

An alternatively spliced KAI1 mRNA is expressed at low levels in human bladder cancers and bladder cancer cell lines and is not associated with invasive behaviour

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Abstract. Levels of the KAI1 metastasis suppressor are reduced in advanced stages of many human cancers, leading to the loss of KAI1 function. A recent study has suggested that the loss of KAI1 function may also occur via alternative splicing of KAI1 mRNA which deletes an exon encoding a critical 28 amino acids from the protein. Using PCR, 20 bladder tumours at differing stage and grade, a non-tumourigenic urothelial cell line (SV-HUC-1) and 17 bladder cancer cell lines were examined for expression of this alternatively spliced (AS) KAI1 mRNA. Full-length KAI1 mRNA was present in all tumour samples and low levels of AS KAI1 mRNA in 15/20 samples. There was no association between the presence or absence of AS mRNA and clinicopathological characteristics of these tumours. Low levels of AS KAI1 mRNA were present in SV-HUC-1 and 14/17 bladder cancer cell lines. There was no association between the presence or absence of AS KAI1 mRNA and tumourigenicity, or *in vivo* invasive abilities of these cell lines. In all cell lines expressing AS KAI1 mRNA, levels were 3- to 5-fold lower than levels of wild-type mRNA, irrespective of wild-type mRNA levels. Low levels of an alternatively spliced form of KAI1 mRNA are present in most bladder cancer tumours and tumour cell lines, but are not associated with invasive behaviour.

Introduction

Reduced levels of the KAI1 metastasis suppressor (1) are common in the advanced stages of many cancer types (reviewed in 2,3) and associated with both a poor patient

outcome (4,5) and disease recurrence (6,7). A large body of experimental evidence has shown the importance of KAI1 to tumour cell behaviour and that a loss of KAI1 function is a major factor in the invasive and metastatic phenotype (reviewed in 2).

Studies of mechanisms underlying KAI1 down-regulation indicate that loss of heterozygosity (8), mutations within the KAI1 gene (8) and KAI1 promoter hyper-methylation (9,10) are unlikely to be involved. However, transient transfection approaches have identified an enhancer within the KAI1 promoter, to which specific proteins (p53, AP1, and AP2) bind what appears to be important for the determination of KAI1 levels (11,12). Other studies have linked the down-regulation of KAI1 in prostate cancer cells to specific chromatin remodelling complexes (13).

Of interest, a loss of KAI1 expression does not occur in all advanced cancers, e.g. 20% of invasive bladder tumours remain KAI1 positive (14). Additionally, an increased overall KAI1 expression is associated with some colon cancer metastases (15) and leukaemias (16). Biphasic expression of KAI1 has been observed in cases of pancreatic and prostate cancers (17,18) and KAI1 levels appear unchanged in non-metastatic versus metastatic oesophageal and gastric cancers (19). Recently, a novel mechanism was proposed that might contribute to a loss of KAI1 function, even if KAI1 is not down-regulated (20). Analysis of KAI1 mRNA expression in gastric cancers by PCR identified a shorter, spliced variant (AS), which was a missing sequence of an exon encoding for 28 amino acids in the large extracellular loop of the KAI1 protein. When compared with full-length KAI1, the truncated protein formed weaker interactions with $\alpha 3 \beta 1$ integrin, did not co-localise with E-cadherin and was unable to bind KITENIN, a protein whose functions in promoting metastasis are normally inhibited by KAI1 (20,21). Stable over-expression of the AS-encoded KAI1 protein in mouse colon cancer cells had no effect on cell proliferation, but was associated with increased *in vitro* invasion (20). Importantly, in an *in vivo* mouse model of colon cancer, even with full-length KAI1 present, expression of the shorter KAI1 protein was associated with increased tumourigenicity and a wider spectrum of tissues targeted by metastasis (20). In human gastric cancer samples, the

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Table I. Incidence of a KAI1 splice variant in bladder tumour samples.

Tumour details	No. of samples	Wild-type KAI1 (%)	AS KAI1 (%)
Superficial (Ta/T1)	7	7/7 (100)	5/7 (71)
Invasive (T2/T3)	13	13/13 (100)	10/13 (77)
G1/2	9	9/9 (100)	7/9 (78)
G3/4	11	11/11 (100)	8/11 (73)
Lymph node status			
pN0	18	18/18 (100)	13/18 (72)
pN+	2	2/2 (100)	2/2 (100)
Recurrence			
0	11	11/11 (100)	9/11 (82)
1	9	9/9 (100)	6/9 (67)

presence of AS variant was associated particularly with metastatic disease having a poor prognosis (20), raising the possibility that alternative splicing of KAI1 might also be an important marker of cancer progression.

