

# DNA methylation and chromosome instability in breast cancer cell lines

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**Abstract** We show that in a series of eight breast cancer cell lines, a direct relationship exists between the overall DNA demethylation and the percentage of rearranged chromosomes, except for cell lines with a highly rearranged genome which can be weakly demethylated. A real time fluorescent detection method was used to quantify by reverse transcription-PCR the expression of the DNA methyltransferase 1 and of the newly discovered DNA demethylase. The overall DNA methylation status seems to result from a complex interplay between the expression of these two genes. Our results suggest that in these tumor cells, the overall DNA demethylation is implicated in one of the mechanisms at the origin of the genome instability and that besides the role of the DNA methyltransferase 1, that of the DNA demethylase may be essential in the control of DNA methylation.

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**Key words:** DNA methylation; Genome instability; DNA methyltransferase 1; DNA demethylase; Breast cancer

## 1. Introduction

Mammalian DNA methylation has been proposed to be an important factor in maintaining genome stability. This notion is based on the observation that genomic aberrations are recurrently associated with global DNA demethylation. For example, in cells treated with the demethylating agent 5-azacytidine [1], in lymphoblastoid cell lines [2] and in cells from patients with the disease ICF (immunodeficiency, centromeric instability and facial abnormalities) [3], a direct relationship was found between DNA demethylation and elongations, breakages and rearrangements involving the juxtacentromeric heterochromatic region of chromosomes 1 and 16. In addition for several types of cancer, DNA demethylation has been implied as being an important factor in the formation of rearranged chromosomes involving the same regions, but up until now, no direct demonstration of this has been provided [4,5]. A role for DNA methylation in the stability of other chromosome regions has also been established. In human tumor cell lines from the colon, a relationship was found between a defect in the capacity of DNA methylation, the proficiency of the mismatch repair mechanisms and the presence of chromosome rearrangements [6]. Finally, in the highly demethylated genome of murine embryonic stem cells nullizygous for the DNA methyltransferase 1 (*Dnmt1*), the major cause of the high rate of deletions observed in an integrated viral thymidine kinase transgene was either mitotic recombination or chromosome loss [7]. These data all suggest that DNA methylation plays an important role in maintaining

genome stability, but the mechanisms which drive the formation of the rearranged chromosomes remain to be established.

The mechanisms regulating DNA methylation in mammalian cells are poorly understood, although several of the participants have now been identified. As demonstrated by gene knock-out experiments in mice, *Dnmt1* is a major determinant of DNA methylation [8]. Overexpression of the human DNA methyltransferase 1 (*DNMT1*) was previously observed in tumors and in tumor cell lines (reviewed in [9]). Although this increased expression could mainly reflect enhanced cell proliferation, when normalized with the expression of genes that measure cell proliferation, little differences in the amounts of *DNMT1* mRNA were observed [10,11]. A second potential DNA methyltransferase has been isolated (*DNMT2*), but its ability to methylate DNA has not yet been shown [12]. More recently, another group of DNA methyltransferases (*DNMT3A* and *3B*) was isolated [13]. *DNMT3A* was considered as significantly overexpressed in a series of tumors [14] although no difference of expression was found between colon tumors and normal tissues in another study [10]. In this latter study, no relationship was found between the expression of *DNMT1*, *DNMT3A* or *DNMT3B* and the methylation status of CpG islands. Adding to the complexity of the system, some DNA methyltransferases display several forms, possibly expressed in a tissue-specific manner, differing either in their translation start site or their splicing sites [14–16]. In contrast to the numerous DNA methyltransferases cloned, a DNA demethylase was only recently cloned [17]. However, the implication of this DNA demethylase in the establishment and maintenance of DNA methylation in normal and tumor cells is not yet known.

In this study, we investigate the relationship between overall DNA methylation, the presence of rearranged chromosomes and the expression of *DNMT1* and DNA demethylase in breast cancer cell lines.

## 2. Material and methods

### 2.1. Biological material

Seven breast cancer cell lines (MDA-MD-134 (passage 33), MDA-MB-231 (passage 24), MDA-MB-361 (passage 47), SK-BR-3 (passage 24), BT-20 (passage 286), ZR-75-1 (passage 83), T-47-D (passage 76)) were obtained from the American Type Culture Collection. H-466B has previously been described [18]. Cells were grown for a few more passages in DMEM medium supplemented with 10% fetal bovine serum and collected during the growth phase before confluence. Five breast tissue samples were obtained from plastic mastectomy with informed consent of the donors.

