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KITENIN increases invasion and migration of mouse squamous cancer cells and promotes pulmonary metastasis in a mouse squamous tumor model

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ABSTRACT

KAI1 C-terminal interacting tetraspanin (KITENIN) is reported to promote metastasis in mouse colon cancer models. We investigated the role of KITENIN on the progression of squamous cell carcinoma (SCC). In a preliminary clinical study using resected tissues from head and neck SCC patients, KITENIN was highly expressed in tumors and metastatic lymph nodes, while KAI1 was more increased in adjacent mucosa than in tumor. KITENIN-transfected mouse squamous cancer (SCC VII/KITENIN) cells showed significantly higher invasion, migration, and proliferation than empty vector-transfected cells. In syngeneic mouse squamous tumor models, more increased tumor volume and enhanced lung metastasis were found in SCC VII/KITENIN cells-injected mice. Thus, KITENIN increases invasion and migration of squamous cancer cells and thereby promotes distant metastasis in mouse squamous tumor models.

1. Introduction

Malignant tumors remain a significant cause of cancer related death despite important developments in cancer treatment. There have been many studies evaluating how best to reduce the progression and metastasis of cancer. Gene therapy targeting with the goal of suppression of tumor metastasis is a promising method of preventing metastatic disease because it aims to inhibit the spread of cancer regardless of the tumorigenicity [1,2]. The presence of regional metastasis in patients with head and neck squamous cell carcinoma (HNSCC) is a common adverse event associated with poor prognosis and high mortality. Treatment or prevention of HNSCC metastasis is a critical clinical point that requires a full understanding of the molecular mechanisms of squamous cancer cell invasion and dissemination [3].

KAI1, a tumor metastasis suppressor gene, is a transmembrane glycoprotein that is a member of the tetraspanins and is down-regulated during the malignant progression of various cancers [4–7]. It was recently found that the metastasis suppressor function was decreased in a spliced variant of KAI1 at the COOH-terminal region; this result suggested that the COOH-terminal region of KAI1 is important for the effects of KAI1 on cell motility [8]. Subsequently, via a yeast two-hybrid system, a protein that interacted with the COOH-terminal cytoplasm domain of KAI1 was identified [9]. This protein was found to be the Vang-like 1 (VANGL1) [10]; whose function has not fully understood in carcinogenesis and renamed as KAI1 C-terminal interacting tetraspanin (KITENIN). The KITENIN-overexpressing CT-26 mouse colon cancer cells showed increased tumorigenicity and early hepatic metastasis *in vivo*, as well as increased invasiveness and adhesion to fibronectin *in vitro*, compared with parental cells [9]. Moreover, the suppression of progression and metastasis of established colon cancer in mice was observed by intravenous delivery of siRNA targeting KITENIN [11], and the functional KITENIN/Dishevelled/PKCδ complex controlled colorectal cancer (CRC) cell invasion, which may contribute to promoting metastasis, as an executor in regard to cell motility [12]. In addition, the expression of KITENIN, a tumor metastasis-enhancing gene, inversely correlated with that of KAI1, a tumor metastasis suppressor gene, in CT-26 mouse colon cancer cells [9] and some bladder cancer cell lines [13].

Abbreviations: CRC, colorectal cancer; HNSCC, head and neck squamous cell carcinoma; KITENIN, KAI1 C-terminal interacting tetraspanin; SCC, squamous cell carcinoma; SCC VII, murine squamous cancer cells.

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However, no data assessing a potential role for KITENIN in squamous cell carcinoma (SCC) has been reported until to date. Therefore, based upon the work performed in a mouse colon cancer model showing that KITENIN affects tumor cell migration and metastasis when overexpressed, we evaluated the role of KITENIN in the progression and distant metastasis of SCC by using specimens from locally advanced HNSCC patients, and a murine squamous cancer cells (SCC VII) and its syngeneic mouse squamous tumor model.

2. Materials and methods

2.1. Assays of tumor tissues from HNSCC patients

The malignant tumors and adjacent mucosal tissues were obtained from 17 patients in which 8 and 9 samples were used for total RNA and protein preparation, respectively; six patients from recurrent supraglottic cancer after chemotherapy and radiation therapy (T3N0), five patients from recurrent glottic cancer after radiation therapy (T1bN0), four patients from supraglottic cancer (T4aN2b), and two patients from recurrent hypopharyngeal cancer after radiation therapy (T3N2c). For analysis of metastasis, metastatic and non-metastatic lymph nodes ($n = 6$) were obtained patients from supraglottic cancer (T4aN2b) and patients from recurrent hypopharyngeal cancer after radiation therapy (T3N2c). The Ethics Committee at Chonnam National University Hospital approved our experimental protocols.

