KAII COOH-Terminal Interacting Tetraspanin (KITENIN), a Member of the Tetraspanin Family, Interacts with KAI1, a Tumor Metastasis Suppressor, and Enhances Metastasis of Cancer

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ABSTRACT

We cloned recently an alternatively spliced variant of KAI1 mRNA that lacked exon 7 at the COOH-terminal region and showed differences in metastasis suppression when compared with the wild-type KAI1. These findings indicated that the COOH-terminal region of KAI1 is critical for its metastasis suppressor function. In this study, we isolated a cDNA clone of VANG1, a member of the tetraspan protein family, which interacted specifically with the COOH-terminal cytoplasmic domain of KAI1 in the yeast two-hybrid system. We renamed it KITENIN COOH-terminal interacting tetraspanin (KITENIN). We found that 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. Moreover, increased levels of KITENIN were observed in a human gastric tumor and its metastatic tissues, compared with the normal adjacent mucosa. Our results indicate that KITENIN promotes adhesion and invasion of cancer cells in vitro and in vivo, and suggest that KITENIN participates in the regulation of the tumor formation and metastasis by interacting with KAI1, a metastasis suppressor and antisense KITENIN strategy that can be used to inhibit metastasis in various cancers.

INTRODUCTION

Cell-cell and cell-matrix adhesion is critical to the establishment and maintenance of normal tissue architecture. A number of cell adhesion molecules, such as members of the immunoglobulin superfamily, cadherins, selectins, integrins, and CD44, contribute to cell adhesion. These proteins also play a critical role in organ development, inflammation, and cancer invasion and metastasis. For many tumor cells, increased cell adhesion is associated with increased tumorigenicity and metastasis (1–3).

KAI1/CD82, identified as a metastatic suppressor gene for prostate cancer, is a member of transmembrane 4 superfamily (tetraspanin). The tetraspanins contain four highly conserved transmembrane domains, two short cytoplasmic domains at the NH₂ and COOH termini, and two relatively divergent extracellular domains, the larger of which contains several conserved amino acid motifs. KAI1 is down-regulated during the malignant progression of various cancers (4–7). Low levels of KAI1 mRNA correlate with an increase in invasive ability in vitro, decreased cell-cell adhesion, and specific adhesion to the extracellular matrix protein fibronectin (8). The expression of KAI1 in cancer cells results in reduced cell motility and invasiveness in vitro and in suppressed experimental metastasis in vivo (9).

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biochemical functions of the tetraspanins are not yet clear, but current data suggest a role for this superfamily in the regulation of cell proliferation, activation, and motility (10). KAI1 associates with other tetraspanins such as CD9, CD63, and CD81 in the plasma membrane, and forms a transmembrane complex, tetraspanin web (11). The tetraspanin complexes that contain KAI1 interact not only with transmembrane molecules but also with intracellular signaling molecules such as protein kinase C and phosphatidylinositol 4-kinase (12).

We have found recently the existence of an alternatively spliced variant of KAI1 in the COOH-terminal region, and compared the functional differences between wild-type KAI1 and spliced-KAI1 (13). It showed that the metastasis suppressor function was decreased in spliced-KAI1 and the functional difference of effects on cell motility and growth between them might be partially explained by the structural differences between the two KAI1 proteins. These findings suggest that the COOH-terminal region of KAI1 appears to be important for the effects of KAI1 on cell motility.

In this study, we tried to identify proteins that interact with the cytoplasmic domain of KAI1 by yeast two-hybrid system on the assumption that the COOH-terminal region is important in regulating the functional characteristics of KAI1. We found a cDNA clone identified as the Yang (Van Gogh, Drosophila)-like 1 (VANG1; Refs. 14, 15) that interacted specifically with the COOH-terminal region of KAI1. VANG1 is also a tetraspanin, having four transmembrane domains and one putative PDZ-domain binding motif. VANG1 is located on human chromosome 1p13, a region that is associated with several types of human cancer (16–19). We renamed it as KAI1 COOH-terminal interacting tetraspanin (KITENIN) protein. We found that KITENIN is associated with promoting invasion and metastasis, and the interaction of two tetraspanins, KITENIN and KAI1, affects cellular motility and invasion and thereby regulates tumor formation and metastasis. There was a positive correlation between the expression of KITENIN and the presence of distant metastasis, indicating that KITENIN can function as a metastasis-inducing gene. Also, the increased expression of KITENIN and the decreased level of wild-type KAI1 could be used as molecular markers for the detection of cancer metastasis.

