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p16^{INK4a}, PTEN, E-cadherin, Bcl-2 and Ki-67 Expression in Prostate Cancer: Its Relationship with the Metastatic Potential and Known Prognostic Factors

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Background : At present, adequate prognostic markers for prostate cancer progression are still lacking, in spite of intensive investigation. Accordingly, our study examined the relationship between expression of candidate biomarkers and metastasis in prostate cancer patients. Correlation of molecular markers with prostate-specific antigen (PSA) level, Gleason sum score and tumor stage were also evaluated. **Methods :** A total of 105 prostate tumor specimens and specimens from 19 cases of nodular hyperplasia were obtained through Yeungnam University Hospital from 2007 to 2008. Immunohistochemical analyses for p16^{INK4a}, phosphatase and tensin homolog (PTEN), E-cadherin, Ki-67 and Bcl-2 were performed. **Results :** Overexpression of Bcl-2 was significantly related to bone ($p = 0.006$) and nodal metastases ($p = 0.017$). Other biomarkers were not related to metastatic potential. There were statistically significant relationships between increased PSA level and loss of expression of PTEN ($p = 0.019$) and E-cadherin ($p = 0.001$). High Ki-67 index was significantly correlated with nodal metastasis ($p = 0.029$) as well as with loss of p16^{INK4a} expression ($p = 0.002$) and high Gleason score ($p = 0.011$). **Conclusions :** High Gleason score, Bcl-2 overexpression and increased Ki-67 labeling have significant predictive value in assessing the potential for prostate cancer metastasis. In addition, a high Ki-67 index is related to high Gleason score and loss of p16^{INK4a} expression.

Key Words : Prostatic neoplasms; bcl-2; PTEN protein, human; Cadherins; Ki-67 antigen

Prostate cancer is the most common cancer in men in Western countries[1] and has recently been increasing in prevalence in Korea.[2] Screening for prostate-specific antigen (PSA) in asymptomatic populations has increased the detection rate of prostate cancer and is useful in following patients after radical prostatectomy. Gleason grade is one of the most widely-used grading systems for predicting the progression of prostate carcinoma. A more aggressive disease is associated with higher Gleason sum scores. However, the pathological grade, serum PSA value and clinical stage have some restrictions that hinder evaluation of the prognosis of prostate carcinoma, although the PSA level combined with the Gleason grading system is still considered the most reliable prognostic marker.[3-5] At present, adequate prognostic or predictive markers for tumor progression are still lacking. Therefore, efforts to identify additional predictive markers are currently focused on prostate cancer, with the aim of developing markers to distinguish, at an early stage, between aggressive high-risk carcinoma and indolent low-risk carcinoma. Recently, candidate molecules involved in diverse processes such as cell proliferation, apoptosis, cellular adhesion, tumor suppression and cell cycle-related factors have been linked to prostate cancer outcome.[5,6] Our study examined p16^{INK4a}, phosphatase and tensin homolog (PTEN), E-cadherin, Ki-67 and Bcl-2 expression pattern in prostate cancer patients by immunohistochemistry. We tried to determine their value as predictive markers for metastatic potential. In addition, the relationship between these molecular markers and known prognostic factors of serum PSA and Gleason grade were evaluated.

MATERIALS AND METHODS

Case selection

Prostate tumors were obtained from needle biopsies (98 cases) or transurethral resection (20 cases) or surgical specimens of

patients (6 cases) at Yeungnam University Hospital between March 2007 and February 2008. Clinical data were obtained from files of prostate cancer patients treated at the Department of Urology. A total of 124 specimens (105 prostate cancers and 19 nodular hyperplasias) were used. Among these samples, evaluation for bone and lymph node metastasis were possible in 72 and 63 cases, respectively. Therefore, we did a correlation study between expression of immunomarkers and metastatic potential. The selected samples were histologically examined by two pathologists. Serum PSA values were determined using an enzyme-linked immunosorbent assay and measured for all samples. All analyzed specimens were divided into three groups depending on serum PSA values and Gleason scale gradation. The PSA values of the patients were evaluated as < 4, 4-20 or > 20 ng/mL. In the microscopic examinations of prostate cancer, Gleason scores were considered as ≤ 6, 7, and 8-10 as previously described, based on a modified Gleason grading system.[7]

