Expression Analysis of DNA Methyltransferases 1, 3A, and 3B in Sporadic Breast Carcinomas

Igor Girault, Sengül Tozlu, Rosette Lidereau, and Ivan Bieche1

Laboratoire d'Oncogénétique–Institut National de la Santé et de la Recherche Médicale E0017 Centre René Huguenin, F-92211 St-Cloud [I. G., S. T., R. L., I. B.], and Laboratoire de Génétique Moléculaire–Unité Propre de Recherche et d’Enseignement Supérieur EA 3618, Faculté des Sciences Pharmaceutiques et Biologiques, Université René Descartes–Paris V, F-75006 Paris [I. B.], France

ABSTRACT

Purpose: Three genes, namely DNA methyltransferase (DNMT) 1, DNMT3A, and DNMT3B, coding for DNMTs that affect promoter methylation status are thought to play an important role in the development of cancers. Little is known of the biological and clinical significance of these genes in human breast cancer.

Experimental Design: We used real-time reverse transcription-PCR assays to quantify the mRNA expression of the three DNMT genes in a series of 130 breast cancer patients. We also sought relationships between mRNA levels of the DNMTs and those of 20 target genes involved in the DNMT pathway (subgroup of 46 breast tumors).

Results: The DNMT3B gene showed the highest range of expression (81.8 compared with 16.6 and 14 for DNMT1 and DNMT3A, respectively). DNMT3B was overexpressed in 30% of the patients (5.4 and 3.1% for DNMT1 and DNMT3A, respectively). DNMT3B overexpression was significantly related to Scarff, Bloom, and Richardson histopathological grade III (P = 0.002), ERn negativity (P = 0.0015), and strong MKI67 expression (P = 3 × 10-6). In univariate analysis, DNMT3B overexpression was associated with poor relapse-free survival in the subgroup of patients who received adjuvant hormone therapy (with or without chemotherapy; P = 0.0064). Although the poor prognosis associated with DNMT3B overexpression was confirmed by univariate analysis in an independent series of 98 postmenopausal women exclusively treated with adjuvant tamoxifen therapy (P = 0.0036), DNMT3B expression status did not persist as an independent prognostic factor in multivariate analysis.

Conclusions: Although we failed to identify underexpression of specific target genes associated with DNMT increasing expression, the frequent overexpression of DNMT3B in this breast tumor series points to DNMT3B as a potential new therapeutic target in breast cancer.

INTRODUCTION

DNA methylation is an epigenetic modification with an important role in the control of gene expression in mammalian cells. This regulation results from covalent addition of a methyl group to the 5-position of cytosine, predominantly within CpG dinucleotides of gene promoter regions (1). Methylation of CpG-rich DNA regions known as CpG islands is thought to silence gene transcription by recruiting protein complexes involved in nucleosome remodeling (2). Gene up-regulation associated with promoter hypomethylation has also been suggested, but this aspect of transcriptional regulation is far less well documented than the silencing mechanism (1, 3).

The transfer of a methyl group is catalyzed by three functional DNMTs2 with distinctive roles. DNMT1 is mainly involved in maintaining the preexisting methylation pattern during replication, whereas DNMT3A and 3B are mainly involved in de novo methylation (1).

Abnormal DNA methylation is thought to be a major early event in the development of tumors characterized by widespread genome hypomethylation leading to chromosome instability and localized DNA hypermethylation; the latter may be important in tumorogenesis by silencing tumor suppressor genes (4, 5).

Recently, Esteller et al. (6) established the promoter hypermethylation profile in various tumor types. They showed that promoter hypermethylation affected all immortalization and transformation molecular pathways, some changes in promoter gene being shared by all tumor types (CDKN2A/P16), and others being specific for a given tumor type (BRCA1 in breast and ovarian carcinomas; TP73 and CDKN2B/P15 in hematological malignancies). Several studies have shown DNMT overexpression (mainly DNMT1 and DNMT3B) in a variety of cancers (7-14). Induced DNMT1 overexpression in cultured cell lines gradually induces CpG hypermethylation and cell transformation (15). However, no clear relationship between DNMT overexpression and target gene underexpression has been demonstrated in vivo (7, 9, 13, 14, 16).

