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Apolipoprotein-B mRNA-editing complex 3B could be a new potential therapeutic target in endometriosis

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This study investigated the correlation of Apolipoprotein-B mRNA-editing complex 3B (APOBEC3B) expression with hypoxia inducible factor 1α (HIF-1α), Kirsten rat sarcoma virus (KRAS) and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) in endometriosis patients, and the inhibitory effects of APOBEC3B knockdown in a human endometriotic cell line. Here, APOBEC3B, HIF-1α, KRAS, and PIK3CA were examined in patients with and without endometriosis using reverse transcription polymerase chain reaction (RT-PCR). The apoptosis, cell proliferation, invasion, migration, and biological function of APOBEC3B knockdown were explored in 12Z immortalized human endometriotic cell line. We observed APOBEC3B, HIF-1α, KRAS and PIK3CA expressions were significantly higher in endometriosis patients ($p < 0.001$, $p < 0.001$, $p = 0.029$, $p = 0.001$). Knockdown of APOBEC3B increased apoptosis, which was 28.03% and 22.27% higher than in mock and control siRNA samples, respectively. APOBEC3B knockdown also decreased PIK3CA expression and increased Caspase 8 expression, suggesting a potential role in the regulation of apoptosis. Furthermore, knockdown of APOBEC3B significantly inhibited cell proliferation, invasion, and migration compared to mock and control siRNA. (Cell proliferation: mock: $p < 0.001$ and control siRNA: $p = 0.049$. Cell invasion: mock: $p < 0.001$ and control siRNA: $p = 0.029$. Cell migration: mock: $p = 0.004$, and control siRNA: $p = 0.014$). In conclusion, this study suggests that APOBEC3B may be a new potential therapeutic target for endometriosis.

Keywords Apolipoprotein-B mRNA-editing complex 3B, Endometriosis, Apoptosis, Potential therapeutic target

Endometriosis is characterized by the presence of ectopic and functional endometrial glands and stroma in the extrauterine cavity, which affects 6%–10% of women of reproductive age[1]. Hypoxia is also recognized as significantly involved in the development of endometriosis, and the most common method to determine tissue hypoxia is through the detection of HIF-1α. A growing body of evidence demonstrates that hypoxia plays an essential role in regulating the disease phenotypes of endometriosis, such as increasing adhesion ability and aberrant production of pro-inflammatory cytokines[2]. It has also been reported that driver mutations in KRAS and PIK3CA genes are expressed in endometriosis and ovarian cancer, but the mechanisms are still poorly understood[3].

We focused on deoxyribonucleic acid (DNA) editing, such as APOBEC3B in endometriosis. APOBEC3 family (A3A, A3B, A3C, A3D, A3F, A3G, and A3H) is an expansive group of proteins that deaminate cytosine (C) to uracil (U) (C→U) in ribonucleic acid (RNA) and single-stranded deoxyribonucleic acid (DNA)[4–6]. DNA editing is a recently identified epigenetic mechanism that regulates of essential genes by altering their amino acid sequences, leading to changes in gene expression[4, 6].

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Endometriosis patients can develop endometriosis-associated ovarian cancer, as shown in many studies. Women with a history of endometriosis had approximately twice the relative risk for clear cell or endometrioid ovarian carcinomas[7–9]. APOBEC3B has also been reported to be involved in endometriosis-associated ovarian cancer, including ovarian clear cell carcinoma[10]. Notably, Revathidevi et al. reported that the relationship between APOBEC3B and PIK3CA involves genetic alterations in endometriosis[11]. Therefore, this study aimed to investigate the correlation of APOBEC3B expression with HIF-1α and PIK3CA in endometriosis patients and the inhibitory effects of APOBEC3B knockdown in human endometriotic cell lines. Understanding the molecular mechanisms underlying endometriosis may lead to new opportunities for diagnostic and potential therapeutic strategies.

Results

Overexpressions of APOBEC3B, HIF-1α, KRAS, and PIK3CA in endometriosis patients

We measured the expression levels of APOBEC3B, HIF-1α, KRAS, and PIK3CA in the endometriosis group ($n = 15$) and non-endometriosis group ($n = 5$) by RT-PCR. APOBEC3B, HIF-1α, KRAS, and PIK3CA expressions were significantly higher in the endometriosis group than in the non-endometriosis group ($p < 0.001$, $p < 0.001$, $p = 0.029$ and $p = 0.001$, Fig. 1A). We also examined the relationship of APOBEC3B, KRAS, and PIK3CA expressions with HIF-1α. The expressions of APOBEC3B and PIK3CA were significantly correlated with HIF-1α (APOBEC3B: $R = 0.779$, R2 $= 0.608$, $p < 0.001$, and PIK3CA: $R = 0.728$, R2 $= 0.530$, $p < 0.001$, Fig. 1B). We also sought the relationship of expressions of APOBEC3B with PIK3CA and KRAS. PIK3CA was found to be significantly correlated with APOBEC3B ($R = 0.919$, R2 $= 0.845$, $p < 0.001$, Fig. 1C).

