Association between *DNMT3L* polymorphic variants and the risk of endometriosis-associated infertility

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Abstract. Endometriosis is considered to be an epigenetic disease. It has previously been reported that the DNA methyltransferase 3-like (*DNMT3L*) rs8129776 single nucleotide polymorphism (SNP) contributes to endometrioma. In the present study, high-resolution melting curve analysis was used to investigate the risks associated with the *DNMT3L* c.910-635A/G (rs8129776), c.832C/T (rs7354779), c.812C/T (rs113593938) and c.344+62C/T (rs2276248) SNPs on stage I-II endometriosis-associated infertility. Included in the present study were patients presenting with stage I-II endometriosis-associated infertility (n=154) and a control cohort of healthy patients with confirmed fertility (n=383). No significant association between the above-listed *DNMT3L* SNPs and the development of endometriosis-associated infertility was identified. The lowest P-values generated from trend analysis were observed in the *DNMT3L* c.832C/T (rs7354779) SNP (P_{trend}=0.114). Furthermore, haplotype analyses of the *DNMT3L* SNPs failed to reveal any risk association between the development of endometriosis-associated infertility and the above-listed polymorphisms, even when the SNPs were present in combinations. Finally, a meta-analysis was performed to examine the association between the *DNMT3L* rs8129776 SNP and the development of endometrioma, from which no association between the two was identified. On the basis of these results, the present study has demonstrated that variations in the *DNMT3L* gene do not contribute to stage I-II endometriosis-associated infertility.

Introduction

Endometriosis is a gynecological disorder, which is characterized by the combined and multifocal localization of ectopic endometrial implants in the abdominal organs and abdominal cavity (1), which are frequently accompanied by clinical manifestations that include severe chronic pelvic pain and infertility (1-3). It is considered that endometrial lesions arise from additive interactions between genetic and environmental factors (2).

The ability of endometrial implants to survive outside of the uterus results from their increased estrogen activity, overactive oncogenic pathways and increased production of prostaglandins, cytokines and metalloproteinases (4,5). Additionally, the survival of endometrial implants can be prolonged due to abnormal immune system functioning, such that the ectopic endometrium does not get flagged for removal from the body (4,5).

There is increasing evidence to suggest that endometriosis is an epigenetic disease (6-9). Epigenetic regulators of gene transcription include DNA methylation patterns and histone modifications, which do not require direct changes to the DNA sequence (10). Several genes have been identified to possess abnormal methylation and expression patterns in women presenting with endometriosis (11-18). DNA methylation is produced by DNA methyltransferase enzymes (DNMTs) (10). These include the maintenance methyltransferase, DNMT1, as well as the *de novo* DNMT3A and DNMT3B enzymes (10). Aberrant expression levels of DNMT1, DNMT3A and DNMT3B have been demonstrated in patients with endometriosis (17). There is an additional member of the human DNMT3 family, DNMT3-like (DNMT3L), which does not possess methyltransferase activity itself, but acts in a co-operative manner with DNMT3A and DNMT3B (19).

The presence of subtelomeric hypermethylated regions of DNA, as well as regions of hypomethylated DNA, which are distributed along the chromosomes, has been demonstrated in all types of endometriosis (20). Additionally, it has been demonstrated that the *DNMT3L* variant, rs113593938, which is situated within exon 10, makes an important contribution to subtelomeric hypomethylation (21). Previous reports have also identified an association between the *DNMT3L* rs8129776 gene variant and the development of endometrioma (22). Therefore, the present study aimed to determine the relative contributions of the *DNMT3L* c.910-635A/G (rs8129776), c.832C/T (rs7354779), c.812C/T (rs113593938) and c.344+62C/T (rs2276248) single nucleotide polymorphisms

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(SNPs) on the development of stage I-II endometriosis. To address this question, women were selected from the Polish population with defined stage I-II endometriosis, according to the revised American Society for Reproductive Medicine classification (23).

