Effect of growth differentiation factor-9 C447T and G546A polymorphisms on the outcomes of *in vitro* fertilization

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Abstract. Single nucleotide polymorphisms (SNPs) in the growth differentiation factor (*GDF*)-*9* gene are associated with premature ovarian failure, insufficient ovarian stimulation and a poor *in vitro* fertilization (IVF) score in women with diminished ovarian reserve. The aim of the present study was to assess the effect of C447T (rs254286) and G546A (rs10491279) SNPs on ovary stimulation response, oocyte quality, fertilization rate and outcome of clinical pregnancy in an infertile population of Polish females (n=86) treated with IVF. The present study also included a group of fertile women (n=202). The P-trend value, calculated for the *GDF-9* C447T transition in infertile women, was statistically significant and were equal to 0.0195. A significant association of the *GDF-9* C447T SNP was observed with infertility for the C/C vs. T/T + C/T model (OR= 2.140; 95% CI=1.043-4.393; P=0.0349). The *GDF-9* G546A SNP was significantly associated with the G/A vs. G/G model with poor ovarian stimulation (OR=9.303; 95% CI=2.568-33.745; P=0.0008) and poor fertilization rate (OR=2.981; 95% CI=1.033-8.607; P=0.0385). For the *GDF-9* C447T SNP, a significant association was observed between the C/C + C/T vs. T/T model and a poor ovarian stimulation response (OR=15.309; 95% CI=0.875-267.83; P=0.0078), and a poor fertilization rate (OR=4.842; 95% CI=1.310-17.901; P=0.0121). The present genetic evaluation revealed associations between IVF outcomes and the *GDF-9* A546G and C447T SNPs. Additionally, these results indicated that the *GDF-9* C447T SNP is a possible candidate genetic risk factor for female infertility in the Polish population.

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Introduction

Previous studies have demonstrated that ~17% of couples are unable to conceive (1). *In vitro* fertilization (IVF) is an efficient assisted reproductive medical technology that is used to treat infertility. The objective of IVF is a full-term pregnancy and the success of IVF is influenced by several factors (2). One of the essential elements of successful IVF treatment is the number of eggs produced following controlled ovarian hyperstimulation with gonadotropins and GnRH analogues (3). However, poor ovarian responses (PORs) have been demonstrated in 9-24% of all IVF cases (4). The mechanism of follicular depletion in a POR remains to be fully understood. Some of the causes of POR include ovarian surgery, single ovary, autoimmune diseases, chemotherapy, radiotherapy, cigarette combustion, unknown infertility and diminished ovarian reserve (DOR) (5-12). A DOR is characterized by a reduction in both oocyte quantity and quality, whereby the oocytes produced are unable to be fertilized, therefore, reducing the possibility of a healthy and successful pregnancy in women of childbearing age (13). The occurrence of DOR is associated with aging, premature ovarian failure and menopause (13,14). It is suggested that ~10% of women suffering from infertility exhibit a DOR (15,16). Infertility and POR are also strongly influenced by genetic background (17).

Several factors regulate follicular growth and depletion. These include bone morphogenetic protein (BMP)-15, anti-Müllerian hormone (AMH), G-protein coupled receptor (GPR)3, newborn ovary homeobox protein and growth differentiation factor (GDF)-9 (18-20). Single nucleotide polymorphisms (SNPs) of these genes are associated with abnormal follicular function and reduced female fertility (21).

GDF-9 contributes to ovarian folliculogenesis as an essential molecule, which controls various granulosa cell processes and ovulation rate. GDF-9 supports proliferation of granulosa cells and the growth of cumulus cells, the halt of follicular apoptosis, and the growth of the oocyte and embryo (22-25).

GDF-9 variants are associated with premature ovarian failure (POF), aberrant follicular growth and the loss of oocytes (26-28). In addition, novel SNPs located in *GDF-9* have been correlated with insufficient ovarian stimulation and a poor IVF score in women with a DOR (29). The

aim of the present study was to evaluate the effects of the C447T (rs254286) and G546A (rs10491279) SNPs on ovary stimulation response, oocyte quality, fertilization rate and the outcome of clinical pregnancy in infertile women treated with IVF.

