RESEARCH COMMUNICATION

Caveolin-1 Promotes Mammary Tumorigenesis: Mutational Profile of the Kashmiri Population

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Abstract

Backgroung: The role of caveolae and the caveolin proteins in cancer has been the subject of extensive research. It has been suggested that caveolin-1 (*Cav-1*) may contribute to certain steps of carcinogenesis. In the present study we focused on its potential clinical relevance in mammary malignancies. Methods: We investigated 130 breast cancer samples along with adjacent normal tissues using allele specific PCR for the mutation status and then conventional PCR-SSCP and sequencing of mutated samples along with the normal adjacent tissues. Results: Caveolin-1 was identified in a screen for genes involved in breast cancer progression and we demonstrated 29.2% mutational status in our Kashmiri ethnic population. We were able to detect 38 mutations out of which 22 were missense, 4 were nonsense, and 12 were frame shifts amongst these 38 we were also able to detect ten novel Cav-1 mutations (missense and frameshift mutations). Conclusion: We conclude that our study suggests that the gene encoding *Cav-1* plays an important role in the promotion of mammary tumorigenesis and are associated with the development and progression of breast cancer.

Keywords: Breast cancer - Cav-1 - mutation - As-PCR - PCR-SSCP

Asian Pacific J Cancer Prev, **11**, 689-696

Introduction

Breast cancer, is the third most common malignancy in the world (Sipetić et al., 2004) with more than 1 million women diagnosed with breast cancer each year (Parkin et al., 2005). Breast cancer has been associated with a variety of risk factors (Kaaks et al., 2005) genetic and epigenetic changes (Xie et al., 2006). But its molecular pathogenesis remains unresolved. Environmental carcinogens have been shown to damage DNA at active fragile sites by disrupting surveillance, which has been shown to be tumorigenic (Arlt et al., 2003; Gasser et al., 2006). Development of human breast cancers is a multistep process, arising from genetic alterations that drive the transformation of normal mammary epithelial cells into highly malignant derivatives(Hanahan and Weinberg, 2000).

Historically, caveolin was first discovered approximately 10 years ago as a major v-Src substrate in Rous sarcoma virus-transformed cells, as the principal structural component of caveolae, identified as a 21- to 24-kDa integral membrane protein and termed caveolin (Glenney et al., 1989; Rothberg., 1992) now referred to as caveolin-1(Scherer et al., 1996). Caveolin-1 is the principal structural component of caveolae micro domains, which represent a sub-compartment of the plasma membrane, Caveolin 1 (*Cav-1*) gene maps to 7q31.1 and encodes a 21- to 24-kDa integral membrane protein. Caveolin interacts directly with heterotrimeric guanine nucleotide binding proteins (G proteins) (Li et al., 1995) and can functionally regulate their activity. Modification and/or inactivation of caveolin-1 expression appears to be a common feature of the transformed phenotype. Three members of the caveolin family (Cav-1, -2, and -3) are essential for the formation of caveolae, Cav-1 is the principal structural protein of caveolae membranes that are found in most cells types, including mammary epithelial cells. Human *Cav-1* gene is a suspected tumor suppressor locus (7q31.1/D7S522) that is deleted in a variety of human cancers, as well as mammary tumors. Mutations in the gene encoding *Cav-1* are associated with the development and progression of breast cancers. Conflicting results on the role of *Cav-1* in human cancers have been reported (Lee et al., 1998; Yang et al., 1998; Engelman et al., 1999; Hurlstone et al., 1999; Hayashi et al., 2001; Lee et al., 2002; Hnasko et al., 2003; Zou et al., 2003; Jones et al., 2004; Chen et al., 2004; Sagara et al., 2004; Williams and Lisanti, 2005; Park et al., 2005; Charafe-Jauffret et al., 2005; Pinilla et al., 2006; Van den Eynden et al., 2006).

