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Advances in Brief

Wild-Type *p53* Suppresses Growth of Human Prostate Cancer Cells Containing Mutant *p53* Alleles[1]

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Abstract

Evidence supporting a broad role for the inactivation of the *p53* gene in human tumorigenesis has been provided by studies showing that the *p53* gene is mutated in many human cancers. In this study, we report on the mutational status of the *p53* gene in prostate cancer cells and provide functional evidence that the wild-type *p53* gene may have a role in suppressing prostatic tumorigenesis. Sequence analysis of exons 5–8 of the *p53* gene reveals that three of five prostate cancer cell lines (TSUPr-1, PC3, DU145) contain mutations which alter the amino acid sequence of this most highly conserved portion of the gene. One of two primary prostatic cancer specimens examined also contained a mutation in this region. Transfection of the wild-type *p53* gene *versus* a mutated *p53* gene into two cell lines with *p53* mutations results in reduced colony formation. Wild-type *p53* gene expression is apparently incompatible with continued growth of these tumor cells inasmuch as none of the colonies which formed after wild-type transfections retain the transfected *p53* sequences. Immunocytochemical data indicate that prostate carcinoma cells expressing the transfected wild-type *p53* gene are growth arrested because they exhibit a reduced level of thymidine incorporation into DNA. This study is the first report of *p53* gene mutations in prostate cancer cells and suggests a functional role for the *p53* gene in suppressing prostatic tumorigenesis.

Introduction

Several lines of evidence support the role of the *p53* gene as a tumor suppressor gene the inactivation of which contributes to the tumorigenic process in many human cancers. Allelic loss in human tumors, a hallmark of tumor suppressor gene inactivation, occurs frequently near the *p53* locus on chromosome 17p in a variety of human tumors including those of the colon, lung, breast, brain, and bladder (1, 2). In nearly all cases where chromosome 17p loss occurs, the remaining allele at the *p53* locus has been found to be mutated suggesting that inactivation of the *p53* gene product may be important in the development of these tumors. More recent experiments have shown that wild-type *p53* can suppress the growth of certain cancer cell types *in vitro* (3, 4). Other studies have indicated that expression of wild-type *p53* protein is necessary for cell growth indicating that the effects of *p53* may be different in different cell types (5, 6).

Prostate cancer is the most common cancer to occur in United States men (>105,000 cases in 1991) and the second leading cause of cancer mortality (>30,000 deaths in 1991) in these men (7). Despite the significant impact of prostate cancer in the United States, the genetic targets of neoplastic transformation in this cancer have not been well characterized. Previous studies of primary prostate cancer have noted that chromosome 17p loss occurs in about one-fifth of clinically resected tumors suggesting a potential role for inactivation of the *p53* gene in human prostatic tumorigenesis (8). To further assess the role of the *p53* gene in this process, we have analyzed the mutational status of this gene in human prostate cancer cells as well as performed functional studies to assess the potential role of the wild-type *p53* gene in suppressing prostate cancer cell growth.

Materials and Methods

Cell Lines and Tumor Samples. Five human prostate cancer cell lines derived from one primary prostate cancer (PC-82, a serially transplantable tumor line maintained in male nude mice) and four metastatic prostate cancers (TSU-Pr1, LNCaP, PC-3, and DU145) were used in this study (9–13). The latter four cell lines were maintained in RPMI supplemented with 10% fetal calf serum. Cells were grown at 37°C in humidified 5% CO_2 atmosphere. Samples of primary prostate cancer were obtained from men undergoing radical prostatectomy for clinically localized prostate cancer (8).

Plasmids. The plasmids used were obtained as gifts from Dr. B. Vogelstein (Johns Hopkins). pCMV-Neo-Bam contains a cytomegalovirus promoter-enhancer upstream of the insertion site for cDNA[3] sequences to be expressed along with a second transcription unit containing a neomycin resistance gene driven by an SV40 promoter-enhancer to allow for selection of colonies in geneticin (3). pC53SN3 is an expression construct containing a wild-type *p53* cDNA inserted into pCMV-Neo-Bam. pC53C3X differs from pC53SN3 by a single nucleotide (C to T) resulting in a valine to alanine substitution at *p53* codon 143.

