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Original Article

Possible involvement of single nucleotide polymorphisms in anti-Müllerian hormone signaling pathway in the pathogenesis of early OHSS in Han Chinese women

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Abstract: To investigate the possible relationship between single nucleotide polymorphisms (SNPs) in the anti-Müllerian hormone (AMH) signaling pathway and the incidence of early OHSS, the genomic DNA was isolated from peripheral blood leukocytes of 122 participants (62 patients with early OHSS and 60 patients without OHSS who underwent IVF/ICSI), and SNPs of the AMH and AMHR2 exons were detected directly. Further more, genotype distribution and allele frequency were analyzed. We found seven types of SNPs in the AMH exons, and two of them were missense mutations (rs10407022 and rs182295886). However, these two missense mutations did not increase the risk of early OHSS (rs10407022, *P*=0.307, OR=1.552, CI 0.668, 3.608; rs182295886, *P*=0.442, OR=0.359, CI 0.026, 4.883). While it was observed that participants with the SNP (rs10407022) had a relatively higher ovarian response than those without the SNP. Further more, we did not find any SNPs in exons of AMHR2. In conclusion, we analyzed the pathogenesis of OHSS by first investigating the SNPs in the AMH signaling pathway. There is no association between SNPs in the AMH/AMHR2 signaling pathway and early OHSS in Han Chinese women.

Keywords: Ovarian hyperstimulation syndrome (OHSS), AMH, AMHR2, single nucleotide polymorphisms (SNPs), controlled ovarian hyperstimulation (COH), assisted reproductive technologies (ART)

Introduction

Ovarian hyperstimulation syndrome (OHSS) is a relatively common iatrogenic complication of controlled ovarian hyperstimulation (COH), which is a part of assisted reproductive technologies (ART) [1]. It is characterized by sudden bilateral ovarian enlargement and an acute shift of intravascular fluid into the third space. The severe form of OHSS is potentially lethal and leads to hospitalization in 0.1%-2% of IVF cases [2, 3].

Presently, the pathogenesis of early OHSS is still not completely understood. It seems that early OHSS primarily results from vasoactive peptides that are released by the large number of granulosa cells (GCs) in hyperstimulated ovaries caused by exogenous Gn and triggered by exogenous human chorionic gonadotropin (hCG) [4]. However, why are these ovaries of OHSS patients hypersensitive to exogenous gonadotropins and so easily to be hyperstimulated? This is still remains unknown.

Anti-Müllerian hormone (AMH) is a member of the transforming growth factor-β (TGF-β) superfamily of growth and differentiation factors [5, 6]. The AMH signaling pathway was first studied for its regulatory role in male sex differentiation [7, 8]. During female fetal development, AMH is responsible for the regression of the Müllerian ducts, from which the female genital tract develops [9]. In recent years, it has been found to play an important role in ovarian functioning [10-12].

At present, both animal and human studies indicate that AMH is highly expressed by the GCs of developing follicles from the early primary stage to the early antral stage (up to 6 mm in diameter) [13]. During follicular development, AMH exerts the following effects [6]: i) the inhibition of the initial recruitment of follicles, preventing primordial follicles from entering the growing follicle pool from the resting states; ii) the attenuation of the sensitivity of the growing follicles to follicle-stimulating hormone (FSH), thus inhibiting FSH-induced follicle development and dominant follicle selection; iii) the inhibition of the growth of GCs in follicles; and iv) the attenuation or inhibition of the aromatase activities and estradiol (E_2) production in the follicles. A recent study demonstrated that in human granulosa-lutein cells, AMH acts through AMHR2 to inhibit FSH-induced adenylyl cylase activation, aromatase expression, and E_2 production [14]. As a result, AMH serves as a suppressive regulator to ensure the balance between the promotion and inhibition of follicle growth and development.

