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p53 Mutations Occur in Clinical, but Not Latent, Human Prostate Carcinoma

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To elucidate the role of the p53 tumor suppressor gene in prostate tumorigenesis, we probed for mutations in latent and clinical prostate cancers using single strand conformation polymorphism (SSCP) and restriction fragment length polymorphism (RFLP) analysis in combination with direct gene sequencing and immunohistochemical methodologies. Fifteen cases of subclinical and 32 cases of clinical carcinoma, the latter graded in stages A through D, were available for study. While p53 point mutations were detected in only 5 of 32 (16%) clinical cancers, no mutations were detected in latent disease. Of the carcinomas in stages B, C and D, 15% (2/13), 29% (2/7) and 9% (1/11) were positive for p53 mutations, respectively. Although no specific mutational patterns were observed, the aberrations found were predominantly single base missense substitutions. The data suggest not only an association of p53 mutation and progression of clinical prostate cancer, but also imply that some other mechanism(s) are at work in latent carcinoma.

Key words: p53 — Human — Prostate carcinoma

Despite the recent increase of prostate cancer in Japan and the high frequency prevalent in Western nations, the genetic events underlying the disease are still poorly understood. Current molecular studies have strongly supported the theory of tumorigenesis as a consequence of multiple genetic mutational events in at least some human malignancies[1]; this mechanism may also operate in the prostate gland.[2] Previous investigations on human prostatic cancers have also demonstrated specific allelic losses on chromosomes 8p, 10q, 16q, and 18q.[3,4]

In man, mutations of the p53 gene, located on chromosome 17p, frequently occur in a variety of solid tissue tumors, including those of the lung, breast, colon and rectum, urogenitalia and brain as well as in leukemias.[5–8] The encoded protein was first identified as a 53 kDa moiety binding to the SV40 large tumor antigen[9] and was later shown to interact as well with both the E1b tumor antigen of adenovirus and the E6 protein of human papilloma virus.[10,11] Initially believed to be an oncogene, p53 is now indicated by mounting evidence to be a tumor suppressor gene.[12,13] In cell transformation cultures, mutated p53 is postulated to exert a dominant inhibitory effect on the growth-restricting function of the normal (also called wild-type) gene products.[14]

Analyses of abnormal p53 in tumors of various tissues suggests that mutational patterns may reflect the characteristic genetic damage induced by endogenous and exogenous agents associated with carcinogenesis. For instance, most base pair transitions in colorectal cancers, leukemias and lymphomas are at CpG dinucleotide mutational "hot spots," while cancers of the lung and liver most frequently exhibit G:C to T:A transversions. Mutations at A:T base pairs are more often seen in esophageal tumors.[5] There is evidence that p53 gene mutations are more closely associated with the subset of advanced and/or highly malignant carcinomas, including those of the prostate.[15–17] Given the current concern over the increasing incidence of prostate cancer in both its clinical and latent forms, it is surprising that there have been so few investigations of p53 mutations in the two disease patterns. We have therefore examined a range of prostate tissue from various stages of frank clinical cancers through latent lesions discovered at autopsy using the combination of immunohistochemistry with single strand conformation polymorphism (SSCP), restriction fragment length polymorphism (RFLP) and gene sequence analyses.

MATERIALS AND METHODS

Prostate tissues Samples of 32 prostate carcinomas were obtained from total prostatectomies and transurethral resections performed at Chiba University and Mie University School of Medicine. Portions of each tumor were frozen at $-70°C$ while other equivalent portions were fixed in formalin and embedded in paraffin; the paraffin-embedded tissue was cut at 3–6 μm and sequential sections were mounted and left unstained for immuno-

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histochemistry or stained with hematoxylin and eosin (HE) for light microscopy, and used to provide a guide for the extraction of DNA from comparable regions of frozen tissue. Tumors were categorized as well, moderately or poorly differentiated adenocarcinomas and graded according to Gleason's histological criteria.[18] Tumors were further staged based on clinical assessment of the primary mass, the presence or absence of metastases to lymph nodes and/or distant sites and histological appraisal using the General Rules for Clinical and Pathological Studies on Prostatic Cancer, Japan.