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Based on the high frequency of deletions at 7q31 (a fragile site known as FRA7G) in human cancers (Nishizuka et al., 1997; Huang et al., 1999; Han et al., 2003). The presence of *Cav-1* gene promoter hypermethylation (Cheb et al., 2004; Engelman et al., 1999), inactivating gene mutations (Hayashi et al., 2001; Chen et al., 2004), and the apparent reduction of *Cav-1* expression in breast carcinomas (Chen et al., 2004; Park et al., 2005), it has been suggested that CAV1 is a potential tumor suppressor gene (Razani and Lisanti, 2001; Hnasko et al., 2003; Williams et al., 2005). Several independent lines of evidence support the notion that caveolin-1 functions as a suppressor of cell transformation. The action of Cav-1 as a tumor suppressor gene is based on its capacity to inhibit the signaling activity of several protooncogene products that impart growth and survival advantages to the cell (Macleod and Jacks, 1999). Reduction in Cav-1 and caveolae appears to be a common event in transformed cell lines, suggesting that Cav-1 might be "inactivated" during tumorigenesis (Engelman et al., 1998; Razani et al., 2000). Loss of Cav-1 disrupts cellular adhesion and consequently can either induce apoptosis when proper cell cycle checkpoints are in place or promote metastasis when synergistically combined with uncontrolled cell growth.

Recently, researchers have suggested that sequestration of several growth-promoting proteins in invaginations of the plasma membrane, named caveolae, serves to potently inhibit their activity (Cohen et al., 2004). Cav-1 functions as a general negative regulator to inhibit the basal activity of many pro-proliferative and oncogenic proteins. Mechanistically, Cav-1 tonically inhibits the activation of multiple signaling molecules, including EGF-R, ERK-1/2, MEK-1/2, and the TGF-β type I receptor, by direct binding via the caveolin-scaffolding domain (Couet et al., 1997; Engelman et al., 1998; Galbiati et al., 1998; Razani et al., 2001). Cav-1 mRNA and protein levels are down-regulated or absent in primary human cancers in oncogenically transformed NIH 3T3 cells, in transgenic mouse models of breast cancer, as well as in several human and mouse transformed mammary epithelial cell lines (Koleske et al., 1995; Lee et al., 1998; Engelman et al., 1998; Racine C et al., 1999; Bender et al., 2000; Razani et al., 2000; Bagnoli et al., 2000; Park et al., 2001). In tumors, caveolin-1, the structural protein of caveolae, constitutes a key switch through its function as a tumor suppressor and a promoter of metastases. Despite important advances in chemotherapy, radiotherapy, and surgery, approximately 30% of patients with breast cancer will relapse and die of the disease. Therefore, complementary therapeutic strategies should be considered for improving the outcome of breast cancer patients.

Materials and Methods

Patients and Tumor Tissue Procurement

All patients included in the study were both male and female, with the histopathological diagnosis of the breast cancer. The patient participation was obtained through informed consent and after approval from the Ethics Committee of Sher-I-Kashmir Institute of Medical Sciences.

A cohort of 130 breast cancer tissue samples were collected consisting of tumor tissues and adjacent normal tissue. Only the tissue samples confirmed by histopathological studies to be cancerous were included in the study.

DNA isolation

Genomic DNA was extracted from tissue samples and peripheral blood samples of breast cancer patients using DNA Extraction Kit (Qiagen, USA). The quality of the resulting genomic DNA was stringently assessed by low percentage agarose gel electrophoresis.

AS-PCR Analysis

A strategy to quickly detect the CAV-1 mutations was designed using allele-specific PCR. The allele specific primers were designed to distinguish the P132L mutant from its wild type counterpart and then the allele specific primers were designed to distinguish the mutant from its wild-type counterpart. Amplification was performed using the allele-specific forward primer and a common reverse primer. PCR was performed in a 25μl total volume reaction mixture containing 50ng of genomic DNA, 100ng of each primer, 100μM of each dNTP, 1.5mM $MgCl_2$, 10X of Taq buffer and 1U of Taq DNA polymerase. The PCR conditions were initial denaturation at 95°C for 5 min followed by 35 to 45 cycles of (denaturation at 95°C for 15 seconds, primer annealing at 60°C for 1 minute,

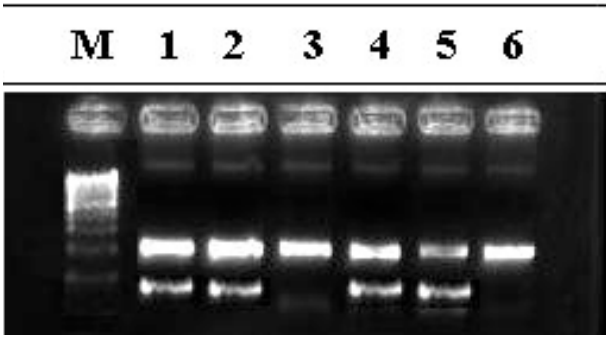

Figure 1. Gel Picture Showing the Amplified Allele Specific PCR Product of Cav-1 (WT-210bp and M-93bpproduct). Lane M, Molecular markers 100bp; Lanes 1,2,4,5, wild type &mutant amplicons having 93bp &210bp bands; Lanes 3,6, showing only 210bp wild type amplicons carrying no mutation

