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Increased expression of CX3CL1 and CX3CR1 in papillary thyroid carcinoma

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Summary. CX3CL1 and its receptor CX3CR1 axis are involved in the development, progression and metastasis of many types of cancers. It has been reported that CX3CL1 and CX3CR1 expression was upregulated in some solid tumors. However, their roles in thyroid cancer remain unknown. In the present study, we investigated the expression of CX3CL1 and CX3CR1 in human papillary thyroid carcinoma (PTC) and their clinical significance. In this study, using immunohistochemistry, we examined the expression of CX3CL1 and CX3CR1 in the tissues of 26 human PTC (including 17 classical or conventional (CPTC) and 9 follicular (FVPTC) variants of PTC; 15 cases without and 11 cases with lymph node metastasis) and 10 cases of nodular goiter (NG). Compared to NG, a significant increase in the expression of CX3CL1 and CX3CR1 was found in PTC overall, as well as in CPTC and FVPTC separately. Higher CX3CL1 expression was found in CPTC than in FVPTC, but there was no significant difference in CX3CR1 expression between these subtypes of PTC. When analyzing their expressions in PTC without and with lymph node metastasis, an increased expression of CX3CL1 and CX3CR1 was observed when compared to NG respectively. There was however no significant difference in CX3CL1 and CX3CR1 expressions in PTC without lymph node metastasis when compared to PTC with lymph node metastasis. Furthermore, when compared to NG, an increased expression of CX3CL1 was correlated with an increased expression of CX3CR1 in PTC. Our data indicate that CX3CL1 and CX3CR1 can be used as tumor markers for PTC and may be potential novel targets for cancer prevention and treatment.

Key words: CX3CL1, CX3CR1, Papillary thyroid carcinoma, Tumor marker, Immunohistochemistry

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Introduction

Thyroid cancer is the most common type of endocrine malignancy worldwide and its incidence has been increasing over several decades (Chen et al., 2016; Siegel et al., 2019). Thyroid cancer derived from follicular cells comprises well-differentiated (papillary and follicular) carcinoma, poorly differentiated and undifferentiated/anaplastic carcinomas. This spectrum of progression has been linked with a pattern of cumulative genetic defects associated with tumor differentiation, metastatic potential and aggressiveness. The increase in the incidence of thyroid cancer has been mainly attributed to the increase of papillary thyroid cancer (PTC) (Ahn et al., 2014). PTC is the most common type of thyroid cancer and constitutes more than 70% of thyroid malignancy (Lloyd et al., 2011). Among PTC, there are numerous pathological variants. The most common variants of PTC include classical or conventional (CPTC), follicular (FVPTC) and tall cell (TCVPTC) variants. Although genetic, environmental and hormonal factors have been linked to the development of thyroid cancer, the pathogenesis of

thyroid cancer is not fully understood.

The tumor microenvironment (TME) plays a pivotal role in cancer development, progression, metastasis, as well as disease prognosis and therapeutic efficacy (Wu and Dai, 2017; Wang et al., 2018). TME comprises cells (such as immune cells, fibroblasts, endothelial cells, etc.), extracellular matrix proteins and secreted factors (such as cytokines and chemokines, etc.). The cross-talk between tumor cells and the surrounding stroma is at least in part mediated by chemokines.

Chemokines or chemotactic cytokines are a family of small proteins that play an important role in directing different immune cell types to the TME. They are subdivided into four subfamilies, C, CC, CXC, and CX3C chemokines. The chemokine (C-X3-C motif) ligand 1 (CX3CL1) and its receptor chemokine (C-X3-C motif) receptor 1 (CX3CR1) have been found to be involved in the pathogenesis, progression and prognosis of various inflammatory diseases and cancers (Borsig et al., 2014; Imai and Yasuda, 2016; Zhuang et al., 2017; Lee et al., 2018). CX3CL1 (also known as Fractalkine or Neurotactin) is the only known member of the CX3C family of chemokines (Bazan et al., 1997; Pan et al., 1997), which is mainly expressed in endothelial cells, neurons (Cook et al., 2001) and epithelial cells (Lucas et al., 2001). CX3CL1 exists in two forms: the membrane-bound form and the soluble form (Liu et al., 2016). It has been found that membrane-bound CX3CL1 can act as an adhesion molecule while soluble CX3CL1 is a chemoattractant (Imai et al., 1997; Fong et al., 1998). CX3CL1 exerts its functions via an interaction with its only known human receptor CX3CR1, which is mainly expressed on T lymphocytes, NK cells (Nishimura et al., 2002), mast cells (Papadopoulos et al., 2000), dendritic cells (DCs) (Kitching, 2014) and many types of cancer cells.

