DNA Methylation Status of Key Cell-Cycle Regulators Such as CDKN2A/p16 and CCNA1 Correlates with Treatment Response to Doxorubicin and 5-Fluorouracil in Locally Advanced Breast Tumors

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Abstract

**Purpose:** To explore alterations in gene promoter methylation as a potential cause of acquired drug resistance to doxorubicin or combined treatment with 5-fluorouracil and mitomycin C in human breast cancers.

**Experimental Design:** Paired tumor samples from locally advanced breast cancer patients treated with doxorubicin and 5-fluorouracil-mitomycin C were used in the genome-wide DNA methylation analysis as discovery cohort. An enlarged cohort from the same two prospective studies as those in the discovery cohort was used as a validation set in pyrosequencing analysis.

**Results:** A total of 469 genes were differentially methylated after treatment with doxorubicin and revealed a significant association with canonical pathways enriched for immune cell response and cell-cycle regulating genes including CDKN2A, CCND2, CCNA1, which were also associated to treatment response. Treatment with FUMI resulted in 343 differentially methylated genes representing canonical pathways such as retinoate biosynthesis, gzi signaling, and LXR/RXR activation. Despite the clearly different genes and pathways involved in the metabolism and therapeutic effect of both drugs, 46 genes were differentially methylated before and after treatment with both doxorubicin and FUMI. DNA methylation profiles in genes such as BRCA1, FOXC1, and IGFBP3, and most notably repetitive elements like ALU and LINE1, were associated with TP53 mutations status.

**Conclusion:** We identified and validated key cell-cycle regulators differentially methylated before and after neoadjuvant chemotherapy such as CDKN2A and CCNA1 and reported that methylation patterns of these genes may be potential predictive markers to anthracycline/mitomycin sensitivity. 

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Introduction

Initially implemented for locally advanced breast cancers, primary (presurgery) systemic therapy is nowadays increasingly used for operable breast carcinomas. Multiple studies have correlated a pathologic complete response to primary chemotherapy with improved prognosis (1). However, although factors like hormone receptor status, HER2 overexpression, and gene expression signatures do correlate with pathologic complete response (2, 3), the predictive power of each parameter is modest, and the cause of drug resistance remains poorly understood (4). TP53 mutation status is another strong prognostic factor, being associated with worse prognosis after treatment with anthracycline as well as mitomycin-containing chemotherapy (5, 6).

Beside genetic abnormalities, epigenetic alterations may contribute to breast carcinogenesis and tumor growth. A large number of genes have been shown to be inactivated in breast cancer through gene silencing by
DNA methylation. Such findings include genes involved in cell-cycle regulation (CDKN2A, CCND2), DNA repair (MGMT, BRCA1, MLH1, GSTP1), regulation of cell transcription (HOXA5), hormone and receptor-mediated cell signaling (ER and THR), apoptosis, and tissue invasion and metastasis (RASSF1A, RAR, TWIST, HIN1, CDH1; ref. 7). Epigenetic silencing of these genes by DNA methylation is frequent and, in contrast with genetic mutation, reversible (8, 9), making them potential candidates for pharmacologic manipulation, for example, through targeted inhibition of DNA methyltransferases. Experimental studies have indicated drug-induced alterations in gene promoter methylation as a potential cause of acquired drug resistance (10).

The aim of this study was to explore alterations in gene promoter methylation as a potential cause of acquired drug resistance to doxorubicin or combined treatment with 5-fluorouracil-mitomycin C (FUMI) in human breast cancers. To do so, we took advantage of our tissue bank providing unique access to paired tumor samples from the same patients before and after primary treatment with doxorubicin or FUMI. We used the Illumina Infinium 27K Human DNA Methylation BeadChip to assess to which degree the genome-wide methylation status is associated with treatment response.

Materials and Methods

Patient cohorts

All tumor samples were collected from two prospective studies described in detail elsewhere (5, 6). Each patient provided written informed consent, and the protocols were approved by the regional ethical committee.

Discovery cohort (genome-wide screening)

Paired tumor samples from locally advanced breast cancer were taken before and after neoadjuvant treatment. The study comprised 19 tumors treated with doxorubicin and 14 tumors treated with FUMI. These patients were admitted to the Haukeland University Hospital in Bergen, Norway between 1991 and 2001 (5, 6). DNA from nine normal breast tissues were collected at the Akershus University Hospital (Lørenskog, Norway) and included as a control of DNA methylation background to which tumor-specific methylation events are compared with.

