Association between frequent CpG island methylation and HER2 amplification in human breast cancers

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The presence of frequent methylation of CpG islands (CGIs), designated as the CpG island methylator phenotype in some cancers, is associated with distinct clinicopathological characteristics, including gene amplification, in individual tumor types. Amplification of HER2 in human breast cancers is an important prognostic and therapeutic target, but an association between HER2 amplification and frequent CGI methylation is unknown. To clarify the association, we here quantified methylation levels of promoter CGIs of 11 genes, which are unlikely to confer growth advantage to cells, in 63 human breast cancers. The number of methylated genes in a cancer did not obey a bimodal distribution, and the 63 cancers were classified into those with frequent methylation (n = 16), moderate methylation (n = 26) and no methylation (n = 21). The incidence of HER2 amplification was significantly higher in the cancers with frequent methylation (11 of 16) than in those with no methylation (2 of 21, P = 0.001). Also, the number of methylated genes correlated with the degree of HER2 amplification (r = 0.411, P = 0.002). Correlation analysis with clinicopathological characteristics and methylation of CDKN2A, BRCA1 and CDH1 revealed that frequent methylation had a significant correlation with higher nuclear grades (P = 0.001). These showed that frequent methylation had a strong association with HER2 amplification in breast cancers and suggested that frequent methylation can be a determinant of various characteristics in a fraction of human breast cancers.

Introduction

Aberrant DNA methylation is deeply involved in the development and progression of human cancers (1–4). Methylation of CpG islands (CGIs) in promoter regions is a major mechanism for inactivation of tumor suppressor genes. At the same time, maintenance of appropriate DNA methylation levels is known to be important for maintenance of genome integrity. DNA hypomethylation can lead to genomic instability and increased tumor incidence in mice (5,6) and is associated with loss of heterozygosity in human cancers (7,8). On the other hand, aberrant DNA methylation precedes loss of heterozygosity in human liver cancers (9).

The presence of frequent methylation of CGIs in a cancer was first described in colorectal cancers and designated as the CpG island methylator phenotype (CIMP) (10). Depending upon tumor tissue types, the presence of frequent CGIs methylation, or CIMP, can be clearly observed and is associated with distinct clinicopathological features. For example, by careful selection of marker genes and their quantitative methylation analysis, CIMP in colorectal cancers was shown to be strongly associated with BRAF mutations (11). In neuroblastosomas, both in Japan and Germany, CIMP was observed as a distinct entity associated with poor prognosis and MYCN amplification (12,13). Remarkably, all the cases with MYCN amplification had frequent methylation, with only one exception. Cases with CIMP but without MYCN amplification had a better prognosis than those that had both and a worse prognosis than those that had neither. This complete containment of tumors with MYCN amplification within CIMP-positive tumors suggested that CIMP could precede gene amplification or that at least the presence of frequent aberrant DNA methylation was associated with gene amplification.

Gene amplification of HER2, which is a member of the epidermal growth factor receptor family (14), is very important in human breast cancers. Initially, HER2 amplification was found to be present in 15–30% of newly diagnosed breast cancer cases and to be associated with increased metastatic potential and decreased overall survival (15). Suppression of HER2 activity was shown to have antitumor activity, and antibodies against HER2 were developed as a therapeutic agent against breast cancers. Now, it is well known that a humanized antibody against HER2, such as trastuzumab, is very effective against breast cancers with HER2 amplification (16,17). Nevertheless, inducers of HER2 amplification remain unknown.

In this study, we aimed to clarify whether or not the presence of frequent CGI methylation was associated with HER2 amplification in human breast cancers. For this end, from the genes silenced in human cancers (18,19), we selected genes whose silencing is unlikely to confer growth advantage and avoided selection bias of cells with methylation. Also, we performed quantitative methylation analysis of their putative nucleosome-depleted regions (20), which are most resistant to DNA methylation (21). Association between frequent CGI methylation and clinicopathological characteristics, including silencing of three tumor-suppressor genes (CDKN2A, BRCA1 and CDH1), was also analyzed.

