Joint effect of glutathione S-transferase genotypes and cigarette smoking on idiopathic male infertility

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Keywords
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Summary
Inconsistent results of association studies investigated the role of glutathione S–transferase genes in idiopathic male infertility may be explained by ethnical differences in gene–gene and gene–environment interactions. In this study, we investigated a joint contribution of \( \text{GSTM1, GSTT1} \) and \( \text{GSTP1} \) gene polymorphisms and cigarette smoking to the risk of idiopathic infertility in Russian men. DNA samples from 203 infertile and 227 fertile men were genotyped by a multiplex polymerase chain reaction (GSTM1 and GSTT1 deletions) and PCR-restriction fragment length polymorphism (GSTP1 I105V) methods. The GSTP1 genotype 105IV was associated with increased risk of male infertility (OR = 1.50 95% CI 1.02–2.20 \( P = 0.04 \)). Genotype combinations GSTP1 1051V/GSTT1 + (G4) were associated with increased disease risk \( (P = 0.001) \). The genotype combinations G3 and G4 showed a significant association with infertility in smokers; however, nonsmokers carriers did show the disease risk. In conclusion, GSTM1, GSTT1 and GSTP1 genes are collectively involved in the development of idiopathic male infertility and their phenotypic effects on the disease risk are potentiated by cigarette smoking.

Introduction
The idiopathic form of male infertility is world widespread disease found in 40–75% of infertile men (Rowe et al., 2000). The aetiology of idiopathic infertility is thought to be multifactorial (Singh & Jaiswal, 2011) meaning that disease susceptibility is determined by complex interactions between a number of genetic and environmental factors acting in cooperation. There is a concern that low-level xenobiotic exposure of the general population may be responsible for increased prevalence of male infertility and decreased sperm quality registered worldwide over last decades (Kunstmann et al., 1995; Auger et al., 2001; Hansen et al., 2010). It is known that oxidative stress, a pathological condition resulting from the enhanced production of reactive oxygen species and decreased activity of antioxidant defence enzymes, emerges as a key mechanism by which environmental xenobiotics impact on male reproductive functions leading to infertility (Agarwal et al., 2006; Tremellen, 2008; Aitken et al., 2014). This means that polymorphic genes encoding antioxidant and biotransformation enzymes determining inter-individual differences in the ability to activate and detoxify chemical substances of the environment may represent potential modifiers of men’s susceptibility to infertility. Pursuing this hypothesis, a number of genetic association studies have been performed to investigate the relationship between polymorphisms in these genes and idiopathic infertility in men (Schuppe et al., 2000; Rubes et al., 2010; Ji et al., 2012; Fang et al., 2014; Sabouhi et al., 2014). Genes for glutathione S-transferases became an object of the most intensive research (Aydemir et al., 2007; Aydos et al., 2009; Finotti et al., 2009; Polonikov et al., 2010; Safarinejad et al., 2010; Tirumala Vani et al., 2010; Jaiswal et al., 2012; Salehi et al., 2012; Lakpour et al., 2013).

Glutathione S-transferases (GST) is an important superfamily of biotransformation and antioxidant defence
enzymes catalysing the conjugation of a large variety of electrophilic and hydrophobic toxic intermediates with reduced glutathione (Hayes et al., 2005). Recent meta-analyses evaluated the associations between GSTM1, GSTTI and GSTP1 gene polymorphisms and risk of idiopathic infertility in diverse ethnicities (Chengyong et al., 2012; Safarinejad et al., 2012; Tang et al., 2012; Kan et al., 2013; Li et al., 2013; Song et al., 2013; Wu et al., 2013; Ying et al., 2013). Although several studies provided a strong evidence for the role of GST genes in the pathogenesis of male infertility, some studies did not report the association of GST gene polymorphisms and disease risk. Although exact reasons of inconsistent results across the genetic association studies in different ethnicities are not yet fully understood, the absence of association between GST genes alone and male infertility risk in some populations of the world may be attributed to gene–gene and gene–environment interactions within the framework of common metabolic pathway of xenobiotic biotransformation determined by the enzymes. In particular, our recent studies (Yarosh et al., 2013, 2014) demonstrated an importance of gene–environment interactions analysis for better understanding the role of genes for xenobiotic-metabolising enzymes in the pathogenesis of idiopathic infertility in men. This study was designed to investigate a comprehensive contribution of GSTM1, GSTTI and GSTP1 gene polymorphisms and cigarette smoking to the risk of idiopathic infertility in Russian men.

