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RESEARCH COMMUNICATION

Genetic association study of *RNF8* and *BRDT* variants with non-obstructive azoospermia in the Chinese Han population

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Abstract

Increasing evidence indicates that polymorphisms in genes relevant to spermatogenesis might modulate the efficiency of reproduction in men. Ring finger protein 8 (*RNF8*) and bromodomain testis-specific (*BRDT*) are two candidate genes associated with spermatogenesis. Here, we considered potential associations of 14 single nucleotide polymorphisms (SNPs) in *RNF8* and *BRDT* genes in Chinese patients with non-obstructive azoospermia (NOA). We analyzed 361 men with NOA and 368 fertile controls by using Sequenom iPLEX technology. Our data did not reveal any variants associated with NOA susceptibility. However, we observed that rs104669 and rs195432 of *RNF8* were in strong linkage disequilibrium. Haplotype analysis of the two SNPs indicated that the haplotype AC reduced the risk of NOA and the haplotype TC significantly evaluated the risk of NOA. Moreover, the *RNF8* variants rs195432 (C/A $p = 0.030$), rs195434 (T/C $p = 0.025$), and rs2284922 (T/C $p = 0.034$) were correlated with the smaller testis volume.

Abbreviations: *RNF8*: ring finger protein 8; *BRDT*: bromodomain testis-specific; SNPs: single nucleotide polymorphisms; NOA: non-obstructive azoospermia; HWE: Hardy Weinberg Equilibrium; OR: odds ratio; LD: linkage disequilibrium; MAF: minor allele frequency(ies)

Keywords

BRDT, male infertility, non-obstructive azoospermia, *RNF8*, single nucleotide polymorphisms (SNPs)

History

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Introduction

Infertility affects about 15% of couples who wish to have children. Male infertility accounts for 50% of all the infertility cases [Brugh and Lipshultz 2004; Hirsh 2003]. Non-obstructive azoospermia (NOA) is one of the infertility subtypes. During spermatogenesis, progenitor cells undergo mitotic and meiotic divisions followed by spermiogenesis yielding spermatozoa. Interruption of any of these phases can result in the failure of spermatogenesis, giving rise to NOA. Besides numerical and structural abnormalities of chromosomes [O'Flynn O'Brien et al. 2010], increasing evidence indicates that of spermatogenesis-related gene, polymorphisms might

provide additional risk factors for the development of NOA [He et al. 2012; Jinam et al. 2013; Ruan et al. 2012].

The ring finger protein 8 (*RNF8*) gene is located on human chromosome 6p21.3. As a RING domain E3 ligase, *RNF8* takes part in histone ubiquitination, which is important for a variety of biological processes [Li et al. 2010; Santos et al. 2010]. Spermatogenesis is arrested in *RNF8*-deficient mice [Ma et al. 2011] and *RNF8* knockout mice fail to generate mature sperm [Santos et al. 2010]. Two potential mechanisms concerning the role of *RNF8* in spermiogenesis were proposed. First, the histone protamine transition during spermiogenesis [Oliva 2006] being induced by *RNF8*-dependent histone ubiquitination [Lu et al. 2010; Ma et al. 2011; Rathke et al. 2007]. The absence of *RNF8* led to incomplete chromatin restructuring resulting in male infertility [Cho et al. 2001; Shirley et al. 2004; Zhao et al. 2004]. Second, sex-linked genes that escaped post-meiotic silencing and activated in round spermatids and throughout spermiogenesis were *RNF8*-dependent [Mueller et al. 2008; Namekawa et al. 2006; Sin et al. 2012a,b] including the *Ssty* gene family. Decreased expression of the *Ssty* gene family was correlated with a reduced number of testes sperm.

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This indicated that RNF8 played an essential role in activating these genes and its absence could result in infertility.

