

# Analyzing the possible involvement of anti-Müllerian hormone and anti-Müllerian hormone receptor II single nucleotide polymorphism in infertility

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## Abstract

**Purpose** We performed TaqMan genotyping assays of anti-Müllerian hormone (AMH) and anti-Müllerian hormone receptor type II (AMHR II) single nucleotide polymorphisms (SNPs) in order to investigate how their frequency and distribution affect infertility treatment outcome.

**Methods** Eighty Japanese women (advanced age:  $n=51$ , endometriosis:  $n=18$ , male infertility as a control:  $n=11$ ) who undertook ART were included in the study, and all couples underwent a full infertility investigation protocol. In order to investigate the natural distribution of SNPs, a naturally pregnant group of 28 subjects was recruited from among women who conceived naturally and subsequently delivered in our department. Genomic DNA was extracted from peripheral blood and genotyping was conducted by TaqMan genotyping assay. The relationship of AMH and AMHR II SNPs and treatment outcome in infertile women. Comparison of allele and genotype frequencies of infertile patients with naturally pregnant women.

**Results** AMHR II -482 A>G homozygote mutation was complicated with ISV 5–6 C>T homozygote mutation and showed a significantly lower oocyte retrieval rate compared with a wild type. Two of 3 cases of AMHR II -482 A>G homozygote mutation were poor responders, and the distribution and frequency of each allele of naturally pregnant women showed no statistical difference compared with infertile women.

**Conclusions** This study revealed the possible involvement of AMHR II -482 A>G polymorphism on the malfunction of follicular development in Japanese women.

**Keywords** AMH · AMHR II · Single nucleotide polymorphism · AMHR II -482 A>G · Oocyte retrieval rate

## Introduction

There are various known parameters for assessing ovarian reserve, including ovarian volume, antral follicle count, follicle stimulating hormone (FSH) and Inhibin B at the beginning of the menstrual cycle, as well as anti-Müllerian hormone (AMH).

Serum AMH, in particular, has been shown to be a better endocrine indicator of a patient's follicular response to controlled ovarian stimulation (COS) with gonadotropins [1–5] in assisted reproductive technology. On the other hand, it has been reported that SNPs of AMH genes also affect follicle counts and folliculogenesis, thus leading to infertility [6]. However, what impact these SNPs have on the clinical outcome of infertile treatment remains unknown. To investigate how the frequency and distribution of AMH and AMHR II SNPs in infertile patients affect infertility treatment outcome, we performed TaqMan genotyping assays of AMH and AMHR II.

**Capsule** AMHR II -482A>G polymorphism may have the potential to cause a malfunction in follicular development

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## Material and methods

### Patients

We included in this study 80 Japanese women who undertook ART between March 2010 and March 2013 in the Department

of Obstetrics and Gynecology at Osaka Medical College. All couples underwent a full infertility investigation protocol, including a laparoscopic examination. Infertile patients were classified into 3 groups: advanced age (35 years old or older), severe endometriosis, and male infertility. In the study, male infertility was allocated as the control group, and unexplained infertility was included in the advanced age group.

Moreover, to investigate the natural distribution of the SNPs, a group of 28 subjects was recruited from among women who conceived naturally and subsequently delivered in our department during the same period. Our institutional review board approved this protocol (No. 71) and its consent form, and we received informed consent for this study from all participants.

#### Protocol for controlled ovarian hyperstimulation (COH)

Controlled ovarian hyperstimulation, hormone assays, follicle monitoring, oocyte retrieval, insemination, embryo culture, embryo transfers and confirming embryo quality were performed as previously reported [7]. Pregnancy was confirmed during ultrasound examination by the identification of an intrauterine gestational sac.

#### DNA preparation and TaqMan® genotyping assay

Genomic DNA was extracted from peripheral blood using a High Pure PCR Template Preparation Kit (Roche Diagnostic GmbH, Mannheim, Germany), and genotyping was carried out by TaqMan® genotyping assay.

TaqMan® SNP Genotyping Assay 5' nuclease technology uses two allele-specific TaqMan® MGB probes and a PCR primer pair to detect the specific SNP target. The probes and primers uniquely align with the genome, enabling TaqMan® genotyping technology to

provide unmatched specificity. Genotypes were decided using Taqman allelic discrimination assays. For the AMH and AMHRII polymorphisms, each probe is shown in Table 1. Each PCR reaction contained 2 ng of dried genomic DNA, 1 µl of TaqMan Universal PCR Master Mix 2 ×, 0.025 µl of the 80 × AMH Assay Mix or 0.1 µl of the 20 × AMHRII SNP mix in a total volume of 2 µl.

