RESEARCH ARTICLE

Chemokine and chemokine receptor patterns in patients with benign and malignant salivary gland tumors: a distinct role for CCR7

Mohammad Reza Haghshenas[1], Mohammad Javad Ashraf[2], Bijan Khademi[3], Abbas Ghaderi[1,4], Nasrollah Erfani[1], Mahboobeh Razmkhah[1]

[1] Shiraz Institute for Cancer Research, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran
[2] Department of Pathology, Khalili Hospital, Shiraz University of Medical Sciences, Shiraz, Iran
[3] Otolaryngology Research Center, Department of Otolaryngology, Shiraz University of Medical Sciences, Shiraz, Iran
[4] Department of Immunology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

Correspondence: A ghaderi, Shiraz Institute for Cancer Research, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran, Department of Immunology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran
<ghaderia@sums.ac.ir> <ghaderia@gmail.com>
M Razmkhah, Shiraz Institute for Cancer Research, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran
<mrazmkhah2@gmail.com> <razmkhahm@sums.ac.ir>

Accepted for publication February 18, 2017

To cite this article: Haghshenas MR, Ashraf MJ, Khademi B, Ghaderi A, Erfani N, Razmkhah M. Chemokine and chemokine receptor patterns in patients with benign and malignant salivary gland tumors: a distinct role for CCR7. *Eur. Cytokine Netw.* 2017; 28(1): 27-35 doi:10.1684/ecn.2017.0388

ABSTRACT. ***Background:*** **To explore the molecular mechanisms involved in pathophysiology of malignant and benign salivary gland tumors (SGTs), we investigated main tumor-inducing chemokines and chemokine receptors, CXCL12/CXCR4/ACKR3 (CXCR7), CXCR3/CXCL10, CCR5/CCL5, CCL21/CCR7, CCL2, CCR4, CXCR5, CCR6, and CXCL8 in tumor tissues.** ***Patients and methods:*** **Parotid tissues were obtained from 30 patients with malignant and benign SGTs. Quantitative real-time polymerase chain reaction (qRT-PCR) was employed to determine the mRNA expression pattern of the mentioned chemokines/chemokine receptors and immunohistochemistry (IHC) was performed to verify the expression of CCR7.** ***Results:*** **Expression levels of CCR7 and CCR4 transcripts were higher in the tumor tissues of malignant cases in comparison to benign ones ($p = 0.03$ and 0.02). Immunohistochemistry analysis confirmed that the protein level of CCR7 concurred with the mRNA expression.** ***CCL2*** **gene transcripts were observed with a higher expression in patients with tumor-free lymph nodes (LN^-) and early stages, whereas CCR7 transcript was higher in LN^+ and late stages of the disease. A significant inverse correlation was found between CXCL10 transcript and tumor size in benign cases. The mRNA expression of CCR7, CCR4, CXCR3, CCL21, CCL5, and CXCL12 was significantly higher in mucoepidermoid carcinoma in comparison to pleomorphic adenoma subtypes ($p < 0.05$).** ***Conclusion:*** **On the basis of the present study, it was determined that malignant and benign SGTs exhibit a distinct pattern of chemokines and chemokine receptors, which are probably associated with known biological and clinical behaviors of these tumors. Significant increased CCR4 and CCR7 expression in malignant SGTs might play a central role in malignant transformation that introduces them as new targets for cancer immunotherapy.**

Key words: salivary gland tumors (SGTs), chemokine, chemokine receptor

Salivary gland tumors (SGTs) are a group of rare and heterogeneous lesions, constituting approximately 3% of head and neck cancers. In the clinical setting, adenoid cystic carcinoma (ACC) and high-grade mucoepidermoid carcinoma (MEC), the two common types of malignant SGTs, exhibit diffuse invasion to the nerve, muscle, and cutaneous tissues as well as metastasis to the regional lymph nodes (LNs) and lungs. Pleomorphic adenoma (PA) is one of the most common benign SGTs, marked by some peculiar clinicopathological characteristics such as high recurrence and malignant potentiality [1, 2]. However, it is unclear that which molecular mechanisms might be responsible for the in unfavorable behavior of these types of head and neck cancer. Recent studies have indicated that tumor tissues of the head and neck region are composed of many different types of immune cells as well as a network of cytokines and chemokines that may influence prognosis of tumor-bearing patients [3, 4]. Our previous studies indicated a significant role of regulatory T (Treg) cells and helper T and cytotoxic T lymphocytes in the pathogenesis of both malignant and benign SGTs [5, 6]. Bachelerie *et al.* have reviewed chemokine nomenclature and their immune functions and introduced chemokines and chemokine receptors as low-molecular weight proteins playing critical roles in tissue homeostasis, inflammation, immune surveillance, as well as cancer progression [7]. In cancer, chemokines and their receptors induce the recruitment of tumor-associated leukocytes to the tumor microenvironment [3, 8, 9]. It has been reported that CCR4-expressing Treg cells are recruited to tumor sites of sinonasal squamous cell carcinoma (SSCC) and ovarian cancer in response to CCL22 produced by tumor

doi: 10.1684/ecn.2017.0388

cells and/or microenvironmental macrophages [10, 11]. Beyond their function in the recruitment of leukocytes, they may contribute to homing of tumor cells to particular sites. CXCL12/CXCR4 and CCL21/CCR7 play crucial roles in the metastasis of tumor cells to the LNs in patients with HNSCC [4]. Despite the numerous studies performed on HNSCC, probable molecular mechanisms involved in the pathophysiology of malignant and benign SGTs have not been completely investigated. To clarify the role of chemokines and chemokine receptors in aggressive biological behaviors as well as multifocal recurrence, which are frequently observed in these tumors, we investigated the expression pattern of chemokines and chemokine receptors including CXCL12/CXCR4/ACKR3 (CXCR7), CXCR3/CXCL10, CCR5/CCL5, CCL21/CCR7, CCL2, CCR4, CXCR5, CCR6, and CXCL8 in tumor tissues of patients with malignant and benign SGTs.

