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ORIGINAL PAPER / OBSTETRICS

LncRNA-loc391533 is involved in the progression of preeclampsia through VEGF

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ABSTRACT

Objectives: Preeclampsia (PE) is a leading cause of maternal death worldwide, which is one of the most major pregnancy complications. The effects of vascular endothelial growth factor (VEGF) and lncRNA-loc391533 on PE were evaluated in the present study.

Material and methods: Expression of VEGF in pregnant women with PE was determined using immunohistochemical and enzyme linked immunosorbent assay (ELISA). The effects of lncRNA-loc391533 knockdown and overexpression on VEGF expression was detected using quantitative polymerase chain reaction (qPCR) and western blotting. Loss/gain-of-function assays were performed to evaluate the role of lncRNA-loc391533 on proliferation, cell cycle and migration of trophoblasts HTR-8/SVneo cells.

Results: We found that VEGF and its receptor VEGFR1/2 were low expressed in PE. Knockdown of lncRNA-loc391533 enhanced VEGF expression, while overexpression of lncRNA-loc391533 downregulated VEGF. Moreover, lncRNA-loc391533 was required for proliferation and migration of HTR-8/SVneo cells.

Conclusions: In conclusion, our findings emphasized that lncRNA-loc391533

exhibited a critical role in progression of PE through VEGF, which might as a novel therapeutic target for PE treatment.

Key words: preeclampsia; VEGF; lncRNA-loc391533; proliferation; migration

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INTRODUCTION

Hypertension is a common complication of pregnancy, which exposes women and their fetuses at disproportionate risk of further complications, as well as lifelong sequelae [1]. The International Society for the Study of Hypertension in Pregnancy has determined that preeclampsia (PE) can be diagnosed when hypertension causes albuminuria, placenta or another maternal organ dysfunction [2]. Generally speaking, PE is divided into early-onset PE and late-onset PE using 34 weeks as a threshold [3]. Preeclampsia usually presents with new hypertension and albuminuria in the 3rd trimester of pregnancy, which can rapidly develop into serious complications, including maternal and fetal death [4]. Furthermore, PE possesses a high morbidity and mortality, which contributed to the death of approximately more than 70 000 pregnant women and 500 000 fetuses every year worldwide [1]. Current treatment of PE includes prenatal counseling, perinatal blood pressure control and monitoring, prenatal aspirin treatment for high-risk women, betamethasone, parenteral magnesium sulfate treatment and postpartum follow-up [5]. Timely delivery of the fetus and placenta is still the only definite treatment, which can solve most signs and symptoms. However, PE can persist after treatment and may even redevelop after treatment in some cases. However, due to the current limited clinical treatment, there is no small molecular target for the treatment of PE to delay preterm delivery. Therefore, it is urgent to actively explore innovative treatments for PE.

Long non-coding RNAs (lncRNAs), widely distributed in mammals, which is a class of RNA molecules with more than 200 bp. Its function is similar to that of RNA and has almost no protein coding ability [6]. The number and type of lncRNAs far exceed the protein-coding mRNAs that encode proteins and regulate gene expression at epigenetic, transcriptional and post-transcriptional levels, including genetic imprinting, genome rearrangement, chromatin modification, cell cycle regulation, transcription, splicing, mRNA decay and translation [7]. Emerging evidence showed that lncRNA plays an important role in normal cellular physiological activities, affecting almost every step of the life cycle in a cis-trans or trans-way [8]. In addition, increasing lncRNAs have been found to be involved in the regulation of a variety of diseases, such as cancer [9], Alzheimer's disease [10], Huntington's disease [11] and cardiovascular disease [12]. At the same time, the abnormal transcriptional spectrum of lncRNAs was also observed in ventricular septal defect and acute renal rejection [13]. In recent years, a number of studies demonstrated that the expression of lncRNA in PE placenta is abnormal, suggesting that lncRNA may be related to the pathogenesis of PE [14]. Additionally, overexpression of lncRNA TCL6 promotes PE progression by regulating PTEN [15]. However, the association between lncRNA-loc391533 and the procession of PE has not been fully elucidated.

Objectives

In this study, the lncRNA-loc391533 was recognized by lncRNA chip expression profile analysis and quantitative polymerase chain reaction (qPCR) verification between PE patients and health volunteers. How lncRNA-loc391533 affects the proliferation and migration of HTR-8/SVneo cells was further evaluated. In general, the effects of lncRNA-loc391533 on trophoblasts and thus PE HTR-8/SVneo were evaluated in the present study.

