J Assist Reprod Genet (2014) 31:873–879
DOI 10.1007/s10815-014-0244-x

GENETICS

Genetic study of Hormad1 and Hormad2 with non-obstructive azoospermia patients in the male Chinese population

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Received: 3 March 2014 / Accepted: 24 April 2014 / Published online: 7 May 2014

Abstract
Purpose To evaluate the association of the *Hormad1* and *Hormad2* single nucleotide polymorphisms (SNPs) variants with non-obstructive azoospermia (NOA) in the Chinese population.
Methods In the present study, we assessed 10 single nucleotide polymorphisms (SNPs) of *Hormad1* and *Hormad2* using Sequenom iplex technology in 361 NOA cases and 368 normal controls from Chinese population.
Results We observed no statistical differences in the distribution of allele frequencies. Further genetic model analysis and haplotype analysis also showed no significant difference between the two groups. However, we found that genotype distribution of rs718772 of *Hormad2* was significantly different between the larger testis group (average testis volume ≥10 ml) and the small testis group (average testis volume <10 ml) in the NOA patients (P=0.035).
Conclusions In conclusion, *Hormad1* and *Hormad2* might not be the susceptible genes for the non-obstructive azoospermia in our study population. However, rs718772 of *Hormad2* variant might be associated with testis development in NOA patients.

Keywords Male infertility · *Hormad1* · *Hormad2* · Non-obstructive azoospermia (NOA)

Introduction

Male infertility accounts for approximately half of the infertility cases, of which Klinefelter's syndrome and Y chromosome

Capsule Hormad1 and *Hormad2* polymorphism might not confer risk to NOA.

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Electronic supplementary material The online version of this article (doi:10.1007/s10815-014-0244-x) contains supplementary material, which is available to authorized users.

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microdeletions occupy about 10 % of male infertility [1, 2]. Non-obstructive azoospermia (NOA) is a severe phenotype of impaired spermatogenesis, only a few patients can benefit from intracytoplasmic sperm injection (ICSI) and finally get their babies. As the causes of NOA remain largely unknown, the genetic risk factors cannot be ignored [2].

HORMA (Hop1, Rev7 and Mad2) domain-containing proteins regulate interactions between homologous chromosomes during meiosis in a wide range of eukaryotes and mammals [3–5]. *Hormad1*, a novel germ cell-specific HORMA domain encoding gene, is a critical component of the synaptonemal complex (SC) and plays vital roles during chromosome synapsis, recombination, meiotic sex chromosome inactivation (MSCI) and transcriptional silencing [6, 7]. *Hormad1* is essential for mammalian gametogenesis as knockout male and female mice lead to infertile [8, 9]. *Hormad1* deficient testis exhibits meiotic arrest in the early pachytene stage. Another meiosis-specific HORMA domain protein, *Hormad2*, is essential for synapsis surveillance during meiotic prophase via the recruitment of ATR (Ataxia Telangiectasia and Rad3) activity [10, 11]. Unlike *Hormad1*, *Hormad2* deficient mice enable infertile only in males. This tip reminds us that *Hormad2* may play a particular role in male infertility. Specially, in the animal model, the weight of the *Hormad2* deficient testis was much lower than that of the wild-type [10, 12].

An impressive association study inquiring into single nucleotide polymorphisms (SNPs) of *Hormad1* in the infertile men was recently available [13]. By direct sequence analysis, the authors detected 3 polymorphisms in the coding regions of *Hormad1* in 30 Japanese men with azoospermia and 80 normal fertile men. They confirmed the two SNPs (c.163A > G and c.501 T > G) were associated with meiotic arrest.

Here, we hypothesized that HORMA domain genes may play a role in the Chinese NOA patients. So we performed a genetic case–control study of *Hormad1* and *Hormad2* in a large cohort of the Chinese population.