Materials and methods

Study subjects. Peripheral blood samples were extracted from infertile women with endometriosis (n=154) and from healthy control individuals (n=383). The patients were recruited through the Gynecologic and Obstetrical University Hospital, Division of Reproduction at Poznan University of Medical Sciences (Poznan, Poland). Suspected cases of pelvic endometrioma were investigated laparoscopically (Table I). Visualization of endometriotic lesions and histopathological criteria were used to diagnose minimal endometriosis in infertile women. Each case of endometriosis was staged according to the revised classification of the American Society for Reproductive Medicine (23). The patients in the experimental cohort in the present study exhibited regular menses, an anatomically intact reproductive tract and infertility spanning at least 1 year, despite the desire for conception. The infertility was confirmed not to be a result of male factor infertility. The control patient cohort in the present study included fertile women of reproductive age, who were identified not to have any malignant disease, endometriosis or adenomyosis following surgical examinations performed during cesarean section. Each of the women in the control group had regular menses and an anatomically intact reproductive tract. Additional inclusion and exclusion criteria for the patient cohorts have been previously described in detail (24). The study subjects were matched by age, and were all Caucasians of Polish descent (Table I). Written informed consent was obtained from all the individuals involved in the present study, and all procedures were approved by the ethics committee of Poznan University of Medical Sciences (Poznan, Poland).

Evaluation of the presence of DNMT3L SNPs. The genomic DNA was isolated from peripheral blood leukocytes by salt extraction. The SNPs used in the present study were selected on the basis of previous results (24), and are shown in Fig. 1. DNA samples were genotyped for four DNMT3L SNPs, including c.910-635A/G (rs8129776), c.832C/T (rs7354779), c.812C/T (rs113593938) and c.344+62C/T (rs2276248; Table I and Fig. 1). Genotyping was performed via high-resolution melting (HRM) curve analysis using the LightCycler 480 system (Roche Diagnostics, Mannheim, Germany) with 5X HOT FIREPol EvaGreen HRM mix (Solis BioDyne, Tartu, Estonia). The PCR program consisted of an initial step at 95˚C for 15 min to activate HOT FIREPol DNA polymerase, followed by 50 amplification cycles of denaturation at 95˚C for 10 sec, primer-dependent annealing at 60.6˚C for 10 sec, and elongation at 72˚C for 15 sec. Amplified DNA fragments were then subjected to HRM with 0.1˚C increments in temperature ranging from 76-97˚C. Primer sequences and conditions for HRM analysis are presented in Table II. The quality of genotyping was evaluated by repeating measurements on a randomly-selected 10% of the samples.

Table I. Clinical characteristics of the patients with endometriosis and control subjects.

Characteristic	Endometriosis	Control
Number	154	383
Age (years)	31 (20-42)[a]	31 (21-39)[a]
Parity	NA	1 (1-2)[a]
Duration of infertility (years)	3 (1-6)[a]	NA
rASRM (stage)	Stage I (n=85) Stage II (n=69)	NA

[a]Data are presented as the median (range). rASRM, revised American Society for Reproductive Medicine classification (23); NA, not applicable.

