Long Non-Coding RNAs Serve as Diagnostic Biomarkers of Preeclampsia and Modulate Migration and Invasiveness of Trophoblast Cells

Authors' Contribution:
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Background: Long non-coding RNAs (lncRNAs) have emerged as crucial regulators of tumor progression. However, the effects of lncRNAs in preeclampsia are not entirely clear. The aim of this study was to demonstrate the potential of lncRNAs to serve as biomarkers in preeclampsia.

Material/Methods: The RNA expression levels of lncRNAs (NR_027457, BC030099, AF037219, NR_024178, AF085938, G43016, G36948, NR_029420, NR_024015, AK002210, NR_026643, and AL049277) in the serum of patients with preeclampsia and in the serum of normal controls were measured by qRT-PCR. The area under the curve (AUC), the optimal cut-off values, the specificity, and the sensitivity of NR_027457, AF085938, G36948, and AK002210 were analyzed by receiver operating characteristic (ROC) curve analysis. We designed RNA interference species to suppress NR_027457 and G36948 and identified the roles of NR_027457 and G36948 in the functions of a trophoblast cell line (HTR-8/SVneo).

Results: The qRT-PCR results indicated that NR_027457 and AF085938 were significantly up-regulated, whereas G36948 and AK002210 were significantly down-regulated in preeclampsia. We found that NR_027457, AF085938, G36948, and AK002210 had potential diagnostic value for the detection of preeclampsia. Furthermore, the levels of NR_027457, AF085938, G36948, and AK002210 in the serum of patients were significantly different before *vs.* after surgery. The silencing of NR_027457 inhibited the proliferation, migration, and invasion abilities of HTR-8/SVneo cells, while the silencing of G36948 promoted the proliferation, migration, and invasion abilities of HTR-8/SVneo cells.

Conclusions: NR_027457, AF085938, G36948, and AK002210 can serve as potential diagnostic biomarkers in preeclampsia, and NR_027457 and G36948 might be involved in the pathogenesis of preeclampsia.

MeSH Keywords: Cell Migration Assays • Pre-Eclampsia • RNA, Long Noncoding

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Background

Preeclampsia, which is characterized by high blood pressure and proteinuria, is a pregnancy-specific disease that can cause a series of problems to both mother and fetus [1,2]. Without proper and timely prevention, it may progress to a worse stage known as eclampsia, which is accompanied by seizures and other severe impairments, including the development of hemolysis, elevated liver enzymes, and low platelet (HELLP) syndrome, hemorrhagic or ischemic stroke, liver damage and dysfunction, kidney injury, and acute respiratory distress syndrome (ARDS) [3–5]. Preeclampsia usually occurs 20 weeks after the beginning of pregnancy, and it can be subdivided into 2 types: that which occurs before 34 weeks of gestation is termed early onset preeclampsia, and late onset preeclampsia, which occurs from ≥34 weeks to delivery [6,7]. Preeclampsia involves multiple risk factors, such as hypertension in pregnancy, diabetes, autoimmune disorders, and previous preeclampsia [8, 9]. The identification of pregnant women at risk for preeclampsia provides an opportunity for its prevention [10]. Unfortunately, most proposed primary prevention agents, including oral antioxidants, dietary, aspirin, and activity restrictions, lack proven efficacy [11–15]. In addition, preeclampsia is a multi-system disease that is associated with adverse maternal and fetal outcomes; therefore, to avoid morbidity and mortality, we cannot misdiagnose true patients with preeclampsia according to the clinical diagnostic criteria. Research by the International Society for the Study of Hypertension in Pregnancy (ISSHP) has also found that the lack of a consensus is the major reason for the controversy regarding the counselling, management, and documentation of pregnancy outcomes [16]. Therefore, preeclampsia has been defined as both hypertension and proteinuria (defined as ≥300 mg per 24 h or a spot urine protein/creatinine ratio of ≥30 mg/mmol) [16,17]. Many studies have been published on the functions and mechanisms of preeclampsia [18–20]. A greater understanding of the genetic and molecular pathways involved in the pathogenesis of preeclampsia is essential and may provide future targets for therapeutic action.

