Association of genetic variants in CFTR gene, IVS8 c.1210-12T[5_9] and c.1210-35_1210-12GT[8_12], with spermatogenetic failure: case–control study and meta-analysis

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ABSTRACT: It has been proposed that the genetic variants of IVS8 c.1210-12T[5_9] and adjacent c.1210-35_1210-12GT[8_12] in cystic fibrosis transmembrane conductance regulator gene might contribute to the spermatogenetic failure, but numerous genetic association studies that aimed to test this hypothesis reported conflicting results. So, in order to clarify such inconsistencies, we first conducted an original case–control study in Chinese Han population that consisted of 126 non-obstructive azoospermia, 169 severe oligospermia and 213 fertile male controls, and subsequently performed a meta-analysis of the available data, including our results. Our case–control study revealed that the frequencies of the T[5] allele and the T[5]+GT[12] combination in patients with non-obstructive azoospermia were both significantly higher than those in the fertile controls (13.1 versus 2.8%, P < 0.01; 97.0 versus 41.7%, P < 0.01, respectively), thus indicating a high risk susceptibility to non-obstructive azoospermia for males with T[5] allele or T[5]+GT[12]. However, as for the patients with severe oligospermia, both the T[5] allele frequency and T[5]+GT[12] did not differ from that for the control subjects (4.4 versus 2.8%, P = 0.01; 53.3 versus 41.7%, P > 0.01, respectively). In addition, our meta-analysis showed a significant increased risk of non-obstructive azoospermia for males with T[5] allele (OR 3.45, 95% CI 2.29–5.20, P = 0.000) and T[5]+GT[12] (OR 7.57, 95% CI 2.53–22.65, P = 0.000) compared with males carrying other alleles. By contrast, neither T[5] allele itself nor T[5]+GT[12] combination had any effects on the risk of severe oligospermia (OR 0.96, 95% CI 0.42–2.21, P = 0.002; OR 1.33, 95% CI 0.64–2.76, P = 0.447). On the basis of these results, it can be concluded that the T[5] allele itself, or in combination with GT[12] repeat, may increase the susceptibility risk of non-obstructive azoospermia, but not that of severe oligospermia.

Key words: CFTR / IVS8 c.1210-12T[5_9] / IVS8 c.1210-35_1210-12GT[8_12] / non-obstructive azoospermia / severe oligospermia

Introduction

Cystic fibrosis transmembrane conductance regulator (CFTR) gene encodes a transmembrane protein that functions as a cyclic adenosine monophosphate-dependent chloride channel, and this gene is widely expressed in the apical membrane of secretory epithelial cells as well as reproductive tissues (Riordan et al., 1989; Trezise and Buchwald, 1991). It is well known that CFTR mutations can result in the typical CF, one of the most common autosomal recessive disorders in Caucasians. Besides typical CF, CFTR mutations have been assumed to be implicated in many other atypical disorders related to male infertility, such as congenital bilateral absence of the vas deferens (CBAVD), non-obstructive azoospermia and oligospermia (Stuhrmann and Dork, 2000; Cuppens and Cassiman, 2004; Clustres, 2005).

Over the years, the IVS8 c.1210-12T[5_9] (polypyrimidine tract in intron 8) and adjacent c.1210-35_1210-12GT[8_12] (TG repeat tracts) in CFTR gene have received much more attention owning to their potential roles in the development of male infertility. The polymorphic IVS8 c.1210-12T[5_9] consists of three common variants, namely T[5], T[7] and T[9], and this locus functions as the acceptor site of alternative splicing of CFTR exon 9 (Chu et al., 1993).
Compared with T[7] and T[9], the shortest T[5] variant results in less efficient exon 9 splicing and hence a reduced expression of functional proteins (Chu et al., 1993). So, the T[5] has been taken as a pathogenic variant with partial penetrance, which may lead to CBAVD or other forms of atypical disorders (Chu et al., 1993; Mak et al., 1997). Besides, such an effect of T[5] on exon 9 splicing can be modulated by another genetic variant c.1210-35_1210-12GT[8_12] adjacent to the IVS8 c.1210-12T[5_9] locus. Several researches have revealed that the T[5] allele, on the background of a longer c.1210-35_1210-12GT[8_12] repeat such as TG[12] or TG[13] would produce a higher proportion of transcripts lacking exon 9, which favors the manifestation of disease phenotype (Niksic et al., 1999; Groman et al., 2004; Hefferon et al., 2004).

