Expression of the CXCR4 and CCR7 chemokine receptors in human endometrial cancer

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Summary

Purpose: The chemokine receptors CXCR4 and CCR7 have been suggested to play an important role in cancer progression but their expression in human endometrial cancer has not been fully characterized. The aim of this study was to investigate CXCR4 and CCR7 expression in endometrial cancers. *Methods*: We immunohistochemically investigated the expression of CXCR4 and CCR7 protein in 166 endometrial cancers and analyzed the correlation with various observed clinicopathological features, including patient outcome. Fresh tumor specimens were obtained from 55 of the 166 endometrial cancer patients, and the expression levels of the CXCR4 and CCR7 genes were also examined in this subgroup. *Results*: Our results indicate that CXCR4 and CCR7 transcripts levels are significantly higher in tumors that express the corresponding protein products. CXCR4 and CCR7 protein expression levels were found to be significantly lower in patients with endometrial tumors of a high grade. Consistent with this, the overall survival rates were significantly better in patients exhibiting higher levels of CXCR4 and CCR7 expression. *Conclusion*: We thus hypothesize that CXCR4 and CCR7 protein levels are suppressed in high-grade endometrial tumors, but that the expression of these receptors per se may not be a crucial role in tumor progression or metastasis in these cancers.

Key words: CXCR4; CCR7; Endometrial cancer.

Introduction

Tumor cell invasion and subsequent metastasis via the bloodstream and lymph vessels are critical steps in the progression of malignant tumors, including endometrial cancer. Chemokines belong to the small-molecule chemoattractive cytokine family and have been assigned to four groups (CXC, CC, CX3C, and C) based on the presence of four conserved N-terminal cysteines [1-3]. Chemokines mediate their chemical effect on target cells through G-protein-coupled hepathelical receptors, which are structurally characterized by seven transmembrane-spanning domains and are involved in the attraction of mononuclear and polymorphonuclear leukocytes. Recent data suggest that chemokine receptors may direct lymphatic and hematogenous spreading and may additionally influence the sites of metastatic growth for different tumors [4]. Moreover, different cancers have been shown to express different CC and CXC chemokine receptors, although the chemokine receptor CXCR4 appears to be expressed by the majority of cancer types [5]. CXCR4 was initially reported to regulate the homing of lymphocytes to inflammatory tissues [6]. In addition, the CXCR4/CXCL12 pathway is involved in stimulating the metastatic processes of many different neoplasms, in which CXCR4 activates various phenomena such as chemotaxis, invasion, angiogenesis, and proliferation [7]. CCR7, which is a receptor for the two major chemokines CCL19 and CCL21, is expressed in naive T cells, memory T cells, B cells, and mature dendritic cells and is considered to play an important role in lymphocyte cell trafficking and homing to the lymph nodes [8. 9]. Recently, we also reported from our laboratory that CXCR4 and CCR7 expression is significantly associated with lymph node metastasis and patient outcome in cervical cancer [10]. Scotton et al. have also demonstrated in another report that CXCR4 is expressed in ovarian cancer cells and may influence cell migration in the peritoneum, a major route for the spread of these tumors [11].

Chemokine receptors are therefore likely to be important during the invasion and metastasis of gynecological cancers. However, their expression in human endometrial cancer has not been well characterized. Therefore, in our present study we have analyzed the mRNA and protein expression levels of both CXCR4 and CCR7 in endometrial cancer specimens by RT-PCR and immunohistochemistry. In addition, the association between CXCR4 and CCR7 protein expression and the clinicopathological features of these cancers, including patient prognosis, was investigated.

Materials and Methods

Patient subjects and tissue samples

In the current study we examined 166 patients with endometrial cancer. Each of these individuals had undergone hysterectomy, bilateral salpigo-oophorectomy, pelvic and/or paraaortic lymphadenectomy and partial omentectomy at the Okayama University Hospital between January 1997 and November 2004.