Reliable biomarkers for prediction and diagnosis would contribute to maternal health, and several promising markers have been suggested, including PlGF, PP13, endothelin-1, plasma-P-selectin, and PAPP-A [21–23]. Although preeclampsia can be diagnosed through the measurement of blood pressure and proteinuria as well as by other biochemical or biophysical markers, the expression of lncRNAs (long non-coding RNAs) and the roles they play in preeclampsia are unclear. LncRNA is defined as a non-protein coding transcript that is longer than 200 nucleotides and that functions as a regulator of gene expression; lncRNAs have vital effects on imprinting control, cell differentiation, immune responses, human diseases, and other biological processes [24,25]. An advantage to targeting lncRNAs over messenger RNAs (mRNAs) is that targeting a single lncRNA has the potential to affect multiple downstream pathways and therefore can amplify the effect of a single target [26]. Targeting multiple pathways may also lead to unwanted adverse effects, so the functions of lncRNAs should be completely characterized before they are used as therapeutic targets. Many different methods are currently being developed to silence lncRNAs in cancer, including siRNAs, antisense transcripts, and aptamers, which have potentially to be adapted for use in the treatment of preeclampsia [24,27].

Thus, we attempted to seek potential and valid lncRNAs to serve as biomarkers for the efficient and precise prediction and diagnosis of preeclampsia. Beyond that, we investigated the impact of alterations in candidate lncRNAs on cell function when their expression deviated from the normal level. The experimental results indicated that NR_027457, AF085938, G36948, and AK002210 may be potential predictive and diagnostic biomarkers for preeclampsia. Moreover, the expression changes in these lncRNAs would cause cell functional disorders.

Material and Methods

Patients and samples

Samples from patients who were diagnosed with preeclampsia were collected between 2011 and 2016 from the Lianyungang Maternal and Children's Hospital; control samples were obtained from normal controls (pregnant women who were completely healthy) and were also provided by Lianyungang Maternal and Children's Hospital. The patient data were fully recorded, and the samples were processed according to standard protocols. The exclusion criteria were as follows: patients with diabetes, chronic nephritis, chronic hypertension, heart diseases, autoimmune diseases, multiple pregnancies, thrombophilic conditions, vaginal delivery, fetal malformations, and HELLP syndrome. The clinical characteristics assessed at enrollment are shown in Table 1. The blood samples were centrifuged for 15 min at 3000×g at 4°C, and then the serum was collected and stored at -80°C. All protocols used in this study were accepted by the patients and were approved by the Ethics Review Board of Lianyungang Maternal and Children's Hospital.

RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA was synthesized using Oligod (T)18 primer, and the synthesis was catalyzed by PrimeScript® RT reagent Kit (Perfect Real Time) (TaKaRa, Japan). The primers used in the qPCR experiments are shown in Table 2. The reaction mixture contained 10 µL of 2×SYBR Premix Ex Taq™ (TaKaRa, Japan),

Table 1. The clinical characteristics assessed at enrollment.

Characteristic	Preeclampsia (n=81)		No Preeclampsia (n=81)		P-value
Age (years)					
≤35	57	(70.37%)	59	(72.84%)	0.43
>35	24	(29.63%)	22	(27.16%)	
BMI at enrollment	26.9±1.48	(22.8–31.7)	24.2±1.12	(21.4–28.6)	<0.001
Blood pressure					
Systolic	113±4.81	(108–119)	110±2.07	(101–114)	<0.001
Diastolic	69±4.74	(62–73)	66±4.43	(61–70)	<0.001
Proteinuria	2.7±0.85	(0.3–6.4)	0.12±0.05	(0–0.3)	<0.001
Gestational age (years)	33.2±2.69	(27–38)	33.3±1.88	(26–38)	0.78
Previous pregnancy	15	(19.75%)	19	(23.46%)	0.28

Table 2. The primer sequences of the related lncRNAs.