The human bromo-domain, testis specific (*BRDT*) gene is located on chromosome 1p22.1 and regulates a suite of previous testis-specific genes during both meiosis and post-meiotic phases of spermiogenesis [Gaucher et al. 2012] and is involved in chromatin remodeling through the post-meiotic phase. Adult *Brdt*^{-/-} mouse testes lack post-meiotic germ cells [Dhar et al. 2012; Gaucher et al. 2012; Jacobson et al. 2000; Plaseski et al. 2012; Pivot-Pajot et al. 2003]. In this study, we genotyped 9 *RNF8* SNPs and 5 *BRDT* SNPs to investigate whether genetic changes of the SNPs were risk factors for NOA.

Results and Discussion

Table 1 summarizes the clinical characteristics of the 368 controls and 361 cases with NOA. The results showed that the testis volumes in NOA were significantly smaller than those in the controls ($p < 0.001$, both sides). There was no statistical difference of body mass index between the cases and the controls ($p = 0.071$).

The genotype distributions of the 14 *RNF8* and *BRDT* SNPs of interest did not deviate from the Hardy Weinberg Equilibrium (HWE) ($p > 0.05$) in the controls. Although we found that the T allele of rs104669 was associated with NOA

($p = 0.038$, odds ratio (OR) = 1.326, 95% confidence interval (CI) = 1.015–1.733, Table 2) and the SNP rs104669 was associated with the risk of NOA in a dominant model ($p = 0.045$, OR = 1.376, and 95% CI = 1.007–1.880, Table 3), both the p values were beyond statistical significance threshold after the Bonferroni correction ($p > 0.05$). In addition, our data revealed no significant associations of the SNPs of the *BRDT* gene with the risk of NOA.

To investigate potential combinatorial effects of the *RNF8* SNPs on susceptibility to NOA, we performed a linkage disequilibrium (LD) analysis. We observed that SNPs rs104669 (SNP7) and rs195432 (SNP8) were in a strong LD (Figure 1). The *RNF8* AC (SNP7,8) haplotype reduced the risk of NOA ($p = 0.043$, OR = 0.759) and the TC (SNP7,8) haplotype significantly evaluated the risk of NOA ($p = 0.003$, OR = 1.693). In addition, the *RNF8* haplotype ATCATCTCT (SNP1-9) and the haplotype TCATCTC (SNP2-8) led to increased risk of NOA ($p = 0.0086$, OR = 4.052; $p = 0.0085$, OR = 1.630, Table 4, respectively).

We stratified the association analysis of the 14 *RNF8* and *BRDT* SNPs as a function of testis volume and serum FSH. On one hand, *RNF8* rs195432 (C > A, $p = 0.03$), rs195434 (T > C, $p = 0.025$), and rs2284922 (T > C, $p = 0.034$) were significantly correlated with testis volume (Table 5). On the other hand the level of serum FSH ($p > 0.05$, data not shown) was not correlated. We compared the relationships between the number of copies of the minor allele at the SNPs of interest and the testis volume in the NOA patients and all the samples. The result showed that there were no significant correlations between the two variables ($p > 0.05$, Table 6).

In this study, we investigated whether *RNF8* and *BRDT* single gene variations could be risk factors for spermatogenic failure in the Chinese population. However, we found no genetic associations between variants of *RNF8* and *BRDT* genes and NOA. Although two independent investigations reported that the SNP rs3088232 in *BRDT* was associated with male infertility in the population of European descent [Aston et al. 2010; Plaseski et al. 2012], we did not recruit rs3088232 into this study because it was beyond the genetic

Table 1. Clinical characteristics of the study population.

| Characteristic | Controls ($n = 368$) ^a | NOA ($n = 361$) ^a | p Value |
|--------------------------------------|--|-----------------------------------|--------------|
| Body mass index (kg/m ²) | 23.61 ± 3.53 | 23.09 ± 3.32 | 0.071 |
| Right testicle volume (ml) | 14.58 ± 2.65 | 8.90 ± 3.51 | 0.000 |
| Left testicle volume (ml) | 14.51 ± 2.70 | 8.95 ± 3.55 | 0.000 |
| FSH | / | 21.42 ± 19.88 | / |

^acontinuous variables were expressed as mean ± SD; NOA: non-obstructive azoospermia; /: no data available; FSH: follicle stimulating hormone. Student's t test was used for the data analysis. Bold values indicated statistical significance ($p < 0.05$).

