

Expression of KITENIN, a KAI1/CD82 binding protein and metastasis enhancer, in bladder cancer cell lines: Relationship to KAI1/CD82 levels and invasive behaviour

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Abstract. KITENIN is a newly identified binding partner of the KAI1/CD82 metastasis suppressor. Recent studies using a mouse model of colon cancer, have suggested that KITENIN might be a metastasis enhancer whose functions are modulated by an interaction with KAI1/CD82. To begin exploration of the possible importance of KITENIN to human cancer, we examined KITENIN mRNA (by RT-PCR) and protein expression (by Western blotting) in a large series of bladder cancer cell lines, and then compared these levels to the expression of KAI1/CD82 and of previously determined *in vitro* invasive behaviour of these same cancer cell lines. We report that KITENIN was uniformly expressed in all cancer cell lines, but those lines in which KAI1/CD82 was not detected, had a higher *in vitro* invasive ability and altered actin organisation (as determined by fluorescence microscopy), than those lines in which KAI1/CD82 was present. Our data suggest that the relationship between KITENIN and KAI1/CD82 may be an important determinant of tumour cell behaviour.

Introduction

A large body of evidence suggests that down-regulation of KAI1/CD82 expression in invasive and metastatic disease, is an important step in the progression of many human malignancies, including prostate and bladder cancer (1). An important avenue of current research is to determine the functional significance of reduced KAI1/CD82 protein expression to the behaviour of cancer cells. The protein encoded by this gene is a 267 amino acid transmembrane glycoprotein of the TM4 super-family (TM4SF) (2). Interactions between

KAI1/CD82, other TM4SF proteins and transmembrane proteins, such as integrins, form a multi-molecular complex termed the 'tetraspanin web' (3). Within this context, KAI1/CD82 is proposed to modulate signals controlling the interactions between cells, and between cells and their extra-cellular environment. Consistent with this function, and a role as a metastasis suppressor, the loss of KAI1/CD82 protein expression is associated with dramatically increased *in vitro* invasive and *in vivo* metastatic behaviour of tumour cells (4-7). Although it is clear that these effects are associated with reduced interactions between cells, and altered interactions between cells and specific extra-cellular matrix components, the underlying basis for these changes is unknown. Recently, a novel KAI1/CD82-binding protein called KITENIN was identified (8). KITENIN is also a TM4SF protein and binds a carboxyl region of KAI1/CD82. A loss of KITENIN binding to KAI1/CD82 abrogates metastasis suppressor activity of KAI1/CD82 (8,9). Interestingly, overexpression of KITENIN increases tumourigenicity, invasiveness and adhesion to fibronectin, of mouse colon cancer cells (8) and specific knock-down of KITENIN inhibits tumour metastasis in a mouse model of colon cancer (10).

Taken together, these data have supported an idea that KITENIN may function as a metastasis enhancer, and that the role of KAI1/CD82 binding, is to modulate this activity. Some support for this model has been obtained from preliminary observations of an inverse relationship between levels of these two proteins in mouse colon cancer cells (8). In a human context, there are scarce data, but overexpression of KITENIN has been reported in metastatic gastric tumours compared with adjacent normal tissue (8). In order to examine the possible importance of KITENIN to human cancer, we have first examined KITENIN levels in a large series of bladder cancer cell lines, for which we have also studied expression of its binding partner, KAI1/CD82, and whose *in vivo* and *in vitro* characteristics we have previously determined. We report that levels of KITENIN were similar in all cell lines examined, but importantly, that loss of KAI1/CD82 in KITENIN-positive cells was clearly correlated with increased invasive ability. These data support the idea that down-regulation of KAI1/CD82 expression may be a major determinant of tumour cell behaviour because a negative regulator of KITENIN function is lost.

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Materials and methods

Cells and cell culture. Growth conditions for bladder cancer cell lines BL13, BL28, BL17/0/x1, BL17/2 and sublines, BL17/5, T24, RT112, J82, 5637 and the SV40-transformed urothelial cell line SV-HUC1 have been described previously (11). All lines were maintained in RPMI-1640 medium containing 10% FBS and 50 U/ml P/S. Media and supplements were from Gibco BRL (Life Technologies, Melbourne, Victoria, Australia).

