# Adenoviral Transduction of *MRP-1/CD9* and *KAI1/CD82* Inhibits Lymph Node Metastasis in Orthotopic Lung Cancer Model

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## Abstract

Conventional therapies still remain less effective for metastasis of lung cancer, thus leading to a poor prognosis for this disorder. Although the processes involved in metastasis have not yet been clearly elucidated, our previous studies have shown that higher expression levels of MRP-1/CD9 and KAI1/CD82 in cancer cells are significantly correlated with less metastatic potency. To determine whether the gene transfer of these tetraspanins into lung tumor cells may be a useful strategy to regulate metastasis, we adopted an orthotopic lung cancer model produced by the intrapulmonary implantation of Lewis lung carcinoma (LLC) cells and evaluated the metastatic growth in the mediastinal lymph nodes using two different methods of gene delivery as follows: (*a*) the implantation of LLC cells preinfected with adenovirus encoding either *MRP-1/CD9* cDNA, *KAI1/CD82* cDNA, or *LacZ* gene into the mouse lung and (*b*) the intratracheal administration of these adenoviruses into the mice orthotopically preimplanted with LLC cells. In both cases, we found that the delivery of either *MRP-1/CD9* or *KAI1/CD82* cDNA dramatically reduced the metastases to the mediastinal lymph nodes in comparison with those of *LacZ* gene delivery, without affecting the primary tumor growth at the implanted site. These results reemphasize the important role of MRP-1/CD9 and KAI1/CD82 in the suppression of the metastatic process and also show the feasibility of gene therapy when using these tetraspanins for lung cancer to prevent metastasis to the regional lymph nodes. This strategy may therefore be clinically applicable as a prophylactic treatment to suppress the occurrence of lymph node metastasis. [Cancer Res 2007; 67(4):1744–9]

## Introduction

Due to the high probability of lymphatic and hematogenic metastases, patients with lung cancer apparently show a poorer prognosis in comparison with other malignancies. Particularly, metastases to lymph nodes are frequently observed in lung cancer even when the primary tumor is small, thus making a complete resection of the tumor extremely difficult. Metastasis of tumor cells consists of several complicated processes, including cell detachment from the primary tumor followed by invasion, intravasation into a vessel, circulation, stasis within a vessel,

extravasation, invasion of the recipient tissue bed, and ultimately proliferation (1–3). At the initiation of the metastatic process, dysregulated cell motility resulting in the invasiveness of tumor cells, undoubtedly, plays a crucial role (4, 5). Among the molecules involved in cell motility, tetraspanins MRP-1/CD9 and KAI1/CD82 have been identified as suppressors of tumor spread (6, 7). The expression of these tetraspanins is observed in almost all normal tissues, and these molecules are believed to mediate migration signals through forming membrane complexes with integrin receptors (8, 9). Supporting these observations, numerous previous studies have shown a reduced expression of either MRP-1/CD9 or KAI1/CD82 in various tumors to correlate with the presence of distant metastasis and a poorer prognosis (10–13). These findings strongly suggest that MRP-1/CD9 and KAI1/CD82 therefore play indispensable roles in the progression of malignant tumors (13–17).

Although the clinical importance of MRP-1/CD9 and KAI1/CD82 on the diagnosis, staging, and prognosis of malignant tumors, including lung cancer, has been established, the therapeutic application of these tetraspanins has not yet been intensively explored (18). Previous studies from our group and others have shown that lung metastases of i.v. injected murine melanoma cells transduced with *MRP-1/CD9* or *KAI1/CD82* cDNA were significantly reduced in comparison with those of parental cells (19, 20). In addition, we have shown that the adenoviral transduction of the *MRP-1/CD9* gene into the primary tumor, which was established by the inoculation of murine melanoma cells into the mouse foot pad, resulted in a significant reduction in the number of lung metastases in comparison with the intratumor injection of control adenovirus (21). These observations clearly indicate that the delivery of *MRP-1/CD9* and *KAI1/CD82* cDNAs into malignant tumors can reduce their metastatic potency, thus raising the question about whether it can be applied for the suppression of metastasis from a primary lung tumor. For the purpose of further investigation, we adopted an orthotopic lung tumor model that was produced by the implantation of Lewis lung carcinoma (LLC) cells into the lung of C56/BL-6 mouse, which subsequently developed huge metastases to the mediastinal lymph nodes. Initially, we intended to see whether the adenoviral delivery of *MRP-1/CD9* or *KAI1/CD82* cDNA into LLC cells before the implantation affected the metastatic growth of the mediastinal lymph nodes. Thereafter, the intratracheal administration of adenovirus encoding *MRP-1/CD9* or *KAI1/CD82* cDNA was attempted after the orthotopic implantation of LLC cells. In both cases, the delivery of either *MRP-1/CD9* or *KAI1/CD82* cDNA dramatically reduced the degree of metastasis to the mediastinal lymph nodes in comparison with that of the control. These data strongly suggest the possibility to prevent lymph node metastasis from primary lung cancer by means of the prophylactic gene transfer of these tetraspanins.

