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Epithelial-Mesenchymal Transition and Inflammation in Head and Neck Squamous Cell Carcinoma

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Abstract

Head and neck squamous cell carcinoma (HNSCC) represents a large majority of cancers arising from the head and neck, especially the oral cavity. Despite advances in therapy, the five-year survival rate remains low due to the number of patients presenting advanced stages of the disease. The role of epithelial-mesenchymal transition (EMT) in tumorigenesis in HNSCC remains unexplored. The current study aimed at investigating the mRNA expression of the three major factors in tumor specimens to define their functional and pathological roles in this malignancy. The expression of E-cadherin, vimentin, and tumor necrosis factor (TNF)-α were examined in 31 tumorous and 31 non-tumorous samples obtained from patients with HNSCC. Total RNA was extracted from all tumors for cDNA synthesis and mRNA expression levels of E-cadherin, vimentin, and TNF-α were assessed by real-time PCR. We showed a significant decrease in E-cadherin expression and increase in vimentin and TNF-α expression in tumorous samples in comparison with non-tumorous ones ($P \leq 0.05$). Also, there was a significant correlation between vimentin mRNA expression and poor differentiation of tumor ($P \leq 0.05$). Since many studies investigated EMT markers in head and neck cell lines, the current study on human samples can unveil the role of these markers in HNSCC and their relationship with patients` clinicopathological features. Therefore, it might be possible to prevent it, and a therapeutic strategy could be effective in the future.

Keywords: HNSCC; EMT; Metastasis; Inflammation; Gene Expression.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide with late diagnosis and poor prognosis [1]. Patients with HNSCC are usually diagnosed with high-grade and -stage tumors

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[2]. Therefore, the identification of specific biomarkers of this cancer would be very substantial. Despite advances in therapy, the five-year survival rate for HNSCC is not ameliorated greatly over the past years [3]. Thus, it is crucial to search for clinicopathological features of patients with HNSCC.

One of the most favorable events that cancerous cells need to spread is metastasis, which could happen by epithelial-mesenchymal transition (EMT). The role of EMT in metastasis is perceptible in many malignancies and there is evidence that HNSCC could occur by EMT.

Studies of epithelial malignancies consistently show that the transition from an epithelial cell to a mesenchymal cell is due to aberrant gene expression [4]. In cancer progression and metastasis, EMT is a pathologenetic event, and cancer cells affected by this process can acquire highly invasive features [5].

Evidence shows that Wnt signaling pathway plays a key role in initiating EMT process, tumor progression, and metastasis [6, 7]. Wnt signaling pathway is known by Wnt-β-catenin pathway since its proteins bind to their transmembrane receptors where β-catenin is a central molecule in this pathway. It acts as a transcriptional cofactor in Wnt signaling [4, 8].

E-cadherin is primarily expressed in epithelial cells to maintain their intracellular adhesion and is frequently inactivated in tumor invasion by multiple mechanisms [9-11]. E-cadherin connects to catenins, similar to β-catenin, by its intracellular domain. The association between E-cadherin and β-catenin makes them a partner in cell-cell adhesion. Therefore, any changes in these proteins can generate tumorigenesis following the cell-cell adhesion damage.

Vimentin is an intermediate filament protein, which acts in mesenchymal cells [10]. Nevertheless, it is expressed in migrator epithelial cells, for example, during wound healing. Its expression in head and neck cancer is pathologically associated with tumor invasion and metastasis [4, 12, 13]. In addition, its promoter is thought to be affected by β-catenin, which recommends its involvement in migration of epithelial cells [14].

The EMT process is tightly connected to inflammation as well. In fact, inflammatory responses have critical roles in all stages of tumor development [15]. Also, studies show that inflammation is a key inducer of EMT during cancer progression [16]. Therefore, assessing EMT and inflammation markers together could be strongly helpful to better understand metastasis, diagnosis, and therapy.