Western blot analysis was used to investigate KITENIN expression in the obtained tissues. The resolved proteins (50 μg) were transferred to a nitrocellulose membrane and blotted with the KITENIN antibody and anti-rabbit immunoglobulin-horseradish peroxidase as described previously [9]. The blot was reprobed with anti-actin antibody (I-19; Santa Cruz Biotechnology, Santa Cruz, CA) as a loading control.

2.2. Cell culture, transfection and RT-PCR

The mouse SCC VII cell line was grown in RPMI 1640 (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (Hyclone) at 37 °C. The cDNA of human KITENIN and the vector only were transfected into the SCC VII cells. The transfections were performed as described previously [8]. Cells were detached using 0.25% trypsin-EDTA solution (Invitrogen, Grand Island, NY) and passed into the selective media containing 5 μg/ml blasticidin (Invitrogen). Two weeks later, the surviving clones were evaluated by Western blot analysis for the expression of the KITENIN protein, and mixed polyclonal cells were used for further analyzes.

Total RNAs were prepared from the adjacent mucosal tissues and malignant tumors of 8 HNSCC patients (four T3N0 and four T1bN0), and non-metastatic and metastatic lymph nodes of 4 HNSCC patients (two T4aN2b and two T3N2c), and the KITENIN- and vector-transfected SCC II cells as described previously [8]. RT-PCR exponential phase was determined to be 32 cycles; this allowed for a quantitative comparison of each cDNA from identical reactions. All of the reactions involved an initial denaturation at 94 °C for 5 min followed by 32 cycles at 94 °C for 50 s, at 58 °C for 50 s, and 72 °C for 60 s using PCR primers The amplification products were analyzed on agarose gels and visualized by UV epifluorescence. The sequence of primers are as follows: KITENIN, forward 5′-CTACAATGTAGATGGCCCC-3′/reverse 5′-AGCCTCATCACTGACAAGCC-3′; MMP-1, forward 5′-TTGGGCTGAAAGTGACTGG-3′/reverse 5′-CCTCCATTACCTGGGCCTGG-3′; COX-2, forward 5′-GCATTCTTTGCCCAGCACTT-3′/reverse 5′-AGACCAGGCACCAGACCAAAG-3′; CD44, forward 5′-ATGGACAAGTTTTGGTGCAC-3′/reverse 5′-CTTCTATGAACCCATACCTGC-3′; CyclinD1, forward 5′-GACCATCCCCCTGACG GCCGAG-3′/reverse 5′-CCGCACGTCGGTGGGTCTGC-3′.

2.3. Cell invasion assay

Cell invasion was measured using a Transwell migration apparatus (Costar Inc., Cambridge, United Kingdom) with minor modification as described previously [8]. To start the assay, 2.4×10^5 cells (120 μl) were loaded onto the upper chamber of the Transwell, whereas 400 μl RPMI 1640/0.2% BSA containing 10 μg/ml or 20 μg/ml human plasma fibronectin (Calbiochem, La Jolla, CA), a chemotactic factor, were loaded onto the lower chamber. The apparatus was incubated for 24 h at 37 °C. The cells on the top surface of the filters were wiped off with cotton balls, and the cells that migrated, on the bottom surface, were counted in six random squares of 0.5×0.5 mm^2 on the microscope field of view. The results were expressed as the mean ± SE of the number of cells/field.

2.4. Cell migration assay

KITENIN-transfected and vector-transfected SCC VII cells were seeded in a 6 well plate (1×10^6 cells/well). Twenty-four hours later, the media was changed to serum-free RPMI 1640 and incubated for 12 h. After the media was removed from the wells, a straight transverse line through the adherent cells was drawn using a ruler and a 1000 μl-tip, resulting a uniform gap. The media was changed to RPMI 1640 supplemented with 2% fetal bovine serum. At 6, 12, 24, and 48 h later, the distances between the gaps were measured in centimeters after capture of six random sites in the microscope fields.