Materials and methods

Study population and diagnosis

All participants of the study provided informed consent before enrolment. The study protocol was approved by the Ethical Review Committee of Kursk State Medical University. A total of 430 unrelated Russian men were recruited from the Family Planning and Reproductive Health Clinic of Kursk Regional Perinatal Centre over a period from 2006 to 2008. The case group included 203 patients with idiopathic male infertility. Criteria for inclusion in the case group were infertility for at least 12 month of regular unprotected intercourse with at least two repeated finding of semen parameter abnormalities and the negative mixed agglutination reaction test. The control group included 227 healthy fertile men (all have normal seminal parameters) who had fathered at least one child. All eligible patients and controls matching the inclusion/exclusion criteria were given the opportunity to be enrolled in the study groups. The mean age of the case group was higher in than in the controls (34.4 years versus 29.2 years; P < 0.01).

Semen parameters were evaluated according to World Health Organization guidelines (Rowe et al., 2000). Semen specimens were collected from all study participants through masturbation into a sterile plastic container after at least 2 days of sexual abstinence. Clinical, laboratory and instrumental investigations by experienced andrologist, endocrinologist, genetics and laboratory assistants excluded all possible causes of male infertility such as varicocele, hypogonadotropic hypogonadism, abnormal sexual ejaculatory functions, obstruction of seminal tract, abnormal karyotypes and microdeletions of chromosome Y. The female infertility factor was excluded by experienced gynaecologists. All study participants recorded interviewer administered questionnaire concerning demographic data, life style factors and cigarette smoking habit (ever or never smokers).

Genotyping

Genomic DNA was purified from whole blood samples through phenol–chloroform extraction and ethanol precipitation according to the standard procedure. Genotyping of polymorphism I105V (rs1695) of the GSTP1 gene was carried out by PCR-RFLP methods, as described in details by Welfare et al. (1999). We used sense 5'-GGCTCTATGGGAAGGACCAGCAGG-3' and antisense 5'-GCACCTCCATCCAGAAACTGGCG-3' primers for amplification of DNA fragment covering the GSTP1 gene polymorphism. The PCR product was then digested with endonuclease BstMAI (Sibenzyme, Russia). Genotyping GSTM1 and GSTTI null genotypes was performed by a multiplex polymerase chain reaction method in the framework of our previous study (Polonikov et al., 2010). The primer sequences for amplifying the GSTM1 null polymorphism were sense 5'-GAACTCCGTAAAGCTAAAGC-3' and antisense 5'-GTTGGGCTCAAATATACGGTGG-3'. The null polymorphism of the GSTTI gene was amplified with the following primers: sense 5'-TTCCCT ACTGGTCCTCACATCTC-3' and antisense 5'-TCACGG GATCATGGCCAGCA-3' was used for genotyping. The primer sequences (sense 5'-CAACCTGATCCAGTGCCACCC-3' and antisense 5'-GAAGAGCCAAGGACAGG TAC-3') of β-globin gene were used as a control of successful PCR. The PCR products were separated on 2% to 3, 5% ethidium bromide-stained agarose gels and visualised under ultraviolet light on the GDS-8000 Computer Detection System (UVP, Upland, CA, USA). Genotyping was carried out blindly to the case–control status, and the repeatability test was conducted for about 10% of the samples, resulting in a 100% concordance rate.
Statistical analyses

