A functional variant in the UBE2B gene promoter is associated with idiopathic azoospermia

Lisha Mou1,2*, Qiang Zhang1,3†, Ruiying Diao1, Zhiming Caí1 and Yaoting Gui2*

Abstract

Background: A variety of genetic variants lead to abnormal human spermatogenesis. The ubiquitin-conjugating enzyme E2B (UBE2B) plays a significant role in spermatogenesis as Ube2b-knockout male mice are infertile.

Methods: In this study, we sequenced the exon and promoter region of UBE2B in 776 patients diagnosed with idiopathic azoospermia (IA) and 709 proven fertile men to examine whether UBE2B is involved in the pathogenesis of IA.

Results: In the exon region, two novel synonymous variants were detected in the patient group. In the promoter region, four known variants and four novel variants were identified in the patient group. Of the novel variants in the promoter region, three were located at the binding site of specificity protein 1 (SP1) transcription factor analyzed by TRANSFAC software. Luciferase assays demonstrated that one heterozygous variant (Chr5:133706925 A > G) inhibited the transcriptional regulation activity of SP1.

Conclusions: A novel variant (Chr5:133706925 A > G) residing in the UBE2B gene promoter region confers a high risk for IA in a Chinese population. These results support a role for UBE2B in the pathogenesis of IA.

Keywords: Male infertility, Idiopathic azoospermia, UBE2B, SP1

Background

Approximately 15 % of couples at child-bearing age are infertile [1–3]. Idiopathic azoospermia (IA), one of the most severe forms of male infertility, affects up to 1 % of all adult men in the general population [4]. Although the genetic causes of IA remain largely unknown in humans, familial case reports and experimental studies in mice have demonstrated that many variants in different genes can result in spermatogenesis defects [5–7]. Recent studies also indicate that genetic variants in the promoter region are associated with IA risk as well [8–10].

The ubiquitin-conjugating enzyme E2B (UBE2B), also known as Rad6b or Hr6b, is located at chr5:133706870–133727799. UBE2B belongs to the Ubiquitin Proteasome System, which mediates H2A and H2B ubiquitylation and function during transcription [11]. Previous studies have shown that the variants of UBE2B are associated with male infertility in humans [12–15]. Other evidence from experimental studies has also shown that Ube2b knockout male mice are infertile with low numbers of predominantly abnormal spermatozoa, abnormal spermatid nuclear condensation, sperm periaxonemal anomalies, damaged synaptonemal complex structure and/or increased cross-over frequency [16, 17].

Because the UBE2B gene is essential for normal spermatogenesis [12–14, 18], the genetic variants that affect UBE2B expression may be involved in the etiology of human IA. Therefore, the objective of this study was to determine the association between UBE2B gene variants and male infertility.

Methods

Ethical approval

The study was approved by the ethics committee of Peking University Shenzhen Hospital. The approval reference number is 20090018. The study was approved...
on July 18th, 2009, initiated on August 1st, 2009 and terminated on December 1st, 2014.

**Patient samples**

A total of 1,880 azoospermic patients were recruited for this study from the Peking University Shenzhen Hospital and the Center of Reproductive Medicine, Tongji Medical College, Huazhong University of Science and Technology. Among them, 776 patients fulfilled the criteria for LA diagnosis as follows: (1) no sperm detected in the pellets of semen samples at three different occasions, (2) no obstruction, inflammation and injury of the reproductive system or pelvic cavity, (3) no endocrinological defect, and (4) no karyotypic abnormality and Y chromosome microdeletion. A total of 709 fertile men from the Center of Physical Examination, Peking University Shenzhen Hospital were recruited as controls: they had fathered at least one child without assisted reproductive techniques such as IVF, ICSI and IMSI. After a panel re-sequencing study and quality control steps, 776 patients aged 24–46 years (average 30.6) and 709 fertile men aged 29–51 years (average 35.6) were available for further analysis. Informed written consent was obtained from each subject.

