Association study of *AMH* and *AMHRII* polymorphisms with unexplained infertility

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Objective: To investigate the association of *AMH* and *AMHRII* polymorphisms with reproductive abilities in a sample of women with idiopathic infertility.
Design: Case-control study.
Setting: University Department of Obstetrics and Gynecology, and University Unit of Clinical Genetics.
Patient(s): 76 women with idiopathic sterility and 100 fertile women as controls.
Intervention(s): Genotyping was performed by high-resolution melt analysis.
Main Outcome Measure(s): Genotype distribution and allele frequency of *AMH* and *AMHRII* polymorphisms. Reconstruction of haplotype alleles to evaluate the linkage disequilibrium between single nucleotide polymorphisms.
Result(s): Allele frequencies of –482 A>G, IVS 5–6 C>T, IVS 10+77 A>G, 146T>G polymorphisms are statistically significantly different in infertile patients compared with controls.
Conclusion(s): Genetic variants of *AMH* and AMHRII genes seem to be associated with infertility, suggesting a role in the pathophysiology of normo-estrogenic and normo-ovulatory infertility. A clearer understanding of their function in ovarian physiology may help clinicians to find a role for antimüllerian hormone measurement in the field of reproductive medicine. (Fertil Steril® 2010;94:1244–8. ©2010 by American Society for Reproductive Medicine.)

Key Words: Antimüllerian hormone, AMH receptor, high-resolution melt analysis, polymorphisms, unexplained infertility

Infertility is an increasing health problem not only in developed countries where one couple out of six undergoes assisted reproduction techniques, but also in developing countries according to more recent reports (1). According to the World Health Organization, in 10% to 15% of cases the cause remains unknown (2, 3). The study of polymorphisms of the genes regulating female reproductive function can help to clarify mechanisms of gonadic function and fertility in humans.

Antimüllerian hormone (AMH), a member of the transforming growth factor-β family, is involved in the regulation of follicular growth (4). It is produced by the granulosa cells of early developing follicles in the ovary. It continues to be expressed in the growing follicles until they have reached the appropriate size and differentiation state, at which point they are selected for dominance by the action of follicle-stimulating hormone (FSH) (5, 6). Studies in *AMH* null mice have demonstrated that, in the absence of AMH, follicles are recruited at a faster rate, and they are more sensitive to FSH. (7). This expression pattern suggests that AMH can inhibit both the initiation of primordial follicle growth and the FSH-induced follicle growth. Therefore, AMH plays an important role in regulating both the primordial follicle recruitment and the cyclic selection (8, 9).

Furthermore, recent studies have demonstrated that serum AMH levels reflect the size of the primordial follicle pool; in fact, they correlate strongly with the number of antral follicles and show a reduction throughout reproductive life (10, 11). Thus, AMH could be a good marker of ovarian aging and thus be of clinical importance in women undergoing in vitro fertilization (IVF) treatment (12, 13).

Recent studies in normo-ovulatory women have demonstrated an association of the polymorphisms of *AMH* and its receptor *AMHRII* with estradiol levels during the early follicular phase of the menstrual cycle, which suggests a role of AMH in the regulation of FSH sensitivity (14). Therefore, genetic variants in the *AMH* and *AMHRII* genes may influence hormone function in folliculogenesis causing infertility. We performed an observational study to investigate the correlation between *AMH* and *AMHRII* polymorphisms and reproductive abilities in a sample of women with idiopathic infertility.

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MATERIALS AND METHODS

Patients

We included in our study Caucasian women who presented to the Departments of Obstetrics and Gynecology of the Padova University and Chieti University between March 2007 and

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TABLE 1

Description of *AMH* and *AMHRII* polymorphisms in this study, primers sequences, amplicon size (bp), and annealing temperature.