Materials and methods

Subjects and sample collection

This study was a hospital-based case–control study. All the infertile men of NOA (n=361) were collected from the First Affiliated Hospital of Anhui Medical University between March 2008 and May 2013. At least repeating two detections for semen samples from each patient were performed to confirm the diagnosis of azoospermia. It was defined as azoospermia when no sperm was found after centrifugation at 3,000 g for 15 min [14]. All the patients were selected based on a comprehensive andrological examination, including medical history, physical examination, semen analysis, hormone analysis, karyotype and Y microdeletion analysis. Patients with history of orchitis, epididymitis, maldescensus of testis, mono or bilateral cryptorchidism, varicocele, hypogonadotrophic hypogonadism, obstruction or absence of vas deferens, chromosomal abnormalities and Y micro-deletions were carefully excluded from this study.

The control group consisted of 368 normozoospermia men who had fathered at least one healthy child. All control donors were drawn from the same hospital where the patients were recruited. The semen analysis for sperm concentration, motility, and morphology was performed following the criterion in the fifth edition of the World Health Organization manuals [14].

Ethics statement

All patients provided written informed consent for the collection of samples and subsequent analysis. This study was conducted in accordance with the tenets of the Declaration of Helsinki and its amendments and approved by the ethics committee of Anhui Medical University (ECAMU No 2008035).

Methods

Extraction of peripheral blood DNA

Blood sample was collected from each subject and stored at −80 °C. Genomic DNA was extracted from the blood using QIAamp DNA Blood Midi Kit (Qiagen Inc, Germany), according to the manufacturer's protocol. The quantitative concentration of DNA was measured by the Nanodrop Spectrophotometer (ND-2000, USA) of full wavelength and standardized to 15–20 ng/μl.

SNP selection and sequenom MassARRAY

Ten tag SNPs of *Hormad1* and *Hormad2* were selected by Haploview software,including 3 SNPs of *Hormad1* (rs1336900 A/G, rs16840074 C/T and rs6694531 A/C) and 7 SNPs of *Hormad2* (rs8135823 G/T, rs11090601 C/A, rs4823073 A/G, rs718772 A/G, rs9620953 T/C, rs9625930 A/G and rs975704 A/G). The 10 candidate SNPs in this study were relatively common SNPs with a minor allele frequency ≥5 % and selected from the HapMap database of the Chinese Han population. Genotyping analysis of the SNPs for fast-track validation analysis was performed using the Sequenom MassArray system. Locus-specific polymerase chain reaction (PCR) and detection primers were designed using the MassARRAY Assay Design 3.0 software (Sequenom, San Diego, California) following the manufacturer's instructions. The DNA samples were amplified by multiplex PCR. The PCR products were then used for locus-specific single-base

Table 1 The Characteristic of the Study Population./: no data available for the controls; Student's *t* test was used for the data comparisons in the cases and controls. *: indicated to be significant compared with the controls ($P<0.05$)

Characteristic	Controls	Cases
Age range (yr)	20–53	19–43
BMI	23.01±3.11	22.91±2.95
Right testicle volume (mL)	14.58±2.65	8.90±3.51*
Left testicle volume (mL)	14.51±2.70	8.95±3.55*
FSH	/	21.42±19.88

extension reactions. The resulting products were desalted and transferred to a 384-element Spectro-CHIP array. Allele detection was performed using MALDI-TOF mass spectrometry. The mass spectrograms were analyzed by the MassARRAY Typer software (Sequenom). The SNP detected with call rate lower than 90 % in the cases and the controls were excluded.

Serum FSH and testis volume calculate

Peripheral blood samples were drawn for hormone measurements. Serum FSH measurement was performed with a commercially available Electro-Chemiluminescence Immunoassay system (Roche Diagnostics, Germany). According to the normal range of FSH level (1.5–12.4 mIU/mL), we devided the patients into two subgroups, high FSH group and normal FSH group.

