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ORIGINAL ARTICLE

Genetic variants in *TP53* and *MDM2* associated with male infertility in Chinese population

Cong Huang[1,2], Wei Liu[1,2], Gui-Xiang Ji[3], Ai-Hua Gu[1,2], Jian-Hua Qu[4], Ling Song[1,2] and Xin-Ru Wang[1,2]

The TP53, a transcriptional regulator and tumor suppressor, is functionally important in spermatogenesis. MDM2 is a key regulator of the p53 pathway and modulates p53 activity. Both proteins have been functionally linked to germ cell apoptosis, which may affect human infertility, but very little is known on how common polymorphisms in these genes may influence germ cell apoptosis and the risk of male infertility. Thus, this study was designed to test whether three previously described polymorphisms 72Arg>Pro (rs1042522) and the Ex2+19C>T (rs2287498) in *TP53*, and the 5′ untranslated region (5′ UTR) 309T>G (rs937283) in *MDM2*, are associated with idiopathic male infertility in a Chinese population. The three polymorphisms were genotyped using OpenArray assay in a hospital-based case–control study, including 580 infertile patients and 580 fertile controls. Our analyses revealed that *TP53* Ex2+19C>T and *MDM2* 309T>G polymorphisms are associated with male infertility. Furthermore, we detected a nearly statistically significant additive interaction between *TP53* rs2287498 and *MDM2* rs937283 for the development of male infertility ($P_{interaction}$=0.055). In summary, this study found preliminary evidence, demonstrating that genetic variants in genes of the TP53 pathway are risk factors for male infertility.
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INTRODUCTION

Infertility is a worldwide health issue that affects almost 10%–15% of couples, among which about half of the cases are due to male factors.[1,2] A couple is considered infertile when they have not conceived after a full year of regular sexual intercourse without contraception. Men are considered infertile if they produce no sperm cells (azoospermia), too few sperm cells (oligospermia), or if sperm cells are abnormal or die before they can reach the egg. However, the causes of most cases remain unknown.[3] Semen quality is an important factor affecting male infertility. Germ cell apoptosis is a normal process during spermatogenesis. For example, the apoptotic Fas/Fasl pathway can mediate ordinary sperm production and affect semen parameters in human.[4,5] Other studies have proved that *TP53* and *MDM2* may play an indispensable role in spermatogenesis.[6] The spontaneous testicular germ cell apoptosis and germ cell quality control in spermatogenesis is TP53-mediated.[7] It has been reported that $TP53^{-/-}$ mice exhibit significantly less mature motile spermatozoa than their $TP53^{+/+}$ counterparts,[8,9] and have testicular giant-cell degenerative syndrome. MDM2, which interacts with TP53 and inhibits TP53 cell-cycle arrest and apoptosis,[10] is critical in regulating TP53 function. It is observed that *MDM2* knockout mice is lethal in early embryogenesis, while mice with simultaneous knockout of *TP53* and *MDM2* can survive and develop normally, indicating that MDM2 is essential in negative regulation of TP53 during development.[11] It is significant that both *TP53* and *MDM2* are haploinsufficient genes, with any change resulting in great impact on the function of the *TP53* pathway.[12,13] Although lots of single nucleotide polymorphisms (SNPs) affecting male infertility have been reported,[2,14–16] few researchers have thought about SNPs in the apoptosis pathway, especially *TP53* and *MDM2*. Researchers have discovered numerous SNPs in the *TP53* pathway, and some of them may alter the function of TP53 protein.[17] For instance, a *TP53* SNP in codon 72 results in a change from Arginine (Arg) to Proline (Pro).[13] It has also been reported that *MDM2* SNP309 can increase MDM2 expression and leads to attenuation of TP53 function.[18]

Although polymorphisms of *TP53* and *MDM2* have an impact on sperm apoptotic mechanism to some degree, no study has clarified whether their polymorphisms are associated with the risk of infertility in Chinese man. In this study, we compared the genotypes of three polymorphisms between infertile men and health controls to explore the risk factor of male infertility for the first time.

