Correlation of neuroendocrine differentiation and PTEN expression with pathologic effects after bicalutamide monotherapy in prostate cancer

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Correlation of neuroendocrine differentiation and PTEN expression with pathologic effects after bicalutamide therapy in prostate cancer

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<ABSTRACT>

Correlation of neuroendocrine differentiation and PTEN expression with pathologic effects after bicalutamide therapy in prostate cancer

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Purpose: The knowledge of molecular changes after neoadjuvant bicalutamide therapy seems to be primary requirement to evaluate the efficacy or the failure of a second-line therapies, which would be applied after progression into hormone-refractory status. Based on these considerations, we have analyzed the expressions of tensin homolog deleted on chromosome 10 (PTEN), human epidermal receptor (HER)-2, and neuroendocrine differentiation in the radical prostatectomy (RP) specimens.

Materials and methods: We evaluated these molecular arrangements in 107 RP specimens from patients who took bicalutamide 150 mg before surgery in terms of pathological regressive changes and assessed whether biochemical failure after RP correlates with the extent of these molecular arrangements and pathologic effects.

Results: The patients showing minimal regression effects after bicalutamide therapy was related to the advanced pathologic stage and tended to have positive chromogranin A (CgA) expression and PTEN inactivation. Only 4 (3.7%) prostatectomy specimens immunostained for HER-2 and there were no HER-2 gene amplifications in all samples. The probability of having positive CgA expression in the PTEN inactivation

group was 2.5-fold (OR 2.5, 95% CI 1.1 to 5.6, p=0.023) greater than in the non-PTEN inactivation group. Cox regression analysis revealed that seminal vesicle invasion, biopsy Gleason score, PTEN/CgA expression were significant variables for the time to biochemical recurrence.

Conclusions: PTEN inactivation together with neuroendocrine differentiation is related to refractoriness for bicalutamide therapy and these results support the hypothesis that neuroendocrine differentiation is caused by activation of serine threonine kinase, AKT pathway, which results from PTEN inactivation.

Key words : prostate cancer, PTEN, HER-2, neuroendocrine differentiation, bicalutamide therapy

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I. INTRODUCTION

Prostate cancer (PC) is the most common noncutaneous cancer among men in western countries.[1] Radical prostatectomy (RP) is the most effective and reliable treatment for patients with locally confined PC. The goal of RP is complete resection of all malignant cells. However, even in carefully selected patients with clinically organ-confined tumors, the positive surgical margin rates range from 10% to 60%.[2-4] Patients with incompletely resected PC are at an increased risk of recurrence and shorter overall survival. To improve the survival results, an approach that combines systemic therapy with local therapy seems warranted. Such a multimodality approach could take the form of neoadjuvant hormonal therapy (NHT) before surgery. However, NHT before the surgery has failed to demonstrate an improvement in long-term outcomes although patients with good pathological effects after NHT tend to have a favorable prognosis.[5,6] Despite these negative effects, the relative short treatment time and the use of different drugs from those used in other trials might still make this regimen attractive in terms of drug-related side effects and effectiveness. In this regard, bicalutamide presents unique characteristics, because it works differently than other antihormonal agents by interfering with both genotropic and nongenotropic mechanisms of androgen receptors.[7] Several hypothesis have been generated to explain the lack of effectiveness of NHT,[8] including relatively short follow-up

periods, studies that were not correctly powered, the relative short time of NHT administration, and the potential effect of androgen deprivation therapy to alter the assessment of positive surgical margins. However, the lack of response might also be the result of a particular molecular arrangement acquired during NHT.

The molecular mechanisms underlying the adaptative phenomenon after an androgen deprivation therapy have not been elucidated, but accumulating evidence indicates that PI3K/Akt signaling pathway is involved.[9-12] Activation of phosphatidylinositol 3-kinase (PI3K) leads to increased serine threonine kinase Akt phosphorylation and activation. In turn, activated Akt inhibits PC cell suicide by blocking many of the key components of the suicide 'machinery'. Although other mechanisms, such as autocrine growth factor loops, may contribute to activation of PI3K/Akt in PC cells, about 30% of PCs exhibit phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a potent tumor suppressor gene. Mutations and the loss of PTEN leads to constitutive activation of the Akt pathway providing a mechanism whereby prostate tumor cells survive after the withdrawal of exogenous trophic factors or androgens.[13-15]

In addition, there are multiple lines of evidence indicating that the human epidermal receptor (HER)-2 is signaling molecules that control many aspects of tumor cell behavior-inducing changes in the survival rate. High levels of HER-2 can be demonstrated in PC, especially after an androgen deprivation therapy.[16,17] The increased expression and activation of this receptor start a signaling cascade resulting in tumor proliferation, cell adhesion and invasion.

Neuroendocrine (NE) differentiation (NED) is also thought to contribute to androgen-independent growth of PC.[18,19] The number of NE cells increases in high grade and high stage tumors and particularly in hormonally treated and androgen-independent tumors. It is hypothesized that hormonal therapy induces NED and the NE cells contribute to androgen-independent growth of PC in the androgen-deprived environment by secreting their products to act on the adjacent

non-NE tumor cells in a paracrine fashion. Moreover, it has been shown recently that Akt is critically involved in NED of PC after androgen deprivation.[20] Therefore, the knowledge of tumor molecular arrangement, especially after pharmacologic treatment, might be used to have information on the probability of survival or to tailor pharmacologic regimens to individual patients.

Based on these considerations, we evaluated these molecular arrangements in the radical prostatectomy specimens in relation to the effects of different durations of neoadjuvant bicalutamide therapy, in terms of regressive pathological changes and assessed whether biochemical failure after RP correlates with the extent of these molecular arrangements and pathologic effects.

II. MATERIALS AND METHODS

1. Patient cohort and treatment

Of 685 patients who underwent RP for PC between August 1995 and June 2007 at our institution, we identified 107 patients received neoadjuvant bicalutamide therapy (150 mg/day) before surgery. During the pre-operative examination, we performed a digital rectal examination, bone scan, and computed tomography or magnetic resonance imaging for all patients. We used the 2002 TNM classification for clinical staging. The length of neoadjuvant bicalutamide therapy was made at the physician's discretion. Serum prostate-specific antigen (PSA) levels were measured before the start of neoadjuvant bicalutamide therapy and on the day before surgery in all patients. All patients underwent bilateral pelvic lymph node (LN) dissection. Patients were followed every 3 months during the first 2 years, every 6 months until year 5 and annually thereafter. The median follow-up period was 16 (2-97) months. A detectable PSA level (0.2 ng/ml or greater) at 6 weeks post-operatively was defined as persistent PSA and two serial detectable PSA levels after reaching the nadir or non-detectable level was defined as biochemical failure. Adjuvant treatment (androgen deprivation and/or radiation) was administered to patients with biochemical failure.

