Original Article

Luteinizing Hormone Receptor Gene and Regulator of G-protein Signaling 2 Gene Expression Level and Association with Oocyte Maturity in *In vitro* Fertilization/Intracytoplasmic Sperm Injection Cycle

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ABSTRACT

Aims: The aim is to study the relation and distribution in gene expression level of the luteinizing hormone receptor (LHR) gene and regulator of G-protein signaling 2 (RGS2) gene expression with oocyte maturation. **Setting and Design:** This cross-sectional study was undertaken in an instruction-based tertiary care infertility unit, department of obstetrics and gynecology. **Materials and Methods:** After controlled ovarian hyperstimulation, cumulus granulosa cells (CCs) from 59 oocytes among 18 women being treated by *in vitro* fertilization/intracytoplasmic sperm injection cycle technique from November 2015 to January 2016 were collected on the day of oocyte retrieval. Total RNA was extracted and converted to cDNA in individual oocytes. LHR and RGS2 gene levels were measured and analyzed using digital droplet polymerase chain reaction. **Statistical Analysis:** Gene expression level was analyzed using software STATA, version 14.0 (College Station, TX: StataCorp LP, USA). **Results:** CCs were obtained from 59 cumulus-oocyte complexes (COC), 46 COC from metaphase II (CC_{MII}), 13 COC from metaphase I, and GV oocyte (CC_{MI+GV}). The RGS2 gene expression level, when compared with the housekeeping gene in CC_{MII} and CC_{MI+GV}, was 0.15 (0.05–0.52) and 0.08 (0.02–0.27), respectively. The LHR gene expression when compared with the housekeeping gene in CC_{MII} and CC_{MI+GV} did not differ and was quite in the same value that was 0.02 (0.00–0.11) and 0.02 (0.00–0.06), respectively. **Conclusion:** This study showed that LHR gene expression did not differ in between oocyte groups. Even though the median of RGS2 gene expression was more in the mature oocyte group, the result was inconclusive due to scattering and overlapping of gene expression data between oocyte groups.

KEYWORDS: *Digital droplet polymerase chain reaction, gonadotropin-releasing hormone antagonist protocol, luteinizing hormone receptor gene, oocyte maturity, regulator of G-protein signaling 2*

INTRODUCTION

Infertility has increased to about 15%[1] in the general population. Therefore, assisted reproductive technology (ART), especially *in vitro* fertilization (IVF)/intracytoplasmic sperm injection cycle (ICSI) is used among these patients because of greater reliability. But to achieve those steps, many factors must be involved. For example, couples must have good quality gametes to produce healthy embryos, and the normal uterine cavity in women is used for successful implantation. Especially, the number and oocyte

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maturation are the most important factors that contribute to be good pregnancy outcomes.

The follicle consists of the oocyte, granulosa cells, and theca cells that surround the oocyte. Without escort cells (granulosa and theca cells), the oocyte could not develop fully and mature.[2] In the primordial follicle stage, one layer of granulosa cells and will gradually increase during the development of oocyte. In antral stage, the granulosa cell that attaches to the oocyte is called "cumulus cell" and serve as somatic cells providing energy, food, and removing waste from the environment and communicating with the oocyte. Finally, the oocyte could grow and develop from the primordial to the preovulatory follicle.[3-5]

The attachment of cumulus cells to the oocyte plays a role in regulating follicular development in meiosis I and oocyte maturation in meiosis II, promotes cumulus cell expansion and renders ovulation after luteinizing hormone (LH) stimulation.[6,7] Treatment with control ovarian hyperstimulation (COH) will produce multiple oocytes, and each oocyte is controlled by a different set of cumulus granulosa cells (CCs) of differing quality and integrity depends on the individual response to gonadotropins administration.

