Epigenetic alterations of *CYP19A1* gene in *Cumulus* cells and its relevance to infertility in endometriosis

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Abstract

Purpose The purpose of the present study was to investigate the epigenetic mechanisms responsible for the aberrant aromatase expression (*CYP19A1*) in *Cumulus* Cells (CCs) of infertile endometriosis patients.

Method *Cumulus* cells were obtained from 24 infertile patients with and without endometriosis who underwent ovarian stimulation for intracytoplasmic sperm injection. Expression of *CYP19A1* gene was quantified using Reverse Transcription Q-PCR. DNA methylation, histone modifications, and binding of Estrogen Receptor, ER β to regulatory DNA sequences of *CYP19A1* gene were evaluated by Chromatin ImmunoPrecipitation (ChIP) assay.

Capsule These epigenetic changes along with differential binding of ER β (as a transcription factor) in *CYP19A1* promoters may impair follicular steroidogenesis, leading to poor Oocyte and embryo condition in endometriosis patients.

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Results *CYP19A1* gene expression in CCs of endometriosis patients was significantly lower than the control group ($P=0.04$). Higher incorporation of MeCP2 (as a marker of DNA methylation) on PII and PI.4 promoters, and hypoacetylation at H3K9 in PII and hypermethylation at H3K9 in PI.4 were observed in *CYP19A1* gene in endometriosis patients ($P<0.05$). Moreover, a decreased level of ER β binding to PII and an increased level of its binding to PI.3 and PI.4 promoters of *CYP19A1* were observed in endometriosis patients when compared to control.

Conclusion Significant reduction of *CYP19A1* gene expression in CCs of endometriosis patients may be the result of epigenetic alterations in its regulatory regions, either by DNA methylation or histone modifications. These epigenetic changes along with differential binding of ER β (as a transcription factor) in *CYP19A1* promoters may impair follicular steroidogenesis, leading to poor Oocyte and embryo condition in endometriosis patients.

Keywords Endometriosis · Epigenetic · *CYP19A1* · Cumulus cell · Estrogen receptor beta

Introduction

Endometriosis which is defined by the presence of endometrial-like tissue outside of the uterine cavity, is one of the most common gynecological disorders reported up to 50 % in infertile women [1, 2]. The association of endometriosis with infertility is proven; however, the mechanisms by which endometriosis affects reproductive function remain as a debatable issue [3]. The impact of endometriosis on fertility and fecundity was obtained from the studies conducted on ovarian cells such as granulosa and *Cumulus* cells, endometrial profiling, follicular microenvironment, and outcomes of in vitro fertilization/intracytoplasmic sperm injection (IVF/

ICSI) [4, 5]. On the basis of previous studies, ovarian dysfunction is one of the most important factors regarding endometriosis and infertility [6]. The proposed mechanisms which account for the fertility deficiency in these cases include endometrial receptivity deficiency, abnormal folliculogenesis, and follicular microenvironment alterations. These factors lead to ovulatory disorders, poor embryo quality, and implantation failure [7, 8].

The presence of proper bidirectional *Cumulus Oocyte* interaction is essential to promote Oocyte cytoplasmic and nuclear maturation and competence acquisition. *Cumulus* cells (CCs) provide nutrients, metabolites, growth factors, amino acids, and other regulatory molecules for Oocyte. These close relationships are also required for the development of the embryo and pregnancy outcome [9–11]. Due to the abovementioned reasons, analyzing CC gene expression could provide information to assess the Oocyte environment and competence [12–14]. One of the major functions of granulosa-*Cumulus* cells, relevant to Oocyte development, is the estrogen biosynthesis from androgen precursors via the enzyme aromatase [15]. Cytochrome P450 aromatase (aromatase), the key enzyme responsible for estrogen biosynthesis, is encoded by the *CYP19A1* gene. Aromatase and its product, intrafollicular estrogen, have an obligatory role in ovarian functions, folliculogenesis, growth, and regulation of follicular development [16]. Estrogen mainly mediates its physiological effects on gene regulation in ovarian cells by activation of estrogen receptor β (ER β) [17]. ER β can act in both up- and downregulation of gene expression via recognizing and binding to the estrogen response element (ERE) on the target gene promoters [18]. Estrogen is one of the major inducer of aromatase gene expression in granulosa cells that provides a positive feedback loop during folliculogenesis to stimulate steroidogenesis [19]. This action of estrogen is also mediated by the activation of ER β [20].

