Transcriptional and translational modulation of KAI1 expression in ductal carcinoma of the breast and the prognostic significance

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Abstract. KAI1, also known as CD82, has been shown to have a potential impact on the invasiveness of cancer cells. In the present study, expression pattern of KAI1, both at transcription and translation levels and the potential clinical value of the expression were explored in a cohort of normal and ductal mammary cancer tissues (n=71). A marked reduction of KAI1 transcript was observed in invasive ductal breast tumours as compared to normal tissues. Expression of KAI1 protein was higher in normal tissues as compared to tumour samples. Though no significant difference of KAI1 expression between different grades of tumour was observed (p=0.064), significant correlation of TNM staging with KAI1 expression has been observed in invasive ductal breast cancer patients (p=0.045). Additionally, it was also observed that patients showing higher expression of KAI1 had a longer 10-year survival rate as compared to a low level or completely negative expression KAI1 (p=0.0136). KAI1 inverse correlation with tumour progression may be used as a strong prognostic marker.

Introduction

Breast cancer is one of the most frequently occurring cancers amongst females across the globe. According to the American Cancer Association, women of all origin have a substantial risk of developing breast cancer (incidence rate in Caucasians 130.8; African Americans 111.5; Asian Americans and Pacific islanders 91.2; American Indians 74.4 per 100,000). Metastasis is the primary cause of death for the patients. Metastasis, a complex biological and clinical event, is influenced by a number of molecules. These molecules, by acting on the breast cancer cells and stromal cells, impact on the cellular behaviours of cancer cells during the metastatic process. KAI1 (Kang ai as from Chinese meaning anticancer) is one of these molecules and is a member of the tetraspanin family that was first identified in a T-cell activation study (1). Metastasis suppression induced by this gene in prostate cancer was explored later on by somatic cell hybridization of highly metastatic and non-metastatic rat prostate cancer cells (2). KAI1 is also termed as SAR2 leukocyte surface antigen R2 and suppressor of tumorigenicity 6 (ST6). Dong *et al*, identified the location of KAI1 gene on chromosome 11p with 10 exons and 9 introns spanning ~80 kb (3). KAI1 protein exists in two isoforms, with 267 residues in isoform-1 and 242 residues in isoform-2 (4). KAI1 plays an important role in cancer largely due to its interesting relationship with other molecules that are strongly linked to the function of cancer cells.

It has been established in recent years that apart from interacting with other members of the tetraspanin family, KAI1 protein molecule interacts with integrins and epidermal growth factor receptor (EGFR) (5,6), in doing so KAI1 aids the internalisation of integrins and EGFR. This may result in loss of reduction of integrins and EGFR, pivotal regulators of cell adhesion and growth in breast cancer. Altered expression of KAI1 ultimately leads to decrease in adhesion and increase invasiveness of cancer cells. KAI1 has an ectopic effect on adhesion by strengthening the interaction among E-cadherin with ß-catenins and reduces the chances of cellular dissemination for the primary tumour (7).

Expressional regulation of these genes in relation to various clinical parameters during cancer progression have been reported in prostate cancer (8), breast (9,10), lung (11), ovarian (12), gastric (13,14), pancreatic (15), oesophagus (16), bladder (17) and cervical cancer (18). Inverse correlation of KAI1 expression with respect to cancer progression has been observed in all these studies. It has been observed that a decreased expression of KAI1 is associated with poor prognosis. The aim of the present study was not only to screen

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Table I. Clinical pathological features of the cohort of the ductal carcinoma of the breast.

Groupings	Number of samples			
Grade	Grade-1 n=9	Grade-2 n=24		Grade-3 n=38
TNM staging	TNM1 n=38	TNM2 n=27		TNM3/4 n=6
NPI status	NPI <3.4 n=35	NPI 3.4-5.4 n=27		NPI >5.4 n=9
Clinical outcome	Disease-free N=52	With metastasis N=7	With local recurrence N=4	Died of breast cancer N=8

expression levels of KAI1 molecule in breast cancer patients but also to find a correlation (if any) with the tumour stage, as well as long-term survival of the patients.

