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Association between cumulus cells—mRNA levels of AMHR2 and FSHR with oocyte maturity

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Abstract

Background: The gene expression of *anti-Mullerian hormone receptor type 2 (AMHR2)* and *follicle stimulating hormone receptor (FSHR)* in cumulus cells (CCs) isolated from mature and immature oocytes was studied as a possible non-invasive approach for determining oocyte maturity and quality. The CCs of 100 infertile women with different etiologic factors were subdivided into control (CCs from MII) and case (CCs from GV) group. Q-PCR was used to evaluate *FSHR* and *AMHR2* mRNA expression levels in CCs from mature and immature oocytes.

Results: *AMHR2* and *FSHR* genes are significantly overexpressed (4–6 fold) in CCs from immature relative to mature oocyte. The expression level of *AMHR2* gene in terms of etiologic subgroups is significantly different (*P* value 0.000). *FSHR* mRNA expression levels in CCs show no significant difference regarding etiologic subgroups (*P* value 0.575).

Conclusion: It seems that determining the expression level of *AMHR2* and *FSHR* genes in CCs could help to understanding molecular mechanism of oocyte maturation process.

Keywords: *AMHR2*, Biomarker, Cumulus cells, *FSHR*, Oocyte maturity

Background

More than three decades after its arrival, human-assisted reproductive technologies (ART) are still routinely and effectively applied to overcome infertility problem. Approximately 15–20% of reproductive age couples suffer from these problems and have significant medical, social and financial implications. One of these problems is that 13–15% of retrieved oocytes in ART cycles are immature [1, 2]. Even at best, if oocyte successfully matures in vitro, the selection of high-quality oocyte and embryos has been one of the main challenges in ART. At present, the selection is based on morphological criteria such as evaluation of cytoplasm, zona pellucida and meiotic spindle, growth rate, and blastocyst formation [3].

But the predictive power of morphological assessment for selecting competent oocyte is still limited.

The emergence of new technologies like ‘omics’ has introduced novel biomarkers that can be applied as a tools for picking out competent oocyte and/or embryo for IVF [4]. It is well proven that oocyte maturation and embryo development depend on the events taking place within the cumulus–oocyte complex (COC) [5, 6]. Therefore, measuring gene expression in cumulus cells (CCs) as a noninvasive indicator could help to select best quality oocyte and/or embryo. Moreover, since these cells in IVF cycles detached from oocytes and then discarded, they are easily available and good candidate for gene expression analysis in order to find a reliable prognostic biomarker for oocyte competence [7–9].

It is proven that oogenesis, folliculogenesis, and oocyte maturation process are heavily controlled by hormones, especially reproductive hormones and gonadotropins include luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Also, the significant role of anti-Müllerian hormone (AMH) in primary follicular

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growth and the expression of it at all stages of folliculogenesis has been reported [10, 11]. Since hormones signal through their receptors, we aimed to investigate whether CC-specific FSH and AMH receptors (*FSHR* and *AMHR2*, respectively) are differentially expressed in CCs surrounding mature or immature oocytes. A few studies have reported differential expression of hormone receptors in CCs separated from oocytes in different maturation stages [12–14]. However, to our knowledge, despite the importance of *FSHR* and *AMHR2* in oocyte maturation signaling, few studies have been conducted in this field. Therefore, we aimed to investigate mRNA expression changes of these two receptors in cumulus cells from oocyte with different maturation level.

Methods

Patients and IVF protocol

This prospective, randomized study was approved by the Ethical Committee of Yazd Reproductive Sciences Institute. The patients undergoing controlled ovarian hyperstimulation (COH) (from February 2016 to May 2018), after signing consent, enrolled in the study. The COC of 100 infertile women classified into control (CCs from MII) and case (CCs from GV) group to study the expression of candidate genes in CCs. The inclusion criteria for both groups were as follows: women ≤ 37 years old without any history of recurrent pregnancy loss (RPL) and genetic disorders. The oocytes with normal morphology according to Rienzi et al. [15] were included. Women with any history of IVF failure, IVF cycles with donor gametes, advanced maternal age (AMA), and severe endocrine conditions were excluded. For all participants, ovarian stimulation was performed using the gonadotropin-releasing hormone (GnRH) antagonist and after 36h, and oocytes were aspirated transvaginally.

