p53 Alteration and Chromosomal Instability in Prostatic High-Grade Intraepithelial Neoplasia and Concurrent Carcinoma: Analysis by Immunohistochemistry, Interphase *In Situ* Hybridization, and Sequencing of Laser-Captured Microdissected Specimens

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p53 mutation has been shown to be associated with chromosomal instability (CI) in many human dysplastic and neoplastic lesions. However, the precise role of p53 in the pathogenesis of prostate carcinoma (Pca) is unknown. Topographic analysis of p53 alteration using immunohistochemistry (IHC) was performed on 35 archived prostatectomy specimens containing Pca foci; high-grade prostrate intraepithelial neoplasia (HPIN) foci intermingled with cancer (HPINI) and situated away (HPINA). Specimens from 2 patients were topographically genotyped using laser capture microdissection, PCR amplification, and direct sequencing of p53 exons 5–9. CI was evaluated in the same tissue foci by interphase *in situ* hybridization (IFISH) using centromere probes for chromosomes 7, 8, and Y. p53 immunoreactivity was found in 20%, 17%, 0, and 0 in Pca, HPINI, HPINA, and benign epithelium, respectively. p53 molecular analysis in the specimens examined confirmed the IHC findings. IFISH revealed numerical chromosomal alterations in keeping with CI in 71% and 25% of p53+ and p53− Pca, respectively ($P = .1$), 67% and 0 of p53+ and p53− HPIN, respectively ($P < .02$), and in 27% and 0 of HPINI and HPINA, respectively. We concluded that p53 mutation is an early change in at least a subset of Pca. HPINI foci tend to have higher overall p53 immunoreactivity and CI than HPINA. The presence of p53 mutation in HPIN was associated with the presence of CI as determined by IFISH. Our study also provided additional evidence in support of the concept that HPIN might be the earliest precursor of cancer. Furthermore, our studies identify genomic similarities in HPINI and Pca, implying that carcinoma may arise from progression of certain HPIN foci that most likely harbor p53 mutation and/or more CI.

KEY WORDS: Chromosomal instability, Immunohistochemistry, *In situ* hybridization, p53 sequencing, Prostate carcinoma, Prostate intraepithelial neoplasia.

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The reported frequency of mutation of the p53 tumor suppressor gene in Pca has varied widely, ranging from 3–72% in carcinomas of the prostate and 0–68% in HPIN (1–8). In the literature, there is controversy about the question of whether p53 alteration is an early or late genetic change (1, 6, 7, 9–15). Striking heterogeneity of p53 mutation in prostate cancer has been reported (16), and different mutated alleles were found among multiple tumor foci in single glands (16, 17). p53 has been found to be associated with genomic instability leading to chromosomal rearrangement, which in turn has been demonstrated to be a feature of many neoplastic and preneoplastic (dysplastic) human epithelia (18–30). The transition from preinvasive disease to invasive carcinoma was shown to be

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associated with changes in the number of chromosome copy and that coincide with the loss of *TP53* function. Whether there is a role of chromosomal instability (CI) in the progression of HPIN foci to invasive cancer and whether is that influenced by heterogeneity in the p53 expression between different HPIN foci is still unknown.

The objectives of this project are as follows. First, to study the p53 mutation pattern in HPIN foci that are intermingled with cancer and to compare them with different isolated HPIN foci situated in the same gland but away from any cancer foci. Second, to study the relation between p53 mutation and CI in precancerous and malignant prostate epithelium.

MATERIAL AND METHODS

Patients

Tissue samples were obtained from prostate carcinoma resected at Toronto General Hospital and Sunnybrook and Women's College Health Sciences Center, Toronto, Ontario, Canada. A total of 35 cases were selected based on the presence of HPIN foci intermingled with cancer and HPIN foci separated from cancer, with no other cancer foci in the serial blocks superior and inferior to that particular foci.

Immunohistochemistry Staining

Immunohistochemistry (IHC) was performed on archival formalin-fixed, paraffin-embedded sections (5 μm) from prostatectomy specimens containing both prostate carcinoma and HPIN foci. The appropriate control was used. Monoclonal antibody to p53 (DO7 clone; Novocastra Laboratories Ltd., Newcastle, United Kingdom) was applied using avidin-biotin peroxidase complex (Elite kit; Vector Laboratories, Burlingame, CA). The positive control for p53 immunoreactivity consisted of formalin-fixed sections from an adenocarcinoma of breast and bladder transitional cell carcinoma. Negative internal controls were stromal cells. Immunoreactivity (IR) was categorized semiquantitatively from 0 to 4+ (0 = no IR, 1+ = 1–10%, 2+ = 11–40%, 3+ = 41–70%, 4+ = 71–100%). Staining was defined as positive whenever any specific nuclear brown staining was detected. In the event of disagreement in quantification, the sample was re-reviewed by both observers, and a consensus score was achieved.

