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The Wnt/β-catenin signaling in endometriosis, the expression of total and active forms of β-catenin, total and inactive forms of glycogen synthase kinase-3β, WNT7a and DICKKOPF-1

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ABSTRACT

Objectives: The cyclical changes in proliferation and differentiation of endometrial cells are regulated by estrogen and progesterone via modulating Wnt/β-catenin signaling. Imbalance in the expression of estrogen and progesterone receptors causes progesterone resistance in endometriosis patients. The aim of this study was to investigate the expression of some main components of Wnt/β-catenin signaling including WNT7a, DKK-1, β-catenin, and GSK-3β in eutopic endometrium and peritoneal endometriotic lesions of endometriosis patients compared to healthy endometrium in the mid-secretory phase of menstrual cycle.

Study Design: This prospective study was performed, during a 12 months period from December 2015 to November 2016, on healthy women as the control group (n = 14) and endometriosis patients (n = 34). We used real-time polymerase chain reaction and Western blot techniques.

Results: Protein and mRNA expression of DKK-1 were significantly down-regulated in both endometriotic lesions and eutopic endometrium of endometriosis group. We also demonstrated that the expression of non-phosphorylated β-catenin (active form) and phosphorylated GSK-3β (inactive form) were up-regulated in endometriosis patients. The mRNA levels of β-catenin, GSK-3β, and WNT7a, as well as the protein levels of total β-catenin, total GSK-3β, and WNT7a in endometriosis group, were not significantly different with those in control group. The patterns of mRNA and protein expression of all interested factors in the lesions were similar to those in the eutopic endometrium of same patients.

Conclusions: It seems that the aberrant activation of Wnt/β-catenin signaling in the secretory phase of the menstrual cycle in endometriosis has two essential elements: excessive inactivation of GSK-3β and suppression of the expression of Wnt signaling inhibitor DKK-1. Interventions in this signaling pathway may allow for the exploration of potential new targets for the control of development and progression of endometriosis.

Introduction

Endometriosis is an estrogen-dependent disorder affecting about 10% of women in reproductive age [1]. Its main symptoms, which impair the quality of life, are infertility and chronic pain [2]. There are several theories regarding the etiology of endometriosis. At present, the most accepted one is Sampson theory. It suggests that endometrial cells reach the peritoneal cavity via the retrograde flow of menstrual fluid and implants in ectopic sites [3]. Endometrial epithelial and stromal cells obtained from endometriosis patients have more proliferative and invasive capacity than those from unaffected controls [4]. These cells can easily attach to intact peritoneal mesothelial cell layer and proliferate rapidly [5].

The normal endometrium undergoes cyclical remodeling under control of sex hormones secreted by ovaries [6]. Estrogen and progesterone exert their function on endometrium via modulating the Wnt/β-catenin signaling [7]. It has been accepted that estrogen activates Wnt/β-catenin signaling and induces nuclear localization

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of β-catenin during the proliferative phase [8]. A significant increase in the progesterone secretion inhibits Wnt/β-catenin signaling, thus induces cell differentiation [7] and prepares the endometrium for embryo reception during the window of implantation period [9]. The rate of pregnancy in endometriosis women who undergo embryo transfer is less than women affected by other infertility factors [10]. The implantation failure in endometriosis patients is associated with a non-receptive endometrium [11] which is connected to progesterone resistance in the eutopic endometrium of endometriosis patients [12]. Resistance to progesterone leads to an aberrant activation of Wnt/β-catenin signaling resulting in over-expression of Wnt target genes [13] involved in the receptivity of endometrium such as Hoxa10 [14] and some matrix metalloproteinases (MMP-9 and MMP-2) [15].

