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Association Between Polymorphisms in the Angiotensin-Converting Enzyme Gene and Non-Obstructive Azoospermia in the Chinese Han Population from Northeast China

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Background: Genetic defects are commonly observed in infertile males, although the majority of cases remain idiopathic. In recent years, the relationship between single-nucleotide polymorphisms (SNPs) and male infertility has received increasing attention. The objective of this study was to explore the relationship between non-obstructive azoospermia (NOA) and single-nucleotide polymorphisms in the angiotensin-converting enzyme gene (*ACE*) using ligase detection reaction (LDR)-PCR.





Material/Methods: A retrospective study was performed and we screened 4 *ACE* SNPs (rs4316, rs4331, rs4343, and rs4362) in 121 NOA cases and 256 control subjects by LDR-PCR. The relationship between SNPs and NOA was analyzed.

Results: *ACE* SNPs were in Hardy-Weinberg equilibrium ($P=0.089$ for rs4331, $P=0.089$ for rs4343, $P=0.089$ for rs4316, and $P=0.381$ for rs4362). The allelic and genotypic frequencies of the 4 SNPs were not significantly different between cases and controls ($P=0.123$ for rs4331, $P=0.123$ for rs4343, $P=0.123$ for rs4316, and $P=0.179$ for rs4362). Haplotype analysis showed the existence of 3 haplotypes, TGAC, CAGT, and TGAT, which showed statistical significance of 0.889, 0.889, and 0.781, respectively, between cases and controls.

Conclusions: No significant association was found between *ACE* SNPs rs4316, rs4331, rs4343, or rs4362 and NOA in the Chinese Han population of Northeast China.

MeSH Keywords: **Azoospermia • Infertility, Male • Polymorphism, Single Nucleotide**

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Background

Approximately 15% of couples suffer from infertility, and it is estimated that male infertility is the cause in 50% of cases [1]. Male infertility can be attributed to several factors such as the obstruction or absence of seminal pathways, varicocele, endocrinological disorders, cryptorchidism, and infections [2]. Genetic alterations are another cause of male infertility, especially in patients with NOA. NOA caused by a genetic factor lacks effective treatment, but a clear diagnosis would play an important role in genetic counseling for patients experiencing difficulties in selecting an appropriate fertility treatment.

Genetic defects commonly observed in infertile males include karyotypic abnormalities (such as 47,XXY) and microdeletions of the Y chromosome, although the majority of cases remain idiopathic [3]. In recent years, the relationship between single nucleotide polymorphisms (SNPs) and male infertility has received increasing attention [4–6]. We previously carried out high-throughput DNA sequencing of 4 SNPs (rs4331, rs4316, rs4343, and rs4362) in the angiotensin-converting enzyme gene (ACE). This revealed that particular genotypes of the 4 SNPs led to changes in the levels of reproductive hormones or seminal plasma biochemical markers in a case–control cohort from Northeast China [7].

ACE is a key enzyme in the renin–angiotensin system (RAS) that converts angiotensin I to angiotensin II. ACE has 2 isoforms: somatic ACE (sACE) and testis-specific ACE (tACE) [8,9]. ACE SNPs have been associated with the risk of preeclampsia in pregnant women [10], hypertrophic cardiomyopathy [11], and migraine [12]. Moreover, the activity of tACE was also associated with infertility in some studies. Animals lacking ACE further suggest a role for the enzyme in male fertility [13,14], and this is supported by the analysis of human samples [15,16]. Additionally, Zalata et al. [17] reported that ACE deletion is associated with abnormal seminal variables. However, Liao et al. [18] showed that ACE polymorphisms were unlikely to contribute to the pathogenesis of male infertility in the Singapore population.

Therefore, in this study, we further explored whether ACE SNPs rs4316, rs4331, rs4343, and rs4362 were associated with NOA Chinese Han patients from Northeast China.

