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Heterogeneity in Intratumor Distribution of p53 Mutations in Human Prostate Cancer

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Prostatic carcinomas from 65 patients have been examined for the occurrence of point mutations in the p53 tumor suppressor gene locus within the region of exons 5 to 8. Overall, only a small fraction of tumors (12.3%) was found to contain p53 mutations. No significant correlation was detected between the presence of the mutant gene and either tumor volume or histopathological grade. However, metastatic prostatic tumors are found to display a higher percentage (21.4%) of p53 mutations compared with primary adenocarcinomas (9.8%). Analysis of the topographical distribution of the p53 mutant genotype revealed two remarkable findings. First, multifocal tumors within a prostate appear to differ in harboring the mutant gene, and, second, evidence is obtained for intratumor heterogeneity in the distribution of the mutant p53 allele. Together these findings appear to explain, at least in part, why there has been a wide discrepancy in the reported detection frequency of p53 mutations in prostate cancer specimens. It appears that the outcome of mutation analysis would depend not only on which tumors but also which regions of the tumors are included in the study. Furthermore, the observed heterogeneous topographical distribution of the mutation, if confirmed to be unique to prostate cancer, may have important implications in the understanding of the biology of prostate carcinogenesis. (Am J Pathol 1995, 147:92–101)

Histopathological analyses of whole-mount sections of cancerous human prostate glands by three-dimensional reconstruction have revealed that, in addition to extensive heterogeneity of cancer cell phenotypes admixed with noncancerous cells, a majority of these glands contain multifocal carcinomas.[1–3] In general, there is a paucity of information on specific genetic mutations that contribute to the genesis and progression of this complex cancer. However, various laboratories have examined the question of the frequency and nature of mutations at the p53 tumor suppressor gene locus.[4] The initial observation that allelic loss of chromosome 17p occurs in approximately one-fifth of primary prostate cancers[5] led to the subsequent examination of the p53 gene itself (which is located in this chromosomal region) in prostatic cancer cell lines and primary tumors.[6] Unlike colorectal, lung, breast, and other cancer types in which the p53 gene is known to be mutated in a majority of the tumors,[4,7,8] findings of p53 abnormalities in prostate tumors on the basis of either immunohistochemical staining or molecular genetic analysis or both reflect incidences ranging from as low as 4% to as high as 42%.[9–16] However, to date, none of the major studies have dealt with the questions of occurrence of heterogeneity in topographical distribution of p53 mutations in prostate cancer or whether the multiple foci of tumors within an individual prostate could vary in harboring p53 aberrations.

To address these important questions, we initially identified p53 mutations in histopathologically well characterized prostate adenocarcinomas and systematically examined selected prostate specimens by mapping the intratumor distribution of malignant cells with mutant p53 genotype within and among malignant foci. Here we present evidence that the topo-

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graphical distribution of p53 mutation in a prostate tumor can be strikingly heterogeneous and that multifocal tumors of the same prostate can vary in terms of detectable p53 mutations.

Materials and Methods

Tissue Specimens

A total of 65 human prostatic carcinomas procured from two different sources were examined. Tissues from 51 patients, obtained from the Department of Pathology, University of Colorado, included multiple microscopic sections of pathologically well characterized formalin-fixed, paraffin-embedded tissues from 29 cases of radical prostatectomies, 12 lymph node, and 1 bone marrow metastases, and 9 cases of freshly frozen surgical specimens.

Radical prostatectomy specimens from patients with clinically localized disease (stages T2a to T2c) were processed by the whole-mount technique.[17] Briefly, after fixation in 10% neutral buffered formalin, the glands were serially sliced in the transverse plane at 4-mm intervals. Each slide was embedded in paraffin and used to prepare 5-μm histological sections that were stained with hematoxylin and eosin (H&E). The H&E-stained slides were examined at 25× to 100× magnification, and the boundaries of carcinomas were dotted on the coverslips with a fine tip permanent pen. The information from the slides was transferred to acetate maps that were used for planimetric estimation of tumor volume and capsule penetration as previously described.[18] Carcinomas separated from each other by more than 4 mm in any direction were assumed to represent independent events. Histological grades for each carcinoma were assigned by the Gleason system.[19] The pathological stage was assigned on the basis of the level of capsule penetration.[20]

For the 29 prostates studied, all tumors in each gland were examined for the presence of p53 mutations. Portions of tissue containing individual carcinomas and adjacent benign tissue (usually benign hyperplasia) were removed from the whole-mount slices and re-embedded. Two 25-μm sections with flanking 5-μm sections were cut. The 5-μm sections were stained with H&E and were used to direct further dissection of the 25-μm sections into benign and malignant components. Malignant areas were selected to contain approximately 90% malignant cells. Benign areas were selected to be completely free of malignant cells.

Frozen specimens of primary tumors, a total of 14, obtained from the Norris Cancer Hospital, University of Southern California, were determined to contain malignant cells varying from 10 to 20% (3 cases) to 40 to 90% (11 cases). For 1 of these 14 cases, a lymph node metastasis was also available for analysis. This lymph node section was determined to contain 90% cancer cells.

