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Association of a miR-34b binding site single nucleotide polymorphism in the 3'-untranslated region of the methylenetetrahydrofolate reductase gene with susceptibility to male infertility

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ABSTRACT. This study aims to explore the possible associations between a genetic variation in the miR-34b binding site in the 3'-untranslated region (UTR) of the methylenetetrahydrofolate reductase (*MTHFR*) gene (rs55763075) with male infertility in a Chinese population. Genotype distributions of the rs55763075 single nucleotide polymorphism were investigated by polymerase chain reaction and direct sequencing in a Chinese cohort that included 464 infertile men with idiopathic azoospermia or oligospermia and 458 controls with normal fertility. Overall, no significant differences in the distributions of the genotypes of the *MTHFR* rs55763075 polymorphism were detected between the infertility and control groups. A statistically significant increased risk of male infertility was found for carriers

of the rs55763075 AA genotype when compared with homozygous carriers of the rs55763075 GG genotype in the azoospermia subgroup (OR = 1.721; 95% CI = 1.055-2.807; P = 0.031). Furthermore, we found that rs55763075 was associated with folate and homocysteine levels in patients with idiopathic azoospermia. Our results indicated that the *MTHFR* 3'-UTR rs55763075 polymorphism might modify the susceptibility to male infertility with idiopathic azoospermia.

Key words: MiR-34b; 3'-UTR; Methylenetetrahydrofolate reductase; Male infertility

INTRODUCTION

Infertility is defined as the failure to achieve a clinically recognized pregnancy despite having 12 months or more of regular unprotected sex (Huang et al., 2012). Infertility is a worldwide reproductive health problem and affects approximately 10-15% of couples. It is estimated that approximately 50% of couple infertility is due to male infertility (De Kretser and Baker, 1999). Although several causes have been identified for impaired male fertility, the etiology of male infertility remains poorly understood. There is a growing body of evidence that genetic abnormalities might account for 15-30% of male factor infertility through affecting the function or expression of proteins involved in male reproduction (O'Flynn O'Brien et al., 2010). Mutation in key testis regulatory genes, for example, in combination with environmental factors, might be responsible for poor sperm quality and reduced sperm numbers (Eloualid et al., 2012). Therefore, it is important to identify genetic polymorphisms and risk factors that are associated with male infertility.

Several genes are known to play roles in orchestrating the complex process of spermatogenesis (Visser and Repping, 2010). Furthermore, folate plays a key role in cellular physiology by participating in DNA synthesis, repair, and methylation, and in the maintenance and stability of the genome. Human methylenetetrahydrofolate reductase (MTHFR) is an integral enzyme in the folate pathway that catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetra-hydrofolate (Goyette et al., 1994). Recent research has further emphasized the role of the MTHFR gene in male fertility. A study on adult mice demonstrated that MTHFR plays a critical role in spermatogenesis (Chen et al., 2001), and it has also been demonstrated that MTHFR deficient mice exhibit hyperhomocysteinemia and compromised spermatogenesis (Kelly et al., 2005). There is increasing evidence that common variants of MTHFR are associated with lower enzyme activity, lower red blood cell and plasma folate, and elevated plasma homocysteine (Shen et al., 2001). Several studies have found that polymorphisms in the MTHFR gene were associated with altered MTHFR enzyme activity and hyperhomocysteinemia, which was considered as a risk factor for different disorders including infertility (Austin et al., 2004). However, studies investigating the role of MTHFR polymorphisms in patients with male infertility have yielded conflicting results. Some studies have suggested that MTHFR polymorphisms (C677T, A1298C, and G1793A) are risks for human male infertility (Gava et al., 2011; Safarinejad et al., 2011), whereas others have not supported such an association (Ravel et al., 2009; Montjean et al., 2011).