PATIENTS AND METHODS

Patients

The parotid tissues were obtained from 30 patients with primary diagnosed SGTs, 15 cases with malignant tumors (7 males and 8 females, mean age ± SD = 53 ± 15), and 15 cases with benign tumors (7 males and 8 females, mean age ± SD = 51.5 ± 14). The specimens were surgically collected prior to any clinical treatment such as chemotherapy or radiotherapy from Khalili Hospital, Shiraz University of Medical Science, Shiraz, Iran. The study was approved by the ethics committee of Shiraz University of Medical Sciences, and all patients provided an informed written consent before participation in this study.

Tissue collection, RNA extraction, and complementary DNA synthesis

Tissue samples were placed in a 2.0 mL cryotube (SPL, Korea), snap-frozen in liquid nitrogen, and stored at –70 °C. Total RNA was extracted from the tumoral region of parotid gland using RNX-Plus (CinnaGen, Iran) and chloroform (Merck, Germany) method. The concentration and the purity of extracted RNA were evaluated by BioPhotometer (Eppendorf, Germany). 1000 ng RNA was treated by using DNase I RNase-free enzyme (Fermentas, Lithuania) and incubated for 30 min at 37 °C, and finally 2 μL EDTA (Fermentas, Lithuania) was added and incubated for 30 min at 65 °C to remove any residual DNase. Complementary DNA (cDNA) was synthesized using 10 μL of extracted RNA based on the manufacturer's instruction (Fermentas, Lithuania).

Quantitative real-time polymerase chain reaction

The abundance of *CXCL12*/*CXCR4*/*ACKR3* (*CXCR7*), *CXCR3*/*CXCL10*, *CCR5*/*CCL5*, *CCL21*/*CCR7*, *CCL2*, *CCR4*, *CXCR5*, *CCR6*, and *CXCL8* gene transcripts were determined by quantitative real-time polymerase chain reaction (qRT-PCR) using ABI thermal cycler (ABI, USA). The cDNA was amplified by 10 μL of 2× SYBR Green Master Mix (ABI, USA), 0.3 μL of each 10 pmol forward and reverse primers (Metabion, Germany), 2 μL of the cDNA product, and 7.4 μL of Diethyl pyrocarbonate (DEPC)-treated water in a total volume of 20 μL as previously reported [12]. Forward and reverse primers were designed and checked by using AlleleID 6 (PREMIER Biosoft, USA) (*table 1*). The reaction mixture for *ACKR3* (*CXCR7*), *CXCR3*, *CXCL10*, *CCR5*, *CCL5*, *CCR7*, and *CCL2* genes was first heated to 95 °C for 10 min and then amplified followed by 40 cycles at 94 °C for 15 s, 57 °C for 30 s, and 60 °C for 60 s. This reaction for CXCL12, CXCR4, CCL21, CXCR5, CCR4, CCR6, and CXCL8 was done by 40 cycles at 94 °C for 15 s and 60 °C for 60 s. The relative expression of target genes was normalized to those for β-*actin* housekeeping gene. Relative quantification was calculated by using $2^{-\Delta\Delta CT}$ formula.

Immunohistochemistry

CCR7 protein expression in the tissue blocks of patients with ACC, MEC, and PA subtypes as well as a normal

Table 1
Primer sequence of chemokine and chemokine receptor genes.

Gene	Sequence	Gene	Sequence
*ACTIN*β	5′-GCCTTTGCCGATCCGC-3′	*CCL21*	5′-CAGGACCCAAGGCAGTGATG-3′
	5′-GCCGTAGCCGTTGTCG-3′		5′-CGGGGCAAGAACAGGATAGC-3′
CXCL12	5′-TGCCAGAGCCAACGTCAAG-3′	*CCR7*	5′-GATGCGATGCTCTCTCATCA-3′
	5′-CAGCCGGGCTACAATCTGAA-3′		5′-TGTAGGGCAGCTGGAAGACT-3′
CXCR4	5′-TGCCAGAGCCAACGTCAAG-3′	*CXCR5*	5′-GGTGATGGGCTGGTGCTA-3′
	5′-CAGCCGGGCTACAATCTGAA-3′		5′-AGGAAGATGACGATGTGGTAGG-3′
CXCR7	5′-ACGTGGTGGTCTTCCTTGTC-3′	*CCR6*	5′-TGCCCACAATGAGCGGGGAAT-3′
	5′-AAGGCCTTCATCAGCTCGTA-3′		5′-ACCTCCTGCAAGGAGCACAGT-3′
CXCR3	5′-AGCTCTGAGGACTGCACCAT-3′	*CCR4*	5′-GTGGTGGTTCTGGTCCTGTT-3′
	5′-CAGTCACTGCTGAGCTGGAG-3′		5′-AGCCCACCAAGACATCCAG-3′
CXCL10	5′-AGGAACCTCCAGTCTCAGCA-3′	*CCL2*	5′-CCCCAGTCACCTGCTGTTAT-3′
	5′-CAAAATTGGCTTGCAGGAAT-3′		5′-TGGAATCCTGAACCCACTTC-3′
CCR5	5′-GGCAAAGACAGAAGCCTCAC-3′	*CXCL8*	5′-TAGCAAAATTGAGGCCAAGG-3′
	5′-AACCTTCTGCAACACCAACC-3′		5′-AAACCAAGGCACAGTGGAAC-3′
CCL5	5′-GAGGCTTCCCCTCACTATCC-3′	–	
	5′-CTCAAGTGATCCACCCACCT-3′		

tissue with parotid origin was assessed by using immunohistochemistry (IHC) staining. The formalin-fixed and paraffin-embedded tumor tissues were sectioned into 3-5 μm slices, deparaffinized in xylene, and rehydrated in graded ethanol. Sections were immersed in a solution of 10% H_2O_2 (20 mL) and methanol (180 mL) for 3 min and followed by incubation at 95 °C for 15 min in the presence of target retrieving solution. After blocking with normal diluent goat serum, sections were stained with rabbit anti-CCR7 antibody or isotype-matched antibody as negative control (Abcam, UK, diluted 1:1000) for 60 min at room temperature. After washing with PBS, the sections were treated with a secondary Horseradish peroxidase (HRP) antibody (Abcam, UK, diluted 1:1000) for 30 min at room temperature. In a sequent step, the color reaction was developed using a 3,3ĭ-diaminobenzidine (DAB) substrate chromogen kit (Dako, Denmark), and sections were counterstained with hematoxylin dye and then dehydrated in alcohol. Paraffin-embedded human normal tonsil was used as positive control. One thousand tumor cells were counted under an OLYMPUS BX41 microscope by a certified pathologist to determine percentage of CCR7-positive tumor cells. The intensity of staining was scored 0 to 3^+ (0 = no staining, 1^+ = weak, 2^+ = moderate, and 3^+ = strong) compared to the positive control staining intensity.