CYP19A1 gene contains ten tissue-specific promoters located in the first exon that are alternatively used in various cell types. Among the various promoters of *CYP19A1*, the promoter II (PII) is the most active one that involves in the aromatase gene expression and function in ovarian cells [21–24].

The roles of aromatase in the etiopathogenesis of endometriosis have shown that endometriosis is an estrogen-dependent disorder [25]. Furthermore, changes in gene expression of aromatase lead to the pathophysiology of infertility in these patients [26].

The regulation of aromatase coding gene and mechanisms that are responsible for the aberrant expression of this gene in ovarian cells of patients with endometriosis are not fully understood. Therefore, in this study, regulatory regions of *CYP19A1* gene were screened to investigate the interactions between epigenetic alterations and aromatase gene expression in endometriosis.

Material and methods

Patient history

This cross-sectional study was approved by the Ethics Committees of Iran University of Medical Sciences (IUMS, No: 23108-April 2014) and the Royan Institute. Also, written informed consent was obtained from all cases and control subjects prior to the Oocyte retrievals. The study was conducted on 24 patients (12 infertile patients with endometriosis and 12 normo-ovulatory patients with tubal factor infertility or egg donors) who underwent ovarian stimulation for ICSI at the ART Center (the Royan Institute, Iran), between November 2014 and April 2015. All women, who were diagnosed with endometriosis either by laparoscopy or pathological examination, constituted the study group. Endometriosis was categorized according to the revised American Fertility Sterility (rAFS) classification (stage III, $n=7$; stage IV, $n=5$).

The criteria for selecting the patients were as follows: being younger than 37 years of age and absence of polycystic ovarian syndrome (PCOS) or other endocrine diseases at the time of Oocyte pickup. The control group was the patients who underwent treatment cycles due to tubal factor infertility ($n=2$) or Oocyte donors ($n=10$), without endometriosis (diagnosed by laparoscopy and histological examination), with regular menstrual cycles, normal hormone profile (FSH, LH, and TSH), and no diabetes or clinical signs of hyperandrogenism. To obtain a sufficient number of samples, two women with tubal factor infertility and inclusion and exclusion criteria were added to the samples. These women had been laparoscopically verified to rule out any degree of endometriosis and also they did not have any other causes of infertility such as ovulatory abnormalities. On the other hand, the strict criteria for the selection of the women with tubal factors were similar to healthy women. Patients with recurrent endometrioma and previous surgical resection of ovarian endometrioma (within 6 months before ovarian stimulation) were also excluded from the endometriosis group.

All couples underwent the first ICSI cycle. Semen analysis of the male partner was performed according to the World Health Organization (WHO) criteria (fifth edition, 2010). Infertile couples with severe male factor infertility, frozen, and surgically retrieved sperm were excluded from the study.

Ovarian stimulation protocols

All endometriosis patients and the control group underwent pituitary downregulation with GnRH agonist (Buserelin Acetate Suprefact, Aventis, Germany) long protocol started in the mid-luteal phase (day 21) of the preceding menstrual cycle. Ovarian stimulation was carried out when pituitary desensitization was achieved (plasma E2 levels ≤ 50 pg/ml, the absence of growing ovarian follicles and endometrial

thickness ≤ 6 mm) using rFSH: follitropine alpha (Gonal-f; Merck Serono, Switzerland) or uFSH + uLH: menotropine (Menopur®; Ferring Pharmaceuticals, Denmark) and was continued until the day of human chorionic gonadotropin (hCG) (Ovitrelle®, Merck-Serono) administration based on ovarian response.

