Analysis of the oncogene *BRAF* mutation and the correlation of the expression of wild-type *BRAF* and *CREB1* in endometriosis

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Abstract. B-Raf proto-oncogene, serine/threonine kinase (*BRAF*) has previously been identified as a candidate target gene in endometriosis. Wild-type and mutated *BRAF* serve important roles in different diseases. The aim of the present study was to explore *BRAF* mutation, the mRNA and protein expression of wild-type *BRAF* (^{wt}*BRAF*) in endometriosis, and the association between the expression levels of ^{wt}*BRAF* and the predicted transcription factor cAMP responsive element binding protein 1 (CREB1). In the present study, *BRAF* mutation was detected using Sanger sequencing among 30 ectopic and matched eutopic endometrium samples of patients with endometriosis as well as 25 normal endometrium samples, and no *BRAF* mutation was detected in exons 11 or 15. A region of ~2,000 bp upstream of the *BRAF* gene was then screened using NCBI and UCSC databases, and *CREB1* was identified as a potential transcription factor of *BRAF* by analysis with the JASPAR and the TRANSFAC databases. Quantitative polymerase chain reaction was used to analysis the mRNA expression levels of ^{wt}*BRAF* and *CREB1*, and the corresponding protein expression levels were evaluated using immunohistochemistry and western blot analysis. The results revealed that the mRNA and protein expression levels of ^{wt}*BRAF* and *CREB1* were significantly upregulated in the eutopic endometrial tissues of patients with endometriosis compared with normal endometrial tissues ($P<0.05$) and no significant difference in ^{wt}*BRAF* and *CREB1* levels was detected between the ectopic and eutopic endometrium ($P>0.05$). In addition, correlation analysis revealed that the protein expression of CREB1 was positively correlated with the transcript level and protein expression of ^{wt}*BRAF*. It is reasonable to speculate that CREB1 may activate the transcription of ^{wt}BRAF through directly binding to its promoter, increasing BRAF expression and regulating the cell proliferation, migration and invasion of endometriosis.

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Introduction

Endometriosis is an estrogen-dependent chronic gynecological disease that is difficult to cure. The main clinical characteristics include pelvic masses, chronic pelvic pain and infertility. Although it is a benign disorder, endometriosis exhibits invasive growth potential, which is similar to that of malignant tumors (1). The incidence of endometriosis is increasing year by year, but its etiology and pathogenesis remain unclear (2). Although the classical theory of endometriosis is Sampson's 'retrograde menstruation theory', which suggests that endometrial fragments undergo retrograde menstruation through the fallopian tubes and implant in the peritoneal cavity, this does not explain why the prevalence of endometriosis is only ~10% in fertile women, the majority of whom experience retrograde menstruation (3). Investigations have shown that cell adhesion, invasion, angiogenesis (4) and apoptosis (5) in the eutopic endometrium in endometriosis are different from that of the normal endometrium, particularly for the secretory endometrium. There is also evidence to suggest that abnormal molecular aberrations in the eutopic endometrium promote the development of endometriosis (6). Thus, an evaluation of specific genes and their associated molecular mechanisms in the eutopic endometrium may provide a new theoretical basis for the pathogenesis of endometriosis. The present study team previously identified 10 upregulated genes in the eutopic endometrium using cDNA representational difference analysis (7). Among them, the abnormal expression of the cofilin 1, methionine adenosyltransferase 2A and LIM domain kinase 1 genes in the eutopic endometrium has been reported (8). Additionally, the present study team also detected that B-Raf proto-oncogene, serine/threonine kinase (*BRAF*) was overexpressed in the eutopic endometrium of endometriosis (7).

BRAF is a component of the RAS-rapidly accelerated fibrosarcoma (RAF)-mitogen-activated protein kinase kinase (MEK)-extracellular signal-regulated kinase (ERK) signaling pathway. Mutations in *BRAF* are associated with the development of malignant tumors (9). *BRAF* mutations have been

detected in exons 11 and 15 in numerous tumors, and the most common mutation is the ^{V600E}*BRAF* mutation (10). *BRAF* mutations may activate the mitogen-activated protein kinase (MAPK) signal pathway constitutively, leading to abnormal cell proliferation, differentiation and tumorigenicity (11). Other studies have shown that the overexpression of wild-type *BRAF* (^{wt}*BRAF*) promotes the activation of the RAS-BRAF-MAPK signaling pathway (12,26). Notably, *BRAF* has been reported to promote cell proliferation through regulation of the Ras/Raf/MAPK signaling pathway in the eutopic endometrial stromal cells of patients with endometriosis (13). Previous studies by the present research team have demonstrated that the mRNA and protein levels of BRAF are markedly overexpressed in the eutopic endometrium tissues of endometriosis compared with normal endometrial tissues (7,14). This suggests that BRAF may regulate the occurrence and development of endometriosis. However, *BRAF* mutations and the mechanism associated with the upregulation of ^{wt}*BRAF* expression in endometriosis remain unclear.