Normally, ovulation occurring in natural cycles usually matures in meiosis stage II, but COH cycles augment oocytes in several stages (germinal vesicle, immature metaphase I, or mature metaphase II) because the response to treatment varies for each oocyte. Currently, many genes, proteins or chemical factors are available, which have a role in oocyte growth, development, and maturation such as growth differentiation factor 9 and bone morphogenetic protein 15.[8,9] These include the regulator of the G-protein signaling 2 (RGS2) gene, which has been reported to play a role in the regulating functions of the luteinizing hormone receptor (LHR) Gene. LHR is a G protein-coupled receptor (GPCR), especially the Gαs protein that is inhibited by the RGS2 protein. As a result, the function of LH is reduced. LH plays a key role in the final step of oocyte maturation and ovulation diverting fully ready to fertilization. On the other hand, many early LH functions may cause premature luteinization that will impact oocyte maturation.

For the study of gene expression of the cumulus cell, the gene expression was mostly in the form of RNA or proteins. The study of RNA for gene expression, called "transcriptomics," consists of mRNA, rRNA, tRNA, and miRNA.[10] The advantage of RNA study of the cumulus cell is it does not directly disturb the developing oocyte which may affect fertilization and maturation of the embryo. Currently, technology used to analyze gene expression in cumulus cells includes microarray and droplet digital polymerase chain reaction (ddPCR) to see copies of gene expression and assess correlation with oocyte/embryo quality and are very advanced. The aim is to improve the quality of oocytes and embryos and then improve pregnancy outcomes and finally live births.

Materials and Methods

Participant population

From November 2015 to January 2016, 18 infertile women, undergoing IVF/ICSI treatment for tubal factor, ovarian factor, male factor, unexplained infertility and other etiologies, were enrolled in an ART program following gonadotropin-releasing hormone (GnRH) antagonist protocol. All women were premenopausal, 25–42 years old with normal hormonal profile. Each received the first IVF/ICSI treatment but had not received ovulation induction or other hormonal treatment within 3 months preceding recruitment. Patients' demographic characteristics (age, body mass index [BMI], and duration of infertility) were recorded before ovarian stimulation protocol. Dose of gonadotropin, duration of ovarian stimulation, number of follicles and oocytes corresponding to each patient were monitored during the treatment cycle. Day 2 hormonal profile (follicle-stimulating hormone [FSH], LH) was measured within the previous 6 months.

Ovulation induction

The protocol was approved by the Ethics Clearance Committee on Human Rights Related to Research Involving Human Subjects, and informed consent was obtained from all the participants. All patients underwent GnRH-antagonist protocol. A baseline ultrasound scan on day 2 of the cycle confirmed the absence of ovarian cysts and endometrial proliferation, low serum E2 levels (<40 pg/ml) and normal serum FSH (<12 mIU/ml). When the above criteria were not met, the stimulation was canceled. As soon as the condition was completed, a fixed 5-day treatment with rFSH followed by GnRH-antagonist at day 6 of stimulation and an adjusted dose of rFSH was thereafter applied. Serum E2 levels were measured on day 5. An ultrasound scan was performed on a daily basis from day 9 and follicular growth along with endometrial thickness was recorded.

A dose of 10,000 IU of human chorionic gonadotropin (hCG) to trigger final oocyte maturation was prescribed once the mean diameter of at least two follicles was >17 mm. Oocytes were retrieved by transvaginal ultrasound-guided ovarian puncture 35–36 h post-hCG injection. Oocyte maturation was assessed under a microscope following the stripping of the cumulus-oocyte

complexes and mature oocytes (metaphase II) were used for ICSI. On day 3, embryos were transferred to the uterus or frozen. Luteal phase support was provided with vaginal progesterone preparation started on the day of oocyte retrieval. Pregnancy was confirmed when serum hCG levels were determined 12 days following embryo transfer, whereas clinical pregnancy was defined as a gestational sac with positive fetal heart activity seen on a transvaginal ultrasound scan 2 weeks later.

