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Original Article

The loss of CD44 and HSP70 overexpression is related to aggressive clinicopathologic factors in prostate cancer

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Abstract: Prostate cancer (PC) is the most common cancer in men with biologically highly heterogeneous clinical outcomes despite early detection. Therefore, the identification of novel molecular markers that are associated with biological aggressiveness is essential for predicting clinical outcomes and deciding the treatment of PC. We examined the expression of cluster of differentiation 44 (CD44) and heat shock protein 70 (HSP70) in PC cells using immunohistochemistry on tissue microarrays and evaluated their clinicopathological significance. A loss of CD44 expression and HSP70 overexpression were observed in 62 (57.9%) and 54 (50.5%) out of 107 cases of PC, respectively. CD44-negative PC showed more vascular invasion, more extra-prostatic extension, more capsular invasion, higher pT stages, higher pathological tumor stages, higher prostate-specific antigen levels (> 20 ng/mL), and higher grades groups. Overexpression of HSP70 was significantly associated with PC with capsular invasion, higher pT stages, and higher pathological tumor stages. The loss of CD44 expression is correlated with tumor invasiveness and higher Gleason grades, reflecting the features of aggressive tumors. Consequently, CD44 could be an important biomarker and a potential therapeutic target.

Keywords: Immunohistochemistry, CD44, HSP70, prognosis, prostate cancer

Introduction

Prostate cancer (PC) is the most commonly diagnosed cancer in men and the second leading cause of cancer-related deaths in the US [1]. Even though most cases of PC are detected in the early localized stages with low or intermediate grades determined by screenings for prostate-specific antigen (PSA) in the serum, PC shows a high biological variability and diverse clinical outcomes [2, 3]. A considerable proportion of PC follows a clinically indolent course without any serious morbidity during the patient's lifetime, while more than 30% of patients undergo a rapid disease progression and relapse [3]. Serum PSA, tumor stage, and Gleason score are still well-known as valuable clinical prognostic parameters for predicting clinical outcomes [4]. Therefore, it is necessary to identify more novel and effective molecular markers that are associated with tumor aggressiveness for the diagnosis, treatment and prognostic prediction of PC.

Cluster of differentiation 44 (CD44), a cell-surface glycoprotein, is expressed in embryonic stem cells and is involved in many cellular processes, including cell-cell adhesion, growth, survival, differentiation, and mortality [5, 6]. Overexpression of CD44 expression has been reported in many human cancers and has been shown to be related to tumor progression and metastasis [7-9]. Thus, CD44 is recognized as a molecular marker for cancer stem cells [10].

Heat-shock proteins (HSPs) are highly conserved proteins found all cell types that promote the folding of newly translated proteins or refolding of denatured proteins to cellular integrity. HSP70 also protects cells from stressful conditions by acting as chaperones [11, 12]. In addition, HSPs are involved in the regulation of cellular growth and apoptosis [13]. HSP70 prevents cell apoptosis under stress and its anti-apoptotic activity is thought to be associated with carcinogenesis [14]. Regarding prognosis, the overexpression of HSP70 has shown diverse

clinicopathologic significances in different types of cancers.

In this study, we aimed to investigate the expression of CD44 and HSP70 to evaluate their association with clinicopathological factors and their prognostic impact on patients treated with radical prostatectomy. The findings may help to predict tumor aggressiveness and prognosis in needle biopsied tissue.

Materials and methods

Patients and tissue samples

From July 2004 to September 2017, 170 consecutive radical prostatectomy specimens from patients with PC were selected and reviewed. Complete baseline and follow-up data were available for 107 patients after excluding the patients with missing information, those with follow-up loss, and those who underwent prior hormonal or radiation therapy. Their medical records were reviewed to determine the most recent follow-up visit, survival status, and pre- and post-operative PSA levels. Resected prostate specimens were fixed for one to two days in 10% neutral-buffered formalin. After removal of the apical and bladder neck margins, the specimen was sectioned transversely at 3- to 4-mm intervals from the apex to the base. Whole sections were stained with hematoxylin and eosin and evaluated with respect to tumor location, tumor size, presence of extra prostatic extension (EPE), lympho-vascular invasion, perineural invasion, lymph node metastasis, surgical margin status, and pathological tumor stage. The Gleason scoring system was applied, based on the International Society of Urological Pathology (ISUP) in 2014 and the new patient-centric grading system [15].

Overall survival (OS) was defined as the time from surgical resection to death or the last follow-up. Disease progression was defined by persistent or rising PSA level, a PSA level of greater than 0.4 ng/mL on at least two occasions, a biopsy-proven local recurrence, or evidence of distant metastasis by bone scan or other tests. The follow-up period ended on March 31, 2018.

