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Expression of tyrosine kinase receptor B in eutopic endometrium of women with adenomyosis

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Abstract
Objective Our study is to investigate whether tyrosine kinase receptor B (TrkB) is expressed in eutopic endometrium of women with adenomyosis and its association with clinical characteristics.
Methods We collected endometrial tissues from 31 women with adenomyosis and 30 adenomyosis-free women undergoing surgery for benign indications. TrkB expression was assessed by immunohistochemistry and reverse-transcription polymerase chain reaction.
Results Immunoreactive staining for TrkB was present as brown flocculent precipitate in the endometrial cells. The average level of TrkB protein (quantitation of immunostaining intensity) in secretory endometrial samples of women with adenomyosis was significantly higher than that in controls ($p < 0.01$). The average level of TrkB messenger RNA (mRNA) expression of women with adenomyosis was significantly higher than that of controls at secretory phase ($p < 0.01$). In addition, the immunostaining quantitation of TrkB protein was positively correlated with the serum CA125 ($r = 0.308$, $p = 0.016$) and dysmenorrhea ($r = 0.393$, $p = 0.002$).
Conclusions Our study revealed elevation of TrkB protein and mRNA expression in the secretory endometrium of women with adenomyosis. Moreover, TrkB protein expression in human endometrium was positively correlated with the serum CA125 and dysmenorrhea. TrkB might contribute to the pathogenesis and progression of adenomyosis.

Keywords Adenomyosis · Eutopic endometrium · Tyrosine kinase receptor B

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Introduction

Adenomyosis is characterized by the presence of ectopic endometrial glands and stroma in the myometrium. It can result in progressive dysmenorrhea and abnormal bleeding. Despite numerous studies on adenomyosis, its etiology, pathogenesis and the better conservative treatment are still not clear to date. Although the morphology of eutopic endometrium from women affected by ectopic foci is similar to that from normal women, its physiology and biochemistry are different, specifically in cell adhesion molecules [1], gene expression [2], invasion [3], proliferation and apoptosis [4].

Therefore, we hypothesized that an imbalance between proliferation and apoptosis signal in the endometrium might contribute to the pathogenesis and progression of the disease. Tyrosine kinase receptor B (TrkB), a neurotrophic receptor, possess extracellular glycosylated polypeptides as well as transmembrane and cytoplasmic tyrosine kinase domains. It contributes to tumor cells' resistance to apoptosis, and acquisition invasive and metastatic abilities [5]. Moreover, overexpression of TrkB has been found in several types of human malignancy, such as prostate

adenocarcinoma [6], multiple myeloma [7] and ovarian cancer [8], and higher level of TrkB expression is generally correlated with more aggressive tumor behavior [9].

Previous studies demonstrated that endometrial cells from women with endometriotic foci exhibit reduced apoptosis compared to healthy women [5, 10–12]. To the best of our knowledge, it is still unclear whether TrkB plays a role in the pathogenesis of adenomyosis. The purpose of our present study was (1) to examine if there was distinctive expression of TrkB in endometrium between women with and without adenomyosis, (2) to determine whether TrkB expression in human endometrium was associated with serum CA125 and visual analog scale (VAS) scores of dysmenorrhea.

Materials and methods

Patients and sample collection

A total of 61 women of reproductive age (36–52 years old) from inpatient Department of Gynecology volunteered for this study. Ethical approval was granted by Ethical Committee of the School of Medicine, Zhejiang University (Zhejiang, China) and written informed consent was obtained from each patient before tissue collection. All participants had normal menstrual cycles (28–32 days) and none had received hormonal treatment for at least 6 months. During the procedure of laparoscopy or laparotomy, pelvic organs were examined carefully excluding the presence of endometriosis in control group, and then endometrial tissues were obtained by endometrial curettage. Shortly after the collection, one part of endometrial tissues was frozen in liquid nitrogen and stored at −80°C for mRNA extraction, and the other part was fixed in 4% paraformaldehyde (for 24 h) for pathological examination and then paraffin embedded for immunohistochemical analysis. Endometrial samples were divided into four groups according to the Noyes pathological diagnosis [13] (hematoxylin–eosin staining): proliferative phase of adenomyosis (17 cases), secretory phase of adenomyosis (14 cases), proliferative phase of control (16 cases), secretory phase of control (14 cases).