2. Evaluation of pathological therapeutic effects

The resected prostatectomy specimens were coated over their entire surface with Indian ink and fixed in 4% buffered formalin and paraffin embedded. The whole-mount step sections were cut transversely at 5 mm intervals from the apex of the prostate to the tips of the seminal vesicles (SVs). Each section was examined for SV invasion, extracapsular extension (ECE), and surgical margins. The total tumor volume was determined by planimetry using a digitizer tablet as described previously (V1: ≤1.0 ml, V2: 1.1-5.0 ml, V3: ≥5.0ml).[21] All areas of

the tumors, including index tumor and all satellite tumors, were used to determine the total tumor volume in each specimen. Assessment of effect of NHT was based on the presence and assessment of nuclear pyknosis, nuclear karyolysis and cytoplasmic vacuolization, reduction in size or number of carcinoma nests, loss of glandular architecture, and stromal hyalinosis and sclerosis.[22-24] Based on the extent of carcinoma cell degeneration on H&E sections, pathological therapeutic effects graded as minimal, moderate, and extensive.[23,24]

3. Construction of tissue microarrays (TMAs)

Tissue microarrays were prepared using archival formation-fixed and paraffin-embedded prostatectomy specimens as described by Kononen et al.[25] To minimize misrepresentation due to tumor heterogeneity, 3-4 cores, 0.6 mm in diameter each, were obtained. The donor paraffin blocks with corresponding hematoxylin & eosin reference slides were analyzed by a pathologist to identify the most representative sections with cancer before core extraction.

4. Reagents

Antibody against HER-2 was obtained from Invitrogen (Invitrogen, Carlsbad, CA, USA) and HER-2 evaluation was performed using HercepTest (DakoCytomation, Glostrup, Denmark). We have selected primary polyclonal antibodies for NED: rabbit anti-human chromogranin A (CgA) from Dako Corporation, Carpinteria, CA, USA. PTEN was purchased from Rockland immunochemicals (Gilbertsville, PA, USA).

5. Immunohistochemical studies (IHC)

Mounted tissues on microarrays (5 μm) were deparaffinized, blocked and processed for immunohistochemical detection of PTEN, NED, and HER-2 using standard techniques, including an avidin-biotinylated peroxidase complex

immunoreactivity (IR) for PTEN and HER-2 was detected on 4 μm tissue sections cut and evaluated blindly by one uropathologist.

PTEN IR was scored according to the following formula: staining index (SI)=(cytoplasmic staining intensity× proportion of immunopositive tumor area). PTEN IR was scored as follows: cytoplasmic staining intensity (0–3) and the proportion of immunopositive tumor cells (≤10%=1; 10–50%=2; ≥50%=3) with a PTEN SI ranging between 0 and 9 and a PTEN index of ≤4 indicating a low expression.

HER-2 IR was scored as follows: 0, <10% of PC cells stained; 1+, >10% of PC cells had faint and incomplete membranous pattern; 2+, >10% of PC cells had weak to moderate and complete membrane staining; and 3+, >10% of PC cells had strong and complete membrane staining pattern. An IHC score of 2+ or greater was considered as high expression for HER-2.

CgA positive tumors were counted in all neoplastic lesions using a gridded eyepiece at 200× magnification. The staining of tumors was classified using the following scoring system: 0= no immunoreactive tumor cells; +1= immunoreactive neoplastic cells <10%; +2= 10–20% immunoreactive tumor cells; +3= >20% immunoreactive neoplastic cells.

6. Fluorescence In Situ Hybridization (FISH)

Two-color FISH was performed on tissues sectioned 4 μm in thickness. Tissue sections were incubated at 56℃ for 24 hr, deparaffined, dehydrated with 100% ethanol, and dried in room air. The tissue slides were treated in 0.2 N HCl for 20 min and washed for 3 min using wash buffer (Vysis Inc., Downers Grove, IL). The tissue slides were incubated in pretreatment buffer (Vysis) at 80℃ for 30 min, washed once with distilled water, and washed with wash buffer two times for 5 min each. The slides were then immersed in Protease solution

(Vysis) at 37℃ for 10 min, washed with wash buffer at 45–50℃, dried in room air for 10 min, and washed again at 40–50℃. Slides were then fixed with 10% formalin for 10 min and washed at 45–50℃. The slides were immersed in denaturation solution (Vysis) at 72℃ for 5 min and dehydrated at 45–50℃ with sequential incubations in 75, 85, and 100% ethanol. Ten microliter of LSI HER-2/CEP17 probe (PathVysion™; Vysis) was added, and the slides were sealed with a coverglass and hybridized overnight with Hybrite (Vysis) at 37℃. The slides were washed with posthybridization wash buffer (Vysis) at 72℃ for 2 min. The nucleus was counterstained with 10 μl 4, 6-diamino-2-phenylindole (Vysis). Copy numbers of centromere 17 (CEP) and HER-2 were counted. Cells that were morphologically normal and had a ratio of pink HER-2 signal to green CEP signal higher than 2 in non-overlapping nuclei were classified as having a gene amplification.

7. Statistical analysis

Statistical analysis was performed using Statistical Software for the Social Sciences, version 12 (SPSS Inc, Chicago, IL, USA). Continuous variables were treated with the Kruskal-Wallis rank test. Nominal variables were analyzed with the chi-square test (or Fisher's exact test when the tables were too sparse) and linear by linear association. Survival curve analysis for the time to biochemical failure was done using the Kaplan-Meier method. A log-rank test was first performed for univariate analysis and then a Cox regression analysis was performed to identify significant variables among the parameters including biopsy Gleason score, initial PSA level, clinical stage, SV invasion, surgical margin, and PTEN/CgA expression. Differences with a probability of <0.05 were considered significant.

III. RESULTS

1. Clinico-pathological characteristics

The effects of bicalutamide therapy were histologically evaluated from the prostatectomy specimen: minimal regression in 66 cases, moderate in 33 and extensive in 8 (Fig. 1). There was a significant difference in the age between 3 groups. However, there were no significant differences in the duration of bicalutamide therapy, clinical stage between 3 groups. The serum PSA levels before the bicalutamide therapy did not correlate to the regression grade, but the serum PSA levels after the bicalutamide therapy were significantly lower in the patients with extensive regression, compared to the patients with minimal or moderate regression. The pathological parameters including biopsy Gleason score, pathological stage, tumor volume, and the incidence of ECE, SV invasion, and positive surgical margin after bicalutamide therapy were related to the pathological regression grade, whereas, no significant difference was noted with regard to LN invasion (Table 1).