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Patients with distant metastasis were excluded from this study. Tumor specimens were obtained at the time of surgery and were immediately fixed in 10% neutral-buffered formalin and embedded in paraffin. Informed consent was obtained from each patient prior to sample collection. Histological cell types were assigned according to the WHO classifications as follows: 159 endometrioid adenocarcinomas, four adenosquamous carcinomas and three serous carcinomas. Histological grades were assigned according to the International Federation of Gynecology and Obstetrics (FIGO) classification as follows: 60 grade 1, 80 grade 2 and 26 grade 3. Surgical staging was reviewed based on the FIGO staging system as follows: 96 Stage I, 14 Stage II, 46 Stage III and ten Stage IV. The median age at the time of surgery was 58 years (range 27-85 years). Patients with a grade 3 tumor, a non-endometrioid histologic subtype, deep myometrial invasion or extrauterine disease were treated with three to six courses of adjuvant combination chemotherapy. Both the disease-free and overall survival rates are defined as the interval between the initial surgery and either clinically or radiologically proven recurrence and death, respectively. The end date of the follow-up study for analysis was July 31, 2005, and the median duration of the follow-up was 29 months (range, 1- 85 months).

Immunohistochemistry and staining evaluation

Sections of 4-μm thickness were obtained from several representative areas of each tumor specimen and were mounted onto glass slides for immunostaining according to the labelled streptavidin biotin procedure of the Dako LSAB kit (Dako North America Inc., CA, USA). Briefly, after each of the slides was dewaxed in xylene and rehydrated in an alcohol series, antigen retrieval was performed in a microwave oven in 10 mM citric acid buffer (pH 6.0) for 3×10 min. The sections were then incubated with 0.3% hydrogen peroxide to block endogenous peroxidase activity, followed by incubation with normal horse serum for 5 min at room temperature. Immunostaining was then performed by incubation with anti-CXCR4 mouse monoclonal antibody (R&D systems Inc., MN, USA) and anti-CCR7 rabbit monoclonal antibody (Epitomics Inc., CA, USA) for two hours at room temperature. The sections were subsequently incubated for 20 min with biotinylated anti-mouse and anti-rabbit immunoglobulin, followed by incubation with peroxidase-conjugated streptavidin for 20 min and 0.05% 3,3'-diaminobenzidine tetrahydrochloride solution (Wako Pure Chemical Industries Ltd., Osaka, Japan) containing hydrogen peroxide for 10 min. Finally, the slides were counterstained with Mayer's hematoxylin and mounted in aqueous mounting medium. At each step the slides were washed carefully in phosphate-buffered saline (pH 7.4).

A specimen was considered to be positive for endometrial cancer if distinct cytoplasmic and/or membrane staining was observed in more than 50% of the cells. Microscopic analyses were conducted independently by two of the authors who had no prior knowledge of the clinical data. The final evaluations of ambiguous cases were performed using a conference microscope.

RNA extraction and RT-PCR

Fresh tumor specimens were obtained from 55 of the 166 endometrial cancer patients included in this study, and the expression levels of the CXCR4 and CCR7 genes were examined in this subgroup. Total RNA was prepared from each specimen and 3 μg aliquots were then used in the RT reactions. For semi-quantitative measurements of CXCR4 and CCR7 mRNA levels, cDNA levels were normalized using ß-actin as the internal standard. In the presence of 1×10^4 copies of ß-actin, the transcribed products were subjected to PCR for CXCR4 and CCR7 with the primers: CXCR4; sense primer, 5'- AGCTGTTGGTGAAAAGGTGGTCTATG-3' and antisense primer, 5'- GCGCTTCTGGTGGCCCTTGGAGTGTG -3' [12], and CCR7; sense primer, 5'- TCCTTCTCATCAGCAAGCTGTC-3' and antisense primer, 5'- GAGGCAGCCCAGGTCCTTGAAG -3' [13]. cDNA amplifications were performed using the following reaction conditions: 30 cycles of 94ºC for 30 sec, 56ºC for 30 sec and 72ºC for 30 sec (CXCR4) and 30 cycles of 94ºC for 30 sec, 55ºC for 1 min and 72ºC for 2 min (CCR7). The amplified PCR products were then resolved by 2% agarose gel electrophoresis and analyzed using Basic Quantifier software (Bio Image, MI, USA).

