**DNMT1, DNMT3A and DNMT3B** gene variants in relation to ovarian cancer risk in the Polish population

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**Abstract** Studies have demonstrated that changes in DNA methylation of cancer related genes can be an elementary process accounting for ovarian tumorigenesis. Therefore, we evaluated the possible association of single nucleotide polymorphisms (SNPs) of DNA methyltransferases (**DNMTs**) genes, including **DNMT1**, **DNMT3B**, and **DNMT3A**, with ovarian cancer development in the Polish population. Using PCR–RFLP and HRM analyses, we studied the prevalence of the **DNMT1** rs8101626, rs2228611 and rs759920, **DNMT3A** rs2289195, 7590760, rs13401241, rs749131 and rs1550117, and **DNMT3B** rs1569686, rs2424913 and rs2424932 SNPs in patients with ovarian cancer (n = 159) and controls (n = 180). The lowest p values of the trend test were observed for the **DNMT1** rs2228611 and rs759920 SNPs in patients with ovarian cancer (p_trend = 0.0118 and p_trend = 0.0173, respectively). Moreover, we observed, in the recessive inheritance model, that the **DNMT1** rs2228611 and rs759920 SNPs are associated with an increased risk of ovarian cancer development [OR 1.836 (1.143–2.949), p = 0.0114, p_corr = 0.0342, and OR 1.932 (1.185–3.152), p = 0.0078, p_corr=0.0234, respectively]. However, none of other nine studied SNPs displayed significant contribution to the development of ovarian cancer. Furthermore, haplotype and multifactor dimensionality reduction analysis of the studied **DNMT1**, **DNMT3B**, and **DNMT3A** polymorphisms did not reveal either SNP combinations or gene interactions to be associated with the risk of ovarian cancer development. Our results may suggest that **DNMT1** variants may be risk factors of ovarian cancer.

**Keywords** Ovarian cancer · Polymorphism · **DNMT1** · **DNMT3A** · **DNMT3B**

**Introduction**

Ovarian cancer includes any malignant growth that may develop in disparate parts of the ovary; however, the majority of ovarian malignancies arise from the ovarian epithelium [1]. This type of cancer usually exhibits vague symptoms and is one of the most lethal gynecological cancers in women in the United States and Europe [2]. Although ovarian cancer has been intensively studied, the underlying cause of this malignant disease remains undetermined [1, 3]. There is a lot of evidence that shows a reduced risk for ovarian cancer in women who use oral contraceptives, have greater parity, or breastfed long-term [3]. Known risk factors of ovarian cancer include infertility, early age of menarche, late age of menopause, inflammation, some environmental factors, and genetic background [3]. The known genetic factors encompass a few high-penetration genes (e.g., **BRCA1**) and numerous moderate and low-penetrance ovarian cancer susceptibility genes [3]. However, these factors alone are not sufficient for ovarian tumorigenesis, which indicates that attention must be paid to the role of epigenetic status changes during ovarian carcinogenesis [4, 5].

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The term epigenetics describes the reversible regulation of gene expression that does not occur due to changes in the DNA sequence [6, 7]. Epigenetic traits include DNA methylation, covalent modification of histones, and expression status of genes modulated by mRNA [6, 7].

DNA methylation in mammalian cells is typically restricted to covalent modification, in which a methyl group is added to a cytosine located in CpG dinucleotides in the genomic DNA [6]. CpG dinucleotides are frequently situated in rich CpG genomic sites designated as CpG islands, which are found in approximately half of all genes in humans [8]. Hypomethylation of regulatory DNA sequences increases the expression of proto-oncogenes and genes encoding proteins involved in genomic instability and malignant cell growth and metastasis [6, 9]. By contrast, hypermethylation of the promoter regions of tumor suppressor genes (TSGs) causes the transcriptional silencing of TSGs [6, 9].

