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Genetic polymorphisms of *CYP2D6*10* and the effectiveness of combined tamoxifen citrate and testosterone undecanoate treatment in infertile men with idiopathic oligozoospermia*

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Abstract: Tamoxifen citrate, as the first line of treatment for infertile men with idiopathic oligozoospermia, was proposed by the World Health Organization (WHO), and testosterone undecanoate has shown benefits in semen values. Our objective was to assess the effectiveness of treatment with tamoxifen citrate and testosterone undecanoate in infertile men with idiopathic oligozoospermia, and whether the results would be affected by polymorphisms of *CYP2D6*10*. A total of 230 infertile men and 147 controls were included in the study. Patients were treated with tamoxifen citrate and testosterone undecanoate. Sex hormone, sperm parameters, and incidence of spontaneous pregnancy were detected. There were no significant differences between the control and patient groups with respect to *CYP2D6*10* genotype frequencies (P>0.05). The follicle-stimulation hormone (FSH), luteinizing hormone (LH), and testosterone (T) levels were raised, and sperm concentration and motility were increased at 3 months and became significant at 6 months, and they were higher in the wild-type allele (C/C) than in the heterozygous variant allele (C/T) or homozygous variant allele (T/T) subgroups (P<0.05). In addition, the percentage of normal morphology was raised at 6 months, and represented the highest percentage in the C/C subgroup (P<0.05). The incidence of spontaneous pregnancy in the C/C subgroup was higher than that in the C/T or T/T subgroups (P<0.01). This study showed that the *CYP2D6*10* variant genotype demonstrated worse clinical effects in infertile men with idiopathic oligozoospermia.

Key words: Infertility, Cytochrome P450, Oligozoospermia, Tamoxifen, Testosterone
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1 Introduction

Tamoxifen (TMX) was used for the treatment of oligospermia for the first time by Comhaire (1976) and has become one of the widely prescribed drugs for idiopathic male infertility until now (Comhaire, 1976; Chua *et al*., 2013). Tamoxifen citrate, as a first line of treatment for infertile men with idiopathic oligozoospermia, was proposed by the World Health Organization (WHO) Working Committee (Rowe *et al*., 2000). Tamoxifen undergoes extensive hepatic and gut wall metabolism in humans for several primary and secondary metabolites that exhibit a range of pharmacologic activity (Squirewell *et al*., 2014). Previous research demonstrated that endoxifen formation

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proceeds stepwise by oxidation of tamoxifen with *N*-desmethyltamoxifen (NDM) as the predominant intermediate (Desta *et al*., 2004), and patients receiving tamoxifen are influenced by cytochrome P4502D6 (*CYP2D6*) genetic variants (Stearns *et al*., 2003; Jin *et al*., 2005). According to the activity of the CYP2D6 enzyme, patients should be grouped into poor metabolizer (PM), extensive metabolizer (EM), and ultra-rapid metabolizer (UM) phenotypes. There are significant inter-ethnic differences in the frequency of the PM phenotype and PM alleles of *CYP2D6*. Except for the non-functional *CYP2D6* PM alleles, intermediate metabolizers (IM) have reduced enzyme activity and are common in Orientals; one of the very common IM alleles is *CYP2D6*10* (Ji *et al*., 2002a; Borges *et al*., 2006). Among Chinese, the most common polymorphism in *CYP2D6* is allelic variant *10, which generates a 188 C to T transition in exon 1, leading to a proline 34 to serine amino acid substitution and resulting in an unstable enzyme with lower catalytic activity (Johansson *et al*., 1994; Garcia-Barcelo *et al*., 2000; Ji *et al*., 2002b).

Recent studies have shown that *CYP2D6*10* genotype affects the efficacy of tamoxifen treatment in Chinese women with breast cancer, although women with the *CYP2D6*10* variant homozygous variant allele (T/T) genotype have a worse clinical outcome (Xu *et al*., 2008). Other studies have shown that giving tamoxifen citrate alone exerts a limited effect on sperm morphology and motility; it appears that the superior functional sperm fraction response was probably related to administration of testosterone undecanoate (Adamopoulos *et al*., 1997; 2003). In this study, we assess the effectiveness of treatment with tamoxifen citrate and testosterone undecanoate in infertile men with idiopathic oligozoospermia, and whether they are affected by polymorphisms of *CYP2D6*10*.

