RESEARCH ARTICLE

Expression Levels of Tetraspanin KAI1/CD82 in Breast Cancers in North Indian Females

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Abstract

Background: Carcinogenesis is a multifaceted intricate cellular mechanism of transformation of the normal functions of a cell into neoplastic alterations. Metastasis may result in failure of conventional treatment and death Hence, research on metastatic suppressors in cancer is a high priority. The metastatic suppressor gene CD82, also known as KAI1, is a member of the transmembrane 4 superfamily which was first identified in carcinoma of prostate. Little work has been done on this gene in breast cancer. Herein, we aimed to determine the gene and protein level expression of CD82/KAI1 in breast cancer and its role as a prognosticator. Materials and Methods: In this study, 83 histologically proven cases of breast cancer and a similar number of controls were included. Patient age ranged from 18-70 years. Quantitative Real Time Polymerase Chain Reaction (q-RT PCR) and immunohistochemistry (IHC) were used to investigate KAI1 expression at gene and protein levels, respectively. Statistical analysis was done to correlate expression of KAI1 and clinicopathological parameters. Results: It was revealed that: (i) KAI1 was remarkably diminished in metastatic vs non metastatic breast cancer both at the gene and the protein levels (P < .05); (ii) KAI1 expression levels were strongly correlated with TNM staging, histological grade and advanced stage (p<0.001) and no association was found with any other studied parameter; (iii) Lastly, a significant correlation was observed between expression of KAI1 and overall median survival of BC patients (P = 0.04). Conclusions: Our results suggest that lack of expression of the KAI1 might indicate a more aggressive form of breast cancer. Loss of KAI1 may be considered a significant prognostic marker in predicting metastatic manifestation. When evaluated along with the clinical and pathological factors, KAI1 expression may be beneficial to tailor aggressive therapeutic strategies for such patients.

Keywords: Breast cancer - prognosis - metastasis - KAI1 - North India

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Introduction

Cancer is the second leading cause of death worldwide. Therefore, the fight against cancer is one of the most important areas of research in medicine, and one that possibly contributes to the increased interest in identifying novel prognostic biomarkers. Breast cancer, a familial, heterogeneous disease, is the second most common cancer among women in the world and, by far 232,340 new cases of invasive breast cancer and 39,620 breast cancer deaths had affected US women in 2013(DeSantis et al., 2013). Incidence and mortality due breast cancer has been increasing for last 50 years, even though there is a lacuna in the diagnosis of breast cancer at early stages. (Donepudi et al., 2014). Approximately 6-10% of new breast cancer cases are initially Stage IV or metastatic ("de novo" metastatic disease). The number of metastatic recurrences are unknown, but are estimated to range between 20-30% of all existing breast cancer cases (O'Shaughnessy, 2005). Metastasis, the major cause of casualty for most cancer patients, remains one of the most imperative and complicated as well as less comprehended aspects of cancer. Metastasis hinges upon a stringently orchestrated cascade of events; therefore, interruption of any step may effectively halts the process (Benjamin et al., 2010). An attractive group of candidates to treat metastasis are the metastasis suppressors, defined by their abilities to inhibit metastasis without blocking orthotopic tumor growth. Hence, molecular suppressors that slow down the metastatic cascade have always attracted focus of researchers all across the globe.

The identification of KAI1 also known as CD82, dates back to 1995 when a gene from human chromosome 11p11.2 was isolated and was shown to suppress

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metastasis when introduced into rat AT6.1 prostate cancer cells (Dong et al., 1995). KAI1, belongs to the transmembrame 4 superfamily (TM4SF) or tetraspanin and being a characteristic feature of the tetraspanins, KAI1 participates in an array of cellular mechanisms like cell proliferation, cellular motility and cell-cell intreraction. (Lazo 2007).

Structurally, KAI1 encodes a 267 amino acid protein and it's expression has been shown to be downregulated during tumor progression of human cancers (Wright et al., 1994). A similar role of the KAI1 gene has also been suggested for cancers of the lung and pancreas, as down-regulation of KAI1 at RNA level correlated with poor survival in patients with lung cancer (Adachi et al., 1996; Adachi et al., 1998). Also Literature reports suggest that Tip60 complex controls the expression of KAI1 and tumor metastatic potential (Kim et al., 2005; Brown et al., 2009). Supplementary data have revealed that other common types of human malignancies also demonstrate decreased expression of KAI1, including bladder, pancreas, hepatocellular, colorectal, ovarian, esophageal, and cervical cancers (Friess et al., 1998; Guo et al., 1998; Lombardi et al., 1999; Liu et al., 2000; Miyazaki et al., 2000; Yang et al., 2000; Liu et al., 2001).