MATERIALS AND METHODS

Subjects

A total of 580 infertile men were recruited from the Centre of Clinical Reproductive Medicine between April 2004 and May 2007. All of the patients received physical tests, semen analyses, serum determination of follicle-stimulating hormone, luteinizing hormone and testosterone, karyotyping, and Y-chromosome microdeletion screening. The inclusion criteria consisted of six terms: (i) azoospermia or severe oligozoospermia (sperm count $<5\times10^6$ ml^{-1}) as demonstrated by

[1]State Key Laboratory of Reproductive Medicine, Institute of Toxicology, Nanjing Medical University, Nanjing 210029, China; [2]Key Laboratory of Modern Toxicology of Ministry of Education, School of Public Health, Nanjing Medical University, Nanjing 210029, China; [3]Nanjing Institute of Environmental Sciences/Key Laboratory of Pesticide Environmental Assessment and Pollution Control, Ministry of Environmental Protection, Nanjing 210042, China and [4]School of Public Health, Nantong University, Nantong 226019, China
Correspondence: Dr XR Wang (xrwang@njmu.edu.cn)
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at least two semen analyses performed according to the World Health Organization criteria (World Health Organization report, 1999); (ii) a normal 46 karyotype with XY sex chromosome; (iii) absence of Y chromosomal microdeletions of AZF region proved by the corresponding molecular analysis;[19] (iv) lack of hypogonadotropic hypogonadism; (v) normal sexual and ejaculatory functions and no seminal tract obstruction or varicocele; (vi) no history of infection or other diseases that could affect fertility. In total, 580 idiopathic infertile men aged from 25 to 38 years were eligible for this study. The control group consists of 580 fertile men who have fathered at least one child without assisted reproductive technologies and have normal semen quality. All the participants in our study are Chinese Han nationality. They all provided informed consent and completed a questionnaire which included information about personal background, cigarette smoking, drinking status, occupational and so on. Finally, 5 ml of peripheral blood was extracted for genomic DNA genotyping. The study was approved by the Ethics Review Board of Nanjing Medical University.

Genotyping

Genomic DNA was extracted from leucocyte pellet by proteinase K digestion and followed by phenol-chloroform extraction and ethanol precipitation. Genotyping was performed using the OpenArray platform (Applied Biosystems, Foster City, CA, USA), and a chip-based TaqMan genotyping technology was employed in the system. Genotyping was conducted and genotype data analysis were made by OpenArray SNP Genotyping Analysis Software V.1.0.3 (Applied Biosystems). For quality control, genotyping was done without knowledge of case/control status of subjects, and a random 5% of cases and controls were genotyped twice by different individuals, and the reproducibility was 100%. To confirm the genotyping results, selected PCR-amplified DNA samples (n=2, for each genotype) were examined by DNA sequencing and the results were also consistent.

Statistical analysis

The differences in genotype distributions of selected characteristics between cases and fertile controls were evaluated using chi-squared analysis. The association between polymorphisms and the risk of male infertility was estimated by comparing the odds ratios (OR) and their 95% confidence intervals (CI) with unconditional univariate and logistic regression models. The potential gene–gene interactions were evaluated by logistic regression analyses and tested by comparing the changes in deviance (−2 log likelihood) between models of main effects with or without the interaction term. All analyses were conducted in the Statistical Analysis System (version 9.13; SAS Institute, Cary, NC, USA), and the $P<0.05$ was used as the criterion of significance.

RESULTS

The frequencies of distributions of the selected characteristics of age, smoking status, pack-years of smoking and drinking status in the study subjects are displayed in **Table 1**. We sought to investigate the effect of drinking status and cigarette smoking on fertility rate in a large, well-defined groups, in which the criteria define people who have ⩾2 cigarette per day as 'smokers' and people who drink ⩾2 times per day as 'drinkers'. No obvious differences were observed between cases and controls with regard to the drinking status and age ($P>0.05$). However, there was a significantly higher percentage of smokers among cases than controls ($P=0.046$). Among smokers, cases also reported greater cigarette consumption than controls, as assessed by the mean number of pack-years ($P=0.019$).

Table 1 Distribution of selected characteristics between cases and fertile controls

Variables	*Controls (n=580)*	*Cases (n=580)*	P[b]
Age (mean±s.d.), year	28.1±3.2	28.2±3.3	0.600
Smoking status, *n* (%)			
Never	306 (52.8)	272 (46.9)	0.046
Ever	274 (47.2)	308 (53.1)	
Pack-years (mean±s.d.)[a], year	4.1±4.3	4.7±4.4	0.019
Drinking status, *n* (%)			
Never	504 (86.9)	500 (86.2)	0.731
Ever	76 (13.1)	80 (13.8)	

[a] Among ever smokers.
[b] *P* values were derived from the chi-squared test for categorical variables (smoking and drinking status) and *t*-test for continuous variables (age and pack-years).

