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# The relationship between DNA methylation and expression of three different DNA methyltransferases in ovarian cancer

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***Background.*** *DNA methylation in mammals is required for embryonic development, X chromosome inactivation and imprinting. Previous studies have shown that methylation patterns become abnormal in malignant cells and may contribute to tumorigenesis. The aim of the study was to ascertain the relationship between overall DNA methylation and the expression of DNMT1, DNMT3A and DNMT3B in ovarian cancer samples.*

***Materials and methods.*** *DNA digestion with either methylation sensitive HpaII, or methylation insensitive MspI restriction endonuclease and quantitative reverse transcription-PCR methods were used to analyse global methylation levels and expression levels of five ovarian cancer and three normal ovarian tissue samples.*

***Results.*** *All five analysed cancer samples were hypomethylated. The differences of methylation levels between normal ovarian tissue and carcinoma samples were statistically significant ($P<0.05$). All five cancer samples showed overexpression of DNMT3A and DNMT3B, and only two ovarian tumour samples showed overexpression of DNMT1. There was no correlation between global demethylation and expression levels for the three different DNMTs.*

***Conclusion.*** *Genome wide hypomethylation facilitates tumour development with predisposition of cells to structural and numerical chromosomal aberrations but the paradox of the global hypomethylation observed in cancer cells and the high levels of DNMTs that are present in these cells still remain to be resolved.*

*Key words: ovarian neoplasms, DNA methylation; methyltransferases*

## Introduction

Investigations into the genetic aetiology of cancer have markedly advanced our understanding of the disease. A growing body of evidence supports the hypothesis that epigenetic events have a prominent role. Mammalian cells possess the capacity to modify epigenetically their genome via DNA methylation. Methylation occurs at the 5 position of the cytosine ring within the context of the

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CpG dinucleotide. CpG islands are short sequences rich in the CpG dinucleotides.[1]

Mammalian DNA methylation has been proposed as an important factor in maintaining genome stability.[2] Human cancer cells typically contain DNA with abnormal CpG dinucleotide methylation patterns. Most often, the cancer cell DNA induces increases in CpG dinucleotide methylation at specific CpG island sequences, accompanied by decreases in CpG dinucleotide methylation at most other sites.[3] There is a growing body of evidence that abnormal methylation of CpG islands in the promoters of tumour suppressor genes can contribute to cancer formation and progression by providing an alternative means to mutational inactivation.

DNA methylation results from a methyl transfer reaction performed by a *trans*-acting enzyme known as DNA methyltransferase (DNMT). Two distinct methyl transfer activities can be distinguished, based on the methylation status of the substrate.[4] The activity which uses hemi-methylated CpG dinucleotides as a substrate is referred to as maintenance methylation activity, whereas *de novo* DNA methylation activity refers to new addition of methyl groups at the sites that were previously unmethylated. Until recently, only one DNA methyltransferase (DNMT1) had been cloned from human cells. It is characteristic of DNMT1 that its relative *de novo* activity is 1-2 orders of magnitude lower than its maintenance activity.[4] Recently, two additional mammalian DNMT genes have been identified, that are referred to as DNMT3A and DNMT3B. These genes differ from DNMT1 in that the encoded polypeptides DNMT3α and DNMT3β have approximately equal ratios of *de novo* DNA methyltransferase activity: maintenance DNA methyltransferase activity.[5]

Ovarian carcinomas are a heterogeneous group of tumours of various cell types. Ovarian epithelial tumours are subdivided into benign (cystadenoma) and malignant (carcinomas) categories. These tumours also include a third category, called tumours of low malignant potential (LMP), which are intermediate between cystadenomas and carcinomas and, like benign tumours, are stable over time. Substantial progress has been made in our understanding of the molecular biology and genetics of ovarian epithelial tumours. Cheng *et al.* report that alterations in DNA methylation are early, but not initial events in ovarian tumourigenesis.[6] Certain global hypomethylation levels are associated with both tumours of low malignant potential and carcinomas, but not with cistadenomas.[7]

One of the proposed causes of changes in the methylation machinery in transformed cells is overexpression of one or more of the three known catalitically active DNMTs. In this study, the relationship between overall DNA methylation and the expression of DNMT1, DNMT3A and DNMT3B in ovarian cancer samples was investigated.

## Material and methods

Five ovarian cancer samples and three normal ovarian tissue samples taken from total hysterectomies performed for prolaps or fibromyoma were analysed. All tissue samples were collected for therapeutic or diagnostic purposes according to ethical rules. Approximately 2g of the surgically removed tissue was frozen immediately in liquid nitrogen and stored at -80°C until DNA and RNA isolation. Total RNA was isolated by the Trzole reagent kit (Life Technologies) and residual DNA contamination was removed using a High Pure RNA isolation kit (Roche). Genomic DNA was isolated by the standard method of protinase K digestion and phenol-chloroform extraction.