## Materials and Methods

**Cell lines and animals.** LLC, a murine non–small cell lung carcinoma (NSCLC) cell line, was maintained in DMEM supplemented with 10% FCS. C57/BL6 mice were purchased from Nippon Clea (Shizuoka, Japan) and kept under laminar airflow conditions. Six- to 8-week-old male mice weighing 23 to 25 g were used to reduce the variability.

**Recombinant adenoviruses.** E1-deleted replication-deficient recombinant adenoviruses encoding human *MRP-1/CD9* cDNA (*rAd-MRP-1/CD9*), *KAI1/CD82* cDNA (*rAd-KAI1/CD82*), and *Escherichia coli LacZ* gene (*rAd-LacZ*) under the control of the human cytomegalovirus immediate early promoter were generated by the usage of an Adenovirus Expression Vector kit (TaKaRa Biomedicals, Otsu, Japan) following the manufacturer's instructions. The production and amplification of the adenoviruses were conducted using the 293 human embryonic kidney cell line. The purification and concentration of adenoviral vectors were carried out using an Adeno-X Virus Purification kit (BD Bioscience Clontech, San Jose, CA) and Centricon Centrifugal Filter Devices (Millipore Corp., Bedford, MA) according to the manufacturers' instructions.

***In vitro*** **transduction of LLC cells with recombinant adenoviruses.** Seventy percent of the confluent LLC cells in a 10-cm plate were infected with $2.0 \times 10^{11}$ particles of adenoviral vector suspended in 150 µL PBS for 60 min at 37°C, and then the adenoviral suspension was replaced with serum-free medium. Twenty-four hours after infection, the cells were suspended in PBS and then examined by flow cytometry [fluorescence-activated cell sorting (FACS)] to detect cell surface MRP-1/CD9 and KAI1/CD82 or underwent a quantitative analysis of the β-galactosidase (β-gal)

expression. The detection of cell surface MRP-1/CD9 and KAI1/CD82 was accomplished by FACS analysis (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ) using M31-15, anti–MRP-1/CD9 monoclonal antibody (mAb; ref. 6), and C33, anti-KAI1/CD82 mAb (22), as primary antibodies (kindly provided by Shionogi Corp., Osaka, Japan) and FITC-conjugated anti-mouse IgG (Sigma, St. Louis, MO) as a secondary antibody. Quantitative analysis of β-gal was done using High Sensitivity β-Galactosidase Assay kit (Stratagene, La Jolla, CA) following the manufacturer's instruction.

**Orthotopic lung cancer model in mice.** Intrapulmonary implantation of LLC cells was done as described previously with some modification (23). Briefly, after anesthetized with i.p. injection of pentobarbital, a 5-mm skin incision was made at ~1 mm tail side from the scapula. Putting fat and muscle aside to observe left lung movement through the pleura, a 30-gauge needle attached to a 0.5 mL insulin syringe was directly inserted through the intercostal space into the left lung at a depth of 4 mm. Cell suspension (20 µL) that contains $6 \times 10^3$ cells and 10 µg Matrigel was injected into the lung parenchyma.