Each SNP assay was designed by Life Technologies (Tokyo, Japan). The TaqMan® genotyping assay was performed in a 48-well StepOne™ Real-Time PCR according to the manufacturer's instructions.

#### Statistical analysis

All experiments were performed in duplicate, and statistical analysis was done with StatMate IV (ATMS Co., Ltd.). Genotype distribution was tested for Hardy-Weinberg equilibrium, and the difference in genotype frequencies was determined/calculated by a chi-square test. A *P* value of <0.05 was considered to be statistically significant.

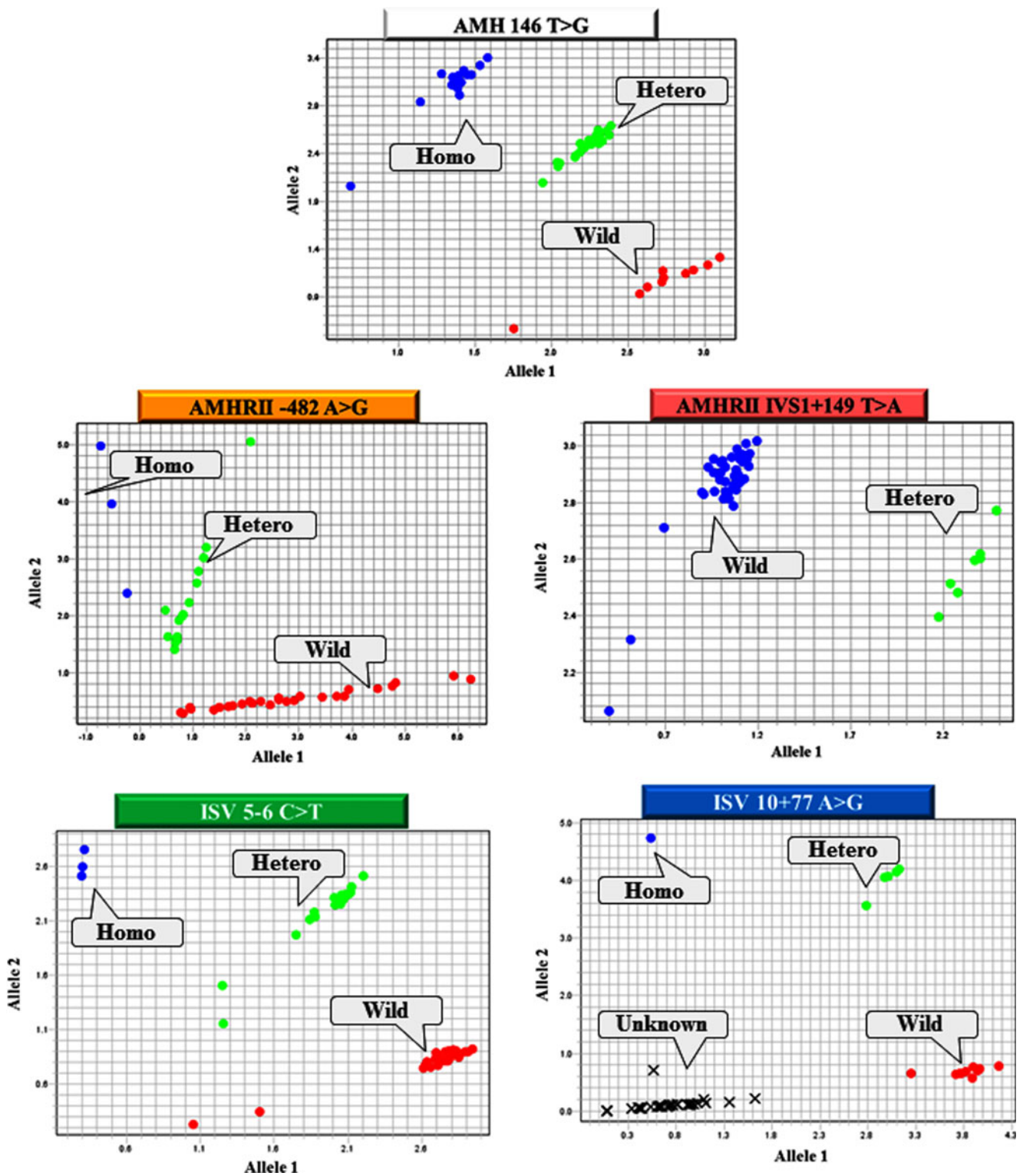
## Results

Comparison of Allele and genotype frequencies of infertile patient with women who delivered following natural conception

