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Expression analysis of *CD44* isoforms *S* and *V3*, in patients with esophageal squamous cell carcinoma

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ABSTRACT

Objective(s): *CD44* is a member of the cell adhesion molecules family. Naturally, *CD44S*, along with *CD44V3* influence the cell motility, migration, and adhesion, while in tumor cells they lead to tumor invasion, progression, and metastasis. The purpose of this research is to evaluate the *CD44S* and *CD44V3* expression in Esophageal Squamous Cell Carcinoma (ESCC) and to reveal their correlations with clinicopathological features of patients.

Materials and Methods: Fresh tumoral and distant tumor-free esophageal tissues were obtained from 50 patients with ESCC. Using quantitative real-time PCR, the expression levels of *CD44S* and *CD44V3* were quantified and compared in both groups of cells. The patients had not received any therapeutic interference, such as chemotherapy or radiation, prior to sampling.

Results: Significant overexpression of *CD44S* and *CD44V3* mRNA was observed in 13 (26.0%, *P*=0.03) and 11 (22.0%, *P*=0.007) tumor specimens, respectively. The expression of the genes were significantly correlated not only with each other (*P*=0.0001), but also with differentiation grade of tumor (*P*=0.033), stage of tumor progression (*P*=0.003), and depth of tumor invasion (*P*=0.00). In addition, low level of *CD44V3* mRNA expression was attended to be associated with tumor invasion.

Conclusion: There is no correlation between *CD44S* expression with clinicopathological features of patients; however, simultaneous expression of these genes has an important effect on tumorigenesis.

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Introduction

Esophageal Squamous Cell Carcinoma (ESCC) is the sixth and fourth most common cancer in the world and in the developing countries, respectively, and also the second most frequent cause of cancer mortality in Iran (1, 2). According to the latest medical reports, the highest rate of ESCC is in Asia, in the so-called "Esophageal Cancer Belt" which is extended from Iran to China (3). Despite the advances in the diagnosis and treatment of ESCC, the rate of ESCC mortality has not yet improved (4, 5), reflecting the late detection of the disease and poor understanding of the cellular and molecular mechanisms for initiation and development of ESCC (6, 7). Tumor progression and development process is mediated by deregulation of different signaling pathways as well as mutual interactions between cancerous and normal cells (8). Different studies have introduced the role of specific molecules such as *CD44* family in tumor progression. *CD44* family is consisted of cell adhesion glycoproteins on the cell surface (9, 10). *CD44* is a single chain glycoprotein consisting of 4 functional domains including a preserved N-terminal extracellular domain, a non-preserved membrane proximal region, a preserved trans-membrane and a cytoplasmic tail domain. The distal extracellular domain is primarily responsible for the binding of hyaluronic acid (HA), while the proximal extracellular domain is the variable region, which can be different in various *CD44* isoforms due to alternative splicing of *CD44* mRNA (11). The cytoplasmic tail of *CD44* molecules exhibits a protein motif, which interacts with cytoskeletal proteins and other intracellular signaling molecules (12). The human *CD44* gene is located on the short arm of chromosome 11 at a position described as 11p13 (13), consisting of 20 exons. Although exons 1 to 5 and 16 to 20 are

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constant and splice together to make *CD44S*, the 10 variable exons (also called v1-v10) will be spliced and enclosed in nursing insertion site between exons number five and sixteen. *CD44* variants are expressed in both normal and tumor cells (14). Previous studies have shown that in some cancers *CD44* isoforms, *CD44S* and *CD44V3* could play a major role in cancer invasion and metastasis (15-18). The expression of *CD44V3* is related to advanced pathological stage and poor prognosis in colorectal cancer and plays an essential role in its invasion and metastasis (19).

In this study, we retrospectively evaluated the level of *CD44* mRNA expression (its standard form and *V3* variant) and its correlation with different clinicopathological features of patients with ESCC.

Materials and Methods

Tissue samples

Fresh tumoral and distant tumor-free esophageal tissues were obtained from fifty patients with ESCC through surgery at the Imam Reza and the Omid Hospitals of Mashhad University of Medical Sciences, Iran. None of patients received any therapeutic interference such as chemo-therapy or radiation, prior to sampling. After dissection, the samples were directly treated in RNAlater solution (Qiagen, Hilden, Germany) and transferred to −20 ˚C till RNA extraction.