**Panel re-sequencing study**

Five micrograms of genomic DNA isolated from peripheral blood samples were sent to the Beijing Genomics Institute at Shenzhen for exome capture and sequencing. The capture procedure was performed in solution with a NimbleGen custom array (Roche NimbleGen, Madison, WI, USA) that is capable of enriching the exonic sequences of 654 infertility- or subfertility-related genes [19]. Most of these genes were reviewed by Matzuk and Lamb [5]. Moreover, we also selected other genes that were shown to cause male reproductive defects in mouse models from studies published between November, 2008 and December, 2010. A panel re-sequencing study was performed on the Illumina platform with pair-end 90 bp reads.

Fastaq sequence files were aligned against the human reference genome (NCBI build 37.1, hg19) with the SOAPaligner software (2.21). Duplicated pair-end reads were removed from the merged data sets. Single nucleotide variants that were different from the hg19 reference genome were filtered out if they met any of the following criteria: Phred-like quality score ≤ 20, overall depth ≤ 8×, estimated copy number ≥ 2 or the genomic distance between two adjacent variants < 5 bp. In addition, the quality score of both the major and minor allele at heterozygous locus should be at least 20. Variants were then annotated using an in-house functional prediction tool and were compared to dbSNP135 and 1000 Genomes databases (as of August, 2010).

**Polymerase chain reaction (PCR) analysis of UBE2B promoter**

Four sets of PCR primers (Table 1) were developed and optimized to amplify the promoter and 5’ UTR region 2551 bp upstream of the UBE2B coding region from HeLa cells by PCR with the following primers: promoter 1, CCCAGGAC AAAAGATGAAc (forward), AGAAATCGCTTGAGACGGAG (reverse); promoter 2, CCTCAACTTCTCGTCTCA (forward), CACCCGCTTTCCCTCTAT (reverse); promoter 3, AGAGGGAAGACGGGTGAC (forward), CGGGTTTAAG AGGTTGAG (reverse); and promoter 4, CCTCTTGGG TAATGTGTGTc (forward), CATCTAGGCGAAGGTGAA (reverse).

Forward and reverse amplification primers were also used as sequencing primers. Sequencing was conducted on an ABI 3730 DNA analyzer. Variants were then annotated using an in-house functional prediction tool and were compared to dbSNP135 and 1000 Genomes databases (as of August, 2010).

**Plasmids construction**

The wild type (WT) and mutant UBE2B promoter bearing one of the identified variants (Chr5.133706771 T > A, Chr5.133706876 T > G, Chr5.133706925 A > G) were amplified by PCR with the primers 5’-CGGGTTACCCAGGGGC AAGGCTGAGGCAATGTT -3’ (forward) and 5’-CTAGC TACGAGGAGGGCCGGAATGTGGT -3’ (reverse) from the patient genome. The PCR products were subcloned into a psiCHECK™-2 vector (C8021, Promega, Madison, WI) with KpnI/Nhel sites. All clones were verified by DNA sequencing.

**Luciferase assay**

HeLa, 293FT and TM4 cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco’s Modified Eagle’s Medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10 % fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C, 95 % humidity and 5 % CO2. Cells were seeded in 24-well tissue culture plates 24 h prior to transfection. Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Equivalent amounts (500 ng) of pCMV-SP1 (OriGene, Rockville, MD, USA), pCMV empty vector were cotransfected with wild type (WT) or mutant UBE2B promoter (Chr5.133706771 T > A, Chr5.133706876 T > G, Chr5.133706925 A > G) plasmids (250 ng), respectively. Cells were harvested 36 h after transfection and assayed for firefly and Renilla luciferase expression using the Dual Luciferase Reporter Assay System (Promega). Renilla luciferase activities were normalized to firefly luciferase activity.

**Histopathology**

Testicular tissues from patients with azoospermia were collected and immediately stored at 4 % paraformaldehyde...
solution. The tissues were sent to the pathology department for sections. Slides were stained with hematoxylin and eosin and then examined using light microscopy.

