Gene Ther Mol Biol Vol 12, 239-246, 2008

Screening of coding region of metastasis suppressor genes KISS1 and KAI-1 for germ line mutations in breast cancer patients

Research Article

Fraz Arshad Malik[1,2,*], Mahmood Akhter Kayani[1], Hina Iqbal[1], Wen G Jiang[2], Rafshan Sadiq[3]

[1]Cancer Genetics Lab, Department of Biosciences, COMSATS Institute of Information Technology, Islamabad, Pakistan
[2]University Department of Surgery, Cardiff University School of Medicine, Cardiff, Wales, United Kingdom
[3]Punjab Institute of Nuclear Medicine, PINUM, Faisalabad, Pakistan

***Correspondence**: Dr Fraz Malik, Department of Surgery, Cardiff University School of Medicine, Cardiff, Wales, CF14 4XN, UK; Tel: 44 29 2074 2895; Fax 44 29 2074 2896; e-mail: Fraz_cemb@yahoo.com

Key words: sporadic breast cancer, KISS1, KAI1, Single strand Conformational polymorphism, SSCP

Abbreviations: Combined Military Hospital of Rawalpindi, (CMH); Metastasis suppressor genes, (MSGs); Nuclear Medicine Oncology and Radiotherapy Institute, (NORI); Punjab Institute of Nuclear Medicine, (PINUM); Single Strand Conformational Polymorphism, (SSCP)

Received: 15 July 2008; Revised: 21 August 2008
Accepted: 5 September 2008; electronically published: October 2008

Summary

Breast cancer is one of the most common female cancers worldwide. Abnormalities of genetic or epigenetic factors are mainly responsible for the development and progression of mammary tumours. In patients with breast cancer, metastasis is the leading cause of death. In recent years, a group of genes has been identified as metastasis suppressor genes (MSGs), which are involved in the suppression of the growth of secondary tumours. Down regulations of MSG expression have been frequently observed in advanced tumours. The present study was designed to screen two of the most frequently down-regulated MSGs (KAI1 and KISS1) for germ line mutations in sporadic breast cancer cases of the Pakistani population. 170 cases of unilateral breast cancer patients, who had no prior history of breast cancer and no other disease in general in their families with age ranging from 35-75yrs, were included in this study. Mutational analysis for the entire coding region of KAI1 and KISS1 (including 10 exons and 3 exons, respectively) was carried out by using the Single Strand Conformational Polymorphism (SSCP) technique. No germ line mutation was observed on the entire coding region in the samples from patients with breast cancer in the Pakistani population. Splice site variants on these genes were also absent in breast cancer patients. Involvement of germ line mutations for these MSGs is thus considered to be an event that occurs less frequently in breast cancer patients of Pakistani population. Conserved coding regions of both MSGs indirectly enlighten the involvement of transacting factor on DNA sequence as major contributor in the progression and aggression of tumours rather than any high risk associated mutation itself. A detailed analysis of regulatory mechanism is required to explore the genetic basis of down regulation of these MSGs for a better understanding of breast cancer progression.

I. Introduction

Metastasis is the leading cause of cancer related death in patients with solid tumours. Metastasis is not a random process and its progression requires contribution of genetic and epigenetic factors in cancer. Metastasis is usually characterized by a chain of events that begins with the dislodging of cancer cells from the primary site, crossing the basement membrane, surviving in circulation and finally its adherence to the secondary site for proliferation. The whole cascade of events is controlled by altered expression of several genes. In recent years a group of genes has been identified and characterized as suppressors of metastasis; termed as metastasis suppressor genes (MSGs) (Steeg et al, 1998). These genes suppress metastasis at certain steps of metastatic cascade without affecting the primary tumour growth. In several types of cancer, frequent down regulation of proteins encoded by these genes have been found in conjunction with clinical progression (Stafford et al, 2008). The two most promising genes in this family of MSGs: KAI1 (Ichikawa et al, 1992)

and KISS1 (Lee et al, 1996) are found to have a tumour suppressor role for different types of cancer. Expressional regulation of these genes in relation to various clinical parameters during cancer progression have been extensively investigated in prostate (Jackson et al, 2003), breast (Stark et al, 2005, Huang et al, 2005), lung (Wang et al, 2005), ovarian (Houle et al, 2002), ocular (Martin et al, 2008), gastric (Tsutsumi et al, 2005; Guan-Zhen et al, 2007), pancreatic (Sho et al, 1998), oesophageal (Ikeguchi M et al, 2004; Farhadieh et al, 2004), bladder (Sanchez et al, 2003; Jackson e al, 2007) and cervical (Schindi et al, 2000) cancers. The *KAI1* gene, also termed as prostate metastasis marker, encodes a glycoprotein with 267 amino acids. KAI1 protein has a transmembrane location (White et al, 1998). KISS1 protein has 138 amino acids, with a cytoplasmic location (Kevin et al, 2006). The mechanism by which KAI1 exerts metastasis suppression has shown to be induced by their interaction with different molecules, integrins and epidermal growth factor receptor EGFR, for example (Odintsova et al, 2000). Forced expression of these proteins results in decreased invasion and adhesion in breast cancer cell lines.

