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Possible involvement of arylamine *N*-acetyltransferase 2, glutathione *S*-transferases M1 and T1 genes in the development of endometriosis

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Wide inter-individual variation of expression of compound metabolic enzymes is determined by polymorphism and may predispose the development of diseases provoked by environmental factors. The combined analysis of phase II detoxification system genes: arylamine *N*-acetyltransferase 2 (*NAT2*), and glutathione *S*-transferases (*GST*) M1 and T1 was carried out in patients with minimal/mild (group I; $n = 36$) and moderate/severe endometriosis (group II; $n = 29$) and controls ($n = 72$) of French origin, using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). The results show a significant difference between patients and controls with regard to *NAT2* gene polymorphism ($P < 0.05$). This is mainly due to the high percentage of slow acetylator genotypes (SA) in patients compared with controls (60.0 versus 38.9%; $P < 0.02$) with a distinct preponderance in subjects with minimal/mild endometriosis (69.4%, $P < 0.005$) where there is a significantly elevated frequency of slow allele S1 (*NAT25) ($P = 0.05$). Significantly increased proportions of *GSTM1*-deficient genotypes were found in both groups of patients, in comparison with the controls (75.0 and 79.3% versus 45.8%; $P < 0.0001$). A preponderance of *GSTT1*-negative subjects among patients was also detected, but did not appear significant. We suggest the involvement of both *NAT2* and *GSTM1* detoxification system genes in the pathogenesis of endometriosis and the possible impact of *NAT2* gene polymorphism in the development of different forms of this disease.**

Key words: arylamine *N*-acetyltransferase2/detoxification/endometriosis/glutathione *S*-transferase/polymorphism

Introduction

Recent ecogenetic and pharmacogenetic studies (Daly *et al*., 1994; Nebert, 1997) show the importance of intraindividual hereditary variations of foreign compound metabolizing enzymes, which contribute to: (i) differences in responses to environmental agents, which may lead to development of environmentally provoked diseases [lung cancer, bladder cancer (Brockmuller *et al*., 1996) and chronic bronchitis (Baranova *et al*., 1997a)]; and (ii) interindividual variation of drug effects (Meyer *et al*., 1997).

Endometriosis is a multifactorial disease with significantly elevated frequency in industrial areas (Nisolle *et al*., 1997) and possible genetic predisposition (Kennedy *et al*., 1996). The important role of numerous environmental toxins, including organochlorines and specially dioxins has been also demonstrated (Osteen *et al*., 1997).

We postulated that the lack of detoxification, which is determined genetically, might be a risk factor for development of endometriosis. Our initial results have suggested the involvement of glutathione *S*-transferase (*GST*) M1 gene (phase II of detoxification) in the pathogenesis of this disease due to the high preponderance of *GSTM1*-deficient subjects among patients (Baranov *et al*., 1996; Baranova *et al*., 1997b). Current investigations are devoted to the other members of phase II detoxification system genes: arylamine *N*-acetyltransferase 2 (*NAT2*) and glutathione *S*-transferase T1 (*GSTT1*).

The polymorphism of *NAT2* gene considerably affects the activity of arylamine *N*-acetyltransferase 2 enzyme and therefore *N*-acetylation and biotransformation of xenobiotics with a primary aromatic amine or a hydrazine structure (Hein *et al*., 1993), such as toxic nitrosamines in tobacco smoke, antioxidants and pesticides. It is also implicated in drug metabolism, including drug–drug interactions (Speilberg, 1996). According to variation of NAT2 enzyme activity, the population is divided in two main groups of slow (SA) and rapid acetylators (RA). SA are homozygous for the recessive forms of *NAT2* gene, have two slow alleles and decreased levels of NAT2 protein up to 20% (Grant *et al*., 1990); RA are characterized by the presence of at least one wild-type *NAT2* fast allele (Cascorbi *et al*., 1995). Of Caucasians, ~50% are SA, but this proportion differs considerably according to geographical regions (Daly, 1994). Numerous studies report the association of *NAT2* slow genotypes with susceptibility to a variety of diseases, including bladder cancer (Brockmuller *et al*., 1996), hepatocellular carcinoma (Agundez *et al*., 1996) and breast cancer (Ambrosone *et al*, 1996).