Materials and methods

Patients and tissue samples

Sixty-three breast cancer tissue specimens were obtained from patients who underwent mastectomy or breast-conserving surgery (stage I 22 cases; stage II 26 cases; stage III 15 cases and stage IV 0 case). Informed consent was obtained from all the patients, and analysis was approved by the institutional review boards. Cancer tissues were frozen after resection and stored at –80°C until extraction of genomic DNA. High-molecular weight DNA was extracted by the phenol–chloroform method. Histological types were evaluated according to the criteria of the Japanese Breast Cancer Society (22).

Bisulfite modification and quantitative methylation-specific polymerase chain reaction

Completely methylated DNA and completely unmethylated DNA were prepared by methylating genomic DNA with SssI methylase (New England Biolabs, Beverly, MA) and amplifying genomic DNA with the GenomiPhi amplification system (GE Healthcare, Buckinghamshire, UK), respectively. Bisulfite modification was performed using 1 μg of BamHI-digested genomic DNA as described previously (23). The modified DNA was suspended in 40 μl of Tris–EDTA buffer, and an aliquot of 1 μl was used for polymerase chain reaction (PCR) with a primer set specific to methylated or unmethylated sequences (supplementary Table 1 is available at Carcinogenesis Online). Using the completely methylated DNA and completely unmethylated DNA, an annealing temperature specific for each primer set was determined. Real-time PCR was performed using SYBR® Green I (BioWhittaker Molecular Applications, Rockland, ME) and an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The number of DNA molecules with methylated sequences and

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that with unmethylated sequences in a test sample were measured by comparing its amplification with those of standard samples that contained 10–10⁶ DNA molecules. The standard samples were prepared by cloning PCR products of methylated and unmethylated sequences into the pGEM-T Easy vector (Promega, Madison, WI) or by purifying their PCR products using the Wizard SV Gel and PCR clean-up system (Promega). The 'methylation level' was calculated as the fraction of methylated DNA molecules among the total DNA molecules.

Fluorescence in situ hybridization analysis of the HER2 amplification

Fluorescence in situ hybridization was performed using a PathVysion kit (Abbot Molecular, Des Plaines, IL) with our modification (24). The HER2 locus

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**Fig. 1.** Methylation levels in the 63 breast cancer samples. The numbers of DNA molecules methylated and unmethylated in promoter CGIs were obtained by quantitative methylation-specific PCR, and a methylation level was calculated as a fraction of methylated DNA molecules among the total DNA molecules. Some cancers had no methylation and the others had various levels of methylation. The methylation level in cancers was considered to represent the fraction of cancer cells in a sample and less occasionally the fraction of cells with methylation among cancer cells. We confirmed that we detected dense methylation of promoter CGIs by sequencing the quantitative methylation-specific PCR products obtained using primers specific to methylated DNA molecules (supplementary Figure 3 is available at Carcinogenesis Online).
and centromere of chromosome 17 (CEP17) were labeled by SpectrumOrange and SpectrumGreen fluorescence, respectively, and nuclei were counterstained with 4′, 6-diamidino-2-phenylindole. HER2 and CEP17 signals were counted in 60 nuclei under a fluorescence microscope. Cancers with HER2:CEP17 ratio ≥2 were determined as HER2 amplification positive.

Analysis of 3OST2 expression on cell growth
MCF7 Tet-Off cell line was purchased from Clontech Laboratories (Mountain View, CA). Full-length 3OST2 complementary DNA, cloned from human mammary epithelial cells, was inserted into the multiple cloning site of pTRE2hyg vector (Clontech Laboratories). The MCF7 Tet-Off cell line was transfected with the vector, and a stable clone was obtained by selection using hygromycin. Growth curves were analyzed by counting the cell numbers for the parental cell line, stable clones transfected with 3OST2-expressing vector and with empty vector (without doxycycline). Overexpression of 3OST2 complementary DNA was confirmed by real-time reverse transcription–PCR analysis.

Sequencing analysis of quantitative methylation-specific PCR products
Quantitative methylation-specific PCR products of seven genes, 3OST2, FLnc, GREM1, THBD, PCDH10, XT3 and LOC346978, were cloned into pGEM-T Easy Vector (Promega). For each sample, ~10 clones were cycle sequenced using T7 primer, 5′-TAATACGACTCACTATAGGG-3′ and an Applied Biosystems 310 sequencer (Applied Biosystems, Foster City, CA).