Information about the VAS scores of dysmenorrhea and the concentration of serum CA125 on the surgery day was taken from the patients' clinical records and laboratory examination. The VAS was used in the dysmenorrhea assessment [14].

Immunohistochemistry

The endometrial samples were sectioned at 4-μm intervals, deparaffinized, dehydrated, and then immersed in a 10 mM citrate buffer(PH = 6.0), boiled in a pressure cooker and jet 30 s for antigen retrieval. After cooling to room temperature, endogenous peroxidase was quenched with 0.3% hydrogen peroxide(vol/vol) for 20 min. Non-specific binding was blocked by 10% (vol/vol) normal goat serum for 30 min at room temperature, and the slides were incubated overnight at 4°C with primary antibody at 1:140 dilution (sc-8316; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After three 5-min washes with PBS, the slides were incubated for 30 min with a secondary antibody (GK500705 EnVision™ Detection Kit; Peroxidase/DAB, Rabbit/Mouse, DakoCy-tomation, Denmark). After another washing and following reaction with diaminobenzidine (GK500705 EnVision™ Detection Kit; Peroxidase/DAB, Rabbit/Mouse, DakoCy-tomation, Denmark), the sections were counterstained with hematoxylin, dehydrated, and mounted in DPX. Slides incubated with PBS as the primary antibody were used as negative controls.

Sections were scanned under bright-field microscopy and images were photographed. Quantitation of the immunostaining intensity for TrkB protein was performed by image analysis using the Image-Pro Plus 5.0 (Media Cybernetics, Silver Spring, MD, USA). Upon calibration of the system, TrkB protein is expressed in pixel units of relative intensity, and the relative optical density of per unit area was determined using the Image-Pro Plus software (Media Cybernetics) [15]. Mean density = integrated optical density (IOD)/area, and its sum was regard as immunostaining quantitation for each sample.

RT-PCR analysis

Total RNA was extracted using Triozol reagent. Reverse transcription was carried out with 3 μg RNA using oligo$(dT)_{18}$ primers and M-MLV reverse transcriptase (#K1622 RevertAid™ First Strand cDNA Synthesis Kit; Fermentas Life Sciences, Fermentas UAB, Canada) according to manufacturer's protocols. The polymerase chain reaction (PCR) reaction was performed in a final volume of 25 μL by using 12.5 μL PCR Master Mix (2×) (#K0171 PCR Master Mix; Fermentas Life Sciences, Fermentas UAB), 2 μL of reverse transcription products, primers and nuclease-free water. The sequences of TrkB primers were as follows: sense, 5′-ATCTCCAACCTCAG ACCACCAC-3′ and anti-sense, 5′-TGTTTCTCATCCTT CCCATACTCAT-3′, amplifying a 240-bp product. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used here for endoreference control. The sequences of GAPDH primers were as follows: sense, 5′-ATCCCATCACCAT CTTCCAG-3′, and anti-sense, 5′-GAGTCCTTCCACG ATACCAA-3′, amplifying a 307-bp product. PCR was performed in a DNA thermal cycle, in which the following conditions were used: 1 cycle of 94°C for 3 min, then 33

cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and finally 1 cycle of 72°C for 10 min. 10 μL PCR products were separated directly electrophoretically on 1.5% agarose gel and were visualized under ultraviolet transillumination. The gels were photographed, and the images were scanned and analyzed with Image-Pro Plus 5.0 (Media Cybernetics). Normalized densities were determined with ratio of band density of TrkB to band density of GAPDH.

Statistical analysis

All data were in normal distribution and statistical analysis was performed by SPSS for Windows 16.0 (SPSS Inc., Chicago, IL, USA) and results were expressed as mean ± SD. Single comparisons were performed using Student's *t* test. Bivariate correlation and linear regression were used to analyze the correlation between TrkB protein expression and serum CA125 and VAS scores of dysmenorrhea. Probability values of <0.05 were regarded as significant.

