MALAYSIAN JOURNAL OF BIOCHEMISTRY & MOLECULAR BIOLOGY

The Official Publication of The Malaysian Society for Biochemistry & Molecular Biology (MSBMB)
http://mjbmb.org

# IDENTIFICATION OF HYALURIC ACID SYNTHESIS 2 (*HAS2*) AND GREMLIN 1 (*GREM1*) GENE EXPRESSIONS IN HUMAN CUMULUS CELLS AS A BIOMARKER FOR OOCYTE QUALITY

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History
Received: 17th April 2019
Accepted: 10th September 2019

Keywords:

*Cumulus cells, HAS2, GREM1, rt-PCR, oocyte quality*

**Abstract**

Nowadays, the criteria for oocyte selection is based on morphological criteria however, it needs further improvement to select the best embryo quality. The gene expression in the cumulus cell plays important role in signaling for follicular development as well as for oocyte quality. The aim of this study is to investigate the present of *HAS2* and *GREM1* gene expression in the cumulus cells that can become a useful marker for oocyte quality. A preliminary study was performed on cumulus cells derived from four different patients that undergo assisted reproductive technique treatment. Cumulus cells were isolated from 4 patients and the expression of *HAS2* and *GREM1* was analyzed by using reverse transcriptase polymerase chain reaction (rt- PCR). The results shown that *HAS2* and *GREM1* were expressed in grade 3 oocytes, whereas, the genes were absent in grade 4 oocytes. This showed that the expression influenced the oocyte quality. Hence, the measurement of *HAS2* and *GREM1* expressions in cumulus cells would reliably useful tool for selecting competence oocytes with greater chances to be fertilized in assisted reproductive technique.

## INTRODUCTION

To date, single embryo transfer (SET) had tremendously increased in assisted reproduction technology (ART). Selection of high potential embryos is one of the major aims in the ART. However, the lack of an accurate objective method in selecting a highly competent oocytes and embryos is the major problem in enhancing the efficiency of ART. This has emerged the searching of biomarkers in oocyte prediction from morphological assessment to biochemical evaluation which mainly focused on gene expression profiling of the cumulus cells (CCs) [1].

Human CCs are the specialized cells that surround and support the oocyte development. Originating from granulosa cells (GCs), CCs are intimately connected within the gap junction during the follicle development and ovulation process [2]. Furthermore, a study reported that CCs metabolize alternative substrates like glucose, cholesterol and amino acids for energy requirement of oocytes development which poorly metabolized by oocyte itself [3].

Moreover, another study also revealed that the cumulus expansion during pre-ovulatory period is the most significant process for oocyte to be fertilized and developed [2]. The literature also reported that this expansion is vital for normal oocyte development *in vivo*, and oocyte not associated with cumulus expansion have reduced the potential of implantation that results in infertility [2,4]. In fact, several genes expressions are responsible in regulation during the expansion of CCs including *HAS2* and *GREM1*. Previously, these gene expressions have been assessed as the marker of oocyte and embryo competence [2,4,5]. The finding showed that there is a correlation between mRNA expression in CCs and oocyte quality as well as differential

expression of transcripts between CCs of oocytes that achieved successful pregnancy or not. Rising of these genes in CCs had contributed to a better blastocyst formation and high quality of embryo. Indirectly, it improved the pregnancy rate especially in ART.

The semi-quantitative measurement of *HAS2* and *GREM1* mRNAs by using rt-PCR had shown that the expression levels of *HAS2* and *GREM1* in isolated CCs from oocytes developed into good quality embryo are higher compared to the expression levels detected from unfertilized oocytes or poor quality oocytes, which ended, developed into poor quality embryos [2]. Results also denoted that the measurement of *HAS2* and *GREM1* expression in CCs is complement to the morphological evaluation. Thus, analysis of CCs *HAS2* and *GREM1* gene expressions can provide an additional tool for selecting high quality of oocyte that leads to successfully development in IVF. The aim of this study was therefore to investigate *HAS2* and *GREM1* gene expressions in CCs in relation to oocyte and embryo quality.

