Association of CC Chemokine Receptor 7 with Lymph Node Metastasis of Esophageal Squamous Cell Carcinoma[1]

Yongzeng Ding, Yutaka Shimada,[2] Masato Maeda, Atsushi Kawabe, Junichi Kaganoi, Izumi Komoto, Yosuke Hashimoto, Masayuki Miyake, Hiroki Hashida, and Masayuki Imamura

Department of Surgery and Surgical Basic Science, Graduate School of Medicine, Kyoto University, Kyoto 606-8507 [Y. D., Y. S., M. M., A. K., J. K., I. K., Y. H., M. I.], and Department V of Oncology and Department of Thoracic Surgery, Kitano Hospital, Tazuke Kofukai Medical Research Institute, Osaka 530-8480 [M. M., H. H.], Japan

ABSTRACT

Purpose: **CC chemokine receptor 7 (CCR7) plays a critical role in the migration of activated dendritic cells to regional lymph nodes. Recent studies have shown that CCR7 is involved in metastasis in some malignant diseases. The role of CCR7 in esophageal squamous cell carcinoma (SCC) has not yet been clarified.**

Experimental Design: **We performed reverse transcription-PCR analysis for CCR7 in 20 esophageal SCC cell lines and immunohistochemical analysis of 96 esophageal SCC samples. We then performed a cell migration assay, F-actin polymerization, and a phagokinetic assay on esophageal SCC cell lines in the presence of CCL21, a ligand of CCR7.**

Results: **CCR7 mRNA was detected in 9 of 20 esophageal SCC cell lines. Immunoreactive CCR7 was found mainly in esophageal cancer cells. High CCR7 expression was significantly correlated with esophageal SCC lymphatic permeation, lymph node metastasis, tumor depth, and tumor-node-metastasis stage and was associated with poor survival. *In vitro* studies demonstrated that CCL21 significantly increased the cell migration ability of esophageal SCC cell lines, and pseudopodia formation was induced by CCL21 stimulation. Furthermore, CCL21 markedly enhanced the motility of esophageal carcinoma cell lines by the phagokinetic assay.**

Conclusions: **The results suggested that the CCR7/CCL21 receptor ligand system may play a role in the lymph node metastasis of esophageal SCC.**

INTRODUCTION

Esophageal carcinoma is one of the most aggressive tumors, and its growth is relatively rapid. In particular, the presence of lymph node metastasis and vascular invasion indicate highly malignant potential in esophageal carcinoma (1). The 5-year survival rate is 20–30% after curative surgery (2). The reason for this poor prognosis is that esophageal cancer exhibits extensive local invasion or frequent regional lymph node metastasis, even at the time of initial diagnosis. Metastasis is the result of several sequential steps and represents a highly organized, nonrandom, and organ-selective process. Our knowledge of the molecular determinants of metastasis is clearly limited.

Chemokines belong to the small molecule chemoattractive cytokine family and are grouped into CXC chemokines and CC chemokines on the basis of the characteristic presence of four conserved cysteine residues (3–5). Chemokines mediate their chemical effect on target cells through G-protein-coupled receptors, which are characterized structurally by seven transmembrane spanning domains and are involved in the attraction and activation of mononuclear and polymorphonuclear leukocytes. Chemokines and their receptors play important roles in angiogenesis and tumor growth (6), however, the role of chemokine receptors in metastasis has only recently been explored (7). Of interest, CCR7[3] is expressed by human adult T-cell leukemia cells with lymph node involvement (8), and CCR7 up-regulation in classical Hodgkin's disease correlates with lymphoid organ dissemination (9). In addition, some human breast and melanoma cell lines also express functional CCR7 (10). Recently, Mashino *et al.* (11) found that CCR7 is associated with lymph node metastasis of gastric carcinoma. Furthermore, the expression of a single chemokine, CCR7, by murine melanoma cells increases metastasis to the lymph nodes (12), raising the possibility that cancer cells may use normal mechanisms of lymph node homing for metastasis dissemination.

To clarify the role of CCR7 in esophageal SCC, we investigated the CCR7 expression using RT-PCR and performed immunohistochemical analysis. We also evaluated the experimental chemotactic activity and motility in the presence of CCL21 in esophageal cancer cell lines.

Received 8/27/02; revised 4/10/03; accepted 4/21/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported, in part, by from the Japanese Ministry of Education, Science and Culture Grants-in-Aid 07671386, 07457271, and 0971301.

[2] To whom requests for reprints should be addressed, at Department of Surgery & Surgical Basic Science, Graduate School of Medicine, Kyoto University, Kawaracho 54 Shogoin Sakyo-KU, Kyoto 606-8507, Japan. Phone: 81-75-751-3227; Fax: 81-75-751-4390; E-mail: shimada@kuhp.kyoto-u.ac.jp.

[3] The abbreviations used are: CCR7, CC chemokine receptor 7; SCC, squamous cell carcinoma; RT-PCR, reverse transcription-PCR; TNM, tumor-node-metastasis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ATLL, adult T-cell leukemia/lymphoma; AGPC, acid guanidium-phenol-chloroform.