Grouping, etiology, and characteristics of patients

The COC of 100 infertile women classified into control (CC from MII) and case (CC from GV) groups. The women aged 31.2 ± 4.6 years have a duration of infertility 6.2 ± 1.6 . The etiology of them was subdivided in 4 following groups: polycystic ovarian syndrome (PCOS, tubal factors (TF, premature ovarian factor (POF), and male factor (MF) (Table 1). The patients went through standard antagonist stimulation protocols with the same dose of FSH to induce multiple follicular development and oocyte retrieval [16].

Cumulus cell collection

After oocyte retrieval and twice washing with G-Mops-V1 (Vitrolife Co., Switzerland), COCs were subjected to enzymatic and mechanical denude methods [17]. The maturity of denuded oocytes was evaluated under

Table 1 The demographic, etiologic, and hormonal information of patients between two control and case groups

Patient ^a	N=100
Duration of infertility (year)	5.5±0.4
Patient age (year)	31.2±4.63
Etiology ^b	
PCO	27
TF	8
POF	36
MF	29
Hormones ^c	
Estradiol (pg/ml)	2290.62±1874.16 (163–6189)
FSH (mIU/ml)	4.96±2.26 (1.8–12.6)
AMH (ng/ml)	4.88±3.73 (0.3–16.4)

PCO polycystic ovarian syndrome, TF tubal factor, POF premature ovarian factor, MF male factor

^a Data are presented as mean±SD

^b Data are presented as total numbers of each variable

^c Data are presented as mean±SD (min-max)

inverted microscope (Olympus Co., Japan) based on the existence of first polar body (PBI) [15]. Then, CCs of each patient were pooled based on maturity grade of oocyte, GV-specific CCs allocated to the test group and mature-specific CCs to the control group. CCs were stored at -80°C in RNA stabilization reagent (Qiagen Co., Europe) after twice washing with PBS.

Total RNA extraction and RT-PCR

The total RNA was extracted by the QuantiTect[®], RNeasy Micro kit (QIAGEN) according to slightly modified company's protocol in a total volume of 14 μl . Concentrations of extracted RNA were measured by NanoDrop spectrophotometer (Thermo Scientific). Subsequently, 1000 ng of the extracted total RNA was reverse-transcribed using the RevertAid First-Strand cDNA synthesis kit (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. For negative control samples, the reverse transcriptase (RT) enzyme or the RNA template was removed from reactions. We store all acquired cDNA at -20°C until PCR running.

Quantitative real-time PCR (q-PCR)

Quantitative real-time PCR (qPCR) was used to validate 2 differentially expressed genes including *AMHR2* and *FSHR* on CCs from both mature and immature groups, and 18s rRNA gene as an internal control was added for normalization. The reason for selecting these genes is their significance and biological role in oogenesis, folliculogenesis, and oocyte maturation. Relative expression of the genes was calculated using QuantiTect SYBER Green

RT-PCR kit (Applied Biosystems, UK) by real-time PCR thermocycler (ABI 7500 Step One). Primer sequences for understudied genes are listed in Table 2. For omitting handling error, amplification of all samples was as duplicated. The specificity of each amplicon was confirmed through investigating of it on 2% gel electrophoresis as well as analyzing melting curve.

Statistical analysis

All comparisons between CCs_{MII} and CCs_{GV} were performed by the non-parametric Mann-Whitney test. The frequency distributions between the groups were analyzed using Fisher’s exact test and chi-square analysis. All hypotheses were two-sided, and the significant level was considered as *p* < 0.05. All statistical analysis was run on SPSS 19 (SPSS Inc., Chicago, IL).

Results

Real-time PCR analysis and gene expression level

Q-PCR was used to determine the relative abundance of FSHR and AMHR2 mRNA expression levels and 18s rRNA as endogenous references in CCs from 100 infertile patients. Expression of 18s rRNA is independent of oocyte maturity and is therefore good candidate for reference gene. The differences in the gene expression of FSHR and AMHR2 between the groups are highly significant (Fig. 1). In terms of the patients’ etiology, we observed significant over expressions in AMHR2 mRNA levels but the expression of FSHR is not statistically significant (Table 3). Moreover, both genes are significantly overexpressed in CCs from immature relative to mature oocyte from PCOS and POF patients. In contrast, the abundance of FSHR and AMHR2 mRNA between CC GV and CC MII in TF and MF patients was insignificant (*p* ≥ 0.05, Fig. 2).