Immunohistochemistry

The specimens were immediately preserved in buffered formalin (phosphate buffer, pH 7.4) with subsequent preparation of paraffin blocks for ordinary histological examination. Immunohistochemical staining was performed for $p16^{INK4a}$, PTEN, E-cadherin, Ki-67 and Bcl-2 in 4 μm-thick paraffin embedded sections of the specimens. After deparaffinization, slides were treated in a microwave oven for 30 minutes for antigen retrieval. Immunohistochemical reactions were done using a Benchmark® XT automated immunostainer system (Ventana Medical Systems, Tucson, AZ, USA) with the solutions and steps done according to the manufacturer's instructions. The expression of proteins on tumor tissues was detected using an UltraView Universal DAB detection kit (Ventana Medical Systems) for antibodies against $p16^{INK4a}$ (1 : 100, Labvision, Fremont, CA, USA), PTEN (1 : 100, Zymed Laboratories, San Francisco, CA, USA), E-cadherin (1 : 300, Zymed Laboratories), Ki-67 (1 : 200, Zymed Laboratories) and Bcl-2 (1 : 300, Zymed Laboratories). Immunostaining for five markers was assessed as positive reactivity when over 50% of the tumor cells stained with moderate to strong intensity. Assessment of Ki-67 immunostaining was performed using a labeling index, wherein the number of positively stained nuclei per 100 nuclei counted was assessed and scored as a percentage. For convenience, Ki-67 index was evaluated as < 10% (group 1), 10-20% (group 2) and ≥ 20% (group 3), based on reference data obtained through a preliminary study including statistical application.

Statistical analyses

Statistical analyses were performed using Pearson's chi-square tests or the Fisher's exact tests where appropriate. Associations between molecular markers and known prognostic factors of prostate cancer were analyzed by one way ANOVA. p-values < 0.05 were considered to indicate statistical significance. All analyses were performed using SPSS ver. 16.0 (SSPS Inc., Chicago, IL, USA).

RESULTS

Correlation between patients' characteristics and metastatic potential

The relationship between known prognostic factors in prostate cancer patients and bone or lymph node metastasis is summarized in Table 1. The mean age of the patients was 69 years old (range, 51 to 84 years). In prostatic cancer patients, the PSA level was 72.6 ± 208.3 ng/mL, whereas the PSA value in nodular hyperplasia was 14.3 ± 35.1 ng/mL. There was a significant difference in serum PSA level (p = 0.037), but not in patient age between prostate cancer and nodular hyperplasia. In prostate carcinoma, the serum PSA value was not significantly correlated with bone or lymph node metastasis. On the other hand, there was a statistically significant association between high Gleason score and nodal metastasis (p = 0.003).

Analysis of immunohistochemical results

Hyperexpression of cytoplasmic Bcl-2 protein was detected in 11 of 105 prostate cancer patients (10%) and in all nodular hyperplasia patients (Fig. 1). Overexpression of Bcl-2 protein was significantly associated with bone metastases (p = 0.027) and nodal metastases (p = 0.017). In relation to lymph node metastasis, Ki-67 was significantly expressed in prostatic cancer (p = 0.029). No statistically significant difference in Ki-67 index was observed when comparing prostate cancer patients who had bone metastasis with those who had no metastasis. Other biomarkers did not have a statistically significant effect on metastatic potential (Table 2). Ki-67 was significantly high in prostatic cancer (9.64%) compared to that of nodular hyperplasia (2.45%) (Fig. 2). PSA values were related to loss of PTEN (p = 0.019) and E-cadherin expression (p = 0.001) (Table 3, Fig. 3). With higher Gleason score, Bcl-2 (p = 0.043) and Ki-