Hypoxia strengthened APOBEC3B and knockdown of APOBEC3B suppressed HIF-1α

As shown in Fig. 2, hypoxia at different concentrations (5%, 1% O_2) activated expressions of HIF-1α, APOBEC3B, KRAS, and PIK3CA in 12Z endometriotic cell line, as measured by RT-PCR. As expected, the 12Z endometriotic cell line cultured under hypoxic conditions (1% O_2) showed a significant increase in HIF-1α expression compared to cultures maintained at 20% O_2 ($p = 0.008$) and 5% O_2 ($p = 0.008$). Similarly, under hypoxic conditions (1% O_2), the 12Z cell line exhibited a marked increase in APOBEC3B expression compared to those at 20% O_2 ($p = 0.044$) and 5% O_2 ($p = 0.037$). Additonally, the impact of APOBEC3B knockdown on the expression of HIF-1α expressions in the 12Z endometriotic cell line was examined. After transfection with APOBEC3B siRNA, HIF-1α expression was significantly decreased compared to the mock sample ($p = 0.042$) (Fig. 3D).

Knockdown of APOBEC3B increased apoptosis and activated apoptosis pathway-related factors

We examined the changes in APOBEC3B expression after the knockdown of APOBEC3B in the 12Z endometriotic cell line. The knockdown efficiency of APOBEC3B was confirmed by RT-PCR, and APOBEC3B expression was significantly decreased following APOBEC3B siRNA transfection into 12Z endometriotic cell line (Mock: $p = 0.029$ and control siRNA: $p = 0.033$, Fig. 3A). After transfection of the APOBEC3B siRNA into the 12Z endometriotic cell line, we also performed an MTS assay to evaluate cell proliferation. The percentages of viable cells decreased by 43.9% (mock) and by 31.0% (control siRNA), compared to those transfected with APOBEC3B siRNA (mock: $p < 0.001$, and control siRNA: $p = 0.001$: Fig. 3B). Representative flow cytometric data revealed that the transfection of APOBEC3B for 48 h increased AnnexinV- FITC and PI-positive signals. APOBEC3B siRNA induced early and late apoptosis in the 12Z endometriotic cell line compared to the proportions in the mock and control siRNA groups of 28.03% and 22.27%, respectively. Therefore, APOBEC3B siRNA likely regulates the early and late apoptosis in the 12Z endometriotic cell line (Fig. 3C).

We examined the effects of APOBEC3B knockdown on the expression of KRAS, PIK3CA, and Caspase 8 in the 12Z endometriotic cell line. After transfection of APOBEC3B siRNA, PIK3CA expression significantly decreased (Mock; $p = 0.045$). In contrast, Caspase 8 expression following transfection of APOBEC3B siRNA significantly increased (Mock; $p = 0.027$). These results suggest that the knockdown of APOBEC3B regulates the apoptosis pathway via suppression of PIK3CA and activation of Caspase 8 (Fig. 4A).

Knockdown of APOBEC3B attenuated cell proliferation, monolayer growth, cell invasion, and migration

We performed cell invasion and migration assays and examined monolayer growth in 12Z endometriotic cell line transfected with mock, control, and APOBEC3B siRNA. The proportions of cells capable of invasion declined by 33.9% (mock) and by 30.7% (control siRNA), compared to those transfected with APOBEC3B siRNA (mock: $p < 0.001$, and control siRNA; $p = 0.029$: Fig. 4B). Moreover, the percentages of cells capable of migration markedly decreased by 59.2% (mock) and by 25.9% (control siRNA) compared to those transfected with APOBEC3B siRNA (mock; $p = 0.001$, control siRNA; $p = 0.014$: Fig. 4C). Furthermore, the knockdown of APOBEC3B significantly inhibited monolayer growth in the 12Z endometriotic cell line compared with mock and control siRNA (Day 6: mock: $p < 0.001$, control siRNA: $p = 0.049$, Fig. 4D). These results demonstrate thatAPOBEC3B potentially affects the proliferation, migratory, and invasive ability of 12Z endometriotic cells.