Allele frequencies of polymorphism I105V of the GSTP1 gene were estimated by gene counting method. The chi-square test was used to identify significant departures from Hardy–Weinberg equilibrium. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to estimate the association between GST genotypes and male infertility risk. Gene–smoking interactions were evaluated by joint categories of GST genotypes and smoking habit with respect to male infertility risk using binary logistic regression analysis. Data on cigarette smoking status were available from 203 infertile men and 164 fertile controls. A two-sided P-value $\leq 0.05$ was considered to be statistically significant. Bonferroni correction for P-values ($P_{adj}$) was applied in cases when multiple tests were performed. The statistical calculations were performed using STATISTICA for WINDOWS (v8.0) software package (StatSoft; Tulsa, OK, USA).

Results

Table 1 shows the distribution of alleles and genotypes of polymorphism I105V of the GSTP1 gene among infertile and fertile men. The GSTP1 genotype frequencies were in Hardy-Weinberger equilibrium ($P > 0.05$) in the case and control groups. No difference in frequency of allele 105V was seen between the study groups. Meanwhile, the heterozygous genotype 105IV of the GSTP1 gene was associated with increased risk of idiopathic male infertility (OR = 1.50 95% CI: 1.02–2.20 $P = 0.04$). The distribution of null genotypes for the GSTM1 and GSTT1 genes in the case and control groups was reported in our previous study (Polonikov et al., 2010). The frequencies of GST genotypes were comparable with those reported previously in our population (Polonikov et al., 2012, 2014). Associations of GST genotype combinations with risk of idiopathic male infertility are shown in Table 2. As can be seen from Table 2, two genotype combinations such as GSTP1 105II $\times$ GSTT1 del and GSTM1 del $\times$ GSTT1 del were absent in infertile men. One patient with idiopathic infertility had a genotype combination GSTM1 + $\times$ GSTT1 del. These genotype combinations were significantly associated with decreased risk of male infertility after adjustment for multiple tests ($P_{adj} \leq 0.003$). Meanwhile, a genotype combination GSTP1 105IV $\times$ GSTT1 + was significantly associated with increased risk of male infertility ($P_{adj} = 0.001$).

As we reported earlier (Yarosh et al., 2013), the case and control groups were comparable with respect to smoking status. Table 3 shows the results of GST genotypes–smoking interactions analysis. For this analysis, we selected only those combinations of genotypes which were found to be significantly associated with disease risk. As can be seen from Table 3, four genotype combinations showed joint effects with cigarette smoking on male infertility risk, however, only two of them were statistically significant after Bonferroni correction for multiple tests. In particular, a genotype combination GSTP1 105IV $\times$ GSTT1 + was associated with increased risk of infertility in smoker men ($P = 0.006$), whereas this genotype combination did show the disease risk in nonsmokers. Absence of genotype combination GSTM1 + $\times$ GSTT1 del showed a significant association with male infertility in smokers ($P = 0.006$), whereas no association was seen in nonsmoker men.

Discussion

The present study was designed to investigate a joint contribution of GSTM1, GSTT1 and GSTP1 gene polymorphisms and cigarette smoking to the risk of idiopathic infertility in Russian men. We found only one paper in the literature that investigated the relationship between variation in the GSTP1 gene and male infertility in which Safarinejad et al. (2010) observed a protective effect of this gene polymorphism on the disease risk. This result is inconsistent with our finding that the variant GSTP1 genotype may be a risk factor for male infertility because the allele 105Val was found to diminish the enzyme

<table>
<thead>
<tr>
<th>Table 1 Distribution of alleles and genotypes of polymorphism I105V of the GSTP1 gene among infertile and fertile men</th>
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<tbody>
<tr>
<td>Alleles/genotypes the GSTP1 gene</td>
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<td>----------------------------------</td>
</tr>
<tr>
<td>Allele frequencies</td>
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<tr>
<td>105I</td>
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<tr>
<td>105V</td>
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<tr>
<td>Genotype frequencies</td>
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<tr>
<td>105II</td>
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<tr>
<td>105IV</td>
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<tr>
<td>105VV</td>
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</table>