In order to analysis the SNP variants with testis development, we devided all the cases into two subgroups according previous studies about the testis volume [15, 16]. Large testis group: mean testis volume larger than 10 ml, small testis group: mean testis volume less than 10 ml. All the testis volume of the controls are lager than 10 ml.

Statistical analysis

The Hardy-Weinberg equilibrium (HWE) was determined for the controls and study groups. Chi-squared test was used for the genotype distributions in the controls and study groups. When the number of genotype were ≤5, Fisher exact test was used. In addition, the genetic models (codominant, dominant and recessive) were analyzed for the detected SNPs. The *P* values reported in the study were based on two-sided probability test with a significance level of $P<0.05$. The statistical software plink v1.07 (http://pngu.mgh.harvard.edu/purcell/plink/) [17] and Stata/SE version 10 were used for statistical analyses (Stata Corp LP, College Station,TX).

Result

Characteristics of the study population

The age of the NOA patients and the controls ranged from 19 to 43 years old (28.82±4.26) and 20 to 53 years old (31.22±5.12), respectively. Main clinical characteristics of the 361 patients of NOA and the 368 controls were shown in Table 1. The testis volume in the NOA group was significantly smaller than that in the controls ($P< 0.05$).

Polymorphisms of the genes and risk of NOA

The genotype distributions of all the SNPs did not deviate from the HWE ($P> 0.05$) in the controls. The minor allele frequency of the 10 SNPs in the cases and the controls were shown in Table 2. We did not find any significant differences between the cases and the controls ($P>0.05$). Furthermore, we summarized the genotype model analysis of the 3 *Hormad1*

Table 2 Minor Allele Frequencies of *Hormad1* and *Hormad2* polymorphisms in the infertility cases and the controls. SNP:single nucleotide polymorphism; MAF: Minor allele frequency; HWE: Hardy-Weinberg equilibrium. HWE and the distribution of MAF were analyzed by plink 1.07 software. No significant difference was found in the comparisons

Gene	SNP rs#	Alleles	Position	HWE		MAF		*P*	OR (95%CI)
				Controls	Cases	Controls	Cases		
Hormad1	rs1336900	A/G	Exon	0.906	0.139	0.332	0.367	0.15	1.171[0.943–1.454]
Hormad1	rs16840074	C/T	Intron	0.297	0.874	0.227	0.212	0.456	0.909[0.708–1.168]
Hormad1	rs6694531	A/C	Intron	0.523	0.587	0.437	0.412	0.518	0.933[0.757–1.150]
Hormad2	rs8135823	G/T	Intron	0.157	0.737	0.090	0.086	0.773	0.948[0.659–1.363]
Hormad2	rs11090601	C/A	Intron	0.802	1	0.119	0.121	0.907	1.019[0.742–1.399]
Hormad2	rs4823073	A/G	Intron	0.181	0.224	0.314	0.316	0.932	1.010[0.809–1.260]
Hormad2	rs718772	A/G	Intron	0.628	0.187	0.122	0.113	0.577	0.913[0.663–1.258]
Hormad2	rs9620953	T/C	Intron	0.526	0.737	0.092	0.086	0.717	0.935[0.651–1.343]
Hormad2	rs9625930	A/G	Intron	1	0.337	0.083	0.090	0.644	1.091[0.754–1.579]
Hormad2	rs975704	A/G	3' UTR	0.409	0.105	0.108	0.112	0.809	1.041[0.750–1.446]

Table 3 Distribution of Genotypes for the *Hormad1* and *Hormad2* variants in the cases and controls. Fisher exact test was used when the number of genotype were ≤5. For others, chi-squared test was used. No significant difference was found in the comparisons ($P>0.05$)