## MATERIALS AND METHODS

### Patient Treatment and Cumulus cells (CCs) Collection

Four patients were recruited from International Islamic University Malaysia (IIUM) Fertility Centre, Malaysia with human ethical approval. Informed consent had been provided to all patients prior to participation in this study. Patients were stimulated with short or antagonist protocol. Patients were injected with recombinant follicle stimulation hormone (rFSH) Gonal-F (Merck Serono) or Puregon (Merck) alone on daily basis or combined with Orgalutron (Merck) started on day 6. Human chorionic gonadotropin (hCG) dose of 10,000 IU Pubergen (Firstline-Pharma) was given to the patient when follicles reached 18mm diameter. Oocyte retrieval was performed under transvaginal ultrasound 36 hours post hCG and CCs were collected for research.

The fresh CCs samples were centrifuged at 1000g for 10 min to have the pellet and the supernatant were removed. The pellet was re-suspended in 1000 µl freezing media containing DMEM with FBS and antibiotic with 10% DMSO. After that, all the individual pool samples were stored at -80 °C in separated cryovials until used. All oocytes used in this study underwent a preliminary morphological examination. The morphological criteria of the oocyte based on the presence, shape and size of polar body, the texture and fine granularity of the cytoplasm and the size of the perivitalline space. The morphological assessment of oocytes was done to verify the results of cumulus cell transcript levels analysis.

### RNA Extraction

Frozen CCs samples from each patient were thawed and transferred into the centrifuge tube. The cells were washed by using PBS with 1:1 ratio and being centrifuged at 2000 rpm, 24 °C for 10 min. The supernatant was removed. After that, 2 mL of ACK lysing buffer was added to each of the cell and being centrifuged at 1000 rpm, 24 °C for 5 min. The supernatant was removed and the previous step was repeated until pellet appeared before proceeded with the extraction. Total RNA of CCs was extracted using PureLink RNA mini kit (Ambion, USA) which involved the process of lysis and homogenization as well as RNA purification. The extraction method was performed according to the protocol provided by the manufacturer. The RNA was then quantified by using NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA) prior to purity test. 2 uL of each RNA sample was required for the quantification. RNAse free water was used as the reference blank for calibration the spectrophotometer. The reading of RNA purity and concentration of each sample were recorded. The purity of RNA, A 260/280 should be within the range of 1.8 to 2.0.

**Table 1.** The details of *HAS2* and *GREM1* primer used for rt-PCR

| Gene | Primer sequences | Product size (bp) | GenBank Acc. No. |
|---|---|---|---|
| Hyaluric Acid Synthesis2 (*HAS2*) | F: 5'-ATCATCCAAAGCCTGTTTGC-3'<br>R: 5'-GGACCCTTTTCGTGGAAGTT-3' | 301 | NM_005328 |
| Gremlin1 (*GREM1*) | F: 5' TGCTGGAGTCCAGCCAAGA 3'<br>R: 5' GCACCAGTCTCGCTTCAGGTA 3' | 65 | NM_013372 |

### The Synthesis of cDNA

The cDNA was generated from individual RNA samples by using SuperScript III First-Strand synthesis system for rt-PCR kit (Invitrogen, USA). All the components were briefly centrifuged before used. The cDNA synthesis involved 2 types of mixture; RNA mixture and cDNA synthesis Mix as mentioned in the provided protocol by the manufacturer. The RNA mixture was first incubated at 65 °C for 5 min in automated thermal cycler (Bio-Rad, USA). Then, it was placed on ice for at least 1 min. 10 µL of cDNA Synthesis Mix were added to the RNA mixture. The mixtures were incubated at 50 °C for 50 min. The reaction was terminated at 85 °C for 5 min and the products were chilled on ice. After that, 1 uL RNase H was added to each tube containing different samples and incubated for 20 min at 37 °C. The products of cDNA synthesis were stored at -20 °C or used for PCR immediately. Control reaction was also done by using the provided RNA which consists of total HeLa RNA (10 ng/µL).

