Research Article

MTHFRC 677T Gene Polymorphism and Homocysteine in North China Patients with Varicocele

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To investigate the correlation between methylenetetrahydrofolate reductase (MTHFR) C677T gene polymorphism, homocysteine, and male infertility with varicocele in North China. One Hundred infertile males with varicocele (VC; grade II-III, VC group) and 100 healthy males with normal semen parameters and no varicocele (NC group) were recruited for PCR microarray, blood and semen testing. Compared with the CC genotype in the NC group, the TT genotype in the NC group and the CC genotype in the VC group showed no significant changes in sperm motility (P = 0.191; P = 0.130), sperm density (P = 0.591, P = 0.643), plasma homocysteine level (P = 0.511; P = 0.677), and seminal plasma MDA (P = 0.752; P = 0.451). In contrast, VC patients with the TT genotype had higher plasma homocysteine level and seminal plasma MDA levels (P < 0.001), lower partial pressure of oxygen in seminal pulse (PO2; P < 0.001) and poorer sperm quality (P < 0.001), as compared with the CC genotype. This suggests that MTHFR C677C>T may not be a risk factor for male Varicocele in North China. However, this may affect the oxidative stress associated with homocysteine expression, which in turn affects semen parameters in VC patients. Larger studies are needed to validate our findings.

1. Introduction

According to global statistics, one-quarter to one-fifth of couples will be affected by fertility problems. Among them, known male and female factors each account for 25%, and unexplained infertility accounts for 50% [1–3]. One-third of the known male infertility factors are related to varicocele (VC). VC is one of the most common diseases of adolescent and reproductive-age men and one of the main causes of male infertility during the reproductive period [4]. VC occurs from a combination of genetic susceptibility and acquired factors [5], but the exact mechanism is unclear [6, 7]. VC affects the pathogenesis of male testicular function and causes infertility, which is related to multiple factors, such as high temperature, hypoxia, and oxidative stress damage [5, 8, 9], of which oxidative stress damage is the core cause of VC-associated infertility. Recent studies have found that homocysteine (Hcy) has many adverse effects such as inducing oxidative stress, promoting inflammation and damage to vascular endothelial cells, stimulating vascular smooth muscle cell proliferation, and promoting thrombosis [10, 11]. Therefore, Hcy was considered to be a potential factor in the occurrence of VC and in the reduction of sperm quality.

The 1-carbon unit cycle is an important amino acid synthesis pathway that includes two cycles of folic acid and methionine, as well as the transsulfuration pathway. During the methionine and folate cycles, Hcy is regulated [12]. Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme involved in the metabolism of folate and methionine and can catalyze the reduction of 5,10-methylenetetrahydrofolate to...
2. Materials and Methods

2.1. Research Subjects. This case-control study recruited 100 male patients with primary infertility and grade II-III varicocele (VC group) from the department of urology, and 100 healthy young volunteers (18–30 years old) (normal control, NC group) at the physical examination centre, The Second Hospital of Tianjin Medical University, from December 2019 to October 2020. Experiments were approved by local ethics committee (NO.:2018-IRB-076), in compliance with national guidelines for the care and use of clinical samples.

The inclusion criteria were as follows: 18 years of age or older, and male infertility for more than 1 year, and second-grade or third-grade VC with positive reflux. The exclusion criteria were as follows: patients with grade I VC, smoking, excessive alcohol use, drug consumption, previous VC repair, the presence of other infertility-related diseases, patients with VC combined with haematological diseases, endocrine diseases, cancer or systemic infectious diseases, patients who had taken vitamin B or E or folic acid within 1 month, or other antioxidant treatments, and patients with VC combined with acute epididymitis or prostatitis.

This study was reviewed and approved by the local medical ethics committee. Only VC patients underwent the microsurgical ligation. VC patients and their families were informed of the possible consequences of performing microsurgical spermatic ligation. Both VC patients and healthy volunteers were required to provide data and samples and to sign an informed consent form.

2.2. Methods

2.2.1. Clinical Data Collection. Clinical data, including age, body mass index (BMI), and distribution of the MTHFR C677T gene and allele frequencies was collected from the two groups.