To further understand the TME, in the present study we investigated CX3CL1 and CX3CR1 expression in PTC. As far as we know, their roles in PTC development, progression and metastasis remain unclear.

Materials and methods

Patients

All pathological specimens of PTC and nodular goiter (NG) that were operated and diagnosed in the First Affiliated Hospital of Jinzhou Medical University, Jinzhou, Liaoning, China from 2006 to 2018 were included in the study. The paraffin-embedded tissues were collected for immunohistochemistry and analysis. Written or oral informed consent was obtained from all patients analyzed in this study. This study was approved by the Ethical Review Board of Jinzhou Medical University, Jinzhou, Liaoning, P. R. China.

Immunohistochemistry

Immunohistochemistry (IHC) was performed according to the antibody manufacturer's recommended protocol and methods (Yu et al., 2011; Zhang et al., 2019) with minor modifications. The sections (4 μm) of human PTC and NG were deparaffinized and dehydrated, followed by being microwaved in Antigen unmasking solution (Vector Laboratories, Burlingame, CA) for 10 minutes. Endogenous peroxidase activity was inactivated with 3% hydrogen peroxidase and the sections were blocked with 5% non-fat milk, 10% goat serum and 0.3% Triton X-100 in PBS for 1 hour. The sections were incubated with primary CX3CL1 and CX3CR1 antibodies respectively (1:150, MyBioSource Inc., San Diego, CA) in blocking solution overnight at 4°C. The sections were incubated in biotinylated goat anti-rabbit antibody (1:300, Vector Laboratories) for 1 hour at room temperature. The avidin-biotin complex (ABC) (VECTASTAIN®, Vector Laboratories) and diaminobenzidine (DAB) (DAB Peroxidase Substrate Kit, Vector Laboratories) were applied to the sections for visualization of the reaction product. For negative controls, the primary antibody was omitted.

Evaluation of staining

Immunohistochemical staining was assessed and confirmed by two pathologists independently according to well established methods (Yu et al., 2011; Zhang et al., 2019). CX3CL1 and CX3CR1 positive and negative cell counts were performed by two authors separately. The values from five random fields per section obtained by the two authors were averaged and expressed as percentage of the number of CX3CL1 or CX3CR1 positive cells/total cells respectively.

Statistical analysis

All of the following analyses were performed using SPSS 20.0. Data was expressed as mean ± standard deviation (SD) and p value. Unpaired t-tests were performed for analyzing the comparison of CX3CL1 and CX3CR1 expression between sex, age and clinical stages (based on the 8th edition of the AJCC/UICC TNM stage). The difference in CX3CL1 and CX3CR1 expression between PTC and NG was also analyzed by the unpaired t-test. One-way ANOVA was performed for analyzing the changes in the expression of CX3CL1 and CX3CR1 among NG, CPTC and FVPTC; and among NG, PTC without and with lymph node metastasis. The correlation between increased CX3CL1 and CX3CR1 expression in PTC compared to NG was analyzed by Spearman's Correlation. p values ≤0.05 were considered statistically significant. Receiver operating characteristic (ROC) analysis was also used to determine the cut-off values for CX3CL1/CX3CR1 IHC staining.

Results

Patients and specimens

There were a total of 36 paraffin-embedded tissues,

26 human PTC and 10 NG. Of the PTC tissue samples, 17 cases were CPTC (8 cases without and 9 cases with lymph node metastasis) and 9 cases were FVPTC (7 cases without and 2 cases with lymph node metastasis). The patients had a mean age of 50.36 (range=15-77) years at diagnosis.