Consistent with these functional findings, numerous population genetic studies have revealed a significant association between IVS8 c.1210-12T[5_9] and c.1210-35_1210-12GT[8_12] and CBAVD development worldwide. Moreover, the spectrum and frequency of CFTR mutations associated with male infertility exhibit ethnic differences. In European Caucasians, the frequency of T[5] has been reported as roughly the second to c.1521_1523delCTT (usual ΔF508) among the three most common CFTR mutations responsible for CBAVD (Hefferon et al., 2004). By contrast, in Asian populations, only a small number of c.1521_1523delCTT carriers were identified, but an exceptional high frequency of T[5] allele was found in CBAVD males (Anzai et al., 2003; Wu et al., 2004, 2005; Chiang et al., 2009). The disease penetrance of IVS8 c.1210-12T[5_9] has been generally assumed to be determined predominantly by the length of c.1210-35_1210-12GT[8_12] repeats. Several studies have reported that the individuals with T[5] alleles were substantially more likely to exhibit a disease phenotype for CBAVD if in combination with a longer GT repeat (12 or 13 GT) (Cuppens et al., 1998; Groman et al., 2004; Chiang et al., 2009).

Although it has been generally believed that infertility in the CBAVD patients are simply due to anatomical obstacle and that the spermatogenesis is normal, there is increasing evidence indicating the possible involvement of IVS8 c.1210-12T[5_9] and c.1210-35_1210-12GT[8_12] in the development of abnormal spermatogenesis such as non-obstructive azoospermia and severe oligospermia. Several studies have shown a developmentally regulated expression of CFTR in both the testis and epididymis of humans and rodents, indicating a potential function of CFTR during spermatogenesis (Trezise and Buchwald, 1991; Trezise et al., 1993b; Patrizio and Salameh, 1998). In particular, it has demonstrated that there was a correlation between the presence of T[5] variant and the increased level of defective alternatively spliced exon 9 in the testicular samples, which again suggests that the T[5] allele may confer substantial susceptibility to abnormal spermatogenesis (Trezise et al., 1993a; Larriba et al., 1998). By contrast, population genetic studies generate conflicting results. Some researches have shown that non-obstructive azoospermia and severe oligospermia are highly associated with T[5] and GT[12] and other CFTR mutations (van der Ven et al., 1996; Tamburino et al., 2008; Gallalt et al., 2009). However, these observations were not confirmed in other studies (Pallares-Ruiz et al., 1999; Stuhrmann and Dork, 2000; Ravnik- Glavac et al., 2001).

Considering the facts that CFTR mutations associated with male infertility exhibit striking ethnic differences and that findings about the association between IVS8 c.1210-12T[5_9] and c.1210-35_1210-12GT[8_12] and spermatogenetic defects remains controversial. We first conducted an original case–control study in Chinese Han population, and second performed an updated meta-analysis of available data, so as to clarify the contribution of IVS8 c.1210-12T[5_9] and c.1210-35_1210-12GT[8_12] to the spermatogenetic failure.

**Materials and Methods**

**Subject selection**

All patient subjects, including non-obstructive azoospermia (n = 126) and severe oligospermia (n = 169), were recruited from the infertile males presenting to the hospital and research center involved in this study (Reproductive Medicine Center, First Affiliated Hospital of Shantou University Medical College and Center for Genetic Medicine, Zhejiang University School of Medicine and Women and Children’s Hospital of Shaoxing, China). Fertile males, with a history of fatherhood and normal semen analysis, were selected from the general population as normal controls (n = 213). For each patient, clinical evaluation (including history taking and physical examination) was performed. Meanwhile, routine semen analysis, serum hormone measurements and scrotal ultrasonography were also conducted. Additionally, special investigations such as testicular biopsy and chromosome analysis were performed as an indicated for a diagnostic confirmation. Infertile males with a sperm concentration of <5 × 10^6/ml were diagnosed as severe oligospermia, and those having normal genital tract but no detectable sperm in the ejaculate was diagnosed as non-obstructive azoospermia if they had concurrently bilateral testicular atrophy and normal semen volume and elevated FSH concentration. For those males suspected of having non-obstructive azoospermia but with normal-sized testicles and/or normal FSH concentration, a diagnostic testicular biopsy was carried out for confirmation of spermatogenetic defects. Azoospermic males with normal spermatogenesis and/or with abnormal genital tract suggestive of obstructive azoospermia were excluded. All the subjects had not any clinical manifestations or family history suggestive of CF or congenital absence of vas deferens. The individuals presenting with chromosomal aberrations and/or Y chromosome microdeletions were also excluded. All the involved subjects gave their informed consents, and the study was approved by the local ethical committees from all the participating units.