MATERIAL AND METHODS

Samples collection and immunohistochemical analysis

This study was approved by the Research Ethics Committee of Second Affiliated Hospital of Nanchang University, and written informed consent was obtained from each participant. The placental tissue was collected from patients with PE (n = 30) and normal control group (n = 30). The expression of vascular endothelial growth factor (VEGF) in placenta was analyzed by immunohistochemistry. Briefly, the tissues were soaked in xylene and alcohol for dewaxing and rehydration in turn. After that, they were boiled in sodium citrate buffer (pH = 6.0) for antigen repair and incubated with 5% animal serum in PBST at room temperature for 30 min. The tissue was incubated with primary antibody (anti-VEGF, Cat. No. ab150375; anti-VEGFR1, Cat. No. ab32152; anti-VEGFR2, Cat. No. ab233693, Abcam, USA) for 2 h at room temperature and then overnight with secondary antibody IgG (Cat. No. ab6721, Abcam, USA,) at 4℃. The color was developed with diaminobenzene for 10 min and then redyed with hematoxylin. Immunohistochemical scores were determined by staining percentage and staining intensity as previously described by Haonon et al. [16]. The tissues were classified into negative (–) and positive (+, ++, +++) based on the sum of the staining intensity and staining extent scores.

Bioinformatics analysis

Total RNA was extracted from 11 placental samples, including 6 cases of severe PE and 5 normal controls. Subsequently, these samples were analyzed by LncRNA chip expression profiling (Human LncRNA Array v2.0, Arraystar). The data was analyzed by Agilent Feature Extraction software (version 11.0.1.1, Agilent Technologies). The chip data were stored in the NCBI gene expression synthesis GEO (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE50783).

Cell culture

Human chorionic trophoblast cells HTR-8/SVneo (Beina, Cat. #BNCC353416) were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco; Thermo

Fisher Scientific, Inc.), 100 U/mL penicillin, and 100 μg/mL streptomycin, which were placed in a cell incubator at 37℃ and 5% CO_2.

Construction of lncRNA-loc391533 RNA interference lentivirus vector and cell transfection

The short hairpin (sh) RNAs specifically targeting lncRNA-loc391533 (shloc391533) and amplified sequence of loc391533 [overexpression (OE)] were designed and produced. Subsequently, the sequences (shloc391533 and OE) were directly inserted into the lentivirus vector BR-V108 (Cell and Molecular Biology Research Center, Shanghai, China). HTR-8/SVneo cells were cultured in 6-well plates (2 × 105 cells/well; Corning, Inc.) at 37℃ for 24 h. Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfected HTR-8/SVneo cells (5×10^5 cells/mL) at a multiplicity of infection of 10 with the second-generation lentivirus BR-V108 (1×10^7 TU/mL) for 40 min at 37℃. Seventy-two hours later, the successfully infected cells were screened by observing the expression of green fluorescent protein under a fluorescence microscope (200× magnification, OLYMPUS). The experiment was divided into follow groups: shCtrl vs shloc391533; NC vs OE. The primer sequence is as follows: shloc391533-1: 5’-TCCCACAGCAATACTCCCTAA-3’; shloc391533-2: 5’-CCTGAAACTTGGAGCTCCCAA-3’; shloc391533-3: 5’-TGCCCTTGCCTTTGCTGATGT-3’; scrambled short interfering (si) RNA (shCtrl): 5’-CCGGAGGGATTGACTTAGAGCAAATCTCGAGATTTGCTCTAAGTCAATCCCTTTTTTG-3’).

qPCR

The total RNA of HTR-8/SVneo was extracted according to the Trizol operation manual of sigma Company. The concentration and quality of the extracted RNA were determined by Nanodrop 100 spectrophotometer. The cDNA was obtained by reverse

transcription of RNA via Hiscript QRT Supermix (+gDNA wiper) (Vazyme, Nanjing). Then, qPCR was carried out and relative mRNA expression was quantified with cycle threshold (Ct) values and normalized by using the $2^{-\Delta\Delta Cq}$ method [17]. The mRNA expression of lncRNA-loc391533 was detected by qPCR. The primer as follows, loc391533: 5’-ACTGGGGTCAGATCAGCAAAC-3’, 5’-ACGCTCCATGTGTGATGCC; FLT1P1: 5’-CTCTGGGAGTTGTAGTCGGC-3’, 5’-GGCTCCCCTGGAAAGGTAAAA-3’; GAPDH (The internal reference): 5’-TGACTTCAACAGCGACACCCA-3’, 5’-CACCCTGTTGCTGTAGCCAAA-3’.