Tissue microarray block construction

Tissue microarray blocks (TMA) were constructed. Two 2-mm tissue cores from the area containing the most dominant Gleason grade pattern, one core from the area with secondary Gleason grade pattern, and one core from an adjacent benign glandular area were constructed.

Immunohistochemistry

Immunohistochemical staining was conducted using the automated Benchmark® platform (Ventana Medical Systems, Tucson, AZ, USA). In brief, TMA sections (4 μm) were deparaffinized and incubated for 32 min at room temperature with a mouse monoclonal CD44 (1:100, clone MRQ-13, Cell marque, USA) using the onboard heat-induced epitope retrieval method in a high pH CC1 buffer. For HSP70, the TMA sections were incubated for 24 min at room temperature with a mouse monoclonal HSP70 (predilution, clone W27, Thermo Fisher scientific, USA) using the onboard heat-induced epitope retrieval method in a standard pH CC1 buffer. Immunoreactivity was detected using the UltraView™ universal DAB detection kit (Automated BenchMark®, Ventana), which included a hydrogen peroxide substrate and a 3,3'-diaminobenzidine chromogen solution. The slides were subsequently counterstained with hematoxylin.

Interpretation of the immunohistochemical staining

The slides were assessed by investigators who were blinded to the patients' clinicopathological information. According to the definition of CD44-positive staining in previous reports, the percentage of positive cells was categorized into five score ranges: 0-5, 6-25, 26-50, 51-75, and 76-100% positive cells. The intensity was scored as 0 for no staining, 1 for barely detectable or weak, 2 for moderate and 3 for strong. An immunoreactivity score (IRS) was calculated by the addition of the percentage and the intensity of the positive cells, resulting in a scale from 0 to 7. The IRS was divided into two groups: negative or loss of staining (IRS 0-4) and positive or high staining (IRS 5-7) [16, 17]. For nonneoplastic prostate tissue, the basal cells and luminal cells showed membranous staining on CD44, and more than moderate intensity. For HSP70, overexpression was defined when 10% or more of the tumor cells revealed more than moderate cytoplasmic staining [18]. Breast cancer and basal cells of

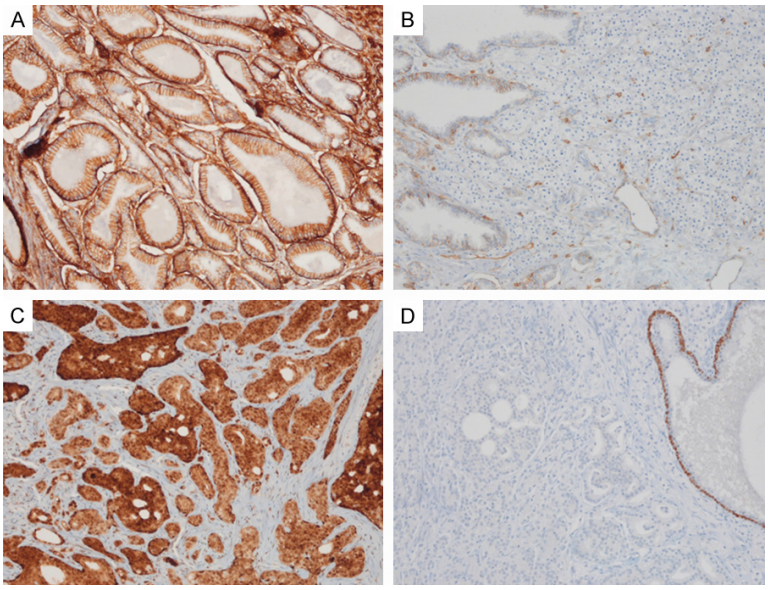

Figure 1. Prostate cancer revealed (A) High expression of CD44, ×200; (B) Loss of CD44 expression, ×200; (C) Overexpression of HSP70, ×100; (D) Negative expression of HSP70, ×100.

nonneoplastic prostate tissue were used as the positive control for HSP70, and the primary antibody with the buffer only were used as the negative control.

Statistical analysis

All comparisons were analyzed using SPSS, version 23.0 (SPSS Inc., Chicago, IL, USA). The comparison of categorical variables was performed using the chi-square test or Fisher's exact test. Overall survival was defined as described above. Disease-free survival (DFS) was defined as the post-operative interval without a known recurrence or metastasis. Univariate survival analyses were conducted using the Kaplan-Meier method. A multivariate analysis using the Cox proportional hazard regression model was used to evaluate the prognostic factors for OS. A value of $P < 0.05$ was considered as statistically significant.