Discussion

The mechanisms involved in endometriosis include aberrant inflammatory responses, abnormal angiogenesis, hypoxia, and genetics. Knockdown of APOBEC3B in an endometriotic cell line affected many important pathological mechanisms. This indicates that APOBEC3B could become a promising treatment for endometriosis. We further confirmed that the expression of APOBEC3B was higher in the endometriosis patient group compared to that of the control.
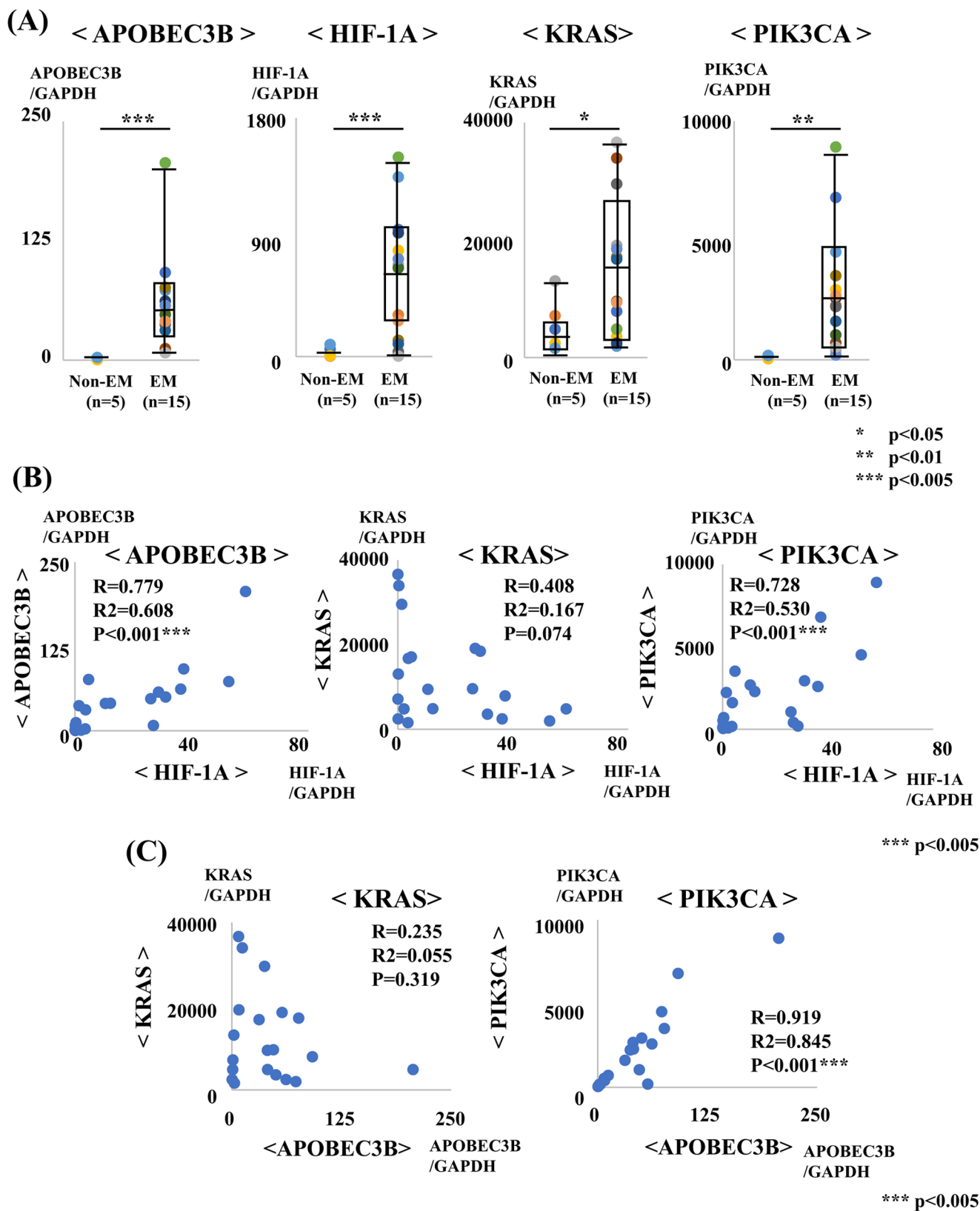
Figure 1. (**A**) Tissue analysis of APOBEC3B, HIF-1α, KRAS, PIK3CA in 5 non-endometriosis and 15 endometriosis patients. (**B**) Regression analysis of HIF-1α with APOBEC3B, KRAS, and PIK3CA in 5 non-endometriosis and 15 endometriosis patients. (**C**) Regression analysis of APOBEC3B with KRAS and PIK3CA in 5 non-endometriosis and 15 endometriosis patients.
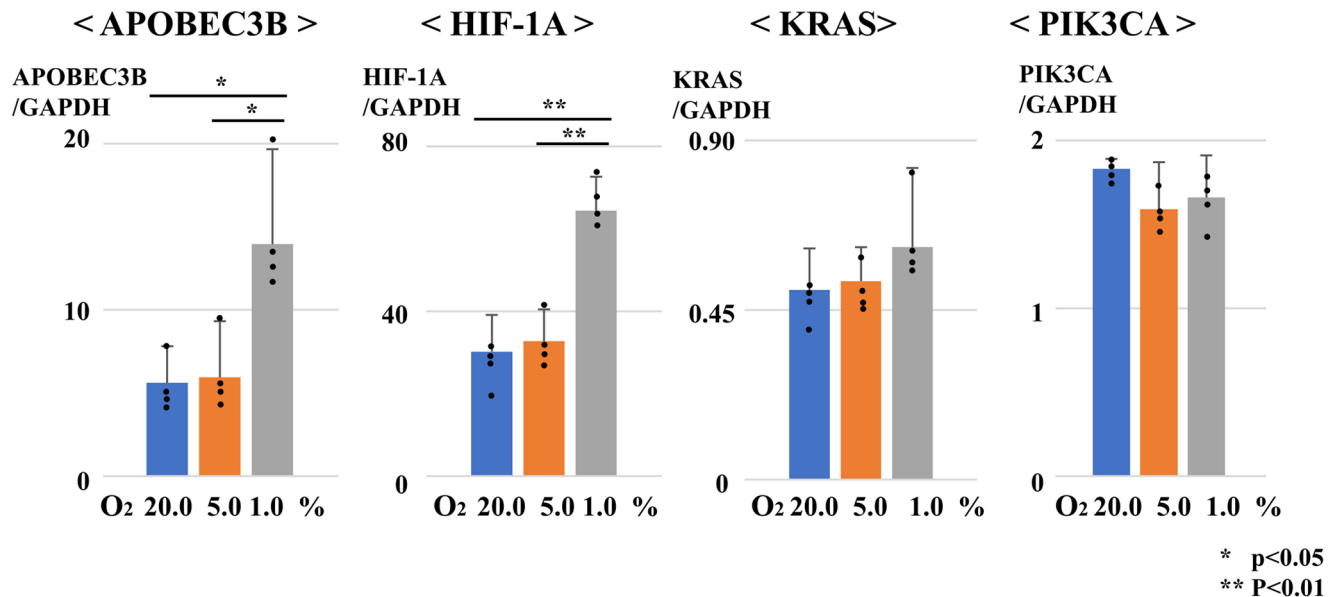
Figure 2. HIF-1α, APOBEC3B, KRAS, and PIK3CA expressions were measured with RT-PCR in the 12Z endometriotic cell line at different concentrations of O_2 (20%O_2, 5% O_2, 1% O_2) for 48 h.