Cumulus-Oocyte complex (COC) collection

Aspiration of COCs from the ovary was performed 34–36 h after hCG injection. The COCs were washed several times with culture medium G1V5™ (Vitrolife AB, Sweden) in order to remove any blood and cell debris, then incubated at 37 °C until denudation. Shortly before ICSI, CCs were stripped from the COC with hyaluronidase for a maximum of 30 s, washed sequentially in several droplets of free enzyme medium; only CC samples isolated from metaphase II oocytes (characterized by the presence of the first polar body) were selected for this study. The collected CCs were washed in cold phosphate-buffered saline (PBS) and centrifuged twice at 800g for 8 min; the cell pellet was resuspended in PBS and equally divided into two aliquots for total RNA and chromatin extraction. Afterwards, the samples were centrifuged, the pellets of *Cumulus* cells were snap frozen in liquid nitrogen then stored at -80 °C.

ICSI and evaluation of clinical parameters

Metaphase II oocytes were submitted to ICSI with ejaculated sperm 2–3 h after oocyte pickup. The following variables related to developmental competence of oocyte were evaluated: (1) perivitelline space (PVS) granularity, (2) PVS size, and (3) cytoplasmic granularity. Indeed, embryo quality was assessed and graded by the following morphological criteria: (i) at 16–18 h after ICSI, the fertilization rate was calculated as the proportion of fertilized oocytes or two pronuclei (2PN) over the number of injected oocytes and (ii) on day 3 post-ICSI, embryo cleavage rate was calculated as the proportion of cleaved embryos over the number of fertilized oocytes (2PN); morphological assessment of embryos were performed by evaluation of blastomeres number, degree of fragmentation, and symmetry of cells in the embryos. Accordingly, the embryos were categorized based on the following parameters: grade A, at least 8 equal size blastomeres with no cytoplasmic fragmentation; grade B, 6–8 blastomeres with less than or equal to 20 % fragmentation; grade C, 4–8 blastomeres with 20–40 % fragmentation; and grade D, 4–8 blastomeres with >40 % fragmentation. Grades A and B are considered as good quality embryos and grades C and D as bad quality embryos [27].

RNA extraction and cDNA synthesis and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA extraction and removal of genomic DNA from the cumulus cells were performed using the RNeasy Micro Kit (Qiagen, cat. no: 74004) according to the manufacturer's instructions. The quantity and quality of RNA were assessed using the NanoDrop spectrophotometer (Thermo Scientific, NanoDrop 2000 spectrophotometer). Twenty nanograms of total RNA was used for cDNA synthesis according to manufacturer's instruction of QuantiTect Whole Transcriptome Kit (Qiagen, Cat.No:207045). RT-PCR was performed with human-specific primers. The primer sets, product size, and annealing temperatures used are listed in Table 1. All experiments included RT controls and negative controls (without cDNA). PCR products were analyzed by gel electrophoresis.

Quantitative RT-PCR (qRT-PCR)

Messenger RNA quantification was performed by qRT-PCR on the Step-One RT-PCR system (Applied Biosystems, USA). Each reaction was run in triplicates. To verify primer specificities, melting curve analyses were performed. Standard curves were obtained for each gene to evaluate primer efficiency using the logarithmic dilution series of total cDNA [28]. 18s rRNA was used as the endogenous control for normalization. Gene expression data were analyzed using $2^{-\Delta\Delta C_t}$ algorithm to calculate the *CYP19A1* mRNA level relative to the level of 18s rRNA [29].

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed using the low cell number ChIP kit (Diagenode, cat. no. C01010072) following the manufacturer's instructions. Briefly, cross-linking of target protein and DNA is performed by adding formaldehyde then quenched by the addition of glycine. Chromatin was sonicated to an average DNA length of 200–500 bp using the Bioruptor sonication system (Diagenode, UCD 200- Bioruptor). One percent of the sheared chromatin solution was saved as input DNA, and the remainder was used for immunoprecipitation with specific antibodies.