Statistical analysis
Increasing or decreasing trends in no methylation, moderate methylation and then frequent methylation cancers were analyzed by the Mantel–Haenszel chi-square test. Differences between the frequent methylation HER2-positive cancers and moderate methylation (or no methylation) HER2-positive cancers were analyzed by the chi-square test. Correlation between the degree of HER2 amplification and the number of methylated genes was analyzed using Pearson’s correlation coefficient. All the analyses were performed using SPSS (SPSS, Chicago, IL).

Results
Quantitative methylation analysis of breast cancers
From 20 and 14 genes that were methylated in human breast and gastric cancers, respectively (18,19), we selected 11 genes (LOC346978, 3OST2, GREM1, XT3, PCDH10, FLnc, THBD, COE2, CLDN3, F2R and AK5) and quantified their methylation levels in 63 breast cancers. These genes, except for 3OST2 and CLDN3, were not expressed in normal human mammary epithelial cells (18,19,25,26), and their silencing was unlikely to confer growth advantage to cells. Also, introduction of 3OST2 complementary DNA into MCF7 cells did not cause growth suppression (supplementary Figure 1 is available at Carcinogenesis Online), and its silencing was unlikely to confer growth advantage. Therefore, the majority of the 11 genes were considered to be suitable to detect the presence of a cellular environment that tends to induce methylation of promoter CGIs. We also analyzed methylation of three tumor suppressor genes (CDKN2A, BRCA1 and CDH1) for clinicopathological analysis.

Quantitative methylation analyses of the 14 genes showed that some cancers had no methylation and the others had various levels

![Fig. 2](image-url)
of methylation (Figure 1). Such distribution of methylation levels was typically observed for FLNc, THBD, CLDN3, F2R and CDKN2A. The presence of such distribution confirmed previous findings that cancer samples could essentially be classified into two groups: cancers with methylation of a specific gene and those without (11,19,27). Counting cancer cells in the tissue section samples showed that two samples with least cancer cells contained cancer cells with fractions of 19.8 ± 5.2% and 22.9 ± 0.3% (mean ± SD). Based on these data, we adopted two cutoff values 10 and 20% to score each cancer sample as positive or negative. When overall distribution of methylation was examined, similar patterns of cancers with methylation were observed using the two cutoff values (Figure 2). Using either value, the number of methylated genes in a cancer did not obey bimodal distribution and looked quite similar (Figure 3). Therefore, we adopted a cutoff value of 20% to score individual cancers as positive or negative for methylation.

Then, the 63 cancers were classified by the frequency of CGI methylation. To avoid biases due to a cutoff number of methylated genes, we classified the cancers into three groups, those with no methylation, moderate methylation and frequent methylation, using two different cutoff numbers for frequent methylation. Using a cutoff number of three methylated genes or more, 16, 26 and 21 cases were classified into those with frequent methylation, moderate methylation and no methylation, respectively. Using a cutoff number of four methylated genes or more, 8, 34 and 21 cases were classified into those with frequent methylation, moderate methylation and frequent methylation, using two different cutoff numbers. To avoid biases due to a cutoff number of methylated genes, we adopted two cutoff values 10 and 20% to score each cancer sample as positive or negative. When overall distribution of methylation was examined, similar patterns of cancers with methylation were observed using the two cutoff values (Figure 2). Using either value, the number of methylated genes in a cancer did not obey bimodal distribution and looked quite similar (Figure 3). Therefore, we adopted a cutoff value of 20% to score individual cancers as positive or negative for methylation.

**Association between frequent CGI methylation and the HER2 amplification**

The presence of HER2 amplification was analyzed by fluorescence in situ hybridization, and 24 of 63 (38%) cancers had HER2 amplification (supplementary Figure 2 is available at Carcinogenesis Online). The extent of amplification ranged from 2.0- to 16.8-fold. Using a cutoff number of three for frequent methylation, the fractions of cancers with HER2 amplification were 11/16, 11/26 and 2/21 in cancers with frequent methylation, moderate methylation and no methylation, respectively (Figure 4A). Using a cutoff number of four, it was 6/8, 16/34 and 2/21, respectively (Figure 4B).

