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Folate and choline metabolism gene variants in relation to ovarian cancer risk in the Polish population

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Abstract Data indicates that genetic factors alone do not account for ovarian tumorigenesis, suggesting that epigenetic status additionally affects this process. Therefore, we assessed the possible contribution of polymorphic variants of genes that may affect DNA methylation to the risk of ovarian cancer incidence in the Polish population. Using PCR-RFLP and HRM analyses, we studied the distribution of *BHMT* (rs3733890), *MTHFD1* (rs2236225), *MTHFR* (rs1801133), *MTR* (rs1805087), *MTRR* (rs1801394) and *TCN2* (rs1801198) genotypes and alleles in patients with ovarian cancer ($n = 136$) and controls ($n = 160$). Moreover, using DNA and methylation-specific PCR (MSP) we also determined the methylation of the *Cadherin 13 (CDH13)* promoter in cancerous tissue from these patients. We did not observe a significant association between all studied gene variants and the incidence of ovarian cancer. The lowest $P_{trend} = 0.1226$ was observed for the *MTHFR* Ala222Val polymorphism. Moreover, the lowest $P = 0.0772$ was found in the comparison of *MTHFR* Ala/Ala versus Val/Val and Val/Ala genotypes in patients and control groups. The multifactor dimensionality reduction analysis also did not indicate a significant interactive genetic effect on ovarian cancer incidence for all analyzed SNPs. However, we observed frequent methylation of the *CDH13* promoter in approximately 21% (29/136) patients with ovarian carcinomas. Our results might suggest that the selected polymorphic gene variants may not contribute to ovarian cancer incidence.

Keywords Ovarian cancer · Polymorphism · Choline · Folate metabolism

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Introduction

Ovarian cancer is the one of the leading causes of malignant deaths in women in Europe and United States [1, 2]. Ovarian carcinogenesis remains unclear, though there is much evidence showing a decreased risk for ovarian cancer in women who use oral contraceptives, have greater parity, or breastfed long-term [3]. In contrast, an increased risk for ovarian cancer can be associated with environmental or inflammatory factors and a few significant-risk genetic components, such as high-penetrance genes (e.g., *BRCA1*) [3]. However, the genetic factors alone have been shown to be insufficient for ovarian carcinogenesis, suggesting the importance of changes in epigenetic status for both ovarian tumorigenesis and the development of other cancers [4–7].

Epigenetic changes include heritable alterations in gene expression that do not affect the DNA sequence. These changes encompass DNA methylation, covalent modification of histones, repositioning of nucleosomes, and micro-RNAs affecting posttranscriptional gene regulation [8, 9]. The transport of a methyl group to the carbon-5 position of cytosines within cytosine-guanine dinucleotides (CpG

islands) leads to the formation of 5-methylcytosine. This methylation level of regulatory DNA sequences and the status of histone covalent modification correlate with the transcriptional activity of genes [10].

The methylation of genomic DNA is conducted by DNA methyltransferases (DNMTs) [10]. Their activity may contribute to aberrant DNA methylation and increased susceptibility to the development of various malignancies, including ovarian cancer [10]. DNMTs use S-adenosylmethionine (AdoMet) as their methyl group donor [10]. This common methyl group donor is biosynthesized from methionine and ATP by methionine adenosyltransferase [11] (Figure 1S, on-line supplementary data).

Intracellular levels of methionine can be maintained by the methylation of homocysteine by betaine-homocysteine methyltransferase (BHMT) (OMIM 602888) or 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR) (OMIM 156570) (Figure 1S, on-line supplementary data). Either 5-methyltetrahydrofolate (5met-THF) or the choline oxidation product, betaine, may serve as a methyl group donor for the MTR and BHMT enzymes, respectively [12, 13]. 5met-THF is biosynthesized during the reduction of 5,10-methylenetetrahydrofolate by 5,10-methylenetetrahydrofolate reductase (MTHFR) (OMIM 607093) [14]. The cellular level of 5met-THF can also be controlled by methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase/formyltetrahydrofolate synthetase (MTHFD1) (OMIM 172460) [15]. Transcobalamin II (TCN II) (OMIM 613441) is a blood plasma protein that functions as the primary transport protein for vitamin B12, which is necessary for MTR functionality [16]. MTR activity also requires reductive activation, which is conducted by methionine synthase reductase (MTRR) (OMIM 602568) [17].

