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Original Article

The predictive effect of ACAA2 and ARID2 gene expressions on oocyte and embryo development

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Abstract: To acquiring high-quality embryos is crucial to the improvement of pregnancy rates for assisted reproductive technology (ART). But how to find a non-invasive approach to early embryo selection and improve the implantation rates from proteins in cumulus cells? Our previously study showed that acetyl-CoA acyltransferase 2 (ACAA2) protein and adenine-thymine (AT)-rich interactive domain-containing protein 2 (ARID2) protein were expressed obviously differentially between mature cumulus cells and immature cumulus cells based on proteomics technique. Furthermore, real-time polymerase chain reaction (PCR) was needed to verify the expressions of the two genes in the mature cumulus cells and the relationship between gene expression and the developmental ability of oocytes and subsequent embryos. Then, the effects of ACAA2 and ARID2 expressions on good quality embryo formation were discussed. Finally, we found that the expression of ARID2 and ACAA2 in the mature cumulus cells was significantly higher than in immature cumulus cells and was positively correlated with the rate of high-quality embryos. So, we drew the conclusions that high expression of ARID2 and ACAA2 may predict the developmental potential of oocytes and thus become predictive biomarkers for embryo quality.

Keywords: ACAA2, ARID2, cumulus cells, embryo development

Introduction

Acquisition of high-quality embryos is crucial to improving the pregnancy rate and reducing the risk of multiple pregnancy in assisted reproductive technology (ART). Currently, the assessment of the oocyte quality and embryo developmental ability primarily relies on morphological criteria and the embryo growth rate during embryo development. However, the morphological criteria are hindered by strong subjectivity and limited reliability and therefore often fail to accurately reflect the developmental potential of embryos. A previous study [1, 2] showed that about 29% embryos with high morphological scores had chromosomal abnormalities. Therefore, the exploration of objective evaluation methods and observation biomarkers to assess the developmental potential of oocytes and embryos has become an area of intense interest in the field of ART. Pre-implantation genetic diagnosis (PGD) on a molecular level and biopsy of the embryo during pre-implantation genetic screening (PGS) have achieved objective assessment standards [3], these approaches are invasive, expensive and accompanied with specific indications [4, 5]. The study of the metabolomics of embryo culture medium revealed that embryo development correlated with the consumption of nutrients such as pyruvate, glucose and amino acids in the culture. The embryo developmental ability might be predicted by the nutrient levels in the culture medium. Unfortunately, such measurements requires complex techniques, large sample sizes and high device configuration requirements, thus rendering it difficult to achieve in clinical practice and application [6]. Recent studies have shown that the microenvironment of follicles determines the quality of oocytes. Mechanisms such as communication, inter-regulation and interactions between cumulus cells and oocytes have been suggested to play an important role in the maturation of the oocyte cytoplasm. In addition, cumulus cells have been shown to affect the developmental potential of

oocytes to a certain extent [7, 8]. Some researchers tested the correlation between certain gene expression levels in cumulus cells and pregnancy outcomes, and found that determination of certain gene expression levels could predict the developmental abilities of oocytes and embryos [9-11].

Previous studies on the function of cumulus cells primarily focused on certain proteins or genes, but in China and other countries, there have been very few studies reporting overall systematic analysis of the functional variance of cumulus cells in the cumulus-oocyte complex (COC) culture in vitro. This study aimed to find a non-invasive approach to early embryo selection for the sake of improving the implantation rates by detecting protein expression levels in cumulus cells. The development and maturation of COCs are closely related to the effects of cytokines and cyclins. The differentiation and proliferation of cumulus cells and the meiosis of oocytes require the involvement and strict regulation of cytokines. These proteins are mainly associated with cell stress, anti-apoptosis, and cell-cycle regulation, suggesting that variations in these two proteins may be one of the major reasons for the different maturation and developmental abilities of oocytes in vitro.