Validation cohort

An enlarged cohort comprising 85 paired tumors treated with doxorubicin and 39 paired tumors treated with FUMI from the same two prospective studies as those in the discovery cohort was used as a validation set. Samples that were used for 27K arrays were also among those in validation cohort. Clinical and molecular parameters were available for all samples. DNA from fresh-frozen tumor tissues was used in both cohorts. Study design is shown in Fig. 1.

Classification of treatment response

The protocols enrolled patients between 1991 and 2001. Response to therapy was classified by the UICC system commonly applied at that time (11) and not the more recent RECIST system (12). Responses were classified as CR (complete response-complete disappearance of all tumor lesions), PR (partial response-reduction ≥50% in the sum of all tumor lesions), PD (progressive disease-increase in the diameter of any individual tumor lesion by ≥25%), and StbD (stable disease; the condition between PR and PD). Similar to what has been done for previous reports on these materials (5, 6), we compared PD tumors as nonresponders with the combined group of tumors classified as StbD/PR as responders.

TP53 analysis

Mutations in the TP53 gene were analyzed with use of genomic DNA and the temporal temperature gradient gel electrophoresis (TTGE) strategy (5, 6). DNA fragments covering exons 2–11 were amplified, all with a GC clamp on one of the primers, and submitted to analysis by TTGE. Mutations of the TP53 gene were correlated with response to chemotherapy with use of the χ² method, including Yates correction for a limited number of observations. In addition, the differences in the distribution of TP53 mutation among patients revealing a PD and the remaining groups were analyzed with the use of Fisher exact test.

Methylation assays

Tumor and normal DNA were isolated using standard phenol/chloroform procedure. Of note, 500 ng of DNA was bisulphite converted using the EpiTect 96 Bisulfite Kit (Qiagen GmbH). Effective bisulphite conversion was verified by absolute quantification assay using Applied Biosystem7900HT/7900HT Fast Real Time PCR System.
Amplification and pairs of primers specific for either converted or unconverted DNA. Aliquote of 4 μL of bisulphate-converted DNA was used to perform genome-wide DNA methylation profiling of 33 paired tumor samples and nine normal breast tissues using the Illumina HumanMethylation27 BeadChip. All steps were performed according to the Infinium protocol. Methylation data are deposited in Gene Expression Omnibus (GEO) repository under accession number GSE59724 and may be viewed at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE59724. Quantitative DNA methylation analysis of bisulphate-treated DNA was performed in 125 tumors by pyrosequencing (13) on 22 selected genes. Thirteen genes (ABCB1, APC, BRCA1, CCNA1, CCND2, CDH1, CDKN2A, ESR1, FOSL1, GSTP1, IGFBP3, MGMT, and RARB2) were chosen for validation from the genome-wide analysis and seven (FOXC1, FOXC2, HMLH1, IGF2, PPP2R2B, PTEN, and RASSF1A) were selected from previous reports from us and others for differential DNA methylation in breast cancer or breast cancer cell lines. Global DNA methylation levels were assessed analyzing the repetitive LINE1 and ALU sequence families as proxy.

Subsequently, quantile normalization was performed for between sample normalization. After preprocessing, seven paired samples treated with doxorubicin were excluded because of low-quality DNA methylation profiles, resulting in 26 paired tumor samples that were further analyzed. Beta values were used for further analysis.

Genes were termed differentially methylated in the discovery cohort when medians between the two selected groups ([median gpA - median gpB] showed a difference of at least 15% methylation. The methylation index of samples (Z-score) was calculated as follows: (methylation level of each sample – mean value of methylation levels)/SD of methylation levels. Then the sum for the genes was calculated giving one single value (Z-score) for each sample.

In the validation cohort, differences in the distribution of methylation between the two selected groups were assessed by the nonparametric Mann–Whitney test (on parameters with two categories) or the Kruskal–Wallis test analysis on parameters with more than two categories. All obtained P values were corrected with the Bonferroni correction method in which the P values are multiplied by the number of comparisons.

Statistical analysis

Dataset preprocessing. All targets that contained a ‘zero’ value in one sample for methylated and unmethylated signals were removed (n = 10). Second, intrasample normalization, which consists of color bias and background level correction, was performed using the lumi R package.

Ingenuity pathway analysis

Data were also analyzed by Ingenuity Pathway Analysis (IPA; ref. 14). Core analyses were performed to determine which genes from our dataset were present in already defined canonical pathways. The significance of the association between our dataset and the canonical pathways are
assessed by: (i) the ratio of the number of molecules from our gene list that map to a canonical pathway; (ii) Fisher exact test $P$ value, $P < 0.05$; and (iii) FDR, FDR $< 0.05$.