APOBEC families regulate the post-transcriptional activity of essential genes by altering their amino acid sequences, leading to changes in gene expression. APOBEC families induce cytosine (C) to uracil (U) (C→U) transitions. APOBEC3B is generally expressed at higher levels compared to other APOBEC3 family members[4, 6]. An analysis of multiple cancers detected enrichment of APOBEC3B in some types of tumor: bladder, breast, head and neck, lung adenocarcinoma, lung squamous cell carcinoma, prostate, clear cell renal, and uterine[12]. High APOBEC3B levels have been reported in bladder, bile duct, lung, gastric, esophageal, neuroendocrine, and ovarian tumors[13–20]. APOBEC3B is over-expressed in breast, cervical, and non-small cell lung cancer, and its overexpression is correlated with survival[14, 21, 22].

A previous study found that endometriotic lesions harbored somatic mutations, including mutations in the well-known cancer driver genes PIK3CA and KRAS[23]. In-vitro assays indicated that KRAS and PIK3CA mutations played causative roles in the aggressive behavior of endometriosis[24]. In the link with APOBEC3B, helical domain hotspot mutations in the PIK3CA gene in multiple cancers were found, and the upregulation of APOBEC3B is correlated with increased mutational load of PIK3CA in esophageal squamous cell carcinoma, breast and cervical cancers[18, 25]. KRAS mutations have been associated with downregulated expression of APOBEC3B in non-small cell lung cancer. The presence of KRAS mutations may influence the expression levels of APOBEC3B, suggesting a potential interplay between KRAS signaling and APOBEC3B activity[14].

Therefore, we confirmed the relationship among expressions of APOBEC3B, HIF-1α, KRAS, and PIK3CA in the endometriotic cell line. We observed the overexpression of APOBEC3B, HIF-1α, KRAS, and PIK3CA in the endometriosis patient group and the correlation of APOBEC3B with HIF-1α and PIK3CA. Accordingly, we conducted investigations of not only these targets in an endometriotic cell line but also HIF-1α and PIK3CA in the APOBEC3B knockdown cell line.

Regarding to APOBEC3B expression in response to hypoxia, Bader et al. also have shown that hypoxic conditions (which include cyclic variations between <0.1% and 2% O_2) lead to the induction of APOBEC3B expression[26]. Cells exposed to fluctuating/cyclic hypoxic conditions stabilize HIF-1α, leading to the increased expression of HIF-target genes, including APOBEC3B. APOBEC3B has been associated with the NF- κB transcription factor, which is known to be active in hypoxic environments[27]. Treatment with an NF- κB inhibitor (Bay11-7085) partially inhibited the induction of APOBEC3B protein in cyclic hypoxia[26]. This suggests a specific role for HIF1α in regulating APOBEC3B expression in response to hypoxia. The present study demonstrates that APOBEC3B, HIF-1α, KRAS, and PIK3CA expressions were significantly higher in endometriotic specimens than non-endometriotic specimens. APOBEC3B and PIK3CA expressions were significantly correlated with HIF-1α in endometriosis patients.