In this study, we have used a series of tumour specimens and tumour-derived cell lines, to determine if there is any evidence of the AS form of KAI1 in bladder cancer. We report here that low levels of AS KAI1 were present in both tumours and tumour cell lines irrespective of overall KAI1 levels, but that expression of AS was not associated with invasive disease or with a poor clinical outcome.

Materials and methods

Tissue samples. Tissue samples were derived from patients who underwent surgery at the Department of Urology, Heinrich-Heine University in Duesseldorf, Germany between 1993 and 2000. Immediately after resection, tumours were macro-dissected and snap-frozen in liquid nitrogen, before storage at -80°C . All samples were confirmed as tumour tissue by the examination of adjacent fresh frozen and paraffin-embedded sections. Haematoxylin/eosin staining of samples for molecular analysis was used to determine relative amounts of tumour cells, benign epithelium and stroma. In specimens for RNA extraction, tumour tissue constituted $>90\%$ of superficial and 50-70% of invasive samples. Of the patients, 16 were male and 4 were female. The mean age was 71 years (range 61-90 years). Tumour, node, metastasis (TNM) designations were made according to the UICC 2002 classification (22). Histological grading was performed as previously described (23). Seven tumours were superficial and patients were treated by transurethral resection; six patients were staged as pTa and one as pT1 (superficial invasive); of the pTa tumours, two were graded as Grade I and four as Grade II. After a mean follow-up of 32 months, 3 of these patients recurred. None progressed to muscle-invasive disease. Another 13 patients underwent radical cystectomy for muscle-invasive or locally advanced bladder cancer. This comprised 4 pT2, 8 pT3 and 1 pT4 tumours; two patients were found on pathological examination to have metastatic

disease in the pelvic lymph nodes (1 pN1, 1 pN2). Considering stage and lymph node status overall, 4 patients had an organ-confined disease. Three tumours were classified as grade II and ten as grade III. Recurrence-free survival was calculated on a retrospective review of outpatient records. Recurrence was defined as a CT- or MRT-confirmed three-dimensional lesion; the time of the first detection (e.g. by bone scan or thoracic X-ray) was considered the time of recurrence. After a mean follow-up of 43 months, 6 out of these 13 patients recurred after a mean time of 6 months and subsequently died of disease. Mean follow-up of the 7 patients without recurrence was 72 months.

Cell culture and maintenance. Origins of all cell lines used have been previously described (24) and their tumourigenic characteristics are summarised in Tables II and III. Growth conditions for transitional cell carcinoma (TCC) bladder cancer cell lines BL13, BL28, BL17/0/x1, BL17/2 and sublines, BL17/5, J82, 5637 and the SV40-transformed urothelial cell line SV-HUC-1 have been previously described (24). Media and supplements were obtained from Life Technologies (Melbourne, Victoria, Australia).

RT-PCR detection of KAI1. Total RNA was isolated from cultured cells using TriReagent, as per manufacturer's instructions. After phenol/chloroform extraction to remove residual DNA, 2 μg of RNA was used to prepare cDNA as previously described (24). Total cellular RNA from frozen tissue specimens was isolated by guanidinium isothiocyanate and phenol-chloroform extraction, followed by chloroform re-extraction and isopropanol precipitation. Purified RNA was dissolved in RNase-free water and quantified by UV spectroscopy scans from 340 nm to 210 nm (UviKon 900; Kontron Instruments, Neufarn, Germany). Integrity of RNA was confirmed by denaturing PAGE. Preparation of cDNA was similar to that used to make cDNA from cell lines, except that 1.5 μg of total RNA was used.

