Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

The polymorphisms of NR5A1 gene in azoospermic men in Sichuan, China

Lanyue Cui
Nanchang University
Jiaoyu He
Sichuan University
Junhang Deng
Sichuan University
Zhilin Song
Sichuan University
Qiufu Li
Sichuan University
Yanru Cui
Sichuan University
Yiran Liu
Sichuan University
Xianping Ding (braiding@scu.edu.cn)
Sichuan University
Yueping Zhou (zqhzyp108@sina.com)
Nanchang University

Research

Keywords: NR5A1, genetic polymorphism, azoospermia, male infertility

Posted Date: July 24th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-37121/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Abstract

Background: Steroidogenic factor 1 (SF1, NR5A1) is a key transcriptional regulator involved in the hypothalamic-pituitary-steroidogenic organ development. Recently, heterozygous mutations in NR5A1 were found may contribute to the male infertility aetiology. Here, we investigated the association of polymorphisms in NR5A1 gene with azoospermic men in Sichuan, China.

Methods: We have performed the NR5A1 gene direct secquencing in a cohort of 102 well-characterised idiopathic Chinese azoospermic infertile men versus 103 fertile men, who were selected by Semen analysis, Karyotype analysis and Y-chromosomal AZF deletion screening. We identified two previously described missense p.

Results: Gly146Ala (rs1110061; c.437 G>C) and p.Arg313His (c.938G>A), and the frequency of 437C ([OR] 1.846, 95% [CI] 1.227-2.778, P=0.003), 437GC (OR =1.884 , 95% CI =1.037-3.422 , P =0.037) and 437CC (OR =3.586 , 95% CI =1.397-9.206 , P =0.006) were found to be increased significantly in azoospermic patients while no mutations in control .Moreover, one novel heterozygous p.Ser322ILe (c.965 G >A) missense mutation was found in 8 patients which highly conserved serine to isoleucine shown in the Beta strand domain on SF-1 protein.

Conclusions: This is the first study, according to our knowledge, to investigate the association between the polymorphisms of NR5A1 gene and azoospermic men in China, and these results suggest that the Gly146Ala polymorphism may be a susceptibility factor for the azoospermic men in Sichuan, China.

Introduction

Human fertility rates are declining all over the world, and one in seven couples have ennnnproblems conceiving [1], and male factor infertility reportedly accounted for approximately 30–40% of these cases[2]. Overall, over 4000 genes are estimated to be involved in human spermatogenetic failure, clinically noted as oligo- or azoospermia[3] and research in the last 20 years has definitively established that male factor infertility has a substantial genetic component, and many cases of azoospermia and severe oligozoospermia that were previously classified as idiopathic are now recognized to be caused by mutations in genes involved in germ cell production and function[4],[5],[6]. However, in the majority of cases, the underlying cause of male infertility is unknown and no candidate gene has been recognised to be administrable in patient care so far[2]. In addition, 5% of all infertile men carried chromosomal anomaly (such as 47, XXY Klinefelter syndrome), and Y-chromosomal AZF microdeletions are present in 10% of azoospermic or severely oligozoospermic (< 1 × 106 sperm/ml) men[7]. Although rodent studies indicate that multiple genes have the potential to cause male infertility, only a few single-gene defects that cause male infertility have been identified in humans. Recently, Bashamboo et al.[8] found mutations in the NR5A1 gene (MIM 184757) in a substantial fraction of 4% of infertile men (N=315) with unexplained reduced sperm counts and all of these were missense mutations leading to reduced protein function in vitro[8]. Ropke et al. identified three missense mutations in the NR5A1 gene associated with

and most likely the cause of the male infertility, according to the in silico analyses, and found the mutation frequency is below 1% (Caucasian German origin, n = 488)[9]. D.Zare-Abdollahi et al. found two heterozygous NR5A1 mutations and in silico analysis of the mutations showed that founded mutations could be detrimental, and the mutation frequency in idiopathic Iranian azoospermic infertile men is only 2.2% (Iranian, n=90)[10] . And Ferlin A et al. still reported that mutations on SF-1 protein impaired transactivation of gonadal promoters[11]. Thus, it can be seen that these studies expanded the phenotypes spectrum of SF-1 protein to include male infertility.

Steroidogenic factor 1 nuclear receptor (SF-1; AD4BP; SPGF8; OMIM 184757), a member of the nuclear hormone receptor superfamily, is an essential transcriptional activator for sexual differentiation and formation of the primary steroidogenic tissues, which is also considered a master regulator of reproduction in mammals[12]. NR5A1 gene, located in chromosome 9q33.3, consists of 7 exons (exons 2-7), encoding a central DNA binding domain (DBD) with two NR C4-type zinc-finger , a variable N-terminal domain with an 'A' box region, a flexible hinge region, and a C-terminal ligand-binding domain (LBD) containing an activation function 2 (AF-2) domain[4],[13]and[14]. NR5A1 binds DNA as a monomer, which is expressed in Sertoli and Leydig cells of the developing testis, in Sertoli cells of the prepubertal and adult testis, as well as in multiple cell types in the fetal, postnatal, prepubertal, and mature ovary[15, 16]. The hinge region is important for controlling SF1 transcriptional activity by stabilizing the LBD and interacts with other proteins. The AF-2 domain recruits cofactors necessary for SF1 transactivation activity[13]. In mammalian testis determination and differentiation, NR5A1 could function as a positive regulator of SOX9 (Sry-box 9) and anti-Müllerian hormone (AMH)[13, 17]. NR5A1 also modulates the expression of many factors involved in cholesterol mobilization and steroid hormone biosynthesis, including HMG-CoA synthase, steroidogenic acute regulatory protein (StAR), 3β-hydroxysteroid dehydrogenase (3βHSD), and several cytochrome P450 steroid hydroxylase (CYP) enzymes[13]. Consistent with its key role in gonadal development, NR5A1 mutations are associated with a wide spectrum of phenotypes, including male infertility.

Here, given the fact that genetic differences in different populations can play an important role in such a prevalent health problem, in this study, our intention is to evaluate the frequency of NR5A1 gene mutations in 102 Chinese with azoospermia and 103 fertile men as control in Sichuan, china.

Materials And Methods

2.1 Patient and the Control Populations

A total of 102 men who had unexplained reduced sperm counts and were seeking infertility treatment were recruited in our study, and all patients recruited were from the Affiliate Reproductive Hospital Genitalia Hygiene Research Center (Sichuan, China) between September 2015 and November 2016. In addition, Infertile men with following situations, including chromosome anomalies, Y chromosome microdeletions, cryptorchidism, hypospadias, occupational hazards, varicocele, and lifestyle factors, such as drug, alcohol, substance abuse, and tobacco use were excluded from this study. Control samples were

obtained from the patients recruited in the same hospital, comprising 103 DNA samples from Chinese populations, who were all fertility men and had fathered at least one healthy child without assisted reproduction. All participants were informed about the study according to a protocol that was approved by the Institutional Ethical Review boards of Sichuan University (Chengdu, China), and all gave their written consent.