Our previous study discovered [1] that COCs contained two types of differentially expressed proteins, including acetyl-CoA acyltransferase 2 (ACAA2) and adenine-thymine (AT)-rich interactive domain-containing protein 2 (ARID2), in mature and immature cumulus cells in vivo.

ACAA2, also known as mitochondrial 3-oxoacyl-coenzyme A thiolase, is located in the mitochondria, and an acyltransferase that participates in fatty acid metabolism. Human ARID2 protein consists of a conserved N-terminal ARID structural zone, a regulatory factor for X box (RFX)-type winged helix, a proline- and glutamine-rich region, and two conserved C-terminal C_2H_2 zinc finger sequences [12]. ARID2 is the second member of the subfamily of the ARID protein family, which involved in various biological events such as embryo development, cell group gene regulation and cell-cycle regulation. ARID2 was a new regulatory protein of the cyclin A1-cyclin-dependent kinase (CDK) 2 complex, but its regulatory mechanism is unclear [13].

Whether the differential protein expression of ACAA2 and ARID2 in cumulus cells can predict the developmental potential of oocytes and embryos remains unanswered. Our previously study applied proteomics techniques to compare the differences in protein expression between COCs in vitro and in vivo. Based on the search for oocyte maturation-related protein families, the present study was intended to use PCR to verify the expression of the two proteins in mature cumulus cells, and explore the mutual relation between and the developmental ability of oocytes and subsequent embryos.

Materials and methods

Research subjects

Forty patients aged 26-37 with a mean of 33.3±3.1 years who received intracytoplasmic sperm injection-embryo transplantation (ICSI-ET) because of pure male infertility factor were selected from the Shenzhen Maternal and Child Health Care (Shenzhen, China) and Shanghai Tongji Hospital Reproductive Centre of Tongji University (Shanghai, China). Females with infertility problems such as polycystic ovary syndrome, endometriosis and ovarian dysfunction were excluded.

Sample collecting and grouping

Ovulation induction: The 40 patients all received the routine standard induction to achieve pituitary down-regulation in the mid-luteal phase as follows. Long-acting triptorelin (1.3-1.875 mg) or daily injection of short-acting triptorelin (0.03-0.05 mg) was administered 7 days after ultrasound-monitored ovulation in a natural cycle. The down-regulation standards included a less than 5 mm thickness of the endometrium, the bilateral ovarian antral follicle counts (AFCs) of not less than 6, serum follicle-stimulating hormone (FSH) levels less than 5 IU/L, luteinising hormone (LH) levels less than 5 IU/L, and estradiol (E2) level less than 50 pg/ml. Fourteen to eighteen days after the down-regulation, exogenous gonadotropin was used to stimulate the ovaries with an initial dose of 150 U-300 U/d continuing 7-14 days. Follicular development was monitored by the same sonologist. The dose of exogenous gonadotropin was adjusted according to the size of the follicles and the patients E2

levels. When more than 3 dominant follicles achieved a mean diameter of 16 mm in combination with the results of a blood hormone E2>200-300 pg/mL/per follicle, 5000-10000 U human chorionic hormone (hCG) was injected intravenously at 9:00 pm of the same day to induce the maturation of the oocytes.

Egg retrieval, cumulus cell collection and grouping: At 34-36 hours after hCG injection, the eggs were retrieved under ultrasound guidance by ovarian follicle puncture through the vagina. The retrieved COCs were classified in a mature group and an immature group. As the number of COCs was very small, the mature COCs were pooled cells from several oocytes classified in the experimental group. COCs in this group were characterized by large size, loose gaps between cells, rich in mucus, loose cell groups and a clear coronal radiation boundary. The immature pooled COCs were classified as the control group. The COCs in this group were characterized by small size and dense distribution with barely any cellular gap or cell mucus block. In addition, the zona pellucid was tightly surrounded by the corona radiata in this group.