**Screening of IVS8 c.1210-12T[5_9] and c.1210-35_1210-12GT[8_12]**

Genomic DNA was extracted from peripheral venous blood using the DNA extraction Kit (Tiangen Company, Beijing, China) according to the manufacturer’s recommendation. The IVS8 c.1210-12T[5_9] variants were analyzed by the nested PCR amplification as described previously (Chillon et al., 1995). The first PCR cycling used primers 9i-5 and 9i-3, and the cycling conditions were as follows with 25 cycles performed: denaturation at 95°C for 30 s, annealing at 54°C for 30 s and extension at 74°C for 40 s. Nested PCR used primers RF9 (5′-CCGCTGTGTGTGTGTGTTGTTTTT-3′) and RR9 (5′-GGATCCAGCAACCGCCAA-3′), and the cycling conditions were the same as the first amplification except that it used first PCR products as templates and 35 cycles were performed. The final PCR products were digested with XmnI enzyme and visualized after 12% non-denaturing polyacrylamide gel electrophoresis. Each PCR product with a gel band pattern indicative of T[5] allele was directly sequenced, and the contribution of other variants was confirmed by sequencing. For each individual with T[5] allele, subsequent direct sequencing of the PCR product was carried out to determine c.1210-35_1210-12GT[8_12] repeat number adjacent to the T[5] allele and phase of T[5] and c.1210-35_1210-12GT[8_12] repeat combinations. The IVS8 c.1210-12T[5_9] and
c.1210-35_1210-12GT[8_12] analysis was performed in the same center to avoid genotyping inconsistencies, and ∼30% of the samples carrying alleles other than T[5] were randomly selected for confirmation by DNA sequencing.

Meta-analysis
A meta-analysis of all available data (including the present and published studies) was conducted as an alternative supplementation to our case–control study. We performed a full search in PubMed for the eligible articles in English until June 2010. The search terms covered medical subject headings and/or text words relating to azoospermia, oligospermia and CFTR. Figure 1 showed the details for the publication selection. Hand-searching reference lists of the retrieved papers and reviews were also conducted to identify additional relevant articles. All studies included should fulfill the following criteria: published in peer-reviewed journal; with sufficient original data for meta-analysis; using healthy males as controls; cases diagnosed properly; the genotyping protocols described. Both data abstraction and quality evaluation were performed by two independent reviewers (Z.L. and J.Y.).

The summary odds ratios (ORs) and their 95% confidence intervals (CI) were estimated under the fixed-effects (Mantel and Haenszel, 1959) or random-effects models (DerSimonian and Laird, 1986) as appropriate. The fixed-effects model (M-H method) would be used if there was no evidence of heterogeneity among studies. Otherwise, the random-effects model (D-L method) would be selected. For each outcome pooled, between-study heterogeneity was assessed by Cochran’s Q test and $I^2$ statistic (Higgins et al., 2003). Publication bias was evaluated using a funnel plot and Egger’s test (Egger et al., 1997).

Statistical analysis
Frequency comparisons between cases and controls were performed using the $\chi^2$ statistic or Fisher exact test as appropriate. All $P$-values were based on two-sided comparisons and $P < 0.05$ indicated statistical significance. The ORs and their 95% CI were estimated for the effect of each allele. All the analyses about our case–control study and meta-analysis were performed using Stata 10.0 software (Stata Corporation, College Station, TX, USA).