Interphase FISH Analysis

Interphase FISH has been performed on 5-μm unstained tissue sections of the same blocks used for the p53 study, using adjacent hematoxylin and eosin (H&E) –stained sections as guidance. Directly labeled VYSIS CEP probes for chromosomes 7, 8, X, and Y were used. Paraffin pretreatment and FISH procedure were performed according to manufacturer instructions (Vysis, Inc., Downers Grove, IL). Dual-probe hybridization was performed. For each probe, 100 nuclei were counted by each observer. Chromosome X was used as an internal hybridization control for chromosome Y to determine whether any apparent loss of Y was caused by inadequate hybridization. The chromosome X signals were not enumerated.

Criteria for Scoring and Evaluation of Numerical Chromosomal Anomalies

In preliminary experiments, the hybridization efficiency of every probe has been tested on prostate tissues. Slides were evaluated according to the accepted criteria (31). Briefly, only sections with hybridization in at least 80% of cells were evaluated. For each probe, two independent investigators counted the number of FISH signals in 200 non-overlapped intact (spherical) interphase nuclei from foci of HPIN. The number of signals per nucleus were scored as in terms of signal per nucleus: 0, 1, 2, 3, 4, and more than 4. Nuclei from stromal element were not enumerated. Normal and hyperplastic glandular epithelium present in the biopsies were counted as internal control. Because of truncation of the nuclei, artifact loss of signals was expected; however, we applied very conservative criteria to detect any significant true numeric changes. Our criteria to evaluate numeric chromosomal abnormality were as follows:

1) Chromosomal gains had been diagnosed when more than 8% of the nuclei exhibited more than two signals (or one for chromosome Y).

2) Chromosomal losses had been diagnosed when more than 50% of the nuclei exhibited a reduction of signal number.

3) Tetraploidy had been suspected when the percentage of nuclei with three and four signals (or two for Y chromosome) was similar for both chromosomes 7 and 8. These cutoff values were adopted from the available literature (32–36). In our study, as in others, no BPH specimens or normal prostate epithelium contained values that exceeded these criteria.

p53 Sequencing: Laser Capture Microdissection and Genomic DNA Extraction

Laser capture microdissection (LCM) was performed with a Pixcell II Laser Capture Microscope (Arcturus Engineering, Mountain View, CA) in the Ontario Cancer Institute. Tissue (4–5-μm thickness) were used, and foci of choice were dissected

as described elsewhere (37, 38). DNA was extracted as previously described (39). DNA was analyzed for p53 mutation by the p53 sequencing method. DNA sequences of p53 (Exons 5–9) were amplified by PCR. Sequencing analysis was done using the p53 Mutation Detection GeneKit (Visible Genetics Inc., Toronto, Ontario, Canada). Each exon has been sequenced separately using 3' primer, and for those with any abnormality, the other, 5' direction was done to confirm the findings.

Statistical Analysis

The McNemar test was used to examine the differences between HPINA and HPINI in the same gland regarding p53 positivity and numeric chromosomal changes. The *z* test was used to analyze the difference between p53+ and p53− HPIN regarding CI. The same test was used to examine the difference between p53+ and p53− Pca regarding CI.

