CCL21 promotes the migration and adhesion of highly lymph node metastatic human non-small cell lung cancer Lu-99 *in vitro*

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Abstract. To develop new therapy strategies for lung cancer, we established an animal model, which reflects the clinical features of mediastinal lymph node metastasis of lung cancer. This study was designed to determine whether CCL21 induced biological functions associated with the metastasis of highly lymph node metastatic human non-small cell lung cancer (NSCLC) selected by our model. Orthotopic intrapulmonary implantation of human NSCLC (Lu-99 and A549) was performed to analyze the metastatic characteristics of these cells. The expression of CCR7, which is a receptor of CCL21, was detected using CCL19 [also called EBI1-ligand chemokine (ELC)]-Fc chimera by flow cytometric analysis. The effects of CCL21 on the migration, adhesion and growth of human NSCLC were investigated. After orthotopic implantation of human NSCLC cell lines, Lu-99, but not A549, metastasized to mediastinal lymph nodes, forming large size nodules, and expressed CCR7 on the surface. Accordingly, its ligand CCL21 induced chemotactic migration and α4ß1-mediated adhesion to VCAM-1 of Lu-99. The expression of CCR7 and vigorous responses to its ligand CCL21 potentially account for lymph node metastasis of a human NSCLC line Lu-99.

Introduction

Human lung cancer, basically classified into non-small cell lung cancer (NSCLC) and small-cell lung cancer (SCLC), preferentially metastasizes to the brain, bone and lymph nodes. Human NSCLC is the major group in lung cancer histology. According to the new TNM revisions, the 5-year survival rate of patients with N2 lung cancer does not exceed 20% even with curative resection of the primary tumor (1,2). However, the mechanism of lymph node metastasis, which is a serious risk factor for the majority of cancer patients, remains to be fully addressed (1-3). Therefore, a suitable animal model that conforms to the clinical features of lung cancer is essential to clarify the mechanism of lymph node metastasis of lung cancer and to develop a new therapeutical strategy for lung cancer (4). Previously, we established a lymph node metastasis model of lung cancer using the orthotopic intrapulmonary implantation of human NSCLC (5-8).

It is now considered that chemokines play a significant role in organ-selective cancer metastasis because tumor cell migration and metastasis share many similarities with leukocyte trafficking (9-12). Chemokines are a family of small cytokines that primarily induce directed migration of hematopoietic cells through interactions with a group of seven transmembranes, G protein-coupled receptors (13,14). Chemokine receptors CXCR4 and CCR7 are highly expressed in human breast cancer cells, and their respective ligands CXCL12 (also called stromal cell-derived factor 1α) and CCL21 (also called secondary lymphoid tissue chemokine) play a critical role in determining the metastatic destination of breast cancer (9).

Here we report that NSCLC cells highly metastatic to lymph nodes in this model express CCR7 and respond to its ligand CCL21, which is known to be highly expressed in lymph nodes, in chemotactic migration and α4ß1 integrin-mediated adhesion to vascular cell adhesion molecule-1 (VCAM-1). Thus, the migration and adhesion induced by CCL21 may explain in part the preferential lymph node metastasis of CCR7-expresssing NSCLC.

Materials and methods

Cell lines and animals. Human NSCLC, Lu-99 and A549, were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). These cell lines were maintained as monolayer cultures in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and L-glutamine in an atmosphere containing 5% CO_2/air at 37˚C. Six-week-old male KSN mice were purchased from SLC, Inc. (Sizuoka, Japan). The animals were maintained in the Laboratory for Animal Experiments,

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Institute of Natural Medicine, University of Toyama. This study was conducted in accordance with the standards established by the Guidelines for the Care and Use of Laboratory Animals of the University of Toyama.

Intrapulmonary implantation procedure. Orthotopic implantation of human NSCLC cells into the lung was performed as described previously (7). Briefly, Lu-99 and A549 cells ($5x10^7$/ml) were suspended in phosphate-buffered saline (PBS; calcium- and magnesium-free) containing 1 mg/ml of Matrigel (Collaborative Biomedical Products, MA, USA). The left chests of anesthetized mice were incised and 20-μl aliquots of cell suspension ($1x10^6$ cells, 20 μg Matrigel) were injected into the lung parenchyma through the intercostal space (~3 mm depth). The skin incision was closed with Autoclips (Becton-Dickinson Co., MD, USA).