MATERIALS AND METHODS

Tissue Samples and Cell Lines. Tissues were obtained from esophageal SCC specimens from 96 patients who underwent esophagectomies at our institution between June 1987 and December 2001. The operative procedures used were as described previously (13). In brief, esophagectomy with lymph node dissection was performed through a right thoracotomy, and subsequent reconstitution was carried out mostly by an esophagogastrostomy using a gastric tube through the retrosternal route. Specimens were fixed in a 10% formaldehyde solution and embedded in paraffin for immunohistochemical analysis. The patients' conditions were followed up every 1 or 2 months after the operation. Primary cancers of the esophagus were classified according to the pathological TNM classification (14). The following 20 esophageal cancer cell lines (KYSE series) established in our laboratory (15) were used in this study: KYSE 70, KYSE 110, KYSE 150, KYSE 170, KYSE 190, KYSE 270, KYSE 350, KYSE 410, KYSE 450, KYSE 510, KYSE 520, KYSE 790, KYSE 850, KYSE 890, KYSE 960, KYSE 1190, KYSE 1850, KYSE 1240, KYSE 1250, and KYSE 2880. Written informed consent was obtained from the patients for surgery and for use of the resected samples for research. (The approval numbers of the Institutional Review Board of Kyoto University were 232 and G48.)

RT-PCR. Total RNA was purified from esophageal squamous cancer cell lines by the AGPC method. Five μg of total RNA were reverse transcribed at 37°C for 60 min in a total 15-μl reaction volume using a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech). cDNA was incubated at 95°C for 5 min to inactivate the reverse transcriptase and served as template DNA for 33 cycles of amplification using the PCR-200 DNA engine (MJ Research, Inc.). PCR was performed in a standard 50-μl reaction mixture, consisting of 10 mM Tris-HCl, 50 mM potassium chloride, 1.5 mM magnesium chloride (pH = 8.3), 0.2 mM deoxynucleoside triphosphates, 50 pmol of each sense and antisense primer, and 2.5 units of TaqDNA polymerase (Life Technologies, Inc.). Amplification was performed for 30 s at 94°C, 1 min at 55°C, and an extension step was carried out for 2 min at 72°C. As a negative control, the RNA template was omitted from the reaction. The amplification products were separated on 2% agarose gels and visualized by ethidium bromide staining. PCR primers for CCR7 cDNA were as follows: forward primer 5′-TCCTTCTCATCAGCAAGCTGTC-3′ and reverse primer 5′-GAGGCAGCCCAGGTCCTTGAAG-3′. According to the CCR7 gene structure, a PCR product of 529 bp was obtained. For GAPDH, the forward primer was 5′-TGGTATCGTGGAAGGACTCATGAC-3′, and the reverse primer was 5′-ATGCCAGTGAGCTTCCCGTTCAGC-3′. A single 189-bp band amplified with primers specific for GAPDH with the same cDNA was detected and used as an internal control under identical conditions.

Immunohistochemical Staining. Immunohistochemical staining was performed using the avidin-biotin complex method. Tissue sections were deparafinized and rehydrated in water. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide for 30 min. Sections were rehydrated, washed, and incubated with a blocking reagent, 5% normal horse serum with 1% BSA in PBS, for 30 min at room temperature to block nonspecific antibody reactions. Sections were incubated overnight at 4°C with antihuman CCR7 monoclonal antibody (BD PharMingen). After four rinses in PBS, sections were incubated for 2 h at room temperature with biotinylated antimouse IgG, followed by four washes with PBS, and then reacted with the avidin-biotin system for 3 min using 0.03% 3,3′-diaminobenzidine tetrahydrochloride as a chromogen. Sections were counterstained with Mayer's hematoxylin. For negative controls, blocking reagent without primary antibody was used as a substitute for the primary antibody of CCR7.

Evaluation of Immunostaining. Specimens were considered immunopositive for CCR7 when ≥1% of the tumor cells had clear evidence of immunostaining. The percentage of positive tumor cells was graded as follows: 0, none; 1, 1–24%; 2, 25–49%; 3, 50–74%; and 4, 75–100%. Immunostaining intensity was rated as follows: 0, none; 1, weak; 2, moderate; and 3, intense. In addition, an immunoreactive score was calculated by multiplying of the score of percentage positive cells and the score of staining intensity as reported previously (16). For example, a specimen containing 40% CCR7-immunopositive tumor cells with strong intensity received a score of $2 \times 3 = 6$. In the case of heterogeneous staining intensities within a sample, each component was scored independently, and the results were summed. Immunostaining was evaluated by two authors (Y. D., A. K.) blinded to patient outcome and other clinical findings. Cases that were evaluated differently after discussion between the two researchers were excluded.

Fluorescence Microscopy. For F-actin location, esophageal cancer cell lines were incubated with 300 ng/ml CCL21 for 20 min, fixed for 20 min in paraformaldehyde in PBS, put on ice, permeabilized for 5 min in 0.2% Triton X-100, incubated with 5 mU/ml FITC-phalloidin (Molecular Probes) for 30 min, washed three times with PBS, and mounted with Antifade (Molecular Probes). Fixed cells were analyzed by confocal microscopy.

Migration Assay for Esophageal Cancer Cell Lines. Cell migration assays were performed in triplicate using 6.5-mm diameter chambers with 12-μm pore filters (Transwell, 24-well culture plates; Costar, Boston, MA). KYSE cell lines were suspended at 1×10^5 cells/ml in serum-free media (RPMI 1640 containing 1% BSA), and 500 μl of the cell suspension were added to the upper chamber. CCL21(300 ng/ml) was placed in the lower well. The chambers were incubated for 12 h at 37°C in a humid atmosphere of 5% CO_2, after which, the cells on the upper surface of the filter were removed using a cotton wool swab. The cells that had migrated to the lower surface were stained using DiffQuik (Dade Behring, Dudingen, Switzerland). For each transwell, the number of cells that had migrated in five medium-power fields (×20) was counted.

