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GENETICS

Association of *AMH* and *AMHR2* gene polymorphisms with ovarian response and pregnancy outcomes in Indian women

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Abstract

Purpose To evaluate the association of single-nucleotide polymorphisms (SNPs) in the anti-Müllerian hormone (*AMH*) and *AMH* type II receptor (*AMHR2*) genes with ovarian response and clinical pregnancy outcomes in women undergoing controlled ovarian hyperstimulation.

Methods In this prospective study, we genotyped *AMH* polymorphisms (c. -649 T > C, c. 146 T > G, c. 252 G > A, and c. 303 G > A) in 365 women and *AMHR2* polymorphisms (c. -482 A > G, c. 622–6 C > T, c. 4952 G > A, c. 10 A > G) in 80 women undergoing controlled ovarian hyperstimulation for IVF.

Results Higher doses of exogenous FSH and lower numbers of preovulatory follicles were noted in women having *AMH* c. -649 T > C and *AMH* c. -146 T > G polymorphisms, respectively. Overall, we found that the presence of a polymorphic genotype (homozygous or heterozygous) at positions c. -649 T > C, c. 146 T > G, c. 252 G > A, and c. 303 G > A in the *AMH* gene was associated with higher doses of FSH for ovulation induction ($p < 0.001$). Interestingly, a higher live birth rate was noted in women with a homozygous polymorphic genotype for all four *AMH* SNPs investigated while none of the women showing a homozygous polymorphic genotype at all *AMHR2* SNPs investigated in this study had a live birth.

Conclusion Our results show that presence of *AMHR2* SNPs (c. 482 A > G, c. 622–6 C > T, c. 4952 G > A, and c. 10 A > G) negatively correlate with live birth rate. However, these findings need to be validated by using larger sample size.

Keywords Anti-Müllerian hormone · Anti-Müllerian hormone type II receptor · Ovary · Hyperstimulation · Embryo · Assisted reproduction

Introduction

Controlled ovarian hyperstimulation (COH) is a critical step in assisted reproductive technology and involves the administration of an exogenous gonadotropin, i.e., follicle-stimulating hormone (FSH) to women so that they can achieve a moderate number of oocytes. High variability of COH outcomes is observed in clinical practice. Some women often exhibit high sensitivity to FSH stimulation which can lead to ovarian hyperstimulation syndrome (OHSS), while others appear to be poor responders. Both circumstances can result in the cancellation of the IVF cycle as well as additional financial expenses. Thus, there is a need for objective markers to predict ovarian response in women undergoing COH.

Individual COH outcomes depend on pharmacological modification of biochemical pathways underlying FSH-induced estrogen and oocyte production [1] and the search for a biomarker that can predict ovarian response is ongoing.

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We and others have previously shown that single-nucleotide polymorphisms (SNPs) in the FSH receptor gene correlate with the amounts of FSH required in COH and can predict both hyper response and poor response [2–4]. However, these associations are highly population-specific and perhaps dependent on genetic backgrounds [5, 6]. Anti-Müllerian hormone (AMH) is expressed in the developing follicle of adult ovaries and its levels in the ovary and serum are altered in women with pathologies such as polycystic ovary syndrome (PCOS) [7]. Although serum AMH is thought to be a biomarker for prediction of ovarian reserve [8], there is no major alteration with ovarian response in women undergoing COH. Polymorphisms in the *AMH* and its receptor (*AMHR*) genes are widely studied as potential predictors of ovarian response. Several clinical studies [9–12] and systematic reviews [13–15] have reported that polymorphisms of *AMH/AMHR2* could influence ovarian stimulation outcomes. Studies focusing on the *AMH* c. 146 T > G and *AMHR2* c. -482 A > G polymorphisms have associated these polymorphisms with elevated follicular phase estradiol levels in normo-ovulatory women [16], unexplained infertility [17], follicle number, and androgen levels in polycystic ovary syndrome [18]. Some studies have also investigated the association of these two polymorphisms in women undergoing COH. In some studies, these polymorphisms are reported to predict ovarian response in women undergoing COH [19, 20], while others fail to support these findings [21, 22]. While the difference in study design and participant selection criteria could be a reason for these differences, the involvement of ethnicity cannot be ruled out. To our knowledge, the association of *AMH* and *AMHR* polymorphisms in Indian women undergoing COH has not been evaluated.

The aim of our study was to analyze SNPs in *AMH* and *AMHR2* genes and their association with the outcomes of COH in women undergoing assisted reproduction. We also aimed to determine if these polymorphisms could predict pregnancy outcomes in women undergoing assisted reproduction.

Materials and methods

Study subjects

The current study was approved by the Ethics Committee for Clinical Research at the National Institute for Research in Reproductive Health, Mumbai (D/ICEC/Sci-13/13/2015). The participants were enrolled for an IVF procedure at Fertility Clinic and IVF Centre, Mumbai, India, and Sushrut Assisted Conception Clinic, Kolhapur, Maharashtra, India. Blood samples and clinical details were collected from the subjects after informed consent. A total of 365 normogonadotropic ovulatory women (menstrual cycle length 25–35 days) with infertility due to male or tubal factor or with unexplained infertility were analyzed for the *AMH* gene. Eighty of these 365 women were also analyzed for the *AMHR2* gene. All subjects were of Indian ethnicity. Informed consent was obtained from all subjects. All recruited subjects were in the age group of 21–44 years with basal serum FSH levels in the normal range (1.2–20.5 mIU/ml). Women with polycystic ovarian syndrome, endometriosis, fibroids, and hyperprolactinemia were excluded from this study.

