

LncRNA MAGI2-AS3 Affects Cell Invasion and Migration of Cervical Squamous Cell Carcinoma (CSCC) via Sponging miRNA-233/EPB41L3 Axis

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Introduction: The incidence of cervical squamous cell carcinoma (CSCC) has expanded in recent years. However, the function of long non-coding RNA (lncRNA) MAGI2-AS3 in the occurrence and progression of CSCC remains unclear. Therefore, the role of lncRNA MAGI2-AS3 in cervical squamous cell carcinoma (CSCC) was investigated in our study.

Methods: We used qRT-PCR analysis to identify the level of MAGI2-AS3 mRNA expression in CSCC clinical samples and cell lines. We investigated cell migration and invasion of CSCC cells transfected with MAGI2-AS3, miR-233 mimic, or EPB41L3 with transwell assays. Bioinformatics analysis and a luciferase reporter assay were employed to predict the interaction between MAGI2-AS3 and miR-233.

Results: We found that MAGI2-AS3 and EPB41L3 were both downregulated in CSCC and the expression of this two was positively correlated. Bioinformatics analysis showed that MAGI2-AS3 might bind to miR-233, which could directly target EPB41L3. In CSCC cells, overexpression of MAGI2-AS3 led to upregulated, while overexpression of miRNA-233 led to downregulated expression of EPB41L3. However, MAGI2-AS3 and miR-233 did not affect the expression of each other. In addition, overexpression of MAGI2-AS3 and EPB41L3 led to inhibited cancer cell invasion and migration, while overexpression of miR-233 played an opposite role and attenuated the effects of overexpressing MAGI2-AS3.

Conclusion: MAGI2-AS3 may sponge miR-233 to upregulate EPB41L3, thereby inhibiting CSCC cell invasion and migration.

Keywords: lncRNA MAGI2-AS3, cervical squamous cell carcinoma, EPB41L3, miR-233

Introduction

As the most common type of cervical cancer, cervical squamous cell carcinoma (CSCC) causes high mortality rate in females.^{1,2} Even worse, young females can be affected by CSCC as well.^{1,2} Infections caused by human papillomavirus (HPV) are the main causes of CSCC.³ In effect, high prevalence of HPV infections is always accompanied by high incidence of CSCC.³ CSCCs are prone to develop tumor metastasis, such as lymph node metastasis and even long-distance metastasis.⁴ As a consequence, most CSCC patients are diagnosed at advanced stages and the survival is poor in most cases.⁵ A better understanding of the pathogenesis of CSCC is critical for the development of therapeutic approaches to treat CSCC.

Erythrocyte membrane protein band 4.1 like 3 (EPB41L3) has tumor suppressive functions in several types of cancer.^{6,7} For example, EPB41L3 suppresses tumor progression by regulating cancer cell behaviors, such as inhibiting tumor cell migration

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and promoting cell apoptosis.^{6,7} However, the mechanism remains poorly understood. EPB41L3 in cancer biology is frequently regulated by non-coding RNAs, such as miRNAs⁸ and long (> 200 nt) non-coding RNAs (lncRNAs).⁹ A recent study reported that miRNA-223 could target EPB41L3 to promote the invasion of gastric cancer cells.⁸ LncRNA MAGI2-AS3 has recently been characterized as a tumor suppressor in several types of cancer.^{10,11} Our preliminary bioinformatics analysis showed that MAGI2-AS3 may interact with miR-233. This study aimed to investigate the interaction between MAGI2-AS3 and miR-233 in CSCC.

Patients and Methods

Patients and Specimens

A total of 60 CSCC patients (females, age range from 34 to 66 years old, and the mean age of 50.2 ± 5.6 years old) were

selected from 121 CSCC patients admitted at the University Of Chinese Academy of Sciences Shenzhen Hospital from August 2016 to April 2019. Inclusion criteria: 1) confirmed by histopathological tests; 2) new diagnosed CSCC cases; 3) willing to donate tissue biopsy; 4) no therapies were initiated. Exclusion criteria: 1) clinical disorders besides CSCC; 2) recurrent cases; 3) history of cancer. All the 60 CSCC were subjected to HPV infection detection and the results showed that all patients were infected by different variants of HPV (HPV positive). The patients were staged according to the standard proposed by AJCC, and 10, 10, 23 and 17 cases were classified to stage I–IV, respectively. All patients received biopsy to collect non-tumor (5 cm around tumor) cervical and CSCC tissues (0.019 to 0.025 g). All tissue specimens were confirmed by histopathological examinations. The whole study was performed in accordance with the Declaration of Helsinki

