Abnormal activation of the sonic hedgehog signaling pathway in endometriosis and its diagnostic potency

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Objective: To investigate the abnormal expression of sonic hedgehog (SHH) signaling molecules in 52 eutopic endometrial tissues and its diagnostic potency in endometriosis.
Design: Retrospective study.
Setting: University hospital.
Patient(s): Twenty-six women with histologically confirmed endometriosis and 26 women with histologically normal endometria who were undergoing curettage or hysterectomy were selected.
Intervention(s): None.
Main Outcome Measure(s): The mRNA and protein levels of molecules in the SHH signaling pathway.
Result(s): The levels of *SHH*, smoothened, GLI family zinc finger 3, and its downstream signaling transcription factor (*GLI1*) not only were upregulated in the eutopic endometrium of endometriosis compared with the control endometrium, but also independently predicted the onset and severity of the disease.
Conclusion(s): This study is the first to reveal differences in the activation of the SHH signaling pathway between women with and without endometriosis and suggests that the SHH signaling pathway has potential in the diagnosis of endometriosis. (Fertil Steril® 2018;110:128–36. ©2018 by American Society for Reproductive Medicine.)
This abstract is available in Spanish at the end of the article.

Key Words: endometriosis, sonic hedgehog, signaling pathway

Endometriosis is characterized by the presence of endometrium-like tissues outside of the uterus leading to pelvic pain, dysmenorrhea and infertility (1). Surgeries due to endometriosis constitute the second largest number of surgeries in premenopausal women. Over the past few decades, endometriosis has been actively and extensively investigated, yet the pathogenesis of endometriosis is largely elusive. Endometriosis was first described by Von Rokitansky in 1860 (2). Then, Sampson proposed the most prevalent theory, namely, retrograde menstruation in 1921 (3). Several other accepted theories included metaplasia from müllerian remnants and distant implantation of menstrual debris (4). At the same time, the eutopic endometria of endometriosis have been reported to facilitate a series of metabolic and molecular abnormalities that include an increase in proliferation and angiogenesis and a decrease in apoptosis, thereby allowing the local production of estrogens and generating progesterone resistance (5). These hypotheses have received widespread attention since they can explain some clinical phenomena of endometriosis; however, arguments against these

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hypotheses are abundant, especially for Sampson's retrograde menstruation theory, which has been highly questioned and challenged. Because endometrial fragment reflux into the peritoneal cavity occurs in 90% of women and because only 10% of women have endometriosis (6), other causative factors likely play roles in the development and progression of the disease.

Sonic hedgehog (*SHH*) is one of a group of mammalian hedgehog (*HH*) proteins (*SHH*, Indian hedgehog, Desert hedgehog) that share a common signaling pathway (7). Ligand binding to the *SHH* receptor, Patched1, activates the *SHH* pathway by relieving Patched1-dependent inhibition of Smoothened (*SMO*). Subsequently, suppressor of fused (*SUFU*) and the GLI family zinc fingers (*GLI2*, *GLI3*) transduce the activation signal into the nucleus to regulate the expression of target genes, such as *GLI1* and vascular endothelial growth factor. Aberrant activation of the SHH signaling pathway plays an oncogenic role in various types of gynecological cancers (8,9). SHH signaling is closely linked to oncogenesis through its involvement in enhancing cell proliferation, stem cell maintenance, and cell differentiation and promoting angiogenesis (10–12), highlighting the multiple pathogenic roles of SHH signaling in gynecological disease. For instance, one study demonstrated that down-regulated *KLF9* was positively associated with ectopic lesion establishment in a mouse model of endometriosis through activated Notch and HH signaling (13). Matsumoto et al. (14) confirmed that *HH* proteins were expressed in the mouse uterus and that recombinant *SHH* protein could promote the proliferation of mouse endometrial mesenchyme cells in vitro. However, to date, the precise relationship between endometriosis and the SHH pathway has not been fully established. Thus, we aimed to explore whether the abnormal activation of SHH signaling within the eutopic endometrium in endometriosis patients was relevant to the etiology of endometriosis.