Fig. 1. A case showing (A) minimal, (B) moderate, (C) extensive response to bicalutamide therapy (H&E, x 100)

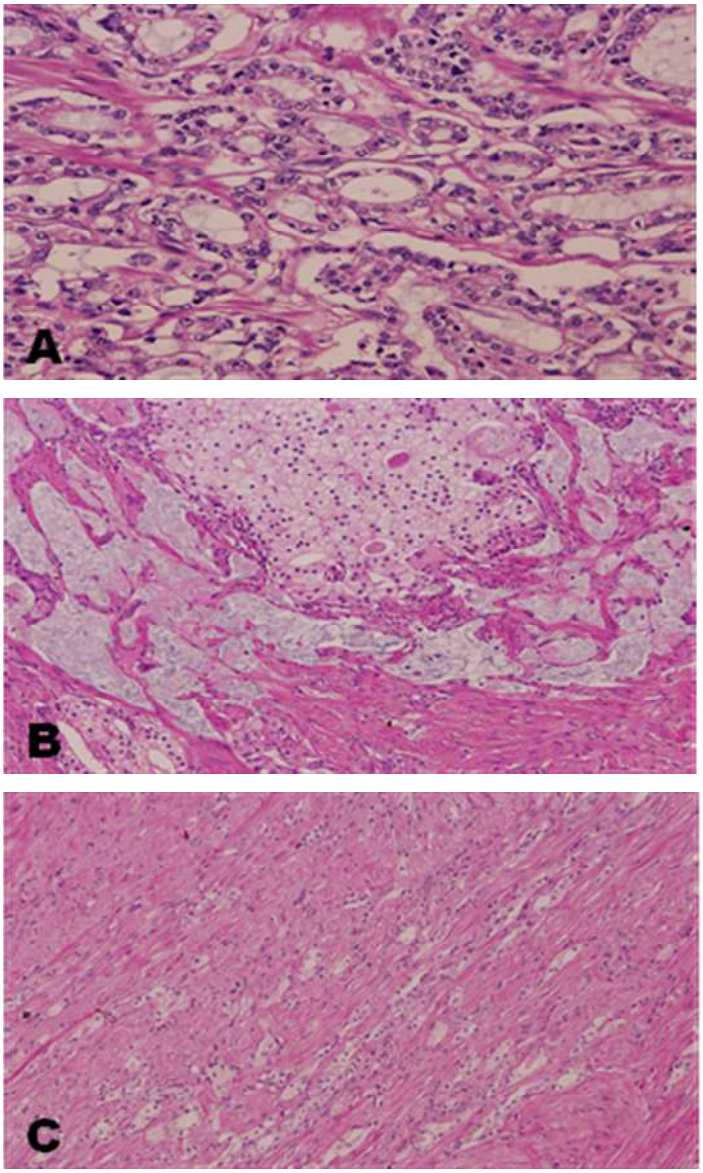


Table 1. Clinico-pathological characteristics

	Pathological therapeutic effects			p-value
	Minimal (n=66)	Moderate (n=33)	Extensive (n=8)	
Median Age (range)	64.7 (51-75)	68.3 (52-79)	67.0 (54-75)	0.016
PSA, mean (range)				
Before NHT	30.9 (4.2-203.0)	84.2 (4.1-1843.0)	23.8 (8.1-84.8)	0.760
After NHT	6.9 (0.04-70.8)	5.5 (0.0-80.2)	1.6 (0.1-6.6)	0.010
HT duration (%)				0.623
≤2 mo	41 (62.1)	17 (51.5)	5 (62.5)	
3 mo	8 (12.1)	5 (15.2)	1 (12.5)	
4-7 mo	10 (15.2)	7 (21.2)	1 (12.5)	
≥8 mo	7 (10.6)	4 (12.1)	1 (12.5)	
Bx Gleason score (%)				0.017
≤6	14 (21.2)	14 (42.4)	4 (50.0)	
7	24 (36.4)	11 (33.3)	2 (25.0)	
≥8	28 (42.4)	8 (24.2)	2 (25.0)	
Clinical stage (%)				0.053
≤cT1c	26 (39.4)	15 (45.5)	5 (62.5)	
cT2	13 (19.7)	9 (27.3)	3 (37.5)	
≥cT3	27 (40.9)	9 (27.3)	0 (0.0)	
Pathologic stage (%)				0.001
pT0	0 (0.0)	0 (0.0)	4 (50.0)	
pT2	28 (42.4)	15 (45.5)	4 (50.0)	
pT3	29 (43.9)	14 (42.4)	0 (0.0)	
pT4	9 (13.6)	4 (12.1)	0 (0.0)	

ECE (%)				<0.001
Negative	15 (22.7)	14 (42.4)	8 (100.0)	
Positive	51 (77.3)	19 (57.6)	0 (0)	
Tumor vol (%)				0.007
V1	23 (34.8)	14 (42.4)	7 (87.5)	
V2	16 (24.2)	10 (30.3)	1 (12.5)	
V3	27 (40.9)	9 (27.3)	0 (0.0)	
SV invasion (%)				0.047
Negative	42 (63.6)	24 (72.7)	8 (100.0)	
Positive	24 (36.4)	9 (27.3)	0 (0.0)	
Surgical margin (%)				0.009
Negative	28 (42.4)	20 (60.6)	7 (87.5)	
Positive	38 (57.6)	13 (39.4)	1 (12.5)	
LN invasion (%)				0.326
Negative	52 (78.8)	29 (89.7)	7 (87.5)	
Positive	14 (21.2)	4 (12.1)	1 (12.5)	

Bx: biopsy, ECE: extracapsular extension, HT: hormonal therapy, LN: lymph node, NHT: neoadjuvant hormonal therapy, SV: seminal vesicle

2. Relationship between pathological therapeutic effects and CgA/PTEN expression

There were various type of CgA expression in the prostatectomy specimens; diffuse, scattered, clustered, negative (Fig. 2). The prostatectomy specimens were divided into CgA positive and CgA negative groups for a comparison of the pathological regression grade. CgA positive cells were observed in 59.8% (64/107) of prostatectomy specimens. The pathological therapeutic effects in the CgA positive group were evaluated as minimal regression in 45 cases, moderate in 17 and extensive in 2, indicating an extensive regression rate of 3.1% (2/64). In the CgA negative group, the effects of bicalutamide therapy were evaluated as minimal regression in 21, moderate in 16 and extensive in 6, for an extensive regression rate of 14.0% (6/43). These results showed that the pathological therapeutic effects were significantly lower in the CgA positive group than in the CgA negative group ($p=0.012$).