2.2. Semen analysis

Semen samples were provided for routine semen analyses performed according to the fifth Edition of the WHO guidelines (WHO, 2010). Fresh semen collected by masturbation method (abstinence 2-6 days) was used to calculate the sperm parameters of motility, vitality and concentration by sperm quality analysis system MIX7.5 after Papanicolaou staining. Semen samples would be regarded as azoospermic men, if their sperm count was zero, and the standard for control/fertile was that the sperm count of one sample is greater than 20×10^6/ml. All samples were examined at least three times according to the fifth edition of World Health Organization (WHO). (Supp. table 1, 2)

2.3. Karyotype analysis

G-banding, standard karyotype analysis method, was performed as previously described in to exclude chromosome aberrations[18]. Briefly, peripheral blood lymphocytes (Stored in heparin sodium) were cultured for 72 h in RPMI-1640 with phytohemagglutinin and fetal bovine serum. Two hours before the completion of culturing, colcemid was added to the medium. G-banding of metaphase chromosomes was performed using Giemsa staining. At least 20 metaphase spreads were analyzed for each patient, and at least 50 metaphase spreads were analyzed to confirm abnormalities. The normalities were reserved for next study.

2.4 Detection of Y microdeletions

Multiplex polymerase chain reaction was performed to detect microdeletions in the Azoospermia Factor (AZF) region for patients with azoospermia. According to previous studies in the Chinese population and the diagnostic criteria of European Academy of Andrology, we chose 6 sequence-tagged site (STS) markers in the AZF region to detect microdeletions: sY86 and USP9Y in AZFa, sY127 and sY134 in AZFb, and sY254 and sY255 in AZFc [19, 20]. The products of polymerase chain reaction amplification were detected with agarose gel electrophoresis, and the detection of AZF microdeletions was described in Supp. table 3.

2.5. Extraction of genomic DNA

The total of genomic DNA was extracted from human peripheral blood leukocytes using the EasyPure Blood Genomic DNA Kit (TransGen Biotech Co., Ltd, China) according to the manufacturer's instructions. Briefly, the total of 200ul blood previously collected in EDTA anticoagulant blood was mixed with 20 mg/ml proteinase K and Binding Buffer 3 (BB3) in clean EP tubes. Uniformly, the mixed was incubated at

room temperature for 10 minutes without moving and then added in Silica gel mould centrifugal column with lysis including CB3(Clean Buffer 3),WB3(Wash Buffer 3) and EB (Elutiong Buffer 3) to wash and elut genomic DNA from the centrifugal column. Ultraviolet spectrophotometer was conducted to detect the concentration of extracted genomic DNA. All of the DNA samples were stored at -20°C until examination.

2.6 Mutational Analysis of *NR5A1*

The coding exons of *NR5A1* (exons 2-7; NM_004959.4), is located on the long arm of HSA9 (9q33.3), were amplified from genomic DNA by polymerase chain reaction(PCR) and all exons of *NR5A1* gene and the parts of flanking intronic sequence were performed using the primers described in Supp. table 4, PCR was using A200 Gradient Thermal cycler (Long Gene) in a total of 25μL volume buffered solution containing 1.5 mM Mg^{2+} (Fermentas International Inc., Burlington, Ontario, Canada), 0.25 mM dNTPs (TransGen, Beijing, China), 0.1 M of each primer (Sangon Biotech, Shanghai, China), 2U of DNA polymerase (Fermentas International Inc., Burlington, Ontario, Canada), approximately 200 ng genomic DNA. All of PCR products were analyzed by electrophoresis using 2% agarose gel. The PCR conditions and the 2% gel electrophoresis conditions were provided. (25 uL PCR amplification reaction system: 11.1 uL double distilled water, 3.0 uL 10×Buffer, 2.5ul dNTPs, 1.0 mol/L F-primers/ R-primers 1ul, 0.4 uL polymerase, 100 μg/μL DNA template 6 μL. PCR reaction conditions: pre-denaturation 95°C 5min, denaturation 95°C 30s, 64°C/62°C annealing (Exon4, Exon7)/(exon2/3, Exon4a, Exon5, Exon6) 30s, 72°Cextension 30s, amplification 30 ~ 35 cycles, 72°C extension 5min. The specificity of the amplified product was tested by 2% agarose gel electrophoresis at 120V for 45min. PCR amplification and electrophoresis were performed three times, and the samples were sent to Shanghai for sequencing. Subsequently, the PCR products has been sequenced twice with ABI3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA) using the BigDye flourescence labeling Terminarorment of the *NR5A1* gene was performed using DNAMAN. Reference sequences of the species were obtained via NCBI (https://www.ncbi.nlm.nih.gov/).

2.7 Statistical analysis and the pathogenicity prediction

Hardy-Weinberg equilibrium and the comparison of genotype frequencies between patients and control groups were performed using the chi-squared (χ^2) test. Using the unconditional logistic regression analysis to calculate odds ratio (OR) and 95% confidence interval (95% CI) were to measure the risk associated with variant genotypes. $P < 0.05$ was considered to be statistically significant. All data were analyzed using Statistical Package for Social Sciences software version 20.0 (SPSS Inc., Chicago, IL, USA). The prediction of the damaging effect of missense mutation to protein structure and function was performed using PolyPhen-2 Bioinformaticprogra (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://sift.jcvi.org/.). Subsequently, we searched the SF-1 protein 3D structure from The Protein Model Portal (http://www.proteinmodelportal.org/) and the secondary structure from UniProtKB (http://www.uniprot.org/uniprot/Q13285), and we also used the ExPASy-ProtScale (http://web.expasy.org/protscale/) to analyze the changes in hydrophobicity of the SF-1 protein due to the mutation.

Results

In the current study, a comprehensive screening comprising Semen analysis, Karyotype analysis, and Detection of AZF microdeletions was performed with 1536 men who were seeking infertility treatment, and confrimed 102 idiopathic azoospermic patients. Besides, 103 healthy controls were recruited in our study (Table 1). Screening for the *NR5A1* gene revealed the presence of 5 synonymous and 3 missense variants (Table 2). The 3 missense variants identified in our study group included 1 novel variant (p.Ser322ILe) and 2 previously reported variants (p.Gly146Ala, p.Arg313His). These substitutions were found in a heterozygous state and none of these substitutions were found in our control population consisting of 206 alleles (excluding the already known p.Gly146Ala polymorphism).

