Biologic markers in prostatic intraepithelial neoplasia : immunohistochemical and cytogenetic analyses

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Abstract : *Objective* : We evaluated the biological properties of High-grade prostatic intraepithelial neoplasia (PIN) by immunohistochemistry and fluorescence in situ hybridization (FISH) analysis in relation to normal tissue and carcinoma lesions.
***Materials and Methods* : Immunohistochemical staining and FISH were performed on 23 formalin-fixed radical prostatectomy specimens taken from patients with PIN. Assays were performed using MIB-1, chromogranin A (CGA) and an anti-androgen receptor antibody (AR). A centromere probe for chromosome 8 was used to test for aneuploidy.**
***Results* : The MIB-1 index of cancerous specimens (16.2±10.5%) was significantly higher than that of benign (1.9±1.6%, p<0.0001) or PIN (4.0±4.5%, p<0.0001) specimens. The percentage of CGA positive cells was significantly lower in normal tissue (1.2±1.8%) than in PIN (3.5±2.9%, p=0.012) or carcinoma (5.4±4.9%, p=0.005) lesions. Positive staining for AR was consistently observed in the nuclei of both benign and malignant epithelial cells, but positive cytoplasmic staining was also seen in PIN epithelial cells. No significant difference in FISH detected anomalies were found between PIN and carcinoma specimens.**
***Conclusions* : Our studies concerning proliferative activity, NE differentiation and chromosomal anomalies of prostatic specimens support the hypothesis that PIN is a biologically intermediate stage in the pathogenesis of prostatic carcinoma. The cellular distribution of AR was altered in PIN cells, but the role of AR in PIN is not yet clear.**
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***Key words* :** *prostatic intraepithelial neoplasia, immunohistochemistry, fluorescence in situ hybridization*

INTRODUCTION

Prostatic intraepithelial neoplasia (PIN) is thought to be a precursor of prostatic carcinoma due to their similar histopathology. Indeed, the presence of PIN in a diagnostic needle biopsy, without any accompanying malignancy, is highly predictive of the presence of carcinoma in subsequent biopsies [1, 2]. However, although some reports have appeared on immunohistochemical or cytogenetic analyses of PIN, its biological properties and clinical significance remain unclear [3, 4].

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Ki-67 is a nuclear protein expressed during the G1, S, G2, and M phases of continuously cycling cells, but not G0 cells. The genetic locus of Ki-67 is not well characterized, and it has been assigned to chromosome 10 [5]. Several studies have shown that cell proliferative activity, as defined by the Ki-67 index, correlates with cell growth fraction. MIB-1 antibody, which detects a formalin-resistant epitope of the Ki-67 antigen, has been a useful index in several studies [6]. Hepburn *et al*. [7] compared the reactivity of MIB-1 antibody in paraffin-embedded sections with that of Ki-67 antibody on frozen sections of prostatic carcinoma, and they reported a significant correlation between the two. Differentiation of prostatic neuroendocrine (NE) cells, which are distributed throughout the prostate gland as well as various ducts and the urethra [8], has been

found to be a good prognostic indicator for cases of prostatic adenocarcinoma [9, 10]. However, the relationship between PIN and NE markers has not been fully elucidated. In addition, the androgen receptor (AR) is widely distributed in tissue and several mutations have been detected in prostate carcinoma [11, 12] suggesting that AR may also play an important role in prostatic carcinogenesis [13, 14].

Recently, fluorescence in situ hybridization (FISH) has been performed on interphase chromosomes in a variety of solid tumors in order to detect genetic aberrations [15, 16]. Brown *et al*. [17] evaluated numeric chromosomal anomalies in localized prostate carcinomas using FISH with centromere probes for chromosomes 4, 6-12, 17, 18, X and Y. They reported that chromosome 8 polysomy was the most frequent aneuploidy in prostate carcinoma. Konig *et al*. [18] also investigated numerical aberrations of chromosomes 1, 7, 8, 10, 18 and Y in both carcinomas and benign hyperplasias. Their results suggested that polysomies of chromosomes 7, 8 and 10 were seen in cancerous tissue, although no evidence of aneuploidy was seen in normal tissue.

