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miR-515-5p suppresses trophoblast cell invasion and proliferation through XIAP regulation in preeclampsia

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ABSTRACT

MicroRNAs (miRNAs) are non-coding small RNA molecules that can be secreted into the circulation and which exist in remarkably stable forms. Circulating miRNAs regulate numerous biological process and are aberrantly expressed in pathological conditions. Differentially expressed circulating miRNAs have received attention as potential biomarkers for many diseases. In this study, we revealed that miR-515-5p was significantly upregulated in maternal serum from preeclampsia patients in comparison to normal pregnant women. Bioinformatics prediction and a dual-luciferase reporter gene assay revealed that miR-515-5p directly targets the X-linked inhibitor of apoptosis protein (XIAP) 3′-untranslated region. In addition, the overexpression of miR-515-5p inhibited the proliferation and invasion of HTR-8/SVneo trophoblast cells. The decreased XIAP expression and reduced epithelial-mesenchymal transition (EMT) were observed in the preeclamptic placenta. Collectively, miR-515-5p may play critical roles in the pathogenesis of preeclampsia through suppression of XIAP, and serum miR-515-5p may act as a potential biomarker for preeclampsia.

1. Introduction

Preeclampsia (PE) is a hypertensive disorder of pregnancy that affects 2–8% of pregnancies (Jeyabalan A, 2013). Severe adverse outcomes in PE pregnancy, such as placental abruption and eclampsia, may occur, which make preeclampsia a serious threat to both maternal and neonatal health. Despite major medical advances, delivery of the fetus and placenta is still the only known cure for preeclampsia. Multiple studies have revealed several theories about the pathogenesis of PE. Disordered placental functions, such as reduced trophoblast invasion and increased apoptosis of trophoblast cells, endothelial dysfunction, inadequate maternal immune tolerance toward the fetus during pregnancy, and other theories have been advocated (Chen SB, et al. 2016; Cindrova-Davies T et al., 2014; Huang N et al., 2020). Among these theories, inadequate trophoblast invasion has been widely accepted by researchers (Pankiewicz K et al., 2021). However, the precise cause and pathogenesis have not been fully elucidated.

X-linked inhibitor of apoptosis protein (XIAP) is an inhibitor of apoptosis protein (IAP) (Holcik M et al., 2001). IAPs were first identified in baculoviruses and shown to be involved in keeping host cells alive during viral infection (LaCasse EC, et al. 1998). To date, eight members of the human IAP family have been identified: cellular IAP1 (cIAP1), cIAP2, X-linked IAP (XIAP), neuronal apoptosis inhibitor protein, survivin, apollon, livin and baculoviral IAP repeat containing 8. Of these, XIAP is the most potent and multifaceted among human IAP family members. XIAP inhibits apoptosis by inhibiting caspase 9 and caspase 3/7. XIAP is also known to modulate autophagy and TNFα-induced necrosis (Hanifeh M et al., 2022). Furthermore, XIAP has been reported to promote cell migration by enhancing epithelial-mesenchymal transition (EMT) (Jin Y et al., 2019). In placenta, XIAP is reportedly present in trophoblasts throughout placental development (Gruslin A et al., 2001). However, little is known about the role of XIAP in placental development or pregnancy-associated disorders.

Recent reports have shown that the expression of XIAP in trophoblasts was decreased in PE, which may be associated with increased apoptosis of placenta (Jeon SY et al., 2013; Arroyo J et al., 2014). However, how XIAP expression is regulated remains unclear.

MicroRNAs (miRNAs) are small non-coding RNAs that bind to the complementary 3′-untranslated regions (UTRs) of target mRNAs and suppress the gene expression via inhibition of the translation of its target mRNA (Ambros V et al., 2001). Previous studies showed that miRNAs play roles in diverse range of cellular events. The human placenta has

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Table 1
Clinical characteristics of normal pregnant women and preeclampsia patients. The details are expressed as mean ± SD, n= number of women in each group.

Characteristics	Normal pregnancy (n = 3)	Preeclampsia (n = 3)	P value
Maternal age(y)	32 ± 3.55	31 ± 2.94	0.77
BMI(kg/m²)	21.7 ± 3.69	37.3 ± 4.54	0.019
Gestational age at delivery (day)	267 ± 4.54	265 ± 3.39	0.75
SBP(mmHg)	100 ± 6.64	163 ± 5.35	<0.01
DBP(mmHg)	63.3 ± 0.94	102 ± 5.24	<0.01
UP/Ucre ratio	None	0.84 ± 0.30	<0.01
50g GCT (mg/dl)	129.3 ± 2.49	69.6 ± 52	0.18

great amounts of miRNAs, and a number of differentially expressed miRNAs have been reported in PE. During the past few years, researchers have focused on the profile of miRNA expression in placental tissues (Ma et al., 2020; Lv Y et al., 2019). In addition, growing numbers of PE studies have focused on extracellular vesicles that harbor cargo molecules, including miRNAs (Ma et al., 2020; Wang D et al., 2020; Xueya Z et al., 2020). Recently, it was proven that miRNAs are stable in the circulation, and there is increasing interest in the possible future application of circulating miRNAs in the diagnosis of several diseases, through diagnostic techniques such as liquid biopsy (McGuire A et al., 2020; Wang W et al., 2020).