Statistical analysis
Data were expressed as the mean ± SD. The Student’s t-test with Bonferroni correction was used to compare the difference in mean between the variants and WT. P < 0.05 was considered statistically significant.

Results
Identification of UBE2B variants in patients with IA
To examine whether UBE2B genetic defects were associated with IA, we screened for UBE2B exonic variants in 776 IA patients and 709 men with proven fertility using the massively parallel sequencing technology. As shown in Table 1, two synonymous variants were detected in the UBE2B exon region. These two variants were not reported as genetic polymorphisms in the public database dbSNP135 or in the 1000 Genome Project dataset. However, no missense variants were identified in our study.

Table 1 UBE2B exonic variants in IA patients and controls

| No. | Chr5.position | Sequence variants | Patients (n=776) | Control (n=709) | Function          |
|-----|---------------|-------------------|-----------------|----------------|------------------|
| 1   | 133712363     | A > G             | 1               | 0              | Synonymous variant |
| 2   | 133724047     | C > A             | 0               | 2              | Synonymous variant |

To examine whether UBE2B promoter variants were associated with IA, we further screened for UBE2B promoter variants by PCR and Sanger sequencing (Fig. 1). Sequence analysis of the UBE2B promoter region in 776 IA patients and 709 men with proven fertility revealed 8 variants (Table 2). As shown in Table 2, four variants had not been reported in either the dbSNP135 database or the 1000 Genome Project dataset and were found to be absent in the normal controls. Further analysis by TRANSFAC software showed that three of these variants (Chr5.133706771 T > A, Chr5.133706876 T > G, and Chr5.133706925 A > G) were located at the binding site of the SP1 transcription factor. We hypothesized that these three variants may affect the DNA-binding ability of SP1 to the UBE2B promoter, thus modifying UBE2B expression. The following study was focused on...
these three variants. In particular, one of these three variant (Chr5. 133706925 A > G) was identified in 32 patients. All of the 32 patients were heterozygous variants.

The other three patient-specific variants (Chr5.133705 424 G > A, Chr5133706626 T > C, Chr5. 133706996 T > G) were not in a regulatory sequence analyzed by TRANSFAC software. Alignment of the UBE2B promoter sequence to its orthologs in different species showed that five patient-specific variants (Chr5.133705424 G > A, Chr5133706626 T > C, Chr5.133706771 T > A, Chr5.133706925 A > G and Chr5. 133706996 T > G) were not conserved (Fig. 2). Only one patient-specific variant (Chr5.133706925 T > G) was conserved (Fig. 2). The subjects with variants listed in Table 2 did not carry mutations in the other assayed genes.

**SP1 failed to activate the UBE2B Chr5.133706925 A > G promoter**

To evaluate whether the identified three variants affect the role of SP1 in UBE2B promoter activation, luciferase reporter constructs containing the UBE2B promoter with these three variants were tested in HeLa, 293FT and TM4 cell lines. The results showed that SP1 significantly increased UBE2B promoter WT, Chr5.133706925 A > G in comparison to the empty vector (Fig. 3).

**Histological analysis**

A patient biopsy (No. 188) with UBE2B Chr5.133706925 A > G confirmed the diagnosis of non-obstructive azoospermia. Moreover, we found that spermatogenic cells were significantly reduced in contorted seminiferous tubules (Fig. 4a, b).

**Discussion**

Previous studies suggested that azoospermia may result from chromosomal aberrations, microdeletions of the Y chromosome, or single gene variants [20–22]. However, the underlying genes and molecular mechanisms are not well identified.

The UBE2B gene is essential for normal spermatogenesis, and defects in this gene will lead to spermatogenic failure. Matzuk’s group found that UBE2B variants exist in oligozoospermic patients, but they did not examine the correlation between UBE2B and IA. In this study, we sequenced the exon and promoter region of UBE2B in patients with IA. The Chr5.133706925 A > G promoter variant, located at the SP1-binding sites, was found in 32 of the 776 patients but was absent in 709 fertile men. However, because only the promoter, exons and flanking regions, which are adjacent to the 5’ end and 3’ end of the gene but not transcribed into RNA, of UBE2B gene were sequenced, it remains to be determined whether there are disease-associated variants at intronic splice junctions.

