

RESEARCH ARTICLE

Loss of Expression and Aberrant Methylation of the CDH1 (E-cadherin) Gene in Breast Cancer Patients from Kashmir

Asia Asiaf¹, Shiekh Tanveer Ahmad², Sheikh Aejaz Aziz⁴, Ajaz Ahmad Malik³, Zubaida Rasool⁵, Akbar Masood¹, Mohammad Afzal Zargar^{1*}

Abstract

Background: Aberrant promoter hypermethylation has been recognized in human breast carcinogenesis as a frequent molecular alteration associated with the loss of expression of a number of key regulatory genes and may serve as a biomarker. The E-cadherin gene (CDH1), mapping at chromosome 16q22, is an intercellular adhesion molecule in epithelial cells, which plays an important role in establishing and maintaining intercellular connections. The aim of our study was to assess the methylation pattern of CDH1 and to correlate it with the expression of E-cadherin, clinicopathological parameters and hormone receptor status in breast cancer patients of Kashmir. **Materials and Methods:** Methylation specific PCR (MSP) was used to determine the methylation status of CDH1 in 128 invasive ductal carcinomas (IDCs) paired with the corresponding normal tissue samples. Immunohistochemistry was used to study the expression of E-cadherin, ER and PR. **Results:** CDH1 hypermethylation was detected in 57.8% of cases and 14.8% of normal adjacent controls. Reduced levels of E-cadherin protein were observed in 71.9% of our samples. Loss of E-cadherin expression was significantly associated with the CDH1 promoter region methylation ($p < 0.05$, OR=3.48, CI: 1.55-7.79). Hypermethylation of CDH1 was significantly associated with age at diagnosis ($p=0.030$), tumor size ($p=0.008$), tumor grade ($p=0.024$) and rate of node positivity or metastasis ($p=0.043$). **Conclusions:** Our preliminary findings suggest that abnormal CDH1 methylation occurs in high frequencies in infiltrating breast cancers associated with a decrease in E-cadherin expression. We found significant differences in tumor-related CDH1 gene methylation patterns relevant to tumor grade, tumor size, nodal involvement and age at diagnosis of breast tumors, which could be extended in future to provide diagnostic and prognostic information.

Keywords: E-cadherin - ER - invasive ductal carcinoma - kashmir - promoter methylation

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Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in females worldwide. Worldwide, there were an estimated 1.7 million cases and 522,000 deaths in 2012 (Ferlay et al., 2013). In the recent past, breast cancer has been rising steadily, and for the first time now, breast cancer is the most common cancer in women in India with an estimated 144,937 incidences and 70218 mortalities due to breast cancer in 2012 (Ferlay et al., 2013; Indian Council of Medical Research, 2013). India has the maximum number of women dying of breast cancer, more than any other country in the world (BCI, 2013). In Kashmir also, the frequency of breast cancer has increased greatly with change in the life style in recent years and is emerging as a major concern in women populations (Wani et al., 2012). Hence, there is an imperative need for molecular biomarkers that will detect

breast cancer at an early, preferably even pre-invasive, stage. Methylation patterns may serve as biomarkers that might very well be appropriate to achieve this goal. Aberrant methylation has been increasingly recognized as a common and frequent molecular alteration in breast cancers. Hypermethylation of the CpG islands in gene promoters is associated with alteration in chromatin structure, delayed replication, inhibition of transcription initiation, and silencing of genes (Baylin and Herman, 2000). Genes involved in cell cycle regulation (p16), cell adhesion (CDH1), DNA repair (MGMT, BRCA1), and cell signaling pathway (ER, RAR β 2) have been reported to undergo hypermethylation (Yang et al., 2001; Szyf et al., 2004; Xiong et al., 2013).