In this study, we aimed to identify a candidate circulating miRNA that regulates the expression of XIAP and its role in the pathogenesis of PE.

2. Materials and methods

2.1. Ethics statement

This study was conducted according to the guidelines laid down in the Declaration of Helsinki. In this study, the collection of human plasma specimens and placenta tissues was performed with the permission of the local ethical committee of the Osaka Medical and Pharmaceutical University (protocol #2558), and written informed consent was obtained from all patients enrolled in this study. Human plasma was collected at delivery from uncomplicated term gestations (n = 3) and PE patients (n = 3). Human placentas were also collected immediately following vaginal or cesarean deliveries from the same patients. Placental tissue samples (<1000 mm^3 each) were randomly collected from different lobules (3 sites) of each placenta sample, according to previous reports (Niu Z et al., 2018, Zhang H et al., 2020). Macroscopic areas of placental infarcts were avoided during sample collection. PE was classified according to the American College of Obstetricians and Gynecologists (ACOG) guidelines. The clinical characteristics of these women are summarized in Table 1.

2.2. miRNA microarray analysis

Total RNA was extracted from 300 μl of serum using 3D-Gene RNA extraction reagent (Toray Industries, Inc., Kanagawa, Japan). A comprehensive miRNA expression analysis was performed using the 3D-Gene miRNA Labeling Kit and the 3D-Gene Human miRNA Oligo Chip (Toray Industries, Inc.), which was designed to detect 2565 mature human miRNA sequences registered in miRBase release 21 (http://www.mirbase.org/). The chip was scanned using a 3D-Gene Scanner, miRNAs with signals higher than the background signal were selected (positive call), and only miRNAs with a positive call were used in subsequent analyses. The miRNA signal values were standardized by global normalization (log conversion of data and median alignment). The relative expression level of each miRNA was validated by a one-way analysis of variance or *t*-test ($p < 0.05$). MicroRNAs for which the expression levels showed an at least 2-fold difference ($|\log_2$ fold change$| > 1$ and $p < 0.05$) in the test sample versus control sample were analyzed.

2.3. Cell culture

The human trophoblast cell lines HTR-8/SVneo was purchased from the American Type Culture Collection. HTR-8/SVneo was cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS) (Equitech-Bio, Inc.) at 37 °C in a humidified incubator under 5% CO_2.

2.4. Transfection of precursor miRNA

Pre-miR miRNA precursor molecules (pre-miR-515-5p; cat.no. AM17100), negative (non-specific) control (pre-miR-negative control #1; cat. no. AM17110) were ordered from Thermo Fisher Scientific, Inc. Pre-miR-negative control #1 is designed to use as a negative control for experiments using Pre-miR miRNA precursor experiments. The transfection of precursor miRNA was performed as previously described (Nakamura M et al., 2020). In brief, HTR-8/SVneo cells were transfected with pre-miR-515-5p or pre-miR-negative control (30 nM) for 24 h. Oligonucleotide transfection was performed using siPORT NeoFX Transfection Agent (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions.

2.5. 3′UTR reporter assay

The full length XIAP 3′UTR was inserted downstream of a Gaussia luciferase (Gluc) reporter in the pEZX-MT05 vector (GeneCopoeia, Inc.). The secreted alkaline phosphatase (seAP) reporter gene was also present in the vector as an internal control for transfection normalization. As a control (pEZX-MT05-CT), miRNA target clone control vector (CmiT000001-MT05) was purchased from GeneCopoeia, Inc. Cells (1×10^5/ml) seeded in 24-well plates were co-transfected using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) complexed with the pEZX-MT05 vector and pre-miR-515-5p or pre-miR-negative control (cont miR) according to the manufacturer's protocol. After transfection for 48 h, the relative luciferase activity (Gluc: seAP ratio) was analyzed using the Secrete-Pair Dual Luminescence Assay kit (GeneCopoeia, Inc.) according to the manufacturer's protocol. Each experiment was performed three times in triplicate wells.

2.6. RNA isolation and RT-qPCR analysis

RNeasy kit (Qiagen, Valencia, USA) was used to isolate total RNA from cells according to the manufacturer's protocol. RNA samples were reverse-transcribed into cDNA using a Super Script II Reverse Transcriptase kit (Thermo Fisher Scientific, Inc.). Subsequently, TaqMan RT-qPCR was performed in triplicate using the StepOne Real-Time PCR system (Thermo Fisher Scientific, Inc.). The mixed primers for TaqMan qPCR used in the present study were purchased from Thermo Fisher Scientific, Inc. (XIAP: Hs00745222_s1). GAPDH (Hs02786624_g1) was used as a housekeeping control gene.

miRNA extraction was performed using a mirVana miRNA isolation kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. miRNA was reverse transcribed using the microRNA reverse transcription kit (Thermo Fisher Scientific, Inc.), then qPCR of miR-515-5p was performed according to the manufacturer's instructions (Thermo Fisher Scientific, Inc.). RNU48 was used as the normalization control. Data analyses were performed using the $2^{-\Delta\Delta Cq}$ method. Each experiment was performed three times in triplicate wells.

