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The Effect of Tumor Resection and Radiotherapy on the Expression of Stem Cell Markers (CD44 and CD133) in Patients with Squamous Cell Carcinoma

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ABSTRACT

Background: Head and Neck Squamous Cell Carcinomas (HNSCCs) are heterogeneous malignancies that comprise 90% of the head and neck cancers. HNSCCs originate from the mucosal lining epithelium of the upper aerodigestive tract. Cancer stem cells (CSCs) that generate HNSCCs with the CD44, CD133, and ALDH phenotype and are resistant to radiotherapy and chemotherapy. In the current, the quantitative alteration in CD44 and CD133 expression pre- and post-tumor resection and radiotherapy was evaluated in HNSCC patients. Moreover, the alterations in the expression of Bax, Bak, Bcl-2, ALDH, and PTEN genes were measured.

Materials and Methods: Flow cytometry was performed to evaluate the alterations in CD44 and CD133 surface markers pre- and posttumor resection and radiotherapy. Quantitative real-time RT-PCR (qRT-PCR) was conducted to investigate the mRNA expression levels of Bax, Bak, Bcl-2, ALDH, and PTEN.

Results: The results indicated that the cancer stem cell CD44 surface marker significantly decreased after tumor resection and radiotherapy in HNSCC cases, while the decrease was insignificant for CD133 marker expression. mRNA expression level of Bcl-2 and ALDH was increased, but Bax and Bak gene expressions were reduced significantly

Conclusion: The results also indicated that the expression of CD44 significantly decreased after tumor resection and radiotherapy. The upregulation of mRNA level of Bcl-2 and ALDH, and the downregulation of Bax and Bak gene expression were noted in these cases when compared to the healthy control group.

Keywords: Head and neck squamous cell carcinoma; Cancer stem cells; Therapy resistance

INTRODUCTION

Head and Neck Squamous Cell Carcinomas (HNSCCs) are the most common malignancies of the upper digestive tract [1, 2]. They originate from the oral cavity, lip, larynx, and pharynx mucosal lining epithelium. Alcohol abuse, smoking, and infection with human papillomavirus (HPV) may be associated with the susceptibility to HNSCCs[3,4]. Despite the possibility of surgical removal of tumor mass, the absence of efficient therapeutic options, as well as the high rates of relapse and metastasis have caused high mortality in these patients. Resistance to radiotherapy and chemotherapy with low survival rates in some patients was associated with the presence of cancer stem cells (CSCs)[5-7]. CSCs with

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CD44[8], CD133[9] , and aldehyde dehydrogenase (ALDH)[2] phenotypes, are the most important cells within carcinoma. They display self-renewal and differentiation properties and also resistance to conventional therapy and environmental changes such as hypoxia and the lack of nutrients [10]. Although the exact molecular mechanism has not been yet identified, it seems that DNA repair capacity and reduced apoptosis by these cells stimulate tumor recurrence and metastasis [11-13]. CSCs are resistant to chemotherapy and CD44 as a key hyaluronan receptor on CSCs plays a vital role in the aggregation, proliferation, and migration of cancerous cells[14]. Cancers are characterized by their ability to evade apoptosis. Alterations in the expression of apoptotic molecules including increased expression of anti-apoptotic (Bcl-2) molecules and decreased or defective expression of pro-apoptotic (Bax and Bak) molecules are associated with cancers[15]. On the other hand, the absence of phosphatase and tensin homologue (PTEN) function, as a tumor suppressor gene, leaves different effects on cancer development (e.g. cell proliferation, apoptosis resistance, and cell migration and metastasis found in 30% of HNSCC cases)[2, 16].

Former findings indicated that the dysregulation of CD44 is associated with tumor relapse and poor clinical outcomes in HNSCC patients[17, 18]. Based on all these properties, finding an efficient method for CSC targeting could be an interesting subject in HNSCCs therapy. In this study, the quantitative changes in CD44 and CD133 expression pre- and post-tumor resection and radiotherapy is investigated. Furthermore, the expression levels of apoptotic (Bax ‹Bak ‹Bcl-2), stemness (ALDH), and tumor suppressor (PTEN) genes alternation in CSCs before treatment are evaluated.

