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Expressions of CXCL12, CXCL10 and CCL18 in Warthin tumors characterized pathologically by having a lymphoid stroma with germinal centers

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Summary. The Warthin tumor is a benign neoplasm of the salivary glands, histologically, the tumor has an oncocytic epithelial component forming uniform rows of cells surrounded by cystic spaces associated with a lymphoid stroma often showing the presence of germinal centers. The lymphoid stroma is a representative microscopic finding. If this lymphocytic accumulation is active, some sort of transmitter should exist between the Warthin tumor cells and lymphocytes. C-X-C motif chemokine ligand (CXCL) 12, CXCL10 and C-C motif chemokine ligand 18 (CCL18) are a chemoattractant for lymphocytes *in vivo*. There is no report on the relationship between these chemokines and Warthin tumors. In this study, we investigated these chemokines expressions in 20 Warthin tumors using immunohistochemistry and reverse transcription polymerase chain reaction (RT-PCR). For comparison, we also enrolled samples of pleomorphic adenoma, which is another benign salivary gland tumor type without prominent lymphocytic infiltration. All Warthin tumors were immunopositive for CXCL12 and CXCL10, and these reactivities were diffuse. Meanwhile, the majority of pleomorphic adenomas were immunonegative for CXCL12 (95%), CXCL10 (80%) and CCL18 (85%). Warthin tumor and pleomorphic adenoma cases were significantly different in these immunostaining expressions (CXCL12, $p<0.001$; CXCL10, $p<0.001$; CCL18, $p=0.024$). We examined *CXCL12, CXCL10* and *CCL18* mRNA expressions of 3 representative Warthin tumor samples, each having these chemokines immunopositive areas detected by RT-PCR. Finding CXCL12 and CXCL10 expressions indicate that these chemokines may play a part in the formation of a lymphoid stroma within Warthin tumors. In regards to this phenomenon, the participation of CCL18 might be restrictive compared to CXCL12 and CXCL10.

Key words: CXCL12, CXCL10, CCL18, Warthin tumor, Salivary gland, Immunohistochemistry, RT-PCR

Introduction

Warthin tumor is most commonly found in male patients at a 4:1 ratio to females and typically occurs between 60 to 80 years of age. Distinct from other benign salivary gland neoplasms, Warthin tumors are often bilateral, about 90% are found in the superficial lobe of the parotid gland, but incidence in females is increasing due to more women smokers, reflecting one among other risks for Warthin tumor development (Chulam et al., 2013). Histologically, the tumor has an oncocytic epithelial component forming uniform rows of cells surrounded by cystic spaces associated with a lymphoid stroma often showing the presence of germinal centers (Chulam et al., 2013). This lymphoid stroma in Warthin tumors is a representative microscopic finding. The most accepted theory among various authors is that the tumor develops from salivary ducts imprisoned within intraparotid lymph nodes during embryogenesis or from heterotopic salivary glands. However, this theory does not support Warthin tumor-like tumor of salivary glands without lymphoid stroma. Other theories credit Warthin tumors' origin to the presence of lymphocytic infiltration in a pre-existing adenoma (Chedid et al., 2011). If these other theories are correct, and because this lymphocytic accumulation is active,

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Abbreviations. CXCL, C-X-C motif chemokine ligand; CCL, C-C motif chemokine ligand; RT-PCR, reverse transcription polymerase chain reaction; FFPE, formalin-fixed, paraffin-embedded; SDF, stromal cell-derived factor, VEGF, vascular endothelial growth factor; MMP, Matrix metalloproteinase.

there should be some sort of transmitter between the Warthin tumor cells and lymphocytes.

Chemokines are a group of small (8-14 kDa), structurally-related molecules that regulate the trafficking of various types of leukocytes through interactions with a subset of 7-transmembrane G-protein-coupled receptors. The C-X-C motif chemokine ligand (CXCL) 12 is a member of the CXC chemokine family and acts as a chemoattractant for T lymphocytes, monocytes and dendritic cells that drives their homing to lymphoid organs (Karin, 2010). Liu et al. in a review article reported that the CXCL10 is a member of the CXC subfamily and performs homing functions to chemoattract macrophages, dendritic cells, NK cells and activated T lymphocytes towards inflammatory, infectious and neoplastic regions (Liu et al., 2011). C-C motif chemokine ligand 18 (CCL18) is a dendritic cell specific chemokine expressed in both T lymphocyte and B lymphocyte areas of secondary lymphoid organs that preferentially attracts naive T lymphocytes (Lindhout et al., 2001).