CgA positive cells were observed in 25.0% (2/8) of the extensive regression group, with a CgA staining score of 1+ in 2 cases. CgA positive rate of the moderate regression group was 51.5% (17/33), with a CgA staining score of 1+ in 12 cases and 2+ in 5, and that of the minimal regression group was 68.2% (45/66), with a CgA staining score of 1+ in 9 cases, 2+ in 23 and 3+ in 13. There was a significant difference in the CgA staining score in relation to the pathological therapeutic effects ($p <0.001$).

PTEN inactivation (low score: ≤ 4) was observed in 59.8% (64/107) of prostatectomy specimens (Fig. 3). The pathological therapeutic effects in the PTEN inactivation group were evaluated as minimal regression in 56 cases, and moderate in 8, indicating an extensive regression rate of 0% (0/64). In the non-PTEN inactivation group, the effects of bicalutamide therapy were evaluated as minimal regression in 10, moderate in 25, and extensive in 8, for an extensive regression rate of 18.6% (8/43). PTEN inactivation was related to the pathological therapeutic effects ($p <0.001$, Table 2).

Fig. 2. CgA immunohistochemistry in the prostatectomy specimens (A) diffuse, (B) scattered, (C) clustered, (D) negative

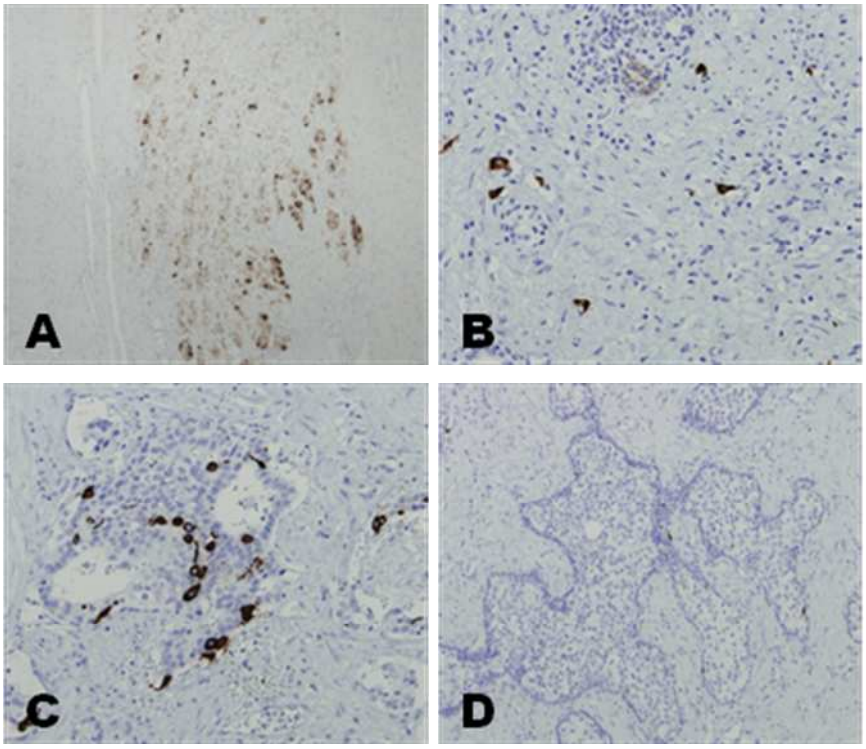


Fig. 3. Immunohistochemistry in the prostatectomy specimens showing PTEN inactivation

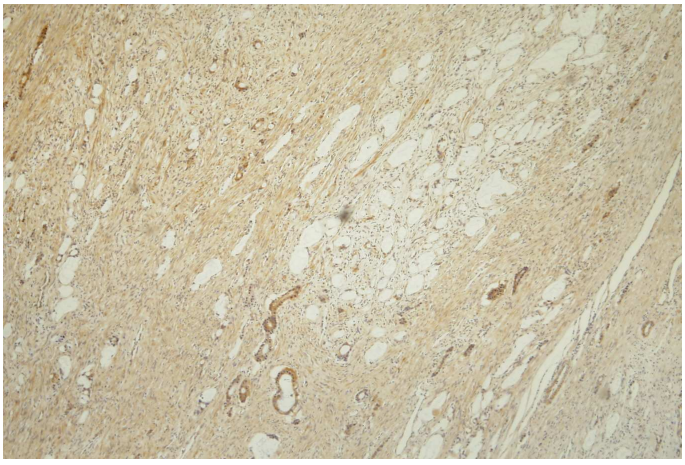


Table 2. Relationship between pathological therapeutic effects and CgA/PTEN expression

	Pathological therapeutic effects			p-value
	Minimal	Moderate	Extensive	
CgA negative (%)	21 (31.8)	16 (48.5)	6 (75.0)	0.012
CgA positive	45 (68.2)	17 (51.5)	2 (25.0)	
CgA score (%)				<0.001
1+	9 (20.0)	12 (70.6)	2 (100.0)	
2+	23 (51.1)	5 (29.4)	0 (0.0)	
3+	13 (28.9)	0 (0.0)	0 (0.0)	
PTEN (%)				<0.001
Low (≤4)	56 (84.8)	8 (24.2)	0 (0.0)	
High (≥5)	10 (15.2)	25 (75.8)	8 (100.0)	

CgA: chromogranin A, PTEN: phosphatase and tensin homolog deleted on chromosome 10

3. Results of HER-2 analysis by IHC and FISH

Only 4 of 107 (3.7%) prostatectomy specimens immunostained for HER-2 and the IHC score of all these 4 specimens were 1+. There were no HER-2 gene amplifications found in any of the samples.

4. Relationship between bicalutamide therapy duration and CgA/PTEN expression

The duration of bicalutamide therapy was significantly longer in the CgA positive cells than in the CgA negative cells ($p < 0.001$). CgA staining score was related to the longer duration of bicalutamide therapy ($p=0.033$). PTEN inactivation (low score: ≤ 4) was not related to the longer duration of bicalutamide therapy ($p=0.928$, Table 3).

