RESEARCH COMMUNICATION

5'-CpG Island Promoter Hypermethylation of the *CAV-1* Gene in Breast Cancer Patients of Kashmir

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Abstract

Background: *Caveolin-1* (*CAV-1*), encoding the structural component of cellular caveolae, is a suggested tumor suppressor gene involved in cell signalling. Aberrant promoter methylation of *CAV-1* is associated with inactivation of expression. We previously observed *CAV-1* mutations in breast cancers and therefore devised this study to examine the hypermethylation status of the promoter region of *CAV-1* with reference to breast cancer progression and development. Methods: Hypermethylation status of *CAV-1* was analyzed by methylation specific PCR. Loss of expression of the *CAV-1* gene was further evaluated by semi-quantitative rt-PCR. Results: 28/130 (21.5%) breast cancer cases showed promoter hypermethylation with reduced *CAV-1* expression levels when compared with adjacent normal breast tissue. *CAV-1* gene hypermethylation was significantly related to menopausal status, histopathological grade and age. Conclusion: The rationale of our study is that *CAV-1* gene is transcriptionally repressed in breast cancer cells due to hypermethylation. Our results reveal that promoter hypermethylation and loss of expression of the *CAV-1* gene is an important alternative mechanism for inactivation of *CAV-1* leading to complete gene silencing.

Keywords: Breast cancer - *CAV-1* - promoter hypermethylation - rt PCR - Kasmir

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Introduction

Breast cancer is the most frequent cancer affecting women all over the world and is third most common malignancy in the world (Hanahan et al., 2000), with more than 1 million women diagnosed with breast cancer each year (Sipetić et al., 2004). Development of human breast cancer arises from genetic and epigenetic alterations that drive the transformation of normal mammary epithelial cells into highly malignant derivatives (Parkin et al., 2005).

The *CAV-1* gene maps to 7q31.1 and encodes a 21- to 24-kDa integral membrane protein (Hnasko et al., 2003; Williams et al., 2005), it is a principal component of caveolae membranes. *CAV-1* gene is a suspected tumor suppressor gene that is deleted in a variety of human cancers, as well as mammary tumors. There is high expression of the protein in terminally differentiated cells such as adipocytes, pneumocytes, chondrocytes and smooth muscle cells (Goetz et al., 2008). In addition, *CAV-1* gene is mutated (P132L) in up to 16% of human breast cancers. Sequencing data have shown that the first and second exons of *CAV-1*are embedded with CpG islands and the CpGs in the promoter region of the gene are methylated in two human breast cancer cell lines which also fail to express the *CAV-1* protein (Engelman et al., 1999). These data cumulatively suggest that regulation of *CAV-1* expression may be controlled, at least in part, by CpG methylation at these sites. *CAV-1* promoter in cancerous and non-cancerous cells, respectively have 25% methylated CpG-island. The frequency of aberrant promoter methylation of breast cancer tissues is significantly higher than non-cancerous tissues ($p<0.05$). There are four types of methylation pattern of *CAV-1* gene in the breast cancer tissues. Hypermethylation of CpG islands in the promoter regions of tumor suppressor genes is one mechanism of tumorigenesis. The GC-rich region of the *CAV-1* promoter is hypermethylated in breast cancer cell lines resulting in transcriptional silencing. Based on the high frequency of deletions of 7q31 (a fragile site known as FRA7G) in human cancers (; Nishizuka et al., 1997; Huang et al., 1999; Han et al., 2003), the arguable presence of *CAV-1*gene promoter methylation (Hnasko et al., 2003; Parkin et al., 2005) and inactivating gene mutations (Hayashi et al., 2001; Chen et al., 2004), and the apparent reduction of *CAV1* expression in breast carcinomas (Chen et al., 2004; Park et al., 2005), it has been suggested that *CAV-1* is a tumor suppressor gene (Razani et al., 2000; Razani et al., 2001; Hnasko et al., 2003; Williams et al., 2005). Aberrant methylation of CpG islands has been suggested to serve as an alternative way of inactivating tumor suppressor genes in certain human cancers, i.e., ones that do not require point mutation or genomic deletions (Jones et al., 1999). Hypermethylation of the *CAV-1* promoter has been associated with decreased caveolin expression in breast cancer cell lines (Lee et al.,

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1998; Goetz et al., 2008).

Since we had previously found the role of genetic mutations of *CAV-1*in breast tumors, suggesting that *CAV-1*could serve as a putative gene in the pathogenesis of breast cancer (Syeed et al., 2010), therefore we chose to examine the methylation status of this gene to know further about the mechanism of this gene in the development and progression of breast cancer. We also showed that the hypermethylation leads to loss of expression in *CAV-1* gene by semi-quantitative rt-PCR.

Materials and Methods

Patients and Tumor Tissue Procurement

A cohort of 130 randomly selected breast cancer patients admitted to the department of General Surgery, Sher-I-Kashmir Institute of Medical Sciences was included in the study. All patients included in the study were both male and female, with the histopathological diagnosis of the breast done under Institutional histopathology department.