Mulligan et al. found a correlation of lymphocytic infiltration in breast carcinoma stroma and CXCL10 (Mulligan et al., 2013). Thus, we think that CXCL12, CXCL10 and CCL18 might be involved in forming a lymphoid stroma within Warthin tumors. There are only few reports like the study of Haghshenas et al. on the relationship of chemokines and benign and malignant salivary tumors (Haghshenas et al., 2017). This study aims to clarify these chemokine expressions in Warthin tumors using immunohistochemistry and reverse transcription polymerase chain reaction (RT-PCR). For comparison, we also enrolled samples of pleomorphic adenoma, which is another type of benign salivary gland tumor, but without prominent lymphocytic infiltration.

Materials and methods

Materials

We collected 20 Warthin tumors and 20 pleomorphic adenomas obtained surgically at the University of Yamanashi Hospital. Two pathologists (K.M. and T.K.) independently reviewed hematoxylin and eosin stained slides blinded to the original pathological diagnosis. The Research Ethics Committee of the Faculty of Medicine, University of Yamanashi approved this study (approval numbers: 2174).

Immunohistochemistry

Sections 4-μm thick were cut from formalin-fixed, paraffin-embedded (FFPE) tissue blocks that were dewaxed and rehydrated. This was followed by immunohistochemical staining performed on representative slides. CXCL12/stromal cell-derived factor 1 (SDF1) (Polyclonal, Abcam plc, Cambridge, UK, dilution 1:400), CXCL10/interferon-γ-induced protein 10 (Polyclonal, Abcam plc, Cambridge, UK, dilution 1:200) and CCL18 (Polyclonal, Abcam plc, Cambridge, UK, dilution 1:800) were used as the primary antibody. We performed antigen retrieval through heat treatment: autoclaving at 121°C for 10 min in citrate buffer pH 6. After inhibiting endogenous peroxidase, we used a positive control (CXCL12, colon cancer; CXCL10, metastatic liver tumor (colon cancer); CCL18, small intestine) to perform the primary antibody reaction. We used the N-Histofine Simple Stain MAX PO (MULTI) (Nichirei Biosciences, Tokyo, Japan) with diaminobenzidine as a chromogen and a light counterstain with hematoxylin to perform immunohistochemistry. Two pathologists (K.M. and T.K.) simultaneously reviewed immunostained sections using a double-headed light microscope.

We used the H-score as immunohistochemical evaluation system, which is calculated by adding the multiplication of the different staining intensities in four gradations with each percentage of positive cells; the H-score was classified as 0=0 to 49 points, 1=50 to 99 points, 2=100 to 199 points, and 3=200 to 300 points, we also defined 1, 2 or 3 classified specimens as positive and sections classified 0 as negative (Specht et al., 2015). The ratio of epithelial component and lymphoid stromal component of Warthin tumors is roughly half and half, we evaluate only their epithelial component.

Microdissection and extraction of RNA from paraffin embedded tissue

Two 10-μm thick serial sections were cut from routinely processed FFPE tissue blocks. The tumor tissue (immunopositive area) was microdissected with a disposable syringe needle and the nucleic acids extracted by standard procedures. To avoid sampling problems, we selected non-necrotic tumor tissue with a considerable number of tumor cells. We used the RNeasy FFPE Kit (QIAGEN, Hilden, German) to extract RNA from the microdissected tissue samples.

RT-PCR

Total RNA was reverse transcripted using iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). All RT reactions were performed in the iCycler Thermal Cycler (Bio-Rad). After the RT reaction, we amplified the cDNA corresponding to CXCL12 (primers: 5'- CTACAGATGCCCATGCCGAT -3' and 5'- CAGCCGGGCTACAATCTGAA -3'; product size: 109bp), CXCL10 (primers: 5'- TGCCATTCTGATTTGCTGCC -3' and 5'- CGTGGA CAAAATTGGCTTGC -3'; product size: 165bp) and CCL18 (primers: 5'- CTTGTCCTCGTCTGCACCAT -3' and 5'- CTGGGGGCTGGTTTCAGAAT -3'; product size: 135bp) using HotStarTaq DNA Polymerase (QIAGEN, Hilden, German). Samples were denatured at 95°C for 15 min followed by 40 three-step cycles (95°C

for 30 s, 58°C for 30 s and 72°C for 1 min), and then at 72°C for 10 min in the iCycler Thermal Cycler (Bio-Rad). We used the amplification of glyceraldehyde-3-phosphate dehydrogenase as a quality control for RNA integrity (primers: 5'-GATGACATCAAGAAG GTGGTGA-3' and 5'-TTCGTTGTCATACCAGGA AATG-3'; product size: 186bp). Amplified fragments were separated on an agarose gel and visualized by Midori Green Advance staining (NIPPON Genetics, Tokyo, Japan).