Statistical analysis

The association between the variables was tested using either the chi-square test, Mann-Whitney *U* test, or via stepwise logistic regression analysis. The survival rates were calculated by the Kaplan-Meier method and differences between the survival curves were examined by the log-rank test. Factors found to be significant were then selected for a stepwise Cox's multivariate proportional hazard model in order to determine their prognostic values. Probability values of less than 0.05 were considered statistically significant.

Results

Association between CXCR4 and CCR7 mRNA expression and clinicopathological factors in endometrial cancer

Of the 166 endometrial cancer patients examined in this study, fresh tumor specimens were obtained from 55 cases in which the expression levels of the CXCR4 and CCR7 genes were then examined. CXCR4 gene expression was observed to be lower in grade 3 tumors, although this finding is not statistically significant ($p = 0.09$). CCR7 gene expression, however, was found to be significantly lower in grade 3 tumors ($p = 0.02$). We could find no association between either CXCR4 or CCR7 gene expression and any other clinicopathological factor in these cancers (data not shown).

Association between the mRNA and protein levels of CXCR4 and CCR7

In our immunohistochemical analyses of endometrial cancer cases, a tumor specimen was considered to be positive for chemokine receptor expression if distinct cytoplasmic and/or membrane staining could be observed in more than 50% of the cancer cells. Based on these criteria, CXCR4 mRNA levels were observed to be significantly higher in tumors with positive CXCR4 protein expression ($p < 0.0001$). The CCR7 mRNA levels were similarly found to be significantly higher in tumors expressing the protein ($p = 0.0002$).

CXCR4 protein expression in endometrial cancer tissue

Figures 1A and 1B show representative immunostaining profiles of CXCR4 in our current endometrial cancer cases. Of the 166 tumor samples under study, 113 (68%) showed positive staining, with the remaining 53 (32%) staining negatively. The association between CXCR4