MicroRNAs (miRNAs) are a class of endogenous noncoding RNAs, 18-25 nt in length,

that can regulate the expression of target mRNAs through base pairing to target sequences in their 3'-UTRs (Bartel, 2004). It is considered reasonable to hypothesize, therefore, that single nucleotide polymorphisms (SNPs) at miRNA-binding sites might alter the strength of miRNA binding, impacting the expression of miRNA targets and hence influencing the development of numerous complex diseases (Sethupathy and Collins, 2008). In addition, SNPs in the pre-miRNA or mature miRNA sequences themselves could modulate miRNA-target interactions through alteration of miRNA expression or maturation (Wang et al., 2008; Ryan et al., 2010). Wu et al. (2013) found that a miR-149 binding site SNP in the 3'-UTR of MTHFR increased coronary heart disease risk through modification of miRNA binding. In a pilot study, we identified a SNP (rs55763075) in a miR-34b binding site in MTHFR using a bioinformatic approach. Increasing evidence has suggested that miR-34b is involved in various biological processes such as spermatogenesis (Abu-Halima et al., 2013a,b). We hypothesized, therefore, that polymorphic variation in the miR-34b binding site might alter the strength of miR-34b binding to regulate the expression of MTHFR and hence influence the process of spermatogenesis.

There are, however, currently no studies that have investigated the association between miR-34b-binding site polymorphisms and idiopathic male infertility. In this study, we test the hypothesis that SNP in miR-34b-binding sites of MTHFR might interfere with the activity of MTHFR and modulate the risk of idiopathic male infertility in a Chinese population.

MATERIAL AND METHODS

Subjects and sample collection

This study was approved by the Ethics Committee of Zhejiang Medical University. We recruited 464 patients with infertility including 253 men with idiopathic azoospermia and 211 with oligospermia (sperm count less than 15 x 10^6/mL) from the Andrology Outpatient Clinic of the Centre of Clinical Reproductive Medicine between March 2011 and April 2013, for a case-control study. Patients having diseases known to affect spermatogenesis, such as maldescensus of the testis, orchitis, or obstruction of the vas deferens and varicocele were excluded from this study. The control group consisted of 458 fertile men who were fathers of at least one child and who reported no history of assisted reproductive technologies. Blood donated from each subject (5 mL) was used to extract genomic DNA; a routine semen analysis was also performed. The analysis of semen was performed according to World Health Organization guidelines (Lu et al., 2010).

Genotyping

Genomic DNA was extracted from peripheral blood mononuclear cells using the QiaAmp DNA Blood Mini kit (Qiagen, Valencia, CA, USA). The genotypes of rs55763075 were determined by direct PCR sequencing. All PCR reagents were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). Primer sequences used for amplifications were as follows: Forward: 5'-TGA TGC CCT TGC GTG TTT TG-3'; reverse: 5'-GCC AGG AGT CTG TGC TCT TT-3'. Reaction conditions were as follows: initial denaturation at 94°C for 3 min; 35 cycles of denaturation at 94°C for 30 s, primer annealing at 58°C for 30 s, extension at 72°C for 1 min; followed by a final extension step of 72°C for 10 min. PCR products were puri-

fied using the QIAQuick PCR purification kit and examined using the automatic sequencer ABI3730XL (Applied Biosystems).

Detection of folate and homocysteine (Hcy) levels

Serum folate levels were determined by enzyme-linked immunosorbent assay (ELISA) (Boster Biotech., Wuhan, China) using an iMark™ microplate absorbance reader (Bio-Rad, Hercules, CA, USA). Plasma Hcy levels were determined by chemiluminescence using an Abbott Laboratories IMX Blood Chemistry Analyzer (Abbott Laboratories, Irving, Texas, USA).

Statistical analyses

Statistical analysis was carried out using the statistical software SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). Infertility risks were estimated as odds ratios (ORs) and their respective 95% confidence intervals (CIs) using unconditional multivariate logistic regression. All P values were two-sided; a P value < 0.05 was considered to be statistically significant.