RESULTS

We identified 35 prostatectomy specimens that have Pca with intermingled HPIN foci and at the same time have HPIN foci that are completely separated from the cancer foci and admixed with benign epithelium (Fig. 1). We performed p53 analyses using IHC (DO7) on representative sections of these specimens (total: 80 HPIN foci and 44 Pca foci). Table 1 summarizes the overall p53 and chromosomal anomalies in Pca, HPINI, HPINA, and benign prostate epithelium. Seven cases (20%) stained positively for p53 in Pca foci (Fig. 2A–C). Immunoreactivity in those positive cases was categorized semiquantitatively as follows: 3 cases as 1+, 2 cases as 2+, and 2 cases as 3+. There was a remarkable similarity between HPINI and Pca in the p53 immunoreactivity because six of those seven cases (86%) also stained positively in the HPINI (Fig. 2A–D). None of those 7 cases showed immunoreactivity in the HPINA in the same glands (Fig. 2E). None of the p53-negative cancers showed positively in the HPIN foci. The normal, atrophic, and hyperplastic tissue situated in the same sections showed negative staining in all the cases (Fig. 2F). The Gleason grades for p53-positive cases were 7 (5 cases) and 8 (1 case) and 9 (1 case). The Gleason grades for p53-negative cases were 6 (11 cases), 7 (15 cases), 8 (1 case), and 9 (1 case). The volume of the tumor as evaluated by the percentage of the tumor in the gland for p53-positive cases was less than 10% in 4 cases and more than 10% in 3 cases. For p53-positive cases, the percentage of tumor was less than 10% in 17 cases and more than 10% in 11 cases. When this results were compared with pathological findings, there was no statistically significant difference between the p53-positive and p53-negative cases regarding Gleason grade, volume of the tumor, perineural invasion, seminal vesicle involvement, and lymph node metastasis. In five of seven p53+ cases, pathological examination showed extraprostatic extension, and that finding was seen in 10 patients out of 28 of the p53-negative cases ($P = .1$). Focal cytoplasmic staining was seen in four cases (Patients 3, 5, 11 and 20) in the Pca and HPIN foci and was counted as negative. Focal (scattered cells) p53 basal staining was seen in about 30% of the cases in the hyperplastic foci but was found very rarely in HPIN foci. p53 sequencing analysis was performed for Exons 5–9 using a laser-captured microdissected specimens from Pca, HPIN foci, and benign epithelium (Fig. 3) from 2 selected patients (one positive and one negative for p53 by IHC). In the patient with p53 immunoreactivity (Patient 34), sequencing analysis revealed that the tumor foci harbored point mutation TGT at Codon 273 instead of wild-type TGC in the highly conserved transcript region at Exon 8 substituting the encoded amino acid from arginine to cystine (Figure 4). The mutation has been confirmed using primers from both 3' and 5' direction. The other patient (Patient 9) with a negative p53 by IHC showed CI in cancer foci, so p53 sequencing analysis has been performed on normal foci, HPIN, and cancer from that patient to see whether cancer foci harbor termination mutation in p53 that might be missed by IHC. The analysis, however, revealed that all those foci harbor normal Exon 5–9 sequences, indicative of wild-type p53. The analysis of these two cases confirmed the IHC findings. IFISH analysis for chromosomes 7, 8, and Y was performed to assess CI in sections from the same blocks used for IHC analysis. Numeric chromosomal aberrations were found in 27% of HPINI and in 47% of Pca (Figs. 5–6). There were no statistically significant differences in the frequency of chromosomal anomalies between HPINI and Pca, and the overall frequencies of numeric chromosomal anomalies between them were similar. Numeric chromosomal aberrations were found in 5/7 and 4/6 of the p53+ Pca and p53+ HPIN, respectively. On the other hand, numeric chromosomal abnormality has been seen only in 2/8 of p53− Pca and in none of the p53− HPIN, including both HPIN that intermingled with cancer and those situated away. However, this finding was not found to be statistically significant. Gain of chromosome 8 was the most frequent change in both HPIN and Pca, followed by gain of chromosome 7. Chromosome Y aneusomy was seen in 2 cases of Pca (in Patient 6 as Y chromosome gain and in Patient 26 as Y chromosome loss), and in both cases the intermingled HPIN foci showed similar changes. No CI has been detected in the normal, hyperplastic, or atrophic epithelium. It