Steps of cumulus granulosa cell collection

1. Prepare PCR tube size 0.2–1.5 ml; add phosphate buffer saline 50–100 µl for cumulus cells
2. Prepare 4-well plate coated with mineral oil; add fertilization medium 200 µl per pit then incubate in chamber 6% CO2, 37°C
3. After oocyte retrieval, move oocytes to 4-well plates, 1 oocyte per pit
4. Collect cumulus granulosa cell with pipette 1:1 oocyte then put in the PCR tube
5. Wash CCs with 50% Percoll gradient[11] and centrifuge at 2000 rpm for 10 min to isolate red blood cells and contaminated cells
6. Final recheck and isolate white blood cell under stereo microscope
7. Store cumulus granulosa cell in −80°C.

RNA extraction and cDNA preparation

CCs were collected on the day of oocyte retrieval. In particular, the cells were segregated from cumulus-oocyte complexes (COCs) through the process of stripping using the mechanical method. Granulosa cells surrounding oocytes were collected from 18 patients (59 COCs) and stored in −80°C before RNA extraction. Total RNA from these cells was extracted using NucleoSpin RNA XS[12] (MACHEREY-NAGEL GmbH and Co. KG, Düren, Germany) and Qiagen RNeasy Micro Kit[13] (Qiagen, Hilden, Germany). cDNA was converted from extracted total RNA using TaKaRa PrimeScript 1st strand cDNA Synthesis Kit[14] (TaKaRa, Japan) and Qiagen Quantitect RT Kit[15] (Qiagen, Hilden, Germany) with Bio-Rad T100 thermocycler (Bio-Rad Laboratories, Hercules, CA)[16] following manufacturer protocols.

Statistical analysis

Statistical analysis was performed using software STATA, version 14.0 (College Station, Texas: Stata Crop LP, USA). First, statistical analysis was performed to verify the homogeneity of the variables at the individual level (age, BMI, and FSH) between the cumulus cells from MII oocytes (CC_{MII}) and $CC_{MI + GV}$ groups presented in mean ± standard deviation and determining significance using Mann–Whitney U-test for continuous data and Student's *t*-test for category data. For continuous data, the level of gene expression was demonstrated at median range. The different levels of gene expression from ddPCR between groups were analyzed using Quantile regression, and the significance level was set at $P < 0.05$. The distribution regarding level of gene expression was demonstrated in ratio data using Pearson's Chi-square to test significance.

Results

The patients allowed to use CCs for gene expression were analyzed. Regarding the cluster of cells from a total of 18 patients, all contained mature oocytes. However, 9 patients had oocytes mixed with immature oocytes. Because every patient had mature oocyte, it made the patient characteristic data of total patients the same as patients with mature oocytes. The average age of total patients was 36.20±+3.56 years, and BMI was in normal range (21.75 ± 1.72 kg/m^2). The average number of years since infertility was 4.0 ± 1.6. The mean level of serum hormone at day 2 of the menstruation cycle (FSH, estradiol) was in normal range of the early follicular phase (FSH D2 = 7.0 ± 1.73 mIU/ml, estradiol D2 = 35.86 ± 14.9 mIU/ml). The mean level of LH at day 6 of stimulation, GnRH antagonist (fixed protocol), was also in normal level before LH surge (1.89 ± 1.78 mIU/ml). The clinical data during stimulation were in accordance as presented in Table 1. The patient characteristic data of the subgroup (immature oocyte group) did not differ from total patient data using statistical analysis.

First, the CCs were collected from a total of 110 oocytes (30 patients), but 44 oocytes were excluded due to a lack of sufficient granulosa cells to extract RNA. As a result, we collected CCs only from 66 oocytes to extract RNA. Finally, after removing 7 oocytes that degenerated, we obtained CCs from 59 oocytes, 18 patients for gene expression analysis (Cumulus Cell from metaphase II, CC_{MII} from 46 oocyte, Cumulus Cell from metaphase I, CC_{MI} from 6 oocytes and Cumulus Cell from Germinal Vesicle, and CC_{GV} from 7 oocyte) [Figure 1]. The major causes of infertility in this study were ovarian factor, unexplained, male factor, tubal factor, and other causes, in rank order. The subgroup (immature oocyte group) also had the same causes of infertility [Table 2].

