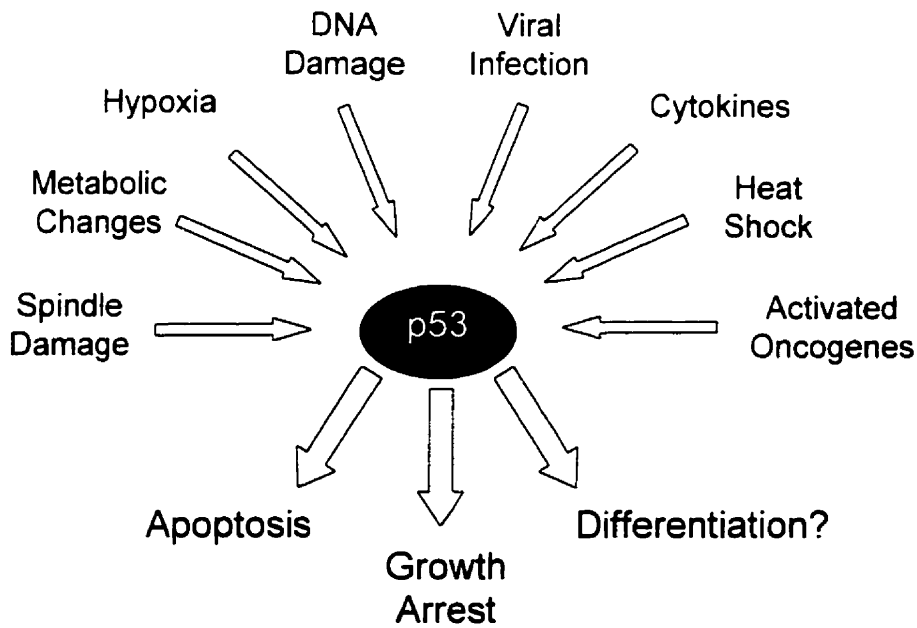


Figure I. 2. 2. 1. Integration of Stress Signals at p53
The p53 pathway is not a simple linear system. Rather, there are many "inputs", which activate the protein and induce its biological activity, as well as many "outputs".

been controversial, because the level of DNA damage associated with the p53 response was very low (perhaps even a single strand break (Huang *et al.*, 1996)). However, there is now evidence that signals not normally thought to be genotoxic are also efficient inducers of the p53 pathway (Figure I. 2. 2. 1.). Examples include hypoxia (Graeber *et al.*, 1996), changes in cell attachment (Nikiforov *et al.*, 1996), oxidative stress (Renzing *et al.*, 1996), changes in cellular growth factor/cytokine milieu (Canman *et al.*, 1995), metabolic depletion of ribonucleotide precursors (Linke *et al.*, 1996), mitotic spindle damage (Knippschild *et al.*, 1997), and the presence of oncogenic, hyperproliferative signals (Pomerantz *et al.*, 1998; Zhang *et al.*, 1998). Indeed, it may be that many other environmental stress signals can induce p53 protein, including heat shock (Zhan *et al.*, 1993) and viral infection (Marx, 1994). Because each of these stresses induces multiple, possibly redundant transcriptional responses in addition to that of p53, there is likely considerable overlap and integration between the signaling pathways.

Cellular p53 function appears to be very tightly controlled at many levels. In *in vitro* studies, p53 induction has been detected as accumulation of the protein, activation of downstream responsive genes, and induction of resultant biological effects such as cell cycle arrest and apoptosis. These studies have revealed an increasing degree of complexity in the control of p53 function (Figure I. 2. 2. 2.). It has been shown that the absolute level of p53 protein can modulate its biological effects, with low levels being

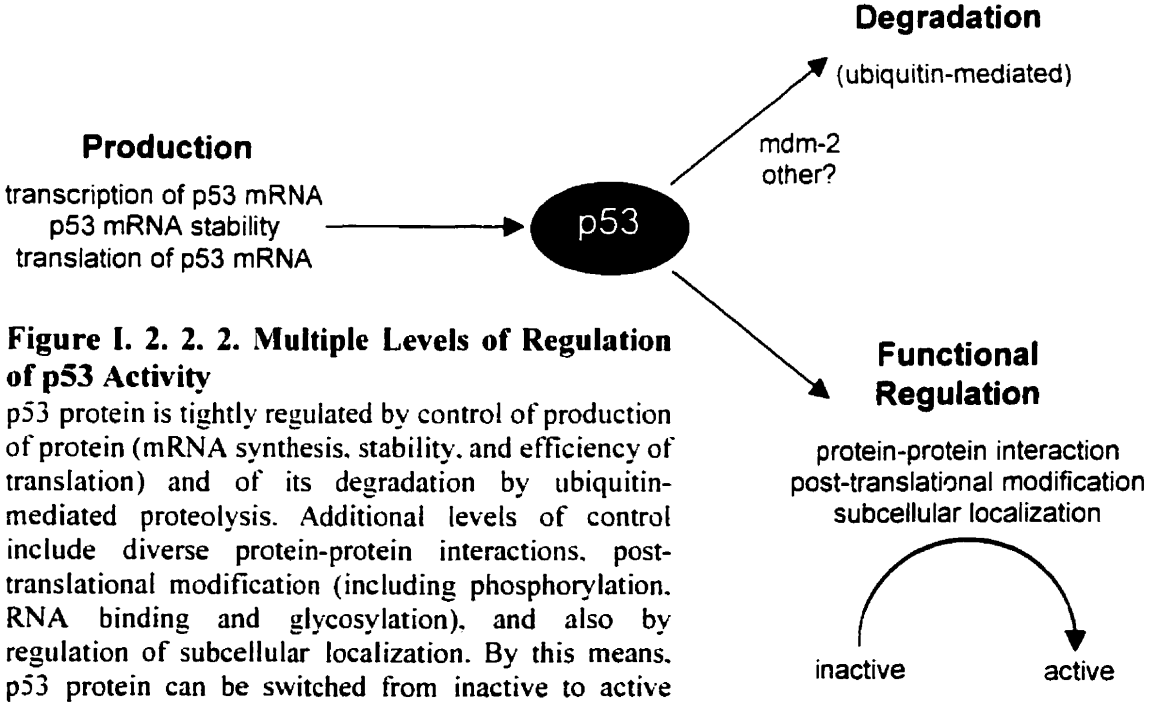


Figure I. 2. 2. 2. Multiple Levels of Regulation of p53 Activity
p53 protein is tightly regulated by control of production of protein (mRNA synthesis, stability, and efficiency of translation) and of its degradation by ubiquitin-mediated proteolysis. Additional levels of control include diverse protein-protein interactions, post-translational modification (including phosphorylation, RNA binding and glycosylation), and also by regulation of subcellular localization. By this means, p53 protein can be switched from inactive to active forms.

antiapoptotic, modest levels inducing growth arrest, and higher levels causing apoptosis (Chen *et al.*, 1996; Lassus *et al.*, 1996). Levels of p53, however, are not the only mechanism of regulation, as demonstrated by other experiments revealing high levels of wild-type, transcriptionally-inactive p53 in teratocarcinoma cells (Lutzker and Levine, 1996), and the post-translational regulation of the DNA-binding function of p53 by phosphorylation of key amino acid residues (Meek, 1998). Following cellular stress, p53 is phosphorylated on a number of sites (Figure I. 2. 2. 3.), increasing its half-life and transcriptional activity. Many different kinase families phosphorylate p53, including DNA-activated protein kinase (DNA-PK), the casein kinase (CK) family, mitogen activated protein kinases (MAPK), cyclin-dependent kinases (CDK), CDK activating kinase (CAK), and others (Meek, 1998). Some phosphorylation events have been directly linked to p53 functional activation. For instance, DNA-PK, which is activated only in the presence of DNA strand breaks, has recently been reported to be required to activate sequence-specific DNA binding following DNA damage via phosphorylation of serine 15 of p53 (Woo *et al.*, 1998), and ATM kinase, encoded by the gene mutated in patients with ataxia-telangiectasia, also phosphorylates p53 on serine 15 and this activity is enhanced in response to ionizing radiation but not UV radiation (Banin *et al.*, 1998). Serine/threonine protein phosphatases have also been shown to dephosphorylate certain residues, causing inhibition of p53 activity (Zuo *et al.*, 1998). Recently, other workers have shown that the acetyltransferases p300 and pCAF can acetylate lysine residues in the C-terminal of p53, leading to increased transcriptional activity of p53 (Sakaguchi *et al.*, 1998). These phosphorylations, dephosphorylations, and acetylations could therefore transmit signals from DNA damage or other stimuli, and the multiple sites available for phosphorylation and acetylation may provide a means for shaping the specificity of p53 activity in response to different types of stress. While modifications of p53 by covalent linkage to RNA (Samad and Carroll, 1991) and O-glycosylation (Shaw *et al.*, 1996) have also been reported, the physiological significance of such changes are presently unclear.

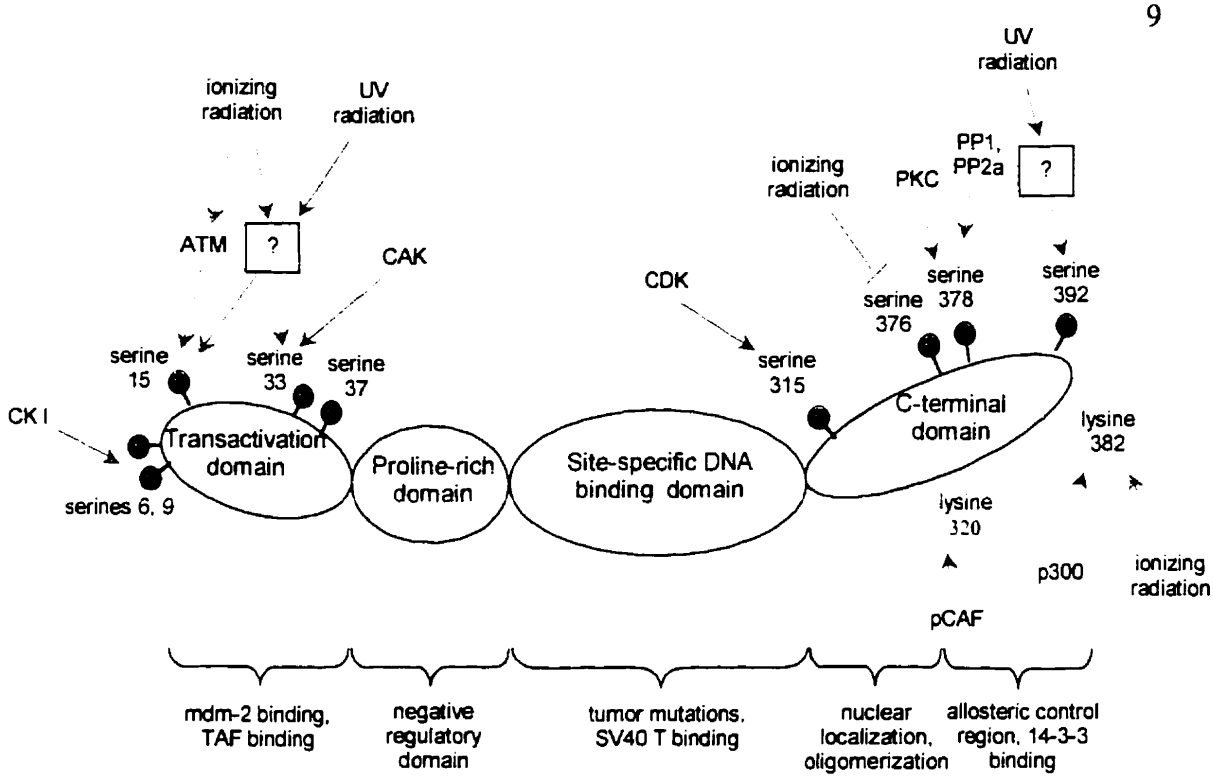


Figure I. 2. 2. 3. Post-translational Modification of p53
Multiple sites on the p53 protein have been shown to be phosphorylated or acetylated. CK I phosphorylates serines 6 and 9. ATM and DNA-PK phosphorylate serine 15 and ionizing raidiation activates ATM kinase activity. CAK phosphorylates serine 33 and ionizing radiation induces serine 33 phosphorylation. CDKs phosphorylates serine 315. PKC phosphorylates and phosphatases 1 and 2a dephosphorylate serine 378. UV radiation induces phosphorylation of serines 15, 33, and 392. Ionizing radiation induces lysine 382 acetylation and serine 376 dephosphorylation. pCAF acetylates lysine 320 and p300 acetylates lysine 382. The major functions of each domain as well as the binding sites of cellular or viral proteins are also shown. (Compiled from Giaccia and Kastan (1998) and Meek (1998)).

Although p53 protein levels are not the sole regulatory control on p53, this mechanism is clearly important and is mediated by p53 binding proteins; mdm2, which is transcriptionally up-regulated by p53 (Thomas and White, 1998), binds to the transcriptional activation domain of p53, thereby blocking the ability of p53 to function as a transcription factor (Momand *et al.*, 1992; Chen *et al.*, 1993). That this interaction is of biological significance is shown by the embryonic lethal phenotype of mdm2 null mice. If mice are engineered to lack both p53 and mdm2, the offspring are fully viable (Montes de Oca Luna *et al.*, 1995). The interaction of p53 and mdm2 has a second and complementary effect, in that the targetting of p53 for ubiquitin-mediated degradation – the primary pathway whereby intracellular levels of p53 protein are decreased (Chowdary *et al.*, 1994) – requires p53-mdm2 interaction (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997).

Furthermore, this interaction may be responsible for the regulation of p53's subcellular localization, which can be either nuclear or cytoplasmic (Moll *et al.*, 1992). In order to bind DNA and activate transcription, p53 must be located in the nucleus, into which newly stabilized p53 and phosphorylated p53 is transported following genotoxic stress (Moll *et al.*, 1996). There is now evidence that nuclear export of p53, an important step towards its inactivation by degradation, may be facililated by its complexation with mdm2, which is an RNA-binding protein (Elenbaas *et al.*, 1996) that can shuttle between the nucleus and cytoplasm (Roth *et al.*, 1998). Interaction between p53 and mdm2 may be inhibited by other signals, including serine 15 phosphorylation subsequent to DNA damage (Hsieh *et al.*, 1997). Other studies, however, suggest that the nuclear export signal may be contained in the p53 C-terminal domain, which is masked by tetramerization following stress (Almog and Rotter, 1998). Taken together, these observations provide an explanation for the widely recognized phenotype of elevated (but nonfunctional) p53 levels in tumors. This p53 overexpression phenotype, strongly associated with both neoplasia and mutation of the p53 gene (Dowell *et al.*, 1994; Hall and Lane, 1994), had been thought to be related to an intrinsic alteration in p53 protein stability, despite the fact that mutations could be widely scattered in the p53 gene. In the new model, the result of the inability of mutant p53 to activate mdm2 gene expression is that p53 levels are no longer down-regulated by mdm2-mediated ubiquitin targeting. While this model is supported by *in vitro* observations of a rapid decline in p53 as a result of its enhanced degradation induced by mdm2 expression in cells with high levels of mutant p53 (Midgely and Lane, 1997), it is not the complete explanation because elevated p53 can occur without p53 mutation (Hall and Lane, 1994) and in light of the wide range of phenotypes of p53 and mdm2 levels in clinical specimens.

The nature of the p53-mdm2 negative feedback loop has recently been expanded to include other molecules which participate in the regulation of p53 activity. In addition to the regulation of mdm2 binding of p53 by serine 15 phosphorylation, studies from Pomerantz *et al.* (1998) suggest that the disruption of the p53-mdm2 interaction may also be mediated by the alternative product of the human INK4a tumor suppressor gene $p14^{ARF}$ (and its muring homolog $p19^{ARF}$). Although the mechanism whereby ARF (alternative reading frame) stabilizes p53 is unclear (Sherr, 1998), it has been found that

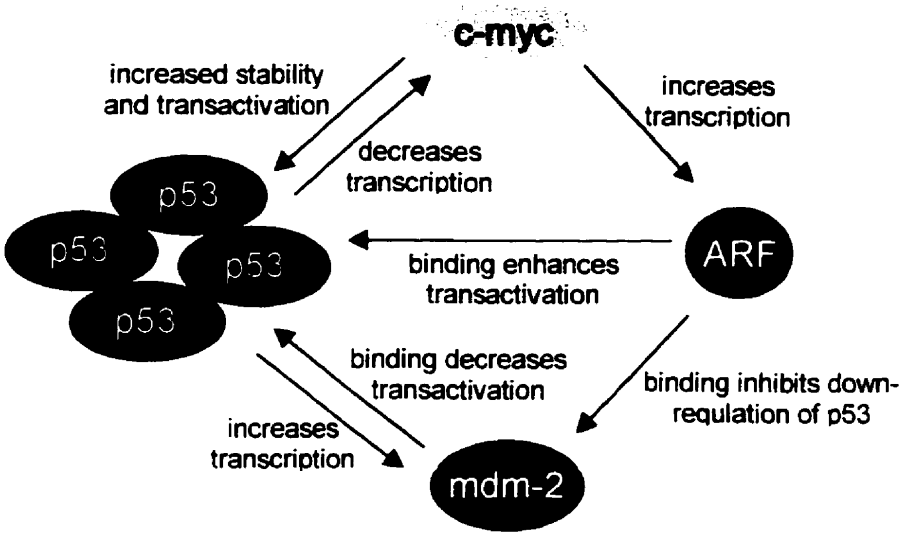


Figure I. 2. 2. 4. Autoregulatory Loops Limiting the Duration of p53 Activation

ARF can physically interact with p53 and/or mdm2 *in vivo*, and its binding blocks both mdm2-induced p53 degradation and transcriptional silencing (Kamijo et al., 1998; Pomerantz *et al.*, 1998; Stott *et al.*, 1998). Interestingly, because p53 can down-regulate ARF gene expression (Kamijo et al., 1998), at least two negative feedback loops may regulate p53 activity (Figure I. 2. 2. 4.): p53 induces mdm2 expression, resulting in the inactivation and degradation of p53, and p53 represses expression of ARF, which promotes stabilization and activation of p53. Further autoregulation may operate between p53 and the product of the proto-oncogene c-myc, which is a proliferative signal contributing toward cellular transformation. Whereas c-myc transcription is down-regulated following p53 activation (Moberg *et al.*, 1992), activation of c-myc increases both the transcription of p53 message and the stability of p53 protein (Hermeking and Eick, 1994), as well as inducing ARF expression (Zindy *et al.*, 1998). Because ARF expression is induced also by members of the E2F family of transcription factors (de Stanchina *et al.*, 1998), it may function to trigger p53-dependent cell cycle arrest or apoptosis in response to proliferative signals such as c-ras or c-myc overexpression, or to inactivation of the Rb tumor suppressor gene by mutation, binding of viral oncoproteins, cyclin D overexpression, or $p16^{INK4a}$ inactivation. A single genetic locus, INK4a/ARF, may therefore link the p53 and Rb tumor suppression pathways (Figure I. 2. 2. 5.). Finally, another proto-oncogene that interacts directly with p53 to regulate its function is the nuclear tyrosine kinase c-abl, which is activated by DNA damage and binds p53, enhancing its sequence-specific transactivation ability (Goga et al., 1995).

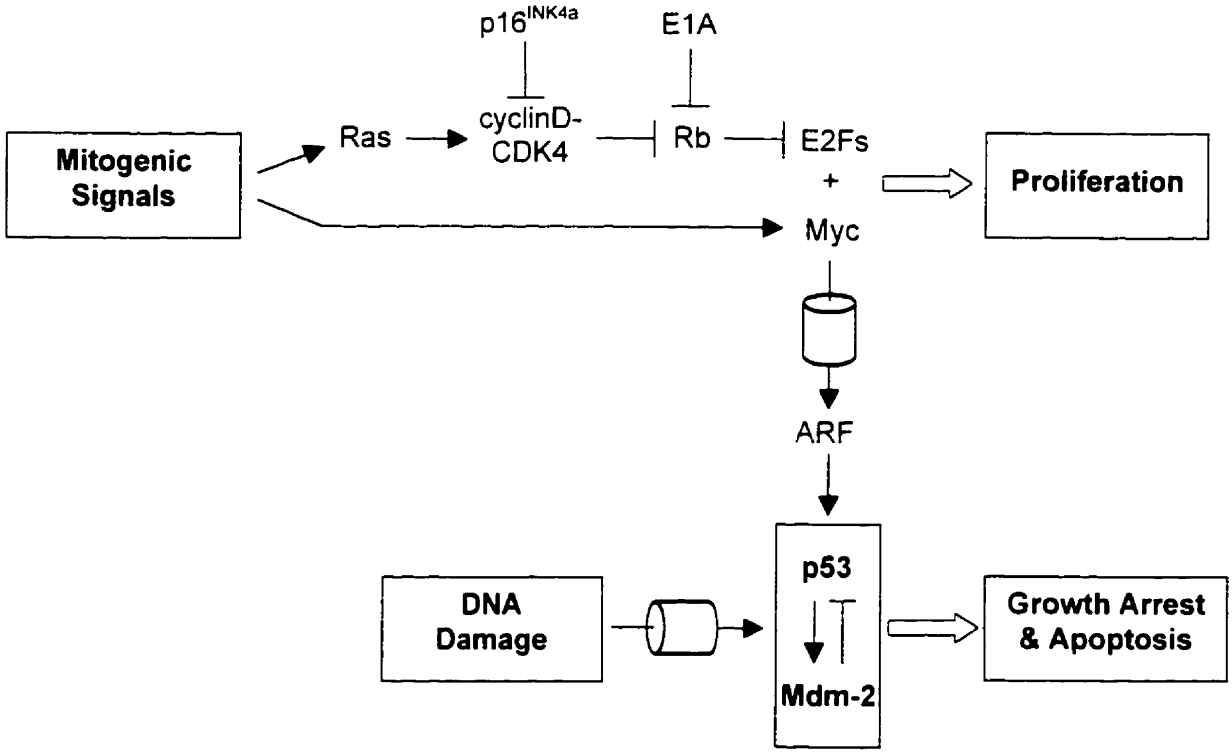


Figure I. 2. 2. 5. Linking the p53 and Rb Tumor Suppression Pathways via ARF
ARF responds to proliferative signals that are normally required for cell proliferation. When these signals exceed a critical threshold, the ARF-dependent checkpoint is activated, and ARF triggers a p53-dependent response that induces growth arrest and/or apoptosis. Signals inducing signaling via the ARF-p53 pathway include myc, E1A, and E2F-1. Upstream oncoproteins, such as products of mutated Ras alleles, constitutively activated receptors, or cytoplasmic signal transducing oncoproteins, might also trigger ARF activity via the cyclin D-CDK4-Rb-E2F or myc-dependent pathways, both of which are normally necessary for S-phase entry. In inhibiting cyclin D-dependent kinases, $p16^{INK4a}$ can dampen the activity of mitogenic signals. E1A is shown to work in part by canceling Rb function, although its ability to inhibit p300 contributes to the response by interfering with mdm2 expression. For simplicity, myc and E2F-1 are shown to activate p53 via ARF although highly overexpressed levels of these proteins can activate p53 in ARF-negative cells. ARF activation of p53 likely depends on inactivation of an mdm2-specific function. Signals through the ARF and DNA damage pathways can synergize in activating p53.

I. 2. 3. Events Downstream of p53

p53 exerts most of its effects within a cell by acting as a transcriptional activator of a wide variety of cellular genes, although the repression of a smaller number of genes has also been described. The physiological functions of the majority of p53 target genes, however, remain unknown. In addition, there may be marked differences in the way a given cell type activates p53 target genes under different circumstances as well as differences in the way that different cell types activate target genes, perhaps depending in both cases on cofactors that enhance or repress the DNA-binding function of p53 or its

interactions with the basal transcription machinery. This notion is supported by recent studies of p53-interacting proteins that modify either p53 or target DNA (Avantaggiati *et al.*, 1997; Jayaraman *et al.*, 1997, 1998). It is also possible that the nature and context of p53 response elements in different genes may have different abilities to respond to particular p53 levels and may be differentially affected by associated partner proteins or by post-translational modifications. Certainly it is clear that different p53 responsive promoters are differentially activated by different p53 mutants (Friedlander *et al.*, 1996).

Table I. 2. 3. 1. 1. Genes Regulated by p53 and their Functions as Potential Mediators of p53 Tumor Suppression

Induced Gene	Function	Outcome
MDM-2	p53 binding	Down-regulation of p53
$p21^{WAF1}$	CDK inhibitor	G1 and G2/M arrest
14-3-3σ	Binds and sequesters CDC25C	G2/M arrest
GADD45	Interacts with CDC2, DNA repair	G2/M arrest
Cyclin G	Cyclin	G1 arrest
Cyclin D1	Cyclin	G1 arrest
BAX	Dimerizes with BCL-2	Promotes apoptosis
IGFBP-3	Inhibits mitogens and survival factors	Apoptosis or growth arrest
PAG 608	Nuclear zinc finger protein	Apoptosis
KILLER/DR5	Membrane receptor in TNFR family	Apoptosis
FAS/APO1	Membrane receptor in TNFR family	Apoptosis
PIG1 to PIG14	Oxidative stress response	Apoptosis or growth arrest
BCL-X	Competes with BAX for BCL-2 binding	Protects against apoptosis
TRID	Antagonist decoy receptor	Protects against apoptosis
TRUNDD	Antagonist decoy receptor	Protects against apoptosis
Suppressed Gene	**Function**	**Outcome**
$p14^{ARF}$	MDM-2 binding	Down-regulation of p53
PCNA	DNA replication and repair	Cell cycle arrest
RB	Sequesters E2F transcription factor	Cell cycle arrest
BCL-2	Survival factor when self-dimerized	Apoptosis

Compiled from Amundson *et al.* (1998) and Kirsch and Kastan (1998).

This is perhaps an explanation for the association of different p53 mutations and variable clinical consequences and disease courses (Blandino *et al.*, 1999).

The list of p53 responsive genes is ever increasing, with a whole series of novel p53-inducible genes being identified using the differential display (Amson *et al.*, 1996; Lehar *et al.*, 1996; Israeli *et al.*, 1997), SAGE (Madden *et al.*, 1997; Polyak *et al.*, 1997), and other methods (Bourdon *et al.*, 1997). Some p53-regulated genes are listed in Table I. 2. 3. 1. 1. Despite the facts that the physiological functions of the currently known p53-regulated genes are diverse and that the biological relevance of most novel genes remain unknown, two well established consequences of p53 activation – cell cycle arrest and programmed cell death – have been described in some detail.

I. 2. 3. 1. Cell Cycle Arrest

A cell can arrest its progression through the cell cycle at a number of points following the detection of DNA damage. p53 has been associated with delays in transit through both G1 and G2, as well as in a mitotic spindle checkpoint. G1 arrest, a prominent outcome of DNA damage, is induced in many cells types by expression of exogenous wild-type p53, and shows a strong concordance with functional p53 status (Hartwell and Kastan, 1994). Depending on the cell type, the G1 arrest may be transient or may permanently remove cells from the cell cycle (Linke *et al.*, 1997). As cells lacking normal p53 function were found to lack DNA damage-induced G1 but not G2 arrest (O'Connor *et al.*, 1997), most early studies focused on the G1 checkpoint. Induction of G1 arrest by p53 is mediated largely by the sequence-specific transactivation of $p21^{WAF1}$ (El-Deiry *et al.*, 1993), which is a CDK inhibitor (Harper *et al.*, 1993) (Figure I. 2. 3. 1. 1.). Exit from G1 and entry into S phase requires the activation of G1 cyclin-CDK complexes, and $p21^{WAF1}$ inhibits phosphorylation and activation of CDK2 associated with cyclin D or cyclin E, preventing the phosphorylation of downstream protein targets required for cell cycle progression (Radford *et al.*, 1994). One of these targets is the retinoblastoma protein (Rb) which, in its hypophosphorylated state, binds and sequesters E2F, a transcription factor required for entry of the cell into S phase (Chellappan *et al.*, 1991). Overexpression of $p21^{WAF1}$ therefore results in G1 arrest through inactivation of cyclin/CDK complexes and the inablity of Rb to release sequestered E2F. Failure to

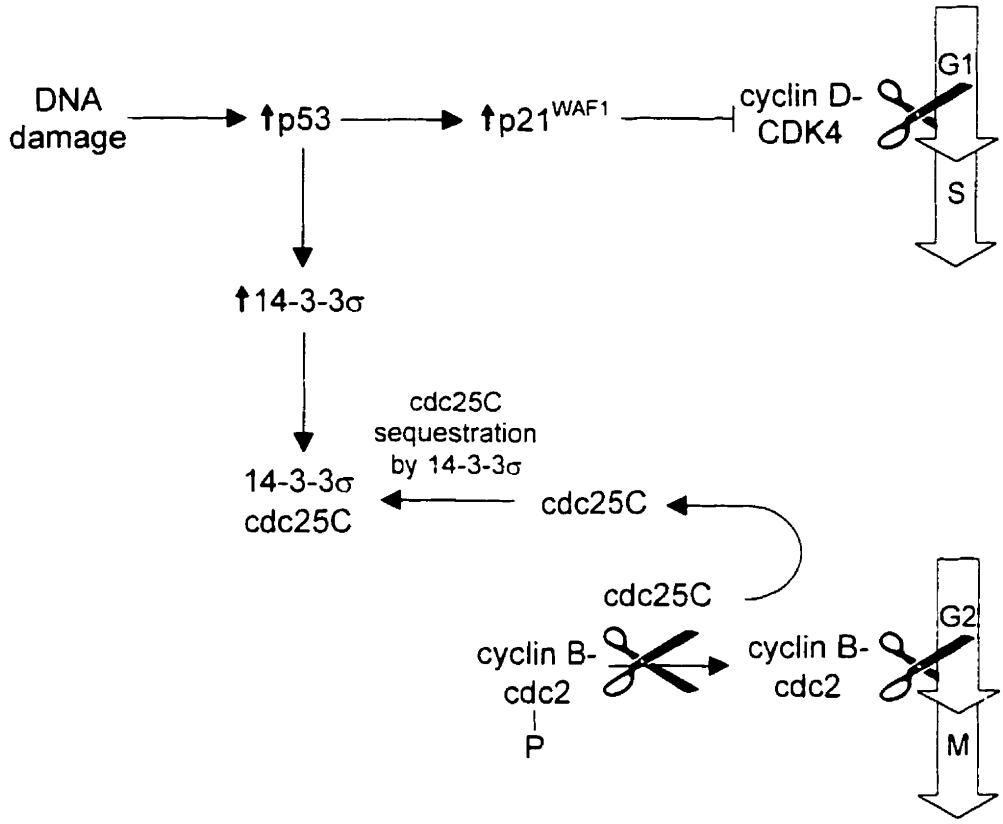


Figure I. 2. 3. 1. 1. p53-Dependent Induction of Genes Controlling Cell Cycle Progression

Activated p53 causes a G1 cell-cycle arrest by increasing the transcription of $p21^{WAF1}$, which binds and inhibits a variety of cyclin-dependent kinases, including CDK4 activated by complexation with its cognate cyclin D. Inactivated CDK4 is unable to phosphorylate the retinoblastoma protein, which consequently remains bound to transcription factors such as E2F and represses their ability to induce expression of genes required for entry of the cell into S phase (not shown). Activated p53 may also contribute to a G2 arrest by increasing the transcription of 14-3-3σ, which sequesters the cdc25C phosphatase in the cytoplasm, preventing it from entering the nucleus and activating cdc2, the CDK that promotes G2/M transition.

arrest in G1 after DNA damage, which occurs in tumor cells with mutant p53 genes, would lead to DNA replication using a damaged template. In addition to binding cyclin/CDK complexes, $p21^{WAF1}$ also blocks DNA replication by binding to proliferating cell nuclear antigen (PCNA) but does not inhibit the ability of PCNA to mediate DNA repair processes (Warbrick *et al.*, 1995). GADD45, another p53 target gene, also interacts with $p21^{WAF1}$ (Kearsey *et al.*, 1995) and PCNA (Hall *et al.*, 1995). Although the precise function of GADD45 remains elusive, it has been speculated that its interaction with $p21^{WAF1}$ may be to act as a molecular chaperone, guiding $p21^{WAF1}$ to PCNA. While

$p21^{WAF1}$ appears to be the major effector of p53-mediated G1 arrest, this CDK inhibitor may also couple the initiation of DNA replication to the completion of mitosis. In this role, $p21^{WAF1}$ might function to prevent damaged cells in G2/M from reinitiating a second round of DNA replication (a phenomenon called endoreduplication), thus preventing mitotic catastrophe and cell death (Waldman *et al.*, 1996).

Although G2 arrest following DNA damage does not require p53, p53-regulated genes can participate in the regulation of G2 arrest. For example, overexpression of $p21^{WAF1}$ has been shown to cause cells to accumulate in both G1 and G2, and is associated with a reduction of cyclin B-associated kinase activity (Niculescu *et al.*, 1998). Exogenous expression of 14-3-3σ, another p53-responsive gene, results in G2 arrest (Hermeking *et al.*, 1997) by binding and sequestering phosphorylated cdc25C, thereby preventing its dephosphorylation and activation of cdc2 (Peng *et al.*, 1997), required for the G2/M phase cell cycle transition (Figure I. 2. 3. 1. 1.).

There is also evidence for cell cycle controls in late G2 and early M phase that may involve p53. Cells will usually not progress through mitosis in the presence of spindle inhibitors, but p53-knockout mouse embryo fibroblasts will undergo multiple rounds of DNA synthesis without completing chromosome segregation, leading to the formation of tetraploid and octaploid cells (Cross *et al.*, 1995). The mechanism whereby this occurs remains uncertain, but may involve the ability of p53 to regulate centrosome duplication (Fukasawa *et al.*, 1996).

I. 2. 3. 2. Apoptosis

p53 can also respond to cellular stress by signaling the apoptotic machinery to induce programmed cell death, which is a process of cell suicide that occurs through characteristic morphologic changes termed apoptosis. These morphologic changes include cell shrinkage, nuclear condensation, DNA fragmentation, and plasma membrane blebbing (Kerr *et al.*, 1972) which can result from the activity of intracellular cysteine proteases called caspases (formerly ICE/ced-3 proteases) (Salversen and Dixit, 1997). Although the mechanism by which p53 initiates apoptosis remains to be fully elucidated (Amundson *et al.*, 1998), several transcriptional targets of p53 have been identified that mechanistically link p53 to caspase activation (Figure I. 2. 3. 2. 1.).

In some cell types, p53 appears to transcriptionally induce Bax (Miyashita and Reed, 1995), which is part of the apoptotic machinery and may cause cell death by forming a pore (Antonsson *et al.*, 1997) in mitochondria that causes release of cytochrome c into the cytosol (Rosse *et al.*, 1998). Cytosolic cytochrome c activates caspases (Liu *et al.*, 1996; Zhivotovsky *et al.*, 1998), which then kill the cell. Other potential transcriptional targets of p53 that may contribute to apoptosis are the cell surface "death receptors" DR5 (Wu *et al.*, 1997) and Fas (Owen-Schaub *et al.*, 1995),

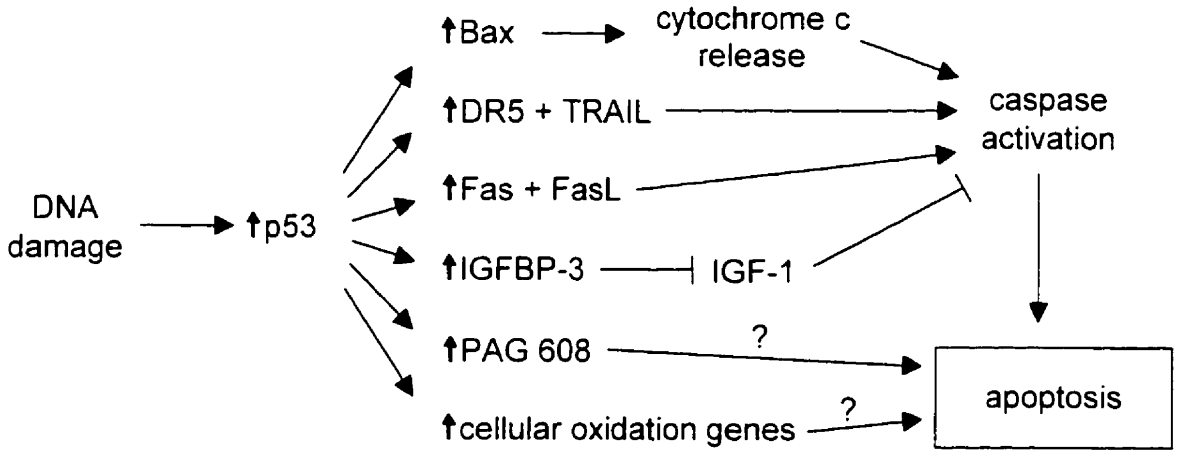


Figure I. 2. 3. 2. 1. Programmed Cell Death Initiated by p53
p53 induction of bax can cause cytochrome c release from mitochondria, which activates caspases that can kill the cell. p53 can also induce the death receptors DR5 and Fas, which can activate caspases after binding to their respective ligands TRAIL and Fas ligand. p53 also increases transcription of IGFBP-3, which binds to IGF-1, preventing it from sending signals that inhibit caspase activation. p53 can also induce PAG608, a protein that can cause apoptosis by an unkown mechanism. Finally, cellular oxidation genes are also induced by p53 and may cause apoptosis through redox mechanisms.

which bind to the extracellular signaling molecules TRAIL (Sheridan *et al.*, 1997) or Fas ligand (Nagata, 1997), respectively, and thereby also activate caspases. Another gene transcriptionally induced by p53, IGFBP-3 (insulin-like growth factor binding protein -3), binds IGF-1 and prevents it from initiating antiapoptotic signals (Buckbinder *et al.*, 1995). An additional p53-induced gene, PAG608, can cause cell death by an unknown mechanism when transfected into cells (Israeli *et al.*, 1997). Finally, a group of p53-inducible genes was recently identified that appear to increase cellular oxidation; when oxidation was blocked, p53-mediated apoptosis was inhibited (Polyak *et al.*, 1997).

I. 2. 3. 3. Factors Affecting Cell Fate Following p53 Induction

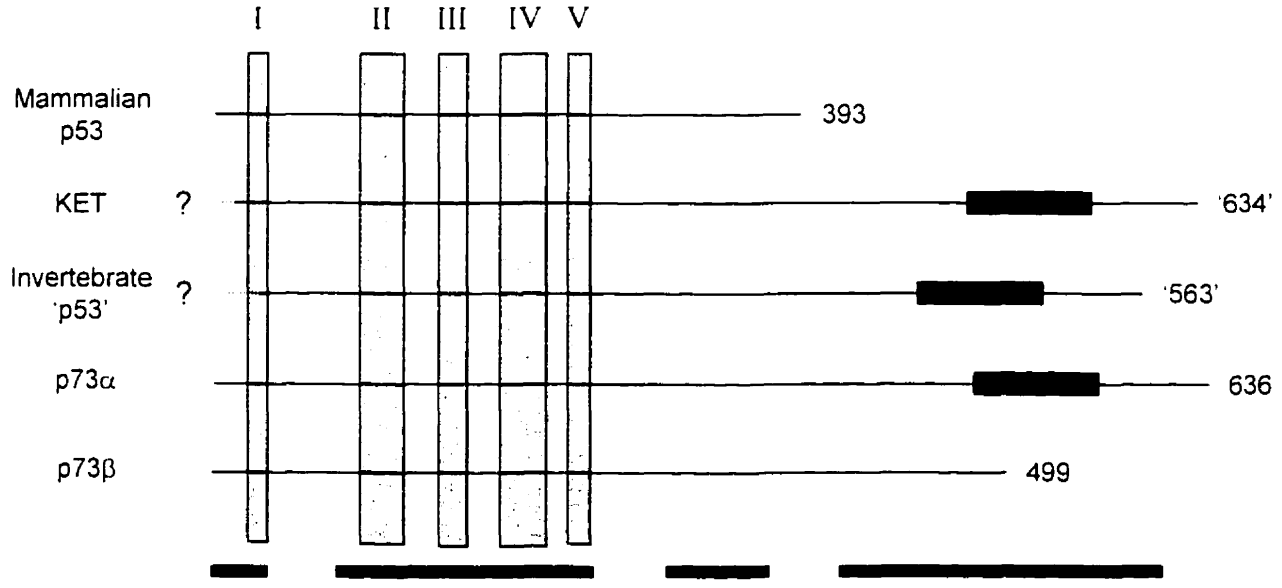
What determines whether a cell responds to a cellular stress with p53-induced cell cycle arrest or with p53-induced apoptosis? One important determinant appears to be cell type. Lymphocytes tend to undergo a rapid p53-dependent cell death following DNA damage, while epithelial cells are more likely to survive and undergo a cell cycle arrest (Slichenmeyer *et al.*, 1993). Even within the same cell type, cellular environment can dictate life or death. For example, DNA damage causes interleukin-3 (IL-3)-dependent lymphocytes to undergo a cell cycle arrest in the presence of IL-3, whereas in the absence of the growth factor the same DNA damage causes p53-dependent programmed cell death (Canman *et al.*, 1995). The response to a cytotoxic stress can also depend on the genetic context. Chemotherapy efficiently induces p53-dependent apoptosis in fibroblasts that express the oncogenes E1A and activated Ras (Lowe *et al.*, 1993a). However, without the expression of these oncogenes, fibroblasts that lack p53 were reported to be more sensitive to certain DNA-damaging agents (Hawkins *et al.*, 1996). This contradiction may be explained either if p53 does not send out an apoptotic signal in the absence of these oncogenes or if the p53 signal cannot be interpreted by the apoptotic machinery in this setting. In this cellular context, p53 may be protecting cells from chemotherapy either by facilitating repair (Hawkins *et al.*, 1996) or by limiting cell death resulting from endoreduplication (Waldman *et al.*, 1996).

I. 2. 4. Homologs of p53

Although it is apparent that p53 is a critical adaptive regulator of stress responses, the discovery of putative p53 homologs, namely p73 (Jost *et al.*, 1997; Kaghad *et al.*, 1997), KET (Schmale and Bamberger, 1997), and possibly p53CP (Bian and Sun, 1997) suggested that its functions may be redundant – a notion supported by earlier observations that p53 null mice developed relatively normally. Functional redundancy has indeed been shown in the case of the Rb tumor suppressor by the Rb-related proteins p107 and p130, which can compensate for the loss of Rb function (Herwig and Strauss, 1997).

The cloning of p73 revealed a high degree of sequence identity to p53, with the homology extensive in the most conserved p53 domains (Figure I. 2. 4. 1.), including the

transcriptional activation domain, the DNA-binding domain, and the oligomerization domain. The p73 gene encodes two polypeptides, the products of an alternatively-spliced mRNA transcript. The longer of the two, the p73α, consists of 636 residues which shows significant homology to a p53-like protein from squid, suggesting that p53 may have evolved from a more phylogenetically ancient p73 protein. Of particular interest is the fact that the p73 gene maps to the chromosome region 1p36, a region frequently deleted in neuroblastomas which, unlike the majority of tumors, rarely carry p53 mutations. It is therefore reasonable to speculate that it is p73 rather than p53 that is performing a tumor suppressor function in these tumors. In a yeast two-hybrid system, the shorter p73β was



Critical residues	F19, L22, W23	R175, G245, R248, R249, R273, R282

p53 versus p73 (54%)	29%	63%	42%	nil
p53 versus KET (55%)	32%	65%	43%	nil
p73 versus KET (57%)	45%	76%	44%	45%
KET versus Loligo p53 (36%)	27%	60%	30%	28%

Figure I. 2. 4. 1. Similarities and Differences Between p53 Homologs
The recognition of possible homologs of p53 in the mammalian genome and the identification of a potential mollusc (Loligo) homolog raise the notion of a family of p53 proteins. All have similarities in the conserved boxes, including conservation of critical contact residues in the N-terminal transactivation domain and in the DNA-binding domain. Similarities in the oligomerization domain also exist, as indicated by the percentage identities listed below the figure. In the long C-terminal extension of p73α, KET, and squid sequence there is a region of homology denoted a SAM domain, which is absent from the splice variant of p73, p73β.

shown to form homotypic and, perhaps more importantly, heterotypic interactions with p53, suggesting a possible role for p73 in regulating p53 function. p73 is capable of $p21^{WAF1}$ induction and growth suppression but is not induced in response to DNA-damaging events. Jost *et al.* (1997) showed that transient expression of p73 in a p53-null cell line promotes apoptosis, but whether this occurred via a p53-independent or a novel p73-dependent pathway remains to be determined. At the same time as the p73 discovery, a group searching for signal transduction components in rat taste receptor cells amplified another p53 homolog termed KET, as it is expressed in keratinocytes (Schmale and Bamberger, 1997). KET is expressed during embryonic development and in certain adult tissues and like p73α, it shows homology close to squid p53, again suggesting that KET and p73 may represent primordial p53 ancestral genes. The restricted expression pattern of KET, especially during embryogenesis, suggests that it is involved in tissue-specific differentiation processes. It is noteworthy that both p73 and KET have remarkable conservation of critical contact residues for mdm2 (equivalent to residues F19, D232 and L26 in p53) and the DNA-binding domain (equivalent to p53 residues R175, G245, R248, R249, R273, and R282) as well as homology in the oligomerization domain. It might be predicted that these other proteins will interact with mdm2, have similar DNA-binding properties, and potentially homo- and hetero-oligomerize. The long C-terminal extension of p73α, KET, and the squid homolog have a motif called the SAM domain which is present in diverse proteins throughout eukaryotic phyla and may function to subserve protein-protein interaction (Schultz *et al.*,1997).

I. 3. Loss of p53 Function and Tumorigenesis

One of the strongest implicators of p53 dysfunction in human tumorigenesis is the very high mutation rate of this gene. Also supporting the involvement of p53 in tumor formation are a wide range of *in vitro* and *in vivo* studies. Convincing evidence was provided by "p53 knockout" mice, which developed normally but which were predisposed to early-onset cancer, most commonly malignant lymphoma with lower frequencies of sarcoma or breast or ovarian cancers (Donehower *et al.*, 1992). Mice heterozygous for p53 inactivation developed fewer lymphomas but had more carcinomas and had a more delayed onset of tumorigenesis that was enhanced by chemical

carcinogen exposure (Harvey *et al.*, 1993a) or by ionizing radiation (Kemp *et al.*, 1994). Mouse embryo fibroblasts from p53 -/- mice had altered growth characteristics compared to wild-type fibroblasts, including shorter doubling time, ability to grow under low cell density, and lack of senescence at high passage (Harvey *et al.*, 1993b). Loss of p53 has also been shown to allow the immortalization of hematopoietic cells by myc or raf oncogenes (Metz *et al.*, 1995). Whether p53 is necessary and/or sufficient for immortalization, or whether the absence of p53 allows other genetic changes to occur which result in immortalization, remains unclear. In skin (Kemp *et al.*, 1993) and prostate (Thompson *et al.*, 1995) cancer model systems, p53 deficiency was found to correlate with increased malignant progression (but not with tumor initiation or promotion) and with a high degree of metastasis, respectively. The absence of wild-type p53 was also associated with aggressive tumor growth and a decrease of apoptosis of brain choroid plexus epithelial cancer, suggesting that p53-dependent apoptosis may act as a check to tumor growth and progression (Symonds *et al.*, 1994).

Other aspects of tumor growth and progression may also be influenced by p53 loss of function. The efficient growth of tumors is dependent on the ability to promote angiogenesis. Wild-type p53 expression results in the secretion of inhibitors of angiogenesis (Dameron *et al.*, 1994), and this may be an additional mechanism by which the presence of wild-type p53 inhibits tumor progression. Hypoxic conditions, such as those in a tumor with inadequate blood supply, induce accumulation of p53 protein (Graeber *et al.*, 1994). The ensuing reducing environment, by redox regulation, would be expected to stimulate the DNA binding ability of p53 (Hainaut and Milner, 1993). Since hypoxia has also been shown to induce p53-dependent apoptosis (Graeber *et al.*, 1996), low oxygen conditions may provide a selective advantage for cells carrying p53 mutation, evidenced by results showing that cells lacking p53 can overtake cells with wild-type p53 after hypoxia treatment. Hypoxia may further favor tumor expansion by loss of expression of anti-angiogenic factors, such as thrombospondin-1 (Dameron *et al.*, 1994), or by increased expression of the angiogenic vascular endothelial growth factor (VEGF) gene (Schweiki *et al.*, 1992).

Missense mutations of p53 might affect its function in several ways, including loss of wild-type p53 function, a trans-dominant effect of mutant over wild-type p53

function (dominant-negative effect), and gain of oncogenic potential. The fact that p53 null mice are highly tumor-prone strongly argues that loss of p53 function contributes to tumorigenesis. A dominant-negative effect of mutant p53 proteins, possibly through oligomerization with wild-type p53, may result in an inhibition of the wild-type ability to bind DNA and activate transcription. Coexpression of several p53 mutants together with wild-type p53 resulted in the suppression of wild-type p53-regulated transcriptional activation (Kern *et al.*, 1992), and indirect evidence from a yeast assay identifying dominant-negative p53 mutants suggests that the majority of mutant p53 proteins found in human tumors have the capacity to function in a dominant-negative fashion (Brachmann *et al.*, 1996). In support of these *in vitro* findings, increased tumor incidence has been found in p53 wild-type mice carrying a dominant-negative transgene (Harvey *et al.*, 1995). Some p53 mutants are capable of conferring increased tumorigenicity, metastatic potential, and tissue invasiveness (Dittmer *et al.*, 1993). Such gain-of-function properties of some mutant p53 proteins may be related to the ability of the mutant, but not the wild-type, p53 proteins to preferentially stimulate transcription from promoters of genes such as MDR1 (Dittmer *et al.*, 1993). A very interesting experiment demonstrating both the dominant-negative and gain-of-function effects was performed with transgenic mice expressing a mutant p53 protein in wild-type and p53-deficient genetic backgrounds. Expression of this mutant increased tumor incidence in mice carrying one or both wild-type alleles but not in mice that were homozygous null for p53 (Harvey *et al.*, 1995).