Enzyme linked immunosorbent assay (ELISA)

We conducted the experiment according to the procedures provided in the literature [18]. ELISA was used to detect the expression of VEGF in the serum of normal control group and pregnant women with PE.

Western blotting analysis

HTR-8/SVneo cells were lysed with ice-cold RIPA lysis buffer (Roche, Alameda, CA, USA), and total proteins were subsequently extracted. A BCA Protein Assay Kit (Beyotime, Jiangsu, China) was used to detect total protein concentration. Equal amounts of proteins (20 μg) in each lane were separated by 10% SDS-PAGE gel and transferred to a poly (vinylidene fluoride) (PVDF) membrane (Millipore, Danvers, MA, USA). Primary antibody against VEGF (1:1000; Cat. No. ab150375; Abcam), FLT-1 (1:1000; Cat. No. ab32152; Abcam) and GAPDH (1:3000; Cat. No. AP0063; Bioworld) were added for incubation overnight at 4℃. The following day, the PVDF membrane was incubated with the corresponding secondary antibodies: HRP-conjugated goat anti-rabbit IgG (1:3000; Cat. No. A0208; Beyotime) at room temperature for 2 h. Each membrane was visualized using the ECL-Plus™ Western blotting system (GE Healthcare Life Sciences), and proteins were detected with an X-ray imaging analyzer (Kodak). Densitometric analysis was performed using ImageJ (version 1.8.0; National Institutes of Health).

CCK8 assay

After the number of HTR-8/SVneo cells in the cell suspension was counted, the cells were inoculated into the 96-well plate. After inoculation, the cells were cultured for 2–4 h to make the cells adhere to the wall, the CCK-8 reagent was added to determine the OD450 nm value. If the OD450 nm value was not determined for the time being, 10 μL 0.1 M HCL solution or 1% w/v SDS solution can be added to each well, and the culture plate can be shielded from light and stored at room temperature. After that, the standard curve was made with the number of cells as abscissa (X axis), OD450 nm value as ordinate (Y axis).

Cell cycle measured by flow cytometry

HTR-8/SVneo cells precipitates were washed by PBS (pH = 7.2~7.4) and fixed by 70% ethanol for at least 1 h. After that, the cells were centrifuged again to remove the fixed liquid. The cell precipitates were stained with 1.5 mL staining solution [40 × PI mother liquor (2 mg/mL): 100 × RNase mother solution (10 mg/mL): 1 × PBS = 25:10:1000]. Finally, the cell cycle distribution was detected by flow cytometry with a pass rate of 200–350 cells/s.

Transwell assay

The required number of compartments were placed in an empty 24-well plate. The 100 μL serum-free medium was added to the chamber and placed in the incubator for 2 h and then removed. The 600 μL medium containing 30% FBS was added to the lower chamber. The HTR-8/SVneo cells were suspended by low serum culture medium counted. After the cells were diluted in serum-free medium, 100 μL of the cell suspension (containing 100000–200000 cell) was added to each compartment. After that, the chamber was transferred to the lower chamber containing 30% FBS medium for 24 h, the culture medium was removed, and the non-metastatic cells were gently removed by cotton swabs. The 400 μL dye solution was added to the hollow

hole of the 24-well plate and the chamber was soaked for 5 min. After the cells were stained on the lower surface of the membrane, the chamber was soaked in the water and washed several times to dry. Finally, 5 fields of view per well were selected randomly under a fluorescence microscope (200× magnification, OLYMPUS), and images were captured for enumeration of the cells.

Statistical analysis

Statistical analyses were performed with either paired t-test or two-way analysis of variance using Graph Pad Prism 8.0 (Graph Pad Software, Inc., San Diego, CA, USA). All experimental data were presented as mean ± SD (n = 3) and significance was accepted at $p < 0.05$.