DNA Amplification by Polymerase Chain Reaction (PCR)

DNA from frozen tissue sections was isolated following standard extraction procedure. Collected materials were deparaffinized and DNA extracted.[21] Phenotypically normal regions or areas of benign hyperplasia were also collected separately for DNA isolation. Exons 5 to 8 of the p53 gene were individually amplified from the isolated DNAs by PCR. Primers used for exon 5 amplification were 5′-GTCTCCTTCCTCTTCCTACAG-3′ and 5′-TCTCCAGCCCCAGCTGCTCA-3′ (encompassing nucleotide positions 13034 to 13259), and primers for exons 6 to 8 were as previously described[22]: exon 6, 5′-GGCCTCTGATTCCTCACTGATT-3′ and 5′-AGAGACCCCAGTTGCAAACC-3′; exon 7,5′-CTTGCCACAGGTCTCCCCAA-3′ and 5′-AGGGGTCAGCGGCAAGCAGA-3′; and exon 8, 5′-TGCTTCTCTCTTTTCCTATCCTGA-3′ and 5′-CGCTTCTTGTCCTGCTTGCT-3′. PCR amplifications were conducted in 50-μl volumes containing 1 μl of template DNA, 5 pmol of each primer, 60 μmol/L of each dNTP, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 20 mmol/L Tris-HCl, pH 8.4, and 1 U of Taq polymerase. After addition of 1 μCi of [^{33}P] dATP (3000 Ci/mmol), the mixtures were denatured at 94°C for 5 minutes, run through 42 cycles each consisting of 1 minute of denaturation at 95°C, 45 seconds of annealing at 55°C, and 45 seconds of chain elongation at 72°C, followed by a final extension at 72°C for 5 minutes. Reactions without added template were run in parallel to detect PCR contaminations.

Single-Stranded Conformational Polymorphism (SSCP) Analysis

PCR products were analyzed for p53 mutations generally following the SSCP procedure as described.[23] Aliquots (3 μl) of PCR products were mixed with mutation detection enhancement (MDE) denaturation buffer (J.T. Baker, Phillipsburg, NJ) in a 1:2 ratio, heated at 98°C for 5 minutes, and then cooled in ice water. Samples (5 μl) were loaded onto a 0.4-mm-thick MDE nondenaturing gel and electrophoresed at 8 watts for 18 hours at room temperature. Gels were

dried at 80°C and autoradiographed overnight at room temperature with Fuji x-ray films.

Direct DNA Sequencing for Identification of Mutations

Band shifts detected by SSCP were analyzed for sequence mutations. Both total DNA and DNA eluted from cut out abnormal SSCP bands were used for sequencing analysis. Aliquots (1 μl) of PCR products were reamplified by an appropriate set of nested primers to produce approximately 125-bp products. The following primers were used for nested PCR amplifications: exon 5 (first part), 5′-GTCTCCTTCCTCCTTCCTACAG-3′ and 5′-TGTGACTGCTTGTAGATGGC-3′; exon 5 (second part), 5′-AGCTGTGGGTTGATTCCACA-3′ and 5′-TCTCCAGCCCCAGCTGCTCA-3′; exon 6, 5′-TCCTCACTGATTGCTCTTAG-3′ and 5′-ACCCC-AGTTGCAAACCAGA-3′; and exon 8, 5′-CTCTTT-TCCTATCCTGAGTAG-3′ and 5′-TTCTTGTCCTG-CTTGCTTAC-3′. The PCR products derived were run in 2% agarose gels, appropriate bands were cut out, and the DNA contents were purified by Qiaex DNA purification kit (Qiagen, Chatsworth, CA). The sequencing reaction was performed according to the manufacturer's protocol (U.S.B. Biochemical, Cleveland, OH) with 7 μl of purified DNA, 1 pmol of sense or antisense primer, and [^{32}P]dATP (ICN, Irvine, CA). Products were separated by 6% denaturing acrylamide gel electrophoresis, the gel was dried, and autoradiographs were produced after an appropriate time of exposure at −70°C.

Selective Ultraviolet Radiation Fractionation (SURF) Analysis

The topographic distribution of p53 mutation within a microscopic tissue section was examined by SURF.[24] Ink dots were placed directly on groups of phenotypically homogeneous cells with a micropipette with the help of a micromanipulator, and the operation was recorded in a video camera. Approximately 100 to 300 cells were protected in a given area, and the unprotected cells were then inactivated with short-wave ultraviolet (254 nm) radiation exposure for 3 to 4 hours. After extraction of SURF DNA from each dotted area, PCR products were made by using appropriate primers and direct sequencing was performed.

Immunohistochemistry

The immunohistochemical detection of mutated p53 was carried out by antigen retrieval and with a cocktail of monoclonal antibodies. Specifically, after deparaffinization, sections were blocked for endogenous peroxidase activity by incubation in 3% hydrogen peroxide for 15 minutes at room temperature. Antigen retrieval was carried out with 0.1 mol/L citrate buffer, pH 6.0, in a microwave oven (650 watts) for two sequential 5-minute intervals at the high setting. After cooling and rinsing, the sections were blocked with 10% normal horse serum. A cocktail of monoclonal antibody clones DO1 (Oncogene Science, Uniondale, NY) and DO7 (Novocastra Laboratories, Newcastle upon Tyne, UK) with each antibody at a dilution of 1:25 was then applied at 4°C, overnight. A Vectastain Elite kit (Vector Laboratories, Burlingame, CA) was used for peroxidase visualization. Mouse ascites fluid from clone NS-1 (Sigma Chemical Co., St. Louis, MO) at a protein concentration equal to the primary cocktail was substituted as a negative control.