Table 1. Correlations between clinical characteristics of patients and metastatic potential

Characteristics	Bone metastasis (n = 72)			p-value	Lymph node metastasis (n = 63)			p-value
	n	(-)	(+)		n	(-)	(+)	
Gleason score								
< 6	13	10	3	0.322	20	20	0	0.003
7	16	11	5		10	8	2	
≥ 8	43	27	16		33	21	12	
PSA level								
< 4	11	9	2	0.065	13	10	3	0.065
4-20	22	19	3		13	12	1	
≥ 20	39	20	19		37	22	15	

PSA, prostate-specific antigen.

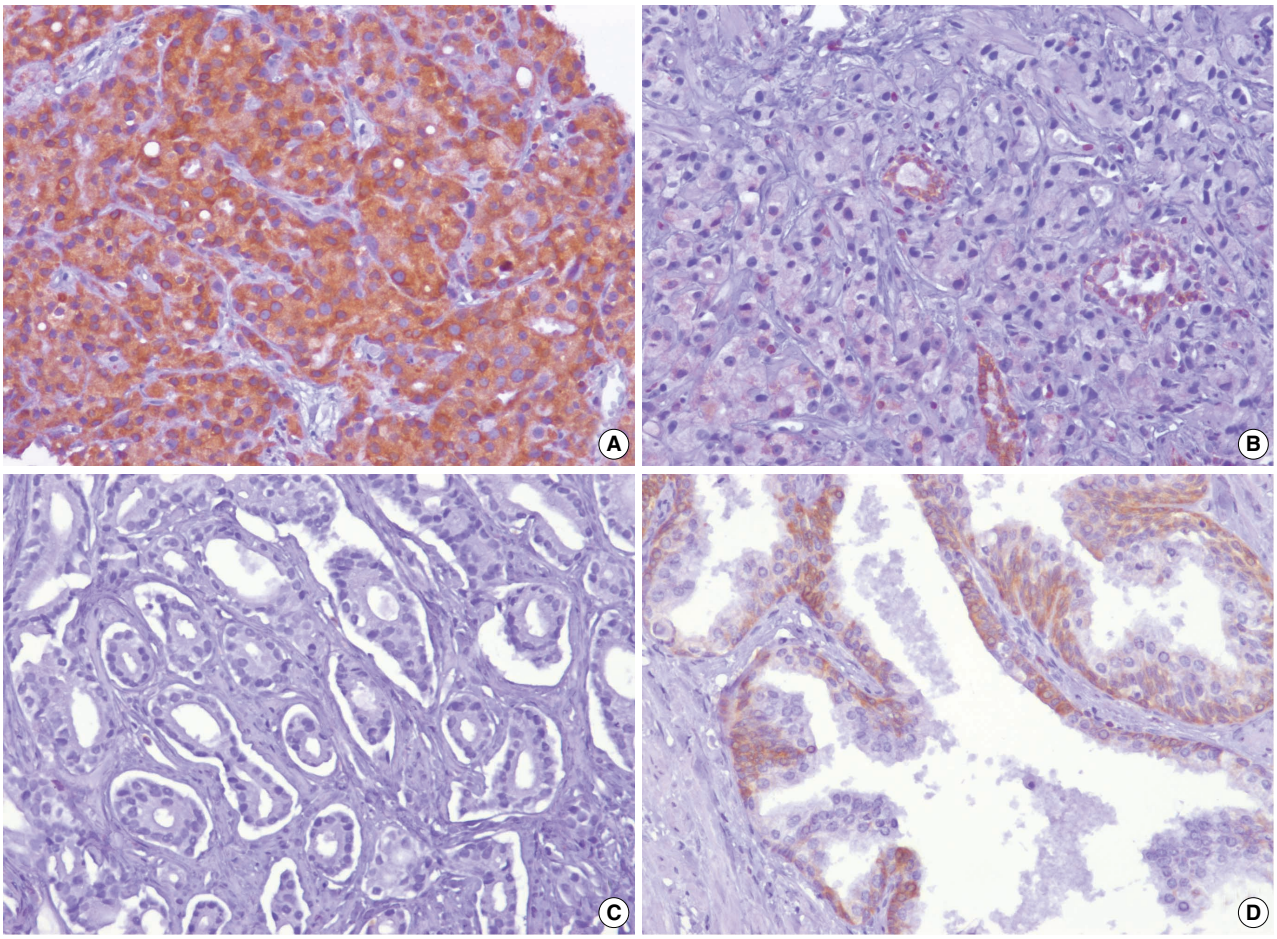

Fig. 1. Bcl-2 expression in prostate carcinoma. There is a diffuse and strong Bcl-2 overexpression in prostate carcinoma with high Gleason grade (A) as well as in nodular hyperplasia (D). Decreased or absent Bcl-2 expression is noted in prostate cancer with low Gleason grade (C) and occasionally with high grade (B).

67 (p = 0.011) were significantly overexpressed in prostate cancer (Table 4). There was a significant correlation between high Ki-67 index and loss of $p16^{INK4a}$ (p = 0.002) (Table 5, Fig. 4).

DISCUSSION

Potential markers involved in numerous biological processes of prostate cancer have been investigated intensively. The difference between aggressive high-risk carcinomas and indolent

Table 2. Correlations between expression of biomarkers and metastatic potential

Biomarkers	Bone metastasis (n = 72)			p-value	Lymph node metastasis (n = 63)			p-value
	n	(-)	(+)		n	(-)	(+)	
Bcl-2								
Positive	10	3	7	0.027	9	3	6	0.017
Negative	62	44	18		54	41	13	
E-cadherin								
Positive	28	19	9	0.714	23	15	8	0.544
Negative	44	28	16		40	29	11	
$p16^{INK4a}$								
Positive	25	18	12	0.427	27	19	8	0.937
Negative	47	29	13		36	25	11	
PTEN								
Positive	47	10	3	0.364	11	10	1	0.094
Negative	25	37	22		52	34	18	
Ki-67 index								
< 10%	52	38	14	0.057	47	37	10	0.029
10-20%	11	4	7		8	3	5	
≥ 20%	9	5	4		8	4	4	

PTEN, phosphatase and tensin homolog.