The first substitution, p.Ser322ILe (c.965 G>A), was found in 8 patients (Fig.1b) , affecting a highly conserved amino acid domain of SF-1 protein, encoded by the *NR5A1* gene, among species from invertebrate to higher species, and is predicted to be damaging to SF1 protein function (Fig. 2b), which localized in the C-terminal ligand-binding domain (LBD)(Fig. 2a). The c.965G>A heterozygous mutation is located in the Beta strand domain (322-324) of SF-1 protein by the secondary structure from UniProtKB (Fig. 3c) and the 3D model obtained from the Protein Model Portal (PMP) (Fig. 3a). Moreover, PolyPhen-2 analysis predicted that the S322I mutation is “probably damaging” with a score of 0.994 on HumVar model (Fig. 4a), and the SIFT results of prediction for position 322 on SF-1 protein also was “harmful” with a score of 0.02(Fig. 4b). In addition, a hydropathy plot of the S322I mutant polypeptide generated with the Kyte-Doolittle algorithm by using an online tool ExPASy-ProtScale demonstrated a further imbalance in its hydrophobicity caused by the S322I mutation and may result in protein structure changes (Fig. 5a and Fig. 5b).

The previously described missense mutation c.437G>C (p.Gly146Ala, rs1110061) (Fig.1a), located in exon 4 and fall within the evolutionarily conserved hinge region (Fig.2a), was present in 70 patients (52 hetero-, 18 homozygous) and 52 controls (44 hetero-, 8 homozygous). The frequencies of C allele (OR = 1.846 , 95% CI =1.227-2.778 , P =0.003) , genotype 437GC (OR =1.884 , 95% CI =1.037-3.422 , P =0.037) and 437CC (OR =3.586 , 95% CI =1.397-9.206 , P =0.006) were all significantly increased among patients compared with controls (Table 3). We identified a previously described missense mutation p.R313H (c.938G>A) in one patient in *NR5A1* gene but otherwise healthy men in controls.

Discussion

Azoospermia is a common cause of male infertility found in subfertile couples and most cases are of unknown origin[21]. It is estimated that in about 30% of eases of male infertility is due to chromosonial abnortnalities or mutalions of genes involved in germ cell production and function[22]. Chromosomal abnormalities, Y microdeletions and gene variation are widely reported to be association with azoospermia [23, 24]. Moreover, it has been reported that defections in the AZF region of the Y chromosome causes 10%-15% of idiopathic NOA[25]. Additionally, among those factors, the chromosomal aberrations (including translocations) and quantitative variances (including semen

concentration) are the most frequent causes of male infertility, affecting nearly 65.9% of infertile men, and the genetic mutations are about 4-10 times more prevalent among men with abnormal semen parameters compared with those with normal semen parameters [26]. Numerous studies have reported a correlation between abnormal karyotypes (chromosomal abnormalities) and azoospermic men, and the prevalence of chromosomal abnormalities is 15.2%, while in non-azoospermic men is 2.3% ,which suggesting that chromosomal abnormalities could be a possible cause of azoospermia.[27, 28]. The human Y chromosome is essential for human sex determination and male germ cell development and maintenance, and normal male development in humans depends on its functions with complete structure ,and the abnormalities in the Y chromosome, such as microdeletions, are associated with male infertility, which is consistent with the fact that Y microdeletions long arm occurs 10%-15% of idiopathic primary testiculopathies (azoospermia and severe oligozoospermia)[24],[29, 30]. Given these current results about male infertility and azoospermia, karyotype analysis and the testing of Y microdeletions are indispensable screening means for male infertility causes, and men with karyotype abnormalities and/or Y microdeletions(AZF region) must be excluded for the case of gene mutation search. In our research, 1536 male who were seeking infertility treatment have been screened by Semen analysis, karyotype analysis and Y microdeletions testing, resulting in Supp.Table 3 and Table 4, and patients with possible causes of azoospermia mentioned above were excluded in the subsequent studies.

Steroidogenic factor 1 is an essential regulator of endocrine development and function and is considered a master regulator of reproduction, and in Human, mutations of *NR5A1* were initially described in patients with 46, XY karyotype and disorders of sex development (DSD), Müllerian structures and primary adrenal failure (MIM 612965)[31]. Recently, *NR5A1* mutations have been related to human male infertility[8],[9],[10]and[11]{Bashamboo, 2010 #13;Röpke, 2013 #14}. In our comprehensive mutation screen of 102 well-characterised patients, we identified only three missense mutations (Table 2) likely causative for the phenotypes of azoospermia according to the *in silico* analyses. Compared with the mutations found in the study by them which were all located in the hinge region (amino acids 95–225) or the *N*-terminal portion of the ligand-binding domain[8],[9],[10]and[11]{Bashamboo, 2010 #13;Röpke, 2013 #14}, consisting with our experimental results that the P.Gly146Ala mutation was found in the hinge region and the two mutations (P.Arg313His and P.Ser322Ile) in the ligand-binding domain of SF1 protein. Nevertheless, in our present study, the C allele frequencies (OR =1.846 , 95% CI =1.227-2.778 , P =0.003) and the frequencies of genotype 437GC (OR =1.884 , 95% CI =1.037-3.422 , P =0.037) and 437CC (OR =3.586 , 95% CI =1.397-9.206,P=0.006) of missense mutation c.437G>C (p.Gly146Ala,rs1110061) were significantly increased compared with controls (Table 3), while the C allele and genotype frequencies were low in their research. Bashamboo et al. [8] reported that the percentage of G146A polymorphism exhibiting in azoospermia (orcryptozoospermia), severe oligozoospermia (OATs < 1×10^6/ml) and moderate oligozoospermia (OATs $1–10 \times 10^6$/ml) were 3.9% (4 in 103), 4.3% (2 in 46) and 2% (1 in 50) ,respectively. Ropke et al. [9] pointed out that the missense mutation c.437G>C (*P*.Gly146Ala, rs1110061) was present in 16 patients and 5 controls, furthermore, neither allele frequencies nor genotype distribution differed significantly between patients and controls. D.Zare-Abdollahi et al. [10] indicated that the c.437G>C polymorphism was only detected in 3 patients and 2 normal

controls(case=90,control=112,Iranian). Alberto Ferlin et al.[11] revealed that the single nucleotide polymorphism of p.Gly146Ala in *NR5A1* gene was detected 11 out of 196 (5.6%) in patients with the phenotype of nonobstructive azoospermia and cryptozoospermia(<1*10^6ejaculate). Moreover, Wuqiang F et al pointed out that this polymorphism causes slightly diminished transactivation activity (about 80% of WT)[32]. Therefore, even if this variation exhibits strong ethnic differences in frequency (from 1.4% in Europeans, to 35.2% in East Asians, to 76.3% in Africans from the ExAC database) and warrants further investigations, it might be of clinical importance [32]. In addition, we provide an overview of the previously published data regarding the p.G146A polymorphism in Asia (Table 4). Comparing previously published results with our data, we noticed a rather high regarding how common the C allele is in the investigated control populations. According to our data, the C allele has a prevalence of 29.1 % in the control population, whereas the frequency of the same allele is low rarely in other control populations which mentioned above. This may be explained by the differences of ethnic group, geographical environment, or simply by the insufficient number of individuals investigated in each study.