There are two kinds of AMH receptors; the AMH type 2 receptor (AMHR2) is highly specific, while the identity of AMH type 1 receptor remains unclear [15]. The length of the human AMH gene is only 2.75 kbp, and this gene consists of 5 exons. AMHR2 has a total length of 8.7 kbp, and its gene consists of 11 exons. We hypothesize that the SNPs of AMH and its receptors could affect the biological activities and signal transduction of hormones, which play important roles in controlling follicle recruitment and development. As a result the above balance may be disrupted, increasing the sensitivities of ovaries to exogenous gonadotropins during ART. Consequently, the patient will be more likely to have OHSS.

Therefore, this study was designed to investigate the association between SNPs of AMH/AMHR2 and early OHSS by sequencing all 5 exons of AMH and all 11 exons of AMHR2 and analyzing the possible clinical significance of genetic variations.

Methods and methods

Patients

All patients undergoing in-vitro fertilization/intra-cytoplasmic sperm injections (IVF/ICSI) from March 2013 to March 2014 at the Reproductive Medicine Center of Tongji Hospital, Huazhong University of Science and Technology, China, were eligible to be included in this study.

The inclusion criteria for this study were as follows: (i) patients who had undergone a first cycle of IVF/ICSI using fresh embryos during the study period; (ii) those ranging in age from 20-35 years; (iii) those whose body mass indices (BMI) ranged from 18.5-25 kg/m^2; (iv) those who were non-smokers (smoking has been proven to reduce the number of retrieved oocytes [16], and serum AMH has been demonstrated to be lower in smokers compared with non-smokers [17]); (v) those who had not been diagnosed with polycystic ovarian syndrome (PCOS) (some SNPs in AMH have been found to be associated with the risk of PCOS, and thus these patients should be excluded [18]) and those with antral follicular count (AFC) between 8 to 20; (vi) those who had been experiencing infertility for less than 10 years; (vii) those with normal basal hormone levels (including FSH, luteinizing hormone (LH), E_2, prolactin (PRL), and testosterone (T)); (viii) those undergoing a long gonadotropin releasing hormone agonist (GnRH-a) protocol for pituitary down-regulation; and (ix) those taking less than 250 IU of gonadotropin per day.

According to their clinical characteristics, we divided these patients into two groups early OHSS group and non-OHSS group. Diagnosis and classification of OHSS were according to the latest criteria [2]. The peripheral blood of the participants was drawn for investigation. A total of 62 patients with early OHSS and 60 controls (non-OHSS patients) were recruited. All were of Han nationality going back three generations without minority ancestry.

Ethical approval

This study was approved by the medical ethics board of the Tongji Hospital of Huazhong University of Science and Technology, and we received informed consent from all participants.

Ovarian stimulation protocol

The patients undergoing IVF/ICSI had received a long protocol using the triptorelin acetate

Table 1. Primer sequences, annealing temperatures, and sizes of amplicons for AMH and AMHR2

Gene exon	Forward primer (5'-3')	Reverse primer (5'-3')	Ann temp (°C)	Size (bp)
AMH1 (1)	ACCTCAGCACCCAGGACATC	GGAGGCCAGTCCAAGTCTTC	56	478
AMH1 (2)	ACCTTCCACTCGGCTCACTT	ACCCGCAACCCAGTCCTA	57	607
AMH2	TGCAGGAAGGCAGCTAAGAG	CCCATCGCCACTGGTTCTA	56	390
AMH3-4	TTGTTGCAGGGTCTGCAGTA	GTGGCGACTCCTCGAGTTC	57	596
AMH5 (1)	CCGCTCTCAACTCCTCCAA	TGTTGGCCTGGTAGGTCTCG	60	667
AMH5 (2)	AGCGCTGCTGCTCCTGAA	GAGAGACAGCGAGCGCTACA	58	627
AMHR2 1	CCCTTTCTTTCCCTGCTTTC	GCCCACTCACCACTCTTCCA	58	356
AMHR2 2	CCATACTGACGCTGGGATGT	TTCTTTCCCACCAGATGTTCTA	56	407
AMHR2 3	AGGGACGCCTCTGATAGAGAA	TCACCAGGGCAACCAACTT	56	438
AMHR2 4-5	AAGTTGGTTGCCCTGGTGAC	GCCCAAACCACTGCATGA	56	702
AMHR2 6	GAGCTGCCTGAGCTGTGTTTC	ATCGAAAGCAGAAGCAATCAAC	56	476
AMHR2 7	GGCCAGCATCCATCAGTGTG	CCCGGCTCTTCCTCCCTCT	56	354
AMHR2 8	CCCACAGGCCAATATAAACC	CTCTGGTGCCATGTACCTCTG	56	416
AMHR2 9-10	GCATTTGGGACATTGCTGAG	CCAGTTCCAGCTGAGCATGA	56	704
AMHR2 11	GAGGCTCCAGGAAAGATCA	CCCAAGTCACTGGTCAGAAC	56	578