Twenty-five (25) samples of latent prostate carcinoma were obtained from autopsies as incidental findings. All of these lesions were fixed in formalin, paraffin-embedded and sectioned at multiple levels. Again, corresponding sections were left unstained for immunohistochemistry and DNA extraction while others were HE-stained for pathological evaluation and as tissue guides. Lesions <3 mm in size were not used in the study.

Immunohistochemistry Formalin-fixed, paraffin-embedded tissues were sectioned at 3 μm and incubated with human p53-specific mouse monoclonal antibody Bp53-12 (Japan Turner Corp., Kobe) using the streptavidin-biotin method as instructed in the kit (Histofine SAB-PO (M) Kit, Nichirei Corp., Tokyo). Briefly, sections were deparaffinized and incubated for 30 min in 0.3% hydrogen peroxide in methanol, rinsed in phosphate-buffered saline (PBS) and incubated again for 20 min with normal rabbit serum. Sections were then rinsed and incubated at 4°C overnight with the monoclonal Bp53-12 diluted 1:20 in PBS. The next day, tissue sections were exposed to a biotin-labeled rabbit anti-mouse IgG/IgA/IgM mixture for 10 min at room temperature, followed by streptavidin for an additional 5 min. Copious washing with PBS between each step was essential. Antigen-antibody complex was visualized via the chromogen 3,3′-diaminobenzidine tetrahydrochloride in the presence of 0.01% hydrogen peroxide. The sections were lightly counter-stained with hematoxylin, dehydrated through graded alcohols and cleared in xylene before being coverslipped for microscopic examination and counting of immunopositive cells. Samples were considered negative if less than 5% of the cells were reactive.

DNA extraction Using corresponding HE-stained sections of each sample as guides, frozen carcinomas were trimmed free from surrounding nontumorous tissue. Tumor tissues were digested with proteinase K and high-molecular-weight genomic DNA was extracted using the phenol-chloroform method. Paraffin-embedded latent carcinomas were cut at 4–6 μm and trimmed free from nontumorous tissue as described above. Tumor tissue collected averaged 1 cm^2; the tissues were deparaffinized in sequential washes of xylene and ethanol, and DNA was extracted by the same method as used for frozen tissue.

PCR-SSCP/PCR-RELP analysis Exons 4–9 of the human p53 gene were amplified by polymerase chain reaction (PCR) and screened for mutations by SSCP analysis performed according to Orita *et al.*,[19] with minor modifications. Six pairs of oligonucleotide primers were designed to span the 6 coding exons, based on the primer sequence data reported by Lehman *et al.*[20] Each 100 μl of the PCR mixture contained 200 ng of DNA template, 22.7 pmol of each primer pair, 1.5 mmol $MgCl_2$, 250 pmol/μl of each deoxyribonucleotide triphosphate, and 2.0 units of *Taq* polymerase. Thirty-five (35) cycles of amplification were carried out using a thermal cycler (Perkin Elmer-Cetus, Norwalk, CT, USA) at 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min. Negative controls without DNA template were used routinely to detect PCR contamination.

For PCR-RFLP analysis, the amplified exon 4 fragments were digested with *Tha I* (GIBCO-BRL, Gaithersberg, MD, USA). Primers used were: 5′-GCCGTCCCAAGCAATGGATG-3′ (upstream) and 5′-AGTCACAGACTTGGCTGTCCCAGA-3′ (downstream).[21] The second base of codon 72 in exon 4 of the p53 gene is known to be normally polymorphic (heterozygous), in that CGC as well as CCC is frequently observed. This polymorphism can be detected using the restriction enzyme *Tha I*; the 265-base pair (bp) segment containing codon 72 will be digested into 2 fragments of 155 and 110 bp. No cleavage in the segment occurs if the CGC polymorphism is not present. The digest was separated by electrophoresis on a 2% agarose gel. The amplified DNA fragments were labeled with oligonucleotide primers and [γ-^{32}P]ATP (3000 Ci/mmol, 10 mCi/ml). Aliquots were heated to 95°C for 2 min, chilled on ice, then loaded onto a 5% polyacrylamide gel containing 10% glycerol. Electrophoresis was performed at 40 W for 3–4 h at 25°C. The gels were exposed to X-ray film (Kodak XAR) at -70°C without intensifying screens. DNA extracts from normal surrounding tissues were also analyzed by PCR-RFLP when the tumor itself showed no codon 72 polymorphism, i.e., was homozygous.

