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Expression of CXCL12 in esophageal high grade dysplasia characterized pathologically by lymphocyte accumulation directly under the lesion

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Summary. Squamous dysplasia of the esophagus is an unequivocal neoplastic alteration of the esophageal squamous epithelium without invasion. Esophageal high grade dysplasia (EHGD) is characterized by >50% epithelial involvement or severe cytological atypia. Frequently, lymphocytes accumulate below EHGD lesions even though there is no invasion. If this lymphocytic accumulation is active, a transmitter should exist between the EHGD cells and the lymphocytes. C-X-C motif chemokine ligand (CXCL) 12, CXCL10 and C-C motif chemokine ligand 18 (CCL18) are all lymphocyte chemoattractants in vivo, but there are no reports on the relationship between these chemokines and EHGDs. In this study, we investigated these chemokines and C-X-C motif chemokine receptor 4 (CXCR4) (receptor for CXCL12) in 30 EHGDs using immunohistochemistry and reverse transcription polymerase chain reaction (RT-PCR). For comparison, we enrolled 30 samples of normal esophageal squamous epithelium (NESE). We confirmed CXCL12 expression (H-score≥50 points) in 70% of EHGD and 0% of NESE samples, CXCL10 expression in 3% of EHGD and 3% of NESE samples, CCL18 expression in 3% of EHGD and 0% of NESE samples, and CXCR4 expression in 53% of EHGD and 0% of NESE samples by immunohistochemistry. EHGD and NESE cases were significantly different in their expressions between the tissue types (CXCL12, $p<0.001$; CXCR4, $p<0.001$). We examined CXCL12 and CXCR4 mRNA expressions of 3 representative EHGD samples, each having their respective immunostained areas detected by RT-PCR. Finding CXCL12 expression may indicate that this chemokine plays a part in the lymphocyte accumulation that occurs directly under EHGDs.

Key words: CXCL12, CXCR4, CXCL10, CCL18, High grade dysplasia, Esophagus, Immunohistochemistry, RT-PCR

Introduction

Squamous dysplasia of the esophagus is an unequivocal neoplastic alteration of the esophageal squamous epithelium, without invasion; esophageal high grade dysplasia (EHGD) is diagnosed when more than half of the epithelium is involved or when severe cytological atypia is present (regardless of the extent of epithelial involvement). EHGD includes the group of lesions also termed “carcinoma in situ” in Japan and other parts of Asia (Takubo and Fujii, 2019).

Frequently, EHGDs have lymphocyte accumulation directly under the lesions in spite of there being no invasion. If this lymphocytic accumulation is active, some sort of transmitter should exist between the EHGD cells and the 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 that performs chemoattractant functions for macrophages, dendritic cells, natural killer cells and activated T lymphocytes (CD4+ T cells and CD8+ T cells) to inflammatory,

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Abbreviations. EHGD, esophageal high grade dysplasia; CXCL, C-X-C motif chemokine ligand; CCL18, C-C motif chemokine ligand 18; RT-PCR, reverse transcription polymerase chain reaction; NESE, normal esophageal squamous epithelium; HE, hematoxylin and eosin; FFPE, formalin-fixed, paraffin-embedded; SDF, stromal-derived factor; CXCR4, CXC motif chemokine receptor 4.

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 and B cell areas of secondary lymphoid organs that preferentially attracts naive T cells (Lindhout et al., 2001).

We think that CXCL12, CXCL10 and CCL18 may be involved in the formation of a lymphoid stroma within EHGDs. We found no previous studies on the relationship between these chemokines and EHGDs. This study aims to clarify CXCL12, CXCL10 and CCL18 expression in EHGDs using immunohistochemistry and reverse transcription polymerase chain reaction (RT-PCR). For comparison, we also examined samples of normal esophageal squamous epithelium (NESE). We performed C-X-C motif chemokine receptor 4 (CXCR4) (receptor for CXCL12) expression as well in both the EHGDs and NESEs (Meng et al., 2018).

Materials and methods

Materials

We collected samples from 30 EHGDs (Table 1) and 30 NESEs obtained endoscopically or surgically at the University of Yamanashi Hospital. Two pathologists (K.M. and T.K.) independently reviewed hematoxylin and eosin (HE) stained slides blinded to the original pathological diagnosis. The Research Ethics Committee of the Faculty of Medicine, University of Yamanashi approved this study (approval number: 2312).

Lymphocytic accumulation

We evaluated lymphocytic infiltration by a representative HE stained slide in each individual case. The HE stained slides were scanned at low magnification (100×). Then 2 pathologists (K.M. and T.K.) together selected areas with the highest density of distinct lymphocyte infiltration (‘hot spot’) under the EHGD sites within each section. They counted the lymphocytes in these ‘hot spots’ within a 400× microscopic field of an Olympus BX53 (Tokyo, Japan) microscope. We defined lymphocytic accumulation as containing more than 100 lymphocytes per field.