In this study, the gene and protein level expression of KAI1 were evaluated and correlated with the clinicopathological parameters of breast cancer. Several clinicopathological parameters have been implicated in prognosis, recurrence and survival in breast cancer. Tumor size, axillary lymph node involvement and extent of metastasis are important prognostic determinants for patients with breast cancer. (Soerjomataram et al., 2008). Estrogen Receptor (ER) expression is long known as a prognostic and a predictive factor for breast cancer (Bevilacqua et al., 2007). Progesterone Receptor (PR) status is also correlated with axillary lymh node involvement and hormone receptor status and remains one of the most significant predictive and prognostic biomarker (Yip et al., 2014). Her2 neu also serves as prognosticator according to earlier reports (Pan et al., 2014). These data together indicate that many clinicopathological parameters may play a key role in breast cancer prognosis and prediction of response to various available therapeutic options.

We herein aim to find out a correlation, between the transcriptomic and translational expression levels of KAI1 gene with the clinicopathological parameters and median overall survival in the BC patients.

Materials and Methods

The study group comprised of 83 histologically proven cases of breast cancer and Adjoining normal breast tissue from the same breast resection specimen.

The samples were collected from Department of Surgical Oncology, King George's Medical University, Lucknow between November 2011 and December 2012. Breast cancer tissue from tumor mass was obtained for the study. Adjacent normal tissue from the mastectomy specimen served as the control tissue. None of the patients received preoperative chemotherapy or radiation therapy. The study protocol was approved by Institutional Ethics Committee at King George's Medical University, Lucknow. Written voluntary informed consent was obtained from all patients before recruitment. KAI1 expression at gene and protein level were studied by RT PCR and immunohistochemistry, respectively. The tissue biopsies were collected in 10x buffered formalin at room temperature for immunohistochemical diagnosis and in RNA later at -80°C until further use for RT PCR.

Quantitative real time PCR

Total mRNA was isolated following single step mRNA isolation method using RNA isolation kit (Invitrogen, USA). Total mRNA (2 μg) was reverse transcribed to cDNA using RT-PCR kit (Applied Biosystems, USA) following manufacturer's instructions. Real time analysis for KAI1 and normalizing gene GAPDH was performed using SYBR GREEN MASTER mix as per the manufacturer's instructions (Applied Biosytems, USA). Analysis were done on Light-cycler 480 (Roche, USA) and fold changes in gene expression were calculated using 2-ΔΔCT method. The q RT PCR primer sequences were 5'-CATGAATCGCCCTGAGGTCACCTA-3' and 5'-GCCTGCACCTTCTCCATGCAGCCC-3' for KAI1; and 5'-AAATCAAGTGGGGCGATGCTG-3' and 5'-GCAGAGATGATGACCCTTTTG-3' for GAPDH.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections were cut into 4 μm-thick sequential sections. After deparaffinization and rehydration, sections were boiled in citrate buffer (0.01 M, pH 6.0) for antigen retrieval. Sections were then incubated with 3% H_2O_2 and 5% serum to block endogenous peroxidase activity and non-specific binding. For KAI1 protein, sections were incubated with rabbit anti-human KAI1 monoclonal antibody (sc-101246). The sections were then incubated with biotinylated secondary antibodies and visualized by DAB. Counterstaining was carried out with hematoxylin. The sections were dehydrated in alcohol and mounted with DPX. For the negative controls, PBS replaced the primary antibody.

Immunohistochemical Scoring for KAI1

IHC evaluation was performed under a microscope by an observer unbiased without the knowledge of clinical outcome. Membranous staining was considered positive for KAI1 expression. The patterns of staining were applied into scales on % of cells with positive immunostaining as 0=complete absence or negative staining, 1=less than 10 % positive cells, 2=greater than 10% and less than 50 % cells and 3=more than 50% cells positive. In general staining in less than 10% was considered as negative staining and more than 10% was considered positive for KAI1.

Statistical analysis

Continuous data were summarized as mean ± SE, while discrete (categorical) in %. Qualitative variables were expressed as numbers and percentages. Comparisons were made between categorical groups by chi-square ($\chi 2$) test. Comparisons were made between two independent

groups by independent Student's t-test. A two tailed p<0.05 was considered statistically significant. Kaplan Meier survival curve was made and survival time was compared using Log rank test. All analysis was performed on SPSS (Windows version 21.0) software.

Results

The study included 83 histologically proven cases of breast cancer and similar number of adjacent normal tissue as control. The median age of the patients was 49 years (range, 18-70 years).