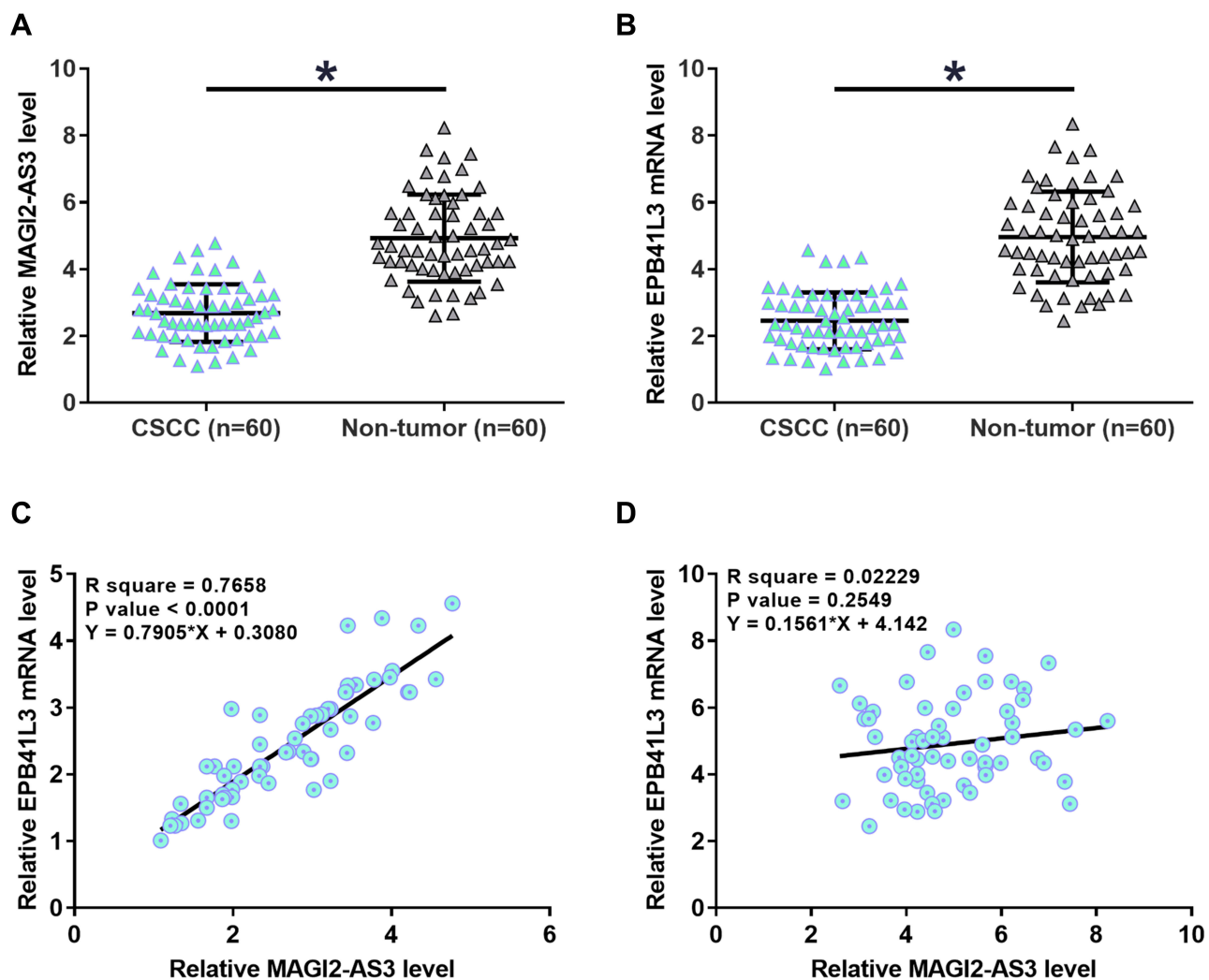


Figure 1 MAGI2-AS3 and EPB41L3 were positively correlated in CSCC. The expression levels of MAGI2-AS3 (**A**) and EPB41L3 (**B**) in two types of tissue were measured by qPCR. Data were compared by performing paired *t* test. Correlations between MAGI2-AS3 and EPB41L3 in CSCC tissues (**C**) and non-tumor tissues (**D**) were analyzed by linear regression (**p* < 0.05).

and was approved by the Ethics Committee of aforementioned hospital. All patients understood the experimental principle and signed the written informed consent.