In the present study, *BRAF* mutations were detected and the potential transcription factors binding to the region upstream of the ^{wt}*BRAF* transcription start site (TSS) were predicted. The correlation of mRNA and protein expression between ^{wt}*BRAF* and the predicted transcription factor was then analyzed. The results may provide a novel insight into the molecular mechanisms of *BRAF* in the regulation of the occurrence and development of endometriosis.

Materials and methods

Tissue collection. Ectopic endometrium and paired eutopic endometrium samples were collected from 30 patients (37.30±6.83 years old) with endometriosis who were undergoing total hysterectomy at the Department of Gynecology, Cancer Hospital of China Medical University (Shenyang, China) from January 2015 to June 2016 as the experimental group. Normal endometrium samples from 25 patients (40.46±5.26 years old) without estrogen-dependent disease were also collected as the control group. All patients had regular menstrual cycles and none had malignant diseases, autoimmune disease, surgical diseases or inflammatory diseases. They also did not receive gonadotrophin-releasing hormone analogs, other hormonal medications or antibiotic therapy in the 6 months prior to the surgery. The tissue samples were all collected in the secretory phase of the menstrual cycle, which was confirmed by pathology. The present study was approved by the China Medical University Research Ethics Committee in accord with the Declaration of Helsinki. Written informed consent was obtained from each patient prior to the surgical procedures. All samples were divided into two groups: One was immersed in 10% formalin solution for immunohistochemistry (IHC), and another was frozen in liquid nitrogen for polymerase chain reaction (PCR) and direct sequencing, quantitative PCR (qPCR) or western blot analysis.

Genomic DNA isolation, PCR and direct sequencing. Genomic DNA was extracted from freshly frozen tissue (100 mg) using the TIANamp Genomic DNA kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocol. Approximately 200 ng genomic DNA was used for PCR in a 20-μl reaction system. The DNA polymerase used for PCR is TaKaRa Ex Taq® (Cat. no. R001A) purchased from Takara Bio, Inc., Otsu, Japan. The primer sequences are shown in Table I. The reaction conditions were as follows: 94˚C for 5 min, 1 cycle; 94˚C for 30 sec, 56˚C (for exon 11)/59˚C (for exon 15) for 30 sec, 72˚C for 30 sec, 35 cycles, and a final extension at 72˚C for 10 min. The integrity of all PCR products was observed by 2% agarose gel electrophoresis. PCR products were analyzed using an ABI 3730xl DNA sequencer following purification (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The positive control, ^{V600E}*BRAF* mutation, was detected using BCPAP thyroid carcinoma cells (provided by the Central Laboratory of Shengjing Hospital of China Medical University).

Prediction of transcription factor binding sites upstream of the ^{wt}BRAF TSS. A region of ~2,000 bp located upstream of the ^{wt}*BRAF* TSS was screened using National Center for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov/) and University of California, Santa Cruz (http://www.genome.ucsc.edu/) databases. Transcription factors that may be able to bind to the ~2,000-bp region upstream of the ^{wt}*BRAF* TSS were then predicted using JASPAR (http://jaspar.genereg.net/) and Transcription Factor (TRANSFAC: http://www.gene-regulation.com/pub/databases.html) datasets.

RNA extraction, reverse transcription and qPCR. Total RNA was extracted from freshly frozen tissues (100 mg) using RNAiso Plus (Takara Bio, Inc.) according to the manufacturer's protocol. RNA purity and concentration were detected by spectrophotometry, and RNA integrity was observed by 1% agarose gel electrophoresis. Total RNA was synthesized into cDNA using the PrimeScript RT Reagent kit (Takara Bio, Inc.) according to the manufacturer's protocol. Primers were designed using PrimerPremier 5.0 (Premier Biosoft International, Palo Alto, CA, Canada), and the sequences used in this study are shown in Table II. qPCR analysis was performed using SYBR Premix Ex Taq (Takara Bio, Inc.) according to the manufacturer's protocol using a LightCycler 480 detection system (Roche Diagnostics International AG, Rotkreuz, Switzerland). The conditions were as follows: One cycle at 95˚C for 5 min, followed by 45 cycles at 95˚C for 10 sec and 60˚C for 30 sec. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as an internal control. All experiments were performed in triplicate. The relative expression of cAMP responsive element binding protein 1 (*CREB1*) and *BRAF* was calculated using the $2^{-\Delta\Delta Cq}$ method (15).

IHC staining. The IHC SP kit (ZSGB-BIO, Beijing, China) was used to detect the protein expression levels of CREB1 and ^{wt}BRAF. Paraffin-embedded specimens were cut into 4-μm sections. The sections were dewaxed with xylene and dehydrated with graded alcohol. Following washing with phosphate-buffered saline (PBS), the sections were incubated in 3% H_2O_2 for 15 min at room temperature followed by microwave antigen retrieval (oven fire to 100%, for 7 min). The sections were blocked with serum (contained in The IHC SP kit) for 30 min at 37˚C and incubated with mouse anti-human BRAF monoclonal antibody (cat. no. sc-5284; 1:50; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and rabbit anti-human CREB1 polyclonal antibody (cat. no. 12208-

Table I. Primer sequences of exons used in the present study.