I. 4. Prevalence and Types of p53 Alterations in Malignancies

Although the overall proportion of human tumors shown to harbor p53 mutations is very high (up to 50%) (Hollstein *et al.*, 1994), the mutation frequency varies depending on the cancer type (Table I. 3. 1.). Allelic losses, missense and frameshift mutations, intragenic deletions, and epigenetic changes have all been shown to occur in the p53 gene of human tumor tissues. Some malignancies, however, such as Wilms tumors, testicular cancers, pituitary tumors, and pheochromocytomas rarely contain p53 mutations. While other tumor suppressor genes are often disrupted by nonsense mutations leading to a truncated protein product, p53 mutations are mostly substitution changes that prevent

Table I. 3. 1. Frequency of p53 Mutations in Different Tumor Types

Tissue or Site	Frequency (%)	Tissue or Site	Frequency (%)
Lung	50	Brain	25
Colorectum	50	Adrenal	23
Esophagus	45	Breast	22
Ovary	44	Endometrium	22
Pancreas	44	Kidney	19
Skin	44	Thyroid	13
Stomach	41	Hematopoietic	12
Head and neck	37	Carcinoid	11
Bladder	34	Melanoma	9
Prostate	30	Parathyroid	8
Hepatocellular	29	Cervix	7

Compliled from Hollstein *et al.* (1994).

sequence-specific transcriptional activation (Vogelstein and Kinzler. 1992). Approximately 40% of these mutations cluster in four of the evolutionarily conserved hotspots which encompass the DNA-binding domain (Cho et al.. 1994). The sites of these mutations in the p53 gene sequence. as well as the timing of their occurance in the neoplastic progression. may offer clues to the etiology of the different types of cancer.

Mutations in several oncogenes and tumor suppressor genes that regulate the pathways of cell growth and death appear necessary for tumor formation and progression. As each mutation arises. the tumor may progress histologically. as has been characterized for the adenoma-adenocarcinoma sequence in colon cancer (Fearon and Vogelstein. 1990). By examining the p53 status of different tumors from distinct histopathologic stages. p53 mutations have been shown to occur in relatively early. benign cancers of the skin (Ziegler *et al.*, 1994). lung (Sozzi *et al.*, 1992). breast (Thor *et al.*, 1992). and head and neck (Boyle *et al.*, 1993). In contrast. p53 mutations have been suggested to occur later in the development of tumors of the colon (Baker *et al.*, 1989). prostate (Navone *et al.*, 1993). ovary (Kohler *et al.*, 1993). bladder (Fujimoto *et al.*, 1992). cervix (Tsuda and Hirohashi. 1992). endometrium (Tsuda and Hirohashi. 1992). and thyroid (Donghi *et al.*, 1993). Interestingly. the former group of tumors is associated with known environmental

carcinogens. A comparison of the types of p53 mutations identified in these tumors demonstrates significant variation between tumor types, but correlates well with the type of DNA damage specific to each environmental carcinogen (Table I. 3. 2.). In fact, specific p53 mutations in tumors may be used to identify carcinogens that contribute to the genesis of a particular type of tumor (Greenblatt *et al.*, 1994). For example, dipyrimidine mutations such as CC→TT transitions occur in approximately 10% of skin cancers, where UV light is the principle carcinogen, but are rare in other malignancies with p53 mutations (Ziegler *et al.*, 1993). Once a keratinocyte has impaired p53 function, it would be unable to arrest in G1 in response to UV-induced DNA damage, thus potentially enhancing the likelihood of accumulating more mutations. The keratinocyte with mutant p53 is also less likely to undergo apoptosis following UV exposure, and therefore sun exposure may preferentially eliminate skin cells with normal p53, while more resistant p53 mutant cells continue to grow. Since loss of p53 function has been linked to suboptimal repair of UV-induced thymine dimers (Ford and Hanawalt, 1995), continued UV irradiation could then promote genetic instability and tumorigenesis by mutating other genes.

Other types of cancer may also have characteristic p53 mutations. The p53 mutation spectrum identified in squamous cell lung cancer has been linked directly to cigarette smoke carcinogens such as the polycyclic aromatic hydrocarbon,

Table I. 3. 2. Distinct p53 Mutations Frequently Observed in Different Tumor Types

Tumor Type	Frequently Observed p53 Mutation	Mutagen Implicated
Squamous cell carcinoma of the skin	C to T transition at consecutive dipyrimidine nucleotides	UV light
Squamous cell carcinoma of the lung	G to T transversion	Benzo[a]pyrene in cigarette smoke
Colorectal cancer	C to T transition at CpG dinucleotides	Endogenous DNA metabolism
Hepatocellular carcinoma	G to T transversion at codon 249	Dietary aflatoxin B1
Angiosarcoma of the liver	AT to TA double transversion	Vinyl chloride

Compiled from Hollstein *et al.* (1998).

benzo[a]pyrene (Denissenko *et al.*, 1996). A benzo[a]pyrene metabolite binds preferentially to guanine at the three positions where p53 is most commonly mutated in lung cancers. Moreover, this carcinogen causes the same type of mutation (guanine to thymine transversion) in animals that occur at p53 mutation hotspots in lung cancer (Ruggeri *et al.*, 1993). Therefore, a direct molecular connection links a carcinogen found in tobacco smoke to specific p53 mutations in lung cancer. The relatively early appearance of p53 mutation in lung cancer contrasts with the mutation of one p53 allele, frequently coupled with the deletion of the second allele – loss of heterozygosity (Baker *et al.*, 1989) – at the relatively late transition from benign adenoma to malignant carcinoma of the colon. Almost two thirds of these p53 mutations in colon cancer are cytosine to thymine transitions, and most of these occur at CpG dinucleotides, sites where cytosine is methylated and can be mutated to thymine as a result of deamination (Greenblatt *et al.*, 1994). Therefore, these mutations in colon cancer may not be a consequence of an exogenous carcinogen but rather may result from endogenous DNA alteration. p53 mutation may be selected for in these tumors after cell proliferation has created an environment of hypoxia and nutrient deprivation, which can cause p53-dependent apoptosis (Graeber *et al.*, 1996).

In other tumors, p53 protein may be inactivated by mechanisms other than p53 gene mutation and deletion. Most cases of cervical cancer are associated with human papilloma virus (HPV) infection (zur Hausen, 1991). The virus produces the E6 protein, which binds to p53 and facilitates its degradation, thereby creating a state of p53 dysfunction similar to p53 mutation in tumors (Scheffner *et al.*, 1990). In addition, in many soft tissue sarcomas, amplification of the mdm2 gene results in mdm2 protein overexpression (Oliner *et al.*, 1992), which functionally simulates a p53-null state in the tumor because of the ability of mdm2 to bind and inactivate p53 (Momand *et al.*, 1992; Barak *et al.*, 1993).

As mentioned above, in a few tumor types, p53 mutation is uncommon. Although several studies have reported overexpressed p53 protein in testicular germ cell tumors (Guillou *et al.*, 1996), no p53 mutations have been detected (Peng *et al.*, 1993). Since these tumors retain wild-type p53, which is capable of signaling to the apoptotic machinery, this may contribute to the high cure rates even for metastatic disease.

Neuroblastoma is another tumor type that frequently retains wild-type p53 (Vogan *et al.*, 1993). In these tumors, however, a region of chromosome 1 to which the p53-related p73 gene has been mapped is frequently deleted (Kaghad *et al.*, 1997). Although no mutations have been detected in the p73 allele, which is not deleted, initial studies suggest that p73 is monoallelically expressed from the maternal chromosome 1 (Kaghad *et al.*, 1997). Therefore, loss of the single expressed allele might be sufficient to prevent p73 expression and function.

The importance of p53 mutations in human tumor development is further illustrated by individuals who inherit germ-line p53 mutations. These patients are at an extremely high risk for developing malignancies at a young age. Germ-line transmission of a mutant p53 allele results in the familial cancer syndrome, Li-Fraumeni, which is characterized by an increased risk for developing a wide variety of cancers, including breast carcinoma, sarcomas, hematologic malignancies, and adrenocortical and brain tumors (Malkin *et al.*, 1990; Srivastava *et al.*, 1990). Inheriting a mutant p53 allele has also been associated with young patients developing multiple primary cancers (Malkin *et al.*, 1992).

I. 5. Methods of Determining p53 Mutational Status

I. 5. 1. Analysis of the p53 Gene Sequence

Direct sequencing of all 11 exons of the p53 gene, amplified by polymerase chain reaction (PCR), is the most precise method for detecting p53 mutations, although sequencing of exons 5 to 8 (codons 126 to 306) alone has been shown to detect more than 85% of all p53 mutational changes (Greenblatt *et al.*, 1994). Sequencing of the p53 gene has been performed on tumor biopsies (Mitsudomi *et al.*, 1993), microdissected cells from tissue specimens (Hedrum *et al.*, 1994), and on the tumor cells present in minute quantities in body fluids such as urine (Hruban *et al.*, 1994) or sputum (Mao *et al.*, 1994), and has shown itself to be useful in determining the presence or absence of a germline p53 allele in family members of patients with Li-Fraumeni syndrome (Malkin *et al.*, 1990) or with other hereditary cancer syndromes (Sameshima *et al.*, 1992; Brugieres *et al.*, 1993). As powerful as the technique has been demonstrated, direct sequencing of the p53 gene has been considered to be unsuitable for routine clinical testing because of

excessive time and labor requirements. However, the introduction of rapid, automated, and high-throughput sequencing instruments promises to overcome these limitations (Bharaj *et al.*, 1998). In one study of breast cancer patients, automated sequencing of the complete coding region of p53 yielded prognostic information and predicted response to adjuvant therapy and radiotherapy (Bergh *et al.*, 1995), and in another study, targeting of sites in the p53 gene encoding residues that directly contact DNA provided better prognostic information for breast cancer patients than mutations occuring elsewhere in the p53 gene sequence (Berns *et al.*, 1998). It has been suggested that sequencing of cDNA generated from p53 mRNA is a simple and rapid alternative to genomic sequencing, although in one comparative study (Williams *et al.*, 1998), only the latter technique was able to detect intron alterations as well as all splice site mutations and stop mutations, both of which were associated in some cases with reduced stability of p53 mRNA.

Genetic abnormalities of p53 have also been demonstrated by less expensive and simpler analyses, the results of which were later usually confirmed by direct DNA sequencing. The altered electrophoretic mobility, under non-denaturing conditions, of single strands of PCR-amplified p53 fragments containing missense mutations can be compared to the migration rate of wild-type fragments in order to detect mutations. This technique, single-strand conformation polymorphism (SSCP) analysis, has a sensitivity and specificity approaching 90% for detecting p53 mutations (Orita *et al.*, 1989), and has been applied for the analysis of cancer cell lines (Murakami *et al.*, 1991) and of most types of tumors (Chang *et al.*, 1992; Hunter *et al.*, 1993; Kupryjanczyk *et al.*, 1993; Lohmann *et al.*, 1993; Kappes *et al.*, 1995; de Witte *et al.*, 1996). p53 mutations have also been detected by other screening methods, including denaturing gradient gel electrophoresis (Beck *et al.*, 1993) and the related constant denaturant gel electrophoresis (Borresen *et al.*, 1991) and temperature gradient gel electrophoresis (Schlechte *et al.*, 1997), all of which, like SSCP, have sensitivities for detecting p53 mutation less than direct sequencing. Recently developed and applied for the detection of p53 gene alterations are newer screening methods such as dideoxy fingerprinting, which is a highly sensitive hybrid technique combining aspects of SSCP and dideoxy sequencing (Martincic *et al.*, 1999), enriched PCR SSCP (Behn *et al.*, 1998), oligonucleotide-

mediated mismatch ligation assay (Hibi *et al.*, 1998), and cDNA microarrays, enabling the measurement of the expression of thousands of genes, in addition to p53, in a single experiment (Kononen *et al.*, 1998).

I. 5. 2. Detection of p53 Protein Accumulation

Wild-type p53 protein has a lifespan of several minutes, leading to nearly undetectable levels of p53 protein in most cells, whereas the much longer half-lives of p53 mutants facilitate their detection (Nigro *et al.*, 1989). The strong correlation between p53 gene mutation and p53 protein accumulation existing under most, but not all, circumstances permits simple immunological methods for p53 protein detection to substitute for the more complex and labor-intensive molecular biology techniques. Most commonly, these methods take the form of routine immunohistochemical techniques which are rapid, technically simple, and able to provide antigen localization with respect to histologic features of the tumor. A compilation of 84 studies in which immunohistochemistry (IHC) and sequencing methods were performed on the same tumor sets revealed that sensitivity for immunostaining for mutant p53 was 75% and the positive predictive value was 63% (Greenblatt *et al.*, 1994). The significant percentages of false-positive and false-negative findings typical of IHC (or of ELISA) may result from the fact that nonsense mutations, which lead to truncated forms of p53, do not result in increased concentrations of p53 intracellularly; moreover, p53 concentrations are increased in some tumors, particularly melanomas and testicular carcinomas, without containing any mutations (Harris and Hollstein, 1993).

Although immunohistochemical techniques have proven invaluable for the rapid screening of essentially every tumor type for p53 accumulation, methodologic differences resulting from various combinations of tissue fixation and antigen unmasking procedures (for formalin-fixed, paraffin-embedded sections), primary anti-p53 antibody, and criteria for designating the p53 expression status of the specimens, have made cross-study comparisons of IHC-detected p53 protein difficult (Baas *et al.*, 1994; Fisher *et al.*, 1994). An alternative to IHC is the measurement of soluble p53 protein in tissue extracts by quantitative immunoassay. Although these ELISA methods likewise suffer from inherent pitfalls, including the requirement for fresh frozen tissue and the inability to localize p53

expression to cellular or tissue components, they may offer several advantages. Relative to IHC, ELISAs may be performed with minimal technical expertise, they generate numerical results amenable to objective and consistent interpretation, and they may provide enhanced specificity owing to the use of two p53-specific antibodies (in the most common assay configuration). ELISAs for p53 have been used to determine p53 concentrations in extracts from tumors of breast (Bartkova *et al.*, 1993; Hassapoglidou *et al.*, 1993; Thomas *et al.*, 1997; Norberg *et al.*, 1998), gastrointestinal (Bartkova *et al.*, 1993; Joypaul *et al.*, 1993), vulval (Bartkova *et al.*, 1993), head and neck (Maass *et al.*, 1997), and lung (Pappot *et al.*, 1996) origins and have been shown to be comparable to immunohistochemistry, when performed in parallel, for the assessment of p53 protein status in breast (Vojtesek *et al.*, 1993; Thomas *et al.*, 1997; Norberg *et al.*, 1998) and gastrointestinal (Joypaul *et al.*, 1993) tumor specimens. Direct comparison of a p53-specific luminometric immunoassay and cDNA sequencing has recently reported the ability of the former method to detect the majority of missense mutations, but not deletions, insertions, and some frameshift mutations in breast cancer specimens (Norberg *et al.*, 1998).

I. 6. Potential Role of p53 in the Management of Cancer

I. 6. 1. p53 as a Diagnostic and MonitoringTool

The high prevalence of p53 mutations in human cancer suggests that p53 could be used as a marker of malignancy. In cytology, suspicious cases frequently occur for which a satisfactory distinction between benign and malignant cannot be made, and it has been suggested that the immunocytochemical detection of p53 may be used for this purpose in those tumor types in which p53 mutation is an early event (Dowell *et al.*, 1994). As p53 mutation is not a consistent finding in all tumors, the sensitivity of this approach would be poor, but the specificity would be high (close to 97%). The detection of p53 alterations would be especially useful in those cancers in which p53 mutational events occur early in tumor progression, such as in tumors of lung (Chiba *et al.*, 1990; Kohno *et al.*, 1999), breast (Thor *et al.*, 1992), and head and neck (Boyle *et al.*, 1993) origin. p53 alteration has also been associated with the progression of Barrett's epithelium to invasive esophageal carcinoma (Casson *et al.*, 1994). Although p53 mutation can occur in the

earliest clinically detectable stages of the neoplastic process in some types of cancer, the fraction of tumors with an altered p53 gene is typically higher in late stage tumors. Alteration of p53 status assessed by molecular diagnostic strategies or immunochemical analyses of tissue biopsy samples, cytologic specimens, or cells collected from nipple aspirates might be an early indicator of response in cancer chemoprevention trials as well as an indicator of disease in screening programs that use cytologic specimens. In fact, the potential of p53 mutational analysis to aid in the early diagnosis of cancer has already been demonstrated in some cases. Both an invasive bladder carcinoma specimen, and a cytologic preparation made from urine collected nine years before surgery, were found to contain the same p53 mutation (Hruban *et al.*, 1994). Bronchial biopsy samples from patients with lung cancer have also been found to harbor p53 mutations (Mitsudomi *et al.*, 1993a), and the sputum of a series of lung cancer patients were frequently found to have p53 mutations, detected one year prior to the diagnosis of lung cancer in one case (Mao *et al.*, 1994). Similar findings of p53 mutations in bronchial washings of patients with early stage non-small cell lung cancer (NSCLC) have recently been reported (Ahrendt *et al.*, 1999). p53 gene status has also been proposed as a marker to discriminate multicentric lung cancers from intrapulmonary metastases (Matsuzoe *et al.*, 1999) and, when determined in serum DNA from patients with colorectal cancer, may be useful for disease monitoring (Hibi *et al.*, 1998).

Carriers of a mutated p53 gene in families affected by Li-Fraumeni syndrome, characterized by an elevated risk of early-onset breast cancer, childhood sarcomas, and other neoplasms (Malkin *et al.*, 1992), have a 50% likelihood of being given a diagnosis of cancer by the age of 30, and the probability increases to nearly 90% by the age of 65. Treatment of the primary cancer with radiation or chemotherapeutic agents may increase the risk of second cancers. Rapid DNA-based screening tests have been developed to detect these germline p53 mutations, but simpler tests of p53 function have also been described to identify carriers (Frebourg *et al.*, 1992; Camplejohn *et al.*, 1995). The potential benefits of predictive testing for germline p53 mutations among cancer prone individuals has been subject to debate, and a number of bioethical and economic issues have also been raised (Li *et al.*, 1992). Although much less strongly linked to cancer risk, a polymorphism at codon 72 of the p53 gene has been associated with an increased odds

ratio for development of adenocarcinoma of the lung (Wang *et al.*, 1999). Development of breast cancer in women with benign breast disease has also been associated with p53 status, as demonstrated in a large nested case-control study (Rohan *et al.*, 1998).

Very soon after p53 protein accumulation was recognized in tumor tissue, mutant p53 proteins, as tumor specific antigens, were proposed to be targets of the host immune system. Examination of serum has shown that some patients with cancer harboring a mutated p53 allele have mounted a humoral immune response to abnormally high levels of p53 resulting from the prolonged stability of the mutant forms released from the dying tumor cells. Serum antibodies against p53 have been found in approximately 10-20% of patients with breast (Crawford *et al.*, 1982; Green *et al.*, 1994), lung (Winter *et al.*, 1992), colon (Angelopoulou *et al.*, 1994; Houbiers *et al.*, 1995), and ovarian (Angelopoulou *et al.*, 1994) cancers but in a smaller percentage of patients with leukemia or with thyroid or prostate cancers (Lubin *et al.*, 1995a). Although the vast majority of sera containing autoantibodies against p53 are from patients whose primary tumor tissues overexpress p53 (Wild *et al.*, 1995), a detectable immune response to p53 protein does not appear to be a consistent phenomenon, since the proportion of tumors with mutant p53 protein is usually considerably higher (Rainov *et al.*, 1995; Vojtesek *et al.*, 1995). Serological diagnosis of cancers such as liver angiosarcoma in workers exposed to vinyl chloride (Trivers *et al.*, 1995) or lung cancer (Lubin *et al.*, 1995b; Trivers *et al.*, 1996) may be possible since the anti-p53 immune response may be early events detectable before clinical manifestation of these diseases. Several studies document the ability of serum titre of p53 autoantibodies to parallel tumor burden (Angelopoulou *et al.*, 1997; Mitsudomi *et al.*, 1998) and response to treatment (Zalcman *et al.*, 1998), suggesting their potential utility for serological monitoring of cancer.

The serum concentration of soluble p53 protein, presumably tumoral in origin, has also been suggested as a potential cancer biomarker (Winter et al., 1992). Application of p53 immunoassays have shown elevated levels of detectable p53 in the sera of patients with lung cancer (Luo *et al.*, 1994), pancreatic cancer (Suwa *et al.*, 1997), hematopoietic cancers (Lehtinen *et al.*, 1996), asbestosis (Partanen *et al.*, 1995; Hemminki *et al.*, 1996), and following exposure to carcinogenic chromium compounds (Hanaoka *et al.*, 1997) or vinyl chloride (Smith *et al.*, 1998), relative to nondiseased or nonexposed controls.

However, in other studies, anti-p53-immunoreactivity was inconclusively related to either the presence of malignancy (Segawa *et al.*, 1997) or to exposure to carcinogens such as asbestos (Krajewska *et al.*, 1998). Furthermore, although the use of HPLC fractionation of putative tumor-associated antigens isolated by novel a gel fiberglass chromatography method has also been proposed to demonstrate p53 protein presence in serum of patients with colon cancer (Zusman *et al.*, 1996; Sandler *et al.*, 1998), the specificity of this method has not been adequately confirmed.

I. 6. 2. Cancer Therapy by p53 Restoration or Modulation

In the treatment of cancer, efforts to restore or modulate p53 function have been based on the rationale that tumor cells endogenously lacking this function might destroy themselves or at least become more susceptible to the effects of DNA damage inflicted by conventional chemotherapy or radiotherapy once p53 function would be restored. Three approaches are particularly promising. The first approach involves virus-mediated gene transfer and expression, the second is the use of a cytolytic virus which can replicate only in cells lacking p53 function, and the third is based on the design of small molecules that can interfere with the negative regulation of p53, pharmacologically activating the p53 response.

A wild-type p53 DNA fragment which has been inserted into a retroviral vector has been used to restore the wild-type p53 gene to lung cancer cells and has been shown to suppress the growth of both lung cancer cell lines and human lung cancers in nude mice (Fujiwara *et al.*, 1994). Similar findings in colorectal cancer cells have been reported using the replication defective adenovirus Ad5/CMV/p53 (Spitz *et al.*, 1996a); it has also been shown that this agent has a synergistic effect when used with cisplatin chemotherapy (Ogawa *et al.*, 1997). In fact, introduction of the same adenovirus into colon cancer cells has been found to potentiate the cytotoxic effects of a variety of anticancer agents (Yang *et al.*, 1996) as well as to ionizing radiation (Spitz *et al.*, 1996b), and its intracerebral injection into gliomas in nude mice caused tumor regression, even in those tumors containing endogneous wild-type p53 (Li *et al.*, 1999). A clinical trial using wild-type p53 gene transfer in nine patients with NSCLC in whom conventional treatment had failed has been reported (Roth *et al.*, 1996). In this study, a retroviral

vector was injected directly into the tumor either percutaneously with radiological guidance or via a brochoscope. *In situ* hybridization and PCR demonstrated vector-p53 sequences in post-treatment biopsies, and apoptosis was more frequent in post-treatment than in pretreatment biopsies. No treatment-related toxicity was noted and tumor regression occurred in three patients. Another phase I trial in advanced NSCLC using a different adenovirus, delivered similarly, has confirmed the expression and safety of wild-type p53 gene therapy (Schuler *et al.*, 1998), proof of principle of which has also been demonstrated in several other cancer models including thyroid (Blagosklonny *et al.*, 1998), ovarian (von Gruenigen *et al.*, 1998), head and neck (Clayman *et al.*, 1998), and colon (Bouvet *et al.*, 1998) cancers. Rather than using viruses as a vehicle to deliver wild-type p53 into lung cancer cells, another group employed cationic liposome-p53 complexes, which were shown to effectively introduce p53 into bronchial epithelium *in vivo* (Zou *et al.*, 1998).

A DNA tumor adenovirus produces a 55 kDa protein from the E1B region of its genome which binds and inactivates p53. It was hypothesized that an adenovirus lacking E1B would not be able to replicate in normal cells but would in cancer cells lacking p53 function. For this reason, ONYX-015, an E1B gene-attenuated adenovirus was compared with normal adenovirus in human colonic cancer cell lines with and without p53 function. As expected, the ONYX-015 virus replicated as efficiently as the normal virus in the cell line lacking p53, but not in the cell line with normal p53 (Bischoff *et al.*, 1996). Subsequent work showed that, while normal human cells are very resistant to the cytolytic effects of ONYX-015, a wide range of human tumor cell lines with either mutant or normal p53 gene sequences are destroyed (Heise *et al.*, 1997). The susceptibility of tumor cells with wild-type p53 may be due either to undetected p53 mutations or to malfunction of other components of the p53 pathway. Although a phase I trial of ONYX-015 in patients with advanced cancer is currently underway (Kirn *et al.*, 1998), doubts have been raised about both its efficacy and safety (Hall *et al.*, 1998; Rothman *et al.*, 1998).

The activity of p53 is dependent, at least in part, on sequence-specific DNA binding which is controlled by at least two negative regulatory domains in the p53 molecule (Hupp *et al.*, 1992; Muller-Tiemann *et al.*, 1998). Neutralization of the C-

terminal regulatory domain by a specific antibody activates p53 (Hupp *et al.*, 1995), and it is likely that stress-related factors which influence the transcriptional activity of p53 act in a similar manner. The same antibody can activate mutant forms of p53 synthesized by human tumor cell lines (Niewolik *et al.*, 1995), and it has been suggested that small molecules might be designed to activate conformationally-altered mutant p53 to a form competent for DNA-binding and transactivation.

I. 6. 3. Prognostic Implications of p53 Mutations

Consistent with findings that p53 alterations often correlate with more aggressive tumor phenotypes, the presence of p53 mutation has been associated with worse clinical outcome in many forms of cancer. Contributing to the poor prognosis of patients with p53 mutation may be the loss of wild-type p53 tumor suppressor function, resistance to radio- or chemotherapy, or the acquisition of oncogenic properties by mutant p53. The rapid progression, including invasion and/or metastasis of follicular lymphomas (Sander *et al.*, 1993), gliomas (van Meyel *et al.*, 1994) and of many other cancers, including brain (Sidransky *et al.* 1992), bladder (Fujimoto *et al.*, 1992), breast (Mazars *et al.*, 1992; Faille *et al.*, 1994), gastric (Kakeji *et al.*, 1993), head and neck (Boyle *et al.*, 1993), prostate (Visakorpi *et al.*, 1992), ovarian (Marks *et al.*, 1991), liver (Hsu *et al.*, 1993), and thyroid (Donghi *et al.*, 1993) carcinomas have been associated with p53 mutations.

Decreased survival of patients with various types of cancer have also been associated with mutations of the p53 gene. In breast cancer, accumulation of p53 protein was correlated with both p53 mutation (a relationship common to most, if not all, malignancies) and shortened survival (Thor *et al.*, 1992; Friedrichs *et al.*, 1993), and has predicted decreased overall survival in node-negative patients (Silvestrini *et al.*, 1993; Falette *et al.*, 1998). This latter finding might prove to be of especial importance since a proportion of axillary lymph node-negative breast cancer patients undergo an accelerated relapse and hence may be good candidates to receive adjuvant chemotherapy, which otherwise might not be administered to these patients. Not all studies of p53 gene mutation or protein accumulation and survival of node-negative breast cancer patients, however, have come to the same conclusion (Rosen *et al.*, 1995). In colorectal cancer, accumulation of p53 was found to correlate with high risk for disease recurrence and

decreased survival time after surgery (Bosari *et al.*, 1994), and in lymph node-positive colon cancer, p53 was shown to be an independent prognostic factor (Zeng *et al.*, 1994). p53 cDNA sequencing was recently shown to offer better prognostic information than p53 immunostaining of colorectal carcinomas (Kressner *et al.*, 1999). p53 mutation and/or protein overexpression has also been reported to predict shortened overall survival in soft tissue sarcomas (Drobnjak *et al.*, 1994), gastric cancer (Martin *et al.*, 1992), chronic myelocytic leukemia blast crisis (Nakai *et al.*, 1994), NSCLC (Mitsudomi *et al.*, 1994), endometrial cancer (Soong *et al.*, 1996), colorectal cancer (Goh *et al.*, 1995), prostate cancer (Bauer *et al.*, 1995), head and neck cancer (Sauter *et al.*, 1995), and renal cell carcinoma (Uhlman *et al.*, 1994). Furthermore, the presence of a humoral immune response to p53, manifested as detectable serum anti-p53 antibodies, has also been associated in some studies with shortened post-operative survival in a wide variety of cancers (Houbiers *et al.*, 1995; Angelopoulou *et al.*, 1996; Bourhis *et al.*, 1996; Lai *et al.*, 1998), although other studies have been unable to confirm the prognostic value of these antibodies (Mitsudomi *et al.*, 1998; Maehara *et al.*, 1999) or have demonstrated them to be associated with improved outcome (Saffroy *et al.*, 1999). Similarly, p53 alterations in some tumor tissues has also been associated with better prognosis (Soong *et al.*, 1997).

I. 6. 4. p53 Mutation and Response to Cancer Treatment

Because both chemotherapy and radiotherapy had been demonstrated to cause DNA damage (Kerr *et al.*, 1994), it was suggested that p53-dependent apoptosis may be responsible for at least part of the therapeutic effects of these treatments (Lowe *et al.*, 1994). Cells transformed by the viral oncogene E1A undergo p53-dependent cell death in response to ionizing radiation or treatment with 5-fluorouracil, etoposide, or doxorubicin (Lowe *et al.*, 1993a). Work using transgenic mice has shown that p53 mutations increase the resistance of hematopoietic cell lineages to gamma-irradiation (Lee and Bernstein, 1993). In addition, X-irradiation at 9.5 days of gestation produces fewer deaths but more embryonic abnormalities in p53-deficient mice compared with wild-type animals (Norimura *et al.*, 1996), reinforcing the notion that normal functioning of the p53 pathway is required for efficient DNA repair or cell death after irradiation. A study of a small series of patients with rectal cancer showed that radiotherapy appeared to increase

the number of apoptotic cells only in tumors expressing wild-type p53 (Hamada *et al.*, 1996). In breast cancer, p53 mutations were correlated with resistance to doxorubicin treatment (Aas *et al.*, 1996). p53 protein overexpression status or gene mutation was also associated with resistance to combination chemotherapy in patients with advanced gastric cancer (Cascinu *et al.*, 1998), esophageal carcinoma (Ribeiro *et al.*, 1998), NSCLC (Kawasaki *et al.*, 1997), ovarian cancer (Marx *et al.*, 1998) and multiple myeloma (Drach *et al.*, 1998). It is also of interest that human tumors that are generally sensitive to radiotherapy or chemotherapy, such as testicular cancer or childhood acute lymphoblastic leukemia, display low rates of p53 mutations (Lowe *et al.*, 1993a; Lutzker and Levine, 1996; Zamble *et al.*, 1998), whereas tumors that very often contain p53 mutations, such as colorectal and lung cancers, tend to respond less well to these treatments. There is also some evidence that p53 status may not always predict outcome reliably. Some reports indicate that inactivation of the p53 gene can actually render cells more sensitive to genotoxic damage (Fan *et al.*, 1995; Xu *et al.*, 1995), and in a study of human squamous carcinoma cells p53 mutations did not correlate with radiosensitivity (Jung *et al.*, 1992).

I. 7. Hypotheses, Rationales, and Objectives

The p53 gene, mutated more frequently than any other in diverse human malignancies (Harris and Hollstein, 1993), functions to maintain genomic stability by the induction of cell cycle arrest or programmed cell death in the presence of cellular stresses such as DNA damage (Levine *et al.*, 1997). Mutational inactivation of p53 is thought to impair this cell cycle checkpoint, resulting in a proliferative advantage and/or escape from apoptotic deletion of cells with potentially cancer-causing genetic changes (Levine *et al.*, 1997). Most p53 mutations lead to the expression of a conformationally-altered p53 protein which accumulates in tumor cell nuclei and can be detected with specific antibodies (Hall *et al.*, 1991). Studies of p53 protein expression in tumors have employed these antibodies almost exclusively as immunostaining reagents (Dowell *et al.*, 1994), despite the possible advantages offered by immunoassays (ELISAs) of p53 protein. For example, an ELISA would be applicable for the quantitative assessment of p53 protein not only in extracts prepared from solid tumors, but also in other biological fluids, including serum, where p53 might prove to have important cancer diagnostic and

monitoring value. The presence of p53 protein in the sera of cancer patients, however, has not been unequivocally demonstrated using the existing detection methods (Luo *et al.*, 1994; Partanen *et al.*, 1995; Zusman *et al.*, 1995, 1996; Hemminki *et al.*, 1996; Lehtinen *et al.*, 1996; Hanaoka *et al.*, 1997). Other clinical implications of p53 might focus on its expression status in tumor tissue, particularly as it relates to cancer prognosis and treatment response. Despite the value of established prognostic markers such as disease stage, additional prognostic markers are often needed in order to identify those patients with relatively localized cancer who will nonetheless relapse and die because of their aggressive cancers. Numerous clinical studies have shown that although p53 alteration typically appears in more advanced cancers (limiting its usefulness for early cancer detection) (Baker *et al.*, 1989; Fujimoto *et al.*, 1992; Donghi *et al.*, 1993; Kohler *et al.*, 1993; Navone *et al.*, 1993), the ability of tumor p53 status to indicate disease aggressiveness and hence patient prognosis remains controversial (Dowell and Hall, 1995; Kirsch *and* Kastan, 1998). Furthermore, although the efficacy of many antineoplastic drugs may depend on their capacity to induce p53-dependent apoptosis, especially as demonstrated *in vitro* (Lowe *et al.*, 1994), the value of p53 status for predicting clinical response to chemotherapy is also unclear (Fan *et al.*, 1995; Xu *et al.*, 1995; Aas *et al.*, 1996; Kawasaki *et al.*, 1997).

The development and careful validation of a tool with which to quantitatively analyze soluble p53 protein was the first objective of the series of studies summarized in this thesis and is described in Chapter II. The immunoassay was designed so as to be applicable not only to lysates prepared from cultured cells or pulverized solid tissues, but also to serum, a sample matrix which frequently contains immunoassay-interfering constituents (Nahm and Hoffmann, 1990). The detection of p53 protein, exogenously added to serum, by the new immunoassay was another early objective addressed in Chapter II and was necessary for a screen of cancer patient serum conducted later. Because the standard approach to detect p53 protein in tumor tissue is by immunohistochemical staining, the second objective was to compare the latter technique, performed on formalin-fixed, paraffin-embedded sections of primary lung tumor tissues, to the new immunoassay applied to extracts of fresh-frozen specimens from adjacent regions of the same tissues. This study, described in Chapter III, was important in order

to provide additional validation for our immunoassay as well as an understanding of the relationship between histological distribution of p53 immunostaining and the concentration of p53 in tissue lysates. Another relationship, that between p53 protein accumulation and p53 gene mutation, was addressed in Chapter IV. The work in this chapter satisfied the third objective, which was to assess ovarian carcinoma tissues both for genetic p53 abnormalities by sequencing of PCR-amplified exons 5 to 9 (within which the majority of mutations have been shown to occur) and for p53 protein accumulation by the new immunoassay. This methods comparison was primarily a further validation for the new assay against which some researchers consider is the definitive technique for ascertaining p53 status. Chapter V describes a study pursuing the fourth objective. Because experiments performed during assay development suggested to us that reports from other groups of measurable quantities of p53 protein in the sera of cancer patients may represent artifactual findings, sera collected from cancer patients before and after surgery, from patients with nonmalignant lung diseases, and from normal individuals were assayed for p53 in an effort to determine the utility of p53 as a circulating tumor marker. Our negative findings prompted us not to consider further studies of p53 in serum. However, studies performed previously employing a "first generation" p53 immunoassay (Hassapoglidou *et al.*, 1993) had indicated that p53 protein accumulation in breast (Levesque *et al.*, 1994, 1995a) and ovarian (Levesque *et al.*, 1995c) tumors was associated with biochemical, histologic, and clinical features of aggressive cancers, confirming a number of other studies (Thor *et al.*, 1992; Friedrichs *et al.*, 1993; Teneriello *et al.*, 1993). In light of the potential prognostic relevance of immunoassay-determined p53, the fifth, sixth, and seventh objectives were to assess the relationship between p53 protein expression and survival outcomes in patients with lung (Chapter VI), breast (Chapter VII), and ovarian (Chapter VIII) malignancies, respectively. Also studied in Chapter VIII was the relationship between the expression levels of a downstream target gene ($p21^{WAF1}$) and patient prognosis, as well as the relationship between p53 status and response to platinum-containing chemotherapy - the latter representing the eighth and final objective. These studies, all of which have been published or submitted for publication, are followed by a general discussion (Chapter IX).

CHAPTER II

DEVELOPMENT OF AN IMMUNOFLUOROMETRIC ASSAY FOR p53 PROTEIN

(A version from Levesque MA, D'Costa M, Angelopoulou K, Diamandis EP. *Clin Chem* 1995; 41: 1720-1729, with permission from Mac Fancher, Publisher)

II. 1. Abstract

Described here is the evaluation of a new "sandwich" immunoassay for p53 protein incorporating modifications to a previously reported method, including the use of microtiter plates coated directly with the anti-p53 monoclonal antibody DO-1, of a detergent- and mouse serum-containing sample diluent, and of a labeled secondary antibody diluent to which goat serum was added. The use of CM-1 antiserum to probe the immunocaptured p53 and the detection of bound complexes by a labeled secondary antibody allows coupling to a time-resolved fluorescence detection system. The new assay yielded p53 concentrations comparable to the previous one in breast tumor cytosols (n=198), in nondiseased breast tissues (n=70), and in five transformed cell lines, but showed differences in p53 values measured in sera from patients without cancer (n=78) which were revealed to be nonspecific interferences affecting the original method, implying that the new immunoassay has improved specificity for serum p53 quantification.

II. 2. Introduction

Correlating very closely with p53 genetic changes is the accumulation of a conformationally-altered mutant p53 protein, usually in the nucleus of transformed and tumor-derived cells (Lane and Benchimol, 1990). Normal cells, in contrast, typically have undetectable wild-type p53 protein levels. Because of the relative ease of the immunochemical detection of p53 protein compared to the approaches targeting changes at the genetic level, immunohistochemical techniques to visualize p53 protein *in situ*, using either monoclonal antibodies against mutant p53 protein or polyclonal antisera, have become the standard tools used in the majority of studies reporting p53 protein overexpression (Hall *et al.*, 1991). Using such methods, it has been demonstrated that the overexpression of p53 protein in tumor tissue may predict reduced patient disease-free survival and/or overall survival for a number of tumor types (Martin *et al.*, 1992; Thor *et al.*, 1992; Levesque *et al.*, 1995c). However, quantitative immunological methods for p53 protein measurement in cellular extracts offer an alternative approach for the quantitation of p53 protein in tumor tissues and in cultured cell lines.

Despite the potential advantages of immunological assay systems (Diamandis and Levesque, 1995), the availability of a large number of p53-specific monoclonal and polyclonal antibodies, and the recognition that more precise quantitative estimations of p53 accumulation in tumor tissues are needed than are presently provided by immunohistochemical methods (Hall and Lane, 1994), few immunoassays for the quantification of soluble p53 protein have been described (Midgley *et al.*, 1992; Vojtesek *et al.*, 1992; Hassapoglidou *et al.*, 1993). Two enzyme-linked immunosorbent assay (ELISA) kits for p53 are, however, commercially available (Oncogene Science Inc., Uniondale, NY). A p53 immunoassay developed in our laboratory (Hassapoglidou *et al.*, 1993) has been used to quantify p53 protein levels in tissue extracts prepared from breast (Hassapoglidou *et al.*, 1993; Levesque *et al.*, 1994; Levesque *et al.*, 1995a) and ovarian (Levesque *et al.*, 1995c) tumors. In this assay design, a mouse monoclonal anti-p53 antibody was immobilized on microtiter plates precoated with anti-mouse immunoglobulin (Ig). The monoclonal antibody-bound p53 is then probed with polyclonal rabbit antisera raised against recombinant p53. The subsequent addition of enzyme-labelled anti-rabbit Ig allowed coupling of the immunocaptured p53 protein with a sensitive time-resolved fluorescence detection system (Christopoulos and Diamandis, 1992).

Although this assay was successful when applied to tumor tissue extracts, preliminary studies with serum specimens indicated nonspecific interference by serum components. In the present study, we have developed and evaluated a new assay which incorporates a member of a new generation of anti-p53 antibodies as the initial capture immunoreagent together with the addition of blocking agents (animal serum and detergent) to sample and labeled antibody diluents. The result is an assay which is suitable for any sample type, including serum.

II. 3. Materials and Methods

II. 3. 1. Production of Monoclonal Antibodies

The DO-1 hybridoma cell line (Vojtesek *et al.*, 1992), provided by Dr. David P. Lane (University of Dundee), was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 mg/L sodium pyruvate and 50 g/L

gentamycin (all from GIBCO BRL, Gaithersburg, MD). Approximately 5 x 10^6 log phase growing cells were collected, washed with phosphate-buffered saline (PBS), and injected intraperitoneally as a 0.5 mL suspension in PBS into adult female BALB/c mice which had been primed 7 days earlier by injection with pristane. Ascites fluid removed after one to three weeks was subjected to Protein A affinity chromatography using Econo-Pac Protein A columns (Bio-Rad Laboratories, Richmond, CA) following the manufacturer's instructions, after which the eluates were desalted by dialysis against 0.1 mol/L $NaHCO_3$. The concentration of DO-1 antibody was determined spectrophotometrically at 280 nm.

II. 3. 2. Production of Recombinant p53 Protein and Preparation of Standard Series

Sf9 insect cells were cultured in Grace's Insect medium with supplements (Invitrogen, San Diego, CA) and infected with recombinant baculovirus for both viral amplification and protein expression according to standard practices (O'Reilly *et al.*, 1994). Low titre stock of a baculovirus vector containing the full-length wild-type human p53 gene (gift of Dr. T. Soussi, INSERM, Institut de Génétique Moléculaire, France) was used to infect log phase Sf9 cells, five days after which the supernatant was harvested and used to reinfect Sf9 cells at a multiplicity of infection between 5 and 10. Infected cells were collected after 48 hours, washed 3 times in PBS, and lysed in a lysis buffer (10 mmol/L Tris, pH 8.0, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 150 mmol/L NaCl, 1.0% NP-40, 1.0% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mg/L pepstatin, and 10 mg/L each of leupeptin and aprotinin) on ice for 30 minutes. The soluble extract was collected following ultracentrifugation at 100,000 g for 30 minutes at 4°C and aliquoted for storage at -80°C. The presence of p53 protein was confirmed by Western blot analysis. This involved electrophoresis of the Sf9 lysate using a pre-cast 8-16% Tris-glycine minigel (Novex, San Diego, CA), transfer to a nitrocellulose membrane, probing with polyclonal CM-1 antiserum (Midgley *et al.*, 1992) (Novocastra Laboratories, Newcastle upon Tyne, UK) diluted 1000-fold in buffer A (50 mmol/L Tris, pH 7.80, 60 g/L bovine serum albumin (BSA), 0.5 g/L NaN_3), and detection using the ECL Western blotting kit (Amersham, Buckinghamshire, UK).

Sf9 lysate containing p53 protein was assayed at several dilutions using our new immunofluorometric procedure (see below) in order to determine the concentration of p53 relative to arbitrary standards prepared from a breast tumor lysate containing a very high level of p53 protein (Levesque *et al.*, 1994). Assay of lysates prepared from uninfected Sf9 cells demonstrated undetectable levels of p53 protein. It was estimated that the concentration of p53 protein in the undiluted Sf9 lysate exceeded 40.000 arbitrary units per litre (U/L), or 130.000 U/g of total extract protein. Dilutions of the p53-quantitated Sf9 lysate in buffer A yielded standard solutions of 0, 2, 10, 50, 200, and 1000 U/L which were aliquoted and stored at -80°C until use. These standards were compared to p53 standards purchased from Oncogene Science. The obtained relationship was as follows: 1 µg/L of p53 protein (Oncogene Science) is approximately equivalent to 13 U/L (our arbitrary standard).

II. 3. 3. Optimization of Assay Conditions

Variations of the previously described immunofluorometric assay for p53 protein (Hassapoglidou *et al.*, 1993; Levesque *et al.*, 1994), hereafter referred to as the "original assay", were first evaluated by assaying in duplicate, 50 µL volumes of p53 standards prepared by dilution of a breast tumor extract. In common with all of the configurations described were the use of 96-well white polystyrene microtiter plates (Dynatech Laboratories, Chantilly, VA) and a programmable plate washer (Adil Instruments, Strasbourg, France). The wash solution consisted of 5 mmol/L Tris, pH 7.80, containing 150 mmol/L NaCl and 0.5 g/L Tween-20 (Sigma, St. Louis, MO). All assay configurations also shared a final enzymatic activity measurement step. This included addition of a stock solution of 0.01 mol/L diflunisal phosphate in 0.1 mol/L NaOH, diluted 10-fold in 0.1 mol/L Tris, pH 9.10, containing 0.15 mol/L NaCl, 1 mmol/L $MgCl_2$ and 0.5 g/L NaN_3 as a substrate for alkaline phosphatase (ALP), conjugated to one of the immunoreagents. In all cases, incubation of the wells with 100 µL volumes of this enzyme substrate for 10 minutes at room temperature (approximately 25°C) on a shaker platform was followed by the addition, without washing the plates, of 100 µL volumes of a developing solution consisting of 1 mol/L Tris, 0.4 mol/L NaOH, 2 mmol/L $TbCl_3$ and 3 mmol/L EDTA for a one minute incubation while shaking, before measuring the

fluorescence at 615 nm in a time-resolved mode on a Cyberfluor-615 Immunoanalyzer (Cyberfluor, Toronto, Canada). Calibration curve fitting and interpolation to determine unknown analyte concentrations were performed by software present in the instrument. The advantages of lanthanide chelates as fluorescence labels and of time-resolved fluorometry have been discussed previously (Diamandis, 1991; Christopoulos and Diamandis, 1992).

To assess differences between direct versus indirect methods of coating the microtiter plates with anti-p53 antibodies, plates were coated overnight at 4°C either directly with 100 µL volumes of monoclonal antibodies PAb240, recognizing the p53 protein in a mutant conformation (Gannon *et al.*, 1990), panspecific PAb421, recognizing both mutant and wild-type p53 (Milner *et al.*, 1991), DO-1, also recognizing both forms, or with a monoclonal against human thyroid stimulating hormone (TSH) (clone 5404, Medix Biochemica, Kauniainen, Finland) as a negative control. In other configurations, wells were coated indirectly by first coating with 100 µL volumes of goat anti-mouse immunoglobulin (GaMIg) (Jackson ImmunoResearch, West Grove, PA). All antibodies for plate coating were diluted in a buffer consisting of 50 mmol/L Tris, pH 7.40, and 0.5 g/L NaN_3, and used at concentrations of approximately 4 mg/L (100 µL per well); antibodies against p53 had been previously purified from murine ascites fluid as described above for DO-1. The hybridoma cells producing PAb240, PAb421 and DO-1 were provided by Dr. David Lane (University of Dundee). Microtiter wells indirectly coated with GaMIg were subsequently incubated with either PAb240, PAb421, or DO-1 cell culture supernatants at 50-fold dilutions in buffer A containing 0.5 mol/L KCl. We have used the configuration consisting of plates coated first with GaMIg in order to capture subsequently added PAb240 as our original assay method. In control wells we added the anti-TSH antibody (2 mg/L) diluted in the same buffer. In the directly coated plates, the p53 standards were added along with assay buffer (50 µL each). In the GaMIg-coated plates, the p53 standards were added simultaneously with the anti-p53 antibody (also 50 µL each). After a three hour incubation with shaking at 37°C, all assays were identical: washing of the wells four times; incubation with 100 µL volumes of polyclonal CM-1 antiserum diluted 5000-fold in buffer A for one hour at 25°C; washing four times; incubation with 100 µL volumes of 0.12 mg/L goat anti-rabbit IgG conjugated to alkaline

phosphatase (GaRIg-ALP) (Jackson ImmunoResearch); washing six times: and finally the detection procedure as described above.

Other experiments were carried out to determine the optimal concentration of DO-1 and duration of incubation for microtitre plate coating, the effect of diluting samples two-fold in the wells with buffer A supplemented with 0.5 mol/L KCl, 1.0% mouse serum, and 0.5% Tween-20 detergent (Sigma), the optimal sample incubation duration and temperature, the optimal duration, temperature, and antibody concentrations (CM-1 and GaRIg-ALP) of subsequent incubation steps, and the effect of adding varying amounts of goat serum to the GaRIg-ALP diluent.

The assay of all standards, as well as controls, cell lysates, and clinical specimens (see below), were performed in duplicate unless otherwise stated.

II. 3. 4. Assay Evaluation

The use of all materials of human origin with which to evaluate the new immunoassay had been approved by the Committee for Research on Human Subjects at the University of Toronto. Clear, turbid or hemolyzed serum samples were spiked with lysates from Sf9 cells expressing recombinant p53 and lysates from COLO 320HSR cells, obtained from the American Type Culture Collection (ATCC), cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. These cells are known to overexpress mutant p53 (Murakami *et al.*, 1991; Hassapoglidou *et al.*, 1993). The COLO 320HSR cell extract was prepared by lysing approximately 10^7 cells for 30 minutes on ice with 300 µL of a buffer containing 50 mmol/L Tris, 150 mmol/L NaCl, 5 mmol/L EDTA, 1.0% NP-40, 10 mg/L PMSF, and 1 mg/L each of leupeptin and aprotinin, followed by centrifugation for 30 minutes at 14,000 g at 4°C to collect the supernatant. Each serum, and buffer A as a control were used to dilute Sf9 lysate 400-fold and COLO 320HSR lysate 10-fold. Recovery of p53 protein in each serum was expressed as the ratio of the p53 protein concentration of the serum in which either cell lysate was added, to the p53 protein concentration of buffer A to which an equivalent amount of either Sf9 or COLO 320HSR lysate was added. Three serum specimens were also spiked with COLO 320HSR lysate and assayed for p53 protein immediately and following a one hour incubation at 37°C. Sera and buffer A which were unspiked were also assayed similarly.

Lysate prepared from 10^7 COLO 320HSR cells was serially diluted in buffer A to yield sixteen p53-containing solutions, each made by successive two-fold dilutions. This dilution series was used to assess the linearity of the new assay. Within-assay precision was determined from the assay of three breast tumor extracts whose p53 protein concentrations were approximately 0.15, 0.75, and 4.5 μg/L.

II. 3. 5. Immunoassay Applications

We compared the new assay with the original assay for the analysis of soluble extracts from breast tumors, nondiseased breast tissue, and transformed cell lines, and of sera collected routinely from hospitalized patients.