MATERIALS AND METHODS

Yeast Two-Hybrid Assay. The KAI1 COOH-terminal domain [amino acids (aa) 201–267] was amplified from full-length wild-type KAI1 cDNA, using PCR primers (sense, 5'-CGGAATTCAGAGTGGCAACCACCCT-3'; antisense, 5'-CCGGATTCTGACTGTTGGACCCTGT-3') and a PCR system (Perkin-Elmer 9600; Foster City, CA). PCR conditions were: 1 cycle of 5 min at 94°C; 35 cycles of 1 min at 94°C, 2 min at 60°C, and 3 min at 72°C; 1 cycle of 72°C for 10 min; followed by storage at 4°C. PCR product was size-fractionated on 0.8% agarose, gel purified, and subcloned into pLexA DNA-binding domain (pBD). This construct was used as bait. The nucleic acids of KAI1 encoding aa 201–267 [pBDKAI1 (201–267)], the splice-KAI1 product [deleted aa residues: 215–242, pBDKAI1(201–267[215–242])], the transmembrane region [aa residues 201–245, pBDKAI1 (201–245)], and the NH₂-terminal portion [aa residues 1–34, pBDKAI1 (1–34)] were also prepared.
by PCR. Each cloned PCR product was sequenced and confirmed as error free. These products were also subcloned into pBD.

The yeast reporter strain EGY48 (pGAL-lacZ) was sequentially transformed with pBD-KAI1 and the pB42AD-human lung cancer cDNA library using a modified lithium acetate method. Positive clones were selected on supplemented minimal galactose medium (Ura-, His-, Trp-, and Leu-). To double-check the positive colonies, qualitative blue/white screening with X-galactosidase (gal) as a substrate for the colony- replica plating assay was done. Plasmid DNA from positive yeast clones was isolated and transformed into *Escherichia coli* strain DH5α.

**Quantification of β-Galactosidase Activity.** For relative quantification of protein-protein interactions, β-galactosidase assays were performed. Yeast strains cotransformed by pLexA and the pB42AD-human lung cancer cDNA library using a modified lithium acetate method. Positive clones were selected on supplemented minimal galactose medium (Ura-, His-, and Trp-) in a shaking atmosphere of 5% CO2 at 37°C, and the clones were grown in supplemented minimal galactose medium (Ura-, His-, and Trp-) in a shaking atmosphere of 5% CO2 at 37°C, and transfection was performed using FuGene 6. Cells were rinsed with PBS three times and fixed with ice-cold 2% buffered paraformaldehyde (pH 7.4) in PBS for 10 min. After washing with PBS and blocking with a buffer containing 0.1% saponin and 0.05% BSA in PBS (pH 7.4), for 30 min, the cells were incubated with anti-KAI1 antibody for 1 h at room temperature and then washed with the blocking buffer. Tetramethylrhodamine-labeled anti-rabbit IgG antibody (dilution 1:250; BD Biosciences) was added to the cells, and they were incubated for 1 h. After washing with PBS three times, the cells were examined with a Laser Scanning Confocal Microscope (Leica Microsystems TCS NT, Leica, Germany).

**Cell Attachment Assay.** The fibronectin-coated 96-well plate was prepared as described (13). Cells were detached from the culture flasks with 5 ml EDTA in PBS, resuspended in culture medium containing 0.02% BSA to 4 × 105 cells/ml, and 100 μl was added to fibronectin (+) or fibronectin (−) wells. All of the cells were assessed in quadruplicate, and adherent cells were then counted in three random areas of each well, using an inverted phase contrast microscope to determine the average number of cells/field of view as described (13). Differences between cell lines were tested for statistical significance using the Student’s t test. A P value of < 0.05 was considered to be significant.

**Cell Invasion Assay.** Cell invasion was measured using the Transwell migration apparatus (Costar Inc., Cambridge, United Kingdom) as described (13).

**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. Subcutaneous CT-26 cells were trypsinized and then suspended in DMEM. The cell suspension (5 × 106 cells in 0.1 ml medium per mouse) was injected s.c. into BALB/c syngeneic mice (n = 14 for each group of CT-26/parent, CT-26/KITENIN, and CT-26/KITENIN/AS-KITENIN). Tumor size was measured daily from the first week to the fourth week after injection, and tumor volume was calculated as described (13). At the fifth week after injection, the presence of metastasis in the liver and lung tissues in each mouse bearing a tumor mass on the back was examined by gross and microscopic examination. After confirming the metastasis, tumor mass and metastatic foci in the lung tissues and liver tissues were excised (n = 5 for each group of CT-26/KITENIN and CT-26/KITENIN/AS-KITENIN), and proteins were prepared for Western analyses.