Exon	Primer sequences (5'→3')	Product length (bp)
Exon 11	F: ATAAGGTAATGTACTTAGGGT GAAACATAA	356
	R: TTTTGTTAGAAACTTTTGGAG GAGTC	
Exon 15	F: GCTTGCTCTGATAGGAAAATG AGA	249
	R: AATGACTTTCTAGTAACTCAG CAGCA	

F, forward; R, reverse.

Table II. Primer sequences used for quantitative polymerase chain reaction in the present study.

Gene	Primer sequences (5'→3')	Product length (bp)
CREB1	F: GGAGTGCCAAGGATTGAAGAAGA	333
	R: TGCTGTGCGAATCTGGTATGTT	
^{wt}*BRAF*	F: GGCAGAGTGCCTCAAAAAGAA	134
	R: AACCAGCCCGATTCAAGGA	
GAPDH	F: GCACCGTCAAGGCTGAGAAC	138
	R: TGGTGAAGACGCCAGTGGA	

CREB1, cAMP responsive element binding protein 1; ^{wt}BRAF, wild-type B-Raf proto-oncogene, serine/threonine kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; F, forward; R, reverse.

1-AP; 1:200; ProteinTech Group, Inc., Chicago, IL, USA) overnight at 4˚C. The sections were washed with PBS and then incubated with secondary antibody (contained in the IHC SP kit) for 30 min at 37˚C. Staining was performed using a diaminobenzidine kit (Beyotime Institute of Biotechnology, Nanjing, China) according to the manufacturer's protocol. Immunostaining results were scored using a light microscope (E100; Nikon, Tokyo, Japan) according to the positive cell percentage and positive staining intensity. First, the extent of the staining was scored according to the positive cell percentage: <5%, 0 points; 5-10%, 1 point; 10-50%, 2 points; >50%, 3 points. Additionally, the intensity of staining was scored as follows: no staining, 0 points; light yellow, 1 point, moderate yellow, 2 points; strong yellow, 3 points. The extent of the staining multiplied by the intensity was the final score; scores of 0-3 and 4-9 were considered negative and positive expression, respectively.

Western blot detection of protein expression levels. To 100 mg frozen endometrial tissue was added lysis buffer (quantity to volume ratio in mg/μl; 1:5) with 1% protease inhibitor, lysed in ice and centrifuged at 12,000 x g and 4˚C for 15 min. A bicinchoninic acid reagent kit (Beyotime Institute of Biotechnology) was used to detect the protein concentration of the supernatant. The protein sample (80 μg) was separated by 8% SDS-PAGE, followed by transfer to a polyvinylidene fluoride (EMD Millipore, Billerica, MA, USA) membrane. After blocking with 5% non-fat milk for 2 h at room temperature, the membranes were incubated with mouse anti-human BRAF monoclonal antibody (cat. no. sc-5284; 1:100; Santa Cruz Biotechnology, Inc.), rabbit anti-human CREB1 polyclonal antibody (cat. no. 12208-1-AP; 1:500) and mouse anti-human GAPDH monoclonal antibody (cat. no. 0004-1-Ig; 1:10,000) (both from ProteinTech Group, Inc.) overnight at 4˚C. GAPDH was used as a loading control. The membrane was washed with TBS with 0.1% Tween-20 buffer three times, and then the matched secondary antibodies (goat anti-mouse; cat. no. A00001-1; 1:2,000; goat anti-rabbit; cat. no. A00001-2; both ProteinTech Group, Inc.) were added for 2 h at room temperature. The binding was detected using a BeyoECL Plus kit (Beyotime Institute of Biotechnology), and the integrated density was analyzed using ImageJ 1.48v software (National Institutes of Health, Bethesda, MD, USA). The ratio of the integrated density between the target band and GAPDH was considered the relative expression level of each protein.

Statistical analysis. All data are presented as mean ± standard deviation. ANOVA and Chi-squared test were used to analyze differences in the mRNA and protein expression of *BRAF* and *CREB1* among eutopic and ectopic endometrium tissues from patients with endometriosis and normal endometrium. Pearson's coefficient correlation was applied to analyze the correlation between the expression of CREB1 protein and the mRNA and protein expression of ^{wt}*BRAF*. Data analysis was performed with the statistical software SPSS 20.0 (IBM Corp., Armonk, NY, USA), and $P<0.05$ was considered to indicate a statistically significant result.