Breast tumor cytosolic extracts prepared for routine steroid hormone receptor analysis, as described previously (Levesque et al., 1994), were provided by a regional service laboratory at Sunnybrook Health Sciences Centre in Toronto, Ontario. Consecutive specimens (n=198) were assayed for estrogen (ER) and progesterone (PR) receptors, by enzyme immunoassay kits (Abbott Laboratories, North Chicago, IL), and stored at -80°C until assaying for p53 by both methods in parallel. All results were expressed relative to the total protein content of the extracts, measured by the Lowry method (Lowry *et al.*, 1951). For both steroid hormone receptors, 10 fmol/mg was used as the cutoff for positive receptor status, following previous work by our group (Levesque *et al.*, 1994). Cytosols were rerun in dilution if the initial p53 protein concentration exceeded 75 μg/L.

Bilateral breast tissue specimens were also obtained from 35 women who underwent cosmetic breast reduction surgery. Representative portions of each of these tissues (n=70), snap-frozen immediately after surgery, were pulverized and extracted as described previously (Levesque *et al.*, 1995c), before assaying for p53 protein. Concentrations of p53 were expressed in units of μg per gram of total protein in the extract, the latter quantity determined by a commercially available kit based on the bicinchoninic acid (BCA) method (Pierce, Rockford, IL).

Extracts from cultured cell lines were prepared as described above for COLO 320HSR. The COLO 320HSR, T-47D, DU-145, and MCF-7 cell lines were obtained from the ATCC while the pRNS-1 cell line was a gift from Dr. Donna Peehl (Stanford

University School of Medicine). Culture media for the COLO 320HSR and T-47D cell lines consisted of RPMI-1640 supplemented with 10% fetal bovine serum and additionally, in the case of T-47D, with 10 mg/L insulin. The MCF-7 and DU-145 cell lines were cultured in Eagle's minimal essential medium with nonessential amino acids supplemented with 1 mmol/L sodium pyruvate, 1 mg/L insulin, and 10% fetal bovine serum. For culturing of the pRNS-1 cells, keratinocyte serum-free medium supplemented with 5 µg/L epidermal growth factor and 50 mg/L bovine pituitary extract was used. All culture reagents were from the same supplier (GIBCO BRL). Both immunoassays were used to quantify p53 protein in the cellular extracts relative to the total protein concentration, measured by a BCA Protein Assay kit (Pierce). Extracts with p53 values above 75 µg/L were repeated in dilution.

Randomly selected serum specimens (n=78) collected for routine bloodwork on hospitalized patients were provided by the Department of Pathology and Laboratory Medicine at Mount Sinai Hospital in Toronto, Ontario. These serum specimens had been stored for no longer than 7 days at 4°C before assaying simultaneously by both p53 immunoassays and by variations of the method in which the PAb240 capture antibody was replaced by a monoclonal antibody against the irrelevant antigen luteinizing hormone (LH) (clone 5301, Medix Biochemica) or when the primary capture antibody was omitted altogether by adding only diluent. The latter two assay configurations served as negative controls with which to reveal nonspecific interferences by serum using the original method.

II. 3. 6. Statistical Methods

Determination of descriptive statistics and Spearman correlation coefficients, and the analysis of 2x2 contingency tables by Chi-square tests, were performed by SAS version 6.02 software (SAS Institute, Cary, NC).

II. 4. Results

II. 4. 1. Assay Configuration

The ability of an anti-p53 monoclonal antibody, directly coated onto microtiter wells, to function in a "sandwich-type" immunoassay was demonstrated in the case of

DO-1 (Figure II. 1.). Unlike PAb240 or PAb421 antibodies, DO-1 permitted a p53-containing standard solution (15 µg/L given as an example) to generate high fluorescence counts when either directly coated to wells or incubated in wells precoated with GaMIg. The fact that DO-1 was as efficient as PAb240 in an assay configuration where immunocaptured p53 protein is detected by polyclonal CM-1 antiserum suggests that DO-1 does not mask a critical epitope recognized by the polyclonal rabbit antibodies. The assay of buffer A alone (sample or standard diluent) gave the background fluorescence

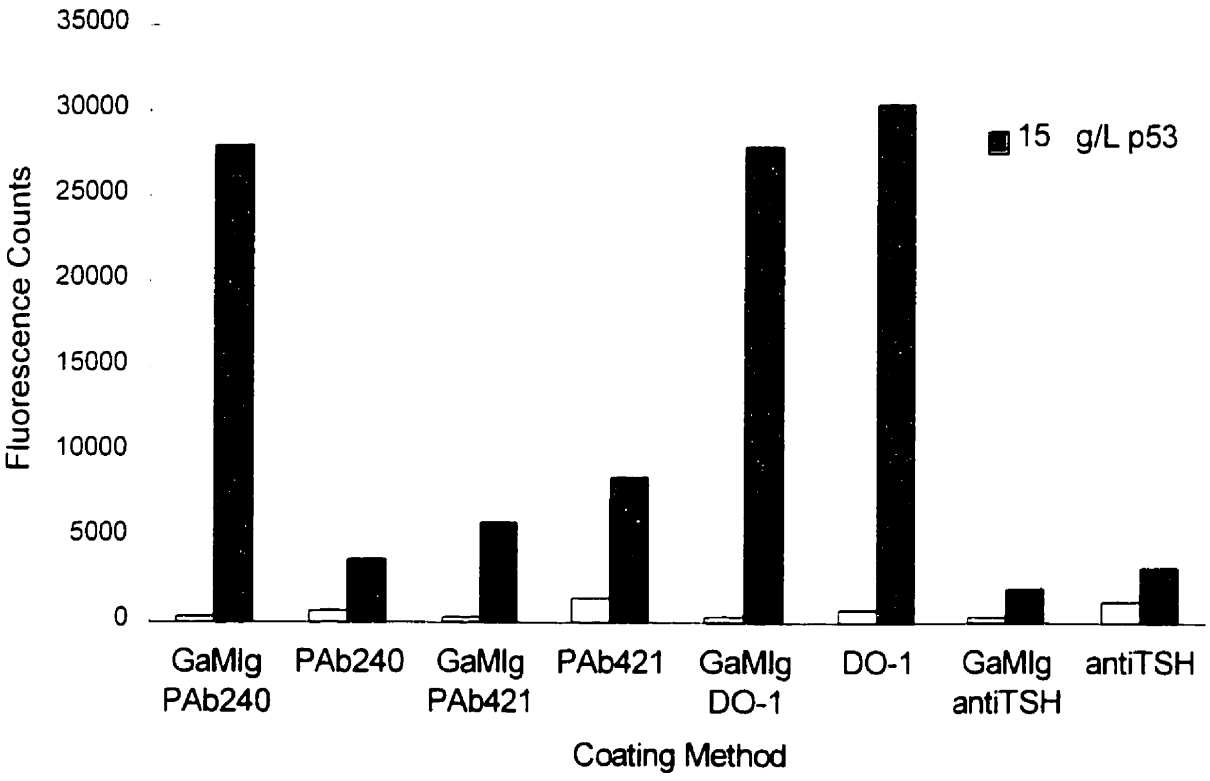


Figure II. 1. p53-Associated Fluorescence from Several Immunoassay Designs Assays involved direct or indirect coating with anti-p53 or irrelevant antibodies. Unshaded bars indicate background fluorescence.

for each assay design which was similar in all cases. As a consequence, the indirect PAb240 and direct DO-1 configurations had comparable signal to background ratios when assaying a given p53 standard solution. When an antibody against human TSH was incorporated into the direct- and indirect-coating assays at concentrations equivalent to that used for the anti-p53 antibodies, the low fluorescence demonstrated the requirement

for p53-specific IgG to generate signal. Separate experiments have shown that optimal coating amounts for DO-1 were 400 ng per well. Optimal incubation times were three hours at 37°C for the first incubation step and one hour at room temperature for the second incubation step. Further optimization of the composition and amounts of other reagents used in the new assay are listed below. A diagrammatic representation of our assay is shown in Figure II. 2.

Like the assay design for the immunofluorometric measurement of prostate-specific antigen (Yu and Diamandis, 1993), this newly developed assay includes the addition of goat serum to the diluent of the GaRIg-ALP to reduce background signal by neutralizing anti-goat antibodies present in some sera. These antibodies would be especially problematic to the indirect microtiter well coating methods used here, due to possible crosslinking of the GaMIg antibodies on the well surface and the subsequently added GaRIg-ALP enzyme-labelled antibodies, resulting in non p53-dependent immobilization of the labelled detection antibody to the wells, hence giving false positive signals. A remedy for this problem was provided by the relative excess of unlabeled goat IgG derived from the goat serum, compared to the ALP-labeled GaRIg in the latter's diluent. We suspect that saturation of free epitope-binding sites of nonspecifically immobilized human anti-goat antibodies by the nonlabeled IgG might therefore contribute to reducing background fluorescence.

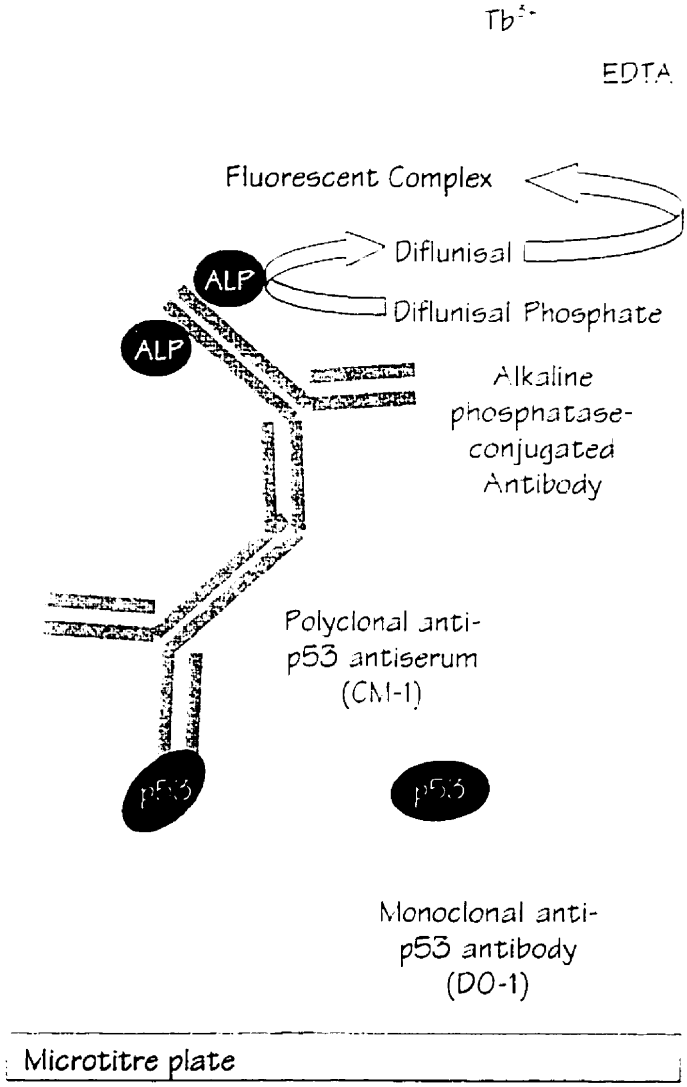


Figure II. 2. Schematic Diagram of New p53 Immunofluorometric Assay

Given the results and practical considerations mentioned above, the assay design selected, as depicted in Figure II. 2., included the following features: microtiter wells coated overnight with 400 ng/100 µL/ well of DO-1: two-fold dilution of samples and standards in the wells (50 µL each) with buffer A containing 0.5 mol/L KCl, 1.0% mouse serum, and 0.5% Tween-20 detergent, and incubation for three hours at 37°C: addition of 100 µL polyclonal CM-1 antiserum at a 5,000-fold dilution in buffer A and incubation for one hour at 25°C: addition of 12 µg/L (100 µL) GaRIg-ALP diluted in buffer A containing 0.5 mol/L KCl and 10% goat serum for a one hour incubation at 25°C: and the addition of enzyme substrate and developing solution as described under "Methods" for time-resolved fluorescence measurement. Figure II. 3. displays a representative calibration curve.

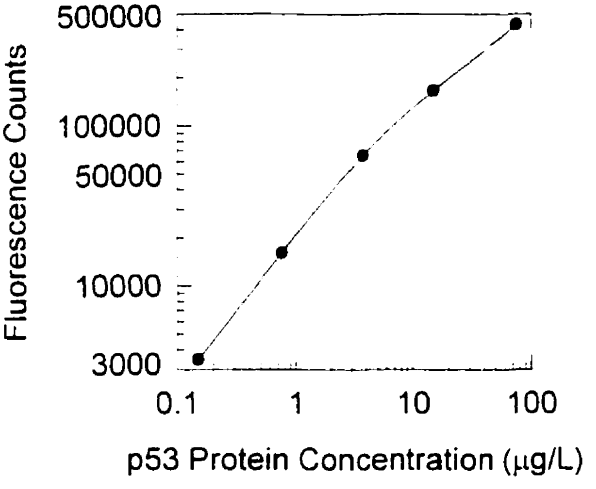


Figure II. 3. Calibration Curve of New Assay

II. 4. 2. Recovery of p53 from Serum

When the new p53 immunoassay was applied to nine patient sera spiked with p53 protein from two different cell lysates (COLO 320HSR and Sf9), the recovery of p53 relative to that in buffer A (sample and standard diluent) ranged from 75 to 131% for the COLO 320HSR lysate, and 72 to 98% for the Sf9 lysate (Figure II. 4.). Due to the higher dilution of Sf9 lysate by each serum specimen, the total amount of p53 protein added to specimens spiked with Sf9 lysate was lower than that added to the same sera spiked with the lysate from the colon carcinoma cell line. The background fluorescence was uniformly low in all sera and only slightly elevated above that of buffer A (data not shown).

II. 4. 3. Analytical Characteristics

The detection limit of the assay, calculated as the p53 concentration that could be discriminated from zero with 99% confidence was found to be 0.5 U/L (approximately 0.04 µg/L of p53). The range of p53 concentrations yielding a linear assay response was

determined to be 2 to 1.000 U/L (corresponding to 0.15 μg/L to 75 μg/L of p53). The calculated values for intra-assay precision at p53 levels of 0.15, 0.75, and 4.5 μg/L were 11, 9, and 7%, respectively; the inter-assay precision at the same levels (10 measurements over one week) was 15, 12, and 8%.

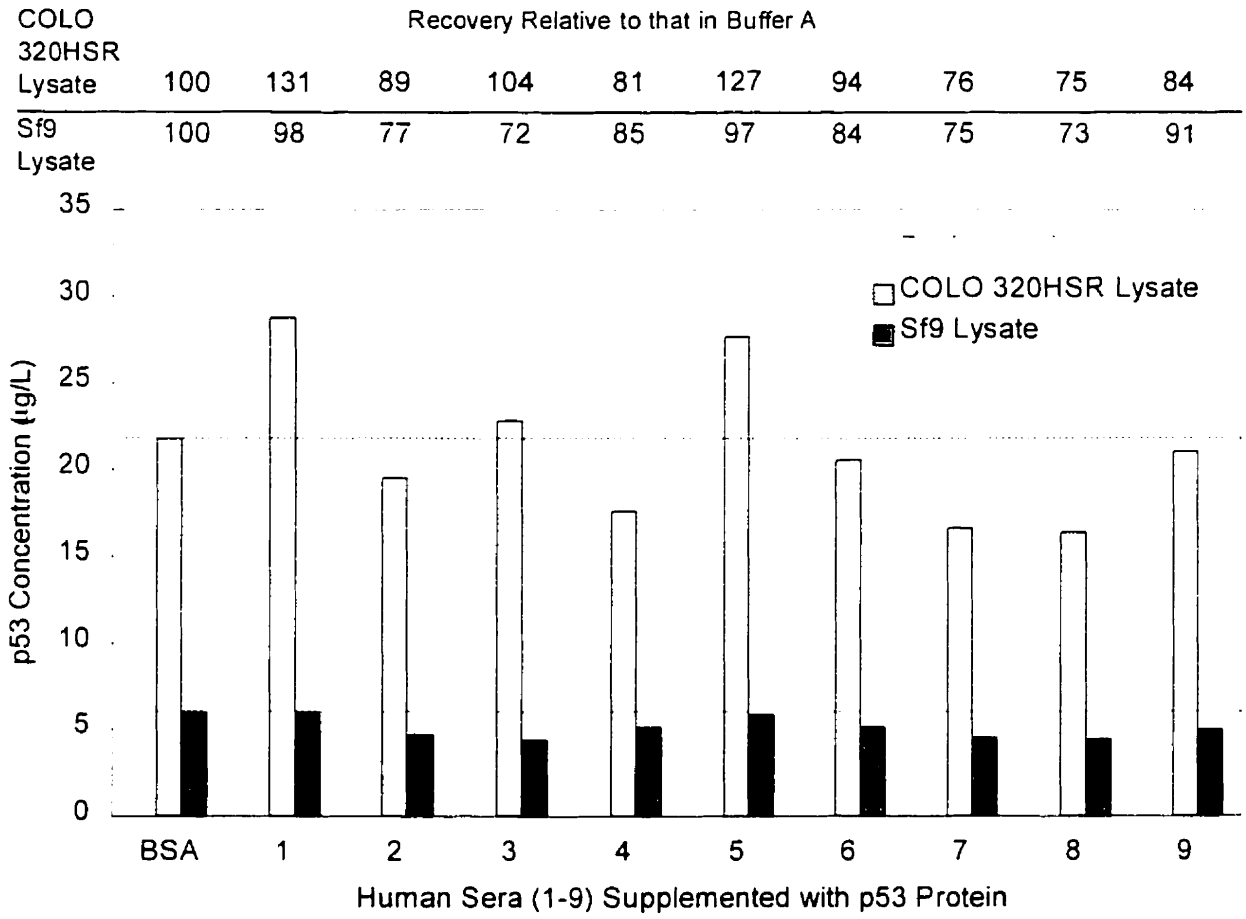


Figure II. 4. Recovery of p53 Protein from Human Sera
p53 was added from lysates of COLO 320HSR cells, or from Sf9 cells infected with a recombinant baculovirus expressing the wild-type p53 gene. The broken lines indicate 100% recovery for each lysate. Recovery from each lysate relative to that from buffer A is listed above chart.

II. 4. 4. Assay of Extracts of Breast Tumors and Nondiseased Breast Tissues

Both the original and new immunoassay configurations were used to assay the cytosolic extracts of 198 breast tumors for p53 protein concentration, the distribution of which, determined by each of the two methods, is summarized in Table II. 1. Spearman correlation analysis indicated good correlation (r_s=0.93, $p<0.001$) between the methods (Figure II. 5.). A frequency distribution of p53 values obtained by the new assay of tumor

Table II. 1. Descriptive Statistics of Clinical Specimens Assayed for p53 Protein

Specimen	N	Mean	SD	Median	Minimum	Maximum
Breast Tumor Tissue Extracts[a]						
Original Assay	198	0.90	6.08	0.10	0	83.33
New Assay	198	0.60	1.80	0.18	0	19.88
Nondiseased Breast Tissue Extracts[a]						
Original Assay	70	< 0.04	0.02	< 0.04	0	0.05
New Assay	68	< 0.04	0.02	< 0.04	0	0.08
Hospitalized Patient Sera[b]						
Original Assay	78	0.75	2.33	0.09	0	15.23
New Assay	78	< 0.04	0.05	0	0	0.38

[a] p53 concentrations expressed in μg per g of total protein.
[b] p53 concentrations expressed in μg per liter.

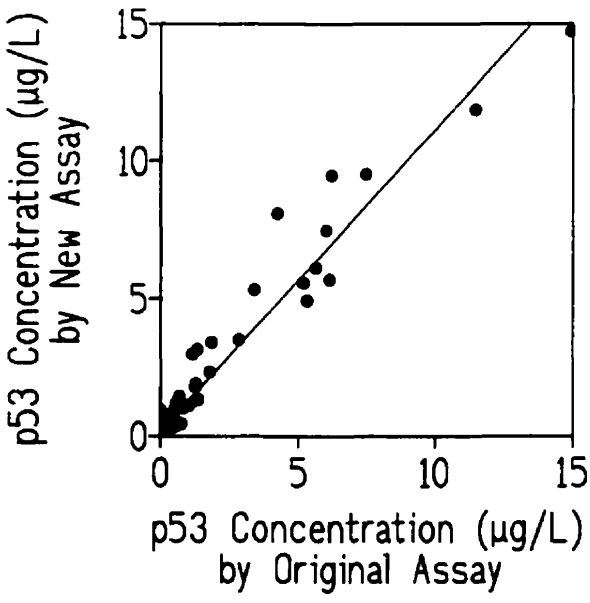


Figure II. 5. Correlation Between p53 Concentrations in Breast Tumor Cytosols by Original and New Assays

extracts is shown in Figure II. 6. When dichotomized based upon an arbitrary cutoff of 0.38 μg per gram of total protein (or 5 U/g as previously described) (Levesque *et al.*, 1995a), the p53-positivity rates were found to be 15% and 23% by the original and new methods, respectively. Regardless of the p53 assay method employed to categorize the specimens, contingency table analyses revealed p53-positivity status to be strongly associated with negative status for

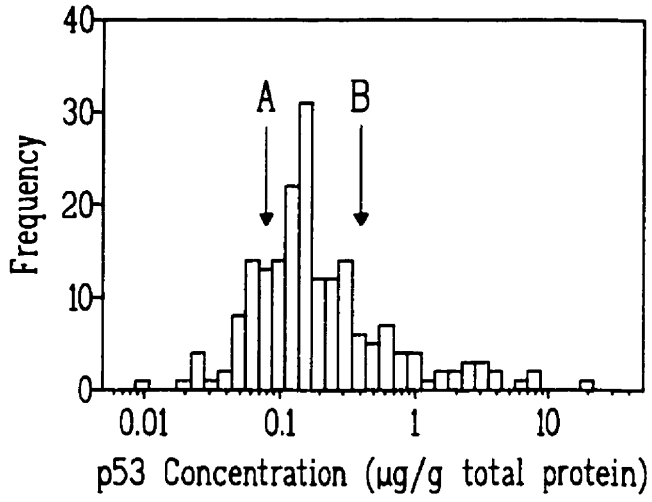


Figure II. 6. Frequency Distribution of p53 Concentrations in Breast Cytosols by New Assay

A. 100th percentile of p53 values in nondiseased breast tissues; B. arbitrary cutoff for p53 overexpression (0.38 µg/g) in breast tumors used in this study

estrogen receptors or progesterone receptors (Table II. 2.).

The assay of nondiseased breast tissue yielded p53 protein values far lower than those obtained from the analysis of breast neoplasms. This is illustrated by the minimal overlap between the p53 concentration frequency distributions generated by the assay of normal and diseased tissues by either assay design (Table II. 1. and Figure II. 6.). Because the measured p53 protein values of all of the nondiseased breast extracts fell below the analytical detection limits of both assay techniques (~0.04 µg/L), the lack of a very strong correlation (r_s=0.40, $p<0.001$) between the original and new methods was not surprising.

II. 4. 5. Assay of Sera of Hospitalized Patients

We were interested in investigating, in a future prospective study, if mutant p53 protein enters the blood circulation and if its concentration is related to the levels of p53 in the tumor. The ability to detect p53 in serum is dependent on a sensitive and specific analytical method. When p53 protein was spiked into the serum the recovery was almost complete (Figure II. 4.). We have further investigated if the analysis of undiluted sera from 78 hospitalized patients without cancer yielded negative results. In contrast to the new assay, which yielded results < 0.15 µg/L for all sera tested except two (with values of 0.23 and 0.38 µg/L), the original assay gave values > 0.15 µg/L in 29 specimens and > 0.38 µg/L in fifteen. Moreover, 87% (65/78) of sera measured by the new assay had p53 values below the detection limit of 0.04 µg/L, compared to only 21% (16/78) when measured by the original method. Consequently, the correlation between the two methods

Table II. 2. Relationships Between p53 Protein Status and Steroid Hormone Receptor Status of Breast Tumors

Receptor Conc., fmol/mg	Number of Specimens (and %)		p-Value
	p53 < 0.38 μg/g	p53 ≥ 0.38 μg/g	
Old Assay			
ER < 10	47 (71.2)	19 (28.8)	
ER ≥ 10	122 (92.4)	10 (7.6)	<0.001
PR < 10	52 (75.4)	17 (24.6)	
PR ≥ 10	117 (90.7)	12 (9.3)	0.004
New Assay			
ER < 10	41 (62.1)	25 (37.9)	
ER ≥ 10	112 (84.8)	20 (15.2)	<0.001
PR < 10	47 (68.1)	22 (31.9)	
PR ≥ 10	106 (82.2)	23 (17.8)	0.025

for the analysis of these sera was intermediate (r_s=0.67, $p<0.001$) between those found for the analyses of breast tumors and nondiseased breast tissues. In order to determine if these sera contained interfering substances, they were assayed in parallel by two methods in which an another irrelevant capture antibody (anti-LH monoclonal instead of PAb240) was utilized or when no capture antibody was employed. The latter two modifications were applied to the original assay configuration in which GaMIg was immobilized on the solid-phase. The results are shown in Figure II. 7. Clearly, these sera, which gave undetectable p53 values by the new assay configuration, contained substances which interfered and generated a signal with both specific and nonspecific monoclonal capture antibodies (anti-p53 or anti-LH, or none at all). We hypothesize that the interfering agents, generated in goats, act by crosslinking the coating GaMIg and detection GaRIg-ALP antibodies. These substances are probably human anti-goat antibodies, given that we found that inclusion of goat serum into the assay buffer diminished but did not

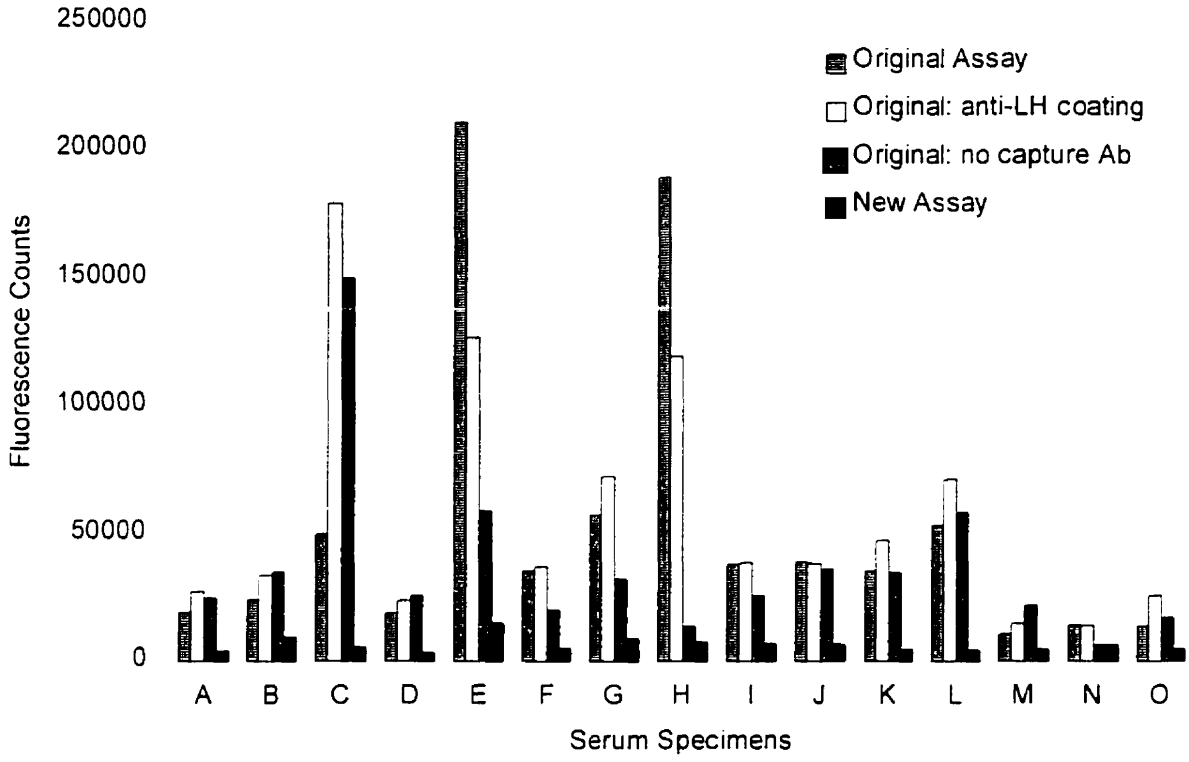


Figure II. 7. Results of Various Methods for Sera Displaying High p53 Values by Original Method

completely eliminate the problem (data not shown). With directly coated monoclonal anti-p53 mouse antibodies this problem is eliminated.

II. 4. 6. Assay of Lysates of Cultured Cells

The two methods were also used to measure p53 in the cell lysates of various cell lines and the results are presented in Table II. 3.

II. 5. Discussion

Investigations aimed at determining the role of p53 in a given malignancy have generally followed one of two parallel paths: either the identification of mutations in the p53 coding sequence. or the demonstration of p53 protein accumulation within affected cells. In either approach. a wide variety of analytical techniques may be applied (Soussi

Table II. 3. p53 Protein Concentrations in Cell Lines

Cell Line	Source	p53 Concentration (μg/g)	
		Old Assay	New Assay
COLO 320HSR	Colon	82.23	49.85
T-47D	Breast	20.46	27.51
DU-145	Prostate	5.70	7.88
MCF-7	Breast	22.95	45.12
pRNS-1	Prostate	107.64	93.05

et al., 1994). While direct sequencing provides unequivocal evidence of mutational events, indirect methods including single strand conformation analysis (SSCP) and constant denaturant gel electrophoresis (CDGE) may also be informative for alterations within the p53 gene. Complexity and cost have limited the ability to sequence all eleven exons of the p53 gene in a large number of clinical specimens. Far more common have been attempts to show p53 protein overexpression, particularly by conventional immunohistochemistry (IHC), in studies questioning the clinical implications of p53 alterations.

IHC also has its limitations for p53 protein detection in tissues (Wynford-Thomas, 1992). Most important is the lack of consensus with regard to the appropriate methodology and scoring system for the interpretation of staining patterns, which may vary considerably depending on the choice of antibodies and fixation methods (Fisher *et al.*, 1994; Lambkin *et al.*, 1994), as well as inter-observer variability when a given scoring system is applied. We have suggested elsewhere (Diamandis and Levesque, 1995) that ELISA-type methods for p53 may overcome many of these pitfalls by reporting quantitative results which are more objective, and possess further advantages making them ideal for use in clinical studies. These include the requirement for p53 protein to bind simultaneously two antibodies thereby enhancing specificity, washing tissue extracts from the wells after initial sample incubation and consequently reducing

background signal, demanding less technical expertise, and the inherent ability to analyze large batches of specimens.

The number of ELISA methods developed for p53 protein quantification remains small. First was that developed by Midgley *et al.* (1992) in which monoclonal antibody PAb421 (recognizing an epitope in the carboxy terminus of p53) was directly coupled to the microtiter well surface. In this assay, the detection of PAb421-bound p53 was accomplished by probing with polyclonal CM-1 rabbit antisera, with the subsequent addition of swine anti-rabbit antibody conjugated to horseradish peroxidase and visualization with tetramethylbenzidine. In the process of evaluating new monoclonal antibodies against p53, the same group reported (Vojtesek *et al.*, 1992) that DO-1 (recognizing an epitope in the amino terminus of p53) was suitable for IHC or immunoblotting procedures and was functional in an ELISA in which DO-1 replaced PAb421 in the assay outlined above. The latter assay has been applied extensively by these workers, including its use in comparing the relative merits of ELISA and IHC for p53 analysis (Joypaul *et al.*, 1993; Vojtesek *et al.*, 1993). More recently, a "sandwich" luminometric immunoassay for p53 protein, with a lower detection limit than conventional microplate ELISAs, has been described (Borg *et al.*, 1995).

An immunoassay against p53 had also been reported by our group using PAb240 (recognizing a cryptic epitope exposed by unfolding of the central domain of p53, as commonly occurs by p53 missense mutation in exons 5 to 8) and CM-1 as the capture and detection antibodies, respectively (Hassapoglidou *et al.*, 1993; Levesque *et al.*, 1994). Because PAb240 performed poorly when coated directly to plates, it was added instead to GAMIg coated wells. With the introduction of newer monoclonal antibodies against p53 demonstrated to have utility for use in a wider range of histological specimens, and because of the difficulties encountered when assaying sera, attempts were made to alter our original configuration of the immunoassay. Our detection system involved the enzymatic hydrolysis of the ALP substrate, diflunisal phosphate, which could enter into a ternary complex with EDTA and the lanthanide metal Tb^{3+}, emitting fluorescence at 615 nm which persists for milliseconds, far longer than the inherent fluorescence of most sample matrices. The same detection system is employed in our new p53 immunoassay. The basic assay configuration in terms of choice and orientation

of anti-p53 antibodies used in our new method is similar to the method of Vojtesek *et al.* (1992), but their method does not incorporate further background signal-reducing measures and therefore it is less sensitive.

The most frequent specimen type to which p53 ELISA methods have been applied are tumor tissue extracts. With our new assay a broad range of p53 values were obtained in breast tumor cytosols. These values were generally higher than those obtained in normal breast tissues, which had undetectable p53 in the great majority of cases. We propose that the p53 values in the latter group of specimens represent the normal reference range for p53 protein in breast tissue. The selection of a cutoff point for p53-positivity exceeding the 100th percentile of this normal range is a highly conservative one and requires validation in future clinical studies. However, categorizing the breast tumor extracts based upon the cutoff of 0.38 μg/g of total protein (5 U/g), the p53-positivity rate of 23% is in general accordance with our earlier observations in breast tumors (Levesque *et al.*, 1994; Levesque *et al.*, 1995a) and by others using ELISA (Bartkova *et al.*, 1993) or a variety of IHC techniques (Andersen *et al.*, 1993; Faille *et al.*, 1994). The use of this cutoff point permitted the demonstration of the negative association between p53 status and ER or PR status, which we have reported previously (Hassapoglidou *et al.*, 1993; Levesque *et al.*, 1994; Levesque *et al.*, 1995a). The determination of negative hormone receptor status is generally thought to predict poor response to endocrine (ie. antiestrogen) therapy (Fisher *et al.*, 1990) and may be associated with an unfavorable prognostic outcome (Chevallier *et al.*, 1988).

The analysis of sera, from non-cancer hospitalized patients, by the our original method uncovered an unexpectedly high p53-positivity rate. The same sera assayed in parallel by the new method exhibited mostly background levels of fluorescence. Control assays confirmed that the high fluorescence signals in these sera, by the original method, were entirely due to nonspecific interference. The results of the assay of the 78 sera by the new method, designed to suppress such serum-associated nonspecificity, may constitute a reference range for hospitalized patients where essentially all of the p53 values fell below 0.38 μg/L. Using a commercial kit (Oncogene Science), Greco *et al* (1994) have reported similar levels of p53 protein in the sera of blood donors. In contrast, other workers (Luo *et al.*, 1994) report concentrations of p53 protein up to 2.30 μg/L in

the sera of hospital control subjects using a similar kit. Clearly, specificity is an important consideration in assessing the applications of ELISA assays to clinical studies involving p53.

The assay for p53 protein in cell lines raises a number of issues of which we presently have only a limited understanding. It has been found that the assay of many cultured cell lysates diluted in a neutral buffer equivalent to PBS results in a nonlinear decrease in p53 levels measured by either immunoassay, implying that components of the extracts may bind p53 and release it only upon further dilution (unpublished data). Furthermore, in this study, there were substantial differences between the p53 concentrations measured by the two methods depending on the cell line tested. For example, both the human colon adenocarcinoma cell line COLO 320HSR (Gannon *et al.*, 1990) and the ductal breast carcinoma cell line T-47D (Bartek *et al.*, 1990) are known to harbor p53 mutations leading to the accumulation of mutant p53 protein. There was concordance between the methods when T-47D was measured, but not in the case of COLO 320HSR. The DU-145 prostate carcinoma cell line, demonstrated elsewhere to show strong nuclear staining for p53 and to harbor a double mutated p53 gene (Carroll *et al.*, 1993), appeared to have lower p53 levels by both assays in comparison to COLO 320HSR or T-47D. Having p53 levels two-fold higher by the modified immunoassay, the MCF-7 breast adenonocarcinoma cell line has been demonstrated to express wild-type p53 protein sequestered cytoplasmically by unknown mechanisms (Takahashi *et al.*, 1993). The highest levels of p53 protein observed using both assay methods occurred in pRNS-1(Lee *et al.*, 1994), a prostate cell line transformed by SV40 and stably expressing large T antigen, which can bind to and thereby increase the half-life of p53 protein. Studies to identify p53-binding factors in cell lysates responsible for the nonlinear assay response to dilutions of cell lysates, and to determine the basis for the widely differing p53 levels in the lysates measured by the two assay methods, must therefore be performed.

We report here on the development of a new, specific, and highly sensitive ELISA technique for p53 protein quantification in biological fluids. Although this new assay is comparable to the immunofluorometric procedure used in our laboratory in a number of earlier studies of p53 protein in breast tumor cytosols, it has a significant

advantage in being virtually free of interferences in human serum specimens. Data by other groups (Greco *et al.*, 1994; Luo *et al.*, 1994) and by a study in our laboratory suggest that p53 protein may be found in the serum of cancer patients, perhaps by its release from p53-overexpressing tumors. The development of a specific assay for p53 protein in serum may therefore prove to be a fundamental tool given the diagnostic, prognostic, and disease-monitoring implications of this finding.

CHAPTER III

COMPARISON OF NEW IMMUNOASSAY TO IMMUNOHISTOCHEMICAL STAINING

(A version from Levesque MA, Tadross L, Diamandis EP, D'Costa M. *Am J Clin Pathol* 1997; 107: 308-316, Copyright © 1997, by the American Society of Clinical Pathologists, Reprinted with permission)

III. 1. Abstract

In this study we compared IHC, performed on formalin-fixed, paraffin-embedded sections of 91 primary lung tumor tissues, with our new quantitative two-site immunofluorometric assay, on extracts of fresh-frozen specimens from adjacent regions of the same tissues. Monoclonal DO-7 antibody, and the related monoclonal DO-1 together with polyclonal CM-1 antibodies, were used for immunostaining and ELISA, respectively. Concentrations of p53 were expressed relative to total protein, while an immunostaining score reflected the proportion of stained malignant cells, intensity of staining and tumor cellularity. There was strong concordance between the two methods by Spearman correlation ($p<0.001$), Wilcoxon Rank Sum ($p<0.001$) and contingency table ($p<0.001$) analyses. The use of ELISA-type assays for p53 quantification in lung tumor tissues may be an alternative to the more labor-intensive histological techniques.

III. 2. Introduction

The strong correlation between p53 gene mutation and p53 protein accumulation existing under most, but not all circumstances (Hall and Lane, 1994) permits simple immunological methods for p53 protein detection to substitute for the more complex and labor-intensive procedures for determining p53 alterations at the nucleic acid level. Most commonly, these immunological methods take the form of well-established immunohistochemical techniques which are rapid, technically non-demanding, and able to provide antigen localization with respect to histologic features of the tumor. However, methodologic differences resulting from various combinations of tissue fixation and antigen unmasking procedures (Bartek et al., 1993; Baas *et al.*, 1994; Fisher *et al.*, 1994; Lambkin *et al.*, 1994), primary anti-p53 antibody (whose performance may be affected by specimen processing) (Bartek et al., 1993; Baas *et al.*, 1994; Lambkin *et al.*, 1994), and criteria for designating the p53 expression status of specimens (Fisher *et al.*, 1994) have made cross-study comparisons of immunohistochemically-detected p53 protein difficult. An alternative to IHC is the measurement of soluble p53 protein in tissue extracts by quantitative immunoassay. Although such ELISA-type methods likewise suffer from inherent pitfalls, including the requirement for fresh frozen tissue and the inability to localize p53 expression to cellular or tissue components, they may offer

several advantages (Diamandis and Levesque, 1995). Most noteworthy of these are that they may be performed with minimal technical expertise, that they generate numerical results amenable to objective and consistent interpretation, and that they provide enhanced specificity owing to the use of two p53-specific antibodies (the most common assay configuration).

Relative to the widespread use of IHC for the demonstration of p53 protein overexpression in human tumor tissues, ELISAs of p53 protein have been employed much less frequently (Midgley *et al.*, 1992; Vojtesek *at al.*, 1992; Hassapoglidou *et al.*, 1993). One of these ELISA methods, among the first to be developed (Vojtesek *at al.*, 1992), has been used to determine p53 protein concentrations in extracts from tumors of breast (Bartkova *et al.*, 1993; Vojtesek *et al.*, 1993), gastrointestinal (Bartkova *et al.*, 1993; Joypaul *et al.*, 1993), and vulval origin (Bartkova *et al.*, 1993;) and has been shown to be comparable to IHC, when performed in parallel, for the assessment of p53 protein status in breast (Vojtesek *et al.*, 1993) and gastrointestinal (Joypaul *et al.*, 1993) tumor specimens. Recently, we have also described a highly sensitive ELISA of p53 protein (Levesque *et al.*, 1995b), incorporating a time-resolved fluorescence detection system. Since p53 protein concentrations in primary lung tumor extracts have not yet been compared to the degrees of immunostaining performed on the same specimens, the purpose of this study was to determine if the levels of p53 expression in lung tumor tissue demonstrated by our immunofluorometric assay were concordent with results obtained by a conventional immunohistochemical technique.

III. 3. Materials and Methods

III. 3. 1. Tumor Specimens

This study received the approval of the ethics and research committee at St. Joseph's Health Centre, Toronto, Ontario, Canada. Surgically resected lung tumor tissues were obtained from 91 patients operated at St. Joseph's Health Centre between June 1993 and March 1995 for the treatment of primary lung carcinoma. Immediately following surgery, representative tissues of each lung tumor were selected and partitioned into two equivalent portions. While one portion was fixed in 10% neutral buffered formalin and embedded in a permanent paraffin block, the other was snap frozen on dry ice and stored

at -80°C for no more than 6 months until the extraction and ELISA procedures (see below).

III. 3. 2. Soluble Extracts

Frozen lung tumor tissues (~0.2 g) were pulverized on dry ice to a fine powder, which was suspended in 1 mL lysis buffer (50 mmol/L Tris, pH 8, 150 mmol/L NaCl, 5 mmol/L EDTA, 10 mL/L NP-40 surfactant, 10 mg/L PMSF, and 1 mg/L each of aprotinin and leupeptin) and incubated for 30 minutes on ice before centrifugation at 14,000 g for 30 minutes at 4°C to collect the supernates. The crude cell lysates were immediately assayed both for p53 protein by immunofluorometry (see below), and for total protein content by a kit based on the BCA method (Pierce Chemical, Rockford, IL). Ten tumor tissues were further sampled by cutting, from each of 3 different surfaces, approximately 0.2 g portions of tissue which were independently pulverized and extracted as above. Extracts from histologically normal lung tissue, cut from the resection margins of 7 lung tumors, served as negative tissue controls.

III. 3. 3. Immunofluorometric Assay

A "sandwich-type" ELISA, briefly outlined below and described in detail above (Levesque *et al.*, 1995b), was used to measure the p53 protein concentrations in the lung tumor extracts. Soluble p53 protein, present in extracts and calibrators diluted two-fold in buffer A (50 mmol/L Tris, pH 7.80, 60 g/L BSA, and 0.5 g/L NaN_3) supplemented with 0.5 mol/L KCl, 10 mL/L mouse serum, and 5 mL/L Tween-20 detergent, was first immobilized in microtiter wells coated with monoclonal DO-1 antibody (gift of Dr. David Lane, University of Dundee, UK), which recognizes the same epitope on the surface of p53 protein as the related DO-7 antibody (Vojtesek *et al.*, 1992) used for the immunohistochemical staining procedure. Following this initial 3 hour incubation step at 37°C, bound p53 protein was then detected by subsequent one hour incubations at room temperature with polyclonal CM-1 antiserum (Novocastra, Newcastle upon Tyne, UK) raised in a rabbit host against recombinant wild-type human p53 and diluted 5000-fold in buffer A, and then with ALP-conjugated GaRIg diluted to 120 µg/L in buffer A containing 0.5 mol/L KCl and 100 mL/L goat serum. Hydrolysis of the enzyme substrate

(0.01 mol/L diflunisal phosphate in 0.1 mol/L NaOH, diluted 10-fold in 0.1 mol/L Tris, pH 9.10, containing 0.15 mol/L NaCl, 1 mmol/L $MgCl_2$ and 0.5 g/L NaN_3), added for 10 minutes at room temperature, yielded a product which entered into a fluorescent complex when the developing solution (1 mol/L Tris, 0.4 mol/L NaOH, 2 mmol/L $TbCl_3$, and 3 mmol/L EDTA) was also added. Fluorescence at 615 nm was measured after one minute by a Cyberfluor-615 Immunoanalyzer (Cyberfluor, Toronto, Ontario, Canada) in a time-resolved mode, which greatly reduces the background fluorescence signal (Christopoulos and Diamandis, 1992). All reagents were added to wells in 100 µL volumes. Concentrations of p53 were interpolated from a calibration curve generated by the simultaneous assay of a dilution series of an extract of Sf9 insect cells infected with a p53-expressing baculovirus (gift of Dr. Thierry Soussi, INSERM, Institut de Génétique Moléculaire, France), as described previously (Levesque *et al.*, 1995b). Values of these calibrators, ranging from 0 to 75 µg/L, were established based on the assay of reconstituted preparations of premeasured, lyophilized recombinant human p53 protein (Oncogene Science, Uniondale, NY, USA). Analytical characteristics of the ELISA include a sensitivity of ~0.04 µg/L and a linear response range from 0.15 to 75 µg/L. Concentrations of p53 protein in the lung tumor extracts were expressed relative to the total protein content. Because the epitope recognized by DO-1 antibody is within an amino terminal domain shared by all conformations of p53 protein (Vojtesek *et al.*, 1992), the ELISA is able to detect both mutant and wild-type p53 protein.

III. 3. 4. Immunohistochemistry

Sections (4-5 µm) of the same paraffin-embedded tissues used to make the histologic diagnosis in each case were placed on silanated slides, dried overnight at 65°C, dewaxed and rehydrated in xylene and graded concentrations of alcohol to distilled water, and treated with methanol hydrogen peroxide. Microwave antigen retrieval (Shi *et al.*, 1991) was performed in a 1.75 kW microwave oven (Litton-Moffat, Rexdale, Ontario, Canada) at high setting for 30 minutes in a 10 mmol/L citrate buffer, pH 6.0, after which the slides were incubated for 30 minutes in the same buffer before being transferred into PBS (150 mmol/L NaCl, 10 mmol/L Na_2HPO_4, 2 mmol/L $NaKH_2PO_4$, pH 7.2). The slides were then incubated for 5 minutes with 2% nonimmune goat serum in PBS before

applying monoclonal DO-7 antibody (Novocastra, Newcastle upon Tyne, UK), diluted 50-fold in Dako Diluent Buffer (Dako, Glostrup, Denmark), for a 1.5 hour incubation. After washing the slides twice in PBS, bound complexes were detected by a subsequent 45 minute incubation with biotinylated GaMIg (Dako, Glostrup, Denmark) diluted 200-fold in PBS, by washing twice as before, and by a 45 minute incubation with horseradish peroxidase-conjugated streptavidin (Dako, Glostrup, Denmark) diluted 300-fold in PBS. All of the above incubations were performed at 37°C in a humidified chamber. Washing of the slides again two times was followed by incubation for 4 minutes at room temperature with the chromogen, a solution of 3,3'-diaminobenzidine tetrahydrochloride (0.5 mg/mL) and 0.009% hydrogen peroxide in Tris-buffered saline (150 mmol/L NaCl, 50 mmol/L Tris, pH 7.6). The slides were then lightly counterstained with Harris' hematoxylin and immersed in acid alcohol, graded alcohols, and xylene, and finally coverslipped. Tissues derived from an astrocytoma, an ovarian carcinoma, and a ductal breast carcinoma, all previously shown to exhibit strong staining for p53 protein using DO-7, were sectioned and processed as above in parallel as positive controls; lung cancer tissues of previously shown p53 overexpression status were unavailable. Negative controls for each lung tumor specimen were provided by omitting the DO-7 antibody.

III. 3. 5. Scoring System

A simple scoring scheme, similar to that used by another group (Joypaul *et al.*, 1993; Vojtesek *et al.*, 1993), was used to semiquantify the degree of immunohistochemical staining of the lung tumor sections. Scoring was performed by examination of each DO-7 antibody stained section by a single pathologist without knowledge of the corresponding p53 ELISA result. Three parameters were used for scoring, namely the proportion of malignant cells staining, the intensity of staining, and the tumor cellularity. To account for differences in tumor cellularity between specimens, a value of 1 (low), 2 (intermediate), or 3 (high) was assigned to the corresponding hematoxylin and eosin stained section, viewed under scanning power (x 4), to categorize it with respect to the amount of neoplastic tissue present (excluding regions of necrosis, stroma etc.). To reflect the proportion of malignant cells stained in each section, 10 nonoverlapping, randomly chosen low power (x 10) fields were each given an integer

value of 1, 2, 3, or 4 corresponding approximately to 0-25%, 26-50%, 51-75%, or 76-100% staining. Ten additional higher power (x 20) fields chosen in a similar manner were also each given a value from 0 (absent) to 3 (dark) to represent the intensity of staining within malignant cells in each section. Summation of the mean of the 10 values reflecting the proportion of stained cells and the mean of the 10 values reflecting the staining intensity yielded the "raw score" (ranging from 1 to 7) in each case. Finally, the overall score (ranging from 1 to 21) was calculated by multiplication of the raw score and the cellularity value in each case.

III. 3. 6. Statistical Analysis

The relationship between the concentrations of p53 protein determined by ELISA and the corresponding cellularity-adjusted IHC scores was examined by Spearman correlation analysis, Wilcoxon Rank Sum tests, and contingency table analysis, all performed using SAS version 6.02 software (SAS Institute, Cary, NC, USA). For the latter two procedures, tumor specimens were classified as p53-negative or p53-positive by each of the detection methods under comparison based on cutoff points for p53-positivity equal to the median protein-adjusted p53 concentration in the case of the ELISA results, and equal to the median cellularity-adjusted staining score in the case of the IHC findings.