Gene	Sequence of primers (5'-3')
NR_027457	F: GTCCTTTGCAGCAACATGGA R: TTCAACCCATGCACCTCTCT
BC030099	F: CAGCTGAGCGGGACATTTAC R: GGTCTGAAAGGATGGAGGCT
AF037219	F: TGAAGCTGTTGAGGGAGGAG R: CCCTCCACCTTCTTTCCCAT
NR_024178	F: ACAGGCTAGGAGAGAAGGGA R: CTTAAGCATCTTCACGGCCC
AF085938	F: GAGCAACACAAGCAGCATCT R: TGAGAGGATATTGTGGCCGA
G43016	F: TCAGTAATGCTCTAGTCCAGGG R: ACAGGTTGCTTGGTCTCAC
G36948	F: AGACCTACCCTCGACCCAG R: TGGGAGTTCTGCTGGATTCA
NR_029420	F: GAGACCTTGGCCAAACACAG R: TTGTCAGCTTTCTTGTGGGC
NR_024015	F: CGCCCATTCTGCTCTTCTTC R: GGAGGTTTCAGAGATGGGCT
AK002210	F: CTACCCCAGCTCGCATCATA R: TGACTTCGATCTGCCCACTT
NR_026643	F: CTGGTACTGGGATTTGGGGT R: TGCTGACCTGGACAAGAGAG
AL049277	F: AGCCTTTCCACTCCCAACTT R: TCTCCCCAGACTTGCCAAAC
GAPDH	F: TATGATGATATCAAGAGGGTAGT R: TGTATCCAAACTCATTGTCATAC

0.4 µL of 50×ROX Reference Dye II, 0.5 µL of 10 µM forward and reverse primers, 2 µL of template cDNA and ddH$_2$O in a final volume of 20 µL. The qPCR reaction was performed with a denaturation step of 10 min at 95°C; followed by 35 cycles at 95°C (10 s), 60°C (40 s), 72°C (30 s), and a final step at 72°C for 10 min. The relative lncRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [28].

Cell culture and siRNA transfection

The human immortalized first trimester trophoblast cell line HTR8/SVneo was used in the Transwell assay as previously depicted [29]. Briefly, HTR8/SVneo cells were cultured in RPMI 1640 medium (Gibco, Thermo Fisher Scientific, USA) with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, USA) and were maintained in a humid atmosphere at 5% CO$_2$ and 37°C. The cells were incubated in serum-free RPMI 1640 medium for different time intervals for serum starvation. Torin1 stimulation was performed by incubating the cells in fresh medium containing different concentrations of Torin1 for 24 h. Transfection of small interfering RNA (siRNA) was performed using Lipofectamine 2000 (Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

Cell proliferation assay

An MTT assay was performed to evaluate the effects of si-lncRNA as previously reported [30]. Briefly, the cells were harvested with trypsin and seeded at a concentration of 2×10^5 cells/well in a 24-well plate. Then, 20 µl of MTT reagent was added to each well, and the cells were incubated at 37°C for 4 h, followed by the addition of 150 µl dimethylsulfoxide (DMSO). The absorbance was determined sequentially at 48 h using a microplate reader at 490 nm (Sunrise™; Tecan Group Ltd., Männedorf, Switzerland).

Cell migration and invasion assay

A Transwell assay was performed to examine the invasion and migration abilities of cells transfected with si-NR_027457, si-AF085938, si-G36948, or si-AK002210. For the invasion assay, 2×10^5 cells were suspended in 200 µl medium and then plated into the upper chamber of the Transwell plate (8 µm pore size; Corning Inc.; NY, USA), which was coated with Matrigel. Then, 0.5 ml medium supplemented with 10% FBS was added to the lower chamber. After incubation at 37°C for 24 h, a portion of cells remained on the surface of the upper membrane; these cells were removed with a cotton swab, and the cells on the surface of the reverse membrane were stained with crystal violet. The stained cells were examined by confocal microscopy, and the numbers of cells were calculated in at least 5 random images, including the upper, lower, left, right, and middle areas of each image. The general manipulation steps of the migration assay were the same as those described above but without Matrigel.

Statistical analysis

All statistical analysis was performed with SPSS version 19.0 (SPSS Inc, Chicago, IL, USA). The t test was used to detect variations in the data between patients and normal controls. Receiver operating characteristic (ROC) curves were generated to estimate the utility of each parameter as a tool for predicting preeclampsia, and the area under the ROC curves and the related parameters were also calculated in order to analyze the diagnostic points. The log-rank test was used to analyze survival, and the Kaplan-Meier method was used to plot the survival curves. In all analyses, p-value<0.05 was considered statistically significant.