Results

The mean ages of women with and without adenomyosis were 45.27 ± 4.71 and 43.50 ± 5.63 years, respectively, and no difference in age was observed between them ($p > 0.05$).

Immunohistochemistry

Tyrosine kinase receptor B immunoreactive staining was evident as brown flocculent precipitate in the cytoplasm and membrane of glandular epithelial and stromal cells in endometrium (Fig. 1). The immunostaining in the glandular epithelium was more noticeable than in stroma's.

By analyzing immunostaining quantitation for each section, we found that the TrkB protein in endometrial samples of women with adenomyosis was significantly higher than that in controls ($p < 0.05$). Furthermore, TrkB protein in secretory endometrial samples of women with adenomyosis (0.29 ± 0.060) was significantly higher than that in controls (0.22 ± 0.054; $p < 0.01$) (Fig. 2), but there

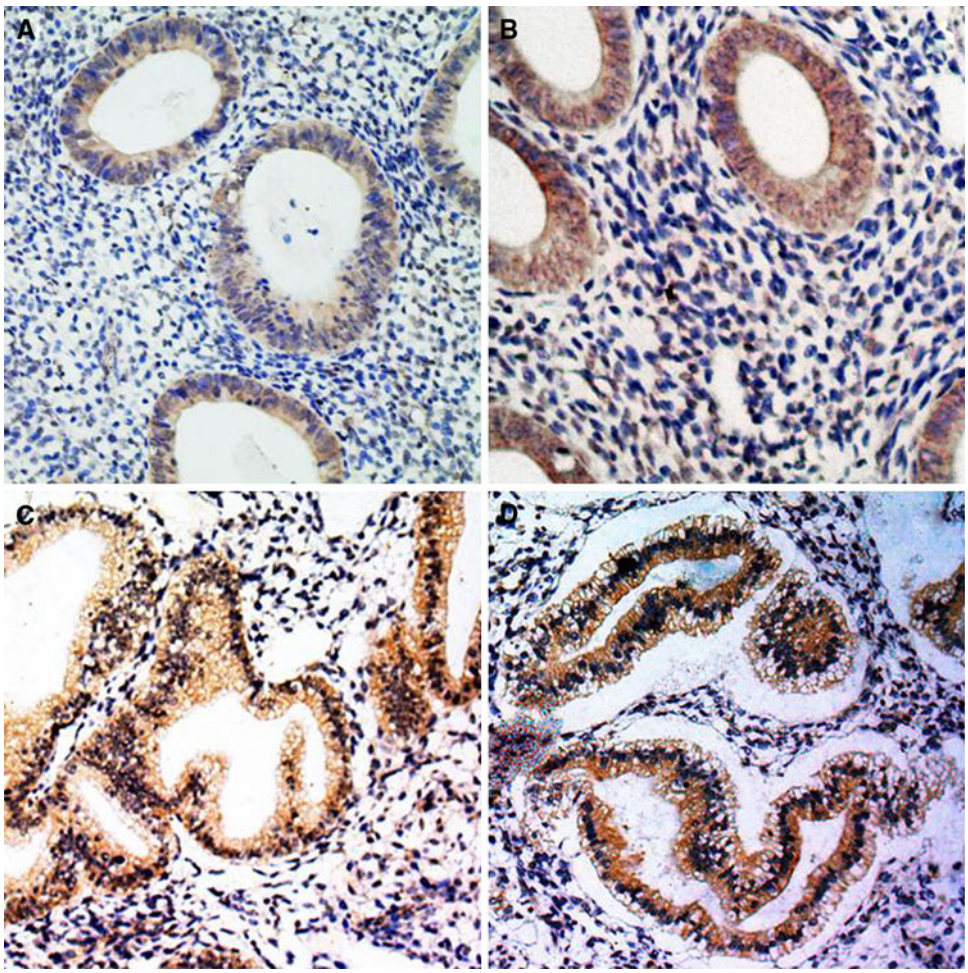

Fig. 1 Positive expression figures of immunohistochemical staining for TrkB in the endometrium of women with and without adenomyosis. **a**, **c** Tissue sections from women without adenomyosis at proliferative and secretory phases of the cycle, respectively. **b**, **d** Tissue sections from women with adenomyosis at proliferative and secretory phases, respectively. Original magnification ×200

