

Research Article

Polymorphisms in the 3' UTR Region of *ESR2* and *CYP19A1* Genes and Its Influence on Allele-Specific Gene Expression in Endometriosis

Beata Smolarz ¹, Tomasz Szaflik,² Hanna Romanowicz,¹ and Krzysztof Szyłło²

¹Laboratory of Cancer Genetics, Department of Pathology, Polish Mother's Memorial Hospital Research Institute, Rzgowska 281/289, 93-338 Lodz, Poland

²Department of Operative Gynaecology and Oncological Gynaecology, Polish Mother's Memorial Hospital Research Institute, Rzgowska-Lodz, Poland

Correspondence should be addressed to Beata Smolarz; smolbea@wp.pl

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Objectives. Endometriosis is supported by hormonal, immunological, and environmental factors. No specific marker for endometriosis has yet been identified. *ESR2* and *CYP19A1* genes play a major role in the hormonal control of endometriosis women, the development of which largely depends on steroid hormones. **Aim.** An analysis of *ESR2* and *CYP19A1* allele-specific gene expressions in the context of the risk for endometriosis occurrence. **Methods.** The study material included paraffin-embedded tissue specimens, collected from patients ($n = 100$) with endometriosis. Blood samples from age-matched, endometriosis-free women ($n = 100$) served as a control. The RT-PCR technique was performed to observe the expression of *ESR2* and *CYP19A1* genes. Moreover, Sanger's sequencing method was applied for polymorphism analysis. **Results.** A set of 4 single-nucleotide polymorphisms (SNPs) was determined; all of them most significantly associated with endometriosis: rs4986938 (G>A) (chromosome 14), rs928554 (A>G) (chromosome 14), rs10046 (C>T) (chromosome 15), and rs4646 (C>A) (chromosome 15). There were no differences in the distribution of genotypes and alleles in the studied groups, taking into account *ESR2* and *CYP19A1* gene expressions. **Conclusion.** The *ESR2* and *CYP19A1* polymorphisms may not be correlated with endometriosis susceptibility. Further analysis is needed to specify the role of these polymorphisms in the pathogenesis of endometriosis.

1. Introduction

Endometriosis is an oestrogen-dependent disease affects 10-15% of women in the reproductive age and 35-50% of women with pains in the pelvis minor and/or with infertility [1]. Aetiology of the disease is complex. Hormonal, immunological, and environmental factors are responsible for its formation [2-4]. In recent years, special attention has been paid to the genetic mechanisms that might have a significant impact on the increased incidence of endometriosis.

CYP19A1, aromatase encoding gene, is an enzyme involved in biosynthesis of oestrogens. Different expression levels of aromatase were determined in the endometriosis

foci in comparison to the eutopic endometrium. The exact basis of observed changes remains unknown [5].

Endometriosis lesions, unlike the eutopic endometrium, demonstrate some activity of the aromatase [6].

The activity of oestrogens on target cells is possible via oestrogen receptors. Oestrogen receptors (ERs) acting as transcription factors play a significant role in the growth and differentiation processes of endometrial cells, as well as in numerous biological functions in the eutopic endometrium and endometriosis. The two following types of oestrogen receptors have thus far been identified: ER α and ER β , encoded by two different genes (*ESR1* and *ESR2*, respectively). ER β is the predominant isoform in patients with endometriosis [7, 8].

TABLE 1: Characteristic features of the patients ($n = 100$) and of the controls ($n = 100$).