4-10 % of breast cancer cases are due to germ line mutations. So far KISS1 and KAI1 have not been screened for germ line mutations in mammary tumours. The only available report for KAI-1 mutation is a study on oesophageal squamous cell carcinoma, in which node metastasis was found to be correlated with KAI1 expression along with mutational screening of KAI1 (Miyazaki et al, 2000). An altered splice variant of KAI1 has also been identified and is known as KITENIN. The carboxy terminal portion of this variant is unable to induce suppression of tumour invasion due to lack of exon 7 coding amino acids (Lee et al, 2004). This suggests the importance of screening KAI1 for any germ line mutations to distinguish whether there is germ line involvement for regulation of this protein. KISS1, termed as metastin or kisspeptin (KP54), is known to have spliced variations and play a role in cancer progression (Rouille et al, 1995). However, germ line variants of the coding region of KISS1 has not been explored in relation to breast cancer. The present study was designed with an intention to screen the above mentioned genes for germ line mutations.

We have recently reported that KISS-1 expression in human breast cancer is aberrant. In the study breast tumours were found to have high levels KISS-1 transcript (but not KiSS-1 receptor), compared with normal mammary tissues. Furthermore, our study demonstrated that highly expressed KISS-1 transcript was linked to nodal metastasis and long term survival (Martin et al, 2005). The study has further shown that over-expression of KISS-1 in breast cancer cell lines rendered the cells more motile and invasive. In a recent study, we have shown that the pattern of KAI-1 expression in human breast cancer cells is inversely linked to the invasiveness of the cells, in that over-expression of KAI-1 reduced the *in vitro* invasion and knocking down of KAI-1 in breast cancer cells resulted in cells becoming more invasive (Malik et al, 2008).

The aim of this study was to explore these MSGs genome for any deletion, insertion or frame shift mutations in breast cancer patients. The prime objective of this study was to assess the presence of any germ line mutations on these genes and also to estimate the penetrance of those mutated alleles, if any, in the breast cancer patients. Allelic variations were assessed to estimate whether there was any correlation to breast cancer patients. Splice sites for each exon of the respective genes were screened for any complete or partial loss of genomic portion.

II. Materials and Methods

All reagents used in the following experiment were purchased from Sigma Chemical Ltd. (Dorset, UK) unless stated otherwise. Primers were synthesized from Invitrogen (Paisley, UK) and BigDye®Terminator used for sequencing was purchased was purchased from ABI (Applied Biosystems, USA).

170 patients at different stages of breast cancer were included in this study. Blood samples were collected with prior approval from the Ethical Committees of the participating oncology institutes and hospitals of Pakistan. Oncologists at the Punjab Institute of Nuclear Medicine (PINUM), Allied Hospital of Faisalabad, Nuclear Medicine Oncology and Radiotherapy Institute (NORI) and Combined Military Hospital of Rawalpindi (CMH) helped in identifying and communicating with the respective patients. Blood samples of 200 healthy normal females of similar age group were also included in this study as controls. Samples from these normal women were used as a positive control and also to observe any polymorphism on the respective genes. Blood sampling was done after stringent initial screening (no family history of breast cancer, age of onset of breast cancer, no other family prevailing disorders, no earlier sampling from any other group for any study). The clinical and histological details of the tumours are given in Table 1. Incidence of breast cancer within the male population was not found to be very frequent and adequate enough to justify the penetration, so only female patients were selected for this study.

A. Sample collection and storage

Blood samples from each case were collected in blood vaccutainer with EDTA as an anticoagulant. For storage, transportation and preservation, recommended guidelines were followed (Anderson et al, 1998). 200 blood samples from normal females exonerated from any disorder were also collected from respective ethnic groups of Punjabi, Pathan, Sindhi and Balochi so that a mutation or polymorphism of the respective genes could be differentiated.

B. DNA isolation and dilution

Genome isolation was carried out following the recommended protocol (Köchl et al, 2005) with minor modifications of ethanol precipitation. DNA isolated was first confirmed by agarose gel electrophoreses, than quantified by using a spectrophotometer for the polymerase chain reaction. DNA samples were stored at -20C°, while dilutions were kept at 4C° for further usage.

C. Amplification and Mutation Screening

Sequences for the coding region of KAI1 and KISS1 available on NCBI with gene IDs as 3732 and 3814, respectively, were included in this study. Primers were designed using the Primer-3 software and intron/ exon junctions were also included in this study for a better identification of splice variants. Primer sequences for respective genes KISS1 (3 exons) and KAI1 (10 exons) involved in this study are given in the Table 2 and Table 3 respectively. After optimization, amplification conditions set for each exon were as 95C° for 4min; 95C°for 30s, 55C° for 30s, 72C° for 1min for 30cycles with a final extension of 72C° for 45

min. Amplified products were then run on 2% agarose gels to confirm chances of non-specificity and the yield of amplified products.