Table 2. Analysis of *RNF8* and *BRDT* polymorphisms associated with the risk of NOA.

| Gene | SNP reference | Position | Nucleotide change | MAF | | HWE | | OR (95%CI) | p Value | P_M |
|------|---------------|----------|-------------------|----------|-------|----------|-------|---------------------|-----------|-------|
| | | | | Controls | NOA | Controls | NOA | | | |
| RNF8 | rs104669 | 37354059 | A > T | 0.162 | 0.204 | 0.336 | 0.517 | 1.326 (1.015–1.733) | 0.038 | NS |
| | rs195379 | 37345704 | T > C | 0.119 | 0.090 | 0.802 | 0.513 | 0.738 (0.526–1.036) | 0.078 | NS |
| | rs195434 | 37360557 | T > C | 0.142 | 0.131 | 0.277 | 0.034 | 0.914 (0.677–1.235) | 0.558 | NS |
| | rs2284922 | 37349033 | T > C | 0.256 | 0.270 | 0.784 | 0.422 | 1.072 (0.848–1.354) | 0.563 | NS |
| | rs2284924 | 37327394 | T > C | 0.135 | 0.118 | 0.824 | 1 | 0.856 (0.628–1.167) | 0.325 | NS |
| | rs9380661 | 37331049 | C > G | 0.066 | 0.059 | 0.661 | 0.341 | 0.883 (0.576–1.354) | 0.567 | NS |
| | rs2269058 | 37324857 | A > C | 0.363 | 0.357 | 0.174 | 1 | 0.976 (0.787–1.209) | 0.822 | NS |
| | rs195432 | 37358022 | C > A | 0.086 | 0.081 | 0.326 | 0.019 | 0.930 (0.641–1.35) | 0.704 | NS |
| | rs169005 | 37341920 | A > G | 0.119 | 0.090 | 0.802 | 0.513 | 0.738 (0.526–1.036) | 0.078 | NS |
| | rs10783071 | 92428495 | A > C | 0.390 | 0.397 | 0.079 | 0.826 | 1.032 (0.836–1.274) | 0.768 | NS |
| BRDT | rs1156281 | 92442695 | A > C | 0.389 | 0.397 | 0.062 | 0.826 | 1.034 (0.837–1.276) | 0.759 | NS |
| | rs11165906 | 92440939 | T > A | 0.370 | 0.369 | 0.043 | 0.910 | 0.998 (0.806–1.236) | 0.987 | NS |
| | rs2183036 | 92477964 | T > A | 0.482 | 0.479 | 0.462 | 1 | 0.988 (0.804–1.215) | 0.910 | NS |
| | rs17576636 | 92447668 | C > T | 0.206 | 0.195 | 0.427 | 0.311 | 0.932 (0.721–1.205) | 0.591 | NS |

NOA: non-obstructive azoospermia; MAF: minor allele frequency; HWE: Hardy-Weinberg equilibrium; P_M : corrected p value after Bonferroni correction; NS: no significance.

Table 3. The genotype distributions of rs104669 in the controls and the NOA cases.

| SNPs | Model | Genotype | Controls | NOA | OR (95%CI) | p Value | P _M |
|----------|------------|----------|---------------|---------------|--------------------|---------|----------------|
| | | | N (frequency) | N (frequency) | | | |
| rs104669 | Codominant | AA | 258(0.709) | 230(0.639) | 1 | | |
| | | AT | 94(0.258) | 113(0.314) | 1.348[0.973–1.869] | 0.072 | NS |
| | | TT | 12(0.033) | 17(0.047) | 1.589[0.743–3.398] | 0.229 | NS |
| | Dominant | AA | 258(0.709) | 230(0.639) | 1 | | |
| | | TT + AT | 106(0.291) | 130(0.361) | 1.376[1.007–1.880] | 0.045 | NS |
| | Recessive | TT | 12(0.033) | 17(0.047) | 1 | | |
| | | AT + AA | 352(0.967) | 343(0.953) | 0.688[0.324–1.462] | 0.328 | NS |

SNPs: single nucleotide polymorphisms; NOA: non-obstructive azoospermia; OR: odds ratio; Codominant model: DD versus Dd and DD versus dd; Dominant model: DD versus (Dd + dd); Recessive model: (DD + Dd) versus dd; d: minor allele; D: major allele. P_M: corrected p value after Bonferroni correction; NS: no significance.