Discussion

Up to now, the assessment of oocytes competence is regularly based on morphology. The morphological scoring is generally based on extra-cytoplasmic and cytoplasmic remarks [18]. A wide range of publications has supported

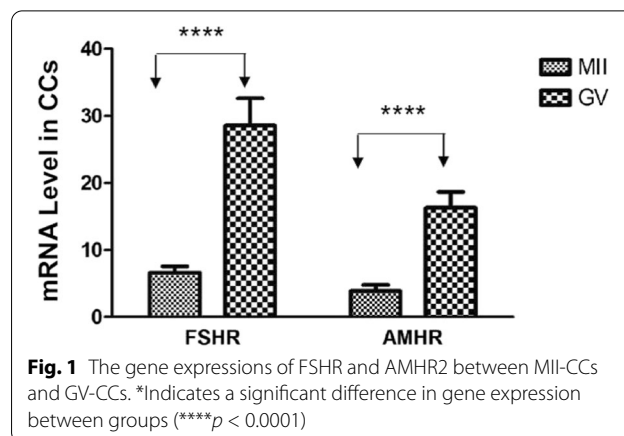


Fig. 1 The gene expressions of FSHR and AMHR2 between MII-CCs and GV-CCs. *Indicates a significant difference in gene expression between groups (*****p* < 0.0001)

the correlation between morphological characteristics of oocyte and the quality of the following stages of embryo development. Tilia et al. [19] concluded that morphology of meiotic spindle is associated with developmental competence of embryo and that the morphological grading could be valuable to achieve successful IVF and pregnancy. However, the power of this morphological grading system in predicting IVF results is still controversial. Therefore, the determination of biomarkers in transcriptome level for the selection of oocytes with good developmental competence is a main research goal [18, 20].

The study of CC gene expression at different stages of oocyte maturity showed that the peak of changes belongs to the stage of oocyte transition from the MI to the MII stage. Also, previous studies reporting considerable transcriptional changes in CCs accompanied by a significant mRNA degradation during oocytes maturity [21–23]. Devjak et al. suggested that the genes involved in signal transduction, cell adhesion, and cell division play an important role in the process of meiosis [12]. With regard to the oocyte-cumulus dialogue and non-invasive access to CCs, these cells are hopeful source of reliable biomarker for evaluating developmental competence of oocyte and embryo quality. The growth and maturity of primary oocytes are strongly regulated by hormones and their receptors. Among them, AMH induces primordial

Table 2 Primer sequences for understudied genes

Gene	Primer sequence	Length	Product size	Tm
18s rRNA	F:AGAAACGGCTACCACATCCA	20	158 bp	59
	R:CCCTCCAATGGATCCTCGTT	20		
AMHR2	F:TGTGTTTCTCCAGGTAATCG	24	164 bp	60
	R:AATGTGGTCGTGCTAGGC	20		
FSHR	F:CAGTGTGATGGTATGGGCT	20	234 bp	60
	R:GAGGAGGACACGATGTTGGG	20		

Table 3 The expression level of AMHR2 and FSHR genes in terms of etiologic subgroups

Variable	PCOS	TF	POF	MF	<i>P</i> value
AMHR	18.30 ± 16.19	8.09 ± 12.30	4.75 ± 9.05	9.76 ± 13.99	0.000 [†]
FSHR	21.27 ± 25.79	12.75 ± 23.84	15.34 ± 20.88	18.11 ± 25.20	0.575

PCOS polycystic ovarian syndrome, TF tubal factor, POF premature ovarian factor, MF male factor

[†] Statistically significance difference between groups (at significance level of *p* < 0.05)

