RESEARCH COMMUNICATION

# Expressional Alterations and Transcript Isoforms of Metastasis Suppressor Genes (KAI1 & KiSS1) in Breast Cancer Patients

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## Abstract

**Background: Metastasis suppressor genes are involved in the inhibition of a cancer cell's ability to metastasize. Down expression of such genes may contribute to pathogenesis of breast cancer. The aim of current study was firstly to evaluate expression of two examples, KAI1 and KISS1, and then to determine relationships with stages of breast cancer in a Pakistani population. Methodology: Fresh biopsy tissues were collected from different hospitals and oncology research institutes. The semi quantitative reverse transcriptase polymerase chain reaction was used to investigate KAI1 and KISS1 expression in 25 breast tumor tissues and 25 normal tissues. Statistical analysis was performed to explore its association with breast cancer risk. Results: The present study revealed that KAI1 and KISS1 mRNA expression was markedly reduced in tissues of breast cancer compared to adjacent normal tissue. In present study a splice variant of KAI1 during a screen for its expression analysis was also observed. This splice variant has not been reported previously. Conclusions: Metastasis suppressor genes demonstrate reduced expression in breast cancers in Pakistan.**

**Key words:** Breast cancer - metastasis suppressor genes - KAI1 - KISS1 - mRNA expression

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## Introduction

Breast cancer is the most common malignancy among Pakistani women, accounting for 34.6% of all female cancers. This malignancy arises from the uncontrolled growth of cells in the breast. It is considered as a heterogeneous disease and the most lethal attribute of a cancer (Barbara et al., 2000; Bhurgri, 2004; Haris et al., 2007). Breast cancer metastasis is a leading cause of death in cancer patients and is a multistep process involving complex interactions between tumor and host cells. To metastasize, tumor cells must invade or migrate from the primary tumor while moving to close or distant secondary sites (Shoji et al., 2010).

KiSS1 and KAI1/CD82 genes are regarded as new metastasis suppressor genes. Human KAI1 gene, located on chromosome 11p11.2, contains 10 exons and 9 introns with its coding region starting in exon 3 and ending in exon 10 (Dong et al., 1995 and Dong et al., 1997). It was first described in 1991 as a 267 amino acid, tetraspanin protein with four membrane-spanning segments and a single major extracellular domain, with three potential N-glycosylation sites, thought to be involved in inhibiting cancer cell migration and invasion (Ichikawa et al., 1991; Wright et al., 1994). Down-regulation of KAI1/CD82 mRNA and protein levels has been reported in the invasive and metastatic stages of various cancers including prostate cancer (Jackson et al., 2003), lung cancer (Wang et al., 2005), gastric cancer (Tsutsum et al., 2005 and Guan-Zhen et al., 2007), ovarian cancer (Houle et al., 2002), bladder cancer (Jackson et al., 2007) and cervical cancer (Schindi et al., 2000). KAI1/ CD82 might function as a negative regulator of colorectal carcinoma metastasis (Liu et al., 2003), and suppress integrin-induced invasion by regulating signaling to c-Met and Src kinases (Sridhar and Miranti, 2006). Down-regulation of KAI1/CD82 expression may be an important step in the progression of many types of human malignancy but the mechanism of the down-regulation remains to be elucidated.

KiSS1 gene has been found to be a novel metastasis suppressor gene (Lee and Welch 1997). It is located on chromosome 1q32, containing 4 exons. First exon is not translated while exon 2 and 3 are coding exons. The primary translation product of the KiSS1 gene is a 145 amino acid polypeptide (Kp-145) (Ohtaki et al., 2001), but shorter kisspeptins (Kp) with 10, 13, 14 or 54 amino acid residues have been discovered (Kotani

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et al., 2001; Stafford et al., 2002; Harms et al., 2003). Kp-54, also termed 'metastin', is the putatively secreted and biologically active form of Kp-145 (Harms et al., 2003). Expression of this gene has been shown to be high in human placenta and testis, followed by the pituitary and spinal cord, with moderate levels in the liver, pancreas and intestines (Ohtaki et al., 2001 and Harms et al., 2003). KiSS1 has been shown to function as a metastasis suppressor gene and reduce the number of metastasis in different cancers including bladder cancer (Sanchez-Carbayo et al., 2003), gastric cancer (Dhar et al., 2004) and prostate cancer (Davies et al., 2004). Expression of KiSS1 like other metastasis suppressor genes is commonly reduced or completely ablated in a variety of cancers via an unknown mechanism (Mitchell et al., 2006). In the present study, expression levels of KAI1 and KiSS1 molecules were studied.