MTERIALS AND METHODS

Patient characteristics and sample preparation

The current study was approved by the Committee of Ethics of Tabriz University of Medical Sciences (ethic code, IR.TBZMED.REC.1400.500), and patients provided informed consent for sample use and data analysis through opt-out agreements. This study enrolled a total of 16 patients (Over 50 years old; 8 women and 8 men) with Squamous Cell Carcinoma (SCC) from September 2020 to September 2021. The degree of disease invasiveness was determined according to the TNM (Tumor, Node, and Metastases) staging that was recorded in the patients' files (stage I-IV). The expression of surface markers and associated genes were evaluated pre- and post-radiotherapy. All patients were followed up within 2 weeks post radiotherapy. The demographics are summarized in Table 1. In terms of tissue samples**,** directly after surgical resection, 100 to 500 mg/kg of the native (not formalin-fixed) tissue was taken from 16 throat and larynx SCC (stage I-IV) (during surgery) patients and transferred to the laboratory at Tabriz University of Medical Sciences. The tissue specimens were minced into 2–4 mm fragments and placed in sterile 15ml centrifuge tubes. The samples were supplemented with 5 mg/ml collagenase I (Gibco) and incubated for 3h at 37C° and the dissociation solutions were discarded. The expression of genes was evaluated pretreatment. 5mL peripheral blood of these cases was obtained pre- and post- radiotherapy. The samples were taken by the Committee of Ethics of Tabriz University of Medical Sciences.

RNA isolation and quantification

The dissociated cells were washed once in FBS solution and total RNA was extracted using a RNA isolation kit (Yekta Tajhiz Azma, Iran). The concentration of RNA samples was calculated with Nano drop (NanoDrop1000; Thermo Fisher Scientific). A Reverse transcription kit (Yekta Tajhiz Azma, Iran) was performed to synthesize cDNA. The cDNA was subjected to qRT-PCR using 2X Real-Time PCR Master Mix (BioFACT) on a light cycler instrument (Roche Molecular Diagnostics). Thermal cycling conditions consisted of an initial activation step for 10 min at 94 °C followed by 40 cycles, including a denaturation step for 10 s at 94 °C, 1 min for each primer pair at the proper annealing temperature, 1 min at 72 °C and finally 10-min for extension at 72 °C. The housekeeping gene, β-actin, was used for normalizing, and relative gene mRNA levels were calculated using the 2-ΔΔCT method. Nucleotide sequences of the primers used for quantitative RT-PCR are listed in Table 2.

Table 1. Tumor stage in patients

Patients	Age	Tumor stage
8 Males	**61-70 Y**	2 patients stage I 2 patients stage II 4 patients stage III
8 Females	**59-64 Y**	1 patient stage I 4 patients stage II 2 patients stage III 1 patients stage IV

Table 2. Nucleotide sequences of the primers used for Real-Time RT-PCR

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Size (bp)
B-actin	AAACTGGAACGGTGAAGGTG	TATAGAGAAGTGGGGTGGCT	174
Bax	TCACTGAAGCGACTGATGTCC	CTCCCGCCACAAAGATGGTC	194
Bcl-2	AGTGAACATTTCGGTGACTT	CTTCCAGACATTCGGAGACC	208
Bak	CGTCCCTAAGCATGTGTCC	CCAGAATCCCTGAGAGTCC	132
PTEN	AGGAAGTGAATCTGTATTGGG	TTGCTGTGTTTCTTACCTATG	183
ALDH	TGTTAGCTGATGCCGACTTG	TTCTTAGCCCGCTCAACACT	175

Antibodies and flow cytometry analysis

Cell surface phenotype was determined by flow cytometry using the monoclonal antibodies (mAbs) including CD44 (PE, colone name, e Bioscience) (5µl/1× 10^6 cells), CD133 (FITC, colone name, e Bioscience) (5µl/1× 10^6 cells). Briefly, the cells were centrifuged at 300g and washed with staining buffer (PBS plus 5% FBS), the appropriate volume of a monoclonal antibody was added and incubated at 4°C for 20 min in the dark. The samples were washed with 1ml of staining buffer, centrifuged at 300g for 5 min, and then re-suspended in 500µl of staining buffer. At least 10,000 to 30,000 events were saved for each sample by using BD caliber (BD e bioscience). The data were analyzed by FlowJo (7.6.1) software.