**Histochemistry of Metastatic Tissue.** We prepared other groups of syngeneic mice, which were injected s.c. with CT-26/parent, CT-26/KITENIN, or CT-26/KITENIN/AS-KITENIN cells (n = 12 for each group). Two weeks after cell injection, internal organs (liver, lung, kidney, spleen, and brain) were excised weekly in each experimental group (n = 3) for histochemistry until 5 weeks. The tissue sections were deparaffinized, rehydrated, and rinsed. They were stained with H&E, examined for metastatic cells, and photographed through a light microscope.

**Cancer Tissue Specimens.** Thirteen gastric cancer tissues were obtained for RNA preparations from surgically removed specimens at Chonnam University Hospital (Kwangju, Korea). We also collected 13 normal and metastatic lymph nodes and 6 peritoneal and 8 hepatic metastases. The tumors were histologically examined, and pathological stage was estimated by Tumor-Node-Metastasis score (stage IV; n = 13). The Ethics Committee of Chonnam University Hospital approved our experimental protocols.

**Reverse Transcription-PCR.** Reverse transcription was performed as described (13). All of the reactions involved an initial denaturation at 94°C for 5 min followed by 26 cycles for KITENIN at 94°C for 30 s, at 58°C for 30 s, and at 72°C for 60 s using PCR primers (sense, 5'-GGAATTCATTGCAGAAGACTA-3'; antisense, 5'-CCGCTGAGGCCCCAGGTTTTCAGCA-3') on a PCR system. The specific conditions and primers for each gene
was as follows: for KAI1, 28 cycles at 94°C for 30 s, at 58°C for 30 s, and at 72°C for 60 s using PCR primers (sense, 5'-GCAAAACGGTTTCCGGAGGAAAG-3'; antisense, 5'-GCTGTTCTGATGCAGTTGGGACCTTGGTGA-3'); for mm23, 30 cycles at 94°C for 30 s, at 58°C for 30 s, and at 72°C for 60 s using PCR primers (sense, 5'-GGTCACTTCTGGGTGAGGATCAAC-3'; antisense, 5'-ATCCAGTGCTAGCACAGTTGCAGG-3'); for KIS1, 38 cycles at 94°C for 5 s, at 55°C for 30 s, and at 72°C for 60 s using PCR primers (sense, 5'-GATCCTCAGGTTCCCGTTG-3'; antisense, 5'-AGTTAGGTGTCAGGATT-3'); for TIMP2, 26 cycles at 94°C for 50 s, at 58°C for 50 s, and at 72°C for 60 s using PCR primers (sense, 5'-CCTCGTGCCTCTGGGCCT-3'; antisense, 5'-TACACGGGGGTGAAGGTT-3'); and for MMP9, 35 cycles at 94°C for 50 s, at 55°C for 60 s using PCR primers (sense, 5'-TGTAGGTCGACAGGT-3'; antisense, 5'-TCTTCTTCTGGGTGATGC-3').

RESULTS

Isolation of a Tetraspanin Protein Interacting with KAI1. In searching for proteins capable of association with the cytoplasmic region of KAI1, we used a polypeptide corresponding to the entire COOH-terminal cytoplasmic region of KAI1 (aa residues 201–267) as bait for a yeast two-hybrid screen. By screening 3×10^6 colonies from a human lung cancer cDNA library, we isolated and sequenced several positive clones that had a specific interaction with the cytoplasmic domain of KAI1. One of the cDNA clones was identified as VANGL1 based on a basic local alignment search tool search. The clone encoded part of the VANGL1 protein, composed of aa residues 80–524 at the COOH-terminal end of the protein (14, 15). We obtained the full-length VANGL1 cDNA using Expand long template PCR with human gastric mucosa RNA.

COOH-Terminal Region of KAI1 Is Important for KAI1-VANGL1 (KAI1-KITENIN) Interaction. To determine which portion of KAI1 is responsible for the KAI1-VANGL1 interactions, we performed β-gal assays. Because β-gal activity in the yeast two-hybrid system assay is a simple measure of the relative interaction between proteins (21), it was used to determine the strength of binding between VANGL1 and several regions of KAI1.