Material and Methods

Subjects

A total of 121 infertile men were collected from the Center for Reproductive Medicine of First Hospital, Jilin University, China between January 2013 and February 2016. Patients were

diagnosed with NOA by semen analysis and testicular fine needle aspiration cytology. A total of 256 control subjects were randomly selected from sperm donors at the Human Sperm Bank of Jilin Province between October 2014 and November 2015. Peripheral blood G-banding karyotype analysis and Y chromosome AZF microdeletion detection were carried out for all cases, and those with chromosomal abnormalities or AZF microdeletions were excluded. All patients and controls were of Han ethnicity and were from Northeast China. This study was approved by the Ethics Committee of the First Hospital of Jilin University, and all participants provided their written informed consent for participation.

Semen analysis

Samples were obtained as previously described [19]. Semen analysis was performed according to the WHO Laboratory Manual for the Examination and Processing of Human Semen (2010) with the following cutoff points for healthy semen: normal sperm morphology 4%, semen volume 1.5 ml, total sperm count 39×10^6 /eja, sperm concentration 15×10^6 /ml, and sperm total motility 40%.

Evaluation of biochemical markers

Semen samples were centrifuged at $3,000 \times g$ for 15 min to remove spermatozoa and were frozen at -20°C until analysis. The total fructose concentration and neutral alpha-glucosidase in the seminal plasma were evaluated by seminal plasma fructose quantitative assay kit (Indole method) and seminal plasma neutral alpha-glucosidase quantitative assay kit (Enzyme method), respectively (Shenzhen Huakang Co., Ltd., Shenzhen, China). The seminal plasma zinc concentration was measured by seminal plasma zinc quantitative assay kit (modified 5-Br-PAPS) (Shenzhen Huakang Co., Ltd.).

Serum reproductive hormone analysis

The levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone (T), estradiol (E_2), prolactin (PRL), and inhibin B (INHB) were measured by electrochemiluminescence immunoassays using the Elecsys 2010 Chemistry Analyzer (Roche, Mannheim, Germany), based on the manufacturer's instructions.

SNP selection and genotyping

Genotyping was performed by Shanghai BioWing Applied Biotechnology Co., Ltd. (Shanghai, China) using LDR–PCR [20]. Genomic DNA was extracted from ethylenediaminetetraacetic acid-anticoagulated blood using standard protocols. Target DNA sequences were amplified in a multiplex PCR method using primers shown in Table 1. PCR was conducted in a 20 μl

Table 1. Primers of target genes used in the PCR.

Primer name	Sequence (5'-3')		PCR length
rs4331	TGCAGAACACCACTATCAAG	GAGCCACACACCTCCTCCA	96
rs4343	GTGAGCTAAGGGCTGGA	CCAGCCCTCCATGCCATAA	109
rs4316	TTCCTGCTGCTCTGCTACG	TGGGTGACTGTCACCTGTTG	74
rs4362	GGAAGTGGAACTGGATGATG	TGGGGCCAGTGGCACAAG	100

Table 2. Target gene probe sequences of LDR.

Probe name	Sequence (5'-3')	LDR length
rs4331_modify	P-GCTGCCCGTTCTAGGTCCTGTTTTTTTTTTTTTTTTTTTTT-FAM	
rs4331_A	TTTTTTTTTTTTTTTTTTCCTCCAGCTCTGGGCAGGCAGT	82
rs4331_G	TTTTTTTTTTTTTTTTTTCCTCCAGCTCTGGGCAGGCAGC	84
rs4343_modify	P-GTGCCATCACATTCGTCAGTTTTTTTTTTTTTTTTTTTTT-FAM	
rs4343_A	TTTTTTTTTTTTTTTTTTTACAGGTCTCATATTTCCGGGAT	87
rs4343_G	TTTTTTTTTTTTTTTTTTTACAGGTCTCATATTTCCGGGAC	89
rs4316_modify	P-GGGACCAGCAGAGGGTGCCTTTTTTTTTTTTTTTTTTTTTT-FAM	
rs4316_C	TTTTTTTTTTTTTTTTTTTTTCTGTTGGGATGCCTCCTGGCTG	102
rs4316_T	TTTTTTTTTTTTTTTTTTTTTCTGTTGGGATGCCTCCTGGCTA	104
rs4362_modify	P-AAGTACCTGGAGCAGAGCGATTTTTTTTTTTTTTTTTTTTTT-FAM	
rs4362_C	TTTTTTTTTTTTTTTTTTTTTTTGGAACTGGATGAAGCTGACG	112
rs4362_T	TTTTTTTTTTTTTTTTTTTTTTTGGAACTGGATGAAGCTGACA	114