In the current report, we also present the identification of a novel missense mutations (p. Ser322Ile) located in the LBD of SF-1 in 8 patients and none of the substitution were found in our control population. The substitution of Ser322Ile was found in a heterozygous state, although, since this specific mutation has previously not been reported, we hypothesized it may play a role in the development of azoospermia. In addition, according to the prediction of PolyPhen-2(score=0.994) and SIFT (score=0.02), the position of it on the Beta strand domain (322-324) of SF-1 protein by the secondary structure and the 3D model, suggesting that it may affect the expression of SF-1 protein. Moreover, quite recently, Ropke et al. [9] reported that a patient with severe oligozoospermia, sperm concentration repeatedly below 0.3 million/ml and no cryptorchidism deceted a mutation(c.968T>C,P.Ile323Thr) in *NR5A1* gene, witch next to the mutation of P.Ser322Ile, which were found in 8 patients(7.8%,8/102) otherwise in healthy men. Concerning the mutation P.Ser322Ile, the possibility cannot be ruled out that this mutation might well be play an important role in the process of spermatogenic. Consequently, to clarify the role of the novel missense mutation (p. Ser 322 ILe) in the disease of azoospermia, additional studies including structure and functional experiments are required. Moreover, in this research, we identified a previously described missense mutation p.R313H(c.938G>A) in 1 patient (0.98,1/102) in *NR5A1* gene but otherwise healthy men in controls, and SIFT results of prediction for position 313 on SF-1 protein also was “harmful ” with a score of 0.00. (Fig.4) Moreover, it has been detected in monozygotic male twins displaying very severe hypospadias in Caucasians[33] and another study reported the p.Arg313Cys mutation in a patient with isolated distal hypospadias [34]. In addition, Allali et al. reported that the p.Arg313Cys *NR5A1* proteins associated with ambiguous genitalia and distal hypospadias showed a marked impairment of the function of the protein, and in vitro and in vivo experiments have previously revealed that functional cooperation between *NR5A1* and a protein partner GATA4 contribute to the proper spatiotemporal expression of the AMH gene during the development of mammalian genitalia[35], so additional studies are required to clarify the role of the missense mutation (p.Arg313His) in the disease of azoospermia.

There are several mechanisms by which mutations in *NR5A1*, and therefore reduced SF-1 protein activity, might determine the alteration of the testicular development and descent and spermatogenesis. Jeyasuria P.et al. reported that the *NR5A1* Leydig cell-specific knockout mice had hypoplastic testes with the lumens of the seminiferous tubules failed to open and spermatogonia never developed into mature sperm, and also showed reduced the expression of *Cyp11a* and *StAR* genes in testosterone biosynthesis[36]. Kojima Y et al. conducted a study of azoospermic patients and found the levels of *NR5A1* expression in gonadal tissue correlated positively with serum testosterone concentrations, suggesting a direct connection between these two factors[37]. Zhao L et al. revealed that mice that lack *NR5A1* show marked hypogonadism with a reduction in testis volume, a decreased number of Leydig cells, and an absence of mature spermatids, resulting in infertility[38]. Bashamboo et al. showed that the *NR5A1* mutants associated with male infertility show impaired activation of two of the *NR5A1* target genes, *AMH* and *Cyp11a1.* Furthermore, the detected p.G146A polymorphism located in the hinge region of SF-1 has previously been described to mildly diminish the SF-1 transactivation function for the adrenal specific cyp11A promoter and the ovary specific cyp19 promoter II by 20% [32]. And the mutations fall within the hinge region (amino acids 95–225) and proximal portion of the LBD, and a number of physical interactions and functional activities have been mapped to this portion of the protein[39]. Phosphorylation of Ser 203 in the hinge region enhances the interaction of GRIP1 and SMRT with the AF1 and AF2 regions of *NR5A1*, whereas sumoylation of lysines within the hinge region increases interactions with DEAD box proteins and results in transcriptional repression [40, 41].

In conclusion, this study is the first, according to our knowledge, to investigate the association between the polymorphisms of *NR5A1* gene and azoospermic men in China. The results of this study revealed that the polymorphisms of the Gly146Ala may be a susceptibility factor for the obstructive azoospermic men in the population in Sichuan, China. However, due to the limited size of the individuals investigated in our study or regionally genetic difference, more evidence studies need to be confirmed with larger groups of participants from different ethnic and geographic origins.

Declarations

6.1 Ethical Approval and Consent to participate

All participants were informed about the study according to a protocol that was approved by the Institutional Ethical Review boards of Sichuan University (Chengdu, China).

6.2 Consent for publication

All subjects participating in the study signed the consent form.

6.3 Availability of supporting data

Seminal parameters statistics in patients and control groups, basic characteristics of case group and control group, 1536 patients with AZF segment microdeletions statistics, and 4 primers for 6 exon

amplification of NR5A1 gene.

6.4 Competing interests

The authors have no conflicts of interest.

6.5 Funding

This work was funded by Key Scientific Research Foundation Projects of Sichuan Province (No. 2018JY0601).

6.6 Authors' contributions

All of the authors listed made substantial contributions to the manuscript and qualify for authorship, and no authors have been omitted. Conception and design, XPD; development of methodology and acquisition of data, LYC, JYH, JHD, ZLS, QFL, YRC and YRL; analysis and interpretation of data, LYC, JYH, JHD, ZLS, QFL, YRC and YRL; writing and revision of the manuscript, LYC, JYH, JHD, ZLS, QFL, YRC, YRL, XPD, YPZ. All the authors read and approved the final manuscript.

6.7 Acknowledgements

The authors thank the Affiliate Reproductive Hospital Genitalia Hygiene Research Center (Sichuan, China) and all patients for their participation in this study.

6.8 Authors' information

Lanyue Cui: cuilanyueliang@qq.com, College of Queen Mary, Nanchang University, Nanchang, China.

Jiaoyu He: 1061355567@qq.com, Key Laboratory of Bio-Resources and Eco-Environment, Ministry of Education; Institute of Medical Genetics, College of Life Science, Sichuan University, China; Bio-resource Research and Utilization Joint Key Laboratory of Sichuan and Chongqing, Sichuan, Chongqing, PR China.

Junhang Deng: 1916358148@qq.com, Key Laboratory of Bio-Resources and Eco-Environment, Ministry of Education; Institute of Medical Genetics, College of Life Science, Sichuan University, China; Bio-resource Research and Utilization Joint Key Laboratory of Sichuan and Chongqing, Sichuan, Chongqing, PR China.

Zhilin Song: Szi9585@126.com, Key Laboratory of Bio-Resources and Eco-Environment, Ministry of Education; Institute of Medical Genetics, College of Life Science, Sichuan University, China; Bio-resource Research and Utilization Joint Key Laboratory of Sichuan and Chongqing, Sichuan, Chongqing, PR China.

Qiufu Li: lqf1192069072@126.com, Key Laboratory of Bio-Resources and Eco-Environment, Ministry of Education; Institute of Medical Genetics, College of Life Science, Sichuan University, China; Bio-resource Research and Utilization Joint Key Laboratory of Sichuan and Chongqing, Sichuan, Chongqing, PR China.