We prepared several fragments of KAI1 fused in frame to pLexA vector (Fig. 1A). LexA-KAI1 (201–267) exhibited a significantly greater strength of interaction with VANGL1, whereas LexA-spliced, LexA-KAI1 (201–245), and LexA-KAI1 (1–34) were completely defective in this interaction (Fig. 1C). These data are consistent with the hypothesis that the COOH-terminal region of KAI1 contains the region of highest affinity to VANGL1.

In searching for proteins capable of association with VANGL1, we used a polypeptide corresponding to the entire length of VANGL1 as bait for a yeast two-hybrid screen with the human lung cancer library. The DNA sequences of the positive clones were identified using basic local alignment search tool alignment, and one of the isolated cDNA clones was a partial sequence of KAI1. The isolated KAI1 clone encoded the COOH-terminal region of the KAI1 protein, composed of aa residues 162–267. This result gives additional support to the idea that the COOH-terminal region of KAI1 is essential for the KAI1-VANGL1 interaction. Thus, we renamed VANGL1 as KAI1 COOH-terminal interacting tetraspanin (KITENIN) protein.

The Full Length of KITENIN Is Required for KAI1-KITENIN Interaction. To know which portion of KITENIN is responsible for the KAI1-KITENIN interaction, we prepared several fragments of KITENIN fused in frame to pLexA vector (Fig. 1B). A LexA-fusion protein containing the whole region of KITENIN exhibited higher strength of interaction with the COOH-terminal region of KAI1 among the several fragments of KITENIN (Fig. 1D). These data indicate that whole region of KITENIN is required to interact with COOH-terminal KAI1.

KITENIN Is Expressed in Cultured Cell Lines and Mouse Tissues. Expression of KITENIN was investigated by Western blot analysis. The expected size of KITENIN protein was ~65 kDa by an in vitro transcription-translation analysis (data not shown). Western blot analysis revealed KITENIN protein in the extracts of most human...
Fig. 2. KITENIN expression in various tissue and cell lines, and interaction and colocalization of KITENIN with KAI1. A. Western blot analysis with anti-KITENIN antibody in various normal tissues. Tissues are brain, heart, kidney, liver, lung, spleen, testis, and thymus. KITENIN (~65 kDa, indicated by an arrow) is expressed in several tissues, with an especially high level in testis. Actin is used as an evidence for the protein loading control. B. Western blot analysis of endogenous KITENIN expression in various cancer cell lines. Cell lines tested were 293 cells (human embryonic kidney); A375SM (human melanoma cells); HCT116 (human colon cancer cells); HeLa (human cervical cancer cells); KM12C, KM1214, and KM12SM (human colon carcinoma cell lines); CT-26 (mouse colon adenocarcinoma cells). KITENIN is expressed in every cell line tested except KM12SM floating cells. C. establishment of cell lines expressing KITENIN and AS-KITENIN cDNAs. Western blot analysis showing that KITENIN expression (indicated by an arrow) was higher in the CT-26/KITENIN cells than in the CT-26/parent cells, whereas KITENIN was nearly absent in the CT-26/KITENIN/AS-KITENIN cells. D. Interaction of KITENIN with KAI1 by immunoprecipitation. Cell lysates from stably expressing cell lines were reacted with anti-KAI1 antibody (C-16; Santa Cruz Biotechnology) and blotted with anti-KITENIN antibody. KITENIN protein (~65 kDa band, indicated by an arrow) was detected in the KITENIN-expressing cells. E. colocalization of KITENIN and KAI1. Fluorescent pEGFPN1-KITENIN construct was transiently transfected into KM12C cells, and immunostaining of KAI1 was performed.

cell lines and mouse tissues, such as testis, spleen, and thymus (Fig. 2, A and B). Although different levels of KITENIN were present in the various human cell lines, KITENIN was a little higher in cancer cells than normal cells (Fig. 2B). CT-26 cells showed a high level of endogenous KITENIN (Fig. 2B). Adherent cancer cells of human colon with high metastatic potential (KM1214) expressed KITENIN more than counterpart colon cancer cells with low metastatic potential (KM12C; Fig. 2B). However, KITENIN was not expressed in floating cancer cells of human colon with high metastatic potential (KM12SM; Fig. 2B). These results suggest that KITENIN is associated with adhesion. For subsequent experiments, CT-26 cells were used to establish KITENIN-expressing cell lines through stable transfection of KITENIN or AS-KITENIN cDNA.