Table 3. Relationship between bicalutamide therapy duration and CgA/PTEN expression

	bicalutamide therapy duration				p-value
	≤2 mo	3 mo	4-7 mo	≥8 mo	
CgA negative (%)	42 (66.7)	1 (7.1)	0 (0.0)	0 (0.0)	<0.001
CgA positive (%)	21 (33.3)	13 (92.9)	18 (100.0)	12 (100.0)	
CgA score (%)					0.033
1+	10 (47.6)	5 (38.5)	7 (38.9)	1 (8.3)	
2+	8 (38.1)	6 (46.2)	8 (44.4)	6 (50.0)	
3+	3 (14.3)	2 (15.4)	3 (16.7)	5 (41.7)	
PTEN (%)					0.928
Low (≤4)	37 (58.7)	10 (71.4)	9 (50.0)	8 (66.7)	
High (≥5)	26 (41.3)	4 (28.6)	9 (50.0)	4 (33.3)	

CgA: chromogranin A, PTEN: phosphatase and tensin homolog deleted on chromosome 10

5. Relationship between biopsy Gleason score and CgA/PTEN expression

Biopsy Gleason score was significantly higher in the CgA positive cells than in the CgA negative cells (p=0.001), but CgA staining score was not related to the higher biopsy Gleason score (p=0.317). There was no significant difference in PTEN expression with regard to the biopsy Gleason score (p=0.224, Table 4).

Table 4. Relationship between Bx Gleason score and CgA/PTEN expression

	Bx Gleason score (%)			p-value
	≤6	7	≥8	
CgA negative (%)	20 (62.5)	14 (37.8)	9 (23.7)	0.001
CgA positive (%)	12 (37.5)	23 (62.2)	29 (76.3)	
CgA score (%)				0.317
1+	7 (58.3)	7 (30.4)	9 (31.0)	
2+	4 (33.3)	9 (39.1)	15 (51.7)	
3+	1 (8.3)	7 (30.4)	5 (17.2)	
PTEN (%)				0.224
Low (≤4)	15 (46.9)	25 (67.6)	24 (63.2)	
High (≥5)	17 (53.1)	12 (32.4)	14 (36.8)	

Bx: biopsy, CgA: chromogranin A, PTEN: phosphatase and tensin homolog deleted on chromosome 10

6. Relationship between pathological stage and CgA/PTEN expression

Pathological stage was not significantly higher in the CgA positive cells than in the CgA negative cells (p=0.433), and CgA staining score was not related to the higher pathological stage (p=0.221). However, there was significant difference in PTEN expression with regard to the pathological stage (p=0.035, Table 5).

Table 5. Relationship between pathological stage and CgA/PTEN expression

	Pathological stage (%)				p-value
	pT0	pT2	pT3	pT4	
CgA negative (%)	3 (75.0)	17 (36.2)	20 (46.5)	3 (23.1)	0.433
CgA positive (%)	1 (25.0)	30 (63.8)	23 (53.5)	10 (76.9)	
CgA score (%)					0.221
1+	1 (100.0)	11 (36.7)	7 (30.4)	4 (40.0)	
2+	0 (0.0)	15 (50.0)	11 (47.8)	2 (20.0)	
3+	0 (0.0)	4 (13.3)	5 (21.7)	4 (40.0)	
PTEN (%)					0.035
Low (≤4)	0 (0.0)	26 (55.3)	29 (67.4)	9 (69.2)	
High (≥5)	4 (100.0)	21 (44.7)	14 (32.6)	4 (30.8)	

CgA: chromogranin A, PTEN: phosphatase and tensin homolog deleted on chromosome 10

7. Relationship between the time to biochemical failure and CgA/PTEN expression

CgA positive cells were found in 44 (68.2%) of 64 tumors in the PTEN inactivation group and only 20 (46.5%) of 43 tumors in the non-PTEN inactivation group. The probability of having CgA positive cells in the PTEN inactivation group was 2.5-fold (OR 2.5, 95% CI 1.1to 5.6; P=0.023) greater than in the non-PTEN inactivation group (Table 6).

After a mean follow-up period of 23.8 months (range 2-97), 40 (37.4%) patients had biochemical failure. After RP, the median time to biochemical failure was 54.9 months (95% CI: 44.7-65.0). The 5-year biochemical failure-free probability was 51.3% (Fig. 4). The 3-year no-biochemical failure rate was 42.6% for the CgA positive group and 77.3% for the CgA negative group, and this difference was significant (p=0.003, Fig. 5A). There was a biochemical failure in 33 of 64 (51.6%) in the CgA positive group and 7 of 43 (16.3%) in the CgA negative group. For the PTEN expression, the 3-year no-biochemical failure rate was 42.8% for the PTEN low (score $\leq$4) group and 72.2% for the PTEN high group (p=0.012, Fig. 5B). There was a biochemical failure in 30 of 64 (46.9%) in the PTEN low group and 10 of 43 (23.3%) in the PTEN high group. In the analysis of combined PTEN/CgA expression, the 3-year no-biochemical failure rate was 28.0% for the PTEN low/CgA positive group, 58.9% for the PTEN high/CgA positive or PTEN low/CgA negative group, and 89.1% for the PTEN high/CgA negative group. The overall difference was significant (p=0.002, Fig. 5C).

Based on Cox regression analysis, SV invasion, biopsy Gleason score and PTEN/CgA expression were statistically significant variable for the time to biochemical failure (p<0.001, 0.012, and 0.032, respectively). Although initial PSA (p=0.004), LN invasion (p<0.001), clinical stage (p=0.064), and positive

surgical margins (p=0.002) were significant by univariate analysis, none of these were statistically significant by Cox regression analysis (Table 7).