III. 4. Results

III. 4. 1. Distribution of p53 Protein Concentrations

In all 91 extracts prepared from the frozen lung tumor tissues, the p53 protein concentrations exceeded the detection limit of the ELISA; the distribution of values ranged from 0.04 μg/L to 70.69 μg/L, with a median, mean, and SD of 0.55 μg/L, 6.03 μg/L, and 12.81 μg/L, respectively. When the p53 protein concentrations were divided by the amount of protein present, the distribution of values expressed as μg/g protein, shown as a histogram in Figure III. 1., had the following characteristics: minimum=0.01 μg/g, maximum=10.97 μg/g, median=0.13 μg/g, mean=1.13 μg/g, and SD=2.15 μg/g. Evidence that these tumor tissues displayed minimal heterogeneity with respect to p53

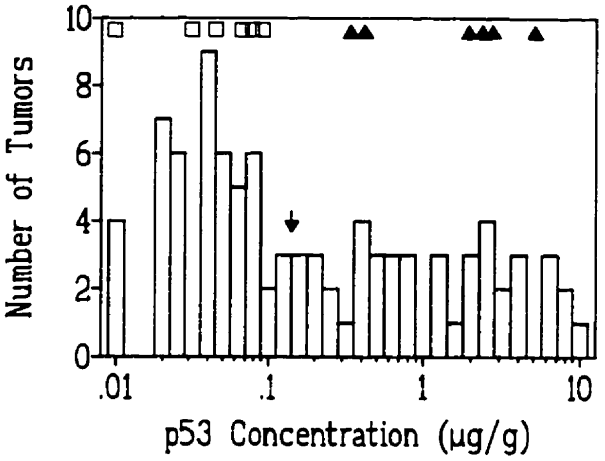


Figure III. 1. Distribution of p53 Protein in Lung Tumor Extracts

Values of p53 protein, expressed relative to the total protein content of the extracts, were determined by the immunofluorometric assay. The arrow indicates the median value used as the cutoff point for p53-positivity. □ indicates p53 levels of ELISA-negative, IHC-positive specimens and ▲ indicates p53 levels of ELISA-positive, IHC-negative specimens.

protein overexpression was provided by further sampling of a subset of tumors (n=10) in which protein-adjusted p53 protein concentrations, determined in three different samples of tissue removed from nonadjacent surfaces and then independently extracted and assayed for p53 and for total protein, were revealed to vary by no more than 10% for each specimen (data not shown). For statistical analysis, the median protein-adjusted p53 concentration of 0.13 µg/g was arbitrarily selected as the cutoff point at which, and beyond, p53 concentrations were considered positive. Compared to these extracts of neoplastic tissues, those of a small number (n=7) of normal tissues bordering regions of overt malignancy differed markedly in p53 protein content, since p53 concentrations in these control specimens were no greater than 0.07 µg/g.

III. 4. 2. Immunostaining of Tissue Sections

Light microscopic examination of the matched formalin-fixed, paraffin-embedded sections on which immunohistochemical staining was performed revealed predominantly nuclear staining, except in occasional cases of squamous cell carcinoma in which only faint cytoplasmic staining was observed. These latter cases were considered to be p53-negative by IHC. In all cases where immunostaining was present, however, it was confined to malignant cells; normal epithelia and stroma were consistently negative for immunostaining. Although some sections which were positive for staining displayed focal clustering of stained tumor cells when viewed under low power (x10) magnification, in others the staining was found to be more diffuse throughout the specimen. There was no obvious relationship between the number of cells staining, the

intensity of staining, and the tumor cellularity in these sections. For comparison, the p53 immunostaining of the astrocytoma, breast carcinoma, and ovarian carcinoma sections used as positive controls were moderately intense. In contrast, immunostaining was completely absent for every one of the specimen controls in which the primary antibody was omitted.

Conversion of these visual findings into numerical scores composed of three categorical variables, tumor cellularity, frequency of malignant cell staining, and intensity of malignant cell staining, allowed the integration of these qualitative features into single values representative of the overall extent of staining of the sections. Like the distribution of p53 protein concentrations measured by the ELISA, that of the immunostaining scores (Figure III. 2.), which ranged from 1 to 20.7, was also skewed positively. Specimens whose scores were equal to or greater than the median value of 3.6 were arbitrarily denoted as p53-positive by IHC. Figure III. 3. shows DO-7 stained sections representative of cases in which weak staining occurs in a small proportion of malignant cells or in which a high proportion of the tumor cells are intensely stained. Since ten separate microscopic fields of each section were examined in order to evaluate the number of stained tumor cells, and ten other fields were used to judge the intensity of staining, estimates of the variability within given specimens with respect to these characteristics could also be made. The majority of sections displayed DO-7 staining patterns which were homogeneous for both the proportion of malignant cells taking up stain and the intensity of such staining (data not shown). While only a few (n=10) sections were in the low tumor cellularity group, most had tumor cellularities which were either intermediate (n=38) or high (n=43). A statistical association

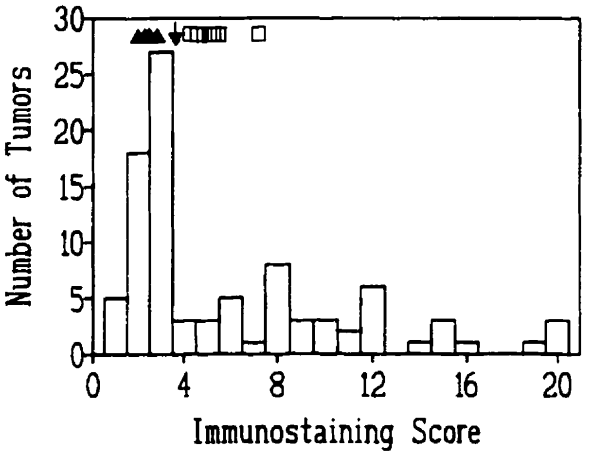


Figure III. 2. Distribution of Immunostaining Scores

Monoclonal antibody DO-7 used on formalin-fixed, paraffin-embedded lung tumor tissues treated for antigen retrieval by microwave heating. The arrow indicates the median score used as the cutoff point for p53-positivity. □ indicates staining scores of ELISA-negative, IHC-positive specimens and ▲ indicates staining scores of ELISA-positive, IHC-negative specimens.

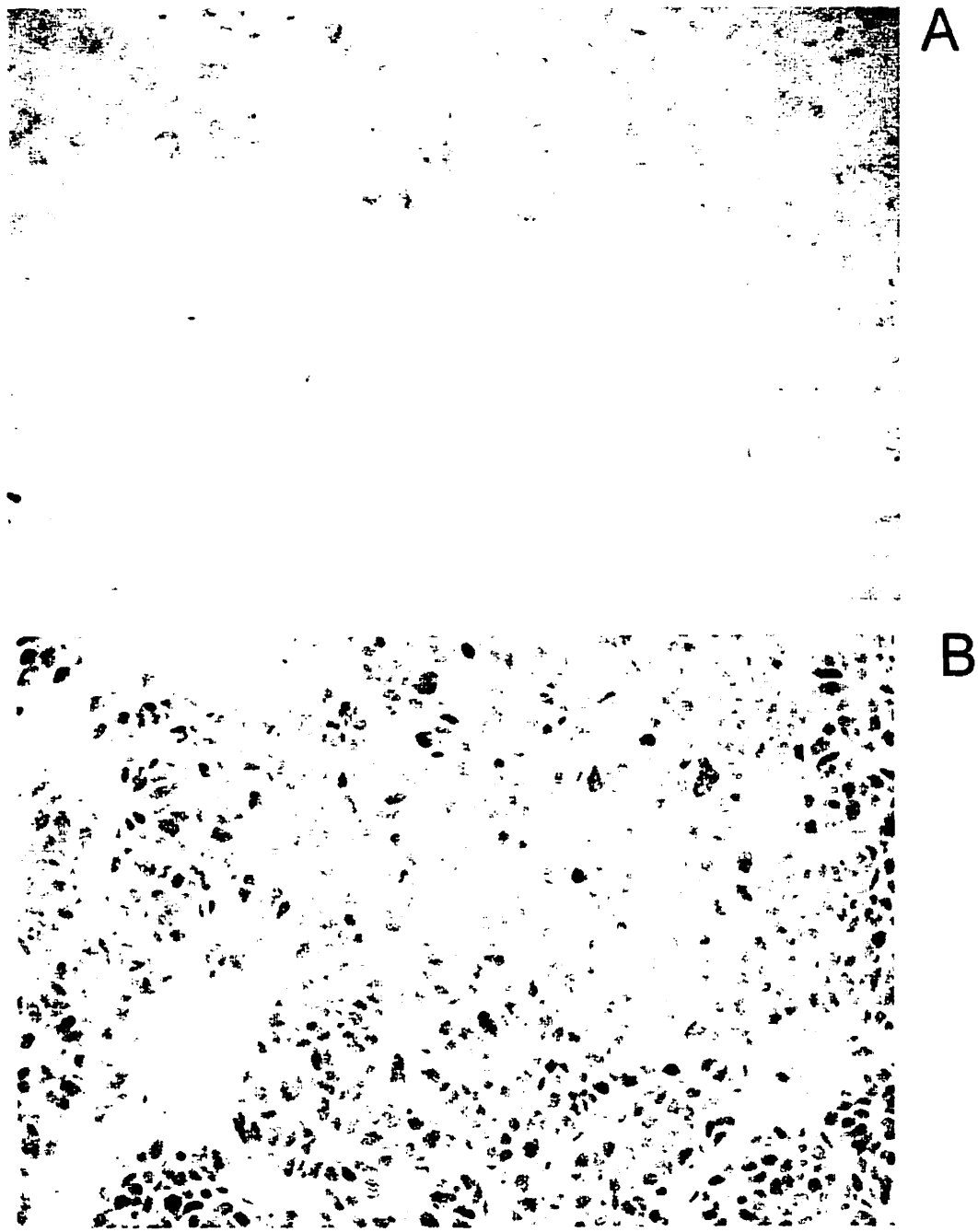


Figure III. 3. Immunohistochemical Staining for p53 Protein
Monoclonal antibody DO-7 was used as the primary antibody, showing A. fewer cells (<25%) with low intensity staining (score=1) and B. many cells (>75%) with high intensity staining (score=3) (original magnification X 500).

could be found between the cellularity values and the corresponding mean proportions of staining cells or mean intensities of staining (data not shown). However, sections in which a large number of tumor cells stained for p53 protein also tended to be those which had intense immunostaining, indicated by the strong correlation (r_s=0.91, p=0.01) between the mean values reflecting these two parameters.

III. 4. 3. Concordence Between the Two Methods

The degree of concordance between the results of the two techniques for p53 protein detection was first examined by Spearman correlation analysis, by which the methods were shown to be positively correlated (r_s=0.65, p<0.001). The Wilcoxon Rank Sum test was also applied in two formats. In the first, specimens divided into two groups on the basis of p53 protein concentrations, determined by ELISA, which were either below (n=46) or above (n=45) the median p53 protein level of 0.13 μg/g were revealed to differ significantly (p<0.001) in terms of their matched IHC scores. Specifically, the group of tumor specimens negative for p53 protein by ELISA had a lower median staining score (equal to 3; range 1-8.4) than the median score (equal to 9.3; range 2-20.7) found in specimens whose p53 protein concentrations were greater than 0.13 μg/g. In the second configuration of the Wilcoxon Rank Sum test, an IHC score of 3.6 was used as the cutoff point to dichotomize specimens into p53-negative (n=43) and p53-positive (n=48) groups, whose respective distributions of p53 protein concentrations were compared. p53 protein levels in specimens categorized as p53-negative by immunostaining were thus shown to be significantly (p<0.001) lower (median=0.06; range 0.01-0.76) than the p53 concentrations detected in the p53-positive group (median=0.75; range 0.01-10.97). Finally, when matched tissue extracts and paraffin sections were simultaneously classified as either p53-negative or -positive by both ELISA and IHC, respectively, contingency table analysis (Table III. 1.) was able to show a significant association (χ^2=35.9, p<0.001) between the two systems of classifying tumors for p53 protein status. Shown in the table, 83.7% of lung tumor specimens whose DO-7 stained sections had scores less than 3.6 yielded extracts in which p53 protein levels were below the cutoff point for p53-positivity by ELISA. Similar agreement was demonstrated by specimens classified as immunohistochemically p53-positive, 79.2% of

Table III. 1. Contingency Table Analysis of p53 Protein Status Determined by Both Methods

	p53 Protein Status by ELISA[a] Number of Patients (and %)		
	Negative	Positive	p-Value
p53 Protein Status by IHC[b]			
Negative score	36 (83.7)	7 (16.3)	
Positive score	10 (20.8)	38 (79.2)	<0.001

[a] Cutoff for ELISA: negative < 0.13 μg/g; positive ≥ 0.13 μg/g.
[b] Cutoff for IHC: negative < 3.6; positive ≥ 3.6.

which had matched frozen tissues found to have p53 protein concentrations greater than 0.13 μg/g. Also shown in the table are the 17 cases in which IHC and ELISA yielded contradictory findings. Neither the 7 IHC-negative, ELISA-positive cases (whose p53 protein concentrations were no greater than 0.76 μg/g) nor the 10 IHC-positive, ELISA-negative cases (whose immunostaining scores ranged from 3.6 to 8.4) could be explained by clustering within a given stage, grade, or histotype (data not shown). Re-examination of the immunostained sections of these discrepant cases confirmed the earlier scoring results. However, 3 of the 10 cases which were p53-negative by ELISA were only borderline positive by IHC, having immunostaining scores equal to the median value, equal to 3.6, used as the cutoff. Of the 7 cases in which positive ELISA findings were accompanied by negative IHC results, one case had a p53 protein concentration equal to the cutoff point of 0.13 μg/g, and 2 cases had p53 protein levels of 0.14 μg/g, just beyond the cutoff point. Another of the same 7 cases had an immunostaining score of 3.4, just below the median value. Five of the 10 ELISA-negative cases were also revealed to have achieved positive overall IHC scores by the multiplication of raw scores, indicating sparse staining of weak to moderate intensity, by high tumor cellularity values. To ensure homogeneity in the sampling of frozen tissues for extraction, 10 of the 17 discordant specimens were sampled at three different sites, re-extracted, and assayed for p53 protein. The variability between the three samples taken from each of the frozen specimens did not exceed 10%. These results clearly show that the parallel assessments of p53

expression status by ELISA and IHC yielded concordant findings in matched lung cancer specimens, all but three of which were nonsmall cell lung carcinomas. The cell extracts prepared from the small cell lung carcinomas had p53 protein concentrations of 0.02 μg/g, 1.49 μg/g, and 33.48 μg/g which were in agreement with the immunostaining scores of 3, 10.4, and 20.4, respectively, determined in the matched paraffin sections.

III. 5. Discussion

Lung cancer is the leading cause of cancer-related deaths in Western countries (Boring *et al.*, 1993). p53 gene mutation and loss of heterozygosity on chromosome 17p13.3 occur in up to 75% of lung tumors (Miller *et al.*, 1992). Genetic abnormalities at the p53 gene locus are usually accompanied by the expression of stabilized mutant p53 protein (Caamano *et al.*, 1991), detected in 30 to 70% of resected lung cancers (McLaren *et al.*, 1992; Quinlan *et al.*, 1992; Fontanini *et al.*, 1993; Passlick et al., 1995) and demonstrated to correlate both with clinicopathologic factors associated with poor prognosis (Caamano *et al.*, 1991; Fontanini *et al.*, 1993) and with resistance to chemotherapy (Rusch *et al.*, 1995), and to be an independent predictor of reduced overall survival (OS) (Quinlan *et al.*, 1992; Ebina *et al.*, 1994). However, some investigators have either failed to find prognostic value in p53 protein overexpression (Caamano *et al.*, 1991; McLaren *et al.*, 1992) or have shown that p53 protein may in fact predict a favorable clinical outcome in subsets of lung cancer patients (Lee et al., 1995; Passlick *et al.*, 1995). An association between lung cancer patient survival and p53 protein levels in tumor tissue might be obscured by the lack of an absolute correlation between intracellular accumulation of p53 and its functional status (Hall and Lane, 1994), which might have a direct influence on tumor aggressiveness. The different conclusions relating to the prognostic utility of p53 protein in lung cancer could be also due to differences in patient populations, especially the histologic types represented, or to the analytical methods used to assess the p53 expression level of the tumor tissues. For the most part, these detection methods have been standard immunohistochemical procedures.

IHC is a rapid and simple technique, routinely practised in most histopathology laboratories. Unlike biochemical analysis, it can identify distinct staining patterns at single cell resolution which could be clinically relevant (Hall and Lane, 1994). Relative

to mutational analysis, the major disadvantage often cited of IHC is the significant rate of false-negative and false-positive predictions of the mutational state of the p53 gene (Wynford-Thomas, 1992). The choice of fixative (Bartek *et al.*, 1993; Fisher *et al.*, 1994) may affect the staining intensity and distribution of stained cells using a single anti-p53 antibody. The section pretreatment, such as by enzymatic digestion or by microwaving to unmask otherwise cryptic p53 epitopes (Baas *et al.*, 1994; Lambkin *et al.*, 1994; Tenaud *et al.*, 1994), is another example. Since monoclonal anti-p53 antibodies differ in their epitope specificities and p53 mutants may vary in terms of epitope expression, selection of the primary immunoreagent is probably the single most important factor determining the success of IHC for p53 protein detection. It was therefore suggested that a cocktail consisting of at least three antibodies, one recognizing each of the three functional domains of p53 protein, enhances detection sensitivity (Tenaud *et al.*, 1994). Monoclonal antibodies are also differentially sensitive to fixation-induced epitope loss. As a consequence, certain antibodies may simply be ineffective on sections processed in particular fixatives (Vojtesek *et al.*, 1992; Bartek *et al.*, 1993), and although antigen retrieval procedures have solved this problem in many cases, these techniques may also differ in effectiveness depending on the antibody used for p53 detection (Lambkin *et al.*, 1994; Tenaud *et al.*, 1994; Resnick *et al.*, 1995). In addition, storage of paraffin slides for prolonged periods of time before staining has been associated with loss of p53-immunoreactivity (Prioleau and Schnitt, 1995). Finally, there is the subjective nature of interpreting IHC, image analysis being the exception, and the subdivision of specimens into groups (often simply p53-negative and p53-positive) based on arbitrary criteria. Numerous scoring systems for designating the p53 status of tumor tissues have been devised which differ widely in complexity. Such lack of consensus as to what constitutes positive p53 expression, together with the wide range of immunohistochemical procedures used in the different studies, has meant that the results of p53-immunostaining in any tissue must be interpreted cautiously (Wynford-Thomas, 1992).

Although ELISA-type immunochemical assays have been applied mostly to serum, they have also been used for other fluid matrices, including cell extracts from pulverized tissues. All ELISAs of p53 protein developed to date (Midgley *et al.*, 1992; Vojtesek *et al.*, 1992; Hassapoglidou *et al.*, 1993; Levesque *et al.*, 1995b; Thomas *et al.*,

1997), including the two which are commercially available (Oncogene Science, Uniondale, NY, USA), are of a "sandwich" configuration in which soluble p53 is immobilized between a solid phase monoclonal antibody recognizing mutant, wild-type, or both forms of p53, and enzyme-labelled polyclonal antibodies. Because the signal intensities are quantitative, ELISAs are far less subjective and obviate the high level of professional training needed to meaningfully interpret the results of immunostaining in a standardized manner. Furthermore, the requirement for p53 protein to simultaneously bind two immunoreagents and the rigorous multiple washing steps between incubations may impart to ELISAs a greater degree of specificity. Greater sensitivity may also result from the reduced background signal in the tumor extracts, due to washing steps but also by the use of a sample matrix containing only soluble components. Antigen unmasking is therefore not required in ELISAs. However, because ELISAs, like IHC methods, are based on the immunochemical detection of analytes, they are susceptible to many of the same limitations as mentioned above in terms of antibody selection and epitope expression. Another major drawback of ELISAs is the requirement for fresh frozen tissue, since they cannot be applied to fixed tissue. Studies based on the immunoassay analysis of tumors must therefore provide cryogenic storage of specimens frequently containing highly heat-labile antigens, a situation unlike the relatively convenient storage of paraffin blocks. Although the smallest mass of tissue (or alternatively the smallest mass of total protein) required for reliable detection of p53 protein by ELISA has not been determined (but likely differs between different assays, extraction conditions, and tissue types), it is conceivable that the amount of specimen available could be a limiting factor given that a certain volume of extract is needed for the assay. Another major disadvantage of ELISAs of p53 is related to specimen processing. Pulverization of the tissue, necessary for evenly distributed cell lysis, destroys all tissue architecture and hence any information regarding the relationship between p53 expression and histologic features. The p53 concentration in each extract simply represents the average p53 protein level throughout the portion of the tissue sampled. This average level may be heavily influenced by the ratio of normal to malignant cells in the tissue sampled, such that a specimen with a small proportion of highly overexpressing tumor cells might be

erroneously considered p53-negative. For this reason, enrichment of tumor cells by histologic examination and selection prior to extraction and immunoassay is essential.

Comparison between ELISA and IHC for p53 protein detection, performed in parallel on the same tumor tissues, has already been reported for breast (Vojtesek *et al.*, 1993), colon (Joypaul *et al.*, 1993), and gastric (Joypaul *et al.*, 1993) cancers. These authors found statistically significant correlations between p53 protein concentrations in frozen tissue, measured by an ELISA method employing DO-1 and CM-1 antibodies, and the p53 immunostaining scores using polyclonal CM-1 antiserum in sections of matched formalin-fixed, paraffin-embedded tissues using a scoring system which we used in this study. Since there were cases in both studies, however, in which ELISA failed to confirm the p53-positive status indicated by IHC, the latter technique was claimed to be more sensitive.

Several studies have demonstrated the utility of monoclonal DO-7 as the primary antibody for p53-immunostaining of formalin-fixed, paraffin-embedded lung carcinomas (Ebina *et al.*, 1994; Tenaud *et al.*, 1994; Lee *et al.*, 1995), especially in conjunction with epitope retrieval techniques (Tenaud *et al.*, 1994; Resnick *et al.*, 1995). Since DO-7 recognizes the same linear sequence of amino acids as DO-1 (amino acids 21-25 in the transactivation domain), and since both are of related immunoglobulin subclasses (IgG2a and IgG2b for DO-1 and DO-7, respectively), and both were shown to be equally suited for immunohistochemical detection of p53 in breast carcinomas, they may be considered equivalent in terms of their affinities for p53 protein (Vojtesek *et al.*, 1992). Because of such similarities between DO-1 and DO-7, the ideal comparative study in which each antibody would have been used in parallel for both ELISA and IHC analyses of matched tissues was not performed. However, some differences between these two antibodies have been demonstrated. The immunoreactivity of DO-1 has been shown to be unaffected by microwaving (Resnick *et al.*, 1995), whereas staining with DO-7 is enhanced by microwave heating (Baas *et al.*, 1994; Lambkin *et al.*, 1994; Tenaud *et al.*, 1994; Resnick *et al.*, 1995) but is impaired by enzymatic digestion of the specimens (Lambkin *et al.*, 1994; Resnick *et al.*, 1995). Because of the epitope specificities of DO-1 and DO-7 primary antibodies, the ELISA and IHC techniques compared in this study could not

distinguish between wild-type and mutant conformations of p53. Therefore, accumulation of p53 in some cases could have been caused by nonmutational mechanisms.

Our finding that p53-immunostaining scores correlated with p53 protein concentrations measured by ELISA, confirmed by Wilcoxon and contingency table analyses, was consistent with the concordance between these methods for detecting p53 protein accumulation reported earlier for both breast (Vojtesek *et al.*, 1993) and gastrointestinal (Joypaul *et al.*, 1993) tumors. In these other studies, however, each method resulted in the classification of a small number of specimens for p53 protein status which were discordant. While neither IHC-negative, ELISA-positive nor IHC-positive, ELISA-negative cases could be explained on the basis of any particular clinicopathologic or histologic feature, some of the discrepant cases could be explained by an artifact related to the selection of cutoffs, with either one of the methods giving results just below or above the cutoff point used to dichotomize specimens as p53-negative or p53-positive. Sampling variation could not explain the ELISA results, although this factor could not be ruled out for IHC since only one sampling site was used. Differences between tumor cellularities assessed in the matched specimens used for extraction and those used for immunostaining were not remarkable (data not shown). In the remaining cases, other factors relating to specimen handling before p53 analyses may have played a part. For example, partitioning of each lung tumor specimen in the operating room into portions which were not equivalent for the amount of p53-expressing cells might have led to discrepant findings between the methods. Degradation of p53 protein by autolysis of fresh tissue not immediately frozen could have led to false-negative results by ELISA despite efforts to freeze each specimen promptly. Conversely, false-negative findings by IHC might have occurred from failure of microwave irradiation to unmask p53 protein, perhaps as a consequence of overfixation. Because controls to determine the effectiveness of antigen retrieval were not performed, we could not address this latter possibility. For determining p53 protein expression status (and not p53 alteration *per se*), there exists no "gold standard" method against which the results of IHC and ELISA could have been compared. However, techniques to reveal the mutational status of the p53 gene or to detect p53 mRNA transcripts would have provided valuable correlates to the two methods compared in this report but were not performed.

Nevertheless, this study indicates that sensitive immunofluorometry for p53 protein in lung tumor tissues gives results which are highly concordant with those obtained by standard IHC and therefore is an alternative approach for the determination of p53 overexpression in malignant lung tissue.

CHAPTER IV

COMPARISON OF NEW IMMUNOASSAY TO DNA SEQUENCE ANALYSIS

(A version from Lianidou ES, Levesque MA, Katsaros D, Angelopoulou K, Yu H, Genta F, Arisio R, Massobrio M, Bharaj B, Diamandis EP. *Anticancer Res* 1999; 19: 799-806, with permission from Dr. John G. Delinassios, Editor and Publisher)

IV. 1. Abstract

Tumor tissues from 55 patients with well or poorly differentiated (grades 1 or 3) primary epithelial ovarian carcinoma were assessed both for p53 protein overexpression by our sensitive time-resolved immunofluorometric assay employing DO-1 and CM-1 antibodies, and for genetic p53 abnormalities by direct sequencing of polymerase chain reaction (PCR)-amplified exons 5 to 9. Sixteen p53 mutations (29%), including 3 deletions causing frameshifts as well as one nonsense and 12 missense point mutations were found in all exons except exon 9. Overexpression of p53 protein, defined as a concentration exceeding the 75th percentile, was found in 15 cases (27%), 10 of which had missense mutations ($p<0.01$). Tumors with nonsense and frameshift mutations were p53-negative by immunoassay. Both p53 mutation ($p=0.04$) and p53 protein accumulation ($p<0.01$) were associated with stage III-IV disease, while p53 mutation was more closely related to grade 3 lesions ($p=0.04$) and serous histotype ($p=0.01$). These results indicate that p53 protein accumulation correlates well with missense point mutation in carcinoma of the ovary and, together with other evidence that p53 abnormality may be prognostic of outcome in this disease, suggest that the immunoassay of p53 protein may have clinical value.

IV. 2. Introduction

The close correlation between p53 genetic abnormalities and p53 protein overexpression has facilitated the use of simple and rapid immunohistochemical techniques to study the diagnostic and prognostic implications of p53 mutation in ovarian cancer. Complete DNA sequencing of the p53 gene, or as more commonly performed, of exons 5 to 9 within which up to 80% of the mutations occur, has been shown to offer greater sensitivity for the detection of mutations (Casey *et al.*, 1996) than either indirect mutational analysis (such as SSCP, CDGE or other screening techniques) or immunohistochemical staining, but it remains more laborious despite the widespread use of automated sequencing instruments. The majority of studies have therefore employed immunohistochemical methods. However, perhaps advantageous to immunostaining in terms of sensitivity, specificity, and reproducibility for the detection of p53 protein in tumor tissue are quantitative immunoassays of p53, several of which have been

developed and applied to extracts of various tumor types (Hassapoglidou *et al.*, 1993; Vojtesek *et al.*, 1993; Levesque *et al.*, 1995b), including ovarian carcinoma (Levesque *et al.*, 1995c). Comparisons between the findings of such ELISA-type assays of p53 protein performed in parallel with p53-immunostaining of breast (Vojtesek *et al.*, 1993), lung (Levesque *et al.*, 1997), gastrointestinal (Joypaul *et al.*, 1993), and ovarian (Levesque *et al.*, 1995c) tissues have demonstrated the general concordance between the two approaches. The evaluations of immunoassays of p53 protein have not yet included comparisons of tumoral p53 protein accumulation to the corresponding mutational status of the p53 gene ascertained by direct DNA sequence analysis. The purpose of this study was therefore to compare the findings of these two methods in a series of 55 well or poorly differentiated (grade 1 or 3) ovarian carcinomas.

IV. 3. Materials and Methods

IV. 3. 1. Ovarian Cancer Patients

Fifty-five patients operated at the Department of Gynecology, Gynecologic Oncology Service of the University of Turin, Turin, Italy between November 1989 and February 1996 for treatment of primary epithelial ovarian carcinoma were included in this study. These patients constituted a subset of a larger patient population and were selected on the basis of having primary epithelial ovarian tumors of either low or high histologic grade (grade 1 or grade 3, respectively). Three patients for whom tumor specimens were available had been excluded, since two had been diagnosed as having germinal ovarian neoplasms and one had had a primary colon cancer metastatic to the ovary. The age range of these patients was 20 to 79 years, with a median age of 57 years. Additional clinicopathologic variables for which the patients had been characterized at the time of surgery, including residual tumor size, stage according to the criteria of the International Federation of Gynecologists and Obstetricians (FIGO) (FIGO, 1987) and histologic grade and type based on World Health Organization (WHO) (Serov and Scully, 1973) criteria, were also assessed. The FIGO staging scheme assumes that an adequate staging operation, described elsewhere (Levesque *et al.*, 1995c), has been performed. Twelve patients were found to have stage I disease, 3 patients were in stage II, 31 patients were in stage III, and 9 patients had stage IV ovarian cancer. Forty of the

tumors were poorly-differentiated (grade 3), whereas 15 were well-differentiated (grade 1). With respect to histologic type, 3 tumors were clear cell, 12 were endometrioid, 7 were mucinous, 23 were serous, 9 were undifferentiated and one tumor had a mixed mullerian histologic type. Postsurgically, 20 patients were apparently free of residual tumor tissue, while 10 patients had residual tumor masses estimated in size to be between 1 and 4 cm in greatest diameter, 13 had masses between 5 and 8 cm in diameter, 11 had masses larger than 9 cm, and residual tumor size was unknown for 1 patient.

IV. 3. 2. Tumor Extraction and Immunofluorometric Assay

Immediately following surgery, a representative portion of each tumor was selected during quick-section procedures, snap-frozen in liquid nitrogen, and stored at -80°C until analysis. Approximately 200 mg of each tumor tissue, which contained more than 70% tumor cells as determined by histologic examination, was pulverized to a fine powder at -80°C. DNA was extracted and purified from the tissue using a conventional phenol-chloroform-based procedure (Strauss, 1995), quantified by absorbance measurements at 260 nm, and stored at 4°C until analysis. For p53 protein analysis, extracts were prepared as described above (Levesque *et al.*, 1995b) and assayed for total protein content by a commercially available method utilizing the BCA reagent (Pierce Chemical Co., Rockford, IL). p53 protein was quantitatively analyzed using a new immunofluorometric procedure as previously described in detail elsewhere (Levesque *et al.*, 1995b).

IV. 3. 3. PCR Amplification

The paired primer sequences flanking each of the exons 5 to 9 of the p53 gene are shown in Table IV. 1. All oligonucleotide primers were synthesized commercially (ACGT Corp., Toronto, Ontario, Canada). PCR amplification of each exon was performed in a final volume of 50 µL, containing approximately 500 ng of template DNA, 10 mmol/L Tris (pH 8.3), 50 mmol/L KCl, 2 units AmpliTaq Polymerase (Hoffmann-La Roche, Basel, Switzerland), 200 µmol/L deoxynucleoside triphosphates, optimized $MgCl_2$ concentrations (1.5 mmol/L for exons 6 and 7, 2.0 mmol/L for exons 8 and 9, and 2.5 mmol/L for exon 5), and optimized concentrations of each primer (0.4

Table IV. 1. Primers for PCR Amplification and DNA Sequencing of p53 Exons 5 to 9

Exon	Strand	PCR Primer Pair	Sequencing Primers
5	Sense	5'-CACTTGTGCCCTGACTTT-3'	5'-TCTTTGCTGCCGTGTTCC-3'
	Antisense	5'-CCTGGGGACCCTGGGCAA-3'	5'-CCTGGGACCCGTTGGTCG-3'
6	Sense	5'-TGTTCACTTGTGCCCTGACT-3'	5'-TGGTTGCCCAGGGTCCCC-3'
	Antisense	5'-GGAGGGCCACTGACAACCA-3'	5'-CCACCCTTAACCCCTCC-3'
7	Sense	5'-GGCGACAGAGCGAGATTCCA-3'	5'-CTCCCCTGCTTGCCACA-3'
	Antisense	5'-GGGTCAGCGGCAAGCAGAGG-3'	5'-TCAGCGGCAAGCAGAGG-3'
8	Sense	5'-GACAAGGGTGGTTGGGAGTAGATG-3'	5'-ATGGGACAGGTAGGACC-3'
	Antisense	5'-GCAAGGAAAGGTGATAAAAGTGAA-3'	5'-CATAACTGCACCCTTGG-3'
9	Sense	5'-GCGGTGGAGGAGACCAAGG-3'	5'-GGAGGAGACCAAGGGTGC-3'
	Antisense	5'-AACGGCATTTTGAGTGTTAGAC-3'	5'-GGAAACTTTCCACTTGA-3'

μmol/L for exons 5, 6, and 9, 0.6 μmol/L for exon 8, and 0.8 μmol/L for exon 7). The thermal cycling profile consisted of a 20 second denaturation step at 94°C, a 30 second annealing step at the optimal temperature determined for each exon (60°C for exons 5, 6, and 9, 62°C for exon 7, and 63°C for exon 8), and a 30 second extension step at 72°C, for a total of 30 cycles. Each PCR was initiated with a 3 minute denaturation at 94°C and terminated with a 3 minute extension at 72°C. Following determination of the approximate yield and purity in each case by agarose gel electrophoresis, the PCR products were incubated first at 37°C for 15 minutes with 10 units of Exonuclease I and 2 units of shrimp alkaline phosphatase (both from Amersham Life Science Inc., Arlington Heights, IL), and then incubated at 80°C for another 15 minutes to inactivate these enzymes. Dilution of the pretreated PCR products 1:5 to 1:10 preceded their sequencing.

IV. 3. 4. DNA Sequence Analysis

The primers used for sequencing the PCR-amplified p53 exons 5 to 9 were designed using Oligo 5.0 software (National Biosciences Inc., Plymouth, MN) based on the genomic p53 sequence deposited into GenBank by Chumakov *et al.* (accession #

X54156). The primers were synthesized, and labeled at the 5'-end with the fluorescent dye Cy5, at National Biosciences Inc., and their sequences are also given in Table IV. 1. The Thermo Sequenase cycle sequencing protocol (Amersham Life Science Inc.) was followed according to the manufacturer's instructions, after which the reaction products were resolved and sequenced using an ALFexpress DNA Sequencer (Pharmacia Biotech AB, Uppsala, Sweden).

IV. 3. 5. Statistical Analysis

The associations of p53 gene mutation with other clinical or pathological variables, including patient age, stage, histologic grade and type, and residual tumor size, as well as with p53 protein concentrations dichotomized on the basis of a 75th percentile cutoff point, were examined using Chi-square or Fisher's Exact tests where appropriate. McNemar's test was also applied to the latter comparison of p53 status assigned by each of the two methods, in order to examine the distribution of discordant pairs. The medians of p53 protein concentrations in tissue extracts among different clinical or pathological groups were compared using Wilcoxon Rank Sum tests. Computer software SAS 6.12 (SAS Institute, Cary, NC) was used for these analyses, and 2-sided tests of significance were used throughout.

IV. 4. Results

IV. 4. 1. Quantitative p53 Protein Analysis

p53 protein concentrations above the detection limit of the immunoassay were found in all except four soluble protein extracts prepared from the 55 ovarian tumor specimens. These values ranged from 0.04 to 196.2 μg/L and had a median, SD, and mean of 2.27 μg/L, 39.71 μg/L, and 19.80 μg/L, respectively. Adjustment of each p53 protein concentration to reflect the particular total protein content in each case yielded a highly skewed distribution (Figure IV. 1.) which ranged from 0.00 to 34.34 μg/g, had a mean of 3.38 μg/g and a SD of 6.23 μg/g, and had 25^{th}, 50^{th}, and 75^{th} percentiles of 0.08 μg/g, 0.94 μg/g, and 3.00 μg/g, respectively. The simple division of patients into two groups, p53-negative and p53-positive, was made on the basis of a cutoff point equal to

the 75th percentile, at which and beyond, total protein-adjusted p53 concentrations were considered p53-positive.

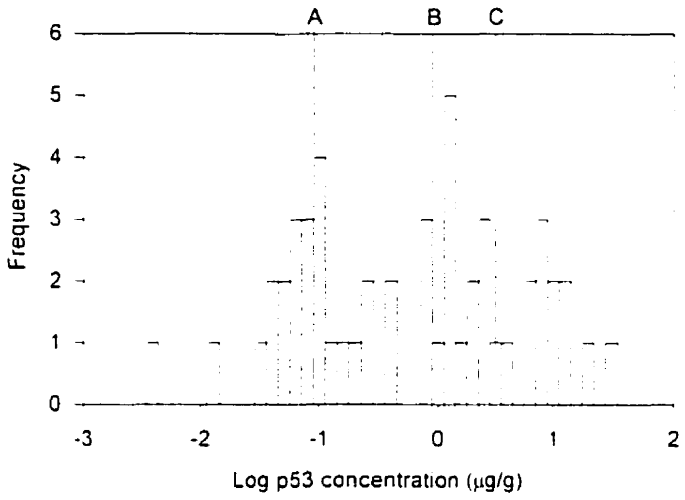


Figure IV. 1. Frequency Distribution of p53 Protein Concentrations in Ovarian Tumor Extracts
The dashed lines A, B, and C indicate the 25th, 50th, and 75th percentiles of the distribution, respectively.

IV. 4. 2. Mutational Analysis

Automated sequencing of the PCR-amplified exons 5 to 9 of the p53 gene in each of the ovarian carcinomas revealed mutations in 16 cases (29%) (Table IV. 2.). These mutations were approximately evenly distributed across exons 5 to 8; exon 9 was found to have a wild-type sequence in every case. In addition to the three mutations detected at codon 248, missense point mutations at other p53 mutational hotspots (codons 175 and 273) were also found. One of the mutations at codon 248, as well as those detected at codons 196 and 282, represented GC→AT transitions at CpG dinucleotides. Five other mutations in specimens 5, 144, 62, 91, and 113, three of them transversions, also occurred at CpG sites encoding arginine residues. In total, substitutions affecting arginine occurred in 8 of the 12 missense cases. There was a slight predominance of transition (n=8) over transversion (n=5) mutations, and G to A or G to T substitutions accounted for 6 of the 16 mutations observed. Translational termination was encoded by a C to T transition within exon 6. Since additional noncancerous tissues were not collected from these patients, the possibility that these genetic changes may have represented germline p53 mutations could not be investigated. The same neutral genetic polymorphism, an A to G transition at the second position of codon 213 in exon 6 encoding an arginine residue, was detected in three tumor specimens. Sequence deletions, predicted to lead to

Table IV. 2. p53 Mutations and Corresponding p53 Protein Expression Levels in Ovarian Carcinomas

Specimen	Exon	Codon	Nucleotide Change[a]	Amino Acid Change	p53 Protein[b]	Grade	Stage
189	5	172	GTT → TTT	Val → Phe	3.84	3	IV
5	5	175	CGC → CAC	Arg → His	3.07	3	III
8	5	175	CGC → CAC	Arg → His	0.00	3	III
205	5	183	Deleted CAGATAGC	Stop at codon 211	0.11	3	II
112	6	193	CAT → GAT	His → Asp	10.76	3	III
88	6	194	CTT → CGT	Leu → Arg	7.86	3	IV
144	6	196	CGA → TGA	Arg → Stop codon	0.02	3	III
68	7	227	Deleted TGAC	Stop at codon 244	0.06	3	IV
77	7	248	CGG → TGG	Arg → Trp	3.08	1	III
104	7	248	CGG → GGG	Arg → Gly	14.52	3	III
114	7	248	CGG → CTG	Arg → Leu	15.35	3	III
75	8	272	GTG → ATG	Val → Met	2.89	3	III
62	8	273	CGT → CAT	Arg → His	4.96	3	IV
91	8	280	AGA → GGA	Arg → Gly	20.31	3	III
113	8	282	CGG → TGG	Arg → Trp	8.70	3	III
175	8	292	Deleted AAAG	Stop at codon 303	1.31	3	III

[a] Sequences flanking deletions given in text.
[b] p53 protein concentrations in units of μg/g; values ≥ 3 μg/g were considered p53-positive.

premature translational termination, were also found to affect exon 5 (8 nucleotides in length), exon 7 (4 nucleotides), and exon 8 (4 nucleotides). Examination of sequences flanking the deletions revealed the presence of short repeats present also in the deleted segments: TGCTC**AGATAGC**GATG in exon 5; GGT**TG**GCTC**TGACTG**TAC in exon 7; and C**AAG**AAAGGGGAG in exon 8, where the deletion is underlined and repeated sequences are shown in bold. Splice-site mutational changes in the p53 gene were not found.

IV. 4. 3. Relationships Amongst p53 Gene Mutation, p53 Protein Accumulation, and Other Variables

Missense p53 mutation and overexpression of p53 protein were closely associated, evident from the observation that of 12 ovarian tumors with such genetic

changes. 10 were found to have p53 protein concentrations above 3 μg/g (Table IV. 2.). The three deletion mutations, however, as well as the point mutation resulting in a stop codon, were all accompanied by much lower p53 protein concentrations - findings consistent with the expression of truncated p53 products either not subject to stabilization or unrecognizable by the immunoassay. The occurrence of any kind of p53 mutation was considered in relation to p53 protein status, as well as to the status of the other

Table IV. 3. p53 Gene Mutation[a] in Relation to Clinicopathologic Variables

Variable	Number of Cases (and %)		p-Value
	No p53 Mutation	p53 Mutation	
Age			
< 57 years	20 (51.3)	7 (43.8)	
≥ 57 years	19 (48.7)	9 (56.2)	0.612[b]
Stage			
I	12 (30.8)	0	
II	2 (5.1)	1 (6.2)	
III	20 (51.3)	11 (68.8)	
IV	5 (12.8)	4 (25.0)	0.037[c]
Histologic Type			
Serous Papillary	12 (30.8)	11 (68.7)	
Endometrioid	12 (30.8)	0	
Undifferentiated	6 (15.4)	3 (18.8)	
All Other Histotypes	9 (23.0)	2 (12.5)	0.013[c]
Grade			
1	14 (35.9)	1 (6.2)	
3	25 (64.1)	15 (93.8)	0.043[b]
Residual Tumor[d]			
<1 cm	18 (46.1)	2 (18.8)	
≥1 cm	21 (53.9)	13 (81.2)	0.057[b]
p53 protein[e]			
Negative	34 (87.2)	6 (37.5)	
Positive	5 (12.8)	10 (62.5)	<0.001[b]

[a] Mutation identified in exons 5 to 9 of the p53 gene.
[b] p-value determined from Chi-square test.
[c] p-value determined from Fisher's Exact test.
[d] Residual tumor size unknown for one patient whose ovarian tumor had a p53 mutation.
[e] p53 protein concentrations equal to or exceeding 3 μg/g were considered p53-positive.

clinicopathologic features for which the patients had been characterized, as shown in Table IV. 3. The categorization of ovarian tumors into p53-negative and p53-positive groups by immunoassay agreed with the findings of mutational analysis in 10 of the 16 cases where mutations were demonstrated, and in 29 of the 34 cases where p53 mutations were not found. Missense p53 mutations were identified in 10 of the 15 cases in which p53 protein was overexpressed above the cutoff point. p53 protein accumulation above 3 μg/g and mutation of conserved p53 exons also agreed with respect to the similar number of cases positive by one method and negative by the other (p=0.763 by McNemar's test). Statistically significant associations were also found between p53 mutation and greater anatomic extent of disease, serous papillary histotype, and high grade. All except one of the 16 mutations identified were from poorly differentiated (grade 3) tumors, all except one were from patients with stage III or IV disease, and all except two were from patients who had been suboptimally debulked at surgery. There was no tendency, however, for the frequency of p53 mutation to increase or decrease with increasing patient age. Similarly, p53 protein levels did not differ significantly between patients of ages above or below the median cutoff or between ovarian tumors of different histologic types (Table IV. 4.). Although the statistical tests were borderline significant, there were tendencies for higher levels of p53 protein to associate with higher histological grade and presence of residual tumor. On the other hand, p53 protein concentrations were shown to be elevated in tumor extracts from patients with stage III or IV compared to stage I or II assignments, and were strongly associated with p53 gene mutation. The distributions of p53 protein concentrations in tumors with and without mutation are shown in Figure IV. 2.

IV. 5 Discussion

At least one in four patients with epithelial ovarian cancer, especially those presenting late at diagnosis, has a primary tumor in which the p53 tumor suppressor gene has been mutated. However, the impact of p53 mutation upon the ovarian cancer patient in terms of the natural history of the disease and the likelihood of therapeutic success remains controversial. Relative to many of the more established markers (stage, grade, residual tumor), p53 alteration has been found to have minor prognostic value (Marks *et al.*, 1991; Kohler *et al.*, 1993; Niwa *et al.*, 1994), although its importance may be greater

Table IV. 4. p53 Protein Concentrations[a] in Relation to Clinicopathologic Variables

Variable	Number	Median	Range	p-Value[b]
Patient Age				
< 57 years	27	0.11	0.03 - 15.35	
≥ 57 years	28	0.38	0 - 34.34	0.395
Stage				
I - II	15	0.11	0 - 1.37	
III - IV	40	1.41	0 - 34.34	0.004
Histologic Type				
Serous Papillary	23	1.49	0 - 20.31	
Endometrioid	12	0.35	0.04 - 34.34	
Undifferentiated	9	0.97	0 - 15.35	
All Other Histotypes	11	0.26	0 - 7.86	0.378
Grade				
1	15	0.26	0 - 3.08	
3	40	1.14	0 - 34.34	0.054
Residual Tumor[c]				
<1 cm	20	0.26	0 - 4.96	
≥1 cm	34	1.14	0 - 34.34	0.065
p53 Mutation				
No	39	0.26	0 – 34.34	
Yes	16	3.46	0 – 20.31	0.015

[a] p53 concentrations expressed in μg/g.
[b] p-values determined from Wilcoxon Rank Sum tests.
[c] Residual tumor size unknown for one patient whose tumor extract was p53-positive.

for patients with particular clinical or pathologic features (Levesque *et al.*, 1995c). On the other hand, recent findings suggest that p53 functional status may be a critical determinant for the success of systemic chemotherapy with drugs which exert their antineoplastic effects by the induction of p53-dependent apoptosis (Perego *et al.*, 1996; Righetti *et al.*, 1996). Among these agents are platinum-containing compounds, and possibly taxol, which are components of regimes typically prescribed for patients whose malignancies exhibit extraovarian extension. In the present absence of accurate predictive factors for platinum-based chemotherapy, many patients with advanced disease are

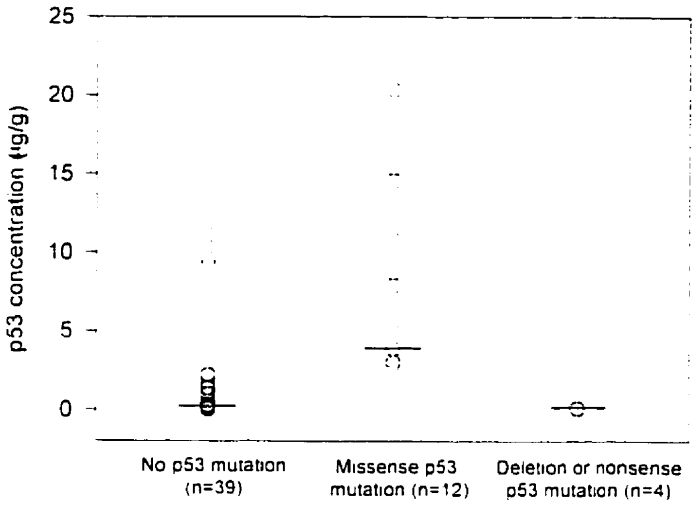


Figure IV. 2. p53 Protein Concentrations in Ovarian Tumors with and without Mutation in Exons 5 to 9 of the p53 Gene

The horizontal bars indicate the median value in each of the three groups. Not shown in the figure is a specimen in which the absence of mutation was accompanied by p53 protein concentration of 34.34 µg/g.

exposed to noxious but often ineffective treatments. A major focus of clinical research on ovarian cancer has therefore been the evaluation of novel prognostic and predictive factors, among which p53 mutational change has been the subject of intense interest.