Macroscopic findings of orthotopic and metastatic cancer formation. The mice injected with Lu-99 (n=5) and A549 (n=5) were sacrificed on day 60. Formation of the primary tumor at the implantation site and the metastatic features of the two cell lines were investigated. The primary tumor and metastatic site, which was the mediastinal lymph node, were weighed.

Reverse transcriptase-polymerase chain reaction (RT-PCR) for CCR7 expression. Total-RNA of Lu-99 and A549 cells was isolated using RNA extracted using an RNeasy Mini kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's directions, and reverse-transcribed to produce complementary DNA using oligo $(dT)_{18}$ primer and SuperScript II reverse transcriptase (Invitrogen Corp., CA, USA). PCR was performed by denaturation (94˚C for 30 sec), annealing (64˚C for 1 min) and extension (72˚C for 1 min 30 sec) using a Takara ExTaq™ PCR kit (Takara Shuzo Co., Ltd., Kyoto, Japan). The following human CCR7 primer sequences were used in PCR amplifications: 5'GTGCCCGCGTCCTTCTCATCAG3' (forward) and 5'GGCCAGGACCACCCCATTGTAG3' (reverse). PCR products were electrophoresed on 1.5% agarose gels and detected by ethidium bromide staining.

Flow cytometry. The following anti-human integrin antibodies were added into Lu-99 and A549 as the primary antibody: anti-ß1 antibody (Beckman Coulter, Inc., FL, USA), anti-α4 (Seikagaku Corp., Tokyo, Japan), anti-ß7 antibody (Santa Cruz Biotechnology, Inc., CA, USA), anti-ß2 (Immunotech, Marseille Cedex, France) and anti-α_L antibody (Nichirei Corp., Tokyo, Japan). After incubation with human integrin antibody, these cells were washed three times with PBS, and then stained with FITC-labeled goat anti-mouse immunogloblin (DakoCytomation, Kyoto, Japan) as the second antibody. To stain for CCR7, Lu-99 or A549 were incubated with ELC-Ig (kindly provided by Dr O. Yoshie, Kinki University School of Medicine, Japan) followed by goat anti-human Ig biotin and streptavidin PE. PE-labeled cells were then analyzed by flow cytometric analysis using FACSCalibur (Becton-Dickinson, CA, USA).

Cell migration assay. The migration of tumor cells was assayed in Transwell cell culture chambers (Costar, Cambridge, MA, USA) as described previously (11,12). The filters (8.0 μm pore size, Nucleopore, Pleasanton, CA, USA) were pre-coated with 125 ng of fibronectin (Iwaki Glass, Tokyo, Japan) on the lower surfaces and dried at room temperature. Lu-99 ($1x10^5$ cells/100 μl) and A549 ($2x10^5$ cells/100 μl) were added to the upper chamber and CCL21 (0-10 nM) (Pepro Tech EC, London, UK) was added to the chamber. After 1 h for Lu-99 or 2 h for A549 at 37˚C, the filters were fixed with methanol and stained with hematoxylin and eosin. Cells on the upper surface of the filters were removed by wiping with cotton swabs. Cells that had migrated to various areas of the lower surface were counted under a microscope at x400 magnification. In some experiments, Lu-99 cells were pre-incubated with various concentrations of pertussis toxin (PTX, List Biological Laboratories, Inc., CA, USA) in RPMI/10% FBS for 2 h prior to the migration assay.

Cytotoxicity assay. Lu-99 and A549 ($1x10^4$/well) were seeded into 96-well plates (Falcon, Becton-Dickinson Co., NJ, USA). After 12 h of pre-incubation, cells were cultured with various concentrations of PTX in RPMI-1640 supplemented with 10% FBS and L-glutamine for 24 h. WST-1 solution (10 μl/well, WST-1 Cell Counting Kit; Wako Pure Chemicals, Osaka, Japan) was added to each well for 2 h before the end of the incubation. Activity was assessed by measuring the absorbance at 450 nm using an immunoplate reader.

Cell adhesion assay. Triplicate wells of 96-well plates were coated with recombinant human VCAM-1 (rhVCAM-1, 500 ng/well, Genzyme Techne, MN, USA) overnight at room temperature. To immobilize CCL21 with rhVCAM-1, 75 μl of CCL21 (0.1-10 nM) was added to the well for 1 h at 37˚C. Lu-99 and A549 ($2x10^4$ cells/100 μl) were pre-incubated in the presence of CCL21 (0.1-100 nM) and anti-α4 (400 nM, clone SG/73, Seikagaku Corp.), anti-ß1 antibody (400 nM, clone 4B4, Beckman Coulter, Inc.) and control mouse IgG1 (DakoCytomation) for 30 min at 37˚C and added to CCL21-immobilized wells for 15 min at 37˚C. After washing, the fluorescence intensity of the lysates of the attached cells to rhVCAM-1 made by the addition of 0.1% Tween-20 in PBS containing Pico Green (Molecular Probes, Inc., Eugene, OR) was measured at 485/535 nm. For PTX inhibition studies, cells were pre-treated with PTX (400 nM) for 2 h at 37˚C before adhesion assay.