2.2.2. Semen Analysis. In the VC and NC groups, patients were asked to practice abstinence for 5–7 days. Semen obtained by masturbation was analyzed using an automatic semen analyzer (BEION, Shanghai, China) for routine semen analysis and examination within half an hour, adopting the WHO (No. fifth edition) semen examination standard. Sperm density was recorded with normal reference $>2.0 \times 10^6 \text{mL}^{-1}$; sperm motility was recorded with normal reference PR+NP > 40%.

2.2.3. Hcy Concentration Detection and Genotype Analysis. In the VC and NC groups, 2 mL of peripheral venous blood was drawn from each patient. A fully automatic biochemical analyzer (Beckman Coulter, USA) was used to determine the Hcy level using a cyclic enzymatic method. The normal reference range is $0–15 \mu \text{mol L}^{-1}$.

2.2.4. Determination of Seminal Plasma Malondialdehyde (MDA). The semen samples collected following the masturbation test were frozen immediately after liquefaction. All samples were transferred to the laboratory while frozen and thawed within a week, and the seminal plasma MDA value was analyzed according to the instructions of the kit (jiancheng, Nanjing).

2.2.5. Determination of the MTHFR Gene Polymorphism. A blood genome extraction kit was used to extract genomic DNA of peripheral venous blood. After PCR amplification, an MTHFR (C677T) gene detection kit (Sanji Bio, Changsha) was used for PCR amplification and sequencing. The patient was genotyped using a biochip reader.

2.2.6. Intraoperative Spermatic Venous Oxygen Saturation PO$_2$ of VC Patients. In the VC group, the spermatic vein was ligated under a microscope under general anesthesia, the arteries and lymph vessels were preserved, and the internal and external spermatic veins were ligated. After the spermatic cord was exposed, the internal spermatic vein was separated and punctured with a blood gas needle to collect blood from the internal spermatic cord for blood gas analysis.

2.3. Statistical Analysis. Statistical analysis was performed using IBM SPSS statistical software (version 22.0). Continuous data related to Hcy levels and sperm density, among others, were expressed as mean $\pm$ standard deviation, $\chi^2$ test was used to assess categorical data related to the distribution and frequency of genetic polymorphisms in order to analyze differences between groups. Independent samples $t$-test or ANOVA was used to compare measurements between groups. $P < 0.05$ indicated statistically significant differences.

3. Results

3.1. General Information. There were 100 cases in the VC group and 100 cases in the NC group. In the VC group, ages ranged from 18 to 37 years, and 75 cases of left-sided varicoceles and 25 cases of bilateral varicoceles, all of which were varicocele above grade I. In the NC group, ages ranged from 18 to 32 years old, and healthy volunteers with normal semen parameters were excluded from the diagnosis of VC by physical examination and color Doppler ultrasound in Standing position.
3.2. MTHFR C677T Gene Polymorphism Test Results. The distribution of MTHFR polymorphisms in each group was analyzed, including 100 VC patients and 100 healthy volunteers with normal semen parameters and no VC. Three genotypes—cytosine–cytosine (CC), cytosine–thymine (CT), and thymine–thymine (TT)—were determined (Figure 1). The results showed that among the 100 patients in the VC group, 37 patients had the CC genotype, 48 patients had the CT genotype, and 15 patients had the TT genotype. The frequency of the T allele was 39%. Among the 100 subjects in the NC group, 35 subjects had the CC genotype, 49 subjects had the CT genotype, 16 subjects had the TT genotype, and the frequency
of the T allele was 40.5% (Table 1). The frequency of each group of genotypes conformed to the Hardy–Weinberg equilibrium law, the sample was representative of the group (CC 37.0% VS 35.0%), CT (48.0% VS 49.0%), and TT (15.0% VS 16.0%), and there was no significant difference ($P > 0.05$). The frequency of the T allele mutation in the NC group was slightly lower than that in the NC group (39.0% VS 40.5%), but the difference was not statistically significant ($P = 0.437$). There was no significant difference between varicoceole grade II and III (Table 2).