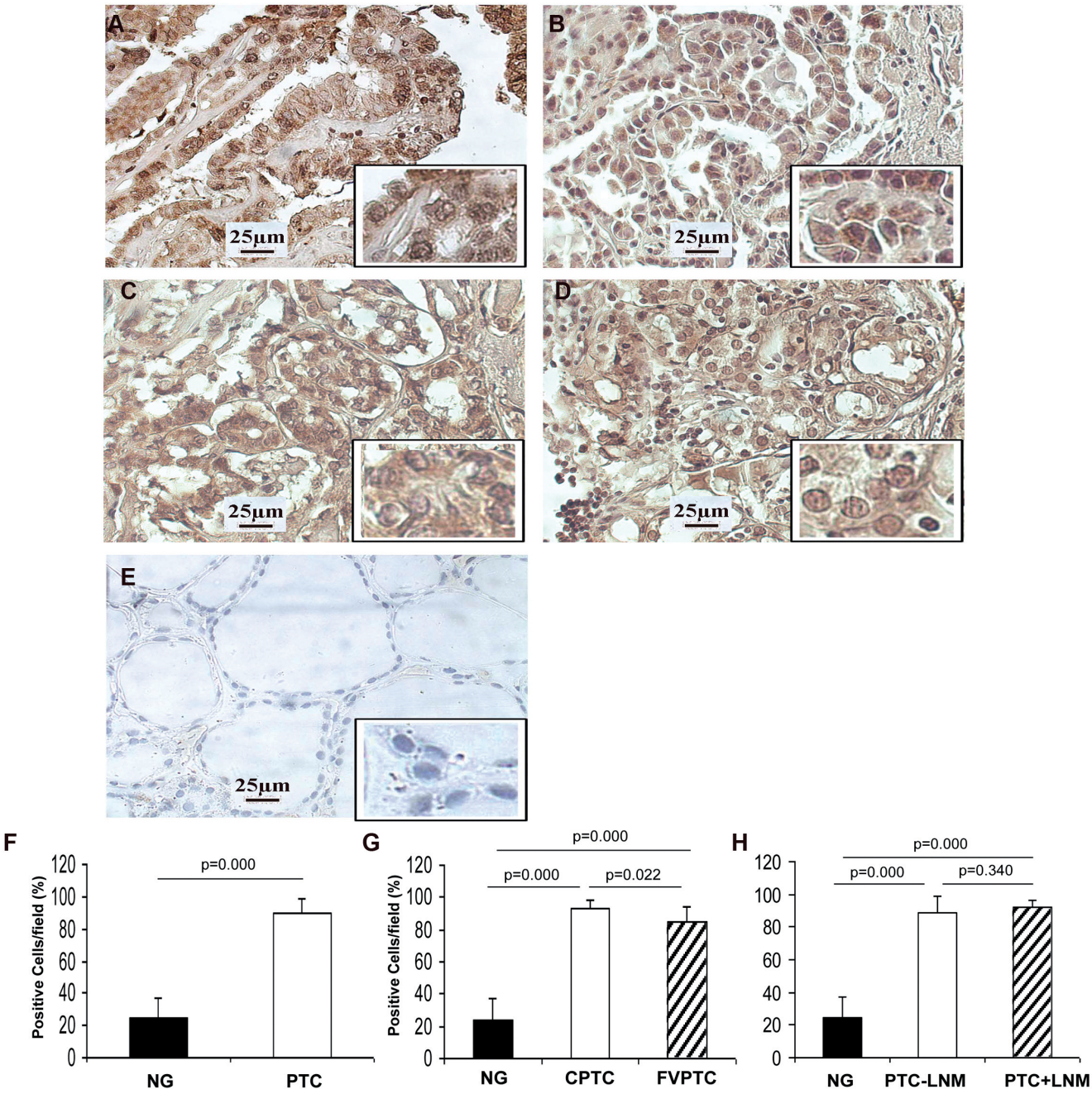

Fig. 1. Increased expression of CX3CL1 in PTC compared to its expression in NG. Immunohistochemistry was performed to analyze CX3CL1 expression in the tissues of human PTC and NG **(A-E)**. CX3CL1 was expressed in all tissues including CPTC without **(A)** CPTC with **(B)**, FVPTC without **(C)**, FVPTC with **(D)** lymph node metastasis (LNM) and NG **(E)**. Increased CX3CL1 expression was found in PTC including CPTC and FVPTC compared to its expression in NG **(F)**. Compared to NG, the expression of CX3CL1 was significantly increased in CPTC and FVPTC respectively. There was significant difference in its expression between these two subtypes of PTC **(G)**. Elevated expression of CX3CL1 was observed in PTC without and with LNM compared to its expression in NG. There was no significant difference in its expression between PTC without and with LNM **(H)**. All data are shown as mean±SD (%). Significant at $p<0.05$.

CX3CL1 expression in PTC compared to NG

CX3CL1 immunostaining was positive in all of the tissues of PTC and NG, but was stronger in PTC than in NG (Fig. 1A-E). The difference was statistically significant, higher in PTC (n=26, 90.2058±8.04337%)