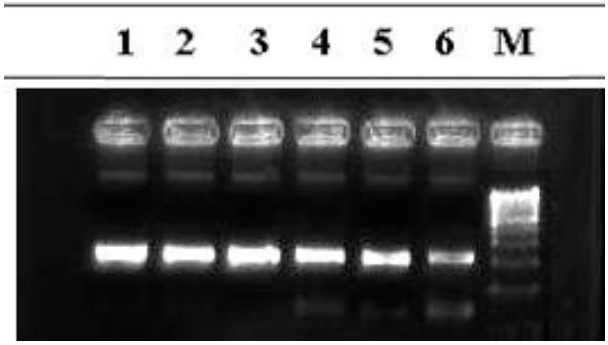

Figure 2. Gel Picture Showing the Amplified Product of Cav-1 (210bp) Product. Lane M, Molecular markers 100bp; Lanes 1-6, Amplicons from breast cancer tissue samples

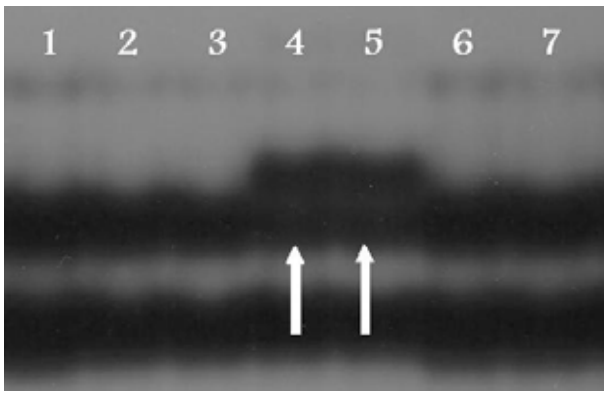

Figure 3. SSCP (Non-radioactive) Analysis of Cav-1 Showing Mobility Shift when Compared to Control

and extension at 60°C for 1 minute) and final extension at 72°Cfor 7 minutes. The PCR products were run on 2% agarose gel and analyzed under UV illuminator, that showed the wild type allele showing a 210 bp band and mutant allele showing 93 bp band as shown in Figure 1.

Conventional PCR-SSCP

The samples that showed mutation by AS-PCR (Figure 1) were then amplified by conventional PCR (Figure 2). The entire region (amino acids 87 to 156) of CAV-1 gene was amplified, and the high-quality amplified products were assessed with SSCP analysis (Figure 3). For conventional PCR, the forward primer and reverse primer were used to amplify a 210-bp DNA fragment corresponding to a 70-amino acid region, which includes the entire transmembrane domain (amino acids 102 to 134) of the Cav-1 gene. Each PCR reaction was performed in a 50ul final volume (containing 20 to 100ng of genomic DNA, 10mmol/L Tris-HCl, 10pmol/L of each primer, 200 umol/L of each dNTP, and 0.2 U of TaqDNA polymerase (Banglore Genei)) in a thermal cycler (Biorad icycler) using the following program: denaturation at 95°C for 5 minutes,followed by 35-40 amplification cycles (denaturation at 95°C for 60 seconds, annealing at 56°C for 60 seconds, and extension at 72°C for 60 seconds), and final extension at 72°C for 10 minutes. Both positive and negative controls were performed in parallel for each PCR reaction. Negative control reactions were performed without DNA template to exclude nonspecific amplification. The PCR products were run on 1.8% agarose gel sand analyzed under a UV illuminator.

The high-quality amplified products were assessed with SSCP analysis .For this assessment, 8µl of each PCR product was added 2µl of loading solution. Next, the tube was immediately heat-denatured at 95°C for 5 min, then cooled on ice for 5 min, and at 4°C for 5 min, respectively, and kept there or on ice until a sample was loaded into the gel. Four microliters of each processed PCR product were electrophoresed on 1X MDE™ gels. The products were run at 600V for 10 to 12h at room temperature in 1X TBE buffer solution (89mM tris-base, 89mM boric acid, 2mM EDTA, pH 8.0).The gels were stained using a non-radioactive silver staining method and results were visualized and photographed with an imaging analyzer. Samples that showed one or two bands separated from the wild-type bands were identified as SSCP positive. All the samples that contained mutations were subjected to the SSCP analysis procedure at least twice to rule out contamination.