The *MTHFR* Ala222Val (rs1801133), *MTR* Asp919Gly (rs1805087), and *TCN2* Arg259Pro (rs1801198) polymorphisms have been linked to hypomethylation of genomic DNA in cancer cells [18–20]. Moreover, the *BHMT* Arg 239Gln (rs3733890), *MTHFD1* Arg653Gln (rs2236225), and *MTRR* Ile22Met (rs1801394) polymorphic variants have been shown to contribute to the incidence of various cancers [21–25].

Therefore, these functional variants of the *BHMT*, *MTHFD1, MTHFR, MTR*, *MTRR* and *TCN2* (Figure 1S, on-line supplementary data) encoding enzymes associated with the biosynthesis of methionine may regulate the cellular AdoMet levels, DNMTs activity, and DNA methylation in cancer cells. We studied the frequency of the genotypes and alleles of these polymorphisms in women with ovarian cancer and controls in a sample from a Polish cohort. The promoter of *Cadherin 13 (CDH13)* has demonstrated an increased frequency of methylation in ovarian cancer patients [26, 27]. Therefore to determine the effect of investigated polymorphic variants on DNA methylation we also evaluated the methylation of the *Cadherin 13 (CDH13)* promoter in cancerous tissues from these tested patients.

Materials and methods

Patients and controls

The patient group was composed of one hundred thirty-six women with histologically recognized ovarian carcinoma according to the International Federation of Gynecology and Obstetrics (FIGO). All patient data including formalin-fixed, paraffin-embedded ovarian cancer samples were collected in the Clinic of Gynecological Surgery, Poznań University of Medical Sciences, Poland. Histopathological classification, including the stage, grade and tumor type, was performed by an experienced pathologist (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. The control group comprised of one hundred sixty unrelated healthy female volunteers who were matched by age to the cancer patients (Table 1). All patients and controls were Caucasian from the Wielkopolska area of Poland.

Table 1 Clinical characteristics of ovarian cancer patients and healthy controls

Characteristic	Patients ($n = 136$)	Controls ($n = 160$)
Mean age ± SD	56.9 ± 10.4	54 ± 8.9
Histological grade		
G1	21 (15.4%)	
G2	37 (27.2%)	
G3	38 (27.9%)	
Gx	40 (29.4.%)	
Clinical stage		
I	38 (27.9%)	
II	28 (20.6%)	
III	44 (32.4%)	
IV	26 (19.1%)	
Histological type		
Serous	35 (25.7%)	
Mucinous	23 (16.9%)	
Endometrioid	43 (31.6%)	
Clear cell	18 (13.2%)	
Brenne	3 (2.2%)	
Mixed	7 (5.1%)	
Untyped carcinoma	7 (5.1%)	

Table 2 Characteristics of the polymorphisms genotyped in the data set

Gene symbol	Gene name	rs no.	Protein effect	Location	MAF
BHMT	Betaine-homocysteine methyltransferase	rs3733890	Arg239Gln	c.716G>A	0.34
MTHFD1	Methylenetetrahydrofolate dehydrogenase 1	rs2236225	Arg653Gln	c.1958G>A	0.49
MTHFR	5,10-Methylenetetrahydrofolate reductase	rs1801133	Ala222Val	c.665C>T	0.36
MTR	5-Methyltetrahydrofolate-homocysteine methyltransferase	rs1805087	Asp919Gly	c.2756A>G	0.20
MTRR	Methionine synthase reductase	rs1801394	Ile22Met	c.66A>G	0.39
TCN2	Transcobalamin II	rs1801198	Arg259Pro	c.776C>G	0.46