Results

Detection of p53 Mutations in Prostate Cancer Specimens

Because of increased metabolic stability of mutated p53 protein relative to wild-type p53, there is generally a good correlation between immunohistochemical detection of p53 protein in the nucleus of various types of tumor cells and mutations in the p53 gene. However, a small but still substantial proportion of tumors that indicated p53 nuclear reactivity have been found by others to lack evidence of genetic mutation.[25] Considering this limitation, we used only SSCP analysis for screening mutations, and, in specific cases when SSCP findings were confirmed by detection of coding sequence mutations, aberrant p53 accumulation was correlated with immunohistochemistry.

Recognizing that most of the point mutations observed in human tumors occur in exons 5 to 8 of the p53 gene,[4] we screened these exons for the presence of mutations. Figure 1 illustrates typical results of SSCP analysis. Band shifts were observed in eight tumors (Table 1). In all cases except one in which direct sequencing was undertaken, SSCP results were confirmed by detection of mutation either leading to amino acid change in conserved codons or, as in one case, frameshifting ending in a premature termination codon. Consistent results were obtained

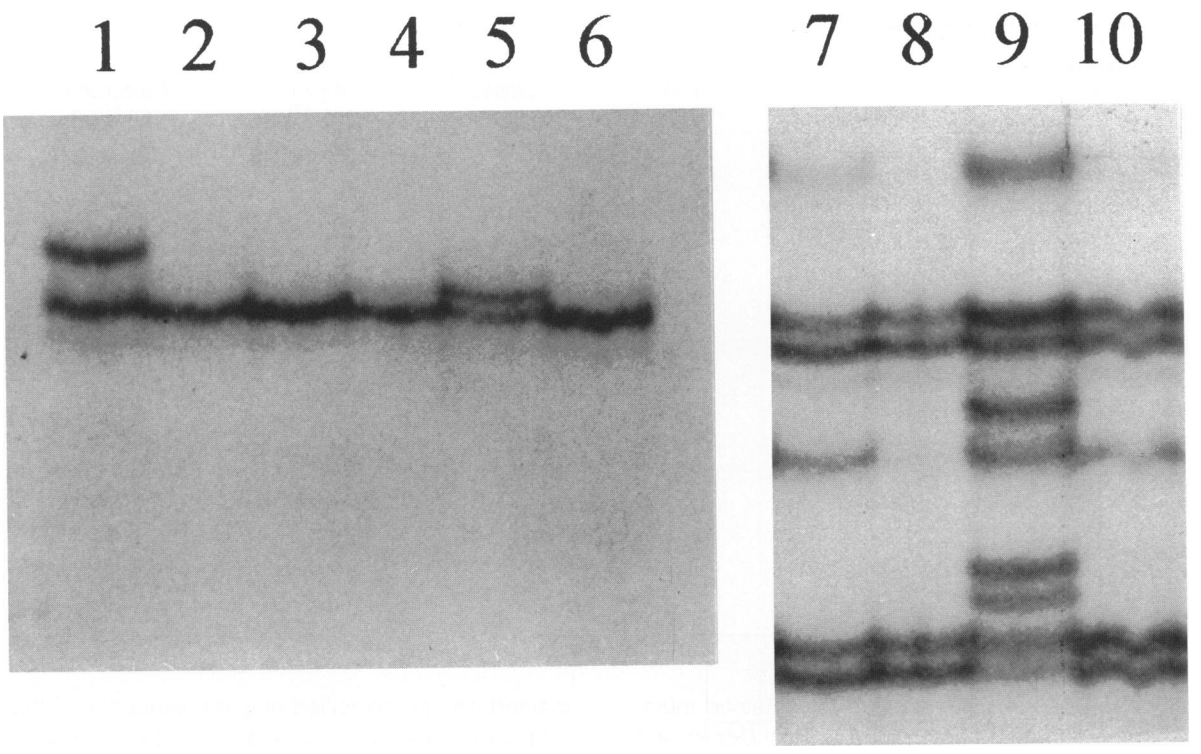

Figure 1. *Detection of p53 intragenic mutations in PCR products of prostate cancer DNA. This figure illustrates the detection of mutations in regions of exon 5* (lanes 1 *to* 6) *and exon 6* (lanes 7 *to* 10) *by SSCP analysis.* Lane 1, *lymph node deposit 91–10-D002;* lane 2, *carcinoma 162-AO1;* lane 3, *carcinoma 162-CO1;* lane 4, *carcinoma 125-DO1;* lane 5, *carcinoma 125-DO3;* lane 6, *BPH 125-DO2;* lane 7, *carcinoma 047-BO1;* lane 8, *carcinoma 059-DO1;* lane 9, *carcinoma 080-CO1; and* lane 10, *carcinoma 080-DO1. Band shifts were observed in* lanes 1, 5, *and* 9.