Materials and methods

Patients. Peripheral blood samples from 88 infertile women treated with an IVF procedure and 202 fertile control women were obtained from the Division of Infertility and Reproductive Endocrinology, University of Medical Sciences (Poznan, Poland). Inclusion criteria for the infertile women included individuals aged <35 with a regular menstrual cycle (28±7 days), a serum concentration of follicle stimulating hormone (FSH) <12 IU/ml at the early follicular phase (menstrual cycle day 2-4), an anatomically intact reproductive tract and infertility spanning at least 2 years, despite the desire and attempts to conceive. The exclusion criteria included male factor infertility, body mass index >25, irregular menstrual cycle (<21 or >35 days), concentration of FSH >12 IU/ml, diabetes, polycystic ovarian syndrome (PCOS), endocrine disorders, habitual smoking of cigarettes and receiving hormone treatment during the 3 months prior to the present study. The exclusion criteria also included ultrasonography (USG)-determined endometriosis, ovarian cysts with diameter >3 mm, fibroids and hydrosalpinx.

The fertile women were aged <35 and gave birth to a child during the 12 months prior to the present study, with a maximum 12 months of desire for conception. These individuals also exhibited regular menses, an anatomically intact reproductive tract and were without any malignancies, endometriosis, or adenomyosis determined during a cesarean section. The fertile and infertile women were matched by age and were all Caucasians of Polish descent (Table I).

Evaluation of ovarian reserve. The concentration of FSH, AMH and Inhibin B were determined in the blood plasma at the early follicular phase (days 2-4 of the menstrual cycle) during the 3 months prior to starting the IVF procedure. Two independent researchers determined the antral follicle counts with a diameter of 2-10 mm during a transvaginal ultrasound examination with a frequency of 7.5 MHz using the Hitachi Aloka's Prosound Alpha 7 instrument (Hitachi Aloka, Wallingford, CT, USA).

Controlled hyperstimulation of the ovaries. The IVF technique performed included the standard long protocol of controlled ovarian stimulation. Briefly, the infertile women were treated with a GnRH analogue (Gonapeptyl 0.1; Ferring Pharmaceuticals, Kiel, Germany). This treatment was initiated on day 21 of the menstrual cycle, preceding the hyperstimulation of the ovaries. Pituitary desensitization was determined by the evaluation of luteinizing hormone (LH) <2 IU/l and 17β-estradiol <50 pg/ml in the peripheral blood plasma, USG assessment of endometrial thickness <5 mm and the lack of an ovarian follicle with a diameter >10 mm. Next, the women were treated with a subcutaneous injection of gonadotropin Gonal F (Merck Serono, Geneva, Switzerland) and/or Menopur (Ferring Pharmaceuticals). The dose of these gonadotropins was dependent on the patient response to USG ovary examination and levels of 17β-estradiol in the peripheral blood plasma. This evaluation commenced from the fifth day of stimulation and ended at the time of human chorionic gonadotropin (hCG) administration. When at least three leading follicles reached 17 mm in diameter, 250 μg hCG (Merck Serono Europe) was used. Premature luteinizing was excluded by assessment of progesterone levels (<1.5 ng/ml). Oocyte retrieval was performed under transvaginal USG guidance 36 h following hCG injection. Oocytes were classified as mature if they were in metaphase II (MII) or immature if they were in metaphase I (MI), or the germinal vesicle stage. After 16-18 h of fertilization intracytoplasmic sperm injection, the quality of the embryos was assessed by the presence of two pronuclei, according to the Alpha Scientists of the Reproductive Medicine and ESHRE Special Interest Group of Embryology (30). After fertilization (72 h) the embryos were transferred into the patient. Supernumerary embryos were evaluated on the fifth day of culture and subjected to a process of vitrification freezing. The patients with embryos were vaginally supplemented with 600 mg progesterone Luteina daily (Adamed, Pieńków, Poland). Pregnancy was confirmed 14 days after embryo transfer using hCG levels in the peripheral blood plasma. Clinical pregnancies were determined by USG examination during the sixth week of pregnancy. POR was designated as the retrieval of ≤3 oocytes and was determined at cycle cancellation. Oocytes of good quality were defined as ≥75% oocytes in metaphase II. The presence of two pronuclei was considered to indicate fertilization. Good fertilization rate was considered as at least 50% fertilization rate was achieved on the third day. Informed consent was obtained in writing from all individuals. The Local Ethical Committee of Poznan University of Medical Sciences approved the procedures used in the present study.

Genotyping. DNA was isolated from the peripheral leucocytes using a salting out procedure (31). A DNA fragment (509 bp) covering the fragment of intron 1 (final 57 bp) and a fragment of exon 2 (first 452 bp) of the GDF-9 gene was amplified using the primers, 5'-CTGCCTGTTGTGTTG ACTGA-3' and 5'-ATAGTGAAGGGAATACCAGC-3'. The *GDF-9* C447T (rs254286) and G546A (rs10491279) polymorphisms were subsequently determined by a Sanger sequencing analysis at the Laboratory of DNA Sequencing and Oligonucleotides Synthesis, Institute of Biochemistry and Biophysics, The Polish Academy of Science (Warsaw, Poland). The results of sequencing were evaluated using FinchTV v.1.4.0 software (Geospiza, Inc., Seattle, WA, USA).