Statistical analysis

The software Graph Pad Prism version 6.01 was used for analyzing the data. Values were expressed as the Means ± SD by triplicate independent experiments. One-way and two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test and Bonferroni's multiple comparisons test were applied to determine the significant difference among groups at $p < 0.05$.

RESULTS

CD44 downregulated after tumor resection and radiotherapy in female patients with HNSCCs

CD44 and CD133 expression were evaluated in HNSCC female patients by flow cytometry before tumor resection and two weeks after treatment. According to the flow cytometry results, the expression of CD44 decreased from 80% to 42% in sample 1, 85% to 39% in sample 2, 73% to 30% in sample 3, 75% to 53% in sample 4, and 77% to 47% in sample 5 after treatment. As shown in Figures 1A and B, no significant change was observed in CD133 expansion after tumor resection and radiotherapy treatment.

CD44 downregulated after tumor resection and radiotherapy in male patients with HNSCCs

CD44 and CD133 expression were evaluated in HNSCC male patients by flow cytometry before tumor resection and two weeks after treatment. According to the flow cytometry results, the expression of CD44 decreased from 83% to 36% in sample 1, 66% to 40% in sample 2, 88% to 29% in sample 3, 81% to 54% in sample 4, and 62% to 61% in sample 5 after treatment. As shown in Figures 2A and B, no significant change was observed in CD133 expansion after tumor resection and radiotherapy treatment.

Genes expression involved in apoptosis, stemness, and tumor suppression was changed in patients with HNSCC

The mRNA expression of apoptotic (Bax, Bak, and Bcl-2), stemness (ALDH), and tumor suppressor (PTEN) genes was evaluated in HNSCC patients by Real-time PCR. The mean changes in gene expression in 16 patients are shown in Figure 3. Expression of Bax and Bak pro-apoptotic genes was decreased while expression of Bcl-2 anti-apoptotic gene was remarkably increased. Also, the ALDH stemness mRNA was increased to 3.05 fold in patients compared to healthy control. Furthermore, PTEN tumor suppressor gene expression was significantly decreased.