2.5. Cell proliferation assay

Proliferation of the cells was measured as described previously [8]. Cells were seeded at 1×10^4 cells/well in 24-well plates and grown for 3 days in the presence of serum. Next the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent was added for 2 h, after which, the DMSO was added. The absorbance at 570 nm was determined 16 h later using a microplate reader with SOFTmax PRO software (Molecular Devices, Sunnyvale, CA). The data were adjusted to the first value of each group.

2.6. In vivo tumor growth

Prior approval of the experimental protocol was obtained from the Chonnam National University Medical School Research Institutional Animal Care and Use Committee. Maintenance of animals and all *in vivo* experiments were performed according to the Guiding Principles in the Care and Use of Animals. Subconfluent SCC VII cells (KITENIN-transfected and vector-transfected) were trypsinized and then suspended in RPMI 1640. The cell suspension (1×10^6 cells in 0.1 ml medium/mouse) was injected subcutaneously into the right flank of eight-week old, female, C3H/HeJ syngeneic mice ($n = 6$ for each group). The tumor size was measured daily with a vernier caliper from the third day to the fourth week after injection. The tumor volume (V) was calculated using the values of the largest (A) and the smallest (B) diameter according to the formula: $V = 0.5 \times AB^2$.

During the fifth week after injection, the presence of metastasis in the lung and liver tissues from each mouse bearing a tumor mass on the back was evaluated by gross and microscopic examination. The tissue sections were deparaffinized, rehydrated, and rinsed. They were stained with H & E and examined for metastatic nodules and cell pathology.

2.7. Statistical analysis

Experimental differences were tested for statistical significance with SPSS 12.0 (Microcal Software Inc., USA). ANOVA and the Stu-

dent's *t*-test were used, and all statistical tests were two-sided, and *P*-values of less than 0.05 were considered statistically significant.

3. Results

3.1. Increased KITENIN expression in the tumors and metastatic lymph nodes from HNSCC patients, while KAI1 is more increased in adjacent normal mucosa than in tumor

At first, in 8 HNSCC patients studied, KITENIN mRNA expressions were increased in the malignant tumors than in adjacent mucosal tissues, but significantly elevated in metastatic lymph nodes compared with non-metastatic lymph nodes (Fig. 1A). Next, in 9 HNSCC patients studied, KITENIN protein expressions were increased in the malignant tumors and significantly higher in metastatic lymph nodes than in adjacent mucosal tissues and non-metastatic lymph nodes, respectively (Fig. 1B and C). In addition, as previously reported [9,14–16], the expression of KAI1 increased in adjacent normal mucosa more than in tumor, but unexpectedly exhibited an elevated level in metastatic lymph nodes (Fig. 1B and D). This observation suggests that the expression level of KITENIN is much better molecular marker for both the malignant tumor and the metastatic lymph nodes of HNSCC patients than that of KAI1. Moreover, it was suggested that the KAI1 gene may not be a useful predictor of prognosis of oral SCC, although decreased KAI1 expression may be associated with increased invasive ability of oral SCC [15]. Overall, these preliminary clinical data from 17 HNSCC patients strongly suggest that KITENIN expression is involved in the progression of SCC. Therefore, we tried to prove this proposal through *in vitro* assays using mouse SCC VII cells and *in vivo* assay using a mouse SCC tumor model.

3.2. Increased invasion, migration and proliferation in KITENIN-transfected squamous cancer cells

At first, to establish squamous carcinoma cells stably expressing KITENIN, SCC VII cells were stably transfected with pcDNA-KITENIN cDNA or empty vector alone. KITENIN expression in mixed clones following selection was then evaluated by Western blot analysis, showing that KITENIN expression was higher in the KITENIN-transfected SCC VII cells, whereas KITENIN was barely detected in the empty vector-transfected SCC VII cells (Fig. 2A). Therefore, polyclonal cells that were employed for omitting problematic clonal variation were subject to further functional assay.