Statistical analysis. The statistical significance of differences between the groups was determined by applying Student's two tailed t-test. Results are presented as the mean ± SD. A p-value <0.05 was considered significant.

Results

Lymph node metastasis of human NSCLC. Human NSCLC cell lines Lu-99 and A549 were orthotopically implanted into the lungs of KSN athymic nude mice according to our previous method (7). Both cells showed similar tumorigenicity as observed by the comparison of pulmonary weights at the injection site on day 60 after implantation (data not shown). Distinct metastatic activity was, however, observed between Lu-99 and A549: A549 did not cause metastasis, while Lu-99

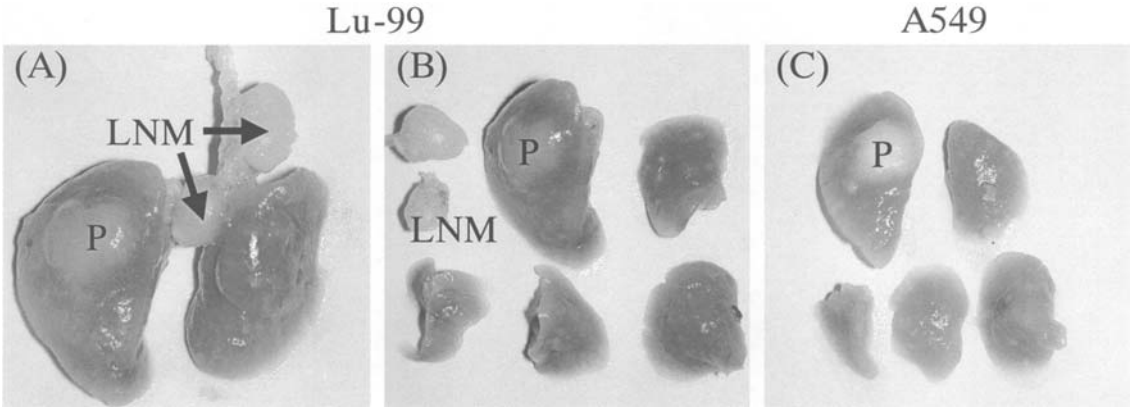

Figure 1. Macroscopic findings of primary cancer and mediastinal lymph node metastasis of human NSCLC. Autopsy of mice was performed on day 60 after orthotopic implantation of Lu-99 (A and B) and A549 (C). Primary cancer at the inoculation site (P). Arrows indicate mediastinal lymph node metastasis (LNM).

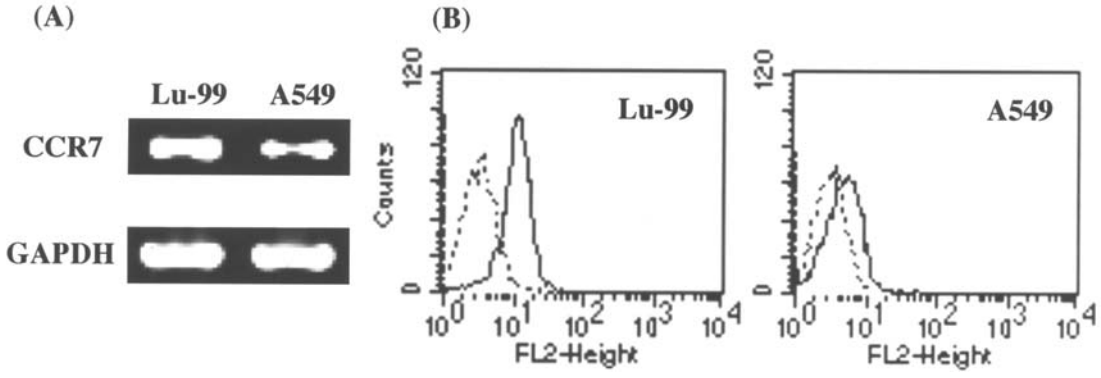

Figure 2. The expression of chemokine receptor in human NSCLC. (A) PCR was performed using specific primers for CCR7. The mRNA-derived PCR products for CCR7 were amplified for 35 cycles. The PCR products were separated on 1.5% agarose gels and detected by ethidium bromide staining. (B) To stain for CCR7, Lu-99 or A549 was incubated with ELC-Ig followed by goat anti-human Ig biotin and streptavidin PE. PE-labeled cells were analyzed using FACSCalibur flow cytometer (dashed lines, isotype control; full lines, human CCR7-specific staining).

developed lymph node metastasis, generating a large mass in the mediastinal lymph node (Fig. 1).