MAF minor allele frequency calculated from the control samples

Genotyping

DNA was isolated from peripheral blood leucocytes by salt extraction technique.. The chosen genes encode enzymes contributing directly or indirectly to the methylation of homocysteine to methionine. The six selected single nucleotide polymorphisms (SNPs) were chosen according to their functionality, location in the coding sequence, and minor allele frequency >0.1 in Caucasian population (Table 2). Genotyping of *MTHFD1* (rs2236225), *MTHFR* (rs1801133), *MTR* (rs1805087), and *TCN2* (rs1801198) was carried out by PCR, followed by restriction fragment length polymorphism (PCR-RFLP) analysis conducted according to the manufacturer's instructions (Fermentas, Vilnius, Lithuania). DNA fragments were separated in 2% agarose gels and visualized by ethidium bromide staining. Starter sequences and conditions for PCR-RFLP analyses are provided in Table 1S on-line supplementary data. The *BHMT* SNP (rs3733890) and *MTRR* SNP (rs1801394) were genotyped by high-resolution melting curve analysis (HRM) on the *LightCycler* 480 system (Roche Diagnostics, Mannheim, Germany) (Table 1S on-line supplementary data). The genotyping quality of all polymorphisms was examined by repeat analysis of approximately 10% of the samples using the initial genotyping method or direct commercial sequencing.

Bisulphite modification of DNA and methylation-specific polymerase chain reaction (MSP)

Genomic DNA was isolated from formalin-fixed, paraffin-embedded tissue sections using QIAamp DNA FFPE Tissue Kit™ Qiagen GmbH, (Hilden, Germany). One μg of genomic DNA was subjected to bisulfite conversion of cytosine to uracil according to EZ DNA Methylation Kit™ procedure from Zymo Research Corp. (Orange, CA). In order to determine *CDH13* gene promoter methylation we used the MSP technique.

PCR amplification was performed by FastStart Taq DNA Polymerase Roche Diagnostic GmbH, (Penzberg, Germany). Since bisulfite conversion of cytosine to uracil results to sequence differences, we used two pairs of primers: one for the unmethylated template and another for the methylated template (Table 1S online supplementary data) [26]. All MSP reactions were repeated and an additional third round of MSP was performed when the results were not consistent. Methylated and non-methylated bisulfite treated DNA from human methylated/non-methylated DNA Set Zymo Research Corp. (Orange, CA) was used as MSP positive control of methylated and unmethylated *CDH13* gene (Fig. 1). In both reactions water was employed as a negative PCR control. For quality control, 20% of the samples were used for direct bisulphite sequencing.

Statistical analysis

The distribution of genotypes in patients and controls was tested for deviation from Hardy–Weinberg equilibrium. The χ^2 square or Fisher exact tests were used to evaluate differences in genotypic and allelic prevalence between patients and controls. All polymorphisms were tested for association with ovarian cancer incidence using the χ^2 test for trend (P_{trend}). Moreover, the odds ratio (OR) and 95% confidence intervals (95% CI) were calculated. Statistical power was evaluated by power and sample size calculation program version 2.1.30 (http://medipe.psu.ac.th/episoft/pssamplesize/). Associations between the studied polymorphisms and the risk of ovarian cancer were tested using the nonparametric and genetic model-free Multifactor

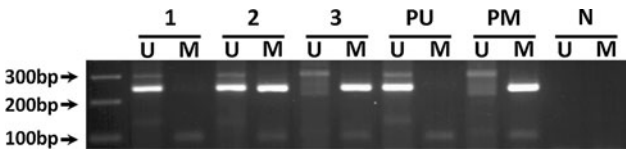

Fig. 1 Representative results of methylation-specific polymerase chain reaction (MSP) analysis of the *CDH13* promoter methylation in ovarian cancer tissues. PCR products in *lane U* represent unmethylated alleles, whereas PCR products in *lane M* correspond to the presence of methylated alleles. *PU* and *PM* represent positive control for unmethylated and methylated templates, respectively, obtained from MSP reaction of bisulfite treated DNA of Human Methylated/Non-methylated DNA Set Zymo Research Corp. (Orange, CA). *N* represents negative PCR control with the addition of water. Numbers *1*, *2*, and *3* correspond to patients

dimensionality reduction (MDR) approach (MDR version 2.0 beta 5). Statistical significance was evaluated using a 1,000-fold permutation test (MDR permutation testing module 0.4.9 alpha). A p value of <0.05 was considered statistically significant.