**Treatment of tumor-bearing mice with adenoviral vectors.** In the preliminary study, the control mice had already micrometastases in mediastinal lymph nodes at day 7 and clear metastases at day 11 after the tumor implantation. Therefore, we decided the first dose of the virus was given only 3 days after the tumor implantation (23). Adenoviral vectors were given intratracheally on days 3, 6, and 9 after the intrapulmonary implantation of LLC cells. To enhance the transgene expression in the lung, each adenoviral vector was complexed with DEAE-dextran as described previously with some slight modifications (24, 25). Briefly, $1 \times 10^{10}$ particles of adenoviral vector were suspended in 50 µL PBS containing 40 µg of
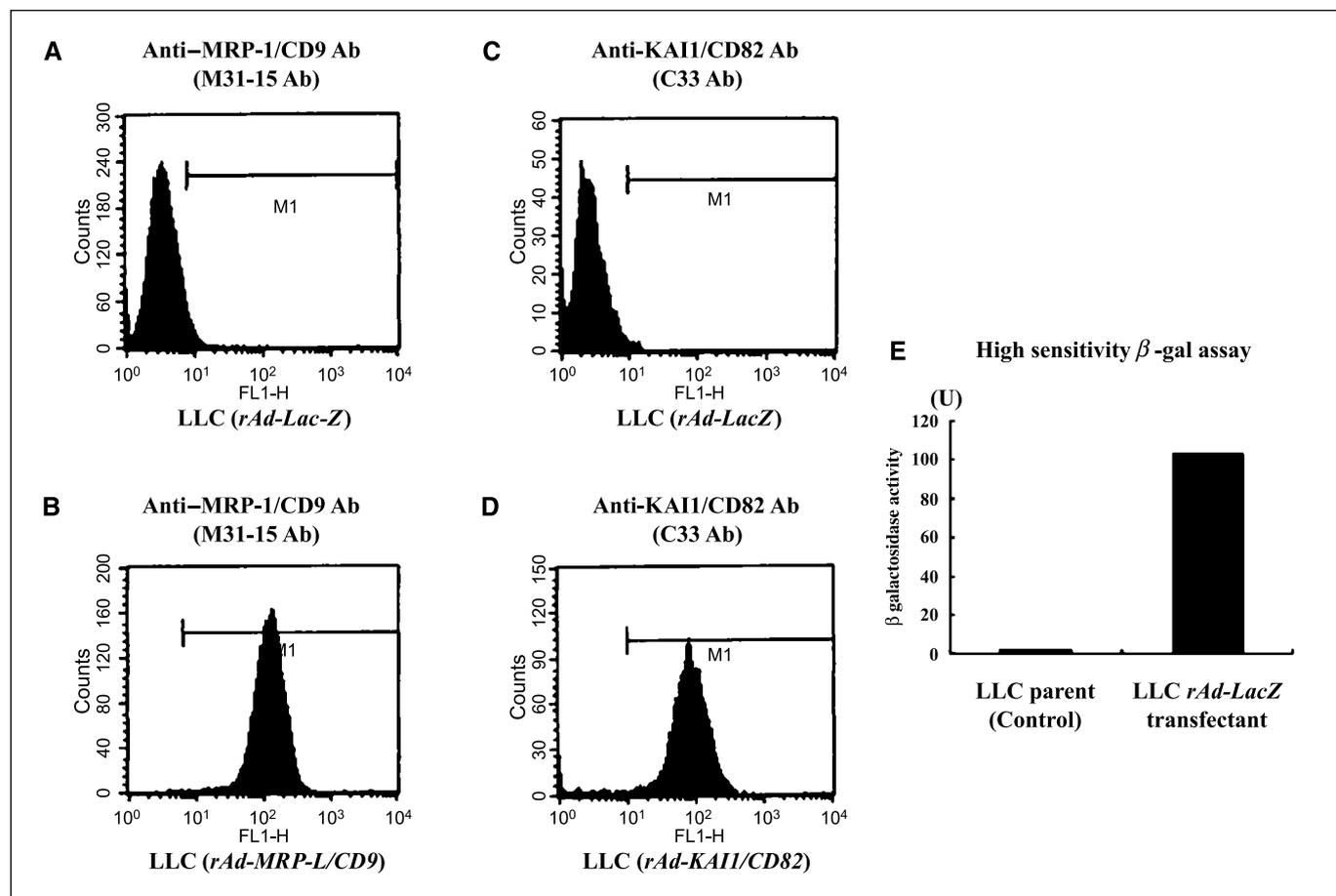


**Figure 1.** Adenoviral transduction of LLC cells with *MRP-1/CD9, KAI1/CD82,* and *LacZ.* Flow cytometric analysis showed that LLC cells were negatively stained with anti–MRP-1/CD9 mAb M31-15 (*A*) and anti-KAI1/CD82 mAb C33 (*C*). After infected with adenoviruses encoding *MRP-1/CD9* and *KAI1/CD82* cDNA, LLC cells turned positive for MRP-1/CD9 (*B*) and KAI1/CD82 (*D*), respectively. β-Gal activity was measured from both untreated LLC cells and LLC cells infected with adenovirus encoding *LacZ* gene. *E,* significantly high activity of β-gal was detected in *LacZ*-transduced LLC cells, confirming good transduction efficiency of adenoviral vectors.
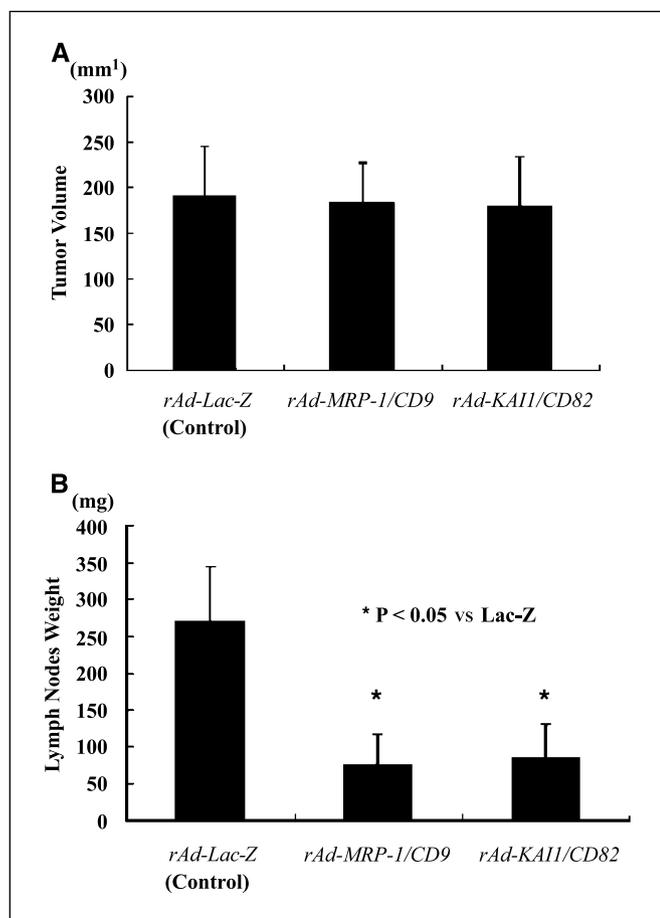
**Figure 2.** Tumor volumes and weights of mediastinal lymph nodes resected from the mice implanted with LLC cells preinfected with *rAd-MRP-1/CD9*, *rAd-KAI1/CD82*, and *rAd-LacZ* were investigated (*n* = 10 in each group) on day 21 after the implantation of LLC cells (6 × 10³/left lung). LLC cells were adenovirally transfected with *MRP-1/CD9, KAI1/CD82*, and *LacZ* before the implantation, which has already been shown in Fig. 1. *A*, about the primary tumor volumes of LLC cells, there was no significant difference among β-gal transfectant group, *MRP-1/CD9*, and *KAI1/CD82* group. *B*, on the other hand, mediastinal lymph node weights of LLC cells transfected with *MRP-1/CD9* and *KAI1/CD82* were significantly lighter than both those of control group and *LacZ* transfectant group, which strongly suggests that the transduction of these tetraspanins into LLC cells has reduced the lymph node metastasis. *Bars,* SD.