## Materials and Methods

*Materials*

RNA later® (Ambion), TRIzol (Invitrogen). SuperScript III First Strand cDNA synthesis kit (Invitrogen), PCR Mastermix (Fermantas) and Agrose (Invitrogen) were used in this study. PCR Primers were designed using Primer 3 input software version 0.40 and BLAST using NCBI PRIMER BLAST, which were then synthesized by Eurofins.

*Tissue collection and storage of samples*

Breast tissue samples were collected from April 2010 to October 2010 with a prior approval from Ethical Committees of both CIIT and hospitals/institutes including PIMS (Pakistan Institute of Medical Sciences) Islamabad and MH (Military Hospital) Rawalpindi. Tumor samples and their adjacent non-cancerous breast tissues were collected from 25 patients immediately after surgery by the concerned surgical team. Each tissue sample was collected in RNA later® and kept at 4°C or at - 80°C for long term storage.

*Tissue processing and RNA isolation*

Breast tissue sample was removed out from freezer and cut into small pieces by using sterile razor blade. All the cut tissue pieces were scraped together into microfuge tubes using a spatula and homogenized using a hand held homogenizer. Total RNA of the tumor and non-tumor tissue samples was extracted with Trizol reagent. Concentration of RNA was confirmed by using UV spectrophotometer (GENESYS).

*Polymerase chain reaction (PCR)*

In order to evaluate expression analysis of KAI1 and KISS1, semi quantitative reverse transcription PCR was used. Extracted RNA was first reverse transcribed to synthesize cDNA using cDNA kit. In the second step, this cDNA was amplified using gene specific primes (KAI1 and KISS1) followed by the expressional analysis of respective genes in control and tumor samples.

**Table 1. Primers for KAI1, KiSS1 and GAPDH**

| Gene | Function | Sequence |
|---|---|---|
| KAI1 | Forward | 5'-TCTGGGACTGAGACCAGGAG-3' |
| | Reverse | 5'-TTACACTAATCCCGCGAAGG-3' |
| KISS1 | Forward | 5'-GGGACCTCGCTGTCCCCGCC-3' |
| | Reverse | 5'-GAAGCGCAGGCCGAAGGAGT-3' |
| GAPDH | Forward | 5'-GAAGGTGAAGGTCGGAGTC-3' |
| | Reverse | 5'-GAAGATGGTGATGGGATTTC-3' |

*Reverse transcription polymerase chain reaction*

RNA was reverse transcribed using oligo (dT) primers and the SuperScript First-Strand Synthesis System (cDNA kit) (Cat no. 11904-018; Invitrogen, USA). cDNA was synthesized in a 10 µl reaction mixture using 3-5 µl of total RNA as per manufacturer's instructions.

*Amplification of KAI1 and KISS1 transcripts*

To conduct the expression analysis of metastasis suppressor genes reaction conditions for PCR amplification of KAI1 and KISS1 were optimized in breast cancer patients of Pakistani population. Semiquantitative reverse transcriptase (RT) PCR was used to screen a number of human breast cancer tissues along with their controls. cDNA was used as the template for PCR. Reaction conditions were 94˚C for 2 min; 94˚C for 15 sec; 55˚C for 30 sec, 72˚C for 1 min for 30 cycles with 2 min final extension at 72˚C. Amplified PCR products were fractionated on to a 2 % agarose gel. The primer pairs are given in Table 1.

*Sequencing*

2% agarose gel electrophoresis showed 274bp fragment that was expressed in 6 samples, in addition to the normal 174 bp transcript of KAI1 gene. PCR products were then sequenced from (MC LAB,US).

*Statistical Analysis*

The $\chi 2$ probability test was used to compare the distribution of individual variables among patients. A P value of $<0.05$ was considered statistically significant.