Gene/SNPs	Genotype	Cases	Controls	OR [95%CI]	*P*
Hormad1					
rs1336900	GG	150(0.419)	161(0.445)	1	
	AG	153(0.427)	162(0.448)	1.014[0.741–1.387]	0.936
	AA	55(0.154)	39(0.108)	1.514[0.949–2.414]	0.099
	AG + AA	203(0.581)	201(0.555)	1.084[0.806–1.457]	0.593
rs16840074	TT	222(0.620)	219(0.607)	1	
	CT	121(0.338)	120(0.332)	0.995[0.727–1.362]	0.974
	CC	15(0.042)	22(0.061)	0.673[0.340–1.331]	0.305
	CT + TT	136(0.380)	142(0.393)	0.945[0.700–1.276]	0.759
rs6694531	CC	117(0.329)	112(0.308)	1	
	AC	179(0.503)	186(0.511)	0.921[0.662–1.282]	0.673
	AA	60(0.169)	66(0.181)	0.870[0.563–1.345]	0.580
	AC + AA	239(0.671)	252(0.692)	0.908[0.663–1.242]	0.576
Hormad2					
rs8135823	TT	301(0.836)	303(0.830)	1	
	TG	56(0.156)	58(0.159)	0.972[0.651–1.450]	0.919
	GG	3(0.008)	4(0.011)	0.755[0.168–3.402]	1
	TG + GG	59(0.164)	62(0.170)	0.958[0.648–1.416]	0.843
rs11090601	AA	277(0.772)	282(0.773)	1	
	AC	77(0.214)	79(0.216)	0.992[0.696–1.415]	1
	CC	5(0.014)	4(0.011)	1.273[0.338–4.789]	0.750
	AC + CC	82(0.228)	83(0.227)	1.006[0.711–1.423]	1
rs4823073	GG	174(0.482)	166(0.455)	1	
	AG	146(0.404)	169(0.463)	0.824[0.606–1.120]	0.241
	AA	41(0.114)	30(0.082)	1.304[0.778–2.186]	0.361
	AG + AA	187(0.518)	189(0.545)	0.944[0.704–1.266]	0.709
rs718772	AA	286(0.794)	280(0.767)	1	
	AG	67(0.186)	81(0.222)	0.810[0.563–1.164]	0.269
	GG	7(0.019)	4(0.011)	1.713[0.496–5.917]	0.545
	AG + GG	74(0.216)	85(0.233)	0.887[0.625–1.258]	0.533
rs9620953	CC	300(0.836)	302(0.827)	1	
	CT	56(0.156)	59(0.162)	0.955[0.641–1.424]	0.839
	TT	3(0.008)	4(0.011)	0.755[0.168–3.402]	1
	CT + TT	59(0.164)	63(0.173)	0.943[0.639–1.392]	0.843
rs9625930	GG	289(0.823)	304(0.840)	1	
	AG	61(0.174)	56(0.155)	1.146[0.770–1.704]	0.544
	AA	1(0.003)	2(0.006)	0.526[0.047–5.832]	1
	AA + AG	62(0.177)	58(0.161)	1.124[0.759–1.392]	0.843
rs975704	GG	288(0.798)	292(0.800)	1	
	AG	65(0.180)	67(0.184)	0.984[0.674–1.436]	1
	AA	8(0.022)	6(0.016)	1.352[0.463–3.945]	0.602
	AG + AA	73(0.202)	73(0.200)	1.014[0.705–1.458]	1

SNPs (rs1336900, rs16840074, rs6694531) in the cases and the controls, but there is still no significant difference were found (Table 3). Similar results were obtained when the 7 SNPs of *Hormad2* (rs8135823, rs11090601, rs4823073, rs718772, rs9620953, rs9625930, rs975704) were interrogated (Table 3). We calculated the power of the sample size with Quanto software (http://hydra.usc.edu/gxe). The power calculation of all the SNP detected reached the research need (>0.80).To address potential combination effects of the 10 SNPs, we performed the further haplotype analysis but failed to find any difference between the two groups, either (Supplement Table S1 and Supplement Table S2).