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 antibodies. We performed antigen retrieval through heat treatment by 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. CXCR4 (clone UMB2, Abcam plc, Cambridge, UK, dilution 1:500) was used as the primary antibody. We performed antigen retrieval through heat treatment by autoclaving at 121°C for 10 min in Tris-EDTA buffer pH 9. After inhibiting endogenous peroxidase, we used a positive control (adrenal gland) 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.

To evaluate the immunohistochemistry results, we used the H-score, which is calculated by differentiating staining intensities in four gradations (0 to 3 with 0 being no staining) and adding the products of gradation x percentage of positive cells within that gradation. The H-score classifications are 0=0 to 49 points, 1=50 to 99 points, 2=100 to 199 points, and 3=200 to 300 points with 1, 2 or 3considered positive and 0 as negative (Specht et al., 2015).

Table 1. Clinicopathologic findings of 30 cases with EHGD.

Case	Age/Sex	EHGD size (mm)	lymphocyte accumulation
1	73/F	13	+
2	65/M	5	-
3	75/M	12	+
4	68/M	60	+
5	72/M	6	+
6	73/M	6	+
7	64/M	5	+
8	64/M	3	+
9	78/M	7	+
10	86/M	10	+
11	57/M	25	+
12	76/M	12	+
13	72/M	5	+
14	70/M	10	+
15	74/F	12	+
16	80/M	20	+
17	63/M	37	+
18	74/M	15	+
19	46/M	6	+
20	79/M	35	+
21	62/M	13	+
22	67/M	9	+
23	70/M	12	+
24	69/F	20	+
25	63/M	6	+
26	74/M	25	+
27	73/M	25	+
28	73/M	10	+
29	70/F	10	+
30	78/M	8	+

EHGD, esophageal high grade dysplasia; F, female; M, male.

Microdissection and extraction of RNA from paraffin embedded tissue

Four 10-µm thick serial sections were cut from routinely processed, FFPE tissue blocks. The EHGD cells from the immunopositive areas were microdissected with Arcturus XT Laser Capture Microdissection System (Thermofisher Scientific, MA, USA) and the nucleic acids extracted in standard procedures. To avoid sampling problems, we selected non-necrotic tissue with a considerable number of 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'- CTACAGATGCCCATGCC GAT -3' and 5'- CAGCCGGGCTACAATCTGAA -3'; product size: 109bp) and CXCR4 (primers: 5'-TGGTCTATGTTGGCGTCTGG -3' and 5'-GTCATTGGGGTAGAAGCGGT -3'; product size: 116bp) using HotStarTaq DNA Polymerase (QIAGEN, Hilden, Germany). 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'-GATGACATCAAGAAGGTGGTGA -3' and 5'-TTCGTTGTCATACCAGGAAATG-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 EHGD and NESE regarding lymphocytic accumulation and immunohistochemical staining of CXCL12, CXCL10, CCL18 and CXCR4. 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

Lymphocytic accumulation

Results of lymphocytic accumulation (Fig. 1A) are summarized in Table 1 and confirmed in 29 EHGD cases (97%). On the other hand, 13 NESE cases (43%) also had lymphocytic accumulation ($p<0.001$).

CXCL12 immunostaining in EHGDs and NESEs

Results of immunohistochemical studies are summarized in Table 2. EHGDs showed the following immunostaining patterns: 30% classified 0, 63% classified 1, 7% classified 2, and 0% classified 3 (Fig. 1C). NESEs showed the following immunostaining patterns: 100% classified 0, 0% classified 1, 2 or 3. Using the two-tailed Pearson's chi-square test, EHGD and NESE cases were significantly different in CXCL12 immunostaining expression ($p<0.001$).

CXCL10 immunostaining in EHGDs and NESEs

Results of immunohistochemical studies are summarized in Table 2. EHGDs showed the following immunostaining patterns: 97% classified 0, 3% classified 1, 0% classified 2 or 3. NESEs showed the following immunostaining patterns: 97% classified 0, 3% classified 1, 0% classified 2 or 3. Using the two-tailed Pearson's chi-square test, EHGD and NESE cases were not significantly different in CXCL10 immunostaining expression ($p=1.000$).

CCL18 immunostaining in EHGDs and NESEs

Results of immunohistochemical studies are summarized in Table 2. EHGDs showed the following immunostaining patterns: 97% classified 0, 3% classified 1, 0% classified 2 or 3. NESEs showed the following immunostaining patterns: 100% classified 0, 0% classified 1, 2 or 3. Using the two-tailed Pearson's chi-square test, EHGD and NESE cases were not significantly different in CCL18 immunostaining expression ($p=0.313$).