Transfections. Cells were transfected with various plasmids in T-75 flasks upon reaching 60–70% confluence. Plasmid DNA (6 μg), dissolved in 900 μl Optimem serum-free medium (BRL), was mixed with Lipofectin reagent (BRL) (60 μl in 900 μl Optimem). After 5 min at room temperature, 7.2 ml Optimem are added, and the mixture is added to the cell layer, which had been washed with serum free medium. After 16 h the medium was replaced with growth medium (RPMI containing 10% fetal calf serum). Twenty-four h later, the cells were trypsinized and seeded into either T-75 flasks for subsequent selection of G-418 resistant colonies or into chamber slides for transient expression and thymidine incorporation analysis. For the latter, cells were labeled 22 h later for 2 h with [^{3}H]thymidine (40 Ci/mmol, 1 μCi/ml). Under these conditions, the number of cells which incorporated label was between 30 and 40%. Cultures were then rinsed with Hanks' balanced salt solution containing unlabeled thymidine (0.1 mg/ml) and fixed with methanol at −20°C for 10 min. The fixed cells were stained with anti-p53 antibodies and subsequently processed for autoradiography (see below). For selection and quantitation of G-418 resistant colonies, transfected cells were cultured in growth medium supplemented with G-418 (500 μg/ml) for 3 weeks. Resistant colonies (>30 cells) were counted and assayed for the presence of *p53* protein by immunostaining (see below).

Immunocytochemistry and Autoradiography. Cells were fixed in cold methanol as described above and incubated with monoclonal antibodies against *p53*. Identical results were obtained with either Pab 421 (Oncogene) or Pab122 (American Type Culture Collection). Primary anti-

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[3] The abbreviations used are: cDNA, complementary DNA; PCR, polymerase chain reaction; VNTR, variable number tandem repeat.

bodies were detected either by immunofluorescence using a fluorescein-conjugated goat anti-mouse IgG or by peroxidase staining using a biotinylated second antibody and avidin-conjugated peroxidase. Following peroxidase staining, tritium-labeled samples were processed for autoradiography by dipping in NBT-2 emulsion, exposure for 1–3 days, and development in D-19 for 1–2 min.

Immunoprecipitations and Western Blot Analysis. Cell lines in 60-mm dishes were metabolically labeled with 100 μCi/ml of [^{35}S]methionine Translabel (1000 Ci/mmol; ICN) in 2.5 ml methionine-free Dulbecco's modified Eagle's medium with 10% dialyzed fetal calf serum for 3.5 h. Cells were lysed in 400 μl extraction buffer (150 mM NaCl-50 mM Tris, pH 8.0-5 mM EDTA-1% Nonidet p-40-1 mM phenylmethylsulfonyl fluoride-50 μg/ml leupeptin). The lysate was scraped from the plate and stored at −70°C. Lysates were thawed on ice, centrifuged at 12,000 × *g* for 30 min at 4°C, and incubated with conditioned media from Pab122 hybridoma cells fro 3–4 h before incubation with protein A-agarose beads. After 5 washings with radioimmunoprecipitation assay buffer, the immune complexes were released from the beads by boiling in sodium dodecyl sulfate sample buffer followed by analysis on 10% polyacrylamide gels. Western blots were performed using ^{125}I-labeled donkey anti-mouse IgG as the detection reagent.

Southern Analysis. Genomic DNA was isolated, digested with restriction enzymes, separated on 0.8% agarose gels, transferred to nylon membrane, and hybridized as described previously (8).