The CDH1 (16q22.1) gene encodes the transmembrane glycoprotein E-cadherin that is important in maintaining homophilic cell-cell adhesion in epithelial tissues (Overduin et al., 1995; Zheng et al., 2012). The

¹Department of Biochemistry, Faculty of Biological Science, University of Kashmir, ³Department Of General Surgery, ⁴Department of Medical Oncology, ⁵Department Of Pathology, Sher-I-Kashmir Institute of Medical Sciences, Srinagar, Kashmir, India, ²Clarke H. Smith Brain Tumour Centre, University of Calgary, Alberta, Canada *For correspondence: mazargar65@gmail.com

cytoplasmic terminus of the E-cadherin molecule has been shown to be linked to the actin cytoskeleton via α -catenin and β -catenin (Cavallaro and Christofori, 2004). In malignant tumors in general, E-cadherin and catenins are strongly expressed in low grade tumors that maintain their cell adhesiveness and are less invasive, whereas their expressions are reduced in high grade tumors which have lost their intercellular adhesion and show strong invasive behavior (Mir et al., 2012; Yuan et al., 2012). Alterations in E-cadherin expression have been related in several cancer types including breast cancer with pathological features such as poor tumoral differentiation, infiltrative growth, lymph node metastasis and decreased patient survival (Oka et al., 1992; Hirohashi, 1998; Yoshida et al., 2001; Brock et al., 2003; Yuan et al., 2012). There are several mechanisms for E-cadherin downregulation in breast cancer. Loss of E-cadherin protein expression is most frequent for infiltrating lobular tumor types and can occur by somatic or germline mutation or by loss of heterozygosity, indicating that E-cadherin acts as a classic tumor suppressor gene (Kanai et al., 1994; Berx et al., 1995; Vos et al., 1997). Loss of E-cadherin expression was reported in 85% of invasive lobular carcinomas (ILCs) and lobular carcinoma in situ (LCIS) (Sarrío et al., 2004; Yoder et al., 2007). However, ductal histology often presents with varying levels of expression associated with epigenetic transcriptional downregulation (Graff et al., 1995; Nass et al., 2000; Prasad et al., 2008). Analysis of CDH1 methylation in breast cancers and other tumor types has shown that aberrant hypermethylation of CpG islands in CDH1 promoter region often occurs prior to invasion, indicating it to be an early event in tumorigenesis (Graff et al., 2000).

To date, the methylation status of CDH1 in breast cancer has been reported in several studies from different populations, but none of which was derived from Kashmiri population, distinct from other areas of India in terms of its unique geographical locale, intracommunity marriages, tradition, culture, food habits and ethnicity (Syeed et al., 2010). Given this background, our study was taken to provide an initial insight into the methylation profile and expression of CDH1 in breast cancer of Kashmiri women. Using methylation-specific polymerase chain reaction (PCR), we examined the methylation status of CDH1. We also tried to delineate more precisely the association of CDH1 methylation and its expression using Immunohistochemistry among breast cancer patients from our ethnic Kashmiri population. We also found association between the major clinicopathologic features of breast cancer and methylation status of CDH1 gene among primary breast cancer cases..

Materials and Methods

Patients

The present study was carried out from December 2011 to May 2013. A total of 128 newly diagnosed breast cancer patients from Kashmiri ethnic population admitted to the Sher-I-Kashmir Institute of Medical Sciences (SKIMS) were included in the study. All patients were recruited after histopathological diagnosis of breast cancer and all were

females. Tissue samples consisting of tumor and adjacent normal (from tissues located at least 3 cm away from the site at which the tumor was sampled) were collected from the Department of General Surgery, SKIMS. The tissue samples were immediately shock-frozen after surgical resection of the tumor or normal tissue and stored at -80°C deep freezer until further analysis. Each sample was histopathologically evaluated to ensure the presence of at least 80% of tumoral cells. Histopathological staging was performed according to AJCC-02 TNM staging system and grading was carried out by surgeon according to the Scarff-Bloom-Richardson classification as GI, GII and GIII. All patients received a patient information sheet and signed a consent form, approved by the Sher-I-Kashmir Institute of Medical Sciences Ethical Committee.