The frequencies of *TP53* 72Arg>Pro (rs1042522), Ex2+19C>T (rs2287498) and *MDM2* 309T>G (rs937283) genotypes in cases and controls and their associations with the risk of idiopathic male infertility are shown in **Table 2**. All observed SNPs were in Hardy–Weinberg equilibrium ($P=0.181$ for rs1042522, $P=0.698$ for rs2287498 and $P=0.170$ for rs937283, respectively). Overall, two SNPs (rs2287498 and rs937283) exhibited a significant association with the risk of male infertility under a dominant model (variant-containing genotypes *vs.* homozygous wild-type genotype). For the *TP53* Ex2+19C>T (rs2287498) polymorphisms, carriers of variant allele (T allele) had a 46% increased risk of male infertility compared with individuals with common homozygous genotype (CC). Similarly, patients with the *MDM2* rs937283 GG genotype exhibited a significantly increased male infertility risk (adjusted OR=1.55; 95% CI=1.11–2.16). However, as to the *TP53* 72Arg>Pro (rs1042522) polymorphisms, no significant differences were observed.

We further evaluated the additive combined effects of the two high-risk genotypes on male infertility by combining the at-risk genotypes of *TP53* rs2287498 and *MDM2* rs937283. As shown in **Table 3**, 46.2% of the cases and 38.1% of the controls carried the four variant genotypes (*TP53* rs2287498 CT/TT and *MDM2* rs937283 TG/GG), and these carriers had an increased risk of male infertility (adjusted OR=1.95; 95% CI=1.32–2.90). Logistic regression analyses revealed nearly statistically significant additive interaction between *TP53* rs2287498 and *MDM2* rs937283 for the development of male infertility (P for additive interaction=0.055).

DISCUSSION

In testis, apoptosis is a way to eliminate damaged germ cells during their development. The loss of functional p53 protein leads to a disruption in the apoptosis process. As essential genes in the apoptosis pathway, TP53 and MDM2 are thought to provide another level of stringency in addition to other spermatogenic 'quality control' mechanisms. Moreover, TP53 contributes to the efficiency of DNA repair during the postmitotic stages of spermatogenesis.[10] If either the TP53 or the MDM2 pathway is abnormal in its function, for example, some SNPs that affect their functions, infertility may occur.

It has been reported that *TP53* 72Arg>pro and *MDM2* 309T>G modify the activity or the levels of the TP53 protein, and polymorphisms of the two genes are associated with the risk of apoptosis disorder diseases, such as various cancers.[20,21] The codon 72Arg allele induces apoptosis better than the codon 72Pro allele to the mitochondria.[22] While considering that the 72Arg may enhance localization of the Arg72 variant to the mitochondria which participates in an important process of apoptosis, we also assumed that germ cell apoptosis may be

Table 2 Genotype and allele frequencies of *TP53* and *MDM2* genes among the patients and controls and their associations with male infertility

Genotype	Cases		Controls		Crude OR	Adjusted OR	P-value[b]	
	n	(%)	n	(%)	(95% CI)	(95% CI)[a]	Genotype	Allele
TP53 72Arg>Pro (rs1042522)							0.361	0.146
GG	154	(26.6)	170	(29.3)	1.00	1.00		
GC	270	(46.6)	273	(47.1)	1.09 (0.83–1.44)	1.05 (0.79–1.38)		
CC	156	(26.9)	137	(23.6)	1.26 (0.92–1.73)	1.20 (0.88–1.65)		
GC+CC	426	(73.4)	410	(70.7)	1.15 (0.89–1.48)	1.10 (0.85–1.42)		
TP53 Ex2+19C>T (rs2287498)							0.0002	<0.001
CC	239	(41.2)	296	(51.0)	1.00	1.00		
CT	254	(43.8)	234	(40.3)	1.34 (1.05–1.72)	1.32 (1.03–1.69)		
TT	87	(15.0)	50	(8.6)	2.15 (1.46–3.17)	2.11 (1.44–3.11)		
CT+TT	341	(58.8)	284	(49.0)	1.49 (1.18–1.88)	1.46 (1.16–1.84)		
MDM2 5′ UTR T>G (rs937283)							0.011	0.004
TT	120	(20.7)	149	(25.7)	1.00	1.00		
TG	295	(50.9)	306	(52.8)	1.21 (0.90–1.61)	1.13 (0.85–1.51)		
GG	165	(28.4)	125	(21.6)	1.65 (1.18–2.31)	1.55 (1.11–2.16)		
TG+GG	460	(79.3)	431	(74.3)	1.31 (1.00–1.72)	1.23 (0.93–1.61)		

Abbreviations: 5′ UTR, 5′ untranslated region; CI, confidence interval; OR, odds ratio.
[a] Adjusted for age, smoking and drinking status.
[b] Two-sided chi-squared test for the distributions of genotype and allele frequencies.
P for Hardy-Weinberg (HW) equilibrium: rs1042522, $P=0.181$; rs2287498, $P=0.698$; rs937283, $P=0.170$.

affected by this pathway. Hence, we deemed that polymorphisms of *TP53* and *MDM2* may be associated with germ cell apoptosis and male infertility.

