Prostatic intraepithelial neoplasia (PIN) is characterized by an intraluminal proliferation of epithelial cells in ducts and acini. PIN frequently coexists with prostatic carcinoma (PCA). PIN is usually multicentric, and is commonly found in the non-transition zone, which is the predominant site of PCA. According to the histologic and cytologic findings, PIN can be divided into high-grade PIN (HGPIN) and low-grade PIN (LGPIN). Previous histopathological study on whole-mount prostatectomy specimens showed that HGPIN lesions were detected in more than 75% cases with PCA. Proximity of PCA and PIN (≤2 mm) was proposed to indicate a precancerous nature of PIN, and our recent study revealed that close association of PIN with PCA was more frequently found in HGPIN (54% of lesions) than in LGPIN (38%). All these findings indicated that HGPIN, but not LGPIN, is the most likely precursor lesion for PCA.

Recent studies revealed that HGPIN and PCA share common cytogenetical features. Allelic loss of chromosome 8p was frequent both in PIN and invasive PCA. Mutations of Ha-ras gene were closely associated with progression of PIN into invasive PCA in transgenic mice, although this was not confirmed in humans.

The p53 gene is a tumor suppressor gene located on the short arm of chromosome 17. It consists of 11 exons and 10 introns and encodes 393 amino acids. In a wide variety of human tumors, p53 gene mutations have been detected mainly in exons 5 through 8, which include the highly conserved domains II-V. Little is known about p53 mutations in PIN due to technical problems, i.e., it is difficult to excise exclusively the PIN lesions, which are fairly small and difficult to define macroscopically. HGPIN frequently coexist with cancer or benign glands in the non-transition zone, which is the predominant site of PCA. Therefore, it is difficult to excise exclusively the PIN lesions, which are fairly small and difficult to define macroscopically. HGPIN frequently coexist with cancer or benign glands in the non-transition zone, which is the predominant site of PCA.
a haphazard fashion in the non-transition zone. Recently we successfully microdissected PIN and PCA lesions, and examined p53 mutations in PIN and PCA.\textsuperscript{15)

Laser capture microdissection under direct microscopic visualization enables rapid one-step procurement of selected human cell populations from a histologic section. This method has made microdissection of selected areas much easier, so that extensive study of specific target lesions is possible. Using this method on the whole-mount samples, we have now selectively microdissected numerous lesions of PIN (both high and low grade) and PCA from the non-transition and transition zones. DNA extracted from each lesion was analyzed for p53 mutations by single-strand conformation polymorphism (SSCP) of polymerase chain reaction (PCR)-amplified DNA fragments, followed by direct sequencing.

**MATERIALS AND METHODS**

**Patients** Twenty-seven patients, who received total prostatectomy for PCA, were selected for the current study: they had been admitted to hospital during the period 1996 to 1998. Fourteen patients received androgen deprivation therapy (castrated): an LH-RH agonist (leuprolide) for 2 to 6 months (mean 3.4 months) together with the anti-androgen agents (flutamide or chlormadinone), and the remaining 13 did not (non-castrated). Then the current cases were conventionally subdivided into preoperatively non-castrated group (13 cases) and medically castrated group (14 cases). None of the 27 patients received preoperative chemotherapy or radiation therapy. Based on the American staging system (modified by Whitmore-Jewett),\textsuperscript{16) pathological stage was determined as follows: 11 cases (41\%) in stage T2 and 16 (59\%) in T3. Histologic specimens were fixed in 10\% neutral buffered formalin and routinely processed for paraffin-embedding. Serial 5-\mu m-thick sections were cut, and every first section was stained with hematoxylin and eosin for histologic diagnosis. Mean number of sections examined was 9.3 per case. Histologic sections were reviewed independently by three pathologists (H.T., M.S. and K.A.) (Fig. 1). In problematical cases, preservation of the basal cell layer, a reliable criterion for benign prostatic lesions, was immunohistochemically examined by using basal cell-specific, high-molecular-weight cytokeratin antibody (34\beta E12). PIN is characterized by the intraluminal proliferation of crowded and stratified cells. HGPIN have features mimicking PCA, i.e., enlargement of the nuclei and the prominent nucleoli as well as a partially disrupted basal layer.