Next, we examined whether overexpressed KITENIN affects on cell invasion of SCC II cells. The number of invading vector-transfected SCC VII cells were 65.0 ± 40.6, whereas for the KITENIN-transfected SCC VII cells they were 158.8 ± 15.8 as measured by the six random squares of the 0.5×0.5 mm^2 microscope fields under conditions of 10 μg/ml fibronectin; the difference between the two was statistically significant ($P = 0.004$). In addition, under the increasing dose of fibronectin (20 μg/ml), the invading cells were 172.0 ± 18.9 in the vector-group and 212.8 ± 24.2 in the KITENIN-group ($P = 0.009$) (Fig. 2B). We also tested whether overexpressed KITENIN affects on cell migration of SCC II cells. The artificial wound gap became significantly narrower in the KITENIN-group as time passed by 12, 24, and 48 h, compared with the empty vector-group ($P = 0.002$) (Fig. 2C). In the KITENIN-group, the gap was completely filled within 48 h, indicating that cell migration was increased in KITENIN-transfected SCC VII cells. Moreover, the proliferating cells, as determined by the absorbance, were significantly increased in the KITENIN-group compared to the empty vector-group on the second and third day of measurement ($P < 0.05$) (Fig. 2D).

3.3. KITENIN positively affects on in vivo tumor growth and distant metastasis in a syngeneic mouse squamous tumor model

Transfected SCC VII cells were injected subcutaneously into the right flank of syngeneic mice and at 6 days after injection, the tumors were large enough to measure. The average tumor size was significantly larger in the KITENIN-group than in the empty vector-group during the time period measured after 12 days following injection ($P < 0.01$) (Fig. 3A). Next, we examined whether overexpressed KITENIN in SCC VII cells promotes the distant metastasis in a mouse squamous tumor model. Lung and liver tissue samples were obtained from the vector-group ($n = 6$) or KITENIN-group ($n = 6$). Pulmonary metastases were observed in lung tissues of 2 out of 6 mice from the vector-group and the number of metastatic nodule is 0.5 ± 0.34/mouse. In contrast, in the KITENIN-group, metastatic nodules were observed in lung tissues of 4 from 6 mice and the number of metastatic nodule was significantly increased (13 ± 5.3/mouse) more than that of vector-group (Fig. 3B). However, no liver metastases were observed in both groups. The pathology of metastasized lung tissue demonstrated the tumor cell nests invading the pleura and alveoli, and also dyskeratosis, frequent mitosis, coarse chromatin, and nuclear pleomorphism were found in high-power field of pleural metastatic tissue, indicating a presence of metastatic squamous tumor in lung tissue (Fig. 3C).

3.4. Increased expression of AP-1 target genes in KITENIN-transfected squamous cancer cells

Recently, a functional KITENIN complex was reported to enhance migration and invasion of intestinal epithelial and CRC cells in an ERK/AP-1-dependent manner [12]. To investigate the relationship between KITENIN and AP-1 axis in squamous cancer cells, some well-known AP-1 target genes were compared between the KITENIN- and empty vector-transfected SCC VII cells, such as cyclin D1, MMP-1, COX-2, and CD44 [17–19]. The mRNAs of all of the investigated AP-1 target genes were highly expressed in KITENIN-transfected SCC VII cells (Fig. 4), indicating the positive relationship between KITENIN and AP-1 axis in squamous cancer cells.

4. Discussion

The *KITENIN/VANGL1* gene is located on human chromosome 1p13. Abnormalities in this 1p13 region have been reported in head and neck cancer, breast cancer and Kaposi's sarcoma [20–22]. In addition, a putative prostate cancer susceptibility gene has been mapped to human chromosome 1p13–q32 [23]. These results indicate that KITENIN is located at a human chromosome locus, which is deleted, mutated or rearranged in several types of human cancer. In this study, KITENIN expression was higher in the tumors and metastatic lymph nodes than in the adjacent mucosal tissues and non-metastatic lymph nodes from patients with locally advanced HNSCC. KITENIN-overexpressing SCC VII cells exhibited increased invasiveness, migration, and proliferation *in vitro*. Moreover, syngeneic mice given KITENIN-overexpressing SCC VII cells showed more increased tumorigenicity and lung metastasis compared with control syngeneic mice given vector-transfected SCC VII cells. Therefore, our present results indicate that KITENIN participates in the tumor progression and metastasis in SCC as well as in CRC and bladder cancer [9,11–13].