There are three types of enzymatically active DNA methyltransferases (DNMTs), designated DNMT1, DNMT3A, and DNMT3B [6, 9]. Abnormal levels of DNMT1, DNMT3A and DNMT3B contributing to changes in the expression of cancer related genes have been found in different types of malignancies [10–13]. The DNMTs levels can be changed by single nuclear polymorphisms (SNPs) situated within their genes and which may affect the development of various cancers [14, 15]. Therefore, we selected 11 SNPs of DNMT1, DNMT3A and DNMT3B located in distinct blocks of linkage disequilibrium (LD) according to HapMap CEU data (http://hapmap.ncbi.nlm.nih.gov/) (Supplemental Table 1; Supplemental Fig. 1a–c). Furthermore, we aimed to study whether these DNMT1, DNMT3A and DNMT3B SNPs can be a genetic risk factor of ovarian cancer.

Materials and methods

Patients and controls

The patients include 159 women with histologically recognized ovarian carcinoma according to the International Federation of Gynecology and Obstetrics (FIGO). Histopathological classification, including the stage, grade and tumor type, was performed by an experienced pathologist (Table 1). The controls included 180 unrelated healthy female volunteers who were matched by age to the cancer patients (Table 1). Written informed consent was obtained from all participating individuals. The procedures of the study were approved by the Local Ethical Committee of Poznań University of Medical Sciences. All women with ovarian cancer and controls were Caucasian from the Wielkopolska area of Poland.

| Characteristic       | Patients | Controls |
|----------------------|----------|----------|
| Mean age ± SD        | 55.6 ± 9.6 | 56.1 ± 8.7 |
| Histological grade   |          |          |
| G1                   | 29 (18.2 %) |          |
| G2                   | 46 (28.9 %) |          |
| G3                   | 44 (27.7 %) |          |
| Gx                   | 40 (25.2 %) |          |
| Clinical stage       |          |          |
| I                    | 44 (27.7 %) |          |
| II                   | 35 (22.0 %) |          |
| III                  | 54 (34.0 %) |          |
| IV                   | 26 (16.3 %) |          |
| Histological type    |          |          |
| Serous               | 44 (27.7 %) |          |
| Mucinous             | 27 (17.0 %) |          |
| Endometrioid         | 47 (29.5 %) |          |
| Clear cell           | 18 (11.3 %) |          |
| Brenne               | 3 (1.9 %) |          |
| Mixed                | 10 (6.3 %) |          |
| Untyped carcinoma    | 10 (6.3 %) |          |

Genotyping

Genomic DNA was isolated from peripheral blood leukocytes by salt extraction. DNA samples were genotyped for the 11 SNPs in DNMT1, DNMT3A and DNMT3B (Supplemental Table 1; Supplemental Fig. 1a–c). SNPs were selected with the use of the genome browsers of the International HapMap Consortium (http://www.hapmap.org/index.html.en), UCSC (http://genome.ucsc.edu) and dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/). SNPs were selected based on functional significance, location in different LD blocks, and minor allele frequency (MAF) >0.1 in the Caucasian population. Genotyping of the DNMT1 rs2228611, rs759920, DNMT3A rs2289195, rs13401241, rs749131, rs1550117, DNMT3B rs2424932 SNPs was performed by high resolution melting curve analysis (HRM) on the LightCycler 480 system (Roche Diagnostics, Mannheim, Germany). Genotyping of the DNMT1 rs8101626, DNMT3A rs7590760, and DNMT3B rs1569686, rs2429432SNPs was performed by PCR, followed by the appropriate restriction enzyme digestion (PCR-RFLP) according to the manufacturer’s instructions (Fermentas, Vilnius, Lithuania). DNA fragments were separated in 2 % agarose gels and visualized by ethidium bromide staining. Primer sequences and conditions for HRM and PCR–RFLP analyses are presented in Supplemental Table 2. Genotyping quality was evaluated by repeated genotyping of 10 % randomly selected samples.
Table 2: Association of polymorphisms in genes encoding DNA methyltransferases with the risk of ovarian cancer