LncRNA–miRNA Interaction Prediction

The interaction between MAGI2-AS3 and miR-233 was predicted using an online program IntaRNA (<http://rna.informatik.uni-freiburg.de/IntaRNA/Input.jsp>). During prediction, miR-233 sequence was used as the “short sequence” and MAGI2-AS3 sequence was used as the “long sequence”.

Cell Culture

In vitro cell experiments were performed using CSCC cell lines SiHa and HeLa (ATCC, USA). SiHa and HeLa are both HPV-positive cell lines, which are consistent with the status of patients included in this study (all the patients were HPV-positive). Eagle’s Minimum Essential Medium (10% FBS) was used as the cell culture medium. Cells were cultured in a 5% CO₂ incubator at 37 °C.

Lipofectamine 2000-Mediated Transient Transfections

Negative control miRNA and miR-233 mimic were obtained from Sangon (Shanghai, China). MAGI2-AS3 and EPB41L3 expression vectors were constructed using pcDNA3 vector by GenePharma (Shanghai, China). Cells (10⁶) were transfected with 35 nM negative control miRNA (negative control group, NC), or 35 nM miR-233 mimic, or 10 nM empty pcDNA3 vector (NC), or MAGI2-AS3/EPB41L3 expression vector using lipofectamine 2000 (Sangon). Cells were collected at 24 h post-transfections to perform the following experiments.

RNA Extractions

Total RNAs were extracted from tissues (0.015 g grounded in liquid nitrogen) or 10⁶ cells with 1 mL Trizol (Invitrogen, USA) to perform. All operations were performed following the protocol from Invitrogen, except that miRNAs were harvested using 80% ethanol instead of 75% ethanol to precipitate and wash RNA samples.

qPCR

All RNA samples were subjected to DNase I digestion to remove genomic DNAs. The iScript cDNA Synthesis Kit (Bio-Rad, USA) was used to perform reverse transcriptions. All qPCR reactions were prepared using the BlazeTaq™

SYBR Green qPCR Mix (Genecopoeia, Guangzhou, China). The expression levels of MAGI2-AS3 and EPB41L3 mRNA were detected with GAPDH as endogenous control. miRNA reverse transcriptions were performed using qScript microRNA cDNA Synthesis Kit (Quantabio, USA). The miScript SYBR Green PCR Kit (QIAGEN) was used to prepare all qPCR mixtures. The expression level of miR-223 was determined with U6 as endogenous control. Three replicate reactions were set for each experiment. The expression levels of MAGI2-AS3, EPB41L3 mRNA and miR-233 were normalized to endogenous controls using 2^{-ΔΔCT} method.

Luciferase Activity Assay

Before transfection, cells were well cultivated in a 48-well plate, and then were co-transfected with MAGI2-AS3 luciferase vector plasmid and miR-233 mimics or control mimics. The Firefly and Renilla luciferase activity was detected by Luc-Pair™ Duo-Luciferase Assay Kit 2.0 (GeneCopoeia, USA) at 48 h after transfection. The proportion of Firefly luciferase to the Renilla luciferase was counted as the relative luciferase activity.

Table 1 Association with MAGI2-AS3 and the Clinical Pathological Characteristics of CSCC Patients

	Num	MAGI2-AS3		P value
		Low	High	
Status of HPV				
Positive	60	35	25	0.035*
Negative	0	0	0	
Age (years)				
>50	36	21	15	0.412
<50	24	14	20	
HPV types				
HPV-16/18	37	21	16	0.041*
Other types	23	14	9	
Tumor diameter				
≥4 cm	33	25	8	0.031*
<4 cm	27	10	17	
Stage				
I	10	5	5	0.039*
II	10	4	6	
III	23	16	7	
IV	17	10	7	

Notes:The mean expression levels were used as the threshold. For analysis of association between MAGI2-AS3 levels and clinical features, Pearson’s χ^2 tests were used *P < 0.05.