RESULTS

Expression of VEGF in pregnant women with PE

We used immunohistochemical to analyze the expression of VEGF, VEGFR1 and VEGFR2 in PE and normal placenta tissue. As shown in the Figure 1A, VEGF, VEGFR1 and VEGFR2 was expressed in the placenta of different pregnant women. Compared with other groups, the expression intensity of VEGF, VEGFR1 and VEGFR2 in PE group was significantly decreased. Moreover, the positive rate of VEGF, VEGFR1 and VEGFR2 in the normal and PE groups was distinguished (Table 1), founding that the positive rate of VEGF, VEGFR1 and VEGFR2 in PE group was significantly lower than that in normal group. Consistently, ELISA results showed that the serum level of VEGF in PE group was significantly lower than that in normal group (Table 2, Figure 1B). Therefore, we concluded that the levels of VEGF and its receptors in the placental tissues of pregnant women were related to PE.

lncRNA-loc391533 regulates the expression of VEGF

We performed miRNA chip analysis on PE and normal samples, identified that lncRNA-loc391533 was the most significant differential expression lncRNAs (Figure 2A). This suggested that the relationship between lncRNA-loc391533 and VEGF and

PE need to be further clarified. Thus, HTR-8/SVneo cells with lncRNA-loc391533 knockdown (shlncRNA-loc391533) and lncRNA-loc391533 OE were constructed to clarify the effects of lncRNA-loc391533 in PE. After transfection with Lipofectmine2000, the results showed that the positive rate of green fluorescent protein in HTR-8/SVneo cells was over 80% (Figure 2B), which demonstrated the successful transfection of lentivirus. In HTR-8/SVneo cells, the knockdown efficiency of lncRNA-loc391533 in shloc391533-3 group was the highest ($p < 0.05$), which was selected for downstream experiments (Figure 2C). Similarly, the successful overexpression of lncRNA-loc391533 in HTR-8/SVneo cells were established. Overexpression group was the cells with overexpression of lncRNA-loc391533, NC was the vector with control scrambled sequence for cell transfection (Figure 2D–E). In addition, previous study reported that VEGF and FLT-1 is required for the development and progression of PE. Aa s consequence, the effect of lncRNA-loc391533 on the expression of VEGF and FLT-1 was explored in this study [19]. We found that VEGF expression was increased in lncRNA-loc391533 knockdown HTR-8/SVneo cells. Similarly, VEGF expression was downregulated in lncRNA-loc391533 overexpressed cells. However, alterations in the expression of lncRNA-loc391533 had no significant effect on the protein expression of FLT-1 (Figure 3A–B). Therefore, the results indicated that lncRNA-loc391533 could regulated the expression of VEGF.

lncRNA-loc391533 is required for proliferation and migration of trophoblast cells

The loss/gain-of-function experiments were conducted to further verify the biological functions of lncRNA-loc391533 in trophoblast cells. As shown in Figure 4A, the absorption curves showed that shloc391533 cells had a significant downward trend compared with shCtrl cells over time ($p < 0.001$), which indicated that knockdown of lncRNA-loc391533 resulted in reduced proliferation of HTR-8/SVneo cells. Simultaneously, overexpression of lncRNA-loc391533 resulted in the promotion effect of cell proliferation ($p < 0.001$). Moreover, the cycle distribution of HTR-

8/SVneo cells was detailed in the column chart (Figure 4B), suggesting that there were relatively more cells in G2 phase in shloc391533 group than that in shCtrl ($p < 0.05$). At the same time, overexpression of lncRNA-loc391533 decreased the percentage of HTR-8/SVneo cells in G2 phase. On the other hand, Transwell results showed that the number of migrated cells in the shloc391533 group was significantly lower than that in the shCtrl group ($p < 0.001$), indicating that the downregulation of lncRNA-loc391533 resulted in a significant inhibition of migration in HTR-8/SVneo cells. In contrast, the overexpression of lncRNA-loc391533 significantly enhanced the migration of HTR-8/SVneo cells ($p < 0.001$) (Figure 4C). In general, knockdown of lncRNA-loc391533 weakened proliferation, arrested the cycle in G2 phase and impaired migration capacity of trophoblast cells. Overexpression of lncRNA-loc391533 promoted the PE-related characteristics of trophoblast cells.