The methylation status of total genomic DNA was established as previously described.[8] DNA was digested with either methylation-sensitive *HpaII*, or methylation insensi-

tive *MspI* restriction endonuclease. The digested DNA samples were separated on 1% agerose gel and blotted. Hybridization was performed with $^{32}$P-labelled total DNA from human placenta. For each lane, the ratio r between the radioactivity present between the molecular weights of 1.8 kilobase (kb) and 2.9 kb and the totality of the smear was calculated. The value of R for *MspI* digestion was expected to be identical for all the DNA. It was 0.17 ± 0.01 for all eight samples. The ratio between R obtained for *MspI* and *HpaII* digestion was calculated for each DNA. This value (RD=relative demethylation) multiplied by 100, theoretically ranges from 0 (the most methylated) to 100 (the least methylated).

A real time fluorescent detection method was used to quantify the mRNA expression of DNMT1, DNMT3A and DNMT3B by RT-PCR.[9] Reverse transcription was performed using the Superscript II enzyme (Life Technologies) with 2µg 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 (Parker-Elmer Applied Biosystem). The following primers were used: 1) DNMT1: 5'-TGGAGAGAA GCTCCCTCTGTTC-3' and 5'-CCGAGCTCAACCTGGTTATGTT-3'which yield a 119 bp fragment; 2) DNMT3A: CAAT-GACCTCTCCATCGTTCAAC-3' and 5'AGC-CGGCCAGTGCCCTCGTAG-3'; DNMT-3B: 5'-CCATGAAGGTTGGCG ACAA-3' and 5'-TGGCATCAATCATCACTGGATT-3' 4) histone H4, partially degenerated primers were used taking into account the published sequences for the different forms (Genebank released 104), 5'-ATYTAYGAGGAGACY-CGCG-3', 5'-CCATGG CKGTGACYGTCTT-3' which gave a 107 bp fragment.

The specific cDNA of interest and reference cDNA (histone H4) were PCR-amplified separately by PCR using a GeneAmp 5700 sequence detection system and a SYBR Green PCR kits (Parker 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. PCR amplification was performed in MicroAmp Optical tubes (Parker Elmer Applied Biosystems) positioned in a 96 well support. The reaction mixture (25 µl) contained the reverse transcription product, 250 nM each primer, 200 µM each dATP, dCTP, and dGTP, 400 µM d UTP, 4mM MgCl2, 5 units of AmpliTaq Gold DNA polymerase, 1 unit of AmpErase uracil N-glycosylase and 1 x SYBR Green PCR buffer containing the SYBR Green dye. Thermal cycling consisted of 1 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 at 95°C for 15 s.

Each assay included a standard curve and nontemplate control and the tested samples, all in duplicate. All the primer pairs used gave an efficiency of amplification higher than 95%. Two reverse transcriptions followed by at least two PCR amplifications were performed for each sample. For each sample (corresponding to 10 ng of total RNA), the cycle number at which the fluorescent signal crossed the 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 tissue was compared to the standard curve and reported in equivalent quantity of total RNA from the reference cell line. Normalisation of RNA amounts was performed using histone H4 expression analysed with the same procedure. The expression ratios DNMT1/H4, DNMT3A/H4 and DNMT3B/H4 were calculated. This method did not give the absolute quantity of mRNA, nor did it allow a quantitative gene to gene comparison of the expression.

## Results

The relative demethylation value (RD) was measured for control DNAs from three normal ovarian tissues. All three samples had similar RDs ranging from 37 to 39 with a mean RD at 37 ± 1. The RD values were much more variable for the five ovarian tumour tissues, ranging from 41 to 57, with a mean RD at 47 ± 6. Thus by comparison with controls, tumour sample DNAs were hypomethylated and there was no overlapping between the RDs for normal and tumour ovarian samples. The differences of global methylation levels between normal and tumour samples were statistically significant ($P<0.05$).

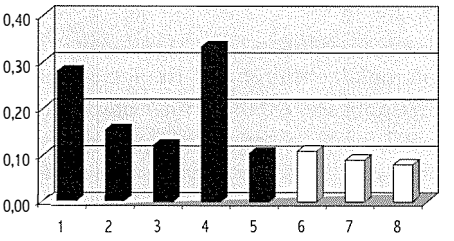


**Figure 1.** The relative expression levels of DNMT1 in five ovarian tumours (black) and normal ovarian samples (white). The expression levels were normalised with expression levels for the proliferation-associated gene H4.

Figure 1 shows the relative expression levels of DNMT1 in five individual ovarian tumours and three normal ovarian samples. We found overexpression in just two ovarian tumours when the proliferation-associated gene H4 was used for normalisation.