Statistical analysis

We used the Pearson's chi-square test to evaluate differences between the Warthin tumor and pleomorphic adenoma samples regarding each immunohistochemical staining of CXCL12, CXCL10 and CCL18. A P-value of less than 0.05 indicates statistical significance. Statistical analysis was carried out using the IBM SPSS Statistics version 22 (IBM Corp., Armonk, NY, USA).

Results

CXCL12 immunostaining in Warthin tumors and pleomorphic adenomas

Results of immunohistochemical studies are summarized in Table 1. Warthin tumors showed the following immunostaining patterns: 0% classified 0, 0% classified 1, 65% classified 2, and 35% classified 3 (Fig. 1C). Pleomorphic adenomas showed the following immunostaining patterns: 95% classified 0, 5% classified 1, 0% classified 2, and 0% classified 3 (Fig. 2C). Using the two-tailed Pearson's chi-square test, Warthin tumor and pleomorphic adenoma cases were significantly different in CXCL12 immunostaining expression ($p<0.001$). A few lymphoid stromal cells were immunopositive for CXCL12 in spots of plural Warthin tumors (Fig. 1C).

CXCL10 immunostaining in Warthin tumors and pleomorphic adenomas

Results of immunohistochemical studies are summarized in the Table 1. Warthin tumors showed the following immunostaining patterns: 0% classified 0, 30% classified 1, 70% classified 2, and 0% classified 3 (Fig. 1D). Pleomorphic adenomas showed the following immunostaining patterns: 80% classified 0, 20% classified 1, 0% classified 2, and 0% classified 3 (Fig. 2D). Using the two-tailed Pearson's chi-square test, Warthin tumor and pleomorphic adenoma cases were significantly different in CXCL10 immunostaining expression ($p<0.001$). No lymphoid stromal cells were immunopositive for CXCL10 in Warthin tumors.

CCL18 immunostaining in Warthin tumors and pleomorphic adenomas

Results of immunohistochemical studies are summarized in the Table 1. Warthin tumors showed the following immunostaining patterns: 45% classified 0, 45% classified 1, 10% classified 2, and 0% classified 3 (Fig. 1E). Pleomorphic adenomas showed the following immunostaining patterns: 85% classified 0, 15% classified 1, 0% classified 2, and 0% classified 3 (Fig. 2E). Using the two-tailed Pearson's chi-square test, Warthin tumor and pleomorphic adenoma cases were significantly different in CCL18 immunostaining expression ($p=0.024$ i.e. $p<0.05$). No lymphoid stromal cells were immunopositive for CCL18 in Warthin tumors.

CXCL12, CXCL10 and CCL18 mRNA expressions in Warthin tumors by RT-PCR

We examined *CXCL12* (Fig. 3A), *CXCL10* (Fig. 3B) and *CCL18* (Fig. 3C) mRNA expressions in 3 representative Warthin tumor samples each having

Table 1. Expressions of CXCL12, CXCL10 and CCL18 in 20 Warthin tumors and 20 pleomorphic adenomas.

Tumor type	H-score (Classification)*				p-value**
	0	1	2	3	
CXCL12					
Warthin tumor (n=20)	0 (0%)	0 (0%)	13 (65%)	7 (35%)	<0.001
Pleomorphic adenoma (n=20)	19 (95%)	1 (5%)	0 (0%)	0 (0%)	
CXCL10					
Warthin tumor (n=20)	0 (0%)	6 (30%)	14 (70%)	0 (0%)	<0.001
Pleomorphic adenoma (n=20)	16 (80%)	4 (20%)	0 (0%)	0 (0%)	
CCL18					
Warthin tumor (n=20)	9 (45%)	9 (45%)	2 (10%)	0 (0%)	0.024
Pleomorphic adenoma (n=20)	17 (85%)	3 (15%)	0 (0%)	0 (0%)	

CXCL12, C-X-C motif chemokine ligand 12; CXCL10, C-X-C motif chemokine ligand 10; CCL18, C-C motif chemokine ligand 18. *0=0 to 49 points; 1=50 to 99 points; 2=100 to 199 points; 3=200 to 300 points. **Pearson's chi-square test.