Statistical analysis

The normality assumptions of the data were verified by the Kolmogorov–Smirnov test. The Mann–Whitney *U* and Kruskal–Wallis *H* non-parametric tests were used for making statistical comparisons between groups using SPSS software package (version 11.5; SPSS Inc, Chicago, IL, USA). GraphPad Prism 6 (GraphPad Software Inc, San Diego CA, USA) was used to evaluate the relative expression of chemokines and chemokine receptors and present the graphs. Values were expressed as mean ± SEM, and p values less than 0.05 were regarded statistically significant. Bonferroni correction was considered as significant in the case of multiple comparison analysis. Non-parametric Spearman rank correlation was used to evaluate the correlation between the relative amounts of each chemokine, chemokine receptor, and tumor size.

RESULTS

The clinicopathological characteristics of the patients with malignant and benign SGTs

Among the malignant cases, four (26.7%) patients were diagnosed at early stages (stages I and II) and 11 (73.3%) patients at late stages (stages III and IV). Eight (53.3%) of these patients had no LN involvement and seven (46.7%) had at least one involved LN. No patient had distance metastasis at the time of diagnosis. On the basis of AJCC cancer manual, six (40%) cases were diagnosed with well-differentiated tumor, four cases (26.7%) with moderately differentiated tumor, and five individuals (33.3%) with poorly differentiated tumor. The most frequent histological tumor type of malignant and benign cases comprised MEC (8/15 (53.3%)) and PA (11/15 (73.3%)). The characteristics of the patients with malignant and benign SGTs are shown in *table 2*.

Chemokine and chemokine receptor gene transcripts in tissue samples of patients with malignant and benign SGTs

Gene expression of CXCL12/CXCR4/ACKR3 (CXCR7), CXCR3/CXCL10, CCR5/CCL5, CCL21/CCR7, CCL2, CCR4, CXCR5, CCR6, and CXCL8 was assessed by qRT-PCR, and the results were compared between tumor tissues of 15 patients with malignant and 15 patients with benign SGTs. As shown in *figure 1*, the CCR7 mRNA expression was significantly higher in malignant (168.12 ± 76.30) versus benign tissues (1 ± 0.28) ($p = 0.03$). In addition, the mRNA expression of CCR4 was found to be significantly upregulated in the malignant tissues (2.62 ± 1.01) in comparison with benign ones (1 ± 0.46) ($p = 0.02$). CXCL8 mRNA expression also showed a tendency towards increased values in malignant tissues (3.78 ± 1.31) compared to benign ones (1 ± 0.49) ($p = 0.05$). The mRNA expression of other chemokines and the chemokine receptors, however, showed neither statistical nor significant difference between the studied groups ($p > 0.05$).

Evaluation of chemokine and chemokine receptor mRNA expression between mucoepidermoid carcinoma and pleomorphic adenoma

The transcript expression of chemokines and chemokine receptors was investigated between MEC, the most common tumor type of malignant tumors, and PA, the most common tumor type of benign tumors. As shown in *figure 2*, mRNA expression of CXCL12, CCL5, CXCR3, CCL21, CCR7, and CCR4 was significantly higher among patients with MEC compared to the PA (0.47 ± 0.21 vs. 0.07 ± 0.02, $p = 0.02$ for CXCL12, 1.4 ± 0.57 vs. 0.69 ± 0.45, $p = 0.04$ for CCL5, 1.77 ± 0.54 vs. 0.92 ± 0.57, $p = 0.009$ for CXCR3, 6.38 ± 3.47 vs. 0.57 ± 0.32, $p = 0.016$ for CCL21, 258 ± 127.63 vs. 0.73 ± 0.30, $p = 0.003$ for CCR7, and 3.29 ± 1.76 vs. 0.63 ± 0.24, $p = 0.005$ for CCR4). Expression of other chemokines and chemokine receptors studied, except for CCR5, were higher among patients with MEC in comparison to those with PA; however, this difference was not statistically significant ($p > 0.05$).

Association of chemokine and chemokine receptor mRNA expression with the grade of differentiation in patients with malignant SGTs

On the basis of a quantitative analysis, none of the investigated chemokines and chemokine receptors showed significant association with the grade of differentiation in patients with malignant tumors.

Association of chemokine and chemokine receptor mRNA expression with tumor size in patients with malignant and benign SGTs

Neither the chemokines nor chemokine receptors showed correlation with tumor size in malignant SGTs. However, a statistically significant negative correlation was observed between CXCL10 and tumor size ($R = –0.6$, $P = 0.02$, $n = 15$) in patients with benign SGTs.

Table 2
The clinicopathological characteristics of the patients with malignant and benign salivary gland tumors.

Characteristic	Classification	Frequency (%)
Malignant tumors (N = 15)		
Age	–	53 ± 15
Histological tumor type	Mucoepidermoid carcinoma	8 (53.3%)
	Malignant polymorphic adenoma (malignant mixed tumor)	1 (6.7%)
	Polymorphic adenoma with malignant transformation (malignant EX mixed tumor)	3 (20%)
	Adenoid cystic carcinoma	2 (13.3%)
	Adenosquamous cell carcinoma	1 (6.7%)
Tumor location	Parotid gland	15 (100%)
Histological grade	Well differentiated	6 (40%)
	Moderately differentiated	4 (26.7%)
	Poorly differentiated	5 (33.3%)
Clinical stage	Early stage (stages I and II)	4 (26.7%)
	Late stage (stages III and IV)	11 (73.3%)
Lymph node (LN) status	Free	8 (53.3%)
	Involved	7 (46.7%)
Metastasis	Yes	0
	No	15 (100%)
Tumor size (cm)	2 (T1)	1 (6.7%)
	2-4 (T2)	6 (40%)
	4-6 (T3)	7 (46.6%)
	>6 (T4)	1 (6.7%)
Benign (N = 15)		
Age	–	51.5 ± 14
Histological tumor type	Pleomorphic adenoma	11 (73.4%)
	Warthin's tumor	2 (13.3%)
	Oncocytoma	2 (13.3%)
Tumor location	Parotid gland	15 (100%)
Tumor size (cm)	2-4 (T2)	11 (73.3%)
	4-6 (T3)	3 (20%)
	>6 (T4)	1 (6.7%)