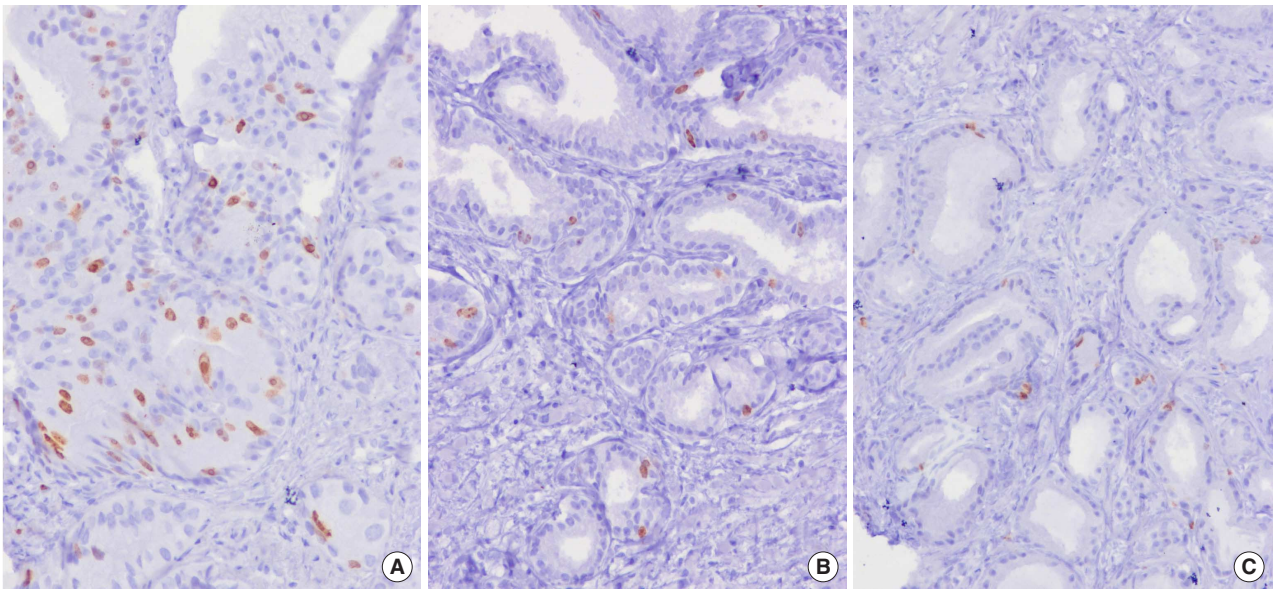

Fig. 2. Ki-67 immunostaining patterns (Ki-67 labeling index). High grade prostate cancer shows significantly increased Ki-67 index (15.1%) (A). On the contrary, low grade prostate cancer (5.2%) (B) and nodular hyperplasia (2.1%) (C) show decreased indices.

low-risk carcinomas is its potential to metastasize. Although many potentially prognostic markers have been studied, few have been incorporated into prognostic models of therapeutic decision making.[8]

Bcl-2 is an oncogene that codes for a protein that suppresses apoptosis.[9] It is well-known that the protein encoded by the *Bcl-2* pro-oncogene can arrest apoptosis of prostate cells induced by p53 protein or other stimuli. In the case of Bcl-2 protein overexpression, it acts as an oncogene and participates in the formation of an androgen-resistant phenotype and tumor resistance to chemotherapeutic compounds.[10] In our study, there was a direct correlation between metastasis and overexpression of Bcl-2 protein. This result and earlier studies[11-13] suggest that Bcl-2 overexpression could be a high-risk marker of prostate carcinoma. These results also suggest that defective signaling of apoptosis likely contributes to high levels of Bcl-2, and, as in many cancers, subsequent development of prostate cancer and treatment failure. A recent study has shown that the expression

Table 3. Expression of potential markers according to serum PSA value

PSA (n = 124)	p16 ^{INK4a}		PTEN		E-cadherin		Bcl-2		Ki-67		
	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	1	2	3
< 4 (n = 23)	10	13	11	12	5	18	13	9	19	1	3
4-20 (n = 46)	22	24	28	18	24	22	38	8	39	5	2
≥ 20 (n = 55)	27	28	41	14	35	20	43	13	38	12	5
p-value	0.672		0.019		0.001		0.065		0.333		

1, 2 and 3 in Ki-67 represents Ki-67 labeling index < 10%, 10-20% and ≥ 20%, respectively.
PSA, prostate-specific antigen; PTEN, phosphatase and tensin homolog.