RNA isolation and cDNA synthesis. Total-RNA was isolated from cell cultures when 80-90% confluent, using TriReagent (Sigma-Aldrich Pty Ltd., Castle Hill, NSW, Australia) as per manufacturer's instructions, but with an extra-phenol and phenol/chloroform extraction to remove residual DNA. For each sample, 2 μ g RNA was used to prepare cDNA in a reaction containing 19 μ l RNase-free water, 1 μ l of 100 μ M random hexamer, 6 μ l of MMLV-RT 5X reaction buffer and 1.5 μ l of 10 mM dNTP mix. After incubation at 72°C for 3 min, 0.6 μ l RNasin (1 U/ μ l) and 2 μ l MMLV reverse transcriptase (13.3 U/ μ l) were added and incubation continued at 37°C for 2 h, then 50°C for 10 min, before finishing with 95°C for 5 min. In each subsequent PCR reaction, 1 μ l of cDNA was used.

RT-PCR analysis. A 291-bp KITENIN mRNA fragment was amplified with forward primer, 5'-GGAATTCCATTCGAAA AAATCTCA-3' and reverse primer 5'-CCGCTCGAGGCC AGGTAGCGTTTGCA-3'. As a control for RNA integrity and loading into cDNA reactions, a 600-bp GAPDH fragment was amplified using primers 5'-CCACCCATGGCAAA TTCCATGGCA-3' and 5'-TCTAGACGGCAGGTCAGGTC CACC-3'. Reactions contained 2.5 μ l of 10X reaction buffer, 0.25 μ l of 25 mM dNTP mix, 0.2 μ l of each primer at 20 pmol/ μ l, 1 μ l cDNA, 0.5 μ l Taq (5 U/ml) and 10 μ l 25 mM MgCl₂ in a total volume of 25 μ l. In preliminary experiments, conditions for linear amplification of KITENIN and GAPDH were established in a Hyaid Touchdown Thermocycler. Linear amplification was achieved between 29-38 (KITENIN) and 18-27 (GAPDH) cycles of amplification. In subsequent analyses, KITENIN and GAPDH were amplified using 94°C 4 min, 94°C 30 sec, 58°C 30 sec and 72°C 30 sec (24 cycles for GAPDH; and 29, 32, 35, 38 cycles for KITENIN), with a final step of 72°C 10 min. All amplifications were performed at least three times, with similar results. Relative levels of KITENIN were obtained by normalising to GAPDH levels. Results are presented as means \pm standard error.

Western blot analysis. Total cell lysates were prepared by the Pierce M-Per system (Pierce Endogen, Rockford, IL) as described by the manufacturer. Protein concentrations were determined by BCA assay (Pierce Endogen). Extracts containing 10 μ g of protein, were separated in 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. After blocking with PBSTM (0.1% Tween-20 in PBS, PBST; containing 5% skimmed-milk) for 1 h at room temperature, filters were probed overnight at 4°C with a rabbit polyclonal serum to KITENIN (1/3000 in PBSTM) (8), a commercially available anti-KAI1/CD82 polyclonal serum (1/1000 in PBSTM; Santa Cruz, C6), or a mouse monoclonal antibody