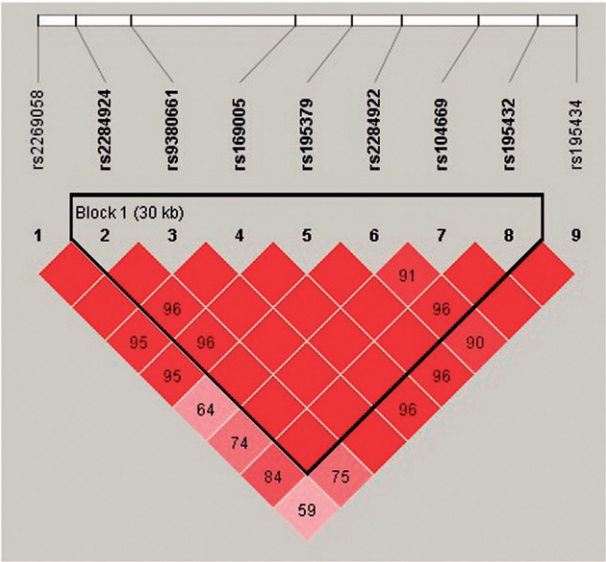


Figure 1. Pairwise linkage disequilibrium (r^2) in the study population.

Table 4. Haplotype distributions in 368 controls and 361 cases^a.

| SNPs | Haplotype | Frequency | | p Value | OR |
|----------------|-----------|-----------|----------|---------------|-------|
| | | Cases | Controls | | |
| SNP1-9 | ATCATCTCT | 0.021 | 0.005 | 0.0086 | 4.052 |
| SNP2-8 | TCATCTC | 0.110 | 0.070 | 0.0085 | 1.630 |
| SNP2,3,4,5,6,8 | CCATTC | 0.029 | 0.021 | 0.2926 | 1.426 |
| SNP2,3,4,5,6,7 | TCATCA | 0.081 | 0.102 | 0.1583 | 0.772 |
| SNP7,8 | AC | 0.796 | 0.837 | 0.0428 | 0.759 |
| SNP7,8 | TC | 0.124 | 0.077 | 0.0031 | 1.693 |
| SNP7,8 | TA | 0.081 | 0.086 | 0.7041 | 0.930 |

^athe number 1–9 of the SNPs in the LD plot were rs2269058, rs2284924, rs9830661, rs169005, rs195379, rs2284922, rs104669, rs195432, and rs195434 of *RNF8*. SNPs: single nucleotide polymorphisms; OR: odds ratio. Bold values indicated statistical significance ($p < 0.05$).

spectrum of the Chinese population minor allele frequency (MAF = 0.022) based on the HapMap database [International HapMap 2005]. Small MAF would decrease statistical power for genetic analysis and likely yield a false negative.

To address the potential combinatorial effects of *RNF8* SNPs on the risk of spermatogenic failure, we conducted a haplotype analysis to reveal associations that would be hidden in the evaluation of an individual SNP. We observed that *RNF8* SNPs rs104669 and rs195432 were in a strong LD. NOA occurred when the Chinese individuals carried the major allele C of rs195432. The potential mechanism with regard to the effect of the *RNF8* variants on spermatogenesis requires further investigation.