DNMT expression in breast cancer has only been studied in cell lines (17). To gain more insight into DNMT expression in breast tumors, we quantified the mRNA expression of the three known functional DNMT genes, namely DNMT1, 3A, and 3B, in

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1 To whom requests for reprints should be addressed, at Laboratoire d’Oncogénétique INSERM E0017, Centre René Huguenin, 35 rue Dailly, F-92211 St-cloud, France. Phone: (33) 1 47 11 15 66; Fax: (33) 1 47 11 16 96; E-mail: i.bieche@stcloud-huguenin.org.

2 The abbreviations used are: DNMT, DNA methyltransferase; RT-PCR, reverse transcription-PCR; SBR, Scarff Bloom and Richardson; ER, estrogen receptor; Ct, cycle threshold; RFS, relapse-free survival; TBP, TATA box-binding protein.
a series of 130 patients with unilateral invasive primary breast tumors, by means of real-time quantitative RT-PCR assays (18). We then sought relationships between the expression of DNMT3B, the only markedly dysregulated DNMT gene in our tumor series, and classical clinical, pathological, and biological parameters, including patient outcome. Clinical outcome was also examined according to DNMT3B expression status in an additional well-characterized series of 98 postmenopausal patients exclusively treated with tamoxifen after surgery.

Finally, we sought relationships between the expression of DNMTs and (a) well-known genes subject to promoter hypermethylated, namely ERα, MLH1, RB1, E-cadherin, CDKN2B/P15, CDKN2AP16, ARF/P14, BRCA1, and MASPIN (19–21); (b) eight genes which expression has been shown to be deregulated in an in vitro model of loss of DNMT expression (22); and (c) genes involved in the DNMT downstream pathway, namely MECP2 (23), HDAC1 (24), and MBD2 (25).

MATERIALS AND METHODS

Patients and Samples

We analyzed samples from two series of women with primary unilateral nonmetastatic breast carcinoma.

The first series consisted of 130 women whose breast tumors were excised at Centre René Huguenin from 1977 to 1989. The patients (mean age 58.5 years, range 34–91) had ERα-positive or -negative breast tumors (100 and 30, respectively) and were both pre and postmenopausal (45 and 85 patients, respectively). Seventy-five patients received adjuvant therapy, consisting of chemotherapy alone in 22 cases, hormone therapy alone in 18 cases, and both treatments in 35 cases. The standard prognostic factors have been reported elsewhere (26). The median follow-up was 8.1 years (range 1–15.9 years). Forty-seven patients relapsed. The first relapse events consisted of local and/or regional recurrences in 13 patients, metastases in 30 patients, and both events in four patients.

The second series consisted of 98 postmenopausal women whose breast tumors were excised at Centre René Huguenin from 1980 to 1994. The patients (mean age 70.7 years, range 54–86) all had ERα-positive breast tumors and received postoperative adjuvant hormone therapy consisting of tamoxifen (20 mg daily for 3–5 years) and no other treatment. The standard prognostic factors are reported in Table 1. The median follow-up was 6 years (range 1.5–17.5 years). Thirty-three patients relapsed. The first relapse events consisted of local and/or regional recurrences in 13 patients, metastases in 26 patients, and both events in four patients.

Both series of tumor samples were placed in liquid nitrogen until total RNA extraction immediately after surgery. Complete clinical, histological, and biological information was available; no radiotherapy or chemotherapy was given before surgery, and full follow-up took place at Centre René Huguenin. The histological type of the tumor and the number of positive axillary nodes were established at the time of surgery. The malignancy of infiltrating carcinomas was scored according to SBR’s histoprognostic system (27). ERα status was determined at both the protein level by the dextran-coated charcoal method until 1988 and enzymatic immuno-assay thereafter and at the mRNA level by real-time quantitative RT-PCR assay (26, 28).

Specimens of normal breast tissue, adjacent to the malignant breast tumor from six patients, were used as sources of normal RNA. The normal breast tissue samples were checked histologically and selected on the basis of their cellular composition, i.e., a large proportion of epithelial cells. Samples with a majority of stroma or inflammatory cells were excluded from the study.