Although endometriosis is a benign disease, it mimics some properties of malignancies such as cell proliferation, adhesion, invasion, and elevated local angiogenesis [16] which may be explained by abnormal activation of Wnt/β-catenin pathway [17]. The expression of some Wnt/β-catenin target genes including Cyclin D1 [18], SOX9 [19], and vascular endothelial cell growth factor [20] are up-regulated in the endometrium of women with endometriosis. The central mediator of this pathway is β-catenin. It is continuously synthesized in the cytoplasm but in a degradation complex, glycogen synthase kinase-3β (GSK-3β) phosphorylates β-catenin and targets it for proteasome destruction which keeps its cytosolic level low. Wnt signaling inhibits GSK-3β activity, leads to stabilization and accumulation of β-catenin within the cytoplasm, and then it enters to the nucleus and activates the transcription of its target genes [21]. The activity of GSK-3β is regulated by several kinases including AKT, PKC, and PKA via an inactivating phosphorylation of GSK-3β at serine 9 position [22]. Over-activation of Wnt signaling pathway has a key role in some cancers such as endometrial [23], breast [24], and colorectal [25].

In a recent study, in vitro inhibition of Wnt/β-catenin signaling repressed the main characteristics of endometrial cells involved in the development of endometriosis including cell migration, proliferation, and invasion [15]. There are controversies regarding the expression of Wnt/β-catenin signaling components in endometriotic lesions and eutopic endometrium of women with endometriosis among researchers. Most of the previous studies focused on the evaluation of the localization of factors such as β-catenin and GSK-3β within the endometrial cells [26–28]. Our objectives were to investigate the expression of total and active forms of β-catenin, total and inactive forms of GSK-3β, WNT7a, as well as DICKKOPF-1 (DKK-1) (a potent Wnt signaling inhibitor) in endometriotic lesions and eutopic endometrium of a homogeneous group of infertile endometriosis patients during the window of implantation.

Materials and methods

This study was a part of a bigger study aimed at understanding the molecular basis of infertility in women with severe endometriosis.

Patients and sampling

Patients undergoing diagnostic laparoscopy for infertility and those undergoing laparoscopy for reversal of tubal ligation were recruited at the Alzahra hospital (Tabriz University of Medical Science, Iran) from December 2015 to November 2016. The initial inclusion criteria were: 1) having regular menstrual cycles; 2) no hormonal treatment for at least 6 months before laparoscopy; 3) age 22–37 years; and 4) body mass index between 18.5 to 30 kg/m^2. The exclusion criteria were: 1) diabetes mellitus, chronic infections or immune system diseases and 2) any pathological finding in uterus cavity such as fibroids and polyps. A total of 46 infertile women of reproductive age who met our initial inclusion criteria and planned to undergo diagnostic laparoscopy, accepted to participate in the study.

In all women, surgery was performed in the mid-secretory phase of the menstrual cycle. Monitoring of urinary LH surge and the date of last menstrual period were used for determination of mid-secretory phase (LH + 6 to LH + 10) which was confirmed by an independent pathologist after endometrial sampling. All sampling processes were done based on the recommendations of World Endometriosis Research Foundation (WERF) Endometriosis Phenome and Biobanking Harmonisation Project (EPHect) [29]. All endometrial tissue samples were obtained with Pipelle curette ((Pipelle de Cornier Mark II, France). Also, we collected samples of the peritoneal endometriotic lesions from all patients with suspected endometriosis. The collected samples were divided into three parts, the first section washed with sterile cold phosphate-buffered saline (PBS) and immediately stored in liquid nitrogen for western blot, the second section immersed in RNAlater (QIAGEN) and stored at −80 °C for RNA extraction, and the third section was fixed in formalin for endometrial dating or confirming endometriotic lesions.

Twelve out of 46 patients were excluded from the study because of other pathological findings including severe hydrosalpinx, subserosal uterine leiomyomas, and ovary cancer (n = 9) and no pathological findings (n = 3) according to their laparoscopic and pathologic examinations. In 39 out of 46 patients, endometriosis was diagnosed. Endometriosis severity was staged based on the revised American Society for Reproductive Medicine classification system [30]. All 34 endometriosis stage III–IV patients were included in the study as endometriosis group. Laparoscopy confirmed no macroscopic pathological finding in 14 fertile women who underwent reversal of tubal ligation; all included in the study as the control group. The samples obtained from the others were excluded from the study.