In mammalian spermatogenesis, the histone post-translational modifications such as acetylation, methylation,
phosphorylation, sumoylation and ubiquitination have been linked to the regulation of cellular activities such as gene transcription, repair, replication and silencing [23, 24]. UBE2B protein, an ubiquitin-conjugating enzyme, can mediate H2A and H2B ubiquitylation and function during transcription in somatic cells as well as germ cells [25–27]. UBE2B is mainly expressed in round spermatids and Sertoli cells, and it is essential for normal spermatogenesis. Ube2b-deficient male mice are infertile as a result of low numbers of predominantly abnormal spermatozoa, abnormal spermatic nuclear condensation, and sperm perianaxonal anomalies [16, 17, 28]. Furthermore, detailed analysis has shown that UBE2B is responsible for proper chromosome crossover in the meiotic recombination of mouse spermatocytes, whereas UBE2B defects lead to arrest of the meiotic prophase and abnormal chromosome synopsis [16, 17, 28]. These results clearly demonstrate the important role of UBE2B in maintaining normal spermatogenesis and the involvement of UBE2B defects in spermatogonic failure. Therefore, it is reasonable to suggest that Chr5.133706925 A > G in the UBE2B promoter may affect gene expression by altering the binding sites of some transcription factors, which in turn results in spermatogonic defects.

To provide additional evidence for our conclusion, we performed further experiments to address the biological functional bases underlying the association between UBE2B promoter variants and IA risk. First, the sequence region around Chr5.133706925 (A > G) was predicted to be a DNA-binding site of SP1, with the allele A to allele G change leading to the alteration and possibly loss of function for this site. Second, the luciferase assay results showed that substitution of A by G at Chr5.133706925 reduced the regulation of SP1 to the UBE2B promoter. In this study, a biopsy of a patient (No. 188) with the UBE2B Chr5.133706925 A > G confirmed the diagnosis of non-obstructive azoospermia and further histopathology showed abnormal spermatogenesis. Therefore, these results collectively indicate that Chr5.133706925 A > G in UBE2B promoter confers susceptibility to IA. It is possible that abrogation of the SP1 binding site due to the presence of Chr5.133706925 (A > G) decreased the expression of UBE2B and thus led to spermatogonic failure.

Studies have identified several alternatively spliced transcripts encoding SP1 isoforms that display stage and cell-type-specific expression profiles in differentiating germ cells of the seminiferous epithelium in the testis [29]. Further studies need to be performed to confirm that the GC box of Chr5.133706925 is actually bound by SP1 in spermatids. Additional functional assays are needed to confirm the effects of SP1 on transcription of the UBE2B variant (Chr5.133706925 A > G) and examine the results of the SP1 factor failure to bind in spermatids with this variant.

Conclusions

In conclusion, with the use of bioinformatics analysis and functional analysis, we identified a variant (Chr5.133706925 A > G) that resides in the UBE2B gene promoter and confers a high risk for IA in a Chinese population, possibly through modification of the DNA-binding ability of SP1 and subsequent reduction in UBE2B expression. Our study also demonstrated that systematic analysis of the genetic variants in large cohorts of patients in addition to subsequent functional assays may provide new insights into the cause of IA in humans.

Abbreviations

IA: Idiopathic azoospermia; UBE2B: Ubiquitin-conjugating enzyme E2B.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

LM designed the study and performed experiments, analyzed and interpreted the data, drafted the manuscript and obtained funding. QZ performed PCR, luciferase experiments and analyzed the data. RD recruited patients and conducted clinical diagnoses. ZC supervised the study design. YG designed the study and obtained funding. All other authors provided technical assistance, and all authors read and approved the final manuscript.