| Gene   | rs no.  | Allelesa | MAFb | Genotypes casesc | Genotypes controlsc | p_allele value | p_recessive value | OR_{dominant} (95 % CI); p value | OR_{recessive} (95 % CI); p value |
|--------|---------|----------|------|------------------|---------------------|---------------|------------------|-------------------------------|-------------------------------|
| DNMT1  | rs8101626 | A/g     | 0.44 | 35/84/40         | 55/92/33            | 0.0456        | 0.0412           | 1.559 (0.954–2.548); 0.0755    | 1.497 (0.890–2.520); 0.1272    |
|        | rs2228611 | A/g     | 0.49 | 28/74/57         | 44/94/42            | 0.0117        | 0.0118           | 1.514 (0.890–2.575); 0.1247    | 1.836 (1.143–2.949); 0.0114    |
|        | rs759920  | A/g     | 0.48 | 32/74/53         | 46/97/37            | 0.0179        | 0.0173           | 1.362 (0.816–2.274); 0.2359    | 1.932 (1.185–3.152); 0.0078    |
|        | rs2289195 | a/G     | 0.45 | 48/70/41         | 61/77/42            | 0.4226        | 0.4502           | 1.185 (0.890–1.874); 0.4667    | 1.142 (0.696–1.874); 0.6002    |
|        | rs7590760 | C/g     | 0.44 | 62/76/21         | 53/96/31            | 0.0728        | 0.0656           | 0.653 (0.415–1.026); 0.0638    | 0.731 (0.401–1.333); 0.3060    |
|        | rs13401241| A/c     | 0.45 | 55/74/30         | 56/85/39            | 0.4110        | 0.4217           | 0.854 (0.542–1.345); 0.4956    | 0.841 (0.494–1.432); 0.5230    |
|        | rs749131  | G/t     | 0.45 | 51/82/26         | 54/91/35            | 0.4983        | 0.4899           | 0.908 (0.572–1.439); 0.6800    | 0.810 (0.463–1.417); 0.4595    |
|        | rs1550117 | a/G     | 0.08 | 135/23/1         | 151/29/0            | 0.9258        | 0.9241           | 0.926 (0.514–1.668); 0.7970    | 3.416 (0.138–4.529); 0.4690f   |
| DNMT3A | rs1569686 | G/t     | 0.42 | 57/73/29         | 59/90/31            | 0.7866        | 0.7878           | 0.873 (0.557–1.368); 0.5520    | 1.072 (0.613–1.874); 0.8066    |
|        | rs2424913 | C/t     | 0.46 | 46/86/27         | 51/91/38            | 0.5372        | 0.5259           | 0.971 (0.606–1.557); 0.9033    | 0.764 (0.442–1.321); 0.3351    |
|        | rs2424932 | a/G     | 0.42 | 62/64/33         | 61/88/31            | 0.8356        | 0.8414           | 0.802 (0.515–1.250); 0.3293    | 1.259 (0.730–2.170); 0.4069    |

Statistically significant results that persisted after correction for multiple testing are highlighted in italic. Experiment-wide significance threshold required to keep type I error rate at 5 %: 0.0166

- **a** Uppercase denotes the more frequent allele in the control samples
- **b** MAF minor allele frequency calculated from the control samples
- **c** The order of genotypes: DD/Dd/dd (d is the minor allele)
- **d** Dominant model: dd + Dd versus DD (d is the minor allele)
- **e** Recessive model: dd versus Dd + DD (d is the minor allele)
- **f** Fisher exact test
Statistical analysis