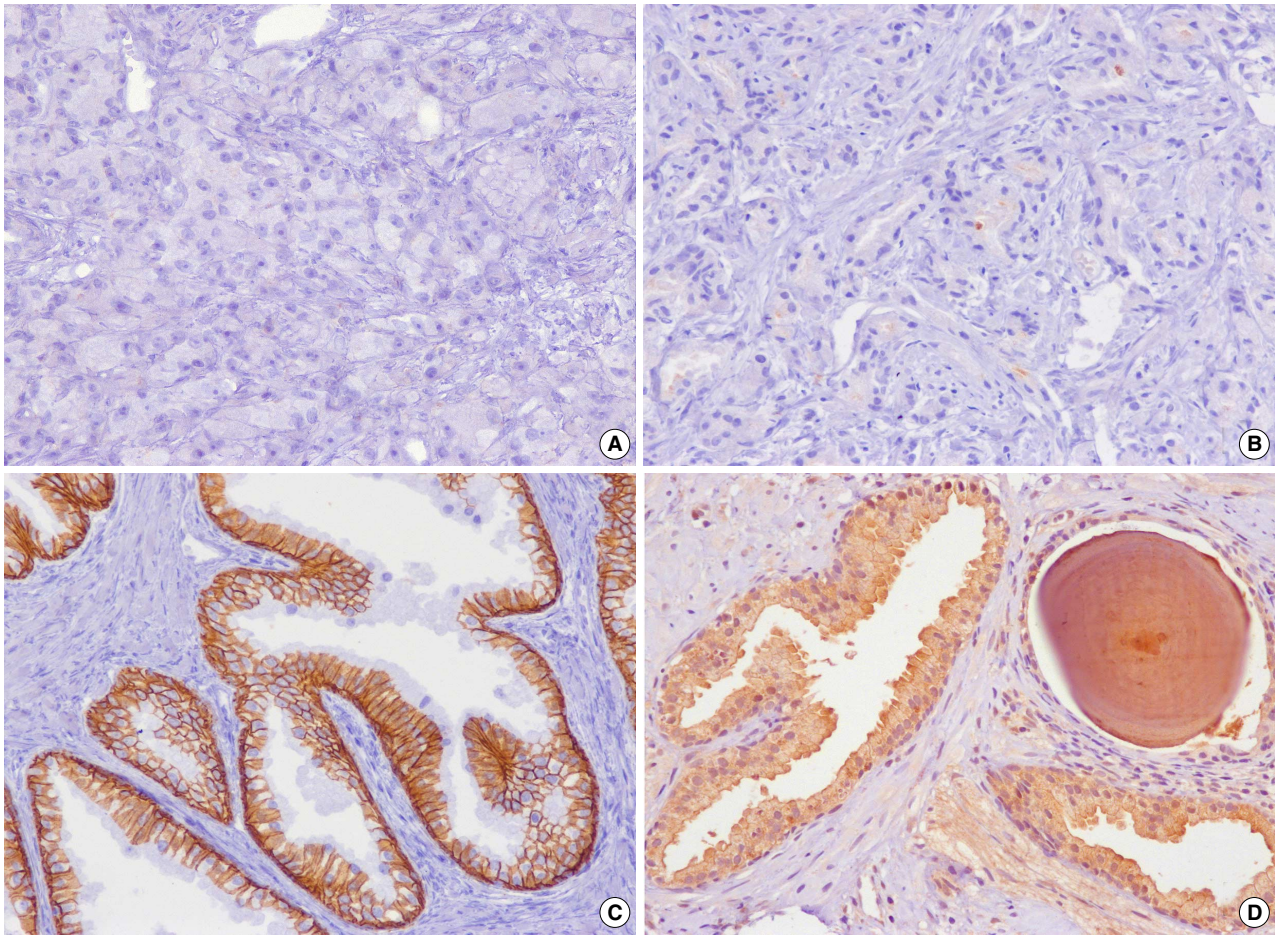

Fig. 3. E-cadherin and phosphatase and tensin homolog (PTEN) expression. Loss of E-cadherin (A) and PTEN (B) expression is noted in prostate carcinoma with high serum prostate-specific antigen (PSA). In contrast, nodular hyperplasia displays diffuse membranous and cytoplasmic expression of E-cadherin (C) and PTEN (D), respectively.