PCR Amplification and Sequencing. A 1.8-kilobase fragment containing exons 5–9 of the *p53* gene was generated by the PCR as described previously (14). Briefly, 500 ng of genomic DNA and 350 ng each of primers (5′-GTAGGAATTCACTTGTGCCCTGACTT-3′ from intron 4 and 5′-CATCGATTCTGGAAACTTTCCACTTGAT-3′ from intron 9) containing *Eco*RI sites were used in a 50-μl reaction mix containing nucleotides, dimethyl sulfoxide, and magnesium in concentrations described previously (15). The PCR consisted of 35 cycles of 30 s at 95°C, 3 min at 58°C, and 2 min at 70°C. Following amplification, the PCR product was cleaved with *Eco*RI, separated on a 1.2% agarose gel, and purified by centrifugation through a Co-Star Spin-X column. PCR products were either sequenced directly or cloned into phage. For the latter, one-half of the recovered product (50 ng) was ligated to Lambda ZAP II vector arms and the ligation reaction was packaged by using GigaPack II Gold packaging extracts (Stratagene) according to the manufacturer's instructions. Phage were used to infect XL-1 blue cells and plated on L-agar plates at a density of 10^3–10^4 plaques/plate. Phage were eluted and single-stranded phagemids were obtained as described previously (1). These phagemids were used to infect XL-1 blue cells and obtain double-stranded plasmids for sequencing with previously described primers (16).

Results and Discussion

To begin our analysis of the *p53* gene in prostate cancer, we chose five prostate cancer cell lines for study. One of these cell lines, PC82, was derived from a primary prostatic cancer and can be maintained only by passage in male nude mice, inasmuch as androgens are required for its growth (11). The remaining four lines, derived from metastatic deposits of prostate cancer, can be maintained in culture, and with the exception of LNCaP, will readily form tumors when injected into nude mice (male or female). LNCaP cells reproducibly form tumors in nude mice only when coinjected with Matrigel or fibroblasts (17).

To examine *p53* gene expression in prostate cancer cell lines, we assayed the synthesis and accumulation of the *p53* protein, respectively, by immunoprecipitation of metabolically labeled cell extracts and Western blot analysis of unlabeled cell extracts. As shown in Fig. 1, both PC-3 and TSU-Pr1 cells are negative for *p53* protein in either analysis. Both DU145 and LNCaP cells synthesize detectable quantities of the protein, but only in DU145 cells does this protein accumulate to sufficient quantities to be detectable by immunoblotting. These data suggested that *p53* synthesized by DU145 cells may have an extended half-life, a property often associated with mutated forms of *p53* (18).

To search for possible genetic alterations which may affect the expression of the *p53* gene, we used Southern blot analysis to examine allelic loss on chromosome 17p, as a hallmark *p53* gene inactivation, in five prostate cancer cell lines. Two highly informative VNTR probes, 144D6 (19) and YNH37.3 (20), mapped to chromosome 17p13, were used to detect allelic deletions; this combination of probes allows for the assessment of allelic loss with greater than 95% certainty even in the absence of normal tissue for comparison (1). Two of the cell lines (TSU-Pr1, PC3) exhibited only one allele at chromosome 17p while the other three cell lines retained both chromosome 17p alleles with each probe. To assess whether there were any gross structural alterations at the *p53* locus, genomic DNA digested with *Bam*HI from the five prostate cancer cell lines was examined by Southern analysis using a 1.8-kilobase *Xba*I fragment of human *p53* cDNA as probe. Only one band at 7.8 kilobases was noted in all samples suggesting that there were

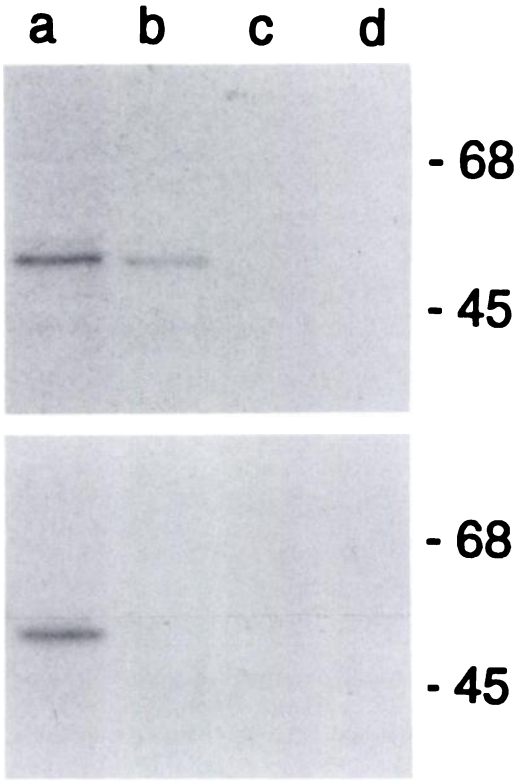

Fig. 1. Synthesis and accumulation of *p53* protein in prostate cell lines. *Lane a*, DU-145; *Lane b*, LNCaP; *Lane c*, PC-3; *Lane d*, TSU-Pr1. *Top*, immunoprecipitation of metabolically labeled cell extracts; *bottom*, Western blot of cell extracts.