Table 6. Relationship between CgA and PTEN expression

Variable	PTEN		p-value	Odds Ratio	95% CI
	Low (≤4)	High (≥5)			
CgA negative	20 (31.3)	23 (53.5)	0.023	2.530	1.138-5.625
CgA positive	44 (68.7)	20 (46.5)			

CgA: chromogranin A, PTEN: phosphatase and tensin homolog deleted on chromosome 10

Fig. 4. Overall biochemical failure-free probability in RP patients with neoadjuvant bicalutamide therapy

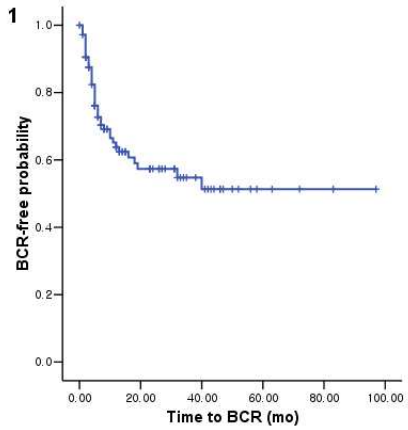
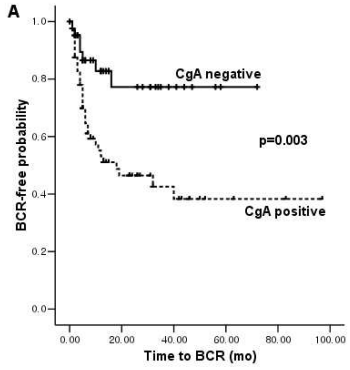
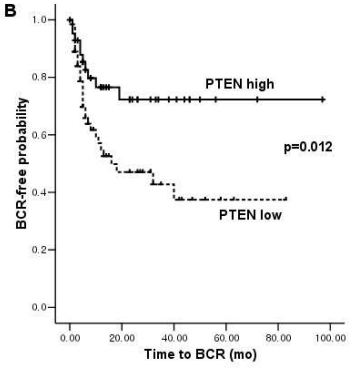


Fig. 5. Biochemical recurrence-free probability according to (A) CgA, (B) PTEN, (C) PTEN/CgA expression





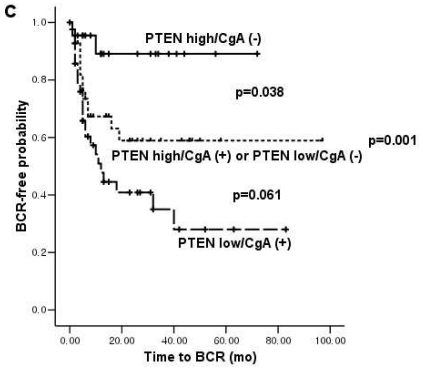


Table 7. Cox regression analysis evaluating predictors of time to biochemical failure after RP in patients who had neoadjuvant bicalutamide therapy

Variable	p-value	Hazards Ratio	95% CI
SV invasion	<0.001	4.507	2.308-8.802
Bx Gleason score			
≤6	0.016	Reference	Reference
7	0.089	2.623	0.863-7.970
≥8	0.006	4.617	1.542-13.822
PTEN/CgA expression			
High/negative	0.044	Reference	Reference
High/positive or low/negative	0.019	5.712	1.338-24.387
Low/positive	0.086	3.699	0.830-16.491
Initial PSA (ng/ml)			
<10	0.191	Reference	Reference
10-20	0.313	0.529	0.153-1.822
>20	0.583	1.318	0.492-3.529
Clinical stage			
≤cT1c	0.981	Reference	Reference
cT2	0.846	0.915	0.372-2.248
≥cT3	0.962	0.979	0.414-2.316
Surgical margin	0.896	1.066	0.408-2.786

Bx: biopsy, CgA: chromogranin A, PSA: prostate-specific antigen, PTEN: phosphatase and tensin homolog deleted on chromosome 10, RP: radical prostatectomy, SV: seminal vesicle

IV. DISCUSSION

Recently, Bicalutamide monotherapy (150 mg/day) is growing in popularity as an alternative in the treatment of locally advanced prostate cancer because of the comparative benefits in relation to quality of life issues and associated morbidity. Nevertheless, a significant number of these patients will progress into hormone-refractory status and need second-line therapies. The knowledge of molecular events induced by the primary therapy may be informative as to the selection and predicted success of subsequent therapies.

One of the unique theoretical advantages in neoadjuvant therapy is that preoperative neoadjuvant therapy offers a clinical milieu in which to evaluate new drugs for their activity (i.e., their specific molecular effects), and for their efficacy as measured against standard pathologic and clinical parameters.[8] Molecular markers or pathologic parameters may be assessed in tumors treated with neoadjuvant therapies. Future regimens may be formulated based on the changes of these molecular or pathologic parameters.

Based on these considerations, we analyzed the expressions of PTEN, HER-2 and NED after bicalutamide therapy. It has been demonstrated that loss of expression of the tumor suppressor PTEN, a common event in prostate cancer, resulted in increased phosphorylation of AKT.[26] AKT, an important signaling molecule in mammalian cells is activated by PI3K and inhibited by tumor suppressor gene PTEN. PTEN, a dual lipid/protein phosphatase, acts as a tumor suppressor by inhibiting the kinase activities of critical tumor-promoting kinases such as PI3K. PTEN inactivation is able to modulate the activation of PI3K-AKT mediated signaling,[26,27] which has been associated with tumor progression,[14,15,28] pharmacological resistance against chemotherapeutics,[10,29] and anti-target drugs.[30] Constitutive activation of the PI3K-AKT pathway in combination with loss of PTEN has been commonly observed in prostate cancer and result in uncontrolled cell proliferation and reduced apoptosis.[31]

HER-2 is a proto-oncogene for a transmembrane tyrosine growth factor with

a chromosomal location of 17q21.32. Basic research has shown that HER-2 overexpression induces cancerous transformation and shows increased aggressiveness.[33] In the absence of androgens, HER-2 has been shown to confer growth advantage to prostate cancer cells.[34] Alternatively, the oncogene HER-2 has been proposed as the survival factor for prostate cancer cells in an androgen-depleted environment.[17,34-36] Furthermore, HER-2 has been linked to the activation of androgen receptor signaling and to the clinical progression of ablation-resistant human prostate cancer.[37,38] It has been suggested that induction and activation of HER-2 occurs in an androgen-depleted environment or as a result of androgen receptor inactivation, promoting ablation-resistant survival of prostate cancer cells.[39]

In addition, many studies have shown that NED may contribute to androgen-independent growth of PC. The epithelial compartment of benign prostate consists of luminal secretory cells, basal cells, and a minor component of NE cells that have neuron-like morphology and secret biogenic amines and neuropeptides.[40] NE cells are also present in PC as scattered individual cells or small nests among the more abundant secretory type cancer cells. The number of NE cells increases in high grade and high stage tumors and particularly in hormonally treated and androgen-independent tumors.[41,42] It is hypothesized that hormonal therapy induces NED and the NE cells contribute to androgen-independent growth of PC in the androgen-deprived environment by secreting their products to act on the adjacent non-NE tumor cells in a paracrine fashion.[18,19,41,43]

After bicalutamide therapy, we observed that the patients with minimal regression effects showed high Gleason score and advanced pathologic stage. The pathological therapeutic effects were related to a decrease in serum PSA levels, a decrease in positive surgical margins, but there was no difference in pre-neoadjuvant therapy serum PSA levels, clinical stage, and decrease in rate of positive LN metastasis between the pathological therapeutic effects.