Protein A/G-coated magnetic beads (Diagenode, cat. no: K02141006/C03010021) were incubated either with the following ChIP-validated antibodies: anti-methyl-CpG-binding domain protein2 (MeCP2; ab2828, Abcam, Cambridge, UK); H3 dimethyl Lys9 (H3K9me2, ab1220, Abcam, Cambridge, UK); H3 acetyl Lys9 (H3K9ac, ab4441, Abcam); or estrogen receptor beta (ER β , ab3577, Abcam). The beads were combined with chromatin extracts of CCs on a rotating wheel at 4 °C overnight. The immunoprecipitated DNA was purified using DNA isolation buffer (Diagenode). The relative levels of DNA methylation and histone modifications of *CYP19A1* PII, PI.3, and PI.4 promoters

Table 1 Sequence of the primers used for qPCR and ChIP-qPCR experiments

Gene	Forward primer (5–3)	Reverse primer (3–5)	Annealing temperature (°C)	Product size (bp)
CYP19A1	AGGAGGTGACCAATGAATCG	AGGAGGTGACCAATGAATCG	60	116
18 s rRNA	GTAACCCGTTGAACCCCAT	CCATCCAATCGGTAGTAGCG	60	151
Primers for ChIP assay				
PII	TGGGCTTCCTTGTTTTACT	ACAGAATCCCCTAAAAGGTTCA	60	142
PI.3	TGGCTTTCAATTGGGAATG	TTGCAGCATTTCTGACCTTG	60	116
PI.4	TGTCCTTGATCCCAGGAAAC	TCTGCCTTCTGTGACCTCAA	60	133

were analyzed by real-time PCR with specific primer sets listed in Table 1. The IP/INPUT ratio of the target sequence was determined using the following formula: $(\% \text{ IP/INPUT} = 2^{[(Ct \text{ (x\% input)} - \log \text{ (x\%)} / \log 2) - Ct \text{ (IP)}] \times 100})$.

Statistical analysis

Statistical calculations were performed using the IBM SPSS statistic 21 software (IBM Corp., Armonk, NY). The Student's *t* test with a two-tailed distribution and Levene's test for equality of variances were used when the distribution of the values was normal, and the non-parametric Mann-Whitney test when the distribution was not normal to compare samples for the normalization of data with statistical significance at the level of $P < 0.05$.

Results

Demographic information and clinical outcomes

All demographic data, clinical characteristics, ICSI outcomes of endometriosis, and control groups are presented in Table 2. There were no significant differences between patients with endometriosis and control group with regard to age, BMI, FSH or LH level, and days of ovarian stimulation ($P > 0.05$). Total gonadotropin dosage and bad quality embryos were significantly higher in endometriosis compared to the control group. At least one morphologic abnormality was observed in 41.74 (43/106) and 67.74 % (63/93) of the oocytes retrieved from the control and endometriosis patients, respectively.

Table 2 Demographic information and clinical outcomes

Variables	Control (<i>n</i> = 12)	Endometriosis (<i>n</i> = 12)	<i>P</i> value
Age (years)	31.58 ± 3.47	31.66 ± 3.52	NS
BMI (kg/m ²)	25.1 ± 3.5	24.99 ± 3.01	NS
FSH (IU/L)	8.32 ± 2.3	9.52 ± 2.5	NS
LH (IU/L)	7.63 ± 1.8	8.7 ± 2.1	NS
AMH (ng/ml)	1.5 ± 0.42	1.2 ± 0.34	NS
AFC	10.41 ± 3.5	8.91 ± 1.5	NS
Duration of stimulation (days)	10.5 ± 1.73	10.91 ± 1.83	NS
Total gonadotropin dose (IU)	1989 ± 411	2987 ± 1156	<i>P</i> = 0.01
Total no. of COCs	106	93	NA
Total no. of MII oocytes	99	87	NA
No. of oocytes retrieved	8.83 ± 4.46	7.75 ± 1.65	NS
Dysmorphic oocytes (%)	41.74 % (43/106)	67.74 % (63/93)	<i>P</i> = 0.04
Fertilization rate (%)	79.94 ± 4.73	77.4 ± 4.27	NS
Cleavage rate (%)	75.46	73.32	NS
No. of embryos produced	7.1 ± 4.1	6.08 ± 2.1	NS
No. of high-quality embryo	5.41 ± 2.4	3.25 ± 2.09	<i>P</i> = 0.02
No. of low-quality embryo	1.25 ± 1.42	2.83 ± 1.52	<i>P</i> = 0.01

Values are expressed as mean ± SD

BMI body mass index, *FSH* follicle-stimulating hormone, *LH* luteinising hormone, *AMH* anti Müllerian hormone, *AFC* antral follicle count, *NS* not significant

