A novel variant of androgen receptor is associated with idiopathic azoospermia

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Abstract. A variety of genetic variants can lead to abnormal human spermatogenesis. The androgen receptor (AR) is an important steroid hormone receptor that is critical for male sexual differentiation and the maintenance of normal spermatogenesis. In the present study, each exon of AR in 776 patients diagnosed with idiopathic azoospermia (IA) and 709 proven fertile men were sequenced using use panel re-sequencing methods to examine whether AR is involved in the pathogenesis of IA. Two synonymous variants and seven missense variants were detected. Of the missense variants, a luciferase assay demonstrated that the R630W variant reduced the transcriptional regulatory function of AR. This novel variant (p. R630W) of AR is the first to be identified in association with IA, thereby highlighting the importance of AR during spermatogenesis.

Introduction

Infertility is experienced by 10-15% of couples. It has previously been estimated that half of these cases are due to male infertility (1,2), a serious form of which is idiopathic azoospermia (IA). Genetic analysis of patients with IA have revealed a variety of causes, predominantly chromosome aberrations or variants of functional genes associated with spermatogenesis (3,4). Understanding the genetic variants may contribute to the diagnosis and management of infertility in men, and is crucial to prevent the passing of genetic defects to offspring in future generations via *in vitro* fertilization procedures.

Androgen receptor (AR) is an important steroid hormone receptor that is critical during male sexual differentiation and for the maintenance of normal spermatogenesis (5-7). AR belongs to a family of nuclear transcription factors that mediate the action of steroid hormones (8). There are 4 domains in the AR protein structure, including the N-terminal transactivation domain, DNA-binding domain, hinge region and carboxyl ligand-binding domain (9,10). Binding of androgens, including testosterone and 5α-dihydrotestosterone, to the ligand-binding domain of AR results in nuclear translocation, where it acts as a transcriptional regulator (10).

Previously, a number of AR mutations or polymorphisms that cause or are associated with a spectrum of hereditary disorders, including complete and partial androgen insensitivity syndrome (CAIS and PAIS, respectively), were identified (11,12). In order to identify variants of the AR gene in patients with IA, the present study sequenced each exon of AR in 776 patients diagnosed with IA and 709 proven fertile men. The results may be important for the accurate diagnosis of IA and useful for genetic counseling.

Materials and methods

Ethical approval. The present study was approved by the ethics committee of Peking University Shenzhen Hospital (Shenzhen, China; approval no. 20090018). The current study was approved on July 18, 2009, initiated on August 1, 2009 and terminated on December 1, 2014.

Patient samples. The patient inclusion criteria was the same as described previously (13), with certain modifications. A total of 1,880 azoospermic patients were recruited for the present study from the Center of Reproductive Medicine,

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Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China) between January 2007 and October 2011. Among the subjects, 776 patients fulfilled the following criteria for IA diagnosis: i) No sperm detected in the pellets of semen samples on three different occasions; ii) no obstruction, inflammation or injury of the reproductive system or pelvic cavity; and iii) no karyotypic abnormality or Y chromosome microdeletion. A total of 709 fertile men from the Center of Physical Examination, Peking University Shenzhen Hospital were recruited as controls. Each had fathered at least one child without assisted reproductive techniques, including *in vitro* fertilization, intracytoplasmic sperm injection or intracytoplasmic morphologically selected sperm injection. Following panel re-sequencing and quality control steps, 776 patients aged 24-46 years (mean, 30.6 years) and 709 fertile men aged 29-51 years (mean, 35.6 years) were available for further analysis. Informed written consent was obtained from each subject.

Panel re-sequencing. Panel re-sequencing was performed as described previously (13). Genomic DNA samples (5 μg) isolated from peripheral blood samples using the E.Z.N.A. Blood DNA kits (Omega Bio-Tek, Inc., Norcross, GA, USA) were sent to the Beijing Genomics Institute (Shenzhen, China) for exome capture and sequencing. The capture procedure was performed in solution with a NimbleGen custom array (Roche Applied Science, Madison, WI, USA) that enriched the exonic sequences of 654 infertility- or subfertility-associated genes. The majority these genes have been reviewed by Matzuk and Lamb (14). Additionally, the present study selected other genes that have been previously demonstrated to cause male reproductive defects in mouse models in studies published between November 2008 and December 2010 (15). Panel re-sequencing was performed using the Illumina platform (Illumina, Inc., San Diego, CA, USA) with 90 bp pair-end reads.