2 Materials and methods

2.1 Subjects

This study included 230 infertile men (mean age (27±3.7) years, range 22–40 years) with idiopathic oligospermia diagnosed at the First Affiliated Hospital of the Medical College of Xi'an Jiaotong University, China. All patients had a history of infertility for at least one year, with their spouses confirmed to have had a normal gynecological evaluation. All of the subjects had sperm concentration below the WHO (1999) threshold (20×10^6 ml^{-1}) on at least two semen analyses. The infertile men with known or demonstrable causes of oligozoospermia (varicocele, infections, autoimmunity, stress, chromosomal abnormalities, environmental factors, or epididymopathy) were excluded. The study also included 147 volunteers with normozoospermic semen (mean age (25±2.5) years, range 23–35 years). Their sperm underwent the same examinations as those of the oligozoospermic men. Written informed consent was obtained from all the patients and controls included in this study. The protocol was approved by the Ethics Committee of the First Affiliated Hospital of the Medical College of Xi'an Jiaotong University, China.

2.2 Protocol

The protocol included two phases. Initial assessment was made during the first phase. The basal follicle-stimulation hormone (FSH), luteinizing hormone (LH), and testosterone (T) were measured during this phase, and at least two semen analyses were taken after 3–5 d of sexual abstinence in all participants. None of the participants had received any medication for at least 3 months before entry into the trial, and each of them had not participated in any recent relevant study. Additional blood samples (3 ml taken into ethylene diamine tetraacetic acid (EDTA) by venipuncture) were obtained from all participants. After collection, the whole blood was immediately stored at −80 °C until use for genomic DNA extraction. In the second phase, all the patients (n=230) received tamoxifen citrate (20 mg/d) and testosterone undecanoate (40 mg/d), and the treatment was prescribed for 6 months. Sex hormone measurement and semen analysis were performed at the 3rd and 6th months, respectively, and the percentage of pregnancies was recorded as well.

2.3 Measurements

The FSH, LH, and T in serum were measured by using a double-antibody recombinant immunoassay (CIS Biointernational, Paris, France, for FSH and LH; Farmos, Oulunsalo, Finland, for T). Semen samples were obtained after 3–5 d of sexual abstinence and were analyzed within 1 h after collection. In all patients, a standard semen analysis was performed, assessing semen

parameters, including sperm concentration, motility, and morphology according to the WHO (1999) guidelines.

2.4 DNA source, genotyping, and definition of phenotypes

An AxyPrep™ Genomic DNA Miniprep Kit (Axygen Biosciences, Union City, CA, USA) was used to isolate genomic DNA from blood samples. The mutant *CYP2D6*10* (C188T) allele was detected by polymerase chain reaction (PCR) amplification using primers: forward, 5'-TCAACACAGCAGGT TCA-3'; reverse, 5'-CTGTGGTTTCACCCACC-3', followed by *Hph*I digestion (5 U, 37 °C) (Sachse *et al.*, 1997). PCR was performed in a 25-μl reaction buffer containing 200 μmol/L dNTPs, 1.5 mmol/L $MgCl_2$, 10 pmol of each primer, approximately 200 ng of template DNA, and 2 U of thermostable Taq DNA polymerase (MBI, Vilnius, Lithuania). After a 5-min pretreatment at 95 °C, the specific conditions used for all PCR amplifications (30 cycles) were denaturation for 45 s at 94 °C, annealing for 45 s at 55 °C, and fragment extension for 45 s at 72 °C, and a final elongation step for 7 min at 72 °C. PCR products digested by restriction enzyme were electrophoresis on a 2% (0.02 g/ml) agarose gel stained with ethidium bromide to verify the correct size of the expected fragments. The mutant *CYP2D6*10* (C188T) allele was detected by *Hph*I digestion after PCR amplification, the 433 bp amplified product was digested into 362 and 71 bp products for wild-type allele (C/C), and 262, 100, and 71 bp products for homozygous variant allele (T/T), and 362, 262, 100, and 71 bp products for heterozygous variant allele (C/T).