DNA isolation

Genomic DNA was isolated from tissue samples using a genomic DNA extraction kit (EZ DNA extraction kit (Zymo Research). Isolated DNA was resuspended in Tris EDTA buffer (pH 8.0) and stored at -20°C until use. The integrity of the resulting genomic DNA was assessed by low percentage agarose gel electrophoresis and concentration was determined by UV spectrophotometer.

Sodium bisulfite treatment

Sodium bisulfite conversion of genomic DNA (10 μl) was performed by EZ DNA Methylation™ Kit (Zymo Research, USA) according to manufactures protocol. Treatment of genomic DNA with sodium bisulfite converts unmethylated cytosines (but not methylated cytosines) to uracil, which are then converted to thymidine during the subsequent PCR step, giving sequence differences between methylated and unmethylated DNA.

Methylation-specific PCR (MSP)

The methylation pattern within the CpG island in exon 1 of the CDH1 (sequence -126 bp to +144 bp relative to transcription start, GenBank accession number D49685) was detected using a nested-PCR approach that has been published previously (Zheng et al., 2012). In the first round of PCR, bisulfite-treated DNA was amplified using the primers 5'-GTTTAGTTTTGGGGAGGGGTT-3' (sense) and 5'-ACTACTACTCCAAAACCCATACTAA-3' (antisense). Briefly, 2 μl of bisulfite modified DNA was amplified in a total volume of 25 μl containing 1X PCR buffer (Fermentas life sciences, Inc. USA), 1 μM of each primer, 2.0 mM MgCl_2 , 0.3 mM deoxyribonucleotide triphosphates (Fermentas Life Sciences, Inc., USA), and 1U of Taq polymerase (Fermentas life sciences, Inc. USA). The size of the product after this initial PCR reaction was 270 bp. PCR was carried out in Thermal cycler (Mastercycle, Ependroff) under the following conditions: 95°C for 10 min, followed by 35 cycles of 95°C for 1 min, 50°C for 30 sec, 72°C for 1 min a final extension at 72°C for 10. For the second PCR, 5 μl of this product was used for MSP as the template. The nested primers for the methylated sequence reaction were 5'-TGTAGTTACGTATTTATTTTGTAGTGGCGTC-3' (sense) and 5'-CGAATACGTCGAATCGAACCG-3' (antisense), and primers sequences

for the unmethylated sequence were 5'-TGGTTGTAGTTATGTATTTATTTTGTAGTGGTGT-3' (sense) and 5'-ACACCAATACAACAAATCAAACAAA-3' (antisense). The PCR parameters were the same listed above, except that the annealing temperature used for both primer pairs was 53°C. From each PCR reaction, 8 µl was loaded onto a 3% agarose gel, stained with ethidium bromide and visualized under UV illumination and photographed with Alpha Imager 1220 v5.5 Camera software. Representative gel pictures are shown in Figure 1. The PCR for all samples demonstrating methylation for the individual genes was repeated to confirm these results. The product sizes of the methylated and unmethylated amplicons were 112 bp and 120 bp, respectively.

Water blanks were used as a negative control for methylated genes. DNA from peripheral blood lymphocytes of healthy volunteers treated with SssI methyltransferase (New England Biolabs, Beverly, MA, USA) and then subjected to bisulfite modification was used as positive controls for methylated alleles. The reaction was performed in a total volume of 50 µl containing 10 µg of genomic DNA, 10 U of SssI methylase, 160mM of S-adenosyl-metionina, 50mM of NaCl, 10mM of Tris-HCl, 10mM of MgCl₂, 1mM of DTT pH 7.9, during 18 hours at 37°C.