Western Blot

RIPA solution (Sangon) was mixed with CSCC cells (1 mL per 10^6 cells) to extract total proteins. A BCA kit (Sangon) was used to measure the concentrations of protein samples. Protein samples were then incubated in boiled water for 5 min to denature proteins. To separate proteins, 12% SDS-PAGE gel was used to carry out electrophoresis. After electrophoresis, proteins were transferred to PVDF membranes, followed by blocking at 23°C for 1 h in PBS containing 5% non-fat milk. The first blotting was performed using GAPDH (1:1300, ab37168, Abcam) and EPB41L3 (1:1300, ab154071, Abcam) rabbit primary antibodies (4 °C for 12 h). The second blotting was performed using HRP goat anti-rabbit (IgG) antibody (1:1000; ab6721; Abcam, 23 °C for 2 h). RapidStep™ ECL detection reagent (EMD Millipore) was dropped onto the membranes to develop signals. All gray values were normalized using Image J v1.48 software.

In vitro Cell Migration and Invasion Assay

Eagle’s Minimum Essential Medium (1% FBS) was mixed with cells with a ratio of 1 mL per 4×10^4 cells. The upper Transwell chamber was added with 0.1 mL mixture containing 4×10^3 cells, and the lower chamber was filled with mixture of 80% Eagle’s Minimum Essential Medium and 20% FBS. Matrigel (Millipore) was used to coat the membranes before invasion assay. Cells were cultivated at 37 °C for 12 h. After that, 0.5% crystal violet (Sigma-Aldrich) was used to stain the membranes and cells were counted using a light microscope.

Data Analysis

Mean values of the 3 biological replicates of each experiment were calculated and used for all data analyses. Paired *t* test and ANOVA (one-way) in combination with Tukey’s test were used to explore differences between two types of