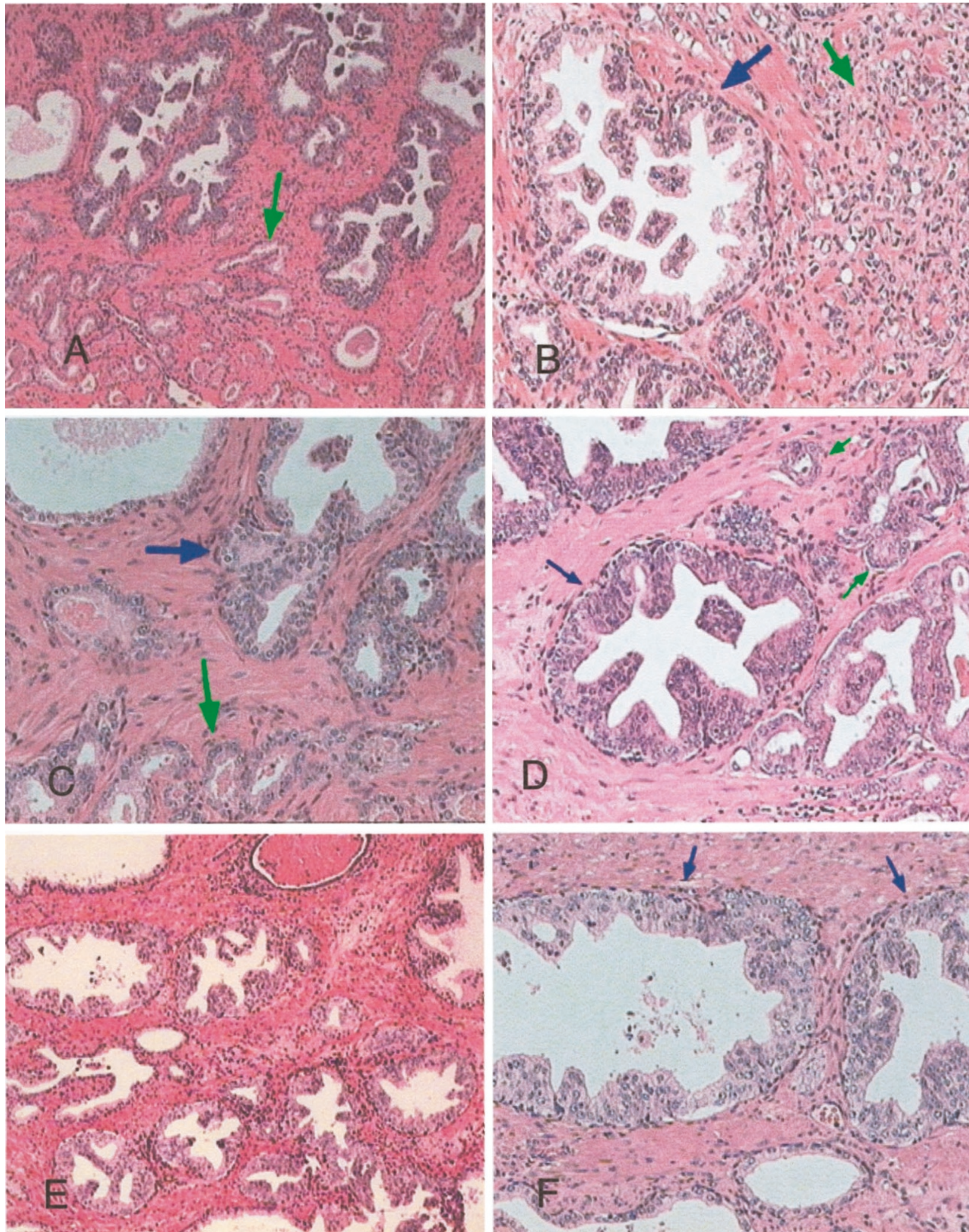

FIGURE 1. **A–D,** H&E sections show foci of HPIN (***blue arrow***) intermingled with invasive cancer (***green arrow***). **E,** H&E section of HPIN surrounded by benign epithelium. **F,** a higher power; HPIN foci (***blue arrow***) and benign epithelium in the lower part of the image.

was interesting to notice occasionally that early stromal invasion, the earliest morphologic indication of carcinomas, occurs at sites of acinar outpouching and basal cell disruption in acini with HPIN.

DISCUSSION

In agreement with results of other studies (6, 7, 40–44) our results showed that p53 mutation occurs relatively infrequently in Pca (20%) compared with the case of other human cancers like colon, esophagus, and lung cancer. However, the presence of HPIN foci with positive staining for p53 indicated that in a subset of Pca, the mutation could occur at an earlier stage of cancer pathogenesis. Our study showed heterogeneity of p53 positivity in the HPIN foci in the same gland, where foci of HPIN intermingled with p53-positive cancer foci tend to have

TABLE 1. Summary of the p53 and Interphase FISH Results on Prostatectomy Specimens

Group	Pca (%)	HPINI (%)	HPINA	Normal
Total	35	35	35	35
P53+ (n:35)	7 (20)	6 (17.1)	0	0
CIN+ (n:15)	7 (47)	4 (27)	0	0
P53+/CIN+	5	4	0	0
P53+/CIN−	2	2	0	0
P53−/CIN+	2	0	0	0
P53−/CIN−	6	9	15	15