In recent years, an elevated preoperative serum cancer antigen 125 (CA125) level has been used as a non-invasive marker for endometriosis. However, the specificity of CA125 level alone is limited (15). Laparoscopy remains the gold standard for diagnosing endometriosis. Since laparoscopy requires anesthesia and an operation theater, most patients decline this diagnostic protocol. Thus, coupled with a lack of specific diagnostic laboratory biomarkers for endometriosis, these factors lead to a mean latency of 8–11 years from the establishment of endometriosis to a clinical diagnosis (16). These diagnostic delays may have serious consequences in terms of disease progression and may have a profound economic impact (17). However, studies on the identification and functional characterization of SHH signaling in gynecological diseases, especially endometriosis, are limited. Thus, we determined whether four of the SHH pathway molecules could discriminate endometriosis and its associated clinical features. The endometriosis score was evaluated to grade endometriosis into different stages (I-IV) according to the revised American Fertility Society (AFS) criteria (18). Based on the inflammatory response in endometriosis, SHH signaling components were examined with the endometriosis score, mean platelet volume and peripheral marker neutrophil/lymphocyte ratio to determine the diagnostic potential of these components in endometriosis (19).

MATERIALS AND METHODS

Patient and Clinical Information

The samples and clinicopathologic data were collected between 2014 and 2017 from the Department of Gynecology at the First Affiliated Hospital of Harbin Medical University (Harbin, PR China). Institutional Review Board approval for this project was provided by the Ethics Committee of Harbin Medical University (2017117). All methods were performed in accordance with the approved guidelines and regulations. Written informed consent in the study was obtained from each subject. To compare the mRNA and protein expression levels, 26 women with pathological reports of confirmed endometriosis and 26 endometriosis-free patients were enrolled in this study. Each patient's preoperative complete blood count was recorded, and the endometriosis score was assessed (20). None of the patients had received any hormonal therapy within six months before surgery. Women suffering from cancer, a benign ovarian cyst other than an endometrioma, severe pelvic inflammation during surgery, or a known endometrial polyp were excluded from this study.

Twenty-six women (mean age 36 y; range 25–45 y) had histologically confirmed endometriosis. Twenty-six women (mean age 33 y; range 28–36 y) undergoing laparoscopy for idiopathic infertility served as controls in the First Affiliated Hospital of Harbin Medical University. All subjects in the control group were negative for endometriosis. Among all 52 patients, 28 were in the proliferation phase of the menstrual cycle (73%), and14 were in the secretory phase of the menstrual cycle (27%). Twenty-eight (54%) had infertility. In addition, 13 endometriosis patients (25%) presented with progressive dysmenorrhea. The detailed clinical characteristics of the cohort are summarized in Supplemental Figure 1.

Reverse Transcription and Quantitative Real-Time Polymerase Chain Reaction

Eutopic endometrial samples (26 endometriosis samples and 26 controls) were frozen in liquid nitrogen until total RNA extraction using the TRIzol reagent (Invitrogen, America). Due to the current lack of stable standard curves for SHH signaling components in tissues, we applied a double standard curve method. Standard samples were obtained using plasmid (*SHH*) and cDNA (*SMO*, *GLI1* and *GLI3*) templates, with GAPDH standard internal controls. The total RNA was used only if the A260/280 ratio of the absorbances ranged between 1.8 and 2.2 as determined by spectrophotometry. cDNA synthesis was performed in a 20 μl SYBR reaction system with the SYBR premix ExTaqTM II kit (TAKARA, Japan). The standard curves of these selected mRNAs had a good amplification efficiency, R2 and slope, indicating that the method was suitable for mRNA quantification. The mRNA expression levels in the tissues were quantified by establishing standard curves with a set of serially diluted