KAI1 sequences were specifically amplified using one of two Primer sets. Primer set I was designed to amplify the full KAI1 cDNA (1031 bp; sense 5'-AGTCCTCCCTGCTGCTGTGTG-3' and anti-sense 5'-TCAGTCAGGGTGGGCAAGAGG-3'). Primer set II was designed to span the spliced region (exon 9, 344 bp; sense 5'-CCCGGCAACAGGACCCAGA GT-3' and anti-sense 5'-TCAGTCAGGGTGGGCAAGAGG 3'). To control for RNA loading, a 600 bp GAPDH fragment was amplified (sense 5'-CCACCCATGGCAAATTCCATG GC A-3' and anti-sense 5'-TCTAGACGGCAGGTCAGGTCC A-3'). Reactions (total 25 μl) contained 2.5 μl 10x reaction buffer, 0.25 μl of 25 mM dNTP mix, 0.65 μl each of sense and anti-sense primer (20 pmol/ μl), 1.0 μl cDNA, 0.5 μl Taq (5 U/ μl) and either 2.0 μl (KAI1) or 3.0 μl (GAPDH) of 25 mM MgCl_2 . Amplifications were performed in a Hybaid touchdown thermocycler. Amplification of KAI1 using Primer set I used conditions of 94°C for 4 min, followed by 94°C for 30 sec, 60°C for 30 sec and 72°C for 70 sec, with a final step of 72°C for 10 min. Conditions for amplification using KAI1 Primer set II and GAPDH were similar, except that the elongation steps were reduced to 30 sec (KAI1) or 45 sec (GAPDH). In preliminary experiments, cycle titration reactions determined numbers of cycles required for optimal



Cell lines ^a	Tumour of origin		Tumourigenicity <i>in vivo</i> (+/-) ^b	Characteristics of <i>in vivo</i> tumours	Relative wild-type KAI1 mRNA ^c	AS KAI1 (+/-) ^d
	Stage	Grade				
SV-HUC-1 ^e	-	-	-	-	++	+
RT112	NR ^f	GII	+	GI -GII TCC	+++++	-
5637	T2-T4	GII	+	ND	+++	+
J82	T3	GIII	+	Poorly differentiated	+	-
BL13/0	T3	GII	+	GII-III	+++	+
BL28	T4b	GII	+	GII-III TCC	++	-
BL17/0/x1	T4b	GIII	+	GIII	+	+
BL17/5	T4b	GIII	+	GIII	++	+

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

^cWild-type KAI1 mRNA levels from (24). ^dSplice KAI1 verified by direct sequencing of nested PCR products, as described in Materials and methods. ^eNon-tumourigenic, SV40-transformed normal uro-epithelial cells. ^fNR, not recorded. ^gND, not determined.

detection of the shorter, spliced form of KAI1 using each Primer set. Subsequently, levels of KAI1 were examined after 34 cycles. Under these conditions amplification of full-length KAI1 reached saturation. Amplification of GAPDH was performed using 24 cycles, which was in the middle of the linear amplification phase. For semi-quantitative analysis of full-length versus AS KAI1, levels were assessed after 31 cycles, which was the midpoint of the linear phase of amplification. Reaction products were separated in 1.5% (for KAI1 Primer set I) or 3.0% (for KAI1 Primer set II) agarose gels, visualised with ethidium bromide and analysed by Kodak digital science 1D image analysis software.

Sequencing of spliced KAI1 PCR products. We used a nested-PCR approach to amplify sufficient AS KAI1 for sequencing reactions needed to validate splicing. After separation in a 3% agarose gel, 280 bp fragments generated using KAI1 Primer set II (corresponding to the predicted size if exon 9 was spliced out) were excised and purified using the Freeze 'n Squeeze™ method (BioRad Laboratories, Hercules, CA), as described by the manufacturers. Purified products were precipitated overnight at -20°C, rinsed in 70% ethanol and then resuspended in 10 µl, TE pH 8.0. These fragments (1 µl) were used as a template for nested PCR reactions using KAI1 Primer set III (208 bp product; sense 5'-CCACCCTGAGGACTGGCC-3' and anti-sense 5'-TCAGTCAGGATGGGCAAGAGG-3'). Conditions were similar to those described for reactions with KAI1 Primer set II, except that 25 cycles of amplification were used. After separation in a 3% agarose gel, the amplified fragments were purified and concentrated, as described above. Fragments were then directly sequenced in forward and reverse orientations, using Primer set III by the dye termination method, at the Sydney University Prince Alfred Macromolecular Analysis Centre (SUPAMAC, Camperdown, NSW, Australia).