Fig. 1 Result of RT-PCR for mRNA expression of *CYP19A1* and 18s rRNA genes in human cumulus cells (DNA ladder 50 bp). **b** *CYP19A1* mRNA expression in human cumulus cells (CCs) of patients with endometriosis and control groups (mean $\pm$ SEM)

CYP19A1 mRNA expression in cumulus cells

All amplified RT-PCR products were at the expected size for *CYP19A1* and *18s rRNA* (housekeeping) genes (Fig. 1a). As shown in Fig. 1b, our data revealed that the mean relative expression of *CYP19A1* gene was significantly lower in CCs of infertile endometriosis patients compared to the control group ($P=0.04$) (Fig. 1b).

DNA methylation and histone modifications of the *CYP19A1* promoters

The alterations of DNA methylation and histone modifications of the *CYP19A1* promoters (PII, PI.3, and PI.4) were evaluated by ChIP assay in the regulatory regions shown in Fig. 2a. The region between -228 and $+51$ bp in the *CYP19A1* promoters was selected because a part of this region contains the

Fig. 2 Analyses of epigenetic profile of *CYP19A1* gene by chromatin immunoprecipitation. **a** Schematic diagram of regulatory regions of the *CYP19A1* promoters (PII, PI.3, and PI.4) monitored by ChIP assay. Regions amplified by qPCR are indicated by arrows, and nucleotide numbers are relative to the transcription start site (TSS). **b** Incorporation

of MeCP2, H3K9me2, and H3K9ac into PII, PI.3, and PI.4 **c** Incorporation of ER β into analyzed regulatory regions of *CYP19A1* gene. The results are expressed relative to a 1/100 dilution of input chromatin (mean $\pm$ SEM)

regulatory element-binding sites which authorize specifically promoter regulation of aromatase expression in the ovarian cells [30]. Also, we found two estrogen receptor half-site sequences (1/2ERE: AGGTCA) in PI.3 and PI.4 promoters; therefore, these regions (details are shown in Fig. 2a) were selected to screen for ER β incorporation and epigenetic alterations.

In CCs of endometriosis patients, incorporation of MeCP2 in PII (twofold) and PI.4 (threefold) promoters of *CYP19A1* was significantly higher than that of the control group ($P=0.02$ and $P=0.01$, respectively). However, the level of MeCP2 binding to the PI.3 promoter did not show a significant change between the two groups ($P>0.05$) (Fig. 2b).

Furthermore, a significant hypoacetylation at lysine 9 of histone 3 (H3K9) of PII promoter was observed in patients affected with endometriosis ($P=0.001$), whereas no significant difference of methylation level at lysine 9 of histone 3 (H3K9me2) was detected between endometriosis and control groups ($P>0.05$) (Fig. 2b).

The H3K9 methylation level in the PI.4 promoter was significantly higher in the endometriosis group ($P=0.02$) (Fig. 2b), whereas the H3K9 methylation level in PI.3 and histone H3 acetylation level in the PI.3 and PI.4 promoters did not show significant difference between control and endometriosis ($P>0.05$) (Fig. 2b).

Differential incorporation of ER β into the *CYP19A1* promoters

ChIP-qPCR analysis of ER β incorporation into the three analyzed promoter regions of *CYP19A1* gene showed a reduced binding of the ER β in the ovarian-specific promoter PII in CCs of patients with endometriosis ($P=0.001$). In contrast, the incorporation of ER β into promoters PI.3 and PI.4 in these patients is significantly higher than in the control group ($P=0.006$ and $P=0.02$, respectively) (Fig. 2c).

Discussion

We evaluate the gene expression of aromatase in cumulus cells of endometriosis patients and the epigenetic mechanisms which are responsible for its aberrant expression. The obtained results revealed that the mean relative expression of *CYP19A1* gene was significantly lower in CCs of infertile endometriosis patients compared to the control group. This data is in compliance with the study of Barcelos et al. that reported a low level of aromatase mRNA in cumulus cells of patients with pelvic endometriosis [26]. However, there are some controversial evidences for the expression of the *CYP19A1* gene in granulosa cells. Harlow et al. and Lu et al. showed that aromatase activity, in vitro estradiol production, and aromatase mRNA expression are reduced in granulosa cells cultured or isolated from patients

with endometriosis [31–33]. However, De Abreu et al. demonstrated that endometriosis does not have a significant impact on *CYP19A1* gene expression in mural granulosa cells [34].