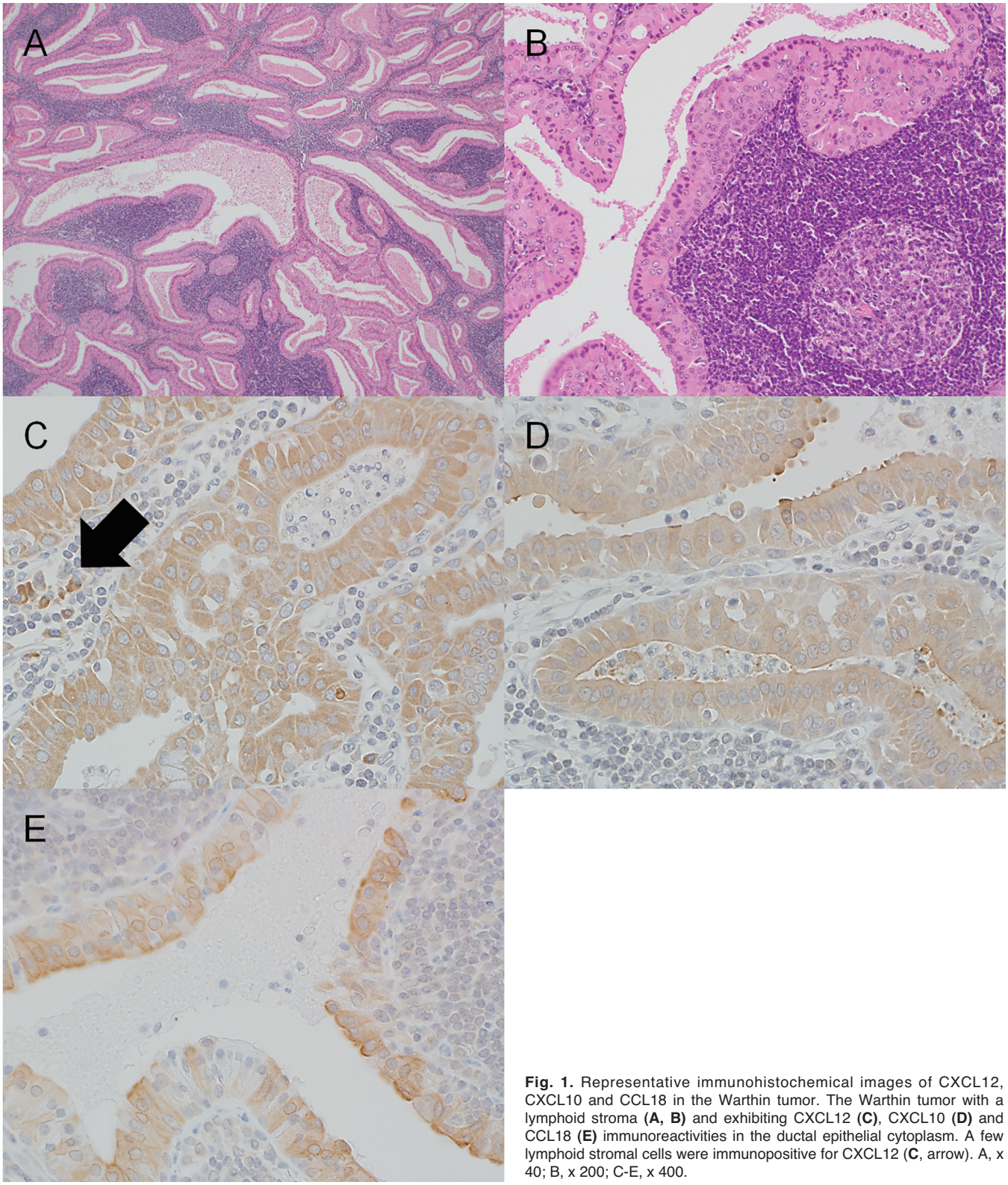

Fig. 1. Representative immunohistochemical images of CXCL12, CXCL10 and CCL18 in the Warthin tumor. The Warthin tumor with a lymphoid stroma **(A, B)** and exhibiting CXCL12 **(C)**, CXCL10 **(D)** and CCL18 **(E)** immunoreactivities in the ductal epithelial cytoplasm. A few lymphoid stromal cells were immunopositive for CXCL12 (**C**, arrow). A, x 40; B, x 200; C-E, x 400.

