The relationship between follicle development and progesterone receptor membrane component-1 expression in women undergoing in vitro fertilization

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Objective: To determine the relationship between progesterone receptor membrane component-1 (PGRMC1) expression and the outcome of IVF treatment.
Design: A prospective study in which PGRMC1 messenger RNA (mRNA) levels, methylation status of the *Pgrmc1* promoter, and the presence of point mutations within *Pgrmc1* were obtained from granulosa (GC)/luteal cells of women undergoing controlled ovarian hyperstimulation (COH).
Setting: Fertility center/basic science laboratory.
Patient(s): Eighty-five patients undergoing IVF treatment and 10 women who were undergoing COH for the purpose of oocyte donation were included in this study.
Intervention(s): None.
Main Outcome Measure(s): The PGRMC1 measurements were correlated with clinical outcomes, such as number of follicles, number of retrieved oocytes, and ongoing pregnancy rates (PR).
Result(s): The PGRMC1 mRNA levels within GC/luteal cells of 18% of IVF patients were >2.25-fold higher than those of oocyte donors. Individuals with elevated PGRMC1 mRNA levels had 30% fewer large follicles and fewer oocytes retrieved. The elevated PGRMC1 mRNA levels were associated with an increase in the methylation of *Pgrmc1* promoter.
Conclusion(s): In patients with elevated PGRMC1 mRNA levels, gonadotropin-induced follicle development is attenuated, although sufficient numbers of follicles develop to allow for ET and subsequent pregnancy. (Fertil Steril® 2012;97:572–8. ©2012 by American Society for Reproductive Medicine.)
Key Words: PGRMC1, granulosa/luteal cells, IVF, controlled ovarian hyperstimulation, follicular development

The ability to conceive and to ultimately deliver a healthy child is an extremely complex process that can be disrupted by genetic and epigenetic events (1, 2). Because of the importance of these genetic and epigenetic events, numerous studies have been conducted to identify genetic and/or epigenetic biomarkers that might be able to predict a successful IVF outcome and/or pregnancy (3–6). One gene that might be useful as a predictor of IVF outcome is progesterone receptor membrane component-1 (*Pgrmc1*). *Pgrmc1* (Gene ID: 10857) is located on the X-chromosome (Xq22-q24) and has three exons that encode a 22-kDa protein (7, 8). In addition, PGRMC1 is highly expressed in human ovarian granulosa cells (GC) (9, 10) and functions to preserve the viability of these cells (9–13).

Consistent with its important role in regulating the viability of GC/luteal cells, *Pgrmc1* deletion and point mutations have been observed in some women with impaired ovarian function (8, 14, 15). For example, a mother and daughter, who were diagnosed with premature ovarian failure (POF), had a deletion mutation that resulted in a 50% reduction in PGRMC1 expression (8). A point mutation within exon 3 of *Pgrmc1*, which results in an alteration in amino acid 165, was detected in a third patient with POF (8). This point mutation is important in that it reduced the functional capacity of PGRMC1 (8). In addition, reduced expression of PGRMC1 was

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observed in women with polycystic ovarian syndrome (PCOS) (14), further implicating PGRMC1 as a regulator of ovarian function.

Based on these observations, the present series of studies were undertaken to determine whether the PGRMC1 messenger RNA (mRNA) levels within the GC/luteal cells of women undergoing controlled ovarian hyperstimulation (COH) protocols could be used to identify a subset of women whose response to gonadotropin treatment was altered in a way that would affected the success of their IVF treatment. In addition to PGRMC1 mRNA levels, the presence of the point mutation within exon 3 was assessed in these women. Finally, the methylation status of the PGRMC1 promoter was evaluated, because hypermethylation suppresses transcription, thereby reducing mRNA levels (16). In addition, gonadotropins regulate the methylation status and thereby the expression of several genes (i.e., *Cyp19A1, Hsd3B,* and *Cyp11A1*) whose expression is regulated in an ovarian cell-specific manner (1).