Patients group		Control group	
Age (range) 21-53 years		Age (range) 26-67 years	
Age (mean) 34.89 ± 7.49		Age (mean) 37.93 ± 6.01	
Patients group		Control group	
Body mass index (BMI) (kg/m^2)	The number (%)	Body mass index (BMI) (kg/m^2)	The number (%)
<25	68 (68%)	<25	29 (29%)
$25 \leq \text{BMI} < 30$	22 (22%)	$25 \leq \text{BMI} < 30$	43 (43%)
≥ 30	10 (10%)	≥ 30	28 (28%)
Deliveries > 0	The number (%)	Deliveries > 0	The number (%)
Yes	47 (47%)	Yes	93 (93%)
-1	21 (21%)	-1	36 (36%)
-2 and more	26 (26%)	-2 and more	57 (57%)
No	53 (53%)	No	7 (7%)
Spontaneous abortions	The number (%)	Spontaneous abortions	The number (%)
Yes (spontaneous)	5 (5%)	Yes (spontaneous)	9 (9%)
No	95 (95%)	No	91 (91%)
Clinical stage	The number (%)		
I	26 (26%)		
II	25 (25%)		
III	17 (17%)		
IV	32 (32%)		

Different expression levels of aromatase were determined in the endometriosis foci in comparison to the eutopic endometrium. [9].

Previous studies have been demonstrated that both ERs are expressed in human endometriotic tissues but it has been shown that there are significantly increased levels of oestrogen receptor ER β and decreased levels of ER α in the endometriotic lesions in comparison with the eutopic endometrium. [10–12].

The literature data showed that a number of specific SNPs in the 3'UTR regions have structural consequences that may result in the emergence of phenotypic manifestations in the form of the disease [13]. 3'UTR fragments are within the regulatory elements of genes and play an important role in the translation and distribution of RNA.

The reported study was aimed at finding out whether there were any correlations between the allele-specific gene expression of the *ESR2* and *CYP19A1* genes and the incidence of endometriosis.

The goals of the study included (a) an analysis of the *ESR2* and *CYP19A1* gene expression levels in patients with endometriosis and in a control group, (b) an analysis of the *ESR2* and *CYP19A1* gene polymorphisms in the 3'UTR region in patients with endometriosis and in a control group, (c) a correlation of the assayed levels of the *ESR2* and *CYP19A1* gene expressions with polymorphic variants, and (d) the assessment of the significance of obtained results in the context of the risk for endometriosis.

2. Materials and Methods

2.1. Patients. 100 tissue specimens, collected from patients with endometriosis ($n = 100$) embedded into paraffin blocks (Archive of the Department of Clinical Pathomorphology, Polish Mother's Memorial Hospital Research Institute in Lodz, Poland) provided the material for studies (Table 1). Blood samples from age-matched, endometriosis-free women ($n = 100$) served as the control. The clinical staging was carried out in the patients by the rASRM (The Revised American Society for Reproductive Medicine classification of endometriosis 1996) (Table 1). The study protocol was approved by the Bioethical Committee of the Polish Mother's Memorial Institute (approval number: 8/2016).

2.2. DNA Isolation. Genomic DNA was prepared, using a QIAamp DNA FFPE Tissue Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instruction. DNA was extracted from the blood, using a commercially available QIAamp DNA purification kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instruction.

2.3. Detection of Polymorphisms in the *ESR2* and *CYP19A1* Genes. A reaction mixture of the following composition was used for PCR: 2.5 μl of 10x concentrated PCR buffer, 1 μl of each primer, 0.2 μl of Taq polymerase with 5 U/ μl activity, 1 μl of a 200 μM mixture of nucleotides (dNTPs), and 100 ng matrix. The reaction mixture was made up to a final volume of 25 μl . The primers were designed using the Primer3 program (Table 2). For all primer pairs, the amplification reactions were carried out using the following thermal

TABLE 2: The nucleotide sequence of the amplified 3'UTR region of the *ESR2* and *CYP19A1* genes.