Results

Differentially methylated genes in tumors before and after treatment with doxorubicin and FUMI revealed by array screen

Genome-wide DNA methylation analysis was performed on 12 and 14 paired tumor samples from locally advanced breast cancer taken before and after neoadjuvant treatment with doxorubicin and FUMI.

A total of 486 CpGs representing 469 genes were found differentially methylated ($\frac{\text{median}_{\text{gpA}} - \text{median}_{\text{gpB}}}{\text{median}_{\text{gpA}}} > 15\%$ of methylation) before and after treatment with doxorubicin; out of these, 230 genes had higher methylation level before and 239 genes after treatment. The DNA methylation status was also compared before and after treatment with FUMI identifying 380 CpGs corresponding to 343 genes to be differentially methylated (215 genes had higher methylation level before and 128 genes higher methylation level after treatment). Among these, 469 and 343 genes were those involved in regulation of cell transcription, immunoregulatory and inflammatory processes, cell-cycle regulators, growth factors, and tumor suppressor genes (Supplementary Table S1a and S1b).

Next, IPA was applied to the lists of differentially methylated genes before/after treatment with both drugs separately. Interestingly, the pathway analysis of the 469 genes differentially methylated before and after treatment with doxorubicin revealed a significant association with the following canonical pathways: LXR/RXR activation, communication between innate and adaptive immune cells (Supplementary Fig. S1), and the role of cytokines in mediating communication between immune cells (Supplementary Table S2a). IPA of the 343 differentially methylated genes before and after treatment with FUMI revealed a significant association with canonical pathways such as retinoate biosynthesis, gtri signaling (Supplementary Fig. S2), LXR/RXR activation, and cardiac $\beta$-adrenergic signaling (the four significant canonical pathways are given in Supplementary Table S2b).

Despite the clearly different genes and pathways involved in the metabolism and therapeutic effect of both drugs, 46 genes were differentially methylated before and after treatment with both drugs. The full list of the 46 genes and their functions are given in Supplementary Table S3a and S3b.

DNA methylated genes from array screen and response to treatment with doxorubicin and FUMI

A total of 1,468 CpGs representing 1,310 genes were found differentially methylated ($\frac{\text{median}_{\text{gpA}} - \text{median}_{\text{gpB}}}{\text{median}_{\text{gpA}}} > 15\%$ of methylation) in tumors progressing on therapy (PD) compared with tumors retaining a stable disease or an objective response after treatment with doxorubicin. Out of these, 639 genes had higher methylation levels in responders compared with nonresponders before treatment. Methylation levels of responders were significantly higher than normal controls both before and after treatment with doxorubicin, whereas methylation levels of nonresponders were close to those in normal controls (Fig. 2A). Differentially methylated genes and their functions are listed in Supplementary Table S1c. These 1,310 genes were then used as input for an IPA. The top significant canonical pathways showed a significant association with two canonical pathways (Supplementary Table S2c). Again, communication between innate and adaptive immune cells came on top, as in the case of most differentially methylated genes found when comparing before and after treatment with doxorubicin.

Differentially methylated genes were also observed in responders versus nonresponders for 1,220 genes represented by 1,424 CpGs in patients treated with 5-FU/mitomycin and 546 genes had higher methylation levels in responders compared with nonresponders. Compared with the normal controls, methylation levels of responders were significantly higher before treatment, whereas methylation levels of nonresponders were close to those in normal controls. Interestingly after treatment, in contrast with what was observed for doxorubicin, methylation levels of nonresponders were significantly higher compared with both, responders and normal controls (Fig. 2B). Differentially methylated genes and their functions are listed in Supplementary Table S1d. All differentially methylated 1,220 genes were then used as input for IPA. The top significant canonical pathways are shown in Supplementary Table S2d. Canonical pathway gtri signaling involved in the $G$-protein cascade was one also found among the most differentially methylated genes before and after treatment with FUMI.

When comparing the differentially methylated genes associated to treatment response to both drugs, the methylation status of 333 genes was associated to response to both treatment types. The full list of genes is given in Supplementary Table S4, including $CD33$, $CD40$, $ESR2$, and $WT1$ as well as $CCNA1$, $CCND2$, and $CDKN2A$, which notably were also found differentially methylated after each treatment. Pathway analysis on 323 genes revealed three significant canonical pathways: cAMP-mediated signaling, G-protein-coupled receptor signaling, and Gtri signaling (Supplementary Table S5).