DEAE-dextran and then incubated for 10 min at room temperature. After the trachea of mouse anesthetized with i.p. pentobarbital was exposed, 50 μL of adenoviral solution with DEAE-dextran were instilled directly into the trachea through a 30-gauge needle.

**Macroscopic assessments of primary lung tumor and mediastinal lymph node metastasis.** The mice were sacrificed by exposure to carbon dioxide on day 21 after the implantation of LLC cells, and lungs, heart, and mediastinal tissues were excised en bloc after the lungs were filled with 1 mL of 10% formalin. The mediastinal lymph nodes were removed and their weights were measured immediately. The long and short diameters of the primary tumor were measured, and its volume was calculated by the following formula: tumor volume (mm³) = 1/2 × (long diameter) × (short diameter)².

**Assessment of transgene expression in inoculated tumor cells.** To confirm the adenovirus-mediated expression of MRP-1/CD9 and KAI1/CD82 in the inoculated tumor cells, mice were sacrificed on day 10 after the implantation of LLC cells (i.e., 1 day after the last administration of adenoviral vectors). All whole lung tissues were excised and then embedded and frozen in OCT compound. Frozen sections were prepared, and the detection of MRP-1/CD9 and KAI1/CD82 was done as described previously

(16). Briefly, the sections were immersed for 30 min in methanol with 0.3% $H_2O_2$ to block the endogenous peroxidase activity and then treated with 5% bovine serum albumin. Sections were incubated for 120 min with M31-15 and C33 mAbs followed by reaction with biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) and avidin-biotin-peroxidase complex (Vector Laboratories). The bondage of antibodies was visualized with 3,3′-diaminobenzidine tetrahydrochloride in the presence of 0.05% $H_2O_2$. After these treatments, the sections were counterstained with hematoxylin, dehydrated, and then mounted.

**Statistical analysis.** The data are shown as the mean ± SE. Mann-Whitney $U$ test was used to show the intergroup differences, and a $P$ value of <0.05 was considered to be significant.