ESR2

Forward 5'-GGGCAGAAAAGGCCTCTCA-3'

Reverse 5'-GAAGCCTCAGCTTTCTACATTGG-3'

Complete nucleotide sequence

```

gggcagaaaaggcctctcaaacac
tcacctattggaatgaagatggagactct
tttgctgaagcaacgatggagc
agtgcaccttaatacaactcggctaaagaaaatctgggtaacat
ttcactcagttccctctgggatcatgtaacatcagaaaaataat
ttaaagaagagtgtaaaacttgaagtt
agttatgtgttaaaaac
cacctctcttctatta
tcaatccaaca
ttgataactgtaaagccta
aagtgaagcggatt
ctctcagatggtctccta
actgccaggcctgc
agatgtctcaccatgagggccacaatgtagaaaagc
tgaggcttc
    
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CYP19A1

Forward 5'-CCTTGCACCCAGATGAGAC-3'

Reverse 5'-CAGAGCCAAGAGTTTGAGG-3'

Complete nucleotide sequence

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ccttgaccagatgagacgggag
ttctggaggctgaa
cacgtggaggcaaacagga
aggtgaagaagaacttatcctatcaggacgga
aggtcctgtgctcgggatctccagacgtcgca
ctcaaatgccctctgaggtcaaggaa
cacaagatggtttgaaatgctga
accgcatacattataacatcac
cagcatcgtcctgaagccatgc
ctgctgccaccatgccagctcctgc
tcctcactggcctcaaaccttggcctctg
    
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profile: pre-PCR→94°C, 4 minutes; PCR→94°C, 20 seconds; 59°C, 20 seconds; 72°C, 40 seconds (35 cycles); post-PCR→72°C, 10 minutes.

2.4. *Sequencing of PCR Products.* The PCR reaction was performed on a GeneAmp 2400 PCR System thermocycler (Perkin Elmer), and the process products were purified, precipitating them with ethanol and EDTA. Automatic sequence reading was performed using the ABI PRISM™ 377 automatic DNA sequencer (Applied Biosystems) and the Big Day™ Terminator Cycle sequencing kit Ready Reaction Kit (Applied Biosystems) according to the manufacturer's instructions. The results were analysed using the Sequencing Analysis Software™ ver. 3.4.1 (Applied Biosystems) and Factura™.

2.5. *RNA Isolation.* Total RNA was isolated with the TRIzol reagent (Ambion, the USA). RNA samples were stored in the temperature of -20°C.

2.6. *RT-PCR Reaction.* A reverse transcription reaction was carried out, using a TaKaRa RNA PCR kit (AMV) ver 3.0 (Takara Bio INC, Japan). The obtained cDNA samples were stored in the temperature of -20°C.

2.7. *PCR with an Analysis of Product Volume Growth in Real Time.* The reaction mixture included 0.5 µl of cDNA, 0.5 µl of 20x TaqMan® Gene Expression Assay (Applied Biosystems, USA), and 5 µl of TaqMan® Universal PCR Master Mix (Applied Biosystems, USA), containing TaqMan® DNA polymerase, dNTP, a reaction buffer, and 4 µl of water. Real-time PCR was carried out in a Mastercycler® ep realplex device (Eppendorf, Germany). The thermal profile of the reaction included a preliminary denaturation in the temperature of 95°C for 10 minutes, followed by 50 cycles of 15-second incubation in 95°C, combined with 1 minute in 60°C. The following, commercially available kits of probes and starters were applied in the real-time PCR: Hs01100353_m1 for *ESR2* gene, Hs00903411_m1 for *CYP19A1* gene, and Hs02800695_m1 for *HPRT1* (hypoxanthine phosphoribosyltransferase 1) gene, being a reference gene. The yield and quality (260/280 optical density ratios) of the RNA products were determined using PicoDrop spectrophotometer (Picodrop Limited, Hinxton, UK) (Figure 1). The purified total RNA was immediately used for cDNA synthesis or stored at -80°C until use.