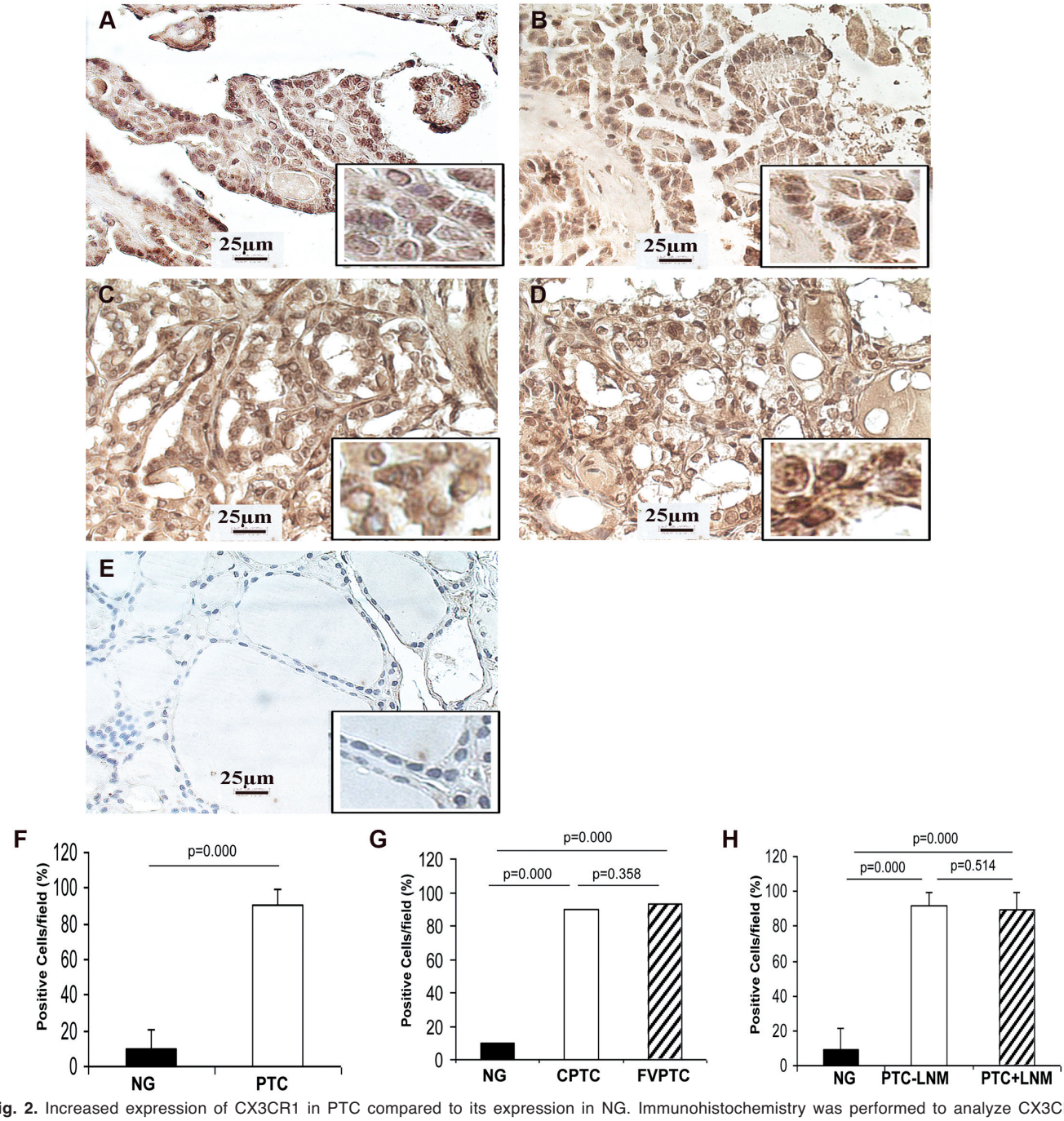

Fig. 2. Increased expression of CX3CR1 in PTC compared to its expression in NG. Immunohistochemistry was performed to analyze CX3CL1 expression in the tissues of human PTC and NG **(A-F)**. CX3CR1 was expressed in almost all tissues, including CPTC without **(A)** CPTC with **(B)**, FVPTC without **(C)** and FVPTC with **(D)** lymph node metastasis (LNM) and NG **(E)**. Increased CX3CR1 expression was found in PTC including CPTC and FVPTC compared to its expression in NG **(F)**. Compared to NG, the expression of CX3CR1 was significantly increased in CPTC and FVPTC respectively. There was no significant difference in its expression between these two subtypes of PTC **(G)**. Elevated expression of CX3CR1 was respectively observed in PTC without and with LNM compared to its expression in NG. There was no significant difference in its expression between PTC without and with LNM **(H)**. All data are shown as mean±SD (%). Significant at $p<0.05$.