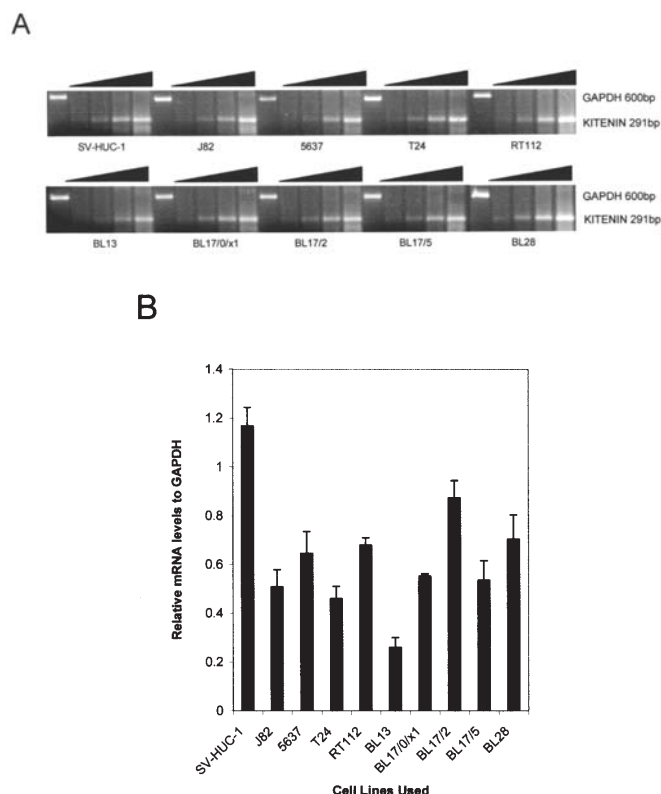


Figure 1. KITENIN mRNA levels in bladder cancer cell lines. Total-RNA was prepared from cultures of the indicated cancer cell lines, converted to cDNA and used in PCR reactions for analysis of KITENIN and GAPDH mRNA levels, as described in Materials and methods. (A), For all samples, GAPDH amplification used 24 cycles and KITENIN amplification used 29, 32, 35 and 38 cycles. (B), For determination of relative KITENIN mRNA levels, the ratio of KITENIN/GAPDH expression was determined using 24 cycles of GAPDH amplification and 35 cycles of KITENIN amplification, which was in the centre of the linear amplification phase for these primers. Data are mean \pm SE of three independent amplifications.

to β -actin (1/1330 in PBSTM; Sigma-Aldrich) as control for protein loading. After rinsing in PBST (3x5 min), filters were incubated with a 1/5000 dilution in PBSTM, of an appropriate HRP-linked secondary antibody, for 1 h at room temperature. After final rinsing with PBST (3x5 min) to remove unbound antibody, specific proteins were visualised by the enhanced chemiluminescence method (Amersham Biosciences, Castle Hill, NSW, Australia) and exposure to Kodak XOMat film.

Fluorescence microscopy. Cells growing on glass cover slips were washed with PBS before fixing in 3.7% paraformaldehyde in PBS, for 10 min at room temperature. After a further wash with PBS, cells were permeabilised with acetone cooled to -20°C, for 5 min. After washing with PBS, cells were blocked for 30 min in PBS containing 1% BSA before each cover slip was covered with 200 μ l of PBS +1% BSA containing 5 μ l of Texas-red conjugated phalloidin (Molecular Probes, Eugene, OR) for 20 min at room temperature. After washing in PBS, each cover slip was air dried and mounted onto glass slides, and sealed with clear nail varnish. Fluorescence was viewed with a 40X oil immersion lens using an Olympus BX51 microscope, fitted with epi-fluorescence and appropriate filters for viewing Texas-red. Images were captured using Q-capture software (QImaging, Burnaby, BC, Canada).



Cell line (ref.) ^a	Tumourigenicity <i>in vivo</i> (\pm) ^b	Relative <i>in vitro</i> invasive ability (12)	KITENIN (\pm) ^c	KAI1/CD82 (\pm) ^c
SV-HUC-1 (13) ^d	-	+	+	+
T24 (14)	+	+	+	+
5637 (15)	+	-	+	+
J82 (16)	+	+	+	+
BL13/0 (17)	+	++	+	+
BL17/5 (unpublished data)	+	+	+	+
BL28 (18)	+	+	+	+
RT112 (19)	+	+++	+	+
BL17/0/x1 (20)	+	+++++	+	-

^aAll tumour cell lines derived from transitional cell carcinomas (TCCs). ^bTumourigenic ability in mice except for T24 (hamster cheek).

^cKITENIN and KAI1/CD82 protein expression determined by Western blotting, as described in Materials and methods. ^dNon-tumourigenic, SV40 transformed normal uro-epithelial cells.