## Results

**Intrapulmonary implantation of LLC cells adenovirally transduced with either MRP-1/CD9 or KAI1/CD8 resulted in remarkably reduced metastatic growth in mediastinal lymph nodes.** Initially, we investigated whether adenoviral transduction of transgene could be achieved in LLC cells. A flow cytometric
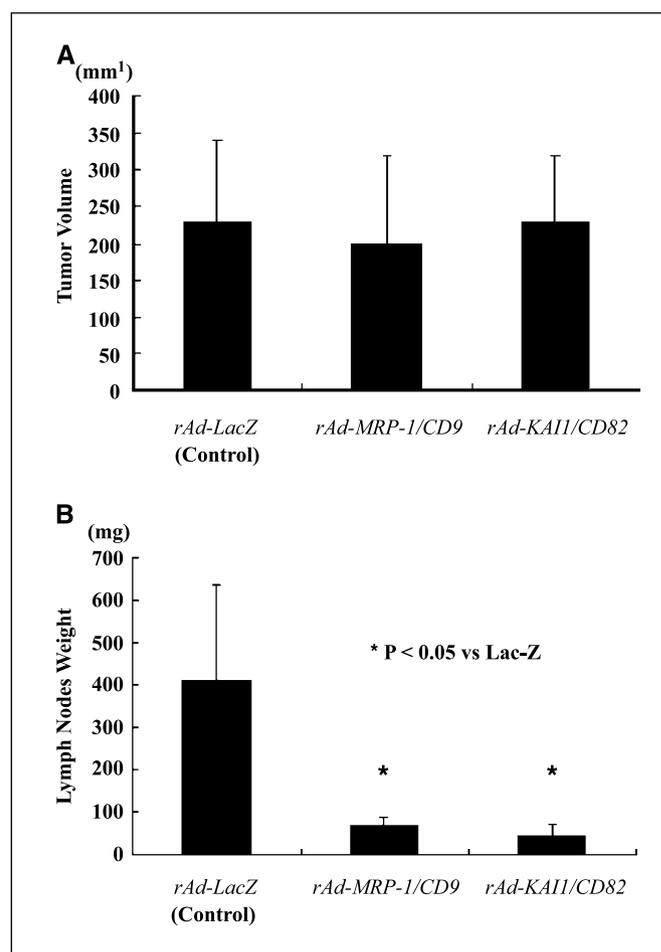


**Figure 3.** Effect of intratracheal adenoviral transfection on the primary tumor volumes and weights of mediastinal lymph nodes on day 21 after intrapulmonary implantation of LLC cells. These recombinant adenoviral vectors were intratracheally introduced on days 3, 6, and 9 after the implantation (*n* = 10 in each group). There was no significant difference between any groups in the primary tumor volumes (*A*), but great reduction in mediastinal lymph node metastasis was observed in MRP-1/CD9-transduced and KAI1/CD82-transduced groups in comparison with both control group and β-gal transfection group (*B*). The results were very close to those with adenoviral pretreatment experiments shown in Fig. 2, which suggests that three-time intratracheal gene delivery was efficient enough for treatment. *Bars,* SD.
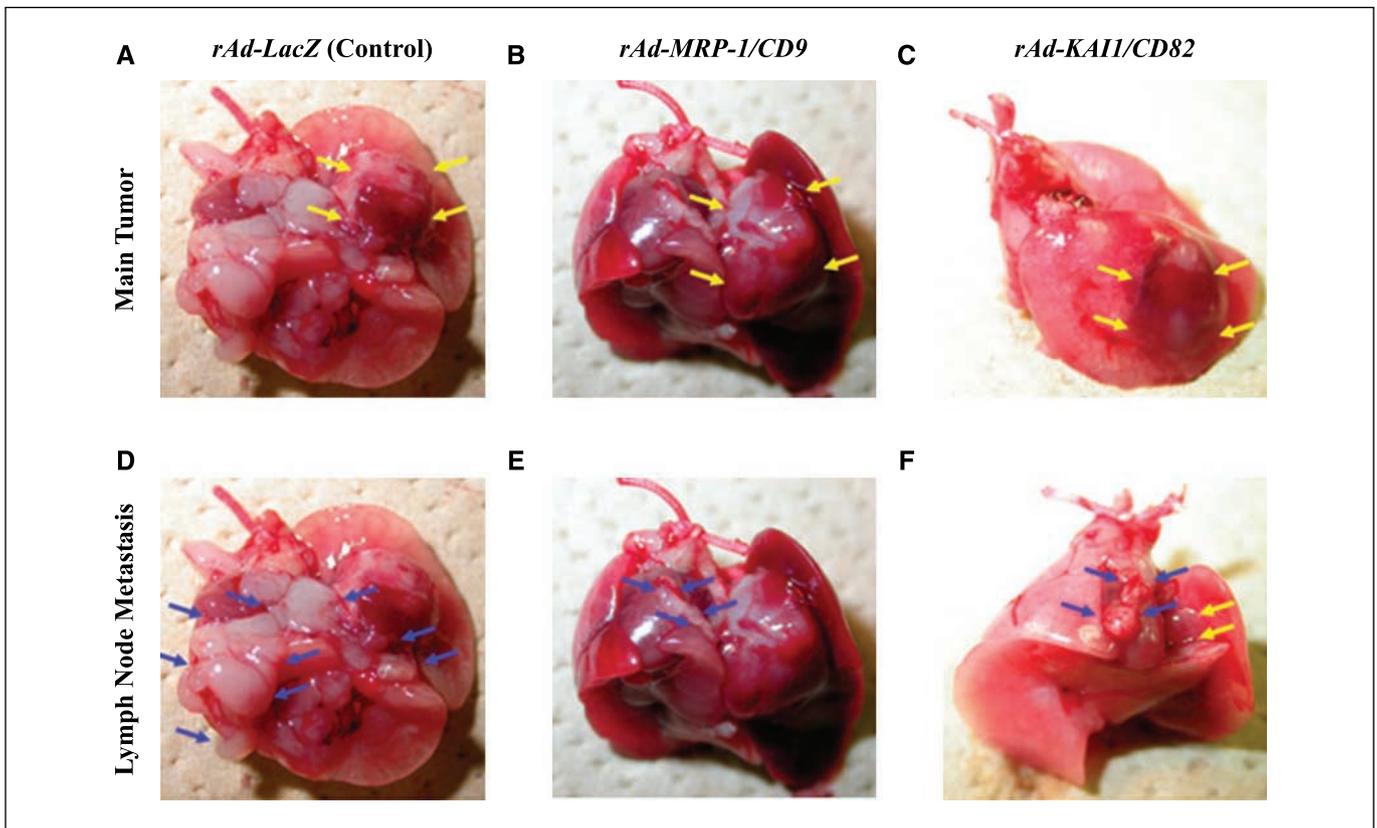
**Figure 4.** Macroscopic findings of resected lung and mediastinum from C57/BL6 mice, which underwent orthotopic implantation of LLC cells followed by intratracheal adenoviral gene deliveries [rAd-LacZ (*A* and *D*), rAd-MRP-1/CD9 (*B* and *E*), and rAd-KAI1/CD82 (*C* and *F*)]. Two pictures are shown for each group to show primary tumor and mediastinal lymph node metastasis separately. *Yellow arrows,* implanted primary tumors; *blue arrows,* mediastinal lymph node metastases. Primary tumor volumes showed no significant difference between any groups (*A–C*), but interestingly, great reduction was observed in the weights of mediastinal lymph node metastasis among those transfected with MRP-1/CD9 (*E*) and KAI1/CD82 (*F*) compared with those of LacZ (*D*).