than in NG (n=10, 24.1191±12.96024%, p=0.000) (Fig. 1F). The cut-off value of CX3CL1 for PTC was found to be 56.22% with a sensitivity of 100% and specificity of 100%. Compared to NG, CX3CL1 expression was increased in both CPTC (n=17, 93.2975 ± 4.92006%, p=0.000) and FVTPC (n=9, 84.3659±9.75092%, p=0.000) respectively, and there was significant difference between these two PTC subtypes (p=0.022) (Fig. 1G). In addition, CX3CL1 was highly expressed in PTC without (n=15, 88.6453±9.86054%, p=0.000) and with (n=11, 92.3336±4.12113%, p=0.000) lymph node metastasis compared to NG. A statistically significant difference in the expression of CX3CL1 between the PTC without and with lymph node metastasis (p=0.340) was not found (Fig. 1H).

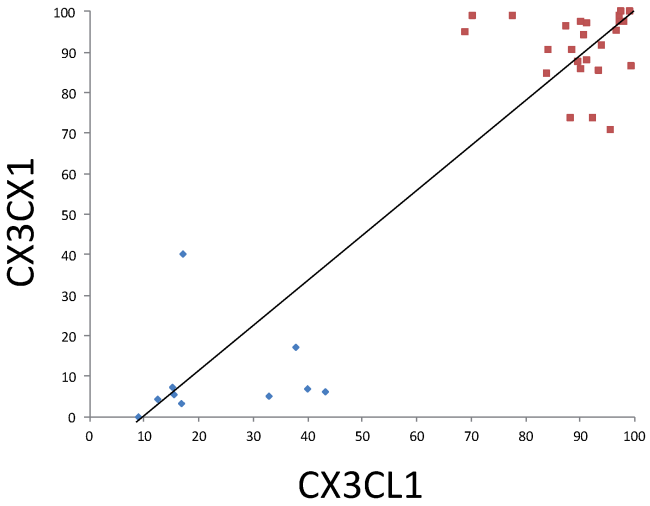

Fig. 3. Elevated CX3CL1 expression was significantly correlated with increased CX3CL1 expression in PTC. Our results showed that expression of CX3CL1 and CX3CR1 was increased in PTC compared to its expression in NG. Here, using Spearman's Correlation we analyzed whether there was correlation between elevated CX3CL1 and increased CX3CR1 expression in PTC (red) compared to their expression in NG (blue). The statistical analysis showed that compared to NG elevated expression of CX3CL1 was significantly correlated with increased CX3CR1 (r=0.670, p=0.000).