Tissue samples consisting of tumor and adjacent normal were collected directly from Operation Theatre of Department of General Surgery. Only histopathological confirmed tumor tissues were included in the study. Peripheral blood samples were also collected from the patients before the surgical resection of the tumor. The study was approved by the Sher-I-Kashmir Institute of Medical Sciences Ethical Committee.

DNA isolation

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

Methylation-Specific Polymerase Chain Reaction

Genomic DNA isolated from tumor and adjacent normal tissues by the protocol described above was bisulphite modified by using commercial kit (Methylation Direct Kit, Zymo Research) according to manufacturer's instructions. Two sets of primers were used; one was specific for DNA methylated at the promoter region of *CAV-1* gene and the other specific for unmethylated DNA. 3μl of bisulphite modified DNA was used in the PCR mix containing 1X PCR buffer, 200 umol/L of each dNTP and 2U Amp gold Taq DNA polymerase, 0.4μM of primer and were amplified using the following reaction conditions: initial denaturation at 95°C for 5 mins followed by 38 cycles at 95°C 50 seconds, 62°C 50 seconds, 72°C 1min, and the final incubation at 72°C for 7 mins. Ten-μl of the PCR products were loaded onto 4 % agarose gel and visualised under UV illuminator, the PCR generated a 353 bp product for methylated and a 275bp product and unmethylated.

Semi-quantitative analysis of mRNA expression of CAV-1 Gene

To assess gene expression, total RNA isolated from breast tumor tissues was reverse transcribed using random primers and the Zymogen first strand RT-PCR kit (Zymogen, research). A semi-quantitative analysis of gene expression was performed with β-actin as control .The PCR products were run on 1.5% agarose gels, visualized under UV illuminator. A tumor was considered to have lost expression when the gene showed complete lack of expression or at least 50% reduction from the normalized values.

Table 1. Effect Of *CAV*-1 Hypermethylation Pattern in the Breast Cancer Patients from Kashmir Valley.

Variable	Total		Methylated	OR; P value; CI (95%)
	N= 130	(%)	N = 28	
Sex				
Males	7	(53.8%)	2	1.5;1.0; 0.3-8.1
Females	123	(94.6%)	26	
Age				
> 50	13	(10.0%)	9	12;0.0001;3.2-41.6
≤ 50	117	(90.0%)	19	
Smoking Status				
Never:	93	(71.5%)	9	0.1;8.5; 0.0-0.3
Ever:	37	(28.5%)	19	
Menopausal Status				
Pre:	36	(27.7%)	13	3.0;0.0; 1.2-7.1
Post:	94	(72.3%)	15	
Nodal Status				
Involved	34	(26.2%)	10	1.8;0.2; 0.7-4.3
Not Involved	96	(73.8%)	18	
Tumor Stage				
II(a+b)	72	(55.4%)	12	0.5;0.1; 0.2-1.2
III(a+b)+ IV	58	(44.6%)	16	
Histopathological Tumor Grade				
PD	25	(96.0%)	11	4.1;0.004; 1.6-11
MD + WD	58+47	(24.1%)	08+09	

Statistical analysis

Pearson's two proportions test was used to compare the determined *CAV-1* gene mutational and methylational status 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

Frequency of CAV-1 Hypermethylation in Breast Carcinomas

In the present study a total of 130 breast cancer patients were studied for the methylational status of *CAV-1*.The promoter region of *CAV-1* in 130 tumor samples showed hypermethylation 21.5 % (28/130) of *CAV-1* gene were methylated. Using statistical analysis, we examined methylation status with regard to clinicopathological parameters of these cancer patients.

Relationship between promoter hypermethylation and gene expression

To evaluate the role of promoter hypermethylation in *CAV-1* gene, we assessed the levels of gene expression by semi-quantitative rt-PCR in 28 tumors with known methylation status. All tumor samples with promoter hypermethylation revealed an absence or down regulated expression of the *CAV-1*gene. The *CAV-1* gene exhibited

down-regulated expression in 26 out of the 28 (92.8%) tumors, including all the tumor samples that showed promoter hypermethylation. Our data, showed loss of *CAV-1* expression in majority of breast cancer cases.

Clinicopathological Association

In the present study, breast tumors that were found to be hypermethylated for *CAV-1*gene were statistically significant with menopausal status, histopathological grade and age. However there was no significant association with nodal status, sex, and smoking status as shown in Table 1.

Discussion

Epigenetic changes differ from genetic changes mainly in that they occur at a higher frequency than genetic changes, and are reversible upon treatment with pharmacological agents and occur at designed regions in a gene. Transcriptional silencing by DNA methylation of promoters can disable tumor-suppressor genes (Jones et al., 1999).