Statistical analysis. Statistical evaluations were performed using SPSS for Windows (version 12.0). Standard statistical procedures, e.g. Pearson's Chi-square test were used. Recurrence-free survival was calculated as the time from

interval between transurethral resection or cystectomy, until any bladder cancer recurrence. Patients who died of other causes or were alive without recurrence at last follow-up were censored. A separate follow-up analysis was performed for patients undergoing a cystectomy (n=13). Kaplan-Meier survival curves were used to summarize treatment outcome with regard to recurrence-free survival. Groups of patients were compared using the log-rank test. All reported p-values are two-sided and p<0.05 was considered significant.

Results

Alternatively spliced (AS) KAI1 mRNA is present in superficial, invasive and recurrent bladder tumour samples. A recent study showed that expression of an alternatively spliced KAI1 mRNA (missing 85 bp of exon 9; hereafter called AS KAI1) and encoding a functionally defective KAI1 protein, is associated with metastasis and poor patient outcome in gastric cancer (20) (illustrated in Fig. 1A). To explore a possible role for this in bladder cancer, we first used PCR primers (Primer set I) to amplify a full-length KAI1 cDNA from 20 bladder tumour samples of varying stage and grade. All samples contained a 1031 bp product corresponding to full-length KAI1, but in 15/20 (75%) samples, an additional fainter band was detected at ~950 bp, consistent with amplification of a sequence missing exon 9 (see for example, results for two tumours in Fig. 1B). To verify this conclusion, we re-analysed all samples using a different set of primers (Primer set II) designed to amplify a smaller fragment spanning exon 9 (Fig. 1A). All expressed a 344 bp fragment corresponding to full-length KAI1, but in the same 15/20 samples as above, a 260 bp fragment predicted to be formed if exon 9 was deleted, was present at low levels (Fig. 1C). The 344 bp and 260 bp fragments were then purified and used as templates in nested PCR reactions using Primer set III, to amplify sufficient product for direct sequencing. This approach confirmed that the 344 bp fragments corresponded to wild-type KAI1, but that the 260 bp fragments corresponded to KAI1 sequence in which exon 9 was specifically deleted (a representative electropherogram is illustrated in Fig. 1C).

Table III. Characteristics of BL17/2 and clonally-derived cancer cell lines, examined for expression of AS KAI1.

Cell line	Tumourigenic in nude mice	<i>In vivo</i> invasive ability	Relative wild-type KAI1 mRNA levels ^a	AS KAI1 (+/-) ^b
B11	-	-	++	+
C10	-	-	+++	+
C3	-	-	++	+
BL17/2 ^c	+	-	++	+
B12	+	-	+/-	+
C1	+	-	+/-	+
D2	+	-	+	+
B8	+	+	+/-	+
B9	+	+	+/-	+
B10	+	+	+/-	+

^aWild-type KAI1 mRNA levels from Jackson *et al* (9). ^bKAI1 splice verified by direct sequencing of nested PCR products, as described in Materials and methods. ^cBL17/2 (25) is derived from the second serial passage in nude mice, of a xenograft from a Grade III, stage T4b tumour biopsy used to generate the BL17/0/x1 cell line (Table 1). All sublines were clonally isolated and characterised previously (25).

We assessed the relationship between the presence or absence of AS KAI1 mRNA and various clinico-pathological characteristics of the tumours. Data summarised in Table I, showed that the occurrence of AS KAI1 mRNA expression did not differ significantly between superficial and invasive bladder cancers (pTa/pT1 vs. pT2-4). Similarly, there was no correlation with tumour grade (G1/2 vs. G3). Although usually associated with systemic disease, pelvic lymph node metastases are believed to precede the formation of distant metastases. There was no correlation between the presence of AS KAI1 expression and lymph node status (pN0 vs. pN+). To determine the impact of AS KAI1 on metastasis development, we analysed the outcome data. There was no significant correlation between the presence of the KAI1 splice variant with regard to recurrence-free survival for the overall patient cohort and for patients undergoing cystectomy with curative intent, respectively. However, in both cohorts the outcome was better for patients with tumours which expressed the KAI1 splice variant.

