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Polymorphisms in inhibin α gene promoter associated with male infertility

Wan-hong Li [a], Lu Chen [a], Shu-xiong Chen [a], Hong-jiao Li [a], Zhuo Liu [a], Li-na Sun [a], Yun Zhao [a], Lian-wen Zheng [b], Chun-jin Li [a,*], Xu Zhou [a,*]

[a] College of Animal Sciences, Jilin University, 5333 Xian Road, Changchun 130062, PR China
[b] The Second Hospital of Jilin University, Reproductive Medical Center, Changchun, Jilin 130062, PR China

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ABSTRACT

Inhibins play important roles in normal gonadal function, including regulation of proliferation, differentiation, and steroidogenesis of Leydig and Sertoli cells via paracrine and autocrine processes. In adult males, circulating inhibin levels are correlated with fertility by regulating the number of Sertoli cells, total sperm count, and testicular volume. Given this important role, inhibin-α subunit (INHA) is a strong candidate gene in male fertility. However, limited data regarding the association of polymorphisms of *INHA* with male fertility are available. This study was based on the hypothesis that polymorphisms in the promoter of *INHA* are associated with male fertility. Han Chinese patients with non-normozoospermia ($n = 153$) and normozoospermia ($n = 72$) from Northern China were screened, and genotypes were analyzed by polymerase chain reaction–restriction fragment length polymorphism after *INHA* promoter was amplified. Statistical analysis results revealed a significant difference in the allele frequency of *INHA* promoter between males with non-normozoospermia and normozoospermia. For c.-124G>A, males carrying c.-124GG genotype and c.-124GA genotype showed an increased risk of non-normozoospermic syndrome. For c.-16C>T polymorphism, no significant difference in allele frequency was observed between the two groups. Therefore, the haplotype AC possibly displayed a considerable reduced risk of non-normozoospermic syndrome.

1. Introduction

Semen quality is an important standard in the evaluation of male fertility. In males, infertility is classified under three major categories, namely, oligozoospermia, asthenozoospermia, and teratozoospermia, based on methods used to assess semen quality. Previous studies showed that at least 30% of male infertility cases are caused by genetic factors, such as Y-chromosome microdeletions (Peterlin et al., 2004), gene mutations, and polymorphisms (Ounis et al., 2014), and biochemical molecular factors, such as testosterone (T) (Oluboyo et al., 2012), follicle-stimulating hormone (FSH) (Babu et al., 2004), luteinizing hormone (LH) (Babu et al., 2004), and inhibin B (Andersson et al., 2004).

Inhibins are heterodimeric gonadal glycoprotein hormones belonging to the transform growth factor-β superfamily; these hormones consist a common inhibin-α subunit (INHA) and an inhibin-βA subunit (INHBA) or inhibin-βB subunit (INHBB), thereby forming inhibin A or inhibin B (Klein et al., 2004). Inhibin subunits are encoded by three separate genes mapped to chromosomes 2q33-36, 2cen-q13, and 7p15-p13 for *INHA*, *INHBB*, and *INHBA*, respectively in humans (Barton et al., 1989). Inhibins biologically function as a suppressor of FSH secretion from the anterior pituitary gland. In males, INHA has been detected in spermatogonia, spermatocytes, early spermatids (Barakat et al., 2008), Leydig cells, and Sertoli cells (Marchetti et al., 2003). Inhibins are also implicated in normal gonadal function, including regulating proliferation, differentiation, and steroidogenesis of Leydig and Sertoli cells via paracrine and autocrine processes (Luisi et al., 2004). In adult males, circulating inhibin levels are correlated with the number of Sertoli cells, total sperm count, and testicular volume (Pierik et al., 1998). In males with testicular dysfunction, serum inhibin B is significantly lower than that in the controls (Toulis et al., 2010).

Given this important role in male reproduction, INHA is a potential candidate gene in male fertility. Several polymorphisms, such as c.-124A>G and c.-16C>T, in the promoter of *INHA* have been reported in premature ovarian failure (POF) (Wood et al., 2009) because this promoter is involved in the negative feedback control of FSH synthesis. In this study, we analyzed the association of the *INHA* promoter sequence variants with male infertility in patients from Northern China by PCR–RFLP analysis.