Results

The expression levels of 12 lncRNAs in the serum of patients with preeclampsia

This study first determined the genome-wide lncRNA expression patterns in placentas derived from patients with preeclampsia using a Human LncRNA Microarray [27]. The results showed that clusters of lncRNAs were aberrantly expressed in placentas from patients with preeclampsia. Simultaneously, this study also demonstrated that LOC391533, LOC284100, and CEACAMP8 might contribute to the underlying mechanism of preeclampsia. In our study, we randomly selected relatively distinct lncRNAs, such as NR_027457, BC030099, AF037219, NR_024178, AF085938, G43016, G36948, NR_029420, NR_024015, AK002210, NR_026643, and AL049277. In this research, serum samples from all 81 patients with preeclampsia were obtained, and 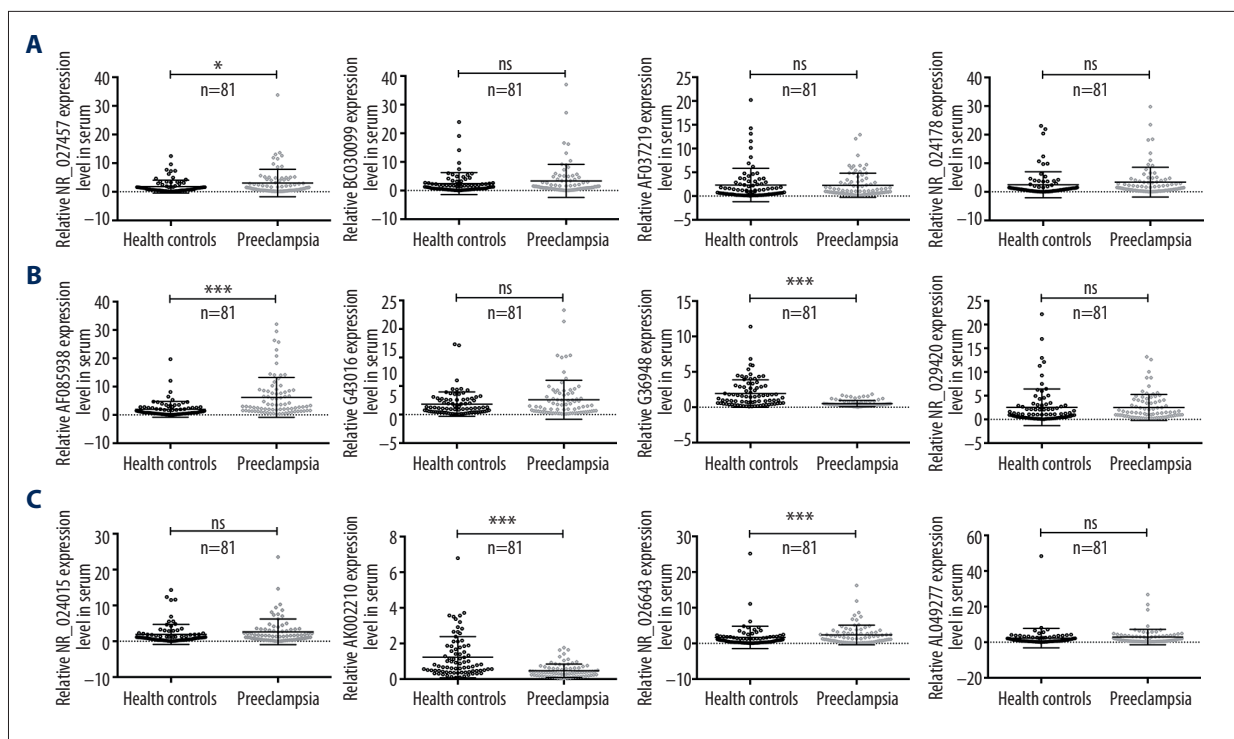the expression levels of 12 various lncRNAs were detected by qRT-PCR according to the method described in a previous study. Compared with the normal controls, 4 lncRNAs were found to be differentially expressed in the serum samples of patients with preeclampsia; these lncRNAs were NR_027457, AF085938, G36948, and AK002210. The qRT-PCR results indicated that NR_027457 and AF085938 were significantly up-regulated, while G36948 and AK002210 were significantly down-regulated, and no significant difference was observed in the remainder of the lncRNAs that were tested (Figure 1A–1C).