Results

BRAF mutation at exons 11 and 15. All DNA specimens were amplified using specific primers and detected using gel electrophoresis. The band size of exon 11 was 356 bp and that of exon 15 was 249 bp (Fig. 1A). No *BRAF* mutations were detected among the 30 cases of ectopic and matched eutopic endometrium samples from patients with endometriosis and the 25 cases of normal endometrium (Fig. 1B), compared with the positive control in which the *BRAF* mutation (T1799A) was detected at exon 15 in the BCPAP papillary thyroid cancer cell line (Fig. 1C).

Prediction of the transcription factor binding sites of the BRAF TSS. A region of ~2,000 bp upstream of the *BRAF* TSS was screened, and transcription factors binding to the ~2,000-bp region were predicted using the JASPAR core transcription factor database. In total, 323 transcription factors (profile score threshold 80%) were identified. Among them, 5 were filtered (relative score >1.000; Table III), including *CREB1* (NCBI Gene ID 1385), Spi-B transcription factor (*SPIB*; NCBI Gene ID 6689), nuclear factor of activated T-cells 2 (*NFATC2*; NCBI Gene ID 4773), zinc finger protein 354C (*ZNF354C*;

Table III. Transcription factor binding sites predicted by the JASPAR database.

Model ID	Model name	Score	Relative score	Start	End	Strand	Predicted site sequence
MA0018.2	CREB1	11.569	1.0000161	1735	1742	-1	TGACGTCA
MA0018.2	CREB1	11.569	1.0000161	1735	1742	1	TGACGTCA
MA0081.1	SPIB	10.470	1.0000147	1599	1605	1	AGAGGAA
MA0152.1	NFATC2	11.360	1.0000115	938	944	1	TTTTCCA
MA0152.1	NFATC2	11.360	1.0000115	1243	1249	-1	TTTTCCA
MA0130.1	ZNF354C	8.916	1.0000095	823	828	1	ATCCAC
MA0442.1	SOX10	8.910	1.0000092	252	257	-1	CTTTGT
MA0442.1	SOX10	8.910	1.0000092	1098	1103	-1	CTTTGT

CREB1, cAMP responsive element binding protein 1; SPIB, Spi-B transcription factor; NFATC2, nuclear factor of activated T-cells 2; SOX10, SRY-box 10.

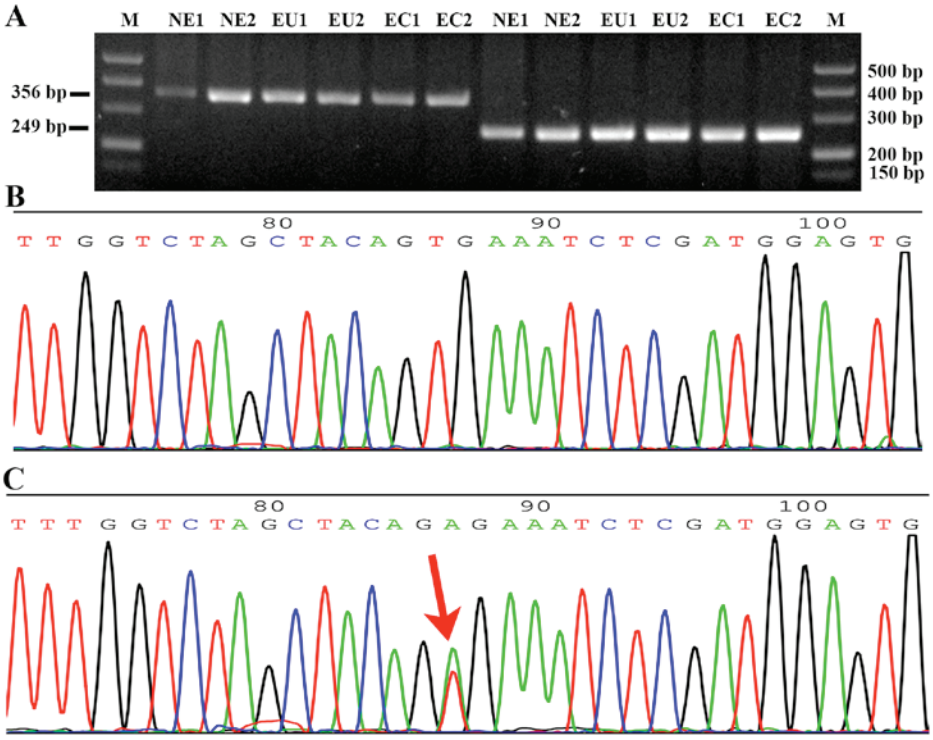

Figure 1. Detection of *BRAF* gene mutation at exons 11 and 15. (A) Gel electrophoresis of the PCR products of exon 11 and 15 of the *BRAF* gene. The PCR product size of exon 11 is 356 bp, and that of exon 15 is 249 bp. Part of a sequence chromatogram from the Sanger sequencing of BRAF from (B) a patient and (C) the positive control. The red arrow indicates a *BRAF* mutation (T1799A) of exon 15 in the BCPAP papillary thyroid cancer cell line. BRAF, B-Raf proto-oncogene, serine/threonine kinase; PCR, polymerase chain reaction; M, DNA marker; NE, normal endometrium; EU, eutopic endometrium of endometriosis; EC, ectopic endometrium of endometriosis.