When correlation between the degree of CGI methylation and fraction of cancers with HER2 amplification was examined by trend analysis, a highly significant increasing trend was observed from cancers with no methylation, to those with moderate methylation and then to those with frequent methylation ($P < 0.001$ for both of cutoff numbers). When cancers with frequent methylation and those with no methylation were compared, the former had a significantly higher fraction ($P = 0.003$ and 0.001 for cutoff numbers of four and three, respectively). Also, the degree of HER2 amplification showed a correlation with the number of methylated genes (correlation coefficient = 0.411, $P = 0.002$) (Figure 5 and supplementary Table 2 is available at Carcinogenesis Online). This demonstrated that frequent CGI methylation had an association with HER2 amplification.
Association between frequent CGI methylation and other clinicopathological features, including methylation of tumor suppressor genes

The correlation between frequent CGI methylation and methylation of three tumor suppressor genes, CDKN2A, CDH1 and BRCA1, was analyzed (Table I). However, none of the three genes showed any correlation ($P = 0.557$, 0.157 and 0.232, respectively). Regarding other clinicopathological characteristics, the degree of frequent CGI methylation correlated with higher nuclear grades ($P = 0.001$). The degree of frequent CGI methylation tended to show correlations with advanced pathological stage ($P = 0.068$) and post-menopausal status ($P = 0.044$). However, no association was observed with lymph node metastasis and negative expression of estrogen receptor (ESR) or progesterone receptor (PGR).

**Discussion**

The present study demonstrated for the first time that frequent CGI methylation in breast cancers had a highly significant association with HER2 amplification. Regarding DNA methylation and HER2 overexpression, Fieg et al. (28) previously found that methylation levels of four genes (CDH13, PGR, HSD17B4 and MYOD1) and one gene (BRCA1), which were selected from 35 genes, correlated with HER2 expression positively and inversely, respectively ($P = 0.01–0.04$). Methylation levels of individual genes in cancers are affected by the content of cancer cells, and, also, the correlation observed in the study was considered to be due to interaction between a function of an individual gene and HER2 overexpression. In contrast, here, we focused on the abnormality in epigenetic regulation in cancers. To estimate its degree, we used marker genes that were unlikely to confer growth advantages even if methylated, scored their methylation as positive or negative and integrated the information from the 11 marker genes into the frequency of methylation in a cancer sample. The cancers were classified into three groups, namely those with frequent methylation, moderate methylation and no methylation. As a result, a very strong association between frequent methylation and HER2 amplification ($P < 0.001$) was demonstrated. Also, the degree of frequent methylation showed a clear correlation with the degree of HER2 amplification. BRCA1 methylation did not correlate with the degree of frequent methylation or HER2 amplification ($P = 0.806$).

The association between frequent methylation and HER2 amplification has clinical implications. It is known that HER2 amplification status can show a discrepancy between primary and metastatic sites in a small fraction of patients (29). There is a possibility that HER2-negative breast cancers at initial diagnosis change into HER2 positive at their recurrence and that the presence of frequent methylation at the initial diagnosis can be used to predict such cases. Since accurate detection of HER2-positive cancers is very important to implement appropriate treatment, including trastuzumab (17), future studies to predict the HER2 amplification status using frequent methylation and to clarify the mechanism of the association are warranted. Also, the effect of frequent methylation on long-term survival is important. So far, only 5 of 63 cases suffered from recurrence (one frequent methylation, three moderate methylation and one no methylation cases), and the effect cannot be statistically analyzed. Since the association between HER2 amplification and poor survival (without trastuzumab) is well established, the effect of frequent methylation on long-term survival seems worth being analyzed in the future.

Some breast cancers with HER2 amplification belonged to the moderate methylation or no methylation groups although the majority of cancers with HER2 amplification belonged to the frequent methylation group. This was in contrast with the case of neuroblastomas, where all the neuroblastomas with MYCN amplification had frequent methylation, CIMP, with only one exception (12,13). Therefore, the relationship between frequent methylation and HER2 amplification in breast cancers seems more complex than the relationship between CIMP and MYCN amplification in neuroblastomas. Not only frequent methylation could lead to HER2 amplification through chromosomal instability (9), which was our initial expectation, but also HER2 amplification could lead to frequent methylation or they might have common inducers.