DISCUSSION

Many studies have shown that abnormal trophoblast infiltration, inadequate uterine spiral artery remodeling and increased trophoblast apoptosis are the main pathological features of PE [20]. As a serious complication of pregnancy, the pathogenesis of PE is associated with many factors [21]. Therefore, it is necessary to strengthen the early identification and clinical intervention of high-risk factors of PE in order to improve the treatment strategy. The clinical treatment of PE is disappointingly slow, but recent research offers new hope that effective treatment is just around the corner. For example, the incidence of PE in high-risk women treated with aspirin is lower, which is of great significance for the prevention of PE in high-risk women [22]. Furthermore, the removal of anti-angiogenic protein by plasma separation can significantly prolong the time of pregnancy and alleviate the symptoms of PE, suggesting that this may replace the treatment of preterm delivery [23].

Preeclampsia is primarily the result of an imbalance between pro-angiogenic factors (such as VEGF-A) and anti-angiogenic factors (such as sFLT-1) [24]. Recently, Xiao et al. [25], pointed that VEGF-A regulates sFLT-1 production in

trophoblast cells through FLT-1 and KDR receptors. In fact, sFLT-1 is responsible for most maternal vascular dysfunction and organ damage [26], and elevated level of sFLT-1 in the blood is an indicator of PE [27]. The application of VEGF coated beads is an effective way to capture sFLT-1 and release endogenous placental growth factor, which provides a new perspective for the treatment of PE [19]. In this study, we identified that the levels of VEGF and its receptors in the placental tissues of pregnant women were related to PE. Moreover, the expression of lncRNA-loc391533 could regulate VEGF expression. We speculated that lncRNA might play a regulatory role in PE through the VEGF pathway, the specific mechanism of which still needs to be further explored. In addition, alterations in the expression of lncRNA-loc391533 had no significant effect on the protein expression of FLT-1. The involvement of FLT-1 in PE could depend on other regulatory pathways, which will be the focus of our attention in the future.

We performed miRNA chip analysis on PE and normal samples, recognized that lncRNA-loc391533 was the most significant differential expression lncRNAs. Moreover, we found that lncRNA-loc391533 could regulated the expression of VEGF. Therefore, the biological functions of lncRNA-loc391533 in trophoblast cells were explored. On the other hand, trophoblast cells physiologically invade the uterus during pregnancy, which is similar to the invasive behavior of tumor cells. A number of studies demonstrated that various lncRNAs have effects on the invasion and migration of trophoblast cells. Silencing of lncRNA induced microvascular invasion in hepatocellular carcinoma inhibits cell proliferation, invasion, migration and angiogenesis in different trophoblast cell lines [28]. Moreover, inhibition of endogenous lncRNA can inhibit the proliferation, migration and microtube formation of HTR-8/SVneo cells [29]. Growing evidence clarified that lncRNA SPRY4-IT1, MEG3, loc391533, loc284100, Malat-1 and TUG1 in placenta play important roles in the pathogenesis of PE [30]. In this study, we confirmed that knockdown/overexpression of lncRNA-loc391533 can inhibit/promote the proliferation and migration of HTR-8/SVneo cells. Therefore, lncRNA-loc391533 is

required for proliferation and migration of trophoblast cells.

CONCLUSION

In this study, we identified that the levels of VEGF and its receptors in the placental tissues of pregnant women were related to PE. Moreover, lncRNA-loc391533 could regulated the expression of VEGF. In conclusion, lncRNA-loc391533 exhibited a critical role in progression of PE, which might as a novel therapeutic target for PE treatment. However, there are some limitations in this study. The mechanism of lncRNA-loc391533 regulating VEGF has not been revealed, which will be the focus of our later studies.

Author contributions

Xiaoju He designed this program. Kangxiang Xu, Bingqi Wu, Yuqi Xu, Dong Ruan, operated the experiments. Xuan Jin conducted the data collection and analysis. Jun Xiong produced the manuscript which was checked by Xuan Jin and Xiaoju He. All the authors have confirmed the submission of this manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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Table 1. The positive rate of VEGF/R1/R2 expression in placenta of pregnant women in the normal group and the severe preeclampsia group

Group	Number	Expression level	-	+	++	+++	Number of positive	Positive rate

								(%)
Normal	30	VEGF	0	3	6	22	30	100
PE	30		22	4	4	0	8	26.67
Normal	30	VEGFR1	0	2	7	22	30	100
PE	30		21	5	4	0	9	30
Normal	30	VEGFR2	1	1	5	23	29	96.67
PE	30		21	7	2	0	9	30