Results

Distribution of *BHMT* Arg239Gln, *MTHFD1* Arg653Gln, *MTHFR* Ala222Val, *MTR* Asp919Gly, *MTRR* Ile22Met and *TCN2* Arg259Pro polymorphic variants with regards to the incidence of ovarian cancer

Genotype analysis of all studied polymorphisms did not display a significant deviation from Hardy–Weinberg equilibrium in the patient and control groups. We did not find a significant association between all studied SNPs and the incidence of ovarian cancer (Table 3). The lowest $P_{\text{trend}} = 0.1226$ was observed for the *MTHFR* Ala222Val polymorphism. Moreover, the lowest $P = 0.0772$ was found in the comparison of *MTHFR* Ala/Ala versus Val/Val and Val/Ala genotypes in patients and control groups. The MDR evaluation also did not display a significant interactive genetic effect on ovarian cancer incidence for all analyzed SNPs (Table 4). All possible two-, three- and four-way SNP interactions were tested using tenfold cross validation in an exhaustive search (considering all possible SNP combinations). All of the "best models" (presenting the highest testing balanced accuracy and cross validation consistency >5 out of ten) did not demonstrate statistical significance, as evaluated by 1,000-fold permutation test. We also did not observe a significant contribution of the studied SNPs to tumor characteristics (data not shown).

CDH13 promoter methylation

We observed methylation of the *CDH13* promoter in approximately 21% (29/136) of our patients with ovarian carcinomas. There was no association between the promoter methylation frequency and histological grade, clinical stage and differentiation. Moreover, we did not find differences in the frequency distribution of the methylation of the *CDH13* promoter in groups of *BHMT*, *MTHFD1*, *MTHFR*, *MTR*, *MTRR*, and *TCN2* genotypes and alleles (data not shown).

Discussion

Ovarian cancers are extremely heterogeneous at the clinical, histopathological, and molecular level [28]. The development of ovarian cancer is associated with an accumulation of genetic alterations and the presence of some polymorphisms in numerous cancer related genes [29]. These cancer related genes encode compounds of the steroid hormone pathway and its metabolic enzymes, components involved in cell cycle control, DNA repair and mismatch repair, and proteins linked to cellular signaling and chromosome segregation [29, 30]. These genetic factors may modulate cellular signaling pathways, the accumulation of genetic mistakes, and may increase cell proliferation, all of which are some of the features of ovarian carcinogenesis [29].

Recent findings have suggested that the initiation of ovarian carcinogenesis requires both genetic and epigenetic aberrations [31, 32]. To date it has been reported that multiple tumor suppressor genes and several cancer-associated genes are silenced via the hypermethylation of DNA regulatory sequences in ovarian cancer [32]**.** Moreover, ovarian tumorigenesis is also accompanied by global DNA hypomethylation, which increases the expression of many oncogenes, proto-oncogenes, cancer-promoting genes and cancer-associated genes [33].

We studied functional variants of genes that encode enzymes involved in either directly or indirectly providing a methyl group for DNA methylation. Our study did not reveal a significant association of *BHMT* Arg239Gln, *MTHFD1* Arg653Gln, *MTHFR* Ala222Val, *MTR* Asp919Gly, *MTRR* Ile22Met and *TCN2* Arg259Pro genotypes or alleles to ovarian cancer incidence. However, in these patients, we found frequent methylation of the *CDH13* promoter in cancerous tissues, which was also observed at a similar level in other studies [26, 27].