### 2.2. DNA methylation measurement

The methylation status of total genomic DNA was established as previously described [19]. DNA was digested by *MspI* or *HpaII*, separated on agarose gel and blotted. Hybridization was performed with <sup>32</sup>P-labelled total DNA from human placenta. For each lane, the ratio *r* between the radioactivity present between the molecular weights of 1.8 and 2.9 kb and the total of the smear was calculated. The ratio *R*,

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between the  $r$  obtained for *MspI* and *HpaII* digestions was calculated for each DNA sample. The total DNA methylation ( $1-R$ ) theoretically ranges from one (fully methylated DNA) to zero (fully demethylated DNA).

### 2.3. Cytogenetic analysis

The percentage of rearranged chromosomes, including all structural abnormalities, was determined on R-banded chromosomes<sup>1</sup> as described [20].

### 2.4. Expression analysis

A real time fluorescent detection method [21] was used to quantify the mRNA expression of *DNMT1* and of the DNA demethylase by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was prepared with the Trizol reagent kit (Life Technologies). Residual DNA contamination was removed using the High Pure RNA isolation kit (Roche). Reverse transcription was performed using the Superscript II enzyme (Life Technologies) with 2 µg of total RNA, 200 ng of oligo-d(T) and 0.5 mM of each dNTP. Primers for PCR were chosen with the assistance of the computer program Primer Express (Perkin-Elmer Applied Biosystems). Primers were purchased from Oligo Express (France). The following primers were used: (1) DNA methyltransferase 1 [22], 5'-TGGAGAGAAGCTCC-CTCTGTTTC-3', 5'-CCGAGCTCAACCTGGTTATGTT-3' which yielded a 119 bp fragment (2) DNA demethylase [17], 5'-GAATGAACAGCCACGTCAGCTT-3', 5'-GCTACCTGGACCAACTCCTTGA-3' which generated a 127 bp fragment, (3) histone H4, partially degenerated primers were used taking into account the published sequences for the different forms (GenBank released 104), 5'-ATYTAYGAGGAGACYCGCG-3', 5'-CCATGGCKGTGACYGTCTT-3' which gave a 107 bp fragment.

The target cDNAs were amplified separately by PCR using a GeneAmp 5700 sequence detection system and a SYBR Green PCR kits (Perkin-Elmer Applied Biosystems). The detection method was based on the property of the SYBR Green dye which fluoresces when bound to double-stranded DNA. At each cycle, the amount of amplified product was measured by monitoring the green light emitted. The PCR reactions were performed in MicroAmp Optical tubes (Perkin-Elmer Applied Biosystems) positioned in a 96 well support. The reaction mixture (25 µl) contained the reverse transcription product, 250 nM of each primer, 200 µM of each dATP, dCTP and dGTP, 400 µM dUTP, 4 mM MgCl<sub>2</sub>, 5 U of AmpliTaq Gold DNA polymerase, 1 U of AmpErase uracil *N*-glycosylase and 1×SYBR Green PCR buffer containing the SYBR Green dye. Thermal cycling consisted of one cycle at 50°C for 2 min and at 95°C for 10 min followed by 40 cycles at 60°C for 1 min and 95°C for 15 s. Each assay included a standard curve, a no-template control and the tested samples, all in duplicate. All the primer pairs used gave an efficiency of amplification higher than 95% (100% corresponding to doubling of the amplified product at each cycle). Two reverse transcriptions followed by at least two PCR amplifications were performed for each sample. Control of the products of amplification by gel electrophoresis and by determining the melting curves demonstrated that the expected products were specifically amplified. For each sample (corresponding to 10 ng of total RNA), the cycle number at which the fluorescent signal crossed a threshold in the exponential phase of the PCR reaction was measured and compared to the standard curve. The standard curve was constructed with serial dilutions of reverse transcription products corresponding to 0.1, 1, 10 and 100 ng of total RNA from a reference cell line (MDA-MB-134). The expression of the other cell lines was compared to the standard curve and reported in equivalent quantity of total RNA from the reference cell line. The normalization of RNA amounts was performed using histone H4 expression analyzed with the same procedure. The expression ratios DNA demethylase 1/histone H4 and *DNMT1*/histone H4 were calculated. This method did not give the absolute quantity of mRNA nor did it allow for a quantitative gene to gene comparison of the expression. In contrast, the method did allow for each gene a comparison between various cell lines.