The association of chemokine and chemokine receptor mRNA expression with lymph node status (LN) in patients with malignant SGTs

Tumor tissues of patients with negative lymph nodes (LN^-) had a significantly higher expression of CCL2 in comparison to those with positive lymph nodes (LN^+) (1.62 ± 0.42 vs. 0.25 ± 0.04, $p = 0.001$). By contrast, CCR7 was highly expressed in tumor tissues of patients with LN^+ compared to those with LN^-; however, the difference was not statistically significant (340 ± 139 vs. 17.59 ± 15.98, $p = 0.23$). The data are presented in *figure 3*.

The association of chemokine and chemokine receptor mRNA expression with clinical stage in patients with malignant SGTs

Quantitative analysis of the chemokine and the chemokine receptor expression indicated no significant difference between early stages (I and II) and late stages (III and IV) in patients with malignant tumors. However, the expression of CCL2 was found to be upregulated at early stages, whereas CCR7 showed higher expression at late stages (data not shown).

Protein expression in tumor tissue sections of patients with benign and malignant SGTs

The protein expression of CCR7 was investigated in tissue sections of primary SGTs by IHC. IHC staining score of CCR7 in tumor tissues of patients with ACC (n = 7), MEC (n = 7), and PA (n = 6) subtypes and status of LN involvement of each patient are presented in *table 3*. As shown in *table 3*, intensity as well as percentage of CCR7-positive tumor cells was found to be higher in ACC and MEC subtypes in comparison to PA subtype. Normal salivary gland tissue with parotid origin did not express CCR7 (*figure 4A*). Microscopic analysis also demonstrated that CCR7 was largely presented in the membrane of tumor cells. Infiltration of lymphocytes was either absent or near absent in tissue blocks of these patients. CCR7 positivity in SGT subtypes is shown in *figure 4*.

DISCUSSION

Tumor cells of head and neck cancer exhibit a specific network of chemokines and/or chemokine receptors, which participate in the molecular mechanisms of cancer development [4]. In this study, we evaluated the

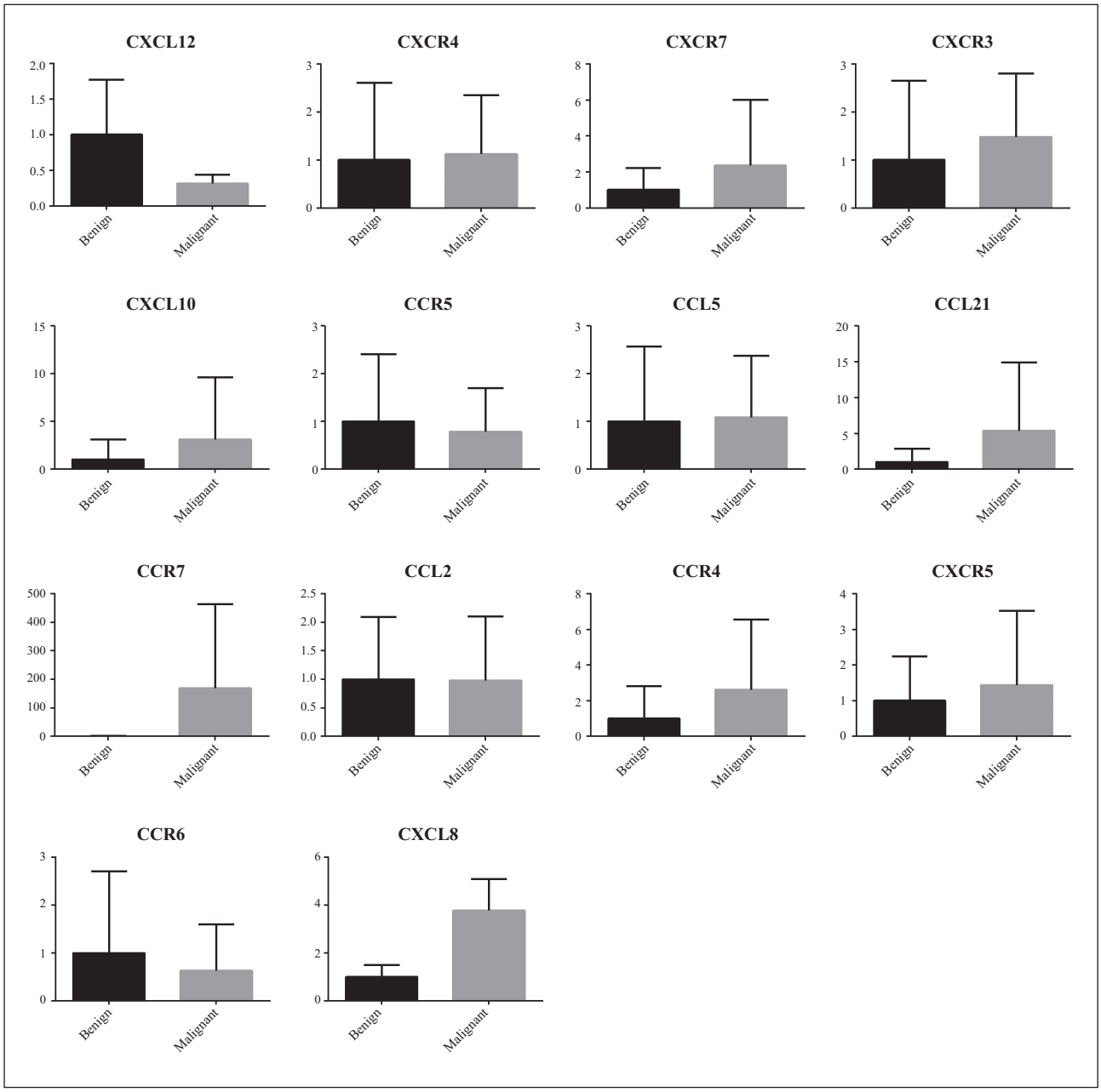

Figure 1

Evaluation of chemokine and chemokine receptor gene transcripts in tissue samples of patients with malignant and benign salivary gland tumors. CCR7 and CCR4 gene expression was found to be significantly higher in malignant cases in comparison to benign ones.