The study was approved by Tehran Medical University Research Ethical Committee (IR.TUMS.REC.1394.476). Participation in the study was voluntary and after being informed that they could leave the study at any stage, the participants gave their informed written consent.

Real-time quantitative polymerase chain reaction (RT-PCR)

We used the RT-PCR method to quantify the transcripts of β-catenin, GSK-3β, DKK-1, and WNT7a. Total RNA was extracted individually using the RNeasy Mini Kit (QIAGEN, 74104). The integrity of mRNA was confirmed with visualizing the proper ribosomal bands after agarose gel (1%) electrophoresis. Four μg RNA was reverse transcribed into cDNA by using RevertAid First Strand cDNA Synthesis Kit (Fermantas, K1622). The PRIMERBLAST software was utilized (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to design the primers sequences. The used primer sequences were as follows: β-catenin: forward, 5′-TGGCTTGGAATGAGACTGCTG-3′, reverse, 5′- GCCACCCATCTCATGTTCCAT-3′; GSK-3β: forward, 5′- AGGACATTTCACCTCAGGAGTG-3′, reverse, 5′-GGTCTCCAGTATTAGCATCTGACG-3′; DKK-1: forward, 5′- TTGACAACTACCAGCCGTACC-3′, reverse, 5′- CAGGCGAGACAGATTTGCAC-3′; WNT7a: forward, 5′- TGCCCGGACTCTCATGAAC-3′, reverse, 5′- GTGTGGTCCAGCACGTCTTG-3′; and GAPDH: forward, 5′-GAAGGTGAAGGTCGGAGTC-3′, reverse, 5′- GAAGATGGTGATGGGATTTC-3′.

The Real-Time PCR System (Applied Biosystems) apparatus was used to quantify all transcripts.

The total reaction volume was 20 μl containing diluted cDNA 1:100, 0.25 mM of each primer, and 12 μl RealQ Plus 2 x Master Mix

Green – Ampliqon. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control housekeeping gene. All samples were analyzed in triplicate. The melting curves analysis confirmed the specificity of PCR reactions. Also, the expected size of PCR products was confirmed by electrophoresis (3% agarose gels stained with ethidium bromide). We used the Livak method ($2^{-\Delta\Delta Ct}$) [31] for quantification of the mRNA expression of β-catenin, GSK-3β, WNT7a, and DKK-1 in each sample relative to the calibrator sample. The $2^{-\Delta\Delta Ct}$ was reported as mRNA fold change.

Western blot

The protein content of tissue samples was extracted by using RIPA lysis buffer containing protease inhibitors (sc-24948, Santa Cruz Biotechnology) supplemented with phosphatase inhibitor cocktail (Sigma-Aldrich). Then, the cell lysates were centrifuged at 13,000 × *g* for15 min at 4 °C. Total protein concentration of supernatants was determined by using Bradford assay (Pierce) based on manufacturer's instruction. Samples were electrophoresed (20 μg of protein per lane) on SDS (sodium dodecyl sulfate)-polyacrylamide gel and, subsequently, proteins were transferred onto a nitrocellulose membrane (Bio-Rad). The membranes were placed into the blocking buffer containing 5% non-fat dry milk in 0.1% TBS-T (Tris-buffered saline/Tween-20) and then were incubated overnight at 4 °C with anti-β-catenin (#8480, Cell Signaling), anti-non-phospho-Ser^{33,37}Thr^{41}-β-Catenin (active β-Catenin) (#8814, Cell Signaling), anti-GSK-3β (sc-7291, Santa Cruz Biotechnology), anti-phospho-Ser^{9}-GSK-3β (inactive GSK-3β) (sc-81495, Santa Cruz Biotechnology), anti-DKK-1 (sc-25516, Santa Cruz Biotechnology), anti-WNT-7a (sc-26360, Santa Cruz Biotechnology), or anti-GAPDH (sc-25778, Santa Cruz Biotechnology). The membranes were washed with TBS-T, incubated with the respective HRP-labeled secondary antibodies against mouse (sc-2005, Santa Cruz Biotechnology), rabbit (sc-2004, Santa Cruz Biotechnology), or goat (sc-2768, Santa Cruz Biotechnology) for 1 h at room temperature and washed again with TBS-T. Immunodetection was achieved by using ECL detection kit (34080, Thermo Scientific) and chemiluminescence was captured using Western Blot Imaging System (Sabz Biomedical). The human colon adenocarcinoma HT29 cell lysate was utilized as a positive control. The signals intensities were quantified using densitometry analysis software (ImageJ version 1.50i) and normalized to GAPDH.