Results

Patients characteristics

The ages of the patients ranged from 49 to 79 years (median 66.6 years), and the patients were followed for a median of 39.9 months (range, 0-131 months). Their serum PAS levels at diagnosis ranged from 3.59 ng/ml to 175 ng/ml (median 19.5 ng/ml). During the follow-up period, all five patients died. Four patients died from other diseases (stomach cancer and myocardial infarction) or from an unexpected event. Only one patient died after experiencing metastasis with PC recurrence.

Immunohistochemical results of CD44, HSP70 and their clinicopathological significance

Out of the 107 cases of PC, 62 (57.9%) were negative for CD44, and 54 (50.5%) were positive for HSP70 (**Figure 1**). The CD44-negative PCs showed more vascular invasion ($P = 0.037$), more EPE ($P = 0.003$), more capsular invasion ($P < 0.000$), higher pT stages ($P = 0.008$), higher pathological tumor stages ($P = 0.038$), higher PSA levels (> 20 ng/mL) ($P = 0.009$), and higher grades groups ($P = 0.010$). Overexpression of HSP70 was significantly associated with PCs showing capsule invasion ($P = 0.008$), higher pT stages ($P = 0.008$) and higher pathological tumor stages ($P = 0.001$), as summarized in **Table 1**.

CD44 and HSP70 expression in relation to patient outcome

No associations were demonstrated between loss of CD44 expression or overexpression of HSP70 and OS. There was not an independent prognostic factor in the multivariate analysis.

Discussion

CD44 is involved in cell-cell and cell-matrix interaction, and its major function is the maintenance of tissue structure [19]. CD44 expression has been studied in various human cancers and its overexpression was generally shown to be associated with aggressive behavior or poor prognosis of cancers including breast, colon, gastric, and lung cancers [9, 10, 20-22]. However, the opposite result, whereby the loss of CD44 is associated with poor prognosis, has been demonstrated in colorectal and esophageal adenocarcinoma [6, 16, 23]. In

Table 1. Correlation between clinicopathologic factors and the expression of CD44 and HSP70 in prostate cancer

Variable	CD44		*p*	HSP70		*P*
	Negative	Positive		Negative	Positive	
Vascular invasion			0.037			0.778
Present	19 (76.0%)	6 (24.0%)		13 (52.0%)	12 (48.0%)	
Absent	43 (52.4%)	39 (47.6%)		40 (48.8%)	42 (51.2%)	
Neural invasion			0.074			0.386
Present	41 (65,1%)	22 (34.9%)		29 (46.0%)	34 (54.0%)	
Absent	21 (47.7%)	23 (52.3%)		24 (53.5%)	20 (45.5%)	
pN stage			0.615			0.053
N0	27 (51.9%)	25 (48.1%)		26 (50.0%)	26 (50.0%)	
N1	3 (75.0%)	1 (25.0%)		0 (0%)	4 (100%)	
Extra-prostatic extend			0.003			0.097
Present	36 (73.5%)	13 (26.5%)		20 (40.8%)	29 (59.2%)	
Absent	26 (44.8%)	32 (55.2%)		33 (56.9%)	25 (43.1%)	
Margin involvement			0.246			0.084
Present	29 (64.4%)	16 (35.6%)		20 (44.4%)	25 (55.6%)	
Absent	33 (53.2%)	29 (46.8%)		38 (61.3%)	24 (38.7%)	
Capsule invasion			0.000			0.008
Present	47 (71.2%)	19 (28.8%)		26 (39.4%)	40 (60.6%)	
Absent	15 (36.6%)	26 (63.4%)		27 (65.9%)	14 (34.1%)	
pT stage			0.008			0.008
pT2	31 (48.4%)	33 (51.6%)		39 (60.9%)	25 (39.1%)	
pT3a	15 (65.2%)	8 (34.8%)		5 (21.7%)	18 (78.3%)	
pT3b	15 (78.9%)	4 (21.1%)		8 (42.1%)	11 (57.9%)	
pT4	1 (100%)	0 (0%)		1 (100%)	0 (0%)	
Stage			0.038			0.001
I	4 (57.1%)	3 (42.9%)		7 (100%)	0 (0%)	
II	27 (47.4%)	30 (52.6%)		35 (61.4%)	22 (38.6%)	
III	27 (71.1%)	11 (28.9%)		14 (36.8%)	24 (63.2%)	
IV	4 (80.0%)	1 (20.0%)		2 (40.0%)	3 (60.0%)	
Serum PSA			0.009			0.339
< 10	27 (50.9%)	26 (49.1%)		27 (50.9%)	26 (49.1%)	
≥ 10 & < 20	12 (44.4%)	15 (55.6%)		16 (59.3%)	11 (40.7%)	
≥ 20	23 (85.2%)	4 (14.8%)		10 (37.0%)	17 (63.0%)	
Grade groups			0.010			0.168
Grade group 1 + 2	23 (47.9%)	25 (52.1%)		29 (60.4%)	19 (39.6%)	
Grade group 3	12 (50.0%)	12 (50.0%)		9 (37.5%)	15 (62.5%)	
Grade group 4 + 5	27 (77.1%)	8 (22.9%)		20 (57.1%)	15 (42.9%)	