**Laser capture microdissection and DNA extraction**

Microdissection of each lesion was performed using a PixCell laser capture microscope (Arcturus Engineering, Santa Clara, CA) according to the previously described methods with some modifications,\textsuperscript{17,18,19} a representative case is shown in Fig. 2. Briefly, histologic sections were dehydrated, and then the histologic fields of interest were selected, overlaid with a thermoplastic film mounted on a transparent cap, and captured by the film through laser energy. The dissected pieces were allowed to adhere to the transparent cap and collected in 0.5 ml Eppendorf tubes. The procured cells were subsequently resuspended in 20–50 \mu l of extraction buffer containing 10 mM Tris (pH 8.0), 2 mM EDTA, 0.2\% Tween 20, and 200 \mu g/ml proteinase K, and incubated overnight at 37\degree C. The mixture was heated at 100\degree C for 10 min to inactivate the proteinase K, and 3–5\% of the solution was used as a template for each PCR. The total number of microdissected lesions from 27 cases was 193; 111 lesions with HGPIN (75 lesions from non-transition and 36 from transition zone), 55 with PCA (30 lesions from non-transition and 25 from transition zone), and 27 with benign glands.

**PCR-SSCP analysis and direct sequencing**

The proce-
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dure was carried out as previously described, with a minor modification to increase efficiency: (1) the amount of microdissected DNA template for PCR amplification was 1 µl, (2) possible mutated bands at SSCP were extracted from gels and incubated with 20 µl of diffusion buffer containing 50 mM Tris-HCl, 1 mM EDTA, and 0.5% Tween 20 at 50°C for 1 h to make templates for subsequent amplification, (3) PCR products were extracted from 2% agarose TBE (Tris-borate, EDTA) gels. Mutations of p53 gene from exon 5 to exon 8 were analyzed.

RESULTS

As shown in Table I, 27 mutations of the p53 gene were detected in 24 lesions from 12 cases (Fig. 3). All mutations were point mutations; 17 were missense, 7 silent, and 2 nonsense. There were no mutational hot spots, although exon 5 was the commonest site. In cases 3, 19 and 20, there were double mutations with different types of nucleotide substitutions in the same exons. Mutations were detected in 6 cases (22.2%) or 13 of 111 lesions (11.7%) with HGPIN and 8 cases (29.6%) or 11 of 55 lesions (20.0%) with PCA. Benign proliferative glands adjoining PIN and/or PCA had no mutations of the p53 gene. The PCA cases with mutations had stage T2 (2 cases) and T3 (6 cases) disease. In cases 19 and 20, two each of the PCA and PIN lesions had different mutations. In cases 6, 16, 17, 18 and 21, each of the HGPIN and PCA lesions had different mutations from each other. Mutations at CpG sites were found in one case (case 21). Regarding patterns of p53 mutations, G-to-A transition was the commonest pattern (6/27; 22.2%), followed by C-to-T transition (5 mutations) and A-to-C transversion (5 mutations).

The frequency of p53 mutation of PCA in the non-transition zone (33.3%) was significantly higher than that in the transition zone (4%) (P<0.05) (Table II). The frequency of p53 mutation in PCA with stage T3 (30.3%), 10 of 33 lesions, was significantly higher than that with stage T2 (4.5%), 1 of 22 lesions (P<0.05). The frequency of p53 mutation of PIN in the non-transition zone (14.7%) was higher than that in the transition zone (5.6%), although the difference was not significant. The frequency rate of p53 mutation in HGPIN close to PCA (≤2 mm of distance) was significantly higher (24%) than that in HGPIN lesions >2 mm from PCA (3%) (P<0.05).