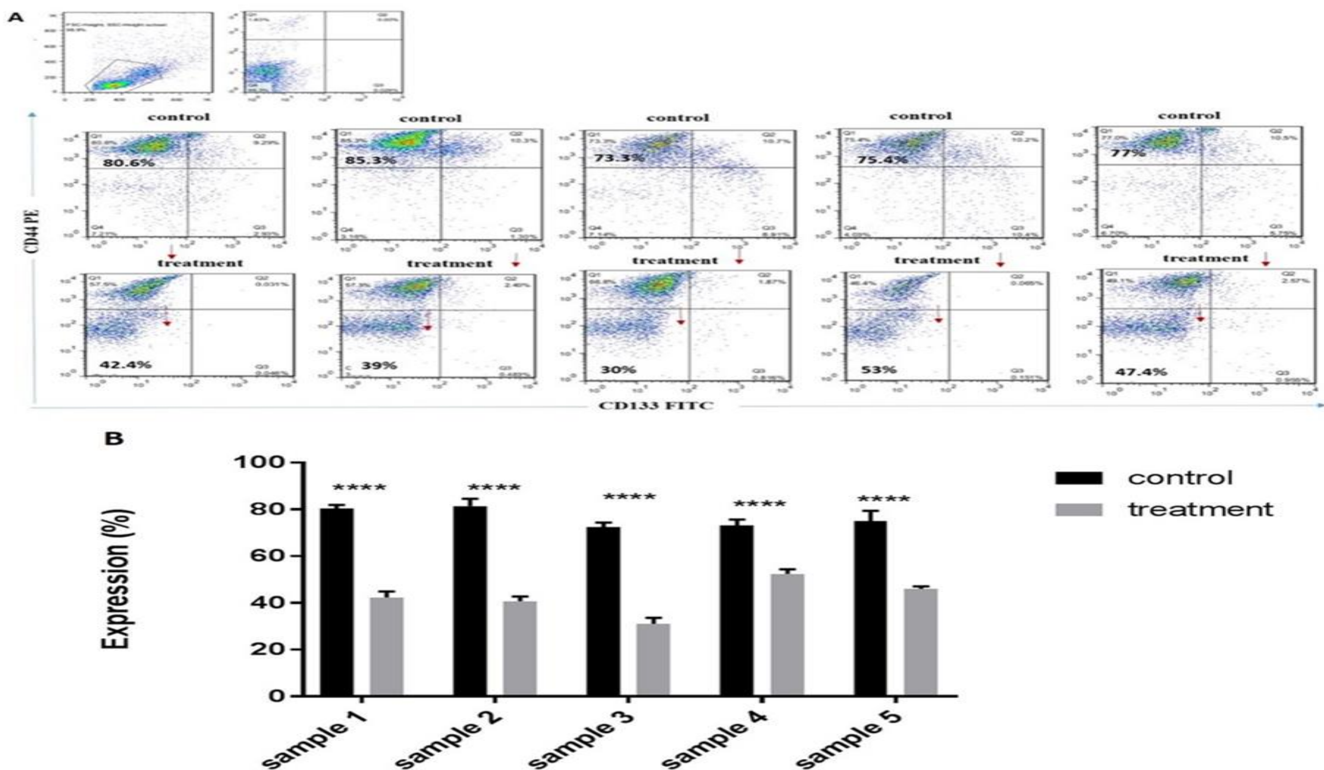

Figure 1. CD44 expression reduced in cancer stem cells following tumor resection and radiotherapy. Representative flow cytometry profile of CD44 and CD133 expression on cancer stem cells in 8 HNSCC female patients (A). The mean ± SD percentage of CD44 expression on cancer stem cells in control and treatment groups (B). Data are representative of 3 independent experiments. The values are illustrated in in mean ± SD.****p < 0.0001.