Yanru Cui: 512927123@qq.com, Key Laboratory of Bio-Resources and Eco-Environment, Ministry of Education; Institute of Medical Genetics, College of Life Science, Sichuan University, China; Bio-resource Research and Utilization Joint Key Laboratory of Sichuan and Chongqing, Sichuan, Chongqing, PR China.

Yiran Liu: 532154290@qq.com, Key Laboratory of Bio-Resources and Eco-Environment, Ministry of Education; Institute of Medical Genetics, College of Life Science, Sichuan University, China; Bio-resource Research and Utilization Joint Key Laboratory of Sichuan and Chongqing, Sichuan, Chongqing, PR China.

Xianping Ding: brainding@scu.edu.cn, Key Laboratory of Bio-Resources and Eco-Environment, Ministry of Education; Institute of Medical Genetics, College of Life Science, Sichuan University, China; Bio-resource Research and Utilization Joint Key Laboratory of Sichuan and Chongqing, Sichuan, Chongqing, PR China.

Yueping Zhou: zqhzyp108@sina.com, College of Queen Mary, Nanchang University, Nanchang, China; Key Laboratory of Preventive Medicine of Jiangxi Province, College of Public Health, Nanchang University, Nanchang, China.

Acknowledgements

The College of Life Science, Sichuan University provided the funding. All authors read and approved the final manuscript and none of the authors declare competing financial interests.

Abbreviations

Steroidogenic factor 1 (SF1), DNA binding domain (DBD), ligand-binding domain (LBD), activation function 2 (AF-2), anti-Müllerian hormone (AMH), steroidogenic acute regulatory protein (StAR), 3β-hydroxysteroid dehydrogenase (3βHSD), cytochrome P450 steroid hydroxylase (CYP), World Health Organization (WHO), Azoospermia Factor (AZF), sequence-tagged site (STS), Binding Buffer 3 (BB3), Clean Buffer 3 (CB3),Wash Buffer 3(WB3), Elution Buffer (EB3), polymerase chain reaction(PCR) and disorders of sex development (DSD).

References

1. Skakkebæk NE, Jørgensen N, Main KM, Meyts ERD, Leffers H, Andersson AM, et al. Is human fecundity declining? International journal of andrology. 2006;29(1):2-11.
2. Tüttelmann F, Rajpert-De Meyts E, Nieschlag E, Simoni M. Gene polymorphisms and male infertility–a meta-analysis and literature review. Reproductive biomedicine online. 2007;15(6):643-58.
3. Venables J, Cooke H. Lessons from knockout and transgenic mice for infertility in men. Journal of endocrinological investigation. 2000;23(9):584-91.
4. Krylova IN, Sablin EP, Moore J, Xu RX, Waitt GM, MacKay JA, et al. Structural analyses reveal phosphatidyl inositols as ligands for the NR5 orphan receptors SF-1 and LRH-1. Cell. 2005;120(3):343-55.

5. Ferlin A. New genetic markers for male fertility. Asian J Androl. 2012;14(6):807-8.
6. Ferlin A, Foresta C. New genetic markers for male infertility. Current Opinion in Obstetrics and Gynecology. 2014;26(3):193-8.
7. McLachlan RI, O'Bryan MK. State of the art for genetic testing of infertile men. The Journal of Clinical Endocrinology & Metabolism. 2010;95(3):1013-24.
8. Bashamboo A, Ferraz-de-Souza B, Lourenço D, Lin L, Sebire NJ, Montjean D, et al. Human male infertility associated with mutations in NR5A1 encoding steroidogenic factor 1. The American Journal of Human Genetics. 2010;87(4):505-12.
9. Röpke A, Tewes A-C, Gromoll J, Kliesch S, Wieacker P, Tüttelmann F. Comprehensive sequence analysis of the NR5A1 gene encoding steroidogenic factor 1 in a large group of infertile males. European Journal of Human Genetics. 2013;21(9):1012-5.
10. Zare-Abdollahi D, Safari S, Mirfakhraie R, Movafagh A, Bastami M, Azimzadeh P, et al. Mutational screening of the NR5A1 in azoospermia. Andrologia. 2015;47(4):395-401.
11. Ferlin A, Santa Rocca M, Vinanzi C, Ghezzi M, Di Nisio A, Foresta C. Mutational screening of NR5A1 gene encoding steroidogenic factor 1 in cryptorchidism and male factor infertility and functional analysis of seven undescribed mutations. Fertility and sterility. 2015;104(1):163-9. e1.
12. Parker KL, Schimmer BP. Steroidogenic factor 1: a key determinant of endocrine development and function. Endocrine reviews. 1997;18(3):361-77.
13. Lin L, Achermann J. Steroidogenic factor-1 (SF-1, Ad4BP, NR5A1) and disorders of testis development. Sexual Development. 2008;2(4-5):200-9.
14. Sablin EP, Blind RD, Krylova IN, Ingraham JG, Cai F, Williams JD, et al. Structure of SF-1 bound by different phospholipids: evidence for regulatory ligands. Molecular endocrinology. 2009;23(1):25-34.
15. Ikeda Y, Shen W-H, Ingraham HA, Parker KL. Developmental expression of mouse steroidogenic factor-1, an essential regulator of the steroid hydroxylases. Molecular endocrinology. 1994;8(5):654-62.
16. Morohashi K-i, Iida H, Nomura M, Hatano O, Honda S-i, Tsukiyama T, et al. Functional difference between Ad4BP and ELP, and their distributions in steroidogenic tissues. Molecular endocrinology. 1994;8(5):643-53.
17. Sekido R, Lovell-Badge R. Sex determination involves synergistic action of SRY and SF1 on a specific Sox9 enhancer. Nature. 2008;453(7197):930-4.
18. Zhang S, Wang Q-m, Ding X-p, Wang T, Mu X-m, Chen Z-y. Association of polymorphisms in PATE1 gene with idiopathic asthenozoospermia in Sichuan, China. Journal of Reproductive Immunology. 2016;118:54-60.
19. Navarro-Costa P, Plancha CE, Gonçalves J. Genetic dissection of the AZF regions of the human Y chromosome: thriller or filler for male (in) fertility? BioMed Research International. 2010;2010.
20. Gong M, Dong W, He T, Shi Z, Huang G, Ren R, et al. MTHFR 677C> T polymorphism increases the male infertility risk: a meta-analysis involving 26 studies. PloS one. 2015;10(3):e0121147.