Gene	Base pair change	Position (aa change)	Frequency reported in literature, %	Primers	Base pair	Temperature	Reference
AMH	146T>G	Exon 1 (I49S)	12	FOR: ACCAGTGGCCTCATCTTCC REV: AGGAAGGCCTGCTCATAGG	152 bp	59°C	(14, 18)
AMHRII	–482 A>G	Promotor	13	FOR: GGTAACCTCTAATATGGGCTGTG REV: TCTCAGGAGGAAACCAATGTG	150 bp	59°C	(14)
AMHRII	IVS1+149 T>A	Intron 1 (–)	13	FOR: CCCTTTGGAAGAGTGGTGAG REV: AGGTGGGAGTGAATGCAGAG	196 bp	56°C	(14, 19)
AMHRII	IVS 5-6 C>T	Intron5 (–)	19	FOR: AGCTGTGTTTCTCCCAGGTG REV: ATGGCAACCAGTTTTCCTTG	197 bp	56°C	(14, 19)
AMHRII	IVS10+77 A>G	Intron 10 (–)	15	FOR: AAGAGGCCTAGGCTGTTGGT REV:CAAGGTTGAGCAGGAGGAAG	241 bp	60°C	(14, 19)

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June 2008 for infertility treatment. We considered a couple to be infertile if the woman had not conceived after 24 months of contraceptive-free intercourse if the woman was aged <35 years or after 12 months if the woman was aged >34 years.

All couples underwent a full infertility investigation protocol (15–17). The women underwent hormonal assessment to evaluate their ovulatory cycles, thyroid function, and prolactin and androgen circulating levels. Ovarian reserve was detected through measurement of serum FSH and an antral follicle count on the third day of the menstrual cycle. Infections, including hepatitis, human immunodeficiency virus (HIV), TORCH complex, *Helicobacter pylori*, *Chlamydia trachomatis,* and *Mycoplasma* infections were investigated by blood samples and vaginal tampons. Finally, all women underwent a complete genetic screening, including chromosomal analysis, *CTFR* mutation screening, and hematologic screening that included thrombophilic polymorphisms.

The men underwent at least two semen analyses according to the World Health Organization criteria as well as measurements of testosterone, luteinizing hormone (LH), and FSH serum levels. They also underwent chromosomal analysis, *CTFR* mutation screening, and Y chromosome molecular analysis. Finally, we excluded infections by use of a blood sample and an uretral tampon.

The couples in whom one of the partners presented an anomaly in one or more of the above tests were excluded from this study. All couples presenting with normal results to our diagnostic protocol were considered to have unexplained infertility and were included in the analysis.

A control group of 100 Caucasian women was casually recruited from among women who delivered infants in our units at the same time an infertile couple was diagnosed and included in the study. The inclusion criteria for the control group were the following: age ± 1 year of the index woman, no history of subinfertility (they had conceived after 6 to 12 months of free-contraceptive intercourse), and no more than one previous spontaneous abortion.

All patients and controls included in the study were aged between 28 and 38 years. The study was approved by the institutional review board (headed by Dr. C. Giaquinto). All the couples and control women gave written informed consent.