of BAD, a pro-apoptotic protein of the Bcl-2 family, is elevated in prostate carcinoma and might play a positive role in prostatic tumor growth.[14,15] However, other studies have shown that Bcl-2 does not have prognostic significance in prostate cancer patients.[16] We found that immunohistochemical expression of Bcl-2 was associated with the Gleason score, but not with the PSA value. A high Gleason score by itself contributed to the prediction of aggressive progression of prostate cancer. Iemelynova *et al*.[17] reported a correlation between Gleason scale and expression of Bcl-2. They emphasized the fact that the Gleason scale has both diagnostic and prognostic value. Our results suggest that a combination of overexpression of Bcl-2, together with high Gleason score, may be a marker for high-risk prostate cancer. In addition, Bcl-2 expression may have predictive value

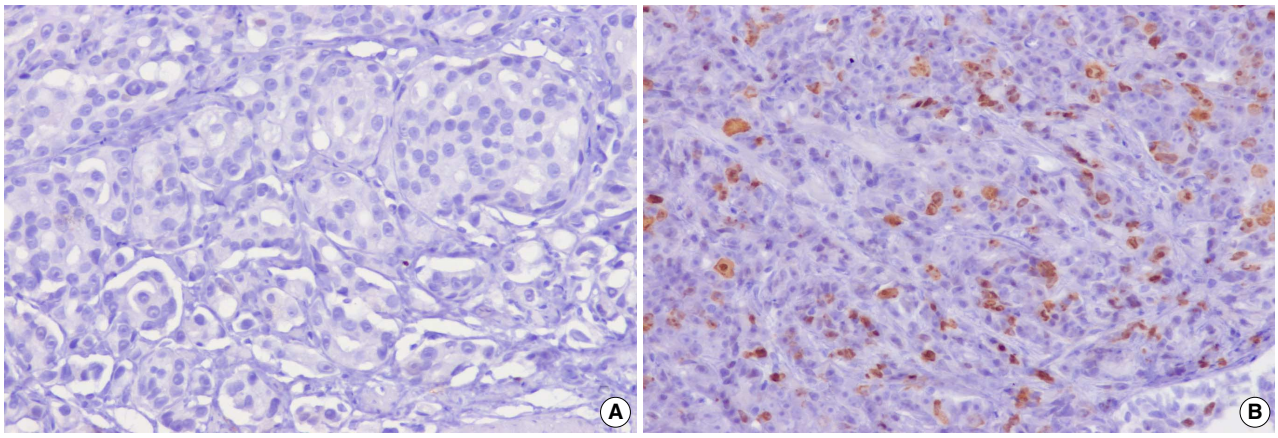

Fig. 4. Correlation between $p16^{INK4a}$ and Ki-67 expression. Loss of $p16^{INK4a}$ (A) is associated with increased Ki-67 labeling index (B) in the same case.

Table 4. Expression of potential markers according to Gleason score (n = 105)

Gleason score	p16 INK4a		PTEN		E-cadherin		Bcl-2		Ki-67		
	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	1	2	3
< 6 (n = 25)	10	10	12	8	9	11	20	0	18	2	0
7 (n = 9)	13	12	18	7	13	12	23	2	23	2	0
≥ 8 (n = 30)	34	26	50	10	42	18	50	10	37	13	10
p-value	0.845		0.090		0.080		0.043		0.011		

1, 2 and 3 in Ki-67 represents Ki-67 labeling index < 10%, 10-20% and ≥ 20%, respectively.
PTEN, phosphatase and tensin homolog.

Table 5. Relationship between potential markers and Ki-67 index (n = 87)

Ki-67 index	p16 INK4a		PTEN		E-cadherin		Bcl-2	
	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)
< 10%	41	21	50	12	39	23	56	6
10-20%	6	9	11	4	11	4	12	3
≥ 20%	1	9	10	0	7	3	8	2
p-value	0.002		0.221		0.711		0.423	

PTEN, phosphatase and tensin homolog.

regardless of Gleason grade. Bcl-2 protein may have potential as an independent prognostic marker.