21. Gulum M, Gumus K, Yeni E, Dogantekin E, Ciftci H, Akin Y, et al. Blood and semen paraoxonase—arylesterase activities in normozoospermic and azoospermic men. Andrologia. 2016.
22. Vogt PH. Molecular genetic of human male infertility: from genes to new therapeutic perspectives. Current pharmaceutical design. 2004;10(5):471-500.
23. Dul E, van Echten-Arends J, Groen H, Dijkhuizen T, Land J, van Ravenswaaij-Arts C. Chromosomal abnormalities in azoospermic and non-azoospermic infertile men: numbers needed to be screened to prevent adverse pregnancy outcomes. Human reproduction. 2012:des222.
24. Hamada AJ, Esteves SC, Agarwal A. A comprehensive review of genetics and genetic testing in azoospermia. Clinics. 2013;68:39-60.
25. Ferlin A, Moro E, Garolla A, Foresta C. Human male infertility and Y chromosome deletions: role of the AZF-candidate genes DAZ, RBM and DFFRY. Human Reproduction. 1999;14(7):1710-6.
26. McAuliffe ME, Williams PL, Korrick SA, Dadd R, Perry MJ. The association between sperm sex chromosome disomy and semen concentration, motility and morphology. Human reproduction. 2012;27(10):2918-26.
27. Dul E, Groen H, van Ravenswaaij-Arts C, Dijkhuizen T, van Echten-Arends J, Land J. The prevalence of chromosomal abnormalities in subgroups of infertile men. Human reproduction. 2012;27(1):36-43.
28. Tunç E, Tanrıverdi N, Demirhan O, Süleymanova D, Çetinel N. Chromosomal analyses of 1510 couples who have experienced recurrent spontaneous abortions. Reproductive biomedicine online. 2016;32(4):414-9.
29. Ferlin A, Moro E, Rossi A, Dallapiccola B, Foresta C. The human Y chromosome's azoospermia factor b (AZFb) region: sequence, structure, and deletion analysis in infertile men. Journal of Medical Genetics. 2003;40(1):18-24.
30. O'Brien KLF, Varghese AC, Agarwal A. The genetic causes of male factor infertility: a review. Fertility and sterility. 2010;93(1):1-12.
31. Ozisik G, Achermann JC, Jameson JL. The role of SF1 in adrenal and reproductive function: insight from naturally occurring mutations in humans. Molecular Genetics and Metabolism. 2002;76(2):85-91.
32. Wuqiang F, Yanase T, Wei L, Oba K, Nomura M, Okabe T, et al. Functional characterization of a new human Ad4BP/SF-1 variation, G146A. Biochemical and Biophysical Research Communications. 2003;311(4):987-94.
33. Adamovic T, Chen Y, Thai H, Zhang X, Markljung E, Zhao S, et al. The p. G146A and p. P125P polymorphisms in the steroidogenic factor-1 (SF-1) gene do not affect the risk for hypospadias in Caucasians. Sexual Development. 2012;6(6):292-7.
34. Allali S, Muller J-B, Brauner R, Lourenço D, Boudjenah R, Karageorgou V, et al. Mutation analysis of NR5A1 encoding steroidogenic factor 1 in 77 patients with 46, XY disorders of sex development (DSD) including hypospadias. PLoS One. 2011;6(10):e24117.
35. Tremblay JJ, Viger RS. A mutated form of steroidogenic factor 1 (SF-1 G35E) that causes sex reversal in humans fails to synergize with transcription factor GATA-4. Journal of Biological

Chemistry. 2003;278(43):42637-42.
36. Jeyasuria P, Ikeda Y, Jamin SP, Zhao L, de Rooij DG, Themmen AP, et al. Cell-specific knockout of steroidogenic factor 1 reveals its essential roles in gonadal function. Molecular endocrinology. 2004;18(7):1610-9.
37. Kojima Y, Sasaki S, Hayashi Y, Umemoto Y, MOROHASHI KI, Kohri K. Role of transcription factors Ad4bp/SF-1 and DAX-1 in steroidogenesis and spermatogenesis in human testicular development and idiopathic azoospermia. International journal of urology. 2006;13(6):785-93.
38. Zhao L, Bakke M, Krimkevich Y, Cushman LJ, Parlow A, Camper SA, et al. Steroidogenic factor 1 (SF1) is essential for pituitary gonadotrope function. Development. 2001;128(2):147-54.
39. Hoivik EA, Lewis AE, Aumo L, Bakke M. Molecular aspects of steroidogenic factor 1 (SF-1). Molecular and cellular endocrinology. 2010;315(1):27-39.
40. Hammer GD, Krylova I, Zhang Y, Darimont BD, Simpson K, Weigel NL, et al. Phosphorylation of the nuclear receptor SF-1 modulates cofactor recruitment: integration of hormone signaling in reproduction and stress. Molecular cell. 1999;3(4):521-6.
41. Lee MB, Lebedeva LA, Suzawa M, Wadekar SA, Desclozeaux M, Ingraham HA. The DEAD-box protein DP103 (Ddx20 or Gemin-3) represses orphan nuclear receptor activity via SUMO modification. Molecular and cellular biology. 2005;25(5):1879-90.

Tables

Due to technical limitations, tables 1-4 are only available as downloads in the supplemental files section.

Figures

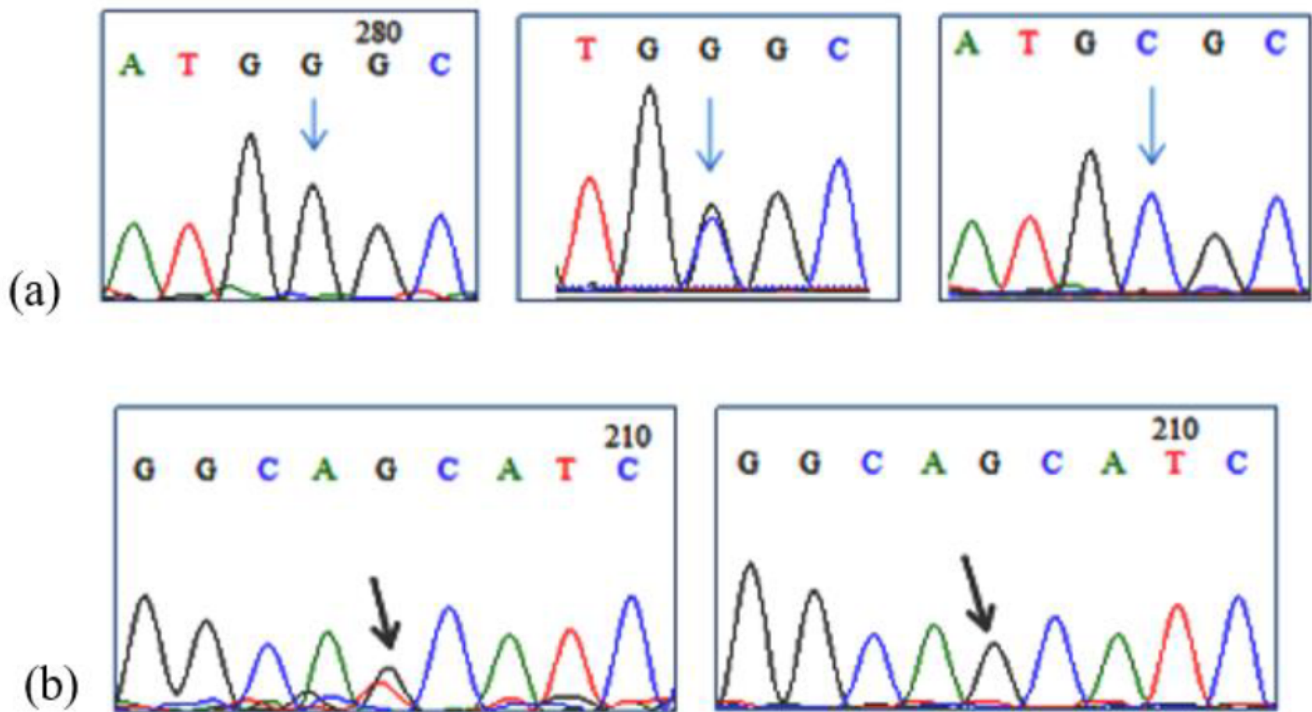
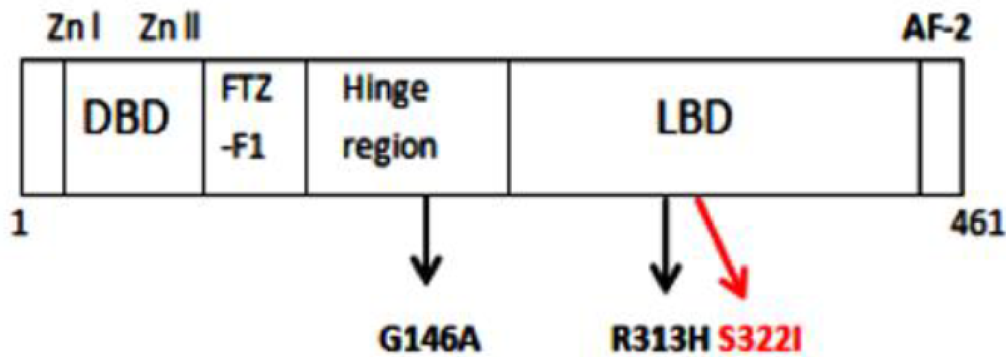