Statistical analyses

The normality analysis of parameters was checked by the Kolmogorov-Smirnov test. Data were described as mean and SEM. Statistical analyses were performed by the independent sample *t* test or paired student's *t*-test using SPSS version 22 software. Significance was set at p value less than 0.05.

Results

The mRNA expression of DKK-1, GSK-3β, WNT7a, and β-catenin in the endometriotic lesions and eutopic endometrium of women with endometriosis

Expression of the interested genes in the endometrium of control group as well as endometriotic lesions and eutopic endometrium of endometriosis group was assessed with RT-PCR. As presented in Fig. 1, the mRNA expression of only DKK-1 in both endometriotic lesions and eutopic endometrium of endometriosis group was significantly lower than its expression in control group. There was no significant difference in expression of DKK-1 between endometriotic lesions and eutopic endometrium of endometriosis patients.

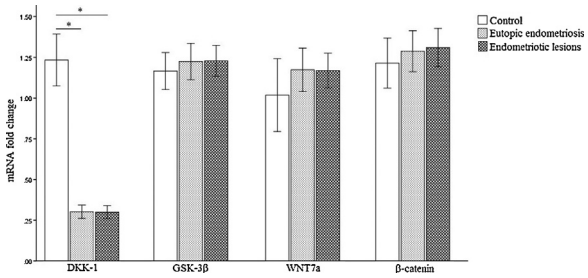

Fig. 1. The mRNA fold change of DKK-1, GSK-3β, WNT7a, and β-catenin in the endometrium of control and both endometriotic lesions and eutopic endometrium of endometriosis groups at the mid-secretory phase of menstrual cycle. The bars illustrate means ± SEM of 14 controls, 34 endometriotic lesions, and 34 eutopic endometriosis samples. *: P < 001.

The protein expression of DKK-1, total GSK-3β, phospho-Ser^{9}-GSK-3β (inactive GSK-3β), WNT7a, total β-catenin, and non-phospho-Ser^{33,37}Thr^{41}-β-catenin (active β-catenin) in endometriotic lesions and eutopic endometrium of women with endometriosis

Expression of the interested proteins in the endometrium of control group as well as endometriotic lesions and eutopic endometrium of endometriosis group was evaluated using western blot analyses. The results showed that the expression level of DKK-1 in the endometriotic lesions and eutopic endometrium of endometriosis patients was significantly lower than in the endometrium of control group, while there was no significant difference in DKK-1 protein expression between endometriotic lesions and eutopic endometrium of endometriosis group. There was no significant difference in expression of WNT7a between the endometrium of control and endometriosis groups as well as between endometriotic lesions and eutopic endometrium of endometriosis group (Fig. 2A and 2B).

As depicted in Fig. 2C and D, the expression level of inactive GSK-3β in both endometriotic lesions and eutopic endometrium of endometriosis group was higher than its level in control group, while there was no significant difference between the three tissues regarding the expression of total GSK3β.

Total β-catenin was expressed in all samples with no significant difference. But the endometriotic lesions and eutopic endometrium of endometriosis patients expressed higher levels of active β-catenin compared to the endometrium of control group. There was no significant difference between endometriotic lesions and eutopic endometrium of endometriosis group in this regard (Fig. 2E and F).