Ovarian stimulation

The dose of exogenous FSH to be administered for controlled ovarian hyperstimulation (COH) was optimized based on age, body mass index (BMI), serum AMH levels, and antral follicle count (AFC). A standard protocol described previously [23] was used for ovulation induction during IVF treatment. Briefly, stimulation with FSH was monitored by measuring serum estradiol levels and follicle growth. The maximum daily dose initiated was 375 IU; however, in a small number of cycles, further increment to 450 and 600 IU became necessary based on the ovarian response. Human Chorionic Gonadotropin (hCG) (10,000 USP) was administered for triggering ovulation triggering final oocyte maturation. Oocytes were retrieved after 36 h under transvaginal ultrasound guidance and mature oocytes (> 14 mm in diameter) were collected. The number of preovulatory follicles and retrieved oocytes and metaphase II (MII) oocytes were recorded for all study subjects.

Clinical and endocrinological parameters

Basal FSH levels on day 3 of the menstrual cycle were measured in one of the cycles before ovarian stimulation. The peak estradiol (E_2) and progesterone levels were those of the day of hCG trigger. Serum levels of FSH, luteinizing hormone (LH), and E_2 were measured by chemiluminescence microparticle assay (Abbott Architect, Abbott Park, IL, USA). Progesterone levels were estimated by chemiluminescence immunoassay (Immulite 2500; Siemens, Golden, CO, USA). AMH was measured by using enzymatically amplified two-site immunoassay (AMH GENII ELISA, A73818, Beckman Coulter, Inc. Brea, CA USA). Samples were analyzed using materials and procedures provided with the kit. Detection limit of AMH was 0.08 ng/ml.

Genotyping

Genomic DNA was extracted from 200 µl of whole blood obtained from each of the subjects ($n = 365$) using a commercial kit (GE Healthcare, Illustra™ blood genomic Prep Mini Spin Kit) according to the manufacturer's instructions.

Amplification of the *AMH* and *AMHR2* genes to screen the polymorphisms was performed as described previously (10) using the PCR core system (Promega, Madison, USA) and direct DNA sequencing at the DNA sequencing core facility of our institute.

Statistical analysis

The Hardy–Weinberg equation was used to calculate the expected numbers and then compared with the actual numbers of each phenotype using Haploview version 3.2 (http://www.broad.mit.edu/mpg/haploview/). Comparison of clinical parameters between the study groups on the basis of *AMH* and *AMHR2* genotypes was performed using the one-way ANOVA and Bonferroni's post-test using GraphPad Prism version 8. The association between *AMH* and *AMHR2* SNPs and clinical pregnancy outcomes was tested using StatCalc program (Epi Info version 6.0.4 CDC Atlanta, GA, USA) by estimation of Fisher's two-tailed *p*-values, odds ratios *p*-values < 0.05 were considered statistically significant.

Results

A total of 365 women undergoing IVF treatment cycles were included in this study. Analysis of the *AMH* polymorphisms was performed in all 365 women while that of the *AMHR2* polymorphisms was performed in 80 of the 365 women.

Genotypic and allelic frequencies

The genotypic and allelic frequencies of polymorphisms in the *AMH* and *AMHR2* genes in women undergoing ART are shown in Table 1. In all, we identified four *AMH* gene polymorphisms. Among these, one polymorphism lies in the promoter region, the c. 146 T > G polymorphism results in a substitution of Ser49 to Ile, and the other two polymorphisms were silent (Table 1). Among the four *AMHR2* gene polymorphisms, the c. -482 A > G polymorphism is located in the non-coding region of the promoter and it can affect the transcription process of *AMHR2*; the c. 622–6 C > T polymorphism is associated with aberrant or modified splicing by affecting the NAGNAG motif acceptors which results in

Table 1 Genotypic and allelic frequencies of *AMH* ($n = 365$) and *AMHR2* ($n = 80$) gene polymorphisms evaluated in this study