2.7. Western blotting

Western blotting was performed as described previously (Ono YJ, et al. 2015). In brief, total proteins from HTR-8/SVneo were prepared

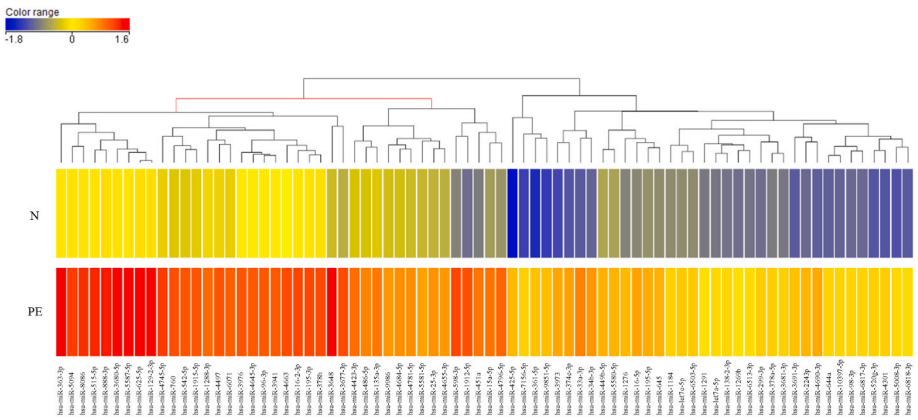

Fig. 1. Heatmap illustrating the expression of miRNAs in the serum of pregnant women.
The microarray analysis was used to identify the miRNA expression in serum from normal pregnant women (N) and PE patients (PE). Expression values (fold change >2.0, $p < 0.05$) are represented in different colors, which indicate expression levels above and below the median expression level across all samples.

using Pierce RIPA Buffer (Thermo Fisher Scientific, Inc.). The protein concentration was quantified by the DC Protein Assay (Bio-Rad Laboratories, Inc.). Protein samples (15 μg/lane) were resolved via SDS-PAGE and transferred to PVDF membranes. After being blocking with 10% bovine serum albumin (New England BioLabs, Inc.) for 1 h at room temperature, the membranes were incubated overnight at 4 °C with anti-XIAP (Cell Signaling, Boston, MA, USA; 14334) or anti-β-actin (Cell Signaling; 4970). After incubation with horseradish peroxide-conjugated anti-rabbit secondary antibody (Santa Cruz Biotechnology, Inc.; sc-2030) at room temperature for 1 h, the blots were visualized using enhanced chemiluminescence (ECL Plus; GE Healthcare Life Sciences). The experiments were performed three times.

2.8. Proliferation assay

Cell proliferation was measured using the CellTiter 96 AQueous (MTS) One Solution Cell Proliferation Assay (Promega, Tokyo, Japan) according to the manufacturer's instructions. Briefly, HTR-8/SVneo cells were transfected for 24 h, and CellTiter 96 AQuenous (MTS) One Solution Reagent was added to each well. The cells were then incubated for 1 h, and absorbance was recorded at 490 nm using the Corona SH-1000 lab absorbance microplate reader (Corona Electric, Ibaraki, Japan). The experiments were performed at least three times.

2.9. Cell invasion assay

The invasive ability of the cells was assessed using the Corning BioCoat Matrigel Invasion Chamber (Corning, NY, USA), in accordance with the manufacturer's instructions. In brief, transfected cells were immersed onto the upper chamber, which contained FBS-free medium, and RPMI-1640 containing 10% FBS for the lower chamber. The upper chamber was swabbed with a cotton swab after culturing for 24 h. Cells that penetrated the membrane were fixed in 4% methanol for 10 min and stained with 0.1% crystal violet. In each insert, four microscopic fields were photographed randomly, and the cell numbers were counted manually. The experiments were performed three times.

2.10. Immunohistochemistry

The tissue samples were fixed in formalin and embedded in paraffin. Deparaffinized and rehydrated sections (4 μm) were heated with microwaves in 0.01 mol/l citrate buffer with a pH of 6.0 for antigen retrieval. After blocking in 3% hydrogen peroxide (H_2O_2) for 10 min, the sections were incubated at room temperature for 60 min with anti-E-cadherin antibodies (Agilent DAKO; M3612, 1:100), anti-N-cadherin antibodies (Thermo Fisher Scientific; 33–3900, 1:300) and anti-Ki-67 antibodies (Nichirei Biosciences Inc.; 418071). The samples were then incubated with secondary antibodies (Histofine Simple Stain MAX PO; Nichirei Biosciences Inc.) for 30 min. Finally, the slides were washed with Tris-HCl buffer and incubated with H_2O_2/diaminobenzidine substrate solution for 5 min. The Ki-67 immunohistochemical specimens were observed with a slide scanner (NIS-Elements; Nikon) and image processing software program (WinROOF 2021; Mitani Corporation). The percentage of the total number of the cells with nuclear staining with Ki-67 in viable regions from 3 randomly selected fields was observed (magnification, × 100).

2.11. Statistical analyses

The statistical analyses were performed using the JMP software program (version.13.1.0). The data represent the mean ± standard deviation of three independent experiments. The statistical analysis was performed using Student's *t*-test. P values of <0.05 were considered to indicate statistical significance.