Materials and methods

RNA-extraction kits and Mastermix for routine PCR and quantitative PCR were obtained from AbGene (Surrey, UK). PCR primers were designed using Beacon designer (Palo Alto, CA, USA) and synthesised by Invitrogen (Paisley, UK). Molecular biology grade agarose and DNA ladder were purchased from Invitrogen.

The first strand cDNA synthesis kit was purchased from Sigma Chemical Ltd. (Dorset, UK). A universal IHC kit was purchased from Vector Laboratories (Peterborough, UK). Low fluorescent 96-well plates were obtained from AbGene and sealing film was purchased from BioRad (Hemel Hempstead, UK).

Breast sample collection. Ductal breast cancer tissues (n=71) and normal tissues (n=31) that were free from cancer cells were collected (with approval from the local ethics committee) immediately after surgery and stored at -80˚C until required. These patients were routinely followed clinically after surgery. Median follow-up was 120 months. Histopathological features, tumour grade, tumour staging and the prognostic index for the patients are shown in Table I.

Tissue processing. Frozen sections of breast tissues were cut using cryostat at a thickness of 5-10 μm and were stored at -20˚C (19,20). Approximately 15-20 sections from each breast tissue sample were homogenized using a hand held homogenizer in ice-cold RNA extraction buffer. Concentration of RNA was confirmed by using UV spectrophotometer (Wolf Laboratories, York, UK).

cDNA synthesis and analysis of the KAI1 transcript. Reverse transcription was carried out by using 1 μg of total RNA from each sample. Oligo-dT primer present in the RT kit was used and cDNA was prepared according to the manufacturer's instructions. Polymerase chain reaction was preformed using this synthesized cDNA. Reaction conditions

were 95˚C for 5 min; 94˚C for 20 sec; 55˚C for 30 sec, 72˚C for 1 min for 38 cycles with 10 min final extension at 72˚C. The following primers were used in the reaction, KAI1 forward: 5'CTGTACTTTGCTTTCCTGCT'3 and KAI1 reverse: 5'CTGTAGTCTTCGGAATGGAC'3. ß-actin was used as internal control in the experiment. Amplified products were visualized, following staining with 2% agarose gel.

Quantitative PCR analysis. Transcripts of all patients were analysed by using IQ real-time quantitative Thermal cycler (BioRad). Specific pairs of primers were designed using Beacon Designer and synthesized by Invitrogen. Sense primers for KAI1 (5'CATTCGAGACTACAACAGCA3') and antisense primer including Z sequence (complementary to universal probe 5'ACTGAACCTGACCGTACATCCAGTTGTAGA AGCTGACC'3) were used for its detection. GAPDH was used as an internal control in this quantitative reaction (primer pair for GAPDH: 5'CTGAGTACGTCGTGGAGTC'3 and 5'ACT GAACCTGACCGTACACAGAGATGATGACCCTTTTG'3). The reaction was carried out using the following reaction conditions: Hot-start Q-master mix (Abgene), 10 pmol of specific forward primer, 1 pmol of antisense primer (containing Z sequence). A probe of 100 pmol concentration of (6 carboxy-fluorescein FAM) was used. The reaction conditions were 94˚C for 120 min; 94˚C for 150 sec, 55˚C for 400 sec and 72˚C for 200 sec with 60 cycles. The data generated was first normalized with the GAPDH molecule.