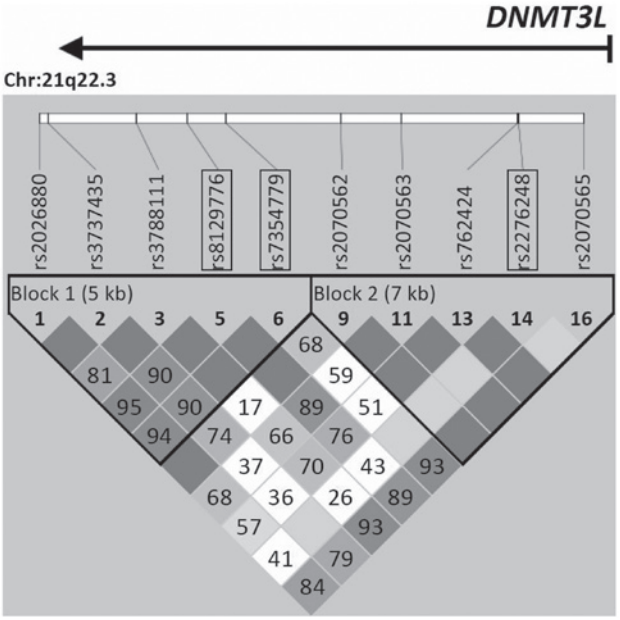

Figure 1. An LD plot of HapMap SNPs within the DNMT3L gene. The plot was generated using the genotypic data from the HapMap CEU samples and the Haploview 4.0 software package (Broad Institute, Cambridge, MA, USA). The names of the SNPs selected for examination are enclosed in boxes, and the asterisks correspond to the SNPs available in HapMap. The numbers within diamonds indicate the percentage of LD between a given pair of SNPs (D' values). LD, linkage disequilibrium; SNP, single nucleotide polymorphism; *DNMT3L*, DNA methyltransferase 3-like.

Statistical analysis. For each SNP, the Hardy-Weinberg equilibrium (HWE) was assessed using Pearson's goodness-of-fit χ^2 statistic. Differences in allele and genotype frequencies between the case and control subjects were evaluated using Fisher's test. The associations between the SNPs selected for the present study and the development of endometriosis were evaluated using the Cochran-Armitage trend test. Statistical analyses were conducted using Statistica version 10 (Stat Soft, Inc., Tulsa, USA). Odds ratios (ORs) and associated 95% confidence intervals (95% CIs) were also determined. The data were analyzed using recessive and dominant inheritance models. Pair-wise linkage disequilibrium (LD) between the selected SNPs was computed as D' and r^2 values

Table II. High-resolution melting curve conditions for genotyping of *DNMT3L* polymorphic variants.

rs no.	Chromosome location	SNP function	Alleles[a]	MAF[b]	Primers for PCR amplification (5'-3')	PCR product length (bp)	Annealing temp. (˚C)	Melt temp. range (˚C)
rs8129776	Chr21:45669629	Intron	A/G	0.38	F: GAACAGAGGTCGTAAGTTCCA R: GTTATGGAGGAGCGGTGA	85	57.8	76-91
rs7354779	Chr21:45670770	Missense p.Arg278Gly	C/T	0.25	F: CACCAGATTGTCCACGAAC R: GGTACCTGTTCCAGTTCCAC	95	57.8	80-95
rs113593938	Chr21:45670790	Missense p.Arg271Gln	C/T	0.01	F: GGGGCTGCCTGGCTTGGGC R: CCTCAGCCCTGCCCCCTCACC	92	70.0	82-97
rs2276248	Chr21:45679258	Intron	C/T	0.02	F: AAATCCACCCACACTCCAGA R: CTGCGGAAACCCTGATTG	111	57.8	80-95

[a]According to the SNP database; the underlining denotes the minor allele in the control samples. [b]MAF, minor allele frequency from the 1000 Genomes project for the Total European Ancestry (EUR) samples. F, forward; R, reverse; SNP, single nucleotide polymorphism; PCR, polymerase chain reaction.

Table III. Association between *DNMT3L* polymorphic variants and the risk of endometriosis.

rs no.	Position	Alleles[a]	Genotype cases[b]	Genotype controls[b]	P_{trend} value	$OR_{dominant}$ (95% CI)[c]; P-value	$OR_{recessive}$ (95% CI)[d]; P-value
rs8129776	c.910-635A>G	A/G	22/74/58	51/175/157	0.509	1.150 (0.783-1.689); 0.476	1.085 (0.633-1.860); 0.767
rs7354779	c.832T>C	C/T	16/70/68	31/155/198	0.114	1.346 (0.925-1.961); 0.120	1.320 (0.700-2.491); 0.390
rs113593938	c.812C>T	C/T	0/2/152	0/2/382	0.342	2.215 (0.351-18.011); 0.324[f]	NA
rs2276248	c.344+62T>C	C/T	0/5/149	0/15/369	0.715	0.826 (0.295-2.313); 0.806[f]	NA