*p < 0.05; PE — preeclampsia; VEGF — vascular endothelial growth factor

Table 2. Serum VEGF levels of pregnant women in normal control group and preeclampsia group

Group	Number	VEGF (pg/mL)
Normal	30	628.05 ± 121.16
PE	30	354.70 ± 66.52
P	—	< 0.01

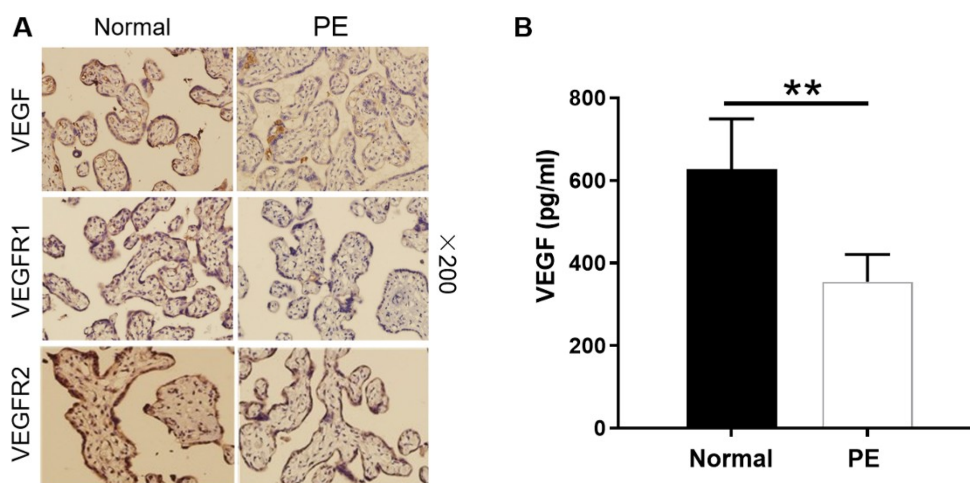

Figure 1. VEGF expression is associated with PE: **A.** The positive rate of VEGF/R1/R2 expression in placenta of pregnant women in the normal group and the PE group was determined using immunohistochemical staining; **B.** The serum level of VEGF in PE group was identified by ELISA. The presented results were representative of experiments repeated at least 3 times. Data was represented as mean ± SD; **p < 0.01; ELISA — enzyme linked immunosorbent assay; PE —