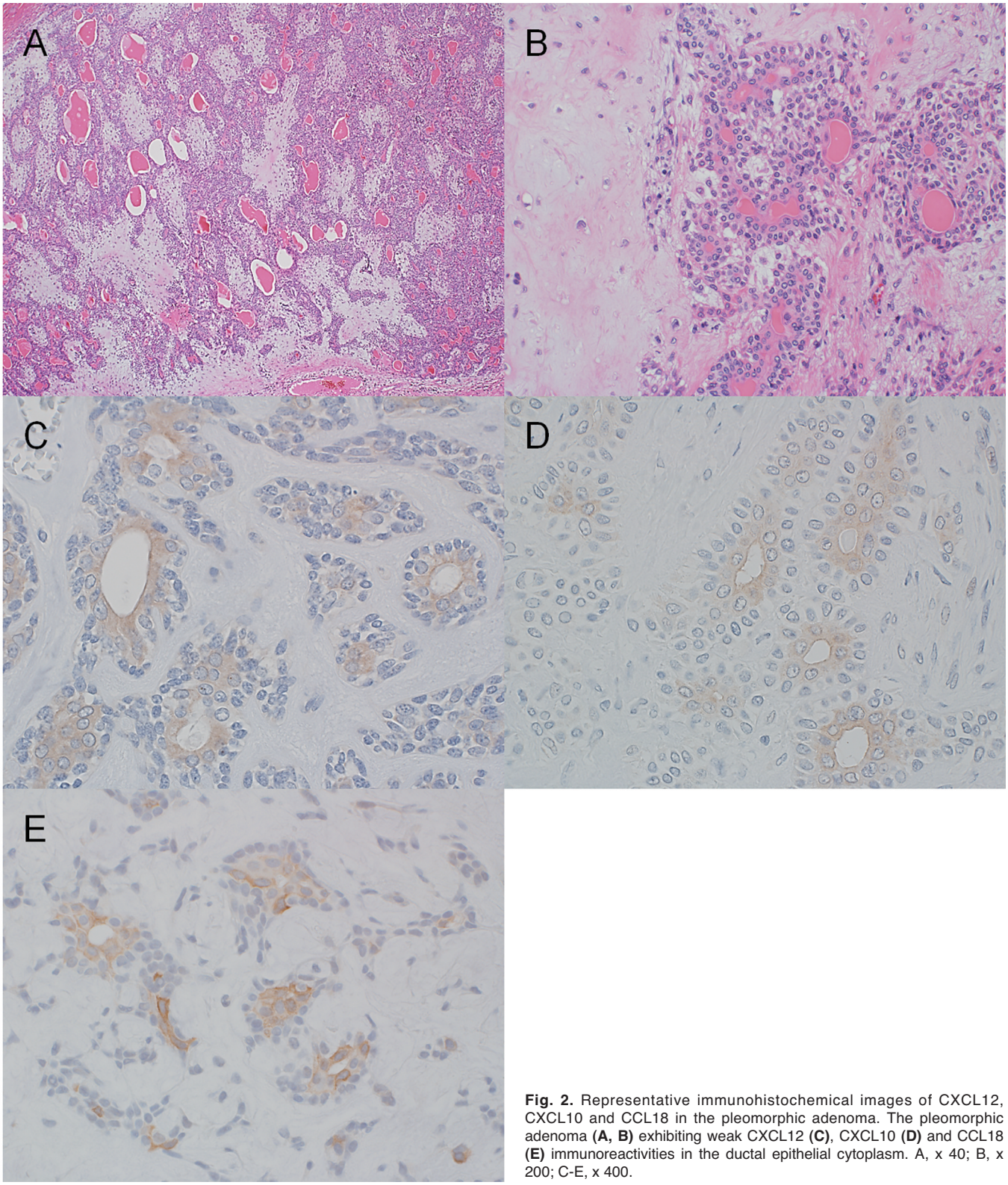

Fig. 2. Representative immunohistochemical images of CXCL12, CXCL10 and CCL18 in the pleomorphic adenoma. The pleomorphic adenoma **(A, B)** exhibiting weak CXCL12 **(C)**, CXCL10 **(D)** and CCL18 **(E)** immunoreactivities in the ductal epithelial cytoplasm. A, x 40; B, x 200; C-E, x 400.

immunopositive areas; lane 1 is DNA size markers (100 bp ladder), lane 2 is a positive control, positive bands are shown in all cases (lanes 3-5), lane 6 is a negative control (water), and lane 7 is a negative control without reverse transcriptase (tissue of lane 5).