3. Results

3.1. The expression level of miR-515-5p was upregulated in serum from PE patients

A comprehensive miRNA microarray analysis with coverage of more than 2500 miRNA transcripts was performed using RNAs extracted from maternal serum from 3 PE patients and 3 normal pregnant women to investigate circulating miRNA profiles in pregnant women and the potential role of miRNA in the pathogenesis of PE. The clinical characteristics of these women are summarized in Table 1. The systolic BP and diastolic BP and BMI of PE patients were significantly higher than in normal pregnant women ($p < 0.05$). The maternal and gestational ages were not significantly different between PE patients and normal pregnant women (Table 1). Seventy-six miRNAs and 72 miRNAs were up- and downregulated by at least 2-fold in PE in comparison to normal pregnant women, respectively. As shown in Fig. 1, miR-515-5p was one of the most significantly upregulated miRNAs in serum from PE patients. This was validated using qRT-PCR (supplementary material). It was previously reported that miR-515-5p is significantly increased in PE placentas (Zhang M et al., 2016), suggesting a relationship with PE. However, serum level of miR-515-5p in pregnant women has not been examined. In this study, we investigated the relationship between serum miR-515-5p and the pathogenesis of PE.

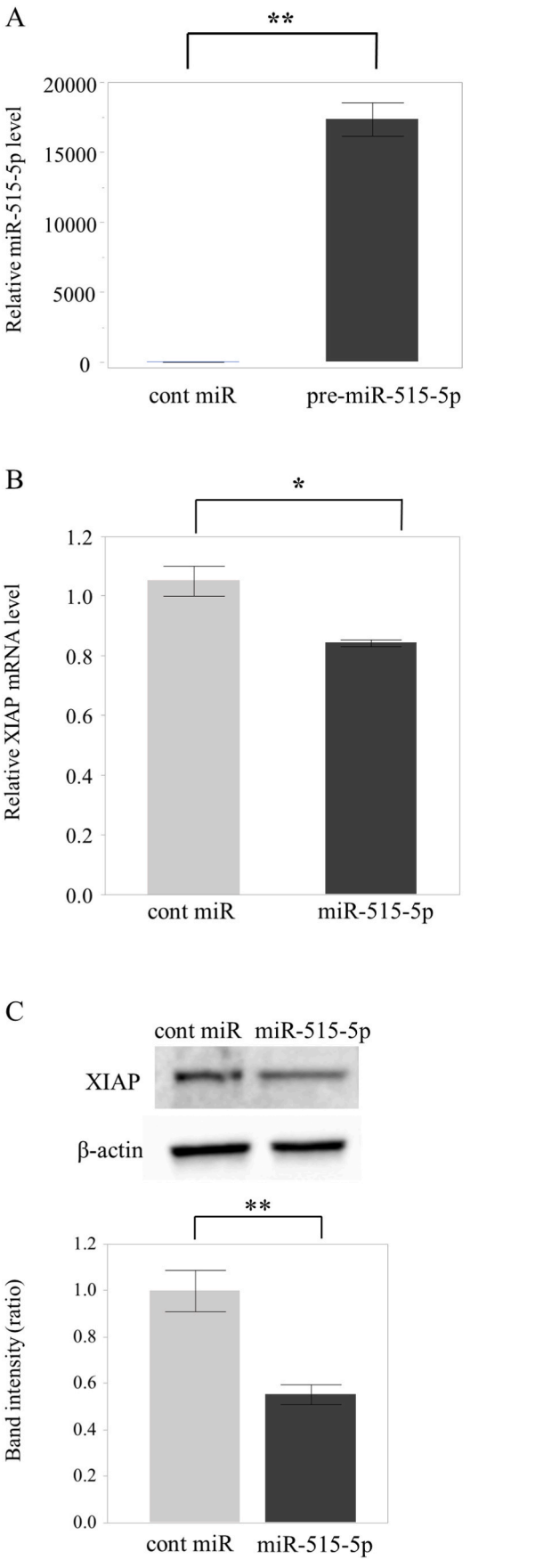

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Fig. 2. Inverse correlation between miR-515-5p and XIAP in HTR-8/SVneo cells.
(A) HTR-8/SVneo cells were transfected with either miR-515-5p or control (cont miR). The relative abundance of miR-515-5p with respect to RNU48 was calculated by RT-qPCR, and relative fold differences in comparison to cont miR are presented. **$p < 0.01$. (B) The overexpression of miR-515-5p inhibited XIAP mRNA in HTR-8/SVneo cells. Relative fold differences in comparison to cont miR are presented. *$p < 0.05$. (C) HTR-8/SVneo was transfected with either miR-515-5p or control (cont miR), and the XIAP protein level was measured by Western blotting. β-actin was used as a loading control. Data are shown as the means ± SD of three independent experiments.

3.2. miR-515-5p targets the expression of XIAP mRNA in HTR-8/SVneo trophoblast cells

We first performed RT-qPCR to evaluate the expression profile of miR-515-5p in HTR-8/SVneo trophoblast cells. The overexpression of miR-515-5p was induced by the transfection of pre-miR-515-5p, and an increased level of miR-515-5p was confirmed by RT-qPCR (Fig. 2A). Next, we examined whether or not the expression of XIAP could be altered by the overexpression of miR-515-5p. As shown in Fig. 2B, the level of XIAP mRNA was inhibited when miR-515-5p was overexpressed. In addition, miR-515-5p negatively modulated the XIAP protein expression (Fig. 2C).