Table 2. Clinical characteristics of all participants after pituitary down regulation and ovarian hyperstimulation

	OHSS group (n=62)	Control group (n=60)	*P*-value
E_2 after down regulation (pg/ml)*	24.80±8.84	22.40±8.31	0.43
LH after down regulation (mIU/ml)*	1.34±0.65	1.42±0.68	0.55
FSH after down regulation (mIU/ml)*	3.33±0.98	3.54±1.00	0.29
P after down regulation (ng/ml)*	0.32±0.16	0.38±0.29	0.18
Days of gonadotropin (Gn) injection	9.5±1.4	10.0±2.0	0.13
Total dose of Gn (IU)	1554.0±415.4	2041.0±673	<0.001
Daily dose of Gn (IU)	162.6±34.8	202.9±49.7	<0.001
E_2 level on the hCG day (pg/ml)	7449.6±3187.3	4070.1±2236.2	<0.001
Number of large follicles (>14 mm)	16.8±4.2	11.1±4.6	<0.001
Number of oocytes retrieved	21.7±5.8	12.2±5.0	<0.001

Data are presented as the means±standard deviation; E_2, estradiol; LH, luteinizing hormone; FSH, follicle stimulating hormone; P, progesterone; hCG, human chorionic gonadotropin. *The serum hormone levels were measured after 14 days of pituitary down regulation.

(Diphereline, IPSEN Pharma Biotech, France or Decapeptyl, Ferring, Switzerland) at a dosage of 0.1 mg daily commencing from day 20 or 21 of the previous menstrual cycle for pituitary down-regulation; the dose was reduced to 0.05 mg once adequate down-regulation was achieved. Adequate pituitary suppression was confirmed by serum E_2 levels <30 pg/ml and serum LH levels <2 mIU/ml. After 10-14 days, ovarian stimulation was performed using recombinant FSH (Gonal-F, Serono, Switzerland or Puregon, Organon, Netherlands) starting with 150-225 IU/d; the dosages were adjusted individually according to ovarian responses as assessed by B-ultrasound and serum hormone levels on the following days. When at least two leading follicles reached mean diameters of 18 mm, the patients received recombinant HCG (Serono, Switzerland). Oocyte retrieval was performed by transvaginal ultrasound-guided needle aspiration at 34-36 h after HCG triggering. The oocytes were fertilized using routine techniques for IVF/ICSI, and 2 embryos were typically transferred on day 3 following oocyte retrieval. High-quality embryos that were not transferred were cryopreserved. For luteal phase support, 60 mg of progesterone (P) was used daily beginning on day of OPU.

Table 3. Details of genotyping assays

Gene	NCBI SNP reference	SNP	Position	Major allele	Minor allele	MAF	Type	Reported frequency
AMH	rs10407022	365 g>t	Exon1	T	G	0.327	mis	0.19
	rs61736572	471 g>a	Exon1	A	G	0.057	syn	-
	rs147472740	519 c>y	Exon1	T	C	0.002	syn	-
	rs61736575	522 g>a	Exon1	A	G	0.062	syn	-
	rs182295886	535 g>r	Exon1	A	G	0.004	mis	-
	rs17854573	765 g>r	Exon2	A	G	0.098	syn	-
	rs7252789	1458 t>a	Exon5	A	T	0.069	syn	-