preeclampsia; SD — standard deviation; VEGF — vascular endothelial growth factor

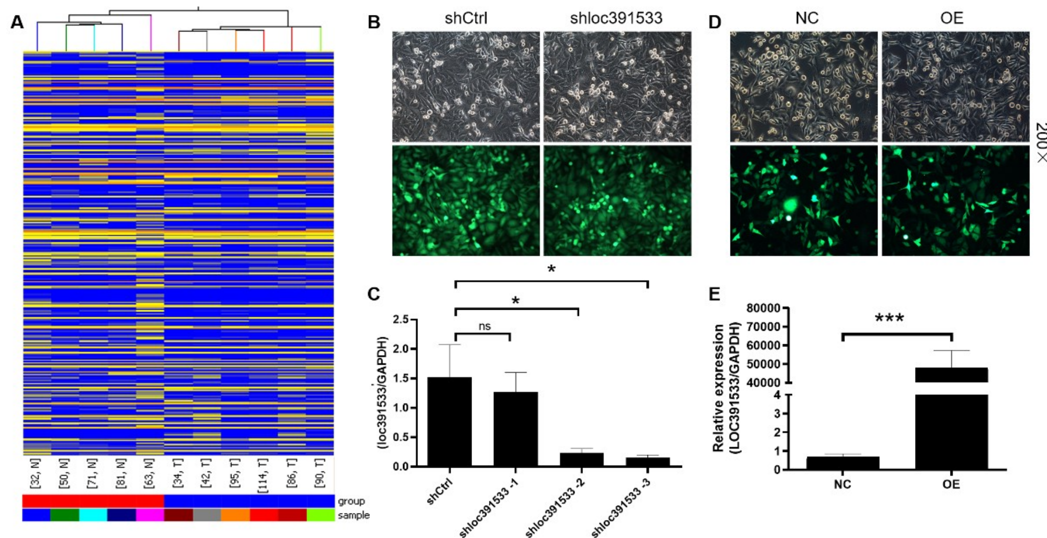

Figure 2. Construction of lncRNA-loc391533 knockdown and overexpression in HTR-8/SVneo cells: **A.** The significant differential expression lncRNAs were identified using miRNA chip analysis on PE and normal samples; **B.** Transfection efficiency of lentivirus shCtrl and shlncRNA-loc391533 in HTR-8/SVneo cells was evaluated by expression of green fluorescent protein 72 h post-infection; **C.** qPCR was used to screen knockdown efficiency of lncRNA-loc391533 in shlncRNA-loc391533-1, shlncRNA-loc391533-2, and shlncRNA-loc391533-3; **D.** Transfection efficiency of lentivirus NC and OE in HTR-8/SVneo cells was evaluated by expression of green fluorescent protein 72 h post-infection; **E.** Expression of lncRNA-loc391533 in NC and OE groups was detected using qPCR. The presented results were representative of experiments repeated at least 3 times. Data was represented as mean ± SD; *$p < 0.05$; ***$p < 0.001$; OE — overexpression; PE — preeclampsia; qPCR — quantitative polymerase chain reaction; SD — standard deviation

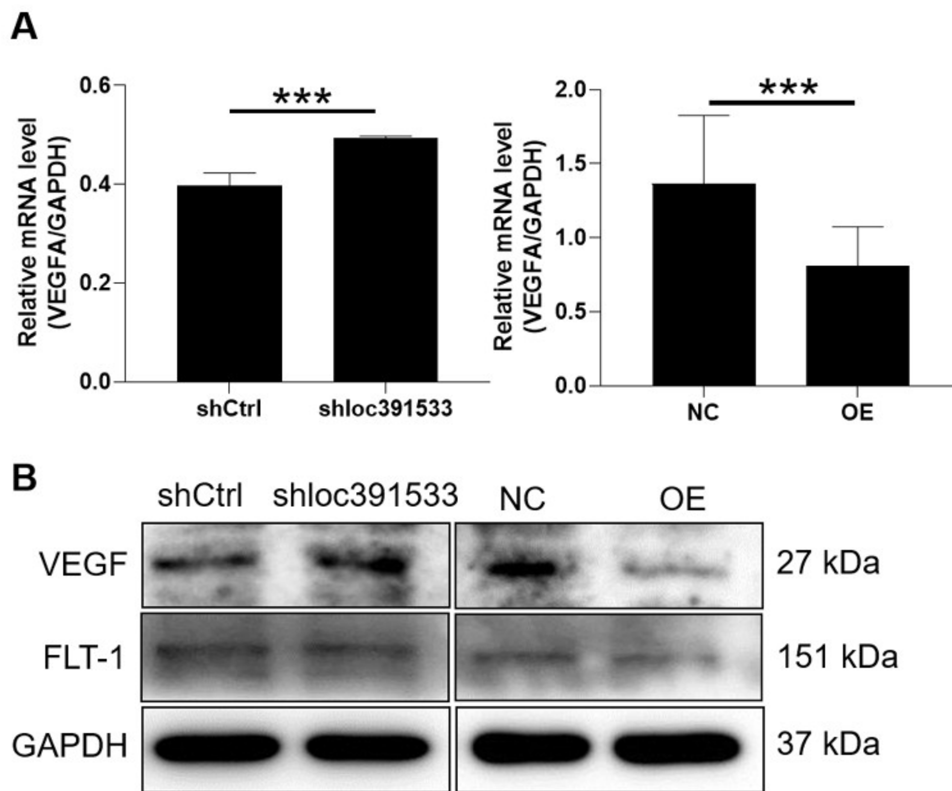

Figure 3. lncRNA-loc391533 regulates the expression of VEGF. The effects of lncRNA-loc391533 knockdown and overexpression on VEGF expression was detected using: **A.** qPCR and **B.** western blotting. The presented results were representative of experiments repeated at least 3 times. Data was represented as mean ± SD; *p < 0.05, ***p < 0.001; qPCR — quantitative polymerase chain reaction; SD — standard deviation; VEGF — vascular endothelial growth factor