FASTQ sequence files were aligned against the human reference genome (NCBI build 37.1, hg19) with Short Oligonucleotide Analysis Package aligner software (version 2.21; www.soap.genomics.org.cn). Duplicated paired-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: i) A Phred-like quality score of ≤20; ii) overall depth of ≤8x; estimated copy number of ≥2; iii) or genomic distance between two adjacent variants of <5 bp. In addition, the quality score of the major and minor allele at heterozygous loci were ≥20. The variants were then annotated using an in-house functional prediction tool, and were compared with the dbSNP Build 135 (www.ncbi.nlm.nih.gov/projects/SNP/) and 1000 Genomes (www.1000genomes.org; as of August 2010). Validation of novel missense variants by Sanger sequencing. To validate the novel missense variants identified by deep sequencing, polymerase chain reaction (PCR) amplifications were performed and the PCR products were sequenced in both directions with a 3730 DNA Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The primers for PCR and Sanger sequencing validation of the AR gene are presented in Table I.

Validation of novel missense variants by Sanger sequencing. As described in Panel re-sequencing, genomic DNA samples (5 μg) were isolated from peripheral blood samples using the E.Z.N.A. Blood DNA kits (Omega Bio-Tek, Inc.). PCR was performed in a volume of 50 μl containing 1 μM of each forward and reverse primer, 50 ng DNA, and 25 μl EmeraldAmp PCR Master mix (Takara Bio Inc., Otsu, Japan). Products were amplified in a thermocycler (MyCycler; Bio-Rad, Hercules CA, USA) with the following conditions: 30 cycles of 10 sec at 98˚C, 30 sec at 60˚C, and 40 sec at 72˚C. Amplicons were extracted from gels and sequenced in both directions with a 3730 DNA Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primers for PCR and Sanger sequencing validation of the AR gene are presented in Table I.

Protein alignment. Multiple protein alignments were performed with MegAlign 7.1.0 (DNASTAR, Inc., Madison, WI, USA). The amino acid sequences of the androgen receptor in humans, chimpanzees, rhesus monkeys, cows, rats, mice and chickens was determined. The identification numbers of androgen receptor protein were as follows: Human, NP_000035.2; chimpanzee, XP_009437511.1; rhesus, NP_001028083.1; cow, NP_001231056.1; rat, NP_036634.1; mouse, NP_038504.1; and chicken, NP_001035179.1).

Evaluation of coding single nucleotide polymorphisms. Sorting Intolerant From Tolerant (SIFT; sift.jcvi.org/) and PolyPhen 2.0 (genetics.bwh.harvard.edu/pph2/) analysis were used for the evaluation of coding single nucleotide polymorphisms (16,17). SIFT is based on the premise that protein evolution is correlated with protein function. SIFT scores ≤0.05 are predicted by the algorithm to be damaging, whereas scores >0.05 are considered tolerant. Predictions are based on a combination of phylogenetic, structural and sequence annotation information characterizing a substitution and its position in the protein. PolyPhen scores >0.85 are predicted by the algorithm to be probably damaging, scores >0.15 are considered possibly damaging, whereas scores <0.15 are considered benign.

Plasmid construction and site-directed mutagenesis. A human AR expression plasmid was provided as a gift from Dr Chawnshang Chang (George H. Whipple Lab for Cancer Research, Departments of Pathology and Urology, University of Rochester Medical Center, Rochester, NY, USA). Site-directed mutagenesis was performed to generate AR expression plasmids bearing the C290R, S495N or R630W variant, as described previously (13). DNA sequencing was performed to confirm the introduced variants. The PCR primers used for site-directed mutagenesis and plasmid construction are presented in Table II. Products were amplified in a thermocycler (Bio-Rad) using an Expand High Fidelity PCR system (Roche, Basel, Switzerland). The 50 μl PCR reaction was conducted with 50 ng templates, 1 μM primer pairs, 200 μM dNTPs and 2 units of DNA polymerase. The extension reaction was initiated by pre-heating the reaction mixture to 94˚C for 3 min; 16 cycles of 94˚C for 1 min, 52˚C for 1 min and 68˚C for 8 min; followed by incubation at 68˚C for 15 min. DNA sequencing was conducted with a 3730 DNA Analyzer (Applied Biosystems) to confirm the introduced variants.