Direct gene sequencing DNA samples showing altered migration patterns in SSCP analysis were purified using the Geneclean II kit (Bio 101 Corp., La Jolla, CA, USA) in preparation for sequencing. Sequencing was performed by the dideoxy procedure[22] utilizing the Circum Vent Thermal Cycle Sequencing kit (New England Biolab, Beverly, MA, USA). Following the manufacturer's instructions, each primer was incubated with [γ-^{32}P]ATP and T4 polynucleotide kinase (Toyobo Inc., Tokyo) at 37°C for 30 min, then 35 cycles of amplification were carried out in a thermal cycler with the end-labeled primer added to the samples. PCR products were electrophoresed on 8% polyacrylamide/7 *M* urea gels for 2–3 h at 1800 V. The gels were dried and exposed to

Table I. p53 Mutation Analysis: Comparison with Expression of Immunoreactive p53 Protein

Case No.	Histology (Gleason)	Stage	SSCP abnormality (codon)	Codon 72 polymorphism		p53 expression
				Informative	LOH	
1	PD (4/5)	D2	—	Yes	Yes	+
2	MD (3/5)	C	—	NT		—
3	WD (2/3)	C	—	NT		—
4	MD (3/4)	C	—	No		—
5	MD (3/3)	B	—	Yes	Yes	—
6	WD (2/4)	B	—	No		—
7	PD (4/5)	B	—	NT		—
8	MD (4/5)	D	—	No		—
9	MD (3/4)	B	—	NT		—
10	MD (3/3)	B	exon 4 (69)	NT		—
11	MD (3/3)	B	—	NT		—
12	MD (4/3)	D2	—	NT		—
13	PD (4/5)	D2	—	NT		—
14	MD (4/3)	A	—	NT		—
15	MD (3/2)	B	exon 7(234)	NT		—
16	PD (4/5)	D2	—	NT		—
17	WD (2/1)	B	—	NT		+
18	PD (4/3)	D2	exon 5(145)	Yes	Yes	+
19	PD (4/5)	B	—	No		—
20	MD (2/3)	B	—	NT		—
21	MD (2/4)	C	exon 7(242)	Yes	Yes	+
22	PD (4/5)	B	—	NT		—
23	PD (4/5)	B	—	Yes	Yes	—
24	WD (2/2)	C	—	NT		—
25	MD (3/4)	D2	—	NT		—
26	PD (5/4)	D1	—	NT		—
27	PD (4/5)	D1	—	NT		—
28	MD (3/4)	D1	—	NT		—
29	MD (3/4)	C	—	Yes	Yes	—
30	PD (4/5)	D2	—	No		—
31	MD (2/3)	B1	—	No		—
32	MD (3/2)	C	exon 4 (60)	No		+

Abbreviations: LOH, loss of heterozygosity; NT, not tested; WD, well-differentiated adenocarcinoma; MD, moderately differentiated adenocarcinoma; PD, poorly differentiated adenocarcinoma.

Kodak X-ray film without intensifying screens at $-70°C$ for 3–6 h.

RESULTS

Immunohistochemistry Of the 32 clinical prostate carcinomas examined, 5 (16%) exhibited positive nuclear staining for p53 (Table I). None of the 15 latent cancers was positive. The fraction of cells within positive tumors showing nuclear staining varied from 5% to 40% (mean 10–20%). Two (2) of the immunoreactive tumors were classified as poorly differentiated adenocarcinoma, 2 were moderately differentiated and 1 was a well-differentiated adenocarcinoma. The p53 immunostaining was highly associated with the clinical stages; 2 positive cancers were stage C, two stage D, and only one stage B.

PCR-RFLP analysis Thirteen cases of frank carcinoma were homozygous at codon 72 in exon 4, while the remaining 19 were heterozygous. The normal surrounding tissues in these 19 cancers heterozygous at codon 72 should be heterozygous. In the 13 homozygous cancers, 6 cases of normal surrounding tissues were heterozygous, i.e., informative (Fig. 1A). However, 100% (15/15) of the latent carcinomas demonstrated heterozygosity (Fig. 1B), i.e., the normal surrounding tissue is non-informative, and no loss of heterozygosity (LOH) was found in latent carcinomas. Of the 6 carcinomas showing LOH, only 2 (cases 18 and 21) were associated with p53 mutations.

