

Full Length Research Paper

Evaluation of methylation pattern in promoter region of E-cadherin gene and its relation to tumor grade and stage in breast cancer

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The epithelial cadherin gene (CDH1) has been identified as a tumor suppressor gene located within the 16q22.1 region. The CDH1 gene encodes a transmembrane glycoprotein involved in cell to cell adhesion and loss of CDH1 expression contributes to increased proliferation, invasion and metastasis in breast carcinoma. No mutation in CDH1 have been identified in invasive ductal carcinoma (IDC), suggesting that, other inactivation mechanisms are responsible for IDC oncogenesis. In order to analyze the role of promoter methylation in CDH1 gene inactivation in breast cancer, the CpG methylation status of E-cadherin promoter region by bisulfite sequencing PCR (BSP) was investigated. 10 CpG sites [nucleotide (nt) 863, 865, 873, 879, 887, 892, 901, 918, 920 and 940] in the promoter region were screened for methylation. The CDH1 methylation was detected in 94% (47 to 50) of breast tumors which was associated with higher tumor grade ($p = 0.035$), tumor stage ($p = 0.000$) and tumor metastasis ($p = 0.000$). There was also a significant correlation between tumor stage, grade and metastatic status with sites of methylation ($p = 0.000$). The data indicate that CDH1 promoter methylation might be a potential mechanism for epigenetic silencing of CDH1 in primary breast cancer suggesting a valuable molecular marker for detection of breast cancer progression.

Key words: Breast cancer, E-cadherin, methylation pattern, tumor stage, tumor grade.

INTRODUCTION

Breast carcinoma is the most common malignancy among women worldwide. The disease arising from the ductal and lobular epithelium of the mammary glands (Oyama et al., 2009) and metastasis represents an important step in the progression of the fatal disease (Sahin et al., 2009). Complex genetic and epigenetic alterations affect the severity of each step in tumor progression. Genetic changes that occur in metastatic cells have been studied at the level of several genes, tissue specific profiles and

whole genome approaches (Hunter and Alsarraj, 2009). Different mechanisms in both genetic and epigenetic changes are involved, including mutation, loss of heterozygosity (Ehdaie and Theodorescu, 2008), transacting pathways (Vesuna et al., 2008), chromatin rearrangement (Rodenhiser and Mann, 2006) and hypermethylation in promoter CpG sites (Choi et al., 2009). Unlike genetic changes, epigenetic alterations in metastasis are less characterized in primary cancers. DNA methylation is the covalent addition of a methyl group to a cytosine base, generally in the context of a CpG dinucleotide. The mammalian genome is predominantly methylated with the exception of CpG-rich regions, which are generally found in gene promoters and

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are known as "CpG islands" (Esteller, 2005). These changes alter gene expression levels primarily through regulation of methylation state-dependent interactions with transcriptional activators or repressors (Conerly and Grady, 2010). The aberrant methylation of gene promoters observed in many cancers is believed to play a key role in the development of different cancers. It is also well understood that hyper-methylation of some regulatory sequences is more frequent than mutational change (Chan et al., 2008). This methylation can silence genes such as CDH1, which can promote enhanced invasiveness (Nakata et al., 2006). CDH1 is a gene localized in the chromosome 16q22.1, that is expressed from epithelial cells and involved in cell to cell interactions. This molecule contains two extra and intra membrane domains. The extra membrane domain interacts with other cells whereas, intra membrane domain participates in structural proteins like α -catenin and β -catenin (Bex and Roy, 2009). CDH1 displays a tumor/invasion suppressor functions and its expression level influences the cell shape, adhesion and biological interactions (Masciari et al., 2007). CDH1 dysfunction due to mutations has been reported in gastric and lobular breast carcinomas (Lascombe et al., 2006). Also, its down regulation due to allelic deletion has been seen in various types of tumors like hepato-cellular carcinomas (Xia et al., 2008) lobular and ductal breast carcinomas (Masciari et al., 2007). For most of the primary breast cancers and cell lines of the ductal histotype, no CDH1 mutations could be identified despite the fact that these tumors often show strikingly reduced CDH1 gene and protein expression (Bex and Roy, 2001). Possible mechanisms to explain this reduced expression include chromatin rearrangement, hypermethylation and alterations in trans-factor binding (Li et al., 2006). Hypermethylation of the CDH1 promoter and overlapping 5' CpG Island has been demonstrated to be correlated with the loss of CDH1 expression at the transcriptional level for various breast cancer cell lines and primary ductal breast cancers (Chao et al., 2010). However, the relation of CDH1 hypermethylation and grade and stage of tumors has not been well understood. In this study, the methylation pattern of CDH1 promoter in breast cancer was investigated. The methylation status was classified in a qualitative manner as full, hemi and non-methylated in tumor and normal tissues. Quantitative analysis of methylation pattern of CpG sites and their relation with tumor's grade and stage were also investigated.