Discussion

Findings of the present study confirmed that eutopic endometrium of women with endometriosis is not able to repress the activity of Wnt/β-catenin signaling in the mid-secretory phase of menstrual cycle. In healthy endometrium, β-catenin expresses in both proliferative and secretory endometrium without any significant difference [32]. Hou et al. indicated that endometrial epithelial cells treated with estrogen exhibit more nuclear localization of β-catenin [33], the insignia of Wnt/β-catenin signaling activation. The endometrial elevated expression of estrogen receptor-α [34] and an inadequate response to progesterone in endometriosis patients lead to the persistence of the proliferative phenotype in the mid-secretory phase of the menstrual cycle [12,35]. The results of our study demonstrate that there is no difference in protein expression of total β-catenin in mid-secretory phase endometrium of endometriosis patients compared to healthy women. This is in disagreement with the findings of Matsuzaki et al. who demonstrated the elevated

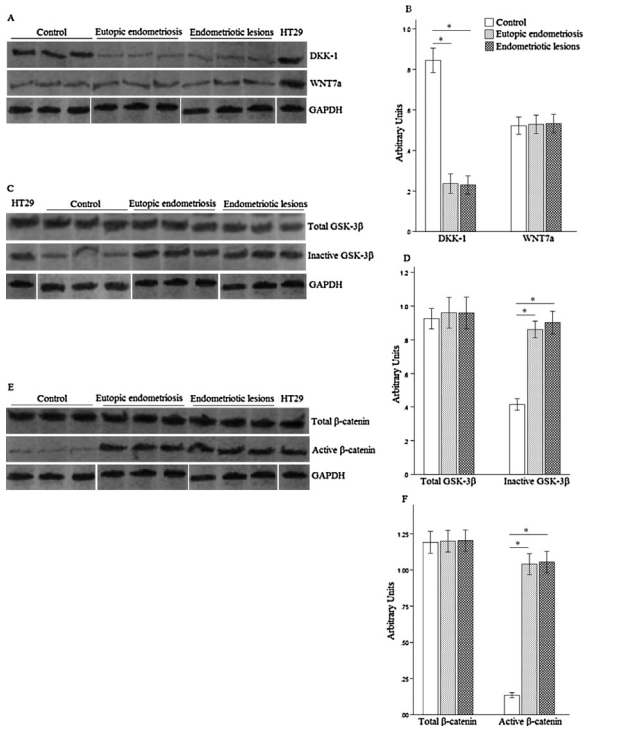

Fig. 2. The protein expression of DKK-1 (35 kDa), WNT7a (39 kDa), total GSK-3β (47 kDa), inactive GSK-3β (phospho-Ser^{9}-GSK-3β) (47 kDa), total β-catenin (92 kDa), and active β-catenin (non-phospho-$Ser^{33,37}Thr^{41}$-β-Catenin) (92 kDa) in the endometrium of control and both endometriotic lesions and eutopic endometrium of endometriosis groups at the mid-secretory phase of menstrual cycle. The panels show three representative samples from each tissue. HT29 cell lysate was used as a positive control. We used GAPDH as a loading control. (A) Western blot analysis and (B) densitometry analyses of DKK-1 and WNT7a bands normalized to the related GAPDH band. (C) Western blot analysis and (D) densitometry analyses of total GSK-3β and inactive GSK-3β bands normalized to the related GAPDH band. (E) Western blot analysis and (F) densitometry analyses of total β-catenin and active β-catenin bands normalized to the related GAPDH band. The bars illustrate means ± SEM of 14 controls, 34 endometriotic lesions, and 34 eutopic endometriosis samples. *: $P < .001$.

expression of total β-catenin in endometriosis patients [26]. The incompatible results may be explained by the different methods (western blot versus immunohistochemistry) used to determine the protein levels of β-catenin in endometrial tissues. It is interesting that in agreement with Matsuzaki et al. [26], there was no significant difference in mRNA expression of β-catenin in the endometrium of endometriosis patients and healthy women, which confirms our findings in the expression of total β-catenin protein. The phosphorylated form of β-catenin is rapidly degraded in the cytoplasm [21]; so we assessed the level of the dephosphorylated form of β-catenin as its active form which can localize in nuclear and activate Wnt signaling. Unlike total β-catenin, a significantly higher level of active β-catenin was shown in endometriosis compared to the healthy endometrium, confirming the results obtained by Matsuzaki et al. [26]. Also, the endometriotic lesions and eutopic endometrium of endometriosis patients showed the same expression patterns of mRNA as well as total and active forms of β-catenin. Changes in the phosphorylation status of GSK-3β can explain the elevated levels of active form of β-catenin in endometriosis. The activity of GSK-3β in deactivation of β-catenin is negatively regulated via phosphorylation of GSK-3β in Ser9 [22]. Estrogens inhibit the activity of GSK-3β [36].