Immunohistochemical staining. Immunohistochemical staining was done using both tumour and normal frozen sections of 6 μm thickness. The sections were mounted on Super Frost Plus microscopic slides and air dried for 20-30 min. These fixed tissue sections were treated in 50% methanol and 50% acetone for 15 min. Sections were then air dried for 10 min and stored at -20˚C (wrapped in foil) for further use or immediately stained. These air dried samples were placed in PBS for 5 min to rehydrate, followed by blocking using a buffer with 10% horse serum. These sections were then treated with primary antibodies. Antibodies used for KAI1 were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA) raised in rabbit. After a 1-h incubation of primary antibodies
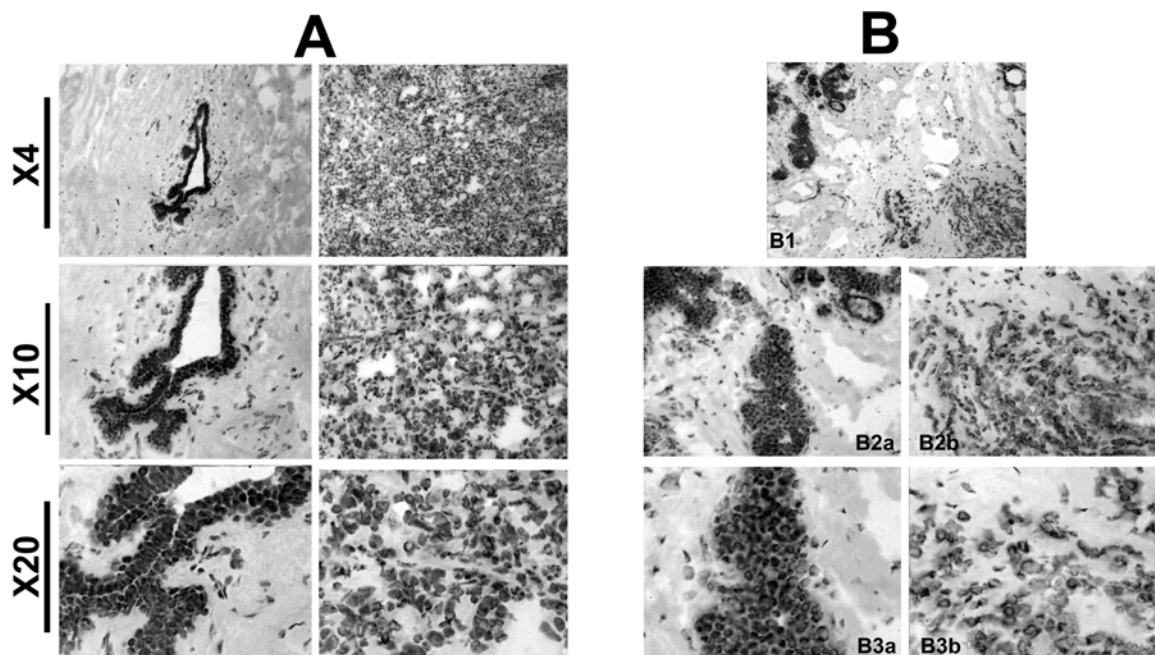
Figure 1. Immunohistochemical staining of KAI1 in frozen sections of unaffected normal mammary tissues and tumour tissues. (A) Comparison between unaffected normal tissues (left panel, ID 136) and an invasive tumour (ID 111). (B) Comparison between residual mammary cells (B2a and B3a) and invasive tumour cells (B2b and B3b) in the same sample (ID 113). Tumour cells showed a marked reduction of KAI1 staining compared with affected mammary epithelial cells.

the slides were subjected to 4 washes with PBS and then treated in universal multi-link biotinylated secondary anti-body. After 30 min of incubation, 4 washes with PBS were performed on these slides. Slides were then treated with avidin biotin complex (ABC, Vector Labs, UK). Diamino-benzidine tetrahydrochloride [3,3-diaminobenzidine (DAB) purchased from Sigma] was added for 5 min to detect the bound antibody. The slides were washed with water for 5 min and treated with Mayer's haematoxylin for 1 min followed by a further wash with water for 10 min. The slides were then treated with methanol (3 times) and clearing in 2 changes of xyline before mounting under a cover slip. PBS is used as negative control in this experiment. Each experiment was repeated thrice for conclusive results.

Statistical analysis was carried out using Mann-Whitney U test (IQR) and survival analysis by Kaplan Meier survival analysis, using SPSS package (SPSS version 16).