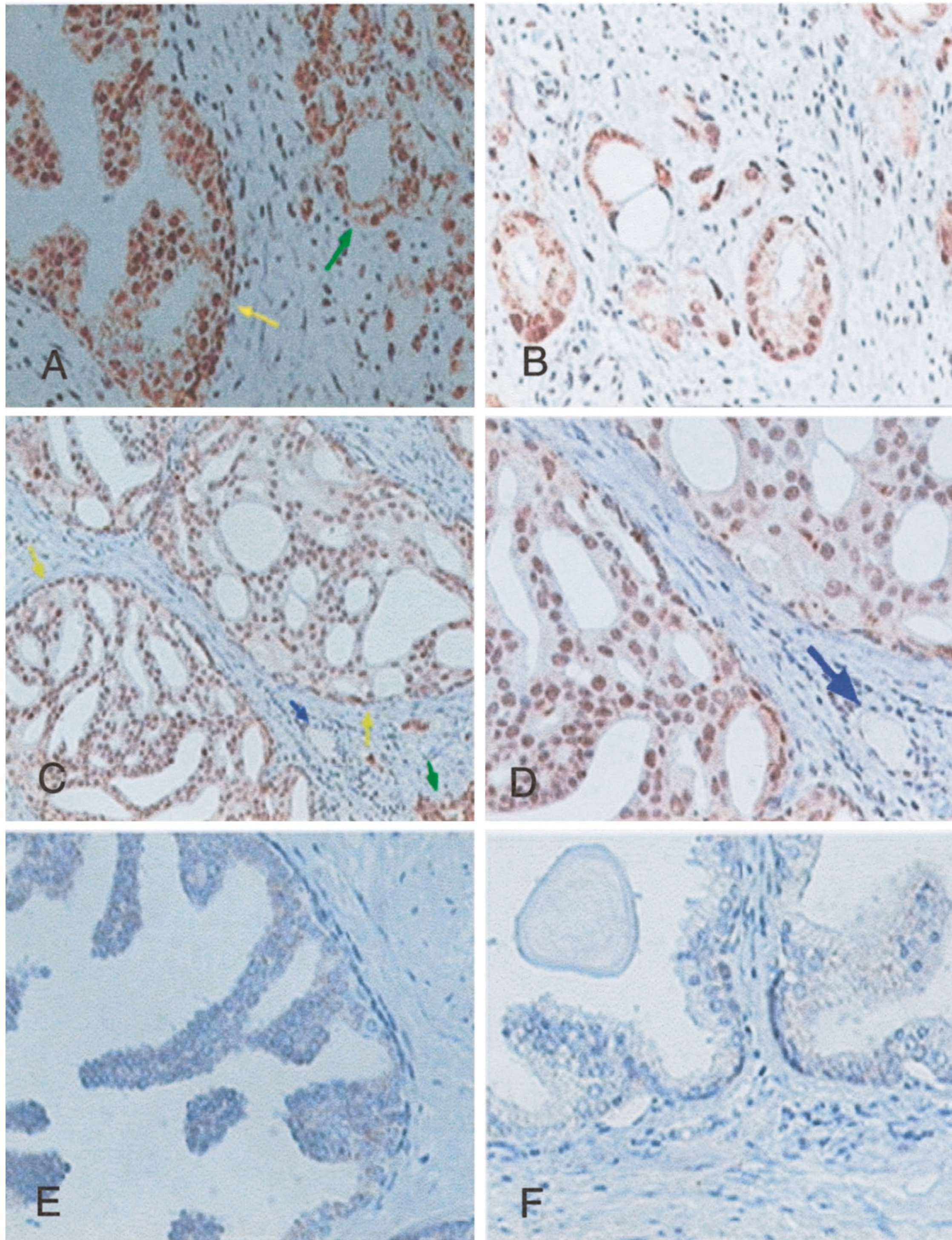

FIGURE 2. p53 IHC (DO7). **A,** positive nuclear staining in invasive cancer (***green arrow***) and in the adjacent HPIN (***yellow arrow***). **B,** positivity in cancer gland. **C,** another case with the same features. ***Blue arrow,*** vessels used as a negative control. **D,** high power. **E,** HPIN away from cancer with negative staining in the same Gland A. **F,** negative staining in a hyperplastic epithelium.

