IMMUNOREACTIVE P53 PROTEIN AS A PROGNOSTIC INDICATOR IN OVARIAN CARCINOMA

by

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Abstract

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In ovarian carcinoma, the prognostic significance of p53 protein accumulation, usually reflecting p53 gene mutation, remains to be established. Using a sensitive immunofluorometric assay for p53 protein quantification, we evaluated p53 overexpression in relation to patient survival and to clinicopathologic factors including age, stage, grade, histotype, tumor size, and presence of residual tumor. In Cox regression analysis, p53-positive patients exhibited two-fold higher risk for both relapse and death, but at the multivariate level, p53 accumulation was not independently associated with patient outcome. After stratification by factors associated with p53 status and outcome, patients with well or moderately differentiated cancers were shown to have higher risk of relapse ($p<0.01$) and death ($p<0.01$) when p53 protein accumulation was present. Kaplan-Meier analyses confirmed these findings, which together indicated that mutant p53 protein in ovarian carcinoma is associated with poor prognosis in patients with low grade tumors, early stage, or absence of residual tumor.

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List of Abbreviations

ABC	avidin-biotin complex
BCA	bicinchoninic acid
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
CBF	CCAATT binding factor
CDK	cyclin-dependent kinase
CEA	carcinoembryonic antigen
CK	creatine kinase
CSF-1	colony stimulating factor-1
DAB	diaminobenzidine
dNTP	deoxynucleoside triphosphate
dsDNA-PK	double-stranded DNA-activated protein kinase
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
FIGO	International Federation of Gynecologists and Obstetricians
GADD45	growth arrest and DNA damage-inducible 45

GaMIg	goat anti-mouse immunoglobulin
GaRIg-ALP	goat anti-rabbit immunoglobulin-alkaline phosphatase conjugate
HPV	human papillomavirus
IHC	immunohistochemistry/ immunohistochemical
IGF-II	insulin-like growth factor-II
IGF-BP3	insulin-like growth factor bindin protein 3
IRF-1	interferon regulatory factor-1
JNK1	jun amino-terminal kinase 1
LOH	loss of heterozygosity
LFS	Li-Fraumeni syndrome
MAPK	mitogen-activated protein kinase
MDM2	murine double minute 2
MDR1	multi-drug resistance 1
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PKC	protein kinase C
PR	progesterone receptor
PSA	prostate-specific antigen

RFLP	restriction fragment length polymorphism
ROC	Receiver Operator Characteristic
RPA	replication protein A
SDS	sodium dodecyl sulfate
SSCP	single-strand conformation polymorphism
STAT	signal transducers and activators of transcription
SV40	simian virus 40
TAF	TATA box-binding protein associated factors
TBP	TATA box-binding protein
TGF-α	transforming growth factor-α
UV	ultraviolet
VEGF	vascular endothelial growth factor
WAF1	wild-type p53 activated fragment 1
WHO	World Health Organization

Chapter 1. Introduction

Carcinoma arising from the germinal epithelium of the ovary has the highest mortality rate among gynecological malignancies and is the 4th leading cause of cancer death for women (American Cancer Society, 1995). Reflecting the lack of warning symptoms, absence of effective screening, and the proliferative capacity of this tissue, approximately 70% of patients present with advanced disease at diagnosis (Cannistra, 1993). Although ovarian cancer has a propensity to remain limited to the abdominal cavity, the prognosis of the patients is generally poor due to persistent residual or rapidly recurrent disease: the 5-year overall survival after surgical removal of malignancy does not exceed 30% for Stage III and 20% for Stage IV (Averette *et al.*, 1995). Moreover, ovarian cancer cells are highly invasive, have a high capacity to induce ascites, and present pleomorphic, usually drug-resistent phenotypes. Notwithstanding the latter fact, all patients, with the exception of those with low grade Stage I tumors, are recommended to receive postoperative chemotherapy. The identification of ovarian cancer patients at higher risk for recurrent disease or early death is of tremendous interest, as these individuals might benefit from more intense or prolonged therapy.

Since clinical outcome is determined primarily by tumor invasion and metastasis, it is not surprising that most prognostic markers are direct or surrogate measures of these events. Although much can be predicted for an individual patient on the basis of the disease stage, presence of ascites, tumor grade, and performance status (Omura *et al.*, 1991), and

on the serum CA-125 level (Bast *et al.*, 1983), there may be situations in which more precise information is required. A number of additional histologic or molecular features of ovarian tumors have been proposed to indicate residual tumor aggressiveness and hence risk for relapse or death. Particular interest has focused on recently described markers of proliferation [DNA index (Demirel *et al.*, 1996), S-phase fraction (Kaern *et al.*, 1994), Ki-67 index (Henriksen *et al.*, 1994), nm23 (Scambia *et al.*, 1996), proliferating cell nuclear antigen (PCNA) (Thomas *et al.*, 1995)], drug resistance and DNA repair [leukocyte platinum adducts (Gupta-Burt *et al.*, 1993), DNA excision repair helicase complexes (Dabholkar *et al.*, 1994), p170 (Kavallaris *et al.*, 1996), multidrug resistance proteins (Izquierdo *et al.*, 1995)], serum cytokine levels [colony stimulating factor-1 (CSF-1) (Scholl *et al.*, 1994), interleukin-6 (Plante *et al.*, 1994)], and signal transduction pathways [epidermal growth factor receptor (Bartlett *et al.*, 1996), c-erbB2 (Fajac *et al.*, 1995), serine-threonine kinases (Bellacosa *et al.*, 1995)] to name but a few. Most of these reports have been based on limited retrospective studies without informative multivariate analysis or confirmation in other studies. None of these new markers has yet proven influential in the management of patients with ovarian cancer.

Very likely, the molecule most studied for its potential to predict disease-free or overall survival, as well as response to postoperative treatment in virtually every human malignancy has been the p53 tumor suppressor protein. The tremendous interest in p53 is due to evidence that: p53 is the most frequently altered gene in human cancer (Hollstein *et al.*, 1991); it normally suppresses the growth of tumor cells containing multiple genetic

changes (Baker *et al.*, 1990); germ line mutation of p53 has been linked to an inherited predisposition to cancer, the Li-Fraumeni syndrome (Malkin *et al.*, 1990); increased amounts of cellular p53 protein after DNA damage have been associated with cell cycle arrest (Kastan *et al.*, 1991) and programmed cell death (Shaw *et al.*, 1992); and mutations or losses of p53 have been associated with gene amplification (Livingstone *et al.*, 1992) and polyploidy (Cross *et al.*, 1995). Observations that 40-80% of epithelial ovarian tumors may harbor p53 gene mutations (Kupryjanczyk *et al.*, 1993; Milner *et al.*, 1993; Kappes *et al.*, 1995), that some of these mutations are inherited in the germline (Buller *et al.*, 1993; Kupryjanczyk *et al.*, 1993), and that the same pattern of p53 alterations often occur at multiple sites of metastasis (Jacobs *et al.*, 1992) have strongly implicated p53 in the pathogenesis of ovarian neoplasia. Although p53 genetic abnormalities, as well as accumulation of mutant p53 protein usually accompanying missense mutations (Finlay *at el.*, 1988), have been shown to be associated with several other prognostic factors in ovarian cancer (Kupryjanczyk *et al.*, 1993; Renninson *et al.*, 1994; Kappes *et al.*, 1995; Klemi *et al.*, 1995), a few studies have demonstrated that p53 has independent prognostic value in this disease (Klemi et al., 1993; Klemi *et al.*, 1995; van der Zee *et al.*, 1995). Agreement has not been unanimous, however, since other studies have failed to correlate p53 alterations with survival of ovarian cancer patients (Kohler *et al.*, 1993a; Hartmann *et al.*, 1994; Niwa *et al.*, 1994; Sheridan *et al.*, 1994). While differences in the study populations may partly explain the discrepancies, methodologic differences, especially in the assessment of p53 overexpression in tumor tissue, may be far more important for studies in which p53 protein accumulation is under evaluation (Wynford-Thomas, 1992).

In contrast to all of these latter studies in which immunohistochemical (IHC) techniques were used to detect p53 protein, we employed a quantitative enzyme-linked immunosorbent assay (ELISA) to measure p53 protein concentrations in extracts of ovarian tumors from 90 patients and related p53 expression status to other clinicopathologic variables and to patient survival.

Chapter 2. Literature Review

2-1. An Historical Perspective of p53

First identified in 1979 by virtue of its association with simian virus 40 (SV40) large T antigen and by its apparently high expression in chemically induced tumors or spontaneously transformed cells (DeLeo *et al.*, 1979; Linzer *et al.*, 1979), p53 was initially classified as a tumor antigen. Further experiments suggested that p53 expression might be dominantly oncogenic, since transfection of cloned p53 cDNA into rodent cells of limited lifespan could result in their immortalization (Jenkins *et al.*, 1984) and p53 could cooperate with activated ras in transforming cells (Parada *et al.*, 1984). Besides SV40 large T antigen, adenoviral E1B 55 kDa protein was also found to form a tight complex with p53 protein (Sarnow *et al.*, 1982), suggesting a common pathway whereby DNA transforming viruses stimulate cell proliferation. Additional insight into the cellular role played by p53 was anticipated from studies showing that p53 production in nontransformed cells was increased after exposure to ultraviolet (UV) radiation (Maltzman *et al.*, 1984) and that p53 was found complexed to a 70 kDa heat-shock protein in transformed cells (Hinds *et al.*, 1987).

In the late 1980's, however, several discoveries defined the normal function of p53 to be anti-oncogenic. Whereas wild-type p53 could inhibit transformation induced by combinations of oncogenes (Finlay *et al.*, 1989) and could suppress the growth of cancer cells (Baker *et al.*, 1990), only mutant p53 could cooperate with ras in cellular

transformation (Hinds *et al.*, 1989). Furthermore, the screening of DNA from colon cancer patients revealed that chromosome 17p losses encompassing the p53 gene were usually found together with mutations affecting the remaining p53 allele, a theoretical hallmark of a tumor suppressor gene (Baker *et al.*, 1989). When these observations were extended to the other major forms of human cancer, p53 was shown to be the most commonly mutated gene in human malignancy (Nigro *et al.*, 1989).

As a result of the many thousands of manuscripts relating to p53 which have been published, especially over the past 5 years, great strides have been taken toward understanding the cellular functions of p53 and the consequences of its inactivation, as well as toward cataloguing the p53 alterations found in human tumors. Applications of this basic knowledge to the care of patients with cancer, in terms of early diagnosis, prediction of clinical outcome and response to treatment, and in the design of novel anticancer therapies, are presently the subjects of intense investigation.

2-2. Molecular Biology of p53

2-2-1. Structure of p53

2-2-1-1. Overview

Human p53 is a gene of approximately 20 kb, which contains 11 exons and codes for a protein of 393 amino acids (Lamb and Crawford, 1986). Sequence analysis of the coding

region has revealed five evolutionarily conserved regions (Soussi *et al.*, 1990). The first and smallest of these regions is located near the amino terminus and spans codons 13 through 19. The other four conserved regions are clustered more centrally in an area bounded by codons 100 to 300. Functionally, the p53 protein can be divided into thirds, containing the amino-terminal activation domain (residues 1-13), the central core which has sequence-specific DNA-binding activity (residues 100-300), and the multifunctional carboxy-terminal domain (residues 300-393).

2-2-1-2. The Amino-Terminal Activation Domain

Fusion of 72 amino acids from the amino terminus of p53 to a heterologous DNA-binding domain can activate transcription of a test gene (Fields and Jang, 1990). The function of this domain in transcriptional activation is also supported by the array of proteins to which it binds *in vitro*, including the TATA box-binding protein (TBP) component of the general transcription factor TFIID (Horikoshi *et al.*, 1995), several TBP-associated factors (TAFs) including *Drosophila* TAF40 and TAF60 (Thut *et al.*, 1995), human TAF31 (Lu and Levine, 1995), and the p65 subunit of the transcription/repair factor TFIIH (Wang et al., 1995). p53 also recognizes the eukaryotic single-stranded DNA-binding replication protein A (RPA) (p70) (Dutta *et al.*, 1993). The product of the MDM2 gene also binds p53 in this region (Oliner *et al.*, 1993). These interactions may be important for transcriptional regulation, as mutation of residues 22 and 23 renders p53 transcriptionally inactive (Lin *et al.*, 1994) and disrupts its interactions with TAFs and MDM2 (Lin *et al.*, 1994; Thut *et al.*, 1995).

2-2-1-3. The Central DNA-Binding Domain

The vast majority of p53 missense mutations in tumors are clustered within the central core region of p53 (Hollstein *et al.*, 1994), which contains the sequence-specific DNA-binding domain (Pavletich *et al.*, 1993). The three-dimensional co-crystal structure of this domain bound to its cognate site has been solved (Cho *et al.*, 1994), revealing a structure not similar to any other known DNA-binding protein. Several specific points, including codons 248 and 273 which are mutational hotspots, directly contact the DNA. Rather than zinc fingers, there are two antiparallel β sheets which anchor three loop-based elements; two of these loops contact the DNA, while the third stabilizes the shape of the region. At least two tumor antigens, SV40 large T antigen (Jenkins *et al.*, 1988) and human papilloma virus (HPV) E6 protein (Mansur *et al.*, 1995), and two cellular proteins, p53BP1 and p53BP2 (Iwabuchi *et al.*, 1994), bind this region.

2-2-1-4. The Multifunctional Carboxy Terminus

The carboxy terminus of p53 can function as an autonomous domain capable of nonspecifically binding to different forms of DNA, including damaged DNA (Lee *et al.*, 1995b), and reannealing complementary single strands of DNA or RNA (Balkalkin *et al.*, 1994). The carboxy terminus can be further subdivided into three regions, a flexible linker (residues 300-320) that connects the DNA-binding domain to the tetramerization domain, the tetramerization domain itself (residues 320-360), and, at the extreme carboxyl terminus, a stretch of 30 amino acids which is rich in basic residues (residues 363-393). The structure of the tetramerization domain consists of a β- sheet-turn-α-helix

motif that can homodimerize; the p53 tetramer contains a pair of such dimers (Jeffrey *et al.*, 1995). Although it is known that the minimal region of mutant p53 necessary for cellular transformation localizes to this oligomerization domain (Shaulian *et al.*, 1992), the normal role of this domain is not clear, since observations that p53 oligomerization is required for DNA binding (Pietenpol *et al.*, 1994) are contradicted by findings that this domain is dispensible for sequence-specific trans-activation (Shaulian *et al.*, 1993).

One mechanism whereby the transcriptional properties of p53 might be regulated is autoinhibition by the last 30 amino acids of the carboxy terminus. Several groups have found that p53 is stimulated to specifically bind DNA when this region is deleted (Hupp *et al.*, 1992), bound by antibody or DNA-activated kinase (Hupp *et al.*, 1992; Halazonetis *et al.*, 1993) or phosphorylated by protein kinases casein kinase II (Hupp *et al.*, 1992) or protein kinase C (Takenaka *et al.*, 1995). DNA binding may also be stimulated by peptides spanning a region within the last 30 amino acids of the carboxy terminus (Hupp *et al.*, 1995). These findings prompted the hypothesis that the carboxy terminus functions to regulate the conversion of p53 between forms which are inactive or active for DNA binding, in a manner similar to allostery. Regulation of p53 by this region is probably more complex, however, given the observations that different domains of p53 recognize single-stranded DNA and internal DNA segments (Bakalkin *et al.*, 1995), and that the p53 carboxy terminal monoclonal antibody PAb421 stimulates sequence-specific DNA binding but inhibits nonspecific interaction and reannealing by p53 (Jayaraman and Prives, 1995). In one configuration, p53 might be inhibited for DNA binding but might

remain active for other activities, whereas the second conformation might allow sequence-specific DNA binding but preclude nonspecific interactions with DNA.

2-2-2. Signaling Pathways Upstream of p53

It is likely that there are multiple pathways by which p53 can be induced, potentially resulting in an increase in the level of p53 and conversion of p53 from an inactive to an activated form for DNA binding. Most cells contain very small quantities of wild-type p53, which displays a turnover rate on the order of minutes. Irradiation of cells either with ionizing radiation or UV light induces p53, most likely post-transcriptionally (Kastan *et al.*, 1991), and the presence of DNA strand breaks appear critical for this response (Nelson and Kastan, 1994). p53 has also been proposed to mediate a more general stress response to suboptimal growth conditions, in light of findings that it is induced by hypoxia, heat, and nutrient starvation (Zhan *et al.*, 1993; Graeber *et al.*, 1994). For instance, p53 induction and an associated G1 arrest was found recently to be induced by ribonucleotide depletion in the absence of DNA damage, prompting the suggestion that p53 serves as a metabolite sensor (Linke *et al.*, 1996).

Although accumulation of p53 in virally transformed cells was thought to be caused by complex formation with viral proteins known to interact with p53, this is not always the case. For instance, SV40 T antigen has been shown to stabilize p53 without physically interacting with it (Bargonetti *et al.*, 1992). Moreover, the expression of either adenovirus E1A or the E7 protein of oncogenic forms of HPV, both of which bind the retinoblastoma

protein but not p53, have also been shown to stabilize p53 (Lowe and Ruley, 1993; Demers *et al.*, 1994b). The cellular product of another tumor suppressor gene, WT1, also stabilizes p53 without direct interaction (Maheswaran *et al.*, 1995).

Studies of HPV have also revealed that it has evolved a mechanism to inactivate p53 by degradation mediated by the virally encoded E6 protein. Formation of a complex of E6, p53, and a cellular protein, E6-AP, targets p53 for ubiquitin-dependent proteolysis (Scheffner *et al.*, 1993). This same pathway may normally degrade p53 in cells, since a cell line with a defect in the ubiquitin pathway was shown to accumulate p53 protein (Chowdary *et al.*, 1994).

Recent experiments showing that the form of p53 inactive for DNA binding may be converted into an active state *in vitro* have suggested that p53 functional activity *in vivo* might be regulated after DNA damage without gross changes in cellular p53 content. For example, transcription from a p53-responsive reporter construct was shown to be stimulated after microinjection of cells with an antibody against a p53 carboxy-terminal epitope (Hupp *et al.*, 1995). Another very interesting possibility is that redox conditions may also regulate the DNA binding ability of p53 such that oxidation inhibits DNA binding while reduction favors it (Hainaut and Milner, 1993). It is thought that oxygen radicals, produced in response to various stresses including ionizing radiation, might alter the redox state of specific cysteine residues in the central core of p53 implicated in zinc

ion coordination and thereby affect the conformation of the DNA binding domain. (Hainaut and Milner, 1993).

Because p53 is phosphorylated at a number of serines and threonines within its amino- and carboxy-termini, the participation of kinases and phosphatases in the regulation of p53 function has also been postulated. Protein kinases shown to phosphorylate p53 include cyclin-dependent kinases (CDKs), casein kinases I and II (CKI and CKII), double-stranded DNA-activated protein kinase (dsDNA-PK), and protein kinase C (PKC) (Meek, 1994). Mitogen-activated protein kinase (MAPK) (Milne *et al.*, 1994), Jun amino-terminal kinase 1 (JNK1) (Milne *et al.*, 1995), and Raf kinase (Jamal and Ziff, 1995) have also been shown to phosphorylate p53. Although it has been reported that hyperphosphorylation of p53 catalyzed by CKII (Hupp *et al.*, 1992), PKC (Takenaka *et al.*, 1995), and CDKs (Wang and Prives, 1995) may increase DNA binding by p53, the overall significance of p53 phosphorylation is uncertain since other studies have found no effect of p53 phosphorylation status on p53 function (Slingerland *et al.*, 1993; Fiscella *et al.*, 1994).

Other intriguing possible mechanisms of p53 regulation include negative autoregulation whereby p53 may bind to its own mRNA and specifically inhibit translation (Mosner *et al.*, 1995), and stimulation of p53 DNA binding by short single strands of DNA of the sort generated during nucleotide excision repair processes (Jayaraman and Prives, 1995).

2-2-3. Regulation of Gene Expression by p53

2-2-3-1. Overview

Since almost every tumor-derived p53 mutant has lost its ability to bind DNA and induce expression of nearby genes (Vogelstein and Kinzler, 1992b), the transcriptional activation function of p53 is most probably a major component of its biological effects. After the determination of the consensus DNA binding site of p53 (El-Deiry *et al.*, 1992), the human genome has been estimated to contain several hundred p53-binding sites (Tokino *et al.*, 1994). A potentially large number of genes may therefore be under the control of p53 transcriptional regulation. The list of p53 target genes compiled to date suggests that p53 may be involved in modulating cell cycle control, apoptosis, DNA repair, differentiation, angiogenesis, and growth factor signalling. However, whether all of these genes are true p53 response genes may be questionable. Discussed below are candidate p53-regulated genes which have functions that are especially relevant to the biological functions of p53.

2-2-3-2. GADD45

Expression of one the *g*rowth *a*rrest and *D*NA *d*amage-inducible genes, GADD45, was shown to be increased when cells are subjected to DNA damage leading to arrest in the G1 phase of the cell cycle (Kastan *et al.*, 1992). A p53 consensus sequence has been found in intronic sequences of GADD45, and in many, but not all, types of cells, GADD45 induction after DNA damage is p53-dependent (Kastan *et al.*, 1992; Lu and

Lane, 1993). It is thought that GADD45 may participate in the cellular response to DNA lesions by interacting with the replication and repair factor PCNA, thereby inhibiting entry of cells into S-phase (Smith *et al.*, 1994).

2-2-3-3. MDM2

The *m*urine *d*ouble *m*inute number *2* gene, MDM2, is amplified in a subset of human sarcomas and encodes a protein that binds to p53 and inhibits p53-mediated transactivation (Momand *et al.*, 1992; Oliner *et al.*, 1993). The MDM2 gene itself contains p53 binding sites in its first intron and can be activated in response to UV radiation (Barak *et al.*, 1993). Taken together, these findings imply an autoregulatory feedback loop between p53 and MDM2 (Barak *et al.*, 1993) which prevents overactivity of p53 transcriptional induction, perhaps important during embryonic development (Jones *et al.*, 1995).

2-2-3-4. WAF1

The *w*ild-type *a*ctivated *f*ragment *1* gene, WAF1, also known variously as p21, cip1, sdi1 and mda6, depending on the reseach focus of the many groups identifying it, was the first gene to provide a molecular link between p53 induction and growth arrest. WAF1 was found to be highly conserved and showed p53-dependent induction in a number of vertebrate cell lines (El-Deiry *et al.*, 1993). The upstream regulatory region of WAF1 contains p53 DNA binding sites (El-Deiry *et al.*, 1993). Expression of the WAF1 protein, which binds to a wide range of cyclin/CDK complexes and to the DNA processivity

factor PCNA (Xiong *et al.*, 1993), has been shown to result in growth suppression of brain, lung, and colon cancer cell lines (El-Deiry *et al.*, 1993). Several studies have demonstrated that p53-dependent G1 arrest in response to irradiation is mediated, at least in part, by p53 induction of WAF1 (El-Deiry *et al.*, 1994; Deng *et al.*, 1995). However, the expression of WAF1 in many tissues of mice in which p53 has been homozygously deleted suggests that it is also transcriptionally regulated by p53-independent mechanisms (Michieli *et al.*, 1994), one of which was recently demonstrated to involve a transcription factor known as interferon regulatory factor (IRF)-1 (Tanaka *et al.*, 1996) activated by signal transducers and activators of transcription (STAT) proteins in response to γ-interferon (Chin *et al.*, 1996).

2-2-3-5. Other Target Genes Activated by p53

Although the volume of data supporting the involvement of GADD45, MDM2, and WAF1 in the mediation of the p53 response to genotoxic stress is impressive, the p53-inducibility of many other genes has also been demonstrated to varying degrees. Reported to be upregulated by p53 transactivation are: an apoptosis-promoting gene, bax (Miyashita and Reed, 1995); the insulin-like growth factor binding protein 3 (IGF-BP3) gene (Buckbinder *et al.*, 1995); the cyclin G gene, of unknown function (Okamato and Beach, 1994); the gene encoding thrombospondin-1, an inhibitor of angiogenesis (Dameron *et al.*, 1994); the gene for a novel transcription factor, HIC1, which is hypermethylated in cancer cells (Makos Wales *et al.*, 1995); the gene for PCNA (Shivakumar *et al.*, 1995); the transforming growth factor-α gene (TGF-α) (Shin *et al.*,

1995); the gene for the apoptosis-associated cell surface receptor FAS/apo1 (Owen-Schaub *et al.*, 1995); the EI24 gene following treatment with the cytotoxic drug etoposide (Lehar *et al.*, 1996); and eight newly isolated genes, of unknown function, expressed in p53-induced apoptosis (Amson *et al.*, 1996). As lengthly as it appears, the above list is not exhaustive.

2-2-3-6. Transcriptional Repression by p53

A number of genes lacking p53 consensus binding sites have been shown to be transcriptionally repressed by wild-type p53. These genes include: bcl-2 (Miyashita *et al.*, 1994); hsp70 (Agoff *et al.*, 1993); c-fos (Kley *et al.*, 1992); Rb (Osifichin *et al.*, 1994); basic fibroblast growth factor (bFGF) (Ueba *et al.*, 1994); multi-drug resistance 1 (MDR1) (Chin *et al.*, 1992); insulin-like growth factor II (IGF-II) (Zhang *et al.*, 1996a); and O^6-methylguanine-DNA methyltransferase (Harris *et al.*, 1996). Early data suggested that that only promoters containing TATA boxes were repressed (Mack *et al.*, 1993), and coupled with evidence that p53 interacts with TBP, a model was proposed in which p53 inhibits transcription by sequestering TBP from the basal transcriptional machinery (Mack *et al.*, 1993). Since p53 can also bind to specific TAFs, these may also be necessary for p53-mediated repression (Sabbatini *et al.*, 1995). In the case of the hsp70 promoter, however, p53-mediated repression likely occurs by an interaction between p53 and CCAAT-binding factor (CBF), an activator of transcription (Agoff *et al.*, 1993).

2-2-4. Induction of Cell Cycle Arrest by p53

Overexpression of wild-type p53 was initially shown to cause growth arrest of colorectal carcinoma cells (Finlay *et al.*, 1989), and later work localized the timing of the p53-specific growth suppression to the G1 phase of the cell cycle (Kastan *et al.*, 1991). When subjected to ionizing radiation, cells containing wild-type p53 show an induction of p53 and subsequent cell cycle arrest in G1 and G1 phases, whereas cells lacking p53 only undergo a G2 arrest (Kastan *et al.*, 1992). The principle pathway leading to cell cycle arrest following DNA damage is thought to involve the p53-mediated upregulation of WAF1, which encodes a CDK inhibitor able to inhibit cyclin E/CDK2 and cyclin A/CDK2 activities (Dulic *et al.*, 1994), allowing the accumulation of hypophosphorylated Rb (Demers *et al.*, 1994b), and resulting in radiation-induced G1 arrrest. Because overexpression of WAF1 itself can lead to arrested growth (Harper *et al.*, 1995), and mouse cells null for the WAF1 gene have defective cell cycle arrest after radiation exposure (Deng *et al.*, 1995), WAF1 is likely a key target of p53.

It was originally theorized that p53 functions as a "guardian of the genome" (Lane, 1992) such that DNA damage would trigger a transient G1 arrest in cells containing wild-type p53, allowing time for the repair of lesions to DNA before continuation of the cell cycle. More recent experiments, however, have shown that DNA damage may result in a prolonged and irreversible G1 arrest (DiLeonardo *et al.*, 1994) and that a significant G2 arrest may also be induced (Agarwal *et al.*, 1995; Aloni-Grinstein *et al.*, 1995). Moreover, other workers (Cross *et al.*, 1995), observing that fibroblasts from p53

knockout mice did not arrest in response to mitotic spindle inhibitors but, rather, would undergo multiple rounds of DNA synthesis without the appropriate chromosome segregation and consequently producing polyploid cells, proposed that p53 might also function as a mitotic checkpoint. Genetic instability associated with p53 loss may also be caused by the multiple copies of functionally competant centrosomes generated during a single cell cycle of p53-deficient mouse embryonic fibroblasts (Fukasawa *et al.*, 1996). Finally, it has been suggested that p53 may induce cell cycle arrest in a transactivation-independent manner though overexpression of the gas1 gene, which blocks the G_0 to S transition (Del Sal *et al.*, 1995).

2-2-5. Triggering of Programmed Cell Death by p53

While cell cycle arrest, mediated by transcriptional activation of p53 target genes, appears to be a consequence of p53 induction in many cell types, p53 also may cause apoptotic cell death in other cell lines (Shaw *et al.*, 1992), especially those of hematopoietic lineages (Yonish-Rouach *et al.*, 1991). These results were confirmed *in vivo* with the cancer-prone p53 knockout mice, thymocytes of which, subjected to ionizing radiation, were resistant to apoptosis in contrast to thymocytes with wild-type p53 (Clarke *et al*, 1993; Lowe *et al.*, 1993b). Mice that were heterozygous for wild-type p53 showed a slight resistance to thymocytic apoptosis, suggesting that precise levels of p53 may be important. Although several stimuli, including DNA damage (Clarke *et al*, 1993; Lowe *et al.*, 1993b), adenovirus E1A expression (Debbas and White, 1993; Lowe and Ruley, 1993), c-myc expression (Wagner *et al.*, 1994), or withdrawal of growth factors (Gottlieb

et al., 1994) can cause p53-dependent apoptosis, programmed cell death may also occur by pathways independent of p53 (Clarke *et al*, 1993b; Lowe *et al.*, 1993b). Inhibition of p53-mediated apoptosis has been shown to occur in the context of bcl-2 or adenovirus E1B 19 kDa protein overexpression (Debbas and White, 1993), as well as by the expression of growth factors including IL-3, IL-6, and erythropoietin (Yonish-Rouach *et al.*, 1993; Gottlieb *et al.*, 1994).

It is generally agreed that p53-mediated apoptosis may be an important mechanism by which oncogene-expressing cells are deleted, thereby suppressing tissue transformation as well as tumor growth and progression (Lowe *et al.*, 1994b; Symonds *et al.*, 1994). For example, exposure of cells to potentially mutagenic physiological conditions, such as hypoxia (Graeber *et al.*, 1996) which almost always occurs in solid tumors, and UV radiation (Ziegler *et al.*, 1994) of the skin, has been shown to induce p53-dependent apoptosis.

In contrast to its function in mediating G1 arrest, the ability of p53 to trigger apoptosis may not require transcriptional activation. Studies demonstrating that p53-dependent cell death occurs in the presence of the transcriptional inhibitor actinomycin D or the translational inhibitor cycloheximide (Caelles *et al.*, 1994; Wagner *et al.*, 1994), and others showing that a p53 mutant defective in translation was still a potent inducer of apoptosis (Haupt *et al.*, 1995) support this hypothesis. However, conflicting results were presented in another study which reported a requirement for the transcriptional activation

function of p53 for apoptosis (Yonish-Rouach *et al.*, 1994). Separate transcription-dependent and -independent modes of cell death may therefore be induced by p53, and the relative importance of each of these pathways may vary with the cell type (Haupt *et al.*, 1996). In fact, recent evidence supports a role for transcriptional repression in mediating apoptosis - two proteins which block p53-dependent apoptosis, E1B 19 kDa protein and bcl-2, were found to inhibit transcriptional repression but not to affect transcriptonal activation by p53. (Shen and Shenk, 1994).

2-2-6. Participation of p53 in DNA Replication and Repair

Data have been accumulating which implicate a function of p53 in DNA replication and repair. While the products of two different p53 target genes, WAF1 and GADD45, have been shown to interact with the replication- and repair-associated PCNA (Flores-Rozas *et al.*, 1994; Smith *et al.*, 1994, Waga *et al.*, 1994) and, in the case of WAF1, to inhibit the function of PCNA in replication (Flores-Rozas *et al.*, 1994; Waga *et al.*, 1994), p53 may itself directly regulate these processes. A number of proteins involved in DNA repair, including the 70 kDa subunit of RPA (Dutta *et al.*, 1993) which plays a role in both replication and repair, and polypeptide components of the transcription-repair factor TFIIH such as the DNA helicases XPD (ERCC2) and XPB (ERCC3) (Wang *et al.*, 1994; Wang *et al.*, 1995), have been shown to bind to p53. Both irradiated DNA and mismatched DNA may be bound by p53 (Lee *et al.*, 1995b). In addition, p53 has been shown to block DNA replication using two experimental systems (Cox *et al.*, 1995; Miller *et al.*, 1995). Reduced repair of DNA was observed in some cases associated with

p53-deficiency (Smith *et al.*, 1995; Wang *et al.*, 1995). Finally, wild-type, but not mutant, p53 protein has very recently been shown to exhibit 3'-to-5' exonuclease activity which has been mapped to the core domain of the protein (Mummenbrauer *et al.*, 1996). As impressive as this collective evidence may be, an equally voluminous collection of findings suggest that p53 is not involved in replication and repair of DNA. For example, other workers have reported that DNA replication may actually be stimulated by p53 *in vivo* (Kanda *et al.*, 1994), that p53 knockout mice have normal DNA repair rates (Sands *et al.*, 1995), and that p53 does not influence DNA repair *in vitro* (Leveillard *et al.*, 1995). These discrepancies clearly indicate the need for more experimentation.