PCR-SSCP analysis All samples of clinical and latent prostatic tumors were screened for p53 mutations in exons 4–9 by PCR-SSCP analysis of each exon. Altered

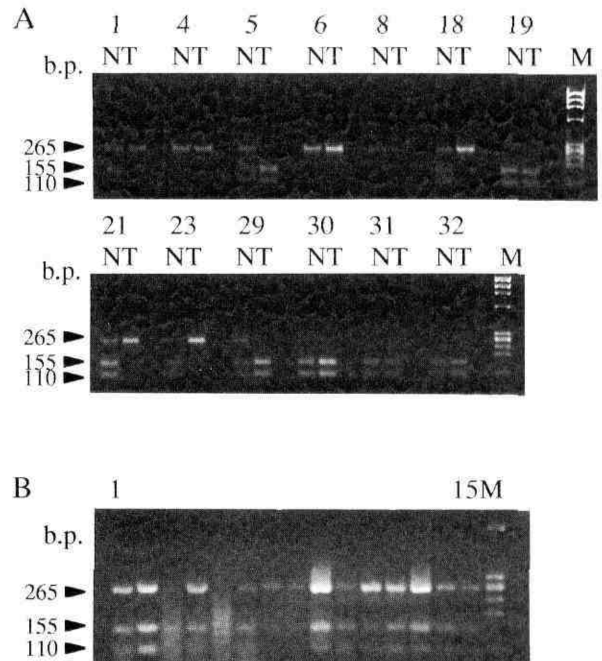

Fig. 1. Loss of heterozygosity for p53 demonstrated by PCR of DNA isolated from normal tissue (N) or from prostatic tumor tissue (T). DNA samples were amplified with a PCR primer flanking a *Tha I* RFLP site in codon 72 of the p53 gene. The PCR products (265 bp) obtained by digestion with *Tha I* were subjected to electrophoresis on an agarose gel. The fragments resulting from cleavage by the restriction enzyme were 155 and 110 base pairs in length. (A) Clinical prostate carcinomas. (B) All latent carcinomas showed codon 72 polymorphism.

mobilities of PCR-amplified products, which suggest the existence of a mutation, were observed in 5 of the 32 (16%) clinical carcinomas: 2 cases had a mutation in exon 4, 1 case in exon 5, and 2 cases in exon 7 (Table I and Fig. 2).

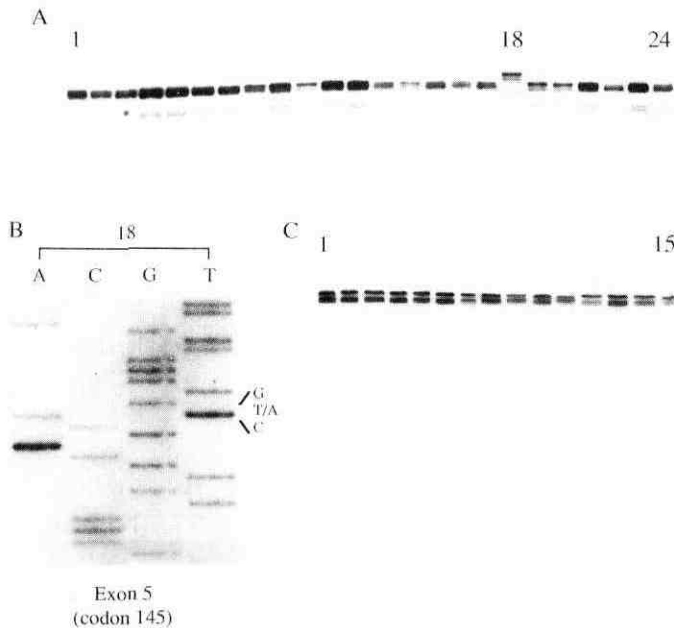

Fig. 2. PCR-SSCP analysis of exon 5 in clinical (A) or latent (C) carcinomas of the prostate. A mutation in case 18 was detected and subsequently identified as a CTG→CAG transversion (codon 145) by direct dideoxy sequencing (B).