A significant correlation between *RNF8* SNPs rs195432, rs195434, and rs2284922 with testis volume was observed ($p < 0.05$). However, when we compared the correlation between the number of copies of each minor allele at the SNPs (rs195432, rs195434, and rs2284922) and testis volume in the NOA patients and all the samples, we found no correlations between the two variables ($p > 0.05$). The reasons remained unknown. However, *Rnf8*^{−/−} male mice present a reduction in testis volume [Li et al. 2010]. Perhaps SNPs rs195432 and rs2284922 perturb splicing, yielding other functional introns and thus alternative exons [Chamary et al. 2006; Chorev and Carmel 2012]. It was estimated that more than 50% of human genetic disorders were caused by the disruption of the normal splicing pattern [Lopez-Bigas et al. 2005; Wang and Cooper 2007]. Alternatively, the minor C allele of SNP rs2284922 is located in a conserved F-SNP (a web-based SNP analysis tool) sequence [Lee and Shatkay 2008]. A minor alteration of the conserved sequences could induce a phenotypic change [Venkatesh et al. 2011; Westerveld et al. 2004]. One must also consider that the variation of the SNP rs195434 in the 3′-UTR region might affect translational progress, as translational control in the 3′-untranslated region (3′-UTR) could influence protein level [de Moor et al. 2005]. Lastly SNP rs2284922 variation might destabilize the *RNF8* mRNA [Chamary et al. 2006].

In conclusion, this was the first study addressing the associations of *RNF8* and *BRDT* variants as a function of the risk of male infertility. Our data indicate that *RNF8* rs104669 and rs195432 are in strong LD. *RNF8* SNPs rs195432 (C/A, $p = 0.03$), rs195434 (T/C, $p = 0.025$), and rs2284922 (T/C, $p = 0.034$) are correlated with testis volume in the patients. Further functional analysis would be helpful to validate the biological mechanisms of *RNF8* polymorphisms in the spermatogenesis failure of Chinese patients.

Table 5. Associations of the selected polymorphisms with testis volume in the NOA patients.

| Gene | Genotype | Testis volume | | <i>p</i> Value | OR (95%CI) |
|------|----------------|----------------|----------------|----------------|--------------------|
| | | <10 ml | ≥10 ml | | |
| RNF8 | rs195432(C>A) | <i>N</i> = 163 | <i>N</i> = 149 | 0.030 | 1 |
| | CC | 133 | 135 | | 0.387(0.185–0.809) |
| | CA | 28 | 11 | | 1.478(0.243–8.986) |
| | AA | 2 | 3 | | |
| RNF8 | rs195434(T>C) | <i>N</i> = 163 | <i>N</i> = 149 | 0.025 | 1 |
| | TT | 124 | 115 | | 1.227(0.701–2.147) |
| | CT | 29 | 33 | | 0.108(0.014–0.856) |
| | CC | 10 | 1 | | |
| RNF8 | rs2284922(T>C) | <i>N</i> = 161 | <i>N</i> = 149 | 0.034 | 1 |
| | TT | 78 | 91 | | 0.67(0.415–1.080) |
| | CT | 64 | 50 | | 0.361(0.150–0.870) |
| | CC | 19 | 8 | | |

The non-obstructive azoospermia (NOA) cases were divided into two groups, <10 ml and ≥10 ml according to testis volume of the patients. Chi-square test was used for data analysis. Fisher exact test was used where the number of genotype was less than five. Bold values indicated statistical significance ($p < 0.05$).

Table 6. The bivariate correlation between the number of copies of the minor allele at the SNPs and testis volume in the NOA patients and all the samples.

| SNP | r1 | r2 | P1 | P2 |
|-----------|--------|--------|-------|-------|
| rs195432 | −0.082 | −0.001 | 0.147 | 0.963 |
| rs195434 | −0.073 | −0.060 | 0.198 | 0.128 |
| rs2284922 | −0.105 | −0.026 | 0.064 | 0.508 |

SNPs: single nucleotide polymorphisms; NOA: non-obstructive azoospermia; r1: correlation index of the two variates in the NOA patients; r2: correlation index of the two variates in all the samples; P1: *p* value in the NOA patients; P2: *p* value in all the samples.

Materials and Methods

Ethics statement

Written informed consents for the sample collection and the subsequent research were provided by all the patients and the controls. We conducted this study according to the tenets of the Declaration of Helsinki and the ethics committee of Anhui Medical University approved this study (reference no. 2008035, 10 January 2008).