Results

Distribution of KAI1 in mammary cells. After immunohisto-chemical staining, the presence of KAI1 protein was confirmed in breast tissue. KAI1 was observed in tumour cells as well as in normal cells. The protein was localized on the cell membrane and in the cytosol. Dark brown staining was observed on the cell boundaries of normal cells indicating the presence of KAI1. KAI1 protein was more abundantly observed in mammary epithelial cells in unaffected tissues (Fig. 1A left panel and B2a) as compared to cancer cells (Fig. 1A right panel and B2b).

Expression of the KAI1 transcripts in breast cancer cells and tissues. Conventional RT-PCR highlights alteration in KAI-1
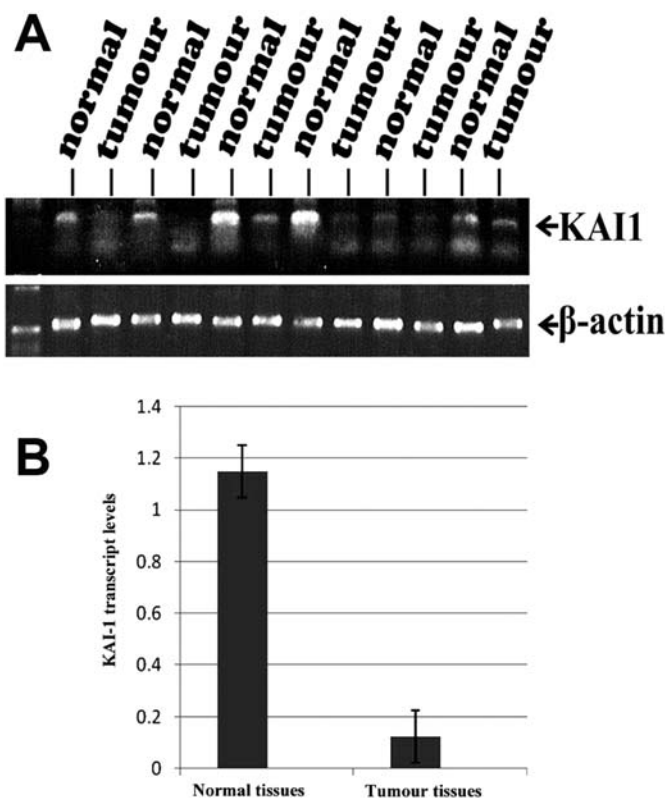

Figure 2. Expression of KAI1 transcripts in human mammary tissues. (A) Expression pattern of KAI1 transcript by using conventional RT-PCR (shown are PCR products separated by 2% agarose gel electrophoresis). Almost complete loss of KAI1 mRNA is clearly evident in ductal breast tumour samples in comparison to ß-actin which was used as internal control. (B) KAI1 expression levels in normal and ductal breast tissue in the cohort by quantitative real-time PCR analysis (shown are medians of KAI1/ GAPDH ratios). Ductal breast tissue samples showed a significant decrease of KAI1 transcript in comparsion to normal tissue samples.

Table II. Summary of KAI-1 transcript levels in the study cohort.[a]

Group	KAI1 transcript (median and IQR)	p-value (Mann-Whitney U test)
Grade		
1	0.0045 (IQR 0.003-0.0353)	
2	0.002 (IQR 0-0.005)	NS
3	0.003 (IQR 0-0.033)	NS
NPI index		
<3.4	0.002 (IQR 0-0.025)	
3.4-5.4	0.003 (IQR 0-0.016)	NS
>5.4	0.041 (IQR 0.001-0.0335)	NS
Clinical outcome		
Disease free	0.004 (IQR 0.001-0.029)	
With metastasis	0.02 (IQR 0-0.04)	NS
With local recurrence	0.021 (IQR 0.006-.041)	NS
Died of breast cancer	<0.0001 (IQR 0-0.0008)	p=0.0136 vs. disease-free