Results
Case–control study
Genotyping analysis of the IVS8 c.1210-12T[5_9] in Chinese Han population identified three types of alleles, namely T[5], T[7] and T[9], and the genotypes for these alleles were mainly presented as T[7]/T[7] and T[5]/T[7], with only one heterogeneous T[7]/T[9] found. All T[5] alleles appeared as heterogeneous T[5]/T[7]. The allele frequencies of IVS8 c.1210-12T[5_9] are shown in Table I. The T[5] allele frequency in males with non-obstructive azoospermia was significantly higher than that in the fertile controls (13.1 versus 2.8%, $\chi^2 = 26.99$, $P = 0.000$). By contrast, the T[5] allele frequency in severe oligospermia did not differ significantly from that in the fertile controls (4.4 versus 2.8%, $\chi^2 = 1.45$, $P = 0.228$). These data clearly indicated that the T[5] allele increased the susceptibility risk for non-obstructive azoospermia (OR 5.20, 95% CI 2.55–11.26), but not for severe oligospermia (OR 1.60, 95% CI 0.69–3.80).

Subsequently, all subjects with T[5] alleles were screened to determine the length of GT repeats. It revealed that almost all the T[5] alleles in non-obstructive azoospermic patients (32 of 33 chromosomes) and most in severe oligospermia patients (8 of 15 chromosomes) were linked to TG[12]. By contrast, of the 12 fertile controls with T[5] alleles, only a few (5 of 12 chromosomes) appeared with GT[12] and the remaining with GT[11]. As shown in Table I, the frequency of T[5]+GT[12] in males with non-obstructive azoospermia was significantly higher than that in the general population (97.0 versus 41.7%, $P = 0.000$), which indicated that males with T[5]+GT[12] have an increased risk for this disease compared with other alleles (OR 44.8, 95% CI 3.87–2077.72). However, T[5]+GT[12] frequency in severe oligospermia did not differ from that in normal controls (53.3 versus 41.7%, $P = 0.547$), indicating not any significant risk of severe oligospermia for males caring T[5]+GT[12] than other alleles (OR 1.6, 95% CI 0.27–9.68).

Meta-analysis
We conducted a meta-analysis of available data, including previous published and our present studies, to determine the overall effect of the IVS8 c.1210-12T[5_9] and c.1210-35_1210-12GT[8_12] on the susceptibility risk for non-obstructive azoospermia and severe oligospermia. The process for publication selection was detailed in Fig. 1. Briefly, of the 85 articles identified by the PubMed search, 7 papers met the inclusion criteria after careful evaluation. Of the seven studies excluded by examination of the full manuscript, four studies had no fertile controls (Kanavakis et al., 1998; Mak et al., 2000; Dohle et al., 2002; Schulz et al., 2006) and two studies selected only oligozoospermatozoospermia as case subjects (Tuerlings et al., 1998; Pallares-Ruiz et al., 1999) and one study just reported alleles other than the IVS8 c.1210-12T[5_9] and c.1210-35_1210-12GT[8_12] (van der Ven et al., 1996).
Detailed characteristics for each included study were described in Table II. These studies were conducted in a wide range of geographical and ethnic settings, including several European countries and China. Overall, most studies were properly designed with normal controls. Moreover, the case diagnostics, subject selection criteria and genotyping method in most studies were stated clearly in general. One study did not give detailed information about case selection (Ravnik-Glavac et al., 2001). The control selections varied a little. Some were population-based, whereas one was not documented clearly (Ravnik-Glavac et al., 2001), and in other two studies the control selection was hospital-based (Cruger et al., 2003; Larriba et al., 2005).

Our meta-analysis revealed that the T[5] variant was significantly associated with an increased susceptibility risk for non-obstructive azoospermia, giving the summary OR 3.45 (95% CI 2.29–5.20, P = 0.000) based on a fixed-effect model (Fig. 2A). The males with T[5] had more than three times the risk of developing non-obstructive azoospermia when compared with individuals carrying other alleles. The heterogeneity among those studies was moderate, but not significant (χ² = 7.94, I² = 37.0%, P = 0.160). Due to the limited number of studies included, we could not explore all possible sources of heterogeneity by subgroup analyses. By contrast, the T[5] allele had not shown any effect on the risk of severe oligospermia susceptibility, with an OR of 0.96 (95% CI 0.42–2.21, P = 0.930) based on the random model (Fig. 2B). The random model was selected due to a significant heterogeneity for studies of severe oligospermia (χ² = 18.55, P = 0.002, I² = 73.0%).