Cellular invasion is stimulated in target cells by a variety of extracellular signals including extracellular matrix, soluble growth factors and chemokines [2]. Activation of the AP-1 transcription factor complex is a universal response of most mammalian cells to a broad range of stimuli and the AP-1 activity is increased in a large number of tumors and transformed cell lines [24,25]. Re-

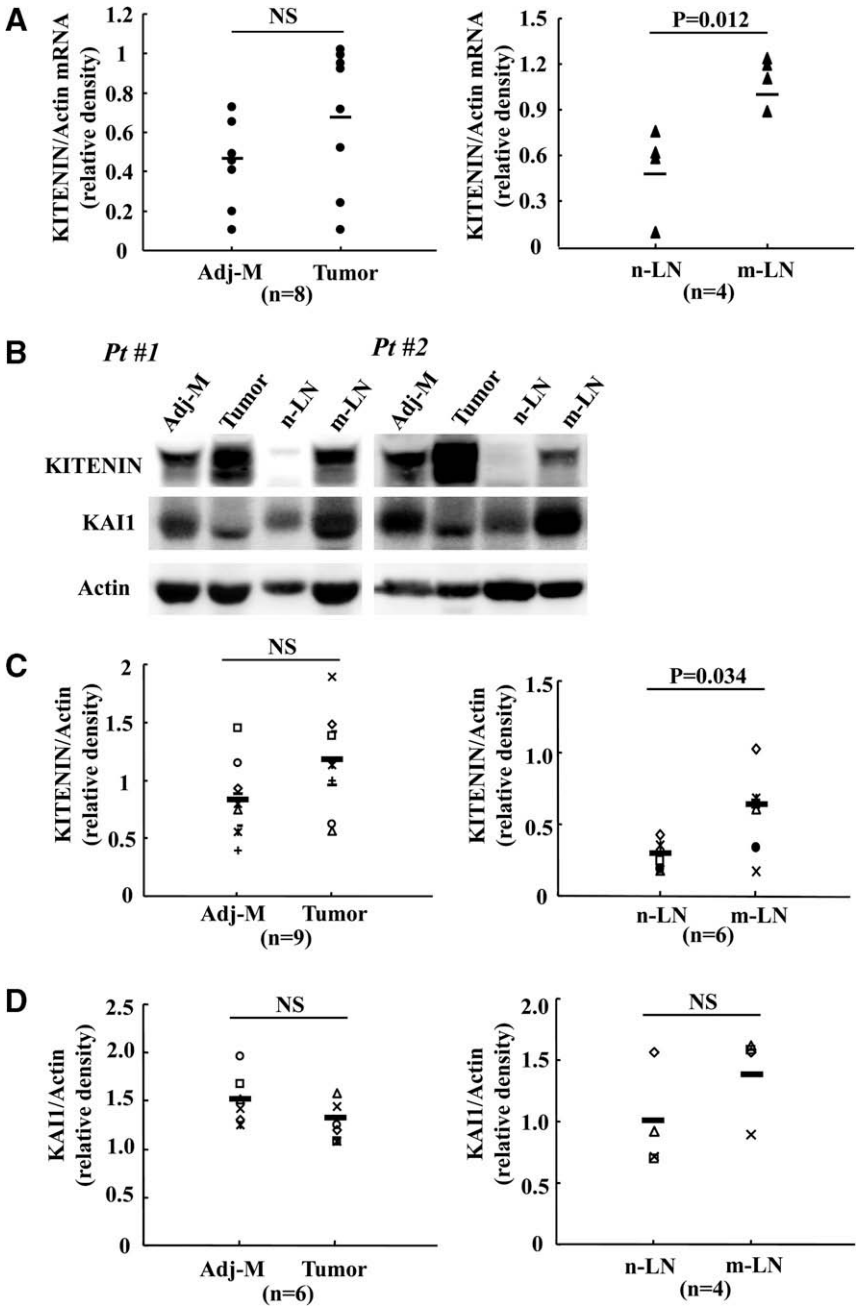

Fig. 1. Expression of KAI1 C-terminal interacting tetraspanin (KITENIN) and KAI1 in resected tissues from head and neck squamous cell carcinoma (HNSCC) patients. (A) Densitometric analyzes of RT-PCR for KITENIN in HNSCC. Each KITENIN level was normalized with respect to the corresponding *actin* level. KITENIN mRNA expression was increased in tumors compared with adjacent mucosa (adj-M) from 8 HNSCC patients (left), while significantly elevated in metastatic lymph nodes (m-LN) more than non-metastatic lymph nodes (n-LN) from 4 HNSCC patients (right). (B) Representative immunoblotting results of KITENIN and KAI1 expression among the resected tissues from 2 HNSCC patients. KAI1 expression inversely correlated with that of KITENIN between adj-M and tumor tissues, but not between n-LN and m-LN. (C) Densitometric analyzes of immunoblot for KITENIN in HNSCC. Each KITENIN level was normalized with respect to the corresponding actin level. KITENIN expression was increased in tumors compared with adj-M from 9 HNSCC patients (left), while significantly higher in m-LN than that of n-LN from 6 HNSCC patients (right). (D) Densitometric analyzes of immunoblot for KAI1 in HNSCC. Each KAI1 level was normalized with respect to the corresponding actin level. KAI1 expression was decreased in tumors compared with adj-M from 6 HNSCC patients (left), while elevated in m-LN than that of n-LN from 4 HNSCC patients (right).

cently, we found that in CRC cells, KITENIN served as a scaffolding molecule that simultaneously recruited both Dishevelled and PKCδ to form a complex that stimulated ERK/AP-1 via a PKCδ component and controlled the invasiveness, and that KITENIN expression was significantly higher in advanced stage CRC tissues [12]. In this study, we observed that the expressions of some AP-1 target genes were higher in the KITENIN-transfected SCC VII cells. This finding suggests that KITENIN could enhance the progression of SCC as a result of activation of the AP-1 signal transduction pathway.

HNSCC is characterized by a marked propensity for local invasion and lymph node metastasis. Understanding the molecular mechanisms that mediate SCC invasion and metastasis may enable the identification of novel therapeutic targets for the management of tumor dissemination. Invasion and metastasis are complex processes in which many molecules identified interact with one another in enabling squamous cancer cells to disseminate from their primary site [3]. One is that signal transduction elements for increased proteolytic degradation of matrix components, which