2.5 Statistical analysis

The semen analyses and sex hormone results before treatment were compared with those at 3- or 6-month using the paired Student's *t*-test. The comparisons of variance on semen analyses and hormone results between genotype groups and between patients taking CYP2D6*10 inhibitors and those not taking CYP2D6*10 inhibitors were performed by use of *t*-tests. Phenotype expression in each defined genotype group was reported as mean±standard deviation (SD). The associations between *CYP2D6*10* genotype subgroups were evaluated by Chi-square test, and $P<0.05$ was considered statistically significant.

3 Results

At the ending of this study, 204 of 230 infertile men with idiopathic oligozoospermia and 147 controls completed the protocols. No statistically significant differences were found between the patients and controls with respect to age, smoking, or alcohol consumption. In addition, no differences were found among the subgroups with respect to the age of the female partners.

3.1 Genotypes of *CYP2D6*10*

The frequencies of the *CYP2D6*10* C/C, C/T, and T/T genotypes in the control and patient groups are shown in Table 1. Both of these two groups did deviate from the Hardy-Weinberg equilibrium law ($P>0.05$). The frequencies of *CYP2D6*10* C/C, C/T, and T/T genotypes were 19.73%, 55.78%, and 24.49%, respectively, in the control group; and 18.14%, 55.88%, and 25.98%, respectively, in the patient group. No significant differences were found between the control and patient groups with respect to the frequencies of the *CYP2D6*10* genotypes ($P>0.05$).

Table 1 Frequencies of *CYP2D6*10* genotypes in the control and patient groups

*CYP2D6*10* genotype	Frequency*		χ^2	*P*-value	OR (95% CI)
	Control	Patient			
Total	147	204			
C/C	29 (19.73%)	37 (18.14%)	0.142	0.707	0.902 (0.525–1.547)
C/T	82 (55.78%)	114 (55.88%)	<0.001	0.985	1.004 (0.655–1.539)
T/T	36 (24.49%)	53 (25.98%)	0.1	0.751	1.082 (0.664–1.765)
HWE (χ^2, *P*-value)	2.053, 0.152	3.165, 0.075			

* Data are expressed as number (percentage). C/C: wild-type allele; C/T: heterozygous variant allele; T/T: homozygous variant allele; HWE: Hardy-Weinberg equilibrium law; OR: odds ratio; CI: confidence interval

3.2 Association of *CYP2D6*10* genotypes with sex hormone and semen parameters

The levels of the FSH, LH and T, sperm concentration, motility, and normal morphology in the participants are shown in Table 2. No significant differences were found with the basal evaluation of FSH, LH, T, density, motility, or normal morphology between the control and patient groups, and among the subgroups in patients with respect to *CYP2D6*10* genotypes (P>0.05).

At the 3rd month, FSH, LH and T levels, motility, and the percentage of normal morphology in the C/C subgroup were all higher than those in the basal evaluation and C/T and T/T subgroups, respectively (P=0.001, P<0.001, P=0.002 for FSH; P=0.001, P=0.009, P=0.001 for LH; P<0.001, P<0.001, P<0.001 for T; P=0.034, P=0.009, P=0.021 for motility; P=0.157, P=0.051, P=0.064 for the percentage of normal morphology). Sperm concentration in the C/C subgroup was higher than those in the basal evaluation and T/T subgroup, respectively (P=0.015, P=0.004).

At the 6th month, we found that FSH, LH, and T levels, sperm concentration, and motility in the C/C subgroup were all higher than those in the basal evaluation and 3-month evaluation, and also C/T and T/T subgroups, respectively (P<0.001, P<0.001, P<0.001, P<0.001 for all). The percentage of normal morphology in the C/C subgroup was also higher than those in the basal evaluation, 3-month evaluation, and C/T and T/T subgroups, respectively (P<0.001, P=0.022, P=0.002, P<0.001).