GSTs play an important role in the defence reactions of the organism by glutathione conjugation with electrophilic

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compounds (Mannervik *et al*., 1985). They are involved in detoxification of polycyclic aromatic hydrocarbons (found in tobacco smoke, food, and combustion fumes) and also pesticides (Chasseaud *et al.*, 1979). *GSTM1* and *GSTT1* genes are polymorphic and the presence of two 0-alleles in each gene corresponds to the presence of deletion with consequent loss of mRNA and protein product (Seidegard *et al*., 1988; Arand *et al*., 1996). The percentage of *GSTM1*-deficient individuals is ~45% in the general Caucasian population, but it is greatly increased in patients with tobacco-induced lung and bladder cancer (Seidegard *et al*., 1990; Brockmoller *et al*., 1996). *GSTT1* deletion is present in 15% of Caucasians. Both *GSTM1* and *GSTT1* genes seem to be involved in pathogenesis of different types of cancers and can be considered as risk modifiers for various environmentally induced diseases (Lear *et al*., 1996; Sarhanis *et al*., 1996).

In the current study, comparative analysis of distribution of *NAT2*-, *GSTM1*- and *GSTT1*- deficient genotypes and precise allelic detection were carried out in patients with different stages of endometriosis and controls in order to estimate possible impact of *NAT2*, *GSTM1* and *GSTT1* gene polymorphisms in development of this disease.

Materials and methods

Patients

Previously described controls ($n = 72$) and patients ($n = 50$), who were analysed for the presence of *GSTM1*0/0 deletion (Baranova *et al.*, 1997) and also 15 new patients with different stages of endometriosis were included in this study, which was carried out in Polyclinique, Centre Hospitalier Université de Clermont-Ferrand, France. All individuals were women of reproductive age of French origin and from Central France. Only patients with clinically, endoscopically and histologically confirmed diagnoses were studied (Canis *et al.*, 1995). According to the revised American Fertility Society (AFS, 1985) classification, all patients were staged into two groups: group 1 = AFS stage I–II; group 2 = AFS stages III–IV. Women who were admitted to the hospital for voluntary abortions and who did not have any malignant pathology were included in the control group. The absence of endometriosis in controls was verified by standard examination and ovarian ultrasound procedure (Canis *et al*., 1992). Anamnestic data were obtained in a 10 min interview by the clinicians and information for statistical analysis was scored as present/absent for: (i) life history factors: smoking (current, regular or occasional; Perriot, 1995), allergy [delayed or intermediated hypersensitivity and type of allergy; (Bousquet *et al.*, 1993)], chronic diseases, previous pregnancies; (ii) clinical features: pelvic pain syndrome, infertility and relapses (recurrence of clinical symptoms) (International Dictionary of Medicine and Biology, 1986; Canis *et al*., 1992). Information about residence (city/village) of all studied individuals was also included.

Laparoscopic treatment and associated procedures for ovulation disorders, if necessary, were applied in patients with infertility. Pain was treated by laparoscopic procedures in combination with postoperative pharmacotherapy (progestins, luteinizing hormone-releasing hormone: Canis *et al*., 1992).

Genetic analysis

Polymorphism in three genes: *NAT2*, *GSTT1* and *GSTM1* of the phase II of detoxification system was detected by polymerase chain reaction (PCR) and PCR restriction fragment length polymorphisms (PCR–RFLP). PCR and PCR–RFLP were performed directly from blood spots, as described previously (Baranov *et al*., 1991).

Correlation between the genotype and enzyme activity for all three genes has been demonstrated in previous studies (Brockmoller *et al.*, 1994; Cascorbi *et al*., 1995; Arand *et al*., 1996) and, therefore, it has not been performed in the present investigation.