expression pattern of main tumor-inducing chemokines and chemokine receptors in tumor tissues obtained from patients with malignant and benign SGTs. Our findings indicated overexpression of CCR7 mRNA as well as protein in the malignant tissues in comparison with benign tissues. Recently, it has been reported that expression of CCR7, a key molecule for LN homing of leukocytes, is significantly correlated with LN metastasis in HNSCC [13, 14]. Such a correlation has also been shown in pancreatic ductal adenocarcinoma as well as esophageal, bladder, and gastric cancers [15-19]. In accordance with these findings, our data indicated that CCR7 expression was higher in tumor tissues of LN^+ compared to LN^- SGT patients; however, the difference was not statistically significant. Higher expression of CCR7 may have an important role in aggressive behaviors of malignant SGTs, and consequently, specific targeting may prevent the spread of tumor and LN metastasis in these patients. Our results also disclosed that CCR4 transcript were abundantly increased in the malignant tissues compared to benign tissues. CCR4 expression in human HNSCC tissues was found to be associated with LN metastasis. CCL22 produced by tumor-surrounding M2 macrophages is able to promote the cell motility and the metastatic activity of CCR4+ HNSCC cells [20]. In addition, new findings introduce CCR4 as a chemokine receptor that tends to increase tumor invasion. In this regard, IHC analysis of CCR4 in gastric tissue revealed that highly invasive cancer cells were strongly positive for CCR4, whereas less aggressive gastric cancer cells weakly expressed this chemokine receptor, and normal adjacent tissues were negative [21]. In our study, although we did not verify CCR4 expression at the protein level, the significant increase of CCR4 transcript in malignant tumors could be explained with the concept that malignant SGTs exhibit more highly invasive and migration capacities such as muscle and nerve invasion as well

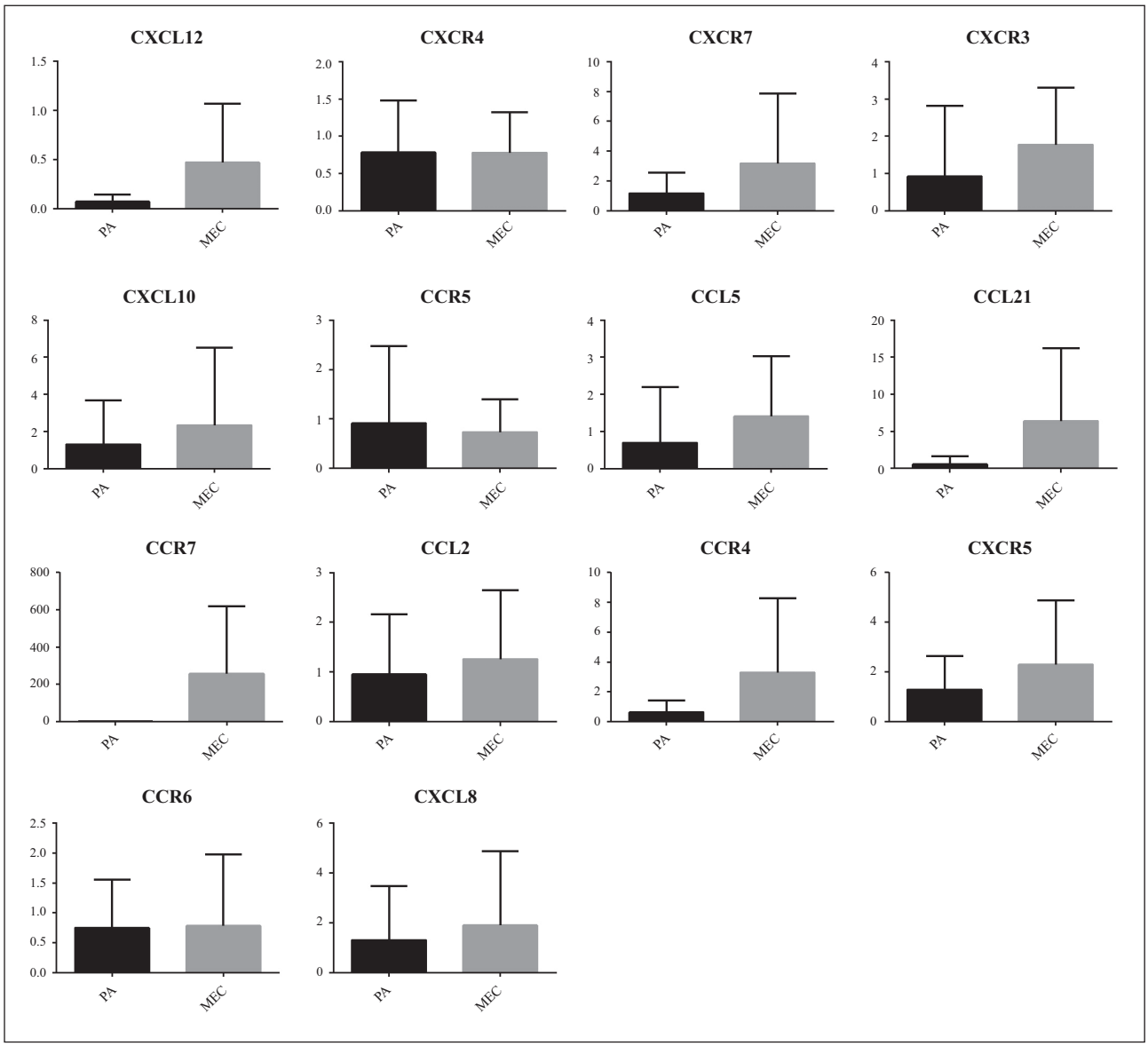

Figure 2
Evaluation of chemokine and chemokine receptor gene expression between patients with mucoepidermoid carcinoma (MEC) and pleomorphic adenoma (PA). The mRNA expression of CXCL12, CCL5, CXCR3, CCL21, CCR7, and CCR4 was observed to be significantly higher among patients with MEC in comparison to patients with PA.