3.3 Association of *CYP2D6*10* genotypes with pregnancy incidence

In this study, the incidence of spontaneous pregnancy was 21.1% (43/204) in the infertile men with idiopathic oligozoospermia, after treatment with tamoxifen citrate and testosterone undecanoate (Table 3). According to the genotypes of *CYP2D6*10*, the incidence of spontaneous pregnancy was 40.5% (15/37) in the C/C subgroup, 20.2% (23/114) in the C/T subgroup, and 9.4% (5/53) in the T/T subgroup. It was shown that the incidence of spontaneous pregnancy in the C/C subgroup was higher than those in the C/T and T/T subgroups, respectively (χ^2=6.152, P=0.013; χ^2=12.198, P<0.001).

Table 2 Sex hormones and sperm parameters in each group

Group	No.	FSH (IU/L)	LH (IU/L)	T (nmol/L)	Sperm concentration ($\times10^6$ ml^{-1})	Motility (%)	Normal morphology (%)
Basal evaluation							
Control	147	8.03±1.01	7.31±1.32	15.01±2.98	56.72±3.07	59.24±4.80	55.39±3.95
Patient	204	7.73±1.88	7.22±1.98	13.70±3.80	9.45±2.35	30.39±3.60	44.52±4.87
C/C	37	7.85±1.12	7.16±1.12	13.77±2.13	9.35±1.43	30.17±5.05	45.02±5.83
C/T	114	7.66±2.06	7.23±2.49	13.87±3.88	9.56±2.93	30.10±3.16	45.49±4.10
T/T	53	7.79±1.92	7.22±1.01	13.14±4.40	9.29±1.24	31.20±3.08	44.09±4.90
Three-month evaluation							
Patient	204	8.02±1.77	7.58±2.44	14.41±3.73[a]	9.74±1.78	31.22±4.43[a]	45.97±4.94
C/C	37	9.29±2.15[a]	8.35±1.34[a]	16.99±3.50[a]	10.23±1.56[a]	33.61±7.62[a]	46.95±6.08
C/T	114	7.72±1.10[c]	7.43±2.98[c]	13.97±3.21[c]	9.73±2.02	30.09±3.28[c]	46.31±4.24
T/T	53	7.91±2.28[c]	7.31±1.43[c]	13.55±4.18[c]	9.40±1.21[c]	31.96±2.34[c]	44.68±5.32
Six-month evaluation							
Patient	204	9.52±4.05[a,b]	8.76±3.16[a,b]	19.28±7.37[a,b]	11.35±4.60[a,b]	34.34±5.74[a,b]	47.35±3.98[a,b]
C/C	37	15.69±4.30[a,b]	13.95±2.77[a,b]	31.85±6.10[a,b]	18.19±1.23[a,b]	40.76±5.21[a,b]	50.17±5.18[a,b]
C/T	114	8.11±1.51[c]	7.66±2.01[c]	17.06±4.02[c]	9.98±4.13[c]	32.84±5.42[c]	47.15±2.79[c]
T/T	53	8.06±3.04[c]	7.50±1.18[c]	15.27±3.46[c]	9.53±1.90[c]	33.10±3.16[c]	45.85±4.28[c]

FSH: follicle-stimulation hormone; LH: luteinizing hormone; T: testosterone; C/C: wild-type allele; C/T: heterozygous variant allele; T/T: homozygous variant allele. [a] P<0.05 vs. baseline value; [b] P<0.05 vs. 3-month value; [c] P<0.05 vs. C/C genotype

Table 3 Incidence of spontaneous pregnancy in each group

Group	No.	Incidence of spontaneous pregnancy*				χ^2	*P*-value
		Starting	Three-month	Six-month	Total		
Patient	204	0	8 (3.2%)	35 (17.2%)	43 (21.1%)		
C/C	37	0	3 (8.1%)	12 (32.4%)	15 (40.5%)		
C/T	114	0	4 (3.5%)	19 (16.7%)	23 (20.2%)	6.152	0.013[a]
T/T	53	0	1 (1.9%)	4 (7.5%)	5 (9.4%)	12.198	<0.001[a]