The primary purpose of this study was to compare two complementary methods of indirectly assessing the functional status of the p53 gene in ovarian carcinomas. These methods investigate p53 at the levels of genomic DNA sequence and protein expression. Structural alterations of the p53 gene, most commonly by missense point mutations within the evolutionarily-conserved DNA-binding domain (Hollstein *et al.*, 1991), are associated with gene products impaired in their ability to function as transcription factors for genes mediating cell cycle arrest (Kastan *et al.*, 1992), programmed cell death (Lowe *et al.*, 1993), or DNA repair (Wang *et al.*, 1995). Failure of these processes following DNA damage is thought to contribute to the genomic instability predisposing to neoplastic transformation and progression. Although DNA sequencing of the entire coding portion of the p53 gene is not usually necessary in order to identify the majority of mutations, which cluster within exons 5 to 8 of the 11 exon gene, it is possible that many of the 10-20% of mutations missed by sequencing only the central DNA-binding domain may also inactivate p53 function (Casey *et al.*, 1996). Nevertheless, most studies have restricted their analyses to p53 exons 5 to 8, alterations of which have usually been initially detected by screening techniques such as SSCP or CDGE prior to DNA sequence analysis of aberrant cases due to the considerable expenditure of resources associated

with the latter procedure. The introduction of more rapid and affordable automated DNA sequencing technologies, targeting the clinical diagnostics setting, has made possible the processing and analysis of the large numbers of specimens required for meaningful epidemiologic studies (Bharaj *et al.*, 1998). Sequence analysis remains, however, technically far more demanding than simpler techniques taking advantage of another consequence of missense p53 mutation, namely, the conformational alteration of mutant p53 protein which confers resistance to normal degradative turnover. Cells harboring mutant p53 alleles usually display nuclear accumulation of the encoded protein, which can be detected by immunochemical methods employing a variety of monoclonal and polyclonal antibodies against epitopes exclusive to mutant p53 or shared by mutant and wild-type conformations of the protein. Most commonly, these antibodies have been applied as primary detection reagents for immunohistochemical staining of fixed or fresh tissue sections, although their use in ELISAs of soluble extracts of tissues has also been reported by a smaller number of groups (Hassapoglidou *et al.*, 1993; Vojtesek *et al.*, 1993; Levesque *et al.*, 1995b). While the advantages and disadvantages of immunoassays of p53 protein compared to conventional p53-immunostaining have been discussed elsewhere (Levesque *et al.*, 1997), use of an immunoassay of p53 protein has not yet been compared to DNA sequence analysis of the p53 core region.

The p53 mutation rate of 29% (16/55) in our series of ovarian carcinomas was somewhat low but within the range of those reported in other studies (Marks *et al.*, 1991; Okamoto *et al.*, 1991; Eccles *et al.*, 1992; Kohler et al., 1993; Kupryjanczyk *et al.*, 1993; McManus et al., 1994; Kim et al., 1995), even though specimens with grade 2 histology had been excluded from the analysis. Omission of moderately differentiated (grade 2) specimens served to enhance the partitioning of p53 mutations into high grade lesions but was unlikely a major source of bias since the 43% rate of p53 abnormality in our previous study (Levesque *et al.*, 1995c), which included grade 2 tumors, was obtained from a similar number of low (n=16) and high (n=49) grade specimens as included in the study reported here. Our previous study had found the p53 overexpression rate of grade 2 ovarian tumors to be intermediate (26%) to those of grade 1 (5%) or grade 3 (69%) tumors, in agreement with other studies (Klemi *et al.*, 1995; Eltabbakh *et al.*, 1997). The overall rate of p53 protein overexpression in the present study (27%), however, is lower

than that obtained in our previous work and may be explained largely by the selection of the cutoff point by which specimens were considered p53-positive – 3 μg/g compared to 0.23 μg/g used earlier. Although the immunoassays used in these two studies differed in their respective primary anti-p53 antibodies, they had been shown to yield highly correlated results when applied to extracts of various cell lines and resected breast tumors (Levesque *et al.*, 1995b).

Comparison of the mutations identified in this study with those reported in a comprehensive database of p53 mutations (Hainaut *et al.*, 1997) revealed several nucleotide changes detected at particular codons to be previously undescribed in ovarian carcinoma (G to T at codons 172 and 248; C to G at codon 193; A to G at codon 280). Our finding of a slight excess of transition over transversion mutations was consistent with the majority of other studies reporting a higher frequency of transitions (Mazars *et al.*, 1991; Okamoto *et al.*, 1991; Kohler *et al.*, 1993; Kupryjanczyk *et al.*, 1993) suggestive of endogenous mutagenic processes rather than exposure to carcinogens. The three deletions found in our series were novel for ovarian cancer and were found at a frequency (19% of identified mutations) similar to that observed in a recent study by our group (Angelopoulou *et al.*, 1998). In contrast to this earlier study, exon 8 was affected by deletions in addition to exons 5 and 7. Since the deletions were flanked by short direct repeats, misalignment of the template strands during DNA replication may have been responsible for their generation (Jego *et al.*, 1993). In spite of the fact that p53 coding sequences outside exons 5 to 9 were not surveyed for mutations, we feel that our analysis likely identified the majority of p53 genetic changes.

The strong relationship between p53 gene mutation, particularly in-frame substitutions, and p53 protein overexpression was confirmed in this study: 34 of the 39 cases without demonstrable p53 mutations in exons 5 to 9 were classified as p53-negative by immunoassay, while 10 of the 12 specimens in which missense mutations were detected were shown to have p53 protein concentrations above the cutoff point. Whether particular missense mutations resulted in greater degrees of p53 protein stabilization than others was not addressable due to the small number of specimens studied and remains unclear in the literature (Blagosklonny, 1997), although virtually all of the approximately 570 different missense p53 mutations (Hainaut *et al.*, 1997) occuring in the central DNA

binding domain have been associated with p53 protein accumulation (Almog and Rotter, 1998). Whether the precise sites and nature of missense mutation in the p53 gene are associated with differences in DO-1 antibody reactivity, similarly, has also not been performed. To account for the eleven cases for which genetic analysis and immunoassay were not in agreement, several explanations might be invoked. Intragenic deletions and translational termination mutations comprised four of the six cases in which p53 mutation was accompanied by low p53 protein expression. The selection of the p53 protein concentration (3 μg/g) above which specimens were considered p53-positive may have resulted in the artifactual misclassification of another tumor in which a missense point mutation was found in exon 8 but in which the p53 protein concentration (2.89 μg/g) was only slightly below the cutoff. However, the other p53-negative tumor in which a missense mutation was found had no detectable p53 immunoreactivity. It is possible that the nonequivalence of tissues, with respect to the histologic distribution of p53 abnormalities, that were used for DNA sequence analysis and for immunoassay in each case might have led to discrepant findings, both for these two p53-negative cases and for the other five in which p53 mutations were not found and yet p53 protein accumulated. Nonmutational stabilization of p53 protein, such as by cytoplasmic sequestration, has been documented in many malignancies (Moll *et al.*, 1992) and has been associated with complexation of p53 with mdm2 (Momand *et al.*, 1992) and a variety of viral oncoproteins (Blagosklonny, 1997; Funk and Galloway, 1998). Since the primary capture antibody used in the immunoassay recognizes an epitope expressed on wild-type as well as missence mutant p53, oncoprotein-stabilized nonmutant p53 would have elicited a signal interpreted as p53-positivity. However, although neither mdm2 expression nor the expression of SV40 large T antigen, adenovirus E1B 55 kDa protein, or other oncoproteins were determined in the discrepant cases, none of these proteins are associated with epithelial ovarian tumorigenesis (Ozols *et al.*, 1997). A less likely explanation would have been the presence of mutations outside exons 5 to 9 leading to the expression of a p53 gene product with an enhanced half-life. Since these explanations were not investigated, they remain speculative.

The results of this study provide evidence that the assessment of p53 abnormalities in epithelial ovarian carcinoma may be performed either by a simple

immunochemical assay or by a more complex DNA sequence analysis. Although the results of p53 immunoassay were not in complete concordance with the sequence-based analysis of p53, the advantages of the former method, especially in terms of its ease of application, may make it more suitable for clinical laboratory use.

CHAPTER V

SCREENING OF LUNG CANCER PATIENT SERA FOR p53 PROTEIN

(A version from Levesque MA, D'Costa M, Diamandis EP. *Br J Cancer* 1996; 74: 1434-1440, Copyright © 1996 by Churchill Livingstone)

V. 1. Abstract

p53 protein, which accumulates intracellularly in over half of all human tumors, has also been reported to be present in the sera of patients with various malignancies, including lung cancer. Using our quantitative immunoassay, we measured p53 protein concentrations in 216 sera from 114 lung cancer patients of whom 75 provided matched lung tumor tissues which were also assayed for p53 protein. p53 protein levels above the detection limit of 0.04 μg/L were detected in only 2 sera from lung cancer patients (0.14 μg/L and 0.27 μg/L) and not in any of 13 sera from non-malignant lung disease patients or in 100 sera from normal non-diseased individuals. The presence of these apparent traces of serum p53 protein concentrations could not be related either to the p53 protein expression status of the primary lung tumors or to the tumor stage, grade, or histological type. By pretreating these two sera with anti-p53 antibody linked to solid phase, and by the addition of mouse serum to neutralize possible heterophilic antibodies, the signals arising from these sera were shown to be nonspecific and possibly due to heterophilic antibodies. We conclude that our data do not support previous reports of p53 protein in the sera of lung cancer patients. Since immunoassays are subject to numerous sources of interference in serum, including heterophilic antibodies, we suggest that the results of p53 protein analysis of serum specimens should be interpreted with caution.

V. 2. Introduction

Whether tumor cells which overexpress p53 protein may release it into the bloodstream has been the subject of only a few investigations to date. A screen of 800 serum specimens collected from patients with a wide range of malignancies was unable to detect p53 protein in any specimen using an immunofluorometric assay (Hassapoglidou *et al.*, 1993). This finding is consistent with those of other workers (Winter *et al.*, 1992) employing a commercially available p53 ELISA method (Oncogene Science, Uniondale, NY) on lung cancer patient sera. More recently, however, elevated levels of p53 protein have been reported in the sera of patients with lung cancer (Luo *et al.*, 1994; Braun *et al.*, 1995) and colon adenomas and carcinomas (Greco *et al.*, 1994; Luo *et al.*, 1995) relative to the sera of control subjects, as well as in the sera of patients

with Hodgkin's disease (Trumper *et al.*, 1994), malignant lymphomas (Lehtinen *et al.*, 1993; Lahdeaho *et al.*, 1994), breast cancer (Rosanelli *et al.*, 1993), and asbestosis with and without lung cancer (Partanen *et al.*, 1995). In all of these recent studies, p53 protein was quantified in sera by the same or similiar ELISA procedure. We report here the use of a recently developed highly sensitive immunoassay of p53 protein (Levesque *et al.*, 1995b) which is suitable for all sample matrices including serum. Because our initial inability to detect p53 protein in the serum of cancer patients (Hassapoglidou *et al.*, 1993) was in conflict with the findings of other groups, we sought to determine if the p53 immunoassay signals arising in serum were truly p53-specific. The results of this study, which compared p53 protein concentrations in sera and tumor tissue extracts of patients with primary lung cancer and reported the measurements of p53 protein levels in sera from non-malignant lung disease patients and from normal individuals, suggests that p53 protein is not detectable in the sera of patients with lung cancer.

V. 3. Materials and Methods

V. 3. 1. Lung Cancer Patients

Matched tumor and serum specimens were obtained from 75 patients who were operated at St. Joseph's Health Centre in Toronto, Ontario, Canada between June 1993 and August 1995 for resectable primary lung carcinoma. This group consisted of 23 males and 52 females, and included 16 individuals less than 59 years of age, 32 between 60 and 69 years, 23 between 70 and 79 years, and 4 greater than 80 years of age at the time of surgery. Only 4 patients lacked a history of tobacco smoking. All but three patients were staged at surgery according to the Tumor-Node-Metastasis (TNM) classification system (Beahrs *et al.*, 1992): 46 were found to have stage I disease, 12 were in stage II, 12 had stage IIIA, one patient had stage IIIB, and one patient had stage IV cancer.

V. 3. 2. Tumor Specimens

All lung tumor specimens were obtained during routine surgery for the treatment of primary lung cancer. This study was approved by the ethics and research committee at St. Joseph's Health Centre, Toronto, Ontario, Canada. Immediately following surgery, a

representative portion of each primary lung tumor was selected during quick-section procedures in the operating room, snap frozen, and stored at -80°C for subsequent p53 immunoassay (see below). Formalin-fixed, paraffin-embedded sections of adjacent tumor tissue were used to routinely establish the grade and histological type in 72 cases following WHO guidelines (World Health Organization, 1982). Well differentiated (G1) tumors were found in 6 patients, 43 had moderately differentiated (G2) tumors, 22 were poorly differentiated (G3), and one was not differentiated (G4). Adenocarcinoma and squamous cell carcinoma, represented by 32 and 34 cases, respectively, accounted for the majority of specimens, while the remainder consisted of carcinoid tumors (n=3), small cell carcinoma (n=2), large cell carcinoma (n=2), and one each of adenosquamous and carcinosarcoma histologies. Further histological examination classified the tumor cellularity of 26 lung tumors as high (n=10), intermediate (n=12), or low (n=4).

Approximately 200 mg of the frozen tumor tissue was pulverized and subjected to cell lysis by incubation on ice for 30 minutes with 1 mL of a buffer containing 50 mmol/L Tris, 150 mmol/L NaCl, 5 mmol/L EDTA, 10 mL/L NP-40 surfactant, 10 mg/L PMSF, and 1 mg/L each of aprotinin and leupeptin. The soluble extracts were obtained following centrifugation at 14,000 g for 30 minutes at 4°C and collection of the supernatant fractions. Extracts were then assayed immediately for p53 protein, and for total protein content by the BCA method (Pierce Chemical, Rockford, IL). In order to determine the degree of heterogeneity for p53 protein accumulation within individual tumor specimens, ten tumor specimens were each further sampled at three different surfaces, and the equivalent masses of tissue were extracted and assayed for p53 protein. Normal lung tissue, free of overt malignant infiltration, was cut from seven surgical specimens at the margins of resection and subsequently pulverized, extracted, and assayed for both p53 protein and total protein. These were designated as control or "normal" tissues.

V. 3. 3. Serum Specimens

For each of the 75 lung cancer patients, preoperative and/or postoperative aliquots of serum specimens were obtained from the routine biochemistry laboratory. A preoperative serum specimen was obtained from 54 patients: 17 on the same day as

surgery, 19 one day before, and 18 two to nine days prior to surgery. At least one postoperative serum was obtained from 64 patients. Multiple postoperative samples were collected from 12 patients up to 12 days after surgery: 4 patients provided 2 specimens, 4 patients had 3 sera, and one patient each provided 4, 5, 6, and 7 specimens, respectively, after surgery. A preoperative serum and at least one postoperative serum were collected from 43 patients. Sixty-eight sera were also obtained from 39 lung cancer patients for whom no matched tumor tissues were available. For these latter specimens, the dates of collection relative to the date of surgery in each case were not known. All sera were stored at -80°C until analysis.

Control sera from 100 individuals from the general population without symptomatic disease were stored for no longer than six months at -40°C. Further controls were provided by 13 sera obtained from patients assessed at St. Joseph's Health Centre for nonmalignant lung diseases including chronic obstructive lung disease (n=5), pulmonary embolism (n=2), sarcoidosis (n=2), or respiratory failure due to other causes (n=5). These specimens were frozen after venipuncture and stored at -40°C prior to immunoassay.

V. 3. 4. p53 Immunofluorometric Assay

Lung tumor extracts and undiluted sera were assayed for p53 protein in duplicate using a "sandwich-type" immunoassay (Levesque *et al.*, 1995b). In lung tumor extracts, p53 levels were expressed relative to the amount of total protein in the cell lysates.

Serum specimens with p53 concentrations consistently exceeding the detection limit on repeated analyses were reassayed after incubation for one hour at room temperature with 30% (v/v) of DO-1 Sepharose (to immunoabsorb p53 protein), anti-digoxin Sepharose (control), or uncoupled Sepharose (control), used as 50% slurries, followed by centrifugation at 14,000 g for 30 minutes to collect the supernatants. The solid phase Sepharose-antibody conjugates were prepared from high titre ascites fluids collected from mice injected with monoclonal DO-1 or anti-digoxin-producing hybridomas using standard procedures (Harlow and Lane, 1988), and from CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) used according to the manufacturer. A lung tumor extract prepared as above, and a serum specimen from a

hospitalized patient without evidence of malignancy which was supplemented with 10% (v/v) lung tumor extract, were also treated as above and then assayed for p53 protein. Serum specimens containing detectable levels of p53 protein were also tested for the presence of heterophilic antibodies, a potential source of spurious background signals in two-site immunoassays (Nahm and Hoffmann, 1990). The fluorescence counts yielded by the reassay of specimens 30 minutes after the addition of mouse serum (50% v/v), with agitation at room temperature, were compared to the counts elicited by assay of the same specimens to which equivalent volumes of 6% BSA (50 mmol/L Tris, pH 7.80, 60 g/L BSA, and 0.5 g/L NaN_3) had been added.

V. 4. Results

V. 4. 1. p53 Expression in Primary Lung Tumors and Normal Lung Tissues

All 75 lung tumor extracts had p53 protein concentrations that exceeded the p53 detection limit of ~0.04 μg/L: values ranged from 0.06 to 70.7 μg/L with a median p53 concentration of 0.52 μg/L. Because of variations in the extraction efficiency, the levels of p53 protein were adjusted for the total protein concentrations in the extracts. The distribution of these adjusted p53 concentrations in the tumor tissues was positively skewed (minimum=0.008 μg/g, maximum=10.97 μg/g, median= 0.13 μg/g, mean=1.10 μg/g, SD=2.20 μg/g). In order to categorize tumor specimens as either p53-negative or p53-positive, the median value of 0.13 μg per gram of total protein was selected as the arbitrary cutoff point. Tumor tissues were roughly homogeneous for p53 protein overexpression, since three independent extractions of tissue cut from different surfaces of a small number of p53-positive (n=5) and p53-negative (n=5) specimens yielded p53 concentrations, adjusted for total protein, which did not vary by more than 10% for each tumor (data not shown). Normal lung tissue cut from the resection margins of seven tumor-containing specimens revealed much lower p53 concentrations which ranged from 0.010 μg/g to 0.070 μg/g with a median of 0.030 μg/g.

V. 4. 2. Serum Levels of p53 Protein

Of all the sera collected from the lung cancer patients, only two had p53 protein levels which consistently exceeded the assay detection limit on repeated analyses,

necessary for the measurement of p53 protein levels in serum specimens because of the random fluctuations of the assay at the beginning of the calibration curve. One of these sera, having a p53 protein concentration of 0.27 μg/L (which exceeds the first p53 protein standard in the linear portion of the calibration curve) and denoted as Patient Serum 1, was identified from a screen of 68 sera collected from patients while they were in hospital for surgical removal of their primary lung carcinomas. The other serum specimen, henceforth referred to as Patient Serum 2, had a p53 protein level of 0.14 μg/L and was collected from a lung cancer patient before surgery. Although it was known that this patient had a poorly differentiated squamous cell carcinoma which had invaded the visceral pleura but did not involve any of the regional lymph nodes (stage I), correlation between the appearance of detectable p53 protein in serum and any clinicopathologic feature was obviously not possible. The relationship between p53 protein expression status by the primary tumor and its detection in serum could similiarly not be addressed: the p53 protein concentration in the extract prepared from this patient's lung tumor was 0.015 μg/g, below the median cutoff point for p53 positivity. None of the 100 sera from asymptomatic members of the general population or the 13 sera from patients with lung diseases other than malignancy had p53 protein concentrations exceeding the assay detection limit.

V. 4. 3. Assessment of Assay Specificity in Apparently p53-Positive Sera

To provide evidence that the fluorescence signals in apparently "p53-positive" sera were related to p53 protein concentrations, Patient Sera 1 and 2 were further investigated by a simple immunoabsorption procedure in which specimens were incubated with Sepharose beads conjugated to DO-1 antibody to clear them of soluble p53 protein prior to assay. The high capacity of DO-1 Sepharose to specifically immunoabsorb p53 protein from a lung tumor extract is shown in Figure V. 1. (panel A): the assay of the tumor extract after treatment with DO-1 Sepharose yielded only 8% of the fluorescence counts generated by the assay of the same extract after incubation with uncoupled Sepharose, used as a dilution control since the conjugates were added as aqueous slurries. The tumor extract, which had a p53 protein concentration of approximately 10 μg/L, was completely cleared of detectable p53 protein as the signal

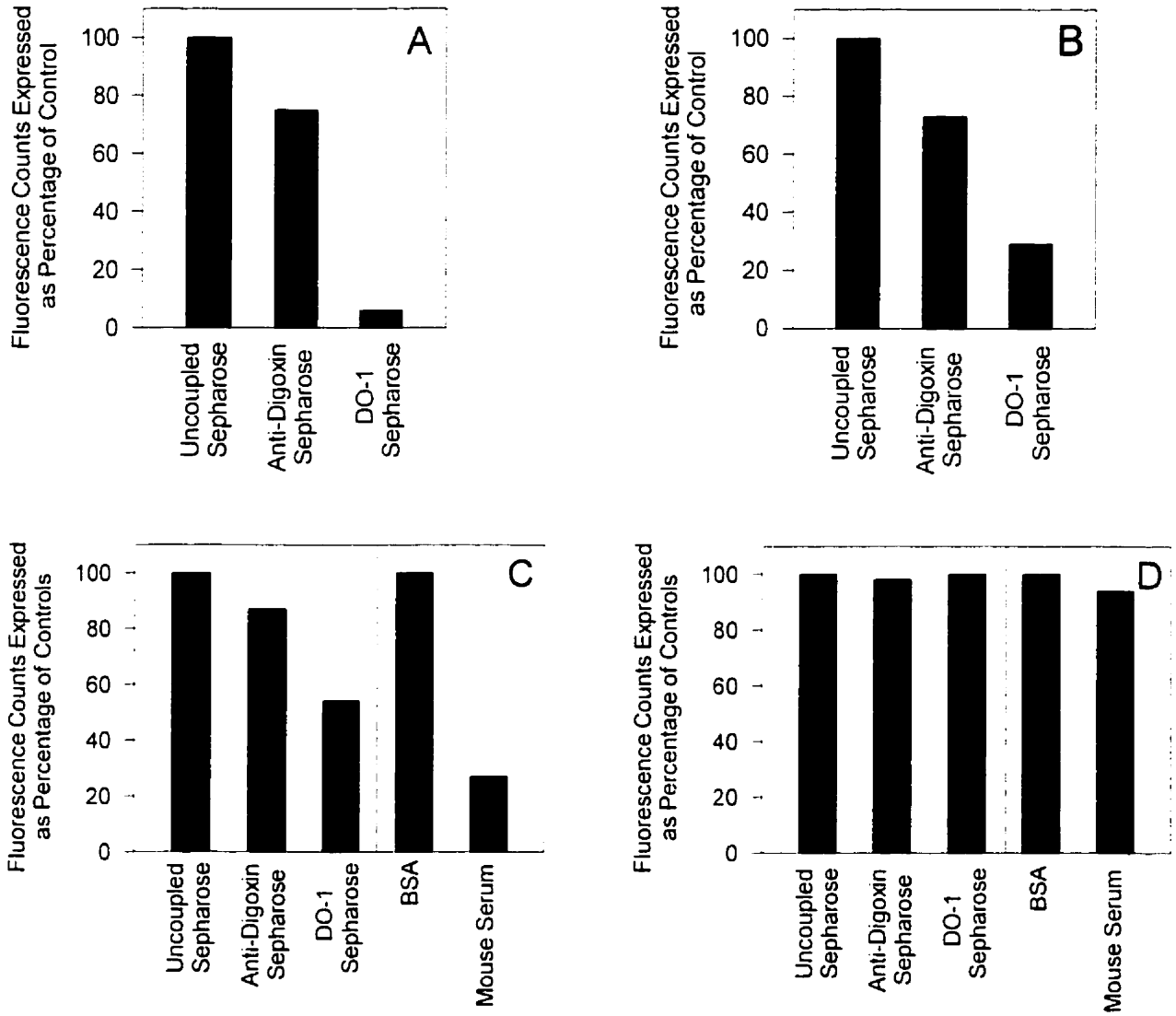


Figure V. 1. Treatment of Sera with Solid-Phase Conjugates and Testing for the Presence of Heterophilic Antibodies

A. lung tumor extract (10 μg/L p53 protein); B. normal control serum spiked with lung tumor extract (0.8 μg/L p53 protein); C. lung cancer patient serum 1 (0.27 μg/L p53 protein); D. lung cancer serum 2 (0.14 μg/L). Controls in each case are uncoupled Sepharose (A and B) or uncoupled Sepharose and mouse serum (C and D).

after Sepharose treatment did not exceed background. Treatment with Sepharose conjugated to an antibody against the irrelevant antigen digoxin resulted in a much smaller reduction of the fluorescence signal. As shown in Figure V. 1. (Panel B), DO-1 Sepharose was also able to remove p53 protein from control sera which was supplemented, to a p53 protein level of 0.8 μg/L, with p53-containing lung tumor extract. Again, background fluorescence counts were achieved in these sera following DO-1 Sepharose treatment. The high recoverability of p53 protein from serum (Levesque *et al.*,

1995b) was confirmed by the observation that dilution of the lung tumor extract 10-fold by the control sera yielded fluorescence signals less than seven times lower than that of the undiluted tumor extract (data not shown). Given these results, the reduction in p53-associated fluorescence after incubation of Patient Serum 1 with DO-1 Sepharose to 58% of the signal produced in the same serum treated with uncoupled Sepharose (Figure V. 1. (Panel C)) was suggestive that this serum specimen contained p53 protein. In contrast, no reduction in fluorescence counts resulted from the treatment of Patient Serum 2 with DO-1 Sepharose (Figure V. 1. (Panel D)).

Because treatment of Patient Serum 1 with DO-1 Sepharose did not reduce the fluorescence signal to a background level, as was the case when DO-1 Sepharose was added to both the tumor extract-spiked control sera and to the extract itself, we investigated the possibility that the signals in these specimens were due to the presence of human antibodies with broad anti-species specificities which might have cross-linked the solid phase DO-1, polyclonal CM-1, or ALP-conjugated antibodies in our immunoassay leading to false positive results. A common practice to neutralize these heterophilic antibodies, which have been reported in the sera of up to 40% of normal subjects (Boscato and Stuart, 1986), is the inclusion of nonimmune serum from a species used to raise one of the analyte-specific antibodies to the sample assay buffer (Nahm and Hoffmann 1990). The large excess of mouse immunoglobulins in mouse serum, added to human serum specimens, would be expected to saturate the binding of any heterophilic antibodies with anti-mouse specificity. Figure V. 1. (Panel C) shows that when added to Patient Serum 1, mouse serum (added as 30% of the total volume) was able to completely suppress the fluorescence signal, unlike equivalent dilution of this serum by the addition of immunoglobulin-free 6% BSA. The same amount of mouse serum added to Patient Serum 2 (Figure V. 1. (Panel D)) or to either the tumor extract or the spiked control sera (data not shown) were without similar effect. These results strongly suggest that Patient Serum 1 contained non-specific reactants with the sandwich immunoassay.

V. 5. Discussion

Alterations to the p53 gene are the most frequent genetic changes revealed so far in human cancer and occur in 43-75% of non-small cell lung cancers (NSCLC)(Marchetti

et al., 1993; Mitsudomi *et al.*, 1993b; Gazzeri *et al.*, 1994) and 32-70% of small cell lung cancers (SCLC)(Miller *et al.*, 1992; Lohmann *et al.*, 1993; Ryberg *et al.*, 1994), demonstrating its importance in the pathogenesis of these malignancies. Determination of the functional status of p53 in cell lines, normal tissues, and in tumors is most often inferred by sequence analysis of p53 coding regions, indirect methods of revealing the genotype, or by the detection of p53 protein in the nuclei of tumor cells by standard immunohistochemical techniques (Soussi *et al.*, 1994). Quantitative immunoassays for p53 protein, however, may offer a number of advantages (Diamandis and Levesque, 1995) and have been applied to soluble cell extracts prepared from breast (Bartkova *et al.*, 1993; Vojtesek *et al.*, 1993; Levesque *et al.*, 1995b), ovarian (Levesque *et al.*, 1995c), gastrointestinal (Bartek *et al.*, 1991; Bartkova *et al.*, 1993; Joypaul *et al.*, 1993), vulval (Bartkova *et al.*, 1993), muscle (Bartek *et al.*, 1991), and more recently, in lung tumors (Pappot *et al.*, 1996). For tumors of three anatomic sites, breast (Vojtesek *et al.*, 1993), stomach (Joypaul *et al.*, 1993) and colon (Joypaul *et al.*, 1993), parallel determinations of p53 protein expression status by IHC and immunoassay have revealed that these methods yield concordent findings.

Procedures less invasive than thoracotomy to remove structures affected by lung cancer have also been shown to directly or indirectly indicate p53 mutational events. SSCP analysis of p53 exons 5 to 8 (Mitsudomi *et al.*, 1993a), where the majority of mutations occur in lung and other cancers (Hollstein *et al.*, 1991; Levine *et al.*, 1991), and immunostaining for p53 protein (Bennettt *et al.*, 1993; Walker *et al.*, 1994), in bronchial biopsy specimens have demonstrated the potential for the early diagnosis of lung cancer. In addition, sputum specimens not cytologically diagnostic for cancer from patients who later developed adenocarcinoma have been shown to contain the same mutations identified in the primary lung lesion up to a year prior to clinical diagnosis (Mao *et al.*, 1994). Circulating antibodies recognizing p53 protein have been detected in the sera of a proportion of both SCLC and NSCLC patients by immunoblotting (Winter *et al.*, 1992; Schlichtholz *et al.*, 1994) and by enzyme immunoassay (Angelopoulou *et al.*, 1994; Lubin *et al.*, 1995). Patients expressing these antibodies have been shown to possess tumors harbouring p53 mutations and to be highly positive for p53 protein (Winter *et al.*, 1992). Because the appearance of anti-p53 antibodies is likely an early

event in lung cancer (Lubin *et al.*, 1995), and the antibody titre may reflect the clinical course of the disease (Angelopoulou *et al.*, 1994; Lubin *et al.*, 1995), measurement of serum antibodies against p53 has been proposed for both diagnosis and monitoring of lung cancer.

Attempts to detect in serum the p53 antigen which elicited this antibody response were initially unsuccessful (Winter *et al.*, 1992; Hassapoglidou *et al.*, 1993). A number of subsequent studies, however, have reported the presence of p53 protein in serum. Over 30% of sera from patients with malignant lymphomas were found to be positive for p53 protein in two Finnish studies correlating serum levels of p53 with either thymidine kinase (Lehtinen *et al.*, 1993) or antibodies directed against the adenovirus 12 E1b protein (Lahdeaho *et al.*, 1994). A 64% p53-positivity rate in the sera of Hodgkin's lymphoma patients was also reported (Trumper *et al.*, 1994). In another study, pre- and postoperative serum specimens collected from 60 cases of primary breast carcinoma and assayed for p53 protein were found to exceed 1 μg/L in 5 cases but did not correlate with immunohistochemical p53-positivity in the matched breast tumors (Rosanelli *et al.*, 1993). Two groups have provided evidence that serum p53 protein may be measurable in patients with neoplasms of the colon. One of these (Greco *et al.*, 1994) has shown statistically significant differences in median serum p53 values between patients with colon adenoma (0.06 μg/L) and adenocarcinoma (0.10 μg/L), although, again, associations between serum p53 and tumor stage, grade, or site could not be demonstrated. Another group (Luo *et al.*, 1995) additionally collected normal plasma controls, which were shown to differ with respect to p53 concentrations from sera obtained from patients with colon adenoma (mean=0.44 μg/L), with a 20% p53-positivity rate, and from patients with colon carcinoma (mean=0.55 μg/L), 32% of which were positive for p53 protein. These same authors have also examined serum levels of p53 protein in 23 cases of lung cancer, an equal number of matched hospital controls, 58 members of the general population, and 4 people with non-malignant lung disease (Luo *et al.*, 1994). The mean p53 level in lung cancer patients (0.55 μg/L) was in fact higher than those of non-malignant lung disease (0.42 μg/L) or of the other control subjects (approximately 0.32 μg/L) for both groups), but these differences did not achieve statistical significance. Even more recent are reports of p53 protein presence in the sera

of patients with asbestosis, some of whom also have lung cancer. While one group (Partanen *et al.*, 1995) was unable to find significant differences in mean serum p53 protein levels between asbestosis patients with (0.33 μg/L) or without (0.29 μg/L) cancer and control subjects (0.61 μg/L), another group (Braun *et al.*, 1995) revealed that uranium miners with lung cancer had higher serum concentrations of p53 (median=0.23 μg/L) than those of control subjects including smokers with lung cancer (median=0.06 μg/L) and individuals without malignancy (median=0.03 μg/L), possibly signaling exposure to radioactive radon. Unlike all of the above investigations which utilized commercial p53 ELISA methods was a study reporting levels of p53 protein in colon cancer patient sera up to 10^6 μg/L, determined by a chromatographic procedure to extract tumor-associated antigens from sera (Zusman *et al.*, 1995). This latter finding deviates tremendously from data obtained by ELISAs and should be interpreted with caution.

In this study we have used a new immunoassay (Levesque *et al.*, 1995b) to measure p53 protein in both tumor tissue and sera. Compared to our original assay configuration, the new assay incorporated modifications, including the use of microtitre plates coated directly with an anti-p53 monoclonal antibody, a detergent and mouse-serum containing sample diluent, and a labelled secondary antibody diluent containing goat serum, which greatly reduced the nonspecific background signals arising in many serum specimens from hospitalized patients without cancer. Freedom from such interference, the high recovery (range 72 to 131%, mean 90%) of p53 protein from serum, and the generation of p53 results concordant with the original method when applied to extracts of non-diseased and malignant breast tissues all indicated that the new immunoassay was equally suited for the analysis of p53 protein in both tissue extracts and serum specimens.

To our knowledge, only one other group (Pappot *et al.*, 1996) has used a quantitative ELISA-type method to detect p53 protein in malignant lung tissue, rather than the more widely-used p53 immunostaining techniques. However, ours is the first report of the use of an immunoassay for the concomitant measurement of p53 protein in both tumors and matched serum specimens. To classify tumor specimens as either p53-negative or p53-positive, we used the median p53 concentration of 0.13 μg/g rather than the preferred Receiver Operator Characteristic (ROC) analysis (Zweig and Campbell,

1993) since a reference method for unequivocally establishing p53 status has not been universally accepted. This practice had also been adopted in work recently reported (Pappot *et al.*, 1996), where a median p53 concentration of 0.10 μg/g was found by the immunoassay of 214 NSCLC extracts whose p53 values ranged from 0 to 0.70 μg/g. The use of the median p53 concentration value provides a more objective determination of p53 expression status than the variety of immunohistochemical scoring schemes used in other studies (Caamano *et al.*, 1991; Quinlan *et al.*, 1992; Brambilla *et al.*, 1993).

Given these findings by a number of groups of p53 protein in the sera of patients with lung (Luo *et al.*, 1994; Braun *et al.*, 1995; Patenan *et al.*, 1995) and other (Lehtinen *et al.*, 1993; Greco *et al.*, 1994; Lahdeaho *et al.*, 1994; Luo *et al.*, 1994) malignancies, our failure to detect p53 in the sera from over 100 lung cancer patients was surprising. In those specimens where p53 concentrations were found to exceed the assay detection limit, subsequent reassay following either immunoabsorption by solid phase anti-p53 antibody or addition of Ig to neutralize possible cross-linking heterophilic antibodies, provided strong evidence that even these serum specimens were devoid of p53-specific immunoreactivity. Confidence in our findings rests on several attributes of our study design. Most importantly, for the measurement of p53 protein concentrations we have used a sensitive, well characterized immunoassay (Levesque *et al.*, 1995b) yielding minimal background signals in serum, a matrix recognized as containing numerous interfering substances. We have also used this assay in conjunction with further efforts to show the dependence of the signals in serum on p53 protein. Finally, the collection of serum specimens from 54 lung cancer patients immediately before surgical removal of their tumors, all of which were also characterized for p53 protein expression, afforded us the greatest opportunity of detecting p53 in serum, assuming that the primary tumor was the source of the circulating serum protein. But as our results show, neither p53-positive nor p53-negative tumor tissues were associated with detectable serum p53 protein. Consequently, we could not determine if p53 levels declined following surgery.

The most likely explanations for the discrepancy between our findings and those of all others reporting p53 protein in patient sera invoke potential differences between the immunoassay methods used to quantitate serum p53 protein. The estimated analytical sensitivity of our method, 0.04 μg/L, is superior to that of either the mutant p53 selective

(0.25 μg/L) or pantropic p53 (0.10 μg/L) ELISA assays (Oncogene Science, Uniondale, NY) commonly employed in studies measuring p53 in serum. Therefore, the relatively low p53 protein concentrations reported in these studies would easily have been detected by our immunoassay. Whether p53 protein is complexed by serum components, masking its detection by the combination of polyclonal CM-1 and monoclonal DO-1 immunoreagents used in our assay yet allowing its detection by polyclonal CM-1 and the monoclonal antibodies used in the other assays, cannot be determined at present. It has been shown that p53 autoantibodies in the sera of cancer patients are directed primarily against epitopes in the amino and carboxy terminii of the p53 protein (Schlichtholz *et al.*, 1992; Lubin *et al.*, 1993), suggesting that serum p53 bound to endogenous anti-p53 antibodies might not be detected by the amino-terminal specific DO-1 antibody used in our immunoassay. Assay of sera for p53 autoantibodies was not performed in this study, and therefore this possibility could not be excluded. Moreover, it should be noted that because the parallel assay of serum specimens by our new ELISA and immunoassays used by other investigators has not been performed, previous findings of the presence of p53 in serum cannot be totally discounted as artifactual. The results of our study, however, in addition to biological and technical considerations, are suggestive of previous artifactual findings.

The potential of the measurement of p53 protein levels in serum for the diagnosis and monitoring of a variety of malignant conditions in which p53 is overexpressed is of obvious clinical interest. The success of such an approach might be limited, however, by three major biological considerations: i) not all tumor tissues, even those in which the p53 gene has been mutated, show accumulation of p53 protein (Dowell *et al.*, 1994); ii) in many patients, the generation of autoantibodies against p53 might mask epitopes of the protein necessary for its detection by ELISA-type immunoassays (Schlichtholz *et al.*, 1992; Lubin *et al.*, 1993); and iii), since p53 is a transcription factor thought to function primarily in the nucleus and is not targeted for extracellular release, it likely could enter the extracellular matrix and ultimately the blood circulation only by apoptotic or necrotic cell death within the tumor (Bates and Vousden, 1999).

Investigators concerned with the measurement of p53 protein in biological fluids should also be aware of possible analytical difficulties. p53 immunoassay designs which

use anti-species antibodies (eg. an antiserum raised in a goat host against mouse IgG) to coat the solid phase are not suitable for serum analysis since they are susceptible to nonspecific interference by heterophilic antibodies, which may be present in up to 30% of normal individuals. These assays will therefore yield false positive results in a large proportion of the patient sera tested. In addition, since all current ELISA-type methods for p53 quantification include rabbit polyclonal anti-p53 detection antibodies and subsequently added enzyme-labeled anti-rabbit antibodies, it is imperative that the anti-rabbit antibody used in each assay has been treated, such as by adsorption to human IgG columns, in order to eliminate any cross-reactivity with human IgG present in serum specimens. Otherwise, in our experience, approximately 30% of human sera assayed will be apparently positive for p53 protein. Once "p53-positive" specimens are identified, it is highly recommended to check for the presence of heterophilic antibodies by treating the sera with mouse IgG and then reassaying them (Levinson, 1986). In some cases, immunoglobulin from two or three species may be necessary to eliminate interferences (Thompson *et al.*, 1986). Alternatively, heat denaturation (at 90°C) or pretreatment with polyethylene glycol, sulfhydryl reducing agents, or detergents could be used to destroy the reactivity of these antibodies (Primus *et al.*, 1988) which, in the case of human anti-mouse antibodies (HAMA), can be detected by commercially available methods (HAMA Survey Group, 1992). Since the ability to confirm the molecular weight of the immunoreactive species in serum by Western blotting is impaired by the relatively low sensitivity of this procedure, other methods to demonstrate the dependence of the assay response upon p53 protein, such as the immunoabsorption experiment described here, are required. Further indirect evidence for the existence of p53 protein in the sera of cancer patients may also be provided by the demonstration that p53 levels in the sera are compatible with those found in the tumor tissues and that serum p53 concentrations decline following surgical removal of the tumor. When all of these stringent criteria were applied, none of the serum specimens assayed for p53 protein in this study were found to have measurable p53 protein levels. The presence of detectable p53 protein in the sera of patients with lung cancer is therefore not supported by the results of this study. In light of our finding, we strongly recommend that before it is concluded that p53 protein is present in the sera of patients with lung or other malignancies (and has clinical utility in these

diseases), stringent criteria such as those outlined above should be used to identify p53-specific assay responses. Immunological assays for p53 protein based only on monoclonal antibodies will likely prove more successful for the assay of serum specimens but as yet await development.

CHAPTER VI

ASSESSMENT OF THE PROGNOSTIC VALUE OF p53 PROTEIN IN NON-SMALL CELL LUNG CANCER

(A version from Levesque MA, D'Costa M, Spratt EH, Yaman MM, Diamandis EP. *Int J Cancer (Pred Oncol)* 1998; 79: 494-501, Copyright © 1998, Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc.)

VI. 1. Abstract

Accumulation of mutant p53 protein occurs frequently in human malignancies, including 40 to 60% of NSCLCs. The implications of such p53 overexpression, usually assessed by immunohistochemical techniques, for the prognosis of lung cancer patients remains controversial. In this study, we used our time-resolved immunofluorometric assay to measure p53 protein concentrations in extracts prepared from 86 primary non-small cell lung tumors, and examined the associations between p53 protein levels (corrected for total protein) and other clinicopathologic variables including post-surgical disease-free survival (DFS) and OS. Contingency tables analyzed by Chi-square tests revealed no significant relationships between p53 status, defined by a median cutoff point, and patient gender, age, disease stage, histologic grade and type, lymph node extension, smoking history, and administration of adjuvant chemotherapy or radiation. However, multivariate Cox proportional hazard regression analysis demonstrated a dose-response relationship between p53 concentration, expressed as a four-level, quartile-divided variable, and increased risks for patient relapse ($p=0.010$) and death ($p=0.016$). Patients whose tumors contained p53 concentrations exceeding the median value had over three-fold higher risks of relapse ($p=0.002$) and death ($p=0.007$) than those whose tumors had lower p53 concentrations. We also provided evidence suggesting that the impact of p53 on survival was greater in patients with squamous cell carcinoma than in those with adenocarcinoma. Although the latter finding needs confirmation by further studies, our results suggest that the application of an immunoassay of p53 protein on non-small cell lung tumor extracts may identify patients at increased risk of unfavorable outcome.

VI. 2. Introduction

Lung carcinoma is a leading cause of premature death in most countries, and is one of several malignancies for which the etiologic role of cigarette smoking has been clearly demonstrated. Unlike small cell lung cancer, which is less prevalent and typically disseminated at diagnosis but initially responsive to primary chemotherapy and radiotherapy, NSCLC accounts for approximately 85% of all cases and presents in one third of these as localized disease treated primarily by surgery (Mountain, 1986). The

prognosis of patients with advanced NSCLC is generally poor, and for this reason adjuvant systemic therapy is usually instituted. Among patients with early stage disease, however, 40-50% have also been shown to relapse within 5 years after undergoing potentially curative resection (Mountain, 1986). Additional prognostic markers would be useful, particularly to identify those patients managed by surgery alone who are at increased risk of early relapse or death and for whom adjuvant therapy may be beneficial.

Mutation of the p53 gene has been shown to occur at very high frequencies in the majority of human cancers, including 40-60% of patients with NSCLC (Chiba *et al.*, 1990; Kishimoto *et al.*, 1992). These mutations, which are predominantly missense and accompanied by nuclear accumulation of conformationally-altered mutant p53 protein, have been detected in preneoplastic lesions of the lung (Sozzi *et al.*, 1992; Bennett *et al.*, 1993), in carcinomas *in-situ* (Bennett *et al.*, 1993), and in both primary tumors and metastases from individuals with extensive disease (Marchetti *et al.*, 1993; Fontanini *et al.*, 1994) – suggesting that p53 alteration may be an early event in NSCLC progression. Moreover, the high percentage of G to T transversion mutations of p53 in lung tumors have been linked to exposure to carcinogens present in cigarette smoke (Suzuki *et al.*, 1992). The cellular consequence of p53 inactivation is thought to be the impairment of a pathway whereby cells harboring damaged DNA are growth arrested (Kastan *et al.*, 1992), or deleted by programmed cell death (Lowe *et al.*, 1993). A possible manifestation of this impairment in NSCLC may be resistance to chemotherapeutic agents, the efficacies of which are based on their abilities to induce p53-dependent apoptosis (Rusch *et al.*, 1995). However, whether p53 mutations predict shortened post-operative survival in these patients more accurately than the existing prognostic factors based on tumor morphologic characteristics remains controversial, despite having been a subject of investigation for many years (McLaren *et al.*, 1992; Quinlan *et al.*, 1992; Mitsudomi *et al.*, 1993b; Lee *et al.*, 1995; Passlick *et al.*, 1995; Nishio *et al.*, 1996).

Until quite recently, most studies of the prognostic implications of p53 in NSCLC have focused on the detection of p53 protein accumulation in tumor tissue rather than on the direct analysis of the p53 gene for structural alterations. Although the simple immunohistochemical techniques commonly employed for this purpose have demonstrated good concordance with the results of DNA sequencing (Top *et al.*, 1995),

the different antibodies, specimen processsing details, and scoring criteria used for immunostaining in these studies have likely contributed to their discordant conclusions. ELISAs of p53 protein have also been described (Vojt©ek *et al.*, 1992; Hassapoglidou *et al.*, 1993; Levesque *et al.*, 1995; Thomas *et al.*, 1997) and may be advantageous in terms of sensitivity and specificity for the quantitative assessment of p53 protein in extracts of tumor tissues. With one recent exception (Pappot *et al.*, 1996), these immunoassays have generally not been used to study the relationship between p53 protein accumulation status and the duration of survival in patients with NSCLC. Another such method, developed in our laboratory, has been used to demonstrate unfavorable prognosis associated with p53 protein overexpression in patients with breast carcinomas (Levesque *et al.*, 1998), and has been shown to yield p53 protein concentrations in extracts of lung tumors in agreement with the findings of immunostaining of the same tissues, performed in parallel (Levesque *et al.*, 1997). Since follow-up information, including that related to survival, was not initially available in the latter study, the prognostic value of ELISA-detected p53 protein in lung cancer could not be evaluated. The purpose of the present study was therefore to determine the relationships between p53 protein concentrations and the risks of relapse and death in a subset of patients who were prospectively monitored for a median of 16 months and whose lung cancers were restricted to NSCLC histologic types.

VI. 3. Materials and Methods

VI. 3. 1. Lung Cancer Patients

In this study, we investigated a cohort of 86 patients operated at St. Joseph's Health Centre, Toronto, Ontario, Canada from July 1993 to March 1995 for treatment of primary non-small cell carcinoma of the lung. The ethics and research committee at this institution had given this study its approval. Excluded from the consecutive series were patients with multiple synchronous lung tumors or other primary malignancies metastatic to the lung, as well as patients whose lung tumors were of a non-invasive or small cell histologic type, were judged to be non-resectable, were of insufficient quantity for p53 protein analysis, or for whom follow-up information was unavailable. Surgery followed diagnosis of lung cancer by an average of nine days and consisted of lobectomy (n=46),

pneumonectomy (n=21), wedge resection (n=12), lobectomy combined with wedge resection (n=5), or biopsy obtained at bronchoscopy (n=2). Ages of these patients, 58 of whom were male and 28 of whom were female, ranged from 42 to 87 years; the median age was 67 years. Among the 76 patients for whom history of tobacco use was known, the majority (n=68) recounted smoking on average the equivalent of one package of cigarettes per day for 45 years (range=10-100 pack-years) while 8 patients remained non-smokers. Other clinicopathologic features for which the majority of tumors had been characterized at the time of surgery included the pathologic stage classification according to the TNM scheme (Beahrs *et al.*, 1992), and the histologic grade and type based on WHO criteria (World Health Organization, 1982). Fifty-two patients (61%) were in stage I disease, sixteen (19%) were in stage II, thirteen (15%) were in stage IIIA, one (1%) was in stage IIIB, and three (4%) patients had stage IV lung cancer. Of the three patients with stage IV disease, metastases to the bone and liver were identified in two cases and one case, respectively. Well differentiated (G1) tumors comprised 7% (n=6) of the specimens for which grade was known, while 62% (n=51) were moderately differentiated (G2) and 30% (n=25) were poorly differentiated (G3). Undifferentiated (G4) tumors were not present in our series. Tumor stage and grade could not be assessed for one, and four patients, respectively. Most specimens were revealed to be either adenocarcinomas (n=43, 50%) or squamous cell carcinomas (n=37, 43%); the remainder consisted of large cell carcinomas (n=5, 6%) and an adenosquamous carcinoma (n=1, 1%). Regional lymph node metastases were identified in 32 of the patients.

For all patients, follow-up was performed by review of medical records at St. Joseph's Health Centre. Extended follow-up was also obtained by a questionnaire sent to other institutions and private practitioners throughout southern Ontario involved in the patients' post-operative care. Informative responses were obtained for 56% of the questionnaires mailed. Forty patients (46%) underwent lung cancer relapse and twenty-two (26%) died of their disease during their respective follow-up periods, which ranged from 1 to 44 months (median=16 months). DFS time, defined as the interval between the dates of tumor resection and the first evidence of recurrent or metastatic disease, ranged from 0 months (stage IV) to 30 months (median=9 months). The corresponding period from surgery to patient death – the OS time – was distributed from 1 to 40 months and

had a median of 11 months. In the DFS analysis, deaths without evidence of recurrence were considered censored observations, as were deaths due to causes other than lung cancer in the OS analysis (see below). Of the 61 patients remaining alive at last follow up, 18 had relapsed and 43 were in remission. Prior to surgery, all patients had been untreated for lung cancer. Post-operatively, 13 patients received radiotherapy, 18 were administered systemic chemotherapy, and 2 patients received both treatment modalities.

VI. 3. 2. Tissue Extraction and Immunofluorometric Assay of p53 Protein

Immediately following surgical excision, lung tumor tissues were snap-frozen and examined histologically, by which representative portions were selected for subsequent analysis. The percentages of tumor cells in these specimens were judged to be low (1-33%), medium (34-66%), or high (67-100%) in 7, 34, and 45 cases, respectively. After storage at -80°C for no more than two months, approximately 200 mg of these tissues were pulverized to a fine powder on dry ice and extracted as described above (Levesque *et al.*, 1997). The crude cell lysates were assayed directly for p53 protein using immunofluorometry and for total protein content using a kit based on the BCA method (Pierce, Rockford, IL). A "sandwich-type" immunoassay of p53 protein, described in detail previously (Levesque *et al.*, 1995b), was used to measure the p53 protein concentrations in the lung tumor extracts.