There are several inflammatory markers identified to trigger EMT [17]. One of the most important inflammatory cytokines is tumor necrosis factor (TNF)-α secreted by tumor-resident macrophages. It is observed that TNF-α can induce EMT from various cancer cells [18]. It can interfere with the EMT process by activating signaling pathways that lead to several gene expressions.

The connection between EMT markers and TNF-α explains the molecular and cellular interaction for the association between inflammation-driven tumor progression and metastasis.

There are several studies on the expression of E-cadherin and vimentin in HNSCC cell lines[19, 20], but the current study aimed at investigating a possible role of these markers with respect to clinicopathological features of patients with HNSCC.

Materials and Methods

31 paraffin-embedded HNSCC biopsy samples and 31 adjacent non-cancerous samples were obtained from the Cancer Institute of Imam Khomeini Hospital Complex affiliated to the Tehran University of Medical Sciences from January 2015 to August 2016. Written informed consent of the study was obtained from all patients.

The total RNA was extracted using TRIzol® Reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. Then, total RNA (1000 µg) was used for cDNA synthesis using Prime¬Script™ RT Master Mix (Takara Bio, Inc., Otsu, Japan) in a 10 µL reaction mixture. Rotor-Gene 6000 (Corbett, now available as the Rotor-Gene Q, Qiagen, Hilden, Germany) was used to perform real-time polymerase chain reaction (PCR). The current study reaction mixture (the final volume reaction of 20 µL per well) included 2 µL of cDNA, 10 µL of 2xSYBR Premix Ex Taq II (Takara Bio, Inc.), 6 µL of H2O, and 2 µL of 0.5 pM primers. The study used a two step method due to the 58˚C annealing temperature. The amplification process was as follows: 95˚C for 30 seconds, 40 cycles of 95˚C for 15 seconds and 58˚C for 25 seconds. Each tissue sample was assayed in duplicate. The efficiency of the PCR amplification process was 98% ± 2%. To evaluate primer specificity, a melting curve analysis was performed for the PCR products of the target genes. Finally, the mean relative mRNA expression of the target gene was calculated using fold changes with the formula 2-ΔΔCt and normalized to β-actin. The difference in mRNA expression was presented as the relative fold between the groups.

GraphPad Prism 7 and SPSS version 16 were employed to analyze the results. The Student t-test was used to determine significant differences between the groups. The Kruskal-Wallis test was used to determine

the correlation between markers of mRNA expression levels and tumor differentiation. The data were expressed as mean ± standard deviation (SD). P <0.05 was considered statistically significant.

Results

All 31 enrolled patients were diagnosed with HNSCC. Tongue (32.2%), lip (19.3%) and submandibular (16.1%) were the three most frequent samples. The mean age of the HNSCC patients was 61.3±10.3. Twenty (64.5%) patients were male (Table 1).

The expression of E-cadherin, vimentin, and TNF-α mRNA was analyzed in 31 tumorous and 31 non-tumorous tissue samples. It was identified that mRNA expression of E-cadherin was downregulated in tumorous tissue samples whereas its expression did not show such changes in non-tumorous samples (Figure 1).

The relative mean of vimentin mRNA expression was significantly higher in tumorous tissue samples than non-tumorous samples (Figure 2). Due to the relative mean expression of E-cadherin and vimentin mRNA, it was found that the tumor margin was not affected by EMT factors when tumorous cells did not spread to the adjacent tissue.

The mRNA expression of TNF-α was similar to that of vimentin. On the other hand, it was observed that the samples obtained from cancerous regions had higher levels of expression compared with the control ones (Figure 3), it meant that cancerous cells had more

Table 1. Clinical characteristics of the study population

Variables	Patients (n = 31)	Non-cancerous adjacent normal cells (n = 31)
Mean age ± SD	61.3 ± 10.3	61.3 ± 10.3
Gender (male/female)	20/11 (64.5/35.5%)	20/11 (64.5%/35.5%)
Smoking status		
Smokers	21 (67.8%)	21 (67.8%)
Non-smokers	10 (32.2%)	10 (32.2%)
Histopathology		
Tongue	10 (32.2%)	-
Lip	(19.3%)	-
Submandibular	(16.1%)	-
Throat	(9.7%)	-
Cheek	(9.7%)	-
Mandibular	(6.5%)	-
Larynx	(6.5%)	-