The present study attempted to determine the expression of these biomarkers in PIN taken from prostatectomy specimens of untreated patients, and to evaluate the biological relationship between normal epithelium, PIN and carcinoma tissue.

MATERIALS AND METHODS

Tissue samples

Twenty-three patients with previously untreated-localized prostate cancer who underwent radical prostatectomy were studied. All tissue specimens selected for this study contained both carcinoma and PIN. Formalin-fixed, paraffin-embedded prostate tissue was obtained from the Department of Urology, The University of Tokushima School of Medicine and its affiliated hospitals. Patients participating in this study ranged in age from 60 to 79 years (mean age 68.3). Tumors were staged according to the UICC classification, and tumor grade was determined based on the Gleason sum score. High-grade PIN samples were further classified by hematoxylin-eosin staining as described by McNeal and Bostwick [19]. Additionally, for cases in which a diagnosis of PIN was not clearly evident, immunostaining was performed for high-molecular-weight cytokeratin using 34βE12 (Enzo Biochemicals, USA, dilution 1 : 50). This antibody acts as a specific marker for basal cells. In the present study, the term PIN is used to indicate high-grade PIN. All slides were reviewed and a representative paraffin-embedded tissue block containing PIN, prostatic adenocarcinoma, and non-cancerous normal prostatic foci, was selected from each case for immunohistochemical examination and FISH analysis. Atypical adenomatous hyperplasia (AAH) was not observed in any of these cases.

Immunohistochemical staining

Immunohistochemical analysis was performed with only slight modifications of previously described methods [20]. Paraffin-embedded 5-μm sections were prepared and deparaffinized in xylene and rehydrated in graded alcohols. Endogenous peroxidase activity was blocked by immersing sections in 0.3% hydrogen peroxide (H_2O_2) in methanol for 30 minutes. Sections were placed in an autoclave for 10 minutes at 120°C and were then allowed to cool at room temperature for 40 min before immunostaining. Pretreatment with normal rabbit serum for 30 min at room temperature was followed by incubation overnight at 4°C with the primary antibodies in a humidified chamber. Subsequently, biotinylated anti-mouse antibody was applied to the sections for 30min. Streptavidin-biotinylated peroxidase complex (LSAB kit ; DAKO, California, USA) was then applied, and the sections were incubated with diaminobenzidine (DAB ; Wako, Osaka, Japan) as chromogen. Following immunohistochemical staining, the sections were counterstained with hematoxylin to enhance nuclear detection. For negative controls, the primary antibody was substituted with phosphate-buffered saline in duplicate sections. The primary antibodies were as follows : anti-MIB-1 (Immunotech S.A., France, dilution 1 : 50), anti-ChromograninA (CGA) (DAKO, Denmark, dilution 1 : 50), and anti-androgen receptor (AR) (Novocastra, UK, dilution 1 : 50).

Labeled cell counts

MIB-1, CGA and AR expression were evaluated under light microscopic observation at X400 magnification. Immunostaining results were interpreted according to the positivity of the immunoreactive product seen in epithelial cells. The percentage of positive cells for each marker was determined by counting at least 800 tumor cells in four selected fields that displayed the highest immunoreactivity.

Fluorescence in situ hybridization

FISH analysis was performed using a slight modi-

fication of the procedure described previously [21]. Serial 5-μm sections were prepared from each specimen for FISH analysis. The slides were deparaffinized, dehydrated, and treated in a 30% concentration of a pretreatment solution (Oncor, Inc. Gaithersburg, USA) at 45°C for 15 minutes. The slides were then washed three times in 2x standard saline citrate (SSC), followed by digestion in pepsin solution (4 mg/ml in 0.9% sodium chloride, PH1.5) at 45°C for 18 min. This was again followed by three washes in 2x SSC. After dehydration in ethanol, air drying, and denaturing of DNA probes and target DNA in an 82°C oven for 5 minutes, 10 μl of a chromosome 8 centromeric probe (Oncor, inc. Gaithersburg, USA) was applied directly to each treated slide. The slides were coverslipped, sealed with rubber cement, and incubated overnight in a 37°C humidified chamber. Coverslips were then removed and the slides were washed in 2 X SSC at 72°C for 5 minutes. Hybridization was detected using fluorescein-isothiocyanate-conjugated (FITC) avidin, and nuclei were counterstained with propidium iodide.