## Results

*Expression of KAI1/CD82, KISS1 and GAPDH molecule in control and tumor breast tissues*

Semi-quantitative PCR highlights expressional levels between paired control and tumor tissues. Uniform expression of KAI1, KISS1 and GAPDH was observed in all control breast tissue samples. No expression variation has been observed in KAI1: GAPDH and KISS1: GAPDH as presented in Figure 1. Uniform expression of GAPDH was observed in all tumor breast tissues, whereas expression of KAI1 and KISS1 varied depending upon the stage of breast cancer. In majority of these paired samples KAI1 and KISS1 levels appeared to be reduced in the tumor tissue as compared to control

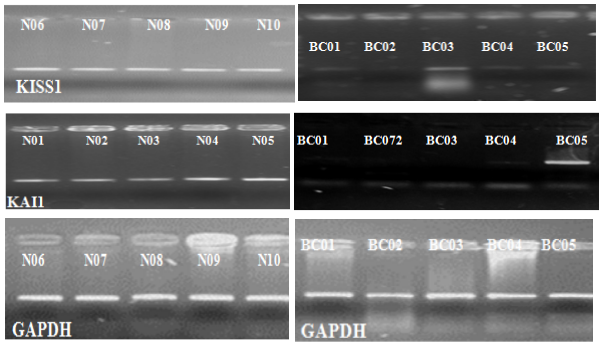


**Figure 1. Findings of 2% Agarose Gel Electrophoresis.** Normal (N) and breast cancer (BC) mRNA expression for KISS1, KAI1 and GAPDH detected by semi quantitative RT-PCR

samples as shown in Figure 1.

*Socio-demographical and clinico-pathological characteristics with reference to KAI1 and KiSS-1 genes*

The present study shows that down-expression of KAI1 and KiSS1 genes is significantly correlated with tumor progression. Tables 2 and 3 explain correlations with socio-demographical and clinic-pathological findings.

*KAI1 and KISS1 expression in different stages and histological types*

The results indicated that KAI1 and KiSS1 expression

**Table 2. Correlation Between Patient Findings and KAI1 Gene Expression Levels in Breast Cancers**

| Variables | | Normal | Low | p Value[a] |
|---|---|---|---|---|
| Age (years) | ≤40 | 4(16%) | 3(12%) | 0.03 |
| | ≤60 | 1(4%) | 14(56%) | |
| | Above | 0 | 3(12%) | |
| Marital Status | Married | 4(16%) | 22(88%) | 0.01 |
| | Single | 2(8%) | 0 | |
| Tumor Side | Left | 4(16%) | 10(40%) | NS |
| | Right | 2(8%) | 9(36%) | |
| Tumor Size (cm) | ≤2 | 4(16%) | 1(4%) | 0.0024 |
| | >2 &<5 | 2(8%) | 7(28%) | |
| | ≥5 | 0 | 11(44%) | |
| Tumor Location | Center | 2(8%) | 13(52%) | 0.018 |
| | Eccentric | 4(16%) | 2(8%) | |
| | Sub areolar | 0 | 4(16%) | |
| Lymphatic Invasion | Yes | 0 | 15(60%) | 0.0016 |
| | No | 6(24%) | 4(16%) | |
| Vascular Invasion | Yes | 0 | 10(40%) | 0.021 |
| | No | 6(24%) | 9(36%) | |
| Skin Involved | Yes | 0 | | 0.002 |
| | No | 6(24%) | | |
| Nipple Involved | Yes | 0 | 10(40%) | 0.021 |
| | No | 6(24%) | 9(36%) | |
| Histological Grade | Well diff | 4(16%) | 1(4%) | 0.0024 |
| | Mod diff | 2(8%) | 7(28%) | |
| | Poor diff | 0 | 11(44%) | |
| Histological Type | Lobular[b] | 3(12%) | 2(8%) | 0.007 |
| | Ductal[b] | 1(4%) | 13(52%) | |
| | Medullary | 2(8%) | 0 | |
| | Other | 0 | 4(16%) | |