Sequencing

Representative variant bands that tested SSCP positive were re-amplified for sequencing. All Cav-1 mutations were confirmed by direct sequencing using the reverse PCR primer. The PCR products were gel-extracted using a Gel Extraction Kit (Qiagen) and then used for direct DNA sequencing. DNA sequencing was carried out at MACROGEN INC, Korea. To minimize the sequencing artifacts with the PCR, products from at least two different PCRs were sequenced using both forward and reverse primers.

Primer Specificity

The primer sequences were checked very carefully, and it was ensured that they did not co-amplify other caveolins, such as Cav-2 or Cav-3. The DNA and protein sequences of the caveolins are actually quite divergent. If Cav-2 or Cav-3 sequences had co-amplified, our processing would have detected them, because they are easily distinguished based on their divergent DNA sequences.

Statistical analysis

Pearson's two proportions test was used to compare the determined Cav-1 gene mutations with various clinical parameters. Differences with $P > 0.05$ were accepted as statistically not significant. Calculations were done using SPSS for Windows, version 11, 5 (SPSS, Chicago, IL, USA).

Results

A rapid and sensitive strategy was established to detect Cav-1 mutations in human breast cancer samples by allele-specific PCR (AS-PCR) and thereafter only mutant samples were amplified by conventional PCR and sequenced. Our study included a total of 130 patients, out of which 38 harbored the mutation, showing an overall percentage of 27.6% in the ethnic Kashmiri population.

In order to increase the sensitivity of mutation detection, we used 1) Allele specific PCR 2) conventional PCR-SSCP and sequencing to validate the study and the Assay both. In the course of direct sequencing, we were able to detect 38 mutations out of which 22 were missense, 4 were nonsense, and 12 were frame shifts (Figure 4).

During our course of study, we were also able to detect ten novel Cav-1 mutations (missense and frameshift) in the same patient cohort. Out of 22 missense mutations six were present in codon 136, four were present in codon 132, four were present in codon 133, three were present in codon 107, three were present in codon 125, two were present in codon 141, two were present in codon 148, one was present in codons 116 and 128.

All the nonsense mutations were present in codon 128 leading to Trp>Stop at codon 128, a total of 12 frameshift mutations were present, out of which four mutations were present in codon 113, four in codon 133, two in codon 125 and two in codon 154. Importantly, Cav-1 sequence