2-2-7. Role for p53 in Embryonic Development

The mere fact that mice lacking both alleles of the p53 gene were viable implied that p53 was dispensible for growth, differentiation, and embryonic development (Donehower *et al.*, 1992). However, a more careful examination of a number of embryonic tissues revealed an unexpectedly high incidence of exencephaly in p53-null embryos, all cases of which were female (Sah *et al.*, 1995). Lethality of a subset of p53-deficient embryos was also observed in another study (Nichol *et al.*, 1995), in which the teratogenicity of embryos was found to be dependent upon the p53 genotype of the pregnant female mice. Embryonic lethality of MDM2-deficient mice has also been shown to be dependent on p53 status, such that mice null for MDM2 but with at least one normal p53 allele die *in utero* while the homozygous deletion of p53 was sufficient to rescue embryos from death associated with MDM2 deletion (Jones *et al.*, 1995; Montes de Oca Luna *et al.*, 1995).

2-3. p53 Dysfunction in Cancer

2-3-1. Prevalence of p53 Alterations in Malignancies

p53 genetic alteration occurs with extraordinarily high frequency in diverse types of human cancer. The analysis of screening data from more than 2500 cancers worldwide revealed that 37% contained mutation of the p53 gene (Greenblatt *et al.*, 1994). Tumor types differed markedly in their p53 mutation rates, where alterations of p53 were commonly present in lung (>50%), colon (>50%), esophageal (45%), ovarian (44%), pancreatic (44%), skin (44%), stomach (41%), head and neck (37%), bladder (34%), sarcoma (31%), prostate (30%), hepatocellular (29%), brain (25%), adrenal (23%), breast (22%), endometrial (22%), mesotheliolomal (22%), renal (19%), thyroid (13%), hematological (12%), carcinoid (11%),, melanoma (9%), parathyroid (8%), and cervical (7%) cancers. In contrast, Wilms tumors, testicular cancers, pituitary tumors, and pheochromocytomas rarely harbored p53 mutations. Not surprisingly, other literature focusing on p53 alterations in specific cancer types of patients in more defined risk groups and geographic locations often have reported p53 mutation rates differing from the summarized data given above.

The Li-Fraumeni syndrome (LFS) is characterized by early-onset malignancy, usually at multiple sites, and by the inheritance of p53 missense mutations (Malkin *et al.*, 1990). Common tumor types affecting LFS families include breast carcinomas, soft tissue sarcomas, brain tumors, osteosarcomas, leukemias, and adrenocortical carcinomas. Later

in life, these individuals are also highly susceptible to the development of lung cancer, prostate cancer, pancreatic cancer, melanoma, skin cancer, lymphoma, and stomach carcinoma.

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. 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 sequence-specific transcriptional activation (Vogelstein and Kinzler, 1992b). Approximately 40% of p53 mutations cluster in four of the evolutionarily conserved hotspots which encompass the DNA binding domain (Cho *et al.*, 1994). Interestingly, the spectrum of DNA base changes constituting mutations of p53 is distinctly different in various types of tumors (Greenblatt *et al.*, 1994). In fact, given evidence that certain mutations are associated with specific carcinogens, some mutagens might leave "fingerprints", depending on the site and type of DNA damage (Vogelstein and Kinzler, 1992a). 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). While G:C→A:T transitions are common in colon cancer, possibly resulting from deamination of methylated CpG sites, they are much less frequently detected in hepatocellular and lung carcinomas (Greenblatt *et al.*, 1994). On the other hand, G:C→T:A transversions are more prevalent in lung carcinoma than in colon and gastric cancers and are associated

with cigarette smoking (Miller *et al.*, 1992). p53 mutation at codon 249 is much more frequent in hepatocellular carcinoma, where hepatitis B and aflatoxin act as synergistic carcinogens, than in other cancers (Hsu *et al.*, 1991).

2-3-2. 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.*, 1993) 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.*, 1993). 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, is presently 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 in this tissue (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), which may also be induced by mutant p53 in synergy with PKC (Kieser et al., 1994).

Misssense 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). Alternatively, or in addition, gain-of-function mutants may associate with cellular proteins p38 or p42 (Chen *et al.*, 1994) or may synergize with PKC in the induction of the VEGF gene (Kieser *et al.*, 1994). 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).

2-3-3. Clinical Implications of p53 Alterations

2-3-3-1. Diagnosis and Monitoring of Cancer

The high prevalence of p53 mutations in human cancer suggests that p53 could be used as a marker of malignancy. In various cancer types, p53 mutational events may occur early or late in tumor progression. p53 mutations have been found in late or advanced stages of gastrointestinal cancer (Fearon and Vogelstein, 1990), prostate cancer (Navone *et al.*, 1993), ovarian cancer (Kohler *et al.*, 1993a), bladder cancer (Fujimoto *et al.*, 1992), cervical cancer (Tsuda and Hirohashi, 1992), endometrial cancer (Tsuda and Hirohashi, 1992), and liver cancer (Nishida *et al.*, 1993). In other cancers, however, including those of lung (Chiba *et al.*, 1990), head and neck (Boyle *et al.*, 1993), and breast (Thor *et al.*, 1992) origins, p53 mutations occur most often in early stages of disease. p53 alteration has also been associated with the progression of Barrett's epithelium to invasive esophageal carcinoma (Casson *et al.*, 1994), and the conversion of benign colonic adenoma to malignant colon carcinoma has been shown to correlate with the acquisition of p53 point mutation, theorized as one of the essential molecular events involved in colon cancer progression (Fearon and Vogelstein, 1992). Although p53 mutation can thus 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 (see below) 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.*, 1993), and the sputum of a series 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).

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. 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) may be possible since the anti-p53 immune response may be early events detectable before clinical manifestation of these diseases.

Carriers of a mutated p53 gene in families affected by LFS, 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).

2-3-3-2. Prognostic Value of p53

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 (Teneriello *et al.*, 1993), 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). 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 is not 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 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), nonsmall cell lung cancer (Mitsudomi *et al.*, 1994), endometrial cancer (Soong *et al.*, 1996), colorectal cancer (Goh *et al.*, 1995), prostate cancer (Bauer *et al.*, 1995), and renal cell carcinoma (Uhlman *et al.*, 1994).

2-3-3-3. p53 and Resistance to Treatment

Given that wild-type, but not mutant, p53 is able to induce programmed cell death under certain, although not yet clearly defined, circumstances, and that the effectiveness of many antineoplastic therapies correlate with their ability to induce apoptosis, it might be expected that the p53 functional status of a tumor would predict radio- or chemosensitivity (Fisher, 1994). Experimental evidence exists to support this hypothesis. Treatment of p53 +/+ mouse thymocytes with radiation resulted in apoptosis, whereas p53 -/- thymocytes were resistant. (Clarke *et al.*, 1993; Lowe *et al.*, 1993b). Similarly, treatment of mouse +/+ fibroblasts transformed with adenoviral E1A protein and the ras oncogene with either γ-irradiation or chemotherapeutic drugs resulted in apoptotic cell

death, whereas p53 -/- fibroblasts were resistant to apoptosis (Lowe *et al.*, 1993a). Using Burkitt lymphoma cell lines, similar results were obtained (Fan *et al.*, 1994). The therapeutic responsiveness of p53-deficient tumors to γ-radiation or adriamycin was found in another study to be impaired relative to tumors expressing wild-type p53 (Lowe *et al.*, 1994a). Using the same EIA- and ras-transformed fibroblasts injected into immunocompromized mice, these authors demonstrated radio- and chemosensitivity of tumors with wild-type p53, which underwent apoptosis and regression, compared to p53-deficient tumors which continued to enlarge and displayed few apoptotic cells. Another study found a correlation between radiosensitivity and the ability ot wild-type p53 to induce G1 arrest in a variety of cell lines; transfection of a dominant-negative p53 mutant into radiosensitive cells abrogated the G1 arrest and led to resistance to ionizing radiation (McIlwrath *et al.*, 1994). Sensitivity of gastric and esophageal cell lines to a number of antineoplastic drugs was also shown to be p53-dependent (Nabeya *et al.*, 1995).

Other evidence, however, suggests that p53 status might not necessarily predict therapeutic outcome. Experiments in which p53 in tumor cells was targeted for degradation by HPV16 E6 did not give rise to radio- or chemoresistance (Wu and El-Deiry, 1996). Some studies have, in fact, shown that inactivation or mutation of p53 renders cells more sensitive to cytotoxic agents whose primary mechanism of action is DNA damage (Fan *et al.*, 1995; Xu *et al.*, 1995). Radioresistance did not correlate with p53 mutation in a series of squamous cell carcinoma cell lines (Jung *et al.*, 1992). Sensitivity of ataxia-telagiectasia cells to cisplatin was also not p53-dependent (Zhang *et*

al., 1996b). Tamoxifen-sensitive breast cancer cells transfected with a mutant p53 cDNA did not become resistant and retained estrogen growth dependence (Elledge *et al.*, 1995). Treatment with tamoxifen, however, has been shown by other workers (Guillot *et al.*, 1996) to dramatically decrease p53 protein levels, leading to a loss of wild-type p53 response to genotoxic treatment. Very recently, however, the radioresistance of p53-deficient cells, which typically retain the ability to arrest at the G2/M transition, has been shown to be circumvented by treatment with caffeine, an inducer of $p34^{cdc2}$ kinase which is the universal determinant of entry into S-phase (Yao *et al.*, 1996). Although the different *in vitro* findings may be accountable by variable experimental conditions and cell types, it is the *in vivo* impact of p53 function that is of great clinical relevance. In one study, the later, drug-resistant stages of non-Hodgkin lymphomas were revealed to have p53 mutations far more frequently than lymphomas which did not progress to an aggressive phenotype (Lo Coco *et al.*, 1993). The mutational status of p53 was found to predict responsiveness to 5-fluorouracil regimens of patients with advanced colorectal cancers (Benhattar *et al.*, 1996), and in patients with breast cancer, p53 mutations affecting specific residues conferred greater resistance to doxorubicin than other mutations (Aas *et al.*, 1996).

2-3-3-4. Gene Therapy for p53

Several strategies to reconstitute normal p53 function in tumor cells which have p53 mutations are presently being explored. One of the most exciting involves the replacement of the p53 gene or its transcriptional targets with viral vectors. Successful

p53 gene therapy to replace lost or dysfunctional endogenous p53 would rely on a number of factors, including efficient deleivery of the expression vector to target tumor cells, sustained functional expression of p53 protein in the tumor cells, cell death following p53 expression, acceptable toxicity to normal cells, absence of a deleterious host immune response, and increased survival of the host. Replication-deficient adenoviral vectors have proven effective vehicles for the delivery of wild-type p53 into several prostatic (Srivastava *et al.*, 1995; Yang *et al.*, 1995), cervical (Hamada *et al.*, 1996), head and neck (Liu *et al.*, 1994), breast (Runnebaum *et al.*, 1995), and ovarian (Santoso *et al.*, 1995) carcinoma cell lines containing mutated p53 and resulted in growth suppression via G1 arrest and/or apoptosis in all cases. In breast cancer cells expressing wild-type p53 protein, recombinant adenoviral expression of wild-type p53 also led to cytotoxicity, mediated by apoptosis, but normal mammary epithelial cells were resistant to cytotoxicity mediated by gene transfer (Katayose *et al.*, 1995). Apoptotic cell death, associated with infection by a p53-expressing adenovirus, was also shown to be induced in breast cancer cells with a multidrug resistance phenotype (Blagosklonny and El-Deiry, 1996). Very recently, the ability of a retroviral vector containing the wild-type p53 gene to promote tumor regression of patients with lung cancer was evaluated in a small pilot study, which noted an absence of clinically significant toxic effects, an increase of apoptotic bodies present in posttreatment biopsies, and tumor regression or growth stabilization in most of the patients studied (Roth *et al.*, 1996b). Rather than using wild-type p53 to replace the absent or defective endogenous p53 of tumor cells, one group of workers have suggested using an engineered "dominant wild-type" p53 protein which

substituted the tetramerization domain for a leucine zipper-like coiled coil and could overcome transdominant inhibition by endogenous mutant p53 (Waterman *et al.*, 1996).

Under certain conditions, mutant p53 proteins can adopt wild-type properties. It was initially assumed that all tumor-derived p53 mutants were defective for sequence-specific transactivation. However, because mutations may affect different structures, and therefore specific properties, of p53 protein, not all p53 mutants would be expected to be equal with respect to normal function. For example, His-273 and Ala-143 mutants may show a degree of wild-type DNA binding and transcriptional activating activity, whereas other mutants are generally inactive in these assays (Chen *et al.*, 1993; Pietenpol *et al.*, 1994). The restoration of DNA binding and/or transcriptional activity of some p53 mutants has been shown to be accomplished by stimulation with PAb421 (Hupp *et al.*, 1992; Zhang *et al.*, 1993), incubation with the bacterial heat shock protein dnaK (Hupp *et al.*, 1992), using artificial, high affinity DNA binding sequences (Pietenpol *et al.*, 1994), or temperature shift (Zhang *et al.*, 1994). Translation of these basic findings into clinically applicable treatments for cancer has not yet been reported. Other interesting approaches of targeting p53 for therapeutic benefit include: 1) using antisense wild-type p53 oligonucleotides, which have been shown to cause growth inhibition when expressed in wild-type p53-containing HeLa cells (Iotsova and Stehelin, 1995), and 2) p53 immunotherapy, illustrated by a study showing that immunization of mice with a p53-expressing poxvirus protects them against the growth of tumors containing mutant p53 (Roth *et al.*, 1996a)

2-4. Analytical Methodology to Determine p53 Functional Status

2-4-1. Methods for Detecting p53 Genetic Abnormalities

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) 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. In one recent 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). The use of solid-phase, automated genomic DNA sequencing on a robotic workstation was demonstrated to detect p53 mutations in basal cell carcinoma of the skin (Hedrum *et al.*, 1994).

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), both of which, like SSCP, have sensitivities for detecting p53 mutation less than direct sequencing.

2-4-2. Methods for Detecting 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 IHC 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 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 IHC 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), gastrointestinal (Bartkova *et al.*, 1993; Joypaul *et al.*, 1993), vulval (Bartkova *et al.*, 1993), 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) and gastrointestinal (Joypaul *et al.*, 1993) tumor specimens.

2-4-3. Methods for Directly Assessing p53 Functional Status

Both mutational analysis of the p53 gene and IHC detection of accumulated p53 protein are, in a sense, surrogate measurements for the ability of the endogenous p53, if expressed at all, to function as a tumor suppressor. Because the p53 gene has been shown to be subject to a broad range of mutational events affecting different functional domains of the protein, various p53 mutants would be expected to differ in their abilities to bind to DNA and activate transcription of target genes or to inhibit the expression of other genes. Functional assays of p53 have been developed to screen for germline mutations in LFS family members at risk of disease although, in principle, they may also be applied for the evaluation of the functional capacity of p53 mutants present in sporadic cancers. Briefly, these methods involve cloning of the patients' cDNA, isolated from fibroblasts or lymphocytes, 1) *in vitro* into an expression vector followed by cotransfection into human

p53-deficient Saos-2 cells together with a reporter plasmid containing a p53-responsive element and the β-galactosidase gene for colony selection (Frebourg *et al.*, 1992), 2) *in vivo* by recombination in *Saccharomyces cerevesiae* yeast cells also containing a plasmid encoding the HIS3 gene expressed under a p53-responsive promoter allowing growth selection in media lacking histidine (Ishioka *et al.*, 1993), or 3) by an adaptation of the previous assay in yeast in which expression of the enzyme ADE2 is driven by p53 transcriptional activation, allowing color selection for identifying wild-type, mutant, and even temperature-sensitive p53 alleles (Flaman *et al.*, 1995). All of these assays were demonstrated to detect most tumor-derived p53 mutations, supporting the contention that the most important property of p53 with regard to tumor suppression is transcriptional activation through sequence-specific DNA binding.

Chapter 3. Hypothesis

Given that mutation of the p53 gene occurs frequently in ovarian carcinoma, that missense mutations are usually accompanied by overexpression of mutant p53 protein, and that loss of p53 function is thought to lead to genetic instability as a consequence of derangements in cell cycle control, apoptosis, and possibly DNA repair, it is hypothesized that the presence of mutant p53 protein accumulation in ovarian carcinoma tissues might indicate aggressive phenotypes and therefore identify patients with poorer prognoses compared with other ovarian cancer patients in whose tumors p53 protein is not overexpressed.

Chapter 4. Objective

A retrospective cohort study was to be performed in order to examine if p53 protein accumulation, determined using a time-resolved immunofluorometric assay of p53, in extracts prepared from primary ovarian carcinoma tissues, could predict reduced disease-free and/or overall post-surgical survival of ovarian cancer patients independently of other clinicopathologic features including disease stage, grade, histological type, residual tumor size, menopausal status, and patient age.

Chapter 5. Materials and Methods

5-1. Study Population

Ninety patients operated consecutively at the Department of Gynecologic Oncology of the University of Turin, Turin, Italy between 1989 and 1993 for treatment of primary epithelial ovarian carcinoma were included in this study. Five patients for whom tumor specimens were available had been excluded for various reasons (one had been diagnosed as having an ovarian lymphoma, two had provided primary breast cancer tissues, one had had a primary colon cancer metastatic to the ovary, and one patient had been lost to follow-up). The age range of these patients was 20 to 78 years, with a median age of 54 years. Patients were followed-up at the same centre for periods ranging from 1.3 months to 55.2 months; the median follow-up time was 22.2 months. Additional clinicopathologic variables for which the patients had been characterized at the time of surgery, including menopausal status, stage according to the criteria of the International Federation of Gynecologists and Obstetricians (FIGO, 1987; Beahrs *et al.*, 1992), and histologic grade and type based on World Health Organization (WHO) (Serov and Souly, 1973) criteria, are summarized for the patient population in **Table 5-1-1**. With the exception of patients with Stage IA/IB disease who did not receive post-surgical chemotherapy, all others were treated with cisplatin-containing regimens.

5-2. Optimization of Tissue Extraction Procedure

In order to identify a simple yet effective procedure to release soluble p53 protein from ovarian tumor specimens, six specimens for which sufficient tissue were available were each processed by three different cellular disruption methods performed in parallel: one used routinely at Sunnybrook Health Sciences Centre in Toronto, Ontario, to isolate estrogen and progesterone receptors from breast tumor tissues prior to their quantification (Dr. Donald J.A. Sutherland, personal communication), and two reported by other groups (Bartkova *et al.*, 1993; Lavigueuer *et al.*, 1989) to result in p53 protein solubilization from breast, colon, skin, gastric, lung, bone, and lymphoid tumors. The protein extraction method yielding the highest p53 levels would subsequently be applied to all of the ovarian tumors. For this experiment, a sample cut from a randomly selected surface of each of the six specimens was first immersed in liquid nitrogen and pulverized to a fine powder, and then partitioned into three approximately equal (0.2 g) portions.

One portion was suspended in 3 mL of homogenization buffer (0.01 mol/L Tris, pH 7.4, 1.5 mmol/L ethylenediaminetetraacetic acid (EDTA)-disodium dihydrate, and 5 mmol/L sodium molybdate) and homogenized on ice with a single 5 second burst of a "polytron" (Pro Scientific Inc. Monroe CT). Between-sample carryover was minimized by washing the rotor-stator in three consecutive volumes of distilled water after homogenization of each sample. Clarification of each homogenate, divided into three 1 mL volumes, was performed either by centrifugation at 100,000 g for 1 hour at 4°C in a Beckman L5-50 ultracentrifuge (Beckman Instruments, Fullerton CA) using a TY-65 rotor, or by

centrifugation at 15,000 g in an Eppendorf 5415C centrifuge (Brinkmann Instruments, Westbury NY) at 4°C either for 30 minutes or for 1 hour. In all cases, the supernatant was carefully removed and stored at -20°C for two days until total protein determination and p53 immunoassay (see below for both procedures).

A second portion was combined with 3 mL of a lysis buffer (50 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Nonidet NP-40, and 1 mmol/L phenylmethylsulfonyl fluoride), vortexed, and incubated on ice for 30 minutes. Equal (1 mL) volumes of each disruptate were either centrifuged at 15,000 g at 4°C for 30 minutes, as suggested by the authors describing this method (Bartkova *et al.*, 1993), or for 1 hour, or were ultracentrifuged at 100,000 g at 4°C for 1 hour as described above. As before, the supernatant fractions were stored at -20°C for 2 days until analysis.

A more harsh treatment of the pulverized tissue involved adding the tissue powder to 3 mL of RIPA buffer (50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mmol/L phenylmethylsulfonyl fluoride, and 0.05 mmol/L aprotinin) followed by homogenization, as described above, and by incubation of the tubes on ice for 30 minutes. Centrifugation of the homogenate, in two 1 mL volumes, was performed at 15,000 g at 4°C for either 5 minutes, as originally described (Lavigueuer *et al.*, 1989) or for 30 minutes, to collect the supernatants, stored afterwards at -20°C.

5-3. Total Protein Determination

A commercially available kit (Pierce Chemical Co., Rockford IL) based on the use of the bicinchoninic acid (BCA) detection reagent was used, as directed by the product literature for performing the "microtitre plate protocol", to assay the ovarian tumor extracts for total protein. Assayed in parallel were protein standard solutions, ranging in concentration from 0.05 to 2 g/L, which were made by serial dilution of a 2 g/L albumin standard with a buffer containing 50 mmol/L Tris, pH 7.4, and 7.5 mmol/L NaN_3. The same buffer was used to dilute tumor extracts whose initial total protein concentrations exceeded 2 g/L. Both standards and samples were assayed in duplicate. Ninety-six well, transparent polystyrene microtitre plates (Dynatech Laboratories Inc., Chantilly VA) served as the reaction vessels. Absorbance at 545 nm (close to the absorbance maximum of the reaction product, 562 nm) was measured on an ELISA plate reader (Bio-Tek Instruments Inc., Winooski VT). Calculation of protein concentrations in the tumor extracts, by interpolation from a linear calibration curve, was performed using Deltasoft ELISA Analysis software (BioMetallics Inc., Princeton NJ).

5-4. Time-Resolved Immunofluorometric Assay of p53 Protein

5-4-1. Brief Description

A noncompetitive "sandwich" immunofluorometric assay developed previously in our laboratory, a schematic of which is shown in **Figure 5-4-1-1**, was used to quantitate mutant p53 protein levels in the ovarian tumor extracts (Hassapoglidou *et al.*, 1993). The

assay involves capture of soluble p53, present in samples and calibrators, by monoclonal PAb240 antibody immobilized onto microtitre wells coated with goat anti-mouse immunoglobulin (GaMIg). Bound p53 is then detected by subsequently added polyclonal CM-1 antiserum and alkaline phosphatase-conjugated goat anti-rabbit antibody (GaRIg-ALP). Dephosphorylation of the enzyme substrate, diflunisal phosphate, yields a product which enters into a complex with Tb^{3+} and EDTA, capable of absorbing UV light at 336 nm and emitting long-lasting fluorescence at 615 nm. The use of lanthanide chelates as fluorescence labels and of time-resolved fluorescence has been shown to facilitate assay designs characterized by low background signals and hence high detection sensitivities (Christopoulos and Diamandis, 1992).

5-4-2. Reagents and Solutions

Dilution of the microtitre well-coating GaMIg antibody was made in 50 mmol/L Tris, pH 7.4, containing 7.5 mmol/L NaN_3. The wash solution was a 5 mmol/L Tris buffer, pH 7.8, containing 150 mmol/L NaCl and 0.5 g/L Tween 20. The blocking solution was a 50 mmol/L Tris buffer, pH 7.8, containing 10 g/L bovine serum albumin (BSA) and 7.5 mmol/L NaN_3. Diluents for the two anti-p53 antibodies, polyclonal CM-1 and monoclonal PAb240, were respectively a 50 mmol/L Tris buffer, pH 7.8, containing 60 g/L BSA and 7.5 mmol/L NaN_3, and the same buffer to which 0.5 mol/L KCl was also added. The latter buffer, supplemented with 10% goat serum, was also used to dilute the GaRIg-ALP. The stock enzyme substrate solution consisted of 0.01 mol/L diflunisal phosphate in 0.1 NaOH. For the assay, the stock substrate solution was diluted 10-fold in

the substrate buffer, a 0.1 mol/L Tris buffer, pH 9.1, containing 0.15 mol/L NaCl, 1 mmol/L $MgCl_2$ and 7.5 mmol/L NaN_3. The developing solution, containing 1 mol/L Tris base, 0.4 mol/L NaOH, 2 mmol/L $TbCl_3$, and 3 mmol/L EDTA, was used without pH adjustment.

5-4-3. Antibodies

With the exception of the PAb240 antibody, all antibodies used in the assay were purchased from commercial suppliers. Both the GaMIg and the GaRIg-ALP were Fc fragment specific, had been affinity purified to remove cross-reactivities with human serum proteins (and also with bovine and horse serum proteins in the case of the GaMIg), and were supplied as ~1 mg/mL preparations (Jackson ImmunoResearch Inc., West Grove PA). CM-1 antiserum, raised in rabbits against recombinant wild-type human p53 protein and recognizing both mutant and wild-type p53 (Midgley *et al.* 1992), was aliquoted and frozen at -20°C upon receipt as suggested by the manufacturer (Novocastra Laboratories Inc., Newcastle upon Tyne, UK) to prolong its stability.

Frozen PAb240 hybridoma cells were generously provided by Dr. David Lane (University of Dundee, Dundee, UK), and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 50 g/L gentamycin (all from Gibco BRL, Life Technologies Inc., Gaithersburg MD) according to standard practice (Harlow and Lane, 1988). Log-phase growing cells, in suspension, were allowed to reach a density of ~10^7 cells/mL before being centrifuged at 1000 g for 10 minutes to collect the supernatants,

which were used in the immunoassay without purification. Monoclonal PAb240 reacts only to p53 protein in a mutant conformation (Gannon *et al.*, 1990).

5-4-4. p53 Standards

A protein standard preparation was not commercially available at the time that this study was performed. In order to assign quantitative values to the results of the assay of the tumor extracts, an ovarian tumor extract with very high p53-associated immunofluorescence was selected as the calibrating material. Dilution of this extract, given an arbitrary p53 concentration of 1000 units per litre (U/L), in 50 mmol/L Tris buffer, pH 7.8, containing 60 g/L BSA and 7.5 mmol/L NaN_3 (PAb240 diluent), yielded standard solutions of 0, 2, 5, 20, 50, and 200 U/L which were aliquoted into small volumes, frozen at -80°C, and thawed immediately before use. Assay of these primary standards along with the extracts of the ovarian tumors allowed the relative p53 levels in the latter specimens to be determined by interpolation from the standard curve, which plotted the background-corrected raw fluorescence counts against the defined concentrations of the calibrator solutions. A series of standards were included in each 96-well microtitre plate. Extracts whose initial p53 protein concentrations exceeded 200 U/L were reassayed after dilution in PAb240 diluent. Assayed also in each run were extracts of breast tumors as controls, prepared by the method determined to be optimal (see above) and aliquoted for storage at -80°C until use, which had p53 protein levels of 1, 3, 10, and 80 U/L. Analytical features of the assay, including linearity, precision, and detection limit have been described previously (Hassapoglidou *et al.*, 1993).

5-4-5. Assay Procedure

Ninety-six well, white polystyrene microtitre plates (Dynatech Laboratories Inc., Chantilly VA) were incubated overnight at 4°C with 100 μL/well of the GaMIg diluted 400-fold in the coating buffer. After six washing cycles, the wells were blocked by the addition of 250 μL of blocking solution at least 30 minutes before the addition of the samples. Two cycles of washing were followed by the addition, in duplicate, of the ovarian tumor extracts to be assayed for p53, of the standards, and of control samples, all in 50 μL volumes, together with 100 μL of PAb240 hybridoma cell culture supernatant diluted 20-fold in the PAb240 diluent. The plates were incubated for 3 hours at 37°C on a shaker platform. All subsequent incubation steps were performed at room temperature, also on a shaker platform. After another six washing cycles, CM-1 antiserum was diluted 5000-fold in its diluent and added in 100 μL volumes to the wells for a 1 hour incubation. The plates were washed again as above before 100 μL of GaRIg-ALP, diluted 5000-fold in its diluent, was added for another 1 hour incubation. The final six cycle washing step was followed by the addition of 100 μL of the diluted enzyme substrate and incubated for 10 minutes. Developing solution was added in 100 μL volumes without washing the wells for a 1 minute incubation before fluorescence measurement.

5-4-6. Instrumentation

Two pieces of equipment were essential for the performance of the p53 immunoassay: an automated, programmable plate washer (Adil Instruments, Strasbourg, France), and a Cyberfluor 615 Immunoanalyzer (Cyberfluor Inc., Toronto, Canada) which could

measure fluorescence in a time-resolved mode directly from the microtitre plates. Calculation of calibration curves, using the cubic spline method, and thereby of p53 concentrations in the samples, was performed by the instrument software.

5-5. Determination of Tissue Specimen Heterogeneity for p53 Expression

To control for potential specimen sampling error, that is, for possible differences in p53 protein accumulation throughout the resected ovarian tumors, three pieces of tissue were cut from different surfaces of each tumor specimen and were independently pulverized, extracted using the optimal method (see above), and assayed both for p53 and total protein as described above. The final p53 protein value reported for each specimen was the mean of the three total protein-adjusted p53 protein concentrations determined in the separate extracts made from each specimen.

5-6. Determination of Reproducibility of Extraction Procedure

Because extracts made from different portions of each tumor might also have differed, to some extent, due to poorly reproducible extraction efficiencies, 10 tumors with consistently high protein-adjusted p53 concentrations (above 15 U/g in all 3 samples from each specimen), 5 cases with consistently low p53 levels (below 1 U/g), and 4 in which the 3 extracts made yielded discrepant p53 assay results, were further subjected to the following experiment. Only specimens with sufficient material available were

included. Approximately 0.50 g of each of the tumors was completely pulverized, divided into 4 equal portions (~0.15 g), and combined with 500 mL of extraction buffer. For each set of 4 portions, one pair was left on ice for 30 minutes, while the other pair was homogenized, as described above, before incubating it on ice for 30 minutes. All extracts were then assayed for both p53 and total protein. As an indicator of the variability of extraction, with and without homogenization, protein-adjusted p53 concentrations were compared within each pair, and between the two pairs prepared for each tumor specimen. Differences between the means of the pairs were examined using a Wilcoxon Signed Rank test. Precision, expressed as %CV, was calculated for each method as described elsewhere (Westgard, 1981).

5-7. Immunohistochemical Detection of p53

5-7-1. Reagents and Solutions

The formol calcium post-fixative consisted of a 4% formaldehyde solution containing 70 mmol/L $CaCl_2$. Phosphate buffered saline (PBS), used to dilute the normal (goat) blocking serum, the primary anti-p53 antibody, and the biotinylated secondary antibody, and also used for all of the washing steps, was a solution containing 150 mmol/L NaCl, 10 mmol/L Na_2HPO_4, 2 mmol/L KH_2PO_4, pH 7.2. The nonimmune goat serum and the horseradish peroxidase-conjugated avidin-biotin complex (ABC) were supplied in the Vectastain Elite ABC kit (Vector Laboratories Inc., Burlingame CA). The ABC solution was prepared by mixing together two reagents, avidin DH and the biotinylated peroxidase

H, respectively, at least 30 minutes before use. The peroxidase substrate solution, containing 3,3'-diaminobenzidine tetrahydrochloride (DAB), H_2O_2, and $NiCl_2$, was prepared from reagents provided separately by the same manufacturer.