DNA preparation and genotyping assay

In total, 122 women were included in this portion of the study after their first IVF/ICSI procedure (including 62 OHSS patients and 60 non-OHSS patients). Genomic DNA was isolated from peripheral blood leukocytes according to standard procedures using a PureLink® Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) and stored at -80°C. DNA was amplified by PCR using specific primers for the AMH and AMHR2 genes (Life Technologies™, Carlsbad, CA, USA) (**Table 1**). PCR was performed in a 25 μl total volume containing 2.5 μl 10× PCR buffer, 0.5 μl 10 mM dNTPs, 0.8 μl 50 mM $MgCl_2$, 1 μl 5 μM of each primer, 0.2 μl Platinum® Taq DNA polymerase (Life Technologies™, Carlsbad, CA, USA), 1 μl DNA, and 18 μl ddH_2O. The PCR conditions were as follows: one cycle of 95°C for five min; 37 cycles consisting of 95°C for 30 s, annealing temperatures according to **Table 1** for 30 s, 72°C for 45 s; and a final cycle of 72°C for 5 min. The amplicons were sequenced using an ABI 3730XL automatic sequencer (Life Technologies Corporation, CA, USA).

Hormonal and biochemical measures

We collected blood samples from all subjects on day 3 of their menstrual cycles and on the hCG day. Serum levels of E_2, LH, FSH, P and T were determined by electrochemiluminescence immunoassay using the ADVIA Centaur XP immunoassay system (Siemens, Germany) according to the manufacturer's instructions.

Statistical analyses

Distributions of demographic characteristics between the cases and controls were examined with Student's *t* test for continuous variables with normal distributions, the Mann-Whitney U-test for continuous variables with non-normal distributions and the chi-squared test for categorical variables. Potential associations between the SNPs found and the state of OHSS were assessed by computing odds ratios (ORs) and their 95% confidence intervals (CIs) using binary logistic regression analyses. Trends in association between the risk of OHSS and the SNPs found were estimated and adjusted for age, BMI, AFC, basal serum level of FSH, LH, E_2 and T using logistic regression. The genotype distribution was tested for its adherence to Hardy-Weinberg equilibrium using the online tool described by Rodriguez et al. The sample size was determined, and a power analysis was performed using the Power and Sample Size Calculation (version 3.0) (Dupont and Plummer, 1990). All analyses were performed using the Statistical Package for Social Sciences (SPSS) version 13.0 (SPSS Inc., Chicago, IL, USA). All tests were two-sided, and a *P* value of <0.05 was considered to be statistically significant.

Results

Clinical characteristics

The current study included 62 patients with early OHSS and 60 patients without OHSS. No differences between the two groups were found with regards to age, BMI, duration of infertility, antral follicular count, basal hormone levels (FSH, LH, PRL, E_2 and T) and basal thickness of the endometrium. The clinical profiles of all participants after pituitary down regulation and ovarian hyperstimulation are shown in **Table 2**. Serum hormone levels after 14 days of pituitary down regulation were similar between the two groups. The total dose of Gn and the daily dose of Gn were significantly lower in the case group compared to the control group, indicating an elevated ovarian response among patients with

Table 4. Association of AMH polymorphisms with OHSS

	Cases (n=62)		Controls (n-60)		Adjusted *P*-value*	Adjusted OR* (95% CI)
	No.	%	No.	%		
rs10407022						
TT (Ile/Ile)	26	41.9	26	43.3		1†
GG (Ser/Ser)	12	19.4	4	6.7		
GT (Ser/Ile)	24	38.7	30	50		
GG+GT (Ser/Ser+Ser/Ile)	36	80.6	34	93.3	0.307	1.552 (0.668, 3.608)
rs182295886						
AA (Ser/Ser)	60	96.8	59	98.3		1†
GG (Gly/Gly)	0	0	0	0		
GA (Gly/Ser)	2	3.2	1	1.7		
GG+GA (Gly/Gly+Gly/Ser)	2	3.2	1	1.7	0.442	0.359 (0.026, 4.883)

OR, odds ratio; CI, confidence interval. *P-value and OR were adjusted for age, body mass index, antral follicle count, serum basal hormone level (follicle stimulating hormone, luteinizing hormone, estradiol and testosterone) using logistic regression. †This group is defined as the reference group.