MATERIALS AND METHODS

Patients and tissue specimens

Samples of breast cancer were obtained from 50 women (mean age of 48.2 ± 10.55 years) who had undergone surgery at the Imam

Reza hospital in Tabriz university of Medical Sciences. The samples obtained during surgery were according to the institutional guidelines. Normal breast tissues ($n = 50$) were also taken from the same patients and breasts that underwent partial or total mastectomy, 3 cm away from the site at which the tumor was sampled.

Staging was performed according to AJCC-02 TNM staging system and grading was carried out according to WHO international protocol by surgeon as GI, GII and GIII.

DNA isolation

DNA was extracted from 25 mg frozen breast tissues at -80°C of 50 tumoral and 50 normal samples using a SDS-proteinase K and phenol-chloroform method as previously described (Pourabbas et al., 2009). 10 CpG sites from the promoter region located at -836 to -962 upstream to transcription start site were analyzed for methylation pattern as described earlier (Ribeiro-Filho et al., 2002).

Bisulfite conversion and BSP analysis

To amplify the interested region of the promoter, DNA was treated with sodium bisulfite as described earlier (Frommer et al., 1992) with some modifications. Briefly, 1 μg of DNA was denatured using NaOH and treated with sodium bisulfite for 8 h in 55°C . Bisulfite treated DNA was amplified with specific primer pairs (forward: 5'-TTTAGTAATTTAGGTTAGA GGGTT-3', nt -836 to -861) and (reverse: 5'-CTAATTAATAAAAATTCACCTACC-3', nt -940 to -965). 2 μl of treated DNA were entered into a 25 μl polymerase chain reaction (PCR) mixture containing 0.4 μM forward and reverse primers each; 1 \times Taq buffer, 0.2 mM four deoxynucleotide triphosphate and 1.2 U Taq polymerase (Fermentas, Letonia). PCR was carried out with the program as followed: a 5 min cycle in 95°C followed by 30 cycles of 30 s in 94°C , 30 s in 54°C , 30 s in 72°C and a final extension cycle of 5 min in 72°C . The untreated breast tissue genomic DNA was used as negative control and universal methylated DNA (invitrogen) sample as ready to use commercial control that underwent bisulfite treatment, was used as positive control. PCR products then, were purified using PCR product purification kit and checked by electrophoreses on 5% polyacrylamide gel. The resulting products were sequenced on an ABI automated sequencer with big dye terminator (Perkin-Elmer, CA).

Classification and quantification of methylation levels

Determination of methylated CpG sites was performed by comparing the C versus T nucleotide peaks in bisulfite treated tumor samples in each 10 specified CpG sites that remained as C and was converted to T in bisulfite treated normal samples. Methylation was considered 100% if all 10 CpG sites were methylated. Methylation levels less than 10 CpG sites were classified as partial methylation and absence of methylation was considered unmethylated.

Statistical analysis

The percentage of methylation was calculated by chi-square test and significance difference between cancer and normal samples, also, cancer grade and stage with methylated CpG sites was analysed by using spearman regression test.