### 3.3. Test Results of Plasma Homocysteine Levels

The VC and NC groups were divided into three subgroups, CC, CT, and TT, according to the MTHFR C677T genotype. The plasma Hcy concentrations in the three subgroups of the NC group were $8.65 \pm 4.65$, $9.51 \pm 5.77$, and $9.68 \pm 4.11 \text{mmol} \text{L}^{-1}$, respectively, and the differences in the data for the CC and TT subgroups were not statistically significant ($P = 0.451$). In the VC group, comparison of the MTHFR gene showed the lowest Hcy concentration in patients with the CC genotype and the highest in the TT genotype. The plasma Hcy concentrations in the three groups were $8.35 \pm 3.17$, $14.79 \pm 7.36$, and $19.84 \pm 6.67 \text{mmol} \text{L}^{-1}$, respectively. Statistically significant differences between the data of the CC and TT subgroups were found ($P < 0.001$). However, there was no significant difference in Hcy levels between VC men with the CC genotype and those in the p-NC group ($P = 0.752$), as shown in Table 3 and Figure 2(a).
Comparison of plasma homocysteine between different genotypes of MTHFR C667T in VC group and NC group

Comparison of sperm motility between different genotypes of MTHFR C667T in VC group and NC group

Comparison of sperm density between different genotypes of MTHFR C667T in VC group and NC group

Comparison of MDA in seminal plasma between different genotypes of MTHFR C667T in VC group and NC group

Comparison of PO2 in spermatic vein between different genotypes of MTHFR C667T in VC group and NC group

**FIGURE 2:** Comparison of plasma homocysteine content, sperm motility, sperm density, MDA in seminal plasma, and PO2 in spermatic vein between different genotypes of MTHFR C667T in VC group and NC group. The difference of plasma homocysteine content, sperm motility, sperm density, and MDA in seminal plasma between the VC–CC and NC–CC groups was not significant (*P > 0.05; a–d), while (a) the
VC–CT and TT groups had higher plasma homocysteine content (*P < 0.05) and (b, c) lower sperm motility and sperm density compared to the NC groups (*P < 0.05). (d) MAD content was not significantly different in the VC–CT and NC–CT groups (*P > 0.05), while the CT–TT group was significantly higher than the NC–TT group (*P < 0.05). (e) And for PO2 levels, VC–CC was significantly higher than both VC–CT and VC–TT groups (*P < 0.05).

3.4. Semen Related Examination Results. The overall data of the VC group were compared with the NC group. There were significant differences in sperm motility (Figure 2(b)), sperm density (Figure 2(c)) and seminal plasma MDA values (Figure 2(d)). However, comparisons between subgroups were slightly different. Patients with the TT genotype in the VC group had decreased sperm viability (P < 0.001), decreased sperm density (P < 0.001), and increased seminal MDA levels (P < 0.001) compared with patients with the CC genotype. However, there were no significant differences in sperm motility, sperm density, and semen MDA levels (P > 0.05) between the CC genotype group in the VC group and the CC genotype group in the NC group. Also, compared to the CC genotype group of the NC group, the TT genotype group did not show significant differences (P > 0.05), as shown in Table 3.

3.5. Results of Spermatic Vein Blood Gas Analysis (BGA) in the VC Operation Group. Compared with that of subjects with the CC genotype, the PO2 in the spermatic vein of the VC Operation Group. Compared with that of subjects with the CC genotype group in the VC group was significantly reduced (P < 0.001). Statistical significance is shown in Table 3 and Figure 2(e).

4. Discussion

Previous studies have shown that the MTHFR gene frequently exhibits multiple single nucleotide polymorphisms (SNPs), with the MTHFR C677T >T mutation leading to tetrahydrofolate reductase dysfunction, resulting in elevated Hcy. And Hcy may be associated with the risk of vascular injury and progression [10, 24]. However, further investigation is needed to determine the correlation between MTHFR C677T >T polymorphisms and the pathological changes of spermatic vein [25]. This study examined the correlation between MTHFR gene polymorphism and the risk of patients with grade II–III VC compared to healthy controls. However, our study showed no significant difference in the frequency of MTHFR C677T genotype between the two groups (P > 0.05). In contrast, patients with MTHFR C677T >T mutation in the VC group had reduced PO2 levels in the seminiferous veins, while those with TT genotype had the lowest PO2 levels (P < 0.05). Therefore, we suggest that polymorphisms in the MTHFR gene might not be associated with VC incidence in North China.