Table 2. Expressions of CXCL12, CXCL10, CCL18 and CXCR4 in 30 EHGDs and 30 NESEs.

Tumor type	H-score (Classification)*				p-value**
	0	1	2	3	
CXCL12					
EHGD (n=30)	9	19	2	0	<0.001
NESE (n=30)	30	0	0	0	
CXCL10					
EHGD (n=30)	29	1	0	0	1.000
NESE (n=30)	29	1	0	0	
CCL18					
EHGD (n=30)	29	1	0	0	0.313
NESE (n=30)	30	0	0	0	
CXCR4					
EHGD (n=30)	14	10	6	0	<0.001
NESE (n=30)	30	0	0	0	

EHGD, esophageal high grade dysplasia; NESE, normal esophageal squamous epithelium; CXCL12, C-X-C motif chemokine ligand 12; CXCR4, C-X-C chemokine receptor 4. *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.

CXCR4 immunostaining in EHGDs and NESEs

Results of immunohistochemical studies are summarized in the Table 2. EHGDs showed the following immunostaining patterns: 47% classified 0, 33% classified 1, 20% classified 2, and 0% classified 3 (Fig. 1D). NESEs showed the following immunostaining patterns: 100% classified 0, 0% classified 1, 2 or 3. Using the two-tailed Pearson's chi-square test, EHGD and NESE cases were significantly different in CXCR4