Quantitative RT PCR: Quantitative mRNA expression was analysed using RT-PCR in 75 breast cancer tumors and same number of controls. The mean fold expression of gene indicated that it was overexpressed 7.68±2.7 fold in breast cancer as compared to controls, whilst in non metastatic cases it was overexpressed 2.8±1.36 fold as compared to metastatic cases. Moreover, KAI1 expression was higher in 61.3% (46/75) patients who exhibited high (>4) KAI1 expression and 38.7% (29/75) had low (≤4) KAI1 expression. The relationship between KAI1 mRNA and clinicopathological features of breast cancer is summarized (Table 1).

Immunohistochemistry: Transcriptomic analysis revealed membranous expression in 61.4% (46/75) cases so we left out the remaining 38.6% samples and did not subject these to IHC. IHC results unveiled KAI1 expression was 1 positive in 26.6% (10/46) breast cancer cases, 2 positive in 26.1% (12/46) breast cases, 3 positive in 40.2% (18/46) cases and negative in 11.9% (6/46)

Table 1. Correlation of KAI1 Gene Expression with Clinical and Histopathological Characteristics Of Breast Cancer Patients

Variables	KAI1 high (>4) (n=46) (61.3%)	KAI1 low (≤4) (n=29) (38.7%)	P value
Age			0.262
≤45	24 (52.1%)	17 (58.6%)	
>45	22 (47.8%)	12 (41.3%)	
Node Size			<0.001
≤2	26 (56.5%)	16 (55.1%)	
>2	20 (43.4%)	13 (44.8%)	
Node Status			<0.001
N0	07 (15.2%)	05 (17.2%)	
N1	11 (23.9%)	04 (13.7%)	
N2	19 (41.3%)	07 (24.1%)	
N3	09 (19.5%)	13 (44.8%)	
Tumor size			<0.001
≤3	38 (82.6%)	21 (72.4%)	
>3	08 (17.4%)	08 (27.6%)	
Tumor Stage			0.001
T0	06 (13.1%)	04 (13.7%)	
T1	09 (19.5%)	03 (10.3%)	
T2	03 (6.5%)	07 (22.4%)	
T3	16 (34.7%)	04 (12.1%)	
T4	12 (26.1%)	11 (43.1%)	
Metastasis status			<0.001
M0	43 (93.4%)	16 (77.6%)	
M1	03 (6.6%)	13 (22.4%)	
ER Status			0.48
-ve	28 (60.8%)	12 (41.3%)	
+ve	18 (39.1%)	17 (58.6%)	
PR status			0.095
-ve	27 (58.6%)	14 (48.2%)	
+ve	19 (41.3%)	15 (51.8%)	
Her2 neu status			0.58
-ve	22 (47.8%)	16 (53.4%)	
+ve	24 (52.1%)	13 (46.6%)	
Histological grade			0.016
Well differentiated	09 (19.5%)	04 (13.7%)	
Moderately differentiated	17 (36.9%)	09 (25.8%)	
Poorly differentiated	20 (43.4 %)	16 (60.4%)	
Stage			<0.001
I	10 (21.7%)	03 (6.8%)	
II	07 (15.2%)	07 (29.3%)	
III	22 (47.8%)	12 (44.8%)	
IV	07 (15.2%)	07 (19.1%)	

Table 2. Correlation of KAI1 Protein Expression with Clinical and Histopathological Characteristics of Breast Cancer Patients

Variables	KAI1 positive (n=25) (61.9%)	KAI1 negative (n=15) (38.1%)	P value
Age			0.217
≤45	14 (56.0%)	12 (80.0%)	
>45	11 (44.3%)	03 (20.0%)	
Node size			<0.001
≤2	22 (88.2%)	03 (20.0%)	
>2	03 (11.8%)	12 (80.0%)	
Node status			<0.001
N0	03 (12.0%)	02 (13.3%)	
N1	07 (28.0%)	02 (13.3%)	
N2	11 (44.0%)	01 (6.7%)	
N3	06 (24.0%)	10 (66.7%)	
Tumor size			0.003
≤3	19 (76.0%)	04 (26.7%)	
>3	06 (24.0%)	11 (73.3%)	
Tumor stage			0.003
T0	02 (8.0%)	02 (13.3%)	
T1	02(8.0%)	02 (13.3%)	
T2	02 (8.0%)	03 (20.0%)	
T3	11 (44.0%)	04 (26.7%)	
T4	07 (28.0%)	04 (26.7%)	
Metastasis status			0.003
M0	22 (88.2%)	13 (86.7%)	
M1	03 (11.8%)	02 (13.3%)	
ER status			0.67
-ve	16 (64.0%)	07 (46.7%)	
+ve	07 (28.0%)	08 (53.3%)	
PR status			0.12
-ve	18 (72.0%)	07 (46.7%)	
+ve	07 (28.0%)	08 (53.3%)	
Her2 neu status			0.53
-ve	13 (52.0%)	09 (60.0%)	
+ve	12 (48.0%)	06 (40.0%)	
Histological grade			0.763
Well differentiated	04 (16.1%)	03 (20.0%)	
Moderately differentiated	12 (48.0%)	07 (46.7%)	
Poorly differentiated	09 (35.9 %)	05 (33.3%)	
Stage			<0.001
I	05 (20.0%)	02 (13.3%)	
II	03 (12.0%)	07 (46.7%)	
III	13 (52.0%)	03 (20.0%)	
IV	04 (16.0%)	03 (20.0%)	