### Reverse Transcriptase (rt-PCR)

**Table 1** showed the primer sequences, amplication product size and accession number for *HAS2* and *GREM1* genes. The sequence of primers had been validated at NCBI website (www.ncbi.nlm.nih.gov/nuccore/NM_013372). PCR was carried out in an automated TechneTC-512 Thermal Cycler (Fisher Scientific, UK), in a final volume of 25 µl, using TopTaq DNA polymerase kit (Qiagen, USA). The dNTP mix and primer solutions were thawed at room temperature. A Master Mix was prepared according to manufacturer with 2 µL of cDNA per reaction. The protocol of 35 cycles rt-PCR for both genes were similar; *HAS2*: 30s at 94°C (denaturation), 1 min at 56°C (annealing), and 1 min at 72°C (extension); *GREM1*: 1 min at 94°C, 1 min at 55.1°C, and 1 min at 72°C.

### Detection and Semi-quantification of rt-PCR Products

The amplification products were subjected to electrophoresis on a 2% agarose gel in TBE buffer containing 0.5µg/mL ethidium bromide. The fragments were visualized on AlphaImager Hp (Alpha Innotech, USA) after electrophoresis at 90 V for 45 min. The image of the gels were recorded and analysed. Band intensity of each samples was assessed by densitometric analysis performed using the public domain NIH Image 1.63 program. The quantification procedure based on direct digitalization of the PCR

product after separation on agarose gel provides a well-established and sensitive method to detect even small differences in amounts of mRNA from different biological samples [5]. Thus, in this experiment, the relative amount of the HAS2 and GREM1 was calculated by dividing the band intensity from each patient by the intensity of positive control (pooled samples) as mentioned in the early publication [5].

**Statistical analysis**

The experiments were carried out in triplicates. Data were presented as ± means relative band intensity. Kruskal-Wallis test was also performed using SPSS software (PASW Statistics 12, SPSS Inc., Chicago, IL) to test the significance between patients with same group of oocyte quality and HAS2 and GREM1 expression in term of relative band intensity. The result was considered non-significantly differences for $p \geq 0.05$.

## RESULTS AND DISCUSSION

Based on **Table 2**, all the patients were reported having grade 3 oocytes except for patient 1 with grade 4 oocytes. Fertilization occurred in patients 1, 3 and 4 but not patient 2. Patient 3 and 4 at least had 1 oocyte not fertilized. In term of embryo grading, most of them were abnormal. Each patient with fertilization oocytes had at least 1 embryo with grade 2. All the patients were reported to have pathological condition; patient 1 with unexplained, patient 2 with polycystic ovarian syndrome (PCOS) and male factor, patient 3 with PCOS and patient 4 with endometriosis. As for ART method, both patient 1 and 4 underwent *in vitro* fertilization (IVF), patient 2 undergone intracytoplasmic sperm injection (ICSI) and testicular sperm extraction (TESE) and patient 3 undergone ICSI only.

**Table 2.** Evaluation reports of IVF patients

| Subjects | Patient 1 (P1) | Patient 2 (P2) | Patient 3 (P3) | Patient 4(P4) |
|---|---|---|---|---|
| Oocytes grading | Grade 4 | Grade 3 | Grade 3 | Grade 3 |
| Embryo grading | E1: Grade 2 E2: Abnormal E3: Abnormal E4: Abnormal | All oocytes no fertilization | E1: Grade 2 E2: Abnormal E3: Abnormal E4: No fertilization | E1: Grade 2 E2: Abnormal E3: Abnormal E4: No fertilization |
| Pathological condition | Unexplained | PCOS & Male factor | PCOS | Endometriosis |
| Types of ART | IVF | ICSI/TESE | ICSI | IVF |

A total of 4 cumulus samples were obtained from 4 women undergo IVF and ICSI treatment in ART. **Figure 1 and 4** illustrated the successful mRNA extraction and rt-PCR in all pooling samples from each patient (based on positive control). In this experiment, pooling samples of CCs from all samples was used for the positive control (+C). Based on the gel electrophoresis result, *HAS2* (**Figure 1**) and *GREM1* (**Figure 2**) expressions were identified in all of the cumulus samples except one sample depicts negative result in which the cumulus originated from grade 4 oocytes. Other samples that showed the expressions of *HAS2* and *GREM1* derived from grade 3 oocytes. **Figure 3** for *HAS2* showed patient 4 has the highest peak density that is 2.49. Then followed by patient 3 (2.28) and patient 2 (1.42). As for *GREM1* (**Figure 4**), patient 3 indicates the highest expression with relative band intensity of 1.52 followed by patient 4 (1.11) and patient 2 (1.10). However, both of these results did not reach statistical significance for $p \geq 0.05$.