2.8. *Statistical Analysis.* The obtained results were statistically processed by means of STATISTICA 11 software (StatSoft, Poland). The significance of differences was analysed at the level of gene expression and mRNA, using nonparametric tests (the Mann-Whitney *U* test and the Kruskal-Wallis test) for a lack of distribution normality of the obtained results, as confirmed by the Shapiro-Wilk test. The *R* Spearman test was used to assess correlations between variables. Statistical analysis of the distribution of genotypes and alleles in the test and control group was carried out after prior confirmation that the obtained systems remain in a state of equilibrium according to the rules of Hardy and Weinberg. Wild type of the genotype and allele was the reference group. The result was

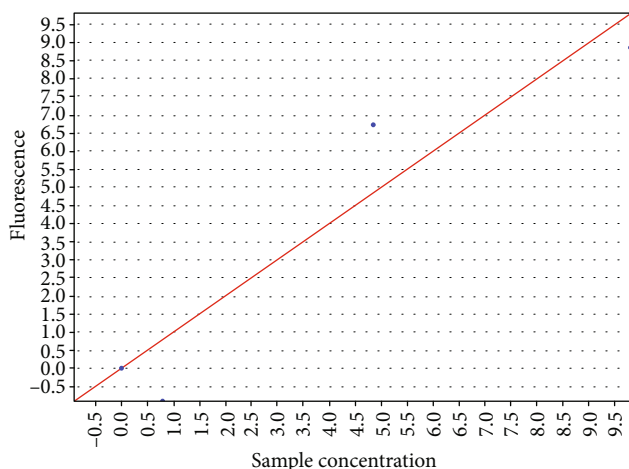


FIGURE 1

TABLE 3: The chromosomal coordinates of the four studied SNPs. (<https://www.ncbi.nlm.nih.gov/genome/guide/human/>).

	Location	Reference
rs4986938	Chromosome 14, NC_000014.9 (64226707..64338631, complement)	GRCh38 38.1/141
rs928554	Chromosome 14, NC_000014.9 (64226707..64338631, complement)	GRCh38.p12
rs10046	Chromosome 15, NC_000015.10 (51208057..51338596, complement)	GRCh38 38.1/141
rs4646	Chromosome 15, NC_000015.10 (51208057..51338596, complement)	GRCh38 38.1/141

TABLE 4: Relative *ESR2* gene expression with regard to the polymorphism rs4986938.

<i>ESR2</i>	Median	25th percentile	75th percentile	<i>p</i> value ^a
GG (73%)	201.27	112.42	365.32	
GA (25%)	169.80	104.73	214.31	0.46
AA (2%)	—	—	—	
<i>p</i> trend ^b	0.35			

^aKruskal-Wallis test. ^bTesting additive genetic model (Cochran-Armitage test for trend).

TABLE 5: Relative *ESR2* gene expression with regard to the polymorphism rs928554.

<i>ESR2</i>	Median	25th percentile	75th percentile	<i>p</i> value ^a
AA (48%)	601.95	151.41	772.82	
AG (52%)	179.73	159.26	557.43	0.11
GG (0%)	—	—	—	
<i>p</i> trend ^b	0.10			

^aMann-Whitney *U* test. ^bTesting additive genetic model (Cochran-Armitage test for trend).

TABLE 6: Relative *CYP19A1* gene expression with regard to polymorphism rs10046.

<i>CYP19A1</i>	Median	25th percentile	75th percentile	<i>p</i> value ^a
CC (37%)	299.24	155.66	1089.46	
CT (63%)	514.13	254.23	614.31	0.23
TT (0%)	—	—	—	
<i>p</i> trend ^b	0.27			

^aMann-Whitney *U* test. ^bTesting additive genetic model (Cochran-Armitage test for trend).

TABLE 7: Relative *CYP19A1* gene expression with regard to polymorphism rs4646.

<i>CYP19A1</i>	Median	25th percentile	75th percentile	<i>p</i> value ^a
CC (48%)	174.13	151.41	572.82	
CA (51%)	209.73	159.26	557.43	0.18
AA (1%)	—	—	—	
<i>p</i> trend ^c	0.62			

^aMann-Whitney *U* test. ^bTesting additive genetic model (Cochran-Armitage test for trend).

considered statistically significant at the significance level *p* less than 0.05.

3. Results