Results

KITENIN expression in bladder cancer cell lines. KITENIN mRNA levels were first examined in a series of nine established bladder cancer cell lines (Fig. 1; Table I) and compared with levels in a non-tumourigenic transformed uro-epithelial cell line (SV-HUC-1). KITENIN mRNA levels were reduced in all cancer cell lines compared with SVHUC-1, with lowest levels in BL13 (20% of levels in SVHUC-1). There was little variation between remaining cancer lines, which all possessed 40-60% of KITENIN mRNA levels in SVHUC-1.

We then examined KITENIN protein levels by Western blotting, using a previously characterised antibody to KITENIN (8). Consistent with PCR data, KITENIN protein expression was high in SVHUC-1, with lower but similar levels of protein present in 8/9 tumour cell lines (Fig. 2A). The only exception to this finding was BL28, in which KITENIN protein was poorly expressed. We also examined levels of KAI1/CD82 protein in these cell lines to determine how expression of KAI1/CD82 compared with KITENIN, and then examined how the presence of these two proteins related to previously determined *in vitro* invasive abilities (12). In contrast to KITENIN, but consistent with a previous study of KAI1/CD82 mRNA levels in these cell lines (12), levels of KAI1/CD82 protein varied widely (Fig. 2A, summarised in Table I). High levels of KAI1/CD82 were present in SVHUC-1, 5637, T24, RT112, BL13, BL28 and BL17/5, with much lower levels in J82. KAI1/CD82 was not detected in BL17/0/x1. When compared with previously determined tumourigenic and *in vitro* invasive ability (12) (Table I), these Western blotting data indicated that there was no relationship between levels of KITENIN, KAI1/CD82, and tumourigenic potential. However, 7/8 cell lines where KITENIN and KAI1/CD82 expression was detected had weak invasive ability, even if KITENIN (BL28) or KAI1/CD82 (J82) levels were very low. The exception to this rule was RT112, which was highly invasive even though it expressed both KITENIN and KAI1/CD82

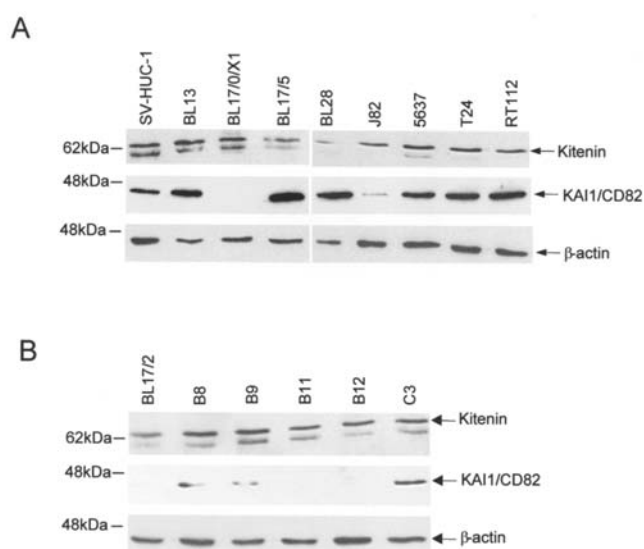


Figure 2. KITENIN and KAI1/CD82 protein levels in bladder cancer cell lines. Lysates of cultured bladder cancer cells (A) or BL17/2 and five clonally derived sublines (B), were examined by Western blotting for KITENIN, KAI1/CD82 and β -actin, as described in Materials and methods. The identity of KITENIN as the uppermost of two bands was verified by comparison with the size of an *in vitro* translated KITENIN protein (data not shown).

strongly. Interestingly, the only cell line in which KITENIN was present and in which KAI1/CD82 was not detected (BL17/0/x1), was also highly invasive, suggesting that the absence of KAI1/CD82 in the presence of KITENIN, might be an important factor in invasive ability.