5-7-2. Antibodies

DO-1 hydridoma cells, the frozen stocks of which were provided by Dr. David Lane (University of Dundee, Dundee UK), were 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, Life Technologies Inc., Gaithersburg MD) following standard techniques (Harlow and Lane, 1988). Cells were grown in log phase until reaching a density of $\sim 10^7$ suspended cells/mL, at which time they were centrifuged at 1000 g for 10 minutes to collect the antibody-containing supernatant. Monoclonal DO-1 antibody recognizes both mutant and wild-type p53 protein and has been shown to be suitable for immunohistochemical localization of p53 on sections fixed with a variety of agents (Vojtesek *et al.*, 1992). The biotinylated GaMIg, reacting against both heavy and light chains of mouse IgG, was provided in the Vectastain Elite ABC kit (Vector Laboratories Inc., Burlingame CA).

5-7-3. Specimen Preparation

Two ovarian tumor specimens were selected on the basis of very high (200 U/g) and low (0.60 U/g) p53 expression determined by immunoassay and subjected to IHC analysis to validate the p53 immunoassay findings. The p53-positive specimen by ELISA was from a

Stage IV, grade G2, serous ovarian carcinoma, whereas the p53-negative specimen was from a Stage III, grade G3, undifferentiated lesion. For each of the two, the entire specimen remaining after pulverization and extractions for both cellular proteins and nucleic acids (see below) was embedded in OCT medium (Miles Inc., Elkhart IN) while still frozen, and 6 μm "thin" sections were cut in a cryostat and placed on Superfrost/Plus slides (Fisher Scientific Co., Pittsburgh PA). After allowing to air dry for 15 minutes, the sections were fixed in acetone for 15 minutes and treated with formol calcium for 15 seconds with gentle agitation. Post-fixation in formol calcium had been demonstrated to increase the nuclear and cytologic detail of frozen sections without destroying sensitive lymphoid surface antigens (Stratis *et al.*, 1988). The sections were rinsed briefly in warm tap water and washed in three consecutive volumes of PBS, in which the slides were left until the immunostaining procedure (see below).

5-7-4. Immunostaining Procedure

Acetone-fixed, formol calcium-postfixed sections of the ovarian tumors were incubated for 15 minutes with goat serum (nonimmune) diluted 60-fold (1.5%) in PBS. All incubation steps were performed at 37°C in a humidified chamber. After blotting excess serum from the sections, they were incubated with the DO-1 primary antibody, present in cell culture supernatant, diluted 50-fold in PBS containing 1.5% goat serum for 20 minutes. The sections were washed vigorously in PBS for 5 minutes before the biotin-labeled secondary antibody, diluted 200-fold in PBS containing 1.5% goat serum, was added for a 20 minute incubation period. The sections were washed for 5 minutes in PBS

as before, and incubated for 20 minutes with the prepared ABC reagent. After a final wash step for 5 minutes in PBS, the sections were incubated with the peroxidase substrate solution for 5 minutes, treated for a few seconds with 5% HCl in ethanol, and washed in warm tap water for 5 minutes. Finally, the sections were dehydrated by consecutive incubations in isopropanols of increasing strength and in xylene, and coverslipped with Permount (Fisher Scientific Co., Pittsburgh PA). Controls consisted of adjacent sections of each tumor in which the primary antibody was omitted (replaced by diluent only).

5-7-5. Instrumentation

Frozen ovarian tumor specimens were sectioned using a Leitz 1720 Digital cryostat (Leica AG, Heerbrugg, Switzerland). All incubation steps in the IHC staining procedure were performed in a heated, humidified slide incubator (Signet Laboratories Inc., Dedham MA). The results of immunostaining were assessed using a Leitz DMR microscope (Leica AG, Heerbrugg, Switzerland) equiped with a Wild MPS 48/52 photographic system.

5-8. Single-Strand Conformation Polymorphism Analysis

5-8-1. Reagents, Solutions, and PCR Primers

DNA was extracted with a 10 mmol/L Tris buffer, pH 8.0, containing 0.1 mol/L EDTA, 20 μg/mL bovine pancreatic RNase A (Sigma Chemical Co., St. Louis MO) and 0.5% SDS. Dialysis after the extraction step was performed using a 50 mmol/L Tris buffer, pH

8.0, containing 10 mmol/L EDTA. Three pairs of oligonucleotide primers, identical to those used by another group (Miyashima *et al.*, 1991) to amplify p53 exons 5-6, 7, and 8, are given in **Table 5-8-1-1** and were made by an automated DNA sequencer at Dalton Chemical Laboratories, Toronto, Canada. The other components of the PCR reaction mixture, including AmpliTaq DNA polymerase (250 Units, 5 Units/μL), each of the deoxynucleoside triphosphates (dNTPs) in 10 mmol/L concentrations, 10X PCR buffer consisting of 500 mmol/L KCl and 100 mmol/L Tris, pH 8.3, and a 25 mmol/L $MgCl_2$ solution, were provided in a kit (Perkin Elmer, Foster City CA). The 10X exchange reaction buffer used for PCR product labeling contained 500 mmol/L imidazole HCl, pH 6.4, 180 mmol/L $MgCl_2$, 50 mmol/L dithiothreitol, 1 mmol/L spermidine HCl, and 1 mol/L EDTA, pH 8.0. The TE buffer used to dissolve the DNA was a 10 mmol/L Tris buffer, pH 8.0, containing 1 mmol/L EDTA. The electrophoresis gel loading buffer for SSCP analysis contained 95% formamide, 20 mmol/L EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol. The TBE running buffer contained 100 mmol/L Tris, pH 8.0, 100 mmol/L boric acid, and 200 mmol/L EDTA.

5-8-2. DNA Extraction

Genomic DNA from ~100 mg of each of nine pulverized ovarian tumor tissues, and from ~5 X 10^7 COLO 320HSR cells (American Tissue Type Collection, Rockville MD), colorectal carcinoma cells overexpressing mutant p53 (Hassapoglidou *et al.*, 1993) and cultured under the same conditions used for the PAb240 cell line (see above), was prepared essentially as described elsewhere (Sambrook *et al.*, 1989). Briefly, harvested

COLO 320HSR cells were resuspended first in ice cold PBS and centrifuged again before they were resuspended in PBS at a concentration of ~5 X 10^7 cells/mL. Ten volumes of DNA extraction buffer were added to the cell suspension and to each of the ovarian tumor tissues for a 1 hour incubation at 37°C, after which proteinase K (Sigma Chemical Co., St. Louis MO) was added to a concentration of 100 μg/mL for a 3 hour incubation at 50°C. An equal volume of phenol equilibrated with 0.5 mol/L Tris, pH 8.9, was added and the two phases were separated by centrifugation at 5000 g after mixing for 10 minutes; this extraction step was repeated two more times. The pooled aqueous phases were finally dialyzed against dialysis buffer until the OD_{270} of the dialysate was less than 0.05. The purity and yield of the DNA were determined spectrophotometrically.

5-8-3. PCR Amplification and Product Labeling

PCR was carried out according to standard practice (Innis *et al.*, 1990), in which 20 μL reaction volumes, containing 5 mmol/L $MgCl_2$, 100 ng of each primer, 200 μmol/L of each dNTP, 100 ng of high molecular weight DNA, and 2 Units of AmpliTaq DNA polymerase, underwent thirty cycles of denaturation (94°C for 30 seconds), annealing (60°C for 2 minutes), and extension (72°C for 3 minutes). A final extension step at 72°C for 5 minutes followed the 30 cycles. Sizes of the amplified DNA fragments, and the approximate yield, were confirmed by electrophoresis on a 3% agarose gel stained with ethidium bromide. Purification of the PCR products from primers and other contaminants was achieved using Centricon-30 spin columns (Amicon Inc., Beverly MA) according to manufacturer's instructions.

Performed as described elsewhere (Sambrook *et al.*, 1989), the purified PCR products were labeled on their 5'-termini with [γ^{32}-P]ATP by combining, for each reaction, 20-50 pmoles of DNA, 5 μL of 10X exchange reaction buffer, 5 μL of 1 mmol/L ADP, 1 μL of 50 nmol/L ATP, 50 pmoles of [γ^{32}-P]ATP (10 μCi/μL), H_2O up to 40 μL, 10 μL polyethylene glycol (24% w/v), and 1 μL T4 polynucleotide kinase (20 Units) (New England Biolabs, Beverly MA). After incubation for 30 minutes at 37°C, 2 μL of 0.5 mol/L EDTA was added, and the DNA was extracted with chloroform and then precipitated with two volumes of ethanol for 15 minutes at 0°C. Centrifugation at 14,000 g for 10 minutes at 4°C allowed recovery of the DNA, which was then dissolved in TE buffer.

5-8-4. Electrophoresis Conditions and Autoradiography

For each specimen, approximately 100 ng of radiolabeled DNA (1-3 μL) was combined with 7-9 μL of gel loading buffer, heat-denatured at 95°C for 3 minutes, chilled on ice, and rapidly loaded onto a 0.4 mm 5% nondenaturing polyacrylamide gel (50:1 acrylamide: *N,N'*-methylene *bis*acrylamide) equilibrated with running buffer containing 10% glycerol. Electrophoresis was performed at 7 W for 12 hours at room temperature. After fixation with a solution of 5% glacial acetic acid and 15% methanol for 15 minutes, the gel was dried at 60°C, and autoradiographed against Kodak XAR-5 film (Eastman Kodak Co., Rochester NY) at -80°C with intensifying screens.

5-8-5. Instrumentation

PCR was performed in a Perkin Elmer DNA Thermal Cycler (Perkin Elmer Inc., Foster City CA). A sequencing type apparatus (Fisher Scientific Inc., Pittsburgh PA), having 35 X 45 cm glass plates and 0.4 mm spacers was used for polyacrylamide gel electrophoresis. Drying of the gel was performed using a heated slab gel drier (Bio-Rad Laboratories Inc., Hercules CA).

5-9. Ethical Issues Addressed

The use of all materials of human origin had been approved by the Ethics and Research Committee at the University of Toronto. Clinical specimens were processed and assayed for p53 protein by immunoassay without knowledge of the corresponding patient clinicopathologic or survival information, provided subsequently by our collaborators in Italy. These workers had also assigned only numbers both to the specimen containers and to the clinicopathologic/survival records, thus protecting patient identities.

5-10. Statistical Analysis

5-10-1. Numerical Data

Continuous numerical data collected for this study included data generated by p53 immunoassay and total protein assay of the ovarian tumor extracts, and also those assembled by our collaborators at the University to Turin, who provided us with the age

of each of the patients. The p53 protein concentration (in U/L) measured in each extract was divided by the total protein content, yielding protein-adjusted p53 protein levels expressed in U/g. The distributions of the unadjusted and protein-adjusted p53 protein concentrations, and of the patient ages, were revealed by frequency distributions (and illustrated by histograms) and summarized by the calculation of descriptive statistics, including the mean, standard deviation, skewness, and kurtosis for each variable.

5-10-2. Categorical Data

The status of several ordinal categorical variables, including tumor stage and grade, were recorded for each patient. Also provided by the Italian investigators was an estimation, made for each patient at the time of surgery, of the diameter of the nonresectable, residual tumor mass. Although a numerical variable, residual tumor size was reported simply as 0 cm, $\leq$5 cm, or >5 cm. Data regarding the histologic type of the tumors, whether or not the patient had reached menopause at the time surgery was performed, and the status of the patient on the last follow-up date with respect to both cancer relapse (relapse versus relapse-free) and survival (alive versus dead), all nominal categorical variables, were also collected in Italy and made available to our group. For statistical analysis, the classification schemes of two variables, stage and histotype, were further collapsed (Stages I and II versus Stages III and IV; endometrioid versus serous versus all other histotypes reported). For the same purpose, patients were also divided into four groups on the basis of age, categorized as <40 years, 40-49 years, 50-59 years, and >60 years of age, and were dichotomized into either p53-negative or p53-positive groups based on the

arbitrary selection of a protein-adjusted p53 protein level as the cutoff point for p53-positivity (see below).

In order to determine if tumors classified as either p53-negative or -positive clustered within groups defined by the tumors' status for each of the other clinicopathologic factors considered in this study (age, menopause, stage, grade, histotype, and residual tumor size) and for relapse-free and overall survival, 2 x 2, 2 x 3, and 2 x 4 contingency tables were constructed and analyzed by Chi square tests. Differences were considered significant when the probability values were 0.05 or less.

5-10-3. Survival Analysis

For survival analysis, two different end points of follow-up - cancer relapse (either local recurrence or distant metastasis) and death - were used to calculate disease-free and overall survival, respectively. Disease-free survival was defined as the time interval between the date of surgery and the date of identification of recurrence or metastatic disease. Overall survival was defined as the time interval between the date of surgery and the date of death due to cancer, other causes of death having been excluded.

The Cox proportional hazard regression model (Cox, 1972) was used to evaluate the strength of the associations (i.e. the hazard ratios and their confidence intervals) between each of the prognostic markers, including p53, and disease relapse or death. This analysis was conducted at both univariate and multivariate levels. In the multivariate analysis, the

presence of mutant p53 protein, stage (which was not included in overall survival analysis because the model did not converge), grade, residual tumor, and age were included in the model. All these variables except age were categorized dichotomously (i.e. p53-positive versus p53-negative, Stages I/II versus Stages III/IV, grades G1/G2 versus grades G3/G4, and the presence of residual tumor versus the absence of residual tumor). Application of the Cox model, to determine the ability of p53 protein accumulation to predict patient relapse or death, was repeated after dichotomous stratifications of the patients, as above, by stage, grade, and residual tumor presence. The latter analyses were performed using both age-adjusted and unadjusted models. Kaplan-Meier survival curves (Kaplan and Meier, 1958) were constructed for p53-positive and -negative patients. Within each p53 category, Kaplan-Meier curves were also calculated for subgroups, defined as above, with different stage, grade, or residual tumor. The log rank test (Mantel, 1966) was used to examine the differences between the Kaplan-Meier curves. Probability values determined from both the Cox analyses and the log rank tests were considered significant at $p \leq 0.05$.

5-10-4. Computer Software

Initially, all data were entered into a Microsoft Excel version 5.0 spreadsheet (Microsoft Corp., Redmond VA) to serve as a platform for export into the statistical analysis software. While SAS version 6.02 (SAS Institute, Cary NC) was used to calculate descriptive statistics and to analyze contingency tables, EGRET (Statistics and Epidemiology Research Corp., Seattle WA) was used for Cox proportional hazard and Kaplan-Meier survival analyses (including log rank tests). All graphical figures were

made using either Word for Windows version 6.0 (Microsoft Corp., Redmond VA) or SigmaPlot version 2.01 (Jandel Scientific Corp., San Rafael CA).

Chapter 6. Results

6-1. Extraction Optimization

Shown in **Figure 6-1-1** are the total protein-adjusted p53 concentrations determined from the immunoassay of the six ovarian tumor specimens plotted against the different methods used to extract proteins from the pulverized tissues. The methods under comparison included those published (Lavigueur *et al.*, 1989; Bartkova *et al.*, 1993) and in routine practice, as well as variations of the three in which the centrifugation conditions used to clarify the extracts were changed. Except in the case of specimen 1, extracts made by the eight extraction procedures applied to each of the specimens did not differ markedly in their p53 protein levels. Because no apparent advantages were conferred by either homogenization of the buffer-suspended tissue powder or ultracentrifugation for 1 hour, compared to chemical lysis and centrifugation at a lower speed (15,000 g) for 30 minutes or 1 hour, the method selected to release p53 from the ovarian tumor specimens was that of Bartkova *et al.*, (**Figure 6-1-1**, method F) modified to include the addition of proteinase inhibitors (10 mg/L phenylmethylsulfonyl fluoride, and 1 mg/L each of leupeptin and aprotinin) to the extraction buffer.

6-2. p53 Assay Performance

6-2-1. Standard Curve and Assay of Controls

Assay of the six standard solutions, given arbitrary p53 concentrations of 0, 2, 5, 20, 50, and 200 U/L, yielded calibration curves from which unknown levels of p53 were determined. A representative calibration curve is shown in **Figure 6-2-1-1**, which demonstrates the almost linear assay response across the range of p53 standard solutions; within a given assay run, standards of successively increasing p53 concentrations yielded consistently two-fold greater fluorescence counts. Since this applied to the two lowest standards, a p53 concentration of 2 U/L could easily be discriminated from zero. The p53 concentrations determined from the control samples did not differ by more than 29%, 15%, 12%, and 10% from the established p53 levels of 1, 3, 10, and 80 U/L, respectively.

6-2-2. Reproducibility of Extraction

In the series of 18 tumor specimens used to assess the variability of the selected extraction method and its comparison to that of the same method including a homogenization step, the protein-adjusted p53 concentrations found in the 4 aliquots of tissue powder differed widely. Since the estimates of precision, expressed as %CV, were 31% and 25%, respectively, both methods suffered from considerable variability. Homogenization, therefore, did not lead to much improved reproducibility of p53 assay results, nor did it increase the overall yield of p53 released since a Wilcoxon Signed Rank test failed to find a net difference between the medians of the two groups of mean

protein-adjusted p53 levels (p=0.44) (data not shown). These results provide further support for our choice of extraction method but indicated its large inherent variability.

6-2-3. Tissue Heterogeneity for p53

Small masses of tissue sampled from different sites of each tumor specimen did not differ markedly with respect to p53 content, judging by the observation that the mean imprecision (%CV) between the triplicate samples was 35%, not very different from the variability expected from tissue extraction (31%), p53 assay (10-30%), and total protein assay (~10%) results.

6-2-4. Distribution of p53 Protein Levels and Selection of a Cutoff Point

The distribution of the mean protein-adjusted p53 concentrations determined from the 3 samples taken from each of the 90 ovarian tumor specimens is shown as a histogram in **Figure 6-2-4-1** and had the following characteristics: minimum=0.35 U/g, maximum=30 U/g, median=2.21 U/g, mean=18.14 U/g, and standard deviation=32 U/g. For statistical analyses, a cutoff point of 3 U/g, at which and beyond, p53 protein concentrations were considered positive was arbitrarily selected based on the frequency distribution. Use of this cutoff resulted in a 43% p53-positivity rate.

6-3. Validation of p53 Assay Findings by Immunohistochemistry

The two tumor specimens which were sectioned while frozen, fixed, and stained with DO-1 antibody against p53 protein both displayed excellent morphological preservation.

The two photomicrographs of **Figure 6-3-1** show the immunostaining results of specimens which were shown previously to be very highly positive, and negative, respectively, for p53 by immunoassay. While both sections had faint staining of stromal components, only the section of the p53-positive tumor had intense nuclear staining and fainter cytoplasmic staining confined to epithelial cells. On an adjacent section of the p53-positive tumor which was processed in parallel, the replacement of the reagent containing DO-1 antibody by its diluent resulted in immunostaining comparable to that of the p53-negative specimen (data not shown).

6-4. Validation of p53 Assay Findings by Single-Strand Conformation Polymorphism Analysis

The results of the SSCP analysis of exons 5 to 8 of the p53 gene amplified from nine ovarian tumors, and from COLO 320HSR cells as a positive control, is displayed in **Figure 6-4-1** and compared with p53 protein concentrations determined by immunoassay in protein extracts of the same tumors in **Table 6-4-1**. Altered electrophoretic mobilities in exons 5-6 in one specimen, in exon 7 in another, and in exon 8 in two tumors were associated with p53 protein overexpression. In contrast, p53 protein did not accumulate in three tumors whose SSCP analysis did not reveal band shifts. For three specimens, however, SSCP and immunoassay provided discrepant findings: p53 protein accumulated in two specimens whose exons 5 to 8 did not show aberrant bands, but was undetectable in a tumor in which a band shift was found in exons 5-6. A faster migrating conformer of exon 7 from COLO 320HSR cells was also observed (data not shown), a finding which

was consistent with the presence of a missense mutation in this exon at codon 248 (Murakami *et al.*, 1991).

6-5. Associations Between p53 and Other Variables

Table 6-5-1 presents the relationships between p53 protein expression status and other clinical or pathologic variables, including patient age, menopausal status, stage, grade, residual tumor size after surgery, and histologic type. Patients with p53-negative tumors tended to be younger than patients with p53-positive tumors, but the difference was not statistically significant. Of all p53-negative patients, 43% were younger than 50 years of age. In the p53-positive group, only 23% of patients were in the same age category. Similar tendency was also observed between p53 and menopausal status, because menopause is an age-dependent event. The presence of mutant p53 protein was also associated significantly with late stage, high grade, presence of residual tumor, and serous histotype.

The positivity rates for p53 per stage were 5% (1 of 21) for Stage I disease, 17% (1 of 6) for Stage II, 57% (29 of 51) for Stage III, and 67% (8 of 12) for Stage IV ovarian cancer. Similarly, the positivity rates for p53 per grade were as follows: 13% (2 of 16) for G1, 40% (10 of 25) for G2, and 55% (27 of 49) for G3. The positivity rates for p53 were 18% (6 of 34) in patients with no residual tumor 5 cm or smaller in size, and 64% (16 of 25) in patients with residual tumor larger that 5 cm. Positivity rates for p53 expression also varied with histologic type: 29% (2 of 7) for clear cell tumors, 10% (2 of 21) for

endometrioid tumors, 38% (3 of 8) for mucinous tumors, 61% (22of 36) for serous tumors, 50% (4 of 8) for unclassified tumors, and 60% (6 of 10) for undifferentiated tumors.

6-6. Associations Between Clinicopathologic Variables and Survival

The associations between these prognostic markers and cancer relapse or death are shown in **Table 6-6-1**. Statistically significant relationships were not observed between either endpoint and patient age or menopausal status, although there were trends for older or postmenopausal women to suffer a relapse or die more frequently. Stage, grade, histotype, and postsurgical residual tumor size were all associated significantly with cancer relapse or death. Patients whose tumors were p53-positive also had higher relapse and death rates in comparison with patients whose tumors were p53-negative, and these differences were statistically significant.

6-7. Cox Proportional Hazard Regression Analysis

The strength of the associations between each individual predictor and disease-free or overall survival are demonstrated by the univariate analysis in **Table 6-7-1**. The presence of residual tumor showed the strongest relationship to cancer relapse and death, and the hazard ratio reached maximum values of 8.3 and 27.5, respectively. Patients with late clinical stage (III or IV) or poorly differentiated (G3) tumors had a two-to-seven-fold increased risk of developing recurrent or metastatic disease or of dying compared with those with early stage (I or II) or with well or moderately differentiated (G1 of G2)

tumors. The hazard ratio for patients with p53-positive tumors was 2 for both disease-free and overall survival. When all these predictors were included in the Cox model (multivariate analysis in **Table 6-7-1**), however, the presence of residual tumor was the only predictor significantly associated with disease-free and overall survival. Mutant p53 protein, stage, and grade were shown to have no independent value for predicting the prognosis of ovarian cancer patients.

6-8. Cox Proportional Hazard Regression Analysis for Subgroups of Patients

The associations between p53 expression status and cancer relapse or death in subgroups of patients who were categorized based on their stage (I or II versus III or IV), grade (G1 or G2 versus G3), or postsurgical residual tumor (presence versus absence) were also examined (**Table 6-8-1**). Patients with well (G1) or moderately (G2) differentiated cancer had a significantly higher risk of developing recurrent disease or of dying if p53 protein was overexpressed in their tumors compared with patients who had tumors of the same grade but were p53-negative. Patients with no postsurgical residual tumor also demonstrated significantly increased risk for relapse or death when the tumors were p53-positive. Although higher risks for relapse and death were observed in patients with p53-positive tumors who had disease of an early stage, the elevated risk did not reach statistical significance. The risks for cancer relapse or death were not significantly different between p53-negative and -positive tumors in patients with late stage, poorly differentiated tumors, or when residual tumor was present.

6-9. Kaplan-Meier Survival Analysis

In agreement with the univariate Cox analyses were log rank tests of differences between the Kaplan-Meier survival curves (**Figure 6-9-1**) which demonstrated that patients with p53-negative tumors had significantly longer disease-free survival compared with patients with p53-positive tumors. A similar tendency was revealed for overall survival, with borderline statistical significance ($p=0.06$). Similarly, comparison of Kaplan-Meier curves for p53-negative and p53-positive patients within subgroups defined by stage, grade, and residual tumor presence confirmed the Cox regression analyses of patients stratified by the same variables. **Figure 6-9-2** shows the disease-free and overall survival curves for cancer patients with grades G1 and G2. In this subgroup, patients with p53-negative tumors had substantially longer disease-free and overall survival than did patients with p53-positive tumors ($p<0.01$). These differences were not observed in patients with poorly differentiated cancer (**Figure 6-9-3**). For patients with residual tumor after surgery (**Figure 6-9-4**) or late stage (**Figure 6-9-5**), there were no differences between p53-positive and p53-negative tumors with respect to survival. Kaplan-Meier curves were not calculated for patients with early stage cancer or with no residual tumor because of the small number of patients in each p53-positive group.

Chapter 7. Discussion

7-1. Molecular Basis of Sporadic Ovarian Cancer

7-1-1. Oncogenes and Tumor Suppressor Genes

There is now overwhelming evidence that most human malignancies are the end result of an accumulation of mutations within growth regulatory genes - oncogenes and tumor suppressor genes - which are normal cellular genes involved in the control of cell proliferation and differentiation. Whereas oncogenes normally act to stimulate cell growth, and specific alteration of a single allele of a proto-oncogene is sufficient for its activation, inactivation of both copies of a tumor suppressor gene is usually required to circumvent the normal contraints to cell proliferation. Recently identified have been a third class of cancer-related genes involved in DNA-mismatch repair, deficiencies of which are thought to contribute to the mutation of growth regulatory genes.

Cellular proto-oncogenes are generally participants in various signal transduction pathways integrating messages from the local external and internal environments and leading to altered expression of genes influencing cell division and differentiation. The most extensively studied oncogenes in ovarian cancer are HER2/neu and K-ras. Overexpression of the HER2/neu gene, which codes for a growth factor receptor structurally similar to epidermal growth factor receptor (EGFR) (Schechter *et al.*, 1984), has been identified in human breast and ovarian carcinomas and has been shown to result

from the presence of multiple copies of the HER2/neu gene in these tumors (Slamon *et al.*, 1989). Increased levels of HER2/neu protein have been found in approximately one-third of ovarian carcinomas (Slamon *et al.*, 1989) and have been associated with poor prognosis (Berchuck *et al.*, 1990). Mutations in the ras proto-oncogene have been identified in a wide range of human adenocarcinomas, as well as in approximately 50% of borderline and invasive mucinous ovarian carcinomas (Mok *et al.*, 1993; Teneriello *et al.*, 1993). Borderline ovarian tumors constitute a unique subgroup of ovarian neoplasms characterized by an unusual degree of epithelial cell proliferation and atypia compared to benign ovarian tumors, but they lack the stromal invasion characteristic of ovarian carcinomas. Although they are known to metastasize within the peritoneal cavity, they rarely result in death. The frequent finding of K-ras mutations in borderline tumors (Pieretti *et al.*, 1995) suggests that K-ras mutations are an early event in the development of carcinoma of the ovary and that borderline tumors are indeed precursors to invasive tumors, a notion that remains subject to controversy (Lawrence, 1995).

Loss of tumor suppressor gene function may occur by means of a number of mechanisms, including deletion of the gene, inactivating mutations, absent gene transcription, or inactivation of a structurally normal gene product. Loss of both functional copies of a tumor suppressor usually results from an inactivating mutation in one allele and deletion of the remaining wild-type allele, a condition referred to as loss of heterozygosity (LOH). Almost a decade ago, cytogenetic analysis had revealed frequent structural aberrations and deletions of chromosomes 1, 2, 6, and 11 (Tanaka *et al.*, 1987), suggesting that

inactivation of genes on these chromosomes may contribute to ovarian tumorigenesis. Molecular genetic techniques have more recently been used to detect submicroscopic loss of genetic material in ovarian tumors. Restriction fragment length polymorphism (RFLP) analysis and microsatellite analysis of ovarian tumor cell lines and clinical tumor specimens has revealed a high frequency of LOH on chromosomes 6p, 6q, 9q, 13q, 17p, and 17q (Okamoto *et al.*, 1991; Tsao *et al.*, 1991; Cliby *et al.*, 1993; Pieretti *et al.*, 1995). Of particular importance appear to be the losses of loci on chromosome 17: on the short arm, LOH and mutations of the p53 gene as well as LOH at a more distal locus (17p13.3) have been observed in a high percentage of ovarian tumors (Phillips *et al.*, 1993); on the long arm, losses of the BRCA1 region and a more distally located locus (17q22-23) are frequently observed (Goodwin *et al.*, 1993; Saito *et al.*, 1993). In one study involving 16 polymorphic markers spanning the length of chromosome 17, LOH for at least one locus on this chromosome was found in approximately 40% of ovarian tumors (Pieretti *et al.*, 1995). More than 80% of the tumors with chromosome 17 loss exhibited loss of all informative markers, suggesting complete loss of an entire chromosome 17 and therefore that the loss of tumor suppressor genes on this chromosome confers a selective growth advantage during tumor progression.

7-1-2. p53 Alterations in Ovarian Cancer

Molecular genetic analysis of p53 has provided insight into ovarian cancer progression. Somewhat controversial has been the notion that ovarian malignancies typically presenting at diagnosis, where tumor is found at multiple sites throughout the peritoneal

cavity, are unifocal in origin. One study comparing the mutational spectrum of the p53 gene in cancer cells at several sites within the same patients (Mok *et al.*, 1992) and another study combining p53 genetic analysis with X chromosome inactivation analysis of primary tumors, metastatic deposits and ascites (Jacobs *et al.*, 1992) strongly support a monoclonal origin of ovarian cancer. The same conclusion was formulated from the findings of a cytogenetic analysis which found that the identical patterns of loss for alleles examined on chromosomes 1, 6, 11, 13, 16, and 17 occured in the multiple tumor sites within a patient with familial ovarian cancer (Gallion *et al.*, 1996).

Although some studies have demonstrated the relative paucity of p53 mutations or chromosome 17p losses in borderline ovarian tumors compared to frankly malignant tumors (Kupryjanczyk *et al.*, 1995; Lee *et al.*, 1995b; Wertheim *et al.*, 1996), the accumulation of p53 protein has been reported to occur in 10-20% of these tumors (Kupryjanczyk *et al.*, 1994; Hutson *et al.*, 1995). Stage I ovarian tumors, however, are found to have p53 protein accumulation at a much higher frequency (~40%) (Kupryjanczyk *et al.*, 1994), reflecting the higher mutation rate of stage I neoplasms (Kupryjanczyk *et al.*, 1995). While the bulk of evidence indicates that the p53 mutation rate increases with the stage of the disease (Mazars *et al.*, 1991; Kohler *et al.*, 1993b), chromosome 17 LOH analysis of ovarian carcinomas with areas of benign or borderline epithelium adjacent to invasive carcinoma has suggested that p53 losses may appear early in ovarian cancer progression. In one study (Zheng *et al.*, 1995), 52% of ovarian carcinomas had p53 mutations, compared to none of the tumors of low malignant

potential and none of the solitary cystadenomas. However, all cystadenocarcinomas with p53 mutations and adjacent morphologically benign cysts harbored the same p53 mutation in both tissues, and were further concordent with respect to LOH at the p53 locus. The simple explanation for this finding is that the small proportion of benign ovarian cysts which have p53 mutation may progress to malignancy, but it is also possible that the p53-mutated benign cysts may be differentiated cells derived from the adjacent carcinoma (Liu and Nuzum, 1995). The latter possibility is quite exciting as it points to a viable treatment alternative to cytotoxic therapy for ovarian cancer patients, that is, the forced differentiation of neoplastic cells achieved perhaps by pharmacological agents.

