

N-acetyltransferase 2 (NAT2) Gene Polymorphisms and the Effectiveness of Infertility Treatment in Patients with Peritoneal Endometriosis

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Abstract

Today, infertility has become a global issue. WHO ranks it the fifth among the major diseases of those below 60 years, after alcoholism, depression, injuries and eyesight disorders. Numerous studies conducted on the problems of infertility in endometriosis still do not offer clear answers regarding the pathogenesis and mechanisms of this disease and its influences on fertility.

According to the survey results, point mutations of the *NAT2* gene (*NAT2*5* and *NAT2*6*) have been identified in 75.6% of the patients with infertility problems and the peritoneal form of endometriosis, that create "slow" allelic variants, which exceed the average index in the population.

The peculiarities of the *NAT2* gene polymorphisms have been proven to be associated with the effectiveness of the infertility treatment of female patients with peritoneal endometriosis. In the group of non-pregnant patients, the presence of c.341T>C, c.481C>T, c.590G>A and c.803A>G heterozygous point mutations are 73.2, 73.2, 5.4, and 62.5%, respectively. The significant difference in the comparison of the allelic polymorphism during the various stages of the endometriosis was not identified. At stage III-IV endometriosis the frequency of three and more point substitutions was significantly higher.

NAT2 gene polymorphisms can find use as an additional criterion for predicting the effectiveness of the infertility treatment of patients with peritoneal endometriosis.

Keywords: endometriosis, infertility, *NAT2* gene polymorphism

Introduction

Currently, infertility is a global issue. WHO ranks it fifth among the major diseases of those below 60 years of age, after alcoholism, depression, injuries and eyesight disorders. The cold temperatures in most regions of the Russian Federation, early sexual debut, the high frequency of sexually transmitted infections and the low degree of contraceptive use result in the highest abortion rate in Europe, greatly reducing the reproductive potential of Russia to critically low numbers.

Unfortunately, the present frequency of gynecological diseases shows no likelihood of decreasing and the frequency of infertile marriages in Russia is currently above 15%. According to WHO, the total fertility rate in recent years is fast approaching the critical value of 1.3 (World's Health Statistics, World's Health Organization, 2011).

Despite the fact that in 30-40% of cases endometriosis is coupled with infertility, a clear understanding of the pathogenesis and mechanisms of the influence of this disease on fertility still remains elusive. Over the recent years, the roles played by inflammation, angiogenesis and oxidative stress were thoroughly studied by many Russian researchers [1].

The manifestation rate of endometriosis is known to affect the growth of the follicles and oocytes, embryo quality and the beginning of the pregnancy in ART [2]. According to

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the classification of the American Fertility Society, patients at stages III-IV endometriosis show significant reduction in the ovarian reserve [3]. Foreign research studies have proven the presence of genetic polymorphism in the cases of endometriosis [4], polycystic ovaries, uterine fibroids, ovarian cancer, cervical cancer, premature ovarian failure, habitual miscarriage and preeclampsia [5,6]. Of great significance, the specific polymorphism observed in endometriosis from the standpoint of evidence-based medicine is the disorder of the gene system noted in the second detoxification phase [7].

According to the specialized literature, the presence of specific point mutations of the *NAT2* gene is associated with widespread stages of endometriosis and extragenital endometriosis [8]. No other studies have been reported examining the correlation between the occurrence of the *NAT2* gene polymorphism in endometriosis and infertility. The need to improve the diagnosis of the causes for infertility that accompanies peritoneal endometriosis became the basis for this study.

Materials and Methods

The present study included 90 female patients with infertility and peritoneal endometriosis, verified during laparoscopy. Laparoscopy was conducted at a clinical site of the Chair of Obstetrics, Gynecology and Reproductive Medicine of Advanced Training Faculty for health workers at Peoples' Friendship University of Russia and at the Department of Reconstructive and Emergency Gynecology of the City Clinical Hospital #79 (Moscow).

Inclusion criteria:

1. Patients with peritoneal infertility and endometriosis, verified during laparoscopy
2. Between 20 and 43 years of age

Elimination criteria:

1. Patients with uterine fibroids and ovarian tumors
2. Patients with malformations of female genital mutilation
3. Male factor infertility

During the study, the patients were segregated into two groups depending on the effectiveness of the treatment: Group 1 included 34 (37.8%) patients, who became pregnant on their own within 2-24 months after the surgery and Group 2, which had 56 (62.2%) patients, who failed to become pregnant during the same period of time. After evaluating the long-term results (uterine pregnancy occurring within 1-2 years), a comparative analysis of the results of the polymorphism of the *NAT2* gene study in both women groups was conducted.