From Table 3, the study demonstrated the result of gene expression from the ddPCR reader regarding CC_{MII} and MI+GV oocytes ($CC_{MI + GV}$). The B2M gene (reference gene) expression showed no significant difference in between groups. The RGS2 gene expression when compared with the reference gene in CC_{MII} and $CC_{MI + GV}$ was 0.15 (0.05–0.52) and 0.08 (0.02–0.27), respectively. It was noteworthy that the LHR gene expression when

Table 1: Patients characteristics, total=18 patients

Data	Result			
***n* (patients)**	**Total**	**CC_{MII}, 18**	**CC_{MI+GV}, 9***	***P* (<0.05)**
Age (year, mean±SD)	36.20±3.56	36.20±3.56	35.8±3.33	NS**
BMI (kg/m², mean±SD)	21.75±1.72	21.75±1.72	21.26±0.98	NS**
Year since infertility (year, mean±SD)	4.0±1.6	4.0±1.6	4.1±0.7	NS**
FSH D2 (mIU/ml, mean±SD)	7.0±1.73	7.0±1.73	7.3±1.5	NS**
Estradiol D2 (mIU/ml, mean±SD)	35.86±14.9	35.86±14.9	32.67±10.48	NS**
LH D6 (mIU/ml, mean±SD)	1.89±1.78	1.89±1.78	2.03±1.12	NS**
Dose of Gn (unit, mean±SD)	2475±862	2475±862	2550±770	NS**
Day of GnRH antagonist (mg, mean±SD)	5.05±0.25	5.05±0.25	5.45±0.7	NS**
Number of follicle (mean±SD)	11±5.49	11±5.49	10.5±5.01	NS**
Number of oocyte (mean±SD)	8.43±5.68	8.43±5.68	8.05±5.46	NS**
Day of stimulation (day, mean±SD)	9.53±0.92	9.53±0.92	9.23±0.88	NS**
Endometrial thickness at day of trigger (mm, mean±SD)	9.35±1.86	9.35±1.86	10.3±0.98	NS**

*9 patients from MII group also have MI oocyte, **Mann-Whitney U-test, the significance level was set at $P<0.05$. SD=Standard deviation; CC=Cumulus cells; NS=Not significant; BMI=Body mass index; FSH=Follicle-stimulating hormone; LH=Luteinizing hormone; GnRH=Gonadotropin-releasing hormone; GN=Gonadotropin

Table 2: Cause of infertility, total 18 patients

Etiology	CC_{MII}, 18	CC_{MI+GV}, 9*
Unexplained	4	1
Tubal factor	2	2
Ovarian factor	8	5
Male factor	3	1
Other factor	1	-