Direct cycle sequencing Mutations in the p53 gene were detected by direct cycle sequencing of the 6 coding exons, 4–9, previously amplified by PCR. Sequencing yielded missense point mutations in 5 cases of clinical carcinoma: A:T to T:A (exon 5, tumor 18) resulting in Leu→Glu substitution (Fig. 2, A and B), G:C to C:G (exon 4, tumor 10) resulting in Ala→Gly substitution, G:C to A:T (exon 4, tumor 32) resulting in Pro→Leu substitution, A:T to T:A (exon 7, tumor 15) resulting in Cys→Gly, and A:T to T:A (exon 7, tumor 21) resulting in Tyr→Asn substitution (Fig. 3). Of these 5 tumors, 4 were diagnosed as moderately differentiated adenocarcinomas of stages B and C; the remaining tumor was diagnosed as poorly differentiated stage D2.

Correlation of immunohistochemistry and molecular screening methods As was previously described, positive immunostaining for p53 protein was demonstrated in 5 of the 32 clinically apparent prostatic adenocarcinomas submitted for evaluation. Point mutations were picked up in 3 of these 5 by direct sequencing. Immunoreactivity showed an association with higher grades of dedifferentiation, in that all positive cancers were of stage B (1) or higher (2 of stage C, and 2 of stage D). All 15 samples tested from latent prostatic cancers were uniformly negative by immunohistochemistry and molecular gene screening (Figs. 1B and 2C).

DISCUSSION

The ability to detect altered p53 gene products in tissues by immunohistochemical methods is a result of increased cellular accumulation of mutant protein with a

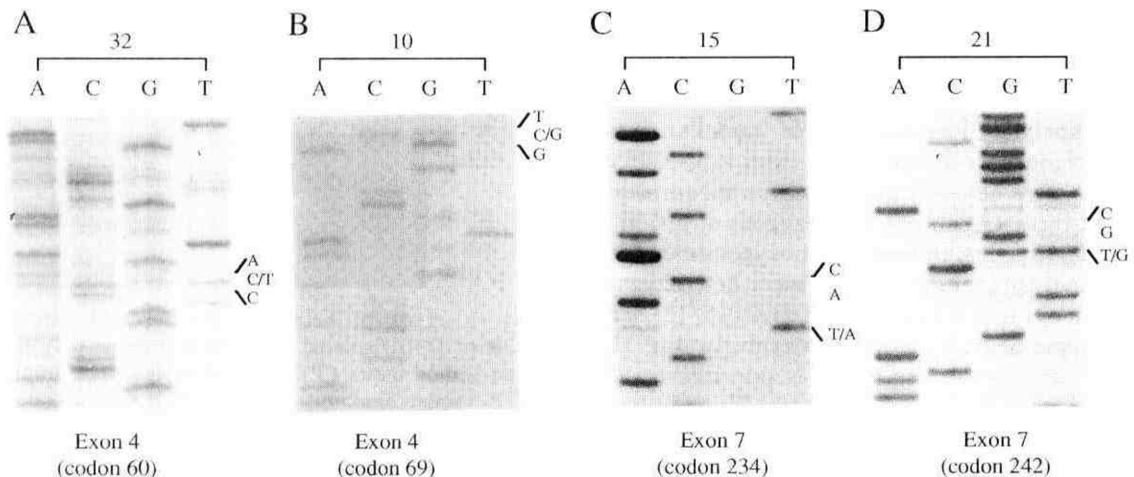

Fig. 3. Demonstration of point mutation in p53 by direct sequencing of PCR-amplified genomic DNA fragments. (A) a CCA→CTA transition in exon 4 (codon 60) in case 32. (B) a GCT→GGT transversion in exon 4 (codon 69) in case 10. (C) a TAC→AAC transversion in exon 7 (codon 234) in case 15. (D) a TGC→GGC transversion in exon 7 (codon 242) in case 21.