Table 5. Association of AMH polymorphism (rs10407022) with ovarian response among all participants

rs10407022	TT (Ref.)	GG+GT	*P*-value
Follicular phase serum E_2 level	41.85±22.32	42.92±15.46	0.76
Serum E_2 level after pituitary down regulation	23.07±7.71	23.96±9.27	0.59
E_2 level on the hCG day	5161.8±2841.8	6252.4±3437.9	0.065
Number of large follicles (>14 mm)	13.3±5.0	14.5±5.4	0.182
Number of oocytes retrieved	16.1±6.9	17.6±7.4	0.258

Data are presented as the mean ± standard deviation. E_2, estradiol; hCG, human chorionic gonadotropin.

early OHSS. The E_2 level on the hCG day, the number of large follicles and the number of oocytes retrieved differed significantly (P<0.05) between the two groups, which is in accordance with the characteristics of OHSS.

Genotype distribution and association with OHSS risk

All the exons of AMH and AMHR2 were directly sequenced. Altogether, 7 SNPs were found in the exons of AMH, and none was found in those of AMHR2. The details of these SNPs are listed in **Table 3**. Out of these SNPs, only rs10407022 and rs182295886 were missense mutations. Thus, we analyzed the genotype distributions of these two particular SNP mutations. **Table 4** shows that after adjustments for basic demographic data of all the participants using logistic regression, we did not observe any protective or negative effects to be conferred by these two SNPs to OHSS risk (rs10407022, *P*=0.307, OR=1.552, CI 0.668,3.608; rs182295886, *P*=0.442, OR=0.359, CI 0.026,4.883). Analyses in Haploview showed that in this population, the two SNPs in AMH were in incomplete linkage disequilibrium (D'=1.0, r^2=0.023). All the genotype distributions were found to follow the Hardy-Weinberg equilibrium.

Clinical characteristics of patients with AMH SNP (rs10407022)

Furthermore, we observed the clinical characteristics of patients with AMH SNP (rs10407022). Although not statistically significant, there was a slightly higher ovarian response among patients with this SNP (GT+GG, rs10407022); these patients had a higher level of E_2 on hCG day (6252.4 pg/ml vs. 5161.8 pg/ml, *P*=0.065), a larger number of mature follicles at OPU day (14.5 vs. 13.3, *P*=0.182) and a larger number of oocytes retrieved (17.6 vs. 16.1, *P*=0.258) (see **Table 5**). However, the clinical significance of these slight disparities is debatable. We further analyzed the potential relationship between this SNP and some detailed characteristics of all OHSS patients

Table 6. Association between AMH polymorphism (rs10407022) and the detailed characteristics of OHSS patients

rs10407022	TT (Ref.) (n=26)	GG+GT (n=36)	*P*-value
Cycle cancellation rate	50.0%	58.3%	0.52
Pregnancy rate	42.31%	25.0%	0.74
Rate of severe OHSS	61.5%	47.2%	0.27
Onset of symptoms (days) after OPU	4.4±3.6	4.1±3.6	0.74
Duration of hospitalization (days)	13.1±11.4	10.4±10.5	0.34
Total cost of hospitalization (RMB)	6190.5±6096.1	5155.6±5873.6	0.51

OHSS, ovarian hyperstimulation syndrome; OPU, ovum pick up; RMB, renminbi.

(see **Table 6**). Similarly, no significant differences were found with regards to any of the clinical characteristics of OHSS between the patients with and without this SNP (rs10407022), though non-significant differences were apparent. OHSS patients with this SNP had a higher cycle cancellation rate and an earlier onset of the OHSS symptoms, which may reflect the possibility that they have hypersensitive ovaries.

Discussion

OHSS is an iatrogenic, potentially life-threatening condition resulting from excessive ovarian stimulation. Lyons et al. first divided this disease into early OHSS and late OHSS according to the onset of symptoms [19]. Later, Mathur et al. defined an exact timeline of early and late OHSS: Early OHSS refers to patients whose symptoms occur within 9 days following ovum pick-up day (OPU day), while late OHSS refers to patients whose symptoms began at least 10 days after OPU day [4].