Fig. 4 Histopathology of testes from a patient (No. 188) with Chr5.133706925 A > G and normal tissue by hematoxylin and eosin staining. a. The patient (No.188). The spermatogenic cells were reduced in contorted seminiferous tubules and there were no mature sperm. b. Testis tissue from a patient with obstructive azoospermia as the control. (Magnification: X200)
Acknowledgments

The authors thank the patients and the family members for their cooperation during the study. This work was supported by grants from the National Natural Science Foundation of China (grant number 31272144, 81200465), Guangdong Natural Science Foundation of China (grant number 2014A03031785), Shenzhen Foundation of Science and Technology (grant number JCYJ20140414140721192, JCYJ20140414140721337), ‘Three Outstanding Projects’ of Shenzhen, the Project of Shenzhen Engineering Center, Clinical Doctor-Basic Scientist Combination Foundation of Shenzhen People’s Hospital and Key Laboratory Project of Shenzhen Second People’s Hospital.

Author details

1. Shenzhen Domesticated Organ Medical Engineering Research and Development Center, First Affiliated Hospital of Shenzhen University, Shenzhen, China. 2. Guangdong and Shenzhen Key Laboratory of Male Reproductive Medicine and Genetics, Institute of Urology, Peking University Shenzhen Hospital, Biomedical Research Institute, Shenzhen PKU-HKUST Medical Center, Shenzhen, China. 3. The people’s hospital of Ankang, Shanxirn, China.