The performed analysis was aimed at determining the significance of new genetic variants as endometriosis risk factors. PCR analysis identifies statistically significant correlations among SNPs localised on chromosomes 14 and 15 (Table 3). A pool of 4 SNPs mostly correlated with endometriosis risk was determined: rs4986938 (G>A) (chromosome 14), rs928554 (A>G) (chromosome 14), rs10046 (C>T) (chromosome 15), and rs4646 (C>A) (chromosome 15).

In the females with endometriosis, GG, GA, and AA genotypes within *ESR2* rs4986938 SNP were observed in 73% (73/100), 25% (25/100), and 2% (2/100) of the studied individuals, respectively (Table 4). In the case of rs928554, AA, GA, and GG genotypes were found in 48% (48/100), 52% (52/100), and 0% (0/100) of the studied patients, respectively (Table 5).

Taking into account the nonendometriosis control females, the GG, GA, and AA genotypes in rs4986938 polymorphism were found in 61% (61/100), 32% (32/100), and 7% (7/100) of the patients, respectively. Regarding rs928554, the AA, AG, and GG genotypes were observed in 51 (51/100), 49% (49/100), and 0% (0/100) of the studied individuals, respectively.

In the females with endometriosis, CC, CT, and TT genotypes within *CYP19A1* rs10046 SNP were observed in 37% (37/100), 63% (63/100), and 0% (0/100) of the studied individuals, respectively (Table 6). In the case of rs4646, CC, CA, and AA genotypes were found in 48% (48/100), 51% (51/100), and 1% (1/100) of the studied patients, respectively (Table 7). Taking into account the nonendometriosis control females, the CC, CT, and TT in rs10046 polymorphism were found in 41% (41/100), 59% (59/100), and 0% (0/100) of the patients, respectively. Regarding rs4646, the CC, CA, and AA were observed in 56% (56/100), 44% (44/100), and 0% (0/100) of the studied individuals, respectively.

However, the current study failed to show any correlation of analysed SNPs with the expression of the *ESR2* and *CYP19A1* genes.

The genotype frequency of the *ESR2* rs4986938 polymorphism in relation to its expression is summarized in Table 2. There were no statistically significant differences in the *ESR2* gene expression in relation to the occurrence of rs4986938 polymorphism (Kruskal-Wallis test *p* = 0.46).

No statistically significant differences were observed in genotype frequencies of *ESR2* rs928554 polymorphism and *ESR2* expression (Mann-Whitney test *p* = 0.11) (Table 3).

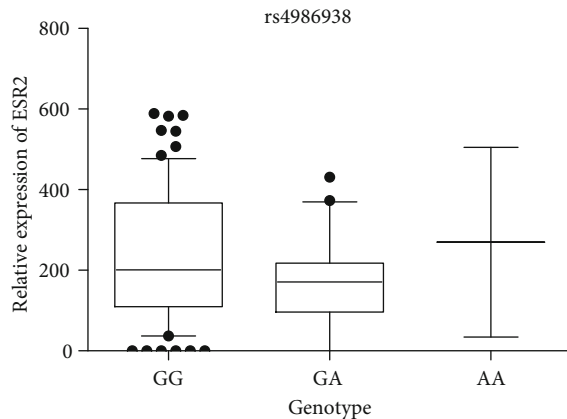


FIGURE 2

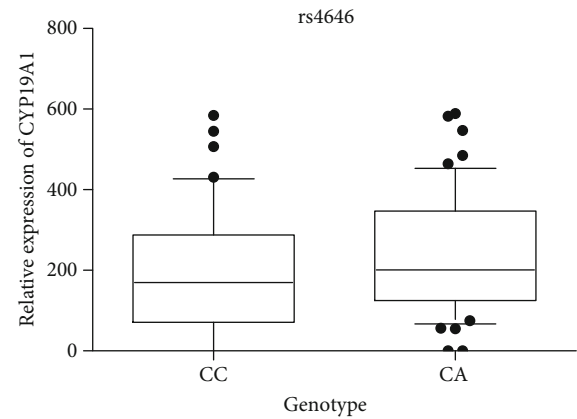


FIGURE 5

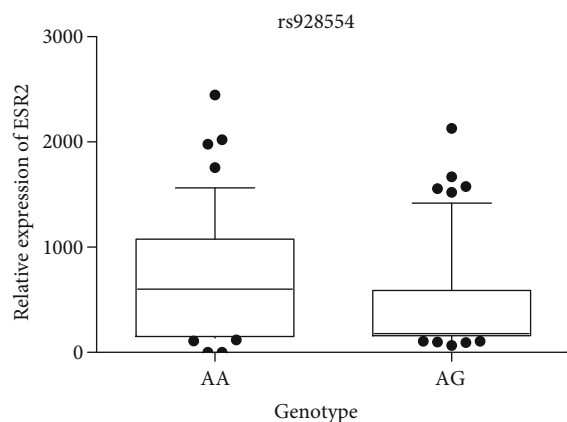


FIGURE 3

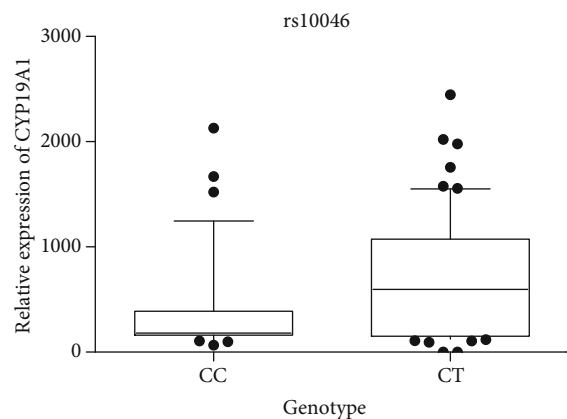


FIGURE 4

There were no statistically significant differences in the *CYP19A1* gene expression in relation to the occurrence of rs10046 polymorphism genotypes (Mann-Whitney test $p = 0.23$) (Table 4).

Our data did not demonstrate any statistically significant correlation between *CYP19A1* rs4646 polymorphisms and *CYP19A1* expression (Mann-Whitney test $p = 0.18$) (Table 5).