MATERIALS AND METHODS

Patients

This study included 85 patients who underwent COH as part of their IVF treatment between March 2010 and November 2010. This study was approved by The University of Connecticut Institutional Review Board. Patients in this study were undergoing either their first or second IVF cycle. No patients contributed data from more than one cycle in this study. Patients were $\leq$38 years, with normal basal serum FSH, LH, and E_2 levels obtained on day 3, and no prior history of low response to ovarian stimulation. A low ovarian response was defined as a peak serum E_2 level of $\leq$850 pg/mL and/or retrieval of less than five oocytes. Severe male factor infertility requiring surgical sperm harvest and patients with PCOS were excluded. Patients were stimulated with either a flexible antagonist or GnRH agonist protocols, as described by Engmann et al. (17). The assignment of the patients to either protocol was made at their physician's discretion. In addition, 10 women undergoing COH for the purpose of oocyte donation were included as controls.

Stimulation Protocols

Briefly, patients were treated with recombinant FSH (Gonal F; Serono) and purified urinary hMG (Menopur; Ferring Pharmaceuticals). In patients undergoing flexible antagonist protocol, ganirelix acetate (0.25 mg SC, Ganirelix, Organon Pharmaceuticals) was started when either the lead follicle measured $\geq$13 mm or E_2 level was >300 pg/mL. Ganirelix acetate was continued until the day of hCG administration. The hCG (Profasi, Serono) was administered SC when at least three follicles had attained or exceeded a mean diameter of 17 mm. Ultrasound imaging was conducted throughout the stimulation cycle to monitor follicle growth and blood samples were periodically taken to determine serum E_2 and progesterone (P4) levels, as previously published (17). Transvaginal oocyte retrieval was performed approximately 36 hours after hCG administration and the GC/luteal cells were obtained from the follicular aspirate.

Isolation of Granulosa/Luteal Cells

After the oocytes were removed, follicular aspirates were pooled and centrifuged at 250 $\times$ *g* for 10 minutes. The cell pellet was suspended in phosphate-buffered saline (PBS), layered on Histopaque-1077 (Sigma Chemical Co.) and centrifuged for 30 minutes at 400 $\times$ *g*. After centrifugation, the opaque interface containing the GC/luteal cells was carefully aspirated and transferred into a 15-mL sterile conical centrifuge tube. The cells were then suspended in 12 mL of PBS and centrifuged at 250 $\times$ *g* for 10 minutes. This was repeated an additional time (9, 10).

Analysis of Exon 3 for Point Mutations

Genomic DNA was extracted from GC/luteal cells using All-Prep DNA/RNA Micro kit from Qiagen and used in a polymerase chain reaction (PCR) protocol to amplify exon 3 of human PGRMC1. This protocol used the following primers: forward: 5′-TTGCAGGCCTCTA ATAAATG-3′; reverse: 5′-CAGCACTG CAGTTCACTTTC-3′. The PCR reaction consisted of 35 cycles of a denaturation phase of 30 seconds at 94°C, an annealing phase of 30 seconds at 55°C, and an extension phase of 60 seconds at 68°C. The PCR product was then run on 1.2% of agarose gel and the 415-bp band extracted and purified using the QIAquick gel extraction kit from Qiagen. The purified PCR product was then send to Agencourt Bioscience Corp. for sequencing. The DNA sequence was analyzed for the presence of any mutation using 4Peaks software program (http://www.mekentosj.com/science/4peaks).

Real-time PCR for PGRMC1 mRNA Measurements

Total RNA was also extracted from GC/luteal cells using All-Prep DNA/RNA Micro kit from Qiagen. This RNA preparation was used in a real time PCR protocol using CFX96 real time PCR system. The RNA samples (1 ng) were resuspended in 25 μL of the SsoFast Probes Supermix (Bio-Rad) containing 300 nm of each PGRMC1 primer: forward 5′-GGTGTTCGATG TGACCAAAG-3′and reverse 5′-GATGCA TCTCTTCCAGCAAA-3′ and the probe: CGCAAATTCTACG GGCCCGA (5′ FAM $\rightarrow$ 3′ BHQ1). Actin was used as an internal control and was detected using the following primers: forward 5′-CACTCTTCCAGCCTTCCTTC-3′ and reverse 5′-GGATGTCCACG TCACACTTC-3′ and the probe: TGCCACA GGACTCCATGCCC (5′ CAL Gold 540 $\rightarrow$ 3′ BHQ1). Gene expression was evaluated with Bio-Rad iCycler software and the PGRMC1 mRNA levels expressed relative to the average value obtained from the oocyte donor samples.