*Nine patients from MII group also have MI oocyte. CC=Cumulus cells

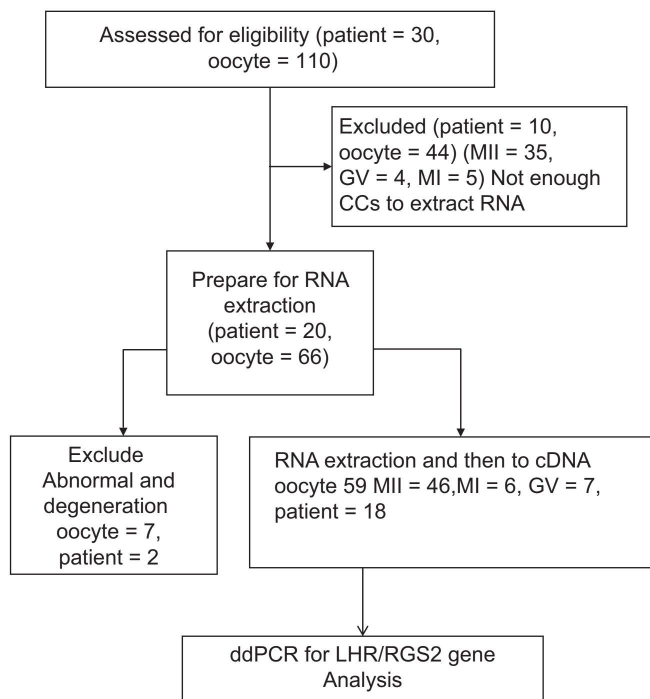

Figure 1: Participants flow

compared with the reference gene in CC_{MII} and CC_{MI+GV} was quite in the same value, that is, 0.02 (0.00–0.11) and 0.02 (0.00–0.06), respectively. Finally, the expression of the RGS2 gene was approximately 8 fold when compared with LHR gene expression in CC_{MI} and about 5 fold in CC_{MI+GV}.

From Table 4, the study demonstrated the proportion of RGS2/B2M gene expression of CC_{MII} and CC_{MI+GV} from total CCs by categorizing in three groups, Group 1: expression of gene < P_{50}, Group 2: expression of gene between P_{50} and P_{75}, and Group 3: expression of gene > P_{75}. In $CC_{MI+GV,}$ we found that nearly about 85% of RGS2/B2M gene expression was < P_{50} and about 15% expressed more than P_{50}. In CC_{MII}, the gene expression more than P_{50} was about 60%.

Discussion

Oocyte immaturity is also a major problem in the treatment by IVF/ICSI method because it means acquiring oocytes that cannot be fertilized. Moreover, having good mature oocytes that cannot be fertilized with sperm is still a major problem making IVF treatment unsuccessful. The data from Stimpfel *et al.*[17] revealed that oocyte immaturity rate remained at a relatively high level around 14%–16% in GnRH agonist and GnRH antagonist protocol. Especially for the GnRH antagonist protocol, that is, widely used today and has many advantages for COH. Advantageous and acceptable methods are available to reduce ovarian hyperstimulation syndrome[18] and prevent premature LH surge. On the other hand, when an overreaction occurs at the target cell, the GnRH antagonist could suppress the activity of the gonadotrope to release endogenous LH, which has an important role to make the oocyte become immature. One study mentioned that the GnRH antagonist protocol may be a contributing factor to

Table 3: Result of gene expression from digital droplet PCR reader

Gene of interest, expression ration	CC_{MII}* (median, range)	CC_{MI+GV}**(median, range)	*P* (<0.05)
RGS2 (copies/20 µl)	16.85 (0.86-220)	15.30 (0.24-91.70)	0.84
LH (copies/20 µl)	1.80 (0.07-21)	1.90 (0.01-14.30)	0.93
B2M (copies/20 µl)	103.80 (11.90-1882)	73.10 (4.20-1192)	0.62
RGS2/B2M	0.15 (0.052-0.517)	0.08 (0.024-0.27)	0.01***
LH/B2M	0.016 (0.003-0.107)	0.017 (0.001-0.057)	0.983
RGS2/LH	8.34 (1.34-58)	5.1 (1-15.33)	0.344
LH/RGS2	0.119 (0.017-0.744)	0.196 (0.065-1)	0.265

*CC from MII oocyte, **CC from MI+GV oocyte, ***Quantile regression test, the significance level was set at $P<0.05$. CC=Cumulus cells; LH=Luteinizing hormone; RGS2=Regulator of G-protein signaling 2

Table 4: Proportion of regulator of G-protein signaling 2/B2M gene expression of cumulus cells$_{MII}$ and cumulus cells$_{MI+GV}$ from total cumulus cells