No relationship was revealed between *ESR2* and *CYP19A1* polymorphisms and rASRM (revised American Society for Reproductive Medicine) classification scores in the study group. The SNPs in the study group did not correlate with either the age of the patients or with their BMI, menopausal status, or with the number of pregnancies in history.

4. Discussion

Our research addressed the role of allele-specific gene expression of *ESR2* and *CYP19A1* genes as a risk factor for endometriosis. Performed genetic analyses were based on the rs4986938, rs928554, rs10046, and rs4646 polymorphisms of the *ESR2* and *CYP19A1* genes.

The allele-specific gene expression was assayed in patients with endometriosis vs. a healthy control group, while also studying their effects on the increased prevalence of endometriosis among Polish women.

Disorders at the level of the *ESR2* and *CYP19A1* gene expressions, observed in endometriosis as related to the types and sites of lesions, associated with the disease, are confirmed by world literature reports [14–20]. Several different studies are conducted in order to evaluate the risk of endometriosis in association with different genes polymorphism: *CYP17A1*, *CYP19A1*, *ESR1*, *ESR2*, *PGR*, *HSD17B1*, and *HSD17B2* [21–26].

Regarding studies of *CYP17* and *ESR2* gene polymorphisms, there are few reports concerning the 3'UTR region, which is important, because it attaches to the miRNA, which regulates gene expression. This region is therefore important for phenotypic changes that may trigger development of endometriosis

While SNPs in *CYP19A1* have been associated with sex-steroid hormone levels and other oestrogen-dependent diseases including endometriosis, the results of previous studies of the associations between various *CYP19A1* polymorphisms and endometriosis have been inconsistent up to a certain degree. Many studies of *CYP19A1* polymorphisms in relation to endometriosis risk have been published; however, their findings were not confirmed by any additional researches on affected populations [27, 28].

Tempfer et al. reported that the *ERβ* gene is associated with increased risk of stage IV endometriosis in Japanese women, while we found polymorphism in all stages of classification of endometriosis of the patients (classification of endometriosis according to the American Society for Reproductive Medicine) [29].

In the other studies, it was found that the allelic frequency of *RsaI* polymorphism of the *ERβ* gene in AG is about nine times higher in patients with endometriosis in comparison to control groups [30].

However, when comparing fertile and infertile subgroups, no significant differences were stated. The study of *ERβ* and its correlation with the risk of endometriosis could help in the explanation of disease's genetic aetiology.

Szaflik et al. identified statistically significant correlations between new SNP, rs4986938 and rs928554, not described earlier, and endometriosis. In the case of rs4986938, the AA genotype decreased the risk of endometriosis. A similar effect was demonstrated in the case of rs928554 polymorphism of the AG genotype. The results, obtained during that analysis, demonstrated that rs4986938 and rs928554 polymorphisms of the *ESR2* gene are associated with the occurrence of endometriosis [31].