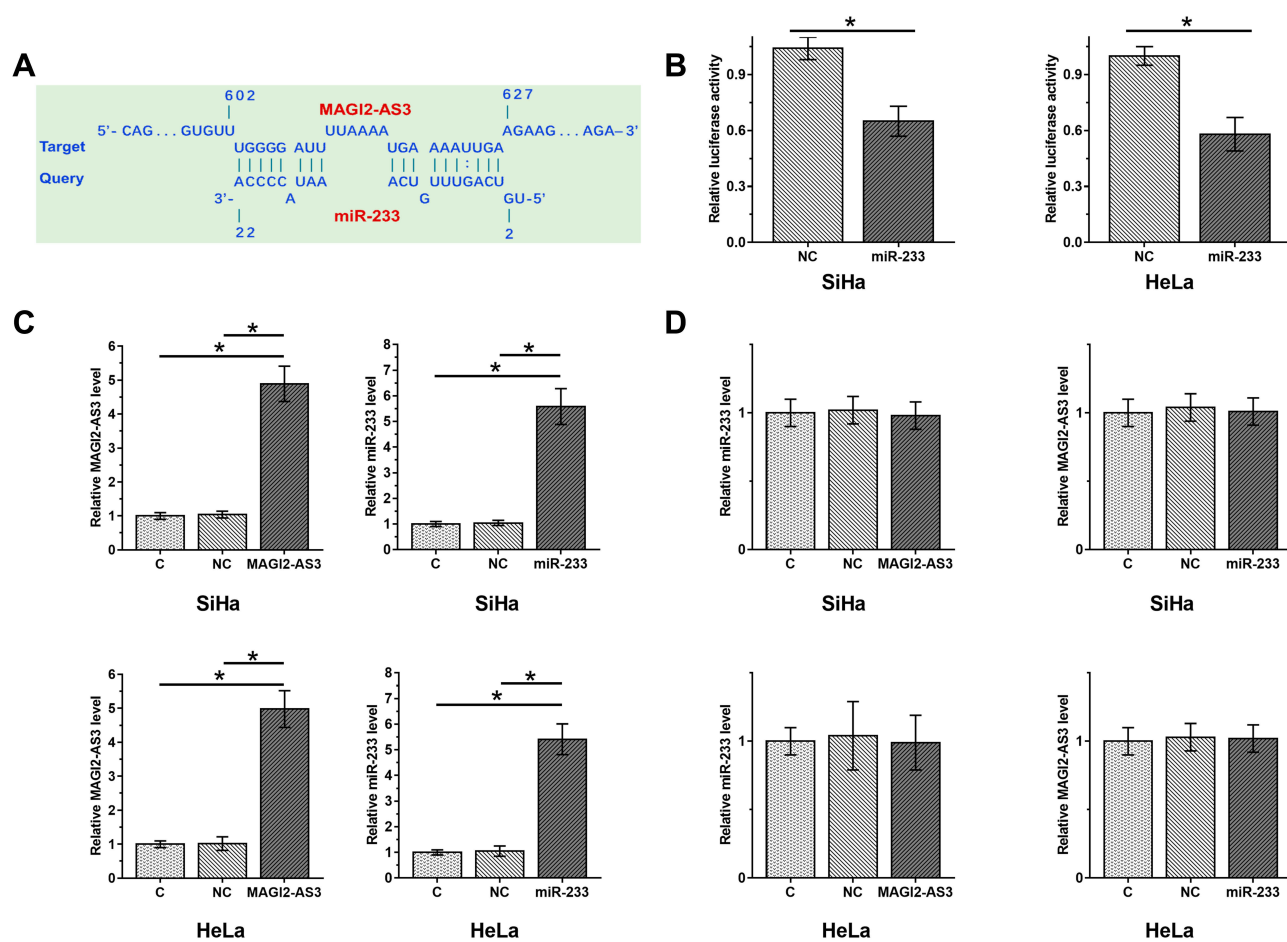


Figure 2 MiR-233 may bind with MAGI2-AS3 but did not regulate its expression. Interaction between miR-233 and MAGI2-AS3 predicted by IntaRNA showed that miR-233 may bind MAGI2-AS3 at position 603 to 626 (A). The luciferase reporter plasmids were co-transfected into CSCC cells with miR-233 mimic or NC. The luciferase activities were measured by dual luciferase assays (B). To further detect their interactions, miR-233 mimic and MAGI2-AS3 expression vector were transfected into SiHa and HeLa cells and their overexpression was confirmed by qPCR at 24 h post-transfections (C). The relationship between MAGI2-AS3 and miR-233 was explored by qPCR (D). Mean values of 3 biological replicates were presented (**p* < 0.05).

tissues or among different cell transfection groups. Correlations were analyzed by linear regression. $P < 0.05$ was considered as statistically significant.

Results

MAGI2-AS3 and EPB41L3 Were Positively Correlated in CSCC

The expression levels of MAGI2-AS3 and EPB41L3 in two types of tissue were measured by qPCR. Data were compared by paired t test. It was observed that the expression of MAGI2-AS3 (Figure 1A) and EPB41L3 (Figure 1B) were both downregulated in CSCC tissues compared to that in non-tumor tissues ($p < 0.05$). Correlations between MAGI2-AS3 and EPB41L3 were analyzed by linear regression. It was observed that the expression of MAGI2-AS3 and EPB41L3 were significantly and positively correlated in CSCC tissues (Figure 1C), but not in non-tumor tissues (Figure 1D). To further investigate the clinicopathological role of MAGI2-AS3 expression in CSCC patients, we

divided 60 patients into low MAGI2-AS3 expression group ($n = 35$) and high MAGI2-AS3 expression group ($n = 25$). As presented in Table 1, the expression levels of MAGI2-AS3 in CSCC tissues were positively correlated with tumor diameter, HPV types and stages. These results indicated that the abnormal expression of MAGI2-AS3 played a crucial role in the progression of CSCC.

MiR-233 May Bind with MAGI2-AS3 but Did Not Regulate Its Expression

MiR-233 can directly target EPB41L3. We predicted the interaction between miR-233 and MAGI2-AS3 by IntaRNA. It can be observed that miR-233 may bind with MAGI2-AS3 at position 603 to 626 (Figure 2A). We constructed luciferase MAGI2-AS3 luciferase vector plasmid and miR-233 mimics or control mimics in both SiHa and HeLa cells. As shown in Figure 2B, the luciferase activity in these cells transfected with MAGI2-AS3 was reduced by miR-233 mimic treatment, compared with