CAV-1 is known to serve as a scaffolding protein where signaling molecules are assembled. It directly interacts with the non-functional forms of the signaling molecules through a common N-terminal domain, termed the caveolins scaffolding domain. Through this interaction, caveolin-1 negatively regulates the activation of many signaling molecules in caveolae, including endothelial nitric oxide synthase, heterotrimetric G protein and MAP kinase (Williams et al., 2005; Razani et al., 2000; Okamoto et al., 1998). *CAV-1* is a membrane associated scaffolding protein that regulates a myriad of signaling molecules. Even though *CAV-1*is linked to tumor promotion it is also hypothesized to be a tumor suppressor because the protein inhibits signaling molecules associated with cell proliferation. Cyclin D is a regulatory unit of cyclin dependent kinases (CDKs) necessary for progression through the cell cycle. Over-expression of *CAV-1*causes transcriptional repression of cyclin-D (Huilt et al., 2000), and knocking out caveolin-1 causes upregulation of cyclin D1 levels (Williams et al., 2003).Continuing research illustrates a growing list of cancers in which *CAV-1* is downregulated by promoter methylation and mutation. Down regulation of *CAV-1* has been reported for invasive ductal carcinoma of the breast (Park et al., 2005).

Elucidation of a role for *CAV-1* in cellular transformation began with the observation that CAV-1 is down-regulated in oncogenically transformed cells (Nishizuka et al., 1997), Lee et al. (Lee et al., 1998) demonstrated that *CAV-1* possesses a transformation suppressor function, as *CAV-1* overexpression in human mammary cancer cells (T47D) results in tumor cell growth inhibition.

As we had found in our earlier study that *CAV-1* gene is 29.2% mutated in the breast cancer patients of Kashmir, therefore we chose to study the role of promoter hypermethylation of *CAV-1* gene in breast cancer patients of Kashmir. We found 21.5 % (28/130) of breast cancer cases hypermethylated. We did not found hypermethylation in any non-cancerous tissues. The difference between tumor and normal breast cancer samples suggests that the entire promoter may be hypermethylated. As has been reported earlier (Issa et al., 1993) the degree of methylation of the CpGs is not site-specific, which suggests that promoter hypermethylation of CpG Islands might be a common event resulting from a primary mechanism such as higher levels of DNA methyltransferase activity in tumor cells over normal cells or may in fact reflect a specific tumor suppressor mechanism of *CAV-1*. Moreover, we had similar results as Pinilla et al., who described an association between *CAV-1* expression and sporadic breast cancers (Pinnila et al., 2006).

Our data shows that *CAV-1* promoter hypermethylation is an important molecular signature in breast cancer. Our findings of hypermethylation of the *CAV-1* promoter, suggests that hypermethylation of *CAV-1* may not only be a strong marker for breast cancer, but also as a disease predictor. Promoter methylation seen in *CAV-1* gene resulted in transcriptional repression. The data also suggest that multiple mechanisms, in addition to the promoter methylation, might play a role in silencing of *CAV-1* gene expression in breast cancer. Our findings suggest that aberrant promoter methylation of *CAV-1* gene is associated with decline of expression.

We infer from our current study that like the mutational status (Syeed et al., 2010), the promoter hypermethylation of *CAV-1* gene plays a significant role for inactivation of *CAV-1* tumor suppressor genes (Baylin et al., 1998). Further studies of the exact mechanisms involved in epigenetic gene silencing of *CAV-1*, may provide important clues in understanding the mechanism involved in the progression of breast cancer.

Clinicopathologically, *CAV-1* gene hypermethylation was found significant with some of the clinico-pathological parameters like histopathological grade, age, and menopausal status. *CAV-1* hypermethylation status was significantly associated with age, as has been suggested earlier that De novo hypermethylation is associated with the aging of cells (Ahuja et al., 1998). *CAV-1* gene hypermethylation was significantly associated with pre-menopausal women suggesting that the premenopausal women are at more risk, than the postmenopausal women, as has been reported earlier that proliferation rate is somewhat lower and relatively stable in post menopausal women (Ferguson et al., 1981; Longrace et al., 1986; Meyer et al., 1997). *CAV-1* gene hypermethylation was significantly associated with poorly differentiated histopathological grade, suggesting that down-regulation of *CAV-1* in mammary tumor cells is a metastasis promoting event.

The relation between the loss of *CAV-1* expression and its hypermethylation was analysed in the present study. Our gene expression analysis by rt-PCR revealed that breast tumors showed down-regulation of mRNA levels in most of the hypermethylated breast tumors, suggesting promoter hypermethylation of *CAV-1* can be considered as one of the possible mechanism involved in the development of breast cancer. This loss of expression could be attributed majorly to hypermethylation of the *CAV-1* gene apart from other mechanisms of gene inactivation such as loss of heterozygosity and gene

deletions. We further found a highly significant ($p<0.0001$) positive correlation between *CAV-1* methylation and expression. This finding demonstrates that methylation of *CAV-1* is an important mechanism for silencing this gene in breast cancer and can be used as a marker for risk assessment.

In, conclusion, our results of a tumor-related epigenetic alterations favour the role of *CAV-1* as a tumor suppressor gene. *CAV-1* may play a crucial role in development and progression of breast cancers and may act as a prognostic factor in patients with breast carcinoma. Moreover, knowledge of the *CAV-1* hypermethylation state in breast cancers may be useful to identify breast tumors at an early stage. However, further research is needed to study the exact mechanism of the *CAV-1* inactivation in breast carcinoma and larger studies will be required to elucidate whether *CAV-1* loss is a useful prognostic biomarker of breast carcinoma or not.

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