Table 1 *p53 gene mutations in prostate cancer*

Cell line	No. of 17p alleles	Codon	Nucleotide change	Amino acid change
TSU	1	126	TAC→TAG	Stop
PC-3	1	138	Deleted a C	Frame-shift
DU145	2	223	CCT→CTT	Pro→Leu
		274	GTT→TTT	Val→Phe
LNCaP	2		None detected	
PC82	2		None detected	
Primary tumor				
23	1	197	GTG→GGG	Val→Gly
29	1		None detected	

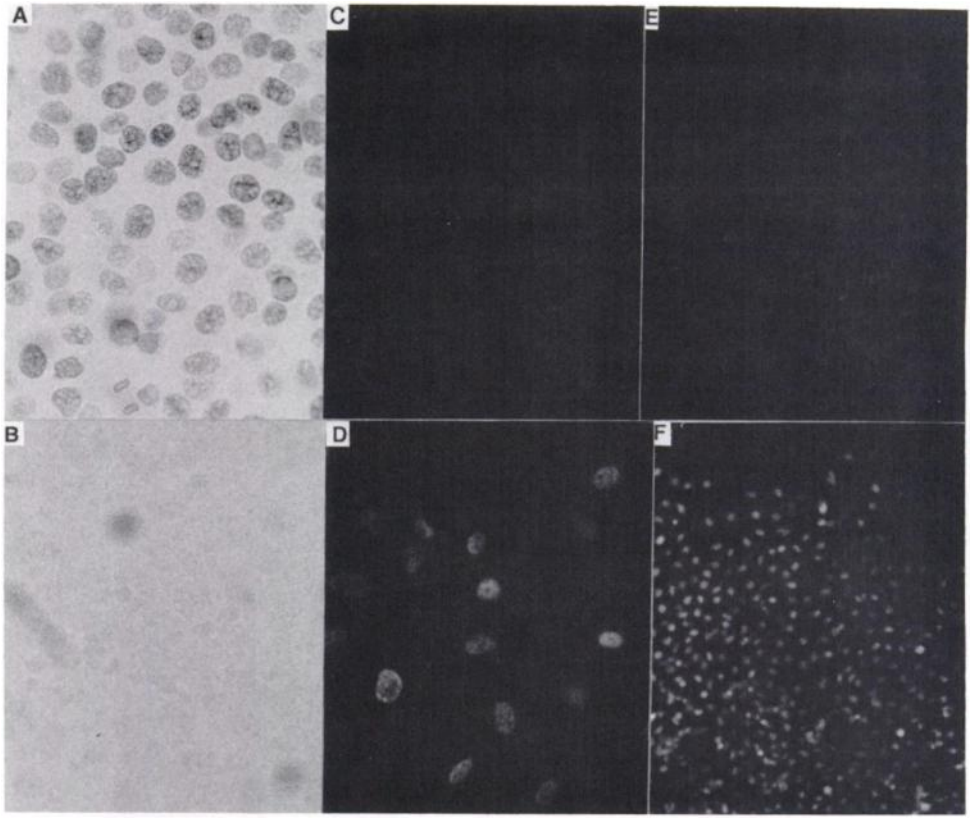

Fig. 2. Immunostaining of *p53* protein in untreated and transfected prostate cancer cells. *A* and *B*, immunoperoxidase; *C–F*, immunofluorescence. *A*, untreated DU-145 cells, positive for *p53* staining; *B*, untreated LNCaP cells, negative for *p53* staining; *C*, TSU-Pr1 cells 3 weeks after transfection with wild-type *p53* cDNA (all cells are negative); *D*, a similar number of TSU-Pr1 cells as *C*, 3 weeks after transfection with mutant *p53* cDNA; *E*, PC-3 cells 3 weeks after transfection with wild-type *p53* cDNA (all cells are negative); *F*, a similar number of PC-3 cells as in *E*, 3 weeks after transfection with mutant *p53* cDNA. All transfected cells are growing in antibiotic, G-418. *A–D*, × 1000; *E* and *F*, × 400.

no gross structural abnormalities of the *p53* gene in these samples (data not shown).