Therefore, bicalutamide appears to reduce the prevalence of positive surgical margins, especially in the patients with extensive regression effects.

In the patients with minimal regression effects, there was significantly high prevalence of PTEN inactivation and positive CgA expression. Moreover, the patients with biopsy Gleason score ≥7 showed statistically high incidence of positive CgA expression and PTEN inactivation. In the PTEN inactivation group, the patients showed the probability of having positive CgA expression 2.5-fold greater than in the non-PTEN inactivation group. Therefore, minimal regression effects after bicalutamide therapy may be correlated with the biopsy Gleason score, and the high incidence of PTEN inactivation expression and positive CgA expression.

In our study, we suggest that refractoriness for bicalutamide therapy is related to the PTEN inactivation and NED. As previously stated, PTEN inactivation is able to modulate the activation of PI3K-AKT mediated signaling.[26,27] Constitutive activation of the PI3K-AKT pathway induced by loss of PTEN has been commonly observed in prostate cancer and resulted in uncontrolled cell proliferation and reduced apoptosis.[31] LNCaP harbors a point mutation in PTEN, which may allow activation of AKT readily in such cells.[44,45] Several studies have shown that the activity of AKT in LNCaP cells is significantly increased after androgen deprivation therapy.[46,47] AKT participates in various cellular processes, including proliferation, apoptosis, and survival, and is considered a key player in many tumors, including PC.[48] Similarly, in most other studies, AKT activation appears to be generally associated with malignant transformation and cell proliferation.[49-51] However, the mechanism of action of AKT in various cancers is not clear and is likely cell type and organ dependent. Recently, it has been recently suggested that activation of the PI3K-AKT pathway may be necessary and sufficient for NED.[20] In this study, authors studied signal transduction pathways of NED in LNCaP cells after androgen withdrawal, and showed that both the PI3K-AKT pathway is activated and is

required for NED. They reported that a constitutively active AKT promotes NED, whereas a dominant negative AKT inhibits it and activation of AKT by insulin-like growth factor-1 (IGF-1) leads to NED, and NED induced by epinephrine requires AKT activation. In addition, they showed that the AKT pathway is likely responsible for NED in DU145, an androgen-independent prostate cancer cell line. Therefore, we hypothesized that activation of PI3K-AKT pathway, mainly caused by PTEN inactivation may induce NED and it appears as minimal regression effects after bicalutamide therapy. Our study showed that by itself, PTEN inactivation was not a good predictor for the time to biochemical recurrence, but in combination with positive CgA expression, it was a significant predictor for the time to biochemical recurrence, and this finding supports that PTEN inactivation together with NED is related to refractoriness for bicalutamide therapy.

It has been shown that PTEN positive prostate cancer cells may develop high levels of AKT through the activation of several growth factor receptors, such as epidermal growth factor receptor, and HER-2.[34,52] However, to date, efforts to conclusively establish that HER-2 overexpression is important to androgen-dependent PC or to progression to androgen independence have failed because of variability in tissue procurement, antibodies, immunostaining procedures, and assessment methods.[16,17,53,54] Also in our data, there were only 3.7% of prostatectomy specimens immnostained for HER-2 and there were no HER-2 gene amplifications found in any of the samples.

Recently, many clinical trials are in the process of study targeting PI3K-AKT pathway in PC based on the results that this pathway may be a key element in malignant transformation and cell proliferation.[49] We suggest that PTEN inactivation together with NED is related to refractoriness for bicalutamide therapy and support the results of the previous study that AKT is critically involved in NED of PC after androgen deprivation therapy. Therefore, combination of hormonal therapy, which induces NED through the PI3K-AKT

pathway, and an agent targeting the PI3K-AKT pathway may suppress the proliferation of PC while inhibiting NED, thus possibly delaying and preventing the emergence of androgen-independent PC.

V. CONCLUSION

The patients showing minimal regression effects after bicalutamide therapy were related to the advanced pathologic stage and tend to have PTEN inactivation and NED. PTEN inactivation and NED is useful as a marker of an unfavorable prognosis by predicting the time to biochemical failure and outcomes after RP in patients who had neoadjuvant bicalutamide therapy. We consider that PTEN inactivation together with NED is related to refractoriness for bicalutamide therapy and these results support the hypothesis that NED may be caused by activation of serine threonine kinase, AKT pathway, which results from PTEN inactivation.

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<ABSTRACT(IN KOREAN)>

전립선암에서 bicalutamide 단일요법후 neuroendocrine differentiation과 PTEN 발현의 병리적 효과와의 연관분석

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함 원식

목적: bicalutamide 단일요법은 삶의 질 유지 및 적은 빈도의 부작용으로 인해 국소적으로 진행된 전립선암에 대한 대체 치료요법으로 최근 각광을 받고 있다. 그러나 상당수의 환자들은 결국 재발하여 이차적인 치료를 요한다. 이러한 경우, 일차 약물요법에 의해 유도되는 분자학적 변화를 파악하는 것은 이후 치료의 선택 및 성공여부를 판단하는 데에 도움이 된다.
술 전 약물요법의 독특한 이론적 장점들 중의 하나는 술 전 약물요법을 시행함으로서 새로운 약제의 독특한 분자학적 효과와 같은 활동성 및 효율성을 기존의 임상 병리적인 변수들과 비교하여 평가할 수 있는 기회를 제공한다는 것이다. 즉 술 전 약물요법을 시행한 종양의 조직표본을 대상으로 분자학적 인자들 및 병리적 변수들을 평가할 수 있고 이러한 분자학적 인자들 및 병리적 변화들을 바탕으로 앞으로의 치료법을 구상할 수 있다는 것이다.
이러한 이론적 배경을 바탕으로 저자들은 bicalutamide 단일요법후 발생하는 분자학적 변화를 파악하기 위해 술 전 bicalutamide 단일요법을 시행받은 전립선암환자들의 근치적 전립선적출술 표본을 대상으로 전립선암의 발생 및 진행 특히 호르몬불응성 전립선암으로의 진행에 관계된다고 알려진 분자학적 인자들인 tensin homolog deleted on chromosome 10 (PTEN), human epidermal receptor-2 (HER-2), 및