Study population

This study recruited 361 infertile men with NOA from the Reproductive Medicine Center, the First Affiliated Hospital of Anhui Medical University, China between March 2008 and July 2013. At least two semen samples from each patient were analyzed to confirm the diagnosis of azoospermia, i.e., no sperm was found after ejaculate centrifugation at $3000 \times g$ for 10 min. All the patients' diagnoses were based on a comprehensive andrological examination, including medical history, physical examination, semen analysis, hormone analysis, karyotype, and Y chromosome microdeletions screening. Patients with a history of orchitis, epididymitis, mono- or bilateral cryptorchidism, varicocele, hypogonadotrophic hypogonadism, obstruction/absence of vas deferens, chromosomal abnormalities, and Y microdeletions were carefully excluded from this study [Lu et al. 2009; Wu et al. 2007].

The controls consisted of 368 normozoospermia men who were in search of assisted reproduction as a result of female infertility. All the controls were drawn from the same reproductive medicine center where the patients were recruited. All patients and controls were from the Anhui province Chinese population. The age of the cases ranged from 19 to 43 and the age of the controls ranged from 20 to 53. The semen analysis for sperm concentration, motility, and morphology was performed twice following the fifth edition of the World Health Organization manuals [Cooper et al. 2010].

Extraction of peripheral blood DNA

Blood was collected from each subject in EDTA and stored at -80°C . Genomic DNA was extracted from peripheral blood using QIAamp DNA Blood MiDi Kit (Qiagen Inc., Hilden, Germany), according to the manufacturer's instructions. DNA purity and concentrations were determined using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, OH, USA) of full wave length and standardized to the concentration of 30 ng/μL.

SNP selection and genotyping

Nine *RNF8* SNPs (rs104669, rs195379, rs195434, rs2284922, rs2284924, rs9380661, rs2269058, rs195432, and rs169005) and five *BRDT* SNPs (rs10783071, rs1156281, rs11165906, rs2183036, and rs17576636) were selected according to the HapMap database of the Chinese population [International HapMap 2005]. MAF of the SNPs were >0.05 . The SNPs were genotyped using the Sequenom MassArray system at State Key Laboratory Incubation, Base of Dermatology, Ministry of National Science and Technology, Hefei, Anhui, China as described elsewhere [He et al. 2012]. Briefly, 15 ng of genomic DNA was used to genotype each sample. Locus-specific PCR and detection primers were designed using the MassARRAY Assay Design 3.0 software (Sequenom, San Diego, California, USA). The DNA samples were amplified by multiplex PCR, and the PCR products were then used for locus-specific single-base extension reactions. The resulting

products were desalted and transferred to 384-element SpectroCHIP arrays. Allele detection was performed using MALDI-TOF MS. The mass spectrograms were analyzed using MassARRAY Typer software (Sequenom).

Data analysis

The t-test was used to compare the clinical characteristics of the controls and the cases, including the body mass index, the testicle volume on both sides, and the FSH level. The associations between the variants and the NOA risk were analyzed by calculating the OR and 95 %CI of the MAF. HWE for each SNP was determined according to the control samples ($p > 0.01$). All analyses for p values were two-sided. SHEsis software (<http://analysis.bio-x.cn/myAnalysis.php>) and SPSS release 17.0 statistical package were used for statistical analyses. Bonferroni correction was used to correct the p values of the allele frequency and the genotype of each SNP. For chi-square test, Fisher's exact test was used where the number of genotype was ≤ 5 . The haplotype analysis was performed by the haploview 4.2 software [Barrett et al. 2005]. Associations with a p value of < 0.05 were considered to be of significance.

Declaration of interest

The authors report no conflicts of interest. This research did not receive any specific grants from any funding agencies in the public, commercial, or not-for-profit sectors. The authors alone are responsible for the content and writing of the paper.

Author contributions

Conception and design: YZ, BS, WD; Collected all samples: JR, XH; Performed the experiments: YZ, BS, XX; Acquisition of data: FZ, XZ; Analyzed the data: XZ, YZ, LY; Preparation of the manuscript: YZ, WD; Final approval of manuscript: WD, YC.

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Notice of Correction:

Changes have been made to Tables 2 and 4 since the original online publication date of November 6, 2014.