The expression of GSK-3β is not dependent on the phase of menstrual cycle [28], but there are controversies regarding the expression level of its inactive form. An Immunofluorescence study showed a lower expression of inactive GSK-3β [28], while the findings of another study, based on western blotting, indicated higher expression of it in secretory phase compared to proliferative phase [37]. Anyway, it is evident that estrogen treatment of endometrial cells increases the phosphorylation of GSK-3β at Ser^{9} while progesterone treatment inverts estrogen-induced phosphorylation of GSK-3β [38]. In the present study, the mRNA and total protein expression of GSK-3β were not different between healthy endometrium and both endometriotic lesions and eutopic endometrium of endometriosis patients, but the major part of GSK-3β in both endometriosis tissues remains phosphorylated. The expression of total and inactive GSK-3β in eutopic endometrium of endometriosis patients, using IHC, had been studied previously by Franco-Murillo et al. [28]. Our results confirmed their findings. We did not find any difference between endometriotic lesions and eutopic endometrium of endometriosis patients regarding the expression of inactive GSK-3β. To our knowledge, the present study is the first in the assessment of expression of GSK-3β in endometriotic lesions.

Consistent with the findings of Kao et al. [39], we demonstrated that the expression of DKK-1 was down-regulated in eutopic endometrium of endometriosis patients compared to the healthy endometrium. Previous studies demonstrated that the expression of DKK-1is changed in human endometrium during the menstrual cycle [32,40]. Its expression is up-regulated by progesterone in the secretory phase [41,42]. DKK-1 binds to the co-receptors of Wnt (LRP5 and LRP6) and represses the Wnt/β-catenin signaling [43]. Brueggmann et al. reported that ovarian endometriosis tissue expresses lower levels of DKK-1 than the eutopic endometrium of endometriosis patients [44]. Our results showed that, unlike endometrioma, there was no difference between peritoneal endometriotic lesions and eutopic endometrium of same patients regarding the expression of DKK-1.

The expression of WNT7a in normal endometrium does not alter during the menstrual cycle [32,45]. It means that its in-vivo expression is not under the estrogen and progesterone regulation. Using RT-PCR and Immunofluorescence methods, Gaetje et al. studied the expression of WNT7a in endometriotic lesions and eutopic endometrium of women with endometriosis compared to heterogeneous control subjects. They reported that the endometriotic lesions express higher levels of WNT7a than the endometrium of women with and without endometriosis [45]. By contrast, our results showed that there was no significant difference in expression of WNT7a between healthy endometrium and both endometriotic lesions and eutopic endometrium of endometriosis patients. The incompatible findings may be related to different patient groups analyzed as well as different technical methods used for detection of protein levels of WNT7a.

In this study, all of the endometriosis patients were stage III–IV and infertile, which may limit the generalizability of the findings to all endometriosis patients.

In summary, the results of this study indicated that the inactivation of a major part of GSK-3β along with a significant repression of DKK-1 expression led to over-activation of β-catenin in eutopic endometrium of endometriosis patients. Also, the peritoneal ectopic endometrium was completely similar to the eutopic endometrium of endometriosis patients in deactivation of GSK-3β, down-regulation of DKK-1 and over-activation of β-catenin. More investigations will reveal whether intervention in the expression of DKK-1 and phosphorylation of GSK-3β can help to modify the endometriosis-related properties of endometriotic cells such as proliferation and invasion.

Declaration of conflicting interests

Authors have no conflicts of interest to declaration.

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