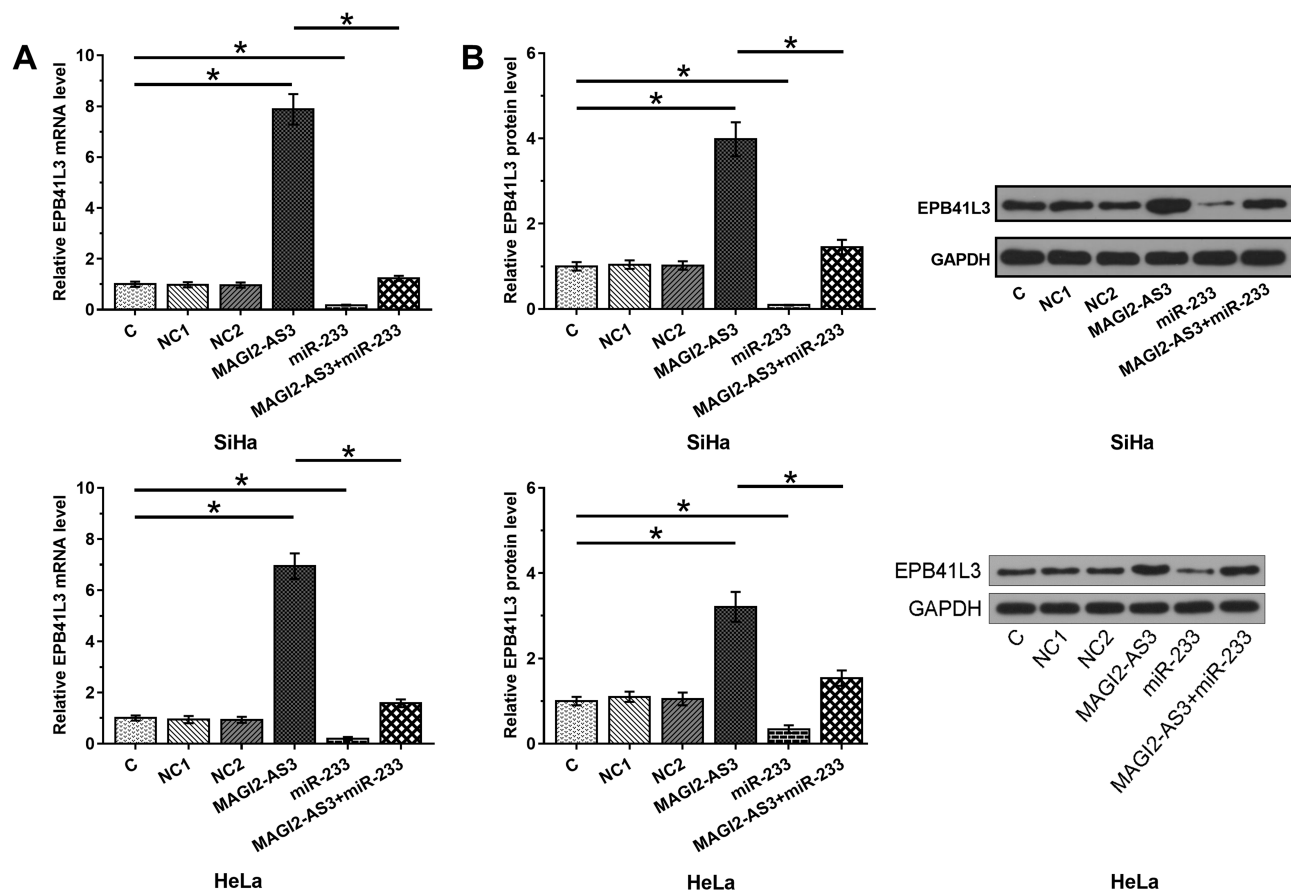


Figure 3 MAGI2-AS3 upregulated EPB41L3 through miR-233. The effects of overexpression of MAGI2-AS3 and miR-233 on the expression of EPB41L3 were analyzed by performing qPCR (A, mRNA) and Western blot (B, protein). Mean values of 3 biological replicates were presented. NC1, empty pcDNA3 transfection; NC2, negative control miRNA transfection ($*p < 0.05$).

that in the control groups (Figure 2B). To further detect their interactions, miR-233 mimic and EPB41L3 expression vector were transfection into SiHa and HeLa cells. Compared to the C and NC group, expression levels of miR-233 and EPB41L3 were both significantly increased at 24 h post-transfections (Figure 2C, $p < 0.05$). However, overexpression of miR-233 did not affect the expression of MAGI2-AS3. Moreover, overexpression of MAGI2-AS3 also did not affect the expression of miR-233 (Figure 2D).