Correlations between DNMTs and target genes mRNA levels were investigated in a subgroup of 46 breast tumors from the first series of 130 patients with a similar range of DNMT3B expression levels.

Real-time RT-PCR

(1) Theoretical Basis. Reactions are characterized by the point during cycling when amplification of the PCR product is first detected, rather than the amount of PCR product accumulated after a fixed number of cycles. The larger the starting quantity of the target molecule, the earlier a significant increase in fluorescence is observed. The parameter Ct is defined as the fractional cycle number at which the fluorescence generated by SYBR Green dye-amplicon complex formation passes a fixed threshold above baseline.

The precise amount of total RNA added to each reaction mix (based on absorbance) and its quality (lack of extensive degradation) are both difficult to assess. We therefore also quantified transcripts of the gene coding for the TBP (a component of the DNA-binding protein complex transcription factor IID) as the endogenous RNA control and normalized each sample on the basis of its TBP content (18).

The relative target gene expression level was also normalized to a calibrator, or 1 × sample, consisting of a pool of normal breast tissue specimens.

Table 1

| Characteristics of the 98 postmenopausal patients with ERα-positive breast tumors and relation to RFS |
|----------------------------------------------------|----------------------------------------------------|
| Age                                               | RFS                                                |
| <70 patients                                      | 49                                                | 20 (40.8) |
| >70 patients                                      | 49                                                | 13 (26.5) |
| Histological grade                                |                                                     |           |
| I patients                                        | 13                                                | 2 (15.4)  |
| II patients                                       | 64                                                | 17 (26.6) |
| III patients                                      | 20                                                | 13 (65.0) |
| Lymph node status                                 |                                                     |           |
| 0 patients                                        | 15                                                | 2 (13.3)  |
| 1–3 patients                                      | 56                                                | 15 (26.8) |
| >3 patients                                       | 27                                                | 16 (59.3) |
| Macroscopic tumor size                            |                                                     |           |
| ≤30 mm patients                                   | 67                                                | 18 (26.9) |
| >30 mm patients                                   | 29                                                | 14 (48.3) |

Note: Information available for 96 patients.

<table>
<thead>
<tr>
<th>p</th>
<th>0.00062</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0014</td>
<td>0.010</td>
</tr>
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</table>

" First relapses (local and/or regional recurrences and/or metastases).
^ b P (Log-rank test). NS, not significant.
^ ^ c SBR classification. Information available for 97 patients.
^ ^ d Information available for 96 patients.
For each PCR run, a master mix was prepared on ice with 1× SYBR Green buffer; 5 mM MgCl$_2$; 0.2 mM dATP, dCTP, and dGTP; 0.4 mM dUTP; 0.3 μM each primer; and 1.25 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer Applied Biosystems). Five microliters of each diluted reverse transcriptase sample were added to 20 μl of the PCR master mix. The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min and 50 cycles at 95°C for 15 s and 65°C for 1 min.

### Statistical Analysis

RFS was determined as the interval between diagnosis and detection of the first relapse (local and/or regional recurrence and/or metastasis). Clinical, histological, and biological parameters were compared using the χ$^2$ test. Spearman’s rank correlation test was used to study relationships between continuous variables. Survival distributions were estimated by using the Kaplan-Meier method (30), and the significance of differences between survival rates was determined using the Log-rank test. Multivariate analysis using Cox’s proportional hazards model was used to assess the independent contribution of each variable to RFS (31). Differences between groups were judged significant at confidence levels >95% ($P < 0.05$).

### RESULTS

#### DNMTs mRNA Expression in Normal Breast Samples

To determine the cutoff point for altered DNMT expression in breast cancer tissue, the $N_{DNMT}$ value for each DNMT was determined in six normal breast RNA samples. $N_{DNMT}$ values fell between 0.65 and 1.61 (1.15 ± 0.34) for $DNMT1$, between 0.63 and 1.53 (1.09 ± 0.31) for $DNMT3A$, and between 0.94 and 1.37 (1.20 ± 0.22) for $DNMT3B$. As $N_{DNMT}$ values for overexpression cutoffs (determined as the mean ± 5 SD) were 2.9, 2.6,
and 2.3 for DNMT1, DNMT3A, and DNMT3B, respectively, a common $N_{DNMT}$ value of 3 was chosen to define overexpression of all of the DNMTs in breast tumor RNA samples.