Immunohistochemistry

Sections of formalin-fixed, paraffin-embedded breast were obtained on poly-L-lysine coated slides. Sections were deparaffinized in xylene, then rehydrated through a graded alcohol series. Antigen retrieval was performed by incubating slides in citrate buffer (pH 6.0) (10 mM) at 95°C for 20 min in microwave oven. Endogenous peroxidase activity was blocked with 3% H₂O₂ for 30 min. To detect protein expression sections were incubated under humid conditions overnight at 4°C with the specific antibodies against estrogen receptor (ER) (dilution 1:100; Santacruz Biotechnology Inc., USA), the progesterone receptor (PgR) (1:100 dilution, clone 1A6, Biocare Medical, USA) and E-cadherin (1: 200 dilution Clone: EP700Y; ThermoScientific). Positive and negative controls for each marker were routinely performed during experiments. Next day, the slides were washed three times in Tris buffers (pH 6.0) and bound primary antibody was detected by MACH1 Universal HRP-Polymer (Biocare medical, USA) for 30 min at room temperature. After

washing in Tris buffer, the immunostaining reaction product was developed using 3,3-diaminobenzidine (Betazoid DAB Plus substrate, Biocare Medical, USA). After immunoreactivity, slides were dipped in distilled water, counterstained with Harris hematoxyline and finally the sections were dehydrated in xylene, mounted with DPX and coverslipped. Tumors were classified by intensity of staining and the percentage of cells showing antibody reactivity. The ER and PR sections were scored for the immunohistochemical signal as follows: weak (1+), moderate (2+), and strong (3+) staining in >10% of the tumor cells or absent (0). Positivity nuclear staining was defined as moderate/strong expression (2+, 3+) in nucleus. For E-cadherin, the strength of the membranous staining was interpreted as either normal (strong) or aberrant (reduced or absent) (Kowalski et al., 2003). Aberrant staining was defined as either negative staining or <70% membranous staining of the population of cells examined. Normal staining was defined as ≥70% membranous staining of the cancer cells.

Statistical analysis

Correlation between CDH1 methylation status and various clinicopathological parameters of the patients was investigated by Pearson's chi square test and Fisher exact test whatever was appropriate. All p values are two-sided and considered statistically significant at the 0.05 level. Unconditional logistic regression was used to assess odds ratios (ORs) and 95% confidence intervals (CIs). All the statistical analysis was done by SPSS version 16.0 (SPSS Inc., Chicago, IL, USA.)

Results

Clinicopathological findings

A total of 128 cases of IDC were included in this study. The mean age of patients was 51.9±1.02 years and mean body mass index (BMI) was 23.6±0.21. Youngest patient was 26 years old and oldest was 80 years old. 56.2% cases were >50 years. Approximately 68.0% were rural. The stage distribution was typical with 38.3, 31.2, 23.4 and 7.0% presenting with Stage I, II, III, and IV, respectively.

CAD1 methylation status in tumor and normal samples

We analyzed the gene promoter methylation status in tumor and corresponding normal tissue from the same patient using nested PCR approach after the treatment of tissue genomic DNA with sodium bisulfite and interpreted data using appropriate statistical tests. In all samples, we detected the amplicon of 120 bp from unmethylated alleles. The amplification product of 112 bp from methylated alleles was observed in 57.8% (74 of 128) of breast cancer tissues and 14.8% (19 of 128) of corresponding normal tissues (non-malignant). There was a significant difference in the methylation of CDH1 between tumor and normal samples with a p value of <0.0001 as shown in Table 2 demonstrating that CDH1 methylation was correlated with the genesis of breast cancer. The methylation of CDH1 increased the risk of breast cancer very strongly (tumor vs normal, odds ratio [OR]=7.86, Confidence interval [CI]: 4.31-14.3 (Table

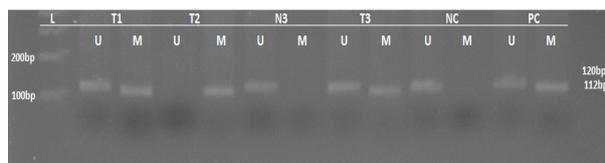


Figure 1. CDH1 Panel. Viewed from Left to Right Shows a 100-bp Ladder as Molecular Weight Marker; N3 Shows the Presence of Unmethylated DNA Detected in Normal Breast Tissue from Patient 3. T1, T2 and T3 show the presence of methylated DNA in tumor tissues of patient 1, 2 and 3. DNA from peripheral blood lymphocytes treated with SssI methyltransferase was used as a positive control (PC) for methylated alleles. NC: negative control. Lane U: unmethylated PCR product. Lane M: methylated PCR product