The Ethics Committee of Mashhad University of Medical Sciences approved the study and enrolled patients formally declared their consent to be recruited in the study. Histopathological characteristics of the neoplasmic samples, such as size, location, and grade of differentiation were recorded. All tumoral tissues were also confirmed histologically to ensure that they contain at least 70% tumor cells (20).

cDNA synthesis and quantitative real-time-PCR

RNA was extracted from the normal and tumoral samples, using the RNeasy Mini kit (Qiagene, Hilden, Germany). Synthesis of cDNA was performed with oligo dT using first-strand synthesis kit (Fermentas, Lithuania) according to the manufacture's procedure. Expressional analysis of *CD44S* and *CD44V3* was performed in triplicate reactions through a comparative threshold cycle/SYBR in experienced methodology (GENET BIO, Korea) in a real-time thermal cycler (StratageneMx3000P, La Jolla, CA) using the primer sequences described in Table 1. Comparative real-time PCR was carried out using SYBR Green PCR Master mix containing 6-Carboxyl-X-Rhodamine (ROX) as a reference dye. The subsequent thermal cycling program was applied: 10 min at 95 ˚C as initial denaturation step, followed by 40 cycles of 15 sec at 95 ˚C, 10 sec at 60 ˚C and 1 min at 72˚C. Data was normalized for glyceraldehyd 3-phosphat dehyrogenase (GAPDH) expression as an internal control by means of comparative threshold cycle method. PCR reactions were accomplished in a 20 µl total volume with 400 ng of cDNA, 10 µl SYBR Green PCR master mix (GENET BIO, Korea), (nuclease-free water, and 0.6 µl forward and reverse primers (10 pmol). Over two-fold increase in mRNA expression was considered as overexpression, whereas minus two-fold decrease was considered as underexpression. Any value in the range between was considered as normal expression.

Statistical analysis

The statistical analysis was performed using SPSS 16.0 statistical package (SPSS, IL, US). The associations between *CD44S/CD44V3* mRNA expression levels and different clinicopathological aspects were analyzed by $\chi 2$ squared test. All *P* values were two-tailed and *P*-values <0.05 were considered statistically significantly.

Results

Fifty patients including 24 males (48%) and 26 females (52%) enrolled in this study. The surgical samples were obtained before any other treatment ensuring that the histopathological characteristics of the samples were not affected with the therapeutic interference. The patients' mean age and standard deviation at the time of diagnosis was 61.73±12.14 years (ages ranged from 30 to 87 years). Tumor sample sizes ranged between 1.5 and 12 cm (4.16±1.89). Samples were dissected from middle (25 of 50), lower (22 of 50) or upper (3 of 50) regions of the esophagus. Most tumor samples (48 of 50) were in stages II or III of tumor growth, and only two samples were in stage I. Based on the histopathological analyses, 8 tumor samples (16%) were classified as poorly differentiated, whereas 10 (20%) and 32 (64%) specimen were well and moderately differentiated, respectively.

Furthermore, 21 of the tumors (42%) had metastasized to the lymph nodes. Table 2 summarized the clinicopathological characteristics of the patients.

Table 1. Primer sequences used for comparative real-time PCR

Primer	Forward	Reverse
CD44S	TCCAACACCTCCCAGTATGACA	GGCAGGTCTGTGACTGATGTACA
CD44V3	GCACTTCAGGAGGTTACATC	CTGAGGTGTCTGTCTCTTTC
GAPDH	GGAAGGTGAAGGTCGGAGTCA	GTCATTGATGGCAACAATATCCACT

Table 2. Correlations between *CD44* isoforms *S* and *V3* genes expression and clinicopathological characteristics of the Esophageal Squamous Cell Carcinoma patients