**DNMTs mRNA Expression Analysis in the Series of 130 Breast Tumors**

**DNMTs mRNA Expression Status.** Transcripts of the three DNMTs were detected and quantifiable in all of the breast tumors. Means, medians, and ranges of mRNA values are shown in Table 3. The highest expression range was observed with DNMT3B (81.8-fold between the tumor with the lowest and the tumor with the highest DNMT3B mRNA levels); DNMT1 and DNMT3A had similar, more moderate expression ranges (16.6- and 14-fold, respectively).

We sought links between continuous mRNA values of each DNMT by using the Spearman rank correlation test. mRNA levels of the three DNMTs correlated strongly with each other, as follows: DNMT1 with DNMT3A ($r = +0.421, P = 2.3 \times 10^{-6}$), DNMT1 with DNMT3B ($r = +0.363, P = 4.3 \times 10^{-5}$), and DNMT3A with DNMT3B ($r = +0.521, P < 10^{-7}$).

Using the overexpression cutoff of $N_{DNMT} > 3$, DNMT1 and DNMT3A overexpression was only observed in 7 (5.4%) and 4 (3.1%) tumors, respectively, whereas DNMT3B overexpression was observed in 39 tumors (30%; Table 3). No DNMT mRNA underexpression ($N_{DNMT} < 2$ SD below the mean for normal breast tissue) was observed.

**Table 4** Relationships between DNMT3B expression status and clinical, pathological, and biological parameters in the 130 breast tumor series

<table>
<thead>
<tr>
<th>DNMT3B expression status</th>
<th>Normal</th>
<th>Overexpression</th>
<th>$P^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total population (%)</td>
<td>130 (100.0)</td>
<td>91 (70.0)</td>
<td>39 (30.0)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\leq 50$</td>
<td>39 (30.0)</td>
<td>30 (33.0)</td>
<td>9 (23.1)</td>
</tr>
<tr>
<td>$&gt; 50$</td>
<td>91 (70.0)</td>
<td>61 (67.0)</td>
<td>30 (76.9)</td>
</tr>
<tr>
<td>Menopausal status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Premenopausal</td>
<td>45 (34.6)</td>
<td>33 (36.3)</td>
<td>12 (30.8)</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>85 (65.4)</td>
<td>58 (63.7)</td>
<td>27 (69.2)</td>
</tr>
<tr>
<td>Histological grade$^b$</td>
<td></td>
<td></td>
<td>0.002</td>
</tr>
<tr>
<td>I</td>
<td>17 (14.1)</td>
<td>15 (18.1)</td>
<td>2 (5.3)</td>
</tr>
<tr>
<td>II</td>
<td>58 (47.9)</td>
<td>45 (54.2)</td>
<td>13 (34.2)</td>
</tr>
<tr>
<td>III</td>
<td>46 (38.0)</td>
<td>23 (27.7)</td>
<td>23 (60.5)</td>
</tr>
<tr>
<td>Lymph node status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Node negative</td>
<td>49 (37.7)</td>
<td>38 (41.8)</td>
<td>11 (28.2)</td>
</tr>
<tr>
<td>Node positive</td>
<td>81 (62.3)</td>
<td>53 (58.2)</td>
<td>28 (71.8)</td>
</tr>
<tr>
<td>Macroscopic tumor size$^c$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\leq 30 \text{ mm}$</td>
<td>89 (72.4)</td>
<td>63 (74.1)</td>
<td>26 (68.4)</td>
</tr>
<tr>
<td>$&gt; 30 \text{ mm}$</td>
<td>34 (27.6)</td>
<td>22 (25.9)</td>
<td>12 (31.6)</td>
</tr>
<tr>
<td>ER(x) status$^d$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>30 (23.1)</td>
<td>14 (15.4)</td>
<td>16 (41.0)</td>
</tr>
<tr>
<td>+</td>
<td>100 (76.9)</td>
<td>77 (84.6)</td>
<td>23 (59.0)</td>
</tr>
<tr>
<td>ER(B) status$^d$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>43 (33.1)</td>
<td>32 (35.1)</td>
<td>11 (28.2)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>44 (33.8)</td>
<td>30 (33.0)</td>
<td>14 (35.9)</td>
</tr>
<tr>
<td>High</td>
<td>43 (33.1)</td>
<td>29 (31.9)</td>
<td>14 (35.9)</td>
</tr>
<tr>
<td>MKI67 status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>43 (33.1)</td>
<td>40 (43.9)</td>
<td>3 (7.7)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>44 (33.8)</td>
<td>33 (36.3)</td>
<td>11 (28.2)</td>
</tr>
<tr>
<td>High</td>
<td>43 (33.1)</td>
<td>18 (19.8)</td>
<td>25 (64.1)</td>
</tr>
</tbody>
</table>