Figure 1

Mutation analysis of the NR5A1 gene. a.Sequencing analysis of the c.437 G >C in the NR5A1 with different allele expressions. b.Sequencing analysis of the c.965 G>T in the NR5A1 with different allele expressions.The position of our reported mutated amino acid (G146A with a blue down-pointing arrow and S322I with black down-pointing arrow) is framed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



(b)

		NCBI Reference Sequence
Human	YRQVQHGKEGSILLVTGQEVE	NP_004950.2
Chimpanzee	YRQVQHGKEGSILLVTGQEVE	XP_520248.3
M.mulatta	YRQVQHGKEGSILLVTGQEVE	XP_002800023.1
Norway rat	YRQVQYGKEDSILLVTGQEVE	NP_001178028.1
House mouse	YRQVQYGKEDSILLVTGQEVE	NP_620639.1
Chicken	YRQLQHGKEHSVLLVTGQEVD	NP_990408.1
Frog	YRQMQHSKENSILLVTGQEIEVS	NP_001139213.1
Cattle	YRQIQHGKEGSILLVTGQEVE	NP_776828.1
Pig	YRQIQHGKEGSILLVTGQEVE	NP_999344.1

Figure 2

(a) Schematic diagram of the functional domains of NR5A1. Arrows indicate the mutations in patients with preserved fertility (details in the main text). Red arrow indicates the mutation of S322I. (b) Homology study revealed that the serine acid residue at codon 322 is highly conserved throughout the species.

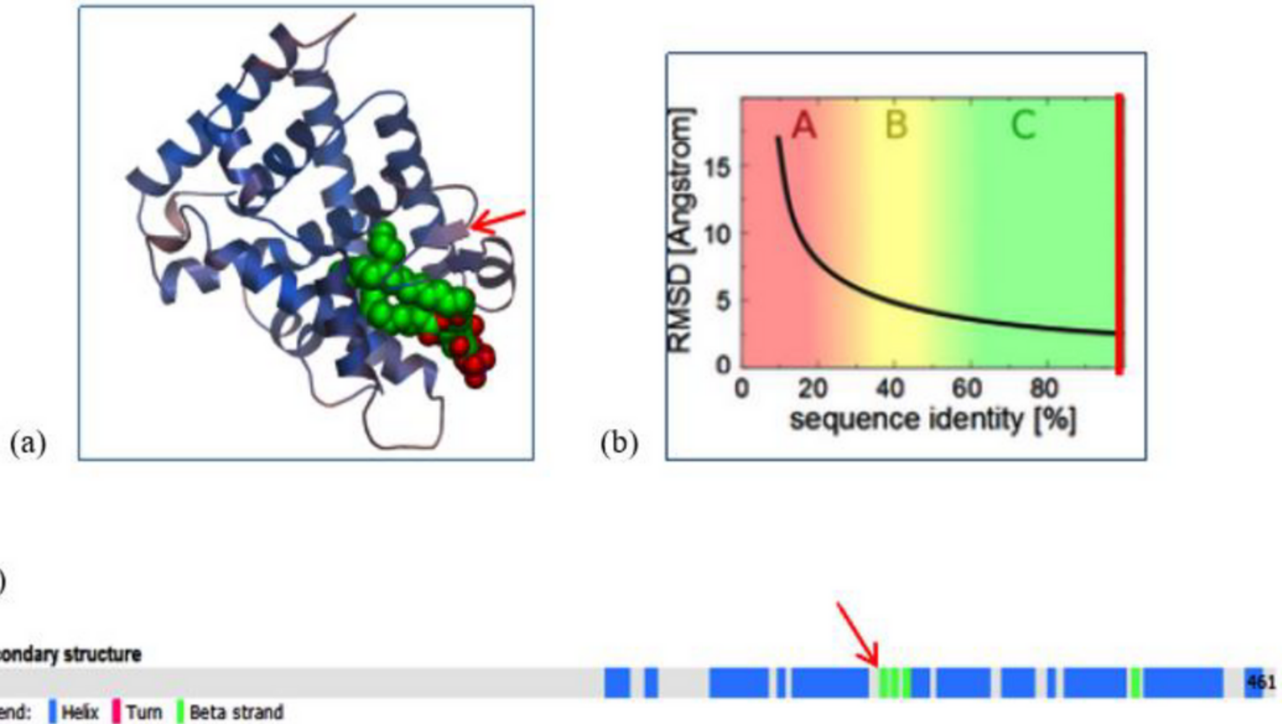
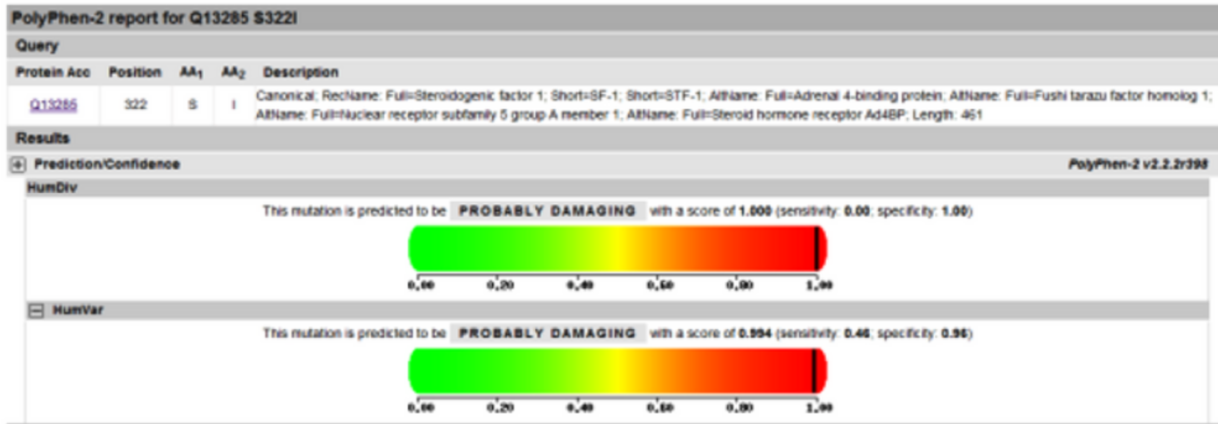