Statistical analysis. For each SNP, the Hardy-Weinberg equilibrium (HWE) was assessed using Pearson's goodness-of-fit χ^2 statistic. The association between the selected SNPs and the analyzed groups was evaluated using the Cochran-Armitage P-trend test (P_{trend}), χ^2 or Fisher tests. The Odds ratios (OR) and associated 95% confidence intervals (CI) were also assessed. The data were analyzed using recessive and dominant inheritance models. P<0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed using Statistica version 10.2011 (StatSoft, Inc., Tulsa, OK, USA).

Table I. Clinical characteristics of infertile and fertile females.

Characteristic	Infertile	Fertile
Number	88	202
Median age, years (range)	31 (23-34)	31 (22-34)
Parity	NA	1 (1-2)
Duration of infertility, years (range)	3 (1-6)	NA

NA, not applicable.

Table II. Distribution of *growth differentiation factor-9* G546A (rs10491279) single nucleotide polymorphism in fertile (n=202) and various subgroups of infertile females (n=88) treated with IVF.

	Genotype number (%)			
Patient/subgroup	G/G	G/A	Adjusted Odds ratio (95% CI)[b]	P-value
Fertile females	165 (81.7)	37 (18.3)	1.228 (0.660-2.284)[c]	0.516[a]
Infertile females treated with IVF				
Good responders	64 (72.7)	11 (12.5)	**9.303 (2.568-33.745)[d]**	**0.0008[b]**
Poor responders	5 (5.7)	8 (9.1)		
Good quality oocytes	47 (53.4)	10 (11.4)	1.923 (0.684-5.404)[e]	0.211[a]
Poor quality oocytes	22 (25.0)	9 (10.2)		
Good fertilization rate	53 (60.2)	10 (11.4)	**2.981 (1.033-8.607)[f]**	**0.0385[a]**
Poor fertilization rate	16 (18.2)	9 (10.2)		
Positive pregnancy	38 (43.2)	10 (11.4)	1.103 (0.399-3.054)[g]	0.850[a]
Negative pregnancy	31 (35.2)	9 (10.2)		

[a]χ^2; [b]Fisher's exact test; [c]Fertile vs. infertile females; [d]Poor vs. good responders; [e]Poor vs. good quality oocytes; [f]Poor vs. good fertilization rate; [g]Negative vs. positive pregnancy. Statistically significant data are highlighted in bold. No A/A genotype was identified in infertile or fertile females. Good responders ≥4 oocytes; good quality oocytes ≥75% oocytes in metaphase II; good fertilization rate ≥50% fertilization at third day; IVF, *in vitro* fertilization; CI, confidence intervals.

Results

Contribution of GDF-9 G546A (rs10491279) and C447T (rs254286) to infertility. The values for the χ^2 test of HWE were equal to 0.358 and 0.525 for the *GDF-9* G546A SNP and 0.414 and 0.935 for the *GDF-9* C447T SNP in the infertile and fertile women, respectively. The frequency of genotypes and results of statistical analyses are shown in Tables II and III. No association between the *GDF-9* G546A SNP was observed with infertility in the dominant (G/A vs. G/G) inheritance model (OR=1.228; 95% CI=0.660-2.284; P=0.516). However, the P-trend value of 0.0195, calculated for the *GDF-9* C447T transition, was statistically significant. A contribution of the *GDF-9* C447T SNP was confirmed for infertility in the recessive (C/C vs. T/T + C/T) inheritance model (OR=2.140; 95% CI=1.043-4.393; P=0.0349). No association was observed between the *GDF-9* C447T SNP and infertility in the dominative (C/C + C/T vs. T/T) inheritance model (OR=1.608; 95% CI=0.944-2.740; P=0.0791).