RESULTS

Characteristics of the study population

The baseline demographics, plasma Hcy levels, and serum folate levels of the study subjects are shown in Table 1. There were no significant differences in age or body mass index between the men with infertility and the normal controls. Serum folate levels were significantly decreased in the men with infertility compared to the controls; in contrast, the men with infertility exhibited increased plasma Hcy levels as compared to the subjects with normal fertility.

Table 1. Characteristics of the study population.

	Infertile men	Normal fertile donors
Number	464	458
Age (years)	36.4 ± 7.2	35.8 ± 6.9
BMI (kg/m^2)	27.4 ± 4.5	26.8 ± 5.1
Folate levels (ng/mL)	7.7 ± 2.8	11.3 ± 3.7
Hcy levels	14.2 ± 4.2	8.5 ± 2.1

Data = means ± SD; BMI = body mass index; Hcy = homocysteine.

Association between the *MTHFR* rs55763075 polymorphism and male infertility

The frequencies of the *MTHFR* 3'-UTR rs55763075 genotypes in patients and controls are shown in Table 2. All the rs55763075 genotype distributions (normal fertile controls: $\chi^2 = 2.204$, P = 0.138; infertile participants: $\chi^2 = 0.296$, P = 0.586) were in agreement with Hardy-Weinberg equilibrium. Overall, no significant differences in the distributions of the *MTHFR* rs55763075 genotypes were observed between the men with infertility and the control group. The infertility group was further stratified into two subgroups: subjects with idio-

pathic azoospermia or with oligozoospermia (Table 3). Compared to the control group, a statistically significant increased risk of male infertility was found in the azoospermia subgroup for carriers of the rs55763075 AA genotype of *MTHFR* when compared with homozygous carriers of rs55763075 GG genotype (OR = 1.721; 95%CI = 1.055-2.807; P = 0.031), while in the oligozoospermia group, the genotype frequency distribution showed no significant difference. These results suggested that the AA genotype of rs55763075 (G>A) might contribute to idiopathic azoospermia.

Table 2. Association of rs55763075 with the risk of male infertility.

Polymorphism	Infertile men (N = 464)	Normal fertile donors (N = 458)	OR (95%CI)	P value
GG	176 (37.9%)	192 (41.9%)	1.0 (Reference)	
GA	224 (48.3%)	220 (48.0%)	1.111 (0.842-1.465)	0.481
AA	64 (13.8%)	46 (10.0%)	1.518 (0.987-2.334)	0.065
G allele	576 (62.1%)	604 (65.9%)	1.0 (Reference)	
A allele	352 (37.9%)	312 (34.1%)	1.183 (0.978-1.431)	0.090

OR = odds ratio; CI = confidence interval.

Table 3. Subgroup analysis of the *MTHFR* gene polymorphism and the risk of male infertility.

Infertility class	Polymorphism	Infertile men	Normal fertile donors (N = 458)	OR (95%CI)	P value
Oligozoospermia	GG	81 (38.4%)	192 (41.9%)	1.0 (Reference)	
	GA	108 (51.2%)	220 (48.0%)	1.193 (0.842-1.691)	0.331
	AA	22 (10.4%)	46 (10.0%)	1.268 (0.725-2.217)	0.465
	G allele	266 (63.0%)	604 (65.9%)	1.0 (Reference)	
	A allele	156 (37.0%)	312 (34.1%)	1.183 (0.978-1.431)	0.090
Idiopathic azoospermia	GG	95 (37.6%)	192 (41.9%)	1.0 (Reference)	
	GA	116 (45.8%)	220 (48.0%)	1.044 (0.749-1.455)	0.866
	AA	**42 (16.6%)**	**46 (10.0%)**	**1.721 (1.055-2.807)**	**0.031**
	G allele	306 (60.5%)	604 (65.9%)	1.0 (Reference)	
	A allele	**200 (39.5%)**	**312 (34.1%)**	**1.265 (1.011-1.584)**	**0.043**

OR = odds ratio; CI = confidence interval; bold text indicates there exist significance between infertile man and normal donors (P < 0.05).