The correlation between the expression levels of 4 lncRNAs and preeclampsia

The differential expression of the lncRNAs NR_027457, AF085938, G36948, and AK002210 was verified by qRT-PCR, suggesting that they may have potential value as diagnostic markers of preeclampsia; moreover, the results clearly confirmed this via the ROC curves and the area under the ROC curves (AUC) of NR_027457, AF085938, G36948, and AK002210 (Figure 2A). Among these, the AUCs of AF085938 (AUC=0.7673, P<0.001), G36948 (AUC=0.7956, P<0.001), and AK002210 (AUC=0.7575, P<0.001) were larger than 0.75, which indicated that the performance of these 3 lncRNAs can be considered "fair" in the prediction of preeclampsia (Figure 2A). However, the results of NR_027457 (AUC=0.5633, P=0.164) were not as good as those for the previously mentioned 3 lncRNAs (Figure 2A). In addition, the Kaplan-Meier analysis showed that patients with higher expression of NR_027457 and AF085938 would be more likely to experience preeclampsia at an earlier time than patients who expressed lower levels; patients with lower expression of G36948 and AK002210 would also be more likely to experience preeclampsia at an earlier time than patients with higher levels. In summary, patients with high expression levels of NR_027457 and AF085938 are more likely to relapse than patients with low expression levels of NR_027457 and AF085938; patients with low expression levels of G36948 and AK002210 are more likely to relapse than those patients with high expression levels of G36948 and AK002210. Therefore, NR_027457, AF085938, G36948 and AK002210 indicate a good prognosis and have the potential to be valid markers for the prediction of preeclampsia (Figure 2B).

The effects of lncRNA NR_027457, AF085938, G36948, and AK002210 knockdown on cell proliferation

The patient-derived serum samples were tested, and the results showed that the lncRNAs NR_027457, AF085938, G36948, and AK002210 had high potential to be valid markers for the prediction of preeclampsia. For further research and for the validation of the availability of selecting these lncRNAs as markers, *in vitro* experiments were conducted. SVneo cells were transfected with the synthesized interfering oligonucleotide

Figure 1. The expression levels of 12 different lncRNAs in the serum samples of patients with preeclampsia were examined by qRT-PCR. (**A–C**) In all, 81 serum samples were collected from patients with preeclampsia and from normal controls. The statistical results for the expression levels of lncRNA were demonstrated in healthy controls and in patients with preeclampsia, and each lncRNA is presented in an individual plot. The X axis shows the categories, the Y axis represents the relative expression levels of the lncRNAs ($2^{-\Delta\Delta Ct}$), and ns indicates no significance (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).
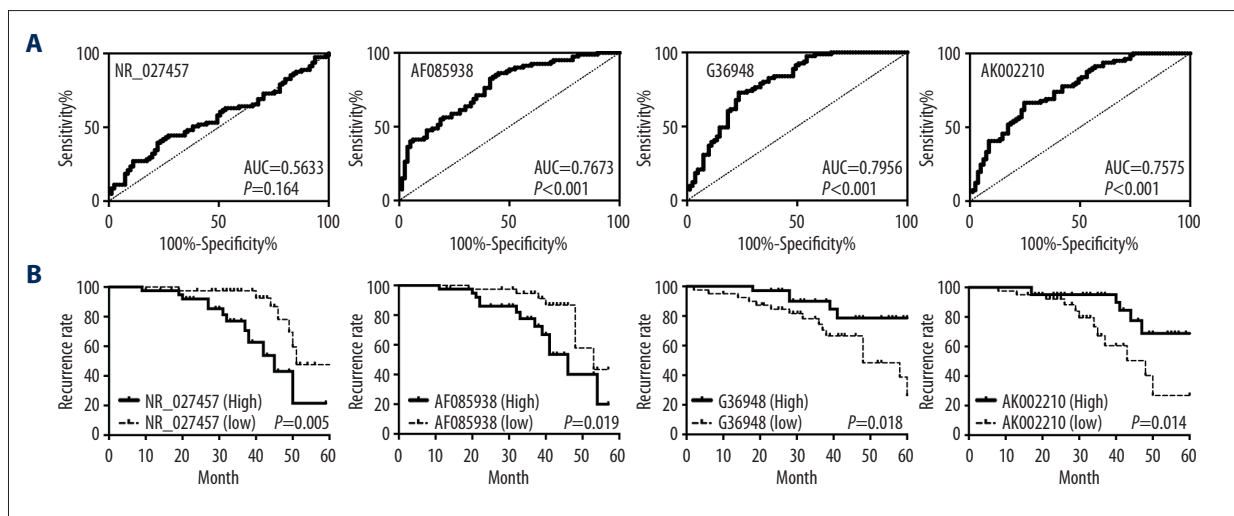
Figure 2. The good prognostic effects of NR_027457, AF085938, G36948, and AK002210 in patients with preeclampsia. (**A**) The ROC curve for the 4 lncRNAs in their ability to predict preeclampsia. (**B**) Kaplan-Meier curve for the same lncRNAs in patients with a high or a low expression level of a particular lncRNA.