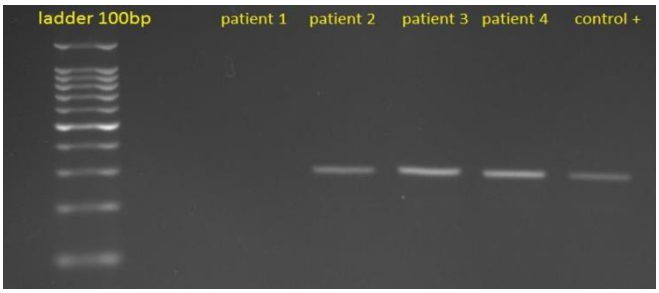


**Figure 1.** Gel electrophoresis results of RT-PCR amplification products for *HAS2* (301 bp). P1 until P4 at the top indicates four different patients. The 100 bp ladder is on the left of the gel. Positive control (+ C) was used for validation of the results.

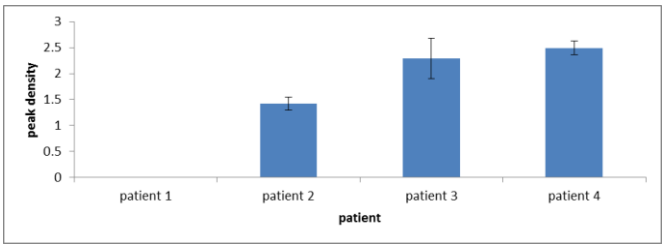


**Figure 2.** Relative band intensity of *HAS2* in cumulus samples from different patients * p-value ≥ 0.05, result was not significant.

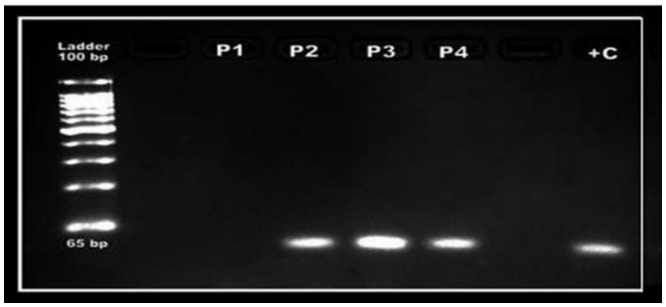


**Figure 3.** Gel electrophoresis results of PCR amplification products for *GREM1* (65 bp). P1 until P4 at the top indicates four different patients. The 100 bp ladder is on the left of the gel. Positive control (+ C) was used for validation of the results.

Information about the oocyte developmental might be a valuable contribution to the existing selection criteria as the oocyte quality is one of the fundamental factor reported in female fertility which reflects the intrinsic development of competence oocyte and comprehensively responsible in both fertilization and further development [6]. Understanding the factors that related to oocyte developmental is crucial so that an additional parameter can be used to support the previous method of oocytes assessment that is lack in effectiveness and efficiency.

The most prominent somatic cell that responsible in oocyte maturation is the CCs since a bidirectional communication occurred between the oocyte and CCs. This intimate relationship enables a continuous exchange of metabolites between both cells

which essentials in supporting and resourcing of the maturing oocyte. Furthermore, CCs can be easily collected without compromising the oocyte especially during metaphase II in which the oocyte are delicate cell, even damaged by repeated morphological examinations. Thus, the analysis of CCs makes them as an attractive target for the development of non-invasive assays of oocyte competence.

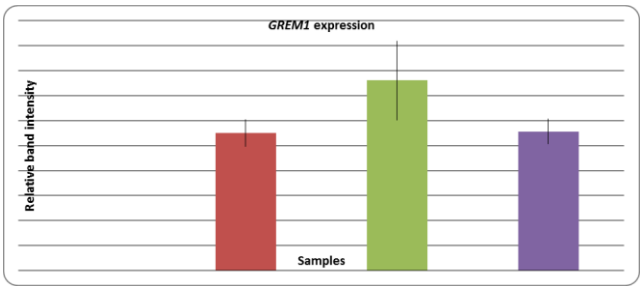


**Figure 4.** Relative band intensity of *GREM1* in cumulus samples from different patients * p- value ≥ 0.05, result was not significant.