3.3. XIAP expression was inhibited by miR-515-5p through direct targeting of its 3′UTR in HTR-8/SVneo cells

We performed an *in silico* analysis using TargetScan (version 7.2; targetscan.org/) to find the potential target site of miR-515-5p on XIAP mRNA. As shown in Fig. 3A, three complementary binding sequences of miR-515-5p existed on XIAP. To verify this, we performed a luciferase reporter assay with the overexpression of miR-515-5p or the reduced expression of miR-515-5p. HTR-8/SVneo cells were co-transfected with either the pre-miR-515-5p or control (cont miR) and with either a plasmid containing a luciferase reporter driven by the wild-type human XIAP 3′ UTR (pEZX-MT05-XIAP; Fig. 3B) or a control plasmid (pEZX-MT05-CT) into HTR-8/SVneo cells. The treatment of HTR-8/SVneo with complexes of pre-miR-515-5p and pEZX-MT05-XIAP significantly reduced the luciferase activity in comparison to the combination of cont miR and pEZX-MT05-XIAP (Fig. 3C, left). The antagonism of miR-515-5p by the transfection of anti-miR515-5p significantly increased the luciferase activity of the XIAP 3′UTR in comparison to the transfection of cont miR (Fig. 3D, left). These observations suggested that miR-515-5p suppressed XIAP levels by directly targeting the XIAP 3′UTR.

3.4. Cell proliferation and invasive ability of HTR-8/SVneo cells were suppressed by the overexpression of miR-515-5p

To investigate the biological function of miR-515-5p in PE pregnancy, we then examined the effect of miR-515-5p on the proliferation and invasive ability of trophoblast cells. An MTT assay revealed the striking inhibition of cell proliferation of HTR-8/SVneo cells transfected with miR-515-5p (Fig. 4A). Transfection of miR-515-5p also inhibited HTR-8/SVneo cell invasion (Fig. 4B). Taken together, these results suggested that the increased expression of miR-515-5p can suppress the cell proliferation and invasive ability of trophoblast cells.

3.5. The expression of XIAP was decreased in the PE placenta

The expression of XIAP in placental tissue was measured in PE patients and normal pregnant women. RT-qPCR showed that the XIAP mRNA level in the placenta was significantly decreased in women with PE in comparison to normal pregnant women (Fig. 5).

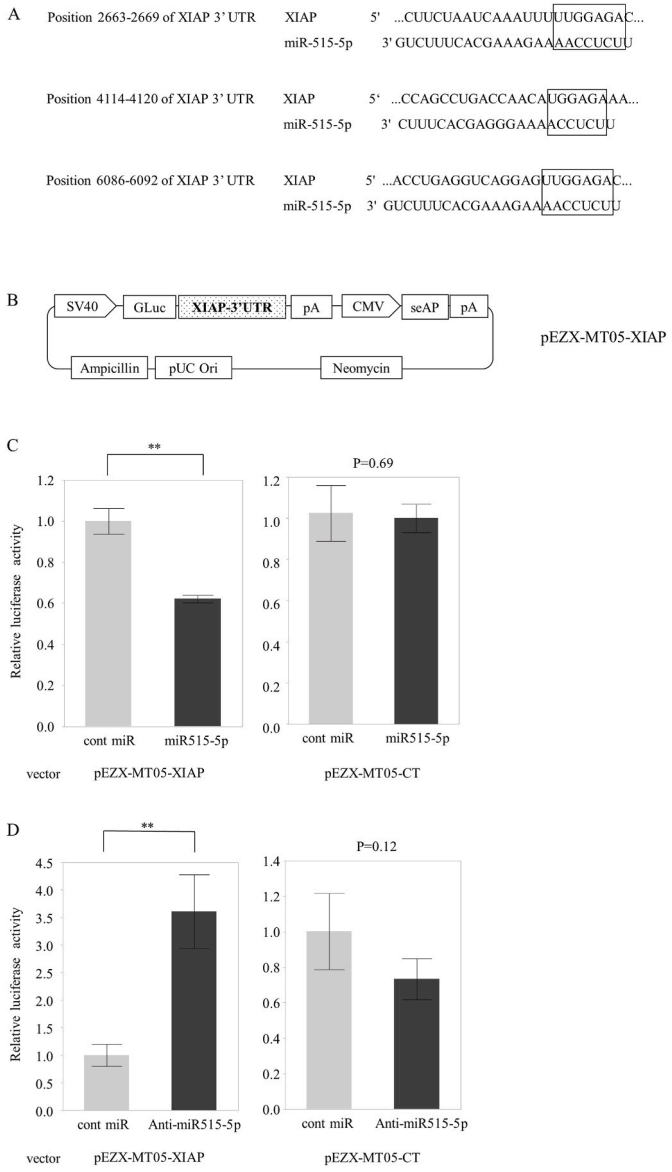


Fig. 3. XIAP is the downstream target of miR-515-5p in HTR-8/SVneo cells. (A) The putative sequences of miR-515-5p and the XIAP 3′ UTR with 3 binding sites. (B) A diagram of the XIAP 3′ UTR-containing reporter construct. (C) The relative Gaussian luciferase-to-seAP signal is shown. HTR-8/SVneo cells were co-transfected with the XIAP 3′ UTR reporter construct (pEZX-MT05-XIAP) or control vector (pEZX-MT05-CT) and with miR-515-5p or cont miR. **$p < 0.01$ (D) HTR-8/SVneo cells were co-transfected with either pEZX-MT05-XIAP or pEZX-MT05-CT and with either inhibitor miR-515-5p (Anti-miR515-5p) or control. **$p < 0.01$. Data are shown as the mean ± SD. Independent experiments were repeated in triplicate.