Concerning the combined T[5]+GT[12] alleles, only four studies reported the association of these genetic variants with non-obstructive azoospermia and severe oligospermia. As shown in Fig. 3, meta-analysis of these four studies (Larriba et al., 2005; Stuppia et al., 2005; Tamburino et al., 2008), together with our present study by M-H model, gave the summary OR 7.57 (95% CI 2.53–22.65, P = 0.000) for non-obstructive azoospermia and OR 1.33 (95% CI 0.64–2.76, P = 0.447) for severe oligospermia, indicating a significant association of T[5]+GT[12] with an increased risk of non-obstructive azoospermia, but not with severe oligospermia. These results suggested that the risk of developing non-obstructive azoospermia in males with the T[5]+GT[12] alleles was increased nearly eight times when compared with males with other alleles. There was no statistical heterogeneity among studies of T[5]+GT[12] with non-obstructive azoospermia (χ² = 3.48, P = 0.323, I² = 13.9%) and severe oligospermia (χ² = 1.40, P = 0.706, I² = 0.0%).

The funnel plot analysis revealed no evidence of publication and related biases concerning all the included studies that related T[5] variant to non-obstructive azoospermia (Fig. 4A) and severe oligospermia (Fig. 4B). Furthermore, there was no statistically significant evidence of bias for these studies using Egger’s method (P = 0.532 and P = 0.146, respectively).

### Table I Frequencies of the IVS8 c.1210-12T[5_9] and c.1210-35_1210-12GT[8_12] in Chinese Han population.

<table>
<thead>
<tr>
<th></th>
<th>c.1210-12T[5_9] n/2N (%)</th>
<th>c.1210-35_1210-12GT[8_12] n/2N (%)</th>
<th>OR (95%CI)/P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-obstructive azoospermia (N = 126)</td>
<td>33/252 (13.1)</td>
<td>219/252 (86.9)</td>
<td>0/252 (0)</td>
</tr>
<tr>
<td>Severe oligospermia (N = 169)</td>
<td>15/338 (4.4)</td>
<td>323/338 (95.6)</td>
<td>0/338 (0)</td>
</tr>
<tr>
<td>Normal controls (N = 213)</td>
<td>12/426 (2.8)</td>
<td>413/426 (97.0)</td>
<td>1/426 (0.2)</td>
</tr>
</tbody>
</table>

* n, allele number; N, subject number; b, T[5]/GT[12] number; a, T[5] allele number.

### Discussion

Given the evidence showing potential roles of CFTR gene in the process of spermatogenesis, it has been proposed that the genetic variants of IVS8 c.1210-12T[5_9] and c.1210-35_1210-12GT[8_12] in CFTR might be implicated in the cause of spermatogenetic failure. However, population genetic studies that attempted to test this hypothesis produced conflicting results. We conducted an original case–control study in Han Chinese population and subsequent meta-analysis of the available data to clarify the associations between IVS8 c.1210-12T[5_9] and c.1210-35_1210-12GT[8_12] and spermatogenetic failure related to non-obstructive azoospermia or severe oligospermia.