Laparoscopy was performed under standard conditions, using the traditional methods and employing the Karl Storz equipment. Surgeries were performed under endotracheal anesthesia. All patients underwent the standard preoperative examination. Contraindications to surgery were not identified. During the surgery the excision or coagulation of endometriosis was performed using different energy types (bipolar, argon plasma, laser).

Genetic research on the presence of polymorphism in the *NAT2* genes was conducted in the Department of Genetics

of the Federal State Institution 'N.F. Gamaleya Scientific-Research Institute of Epidemiology and Microbiology' of the Ministry of Health and Social Development of the Russian Federation. Informed consent for the use of blood for research was obtained from all the patients.

DNA samples isolated from the peripheral blood leukocytes of the patients served as the study material. 1 ml of 0.5M EDTA, pH 8.0 was used as the preservative. The first step was DNA extraction employing a set of reagents for the *DLAtom™ DNA Prep100* isolation according to manufacturer's protocol. To 200µl of blood in a 1.5ml test tube, 800µl of lysis reagent was added and all the test tube contents were thoroughly mixed by inverting it (5-10 times). Using a thermostat the test tube with the mixture was kept aside for 5-7 min at 65°C. Next, the test tube with the mixture was centrifuged for 10 seconds at 5000g, after which the clear supernatant was transferred to a clean test tube. A suspension of 30-40µl of the sorbent *NucleoS™* was then added. The test tube was then placed on a rotator and mixed for 10 min and centrifuged again for 10 seconds at 5000g. The supernatant was removed without unsettling the sediment. Next, 400µl of lysis reagent was added to the sediment and vortexed thoroughly to reach a completely homogeneous state. Then, 1 ml of saline buffer and the test tube contents were added to this test tube and then shaken well 5-10 times, before being centrifuged for 10 seconds at 5000g. The supernatant was removed without disturbing the sediment. Saline buffer 1 ml was added to the test tube and the entire contents were mixed together and centrifuged once more for 10 seconds at 5000g, after which the supernatant was carefully removed. The whole process was repeated 12 times. Next, the sediment was dried at a temperature of 65°C for 4-5 min. After this, 100-200µl of bi-distilled water or *ExtraGene* was added into the same test tube. Next, the content of the tube was suspended on a vortex for 5-10 sec to obtain a homogenous suspension. After that, it was thermostated for 5-10 seconds at 65°C. Once again, it was suspended and centrifuged for 2 minutes at 10000g. The supernatant with the DNA was transferred into a clean test tube and stored at $t = -20^{\circ}\text{C}$. Next, the amplification of all the tested DNA fragments using the PCR method in 25µl volume of reaction mixture was performed. The concentration of MgCl_2 in 1 x reaction buffer and annealing temperature were individually selected. The PCR was performed on a programmable thermal cycler *MC2* manufactured by the DNA technology company with the following parameters:

Initial denaturation at 95°C for 5 minutes

28-30 cycles $\left\{ \begin{array}{l} 94^{\circ}\text{C} - 45 \text{ seconds} \\ t, ^{\circ}\text{C} - 45 \text{ seconds} \\ 72^{\circ}\text{C} - 45 \text{ seconds} \end{array} \right.$

Final fill at 72°C for 7 minutes.

$t, ^{\circ}\text{C}$ - the annealing temperature of specific primers.

The sequences of the primers used in this work were selected based on the nucleotide sequences of the DNA fragments analyzed that were available in the GenBank database. The next step in the DNA-diagnostics following the PCR was the restriction analysis (this was performed in a separate room). After centrifugation in a Vortex microcentrifuge for 3-10 seconds, 5-10µl of the amplificate

was transferred using individual tips from mineral oil into new, pre-labeled test tubes.

The results of the amplification were analyzed by vertical electrophoresis in polyacrylamide gel, followed by staining the gel with ethidium bromide solution and registration in UV of 312 nm.

A 7% gel in a 29 to 1 ratio of acrylamide to bis-acrylamide was used for the analysis of the electrophoretic mobility of the fragments of amplification. Ammonium persulfate and TEMED were the catalysts used to polymerize the gel. Electrophoresis was performed in 1 x TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA) in an electric field intensity of 16 V/cm at room temperature. The samples were mixed with the stain (0.5% bromophenol blue and 0.5% xylene cyanol) in a ratio of 5:1 prior to gel application for clear visual inspection.