Phagokinetic Assay. For the phagokinetic assay (17), 1000 trypsinized cells were plated on uniform carpets of gold particles prepared on glass coverslips coated with BSA and incubated for 24 h. The phagokinetic tracks were visualized by low-power, dark-field microscopy using side illumination. The area cleared of gold particles was measured after photography, and the mean value for 20 cells was calculated in each experiment.

Statistical Analysis. Relationships between the immunostaining results of CCR7 and clinical factors were analyzed

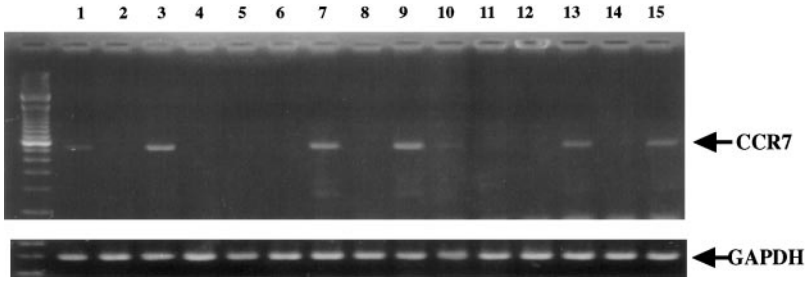

Fig. 1 Representative RT-PCR analysis on RNA isolated from human lymph node and human esophageal cancer cell lines using a primer set specific for CCR7. Another primer set specific for GAPDH was used for normalization. *Lanes 1–15* represent human lymph node, KYSE 70, KYSE 110, KYSE 150, KYSE 170, KYSE 190, KYSE 270, KYSE 350, KYSE 410, KYSE 450, KYSE 510, KYSE 520, KYSE 790, KYSE 890, and KYSE 960, respectively.

using the Pearson's χ^2 test and Fisher's exact test. Survival curves of the patients were calculated using the Kaplan-Meier method and analyzed using the log-rank test and Breslow-Gehan-Wilcoxon test. Cox's proportional hazard model was used for multivariate analysis. For the multivariate analysis, cases with an immunoreactive score ≥ 8 were designated "1," and cases with an immunoreactive score < 7 were designated "0." pT, pN, histological grade, age, and gender were divided into the following categories: (pT1:0, pT2, pT3, pT4:0); (pN0:0, pN1:1); (G1, G2:0, G3, G4:1), ($<$63 years old: 0; $\geq$63 years: 1); and (female: 0, male: 1). Results of the migration assay and the phagokinetic assay were assessed with the Student's *t* test.

StatView J-4.5 (Abacus Concepts, Inc., Berkeley, CA) and JMP version 4 (SAS Institute, Inc., Cary, NC) were used for statistical analysis. Differences with $P < 0.05$ were accepted as significant.

RESULTS

CCR7 mRNA Expression in Esophageal SCC Cell Lines. We performed RT-PCR using specific primers, as described in "Materials and Methods." CCR7 mRNA expression was detected in the following 9 of the 20 esophageal SCC cell lines: KYSE 110, KYSE 270, KYSE 410, KYSE 450, KYSE 790, KYSE 850, KYSE 960, KYSE 1190, and KYSE 1240. Human lymph node was used as a positive control (18; Fig. 1).

Immunohistochemical Staining Analysis of CCR7 in Esophageal SCC. The distribution of CCR7 protein expression in esophageal SCC specimens was examined by immunohistochemical analysis. To confirm whether fixation alters the CCR7 protein expression, we performed CCR7 immunohistochemical staining using 10 frozen esophageal SCC sections. We found that the CCR7 epitope expression and staining patterns of the frozen sections were consistent with those of the paraffin sections (data not shown). Fig. 2*A* shows representative immunostainings of esophageal SCC. Staining of the CCR7 protein was identified in the cytoplasm and cell membrane of cancer cells but was not detected in the normal esophageal epithelium (data not shown). Strong staining for CCR7 protein was observed in some infiltrating inflammatory cells in the specimens. Ninety of the 96 cases were positive for CCR7 protein (93.8%). Six cases were completely negative (6.2%). In addition to primary tumors, regional lymph node metastasis exhibited strong CCR7 expression in the tumor cells (Fig. 2*B*). The immunoreactive score ranged between 0 and 12, and the mean score was 7.24. Table 1 shows the expression patterns in terms of the score of positive tumor cells, staining intensity, and immunoreactive score.