In this study, we showed that the knockdown of APOBEC3B in the endometriotic cell line promoted apoptosis. This result is consistent with the studies which demonstrated that the downregulation of APOBEC3B increased apoptosis in biliary tract cancer and chondrosarcoma[28, 29].

Furthermore, APOBEC3B may negatively regulate apoptosis in cervical cancer cells via apoptosis-associated factors such as caspase[30]. Similarly, caspase-3, -8, and −9 activity were significantly increased in chondrosarcoma cells with APOBEC3B knockdown[29]. Our study also observed that knockdown of APOBEC3B decreased PIK3CA and increased Caspase 8 expressions in the endometriotic cell line. This is consistent with other studies that indicated that upregulation of APOBEC3B is correlated with increased PIK3CA[18, 25]. Therefore, we hypothesized that PIK3CA may act as an essential factor for APOBEC3B to regulate apoptosis-related pathways.
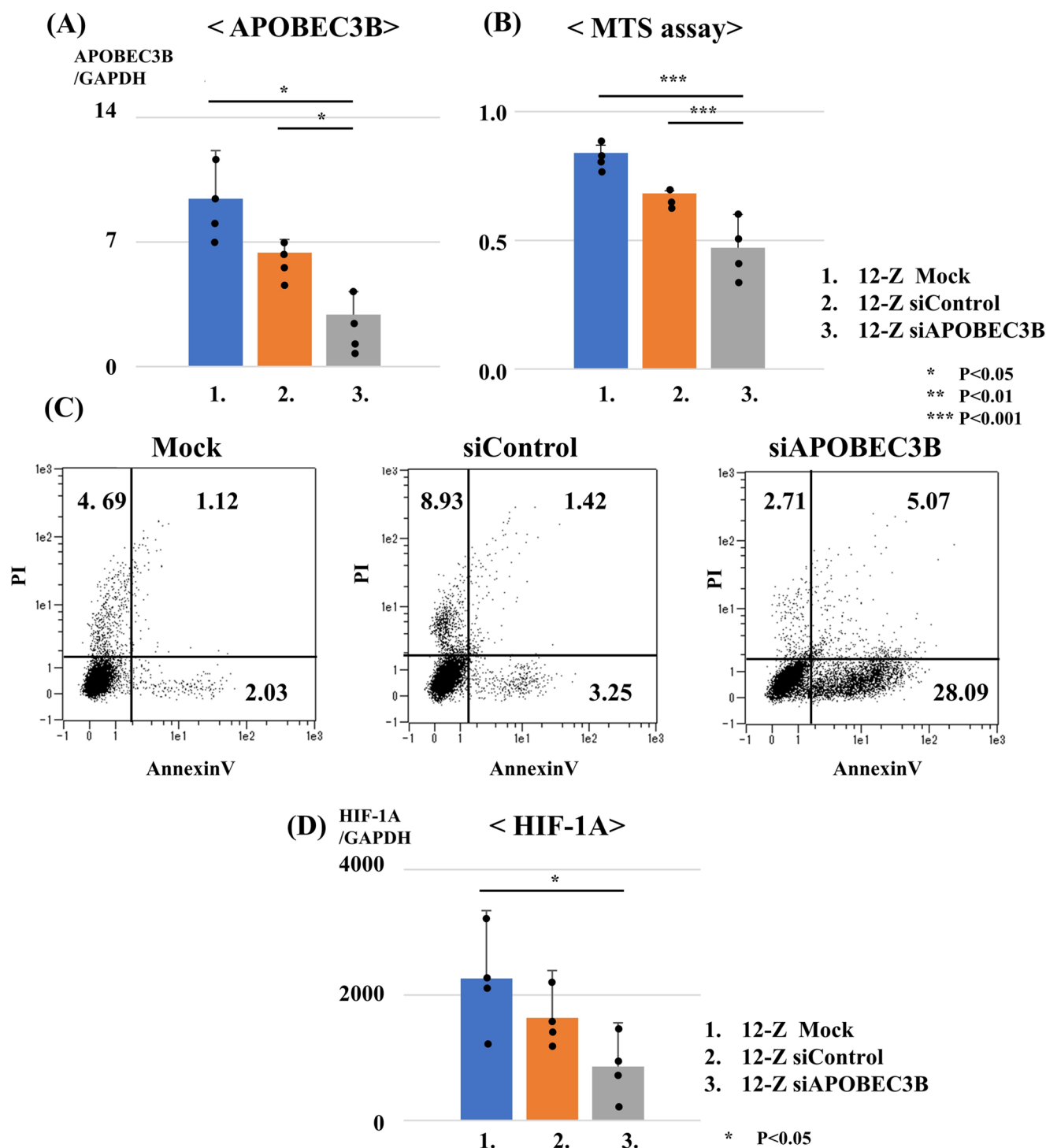
Figure 3. After transient transfection of the mock, control siRNA (siCon) and APOBEC3BsiRNA (siAPOBEC3B) into 12Z endometriotic cell line for 48 h: (**A**) RT-PCR analysis of the APOBEC3B expression levels in mock, siCon and siAPOBEC3B cells. (**B**) MTS assays of mock, siCon, and siAPOBEC3B cells. (**C**) Representative flow cytometric data for apoptosis levels in mock, siCon, and siAPOBEC3B cells. (**D**) RT-PCR analysis of the HIF-1α expression levels in mock, siCon, and siAPOBEC3B cells.