Table 3. Clinical characteristics of control subjects and cases.

Variables	Patients (121)	Controls (256)	P value
Age (years)	30.00±5.69	25.35±5.51	<0.001
BMI (kg/m ²)	25.31±5.13	22.89±3.83	<0.001
pH	7.48±0.41	7.51±0.06	0.314
Sperm density (10 ⁶ /mL)	0	66.88±11.11	<0.001
Sperm volume (mL)	2.74±1.49	3.69±1.29	<0.001
Fructose (μmol/eja)	66.47±57.96	20.40±12.88	<0.001
Alpha-glucosidase (mIU/eja)	41.25±43.95	30.32±18.99	0.004
Seminal plasma zinc (μmol/eja)	7.45±5.31	2.91±2.88	<0.001
FSH (mIU/mL)	19.42±11.62	3.35±1.58	<0.001
LH (mIU/mL)	9.17±5.20	4.96±2.05	<0.001
PRL (μIU/mL)	305.11±168.37	409.28±215.19	<0.001
E ₂ (pg/mL)	35.46±25.32	34.21±11.85	0.520
T (nmol/L)	12.46±6.90	17.72±6.43	<0.001
INHb (pg/mL)	51.90±84.52	200.93±71.27	<0.001

P<0.05 has statistical significance.

Table 4. Genotypes and dominant/recessive model analysis of NOA-associated SNPs.

dbsnp code	Cases (n=121)		Controls (n=256)		OR (95% CI)	P value
	n (%)	MAF%	n (%)	MAF%		
rs4331						
A A	14		42			
A G	61	(153) 63.20	107	(321) 62.70	0.978 (0.712~1.342)	0.889
G G	46		107			
A>G	60 (24.80)		149 (29.10)		0.799 (0.489~1.239)	0.123*
GG+AG/AA	107/14		214/42		0.987 (0.956~1.019)	0.417*
GG/AG+AA	46/75		107/149		/	/
rs4343						
A A	46		107			
A G	61	(153) 63.20	107	(321) 62.70	1.022 (0.745~1.404)	0.889
G G	14		42			
G>A	60 (24.80)		149 (29.10)		0.799 (0.489~1.239)	0.123*
AA+AG/GG	107/14		214/42		0.987 (0.956~1.019)	0.417*
AA/AG+GG	46/75		107/149		/	/
rs4316						
C C	14		42			
C T	61	(153) 63.20	107	(321) 62.70	0.978 (0.712~1.342)	0.889
T T	46		107			
C>T	60 (24.80)		149 (29.10)		0.799 (0.489~1.239)	0.123*
TT+CT/CC	107/14		214/42		0.987 (0.956~1.019)	0.417*
TT/CT+CC	46/75		107/149		/	/
rs4362						
C C	40		96			
C T	63	(143) 59.10	116	(308) 60.20	0.957 (0.700~1.306)	0.780
T T	18		44			
T>C	58 (23.97)		140 (27.34)		1.303 (0.807~2.106)	0.179*
CC+CT/TT	103/18		212/44		0.986 (0.953~1.020)	0.408*
CC/CT+TT	40/81		96/160		0.090 (0.000~)	0.415*

* Binary logistic regression analysis; P<0.05 has statistical significance.