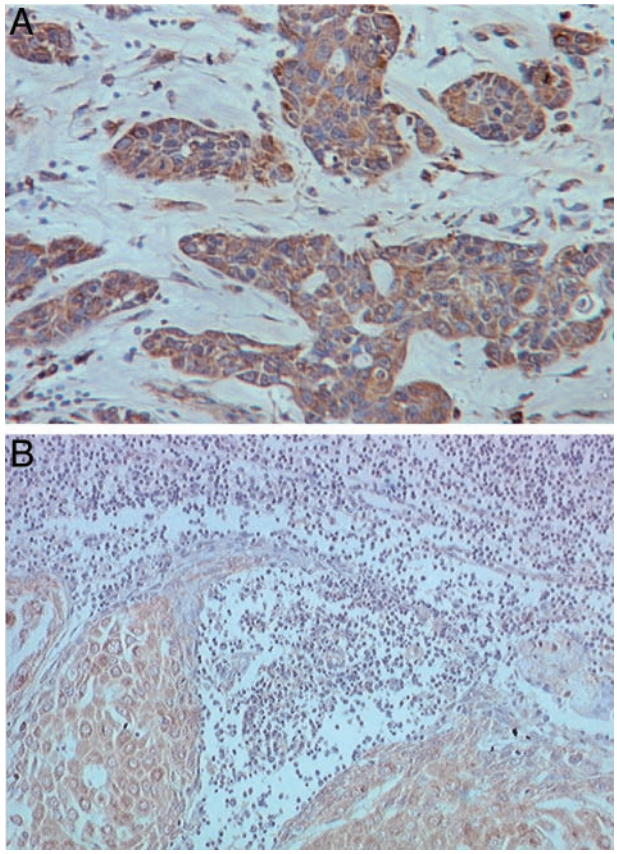

Fig. 2 Immunohistochemical staining of CCR7 in esophageal SCC and metastatic lymph node. *A*, anti-CCR7 staining of esophageal SCC showing that CCR7 was observed mainly in esophageal SCC cells in the tumor nests. CCR7 was immunolocalized strongly in both the cytoplasm and membrane of cancer cells (original magnification, ×160). *B*, CCR7 staining was detected in cancer cells in metastatic lymph node (original magnification, ×80).

Correlation between the Expression of CCR 7 and Clinicopathological Findings. To evaluate the association between CCR7 expression and clinicopathological findings, patients were divided into low CCR7 expression (score 0–7) and high CCR7 expression (score 8–12). The mean immunoreactive score of 7.24 was taken as the cutoff score. Table 2 summarizes the relationship between the CCR7 expression and clinicopathological features of the 96 esophageal SCC. The CCR7 expression was significantly higher in patients with lymph node me-

Table 1 Expression of CCR7 in 96 esophageal SCC by immunohistochemistry

A. Score of positive tumor cells[a]												
Score	0	1	2	3	4							
Patients	6	9	8	20	53							
B. Score of staining intensity[b]												
Score	0	1	2	3								
Patients	6	18	27	45								
C. Immunoreactive score[c]												
Score	0	1	2	3	4	5	6	7	8	9	10	11	12
Patients	6	1	11	5	5	0	8	5	13	11	9	3	19

[a] Percentage of positive cells: 0 (None); 1 (1–24%); 2 (25–49%); 3 (50–74%); and 4 (75–100%).
[b] Staining intensity: 0 (none); 1 (weak); 2 (moderate); and 3 (intense).
[c] Immunoreactive score: calculated by multiplying scores A and B.

Table 2 Clinicopathological characteristics regarding CCR7 expression in esophageal carcinoma

Term	CCR7 immunoreactive score ≧ 8 (55)	CCR7 immunoreactive score < 8 (41)	*P*
Age (yr ± SD)	61.18 ± 1.57	61.80 ± 1.81	
Sex			
Male	48	33	
Female	7	8	0.366[a]
Histological grade			
Well[b]	11	6	
Mod	26	26	
Por	18	9	0.289[c]
pT			
1	5	15	
2	15	9	
3	22	11	0.011[c]
4	13	6	
Vessel permeation			
Absent	24	20	
Present	31	21	0.617[a]
Lymphatic permeation			
Absent	11	20	
Present	44	21	0.001[a]
pN			
pN_0	11	28	
pN_1	44	13	<0.001[a]
Stage			
1	2	13	
2a	8	12	
2b	11	4	
3	21	9	
4a	6	1	
4b	7	2	<0.001[c]

[a] Fisher's exact probability.
[b] Well, well differentiated; Mod, moderately differentiated; Por, Poorly differentiated.
[c] Pearson's χ^2 test of equality.

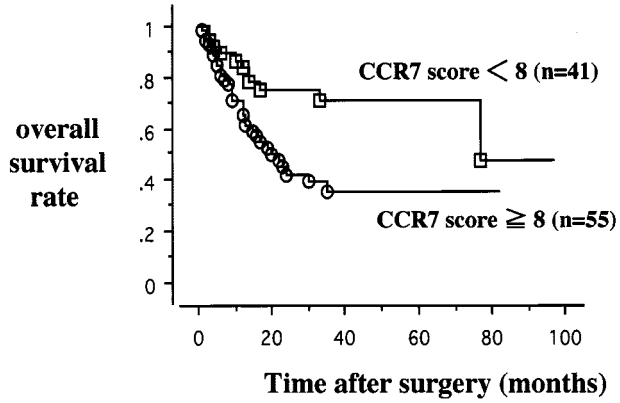

Fig. 3 Overall survival of the 96 patients with esophageal SCC who underwent curative surgery. The patients with high CCR7 expression (CCR7 immunoreactive score ≥ 8) showed a significantly poorer long-term survival rate than those with low CCR7 expression (CCR7 immunoreactive score < 8) by the log-rank test ($P = 0.006$) and Breslow-Gehan-Wilcoxon test ($P = 0.011$).

Table 3 Cox multivariate analysis

Term	Estimate	RR[a]	95% CI	*P*
Age	0.781	2.184	1.139–4.437	0.018
Gender (male)	0.251	1.286	0.557–3.497	0.576
pT(2,3,4)	0.852	2.345	0.762–10.282	0.148
pN (1)	1.02	2.785	1.194–7.070	0.0171
Histological grade	0.028	1.028	−0.658–0.681	0.934
CCR7 (+)	−0.212	0.809	0.392–1.765	0.582

[a] RR, risk ratio; CI, confidence interval.

tastasis compared with those without lymph node metastasis ($P < 0.001$) and was associated with lymphatic permeation ($P = 0.001$), tumor depth ($P = 0.011$), and TNM stage ($P < 0.001$). The patients with high CCR7 expression showed a poorer survival rate compared with those who showed low CCR7 expression by the log-rank test ($P = 0.006$) and Breslow-Gehan-Wilcoxon test ($P = 0.011$; Fig. 3). Multivariate analysis revealed that age and pN were independent prognostic factors and that gender, pT, histological grade, and CCR7 were not independent prognostic factors (Table 3).