analysis revealed that LLC cells were negatively stained for M31-15 and C33 mAbs (Fig. 1*A* and *C*). Next, the LLC cells were infected with *rAd-MRP-1/CD9, rAd-KAI1/CD82*, and *rAd-LacZ* as described in Materials and Methods. As shown in Fig. 1*B* and *D*, these infected cells were positive for M31-15 and C33 mAbs and expressed a significantly larger amount of β-gal in comparison with that in noninfected cells. Next, to see the difference among the LLC cells infected with *rAd-MRP-1/CD9, rAd-KAI1/CD82*, and *rAd-LacZ*, in an orthotopic lung cancer model, these cells were implanted into the left lungs of C57/BL6 mice. The volume of the tumor at the implanted site and the weight of the mediastinal lymph nodes were measured on day 21 after the implantation. As shown in Fig. 2*A*, about tumor volumes, there were no significant differences among these groups. On the other hand, the weights of the mediastinal lymph nodes were remarkably reduced in the mice implanted with LLC cells transduced with MRP-1/CD9 and KAI1/CD82 in comparison with those of the cells transduced with β-gal (Fig. 2*B*). The mediastinal lymph node weights did not differ between the cells transduced with MRP-1/CD9 and KAI1/CD82. The intrapulmonary implantation of the cells transduced with MRP-1/CD9 and KAI1/CD82 was shown to result in a reduced metastatic growth in the mediastinal lymph nodes without suppressing the tumor volume at the implanted site.

**Intratracheal administration of adenovirus encoding either MRP-1/CD9 or KAI1/CD82 into tumor-bearing mice reduced metastasis to the mediastinal lymph node.** To investigate whether the adenoviral transduction of transgenes affects tumor

growth and lymphatic metastasis, we gave *rAd-MRP-1/CD9, rAd-KAI1/CD82*, and *rAd-LacZ* intratracheally into the mice orthotopically implanted with LLC cells. Adenoviruses were instilled on days 3, 6, and 9 after the implantation, and on day 21, the tumor volume at the implanted site and weight of the mediastinal lymph nodes were evaluated. As shown in Fig. 3*A*, the primary tumor volumes did not differ among the four groups (i.e., *MRP-1/CD9, KAI1/CD82*, and *LacZ*). On the other hand, a dramatic reduction in the weights of the mediastinal lymph nodes was observed in *MRP-1/CD9* and *KAI1/CD82* groups in comparison with those of *LacZ* groups (Fig. 3*B*). There was no significant difference in the lymph node weight between the *MRP-1/CD9* and *KAI1/CD82* groups. The typical appearance of the tumors at the implanted site and the mediastinal lymph nodes in these four groups are shown in Fig. 4.