All genotypes, except for IVS10+77 A>G of the AMH and AMHRII genes, were easily confirmed by TaqMan® genotyping assays. In IVS10+77 A>G, there were many cases of unclassifiable genotypes (Fig. 1). Table 2 shows the distribution of each genotype in infertile patients, the male infertility

**Table 1** For investigating the AMH and AMHRII polymorphisms, genotypes were decided using Taqman allelic discrimination assays

Gene	Base pair change	Position (aa change)	Primers	Base pair	Tm (C°)
AMH	146 T>G	Exon1	F:ACCAGTGGCCTCATCTTCC R:AGGAAGGCCTGCTCATAGG	152	59
AMHR-II	-482 A>G	Promoter	F:GGTAACCTCTAATATGGGCTGTG R:TCTCAGGAGGAAACCAATGTG	150	59
AMHR-II	IVS1+149 T>A	Intron 1 (-)	F:CCCTTTGGAAGAGTGGTGAG R:AGGTGGGAGTGAATGCAGAG	196	56
AMHR-II	IVS5-6 C>T	Intron 5 (-)	F:AGCTGTGTTTCTCCCAGGTG R:ATGGCAACCAGTTTTCTTG	197	56
AMHR-II	IVS10+77 A>G	Intron 10 (-)	F:AAGAGGCCTAGGCTGTTGGT R:CAAGGTTGAGCAGGAGGAAG	241	60



**Fig. 1** All genotypes, except for IVS10+77 A>G of the AMH and AMHRII genes, were easily confirmed by TaqMan genotyping assays. In IVS10+77 A>G, there were many cases of unclassifiable genotypes

control, and the natural pregnancy group (Table 2). The distribution and frequency of each allele in women who

conceived naturally showed no statistical difference compared with infertile women (Table 3).

**Table 2** The distribution of each genotype of women with endometriosis, advanced age, and natural pregnancy as control

	AMH 146 T>G			AMHR -482 A>G			AMHR IVS1+149 T>A T>G			AMHR ISV 5-6 C>T			AMHR ISV 10+77 A>G		
	Homo	Hetero	Wild	Homo	Hetero	Wild	Homo	Hetero	Wild	Homo	Hetero	Wild	Homo	Hetero	Wild
Endometriosis	3	8	4	3	2	12	0	6	11	3	2	7	2	2	6
Advanced age	8	26	14	0	27	23	0	9	25	1	25	21	0	13	8
Male infertility	2	18	21	3	13	18	0	8	27	4	11	17	2	2	6
Natural pregnancy	1	11	9	3	9	11	0	7	17	3	8	11	2	0	0

**Table 3** The distribution and frequency of each allele in women who conceived naturally showed no statistical difference compared with infertile women

	Infertility	Natural pregnancy	$\chi^2$ test ( <i>P</i> value)
AMH 146 T>G			
T:G	90:54	29:13	NS
T/T:T/G:G/G	25:40:2	9:11:1	NS
AMHR -482 A>G			
A:G	118:38	31:15	NS
A/A:A/G:G/G	43:32:3	11:9:3	NS
AMHR IVS1+149 T>A			
T:A	148:6	41:7	NS
T/T:T/A:A/A	66:16:0	17:7:0	NS
AMHR ISV5-6 C>T			
A:G	57:21	28:10	NS
A/A:A/G:G/G	20:17:2	11:8:3	NS
AMHR ISV10+77 A>G			
C:T	100:40	30:14	NS
C/C:C/T:T/T	35:30:5	11:6:0	NS

Clinical characteristics of AMH and AMHR II SNP in infertile women

AMHR II -482 A>G homozygote mutation, all of which were accompanied with ISV 5-6 C>T homozygote mutation,

**Table 4** AMHR II -482 A>G homozygote mutation, all of which were accompanied with ISV 5-6 C>T homozygote mutation, showed a statistically lower oocyte retrieval rate (28.5 %) and pregnancy rate (0 %) in