Contribution of GDF-9 G546A (rs10491279) to ovary stimulation response, oocyte quality, fertilization rate and pregnancy outcome. The genotype distribution for good/poor responders, good/poor quality oocytes, good/poor fertilization rates and the outcome of clinical pregnancy for the *GDF-9* G546A SNP are shown in Table II. The A/A genotype was not identified in infertile and fertile women. A significant association between the dominant (G/A vs. G/G) inheritance model and the group of poor ovary stimulation responders (OR=9.303, 95% CI=2.568-33.745, P=0.0008) was demonstrated. An association was also demonstrated between the *GDF-9* G546A SNP with a poor fertilization rates for G/A vs. G/G (OR=2.981; 95% CI=1.033-8.607; P=0.0385). However, the *GDF-9* G546A SNP revealed no influence on poor quality oocytes (OR=1.923; 95% CI=0.684-5.404; P=0.211) and outcome of clinical pregnancy (OR=1.103; 95% CI=0.399-3.054; P=0.850).

Contribution of GDF-9 C447T (rs254286) to ovary stimulation response, oocyte quality, fertilization rate and pregnancy outcome. The genotype distribution for good/poor responders, good/poor quality oocytes, good/poor fertilization rates and the outcome of clinical pregnancy for the *GDF-9* C447T SNP are shown in Table III. For poor ovary stimulation responders, a P-trend of 0.256, calculated for the *GDF-9* C447T

Table III. Distribution of *growth differentiation factor-9* C447T (rs254286) single nucleotide polymorphism in fertile (n=202) and various subgroups of infertile females (n=88) treated with IVF.

	Genotype number (%)					
Patient/subgroup	T/T	T/C	C/C	Adjusted Odds ratio (95% CI)[b]	P-value	P-trend
Fertile females	84 (41.6)	99 (49.0)	19 (9.4)	**2.140 (1.043-4.393)[a,e]** 1.608 (0.944-2.740)[b,e]	**0.0349[c]** 0.0791[c]	**0.0195**
Infertile females treated with IVF						
Good responders	27 (30.7)	33 (37.5)	15 (17.1)	0.333 (0.0401-2.770)[a,f]	0.448[d]	0.256
Poor responders	0 (0.0)	12 (13.6)	1 (1.1)	**15.309 (0.875-267.83)[b,f]**	**0.0078[d]**	
Good quality oocytes	17 (19.3)	28 (31.8)	12 (13.6)	0.556 (0.163-1.898)[a,g]	0.400[d]	0.491
Poor quality oocytes	10 (11.4)	17 (19.3)	4 (4.6)	0.893 (0.348-2.292)[b,g]	0.813[c]	
Good fertilization rate	24 (27.3)	25 (28.4)	13 (14.8)	0.492 (0.128-1.896)[a,h]	0.375[d]	0.270
Poor fertilization rate	3 (3.4)	20 (22.7)	3 (3.4)	**4.842 (1.310-17.901)[b,h]**	**0.0121[d]**	
Positive pregnancy	14 (15.9)	24 (27.3)	10 (11.4)	0.671 (0.220-2.041)[a,g]	0.584[d]	0.534
Negative pregnancy	13 (14.8)	21 (23.9)	6 (6.8)	0.855 (0.345-2.122)[b,g]	0.736[c]	

[a](C/C vs. C/T + T/T); [b](C/C + C/T vs. T/T); [c]χ^2; [d]Fisher's exact test; [e]Fertile vs. infertile females; [f]Poor vs. good responders; [g]Poor vs good quality oocytes; [h]Poor vs. good fertilization rate; [g]Negative vs. positive pregnancy. Statistically significant data are highlighted in bold. Good responders ≥4 oocytes; good quality oocytes ≥75% oocytes in metaphase II; good fertilization rate ≥50% fertilization at third day; IVF, *in vitro* fertilization; CI, confidence intervals.

SNP, was not statistically significant. However, a significant association with poor ovarian stimulation responders in the dominant (C/C + C/T vs. T/T) inheritance model (OR=15.309; 95% CI=0.875-267.83; P=0.0078) was revealed. For the poor fertilization rate, a P-trend of 0.270, calculated for the *GDF-9* C447T SNP, was not statistically significant. However, a significant association was observed with a poor fertilization rate in the dominant (C/C + C/T vs. T/T) inheritance model (OR=4.842; 95% CI=1.310-17.901; P=0.0121). No significant contribution of the *GDF-9* C447T SNP was revealed in the recessive (C/C vs. C/T + T/T) inheritance model to poor ovarian stimulation response (OR=0.333; 95% CI=0.0401-2.770; P=0.448) or a poor fertilization rate (OR=0.492; 95% CI=0.128-1.896; P=0.375). Additionally, no association of the *GDF-9* C447T SNP was demonstrated with poor quality oocytes (P_{trend}=0.491), for the recessive (C/C vs. C/T + T/T) inheritance model (OR=0.556; 95% CI=0.163-1.898; P=0.400) and for the dominant (C/C + C/T vs. T/T) inheritance model (OR=0.893; 95% CI=0.348-2.292; P=0.813). In addition, no involvement of the C447T SNP was detected in the outcome of clinical pregnancies (P_{trend}=0.534) for the recessive (C/C vs. C/T + T/T) inheritance model (OR=0.671; 95% CI=0.220-2.041; P=0.584) or for the dominant (C/C + C/T vs. T/T) inheritance model (OR=0.855; 95% CI=0.345-2.122; P=0.736).