$^a$ $P$ chi-square test.
$^b$ SBR classification. Information available for 121 patients.
$^c$ Information available for 123 patients.
$^d$ Bieche et al. (26).

Relationships between DNMT3B Expression Status and Clinical, Pathological, and Biological Parameters. As few tumors overexpressed DNMT1 or DNMT3A, we focused on the DNMT3B gene and sought links between DNMT3B mRNA expression status and clinical, pathological, and biological parameters (Table 4). DNMT3B overexpression was significantly associated with SBR histological grade III ($P = 0.002$), ER\(x\) negativity ($P = 0.0015$), and strong expression of the proliferation marker MKI67 ($P = 3 \times 10^{-6}$). We found no link between DNMT3B expression status and age, menopausal status, lymph node involvement, macroscopic tumor size, or ER\(B\) status.

Prognosis Value of DNMT3B Expression Status. In the series of 130 patients, the 39 patients whose tumors overexpressed DNMT3B tended to have shorter RFS ($P = 0.12$, data not shown).

We then investigated the prognostic value of DNMT3B expression status according to adjuvant treatment. In the subgroup of patients who received chemotherapy-based adjuvant treatment (57 patients, 35.1% of whose tumors overexpressed DNMT3B), patient outcome was not significantly associated with DNMT3B expression status (Log-rank test, $P = 0.18$; Fig. 1A). In contrast, among the 53 women who received hormone therapy, the 17 whose tumors overexpressed DNMT3B had significantly shorter RFS than the 36 patients with normal DNMT3B expression (Log-rank test, $P = 0.0064$; Fig. 1B) [5-year RFS, 58.8% (46.9–70.7) versus 88.6% (83.2–
node status, and macroscopic tumor size (Table 1). The prog-
significant in this series, namely histopathological grade, lymph
bination with the three other prognostic parameters that were
DNMT3B
prognostic significance of
83.63); 10-year RFS, 38.5% (27.36
–
–
–

clusively received tamoxifen as hormone therapy.

ER-positive breast tumors who ex-
menopausal women with ER
studied an independent well-characterized series of 98 post-
status in patients who receive adjuvant hormone therapy, we
RFS in this independent series (Log-rank test,

Fig. 1 RFS analysis according to DNMT3B expression status in a
subgroup of 57 patients (A; from the first series of 130 patients) who
received adjuvant chemotherapy (normal expression in 37, overexpres-
sion in 20), a subgroup of 53 patients (B; also from the first series of 130
patients) who received adjuvant hormone therapy (normal expression in
36, overexpression in 17), and postmenopausal patients from the second
series of 98 patients, who exclusively received tamoxifen adjuvant
hormone therapy (C; normal expression in 78, overexpression in 20).

To confirm the prognostic value of DNMT3B expression status in patients who receive adjuvant hormone therapy, we
studied an independent well-characterized series of 98 post-
menopausal women with ERα-positive breast tumors who ex-
clusively received tamoxifen as hormone therapy.