Table 4 Stratified Association Analysis of the SNPs of *Hormad1* and *Hormad2* by clinical subgroups. Chi-squared test was used for the association analysis. *: Significant difference when compared between the subgroups($P<0.05$)

		Testis Volume			FSH level		
SNP	genotype	<10 ml	≥10 ml	*P*-value	Normal	High	*P*-value
rs1336900				0.09			0.456
	AA	25	22		25	8	
	AG	78	56		62	31	
	GG	58	71		30	34	
rs168400				0.902			0.955
	CC	7	8		7	3	
	CT	55	49		46	24	
	TT	99	92		94	46	
rs6694531				0.305			0.483
	AA	24	27		23	15	
	AC	75	78		74	39	
	CC	60	44		48	19	
rs718772				0.035*			0.58
	AA	134	117		123	57	
	AG	28	27		23	13	
	GG	0	6		3	3	
rs8135823				0.155			0.455
	GG	0	3		1	2	
	GT	22	24		21	10	
	TT	140	123		127	61	
rs11090601				0.507			0.081
	AA	129	112		119	53	
	AC	30	26		29	18	
	CC	2	2		0	2	
rs4823073				0.635			0.557
	AA	19	16		19	12	
	AG	62	65		56	30	
	GG	82	69		74	31	
rs9620953				0.159			0.459
	CC	139	123		126	61	
	CT	22	24		21	10	
	TT	0	3		1	2	
rs9625930				0.462			0.26
	AA	1	0		0	1	
	AG	24	28		24	15	
	GG	131	121		121	56	
rs975704				0.123			0.885
	AA	7	1		6	2	
	AG	30	27		29	14	
	GG	126	122		114	57	

Association of the SNP variants with clinical characteristics of patients

We investigated the relationship between the SNP variants and the clinical phenotype (testis volume and FSH level) in the NOA patients. We failed to find any difference of genotype distribution of the 10 detected SNPs between the high FSH group and the normal FSH group. Interestingly, we found that the genotype distribution of rs718772 of *Hormad2* was significantly associated with testis volume ($P=0.035$) (Table 4). The individuals with homozygosis GG genotype of rs718772 had lower risk susceptibility to smaller testes in the NOA subgroup ($P=0.011$) (Table 5). But no significant difference was found when compare the small testis group with controls

Table 5 Distribution of genotypes for rs718772 in subgroups of testes volume. Codominant, DD versus Dd and DD versus dd; dominant model, DD versus (Dd, dd); recessive model (DD,Dd) versus dd; d, minor allele; D, major allele. Fisher exact test was used when the number of genotype were ≤5. For others, chi-squared test was used. *: Significant difference when compared between the subgroups ($P<0.05$)

Model	Genotype	<10 ml in NOA	>10 ml in controls				>10 ml in general population	
		n(%)	*n*(%)	*P*	*n*(%)	*P*	*n*(%)	*P*
Codominant	AA	134 (0.827)	117 (0.780)		280 (0.767)		397 (0.771)	
	AG	28 (0.173)	27 (0.180)	0.767	81 (0.222)	0.201	108 (0.210)	0.313
	GG	0 (0.000)	(0.040)	0.011	4 (0.011)	0.311	10 (0.019)	0.130
Dominant	AA	134 (0.827)	(0.780)		280 (0.767)		397 (0.771)	
	AG + GG	28 (0.173)	33 (0.220)	0.319	85 (0.233)	0.135	118 (0.229)	0.154
Recessive	GG	0 (0.000)	(0.040)		4 (0.011)		10 (0.019)	
	AA + AG	162 (1.000)	144 (0.960)	0.012	(0.989)	0.318	505 (0.981)	0.129

(All the testis volume of the controls were lager than 10 ml) or the general larger testis group (Testis volume were larger than 10 ml in both NOA and controls).

