Full Length Research Paper

Evaluation of methylation modification in E-cadherin gene and its application in the improvement of breast cancer

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CDH1 (E-cadherin), which mediates cell-cell interaction and polarity, is known as glycoprotein in cytoskeleton. The objective of this study was to evaluate CDH1 expression loss as the metastatic marker by defining the methylation pattern in the promoter region and determining whether or not the methylation pattern changes in correlation with a kind of tumor, grade and metastatic status. Fifty patients with breast carcinoma were enrolled in this study and fifty normal breast tissues were obtained from an adjacent tumor area as control from the same patients’ breast. All of these patients had different grades, metastasis status and tumor kind. Fresh tissue sections of breast cancers were obtained and their DNA was isolated, bisulfite treated, PCR amplified and analyzed for sequencing. The loss of CDH1 was assessed as percentage of methylation (full, partial and non-methylated) in the promoter region, and the number of CpG sites involved in methylation was assessed as the methylation pattern. The percentage of CDH1 gene promoter methylation in the tumor samples was 44% for full methylation, 50% for partial methylation and 6% for non-methylated. There was significant difference between normal and tumor tissues in methylated CpG sites and also between different grades and kinds of tumor. More so, there was no significant variation in the recurrence state of tumor. Even though loss of CDH1 expression in breast cancer has been established before, its critical role in cell-cell contact can reflect the metastatic effect of the lost expression during the metastatic phase of cancer. However, methylation pattern significantly differs in high grade tumor samples (p<0.00).

Key words: E-cadherin, promoter, methylation, breast cancer, prognosis.

INTRODUCTION

CDH1 (E-cadherin) acts as a transmembrane glycoprotein that is important in epithelial cell-cell interactions (Kim and Sahin, 2005), and also interacts with α and β catenin together with actin in cytoskeleton (Lombaerts et al., 2006). CDH1 plays a critical role for establishment and maintenance of polarity and differentiation of epithelium during the development period (Wilson et al., 2004). Also, it plays an important role in signal transduction, differentiation, gene expression, cell motility and inflammations (Slaus, 2003). However, somatic loss of CDH1 expression is associated with lobular breast cancer (Goldstein et al., 2001). Besides the mutational changes (Mastracci et al., 2005) observed in gene expression, there are evidences for epigenetic silencing of CDH1 gene including promoter region methylation in CpG sites (Triscoof and Tannapfel, 2008). Loss of CDH1 expression in cancer cells leads to a diffused growth pattern and metastasis potential in tumors; thus, CDH1 generally acts as a tumor suppressor gene (Maruyama et al., 2001).

CDH1 gene expression down-regulation is mediated by several mechanisms that can be evaluated by different
approaches (Masciari et al., 2007). One way is to search for methylation pattern in the promoter region (Dhillon et al., 2004). For this purpose, we performed a methylation pattern study on 50 breast cancer samples and 50 normal adjacent normal breast tissues with a known breast cancer and evaluated them for different kinds of tumor, grade (according to TNM grading system) and metastasis status.

MATERIALS AND METHODS

Sample collection and DNA extraction

Fresh surgery samples from known breast carcinoma (n=50) were obtained from the general surgery department of Imam Reza Hospital dependent of Tabriz university and private Nour-najat hospital during one year. The tumors were classified according to the three-tier WHO histological grading system (Helpap, 2002; Sobin and Wiltenkind, 2002). The patients’ age ranged from 23 to 74 (average 48), and none of them underwent chemotherapy. Clinical and pathological data were documented and entered into a specific tumor registry grading and other markers. This study was designed as an historical prospective study (all samples are obtained from discarding pathological samples). DNA was extracted from fresh frozen tissues after overnight digestion with proteinase K in TBE buffer and purification with sodium acetate.

Bisulfite specific polymerase chain (PCR) and sequencing

Bisulfite specific PCR (BSP) reaction was performed with DNA and treated with sodium bisulfite as described previously (Clark et al., 1994). Briefly, 1 µl of genomic DNA was denatured by incubation with 0.2 M NaOH for 10 min at 37°C. Aliquots of 10 mM hydroquinone (30 µl:Sigma) and 3 M sodium bisulfate (PH 5.0, 520 µl: Sigma) were added, and the solution was incubated at 56°C for 8 h. Treated DNA was purified by use of DNA purification kit (fermentaz.corp), desulfonated with 0.3 M NaOH, precipitated with ethanol, and resuspended in water. Modified DNA was stored at -70°C. One set of primers was used to amplify the region of interest regardless of the methylation status. Negative control samples with genomic DNA were included for each set of PCRs, while the universal methylated DNA prepared commercially was used as positive control. PCR products were analyzed on 6% poly acrylamide gels. The primer set for bisulfite specific PCR is designed in such a way that all methylated and non methylated samples had PCR product (Figure 1).