Validated genes differentially methylated before and after treatment

Validation experiment was performed using the highly quantitative pyrosequencing method in 85 paired tumors treated with doxorubicin and 39 paired tumors treated with FUMI from the same two prospective studies as those in the discovery cohort. Of the 22 genes selected for validation, 13 and six genes remained significantly differentially methylated either before or after treatment with doxorubicin and FUMI, respectively (Table 1 and 2). After correction for multiple testing (Bonferroni correction), the observed differences in DNA methylation levels before and after treatment with doxorubicin were still significant for all discovered genes except for $GSTP1$ (marked in Table 1) and for all initially found genes.
before and after treatment with FUMI. Three genes (CCNA1, CCND2, and CDKN2A) were consistently reported with the same results in the doxorubicin discovery and validation cohorts, whereas CDKN2A was found both in the FUMI discovery and validation cohort.

Validated genes associated with response to treatment

Pyrosequencing analysis confirmed 15 and 13 genes differentially methylated either before or after treatment between responders and nonresponders in the doxorubicin and FUMI-treated tumors, respectively (Table 2). After correction for multiple testing (Bonferroni correction), differences in DNA methylation levels between responders and nonresponders were significant for all initially observed genes except for APC, CCND2, and PTEN before treatment and CDKN2A, CCNA1, and PTEN after treatment with FUMI (marked in Table 2). Six genes (ABCB1, BRCA1, CCNA1, CCND2, CDKN2A, and RARB) were discovered in the genome-wide analysis and confirmed by pyrosequencing.

The DNA methylation status of cell-cycle regulatory genes, CCNA1, CCND2, and CDKN2A was associated to treatment response for both doxorubicin and FUMI (Fig. 3). These genes were significantly differentially methylated before/after treatment with doxorubicin and before/after treatment between responders. Lower levels of methylation were observed after treatment in the responder groups. Changes of methylation levels in nonresponders were observed after treatment in the responder groups. The DNA methylation levels were observed before and after treatment with FUMI. The main difference was higher levels of methylation of nonresponders compared with responders of the CCNA1 gene.
Correlation with TP53 mutations and gene expression profiles

We compared the observed DNA methylation profiles with the TP53 mutations status before and after treatment in both patient cohorts. In the patient cohort treated with FUIMI, TP53-mutated tumors had significantly lower DNA methylation levels both before and after treatment in three genes: ABCB1, CCND2, and PPP2R2B (Fig. 4A). In addition, six genes were significantly differentially methylated between TP53 wild-type and mutated tumors before treatment only (Supplementary Table S6). In the doxorubicin-treated cohort, tumors with TP53 mutations had significantly lower DNA methylation levels in CCND2 and RASSF1A before treatment \((P = 0.023 \text{ and } 0.035)\).

We then compared the observed methylation patterns with well-defined tumor subclasses. Interestingly, only CDKN2A, which was found associated both with degree of methylation before and after treatment as well as with treatment response to FUIMI and doxorubicin, was also differentially methylated between different molecular subclasses, both before/after doxorubicin treatment. However, although the methylation levels of CDKN2A in Luminal A and Luminal B did not differ significantly before treatment, the methylation level of this gene was significantly decreased after treatment in the Luminal B subtype. Significant differences in methylation levels were observed between Luminal A and ERBB2 enriched subtype both before/after treatment. In all cases, the methylation levels were lower after treatment but the fold change of methylation levels was strongest in the Luminal B and a fraction of the basal-like subtype. The changes were less among tumors belonging to the Luminal A subtype (Fig. 4B).

Discussion

The use of neoadjuvant chemotherapy for patients with large and locally advanced breast cancer before surgery has become an established practice allowing shrinkage of the primary tumor before surgery. Whether the mechanisms of
primary and acquired resistance are similar is still unknown. The few studies conducted on potential associations between gene expression profiles and chemoresistance have not provided definite answers (15, 16). Genes are expressed to influence whole “pathways”; thus, it is likely that resistance is not related to either individual factors or expression, but rather, should be considered in the context of “functional cascades” (17).