A large number of studies have documented frequent allele loss and mutation of p53 in human ovarian carcinomas and have related these genetic events to p53 protein overexpression, phenotypic changes, and expression of other cancer-related genes. Chromosome 17p13 LOH has been observed in 50-90% of ovarian cancers of various histologic types, determined primarily by RFLP analysis (Okamoto *et al.*, 1991; Tsao *et al.*, 1991; Foulkes *et al.*, 1993; Phillips *et al.*, 1993; Frank *et al.*, 1994; McManus *et al.*, 1994). Reflecting the likelihood that such chromosome losses might have targetted other tumor suppressors near the p53 locus, p53 gene mutations affecting the protein coding sequence have been found at slightly lower frequencies, ranging from 30 to 80% and detected by SSCP followed by sequencing of aberrant cases (Okamoto *et al.*, 1991; Kihana *et al.*, 1992; Naito *et al.*, 1992; Kupryjanczyk *et al.*, 1993; Milner *et al.*, 1993;

Kim *et al.*, 1995), by chemical mismatch cleavage (Sheridan *et al.*, 1993), or by temperature-gradient gel electrophoresis followed by direct sequencing (Kappes *et al.*, 1995). These studies have revealed that gross rearrangements of the p53 gene occur infrequently in ovarian tumor specimens (Tsao *et al.*, 1991), in contrast to ovarian cancer cell lines in which small p53 deletions and insertions were found in over half of the cell lines examined (Yaginuma and Westphal., 1992; Runnebaum *et al.*, 1994). p53 genetic abnormalities are usually absent in benign ovarian tumors and in normal ovarian epithelium (Naito *et al.*, 1992). Identified in sporadic ovarian carcinomas have been missense (mostly transition base substitutions), nonsense, and frameshift p53 mutations, as well as small (1-2 bases) intragenic deletions and insertions. These mutations have been found to cluster in exons 5 and 7 (Mazars *et al.*, 1991; Sheridan *et al.*, 1993; Niwa *et al.*, 1994; Kim *et al.*, 1995), although mutations in other exons as well as polymorphisms have also been reported (Kohler *et al.*, 1993b; Kim *et al.*, 1995). Interestingly, one recent study reported an incidence of null mutations, including deletions, insertions, splice junction changes, and nonsense mutations leading to truncation or complete absence of the p53 gene product, equal to 20% in ovarian carcinomas, which is far greater than that observed in the majority of other human cancers (Skilling *et al.*, 1996) and suggests that p53 protein accumulation might not corrrelate closely with p53 mutation (see below). The predominance of transition mutations suggests that p53 mutational events in ovarian cancer likely occur because of spontaneous errors in DNA synthesis and repair rather than by the direct interaction of carcinogens with the p53 DNA sequence (Greenblatt *et al*, 1994). While the screening of hereditary ovarian cancer patients in one study did not

reveal any germline p53 mutations but found only silent polymorphic base substitutions (Buller *et al.*, 1995), another group reported the presence of a splice site p53 mutation in a family with hereditary breast-ovarian cancer syndrome (Jolly *et al.*, 1994).

As in other cancers, overexpression of p53 protein in ovarian cancer appears to correlate closely with the presence of p53 gene mutations, particularly those in the highly conserved central domain of p53 (Marks *et al.*, 1991; Kupryjanczyk *et al.*, 1993; Kappes *et al.*, 1995). A similar general concordance has also been demonstrated between p53 protein accumulation and allelic loss on chromosome 17p13 (Eccles *et al.*, 1992; Frank *et al.*, 1994; McManus *et al.*, 1994; Sheridan *et al.*, 1994). In all of these studies, p53 accumulation was predominantly nuclear and was detected by standard IHC techniques coupling monoclonal or polyclonal immunoreagents against p53 to enzyme-driven chromogen deposition in fresh frozen (Eccles *et al.*, 1992; Sheridan *et al.*, 1994) or formalin-fixed, paraffin-embedded (Marks *et al.*, 1991; Kerns *et al.*, 1992; Kupryjanczyk *et al.*, 1994; Renninson *et al.*, 1994) ovarian tumor tissues.

A number of biochemical, histologic, and anatomic characteristics of ovarian tumors thought to indicate the tissue proliferative capacity and degree of differentiation have been associated with p53 gene mutation and/or protein overexpression. Significantly correlated with p53 abnormalities have been DNA aneuploidy (Marks *et al.*, 1991; Kihana *et al.*, 1992), Ki-67 expression (Henriksen *et al.*, 1994), high histologic grade (Kupryjanczyk *et al.*, 1993; Hartmann *et al.*, 1994; Kupryjanczyk *et al.*, 1993; Kim *et al*,

1995), serous histotype (Kupryjanczyk *et al.*, 1993; Fujita *et al.*, 1994; Rennison *et al.*, 1994), large tumor size (Kohler *et al.*, 1993b), high S-phase fraction (Klemi *et al.*, 1995), and late stage (Marks *et al.*, 1991; Niwa *et al.*, 1994; Kim *et al.*, 1995). Demonstration of these associations has not been consistent, however, since other studies have not shown p53 mutation or overexpression to be related to some these factors (Marks *et al.*, 1991; Renninson *et al.*, 1994; Niwa *et al.*, 1994, Klemi *et al.*, 1995). On the other hand, two studies comparing the presence of p53 mutations and activating mutations in K-ras were in agreement that these two events occur independently of each other (Teneriello *et al.*, 1993; Fujita *et al.*, 1994). The possibility that p53 abnormalities might associate with at least some of these other factors known or suspected to predict survival of ovarian cancer patients led to studies examining the prognostic potential of p53 mutations and protein overexpression. These studies too have not been concordent given that disease recurrence or overall survival was not significantly related to either p53 mutation (Niwa *et al.*, 1994; Sheridan *et al.*, 1994) or to p53 protein accumulation (Marks *et al.*, 1991; Kohler *et al.*, 1993a; Hartmann *et al.*, 1994) in many of the multivariate survival analyses performed to date, while in a few other studies, p53 immunostaining was an independent predictor of survival in patients with serous ovarian tumors (Klemi *et al.*, 1994) and in patients with a variety of malignant epithelial ovarian neoplasms (Klemi *et al.*, 1995; van der Zee *et al.*, 1995). Clearly, further studies are needed to clarify the prognostic utility of p53 alteration in ovarian cancer.

7-2. p53 Protein Accumulation in Tumor Tissue Detected by ELISA

IHC staining of tumor tissues for p53 protein is a rapid and simple technique which, unlike biochemical analysis, can identify distinct staining patterns at single cell resolution. An often cited disadvantage of p53 immunostaining, the significant rate of false-negative and false-positive predictions of the mutational state of the p53 gene, would be expected to be shared by any method of p53 protein detection and may be dependent, at least to some extent, on the relative immunoreactivities of the antibodies employed in these methods to the altered p53 proteins produced by the mutant p53 genes. However, the choice of tissue (Bartek *et al.*, 1993; Fisher *et al.*, 1994) may also 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 (Baas *et al.*, 1994, Vojtesek *et al.*, 1992), 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). 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 IHC 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), 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, the major limitation of ELISAs is the requirement for fresh frozen tissue, since they cannot be applied to fixed tissue. 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. Accurate interpretation of ELISA results, like those of IHC, must take into account the specificity of the primary antibody, which may recognize wild-type and/or mutant p53 protein and which may, theoretically, display different affinities for p53 proteins mutated at different sites.

Comparison between ELISA and IHC for p53 protein detection, performed in parallel on the same tumor tissues, has 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. 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. Although it was not the purpose of the present study to address this issue, when a standard IHC procedure for p53 protein was performed using a different anti-p53 antibody on frozen sections of two ovarian tumors for which extracts were also prepared and assayed for p53 by our immunofluorometric assay (in order to validate the latter method when applied to this tissue), very good concordance was shown between immunostaining results and the p53 concentrations measured by our ELISA.

To further validate the findings of our immunoassay, the p53 protein expression status of nine other ovarian tumors was compared with the alterations in the p53 gene sequence in the same specimens suggested by SSCP analysis. Aberrant band shifts were found in four out of six specimens in which p53 protein accumulation occured but were also found in one of the three p53-negative ovarian tumors. Numerous explanations could have accounted for the imperfect concordance between the SSCP and ELISA analyses. For example, it is possible that mutant p53 genes leading to overexpression of conformationally-altered p53 protein, detectable by PAb240 antibody in our immunoassay, may have escaped detection by SSCP analysis if the p53 mutation led to single-stranded conformers not resolvable from those of wild-type p53. Furthermore, the expression of a stabilized p53 protein may also have resulted from mutation outside of the range of p53 exons examined (5 to 8) or as a consequence of nonmutational

mechanisms. Conversely, an SSCP-positive result reflecting true p53 mutation may have been accompanied by the absence of detectable p53 protein if the mutation caused a reading frameshift leading to early transcription termination and thus to an unstable, truncated protein product which might have been rapidly degraded or might simply not have expressed the cryptic PAb240 epitope necessary for its detection by our ELISA. In the latter case, modification of our ELISA by replacing the primary capture antibody, PAb240, by a monoclonal antibody such as DO-1 which recognizes an amino terminal epitope expressed on both mutant and wild-type conformations of p53 protein might have led to improved agreement between ELISA and SSCP. Finally, SSCP mobility shifts in the absence of p53 protein accumulation might also have resulted from genetic polymorphism, as has been reported elsewhere (Murakami *et al.*, 1991). Although all of these possibilities are supported by observations in the literature, they remain purely speculative since further investigations were not carried out. Indeed, although sequencing all of the nine cases might have eliminated or demonstrated at least some of the invoked explanations, it was not performed in this study. Similar discordance in some cases has been noted by other workers comparing p53 protein expression and SSCP findings in ovarian cancers (Kupryjanczyk *et al.*, 1993; Kappes *et al.*, 1995).

This study represents the first application of a p53 immunoassay developed earlier in our laboratory (Hassapoglidou *et al.*, 1993) to the quantification of p53 in ovarian carcinomas and is also the first study, to our knowledge, in which p53 protein concentrations measured by an ELISA were related to clinicopathologic features and to survival of

patients with a malignant disease. Previously, the same immunoassay had been used in two studies to determine p53 protein levels in cytosolic extracts of breast tumors, but patient clinical information was not available for either of the populations studied, thus precluding survival analysis (Hassapoglidou *et al.*, 1993; Levesque *et al.*, 1994). In one of these studies (Levesque *et al.*, 1994), concentrations of p53 protein were found to be negatively associated with those of estrogen (ER) and progesterone (PR) receptors and also with those of carcinoembryonic antigen (CEA), but were not significantly associated with prostate-specific antigen (PSA) levels, in over 950 breast tumor tissue extracts prepared for steroid hormone receptor analysis. Another study of 200 breast tumor extracts from patients whose cancers were characterized for p53 protein levels by our ELISA and for the expression of PSA, ER, PR, EGFR, cathepsin D, and HER-2/neu, as well as for S-phase fraction and DNA ploidy, demonstrated a number of significant associations between p53 expression status and these other variables (Levesque *et al.*, 1995a). Breast tumor specimens in which p53 protein concentrations exceeded a cutoff point of 5 U/g were found to have reduced levels of both steroid hormone receptors, to have elevated expression of EGFR and HER-2/neu, to have higher S-phase fractions, and to be more likely of aneuploid DNA content, compared to p53-negative breast tumor specimens. These findings were highly suggestive that p53 protein accumulation, determined by an ELISA method, tended to occur in breast tumors which lacked steroid hormone growth dependence, expressed oncogene products known to be associated with aggressive tumor phenotypes, and displayed increased proliferative activities compared to breast tumors not overexpressing p53 protein. A study employing an improved

immunofluorometric assay of p53 protein (Levesque *et al.*, 1995b) for assessing the prognostic utility of p53 in a much larger series of breast cancer patients for whom detailed follow-up information, including survival, is available is currently in progress (Levesque *et al.*, 1996).

The previous studies relating p53 expression to other features of breast cancer used extracts prepared from the pulverized breast tumors by vigorous homogenization without the addition of enzymes to digest the tissue matrix, followed by ultracentrifugation. This method had been shown to be optimal for the isolation of steroid hormone receptors prior to their quantification in breast tissue, but its application to ovarian tumor tissue for the analysis of p53 might not have been appropriate. p53 protein has different physicochemical properties from those of ER or PR, and compared to breast tumors, which usually have a prominant adipose tissue component, tumors of the ovarian epithelium tend to be more fibrous. For these reasons, variations of three different extraction procedures for the release of soluble p53 protein from six resected ovarian tumors were compared in a simple experiment, from which one method was selected to be applied to all 90 specimens included in the study. Since the more labor-intensive homogenization or ultracentrifugation did not significantly improve the yield of p53 released from most of the specimens tested, the simplest procedure was chosen and was based on detergent-mediated cell lysis and moderate-speed centrifugation. Additional experiments, however, such as comparison of total protein extraction efficiencies between the eight methods differing in their use of buffers, centrifugation speeds, and proteinase

inhibitors, and recovery experiments of protein added to each of the buffers, were not performed. One variation of the selected method which was investigated, the inclusion of a homogenization step before incubation with cell lysis buffer, was shown not to noticeably improve the reproducibility of the p53 protein concentrations measured in 18 pairs of identical suspensions of ovarian tissue powders in lysis buffer. Sufficient tumor tissue was available for each of the specimens so that the tissue remaining after sampling could be stored for future studies. In order to avoid any kind of bias in sampling from each of the 90 tumors and to ensure that the extracts used for p53 analysis were representative of the specimens, three pieces of tissue were cut from each tumor and were independently pulverized, extracted and assayed for p53 and for total protein so that the concentrations of the former analyte were divided by those of the latter to provide p53 protein levels adjusted for the extraction efficiencies.

For the statistical analysis in which associations between p53 expression and other variables, including survival, were examined, protein-adjusted concentrations of p53 were assigned p53-negative or p53-positive status based on a cutoff level of 3 U/g. While there was no *a priori* biological rationale for the selection of this cutoff value, which resulted in a 43% p53-positivity rate, its use was convenient and suggested by the bimodality of the distribution of log-transformed p53 concentrations. Selection of other cutoff points within the range of 2-10 U/g would not have markedly changed the p53-positivity rate since only seven specimens had p53 concentrations in this range. The application of the Receiver Operator Characteristic (ROC) analysis (Zweig and Campbell, 1993) would

have been inappropriate in this case due to the lack of a "gold standard" method for p53 protein detection. Selection of a reasonable cutoff point for p53-positivity which exceeded a certain percentile of a distribution of p53 in extracts of normal epithelial ovarian tissue was not possible due to the unavailability of such tissues.

7-3. p53 Protein Status and Survival of Ovarian Cancer Patients

In this study, groups of ovarian tumors defined on the basis of stage, grade, residual tumor presence, or histotype classifications were found to differ in their p53-positivity rates, suggesting associations, between p53 overexpression status and the status of these other variables, which might prove to have confounding influences on the relationship between p53 and patient survival. Consistent with other workers who reported that the p53 mutational frequency is lower in early stages of ovarian cancer (Mazars *et al.*, 1991; Kohler *et al.*, 1993a; Hartmann *et al.*, 1994; Niwa *et al.*, 1994; Kim *et al.*, 1995), we observed increasing positivity rates for p53 protein as stage increased (ie. 5%, 17%, 57%, and 67% in Stages I, II, III, and IV, respectively). The increase in p53-positivity rate with the increasing anatomic extent of the malignancy may indicate that aberrant p53 expression may be a relatively late event in ovarian carcinogenesis that occurs during tumor invasion and metastasis, or that ovarian cancers without p53 mutation represent a different and perhaps less aggressive subset of tumors which metastasize less frequently. The relationship between p53 and stage also may have accounted for the association observed between p53 and the estimated size of the tumor remaining after laparotomy, assuming that more extensive disease is less likely to have been completely eradicated by

surgery. With respect to histologic type, the highest percentage of p53-positive specimens occurred in serous tumors (61%), followed by undifferentiated (60%), unclassified (50%), mucinous (38%), clear cell (29%), and endometrioid (10%) tumors. This finding is also in agreement with previous reports which demonstrated a greater prevalence of p53 mutations and/or protein accumulation in serous carcinomas compared to those of endometrioid or mucinous histotypes (Eccles *et al.*, 1993; Kupryjanczyk *et al.*, 1993; Milner *et al.*, 1993; Renninson *et al.*, 1994; Klemi *et al.*, 1995) and suggested that factors relating to disease etiology, associated with the histological types, may determine the frequency of functional inactivation of the p53 gene. Our finding that the p53-positivity rates increased also as the tumors became less tissue-specifically differentiated (ie. high grade) confirmed earlier work reported elsewhere (Kim *et al.*, 1995; Klemi *et al.*, 1995) and was not surprising given the inverse relationship generally attributed between tumor grade and proliferative activity.

In a preliminary analysis to demonstrate associations between relapse or death and status for the potential prognostic markers, including p53, it was found that patients who had relapsed during their respective follow-up periods were more likely to be post-menopausal, to have advanced stage and high grade disease, to have tumors of serous histological appearance, to have residual tumor remaining after cytoreductive surgery, and to have p53-positive extracts prepared from their ovarian tumors. Similarly, patients who had died during follow-up tended to be those with late stage, serous tumors which were not completely resected and which overexpressed p53 protein. Because this analysis

did not control for the variable lengths of follow-up between individual patients, many of whom became censored before the time of study termination and data analysis, it merely suggested that these variables were predictive of disease outcome. Some of the associations implied by this analysis were confirmed, however, in the more appropriate Cox proportional hazard regression analysis, performed at both univariate and multivariate levels.

Initial univariate analysis revealed that the detection of mutant p53 protein in ovarian cancer was significantly associated with increased frequency of relapse and death. Patients with late disease stage, poorly differentiated tumors, or residual cancer diagnosed after surgery also had significantly higher risk for both relapse and death. In multivariate analysis, where the contribution of all other factors in the model were controlled, only the presence of postsurgical residual tumor was associated significantly with survival, whereas none of the other markers, including p53, showed any independent predictive value for patient prognosis. In another study, it was proposed that such predictive value may be established at least for some of these parameters if longer follow-up periods are evaluated (Hartmann *et al.*, 1994). Failure of p53 immunostaining or mutation to predict survival of ovarian cancer patients in multivariate analysis has been an almost universal finding in the literature (Marks *et al.*, 1991; Kohler *et al.*, 1993a; Hartmann *et al.*, 1994; Niwa *et al.*, 1994; Sheridan *et al.*, 1994), although a few studies did implicate p53 protein accumulation with poor overall survival (Klemi *et al.*, 1994; Klemi *et al.*, 1995) or with both progression-free survival and overall survival (van der Zee *et al.*, 1995).

Because postsurgical residual tumor presence, disease stage, and histologic grade were all associated significantly with both p53-positivity and survival outcome, the possible existence of interactions between these factors on patient survival were considered because such interactions may lead to improper observation of the associations of these markers with the outcome variables (Rosner, 1995). To avoid the possible effects of these interactions and simultaneously to control for the confounding influence of other factors when the predictive value of p53 was examined, we evaluated the relationship between p53 expression and survival in patients who were classified into groups based on their stage, grade, and residual tumor categories. In this analysis, a strong and significant association was found between the presence of p53 accumulation and increased risk for cancer relapse and death in well or moderately differentiated tumors (G1 or G2), but not in poorly differentiated tumors (G3). This association was also observed in the subgroup of patients who had no residual tumor. Similar predictive value of p53 was also suggested, although not to a statistically significant degree, in patients with early stage disease (I or II) but not in those with late stage cancer (III or IV). These observations were further demonstrated by the Kaplan-Meier survival curves of patients subclassified by grade, stage, or residual tumor presence with the exceptions that such survival plots could not be constructed for the subgroups of patients with Stage I-II disease or with no residual tumor because of the small number of patients in each p53-positive category who developed cancer relapse or death.

To our knowledge, this study is the first to demonstrate a statistically significant relationship between the accumulation of p53 protein and poor outcome in a subset of ovarian cancer patients, in this case those who had either well or moderately differentiated carcinoma. The association between p53 overexpression and reduced survival either in all patients or in the G1/G2 subgroup could not be explained by a tendency of p53-negative patients to receive potentially life-extending postoperative therapies, since all but one of the 39 p53-positive patients received cisplatin-containing regimens while only 30 of the 51 p53-negative patients were treated. In one of the two studies that examined p53 protein overexpression in early stage ovarian cancer (Hartmann *et al.*, 1994), no association was found between p53 abnormality and overall survival of Stage I patients in multivariate analysis, although the relationship approached statistical significance at the univariate level. Another study also reporting that p53 overexpression was not associated with adverse outcome in early stage (I-II) patients confirmed the IHC findings in many cases by direct sequencing of the p53 gene. (Kohler *et al.*, 1993a). The only other study in which the prognostic significance of p53 was evaluated in a subgroup of patients considered the survival of patients with Stage III/IV tumors, who were revealed to exhibit similar unfavorable outcome regardless of p53 status (van der Zee *et al.*, 1995), an observation in agreement with our findings.

Our results prompted us to speculate that in tumors that are not well differentiated, in addition to or independent of the presence of p53, the function of other genes may confer an aggressive phenotype to the ovarian tumors which ultimately lead to the relapse and

demise of the patient. These genes of uncertain identities may not be expressed in G1 or G2 malignant lesions, and in this case, the unfavorable aspect of p53 inactivation may become manifest. Alternatively, it is also possible that ovarian neoplasms which are well or moderately differentiated and nonmetastatic at the time of diagnosis represent a different, less aggressive subset of ovarian tumors which are less likely to have acquired p53 mutations. The lack of an easily identifiable premalignant lesion in the ovarian epithelium, in contrast to breast and colon cancers, is a major obstacle in determining whether aberrant p53 expression is an early or late event in ovarian carcinogenesis.

It is recognized that most patients with ovarian cancer have advanced disease at the time of diagnosis and will therefore receive postoperative chemotherapy regardless of tumor grade of other features. Our findings might be useful in guiding treatment decisions in the small number of patients who present with early stage, low grade disease. In these patients, assessment of p53 gene alterations or p53 protein overexpression might identify those at higher risk of relapse and/or death, and hence for whom adjuvant treatment would be warranted.

7-4. Conclusions

Using a time-resolved immunofluorometric assay to quantitate p53 protein expression in extracts of 90 epithelial ovarian carcinomas, and the application of Cox proportional hazard regression and Kaplan-Meier survival analyses, the presence of p53 protein accumulation in patients with well or moderately differentiated disease was shown to be a

strong and significant indicator of reduced disease-free and overall survival. In addition, ovarian cancer patients with no diagnosable postsurgical malignancy displayed significantly worse disease-free outcome when p53 protein was overexpressed in their tumors, and a trend was similarly found between p53-positivity and disease-free survival of patients with early stage disease. In poorly differentiated or late stage ovarian cancer, however, other gene products may be linked to the invasive and metastatic phenotype and hence may be of use as prognostic indicators.

Chapter 8. References

AAS T, BORRESEN AL, GEISLER S, SMITH-SORENSEN B, JOHNSEN H, VARGHAUG JE, AKSLEN LA, AND LONNING PE. (1996). Specific p53 mutations are associated with de novo resistance to doxorubicin in breast cancer patients. *Nature Med.*, **2**, 811-813.

AGARWAL ML, AGARWAL A, TAYLOR WR, AND STARK GR. (1995). p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 8493-8497.

AGOFF SN, HOU JH, LINZER DIH, AND WU B. (1993). Regulation of the human hsp70 promoter by p53. *Science*, **259**, 84-87.

ALONI-GRINSTEIN R, SCHWARTZ D, AND ROTTER V. (1995). Accumulation of wild-type p53 protein upon γ-irradiation induces a G2 arrest-dependent immunoglobulin κ light chain gene expression. *EMBO J.*, **14**, 1392-1401.

AMERICAN CANCER SOCIETY. (1995). *Cancer Facts and Figures.* p 6. American Cancer Society: Atlanta.

AMSON RB, NEMANI M, ROPERCH JP, ISRAELI D, BOUGUELERET L, LE GALL I, MEDHIOUB M, LINARES-CRUZ G, LETHROSNE F, PASTURAUD P, PIOUFFRE L, PRIEUR S, SUSINI L, ALVARO V, MILLASSEAU P, GUIDICELLI C, BUI H, MASSART C, CAZES L, DUFOUR F, BRUZZONI-GIOVANELLI H, OWADI H, HENNION C, CHARPAK G, DAUSEET J, CALVO F, OREN M, COHEN D, AND TELERMAN A. (1996). Isolation of 10 differentially expressed cDNAs in p53-induced apoptosis: activation of the vertebrate homologue of the Drosophila seven in absentia gene. *Proc. Natl. Acad. Sci. U.S.A.*, **93**, 3953-3957.

ANGELOPOULOU K, DIAMANDIS EP, SUTHERLAND DJA, KELLEN JA, AND BUNTING PS. (1994). Prevalence of serum antibodies against the p53 tumor suppressor protein in various cancers. *Int. J. Cancer*, **58**, 480-487.

AVERETTE HE, JANICEK MF, AND MENCK HR. (1995). The national cancer data base report on ovarian cancer. *Cancer*, **76**, 1096-1103.

BAAS IO, MULDER JWR, OFFERHAUS GJA, VOGELSTEIN B, AND HAMILTON SR. (1994). An evaluation of six antibodies for immunohistochemistry of mutant p53 gene product in archival colorectal neoplasms. *J. Pathol.*, **172**, 5-12.

BAKER SJ, FEARON ER, NIGRO JM, HAMILTON SR, PREISINGER AC, JESSUP JM, VAN TUINEN P, LEDBETTER DH, BARKER DF, NAKAMURA Y, WHITE R, AND VOGELSTEIN B. (1989). Chromosome 17 deletions and p53 gene mutations in colorectal carcinoma. *Science*, **244**, 217-221.

BAKER SJ, MARKOWITZ S, FEARON ER, WILLSON JKV, AND VOGELSTEIN B. (1990). Suppression of human colorectal carcinoma cell growth by wild-type p53 protein. *Science*, **249**, 912-915.

BALKALKIN G, SELIVANOVA G, YAKOVLEVA T, KISELEVA E, KASHUBA E, MAGNUSSON KP, SZEKELY L, KLEIN G, TERENIUS L, AND WIMAN KG. (1995). p53 binds single-stranded DNA ends through the C-terminal domain and internal DNA segments via the middle domain. *Nucl. Acids Res.*, **23**, 362-369.

BALKALKIN GT, YAKOVLEVA T, SELIVANOVA G, MAGNUSSON KP, SZEKELY L, KISLEVA E, KLEIN G, TERENIUS L, AND WIMAN KG. (1994). p53 binds single-stranded DNA ends and catalyzes DNA renaturation and strand transfer. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 413-417.

BARAK Y, JUVEN T, HAFFNER R, AND OREN M. (1993). mdm2 expression is induced by wild type p53 activity. *EMBO J.*, **12**, 461-468.

BARGONETTI J, REYNISDOTTIR I, FRIEDMAN PN, AND PRIVES C. (1992). Site-specific binding of wild-type p53 to cellular DNA is inhibited by SV40 T antigen and mutant p53. Genes & Dev., 6, 1886-1897.

BARTEK J, BARTKOVA J, LUKAS J, STASKOVA Z, VOJTESEK B, AND LANE DP. (1993). Immunohistochemical analysis of the p53 oncoprotein on paraffin sections using a series of novel monoclonal antibodies. *J. Pathol.*, **169**, 27-34.

BARTKOVA J, BARTEK J, VOJTESEK B, LUKAS J, REJTHAR, KOVARIK J MILLIS RR, LANE DP, AND BARNES DM. (1993). Immunochemical analysis of the p53 oncoprotein in matched primary and metastatic human tumors. *Eur. J. Cancer*, **29A**, 881-886.

BARTLETT JM, LANGDON SP, SIMPSON BJ, STEWART M, KATSAROS D, SISMONDI P, LOVE S, SCOTT WM, WILLIAMS AR, LESSELLS AM, MACLEOD KG, SMYTH JF, AND MILLER WR. (1996). The prognostic value of epidermal growth factor receptor mRNA expression in primary ovarian cancer. *Br. J. Cancer*, **73**, 301-306.

BAST RC, KLUG TL, ST JOHN E, JENISON E, NILOFF JM, LAZARUS H, BERKOWITZ RS, LEAVITT T, GRIFFITHS CT, PARKER L, ZURAWSKI VR, AND KNAPP RC. (1983). A radioimmunoassay using a monoclonal antibody to monitor the course of epithelial ovarian cancer. *J. Clin. Invest.*, **68**, 1331-1337.

BAUER JL, SESTERHENN IA, MOSTOFI KF, MCLEOD DG, SRIVASTAVA S, AND MOUL JW. (1995). p53 nuclear protein expression is an independent prognostic marker in clinically localized prostate cancer patients undergoing radical prostatectomy. *Clin. Cancer Res.*, **1**, 1295-1300.

BEAHRS OH, HENSON DE, HUTTER RVP AND KENNEDY BJ. (1992). *Manual for Staging of Cancer*, 3rd ed. pp 167-169. JB Lippincott: Philadelphia.

BECK JS, KWITEK AE, COGEN PH, METZGER AK, DUYK GM, AND SHEFFIELD VC. (1993). A denaturing gradient gel electrophoresis assay for sensitive detection of p53 mutations. *Hum. Genet.*, **91**, 25-30.

BELLACOSA A, DE FEO D, GODWIN AK, BELL DW, CHENG JQ, ALTOMARE DA, WAN M, DUBEAU L, SCAMBIA G, AND MASCIULLO V. (1995). Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. *Int. J. Cancer*, **64**, 280-285.

BENHATTAR J, CEROTTINI JP, SARAGA E, METTHEZ G, AND GIVEL JC. (1996). p53 mutations as a possible predictor of response to chemotherapy in metastatic colorectal carcinomas. *Int. J. Cancer (Pred. Oncol.)*, **69**, 190-192.

BERCHUCK A, KAMEL A, WHITAKER R, KERNS B, OLT G, KINNEY R, SOPER JT, DODGE R, CLARKE-PEARSON DL, AND MARKS P. (1990). Overexpression of HER2/neu is associated with poor survival in advanced epithelial ovarian cancer. *Cancer Res.*, **50**, 4087-4091.

BERGH J, NORBERG T, SJOGREN S, LINDGREN A, AND HOLMBERG L. (1995). Complete sequencing of the p53 gene provides prognostic information in breast cancer patients, particularly in relation to adjuvant systemic therapy and radiotherapy. *Nature Med.*, **1**, 1029-1034.

BLAGOSKLONNY MV AND EL-DEIRY WS. (1996). In vitro evaluation of a p53-expressing adenovirus as an anti-cancer drug. *Int. J. Cancer*, **67**, 386-392.

BORRESEN AL, HOVIG E, SMITH-SORENSEN B, MALKIN D, LYSTAD S, ANDERSEN TI, NESLAND JM, ISSELBACHER KJ, AND FRIEND SH. (1991). Constant denaturant gel electrophoresis as a rapid screening technique for p53 mutations. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 8405-8409.

BOSARI S, VIALE G, BOSSI P, MAGGIONI M, COGGI G, MURRAY JJ, AND LEE AK. (1994). Cytoplasmic accumulation of p53 protein: an independent prognostic indicator in colorectal adenocarcinomas. *J. Natl. Cancer Inst.*, **86**, 681-687.

BOYLE JO, HAKIM J, KOCH W, VAN DER RIET P, HRUBAN RH, ROA RA, CORREO R, EBY YJ, RUPPERT JM, AND SIDRANSKY D. (1993). The incidence of p53 mutations increases with progression of head and neck cancer. *Cancer Res.*, **53**, 4477-4480.

BRACHMANN RK, VIDAL M, AND BOEKE JD. (1996). Dominant-negative p53 mutations selected in yeast hit cancer hot spots. *Proc. Natl. Acad. Sci. U.S.A.*, **93**, 4091-4095.

BRUGIERES J, GARDES M, MOUTOU C, CHOMPRET A, MERESSE A, MARTIN A, POISSON N, FLAMANT F, BONAITI-PELLIE C, LEMERLE J, AND FEUNTEUN J. (1993). Screening for germ line p53 mutations in children with malignant tumors and a family history of cancer. *Cancer Res.*, **53**, 452-455.

BUCKBINDER L, TALBOTT R, VELASCO-MIGUEL, TAKENAKA I, JACKS T,, AND HANNON GJ. (1995). Induction of the growth inhibitor IGF-binding protein 3 by p53. *Nature*, **377**, 646-649.

BULLER RE, ANDERSON B, CONNOR JP, AND ROBINSON R. (1993). Familial ovarian cancer. *Gynecol. Oncol.*, **51**, 160-166.

BULLER RE, SKILLING JS, KALISZEWSKI S, NIEMANN T, AND ANDERSON B. (1995). Absence of significant germ line p53 mutations in ovarian cancer patients. *Gynecol. Oncol.*, **58**, 368-374.

CAELLES CA, HELMBERG A, AND KARIN M. (1993). p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes. *Nature*, **370**, 220-223.

CAMPLEJOHN RS, PERRY P, HODGSON SV, TURNER G, WILLIAMS A, UPTON C, MACGEOCH C, MOHAMMED S, AND BARNES DM. (1995). A possible screening test for inherited p53-related defects based on the apoptotic response of peripheral blood lymphocytes to DNA damage. *Br. J. Cancer*, **72**, 654-662.