For mutation detection, SSCP technique (Lallas et al, 1997) with few modifications was used and samples were screened for any mobility shift in their banding pattern. This change in mobility shift, either predicting any frame shift alterations or base substitution in the specified region, was confirmed by running normal controls along with the samples from the patients. To check and confirm findings, DNA sequencing of the suspected samples was carried out using BigDye®Terminator Reaction kit (ABI, USA). Bio Edit software was use to compare normal and suspected samples.

Table 1. Clinical stages and age group of breast cancer patients

Type of Breast Cancer	Stages of cancer (number of samples)	Age ranges among patients
IDC	Stage 1 (35) Stage 2 (82) Stage 3 (12) Stage 4 (8)	23 ≥ to ≤ 45
ILC	Stage1 (12) Stage 2 (15) Stage 3 (6)	26 ≥ to≤ 53

IDC: Invasive Ductal carcinoma, ILC: Invasive Lobular Carcinoma

Table 2. Primer sequences for KISS1 genomic sequence

	EXONS		PRIMER Sequences(5'-to-3')	Product sizes
KISS1	EXON1	F	TCTTCAGGAGGGTCTGAGGAG	371
		R	GGCTGGTAAACAGGAAAGATCA	
	EXON2	F	CTCTACCAGGAGCCTCCAAAG	395
		R	TAGATTTCCACCAAATGCAATG	
	EXON3	F	AGGTCAAGGAAGGAAAAGAAGG	675
		R	GCACTGACCTTAATGACACCAG	

Table 3. Primer Sequences for KAI1 genomic sequence

	EXONS		PRIMER Sequences(5'-to- 3')	Product sizes
KAI1	EXON1	F	GTTGGGGTACGGCCATAGTG	458
		R	CCTGTCACTAGTTCCGAGGAAAG	
	EXON2	F	GGGAGCCTGGATTTAAAGTGA	381
		R	AATGCTGTAGGAGCCAGAGAAG	
	EXON3	F	ACAGGGTTAGTACCCACCTCCT	458
		R	CTCAGTCCCTACCCACCAAAT	
	EXON4	F	GACTTGGGTTCCAGGGACAG	261
		R	AAAAGCAACAGGCATTGAAG	
	EXON5	F	CAATCCTGAGAAGCCTTACGAA	492
		R	ATGCTCTCCACCCGATGTTAC	
	EXON6	F	CTGCCCATTTCCTCTCATCC	381
		R	CCCCATTTGATCACAAGGAATA	
	EXON7	F	GGGGGAGGCAGTTTAAGTAGG	278
		R	CAGATGCCAGTCCCAGACAT	
	EXON8	F	GTGGCATATCTCAGTCTCTGTCC	478
		R	GCTCTGCCTGTTCCTTGAATAA	
	EXON9	F	CAGCAATTCCTTCCTGCATTTA	373
		R	CACCTCCCCAAGGAGATCAG	
	EXON 10	F	TCAAGTTGAGGATCCACTTAATC	875
		R	GCCTTATCTAACGCCCTTCC	

III. Results

After extensive screening for the whole coding region of both KISS1 and KAI1, we were unable to find any mobility shift as mentioned in **Figures 1** and **2** for KAI1 and KISS1 genes respectively. Blood samples from normal females with no previous history of any type of cancer were included as positive controls. Suspected samples after sequencing were also found to be negative for frame shift deletions or insertions. Splice sites for coding sequences of both genes were screened by using the same set of primers employed for amplifying the respective exons as they also included an intronic portion. Primer binding sites on KISS1 and KAI1 genes were shown in **Figure 3** and **4** respectively. No partial or complete loss of any exonic portion for both genes had been observed in the respective breast cancer samples. No splice site variations were observed either on KISS1 or KAI1 genomic sequence with respect to the control group. The finding that there was almost no germ line mutation in these genes in sporadic breast cancer patients highlights the following features:

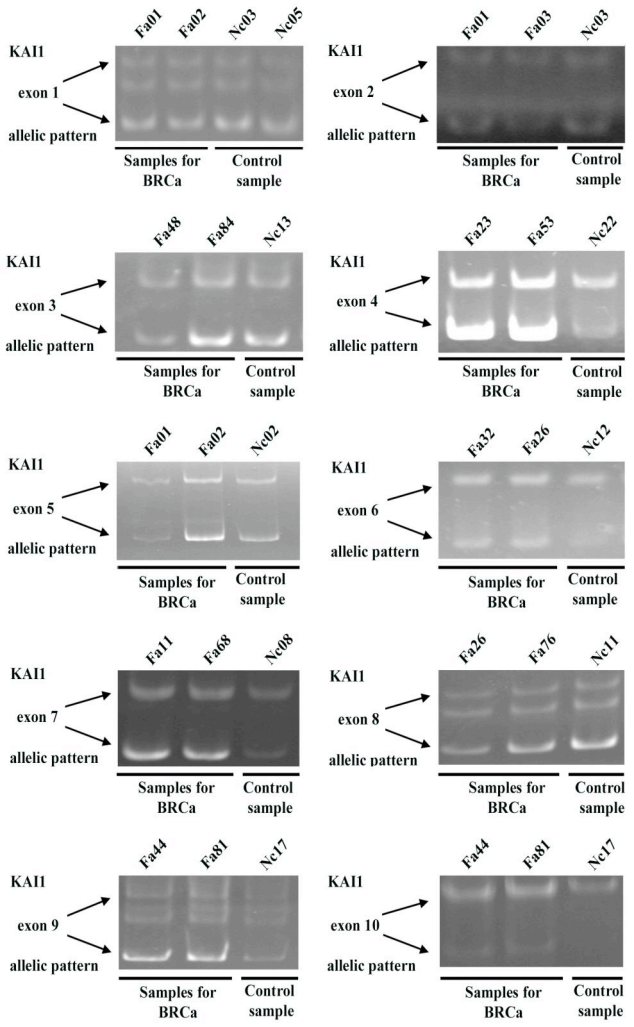

Figure 1. SSCP Gel Stained with ethidium bromide for KAI1. All exons of KAI1 show normal banding pattern among normal and mammary tumour patients on 8% non-denaturing polyacrylamide gel.

Germ line mutations of both KAI1 and KISS1 are less frequent in sporadic breast cancer patients.

Although genetic instability is a well established fact in breast cancer cases, the coding regions of KAI1 and KISS1 genes probably contribute little to the genetic instability, as no partial or complete loss of any coding region has been observed.

Coding regions of these genes are conserved among the patients with breast cancer and the controls.

Splice site variations of KAI1 and KISS1 are less common in sporadic breast cancer patients of Pakistani population.

Almost complete absence of any germ line mutations for these MSGs indirectly indicates the involvement of some other regulatory mechanisms responsible for down-regulation of the genes. Thus, metastasis suppression aberration can be attributed more towards these regulatory factors rather than the impaired coding genome. Contribution of germ line mutation of these MSGs is less likely responsible for their down-regulation in cancer.

IV. Discussion

Breast cancer is a polygenic disease, in which coordinated aberrations in expression of several genes are responsible for the disease spread. Genomic instability is a hallmark feature of breast cancer progression and is induced by a number of factors out of which germ line or

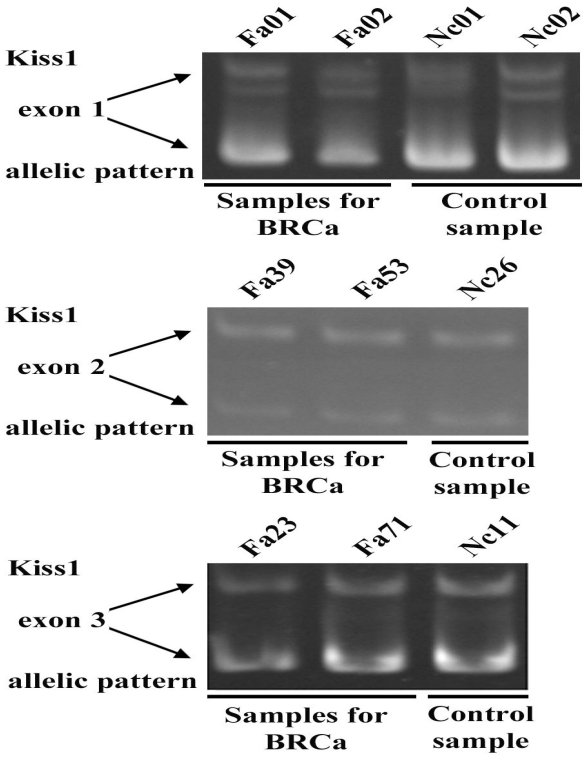

Figure 2. SSCP Gel Stained with Ethidium Bromide for KISS1. All exons of KISS1 show banding pattern among normal and mammary tumour patients on 8% non-denaturing polyacrylamide gel.

Primers binding sites for KISS1 Genomic Sequence

Exon1:

tcccgcctcggagggctcgggtggggcaggaggagaggctgcctccagtcacagag
cccggaggcagaggcctcccaaggtgccatgcTCTTCAGGAGGGTCTGAGGAGgag
ggaggggcggcagagtgggcctgtgcttggagacgtcaccccgggctttataaaag
ggatgtgatcagGGAGCTGGGGAGAACTCTTGAGACCGGGAGCCCAGCTGCCCACC
CTCTGGACATTCACCCAGCCAGGTGGTCTCGTCACCTCAGAGGCTCCGCCAGACTC
CTGCCCAGGCCAGGACTGAGGCAAGgtaggcacactgcagtgtccacccctgggaa
gggggtctgccctgacctggggatgctctggaggggagcagggaatccgttctaag
acggacagatgcatccaggcaggcaaaggcaggggagggagacactgatctttcct
gtttaccagcctggccagattatcctgggatg