To date little is known of the *BHMT* Arg239Gln and *TCN2* Arg259Pro polymorphism contribution to ovarian cancer. The lack of an association to ovarian cancer of the *BHMT* Arg239Gln polymorphism in our study can be supported by the findings of Kotsopoulos et al., who did not find that dietary betaine and choline intake affected ovarian cancer incidence [34]. However, the *BHMT* 239Gln gene variant had previously been associated with the risk of cervical cancer incidence in a cohort from the Polish population [35]. The *BHMT* 239Gln gene variant has been also found to be a factor that reduces the risk of breast cancer mortality and the incidence of colorectal adenoma in patients with high dietary intake of folate and methionine and low alcohol consumption [22, 23, 36]. By contrast, the *TCN2* Arg259Pro polymorphism has been found in linkage with the CpG island methylator phenotype in colorectal cancer [19].

Currently there has been no reported contribution of the *MTHFD1* Arg653Gln, *MTR* Asp919Gly and *MTRR* Ile22Met polymorphisms to ovarian cancer [37]. Moreover, this observation was partially supported by Kotsopoulos et al., who did not indicate a clear relationship between supplemental intake of folate and methionine and the incidence of ovarian cancer [38]. However, the *MTHFD1* Arg653Gln polymorphism has been

Table 3 Contribution of *BHMT*, *MTHFD1*, *MTHFR*, *MTR*, *MTRR*, and *TCN2* polymorphisms to ovarian cancer incidence

Gene	rs no.	Genotype	Cases (frequency)	Controls (frequency)	Odds ratio (95% CI)	P^a	Power (%)[b]	P_{trend}[c]
BHMT	rs3733890	GG	64 (0.48)	67 (0.42)	Referent	–		0.9362
		GA	47 (0.35)	76 (0.47)	0.647 (0.393–1.067)	0.0874	45	
		AA	23 (0.17)	17 (0.11)	1.416 (0.693–2.894)	0.3384	17	
		GA + AA	70 (0.52)	93 (0.58)	0.788 (0.496–1.251)	0.3119	17	
Minor allele frequency			0.35	0.34	0.986 (0.701–1.387)	0.9339	5	
MTHFD1	rs2236225	GG	33 (0.25)	39 (0.24)	Referent			0.9653
		GA	72 (0.53)	85 (0.53)	1.001 (0.572–1.753)	0.9970	5	
		AA	30 (0.22)	36 (0.23)	0.985 (0.504–1.926)	0.9644	5	
		GA + AA	102 (0.75)	121 (0.76)	0.996 (0.584–1.698)	0.9890	5	
Minor allele frequency			0.49	0.49	1.007 (0.728–1.392)	0.9665	5	
MTHFR	rs1801133	CC	67 (0.50)	63 (0.39)	Referent	–		0.1226
		CT	55 (0.41)	79 (0.50)	0.655 (0.402–1.065)	0.0873	43	
		TT	13 (0.09)	18 (0.11)	0.679 (0.307–1.500)	0.3366	16	
		CT + TT	68 (0.50)	97 (0.61)	0.659 (0.415–1.047)	0.0772	42	
Minor allele frequency			0.30	0.36	1.309 (0.926–1.851)	0.1271	36	
MTR	rs1805087	AA	80 (0.59)	104 (0.65)	Referent	–		0.1722
		AG	53 (0.39)	47 (0.29)	1.466 (0.899–2.391)	0.1246	34	
		GG	3 (0.02)	9 (0.06)	0.433 (0.114–1.653)	0.2440[d]	26	
		AG + GG	56 (0.41)	56 (0.35)	1.300 (0.811–2.083)	0.2749	19	
Minor allele frequency			0.22	0.20	0.920 (0.619–1.368)	0.6812	7	
MTRR	rs1801394	AA	47 (0.35)	63 (0.39)	Referent	–		0.9656
		AG	68 (0.51)	68 (0.43)	1.340 (0.808–2.223)	0.2557	24	
		GG	19 (0.14)	29 (0.18)	0.878 (0.440–1.753)	0.7125	7	
		AG + GG	87 (0.65)	97 (0.61)	1.202 (0.747–1.935)	0.4479	12	
Minor allele frequency			0.40	0.39	0.965 (0.712–1.384)	0.9651	5	
TCN2	rs1801198	CC	50 (0.37)	48 (0.30)	Referent	–		0.2488
		CG	58 (0.43)	77 (0.48)	0.723 (0.429–1.219)	0.2234	28	
		GG	26 (0.20)	35 (0.22)	0.713 (0.375–1.358)	0.3026	20	
		CG + GG	84 (0.63)	112 (0.70)	0.720 (0.442–1.172)	0.1852	26	
Minor allele frequency			0.41	0.46	1.220 (0.879–1.694)	0.2336	22	