Figure 3

Protein structure model of NR5A1 was given from The Protein Model Portal(PDB ID: 4QJR) . (a).The location of mutation (S322I) in SF-1 protein 3D structure. (b).This model is based on target-template sequence alignment of 99% sequence identity (C). (c). The location of S322I in SF-1 protein secondary structure.The red down-pointing arrow indicates the position of S322I on SF-1 protein.

(a)



(b)

pos		A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
316Q	0.84	0.65	0.17	0.17	0.27	0.46	0.21	0.34	0.59	0.27	0.74	0.23	0.23	0.20	0.26	0.26	0.32	0.48	1.00	0.12	0.59
317H	0.84	0.04	0.09	0.02	0.02	0.07	0.02	1.00	0.03	0.02	0.05	0.02	0.02	0.01	0.02	0.03	0.20	0.03	0.04	0.01	0.38
318G	0.84	0.16	0.00	0.02	0.03	0.01	1.00	0.08	0.01	0.03	0.01	0.01	0.11	0.01	0.02	0.05	0.27	0.07	0.03	0.00	0.01
319K	0.84	0.01	0.00	0.00	0.02	0.00	0.06	0.01	0.01	1.00	0.01	0.00	0.03	0.01	0.10	0.21	0.01	0.01	0.08	0.00	0.01
320E	0.82	0.01	0.00	0.20	1.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00
321G	0.82	0.02	0.00	0.35	0.02	0.00	1.00	0.14	0.00	0.02	0.00	0.00	0.15	0.01	0.01	0.01	0.13	0.02	0.00	0.00	0.01
322S	0.82	0.09	0.24	0.03	0.09	0.01	0.03	0.01	0.02	0.03	0.04	0.01	0.05	0.04	0.02	0.02	1.00	0.41	0.04	0.00	0.01
323I	0.84	0.00	0.00	0.00	0.00	0.01	0.00	0.00	1.00	0.00	0.43	0.16	0.00	0.00	0.00	0.00	0.00	0.00	0.21	0.00	0.00
324L	0.84	0.01	0.07	0.00	0.00	0.14	0.00	0.00	0.12	0.00	1.00	0.11	0.00	0.00	0.01	0.00	0.00	0.01	0.03	0.00	0.21

Figure 4

Pathogenic prediction of SF-1 protein. (a).PolyPhen-2 analysis predicting the pathogenicity of the p.S322I substitution on the SF-1 protein scored 0.994. (b). SIFT results of prediction for position 322 on SF-1 protein with a score of 0.02. SIFT: numerical range: 0 -1, scores < 0.05, predicted amino acid substitution is harmful; or it is harmless.

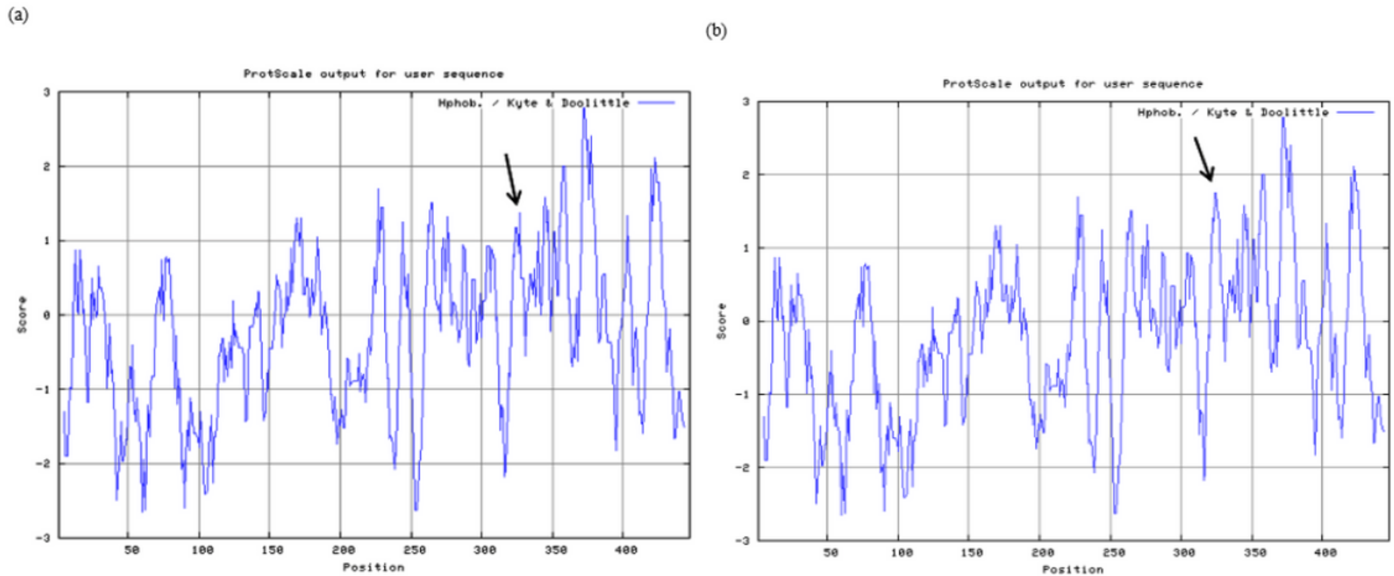


Figure 5

Hydropathy plot for the SF-1 protein prepared in the Expasy ProtScale Website according to the Kyte and Doolittle algorithm. The hydrophobicity of the wild-type SF-1 protein. (a). is compared to the mutant form, including the novel p.S322I mutation. (a).The hydrophobicity scores of p.S322I of the SF-1 protein are higher than the wild type. The change caused by mutated site is indicated with a black arrow.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementarymaterial.pdf
- table.pdf