as LN metastasis. Similarly, lower mRNA expression of CCR4 in benign tumor might be related to less aggressive phenotype of these tumors. In addition, a significant increase in the mRNA expression of CCL2 was revealed among tumor tissue of LN^- compared to LN^+ ones. However, its expression was non-significantly higher in early stages than in later stages of malignant SGTs. Interestingly, a recent study conducted in our laboratory with a larger sample size of patients with SGTs indicated a higher expression of CCL2 levels in the serum of those malignant patients, which were in early clinical stage, LN-, and had lower tumor size and lower tumor grade, suggesting that both of CCL2 mRNA and protein probably reduce during disease progression [22]. Reduced levels of CCL2 mRNA and protein in advanced clinical stage of patients with malignant SGTs suggest a possible evidence of the inadequate recruitment of tumor-associated leukocytes to tumor microenvironment. Contrarily, high expression of CCL2 is associated with an increase in the number of Th2 lymphocytes and tumor-associated macrophages (TAMs) [23]. Therefore, it is suggested that CCL2 expression may participate in immune suppression before LN involvement. A dual role for CCL2 is proposed in patients with head and neck cancer regarding various subtypes and complexity of tumor microenvironment. Furthermore, subdividing the malignant and benign tumors by histological tumor type revealed that in addition to CCR7 and CCR4, mRNA transcript expression of CXCL12, CCL21, CCL5, and CXCR3 were significantly higher in tissue samples of patients with MEC in comparison to PA. Interestingly, an increase in CCL21 expression was simultaneously accompanied by an increase in its receptor CCR7. Several studies indicated a significant association of CXCL12/CXCR4 and CCL21/CCR7 expression with tumor cell migration, invasion, LN involvement, cancer progression, and poor prognosis in oral squamous cell carcinoma (OSCC) and HNSCC [4]. Previous studies demonstrated that CXCL12 mediates the proliferation and migration of tumor cells from salivary gland origin via its interaction with CXCR4 [24, 25]. In the context of oral cavity, one investigation

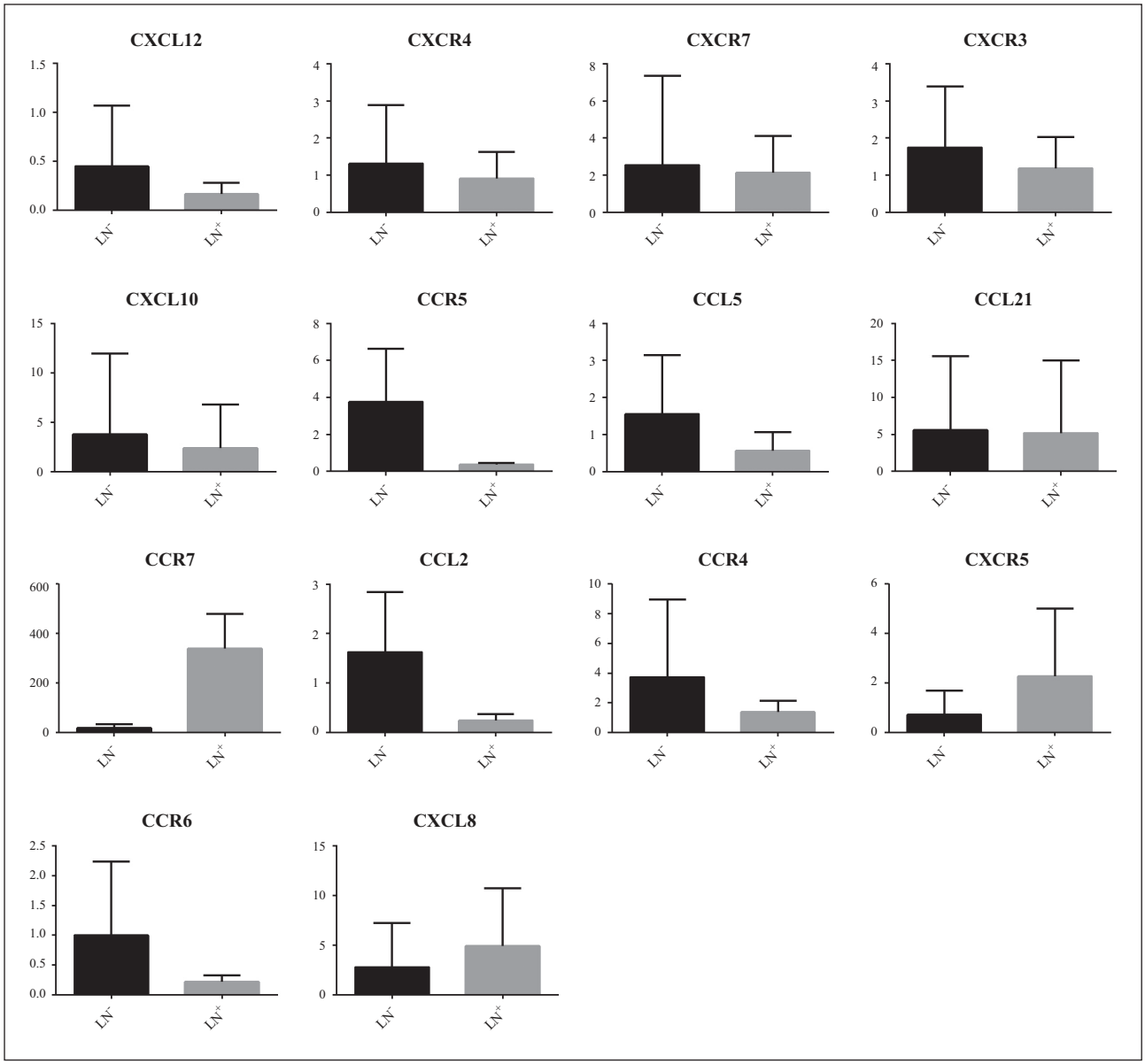

Figure 3

Association of the expression of chemokines and chemokine receptors with lymph node (LN) status in patients with malignant salivary gland tumors. Tumor tissues of patients with negative lymph nodes (LN^-) had a significantly higher expression of CCL2 in comparison to those with positive lymph nodes (LN^+). CCR7 was higher in tumor tissues of patients with LN^+ as compared to those with LN^-; however, the difference was not statistically significant.