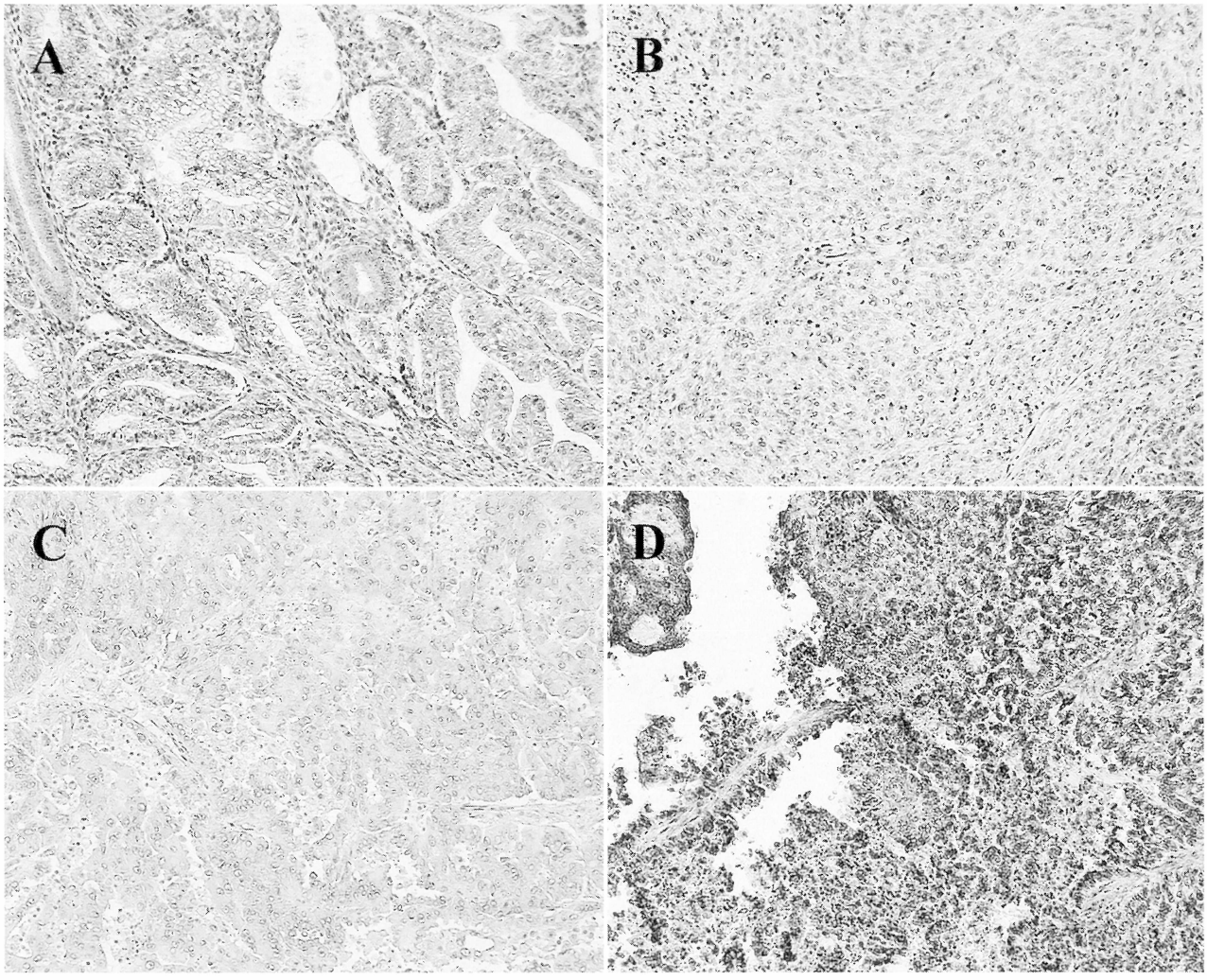

Figure 1. — Immunohistochemical staining of CXCR4 and CCR in endometrial cancer specimens. A) Positive CXCR4 staining in a grade 1 tumor. B) Negative CXCR4 staining in a grade 3 tumor. C) Positive CCR7 staining in a grade 1 tumor. D) Negative CCR7 staining in a grade 2 tumor (original magnification x 200).

expression and clinicopathological factors is shown in Table 1. CXCR4 expression levels were significantly lower in patients with tumors of an advanced FIGO stage ($p = 0.004$) and high-FIGO grade ($p < 0.0001$), and also with cancers showing deep myometrial invasion ($p = 0.049$), lymph node metastasis ($p = 0.032$), ovarian metastasis ($p = 0.003$), or positive peritoneal cytology ($p = 0.001$). Multivariate analysis revealed the FIGO grade to be the most independent factor influencing CXCR4 expression (95% CI, 0.027-0.259; $p < 0.0001$).

CCR7 protein expression in endometrial cancer tissue

Characteristic immunostaining profiles of CCR7 in endometrial cancers were also determined (Figures 1C and D). Of the 166 tumor samples analyzed in this study, 79 (48%) showed positive staining, and 87 (52%) displayed negative staining for CCR7. The association between CCR7 expression and clinicopathological factors is shown in Table 2. CCR7 expression levels were significantly lower in patients with endometrial tumors of a high-FIGO grade ($p = 0.018$), but there was no association found between CCR7 expression and any other clinicopathological factor.