SNPs of the *AMH* gene	Protein	Genotype	*n*	Genotypic frequency	Allele frequency	$\chi 2_{HW}$ *	pHW
c. -649 T > C/ rs4807216	-	TT	232	0.63	T 0.7836	5.94	0.023
		TC	108	0.29	C 0.2164		
		CC	25	0.06			
c. 146 T > G/ rs10407022	p.Ser49Ile	TT	223	0.61	T 0.7795	0.14	0.78
		TG	123	0.33	G 0.2205		
		GG	19	0.05			
c. 252 G > A/ rs61736572	p.Leu84 =	GG	307	0.84	G 0.9096	10.19	0.009
		AG	50	0.13	A 0.0904		
		AA	8	0.02			
c. 303 G > A/ rs61736575	p.Gly101 =	GG	307	0.84	G 0.9096	10.19	0.009
		AG	50	0.13	A 0.0904		
		AA	8	0.02			
SNPs of the *AMHR2* gene	**Protein**	**Genotype**	***n***	**Genotypic frequency**	**Allele frequency**	**$\chi 2_{HW}$**	**pHW**
c. -482 A > G/ rs2002555	-	AA	62	0.77	A 0.875	0.58	0.68
		AG	16	0.2	G 0.125		
		GG	2	0.02			
c. 622–6 C > T/ rs2071558	-	CC	60	0.75	C 0.8625	0.21	0.89
		CT	18	0.22	T 0.1375		
		TT	2	0.02			
c. 4952 G > A/ rs3741664	-	GG	61	0.76	G 0.8625	1.96	0.31
		AG	16	0.2	A 0.1375		
		AA	3	0.03			
c. 10 A > G/ rs11170555	-	AA	60	0.75	A 0.8625	0.21	0.89
		AG	18	0.22	G 0.1375		
		GG	2	0.02			

*Chi-square test of independence

the insertion of glutamine in the protein. The c. 4952 G > A and c. 10 A > G polymorphisms lie in intron 6 and intron 10 of the *AMHR2* gene, respectively. All polymorphisms in the *AMH* and *AMHR2* genes except *AMH* c. 252 G > A and *AMH* c. 303 G > A were in linkage equilibrium.

Clinical and endocrinological parameters

To gain insight into the relationship of *AMH* and *AMHR2* polymorphisms on ART outcomes, the clinical, ultrasonographic, and endocrinological parameters of the women in our study population were compared based on their genotypes (Supplementary Table 1).

For the polymorphisms c. -649 T > C and c. 146 T > G, BMI, number of antral follicles, number of retrieved oocytes, and number of mature oocytes of subjects did not show any statistically significant differences based on genotypes.

For the c. -649 T > C polymorphism, women with the polymorphic genotype had significantly lower numbers of preovulatory follicles and required significantly higher amounts of exogenous FSH but the numbers of MII phase oocytes and estradiol level post-hCG administration did not differ significantly (Fig. 1).

For the c. 146 T > G polymorphism, women with polymorphic genotype had fewer preovulatory follicles as compared with the wild-type genotype. The women with homozygous G allele also required higher amounts of FSH for ovarian stimulation ($p < 0.0196$). However, the numbers of MII oocytes and estradiol levels post-hCG stimulation did not vary. A statistically significant difference was observed in the age of women with the TT genotype (Mean ± SD 31.72 ± 0.29 years) and those with a heterozygous TG genotype (Mean ± SD 32.9 ± 0.34 years) ($p = 0.0282$) at *AMH* position c. 146. Lower numbers of preovulatory follicles were observed in women with the CC genotype (Mean ± SD 8.04 ± 1.00) at *AMH* position c. -649 as compared to those with the heterozygous genotype TC (Mean ± SD 11.43 ± 0.49) ($p = 0.0464$) and wild-type genotype TT (Mean ± SD 11.26 ± 0.35).

Numbers of preovulatory follicles were also significantly lower in women with the GG genotype (Mean ± SD 7.47 ± 0.76) at *AMH* position c. 146 as compared to those with the TG (Mean ± SD 11.03 ± 0.47) ($p = 0.0439$) and TT genotypes (Mean ± SD 11.43 ± 0.36) ($p = 0.0083$). Lack of statistically significant differences was observed in levels of basal FSH, LH, and AMH required by women in our study population based on their *AMH* genotypes. However, higher doses of recombinant FSH were required for ovulation induction in women showing the polymorphic genotypes at *AMH* c. -649 and c. 146 T > G. A statistically significant difference in dose of recombinant FSH required for ovulation induction was observed in women with the CC genotype (Mean ± SD 3569 ± 182.1 IU) and TC genotype (Mean ± SD 2729 ± 90.91 IU) at *AMH* c. -649 as compared to those having the TT genotype (Mean ± SD 2679 ± 64.2) ($p < 0.0001$). This was also observed in women with GG genotype (Mean ± SD 3312 ± 185.5 IU) at *AMH* position c. 146 as compared to those with the TT genotype (Mean ± SD 2672 ± 63.22) ($p < 0.0196$) (Fig. 1). Based on one-way ANOVA, followed by Bonferroni's post-test, no significant differences were found in any of the clinical. endocrinological, or biochemical parameters when the women were segregated based on c. 252 G > A and c. 303G > A polymorphism of *AMH* gene (Supplementary Tables 3 and 4).

Women with a polymorphic genotype at all four *AMH* polymorphisms also required lower levels of estradiol before and post-ovulation induction; however, the differences were not statistically significant. Significantly lower levels of progesterone on the day of hCG administration were observed in women with a TT genotype (17.16 ± 1.59 ng/ml) as compared to those with a GG genotype (32.09 ± 6.36 ng/ml) ($p = 0.0370$) at *AMH* position c. 146 (Supplementary Table 2).