Cumulus cell collection: COCs were incubated in an incubator for 4 hours. Before intracytoplasmic sperm injection (ICSI), COCs were placed in an 80 U/mL hyaluronic acid enzyme solution and pipetted repeatedly with a Bast straw to remove the cumulus cells around the COCs. After removal of the cumulus cells, the maturity of the oocytes was observed under a microscope.

Observation of oocytes and subsequent developmental competence of embryos

ICSI was performed on the mature oocytes (Only metaphase II oocytes were used for ICSI) under the inverted microscope after removing cumulus cells.

According to the modified PETER cleavage stage embryos scoring system for the assessment of the quality of day 3 embryos, the uniformity of blastomere size and the number of fragments, the embryo was classified in four grades after 72-h fertilization. According to the criteria, the day 3, 6-10 cells embryos rated grade I and II embryos were defined as good-quality embryos [14].

Two good-quality embryos were transferred routinely on day 3. Pregnancy was defined as a positive human chorionic gonadotrophin (HCG) test in urine on day 12 post transfer. The implantation rate was defined as the number of fetal sacs on the ultrasound at gestational week 7, per number of transferred embryos. The recorded data, including the fertilization rate, the cleavage rate, the high-quality embryo formation rate and the embryo implantation rate, were used to evaluate the developmental ability of the oocytes and subsequent embryos.

If a pregnancy occurred, ultrasound evaluation was performed to ensure the presence of an intrauterine gestational sac (clinical pregnancy); otherwise the pregnancy was registered as 'biochemical'. The implantation rate was defined as the fraction of transferred embryos resulting in an implanted embryo or gestational sac.

Statistical analysis

Statistical Package for Social Science (SPSS) 13.0 was used for statistical analysis. The mRNA expression levels were represented as the mean ± standard deviation (x±s). Quantitative data were examined using one way ANOVA and the unpaired Student's t-test was applied for the comparison of data among the groups. A *P*-value of <0.05 was regarded as statistically significant.

Results

A total of 116 immature eggs and 308 mature eggs were acquired from 40 patients. The proportion of initial oocytes mature/immature was 2.66.

RT-PCR relative quantification results

The gene expression of both ACAA2 and ARID2 was detected in cumulus cells. All the peaks on the solvation curve were specific single peaks, and the melting points (the temperatures corresponding to the peaks) were all above 82°C. The β-actin amplification products of both target and reference genes were specific.

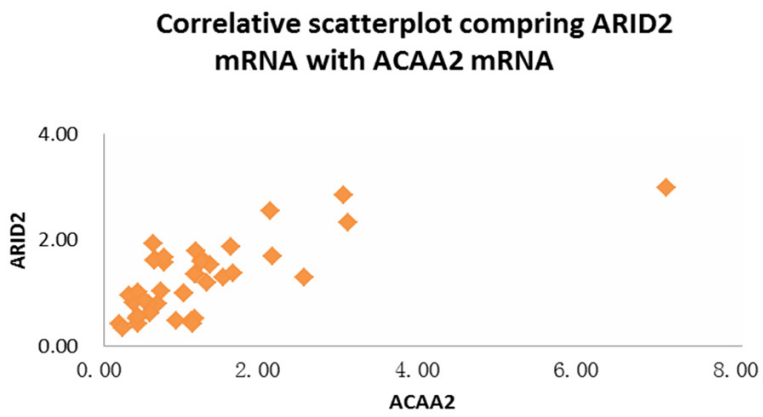

Figure 1. Correlative scatterplot of the relation between ARID2 and ACAA2 mRNA expression. The two genes were positively correlated (r=0.504, P=0.024).