Abbreviations: T, Testosterone; E, Estradiol; FSH, Follicle stimulating hormone; LH, Luteinizing hormone; INHA, Inhibin-α subunit; INHBA, Inhibin-βA subunit; INHBB, Inhibin-βB subunit; POF, Premature ovarian failure; Azoo, Azoospermia; OA, Oligoasthenozoospermia; AS, Asthenozoospermia; OAT, Oligoasthenoteratozoospermia

* Corresponding authors.
E-mail addresses: 285924940@qq.com (C. Li), xzhou65@vip.sina.com (X. Zhou).

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2. Material and methods

2.1. Patient information

A total of 225 male subjects, including 153 non-normozoospermic and 72 normozoospermic, were randomly recruited from the Reproductive Medical Center of the Second Hospital Affiliated of Jilin University from April 2012 to January 2013. Patients with pathologies of epididymis or vas deferens, cryptorchidism, retrograde ejaculation, and chromosomal abnormalities were excluded from this study. Patients with occupational environmental exposures to potential reproductive toxins or those under treatment with spermatogenesis-impairing medication were also excluded. Semen samples were collected after 3 d to 6 d of sexual abstinence. Semen was analyzed according to World Health Organization (1999) guidelines by computer-aided semen analysis. The samples were divided into two groups: (1) normozoospermia spermiograms (rapid motility > 25% or progression > 50%, morphologically normal sperm > 30%, and concentration > 20 million spermatozoa/mL in fresh semen sample) and (2) non-normozoospermia spermiograms that included males with azoospermia (Azoo; no spermatozoa in the ejaculate; $n = 20$), oligoasthenozoospermia (OA; concentration < 20 million spermatozoa/mL and motility < 25%; $n = 58$), asthenozoospermia (AS; rapid motility < 25% or progression < 50% and concentration > 20 millionspermatozoa/mL; $n = 65$), and oligoasthenoteratozoospermia (OAT; concentration < 20 million spermatozoa/mL, progression < 50%, and morphologically normal sperm < 30%, $n = 10$). Peripheral blood samples were collected to detect plasma hormone levels and to isolate DNA. Plasma hormone levels were determined by chemiluminescent immunoassay (Beckman Coulter Access 2, America). This study was approved by the Ethics Review Board of Jilin University; all of the patients provided informed consent.

2.2. DNA isolation and genotype analysis

Genomic DNA was extracted from blood samples by using Multisource Genomic DNA Miniprep Kit (Sangon Biotech, Shanghai, China) according to the manufacturer's protocol. The DNA samples were dissolved in TE buffer (10 mM Tris–HCl, pH 8.0, and 1 mM EDTA) and stored at −20 °C before use.

c.-124G>A (rs11893842) and c.-16C>T (rs35118453) of IN*HA* polymorphisms were genotyped by PCR–RFLP. The genomic sequence (Gene ID: 3623) was obtained from the NCBI website. The following oligonucleotide primers were used to determine c.-124G>A: forward 5′-AGGTCGCTTGAGGCGAAATCCTTCC-3′ and reverse 5′-TCCCACACCC ACCCTCTTCTACCCTTCTGA-3′ (Wood et al., 2009). The following oligonucleotide primers were used to determine c.-16C>T: forward 5′-TGCGTCAGAGATAGGAGGTCTCA-3′ and reverse 5′-TCCTCCTCTTCC TCG GGCTCA-3′. PCR was performed under the following conditions: preheating at 94 °C for 10 min; 35 cycles at 94 °C for 20 s, 63 °C for 20 s, and 72 °C for 30 s; and a final extension at 72 °C for 5 min. The amplified PCR fragments (196 bp for *INHA* c.-124G>A and 433 bp for *INHA* c.-16C>T) were digested with Sau3AI restriction enzymes (Sangon Biotech, Shanghai, China) for *INHA* c.-124G>A and SpeI restriction enzymes (Sangon Biotech, Shanghai, China) for *INHA* c.-16C>T for 2 h; afterward, these fragments were subsequently identified by electrophoresis on 3% agarose gel. PCR products with different genotypes were then purified using a spin column DNA gel extraction kit (Sangon Biotech, Shanghai, China) and sequenced using ABI 3730X DNA sequence at BGI (BGI, Tianjin, China).