Table 1. Association between the clinicopathological characteristics and expression of CX3CL1 and CX3CR1 in thyroid papillary carcinoma.

	N	CX3CL1 (%)*	p	CX3CR1 (%)*	p
Gender			0.924		0.767
Male	10	90.0102±8.0769		90.1324±7.5796	
Female	16	90.3280±8.2851		91.1586±8.9868	
Age			0.465		0.354
≥55	10	88.7122±9.3566		92.7028±6.0028	
<55	16	91.1393±7.2720		89.5408±9.4814	
Clinical Stage			0.368		0.238
I	23	89.6816±8.3575		90.0564±8.5152	
II	3	94.2242±3.6039		96.1884±4.4712	

*Data are expressed as mean ± SD, analyzed by Unpaired t-test.

CX3CR1 expression in PTC compared to NG

Using immunohistochemistry, we found that CX3CR1 was expressed in almost all of the tissues of PTC and NG, and its immunostaining was stronger in PTC than in NG tissues (Fig. 2A-E). High expression of CX3CR1 was observed in PTC (n=26, 90.7639±8.33062%) compared to in NG (n=10, 9.5288±11.56906%, p=0.000) (Fig. 2F). The cut-off value of CX3CR1 for PTC was found to be 55.29% with a sensitivity of 100% and specificity of 100%. Compared to its expression in NG, CX3CR1 expression was significantly increased in CPTC (n=17, 89.5243±9.34616%, p=0.000) and FVPTC (n=9, 93.1054±5.72167%, p=0.000) respectively, but there was no significant difference between CPTC and FVPTC (p=0.358) (Fig. 2G). Similar to the expression of its ligand CX3CL1, CX3CR1 was highly expressed in PTC without (n=15, 91.8029±7.37195, p=0.000) and with (n=11, 89.3471±9.67402, p=0.000) lymph node metastasis compared to NG respectively. However no significant change of CX3CR1 expression was observed in PTC between without and with lymph node metastasis (p=0.514) (Fig. 2H).

Correlation of increased CX3CL1 with elevated CX3CR1 expression in PTC compared to NG

Our results showed that expression of CX3CL1 and CX3CR1 was increased in PTC compared to its expression in NG. Here, we found a significant correlation between the increased expression of CX3CL1 and CX3XR1 in PTC compared to NG (Fig. 3, r=0.670, p=0.000).

Association of CX3CL1 and CX3CR1 expression with gender, age and clinical stage of the patients with PTC

There was no statistically significant association of CX3CL1 and CX3CR1 expression with gender, age, and clinical stages of patients with PTC. However, a slight increase in the expression of CX3CL1 and CX3CR1 was observed in clinical stage II (n=3, 94.2242±3.6039 (CX3CL1); 96.1884±4.4712 (CX3CR1)) relative to its expression in clinical stage I (n=23, 89.6816±8.3575 (CX3CL1); 90.0564±8.5152 (CX3CR1)) (Table 1).

Discussion

To date, there has been no study on the role of CX3CL1 and CX3CR1 in human PTC. We found that the expression of CX3CL1 and CX3CR1 was increased in PTC (including CPTC and FVPTC) compared to NG, a benign thyroid disease. This has also been found in many other cancers. CX3CL1 and CX3CR1 were both