CANNISTRA SA. (1993). Cancer of the ovary. *New Engl. J. Med.*, **329**, 1550-1559.

CASSON AG, MANOLOPOULOS B, TROSTER M, KERKVLIET N, O'MALLEY F, INCULET R, FINLEY R, AND ROTH JA. (1994). Clinical implications of p53 gene mutation in the progression of Barrett's epithelium to invasive esophageal cancer. *Am. J. Surg.*, **167**, 52-57.

CHANG YS, LIN YJ, TSAI CN, SHU CH, TSAI MS, CHOO KB, AND LIU ST. (1992). Detection of mutations in the p53 gene in human head and neck carcinomas by single strand conformation polymorphism analysis. *Cancer Lett*, **67**, 167-174.

CHEN JY, FUNK WD, WRIGHT WE, SHAY JW, AND MINNA JD. (1993). Heterogeneity of transcriptional activity of mutant p53 proteins and p53 DNA target sequences. *Oncogene*, **8**, 2159-2166.

CHEN Y, CHEN PL, AND LEE WH. (1994). Hot-spot p53 mutants interact specifically with two cellular proteins during progression of the cell cycle. *Mol. Cell. Biol.*, **14**, 6764-6772.

CHIBA I, TAKAHASHI T, NAU MM, D'AMICO D, CURIEL DT, MITSUDOMI T, BUCHHAGEN DL, CARBONE D, PIANTADOSI S, AND KOGA H. (1990). Mutations in the p53 gene are frequent in primary resected non-small-cell lung cancer. *Oncogene*, **5**, 1603-1610.

CHIN KV, UEDA K, PASTAN I, AND GOTTESMAN MM. (1992). Modulation of activity of the promoter of the human MDR1 gene by ras and p53. *Science*, **255**, 459-462.

CHIN YE, KITAGAWA M, SU WCS, YOU ZH, IWAMOTO Y, AND FU XY. (1996). Cell growth arrest and induction of cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ mediated by STAT1. *Science*, **272**, 719-721.

CHOY, GORINA S, JEFFREY PD, AND PAVLETICH NP. (1994). Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. *Science*, **265**, 346-355.

CHOWDARY DR, DERMODY JJ, JHA KK, AND OZER HL. (1994). Accumulation of p53 in a mutant cell line defective in the ubiquitin pathway. *Mol. Cell. Biol.*, **14**, 1997-2003.

CHRISTOLOPOULOS TK AND DIAMANDIS EP. (1992). Enzymatically-amplified time-resolved fluorescence immunoassay with terbium chelates. *Anal. Chem.*, **64**, 342-346.

CLARKE AR, PURDIE CA, HARRISON DJ, MORRIS RG, BIRD CC, HOOPER ML, AND WYLLIE AH. (1993). Thymocyte apoptosis induced by p53 dependent and independent pathways. *Nature*, **362**, 849-852.

CLIBY W, RITLAND S, HARTMANN L, DODSON M, HALLING KC, KENNEY G, PODRATZ KC, AND JENKINS RB. (1993). Human epithelial ovarian cancer allelotype. *Cancer Res.*, **53**, 2393-2398.

COX DR. (1972). Regression models and life tables. *J. R. Stat. Soc.(B)*, **34**, 187-202.

COX LS, HUPP T, MIDGLEY CA, AND LANE DP. (1995). A direct effect of activated human p53 on nuclear DNA replication. *EMBO J.*, **14**, 2099-2105.

CRAWFORD LV, PIM DC, AND BULBROOK RD. (1982). Detection of antibodies against the cellular protein p53 in sera of patients with breast cancer. *Int. J. Cancer*, **30**, 403-408.

CROSS SM, SANCHEZ CA, MORGAN CA, SCHIMKE MK, RAMEL S, IDZERDA RL, RASKIND WH, AND REID BJ. (1995). A p53-dependent mouse spindle checkpoint. *Science*, **267**, 1353-1356.

DABHOLKAR M, VIONNET J, BOSTICK-BRUTON F, YU JJ, AND REED E. (1994). Messenger RNA levels of XPAC and ERCC1 in ovarian cancer tissue correlate with response to platinum-based chemotherapy. *J. Clin. Invest.*, **94**, 703-708.

DAMERON KM, VOLPERT OV, TAINSKY MA, AND BOUCK N. (1994). Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. *Science*, **265**, 1582-1584.

DEBBAS M AND WHITE E. (1993). Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. *Genes & Dev.*, **7**, 546-554.

DELEO AB, JAY G, APPELLA E, DUBOIS GC, LAW LW, AND OLD LJ. (1979). Detection of a trnasformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse. *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 2420-2424.

DEL SAL GD, RUARO EM, UTRERA R, COLE CN, LEVINE AJ, AND SCHNEIDER C. (1995). Gas1-induced growth suppression requires a transactivation-independent p53 function. *Mol. Cell. Biol.*, **15**, 7152-7160.

DEMERS GW, FOSTER SA, HALBERT CL, AND GALLOWAY DA. (1994a). Growth arrest by induction of p53 in DNA damaged keratinocytes is bypassed by human papillomavirus 16 E7. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 4382-4386.

DEMERS GW, HALBERT CL, AND GALLOWAY DA. (1994b). Elevated wild-type p53 protein levels in human epithelial cell lines immortalized by human papillomavirus type 16 E7 gene. *Virology*, **198**, 169-174.

DEMIREL D, LAUCIRICA R, FISHMAN A, OWENS RG, GREY MM, KAPLAN AL, AND RAMZY I. (1996). Ovarian tumors of low malignant potential: correlation of DNA index and S-phase fraction with histopathologic grade and clinical outcome. *Cancer*, **77**, 1494-1500.

DENG C, ZHANG P, HARPER JW, ELLEDGE SJ, AND LEDER P. (1995). Mice lacking $p21^{CIP1/WAF1}$ undergo normal development but are defective in G1 checkpoint control. *Cell*, **82**, 675-684.

DE WITTE HH, FOEKENS JA, LENNERSTRAND J, SMID M, LOOK MP, KLUN JGM, BENRAAD TJ, AND BERNS EMJJ. (1996). Prognostic significance of TP53 accumulation in human primary breast cancer: comparison between a rapid quantitative immunoassay and SSCP analysis. *Int. J. Cancer (Pred. Oncol.)*., **69**. 125-130.

DiLeonardo A, Linke SP, Clarkin K, and Wahl GM. (1994). DNA damage triggers a prolonged p53-dependent G_1 arrest and long-term induction of Cip1 in normal human fibroblasts. *Genes & Dev.*, **8**, 2540-2551.

Dittmer D, Pati S, Zambetti G, Chu S, Teresky AK, Moore M, Finlay C, and Levine AJ. (1993). Gain of function mutations in p53. *Nature Genet.*, **4**, 42-45.

Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA, Butel JS, and Bradley A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature*, **356**, 215-221.

Donghi R, Longoni A, Pilotti S, Michieli P, Della Porta G, and Pierotti MA. (1993). Gene p53 mutations are restricted to poorly differentiated and undifferentiated carcinomas of the thyroid gland. *J. Clin. Invest.*, **91**, 1753-1760.

Drobnjak M, Latres E, Pollack D, Karpeh M, Dudas M, Woodruff JM, Brennan MF, and Cordon-Cardo C. (1994). Prognostic implications of p53 overexpression and high proliferative index of Ki-67 in adult soft tissue sarcomas. *J. Natl. Cancer Inst.*, **86**, 549-554.

Dulic V, Kaufmann WK, Wilson SJ, Tlsty TD, Lees E, Harper JW, Elledge SJ, and Reed SI. (1994). p53 dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. *Cell*, **76**, 1013-1023.

Dutta A, Ruppert SM, Aster JC, and Winchester E. (1993). Inhibition of DNA replication factor RPA by p53. *Nature*, **365**, 79-82.

Eccles DM, Brett L, Lessells A, Gruber L, Lane D, Steel CM, and Leonard RC. (1992). Overexpression of the p53 protein and allele loss at 17p13 in ovarian carcinoma. *Br. J. Cancer*, **65**, 40-44.

El-Deiry WS, Harper JW, O'Connor PM, Velculescu VE, Canman CE, Jackman J, Pietenpol JA, Burrell M, Hill DE, Wang Y, Wiman KG, Mercer WE, Kastan MB, Kohn KW, Elledge SJ, Kinzler KW, and Vogelstein B. (1994). WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res.*, **54**, 1169-1174.

El-Diery WS, Kern SE, Pietenpol JA, Kinzler KW, and Vogelstein B. (1992). Definition of a consensus binding site for p53. *Nature Genet.*, **1**, 45-49.

El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, and Vogelstein B, (1993). WAF1, a potential mediator of p53 tumor suppression. *Cell*, **75**, 817-825.

ELLEDGE RM, LOCK-LIM S, ALLRED DC, HILSENBECK SG, AND CORDNER L. (1995). p53 mutation and tamoxifen resistance in breast cancer. *Clin. Cancer Res.*, **1**, 1203-1208.

FAILLE A, DE CREMOUX P, EXTRA JM, LINARES G, ESPIE M, BOURSTYN E, DE ROCQUANCOURT A, GIACCHETTI S, MARTY M, AND CALVO F. (1994). p53 mutations and overexpression in locally advanced breast cancers. *Br. J. Cancer*, **69**, 1145-1150.

FAJAC A, BENARD J, LHOMME C, REY A, DUVILLARD P, ROCHARD F, BERNAUDIN JF, AND RIOU G. (1995). c-erbB2 gene amplification and protein expression in ovarian epithelial tumors: evaluation of their respective prognostic significance by multivariate analysis. *Int. J. Cancer*, **64**, 146-151.

FAN S, EL-DEIRY WS, BAE I, FREEMAN J, JONDIE D, BHATIA K, FORNACE AJ, MAGRATH I, KOHN KW, AND O'CONNOR PM. (1994). p53 gene mutations are associated with decreased sensitivity of human lymphoma cells to DNA damaging agents. *Cancer Res.*, **54**, 5824-5830.

FAN S, SMITH ML, RIVET DJ, DUBA D, ZHAN Q, KOHN KW, FORNACE AJ, AND O'CONNOR PM. (1995). Disruption of p53 function sensitizes breast cancer MCF-7 cells to cisplatin and pentoxifylline. *Cancer Res.*, **55**, 1649-1654.

FEARON ER AND VOGELSTEIN B. (1990). A genetic model for colorectal carcinogenesis. *Cell*, **61**, 759-767.

FIELDS S AND JANG SK. (1990). Presence of a potent transcription activating sequence in the p53 protein. *Science*, **249**, 1046-1049.

FINLAY CA, HINDS PW, AND LEVINE AJ. (1989). The p53 proto-oncogene can act as a suppressor of transformation. *Cell*, **57**, 1083-1093.

FINLAY CA, HINDS PW, TAN, TH, ELIYAHU D, OREN M, AND LEVINE AJ. (1988). Activating mutations for transformation by p53 produce a gene product that forms an hsc70-p53 complex with an altered half-life. *Mol. Cell. Biol.*, **8**, 531-539.

FISHER CJ, GILLETT CE, VOJTESEK B, BARNES DM, AND MILLIS RR. (1994). Problems with p53 immunohistochemical staining: the effect of fixation and variation in the methods of evaluation. *Br. J. Cancer*, **69**, 26-31.

FISHER DE. (1994). Apoptosis in cancer therapy: crossing the threshold. *Cell*, **78**, 539-542.

FLAMAN JM, FREBOURG T, MOREAU V, CHARBONNIER F, MARTIN C, CHAPPUIS P, SAPPINO AP, LIMACHER JM, BRONS L, BENHATTAR J, TADA M, VAN MEIR EG,

ESTRICHER A, AND IGGO RD. (1995). A simple functional assay for screening cell lines, blood, and tumors. *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 3963-3967.

FLORES-ROZAS H, KELAMN Z, DEAN FB, PAN ZQ, HARPER JW, ELLEDGE SJ, O'DONNELL M, AND HURWITZ J. Cdk-interacting protein 1 directly binds with the proliferating cell nuclear antigen and inhibits DNA replication catalyzed by the DNA polymerase δ holoenzyme. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 8655-8659.

FOULKES WD, BLACK DM, STAMP GW, SOLOMON E, AND TROWSDALE J. (1993). Very frequent loss of heterozygosity throughout chromosme 17 in sporadic ovarian carcinoma. *Int. J. Cancer*, **54**, 220-225.

FRANK TS, BARTOS RE, HAEFNER HK, ROBERTS JA, WILSON MD, AND HUBBELL GP. (1994). Loss of heterozygosity and overexpression of the p53 gene in ovarian carcinoma. *Mod. Pathol.*, **7**, 3-8.

FREBOURG T, BARBIER T, KASSEL J, NG YS, ROMERO P, AND FRIEND SH.(1992). A functional screen for germ line p53 mutations based on transcriptional activation. *Cancer Res.*, **52**, 6976-6978.

FRIEDRICHS K, GLUBA S, EIDTMANN H, AND JONAT W. (1993). Overexpression of p53 and prognosis in breast cancer. *Cancer*, **72**, 3641-3647.

FUJIMOTO K, YAMADA Y, OKIJIMA E, KAKIZOE T, SASAKI H, SUGIMURA T, AND TERADA M. (1992). Frequent association of p53 gene mutation in invasive bladder cancer. *Cancer Res.*, **52**, 1393-1398.

FUJITA M, ENOMOTO T, INOUE M, TANIZAWA O, OZAKI M, RICE JM, AND NOMURA T. (1994). Alteration of the p53 tumor suppressor gene occurs independently of k-ras activation and more frequently in serous adenocarcinomas than in other common epithelial tumors of the human ovary. *Jpn. J. Cancer Res.*, **85**, 1247-1256.

FUKASAWA K, CHOI T, KURIYAMA R, RULONG S, AND VANDE WOUDE GF. (1996). Abnormal centrosome amplification in the absence of p53. *Science*, **271**, 1744-1747.

GALLION HH, GUARINO AN DEPRIEST PD, VAN NAGELL JR, VACCARELLO L, BEREK JS, AND PIERETTI M. (1996). Evidence for a unifocal origin in familial ovarian cancer. *Am. J. Ostet. Gynecol.*, **174**, 1102-1108.

GANNON JV, GREAVES R, IGGO R, AND LANE DP. (1990). Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. *EMBO J.*, **9**, 1595-1602.

GOH HS, YAO J, AND SMITH DR. (1995). p53 point mutation and survival in colorectal cancer patients. *Cancer Res.*, **55**, 5217-5221.

GODWIN AK, VANDERVEER L, SCHULTZ DC, LYNCH HT, ALTOMARE DA, BUETOW KH, DALY M, GETTS LA, MASNY A, AND ROSENBLUM N. (1993). A common region of deletion on chromosome 17q in both sporadic and familial ovarian tumors distal to BRCA1. *Am. J. Hum. Genet.*, **55**, 666-677.

GOTTLIEB ER, HAFFNER R, VON RUDEN T, WAGNER EF, AND OREN M. (1994). Down-regulation of wild-type p53 activity interferes with apoptosis of IL-3-dependent hematopoietic cells following IL-3 withdrawal. *EMBO J.*, **13**, 1368-1374.

GRAEBER TG, OSMANIAN C, JACKS T, HOUSMAN DE, KOCH CJ, LOWE SW, AND GIACCIA AJ. (1996). Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature*, **379**, 88-91.

GRAEBER TG, PETERSON JF, TSAI M, MONICA K, FORNACE AJ, AND GIACCIA AJ. (1994). Hypoxia induces accumulation of p53 protein, but activation of a G1-phase checkpoint by low oxygen conditions is independent of p53 status. *Mol. Cell. Biol.*, **14**, 6264-6277.

GREEN JA, MUDENDA B, JENKINS J, LEINSTER SJ, TARUNINA M, GREEN B, AND ROBERTSON L. (1994). Serum p53 auto-antibodies: incidence in familial breast cancer. *Eur. J. Cancer*, **30A**, 580-584.

GREENBLATT MS, BENNETT WP, HOLLSTEIN M, AND HARRIS CC. (1994). Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res.*, **54**, 4855-4878.

GUILLOT C, FALETTE N, COURTOIS S, VOELTZEL T, GARCIA E, OZTURK M, AND PUISIEUX A. (1996). Alteration of p53 damage response by tamoxifen treatment. *Clin. Cancer Res.*, **2**, 1439-1444.

GUPTA-BURT S, SHAMKHANI H, REED E, TARONE RE, ALLEGRA CJ, PAI LH, AND POIRIER MC. (1993). Relationship between patient response in ovarian and breast cancer and platinum drug-DNA adduct formation. *Cancer Epidemiol. Biomarkers Prev.*, **2**, 229-234.

HAINAUT P AND MILNER J. (1993). Redox modulation of p53 conformation and sequence-specific DNA binding in vitro. *Cancer Res.*, **53**, 4469-4473.

HALAZONETIS TD, DAVIS LJ, AND KANDIL AN. (1993). Wild-type p53 adopts a "mutant"-like conformation when bound to DNA. *EMBO J.*, **12**, 1021-1028.

HAMADA K, ALEMANY R, ZHANG WW, HITTELMAN WN, LOTAN R, ROTH JA, AND FOLLEN MITCHELL M. (1996). Adenovirus-mediated transfer of a wild-type p53 gene and induction of apoptosis in cervical cancer. *Cancer Res.*, **56**, 3047-3054.

HARLOW E AND LANE D. (1988). *Antibodies: A Laboratory Manual*, pp 53-287. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.

HARPER JW, ELLEDGE SJ, KEYOMARSE K, DYNLACHT B, TSAI LH, ZHANG P, DOBROWOLSKI S, BAI C, CONNELL-CROWLEY L, SWINDELL E, FOX MP, AND WEI N. (1995). Inhibition of cyclin-dependent kinases by p21. *Mol. Cell. Biol.*, **6**, 387-400.

HARRIS CC AND HOLLSTEIN M. (1993). Clinical implications of the p53 tumor suppressor gene. *N. Engl. J. Med.*, **329**, 1318-1327.

HARRIS LC, REMACK JS, HOUGHTON PJ, AND BRENT TP. (1996). Wild-type p53 suppresses transcription of the human O^6-methylguanine-DNA methyltransferase gene. *Cancer Res.*, **56**, 2029-2032.

HARTMANN LC, PODRATZ KC, KEENEY GL, KAMEL NA, EDMONSON JH, GRILL JP, SU JQ, KATZMANN JA, AND ROCHE PC. (1994). Prognostic significance of p53 immunostaining in epithelial ovarian cancer. *J. Clin. Oncol.*, **12**, 64-69.

HARVEY M, MCARTHUR MJ, MONTGOMERY CA, BUTEL JS, BRADLEY A, AND DONEHOWER LA. (1993). Spontaneous and carcinogen-induced tumorigenesis in p53-deficient mice. *Nature Genet.*, **5**, 225-229.

HARVEY M, SANDS AT, WEISS RS, HEGI ME, WISEMAN RW, PANTAZIS P, GIOVANELLA BC, TAINSKY MA, BRADLEY A, AND DONEHOWER LA. (1993). In vitro growth characteristics of embryo fibroblasts isolated from p53 deficient mice. *Oncogene*, **8**, 2457-2467.

HARVEY M, VOGEL H, MORRIS D, BRADLEY A, BERNSTEIN A, AND DONEHOWER LA. (1995). A mutant p53 transgene accelerates tumour development in heterozygous but not nullizygous p53-deficient mice. *Nature Genet.*, **9**, 305-311.

HASSAPOGLIDOU S, DIAMANDIS EP, AND SUTHERLAND DJA. (1993). Quantification of p53 protein in tumour cell lines, breast tissue extracts and serum with time-resolved immunofluorometry. *Oncogene*, **8**, 1501-1509.

HAUPT T, BARAK Y, AND OREN M. (1996). Cell type specific inhibition of p53-mediated apoptosis by mdm2. *EMBO J.*, **15**, 1596-1606.

HAUPT Y, ROWAN S, SHAULIAN E, VOUSDEN K, AND OREN M. (1995). Induction of apoptosis in HeLa cells by transactivation-deficient p53. *Genes & Dev.*, **9**, 2170-2183.

HEDRUM A, PONTEN F, REN Z, LUNDEBERG J, PONTEN J, AND UHLEN M. (1994). Sequence-based analysis of the human p53 gene based on microdissection of tumor biopsy samples. *Biotechniques*, **17**, 118-129.

HENRIKSEN R, STRANG P, BACKSTROM T, WILANDER E, TRIBUKAIT B, AND OBERG K. (1994). Ki-67 immunostaining and DNA flow cytometry as prognostic factors in epithelial ovarian cancers. *Anticancer Res.*, **14**, 603-608.

HINDS P, FINLAY C, AND LEVINE AJ. (1989). Mutation is required to activate the p53 gene for cooperation with the ras oncogene and transformation. *J. Virol.*, **63**, 739-746.

HINDS PW, FINLAY CA, FREY AB, LEVINE AJ. (1987). Immunological evidence for association of p53 with a heat shock protein, hsc70, in p53-plus-ras-transformed cell lines. *Mol. Cell. Biol.*, **7**, 2863-2869.

HOLLSTEIN M, RICE K, GREENBLATT MS, SOUSSI T, FUCHS R, SORLIE T, HOVIG E, SMITH-SORENSEN B, MONTESANO R, AND HARRIS CC. (1994). Database of p53 gene mutations in human tumors and cell lines. *Nucleic Acids Res.*, **22**, 3551-3555.

HOLLSTEIN M, SIDRANSKY S, VOGELSTEIN B, AND HARRIS CC. (1991). p53 mutations in human cancers. *Science*, **253**, 49-53.

HORIKOSHI N, USHEVA A, CHEN J, LEVINE AJ, WEINMANN R, AND SHENK T. (1995). Two domains of p53 interact with the TATA-binding protein, and the adenovirus 13S E1A protein disrupts the association, relieving p53-mediated transcriptional repression. *Mol. Cell. Biol.*, **15**, 227-234.

HOUBIERS JGA, VAN DER BURG SH, VAN DE WATERING LMG, TOLLENAAR RAEM, BRAND A, VAN DE VELDE CJH, AND MELIEF CJM. (1995). Antibodies against p53 are associated with poor prognosis of colorectal cancer. *Br. J. Cancer*, **72**, 637-641.

HRUBAN RH, VAN DER RIET P, EROZAN YS, AND SIDRANSKY D. (1994). Molecular biology and the early detection of carcinoma of the bladder - the case of Hubert H. Humphrey. *N. Engl. J. Med.*, **330**, 1276-1278.

HSU HC, TSENG HJ, LAI PL, LEE PH, AND PENG SY. (1993). Expression of p53 gene in 184 unifocal hepatocellular carcinomas: association with tumor growth and invasiveness. *Cancer Res.*, **53**, 4691-4694.

HSU IC, METCALF RA, SUN T, WELSH JA, WANG NJ, AND HARRIS CC. (1991). Mutational hotspot in the p53 gene in human hepatocellular carcinomas. *Nature*, **350**, 427-431.

HUNTER SB, BANDEA C, SWAN D, ABBOTT K, AND VARMA VA. (1993). Mutations in the p53 gene in human astrocytomas: detection by single-strand conformation polymorphism analysis and direct sequencing. *Mod Pathol*, **6**, 442-445.

HUPP TR, MEEK DW, MIDGLEY CA, AND LANE DP. (1992). Regulation of the specific DNA binding function of p53. *Cell*, **71**, 875-886.

HUPP TR, SPARKS A, AND LANE DP. (1995). Small peptides activate the latent sequence-specific DNA binding function of p53. *Cell*, **83**, 237-245.

HUTSON R, RAMSDALE J, AND WELLS M. (1995). p53 protein expression in putative precursor lesions of epithelial ovarian cancer. *Histopathol.*, **27**, 367-371.

INTERNATIONAL FEDERATION OF GYNECOLOGY AND OBSTETRICS. (1987). Changes in definition of clinical staging for carcinoma of the cervix and ovary. *Am. J. Obstet. Gynecol.*, **56**, 263-264.

IOTSOVA V AND STEHELIN D. (1995). Antisense p53 provokes changes in Hela cell growth and morphology. *Eur. J. Cell Biol.*, **68**, 122-132.

ISHIOKA C, FREBOURG T, YAN YX, VIDAL M, FRIEND SH, SCHMIDT S, AND IGGO R. (1993). Screening patients for heterozygous p53 mutations using a functional assay in yeast. *Nature Genet.*, **5**, 124-129.

IWABUCHI K, BARTEL PL, LI B, MARRACCINO R, AND FIELDS S. (1994). Two cellular proteins that bind to wild-type but not mutant p53. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 6098-6102.

IZQUIERDO MA, VAN DER ZEE AG, VERMORKEN JB, VAN DER VALK P, BELIEN JA, GIACCONE G, SCHEFFER GL, FLENS FJ, PINEDO HM, AND KENEMANS P. Drug-resistance associated marker Lrp for prediction of response to chemotherapy and prognoses in advanced ovarian carcinoma. *J. Natl. Cancer Inst.*, **87**, 1230-1237.

JACOBS IJ, KOHLER MF, WISEMAN RW, MARKS JR, WHITAKER R, KERNS BA, HUMPHREY P, BERCHUCK A, PONDER BA, AND BAST RC. (1992). Clonal origin of epithelial ovarian carcinoma: analysis by loss of heterozygosity, p53 mutation, and X-chromosome inactivation. *J. Natl. Cancer Inst.*, **84**, 1793-1798.

JAMAL S AND ZIFF EB. (1995). Raf phosphorylates p53 in vitro and potentiates p53-dependent transcriptional transactivation in vivo. *Oncogene*, **10**, 2095-2101.

JAYARAMAN L AND PRIVES C. (1995). Activation of p53 sequence-specific DNA binding by short single strands of DNA requires the p53 C-terminus. *Cell*, **81**, 1021-1029.

JEFFREY PD, GORINA S, AND PAVLETICH NP. (1995). Crystal structure of the tetramerization domain of the p53 tumor suppressor at 1.7 angstroms. *Science*, **267**, 1498-1502.

JENKINS JR, CHUMAKOV P, ADDISON C, STURZBECHER HW, AND WADE-EVANS A. (1988). Two distinct regions of the murine p53 primary amino acid sequence are implicated in stable complex formation with simian virus 40 T antigen. *J. Virol.*, **62**, 3903-3906.

JENKINS JR, RUDGE K, AND CURRIE GA. (1984). Cellular immortalization by a cDNA clone encoding the transformation-associated phosphoprotein p53. *Nature*, **312**, 651-654.

JOLLY KW, MALKIN D, DOUGLAS EC, BROWN TF, SINCLAIR AE, AND LOOK AT. (1994). Splice-site mutation of the p53 gene in a family with hereditary breast-ovarian cancer. *Oncogene*, **9**, 97-102.

JONES SN, ROE AE, DONEHOWER LA, AND BRADLEY A. (1995). Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. *Nature*, **378**, 206-208.

JOYPAUL BV, VOJTESEK B, NEWMAN EL, HOPWOOD D, GRANT A, LANE DP, AND CUSCHIERI A. (1993). Enzyme-linked immunosorbent assay for p53 in gastrointestinal malignancy: comparison with immunohistochemistry. *Histopathol.*, **23**, 465-470.

JUNG M, NOTARIO V, AND DRITSCHILO A. (1992). Mutations in the p53 gene in radio-sensitive and -resistant human squamous cell carcinoma cells. *Cancer Res.*, **52**, 6390-6393.

KAERN J, TROPE CG, KRISTENSEN GB, AND PETTERSEN EO. (1994). Flow cytometric DNA ploidy and S-phase heterogeneity in advanced ovarian carcinoma. *Cancer*, **73**, 1870-1877.

KAKEJI Y, KORENAGA D, TSUJITANI S BABA H, ANAI H, MAEHARA Y, AND SUGIMACHI K. (1993). Gastric cancer with p53 overexpression has high potential for metastasizing to lymph nodes. *Br. J. Cancer*, **67**, 589-593.

KANDA T, SEGAWA K, OHUCHI N, MORI S, AND ITO Y. (1994). Stimulation of polyomavirus DNA replication by wild-type p53 through the DNA-binding site. *Mol. Cell. Biol.*,**14**, 2651-2663.

KAPLAN EL AND MEIER P. (1958). Nonparametric estimation from incomplete observations. *J. Am. Stat. Assoc.*, **53**, 457-481.

KAPPES S, MILDE-LANGOSCH K, KRESSIN P, PASSLACK B, DOCKHORN-DWORNICZAK B, ROHLKE P, AND LONING T. (1995). p53 mutations in ovarian tumors, detected by temperature-gradient gel electrophoresis, direct sequencing and immunohistochemistry. *Int. J. Cancer*, **64**, 52-59.

KASTAN MB, ONYERKWERE O, SIDRANSKY D, VOGELSTEIN B, AND CRAIG RW. (1991). Participation of p53 protein in the cellular response to DNA damage. *Cancer Res.*, **53**, 6304-6311.

KASTAN MB, ZHAN Q, EL-DEIRY WS, CARRIER F, JACKS T, WALSH WV, PLUNKETT BS, VOGELSTEIN B, AND FORNACE AJ. (1992). A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell*, **71**, 587-597.

KATAYOSE D, GUDAS J, NGUYEN H, SRIVASTAVA S, COWAN KH, AND SETH P. (1995). Cytotoxic effects of adenovirus-mediated wild-type p53 protein expression in normal and tumor mammary epithelial cells. *Clin. Cancer Res.*, **1**, 889-897.

KAVALLARIS M, LEARY JA, BARRETT JA, AND FRIEDLANDER ML. (1996). MDR1 and multidrug resistance-associated protein (MRP) gene expression in epithelial ovarian tumors. *Cancer Lett.*, **102**, 7-16.

KEMP CJ, DONEHOWER LA, BRADLEY A, AND BALMAIN A. (1993). Reduction of p53 gene dosage does not increase initiation or promotion but enhances malignant progression of chemically induced skin tumors. *Cell*, **74**, 813-822.

KEMP CJ, WHELDON T, AND BALMAIN A. (1994). p53-deficient mice are extremely susceptible to radiation-induced tumorigenesis. *Nature Genet.*, **8**, 66-69.

KERN SE, PIETENPOL JA, THIAGALINGAM S, SEYMOUR A, KINZLER KW, AND VOGELSTEIN B. (1992).Oncogenic forms of p53 inhibit p53-regulated gene expression. *Science*, **256**, 827-830.

KERNS BJ, JORDAN PA, MOORE MB, HUMPHREY PA, BERCHUCK A, KOHLER MF, BAST RC, IGLEHART JD, AND MARKS JR. (1992). p53 overexpression in formalin-fixed, paraffin-embedded tissue detected by immunohistochemistry. *J. Histochem. Cytochem.*, **40**, 1047-1051.

KIESER A, WEICH HA, BRANDNER G, MARME D, AND KOLCH W. (1994). Mutant p53 potentiates protein kinase C induction of vascular endothelial growth factor expression. *Oncogene*, **9**, 963-969.

KIHANA T, TSUDA H, TESHIMA S, OKADA S, MATSUURA S, AND HIROHASHI S. (1992). High incidence of p53 gene mutation in human ovarian cancer and its association with nuclear accumulation of p53 protein and tumor DNA ploidy. *Jpn. J. Cancer Res.*, **83**, 978-984.

KIM JW, CHO YH, KWON DJ, KIM TE, PARK TC, LEE JM, AND NAMKOONG SE. (1995). Aberrations of the p53 tumor suppressor gene in human epithelial ovarian carcinomas. *Gynecol. Oncol.*, **57**, 199-204.

KLEMI PJ, PYLKKANEN L, KIILHOLMA P, KURVINEN K, AND JOENSUU H. (1995). p53 protein detected by immunohistochemistry as a prognostic factor in patients with epithelial ovarian cancer. *Cancer*, **76**, 1201-1208.

KLEMI PJ, TAKAHASHI S, JOENSUU H, KIILHOLMA P, NARIMATSU E, AND MORI M. (1994). Immunohistochemical detection of p53 protein in borderline and malignant serous ovarian tumors. *Int. J. Gynecol. Pathol.*, **13**, 228-233.

KLEY N, CHUNG RY, FAY S, LOEFFLER JP, AND SEIZINGER BR. (1992). Repression of the basal c-fos promoter by wild-type p53. *Nucleic Acids Res.*, **20**, 4083-4087.