Criteria for FISH anomalies

The following criteria were applied for interpretation of the results [22]. 1) Overlapping nuclei were not counted. 2) Multiple stains within one nucleus have more or less the same size and intensity. Non-specific, smaller stains were not counted. 3) Paired spots were counted as one stain. Stains were enumerated in at least 200 epithelial nuclei from histologically benign regions of each of the 23 prostates. The mean ± SD percentage of nuclei with three or more stains was found to be 4.1±3.3% (ranging from 1.0 to 15.0%), and a cut-off value of 17.3% (mean+4SD) was used to define FISH detected anomalies [23, 24]. If the percentage of cells with 3 or more stains exceeded this number, the foci of PIN or carcinoma were considered to have nuclei with polysomies for chromosome 8.

Statistical analysis

A Student's *t* test and Chi-squared test were used for statistical analysis of the results. P values below 0.05 were considered statistically significant.

RESULTS

Of the 23 radical prostatectomy specimens, 9 were stage pT2 and 14 were stage pT3. The Gleason sum score of the 23 tumors varied from 2 to 9, with 14 tumors classified as low grade (Gleason sum score 2 to 6) and 9 as high grade (Gleason sum score 7 to 9).

MIB-1 positive nuclei were randomly distributed in individual glands comprising normal epithelia, PIN, and carcinoma tissue (Fig.1). The MIB-1 index increased from a mean of 1.9±1.6% (mean±SD) in benign to 4.0±4.5% in PIN to 16.2±10.5% in carcinoma tissue. The MIB-1 index of carcinoma tissue was significantly larger than that of benign or PIN tissue ($p<0.0001$). In addition, there was a significant difference in the mean MIB-1 indexes between high grade (21.2±11.3) and low grade (12.6±8.3) carcinomas ($p<0.05$). Although there was no significant correlation, PIN tended to have a higher MIB-1 index than benign epithelium ($p=0.052$) (Table 1).

Immunohistochemical staining for CGA was observed in the cytoplasm of NE cells in normal epithelium, PIN, and carcinoma tissue. NE cells were relatively large and located in the basal layer (Fig.2). The percentage of NE cells increased from a mean of 1.2±1.8% in benign to 3.5±2.9% in PIN to 5.4±4.9% in carcinoma tissue. The percentage of NE cells was significantly lower in benign glands than in PIN ($p=0.012$) and carcinoma tissue ($p=0.005$, Table 1). However, there was no significant difference in the percentage of NE cells between PIN and carcinoma tissue or between high grade and low grade carcinomas.

AR-positive staining was observed in the nuclei of benign epithelium and stromal cells. However, the basal cell layer did not express AR. In malignant tissue, AR immunoreactivity was observed in the nuclei of carcinoma cells. On the other hand, while AR expression was present in PIN, the nuclear staining intensity was weak in general, and cytoplasmic staining was occasionally observed (Fig.3). The percentage of AR positive cells did not differ significantly between normal epithelia, PIN, and carcinoma tissue (65.9±14.5%, 54.2±15.7% and 67.3±17.0%, respectively) (Table 1).

The FISH results for the chromosome 8 centrometric probe, observed in the foci of normal epithelia, PIN, and carcinoma tissue, are summarized in Table 2. The mean percentage of nuclei with three or more stains 4.1%, 21.3% and 31.5% for normal epithelia, PIN, and carcinoma tissue, respectively. As a result, chromosome 8 polysomies were found in 64.3% (9 of 14) of PIN foci and 61.1% (11 of 18) of carcinoma foci. No significant difference in FISH-detected anomalies were found between PIN and carcinoma foci (Table 2).