Previous reports suggested that APOBEC3B enhanced cell proliferation and migratory and invasive abilities of hepatocellular carcinoma and cervical cancer cells[30, 31]. We examined if this was also true for the endometriotic cell line. Then, we performed the cell proliferation, migration, and invasion assays following the transient transfection of APOBEC3BsiRNA in the endometritic cell line. Knockdown of APOBEC3B in endometriotic cells suppressed the cell proliferative, migratory, and invasive abilities.
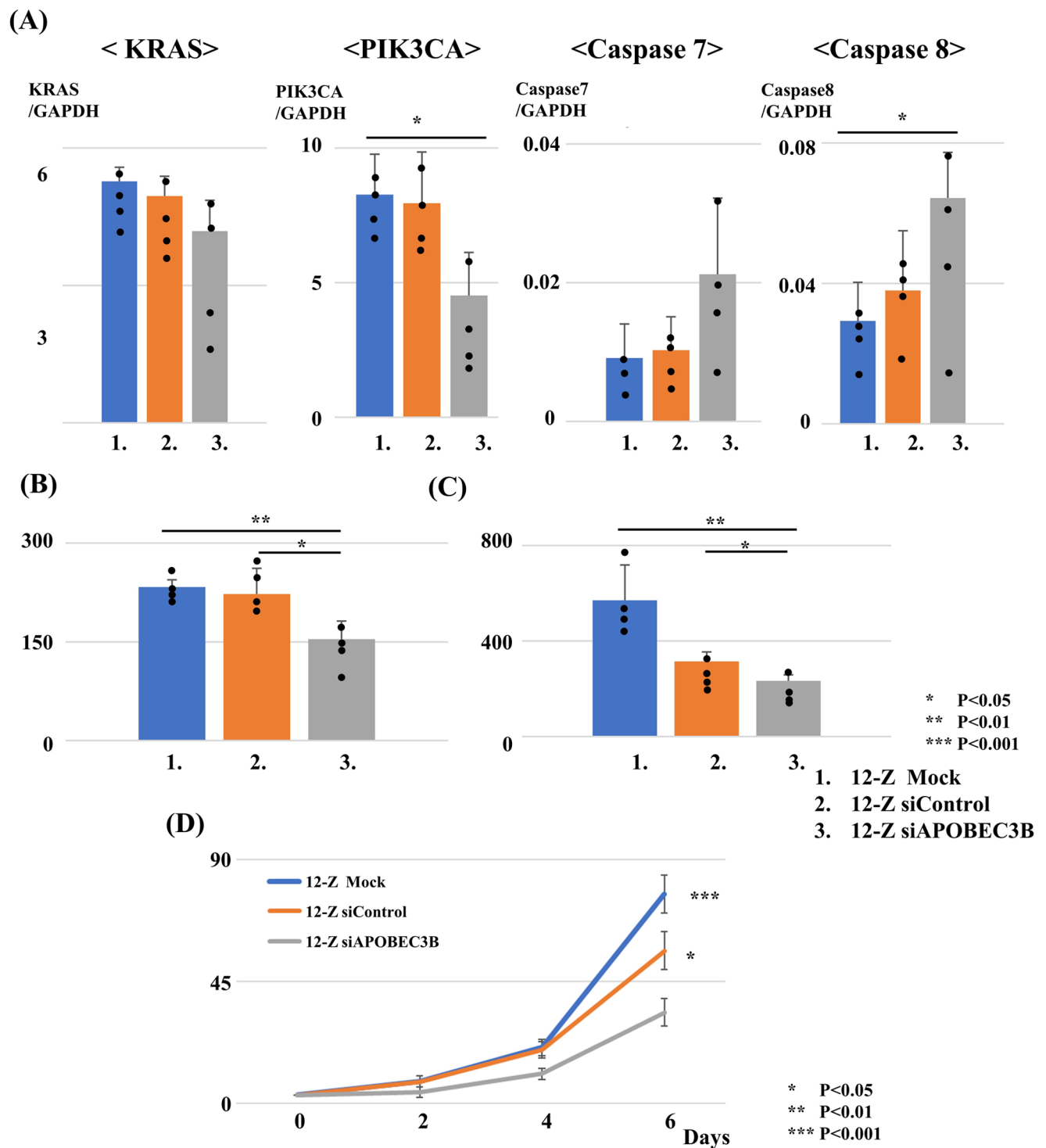
Figure 4. After transient transfection of the mock, control siRNA (siCon) and APOBEC3BsiRNA (siAPOBEC3B) into 12Z endometriotic cell line for 48 h: (**A**) RT-PCR analysis of KRAS, PIK3CA, Caspase 7 and Caspase 8 expressions compared between mock, siCon and siAPOBEC3B cells. (**B**) Cell invasion assay compared between mock, siCon, and siAPOBEC3B transfected cells. (**C**) Cell migration assay compared between mock, siCon, and siAPOBEC3B transfected cells. Cell invasion and migration assays were performed in triplicate. (**D**) Cell growth in monolayers was compared between mock, siCon, and siAPOBEC3B transfected cells using DMEM/ham's F12 medium supplemented with 10% FBS for 2, 4, and 6 days. The numbers represent data from triplicate experiments.