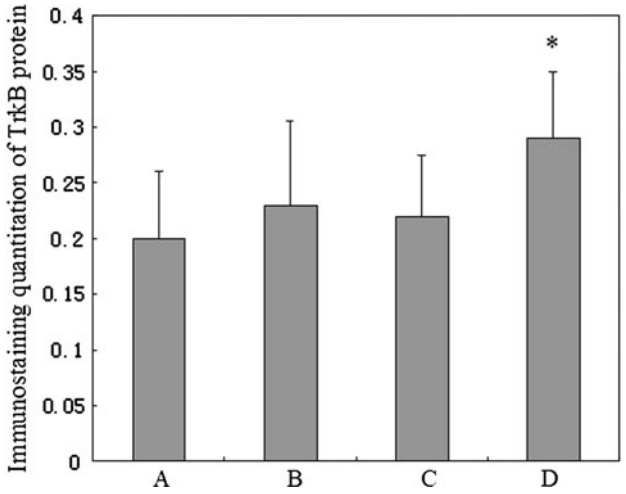

Fig. 2 Immunostaining quantitation of TrkB protein in the endometrium of women with and without adenomyosis. Immunostaining quantitation of TrkB protein was assessed in each section (mean ± SD). *A*, *C* Average level of TrkB immunostaining quantitation from women without adenomyosis at proliferative and secretory phases of the cycle, respectively. *B*, *D* Average level of TrkB immunostaining quantitation from women with adenomyosis at proliferative and secretory phases, respectively. $*p = 0.005$, compared with the secretory endometrial tissues of controls

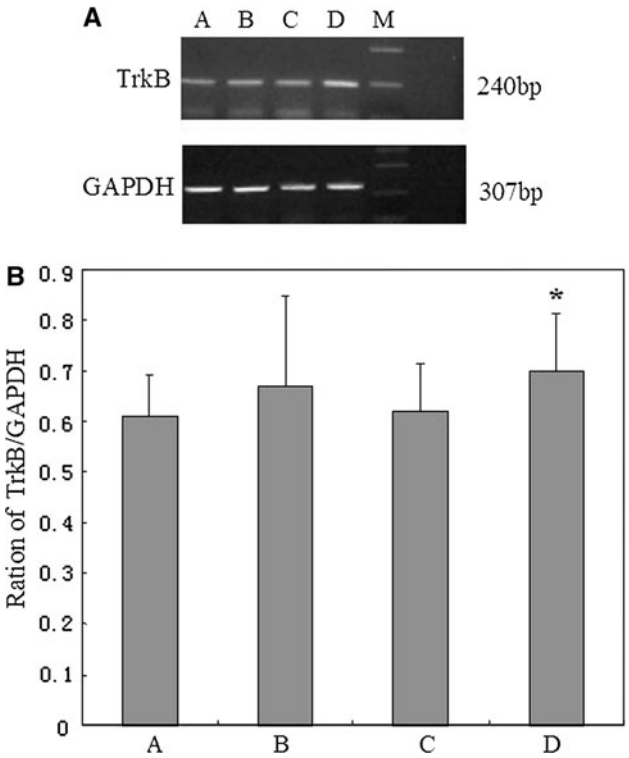

Fig. 3 Expression of TrkB mRNA was assessed by RT-PCR in endometrial samples. **a** A 240-bp product of TrkB mRNA was visualized with ethidium bromide after agarose gel electrophoresis. GAPDH (307 bp) was used as a endoreference to assess the amount of RNA in each sample. Molecular DNA markers (*lane M*), proliferative endometrium from control (*lane A*), proliferative endometrium from women with adenomyosis (*lane B*), secretory endometrium from control (*lane C*), and secretory endometrium from women with adenomyosis (*lane D*). **b** Semi-quantification of TrkB mRNA level was analyzed by using the GAPDH as an endoreference (mean ± SD). $*p = 0.002$, compared with the secretory endometrial tissues of controls

was no significant difference between two groups at proliferative phase ($p > 0.05$) (Fig. 2).