Table1. Immunostaining results in normal epithelium, PIN and carcinoma

	mean±SD(%)		
	Normal	PIN	Carcinoma
MIB-1	1.9±1.6	4.0±4.5	16.2±10.5※
CGA	1.2±1.8*	3.5±2.9	5.4±4.9
AR	65.9±14.5	54.2±15.7#	67.3±17.0

CGA : chromogranin A, AR : anti-androgen receptor antibody, PIN : prostatic intraepithelial neoplasia
※carcinoma vs normal epithelium or PIN ($p<0.0001$),
*normal epithelium vs PIN or carcinoma ($p<0.05$)
#weak nuclear staining and cytoplasmic staining

Table 2 . FISH for Chromosome 8

	mean±SD (%)*			
	≦monosomy	disomy	trisomy≦	polysomic foci (%)※
Normal	24.7±14.1	71.5±14.5	4.1±3.3	
PIN	21.0±16.0	62.2±10.1	21.3±14.4	9/14 (64.3%)
Carcinoma	20.9±18.4	48.8±14.3	31.5±23.9	11/18 (61.1%)

*Percentage of chromosomal anomaly for chromosome 8, ※Cutoff percentage : mean + 4SD = 17.3%, NS

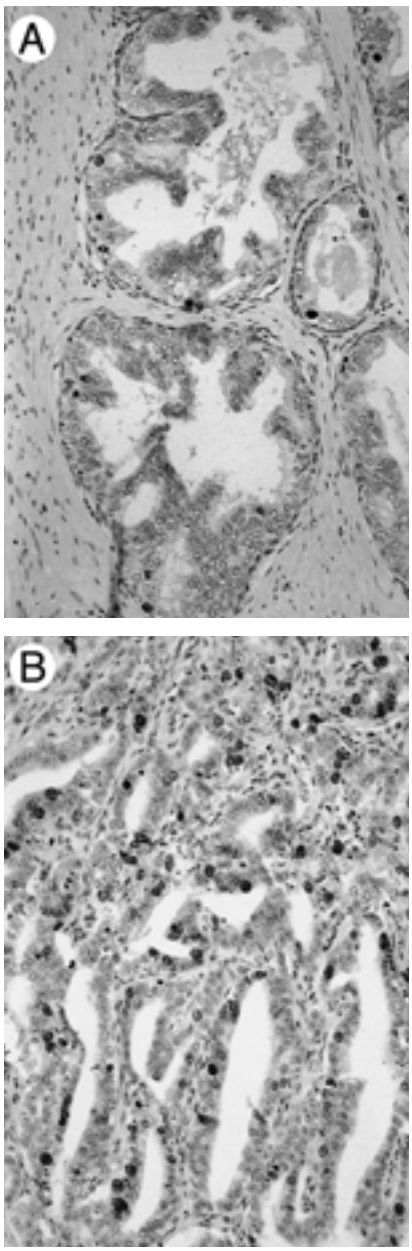

Fig.1. Immunohistochemical staining for MIB-1 in PIN (A) and carcinoma tissue (B) (X200). The MIB-1 index of carcinoma was significantly greater than that of PIN.