Polymorphism ( <i>N</i> =80)	Aspirated follicle	Retrieved oocyte	Oocyte retrieved rate	Fertilization rate (%)	Pregnancy rate (%)
AMH 146 T>G	Homo ( <i>n</i> =12)	96	48	50.0	27.2
	Wild ( <i>n</i> =27)	266	155	58.2	29.3
AMHR -482 A>G	Homo ( <i>n</i> =3)	21	6	28.5 <sup>a</sup>	0 <sup>b</sup>
	Wild ( <i>n</i> =45)	503	276	54.8	28.1
AMHR ISV5-6 C>T	Homo ( <i>n</i> =5)	49	22	44.8	0 <sup>b</sup>
	Wild ( <i>n</i> =36)	436	245	56.8	19.4
AMHR ISV10+77 A>G	Homo ( <i>n</i> =3)	17	7	41.1	33.3
	Wild ( <i>n</i> =21)	249	126	50.6	14.2

$\chi^2$  test  
<sup>a,b</sup> *p*<0.05

showed a statistically lower oocyte retrieval rate (28.5 %) and pregnancy rate (0 %) in comparison with the wild type; however, other homozygote mutations of AMH and AMHR II showed no statistical difference in their oocyte retrieval rate, fertilization rate, and pregnancy rate (Table 4).

As well, there was no statistical difference in the serum hormonal value of AMHR II -482 A>G homozygote mutation compared with the wild type. Additionally, total dose of hMG/FSH to retrieve the oocyte showed no statistical difference between the homozygote mutation and wild types in each SNP (Table 5).

Patients background of AMHR II -482 A>G homozygote mutation

All of AMHR II -482 A>G homozygote mutations were found in the presence of endometriosis, and two of them were complicated with diminished ovarian reserve (DOR). All cases of AMHR II -482 A>G homozygote mutation were also accompanied by ISV 5-6 C>T homozygote mutation. In the natural pregnancy group, a homozygote mutation was confirmed in all SNPs. -482 A>G homozygote mutation cases consisted of two patients with a past history of repeated pregnancy loss (RPL) and one case of fetal growth restriction (FGR). All cases of -482 A>G homozygote mutation were

comparison with the wild type; however, other homozygote mutations of AMH and AMHR II showed no statistical difference in their oocyte retrieval rate, fertilization rate, and pregnancy rate

**Table 5** There was no statistical difference in the serum hormonal value of AMHRII -482 A>G homozygote mutation compared with the wild type. Additionally, total dose of hMG/FSH to retrieve the oocyte showed no statistical difference between the homozygote mutation and wild types in each SNP

	AMHR -482 A>G Homo mutant N=3	AMHR -482 A>G Wild type N=45	P value
Age(y)	38.7±4.16	38.0±4.65	NS
LH (mIU/ml)	2.53±0.95	7.47±6.4	NS
FSH (mIU/ml)	3.4±1.3	9.4±5.7	NS
Estradiol	21.4±25.4	67.6±107.8	NS
Total dose of hMG/FSH (IU)	3100.1±3408.5	3041.6±1359.0	NS

Values presented as mean ± SD

accompanied by a ISV 5–6 C>T homozygote mutation (Table 6).

**Discussion**

Anti-Mullerian hormone (AMH), also known as Mullerian inhibiting substance, is a member of the transforming growth factor-β family [8]. During folliculogenesis, AMH expression starts in the granulosa cells of primary follicles, is highest in granulosa cells of preantral and small antral follicles, and gradually diminishes in the subsequent stages of follicular development. As well, AMH is produced by the granulosa cells of early developing follicles in the ovary, and these cells keep expressing AMH until the follicle reaches the appropriate size and differentiation. In this differentiated state, follicles are selected for dominance by the action of FSH [9–11]. Studies of AMH knockout mice have revealed that follicles are recruited at a faster rate than usual and that they become more sensitive to FSH [12]. This indicates that AMH plays an inhibitory role in the recruitment of primordial follicles and that the absence of AMH leads to a prematurely exhausted follicle pool and, subsequently, an earlier cessation of the estrus cycle. Consequently, follicles might be more sensitive to FSH and may be selected for dominance in advance [13]. It has recently been reported that oocytes from

early preantral, late preantral, and preovulatory follicles up-regulate AMH mRNA levels in granulosa cells in a manner that is dependent on the development stage of the oocyte itself.