Functional pathways may be inactivated not only by genetic, but also by epigenetic events. These may either be de novo or acquired in response to therapy, mutation effects, or cellular selection. Here, we took advantage of the fact that we have at hand before and after treatment samples from two prospective studies. We found genes differentially methylated between responders and nonresponders working in the same pathway. Notably, these relate to immune response and cell-cycle control. Emerging evidence indicates that many chemotherapeutic agents exert immune stimulatory effects (18, 19). Studies of immunogenic cell death after chemotherapy revealed that doxorubicin is involved in processes which lead to expression of toll-like receptor 4 (TLR4) by dendritic cells (20, 21). It was also shown that TLR4 expressed on tumor cells contributes to tumor progression by promoting tumor cell proliferation, (22, 23), and that silencing of TLR4 could reduce the metastatic potential of metastatic tumor cells (24). In the present study, TLR4 was highly methylated in responders compared with nonresponders, implying that silencing of this gene by promoter methylation may predict better prognosis. Furthermore, studies are warranted to address whether epigenetics changes in other genes acting up or downstream in this pathway may be related to outcome as well.

In the present study, we identified genes differentially methylated before and after neoadjuvant chemotherapy involved in cell-cycle control such as CDKN2A, CCNA1, and CCND2. To our knowledge, this is the first report on the DNA methylation level of these genes and treatment response in locally advanced breast cancer treated with doxorubicin and FUMI. CDKN2A promoter methylation has been detected in breast and many other human cancers (25). We previously showed that hypermethylation of CDKN2A is a late event during breast carcinogenesis, leading to its inactivation in late stage breast cancers (26). Expression of CCND2 is lost in the majority of breast cancers, and a mechanism underlying this loss is the promoter hypermethylation of cyclin D2 (27). In addition, hypermethylation of CCNA1 has been correlated to breast cancer progression (28). Again, further studies are needed to validate these findings. A study on the DNA methylation...
profiles in breast cancer cell lines and their doxorubicin-resistant counterpart showed that methylation levels of some genes had a directional tendency when acquiring doxorubicin resistance (10). Some genes (ABCB1, APC, GSTP1) were becoming hypomethylated and some (BRCA1, CDH1, ESR1) got hypermethylated in breast cancer cells resistant to the drug. In the present study, methylation levels of ABCB1 and APC were lower in nonresponders compared with the responders, whereas the methylation level of CHD1 was higher in nonresponders compared with the responders.

In the present study, we observed lower levels of methylation in TP53-mutated tumors compared with wild-type for some genes both before and after treatment with both drugs. It was previously shown that TP53 mutations, together with DNA methylation, play a role in keeping large families of interspersed (SINEs) and tandem repeats transcriptionally dormant (29). The authors demonstrated that TP53-mutated cells treated with a DNA demethylating agent show transcriptional activation of normally silent SINEs. Activation of repeats resulting from combined lack of TP53 function and DNA methylation was followed by induction of the type I IFN signaling pathway (29). Loss of TP53 function and hypomethylation occur naturally in tumors, and transcription of normally silent and heavily methylated sequences that represent repeats has been shown to occur in tumors (30, 31). In present study, we showed that ALU (SINEs) and LINE1 had significantly lower methylation levels in TP53-mutated tumors compared with wild-type. Identification of these processes in tumors could be used for diagnosis of specific tumor types and stages of progression of the given tumor.

Studies on different expression subtypes in breast cancer have shown that different subtypes have a different underlying biology strongly influenced by TP53 mutation status which is reflected in the methylation profile. Thus, basal-like tumors contain in general a low degree of DNA methylation.

Figure 4. A, boxplots illustrating significant association between methylation status and TP53 mutation status. For each gene, difference in methylation between wild-type and TP53-mutated tumors before and after treatment is given. B, boxplots illustrating the differential methylation of CDKN2A between different molecular subtypes before and after treatment with doxorubicin.
methylation but frequently harbor TP53 mutations (32). Interestingly, Luminal B tumors and some of the basal-like tumors had significantly lower methylation levels after treatment. Patients with luminal B tumors were among the better responders so patients of this subtype might benefit from treatment with doxorubicin.

Conclusion

We identified and validated key cell-cycle regulatory genes differentially methylated before and after neoadjuvant chemotherapy such as CDKN2A and CCNA1. Thus, methylation patterns of these genes may be potential predictive markers to anthracycline/mitomycin sensitivity. Furthermore, we identified genes differentially methylated between TP53-mutated and wild-type tumors among which are LINE1 and ALU elements and suggested that methylation levels of these genes together with TP53 status could be of value in predicting response to chemotherapy treatment. However, further studies are necessary requiring additional patient cohorts treated with doxorubicin and FUMI to confirm predictive value of all genes mentioned above.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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