Establishment of Cell Lines Expressing KITENIN and AS-KITENIN cDNAs. To analyze the interaction between KITENIN and KAI1 in cell motility and invasion of colon cancer cells, stable KITENIN-expressing cell lines were established using CT-26 cells. CT-26 cells were stably transfected with pcDNA-KITENIN cDNA (CT-26/KITENIN), and also CT-26/KITENIN cells were stably transfected again with pREP4-antisense-KITENIN cDNA (CT-26/KITENIN/AS-KITENIN). KITENIN expression in selected clones was then analyzed by Western blot analysis. Western blot analysis showed that KITENIN expression was higher in the CT-26/KITENIN cells than in the CT-26/parent cells, whereas KITENIN was nearly not detected in the CT-26/KITENIN/AS-KITENIN cells (Fig. 2C). This result showed that all of the transfected KITENIN gene constructs were expressed well. Other stable cell lines expressing both KAI1 and KITENIN (CT-26/KAI1/KITENIN, CT-26/KAI1/KITENIN/AS-KITENIN, and CT-26/KITENIN/KAI1) were established.

KITENIN and KAI1 Interact and Colocalize. To confirm the interaction of KITENIN with COOH-terminal KAI1 in mammalian cells, an immunoprecipitation assay was performed using stable KITENIN-expressing cell lines with anti-KAI1 antibody and blotted with anti-KITENIN antibody. KITENIN protein appeared as a ~65 kDa band. Association of KITENIN with KAI1 was observed in CT-26/parent and CT-26/KITENIN cells, whereas association of KITENIN with KAI1 disappeared in CT-26/KITENIN/AS-KITENIN cells (Fig. 2D). This result demonstrated that KITENIN interacted directly with KAI1 in the cultured cells.

To detect the localization of KITENIN in the cell, we transiently transfected KM12C cells with pEGFPN1-KITENIN cDNA. We compared the localization of KITENIN with KAI1. KAI1 is normally found at the adherens junctions of cell surface (13). The staining patterns of KAI1 overlapped with fluorescent KITENIN at the edges of the KM12C cells, indicating that KITENIN was present on the cell surface and colocalized with KAI1 (Fig. 2E, bottom).

KITENIN-Transfected CT-26 Clones Have Morphological Changes. Parental CT-26 cells transfected with KITENIN cDNA into CT-26/KITENIN had cellular morphology (Fig. 3A, top left) that was similar to that of control vector-transfected CT-26 cells (data not shown). CT-26/wild-KAI1 cells actually had a longer process than parental CT-26 cells (13); however, transfection of KITENIN into CT-26/wild-KAI1 cells resulted in a process of similar length compared with CT-26/KITENIN cells (Fig. 3A, top right). In contrast, the transfection of AS-KITENIN cDNA into CT-26/parent or CT-26/KAI1 was associated with a longer process than CT-26/parent or CT-26/KITENIN cells (Fig. 3A, bottom). The cellular morphology of CT-26/parent cells expressing AS-KITENIN resembled that of CT-26/wild-KAI1 cells. Thus, the reduced expression of KITENIN was associated with more pleiotropic morphology. This result indicated that the interaction between KAI1 and KITENIN might be able to affect the signaling cascade that mediates actin reorganization at the plasma membrane and thereby change the morphological shape of cells.

KITENIN Expression Increases Binding of Colon Cancer Cells to Extracellular Matrix (ECM). Tumor cells interact with ECM components and basement membranes, an essential initial event during the process of invasion. The adhesion of CT-26 cells to fibronectin
was higher than their adhesion to collagen I, collagen IV, or laminin (22). To analyze the effect of KITENIN expression on the binding of tumor cells to fibronectin, KITENIN-expressing cell lines were examined by a cell attachment assay. In CT-26 cells, wild-type KAI1 expression decreased cell-fibronectin binding compared with parental CT-26 cells (13). Fibronectin exhibited a significantly greater effect on the cell attachment of CT-26/KITENIN and CT-26/KAI1/KITENIN cells than on the CT-26 parental and CT-26/KAI1 cells, respectively (Figs. 3B, top; 3C). In contrast, CT-26/KITENIN/AS-KITENIN cells had significantly decreased adhesion to fibronectin compared with CT-26/KITENIN and CT-26/KAI1/KITENIN cells, respectively (Figs. 3B, bottom; 3C). Thus, the reduced expression of KITENIN decreased cell-fibronectin adhesion. Also, the fact that cell-fibronectin adhesion was even lower in CT-26/KITENIN/AS-KITENIN cells than CT-26/KAI1 showed that AS-KITENIN had a greater effect on reducing cell-ECM binding than did KAI1 (Fig. 3C). Comparison of cell-fibronectin attachment among CT-26/parent, CT-26/KAI1, and CT-26/KAI1/KITENIN cells indicates that the positive role of KITENIN in cell-ECM adhesion is greater than the negative role of KAI1 (Fig. 3C).