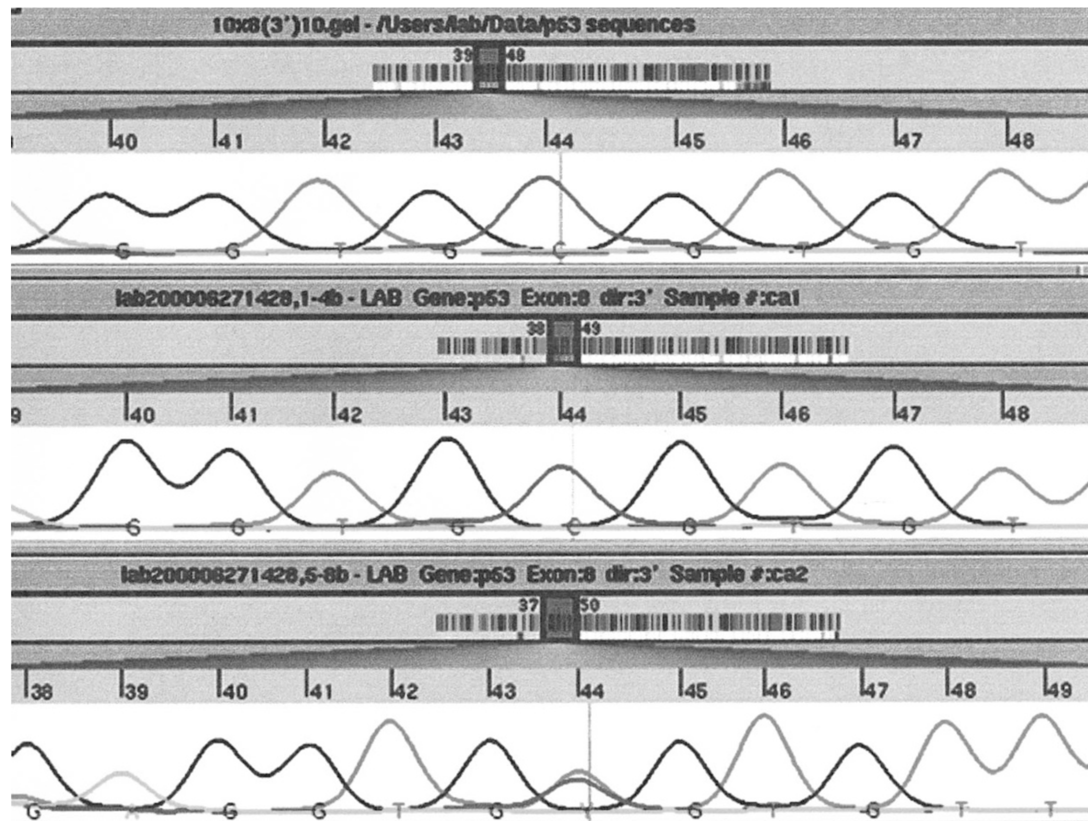

FIGURE 3. p53 sequencing analysis. ***Top picture,*** a normal sequence of Exon 8. ***Middle picture,*** a wild-type p53 (Patient 9). ***Bottom picture,*** mutated p53 with a change in Codon 273, changing the wild-type TGC to TGT and changing the amino acid from arginine to cystine. The corresponding IHC for p53 is on the left side.

higher incidence of p53 alteration than do isolated HPIN situated away from cancer and admixed with benign epithelium. Although 86% of HPINI in p53+ Pca showed p53 positivity, none of the HPINA were positive ($P < .05$). That may explain some of the controversy in the literature regarding the incidence of p53 mutation in HPIN. Our study did not show positive nuclear staining in the adjacent normal, hyperplastic, or atrophic foci, including those tissues adjacent to or intermingled with cancer foci in any of the cases. In addition, CI was not observed in normal, hyperplastic, or atrophic epithelium. Taken together, these findings are not in keeping with the recently proposed idea that atrophy may give rise to carcinoma (45). More than 98% of all p53 mutations are located in Exons 5–9 (46, 47). We have performed p53 sequence analysis for Exons 5–9. Sequencing analysis has been performed using laser-captured microdissected specimens from Pca, HPIN foci, and benign epithelium. It has been done on a subset of cases (samples from 2 patients) to confirm the IHC findings. We have applied a laser capture microdissection technique that enables us to dissect very pure Pca and HPIN foci with no contamination. The discrepancies between IHC and PCR-SSCP that have been reported by some researchers in Pca could be caused by contamination by normal tissue or foci without an apparent mutation because of the heterogeneity of Pca. Still, IHC does not detect all alteration that may affect p53 function, such as loss of heterozygosity at the p53 locus, nonsense or splice site mutations, or amplification of the MDM-2 gene, but all of these are very rare in prostate cancer. Generally, a good correlation between p53 alteration detected by IHC and molecular studies has been noted in prostate cancer (4, 7, 44, 48, 49). Hall *et al.* (44) found complete agreement between IHC and TP53 SSCP analysis. Wertz *et al.* (48) reported 85% overall agreement between the two methods, whereas the concordance was 76.7% by Salem *et al.* (7). In one of our cases, a point mutation has been seen at Codon 273, changing the amino acid from arginine to cystine. p53 mutation at Codon 273 has been described in Pca (10, 50–52). G:C-to-A:T transitions were the most common point mutations (64%) in prostate cancer (10). Six (55%) of 11 G:C-to-A:T transitions occurred at CpG dinucleotides in five hot-spot codons (175, 245, 248, 273, and 282), and it was suggested that specific p53 mutations participate in the progression of human prostate cancer and may be predictive of metastasis (10).

This study, in addition to some other recent studies (both *in vitro* and *in vivo),* has demonstrated