Our population study detected a significant higher frequency of the T[5] allele in the males with non-obstructive azoospermia (13.1% chromosomes) when compared with that in the matched fertile controls (2.8% chromosomes), which suggests that the T[5] allele may increase the susceptibility risk for non-obstructive azoospermia in Han Chinese population (OR 5.20, 95% CI 2.55–11.26). Our results are in accordance with those published in Portuguese patients (15.9%) (Grangeia et al., 2004), Italian patients (19.6 and 9.9%) (Stuppia et al., 2005; Tamburino et al., 2008) and patients of Swiss origin (6.03%) (Gallati et al., 2009), but contrary to others published in a group of patients consisting of 39 Caucasians, one black and five Asian (Mak et al., 2000), in Slovenia (5.26%) (Ravnik-Glavac et al., 2001) and Spain (4.17%) (Larriba et al., 2005). Moreover, similar to most reports published previously (Ravnik-Glavac et al., 2001; Cruger et al., 2003; Larriba et al., 2005; Gallati et al., 2009), our study did not reveal any significant difference of the T[5] frequency between severe oligospermic men and the fertile controls. However, these studies are not consistent with the one that reported a higher frequency of T[5] in severe oligospermia (9.24%, 17/184 chromosomes) than that in normal controls (4.38%, 21/480 chromosomes) (Tamburino et al., 2008).
### Characteristics of the studies included in the meta-analysis.

<table>
<thead>
<tr>
<th>Author (year)</th>
<th>Geography/ethnicity</th>
<th>Subject selection</th>
<th>DNA extraction/genotyping method</th>
<th>T[5] allele</th>
<th>T[5]/GT[12]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ravenik-Glavac et al. (2001)</td>
<td>Slovenia</td>
<td>Non-obstructive azoospermia (n = 80) Healthy individuals (n = 95)</td>
<td>From peripheral blood/nest-PCR amplification and gel electrophoresis</td>
<td>8/160</td>
<td>8/190</td>
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<tr>
<td></td>
<td></td>
<td>Severe oligozoospermia (n = 50)</td>
<td></td>
<td>4/100</td>
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<td></td>
<td></td>
<td>Details for case diagnosis unavailable</td>
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<tr>
<td>Cruger et al. (2003)</td>
<td>Denmark</td>
<td>Severe oligospermia (n = 139)</td>
<td>DNA extraction information unavailable/PCR-based analysis</td>
<td>5/278</td>
<td>4/200</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fertile men (n = 100) working in the hospital, being the fathers of at least two children</td>
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<tr>
<td>Grangeia et al. (2004)</td>
<td>Portuguese</td>
<td>Non-obstructive azoospermia (n = 22)</td>
<td>From peripheral blood/nest-PCR amplification followed by DNA sequencing</td>
<td>7/44</td>
<td>8/228</td>
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<tr>
<td></td>
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<td>Fertile males (n = 114) from the general population</td>
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<td>Patients evaluated by physical examination, genit-urinary ultrasound analysis, spermogram analysis and hormone assays, and excluding chromosomal aberrations or Y chromosome microdeletions</td>
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<tr>
<td>Larriba et al. (2005)</td>
<td>Spain</td>
<td>Non-obstructive azoospermia (n = 36)</td>
<td>DNA extraction information unavailable/PCR amplification and SSCP/heteroduplex technique, followed by DNA sequencing</td>
<td>3/72</td>
<td>4/174</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fertile men (n = 87) that consulted for contraception</td>
<td></td>
<td>2/3</td>
<td>2/4</td>
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<tr>
<td></td>
<td></td>
<td>Severe oligozoospermia (n = 47)</td>
<td></td>
<td>2/94</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diagnostic procedures including physical examination, semen analyses, testicular biopsy, scrotal ultrasonography and chromosomal analysis; four cases with abnormal karyotype and five cases with Yq microdeletions; CF and CAVD excluded</td>
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<tr>
<td>Stuppia et al. (2005)</td>
<td>Italy</td>
<td>Non-obstructive azoospermia (n = 6)</td>
<td>From peripheral blood/a reverse dot blot-based commercial kit and PCR amplification followed by DNA sequencing</td>
<td>NA</td>
<td>4/6</td>
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<tr>
<td></td>
<td></td>
<td>Fertile men (n = 24) with normal sperm count, selected from unexplained infertility couples and all males carrying ST allele</td>
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<td>7/24</td>
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<tr>
<td>Study</td>
<td>Location/Population</td>
<td>Population and Diagnosis</td>
<td>Study Details</td>
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<tr>
<td>Tamburino et al. (2008)</td>
<td>Italy</td>
<td>Oligozoospermia ($n = 24$) All cases carrying T[5] allele and azoospermia diagnosis unclear</td>
<td>Male ($n = 240$) with normal semen parameters From peripheral blood/commercial kits and PCR amplification followed by DNA sequencing</td>
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<td>Gallati et al. (2009)</td>
<td>Swiss</td>
<td>Non-CAVD azoospermia ($n = 116$) The diagnostic process not stated clearly, but cases with CAVD, chromosomal aberrations and Y chromosome microdeletions excluded</td>
<td>Male ($n = 96$) from general population, having at least one healthy child From peripheral blood/PCR amplification and SSCP/heteroduplex technique, followed by direct sequencing</td>
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<tr>
<td>Our present study</td>
<td>China/Chinese Han population</td>
<td>Non-obstructive azoospermia ($n = 126$) The diagnosis based on physical examination, ultrasonography and semen analysis, excluding CF, chromosomal aberrations and Y chromosome microdeletions</td>
<td>Male ($n = 213$) from general population, having at least one healthy child</td>
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The disease penetrance of T[5] allele has been generally assumed to be determined predominantly by the length of GT[8_12] repeats (Cuppens et al., 1998; Groman et al., 2004), and the T[5] allele is more likely appearing at a higher prevalence with the longer GT[8_12]. Thus, although the T[5] itself may not have deleterious consequences, this allele in combination of the longer GT[8_12] such as TG[12] or TG[13] would probably result in an increased risk for disease susceptibility. In accordance with such an assumption, several studies have identified a significant association of the T[5]+GT[12] combination with male infertility caused by non-obstructive azoospermia (Stuppia et al., 2005; Tamburino et al., 2008). Similarly, our study also showed that the T[5]+GT[12] exhibited a significant increased frequency in non-obstructive azoospermia, compared with that in the fertile population. Our results indicate that males with the T[5]+GT[12] combination are more likely to have non-obstructive azoospermia than those with other alleles. In males with severe oligospermia, the frequency of the T[5]+GT[12] did not differ from that in the fertile controls, thus it may be unlikely that T[5]+GT[12] variants have any association with severe oligospermia. Since males with severe oligospermia have, in general, a spectrum of origins and are very probable to be more heterogeneous than those with non-obstructive azoospermia, which would probably mask the true relationship between T[5]+GT[12] and severe oligospermia, our conclusion for severe oligospermia should be taken with caution.