Discussion

In addition to the various intra- and extra-ovarian components, which contribute to the growth of follicles, including FSH, LH and estrogen, paracrine factors produced by oocytes appear to be crucial to the growth of follicles (18). GDF-9, in combination with BMP6, BMP15 and fibroblast growth factors, belong to a transforming growth factor sβ superfamily of proteins, which are the mammalian oocyte-derived paracrine factors (18). These molecules control the function and differentiation of somatic granulosa cells and the growth of the ovarian follicle (18). The essential role of GDF-9 in folliculogenesis and fertility has been well documented in a murine model study by Dong *et al* (32). The authors observed that GDF-9-knockout mice were infertile, exhibiting smaller ovaries compared with the wild-type mice. These mice also lacked normal follicles beyond the primary follicle and no theca cells. The oocytes failed cross normal meiosis *in vitro*. The serum of GDF-9-knockout mice exhibited increased levels of FSH and LH, which is characteristic of hypergonadotropism and hypogonadism (32). Additionally, the synergistic role of GDF-9 and BMP15 on the growth and functions of granulosa cells and follicles has been demonstrated in murine and sheep models (33-35). Additionally, the crucial role of GDF-9 in folliculogenesis was confirmed in a previous study with $Bmp15^{-/-}$ mice, which displayed a relatively mild phenotype until an additional deletion in one allele of the GDF-9 gene, which lead to severe infertility (33,34). Therefore, genetic variants of GDF-9 may effect infertility, as well as IVF outcomes.

The present study revealed that the *GDF-9* 546 G/A heterozygous genotype and the *GDF-9* 447 (CC + C/T) genotype were significantly associated with poor ovarian stimulation response and a poor fertilization rate in infertile women treated with IVF.

Wang *et al* (29) demonstrated that the *GDF-9* G546A polymorphism is associated with a poor ovarian response and poor IVF outcomes, as evidenced by a poor fertilization rate, poor oocyte and embryo quality, and a low pregnancy rate in a population of Chinese women with DOR (29). However, this previous study failed to identify the effect of the *GDF-9* C447T SNP on ovarian response and IVF outcomes (29). These

discrepancies between the present study and the previous study may be due to patient differences in the causes of infertility or to differences in distinct genetic heterogeneity interactions with disparate environmental factors.

Previously, Ma *et al* (36) found that the *GDF-9* 546A gene variant is a risk factor for POF in the Chinese Hui population. In addition, the elevated frequencies of certain *GDF-9* heterozygous variants, including the C447T SNP, have been identified in European, Caucasian and Asian women with POF failure (26,28,37,38), however, not in New Zealander or Japanese woman (27,38). A tandem duplication of 475 bp in the *GDF-9* promoter was previously identified as a causative factor of primary ovarian insufficiency (39). The *GDF-9* variants have also been associated with the risk of PCOS (40) and have been associated with hirsutism scores and parity in patients with PCOS (41).

The present study identified that the *GDF-9* C447T (rs254286) SNP was a possible candidate genetic risk factor for female infertility. This SNP may be associated with certain unidentified missense mutations, resulting in abnormal protein expression of GDF-9 and loss of GDF-9 function. Previously, it was reported that GDF-9 forms a heterodimer with BMP15, which is 10-3,000-fold more biologically active compared with the homodimers of these two proteins (42). Therefore, a missense mutation, either in *GDF-9* or *BMP15*, may result in the destabilization of this complex, causing female infertility.

The present genetic analysis confirmed the *GDF-9* A546G SNP as a genetic factor associated with a poor ovarian stimulation response and a poor fertilization rate in infertile women treated with IVF. Additionally, the present study is the first, to the best of our knowledge, to identify the *GDF-9* C447T (rs254286) SNP as a possible candidate genetic risk factor for female infertility and to associate this SNP with a poor ovarian stimulation response and a poor fertilization rate in infertile women treated with IVF. However, the present study is limited by a low statistical power, therefore the conclusions require confirmation in future studies with other larger independent ethnicities.

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