Reverse-transcription PCR analysis

A 240-bp product of TrkB mRNA by reverse-transcription PCR (RT-PCR) was visualized with ethidium bromide after electrophoresis on agarose gel, and GAPDH was used as an endoreference to assess the volume of mRNA in each sample (Fig. 3a). Semi-quantitative analysis showed that the average level of TrkB mRNA expression in endometrial samples of women with adenomyosis was significantly higher than that in controls ($p < 0.05$). Furthermore, TrkB mRNA expression in secretory endometrial samples of women with adenomyosis (0.70 ± 0.112) was significantly higher than that in controls (0.62 ± 0.093; $p < 0.01$) (Fig. 3b), but there was no significant difference between two groups at proliferative phase ($p > 0.05$) (Fig. 3b).

Correlation between TrkB protein expression in human endometrium and the serum levels of CA125

Serum CA125 concentrations ranged from 5.0 to 210.0 U/ml, and it were remarkedly elevated in patients with adenomyosis (53.97 ± 44.49) comparing to the controls (18.27 ± 11.77) ($p < 0.01$). There was a positive correlation between TrkB protein expression in human endometrium and serum CA125 ($p < 0.05$) (Fig. 4). The Pearson's correlation coefficient (r) was 0.308. The linear equation was y (immunostaining quantitation of TrkB protein) = 0.216 + 0.001x (serum CA125).

Correlation between TrkB protein expression in human endometrium and dysmenorrhea

The VAS scores of dysmenorrhea ranged from 0 to 10, and it were significantly increased in adenomyosis patients (8.27 ± 1.26) comparison to the control group (1.07 ± 1.14) ($p < 0.01$). There was a positive correlation between TrkB protein expression in human endometrium and the VAS scores of dysmenorrhea ($p < 0.01$) (Fig. 5). The Pearson's correlation coefficient (r) was 0.393. The linear equation was y (immunostaining quantitation of TrkB protein) = 0.203 + 0.007x (VAS scores).

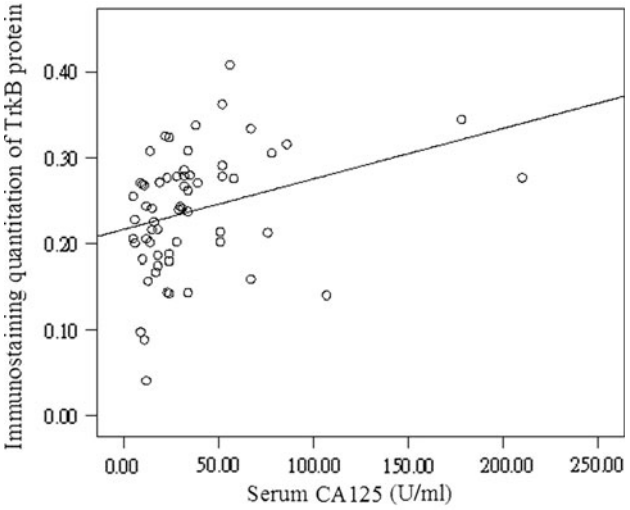

Fig. 4 There was a positive correlation between TrkB protein expression and serum CA125 ($n = 61$, $r^2 = 0.095$, $p = 0.016$)

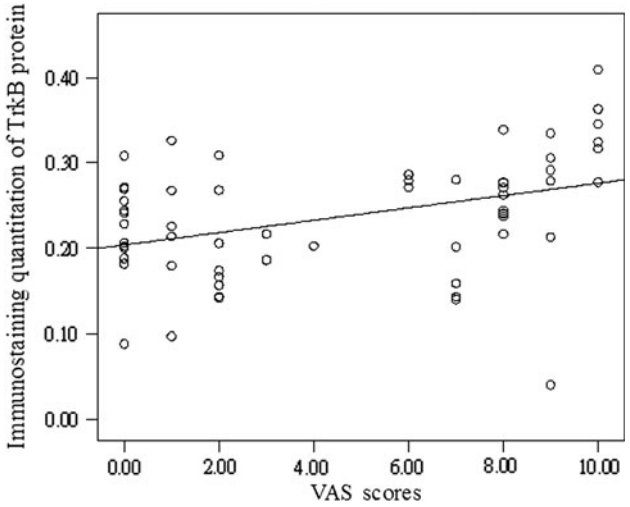

Fig. 5 There was a positive correlation between TrkB protein expression and dysmenorrhea ($n = 61$, $r^2 = 0.154$, $p = 0.002$)