Table 3. Median Survival in Months

Variables	Median survival in months	P value
KAI1 high expression	19.17	0.047
KAI1 low expression	16.28	

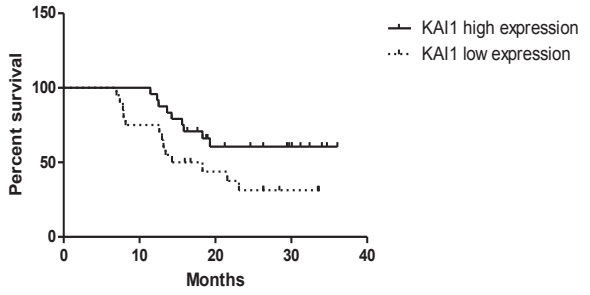

Figure 1. Kaplan Meier Survival Curve of Patients after 3 Years Follow-up. Significant association of KAI1 level (p=0.04) in negative KAI1group versus positive KAI1 group had been observed

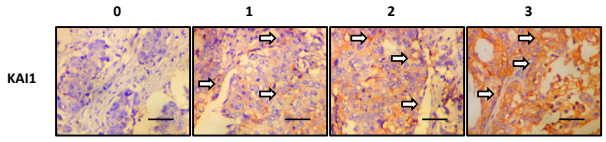

Figure 2. Representative Staining Results for KAI1. Sections of formalin-fixed paraffin-embedded specimens were stained for KAI1 antibodies. Positive membranous staining for the KAI1 antibody is shown, illustrating 0 to 3+ staining scale. Arrows point to the stained cells. x20

breast cases. Representative images have been shown in figure 1. The relationship between KAI1 mRNA and clinicopathological features of breast cancer has been summed up (Table 2).

Clinico pathological characteristics with reference to KAI1 gene: The present study showed that downregulation of KAI1 at the gene and protein level is significantly correlated with advanced TNM categories and higher stage. No significant correlation was observed between KAI1 expression and age, ER, PR, Her2neu and menopausal status (Tables 1 & 2).

KAI1 expression in different stages and histological grade: The mRNA analysis showed that KAI1 expression was low in Advanced (Stage II & IV) vs Early stage (Stage I & II) (2.87 ± 0.63, p<0.05). Similar results were found in protein analysis wherein KAI1 expression levels were lower in advanced as compared to early stage of the disease (1.35 ± 0.24, p<0.05).

We were unable to identify any significant correlation of KAI11 transcript level between grade 1 (well differentiated) and grade 2 (moderately differentiated) and also between grade 3 (poorly differentiated) and grade 2 breast cancer tissues. The mRNA analysis showed that KAI1 expression was low in poor vs well differentiated tissues (2.32 ± 1.08, P<0.05). Protein analysis also corroborated similar findings wherein KAI1 expression levels were lower in poorly differentiated tumors as compared to well differentiated tumors (0.67 ± 0.17, p<0.05) (Tables 1 & 2).

KAI1 expression and its correlation with median overall survival: According to the Log rank test median OS, 19.17 months was the median survival for KAI1 high/positive patients as compared to 16.28 months for KAI1 low/negative patients. This difference was statistically significant (P=0.047) (Figure 1 & Table 2).

Discussion

The abrupt rise of breast cancer incidence among women has made it the leading cause of morbidity and mortality globally leaving behind cervical cancer (Asthana et al., 2014). This unfortunate development has triggered a disquieting need for the identification of novel markers (diagnostic, predictive & prognostic) which can be fruitful in designing remedial measures for breast carcinogenesis. Metastasis plays an important role in cancer-related fatality. Despite recent advances in cancer treatment, including improved surgical excision techniques, radiotherapy and chemotherapy, metastatic recurrence represents a tremendous clinical obstacle for the successful treatment of BC.