To determine whether the reduced aromatase gene expression in the presence of endometriosis was due to epigenetic alterations or not, we then analyzed epigenetic modifications of the *CYP19A1* gene promoters.

Two main epigenetic mechanisms, DNA methylation and post-translational modifications of histone residues, are known to regulate the gene expression and their alterations being the major causes of human diseases [35]. Epigenetic gene suppression/activation is altered with the chromatin structure condensation or recruitment of histone-modifying enzymes.

DNA methylation involves the transfer of a methyl group onto the C5 position of the cytosine so as to form 5-methylcytosine. The recruitment of MeCP2, as a part of the methyl-binding protein family (MBDs), to methylated CpG dinucleotides represents a major mechanism by which DNA methylation can repress transcription [36, 37]. MeCP2 has been shown to function as a repressor of transcription via its functional domains: a methyl-binding domain is necessary for its binding to 5-methylcytosine and a transcriptional repression domain that interacts with histone-modifying enzymes. MeCP2 binds to methylated DNA sequences and changes in chromatin compaction. These modifications cause transcription repression. Furthermore, MeCP2 can recruit histone-modifying enzymes such as histone deacetylases to areas of methylated DNA. These enzymes can deacetylate lysine residues of histone tails, as these interactions can further repress gene activity [36, 38]. The present study demonstrated that MeCP2 level in PII is twofold higher in the endometriosis group compared to the control group; it is concluded that promoter PII region of *CYP19A1* gene is hypermethylated, suggesting that reduced expression of *CYP19A1* in endometriosis is caused by promoter hypermethylation.

Histone H3 lysine 9 acetylation (H3K9ac) and dimethylation (H3K9me2) are the most studied histone modifications which are related to activation and repression of transcription, respectively [39, 40]. Our results revealed that, in the presence of endometriosis, the hypoacetylation of H3K9 at the promoter II region occurs in cumulus cells. The hypoacetylation of promoter PII region in the endometriosis confirmed that the MeCP2 may inhibit gene expression through recruitment of histone deacetylase to this area.

The major inducer of aromatase gene expression in granulosa cells during folliculogenesis is FSH. FSH-induced aromatase expression is enhanced by estrogen. This action of estrogen is mediated by the activation of ER β in follicular cells [30, 41].

Because the regulatory region of *CYP19A1* does not have a consensus ERE site [30], we searched for sequences in the promoters that might be responsible for the regulation of aromatase expression through ER β in cumulus cells. We

recognized an estrogen receptor half-site sequence (AGGTCA) in the promoter PI.3 and PI.4 regions. The binding affinity of estrogen receptors to this 1/2ERE sequence is well known [42]. Moreover, some studies demonstrated that aromatase expression in granulosa cells is regulated via the PI.3 and PI.4 promoters, although to a lesser extent than using ovarian-specific PII [43]. Thus, we focused our attention on these promoters in addition to PII in the following ER β and epigenetic marks-DNA binding studies (Fig. 2a).

By investigating ER β chromatin binding to the three analyzed promoter regions, we found a decreased binding of the ER β in the promoter PII of the *CYP19A1* gene that also contributes to further repressing of aromatase gene in addition to epigenetic alterations in endometriosis patients. Interestingly, the incorporation of ER β into promoters PI.3 and PI.4 in CCs of patients with endometriosis is significantly higher than of the control group (Fig. 2c). Moreover, epigenetic modifications in these promoters were also analyzed and we identified higher DNA and histone3 K9 methylation in promoter PI.4 (Fig. 2b). In normal cumulus cells, ER β was mainly occupied in the PII region of the *CYP19A1* gene. In endometriosis, however, ER β effectively binds to PI.3 and PI.4 regions of the *CYP19A1* gene rather than PII. On the basis of the literature, we do not exactly know how endometriosis change the promoter selectivity of ER β in cumulus cells and further experiments should be carried out. However, promoter switching is a possible event underlying the aberrant expression of aromatase in pathological conditions such as breast adipose cancer and endometriosis. It seems that this differential binding of ER β to *CYP19A1* promoters is a feature of endometriosis. Recently, epigenetic control of some estrogen-responsive genes by ER β as well as ER β -induced promoter switching has been clearly demonstrated [44, 45]. Therefore, it is possible that differential binding of ER β cause a shift from transcriptional activation status to epigenetic suppression status of *CYP19A1* gene. On the other hand, ER β plays key role in the regulation of aromatase through the interaction with other transcription factors such as specific protein-1 (SP1), COX2, and activator protein-1(AP-1) [46]. So the study of these interactions and possibly other abnormalities that interfere the balance between these factors in patients with endometriosis can provide a better insight into the mechanisms controlling promoter switching in *CYP19A1* gene.