reaction mixture containing 1 × buffer, 50 ng of genomic DNA, 0.6 μl Mg²⁺ (3.0 mmol/l), 2 μl of deoxynucleotide triphosphates (2.0 mmol/l), 2.0 μl of primer mix, 0.3 μl of 1U Taq Polymerase (Shanghai BioWing Applied Biotechnology Co., Ltd), and 12.2 μl of ddH₂O. The reaction was run in a Gene Amp PCR System 9600 thermal cycler (Perkin-Elmer, Norwalk, CT, USA) as follows: primary denaturation at 95°C for 2 min, then 40 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 90 s, and extension at 65°C for 30 s, followed by an extra extension at

65°C for 10 min. Primary information for the probes is shown in Table 2. LDR was performed in a 10 μl reaction mixture containing 1×buffer, 1 μl of 2 pmol/μl each probe mix, 0.05 μl of 2 U of Taq DNA ligase (NEB Biotechnology, Shanghai, China), 4 μl ddH₂O, and 4 μl multi-PCR product. The fluorescent products of LDR were distinguished by an ABI PRISM 377, and the results were analyzed with GeneMapper software (Applied Biosystems, Norwalk, CT, USA). The quality of the genotype was controlled under blinded blood duplications.

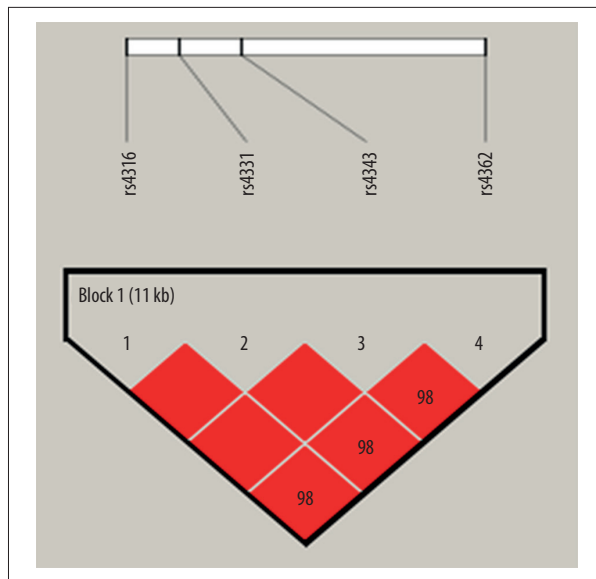


Figure 1. Haplotype analysis of NOA-associated SNPs in chromosome 17. [*ACE* c.81C>T (rs4316), *ACE* c.471A>G (rs4331), *ACE* c.606G>A (rs4343), and *ACE* c.1665T>C (rs4362)]. Red squares indicate a complete chain.

Statistical analysis

Clinical data were assessed using the *t*-test with SPSS Statistics software v. 17.0 (IBM Corporation, Armonk, NY, USA). The Hardy-Weinberg equilibrium (HWE) assumption was determined online using SHEsis Software (<http://analysis.bio-x.cn>) for each SNP in cases and controls separately. Allele frequencies were compared using chi-square analyses and Fisher's exact test (two-sided). SNP genotype frequencies and dominant/recessive model analysis of cases and controls were calculated by logistic regression analysis. *P* values <0.05 were considered to be statistically significant. Haplotype analysis was conducted using SHEsis Software (<http://analysis.bio-x.cn/SHEsisMain.htm>) [21,22]. Linkage disequilibrium analysis was examined using Haploview Software (<http://www.broadinstitute.org/haploview/>).

Results

Clinical data analysis

We identified significant differences in age, body mass index (BMI), sperm concentration, semen volume, seminal FSH, LH, T, fructose, α -glucosidase, zinc, and INHB between cases and controls ($P<0.05$, Table 3). There was no significant difference in semen pH or E_2 between the 2 groups ($P>0.05$, Table 3).