Analysis of Methylation of the *Pgrmc1* Promoter

A 1-kb segment of DNA, which was rich in cytosine nucleotides that were next to a guanine nucleotide separated by a phosphate (i.e., CpG), was identified within the *Pgrmc1* promoter (18). This CpG island spanned the transcriptional start site of PGRMC1. Because the methylation of these cytosine

residues can suppress gene transcription, the Methyl-Profiler DNA Methylation qPCR assay kit was used to determine the methylation status of this CpG island. Briefly, genomic DNA was aliquoted into four equal portions and subjected to either a mock digestion, a methylation sensitive digestion, a methylation-dependent digestion, or a double digestion. After restriction digestion, the PCR reaction was run using primers designed by SABiosciences to amplify this CpG island. The percentage of hypermethylated DNA was then calculated using the software provided by SABiosciences.

Clinical Outcomes

In vitro insemination or intracytoplasmic sperm injection (ICSI) was performed on retrieved oocytes. Of the 85 patients, 93% opted for the ICSI protocol. Eighteen hours after insemination or sperm injection, the oocytes were examined for the presence of pronuclei and those oocytes that possessed two pronuclei (2PN) were considered fertilized. The number of good-quality embryos was assessed after 3 days of culture. Only embryos with $\geq$6 cells and $\leq$20% fragmentation were classified as good-quality embryos. The implantation rate was defined as the number of sacs on transvaginal ultrasound divided by the number of embryos transferred. The ongoing pregnancy rate (PR) per started cycle was assessed after 20 weeks of gestation.

Statistical Analysis

The PGRMC1 mRNA levels obtained from GC/luteal cells of oocyte donors were used to establish a normal range as defined as the mean $\pm$ 2 SD. Using the values from the oocyte donors, PGRMC1 mRNA levels from patients undergoing IVF were classified as either being within or outside the normal range. Statistical comparison of end points for patients undergoing IVF with normal and elevated PGRMC1 mRNA levels was performed using the statistical package for the Social Sciences (release 17.0, SPSS Inc.). Student's *t*-test was used for comparison of continuous variables, whereas Fisher's exact test was used for comparison of proportions. A power analysis for Student's *t*-test and Fisher's exact test revealed a power value of 0.82 and 0.81, respectively. The relationship between PGRMC1 mRNA levels and serum steroid levels was assessed by Spearman correlation analysis. Regardless of the statistical analysis used, $P<.05$ was considered statistically significant.

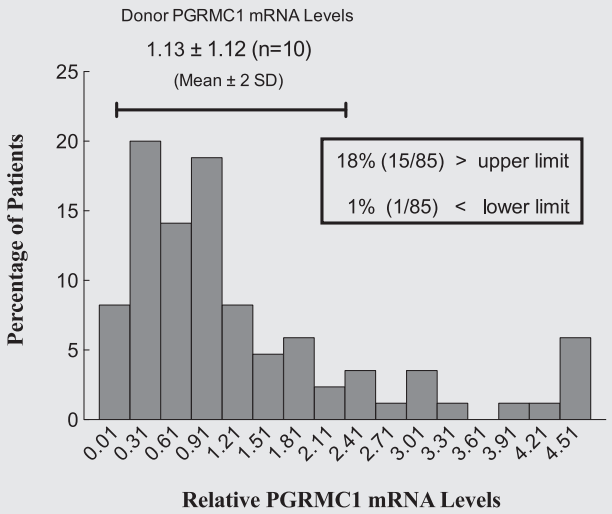
RESULTS

The levels of PGRMC1 mRNA in GC/luteal cells obtained from patients undergoing IVF after gonadotropin treatment were not normally distributed and significantly skewed to the right as determined by Kolmogorov-Smirnov test for normality ($P<.0001$). To define a normal range, PGRMC1 mRNA levels obtained from oocyte donors were used. This analysis revealed that the donor values were normally distributed with the 95% lower and upper limits being defined by the mean $\pm$ 2 SD (bracketed line in Fig. 1). Of the 85 patients undergoing IVF, one had a PGRMC1 mRNA level that was less than the lower limit, 69 (82%) had PGRMC1 mRNA levels that were within the normal range, and 15 (18%) had PGRMC1 mRNA levels that were more than the upper limit of the oocyte donor population. Interestingly, patients undergoing IVF with elevated PGRMC1 mRNA levels had demographic and clinical characteristics comparable with those of patients undergoing IVF with PGRMC1 mRNA levels within the normal range (Table 1).