**Immunohistochemical findings of resected primary lung tumors and mediastinal lymph nodes.** To determine whether MRP-1/CD9 and KAI1/CD82 were transduced in the primary tumor following the intratracheal administration of *rAd-MRP-1/CD9* and *rAd-KAI1/CD82*, we did immunohistochemical staining of the excised lungs with anti–MRP-1/CD9 and anti-KAI1/CD82 mAbs. As shown in Fig. 5*A* and *C*, the intratracheal administration of adenoviruses encoding cDNA of these tetraspanins resulted in the strong surface staining accompanied by weak cytoplasmic staining in the tumor cells at the implanted site. This staining pattern was shown to be characteristic for tetraspanins. In contrast, no stained cells were detected in the tumors excised from the LLC cell–implanted mice treated with *rAd-LacZ* (Fig. 5*B* and *D*).
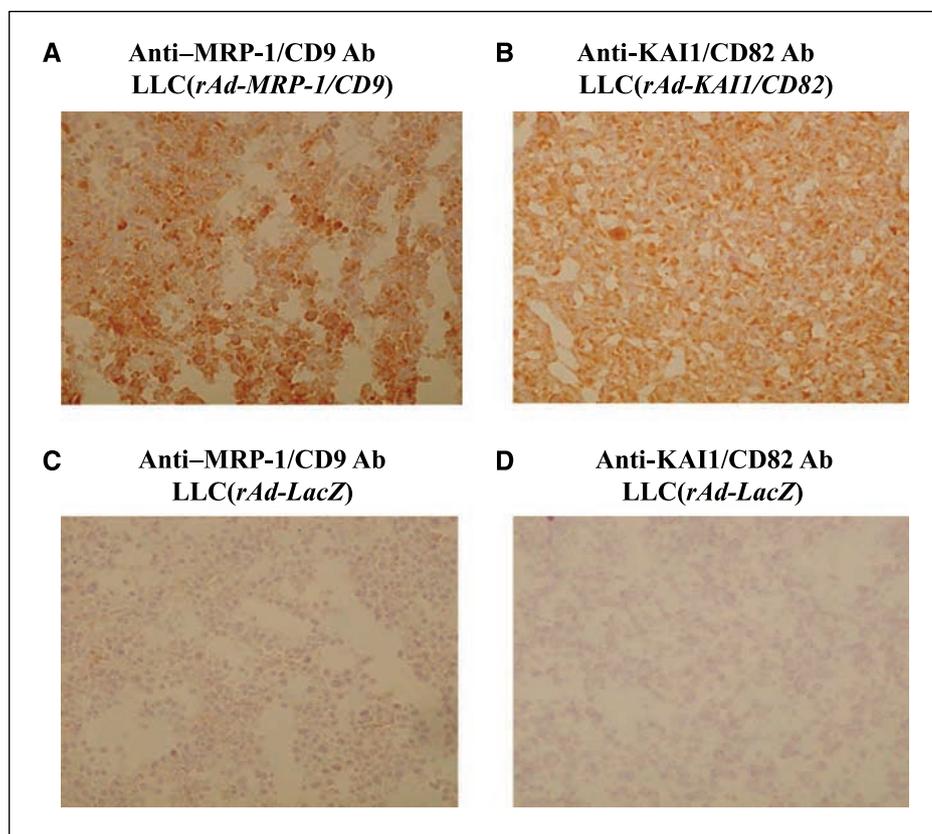
**Figure 5.** Immunohistochemical findings of resected primary lung tumors. Frozen specimens were used for immunohistochemical staining procedures. M31-15 and C33 mAbs were used to detect MRP-1/CD9 (*A* and *C*) and KAI1/CD82 (*B* and *D*), respectively, as described in detail in Materials and Methods. The strongest staining on the surface of tumor cells accompanied by relatively weaker cytoplasmic staining was observed in both specimens obtained from mice treated by *rAd-MRP-1/CD9* (*A*) and *rAd-KAI1/CD82* (*B*), indicating that intratracheal adenoviral gene deliveries were successful. Tumors obtained from mice treated by *rAd-LacZ* did not show significant staining for both MRP-1/CD9 (*C*) and KAI1/CD82 (*D*).

## Discussion

Lung cancer is notorious for its high probability of lymphatic metastasis even in its early stage. A previous study reported that nodal micrometastases were found in up to 36% of the resected lungs from the patients with peripheral NSCLC and the presence of metastases to the lymph nodes has been shown to immensely reduce the survival rates (26–28). Consequently, the development of a strategy to suppress lymphatic metastasis seems to be critical in the treatment of lung cancer patients. Among the numerous factors associated with the metastatic process, a growing body of evidence has shown that tetraspanins, such as MRP-1/CD9 and KAI1/CD82, serve as metastasis-suppressing proteins through the inhibition of cell motility (11, 19). In fact, our previous studies have shown that a higher expression level of MRP-1/CD9 and KAI/CD82 in lung cancer cells is correlated with a lower frequency of metastasis and good prognosis (13, 15, 16). Based on these observations, we produced adenoviral vectors encoding *MRP-1/CD9* and *KAI1/CD82* and applied these vectors to an orthotopic lung cancer model, which develops a huge metastatic growth in the mediastinal lymph nodes. The results presented in the current study showed that gene transduction of *MRP-1/CD9* and *KAI1/CD82* has a remarkable effect to reduce metastasis to the mediastinal lymph nodes (Figs. 2 and 3). Of particular interest, however, the transduction of these tetraspanins into the cancer cells did not suppress the tumor growth at the implanted site. These findings confirm that MRP-1/CD9 and KAI1/CD82 play a crucial role in suppressing the metastatic process while also suggesting that they do not inhibit the proliferation of tumor cells (Fig. 2).