indicated that CCL5/CCR5 axis plays a crucial role in the perineural invasion (PNI) of ACC cases [26]. The prevalence of PNI was previously indicated as a risk factor for recurrence and poor survival in ACC and MEC of SGTs [27, 28]. CXCR3 was reported to be associated with poor prognosis in breast, melanoma, and colon cancers [29]. Besides the findings regarding expression of chemokines and receptors in the patients with malignant SGTs, Spearman rank correlation analysis showed a significant negative correlation between CXCL10 and tumor size in patients with benign SGTs. It is well known that large tumors of the salivary gland region tend to have a poor prognosis in comparison to those with small mass [1]. Similar with our study, CXCL10 mRNA expression was found to be inversely correlated with tumor size in renal cell carcinoma [30]. CXCL10 is an anti-angiogenic factor and may participate in the regulation of angiogenesis during inflammation and tumorigenesis [31]. CXCL10 acts not only anti-tumorally because it is angiostatic, but it is also a potent attractant for anti-tumoral leukocytes including CD8+ T cells and activated NK cells [31]. Our result suggests that higher expression of CXCL10 may promote tumor regression and influence outcome of patients with benign SGTs. Our findings collectively show that difference in the expression pattern of chemokines and chemokine receptors between malignant and benign tumor tissues may biologically and clinically affect known behaviors of these tumors. Our results also propose that any change in the expression profile of chemokines and chemokine receptors may impact cancer development. In this regard, CCL2 might contribute to tumor growth, as well as immune suppression at the initiation of disease and before LN involvement, whereas CCR7 seems to promote cancer progression at later time points of disease.

Collectively, these findings suggest that specific targeting of chemokines and chemokine receptors, particularly

Table 3
Protein expression of CCR7 in tissue sections of patients with benign and malignant salivary gland tumors. Intensity score was graded in a 0 to 3 scale compared to the positive control staining intensity. Percentage was determined on count of one thousand tumor cells. N1 = lymph node involved by tumor; N0 = lymph node free of tumor.

Tumor type	Status of lymph node involvement (N)/intensity/percentage of CCR7-positive tumor cells						
	Patient 1	**Patient 2**	**Patient 3**	**Patient 4**	**Patient 5**	**Patient 6**	**Patient 7**
Pleomorphic adenoma (n = 6)	N0/2$^+$/50%	N0/1$^+$/80%	N0/2$^+$/60%	N0/2$^+$/60%	N0/1$^+$/50%	N0/2$^+$/70%	–
Mucoepidermoid carcinoma (n = 7)	N1/3$^+$/100%	N1/3$^+$/>90%	N0/2$^+$/80%	N1/3$^+$/>90%	N1/2$^+$/>90%	N0/2$^+$/90%	N1/2$^+$/80%
Adenoid cystic carcinoma (n = 7)	N1/2$^+$/>90%	N0/3$^+$/100%	N0/2$^+$/80%	N0/2$^+$/80%	N0/3$^+$/>90%	N0/2$^+$/80%	N1/2$^+$/>90%

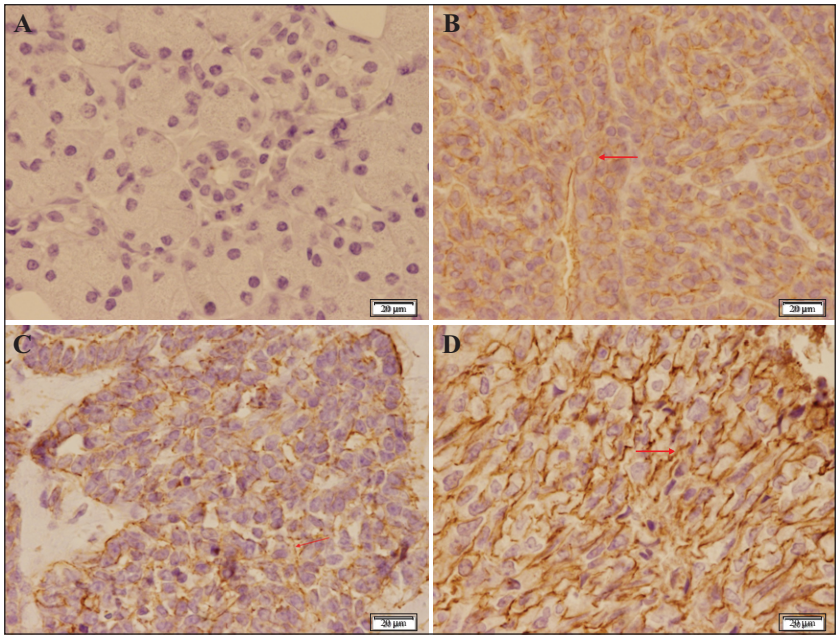

Figure 4
CCR7 expression in tissues sections of salivary gland tumors. **A.** No expression of CCR7 in normal tissue of salivary gland with parotid origin (×400). **B.** Weak staining intensity of tumor cell membrane in a case of pleomorphic adenoma, Immunoperoxidase stain (×400). **C.** Adenoid cystic carcinoma with moderate staining of tumor cell membranes, Immunoperoxidase stain (×400). **D.** Strong linear positivity of CCR7 protein in membrane of mucoepidermoid carcinoma cells, Immunoperoxidase stain (×400).

CCR7, may be a promising approach in SGT therapy trials. However, more investigations are required to achieve appropriate strategies for treatment of salivary gland tumors. Furthermore, assessment of the mRNA expression of chemokines and their receptors served as a preliminary study, which must be verified by more specimens. These data will certainly be more convincing when confirmed at protein level. The low prevalence of malignant tumors and the ensuing possibility to increase the number of cases must be stated as limitations of the current study.

Acknowledgment This work was supported by a grant from Shiraz University of Medical Sciences, Shiraz, Iran, and Shiraz Institute for Cancer Research (Grant no. 92-6859). The present study was a part of the PhD thesis written by Mohammad Reza Haghshenas, Shiraz Institute for Cancer Research, Shiraz University of Medical Sciences, Shiraz, Iran.