This study has several limitations. First, it is a single-center retrospective analysis. Second, we used only one endometriotic cell line (12Z) instead of primary cell cultures, which may not fully reflect the heterogeneity of endometriotic tissues. Additionally, we acknowledge the limited sample size and absence of protein expression data, which may restrict the depth of our conclusions. Based on our findings of key regulatory molecules in the APOBEC3B pathway at the RNA level, future larger-scale studies with expanded sample sizes and protein-level investigations will be considered to draw more comprehensive conclusions about its role in endometriosis.

To the best of our knowledge, this study is the first to explore APOBEC3B in patients with endometriosis. It was found that APOBEC3B expression was significantly higher in these patients and correlated with HIF-1α and PIK3CA. Knocking down APOBEC3B led to an increase in apoptosis but a decrease in cell proliferation, invasion, and migration. These findings suggest that APOBEC3B could be a therapeutic target for endometriosis treatment. Detailed insights into the mechanism of action of APOBEC3B have led to new diagnostic and therapeutic strategies for endometriosis.

Materials and methods
Patients and tissue specimens
The study received approval from the institutional ethics committee of Okayama University (approval number: K2212-042). Informed consent was obtained from all participants, and all procedures adhered to relevant ethical standards and institutional regulations. Endometriosis lesions were collected from the peritoneum in the endometriosis group, while peritoneal tissues removed during hysterectomy for benign diseases like uterine fibroids were gathered in the control group. Endometriotic lesions were visually diagnosed by using ASRM classification for endometriosis and confirmed via histopathology. Twenty female patients of reproductive age who underwent surgical treatment at Okayama University Hospital between April 2014 and October 2022 participated in this study after providing informed consent. The subjects were classified into two groups: the endometriosis group ($n = 15$) and the control group ($n = 5$).

RNA isolation and real-time quantitative PCR analyses
Total RNA was isolated from the samples of patients and endometrial cell lines using the RNeasy Lipid Tissue Mini kit (QIAGEN, Hilden, Germany). The iTaq Universal SYBR Green OneStep Kit and the MiniOpticon Real-Time PCR System (Bio-Rad, CA, USA) were used for gene expression analysis by real-time quantitative PCR. Primer sequences for APOBEC3B, HIF-1α, KRAS, PIK3CA, Caspase 7, Caspase 8, and Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH) genes are shown in Supplementary Table 1. All procedures were done following the manufacturer's instructions. After an initial denaturation step at 95 °C for 10 min, 39 amplification cycles were conducted with annealing/elongation at 56 °C for 50 s for all primer pairs. GAPDH was used as a normalization control, and the relative expression of each mRNA was determined using the ΔΔCt method.

Cell culture
12Z immortalized human endometriotic cell line was obtained from Applied Biological Materials (Vancouver, Canada). 12Z cell line was created by transforming epithelial-like cells from peritoneal endometriosis lesions with SV40T antigen. Research has demonstrated that these cells closely mimic the gene expression and biological function found in endometriosis patients, making them a good in-vitro model for studying endometriosis[32–34]. 12Z endometriotic cell line was maintained in Dulbecco's modified eagle's medium (DMEM)/F12 phenol red-free (Life Technologies, CA, USA), supplemented with 10% fetal bovine serum (FBS). The cell line was trypsinized and then plated in culture dishes in a humidified incubator containing 5% CO_2 at 37 °C or exposed to 1% and 5% O_2 hypoxic conditions using a BIONIX hypoxic cell culture kit (Sugiyamgen, Tokyo, Japan). These cells were used for functional experiments within three months of being passaged after receipt.