Expression of CCR7 by human NSCLC. To understand the differential metastasis of Lu-99 and A549, we compared their expression of CCR7 by RT-PCR and flow cytometry (Fig. 2). In both RT-PCR (A) and flow cytometry (B), Lu-99 expressed CCR7 at higher levels than A549.

CCL21-induced migration of Lu-99. We next examined the migratory responses of Lu-99 to the CCR7 ligand CCL21 in a chemotaxis assay. As shown in Fig. 3A, CCL21 significantly enhanced the migration of Lu-99 cells into lower chambers but was ineffective for cells pretreated with PTX, which is a specific inhibitor of G protein-coupled receptor. No cytotoxic effect was observed in the 24-h incubation of Lu-99 with PTX at this concentration (data not shown). In sharp contrast, CCL21 induced no migration in A549 even at 10 nM.

CCL21-induced adhesion of Lu-99 to VCAM-1. Next, we examined CCL21-induced activation of integrins in lung cancer cells, first assessing the surface expression of integrins on

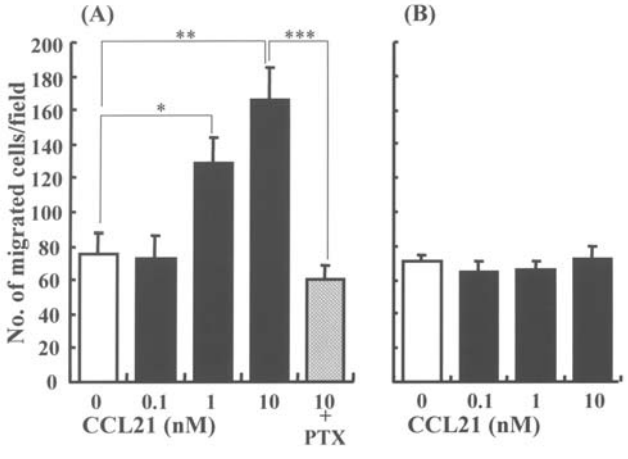

Figure 3. CCL21-induced migration of lymph node metastatic human NSCLC. Lu-99 (A) and A549 (B) were seeded onto filters pre-coated with fibronectin on the lower surface and CCL21 was added to the lower compartment of Transwell chambers. After 2-h incubation, the cells that had migrated to the lower surface were visually counted. For inhibition studies, Lu-99 were pre-treated with PTX before the assay. Data are expressed as the mean ± SD of a group of triplicate cultures. $^{*}p<0.05$, $^{**}p<0.01$, compared with the CCL21-untreated control, $^{***}p<0.002$, compared with 10 nM CCL21-treated culture by the Student's two-tailed t-test.