Its pathophysiology involves increased capillary permeability, leading to the leakage of fluid from vascular compartments with third-space fluid accumulation and intravascular dehydration. Although its exact cause has not been completely elucidated, it seems likely that the release of vasoactive substances that are secreted by the hyperstimulated ovaries may play a key role. These findings bring about a key question regarding the hypersensitivities of the ovaries of OHSS patients to the exogenous gonadotropins that are used in IVF/ICSI.

Studies involving AMH knockout mice have revealed that follicles are recruited at a faster rate than usual and that they become more sensitive to FSH [20]. Additionally, a recent study demonstrated that in human granulosa-lutein cells, AMH acts through AMHR2 to inhibit FSH-induced adenylyl cyclase activation, aromatase expression, and E_2 production [14]. These results indicate that AMH plays an inhibitory role in the recruitment of primordial follicles and that the absence of AMH leads to prematurely exhausted follicle pool, and subsequently, an earlier cessation of estrus cycle. Thus, there is a balance between promoting and inhibiting the recruitment of follicles. For example, FSH promotes the recruitment of primordial follicles; however, AMH inhibits this process. Consequently, disorders in the AMH/AMHR2 signaling pathway will disrupt the balance, and the follicles may become more sensitive to FSH and be selected for dominance in advance.

The SNPs of AMH and its receptors could affect the biological activities and signal transduction of hormones, which play important roles in controlling follicle recruitment and development. Thus, in this study, we investigated the possible relationship between genetic variation of AMH/AMHR2 and the development of early OHSS in a Han population. Our data show that there are seven SNPs in the exons of AMH, while there are none in the exons of AMHR2. AMH 365 g>t (rs10407022) and AMH 535 g>r (rs182295886) are missense mutations. Our analysis indicated that these two SNPs did not increase the risk of OHSS. The other five SNPs of AMH are synonymous codons, therefore, we did not analyze their associations with OHSS risk in this study.

A previous study [21] investigated the correlations between the SNPs (rs10407022 and rs4807216) of AMH and the ovarian stimulation outcome (high or low response) and showed these SNPs did not determine whether the response to ovarian stimulation was high or low. This was similar with our data, while the previous study did have some limitations. For example, smoking status, serum estrogen and antral follicle count data were lacking, which are critical for evaluating individual ovarian response. Additionally, patients in the high-response group were significantly younger than

the patients in the control group, which could be confounding the result. Furthermore, patients with early and late OHSS were analyzed together, which may lead to a possible biased interpretation of the association between AMH/AMHR2 SNPs and high responders; because the clinical symptoms of most patients with late OHSS were mainly due to embryo implantation and sometimes to multiple pregnancy, these patients may have actually had normal ovarian response.

In this study, we also found that a slightly higher response among the population with GT or GG in one SNP (rs10407022) of AMH was seen as evidenced by a higher E_2 level on the hCG day, a larger number of follicles (>14 mm) and oocytes retrieved, a higher rate of cycle cancellation and an earlier onset of clinical symptoms. However, while such differences were not statistically significant, they were in accordance with Kevenaar's work [22]. He found that patients with this SNP (rs10407022) showed an elevated serum concentration of E_2 in the follicular phase without an increased antral follicle count, meaning that the rise in E_2 can be attributed to an enhanced sensitivity of the growing follicles to FSH. To our knowledge, this is the first study to investigate the possible relationship between genetic variation of AMH/AMHR2 and the pathogenesis of early OHSS in a Han population.

In conclusion, we analyzed the pathogenesis of OHSS by first investigating the SNPs in the AMH signaling pathway. Although SNPs in the exons of AMH, they did not influence the risk of OHSS. There is no association between SNPs in the AMH/AMHR2 signaling pathway and early OHSS in Han Chinese women. It seems there is no need to detect these SNPs when applying the serum concentration of AMH as a predictor of early OHSS. Further study (including the expression of AMH and AMHR2 in GCs, the levels of AMH and E_2 in follicular fluid, and so on) is needed to analyzing the possible involvement of the AMH/AMHR2 signaling pathway in the pathogenesis of early OHSSS.

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Disclosure of conflict of interest

None.

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