DNMT3B overexpression was again associated with shorter
RFS in this independent series (Log-rank test, \( P = 0.0036 \); Fig.
1C) [5-year RFS, 55% (43.88–66.12) versus 78.7% (73.77–
83.63); 10-year RFS, 38.5% (27.36–49.64) versus 71% (64.76–
77.24)].

Using a Cox proportional hazards model, we assessed the
prognostic significance of DNMT3B expression status in com-
combination with the three other prognostic parameters that were
significant in this series, namely histopathological grade, lymph
node status, and macroscopic tumor size (Table 1). The prog-
nostic significance of histopathological grade and lymph node
status persisted, whereas that of macroscopic tumor size and
DNMT3B status disappeared (data not shown).

Relationships between mRNA Levels of DNMTs and Genes Involved in DNA Methylation Pathways. We then
sought relationships between mRNA levels of the three DNMTs and genes involved in the DNMT pathway in a subgroup of 46 breast tumors from the series of 130 patients (Table 5).

mRNA levels of the DNMTs and genes with CpG islands showed a single negative relationship, between DNMT3B and
ERα. DNMT1 mRNA levels correlated positively with those of
E-cadherin, whereas DNMT3A mRNA levels correlated posi-
tively with those of MASPIN and RB1.

Among the genes whose expression has been found to be
dysregulated in an in vitro model of DNMT1 expression loss
(22), DNMT3A mRNA levels correlated positively with those of
JUNB and MYCN, whereas DNMT3B mRNA levels correlated positively with those of EREG.

Among the genes involved in the DNMT downstream path-
way, MECP2 mRNA levels correlated positively with those of
both DNMT1 and DNMT3A, whereas HDAC1 only correlated
with DNMT3A.

Finally, MKI67 mRNA levels correlated positively with those of both DNMT3A and 3B but not with those of DNMT1.
MKI67 mRNA levels also correlated positively with EREG
mRNA levels but negatively with ERα.

DISCUSSION

Epigenetic events such as DNA methylation are thought to
be a major early event in tumor development (1). During breast
tumorigenesis, dysregulated expression of the three functional
DNMTs, which catalyze cytosine methylation, may therefore be
of importance in dysregulating gene expression and especially
that of tumor suppressor genes.

Here, we used real-time quantitative RT-PCR assays to
study the mRNA expression of the three functional DNMTs in
a series of 130 primary unilateral nonmetastatic breast carcino-
as. A major advantage of real-time RT-PCR in this setting is
its wide linear dynamic range, which is suitable for analyzing
genomes that show a wide range of expression among tumors.

We found that DNMT1, 3A, and 3B mRNA levels corre-
lated positively with each other, suggesting a common regula-
tion pathway. mRNA levels of DNMT3B showed the highest
expression range (81.8-fold), DNMT3B overexpression status
being observed in 30% of the 130 patients; in contrast, only 5.4
and 3.1% of the patients’ tumors overexpressed DNMT1 and
DNMT3A, respectively. Previous studies have shown that
DNMT overexpression is common in different tumor types
(7–14). Our results, which are consistent with these previous
reports, point to a predominant role of DNMT3B in breast
tumorigenesis, relative to DNMT1 and DNMT3A. We also in-
vestigated the mRNA expression of the recently discovered
DNMT3L gene, which may regulate the DNA methylation pro-
cess (32). DNMT3L mRNA levels were very low, only detect-
able but not quantifiable by means of real-time RT-PCR in a
subgroup of 46 breast tumors from this series (data not shown),
arguing against a role of DNMT3L in breast tumorigenesis.

We sought relationships between DNMT3B expression sta-
DNMT1 expression was associated with up-regulation of MKI67. The negative relationship between DNMT1 and MKI67 has been observed in various studies investigating CpG island methylation status in various cancers have failed to confirm this relationship (7, 13, 14, 16). Studies investigating DNMT1 expression, but most in vivo studies investigating CpG island methylation status in various cancers have failed to confirm this relationship (7, 13, 14, 16). Here, we investigated the influence of DNMT1 mRNA expression status on target gene expression. We first sought relationships between each of the three DNMTs and well-known genes in which CpG islands are associated with the promoters. Few significant relationships were found. The expected relationship between DNMT overexpression and gene silencing, reflected by a negative correlation, was only observed between DNMT3B and ERα. However, this relationship may simply be attributable to: (a) the positive relationship between DNMT3B and MKI67; and (b) the negative relationship between MKI67 and ERα (Table 5). Three unexpected links were also observed, namely DNMT1 with E-cadherin and DNMT3A with MASPIN and RB1.