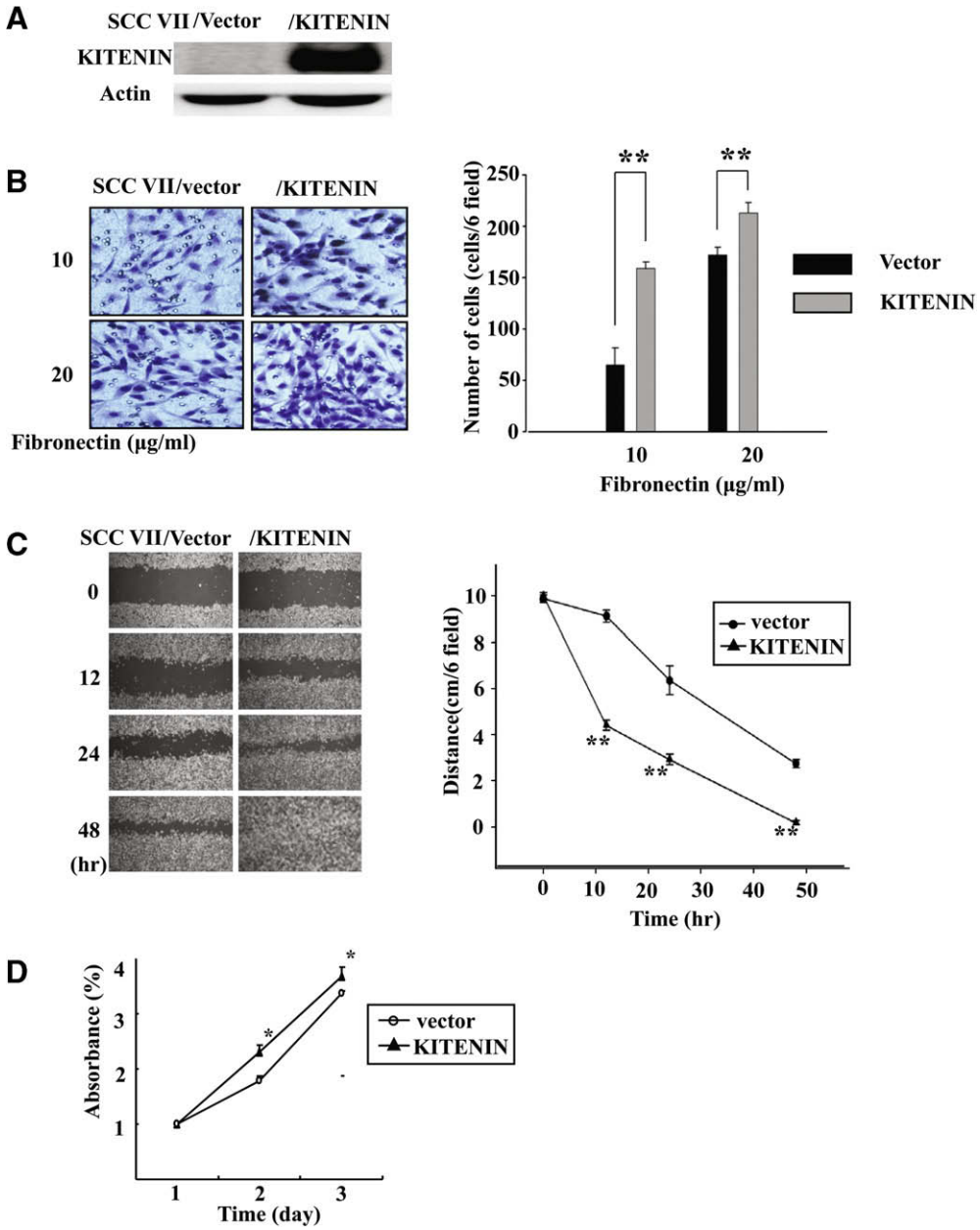

Fig. 2. KITENIN enhances cell invasion, migration and proliferation of murine squamous cancer cells (SCC VII). (A) Establishment of the stably KITENIN-transfected SCC VII (SCC VII/KITENIN) polyclonal cells. (B) Invasion assay of SCC VII cells using fibronectin as a chemoattractant. Invading cells were larger in the KITENIN-transfected SCC VII cells than in the empty vector-transfected SCC VII cells (left). Stained invading cells were counted and are represented as a bar graph between groups (right) (mean ± SEM, $n = 3$; $^{**}p < 0.01$). (C) The effects of the overexpressed KITENIN on cell migration. The empty vector- or KITENIN-transfected SCC VII cells were subjected to a wound healing assay (left), and cell migration are displayed as relative healing distances measured in six random sites (right). Values are mean ± SEM for three independent experiments ($^{**}p < 0.01$). Cell migration was markedly increased in the KITENIN-transfected SCC VII cells. (D) The effects of the overexpressed KITENIN on cell proliferation. The absorbance indicating proliferating viable cells was higher in the KITENIN-transfected SCC VII cells ($n = 3$, mean ± SEM, $^{*}p < 0.05$).