The assessment of the certain gene alteration patterns in women with fertility disorders such as patients affected by endometriosis and understanding how this disease disrupts all aspects of the reproductive process could improve the molecular insights into the reasons for their infertility [47]. Some studies showed that the intrinsic ovarian problems is accompanied with reduced endometrial receptivity, and this is the cause of low rate of pregnancy in endometriosis patients [48, 49]. Given the fact that the physiological role of aromatase and subsequently estrogen production by granulosa and cumulus cells in follicular microenvironment are fundamental for the oocyte

cytoplasmic maturation, fertilization process, and early-stage embryo development [50–52], it is not improbable to speculate that altered expression and disturbed such functions of aromatase might contribute to follicular growth deficiency and decreased oocytes quality. In this context, Barcelos et al. demonstrated that the reduced expression of aromatase in CCs of endometriosis patient might be responsible for the oocyte deficiency, lower number of fertilized oocytes, and cleaved embryos and, subsequently, the infertility in such patients [26]. Also, higher level of estrogen as well as an increased aromatase activity of granulosa cell is associated directly with a larger number of high-grade embryo and pregnancy rates in IVF treatment cycle [53, 54]. In the present study, higher percentage of morphological abnormalities in oocytes retrieved from endometriosis patients is a marker for decreased oocyte quality related to this disease (Table 2). Despite the fact that oocyte-retrieved count and fertilization rate were similar in both endometriosis and control groups of the present study, there was a substantial difference in the number of high- and low-quality embryos between groups (Table 2). This is consistent with other studies that showed a significant increase in the percentage of arrested embryos and aberrant zygote formation in patients with endometriosis compared with control group [49, 55]. Indeed, our clinical analysis revealed that women with endometriosis required higher dosage of gonadotropins during ovarian stimulation (Table 2). It might be possible that increasing the dose of gonadotropin administration in patients with endometriosis can compensate their ovarian responses to stimulation, which leads to the increase in the number of retrieved oocytes.

Nevertheless, the presence of endometriosis may negatively influence intrafollicular signals such as FSH-mediated signaling networks for the steroidogenesis and other functions of the ovarian cells from the point of view of the oocyte maturation and embryo development [56]. Therefore, incorporation of estrogen co-administration into ovarian stimulation cycles or improvement of embryo culture conditions by supplementation of estrogen to culture medium may have positive effects on the oocyte quality or the development of embryo in women with endometriosis. Further investigations using a large number of patients are required to confirm the clinical outcome and conclusions about implantation, fertilization, and pregnancy rates.

According to the present study, several factors can contribute to affecting aromatase expression in cumulus cells of patients with endometriosis that include the lower incorporation of ER β into promoter PII which may diminish aromatase stimulation by FSH and so aromatase gene expression does not augment estrogen production in response to FSH. Moreover, higher DNA methylation and reduced histone acetylation in this regulatory region of *CYP19A1* can cooperate to suppress the gene in endometriosis patients that may contribute to estrogen deficiency in the ovaries. These conditions might reduce oocyte maturity and embryo quality. We postulated that all of these factors may contribute to impair fertility in endometriosis patients.

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Compliance with ethical standards This cross-sectional study was approved by the Ethics Committees of Iran University of Medical Sciences (IUMS, no: 23108-April 2014) and the Royan Institute. Also, written informed consent was obtained from all case and control subjects prior to the oocyte retrieval.

Conflict of interest There is no conflict of interest in this study.

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