MAGI2-AS3 Upregulated EPB41L3 Through miR-233

The effects of overexpression of MAGI2-AS3 and miR-233 on the expression of EPB41L3 were assessed by Western blot and qPCR. Comparing to C and NC (NC1, empty pcDNA3 transfection; NC2, negative control miRNA transfection) groups, overexpression of MAGI2-AS3 upregulated, while overexpression of miR-233 downregulated expression of EPB41L3 at both mRNA (Figure 3A) and

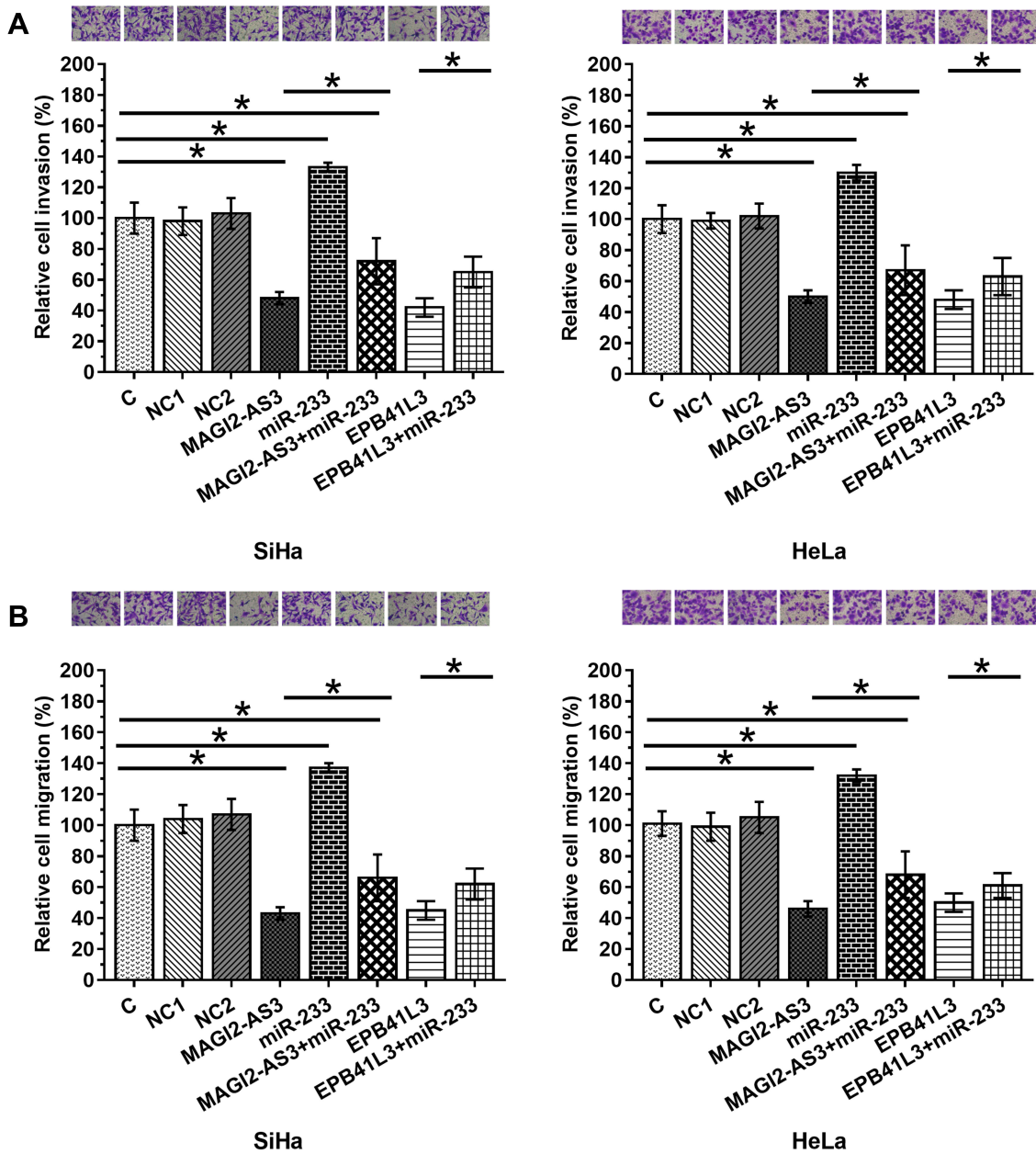


Figure 4 MAGI2-AS3 inhibited SiHa and HeLa cell invasion and migration through EPB41L3 and miR-233. After transfections, cell invasion (A) and migration (B) were analyzed by Transwell assays. Mean values of 3 biological replicates were presented. NC1, empty pcDNA3 transfection; NC2, negative control miRNA transfection ($*p < 0.05$).

protein (Figure 3B) levels ($p < 0.05$). In addition, overexpression of miR-233 reduced the effects of overexpressing MAGI2-AS3 ($p < 0.05$).

MAGI2-AS3 Inhibited SiHa and HeLa Cell Invasion and Migration Through EPB41L3 and miR-233

After transfections, cell invasion and migration were evaluated by transwell assays. Comparing to C and NC (NC1, empty pcDNA3 transfection; NC2, negative control miRNA transfection) groups, overexpression of MAGI2-AS3 and EPB41L3 led to inhibited cancer cell invasion (Figure 4A) and migration (Figure 4B), while overexpression of miR-233 attenuated the effects of overexpressing MAGI2-AS3 or EPB41L3 ($p < 0.05$).

Discussion

Cervical squamous cell carcinoma is the most prevalent gynecologic tumor caused by HPV infection. Even with extensive studies, the mechanisms underlying the tumor initiation and progression remain largely unknown. Patients are confronted with limited clinical application including surgical operations and chemotherapies, which are usually lagged behind, as well as effective biomarkers. This study was the first to show the role of MAGI2-AS3 in CSCC as a bona fide tumor suppressor and also a biomarker for clinical diagnosis. We found that MAGI2-AS3 may sponge miR-233 to upregulate EPB41L3, thereby inhibiting the invasion and migration of CSCC cells. The tumor suppressive function of MAGI2-AS3 has been studied in several types of cancer.