[a]χ2-test; NS, not significant; [b]Invasive

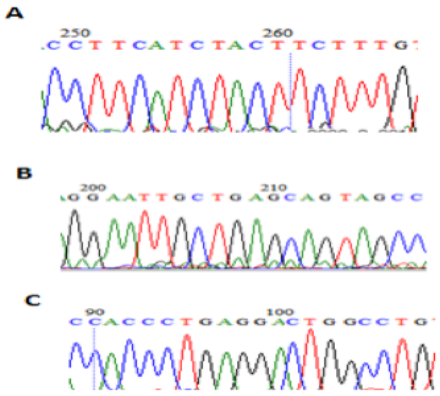


**Figure 2. Sequencing Electropherogram showing analysis of KAI1 variant (A, B) Wild KAI1(C).** Reverse primer was used for sequencing. Insertion of 274 bp intronic region was found between primer pair

decreased with the increase in stage of the disease. The differences in expression were statistically lower and different between stages. Interestingly, KAI1 and KiSS1 expression was considerably lower in invasive ductal carcinoma compared to lobular and other types of breast cancer.

*Sequencing results of KAI1 gene*

Sequencing results are illustrated in Figure 2. A variant was observed in 6 samples with band size of approximately above 400 bp as compared to the desired

**Table 3. Correlation Between Patient Findings and KISS1 Gene Expression Levels in Breast Cancers**

| Variables | | Normal | Low | p Value[a] |
|---|---|---|---|---|
| Age (years) | ≤40 | 2(8%) | 5(20%) | 0.022 |
| | ≤60 | 1(4%) | 14(56%) | |
| | Above | 0 | 3(12%) | |
| Marital Status | Married | 1(4%) | 22(88%) | 0.0003 |
| | Single | 2(8%) | 0 | |
| Tumor Side | Left | 2(8%) | 12(48%) | 0.015 |
| | Right | 1(4%) | 10(40%) | |
| Tumor Size (cm) | ≤2 | 2(8%) | 3(12%) | 0.012 |
| | >2 &<5 | 1(4%) | 8(32%) | |
| | ≥5 | 0 | 11(44%) | |
| Tumor Location | Center | 1(4%) | 14(56%) | 0.022 |
| | Eccentric | 2(8%) | 5(20%) | |
| | Sub areolar | 0 | 3(12%) | |
| Lymphatic Invasion | Yes | 1(4%) | 14(56%) | 0.0001 |
| | No | 2(8%) | 8(32%) | |
| Vascular Invasion | Yes | 0 | 10(40%) | 0.0089 |
| | No | 3(12%) | 12(48%) | |
| Skin Involved | Yes | 1(4%) | 16(64%) | 0.0098 |
| | No | 2(8%) | 6(24%) | |
| Nipple Involved | Yes | 0 | 10(40%) | 0.0089 |
| | No | 3(12%) | 12(24%) | |
| Histological Grade | Well diff | 2(8%) | 3(12%) | 0.0122 |
| | Mod diff | 1(4%) | 8(32%) | |
| | Poor diff | 0 | 11(44%) | |
| Histological Type | Lobular[b] | 1(4%) | 4(16%) | NS |
| | Ductal[b] | 2(8%) | 12(48%) | |
| | Medullary | 0 | 2(8%) | |
| | Other | 0 | 4(16%) | |

[a]χ2-test; [b]Invasive


**Figure 3. The 274 bp Additional Sequence, Matched to the Intronic Portion Present between Exons 8 and 9.** Blue, forward primer; green, reverse primer; red, exons; black intronic region

band of 174bp. Suspected 6 samples were sent for sequencing. The results showed that in addition to 174bp present in transcript cDNA, an additional sequence was present between primer pairs. This sequence was aligned to the DNA sequence present between primer pair using EMBOSS pair wise alignment tool. The additional sequence matched to the intronic portion present between exon 8 and 9. A 274 bp intronic insert was present in variants observed (Figure 3). This transcript variant observed with the intronic insert of 274 bp is reported for the first time. Translation of this sequence using ExPASy Proteomics Server shows that it contains stop codon which may serve as premature termination signal for the protein product; hence no protein product would be formed.