Table 1. *p53 Mutations Detected in Prostate Cancers*

Specimen/ block number	Tumor description	SSCP abnormality	Mutated codon	Nucleotide sequence change	Amino acid change
080-CO1	Primary, Gleason sum 8, 9.05 cm^3	Exon 6	191	CCT → CGT	Pro → Arg
125-DO3	Primary, Gleason sum 6, 0.66 cm^3	Exon 5	133	ATG → AAG	Met → Lys
209-GO1	Primary, Gleason sum 7, 0.61 cm^3	Exon 5	141	TGC → AGC	Cys → Ser
251-EO2	Primary, Gleason sum 8, 0.50 cm^3	Exon 8	ND		
332-EO2	Primary, Gleason sum 8, 1.00 cm^3	Exon 8	303	AGC → TGC	Ser → Cys
91-10-D002	Primary, needle biopsy	Exon 5	176	TGC → AGC	Cys → Ser
91-10-D002 (LN)	Lymph node metastasis	Exon 5	176	TGC → AGC	Cys → Ser
S-92-13773(1)	Bone metastasis	Exon 5	138	1 base deletion	Frameshift (stop at codon 169)
S-115-91FS1	Lymph node metastasis	Exon 8	274	GTT → TTT	Val → Phe

ND, not determined.

from sequence analysis with either DNA extracted from cut out abnormal SSCP bands or DNA representing total genomic DNA of the corresponding tumors (Table 1). In most cases for which simultaneous analysis of adjacent benign prostate hyperplasia (BPH) regions was performed, no mutations were present, indicating the absence of germline mutation (Table 2).

A total of 65 primary carcinomas were examined for mutations. Of these, only 5 were found to be positive. No mutations were found among the 24 tumors with Gleason sums of 2, 3, 4, or 5. Mutations were present in 1 of 14 Gleason sum 6, 1 of 3 Gleason sum 7, and 3 of 20 Gleason sum 8 carcinomas. The 4 carcinomas with Gleason sums of 9 or 10 were negative for mutations. Mutations were found across a wide range of

Table 2. *Tumors Exhibiting Heterogeneity in the Distribution of p53 Mutation*

Specimen/ block number	Tissue	Tumor number	Gleason sum	Tumor volume (cm^3)	Detection of mutation
080-CO1	Carcinoma	1	8	9.05	+
-DO1	Carcinoma	1	8	9.05	–
125-DO1	Carcinoma	2	8	0.95	–
-DO2	BPH				–
-DO3	Carcinoma	1	6	0.66	+
-DO4	BPH				–
209-DO1	Carcinoma	1	2	0.02	–
-DO2	BPH				–
-GO1	Carcinoma	2	7	0.61	+
-GO2	BPH				–
251-EO1	BPH				–
-EO2	Carcinoma	2	8	0.50	+
-EO3	BPH				–
-EO4	Carcinoma	1	4	0.63	–
-GO1	Carcinoma	1	4	0.63	–
-GO2	BPH				–
-GO3	Carcinoma	2	8	0.50	–
-GO4	BPH				–
332-EO1	BPH				–
-EO2	Carcinoma	1	8	1.00	+
-FO1	BPH				–
-FO2	Carcinoma	1	8	1.00	–

tumor volumes; 4 carcinomas that contained mutations were less than 1 cm^3 (range, 0.3 to 1.0 cm^3), and the 5th had a volume of 9.05 cm^3.

Heterogeneous Distribution of the Mutations in Tumors of the Same Prostate

Possible heterogeneity in the topographical distribution of a mutant p53 genotype was indicated by the detection of SSCP band shifts in tissue sections from one part of a tumor but not from other parts as well as by the variation in the detection of abnormal SSCP bands in the multifocal tumors of the same prostate (Table 2). To study this interesting property further, we selected prostate number 125 for detailed analysis. This prostate contained two major tumors (1 and 2) for which a topographical map of the distribution of the tumors within the prostate was available (Figure 2). Various areas representing specific tumors, BPH nodules, atrophy, etc were microdissected from serial slices of this prostate and analyzed for the presence or absence of the p53 mutant gene already identified to harbor a missense (ATG → AAG, Met → Lys) mutation at codon 133 in one region of tumor 1 (Table 1). Results of this analysis are also described in Figure 2 in which microdissected areas containing the mutant alleles are indicated by (+) and those lacking the mutation by (–). The same mutation at codon 133 was detected in only three areas, all of which represented portions of tumor 1. However, not all areas of tumor 1 contained the mutation. For example, multiple regions marked with (–) exhibited only the sequence of the wild-type p53 allele (ie, ATG at codon 133). Similarly, none of the areas from tumor 2 examined nor any of the noncancerous regions examined showed the presence of the mutation.