As for genetic association studies, replication with large samples of different independent populations has been generally regarded as a key criterion for a convincing conclusion. To our knowledge, this is the first study of a Chinese Han population that attempts to relate IVS8 c.1210-12T[5_9] and c.1210-35_1210-12GT[8_12] variants in CFTR with spermatogenic failure. Moreover, as a major strength, this study also involved large-scale samples of cases and matched controls, with a relatively high power that may ensure significant conclusion. Finally, the infertile cases with chromosome abnormalities or microdetection were excluded in our study so as to ensure more homogeneous case subjects, and the normal controls were selected...
CFTR variants and spermatogenetic failure

Figure 4 Funnel plot for studies on the T[5] variant with non-obstructive azoospermia (A) and severe oligospermia (B).

from general population of the same ethnic background as the cases. So, all these strategies will make our results more convincing.

Due to the inconsistencies regarding the association of IVS8 c.1210-12T[5_9] and c.1210-35_1210-12GT[8_12] variants with spermatogenetic failure among different reports, we conducted a meta-analysis by combining our present study and other six articles previously published. The meta-analysis also revealed significant associations of both T[5] and T[5]+GT[12] combination with non-obstructive azoospermia risk (OR = 3.45, P = 0.000 and OR = 7.57, P = 0.000, respectively). By contrast, neither T[5] allele itself nor T[5]+GT[12] combination had any effects on the risk of severe oligospermia (OR = 0.96, P = 0.930 and OR = 1.33, P = 0.447, respectively). Therefore, the risk for developing non-obstructive azoospermia will probably increase by about three times for T[5] carrier and nearly eight times for T[5]+GT[12] carrier, compared with males with other alleles. These results are consistent with those of our case-control study.