Because loss of suppressor gene function can be achieved by subtle alterations such as point mutations or small deletions which result in mutant protein, we sequenced exons 5–8 of the *p53* gene in all five cell lines. These exons contain the most highly conserved areas of the *p53* gene which have been previously documented to harbor most *p53* mutations which occur in human tumors (1). Sequencing revealed that three of the cell lines, TSU, PC-3, and DU145, contained mutant *p53* alleles (Table 1). The DU145 cell line contained two different mutations, present on different chromosomes (indicated by sequencing individual cloned PCR products). No mutations were detected in exons 5–8 in the LNCaP or PC82 cell lines. Interestingly, these latter two cell lines are either minimally tumorigenic (LNCaP) or require the presence of androgens for tumorigenicity (PC82) when injected into nude mice, as mentioned above.

In addition to prostate cancer cell lines, two samples of primary prostate cancer were assayed for the presence of *p53* gene mutations. In both of these samples, allelic loss of sequences on chromosomes 17p had been detected in a previous study (8). A mutation (T→G) resulting in a glycine for valine substitution was detected at codon 197 (exon 6) in one of the tumor samples (No. 23).

The detection of a mutated *p53* gene in three of five prostate cancer cell lines as well as in a primary prostatic cancer suggested a potential functional role for this gene in suppressing prostatic tumorigenesis. In order to assess the functional significance of wild-type *p53*, we transfected both wild-type and mutant *p53* cDNAs into the two prostate cancer cell lines containing only mutant *p53* alleles, TSU-Pr1 and PC-3. As seen in Table 2, after 3 weeks of selection in G-418, in both cases, cells transfected with the wild-type vector, pC53-SN3, formed colonies 3 to 6 times less efficiently than those transfected with the mutant *p53* vector, pC53-SCX3. These data suggested that the clonal growth of these cell lines was inhibited by expression of the wild-type *p53* gene.

To examine the expression of the transfected *p53* cDNA sequences in the G-418 resistant colonies, cultures were stained with antibody to *p53* (Fig. 2). Although transfection of wild-type *p53* cDNA in transient assays results in readily detectable *p53* protein (see below), no colonies were positive for *p53* 3 weeks after transfection with this construct. In contrast, over one-half of the colonies which arose after transfection with

Table 2 *Transfection of p53 gene into prostatic cancer cells*

Cell line	*p53* cDNA	*Stable transfection*[a] No. of colonies formed Experiment 1	Experiment 2	No. of colonies positive for *p53* expression[b]	*Transient transfection*[a] No. of cells positive for *p53*/for no. of cells counted (% positive)	No. of cells positive for *p53* and DNA synthesis (% positive)
TSU	*wt*	105	143	0/80	98/1500 7%	7 7%
	mutant	424	532	43/80	203/1500 13%	67 33%
PC-3	*wt*	40	24	0/40	76/1500 5%	3 4%
	mutant	212	164	61/80	167/1500 11%	43 26%

[a] Colonies were analyzed 3 weeks after transfection (stable); single cells were analyzed 48 hours after transfection (transient).
[b] Expression of *p53* was determined by immunofluorescence.

mutant *p53* cDNA were positive for this protein (Table 2).

To determine whether the colonies that formed after transfection with the wild-type *p53* cDNA retained the transfected sequences, genomic DNA from individual as well as pooled colonies was subjected to Southern analysis. Transfected *p53* sequence can be readily distinguished from the endogenous *p53* gene following digestion of genomic DNA with *Bam*HI, resulting in a 7.8-kilobase fragment for the latter and a 1.8-kilobase band for the former. No exogenous *p53* sequence was detected in any of eight individual clones as well as pooled clones from the wild-type *p53* transfection of the PC3 cell line, whereas seven of eight individual as well as pooled clones from mutant *p53* transfections retained exogenous *p53* sequence. Similar data have been obtained with the TSU-Pr1 cell line (data not shown). These data suggested that retention of the wild-type *p53* gene was not compatible with continued growth of these prostate cancer cells.