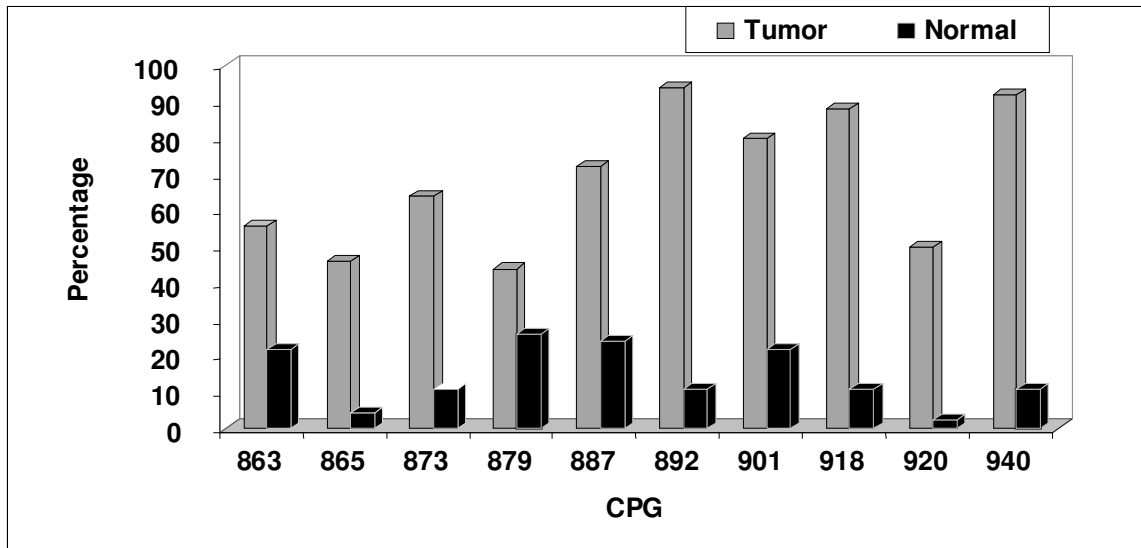


Figure 1. Comparison of methylation pattern of CDH1 promoter region in breast cancer and its adjacent normal tissues.

RESULTS

Methylation status in tumor and normal samples

Among breast cancer samples, 44% (22 of 50) were completely methylated, 50% (25 of 50) were partially methylated and 6% (3 of 50) were unmethylated. The overall hypermethylation rate in breast cancer tissues was 94% (47 of 50). On the other hand, the majority of the normal samples that is 76% (38 of 50) were unmethylated. There were significant differences between tumor and normal samples in the methylated CpG sites (methylation pattern) ($p = 0.000$) (Figure 1).

Methylation of the CDH1 promoter and its relation to grade of tumor

Of 45 tumor samples 10 were classified as grade 1 (well differentiated), 30 grade 2 (moderately differentiated) and 5 grade 3 (poorly differentiated). 5 samples had unknown grade and were excluded from the calculations. Analysis for methylation showed that among 10 grade 1 tumors, 2 (20.0%) were fully methylated and 8 (80.0%) were partially methylated. Among 30 grade 2 tumors, 12 (40.0%) were fully methylated, 15 (50%) partially methylated and 3 (10.0%) were non-methylated. Finally, of 5 grade 3 tumors, 4 (80.0%) were full methylated and 1 (20.0%) partially methylated. Statistical analysis showed a significant direct relationship between full-methylation and grade of tumor samples ($p < 0.022$ for partial and $p <$

0.035 for full methylation) (Figure 2).

Methylation pattern of the CDH1 promoter CpG sites and its relation to grade of tumor

The spearman regression test analysis showed the highest significant relationship between grade of tumor and methylation at 887 nt ($p = 0.007$). There were also an association (statistically non-significant) between methylation at 920, 879 and 865 nts with grade of tumors ($p = 0.022$, 0.035 and 0.031, respectively) (PV was set in 0.05 level, 2 tailed) (Table 1 and Figure 3).

Methylation of the CDH1 gene and its relation to tumors' stage

Among the 7 different stages that have been reported in different studies, in this study, only 3 stages were found in tumor samples including stages 1, 2 and 3a. There were 3 samples in stage 1, 29 in stage 2 and 13 in stage 3a. Of the 3 stage 1 tumor sample, all (100%) were partially methylated, of 33 stage 2 tumors, 8 (24.2%) were full methylated, 22 (66.7%) partial and 3 (9.1%) were not methylated. Finally, of the 14 stage 3a tumors, 14 (100%) were full methylated. There was a significant correlation between tumors' stage and methylation status ($p = 0.000$, in full and partial methylation), which indicated the presence of a meaningful direct relation between stage of tumor and full methylation (Figure 4).