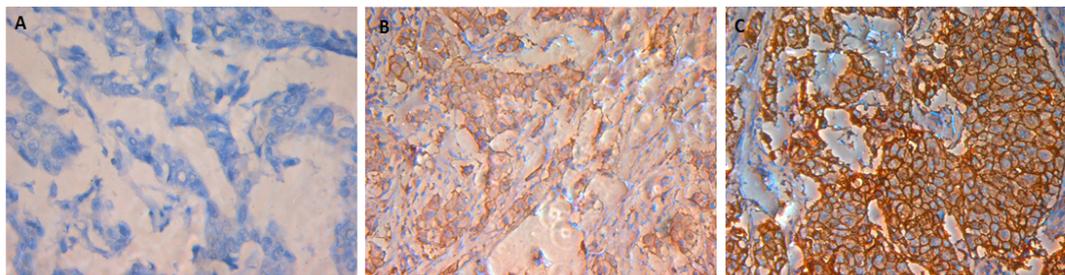


Figure 2. Immunohistochemical Detection of E-Cadherin Expression in Ductal Breast Carcinomas. Paraffin embedded tissue sections from invasive ductal carcinoma of breast were used for immunohistochemical analysis of E-cadherin protein using monoclonal antibody against E-cadherin as described in Materials and methods, sections were counter stained with hematoxylin. (A) IDC showing no detectable E-cadherin immunostaining and (B) IDC showing reduced expression of E-cadherin protein from patient harboring methylated alleles of CDH1 gene; (C) IDC from patient having unmethylated CDH1 showing normal expression of E-cadherin protein

Table 1. Frequency of Methylation in Breast Cancer Tissues and Corresponding Non-Malignant Breast Tissues from the Same Patients

	CDH 1 Methylated	CDH 1 Unmethylated	OR	CI (95%)	p-value
(a) Frequency of methylation in breast cancer tissues and corresponding non-malignant breast tissues from the same patients					
Normal Tissue (%)	19 (14.8)	109 (85.2)	1(reference)		
Breast Ca. Tissue (%)	74 (57.8)	54 (42.2)	7.86	4.31-14.33	<0.0001
(b) Association between methylation of CDH1 and the E-cadherin expression analyzed by Immunohistochemistry					
Normal expression (%)	13 (17.6)	23 (42.6)	1(reference)		
Aberrant expression (%)	61 (82.4)	31 (57.4)	3.48	1.55-7.79	0.0026

1(a)).

Correlation between methylation of the CDH1 promoter and clinicopathological features

In the present study when we analyzed the patient methylation data for its association with various clinicopathological features we found that hypermethylation in CDH1 gene was significantly associated with age (OR=2.30, CI: 1.12- 4.73, p=0.03) and nodal involvement (OR=2.21, CI: 1.04- 4.68, p=0.043). A very significant correlation was observed between CDH1 methylation and advanced tumor grade with CDH1 methylation in 11 (37.9%) of 29 grade I, 48 (60.8%) of 79 grade II tumors and 15 (75%) of 20 grade III tumors (p=0.024). Similarly CDH1 methylation increased with increase in tumor size (p=0.008). However, there was no relationship between CDH1 hypermethylation and menopausal status, dwelling, ER/PR status and tumor stage of patients (Table 2).