One factor that could account for an increase in PGRMC1 mRNA levels is the hypermethylation status of the *Pgrmc1* promoter. In patients with normal levels of PGRMC1 mRNA, 21% $\pm$ 0.02% of the cytosine residues within the CpG island of the *Pgrmc1* promoter was hypermethylated. In contrast only 16% $\pm$ 0.01% of the cytosine residues within the CpG island of the *Pgrmc1* promoter was hypermethylated in those patients with elevated PGRMC1 mRNA levels. In addition, a point mutation at amino acid 165 was not observed in any of the patients undergoing IVF, including all of the patients with elevated PGRMC1 mRNA levels.

Since only one patient undergoing IVF had abnormally low PGRMC1 mRNA levels, her data were not analyzed further. Accordingly, data of patients undergoing IVF with elevated PGRMC1 levels were compared with those with normal levels. Importantly, the stimulation protocols used in both groups were comparable (Table 2). There were also no difference in the length of stimulation and the total dose of gonadotropin (Table 2). Although 18% of the infertility patients had elevated PGRMC1 mRNA levels, their ability to response to gonadotropin with an increase in serum E_2 levels was neither enhanced nor inhibited. Similarly, the amount of PGRMC1 mRNA expressed by GC/luteal cells did not affect

FIGURE 1



Distribution of relative progesterone receptor membrane component-1 (PGRMC1) messenger RNA (mRNA) levels obtained from granulosa (GC)/luteal cell of patients undergoing ovulation induction for the treatment of infertility. Values are shown relative to the mean values obtained from oocyte donors. Values obtained from infertility patients were considered to be in the normal range if they were within the 95% confidence interval of oocyte donors. This confidence interval is shown as a bracketed line.

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TABLE 1

Demographic and clinical characteristics of infertility patients with normal and elevated progesterone receptor membrane component-1 messenger RNA levels within their granulosa/luteal cells obtained after gonadotropin stimulation.

	Normal (n = 69)	Elevated (n = 15)	*P* value
Age (y)	34.6 ± 3.9	35.3 ± 2.6	.35
BMI (kg/m^2)	26.4 ± 6.3	25.2 ± 6.8	.52
Day 3 FSH (mIU/mL)	6.5 ± 2.4	7.8 ± 2.4	.08
Day 3 LH (mIU/mL)	4.8 ± 2.4	4.6 ± 2.1	.79
Day 3 E_2 (pg/mL)	47.5 ± 17.4	38.7 ± 16.8	.08
Parity	0.29 ± 0.55	0.27 ± 0.59	.87
Etiology of infertility (%)			
Male factor	32	33	1.0
Tuboperitoneal factor	19	20	1.0
Unexplained	23	27	.74
Endometriosis	12	7	1.0
Ovulation disorders	14	13	1.0

Note: Values are expressed as either a mean ± SD or as a percentage. For those values expressed as a mean ± SD, statistical comparisons were made using a Student's *t*-test. A Fisher's exact test was used to compare percentage values. BMI = body mass index.

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the serum levels of E_2 and P4 observed after hCG administration (Table 2). Finally, PGRMC1 mRNA levels were not correlated with serum E_2 or P4 at any time as assessed by Spearman correlation analysis (*r* values ranged from -0.03 to -0.12; all correlations had *P* values >.05).

The only aspect of ovarian function that was related to PGRMC1 mRNA levels was follicular growth. Although the initial number of antral follicles was not significantly different, the number of follicles >14 mm in diameter that developed after gonadotropin stimulation was reduced by 35% in patients undergoing IVF with elevated PGRMC1 mRNA levels (Table 3). Consistent with this finding, the number of oocytes retrieved and the number of mature and fertilized oocytes were reduced by approximately 33% in this group (Table 3). However, the maturation, fertilization, and implantation rates were not significantly different between the two groups. The ongoing PR per started cycle for women in which PGRMC1 mRNA levels were elevated was lower than those with PGRMC1 mRNA in the normal range, but this did not reach statistical significance (Table 3). Finally, the number of cryopreserved embryos was lower in patients with elevated levels of PGRMC1 (Table 3).

DISCUSSION

The present study is the first to examine the expression of PGRMC1 in GC/luteal cells of women undergoing gonadotropin ovarian stimulation as part of their IVF treatment. Although alternations in PGRMC1 mRNA levels were not correlated with fertilization or ongoing PRs, a large subset of patients (i.e., 18%) respond to gonadotropin treatment by overexpressing PGRMC1. The reason for the overexpression is not known. In previous animal studies gonadotropin treatment has been shown to increase mRNA and protein levels of PGRMC1 (12, 19), thus it was expected that patients with elevated PGRMC1 mRNA levels would have received higher amounts of gonadotropins. However, in this study overexpression of PGRMC1 cannot be explained by excessive gonadotropin stimulation, as neither the amount nor the duration of gonadotropin treatment was different between these two groups. Similarly, there were no differences in the average age of the patients and the stimulation protocols.