In this study, three functional polymorphisms (*TP53* 72Arg>Pro (rs1042522), *TP53* 5′ untranslated region (5′ UTR) Ex2+19C>T (rs2287498) and *MDM2* 5′ UTR 309T>G (rs937283)) in apoptosis pathway genes were genotyped for investigation of their roles in male fertility. To summarize, we found that the functional polymorphisms of *TP53* Ex2+19C>T and *MDM2* 309T>G have a higher risk of male infertility. The results are consistent with previous data in cancer.[23,24] Previous studies have shown that the GG type of *MDM2* SNP309 is associated with an elevated expression of MDM2 protein.[25] The elevated expression of *MDM2* downregulates *TP53* which leads to the inhibition of the *TP53*-induced apoptotic pathway. Although our results suggest that the SNP of *TP53* 72Arg>Pro does not directly cause idiopathic male infertility, they may perhaps affect male fertility by combining with some additional polymorphisms in other genes or do not have inherited risk factors.

Another interesting finding in our study was that smoking status has a negative effect on reproduction ($P<0.05$). To date, cigarette consumption is a worldwide health problem. However, studies investigating the relationship between smoking and infertility have provided some inconsistent results. A population study of Jordanians found that smokers had significantly lower sperm concentration and motility values and higher serum testosterone and luteinizing hormone levels than non-smokers.[26] Another population study in Shandong, China, showed that the sperm density, viability and forward progression, and the seminal plasma Zn, Cu and superoxide dismutase levels were negatively correlated with the amount and duration of cigarette smoking ($P<0.01$).[27] It is more likely that smoking is an extrinsic factor for sperm DNA damage, which is associated with a lower pregnancy rate and an increase of pregnancy loss in assisted reproduction treatments.[28] Cigarette smoke contains a variety of reactive oxygen species, which would cause sperm DNA damage.[29,30] Smoking may also damage the chromatin structure and produce endogenous DNA strand breaks in human sperm, resulting in a reduced semen quality.[31] Levels of DNA damage tend to be higher in smokers.[32] While in other population studies, for example, Gu *et al.*[33] and Ji *et al.*[34] found that tea consumption may affect sperm quality but not smoking, researchers have obtained different conclusions. This division may be explained by different study populations.

In addition to genotype changes affecting male reproductive health, we cannot ignore the importance of genotype–environment interactions. For examples, in Chinese workers, semen quality is affected by environment factor such as organophosphate pesticide exposure, and genetic factor such as polymorphisms in the paraoxonase gene, which is involved in the metabolism of these pesticides.[35] An additional example of gene–environment interaction is the C677T polymorphism in the gene encoding for methylenetetrahydrofolate reductase, and it showed that the positive effect of folic acid and zinc sulphate

Table 3 Combined effect of *TP53* Ex2+19C>T and *MDM2* 5′ UTR T>G on male infertility risk

TP53 *Ex2+19C>T (rs2287498)*	MDM2 *5′ UTR T>G (rs937283)*	*Cases,* n *(%)*	*Controls,* n *(%)*	*OR (95% CI)*[a]
CC	TT	47 (8.1)	86 (14.8)	1.00
CC	TG/GG	192 (33.1)	210 (36.2)	1.57 (1.05–2.35)
CT/TT	TT	73 (12.6)	63 (10.9)	1.99 (1.22–3.23)
CT/TT	TG/GG	268 (46.2)	221 (38.1)	1.95 (1.32–2.90)
$P_{interaction}$[b]				0.055

Abbreviations: 5′ UTR, 5′ untranslated region; CI, confidence interval; OR, odds ratio.
[a] Odds ratio was adjusted for age, smoking and drinking status.
[b] $P_{interaction}$ for additive interaction.

supplementation on sperm concentration was seen only in subjects without the T variant (CC homozygotes). However, in the present study, the polymorphism itself was not a risk factor for male subfertility.[36]

In summary, our study showed for the first time that *TP53* Ex2+19C>T and *MDM2* 309T>G polymorphisms are associated with male infertility, based on a Chinese population including 580 infertile men and 580 health controls. We will explore genotype–environment interactions on Chinese male infertility in the future. A larger sample size is needed to confirm our results, and *in vivo* functional studies are also necessary to identify the biological mechanisms.

AUTHOR CONTRIBUTIONS

CH was the main investigator, wrote the article and participated in the conception of the study. GXJ performed the acquisition, analysis of data and the patient recruitment, follow-up and drafting of the article. WL and JHQ participated in the execution of the study. AHG made important contributions to the study design and sample collection. LS assisted in performing the experiment. XRW designed the study and is the corresponding author of the article. All authors read and approved the final manuscript.

COMPETING FINANCIAL INTERESTS

The authors confirm that there is no conflict of interest.

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