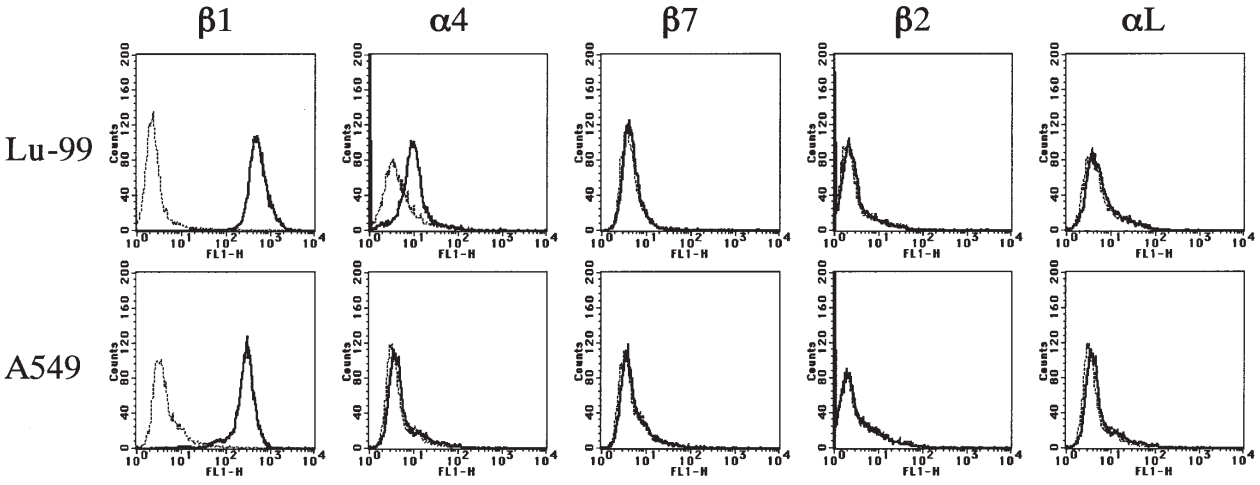

Figure 4. FACS analysis of integrins expressed on lymph node metastatic human NSCLC and non-lymph node metastatic human NSCLC. Lu-99 and A549 were incubated with antibodies (anti-α4, anti-α_L, anti-ß1, anti-ß2 and anti-ß7 integrin) followed by FITC-labeled anti-mouse IgG. FITC-labeled cells were analyzed using FACSCalibur flow cytometer (dashed lines, isotype control; full lines, ß1, α4, ß7, α_L and ß2 integrin-specific staining).

Lu-99 and A549. As shown in Fig. 4, Lu-99 expressed α4ß1 integrin, whereas A549 only expressed the ß1 subunit. Therefore, we examined whether CCL21 could induce the adhesion of Lu-99 to rhVCAM-1, and this was found to significantly enhance the adhesion of Lu-99 to rhVCAM-1 by CCL21, as shown in Fig. 5A; futhermore, this adhesion was effectively blocked by PTX, anti-α4, and anti-ß1 (Fig. 5B). Thus, CCL21 induced the adhesion of Lu-99 to rhVAMC-1 via activation of the α4ß1 integrin through a PTX-sensitive G protein-coupled receptor.

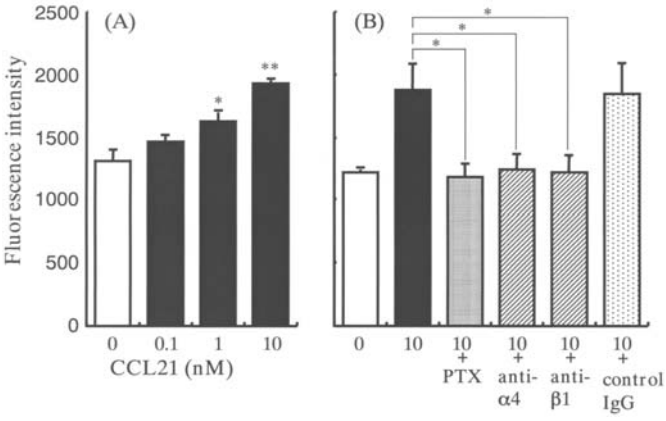

Figure 5. Effect of CCL21 on the adhesion of lymph node metastatic human NSCLC to VCAM-1 and inhibition of CCL21-induced adhesion by PTX. (A) Lu-99 were pre-incubated with CCL21 for 30 min and added to CCL21-immobilized wells (pre-coated with rhVCAM-1) for 15 min at 37˚C. Cells attached to rhVCAM-1 were measured by fluorescence intensity (485/535 nm). Data are expressed as the mean ± SD of a group of quadruplicate cultures. $^{*}p<0.05$, $^{**}p<0.01$, compared with the CCL21-untreated control by Student's two-tailed t-test. (B) Lu-99 were pre-incubated with or without CCL21 for 30 min and anti-α4 and anti-ß1 antibody. For PTX inhibition studies, cells were pre-incubated with PTX before the assay. Data are expressed as the mean ± SD of a group of quadruplicate cultures. $^{*}p<0.05$, compared with the 10 nM CCL21-treated culture by Student's two-tailed t-test.

Discussion

The critical problem in lung cancer therapy is lymphatic metastasis (1-3). Although several experimental models of human lung cancer have been designed, these models have potential shortcomings such as ectopic implantation. Recently, we have developed a lung cancer model involving the orthotopic intrapulmonary implantation of human NSCLC in nude mice (5). Our model is a superior model of human NSCLC because of the formation of a solitary pulmonary cancer and spontaneous lymph node metastasis. Using this model, we demonstrated two human NSCLC lines, Lu-99 and A549, as lymph node metastatic and non-metastatic cells, respectively (Fig. 1).