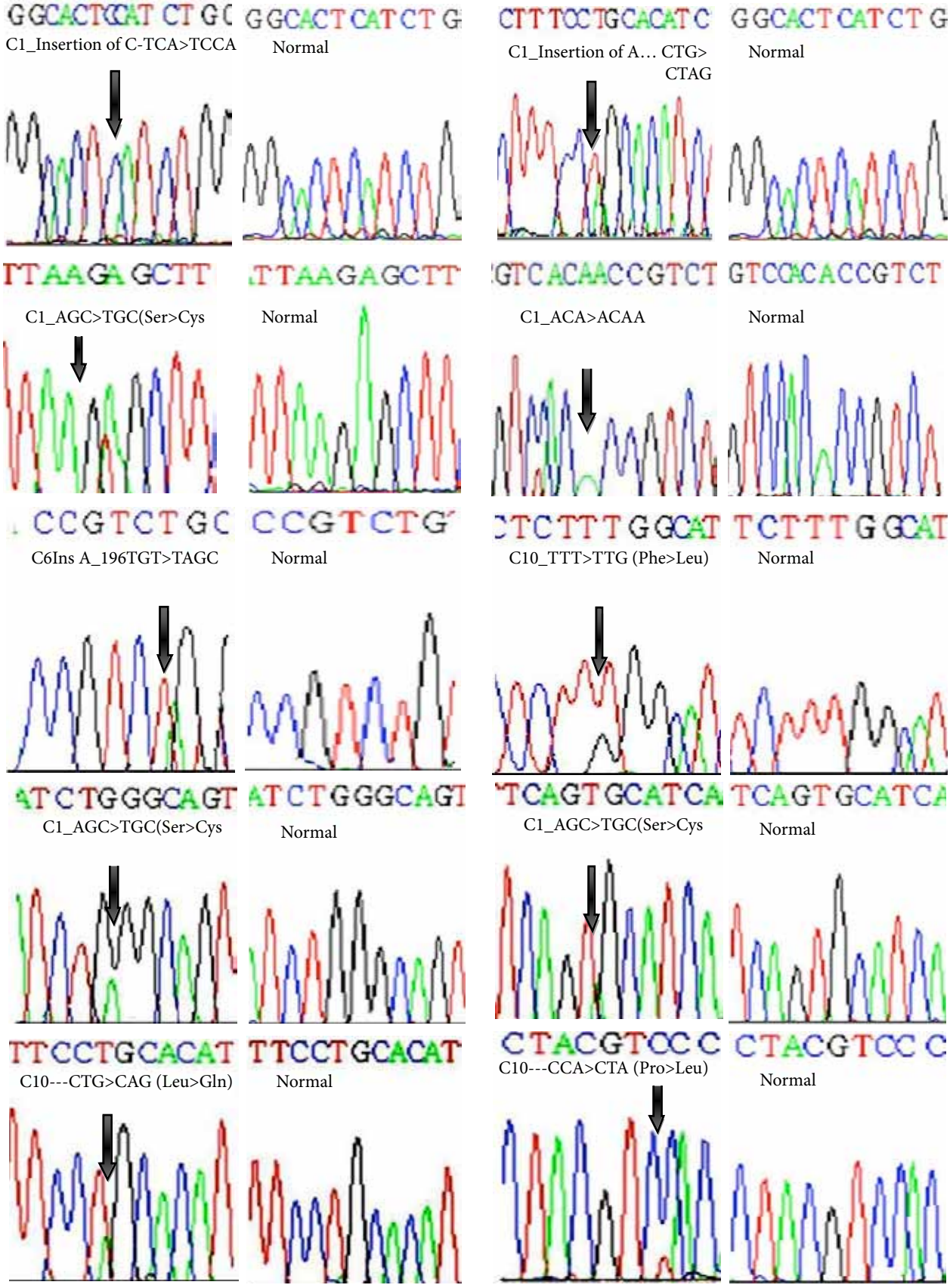

Figure 4. Partial Electropherograms Representing Mutants (shown by arrows) and Corrresponding Normals

changes were exclusively associated with isolated breast cancer cells; they were not observed in mammary epithelial cells isolated from adjacent normal breast tissue.

Discussion

The human Cav-1 gene maps to chromosome locus 7q31.1 near the D7S522 genetic marker. D7S522 encompasses a known fragile site (FRA7G) within 7q31.1 suggesting that Cav-1 may indeed represent the tumor

Table 1. Details of Cav-1 Mutations in Breast Cancer Patients from Kashmir Valley

Affected Codon	Base Change[a]	Amino acid Change	Mutation Effect[b]	Previously described[c]
113	TCA>TCCA	Ins C	FS	-
125	CTG> CTAG	Ins A	FS	-
136	AGC>TGC	Ser>Cys	MS	+
154	ACC>AACC	Ins A	FS	-
133	TGC>CGC	Cys>Arg	MS	-
125	CTG>CAG	Leu>Gln	MS	-
113	TCA>TCCA	Ins C	FS	-
136	AGC>TGC	Ser>Cys	MS	+
132	CCA>CTA	Pro>Leu	MS	-
154	ACC>AACC	Ins A	FS	-
107	TTT>TTG	Phe>Leu	MS	-
116	GGC>AGC	Gly>Ser	MS	-
128	TGG>TAG	Trp><Stop	NS	+
136	AGC>TGC	Ser>Cys	MS	+
133	TGC>TAGC	Ins A	FS	-
132	CCA>CTA	Pro>Leu	MS	-
113	TCA>TCCA	Ins C	FS	-
125	CTG>CTAG	Ins A	FS	-
148	TAT > CAT	Tyr>His	MS	+
136	AGC>TGC	Ser>Cys	MS	+
133	TGC>TAGC	Ins A	FS	-
136	AGC>TGC	Ser>Cys	MS	+
133	TGC>TAGC	Ins A	FS	-
128	TGG>TAG	Trp><Stop	NS	+
141	ATT>TTT	Ile>Phe	MS	+
132	CCA>CTA	Pro>Leu	MS	-
107	TTT>TTG	Phe>Leu	MS	-
133	TGC>TAGC	Ins A	FS	-
125	CTG>CAG	Leu>Gln	MS	-
132	CCA>CTA	Pro>Leu	MS	-
107	TTT>TTG	Phe>Leu	MS	-
136	AGC>TGC	Ser>Cys	MS	+
128	TGG>TAG	Trp><Stop	NS	+
141	ATT>TTT	Ile>Phe	MS	+
148	TAT>CAT	Tyr>His	MS	+
128	TGG>TAG	Trp><Stop	NS	+
113	TCA>TCCA	Ins C	FS	-
125	CTG>CAG	Leu>Gln	MS	-