There were no statistically significant differences in the *AMHR2* genotype frequencies concerning age, body mass index, numbers of antral follicles, preovulatory follicles, retrieved oocytes and mature oocytes, basal FSH, LH and AMH levels, amount of exogenous FSH, administered for ovulation induction, and estradiol and progesterone levels before and after hCG administration based on one-way ANOVA followed by Bonferroni's post-test (Fig. 2).

Association of genotypes with live birth rate

Data on the pregnancy outcomes of 338 women with *AMH* gene polymorphisms was available. One hundred thirty-one of 338 (38.75%) women had live birth, 207 had negative outcomes, and the remaining 27 women either did not proceed with embryo transfer or had missed abortions or had only biochemical pregnancies. The genotype distribution and allele frequency of the different *AMH* and *AMHR2* polymorphisms between negative and positive clinical pregnancy are summarized in Table 2. Although we did not observe any association of *AMH* polymorphisms with clinical outcomes of pregnancy, women with a polymorphic genotype were almost half as likely to have a clinical pregnancy compared with women with wild-type or heterozygous genotypes (Table 2.). No significant difference was found between wild-type and heterozygous genotypes in pregnancy.

Of the 215 women showing wild-type TT genotype at *AMH* position -649, 38% (82/215) had a live birth. Similarly, of the 100 women showing a heterozygous TC genotype at *AMH* position -649, 36% (36/100) had a live birth and of the 23 women with a polymorphic genotype CC at *AMH* position, 56% (13/23) had a live birth. Similarly, 38% of women with wild-type genotype, 38% women with heterozygous