Effect of CCL21 on Chemotaxis and Motility of Esophageal Cancer Cell Lines. A transwell migration assay was performed to examine the effects of CCL21 on the motility of esophageal cancer cell lines. At a concentration of 300 ng/ml, CCL21 significantly induced chemotaxis of KYSE 110, KYSE 450, and KYSE 960, which all expressed CCR7 mRNA compared with prestimulation. On the other hand, 300 ng/ml CCL21 did not significantly induce chemotaxis of KYSE 170, KYSE 510, and KYSE 520, all of which did not express CCR7 mRNA

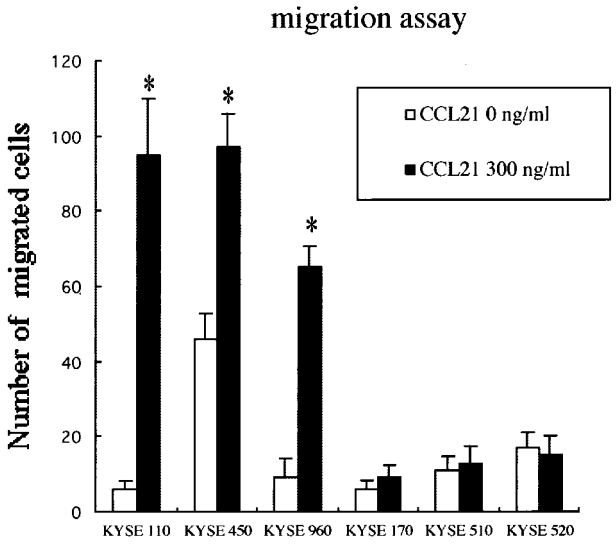

Fig. 4 Effect of CCL21 on chemotaxis of esophageal carcinoma cell lines. To assess the chemotactic responses of esophageal carcinoma cell lines to CCL21, esophageal carcinoma cell lines were plated onto the upper compartment with a 12-μm pore size membrane, and CCL21 was added to the lower chamber at the concentration of 300 ng/ml. After 12 h, the cells on the lower side of the membrane were counted by microscopy in five fields. Data are expressed as the mean number of migrated cells/well in a representative experiment performed in triplicate and repeated three times with similar results. Mean + SD (Student's *t* test, ∗, $P < 0.01$).

(Fig. 4). Phagokinetic motility of esophageal carcinoma cell lines was tested using the gold particle coating method described previously (17). The areas of particle-clear zones for KYSE 520 and KYSE 960 were measured after a 24-h incubation period, and the average track area was calculated. The clearance areas of KYSE 960 were significantly larger in the presence of CCL21 (300 ng/ml; 1546 ± 103 μm^2/20 cells) than those in the absence of CCL21 (300 ng/ml; 409 ± 87 μm^2/20 cells; $P < 0.01$). In contrast, CCL21 did not significantly affect the KYSE 520 motility (area of prestimulation: 490 ± 94 μm^2/20 cells; area of poststimulation: 471 ± 110 μm^2/20 cells; $P = 0.87$).

Fluorescence Microscopy. Reorganization of the actin cytoskeleton is an early event in the migratory response to chemokines (19). To investigate whether actin polymerization could be observed in carcinoma cell lines stimulated by a chemokine, we examined the changes in the actin cytoskeleton of KYSE 520 and KYSE 960 in response to CCL21 by confocal microscopy. Distinct pseudopodia formation was observed in KYSE 960 after 20 min of CCL21 stimulation, however, no remarkable morphological changes were observed in KYSE 520 (Fig. 5).

Relationship between the Primary Tumor and the Cell Lines. The primary tumors of KYSE 520 and KYSE 960 were included in the 96 primary tumors investigated by immunohistochemical analysis. The CCR7 protein expression was very strong in the primary esophageal cancer specimen from which KYSE 960 was derived, however, no staining was detected in the specimen from which KYSE 520 was derived.

DISCUSSION

It has been proposed that molecules regulating the metastatic dissemination of tumor cells to specific anatomical sites need to fulfill the following criteria (20, 21). First, they have to be constitutively expressed at the principal sites of metastasis. Second, adhesion of target cells to the endothelium and transendothelial migration need to be promoted. Third, these molecules must be capable of mediating the invasion of cells into tissues that provide supportive microenvironments. Last, this process requires the expression of a distinct receptor repertoire by the target cells, depending on their metastatic profile. Given their well-established roles in leukocyte trafficking and homeostasis, chemokines are perfectly positioned to fulfill these criteria (22–25).

It has been demonstrated that activated dendritic cells home to lymph nodes through lymphatic vessels by a CCR7-dependent mechanism, and CCL21 and its receptor CCR7 play crucial roles in the homing of lymphocytes into the secondary lymphoid organs. A natural mutation in mice, designated plt (for paucity of lymph node T cells), which results in the loss of one of the forms of mCCL21 and disruption of the CCR7 gene, causes impaired homing of native T cells to the secondary lymphoid organs (26–28). Thus, the abundant expression of the homeostatic chemokine CCL21 in lymph nodes makes it a likely candidate to attract CCR7-positive tumor cells.