Table 2. Mutation Pattern and Mutation Effect of Cav-1 Mutations in the Breast Cancer Patients from Kashmir Valley

Variable	Total N	(%)	Mutants M	(%)	P value
Sex					
Males	7	(28.6%)	2		NS
Females	123	(29.2%)	36		
Age					
> 50	13	(38.4%)	5		NS
≤ 50	117	(28.20%)	33		
Dwelling					
Rural:	45	(62.2%)	28		< 0.01
Urban:	85	(11.7%)	10		
Smoking Status Ever:					
Never:	93	(19.3%)	18		NS
	37	(54.0%)	20		
Menopausal Status					
Pre:	36	(61.1%)	22		<0.01
Post:	94	(17.0%)	16		
Nodal Status					
Involved	34	(76.4%)	26		<0.01
Not Involved	96	(12.5%)	12		
Breast Involved					
R	35	(0.8%)	28		<0.01
L	95	(10.5%)	10		
Tumor Stage					
II(a+b)	14+58	(36.1%)	26		NS
III(a+b)+ IV	46+12	(20.7%)	12		
Histopathological Tumor Grade					
WD	47	(42.5%)	20		
MD	58	(10.3%)	6		<0.05
PD	25	(48.0%)	12		

suppressor role in this fragile genomic region (Engelman et al., 1998). A large number of epithelial cancers (e.g., breast, prostate, ovarian, and renal) have deletions distributed around this D7S522 marker (Shridhar et al., 1997; Jenkins RB et al., 1998; Lee et al., 2002) suggesting that a tumor suppressor gene resides within this region.

Since the initial report of Japanese study in 2001(Yang et al., 1998), in which 16% of mutations in breast cancer tumor samples within the human *Cav-1* gene were reported, which reflects that Cav-1's distinctive mutational profile might be population specific. Hence we formulated our study as a population based study. Kashmir constitutes a unique ethnic population with majority of consanguineous marriages thus resulting in the concentration of genetic pool, besides Kashmir has a socio-economic trend of late marriages occurring at the age of 30 or above. This might explain the results that are somewhat different from those in other studies from different demographics in the world. It is also noteworthy that several other groups have failed to confirm the existence of the Cav-1 mutations in human breast cancers (Hurlstone et al., 1999; Zenklusen et al., 2001; Chen et al., 2004).

We found 22/38 (57.8%) missense mutations, 12/38 (31.5%) frameshifts and 4/38 (10.5%) nonsense mutations, amongst theses we detected nine novel mutations (Table 1). Frameshift and nonsense mutations in the Cav-1 gene have rarely been found. Furthermore, the Cav-1 (I141T) mutation that we identify here is similar to the I141F mutation previously identified in human squamous cell carcinoma samples (Han et al., 2004). We also detected multiple Cav-1 mutations within the same tumor sample (Table 1) which suggests that the different Cav-1 mutations are occurring singly, but within different Cav-1 alleles. Our study is the first to report the identified nine novel mutations that includes five missense and four frameshift mutations. Interestingly, all of the mutations we detected were "heterozygous," consistent with the hypothesis that the Cav-1 mutation behaves in a dominant-negative fashion. This is the first report to evaluate Cav-1 mutations

in Kashmiri patients with breast cancer.

We found a statistically significant correlation between mutational status of Cav-1 with histopathological grading and tumor stages as shown in Table 2 suggesting that Cav-1 may be up regulated during metastasis, and a distinctive mechanism is responsible to inactivate the tumor suppressor function of caveolin-1 resulting in increased cell-invasiveness, and increased chemotaxis (Hayashi et al., 2001).