^{10–12} In breast cancer, MAGI2-AS3 targets the Fas/FasL signaling pathway to suppress the proliferation of cancer cells.¹⁰ In bladder cancer, MAGI2-AS3 inhibits cancer progression by regulating the expression of CCDC19.¹¹ In hepatocellular carcinoma, MAGI2-AS3 inhibits both cell migration and proliferation by interacting with miR-374b-5p and SMG1.¹² To the best of our knowledge, this study is the first to report the downregulation of MAGI2-AS3 in CSCC. Our results also showed inhibited CSCC cell invasion and migration after the overexpression of MAGI2-AS3. Our data suggest the tumor suppressive role of MAGI2-AS3 in CSCC.

MiRNAs target gene expression by direct cleavage or translation inhibition.^{13,14} For the regulation of lncRNA expression, cleavage might be the only mechanism.¹⁵ Our study showed that miR-233 can bind with MAGI2-AS3. However, overexpression experiments showed that overexpression of MAGI2-AS3 has no significant effects on

the expression of miR-233. Recent studies have revealed that lncRNAs can sponge miRNAs to inhibit their functions.^{16,17} It has been reported that MAGI2-AS3 can sponge miR-15b-5p to participate in bladder cancer.¹¹ In the present study, we observed that MAGI2-AS3 may sponge miR-233 to upregulate EPB41L3. It was reported that miR-233 can directly target tumor suppressive EPB41L3 in gastric cancer.⁸ Our results showed that miR-233 may also target EPB41L3 in CSCC. Therefore, our study identified a novel MAGI2-AS3/miR-233/EPB41L3 pathway in CSCC. However, miR-233 can also target other genes, such as Irak1.¹⁸ Our future studies will investigate the involvement of other targets of miR-233 in CSCC.

In conclusion, MAGI2-AS3 plays a tumor suppressive role in CSCC by sponging miR-233 to upregulate EPB41L3, thereby inhibiting the invasion and migration of CSCC cells.

Disclosure

The authors report no conflicts of interest in this work.

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