longer half-life.[23] Previous studies of p53 in clinical prostate cancer have found no significant relationship between tumor grades or stages and protein expression. Reported frequencies of immunopositive tumors of various tissues have ranged from 6%[24] to 17%.[25,26] These numbers may reflect the impact of numerous variables, including the antibodies used, the sample quality, and, perhaps most importantly, the inherent features of the tumors examined. Visakorpi *et al.* were able to demonstrate that higher p53 levels were correlated to both a higher histologic grade and to a higher cell proliferation rate, as determined by flow cytometric S-phase analysis and proliferating cell nuclear antigen expression.[26] This correlation would suggest that mutations in p53 may confer a proliferative advantage leading to malignancy. In another recent study, analysis of tissues from different areas of a single primary prostate adenocarcinoma revealed intra-tumor heterogeneity of p53 expression, while lymph node metastases from the same patient showed a homogeneous pattern.[27] It was postulated that a clone of cells with p53 mutation(s) acquired a greater malignant potential and thus was able to metastasize exclusively or at a more rapid rate than the subset of tumor cells without the mutation. When molecular screening methods, such as PCR-SSCP and gene sequencing, have been employed in combination with immunohistochemistry, mutant p53 has been found in 20–25% of advanced cancers[17] and mutations in the gene have been linked with advanced stage, increased dedifferentiation, and the transition from androgen-dependent to independent growth.[15]

A survey of prostatic tumorigenesis in Japanese men has concluded that p53 mutations occur infrequently in this population[28]; however, it must be pointed out that this study lacked the genetic analyses we employed to detect mutation that immunohistochemistry did not always reveal. Indeed, p53 mutations in human prostate carcinomas may prove to be a more frequent finding than immunohistochemical studies alone would suggest. The specificity of antibodies used will vary, and the monoclonal used in this investigation, Bp53-12, recognizes different amino acid sequences than do those used in other reports.[29] Some mutations in the gene, such as those resulting in stop codons or in gene deletions, will not lead to abnormal protein accumulation and thus may not be detected by immunohistochemical methods alone.[30] In the present study, 2 clinical adenocarcinomas of the prostate were found to have p53 mutations by SSCP and direct gene sequencing but were immunohistochemically negative.

The peculiarities of the p53 gene itself are noteworthy. There appears to be a naturally occurring polymorphism in codon 72 of exon 4, which is the first coding exon. The polymorphic form is considered to exert a "dominant suppressor" effect, since cells with one mutated allele and one wild type express a transformed phenotype.[1,12] By using PCR-RFLP, 6 of 13 clinical carcinomas probed showed LOH at codon 72 and half of these were also positive for immunoreactivity to p53. Two (2) of these homozygous and immunoreactive tumors were additionally found to have point mutations, each in a different exon, detected by SSCP and defined by gene sequencing. This indicates that some subset of clinical prostate cancers may have the ability to progress with only 1 defective allele. However, 100% of the latent carcinomas were heterozygous, but were routinely negative for p53 by immunohistochemistry, which may have been due to the long formalin fixation. In addition, no mutations were detected in the samples of latent cancer by PCR-SSCP.

The latent form of prostatic carcinomas has been thought to be the precursor to aggressive clinical disease. However, the recent increases of latent and clinical cancer, which still remain low in incidence in comparison to that in Western nations, do not parallel each other.[31] Epidemiological studies clearly imply an important role for environmental factors in prostate tumorigenesis.[31,32] The very few molecular studies performed on latent neoplasias of the prostate have tended to concentrate on detection of the products of specific oncogenes.[33,34] There has been no previous report of examination of latent prostatic carcinoma for p53 mutations by PCR-SSCP analysis combined with immunohistochemistry. The current study suggests that p53 plays an insignificant role in latent disease and implies that other factors may be required for malignant progression.

The data generated by this study indicate an association between abnormal p53 and clinically presenting prostate carcinoma, in that mutations in the gene appear to occur as later events in tumor progression; mutations of some kind were detected in 25% (1/4) of well-differentiated tumors, in 35% (6/17) of moderately differentiated tumors and in 27% (3/11) of poorly differentiated tumors. In contrast, p53 appears to play no detectable role in latent cancers of the gland. While the data presented suggest an association between p53 mutation(s) and progression of clinical disease, there appears to be no significant positive correlation with the degree of tumor differentiation or with the Gleason tumor grading or staging criteria.

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