Patients	50	*CD44S/CD44V3* overexpression (*P*- value)
Mean age (mean±SD)	61.73±12.14years	NS
Size (mean ± SD)	4.158±1.88 cm	NS
Sex		
Male	24 (48.0%)	S (0.003)
Female	26 (52.0%)	S (0.047)
Location		
Lower	22 (42.0%)	NS
Middle	25 (50.0%)	NS
Upper	3 (8.0%)	NS
Grade		
PD	8 (16.0%)	NS (> 0.05)
MD	32 (64.0%)	NS (> 0.05)
WD	10 (10.0%)	S (0.033)
Lymph node		
Yes	23 (46.0%)	S (0.04)
No	27 (54.0%)	S (0.05)
Stage		
I	2 (4.0%)	NS (> 0.05)
II	28 (56.0%)	S (0.05)
III	40 (40.0%)	S (0.003)
Depth of tumor invasion (T)		
T1	9 (18.0%)	NS (> 0.05)
T2	41 (82.0%)	S (0.00)

WD: well differentiated; MD: moderately differentiated; PD: poorly differentiated; NS: non- significant, S: significant

Upregulation of CD44S and CD44V3 in ESCC samples

We analyzed *CD44S* and *CD44V3* mRNA expressions in fifty tumor samples compared to their paired non-neoplastic esophageal epithelium by quantitative real time-PCR. The pattern of genes expression is illustrated using a scatter plot in Figure 1.

Significant overexpression of *CD44S* and *CD44V3* mRNA was observed in 13 (26.0%, P=0.03) and 11 (22.0%, P=0.007) specimens, respectively. The minimum and maximum levels of mRNA expression fold change for *CD44S* were -3.96 and 13.80 fold, respectively (mean±SD, 1.22±2.59), while these levels for *CD44V3* were -5.53 and 11.30 fold (0.85±2.89). Eight samples (16.0%) showed overexpression of both genes, whereas 34 samples (68.0%) showed normal or under expression of both *CD44V3* and *CD44S* genes (Table 3).

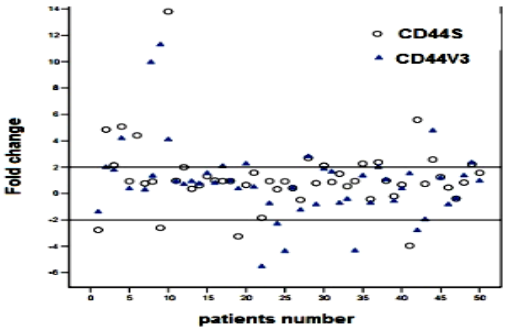

Figure 1. Scatter plot describe the level of CD44S and CD44V3 mRNA expression in ESCC patients. The Y-axis displays fold change of gene expression, and the X-axis demonstrates the number of patients. A two-fold increase in gene expression in tumor samples was considered to be overexpressed state, whereas a minus two-fold decrease was considered as underexpression. The average was thought as neither change nor normal expression

Association between *CD44S* and *CD44V3* gene expression

There is a significant correlation between *CD44S* and *CD44V3* mRNA expression among ESCC specimens (P=0.002). By analyzing concomitant gene expression, we found that the samples with high level of *CD44V3* gene expression, also expressed high level of *CD44S* (Figure 2).

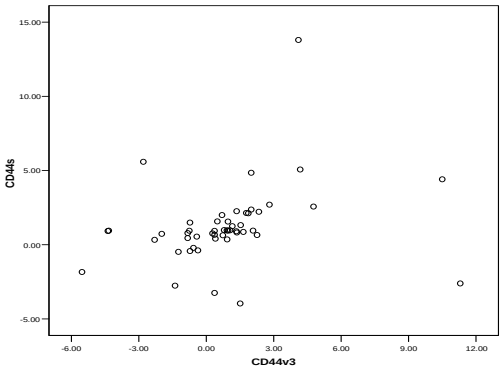

Figure 2. Regression plot representing correlation between CD44S / CD44V3 genes expression (P=0.002)

Association between *CD44S*/*CD44V3* expression and clinicopathological variables

To estimate the impact of *CD44S* and *CD44V3* mRNA expression on clinicopathological features, we analyzed the correlation between *CD44S* and *CD44V3* expression and various clinicopathological variables (Table 2). A significant correlation was detected