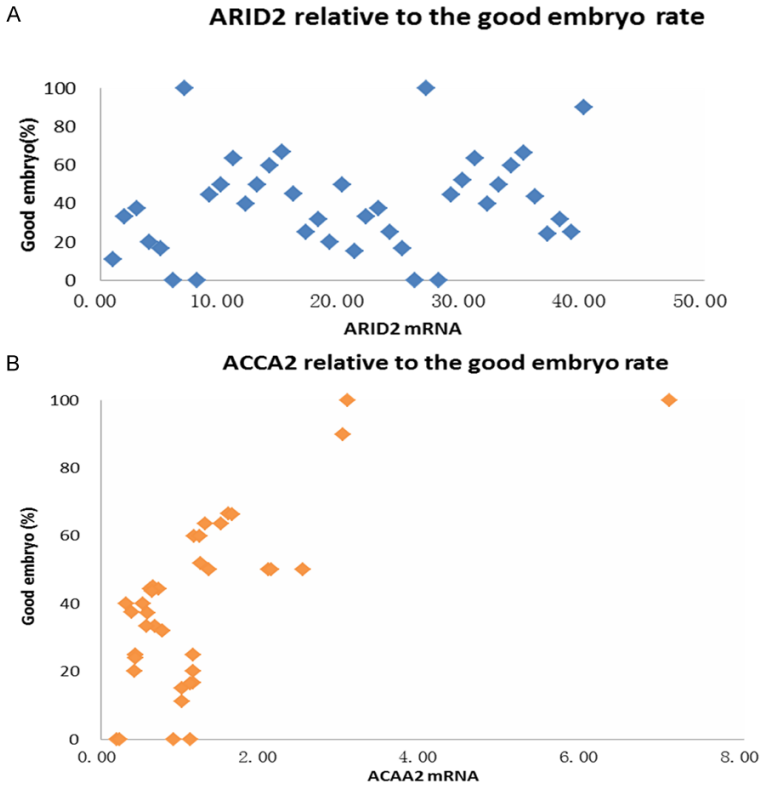

Figure 2. A. The correlation between ARID2 and high-quality embryos, r=0.471, P=0.036. B. ACCA2 was even positively correlated with the high-quality embryo rate, r=0.849, *P*=0.000.

The mRNA expression level of the two proteins and their correlations with fertilization rate, egg cleavage rate, high-quality embryo rate and embryo implantation rate

The relative expression levels of *ACAA2* mRNA and *ARID2* mRNA were positively correlated with the high-quality embryo rate (**Figure 2A**, r=0.471, P=0.036; **Figure 2B**, r=0.849, P=0.000). No significant correlation was observed between the mRNA expression level and the fertilization rate, egg cleavage rate or embryo implantation rate (r=0.081, P=0.736; r=-0.075, P=0.754; r=0.283, P=0.240; r=0.194, P=0.413; r=0.184, P=0.436; r=0.201, P=0.394). The mRNA expression level of the two proteins was not significantly correlated with the number of oocytes, infertile age of patients, the number of days, the total dose of gonadotropin administered, or the baseline sex hormone levels.

Comparison of the relative mRNA expression levels of the two proteins in the pregnant and non-pregnant groups

The *ACAA2* mRNA and *ARID2* mRNA expression levels in cumulus cells were higher in the pregnant group than those in the non-pregnant group, but the differences were not *statistically* significant (**Table 1**, P=0.411, P=0.293).

Discussion

The relative expression level of *ACAA2* mRNA and ARID2 mRNA was 1.3388±1.6907 and 1.4712±0.8401, respectively. In addition, the expression levels of ARID2 mRNA and *ACAA2* mRNA were positively correlated with each other (**Figure 1**, r=0.504, P=0.024).

The advent of ART has brought a great fortune to many infertile families. To date, ART has achieved tremendous technical breakthroughs. However, even when multiple embryos are implanted, the clinical pregnancy rate based on ART is still relatively low. Premature delivery

Table 1. Comparison of five gene expression levels between pregnancy group and non-pregnancy group

Group	Pregnant group (n=28)	Non-pregnant group (n=12)	*t*	*P*
ACAA2 mRNA	1.55±1.99	0.85±0.34	0.84	0.41
ARID2 mRNA	1.60±0.91	1.16±0.59	1.08	0.29

and multiple pregnancy are two major problems often associated with ART. The key to a successful assisted pregnancy is to select high-quality embryos for implantation, which is conducive to increasing the pregnancy rate, reducing complications in mothers and infants [15, 16], and reducing the number of embryos that need to be implanted. Cumulus cells can form functional and structural complexes with oocytes through gap junction and paracrine secretion [17]. In this way, cumulus cells directly affect the development, maturation and embryo potential of oocytes [18]. Therefore, studying the variation in gene expression levels in cumulus cells can provide a more objective, accurate and non-invasive approach to assist embryo implantation.