KITENIN and AS-KITENIN Differentially Affect Cell Invasiveness. The invasiveness of tumor cells is one of several important properties necessary for metastasis. To analyze the effect of KAI1 and KITENIN on in vitro cell invasion, a cell invasion assay was carried out using the Transwell migration apparatus. KAI1 interacts with integrin α3β1, which is a multiple ligand receptor that binds laminin, fibronectin, and kalinin/epiligrin (5, 10). Thus, cell motility was measured using fibronectin as a chemotactic factor. CT-26/KITENIN cells showed significantly increased in vitro motility and invasive potential induced by fibronectin compared with CT-26 parental or CT-26/KAI1 cells (Fig. 3D, top; 3E). Furthermore, CT-26/KITENIN and CT-26/KAI1/KITENIN/AS-KITENIN cells had lower invasive potential compared with parent CT-26, CT-26/KITENIN, or CT-26/KAI1/KITENIN cells (Fig. 3D, bottom; Fig. 3E). These results indicate that having less KITENIN results in less invasive potential, despite stimulation by chemotactic factors. In particular, CT-26/KAI1/KITENIN cells showed significantly greater invasive ability than CT-26/KAI1 cells. In contrast, CT-26/KITENIN/KAI1 cells did not show the decreased invasive ability, but rather showed increased invasion, similar to the level for CT-26/KAI1/KITENIN cells (Fig. 3E). These results indicate again that KITENIN can overcome the suppressive action of KAI1 on cell invasion, just as it did on cell-ECM binding (Fig. 3C).

KITENIN Expression Inversely Correlates with Expressions of KAI1 and Other Metastasis Suppressor Genes. The forced expression of KITENIN in the CT-26/parent and CT-26/KAI1 cells resulted in the decreased expression of wild-type KAI1 mRNA (Fig. 4A) or KAI1 protein (Fig. 4B) compared with nontransfected corresponding cells. However, the expression of KAI1 mRNA or KAI1 protein seemed to restore in the CT-26/KITENIN/AS-KITENIN and CT-26/KAI1/KITENIN/AS-KITENIN cells compared with CT-26/KITENIN and CT-26/KAI1/KITENIN cells, respectively (Fig. 4, A and B). Also, the CT-26 cells stably expressing spliced-KAI1 showed higher KITENIN expression than wild-type KAI1 expressing cells. Thus, there was an inverse correlation between the expressions of KITENIN and KAI1. We also examined whether there is an inverse relationship between the expression of KITENIN and other metastasis suppressor genes. TIMP2 expression was a little decreased, but MMP9 was increased after the overexpression of KITENIN (Fig. 4A). However, nm23 and KISS1 transcripts, the other reported metastasis suppressor gene, were increased after the AS-KITENIN cDNA transfection (Fig. 4A). Also, in the Western blot analysis, the expression of nm23 decreased in CT-26/KITENIN cells but increased in CT-26/KITENIN/AS-KITENIN.
KITENIN might influence the metastasis of tumor cells, because results indicated that KITENIN could induce an increase in tumor inoculated with CT-26/KITENIN/AS-KITENIN cells (Fig. 5B, middle), whereas the liver was intact in the mice injected with CT-26/parent cells (0 of 3 mice; Fig. 5B, top) and CT-26/KITENIN/AS-KITENIN cells (0 of 3 mice; Fig. 5B, bottom). Three weeks after cell injection, liver metastases were detected in mice injected with CT-26/parent cells (3 of 3 mice; Fig. 5B, top) as well as in mice with CT-26/KITENIN cells (3 of 3 mice; Fig. 5B, middle), whereas the liver was still intact in the mice injected with CT-26/KITENIN/AS-KITENIN cells (0 of 3 mice; Fig. 5B, bottom). Five weeks after injection, liver metastases were still not detected in mice injected with CT-26/KITENIN/AS-KITENIN cells (0 of 3 mice; Fig. 5B, bottom). We also observed small metastatic foci in the lungs of CT-26/parent (1 of 3 mice) and CT-26/KITENIN (1 of 3 mice) cell-injected mice. Thus, liver metastasis was promoted in the mice injected with CT-26/KITENIN cells compared with the mice injected with CT-26/parent cells, whereas liver metastases were not detected in mice injected with CT-26/KITENIN/AS-KITENIN cells after 5 weeks of cell injection.