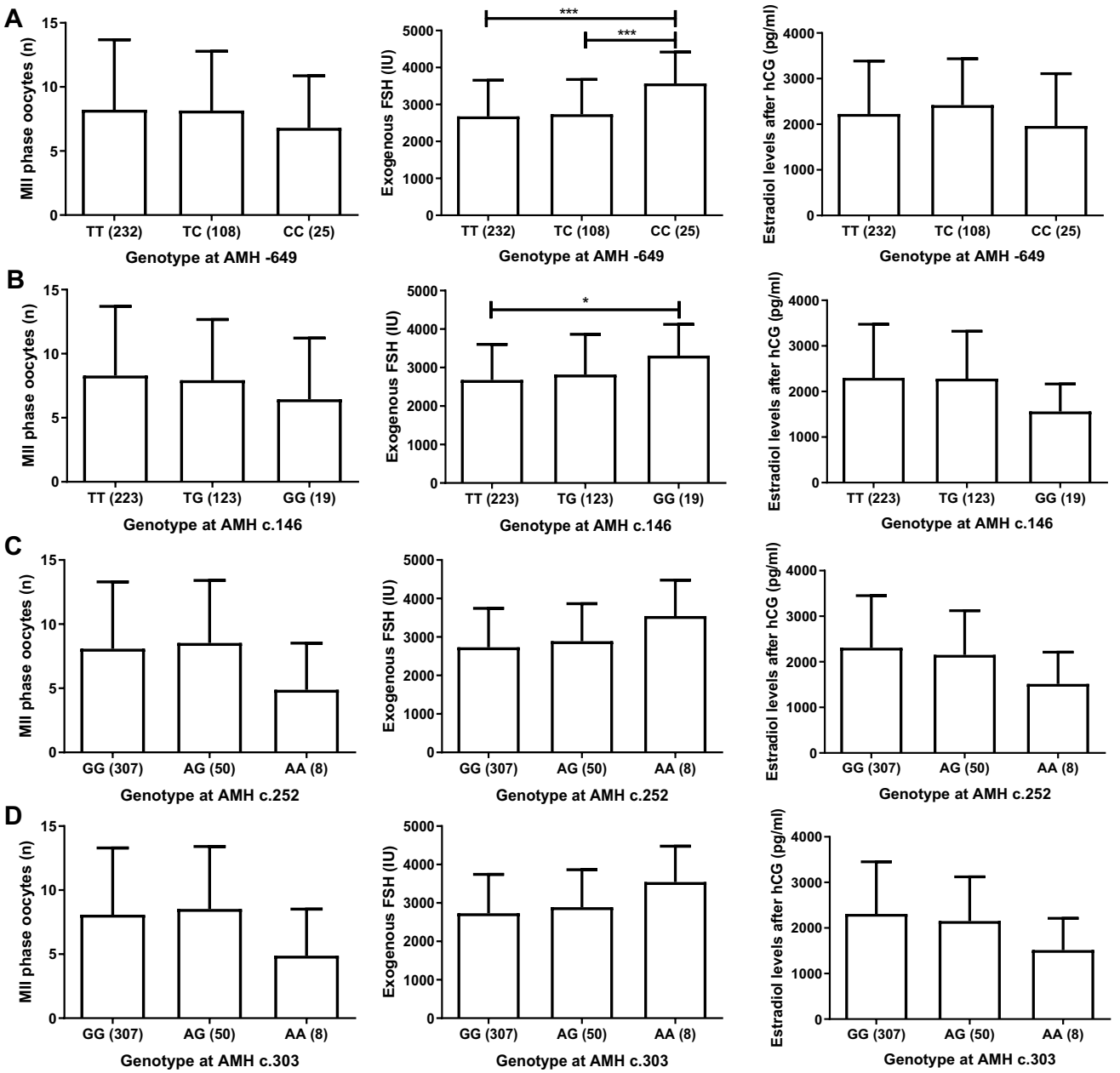

Fig. 1 Comparison of ultrasonographic and endocrinological parameters of subjects undergoing IVF based on polymorphisms of the *AMH* gene. The study subjects were segregated based on their genotypes at each *AMH* polymorphism and number of mature oocytes retrieved, level of exogenous FSH required for ovarian stimulation, and levels of estradiol on the day of hCG administration were assessed. Number of women showing each genotype (indicated in brackets) are shown on the *X* axis and parameters investigated are shown on the *Y* axis. One-way ANOVA followed by Bonferroni's post-test was used for analysis. *** indicates $p<0.001$, ** indicates $p<0.01$, and * indicates $p<0.05$. **A** *AMH* -649 T>C. Amount of exogenous FSH was significantly different among women with TT and CC ($p<0.001$) and TC and CC ($p<0.001$) genotypes. **B** *AMH* c. 146 T>G. Amount of exogenous FSH was significantly different among women with TT and GG ($p<0.05$) genotypes. **C** *AMH* c. 252 G>A. **D** *AMH* c. 303 G>A

genotype, and 47% women with a polymorphic genotype at *AMH* position c. 146 had a live birth. In the case of women with polymorphisms at the *AMH* c. 252 and c. 303 positions, 38% of women with wild-type genotype, 40% women with heterozygous genotype, and 63% women with a polymorphic genotype had a live birth. Notably, more women had a live birth when they had a homozygous polymorphic genotype at all four *AMH* polymorphisms investigated (Fig. 3).

Similarly, we analyzed pregnancy outcomes of 80 women showing polymorphisms in the *AMHR2* gene. Twenty-eight of 80 (35%) women showed a live birth and 52 women had negative outcomes (Table 2). Interestingly, none of the