Metastasis is the most lethal attribute of this disease and has a complicated multistage process that requires the coordination of multiple genes, including both metastasis promoting genes and metastasis suppressor genes (Aznavoorian et al., 1993). Breast cancer progression results from a series of genetic changes (Sato et al., 1990).

Clinicopathological parameters have not been assessed in detail in a single population in context to associating with metastatic markers and metastatic propensity. No Indian study has been reported on this topic. In this cohort study, individuals from North Indian patients with breast cancer were enrolled. The cancer and non cancerous tissues when compared for KAI1 expression levels were dissimilar in the studied groups (P< 0.05), suggesting that these markers individually may confer metastatic propensity to the breast cancer patients in our population. We found differences by comparing the results of KAI1 expression between the cancer group and the normal tissue group (P<0.05) and in the cancer group between the metastatic cases vs non metastatic (P=0.04).

We found decreased expression of KAI1 in non cancerous as compared to breast cancer tissue (P<0.05). In a separate experimental setting, conventional RT-PCR highlighted similar alteration in KAI-1 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 in breast cancer (Malik et al., 2009). However, on the other hand, significantly high levels of KAI1 expression were found in normal breast tissues and benign breast tumors from patients with breast cancer (Yang et al., 2000). Similar decreasing expression of KAI1 was observed in cancer pancreatic tissue vs normal (P<0.05) (Huang et al., 2016).

KAI1 expression was higher in non metastatic breast tissue vs metastatic breast tissue (P<0.05). Decreased KAI1 protein expression was also found to be associated strongly with the progression of endometrial cancers from hyperplasia to metastasis (P<0.001) (Liu et al., 2013). The rate of KAI1 mRNA expression in gastric cancer patients with lymph node metastasis was markedly decreased

compared with gastric cancer patients without lymph node metastasis, and the difference was statistically significant (P<0.05) (Guo et al., 2015).

KAI1 levels were attenuated in advanced T category vs early T category in our study (P<0.05).

Similar conclusions were derived by Guo and his coworkers (Guo et al., 2015) where they found a significant negative correlation between the TNM stage and KAI1 mRNA expression in gastric cancer patients. Likewise, KAI1 expression was negatively associated with the clinical stage in cervical cancer patients (P<0.05) (Zhuo et al., 2015). In a previous study on breast cancer by Malik and his coworkers, (Malik et al., 2000) they showed that early stage breast tumours (TNM1) had a significantly highly levels of KAI1 transcripts compared with late stage tumours (TNM2, 3 and 4) which was in similar to our results.

We also found lower KAI1 expression in poorly differentiated in comparison to well differentiated ones (P<0.05). In an investigation by Yang on breast tissues, (Yang et al., 2000) the group also found analogous results and concluded that KAI1 expression was also inversely correlated with the severity of tumor which are consistent with most of the current literature and hence stated that KAI1 is a favorable prognostic factor for a variety of human cancers. However, in sharp contrast to these results, Malik and group (Malik et al., 2009) 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) invasive ductal breast cancer tissues.

Patients who were alive had significantly higher levels of KAI1 transcripts than those who died of breast cancer (p=0.047). Using the Kaplan-Meier survival model, we found that patients with high levels of KAI1 transcripts had a significantly longer survival (19.17) than patients with low level of KAI1 (16.28) (P<0.05). Literature reports also confirm this finding. It was also found that patients with KAI1- negative tumors had a lower survival rate than those with KAI1 positive tumors (Liu et al., 2003). The findings of this study also revealed a higher survival rate in KAI1 positive breast cancer patients than KAI1 negative breast cancer patients. Distant metastasis was observed at a lower rate in KAI1 positive breast cancer patients than in KAI1 negative breast cancer patients, suggesting that KAI1 is positive indicator of a favorable breast cancer prognosis.

In conclusion, the decreased expression of KAI1 protein, endows cancer with high aggressiveness and a poor prognosis. 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 signaling pathways is an area that requires further investigation. Conclusively, these results provide clinical evidence to support that KAI1 is a breast carcinoma MSG. Measuring KAI1 expression will help to identify those breast cancer patients with metastatic propensity and hence guide clinicians to risk stratify their patients and need for close follow up and aggressive treatment plan. Further functional studies are needed to elucidate the mechanism of metastasis suppression of KAI1 and to confirm its metastasis suppression function in other tumor types and models. The clinical significance of KAI1 mRNA expression in breast cancer ratifies clinical evaluation of KAI1 on a larger BC population size.

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