For each SNP, the Hardy–Weinberg equilibrium (HWE) was assessed by Pearson’s goodness-of-fit Chi square ($\chi^2$) statistic. The differences in the allele and genotype frequencies between cases and controls were determined using standard $\chi^2$ or Fisher tests. SNPs were tested for association with ovarian cancer using the Cochran-Armitage trend test. The odds ratio (OR) and associated 95 % confidence intervals (95 % CI) were also calculated. The data were analyzed under recessive and dominant inheritance models. To adjust for the multiple testing, we employed a correction factor of 3 ($0.005/3 = 0.0167$) to take into consideration the number of genes evaluated. This represents a generally accepted correction strategy; one not as stringent as the Bonferroni correction, yet still taking the number of totally independent analyses into account. Pair-wise LD between selected SNPs was computed as both $D'$ and $r^2$ values using HaploView 4.0 software (Broad Institute, Cambridge, MA). Haplotype analysis was performed using the UNPHASED 3.1.5 program with the following analysis options: all window sizes, full model and uncertain haplotype option [16]. Haplotypes with a frequency below 0.01 were set to zero. The $p$ values for both global and individual tests of haplotype distribution between cases and controls were determined. Statistical significance was assessed using the 1,000-fold permutation test.

High order gene–gene interactions among all tested polymorphic loci were studied by the multifactor dimensionality reduction (MDR) approach (MDR version 2.0 beta 5). A detailed explanation on the MDR method has been described elsewhere [17]. Based on the obtained testing balanced accuracy and cross-validation consistency values, the best statistical gene–gene interaction models were established. A 1,000-fold permutation test was used to assess the statistical significance of MDR models (MDR permutation testing module 0.4.9 alpha).

Results

Association of DNMT1, DNMT3A and DNMT3B SNPs with ovarian cancer development

The frequency of all studied genotypes did not exhibit deviation from the HWE between all investigated groups ($p > 0.05$). The lowest (0.08) and highest (0.49) MAF found for the control samples was for DNMT3A rs1550117 and DNMT1 rs2228611 SNPs, respectively (Table 2). The number of genotypes, OR, and 95 % CI calculations for the 11 studied DNMT1, DNMT3B, and DNMT3A SNPs are presented in Table 2. The lowest $p$ values of the trend test were observed for the DNMT1 rs2228611 and rs759920 SNPs in patients with ovarian cancer ($p_{\text{trend}} = 0.0118$ and $p_{\text{trend}} = 0.0173$, respectively) (Table 2). The statistical significance for multiple testing determined by correction of genes number was $p = 0.0167$. Therefore, we observed, in a recessive inheritance model, that DNMT1 rs2228611 and rs759920 SNPs are associated with increased risk of ovarian cancer development [OR 1.836 (1.143–2.949), $p = 0.0114$, and OR 1.932 (1.185–3.152), $p = 0.0078$, respectively] (Table 2). However, none of the other nine studied DNMT1, DNMT3B and DNMT3A SNPs exhibited significant association either in dominant or recessive inheritance models with ovarian cancer development (Table 2).

Association of DNMT1, DNMT3B, and DNMT3A haplotypes with ovarian cancer development

Haplotype analysis of DNMT1, DNMT3B, and DNMT3A polymorphisms did not reveal SNP combinations associated with the risk of ovarian cancer development (Table 3). In patients, the lowest global $p = 0.0502$ was observed for haplotypes composed of the DNMT1 rs2228611 and rs759920 SNPs (Table 3). However, these results were not statistically significant when permutations were used to generate empiric $p$ values. The empirical 5 % quintile of the best $p$ value after 1,000 permutations was 0.002566. The selected 11 SNPs situated in distinct regions of DNMT1, DNMT3B, and DNMT3A were either in weak or strong pairwise LD. This was calculated from the control samples, and had $D'$ ranges of 0.934–0.976 for DNMT1 SNPs, 0.109–1.000 for DNMT3A SNPs, and 0.626–0.943 for DNMT3B SNPs (Supplementary Table 3).

MDR analysis of gene–gene interactions among the studied DNMT1, DNMT3A and DNMT3B SNPs

Exhaustive MDR analysis evaluating two- to four-loci combinations of all studied SNPs for each comparison did not reveal statistical significance in predicting susceptibility to ovarian cancer development (Table 4). The best combination of possibly interactive polymorphisms was observed for rs759920 of DNMT1, rs2289195 and rs7590760 of DNMT3A and rs2424932 of DNMT3B (testing balanced accuracy = 59.03 %, cross validation consistency of eight out of ten, permutation test $p = 0.0940$).