It is now known that molecules such as adhesion molecules and growth factors play important roles in the hematogenous metastasis of cancer; however, there are few reports on the molecules critically involved in lymph node metastasis (3). These two cell lines therefore provided an excellent opportunity to identify the molecular mechanisms involved in lymph node metastasis. Here we demonstrated that highly lymph node metastatic human NSCLC, Lu-99, but not A549, expressed CCR7 at high levels on the surface and responded to its ligand CCL21 in chemotaxis (Figs. 2 and 3) (15,16).

Several papers reported that chemokines promoted the migration potency of cancer. Muller *et al* described a landmark finding that CXCL12 induced human breast cancer migration in an *in vitro* study, and that lung metastasis was suppressed by the administration of neutralizing anti-human CXCL12 receptor, CXCR4 antibody, in a mouse model (9).

Two studies concerning the association of CCR7 and lymph node metastasis of cancer have been reported. Although CCR7-overexpressing murine melanoma cells (CCR7-B16 melanoma cells) enhanced lymph node metastasis compared to wild-type cells in a mouse model (17), the CCL21-induced migration of CCR7-B16 melanoma cells was not shown in *in vitro* study. In clinicopathological features, CCR7 expression in gastric cancer was associated with lymph node metastasis and poor prognosis was confirmed in an immunohistochemical study. Furthermore, CCL21 was shown to induce biological functions

in vitro such as the induction of calcium flux, actin polymerization and migration, in several gastric cancer cell lines (18). However, the metastatic behavior of these cell lines has not been investigated in animal models.

In the present study, we demonstrated CCL21-induced migration *in vitro* and the metastatic behavior of human NSCLC in an animal model.

We also showed that CCL21 promotes the adhesion of Lu-99 via integrin activation (Fig. 5). Organ selectivity of cancer metastasis is regulated not only by cancer migration but also its adhesion at the target organs of cancer metastasis (19,20).

Integrins play roles in each step of the metastatic cascade, and sustain cell survival and interaction in target organs (21). In metastatic lymph nodes, α4ß1 integrin was strongly expressed in cancer. These data show that α4ß1 integrin plays an important role, but the mechanism of the interactions between lung cancer and its host remains unclear (22).

Chemokines regulate not only migration but also the adhesion of several cells. The activation of integrins is also induced by chemokines, and subsequent cell adhesion occurrs through interactions between integrin and its counterpart molecules (13,14). For example, intercellular adhesion molecule-1 (ICAM-1), VCAM-1 and mucosal addressin cellular adhesion molecule-1 (MadCAM-1) are expressed by several cells as cell adhesion molecules of lymphocytes, and α_Lß2, α4ß1 and α4ß7 integrin are identified as the respective counterparts of these molecules. It has been reported that this integrin-mediated adhesion of lymphocytes was induced by CCL21 and that the adhesion regulated the migration of lymphocytes to the lymph node (23,24).

There are a few reports that CXCL12 triggers cancer cell adhesion via integrin; however, there is no report about the relationship between CCL21 and cancer cell adhesion.

In fact, Lu-99 expressed α4ß1 integrin but not α_Lß2 and α4ß7. In contrast, A549 did not express these integrins (Fig. 4). We also confirmed that CCL21-mediated α4ß1 integrin activation promoted the adhesion of Lu-99 to VCAM-1 (Fig. 5).

Given that CCL21 is expressed constitutively in the endothelium of lymphatic vessels, and high endothelial venules (HEV) and stromal cells in parafollicular T cell zone of lymph nodes (23,24), Lu-99 transplanted into the lung might be guided and adhere to lymph nodes by CCL21.

The expression of CCR7 mRNA in NSCLC is shown to be an independent predictor of lymph node metastasis by multivariate analysis in clinical study (25). Recently, the combined use of all three biomarkers (CXCR4, CCR7 and HER2-neu) was reported to further progress the prediction of lymph node metastasis, although the chemokine receptor CCR7 was reported to be a novel prediction biomarker of metastasis in breast cancer (26). Therefore, future study including the immunohistochemical analysis of both CCR7 and integrin might be useful to predict lymph node metastasis in NSCLC.

In conclusion, we here showed for the first time that the interaction with CCL21 could multiply the effect of the tumor metastasis, i.e. migration and adhesion through α4ß1 integrin in association with lymph node metastasis of human highly lymph node metastatic NSCLC. Therefore, the present study suggests that CCR7 and α4ß1 integrin might be targeting molecules for human NSCLC therapy of metastasis to lymph nodes.

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