Discussion

Chemotactic cytokines or chemokines are a large group of low molecular weight proteins that promote migration and adhesion of their target cell populations. Structurally, they are divided into four groups (C, CC, CX3C and CXC) based on the position of their conserved NH2-terminal cysteine residues. Functionally, chemokines can be divided into inflammatory or homeostatic chemokines based on their inducible or constitutive production, respectively. One such homeostatic CXC chemokine is CXCL12 (Janssens et al., 2018). CXCL12, also known as SDF1, is widely secreted in different tissues by stromal cells, fibroblasts and epithelial cells in six different isoforms encoded on chromosome 10q11 (Meng et al., 2018). Vascular endothelial growth factor (VEGF) is also an important cytokine that induces tumor angiogenesis and promotes tumor metastasis and can induce endothelial cells to express matrix metalloproteinase (MMP)-2 and MMP-9 that stimulate chemotaxis of endothelial cells and the formation of capillary channels, thus indirectly regulating angiogenesis (Meng et al., 2018). Salvucci et al. showed that CXCL12 can induce endothelial cells to express VEGF, and VEGF in turn can promote the expression of CXCL12 in vascular endothelial cells (Salvucci et al., 2002). So CXCL12 can stimulate angiogenesis directly and indirectly. Błochowiak et al. showed that salivary VEGF concentration was significantly higher in patients with pleomorphic adenoma and Warthin tumor (Błochowiak et al., 2019). Additionally, Tan et al. found that fine-needle aspiration, although a common and useful preoperative diagnostic technique, can sometimes lead to ischemic injury and result in an infarction of a Warthin tumor (Tan et al., 2016). Moreover, Warthin tumors of salivary glands are composed of oncocytic cells containing excessive numbers of mitochondria with frequent structural abnormalities and reduced metabolic function. Lewis et al. postulated that in Warthin tumors, oncocytes might be the result of increased oxidative damage to mtDNA and decreased oxidative phosphorylation. Mitochondrial proliferation might then result as the cell tries to compensate for a decline in adenosine triphosphate level (Lewis et al., 2000). This might indicate that Warthin tumors have a chronic shortage of feeding vessels or vascular flow and have an ongoing need for angiogenesis to grow. Immunohistochemistry showed 100% of the Warthin tumor samples had a very high rate of CXCL12 expression (H-score ≥50 points), and reactivity for CXCL12 was consistently diffuse and of strong intensity. These findings might indicate that CXCL12 plays an important role in VEGF production in Warthin tumors to increase angiogenesis for nutrient supply to tumor cells and efficient excretion of tumor cell metabolites. Therefore, in its primary role in angiogenesis, the chemoattractant properties of CXCL12 also might be causing the lymphoid stroma to form within Warthin tumors as a secondary effect; there does not seem to be any primary purpose for lymphoid stromal structuring in Warthin tumors. Meanwhile, there was only focal and weak immunopositivity of CXCL12 in pleomorphic adenomas, another benign salivary gland tumor type, but without prominent lymphocytic infiltration ($p<0.001$).

On the other hand, immunohistochemistry showed 100% of the Warthin tumor samples had a very high rate of CXCL10 expression (H-score ≥50 points), and there was a very low rate of CXCL10 expression in pleomorphic adenomas ($p<0.001$). Consequently, CXCL10 might also lead to lymphocytic accumulation within Warthin tumors. However, Liu et al. in a review article reported that CXC chemokines have dual effects on angiogenesis, depending on the presence of the Glu-Leu-Arg (ELR) motif; ELR-negative CXCL10 is an angiostatic chemokine that inhibits angiogenesis and is