We asked whether the expression of CCR7 could play a role in esophageal lymph node metastasis. In this study, we used an immunohistochemical method to examine the CCR7 expression in esophageal SCC. Immunoreactive CCR7 was found in the cytoplasm and cell membrane of esophageal SCC cancer cells, and CCR7 protein was found mainly in the cancer cells, although it was not expressed in normal esophageal epithelium. Of more importance, the strong CCR7 protein expression observed in esophageal lymph node metastasis was consistent with findings in gastric carcinoma (11). High CCR7 expression was associated with lymphatic permeation, lymph node metastasis, tumor depth, and TNM stage. Next, we found that some esophageal SCC cell lines expressed functionally active CCR7, which triggered pseudopodia formation. Furthermore, CCR7 enhanced the motility and directional migration of the esophageal SCC cell lines in the presence of CCL21. Taken together with the distinct tissue distribution of its ligand, CCL21, which is produced constitutively by the lymphatic endothelial cells (29) and T-cell paracortical regions in the lymph node (27), these findings suggested that CCR7 may play a role in lymph node metastasis in esophageal SCC.

The role of CCR7 in metastasis has also been explored in other malignant diseases. In a study of ATLL, Hasegawa *et al.* (8) showed that ATLL cells from patients with lymph node involvement had increased expression of CCR7 and enhanced functional response to CCL21, suggesting that the expression of CCR7 may alter the trafficking patterns of ATLL cells by recruiting these cells via CCL21-enriched high endothelial venules within the lymph node. Till *et al.* (30) showed that CCR7 engagement by CCL21 and/or CCL19-stimulated chronic lymphocytic leukemia cell entry

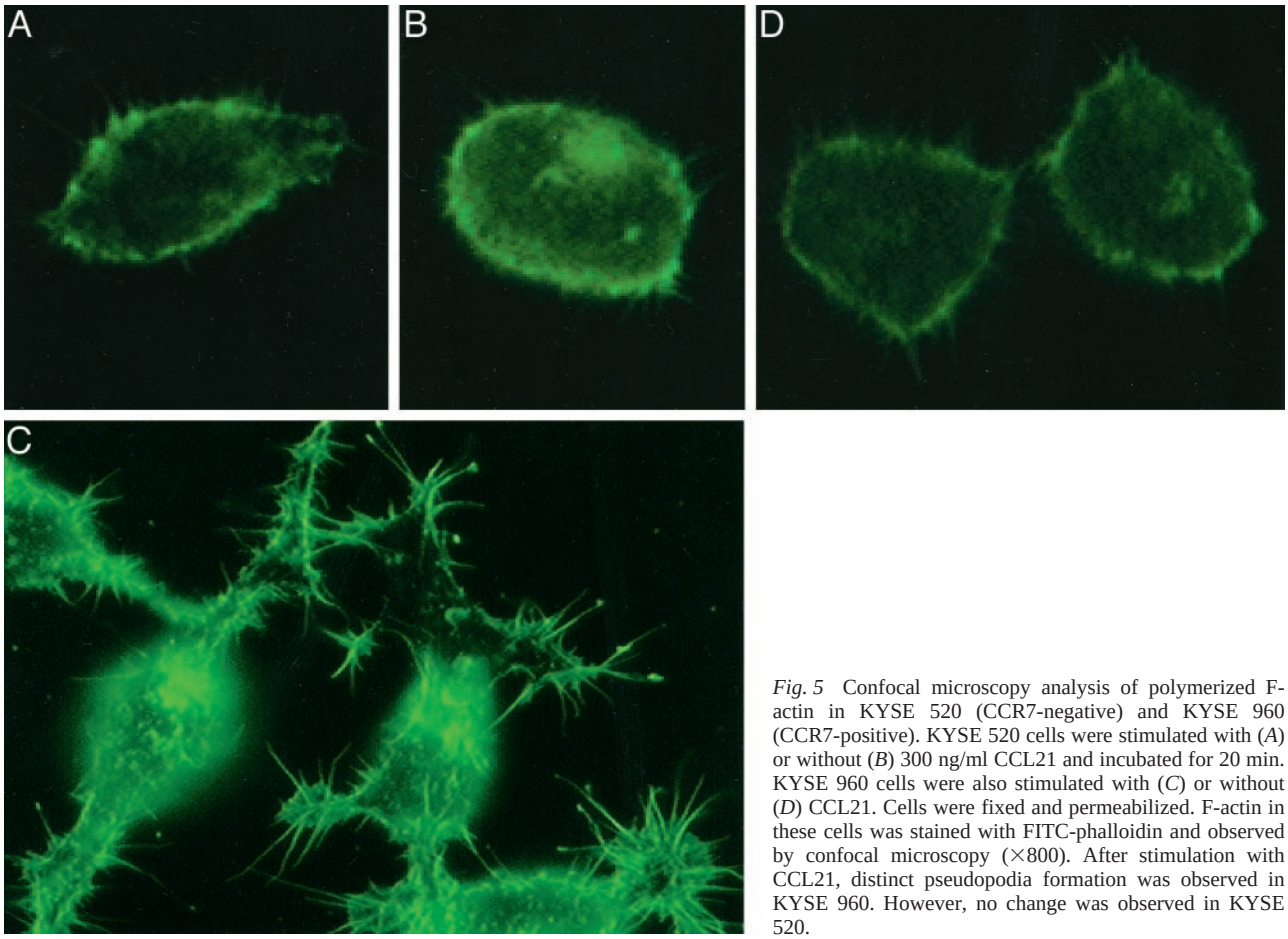

Fig. 5 Confocal microscopy analysis of polymerized F-actin in KYSE 520 (CCR7-negative) and KYSE 960 (CCR7-positive). KYSE 520 cells were stimulated with (*A*) or without (*B*) 300 ng/ml CCL21 and incubated for 20 min. KYSE 960 cells were also stimulated with (*C*) or without (*D*) CCL21. Cells were fixed and permeabilized. F-actin in these cells was stained with FITC-phalloidin and observed by confocal microscopy (×800). After stimulation with CCL21, distinct pseudopodia formation was observed in KYSE 960. However, no change was observed in KYSE 520.

into the lymph nodes. Muller *et al.* (10) showed that chemokines and their receptors such as CXCR4 and CCR7 had a critical role in determining the metastatic destination of breast cancer cells and melanoma cell lines.