[a] χ^2 analysis

[b] Statistical power and sample size were calculated using the power and sample size calculation program v. 2.1.30 based on uncorrected χ^2 test procedure

[c] χ^2 test for trend

[d] Fisher exact test

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

Genes and rs numbers	Testing balanced accuracy[a]	Cross validation consistency[b]	P value[c]
*BHMT*_ rs3733890	0.4689	6/10	0.9970
*MTRR*_ rs1801394, *TCN2*_ rs1801198	0.5041	7/10	0.8260
*MTHFD1*_ rs2236225, *MTRR*_ rs1801394, *TCN2*_ rs1801198	0.4813	6/10	0.9460
*BHMT*_rs3733890, *MTHFD1*_ rs2236225, *MTRR*_ rs1801394, *TCN2*_ rs1801198	0.4662	4/10	0.9990

[a] Testing balanced accuracy is the accuracy of classification of cases and controls in the testing dataset calculated as (Sensitivity + Specificity)/2

[b] Cross validation consistency is the number of times the model was selected as the best model after tenfold cross validation runs

[c] Significance of accuracy (empirical P value based on 1,000 permutations)

associated with head and neck cancer (HNSCC) in patients that smoke and abuse alcohol [39]. Individuals with the *MTHFD1* 653Gln variant demonstrated a lower probability of event-free survival in childhood acute lymphoblastic leukemia (ALL) [40]. The *MTR* 919Gly gene variant was found to be a risk factor of HNSCC, breast cancer and colorectal cancer occurrence in alcoholics, and increase the susceptibility to retinoblastoma, multiple myeloma and non-Hodgkin's lymphoma (NHL) [41–46]. The *MTRR* 22Met gene variant has been linked to HNSCC, lung cancer in smokers, esophageal squamous cell, meningioma, and colorectal carcinomas, and pancreatic cancer in patients with a drinking habit [24, 25, 47–50]. The *MTRR* Ile22Met polymorphism has also been associated with breast cancer and folate intake in postmenopausal women [51].

In contrast to the other studied polymorphisms, the *MTHFR* Ala222Val polymorphism was found as risk for ovarian cancer development [52, 53], while this finding was not observed by Terry et al. and Kelemen et al. [37, 54]. Gershoni-Baruch et al. showed an association between the *MTHFR* Ala222Val polymorphism and ovarian cancer risk in Jewish women [52]. Moreover, Jakubowska et al. demonstrated a statistically significant higher frequency of the *MTHFR* 222Val gene variant in patients with ovarian cancer in a Polish cohort from the Western Pomeranian area of Poland [53]. We found a higher *MTHFR* Val/Val and Val/Ala genotype frequency in patients with ovarian cancer than controls in a Polish population from the Wielkopolska region of Poland. However, the lack of statistical significance in our study can be due to the small sample size. The *MTHFR* 222Val gene variant has also been found to be a risk factor in the incidence of HNSCC, esophageal, lung, breast, stomach, and proximal colon cancers, NHL and ALL, meningioma and glioma [46, 48, 55–61].

Our study did not show an association between ovarian cancer and the six studied SNPs in folate and choline metabolism genes, namely *BHMT*, *MTHFD1*, *MTHFR*, *MTR*, *MTRR*, and *TCN2*. Moreover, we did not observe an interaction between these polymorphic variants in patients with ovarian cancer. Our genetic study was performed on a limited number of women with ovarian cancer and controls, therefore the role of these genetic variants should be further studied in other populations.

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