[a]Shown are median levels and interquartile range (IQR) of KAI1/GAPDH ratio in the respective group. NPI, Nottingham Prognostic Index; NS, not significant.
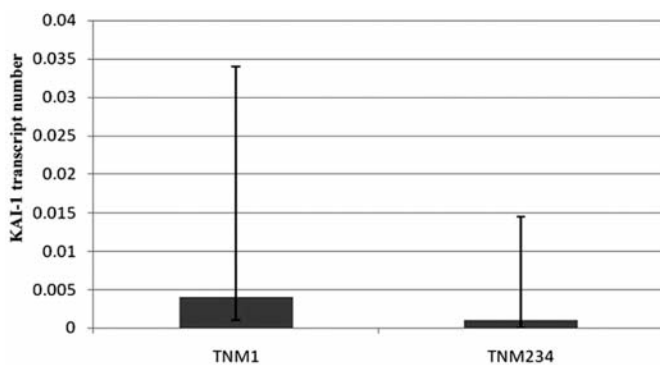

Figure 3. Correlation of KAI1 transcripts with tumour staging of ductal breast cancer. Significant correlation of KAI1 transcript number normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) against tumour, node and metastasis (TNM) staging were observed (p=0.0455). Shown are median and interquartile range (IQR) of KAI1/GAPDH ratio in the respective group.

expressional levels between paired normal and tumour tissues. In majority of these paired samples KAI1 levels appear to be reduced in the tumour tissue as compared to normal samples as shown in Fig. 2A. Further verification was done through quantitative real-time PCR that showed a decrease of KAI1 expression in all breast cancer samples as shown in Fig. 2B. The data presented are after normalizing with GAPDH.

Correlation of KAI1 transcripts with nodal status, grade and tumour staging. We were unable to identify any significant correlation of KAI1 transcript level between grade 1 (well-differentiated), grade 2 (moderately differentiated) and grade 3 (poorly differentiated) of invasive ductal breast cancer tissues. No significant correlation with NPI (Nottingham Prognostic Index) has been observed (Table II). Early stage breast tumours (TNM1) had a significantly highly levels of KAI1 transcripts compared with late stage tumours (TNM2, 3 and 4) (p=0.045, Fig. 3).

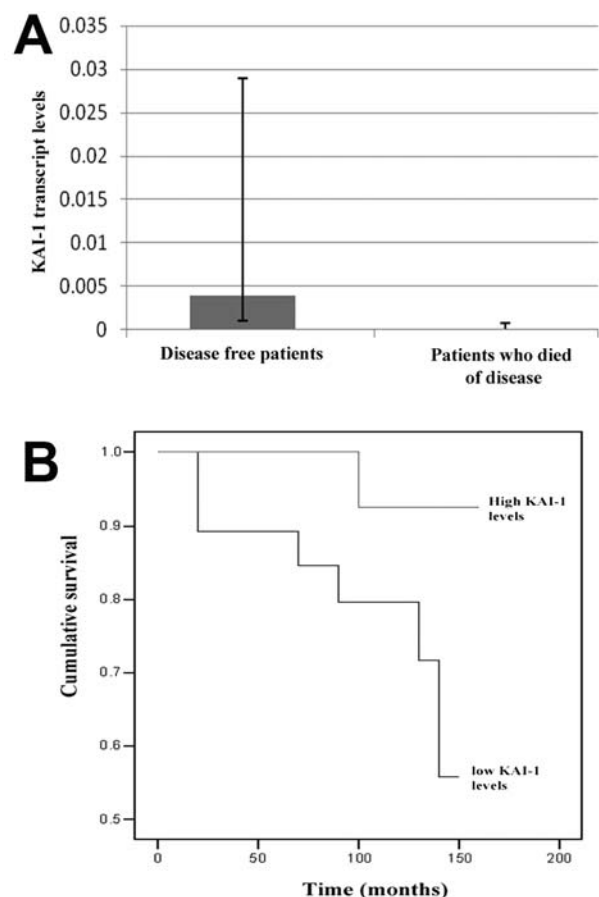

Figure 4. KAI1 transcript levels in ducal carcinoma linked to long-term survival of the patients. (A) Graphical illustration of survival rate in patients with KAI1 expression. Increase survival of breast cancer patients were observed in patients showing strong KAI1 expression in comparison to KAI1 negative patients. Shown are median and interquartile range (IQR) of KAI1/GAPDH ratio in the respective group. (B) Kaplan-Meier survival curve after 10 years of follow-up. Significant association of KAI1 level (p=0.0136) based on KAI1/glyceraldehyde 3 phosphate dehydrogenase (GAPDH) ratio indicating survival of patients with ductal carcinoma had been observed.