Immunohistochemical assessment of E-cadherin and correlation with promoter methylation

To investigate the relationship between aberrant methylation and E-cadherin silencing, E-cadherin protein expression was examined using immunohistochemical analysis in 128 tumors. Out of 128 samples, 92 (71.9%) and 36 (28.1%) had an aberrant/reduced and normal E-Cadherin expression, respectively (Figure 2). After comparing ECAD expression levels between the samples displaying methylated and unmethylated CAD1 genes, for those patients where both expression and MSP data were available, we found a significant correlation between gene methylation and protein expression of E-cadherin (OR=3.48, CI: 1.55- 7.79, p <0.05, Table 1(b)) (Figure 3). The expression of the E-cadherin decreased remarkably

Table 2. Association of CDH1 Hypermethylation with Clinicopathological Parameters in the Breast Cancer Patients from Kashmir Valley

Variable	CAD1 methylation		p value	OR (CI)
	Presence N (%)	Absence N (%)		
Age				
≤50	26 (46.4)	30 (53.6)	0.03	2.30 (1.12- 4.73)
>50	48 (66.7)	24 (33.3)		1 (reference)
Menopausal status				
Premenopausal	48 (61.5)	30 (38.5)	0.359	1.48 (0.72- 3.03)
Postmenopausal	26 (52.0)	24 (48.0)		1 (reference)
Nodal status				
Positive	34 (69.4)	15 (30.6)	0.043	2.21 (1.04- 4.68)
Negative	40 (50.6)	39 (49.4)		1 (reference)
Tumor size				
≤2.0 cm	7 (31.8)	15 (68.2)		1(reference)
2.1- 4.9 cm	41 (69.5)	18 (30.5)	0.008a	4.88 (1.7-14.01)
≥5 cm	26 (55.3)	21 (44.7)		2.65 (0.91-7.70)
Tumor stage				
I	29 (59.2)	20 (40.8)		1(reference)
II	25 (62.5)	15 (37.5)	0.580a	1.14 (0.48-2.70)
III& IV	20 (51.3)	19 (48.7)		0.73 (0.31-1.69)
Tumor Grade				
I	11 (37.9)	18 (62.1)		1(reference)
II	48 (60.8)	31 (39.2)	0.024a	2.53 (1.05-6.08)
III	15 (75.0)	5 (25.0)		4.9 (1.39-17.30)
Dwelling				
Rural	53 (60.9)	34 (39.1)	0.56	1.28 (0.60- 2.71)
Urban	21 (48.8)	20 (51.2)		1(reference)
Estrogen receptor				
Negative	24 (61.5)	15 (38.5)	0.7	1.25 (0.58- 2.69)
Positive	50 (56.8)	39 (43.8)		1(reference)
Progesteron receptor				
Negative	23 (53.5)	20 (46.5)	0.57	0.77 (0.36- 1.60)
Positive	51 (60.0)	34 (40.0)		1(reference)

^aChi square for trend; p-value (two sided) Fisher's exact test; Bold values indicate statistical significance p<0.05

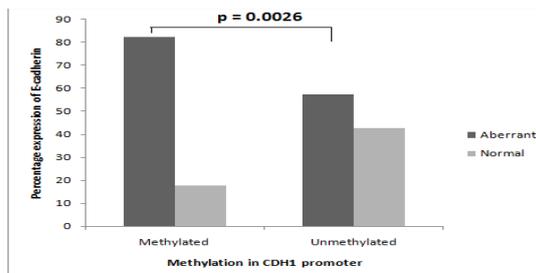
Loss of Expression and Aberrant Methylation of the CDH1 (E-cadherin) Gene in Breast Cancer Patients from Kashmir

Figure 3. Relationship between Methylation Status and Immunohistochemical Expression Analysis of E-Cadherin in IDCs of Breast. The bar diagram shows the correlation between the methylation induced silencing of CDH1 gene and loss of expression of E-cadherin proteins

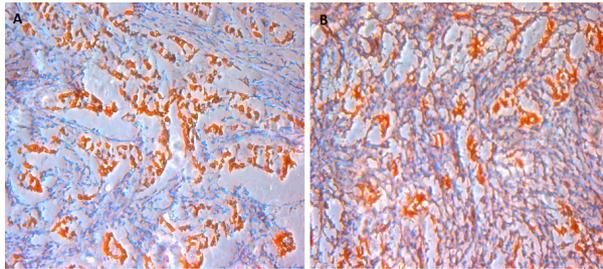


Figure 4. Immunohistochemical Staining Pattern of the Hormone Receptors. A) Estrogen receptor; B) progesterone receptor. Paraffin embedded tissue sections from invasive ductal carcinoma of breast were used for immunohistochemical analysis of Estrogen and Progesterone receptors using specific antibodies against corresponding proteins as described in Materials and methods, sections were counter stained with hematoxylin

in the patient with CDH1 methylation thus confirming the likely role of DNA methylation in the loss of expression of E-Cadherin protein.