2.3. Statistical analysis

Statistical analysis was carried out with SPSS version 13.0. The significance of age, height, weight, body mass index (BMI), plasma hormones, and semen parameters was analyzed by Student's *t*-test. Normal distribution of continuous parameters was evaluated by the Kolmogorov–Smirnov test. Hardy–Weinberg equilibrium of genotypic distribution was evaluated by chi-square test. Significant difference in genotype distribution and allele frequency in *INHA* polymorphism in normozoospermic and non-normozoospermic groups was analyzed by the Pearson chi-square test or Fisher's exact test. Odds ratios and 95% confidence intervals were calculated using a logistic regression model. Haplotypes were analyzed with SHEsis online (http://analysis.bio-x.cn/myAnalysis.php). The lowest frequency threshold of haplotype analysis was 0.01, that is, all frequencies <0.01 were negligible. Significant differences in rates of smokers and drinkers were also evaluated by the Pearson chi-square test. Statistical significance was accepted at $p \leq 0.05$ (two-tailed).

3. Results

3.1. Patient characteristics

The study population consisted of 72 normozoospermic and 153 non-normozoospermic males. Demographic characteristics and plasma FSH, LH, estradiol (E), and T levels of the participants are described in Table 1. T and FSH levels in semen were significantly higher in non-normozoospermic males (T = 3.98 ± 0.51 ng/mL; FSH = 10.55 ± 1.61 IU/L) than in normozoospermic males (T = 1.51 ± 0.17 ng/mL; FSH = 3.74 ± 0.44 IU/L; $p < 0.01$). No significant differences in age, height, weight, BMI, LH, E, smoking, and drinking were observed between normozoospermic and non-normozoospermic males. Sperm motility (74.91 ± 1.71%) and density (66.45 ± 7.32 × 10^6/mL) were significantly higher in males with normozoospermia than in males with non-normozoospermia (motility = 31.89 ± 2.46%; sperm density = 36.52 ± 6.70 × 10^6/mL; $p < 0.01$).

3.2. Genotype analysis

The direct DNA sequencing results of homozygous and heterozygous variants are shown in Fig. 1. PCR–RFLP was performed to analyze c.-124G>A and c.-16C>T polymorphisms in the *INHA* promoter. The genotypes were analyzed according to differences in fragment length (c.-124G>A: GG 196 bp, GA 196 bp and 164 + 32 bp, and AA 164 + 32 bp, Fig. 2; c.-16C>T: CC 433 bp, CT 433 bp and 186 + 247 bp, and TT 186 + 247 bp, Fig. 3) after these fragments were digested by restriction enzymes. The genotype frequencies of c.-124G>A and c.-16C>T in the *INHA* promoter of normozoospermic and non-normozoospermic males are presented in Table 2. No

Table 1
Baseline demographics, plasma hormones and semen parameters in non-normozoospermic men and normozoospermic men.

Characteristics	Non-normozoospermia	Normozoospermia	p-Value (two-tailed)
Age (year)	32.26 ± 0.51	32.37 ± 0.99	NS
Height (cm)	172.67 ± 4.83	172.85 ± 4.28	NS
Weight (kg)	74.83 ± 11.2	73.58 ± 13.87	NS
BMI (kg/m^2)	25.12 ± 0.39	24.67 ± 0.90	NS
Smoker (%)	52.1	53.8	NS
Alcoholist (%)	51.1	69.2	NS
Plasma hormones			
T (ng/mL)	3.98 ± 0.51	1.51 ± 0.17	<0.01
LH (IU/L)	6.52 ± 0.78	4.36 ± 0.76	NS
FSH (IU/L)	10.55 ± 1.61	3.74 ± 0.44	<0.01
E (pg/mL)	35.88 ± 3.75	36.61 ± 3.10	NS
Semen parameters			
Sperm density (×10^6/mL)	36.52 ± 6.70	66.45 ± 7.32	<0.01
Motility (%motile)	31.89 ± 2.46	74.91 ± 1.71	<0.01

Note: BMI, body mass index; T, testosterone; FSH, follicle stimulating hormone; LH, luteinizing hormone; E, estradiol.
Values are means ± M.S.E. NS = not significant.