AS KAI1 mRNA in bladder cancer cell lines. Since the levels of AS KAI1 in our biopsy samples were very low and the proportion of tumour tissue within these samples varied from >90% (superficial) down to 50-70% (invasive), it was possible that contaminating normal tissue was masking the true incidence and levels of AS KAI1, so we repeated our analyses using cDNA derived from a series of cell lines, including a non-tumourigenic transformed urothelial line (SV-HUC-1) and a range of TCC lines (summarised in Table II). Previous studies have also determined relative levels of full-length KAI1 mRNA within these cell lines (24) (summarised in Table II). Using KAI1 Primer sets I and II and PCR conditions to clearly detect AS, full-length KAI1 was clearly present in all cell lines. Low levels of AS KAI1 mRNA were detected in SV-HUC-1, as well as in 4/7 TCC cell lines (Fig. 2). There was no clear relationship between the presence or absence of AS KAI1 mRNA and either stage and/or grade of the tumour of origin of these lines. Additionally, there was no relationship between the absence or

presence of the AS variant and previously determined relative levels of full-length KAI1 mRNA. Thus, in four cell lines with high levels of KAI1 (SV-HUC-1, BL13, BL28 and 5637) AS KAI1 was present in SV-HUC-1 and BL13 but not in either BL28 or 5637 (even after extending PCR amplification to >40 cycles; data not shown). On the other hand, when considering cell lines which express very low levels of KAI1 mRNA (BL17/0/x1 and J82), we clearly detected AS KAI1 in BL17/0/x1, but not in J82.

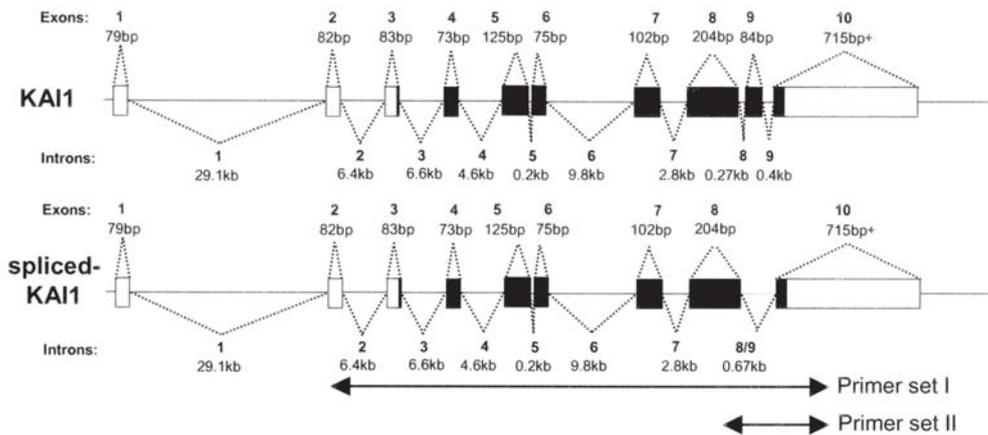
We also examined the incidence of AS KAI1 mRNA in BL17/2 and nine clonally-derived sublines, whose tumourigenicity, invasive abilities *in vivo* and relative wild-type KAI1 mRNA levels were previously analysed (24,25) (summarised in Table III). Again, under the PCR conditions used for detecting the AS form, full-length KAI1 was detected in all cell lines. Interestingly, low levels of AS KAI1 mRNA were also detected in all lines (Fig. 2; summarised in Table III). Thus, AS KAI1 mRNA was found in 3/3 non-tumourigenic sublines, 7/7 lines which were tumourigenic *in vivo*, 7/7 lines which were non-invasive *in vivo* and 3/3 cells with *in vivo* invasive ability.

In order to determine if relative levels of AS KAI1 mRNA relative to the full-length form might be associated with more aggressive behaviour, we performed a semi-quantitative PCR analysis of KAI1 mRNA expression in our AS-positive bladder cancer cell lines (Fig. 3B, C). There was no difference in relative levels of full-length KAI1 versus AS KAI1 between non-tumourigenic SV-HUC-1 and four tumourigenic cell lines (Fig. 2), or between non-tumourigenic, tumourigenic and non-invasive or tumourigenic and invasive cell lines in the BL17/2 series (Fig. 2). In all cases, levels of AS KAI1 mRNA were ~3- to 5-fold lower than those of the wild-type species.