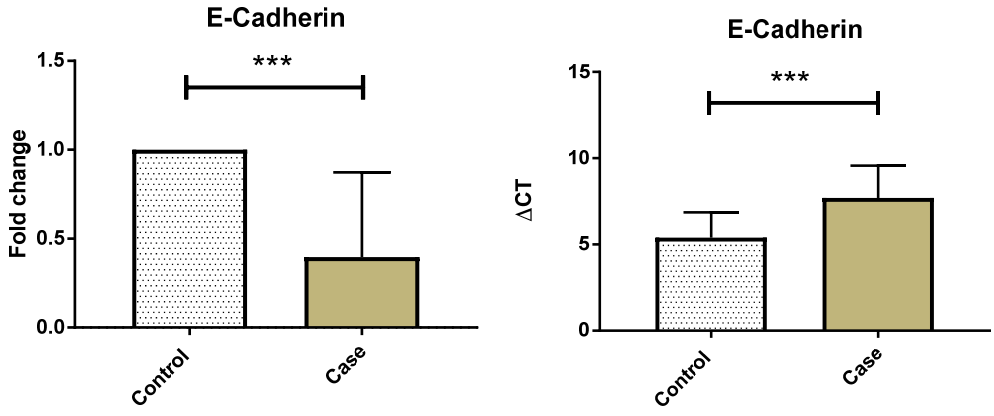

Figure 1. Gene expression of E-cadherin between case and control samples. mRNA levels of E-cadherin were measured using real-time PCR. Data are represented as mean ±SEM of at least three separate experiments ***<0.001.

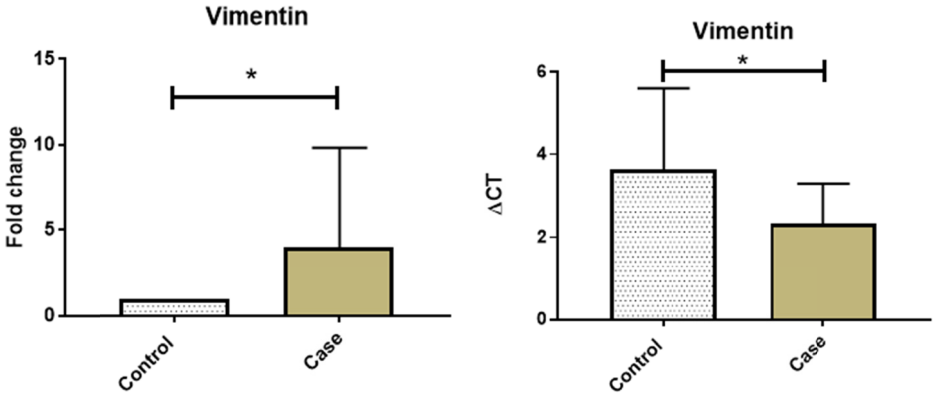

Figure 2. Gene expression of vimentin between case and control samples; mRNA levels of vimentin were measured using real-time PCR. Data are represented as mean ±SEM of at least three separate experiments.*<0.05.