The human AMH gene was cloned in 1986 and is located on the tip of the short arm of chromosome 19p13.3. The length of the human AMH gene is only 2.75kbp and consisted of 5 exons. At the time of this study, 38 different mutations of the AMH gene have been detected. AMH polymorphisms could affect hormone biological activities, which play an important role in controlling follicle recruitment and development. However, in this study, AMH homozygote mutation showed no apparent effect on clinical ART outcomes. AMH binds specifically to AMHR receptor type II (AMHRII) and was cloned in 1994 by two different groups [8, 14]. AMHRII is located on the chromosome 12q13, has a total length of 8.7kbp, and consists of 11 exons. AMHRII is a specific type II receptor, ALK2, 3 and 6 are type I receptors, and Smad 1, 5, 8 are cytoplasmic effectors. Cofactors, coactivators and corepressors for AMH transduction and its relationship with other transduction pathways is not known at the present time.

Most target genes are repressed by AMH action [15], and AMH 146 T>G and AMHRII -482 A>G polymorphisms contribute to the individual variation in the FSH threshold of the ovary [16]. Recent studies in normo-ovulatory women have revealed an association of SNPs of AMH and AMHRII with

**Table 6** All of AMHRII -482 A>G homozygote mutations were found in the presence of endometriosis, and two of them were complicated with diminished ovarian reserve (DOR). All cases of AMHRII -482 A>G homozygote mutation were also accompanied by ISV 5-6 C>T homozygote mutation. In the natural pregnancy group, a homozygote mutation

was confirmed in all SNPs. -482 A>G homozygote mutation cases consisted of two patients with a past history of repeated pregnancy loss (RPL) and one case of fetal growth restriction (FGR). All cases of -482 A>G homozygote mutation were accompanied by a ISV 5-6 C>T homozygote mutation

Age	Gravity & Parity	Diagnosis or Obstetric outcome	AMH 146 T>G	AMHR -482 A>G	IVS1+149 T>A	ISV5-6 C>T	ISV10+77 A>G
40	G0P0	Endometriosis poor-responder	Hetero	Homo	Hetero	Homo	Homo
40	G0P0	Endometriosis poor-responder	Hetero	Homo	Hetero	Homo	unknown
34	G0P0	Endometriosis	unknown	Homo	Hetero	Homo	unknown
40	G6P1	37 wks 2,948 g F	Hetero	Homo	Hetero	Homo	Homo
40	G6P1	37 wks 3282 g M	Hetero	Homo	Hetero	Homo	unknown
34	G0P0	37 wks 1200 g M	unknown	Homo	Hetero	Homo	unknown

wks weeks, F female, M male

estradiol levels during the early follicular phase of the menstrual cycle, thus suggesting AMH's role in the regulation of FSH sensitivity.

Moreover, in patients suffering from normogonadotrophic normo-estrogenic anovulatory infertility, these polymorphisms might become valuable predictors which decide the individual FSH threshold. Distinct differences in the amount of exogenous FSH required to induce an ovarian response might lead to the risk of hyper-response and its subsequent complications such as ovarian hyper-stimulating syndrome (OHSS) and multiple pregnancy [17, 18]. The  $-482\text{ A>G}$  homozygote mutation could result in diminished AMH signaling [16] and, because the  $-482\text{ A>G}$  SNP is located at the promoter region, it could possibly cause a disequilibrium with several other SNPs [16]. However, the detail of disequilibrium with other SNPs remains unknown.

The  $-482\text{ A>G}$  SNP is located at a potential c-Myb and c-Myc transcription factor-binding site and, therefore, may modify promoter activity [19].

In this study,  $-482\text{ A>G}$  homozygote mutation was associated with ISV5-6 C>T homozygote mutation, and the oocyte retrieval rate of  $-482\text{ A>G}$  homozygote mutation was statistically lower than in the control group and in other SNPs, despite the result that total dose of hMG/FSH for oocyte retrieval in  $-482\text{ A>G}$  homozygote mutation cases showed no statistical difference compared with the wild type. These results suggest that  $-482\text{ A>G}$  homozygote mutation could cause poor follicular development. In the normal pregnancy group, all cases of  $-482\text{ A>G}$  homozygote mutation were associated with ISV5-6 and showed poor obstetric outcome. The inclusion of more cases is necessary to determine whether endometriosis and advanced age affect the frequencies of AMH and AMHR SNPs and whether obstetric outcome is affected by these SNPs.

In conclusion, additional replication and functional studies are necessary to secure definite conclusions as to the effect of AMHR II  $-482\text{ A>G}$  polymorphism on infertility and obstetric outcome. However, this study revealed the possible involvement of AMHR II  $-482\text{ A>G}$  polymorphism on the malfunction of follicular development in Japanese women.

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