Received: 19 March 2015 Accepted: 10 July 2015

Published online: 30 July 2015

References

1. Krausz C. Male infertility: pathogenesis and clinical diagnosis. Best Pract Res Clin Endocrinol Metab. 2011;25(2):271–85.
2. Jungwirth A, Gwercman A, Tournaye H, Diemer T, Kopa Z, Dohle G, et al. European Association of Urology guidelines on Male Infertility: the 2012 update. Eur Urol. 2012;62(2):324–32.
3. McLaren JF. Infertility evaluation. Obstet Gynecol Clin North Am. 2012;39(4):653–68.
4. Hu Z, Xia Y, Guo X, Dai J, Li H, Hu H, et al. A genome-wide association study in Chinese men identifies three risk loci for non-obstructive azoospermia. Nat Genet. 2012;44(2):183–6.
5. Matzuk MM, Lamb DJ. The biology of infertility: research advances and clinical challenges. Nat Med. 2008;14(11):197–213.
6. O’Flynn O’Brien KL, Varghese AC, Agarwal A. The genetic causes of male infertility: a review. Fertil Steril. 2010;93(1):1–12.
7. Harburz R, Zouari R, Pierre V, Ben Khelifa M, Kharouf M, Coutton C, et al. A recurrent deletion of DFY19L2 causes infertility in man by blocking sperm head elongation and acrosome formation. Am J Hum Genet. 2011;88(3):351–61.
8. Sassi E, Aleyasin A, Mowla SJ, Sahelkharash H. Study of GT-repeat expansion in Heme oxygenase-1 gene promoter as genetic cause of male infertility. J Assist Reprod Genet. 2011;28(8):737–41.
9. Guarduci E, Nuti F, Becherini L, Rotondi M, Balecia G, Forti G, et al. Estrogen receptor alpha promoter polymorphism: stronger estrogen action is coupled with lower sperm count. Hum Reprod. 2006;21(4):994–1001.
10. Li Z, Yu J, Zhang T, Li H, Ni Y, et al. A functional variant in ATM gene promoter, is associated with idiopathic nonobstructive azoospermia. Fertil Steril. 2010;93(1):1–12.
11. Koken MH, Hoogerbrugge JW, Jasper-Dekker I, de Wit J, Willemens R, Roest HP, et al. Expression of the ubiquitin-conjugating DNA repair enzymes HRH6A and B suggests a role in spermatogenesis and chromatin modification. Dev Biol. 1996;173(1):119–22.
12. Hy Y, Wen W, Yu JG, Song SS, Liu J, et al. Genetic association of UBE2B variants with susceptibility to male infertility in a Northeast Chinese population. Genet Mol Res. 2012;11(4):4226–34.
13. Huang J, Emery BR, Christensen GL, Griffin J, Peterson CM, Carroll DT. Novel UBE2B-associated polymorphisms in an azoospermic/oligozoospermic population. Asian J Androl. 2008;10(3):461–6.
14. Suryavathi V, Khatri A, Gopal K, Rani DS, Panneerdoss S, Gupta NJ, et al. Novel variants in UBE2B gene and idiopathic male infertility. J Androl. 2008;29(5):564–71.
15. Yatsenko AN, Georgiadis AP, Murthy LJ, Lamb DJ, Matzuk MM. UBE2B mRNA alterations are associated with severe oligozoospermia in infertile men. Mol Hum Reprod. 2013;19(6):388–94.
16. Escalier D, Bai XY, Silvius D, Xu PX, Xu X. Spermatic nuclear and sperm periaxonemal anomalies in the mouse Ube2b null mutant. Mol Reprod Dev. 2003;65(3):296–308.
17. Baarends WM, Wassenaar E, Hoogerbrugge JW, van Cappellen G, Roest HP, Vreeburg J, et al. Loss of HR6B ubiquitin-conjugating activity results in damaged synaptonemal complex structure and increased crossing-over frequency during the male meiotic prophase. Mol Cell Biol. 2003;23(6):1151–62.
18. Mou L, Zhang Q, Wang Y, Sun L, Li C, Huang W, et al. Identification of Ube2b as a novel target of androgen receptor in mouse seminal cells. Biol Reprod. 2013;89(2):1–11.
19. Li Z, Huang Y, Li H, Hu J, Liu X, Jiang T, et al. Excess of rare variants in genes that are key epigenetic regulators of spermatogenesis in the patients with non-obstructive azospermia. Sci Rep. 2015;5:8785:1–7.
20. Tuttelfmann F, Rajpert-De Meyts E, Nieschlag E, Simoni M. Gene polymorphisms and male infertility—a meta-analysis and literature review. Reprod Biomed Online. 2007;15(6):643–58.
21. Ferlin A, Raicu F, Gatta V, Zucconeello D, Palka G, Foresta C. Male infertility: role of genetic background. Reprod Biomed Online. 2007;14(6):734–45.
22. Lee JY, Dada R, Sabanegh E, Carpi A, Agarwal A. Role of genetics in azoospermia. Urology. 2011;77(3):598–601.
23. Zhou W, Wang X, Rosenfeld MG. Histone H2A ubiquitination in transcriptional regulation and DNA damage repair. Int J Biochem Cell Biol. 2009;41(1):12–5.
24. Das C, Tyler JK. Histone exchange and histone modifications during transcription and aging. Biochim Biophys Acta. 2013;1819(3–4):332–42.
25. Kote SK, Feil E. Epigenetic transitions in germ cell development and meiosis. Dev Cell. 2010;19(3):675–86.
26. Mulugeta Achame E, Wassenaar E, Hoogerbrugge JW, Sleedens-Linkels E, Ooms M, Sun ZW, et al. The ubiquitin-conjugating enzyme HR6B is required for maintenance of X chromosome silencing in mouse spermatocytes and spermatids. BMC Genomics. 2010;11:367.
27. Inagaki A, Sleedens-Linkels E, Wassenaar E, Ooms M, van Cappellen WA, Hoejmovers JH, et al. Mesiotic functions of RAD18. J Cell Sci. 2011;124(Pt 16):2837–50.
28. Roest HP, van Klaveren J, de Wit J, van Gorp CG, Koken MH, Vormy M, et al. Inactivation of the Hr6b ubiquitin-conjugating DNA repair enzyme in mice causes male sterility associated with chromatin modification. Cell. 1996;86(5):799–810.
29. Thomas K, Wu J, Sung DY, Thompson W, Powell M, McCarrey J, et al. SNP transcription factors in male germ cell development and differentiation. Mol Cell Endocrinol. 2007;270(1–2):1–7.