[a]According to the Single Nucleotide Polymorphism database; the underlining denotes the minor allele in the control samples. [b]Genotypes (dd/Dd/DD), with d as the minor allele in the controls. [c]Dominant model (dd+Dd, vs. DD) with d being the minor allele. [d]Recessive model (dd, vs. Dd+DD) with d as the minor allele. Fisher's exact test was used to determine the OR, 95% CI and P-values. CI, confidence interval; NA, not applicable; OR, odds ratio.

Table IV. Haplotype analysis of *DNMT3L* polymorphic variants.

Polymorphism	Haplotype	Frequency	Case, control ratio	χ^2	P-value	P_{corr}-value[a]
rs8129776_rs7354779	GT	0.364	0.374, 0.359	0.214	0.644	0.887
	AT	0.340	0.295, 0.358	3.994	0.046	0.109
	AC	0.291	0.322, 0.278	2.112	0.146	0.301
rs7354779_rs113593938	TC	0.703	0.666, 0.717	2.837	0.092	0.097
	CC	0.294	0.328, 0.280	2.445	0.118	0.122
rs113593938_rs2276248	CT	0.978	0.977, 0.978	0.004	0.953	1.000
	CC	0.019	0.016, 0.020	0.131	0.717	0.930
rs8129776_rs7354779_rs113593938	GTC	0.362	0.371, 0.359	0.139	0.709	0.941
	ATC	0.340	0.295, 0.358	3.992	0.046	0.094
	ACC	0.288	0.319, 0.276	2.027	0.155	0.331
rs7354779_rs113593938_rs2276248	TCT	0.684	0.649, 0.698	2.402	0.121	0.188
	CCT	0.294	0.328, 0.280	2.442	0.118	0.185
	TCC	0.019	0.016, 0.020	0.131	0.717	0.992
rs8129776_rs7354779_rs113593938_	GTCT	0.348	0.356, 0.345	0.124	0.725	0.985
rs2276248	ATCT	0.339	0.294, 0.358	3.965	0.047	0.131
	ACCT	0.285	0.318, 0.272	2.364	0.124	0.361
	GTCC	0.014	0.015, 0.014	0.007	0.933	1.000

[a]P-value calculated using the permutation test with a total of 1,000 permutations.

Table V. Linkage disequilibrium between polymorphic variants of the *DNMT3L* gene in the control samples.

	Polymorphism			
Polymorphism	rs8129776	rs7354779	rs113593938	rs2276248
rs8129776	-	0.960[a]	1.000[a]	0.554[a]
rs7354779	0.209	-	1.000	0.603[a]
rs113593938	0.001[b]	0.007[b]	-	1.000[a]
rs2276248	0.011[b]	0.003[b]	0.000[b]	-

[a]D' values; [b]r^2 values.

using HaploView 4.0 software (http://www.broadinstitute.org//scientific-community/software). HaploView 4.0 software was also used for haplotype assessment. Significant P-values were corrected using a 1,000-fold permutation test.

Meta-analysis of the c.910-635A/G (rs8129776) SNP. A meta-analysis of two independent datasets, of Borghese *et al* (22) and the present study, was performed using either fixed- or random-effect modeling. A fixed-effect model was used when no evidence of significant heterogeneity was identified between the two datasets, and a random-effect model was used when heterogeneity was observed. The heterogeneity between the two studies was assessed using χ^2-based Cochrane Q and I^2 statistics (25,26). P-values generated using Cochrane's Q test that were <0.10 were considered to indicate the presence of heterogeneity between the two datsets. I^2 values of 25, 50 and 75% were considered to indicate low, moderate and high levels of heterogeneity, respectively. The effects of contrast between alleles (G, vs. A) and between the dominant (AG+GG, vs. AA) and recessive (GG, vs. AG+AA) models were also estimated. All statistical calculations were conducted using Comprehensive Meta Analysis version 2.0 software (www.Meta-Analysis.com).