The results of this meta-analysis are relatively convincing, given that no potential publication bias was observed. Just like many others, our meta-analysis has several limitations. The heterogeneity across the studies of severe oligospermia is statistically significant. Moreover, the heterogeneity among studies for non-obstructive azoospermia, although not significant statistically, may also be plausible with respect to case compositions (with or without chromosome abnormality) and bias of control selection as discussed already (Ravnik-Glavac et al., 2001; Cruger et al., 2003; Larriba et al., 2005). These differences may attribute to the inconsistencies that have been observed among different studies. Second, a limited number of studies were included with relatively small sample size (especially for T[5]+GT[12]), which might be not enough to reach a high statistical power to conduct bias analyses. The small sample size also limited the ability to conduct a more meaningful subgroup analyses. Due to these potential confounding factors, the results of this meta-analysis should be taken with caution. Moreover, replication studies, especially those using a larger number of samples and well designed with matched controls from general population, are required for more conclusive results.

Other evidence from functional studies that suggest the implications of IVS8 c.1210-12T[5_9] and c.1210-35_1210-12GT[8_12] variants in the spermatogenetic pathogenesis have been well documented (Niksic et al., 1999; Groman et al., 2004; Hefferon et al., 2004). Therefore, population genetic finding of the effect of T[5] and T[5]+GT[12] on non-obstructive azoospermia risk has a relatively sound base. Thus, our findings together with others may at least shed light on, if not exactly clarify, the pathogenic mechanism of IVS8 c.1210-12T[5_9] and c.1210-35_1210-12GT[8_12] that underlies the development of spermatogenetic failure. However, it should be noted that a discrepancy among different studies appears and the underlying reasons remain largely unclear. Therefore, our finding is far from conclusive and needs further confirmation. Independent replication studies on large, homogenous patients will strengthen the present finding greatly.

Besides T[5] and GT[12], some other genetic variants in CFTR gene have also been detected in males with isolated infertility. In general, the distribution and frequencies of CFTR variants are markedly different between isolated infertility (due to CBAVD or non-CBAVD infertility) and CF (Cuppens and Cassiman, 2004; Claustres, 2005). It seems that the homozygous or two heterozygous severe mutations primarily found in CF might not play a major role in isolated infertility, but the mild variants that cannot result in CF would be very likely responsible for spermatogenetic failure (Cuppens and Cassiman, 2004; Claustres, 2005). The most common mutations detected in Caucasian CBAVD males are c.1521_1523delCTT, T[5] variant and c.350G>A (usual R117H). By contrast, in Asian or non-Caucasian populations where CF is rare, only a few carriers with c.1521_1523delCTT or c.350G>A were identified, but an exceptional high frequency of the T[5] carriers was found (Claustres, 2005; Wu et al., 2005; Chiang et al., 2009; Sharma et al., 2009). Besides CBAVD, CFTR variants have also been reported to be associated with other forms of infertility in European populations, of which T[5] and c.1408A>G (usual M470V) may be the most common ones with a higher frequency (Cuppens and Cassiman, 2004; Larriba et al., 2005; Tamburino et al., 2008; Gallati et al., 2009). Since data remain scarce for the non-CBAVD infertile patients of Asian origin, our report would add more important information on this topic.

In conclusion, we have characterized for the first time the type and frequency of IVS8 c.1210-12T[5_9] and c.1210-35_1210-12GT[8_12] variants in Chinese Han population, and found that both the T[5] variant itself and T[5]+GT[12] combination have a modest effect on the susceptibility to non-obstructive azoospermia, but may not be related to severe oligospermia risk. A subsequent meta-analysis of all the available data is consistent with our case-control study. Meanwhile, it also reveals that small sample size, population heterogeneity and potential subject selection bias may have contributed to inconsistencies in previous studies. Therefore, our results may shed...
light on the pathogenic mechanisms of IVS8 c.1210-12T[5_9] and c.1210-35_1210-12GT[8_12] underlying the development of non-obstructive azoospermia. However, replication studies are still needed to bring further validation to these conclusions.

**Authors’ roles**

J.Y. performed experiments and data analysis, and was involved in the final approval of manuscript; Z.C. assisted with experiments and data analysis; T.Z. played a role in experiments and data collection; Z.L. assisted with study planning and data analysis; Y.N. was involved in study planning and manuscript drafting; Z.L. undertook study design, data analysis and article writing, and was involved in the final approval of manuscript.

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