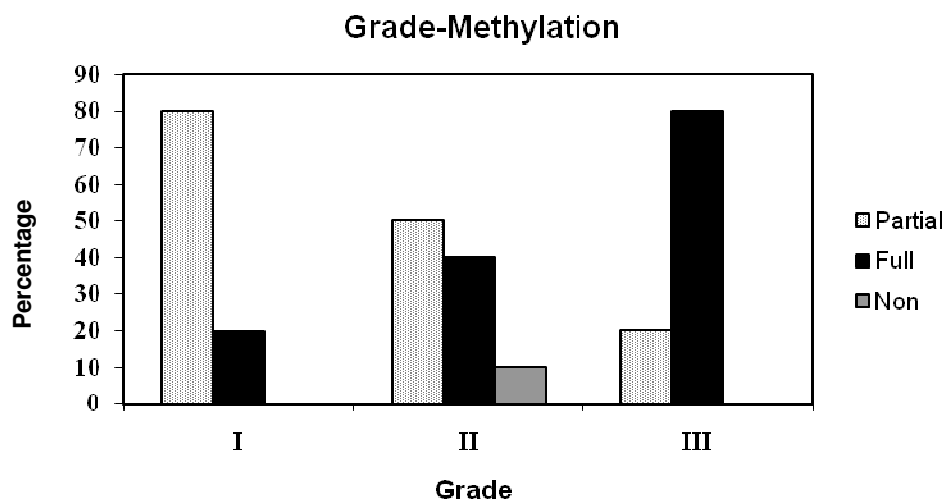


Figure 2. Comparison of tumor grade and methylation status in tumor and normal samples.

Table 1. Methylation pattern and tumor grade in breast tumor samples.

Grade	CpG site	Correlation coefficient	Significance (2-tailed)
Grade	863	0.29	0.054
	865	0.32*	0.031
	873	0.25	0.091
	879	0.31*	0.035
	887	0.40**	0.007
	892	-0.06	0.686
	901	0.19	0.204
	918	0.03	0.844
	920	0.34*	0.022
	940	-0.07	0.637

** Correlation is significant at the 0.01 level (2-tailed); *correlation is significant at the 0.05 level (2-tailed).

Methylation pattern of the CDH1 promoter and its relation to stage of tumor

There were a significant direct relationship between stage of tumor and methylation at nucleotides 863, 865, 873, 879, 887 and 920 ($p = 0.000, 0.000, 0.001, 0.000, 0.002$ and 0.000 , respectively) and in some degree with nucleotide 901 ($p = 0.032$) (PV was set at 0.005 level, 2 tailed) (Table 2).

In the evaluation of methylation patterns in ductal tumor and different tumor stage, the higher methylation rate was observed in stage 3a and among the different tumor grades, the highest rate of CpG methylation was observed in grade 2 of ductal type breast cancer samples (Data not shown).

DISCUSSION

CDH1 hypermethylation is a candidate mechanism responsible for silencing CDH1 association with several types of malignancies (Lo and Sukumar, 2008) but underlying mechanism for silencing of CDH1 is not clearly understood. In this study, CDH1 promoter methylation status was analyzed in breast cancer tissues of 50 individuals. From each patient, a breast cancer sample and normal breast tissue (from neighboring site of tumor) were evaluated. With this design, pair of cancer and normal samples was available for each case. The comparison of normal and malignant tissues showed a statistically significant ($p = 0.006$) difference in the methylation pattern. This finding is consistent with several