Allele and genotype association analysis

We genotyped the *ACE* SNPs rs4316, rs4331, rs4343, and rs4362 in NOA cases and controls after removing failed genotype samples. Genotype and allele frequencies are summarized in Table 4. All SNPs were in HWE ($P=0.089$ for rs4331, $P=0.089$ for rs4343, $P=0.089$ for rs4316, and $P=0.381$ for rs4362). However, our case-control analysis of SNP data showed no significant differences between cases and controls ($P>0.05$). To further explore the genotypes of NOA-related SNPs, binary logistic regression analysis was used for dominance and recessive model analysis with SPSS v.17.0 software (IBM Corporation, Armonk, NY, USA), NOA pathogenicity as a dependent variable, and genetic model as an independent variable. Table 4 shows that there was no association between the 4 SNPs and cases and controls in recessive and dominant genetic models.

Haplotype analysis

Haploview software was used to analyze the haplotypes of the 4 *ACE* SNPs distributed on chromosome 17 (Figure 1). The SNPs formed a haplotype block with a coverage of 11 kb in the order *ACE* c.81C>T (rs4316), *ACE* c.471A>G (rs4331), *ACE* c.606G>A (rs4343), and *ACE* c.1665T>C (rs4362). Three haplotypes were identified in the block: TGAC, CAGT, and TGAT. A comparison of various haplotype frequencies in cases and controls is shown in Table 5; no haplotype was significantly associated with NOA in the present study.

Discussion

ACE was first identified as a key component of the RAS, and *ACE* on 17q23 encodes 2 isoforms in mammals. *sACE* is expressed

Table 5. Haplotype analysis of NOA-associated SNPs (rs4316, rs4331, rs4343, rs4362).

Haplotype	Cases frequency(%)	Controls frequency(%)	P value
TGAC	59.1	59.8	0.860
CAGT	36.8	36.9	0.971
TGAT	4.1	2.9	0.389

$P<0.05$ has statistical significance.

widely in vascular endothelial cells, the kidney, and brain and is associated with blood pressure regulation [8,13,23]. *sACE* is expressed in testicular Leydig cells, but not expressed in germinal cells [13,24]. In contrast, *tACE* is specifically expressed in post-meiotic germinal cells during spermiogenesis and in mature spermatozoa [25].

Although there is now a body of evidence implicating the RAS in pathophysiologies associated with reproductive function, the overall importance of the RAS in normal reproductive function is unclear [26]. Previous studies using gene knock-out techniques showed that *tACE* played an important role in male fertility [15]. Moreover, *tACE* from healthy functional spermatozoa was reported to have fertilizing ability [23].

The role of ACE gene polymorphism in male infertility patients has been paid attention. A hypothesis has been proposed that SNP polymorphism can lead to changes in the expression of *tACE* gene, leading to fertilization failure [27]. Novel *tACE* polymorphisms were identified in European-Americans and African-Americans [28], while Kucera et al. [29] detected different allele frequencies of ACE insertions/deletions in men with a pathological sperm count compared with men with normal fertility. Additionally, Li et al. [27] reported that the ACE SNP rs4316 might be associated with infertility. However, Liao et al. [18] found no significant difference in the frequency of 5 *tACE*

SNPs between patients and controls from Singapore. In our study, 4 *tACE* SNPs were not found to be associated with NOA.

An interesting phenomenon is that ACE inhibitors improve sperm count and motility in a dose-dependent manner. Infertile males with poor semen quality can be improved by low-dose ACE inhibitors [30]. However, it is not clear whether the effect of this inhibitor is related to changes in *tACE* activity. The change of ACE SNPs in male infertility is still controversial. Hence, these issues deserve further study in the future.

Conclusions

The present study detected no significant differences in genotype and allele frequencies, or haplotype analysis of ACE SNPs rs4316, rs4331, rs4343, or rs4362 between NOA patients and healthy controls in a Chinese Han population from Northeast China. Together, these findings suggest the existence of ethnic differences in the distribution of SNPs, although larger collaborative studies involving other ethnicities are needed to confirm this.

Conflict of interest

None.

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