VI. 3. 3. Statistical Analysis

All statistical procedures, performed by SAS for Windows version 6.12 software (SAS Institute, Cary, NC), were non-parametric and based on two-sided tests of significance. Relationships between p53 protein concentrations and other continuous variables such as age, number of pack-years smoked, and the numbers of months until recurrence, death, and last follow-up, were examined by calculation of Spearman correlation coefficients (r_S). These associations, as well as those between p53 and several categorical variables, were also investigated by Chi-square tests applied to contingency tables. In these tables, the distributions of p53-negative and p53-positive tumors (using a cutoff point equal to the median p53 concentration, 0.14 μg/g) were compared between patients differing with respect to age (less than 66 years vs. greater than or equal to 66

years), pathologic stage (I-II vs. III-IV), histologic grade (G1-G2 vs. G3) and type (adenocarcinoma vs. squamous cell carcinoma vs. other histotypes), lymph node involvement (absent vs. present), smoking history (no vs. yes), post-operative treatment (not treated vs. treated with chemotherapy and/or radiotherapy), and whether each of the clinical endpoints were reached during their respective follow-up periods (non-relapsed vs. relapsed, and alive vs. died of lung cancer). The prognostic roles of p53, alone and in conjunction with other clinico-pathologic factors, in determining DFS and OS were evaluated by the hazards ratios (relative risks (RR) for relapse or death) and their 95% confidence intervals (CI), which were calculated from fitted Cox proportional hazards regression models. In the multivariate analysis, the regression models were adjusted for age, stage, grade, node status, and smoking history classified by the criteria given above. p53 protein concentration was tested in the models separately as a continuous variable, as a quartile-divided, four-level ordinal variable, and as a dichotomous variable categorized by the median percentile cutoff point. Univariate and multivariate regression models were also used to evaluate the association between p53 protein concentrations exceeding the median value and post-surgical survival in the subgroups of patients diagnosed with adenocarcinoma, squamous cell carcinoma, early stage (I-II) disease, and in patients with late stage (III-IV) lung cancer. p53-by-histotype and p53-by-stage interactions in the univariate Cox models were evaluated separately in order to determine if the effects of p53 on outcome were mediated by either stage or histotype. Each of the other dichotomous variables, including age, smoking history, grade, stage, and node status were similiarly tested for their impact on prognosis by Cox regression analysis. The median p53 cutoff was also used in survival curves, examining the relapse or death rates of the p53-negative and p53-positive patients, which were plotted by the method of Kaplan and Meier and compared using log-rank statistics.

VI. 4. Results

VI. 4. 1. Relationships Between p53 and Other Clinicopathologic Variables

Immunoassay of the 86 non-small cell lung tumor extracts revealed widely distributed p53 protein concentrations (mean=6.28 μg/L, SD=12.99 μg/L, median=0.56 μg/L, minimum=0.04 μg/L, maximum=70.69 μg/L), all of which exceeded the assay

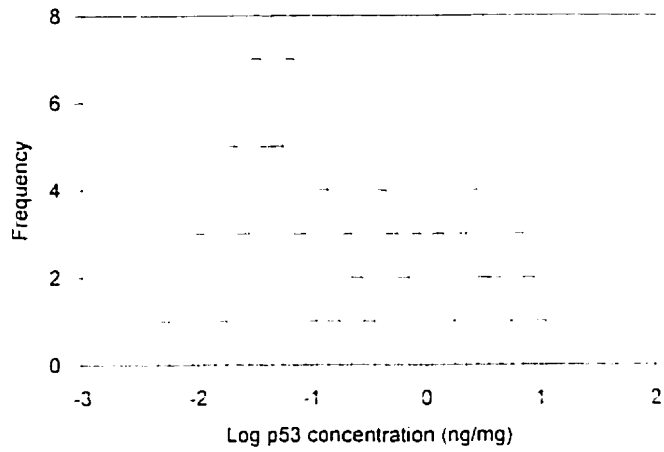


Figure VI. 1. Frequency Distribution of p53 Protein Concentrations in Lung Tumor Extracts
From left to right, dashed lines indicate the 25th, 50th, and 75th percentiles of the distribution.

detection limit. The adjustment of these results for the total amount of protein extracted in each case led to a similiarly positively-skewed, somewhat bimodal distribution (Figure VI. 1.) which had a mean of 1.22 μg/g, a SD of 2.24 μg/g, and ranged from 0.005 μg/g to 10.97 μg/g. The 25th percentile, median, and 75th percentile of this distribution were 0.044 μg/g, 0.14 μg/g, and 1.29 μg/g, respectively. Protein-adjusted p53 concentrations were found not to be significantly correlated to patient age (r_s=0.14, p=0.198) or to the number of pack-years of cigarette smoking (r_s=0.14, p=0.229), nor were they significantly associated with these variables and others, including patient gender, disease stage, histologic grade, histologic type, lymph node metastasis, and whether or not adjuvant therapy was given, in contingency tables in which p53-positivity status was based on a median cutoff point (Table VI. 1.). The same analysis performed using the 25th and 75th percentiles of the p53 protein distribution as cutoff points, as well as Mann-Whitney and Kruskal-Wallis tests, also did not yield statistically significant associations (data not shown), perhaps as a consequence of the small size of the study population.

VI. 4. 2. Relationships Between p53 and Patient Survival

The relationships between p53 protein concentrations and patient DFS and OS were initially examined without considering the variable follow-up times, with which p53 was negatively correlated (r_s=-0.22, p=0.039). Levels of p53 protein were thus also found to be negatively correlated with the lengths of the time intervals from surgery to first recurrence (r_s=-0.36, p=0.023), but not with those leading to patient death (r_s=-0.11, p=0.586). A stronger association between p53 protein status, defined by a median cutoff point, and patient relapse was also suggested in contingency tables (Table VI. 1.),

Table VI. 1. Associations Between p53 Status[a] and Other Clinicopathologic Variables

Variable	p53 < Median Patients (%)	p53 ≥ Median Patients (%)	p-Value[b]
Sex			
Female	11 (39)	17 (61)	
Male	32 (55)	26 (45)	0.17
Age (years)			
< 66	23 (60)	15 (40)	
≥ 66	20 (42)	28 (58)	0.08
Stage[c]			
I-II	35 (51)	33 (49)	
III-IV	8 (47)	9 (53)	0.75
Histologic Grade[d]			
G1-G2	31 (54)	26 (46)	
G3	11 (44)	14 (56)	0.39
Histologic Type[e]			
Adenocarcinoma	19 (44)	24 (56)	
Squamous Cell Carcinoma	20 (54)	17 (46)	
Others	4 (67)	2 (33)	0.47
Lymph Node Metastasis			
No	26 (48)	28 (52)	
Yes	17 (53)	15 (47)	0.66
Smoking History[f]			
No	6 (75)	2 (25)	
Yes	34 (50)	34 (50)	0.18
Postoperative Treatment[g]			
Not Treated	28 (53)	25 (47)	
Treated	15 (45)	18 (55)	0.51
Recurrence			
No	27 (59)	19 (41)	
Yes	16 (40)	24 (60)	0.08
Death			
No	35 (55)	29 (45)	
Yes	8 (36)	14 (64)	0.14

[a] p53 expression status based on median cutoff level of 0.14 μg/g.
[b] p-values calculated from χ^2 tests.
[c] Pathologic stage unknown for 1 patient.
[d] Histologic grade unknown for 4 patients.
[e] Other tumors were of large cell and adenosquamous histotypes.
[f] Smoking history unknown for 10 patients.
[g] Included treatment with chemotherapy, radiotherapy or both.

although neither the tendency of patients who relapsed, nor of those who subsequently died, to have had p53-positive tumors reached statistical significance. Selection of either 25th or 75th percentiles as cutoff points in the contingency tables similiarly did not lead to

Table VI. 2. Associations Between p53 Status and Disease-Free and Overall Survival

p53 Status	Disease-Free Survival			Overall Survival		
	RR[a]	95% CI[b]	p-Value	RR[a]	95% CI[b]	p-Value
Univariate Analysis (n=86)						
Based on Quartiles[c]						
1st Quartile	1.00			1.00		
2nd Quartile	0.93	0.35 – 2.48		0.83	0.20 – 3.37	
3rd Quartile	2.05	0.83 – 5.06		1.55	0.41 – 5.76	
4th Quartile	2.09	0.85 – 5.19		3.31	1.00 – 10.97	
p-Value for Trend			0.025			0.021
Based on Median Value						
Negative	1.00			1.00		
Positive	2.19	1.15 – 4.15	0.016	2.47	1.03 – 5.94	0.043
Multivariate Analysis (n=75)[d]						
Based on Quartiles[c]						
1st Quartile	1.00			1.00		
2nd Quartile	1.27	0.47 – 3.48		0.97	0.21 – 4.45	
3rd Quartile	3.74	1.25 – 11.17		2.16	0.51 – 9.18	
4th Quartile	4.00	1.18 – 13.53		17.16	2.12 – 139.14	
p-Value for Trend			0.010			0.016
Based on Median Value						
Negative	1.00			1.00		
Positive	3.02	1.50 – 6.06	0.002	3.72	1.44 – 9.57	0.007

[a] RR is hazard ratio estimated by the Cox proportional hazards regression model.
[b] CI is the confidence interval of the estimated RR.
[c] Estimated HR for second, third and fourth quartiles relative to first quartile are given.
[d] Included in the multivariate models were patient age, stage, histologic grade, lymph node status, and smoking history.

significant p-values (data not shown). Univariate Cox regression analysis, however, in which p53 concentrations were used as a four-level categorical variable or a dichotomous variable revealed that increasing amounts of p53 protein in the lung tumor extracts were associated with progressively reduced disease-free and overall survival probabilities (Table VI. 2.). Although the use of p53 as a continuous variable in the regression analysis showed trends suggesting a dose-response relationship between p53 and risks for relapse (RR=1.01, p=0.097) and death (RR=1.01, p=0.050), these relationships were also evident from the analysis presented in the table in which patients were divided into four groups by the three quartiles of the frequency distribution of p53 protein concentrations. The simpler division of patients into p53-negative and p53-positive groups of equal size by merging the two lower and two upper quartile-based groups demonstrated over two-fold

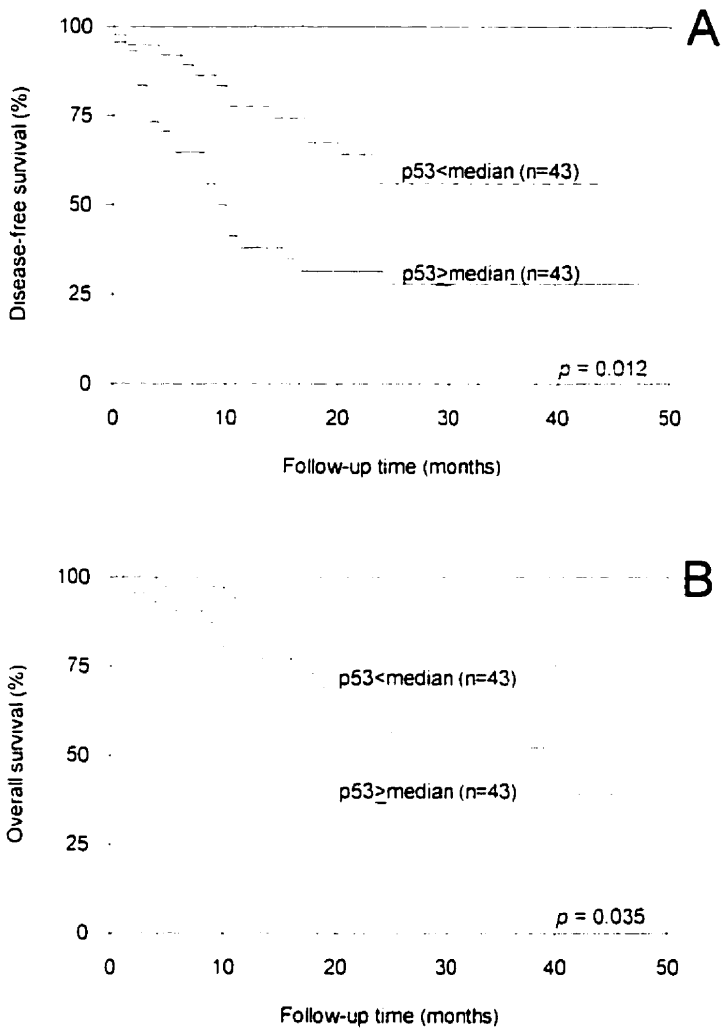


Figure VI. 2. Kaplan-Meier Analysis of Disease-Free and Overall Survival

A. DFS and B. OS of the 86 NSCLC patients. The median p53 concentration was used as the cutoff point for p53-positivity. p-values were calculated by log-rank tests.

increased risks of relapse and death in p53-positive compared to p53-negative patients. These differences in the survival rates between the median-dichotomized p53-negative and p53-positive patients were also shown by Kaplan-Meier survival plots (Figure VI. 2.). Adjustment for the effects of age, stage, grade, node status, and smoking history in multivariate Cox models of DFS and OS revealed that p53 protein concentrations elevated above the median indicated significantly reduced survival independently of these other factors (Table VI. 2.). Inclusion of treatment in the same multivariate models resulted in p53 losing its significance for OS (RR=2.33, p=0.12) but not for DFS (RR=2.80, p=0.009). The magnitudes of the increased risks of relapse or death conferred by p53-positivity in multivariate analysis were comparable to those associated with stage III-IV disease, but of less statistical significance than the risks associated with lymph node metastases (Table VI. 3.). In contrast, patient age, smoking history, and histologic grade dichotomized as above were of no prognostic value in our series of 86 patients.

Table VI. 3. Associations Between Status of Other Clinicopathologic Variables and Patient Survival

Variable Status	Disease-Free Survival			Overall Survival		
	RR[a]	95% CI[b]	p-Value	RR[a]	95% CI[b]	p-Value
Univariate Analysis (n=86)						
Age (years)						
≥66 vs. <66	1.17	0.63 – 2.19	0.61	0.79	0.34 – 1.86	0.59
Smoking History[c]						
Yes vs. No	0.81	0.32 – 2.07	0.65	0.67	0.19 – 2.33	0.53
Histologic Grade[d]						
G3 vs. G1-G2	1.15	0.57 – 2.32	0.69	1.40	0.57 – 3.45	0.46
Stage[e]						
III-IV vs. I-II	2.63	1.29 – 5.39	0.008	3.80	1.56 – 9.26	0.003
Lymph Node Status						
Positive vs. Negative	3.10	1.65 – 5.83	<0.001	6.63	2.55 – 17.22	<0.001
Multivariate Analysis (n=75)						
Stage[e,f]						
III-IV vs. I-II	3.18	1.41 – 7.17	0.005	4.45	1.55 – 12.81	0.006
Lymph Node Status[f]						
Positive vs. Negative	4.32	2.11 – 8.83	<0.001	10.68	3.50 – 32.59	<0.001

[a] RR is hazard ratio estimated by the Cox proportional hazards regression model.
[b] CI is the confidence interval of the estimated RR.
[c] Smoking history unknown for 10 patients.
[d] Histologic grade unknown for 4 patients.
[e] Pathologic stage unknown for 1 patient.
[f] Multivariate models were adjusted for age, smoking history, and p53 status.

VI. 4. 3. p53 and Prognosis in Patient Subgroups

Since the relationships demonstrated between p53-positivity and shortened DFS and OS may have been restricted to patients whose tumors differed with respect to histologic type or anatomic extent, the risks of each outcome associated with p53-positivity were examined separately in subgroups defined by histotype and stage (Table VI. 4.). For patients with adenocarcinoma of the lung, the increased hazards of relapse and death estimated from the models for individuals with p53-positive tumor extracts were not significantly different from p53-negative patients. However, the more than four-fold elevated risks of both endpoints for patients with p53-positive squamous cell carcinomas were of greater statistical significance. Although p53-positivity did confer

increased likelihoods of relapse and death for both early stage (I-II) and late stage (III-IV) patients, the results were of greater statistical significance in early stage lung cancer. In the more homogeneous subgroup of patients with stage I disease (n=52), other trends similiarly suggested that p53-positivity was associated with increased risks of relapse (RR=2.21, p=0.12) and death (RR=4.33, p=0.19). In patients with squamous cell carcinoma or with early stage disease (Table VI. 4.), but not in those with adenocarcinoma or in stages III or IV (data not shown), p53-positivity remained an indicator of unfavorable prognosis in multivariate regression models. These models, which adjusted the relationships between p53 and survival outcomes for the influences of age, stage, and lymph node status, revealed that patients with p53-positive squamous cell

Table VI. 4. Associations Between p53 Status[a] and Survival in Patient Subgroups

p53 Status	Disease-Free Survival			Overall Survival		
	RR[b]	95% CI[c]	p-Value	RR[b]	95% CI[c]	p-Value
Univariate Analysis						
Adenocarcinoma Patients (n=43)						
Positive vs. Negative	1.62	0.68 – 3.88	0.280	2.07	0.69 – 6.21	0.20
Squamous Cell Carcinoma Patients (n=37)						
Positive vs. Negative	4.41	1.40 – 13.88	0.011	4.64	0.91 – 23.73	0.066
Patients in Stages I or II (n=68)						
Positive vs. Negative	3.02	1.36 – 6.72	0.007	3.05	0.93 – 10.02	0.066
Patients in Stages III or IV (n=17)						
Positive vs. Negative	2.97	0.69 – 12.77	0.14	4.42	0.81 – 23.97	0.085
Multivariate Analysis						
Squamous Cell Carcinoma Patients (n=37)[d]						
Positive vs. Negative	11.68	2.89 – 47.20	<0.001	35.69	2.85 – 447.86	0.006
Patients in Stages I or II (n=68)[e]						
Positive vs. Negative	4.82	1.98 – 11.76	<0.001	5.11	1.43 – 18.21	0.012

[a] p53 expression status based on median cutoff level of 0.14 μg/g.
[b] RR is hazard ratio estimated by the Cox proportional hazards regression model.
[c] CI is the confidence interval of the estimated RR.
[d] Multivariate models were adjusted for patient age, stage, and lymph node status.
[e] Multivariate models were adjusted for patient age and lymph node status.

tumors had an 11-fold increased risk for relapse and a 35-fold increased risk for death compared to p53-negative patients with the same histologic type. The greater effect of p53 upon survival in squamous cell carcinoma patients compared to patients with adenocarcinoma could not likely be explained by differences in power, since there were no marked differences in the numbers and proportions of patients relapsing or dying between the two subgroups: 21 and 14 out of 43 adenocarcinoma patients relapsed and died, respectively, whereas 16 and 8 out of 37 squamous cell carcinoma patients relapsed and died, respectively. However, the results of formal tests of interaction did not support a difference in the prognostic value of p53 between the two histologic classifications, since the p53-by-histotype interaction term was not statistically significant for either relapse (p=0.23) or death (p=0.83). p53-by-stage interaction was similiarly not significant in univariate Cox models.

VI. 5. Discussion

Point mutations and allelic loss of the p53 gene are among the most common genetic lesions described in NSCLC, exceeded only by deletions on chromosome 3p (Brauch *et al.*, 1987). Abundant evidence has suggested that this relatively high mutation rate results from the selective growth of tumor cell clones in which p53 has been functionally inactivated (Sozzi *et al.*, 1995). Since p53 mutation appears to be an early, although not necessarily an initiating or even a required event in lung tumorigensis (Sozzi *et al.*, 1992; Bennett *et al.*, 1993; Fontanini *et al.*, 1994), it may lead to a tumor phenotype which is unrelated to the local or systemic spread of the disease, represented by the stage classification. Pathologic stage is currently the major determinant for the receipt of adjuvant chemotherapy or radiotherapy; patients in early stage disease are usually spared such treatment. The need for supplementary prognostic factors in NSCLC arises from the large proportion of patients with early stage tumors, especially those without regional lymph node involvement, who nevertheless relapse and for whom aggressive adjuvant treatment may be warranted (Mountain, 1986). While a number of other morphologic and molecular features of NSCLC have been proposed to provide accurate prognostic information on which to base treatment decisions, none has emerged as unequivocally more valuable than stage alone. With regard to p53, some studies have

shown mutation of the p53 gene (Mitsudomi *et al.*, 1993) or overexpression of p53 protein (Quinlan *et al.*, 1992) to be indicative of shorter survival of NSCLC patients, but others have been unable to demonstrate these associations (McLaren *et al.*, 1992; Lee *at al.*, 1995; Passlick *et al.*, 1995; Nishio *et al.*, 1996). Differences in statistical power resulting from variable population sizes, as well as inherent differences in the populations themselves, may certainly have underlied many of these discordant findings. However, also contributing to the lack of consensus among these studies may have been the differing sensitivities and specificities of the methods used to ascertain p53 mutational status.

The immunohistochemical detection of p53 protein accumulation in the nuclei of tumor cells, including those from NSCLCs, has generally been accepted as evidence of p53 gene mutation. It has been estimated, however, that approximately 10-20% of p53 point mutations occur outside the most frequently mutated exons 5 to 8 and are not accompanied by p53 protein overexpression (Casey *et al.*, 1996). Nonsense mutations, as well as nucleotide deletions and insertions, similiarly are not associated with stabilization of p53 protein, which may also occur by non-mutational mechanisms. Given the imperfect concordance between genetic alterations of p53 and accumulation of its protein product, and the relative insensitivity of indirect techniques of screening for p53 mutations such as SSCP analysis, it has been suggested that complete sequencing of all 11 exons is required to detect all p53 sequence changes (Casey *et al.*, 1996). *In-vitro* functional assays of p53 cloned from tumor cells to assess the ability of p53 mutants to regulate gene transcription in a sequence specific manner, though biologically relevant, remain inappropriate for prognostic studies involving large numbers of patients. In contrast, immunohistochemical methods of detecting p53 protein have been predominantly used to demonstrate associations between p53 abnormalities and survival of cancer patients. Compared to conventional immunostaining techniques, however, densitometric image analysis of stained tissues (Charpin *et al.*, 1996) and ELISA-type assays of tissue extracts (Vojt © ek *et al.*, 1992; Hassapoglidou *et al.*, 1993; Levesque *et al.*, 1995; Thomas *et al.*, 1997) may offer improved reproducibility. In addition, immunoassays, in their most common configuration, may possess greater specificity due to their use of two p53-directed antibodies. Despite these potential advantages, the use of

ELISAs of p53 protein have been largely restricted to serum analysis (Luo *et al.*, 1994), possibly reflecting the firm establishment of p53-immunostaining as a research tool in many histology laboratories, the inability of ELISAs to localize p53 accumulation in the context of histologic features, and the requirement for fresh-frozen tissue specimens. General concordance between the findings of immunoassay and immunohistochemical analyses of several tissues has been reported (Vojt©ek *et al.*, 1992). In a recent study of 91 lung carcinomas of various histologic types, we found a strong correlation (r_s=0.65, $p<0.001$) between the p53 protein concentrations determined by our immunofluorometric assay in extracts of the fresh-frozen tissues, and the immunohistochemical scores reflecting the proportion of stained malignant cells, intensity of staining, and tumor cellularity of the matched formalin-fixed, paraffin-embedded tissues (Levesque *et al.*, 1997). Since there were cases in that study for which the two methods were in disagreement, we considered it possible that p53 ELISA might yield prognostic information different from that of p53 immunostaining. The present study includes 82 NSCLC specimens assessed previously by the two methods, and utilizes essentially the same median cutoff point for p53-positivity, equal to 0.13 μg/g and 0.14 μg/g in the previous and present studies, respectively. Selection of the median value as the cutoff was arbitrary, but reasonable given the bimodal frequency distribution of p53 protein concentrations (Fig. 1) and consistent with the range of p53-positivity rates (40-80%) reported in the literature (McLaren *et al.*, 1992; Bennett *et al.*, 1993; Fontanini *et al.*, 1994; Harpole *et al.*, 1995; Lee *et al.*, 1995; Passlick *et al.*, 1995; Top *et al.*, 1995; Nishio *et al.*, 1996). Furthermore, we have shown that a small number of histologically normal lung tissues displayed p53 contents no greater than 0.07 μg/g (Levesque *et al.*, 1997).

Using a commercially-available pantropic p53 ELISA kit (Oncogene Research, Cambridge, MA), Pappot *et al.* (1996) did not find a statistically significant difference in OS of 228 NSCLC patients whose tumor extracts had p53 levels below vs. above the median cutoff point. These workers revealed, however, significantly higher p53 protein concentrations in squamous cell carcinomas than in adenocarcinomas, although the prognostic values of p53 within these subgroups were similiar, as well as higher p53 levels in patients who were older and who had large, late stage tumors. Several other studies have also shown a slightly greater percentage of squamous cell carcinomas to

exhibit p53-immunostaining than adenocarcinomas (Mitsudomi *et al.*, 1993b; Lee *et al.*, 1995; Nishio *et al.*, 1996), a relationship not consistent with our finding of a trend suggesting higher p53 levels in adenocarcinomas compared to squamous cell carcinomas. This latter relationship, however, was not statistically significant and should be confirmed in further studies of ELISA-detected p53 protein and NSCLC patient prognosis. Although a few studies have reported p53 accumulation in NSCLC to be unrelated to any other clinicopathologic variable (Chiba *et al.*, 1990), a relationship between p53 abnormalities and lifetime exposure to cigarette smoke has been a finding shared by a large number of studies (Suzuki *et al.*, 1992; Nishio *et al.*, 1996). In survival analysis, levels of p53 protein exceeding the median of our series of 86 extracts of NSCLC tissues were also associated with reduced DFS and OS. Moreover, we presented evidence indicating that progressively increasing p53 concentrations were associated with correspondingly increasing risks for relapse or death. That our findings differed from those reported of the only other study of the prognostic utility of ELISA-detected p53 protein in NSCLC (Pappot *et al.*, 1996) was surprising, since the median p53 concentration (0.10 μg/g) used in the latter study was comparable to our median value, the median ages and proportions of patients in the various stages and histologic types were similiar, although the durations of follow-up for the larger number of patients in that study were apparently longer. Other factors possibly accounting for the different conclusions of the two studies might also have been differences in post-operative treatment or other undefined demographic or clinical differences between the patient populations which might have modified the impact of p53 on survival. Since our series of NSCLC patients was not adequately characterized in terms of post-operative chemotherapy and radiotherapy administration, further studies of larger patient samples with detailed adjuvant treatment profiles are needed to determine the prognostic impact of ELISA-determined p53 protein in patients receiving different post-operative treatments.

In multivariate analysis, the increased risks for relapse and death associated with p53-positivity were also shown to be of much greater magnitude in patients with squamous cell carcinomas than in those with adenocarcinomas. Previous studies had also suggested interaction between the histologic type and p53 accumulation status, but in

such a way that it was in adenocarcinoma patients and not in those with squamous cell carcinoma that p53 was shown to be a statistically significant prognostic indicator (Nishio *et al.*, 1996; Fukuyama *et al.*, 1997). Taken together with our finding of a slight trend of greater p53-positivity in adenocarcinomas, the direction of the subgroup differences in the prognostic effect of p53 was quite surprising. Statistically significant interaction between p53 and histotype could not be demonstrated in Cox models of either relapse or death, and it remains possible that differences between patients with which were not included in the multivariate models might have had a confounding influence on the relationships between p53 and survival. For example, differences between subgroups in terms of chemotherapeutic agents received post-operatively could have led to our findings, although, in standard practice, adenocarcinoma and squamous cell carcinoma patients usually receive the same chemotherapy regimens (Ginsberg *et al.*, 1997). Although power calculations were not performed, the similiar numbers of patients and events within the two subgroups did not imply differences in power as an explanation. In contrast to the apparent differences with respect to p53 prognostic value between the two most common NSCLC histologic groups, patients with p53-positive tumors had similiarly elevated risks for both outcome events irrespective of whether they had early (I-II) or late (III-IV) stage disease. Only those in the early stage group were found in multivariate analysis to relapse or die at rates related to their p53 status, but we feel that this finding is likely due more to the small number of patients in the late stage group than to any true interaction between p53 status and stage. When the analysis was restricted to patients in stage I, who constituted 60% of the cohort, the same trends for poor survival associated with p53-positivity were evident. Other workers have also demonstrated p53 alteration to be indicative of poor prognosis in stage I or II NSCLC (Quinlan *et al.*, 1992; Harpole *et al.*, 1995) but not in stages III or IV (Passlick *et al.*, 1995), while Mitsudomi *et al.* (1993b) found p53 to be related to survival only in late stage patients. The lack of consensus evidently points to a need for further investigation of larger, well-characterized patient series assessed for p53 abnormalities by reproducible techniques.

In this study, we have shown that the quantitative assay of p53 protein by a simple ELISA applied to extracts prepared from surgically-resected NSCLCs may identify patients at increased risks of unfavorable outcome. We have also provided evidence that

the utility of p53 quantification may be particularly relevant for the prognosis of patients with squamous cell carcinoma of the lung. Since our data confirm a number of previous reports of the prognostic value of p53 overexpression in this disease, we propose that the detection of p53 protein by ELISA may serve as an alternative to the more commonly employed IHC. Given the small size and heterogeneity of our sample of patients, however, further studies are needed to unequivocally demonstrate the prognostic value of ELISA-detected p53 protein accumulation in patients with NSCLC.

CHAPTER VII

ASSESSMENT OF THE PROGNOSTIC VALUE OF p53 PROTEIN IN BREAST CANCER

(A version from Levesque MA, Yu H, Clark GM, Diamandis EP. *J Clin Oncol* 1998; 16: 2641-2650, with permission from W.B. Saunders Company)

VII. 1. Abstract

This study was designed to evaluate whether patients with unfavorable breast cancer prognosis could be identified by p53 protein overexpression, detected by a quantitative ELISA rather than by conventional IHC. Extracts from 998 breast carcinomas were assayed for p53 protein by an ELISA utilizing both DO-1 monoclonal and CM-1 polyclonal antibodies. Associations between p53 concentrations and patient age, tumor size, S-phase fraction, ER and PR concentrations, DNA ploidy, and lymph node metastases were examined. Hazards for cancer relapse and death after 6 years of follow-up for patients with tumors positive for p53 based on different dichotomization criteria were determined by Cox regression, adjusting for all parameters in multivariate analyses. DFS and OS probabilties of p53-positive and -negative groups, using a median cutoff, were also estimated using the Kaplan-Meier method and the log-rank test. These analyses were performed for all patients and for subgroups defined by ER status, node status, and primary postoperative treatment. Statistically significant associations were found between p53 overexpression and ER and PR negativity ($p<0.01$), aneuploidy ($p<0.01$), and elevated S-phase fraction ($p=0.04$) but not between p53 and tumor size, node status, or patient age. Univariate analysis showed that p53 concentrations exceeding the median indicated significantly increased risks for relapse ($p<0.01$) and death ($p=0.02$). Multivariate analyses confirmed these observations (RR=1.40, $p=0.02$ for DFS and RR=1.50, $p<0.01$ for OS), revealed trends for increasing risks for relapse ($p=0.02$) and death ($p=0.06$) when p53 was considered as a four-level categorical variable, and identified p53-positivity as a significant predictor of outcome in node-positive patients (RR=1.67, $p<0.01$ and RR=2.10, $p<0.01$ for DFS and OS, respectively), ER-positive patients (RR=1.45, $p=0.02$ and RR=1.50, $p=0.01$ for DFS and OS, respectively), and in patients treated with chemotherapy (RR=1.73, $p=0.04$ for relapse and RR=2.04, $p=0.03$ for death). Assessment of p53 overexpression by an ELISA, easily incorporated into the routine biochemical workup of breast tumors, may be an independent predictor of reduced survival of breast cancer patients.

VII. 2. Introduction

Rational treatment decisions for breast cancer patients have traditionally been guided by the presence or absence of axillary lymph node metastases, tumor size, steroid hormone receptor expression, and histologic type. It has become the clinical consensus in recent years that more reliable prediction of breast cancer outcome, and of response to adjuvant therapies, may be facilitated by the integration of the traditional prognostic factors into multiparametric schemes together with additional markers of biological relevance (McGuire, 1991). Although the mutational status of the p53 tumor suppressor gene has been the most extensively studied of these newer markers, conflicting evidence has emerged regarding its ability to identify patients at increased risks of unfavorable outcomes independently of the more established clinicopathologic features of breast cancer (Thor *et al.*, 1992; Friedrichs *et al.*, 1993; Silvestrini *et al.*, 1993; Pietilainen *et al.*, 1995; Rosen *et al.*, 1995; Sjogren *et al.*, 1996).

Mutation of the p53 gene has been reported to occur in 20-50% of sporadic breast carcinomas among women in Western countries (Nigro *et al.*, 1989; Runnebaum *et al.*, 1991) and may occur at higher frequencies or with a different pattern in other populations (Sommer *et al.*, 1992; Shiao *et al.*, 1995). The consequences of these predominantly missense mutations, which are usually accompanied by loss of heterozygosity, is the disruption of the normal, pleiotropic function of the p53 protein. Since p53 is thought to be a critical determinant in the induction of cell cycle arrest (Kastan *et al.*, 1992), programmed cell death (Lowe *et al.*, 1993), and possibly DNA repair (Lee *et al.*, 1995b) in response to cellular stresses which may lead to DNA damage, abrogation of p53 function in breast carcinoma has not surprisingly been associated with genomic instability (Eyfjord *et al.*, 1995), higher proliferative rates (Isola *et al.*, 1992), and resistance to conventional antineoplastic agents whose cytotoxic effects are mediated by p53 (Elledge *et al.*, 1995). A second consequence of p53 mutation is usually, but not always, a conformational change in the expressed protein, resulting in its accumulation in tumor cell nuclei and enabling its detection immunohistochemically (Nigro *et al.*, 1989). Findings that both p53 protein overexpression and mutation of the p53 gene often present contemporaniously with a number of clinical, cytologic, and molecular indicators of aggressive tumor phenotypes (Lipponen *et al.*, 1993; Martinazzi *et al.*, 1993) are

consistent with studies demonstrating that p53 abnormalities are predictive of reduced survival of breast cancer patients (Thor *et al.*, 1992; Friedrichs *et al.*, 1993; Silvestrini *et al.*, 1993; Sjogren *et al.*, 1996). Other studies, however, have not found p53 status to be of independent prognostic value in this disease (Isola *et al.*, 1992; Pietilainen *et al.*, 1995; Rosen *et al.*, 1995). At the heart of such discordant conclusions are likely methodologic differences between studies, particularly with respect to techniques used to determine p53 mutational status. While direct sequencing of all 11 exons of the p53 gene may provide the most accurate assessment (Sjogren *at al.*, 1996), its use, even when confined to screening the most frequently mutated exons 5-8, may be inappropriate in some settings. Far more suitable for routine determination of p53 status are immunochemical methods, which detect p53 protein in tumor tissue and most often take the form of immunohistochemical staining procedures. However, the variable combinations of immunoreagents, specimen processing details, and subjective immunostaining scoring systems reported have fueled disagreement between studies for which immunohistochemical methods were used to detect p53 (Wynford-Thomas, 1992).

The purpose of this study was to evaluate the prognostic value of p53 accumulation in breast carcinoma using ELISA, another immunochemical approach which has been less commonly employed. Quantitative immunoassays of p53 have been applied to soluble extracts derived from various tumor types (Bartkova *et al.*, 1993) and have recently demonstrated utility for breast cancer prognosis by measuring p53 protein in cytosolic extracts prepared for steroid hormone receptor analyses (Borg *et al.*, 1995; de Witte *et al.*, 1996), thereby obviating the need for additional tumor tissue to perform these studies. A larger, North American patient population and a well-characterized immunofluorometric assay distinguished our study, which also related p53 accumulation to survival in patients stratified into groups based on important prognostic categories: ER status, lymph node status, and receipt of adjuvant chemotherapy, endocrine therapy, or no postoperative treatment.

VII. 3. Materials and Methods

VII. 3. 1. Study Population

Tumor specimens from 1000 female patients operated on for primary invasive breast carcinoma were obtained from the Breast Cancer Tissue Resource (BCTR), a collaborative project between the University of Texas Health Science Center at San Antonio, San Antonio, TX and Nichols Institute Research Laboratories (NIRL), San Capistrano, CA. BCTR maintains a large collection of breast tumor specimens at -70°C which had been sent from health care institutions throughout the United States to NIRL for routine quantification of ER and PR and for flow cytometric analyses (see below). Tumor tissue had been frozen in liquid nitrogen or on dry ice immediately after surgery for shipment to NIRL. Criteria for the selection of specimens from the BCTR archive included the availability of at least 0.25 g of tumor tissue for p53 protein analysis and information regarding the status of other clinical and pathologic variables, determined at NIRL (ER and PR concentrations, DNA ploidy, and S-phase fraction). The status of one or more of the demographic or clinical factors (patient age at surgery, number of lymph nodes positive for malignancy, tumor size, and postoperative therapy) provided by the institutions from which the specimens originated was unknown for 124 patients.

The patient cohort was drawn from 165 hospitals which were widely distributed geographically and where surgery had been performed from August 1985 to October 1991. Age at surgery was known for all but one patient and ranged from 22 to 94 years, with a median age of 61 years. Lymph node metastases had been detected in 530 (55%) of the 959 patients for whom the presence or absence of this disease feature had been reported. The mean and median number of affected lymph nodes were 2.3 and 0, respectively, and the number ranged from 0 to 46. Resected tumor size ranged from 1 to 145 mm, with a median size of 23 mm. The percentages of malignant cells in 797 of the tumor specimens were estimated histopathologically by a single pathologist blinded to the results of the p53 protein, steroid hormone receptor, and flow cytometric analyses, and to patient survival status; 4% of the specimens had tumor cellularities from 0-10%, 21% had from 11-30%, 46% had from 31-70%, and 29% had tumor cellularities exceeding 70%. Tumor grade and histologic classification of the specimens were unavailable.

All patients had been previously untreated for breast cancer. Primary surgical therapy consisted of modified radical mastectomy with axillary lymph node dissection (97%), incisional biopsy (2%), or lumpectomy without axillary lymph node dissection (1%). Histopathologic examination of the resected tumor tissues confirmed the diagnosis of primary breast cancer in all cases. Of the 948 patients for whom the modalities of postoperative treatment were reported, 39% received no additional treatment, 9% received locoregional radiotherapy alone, 16% were given only adjuvant chemotherapy, 17% were treated with endocrine therapy alone, 4% received endocrine therapy and radiotherapy, 6% were given both systemic adjuvant therapies, 7% received chemotherapy and radiotherapy, and 2% received all three treatment modalities. Information regarding lymph node status or other disease features of patients receiving each treatment modality was unavailable.

Patient follow-up information, including survival status (alive or deceased) and disease status (disease-free or recurrence/metastasis) at last follow-up, together with the dates and circumstances of relapse and death, if applicable, was available for 997 patients, and was updated annually by the instititions which submitted the specimens to NIRL. Treatment failure, defined the first documented evidence of local recurrence, regional axillary relapse, distant metastasis, new ipsilateral or contralateral breast cancer, as revealed by clinical, radiological or histological evaluations, occured in 213 (21%) of the patients. One hundred and ninety-nine (20%) patients died during their respective follow-up periods. The distribution of follow-up times for patients still alive at the time of analysis ranged from 28 to 112 months with a median of 77 months; only 33 and 9 patients had been followed less than 48 and 36 months, respectively.

This study had been approved by the Ethics and Research Committee at the University of Toronto and by the Institutional Review Board at the University of Texas Health Science Center at San Antonio which assured patient confidentiality at every stage of the investigation.

VII. 3. 2. Flow Cytometric and Steroid Hormone Receptor Analyses

All 1000 breast tumor specimens were subjected to DNA flow cytometry as described elsewhere (Dressler *et al.*, 1988). Briefly, tumor tissue was gently

homogenized, filtered, centrifuged through a double cushion of sucrose, and the cells were resuspended and counted before being simultaneously lysed and stained with propidium iodide. Nuclei were collected and 50,000 were analysed on an Epics V flow cytometer (Coulter Electronics, Hialeah, FL). DNA content and S-phase fraction were determined from the DNA histograms, in which diploid populations were defined as having a DNA index of 1.0, and the percentage of cells in S-phase considered a favorable prognostic indicator was less than 6.7%. The optimal cutoff value for S-phase fraction had been previously determined by cutpoint analysis (Clark *et al.*, 1989).

Tumor specimens (n=1000) were pulverized in liquid nitrogen, homogenized in buffer, and the cytosolic fractions were obtained by ultracentrifugation and quantified for steroid hormone receptors as described by Dressler *et al.* (1988). The results of the dual ligand-binding assay, in which dextran coated charcoal was used to separate bound from free ligand, were interpreted by Scatchard analysis (Scatchard, 1949). Protein concentrations of the cytosols were determined by the Lowry method (Lowry *et al.*, 1951). Cutoff levels for positivity were greater than or equal to 3 fmol/mg and greater than or equal to 5 fmol/mg for ER and PR, respectively, as optimized perviously (Clark and McGuire, 1983; Clark *et al.*, 1983).

VII. 3. 3. Quantitative p53 Protein Analysis

Frozen breast tumor tissues (~0.2 g) were pulverized and extracted as described previously (Levesque *et al.*, 1997). The crude cell lysates were immediately assayed both for p53 protein by immunofluorometry (Levesque *et al.*, 1995b), and for total protein content by a kit based on the BCA method (Pierce Chemical, Rockford, IL). Clinical specimens were assayed for p53 protein without knowledge of the corresponding patient clinicopathologic or survival information.

VII. 3. 4. Statistical Analysis

The statistical analysis, performed using SAS version 6.03 software (SAS Institute, Cary, NC), examined associations between the total protein-adjusted p53 ELISA results and clinical outcome, as well as between the p53 concentrations and other measurements or characteristics of the breast tumor population. Monotonic relationships

between p53 protein, as a continuous variable, and patient age, tumor size, S-phase fraction, ER, PR, and number of involved lymph nodes were demonstrated by the calculation of Spearman correlation coefficients, appropriate given the non-Gaussian distribution of p53 concentrations. All other statistical procedures were similarly nonparametric and based on two-tailed tests of significance. To further investigate associations between p53 and the other clinicopathologic factors, Chi-square tests were applied to contingency tables after dichotomization of all variables: p53 (negative versus positive using a cutoff point equal to the 50th percentile of the ELISA results distribution); age (less than 50 years versus greater than or equal to 50 years); tumor size (less than or equal to 2 cm versus greater than 2 cm); nodal status (no lymph nodes involved versus at least one node with histologic evidence of metastatic spread); S-phase fraction (less than 6.7% versus greater than or equal to 6.7% of tumor cells in S-phase); ER status (negative versus positive using 3 fmol/mg as the cutoff point); PR status (negative versus positive using a 5 fmol/mg cutoff); DNA ploidy (diploid versus aneuploid); endocrine therapy (not treated with tamoxifen versus treated with tamoxifen alone or in combination with other therapies); chemotherapy (not given chemotherapy versus treated with chemotherapy alone or in combination with other therapies); and radiotherapy (not treated with radiotherapy versus treated with radiotherapy alone or together with other therapies).

DFS and OS times were calculated from the dates of surgical resection of the tumors to the dates of occurrence of the two endpoints of interest - earliest diagnosis of recurrence or metastasis, and patient death, respectively. Deaths without evidence of disease were considered censored for both DFS and OS. The prognostic roles of p53, singly or in conjunction with the other clinicopathologic factors, in determining DFS and OS were evaluated by fitting Cox proportional hazard regression models. The hazard ratios and their 95% confidence intervals were calculated from the models using the best category, p53-negativity, as the reference in each case. In the multivariate analysis, the regression models were adjusted for age, tumor size, nodal status, S-phase fraction, DNA ploidy, and status for the steroid hormone receptors, all of which were considered as dichotomous variables using the classification criteria given above. In the univariate and multivariate models, p53 was examined separately as a continuous variable after

transformation into ascending ranks, and as a dichotomous variable categorized by the median percentile cutoff point. In addition, the dose-response relationships between p53 and DFS and OS were evaluated using the quartiles of the p53 distribution. These dose-response effects were also examined by incorporating p53 into univariate and multivariate models as a quartile-divided, four-level continuous variable. Similar analyses were also performed in subgroups of patients stratified by the dichotomous status of three potentially confounding variables, namely, lymph node involvement, ER positivity, and receipt of principle postoperative breast cancer treatments. Univariate and multivariate Cox models assessing median-dichotomized p53 were then constructed in each stratum. The p53-by-ER, p53-by-nodes, p53-by chemotherapy, and p53-by-endocrine therapy interaction terms were also determined from univariate Cox models. The effects of p53-by-nodes interaction upon the risks for relapse and death conferred by p53-positivity were determined among the patients who received chemotherapy. The estimated power to uncover differences in survival between p53-negative and -positive patients in each subgroup was calculated using STPLAN (shareware from Dr. Barry W. Brown, University of Texas M.D. Anderson Cancer Center, Houston, TX) based on a one-sided test at the 5% level of significance and assuming that patients were accrued for one month and followed for 77 months thereafter. The median p53 cutoff point was also used in survival curves, examining relapse and death rates of all p53-positive and p53-negative patients and for those within each of the above-mentioned strata, which were plotted by the method of Kaplan and Meier and compared using log-rank statistics.

VII. 4. Results

VII. 4. 1. Distribution of p53 Protein Concentrations and Relationships to Other Clinicopathologic Variables

The distribution of the p53 concentrations in the 998 breast tumor extracts, ranging from 0 to 110 μg/L, was positively skewed and had a mean, SD and median of 1.89 μg/L, 7.78 μg/L, and 0.19 μg/L, respectively. Twelve % of the extracts had p53 concentrations below the assay detection limit. Expressed relative to the protein content of the extracts, the p53 concentrations considered in statistical analysis were similarly distributed (Figure VII. 1.) with a mean of 1.25 μg/g, a SD of 4.46 μg/g, a median of 0.16

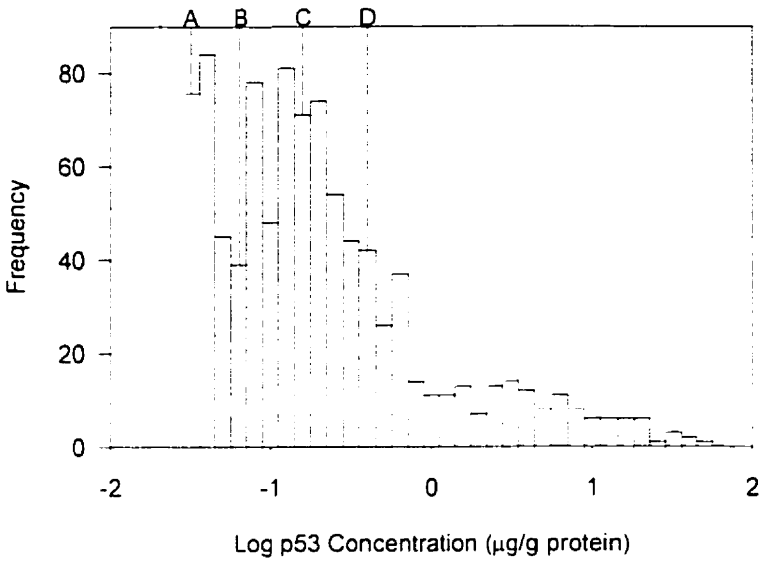


Figure VII. 1. Frequency Distribution of p53 Concentrations in Breast Tumor Extracts
Shown are p53 concentrations in the 876 (of 998) breast tumor extracts that had p53 levels that exceeded the assay detection limit. The dashed lines indicate (A) the detection limit, (B) the 25th, (C) 50th, and (D) 75th percentiles of the frequency distribution.

µg/g, and a range from 0 to 58 µg/g. The 25th, 50th, and 75th percentiles of this distribution were 0.06 µg/g, 0.16 µg/g, and 0.41 µg/g, respectively. The other numerical variables followed somewhat less skewed distributions: ER (mean=142 fmol/mg, SD=186 fmol/mg, median=73 fmol/mg, range=0-1786 fmol/mg); PR (mean=218 fmol/mg, SD=348 fmol/mg, median=71 fmol/mg, range=0-3090 fmol/mg); and S-phase fraction (mean=7.9%, SD=6.48%, median=5.8%, range=0.2-65%). The distributions of patient age, tumor size, and number of lymph nodes have been described above.

Since the associations between p53 accumulation and patient survival times may have been affected by interactions between p53 and the other predictor variables, it was of interest to determine the relationships between other measurements or characteristics of the study population and the levels of p53 protein. Breast tumor extracts with p53 concentrations above the median cutoff point were more frequently below the cutoff points for ER-positivity and PR-positivity (Table VII. 1.) - well established relationships (Caleffi *et al.*, 1994) accompanied by significant, but very weak, negative correlations between p53 and ER (r_s=-0.09, $p<0.01$) and PR (r_s=-0.06, $p=0.04$). Table VII. 1. also indicates that specimens positive for p53 protein were also associated with elevated S-phase fraction (r_s=0.18, $p<0.01$) and DNA aneuploidy, but that significant relationships were not revealed between p53 and tumor size or nodal status, findings confirmed by correlation analysis (data not shown), or between p53 status and whether or not the patients received endocrine therapy, chemotherapy, or radiotherapy as part of their

Table VII. 1. Associations Between p53 Protein Status and Other Clinicopathologic Variables

Factor[a]	Number of Tumors (and %) p53-Negative	p53-Positive	p-Value[b]
Age (years)			
< 50	127 (45.9)	150 (54.1)	
≥ 50	369 (51.2)	351 (48.8)	0.13
Tumor Size (cm)			
≤ 2	234 (52.0)	216 (48.0)	
> 2	242 (47.7)	265 (52.3)	0.19
Nodal Status			
Negative	260 (49.2)	269 (50.8)	
Positive	217 (50.7)	211 (49.3)	0.63
S-phase Fraction (%)			
< 6.7	357 (52.0)	330 (48.0)	
≥ 6.7	140 (45.0)	171 (55.0)	0.04
DNA Ploidy			
Diploid	274 (58.6)	194 (41.4)	
Aneuploid	223 (42.1)	307 (57.9)	<0.01
ER Status			
Negative	54 (39.4)	83 (60.6)	
Positive	443 (51.5)	418 (48.5)	<0.01
PR Status			
Negative	126 (44.8)	155 (55.2)	
Positive	371 (51.7)	346 (48.3)	0.05
Endocrine Therapy			
Not Treated	344 (51.3)	327 (48.7)	
Treated[c]	128 (46.6)	147 (53.4)	0.19
Chemotherapy			
Not Treated	329 (50.8)	319 (49.2)	
Treated[d]	143 (48.0)	155 (52.0)	0.43
Radiotherapy			
Not Treated	381 (51.4)	361 (48.6)	
Treated[e]	91 (44.6)	113 (55.4)	0.09

[a] See Patients and Methods section for details of patient dichotomization by p53, age, tumor size, nodal status, S-phase fraction, DNA ploidy, ER status, and PR status.
[b] P-values determined from Chi-square tests.
[c] Patients treated with endocrine therapy alone or in combination with chemotherapy and/or radiation.
[d] Patients treated with chemotherapy alone or in combination with endocrine therapy and/or radiation.
[e] Patients treated with radiotherapy alone or in combination with endocrine therapy and/or chemotherapy.

postsurgical management. Trends were evident, however, suggesting that p53-positive malignancies were more likely to have been treated with radiation or tamoxifen. While patient age was not associated with p53 accumulation status in the contingency table, the two variables were weakly correlated (r_s=-0.12, $p<0.01$). Contingency tables comparing the proportions of p53-negative and -positive tumors, defined by 25^{th}, 50^{th} or 75^{th} percentile cutoffs, between four groups of specimens with tumor cellularities of 0-10%, 11-30%, 31-70%, or 71-100% revealed no significant association, consistent with the findings of other workers (Vojtesek *et al.*, 1993) and possibly due to variable proportions of malignant cells overexpressing p53 protein within the specimens (Hall and Lane, 1994).