Percentile	Oocyte group		Total CC
	CC_{MI+GV}	CC_{MII}	
$<P_{50}$	11	18	29
	6.4	22.6	29
	84.62%	39.13%	49.15%
P_{50}-P_{75}	0	15	15
	3.3	11.7	15
	0%	32.61%	25.42%
$>P_{75}$	2	13	15
	3.3	11.7	15
	15.38%	28.26%	25.42%

Pearson χ^2=9.1663, P=0.01 (significant $P<0.05$). CC=Cumulus cells

make the oocyte become not fully mature, especially in repeated IVF treatment cycle.[19]

Maturation arrest can be caused by many factors. Levran *et al*.[20] suggested that failure of maturation *in vivo* was due to one of the following three causes. First, is the absent or incomplete LH effect. Second is derangement in the signaling mechanism from the surrounding cumulus cells. Finally, intrinsic oocyte factor can affect maturity. Recently, Feuerstein *et al*.[21] found that the RGS2 gene is an important biomarker to indicate oocyte maturation competence. Later, Lee *et al*.[22] found that the function of the LH receptor is associated with the RGS2 protein. In a rat ovary, supplementing LH/HCG treatment resulted in the rapid and transient stimulation of RGS2 gene expression. In human granulosa cells, Wu *et al*.[23] found that overexpression of the RGS2 gene could deactivate LH receptor function by a mediated signal in COX2 transcription. In addition, the RGS2 protein can interact with Gαs and Gαq proteins, found at the GPCR, by causing guanosine triphosphate hydrolysis to guanosine diphosphate.[23] Moreover, from the knowledge that has come before, the LH receptor was one of the GPCRs. Therefore, it could be said that the RGS2 protein is one part that controls LH receptor function.

The strengths of this study are as follows: first, we could demonstrate the expression level of the RGS2/B2M and LHR/B2M gene in CCs from oocytes of humans using controlled ovarian stimulation with GnRH antagonist protocol by ddPCR technique. Further evaluation found that most of the CCs from MII oocytes had RGS2/*B2M* gene expression more than P_{50} (about 60%) and significantly differed. However, it did not differ regarding LH/B2M gene expression between immature and mature oocyte groups. As mentioned above, the relation was observed between the LH receptor and RGS2 protein. In the past, *in vitro* studies found that the LHR gene was more expressed in mature oocytes[24] and was one of the biomarkers to predict ovarian response at least in terms of duration of stimulation and prediction of pregnancy success in the long luteal GnRH agonist downregulation protocol. However, in this study, the GnRH antagonist protocol program showed that maturity did not mainly depend on function of the LH receptor; on the other hand, it also depended on the function of the RGS2 gene function. The mechanism of oocyte maturation was suggested by Levran *et al*.[20] That study suggested mechanisms other than the LH effect could achieve a similar level of LHR/B2M gene expression in our study. Oocyte maturation involved the signaling mechanism from the surrounding cumulus cells in which RGS2 gene function could explain this mechanism and may perform a key role to control oocyte maturation by controlling the ovarian stimulation cycle and details of cascade mechanism had to be further studied. Second, the technique that detected gene expression in this study was a digital droplet PCR or ddPCR (Bio-Rad Laboratories, Hercules, CA, USA). It was the newest technique as PCR is generally used to detect point mutations or small sequence of RNA due to its high selectivity and sensitivity.[13] The small partitions in nanofluid volumes were created by emulsion droplets. Thus, the PCR carried out one reaction per single droplet, and this separation allowed a more reliable collection and high sensitivity to measure the amount of nucleic acid. Apart from that this technique directly quantified and clonally amplified nucleic acid in one method with known specific primer,

so it was not necessary to validate gene expression by another method. Third, this study was designed to analyze transcriptome in CCs surrounding oocytes at different stages of maturation. The advantage of the transcriptomic study being a noninvasive method that could evaluate the viability of associated oocytes.[25]