In one of the slices (section D) from case 125, tumor 2 was subjected to finer analysis for determining the presence or absence of the mutant genotype. Multiple paraffin sections of this region of tumor 2 were analyzed by SURF to intensely search for the mutation in various fractions of the tumor cells within this segment. The findings were, however, negative as illustrated in Figure 3. Nine different carcinoma areas, of which only four are shown in Figure 3, were examined, but none indicated the presence of a p53 mutation at codon 133.

A similar SURF survey, however, readily revealed the presence of the mutant genotype in paraffin sections of the same region (section D) within tumor 1. This aspect is illustrated in Figure 4. Six areas, of which five are depicted in this figure, were examined by SURF for the mutation. Except for one area (middle dotted area) that was marked for BPH, all other areas were dotted to represent homogeneous groups of cancer cells. All areas identified within this microscopic section that contain malignant cells were indeed found to be positive for the T → A sequence shift at codon 133. As shown in Figure 4, two right side dots as well as two left side dots (one of which is partly shown, but results of the analysis are not included in the figure) demonstrated the detection of both mutant

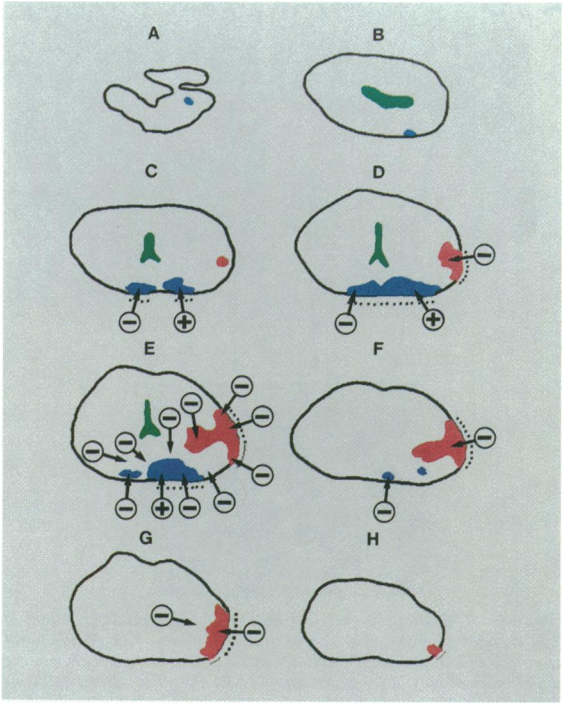

Figure 2. *Digitized maps of serial, sagittal, whole-mount sections* A *(apex) through* H *(base) of prostate 125. The solid black line represents the prostate border. The prostatic urethra is green, tumor 1 is blue, and tumor 2 is red. The arrows indicate regions that were sampled for mutation analysis with + designating those areas in which the codon 133 (Met → Lys) mutation of the p53 gene was detected and − designating the absence of mutation. Coarse dots outside the prostate indicate areas of capsule invasion; fine dots represent capsule penetration.*

and wild-type alleles. The BPH area displayed only the wild-type sequence pattern whereas, interestingly, a second spot from the left of this figure appeared to indicate the presence of only mutant but not wild-type allele. This could potentially be a result of loss of heterozygosity in a highly localized region or, alternately, an artifact of preferential PCR amplification of one allele relative to the other as a result of some unknown factors. This was, however, an isolated case, and no other attempts were made to elucidate this issue in the present work.

Immunohistochemical localization of p53 revealed the presence of abnormal accumulation of the antigen in the nuclei of a small population of malignant cells within tumor 1 (Figure 5). However, stains of tumor 2 failed to reveal the antigen in any malignant cells. Interestingly, tumor 1 is a relatively low grade (Gleason pattern 3) lesion whereas tumor 2 is a high grade (Gleason pattern 5) lesion.

Another set of tumors for which we had a lymph node metastatic deposit (91–10-D002) and fine needle biopsies of the corresponding primary cancer, was investigated for the distribution of the p53 mutant genotype. There were two needle biopsies, one collected from the left side and the other from the right side of the primary tumor. Although the right side biopsy was totally used for mutation comparison with the lymph node deposit (Figure 6C), the left side biopsy was available for SURF analysis. Clearly, the SURF technique can be applied to needle biopsies to examine clusters of cells (Figure 6, A and B). In this particular case, it appeared that all regions examined, including a lymph node deposit, the right needle biopsy, and six different regions of the left needle biopsy, contained both wild-type and codon 176 (TAC → AGC, Cys → Ser) mutant alleles. Although the survey was limited, mutation distribution in this tumor could potentially be wider than that reflected in case 125.