NAT2 *gene*

NAT2 gene polymorphism was detected in four polymorphic sites, as previously described (Spurr *et al*., 1995) with slight modifications and adoption of PCR–PFLP procedure for direct analysis without DNA extraction. Three most common slow alleles: S1, S2, S3 (*NAT2**5, *NAT2**6, *NAT2**7) respectively according to a new classification (Cascorbi *et al*., 1995); and one wild-type fast allele: F1 (*NAT2**4) were identified. Single PCR amplification was performed with the primers: P1: 5′-GCTGGGTCTGGAAGCTCCTC-3′ and P2: 5′-TTGGGTGATACATACACAAGGG-3′ in 50 µl of PCR mixture, containing: 2.5 µl of 10 mM dNTP, 5 µl of 10× buffer (GibcoBRL; Life technologies SARL, Cedex, France), 4 µl of 50 mM $MgCl_2$ (GibcoBRL), 0.5 µl of each primer (50 mM), 5 IU *Taq* DNA polymerase (GibcoBRL), H_2O up to 50 µl. A small fragment of a blood spot (1 mm^2) was plunged in the mixture, which was then overlaid with one drop of mineral oil. PCR was started by 5 min of initial denaturation followed by 35 cycles of 94°C for 40 s; 58°C for 1 min; 72°C for 1 min and final extension for 7 min. Amplified products were restricted with the enzymes: *Kpn*I (for the detection of C⟨⟨T point mutations at nucleotide (nt) position 481), *Dde*I (for A⟨⟨G at nt position 803), *Taq*I (for G⟨⟨A at nt position 590) and *Bam*HI (for G⟨⟨A at nt position 857). Electrophoresis was carried out in 7% of polyacrylamide gel at 200 V for 90 min stained with ethidium bromide (Figure 1).

GSTT1 *gene*

*GSTT1*0/0 deletions were detected by internal standard-controlled PCR in 25 ml of PCR mixture of: 1.5 µl of 10 mM dNTP, 2.5 µl of 10/ buffer (GibcoBRL; Life Technologies SARL, Cedex, France), 2.5 µl of 50 mM $MgCl_2$ (GibcoBRL), 0.5 µl of each primer (50 mM), 2.5 IU *Taq* DNA polymerase (GibcoBRL), H_2O up to 25 µl. *GSTT1* forward primer: 5′–TTCCTTACTGGTCCTCACATCTC-3′ and GSTT1 reverse primer: 5′–TCACCGGATCATGGCCAGCA–3′ (Arand *et al*., 1996) were applied for amplification of 450 bp fragment, which corresponds to *GSTT1* gene and primers 1A1S: 5′-GAACTGCCACTTCAGCTGTCT-3′, 1A1AS*Hinc*II: 5′-GAAAGACCTCCCAGCGGTCA-3′ for the *CYP1A1* gene with result product of 187 bp (Oyama *et al*., 1995) were used as internal control. PCR parameters were as following: 5 min of initial denaturation; 33 cycles of 94°C for 55 s, 64°C for 1 min, 72°C for 1 min; and a final extension for 7 min.

This approach enabled the peculiarities of *CYP1A1* Ile–Val gene polymorphism to be studied by following restriction of the amplified product with *Hinc*II restriction enzyme. Electrophoresis was carried out in 7% of polyacrylamide gel at 200 V 90 min stained with ethidium bromide (Figure 2).

GSTM1 *gene*

GSTM1 gene deficiency (*GSTM1*0/0 genotype) and precise alleleic detection (*GSTM1*A*, *GSTM1*B* and *GSTM1*0*) in our first 50 patients and 72 controls were identified as described (Baranova *et al*, 1997). These results are included in current combined analysis of genetic polymorphisms and life history factors.