## 3. Results

The karyotypes of the eight breast cancer cell lines were established. Various types of chromosome rearrangement were observed (deletions, fusions, homogeneous staining regions). No recurrent site of instability was found except for the juxtacentromeric region of chromosome 1, which was implicated in a chromosome rearrangement in five of the eight cell lines (not shown). For all cell lines, the total DNA methylation was determined and reported as a function of the percentage of rearranged chromosomes (Fig. 1). For six cell lines, (MDA-MB-134, ZR-75-1, H-466B, T-47-D, MDA-MB-231 and SK-BR-3), a linear decrease of the total DNA methylation was observed as a function of the percentage of rearranged chromosomes. In this group, lower levels of methylation were correlated with a higher percentage of rearranged chromosomes. Two of the cell lines analyzed did not follow this relationship. For MDA-MB-361 and BT-20, a highly rearranged genome was associated with a weakly demethylated DNA. The level of methylation for some of the cell lines (MDA-MB-134, MDA-MB-361 and BT-20) was not significantly different from that found in normal breast tissues,

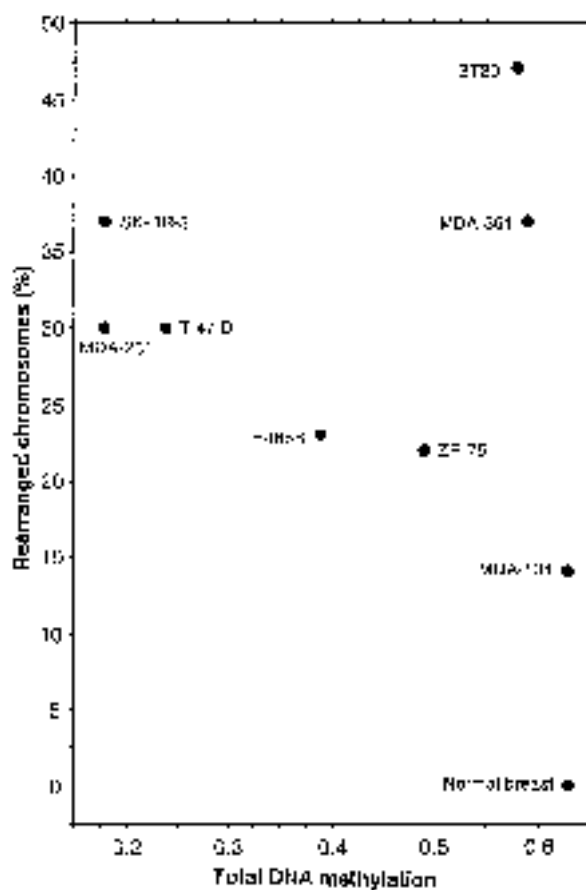


Fig. 1. Relationships between the total DNA methylation status and the percentage of rearranged chromosomes in eight breast cancer cell lines. Values of total DNA methylation for cell lines are the mean values of at least three independent measurements. For normal breast tissues (normal breast), the mean value established with five unrelated samples analyzed at least twice is presented. Total DNA methylation values theoretically range from one (fully methylated DNA) to zero (fully demethylated DNA), experimental variations were  $\pm 0.03$ .

<sup>1</sup> Karyotypes are available on request.

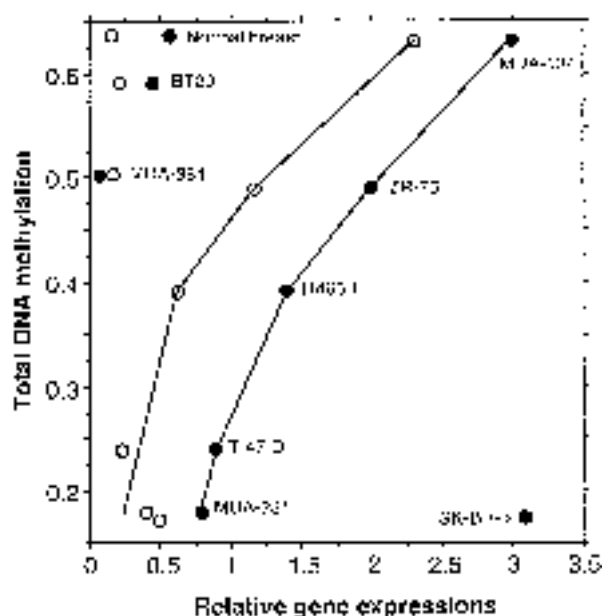


Fig. 2. *DNMT1* (○) and DNA demethylase (●) expression in eight breast cancer cell lines. RNA expression was measured by quantitative real time RT-PCR. Expressions were normalized using histone H4. The ratios between expression of *DNMT1* or DNA demethylase and histone H4 were plotted as a function of the total DNA methylation (see Fig. 1). The name of the cell line refers to the expression of the two genes plotted at the same horizontal level. The values for normal breast are the mean established for five unrelated samples. Each point was the mean of at least four analyses (two reverse transcriptions followed by two PCRs). Ratio variations were in a  $\pm 0.3$  range.

whereas the most demethylated cases observed in this study reached the highest values of demethylation reported in certain breast tumors or in transformed lymphoblastoid cells [2,19]. No relation was found between DNA methylation and the number of chromosomes (not shown).