Figure 2 shows the relative expression levels of DNMT3A and DNMT3B in the same samples as described above when the proliferation associated gene H4 was used for normalisation. The expression levels of DNMT3A and DNMT3B for each tumour versus the mean expression level for each gene in the normal ovarian samples were calculated. We found overexpression of DNMT3A and DNMT3B in all five ovarian tumours. The

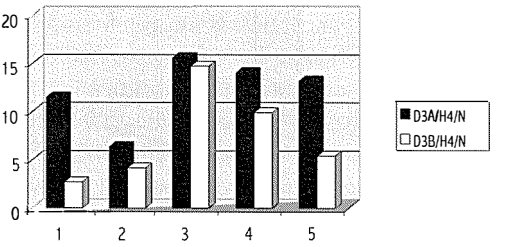


**Figure 2.** The relative expression levels of DNMT3A and DNMT3B normalised with expression levels for H4. The expression levels for each ovarian tumour were such that the mean expression level of three normal ovarian samples equals a value 1.

overexpression means that the expression for DNMT in tumour tissue is at least 2-fold higher than the mean expression level in 3 normal ovarian samples.

No relations were found between global DNA methylation and the expression of DNMT1, DNMT3A and DNMT3B.

## Discussion

The phenotypic characteristics of every living cell are determined primarily by the nucleotide sequence of their respective genome. However, several epigenetic mechanisms may modulate genomic activity and further contribute to phenotypic variation. DNA methylation is the only known covalent epigenetic modification of mammalian DNA.[10] Patterns of methylation are heritable, undergo characteristic changes during embryological development and are tissue specific. The degree of DNA methylation is generally inversely correlated with transcription activity when it occurs within the promoter region of a gene.

A growing body of evidence suggests that alterations in DNA methylation play a major role in the development of human cancers.[11] Transformed cells of virtually all types often simultaneously have widespread loss of methylation from normal methylated sites, increased total activity of DNMT and more regional areas of hypermethylated DNA.[12]

In our study, all five ovarian cancer samples were hypomethylated in comparison with global methylation status of normal ovarian tissue. Qu *et al.* described hypomethylation in satellite 2 DNA of chromosome 1 and 16 of most ovarian carcinomas and LPM.[13] Cheng and co-workers compared the levels of DNA methylation in ovarian cystadenoma, LMP tumours and carcinomas.[6] They reported that mean global levels of DNA methylation showed significant differences among the three ovarian tumour subtypes. They also measured 5 mC levels in four samples of normal ovarian tissues. As in our study, the methylation levels of normal ovarian tissue samples were significantly higher than in tumours. All these findings are consistent with the hypothesis that the genome wide hypomethylation facilitates tumour development with a predisposition of cells to structural and numerical chromosomal aberrations.

The enzymatic methylation machinery itself is composed of three known catalitically active DNA methyltransferase, DNMT1, 3A and 3B. DNMT1 is targeted to replication foci and has a 10-40-fold preference for hemimethylated DNA substrates.[14] The newly identified DNMT3 enzymes are essential for embryonic development and are responsible for the wave of *de novo* methylation seen during embryogenesis which establishes the somatic methylation pattern for the organism.[5]

The exact nature of the methylation defect in cancer cells is not known; however, it has been noted by several groups that DNMT1 is overexpressed in tumour cells and it has been shown more recently that the DNMT3 family can be overexpressed, too. The degree of overexpression varies depending of the tumour type and the method of analysis. Not all tumours, however, overexpress the DNMTs, though overexpression may be necessary; in many cases, it is probably not sufficient to cause the methylation defects observed in cancer cells.[15] In our study, we found overexpression of DNMT3A and DNMT3B in all five ovarian cancers. The mean expression levels were 12-fold higher for DNMT3A and 7-fold higher for DNMT3B than the mean expression levels for normal ovarian tissue. For DNMT1, we found overexpression in just two cases.

The paradox that remains to be resolved is the global hypomethylation observed in cancer cells notwithstanding the high levels of DNMTs that are present in these cells. One possible explanation is that cancer cells also express high levels of a demethylase, which actively removes methyl groups from the DNA. We did not find any correlation between global hypomethylation and DNMT expression in ovarian tumours.

Abnormal hypermethylation recurrently associated with gene silencing has been reported for tumour suppressors genes Rb1 [16], VHL [17], and CDKN2/p16.[18] Hypomethylation of several oncogenes was also reported in tumours. In ovarian cancer, Cheng *et al.* found methylation of the *MyoD1* locus in five of ten ovarian carcinomas, but none of the five normal ovarian tissue samples showed methylation of these sites.[6] McCluskey *at al.* reported that p16 silencing is also important for the development of ovarian carcinoma.[7]

Identification of specific genetic targets for methylation changes in ovarian epithelial tumours may not only lead to a better understanding of the molecular mechanisms and determinants of their development, but may also facilitate the use and monitoring of methylation-targeting drugs in the treatment of ovarian cancer patients.

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