elevated in pancreatic ductal adenocarcinoma (PDAC) tissues, especially in the metastatic samples and were correlated with PDAC severity. Overexpression of CX3CL1 was also found to stimulate PDAC cell proliferation and migration, while knockdown of CX3CR1 inhibited the above effects of CX3CL1 (Huang et al., 2017). An increased expression of CX3CR1 in PDAC was associated with a marked perineural invasion and with earlier local tumor recurrence (Marchesi et al., 2008). In gastric cancer tissues, CX3CR1 was highly expressed and associated with lymph node metastasis, higher clinical TNM stage and larger tumor size (Wei et al., 2015). In osteosarcoma tissues, the increased expression of CX3CL1 was correlated with clinical stage and promoted cell migration (Liu et al., 2017). A higher expression of CX3CR1 was also found in spinal metastatic tissues of breast cancer than in para-tumor tissues and CX3CL1 expression was higher in normal spinal cancellous bones than that in the extremities. It was postulated that a high level of CX3CL1 in the spine might attract high CX3CR1 expressing breast cancer cells, causing metastasis likely via the Src/FAK pathway (Liang et al., 2018). The finding of CX3CR1 directly involved in metastasis to the skeleton was observed in animal models of breast cancer (Jamieson-Gladney et al., 2011). One study showed that CX3CR1 was overexpressed in prostate cancer (Jamieson et al., 2008). Another reported that an overexpression of CX3CL1/CX3CR1 was again found in prostate cancer and both were higher in tissues with spinal metastasis than in primary tumor. Over-expression of CX3CL1 and CX3CR1 was found to induce prostate cell proliferation, migration and invasion. CX3CL1/CX3CR1 enhanced prostate cancer spinal metastasis also by activating the Src/FAK pathway (Liu et al., 2018).

However, some studies have also shown that CX3CL1 has an antitumor function via chemoattracting immune cells (such as T cells, NK cells and DCs etc.) to tumor tissues. A high expression of CX3CL1 in breast cancer was found to correlate with good prognosis and CX3CL1 expressed by tumor cells appeared to enhance recruitment of CD8+T cells, NK cells and DCs (Park et al., 2012). CX3CL1 expressed in colorectal cancer, hepatocellular carcinoma and gastric adenocarcinoma appears to recruit cytotoxic T cells and NK cells to the tumor site, resulting in a better prognosis (Ohta et al., 2005; Matsubara et al., 2007; Hyakudomi et al., 2008). Increased CX3CL1 mRNA level was a positive prognostic factor with the patients with lung adenocarcinoma (Liu et al., 2019). The antitumor effect of CX3CL1 was also demonstrated in the animal model of metastatic colon cancer (Vitale et al., 2007).

The evidence for antitumor functions is mostly in prognostic studies while the evidence for protumor function tends to be in tumor development studies. As such, the role of the CX3CL1/CX3CR1 chemokine system in cancer biology may be a double-edged sword. CX3CL1 can exert antitumor or protumor effect on tumor development via recruiting immune cells to TME and CX3CR1-expressing tumor cells can migrate under the CX3CL1 chemotaxis effect leading to metastasis.

In this study, we found higher expression of CX3CL1 in CPTC than in FVPTC, but no significant difference in CX3CR1 expression between CPTC and FVPTC. An increased expression of CX3CL1 and CX3CR1 was respectively observed in PTC without and with lymph node metastasis compared to NG. There was no significant association of elevated expression of CX3CL1/CX3CR1 with lymph node metastasis. Moreover, we showed that CX3CL1 and CX3CR1 expressions, when compared to their cognate expression in NG, were statistically correlated in PTC. Although previous studies have shown increased expression of CX3CL1 and/or CX3CR1 in certain types of cancer tissues, the correlation between their expression remains unknown. Taken together, these findings indicate that CX3CL1/CX3CR1 might exert protumor functions in PTC.

In this study, we did not find significant association of CX3CL1 and CX3CR1 expression with gender, age and clinical stages of patients with PTC, although a slight increase in expression of CX3CL1 and CX3CR1 was found in clinical stage (II) relative to its expression in clinical stage (I).

In addition, using ROC analysis, we found that the cutoff values of CX3CL1 and CX3CR1 for PTC were 56.22% and 55.29% respectively, both of which had a sensitivity of 100% and specificity of 100%. These findings undoubtedly further support CX3CL1 and CX3CR1 as tumor markers of PTC. However, the number of samples was limited in our study and this conclusion needs further confirmation with large samples.

In conclusion, our findings suggest that CX3CL1 and CX3CR1 can be used as potential tumor markers for PTC. We highlighted the potential involvement of CX3CL1 and CX3CR1 in the pathogenesis of PTC, which may serve as novel therapeutic targets. Of course, further studies will be needed to verify the role of CX3CL1/CX3CR1 in other subtypes of thyroid cancer.

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Conflict of Interest. The authors declare that they have no conflict of interest.

Ethical approval. The study was approved by the Ethics Committee of Jinzhou Medical University.

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