KOHLER MF, KERNS BJ, HUMPHREY PA, MARKDS JR, BAST RC, AND BERCHUCK A. (1993a). Mutation and overexpression of p53 in early-stage epithelial ovarian cancer. *Obstet. Gynecol.*, **81**, 643-650.

KOHLER MF, MARKS JR, WISEMAN RW, JACOBS IJ, DAVIDOFF AM, CLARKE-PEARSON DL, SOPER JT, BAST RC, AND BERCHUCK A. (1993b). Spectrum of mutation and frequency of allelic deletion of the p53 gene in ovarian cancer. *J. Natl. Cancer Inst.*, **85**, 1513-1519.

KUPRYJANCZYK J, BELL DA, DIMEO D, BEAUCHAMP R, THOR AD, AND YANDELL DW. (1995). p53 gene analysis of ovarian borderline tumors and stage I carcinomas. *Hum. Pathol.*, **26**, 387-392.

KUPRYJANCZYK J, BELL DA, YANDELL DW, SCULLY RE, AND THOR AD. (1994). p53 expression in ovarian borderline tumors and stage I carcinomas. *Am. J. Clin. Pathol.*, **102**, 671-676.

KUPRYJANCZYK J, THOR AD, BEAUCHAMP R, MERRITT V, EDGERTON SM, BELL DA, AND YANDELL DW. (1993). p53 gene mutations and protein accumulation in human ovarian cancer. *Proc. Natl. Acad. Sci. USA.*, **90**, 4961-4965.

LAMB P AND CRAWFORD L. (1986). Characterization of the human p53 gene. *Mol. Cell. Biol.*, **6**, 1379-1385.

LAMBKIN HA, MOTHERSILL CM, AND KELEHAN P. (1994). Variations in immunohistochemical detection of p53 protein overexpression in cervical carcinomas with different antibodies and methods of detection. *J. Pathol.*, **172**, 13-18.

LANE DP. (1992). p53. Guardian of the genome. *Nature*, **358**, 15-16.

LAVIGUEUR A, MALTBY V, MOCK D, ROSSANT J, PAWSON T, AND BERNSTEIN A. (1989). High incidence of lung, bone, and lymphoid tumors in transgenic mice overexpressing mutant alleles of the p53 oncogene. *Mol. Cell. Biol.*, **9**, 3982-3991.

LAWRENCE D. (1995). The borderland between benign and malignant surface epithelial ovarian tumors. *Cancer*, **76**, 2138-2142.

LEE JH, KANG YS, PARK SY, KIM BG, LEE ED, LEE KH, PARK KB, KAVANAGH JJ, AND WHARTON JT. (1995a). p53 mutation in epithelial ovarian carcinoma and borderline ovarian tumor. *Cancer Genet. Cytogenet.*, **85**, 43-50.

LEE S, ELENBAAS B, LEVINE A, AND GRIFFITH J. (1995b). p53 and its 14 kDa C-terminal domain recognize primary DNA damage in the form of insertion/deletion mismatches. *Cell*, **81**, 1013-1020.

LEHAR SM, NACHT M, JACKS T, VATER CA, CHITTENDEN T, AND GUILD BC. (1996). Identification and cloning of EI24, a gene induced by p53 in etoposide-treated cells. *Oncogene*, **12**, 1181-1187.

LEVEILLARD T, ANDERA L, BISSONNETTE N, SCHAEFFER L, BRACCO L, EGLY JM, AND WASYLYK B. (1995). Functional interactions between p53 and the TFIIH complex are affected by tumour-associated mutations. *EMBO J.*, **15**, 1615-1624.

LEVESQUE MA, CLARK GM, AND DIAMANDIS EP. (1995a). Immunofluorometric analysis of p53 protein and prostate specific antigen in breast tumors and their association with other prognostic factors. *Br. J. Cancer*, **72**, 720-727.

LEVESQUE MA, D'COSTA M, ANGELOPOULOU K, AND DIAMANDIS EP. (1995b). Time-resolved immunofluorometric assay of p53 protein. *Clin. Chem.*, **41**, 1720-1729.

LEVESQUE MA, DIAMANDIS EP, AND SUTHERLAND DJA. (1994). Quantitative analysis of mutant p53 protein in breast tumor cytosols and study of its association with other biochemical prognostic indicators in breast cancer. *Breast Cancer Res. Treat.*, **30**, 179-195.

LEVESQUE MA, YU H, CLARK GM, AND DIAMANDIS EP. (1996). p53 protein accumulation detected by a sensitive ELISA technique is a predictor of reduced survival of a large cohort of breast cancer patients. (manuscript in preparation).

LI FP, GARBER JE, FRIEND SH, STRONG LC, PATENUADE AF, JUENGST ET, REILLY PR, CORREA P, FRAUMENI JF. (1992). Recommendations on predictive testing for germ line p53 mutations among cancer-prone individuals. *J Natl. Cancer Inst.*, **84**, 1156-1160.

LIN J, CHEN J, ELENBAAS B, AND LEVINE AJ. (1994). Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptonal activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein. *Genes & Dev.*, **8**, 1235-1246.

LINKE SP, CLARKIN KC, DI LEONARDO A, TSOU A, AND WAHL GM. (1996). A reversible, p53-dependent G_0/G_1 cell cycle arrest induced by ribonucleotide depletion in the absence of detectable DNA damage. *Genes & Dev.*, **10**, 934-947.

LINZER DIH AND LEVINE AJ. (1979). Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell*, **17**, 43-52.

LIU E AND NUZUM C. (1995). Molecular sleuthing: tracking ovarian cancer progression. *J. Natl. Cancer Inst.*, **87**, 1099-1101.

LIU TJ, ZHANG WW, TAYLOR DL, ROTH JA, GOEPFERT H, AND CLAYMAN GL. (1994). Growth suppression of human head and neck cancer cells by the introduction of a wild-type p53 gene via a recombinant adenovirus. *Cancer Res.*, **54**, 3662-3667.

LIVINGSTONE LR, WHITE A, SPROUSE J, LIVANOS E, JACKS T, AND TLSTY TD. (1992). Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. *Cell*, **70**, 923-935.

LO COCO F, GAIDANO G, LOUIE DC, OFFIT K, CHAGANTI RS, AND DALLA-FAVERA R. (1993). p53 mutations are associated with histologic transformation of follicular lymphoma. *Blood*, **82**, 2289-2295.

LOHMANN DR, FUNK A, NIEDERMEYER HP, HAUPEL S, AND HOFLER H. (1993). Identification of p53 gene mutations in gastrointestinal and pancreastic carcinoids by nonradioactive SSCA. *Virchows Archiv B Cell Pathol*, **63**, 293-296.

LOWE S AND RULEY HE. (1993). Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. *Genes & Dev.*, **7**, 535-545.

LOWE SW, BODIS S, MCCLATCHEY A, REMINGTON L, RULEY HE, FISHER DE, HOUSMAN DE, AND JACKS T. (1994a). p53 status and the efficacy of cancer therapy in vivo. *Science*, **266**, 807-810.

LOWE SW, JACKS T, HOUSMAN DE, AND RULEY HE. (1994b). Abrogation of oncogene-associated apoptosis allows transformation of p53-deficient cells. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 2026-2030.

LOWE SW, RULEY HE, JACKS T, AND HOUSMAN DE. (1993a). p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell*, **74**, 957-967.

LOWE SW, SCHMITT EM, SMITH SW, OSBORNE BA, AND JACKS T. (1993b). p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature*, **362**, 847-849.

LU H AND LEVINE AJ. (1995). Human TAF31 protein is a transcriptional coactivator of the p53 protein. *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 5154-5158.

LU X AND LANE DP. (1993). Differential induction of transcriptionally active p53 following UV or ionizing radiation: defects in chromosome instability syndromes? *Cell*, **75**, 765-778.

LUBIN R, SCHLICHTHOLZ B, TEILLAUD JL, GARAY E, BUSSEL A, WILD CP, AND SOUSSI T. (1995a). p53 antibodies in patients with various types of cancer: assay, identification, and characterization. *Clin. Cancer Res.*, **1**, 1463-1469.

LUBIN R, ZALCMAN G, BOUCHET L, TREDENIEL J, LEGROS Y, CAZALS D, HIRSCH A, AND SOUSSI T. (1995b). Serum p53 antibodies as early markers of lung cancer. *Nature Med.*, **7**, 701-702.

MACK DH, VARIKAR J, PIPAS JM, AND LAIMINS LA. (1993). Specific represison of TATA-mediated but not initiator-mediated transcription by wild-type p53. *Nature*, **363**, 281-283.

MAHESWARAN S, ENGLERT C, BENNETT P, HEINRICH G, AND HABER DA. (1995). The WT1 gene product stabilizes p53 and inhibits p53-mediated apoptosis. *Genes & Dev.*, **9**, 2143-2156.

MAKOS WALES M, BIEL MA, EL-DEIRY W, NELKIN DB, ISSA JP, CAVANEE WK, KUERBITZ SJ, AND BAYLIN SB. (1995). p53 activates expression of HIC1, a new candidate tumour suppressor gene on 17p13.3. *Nature Med.*, **1**, 570-577.

MALKIN D, LI FP, STRONG LC, FRAUMENI JF, NELSON CE, KIM DH, KASSEL, MAGDALENA AG, BISCHOFF FZ, TAINSKY MA, AND FRIEND SH. (1990). Germ

line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. *Science*, **250**, 1233-1238.

MALTZMAN W AND CZYZYK L. (1984). UV irradiation stimulates levels of p53 tumor antigen in nontransformed mouse cells. *Mol. Cell. Biol.*, **4**, 1689-1694.

MANSUR CP, MARCUS B, DALAL S, AND ANDROPHY EJ. (1995). The domain of p53 required for binding HPV 16 E6 is separable from the degradation domain. *Oncogene*, **10**, 457-465.

MANTEL N. (1966). Evaluation of survival data and two new rank order statistics arising in its consideration. *Cancer Chemother. Rep.*, **50**, 163-170.

MAO L, HRUBAN RH, BOYLE JO, TOCKMAN M, AND SIDRANSKY D. (1994). Detection of oncogene mutations in sputum precedes diagnosis of lung cancer. *Cancer Res.*, **54**, 1634-1637.

MARKS JR, DAVIDOFF AM, KERNS BJ, HUMPHREY PA, PENCE JC, DODGE RK, CLARKE-PEARSON DL, IGLEHART JD. BAST RC, AND BERCHUCK A. (1991). Overexpression and mutation of p53 in epithelial ovarian cancer. *Cancer Res.*, **51**, 2979-2984.

MARTIN HM, FILIPE MI, MORRIS RW, LANE DP, AND SILVESTRE F. (1992). p53 expression and prognosis in gastric carcinoma. *Int. J. Cancer*, **50**, 859-862.

MASHIYAMA S, MURAKAMI Y, YOSHIMOTO T, SEKIYA T, AND HAYASHI K. (1991). Detection of p53 gene mutations in human brain tumors by single-strand conformation polymorphism analysis of polymerase chain reaction products. *Oncogene*, **6**, 1313-1318.

MAZARS R, PUJOL P, MAUDELONDE T, JEANTEUR P, AND THEILLET C. (1991). p53 mutations in ovarian cancer: a late event? *Oncogene*, **6**, 1685-1690.

MAZARS R, SPINARDI L, BENCHEIKH M, SIMONY-LAFONTAINE J, JEANTEUR P, AND THEILLET C. (1992). p53 mutations occur in aggressive breast cancer. *Cancer Res.*, **52**, 3918-2923.

MCILWRATH AJ, VASEY PA, ROSS GM, AND BROWN R. (1994). Cell cycle arrests and radiosensitivity of human tumor cell lines: dependence on wild-type p53 for radiosensitivity. *Cancer Res.*, **54**, 3718-3722.

MCMANUS DT, YAP EP, MAXWELL P, RUSSELL SE, TONER PG, AND MCGEE JO. (1994). p53 expression, mutation, and allelic deletion in ovarian cancer. *J. Pathol.*, **174**, 159-168.

MEEK D. (1994). Post-translational modification of p53. *Semin. Cancer Biol.*, **5**, 203-210.

METZ T, HARRIS AW, AND ADAMS JM. (1995). Absence of p53 allows direct immortalization of hematopoietic cells by the myc and raf oncogenes. *Cell*, **82**, 29-36.

MICHIELI PM, CHEDID M, LIN D, PIERCE JH, MERCER WE, AND GIVOL D. (1993). Induction of WAF1/CIP1 by a p53-independent pathway. *Cancer Res.*, **54**, 3391-3395.

MIDGLEY CA, FISHER CJ, BARTEK J, VOJTESEK B, LANE DP, AND BARNES BM. (1992). Analysis of p53 expression in human tumors: an antibody raised against human p53 expression in *Escherichia coli*. *J. Cell Sci.*, **101**, 183-189.

MILLER CW, SIMON K, ASLO A, KOK K, YOKOTA J, BUYS CHCM, TERADA M, AND KOEFFLER HP. (1992). p53 mutations in lung cancer. *Cancer Res.*, **52**, 1695-1698.

MILLER SD, FARMER G, AND PRIVES C. (1995). p53 inhibits DNA replication in vitro in a DNA-binding-dependent manner. *Mol. Cell. Biol.*, **15**, 6554-6560.

MILNE DM, CAMPBELL DG, CAUDWELL FB, AND MEEK D. (1994). Phosphorylation of the tumor suppressor protein p53 by mitogen-activated protein kinases. *J. Biol. Chem.*, **269**, 9253-9260.

MILNE DM, CAMPBELL LE, CAMPBELL DG, AND MEEK D. (1995). p53 is phosphorylated in vitro and in vivo by an ultraviolet radiation-induced protein kinase characteristic of the c-jun kinase, JNK1. *J. Biol. Chem.*, **270**, 5511-5518.

MILNER BJ, ALLAN LA, ECCLES DM, KITCHENER HC, LEONARD CF, KELLY KF, PARKIN DE, AND HAITES NE. (1993). p53 mutation is a common genetic event in ovarian carcinoma. *Cancer Res.*, **53**, 2128-2132.

MITSUDOMI T, LAM S, SHIRAKUSA T, AND GAZDAR AF. (1993). Detection and sequencing of p53 gene mutations in bronchial biopsy samples in patients with lung cancer. *Chest*, **104**, 362-365.

MITSUDOMI T, OYAMA T, KUSANO T OSAKI T, NAKANISHI R, AND SHIRAKUSA T. (1994). Mutations of the p53 gene as a predictor of poor prognosis in patients with non-small-cell lung cancer. *J. Natl. Cancer Inst.*, **85**, 2018-2024.

MIYASHITA T, HARIGAI M, HANAKA M, AND REED JC. (1994). Identification of a p53-dependent negative response element in the bcl-2 gene. *Cancer Res.*, **54**, 3131-3135.

MIYASHITA T AND REED JC. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell*, **80**, 293-299.

MOK CH, TSAO SW, KNAPP RC, FISHBAUGH PM, AND LAU CC. (1992). Unifocal origin of advanced epithelial ovarian cancers. *Cancer Res.*, **52**, 5119-5122.

MOK SCH, BELL DA, KNAPP RC, FISHBAUGH PM, WELCH WR, MUTO MG, BERKOWITZ RS, AND TSAO SW. (1993). Mutation of K-ras protooncogene in human ovarian epithelial tumors of borderline malignancy. *Cancer Res.*, **53**, 1489-1492.

MOMAND J, ZAMBETTI GP, OLSON DC, GEORGE D, AND LEVINE AJ. (1992). The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transctivation. *Cell*, **69**, 1237-1245.

MONTES DE OCA LUNA R, WAGNER DS, AND LOZANO G. (1995). Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature*, **378**, 203-206.

MOSNER J, MEMMEMBRAUER T, BAUER C, SCZAKIEL G, GROSSE F, AND DEPPERT W. (1995). Negative feedback regulation of wild-type p53 biosynthesis. *EMBO J.*, **14**, 4442-4449.

MUMMENBRAUER T, JANUS F, MULLER B, WIESMULLER L, DEPPERT W, AND GROSSE F. (1996). p53 protein exhibits 3'-to-5' exonuclease activity. *Cell*, **85**, 1089-1099.

MURAKAMI Y, HAYASHI K, AND SEKIYA T. (1991). Detection of aberrations of the p53 alleles and the gene transcript in human tumor cell lines by single-strand conformation polymorphism analysis. *Cancer Res.*, **51**, 3356-3361.

NABEYA Y, LOGANZO F, MASLAK P, LAI L, DE OLIVEIRA A, SCHWARTZ GK, BLUNDELL ML, ALTORKI NK, KELSEN DP, AND ALBINO AP. (1995). The mutational status of p53 protein in gastric and esophageal adenocarcinoma cell lines predicts sensitivity to chemotherapeutic agents. *Int J. Cancer*, **64**, 37-46.

NAITO M, SATAKE M, SAKAI E, HIRANO Y, TSUCHIDA N, KANZAKI H, ITO Y, AND MORI T. (1992). Detection of p53 gene mutations in human ovarian and endometrial cancers by polymerase chain reaction-single strand conformation polymorphism analysis. *Jpn. J. Cancer Res.*, **83**, 1030-1036.

NAKAI H, MISAWA S, TANIWAKI M, HORIIKE S, TAKASHIMA T, SERIU T, NAKAGAWA H, FUJII H, SHIMAZAKI C, AND MARUO N. (1994). Prognostic significance of loss of a chromosome 17p and p53 gene mutations in blast crisis of chronic myelocytic leukaemia. *Br. J. Haematol.*, **87**, 425-427.

NAVONE NM, TRONCOSO P, PISTERS LL, GOODROW TL, PALMER JL, NICHOLS WW, VON ESCHENBACH AC, AND CONTI CJ. (1993). p53 protein accumulation and gene mutation in the progression of human prostate carcinoma. *J. Natl. Cancer Inst.*, **85**, 1657-1699.

NELSON WG AND KASTAN MB. (1994). DNA strand breaks: the DNA template alterations that trigger p53-dependent DNA damage response pathways. *Mol. Cell. Biol.*, **14**, 1815-1823.

NICHOL CJ, HARRISON ML, LAPOSA RR, GIMELHSTEIN IL, AND WELLS PG. (1995). A teratologic suppressor role for p53 in benzo[a]pyrene-treated transgenic p53-deficient mice. *Nature Genet.*, **10**, 181-187.

NIGRO JM, BAKER JS, PREISINGER AC, JESSUP JM, HOSTETTER K, CLEARY K, BIGNER SH, DAVIDSON N, BAYLIN S, DEVILEE P, GLOVER T, COLLINS FS, WESTON A, MODALI R, HARRIS CC, AND VOGELSTEIN B. (1989). Mutations in the p53 gene occur in diverse human tumour types. *Nature*, **342**, 705-708.

NISHIDA N, FUKUDA Y, KOKURYU H, TOGUCHIDA J, YANDELL DW, IKENEGA M, IMURA H, AND ISHIZAKI K. (1993). Role and mutational heterogeneity of the p53 gene in hepatocellular carcinoma. *Cancer Res.*, **53**, 368-372.

NIWA K, ITOH M, MURASE T, MORISHITA S, ITOH N, MORI H, AND TAMAYA T. (1994). Alteration of p53 gene in ovarian carcinoma: clinicopathologic correlation and prognostic significance. *Br. J. Cancer*, **76**, 1191-1197.

OKAMOTO K AND BEACH D. (1994). Cyclin G is a transcriptional target of the p53 tumor suppressor protein. *EMBO J.*, **13**, 4816-4822.

OKAMOTO A, SAMESHIMA Y, YOKOYAMA S, TERASHIMA Y, SUGIMURA T, TERADA M, AND YOKOTA J. (1991). Frequent allelic losses and mutations of the p53 gene in human ovarian carcinoma. *Cancer Res.*, **51**, 5171-5176.

OLINER JD, PIETENPOL JA, THIAGALINGAM S, GYURIS J, KINZLER KW, AND VOGELSTEIN B. (1993). Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. *Nature*, **362**, 857-860.

OMURA GA, BRADY MF, HOMESLEY HD, YORDAN E, MAJOR FJ, BUCHSBAUM HJ, AND PARK RC. (1991). Long-term follow-up and prognostic factor analysis in advanced ovarian carcinoma: The Gynecologic Oncology Group experience. *J. Clin. Oncol.*, **9**, 1138-1150.

ORITA M, IWAHANA H, KANAZAWA H, HAYASHI K, AND SEKIYA T. (1989). Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 2766-2770.

OSIFCHIN NE, JIANG D, OHTANI-FUJITA N, FUJITA T, CARROZA M, KIM SJ, SAKAI T, AND ROBBINS PD. (1994). Identification of a p53 binding site in the human retinoblastoma susceptibility gene promoter. *J. Biol. Chem.*, **269**, 6383-6389.

OWEN-SCHAUB LB, ZHANG W, OHTANI-FUJITA T, CARROZA M, KIM SJ, SAKAI T, AND ROBBINS PD. (1995). Wild-type human p53 and a temperature-sensitive mutant induce fas/APO-1 expression. *Mol. Cell. Biol.*, **15**, 3032-3040.

PAPPOT H, FRANCIS D, BRUNNER N, GRONDAHL-HANSEN J, AND OSTERLIND K. (1996). p53 protein in non-small cell lung cancer as quantified by enzyme-linked immunosorbent assay: relation to prognosis. *Clin. Cancer Res.*, **2**, 155-160.

PARADA LF, LAND H, WEINBERG RA, WOLF D, AND ROTTER V. (1984). Cooperation between gene encoding p53 tumour antigen and ras in cellular transformation. *Nature*, **312**, 649-651.

PAVLETICH NP, CHAMBERS KA, AND PABO CO. (1993). The DNA-binding domain of p53 contains the four conserved regions and the major mutation hot spots. *Genes & Dev.*, **7**, 2556-2564.

PHILLIPS N, ZIEGLER M, SAHA B, AND XYNOS F. (1993). Allelic loss on chromosome 17 in human ovarian cancer. *Int. J. Cancer*, **54**, 85-91.

PIERETTI M, CAVALIERI C, CONWAY PS, GALLION HH, POWELL DE, AND TURKER MS. (1995). Genetic alterations distinguish different types of ovarian tumors. *Int. J. Cancer (Pred. Oncol.).*, **64**, 434-440.

PIETENPOL JA, TOKINO T, THIAGALINGAM S, EL-DIERY WS, KINZLER KW, AND VOGELSTEIN BS. (1994). Sequence-specific transcriptional activation is essential for growth suppression by p53. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 1998-2002.

PLANTE M, RUBIN SC, WONG GY, FEDERICI MG, FINSTAD CL, AND GASTL GA. (1994). Interleukin-6 level in serum and ascites as a prognostic factor in patients with epithelial ovarian cancer. *Cancer*, **73**, 1882-1888.

PRIOLEAU J, AND SCHNITT SJ. (1995). p53 antigen loss in stored paraffin slides. *N. Engl. J. Med.*, **332**, 1521-1522.

RENNINSON J, BAKER BW, MCGOWN AT, MURPHY D, NORTON JD, FOX BW, AND CROWTHER D. (1994). Immunohistochemical detection of mutant p53 protein in epithelial ovarian cancer using polyclonal antibody CM1: correlation with histopathology and clinical features. *Br. J. Cancer*, **69**, 609-612.

ROSEN PP, LESSER ML, ARROYO CD, CRANOR M BORGEN P, AND NORTON L. (1995). p53 in node-negative breast carcinoma: an immunohistochemical study of epidemiologic risk factors, histologic features, and prognosis. *J. Clin. Oncol.*, **13**, 821-830.

ROSNER B. (1995). *Fundamentals of Biostatistics*, 4th ed. pp 585-631. International Thomson Publishing: London.

ROTH J, DITTMER D, REA D, TARTAGLIA J, PAOLETTI E, AND LEVINE AJ. (1996a). p53 as a target for cancer vaccines: recombinant canarypox virus vectors expressing p53 protect mice against lethal tumor cell challenge. *Proc. Natl. Acad. Sci. U.S.A.*, **93**, 4781-4786.

ROTH JA, NGUYEN D, LAWRENCE DD, KEMP BL, CARRASCO CH, FERSON DZ, HONG WK, KOMAKI R, LEE JJ, NESBITT JC, PISTERS KMV, PUTNAM JB, SCHEA R, SHIN DM, WALSH GL, DOLORMENTE MM, HAN CI, MARTIN FD, YEN N, XU K, STEPHENS LC, MCDONNELL TJ, MUKHOPADHYAY T, AND CAI D. (1996b). Retrovirus-mediated wild-type p53 gene transfer to tumors of patients with lung cancer. *Nature Med.*, **2**, 985-991.

RUNNEBAUM IB, TONG XW, MOEBUS V, HEILMANN V, KIEBACK DG, AND KREIENBERG R. (1994). Multiplex PCR sceening detects small p53 deletions and insertions in human ovarian cancer cell lines. *Hum. Genet.*, **93**, 620-624.

RUNNEBAUM IB, WANG S, AND KREIENBERG R. (1995). Retrovirally mediated wild-type p53 restores S-phase modulation without inducing WAF1 mRNA in breast carcinoma cells containing mutant p53. *J. Cell. Biochem.*, **59**, 537-544.

SABBATINI P, LIN J, LEVINE AJ, AND WHITE E. (1995). Essential role for p53-mediated transcription in E1A-induced apoptosis. *Genes & Dev.*, **9**, 2184-2192.

SAH VP, ATTARDI LD, MULLIGAN GJ, WILLIAMS BO, BRONSON RT, AND JACKS T. (1995). A subset of p53-deficient embryos exhibit exencephaly. *Nature Genet.*, **270**, 15915-15918.

SAITO H, INAZAWA J, SAITO S, KASUMI F, KOI S, SAGAE S, KUDO R, SAITO J, NODA K, AND NAKAMURA Y. (1993). Detailed deletion mapping of chromosome 17q in ovarian and breast cancers: 2 cM region on 17q21.3 often and commonly deleted in tumors. *Cancer Res.*, **53**, 3382-3385.

SAMBROOK J, FRITSCH EF, AND MANIATIS T. (1989). *Molecular Cloning: A Laboratory Manual*. pp. 10.59-10.67. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.

SAMESHIMA Y, TSUNEMATSSU Y, WATANABE S, TSUKAMOTO T, KAWA-HA K, HIRATA Y, MIZOGUCHI H, SUGIMURA T, TERADA M, AND YOKOTA J. (1992). Detection of novel germ-line p53 mutations in diverse cancer-prone families identified by selecting patients with childhood adrenocortical carcinoma. *J. Natl. Cancer Inst.*, **84**, 703-707.

SANDER CA, YANO T, CLARK HM, HARRIS C, LONGO DL, JAFFE ES, AND RAFFELD M. (1993). p53 mutation is associated with progression in follicular lymphomas. *Blood*, **82**, 1994-2004.

SANDS AT, SURAOKAR MB, SANCHEZ A, MARTH JE, DONEHOWER LA, AND BRADLEY A. (1995). p53 deficiency does not affect the accumulation of point mutations in a transgene target. *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 8517-8521.

SANTOSO JT, TANG DC, LANE SB, HUNG J, REED DJ, MULLER CY, CABONE DP, LUCCI JA, MILLER DS, AND MATHIS JM. (1995). Adenovirus-based gene therapy in ovarian cancer. *Gynecol. Oncol.*, **59**, 171-178.

SARNOW P, HO YS, WILLIAMS J, AND LEVINE AJ. (1982). Adenovirus E1b-58kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54 kd cellular protein in transformed cells. *Cell*, **28**, 387-394.

SCAMBIA G, FERRANDINA G, MARONE M, PANICI P, GIANNITELLI C, PIANTELLI M, LEONE A, AND MANCUSO S. (1996). nm23 in ovarian cancer: correlation with clinical outcome and other clinicopathologic and biochemical prognostic parameters. *J. Clin. Oncol.*, **14**, 334-342.

SCHECHTER AL, STERN DF, VAIDYANATHAN L, DECKER SJ, DREBIN JA, GREEN MI, AND WEINBERG RA. (1984). The neu oncogene: an erb-B-related gene encoding a 185,000 Mr tumor antigen. *Nature*, **312**, 513-516.

SCHEFFNER M, HUIBREGTSE JM, VIERSTRA RD, AND HOWLEY PM. (1993). The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell*, **75**, 495-505.

SCHOLL SM, BASCOU CH, MOSSERI V, OLIVARES R, MAGDELANAT H, DORVAL T, PALANGIE T, VALIDIRE T, POUILLART P, AND STANLEY ER. (1994). Circulating levels of colony-stimulating factor 1 as a prognostic indicator in 82 patients with epithelial ovarian cancer. *Br. J. Cancer*, **69**, 342-346.

SCHWEIKI D, ITIN A, SOFFER D, AND KESHET E. (1992). Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-mediated angiogenesis. *Nature*, **359**, 843-845.

SEROV SF AND SOULLY RF. (1973). Histological typing of ovarian tumors. In: *International Histological Classification of Tumors*, pp 10-32. World Health Organization: Geneva.

SHAULIAN E, ZAUBERMAN A, GINSBERG D, AND OREN M. (1992). Identification of a minimal transforming domain of p53: negative dominance through abrogation of sequence-specific DNA binding. *Mol. Cell. Biol.*, **12**, 5581-5592.

SHAULIAN E, ZAUBERMAN A, MILNER J, DAVIES EA, AND OREN M. (1993). Tight DNA binding and oligomerization are dispensible for the ability of p53 to transactivate target genes and suppress transformation. *EMBO J.*, **12**, 671-680.

SHAW P, BOVEY R, TARDY S, SAHLI R, SORDAT B, AND COSTA J. (1992). Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 4495-4499.

SHEN Y AND SHENK T. (1994). Relief of p53-mediated transcriptional repression by the adenovirus E1B 19-kDa protein of the cellular Bcl-2 protein. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 8940-8944.

SHERIDAN E, HANCOCK BW, AND GOYNS MH. (1993). High incidence of mutations of the p53 gene in ovarian tumors by the use of chemical mismatch cleavage. *Cancer Lett.*, **68**, 83-89.

SHERIDAN E, SILCOCKS P, SMITH J, HANCOCK BW, AND GOYNS MH. (1994). p53 mutation in a series of epithelial ovarian cancers from the U.K., and its prognostic significance. *Eur. J. Cancer*, **30**, 1701-1704.

SHIN TH, PATERSON AJ, AND KUDLOW JE. (1995). p53 stimulates transcription from the human transforming growth factor α promoter: a potential growth-stimulatory role for p53. *Mol. Cell. Biol.*, **15**, 4694-4701.

SHIVAKUMAR CV, BROWN DR, DEB S, AND DEB SP. (1995). Wild-type human p53 transactivates the human proliferating cell nuclear antigen promoter. *Mol. Cell. Biol.*, **15**, 6785-6793.

SIDRANSKY D, MIKKELSEN T, SCHWECHHEIMER K, ROSENBLUM ML, CAVANEE W, AND VOGELSTEIN B. (1992). Clonal expansion of p53 mutant cells is associated with brain tumor progression. *Nature*, **355**, 846-847.

SILVESTRINI R, BENINI E, DAIDONE MG, VENERONI S, BORACCHI P, CAPPELLETTI V, DI FRONZO G, AND VERONESI U. (1993). p53 as an independent prognostic marker in lymph node-negative breast cancer patients. *J. Natl. Cancer Inst.*, **85**, 965-970.

SKILLING JS, SOOD A, NIEMANN T, LAGER DJ, AND BULLER RE. (1996). An abundance of p53 null mutations in ovarian carcinoma. *Oncogene*, **13**, 117-123.

SLAMON DJ, GODOLPHIN W, JONES LA, HOLT JA, WONG SG, KEITH DE, LEVIN WJ, STUART SG, UDOVE J, AND ULLRICH A. (1989). Studies of HER2/neu proto-oncogene in human breast and ovarian cancer. *Science*, **244**, 707-712.

SLINGERLAND JM, JENKINS JR, AND BENCHIMOL S. (1993). The transforming and suppressor functions of p53 alleles: effects of mutations that disrupt phosphorylation, oligomerization and nuclear translocation. *EMBO J.*, **12**, 5320-5337.

SMITH ML, CHEN IT, ZHAN Q, BAE I, CHEN CY, GILMER TM, KASTAN MB, O'CONNOR PM, AND FORNACE AJ. (1994). Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. *Science*, **266**, 1376-1380.

SOONG R, KNOWLES S, WILLIAMS KE, HAMMOND IG, WYSOCKI SJ, AND IACOPETTA BJ. (1996). Overexpression of p53 protein is an independent prognostic indicator in human endometrial carcinoma. *Br. J. Cancer*, **74**, 562-567.