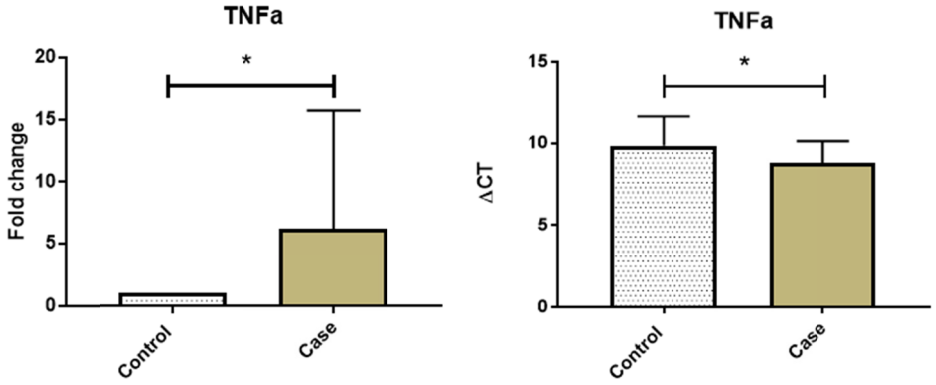

Figure 3. Gene expression of TNF-α between case and control samples. mRNA levels of TNF-α were measured using real-time PCR. Data are represented as mean ±SEM of at least three separate experiments. *<0.05.

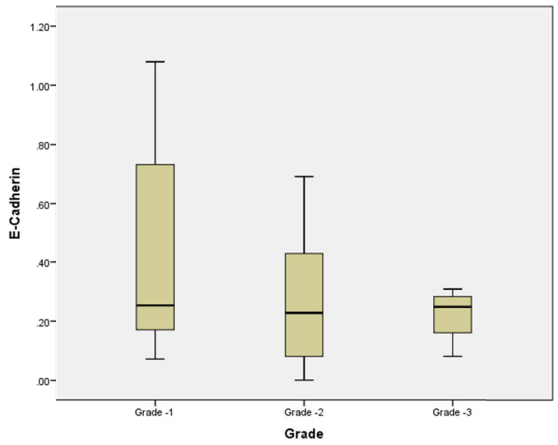

Figure 4. Gene expression of E-cadherin between three different tumor grades. mRNA levels of E-cadherin were measured in three different tumor grades (grade1, grade2, and garde3) using real-time PCR. Data are represented as mean ±SEM of at least three separate experiments.

expression levels of TNF-α mRNA, which was a kind of inflammation marker.

To evaluate the clinical importance of changes in the mRNA expression levels of E-cadherin, vimentin, and

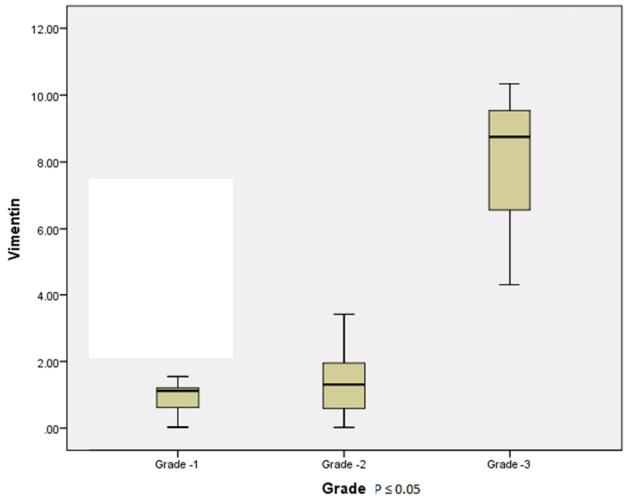

Figure 5. Gene expression of vimentin between three different tumor grades. mRNA levels of vimentin were measured in three different tumor grades (grade1, grade2, and garde3) using real-time PCR. Data are represented as mean ±SEM of at least three separate experiments. *<0.05.

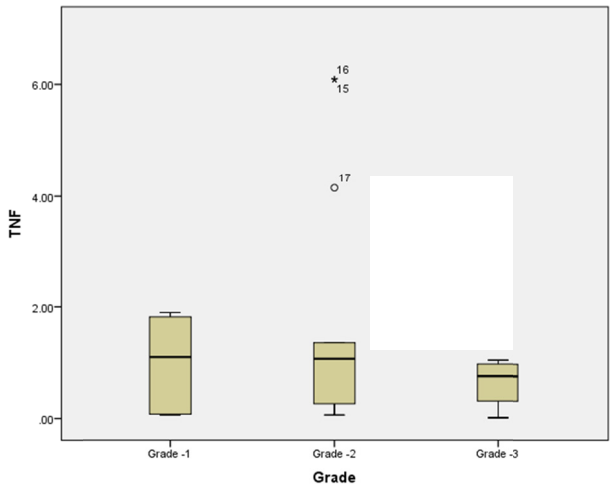

Figure 6. Gene expression of TNF-α between three different tumor grades. mRNA levels of TNF-α were measured in three different tumor grades (grade1, grade2, and garde3) using real-time PCR. Data are represented as mean ±SEM of at least three separate experiments.