## Discussion

Breast cancer is the commonest malignancy of females world wide and second leading cause of death due to cancer among females. In Pakistani population it is the leading cancer in females and accounts for 34.6% of all cancers. 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 stimulating genes and metastasis suppressor genes (Aznavoorian et al., 1993). It is most life-threatening aspect of cancer that requires cancerous cells to escape the primary tumor, intravasate and extravasate the circulatory system, and then penetrate to peripheral sites and grow. Metastatic cells must have the capacity to adhere to basement membranes and degrade membranes (Yang et al., 1997). Breast cancer progression results from a series of genetic changes (Sato et al., 1990).

Metastasis suppressor genes increase the chances of resistance against the metastasis; role of metastasis suppressor genes is now well established in various cancers like breast and ovarian cancer, lung cancer, prostate cancer, gastric cancer, and hepatocellular carcinoma (Dong et al., 1996; Maurer et al., 1999; Lombardi et al., 1999). Many MSG's have been found in different malignant tumors as NM23, KISS1, KAI1, DRG1, BRMS1 etc (Horak et al., 2008). Several lines of evidence have pointed to the existence of metastasis suppressor genes for breast cancer. Inactivation of these genes plays a crucial role in breast cancer metastasis (Welch et al., 2000; Debies and Welch, 2002). If breast cancer is diagnosed and treated before spreading, chances of 5years survival approaches 100%. However, if metastasis has developed, survival is poor. Therefore, metastasis is the most critical parameter determining patient survival from breast cancer (Yang et al., 1997).

Limited number of studies has been published in context to Pakistani population on the metastasis suppressor genes. The objective of the present study was to identify expression of KAI1 and KISS1 gene in breast cancerous tissues, followed by semi-quantitative PCR analysis to measure the mRNA expression of eachrespectively.

Metastasis remains an important clinical problem for most of the malignancies, including breast cancer. Single metastasis suppressor genes can be connected to specific steps in the metastatic cascade. KAI1 is assumed to have the potential to suppress cell detachment from the primary tumor and cell migration. KISS1 is assumed to be able to suppress metastatic growths at the secondary sites (Stark et al., 2004).

KAI1 is a tumor metastasis suppressor gene that is capable of inhibiting the metastatic process in humans (Mashimo et al., 1998). It is a member of the transmembrane-4 superfamily (TM4SF), is located at chromosome 11p11.2 and encodes a transmembrane protein of 267 amino acids (Dong et al., 1995).

In the present study we report that high mRNA expression of KAI1 is a feature of many breast cancer cells with low metastatic potential. KAI1 mRNA expression is substantially lower in the most aggressive and metastatic breast cancer tissues. In order to evaluate the importance of KAI1 mRNA expression in the process of human breast cancer metastasis, mRNA level of KAI1 was compared between tumor and control tissue. Decreased expression of KAI1 has been consistently reported in breast cancer progression in many of earlier studies (Adachi et al., 1998; Yang et al., 2000; Huang et al., 2005). Results of the present study are in accordance with many earlier studies where it has been shown that decreased KAI1 mRNA expression is a useful marker for metastatic/invasive potential in a series of human tumor types. Down regulation of KAI1 is associated with tumor progression and metastasis in multiple tumors of the lung (Adachi et al., 1996), bladder (Yu et al., 1997), colon (Maurer et al., 1999 and Lombard et al., 1999) and esophagus (Uchida et al., 1999 and Miyazaki et al., 2000). Stark et al (2004) found that expression of KAI1 reduces as stages of breast cancer increases and metastasize. In this study, partially lost or ablated expression of KAI1 gene was observed in 5 samples of advanced stages, which is in accordance with the findings of earlier reported studies (Motozawa et al., 2008). Two other studies also revealed that KAI1 expression is down regulated in advanced breast and colorectal cancer (Yang

et al., 1997 and Yang et al., 2000).

Down-regulated KAI1/CD82 expression does not result from loss of heterozygosity (Kawana et al., 1997 and Tagawa et al., 1999), the hyper-methylation of the CpG island within KAI1/ CD82 promoter region (Jackson et al., 2000 ), or the mutations within the KAI1/ CD82 coding region (Miyazaki et al., 2000). It is more likely due to increased transcription repressor activity or decreased activator activity. So far the conducted studies have shown a direct correlation between KAI1 down regulation and p53 mutation in cancers (Liu and Zhang, 2006 and Guan-Zhen et al., 2007). Some believe that there is no correlation between KAI1 and p53 (Jackson et al., 2003 and Farhadieh et al., 2004). A recent study reported that KAI1 promoter activity is dependent on p53, JunB and AP2 in prostrate cells ( Marreiros et al., 2005).