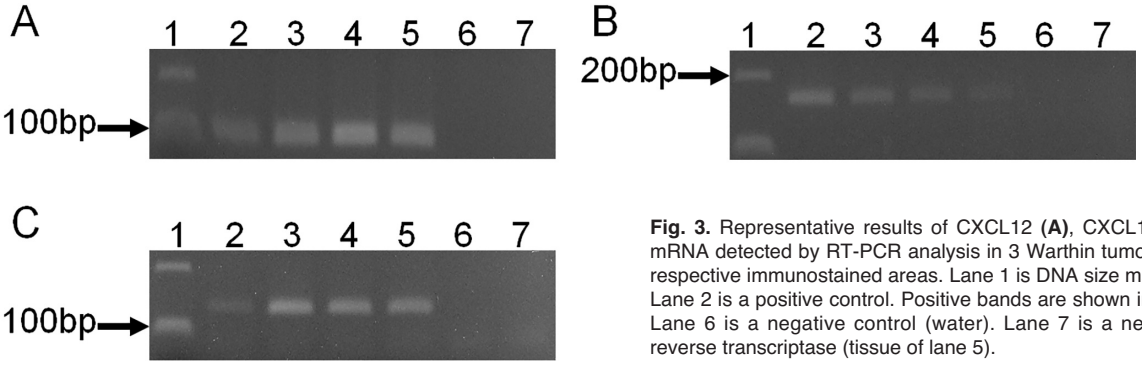

Fig. 3. Representative results of CXCL12 **(A)**, CXCL10 **(B)** and CCL18 **(C)** mRNA detected by RT-PCR analysis in 3 Warthin tumor samples having their respective immunostained areas. Lane 1 is DNA size markers (100 bp ladder). Lane 2 is a positive control. Positive bands are shown in all cases (lanes 3-5). Lane 6 is a negative control (water). Lane 7 is a negative control without reverse transcriptase (tissue of lane 5).

associated with its anti-tumor activities (Liu et al., 2011). This shows that the production of CXCL10 is not beneficial to Warthin tumors. Additionally, Liu et al. in a review article reported that using an *in vitro* model of cultured cortical neurons, neuronal CXCL10 expression recruits glial cells during embryogenesis, indicating that CXCL10 may be involved in apoptosis during the development of the nervous system; CXCL10 also significantly increased the apoptotic rate of cancer cells in cervical carcinoma (Liu et al., 2011). Apoptosis is an inborn process preserved during evolution; it allows cells to systematically inactivate, destroy and dispose of their own components thus leading to their death; this cycle can be activated by both intra and extracellular mechanisms; the intracellular components involve a genetically defined development process, while the extracellular aspects depend on endogenous proteins, cytokines and hormones, as well as xenobiotics, radiation, oxidative stress and hypoxia (Stoian et al., 2014). Meanwhile, cystic spaces filled with cellular (necrotic) debris in Warthin tumors also is a representative microscopic finding. As discussed earlier, there is a possibility that Warthin tumors have a chronic shortage of feeding vessels (vascular flow) and an ongoing need for angiogenesis to grow. Thus, the necrotic debris might be a result of apoptosis through CXCL10 induced by hypoxia within Warthin tumors.

We confirmed CCL18 expression (H-score ≥50 points) in 55% of Warthin tumors and 15% of pleomorphic adenoma samples with statistical difference between Warthin tumors and pleomorphic adenomas ($p=0.024$ i.e. $p<0.05$). Thus, in Warthin tumors CCL18 immunoexpression was lower than CXCL12 and CXCL10 immunoexpressions. Although CCL18 might also lead to lymphocytic accumulation within Warthin tumors, the participation of CCL18 regarding this phenomenon in Warthin tumors might be restrictive compared to CXCL12 and CXCL10. Nevertheless, recent studies have indicated that CCL18 and VEGF synergistically promoted the migration and angiogenesis of endothelial cells both *in vitro* and *in vivo* (Lin et al., 2015).

There are also recent studies discussing the importance of immunological reactions during the formation of Warthin tumors; a hypersensitive/allergic reaction may play a role in epithelial proliferation stimulating the reactivity of the germinal centers in the lymphoid stroma as shown at histological examination. Orabona et al. showed that the incidence rate of autoimmune thyroiditis in their Warthin tumor cases (9.5%) was significantly greater than that of the general population (0.58%) ($p<0.001$). This study supports the hypothesis that this tumor may be the result of an autoimmune reaction (Orabona et al., 2015).

In conclusion, our results showed that CXCL12 and CXCL10 expressed at a high rate in Warthin tumors suggesting that these chemokines may play a part in the formation of a lymphoid stroma within Warthin tumors. In regards to this phenomenon, the participation of CCL18 might be restrictive compared to CXCL12 and CXCL10.

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