Exon2:

tccccagtcactcctatatatggcatctcaccccacctttctcaaacattcctctc
aagcaaccctgagattcagaatcctggccattctctgacccaagcagtcctgcccc
ttccaccaaattgggtctttccttttttcctaactttactgtcctcacctcctcca
ggaagcctccctgatgaccctcacctagcatgaattcagctttaactacactgtg
taaagagtctcaatccCatttcactgatatatttgtttagactcatccCTCTACCA
GGAGCCTCCAAAGgcaagaacttgcccctctcttggaggactgtccctttgcact
gggctctgccctcagcacccagcccagatcctgtgcctgacctagtctttgttccc
tctctctgtctcagCCTCAAGGCACTTCTAGGACCTGCCTCTTCTCACCAAGATGA
ACTCACTGGTTTCTTGGCAGCTACTGCTTTTCCTCTGTGCCACCCACTTTGGGGAG
CCATTAGAAAAGGTGGCCTCTGTGGGGAATTCTAGACCCACAGgtatgtatcctct
ggggaaaggagtgggagggagcaagtgggttgttgcaaaatgagctttcccgtatt
ttccatctagtcgactggtgtgagtttaacattgcatttggtggaaatctaagact
ggcacacatgcaactcattgaggggtcctgttaaccctgcaagtgacggatgcct
tgcctctgtgatggcctcagtccgcccacctaggttccagggcttattaacc

Exon3:

aagagggaggcgcctagggcctaatcatcccagctaaggtgatcgtgctggttcgc
atggcactgagctccgagcttccaaaAGGTCAAGGAAGGAAAAGAAGGataggatg
ggatgacaggaggtgttgcaaagccatctttcccggaccccggcctcatctttctg
tgccctctgtcctagGCCAGCAGCTAGAATCCCTGGGCCTCCTGGCCCCCGGGGAG
CAGAGCCTGCCGTGCACCGAGAGGAAGCCAGCTGCTACTGCCAGGCTGAGCCGTCG
GGGGACCTCGCTGTCCCCGCCCCCCGAGAGCTCCGGGAGCCCCCAGCAGCCGGCCC
TGTCCGCCCCCCACAGCCGCCAGATCCCCGCACCCCAGGGCGCGGTGCTGGTGCAG
CGGGAGAAGGACCTGCCGAACTACAACTGGAACTCCTTCGGCCTGCGCTTCGGCAA
GCGGGAGGCGGCACCAGGGAACCACGGCAGAAGCGCTGGGCGGGGCTGAGGGCGCA
GGTGCGGGGCAGTGAACTTCAGACCCCAAAGGAGTCAGAGCATGCGGGGCGGGGGC
GGGGGGCGGGGACGTAGGGCTAAGGGAGGGGGCGCTGGAGCTTCCAACCCGAGGCA
ATAAAAGAAATGTTGCGTAACTCactgacgtgtggtctgcggctcatttttgcaag
cagctctgatgaggctgttccccttccatcctcaatggatggttagctgatttcaa
gaggcctctggtgtcattaaggtcagtgcttctcacactagtgtaatgaaggaac
aggcggttagttctccaatccattgcagattggtacttaaaaaaaaaaattaaaat

Figure 3. Primer binding sites on KISS1 Genomic Sequence. Exon sequences are in upper case over orange background and introns in plain text. Forward primers are marked in bold underlined upper case over a grey background. Reverse primers are in bold underlined lower case over a grey background.

Primers binding sites for KAI1 Genomic Sequence

Exon1:

cgggggcgaggctgGTTGGGGTACGGCCATAGTGggcggggcctggccggcgggag
cgcaccgcctttcccaaagggctcgggggcgggcccggcggaggggggcgtgtcttct
gggggcggGGCCTGCCGAGTCCGCGGCGTTCCCCGGCTGCAGCCGGGAGGGGGCCG
AGGAGTGACTGAGCCCCGGGCTGTGCAGTCCGACGCCGACTGAGGCACGAGCGGGT
GACGCTGGGCCTGCAGCGCGGAGCAGAAAGCAGAACCCGCAGgtgagcaaggggc
agcgggccggggtagcctgccggacaaccataggcaaagttagttttagccccgc
ttctgcggtccggctcggacaactttcctcggaactagtgacaggtcggggcagct
ggtgggtgaaaaggagctttagccaccagtcctgaaccccagttctcacactcgg
ccgtgcacagctgcgtgatcctgggtccgtcc

Exon2:

tctgagccaaggcttgtgggaaaatacgggaaaagagtaattctggcagagaaacc
cttttgcaaaggccctcaggtaggtataaggacctggggcctgatataccaggaca
acaggaaagaggctacatgagcctgtgggggggaagaggacagaggaatggcaaccc
tggcggggccccgccggccacacgGGGAGCCTGGATTTAAAGTGAgtatgtcttca
gccacattgccttggagagtcacagacaggagtgacgagatctgggtcgtgtttt
tcagAGTCCTCCCTGCTGCTGTGTGGACGACACGTGGGCACAGGCAGAAGTGGGCC
CTGTGACCAGCTGCACTGGTTTCGTGGAAGgtaagtcctgggctgagaggagtggt
gggttggaggggacacacttgggctgtgaggcgaggaaggtggagaccgagcatgg
ccctcaggtgttcagtctgagccaccaggtgggtggagggaccacttctttccctt
ctctggctcctacagcattagagtcctggagtgct