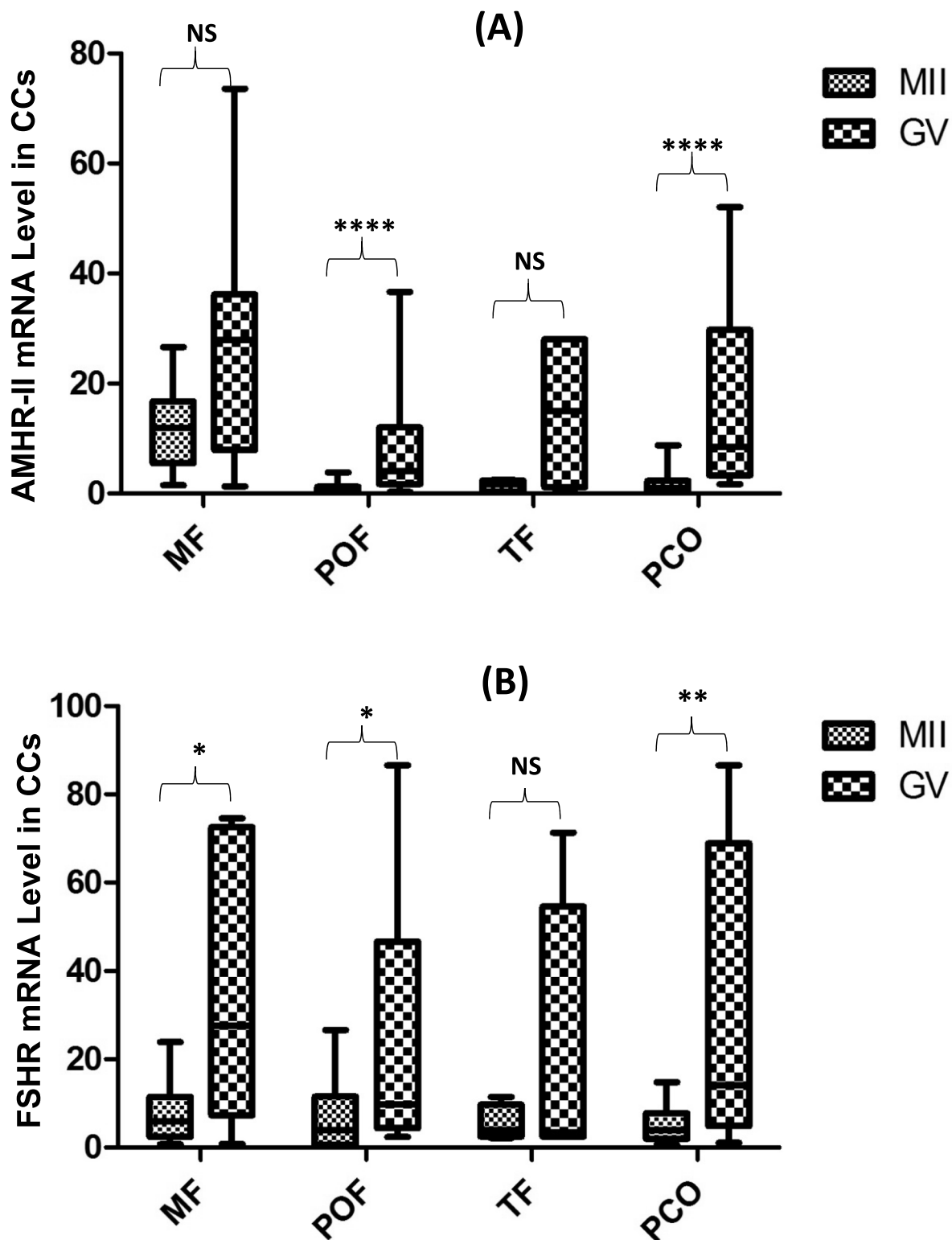


Fig. 2 Comparison of gene expression levels in CCs between groups based on their etiologic patient subgroups. PCOS, polycystic ovarian syndrome; TF, tubal factor; POF, premature ovarian factor; MF, male factor

follicle recruitment through inhibitory effects on follicle sensitivity to FSH. AMH signaling is controlled by two type receptors containing AMHR1 and AMHR2 which AMH and AMHR2 are reciprocally specific. The function of AMH is started by the binding of AMH to AMHR2, which stimulates AMHR1 then phosphorylates the cytoplasmic proteins and transcription factors to regulate related genes [24–26]. Moreover, FSHR plays important role in oocyte maturation and has high expression in CCs. The *FSHR* expression declines along with the maturity of bovine oocytes in vitro and in vivo [27, 28].