Given that the rate of genetic mutations in cancer cells is much higher than that in normal cells, perhaps genetic or transcriptional changes lead to the enhanced expression of CCR7, as demonstrated by Muller *et al.* (10). On the basis of our understanding of dendritic cell migration, it is conceptually clear how CCR7 expression may work to recruit tumor cells to lymphatic channels that constitutively express CCL21 (29). After passive transport of tumor cells to the draining lymph nodes, CCR7 may help to retain malignant cells in the lymph nodes where the CCR7 ligands CCL21 and CCL19 are rich. Recently, Wiley *et al.* (12) demonstrated that the expression of a single chemokine, CCR7, by B16 murine melanoma cells increased metastasis to the regional lymph nodes. This strongly supported the hypothesis that malignant cells may use CCR7 in lymph node metastasis dissemination.

Our data suggested that CCR7/CCL21 was associated with esophageal lymph node metastasis. However, the possibility cannot be ruled out that other chemokines such as CXCR4 may be involved in esophageal lymph node metastasis because CXCL12, a ligand of CXCR4, is also abundantly expressed in the lymph nodes (10) and enhances the migration and invasion of breast cancer, melanoma, pancreatic cancer, prostate cancer, and ovarian epithelial tumors (10, 31–33). Next, it should be clarified whether CXCR4 plays a role in esophageal SCC lymph node metastasis.

There is increasing evidence that the inflammatory cells, cytokines, and chemokines found in human tumors are more likely to contribute to tumor growth, progression, and immunosuppression than they are to mount an effective antitumor response (34). Over the past 10 years, study of the cytokine and chemokine networks has led to the development of a range of antagonists for the treatment of inflammation and allergy. We suggest that such agents may also be of benefit in the treatment of malignant disease.

In summary, our investigations suggested that CCR7 could be associated with lymph node metastasis of esophageal SCC. The mechanism by which the tumor cells escape from the lymphocyte immune response in the lymph node metastasis should be further clarified.

ACKNOWLEDGMENTS

We thank Toby Cavanauga and Daniel Mrozek for proofreading the manuscript. We also thank Sakiko Shimada for technical assistance.

REFERENCES

1. Sugimachi, K., Matsuoka, H., Ohno, S., Mori, M., and Kuwano, H. Multivariate approach for assessing the prognosis of clinical oesophageal carcinoma. Br. J. Surg., *75:* 1115–1118, 1998.

2. Isono, K., Sato, H., and Nakayama, K. Results of a nationwide study on the three-field lymph node dissection of esophageal cancer. Oncology, *48:* 411–420, 1991.

3. Baggiolini, M., Dewald, B., and Moser, B. Human chemokines: an update. Annu. Rev. Immunol., *15:* 675–705, 1997.

4. Rollins, B. J. Chemokines. Blood, *90:* 909–928, 1997.

5. Kelner, G. S., Kennedy, J., Bacon, K. B., Kleyensteuber, S., Largaespada, D. A., Jenkins, N. A., Copeland, N. G., Bazan, J. F., Moore, K. W., and Schall, T. J. Lymphotactin: a cytokine that represents a new class of chemokine. Science (Wash. DC), *266:* 1395–1399, 1994.

6. Rottman, J. B. Key role of chemokines and chemokine receptors in inflammation, immunity, neoplasia, and infectious disease. Vet. Pathol., *36:* 357–367, 1999.

7. Rossi, D., and Zlotnik, A. The biology of chemokines and their receptors. Annu. Rev. Immunol., *18:* 217–242, 2000.

8. Hasegawa, H., Nomura, T., Kohno, M., Tateishi, N., Suzuki, Y., Maeda, N., Fujisawa, R., Yoshie, O., and Fujita, S. Increased chemokine receptor CCR7/EBI1 expression enhances the infiltration of lymphoid organs by adult T-cell leukemia cells. Blood, *95:* 30–38, 2000.

9. Hopke, U. E., Foss, H. D., Meyer, D., Hinz, M., Leder, K., Stein, H., and Lipp, M. Up-regulation of the chemokine receptor CCR7 in classical but not in lymphocyte predominant Hodgkin disease correlates with distinct dissemination of neoplastic cells in lymphoid organs. Blood, *99:* 1109–1116, 2002.

10. Muller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan ME., McClanahan T., Murphy E., Yuan W., Wagner, S. N., Barrera, J. L., Mohar, A., Verastegui, E., and Zlotnik, A. Involvement of chemokine receptors in breast cancer metastasis. Nature (Lond.), *410:* 50–56, 2001.

11. Mashino, K., Sadanaga, N., Yamaguchi, H., Tanaka, F., Ohta, M., Shibuta, K., Inoue, H., and Mori, M. Expression of chemokine receptor CCR7 is associated with lymph node metastasis of gastric carcinoma. Cancer Res., *62:* 2937–2941, 2002.