One factor that may have influenced the expression of PGRMC1 is the methylation status of the PGRMC1 promoter. The PGRMC1 promoter possesses a single CpG island that is

TABLE 2

Gonadotropin stimulation and resulting steroid levels of infertility patients with normal and elevated progesterone receptor membrane component-1 messenger RNA levels within their granulosa/luteal cells obtained after gonadotropin stimulation.

	Normal (n = 69)	Elevated (n = 15)	*P* value
Stimulation protocol			
Antagonist (% of patients)	64	53	.56
Agonist (% of patients)	36	47	.56
No. of stimulation days	10.19 ± 1.6	10.73 ± 2.2	.37
Total gonadotropins (U)	3,201 ± 1,817	4,125 ± 2,247	.15
Peak E_2 (pg/mL)	1,934 ± 947	1,918 ± 935	.95
Peak E_2 1 day after hCG administration (pg/mL)	2,445 ± 1,074	2,185 ± 1,174	.44
P4 1 day after hCG administration (ng/mL)	3.7 ± 2.0	3.1 ±2.0	.26

Note: Values are expressed as either a mean ± SD or as a percentage. For those values expressed as a mean ± SD, statistical comparisons were made using a Student's *t*-test. A Fisher's exact test was used to compare percentage values.

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TABLE 3

Follicle growth and oocyte characteristics of infertility patients with normal and elevated progesterone receptor membrane component-1 messenger RNA levels within their granulosa/luteal cells obtained after gonadotropin stimulation.

	Normal (n = 69)	Elevated (n = 15)	*P* value
No. of antral follicles at day 3	10.9 ± 5.0	12.3 ± 6.4	.49
No. of follicles >14 mm on day of hCG administration	11.6 ± 6.0	7.5 ± 2.7	.004
No. of oocytes retrieved	13.4 ± 6.9	9.2 ± 3.7	.002
No. of mature oocytes	10.0 ± 5.6	7.1 ± 3.9	.02
Maturation rate (%)	76	77	.83
No. of oocytes with 2PN	7.3 ± 4.8	4.5 ± 3.4	.01
Fertilization rate (%)	69	61	.36
No. of available embryos for transfer on day 3 of culture	6.3 ± 4.5	3.9 ± 3.2	.02
No. of good quality embryos	1.8 ± 2.2	1.3 ± 1.8	.4
No. of embryos transferred	2.2 ± 0.7	1.9 ± 1.2	.46
Implantation rate (%)	34	36	.83
Ongoing pregnancies/started cycle (%)	52	33	.19
No. of embryos frozen	1.0 ± 0.2	0.3 ± 0.2	.02

Note: Values are expressed as either a mean ± SD or as a percentage. For those values expressed as a mean ± SD, statistical comparisons were made using a Student's *t*-test. A Fisher's exact test was used to compare percentage values. 2PN = 2 pronuclei.

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approximately 1 kb and spans the transcriptional start site (18). As such, the methylation status of the cytosine residues within this CpG island may play a role in the transcription of PGRMC1. Our analysis revealed that on average 16% of the cytosine residues within the CpG island were hypermethylated in women with elevated PGRMC1 mRNA levels compared with 21% in women with normal PGRMC1 levels. Because hypermethylation makes the initiation of transcription more difficult (16), it is possible that the lower level of hypermethylation could account in part for the higher expression of PGRMC1. How much of the increased PGRMC1 expression can be attributed to this 5% decrease in methylation of the PGRMC1 promoter remains to be determined experimentally.

In the current study hypermethylation of the PGRMC1 promoter was associated with attenuated follicular growth. Thus, the hypermethylation likely occurs during follicular development that is induced by gonadotropin (i.e., FSH) stimulation. Interestingly, FSH in the presence of cumulus cell–oocyte complexes induces the expression of DNA methytransferases (20). Because DNA methytransferases stimulate the methylation of CpG islands (1), defects or alterations in the FSH signal transduction pathway could be responsible for less methylation of the PGRMC1 promoter and subsequently elevated PGRMC1 mRNA levels.