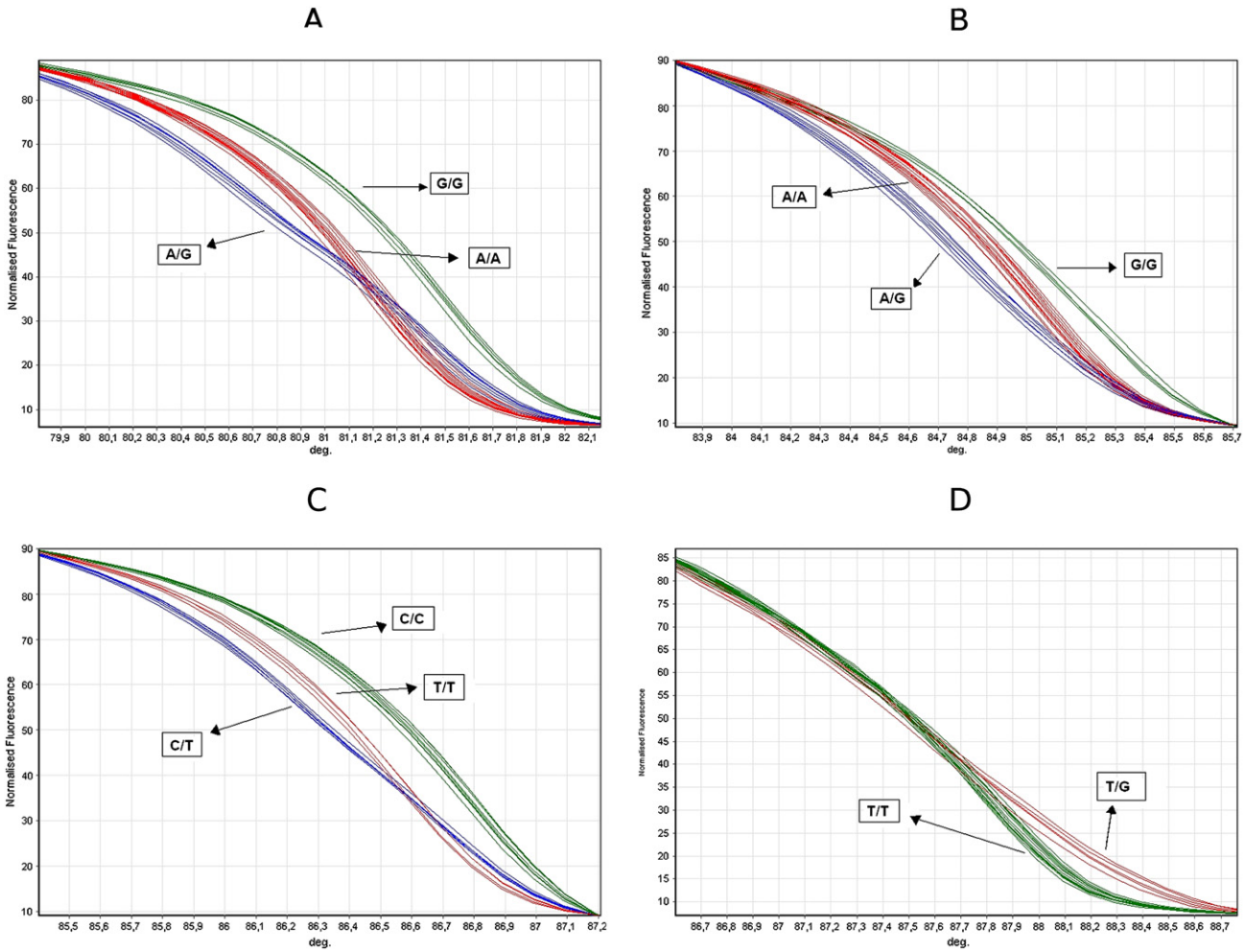
Genotyping

Genomic DNA was extracted from peripheral blood using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany). The polymorphisms examined are found in Table 1. Primers were designed using the OpenSource Primer 3 software (http://primer3.sourceforge.net). The primers sequences and amplicon information can be found in Table 1. Genotypes were determined by high-resolution melt analysis (HRM) (20–22).

The polymerase chain reaction (PCR) cycling and HRM analysis were carried out sequentially on a Rotor Gene (Corbett Research, Mortlake, Australia) using Takara Ex Taq

FIGURE 1

Rotor Gene 6000 HRM normalized graph. (**A**) *AMHRII* –482 A>G polymorphism; green, homozygous for the G allele (GG); red, homozygous for the A allele (AA); blue, heterozygous (AG). (**B**) *AMHRII* IVS 10+77 A>G polymorphism; green, homozygous for the G allele (GG); red, homozygous for the A allele (AA); blue, heterozygous (AG). (**C**) *AMHRII* IVS5-6 C>T polymorphism; green, homozygous for the C allele (CC); red, homozygous for the T allele (TT); blue, heterozygous (CT). (**D**) *AMH* polymorphism 146 T>G; green, homozygous for the T allele (TT); red, heterozygous (TG). Y axis, normalized fluorescence; X axis, temperature (°C).