sequences si-NR_027457, si-AF085938, si-G36948, and si-AK002210 or with the corresponding control siRNA. As the results showed, the expression of all 4 lncRNAs in SVneo cells transfected with siRNAs was dramatically decreased compared

with the control groups transfected with the negative control (si-NC) (Figure 3A). The results of the MTT assay showed that the proliferation rate of si-NR_027457- and si-AF085938-transfected cells was significantly inhibited, which suggests that the
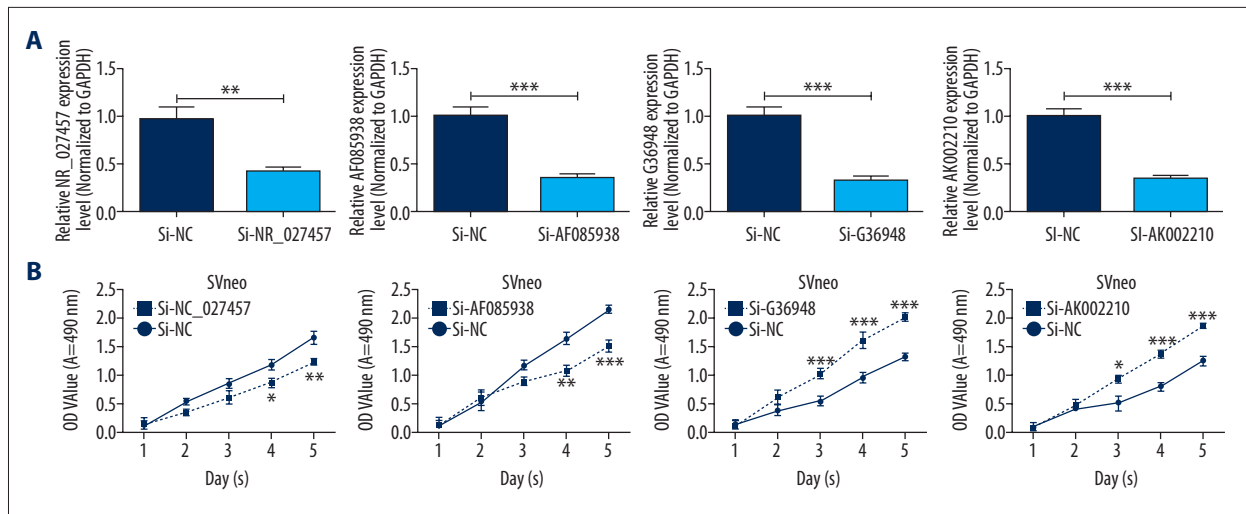
Figure 3. The silencing of NR_027457, AF085938, G36948, and AK002210 affected the proliferation abilities of SVneo cells. SVneo cells were transfected with negative control siRNAs (si-NC), NR_027457 siRNAs (si-NR_027457), AF085938 siRNAs (si-AF085938), G36948 siRNAs (si-G36948), or AK002210 siRNAs (si-AK002210). (**A**) The expression levels of NR_027457, AF085938, G36948 and AK002210 were detected by qRT-PCR assays (** *P*<0.01, *** *P*<0.001). (**B**) The proliferation abilities were measured by MTT assay in treated SVneo cells (* *P*<0.05, ** *P*<0.01, *** *P*<0.001).

silencing of NR_027457 and AF085938 suppressed the proliferation of SVneo cells. In contrast, the proliferation rate of si-G36948- and si-AK002210-transfected cells was significantly promoted, which suggests that the silencing of G36948 and AK002210 promotes the proliferation of SVneo cells (Figure 3B). In summary, NR_027457, AF085938, G36948, and AK002210 play roles in cell proliferation.