Exon3:

agccccagccccagccccagccccagtgcagtggctgatccggatcctaactgcca
agcatcaagtgcctggcacacaagactgctcgtgggacctcatttcctagctgtgt
ggctttgggctagttgtctaacctctctgggcctctgcatcctctaaggcagggAC
AGGGTTAGTACCCACCTCCTggggttgctgcaagggcagactgagctgatcccctc
actggcctgcctgccttctctcttccagGAAGCTCCAGGACTGGCGGGATGGGCTC
AGCCTGTATCAAAGTCACCAAATACTTTCTCTTCCTCTTCAACTTGATCTTCTTTg
taagtatgtcccatgccgtcctgaccacctccgaaaagatttcctttccaggggca
tcccagcctgagcctttccagagccgaccgggccggggattggggggatttggtgg
gtagggactgaggacgaactatctccaacgga

Exon4:

gaagggtggatgtggtgccctctgtggccccctgccctgcccaccctGACTTGGGT
TCCAGGGACAGctggcagggggagggctatctgctcttggctccccattaactgct
cccttctccttccagATCCTGGGCGCAGTGATCCTGGGCTTCGGGGTGTGGATCCT
GGCCGACAAGAGCAGTTTCATCTCTGTCCTGCgtaaggacccctcagcttccccag
acccaggcccactgaagaggggagggccagggcgcagatacagctgctcccataag
tgcttcggcttcaatgcctgttgcttttcccgctgacctcagggatgtggcgaggt
cctgctgcccaccactgcccatgg

Exon5:

agtgaaatgtagtctattCAATCCTGAGAAGCCTTACGAAgtaaaaaataaagccg
ggaaggggcttaggctggatgagggtgtggattgcaatttttactgggggagggaca
caagtggggatgggggtgacctggttcccttgtgggcagtgagaagccagcagggg
aatgcagctgaccccaacaccctggctccaaagaggggatccggaagaagacctgg
acggttaggccaggtgtttatacctgctgtgcccactgattttgtacttcttcttc
cccctagAAACCTCCTCCAGCTCGCTTAGGATGGGGGCCTATGTCTTCATCGGCGT
GGGGGCAGTCACTATGCTCATGGGCTTCCTGGGCTGCATCGGCGCCGTCAACGAGG
TCCGCTGCCTGCTGGGGCTGgtgagtacggatccctccgcagctgcctgcccattt
cctctcatccagccgagtgcagcctgaccgcggcgctggccgtaacatcgggtgga
gagcatctctcggggcacgcaggctgggt

Exon6:

ggatccctccgcagctgcCTGCCCATTTCCTCTCATCCagccgagtgcagcctgac
cgcggcgctggccgtaacatcgggtggagagcatctctcggggcacgcaggctggg
tgcacctggtcggggaccctcagctgactttgtgcctgctctgtgtccccagTACT
TTGCTTTCCTGCTCCTGATCCTCATTGCCCAGGTGACGGCCGGGGCCCTCTTCTAC
TTCAACATGGGCAAGGtaagccccctctccctccctcttcactgggctggaccaa
ccatgggggtgattgactgagtgtgggggatggacaaggaaccccccagttgtca
cagacagatccagtaggtgtcagggacggcctcctggacactattccttgtgatca
aatgggggagcgttagaagagaaggcgg

Exon7:

ggcaggagtggttggcactgcctgcatcgggcaccctgggagcgccaggaaagtgg
ctggagccatgagcgtgtcccagggttgctgaggggaacgGGGGGAGGCAGTTTAA
GTAGGggtgaccacaggtgggcacgggtttcaggaaatctgaccctgacctttgtc
ctccccctgcaGCTGAAGCAGGAGATGGGCGGCATCGTGACTGAGCTCATTCGAG
ACTACAACAGCAGTCGCGAGGACAGCCTGCAGGATGCCTGGGACTACGTGCAGGCT
CAGgtgaggtggggcggggctgcaggaggctctctggcctgggtgtccctgcattt
ggggctctgtgcacccacatgtctgggactggcatctgcagtgctcgtgtgtgcct
gacagtttgtaggagagtgtgcttctat