Univariate and multivariate survival analyses

Figure 2A indicates the disease-free survival curves of the 166 patients in this study, based on the CXCR4 expression status of their tumors. The disease-free survival rate for patients exhibiting CXCR4 expression was higher than in patients lacking this expression, although this was found not to be statistically significant ($p = 0.089$). The overall survival rate of the patients exhibiting CXCR4 expression was also significantly higher than the CXCR4-negative cases, and this is statistically significant ($p = 0.035$) (Figure 2B). Both the disease-free and overall survival rates for patients exhibiting CCR7 expression

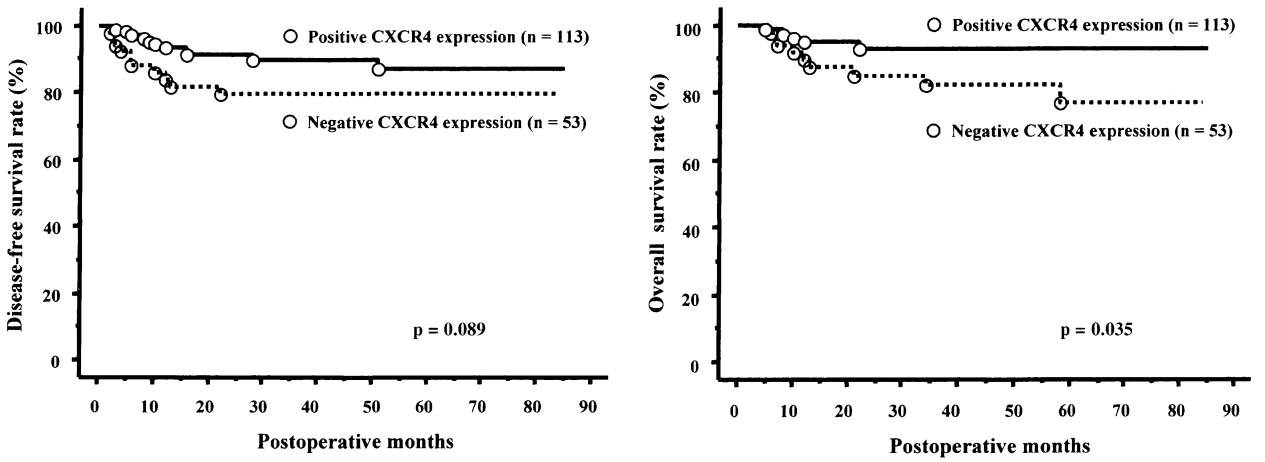

Fig.

Figure 2. — Disease-free A) and overall B) survival curves of the 166 endometrial cancer patients included in this study, according to their CXCR4 expression status.

were significantly higher than their CCR7-negative counterparts (p = 0.019 and p = 0.004, respectively) (Figure 3). Our subsequent Cox's multivariate analyses showed, however, that neither of the CXCR4 nor CCR7 expression status is an independent prognostic factor for endometrial cancer patient survival.

Discussion

The chemokine receptors CXCR4 and CCR7 are the physiological receptors for CXCL12, and for CCL19 and CCL21, respectively. In our present study, we demonstrated that CXCR4 and CCR7 protein expression (deter-

Table 1. — *Association between CXCR4 expression and clinicopathological factors in endometrial cancers.*

	CXCR4 expression		
Variables	(–)	(+)	p value[a]
Age (years)			0.145
< 60	26	69	
≧ 60	27	44	
FRIGO stage			0.004
I + II	27	83	
III+IV	26	30	
FRIGO grande			< 0.0001
1	10	50	
2	23	57	
3	20	6	
Depth of myometrial invasion			0.049
None	3	18	
Inner half	27	64	
Outer half	23	31	
Cervical involvement			0.376
negative	40	92	
positive	13	21	
LVS involvement			0.054
negative	30	81	
positive	23	32	
Lymph node metastasis			0.032
negative	42	103	
positive	11	10	
Ovarian metastasis			0.003
negative	43	108	
positive	10	5	
Peritoneal cytology			0.001
negative	35	99	
positive	18	14	

LVS, lymph-vascular space; [a]Chi-square test.