KITENIN and KAI1/CD82 in clonally derived cell lines with distinct properties. To further examine how levels of KITENIN and KAI1/CD82 might relate to tumour cell behaviour, we examined KITENIN and KAI1/CD82 protein levels in BL17/2 and five clonally derived sub-lines, whose individual *in vitro*

Table II. Characteristics of BL17/2 and clonally-derived cell lines examined for KITENIN and KAI1/CD82.

Cell line ^a (21)	Tumourigenicity in nude mice	Invasive ability <i>in vivo</i> (20)	Relative <i>in vitro</i> invasive ability (12)	KITENIN (±) ^b	KAI1/CD82 (±) ^b
BL17/2 ^c	+	-	++++	+	-
B11	-	-	++++	+	-
B12	+	-	+++	+	-
B8	+	+	++	+	+
B9	+	+	+++	+	+
C3	-	-	++	+	+

^aAll tumour cell lines derived from transitional cell carcinomas (TCCs). ^bKITENIN and KAI1/CD82 protein expression determined by Western blotting, as described in Materials and methods. ^cBL17/2 is a cell line derived from the second serial passage in nude mice, of a xenograft from a grade III, T4b tumour biopsy used to generate the BL17/0/x1 cell line (Table I).

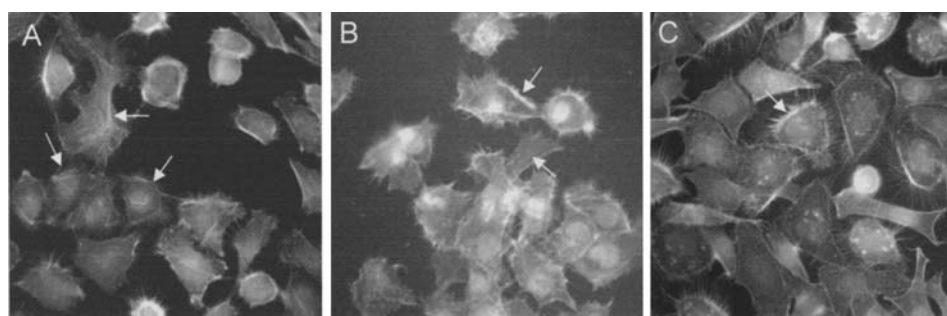


Figure 3. Fluorescence staining of actin filaments in (A) BL28, (B) T24 and (C) BL17/0/x1 bladder cancer cell lines. BL28 and T24 express both KITENIN and KAI1/CD82, whereas BL17/0/x1 expresses KITENIN but not KAI1/CD82. Cells were fixed, stained with Texas-red phalloidin, and examined as described in Materials and methods. Arrows indicate staining of actin filaments. Magnification x400.

and *in vivo* invasive abilities have been determined (Table II) (12,21). All lines examined possessed similar levels of KITENIN (Fig. 2B). In contrast, KAI1/CD82 was only detected in 3/6 lines. Very low KAI1/CD82 levels were present in B8 and B9, and KAI1/CD82 was only clearly seen in C3. As indicated in Table II, there was no relationship between KITENIN and KAI1/CD82 protein levels, and tumourigenic ability, however, all had relatively high invasive ability *in vitro*. Importantly, those lines in which KAI1/CD82 was present (B8, B9, C3), generally had a lower invasive ability than those lines in which KAI1/CD82 was not detected (BL17/2 parent, B11 and B12). Of interest, the two cell lines with an ability to invade bone, in an *in vivo* model of metastasis (B8 and B9), both expressed KITENIN and very low levels of KAI1/CD82.

When data from all cell lines were considered, our findings suggested that the presence of both KITENIN and KAI1/CD82 was associated with a weak *in vitro* invasive ability (9/11 lines). In contrast, a loss of KAI1 in cells expressing KITENIN was associated with high invasive ability (4/4 lines).