Disclosure. *Financial support: none. Conflict of interest: none.*

REFERENCES

1. Speight PM, Barrett AW. Salivary gland tumours. *Oral Dis* 2002; 8: 229-40.
2. Jaso J, Malhotra R. Adenoid cystic carcinoma. *Arch Pathol Lab Med* 2011; 135: 511-5.
3. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell* 2012; 21: 309-22.
4. da Silva JM, Soave DF, Moreira Dos Santos TP, *et al*. Significance of chemokine and chemokine receptors in head and neck squamous cell carcinoma: a critical review. *Oral Oncol* 2016; 56: 8-16.
5. Haghshenas MR, Khademi B, Ashraf MJ, Ghaderi A, Erfani N. Helper and cytotoxic T-cell subsets (Th1, Th2, Tc1, and Tc2) in benign and malignant salivary gland tumors. *Oral Dis* 2016; 22: 566-72.
6. Haghshenas MR, Khademi B, Faghih Z, Ghaderi A, Erfani N. Immune regulatory cells and IL17-producing lymphocytes in patients with benign and malignant salivary gland tumors. *Immunol Lett* 2015; 164: 109-16.
7. Bachelerie F, Ben-Baruch A, Burkhardt AM, *et al*. International Union of Basic and Clinical Pharmacology. Update on the extended family of chemokine receptors and introducing a new nomenclature for atypical chemokine receptors. *Pharmacol Rev* 2014; 66: 1-79.
8. Balkwill FR. The chemokine system and cancer. *J Pathol* 2012; 226: 148-57.

9. Chow MT, Luster AD. Chemokines in cancer. *Cancer Immunol Res* 2014; 2: 1125-31.

10. Lou H, Fang J, Li P, *et al*. Frequency, suppressive capacity, recruitment and induction mechanisms of regulatory T cells in sinonasal squamous cell carcinoma and nasal inverted papilloma. *PLoS One* 2015; 10: e0126463.

11. Curiel TJ, Coukos G, Zou L, *et al*. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 2004; 10: 942-9.

12. Razmkhah M, Jaberipour M, Safaei A, Talei AR, Erfani N, Ghaderi A. Chemokine and chemokine receptors: a comparative study between metastatic and nonmetastatic lymph nodes in breast cancer patients. *Eur Cytokine Netw* 2012; 23: 72-7.

13. Liu FY, Safdar J, Li ZN, *et al*. CCR7 regulates cell migration and invasion through MAPKs in metastatic squamous cell carcinoma of head and neck. *Int J Oncol* 2014; 45: 2502-10.

14. Ueda M, Shimada T, Goto Y, *et al*. Expression of CC-chemokine receptor 7 (CCR7) and CXC-chemokine receptor 4 (CXCR4) in head and neck squamous cell carcinoma. *Auris Nasus Larynx* 2010; 37: 488-95.

15. Liu XY, Song L, Wang Z. CCR7: A metastasis and prognosis indicator of postoperative patients with esophageal carcinoma. *Hepatogastroenterology* 2013; 60: 747-50.

16. Du P, Liu Y, Ren H, *et al*. Expression of chemokine receptor CCR7 is a negative prognostic factor for patients with gastric cancer: a meta-analysis. *Gastric Cancer* 2017; 20: 235-45.

17. Shi M, Chen D, Yang D, Liu XY. CCL21-CCR7 promotes the lymph node metastasis of esophageal squamous cell carcinoma by up-regulating MUC1. *J Exp Clin Cancer Res* 2015; 34: 149.

18. Chen J, Cui YU, Liu L, *et al*. CCR7 as a predictive biomarker associated with computed tomography for the diagnosis of lymph node metastasis in bladder carcinoma. *Oncol Lett* 2016; 11: 735-40.

19. Sperveslage J, Frank S, Heneweer C, *et al*. 215: The role of chemokine receptor CCR7 and Its ligands CCL19 and CCL21 in tumor progression of pancreatic ductal adenocarcinoma. *Cytokine* 2013; 63: 302.

20. Tsujikawa T, Yaguchi T, Ohmura G, *et al*. Autocrine and paracrine loops between cancer cells and macrophages promote lymph node metastasis via CCR4/CCL22 in head and neck squamous cell carcinoma. *Int J Cancer* 2013; 132: 2755-66.

21. Yang Y, Du L, Yang X, *et al*. Aberrant CCR4 expression is involved in tumor invasion of lymph node-negative human gastric cancer. *PLoS One* 2015; 10: e0120059.

22. Mardani M, AndishehTadbir A, Khademi B, Melekzadeh M, Vaziri L. Decreased serum monocyte chemoattractant protein1 in salivary gland tumor patients. *Asian Pac J Cancer Prev* 2016; 17: 3601-4.

23. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. *J Interferon Cytokine Res* 2009; 29: 313-26.

24. Suzuki S, Maeda A, Miura M, Ozeki S. Involvement of chemokine receptor 4/stromal cell-derived factor 1 system in human salivary gland carcinoma motility. *Oral Sci Int* 2006; 3: 13-20.

25. Uchida D, Kuribayashi N, Kinouchi M, *et al*. Expression and function of CXCR4 in human salivary gland cancers. *Clin Exp Metastasis* 2013; 30: 133-42.

26. Shen Z, Li T, Chen D, *et al*. The CCL5/CCR5 axis contributes to the perineural invasion of human salivary adenoid cystic carcinoma. *Oncol Rep* 2014; 31: 800-6.

27. Lanzel E, Robinson RA, Zimmerman MB, Pourian A, Hellstein JW. The use of immunohistochemistry in detection of perineural invasion in mucoepidermoid carcinoma. *Oral Surg Oral Med Oral Pathol Oral Radiol* 2016; 121: 636-42.

28. Barrett AW, Speight PM. Perineural invasion in adenoid cystic carcinoma of the salivary glands: a valid prognostic indicator? *Oral Oncol* 2009; 45: 936-40.

29. Ma B, Khazali A, Wells A. CXCR3 in carcinoma progression. *Histol Histopathol* 2015; 30: 781-92.

30. Kondo T, Ito F, Nakazawa H, Horita S, Osaka Y, Toma H. High expression of chemokine gene as a favorable prognostic factor in renal cell carcinoma. *J Urol* 2004; 171: 2171-5.

31. Liu M, Guo S, Stiles JK. The emerging role of CXCL10 in cancer (Review). *Oncol Lett* 2011; 2: 583-9.