The quantitative evaluation of *DNMT1* expression has led to conflicting results [9]. However, experiments employing RNase protection and quantitative RT-PCR [10,11] have demonstrated that relevant results can be obtained when the expression is normalized with a cell proliferation-associated gene. The cell proliferation dependence of the DNA demethylase is not known, but assuming that its behavior could be similar to that of *DNMT1*, we retained histone H4 as the normalization gene. Therefore, we analyzed the expression of *DNMT1* and DNA demethylase by real time RT-PCR. The levels of expression found, normalized to that of histone H4, are reported as a function of the total DNA methylation (Fig. 2). Two highly rearranged cell lines (MDA-MB-361 and BT-20) displayed at the same time low DNA demethylation and a low expression of the two genes. These low levels of expression were also found in normal breast tissue. For five of the six cell lines for which the DNA methylation was correlated with percentage of rearranged chromosomes (MDA-MB-231, T-47-D, H-466B, ZR-75-1, MDA-MB-134), the increase in the expression of both *DNMT1* and DNA demethylase was associated with an increase in the total DNA methylation. For example, the weakly demethylated cell line MDA-MB-134 had a level of expression of the two genes 5–10 times higher than normal breast tissue. The sixth cell line (SK-BR-3) was strongly demethylated, had a low expression

of *DNMT1* and a level of expression of the DNA demethylase as high as MDA-MB-134. No relation was found between the expression of *DNMT1* (localized at 19p13.3p13.2 [22]) or of the DNA demethylase (18q21.1 [17]) and the gain or loss of the corresponding chromosome arm (not shown).

#### 4. Discussion

In the breast cancer cell lines studied here, the levels of DNA methylation were very widespread. The actual methylation status of these cell lines may be the addition of events that occurred during the development of the original tumor and in culture. Despite this complex situation, distinct behaviors could be observed from cell line to cell line.

Two cell lines with highly rearranged genomes (MDA-MB-361 and BT-20) displayed at the same time a low DNA demethylation and a low expression of *DNMT1* and of the DNA demethylase. Similar low levels of expression were found in the normal breast tissue. The concomitant low level of expression of the two genes appears sufficient to maintain a normal or near normal level of global methylation of the genome. The similarity in expression between the normal tissue and the highly rearranged cell lines suggests that in these later cases, the DNA methylation mechanisms were not perturbed and that DNA methylation was not involved in their karyotype evolution. In MDA-MB-134, ZR-75-1, H-466B, T-47-D, MDA-MB-231 and SK-BR-3 cells, the linear relationship between the changes in overall DNA demethylation and the percentage of rearranged chromosome suggests a contribution of DNA hypomethylation in the formation of chromosome aberrations. The widespread repartition of the sites of breakage indicates a global instability of the genome which is at the origin of the rearranged chromosomes selected during cell transformation. The recurring involvement of the juxta-centromeric region of chromosome 1 in rearrangements has already been established in breast cancer cells. This instability could be related to the demethylation of the classical satellite 2 localized in this region [2–5]. The levels of expression of *DNMT1* and of the DNA demethylase were low in three of these cell lines (SK-BR-3, MDA-MB-231 and T-47-D). A high up-regulation of the DNA demethylase, without concomitant *DNMT1* overexpression, was associated with a low methylation of the DNA (SK-BR-3), indicating that the demethylase activity may control the global methylation level. Moreover, even a weak overexpression of the DNA demethylase seems sufficient to exceed the methylation capacity of the cells (MDA-MB-231, T-47-D). In the case of *DNMT1* overexpression (H-466B, ZR-75-1, MDA-MB-134), both DNA demethylase expression and DNA methylation followed the level of *DNMT1* expression. Thus in these cases, the increase in the demethylation activity that could be linked to the up-regulation of the DNA demethylase was exceeded by the overexpression of the DNA methyltransferase. In this group of breast cancer cell lines, total DNA methylation seems to result from an interplay between the expression of *DNMT1* and DNA demethylase. However, the level of mRNA expression of either of these genes could be by itself unequivocally associated with the methylation status of the genome. Other factors have to be considered, such as factors involved in the post-transcriptional regulation of the genes and other proteins engaged in the methylation/demethylation process or in the modulation of the accessibility of DNA.

We conclude that overall DNA demethylation is implicated in one of the mechanisms at the origin of genome instability in breast cancer cells and that besides the role of DNA methyltransferase 1, that of the newly discovered DNA demethylase may be essential in the control of DNA methylation in these tumor cells.

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