TNF-α in HNSCC tissue, the correlation between mRNA expression of these genes and clinicopathological features of patients with HNSCC were analyzed. It was observed that only vimentin mRNA expression levels were significantly correlated with higher grades of tumors (Figure 4).

Although the poorly differentiated sample size was small, it showed that the vimentin expression levels were more, the tumor differentiation was less. The levels of mRNA expression of E-cadherin and TNF-α did not significantly correlate with tumor differentiation (P = 0.74, P = 0.32, respectively) (Figures 5 and 6).

Discussion

EMT is a cellular mechanism that by means of epithelial cells gains motile features similar to those of mesenchymal cells. In cancer settings, such changes are necessary to make tumor progression, invasion, and metastasis [21]. In several tumors, EMT is associated with poor prognosis and low survival rate [22]. During the EMT process, epithelial cells can gain mesenchymal properties and show reduced-epithelial features such as increased motility and decreased intercellular adhesion.

To date, much evidence shows that this process plays a crucial role in tumor invasion and metastasis. Many

transcriptional factors, such as E-cadherin, are observed in the regulation of EMT event in several cancers such as breast, colon, and liver cancers, as well as HNSCC [9].

The EMT process involves many interactions, but the key step is believed to be the downregulation of E-cadherin and upregulation of vimentin, which lead to phenotypic changes. It means that these changes allow the cancer cells to move through the extracellular matrix [23].

There are several studies on the expression of E-cadherin and vimentin in HNSCC cell lines, but the current study aimed at investigating the possible role of these markers with respect to clinicopathological features of patients with HNSCC.

Despite the fact that many publications showed the relationship between E-cadherin expression and patients` clinical features [24-29], in the current study, no significant correlation was observed between this marker and tumor differentiation. The current study identified that E-cadherin was downregulated in tumorous tissue samples in mRNA expression levels compared with non-tumorous ones, thus, it shows that the cellular adhesion was decreased in tumorous samples and this the EMT process was initiated. Nevertheless, there was no significant evidence about the relationship between E-cadherin expression and tumor grades.

The current study results indicated that high vimentin expression was prognostic for poorly differentiated tumors, on the other hand, vimentin overexpression was observed in cancerous tissue samples compared with noncancerous ones, and also mRNA expression of vimentin was higher in grade III than grade I tumors. Thus, high vimentin expression levels could be served as a prognostic marker of tumor differentiation in patients with HNSCC.

Similar to the current study results, many studies on human epithelial carcinomas, such as hepatocellular and colon carcinoma, as well as breast cancer and prostatic adenocarcinoma showed that vimentin expression is with poor prognosis [4, 30-35].

As many publications note the relationship between cancer and inflammation, and there are consistent associations between chronic inflammatory situation and tumorigenesis [36], it was decided to conduct the current study on one of the most inflammatory factors. Also, there was evidence to prove the effect of TNF-α on EMT setting initiation[37]; thus, the mRNA expression level of TNF-α in patients with HNSCC was examined to show its expression changes between tumorous and non-tumorous samples and its relationship with tumor differentiation.

The overexpression of TNF-α was observed in tumorous samples in comparison with non-tumorous ones, i.e., inflammation was happened along with the EMT process, but there is no significant correlation was observed between TNF-α and tumor differentiation.

Collectively, the downregulation of E-cadherin and upregulation of vimentin could be considered as a hallmark of the EMT process in HNSCC. The current study also showed a positive correlation between vimentin and tumor grades in HNSCC tissue samples, but it failed to show a significant relationship between tumor differentiation, and E-cadherin and TNF-α as well.

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