Data analysis

A comparison of the proportion was done using χ² test or Fisher’s exact method. To compare the extent of methylation for the normal and tumor group with grade metastasis and kind of tumor, the data were calculated using spearman-rank regression method. The existence of the metastasis-free interval was observed before the second operation which confirmed the first evidence of metastasis or the last evidence of follow-up of patients who remained alive and were metastasis-free. Cox proportional model was applied for the multivariant analysis, where p <0.05 was defined as being statistically significant (Figure 1). CDH1 gene methylation status was shown by BSP in the human breast cancer. Normal (N) and Tumor (T) samples from the same patient were analyzed with one set of BSP primers (M=50 bp marker). When the primer set was used, both methylated and non methylated samples resulted in amplification, but were not bisulfite.

RESULTS

Frequency of methylation in breast cancer

Among 50 breast cancers, the methylation frequency was as follows: a total of 94% was methylated (50% partial methylation and 44% full methylation), while only 6% was non-methylated. In normal samples, there were 76% non-methylated samples and 24% methylated samples (22% was partially methylated and 2% was fully methylated). However, there was meaningful differentiation between tumor and normal samples in methylation rate (p=0.000) (Figure 2). Also, there was significant difference in methylation pattern between tumor and normal tissues (p=0.006). A sample of one chromatogram for comparison of tumor and normal sample methylation pattern differences is shown in (Figure 2).
**Figure 2.** Examples of direct sequencing chromatograms. Bisulfate-modified DNA was amplified and then sequenced. a) Complete methylation in high grade breast cancer sample. b) No methylation in normal breast tissues.

**Figure 3.** Correlation of methylation status in different tumor grades and kind of tumor. Tumors were divided into 3 grade groups (I, II, III) that get higher as number increases. Tumor's kind are divided to ductal, lobular and insitu-ductal.

**Correlation of methylation and risk factors**

Figure 3 illustrates the correlation of methylation frequencies with factors of increased risk including tumor grade and growth pattern (kind of tumor). It could be found that the highest methylation occurred in ductal kind.
Table 1. Comparison of different methylation statuses according to tumor’s grade in E-cadherin promoter region in breast cancer of tumor samples.

<table>
<thead>
<tr>
<th>Methylation_state</th>
<th>Grade</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Non</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Count</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>%</td>
<td>0.00</td>
<td>10.00</td>
</tr>
<tr>
<td>Partial</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Count</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>%</td>
<td>80.00</td>
<td>50.00</td>
</tr>
<tr>
<td>Full</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Count</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>%</td>
<td>20.00</td>
<td>40.00</td>
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<tr>
<td>Total</td>
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<td></td>
</tr>
<tr>
<td>Count</td>
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<td>30</td>
</tr>
<tr>
<td>%</td>
<td>100.00</td>
<td>100.00</td>
</tr>
</tbody>
</table>

Figure 4. Comparison of methylation pattern in different sites of CDH1 gene promoter region in different grade in ductal form.

According to the results in grade I, the highest percent in methylation status is about partial methylation (80%); in grade II, the highest percent is about partial methylation (50%), but in grade III, the highest percentage is about the full methylation state (80%). Absolutely, in the full methylation state, there is direct and meaningful correlation between the grade of tumor and intensity of methylation (p=0.035) (Table 1). Any meaningful correlation were found in tumor’s adjacent normal tissues with methylation status at all (Data not shown).

Correlation between tumor kind and grade with methylation pattern

In correlating the kind of tumor (ductal form) with methylation pattern, there was significant variation between full methylation and growth pattern (p=0.049), with the highest correlation found at 892.nt methylation (Figure 4). As we evaluated a methylation status in two
aspects, one in percent of methylated CpG sites (non, partial and full methylation) and the other in the kind of methylated CpG sites (as pattern of methylation), in some CpG sites were marked with nucleotide number (for example 863.nt, 865.nt,…), there is meaningful correlation between the kind of CpG site and its position in the promoter region and grade of tumor, that is, some CpG sites have greater prevalence in methylation than others. The highest methylation prevalence is seen between 887.nt (as CpG site number in the promoter region) and the high grade tumor (Table 2). There is also a meaningful variation between growth pattern and methylation state (ductal carcinoma type, p=0.046) (Table 2). In evaluating the metastasis with methylation state, there was considerable variation between different statuses of metastatic and non-metastatic samples (p=0.028), but not with the other two risk factors: chemotherapy and recurrence status (p=0.741, 0.812, respectively). Also, 940.nt had the significant variation as the most methylated site in metastatic tumors.