3.6. Characteristic of epithelial to mesenchymal transition (EMT) was declined in the PE placenta

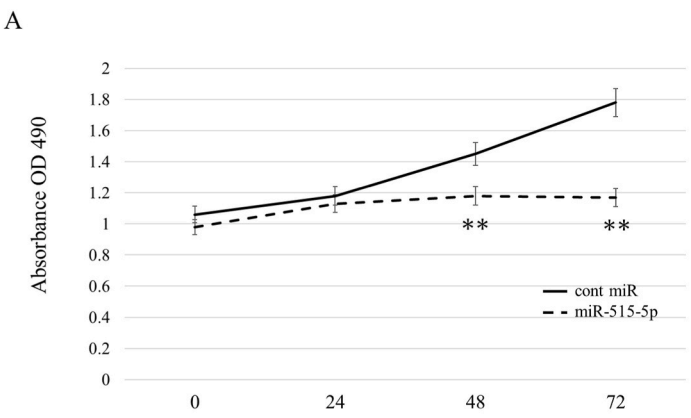
Ki-67 is seen in the nucleus of proliferating cells and is a marker of proliferation. To discuss the proliferation in placental samples, immunohistochemistry of Ki-67 was performed. The levels of Ki-67 tended to be lower in PE placenta than in normal pregnant placenta: however, there was no significant difference (Fig. 6A). We also assessed the invasive markers in placental samples. As shown in Fig. 6B, the loss of the epithelial marker E-cadherin and gain of the mesenchymal marker N-cadherin were observed in normal pregnant placenta but not in PE placenta.

4. Discussion

Preeclampsia is a multisystem disorder during pregnancy that is one of the leading causes of perinatal morbidity and mortality. The molecular mechanisms during the pathogenesis of preeclampsia are still not fully understood. Recent studies have shown that PE is associated with the dysregulation of the miRNA expression in the placenta, and may be involved in the pathogenesis of PE (Chen D et al., 2013). In addition, it has been found that miRNAs remain relatively stable and evade the enzymatic cleavage activity of RNases in the blood (Calin and Croce, 2006; Richard V et al., 2022). Therefore, there is a growing interest in serum miRNAs as a diagnostic biomarker or a serial monitoring application of the therapeutic response in diseases.

miR-515-5p was originally identified as a placenta-specific miRNA that is involved in fetal growth restriction in pregnancy (Higashijima A et al., 2013). Zhang et al. recently reported that miR-515-5p levels were significantly increased in the placenta of women with PE (Zhang M et al., 2016). However, the role of miR-515-5p in either fetal growth restriction or PE has not been elucidated. In our small study, we demonstrated that serum miR-515-5p was significantly increased in PE patients in comparison to normal pregnant women. Functionally, the overexpression of miR-515-5p suppressed trophoblast cell proliferation and invasion, as observed in HTR-8/SVneo trophoblast cells. Using luciferase reporter assays, XIAP was identified as a direct target gene of miR-515-5p, and the overexpression of miR-515-5p was found to decrease the level of XIAP in HTR-8/SVneo cells. In addition, in the placental tissue samples, we found that the levels of XIAP and EMT were decreased in PE placentas. Taken together, these data indicated the involvement of increased miR-515-5p and subsequent XIAP reduction in the dysregulated function of trophoblast cells in pregnant women with PE.

XIAP plays an important role in apoptosis pathways through its suppression of the levels of caspase-9, caspase-3 and caspase-7, as well as mitochondria-dependent apoptosis pathways, exerting an anti-apoptotic effect (Yu Y et al., 2018). Several reports have shown that the downregulation of XIAP is related to increased apoptosis in trophoblast cell lines. In human trophoblast 3A cells, the overexpression of miR-23a induced apoptosis by reducing the expression of XIAP (Li L et al., 2017). Hypoxia-induced downregulation of XIAP induced apoptosis of HTR-8/SVneo trophoblast cells through interaction with increased immortalization-upregulated protein-2 (IMUP-2) (Jeon et al., 2013). In addition to the studies elucidating the mechanisms of the anti-apoptotic function of XIAP, recent studies have revealed non-apoptosis-related functions, such as the promotion of cell growth and cell invasion in various cancers (Cao Z et al., 2013; Fang Y et al., 2021). Interestingly, the level of XIAP has been reported to be decreased in the placenta of women with PE (Jeon et al., 2013). PE is characterized by impaired trophoblast invasion and damaged spiral artery remodeling. However, the functions of XIAP in trophoblast cell invasion have not yet been elucidated. The results obtained from our current study showed that the miR-515-5p-mediated suppression of XIAP attenuated the invasion of HTR8/SVneo human trophoblast cells. In addition, our immunostaining data showed reduced EMT in PE placenta compared with normal pregnant placenta, indicating decreased invasion of trophoblast cells in PE. To our knowledge, this is the first report to show that increased serum miR-515-5p in preeclampsia is involved in insufficient trophoblast proliferation and invasion through the suppression of XIAP. In the current study, all PE samples were obtained from late-onset PE that developed after 34 weeks' gestation. Although the deficient invasion of trophoblast cells with subsequent placental hypoxia is considered to be more strongly associated with the pathophysiological features of early-onset PE than late-onset PE, the etiology of PE remains enigmatic and likely involves multiple factors (Maršál, 2017). Indeed, the ratio of soluble fms-like tyrosine kinase 1 (sFlt-1) to placental growth factor (PlGF), which is considered to represent endothelial dysfunction and hypoxia due to inadequate trophoblast invasion and is associated with