NCBI Gene ID 30832) and SRY-box 10 (*SOX10*; NCBI Gene ID 6663). Combined analysis with the JASPAR and TRANSFAC databases predicted a cAMP-responsive element (CRE) binding site (TGACGTCA) at -266 to -259 bp upstream of the ^{wt}*BRAF* TSS (Fig. 2). Additionally, in a previous study, it was detected that *CREB1* mRNA expression was significantly higher in the eutopic endometrium of patients with endometriosis compared with that in normal endometrium (16). These findings suggest that *CREB1* may activate *BRAF* gene transcription by directly binding to the CRE sequence of the *BRAF* promoter region.

mRNA expression of ^{wt}BRAF and CREB1 in endometrial tissues. qPCR was used to evaluate the mRNA expression levels of ^{wt}*BRAF* and *CREB1* in the ectopic and eutopic endometrium of endometriosis patients. The mRNA and protein levels are shown in (Fig. 3). In the eutopic endometrial tissues, the relative mRNA expression levels of ^{wt}*BRAF* and *CREB1* were significantly higher than those in the normal endometrial tissues ($P<0.001$). However, no significant difference in ^{wt}*BRAF* and *CREB1* mRNAs was detected between the ectopic and eutopic endometrium ($P=0.989$ and $P=0.548$, respectively; Fig. 3A).

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==========================================================================================
seq( 1680.. 1739)     ccctccccggacgacggcgcgaaggcgcgcctcagattacgtcacgcaccggggtgacgt
Segments:
2.3.1.0     1679 1689  ====YY1===
2.3.1.0     1703 1712                 ====Sp1===
9.9.51      1715 1724                      ====ATF===
1.1.1.6     1716 1725                       ==CRE-BP1=
1.1.1.1     1732 1741                                  ===c-Jun
1.1.1.6     1732 1741                                  ==CRE-BP
1.1.2.0     1732 1741                                  ====CREB
2.3.3.0     1733 1742                                   =CPE_bi
==========================================================================================
seq( 1740.. 1799)     cacgaccgtgacacgcgggtgacgccgttgccgcggcgacttctcgtcgtctccgccccc
Segments:
1.1.1.1     1732 1741  ==
1.1.1.6     1732 1741  1=
1.1.2.0     1732 1741  ==
2.3.3.0     1733 1742  nd=
2.3.1.0     1790 1804                                        =======Sp1
2.3.1.0     1796 1805                                            ====
==========================================================================================
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Figure 2. The CRE sequence site predicted by the TRANSFAC database. The arrow represents the CRE binding site (TGACGTCA) from -266 to -259 bp upstream of the wild-type B-Raf proto-oncogene, serine/threonine kinase transcription start site. CRE, cAMP-responsive element.