Discussion

The significance of this work is twofold. First, our analysis of microscopic regions of carcinoma of the prostate reveals the occurrence of heterogeneity in the distribution of mutant p53 alleles in multifocal tumors. Several cancerous prostates that had at least two physically distinct tumors, differing in grade and size, contained a mutant p53 allele (eg, 125, 209, and 251) in one but not in the other tumor(s) (Table 2). Detailed analysis of this tissue in prostate 125 clearly justifies this perception. Tumor 1 of case 125 displays a mutation in p53 Met → Lys at codon 133. However, multiple tissue sections or microscopic regions of its second tumor, when analyzed for the same mutation, failed to reveal its presence or the presence of any other mutations in the p53 gene, at least in the region of exons 5 to 8 investigated in this study. Perhaps a more remarkable observation is that, even within tumor 1, the occurrence of the mutant allele is not topographically uniform. It appears that, although focal regions of the tumor exhibit the mutation, the majority does not. The finding of intratumor mutation heterogeneity in tumor 1 of case 125 is also supported by the observations made in other cases, eg, 080, 251, and 332. Although these other tumors were not examined as systematically as case 125, two different carcinoma regions in each case varied in retention of the mutant genotype (Table 2). These findings lend support to an earlier isolated report that described two distinct regions of a prostatic primary carcinoma, one bearing mutant p53 and the other only the wild-type allele.[26] Similarly, heterogeneity within prostatic adenocarcinoma has been inferred from other types of analyses, as for example, DNA flow cytometric

mapping[27] and detection of allelic losses.[28] Although the issue of distribution of specific point mutations within various human primary cancers has not been widely investigated, at least in adenocarcinoma of the lung and sporadic gastric cancer, it seems that detection of p53 mutations in malignant cells is homogeneous throughout the majority of these tumors.[29,30] In this respect, our observation of such heterogeneity in prostate cancers is unique.

Second, our observations may have profound implications in the interpretation of the p53 gene alterations in the progression of prostate cancer. Although most of the published work on this matter appears to be based on generally well founded experiments, there is a striking disparity in the frequency of detection of p53 mutant alleles in various prostate cancer specimens.[9–16] In view of the heterogeneity described above, it is logical to think that the outcome of incidence would depend on not only which tumors but also which regions of the tumors are being analyzed. Analysis of single thin sections of carcinomas would be expected to yield a low incidence rate when compared with the use of larger tumor masses. This may indeed be an explanation for the apparent discrepancy in the published literature. For example, a well conducted study[13] of 3-μm sections of archival tumor materials revealed only 12.7% overall incidence of p53 mutation in prostate cancer whereas analysis of much larger portions of frozen tumors, especially when RNA was extracted for RNA/PCR work,[15] showed as high as 42% frequency of p53 aberrations. In the present study, we find a mutation frequency that is also low (12.3%), a rate comparable with similar analyses with thin tissue sections.[13] A limited correlation between p53 mutant genotype and histological grade was found. Only tumors with Gleason sums of 6 or more contained mutations. However, 32 of 37 carcinomas with Gleason sums of 6, 7, or 8 as well as 4 of 4 carcinomas with Gleason sums of 9 or 10 contained only wild-type sequences. Therefore, although the mutations that were found occurred in

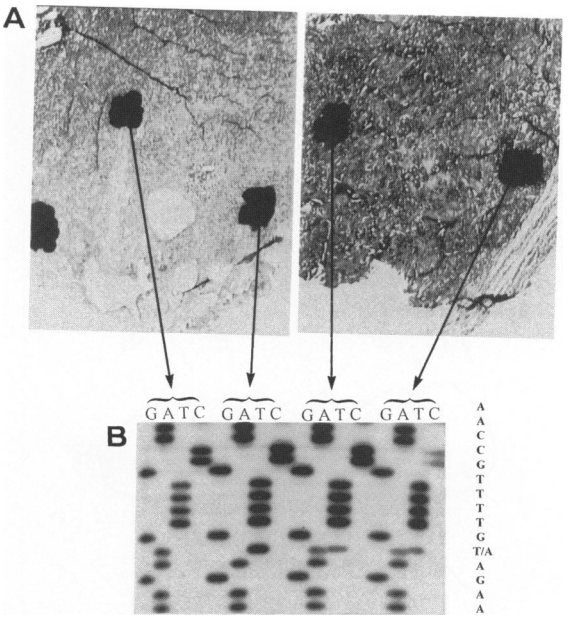

Figure 4. *SURF analysis for distribution of p53 mutation within a thin tissue section (segment D) of tumor 1 of case 125.* **A:** *Photomicrograph of a microscopic section (125-DO3) is shown with specific areas marked by ink for SURF analysis.* **B:** *Results of direct DNA sequencing with lines indicating the corresponding sources of SURF DNA.*