facilitate invasion and metastasis of SCC cells; the integrin $\alpha_v\beta_6$ heterodimer complexes with fibronectin and activates Fyn, which, in turn, activates a matrix-degrading pathway involving urokinase plasminogen activator, MMP-3, and MMP-9. The other is that signal transduction elements for enhanced cell motility; ligand binding to the epidermal growth factor receptor induces a sequential downstream signaling in which actin-binding proteins, calcium, and protein kinase C serve to increase cell motility through adhesion and actin cytoskeleton alterations. In addition, it was reported that the immunohistochemical KAI1 staining was decreased in tumor tissues from oral and esophageal SCC patients [14–16]. In this study, KAI1 expression inversely correlated with that of KITENIN between normal adjacent mucosa and tumor tissues, but not between non-metastatic and metastatic lymph nodes. Unexpectedly, compared with non-metastatic lymph nodes, an elevated level of KAI1 but not significant was observed in metastatic lymph nodes. Although, the number of patients in our present analyzes is small, these results suggest that the expression level of KITENIN, a tumor metastasis-enhancing gene, is more reliable molecular marker for the detection of lymph node metastasis from HNSCC patients rather than that of KAI1, a tumor metastasis suppressor gene. Therefore, in addition to epidermal growth factor receptor, our present results suggest that the KITENIN/AP-1 axis also participates in SCC invasion and metastasis as another signal transduc-