Discussion

As with other cancer types, aberrant DNA methylation encompassing CpG island hypermethylation and global
hypomethylation of heterochromatin has been demonstrated in ovarian cancer [4, 5, 12, 18]. To date, it seems several genes have been found to be down-regulated in ovarian cancer due to their hypermethylation [19–23]. These genes include classical TSGs and genes encoding cell adhesion molecules, proapoptotic proteins, and other proteins supporting ovarian tumorigenesis [19–23]. DNA hypomethylation in ovarian cancer has been found in chromosome 1 satellite 2 and long interspersed nuclear element-1 (LINE-1) repetitive elements [18, 24]. In addition to these findings, many genes are hypomethylated and overexpressed in this type of cancer. Among these are genes encoding activators of the mitogen-activated protein kinases, insulin-like growth factor-2, and proteins associated with chemoresistance [18, 25–27].

Increased levels of some DNMTs have been observed in ovarian cancer cell lines and primary ovarian cancerous tissues as compared to normal ovarian cells [10, 11, 28]. Ahluwalia et al. [10] found significantly higher DNMT1 and DNMT3B mRNA levels in ovarian cancer cells than in normal ovarian surface epithelial cells. Moreover, Chen et al. [28] observed significantly elevated DNMT1 and DNMT3B mRNA levels in primary and recurrent epithelial ovarian carcinoma compared to normal epithelial ovarian cells. Recently, Cheng et al. (2011) demonstrated that DNMT1, but not DNMT3A or DNMT3B, levels were increased in serous borderline ovarian tumor cells. They also demonstrated that DNMT1 levels correlated with E-cadherin promoter methylation [11].

In our study we found that the DNMT1 rs2228611 and rs759920 SNPs can be risk factors of ovarian cancer in a Polish population. To date, the DNMT1 rs2228611 SNP has been associated with sporadic infiltrating ductal breast carcinoma among Chinese Han women in the Heilongjiang Province [29]. Moreover, the DNMT1 rs2228611 SNP modified associations between urinary cadmium and hypomethylation of LINE-1 repeated sequences [30]. In addition to these findings, the two intronic polymorphisms rs2241531 and rs4804490 of DNMT1 may be associated with hepatitis B virus (HBV) clearance and protection from the development of hepatocellular carcinoma [31].

Our studies did not demonstrate an association of the DNMT3B rs1569686, rs2424913 and rs2424932 SNPs with ovarian cancer. To date, the DNMT3B rs2424913 polymorphism has been demonstrated to be a risk factor of lung, breast, and head and neck cancers and age of onset of hereditary nonpolyposis colorectal cancer (CRC) [32–35]. Furthermore, the DNMT3B rs2424913 SNP may contribute to reduced methylation of CpG islands of the MYOD and MLH1 genes in normal colonic mucosa of patients with CRC, and with increased promoter methylation of TSGs related to the development of prostate cancer [36, 37]. Another polymorphism of DNMT3B, rs2424909, being in great LD with rs2424913 has been found to be a genetic