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R-PCR custom (Takara Bio Europe SAS, Saint-Germain-en-Laye France). The PCR was made up in a 25-μL total volume containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 300 nM of each primer, 1.5 μM EvaGreen, 1.25 IU Taq DNA polymerase (Takara Bio), and 3×10^9 copies/μL DNA.

The HRM analysis was performed with the temperature ramping (70° to 95°C, rising by 0.1°C) and fluorescence acquisition setting recommended by the manufacturer. The melting curves were normalized through calculation of the "line of best fit" of two normalization regions before and after the major fluorescence drop, corresponding to the melting of the PCR product using the software provided with the Rotor Gene 6000 (Corbett Research).

The HRM can detect DNA sequence variations but does not succeed in their characterization, so initially we did an analysis of 10 samples for each studied polymorphism. We obtained three different melting profiles for each except one (*AMH* 146 T>G). We sequenced two samples for each melting profile of each polymorphism, confirming that different profiles corresponded to different sequences.

Sequence analysis revealed that heterozygotes and homozygotes have characteristic melting profiles that give rise to differently shaped melting curves. Therefore, in the following HRM analyses we used the curves of homozygotes and heterozygotes that were confirmed by sequencing as the reference for the genotype analysis of the unknown samples.

TABLE 2

Absolute, allele, and genotype frequencies of *AMH* and *AMHRII* polymorphisms.

	Ratio counts		Frequencies			
Polymorphism	**Case**	**Control**	**Case**	**Control**	**Chi square**	***P* value**
146 T>G (AMH)						
T:G	140:12	170:30	0.921	0.855	5.34	.020
T/T, T/G, G/G	64:12:0	70:30:0	0.8421:0.1579:0.0	0.7:0.3:0.0	7.3	.00815
–486 A>G (AMHRII)						
T:G	130:22	157:43	0.855	0.785	4.44	.035
A/A, A/G, G/G	56:18:2	63:31:6	0.737:0.237:0.026	0.63:0.31:0.06	4.12	.127
IVS1+149 T>A (AMHRII)						
T:A	130:22	164:36	0.855	0.820	1.28	.2579
T/T, T/A, A/A	56:18:2	68:28:4	0.737:0.237:0.026	0.68:0.28:0.04	1.21	.546
IVS5-6 C>T (AMHRII)						
C:T	130:22	157:43	0.855	0.785	4.44	.035
C/C, C/T, T/T	56:18:2	63:31:6	0.737:0.237:0.026	0.63:0.31:0.06	4.12	.127
IVS10+77 A>G (AMHRII)						
A:G	130:22	157:43	0.855	0.785	4.44	.035
A/A, A/G, G/G	56:18:2	63:31:6	0.737:0.237:0.026	0.63:0.31:0.06	4.12	.127

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The PCR products were sequenced using the Big Dye Terminator kit (Applied Biosystems Inc., Carlsbad, CA) and resolved on a 310 ABI Prism genetic analyzer (Applied Biosystems Inc.).

Statistical Analysis

Genotype distributions in patients and controls were tested for Hardy-Weinberg equilibrium, and differences in genotype frequencies between patients and control group were tested using a chi-square test for independence. Analysis of variance (ANOVA) was performed to determine differences between genotypic groups.

Haplotypes of *AMHRII* gene were constructed using the PHASE program, version 2.1 (http://www.stat.washington.edu/stephens/software.html). This program implements a Bayesian statistical method for reconstructing haplotypes from population genotype data. It provides an estimation of the most probable haplotypic combinations for each individual, and it allows for the testing of the H_0 hypothesis (cases = controls) (23).

The pair-wise linkage disequilibrium coefficient (D') and the correlation coefficient (r^2) were calculated by Haploview version 3.2 (http://www.broad.mit.edu/mpg/haploview) to estimate the linkage disequilibrium between single nucleotide polymorphisms (SNPs). The software also enables case-control associations and evaluation of chi-square values as well as the haplotype frequencies for each haplotype observed in the target population.

RESULTS

All genotypes except SNP IVS 1 + 149 of the *AMHRII* gene (homozygotes and heterozygotes) were easily identified by HRM analysis and sequencing.