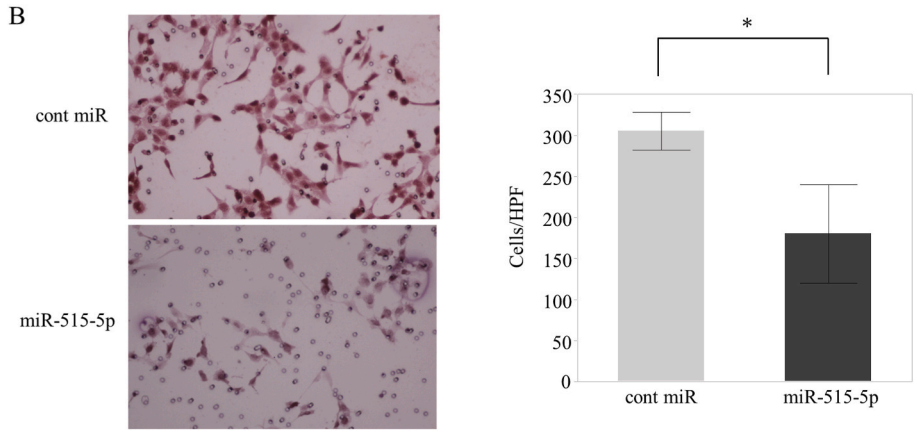


Fig. 4. The overexpression of miR-515-5p inhibits the proliferation and invasion of HTR-8/SVneo cells. (A) HTR-8/SVneo cells were transfected with miR-515-5p or cont miR, then their proliferation was measured by an MTS assay. **p < 0.01 (B) HTR-8/SVneo cells were transfected with miR-515-5p or cont miR, then their invasive ability was measured by a Trans-well assay. Representative images of the invasion assays are shown (left). The quantitative analysis of the invasion potential of HTR-8/SVneo cells is shown (right). *p < 0.05. Data are shown as the mean ± SD. Independent experiments were repeated in triplicate.

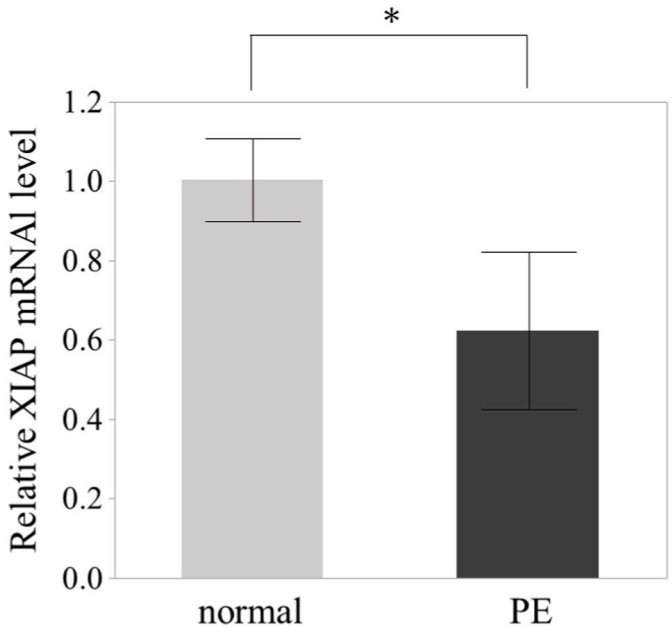


Fig. 5. The XIAP level in the placenta is decreased in PE patients in comparison to normal pregnant women.
RT-qPCR was performed to measure the expression of XIAP in placenta derived from normal pregnant women (n = 3) and PE patients (n = 3). (*p < 0.05).

an increased risk of early-onset PE, was slightly but significantly higher in women with late-onset PE than in normal pregnant women, suggesting that hypoxic factors are also related to placental dysfunction in the case of late-onset preeclampsia (Verlohren S et al., 2010, Schaarschmidt W et al., 2013). Staff et al. also showed that placental malperfusion and dysfunction cause both early- and late-onset PE (Staff AC et al., 2018). Although the placenta samples in the current study may not strictly reflect the phenomena in early pregnancy, the results are expected to help us understand the changes that might be happening in the pathogenesis of PE.

The present study was associated with some limitations, including the fact that there was a difference in the BMI between normal pregnant women and PE patients because of the small sample size. In terms of obese pregnancy and miRNA profiles, a recent report showed that the level of 108 miRNAs, including 5 placenta-specific miRNAs, but not miR-515-5p, were different in fetal umbilical cord blood samples between high-BMI pregnant patients and normal-BMI pregnant patients (Jing J et al., 2020). There have been no reports that a high BMI affects the miR-515-5p or XIAP expression thus far. Subsequent large-scale studies with no significant difference in the BMI between groups are needed. In addition, further *in vivo* experiments are needed in order to clarify the effects of miR-515-5p/XIAP on preeclampsia in a natural setting and to examine whether the serum miRNA level can be successfully used as a biomarker.