When p53 mutations were evaluated in non-castrated cases (Table III), the mutation frequency of PIN was the same as that of PCA, i.e., 4 of 13 cases (30.8%). Five of 19 lesions with PCA and 8 of 36 lesions with PIN in the non-transition zone showed mutation, but none of PCA and PIN in the transition zone did. Nine of 31 HGPIN lesions close to PCA (≤2 mm) showed mutations, but none of HGPIN >2 mm from PCA did.

DISCUSSION

The current series consisted of 14 castrated and 13 non-castrated patients. The mutation frequency was similar in the castrated and non-castrated group, so they are discussed below as a total. The frequency rate of p53 gene mutations in the current PCA lesions with mostly stage T2 or T3 disease (20%) was close to that in our previous study (17%) and also that in reported cases with advanced PCA. P53 gene mutations were found in 12% of the current HGPIN lesions, which is also similar to that of our previous study (9%).

Difference in mutation frequency by stage of PCA could not be evaluated in our previous study because of the relatively small number of PCA lesions examined. In
Table 1  
*p53* Mutations in PIN and Concurrent PCA

| Case | Zone of lesion | Distance from PCA (mm) | Histology/pathological stage | Exon/codon | Mutation | Nucleotide substitution | Pattern |
|------|----------------|------------------------|-----------------------------|------------|----------|-------------------------|---------|
| Castrated group (*n=14*)  
3  | N-T  | PCA/Gleason 2, T3 7/235 | Asp (AAC) → His (CAC) | A to C | Tv missense |
| 18 | N-T  | 7/237 | Met (ATG) → Arg (AGG) | T to G | Tv missense |
| 21 | N-T  | 5/145 | Leu (CTG) → Leu (CTA) | G to A | Ts silent |
| 18 | N-T  | 5/151 | Pro (CCC) → Pro (CCG) | C to G | Tv silent |
| 21 | N-T  | 5/165 | Gln (CAG) → Gln (CAA) | G to A | Ts silent |
| 24 | T    | 8/285 | Glu (GAG) → Stop (TAG) | G to T | Tv nonsense |
| 25 | N-T  | 8/279 | Gly (GGG) → Gly (GGT) | G to T | Tv silent |
| 26 | N-T  | 5/151 | Pro (CCC) → Pro (CCG) | C to G | Tv silent |
| Non-castrated group (*n=13*)  
6  | N-T  | PCA/Gleason 2, T3 7/235 | Asp (AAC) → Thr (ACC) | A to C | Tv missense |
| 9  | N-T  | 8/296 | His (CAC) → Asn (AAC) | C to A | Tv missense |
| 16 | N-T  | 7/243 | Cys (TGC) → Tyr (TAC) | G to A | Ts silent |
| 17 | N-T  | 5/145 | Gly (GGC) → Asp (GAC) | C to G | Ts silent |
| 19 | N-T  | 8/285 | Gln (CAG) → Gln (CAA) | G to A | Ts silent |
| 20 | N-T  | 5/151 | Pro (CCC) → Pro (CCG) | C to G | Tv silent |
| PIN, prostatic intraepithelial neoplasia; PCA, prostatic carcinoma; Ts, transition; Tv, transversion; T, transition zone; N-T, non-transition zone.  
*a*) Mutation at CpG sites.