SOUSSI T, DE FROMENTEL CC, AND MAY P. (1990). Structural aspects of the p53 protein in relation to gen evolution. *Oncogene*, **5**, 945-952.

SRIVASTAVA S, KATAYOSE D, TONG YA, CRAIG CR, MCLEOD DG, MOUL JW, COWAN KH, AND SETH P. (1995). Recombinant adenovirus vector expressing wild-type p53 is a potent inhibitor of prostate cancer cell proliferation. *Urology*, **46**, 843-848.

STRATIS M, BAILEY D, WAHID S, AND OEY A. (1988). Improvement of cellular and nuclear morphology on lymph node frozen section immunopathology. A postfixative technique. *J. Immunotechnol.*, **11**, 139-141.

SYMONDS H, KRALL L, REMINGTON L, SAENZ-ROBLES M, LOWE S, JACKS R, AND VAN DYKE T. (1994). p53-dependent apoptosis suppresses tumor growth and progression in vivo. *Cell*, **78**, 703-711.

TAKENAKA I, MORIN F, SEIZINGER BR, AND KLEY N. (1995). Regulation of the sequence-specific DNA binding function of p53 by protein kinase C and protein phosphatases. *J. Biol. Chem.*, **270**, 5405-5411.

TANAKA K, BOICE CR, AND TESTA JR. (1987). Chromosome aberrations in nine patients with ovarian cancer. *Cancer Genet. Cytogenet.*, **43**, 1-14.

TANAKA N, ISHIHARA M, LAMPHIER MS, NOZAWA H, MATSUYAMA T, MAK TW, AIZAWA S, TOKINO T, OREN M, AND TANIGUCHI T. (1996). Co-operation of the tumour suppressors IRF-1 and p53 in response to DNA damage. *Nature*, **382**, 816-818.

TENAUD C, NEGROESCU A, LABAT-MOLEUR F, LEGROS Y, SOUSSI T, AND BRAMBILLA E. (1994). p53 immunolabeling in archival paraffin-embedded tissues: optimal protocol based on microwave heating for eight antibodies on lung carcinomas. *Mod. Pathol.*, **7**, 853-859.

TENERIELLO MG, EBINA M, LINNOILA RI, HENRY M, NASH JD, PARK RC, AND BIRRER MJ. (1993). p53 and Ki-ras mutations in epithelial ovarian neoplasms. *Cancer Res.*, **53**, 3103-3208.

THOMAS H, MASIM MM, SARRAF CE, ALISON MR, LOVE S, LAMBERT HE, AND PRICE P. (1995). Proliferating cell nuclear antigen (PCNA) immunostaining - a prognostic factor in ovarian cancer? *Br. J. Cancer*, **71**, 357-362.

THOMPSON TC, PARK SH, TIMME TL, REN C, EASTHAM JA, DONEHOWER LA, BRADLEY A, KADMON D, AND YANG G. (1995). Loss of p53 function leads to metastasis in ras+myc-initiated mouse prostate cancer. *Oncogene*, **10**, 869-879.

THOR AD, MOORE DH, EDGERTON SM, KAWASAKI ES, REISHAUS E, LYNCH HT, MARCUS JN, SCHWARTZ L, CHEN LC, MAYALL BH, AND SMITH HS. (1992). Accumulation of p53 tumor suppressor gene protein: an independent prognostic marker in breast cancers. J. *Natl Cancer Inst.*, **84**, 845-855.

THUT C, CHEN JL, KLEMM R, AND TJIAN R. (1995). p53 transcriptional activation mediated by coactivators TAFII40 and TAFII60. *Science*, **267**, 100-104.

TOKINO T, THIAGALINGAM S, EL-DEIRY WS, WALDMAN T, KINZLER KW, AND VOGELSTEIN B. (1994). p53 tagged sites from human genomic DNA. *Hum. Mol. Genet.*, **3**, 1537-1542.

TRIVERS GE, CAWLEY HL, DEBENEDETTI VMG, HOLLSTEIN M, MARION MJ, BENNETT WP, HOOVER ML, PRIVES CA, TAMBURRO CC, AND HARRIS CC. (1995). Anti-p53 antibodies in sera of workers occupationally exposed to vinyl chloride. *J. Natl. Cancer Inst.*, **87**, 1400-1407.

TSAO SW, MOK CH, OIKE K, MUTO M, GOODMAN HM, SHEETS EE, BERKOWITZ RS, KNAPP RC, AND LAU CC. (1991). Involvement of p53 gene in the allelic deletion of chromosome 17p in human ovarian tumors. *Anticancer Res.*, **11**, 1975-1982.

TSUDA H AND HIROHASHI S. (1992). Frequent occurrence of p53 gene mutation in uterine cancers at advanced clinical stage with aggressive histological phenotypes. *Jpn. J. Cancer Res.*, **83**, 1184-1191.

UEBA T, NOSAKA T, TAKAHASHI JA, SHIBATA F, FLORKIEWICZ RZ, VOGELSTEIN B, ODA Y, KIKUCHI H, AND HATANAKA M. (1994). Transcriptional regulation of basic fibroblast growth factor gene by p53 in human glioblastoma and hepatocellular carcinoma cells lines. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 9009-9013.

UHLMAN DL, NGUYEN PL, MANIVEL JC, AEPPLI D, RESNICK JM, FRALEY EE, ZHANG G, AND NIEHANS GA. (1994). Association of immunohistochemical staining for p53

with metastatic progression and poor survival in patients with renal cell carcinoma. *J. Natl. Cancer Inst.*, **86**, 1470-1475.

VAN DER ZEE AGJ, HOLLEMA H, SUURMEIJER AJH, KRANS M, SLUITER WJ, WILLEMSE PHB, AADLDERS JG, AND DE VRIES EGE. (1995). Value of p-glycoprotein, glutathione S-transferase pi, c-erbB-2, and p53 as prognostic factors in ovarian carcinomas. *J. Clin. Oncol.*, **13**, 70-78.

VAN MEYEL DJ, RAMSAY DA, CASSON AG, KEENEY M, CHAMBERS AF, AND CAIRNCROSS JG. (1994). p53 mutation, expression, and DNA ploidy in evolving gliomas: evidence for two pathways of progression. *J. Natl. Cancer Inst.*, **86**, 1011-1017.

VISAKORPI T, KALLIONIEMI OP, HEIKKINEN A, KOIVULA T, AND ISOLA J. (1992). Small subgroup of aggressive, highly proliferative prostatic carcinomas defined by p53 accumulation. *J. Natl. Cancer Inst.*, **84**, 883-887.

VOGELSTEIN B AND KINZLER KW. (1992a). Carcinogens leave fingerprints. *Nature*, **355**, 209-210.

VOGELSTEIN B AND KINZLER KW. (1992b). p53 function and dysfunction. *Cell*, **79**, 523-526.

VOJTESEK B, BARTEK J, MIDGLEY CA, AND LANE DP. (1992). An immunochemical analysis of human nuclear phosphoprotein p53: new monoclonal antibodies and epitope mapping using recombinant p53. *J. Immunol. Methods*, **151**, 237-244.

VOJTESEK B, FISHER CJ, BARNES DM, AND LANE DP. (1993). Comparison between p53 staining in tissue sections and p53 protein levels measured by an ELISA technique. *Br. J. Cancer*, **67**, 1254-1258.

WAGA S, HANNON GJ, BEACH D, AND STILLMAN B. (1994). The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature*, **369**, 574-578.

WAGNER AJ, KOKONTIS JM, AND HAY N. (1994). Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and ability of p53 to induce $p21^{waf1/cip1}$. *Genes & Dev.*, **8**, 2817-2830.

WANG XW, FORRESTER K, YEH H, FEITELSON MA, GU JR, AND HARRIS CC. (1994). Hepatitis B virus X protein inhibits sequence-specific DNA binding, transcriptional activity, and association with transcription factor ERCC3. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 2230-2234.

WANG XW, YEH H, SCHAEFFER L, ROY R, MONCOLLIN V, EGLY JM, WANG Z, FREIDBERG EC, EVANS MK, TAFFE BG, BOHR VA, WEEDA G, HOEIJMAKERS JHJ, FORRESTER K, AND HARRIS CC. (1995). p53 modulation of TFIIH-associated nucleotide excision repair activity. *Nature Genet.*, **10**, 188-193.

WANG Y AND PRIVES C. (1995). Increased and altered DNA binding of human p53 by S and G2/M but not G1 cyclin-dependent kinases. *Nature*, **376**, 88-91.

WATERMAN MJF, WATERMAN JLF, AND HALAZONETIS TD. (1996). An engineered four-stranded coiled coil substitutes for the tetramerization doamin of wild-type p53 and alleviates transdominant inhibition by tumor-derived p53 mutants. *Cancer Res.*, **56**, 158-163.

WERTHEIM I, TANGIR J, MUTO MG, WELCH WR, BERKOWITZ RS, CHEN WY, AND MOK SC. (1996). Loss of heterozygosity of chromosome 17 in human borderline and invasive epithelial ovarian tumors. *Oncogene*, **12**, 2147-2153.

WESTGARD JO. (1981). Precision and accuracy: concepts and assessments by method evaluation testing. *C.R.C Crit. Rev. Clin Lab. Sci.*, **13**, 282-330.

WILD CP, RIDANPAA M, ANTTILA S, LUBIN R, SOUSSI T, HUSGAFVEL-PURSIAINEN K, AND VAINIO H. (1995). p53 antibodies in the sera of lung cancer patients: comparison with p53 mutation in the tumor tissue. *Int. J. Cancer (Pred. Oncol.)*, **64**, 176-181.

WINTER SF, MINNA JD, JOHNSON BE, TAKAHASHI T, GAZDAR AF, AND CARBONE DP. (1992). Development of antibodies against p53 in lung cancer patients appears to be dependent on the type of p53 mutation. *Cancer Res.*, **52**, 4168-4174.

WU GS AND EL-DEIRY WS. (1996). Apoptotic death of tumor cells correlates with chemosensitivity, independent of p53 of Bcl2. *Clin. Cancer Res.*, **2**, 623-633.

WYNFORD-THOMAS D. (1992). p53 in tumour pathology: can we trust immunocytochemistry? *J. Pathol.*, **166**, 329-330.

XIONG Y, HANNON GJ, ZHANG H, CASSO D, KOBAYASHI R, AND BEACH D. (1993). p21 is a universal inhibitor of cyclin kinases. *Nature*, **366**, 701-704.

XU C, MEIKRANTZ W, SCHLEGEL R, AND SAGER R. (1995). The human papilloma virus 16 E6 gene sensitizes human mammry epithelial cells to apoptosis induced by DNA damage. *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 7829-7833.

YAGINUMA Y, AND WESTPHAL H. (1992). Abnormal structure and expression of the p53 gene in humanovarian cancer cell lines. *Cancer Res.*, **52**, 4196-4199.

YANG C, CIRELLI C, CAPOGROSSI MC, AND PASSANITI A. (1995). Adenovirus-mediated wild-type p53 expression induces apoptosis and suppresses tumorigenesis of prostatic tumor cells. *Cancer Res.*, **55**, 4210-4213.

YAO SL, AKHTAR AJ, MCKENNA KA, BEDI GC, SIDRANSKY D, MABRY M, RAVI R, COLLECTOR MI, JONES RJ, SHARKIS SJ, FUCHS EJ, AND BEDI A. (1996). Selective radiosensitization of p53-deficient cells by caffeine-mediated actiation of $p34^{cdc2}$ kinase. *Nature Med.*, **2**, 1140-1142.

YONISH-ROUACH E, BORDE J, GOTTLEAND M, MISHAL Z, VIRON A, AND MAY E. (1994). Induction of apoptosis by transiently transfected metabolically stable wt p53 in transformed cell lines. *Cell Death & Differ.*, **1**, 39-47.

YONISH-ROUACH E, RESNITSKY D, LOTEM J, SACHS L, KIMCHI A, AND OREN M. (1991). Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. *Nature*, **352**, 345-347.

ZENG ZS, SARKIS AS, ZHANG ZF, KLIMSTRA DS, CHARYTONOWICZ E, GUILLEM JG, CORDON-CARDO C, AND COHEN AM. (1994). p53 nuclear overexpression: an independent predictor of survival in lymph node-positive colorectal cancer patients. *J. Clin. Oncol.*, **12**, 2043-2050.

ZHAN QF, CARRIER F, AND FORNACE AJ. (1993). Induction of cellular p53 activity by DNA-damaging agents and growth arrest. *Mol. Cell. Biol.*, **13**, 4242-4250.

ZHANG L, KASHANCHI F, ZHAN Q, ZHAN S, BRADY JN, FORNACE AJ, AND SETH P. (1996a). Regulation of insulin-like growth factor II P3 promoter by p53: a potential mechanism for tumorigenesis. *Cancer Res.*, **56**, 1367-1373.

ZHANG N, SONG Q, LU H, AND LAVIN MF. (1996b). Induction of p53 and increased sensitivity to cisplatin in ataxia-telangiectasia cells. *Oncogene*, **13**, 655-659.

ZHANG W, FUNK WD, WRIGHT WE, SHAY JW, AND DEISSEROTH AB. (1993). Novel DNA binding of p53 mutants and their role in transcriptional activation. *Oncogene*, **8**, 2555-2559.

ZHANG W, GUO XY, HU GY, LIU WB, SHAY JW, AND DEISSEROTH AB. (1994). A temperature-sensitive mutant of human p53. *EMBO J.*, **13**, 2535-2544.

ZHENG J, BENEDICT WF, XU HJ, HU SX, KIM TM, VELICESCU M, WAN M, COFER KF, AND DUBEAU L. (1995). Genetic disparity between morphologically benign cysts contiguous to ovarian carcinomas and solitary cystadenomas. *J. Natl. Cancer Inst.*, **87**, 1146-1153.

ZIEGLER A, LEFELL DJ, KUNALA S, SHARMA HW, GAILANI M, SIMON JA, HAPLEIN AJ, BADEN HP, SHAPIRO PE, BALE AE, AND BRASH DE. (1993). Mutation hotspots due to sunlight in the p53 gene of nonmelanoma skin cancers. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 4216-4220).

ZIEGLER A, JONASON AS, LEFFELL DJ, SIMON JA, SHARMA HW, KIMMELMAN J, REMINGTON L, JACKS T, AND BRASH DE. (1994). Sunburn and p53 in the onset of skin cancer. *Nature*, **372**, 773-776.

ZWEIG MH AND CAMPBELL G. (1993). Receiver-operator characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. *Clin. Chem.*, **39**, 561-577.

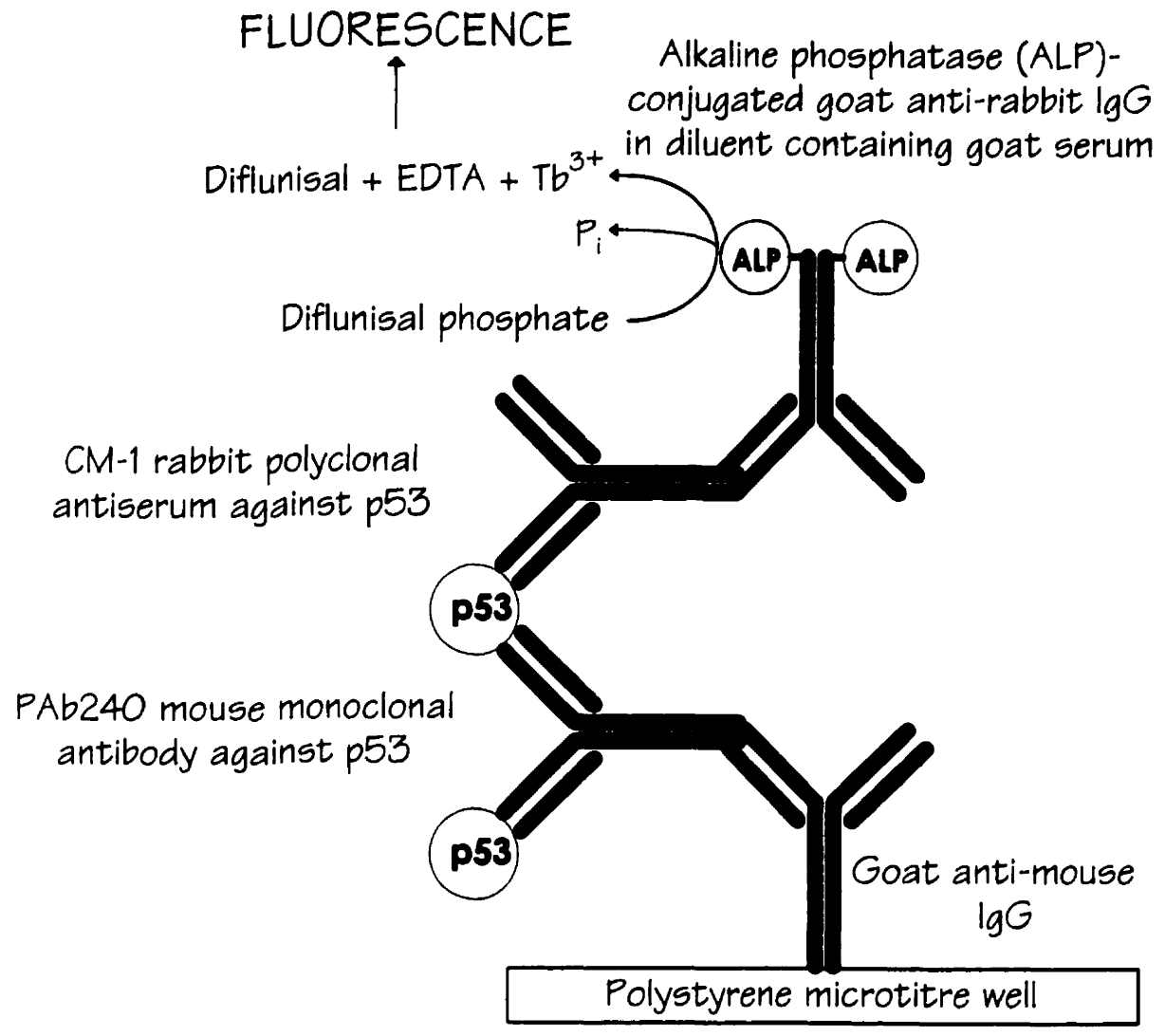

Figure 5-4-1-1. Schematic of p53 Immunofluorometric Assay Configuration

For details of reagent concentrations, incubation times, washing steps, and instrumentation, see Chapter 5-4. and refer to Hassapoglidou *et al.*, 1993. See also Chapter 6-2. for comments on assay performance.

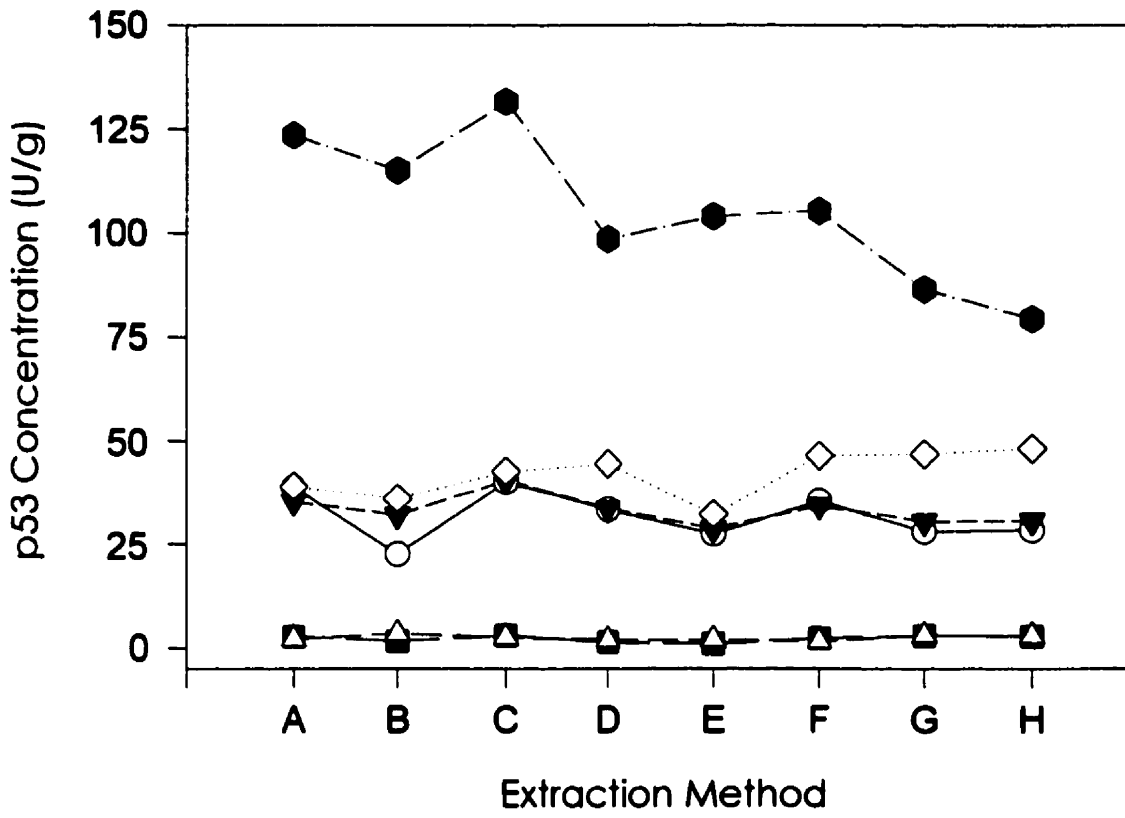

Figure 6-1-1. Comparison of Extraction Methods for p53 Solubilization from Ovarian Tumors

Extracts prepared by eight different protein extraction methods from six ovarian tumors were assayed for p53 by immunofluorometric assay. Each tumor is represented by a single symbol.

Methods used were:

A steroid hormone receptor extraction buffer, spun 100,000 g for 1 hour
B steroid hormone receptor extraction buffer, spun 15,000 g for 1 hour
C steroid hormone receptor extraction buffer, spun 15,000 for 30 minutes

Selected Method →
D buffer of Bartkova *et al.*, 1993, spun 100,000 g for 1 hour
E buffer of Bartkova *et al.*, 1993, spun 15,000 g for 1 hour
F buffer of Bartkova *et al.*, 1993, spun 15,000 g for 30 minutes

G buffer of Lavigueur *et al.*, 1989, spun 15,000 g for 30 minutes
H buffer of Lavigueur *et al.*, 1989, spun 15,000 g for 5 minutes

For details of the experimental procedure, see Chapter 5-2. For comments on the results, see Chapter 6-1.

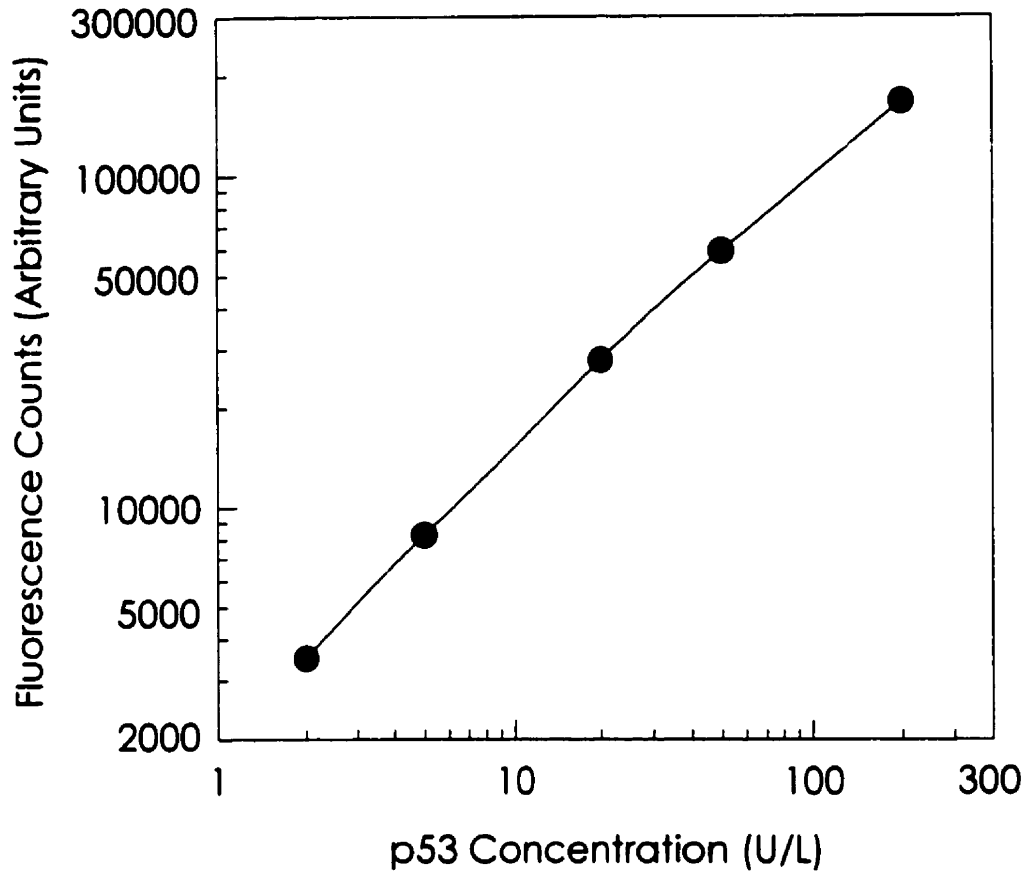

Figure 6-2-1-1. Calibration Curve of p53 Immunofluorometric Assay

p53 standard solutions, assayed in duplicate, were dilutions of an ovarian tumor extract with high p53 immunofluorescence assigned an arbitrary p53 concentration of 1000 U/L. See Chapter 5-4-4. for details.

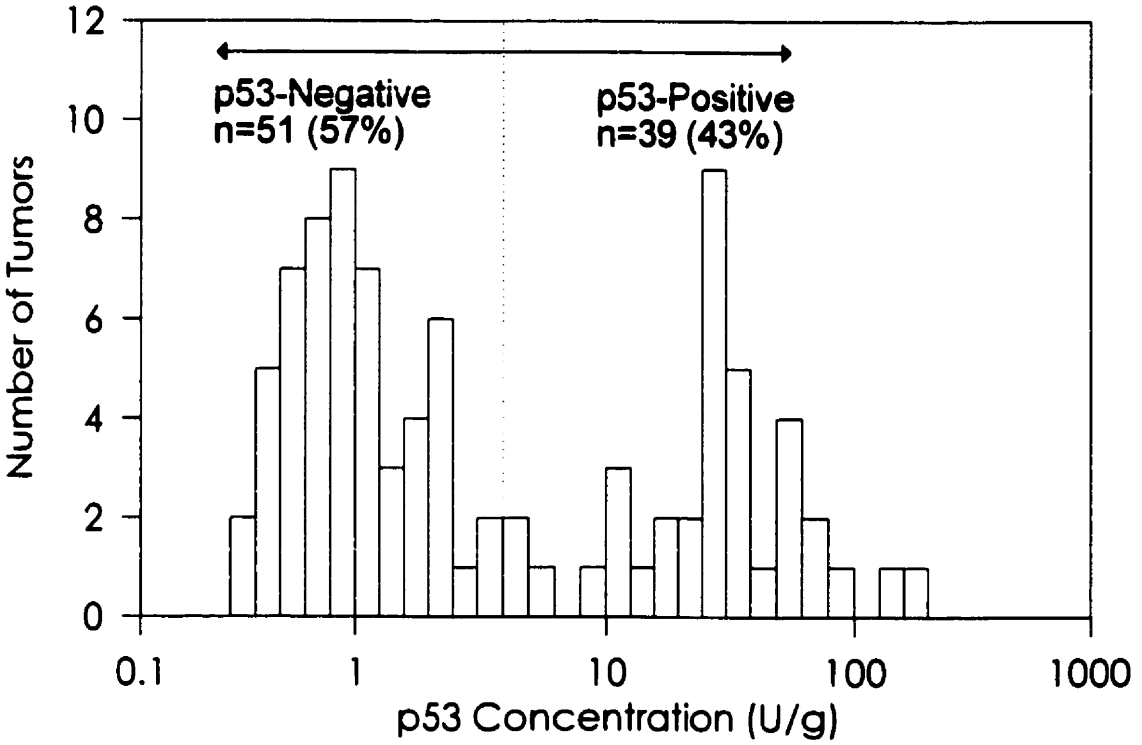

Figure 6-2-4-1. Distribution of p53 Concentrations in Ovarian Tumor Extracts

Values are expressed relative to the total protein content of the 90 extracts assayed. The dotted line represents the p53 concentration (3 U/g) used as the cutoff point for p53-positivity.

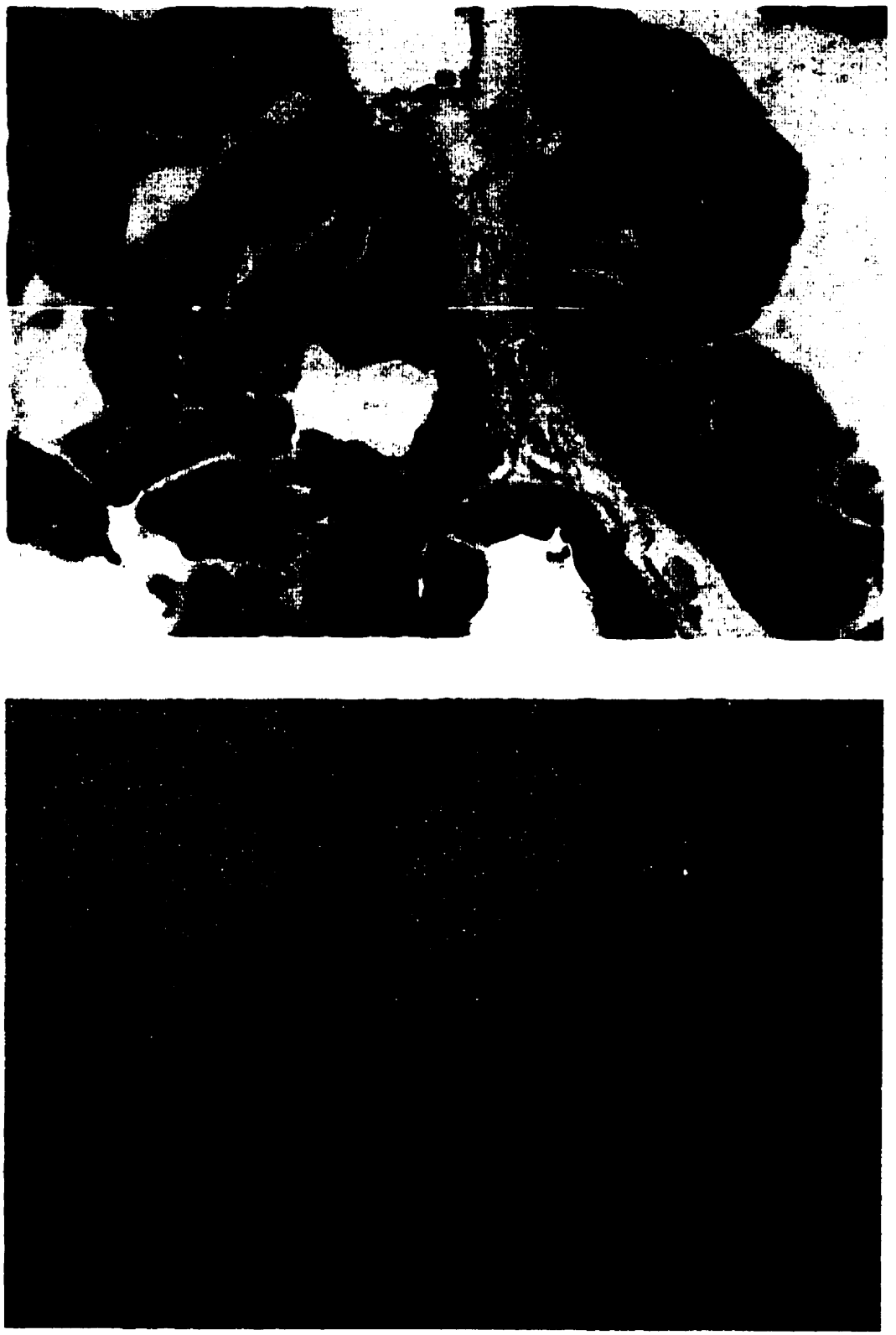

Figure 6-3-1. Immunohistochemical Staining for p53 Protein
Frozen sections of two ovarian tumor tissues shown to be highly p53-positive (panel A) and p53-negative (panel B) by immunofluorometric assay were immunostained with DO-1 antibody and an avidin-biotin-peroxidase complex detection system (x400 magnification). See Chapter 5-7. for details of the experimental procedure and Chapter 6-3. for comments on the results.