Luciferase assay. Luciferase analysis was performed as described previously, with certain modifications (18). HeLa and TM4 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin (Gibco; Thermo Fisher Scientific, Inc.) and 100 μg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C, 95% humidity and 5% CO_2 atmosphere. Cells were seeded in 24-well tissue culture plates for 24 h prior to transfection. Equivalent concentrations (100 ng) of wild-type (WT) or mutant AR expression plasmids were cotransfected with mouse mammary tumor virus (pMMTV) long terminal repeat plasmids (a gift from Dr Chawnshang Chang) into HeLa and TM4 cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Cells were treated with or without 100 nM testosterone (Sigma-Aldrich) after 6 h of transfection and harvested 24 h after treatment. Firefly and *Renilla* luciferase expression was assessed using a Dual-Luciferase Reporter Assay System (cat no. E1910; Promega Corporation, Madison, WI, USA). *Renilla* luciferase activity was normalized to that of firefly luciferase. The Modulus/ 9200-003 luminometer (Turner Biosystems, Sunnydale, CA, USA) was used in this study. Following normalization for transfection efficiency, induction factors were calculated as the ratio of the mean luciferase value for testosterone-stimulated versus non-testosterone-stimulated (ethanol vehicle-treated) samples.

Ultrasound examinations. All ultrasonographic measurements were conducted by the same ultrasonographer. Patients with novel AR variants were examined with scrotal ultrasonography to test the size of testes. All examinations were performed using ultrasound scanner (EUB-7500, Hitachi Medical Corporation, Tokyo, Japan) equipped with a linear array transducer (6.5 MHz; Model EUP-L65; Hitachi Medical Corporation).

Statistical analysis. All experiments were repeated at least three times. Data of the luciferase assay are expressed as the mean ± standard deviation. SPSS version 17.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Analysis of variance (ANOVA) and Dunnett's t-test were used to compare the difference in the means between the variants and WT. $P<0.05$ was considered to indicate a statistically significant difference. Data were log-transformed before ANOVA to satisfy the equal variances assumption.

Table I. Primers for polymerase chain reaction and Sanger sequencing validation of the AR gene.

Primer	Sequence (5'-3')
AR mut1	
Forward	CTCCGCTGACCTTAAAGACATCCT
Reverse	CTCGCCTTCTAGCCCTTTGGTGTA
AR mut2	
Forward	CTCCCCATCCCCACGCTCGCATCA
Reverse	ATCCAGGGGCCCATTTCGCTTTTG
AR mut3	
Forward	GGTTTAGCAGGTATTTGGGATGAT
Reverse	GAGTCGGGCTGGTTGTTGTC

AR, androgen receptor.

Table II. Primers for polymerase chain reaction used for site-directed mutagenesis and plasmid construction.

Primer	Sequence (5'-3')
hAR mut1 (T-C)	
Forward	TGCCCCATTGGCCGAA cGCAAAGGTTCTCTGCT
Reverse	AGCAGAGAACCTTTGC gTTCGGCCAATGGGGCA
hAR mut2 (G-A)	
Forward	CTGGCGGGCCAGGAAA aCGACTTCACCGCACCT
Reverse	AGGTGCGGTGAAGTCG tTTTCCTGGCCCGCCAG
hAR mut3 (C-T)	
Forward	ATGACTCTGGGAGCC tGGAAGCTGAAGAAACTT
Reverse	AAGTTTCTTCAGCTTCC aGGCTCCCAGAGTCAT

Lower case bases indicate the mutant sites. AR, androgen receptor.

Results

Identification of AR variant in patients with IA. To examine whether AR genetic defects were associated with IA, the present study screened for AR exonic variants in 776 patients with IA and 709 fertile men using massive parallel sequencing technology. As demonstrated in Table III, seven missense variants and two synonymous variants were detected in AR. Two of these missense variants (F827L and M887V) have been previously reported in the ExAC database. Another variant at amino acid 290 (C290W) has been previously described in the ExAC database, however, this variant was different from the variant identified in the present study (C290R). The three patient-specific missense variants (C290R, S495N and R630W) have not been previously reported in the dbSNP135 database, 1000 Genome Project dataset or ExAC database. These three novel patient-specific missense variants were further confirmed by Sanger sequencing (Fig. 1). Alignment of the amino acid sequence of AR to its orthologs in different species demonstrated that the R630W variant affected a highly conserved amino acid (Fig. 2). Based on this conservation behavior, the variant R630W was predicted to be possibly damaging to the protein, according to SIFT and PolyPhen-2.0 analysis (Table IV). The locations of these three missense variants within the AR protein are demonstrated in Fig. 3.

Effects of AR variants on AR function. To evaluate whether the identified patient-specific missense variants of AR inhibit its

Table III. Androgen receptor variants and single nucleotide polymorphisms identified in patients with idiopathic azoospermia (IA; n=776) and fertile controls (n=709).