Both in vivo and in vitro studies on COCs have revealed that cumulus cells exhibit apoptosis during the natural maturation process of oocytes. In addition, the apoptosis rate of cumulus cells was shown to affect the developmental potential of oocytes and their subsequent embryos [19, 20]. In addition, the maturation and development of COCs require ATP-generated energy that is produced by fatty acid metabolism in the mitochondria. Therefore, in this study, the down-regulation of ACAA2 in the control group and the significant up-regulation of ACAA2 in the experimental group with mature COCs suggest that the high expression levels of ACAA2 in cumulus cells could serve as an important factor for the assessment of oocyte maturation in COCs, while immature cumulus cells with a low level of ACAA2 expression might be one of the reasons for the low maturation rate of in vitro maturation (IVM) oocytes.

There was no significant correlation between the expression level of ACAA2 and the fertilization rate, egg cleavage rate or embryo implantation rate but was positively correlated with the high-quality embryo rate. The expression of ACAA2 in mature cumulus cells was significantly higher than that in the immature cumulus cells, suggesting that ACAA2 exhibited good activity only in mature COCs in vivo. This finding is in agreement with the animal studies [21]. ACAA2 was also shown to protect mitochondria against stress damage, enhance oxidative phosphorylation of mitochondria, increase ATP production and reduce cell apoptosis [22]. Mitochondria played an important role in the events of oocyte maturation, fertilization and embryo formation [23]. The results of this study suggest that ACAA2 could be associated with the increased fatty acid oxidation activity and promote the metabolic activity of COC cells. Similarly, ACAA2 was suggested to enhance the protective mechanisms of mitochondria, inhibit cumulus cell apoptosis, and provide sufficient nutrients and energy for the cytoplasmic maturation of oocytes. This process could benefit the subsequent embryo formation and increase the high-quality embryo rate.

Our previously study also revealed that ARID2 protein was only expressed in the experimental group [1]. This study also demonstrated a positive correlation between ARID2 expression and the high-quality embryo rate. ARID2 belongs to the ARID protein family and participates in a series of biological functions such as chromatin remodelling, cell proliferation and fission, cell development and gene transcription regulation [24]. ARID2 protein was up-regulated only in mature cumulus cells, and its expression was positively correlated with the high-quality embryo rate, suggesting that ARID2 was involved in cell-cycle regulation events such as cellular DNA synthesis, chromatin assembly and nucleus formation during the maturation process of COCs. The ARID2 protein was also suggested to promote the differentiation and proliferation of cumulus cells and the meiosis of oocytes, thereby increasing the count and rate of high-quality embryos.

This study also discovered that the expression levels of ARID2 mRNA and ACAA2 mRNA were positively correlated with each other, suggesting that ARID2 and ACAA2 might react and communicate with each other in COCs. Taken together, these proteins promote the cytoplasmic and nuclear maturation of oocytes, increase the intrinsic potential of subsequent embryo development, and enhanced the num-

ber of high-quality embryos for implantation. However, the mechanisms through which these proteins enact their effects remain unclear, and further study are needed to clarify these mechanisms.

In summary, ARID2 and ACAA2 gene expression increased the formation of high-quality embryos and significantly improved the clinical pregnancy outcomes. But as the sample size of the present study is limited, and larger-sample studies are needed to confirm the findings and conclusions.

Disclosure of conflict of interest

None.

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