Table 3. Association between *CD44S* and *CD44V3* gene expression

Expression pattern	*CD44V3*		*P*- value
CD44S	Overexpression	Normal/underexpression	
			0.002
Overexpression	8	5	
Normal/underexpression	3	34	

between the expression of both genes and the depth of tumor invasion (P=0.001). Furthermore, expression of both genes was significantly correlated to the stage of tumor progression (P=0.03). Another valuable significant correlation was revealed between *CD44S* and *CD44V3* genes expression and the grade of tumor differentiation (P=0.033). In fact, 2 of 13 (15.38%) tumor samples, which overexpressed *CD44S* and 3 of 11 (27.27%) tumor samples, which overexpressed *CD44V3* were well differentiated (WD), indicating the early expression of these genes in the process of dedifferentiation. In addition, expression of both genes was significantly associated with the lymph node metastasis (P=0.04). Five of 13 *CD44S* overexpressed tumors (38.46%) and 6 of 11 *CD44V3* overexpressed tumors (54.54%) did not show any metastasis to the lymph nodes, while 2 of 13 *CD44S* overexpressed tumors (15.38%) and 1 of 11 *CD44V3* overexpressed tumors (9.09%) had lymph node metastasis. Overexpression of both genes was significantly higher in males (P=0.03) in comparison to females (*CD44S*: mean±SD: 1.84±3.08 and 0.63±1.91, respectively and *CD44V3*: 1.15±2.76 and 0.55±3.04, respectively). *CD44V3* mRNA expression was inversely correlated to the depth of tumor invasion (P=0.015). Five of 11 tumors (45.45%), which overexpressed *CD44V3* were T1, and 6 of 11 (54.54%) tumors that overexpressed *CD44V3* were T2. There were no significant associations between *CD44S* and *CD44V3* expressions alone and other clinicopathological variables such as age and tumor size (P>0.05).

Discussion

ESCC is an extremely invasive tumor of the gastrointestinal system, and novel prognostic, diagnostic and therapeutic modalities are immediately required. These requirements can lead to explore key markers involved in the development and progression of the ESCC. In this study, we analyzed mRNA expression of the stem cell markers *CD44S* and *CD44V3* in tumoral and adjacent non-tumoral tissues of fifty patients with ESCC. Our results showed significant overexpression of both genes in affected cells. Alternative splicing mechanisms lead to the production of different isoforms of CD44 gene. Previous studies have shown that the specific molecular structure of each isoform can influence the cell behavior uniquely. The isoforms vary according their position of N/O-linked glycosylation modification, which exist in the extracellular domain. Changing in these positions influences the affinity of isoforms with various ligands, leading to different modulations of intracellular signaling pathway (21).

Several studies have shown the overexpression of *CD44* and its variants in variety of malignancies. It has been shown that the expression of this gene and its isoforms is increased significantly in different malignant tissues including breast cancer (22), leukemic hematopoiesis (23), head and neck squamous cell carcinoma (17), oral cancer (24) and Barrett's esophagus (25). Takahashi *et al* have shown that there is a significant correlation between *CD44* and grade of tumor cell differentiation in prostate cancer (26).

In this study, we also observed overexpression of *CD44S* (26%) and *CD44V3* (22%) in ESCC patients. Many studies have shown the correlation between *CD44* variants gene expression and depth of tumor cells invasion and metastasis in human cancers (27). Although Wang *et al* have illustrated that the expression of *CD44V3* has a direct correlation with tumor invasion in head and neck cancer (17), nonetheless, Suzuki *et al* have described that decreased expression of *CD44V3* and *V6* is associated with depth of tumor invasion in lung cancer (28).

Conclusion

We observed a significant inverse association between *CD44V3* expression and depth of tumor invasion. This may be due to specific physiological state of esophagus and micro environment of tumor cells. We showed that the simultaneous increased expression of *CD44S* and *CD44V3* genes was associated with clinical features of ESCC patients. Further detailed studies on a large patient population are required to elucidate the exact role of *CD44S* and its variants expression to introduce a specific marker for prognosis of esophageal carcinoma.

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