Table 2. — *Association between CCR7 expression and clinicopathological factors in endometrial cancers.*

	CCR7 expression		
Variables	(–)	(+)	p value[a]
Age (years)			0.487
< 60	52	43	
≧ 60	35	36	
FRIGO stage			0.549
I + II	56	54	
III+IV	31	25	
FRIGO grande			0.018
1	31	29	
2	36	44	
3	20	6	
Depth of myometrial invasion			0.321
None	13	8	
Inner half	43	48	
Outer half	31	23	
Cervical involvement			0.219
negative	66	66	
positive	21	13	
LVS involvement			0.473
negative	56	55	
positive	31	24	
Lymph node metastasis			0.162
negative	73	72	
positive	14	7	
Ovarian metastasis			0.246
negative	77	74	
positive	10	5	
Peritoneal cytology			0.928
negative	70	64	
positive	17	15	

LVS, lymph-vascular space; [a]Chi-square test.

Fig. A

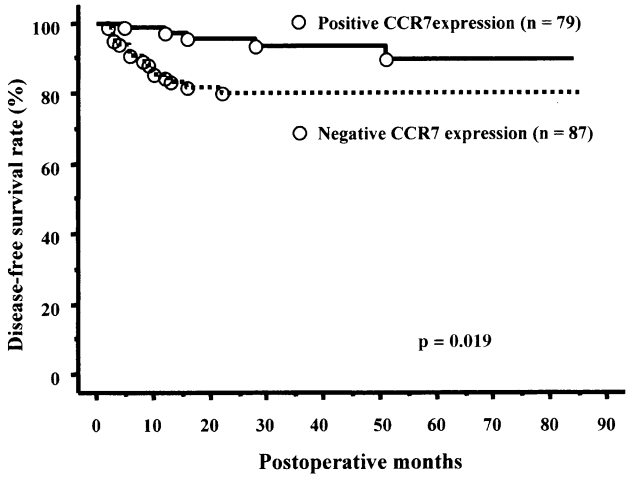

Fig.

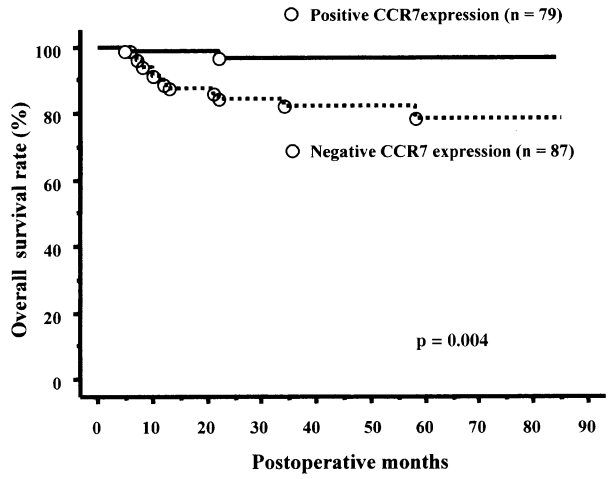

Figure 3. — Disease-free A) and overall B) survival curves of the 166 endometrial cancer patients included in this study, according to their CCR7 expression status.

mined by immunohistochemistry) and mRNA expression (determined by RT-PCR) can be correlated, thus indicating that both genes are regulated at the transcriptional level. We next investigated the CXCR4 and CCR7 protein expression profiles in a considerably larger cohort of endometrial cancer patients. Our findings show that CXCR4 protein expression is at significantly lower levels in advanced-stage endometrial cancers and higher-grade tumors. CXCR4 levels are also lower in endometrial tumors that are characterized by deep myometrial invasion, lymph-vascular space involvement, lymph node metastasis, ovarian metastasis or positive peritoneal cytology. Multivariate analysis further revealed that histological grade was the most independent factor that influences CXCR4 expression. Interestingly, we noted that CXCL12 mRNA levels are low in grade 3 tumors (data not shown) and this is consistent with an earlier study of Mizokami *et al.* who also reported that CXCR4, and its ligand CXCL12, were at significantly low levels in grade 3 endometrial cancers [14]. We additionally demonstrated that CCR7 expression levels are significantly lower in higher-grade tumors only.