Discussion

The identification of an alternatively spliced form of KAI1 mRNA, encoding a functionally defective form of the KAI1 protein, in human gastric cancer samples (20) has raised the possibility that a loss of KAI1 function may occur in human

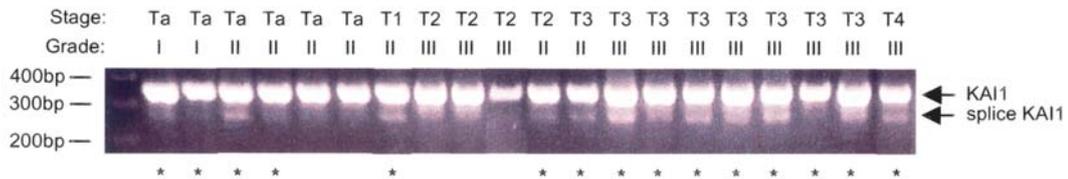
A



B



C



D

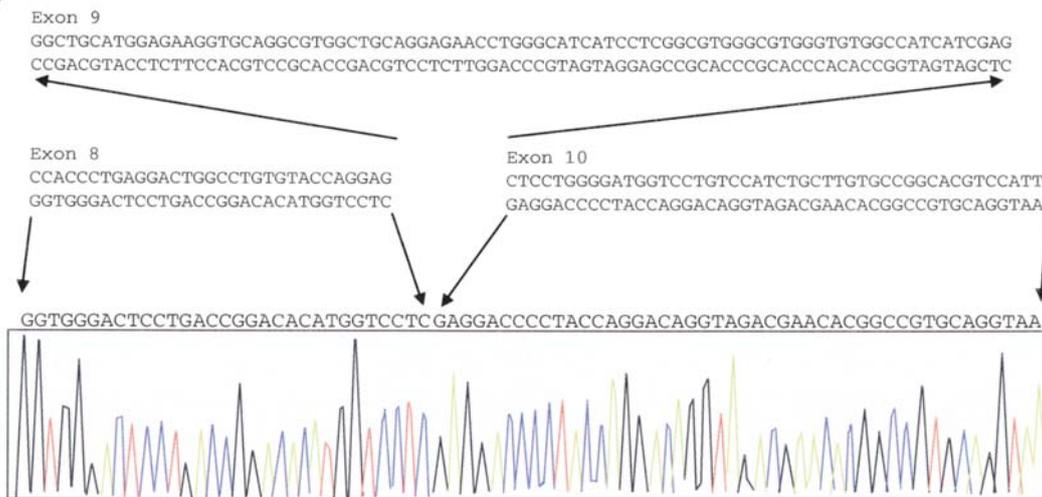


Figure 1. Structure of the KAI1 gene and expression of a spliced form of KAI1 in bladder tumours (A) Schematic illustration of the exon/intron composition of the KAI1 gene showing full-length KAI1 mRNA and a recently identified alternatively spliced form (AS) KAI1 lacking exon 9 (20). Exon/intron numbering is according to previously published data (29) and information available at www.ensembl.org/homo_sapiens/exonview. Double-headed arrows indicate regions of KAI1 spanned by PCR Primer sets. (B) Representative PCR analyses of cDNA from two bladder tumour samples using KAI1 Primer set I, and showing the presence of two amplified products. (C) PCR analysis of KAI1 in all bladder tumour samples using Primer set II, as described in Materials and methods; * indicates samples with AS KAI1. (D) Representative results of sequencing reactions performed on nested purified PCR products, confirming deletion of sequences encoding exon 9 from the AS form of KAI1 mRNA.