To further assess the growth suppression potential of the wild-type *p53* gene in prostate carcinoma cells, a transient expression assay was performed, using the TSU-Pr1 and PC3 cell lines. The results of this assay are shown in Table 2. This assay examines the effect of expression of either wild-type or mutant *p53* protein on the ability of the cell to undergo DNA synthesis. Each cell line was separately transfected with both the mutant and wild-type *p53* cDNA. At 46 h after transfection, cells were labelled with [^{3}H]thymidine for 2 h. Subsequently, the cells from each flask were fixed, stained with an antibody to *p53* protein, and autoradiographed (see Fig. 3). Expression of the wild-type and mutant transfected *p53* cDNA was readily detected in each cell line by immunoperoxidase staining. Scoring for the number of cells simultaneously expressing *p53* protein and synthesizing DNA reveals that, in both cell lines, expression of the wild-type *p53* gene had an inhibitory effect on DNA synthesis as compared to expression of the mutant gene (Table 2). In TSU-Pr1, one-third of cells expressing the mutant gene incorporated labeled thymidine, compared to only 7% of cells expressing the wild-type gene. A similar decrease was seen in the PC-3 cells.

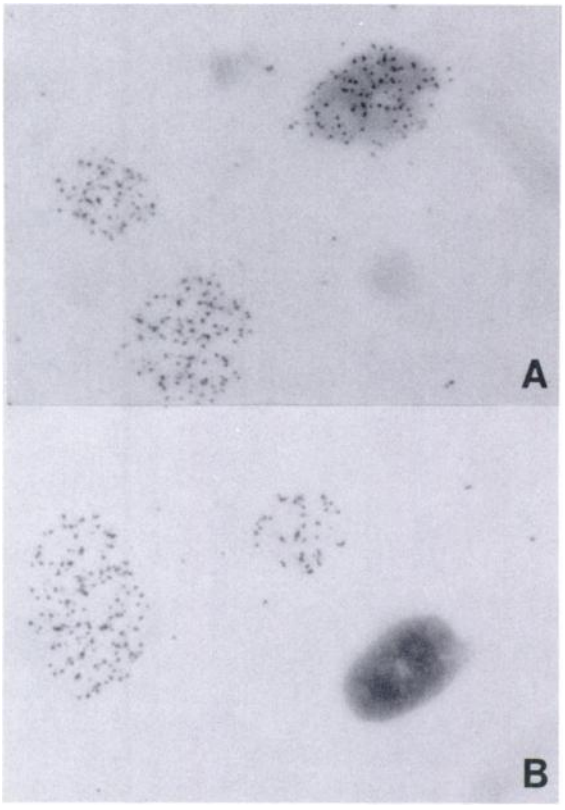

Fig. 3. Examples of thymidine incorporation into prostate cancer cells expressing *p53* in transient transfection assay. Thymidine incorporation is indicated by silver grains and *p53* expression by peroxidase staining. In *A*, a cell expressing mutant *p53* also incorporates thymidine. IN *B*, example of a wild-type *p53* expressing cell which does not incorporate thymidine. × 1000.

In summary, our data demonstrate that *p53* mutations can be detected both in primary prostatic cancer cells and in cell lines derived from metastatic deposits and that wild-type *p53* can suppress the growth of prostatic carcinoma cells *in vitro*. Taken together these data suggest a functional role for *p53* as a suppressor of prostatic tumorigenesis. Given that prostate cancer is the most common cancer in United States men, the implication of *p53* mutations in the pathogenesis of this cancer further supports the view of *p53* as a tumor suppressor gene which is inactivated in the majority of human cancers. Future studies which examine *p53* mutations at various stages in the progression of prostate cancer will reveal the timing and overall frequency of *p53* gene inactivation in this common cancer.

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