Results

Distribution of DNMT3L c.910-635A/G (rs8129776), c.832C/T (rs7354779), c.812C/T (rs113593938) and c.344+62C/T (rs2276248) polymorphisms in patients with endometriosis-associated infertility. No divergence from the HWE was identified in the frequency of any of the genotypes examined in any of the groups (P>0.05). The number of genotypes, OR and 95% CI calculations for each of the four *DNMT3L* SNPs are shown in Table III. The lowest P-values determined by the trend test were observed in the *DNMT3L* c.832C/T (rs7354779) SNP (P_{trend}=0.114). No association was

Table VI. Meta-analysis of the association between the *DNMT3L* rs8129776 polymorphism and endometriosis risk.

Model	Model and study	Odds ratio	Lower limit	Upper limit	Z-value	Heterogeneity			
						P-value	Q-value[a]	P-value[a]	I^2-value
	Dominant model (AG+GG, vs. AA)								
	Borghese *et al* (22)	0.791	0.472	1.327	-0.888	0.375	1.293	0.255	22.675
	Present study	1.150	0.783	1.689	0.712	0.476			
Fixed-effect		**1.007**	**0.739**	**1.370**	**0.042**	**0.967**			
	Recessive model (GG, vs. AG+AA)								
	Borghese *et al* (22)	0.313	0.155	0.632	-3.242	0.001	7.569	0.006	86.789
	Present study	1.085	0.633	1.86	0.296	0.767			
Random-effect		**0.596**	**0.176**	**2.010**	**-0.835**	**0.404**			
	Allelic model (G, vs. A)								
	Borghese *et al* (22)	0.622	0.436	0.889	-2.610	0.009	6.120	0.013	83.659
	Present study	1.096	0.834	1.440	0.661	0.509			
Random-effect		**0.836**	**0.480**	**1.456**	**-0.633**	**0.527**			

[a]χ^2-based Cochrane-Armitage Q-test.

identified between any of the four *DNMT3L* SNPs and endometriosis-associated infertility (Table III). In the dominant and recessive inheritance models for the c.910-635A/G polymorphism, the ORs were 1.150 (95% CI=0.783-1.689; P=0.476) and 1.085 (95% CI=0.633-1.860; P=0.767), respectively. In evaluating the same inheritance models for the c.832C/T SNP, the ORs were 1.346 (95% CI=0.925-1.961; P=0.120) and 1.320 (95% CI=0.700-2.491; P=0.390), respectively. In the dominant model for the c.812C/T and the c.344+62C/T SNPs, the ORs were measured as 2.215 (95% CI=0.351-18.011; P=0.324) and 0.826 (95% CI=0.295-2.313; P=0.806), respectively.

Association between DNMT3L haplotypes and endometriosis-associated infertility. The haplotype analyses of the *DNMT3L* SNPs did not suggest any polymorphism combination to be a risk factor for the development of endometriosis-associated infertility (Table IV). The lowest overall P-values; P=0.046, (P_{corr}=0.094) and P=0.046, (P_{corr}=0.109), were observed for haplotypes ATC (rs8129776/rs7354779/rs113593938) and AT (rs8129776/rs7354779). The *DNMT3L* SNPs featured in the present study ranged between complete and weak pairwise LD values. The D' and r^2 values, as calculated from the control samples, ranged between 0.001 and 1.000 (Table V).