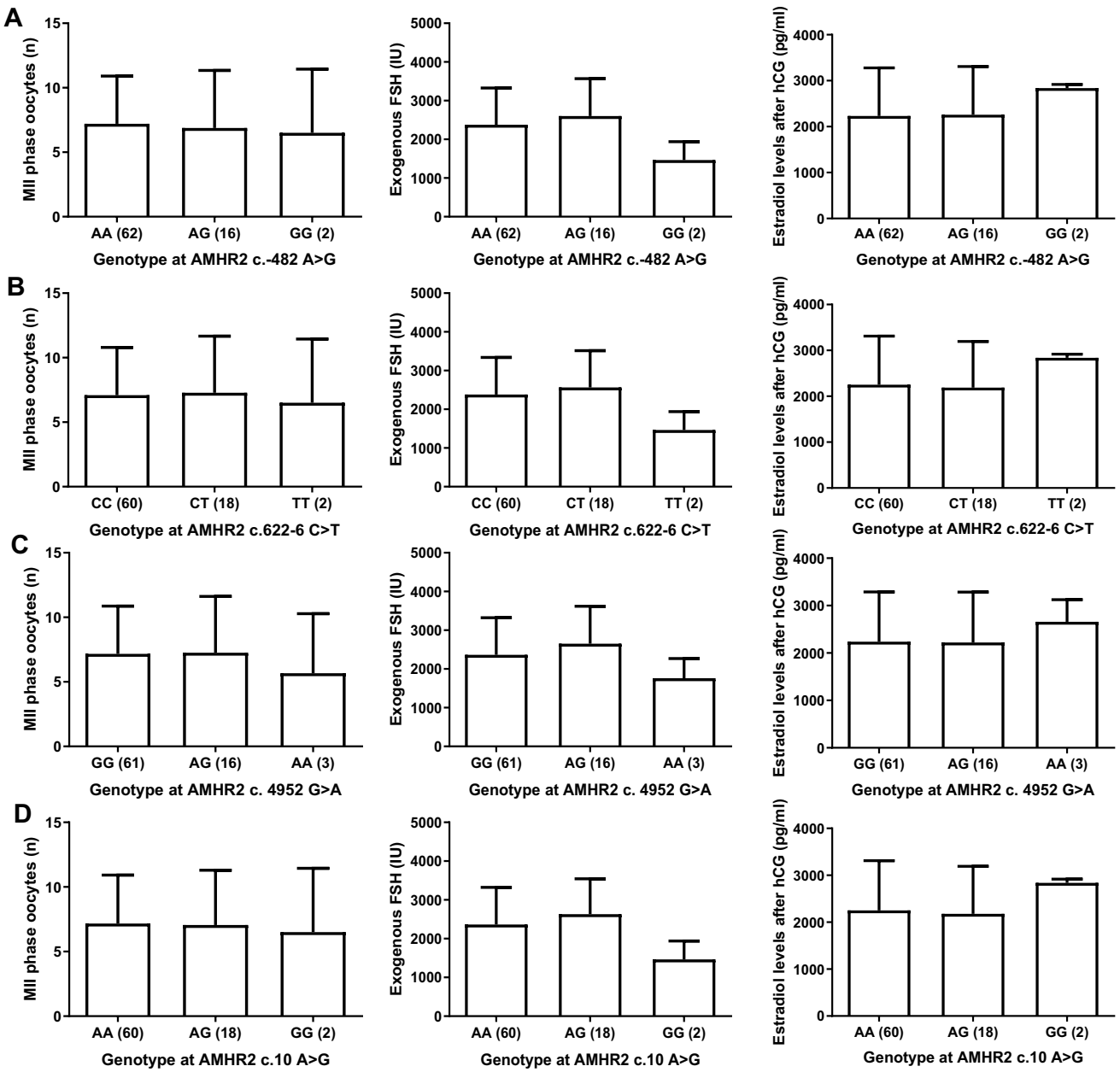

Fig. 2 Comparison of ultrasonographic and endocrinological parameters of subjects undergoing IVF based on polymorphisms of the *AMHR2* gene. The study subjects were segregated based on their genotypes at each *AMHR2* polymorphism and number of mature oocytes retrieved, level of exogenous FSH required for ovarian stimulation, and levels of estradiol on the day of hCG administration were assessed. Number of women showing each genotype (indicated in brackets) are shown on the *X* axis and Mean ± SD values of parameters investigated are shown on the *Y* axis. One-way ANOVA followed by Bonferroni's post-test was used for analysis. *** indicates $p < 0.001$, ** indicates $p < 0.01$, and * indicates $p < 0.05$. **A** *AMHR2* c. 482 A > G. **B** *AMHR2* c. 622–6 C > T. **C** *AMHR2* c. 4952 G > A. **D** *AMHR2* c. 10 A > G

women showing polymorphic genotypes at all four *AMHR2* polymorphisms had a live birth. The highest live birth rates were noted in women with the wild-type genotype at all four *AMHR2* polymorphisms (c. 482 A > G – 40%, c. 622–6 C > T – 40%, c. 4952 G > A – 41%, and c. 10 A > G – 42%) while women with heterozygous genotype showed a live birth rate ranging between 17 and 22% (c. 482 A > G – 18%, c. 622–6 C > T – 22%, c. 4952 G > A – 18%, and c. 10 A > G – 17%).

Lastly, to delineate the effects of polymorphisms in the *AMH* gene on different IVF parameters, we segregated the women based on the presence of wild-type (209) or polymorphic [8] genotype at any of the four *AMH* SNPs. A statistically significant difference in the amount of recombinant

Table 2 Pregnancy outcomes of the women based on *AMH* ($n=338$) and *AMHR2* ($n=80$) genotypes

AMH polymorphisms		Negative ($n=207$)	Positive ($n=131$)	Fisher's 2-tailed *p*-values
c. -649 T > C	TT	133	82	0.81
	TC	64	36	0.54
	CC	10	13	0.07
c. -146 T > G	TT	128	79	0.81
	TG	70	44	1.00
	GG	9	8	0.61
c. 252 G > A	GG	177	108	0.44
	AG	27	18	0.87
	AA	3	5	0.26
c. 303 G > A	GG	177	108	0.44
	AG	27	18	0.87
	AA	3	5	0.26
***AMHR2* polymorphisms**		**Negative ($n=52$)**	**Positive ($n=28$)**	**Fisher's 2-tailed *p*-values**
c. 482 A > G	AA	37	25	0.09
	AG	13	3	0.15
	GG	2	0	0.53
c. 622–6 C > T	CC	36	24	0.11
	CT	14	4	0.26
	TT	2	0	0.53
c. 4952 G > A	GG	36	25	0.05
	AG	14	3	0.15
	AA	2	0	0.53
c. 10 A > G	AA	35	25	0.08
	AG	15	3	0.09
	GG	2	0	0.53

FSH to induce ovulation in women with a polymorphic genotype ($p<0.001$) was observed (Fig. 4). Estradiol levels after ovulation induction or the number of MII phase oocytes retrieved did not differ significantly between women having the *AMH* polymorphism as compared to those who were wild type. A similar analysis could not be performed for women showing *AMHR2* polymorphisms because only three women were polymorphic for the *AMHR2* loci investigated while 60 women showed wild-type genotypes at all four *AMHR2* loci, making such an analysis statistically unsound.

Discussion

In assisted reproduction, response to exogenous FSH for ovulation induction is highly variable and unpredictable among individuals [24–26]. A marker that can predict a hyper or hypo response to exogenous FSH in COH would prove to be a beneficial clinical asset. Levels of serum FSH, inhibin β, 17-β-estradiol, and AMH and/or ultrasound-based measures such as ovarian volume, AFC, and ovarian stromal blood flow have been proposed as predictors of ovarian response [27]. In addition, measurement of serum AMH has also been proposed as a potential marker to predict ovarian reserve [8] and response to FSH [8]. *AMH*, also called Mullerian-inhibiting substance, is a dimeric glycoprotein and a member of the transforming growth factor beta family of secreted signalling proteins [28]. *AMH* is synthesized by the granulosa cells of pre-antral and small antral follicles in the ovary and is expressed continually in the growing follicles until these follicles have reached a size of 4–6 mm and a differentiation state at which *AMH* becomes receptive for exogenous FSH [29, 30].