Association between the *MTHFR* rs55763075 polymorphism and serum folate and Hcy levels

Rs55763075 might modify the binding of human miR-34b to *MTHFR* the as the variant creates a miR-34b binding site in the *MTHFR* 3'-UTR (Figure 1A). Therefore, we detected the serum folate and Hcy levels in patients with azoospermia with different rs55763075 genotypes. As shown in Figure 1B and C, we observed that the presence of the rs55763075 AA genotype was associated with decreased folate levels and increased Hcy levels in patients with azoospermia. The results indicated that the SNP rs55763075 might influence MTHFR function.

DISCUSSION

Although genetic factors play a role in the etiology of idiopathic male infertility, the biochemical abnormalities underlying the predisposition to infertility and the exact pathophysiology of impaired spermatogenesis remain to be elucidated. Several studies have shown

that individual susceptibility to infertility is likely due to *MTHFR* polymorphism (Mfady et al., 2014). In current study, we found that a single nucleotide polymorphism (rs55763075) in a miR-34b binding site in the *MTHFR* 3'-UTR was associated with idiopathic azoospermia in a Han Chinese population.

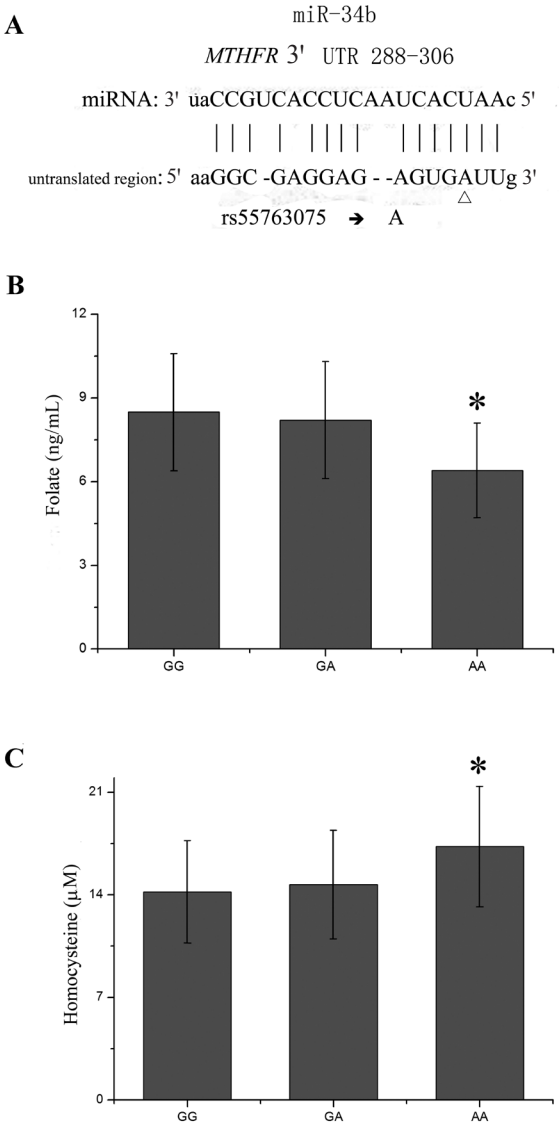

Figure 1. Association between the *MTHFR* rs55763075 polymorphism and folate and Hcy levels. **A.** miRNASNP 2.0 (http://www.bioguo.org/miRNASNP/) predicted that rs55763075 could create a miR-34b-mRNA binding site in the MTHFR 3'-UTR. **B.** Serum vitamin folate levels in patients with azoospermia. **C.** Plasma Hcy levels in patients with azoospermia. Open triangle = the site of polymorphism; *P < 0.05.