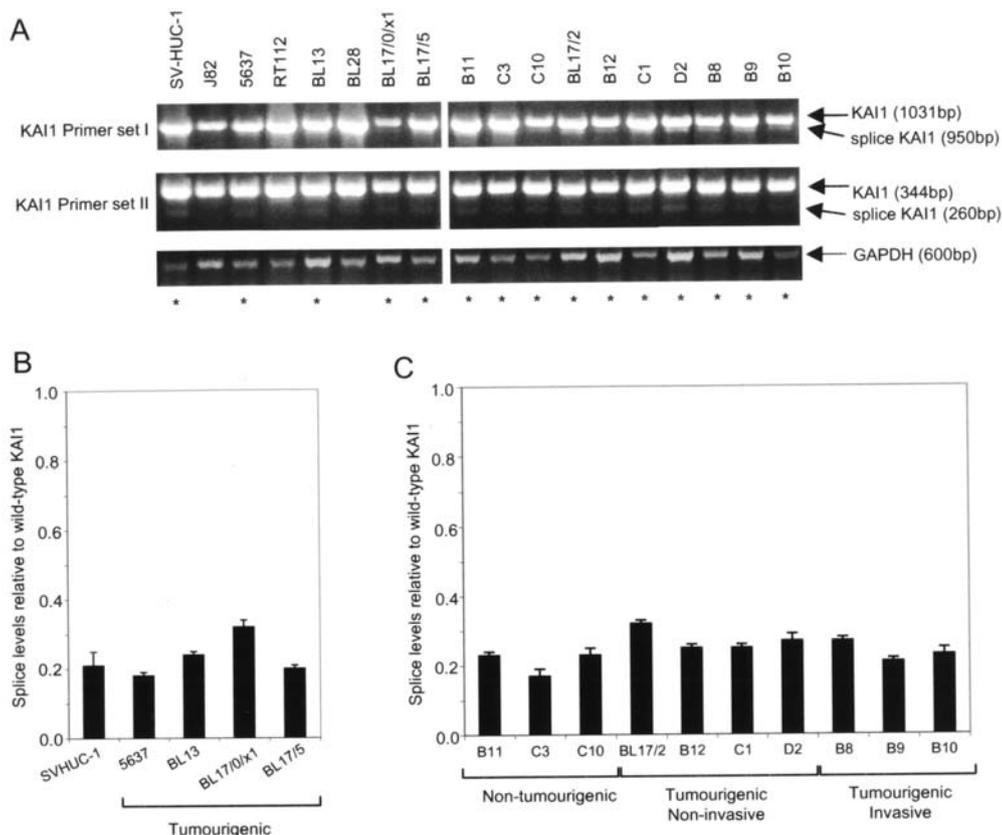


Figure 2. AS KAI1 mRNA expression in bladder cancer cell lines. Representative PCR analyses of cDNA derived from bladder cancer cell lines using KAI1 Primer sets I and II, and GAPDH primers. In both panels, * indicates cell lines with AS KAI1 (A). Semi-quantitative PCR analysis of AS versus wild-type KAI1 in non-tumourigenic versus tumourigenic lines (B) and in BL17/2 and clonally-derived sublines (C). Results are mean with standard error of three independent PCR reactions.

cancer types in which down-regulation of KAI1 has not previously been identified. A loss of overall KAI1 expression is common in advanced bladder tumours (14), but a small proportion of these tumours (~20%) retain KAI1, raising the possibility that alternative splicing of KAI1 could be responsible for the loss of normal KAI1 function in these tumours. Consistent with this possibility, we detected low levels of AS KAI1 in 15 out of 20 bladder tumour samples examined and in which wild-type KAI1 mRNA was clearly present. We found no association between the presence or absence of the AS mRNA species and either stage, grade or invasive ability of these tumours. Additionally, there was no correlation between patient recurrence-free survival and the presence or absence of the AS mRNA form. Our data need to be interpreted with caution since the number of samples examined was small and does not allow clear cut conclusions. However, in contrast to previous reports in other tumours (20), we observed that patients with tumours which expressed the AS variant had an increased recurrence-free and tumour-specific (data not shown) survival compared to those without the AS KAI1 variant.

The AS KAI1 mRNA was also detected in the majority (14/17) of bladder cancer lines we investigated. Interestingly, the AS KAI1 mRNA was also detected in non-tumourigenic cell lines (SV-HUC-1, B11, C3 and C10), as well as tumourigenic cell lines with both high (5637, BL13) or very low (BL17/0/x1, B8, B9 and B10) levels of KAI1 mRNA overall, and the proportion of spliced-KAI1 mRNA to wild-type

KAI1 mRNA was low (3- to 5-fold lower) across all cell lines. Again there was no clear relationship between the presence or absence of spliced mRNA and tumourigenic or invasive behaviour of these cell lines. Whilst these studies are preliminary, our data suggested that in contrast to human gastric cancer and certain murine colon cancer cell lines (20), the AS KAI1 mRNA was not specifically associated with either tumourigenesis or more aggressive tumour cell behaviour in bladder cancer.

In conclusion, we propose that alternative mechanisms may inactivate normal KAI1 functions in those tumours and tumour cell lines which retain KAI1 protein expression. These may include post-translational modifications such as glycosylation and palmitoylation, which have previously been shown to have a profound impact on KAI1 function (26-28).

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