In human granulosa cells, AMH through its action via AMHR2 leads to inhibition of FSH-induced adenylyl cyclase activation, aromatase expression, and E2 production [31] suggesting that AMH plays an inhibitory role in the recruitment of primordial follicles while FSH promotes the recruitment of primordial follicles. Absence of AMH may thus lead to a prematurely exhausted follicle pool (the follicles may become more sensitive to FSH and be selected for dominance early) and disorders in the *AMH/AMHR2* signalling pathway may disrupt the AMH-FSH balance. Indeed, studies in normo-ovulatory women have demonstrated an association of *AMH* gene polymorphisms with estradiol levels during the follicular phase of the menstrual cycle, suggesting

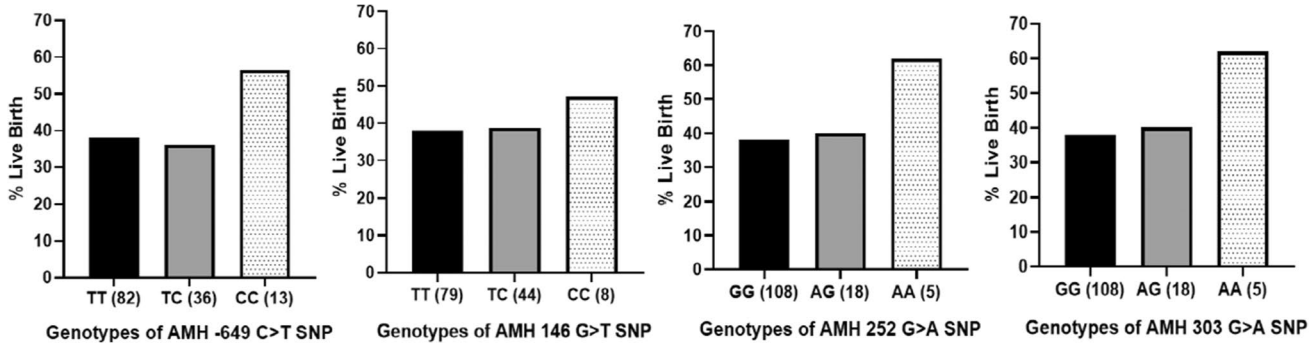

Fig. 3 Live birth rate in women undergoing IVF treatment based on different genotypes of the *AMH* gene

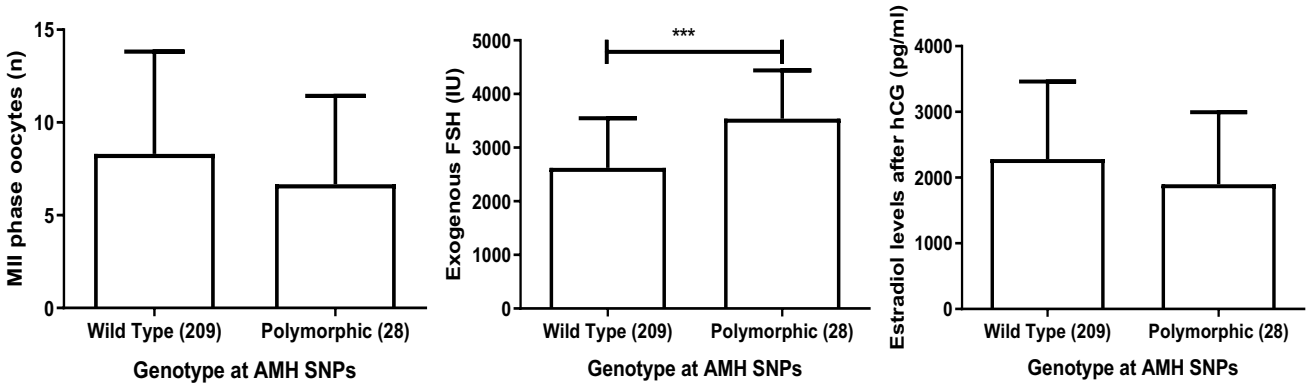

Fig. 4 Exogenous FSH, estradiol levels after ovulation induction, and number of MII phase oocytes in women showing wild-type and polymorphic genotypes at the different *AMH* genes (*** indicates $p < 0.001$)

that AMH plays a role in regulating FSH sensitivity [16]. Thus, genetic variations in *AMH* and *AMHR2* genes may influence the hormonal function in folliculogenesis, resulting in infertility.