Taken together, our current data thus suggest that CXCR4 and CCR7 expression is down regulated in grade 3 tumors and therefore may not contribute to the progression of these tumors. Kwak *et al.* also reported previously that CXCR4 and CCR7 are weakly expressed in undifferentiated human gastric cancers [15]. Although little is known about the epigenetic regulation of the CXCR4 and CCR7 genes, it has also been recently demonstrated that DNA methylation influences CXCR4 expression in both pancreatic cancer and melanoma cells [16, 17]. Sato et al. have also reported that CXCR4 gene methylation is more frequent in poorly differentiated pancreatic cancers than in either the well or moderately differentiated cancers of this type [17]. Further studies will be needed to clarify whether DNA methylation is common in grade 3 endometrial cancers.

We also found in our present analysis that there is no correlation between CXCR4 expression levels and either tumor progression or metastasis in endometrial cancers, even in the cases of grade 1 and 2 tumors (data not shown). These present data are therefore not consistent with previous reports that have described a positive correlation between CXCR4 expression and tumor progression or metastasis in a variety of cancer types including breast, non-small cell lung, pancreaticr, prostate, thyroid, cervical, ovarian, nasopharyngeal and also malignant melanoma and osteosarcoma [10, 18-26]. CXCL12, which is the only ligand for CXCR4, is cleaved at its amino-terminus by a variety of enzymes, including CD26, cathepsin G, neutrophil elastase and matrix metalloproteinases [27-30]. These enzymes remove two to five amino acids from CXCL12, thereby inactivating it. Mizokami et al. reported that CD26 is frequently expressed in grade 1 and 2 endometrial cancers and may degrade CXCL12 [14]. They speculated that an imbalance between CD26 and CXCR4 influences tumor progression and this may explain why there is no association between CXCR4 expression and tumor progression in endometrial cancer.

We also detected in our current analyses that there is no correlation between CCR7 expression and either tumor progression or metastasis in cases of grade 1 and 2 tumors (data not shown). A positive correlation has been reported between CCR7 expression and tumor progression in cases of cervical cancer, breast cancer, non-small cell lung cancer, gastric cancer, colorectal cancer and esophageal cancer [10, 31-35]. However, conflicting results exist regarding the role of CCR7 in metastasis [15, 36, 37]. Furthermore, as the role of chemokines and their receptors in human cancers is undoubtedly complex, it may be too simplistic to assume that chemokine receptors alone determine the extent of tumor progression or metastasis. We speculate that other receptor/ligand systems may in fact be more important during the progression of endometrial cancer.

Thus far, no previous studies have reported the clinical outcome of endometrial cancer cases showing positive CXCR4 and CCR7 expression in endometrial cancer. The prognoses for overall survival among our 166 patients were found to deteriorate with decreasing CXCR4 and CCR7 expression levels. However, Cox's multivariate analyses showed that neither the CXCR4 nor CCR7 expression status is an independent prognostic factor for survival in these patients. This may be explained by the fact that CXCR4 and CCR7 expression is down-regulated in high-grade endometrial tumors.

Conclusion

We hypothesize that CXCR4 and CCR7 expression may be suppressed in high-grade endometrial cancers. Our current findings also provide evidence that CXCR4 and CCR7 expression *per se* may not be a crucial role in tumor progression or metastasis in endometrial cancer unlike other tumor types.

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