E-cadherin is a transmembrane glycoprotein that mediates cell-cell adhesion. While the expression of E-cadherin has been extensively studied in prostate tumors,[18-21] the results are rather controversial. In low-risk cancer, E-cadherin is expressed as in normal tissue. Expression of E-cadherin has been reported to be decreased in high-risk carcinomas and carcinomas with a positive surgical margin.[22] Furthermore, metastatic tissues showed strong E-cadherin staining, despite the absence of E-cadherin expression in primary tumors. These results indicate that the loss of E-cadherin is a transient event occurring during invasion. We found no statistically significant difference in the loss of E-cadherin between metastatic and non-metastatic prostate carcinomas. As previously reported,[20-22] we found that the loss of E-cadherin expression was correlated with serum PSA. The PSA value is known to be related to the post-operative parameter of prostate cancer recurrence. Our results indicated that there was a tendency, albeit insignificant, towards an increased Gleason score in patients with a loss of E-cadherin. This result indicates that E-cadherin expression may not be used as an individual marker. It is reasonable to use E-cadherin expression data in combination with other markers suggesting metastasis.

$p16^{INK4a}$ expression was noted not only in prostate carcinoma, but also in nodular hyperplasia and normal prostate. Our study demonstrates that loss of $p16^{INK4a}$ expression is not significantly associated with distant metastasis or with any known prognostic indicators. However, low expression of $p16^{INK4a}$ was strongly correlated with increased Ki-67. Considering $p16^{INK4a}$ is an inhibitor the cell cycle, this result indicates that a functional disorder of or inactivation of the *p16*^{INK4a} gene can lead to loss of control of mitosis. Low levels or loss of $p16^{INK4a}$ protein were

associated with a significantly higher risk of distant metastasis and adverse clinical outcomes in most of the studies reported.[23,24] In contrast, Zhang *et al*.[25] reported that $p16^{INK4a}$-positive cells were detected in premalignant lesions (19%), low grade carcinoma (25%) and high grade carcinoma (43%), but not in normal or benign tissue. Further, a recent study reported that overexpression of $p16^{INK4a}$ has an effect on formation and differentiation of prostate cancer and was associated with adverse prognosis in prostate cancer.[26] These conflicting results could be secondary to heterogeneity of prostate cancer and a lack of an interpretation guideline for the evaluation of immunostaining of biomarkers.

PTEN, which is a dual lipid/protein phosphatase, acts as a tumor suppressor by inhibiting the kinase activities of critical-tumor-promoting kinases such as phosphoinositide 3-kinase. Previous studies showed that loss of PTEN expression is an important factor in progression towards metastatic disease, potentially serving as an early prognostic marker for prostatic cancer metastasis.[27,28] In addition, the loss of PTEN function in prostate cancer specifically facilitates bone metastasis.[29] In our study, PTEN was not related to metastatic potential. However, loss of PTEN expression was correlated with a high serum PSA level, suggesting its potential role in prostate cancer progression. A previous study showed that the loss of PTEN expression in combination with a high Gleason score has significant predictive value for biochemical recurrence.[27]

The Ki-67 index is considered to have prognostic significance for prostatic cancer. In this study, Ki-67 expression was correlated with an adverse clinical outcome in prostate cancer by a significant association with lymph node metastasis, high stage and increased Gleason score, which was also consistent with earlier studies.[11,30]

The results of our study suggest that a combination of Bcl-2 overexpression, increased Ki-67 index and high Gleason score is of significant predictive value for the progression of prostate cancer to metastasis. In addition, loss of PTEN and E-cadherin expression was significantly associated with increased PSA level. Our data also confirm the prevalent opinion of the importance of known prognostic indicators. Further study is needed to validate these markers with relation to stage grouping in prostatectomy specimen and to analyze multiple molecular marker assays that are useful in practice. In particular, analyzing a combination of potential markers is expected to be a better approach toward discriminating high- from low-risk tumors in an early stage of prostate cancer, because of the limitation associated with individual markers.

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