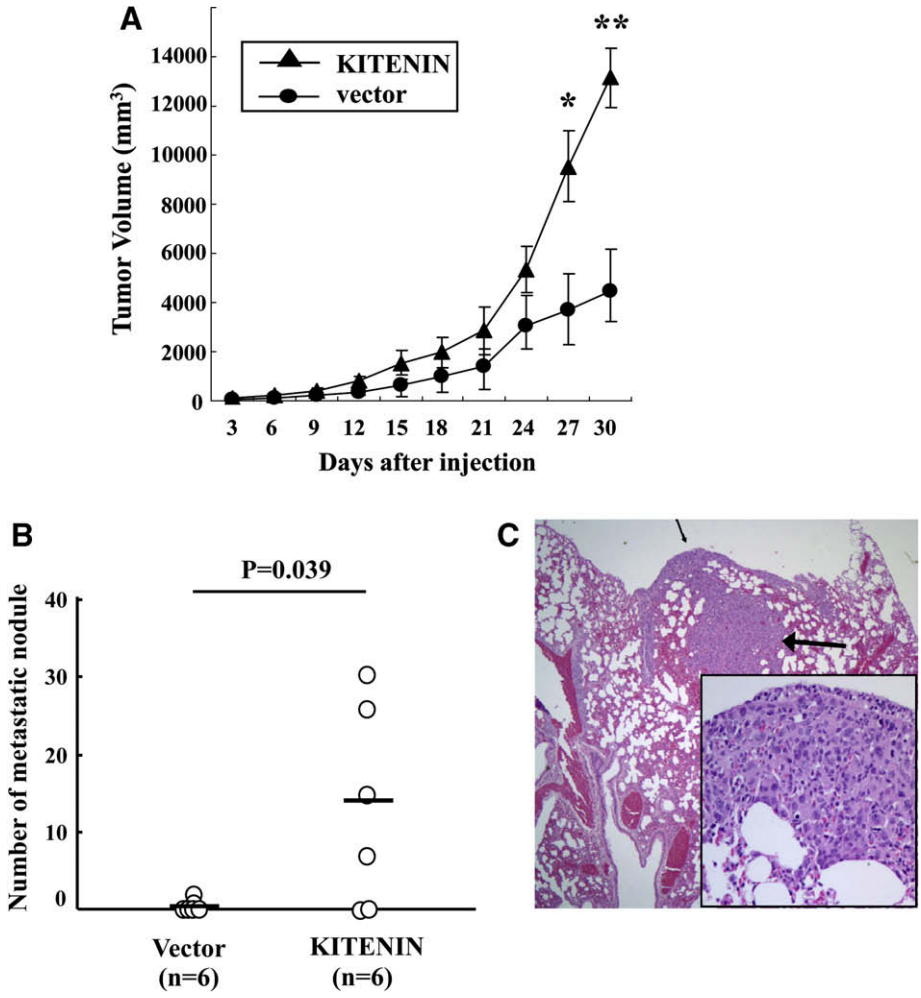

Fig. 3. Promoting effects of the overexpressed KITENIN on *in vivo* tumor growth and distant metastasis. (A) Transfected SCC VII cells were injected subcutaneously into the right flank of syngeneic mice and tumor size was measured daily. Tumor growths were increased in the KITENIN-transfected SCC VII-injected C3H/HeJ syngeneic mice compared to the vector-transfected SCC VII-injected mice ($n = 6$, mean ± SEM, $^{**}p < 0.01$). (B) The number of metastatic nodule was significantly increased in the KITENIN-group more than that of vector-group. (C) H & E stained metastasized lung tissue from the KITENIN-transfected SCC VII-injected C3H/HeJ syngeneic mice. The representative tumor cell nest invading the pleura (a thin arrow) and alveoli (a thick arrow) was shown (×40). Dyskeratosis, frequent mitosis, coarse chromatin, and nuclear pleomorphism were found in a high-power field view of pleural metastatic tissue (*inlet*, H & E stain, ×400).

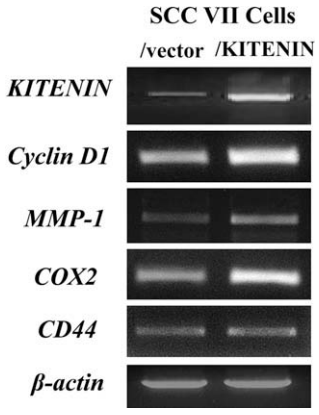

Fig. 4. Increased expressions of AP-1 target genes in the KITENIN-transfected SCC VII cells. Expressions of *cyclin D1*, *MMP-1*, *COX-2*, and *CD44* were higher in the KITENIN-transfected SCC VII cells.

tion element for enhanced cell motility, and raising the possibility that KITENIN-knockdown strategy has the therapeutic potential for inhibiting the distant metastasis in SCC.

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