Exon8:

ttcggctgggaccaggggcctggaaGTGGCATATCTCAGTCTCTGTCCtggggagg
tcctccctctgccaggagggcagcctgcctagggtgagccgtgagcacaagcagtc
tgtccctgccttgcccagGTGAAGTGCTGCGGCTGGGTCAGCTTCTACAACTGGA
CAGACAACGCTGAGCTCATGAATCGCCCTGAGGTCACCTACCCCTGTTCCTGCGAA
GTCAAGGGGGAAGAGGACAACAGCCTTTCTGTGAGGAAGGGCTTCTGCGAGGCCCC
CGGCAACAGGACCCAGAGTGGCAACCACCCTGAGGACTGGCCTGTGTACCAGGAGg
tgtgcgggggctgcggatcgggggcggggctccgagggcgttgggggccatctgg
gctactgctcagcaattccttcctgcatttagttccttccctaattcatctgtca
tttgtaccttcatctacttctttgttaatgtatttattcaaggaacaggcagagc
ctctgtaggggcttcc

Exon9:

cgagggcgttgggggccatctgggctactgctCAGCAATTCCTTCCTGCATTTAgt
tccttccctaattcatctgtcatttgtaccttcatctacttctttgttaatgtat
ttattcaaggaacaggcagagccctctgtaggggcttccgggctgggactgggggg
ctctcggtggttctgcatggcggggtgggatggtgcagagcggggtgatgtgaccg
cattctgcccttgcagGGCTGCATGGAGAAGGTGCAGGCGTGGCTGCAGGAGAACC
TGGGCATCATCCTCGGCGTGGGCGTGGGTGTGGCCATCATCGAGgtctgagccccc
tcccccatcccttcccatcccaggtcctcctgggttgtctctgttctgctgatctc
cttggggaggtggggcc

Exon10:

tggttgtgaggcTCAAGTTGAGGATCCACTTAATCcccatgtaaacctggatggtg
aggctggggcgtctgaggccgggacacccagcctccctctgactctccgcctctcc
ccacagCTCCTGGGGATGGTCCTGTCCATCTGCTTGTGCCGGCACGTCCATTCCGA
AGACTACAGCAAGGTCCCCAAGTACTGAGGCAGCTGCTATCCCCATCTCCCTGCCT
GGCCCCCAACCTCAGGGCTCCCAGGGGTCTCCCTGGCTCCCTCCTCCAGGCCTGCC
TCCCACTTCACTGCGAAGACCCTCTTGCCCATCCTGACTGAAAGTAGGGGGCTTTC
TGGGGCCTAGCGATCTCTCCTGGCCTATCCGCTGCCAGCCTTGAGCCCTGGCTGTT
CTGTGGTTCCTCTGCTCACCGCCCATCAGGGTTCTCTTAGCAACTCAGAGAAAAAT
GCTCCCCACAGCGTCCCTGGCGCAGGTGGGCTTGACTTCTACCTGCCCTCAAGGGT
GTGTATATTGTATAGGGGCAACTGTATGAAAAATTGGGGAGGAGGGGGCCGGGCGC
GGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGCCGAGGCGGGTGGATCACGA
GGTCAGGAGATCGAGACCATCCTGGCTAACATGGTGAAACCCCGTCTCTACTAAAA
ATACAAAAAAAAATTTAGCCGGGCGCGGTGGCGGGCACCTGTAGTCCCAGCTACTTG
GGAGGCTGAGGCAGGAGAATGGTGTGAACCCGGGAGCGGAGGTTGCAGTGAGCTGA
GATCGTGCTACTGCACTCCAGCCTGGGGGACAGAAAGAGACTCCGTCTCaaaaaaa
aaaaaaaaaaaaaaaaaaaattggggagggaagggcgttagataaggcactctgggc
tgtc

Figure 4. Primer binding sites on KAI1 genomic sequence. Exon sequences are in upper case over orange background and introns in plain text. Forward primers are marked in bold underlined upper case over a grey background. Reverse primers are in bold underlined lower case over a grey background.

somatic mutations also impart their substantial contribution. Genome instability can be the result of either progressive genome damage within a tumour cell or accumulation of all sorts of mutation resulting in the formation of cells with a metastatic potential (Rubin et al, 2001). 4-10% of breast cancer cases are due to germ line mutations in various genes. The current study assessed the potential involvement of possible mutations of KAI1 and KISS1 in breast cancer. We did not observe any mutation in these genes but this does not overlook involvement of these MSGs in metastasis suppression.

There are several techniques used for mutation detection including Single Strand Conformational Polymorphism (SSCP), Allele Specific Oligonucleotides, protein truncation test, Temperature Gradient Gel Electrophoresis (TGGE), Denaturing Gradient Gel Electrophoresis, Hetro duplex analysis and DHPLC (Gasser, 1997). These techniques generally rely on altered chemical or physical features of DNA due to variations in one or more nucleotides in the sequence. We used single strand confirmation polymorphism technique because of its simplicity, sensitivity, high throughput and low cost efficiency. The main principle of SSCP technique is largely based on the knowledge that the mobility of single strand DNA molecule depends on its size and structure in a non denaturing gel. During electrophoresis single strand DNA usually forms secondary and tertiary structures. Several factors may influence the mobility pattern of DNA strands including temperature, buffer pH during electrophoresis, percentage of polyacrylamide gel, specificity of the amplified product and sequences. These factors may influence results interpretation and have been taken into consideration which ultimately leads to an increase in SSCP sensitivity as mentioned in previously published reports (Kukita et al, 1997; Noullau and Wagener, 1997). After following a stringent parameter for technique optimization and also sequencing the suspected samples, we concluded that the presence of any germ line mutations of these MSGs in samples from patients with breast is unlikely. The study thus strongly suggests that frequent down-regulation of these proteins in cancer progression is unlikely due to any germ line mutations.