In the present work, a pool of 4 SNPs was determined, all of them most importantly associated with endometriosis: rs4986938 (G>A) (chromosome 14), rs928554 (A>G) (chromosome 14), rs10046 (C>T) (chromosome 15), and rs4646 (C>A) (chromosome 15).

The study involved 100 endometriosis patients. A control group consisted of 100 nonendometriosis women. In the patients with endometriosis, GG, GA, and AA genotypes within *ESR2* rs4986938 SNP were observed in 73% (73/100), 25% (25/100), and 2% (2/100) of the studied individuals, respectively (Table 4). In the case of rs928554, AA, AG, and GG genotypes were found in 48% (48/100), 52% (52/100), and 0% of the studied patients, respectively (Table 5). In the females with endometriosis, CC, CT, and TT genotypes within *CYP19A1* rs10046 SNP were observed in 37% (37/100), 63% (63/100), and 0% of the studied individuals, respectively (Table 6). In the case of rs4646, CC, CA, and AA genotypes were found in 48% (48/100), 51% (51/100), and 1% (1/100) of the studied patients, respectively (Table 7). Neither were any relationships demonstrated between the *ESR2/CYP19A1* gene expression levels and rs4986938, rs928554, rs10046, and rs4646 polymorphisms ($p > 0.05$) (Figures 2–5).

We did not find a relationship between allele-specific genes expression and risk of endometriosis. This study is limited by the sample sizes, and it is possible statistical power may be not enough to detect smaller expression differences among the comparison groups. The study results will be useful for further study design with larger samples sizes.

Therefore, further studies would be justified and needed to reveal the common involvement mechanisms of *ESR2* and *CYP19A1* SNPs in endometriosis formation.

However the results, obtained in the study, contribute to better knowledge of and information on the molecular mechanisms which support the development of endometriosis.

5. Conclusions

There are no statistically significant differences in the *ESR2* and *CYP19A1* allele-specific gene expressions based on the comparisons of the 100 cases vs. the 100 controls, which lead us to conclude that the two genes do not play significant roles in the pathogenesis of endometriosis.

Data Availability

The datasets supporting the conclusions of this article are included within the article.

Ethical Approval

This work was supported by the Institute of Polish Mother's Memorial Hospital, Lodz, Poland, from the Statutory Development Fund. All procedures performed in studies involving human participants are in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent

All the study participants gave a written informed consent. A formal consent was also issued by the Bioethical Committee of the Institute of the Polish Mother's Memorial Hospital in Lodz (Approval number, 8/2016).

Conflicts of Interest

The authors declare no conflict of interest with regards to the reported study.

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