Variant no.	Sequence variant	Amino acid change	IA patients, n	Fertile controls, n	Reported	Sample ID
Missense						
1	c.868 T>C	p.C290R	1	0	No	w320
2	c.1484 G>A	p.S495N	1	0	No	w691
3	c.1888 C>T	p.R630W	1	0	No	w530
4	c.569 C>T	p.T190I	0	1	No	262
5	c.616 A>G	p.S206G	0	1	No	285
6	c.2481 C>A	p.F827L	0	1	Yes	81
7	c.2659 A>G	p.M887V	0	1	Yes	57
Synonymous						
8	c.639 G>A	None	0	1	Yes	177
9	c.1149 C>T	None	2	0	No	w325,w671

regulatory function, luciferase reporter constructs containing the androgen responsive elements (MMTV promoter) were transfected into HeLa and TM4 cell lines. AR WT, C290R and S495N, but not R630W variants, significantly increased MMTV promoter activity compared with empty vector in both cell types ($P<0.05$; Fig. 4). These results indicate that C290R and S495N did not affect the transcriptional regulation activity of AR. However, the transcriptional regulation activity of AR was inhibited by the R630W variant.

Clinical variable and hormone analysis. Scrotal color Doppler ultrasonography of the three patients with novel AR variants revealed small testes in the scrotal sac, with a homogeneous echotexture and wide hypoechogenicity. No solid or cystic lesions were observed. Table V summarizes the clinical and hormone data of these three patients, none of which had a family history of male infertility, CAIS or PAIS.

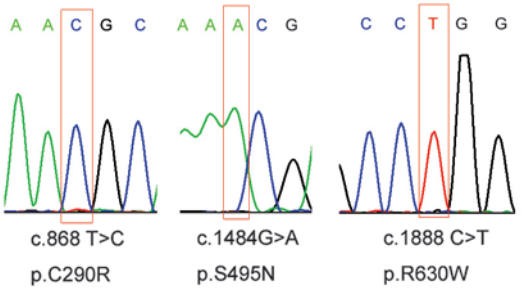

Figure 1. Three missense variants of the androgen receptor identified in patients with idiopathic azoospermia. Chromatogram traces from Sanger sequencing demonstrate the validated missense variants.

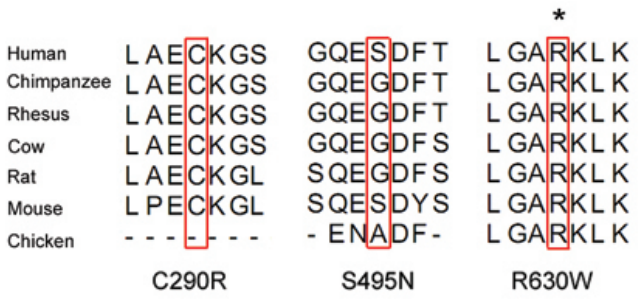

Figure 2. Evolutionary conservation of amino acids affected by the missense variants. Multiple protein alignments were performed with MegAlign. The identification numbers of androgen receptor protein were as follows: Human, NP_000035.2; chimpanzee, XP_009437511.1; rhesus, NP_001028083.1; cow, NP_001231056.1; rat, NP_036634.1; mouse, NP_038504.1; and chicken, NP_001035179.1). The variant alleles are boxed, and the * indicates the conserved residue.

Discussion

Accumulating evidence indicates that AR is a ligand-dependent transcription factor that regulates the expression of androgen-responsive genes (19). Androgens and AR are essential in male spermatogenesis and fertility (20,21).

Although the AR gene has >700 reported mutants and polymorphisms, only 5 mutants located in exon 1 have been observed in azoospermia patients with varying degrees of impaired spermatogenesis or mild AIS (22). In agreement with the present study, all previous subjects presented normal external genitalia, complicating the clinical diagnosis. Additionally, a previous study observed that male mice with total AR knockout exhibited female-typical external appearance, which was similar to human AIS or testicular feminization variant mice (23).

The current study sequenced the coding sequence of AR in a large group of patients with IA. The R630W variant was observed in 1 of the 776 patients with IA, but was not detected in any of the 709 fertile men sequenced or in other individuals previously reported in the public databases. The R630W variant was localized in the conserved DNA-binding domain of AR, which is in accordance with the abolished transcriptional activity of AR caused by this mutation. Computerized analysis using PolyPhen-2.0 and SIFT software classified the R630W variant as damaging, predicting it to have a deleterious effect on the protein structure. Additionally, local alignment analysis of the amino acid sequences of AR demonstrated that the affected arginine residue was highly conserved in multiple species, including

Table IV. List of missense variants predicted to be functionally significant by SIFT and PolyPhen 2.0 programs.