Mutations of functional domains and antagonist variants have all been reported in a number of cancer related genes. For example, CHEK2 germ line mutations have adverse affects on prognosis of breast cancer patients (Meyer et al, 2007). Similarly, germ line mutations of several other genes responsible for metastasis like p53, SIPA1 (signal induced proliferation Associated gene1) are also shown to increase disease progression and metastasis in mammary tumours patients (Hsieh et al, 2006; Guénard et al, 2007). The mutational spectrum of CDH1 gene encoding E-Cadherin (another well known MSG) has also substantially increased the risk (70-80%) of cancer progression (Hsieh et al, 2006).

The present study also screened whether there are any mutated alleles present on these MSGs. In this study, the objective was, *first* to assess whether there are any germ line mutations prevailing on these genes and *second* to see what proportion they are present in breast cancer patients. According to the authors' knowledge, no such study has previously been reported in relation to mammary tumours in any population. Expression dysregulation for both genes was previously observed in several cancers (Stafford et al, 2008). This dysregulation in expression may be attributed to other modulating factors, such as binding of p53 at the promoter binding site along with AP1 and AP2, leading to increased KAI1 protein at the intracellular level (Mashimo et al, 1998; Briese et al, 2008). Although correlation of p53 with KAI1 molecules has not been studied in relation to breast cancer, yet the altered modulation is justified by combination of various proteins bindings at the promoter and upstream binding region of KAI1 molecule (Tonoli et al, 2005). In another study conducted on 52 ovarian carcinomas, only one missense somatic mutation of KAI1 was observed and the authors similarly concluded that the mutation frequency is less likely responsible for protein down-regulation in metastasis progression in ovarian cancer. This lack of germ line mutations also emphasizes the conserved sequence of KAI1, which is also consistent with previous findings in which it has been observed that KAI1 is 76% identical to other members of the tetraspanin family (Nagira et al, 2005).

Similarly, signaling of KISS1 is regulated by CRSP1 gene located at chromosome 6 which is frequently lost during cancer progression. Up-regulation of CRSP1 protein results in inhibition of metastasis by elevating the level of KISS1 (Goldberg et al, 2003). KISS1 has two dibasic cleavages and an amidation cleavage site. Posttranslational processing results in formation of KP54 and a matured and processed form of KISS1 gene termed as metastin, leading to its role in regulating metastasis (Rouille et., 1995; Karges et al, 2005). The carboxy terminus of KISS1 protein was found to be conserved among human and mouse, resulting in an increase in specific binding for its cognate G protein coupled receptor (Terao et al, 2004). The protease responsible for posttranslational modification KISS1 has yet to be identified. Hence, the mechanism of transcriptional regulation is an area that requires further investigation.

Breast cancer cells may be predisposed to high tendency of metastasis, via control mechanisms such as the loss of balance between metastasis stimulating and inhibiting genes. Although germ line mutations of different genes lead to impaired expression of downstream regulatory genes (Marreeiros et al, 2005), levels of proteins and transcripts from these genes are also important in the control of metastatic behaviour of cancer cells. No partial or complete loss of coding region of KAI1 and KISS1 as shown in the present study provides a strong evidential support that down-regulation of these gene products are more likely to be the results of transacting factor rather than mutations. Hence, progression of metastasis with down regulation of both KAI1 and KISS1 proteins is mainly regulated by various cellular proteins rather than germ line mutations in their coding sequences.

V. Conclusion

Mutational spectrum of the MSGs, KAI1 and KISS1, is substantially low and their down regulation in breast cancer patients may be attributed to by other regulatory

mechanisms. Results are consistent with a previously conducted research on KAI1 molecules in squamous cell carcinoma of oesophagus (Miyazaki et al, 2000) and KISS1in breast cancer (Martin et al, 2005). Further studies regarding the expressional variation detection will be helpful in defining a better prognostic marker for early prognosis of mammary tumor patients. Involvement of regulatory proteins as major contributor in the suppression of these vital genes leading to progression of metastasis and also a poor prognosis on patient survival is an area requisite of further investigation. These regulatory pathways also provide the opportunity for potential therapeutic intervention.

Acknowledgement

We are extremely grateful to all patients and Oncologists for their immense help and support in this research. The authors also like to acknowledge the Higher Education Commission of Pakistan for providing funds to carry out this collaborative research work and Dr Richard J Ablin for critical reading of the manuscript. In the last but not least we also extend our gratitude to the reviewers for their valuable time and suggestions for this manuscript.

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