*HAS2* is essential for cumulus expansion as its product; hyaluronic acid is the main component of the matrix. It is also related with maturation of oocyte. Hyaluronic acid was formed during cumulus expansion in response to the ovulatory LH surge [5]. The surge of the hormone caused the *HAS2* expressed as a predictive for oocyte maturity [4]. *GREM1* plays an important role in signalling the GDF9 and BMP15 which essential for oocyte developmental. All mouse oocytes showed the expression of GDF9 not only at the beginning of the follicle stage; antral follicle, but also required for later-stage of follicular development [4].

In this study, our objective is to determine the expression of *HAS2* and *GREM1* genes in CCs and in relation to oocytes quality and embryo competence. Our result has shown that all patients had *HAS2* and *GREM1* gene expressions except for patient 1. This may be due to different oocyte grading where the patient was having the lowest oocytes grade (grade 4) compared to other patients with grade 3 oocytes. Even though there is difference in the peak density between patient 2, patient 3 and patient 4 but there is no significant differences ($p>0.05$) between these patients (**Figure 3 and 4**). So it can be concluded that all the patients have the same oocyte quality (grade 3 oocytes). There is other external factor influence the oocyte quality. Patient 2 and 3 have polycystic ovarian syndrome (PCOS), meanwhile patient 4 has endometriosis. This shows that the patients actually have good quality oocyte but endometriosis and PCOS have affected the quality of the oocyte. Moreover, other factors might influence the fertilization and subsequent embryo quality such as in patient 2 done ICSI using TESE technique to obtained sperm with very poor quality from obstruction azoospermic patient. It showed that the problem arisen from the sperm.

From the findings, it can be confirmed that human CCs expressed *HAS2* and *GREM1* as revealed by the literature [2,4,5,7,8,9,10]. The predictive role of *HAS2* and *GREM1* expression in CCs has been identified as useful marker for oocyte competence both *in vivo* and *in vitro* [2,4,10]. This supported the hypothesis that *HAS2* and *GREM1* expression is associated with the quality of oocyte in which the up regulation of *HAS2* and *GREM1* transcription was highlighted as having expressions patterns indicative of good quality oocytes [7,11]. For this reason, oocyte with grade 3 quality was better than grade 4.

In addition, the patients had been reported to have pathological conditions which associated with poor oocyte quality such as polycystic ovarian syndrome and endometriosis. The theory of a damage oocyte condition is coming from ovaries of endometriosis-affected woman [12]. Oocyte development is conditioned by correct function of ovarian cell [13]. Thus, good quality oocyte originated from follicle with an adequate environmental condition by the follicular fluid (FF) and the neighboring cells which are able to influence their progression [14]. *HAS2* mRNA expression was greater in cumulus cells isolated from oocyte that developed into high quality embryo compared to cumulus cell isolated both from oocyte developing into poor quality embryo and those that failed to be fertilized [2]. The variation of GDF9 has been associated with polycystic ovarian syndrome (PCOS), premature ovarian failure, diminished ovarian reserve (DOR) and poor IVF outcomes [9]. Therefore, GDF9 function probably being disrupted especially in CCs of patient 1 as there was no band expression seen on the gel which indirectly alleviated the mRNA expression of *GREM1*. Hence, it is possible that patients with markedly low or negative *HAS2* and *GREM1* expression may have an underlying defect in oocyte quality in CCs. This suggested a mechanism of reproductive compromise as mentioned by Mc. Kenzie *et al*. [4].

In conclusion, *HAS2* and *GREM1* genes were expressed in CCs of grade 3 oocyte quality. The analysis using rt-PCR provided us the opportunity to analyse CCs expression profile and permitted significant progress in understanding the molecular events involved in the process governing oocyte maturation. This study provided an insight into the follicular microenvironment as a novel biomarker assessment of oocyte quality which is important in selection of optimal embryo transfer so that successful pregnancy can be achieved.

## ACKNOWLEDGEMENTS

This study was financially supported by the International Islamic University Malaysia with grant number FRGS15-253-0494.

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