Loss of actin filaments in representative KITENIN-positive but KAI1/CD82-negative bladder cancer cell lines. Given that KITENIN is homologous to VANG1, a protein proposed

to have a role in regulating architecture of the actin cytoskeleton (8), we were interested to examine localisation of KITENIN and KAI1/CD82 within our bladder cancer cell lines, as well as the overall organisation of the cytoskeleton. In spite of exhaustive attempts, we found that both KITENIN and KAI1/CD82 antibodies used for Western analysis were unsuitable for immunofluorescence analysis (data not shown). In order to determine if we could detect any gross changes to the actin cytoskeleton, which might be related to KITENIN and KAI1/CD82, we stained representative lines BL28 and T24 (KITENIN-positive and KAI1/CD82-positive) with Texas-red phalloidin (which specifically stains polymeric actin) and then compared staining to that present in BL17/0/x1 (KITENIN-positive but KAI1/CD82-negative). We observed many actin filaments throughout the cytoplasm, and cell peripheries in both BL28 and T24 cells (Fig. 3A and B). In contrast, we found few filaments in BL17/0/x1 cells, and these tended to be thinner than those in either BL28 or T24 cells. The cytoplasm appeared devoid of filaments, which were confined to the cell periphery (Fig. 3C). These data suggested the possibility that a loss of KAI1 in cells expressing KITENIN, might be associated with an altered cytoskeletal structure, which might then contribute to an increased invasive ability.

In this study, we examined expression of a newly identified binding partner of the KAI1/CD82 metastasis suppressor protein, called KITENIN, in a series of 15 bladder cancer cell lines, and investigated a possible association between expression of KITENIN, KAI1/CD82 and bladder tumour cell invasive behaviour. We report herein that KITENIN mRNA and protein levels were similar in all cell lines examined, and there was no association between KITENIN expression and a more invasive phenotype either *in vitro* or *in vivo*. However, a loss of KAI1/CD82 protein expression in four of these cell lines was clearly associated with increased *in vitro* invasive ability. Whilst these data do not provide conclusive evidence for the functional importance of an interaction between KITENIN and KAI1/CD82 (8,10), our findings are consistent with the possibility that KAI1/CD82 functions to modulate invasive behaviour promoted by KITENIN. This conclusion is also supported by results of more extensive studies, in which overexpression or specific knock-down of KITENIN expression was associated with increased or decreased invasive and metastatic potential, respectively, in a mouse model of colon cancer (8,10).

How might the interaction between KITENIN and KAI1/CD82 modulate tumour cell behaviour? The *KITENIN* gene, was originally isolated as *VANGL1*, and is a human homologue of the *Drosophila* tissue gene *strabismus/Van Gogh (Vang)* (22), whose encoded protein is a component of a non-canonical *wnt*-signalling pathway in *Drosophila*. Importantly, this pathway functions to modulate organisation of the actin cytoskeleton essential for planar cell polarity (23). Altered cytoskeletal organisation might reasonably be expected to have profound effects on cell-cell and cell-matrix interactions, which could, in turn, contribute to altered tumour cell behaviour. Interestingly, we found that in cell lines expressing KITENIN, but not KAI1/CD82, there were dramatically reduced numbers of actin filaments. Of relevance, previous studies have shown that overexpression of KITENIN increases cellular adhesion to fibronectin (8), whilst down-regulation of KAI1/CD82 is associated with both altered adhesion of tumour cells to fibronectin and markedly reduced cell-cell interactions (4,12), suggesting that the interaction between KITENIN and KAI1/CD82 might be an important factor in modulating these interactions.

Our results for RT112 cells indicated that increased invasive ability can occur in cells expressing both KITENIN and KAI1/CD82. One possible explanation for this observation is that the interaction between KITENIN and KAI1/CD82 might be abrogated in these cells. Altered glycosylation (24) and palmitoylation (25) of KAI1/CD82 impair the ability of KAI1/CD82 to function as a metastasis suppressor. Although there is no data available concerning these modifications to KITENIN, it is conceivable that altered patterns of glycosylation and palmitoylation on KAI1/CD82 may prevent binding to KITENIN, which then results in a loss of the modulating effect of KAI1 on the metastasis promoting function of KITENIN. Important goals of future research will be to define more precisely the functions of KITENIN and the effects of post-translational modifications on the interaction between KITENIN and KAI1/CD82.

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