VII. 4. 2. ELISA-Detected p53 Protein as a Predictor of Breast Cancer Patient Survival

Several approaches were employed in the survival analysis to demonstrate associations between p53 and patient prognosis, including the use of the Cox proportional hazard regression method where p53 was expressed continuously or categorically and its contributions to DFS and OS were considered first singly, and then jointly with the other predictive factors (Table VII. 2.). p53 protein levels expressed as their ranks were able to generate statistically significant hazard ratios, but the incremental risk differences were very small. The relative risks for both relapse and death were significantly increased for p53-positive patients when p53 was classified into two groups based on the median. The use of the median cutoff point also indicated 40% and 50% increased risks for relapse and death, respectively, of p53-positive patients in multivariate analysis adjusted for all of the other variables stated in the table. Also predictive of patient outcome in multivariate analysis were lymph node positivity, associated with 2-fold higher risk for relapse [95% CI=1.51-2.71, $p<0.01$] and 92% higher risk for death [95%CI=1.42-2.60, $p<0.01$], and tumor size larger than 2 cm, which yielded relative risks of 1.51 for both relapse [95%CI=1.12-2.03, $p<0.01$] and death [95%CI=1.11-2.06, $p<0.01$]. The S-phase fraction, DNA ploidy, ER, and PR variables were not significant prognostic factors in our series of breast cancer patients. In addition to simply dichotomizing patients on the basis of p53-negativity or -positivity, they were also classified into four groups based on the

Table VII. 2. Associations Between p53 Protein and Disease-Free and Overall Survival

p53 Status	Disease-Free Survival RR[a]	95% CI[b]	p-Value[c]	Overall Survival RR[a]	95% CI[b]	p-Value[c]
Univariate Analysis[d]						
Expressed as Continuous Variable[e]						
Rank	1.00			1.00		
Rank + 1	1.00	1.00-1.00	<0.01	1.00	1.00-1.00	0.02
Based on Median Cutoff Point						
Negative	1.00			1.00		
Positive	1.47	1.12-1.93	<0.01	1.41	1.06-1.87	0.02
Based on Quartiles[f]						
First Quartile	1.00			1.00		
Second Quartile	1.09	0.71-1.68		0.97	0.62-1.52	
Third Quartile	1.35	0.89-2.05		1.39	0.91-2.13	
Fourth Quartile	1.57	1.05-2.35		1.45	0.96-2.20	
p-Value for Trend			0.01			0.07
Multivariate Analysis[g]						
Expressed as Continuous Variable[e]						
Rank	1.00			1.00		
Rank + 1	1.00	1.00-1.00	0.02	1.00	1.00-1.00	<0.01
Based on Median Cutoff Point						
Negative	1.00			1.00		
Positive	1.40	1.06-1.86	0.02	1.50	1.11-2.02	<0.01
Based on Quartiles[f]						
First Quartile	1.00			1.00		
Second Quartile	1.18	0.03-1.35		1.13	0.98-1.30	
Third Quartile	1.39	0.06-1.82		1.28	0.96-1.69	
Fourth Quartile	1.64	1.09-2.46		1.44	0.94-2.20	
p-Value for Trend			0.02			0.08

[a] Relative risk estimated using the Cox proportional hazard regression model.
[b] 95% confidence interval.
[c] p-values are two-sided.
[d] 997 patients included in univariate survival analyses.
[e] p53 concentrations were ranked in ascending order
[f] Estimated RR for second, third, and fourth quartiles compared to the first quartile are given. P values are based on 1-degree of freedom tests of monotonic association.
[g] 920 patients included in multivariate analysis, which was adjusted for age, tumor size, nodal status, S-phase fraction, DNA ploidy, ER status, and PR status.

quartiles of the p53 distribution, by which patients in the second, third, and fourth quartiles were shown to have successively increasing risks for relapse compared to patients in the first quartile. In the corresponding multivariate models, this dose-response

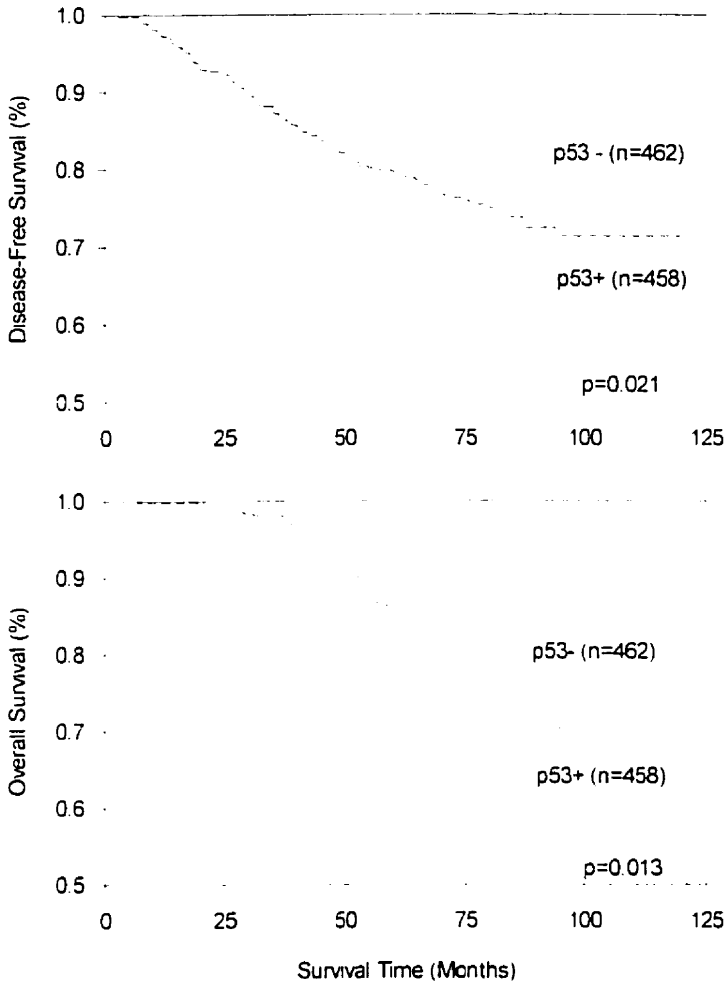


Figure VII. 2. Kaplan-Meier Disease-Free and Overall Survival of All Patients in the Cohort
p53 status was based on the median cutoff point. Differences between curves were determined by log-rank tests, the p-values of which are shown.

trend remained significant. Similar trends were also observed in the analysis of OS, but the p-values for the trends did not reach statistical significance.

It was also of interest to determine the prognostic value of p53 within subgroups of patients recognized as having different prognostic or therapeutic implications. Stratification of patients by nodal status, ER status, and type of postoperative treatment would also have served to eliminate possible confounding effects on outcome between p53 and these other variables. Axillary node-positive patients, but not those without lymph node involvement, had significantly increased risks of 66% and 105%, respectively, for relapse and death if their tumors had p53 concentrations above the median cutoff level (Table VII. 3.). Similarly, patients whose tumors expressed ER had higher risk for both endpoints when their tumors were p53-positive. Formal tests for interaction revealed significant p53-by ER interaction for DFS [RR=1.18, p=0.02] and p53-by-node interaction for OS [RR=1.22, p=0.01]. Trends somewhat suggestive of p53-by-ER interaction for OS [RR=1.12, p=0.11], and p53-by-nodes interactions for DFS [RR=1.12, p=0.13] were also shown by this analysis. For patients who received postoperative treatment, p53 also initially appeared to have values

Table VII. 3. Associations Between p53 Protein and Disease-Free and Overall Survival in Patient Subgroups

p53 Status[a]	Disease-Free Survival RR[b]	95% CI[c]	p-Value[d]	Overall Survival RR[b]	95% CI[c]	p-Value[d]
Univariate Analysis						
Node-Negative Patients (n=505)						
Negative	1.00			1.00		
Positive	1.15	0.74-1.81	0.54	0.95	0.63-1.55	0.95
Node-Positive Patients (n=415)						
Negative	1.00			1.00		
Positive	1.66	1.15-2.39	<0.01	2.05	1.37-3.06	<0.01
ER-Negative Patients (n=129)						
Negative	1.00			1.00		
Positive	1.11	0.54-2.29	0.77	1.51	0.67-3.40	0.32
ER-Positive Patients (n=791)						
Negative	1.00			1.00		
Positive	1.44	1.05-1.95	0.02	1.45	1.05-2.00	0.02
Patients Treated with No Postoperative Therapy (n=274)						
Negative	1.00			1.00		
Positive	1.85	0.93-3.70	0.08	0.94	0.52-1.69	0.83
Patients Treated with Endocrine Therapy +/- Radiation (n=168)						
Negative	1.00			1.00		
Positive	1.44	0.72-2.88	0.30	1.45	0.77-2.76	0.25
Patients Treated with Chemotherapy +/- Radiation (n=184)						
Negative	1.00			1.00		
Positive	1.74	1.03-2.93	0.04	2.04	1.08-3.86	0.03
Multivariate Analysis[e]						
Node-Positive Patients (n=415)						
Negative	1.00			1.00		
Positive	1.67	1.16-2.41	<0.01	2.10	1.40-3.13	<0.01
ER-Positive Patients (n=791)						
Negative	1.00			1.00		
Positive	1.45	1.06-2.00	0.02	1.50	1.09-2.07	0.01
Patients Treated with Chemotherapy +/- Radiation (n=184)						
Negative	1.00			1.00		
Positive	1.73	1.03-2.92	0.04	2.04	1.08-3.86	0.03

[a] p53 status based on median cutoff point.
[b] RR, relative risk estimated using the Cox proportional hazard regression model.
[c] CI, confidence interval.
[d] p-values are two-sided.
[e] Multivariate analysis adjusted for age, tumor size, S-phase fraction, DNA ploidy, ER status, and either ER status (for estimating RR in node-positive patients) or nodal status (for estimating RR in ER-positive patients), or both (for estimating RR in patients treated with chemotherapy +/- radiation).

in predicting the response to chemotherapy. p53-positivity was a predictor of poor prognosis in patients to whom chemotherapy was administered with or without accompanying radiotherapy, but not in those treated with endocrine therapy alone or in combination with radiation, or in patients given only palliative care after surgery to remove their breast tumors. Within the node-positive, ER-positive, and chemotherapy-treated subgroups, p53 status retained its ability to indicate significantly elevated hazard ratios in multivariate analysis (Table VII. 3.). Consistent with the results of the Cox regression analysis, Kaplan-Meier plots also revealed that p53-positive status was an indicator of poorer survival in patients with node-positive or ER-positive breast cancer, or in patients given postoperative chemotherapy (Figures VII. 2. – VII. 5.). However, with respect to the implied ability of p53 to predict outcome only in chemotherapy-treated patients, the lack of significant p53-by-chemotherapy interaction [RR=1.01, p=0.87 for DFS and RR=1.11, p=0.17 for OS] suggested that the differences observed between treatment groups might have been due to the relatively few outcome events in the endocrine therapy-treated and untreated cohorts of patients. Furthermore, after multivariate adjustment of the effects of p53 on survival of patients given chemotherapy by including a p53-by-nodes interaction term [RR=1.28, p=0.03 for DFS and RR=1.33, p=0.04 for OS], the impact of p53 became insignificant [RR=1.24, p=0.37 for DFS and RR=1.49, p=0.17 for OS]. Taken together, these data

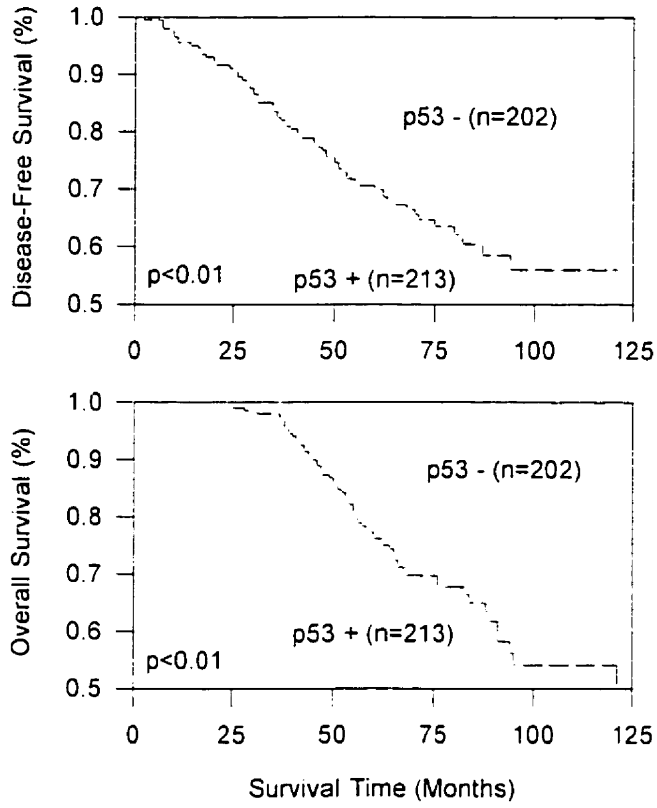


Figure VII. 3. Kaplan-Meier Disease-Free and Overall Survival of Node-Positive Patients
p53 status was based on the median cutoff point. Differences between curves were determined by log-rank tests, the p-values of which are shown.

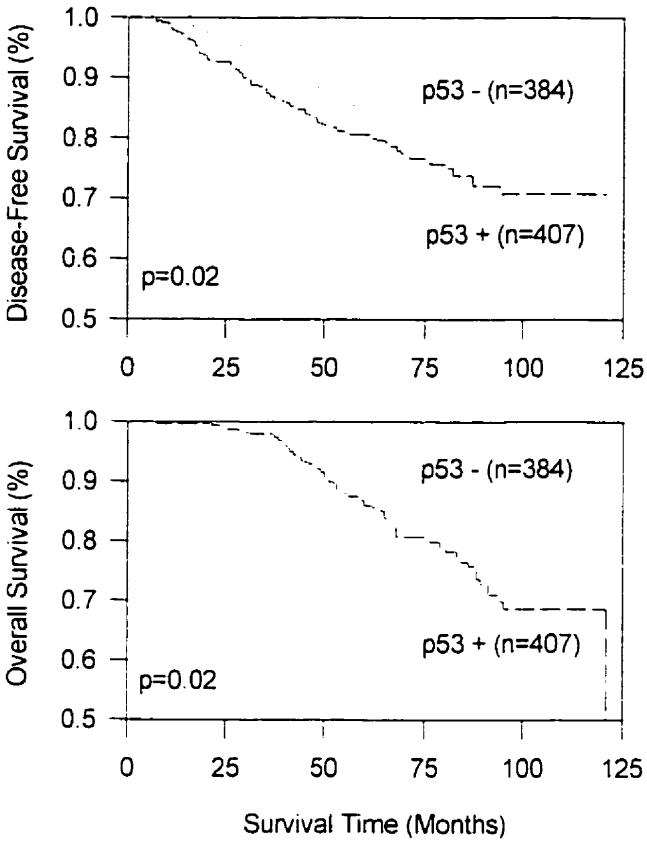


Figure VII. 4. Kaplan-Meier Disease-Free and Overall Survival of Estrogen Receptor-Positive Patients

p53 status was based on the median cutoff point. Differences between curves were determined by log-rank tests, the p-values of which are shown.

indicate that the assessment of the prognostic value of p53 within subgroups given different postoperative treatments, but not controlled for node status, must be interpreted cautiously.

VII. 5. Discussion

The relationship between p53 abnormality and breast cancer prognosis remains unclear, despite the large body of literature focused on the topic. Studies investigating the prognostic value of p53 protein accumulation assessed by immunohistochemical techniques comprise the bulk of the research but often differ substantially with respect to procedural details and scoring criteria, and may report prognostic information inferior to that obtainable by DNA sequence-based methods (Kovach *et al.*, 1996; Sjogren *et al.*, 1996). Quantitative analysis of p53 protein accumulation, implemented by densitometric image analysis of immunostained tissue (Charpin *et al.*, 1996) and by ELISA-type assays of tissue extracts (Bartkova *et al.*, 1993; Borg *et al.*, 1995; Levesque *et al.*, 1995b) may offer improved reproducibility and might therefore serve as alternatives to conventional IHC. However, the ability to assay cytosolic extracts already prepared for ER and PR assays may make ELISAs particularly suitable for p53 protein measurement in breast tumor tissues. Using the commercially available LIA-mat p53 luminometric immunoassay (Sangtec Medical AB, Bromma, Sweden), Borg *et al.* (1995) and de Witte *et al.* (1996) have demonstrated the prognostic value of tumoral p53 concentration on the survival of 205 and 142 breast cancer patients, respectively. The results of our study confirm their findings in a larger patient population and validate an

immunofluorometric assay developed in our laboratory (Levesque *et al.*, 1995b) for clinical application.

Compared to studies in which p53 status is represented by a small number of groups (usually two), our use of a quantitative assay permitted more flexible data manipulation. In one approach which enabled full use of the data, the p53 assay results were used as a continuous variables in the regression analysis. In another approach, p53 was considered as a categorical variable divided into four levels by the quartiles of the p53 distribution. Relative risks for developing each outcome event in patients in the second, third, and fourth quartiles compared to the risks in the first quartile were also determined. Associated with the greatest information loss was the final approach, where p53 was used as a dichotomous variable using an arbitrarily selected cutoff. Since no *a priori* assumptions were made for selecting a particular cutpoint, and a "minimum p-value approach" (Altman *et al.*, 1994) was not employed, the median p53 value (0.16 µg/g) was adopted in the multivariate analysis, in all regression analyses within subgroups of patients, and in the Kaplan-Meier analyses. Interestingly, this median value is very close to the "optimal" cutoff of 0.15 µg/g defining 30% of the specimens to be p53-positive in the series of Borg *et al.* (1995). While de Witte *et al.* (1996) similarly classified 28% of specimens as p53-positive, the cutoff concentration used was over 15-fold higher. Taken together, our results demonstrated modest "dose-response" effects between p53 protein concentration and

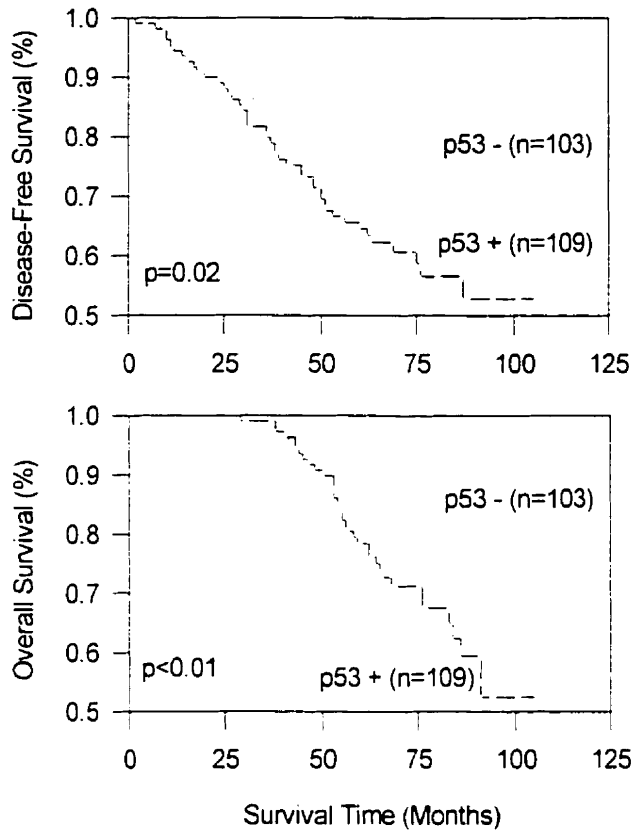


Figure VII. 5. Kaplan-Meier Disease-Free and Overall Survival of Patients Treated with Chemotherapy

p53 status was based on the median cutoff point. Differences between curves were determined by log-rank tests, the p-values of which are shown.

risks for relapse and death. Furthermore, multivariate analysis using median-dichotomized p53 showed that these effects were not dependent on any other factor for which the tumors had been characterized.

Major objectives in breast cancer research in recent years have been the identification of factors capable of distinguishing node-negative patients at reduced risk of relapse who might be spared adjuvant treatment, as well as of factors predictive of adjuvant therapeutic success. In agreement with some workers (Isola *et al.*, 1992; Pietilainen *et al.*, 1995; Rosen *et al.*, 1995) but not with others (Allred *et al.*, 1993; Silvestrini *et al.*, 1993; Silvestrini *et al.*, 1996b), p53 was found not to be a significant prognostic indicator in our series of 505 node-negative patients. p53 protein accumulation was, however, independently associated with poor outcome in the 415 node-positive patients in our study, an observation which had been reported previously (Silvestrini *et al.*, 1996a). The significant interaction for OS and a trend for DFS between p53 and nodes, as well as the demonstration of adequate power in the node-negative subgroup (86%), supports our observation that p53 had prognostic value only in the node-positive patients in our series. When patients were stratified by estrogen receptor status, indicative of endocrine therapy-responsiveness under many circumstances, p53-positivity was found to be associated with increased risks for relapse and death only in estrogen receptor-positive patients, that is, in women who might otherwise be expected to have favorable outcomes (Thor *et al.*, 1992; Friedrichs *et al.*, 1993). This finding is in contrast to that of Caleffi *et al.* (1994), who earlier reported p53 mutation to be of prognostic value neither in estrogen receptor-negative nor -positive patients. Our results, suggesting that the impact of p53 on survival may have been mediated by ER-positivity, are consistent with evidence for interaction between p53 and ER, especially with respect to DFS. The relatively low relapse rate, however, among the 129 ER-negative patients made it unlikely that statistically significant differences in survival between p53-negative and -positive patients would have been found; the power in this subgroup was only 38%. Stratification of our study population into three treatment groups that had received endocrine treatment alone or in combination with radiation, chemotherapy with or without additional radiation, or no postsurgical medical intervention, revealed the assessment of p53 status to be of no apparent prognostic value in patients given endocrine

treatment or who were not given adjuvant therapy, but to be highly significant in the survival analysis of patients treated with chemotherapeutic drugs. Since significant p53-by-chemotherapy interaction could not be demonstrated, and although this analysis was probably lacking in power, the most likely explanation for the apparent differences in effect of p53 between treatment groups lies in the small number of patients in the untreated or endocrine therapy-treated cohorts who underwent relapse. The majority of these patients would have been expected to be node-negative, in contrast to patients in the subgroup that received chemotherapy. Our data suggest that it was the relationship between p53 and node status within the chemotherapy-treated subgroup which led to the apparent prognostic value of p53 overexpression. Given that the patients in our study were not randomized to the treatments they received, we were unable to unambiguously determine whether the prognostic value of p53 protein accumulation is dependent on any particular treatment administered. Other studies, however, have provided *in vitro* and clinical evidence implicating p53 as a mediator of apoptosis induced by cancer chemotherapeutic agents and radiation (Lowe *et al.*, 1994; Aas *et al.*, 1996). It has been proposed that tumors lacking functional p53 may be unable to activate the apoptotic cascade, leading to treatment failure and earlier patient death. The responsiveness of breast tumors to cancer chemotherapeutic agents might therefore be predicted by p53 functional status, reflected by accumulation of p53 protein. Although studies of the association between p53 alteration and chemosensitivity have not yielded consistent findings (Elledge *et al.*, 1995; Jansson *et al.*, 1995; Makris *et al.*, 1995; Barbareschi *et al.*, 1996), sequencing of the entire p53 coding region has recently demonstrated systemic therapy and radiotherapy to be of less therapeutic value for p53-mutated breast tumors (Bergh *et al.*, 1995). Additional work must certainly be performed to establish p53 status as a predictive factor for adjuvant therapy in breast cancer.

The study of p53 alterations in relation to breast cancer survival and treatment response probabilities has been greatly facilitated by the close correlation between p53 gene mutational changes and accumulation of mutant p53 protein, detected in the majority of studies by IHC. The results of this study indicate that a simple and sensitive ELISA for p53 protein may also provide prognostic information for breast cancer patients. Furthermore, we have identified the subgroups of patients with lymph-node-

positive and possibly ER-positive disease for whom the prognostic significance of p53 may be particularly relevant.

CHAPTER VIII

PREDICTION OF OVARIAN CANCER PROGNOSIS AND RESPONSE TO CHEMOTHERAPY BASED ON p53 (AND $p21^{WAF1}$) EXPRESSION STATUS

(A version from Levesque MA, Katsaros D, Massobrio M, Genta F, Yu H, Ricchiardi G, Pia Mano M, Fracchioli S, Durando A, Arisio R, Diamandis EP. *Clin Cancer Res* (submitted))

VIII. 1. Abstract

This study was designed to determine whether patients who received adjuvant chemotherapy for epithelial ovarian cancer could be stratified into groups with different risks for relapse or death on the basis of the expression in tumor tissue of two proteins, p53 and its downstream mediator WAF1 (wild-type p53-activated factor-1) (CIP1/SDI1), quantified by immunoassay rather than by conventional immunostaining. Extracts from 120 epithelial ovarian carcinomas were assayed both for p53 protein by an immunofluorometric assay developed by the authors and for WAF1 protein by a commercially available immunoassay. Relationships between p53 and WAF1 concentrations, and between each of these and other clinicopathologic variables, were determined by Spearman correlation and Wilcoxon Rank Sum tests. Using contingency tables, response to chemotherapy was compared between patients with different status for each variable. RR for cancer relapse and death after 24 months of follow-up for patients with p53-positive or WAF1-positive tumors, categorized by median cutoff points, were determined by Cox regression analysis in which multivariate models were adjusted for patient age, stage, grade, and residual tumor size. Also evaluated in Cox models were a four-level p53 variable and a three-level composite variable of p53 and WAF1 combined. Differences in DFS and OS probabilities of p53-negative and p53-positive patients, and of WAF1-negative and WAF1-positive patients, were also estimated by the Kaplan-Meier method and log-rank tests. Concentrations of p53 were elevated in patients with advanced stage disease ($p=0.02$) or poorly differentiated ($p=0.03$), suboptimally debulked tumors ($p=0.02$), as well as in patients who failed to respond to chemotherapy ($p=0.03$). Despite the absence of statistically significant associations between concentrations of p53 and WAF1, higher concentrations of WAF1 were more common together with younger age, earlier stage, and the absence of residual tumor, although these relationships did not reach statistical significance and no relationship to treatment response was demonstrated. Univariate analysis showed that p53 concentrations above the median indicated significantly higher risks for relapse ($p=0.04$) and death ($p<0.01$) and showed trends for increasing risks for relapse ($p=0.04$) and death ($p<0.01$) when p53 was considered as a four-level categorical variable. Multivariate analyses confirmed these observations (RR=1.50; $p=0.05$ for DFS and RR=1.92; $p=0.03$ for OS) for median-dichotomized p53,

but the trends were of borderline significance (p=0.09 for DFS and p=0.07 for OS). In contrast, WAF1-positivity was not a significant predictor of favorable outcome in univariate survival analysis, and use of a three-level variable combining positivity or negativity status for both p53 and WAF1 did not yield greater separation of patients into risk groups (p=0.07 for DFS and p=0.06 for OS) than the use of p53 alone. Assessment of p53 expression may be an independent indicator of poor prognosis in ovarian cancer patients treated with adjuvant chemotherapy. The prognostic value of WAF1 expression, however, could not be demonstrated in our series of ovarian cancer patients.

VIII. 2. Introduction

Epithelial ovarian cancer is the most lethal gynecologic malignancy in Western countries (Wingo *et al.*, 1995). Approximately 80% of patients are diagnosed with advanced stage disease (Ozols *et al.*, 1997), associated with a 5-year survival rate of only 30% despite improvements in long-term survival gained by the use of combination chemotherapy, principally with cisplatin and more recently with paclitaxel (Stewart *et al.*, 1992). A number of factors contribute to the poor prognosis of patients with advanced stage ovarian carcinoma, including the failure of aggressive cytoreductive surgery to completely eradicate metastatic disease in over 75% of cases, the instrinsic resistance to adjuvant chemotherapy in over half of these patients, as well as the development of chemoresistance in nearly half of the initially responsive patients during the course of their treatment (Perez *et al.*, 1993). Although clinicopathologic characteristics of ovarian cancer other than disease stage, such as volume of residual disease after debulking surgery, histologic grade and type, lymph node status, and presence of ascites are also of demonstrated prognostic value (Kosary, 1994), individual patients may show significant differences in chemosensitivity even though they share identical clinicopathologic features. In light of evidence indicating that most anticancer agents induce tumor regression by triggering apoptosis (Thompson, 1995), it is possible that new variables reflecting the apoptotic potential of ovarian neoplasms may offer more accurate prognostic information for patients treated with chemotherapy.

Among the determinants for the induction of some forms of apoptosis is the status of the p53 tumor suppressor gene, the translated product of which has been shown to

transcriptionally up-regulate and down-regulate bax (Miyashita *et al.*, 1994) and bcl-2 (Halder *et al.*, 1994), respectively - two key components of the triggering mechanism for programmed cell death. Functional loss of p53 by mutations that interfere with its ability to induce apoptosis has been shown to facilitate the development of neoplastic clones resistant to different chemotherapeutic drugs (Lowe *et al.*, 1993a). These mutations, which are mostly missense and occur within conserved sequences of the p53 gene, are the most common genetic alterations in human malignancy (Levine *et al.*, 1991) and have been detected in 40-80% of epithelial ovarian cancers (Marks *et al.*, 1991; Kupryjanczyk *et al.*, 1993; Milner *et al.*, 1993). Rather than impeding p53 protein expression, missense mutations usually confer an altered conformation to the mutant p53 protein and are associated with its predominantly nuclear accumulation (Gannon *et al.*, 1990), in contrast to normal cell nuclei in which p53 protein is expressed at very low levels. Besides its diminished capacity to trigger apoptosis, mutant p53 protein is typically also deficient in its ability to induce cell cycle arrest by the transactivation of other target genes. The first identified of these was WAF1, a protein that binds and inhibits several cyclin/cyclin-dependent kinase complexes (Xiong *et al.*, 1993; El-Deiry *et al.*, 1994) as well as components of the DNA replication machinery (Waga *et al.*, 1994). Despite observations that expression of WAF1, like p53, can cause growth suppression of a variety of cell types *in vitro* (El-Deiry *et al.*, 1993; El-Deiry *et al.*, 1994) and *in vivo* (Yang *et al.*, 1995), WAF1 mutations rarely occur in human cancers (Koopman *et al.*, 1995; Wan *et al.*, 1996), suggesting that derangement of WAF1 function does not contribute to clinical disease. However, WAF1 protein expression has been shown to be highly variable in several tumor types (Barboule *et al.*, 1995; Diab *et al.*, 1997; Gomyo *et al.*, 1997) and to be subject to both p53-dependent and p53-independent transcriptional regulation (Michieli *et al.*, 1994). Unlike the large number of studies investigating the relationship between p53 alteration in diverse malignancies, including ovarian cancer (Bosari *et al.*, 1993; Hartmann *et al.*, 1994; Klemi *et al.*, 1995; Levesque *et al.*, 1995c; Eltabbakh *et al.*, 1997;), and unfavorable prognostic outcome, there have been fewer studies examining WAF1 expression in relation to patient prognosis (Ito *et al.*, 1996; Diab *et al.*, 1997; Erber *et al.*, 1997; Gomyo *et al.*, 1997; Jiang *et al.*, 1997). Moreover, to our knowledge,

the prognostic and predictive implications of p53, considered together with its downstream mediator WAF1, in epithelial ovarian cancer have not yet been reported.

Conventional tools to identify p53 abnormalities have consisted of DNA sequencing methods, indirect screening methods for determining DNA sequence changes, and immunohistochemical staining techniques using monoclonal or polyclonal antibodies to detect p53 protein overexpression. The latter approach, while lacking sensitivity and specificity for demonstrating p53 changes at the genetic level (Casey *et al.*, 1996), nonetheless has been shown to provide useful information regarding the prognosis of patients with ovarian carcinoma (Bosari *et al.*, 1993; Hartmann *et al.*, 1994; Klemi *et al.*, 1995;) at a fraction of the technical costs. Use of the same antibodies as reagents in immunoassays of p53 constitutes an alternative to p53 immunostaining that may offer advantages in terms of more objective results interpretation and relative ease of quantitation. Such immunoassays have been applied to the measurement of p53 concentrations in extracts prepared from a variety of tissues and have yielded results highly concordant with those obtained by immunostaining (Joypaul *et al.*, 1993; Vojtesek *et al.*, 1993; Levesque *et al.*, 1997). Only immunohistochemical methods, however, have been employed for the detection of WAF1 protein in clinical specimens (Barboule *et al.*, 1995; Ito *et al.*, 1996; Diab *et al.*, 1997; Erber *et al.*, 1997; Gomyo *et al.*, 1997; Jiang *et al.*, 1997;), in spite of the possible advantages of commercially available immunoassays.

In this study, we report the use of simple yet sensitive immunoassays of p53 and WAF1 proteins, rather than immunostaining, to determine their respective concentrations in extracts of 120 epithelial ovarian carcinomas obtained from chemotherapy-treated patients residing in the Piedmont region of Northern Italy. The expression levels of p53 and WAF1 were related to each other, to other prognostic features, to patient response to administered chemotherapy, and to DFS and OS.

VIII. 3. Materials and Methods

VIII. 3. 1. Ovarian Cancer Patients

This study had been approved by the Ethics and Research Committees at the University of Toronto and the University of Turin that assured patient confidentiality at every stage of the investigation. One hundred and twenty patients with primary epithelial

ovarian carcinoma, operated at the Department of Gynecology, Gynecologic Oncology Service of the University of Turin, Turin, Italy between April 1988 and January 1997, were included in this study. Excluded from the consecutive series had been patients with benign (n=4) or germline (n=4) ovarian neoplasms, patients with other primary malignancies metastatic to the ovary (n=19), and patients with cancer of the ovarian epithelium who had tumor specimens which were either of borderline histologic grade (n=16) or of insufficient quantity for p53 protein analysis (n=6). Three patients lost to follow-up, and four who did not receive adjuvant chemotherapy, were also excluded. The patients studied were of ages ranging from 26 to 77 years: the median and mean ages were both 55 years. Patients were followed up at the same institution for periods ranging from 3 to 119 months (median and mean were 24 and 30 months, respectively), during which 66 (55%) were diagnosed with ovarian cancer relapse and 44 (37%) died of their disease. DFS time, defined as the number of complete months elapsed from the date of tumor resection to that of the first evidence of recurrent disease or distant metastasis in each case, was distributed from 0 months (stage IV) to 67 months with a mean and median of 15 and 11 months, respectively. Of the 66 patients who relapsed, 12 underwent remission followed by subsequent relapse. Three patients had a third relapse. The time interval between primary surgical treatment and patient death confirmed to be due to complications of ovarian carcinoma – the OS time – ranged from 3 to 79 months and had a mean and a median of 23 and 20 months, respectively. Patient deaths due to other causes were considered censored events. Of the patients remaining alive at the termination of the study in April 1998, recurrent disease or metastasis was identified in 22 patients (29%) but undetectable in 54 patients (71%).

Patients were characterized for a number of clinicopathologic variables at the time of surgery. These included stage classified according to the International Federation of Gynecologists and Obstetricians (FIGO, 1987), by which 17 patients were found to have stage I disease, 7 patients were in stage II, 88 patients were in stage III, and 8 patients had stage IV ovarian cancer. As required by the FIGO staging scheme, extensive surgical and cytologic assessment of the disease extent was performed in all cases. These procedures included collection of ascites or peritoneal washings from the pelvis, gutters, and diaghragm for cytologic studies; total abdominal hysterectomy and bilateral salpingo-

oophorectomy; infracolic omentectomy and appendectomy; selective pelvic and paraaortic lymphadenectomy; and debulking of all gross tumor tissues. If obvious macroscopic tumor was not present, the following procedures were performed: biopsy of any lesion suspected of being a tumor metastasis or any adhesion adjacent to the primary tumor; blind biopsies of bladder peritoneum and cul-de-sac, right and left paracolic gutter, and pelvic side walls; and biopsy or smear of right hemidiaphragm. Histologic grade and type based on WHO criteria (World Health Organization, 1982) were also determined. Thirteen specimens were well-differentiated (grade 1), 31 were moderately-differentiated (grade 2), and 76 were poorly-differentiated (grade 3). Tumors of serous papillary histotype (n=47) constituted the largest group, while 20 were endometrioid, 21 were undifferentiated, 18 were clear cell, 8 were mucinous, and 6 were mullerian. The success of surgical debulking was judged to be optimal for 50 patients, of whom 48 had no grossly apparent residual tumor; the remaining 72 patients had residual tumor masses estimated to be 1 to 3 cm (n=33), 4 to 8 cm (n=26), or greater than or equal to 9 cm (n=13) in maximal diameter.

All patients had been previously untreated for ovarian cancer. Administered as first-line chemotherapy to all patients were combinations of chemotherapeutic agents including cisplatin (given to 76 patients), carboplatin (n=44), cyclophosphamide (n=62), taxol (n=25), epirubicin (n=19), adriamycin (n=11), alkaran (n=1), and methotrexate (n=1). All patients had received either cisplatin or carboplatin. Three patients additionally received radiotherapy, and another two were given hormonotherapy. Assessment of treatment response in the 72 patients with residual tumor size greater than 1 cm was performed after the last cycle of chemotherapy and was based on the following criteria (Menzin, 1996): resolution of all evidence of disease for at least one month was considered a complete response; a decrease of greater than or equal to 50% in the product of the diameters (maximal and minimal) of all measurable lesions lasting at least one month without the development of new lesions was considered a partial response; a decrease of less than 50% or an increase of less than 25% in the product of the diameters of all measurable lesions was considered stable disease; and an increase of greater than or equal to 25% in the measurable lesions as described above or the identification of new lesions was considered progressive disease. The majority of patients initially responded

completely (n=84) or partially (n=22) to first-line chemotherapy, whereas others experienced no change (n=7) or progressive disease (n=7). Second-line chemotherapy following initial treatment failure was given to 67 patients and included cisplatin (n=6), carboplatin (n=12), cyclophosphamide (n=9), taxol (n=25), epirubicin (n=11), and adriamycin (n=4). Twelve patients required third-line chemotherapy, consisting of cisplatin (n=3), carboplatin (n=1), cyclophosphamide (n=1), and taxol (n=7).

VIII. 3. 2. Tumor Extraction and p53 Immunofluorometric Assay

Tumor tissues were snap-frozen in liquid nitrogen immediately following surgery. Representative portions of each tumor containing greater than 70% tumor cells were selected by histologic examination of frozen sections, and stored at -80°C until analysis. A subset of randomly selected tumors (n=27) were sampled at two different surfaces to yield portions which were separately pulverized and extracted as described previously (Levesque *et al.*, 1997). The crude lysates were assayed immediately and concurrently for p53 protein by immunofluorometry, WAF1 protein by colorometric immunoassay, and total protein content by a kit based on the BCA method (Pierce Chemical, Rockford, IL).

Concentrations of soluble p53 protein in the ovarian tumor extracts were determined without knowledge of the corresponding patient clinicopathologic or survival information by a quantitative, sandwich-type immunoassay described in detail elsewhere (Levesque *et al.*, 1995b).

VIII. 3. 3. Immunoassay of WAF1 Protein

The WAF1 Quantitative ELISA Assay™ (Oncogene Research, Cambridge, MA) was used to measure WAF1 concentrations in the ovarian tumor extracts, following the manufacturer's instructions. All necessary reagents were provided in the kits. Features of this sandwich-type immunoassay include a rabbit polyclonal anti-WAF1 antibody immobilized onto microtiter plates, a biotinylated mouse monoclonal antibody specific for human WAF1 protein added following sample addition, and detection by streptavidin conjugated to horseradish peroxidase which catalyzes the conversion of tetramethylbenzidine into a colored product. Dual wavelength absorbances at 450 nm and 540 nm were determined using a microplate spectrophotometer (Labsystems, Helsinki,

Finland). Using dedicated software, concentrations of WAF1 were interpolated from calibration curves constructed from the assay of lyophilized WAF1 standards ranging in concentration from 0 to 20 Units/mL. Calibrators and ovarian tumor extracts were assayed in duplicate and in parallel. Concentrations of WAF1 greater than the reported lower limit of detection of 0.1 Units/mL were expressed as Units/mg, adjusting for the variable protein contents of the extracts. Extracts prepared from breast carcinoma cells (MCF-7, T-47D), obtained from the ATCC, cultured as described elsewhere (Levesque *et al.*, 1995b), and for which the WAF1 expression status had been characterized previously (Ozcelik *et al.*, 1995), were assayed in parallel as qualitative positive and negative controls, respectively.

VIII. 3. 4. Statistical Analysis

The statistical analysis, performed using SAS version 6.12 software (SAS Institute, Cary, NC), examined associations between the total protein-adjusted p53 and WAF1 immunoassay results and DFS and OS, as well as between the p53 and WAF1 concentrations and other measurements or characteristics of the sample of ovarian cancer patients. All procedures were nonparametric and based on two-tailed tests of significance. Monotonic relationships between p53 and WAF1 as continuous variables were shown by the calculation of the Spearman correlation coefficient. Continuity-corrected Wilcoxon Rank Sum tests or Kruskal-Wallis tests were used to compare the distributions of p53 and WAF1 concentrations, one at a time, between patient subgroups defined by their status for the other protein marker (WAF1 or p53, each classified as negative or positive using cutoff points equal to the 50^{th} percentiles of the respective distributions) and for each of the clinicopathologic variables: age (<55 years vs ≥55 years), FIGO stage (stages I or II vs stages III or IV), histologic grade (grade 1 vs grade 2 vs grade 3), histologic type (serous papillary vs all other histotypes), and residual tumor size (<1 cm vs ≥1 cm). Differences in p53 and WAF1 expression status, as well as differences in patient age, tumor grade, and histologic type classified as above, between patients with assessable post-operative disease who exhibited either complete response to first-line chemotherapy, partial response to such treatment, stable disease, or progressive disease despite having received first-line chemotherapy, were determined by 2-tailed Fisher Exact tests.

Wilcoxon Rank Sum tests were also used to examine the occurrences, during follow-up, of ovarian cancer relapse and patient death in relation to p53 and WAF1 concentrations.

The relationships of p53, WAF1, and other clinicopathologic variables to DFS and OS were evaluated by the RRs for relapse and death and their 95% CIs, which were calculated from fitted Cox proportional hazards regression models. In the multivariate analysis, the models were adjusted for age, stage, grade, and residual tumor size, all of which were considered dichotomous or three-level variables defined by the classification criteria given above. In both univariate and multivariate models, p53 was examined separately as a dichotomous variable categorized by the median percentile cutoff point, and as a quartile-divided, four-level ordinal variable. The prognostic value of median-dichotomized WAF1 was determined by fitting a univariate Cox model. In order to determine the prognostic impact of p53 and WAF1 assessed in combination, a three-level ordinal variable was created and evaluated in Cox models of DFS and OS. The first level of this new variable included patients whose tumor extracts were concurrently p53-negative and WAF1-positive. The second level consisted of patients whose tumors were either positive for both markers or negative for both markers. Patients in the third level had tumors which were p53-positive and WAF1-negative. Kaplan-Meier survival curves were also constructed to demonstrate the effects of p53 concentrations exceeding the median percentile on DFS and OS probabilities, differences over time for which were evaluated using log-rank tests. The same Kaplan-Meier analyses were performed to reveal differences in survival between WAF1-negative and WAF1-positive patients.

VIII. 4. Results

VIII. 4. 1. Distributions of p53 and WAF1 Concentrations

Of the 120 ovarian tumor extracts, all except 2 had detectable p53 protein concentrations. When adjusted for the total protein contents of the extracts, these concentrations ranged from 0.005 to 102.51 μg/g and were bimodally distributed with a mean of 5.24 μg/g, an SD of 12.76 μg/g and 25^{th}, 50^{th}, and 75^{th} percentiles of 0.10 μg/g, 0.42 μg/g, and 5.05 μg/g, respectively (Figure VIII. 1. (Panel A)). The high degree of concordance (r_s=0.87, p=0.0001) between p53 concentrations measured in 27 pairs of extracts prepared from two different portions of the same tumors suggested that p53

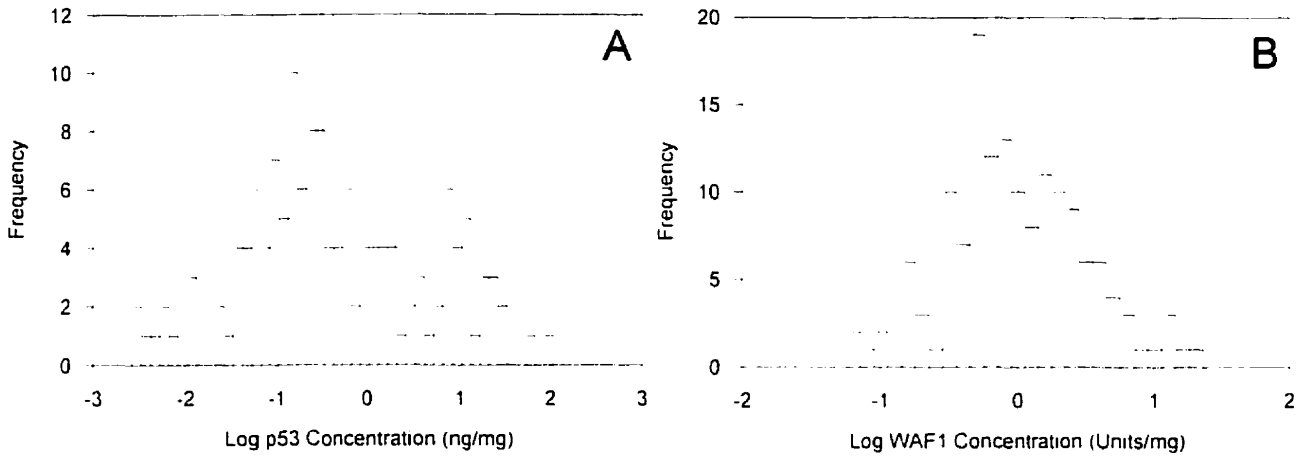


Figure VIII. 1. Frequency Distributions of p53 and WAF1 Protein Concentrations in Ovarian Cancer Patients

Shown are the p53 (A) and WAF1 (B) concentrations in 118 (of 120) and 118 (of 118) ovarian tumor extracts, respectively, that had p53 and WAF1 levels exceeding the assay detection limits. From left to right, the dashed lines indicate the 25th, 50th, and 75th percentiles of each distribution.

accumulation throughout each tumor specimen used for analysis was roughly homogeneous. WAF1 concentrations in the 27 pairs of extracts were also correlated (r_s=0.63, p=0.006), but indicated that WAF1 exhibited greater intra-tumor variability. In the extracts of all 118 tumors assayed for WAF1, the concentrations of this analyte exceeded the assay detection limit in all cases. Adjustment of these values for the total protein contents of the extracts yielded a distribution which ranged from 0.07 to 24.54 Units/mg and had a mean, SD, and median of 1.93 Units/mg, 3.08 Units/mg, and 0.82 Units/mg, respectively (Figure VIII. 1. (Panel B)). The 25th and 75th percentiles of the WAF1 distribution were 0.52 Units/mg and 2.08 Units/mg, respectively.

VIII. 4. 2. Relationships Between p53, WAF1 and Other Clinicopathologic Variables

Given the ability of functional, non-mutant p53 to induce WAF1 expression, and in order to identify possible interaction between the two proteins in survival analysis, it was of interest to determine whether the bulk tumor tissue concentrations of p53 and WAF1 were associated with each other. Concentrations of p53 and WAF1 were not correlated (r_s=0.07, p=0.46). Moreover, neither the difference in p53 concentrations between median-dichotomized WAF1-negative and WAF1-positive specimens (Table VIII. 1.), nor the difference in WAF1 concentrations between tumor extracts classified as

p53-negative and p53-positive using the median p53 value (Table VIII. 2.), were statistically significant by Wilcoxon Rank Sum tests. Using the same analysis, associations between each of these proteins and the status of the other clinicopathologic features for which the ovarian tumors were characterized were also examined because of possible confounding influences of these other variables upon the relationships between patient survival times and p53 or WAF1 concentrations. Table VIII. 1. shows that although p53 concentrations did not differ significantly between the two groups of patients who were younger, and older, respectively, than the median age, concentrations of p53 were higher in extracts prepared from ovarian cancers which were more advanced (stages III-IV), less well-differentiated (grade 3), and suboptimally debulked (residual

Table VIII. 1. p53 Concentrations[a] in Relation to Other Clinicopathologic Variables

Factor[b]	Number	Median	Range	p-Value[c]
WAF1 (Units/mg)				
< median	61	0.42	0 – 102.51	
≥ median	57	0.30	0 – 66.61	0.54
Age (years)				
< 55	57	0.41	0 – 102.51	
≥ 55	63	0.23	0 – 34.34	0.36
Stage				
I - II	24	0.21	0 – 12.16	
III - IV	96	0.60	0 – 102.51	0.02
Grade				
1	13	0.20	0 – 1.73	
2	31	0.33	0 – 102.51	
3	76	0.60	0 – 66.61	0.03
Histologic Type				
Serous	47	0.68	0 – 102.51	
Others	73	0.31	0 – 34.34	0.14
Residual Tumor (cm)				
< 1	48	0.25	0 – 66.61	
≥ 1	72	0.60	0 – 102.51	0.02
Patient Relapse				
No	54	0.23	0 – 66.61	
Yes	66	0.63	0 – 102.51	0.04
Patient Death				
No	76	0.19	0 – 102.51	
Yes	44	1.20	0 – 29.07	<0.01

[a] p53 concentrations expressed in μg/g.
[b] See Materials and Methods section for details of patient stratification by each variable.
[c] p-values determined from Wilcoxon Rank Sum tests with continuity correction, or Kruskal-Wallis tests, where appropriate.