### The effects of lncRNA NR_027457, AF085938, G36948, and AK002210 knockdown on cell invasion and migration

To explore the effects of NR_027457, AF085938, G36948, and AK002210 on the invasion and migration abilities of the cells, a Transwell assay was performed using SVneo cells transfected with si-NR_027457, si-AF085938, si-G36948, and si-AK002210 or with the negative control siRNA. The results showed that the knockdown of NR_027457 and AF085938 significantly suppressed the cell migration and invasion abilities *in vitro* (Figure 4A, 4B), while the silencing of G36948 and AK002210 in SVneo cells significantly promoted the cell migration and invasion abilities (Figure 4C, 4D). These results suggest that NR_027457, AF085938, G36948, and AK002210 act as important regulators in the SVneo cell line.

Discussion

Previous studies on the prediction, diagnosis, and treatment of preeclampsia have mainly concentrated on physiological and biochemical indexes [24,31–33]. However, no single biomarker can be regarded as a standard and valid diagnostic marker [34,35]. Some new predictive markers and therapeutics have been explored, and angiogenic factors, including soluble fms-like tyrosine kinase 1, an endogenous vascular endothelial growth factor inhibitor, have been implicated [36–39]. We attempted and managed to solve this problem by other approaches using lncRNAs as new diagnostic targets. LncRNAs have been demonstrated to be reliable biomarkers and are commonly used as biomarkers for the prediction and diagnosis of cancers [24,40,41]. To investigate the roles of lncRNAs in preeclampsia, above all, we explored numerous prior studies of lncRNA and preeclampsia to find some clues. The research by X He et al. provided a series of lncRNAs that were up-regulated or down-regulated in patients who were clinically diagnosed with preeclampsia by microarray [27]. From those lncRNAs, we selected NR_027457, BC030099, AF037219, NR_024178, AF085938, G43016, G36948, NR_029420, NR_024015, AK002210, NR_026643, and AL049277 as our research objects.

To confirm the different expression levels of these lncRNAs, serum samples were collected from patients with preeclampsia and from healthy controls and were tested by qRT-PCR. The results showed that the expression levels of NR_027457, AF085938, G36948, and AK002210 in the serum derived from patients with preeclampsia deviated from their normal expression levels, in accordance with a previous study [27]. Then, the data were further analyzed using the ROC curve and Kaplan-Meier analysis. As we expected, the AUC of these 4 lncRNAs were all larger than 0.5, and the AUC of AF085938, G36948, and AK002210 were even larger than 0.75 according to the ROC curves. Moreover, the significant potential of
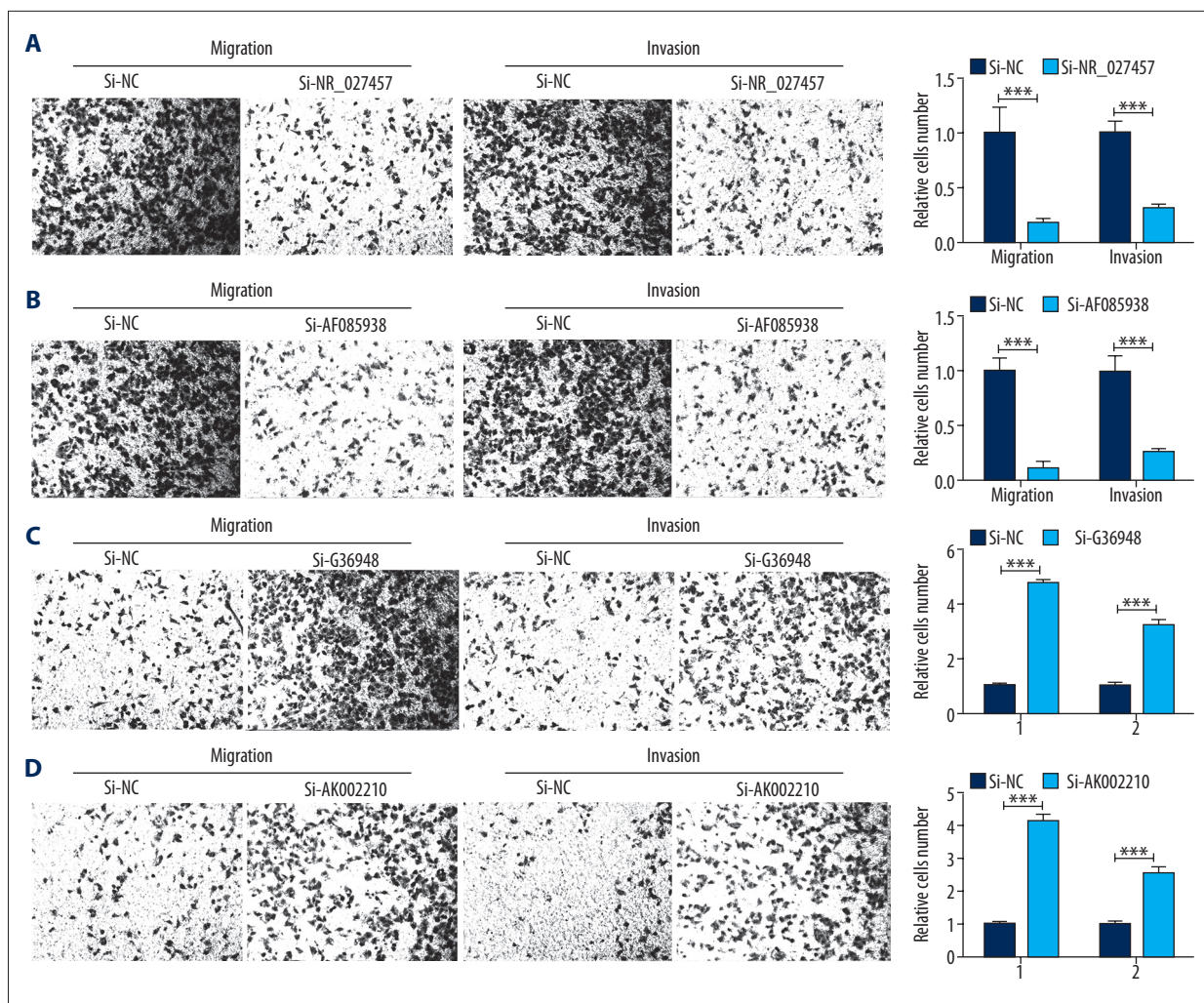
Figure 4. The results of the Transwell assays revealed that the silencing of NR_027457, AF085938, G36948, and AK002210 suppressed the cell migration and invasion capabilities *in vitro*. Images obtained from the Transwell assay illustrate the migratory and invasive abilities of treated cells compared with those of cells treated with si-control; the statistical results are shown alongside the images. (**A**) The result of si-NR_027457 and the corresponding control. (**B**) The result of si-AF085938 and the corresponding control. (**C**) The result of si-G36948 and the corresponding control. (**D**) The result of si-AK002210 and the corresponding control (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