Discussion

Spermatogenesis is a complex process involving mitosis of spermatogonia, two meiotic divisions of spermatocytes and a series morphology change from round spermatids into mature spermatozoa. This process is highly regulated by a train of genes [18]. Increasing evidences indicated that genetic variations or susceptibility might play a crucial role in male infertility [19–21]. The study in mice demonstrated that HORMA domain genes were required for DNA double strand break repair and chromosome synapsis, recombination during meiosis, thus *Hormad1* and *Hormad2* were considered as two candidate genes in conferring NOA risk.

Only one genetic association study of *Hormad1* gene with regard to susceptibility of NOA had been available so far. In this small cohort study of the Japanese population, the authors stated that two SNPs of *Hormad1* (c.163A > G and c.501 T > G) might be associated with human azoospermia [13]. In our study, we tested 10 tag SNPs in the *Hormad1* and *Hormad2* in 361 NOA cases and 368 normal controls with Sequenom iplex technology, but failed to find any tested SNPs were associated with NOA patients in the Chinese population. The disputed conclusion might be attributed to the following reasons. Firstly, SNP and haplotype frequencies can vary widely between different ethnic groups and the specific subtype groups of NOA [2]. Moreover, in this study, we recruited a larger number of the NOA cases than the previous investigation, it would present more statistical power. It also should be mentioned that the excluding criteria such as chromosome abnormalities and Y chromosome microdeletion would interface the study results.

In order to confirm whether GG genotype of rs718772 had lower risk susceptibility to smaller testis in the only NOA patients group or general population (NOA patients group and control group), we compared the genotype distribution of rs718772 in the small testis group of NOA with the the larger testis group of NOA, as well as the control group and the testis volume >10 ml in general population (NOA patients group and control group). Interestingly, genotype GG of rs718772 was only found in the population with the testis volume larger than 10 ml. The genotype distribution was only statistically different in the subgroup of NOA patients, no significant difference was found when compare the small testis group with controls or the general larger testis group. Taking account of the results together, we inferred that GG genotype of rs718772 might play a protective role in testis development particularly in NOA patients. As animal model data showed that the weight of the *Hormad2*-deficient testis was <1/3 of the wild-type testis [10], we infer that *Hormad2* may play a role in testis development in the NOA patients. As rs718772 is an intronic SNP which is located between exon5 and exon 6, we applied the NetGene2 program (www.cbs.dut.dk/services/NetGene2) and Spidey program (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey) to evaluate whether this SNP could affect splicing activity. The data of NetGene2 and Spidey analysis showed that this SNP does not change any conserved nucleotides of the branch site in intron 5 or affect splicing activity. Therefore, we presumed that rs718772 might be in linkage disequilibrium with other mutations or variations that could play a role in testis development of NOA patients.

Clinically, surgical sperm retrieval (SRR) in combination with ICSI offered a desirable probability for the NOA patients who were eager to father a child. However, sperm retrieval rates were associated with distinct pattern of prognostic factors [22, 23]. Previous studies indicated that testicular sperm retrieval rates were poor in NOA patients with small testicular volume and elevated FSH levels [24, 25]. In combination with our findings, we hypothesize that GG genotype of rs718772 might become an additional valuable molecular predictive

marker for assessing the sperm retrieval rate of NOA patients. Obviously, further studies were needed to confirm this hypothesis.

In conclusion, this was the first study investigating the genetic susceptibility and clinical relationship between HORMA domain genes (*Hormad1* and *Hormad2*) with Chinese idiopathic NOA. Our data indicated that genetic variants of *Hormad1* and *Hormad2* might not confer risk to NOA in our study population, *Hormad2* variants rs718772 might affect the testis development. However, additional functional research on the variant is needed to clarify the biological mechanism of *Hormad2* in human testis development.

Acknowledgements This work was supported by the National Natural Science Foundation of China (81300538).The authors thank all the men for their cooperation, and thank the clinicians who provided these patients.

Declaration of interest The authors report no declaration of interest.

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