Among the different polymorphisms in the *AMH* and *AMHR2* genes, the most studied are those in the c. 146 T > G position of the *AMH* gene and the c. -482 A > G position of the *AMHR2* gene. These polymorphisms are reportedly more common in women with PCOS [11, 14] and are associated with follicular phase E2 levels [16], menopausal age related to parity [32, 33], and infertility [17]. The *AMH* c. 146 polymorphism is associated with lower number of retrieved oocytes [13], higher basal FSH levels, and lower fertilization rates [19]. The *AMHR2* c. -482 A > G polymorphism positively correlates with serum FSH and number of oocytes visualized at ultrasound [12, 34]. Other *AMHR2* polymorphisms are positively associated with AMH, estradiol, and FSH levels [9], AFC and degree of oocyte maturation, and number of embryos produced in women undergoing ART [9]. However, there are inconsistencies in these findings [14, 35]. In this study, we have investigated the association of *AMH* and *AMHR2* polymorphisms with ovarian response in the subjects undergoing ART.

We first evaluated the frequency distributions of all four *AMH* and *AMHR2* polymorphisms in women undergoing ART. Genotype frequencies for *AMH* c. -649 T > C and c. 146 T > G and *AMHR2* (c. 482 A > G, c. 622–6 C > T, c. 4952 G > A, c. 10 A > G) polymorphisms observed in this study were in accordance with the Hardy–Weinberg equilibrium. On evaluating the clinical, endocrinological, and ultrasonographic parameters of women undergoing ART in our study, we found lower numbers of preovulatory follicles in women having a polymorphic genotype at c. -649 T > C and c. -146 T > G positions of the *AMH* gene. Interestingly, in our study, the amount of exogenous FSH required for ovulation induction in women with the polymorphic genotype at *AMH* c. -649 and c. -146 positions was greater than that required by women with wild-type genotype. This suggests that the presence of these polymorphisms confers higher resistance to exogenous FSH. Indeed, studies have shown that the polymorphic genotype of *AMH* c. -146 is less effective in reducing individual FSH sensitivity of antral follicles with in vitro studies demonstrating that mutant protein bioactivity is reduced compared with the wild-type protein [16]. This is because the c. -146 polymorphism lies in the *AMH* gene promoter, a region responsible for protein stability and folding (isoleucine to serine change in position 49 of the AMH protein).

Wu et al. [38] have reported that women showing polymorphic genotype at *AMH* c. 146 position were half as likely to have a clinical pregnancy compared with women with wild-type genotype (OR = 0.55, 95% CI: 0.34–0.88, $p = 0.014$) and had a lower clinical pregnancy rate (T/T: 55.0%, T/G:51.8%, G/G: 40.0%; $p < 0.05$). In our study, we also found that women with a polymorphic genotype were half as likely of achieving pregnancy as compared to those women showing a wild-type or heterozygous genotype at *AMH* c. 146 position.

AMH exerts its biological activity via two receptors, AMHRI and AMHRII (*AMHR2*). Upon ligand binding, AMHR2 recruits and phosphorylates AMHRI, which, in turn, induces subsequent downstream activation of effector proteins such as Smads 1, 5, and 8. The Smads act as messengers of hormonal signal to the cell nucleus. It is thought that the presence of *AMHR2* polymorphisms interferes in this signalling pathway. In our study, we were unable to identify any statistically significant difference in endocrinological and ultrasonographic parameters of women undergoing ART women with polymorphisms in the *AMHR2* gene. However, we observed that none of the women showing a homozygous polymorphic genotype at all four *AMHR2* polymorphisms had a positive clinical pregnancy, i.e., live birth. This suggests that the *AMHR2* polymorphisms investigated in our study are associated with negative pregnancy

outcomes in our study population. However, a study from Taiwan has reported positive pregnancies in women with the *AMHR2* − 482 A > G and *AMHR2* IVS1 + 149 T > A polymorphisms [36]. Studying a larger sample size will prove to be beneficial in deciphering the role of *AMHR2* polymorphisms on ovarian response.

Lastly, we examined the effect of wild-type and polymorphic alleles at different polymorphic sites in the *AMH* gene on number of mature oocytes retrieved, levels of exogenous FSH required for ovulation induction, and estradiol levels after hCG administration. We found that higher amounts of recombinant FSH were required to induce ovulation in women with a polymorphic genotype at one or more *AMH* loci as compared to women having a wild-type genotype at these *AMH* loci. These findings suggest that the effect of *AMH* polymorphisms is largely restricted to exogenous FSH dosage and not on other endocrinological or ultrasonographic parameters.

Ours is a prospective observational study and some of the probable reasons for discordance in our results and those reported by others could be regional or ethnic difference among the studies since different ethnicities may have different allelic frequencies. Another reason may be different genotyping techniques used; we have studied the genotype of the *AMH* and *AMHR2* genes by direct DNA sequencing while others [9, 22] have used TaqMan or PCR-based assays. A smaller sample size, absence of a control cohort, and differences in the age of the participants and types of treatment protocols could also have contributed to the high heterogeneity in results.

Conclusion

Among women of Indian ethnic background, polymorphisms in *AMH* c. -649 and c. 146 are associated with increased exogenous FSH requirements for ovulation induction while the *AMH* c. 252 and c. 303 polymorphisms show lack of association with ovarian response in this study. Genotyping of the *AMHR2* polymorphisms may be useful as a predictor of ovarian reserve or ovarian response and treatment outcomes.

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• Data regarding any of the subjects in the study has not been previously published unless specified.
• Data will be made available to the editors of the journal for review or query upon request

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