A striking finding in the present study is that the intratracheal administration of adenoviral vectors encoding *MRP-1/CD9* and *KAI1/CD82* cDNA into tumor-bearing mice was effective at reducing the metastasis to the mediastinal lymph nodes (Figs. 3 and 4). Immunohistochemical staining with anti–MRP-1/CD9 and anti-KAI1/CD82 antibodies showed a significant expression of these tetraspanins in the tumor, thus suggesting the efficient gene delivery of adenoviral vectors to the primary tumor (Fig. 5). This finding indicates that, for the purpose of suppressing lymphatic metastasis, the gene delivery of these tetraspanins via the intratracheal route is sufficient and the direct injection of vectors into the tumor is not necessary. The gene transduction of lung tumor with *MRP-1/CD9* and *KAI1/CD82* may therefore be readily applicable in the clinical treatment. In the patients with NSCLC, the bronchoscopic instillation of vectors through the bronchus connecting to the main tumor could effectively reduce the risk of lymphatic metastasis. Another approach, such as the aerosol delivery of vectors, could possibly be adopted. A previous study showing that the delivery of aerosolized adenoviral vector encoding *LacZ* gene into the tumor-bearing mice resulted in a greater β-gal expression in the tumors than in normal lung tissues also supports the feasibility of this approach (24, 25).

Another finding that surprised us is that the implantation of LLC cells adenovirally transduced with *MRP-1/CD9* and *KAI1/CD82* also resulted in a dramatic absence of lymphatic metastasis without affecting the tumor growth at the implanted site. Because the adenoviral vectors we used in the current study were replication deficient, the transgene could not have been transferred into the replicated cells and the portion of the cells transduced with *MRP-1/CD9* and *KAI1/CD82* became smaller while the tumor grew.

Consequently, the metastasis-suppressing effect of these tetraspanins in the tumor should have become efficient. We cannot provide a clear answer for how these tetraspanins worked to suppress lymphatic metastasis in this model; however, we can speculate that transduction of tumor cells with MRP-1/CD9 and KAI1/CD82 in the small portion of the tumor is sufficient to reduce the risk of lymphatic metastasis.

In the current study, we used an adenoviral vector as a gene delivery vehicle. Because of the significant degree of toxicity, mainly due to the strong immune response against adenovirus, the usage of this vector on metabolic and hereditary diseases has thus far been avoided. However, recent promising results in human cancer trials have confirmed that adenoviruses can be very useful in oncology. In fact, more than 670 cancer patients have been treated with adenovirus intratumorally, intra-arterially, i.p., and i.v. with very manageable adverse effects and no unexpected severe or lethal toxicity (29). In addition, newer adenoviral vectors, such as the vectors driven by tumor-specific promoters and replication-competent adenoviruses, are now currently being developed (30). Although the first-generation adenoviral vectors were given intratracheally to prove the concept in the present study, we have to consider the usage of improved adenoviral vectors and the change of the administration route when clinical application is sought. In a therapeutic point of view, the character that MRP-1/CD9 or KAI1/CD82 cDNA did not affect tumor growth but suppressed metastases is not desirable. However, this modality to suppress

lymphatic metastasis could be applied to clinical gene therapy of lung cancers, especially for the prevention of metastasis during radiotherapy, including carbon ion radiotherapy (31), as a local control of stage I NSCLC patient without surgical tolerability. Considering the improvement in the local control of the early stage of lung cancer due to the progress in interventional radiology, such as ion beam radiotherapy, three-dimensional conformal radiotherapy, and radiofrequency ablation, the prevention of lymphatic metastasis is getting more important than ever.

In conclusion, we showed the successful suppression of lymphatic metastasis by methods of adenoviral transduction with *MRP-1/CD9* and *KAI1/CD82*. The data presented in this study reemphasize the important role of these tetraspanins in the suppression of the metastatic process, and we propose that gene therapy using these tetraspanins for lung cancer can thus be clinically applicable to either prevent or suppress metastasis to the regional lymph nodes.

## Acknowledgments

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# **Adenoviral Transduction of *MRP-1/CD9* and *KAI1/CD82* Inhibits Lymph Node Metastasis in Orthotopic Lung Cancer Model**

Takayuki Takeda, Noboru Hattori, Takahiro Tokuhara, et al.

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