Statistical analysis

The BMDP4F program (Dixon *et al*., 1992) was applied to calculate the Pearson χ^2 test of independence and, if the minimum expected

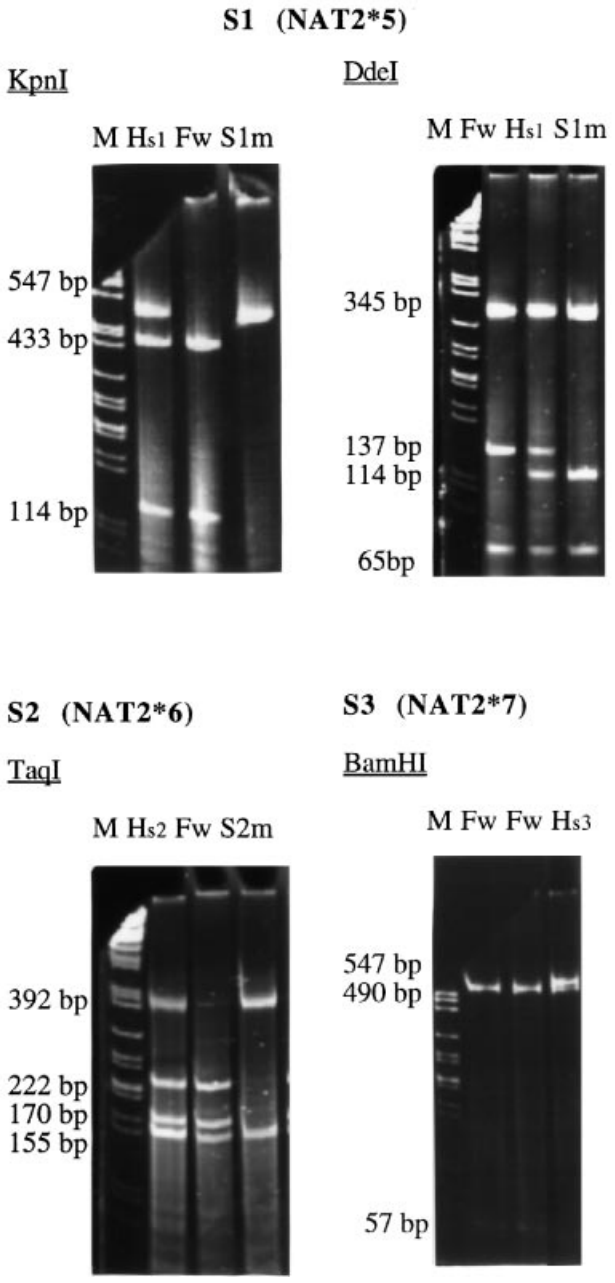

Figure 1. Detection of *NAT2* alleles by direct polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP). M = Marker: DNA digested with *Pst*1; Hs1 = heterozygote for S1 slow allele; Fw = homozygote for wild-type fast allele (*NAT2**4); S1m = homozygote for S1 slow allele; S2m = homozygote for S2 slow allele; Hs2 = heterozygote for S2 slow allele; Hs3 = heterozygote for S3 slow allele.

frequency was too low, Fisher's exact test was used. Mantel–Haenszel analysis (Mantel *et al.*, 1959) was performed to estimate the odds ratio common to different levels and to test whether it was equal to one.

Results

The results of genetic analysis are summarized in Tables I–III and show the significant impact of *GSTM1* and *NAT2* gene polymorphisms, but not of *GSTT1* gene polymorphisms in development of endometriosis ($P = 0.001$; $P = 0.031$ and not significant respectively; Table I).

NAT2 *gene polymorphism*

Polymorphic effects of *NAT2* gene mainly corresponded to the high proportion of SA genotypes in patients: 60.0 versus 38.9% in controls ($P = 0.017$) (Table I).

Precise statistical analysis of each group of patients compared to controls revealed that the highest percentage of SA genotypes was present in the group of patients with minimal/mild endometriosis (69.4%; versus 38.9% in controls; $P = 0.004$), but not that with moderate/severe endometriosis (48.3% versus 38.9% in controls; not significant) (Table I). Also, the number of heterozygotes for *NAT2* gene appeared to be significantly low in patients with minimal/mild forms of disease (27.8% versus 48.6% in controls; $P = 0.042$), but again not those with moderate/severe forms (44.8%; versus 48.6% in controls; not significant). Finally, for development of different stages of endometriosis the impact of *NAT2* gene polymorphism was found to be marginally statistically significant ($P = 0.065$).