neuroendocrine differentiation (NED)의 발현양상을 분석하고자 하였다.
PTEN은 phosphatidylinositol 3-kinase (PI3K)과 같은 주요한 종양유발 kinase를 억제하는 종양억제유전자로 PTEN 발현감소는 전립선암에서 흔히 관찰되는 현상으로 serine threonine kinase AKT phosphorylation 증가를 유발한다고 알려져 있다. PTEN 발현 감소와 연관된 PI3K-AKT경로의 구조적 활성화는 전립선암에서 흔히 관찰되며, 조절되지 않는 세포증식 및 세포자멸사 감소를 야기한다.
HER-2는 transmembrane tyrosine growth factor에 대한 proto-oncogene으로 HER-2의 과도한 발현은 악성종양으로의 변형을 유발하고, 종양의 증가된 공격성을 나타낸다고 하며, 남성호르몬결핍환경에서 HER-2는 전립선암세포의 생존에 기여하는 것으로 알려져 있다.
NED는 전립선암의 호르몬 비의존성 성장에 기여하는 것으로 알려져 있다. 호르몬요법이 NED를 유발하고, neuroendocrine 세포들이 주위의 non-neuroendocrine 세포들에 paracrine 방식으로 그들의 산물들을 분비하여 남성호르몬 결핍환경에서 전립선암의 생존에 기여하는 것으로 생각되고 있다.
재료 및 방법: 다양한 기간동안의 술 전 bicalutamide 150 mg 단일요법을 시행받은 107명의 환자에서 적출한 근치적 전립선적출술 표본을 대상으로 앞서 언급한 분자학적 인자들의 변화양상과 병리적 퇴행과의 상관관계를 분석하였고, 술 후 생화학적 재발이 이러한 분자학적 변화 및 병리적 퇴행 정도와 연관있는지 분석하였다.
병리적 인자들로는 생검 Gleason 점수, 병리적 병기, 정낭침범, 전립선 피막외 침범여부, 양성수술절제면 및 조직표본내 종양용적에 대해 평가하였고, 병리적 퇴행 정도는 minimal, moderate, extensive의 3단계로 분석하였다. Tissue microarray를 시행한 후에 PTEN, HER-2, 그리고, NED의

대표적 표지자인 chromogranin-A (CgA)에 대한 면역화학염색을 시행하였다. PTEN 면역화학염색정도는 0-9의 10단계로 나누어 4이하인 경우를 낮은 발현으로 정의하였고, CgA의 면역화학염색정도는 0-3+ 의 4단계로 나누어 평가하였다. HER-2의 경우 면역화학염색정도를 0-3+ 의 4단계로 나누어 2+ 이상인 경우를 높은 발현으로 정의하였으며, HER-2 유전자의 증폭여부를 평가하기 위해 fluorescence in situ hybridization (FISH)를 시행하였다.
결과: 생검 Gleason 점수 (p=0.017), 병리적 병기 (p=0.001), 종양용적 (p=0.007), 전립선 피막외 침범 (p<0.001), 정낭침범 (p=0.047), 및 양성수술절제면 발생률 (p=0.009)은 술 전 bicalutamide요법을 시행한 후의 병리적 퇴행 정도와 유의한 연관성을 보였다. 병리적 퇴행 정도가 약한 경우 CgA 양성률 (p=0.012) 및 PTEN inactivation의 발생률 (p<0.001)이 유의하게 증가하였다. 술 전 bicalutamide요법 시행기간이 길수록 CgA양성률은 유의하게 증가하였으나 (p<0.001), PTEN inactivation과의 유의한 연관성은 없었다 (p=0.928). 생검 Gleason 점수 7이상인 경우 CgA양성률 및 PTEN inactivation의 발생률이 증가하였다.
HER-2양성인 경우는 4개 (3.7%)의 조직표본에서만 관찰되었고, 4개의 조직표본 모두에서 1+ 의 면역화학염색정도를 나타내었다. 모든 경우에서 HER-2 유전자의 증폭은 관찰되지 않았다.
PTEN inactivation인 64개의 표본들 중 44 (68.2%)개에서 CgA 양성으로 나타난 반면, non-PTEN inactivation인 43개의 표본들 중 20 (46.5%)개만이 CgA양성으로 나타나, non-PTEN inactivation인 경우에 비해 PTEN inactivation인 경우 CgA양성일 가능성이 2.5배 높은 것을 확인하였다 (OR2.5, 95%CI 1.1-5.6, p=0.023). 평균 23.8개월의 추적관찰기간동안 40명에서 생화학적 재발이 발생하였고, 5년 생화학적 재발 자유가능성은 51.3%였다. CgA양성 (p=0.003) 및 PTEN

inactivation여부 (p=0.012)에 따라 생화학적 재발까지의 시간에 유의한 차이가 있었고, PTEN inactivation과 CgA양성 여부를 함께 조합하여 분석하였을 때도 생화학적 재발까지의 시간에 대해 유의한 차이가 있었다 (p=0.002). 생화학적 재발까지의 시간에 영향을 미치는 인자들에 대한 다변량 분석결과 정낭침범 (p<0.001), 생검 Gleason 점수 (p=0.012) 및 PTEN/CgA 발현양상 (p=0.032)이 유의한 인자들로 나타났다.

결론: 술 전 bicalutamide 요법시행 후 병리적 퇴행정도가 약한 경우 나쁜 술 후 병리적 결과를 나타내며, PTEN inactivation 및 NED를 나타내는 경향이 있는 것으로 나타났다. 대부분의 경우 HER-2 단백질 및 유전자의 증폭이 관찰되지 않아 bicalutamide 요법 후의 HER-2의 발현양상에 대해서는 추가적인 연구가 필요할 것으로 생각된다. PTEN inactivation 및 NED는 술 전 bicalutamide요법을 시행 후 근치적 전립선적출술을 시행받은 후 나타나는 술 후 생화학적 재발까지의 시간을 예측하는데 있어 나쁜 예후를 나타내는 예측인자로 유용할 것이다. PTEN inactivation과 NED의 발생은 bicalutamide요법에 대한 불응성과 연관될 것으로 생각되며, 이는 PTEN inactivation으로부터 발생하는 serine threonine kinase AKT경로의 활성화에 의해 NED가 발생할 수 있다는 가설을 지지하며, PI3K-AKT경로를 통해 NED를 유발하는 호르몬요법을 시행할 때 PI3K-AKT경로를 차단하는 약제를 병용하여 치료함으로서 NED를 억제하면서 전립선암의 증식을 억제하여 남성호르몬 비의존성 전립선암의 출현을 연기하거나, 방지할 수 있을 것으로 생각된다.

핵심되는 말: 전립선암, PTEN, HER-2, neuroendocrine differentiation, 술 전 bicalutamide요법

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