*Absolute number and percentage of individuals with particular allele/genotype; *Means a statistically significant association.
activity towards many xenobiotics, leading to the increased urinary 1-hydroxypyrene excretion (a biomarker of polycyclic aromatic hydrocarbons exposure) (Gaspari et al., 2003; Chen et al., 2007) and increased formation of DNA adducts (Brockstedt et al., 2002). Therefore, we suggest that men with a genotype 105VV of the GSTP1 gene may be susceptible to environmental toxicants affecting the reproductive system through the induction of chemical and oxidative stress, as it has been demonstrated by a number of studies (Auger et al., 2001; Tremellen, 2008; Hansen et al., 2010; Rubes et al., 2010; Aitken et al., 2014).

As male infertility is a multifactorial disease, the disease phenotype may not accurately be predicted from the knowledge of the effects of individual GST genes because of possible epistatic and additive interactions between the loci determining the common detoxication pathway. This means that the investigation of glutathione-S-transferase genes M1, T1 and P1 in combination may provide a more comprehensive picture of contribution of the genes to the development of male infertility. However, a few genetic association studies (Finotti et al., 2009; Safarinejad et al., 2010; Jaiswal et al., 2012; Salehi et al., 2012) have been designed so far to investigate the role of interactions between GST genes in idiopathic male infertility. In particular, Jaiswal et al. (2012) observed that combination of null genotypes of GSTM1 and GSTT1 confers protective effect regarding male infertility risk in North Indian population, a finding which is in agreement with our results of the protective effect of genotype combination GSTM1 del × GSTTI del. Meantime, these data are inconsistent with results of other studies in which a combination of GSTM1 and GSTTI null genotypes was found to be associated with increased risk of male infertility in a population of Brazil (Finotti et al., 2009), two Iranian populations (Safarinejad et al., 2010; Salehi et al., 2012) and in recent meta-analysis encompassing 6934 men (Wu et al., 2013). Moreover, SAFarinejad et al. (2010) observed GSTP1 wild-type genotype in combination with GSTM1 null or GSTTI null genotype increased probability for male infertility. In contrast, we found that the GSTP1 105II × GSTTI del genotype combination is associated with decreased risk of male infertility, whereas a genotype combination GSTP1 105IV × GSTTI + possessed a protective effect on the disease risk. The last relationship can be explained by a strong negative association of the GSTTI expressor genotype with infertility risk found in previous study in our Russian population (Polonikov et al., 2010), and then it was confirmed in an independent study in North Indian population (Jaiswal et al., 2012). Such contradictory results across the studies may be explained, on the one hand, by genetic diversity between the studied populations, on the other hand, by heterogeneity of disease pathogenesis in different

### Table 2: Associations of GST genotype combinations with risk of idiopathic male infertility

<table>
<thead>
<tr>
<th>Combinations of genotypes</th>
<th>Infertile men, N = 203</th>
<th>Fertile men, N = 227</th>
<th>Chi-square (P-value)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTP1 105II × GSTTI del</td>
<td>0 (0.0)</td>
<td>13 (5.7)</td>
<td>10.11 (0.001)*</td>
<td>0.04 (0.00–0.66)</td>
</tr>
<tr>
<td>GSTP1 105IV × GSTTI +</td>
<td>95 (46.8)</td>
<td>72 (31.7)</td>
<td>10.26 (0.001)*</td>
<td>1.89 (1.28–2.80)</td>
</tr>
<tr>
<td>GSTM1 + × GSTTI del</td>
<td>1 (15.5)</td>
<td>15 (6.6)</td>
<td>7.73 (0.01)</td>
<td>0.12 (0.02–0.64)</td>
</tr>
<tr>
<td>GSTM1 del × GSTTI +</td>
<td>114 (55.2)</td>
<td>106 (46.7)</td>
<td>3.84 (0.05)</td>
<td>1.46 (1.00–2.14)</td>
</tr>
<tr>
<td>GSTM1 del × GSTTI del</td>
<td>0 (0.0)</td>
<td>14 (6.2)</td>
<td>11.06 (0.001)*</td>
<td>0.04 (0.00–0.64)</td>
</tr>
</tbody>
</table>

*The bold values mean a statistically significant association after adjustment for multiple tests (P<0.05 ≤ 0.003).