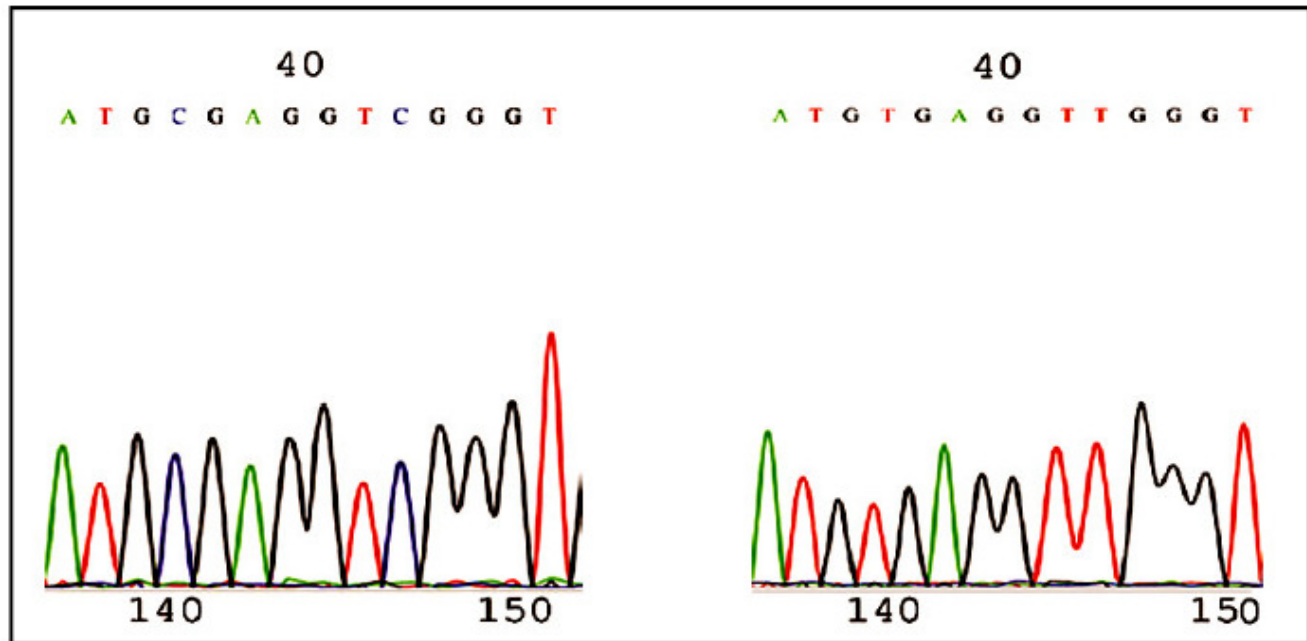


Figure 3. Examples of direct sequencing chromatograms. Bisulfite treated DNA was amplified and sequenced on ABI automated sequencer. The sequences represents CpG sites one of which marked by arrows. 3a) complete non-conversion of cytosine to thymine (complete methylated). 3b) complete conversion of cytosine to thymine (non-methylated).

studies indicating the role of CDH1 methylation in various tumors. Kordi-Tamandari et al. (2010) found that, 61.8% of oral cavity tumors had methylated CDH1 gene. Hiraki et al. (2010) found that CDH1 gene methylation in the peritoneal fluid is a potential marker predicting peritoneal invasion of gastric cancer which varies significantly depending on the extent of the cancer invasion. Methylation of CDH1 in primary human colon cancer and its association with cancer progression and metastasis has also been reported (Kim et al., 2006). Feng et al. (2010) found CDH1 methylation in 23.7% of lymph nodes in metastatic breast cancer when compared with 18.4% in primary cancers and reported that, CDH1 methylation is correlated with lymph node metastasis rate. It has also been demonstrated that, down regulation of CDH1 expression is often accompanied with methylation of the 5'CpG island of CDH1 in prostate, lung, liver, bladder, gastric, thyroid and breast carcinoma cell lines (Bornman et al., 2001; Ehdai and Theodorescu, 2008; Brooks-Wilson et al., 2004).

Because of the small number of *in situ* and lobular form of tumors in this study, evaluation of a possible increasing trend of CDH1 methylation in malignant progression was not possible. The CDH1 methylation status did not seem to correlate with tumor number or with chemotherapy. There was no significant correlation between tumor grade and stage in this study, indicating lack of common methylated site related to both aspects of the tumor.