DNA methylation is the primary epigenetic phenomenon regulating gene expression and genome integrity and is critical for the correct development of sperm. MTHFR is one of the key regulatory enzymes of DNA synthesis and methylation metabolism. There is an increasing number of published reports on the association between MTHFR polymorphisms and the risk of unexplained male infertility. C677T, the most commonly studied MTHFR SNP, has been demonstrated to be associated with male infertility in certain populations but not in others (Bezold et al., 2001; Stuppia et al., 2003; A et al., 2007; Chellat et al., 2012; Vani et al., 2012). Several meta-analyses have suggested that C677T increases the risk of infertility (Tuttelmann et al., 2007; Gupta et al., 2011); however, a recent meta-analysis from Wu et al. (2012) suggested that this SNP was only significantly associated with a susceptibility to male infertility in Asians, but not in Caucasians. Another study involving infertile and fertile and/or normospermic individuals of Korean ancestry found that the MTHFR C677T, Methionine Synthase Reductase (MTRR) A66G and methionine synthase (MS) A2756G genotypes were independently associated with male infertility (Lee et al., 2006). Conversely, a study of 77 subfertile men of Dutch ancestry found the C allele of the C677T polymorphism was not associated with male factor infertility (Ebisch et al., 2003). One explanation for these conflicting results from studies on different geographic populations suggested that the role of the C677T variant in the susceptibility to male infertility might depend on other factors such as total folate intake level, DNA collection and genotyping method, sample size, and ethnic group (Ebisch et al., 2007). In the present study, we found that a genetic variation in the miR-34b binding site in the 3'-UTR of *MTHFR* (rs55763075) was a risk factor for male infertility with idiopathic azoospermia. Therefore, our results indicted that MTHFR polymorphism was associated with spermatogenic impairment.

MiRNAs are a family of small noncoding RNAs of approximately 22 nucleotides that are recognized as endogenous physiological regulators of gene expression via base pair binding to the 3'-UTR of their target mRNAs (Stark et al., 2008). There is increasing evidence that suggests that miRNAs might also play important roles in mammalian spermatogenesis, and in fact a series of miRNAs are expressed abundantly in male germ cells throughout spermatogenesis (He et al., 2009; Yan et al., 2009). The term miR-SNP refers to SNPs in the miRNA coding sequence, whereas miR-TS-SNP is defined as a SNP occurring in the miRNA target site (TS) or binding site. Many studies have revealed that genetic variances in microRNA genes and their TSs are associated with complex genetic diseases (Sethupathy et al., 2007; Saetrom et al., 2009). However, the association between miR-SNPs or miR-TS-SNPs with male fertility remains unclear. In a study from Zhang et al. (2011), SNPs in the miRNA-binding sites of genes for male infertility were analyzed to explore the possible association between SNPs occurring at miRNA-binding sites and idiopathic male infertility. Their results indicated that SNPs residing in miRNA-binding sites of Chorionic Gonadotropin Alpha Polypeptide (CGA) increased the risk of idiopathic male infertility. Recently, several studies have found that miRNA-34b in particular was associated with male infertility. A study from Abu-Halima et al. (2013b) demonstrated that miR-34b, miR-122, and miR-1973 exhibited the highest fold changes in samples from asthenozoospermic men. In the present study, using the online bioinformatic analysis tool TargetScan, we identified a single nucleotide polymorphism (rs55763075) in a miR-34b binding site in the *MTHFR* gene 3'-UTR and assessed whether there was any association between rs55763075 genotype and male fertility status. Our results demonstrated a statistically significant increased risk of male infertility in the azoospermia subgroup for car-

riers of the *MTHFR* rs55763075 AA genotype when compared with homozygous carriers of the rs55763075GG genotype. Furthermore, the rs55763075 AA genotype was associated with decreased folate levels and increased Hcy levels in patients with azoospermia as well. Our findings indicated that the SNP rs55763075 might influence MTHFR function.

In conclusion, in the present study, we found that genetic variation in a miR-34b binding site in the 3'-UTR of *MTHFR* is a risk factor for azoospermia. The validation of our results in larger patient groups might provide evidence for the contribution of rs55763075 genotypes to the efficiency of spermatogenesis.

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