		SIFT[a]		PolyPhen-2.0[b]	
Nucleotide change	Amino acid change	Score	Prediction	Score	Prediction
c.868 T>C	p.C290R	0.340	Tolerated	0.995	Possibly damaging
c.1484G>A	p.S495N	0.480	Tolerated	0.066	Benign
c.1888 C>T	p.R630W	0.000	Damaging	1.000	Probably damaging

[a]SIFT score: ≤0.05, damaging; >0.05, tolerated. [b]PolyPhen-2.0 probably damaging: probabilistic score: >0.85; possibly damaging probabilistic score: >0.15; and benign: probabilistic score: <0.15. SIFT, Sorting Intolerant From Tolerant.

Table V. Clinical and hormone profile of patients with idiopathic azoospermia with novel androgen receptor missense variants.

Sample ID	Age, years	FSH, IU/l[a]	LH, IU/l[b]	Estradiol, pg/ml[c]	Testosterone, ng/ml[d]
w320	25	27.19	14.51	29.64	4.23
w530	30	8.12	11.46	66.61	8.75
w691	24	25.18	8.40	20.59	3.15

Normal ranges are [a]1.5-12.5 IU/l, [b]1.7-8.6 IU/l, [c]7.63-42.6 pg/ml, [d]2.8-8.0 ng/ml. FSH, follicle stimulating hormone; LH, luteinizing hormone.

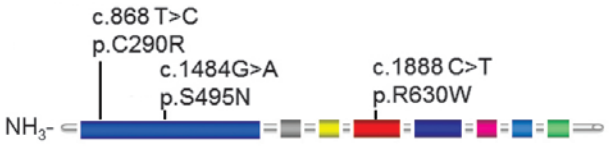

Figure 3. Variants identified in the androgen receptor (AR) gene. The positions of C290R, S495N and R630W variants are indicated on a modeled structure of the AR protein.

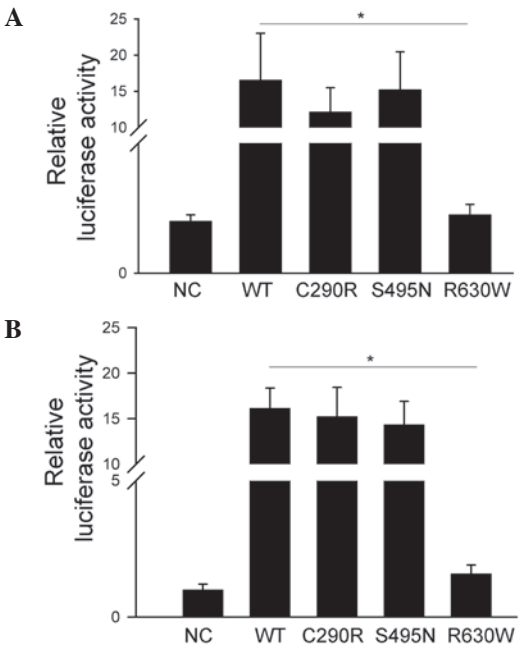

Figure 4. Effect of the androgen receptor (AR) variants on AR function. The WT or mutant AR expression vectors were cotransfected with testosterone-inducible pMMTV-LUC plasmid into (A) HeLa and (B) TM4 cells. NC plasmid (pcDNA3.1-HA) was cotrasfected with the pMMTV-LUC plasmid. Luciferase activities were measured with or without testosterone treatment. Compared with WT and other variants, the AR R630W variant did not increase pMMTV activity in the presence of testosterone. Results are presented as fold-change of testosterone- versus vehicle-treated samples. Values are presented as the mean ± standard deviation. *P<0.05. NC, negative control; WT, wild-type.

chickens. The evolutionary preservation of the entire region around this residue across multiple species indicates that variants in this region may affect the normal functions of the AR protein.

When evaluating the pathogenic effect of AR variants in patients with fertility issues, an important question to address is whether the variant affects transcriptional regulatory function of AR. The present study demonstrated that the R630W variant affected the transcriptional regulatory function of AR at the MMTV promoter.

The hormonal profile of the patient carrying the R630W mutation (w530) was different to the other two patients with novel AR variants. Compared with patients w320 and w691, patient w530 exhibited low follicle stimulating hormone, and high estradiol and testosterone. Thus, the R630W mutation potentially reduces the transcriptional activity of AR, which leads to an imbalance of the hormonal profile.

In conclusion, the current study identified seven missense variants and two synonymous novel variants of AR using massive parallel sequencing technology. Functional analysis confirmed that the R630W variant suppressed the normal transcriptional regulatory function of AR. These results suggested that AR was important in human spermatogenesis. This study also demonstrated that systematic analysis of the genetic mutations in large cohorts of patients complemented by subsequent functional assays may provide novel insights into the cause of IA in humans.

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