Moreover, comparative analysis of allelic frequencies showed a significant difference in distribution of slow and fast alleles between all patients and controls ($P = 0.01$) (Table II). Interestingly, this difference contributed to the group I of patients with minimal/mild endometriosis, but not to the group II (Table II). So, the proportion of S1 was higher in patients with minimal/mild endometriosis (0.53 versus 0.38 in controls; $P = 0.05$), but not in patients with moderate/severe forms (0.40; not significant). The significantly decreased frequency of allele F was also observed in group I of patients (0.17 versus 0.37 in controls; $P = 0.01$), but not in group II (0.29; not significant). The proportions of S2 and S3 were nearly equal in all studied groups.

GSTM1 *gene polymorphism*

The analysis of 15 additional patients did not affect previously reported results (Baranova *et al.*, 1997b). The proportion of *GSTM1*-negative subjects among patients remained highly significant (76.9 versus 45.8%; $P = 0.0001$) and did not vary much between the two groups of patients (group I: 75.0%; group II: 79.3%). The significantly decreased number of individuals with *GSTM1*A/B and *GSTM1*A/0 (or A/A) also seems to be common to both groups of patients, compared with the controls (Table I).

GSTT1 *gene polymorphism*

The preponderance of *GSTT1*-negative subjects was observed in both groups of patients, but did not appear to be significant (20.0% in all patients against 9.7% in controls; not significant) (Table I).

Combined analysis of the deficiencies in GSTM1*,* GSTT1 *and* NAT2

The combined analysis of the deficiencies in studied genes did not reveal any increased risk, synergistic or antagonistic effect on predisposition and development of endometriosis. No correlation between *NAT2* polymorphic effects, life history factors (smoking, chronic pathology, allergy, age) and severity of the disease has been found up to date.

Table I. Distribution of patients with endometriosis and controls according to *GSTM1*, *GSTT1* and *NAT2* genotypes and the impact of polymorphic effects of each gene in development of endometriosis. Figures in parentheses are percentages

Genetic factors Diagnostic groups	GSTM1				GSTT1	NAT2		
	GSTM1 0/0 deletion	GSTM1 A/B	GSTM1 A/A or GSTM1 A/0	GSTM1 B/B or GSTM1 B/0	GSTT1 0/0 deletion	NAT2 slow homozygotes	NAT2 heterozygotes	NAT2 wild-type homozygotes
Group I ($n = 36$)	27 (75.0)	2 (5.5)	4 (11.1)	3 (8.3)	7 (19.4)	25 (69.4)	10 (27.8)	1 (2.8)
Group II ($n = 29$)	23 (79.3)	0 (0.0)	5 (16.7)	1 (3.3)	6 (20.7)	14 (48.3)	13 (44.8)	2 (6.9)
Total ($n = 65$)	50 (76.9)	2 (3.1))	9 (13.8)	4 (6.2)	13 (20.0)	39 (60.0)	23 (35.4)	3 (4.6)
Controls ($n = 72$)	33 (45.8)	13 (18.1)	21 (29.2)	5 (6.9)	7 (9.7)	28 (38.9)	35 (48.6)	9 (12.5)
Probability[a]	$P = 0.0001$	$P = 0.006$	$P = 0.039$	NS	NS	$P = 0.017$	NS	NS
P[a] (for each gene)	$P = 0.001$				NS	$P = 0.031$		

[a]All patients versus controls.

Life history factors and anamnestic data

The results of current analysis of patients and controls relative to age, allergy, chronic pathology, smoking and pregnancies did not differ from that previously performed and were significant for age ($P < 0.001$) and smoking ($P < 0.0135$) (Baranova *et al*., 1997b).