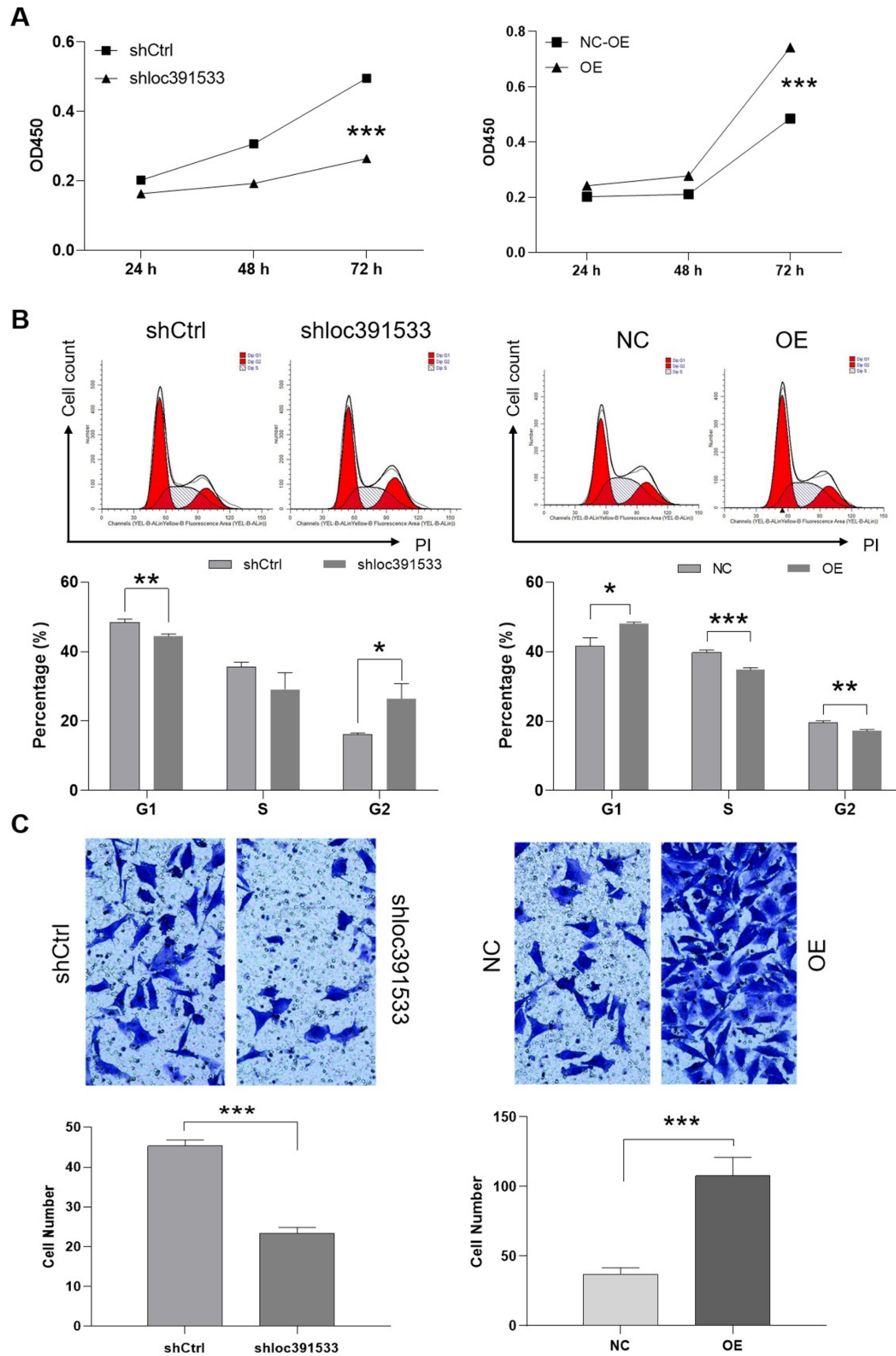

Figure 4. Effects of lncRNA-loc391533 on proliferation, migration and cell cycle of HTR-8/SVneo cells: **A.** CCK8 assay was used to detect proliferation of HTR-8/SVneo cells after lncRNA-loc391533 knockdown and overexpression; **B.** Flow cytometry analysis based on Annexin V-APC staining was utilized to detect cell cycle

distribution of HTR-8/SVneo cells after lncRNA-loc391533 knockdown and overexpression; **C.** The effect of lncRNA-loc391533 knockdown and overexpressed Htr-8 /SVneo cells on migration was detected by Transwell assay. The presented results were representative of experiments repeated at least 3 times. Data was represented as mean ± SD; *p < 0.05; ***p < 0.001