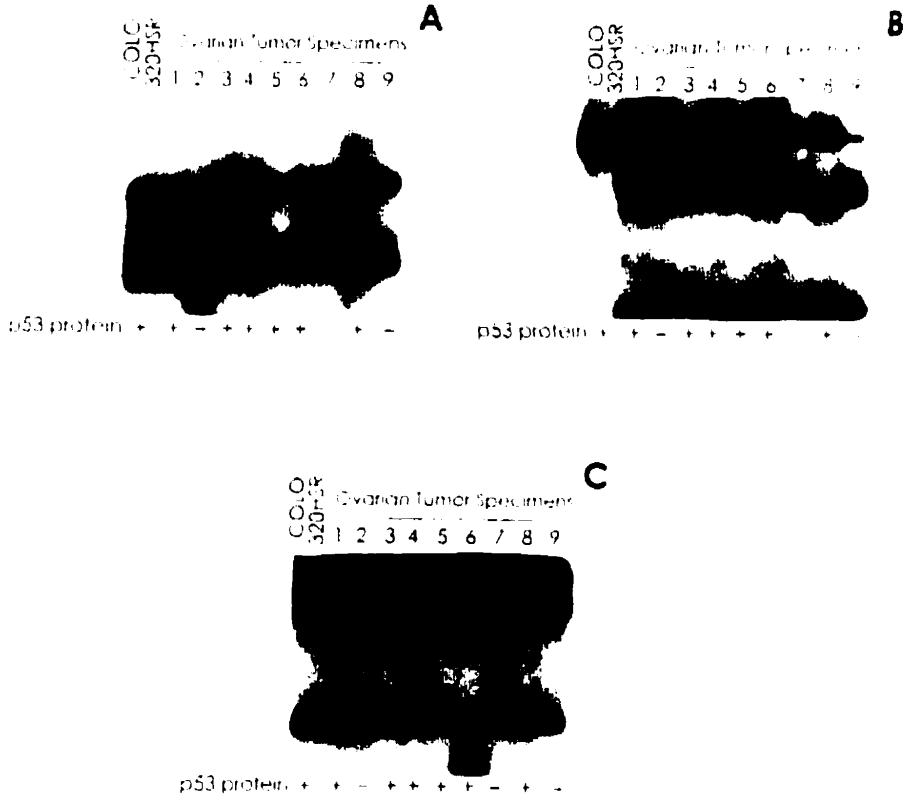

Figure 6-4-1. Single-Strand Conformation Polymorphism Analysis of the p53 Gene

DNA from nine ovarian tumors and from COLO 320HSR cells were subjected to SSCP analysis following PCR amplification. Autoradiographs showing exons 5-6, 7, and 8, are in panels A, B, and C, respectively. See Chapter 5-8. for details of experimental procedure and Chapter 6-4. for comments on the results.

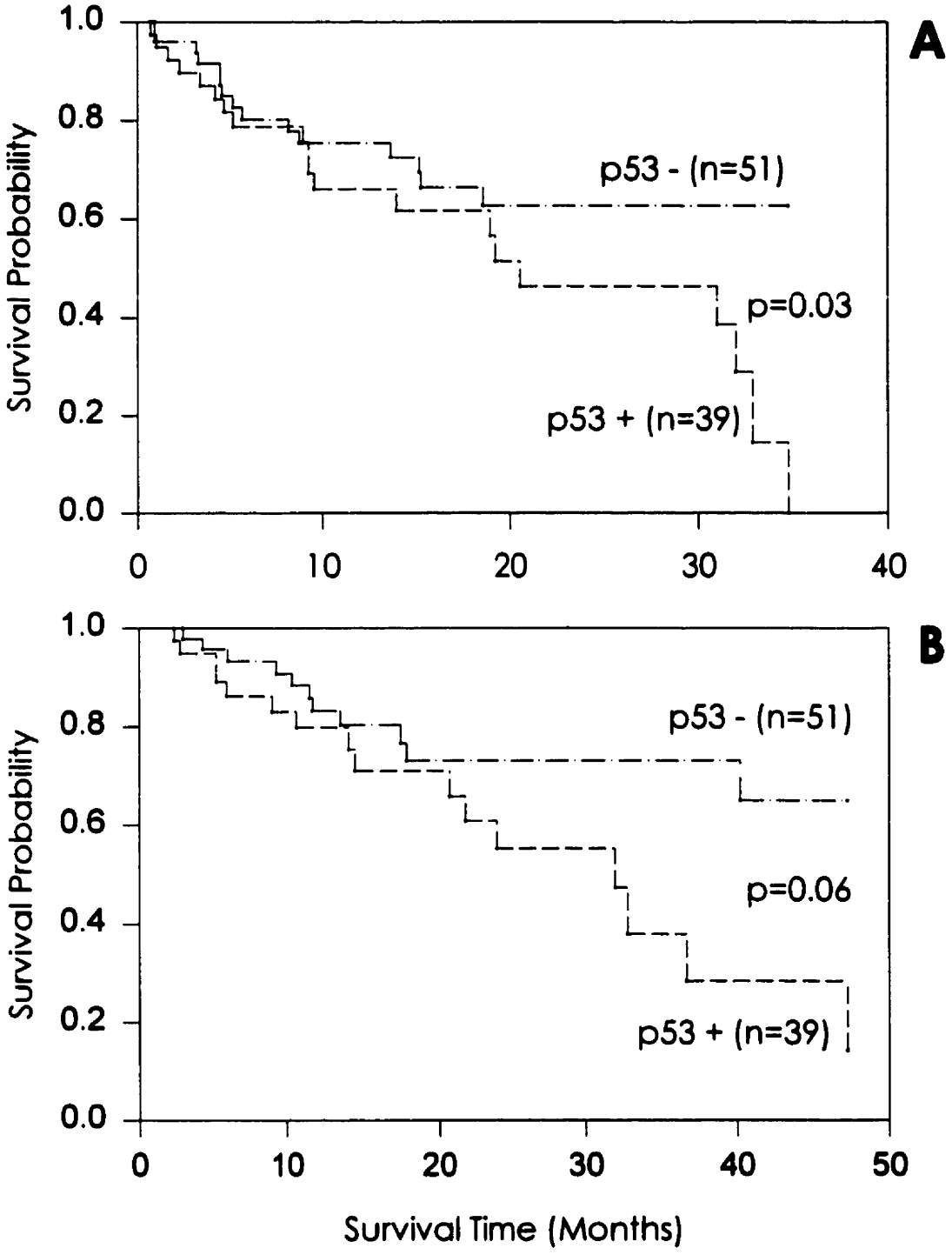

Figure 6-9-1. Kaplan-Meier Survival Analysis for All Patients

Panel A shows disease-free survival while panel B show overall survival for all p53-negative and p53-positive patients without subclassification. Differences between curves were evaluated by the log rank test. See Chapters 5-10-3. and 6-9 for details.

n: number of patients

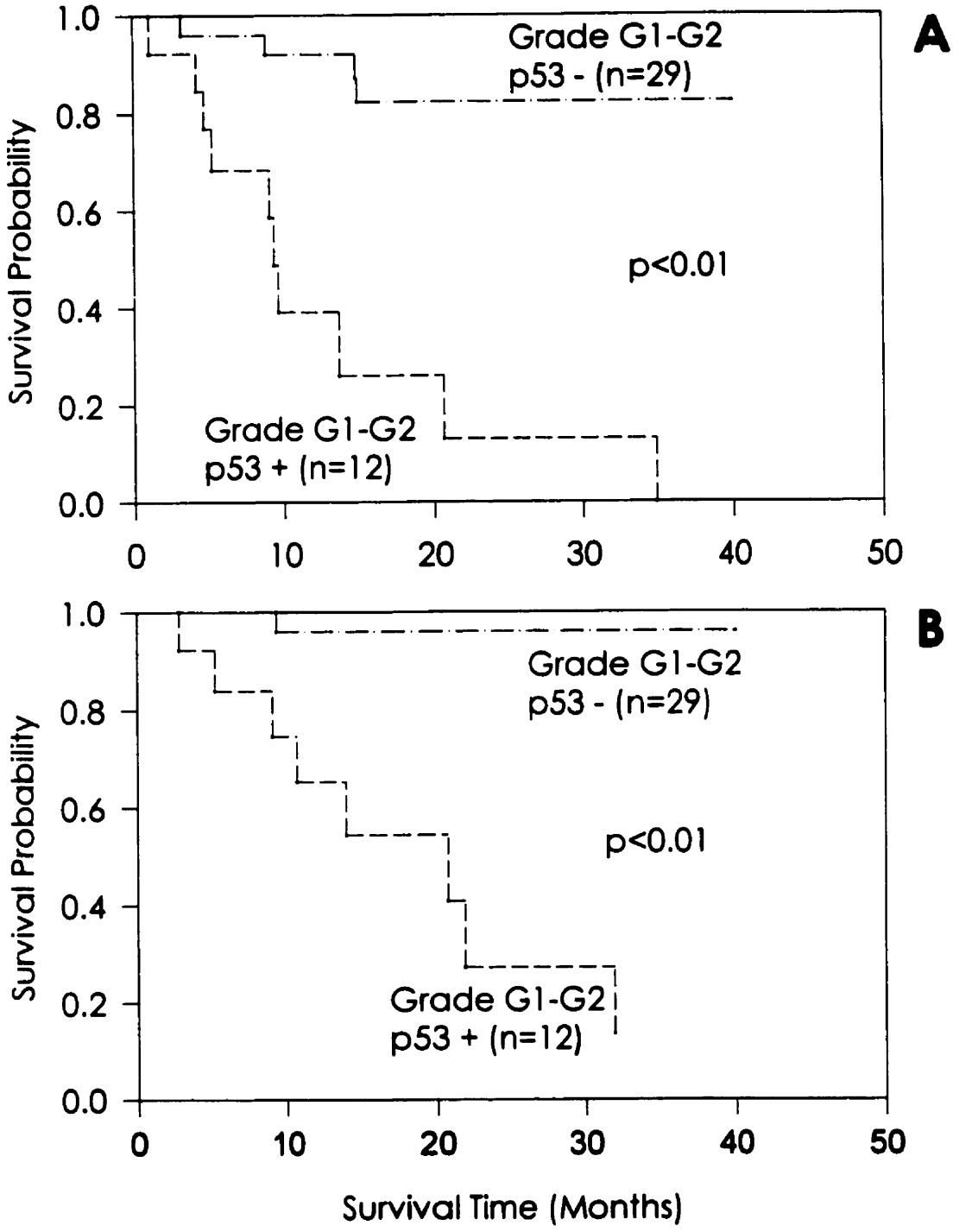

Figure 6-9-2. Kaplan-Meier Survival Analysis for Patients with Low Grade Tumors

Panel A shows disease-free survival while panel B show overall survival for all p53-negative and p53-positive patients with Grade G1-G2 disease. Differences between curves were evaluated by the log rank test. See Chapters 5-10-3. and 6-9 for details.

n: number of patients

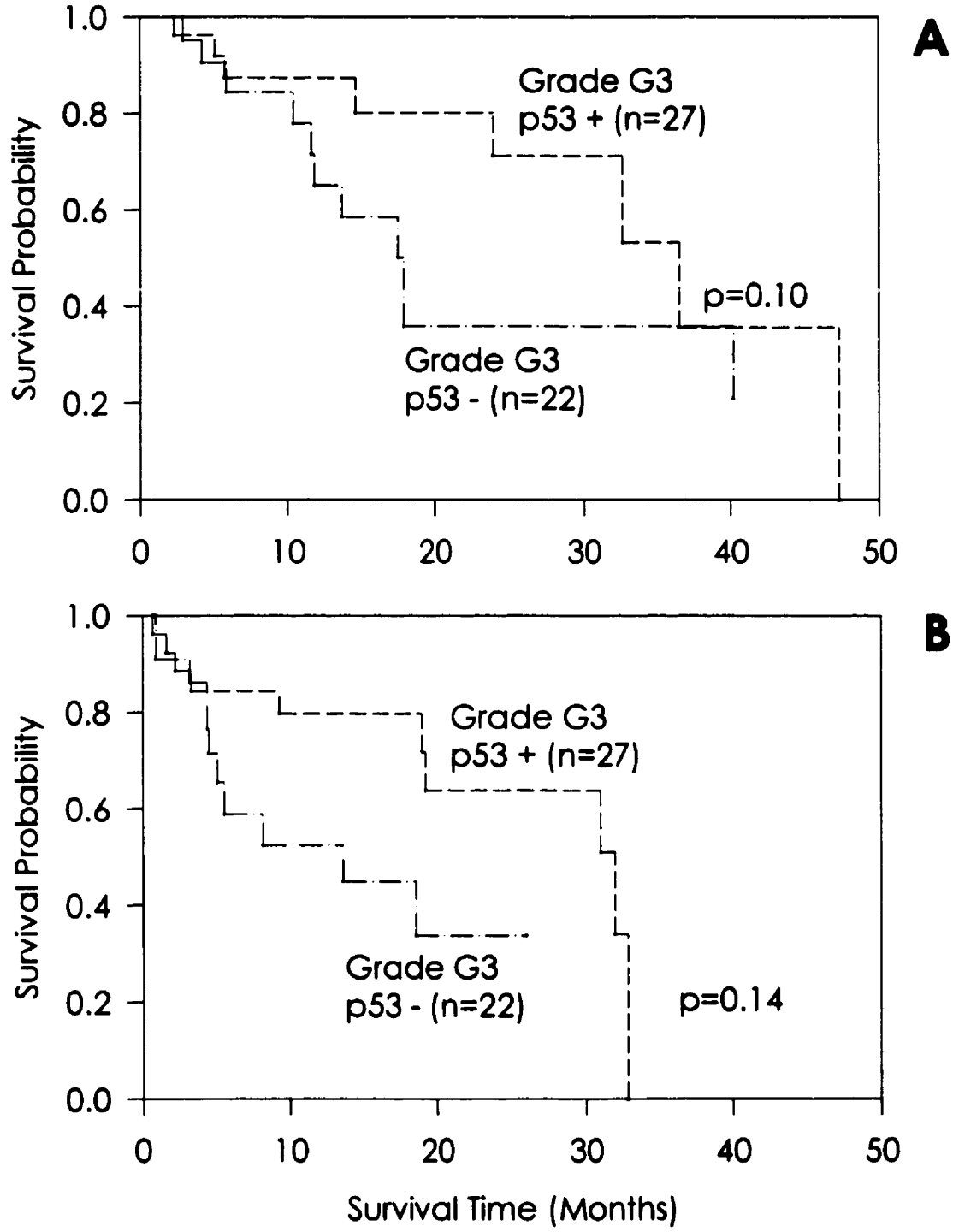

Figure 6-9-3. Kaplan-Meier Analysis for Patients with High Grade Tumors

Panel A shows disease-free survival while panel B show overall survival for all p53-negative and p53-positive patients with Grade G3 disease. Differences between curves were evaluated by the log rank test. See Chapters 5-10-3. and 6-9 for details.

n: number of patients

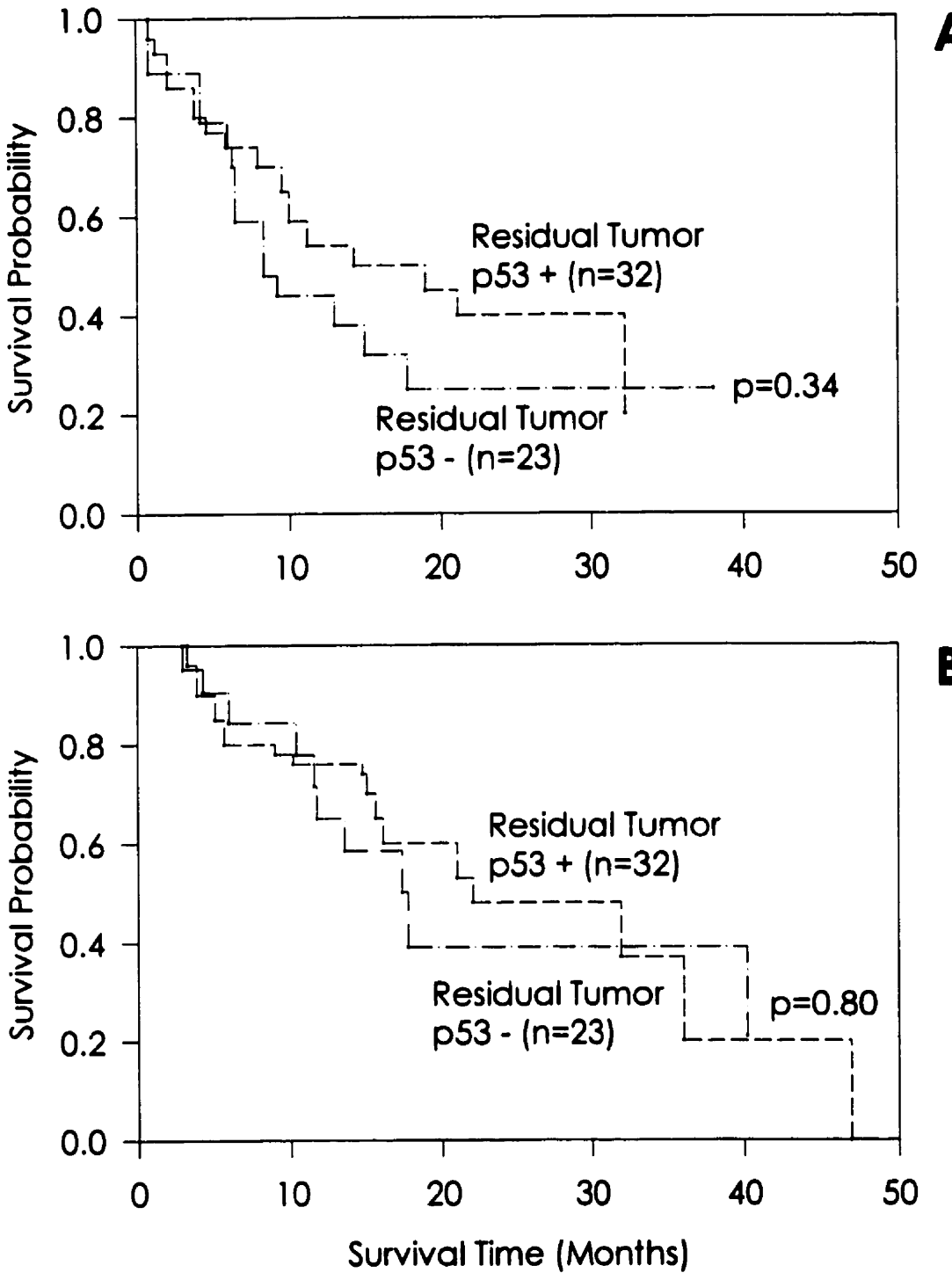

Figure 6-9-4. Kaplan-Meier Analysis for Patients with Residual Tumors

Panel A shows disease-free survival while panel B show overall survival for all p53-negative and p53-positive patients with residual tumor. Differences between curves were evaluated by the log rank test. See Chapters 5-10-3. and 6-9 for details.

n: number of patients

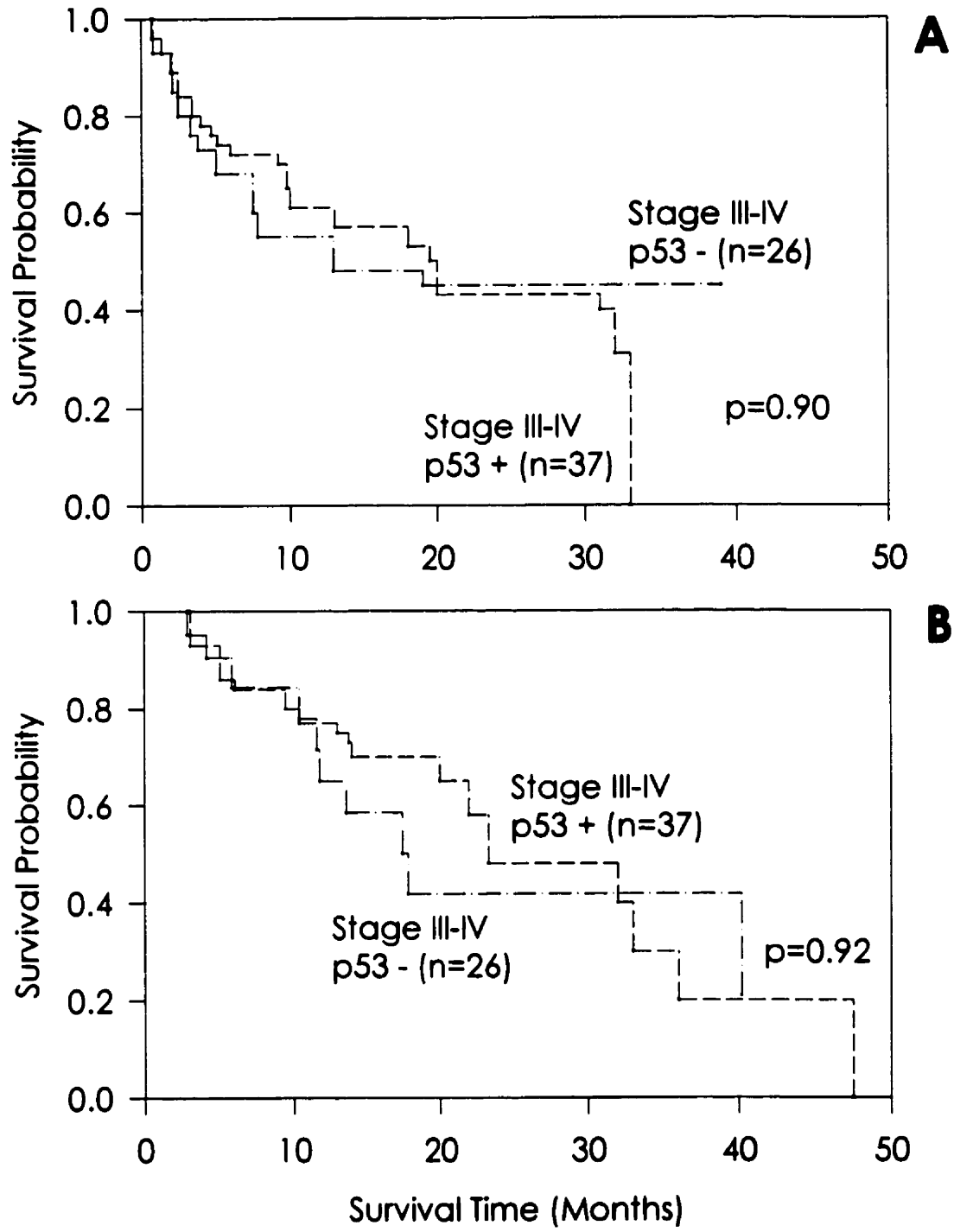

Figure 6-9-5. Kaplan-Meier Survival Analysis for Patients with Late Stage Tumors

Panel A shows disease-free survival while panel B show overall survival for all p53-negative and p53-positive patients with Stage III-IV disease. Differences between curves were evaluated by the log rank test. See Chapters 5-10-3. and 6-9 for details.

n: number of patients

Table 5-5-1. Characteristics of Patient Population

Variable	Number of Patients
Menopausal Status[1]	
Pre	36
Post	53
Stage	
I	21
II	6
III	51
IV	12
Grade	
G1	16
G2	25
G3	49
Histologic Type	
Serous	36
Endometrioid	21
Mucinous	8
Clear Cell	7
Undifferentiated	10
Unclassified	8

1. Menopausal status unknown for one patient.

Table 5-8-1-1. p53 Exons Amplified and Primers Used in PCR

Amplified p53 Exon			Primers Used
Exon	**Codons Included[1]**	**Length[2]**	**Sequence**
Exon 5-6[3]	126 - 201	331	5'-GGAATTCTTCCTCTTCCTGC AGTACTC-3' 5'-GGAATTCGCAAATTTCCTTC CACTCGG-3'
Exon 7	225 - 261	145	5'-GGAATTCGTGTTGTCTCCTA GGTTGGC-3' 5'-GGAATTCCAAGTGGCTCCTG ACCTGGA-3'
Exon 8	262 - 316	167	5'-GGAATTCCCTATCCTGAGTA GTGGTAA-3' 5'-GGAATTCGTCCTGCTTGCTT ACCTCGC-3'

1. Range indicates codon positions.
2. Length given in number of bases.
3. Amplified fragment includes all of exon 5 (codons 126-187) and part of exon 6 (codons 188-201).

Table 6-4-1. Summary of SSCP Analysis of p53 Gene and Comparison with p53 Immunofluorometric Assay Results

Specimen	SSCP Analysis	Immunofluorometric Assay	
	Migration of PCR Product	p53 Conc. (U/g)	Status[1]
Cell Line			
COLO 320HSR	Shift in Exon 7 Fragment	82.23	Positive
Ovarian Tumors			
1	Normal	37.39	Positive
2	Shift in Exon 5-6 Fragment	0.513	Negative
3	Shift in Exon 5-6 Fragment	68.57	Positive
4	Shift in Exon 8 Fragment	64.48	Positive
5	Normal	19.27	Positive
6	Shift in Exon 8 Fragment	81.37	Positive
7	Normal	1.30	Negative
8	Shift in Exon 7 Fragment	9.26	Positive
9	Normal	0.43	Negative

1. p53-positivity status based on cutoff point of 3 U/g.

Table 6-5-1. Associations Between p53 and Other Clinicopathologic Variables

Variable	Number of patients (%)		p value[1]
	p53-negative	p53-positive	
Age (years)			
<40	8 (80.0)	2 (20.0)	
40-49	14 (66.7)	7 (33.3)	
50-59	16 (48.5)	17 (51.5)	
60+	13 (50.0)	13 (50.0)	0.22
Menopause[2]			
Pre	25 (69.4)	11 (30.6)	
Post	26 (49.1)	27 (50.9)	0.06
Stage			
I-II	25 (92.6)	2 (7.4)	
III-IV	26 (41.3)	37 (58.7)	<0.01
Grade			
G1	14 (87.5)	2 (12.5)	
G2	15 (60.0)	10 (40.0)	
G3	22 (44.9)	27 (55.1)	0.01
Residual tumor (cm)[3]			
0	28 (82.4)	6 (17.6)	
≤5	14 (46.7)	16 (53.3)	
>5	9 (36.0)	16 (64.0)	<0.01
Histotype			
Endometrioid	19 (90.5)	2 (9.5)	
Serous	14 (38.9)	22 (61.1)	
Others	18 (54.5)	15 (45.5)	<0.01

1. p values determined from Chi-square tests.
2. Menopausal status unknown for one p53-positive patient.
3. Residual tumor unknown for one p53-positive patient.

Table 6-6-1. Associations Between Clinicopathologic Variables, p53 and Cancer Relapse or Death[1]

Variable	Number of patients (%)		p value[2]	Number of patients (%)		p value[2]
	Relapse	Relapse-free		Dead	Alive	
Age (years)						
<40	2 (20.0)	8 (80.0)		2 (20.0)	8 (80.0)	
40-49	8 (38.1)	13 (61.9)		7 (33.3)	14 (66.7)	
50-59	13 (39.4)	20 (60.6)		7 (21.2)	26 (78.8)	
60+	12 (46.2)	14 (53.8)	0.55	11 (42.3)	15 (57.7)	0.30
Menopause[3]						
Pre	10 (27.8)	26 (72.2)		9 (25.0)	27 (75.0)	
Post	25 (47.2)	28 (52.8)	0.07	18 (34.0)	35 (66.0)	0.37
Stage						
I-II	3 (11.1)	24 (88.9)		0	27 (100)	
III-IV	32 (50.8)	31 (49.2)	<0.01	27 (42.9)	36 (57.1)	<0.01
Grade						
G1	1 (6.2)	15 (93.8)		2 (12.5)	14 (87.5)	
G2	12 (48.0)	13 (52.0)		6 (24.0)	19 (76.0)	
G3	22 (44.9)	27 (55.1)	0.01	19 (38.8)	30 (61.2)	0.10
Residual tumor (cm)[4]						
0	4 (11.8)	30 (88.2)		1 (2.9)	33 (97.1)	
≤5	15 (50.0)	15 (50.0)		10 (33.3)	20 (66.7)	
>5	15 (60.0)	10 (40.0)	<0.01	15 (60.0)	10 (40.0)	<0.01
Histotype						
Endometrioid	3 (14.3)	18 (85.7)		2 (9.5)	19 (90.5)	
Serous	16 (44.4)	20 (55.6)		11 (30.6)	25 (69.4)	
Others	16 (48.5)	17 (51.5)	0.03	14 (42.4)	19 (57.6)	0.04
p53						
Positive	20 (51.3)	19 (48.7)		16 (41.0)	23 (59.0)	
Negative	15 (29.4)	36 (70.6)	0.04	11 (21.6)	40 (78.4)	0.05

1. In this analysis, the follow-up time for each patient was not taken into consideration.
2. p values determined from Chi-square tests.
3. Menopausal status unknown for one p53-positive patient.
4. Residual tumor unknown for one p53-positive patient.

Table 6-7-1. Cox Proportional Hazard Regression Analysis

Variable	Relapse		p value	Death		p value
	HR[1]	95% CI[2]		HR[1]	95% CI[2]	
Univariate Analysis:						
p53[3]	2.03	1.06-3.89	0.03	1.95	0.96-3.97	0.06
Age	1.02	0.99-1.05	0.10	1.03	1.00-1.07	0.04
Grade[4]	2.23	1.11-4.47	0.02	3.22	1.42-7.30	0.01
Stage[5]	7.28	2.20-24.11	<0.01	0[7]		
Residual[6] Tumor	8.31	2.90-23.83	<0.01	27.48[8]	2.70-204.4	<0.01
Multivariate Analysis:						
p53[3]	0.84	0.41-1.72	0.63	0.86	0.40-1.87	0.72
Age	1.03	0.99-1.06	0.11	1.04	1.00-1.09	0.05
Grade[4]	0.79	0.38-1.67	0.54	1.00	0.42-2.34	0.99
Stage[5]	2.69	0.55-13.15	0.22	0[7]		
Residual[6] Tumor	5.35	1.42-20.14	0.01	30.89[8]	3.83-248.8	<0.01

[1] Hazard Ratio.
[2] Confidence Interval.
[3] p53 presence versus p53 absence.
[4] Grade G1 and G2 versus G3.
[5] Stage I and II versus stage III and IV.
[6] Residual tumor presence versus residual tumor absence.
[7] No death in the group of patients with stage I-II.
[8] Only one death in the group of patients with no residual tumor.

Table 6-8-1. Cox Proportional Hazard Regression Analysis for Subgroups of Patients

Variable	Relapse		p value	Death		p value
	HR[1]	95% CI[2]		HR[1]	95% CI[2]	
Grade 1-2:						
p53[3]	11.07	3.28-37.34	<0.01	25.03	3.06-204.7	<0.01
p53[4]	9.43	2.73-32.54	<0.01	20.67	2.52-169.3	0.01
Grade 3:						
p53[3]	0.54	0.24-1.24	0.15	0.48	0.20-1.17	0.11
p53[4]	0.55	0.24-1.26	0.16	0.46	0.18-1.13	0.09
Stage I-II:						
p53[3]	5.82	0.51-66.81	0.16	0[5]		
p53[4]	8.43	0.46-154.2	0.15	0[5]		
Stage III-IV:						
p53[3]	1.04	0.53-2.05	0.90	0.97	0.47-1.98	0.92
p53[4]	0.94	0.46-1.90	0.86	0.76	0.35-1.62	0.76
No residual tumor:						
p53[3]	14.17	1.45-138.3	0.02	0[6]		
p53[4]	8.49	0.85-85.08	0.07	0[6]		
Residual tumor:						
p53[3]	0.71	0.36-1.43	0.34	0.91	0.42-1.94	0.80
p53[4]	0.69	0.34-1.37	0.29	1.04	0.38-1.76	0.60

[1] Hazard Ratio.
[2] Confidence Interval.
[3] Unadjusted analysis.
[4] Age adjusted analysis.
[5] No death in the group of patients with stage I-II.
[6] Only one death exists in the group of patients with no residual tumor, and convergency could not be achieved in the calculation of model parameters.