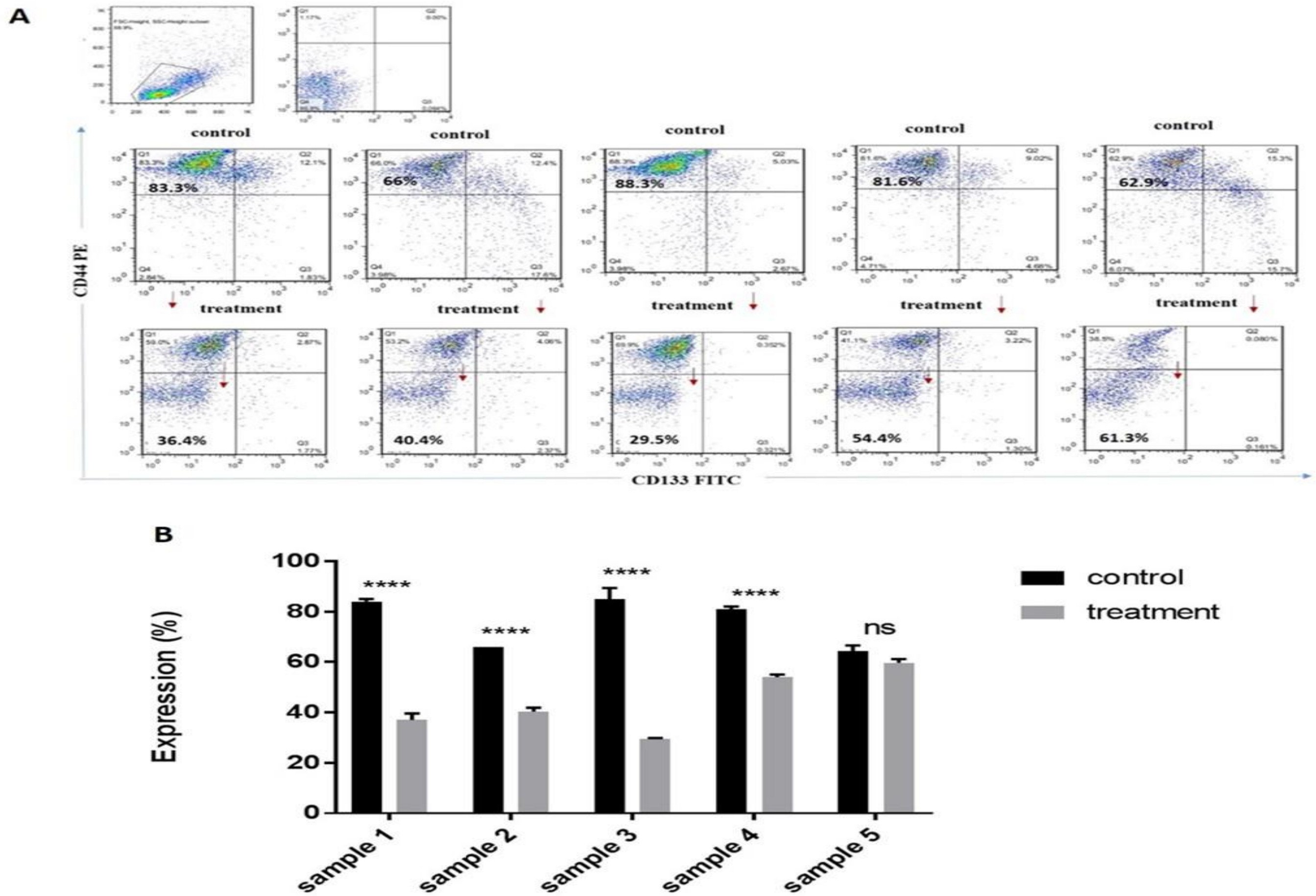

Figure 2. CD44 expression reduced in cancer stem cells following tumor resection and radiotherapy. Representative flow cytometry profile of CD44 and CD133 expression on cancer stem cells in 8 HNSCC male patients (A). The mean ± SD percentage of CD44 expression on cancer stem cells in control and treatment groups (B). Data are representative of 3 independent experiments. The values are illustrated in in mean ± SD.****p < 0.0001.

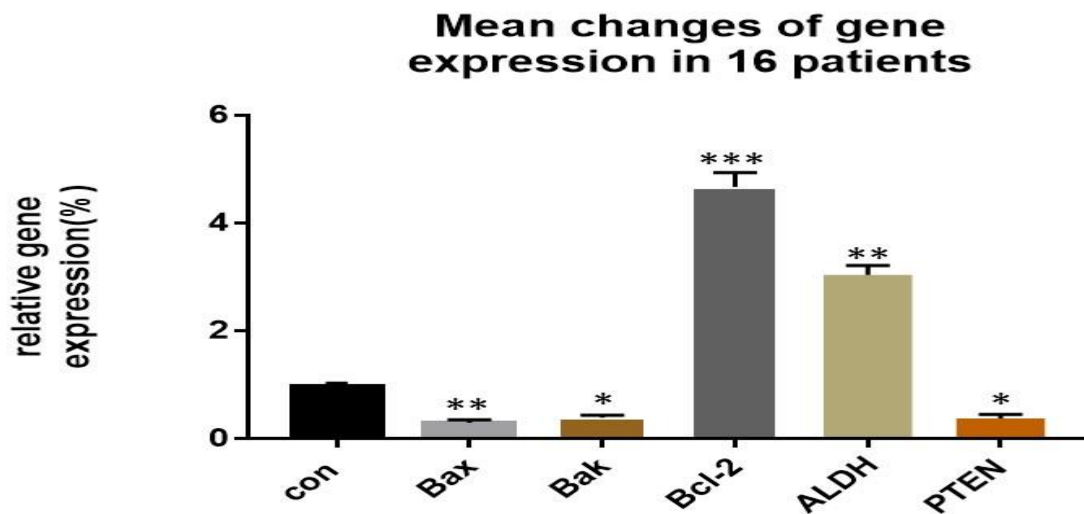

Figure 3. Expression of the genes involved in apoptosis, stemness and tumor suppressor in 16 HNSCC patients. Using RT-PCR analysis, relative mRNA level of apoptotic (Bax, Bak and Bcl-2), stemness (ALDH) and tumor suppressor genes were measured in 16 HNSCC patients, β-actin used as a loading control. Data shown are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