There was no significant difference in distribution of all studied individuals according to their residence and 69.2% of patients and 79.2% of controls lived in urban areas. Differencies in the clinical picture in patients with minimal/mild and moderate/severe forms of endometriosis and the effects of *GSTM1* and *NAT2* deficiencies are presented in Table III. The comparison of the two groups revealed a significant difference for the presence of relapses ($P = 0.01$), but not for the pain syndrome and infertility. Nevertheless, patients without any positive effect after treatment of pain syndrome represented only 16.7% in the group with minimal/mild endometriosis compared with 34.5% in the group with moderate/severe forms. Interestingly, the number of patients with no positive effect after treatment of infertility was elevated in group I: 36.1% (13 cases) versus 27.6% (eight cases).

The analysis of patients according to clinical symptoms and distribution of *GSTM1*- and *NAT2*-deficient genotypes revealed that the proportions of *GSTM1* deletion in patients without positive effect after infertility treatment were nearly equal between group I and II, but the percentages of *NAT2* SA genotypes were very different (92.3% in group I versus 62.5% in group II). The same trends were observed for relapses and presence of the pain syndrome (Table III). The small number of studied cases means that these differences cannot be considered to be significant and the results are mostly descriptive, but nevertheless, these first data indicate possible *GSTM1* and *NAT2* polymorphic effects in endometriosis patients according to the form of disease.

Discussion

Peculiarities of NAT2 *and* GSTM1 *gene polymorphisms in endometriosis patients*

NAT2 *gene*

The over-representation of slow *NAT2* alleles, particularly S1 and under-representation of fast allele and consequently increased number of SA genotypes in patients with endometriosis, suggest a significant impact of the *NAT2* gene in development of this disease.

Table II. Frequencies of detected *NAT2* alleles and comparative analysis of their distribution in patients and controls

Alleles studied groups	S1	S2	S3 (total)	S	F
Group I	0.53	0.28	0.02	0.83	0.17
Group II	0.40	0.29	0.02	0.71	0.29
Total	0.47	0.29	0.02	0.78	0.22
Controls	0.38	0.23	0.02	0.63	0.37
Controls and group I	$P = 0.05$	NS	NS	$P = 0.01$	$P = 0.01$
Controls and group II	NS	NS	NS	NS	NS
Controls and all patients	NS	NS	NS	$P = 0.01$	$P = 0.01$

Unexpectedly, the preponderance of *NAT2* SA genotypes was related mainly to the patients with minimal/mild endometriosis. Moreover, the frequencies of slow alleles were nearly equal in patients with moderate/severe endometriosis and controls. The descriptive analysis of clinical symptoms and *NAT2* polymorphic effects in patients also shows trends towards diversion in the two studied groups (Table III). Interestingly, other investigations also indicate notable differences between minimal/mild and moderate/severe endometriosis according to clinical symptoms, especially pelvic pain and infertility (Thornton *et al*., 1997), immune status (Arichi *et al*., 1996), role of growth factors (Shifren *et al.*, 1996) and morphological criteria (Nisolle *et al*., 1997).

Our results are the first indication that *NAT2* deficiency might be involved in development of different forms of endometriosis.

GSTM1 *gene and* GST *status*

Interestingly, the impact of *GSTM1* gene polymorphisms in endometriosis does not appear to be similar to that of *NAT2*. The proportions of GSTM1 negative subjects do not differ significantly between the groups I and II of patients and are increased with the severity of disease, whereas the percentage of active *GSTM1* genotypes is decreased. Finally, no patient with *GSTM1*A/B genotype, which corresponds to the highest activity of GSTM1 enzyme was found in group II (moderate/severe endometriosis; Table I). These data are consistent with our earlier findings (Baranova *et al*., 1997b). Distribution of clinical symptoms according to the *GSTM1* gene polymorphism

Table III. Distribution of clinical symptoms relative to *GSTM1* and *NAT2* genotypes in endometriosis patients. Figures in parentheses are percentages