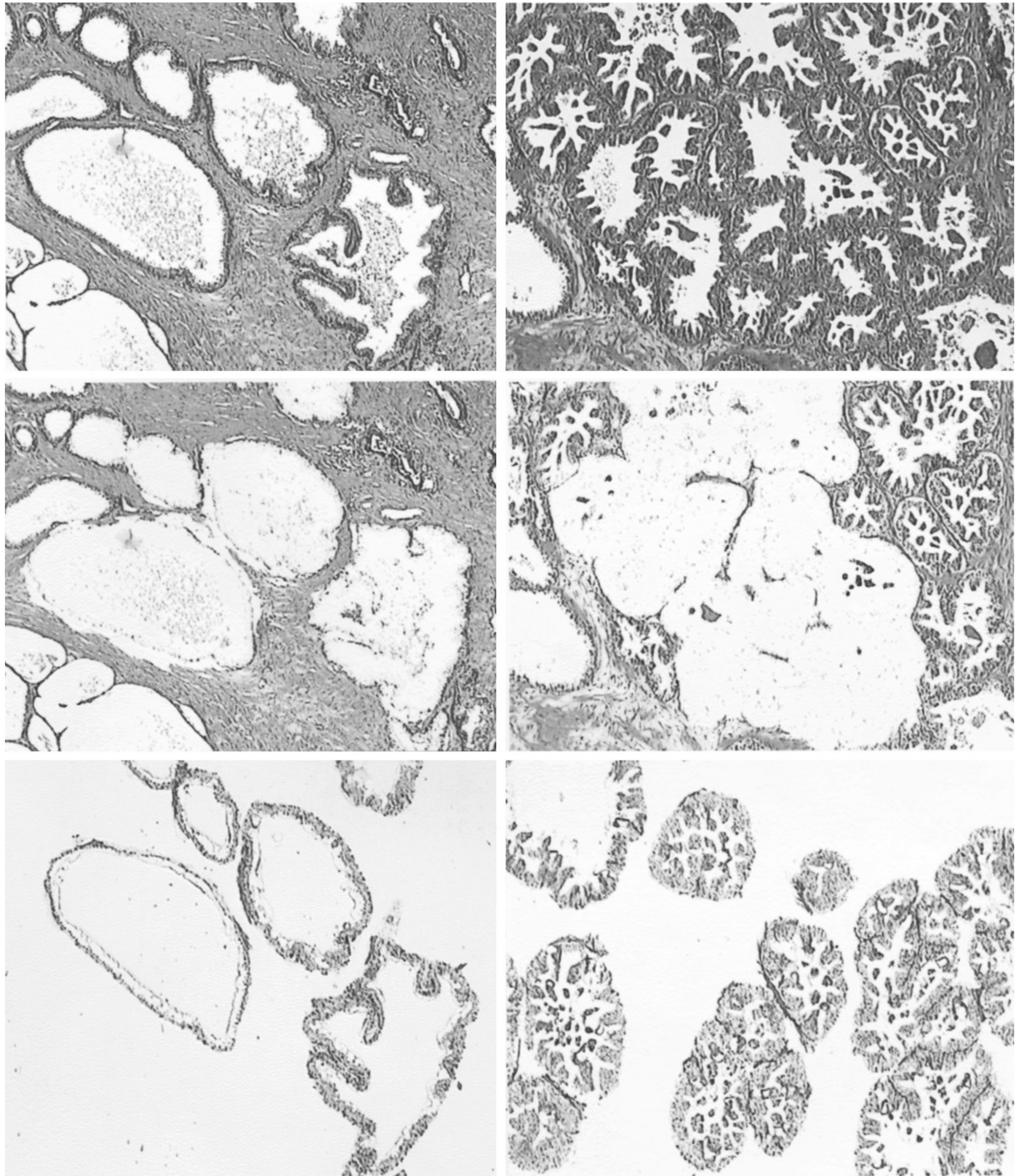

FIGURE 4. H&E sections show an example of LCM. Dissection of benign epithelium (***left side***) and of HPIN (***right side***). ***Top*** pictures represent the tissue before dissection, ***middle*** pictures are after dissection, and ***bottom*** pictures are the cap tissues, which were used for p53, automated sequencing analysis.

correlation between loss or mutation of p53 and the presence of CI (53–63). More recently, centrosome hyperamplification was found to be the major mechanism responsible for CI *in vitro* and *in vivo* (58, 59, 64–66). Centrosome is the major microtubule-organizing center and is required for spindle bipolarity, spindle microtubule assembly, and balanced segregation of the chromosomes (67). A very strong correlation has been found between p53 loss or mutation and centrosome hyperamplification (27, 55, 59, 67). Breast carcinoma and squamous cell carcinoma of the head and neck with either p53 deletion or mutation show centrosome hyperamplification (58, 64, 65).

IFISH analysis for chromosomes 7, 8, and Y was performed to assess CI. We used these chromo-

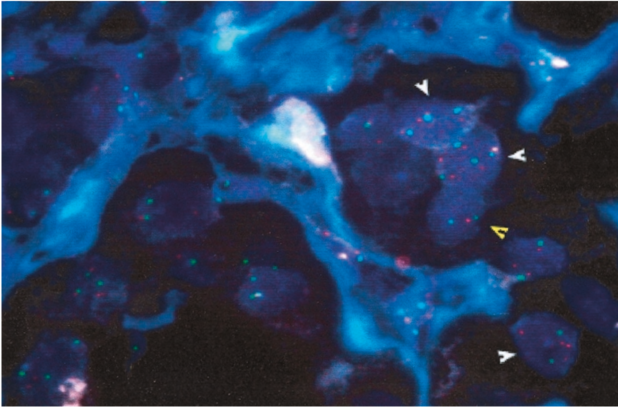

FIGURE 5. Interphase FISH on a focus of invasive prostate carcinoma using dual-centromere probes. Some cells (***white arrowheads***) show more than 2 green and more than 2 red signals, consistent with a gain of chromosomes 7 and 8. The ***yellow arrowhead*** shows cells with 2 green and 1 red signals.