The first analysis with HRM of the SNP in intron 1 of *AMHRII* gene (IVS1+149 T>A) showed that homozygotes T/T and homozygotes A/A had the same melting temperature. Therefore, a complete genotyping was possible by adding a known homozygote T/T DNA to the unknown samples. The homozygotes T/T did not change their melting curves adding exogenous DNA, but homozygotes A/A produced heteroduplexes, which can be correctly identified. The different plots produced by the HRM analysis are shown in Figure 1.

A novel T>C transition in *AMHRII* promoter at position –450 relative to the ATG start codon has been identified in two controls through HRM analysis and sequencing. Table 2 shows the absolute allele and genotype frequencies observed in the target population. Genotype distributions in patients and controls were in Hardy-Weinberg equilibrium.

Allele frequencies of *AMHRII* gene polymorphisms (–482 A>G, IVS 5–6 C>T, IVS 10+77 A>G) were statistically significantly increased in infertile patients compared with controls ($P<.05$). The IVS 1 + 149 T>A polymorphism showed no differences in allele frequency between patients and controls. The genotype frequencies of the *AMHRII* gene polymorphisms were similar in distribution for patients and controls. Analysis of the *AMH* gene polymorphism (146T>G) showed increased frequencies of the T allele and T/T genotype in patients compared with controls ($P<.05$) (Table 2).

Multivariate ANOVA found no correlation between *AMH* and *AMHRII* genotype distributions ($P=.235$).

Haplotype analyses performed for *AMHRII* gene using Phase and Haploview software showed no statistically significant differences ($P>.05$) between patients and controls for

haplotype frequencies. There was a complete linkage disequilibrium between the polymorphisms –482 A>G, IVS 5–6 C>T, IVS 10+77 A>G (D' = 1, LOD = 58.38, r^2 = 1), but the polymorphism IVS1+149 T>A was not in complete linkage disequilibrium with the others SNPs (D' = 1, LOD = 45.52, r^2 = 0.871) in contrast with previous findings (14).

DISCUSSION

We analyzed the polymorphisms in two genes of the AMH signal transduction pathway (i.e., *AMH* and its specific type II receptor *AMHRII*) in women presenting with idiopathic infertility. In the ovary, AMH is produced by granulosa cells of early developing follicles and seems to be able to inhibit the initiation of primordial follicle growth and FSH-induced follicle growth. *AMH* polymorphisms could modify hormone biological activities, playing a role in controlling follicle recruitment and growth, and finally in inducing infertility.

The HRM technique we used in this study enables a clear distinction to be made between the homozygous and heterozygous genomic DNA samples. Thus, HRM represents a great promise for the future because it enables rapid genotyping of individual polymorphic loci. Also, HRM characterizes samples in an easier, less expensive way than probes based on genotyping assays; unlike conventional methods, HRM does not require post-PCR processing.

The results of our analyses show that *AMH* and *AMHRII* polymorphisms, except IVS 1 + 149 T>A, are more frequent in infertile women. Allele frequencies of –482 A>G, IVS 5–6 C>T, IVS 10 + 77 A>G polymorphisms are statistically significantly increased in the infertile women compared with the controls ($P<.05$), but the genotype frequencies are similarly distributed for patients and controls; thus, an increment in the sample size is necessary for definitive conclusions.

Because AMH has an inhibitory effect on the FSH-sensitivity of follicles, polymorphisms on the *AMH* gene or its receptor *AMHRII* might reduce the biological activity of the hormone. Therefore, follicles might be more sensitive to FSH and might be previously selected for dominance (14). Reconstruction of haplotype alleles in the Phase and Haploview programs revealed that *AMHRII* polymorphisms are not in complete linkage disequilibrium as had been previously reported in the literature (14).

In conclusion, genetic variants of *AMH* and *AMHRII* genes seem to be associated with infertility, suggesting their role in the pathophysiology of normoestrogenic and normo-ovulatory infertility. A clearer understanding of their function in ovarian physiology may help clinicians to find a role for AMH measurement in the field of reproductive medicine.

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