BSP product sequencing of tumor samples showed considerably higher CDH1 methylation levels when compared with normal samples at all CpG sites of the promoter region (nt 863, 865, 879, 892, 901, 918, 920 and 940). However, nucleotide 892 (94%) and 940 (92%) demonstrated higher methylation prevalence. This might be related to the higher predisposition of some CpG sites to methylation which has a key role in cancer progression. It has been shown that, CpG sites contain SP1 elements that are located upstream of the transcription initiation site (Varshochi et al., 2005) and their cis-acting property prevent CpG islands methylation (Goldstein, 2002). Disruption of these elements may be responsible for the CpG methylation and gene silencing.

Notable finding of this study was that, the sites of methylated CpGs corresponding to the stage and grade of tumor were different. Even low grade and early stage tumors showed some degree of CDH1 methylation (Figure 2 and 4), confirming previous reports and show that, epigenetic alteration is an early event in malignancy that may take place before tumor invasion (Duffy et al., 2009).

In summary, this study showed the CDH1 gene hypermethylation in breast cancer. There was a specific CpG methylation pattern that might be critical in the silencing of the CDH1 gene in breast cancer. These results are important in evaluating the mechanisms involved in CDH1 silencing through hypermethylation of specific CpG

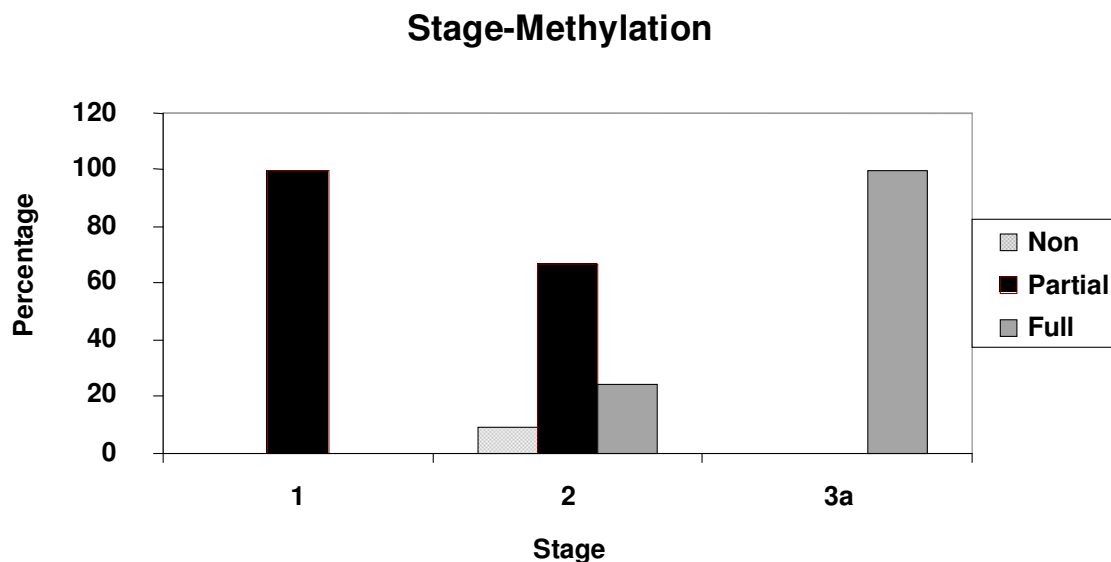


Figure 4. Comparison of tumor stage and methylation status in tumor and normal samples.

Table 2. Relation between promoter methylation pattern in different tumor stage in breast cancer samples in CDH1 gene promoter region.

Stage	CpG site	Correlation coefficient	Significance (2-tailed)
	CPG-863	0.588**	0.0
	CPG-865	0.617**	0.0
	CPG-873	0.469**	0.001
	CPG-879	0.699**	0.0
Stage	CPG-887	0.420**	0.002
	CPG-892	0.116	0.423
	CPG-901	0.304*	0.032
	CPG-918	0.169	0.24
	CPG-920	0.579**	0.0
	CPG-940	0.135	0.349

** Correlation is significant at the 0.01 level (2-tailed);

*correlation is significant at the 0.05 level (2-tailed).

sites, which may provide a marker for diagnosis and prognosis of breast cancer. The gene expression study in the protein level by western blotting method is recommended for confirmation of these results.

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