Mice Inoculated with KITENIN-Expressing CT-26 Cell Lines Have Greater Expression of Spliced KAI1 and KITENIN in Metastatic Tissues. We also examined whether there were differences in the expression of KITENIN and KAI1 in the primary and metastatic tumors. The tumor and peritoneal tissues obtained from the mice in the tumor volume measurement group after 5 weeks were immunoblotted with anti-KITENIN and anti-KAI1 antibody. We observed increased expression of spliced-KAI1 and KITENIN in the metastatic liver and lung tissues, as well as tumor tissues of the mice injected with CT-26/parent and CT-26/KITENIN cells (Fig. 5C). In particular, the expression of spliced-KAI1 was dominant in metastatic liver tissue in the mice with CT-26/parent (Fig. 5C, top) and CT-26/KITENIN cells (Fig. 5C, middle). In contrast, KITENIN and spliced-KAI1 were not expressed in the lung and liver tissues of the mice with CT-26/KITENIN/AS-KITENIN cells (Fig. 5C, bottom). Together with the histological data, this result indicated that KITENIN was associated with promoting metastasis in vivo.

Gastric Cancer Patients Have Higher Expression of KITENIN in Tumor Mucosa and Metastatic Tissues. Samples of normal mucosa and metastatic tumor tissues were obtained from 13 gastric cancer patients. The metastatic liver tissue had higher expression of KITENIN than did the normal extraneoplastic mucosa (13 of 13, Fig. 5D). Similarly, the regional metastatic lymph nodes had higher expression of KITENIN than did nonmetastatic lymph nodes. Thus, the results in gastric cancer specimens also support the idea that KITENIN promotes cancer metastasis.

**DISCUSSION**

The localization and clustering of cell surface receptors to specific subcellular positions can be critical for their proper signaling. We hypothesized that the interaction between the tetraspanins might be able to affect the signaling cascade that controls actin reorganization and thereby change the morphology and motility of cells (Fig. 6). The integrin α3β1-KAI1 complex was reported to suppress fibronectin/α3β1-induced cell invasion through inhibition of the cytoskeletal system (12). The conventional isoforms of protein kinase C participate in cell adhesion mediated by β-integrins and activate protein kinase C-α interaction with the COOH-terminal region of KAI1 (23). One
possibility is that KITENIN induces cell invasion by interfering in the metastasis-suppressive function of KAI1 through the interaction with KAI1. The CT-26/KITENIN cells showed a higher adhesiveness to fibronectin than CT-26/parent cells, and KITENIN did not interact with integrin α3β1 (data not shown). The binding of KITENIN with KAI1 might interfere in the interaction of KAI1 and integrin α3β1, which then mediates increased cell adhesion and invasion. Perhaps when wild-type KAI1 interacts with KITENIN it loses its ability to suppress metastasis.

The other possibility is that KITENIN activates a cytoplasmic signaling pathway toward invasion through its putative PDZ domain-binding motif at the COOH-terminal end. Signaling proteins that have PDZ domains may bind to the COOH-terminal end of KITENIN and associate with other functional modules. These interactions may be involved in the formation of multimeric protein complexes that influence cytoskeletal proteins or downstream signaling effectors. Therefore, the sum of these processes ultimately affects the expression of a number of additional genes and gene products that play a role in the invasive properties of the tumor cell. In fact, preliminary results from cDNA array gene expression analysis show differences in the gene expression profiles between the CT-26/parent and CT-26/KITENIN cells. Among the genes differentially expressed were several encoding proteins believed to play a role in invasion and metastasis.4

In a previous study (13), we observed that spliced-KAI1, which lacks the COOH-terminal region, had less metastasis suppressor function. We also documented higher expression of spliced-KAI1 in the metastatic tissues of gastric cancer patients who had poor prognosis. In this study, we also observed that the expression of