### Table 3: GST genotype-smoking interactions and susceptibility to idiopathic male infertility

<table>
<thead>
<tr>
<th>Combinations of genotypes</th>
<th>Infertile men, N (%)</th>
<th>Fertile men, N (%)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smokers (N = 135)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non smokers (N = 68)</td>
<td></td>
<td></td>
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<tr>
<td>GSTP1 105II × GSTTI del</td>
<td>0 (0.0)</td>
<td>6 (5.2)</td>
<td>0.06 (0.00–1.13)</td>
</tr>
<tr>
<td>GSTP1 105IV × GSTTI +</td>
<td>60 (44.4)</td>
<td>32 (27.6)</td>
<td>2.10 (1.24–3.57)*</td>
</tr>
<tr>
<td>GSTM1 + × GSTTI del</td>
<td>134 (99.3)</td>
<td>106 (91.4)</td>
<td>8.84 (1.57–49.9)*</td>
</tr>
<tr>
<td>GSTM1 del × GSTTI del</td>
<td>0 (0.0)</td>
<td>5 (4.3)</td>
<td>0.07 (0.00–1.37)*</td>
</tr>
</tbody>
</table>

*The bold values mean a statistically significant association (P = 0.006) after adjustment for multiple tests (P<0.05 ≤ 0.013).
ethnicities and specificity of gene–gene and gene–environment interactions underlying susceptibility to male infertility in distinct human populations.

Over the last years, gene–environment interactions analysis became an emerging branch in genetic research of male factor infertility (Aydos et al., 2009; Rubes et al., 2010; Yarosh et al., 2013). This approach was proposed to overcome a problem of contradictory results between genetic association studies of male infertility and to better understand the mechanisms by which environmental factors provoke the disease in genetically susceptible men. Although such investigations are yet in their infancy, the results of our recent studies clearly demonstrated that oxidant and antioxidant exposures of the environment may potentiate the development of male infertility and their effects mediated by polymorphic genes for antioxidant and xenobiotic-metabolising enzymes (Yarosh et al., 2013, 2014). It is known that smoking status may mask the relationship between polymorphism of GST genes and male infertility risk. In particular, Aydos et al. (2009) observed that GSTM1 null genotype was a significant risk factor for male infertility only after adjustment for smoking status. Although many other studies controlled the observed associations of GST genotypes with male infertility risk for smoking habits and other confounding variables, none of them provided a stratified association analysis separately in smoker and nonsmoker men.

We found for the first time that GSTM1, GSTT1 and GSTP1 genes are collectively involved in the development of idiopathic male infertility and their phenotypic effects on the disease risk are potentiated by chemicals of cigarette smoke. Although the contribution of GST genes alone to the development of male infertility and abnormal semen parameters has been well documented in the literature, our study results complement these data with a new finding that genetically determined discoordination in the functioning of glutathione S–transferase genes may represent an important part of polygenic background of susceptibility to male infertility, a classic example of multifactorial disease. Taking the study results into account, it is possible to propose that quantity and quality of glutathione S-transferases in male reproductive organs and tissues is likely to be important in an individual’s response to environmental chemicals with reproductive toxicities and risk of developing infertility. This means that damaging effects of environmental exposures such as chemicals of cigarette smoke and air pollution on male reproductive system are potentiated by several glutathione S-transferases possessing diminished detoxication capacity towards xenobiotics-derived intermediates. The study findings justify the need for further investigations which should be focused on a comprehensive evaluation of the pathogenetic role for a number of biotransformation and/or antioxidant genes in combination with different oxidant exposures to identify gene–gene and gene–environment interactions underlying possible toxicogenic mechanisms of male infertility in the modern world.

References