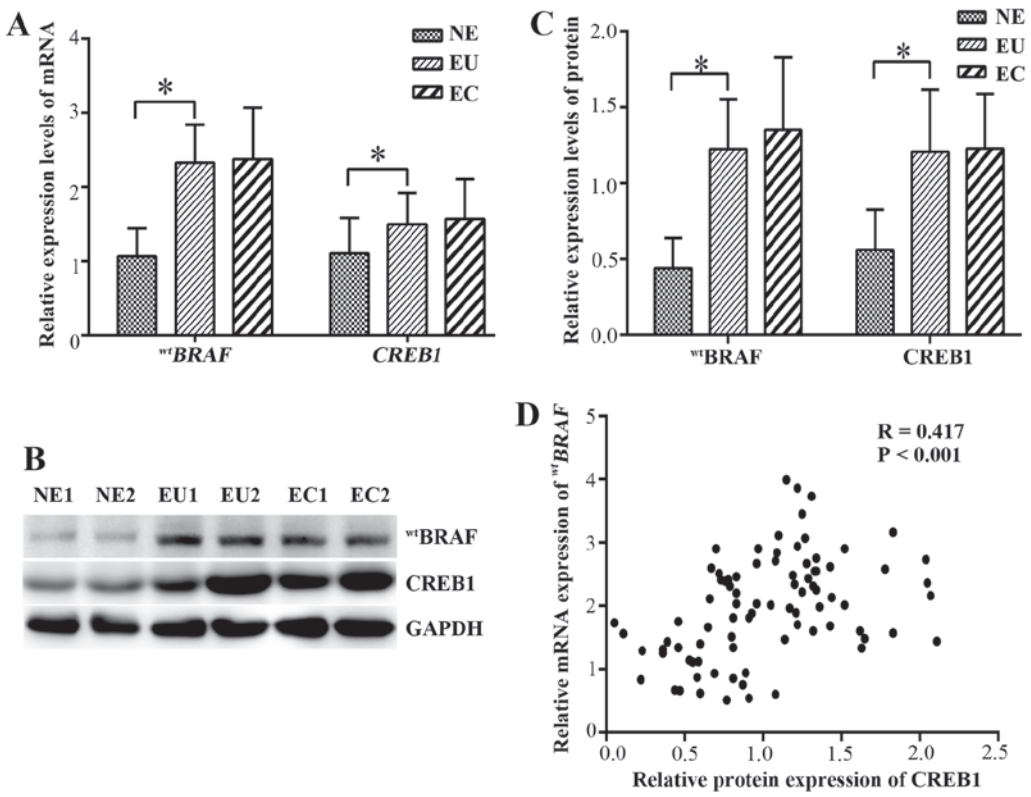

Figure 3. Expression of the mRNA and protein of ^{wt}BRAF and CREB1. (A) Relative mRNA expression levels of ^{wt}*BRAF* and *CREB1*. (B) Western blotting of ^{wt}BRAF and CREB1 proteins. GAPDH was used as a control. (C) Relative protein expression levels of ^{wt}BRAF and CREB1 in different endometrial tissues. (D) Correlation analysis between CREB1 protein and ^{wt}*BRAF* transcript levels in all tissues. *P<0.001 as indicated. ^{wt}BRAF, wild-type B-Raf proto-oncogene, serine/threonine kinase; CREB1, cAMP responsive element binding protein 1; NE, normal endometrium; EU, eutopic endometrium of endometriosis; EC, ectopic endometrium of endometriosis.

Protein expression of ^{wt}BRAF and CREB1 in endometrial tissues by IHC. The location and expression of ^{wt}BRAF and CREB1 in the ectopic and eutopic endometrial tissues of patients with endometriosis and the normal endometrial tissues of the control group were detected using IHC (Fig. 4). The results revealed that the ^{wt}BRAF protein was located in the cytoplasm of the epithelial and stromal cells, while CREB1 was located in the nuclei of these cells. In the normal, eutopic and ectopic