Discussion

In the present study, we revealed elevation of TrkB protein and mRNA expression in the secretory endometrium of women with adenomyosis. This result provides us a clue that the endometrium at secretory phase is more critical to the pathogenesis of adenomyosis. Although the pathology of adenomyosis is benign, malignant biologic activation of endometriotic cell is often seen according to the clinical symptoms. Previous research showed that endometriotic cells have invasive potentiality in invasion assays in vitro [16], the capability of endometrial cells for intramyometrial implantation and intramyometrial spread appears to be a pathogenic mechanism for adenomyosis. Increasing researchers demonstrate that endometrial cells from women with endometriotic lesion exhibit reduced apoptosis when compared with healthy women [5, 10–12]. Accumulated evidence suggests that apoptosis helps to maintain cellular homeostasis during the menstrual cycle by eliminating senescent cells from the functional layer of the uterine endometrium during the late secretory and menstrual phase of the cycle [17]. By binding with its ligand brain-derived neurotrophic factor (BDNF) and/or overexpression inducing autophosphorylation, TrkB triggered the activation of PI3K/AKT pathway, and play a role of resistance to apoptosis, acquisition invasive and metastatic abilities [6]. Our results suggest that TrkB may block endometrial stroma and glands from apoptosis, facilitate their invasion into the myometrium and induce the process of adenomyosis in the myometrium, and then may have an effect on the development of adenomyosis.

Furthermore, our study showed that the serum CA125 concentrations were remarkedly elevated in patients with adenomyosis, but not in the controls. CA125, a high molecular weight membrane glycoprotein, is expressed in all tissues derived from embryonic coelomic epithelium. It is the most widely used serum marker for diagnosis of endometriosis and adenomyosis, and it is correlated with disease stage [18]. It is clinically used not only for diagnosing disease but also for monitoring disease progression and responding to medical or surgical treatment. We also found that there was a positive correlation between endometrial TrkB protein expression and the serum CA125. Therefore, TrkB may be associated with the progression of adenomyosis.

In addition, the VAS scores of dysmenorrhea were significantly increased in adenomyosis patients comparison to the control group. We also found there was a positive correlation between endometrial TrkB protein expression and the VAS scores. Researchers identified TrkB as a potential candidate gene that might be involved in endometriosis-related pain [19, 20]. Gonadotropin-releasing hormone (GnRH) agonists or continuous progestins [21] can alleviate pain associated with deep infiltrating endometriosis (DIE).The expression levels of TrkB mRNA in epithelial cells from DIE were significantly decreased in patients with pre-operative GnRH agonist or progestin [22]. The theory named “tissue injury and repair” involving local estrogen production explained the physiological mechanism of endometriosis and adenomyosis [23] from a novel aspect. Researchers observed that blocking TrkB signaling in the $TrkB^{F616A}$ knock in mice significantly prevented the development of tissue and nerve injury-induced persistent pain [24]. Taken together, these evidence provide us that the increased expression of TrkB may maintain the sensitivity of injury-induced persistent pain, thus due to the development and progress of dysmenorrhea. It is interesting to investigate TrkB-associated

dysmenorrhea in adenomyosis besides uterine prostaglandins and the relationship between them in our future research.

In conclusion, our study demonstrated for the first time a significant increase of TrkB expression in the secretory endometrium of women with adenomyosis as well as a relationship between TrkB expression and serum CA125 and VAS scores of dysmenorrhea. TrkB therefore may play a role in the pathogenesis and progression of adenomyosis. Further investigation of TrkB as a clinical marker and as a therapeutic target appears to be warranted.

Conflict of interest The authors have no conflicts of interest to declare in relation to this article.

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