In summary, upregulated serum miR-515-5p in patients with PE may be involved in the pathogenesis of PE via the targeting of XIAP. Our data may improve our insights on PE, and will hopefully advance research on treatments and the early diagnosis of PE.

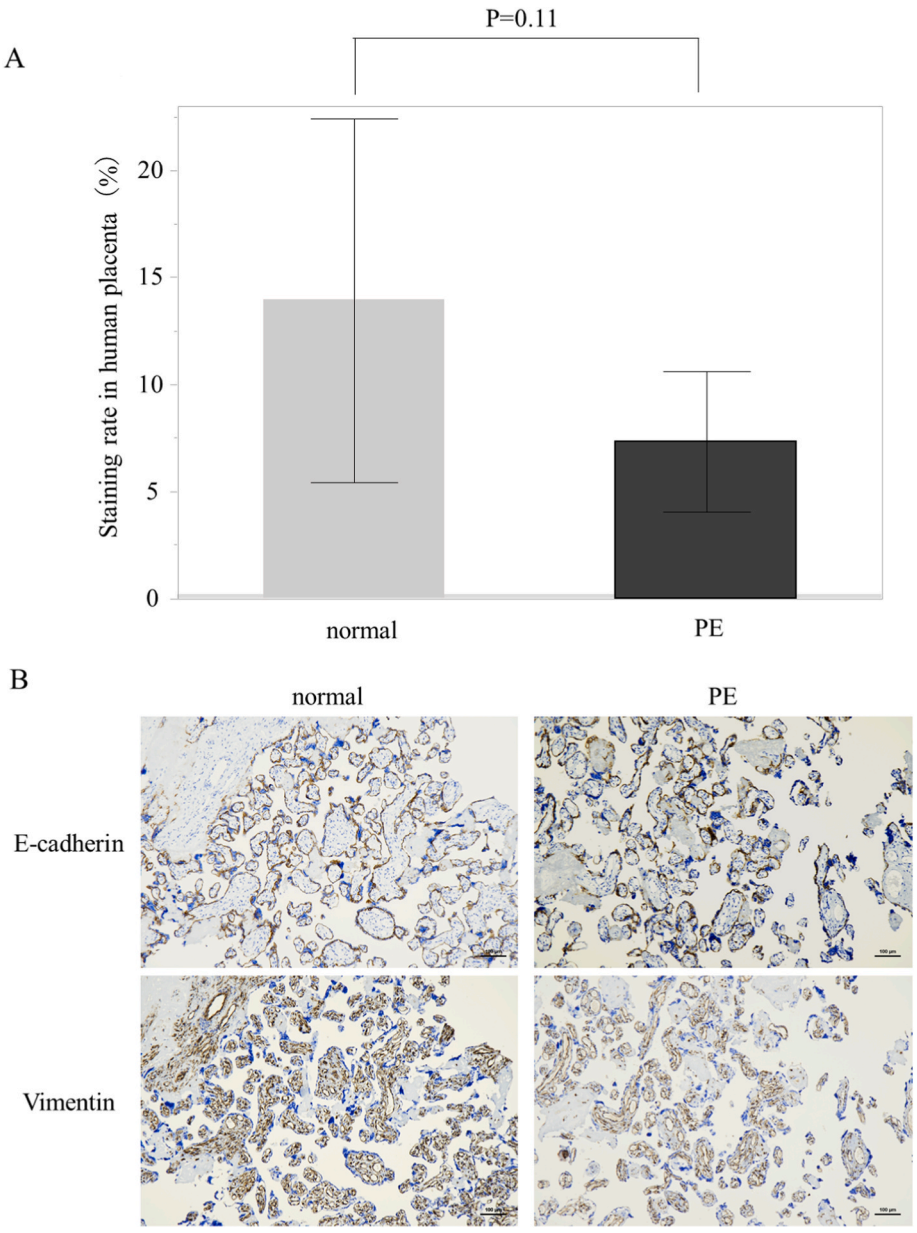


Fig. 6. The proliferation index and EMT expression in the placenta.
(A) Three fields were randomly selected, and the percentage of Ki67-positive nuclei in tumor cells was calculated. The height of the bars indicates the mean values of the percentage of positive immunostaining ± SD.
(B) Immunoreactivity photographs at 100 × magnification. Decreased E-cadherin expression and intense staining of N-cadherin were observed in normal pregnant placenta compared with PE placenta. Scale bar: 100 μm.

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CRediT authorship contribution statement

Misa Nunode: Formal analysis, conceived and designed the experiments and contributed to the analysis and interpretation of all data, were responsible for the collection of samples and the data analysis. **Masami Hayashi:** Formal analysis, conceived and designed the experiments and contributed to the analysis and interpretation of all data, drafted the manuscript. **Yoko Nagayasu:** Formal analysis, were responsible for the collection of samples and the data analysis. **Masami Sawada:** Formal analysis, were responsible for the collection of samples and the data analysis. **Mayumi Nakamura:** Formal analysis, provided technical assistance and contributed to the data analysis. **Takumi Sano:** Formal analysis, were responsible for the collection of samples and the data analysis. **Daisuke Fujita:** Formal analysis, were responsible for the collection of samples and the data analysis. **Masahide Ohmichi:** Formal analysis, conceived and designed the experiments and contributed to the analysis and interpretation of all data. All authors contributed to the revision of the manuscript and approved the final version of the manuscript.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mce.2022.111779.

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