Previous studies (Han et al., 2004) have suggested that mammary tumorigenesis is commonly initiated during pre-menopausal stages. Confirming these findings, our studies found statistically significant correlation between the Cav-1 mutational status and pre-menopausal status.

We also found a significant correlation between Cav-1 mutation Status with lymph node involvement and the right breast involved.

Further we also found significant correlation between Cav-1 mutational status with rural dwellers than urban. The difference is possibly due to the fact that women in rural areas face substantial barriers in receiving preventive health care services.

The mode of action of Cav-1 as a tumor suppressor gene is based on its competence to restrain the signaling activity of several proto-oncogene products that impart growth and survival advantages to the cell (Macleod and Jacks, 1999). The first supposition of the role of Cav-1 in cancer was that Cav-1 is tyrosine-phosphorylated in v-Src-transformed fibroblasts (Glenney, 1989), followed by further observation that oncogene-mediated transformation of murine NIH 3T3 fibroblasts results in the transcriptional downregulation of Cav-1 and a loss of identifiable caveolae organelles (Koleske et al., 1995). Indeed, numerous oncogenes abrogate the transcription of Cav-1(Koleske et al., 1995; Engelman et al., 1997; Engelman et al., 1998), but the exact mechanism of this transcriptional repression remains to be determined. The loss of Cav-1 disrupts cellular adhesion and consequently can either induce apoptosis when proper cell cycle checkpoints are in place or promote metastasis correlated with that of cyclin D1, a positive regulator of cell growth, in transformed cells (Hulit et al., 2000).

There is a growing body of evidence that caveolin-1 plays a significant role in mammary tumorigenesis. Mutations in the gene encoding *Cav-1* are associated with the development and progression of breast cancers. We found certain mutations that were already reported (Lee et al., 2002) and in addition to them we found nine novel mutations that are not yet reported. Although some researchers (Hurlstone et al., 1999) have reported previously that there was no mutation in the *Cav-1* gene in human cancers, we sought the mutation more intensively, focusing specifically on human breast cancers. We also found a specific Cav-1 point mutation that has been reported by other researchers which produces a protein (i.e., P132L) that acts in a dominant negative manner and results in retention in the Golgi apparatus thereby modulating the wild-type Cav-1 and promoting its intracellular accumulation.

From the above studies, it is clear that Cav-1 assumes a dynamic role in regulating mammary epithelial cell proliferation.We,clearly demonstrate that the Cav-1 (P132L) mutation reported in this and other previous studies behaves in a dominant-negative manner, and together with the other novel mutations causing the mislocalization and intracellular retention of wild type Cav-1. Taken together, our data associates loss of functional Cav-1 protein expression (by deletion or mutation) in the pathogenesis of mammary tumorigenesis. Our findings in this study may provide experimental basis for additional analysis of caveolin-1 mutation in human cancers. Our data have provided evidence for the existence of naturally occurring mutations of caveolin-1 that appears to have a role in human breast cancer. These findings are consistent with similar previously reported results except for the additional novel mutations identified in our study. The results of our study provide evidence that the mutation of caveolin-1 have a dominant negative effect on cell transformation and invasiveness. In addition, these findings indicate that caveolin-1 is likely to function as a tumor suppressor gene. We speculate that other effective caveolin-1 mutations, which we have not been identified yet, might exist, because there are other consensus sites for caveolin. *Cav-1* gene promoter is heavily methylated in human prostate cancer samples (Pflug et al., 1999; Cui et al., 2001). Thus, it is likely that dominant-negative mutations in the *Cav-1* gene will soon be identified in other forms of human cancer. In addition, investigation of signaling pathways affected by caveolins should provide additional insights into the molecular pathogenic action of caveolae disorders. We conclude that the overall incidence of Cav-1 mutations in our limited patient sample is 29.2%. This needs to be corroborated by future studies with a larger patient population.

In conclusion, our investigation suggests that Cav-1 mutations are not much common in breast cancer but these mutations plays an important role in the promotion of mammary tumorigenesis. The mutations in the gene encoding *Cav-1* are associated with the development and progression of breast cancers. However, the exact functional role of caveolin-1 still remains controversial.

Acknowledgements

The present research was not funded by any funding agency. The collection of cancer samples used in this study was supported by the Departments of Immunology and Molecular Medicines and Department of General Surgery, Sher-I-Kashmir Institute of Medical Sciences.

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