Regarding the limitations of this study, first, a bias was incurred from the sample size for analysis. It designated that one limitation of study was not using the RNA analysis method (by ddPCR, Bio-Rad Laboratories, Hercules, CA, USA), but rather, the method for RNA extraction. Because the number of granulosa cells was very few, we had to collect granulosa cells at least 10^5 cells for RNA extraction using RNA Isolation Kit, Nucleospin® (Macherey-Nagel, Bethlehem, PA, USA). As a result, we could not analyze data from 44 oocytes that did not provide sufficient granulosa cells to use the RNA extraction method. That made this study analyzes data from 59 oocytes instead of 110 oocytes (54% from total enrollment). The consequence in decreased oocytes for analysis not only dispersed the data of gene expression in each maturity group but also increased the difference in numbers between oocyte groups (mature: immature = 46:13). Consequently, RGS2/B2M gene expression in CC_{MII} and $CC_{MI + GV}$ was 0.15 (0.05–0.52) and 0.08 (0.02–0.27), respectively, and LHR/B2M gene expression in CC_{MII} and $CC_{MI + GV}$ 0.02 (0.00–0.11) and 0.02 (0.00–0.06), respectively. The wide range of data created a high variation and large overlap between mature and immature oocyte groups. That made the result unreliable and the results could not clearly discriminate maturity from the gene expression. Second, notably, the LHR/B2M gene in CC_{MII} and $CC_{MI + GV}$ was quite low (compared with Jeppesen *et al*., 2012[25]) and in the same value. It might be that LHR was downregulated due to timing for analysis of granulosa cells, and because the period of oocyte triggering until denuded was quite long, the mRNA level may have already been a downstream signal. Second, transcriptomic analysis of only a surrogate outcome assumes the final product of LHR and RGS2 gene would be the LH receptor and RGS2 protein, respectively. Therefore, it was not the exact action product at the molecular level. In this study, we could not identify the LHR or RGS2 gene expression in multistage of maturity in one oocyte due to the real clinical setting not *in vitro* maturation, so we exposed oocytes to just the postovulatory phase (after oocyte retrieval). Thus, we could not directly compare the level of gene expression especially LHR in pre- and post-ovulatory phases. In addition, we assume that the different level of gene expression in pre- and postovulatory phases of oocytes may be one factor to determine the maturity of the oocyte. The gene expression between mature and immature oocytes may be a problem for clinical application. For further study, the methods to isolate granulosa cells should be more purified using flow cytometry and also testing for white blood cell contamination with specific white blood cell gene markers[11] to increase data reliability. This is because the RGS2 gene also increased in cells that had active biological activities, including cardiovascular function, immune response, bone formation, and cancer.[26] The other clinical outcomes such as clinical pregnancy rate, ongoing pregnancy rate, miscarriage rate, and live birth rate should be included in addition to this genetic result in our study. This research constituted an early study in the area of reproductive medicine to use ddPCR in transcriptomic analysis, so the reproducibility created the issue of taking time to accumulate data. Future studies should be correlated with embryo morphology, aneuploidy, or even comparing the result with other methods such as microarray or quantitative polymerase chain reaction.

Conclusion

Finally, the GnRH antagonist protocol was explored using a multiple-dose regimen. Although effective in controlling premature LH surge in several related studies, the immaturity of the oocytes was a problem that could not be completely overcome. This study showed that RGS2/B2M may play a role in oocyte maturation that was observed in that most of the CCs from the MII oocyte had gene expression distributed more than P_{50} (about 60%). However, the median of RGS2 gene expression differed between groups of oocyte maturity and the data were highly scattered and overlapped. As a result, it was difficult to confirm more expression of the RGS/B2M gene in the mature oocyte group. No difference was observed in LHR gene expression both in the distribution of the gene above P_{50} or median between groups of oocyte maturity. The reason may have resulted by inappropriate timing for mRNA analysis. Therefore, the benefit of and appropriate timing to detect the LHR gene requires further study.

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Conflicts of interest

There are no conflicts of interest.

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