The degree of frequent methylation also correlated with higher nuclear grades. It also tended to show association with advanced stages and post-menopausal status. It has been reported that CDH1 methylation was associated with negative ESR and PGR expressions ($P = 0.06$ and 0.09, respectively) and that frequent methylation of seven tumor suppressor genes was associated with poor differentiation (30). It has also been reported that PGR expression was negatively associated with ESR1, TGFBR2, PPTG52 and CDH13 methylation ($P = 0.01–0.04$) (31) and that ESR and PGR expressions were positively and negatively associated with HIN-1RASSF1A and RIL/CDH13 methylation, respectively (32). Taken together, the frequent methylation in breast cancers was weakly associated with advanced stages, negative PGR and ESR expressions and poor differentiation (higher nuclear grades). Nevertheless, the correlation between frequent methylation and HER2 amplification was much stronger than these associations in our study. It was considered that quantitative analysis of marker genes was advantageous to clarify the strong association.

![Fig. 5. The correlation between the number of methylated genes and degree of HER2 amplification. The degree of HER2 amplification showed a clear correlation with the number of methylated genes (correlation coefficient = 0.411, $P = 0.002$).](image)

**Table I.** Association between frequent CGI methylation and clinicopathological features, including methylation of tumor suppressor genes

<table>
<thead>
<tr>
<th>No methylation</th>
<th>Moderate methylation</th>
<th>Frequent methylation</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDKN2A methylation (+/−)</td>
<td>2/19</td>
<td>0/26</td>
<td>1/15</td>
</tr>
<tr>
<td>BRCA1 methylation (+/−)</td>
<td>1/20</td>
<td>0/26</td>
<td>0/16</td>
</tr>
<tr>
<td>CDH1 methylation (+/−)</td>
<td>0/21</td>
<td>0/26</td>
<td>1/15</td>
</tr>
<tr>
<td>Menopausal (pre/post)</td>
<td>12/9</td>
<td>9/17</td>
<td>4/12</td>
</tr>
<tr>
<td>Stage (I/II/III)</td>
<td>9/11/1</td>
<td>9/8/9</td>
<td>4/7/5</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>6/15</td>
<td>13/13</td>
<td>7/9</td>
</tr>
<tr>
<td>(positive/negative)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESR (positive/negative)</td>
<td>15/6</td>
<td>14/12</td>
<td>11/5</td>
</tr>
<tr>
<td>PGR (positive/negative)</td>
<td>17/4</td>
<td>14/12</td>
<td>9/7</td>
</tr>
<tr>
<td>Nuclear grade (1/2/3)</td>
<td>4/10/7</td>
<td>2/7/17</td>
<td>0/2/14</td>
</tr>
</tbody>
</table>

Frequent methylation was defined as breast cancers with methylation of three or more genes. Increasing or decreasing trends were tested by Mantel–Haenszel chi-square.
The cutoff value of methylation levels to score cancer samples as positive or negative for methylation was determined based upon the fraction of cancer cells in two samples with their smallest contents (20%). To count methylation in a fraction of cancer cells, we also tested a cutoff value, 10%, but quite similar results were obtained (Figure 3). Regarding the cutoff number for frequent methylation, we tried three and four but observed a highly significant association using both numbers (Figure 4). This excluded a possibility that a false-positive association between frequent methylation and HER2 amplification was observed due to arbitrary cutoff values or numbers. Also, we confirmed that we detected dense methylation of promoter CGIs by our quantitative methylation-specific PCR analysis by sequencing the PCR products. Almost all the CpG sites in the products were densely methylated (Supplementary Figure 3 is available at Carcinogenesis Online). Finally, we confirmed that the methylation detected in cancer tissues originated from cancer cells. Methylation levels of nine genes that showed high methylation levels (>10%) in some cancer samples were measured in 11 pairs of non-cancerous breast and cancer tissues (Supplementary Figure 4 is available at Carcinogenesis Online). The methylation levels of all the genes were elevated only in cancer tissues, and the methylation we detected was considered to originate from cancer cells.

In summary, frequent methylation in breast cancers had a strong association with HER2 amplification.

Supplementary material
Supplementary Tables 1 and 2 and Figures 1–4 can be found at http://cancin.oxfordjournals.org/

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References

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