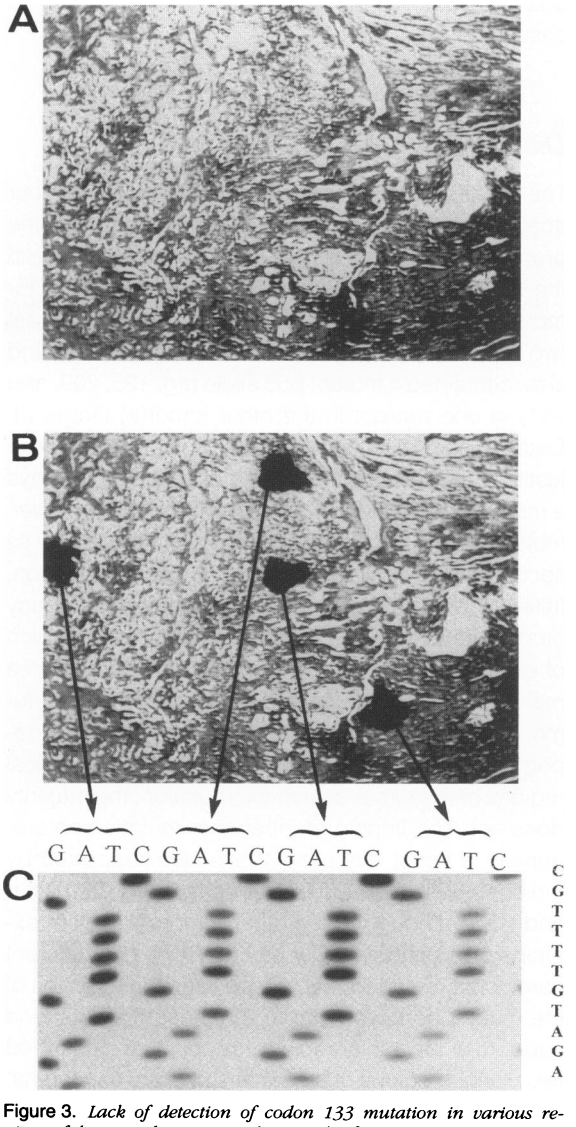

Figure 3. *Lack of detection of codon 133 mutation in various regions of the second carcinoma (tumor 2) of case 125. A tissue section (125-DO1) of tumor 2 (segment D) is shown before* (A) *and after* (B) *marking various cancer cell cluster areas by ink dots for SURF analysis.* **C:** *Autoradiograph showing results of direct sequencing from the indicated areas. No mutation was detected in these regions or other regions examined (not shown).*

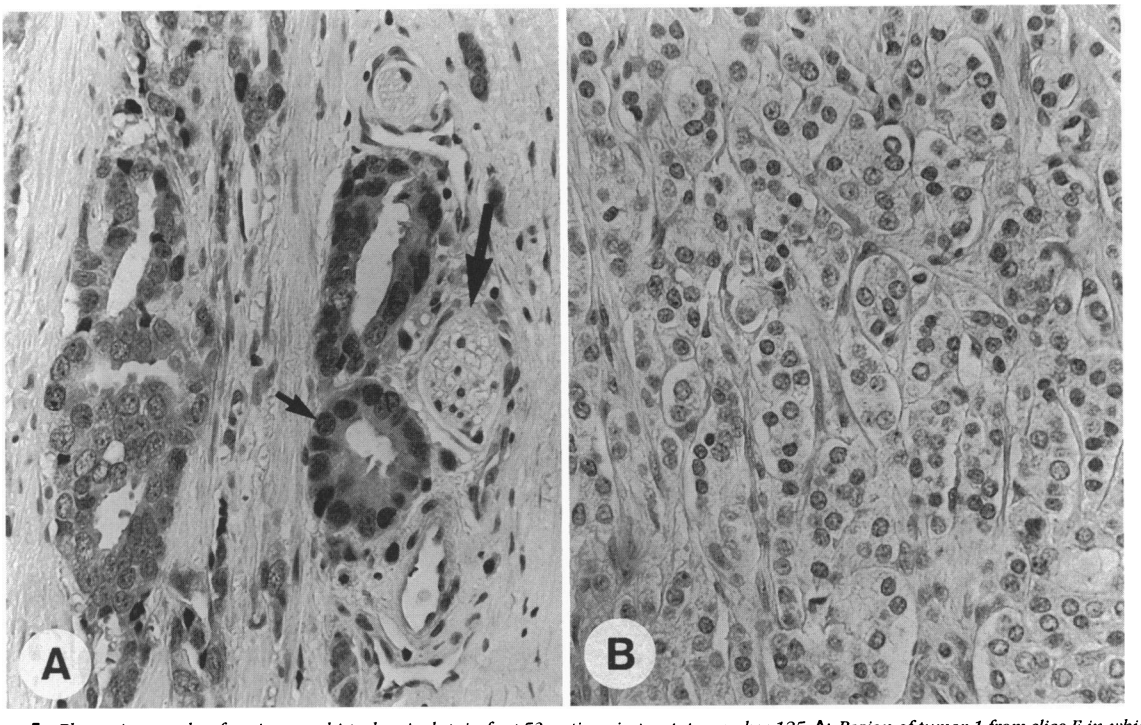

Figure 5. *Photomicrographs of an immunohistochemical stain for p53 antigen in prostate number 125.* **A:** *Region of tumor 1 from slice E in which SSCP analysis revealed a codon 133 (Met → Lys) mutation of the p53 allele (×250). Small arrow indicates positively stained malignant nuclei. Large arrow indicates a small nerve bundle. Note the heterogeneity of staining between the malignant glands on the left compared with the area of perineural invasion by malignant glands on the right.* **B:** *Region of tumor 2 from slice E (×250). Note the absence of immunostaining within the nuclei of this poorly differentiated area of malignant cells.*