It has been reported previously that KAI1 molecule contains atleast one alternatively spliced form in which Exon 7 is deleted. As a result this spliced form lacks the distal part of the second extracellular loop and part of the fourth transmembrane region (Lee et al., 2003). In present study splice variant of KAI1 during a screen for its expression analysis was found, with internal 274 bp intronic insert between Exon 8 and 9. This alternatively spliced transcript variant has not reported previously. When the mRNA of this transcript variant was translated using bioinformatics tool, the intronic insert contained stop codon. This leads us to the assumption that this stop codon may serve as pre-mature stop signal and hence, aberrant protein would be formed. Insertion of 274 bp intronic region may make RNA unstable and leads to structural differences between wild and variant KAI1 which may affect its functional characteristics. Thus, wild type KAI1 suppresses cell growth, whereas variant KAI1 does not have this characteristic. Present results indicate that the metastasis suppressor function decreases in variant KAI suggesting that it might promote cell motility and invasion.

Kotani et al (2001) and Ohtaki et al (2001) indicated that KiSS1 might act as a metastasis suppressor gene through the activation of a novel human G-protein coupled receptor (GPCR) named as GPR54. Stafford et al (2002) found that activation of the receptor by the direct coupling of the KiSS1 peptide to the Gαq-mediated phospholipase C-Ca2+ signaling pathway. Stimulation of this pathway induces the inhibition of cell proliferation and cell migration.Results from this study present that average level of the KiSS1 gene does not change among control breast tissues; as a significant low level of KiSS1 mRNA expression was detected in 23 breast tissue samples. KiSS1 expression was down regulated more with tumor progression. Correlations between KiSS1 expression and the clinicopathologic characteristics are shown in Table 8 and 9. All histopathalogical parameters given in section 3 presented highly significant ($P<0.05$) correlation with KISS1 expression except histological type of cancer ($P>0.05$).

Loss of KiSS1 expression has been found associated with tumor progression and poor patient survival (Sanchez- Carbayo et al., 2003). Shirasaki (2001) reported that loss of KiSS1 mRNA expression is strongly associated with loss of heterozygosity of 6q16.3- q23 in melanoma. In our study mRNA expression of KISS1 is elevated in low grade breast cancers tissues, but is reduced in high grade metastic tumor. Mitchell (2005) suggested a possible model for transcriptional activation of KiSS1 in breast cells. Results indicated that AP-2α and Sp1 are strong transcriptional regulators of KiSS1 and that loss or decreased expression of AP-2α in breast cancer may account for the loss of tumor metastasis suppressor KiSS1 expression and thus increased cancer metastasis.

The results from our study show that down regulation of KiSS1 expression produces tumor invasion and metastasis. Our results are in conflict with other several studies which demonstrated that over expression of KISS1 in thyroid, hepatocellular and breast cancer is associated with disease progression (Ringel et al., 2002; Ikeguchi et al., 2003; Martin et al., 2005). Preliminary findings suggested that the KISS1 gene negatively regulates the metastasis of human breast cancer because reduced mRNA expression is seen more frequently in metastatic breast cancer than localized cancer. Its function is actually controversial in breast cancers and in other tumors. It is evident that further research is necessary before the true role and effect of KiSS1 in breast cancer can be elucidated.

To the best of our knowledge, the present study is the first of its kind in breast cancer in Pakistani population, in which it has been shown that down regulation of KAI1 and KiSS-1 expression produces frequent tumor invasion and may become a strong prognostic determinant in breast carcinoma patients. Role of KAI1 in various cellular signaling pathways, involved in tumor progression, is an area that requires further investigation. For KiSS1 gene, there are several possibilities such as homozygous deletion, promoter methylation, or mutations which might be responsible for down regulation of this gene in tumors and needs further studies for clarification.

In conclusion, KAI1 and KISS1 expression is decreased in human breast cancer, particularly in patients with aggressive tumors and with mortality. It is evident that further research is necessary before the true role and effect of KISS1 and KAI1 in breast cancer can be elucidated.

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