According to ingenuity pathway analysis, *AMHR2* and *FSHR* belong to the top gene network that differentially expressed in CCs at all stages of oocyte maturity [13, 14]. Genes were chosen for the following reasons: first, critical role of both genes in oocyte maturation; second, upregulation of them in CCs; and third, induction of their expression by the LH peak as well as their relationship with reproductive hormones such as FSH and AMH. We studied the expression of *AMHR2* and *FSHR* genes in human CCs from GV and MII oocytes in order to introduce a predictive biomarker of oocytes maturity. A clinical prospective study aimed at finding genes with differentially expression profile in CCs with different degree of oocyte maturity, under gonadotropin-releasing hormone (GnRH) agonists or antagonist stimulation protocol showed significant increase in the expression of *AMHR2* and *FSHR* in CCs_{MI} oocytes compared to CCs_{MII} oocytes [12]. In line with their result, we have observed a higher expression of both *AMHR2* and *FSHR* genes in CC_{GV} in comparison with CC_{MII} oocytes. The expression of both receptor genes in CCs has been reported to be significantly associated either to each other or to the expression level of AMH and androgen receptor (AR) [26, 29, 30]. Moreover, the statistical analysis of the etiological subgroups revealed that the expression of both genes in CCs_{GV} compared to CCs_{MII} significantly increased in PCOS and POF but not in TF and MF (Fig. 2). In this regard, Cateau-Jonard et al. reported that *FSHR*, *AMHR2*, AMH, and AR are upregulated in granulosa cells from stimulated follicles of PCOS women signifying a defect in oocyte maturation [13].

Conclusion

The oocyte quality is decisive for the subsequent embryo development; thus, the priority is the identification of specific biomarkers to select best competent oocytes. The analysis of transcriptomes of COC could be used as noninvasive biomarkers of oocyte quality and maturity, being a more effective system to select high-quality oocytes compared to morphological criteria. In this context, the purpose of this study was to evaluate the relative

gene expression of cumulus cells (CCs) as non-invasive biomarkers of oocyte maturity in order to enhance the final efficiency of the IVF cycles [18, 20]. For the reasons mentioned, the gene expression of *AMHR2* and *FSHR* were targeted as a possible non-invasive biomarker for identifying oocyte maturity and quality. According our findings, it appears the expression level of these genes in cumulus cells have the potential to be a predictive biomarker to select good competent oocyte, thereby improving IVF procedures. Our findings could be useful in reproductive biotechnology to other species, as well. Therefore, further studies in this field especially on the correlation with implantation and pregnancy rate are valuable and important.

Abbreviations

ART: Assisted reproductive technologies; COC: Cumulus–oocyte complex; CCs: Cumulus cells; LH: Luteinizing hormone; FSH: Follicle-stimulating hormone; AMH: Anti-Müllerian hormone; FSHR: FSH receptor; AMHR2: AMH receptor 2; COH: Controlled ovarian hyperstimulation; RPL: Recurrent pregnancy loss; AMA: Advanced maternal age; GnRH: Gonadotropin-releasing hormone; PCOS: Polycystic ovarian syndrome; TF: Tubal factors; POF: Premature ovarian factor; MF: Male factor.

Acknowledgements

This original paper was extracted from Fateme Montazeri' Ph.D. thesis in Molecular Genetics. We sincerely appreciate our colleagues in the ART section of Yazd Reproductive Sciences Institute and Yazd Genome laboratory for their kindly collaboration.

Authors' contributions

Foroughmand AM and Kalantar SM participated in the study design, and also, they were responsible for overall supervision and scientific counseling. Montazeri F performed the in vitro maturation of oocytes and sample collection (CC from GV and MII oocyte) for investigating gene expression of AMHR and FSHR, extraction, Q-PCR, statistical analysis, etc. Fesahat F. and Hoseini SM contributed to the laboratory experiment and scientific counseling. All authors performed the editing and approving the final version of this paper for submission, also participated in the finalization of the manuscript, and approved the final draft.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Declarations

Ethics approval and consent to participate

This experimental study was approved by the Ethical Committee of Yazd Reproductive Sciences Institute. The study was conducted in accordance with the Declaration of Helsinki. An informed consent was obtained from all individual participants included in the study.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Received: 1 May 2022 Accepted: 11 August 2022

Published online: 30 August 2022

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