intermediate to high grade carcinomas, p53 mutations are apparently not necessary for carcinomas to become high grade. No distinct relationship was noted between the presence of mutations and tumor volume. Although mutation was detected in 1 large (9.05 cm^3) carcinoma, 13 other carcinomas with similar or greater volumes contained only wild-type sequences. Mutations were present in 4 of 27 carcinomas with tumor volumes less than 1 cm^3. Thus, although p53 mutations can occur during the early growth of primary prostatic carcinomas, they are not uniformly found at this stage. Of more interest is the observation that p53 mutations are not a frequent event in large volume carcinomas, suggesting that such changes do not necessarily accompany tumor progression in this disease. In agreement with the results of Bookstein et al,[13] a higher incidence of the occurrence of mutation was found in advanced stage cancer as 3 of 14 metastases (21.4%) carried a p53 mutant allele. However, in view of the heterogeneity in topographical distribution of the mutant allele that we have observed, it is realized that a meaningful association could not be derived from analysis of only limited sections of individual tumors. Thus, unless each prostate cancer is analyzed in a systematic and complete manner, both frequency of occurrence and clinicopathological correlates of p53 mutation in human prostatic carcinoma will remain difficult to evaluate.

Heterogeneous distribution of p53 gene mutation raises still other questions. Do multifocal tumors have diverse origins? Why does one part of a tumor differ from another part in the p53 genotype? Secondary carcinomas within a given prostate may be caused by field effects in which other sets of mutations contribute to independent proliferative advantage. In regard to this issue, we carried out an extensive analysis of all of the prostate cancer specimens of this study for the presence of any mutations in two other genes, namely, WAF1 (or p21)[31,32] and MTS1 (or p16),[33,34] which function, like p53, in cell cycle regulation.[31,34,35] However, none of the samples indicated the occurrence of somatic mutations in the coding sequences of these genes. Although other genetic changes, yet to be defined, may be involved in potential independent origin of secondary carcinomas, it is more difficult to explain the observed intratumor heterogeneity of p53 mutations in prostate cancer. One speculation is that mutant p53 alleles originate in focal areas with the progression of the tumor. Alternatively, the heterogeneity may be a result of loss or reversion of the mutant p53 allele during

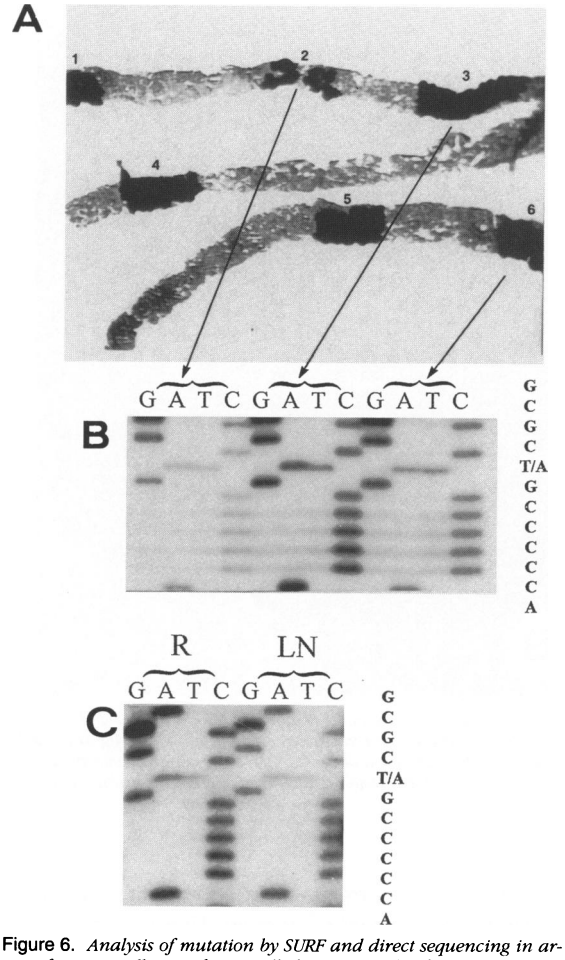

Figure 6. *Analysis of mutation by SURF and direct sequencing in areas of cancer cells in a fine needle biopsy sample of a primary prostate tumor.* **A:** *Photomicrograph of the needle biopsy from the left side of the primary tumor (91–10-D002) with black areas indicating the positions of ink marks.* **B:** *Autoradiograph exhibiting the sequence data from dotted areas 2, 3, and 6. The expected point mutation at codon 176 (TGC → AGC) is seen along with the wild-type allele sequence. Areas 1, 4, and 5 (not shown here for sequence) also contained both normal and mutant alleles.* **C:** *Direct DNA sequencing results of PCR products from total DNA from a fine needle biopsy from the right side of the same tumor (designated as R) and lymph node metastatic deposits (LN) in the same patient. Again, retention of both wild-type and mutant alleles is seen in each case.*

tumor growth. At this time, however, there are no examples in the literature to indicate that tumor cells bearing a mutant p53 allele can naturally regain wild-type p53 genotype.

In conclusion, our results provide direct evidence that prostate cancer has heterogeneity in the topographical distribution of the p53 mutant allele in malignant cells. Intratumor heterogeneity is further complicated by the occurrence of multifocal tumors that may differ in the detection of the mutant genotype. Because of this complexity, it is difficult to determine the broad implications of p53 mutation in prostate tumorigenesis with the existing data on mutation frequency.

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