Recently, Jackson-Grusby et al. (22) showed that loss of DNMT1 expression was associated with up-regulation of EIA, EREG, GRO1, and JUNB but also with down-regulation of COX26, H19, IGF2, and MYCN. No such relationships were observed with DNMT1 in our breast tumor series, but DNMT3A and MYCN expression showed similar trends, in partial agreement with the in vitro model. We observed two positive correlations, namely DNMT3A with JUNB and DNMT3B with EREG. This latter relationship was probably caused by the positive correlation between EREG and MKI67. These results suggest that, although DNMTs may cause gene inactivation through CpG island hypermethylation, increased DNMT expression is not alone sufficient to repress target gene expression (in a dose-dependent manner) and that other factors are therefore involved in gene silencing.

Methyl CpG binding protein or histone deacetylase family genes, along with DNMTs, may also be of importance in tumorigenesis (36). Here, MECFP2 expression was found to correlate positively with both DNMT1 and DNMT3A expression, whereas HDAC1 only correlated positively with DNMT3A. It is noteworthy that these positive links were independent of proliferation status, as MKI67 was not concomitantly up-regulated. These results suggest that DNMTs and genes involved in the regulation of nucleosome remodeling may cooperate in a specific manner.

The shorter RFS associated with DNMT3B overexpression was first observed in a subgroup of patients who had received at least one adjuvant hormone therapy (Log-rank test, \( P = 0.0064 \)). Although the prognostic value of DNMT3B overexpression was confirmed in an independent well-characterized series of postmenopausal patients with ERα-positive breast tu-

<table>
<thead>
<tr>
<th>Genes</th>
<th>GenBank accession no.</th>
<th>DNMT1</th>
<th>DNMT3A</th>
<th>DNMT3B</th>
<th>MKI67</th>
</tr>
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<tbody>
<tr>
<td>BRCA1</td>
<td>NM_007294</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CADH1 (E-cadherin)</td>
<td>NM_004360</td>
<td>0.0049</td>
<td>0.686</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td>ESR1 (ERα)</td>
<td>NM_000125</td>
<td>NS</td>
<td>NS</td>
<td>0.0061</td>
<td>-0.398</td>
</tr>
<tr>
<td>SERPINB5 (MASPIN)</td>
<td>NM_002639</td>
<td>0.017</td>
<td>0.348</td>
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<td>NS</td>
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<tr>
<td>MLH1</td>
<td>NM_000249</td>
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* P, Spearman rank test.
* p, Correlation coefficient, Spearman rank test.
* LocusLink symbol.
* Jackson-Grusby et al. (22).

Table 5. Relationships between DNMT1, 3A, 3B, and target genes mRNA levels in 46 breast tumors.
mors who exclusively received tamoxifen as adjuvant therapy (Log-rank test, \( P = 0.0036 \)), it did not persist in multivariate analysis. This may be explained by the relationship between DNMT3B overexpression and histopathological tumor aggressiveness (SBR grade III; Table 1).

In conclusion, tumor DNMT expression status may have important therapeutic implications. Indeed, re-expression of promoter-methylated genes has been observed with DNMT inhibitor treatment of cell lines in vitro, suggesting that DNMT pathway antagonists could be of therapeutic use (37). We found that DNMT3B was the most frequently overexpressed DNMT gene in primary breast tumors but failed to identify target genes with corresponding underexpression. Further study of expression pattern of other genes involved in the DNMTs pathway and the relationship with gene silencing during breast tumorigenesis is necessary.

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REFERENCES


Expression Analysis of DNA Methyltransferases 1, 3A, and 3B in Sporadic Breast Carcinomas

Igor Girault, Sengül Tozlu, Rosette Lidereau, et al.


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