12. Wiley, H. E., Gonzalez, E. B., Maki, W., Wu, M. T., and Hwang, S. T. Expression of CC chemokine receptor-7 and regional lymph node metastasis of B16 murine melanoma. J. Natl. Cancer Inst. (Bethesda), *93:* 1638–1643, 2001.

13. Imamura, M., Ohishi, K., Mizutani, N., Yanagibashi, K., Naito, M., Shimada, Y., Hattori, Y., Satomura, K., and Tobe, T. Retrosternal esophagogastrostomy with EEA staple after subtotal resection of the esophagus: application and results. Dig. Surg., *4:* 101–105, 1987.

14. Sobin, L. H., and Wittekind, C., (eds.). TNM Classification of Malignant Tumors. International Union Against Cancer, Ed. 5. New York: Wiley-Liss, 1997.

15. Shimada, Y., Imamura, M., Wagata, T., Yamaguchi, N., and Tobe, T. Characterization of 21 newly established esophageal cancer cell lines. Cancer (Phila.), *69:* 277–284, 1992.

16. Krajewska, M., Krajewski, S., Epstein, J. L., Shabaik, A., Sauvageot, J., Song, K., Kitada, S., and Reed, J. C. Immunohistochemical analysis of *bcl-2*, *bax*, and *mcl-1* expression in prostate cancers. Am. J. Pathol., *148:* 1567–1576, 1996.

17. Albrecht-Buehler, G. The phagokinetic tracks of 3T3 cells. Cell, *11:* 395–404, 1977.

18. Yoshida, R., Imai, T., Hieshima, K., Kusuda, J., Baba, M., Kitaura, M., Nishimura, M., Kakizaki, M., Nomiyama, H., and Yoshie, O. Molecular cloning of a novel human CC chemokine EBI1-ligand chemokine that is a specific functional ligand for EBI1, CCR7. J. Biol. Chem., *272:* 13803–13809, 1997.

19. Baggiolini, M. Chemokines and leukocyte traffic. Nature (Lond.), *392:* 565–568, 1998.

20. Nicolson, G. L. Paracrine and autocrine growth mechanisms in tumor metastasis to specific sites with particular emphasis on brain and lung metastasis. Cancer Metastasis Rev., *12:* 325–343, 1993.

21. Wang, J. M., Deng, X., Gong, W., and Su, S. Chemokines and their role in tumor growth and metastasis. J. Immunol. Methods, *220:* 1–17, 1998.

22. Zlotnik, A., and Yoshie, O. Chemokines: a new classification system and their role in immunity. Immunity, *12:* 121–127, 2000.

23. Campbell, J. J., and Butcher, E. C. Chemokines in tissue-specific and microenvironment-specific lymphocyte homing. Curr. Opin. Immunol., *12:* 336–341, 2000.

24. Hedrick, J. A., and Zlotnik, A. Identification and characterization of a novel β chemokine containing six conserved cysteines. J. Immunol., *159:* 1589–1593, 1997.

25. Campbell, J. J., Hedrick, J., Zlotnik, A., Siani, M. A., Thompson, D. A., and Butcher, E. C. Chemokines and the arrest of lymphocytes rolling under flow conditions. Science (Wash. DC), *279:* 381–384, 1998.

26. Gunn, M. D., Kyuwa, S., Tam, C., Kakiuchi, T., Matsuzawa, A., Williams, L. T., and Nakano, H. Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. J. Exp. Med., *189:* 451–460, 1999.

27. Gunn, M. D., Tangemann, K., Tam, C., Cyster, J. G., Rosen, S. D., and Williams, L. T. A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes. Proc. Natl. Acad. Sci. USA, *95:* 258–263, 1998.

28. Vassileva, G., Soto, H., Zlotnik, A., Nakano, H., Kakiuchi, T., Hedrick, J. A., and Lira, S. A. The reduced expression of 6Ckine in the plt mouse results from the deletion of one of two 6Ckine genes. J. Exp. Med., *18:* 1183–1188, 1999.

29. Saeki, H., Moore, A. M., Brown, M. J., and Hwang, S. T. Cutting edge: secondary lymphoid-tissue chemokine (SLC) and CC chemokine receptor 7 (CCR7) participate in the emigration pathway of mature dendritic cells from the skin to regional lymph nodes. J. Immunol., *162:* 2472–2475, 1999.

30. Till, K. J., Lin, K., Zuzel, M., and Cawley, J. C. The chemokine receptor CCR7 and α4 integrin are important for migration of chronic lymphocytic leukemia cells into lymph nodes. Blood, *99:* 2977–2984, 2002.

31. Koshiba, T., Hosotani, R., Miyamoto, Y., Ida, J., Tsuji, S., Nakajima, S., Kawaguchi, M., Kobayashi, H., Doi, R., Hori, T., Fujii, N., and Imamura, M. Expression of stromal cell-derived factor 1 and CXCR4 ligand receptor system in pancreatic cancer: a possible role for tumor progression. Clin. Cancer Res., *6:* 3530–3535, 2000.

32. Taichman, R. S., Cooper, C., Keller, E. T., Pienta, K. J., Taichman, N. S., and McCauley, L. K. Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. Cancer Res., *62:* 1832–1837, 2002.

33. Scotton, C. J., Wilson, J. L., Milliken, D., Stamp, G., and Balkwill, F. R. Epithelial cancer cell migration: a role for chemokine receptors? Cancer Res., *61:* 4961–4965, 2001.

34. Balkwill, F., and Mantovani, A. Inflammation and cancer: back to Virchow. Lancet, *357:* 539–545, 2001.