DISCUSSION

HNSCC is respectively the fourth and second most common cancer incidence and cancer-related mortality worldwide. Recently, it has been reported that the presence of cancer stem cells with specific properties is the main reason for metastasis and cancer relapse in HNSCC patients[19]. The high expression of CD44 is a typical biomarker in HNSCC [20, 21]. Although CD44 is expressed in the normal oral mucosa[22], the high expression of this receptor was reported in many tumors[23]. According to the researchers of the field the upregulation of CD44 stimulates proliferation and migration in the CSCs and promotes tumor invasion [14,17]. The interaction of CD44-hyaluronan stimulates tyrosine kinase (TK) activity of HER2. Furthermore, CD44-hyaluronan interactions with cytoskeletal proteins such as ankyrin promote RHOA and Rac1 activation[24,25]. Taken together, these signaling pathways stimulate CSCs migration and invasion. CD44 interaction with EGFR induces proliferation and chemotherapy resistance in HNSCC patients [26]. According to the evidence the suppression of CD44 hampers tumor growth in different types of cancer [27]. In the current study, it was found that the expression of CD44 decreased in treated patients. Moreover, the alterations in the expression of Bax, Bak, Bcl-2, ALDH, and PTEN genes before treatment was measured in the present study. PTEN expression as a negative regulator of the EGFR-PI3K-Akt-mTOR pathway reduced in HNSCCs. Costa et al.[28] previously reported that the absence of PTEN was associated with disease progression. Losing PTEN causes worse survival and poor prognosis in HNSCC[29]. The high expression of ALDH (aldehyde dehydrogenase-1) is related to chemotherapy resistance and poor clinical outcomes in HNSCC patients[23]. In addition, the high expression of ALDH is associated with the high mortality rate in HNSCC patients[30]. There is a direct association between ALDH overexpression and higher grades of disease. The deregulation of apoptosis is an important mechanism for tumorigenesis. Apoptosis is regulated by the balance of anti-apoptotic and pro-apoptotic proteins[31]. In several types of cancer, the high expression of the anti-apoptotic gene (Bcl-2) causes evasion of apoptosis and promotes the progression of malignancies[32]. Moreover, aberrant Bcl-2 expression

is related to severe grade in Laryngeal Squamous Cell Carcinoma (LSCC)[33]. The current investigation showed that the expression of anti-apoptotic gene (Bcl-2) increased and the expression of the pro-apoptotic gene (Bax and Bak) decreased in HNSCC. The probable reason is that the anti-apoptotic factor (Bcl-2) binds to pro-apoptotic members and prevents Bax and Bak translocation into the mitochondria and suppresses of apoptotic signals[34]. Additionally, Bcl-2 overexpression is associated with tumor grade[33]. Altogether, the present findings suggested that the expression of indicated genes might be associated with the grade and location of tumors.

CONCLUSION

In summary, the expression of CD44 significantly decreased after tumor resection and radiotherapy. The upregulation of mRNA levels of Bcl-2 and ALDH, and the downregulation of Bax, Bak, and PTEN gene expression were noted in the HNSCC patients.

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CONFLICT OF INTEREST

Authors declare that they do not have any conflict of interest.

Ethics approval

This study was approved by Ethical Committee of Tabriz University of Medical Sciences (The Ethic code, IR.TBZMED.REC.1400.500). The study was performed by the Declaration of Helsinki and its later amendments.

Consent to participate

Informed consent was obtained for all individual participants included in the study.

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