**DISCUSSION**

In the last decade, there is a large body of evidences that imply the importance of DNA methylation in the promoter region, in different human cancers (Chiles et al., 2003). This region has significant effects on gene expression level (Chen et al., 2005) characterized by methylation profiles of individual tumor types (Esteller, 2005). There are relatively few studies about methylation pattern of CDH1gene in breast cancer (Brex and Roy, 2001). E-cadherin’s role in tumor development is now well established (Maruya et al., 2004) in different kinds of human carcinomas such as: prostate, colon, thyroid, gastric and breast, which show reduced E-cadherin expression relative to adjacent normal tissues (Corn et al., 2001). Besides the mutational loss of function, there are evidences for epigenetic basis (like methylation pattern changes in promoter region) which can also reduce or lost CDH1 expression (Droufakou et al., 2001).Whatever is the cause, loss of CDH1 in tumor cells means the capability of tumor cells to spread far away from the original site of malignancy/metastasis. In most carcinomas, this lost is usually a late event that is associated with invasion and metastasis (Bornman et al., 2001), but this may happen in the early steps. CDH1 acts as a tumor suppressor gene, with the loss of expression of wild type CDH1 allele usually occurring through promoter hypermethylation as a second hit (Yang et al., 2001; Shaw, 2006). In this study, most of the tumor kinds are in ductal form (99%). In evaluation of methylation changes, there is a direct relation between methylation intensities (from non-methylated in any CpG sites to fully-methylated in all CpG sites), that is, as the tumor grad increases, the methylation status trend becomes fully methylated (Figure 3). In the ductal type of tumor, the highest full methylation rate is seen as follows: 25, 68.8 and 6.3% for grades III, II and I, respectively. Generally, there is a meaningful variation in the growth pattern (ductal) and grade of tumors in comparison with the methylation state (p=0.043), but it must be noted that methylated CpG sites are completely different in the grades and kinds of tumor or metastatic status of tumors. This implies that they could not be correlated as common markers in all tumor invasiveness risk factors. Although, it is interesting to note that the highest degree of methylation is observed in high tumor grade, methylation is seen even in an *in situ* form which reflects the methylation changes in malignant tissues beginning from the initial steps that may have a contributing effect on the cancer characteristics in cells. There was also a significant variation between CpG sites methylation pattern in

**Table 2. Correlation between methylation pattern in breast cancer tumor samples and tumor’s grade.**

<table>
<thead>
<tr>
<th>Selected methylation pattern(sites) in E-cadherin gene promoter region</th>
<th>Correlation coefficient</th>
<th>Sig. (2-tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>863</td>
<td>0.29</td>
<td>0.054</td>
</tr>
<tr>
<td>865</td>
<td>0.32(*)</td>
<td>0.031</td>
</tr>
<tr>
<td>873</td>
<td>0.25</td>
<td>0.091</td>
</tr>
<tr>
<td>879</td>
<td>0.31(*)</td>
<td>0.035</td>
</tr>
<tr>
<td>887</td>
<td>0.40(**)</td>
<td>0.007</td>
</tr>
<tr>
<td>892</td>
<td>-0.06</td>
<td>0.686</td>
</tr>
<tr>
<td>901</td>
<td>0.19</td>
<td>0.204</td>
</tr>
<tr>
<td>918</td>
<td>0.03</td>
<td>0.844</td>
</tr>
<tr>
<td>920</td>
<td>0.34(*)</td>
<td>0.022</td>
</tr>
<tr>
<td>940</td>
<td>-0.07</td>
<td>0.637</td>
</tr>
</tbody>
</table>
kind of tumor (ductal form), but no significant variation was observed between the kind of CpG sites and the growth pattern. However, the expression of CDH1 gene can be affected by these changes in methylation pattern of the promoter region in different tumor kinds, grades and metastatic statuses; although more clear evidences are yet to be studied. In metastasis evaluation of tumor with methylation state, there is significant variation between the existence of metastasis and methylation rate directly, which may explain the E-cadherin’s role in maintenance of cell-cell interaction in cytoskeleton. It seems that there is no correlation with chemotherapy history or recurrence of breast carcinoma with methylation changes. Nevertheless, a small number of samples for analysis need to be clarified in the next studies.

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REFERENCES