Small-interfering RNA (siRNA) transfection
APOBEC3B siRNA (sc-72515) and control siRNA (sc-37007) were obtained from Santa Cruz Biotechnology. When the cells reached approximately 50% confluency, they were transfected with annealed APOBEC3B siRNA, control siRNA, and an empty vector (mock) for gene silencing using a siRNA transfection reagent.

MTS assay
The effect of APOBEC3B siRNA on the cell proliferation of the 12Z endometriotic cell line was evaluated using the MTS assay (Promega). The cell line was collected and suspended in phenol red-free DMEM/F12 medium with 10% FBS before being seeded into 96-well culture plates at a concentration of 5×10^3 cells per well. After overnight incubation in phenol red-free DMEM/F12 medium with 10% FBS, the cells were deprived of serum and then transiently transfected with control siRNA and APOBEC3B siRNA for 48 h. Subsequently, absorbances were measured at a wavelength of 490 nm using an ELISA plate reader (Bio-Rad, CA, USA) following exposure to MTS for one hour.

Apoptosis assay
Apoptosis was measured by staining with fluorescein isothiocyanate (FITC)-conjugated annexin V using a MEBCYTO Apoptosis kit (MBL International Corp., MA, USA). Cells were analyzed using a flow cytometer (MACSQuant Analyzers, Mitenyi Biotec, Bergisch Gladbach, Düsseldorf, Germany). Annexin V binds to phosphatidylserine on the outer membranes of cells, which becomes exposed on the surfaces of apoptotic cells. Therefore, AnnexinV-positive cells are considered apoptotic. Propidium Iodide is an intercalating agent that cannot permeate through the cell membranes of viable or early apoptotic cells.

Cell invasion assay

According to the manufacturer's instructions, the cell invasion assay was performed using CytoSelect™ 24-Well Cell Invasion Assay, Basement Membrane (Cell Biolabs, CBA-111, San Diego, CA, USA). The 12Z cell line was transiently transfected with mock, control siRNA, and APOBEC3B siRNA for 48 h. After transfection, the cells were seeded at a density of 5×10^5 cells per polycarbonate membrane inserted in 300 µl of medium containing 0.1% FBS. Before seeding, the lower well of the plate was filled with 500 µl of medium containing 10% FBS. The culture was then incubated for an additional period of 48 h. During this time, invasive cells passed through the basement membrane layer and adhered to the bottom surface of the insert membrane. Cell quantification was performed by fluorescence using a microplate reader. The cell invasion values (folds) were normalized to control conditions and represent the average results from three independent experiments plus standard deviation.

Cell migration assay

Cell migration was measured using the Cytoselect™ 24-Well Cell Migration Assay kit, 8 µm (fluorometric quantitation) (Cell Biolabs, CBA-101, San Diego, CA, USA) following the manufacturer's protocol. The 12Z cell line was transiently transfected with mock, control siRNA, and APOBEC3B siRNA for 48 h. After transfection, the cells were seeded at a density of 5×10^5 cells per polycarbonate membrane inserted in 300 µl of medium containing 0.1% FBS. Before seeding, the lower well of the plate was filled with 500 µl of medium containing 10% FBS. After 24 h of incubation at 37 °C, migratory cells were quantified by fluorescence using a microplate reader. The cell migration values (folds) were normalized to control conditions and represent the average results from three independent experiments plus standard deviation.

Cell growth in monolayers

For evaluation of cell growth in monolayers, cells were plated at a density of 3×10^4 cells/well in 6-well plates containing DMEM/F12 supplemented with 10% FBS. The cell numbers were counted by a hemocytometer in triplicate after 2, 4, and 6 days to assess cell proliferation.

Statistical analysis

Statistical analyses were performed using the Mann–Whitney U-test for comparisons with controls and one-factor ANOVA followed by Fisher's protected least significance difference test for all pairwise comparisons. The analyses were performed using StatView version 26.0 (Abacus Concepts, Berkeley, CA). Differences were considered significant at $p < 0.05$.

Data availability

All data generated or analyzed during this study are included in this published article.

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Author contributions

Conceived and designed experiments: K.N., T.H.V.; performed experiments: T.H.V., K.N., K.S., C.K.; analyzed data: T.H.V., K.O., C.K.; contributed reagents, materials, and other analytical tools: T.H.V., K.N., K.S., K.O., C.K., K.K., Y.K.; wrote the manuscript: T.H.V., K.N., K.S., K.O., C.K., H.M. All authors have read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate

Written informed consent was obtained from each patient, and the institutional review board (The Ethics Committee of the Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences and Okayama University Hospital) approved the study (K2212-042).

Competing interests

The authors declare no competing interests.

Additional information

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