Statistical analysis was performed using the statistical computer program SPSS (version 10.0.7) and «Statistica» (version 6.0) for Windows. A value of $P < 0.05$ was considered statistically significant.

Results

Clinical characteristics of the patients

The women examined ranged in age from 23 to 39 (30.2±0.4) years. The entire group of females was concerned with infertility. Among them 59 (65.5%) had primary infertility while 31 (34.5%) had secondary infertility. The average duration of infertility in the primary infertility female patients was 3.6±0.7 years, whereas those with the secondary type showed 3.2±1.3 years, revealing no significant difference between both groups.

Analysis of the reproductive anamnesis revealed that the first group included 9 (26.5%) patients with primary infertility and 25 (73.5%) with secondary, whereas the second group had 34 (64.3%) patients with primary and 22 (35.7%) with secondary. The frequency of pregnancy before secondary infertility in both patient groups is as follows: labor – 4 (8.5%) patients, medical abortions -11 (23.4%), spontaneous abortions – 16 (34.0%), medical and spontaneous abortions – 2 (4.3%).

On analysis of their gynecological status it was evident that the alvus during bimanual examination corresponded to normal size in both patient groups, and no pathological changes on the uterine appendages were revealed. On palpation of the area of the patients' uterosacral ligaments and the posterior vaginal fornix, there was evidence of their shortening and compaction, as well as soreness expressed during the study. All the patients examined were of requisite physique and their physical development was consistent with the age norm.

In one-half of the cases, both the first and the second patient groups had digestive disorders (chronic gastritis, peptic ulcer disease). The second group in anamnesis had three times more often indications of respiratory diseases. Also, these patients showed an increase in the frequency of the pustular skin lesions and neurodermatitis (by two times compared with the group of women who became pregnant). Perhaps this was due to the endogenous intoxication and changes in the

antioxidant status, although in the immune system this is due to a violation of the xenobiotic or drug metabolism [9,10].

Burdened family anamnesis was recorded in 12 (35.3%) patients of the first group and 41 (73.2%) of the second. Among them, in the first group, it was due to oncological diseases in 2 (16.7%) patients, due to respiratory diseases in 3 (25.0%) patients, and as a result of diseases of the cardiovascular system in 6 (50.0%) and diabetes in one (8.3%) of them. In the second group: due to oncological diseases in 9 (21.9%) patients, respiratory disease in 21 (51.2%) patients, diseases of the cardiovascular system in 9 (21.9%) patients and diabetes in 3 (7.3%). Therefore, patients of the second group in the anamnesis had 1.5 times more frequent cases of cancer and had two times more diseases of the respiratory system in the immediate family of the first and second lines of relatedness. Compared with the first group, they had two times less number of recorded instances of cardiovascular system diseases.

The second group included 49 (87.5%) patients who had aggravated allergic anamnesis, viz., of these 19 (38.7%) had allergy to drugs, 16 (32.7%) had allergy to household chemicals whereas 14 (28.6%) had it to certain foods. Aggravated allergic anamnesis was also present in four (11.8%) patients of the first group.

Family gynecological anamnesis was aggravated in 18 (52.9%) patients of the first group and in 43 (76.8%) patients of the second group. Among them 10 (55.6%) patients and 29 (67.4%) patients had endometriosis (adenomyosis, peritoneal endometriosis or endometrial ovarian cysts), 6 (33.3%) and 9 (20.9%) patients had hysteromyoma, 2(11.1%) and 5 (11.6%) of them had a pathology of the endometrium. Therefore, the family gynecological anamnesis of the pregnant women in the first group was not significantly different from that of the second group.

No evidence of significant differences was observed between the stages of endometriosis in both groups. Among them 52 % of the pregnant patients had stages I – II endometriosis, 48% had stage III-IV, whereas in the group of non-pregnant patients the figures were 45% and 55%, respectively. The prevalence and incidence of an adhesive process in the pelvis was also not different in both groups.

Features of the NAT2 gene polymorphism

75.6% of the female patients with infertility and peritoneal endometriosis, who participated in this study, had point mutations in the *NAT2* gene (*NAT2**5: c.341T>C, c.481C>T, c.803A>G and *NAT2**6: c.590G>A), which exceeded the average rates in the population. Mutation of *NAT2**7 (c.857G>A) were not identified (Table 1).