PC, De Marzo et al. reported that CD44 was down-regulated in PCs with higher Gleason grades and lymph node or bone metastasis [17]. In contrast, Patrawala et al. demonstrated that CD44-positive PCs showed more aggressive behavior, such as tumor proliferation, and metastasis [24]. In accordance with the previous results, we found that the loss of CD44 expression was significantly associated with tumor invasiveness (vascular invasion, capsule invasion, EPE, and pT3 and pT4), higher stages (III and IV), and higher PSA levels. The grade groups (4 and 5) revealed a significant loss of CD44 expression. Some studies have been performed on CD44 down-regulation in PC and revealed that methylation and hypermethyl-

ation of the CD44 gene promoter were correlated with a loss of CD44 expression and tumor progression [25]. Furthermore, the degree of methylation was higher in stage III compared to that in stage II and higher when the Gleason grade was ≥ 7 than when the Gleason score was ≤ 6 [26]. Bisson et al. reported that WNT activity regulates the self-renewal of PC cells with stem cell characteristics independently of AR activity [27]. Therefore, the inhibition of the WNT signaling pathway can reduce the uncontrolled self-renewal of PC cells that drive cancer, in turn improving the therapeutic outcome. CD44 is a well-known downstream target of the WNT signaling cascade, Therefore, the loss of CD44 expression in PC may be a biomarker for aggressive tumor features and a treatment target.

The important role of HSP70 is the protection and maintenance of cellular viability against environmentally and pathophysiologically stressful stimuli [11, 12]. Cancer cells require higher levels of HSP70 because they are exposed to oncogenic stress with overexpressed abnormal oncoproteins and higher metabolic requirements. HSP70 overexpression participates in tumor growth, an increase in metastasis, and resistance in chemotherapy [13]. Previous studies have shown that HSP70 overexpression is associated with a worse prognosis in gastric cancer, breast cancer, colon cancer, hepatocellular carcinoma, and esophageal squamous cell carcinoma [18, 28-32]. In contrast, Tavassol et al. reported that HSP70 overexpression was associated with a favorable prognosis in oral cancer [33]. Reduced HSP70 expression was also reported to be associated with esophageal squamous cell carcinoma [34]. In this study, HSP70 was significantly overexpressed in PC with capsule invasion, pT3 stage, and tumor stage III and IV. These results support that HSP70 plays a crucial role in protecting tumor cells from oncogenic stresses and promotes tumor growth and invasion. Strategies targeting HSP70 are being developed in cancer therapy [35]. Goloudina et al. reported that HSP70 inhibitors could act as anti-cancer agents by inducing chemosensitization or by activating a specific anti-cancer immune response [13]. Biomarkers are essential for detecting tumors, monitoring tumor growth, and assessing the effectiveness of anti-cancer therapies [35]. Loss of CD44 and HSP70 overexpression may be important biomarkers for the aggressiveness of PCs.

However, there is a limitation in our study. We could not draw meaningful results in relation to patient outcome because of the relatively short follow-up periods, and only one patient's death was PC-related. Nevertheless, our study suggests that the loss of CD44 expression and HSP70 overexpression reflects aggressive tumor invasiveness and higher grade groups. To confirm the correlation with worse clinical outcome, larger studies with sufficient survival data are required.

In summary, loss of CD44 expression was associated with tumor invasiveness and higher grade groups. Although further studies with large cohorts with sufficient survival data are needed to confirm our findings, the loss of CD44 expression in PC may be a biomarker for aggressive tumor features and a future treatment target.

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The study and permission to use the tissue for research were approved by the Human Ethics Review Board of Yeungnam University Hospital (2018).

Disclosure of conflict of interest

None.

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