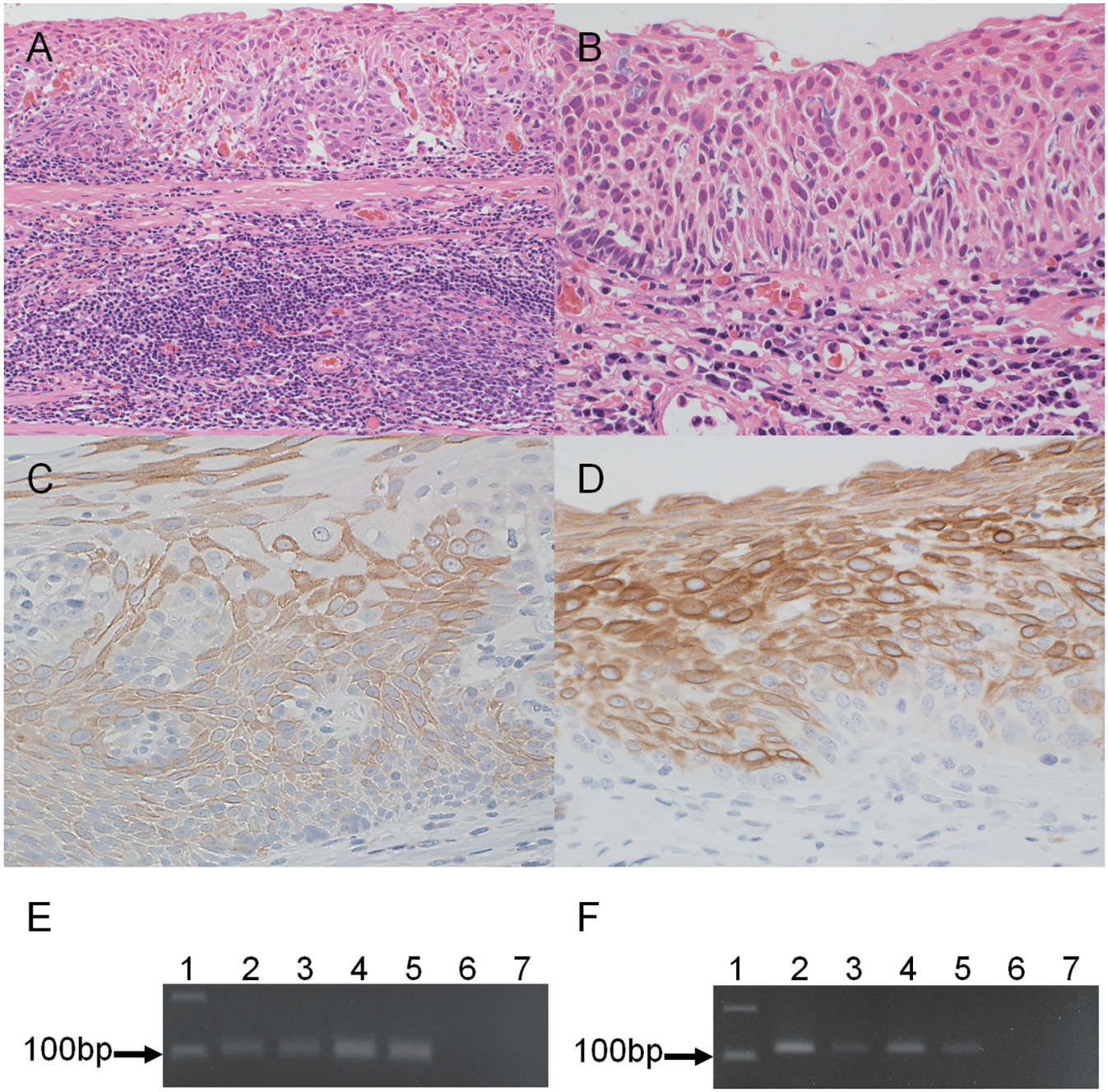

Fig. 1. Representative immunohistochemical images of CXCL12 and CXCR4 in EHGD. EHGD with lymphocyte accumulation directly under the lesion **(A, B)** and exhibiting CXCL12 **(C)** and CXCR4 **(D)** immunoreactivities in the cytoplasm. Representative results of CXCL12 **(E)** and CXCR4 **(F)** mRNA detected by RT-PCR analysis in 3 EHGDs showing 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). A, x 200; B-D, x 400.

immunostaining expression ($p<0.001$).

CXCL12 and CXCR4 mRNA expressions in EHGDs by RT-PCR

We examined CXCL12 and CXCR4 mRNA expressions in 3 representative EHGD samples, each having their respective immunostained areas detected by RT-PCR (Fig. 1E,F).

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 NH_2-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). CXCL12 acts as a chemoattractant for T lymphocytes, monocytes and dendritic cells to drive their homing to lymphoid organs (Karin, 2010). Meanwhile, CXCL12 regulates many essential biological processes, including cardiac and neuronal development, stem cell motility, neovascularization, angiogenesis, apoptosis, and tumorigenesis (Burns et al., 2006). Furthermore, determination of CXCL12 expression has the potential as a cancer biomarker and for adding prognostic information in various cancer types (gastrointestinal cancer, gynaecological cancer, breast cancer, urological cancer and lung cancer) (Samarendra et al., 2017).

Sasaki et al. used immunohistochemistry to show a positive expression rate of 53.7% for CXCL12 and 84.6% for CXCR4 in 214 patients with esophageal squamous cell carcinoma (ESCC). Positive CXCL12 expression correlated significantly with lymph node metastasis, tumor stage, gender and lymphatic invasion: the overall and disease-free survival rates were significantly lower in patients with positive CXCL12 expression than in those with negative CXCL12 expression (Sasaki et al., 2009). Moreover, Uchi et al. showed positive immunohistochemistry expressions for CXCR4 and CXCL12 of 61% and 78%, respectively, in 79 patients with ESCC. The MIB-1 proliferation index was markedly higher in ESCC samples having a positive expression of CXCR4 or CXCL12, and positive CXCL12 expression correlated significantly with lower recurrence-free survival ($p=0.02$) (Uchi et al., 2016). Mechanistically, Wang et al. demonstrated that CXCL12/CXCR4 activated the ERK1/2 pathway and thereby maintained the characteristics of high-level invasion and metastasis of esophageal cancer stem cells (Wang et al., 2017).

Our immunohistochemical results showed 70% of the EHGD samples had a high rate of CXCL12 expression (H-score ≥50 points) and a significant difference in CXCL12 expression between EHGDs and NESEs ($p<0.001$). This may indicate that CXCL12 plays an important role in tumor progression in EHGDs by increasing tumor survival and proliferative ability. Therefore, in addition to its primary role in tumor progression, the chemoattractant properties of CXCL12 also may be causing a secondary effect of lymphocyte accumulation directly under the EHGD. Alternatively, the lymphocyte accumulation may be part of the actual process for invasion into stromal tissue since this accumulation seems to cause embrittlement of the surrounding tissue. On the other hand, we confirmed a high rate of CXCR4 expression (H-score ≥50 points) in 53% of EHGD samples by immunohistochemistry, which also indicates that the autocrine system of CXCL12 and CXCR4 produced by these cells exist.

Expressions of CXCL10 and CCL18 in ESCCs have been reported. Wang et al. showed that overexpression of CCL18 in ESCC tissues was associated with a worse survival rate in patients with ESCC (Liu et al., 2015; Wang et al., 2019). Although we showed that the EHGD samples had a very low rate of CXCL10 (3%) and CCL18 (3%) expressions (H-score ≥50 points), there was no significant difference between EHGDs and NESEs in their expression rates ($p=1.000$ and $p=0.313$, respectively) indicating that the relationship between lymphocytic accumulation in EHGD areas (early phase lesion of squamous epithelial neoplasia) and CXCL10 and CCL18 expressions is not significant.

In conclusion, our results may show that CXCL12 plays a part in the accumulation of lymphocytes directly under EHGDs.

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