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4 Unpublished observations.
KITENIN and spliced-KAI1 were increased in the metastatic lung and liver tissues, as well as tumor tissues of mice, and CT-26 cells stably expressing spliced-KAI1 showed higher KITENIN expression than wild-type KAI1 expressing cells. Also, we found that nm23, KISS1, and TIMP2 mRNA expressions were increased in KITENIN underexpressing cells. Our data reveal that depending on the expression level of KITENIN, the expression of different metastasis suppressor genes is affected. These results together indicate that KITENIN is inversely associated with expression of metastasis suppressor genes, and KITENIN might directly or indirectly affect the loss of metastatic repression. Thus, the study of the action mechanism of KITENIN may shed new light on the negative regulation of metastasis suppressors during malignant progression of cancer. The present results suggest that spliced-KAI1 does not interact with KITENIN and thereby may not effectively suppress the metastasis-enhancing effects of KITENIN. Therefore, the expression of KITENIN and/or spliced KAI1, as well as a decrease in the level of wild-type KAI1, could be used as markers for poor prognosis and metastasis in a variety of cancers.

We observed that the ability of AS-KITENIN to reduce cell-ECM binding was greater than that of KAI1, and CT-26/KAI1/KITENIN cells showed significantly greater invasive ability than CT-26/KAI1 cells, whereas CT-26/KITENIN/KAI1 cells did not show the decreased invasive ability, but rather showed increased invasion just as CT-26/KAI1/KITENIN cells. These results indicate that the positive effect of KITENIN is greater than the suppressive function of KAI1 on adhesion and invasion. It suggests that KITENIN can overcome the suppressive action of KAI1 on cell invasion. Thus, it seems that an antisense KITENIN strategy would be more powerful than gene delivery for overexpression of KAI1 for the therapeutic inhibition of metastasis. Moreover, there was a positive relationship between the expression of KITENIN and the presence of distant metastasis in vivo. After all, antisense KITENIN strategy and gene cassette for overexpression of KAI1 can be used together to inhibit the distant metastasis in various cancers.

The KITENIN/VANGLI 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 (16–18). In addition, a putative prostate cancer susceptibility gene is mapped to human chromosome 1p13-q32 (19). These results indicate that KITENIN is located in a human chromosomal locus deleted, mutated, or rearranged in several types of human cancer. In addition, VANGLI (Strabismus 2) was also cloned as a human homologue of Drosophila tissue polarity gene strabismus/Van Gogh (24). It is highly expressed in gastric and pancreatic cancer cell lines, whereas significantly down-regulated in several cancer cell lines and primary tumors. A Xenopus homologue of VANGLI regulates negatively the WNT-β-catenin signaling pathway (25) in which loss-of-function mutations of these negative regulators lead to carcinogenesis (26). On the basis of functional aspects and human chromosomal loci, VANGLI was predicted to be potent tumor suppressor gene candidate. However, our present results indicate that KITENIN/VANGLI acts as a metastasis-inducing gene. Thus, additional study is needed to determine the mechanisms by which KITENIN enhances invasion and distant metastasis in various cancers.

EW12/PGRIL, an immunoglobulin superfamily member, was reported recently to associate with KAI1/CD82 (27). Consistent with the wide distribution of KAI1/CD82, EW12/PGRIL is expressed ubiquitously in human tissues. Overexpression of EW12/PGRIL in Du145 metastatic prostate cancer cells inhibits cell migration on both fibronectin- and laminin-coated substrates, and EW12/PGRIL synergizes KAI1/CD82 in inhibiting cell migration, indicating that EW12/PGRIL is likely required for KAI1/CD82-mediated suppression of cancer cell migration. Although the functions of KITENIN and EW12/PGRIL, two KAI1-interacting transmembrane proteins, are opposite in regards to cell migration, they both contribute to the elucidation of the mechanism of KAI1/CD82-mediated metastasis suppression.

In summary, our results indicate that the expression of KITENIN affects cellular morphology and motility to facilitate cell invasion, and thereby enhances metastasis. These effects may be derived from the decreased metastasis suppressor functions of KAI1 and/or other metastasis suppressor genes and also from a cytoplasmic signaling pathway that shifts the invasive/anti-invasive balance toward invasion.

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KAI1 COOH-Terminal Interacting Tetraspanin (KITENIN), a Member of the Tetraspanin Family, Interacts with KAI1, a Tumor Metastasis Suppressor, and Enhances Metastasis of Cancer

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