Discussion

Aberrant DNA hypermethylation in CDH1 is a candidate mechanism responsible for silencing CDH1 associated with several types of malignancies (Lo and Sukumar, 2008). Reduced expression of E-cadherin is regarded as one of the main molecular events involved in the dysfunction of the cell-cell adhesion system, triggering cancer invasion and metastasis (Debies and Welch, 2001). Hypermethylation in CDH1 gene and its reduced expression in gut, lung, prostate and breast cancers, which could be due to the disruption of intercellular adhesion and impairment of β -catenin mediated transactivation of cadherin-catenin complex (Graff et al., 1995; Sadot et al., 1998; Tamura et al., 2000; Hu et al., 2002; Caldeira et al., 2006; Prasad et al., 2008; Zheng et al., 2012) has been reported.

In this study, we showed aberrant methylation in CDH1 in breast cancer tissues of 128 individuals. From each patient, a breast cancer sample and the adjacent normal breast tissue were evaluated. With this design, pair of cancer and normal samples was available for each case. The frequency of CDH1 promoter methylation in breast cancer patients in our study was 57.8% as against 14.8% of the corresponding normal breast samples. The frequency of CDH1 methylation in our study is similar to other studies conducted on breast cancer patients (Shinozaki et

al., 2005; Prasad et al., 2008). Other previous studies have shown varying frequencies of CDH1 methylation viz; 72% (Caldeira et al., 2006), 26.1% (Hu et al., 2002), 21.7% (Sebova et al., 2011), 94% (Shargh et al., 2011) in breast cancer tissues from different population. The comparison of normal and malignant tissues showed a statistically significant ($p < 0.0001$) difference in the methylation pattern, thus there was significant eightfold increase in the risk to breast cancer in the subjects with methylated CDH1 promoter. Presence of hypermethylation in some adjacent non-cancerous tissues represents field defect of premalignant changes that occurs early in carcinogenesis. The fact that the adjacent normal breast tissues lack microscopic evidence of malignancy suggests that these changes are non-transforming themselves. However, they might allow the prospective acquisition and accumulation of other genetic and epigenetic changes that lead to malignancy in spatio-temporal manner. These results suggest that the detection of CDH1 hypermethylation using MSP might also provide potential new molecular diagnostic biomarkers of breast carcinomas at an early premalignant stage during multistep breast carcinogenesis. A better understanding of methylation frequencies detectable in 'normal' tissue is warranted that will aid in the selection of the appropriate source for normal tissue (i.e. proximal to the tumor, from the contralateral breast etc.) to use as control in comparative studies.

We investigated the association of promoter hypermethylation with various clinicopathological parameters. CDH1 gene hypermethylation was found significantly associated with some of the clinicopathological parameters like age, lymph node involvement, tumor size and tumor grade. Promoter methylation in several genes increases with age in normal tissues, although the exact mechanism of age-related methylation status remains unknown (Issa and Ahuja, 2000). It has been hypothesised that several factors may modulate age-related methylation, such as exogenous carcinogens, radiation, endogenously generated reactive oxygen species, and genetic background (Issa and Ahuja, 2000). In our study, CDH1 methylation increased with increase in the age of patients. In our study, the prevalence of CDH1 methylation was also significantly associated with tumor histological grade, thus indicating that this event occurs at a later stage and promotes the progression to higher histological grades. Therefore, our finding is in agreement with earlier studies, which reported associations between CpG island methylation and poor histological differentiation of breast tumors (Nass et al., 2000; Raish et al., 2009; Tao et al., 2009; Shargh et al., 2011). CDH1 gene hypermethylation was also found significantly associated with lymph node metastasis suggesting that the breast tumors with CDH1 CpG-island hypermethylation may possess a biologically aggressive phenotype, as suggested in previous study (Shinozaki et al., 2005).