Impact on patients survival. Patients who were disease-free had a significantly higher levels of KAI1 transcripts than those who died of breast cancer (p=0.0136, Fig. 4A). Using the Kaplan-Meier survival model, we found that patients with high levels of KAI1 transcripts had a significantly longer survival [mean survival 152 (95% confident interval 144-160 months)] than patients with low level of KAI1 [120 (95%CI 104-137) months)] (p=0.0136) (Fig. 4B).

Discussion

Metastasis suppression character of KAI1 was first discovered in prostate cancer which was later extrapolated in other types of cancer. The most significant findings of the present study are the correlation with KAI1 transcript and patients long-term survival. After 10 years of follow-up it was observed that patients showed a direct correlation between expression of KAI1 and survival. Disease-free survival rate was significantly higher in patients showing higher levels of KAI1 at the initial stage of mammary tumours compared to those who died as a result of cancer. This relationship of KAI1 expression with patients survival rate was also mentioned (p=0.0091 in 62 patients) in an earlier study (21). Similarly in another study, the 5-year disease-free survival rate of KAI1 positive tumour patients was more than KAI1 negative tumour patients (p=0.0292) (9). Disease-free survival rate of KAI1 positive tissue has also been observed in relation to other types of cancers such as epithelial ovarian carcinoma (22), lung cancer (11), pancreatic cancer (15) and gastric cancer (12). As overall survival rates of lung cancer patients was significantly better in patients showing increased expression of KAI1 compared to patients showing a decrease in KAI1 as observed by Adachi *et al* (23). Similarly decreased level of KAI1 protein in advance stage gastric cancer patients also increase cancer-related death (12) providing a strong evidential usage of KAI1 along with other markers as a potential prognostic marker.

Decreased expression of KAI1 was consistently reported in breast cancer progression (9,10,22,24) but significant correlation with respect to clinical progression of breast cancer stages was lacking. In a study on a Korean population Son *et al* showed a consistent decrease of KAI1 in infiltrating ductal carcinoma of breast but did not find any significant correlation with clinicopathological features (21). In the present study, a correlation of KAI1 with tumour node and metastasis system was identified which may later along with other markers be used as a better prognostic marker. These findings are also in accordance with a previous study on lung cancer (25) indicating a significant relationship with differentiated degree of tumour TNM stages and lymph node metastasis.

Down-regulation of KAI1 is attributed more toward expressional regulation rather than the involvement of coding region mutations. As mentioned also in previous studies the chance of somatic as well as germline mutations on coding region of KAI1 molecule is an extremely rare event (22,26,27). KAI1 molecule has a transmembrane localization forming several interactions with integrin (28), chemokines (29), EGFR (30) and other tetraspanin (31). Suppression of cancer cells is actually induced by internalization of EGFR and also by accelerating desensitization of EGF signalling

(5). These interactions are responsible for cell-cell interaction, cell motility, and adhesion cell signalling. Increased KAI1 level both at transcript and translational scale actually reduce invasion of the breast cancer cells as mentioned in several previous reports (32-34). Thus, down-regulations of KAI1 both at transcript as well as translation level in clinical cases ultimately lead to poor prognosis.

In conclusion, the association of KAI1 expression with breast cancer progression is a potential prognostic candidate marker. KAI1 metastatic suppression ability in conjunction with other markers can also be used as a marker of therapeutic potential. Apart from clinical trials, the role of KAI1 in various cellular signalling pathways is an area that requires further investigation.

Acknowledgements

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