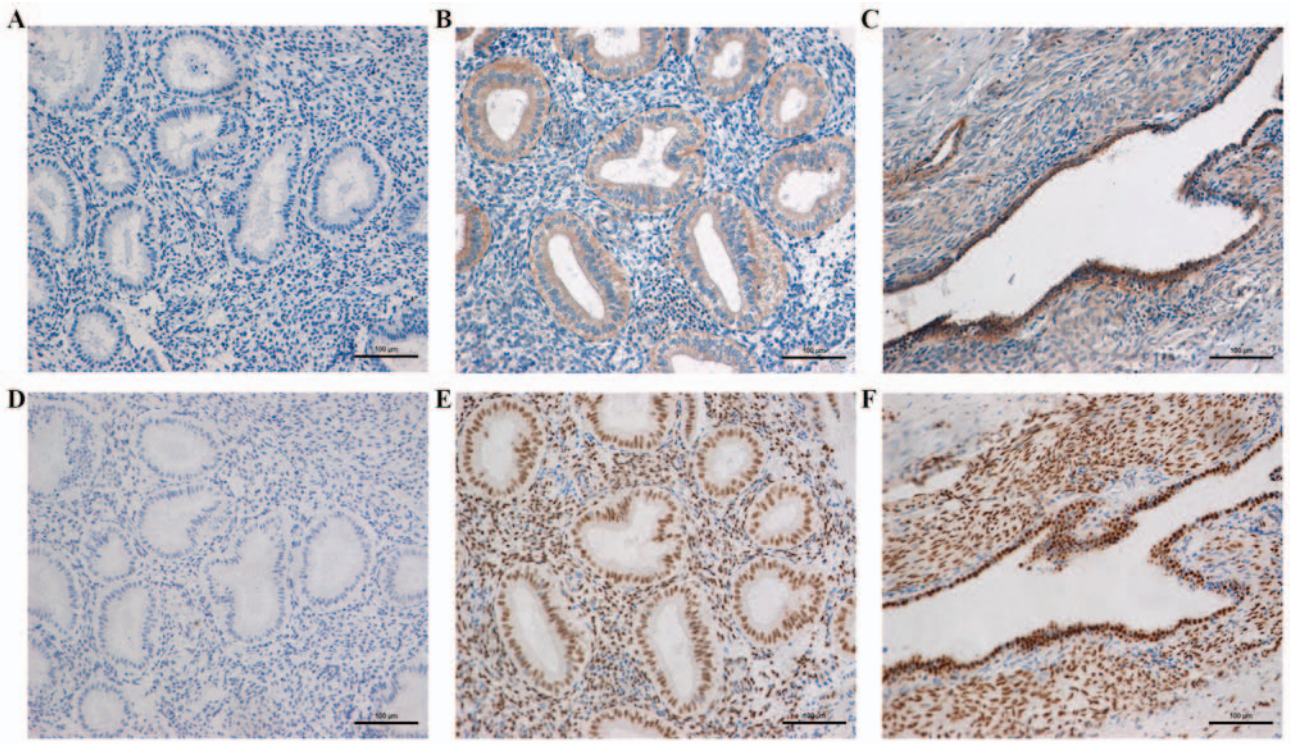

Figure 4. Immunostaining of ^{wt}BRAF and CREB1 in different endometrial tissues. Representative images of (A-C) ^{wt}BRAF and (D-F) CREB1 staining. (A and D) Normal endometrium from the control group, (B and E) eutopic endometrium from patients with endometriosis and (C and F) ectopic endometrium from patients with endometriosis (magnification, x200). ^{wt}BRAF, wild-type B-Raf proto-oncogene, serine/threonine kinase; CREB1, cAMP responsive element binding protein 1.

Table IV. Protein expression of ^{wt}BRAF and CREB1 in different endometrial tissues determined by immunohistochemistry.

	Tissue samples, n (%)			
Protein	EC (n=30)	EU (n=30)	NE (n=25)	P-value
^{wt}BRAF				
Positive	19 (63.3)	21 (70)	6 (24)	0.584[a]
Negative	11 (36.7)	9 (30)	19 (76)	<0.001[b]
CREB1				
Positive	22 (76)	23 (80)	9 (36)	0.766[a]
Negative	8 (24)	7 (20)	16 (64)	0.002[b]

[a]EC vs. EU; [b]EU vs. NE. EC, ectopic endometrium; EU, eutopic endometrium; NE, normal endometrium; CREB1, cAMP responsive element binding protein 1; ^{wt}BRAF, wild-type B-Raf proto-oncogene, serine/threonine kinase.

Table V. Correlation between ^{wt}BRAF and CREB1 protein expression in endometriosis.

	^{wt}BRAF (cases)			
CREB1 (cases)	Positive	Negative	R	P-value
Positive	40	14	0.529	P<0.001
Negative	6	25		

CREB1, cAMP responsive element binding protein 1; ^{wt}BRAF, wild-type B-Raf proto-oncogene, serine/threonine kinase.

endometrial tissues, the positive expression rates of ^{wt}BRAF were 24, 70 and 63.3% and those of CREB1 were 36, 80 and 76%, respectively. The positive rates of ^{wt}BRAF and CREB1 in the eutopic endometrial tissue were significantly higher than those in normal tissues (P<0.05), while no significant difference was detected between the eutopic and ectopic endometrial tissues (P=0.584 and P=0.766, respectively; Table IV).

Protein expression of ^{wt}BRAF and CREB1 in endometrial tissues by western blot analysis. The protein expression of ^{wt}BRAF and CREB1 in the endometriosal tissues was further detected using western blot analysis (Fig. 3B and C). In the eutopic endometrium tissues, the ^{wt}BRAF and CREB1 expression levels were significantly upregulated compared with those in the normal endometrial tissue (P<0.001), while no significant differences in expression were observed between the eutopic and ectopic endometrium (P=0.566 and P=0.811, respectively).

CREB1 positively correlates with ^{wt}BRAF expression. The correlation between ^{wt}BRAF and CREB1 protein expression as determined by IHC was evaluated (Table V). The results indicated that ^{wt}BRAF immunostaining was positively correlated with that of CREB1 (correlation coefficient R=0.529, P<0.001). Furthermore, correlation of the western blotting and qPCR data suggested that there was a positive correlation between

CREB1 protein and the ^{wt}*BRAF* transcript level in all tissues (correlation coefficient R=0.417, P<0.001; Fig. 3D).

Discussion

In the present study, no *BRAF* mutations were detected in exons 11 or 15 in the ectopic and eutopic endometrium samples of patients with endometriosis. However, significant overexpression of ^{wt}*BRAF* and *CREB1* mRNA and protein was detected in the eutopic endometrium of these patients compared with normal endometrium. However, no significant difference was identified between the ectopic and eutopic endometrium. In addition, analysis of the protein expression of CREB1 indicated that it was positively correlated with the transcript level and protein expression of ^{wt}*BRAF*.