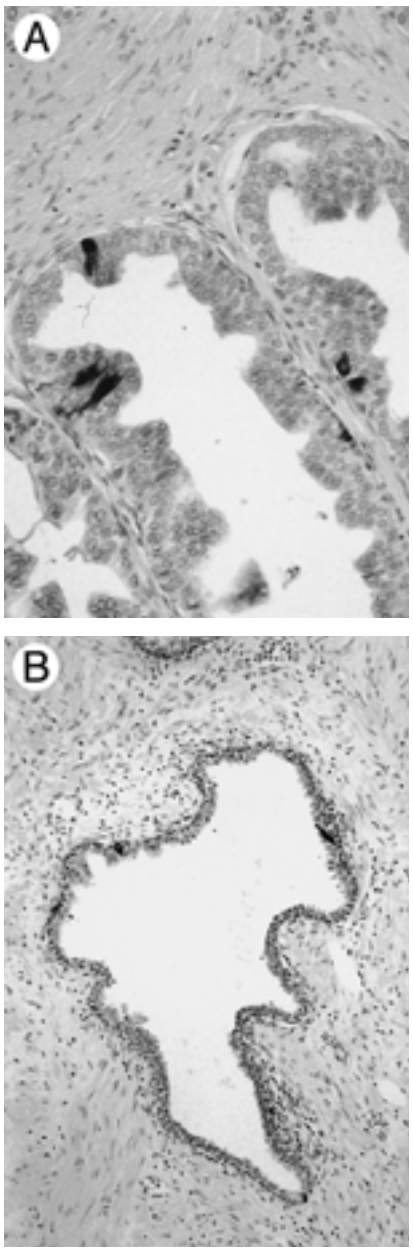

Fig.2. Immunohistochemical staining for Chromogranin A observed in cytoplasm of neuroendocrine cells. Number of NE cells in PIN (A) (X 200) is larger than in normal epithelium (B) (X100).

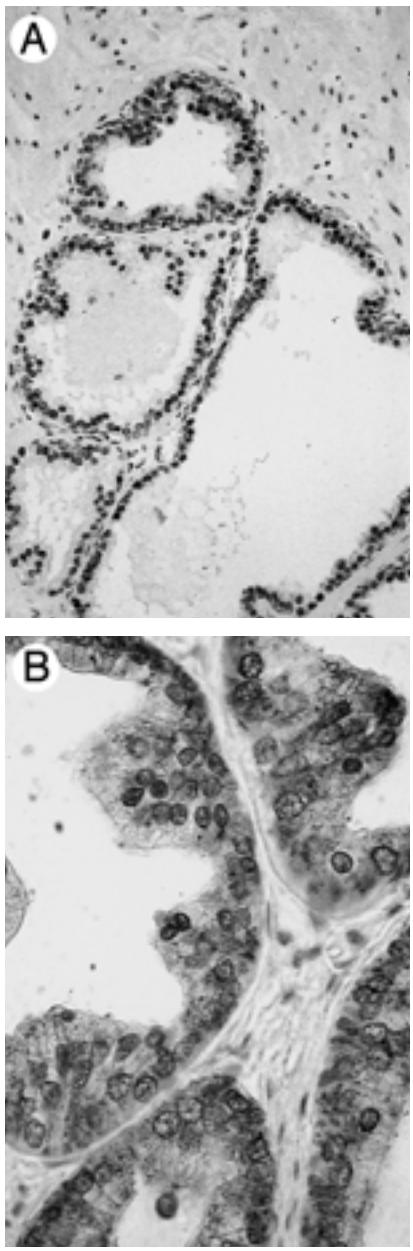

Fig.3. Immunohistochemical staining for AR observed in nuclei of normal epithelium and stromal cells (A) (X100). Nuclear staining intensity in PIN is weak. Occasionally cytoplasmic staining is observed (B) (X400).

DISCUSSION

The clinical importance of recognizing PIN stems from its strong association with carcinoma, which most studies to date have confirmed [1-4]. The frequency and extent of PIN are significantly increased in cancerous prostate specimens compared with non-cancerous prostates [1, 2]. Approximately 57% of patients show carcinoma on repeat biopsy following the appearance of high-grade PIN on a previous biopsy [25]. Therefore, when PIN is encountered in prostate specimens, all tissue should be embedded and evaluated.