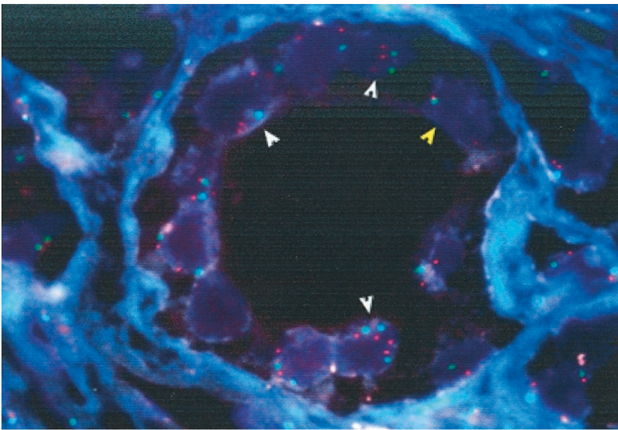

FIGURE 6. Interphase FISH on a focus of invasive prostate carcinoma using dual probes. Some cells (***white arrowheads***) show 3 green signals and 4 red signals, consist with a gain of chromosomes 7 and 8. Other cells (***yellow arrowhead***) show only one red and one green signal.

somes to assess CI because they are the most frequently affected chromosomes in prostate cancer pathogenesis. Although CI represents generalized changes in the cellular chromosomes, it is selective for certain chromosomes in carcinogenesis of different organs. Our finding revealed numeric chromosomal aberrations in 5/7 and 2/8 of p53-positive and p53-negative Pca, respectively ($P = .1$). However, the presence of any numeric chromosomal abnormality has been seen in 4/6 and 0/9 of p53-positive and p53-negative HPIN ($P < .02$). Generally, none of the p53− intermingled and away HPIN foci showed any chromosomal abnormality. So generally, HPINI tend to have more CIN than those situated away (4/15 *versus* 0/15), with statistically significant difference ($P < .05$). No CI has been detected in the normal, hyperplastic, or atrophic epithelium and those areas showed no p53 alteration either. This suggest that those HPINI foci may represent the source of the adjacent invasive component, whereas the other isolated HPINA foci that admixed with benign epithelium may still be in the early stages of the carcinogenesis pathway and probably require more CI to progress to invasive cancer. This also suggested that p53 mutation may play a role in the progression of HPIN to invasive cancer, and this could happen through induction of CI.

We applied IFISH on sections from the same blocks that have been used for p53 IHC and that enabled us to compare the findings of the two assays in the same foci of tissue. IFISH has higher sensitivity than other methods used for this purpose, such as CGH, which detects copy number changes if they are present in more than 50% of the cell population (21). IFISH can identify CI in small subpopulations of interphase cells (68), allowing the detection of infrequent, possibly random changes before they lead to clonal expansion (20). Using IFISH on pretreatment and post antiandrogen therapy prostate cancer specimens, Karashima *et al.* (69) found a remarkable reduction in the number of cells with extra copies of chromosomes 7 and 8.

Our IFISH results showed that gain of chromosome 8 is the most frequent finding in both HPIN and Pca. c-Myc gene is located in the 8q arm, and gain of chromosome 8 indicated an extra copy of that important oncogene. The role of c-Myc in the mechanism of CI has been recently described. Extra copies of the c-Myc gene were identified in 52 and 44% of the high-grade PIN and carcinoma foci, respectively (70), and by Mark *et al.* (71) in 31% of Pca. In some cancers displaying CI, the loss of the checkpoint was associated with the mutational inactivation of a human homologue of the yeast BUB1 gene. BUB1 controls mitotic checkpoints and chromosome segregation in yeast (72). Disruption of the mitotic spindle checkpoint is one of the underlying mechanisms leading to aneuploidy and alterations of hsMAD2 and hBUB1. This mechanism, assumed to take part in the spindle checkpoint in human cells, has been found to be associated with CI in some tumor cell lines (8). However, there is no study on these genes in prostate tumors. Other possible mechanisms may be involved in the causation of CI, such as shortened telomeres, hypomethylation, activation of certain genes or inactivation of tumor suppressor genes.

CONCLUSION

We demonstrated that p53 mutation is an early change in at least a subset of Pca. HPINI foci tend to have higher overall p53 immunoreactivity and CI than HPINA. The presence of p53 mutation in HPIN

was associated with the presence of CI as determined by IFISH. Also, our study provided additional evidence in support of the concept that HPIN is the earliest precursor of cancer. Furthermore our studies identify genomic similarities in HPINI and Pca, implying that carcinoma may arise from progression of certain HPIN foci that most likely harbor p53 mutation and/or elevated levels of CI.

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