these lncRNAs to be diagnostic markers of preeclampsia was also shown by the Kaplan-Meier analysis. Since most diseases involve the dysfunction of cells, and preeclampsia is no exception, we sought to determine the roles of these 4 lncRNAs in cells that may be affected in preeclamptic patients by performing *in vitro* cell experiments (MTT analysis and Transwell assay). The expression of these 4 lncRNAs was knocked down *in vitro* by transfection of si-lncRNAs. The results showed that in the siRNA-transfected cells, the capacity of the cells to proliferate, migrate, and invade was remarkably affected. A large number of studies have drawn similar conclusions, and the lncRNA SPRY4-IT1 was demonstrated to modulate proliferation, migration, apoptosis, and network formation in the trophoblast cell line HTR-8SV/neo [42]. The lncRNA MEG3 was found

to promote apoptosis and suppress the migration of trophoblast cells [43]. The lncRNA CCAT1 promotes colon cancer cell proliferation and invasion [44]. All these results indicate that NR_027457, AF085938, G36948, and AK002210 can be selected as biomarkers for the diagnosis of preeclampsia. Although the results were promising, some issues still need to be resolved. *In vitro* experiments have demonstrated that these 4 lncRNAs play roles in cell proliferation, migration, and invasion, but no *in vivo* data were obtained; therefore, the mechanism is still unclear. In addition, the number of included patients was too small, and more data are required to validate the conclusion of this study. Still, this research may provide a new perspective and a useful reference in the diagnosis of preeclampsia.

Conclusions

We demonstrated that NR_027457, AF085938, G36948, and AK002210 may serve as potential diagnostic biomarkers for preeclampsia. However, further studies are needed to validate this observation on the prognostic and therapeutic effects of NR_027457, AF085938, G36948, and AK002210 in preeclampsia,

and *in vivo* studies are also needed. Moreover, it will be necessary to further explore the functions and mechanisms of NR_027457, AF085938, G36948, and AK002210 in preeclampsia.

Conflict of interests

None.

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