MTHFR gene also plays a crucial role in folate metabolism following spermatogenesis. However, studies conducted on different populations have yielded conflicting results regarding the association between MTHFR C677CT polymorphisms and male infertility [26–28]. In our study, patients with VC exhibited higher levels of Hcy and MDA, as well as lower sperm viability and concentration compared to the control group. However, subgroup analysis demonstrated that these differences were not present in individuals with the CC genotype between the two groups. Patients with the MTHFR C677C >T mutation in the VC group had higher levels of MAD and Hcy, as well as poorer sperm quality, which was particularly significant in those with the TT genotype (P < 0.05). Conversely, there was no significant difference observed in the NC group (P > 0.05). This finding deviates from previous studies [29] and offers a fresh perspective to further elucidate the existing research contradictions.

Aliakbari et al. [18] and H. Nikzad et al. [30] conducted two meta-analyses on various national and regional population studies, which concluded that the MTHFR C677T >T mutation is significantly associated with the risk of male infertility [18, 30]. Additionally, Aliakbari et al.’s [18] study found a significant association between the MTHFR C677T >T polymorphism and male infertility in the Asian population. In contrast, the findings of Wei et al. [26] were different, especially in the Asian population. These discrepancies may be attributed to the presence of other causative genes, a combination of environmental and disease factors contributing to male infertility, racial disparities, and selection bias. The results of this study suggest that VC may play a key role in the significant association of C677T >T polymorphism with male infertility risk in the MTHFR Asian population.

Oxidative stress is one of the main causes of low sperm quality. Reactive oxygen species (ROS), including superoxide anion (O2−), hydroxyl radical (OH), hydrogen peroxide (H2O2), hypochlorite ion (OCl−), etc., together with superoxide dismutase (SOD), glutathione (GSH), and catalase (CAT), maintains redox homeostasis in vivo [31]. VC leads to poor blood reflux in the testicular veins. Some hormones or metabolites and hypoxia lead to increased oxygen radical production. In addition, the MTHFR 677C >T mutation leads to increased levels of Hcy in vivo, which produces a series of reactive oxygen species as described above during its own oxidation. Increased ROS production exceeds the body’s clearance rate, disrupting redox homeostasis, generating polyunsaturated fatty acids, and inducing lipid peroxidation such as MDA attack on biofilms, leading to testicular germinal epithelial damage and apoptosis of spermatogonial cells [32]. In the present study, sperm quality was significantly worse in the TT genotype of VC group, but there was no significant change in the TT genotype of NC group and CC genotype of VC group. This might indicate that the VC alone or the MTHFR 677C >T mutation alone in the present study did not go beyond the regulation of redox homeostasis in the subjects.

This article also has other limitations. First of all, the follow-up of patients with VC infertility after surgery is still ongoing, and the evaluation of surgical outcomes and the relevance of MTHFR C677T >T polymorphism on infertility after the removal of VC as a disease factor need to be
improved. In addition, the sample size of this study is relatively small and the area is limited. We plan to conduct a larger multicenter study in conjunction with other regional medical institutions.

In conclusion, the present study supports the possibility that MTHFR C677C >T polymorphism may be a marker of male infertility susceptibility to VC in North China, without significant correlation with VC occurrence. However, a more comprehensive study is needed to validate our findings.

5. Conclusion
According to our research, MTHFR C677C >T mutation is not associated with the development of varicocele in men in North China, but may affect its progression. It may also lead to poor semen parameters in VC patients by affecting Hcy-related oxidative stress. Larger studies are needed to validate our findings.

Data Availability
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest
The authors declare that there is no conflict of interest.

Authors’ Contributions
Xin Zhongcheng and Feng Yuhong contributed in the conceptualization. Xiu Yiping, Cao Zhiqiang, Yu Dongyang, Yang Xin Zhongcheng and Feng Yuhong contributed in the funding acquisition. All authors contributed in the formal analysis. Pan Jiancheng contributed in the data curation. Pan Jiancheng contributed in the original draft and Writing-review and editing. Cao Zhiqiang and Xiu Yiping these authors contributed equally to this study as co-first authors.

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