Another interesting finding in our study was that serum E_2 and P4 levels were not correlated with PGRMC1 mRNA levels. The PGRMC1 is thought to enhance steroidogenesis by increasing cholesterol synthesis through two distinct mechanisms. First, PGRMC1 can directly bind two proteins, insulin-induced gene-1 and sterol regulatory element-binding protein cleavage-activating protein (21), and this interaction increases the transcriptional action of sterol regulatory element-binding protein, and ultimately the expression of steroidogenic acute regulatory protein (22), an essential factor in steroid synthesis. The second mechanism by which PGRMC1 could influence steroidogenesis, is through its ability to activate the cytochrome P450 enzymes (23), such as cytochrome P450 (21), which plays an essential role in steroid synthesis (24). Apparently, the elevated PGRMC1 mRNA observed in some patients undergoing IVF was not sufficient to activate either of these two mechanisms to the point that affects serum steroid levels.

In addition to changes in mRNA levels, the biological activity of PGRMC1 can be influenced by the presence of a point mutation at amino acid 165. Specifically, mutated PGRMC1, which has an arginine instead of a histidine at amino acid 165, cannot bind to cytochrome P450 7A1 (CYP7A1), a key member of the P450 family that catalyzes a rate-limiting step in cholesterol metabolism (25). If present, this would have likely reduced the overall functional capacity of PGRMC1 in patients undergoing IVF with elevated PGRMC1 mRNA. However, this mutation was not observed in any of the patients undergoing IVF, including those with elevated PGRMC1 mRNA levels. Thus, the reason why elevated PGRMC1 did not influence steroidogenesis remains to be determined.

Although without an affect on serum steroid levels, the elevations in PGRMC1 expression adversely affected gonadotropin-induced follicle growth. This is because those patients with elevated PGRMC1 mRNA levels develop fewer large follicles after gonadotropin treatment. The PGRMC1 has numerous sites of action that could influence follicular growth (26, 27). The most likely mechanism relates to PGRMC1's ability to interact with the mitotic spindle apparatus of GCs (28). For GCs to undergo mitosis and thereby contribute to follicular growth, PGRMC1 intermittently associates and dissociates with the mitotic spindle (28). Elevations in PGRMC1 could disrupt this dynamic interaction with the mitotic spindle and thus slow or inhibit GC mitosis and, in turn, follicle development. In addition, prolonging mitosis could trigger a "mitotic catastrophe" and result in GC apoptosis. It is important to appreciate that PGRMC1 depletion in GCs also leads to GC

apoptosis (11). Thus it appears that PGRMC1 must be maintained at an optimal level that is sufficient to preserve cell viability and allow for mitotic events to occur in a timely coordinated manner.

The reduction in follicular growth is also reflected in fewer oocytes retrieved, fewer mature oocytes, fewer fertilized oocytes, and fewer embryos available for transfer. Importantly, oocytes retrieved from women with elevated PGRMC1 levels have the same capability to mature, to be fertilized, to undergo embryonic development in vitro, and to implant and ultimately develop into viable embryos, as do those derived from women with normal levels.

Although these findings may cast doubt on an association between PGRMC1 expression and IVF success, this conclusion may not be completely justified. Patients with elevated PGRMC1 mRNA levels had fewer embryos available for transfer than patients with normal levels. In addition, the number of cryopreserved embryos in this group was significantly less than patients with normal levels. Thus, the resulting cumulative PR per retrieval might be reduced in patients with elevated PGRMC1 mRNA levels.

Finally, the present study revealed that patients with elevated PGRMC1 mRNA levels respond to gonadotropin with a normal increase in serum E_2 level, but a reduced number of large antral follicles and retrieved oocytes. Interestingly, the number of oocytes retrieved from "poor responders" is similar to that of patients undergoing IVF with elevated PGRMC1 mRNA levels (29). In addition, serum E_2 level is increased in poor responders to the same level as those patients undergoing IVF in the present study, although more gonadotropin is required to induce both serum E_2 levels and follicular development (29). These observations are consistent with the idea that follicular growth and steroid secretion are independent responses to gonadotropin treatment and that elevated PGRMC1 mRNA levels is only associated with attenuated follicular development. If so, then the elevated level of PGRMC1 mRNA might be a genetic biomarker of a patient undergoing IVF who either is or will become a poor responder. This possibility merits further consideration.

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