### Table 3: Haplotype analysis of the DNMT1, DNMT3A and DNMT3B gene polymorphisms

| Gene  | SNP combination                  | $\chi^2$ | Global p value |
|-------|----------------------------------|----------|----------------|
| DNMT1 | 2-Marker window                  |          |                |
|       | rs8101626 rs2228611              | 5.6892   | 0.0582         |
|       | rs2228611 rs759920               | 5.9849   | 0.0502         |
|       | 3-Marker window                  |          |                |
|       | rs8101626 rs2228611 rs759920     | 8.3052   | 0.0810         |
| DNMT3A| 2-Marker window                  |          |                |
|       | rs2289195 rs7590760              | 3.3940   | 0.3348         |
|       | rs7590760 rs13401241             | 2.8982   | 0.4076         |
|       | rs13401241 rs749131              | 6.5006   | 0.0896         |
|       | rs749131 rs1550117               | 0.6357   | 0.8882         |
|       | 3-Marker window                  |          |                |
|       | rs2289195 rs7590760 rs13401241   | 4.1816   | 0.7586         |
|       | rs7590760 rs13401241 rs749131    | 7.1676   | 0.3056         |
|       | rs13401241 rs749131 rs1550117    | 7.1785   | 0.2077         |
|       | 4-Marker window                  |          |                |
|       | rs2289195 rs7590760 rs13401241   | 14.099   | 0.3669         |
|       | rs7590760 rs13401241 rs749131    | 7.9432   | 0.4390         |
|       | rs13401241 rs749131 rs1550117    | 15.178   | 0.4387         |
|       | 5-Marker window                  |          |                |
|       | rs2289195 rs7590760 rs13401241   | 15.178   | 0.4387         |
|       | ds749131 rs1550117               | 15.178   | 0.4387         |
| DNMT3B| 2-Marker window                  |          |                |
|       | rs1569686 rs2424913              | 4.0008   | 0.2614         |
|       | rs2424913 rs2424932              | 1.3364   | 0.7205         |
|       | 3-Marker window                  |          |                |
|       | rs1569686 rs2424913 rs2424932    | 8.7324   | 0.1202         |

**a** Empirical 5 % quantile of the best p value 0.002566

### Table 4: Results of gene–gene interactions analyzed by MDR method

| Genes and rs numbers | Testing balanced accuracy | Cross validation consistency (%) | p value<sup>a</sup> |
|----------------------|--------------------------|---------------------------------|---------------------|
| DNMT1 rs759920,      |                          |                                 |                     |
| DNMT3A rs7590760     | 0.5866                   | 80                              | 0.1160              |
| DNMT1 rs8101626,     |                          |                                 |                     |
| DNMT3A rs2289195,    | 0.4998                   | 30                              | 0.8530              |
| DNMT3B rs1569686     | 0.5903                   | 80                              | 0.0940              |
| DNMT1 rs759920,      |                          |                                 |                     |
| DNMT3A rs2289195,    | 0.5903                   | 80                              | 0.0940              |
| DNMT3B rs2424932     | 0.4998                   | 30                              | 0.8530              |

<sup>a</sup Significance of accuracy, empirical p value based on 1,000 permutations
risk factor of gastric cancer, CRCs, and gallbladder carcinoma [38–40].

It has recently been found that the DNMT3A polymorphism rs1550117 alters the promoter activity and risk of gastric cancer and CRC [14, 15]. However, we did not find an association of any of the five studied DNMT3A SNPs with ovarian cancer development. Frequent mutations in DNMT3A have been demonstrated in human T cell lymphoma, myelodysplastic syndromes, as well as myeloid and monocytic leukemias [41–44]. Moreover, the DNMT3A intronic polymorphism rs13428812, situated in the same LD block with our studied rs7590760, has been shown to contribute to Crohn’s disease [45].

DNMT3A and DNMT3B carry out de novo methylation [7, 13]. DNMT3A and DNMT3B are involved in imprinting of germ cells and normal embryonic development [46, 47]. Moreover, DNMT3A contributes to the differentiation of neural progenitors and hematopoietic stem cells [48, 49]. DNMT1 is responsible for the maintenance of the hemimethylated DNA methylation pattern during DNA replication, and also has a role in gene silencing [13, 50].

Our study may suggest that Polish women bearing either the DNMT1 rs2228611 or rs759920 SNP may have an increased risk of ovarian cancer. Neither of these polymorphisms is a functional variant, and their association with ovarian cancer may be due to LD with one or more functional polymorphisms of DNMT1. Therefore, to confirm the role of these SNPs in ovarian cancer, this study should be repeated in a larger and independent cohort, and functional studies of these SNPs must be conducted. Moreover, a future genomewide SNPs genotyping study in our sample collection will additionally provide more knowledge of the genes contributing to the onset of this disease.

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Conflict of interest The authors declare that they have no conflict of interest.

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