In current clinical practice, the response to hormone therapy is associated with the levels of both estrogen receptors (ERs) and progesterone receptors (PRs) in both primary (Fisher et al., 1983; Rose et al., 1985; Harvey et al., 1999) and advanced (Valavaara et al., 1990; Harvey et al., 1999; Elledge et al., 2000) breast cancers. In the current

study, 69.5% of tumors were found to be ER-positive and 66.4% of tumors were found to be PR positive (Figure 4). Our results of hormone receptor positivity are almost similar to an earlier investigation conducted on the breast cancer patients from Kashmir (Sofi et al., 2012). However, we did not find any correlation between methylation of CDH1 and estrogen or progesterone receptor status although previous studies have reported such associations (Shinozaki et al., 2005; Caldeira et al., 2006; Li et al., 2006; Ronneberg et al., 2011).

Significantly higher methylation of CDH1 in tumors with bigger size or higher grade, lymph node involvement, makes it a good candidate as a prognostic factor in patients with ductal cell carcinoma of breast.

Structural and functional imbalance initiated due to E-cadherin down regulation results in direct effects on cellular proliferation and invasion. E-cadherins act as tumoral suppressor proteins by their ability to block not only the uncontrolled proliferation but also the cellular differentiation toward a malignant phenotype (Andrews et al., 2012). Previous immunohistochemical studies have revealed the loss or aberrant expression of E-cadherin at the cell membrane is associated histopathological tumor characteristics (differentiation, aggressiveness, metastasis and poor prognosis) in different cancer types (Mitselou et al., 2010; Sun et al., 2011). Loss of E-cadherin protein expression is uniform and more frequent in invasive lobular carcinomas (ILCs) and is often a biallelic event resulting from any combination of gene promoter hypermethylation, mutation, or allelic loss, whereas ductal histology often presents with varying levels of expression (Graff et al., 2000; Acs et al., 2001). In our study, we observed aberrant expression of E-cadherin (weak or negative staining in cell membranes) in 71.9% of IDC. Intensity of E-cadherin staining decreased with methylation of CDH1 gene as 82.4% (61 out of 74) breast tumors showed concomitant aberrant levels of E-cadherin and CDH1 hypermethylation, demonstrating statistically significant association of CDH1 hypermethylation with its reduced expression in these breast tumors. However, aberrant immunostaining in 57.4% of unmethylated CDH1 cases may be due to other mechanisms, such as gene mutation, loss of heterozygosity, changes in structure of chromatin (Hennig et al., 1996), and alterations of specific transcription pathways regulating the expression of the CDH1 gene (Hajra et al., 1999; Peinado et al., 2004). Besides, of the methylated tumors, 13 showed positive E-cadherin immunostaining. In these cases, methylation specific PCR fragment might be the result of contamination with infiltrating leukocytes, making the detection and interpretation of tumor-associated distorted methylation patterns more complicated (Lombaerts et al., 2004). Overall, our data corroborate the findings reported by earlier studies that CDH1 promoter hypermethylation is responsible for its heterogeneous downregulation in ductal cell carcinoma (Graff et al., 2000; Caldeira et al., 2006; Prasad et al., 2008).

In conclusion, our present study provided an initial insight into the methylation and expression of CDH1 genes in breast tumor tissues derived from Kashmiri women. In conclusion, abnormal CDH1 methylation not

only occurs in high frequencies in invasive ductal cancers but also may have prognostic significance in breast cancer as it has significant impact on the clinical outcomes of breast cancer such as tumor size, tumor grade and rate of node positivity or metastasis. Therefore, methylated CDH1 could be extended in future to provide prognostic information in breast cancer. However, our preliminary findings needed to be confirmed by new studies with large sample sizes to reach a definite conclusion.

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