In PIN foci, the majority of nuclei are enlarged and prominent nucleoli are frequently observed. The morphologic continuum of cellular proliferations with cytological changes mimics cancer. Bostwick *et al*. [26] identified 4 common patterns of PIN: tufting, micropapillary, cribriform and flat. However, there is no prognostic difference between these morphological patterns of PIN, and this distinction appears to have only diagnostic utility. On the other hand, atypical adenomatous hyperplasia (AAH) is a localized proliferation of small glands, which may be mistaken for adenocarcinoma. Generally, adenocarcinoma glands do not have basal cells, but AAH glands are surrounded by a fragmented layer of basal cells, as with PIN. Presently, the evidence that AAH is a cancerous precursor is unconvincing [27]. Therefore, PIN and AAH show some of the morphologic features of carcinoma, although they lack stromal invasion. 34βE12 is specific for high-molecular-weight cytokeratins 1, 5, 10 and 14, which correspond to molecular weights of 68, 58, 56.5 and 50kd, characteristically found in complex epithelium. This antibody serves as a specific marker for basal cells in the prostate gland, and may have a potential diagnostic use in distinguishing between PIN or AAH and malignant glands of the prostate [26, 28, 29].

Tamboli *et al*. [30] compared the nuclear proliferative activities of benign prostatic tissue, PIN, and prostate cancer in non-hormone treated patients using the MIB-1 antibody. They reported that the MIB-1 index consistently increased in benign, PIN, and malignant epithelium. In the present study, the MIB-1 index also increased from normal epithelium to PIN to neoplastic prostatic tissue. Additionary, the MIB-1 index also significantly increased with increasing tumor grade. However, the proliferative activity of PIN was only slightly higher than that of normal epithelium compared with carcinoma. This finding suggests that the aggressive potential of PIN is not as high as that of carcinoma.

There is now increasing evidence that NE differentiation occurs frequently in prostatic carcinoma. This may have prognostic and therapeutic implications [8-10, 31]. CGA is an acidic, water-soluble protein present in the secretory vesicles of NE cells [32]. Weinstein *et al*. [9] reported that NE differentiation, as measured by the presence of CGA, is very common in prostatic carcinoma, and they suggested that NE differentiation in carcinoma correlates with poor prognosis. The regulatory role played by the NE cell in the prostate is unknown, although it is highly likely that these cells regulate both growth and differentiation [33]. In the present study, CGA positive cells were more frequent in PIN and carcinoma than in normal epithelium. This finding suggests that PIN shares some biological characteristics with carcinoma.

Several studies have shown either the existence of point mutations in the AR gene or AR gene amplification occurring during cancer progression [11, 12, 34]. However, immunohistochemical staining levels of AR protein in normal epithelium, PIN and carcinoma tissue are still controversial [13, 35]. In this study, immunohistochemistry showed the presence of nuclear AR in both prostatic stromal cells and glandular epithelium. Similarly, prostatic adenocarcinoma tissue was comprised of a majority of AR positive cells. On the other hand, there was weak nuclear staining and occasionally cytoplasmic staining observed in PIN tissue. Our data bears a striking resemblance to a report by Magi-Galluzzi *et al*. [36], who found both uniformly decreased nuclear staining and cytoplasmic localization of AR in high-grade PIN. From these studies, we speculate that the distribution of AR protein has been altered in PIN tissue.

FISH has been recognized as a reliable method of detecting aneuploidy. Persons *et al*. [37] evaluated the FISH ploidy patterns of prostate tumors using chromosome 8 and 12 centromere probes, and compared their results to flow cytometry (FCM) ploidy patterns. They reported that FISH was more sensitive than FCM for detection of aneuploidy. Qian *et al*. [24] studied numerical chromosomal anomalies and gene copy number anomalies in PIN and carcinoma tissue using DNA probes for chromosomes 7, 8, 10, 12, and Y, and for chromosome region 8q24 (*c-myc*). Their results revealed that aneuploidy, especially of chromosome 8, as well as extra copies of *c-myc* gene were identified both in

PIN and in carcinoma tissue. Our study also demonstrated that polysomy of chromosome 8 was detected in the majority of PIN and carcinoma foci. From these findings, it would seem that PIN and prostate carcinoma show similar aneuploid characteristics.

In conclusion, the present study concerning proliferative activity, NE differentiation and chromosomal anomaly of prostate specimens supports the hypothesis that PIN is a biologically intermediate stage in the pathogenesis of prostatic carcinoma. However, since the distribution of AR protein was different in PIN from that in normal epithelium or carcinoma, more studies are needed to elucidate the role of AR in PIN.

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