Meta-analysis. The association between the *DNMT3L* rs8129776 polymorphism and the risk of endometriosis is shown in Table VI. The meta-analysis was performed between two datasets, comprising a French population from a study by Borghese *et al* (22) and the Polish population in the present study. Under the assumption of a dominant model, no heterogeneity was identified between the studies (Q test P-value=0.255; I^2=22.7%). The OR (fixed-effect model) for patients with endometriosis with AG+GG, vs. AA was 1.007 (95% CI: 0.739-1.370; P=0.967). Under the assumption of a recessive and allelic model, a high level of heterogeneity was observed between the two datasets (Q test P-value=0.006 and 0.013, respectively; I^2>83%). The OR (random-effect model) for patients with endometriosis with the GG, vs. AG+AA was 0.596 (95% CI: 0.176-2.010; P=0.404). The OR (random-effect model) for the G allele in patients with endometriosis was 0.836 (95% CI: 0.480-1.456; P=0.527).

Discussion

Endometriosis is an estrogen-dependent inflammatory disease, which is considered to arise in response to epigenetic changes (13,16,27). Such changes lead to enhancements in the estrogen activity and invasiveness of endometriotic cells, and may be associated with hypomethylation of the steroidogenic factor-1 (*SF-1*), aromatase, estrogen receptor 2 and E-cadherin (13,16,27,28) genes. By contrast, infertility in women presenting with endometriosis may be associated with the hypermethylation of the homeobox A10 (*HOXA10*), *HOXA11* and progesterone receptor (*PR-B*) genes (14,15,28,29).

DNMT3L is one of the essential molecules involved in *de novo* DNA methylation during the epigenetic reprogramming of cells (30). DNMT3L interacts with either DNMT3A or DNMT3B to effect *de novo* DNA methylation. DNMT3L may also bind to selective chromatin regions that have specific histone modifications capable of modulating DNMT3A action (31-33). The ability of DNMT3L to bind to histone deacetylase 1 further suggests the active role that DNMT3L has in regulating transcriptional repression at the chromatin level (34). It has also been suggested that hyperacetylation of histones may account for the overexpression of G-protein-coupled estrogen receptor 1, *SF-1* and hypoxia-inducible factor-1α in endometrial lesions (35-38). Furthermore, decreased expression and hypoacetylation of histones have been observed in endometriotic cells for the CCAAT/enhancer-binding protein, cyclin-dependent kinase *(CDK)* inhibitor 2A, CDK inhibitor 1A, CDK inhibitor 1B, checkpoint kinase 2, death receptor 6, E-cadherin and *HOXA10* genes (8,35,38,39).

The *DNMT3L* c.812C/T transition (rs113593938), associated with subtelomeric hypomethylation, leads to the replacement of an arginine residue with glutamine at position 271 of the DNMT3L protein, which can effect the stimulation of DNMT3A (21). No significant associations between the *DNMT3L* rs8129776, rs7354779, rs113593938 or rs2276248 polymorphisms and stage I-II endometriosis-associated infertility were identified in the present study, either on an individual SNP basis or in combinations of SNPs. By contrast, Borghese *et al* (22) demonstrated an association between rs8129776 and the development of endometrioma (22), and it was further demonstrated that the ACCC+T haplotypes for rs8129776, rs7354779, rs113593938 and rs2276248 served as risk factors for endometrioma (22). Such discrepancies between studies may be due to the use of patient cohorts presenting with different classes of endometriosis. Neither the meta-analyses performed by Borghese *et al* (22) nor the meta-analysis performed in the present study revealed any association between the rs8129776 SNP and the development of endometriosis.

In addition, two genome-wide association studies (GWAS), which were performed in Caucasian and Japanese populations failed to demonstrate an association between *DNMT3L* SNPs and endometriosis (40,41). However, the Caucasian patients included in these studies presented with an assortment of disparate categories of endometriosis (40), although no histological analyses were performed to confirm the presence of systematic endometriosis in the Japanese patient cohort (41). The GWAS also eliminated several of the genetic variants, which were demonstrated to be associated with disease, due to low significance.

In conclusion, the present study demonstrated that the *DNMT3L* SNPs; rs8129776, rs7354779, rs113593938 and rs2276248, are not risk factors for endometriosis. The present study was performed on patients presenting with infertility and stage I-II endometriosis, and further investigations are required in the future to include additional categories of endometriosis, using a larger groups of patients from disparate cohorts.

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