BRAF is a proto-oncogene that is also known as v-raf murine sarcoma viral homolog B1. It belongs to the Raf kinase family and was first discovered in 1988 (17). *BRAF* mutations are associated with numerous types of malignant tumor, where they activates the MAPK signaling pathway constitutively, resulting in uncontrolled cellular proliferation and survival (18). The most common *BRAF* point mutation, ^{V600E}*BRAF*, accounts for ~90% of all *BRAF* mutations, and derives from a point mutation that results in an amino acid change from valine to glutamic acid (19). However, other malignant tumors, including primary uveal melanoma and uveal melanoma demonstrate a lack of *BRAF* mutations (20,21). Zannoni *et al* (22) found no mutations in the hotspot regions of *BRAF* (i.e. exon 15) in primary clear cell ovarian carcinoma. However, several studies have shown the overexpression of the ^{wt}*BRAF* gene in other cancer types. For example, one study found that the overexpression of *BRAF* activated the RAS-BRAF-MAPK signaling pathway (12). Furthermore, *BRAF* has been demonstrated to be overexpressed in advanced hepatocellular carcinoma (23). The overexpression of *BRAF* may participate in the molecular mechanisms of thyroid papillary carcinoma, and detection of the expression of the *BRAF* gene has been shown to predict the cell invasion ability of papillary thyroid carcinoma (24). In previous studies, the high expression of *BRAF* increased the activity of ERK in the Rat-1 cell line (25), and also stimulated the growth of malignant melanoma cells (26). It has been suggested that BRAF serves an important role in tumor development by binding to specific molecular signaling molecules. Previous studies by the present research team demonstrated that *BRAF* is a candidate gene in the development of endometriosis (7), and preliminarily verified that *BRAF* mRNA and protein levels are significantly upregulated in the eutopic endometrium of patients with endometriosis compared with normal endometrium (14). In the present study, no *BRAF* mutation was detected in exons 11 or 15 among the 30 pairs of ectopic and matched eutopic endometrium samples from patients with endometriosis. This is consistent with another study that screened for *BRAF* mutations in ectopic endometrial tissue (27). Additionally, it was observed in the present study that the mRNA and protein expression levels of ^{wt}*BRAF* in the eutopic endometrial tissues from patients with endometriosis were significantly higher than those in normal endometrium, which further suggests a role for *BRAF* in endometriosis. However, no significant difference was detected between the eutopic and matched ectopic endometrial tissues from patients with endometriosis. There are two possible reasons for this observation: i) The *BRAF* gene promotes the occurrence of endometriosis, but does not serve a role in its development; ii) heterogeneity exists in the ectopic endometrium of endometriosis cases, and the quantity of ectopic endometrium tissues obtained from ectopic focus is too little to influence the research results. Further study is required to investigate these hypotheses.

To explore the mechanism of ^{wt}*BRAF* overexpression, a region of ~2,000 bp upstream of the ^{wt}*BRAF* TSS was selected. Using the JASPAR and TRANSFAC databases, a CRE binding site (TGACGTCA) at -266 to -259 bp upstream of the ^{wt}*BRAF* TSS was predicted. In addition, a previous study by the present research team found that 1,216 mRNAs were expressed differentially between eutopic and normal endometrium by long non-coding RNA microarray, among which the function of cyclin-dependent kinase 6 in endometriosis has been preliminarily validated, and *CREB1* was an overexpressed mRNA (16). These findings suggest that *CREB1* may function as a transcription factor of ^{wt}*BRAF*, and is involved in the regulation of the development of endometriosis.

CREB1 is a proto-oncogenic transcription factor that is involved in oncogenesis in numerous organs. *CREB1* increases the expression of its target genes, which are involved in various cell functions, including metabolism, the cell cycle, cell survival and DNA repair (28). As a potent oncogene, *CREB1* promotes tumorigenesis by significantly impacting the growth, proliferation, survival, metastasis and invasion of tumor cells. The overexpression of *CREB1* promotes these functions, and is also closely associated with the recurrence and poor prognosis of diseases (23). For example, it has been reported that *CREB1* is overexpressed in gastric cancer and increases gastric adenocarcinoma cell growth (29). In addition, Yang *et al* (30) demonstrated that the expression of *CREB1* was associated with the migration of hepatocellular carcinoma cells. Furthermore, the overexpression of *CREB1* has been reported to be associated with poor prognosis in non-smokers with non-small cell lung cancer and in patients with breast cancer (31,32). In the present study, the expression of *CREB1* mRNA and protein in the eutopic endometrium of patients with endometriosis was significantly higher than that in normal endometrium. In addition, it has been suggested that the CREB protein may act as a transcription regulator of aromatase in breast cancer cells to increase the synthesis of estrogen (31). Breast cancer and endometriosis are typical estrogen-dependent diseases. The present study found that the expression of *CREB1* was significantly higher in eutopic endometrium than in normal endometrium, which suggests that *CREB1* may be a candidate gene for endometriosis intervention and treatment. However, no significant difference in *CREB1* expression was detected between the ectopic and eutopic endometrium; this difference remains to be confirmed by microdissection or cell sorting. The correlation analysis conducted in the present study revealed that the protein expression of CREB1 was positively correlated with the transcript level and protein expression of ^{wt}*BRAF*. It is reasonable to speculate that CREB1 may activate the transcription of ^{wt}*BRAF* through directly binding to its promoter, increasing *BRAF* expression and regulating the cell proliferation, migration and invasion of endometriosis.

However, additional studies are required to further verify the exact molecular mechanism by which *CREB1* regulates the expression of ^{wt}*BRAF*.

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