	Patients	Relapses	Pelvic pain	Pelvic pain no change after treatment	Infertility no change after treatment
Distribution of studied clinical symptoms	group I (n = 36)	8 (22.2)*	17 (47.2)	6 (16.7)	13 (36.1)
	group II (n = 29)	16 (55.2)*	21 (72.4)	10 (34.5)	8 (27.6)
	total (n = 65)	24 (36.9)	38 (58.5)	16 (24.6)	21 (32.3)
GSTM1 deletion	group I	5 (62.5)	11 (64.7)	4 (66.7)	10 (76.9)
	group II	12 (75.0)	16 (76.2)	6 (60.0)	6 (75.0)
	total	17 (70.8)	27 (71.1)	10 (62.5)	16 (76.2)
NAT2 slow	group I	5 (62.5)	11 (64.7)	4 (66.7)	12 (92.3)
	group II	5 (31.3)	10 (47.6)	6 (60.0)	5 (62.5)
	total	10 (41.7)	19 (50.0)	10 (62.5)	17 (81.0)

*Significantly different (P = 0.01).

appears to follow a reverse pattern when compared with the impact of *NAT2* deficiency, particularly for relapses, presence of pain syndrome and infertility. The detection of *GSTT1* deletion indicates certain, but non-significant preponderance of *GSTT1*-negative subjects among patients, which has the same trend as *GSTM1* deficiency and is correlated with earlier results (Chen *et al*., 1996) obtained in Caucasians.

Other investigations of GSTs and xenobiotic pathways in endometriosis patients are necessary. However, there is a high likelihood that the lack of such an important metabolic pathway as glutathione conjugation might be a risk factor for susceptibility to endometriosis, so analysis of GST status, particularly detection of *GSTM1* deficiency, could have a prognostic significance.

Biotransformation process and endometriosis

Metabolic pathways of xenobiotics include their activation during the phase I of the biotransformation process followed by conjugation of highly toxic intermediate metabolic products during phase II. Therefore, expression of phase I and II enzymes must be well co-ordinated.

The genes which code for foreign compound metabolizing enzymes are highly polymorphic, so the presence of deletions or slow alleles can provoke imbalanced interactions of phase I and II. In relation to environmentally induced diseases and drug metabolism, the supergene families of cytochrome *P*-450 (*CYP*) (phase I), *GST*, and *NAT* (phase II) play a key role. Several investigations reported the dramatically increased risk for cancer susceptibility in the case of association of some alleleic variants in *CYP*, *GST* and *NAT2* genes (Warwick *et al*., 1994; Brockmuller *et al*., 1996). Interestingly, the *CYP1A1*, *CYP1A2*, *CYP1B1* of phase I, which are known as dioxin inducible genes, seem to play a role in development of endometriosis (Johnson *et al*, 1997). *NAT* and *GST* act on the products of phase I and in case of altered mechanism might be involved in adverse immune reactions. Thus, slow acetylation as a result of *NAT2* gene polymorphism is believed to be a risk factor for the induction of cytotoxity and immune response to neoantigens because of increased covalent binding of reactive metabolites (Spielberg, 1996). The involvement of the immune system in the development of endometriosis due to participation of natural killer (NK) cells (Provinciali *et al*., 1995) and activated macrophages (Kligman *et al*., 1996) has been suggested by other authors (Rier *et al*., 1997). These reactions might be provoked by environmental toxins either directly or by formation of intermediate toxic endogenous compounds (Osteen *et al*., 1997). The changes in NK activity and their correlation with the severity of endometriosis are well known (Koninckx *et al*., 1994). Recent investigations demonstrate that this phenomenon might be also connected with decreased glutathione content, which contributes to disorders in thr cytoskeleton components with consequent impairment of NK cell activity (Malorni *et al*., 1997). From these data we can speculate on possible involvement of acetylation and gluthatione conjugation reactions in pathogenesis of endometriosis.

In conclusion, we suggest the participation of at least two genes of the phase II detoxification system in development of endometriosis.

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