Table 1

Incidence of the *NAT2* gene point mutations in comparison with literature data

Allele	Research results		Literature data* (degree of incidence)
	Abs.	Frequency	
T341C	68	0.756	0.227-0.458
C481T	55	0.611	0.388-0.442
G590A	11	0.122	0.268-0.317
A803G	67	0.744	0.395-0.428
G857A	0	0	0.020-0.034

Note: * - Lin et al., 1994 [20]; Brockmoller et al., 1996 [21]; Woolhouse et al., 1997 [22]; Gröss et al., 1998 [23]; Anitha, Banerjee, 2003 [24]; Gaikovitch et al., 2003 [25].

The degrees of incidence of the point mutations c.341T>C, c.481C>T, c.590G>A and c.803A>G were compared with respect to the stage of endometriosis (EM) and no significant differences were observed (Table 2).

Table 2

Distribution of point mutations at different stages of endometriosis

Stage of EM	Point mutation							
	c.341T>C (n=68)		c.481C>T (n=55)		c.803A>G (n=67)		c.590G>A (n=11)	
	n	%	n	%	n	%	n	%
I	15	22.1	11	20.0	17	25.4	2	18.2
II	16	23.5	13	23.6	15	22.4	3	27.2
III	22	32.4	16	29.1	17	25.4	3	27.3
IV	25	36.7	15	27.3	18	26.8	3	27.3
p	> 0.05		> 0.05		> 0.05		> 0.05	

It is important to note that the presence of the heterozygous mutation variants *NAT2*5A* (c.341T>C, c.481C>T), *NAT2*5B* (c.341T>C, c.481C>T and c.803A>G), *NAT2*5C* (c.341T>C, c.803A>G) in stage I-II endometriosis was significantly higher than the presence of the homozygous mutation variants, although in stage III-IV both variants were present equally. Furthermore, the homozygous type of point mutation of *NAT2*6B* (c.590G>A) in stage III-IV is higher than that of the heterozygous type. Data evaluation on the hetero- and homozygous point mutations depending on the stage of endometriosis revealed significant differences (Table 3).

Table 3

The frequency of hetero- and homozygous point mutation variants depending on the stage of endometriosis

Stage of EM		c.341T>C (n=68)		c.481C>T (n=55)		c.803A>G (n=67)		c.590G>A (n=11)		p
		CT	CC	CT	TT	AG	GG	GA	AA	
I-II	n	36	4	43	1	33	5	3*	2*	<0.05
	%	52.9	5.9	78.1	1.8	49.3	7.4	27.3	18.2	
III-IV	n	18	10	6	5	12	17	1	5	>0.05
	%	26.5	14.7	10.9	9.2	17.9	25.4	9.1**	45.4**	
p		<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	

* - $p > 0.05$; ** - $p < 0.05$.

On analyzing the combinations of the mutations, it was been found that in stage III-IV endometriosis the frequency of three and more point substitutions is significantly higher (Table 4).

Table 4

The frequency of different combinations of point mutations at various stages of endometriosis

Stage of EM	Combinations of discovered point mutations			
	1-2 (n=42) <i>NAT2*5A/NAT2*5C/ NAT2*6B</i>		3 and more (n=26) <i>NAT2*5B/NAT2*5C/ NAT2*6B</i>	
	n	%	n	%
I-II	34	80.9	3	11.5
III-IV	8	19.1	23	88.5
p	< 0.05		< 0.05	

Analysis of the *NAT2* gene polymorphisms in (Table 5) showed that the first group (those who became pregnant) mostly included patients without the mutations which create the “slow” allelic variants of the *NAT2* gene. In the patient group which did not become pregnant after treatment, the presence of the heterozygous variant of the point substitutions 341T>C, 481C>T, c.590G>A and c.803A>G was 73.2, 73.2, 5.4 and 62.5 %, respectively ($P < 0.05$).