* Data are expressed as number (percentage), [a] Compared with the C/C genotype

4 Discussion

Tamoxifen is a trans isomer of clomiphene citrate, which is a combination of two isomers that exert both anti-estrogenic and estrogenic effects simultaneously and human studies have confirmed its estrogenic activity to be minimal or negligible (Lin *et al*., 2010; Lu *et al.*, 2012). Tamoxifen citrate enhances spermatogenesis by increasing FSH, leydig cells sensibility to LH, and testosterone levels, which lacks an intrinsic oestrogenic effect, so it may be more appropriate to use in male infertility (Buvat *et al.*, 1983; Kadioglu *et al*., 1999). Previous studies demonstrated that tamoxifen significantly increased sperm concentration in infertile men with oligozoospermia, but does not affect other semen values, such as volume, pH, motility, morphology, or viability, because of tamoxifen's effectiveness on the seminiferous tubules during the early stages of spermatogenesis (Vermeulen and Comhaire, 1978; Kotoulas *et al*., 1994). Other studies have shown that given tamoxifen citrate alone exerts a limited effect on sperm morphology and motility; it appears that the superior functional sperm fraction response was probably related to administration of testosterone undecanoate (Adamopoulos *et al*., 1997; 2003). In this study, the sperm concentration, motility, and percentage of normal morphology were improved in infertile men with oligozoospermia after treatment with tamoxifen citrate and testosterone undecanoate.

Endoxifen is one of the most important metabolites of tamoxifen, and plasma concentrations of endoxifen appeared to be influenced by the patient's *CYP2D6* genotype. Previous studies have shown that plasma concentrations of endoxifen were statistically significantly lower in patients who were carriers of non-functional *CYP2D6* allelic variants, compared with those having two functional wild-type alleles, and the *CYP2D6*10* genotype is the most common polymorphism of *CYP2D6* in the Chinese population (Stearns *et al.*, 2003; Jin *et al.*, 2005). The percentage of homozygous variant T/T genotype was 24.49% in the control group and 25.98% in the patient group in our present study, which was similar to previous reports (Johansson *et al.*, 1994; Garcia-Barcelo *et al.*, 2000). *In vitro* experiments have shown that the catalytic activity of the homozygous variant T/T genotype is 1/40 of the activity of the wild-type C/C genotype (Johansson *et al*., 1994). In our present study, it was shown that the levels of the FSH, LH and T, sperm concentration, motility, percentage of normal morphology, and the incidence of spontaneous pregnancy in patients with the T/T and C/T genotypes had a lower outcome than those in patients with the C/C genotype.

5 Conclusions

In summary, we found that the *CYP2D6*10* mutant genotype had a worse clinical outcome in the combined treatment of tamoxifen citrate and testosterone undecanoate in infertile men with idiopathic oligozoospermia. Analyses of *CYP2D6*10* genotype may be useful for patients with idiopathic oligozoospermia, and may benefit from treatment when combining tamoxifen citrate with testosterone undecanoate. Nevertheless, our study does have certain limitations. Our results should be carefully interpreted and the *CYP2D6*10* genotypes should not apply in clinical practice until the data from more center studies are available.

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Compliance with ethics guidelines

Kai-fa TANG, Yi-li ZHAO, Shang-shu DING, Qi-fei WU, Xing-yang WANG, Jia-qi SHI, Fa SUN, and Jun-ping XING declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study. Additional informed consent was obtained from all patients for whom identifying information is included in this article.

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中文概要

题　目：*CYP2D6*10*基因多态性对他莫昔芬联合十一酸睾酮治疗特发性少精男性不育症的疗效影响

目　的：探讨细胞色素 P450 2D6*10（*CYP2D6*10*）基因遗传多态性，并评估其对他莫昔芬联合十一酸睾酮治疗特发性少精男性不育症患者血清性激素、精液参数及自然妊娠率的影响。

方　法：该病例对照研究包括 230 例特发性少精男性不育患者和 147 例正常对照。病例组服用枸橼酸他莫昔芬 20 mg/d 和十一酸睾酮 40 mg/d，疗程共 6 个月。采用 *Hph*I 内切酶对 *CYP2D6*10* 基因聚合酶链式反应（PCR）产物进行内切后，从而对其分型。分别于研究开始时、3 月及 6 月分别检测研究对象性激素水平、精液参数及配偶自然妊娠率。

结　论：*CYP2D6*10* 基因突变型特发性少精男性不育患者接受他莫昔芬联合十一酸睾酮疗效较基因野生型组差。

关键词：男性不育症；细胞色素 P450；少精子症；他莫昔芬；性激素