Fig. 3. PCR-single-strand conformation polymorphism (SSCP) analysis of *p53* mutation. Nonradioactive SSCP analysis of exon 7. Aberrant migration patterns (arrow) were seen in lane 1 (case 5, PCA), lane 2 (case 3, PCA), and lane 3 (case 19, PIN). Wild-type SSCP bands are shown in lane 4 (case 19, benign prostatic hyper trophy).
the current series, there is a difference in mutation frequency of PCA lesions by stage of disease: one of 22 lesions (4.5%) in T2 and 10 of 33 lesions (30.3%) in T3 showed mutations. This suggests that p53 gene mutations might be involved in the progression of PCA. Berner et al. (1995) reported that C-to-G transversion at codon 273 was frequently found in PCA. They suggested that this might be a mutational hotspot in the progression of PCA. Although exon 5 was the commonest site for mutations in our previous and the current series, there were no mutational hotspots. Mutation at the CpG site was found in one case (case 21). G-to-A transition and C-to-G transversion substitution were most common in our series. In 8 of 12 cases with PCA and/or PIN lesions, p53 mutations had at least one mutation that changed an amino acid, which might have provided the selection pressure for expansion.

Table II. Summary of p53 Mutations in PCA and PIN

| Case | Lesion | Stage T2 | Stage T3 | Zone Transition | Zone Non-transition | Distance from PCA ≤2 mm | >2 mm |
|------|--------|---------|---------|----------------|---------------------|------------------------|-------|
| PCA  | 8/27   | 11/55   | 1/22    | 10/33          | 1/25                | 10/30                  | a)    |
|      | (29.6) | (20.0)  | (4.5)   | (30.3)         | (0.0)               | (33.3)                 |       |
| PIN  | 6/27   | 13/111  | 4/33    | 9/78           | 2/36                | 11/75                  | a)    |
|      | (22.2) | (11.7)  | (12.1)  | (11.5)         | (5.6)               | (14.7)                 |       |

a) P<0.05.

Table III. Frequency of p53 Mutations in Non-castrated Group

| Case | Lesion | Stage T2 | Stage T3 | Zone Transition | Zone Non-transition | Distance from PCA ≤2 mm | >2 mm |
|------|--------|---------|---------|----------------|---------------------|------------------------|-------|
| PCA  | 4/13   | 7/24    | 0/5     | 7/19           | 0/5                | 7/19                   | a)    |
|      | (30.8) | (29.2)  | (0)     | (36.8)         | (0)                | (36.8)                 |       |
| PIN  | 4/13   | 8/56    | 2/14    | 6/42           | 0/20               | 8/36                   | b)    |
|      | (30.8) | (14.3)  | (14.3)  | (14.3)         | (0)                | (22.2)                 |       |

a) P<0.05.
b) P<0.01.

The non-transition zone is known to be the predominant site for mutations of p53 gene in the HGPin, like PCA, is sensitive to androgen deprivation therapy.
(i.e., the non-transition and transition zone). The current study revealed that the frequency of p53 mutations in PCA lesions was significantly higher in the non-transition than in the transition zone. As for HGPIN, p53 mutations in the non-transition zone were significantly more frequent than those in the transition zone among non-castrated cases. These findings suggest that p53 gene mutations play a role in the development of precancerous and cancerous lesions in the non-transition zone, but not in the transition zone.

The distance between the PCA and HGPIN was reported to be frequently within 2 mm. Bostwick and Brawer reported that the frequency of appearance of HGPIN was increased in cases with PCA compared to those without PCA. In our previous study close association (distance within 2 mm) of HGPIN with PCA was more frequently found in the non-transition zone (63% of lesions) than in the transition zone (38% of lesions). These findings provided support for the precancerous nature of HGPIN, especially in the non-transition zone. Indeed, the frequency rate of p53 mutations in HGPIN lesions close to PCA (24% of lesions in the total and 29% in the non-castrated cases) was significantly higher than that in lesions distant from PCA (>2 mm) (3% in the total and none in the non-castrated cases). These findings further support the precancerous nature of the HGPIN close to PCA.

Our previous study suggested a relationship between HGPIN and PCA with regard to p53 mutation and multi-clonal development of prostatic precancerous lesions. The current study using the laser capture microdissection method clearly showed a significant involvement of p53 gene mutations in the development of HGPIN and PCA in the non-transition zone, and suggests a precancerous nature of HGPIN located close to PCA.

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