Table 5

The distribution of different variants of point mutations in the *NAT2* gene among women of the Group 1 and Group 2

Genotypes	Group 1 n=34		Group 2 n=56		p
	n	%	n	%	
	c.341 T>C				
TT	16	47.1	6	10.7	< 0.05
CT	13	38.2	41	73.2	< 0.05
CC	5	14.7	9	16.1	> 0.05
c.481 C>T					
CC	24	70.6	11	19.6	< 0.05
CT	8	23.5	41	73.2	< 0.05
TT	2	5.9	4	7.2	> 0.05
c.590 G>A					
GG	33	97.1	46	82.1	> 0.05
GA	1	2.9	3	5.4	> 0.05
AA	0	0	7	12.5	< 0.05
c.803 A>G					
AA	18	52.9	5	8.9	< 0.05
AG	10	29.5	35	62.5	< 0.05
GG	6	17.6	16	28.6	> 0.05
c.857 G>A					
GG	23	100	37	100	> 0.05
GA	0	0	0	0	0
AA	0	0	0	0	0

The frequency of the homozygous variant differed significantly only in the case of c.590G>A mutation. In the patient group which included those who did not become pregnant, this frequency was 12.5%, while patients who became pregnant did not have this mutation at all ($P < 0.05$).

Discussion

According to the specialized literature, infertility in every fourth woman is combined with endometriosis [11,12]. Therefore, it should be also noted that the patients having received surgical treatment of the peritoneal endometriosis became pregnant 1.5 times more frequently than those patients who received conservative treatment [13]. In this study, the *NAT2* gene polymorphism was identified in 68 (75.6%) of all the patients with peritoneal endometriosis and infertility: *NAT2*6B* (c.590G>A) in 15 (22.3%) patients, *NAT2*5A* (c.341T>C, c.481C>T) or *NAT2*5C* (c.341T>C, c.803A>G) in 27 (39.7 %) patients, *NAT2*5B* (c.341T>C, c.481C>T and c.803A>G) or combination *NAT2*5* and *NAT2*6* in 26 (38.2 %) patients. Studies examining the inter-relationship of the number of point mutations in the *NAT2* gene and the

pregnancy frequency in patients with gynecological diseases are not available in the literature.

It has been proven that the allelic variants of the *NAT2* gene are characterized by the different activity of the N-acetyltransferase enzyme. Based on this factor, the carriers of the respective allelic variants are distinguished as ‘fast’, ‘intermediate’ and ‘slow’ acetylators [14]. Experimental point mutations (*NAT2*5* and *NAT2*6*) lead to the development of the ‘slow acetylation phenotype’, which, according to the literature, help to reduce the level of the enzyme N-acetyltransferase, thus slowing down the conversion of the acetyl-CoA to acetoacetyl-CoA [15]. The metabolism of xenobiotics is disturbed during such changes and this increases the level of endogenous intoxication.

Data available in the literature states that the changes in the activity of the enzymes that metabolize the xenobiotics enhance the risk of cancer. This is due to the bioactivation of a large number of procarcinogens and a disruption of the detoxification process of the external oncogenic substances. Thus, it is concluded that the genetic factors, detoxification system and oxidative stress play definite roles in the case of endometriosis and infertility [17]. It is also interesting to note that the disruptions in the estrogen metabolism in the endometrium in the case of endometriosis, which is a result of an imbalance of the detoxification phases I and II, lead to the build-up of the free radicals and a stimulation of the proliferation of the ectopic endometrium [18]. It is possible that these changes, conditional on the genetic polymorphism of the detoxification genes, are an additional factor for uterine infertility among the patients with endometriosis. The relationship between genetic polymorphism and male infertility is proven, although the precise mechanisms are yet to be studied [19].

Thus, in summary,

- As a result of this study, the point mutations that create the “slow” allelic variants of the *NAT2* gene were identified in 75.6% of patients with infertility and peritoneal endometriosis, which is almost 1.5 times higher than the average statistical rate in Russia.

- No significant differences were observed in patients with the *NAT2* gene polymorphism depending on the stage of endometriosis; however, the relationship between the frequency of the homozygous variants of the point substitutions, the number of mutation combinations and the stage of endometriosis were recorded. The presence of the heterozygous point substitutions c.341T>C, c.481C>T and c.803A>G was significantly higher in the case of stages I-II endometriosis than in its common forms.

- The patients with peritoneal endometriosis, who became pregnant due to the treatment, had three times lower degree of incidence of the “slow” allelic variant of the polymorphism in the *NAT2* gene (*NAT2*5*: c.341T>C, c.481C>T and c.803A>G; *NAT2*6*: c.590G>A) than those who did not become pregnant.

- Certain features of the in *NAT2* gene polymorphisms (‘acetylation phenotype’) are associated with the effectiveness of the infertility treatment in women with peritoneal endometriosis. Patients without the point mutations, which

create “slow” allelic variants of the *NAT2* gene, can be considered the group with the favorable prognosis.

Competing interests

The authors declare that they have no competing interests.

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