Table VIII. 2. WAF1 Concentrations[a] in Relation to Other Clinicopathologic Variables

Factor[b]	Number	Median	Range	P-Value[c]
p53 (μg/g)				
< Median	58	1.14	0.09 – 24.54	
≥ Median	60	0.94	0.11 – 17.10	0.68
Age (years)				
< 55	61	1.17	0.09 – 24.54	
≥ 55	57	0.82	0.07 – 13.25	0.17
Stage				
I - II	24	1.65	0.09 – 24.54	
III - IV	94	0.83	0.07 – 10.49	0.13
Grade				
1	13	1.84	0.09 – 24.54	
2	30	0.77	0.09 – 17.10	
3	75	0.88	0.07 – 8.78	0.22
Histologic Type				
Serous	46	0.82	0.16 – 13.26	
Others	72	1.11	0.09 – 24.54	0.42
Residual Tumor (cm)				
< 1	47	1.23	0.09 – 24.54	
≥ 1	71	0.81	0.07 – 8.78	0.13
Patient Relapse				
No	54	0.96	0.09 – 24.54	
Yes	64	0.78	0.07 – 15.62	0.21
Patient Death				
No	74	1.17	0.09 – 24.54	
Yes	44	0.76	0.07 – 15.62	0.05

[a] WAF1 concentrations expressed in Units/mg.
[b] See Materials and Methods section for details of patient stratification by each variable.
[c] p-values determined from Wilcoxon Rank Sum tests with continuity correction, or Kruskal-Wallis tests, where appropriate.

tumor diameter greater than 1 cm). A trend suggesting that serous ovarian carcinomas may have had higher p53 concentrations than all other histologic types was also revealed. As shown in Table VIII. 2., none of these other clinicopathologic variables was significantly associated with WAF1 concentrations by Wilcoxon Rank Sum analysis, but trends indicated possible associations between WAF1 expression and patient age less than 55 years, early stage (I-II) disease, and small (<1 cm) residual tumor size.

VIII. 4. 3. Relationships Between Each Clinicopathologic Variable and Patient Response to Treatment

The assessment of clinical response to platinum-based adjuvant treatment of 72 patients with measurable (≥ 1 cm) post-operative lesions enabled comparison of the distributions of p53-negative and p53-positive specimens between patients who exhibited complete response to chemotherapy, partial response, stable disease, or progressive disease. Table VIII. 3. presents this comparison, which demonstrated that tumor extracts containing p53 protein at levels exceeding the median concentration were more frequently obtained from patients who did not respond to treatment. In contrast, WAF1-positivity status, patient age group, histologic grade and histologic type were statistically unrelated to the classification of patients into treatment response groups. The relationship between disease stage and response could not be statistically evaluated because all patients in stages I or II had complete response to adjuvant chemotherapy (data not shown).

Table VIII. 3. Associations Between Clinicopathologic Variables and Response to Adjuvant Chemotherapy

	Number Responding (and %)				
Factor[a]	Complete Response	Partial Response	Stable Disease	Progressive Disease	p-Value[b]
p53 Status					
< Median	20 (63)	10 (31)	2 (6)	0 (0)	
≥ Median	16 (40)	12 (30)	5 (13)	7 (17)	0.03
WAF1 Status					
< Median	19 (54)	11 (31)	3 (9)	2 (6)	
≥ Median	17 (46)	11 (30)	4 (11)	5 (13)	0.76
Age (years)					
< 55	16 (52)	11 (35)	3 (10)	1 (3)	
≥ 55	20 (49)	11 (27)	4 (10)	6 (14)	0.48
Grade					
1	2 (100)	0 (0)	0 (0)	0 (0)	
2	12 (80)	2 (13)	1 (7)	0 (0)	
3	22 (40)	20 (36)	6 (11)	7 (13)	0.11
Histologic Type					
Serous	13 (38)	15 (44)	3 (9)	3 (9)	
Others	23 (61)	7 (10)	4 (10)	4 (10)	0.12

[a] See Materials and Methods section for details of patient stratification by each variable.
[b] p-values determined from 2-tailed Fisher Exact tests.

VIII. 4. 4. p53 and WAF1 as Indicators of Ovarian Cancer Survival

Several approaches, including comparisons of Kaplan-Meier survival plots and fitting of Cox proportional hazards regression models, were used to show associations between patient post-operative prognosis and concentrations of p53 and WAF1, considered individually, in combination with each other, and jointly with the other prognostic factors. A relationship between p53 and patient survival had already been suggested by findings that p53 concentrations were higher in tumor extracts from patients who relapsed or died during their follow-up periods (Table VIII. 1.). The similar analysis for WAF1, shown in Table VIII. 2., revealed reduced levels of WAF1 in tumors of patients who died of ovarian cancer. Consistent with these preliminary results with respect to p53 were the findings of regression analysis, by which the RRs for both relapse and death were shown to be significantly increased for p53-positive patients when p53 was classified into two groups based on the median (Table VIII. 4.). The use of the median cutoff for p53 also indicated 50% and over 90% increased risks for relapse and death, respectively, of p53-positive patients in multivariate analysis adjusted for all of the other variables except histologic type. Furthermore, by classifying patients into four groups based on the quartiles of the p53 distribution, it was shown that patients in the second, third, and fourth quartiles had successively increasing risks for both relapse and death compared with patients in the first quartile. However, in the corresponding multivariate models, these dose-response trends did not reach statistical significance. The differences in the survival rates over time between p53-negative and p53-positive patients are shown in Figure VIII. 2. Whereas these results established p53 to be an independent prognostic factor in our series of ovarian cancer patients, both univariate Cox regression (Table VIII. 4.) and Kaplan-Meier analysis (Figure VIII. 3.) revealed that WAF1-negativity based on a median cutoff value was not associated with relapse and death rates. Use of either the 25^{th} or 75^{th} percentiles as cutoff points for defining WAF1-positivity similarly did not lead to significant differences in DFS or OS between WAF1-negative and WAF1-positive patients (data not shown). On the other hand, because there was evidence of a trend for median-dichotomized WAF1-negative patients to have increased risk for death, and given the prognostic value of median-dichotomized p53, a composite three-level variable was created and evaluated in the Cox regression analysis.

Table VIII. 4. Associations Between p53 and WAF1 Concentrations and Disease-free and Overall Survival

p53 or WAF1 Status[a]	Disease-Free Survival			Overall Survival		
	RR[b]	95% CI[c]	p-Value[d]	RR[b]	95% CI[c]	p-Value[d]
Univariate Analysis of p53 (n=120)						
Based on Median Cutoff Point						
Negative	1.00			1.00		
Positive	1.64	1.01 – 2.67	0.04	2.75	1.41 – 5.32	<0.01
Based on Quartiles[e]						
First Quartile	1.00			1.00		
Second Quartile	0.66	0.31 – 1.41		0.77	0.25 – 2.45	
Third Quartile	1.10	0.72 – 1.44		1.43	0.89 – 2.30	
Fourth Quartile	1.28	0.96 – 1.47		1.51	1.06 – 1.97	
p-Value for Trend			0.04			<0.01
Univariate Analysis of WAF1 (n=118)						
Based on Median Cutoff Point						
Positive	1.00			1.00		
Negative	1.14	0.68 – 1.78	0.56	1.35	0.75 – 2.44	0.17
Univariate Analysis of p53-WAF1 (n=118)[f]						
p53(–), WAF1(+)	1.00			1.00		
p53(+), WAF1(+) or p53(–), WAF1(–)	1.35	0.76 – 1.93		1.16	0.52 – 2.57	
p53(+), WAF1(–)	1.44	0.96 – 2.73		1.38	0.92 – 2.08	
p-Value for Trend			0.07			0.06
Multivariate Analysis of p53 (n=120)[g]						
Based on Median Cutoff Point						
Negative	1.00			1.00		
Positive	1.50	0.63 – 2.17	0.05	1.92	0.93 – 3.96	0.03
Based on Quartiles[e]						
First Quartile	1.00			1.00		
Second Quartile	0.73	0.31 – 1.72		0.69	0.11 – 4.30	
Third Quartile	1.12	0.76 – 1.46		1.52	0.94 – 3.16	
Fourth Quartile	1.31	0.98 – 2.75		1.77	0.99 – 3.35	
p-Value for Trend			0.09			0.07

[a] p53-positivity and WAF1-positivity based on median cutoff points.
[b] RR, relative risk estimated using the Cox proportional hazard regression model.
[c] CI, confidence interval.
[d] p-values are two-sided.
[e] Estimated RR for second, third, and fourth quartiles compared with the first quartile are given. p-values are based on 1 degree of freedom tests of monotonic association.
[f] Estimated RR for second and third groups compared to first group are given. p-values are based on 1 degree of freedom tests of monotonic association.
[g] Multivariate analysis adjusted for age, stage, grade, and residual tumor size.

Patients in the first category, expected to have the best prognosis, were defined as having tumors that were p53-negative and WAF1-positive. Patients who had either p53-positive, WAF1-positive tumors, or p53-negative, WAF1-negative tumors, were members of the

second group. Having the anticipated worst prognosis were patients in the third group, whose tumor extracts were p53-positive and WAF1-negative. As shown in Table VIII. 4., although the P for trends were of borderline significance, this analysis suggested that the combination of increasing p53 concentrations and decreasing WAF1 concentrations was associated with higher risks for relapse and death. In addition to p53-positivity, other clinicopathologic features indicative of poor prognosis in multivariate Cox models were late stage (II-IV) malignancy, associated with a RR for relapse of 9.08 (95% CI, 3.89 to 21.20; p<0.01) and a RR for death of 33.44 (95% CI, 4.59 to 243.43; p<0.01), poorly differentiated (grade 3) tumors, associated with RRs for relapse and death of 9.14 (95% CI, 2.87 to 29.11; p<0.01) and 16.01 (95% CI, 2.21 to 116.56; p<0.01), respectively, and residual tumor size greater than 1 cm, associated with RRs of 11.25 (95% CI, 5.89 to 21.51; p<0.01) and 23.30 (95% CI, 7.20 to 75.38; p<0.01) for relapse and death, respectively.

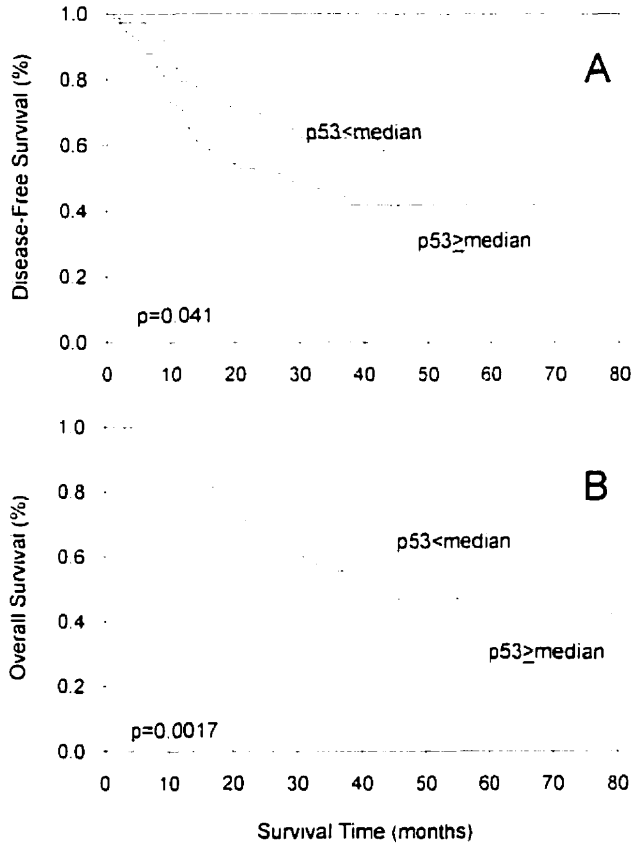


Figure VIII. 2. Kaplan-Meier Analysis of p53-Negative and p53-Positive Patients
DFS (A) and OS (B) of 120 patients treated with first-line chemotherapy were calculated. The median p53 concentration was used as the cutoff point for p53-positivity. p-values were determined by log-rank tests.

VIII. 5. Discussion

The majority of patients treated surgically for epithelial ovarian cancer subsequently receive systemic therapy, most often with platinum-containing antineoplastic regimens although taxol, cyclophosphamide, or other agents are also employed individually or as polychemotherapy. Resistance to these drugs may be reflected, in part, by higher rates of relapse and death, and is thought to be multifactorial

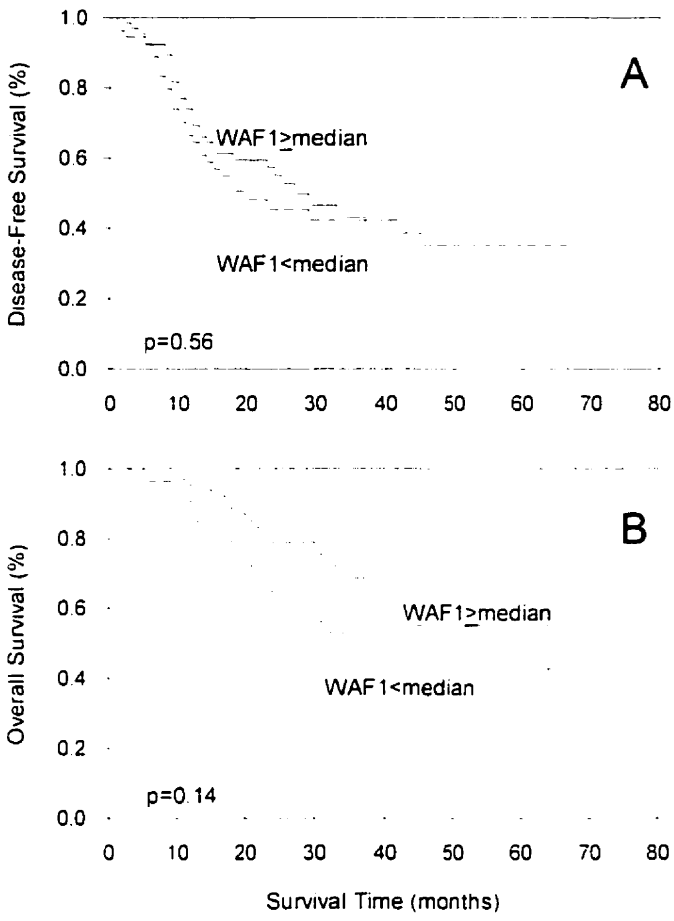


Figure VIII. 3. Kaplan-Meier Analysis of WAF1-Negative and WAF1-Positive Patients
DFS (A) and OS (B) of 120 patients treated with first-line chemotherapy were calculated. The median p53 concentration was used as the cutoff point for p53-positivity. p-values were determined by log-rank tests.

in origin (Coukos and Rubin, 1998). Several molecular factors likely contributing to loss of chemosensitivity in ovarian carcinoma have been identified, including proteins mediating the transport and cellular turnover of drugs as well as those involved in DNA repair and other nonspecific defense mechanisms. It has become apparent that conventional chemotherapeutic agents exert their function ultimately via the cellular machinery governing cell cycle progression and programmed cell death, and that the pathways regulating these processes are fundamentally perturbed in cancer cells (Hickman, 1996). Playing a central role in both processes is p53, alterations of which are strongly associated with chemoresistance and radioresistance in hematologic malignancies (Wattel *et al.*, 1994). In the majority of solid tumors, however, a correlation between p53 mutation and prognosis or chemotherapy response has not been consistently demonstrated. For instance, although a number of studies have shown an association between p53 alteration and poor prognosis of ovarian cancer patients (Klemi *et al.*, 1995; Levesque *et al.*, 1995), other studies have contradicted these findings (Hartmann *et al.*, 1994; Eltabbakh *et al.*, 1997). Similarly, evidence implicating the involvement of p53 in resistance of ovarian neoplasms to chemotherapy, provided primarily by the detection of mutations or

deletions in the p53 gene in chemoresistant human ovarian cancer cell lines (Eliopoulos *et al.*, 1995; Fajac *et al.*, 1996; Perego *et al.*, 1996;), has been accompanied by other reports showing that chemotherapy-induced apoptosis may occur in the absence of functional p53 (Zaffaroni *et al.*, 1995; De Feudis *et al.*, 1997) and that cisplatin resistance may develop independently of p53 alterations (Brown *et al.*, 1993; Vikhanskaya *et al.*, 1997). Clinical studies of the effect of p53 gene status on the response of ovarian cancer patients to adjuvant cisplatin-based chemotherapy have also emerged and have yielded findings suggestive of a role for p53 as a determinant of chemosensitivity (Righetti *et al.*, 1996; Buttitta *et al.*, 1997). The effect of cisplatin-paclitaxel combination treatment for advanced ovarian cancer, on the other hand, was shown not to be influenced by p53 mutation in another study (Smith-Sorensen *et al.*, 1998). To the best of our knowledge, none of these clinical studies has additionally assessed the expression of the WAF1 protein, high levels of which have paradoxically been associated with chemoresistance in acute myelogenous leukemia patients (Zhang *et al.*, 1995). Because WAF1 has been shown to be induced by cisplatin in both chemosensitive and chemoresistant human ovarian carcinoma cell lines (Delmastro *et al.*, 1997) and to be not absolutely correlated with p53 expression levels in normal and malignant ovarian epithelial cells (Elbendary *et al.*, 1995), it remained possible that WAF1 expression in ovarian tumors might predict cisplatin responsiveness independently of p53 expression. Considering this possibility, we studied the prognostic and predictive implications of both p53 and WAF1 expression levels in epithelial ovarian cancer.

Quantitative immunoassays were used to determine the expression levels of p53 and WAF1 in 120 tumors from patients treated with platinum-based adjuvant chemotherapy. For each protein studied, a continuous distribution of concentrations was revealed to be present in the tumor extracts. The immunofluorometric procedure used to assay p53 levels in these extracts was developed in our laboratory (Levesque *et al.*, 1995b), and has been validated by comparison of its findings to p53-immunostaining of matched formalin-fixed, paraffin-embedded lung tumors (Levesque *et al.*, 1997) and to sequencing of exons 5 to 9 of the p53 gene in ovarian carcinomas (Lianidou *et al.*, 1999). Comparisons of p53 concentrations between patients with different pathologic features, treatment responses as defined by standard criteria, and risks for relapse and death

estimated by Cox regression analysis demonstrated significantly increased p53 concentrations in tissues from patients with more aggressive, treatment-refractory ovarian cancers. Comparisons of WAF1 concentrations between the same groups of patients did not reveal significant differences, suggesting that tumor tissue levels of this protein may not have been deterministic of prognosis or chemotherapy response in the patients studied. Our findings are concordant with those of other groups reporting the independent prognostic value of p53 protein expression in ovarian carcinoma (Bosari *et al.*, 1993; Klemi *et al.*, 1995), as well as with our own previous study of a smaller sample of epithelial ovarian cancer patients for whom details of the chemotherapy regimens and responses were unavailable (Levesque *et al.*, 1995c). Also in agreement with our previous study and with those of other workers (Kupryjanczyk *et al.*, 1993; Milner *et al.*, 1993) were the higher concentrations of p53 protein measured in serous ovarian carcinomas compared to other histotypes. Our results also complement the small number of recent studies which have suggested a correlative relationship between p53 alterations and clinical response of ovarian cancer to chemotherapeutic agents (Righetti *et al.*, 1996; Buttitta *et al.*, 1997). However, our other findings that neither the assignment of treatment response category nor the probability of DFS or OS were shown to be affected by the WAF1 levels in the ovarian tumor extracts are novel, but consistent with the lack of an absolute negative correlation between WAF1 and p53 expression levels found here and elsewhere (Elbendary *et al.*, 1995; Ozcelik *et al.*, 1995), as well as with *in vitro* observations that anticancer drug sensitivity is not always dependent on WAF1 expression (Delmastro *et al.*, 1997). Also novel, in the authors' opinion, is the detection of WAF1 protein in ovarian tumor extracts by an immunoassay instead of immunostaining. Although the two procedures were not performed in parallel in order to validate the results of the commercially-developed WAF1 immunoassay, our confidence in the latter's results, at least qualitatively, was provided from the assay of extracts prepared from cell lines for which the expression status of WAF1 was already known.

The relationship between the p53 overexpression status of primary ovarian carcinoma specimens obtained at surgery and the subsequent designation of response to first-line chemotherapy was examined in a subset of patients. Although our results suggest that patients with elevated p53, arbitrarily defined as having p53 concentrations

exceeding the median value, were more likely to exhibit treatment failure, they must be interpreted cautiously. Because the majority of patients received cytotoxic agents in addition to cisplatin or carboplatin, it remains possible that the effects of these other drugs may have modulated the treatment responses independently of p53 status. Moreover, our demonstration in multivariate regression analysis adjusted for stage, residual tumor presence, age, and histotype that p53 was an independent prognostic factor in our sample of chemotherapy-treated patients does not necessarily lead to the conclusion that p53 is predictive of treatment response. Over half of the patients in our series, in fact, received second-line chemotherapy with various agents which might have contributed to relapse-free and overall survival. For these reasons, the results of our investigation must be confirmed by other studies of epithelial ovarian cancer patients receiving single agent therapy.

In summary, the quantitative analysis of p53 and WAF1 proteins in extracts of ovarian carcinomas confirmed the prognostic value of p53 and provided evidence that p53 protein accumulation may predict responsiveness to post-operative chemotherapy. The assessment of WAF1 expression in ovarian cancer, however, was shown to be of questionable clinical value. Despite these latter observations, future studies of larger numbers of ovarian carcinoma patients with more restricted treatment regimens might clarify the prognostic and predictive values of WAF1 and p53 in combination.

CHAPTER IX

GENERAL DISCUSSION

IX. 1. ELISA as an Alternative to Immunostaining and DNA Sequencing for the Detection of p53 Alterations

Since p53 mutations have been found in about half of all common cancers and because p53 plays a central role in cellular responses to DNA damage and other stresses, numerous studies have been conducted to examine if p53 status has clinical implications for patients with various malignancies. Clinical studies are often limited by access to sufficient numbers of suitable specimens, and therefore misclassification of even a few tumors can dramatically affect the conclusions, obscuring circumstances where a correlation might otherwise exist, or suggesting weak associations when in fact there are none (Elledge, 1996). Consequently, proper classification of tumors is central to the interpretation and utility of studies relating p53 status to diagnosis, prognosis or as a predictive factor for therapy response. The methods used to determine p53 status are limited as to which types of mutations or alterations in p53 they will detect. p53 function can be eliminated by several mechanisms, including mutation, deletion, heteromeric protein interactions or by extragenic mutations in the p53 pathway (Wallace-Brodeur and Lowe, 1999)

It has become apparent that methods which detect p53 protein accumulation, as indirect measures of p53 mutational status, are not completely accurate. Both ELISA and IHC are based on the fact that p53 mutations often stabilize the protein, leading to higher steady-state p53 levels than in wild-type cells (Davidoff *et al.*, 1991). However, not all mutations stabilize p53; consequently, ELISA and IHC detection would tend to underestimate mutation frequency in tumors with deletions, frameshift, or nonsense p53 mutations (Casey *et al.*, 1996). By contrast, wild-type protein is upregulated in response to DNA damage, hypoxia or activated oncogenes; this might produce an overestimate of p53 mutation frequency (Barnes *et al.*, 1992; Sjogren *et al.*, 1996). Regulatory stabilization of p53 may also occur by binding of mdm2 (Wu *et al.*, 1993). Our own results in a relatively small series of ovarian carcinomas support this imperfect concordance between p53 mutation *per se* and p53 protein accumulation; although 10 out of 12 missense mutations (of 16 mutations in total) of p53 in exons 5 to 9 were accompanied by p53 overexpression, increased p53 levels were also present in extracts prepared from 5 of 39 ovarian tumors without demonstrated p53 mutations. While the

correlation between p53 mutation and p53 protein overexpression is seldom 100%, it may be dependent, at least in part, on the techniques used to detect p53 protein accumulation.

Wynford-Thomas (1992) was the first to point out that IHC analysis for p53 suffers from a lack of technical and classification standards resulting in subjective evaluation of results. There are no standard criteria for defining positive p53 immunostaining, so a tumor that is classified as mutant in one study may be defined as wild-type in another study. An arbitrary threshold must be set for the percentage of cells staining which distinguishes p53-positivity and the position of this threshold varies and in many reports is not clearly defined. In many other studies, the threshold is chosen to give the best separation between categories with respect to clinical outcome – a practice of questionable validity (Altman *et al.*, 1994). In addition, different p53 antibodies do not always give the same results (Jacquemier *et al.*, 1994). Indeed, the monoclonal antibodies commonly used for p53 immunostaining often recognize different epitopes present on different domains of the p53 protein that might not be exposed in a given specimen due to the effects of tissue fixation (Vojtesek *et al.*, 1992; Bartek *et al.*, 1993) or to subtle differences in the conformational effects of different mutations (Stephen and Lane, 1992; Legros *et al.*, 1994). Because studies use different antibodies and criteria to define p53 status by IHC, it is difficult to compare results between them, and this may account for some of the discrepancies in correlating p53 to prognosis or therapy response.

Possibly free from some, but not all, of the pitfalls of IHC are ELISA methods to ascertain p53 status (Diamandis and Levesque, 1995). In principle, these procedures have greater analytical specificity due to the rigorous washing steps, effective immunopurification of antigen from background signal-eliciting tissues, and the use of two p53-specific antibodies rather than the single p53-specific antibody used in IHC. Tissue fixation variability and pretreatment effects are also eliminated. Moreover, the results of quantitative ELISAs are inherently more objective because they can be evaluated by numerical cutoff values, simplifying the statistical analysis and quality control. As in the case of IHC, however, the selection of this cutoff point should be made on objective, rather than arbitrary, criteria in order for the findings to have applicability outside of the particular dataset studied. Because of these potential advantages over IHC, combined with the recognized expertise in the development and clinical applications of

immunoassays present in our laboratory, we developed two p53-specific ELISAs – an original method evaluated in numerous cell lines (Hassapoglidou *et al.*, 1993) and in breast (Hassapoglidou *et al.*, 1993; Levesque *et al.*, 1994, 1995a) and ovarian (Levesque *et al.*, 1995c) tumor specimens, and a newer method described in this thesis and evaluated in breast (Levesque *et al.*, 1998b, 1998c), lung (Levesque *et al.*, 1997, 1998a), and ovarian (Levesque *et al.*, 1999) tumors as well as in patient serum specimens (Levesque *et al.*, 1996). A small number of other ELISA methods for p53 quantification have been developed by other groups (Vojtesek *et al.*, 1992; Borg *et al.*, 1995; Thomas *et al.*, 1997), and commercial methods are also available (Oncogene Research, Cambridge, MA and Dianova, Hamburg, Germany). Direct comparisons of these immunoassays regarding analytical performance have not yet been performed, although the published detection limits of our new method and the luminometric ELISA developed by Borg *et al.* (1995) are similar. These same two immunoassays have also been performed in parallel with IHC. Our comparison between ELISA and IHC methods revealed a 65% correlation between p53 concentrations and numerical immunostaining scores, respectively, and found 38 out of 43 ELISA-determined p53-positive lung cancer cases to be p53-positive by IHC. Norberg and coworkers (1998), although not directly comparing the results of luminometric immunoassay and IHC performed on breast tumors, presented data suggesting a general concordance between the methods when several p53 cutoffs for the immunoasay and IHC scores were used to stratify the patients. Other comparisons between ELISA and IHC likewise found the methods to be in close, but not absolute, agreement (Joypaul *et al.*, 1993; Vojtesek *et al.*, 1993) or even suggested that ELISA was more sensitive than IHC (Thomas *et al.*, 1997). There are at present only two published comparisons between a p53 ELISA and DNA sequencing of the p53 gene. In addition to comparing ELISA to IHC, Norberg *et al.* (1998) reported that the sensitivity and specificity of their luminometric assay for p53 mutations, identified by cDNA sequencing, were 65% and 90%, respectively, while the corresponding values were 72% and 92% for IHC. In their study, however, the cutoff points for both ELISA and IHC were optimized by assessing the prognostic values of arbitrarily selected cutoffs, whereas in our study described in this thesis (Levesque *et al.*, 1999), where the sensitivity and specificity of ELISA for DNA sequencing of exons 5 to 9 were 63% and 87%,

respectively, the cutoff point for ELISA was chosen arbitrarily on the basis of the distribution of p53 concentrations without knowledge of the clinical implications. As mentioned above, complicating the interpretation of all of these comparative studies are the variations at every step in the IHC methodologies employed. This caveat is true also for the different immunoassays, as they are based upon different tissue extraction methods, antibodies, detection systems, and cutoff levels for classification of p53 overexpression status. What is evident, however, is that ELISAs are an alternative to IHC for detection of p53 protein accumulation in tumor tissue. Important disadvantages of ELISA methods relative to IHC are the requirement for fresh, frozen tissue with which to prepare extracts of soluble proteins, and the loss of information relating p53 expression to cellular and histologic structures resulting from the gross homogenization of the tissues.

Direct sequence analysis is perhaps the most precise method for determining p53 mutation status. In some instances, direct sequencing has identified correlations between p53 and prognosis when IHC has not (Sjogren *et al.*, 1996), or that missense mutations occuring at particular codons encoding key residues in the p53-DNA interaction were the most strongly related to prognosis (Berns *et al.*, 1998). However, sequence analysis for p53 remains more expensive, time-consuming, and sensitive to normal cell contamination. Furthermore, although p53 is most frequently inactivated by a point mutation affecting one of more than 100 different codons (Greenblatt *et al.*, 1994) and producing an altered protein with a single amino acid substitution, there is evidence that many mutant proteins do not completely abolish the activity of wild-type p53 (Fearon and Vogelstein, 1990). An alternative technique that utilizes p53 gene sequence information is the yeast functional assay (Flaman *et al.*, 1995), which takes advantage of the fact that functional p53 transactivates reporter genes when expressed in yeast. p53 cDNA can be isolated from tumor cells and expressed in yeast – a color change indicates whether the tumor-derived p53 is transcriptionally active and hence, whether it is mutant or wild-type. This technique is able to distinguish between silent p53 mutations and those which disrupt p53 transcription, but it does not identify mutations in modifiers of p53 activity or downstream effectors and requires total RNA, not easily isolated from archival specimens. Immunostaining for both p53 and the p53-regulated gene $p21^{WAF1}$ has also been proposed to indirectly differentiate mutant p53 tumors (p53-positive, $p21^{WAF1}$-

negative) from tumors with activated wild-type p53, which also have increased expression of p53-inducible genes (p53-positive, $p21^{WAF1}$-positive) (Bukholm *et al.*, 1997). Although this approach would be expected to increase the predictive value of p53 immunostaining for p53 mutation detection and thereby for clinical outcome, in our study of 120 ovarian carcinoma patients, ELISA-detected $p21^{WAF1}$ combined with similarly-determined p53 did not lead to enhanced prediction of relapse or death rates compared to the prognostic information provided by p53 alone.

IX. 2. Serum p53 Protein as a Tumor Marker

Major contributions of the clinical laboratory to the management of cancer patients include the applications of immunoassays, which are used primarily for the detection of serum tumor markers such as prostate-specific antigen (Chan *et al.*, 1987), carcinoembryonic antigen (McPherson *et al.*, 1973), α-fetoprotein (Alpert, 1975), CA125 (Shelley and Fish, 1986) and many other proteins (Chan and Sell, 1994), although they may also be applied to extracts of tumors for the quantification of steroid hormone receptors (Raam and Vrabel, 1986; Metaye *et al.*, 1987) or oncogenes such as HER2/neu (Sias *et al.*, 1990) and c-myc (Spandidos *et al.*, 1989). It is because of their potential to provide information relatively noninvasively, and therefore for early diagnosis and monitoring of cancer, that serum tumor markers have fostered so much interest despite the fact that none of the existing markers are truly cancer- or tissue-specific. Studies showing that mutant p53 protein accumulates in the majority of malignant tissues (Hollstein *et al.*, 1991) raised the possibility that this nuclear protein might enter the general circulation, perhaps as a consequence of cellular necrosis or apoptosis within the tumor (Winter *et al.*, 1992). Athough the notion of p53 as a tumor marker has not gained widespread acceptance, some investigators have reported the presence of serum p53, detected by immunoassays, in a variety of cancer types including breast (Rosanelli *et al.*, 1993), lung (Luo *et al.*, 1994; Braun *et al.*, 1995; Partanen *et al.*, 1995), colon (Greco *et al.*, 1994), pancreatic (Suwa *et al.*, 1997), and hematologic (Lehtinen *et al.*, 1996) malignancies. Other studies (Winter *et al.*, 1992; Hassapoglidou *et al.*, 1993), however, had failed to reproduce these findings. Considering our previous observation of spurious background signals elicited by the application of our original immunoassay to serum

specimens, together with the disagreement in the literature and lack of unequivocal evidence of serum p53 even in those studies which did report its presence, we undertook a careful screen of cancer patient sera for the presence of p53 protein using a new "serum-optimized" immunoassay. Our negative findings were consistent with those recently reported by other workers (Segawa *et al.*, 1997; Krajewska *et al.*, 1998) and suggested that previous positive findings may have been due to heterophilic antibodies or other sources of nonspecific interferance in serum. To our knowledge, this possibility remains to be tested. Application of our new immunoassay to serum specimens in parallel to their assay by methods employed by other authors reporting the presence of serum p53 has not been performed. Therefore, our speculation that these other immunoassays are more prone to artifactual signals in sera has not been empirically demonstrated.

IX. 3. Prognostic and Predictive Values of p53 Protein Accumulation

Besides the combination of general interest in alterations of p53 as a key molecular abnormality in a very high proportion of human neoplasms, and the widespread availability of robust immunological reagents for the detection of p53 overexpression, there is strong theoretical justification for analysis of p53 to be a potentially important prognostic and predictive factor. p53 functions as a stress response gene, whose product acts to maintain genetic stability (Janus *et al.*, 1999) and to induce cell cycle arrest and apoptosis in the presence of genotoxicity and other stresses (Hartwell and Kastan, 1994). Loss of p53 function, by allelic loss or by mutation, has been shown to result in increased genetic instability and facilitates gene amplification and consequent changes in ploidy (Livingston *et al.*, 1992). The involvement of p53 in the induction of apoptosis after genotoxic insult would also lead to the proposal that loss of p53 function, and loss of the apoptotic response, would allow the survival of cells with an increased mutational load. Consequently, it would be reasonable to expect that loss of p53 function would be associated with tumor progression. Elegant confirmation of this idea has come from the analysis of transgenic animals (Symonds *et al.*, 1994) and is supported by observations in humans (Ziegler *et al.*, 1994). Finally, accumulating evidence indicates that a functional p53 pathway may be required for efficient cell killing by chemotherapeutic agents (Lowe *et al.*, 1994). Although these model systems demonstrate

the potential for p53 status to affect prognosis and chemosensitivity, the relevance of p53 in clinical tumors is more difficult to correlate because of the additional alterations within tumors that may effect outcome. Given that the ultimate goal of identifying prognostic and predictive indicators is that they might eventually guide oncologists in the design of appropriate treatment regimens, it has been proposed that those indicators that are most directly related to tumor progression and chemosensitivity at the molecular level should be the most extensively studied in the clinic (Wallace-Brodeur and Lowe, 1999).

The hypothesis that abnormalities of p53 may be indicative of a poor prognosis has been tested by the analysis of many cohorts of patients with particular neoplasms. The interpretation of these studies must take into account the technical and other caveats mentioned above that may confound their results. Also important for the interpretation of these studies is the demonstrated independence of p53 as a prognostic variable from other better-characterized predictors of tumor behavior. For example, it is possible that assessing p53 may merely substitute for analyzing other variables, such as histologic grade or stage, which are parameters of known prognostic significance in themselves. Multivariate analysis on data derived from statistically meaningful populations is therefore essential. Moreover, additional support for associations between p53 alteration and prognosis of a given malignancy would be provided by testing a second independent cohort of patients, using the same analytical methods, reagents, interpretive criteria, and statistical data handling used in the first patient cohort. Based on a literature review of almost 400 papers focused on p53 and cancer prognosis, Dowell and Hall (1995) noted that the majority of studies published up to 1994 were inadequate in regard to the caveats mentioned above. The same authors also observed a correlation between the number of cases studied and the likelihood of p53 being a prognostic factor: of the smaller studies, a high proportion found p53 to be of no prognostic value. In contrast are other multivariate studies of larger patient populations of p53 alterations in breast cancers using IHC (Silvestrini *et al.*, 1992, 1996a, 1996b; Allred *et al.*, 1993; Friedrichs *et al.*, 1993) or molecular techniques (Bergh *et al.*, 1995; Sjogren *et al.*, 1996) that did report p53 to have independent prognostic value, emphasizing the need for large study groups with high statistical power and implying that p53 is only a relatively weak prognostic factor. But even when these criteria are met, some studies of p53 in defined breast cancer

populations were unable to demonstrate significant independent associations between p53 and survival (Isola *et al.*, 1992; Caleffi *et al.*, 1994; Pietilainen *et al.*, 1995; Rosen *et al.*, 1995). In part, this may reflect the same caveats, as well as differences in clinical endpoints of interest. For example, in some studies p53 was found to be a prognostic factor for relapse-free survival, but not for overall survival, or vice versa (Gasparini *et al.*, 1994; Marks *et al.*, 1994). A similar discussion could have been based on the larger studies of tumors at other anatomical sites (Goh *et al.*, 1995; Soong *et al.*, 1996; Eltabbakh *et al.*, 1997; Kawasaki *et al.*, 1997; Marx *et al.*, 1998), and relates equally to clinical studies of the relationship between p53 and treatment response (Weller, 1998).

The studies described in this thesis which related p53 protein concentrations to patient prognosis satisfied, on an individual basis, most if not all of the attributes of an informative clinical study. That is, they employed multivariate statistical analysis and examined patient cohorts of moderate (n=86) to very large sizes (n=998) which were independent of cohorts of breast (Levesque *et al.*, 1998b) and ovarian (Levesque *et al.*, 1995c) cancer patients studied previously using the same or very similar methodology. Unlike those IHC studies which did not incorporate computerized image analysis in order to provide objective quantification of immunostaining results, each of the studies reported here were able to demonstrate approximate dose-response relationships between p53 protein expression and risks for cancer relapse and death. These risks for unfavorable outcomes differed depending on the tumor type, being greatest for non-small cell lung tumors [400-1700% increased risks for DFS and OS, comparing patients in the fourth to the first quartiles] and being least for breast tumors [40-60% increased risks] – consistent with other studies suggesting that the prognostic significance of p53 may be lower in breast cancers compared to other solid tumors (Kirsch and Kastan, 1998). Furthermore, analyses of clinically important patient subgroups revealed that increased p53 expression was of greater prognostic impact in squamous cell carcinomas than in adenocarcinomas of the lung, and in ER-positive, lymph-node-positive, and chemotherapy-treated breast cancer patients. That p53 protein levels were more strongly related to survival in breast cancer patients who had received a variety of chemotherapeutic drugs was of especial interest given the abundant *in vitro* and growing clinical evidence that p53 status may be partly deterministic of the efficacy of several antineoplastic agents in breast cancer (Lowe

et al., 1993, 1994; Aas *et al.*, 1996; Berns *et al.*, 1998a; Clahsen *et al.*, 1998). These latter findings partially supported the notion that chemotherapy response was p53-dependent, but because the patients had not been randomized to the different treatments, whether the effects of p53 were "mediated" by a particular class of drugs could not be determined. In a study of an even more well-characterized population of epithelial ovarian cancer patients, all of whom had received adjuvant platinum-based (cisplatin and/or carboplatin) chemotherapy, we demonstrated that p53 protein levels were indicative of not only DFS and OS, themselves indirect evidence for success of treatment, but also of clinical response assessed on the basis of changes in residual tumor sizes before and after treatment. Although the statistical analysis of clinical response was not multivariate, and therefore other factors, such as histologic type or grade (or other variables not assessed) could possibly have had confounding influences on the apparent relationship between p53 status and response to first-line chemotherapy, our data agree with other recent studies linking p53 mutations to platinum drug resistance of ovarian cancer cell lines (Pestell *et al.*, 1998) and of patients with the disease (Marx *et al.*, 1998). If future studies confirm that p53 mutations are associated with poor outcome or confer a worse prognosis when a particular cytotoxic agent is used, then identifying patients with tumors that carry these alterations may improve treatment selection.

IX. 4. Possible Future Studies

Although the data presented in this thesis provided evidence that the immunofluorometrically-determined p53 protein status of primary tumors may be useful for identifying lung, breast, and ovarian cancer patients at increased risks of relapse and death and/or of treatment failure, a number of questions were raised as well. These questions focus on: 1) the ability of p53 protein assessment by ELISA to indicate cancer outcome as accurately as p53 genetic analysis by rapid DNA sequencing technologies; 2) the possibility of more accurate cancer prognostication by considering p53 status in combination with additional histological, biochemical, and molecular factors; 3) the potential clinical utility of detectable p53 protein in as yet unassessed biological fluids; and 4) the relationship between p53 status and response to other anticancer agents in ovarian cancer and other neoplastic diseases. The following are some of the possible

studies that could be pursued and which may lead to a more clearly defined role for the immunoassay analysis of p53 in the management of cancer patients.

IX. 4. 1. Comparison of ELISA, Immunostaining, and DNA Sequencing for the Detection of p53 Alterations in Relation to Patient Prognosis

Our studies of the concordance between the new immunoassay and immunohistochemistry for assigning p53 status in lung tumors, and between the new assay and DNA sequencing for categorizing ovarian tumors on the basis of p53 status, were unable to evaluate the three methods with respect to the relative accuracies of the prognostic information they offered. Whereas in the first study follow-up information for the lung cancer patients was unavailable, in the second study the number of ovarian cancer patients included was judged to be too small (n=55) for meaningful survival analysis. Although in both studies the ELISA findings were in general agreement with those of the other method, there were numbers of discordant cases possibly sufficient to cause differences in survival analysis had it been performed. One future study would assess p53 status by several methods (the new ELISA, quantitative IHC, DNA sequencing of the most commonly mutated exons, enriched SSCP, or cDNA sequencing) performed in parallel on tumor specimens from a patient cohort of sufficient size and accompanied by adequate clinicopathologic and follow-up information to permit valid survival analysis. Recently, Norberg *et al.* (1998) reported the comparison of a luminometric immunoassay to immunostaining and cDNA sequencing in a series of 226 breast carcinomas and suggested that cDNA sequencing yielded superior prognostic information, in agreement with another study comparing cDNA sequencing to immunostaining (Sjogren *et al.*, 1996). As the luminometric immunoassay has not been directly compared to our immunofluorometric assay, it remains possible that our method may compare favorably to a DNA-based method in terms of clinical utility.

IX. 4. 2. Incorporation of ELISA-Determined p53 Status into Multivariate "Prognostic Panels"

Multivariate regression models of censored outcomes can be used not only to show that a variable has a statistically significant effect on outcome which is unaffected

by the influences of other variables, but can also determine the combination of variables that best explain the observations. There is growing interest in devising statistical models that could combine the prognostic information of dozens or even hundreds of individual variables (evaluable only in massive datasets), perhaps into an artificial neural network (Burke *et al.*, 1997; Mariani *et al.*, 1997; Naguib *et al.*, 1998). While the relative contribution of ELISA-determined p53 to survival of cancer patients could be examined in one of the large ongoing cohort studies, other studies restricted to a smaller number of potential prognostic factors could also be performed. For example, analogous to the evaluation of the hybrid p53-$p21^{WAF1}$ variable in patients with platinum-treated ovarian carcinoma presented herein, another study by our group assessed the prognostic value of a combined p53 and prostate-specific antigen variable and found it to lead to an enhanced ability to identify breast cancer patients at risk of unfavorable outcome (Yu *et al.*, 1998). Although p53 status could be combined similarly with any one of several biochemical, genetic, histological, or demographic variables of interest, a systematic and logical selection of factors will be necessary. In this regard, it has been suggested that the functional status of p53 homologs (Kaghad *et al.*, 1997), other participants in the "p53 pathway" such as Bax (Krajewski *et al.*, 1997; Ye *et al.*, 1998) and Bcl-2 (Bonetti *et al.*, 1998), proteins which bind and inactivate p53 such as Mdm2 (Leach *et al.*, 1993) and HPV E6 protein (Howley, 1991), proteins that modulate p53 such as ARF (Kamijo *et al.*, 1997), proteins in the "p16/Cyclin D/Rb pathway" (Dong *et al.*, 1997; Bukholm *et al.*, 1998), multi-drug resistance proteins (Kawasaki *et al.*, 1998; Siu *et al.*, 1998), tyrosine kinases such as HER2/neu (Thor *et al.*, 1998), telomerase (Roos *et al.*, 1998), and markers of proliferation such as MIB-1 (Domagala *et al.*, 1996; Siu *et al.*, 1998) should be considered along with p53. It will also be of interest to evaluate the prognostic value of $p21^{WAF1}$ in other cancer types, given that its expression in breast (Caffo *et al.*, 1996; Jiang *et al.*, 1997; Wakasugi *et al.*, 1997) and non-small cell lung (Bennett *et al.*, 1998; Caputi *et al.*, 1998) carcinomas has been associated with reduced risks for relapse and death, as well as to validate the $p21^{WAF1}$ assay results against other methods such as quantitative immunostaining.

IX. 4. 3. Screening of Other Biological Fluids for p53 Protein

The absence of p53 protein in serum does not preclude its presence in other biological fluids collected for cytologic examination. While p53 mutations have been detected in DNA from the cellular fraction of sputum (Mao *et al.*, 1994; Marchetti *et al.*, 1997), urine (Hruban *et al.*, 1994), bronchoalveolar fluid (Ahrendt *et al.*, 1999), and fine needle aspirates of breast tissue (Lavarino *et al.*, 1998; Rao *et al.*, 1998), and the value of p53 immunostaining has been assessed in urine cytology (Righi *et al.*, 1997) and fine-needle aspiration biopsy procedures (Sato *et al.*, 1997), to our knowledge p53 protein has not been examined in these specimen types by immunoassay. However, p53 protein has been detected in a small fraction (7%) of ascites fluids from ovarian cancer patients, but the clinical relevance of its presence was not investigated (Angelopoulou and Diamandis, 1997). More recently, an unpublished pilot study performed in our laboratory was unable to reveal the presence of detectable p53 protein in bronchial epithelial cells obtained by bronchoalveolar lavage of 10 patients with suspected lung carcinoma, although these negative findings might have been due to the low prevalence of malignancy among the patients studied. Lysates of cells obtained from additional lavage specimens from patients with documented bronchogenic carcinoma, or from the cellular components of ascites fluid associated with ovarian carcinoma or sputum associated with lung carcinoma, could be assayed for p53 protein by the new immunoassay in an effort to assess the diagnostic, staging, or prognostic utility of p53 protein in these cancers.

IX. 4. 4. Assessment of Relationship Between p53 Status and Resistance to Other Chemotherapeutic Agents or Radiotherapy

The dependence of cisplatin-sensitivity of ovarian carcinomas on functional p53, suggested by the results presented in this thesis together with the recent results of other clinical (Marx *et al.*, 1998) and *in vitro* (Vikhanskaya *et al.*, 1997; Pestell *et al.*, 1998; Poulain *et al.*, 1998) studies, supports the notion that the efficacy of other antineoplastic drugs might also be predicted by the p53 mutational and/or overexpression status. Findings that p53 accumulation was associated with response to fluorouracil, doxorubicin, and cyclophosphamide combination therapy in node-negative breast cancer (Clahsen *et al.*, 1998), and with response to tamoxifen in recurrent breast carcinoma

(Berns *et al.*, 1998) are consistent with this notion. However, this notion remains controversial because of the equally substantial number of studies in which p53 was unrelated to the responsiveness of a variety of chemotherapeutic agents when tested on cancer cell lines (Weller *et al.*, 1998) or in the clinical setting (Bonetti *et al.*, 1998). For example, sensitivity to taxanes has been found to be independent of p53 status in ovarian cancer cell lines (Perego *et al.*, 1998), astrocytic tumors (Iwadate *et al.*, 1998), and breast carcinomas (Makris *et al.*, 1997). The disagreement between these studies points to the need for the study of the relationship between p53 status and sensitivity to particular agents in well-defined patient groups.

IX. 5. Conclusion

The findings summarized in this thesis demonstrated the clinical utility of a new, well-characterized immunofluorometric assay for p53 protein applied to surgically-resected primary tumor specimens from patients with breast, ovarian, and non-small cell lung carcinomas. In each of these malignancies, p53 accumulation, as detected by elevated p53 concentrations in the tumor extracts, was associated with clinicopathologic factors indicative of aggressive disease. Furthermore, increasing p53 accumulation was shown to be associated with increasingly higher risks for cancer relapse and death, and the impact of p53 upon patient survival was shown in multivariate analyses to be independent of its associations with other markers of prognosis and possibly of greater magnitude in particular subgroups of patients. One of these subgroups of patients were those who received adjuvant chemotherapy for breast cancer. Additional evidence linking p53 and chemotherapy response was also suggested, in that failure of ovarian carcinoma patients to achieve a clinical response to platinum-containing chemotherapy may be related to p53 alteration status. It was therefore in tumor tissue, and not in serum, that the clinical utility of p53 protein could be shown, despite the fact that the immunoassay had been designed as a tool to exploit the potential role of p53 as a serum tumor marker. Our inability to detect specific p53 protein immunoreactivity in a large series of serum specimens from patients with lung cancer suggested that p53 is not present in cancer patient sera in sufficient amounts to facilitate its use in cancer diagnosis or monitoring. Because we have shown that the information yielded by the new immunoassay is

comparable, although not identical, to that provided by immunostaining and sequencing of the p53 gene, we recommend that further studies to clarify the prognostic and predictive values of p53 in defined patient subgroups should employ the new method.

CHAPTER X

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