

## Original Paper

# MiR-543 Promotes Migration, Invasion and Epithelial-Mesenchymal Transition of Esophageal Cancer Cells by Targeting Phospholipase A2 Group IVA

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**Key Words**

MiR-543 • PLA2G4A • Esophageal squamous cell carcinoma

**Abstract**

**Background/Aims:** The aim of this study was to investigate the roles of miR-543 and phospholipase A2 group IVA (*PLA2G4A*) in cell mobility and the invasiveness cascade in esophageal squamous cell carcinoma (ESCC) and to validate the interactive relationship between miR-543 and *PLA2G4A*. **Methods:** Microarray analysis showed the different expression levels of *PLA2G4A* in two ESCC cell lines (KYSE30 and KYSE180). The expression levels of miR-543 and *PLA2G4A* in ESCC tissues were confirmed by qRT-PCR and Western blotting. The targeted relationship between miR-543 and *PLA2G4A* was studied and verified by a luciferase activity assay. Then, the invasion and metastasis ability of ESCC cell lines transfected with miR-543 mimics, miR-543 inhibitor, or *PLA2G4A* and miR-543 mimics were analyzed separately by Transwell migration and invasion assays. In addition, the roles of miR-543 and *PLA2G4A* in the expression of E-cadherin and vimentin were also investigated. **Results:** *PLA2G4A* up-regulated the level of E-cadherin and down-regulated the level of vimentin, which curbed ESCC cell mobility and invasion. In ESCC cells, the expression of miR-543 was significantly higher, whereas the expression of *PLA2G4A* was markedly lower. MiR-543 facilitated ESCC cell mobility and invasion by repressing *PLA2G4A*. **Conclusions:** MiR-543 enhanced the cell mobility and the invasiveness cascade in ESCC cells via the down-regulation of *PLA2G4A* expression.

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**Introduction**

Esophageal squamous cell carcinoma (ESCC) is recognized as a prevalent malignancy with a high morbidity rate [1]. It is the cancer with the sixth highest mortality rate among males and the ninth highest mortality rate among females worldwide. In addition, the

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total five-year survival rate of ESCC is less than 25 % because of massive invasion into the lymphatic system and distant metastases [2]. The current treatment of ESCC is not effective against invasion and migration. The main reason for the lack of an effective treatment is that the processes of invasion and migration of ESCC cells are complex, with numerous events that are not clearly understood [3, 4]. Our experiment aimed to deepen the understanding of the process regulating invasion and migration in ESCC.

MicroRNAs (miRNAs) are a class of endogenous and non-coding RNAs [5]. They up-regulate or down-regulate their target genes' expression post-transcriptionally by binding to the 3'-untranslated regions (3'-UTRs) of the mRNAs [6]. Several studies have identified by there are many kinds of miRNAs with variable expression in human tumor tissues [7]. As we know from other studies, some miRNAs act as tumor facilitators, whereas others act as inhibitors [8]. Although these two types of miRNAs lead to different results, they both play decisive roles in various aspects of the progression of carcinogenesis, including invasion and migration [3, 9]. Previous studies on miR-543 showed that it exerts an inhibitive influence in several cancers such as breast cancer and endometrial cancer, while in malignant hepatoma, it acts as a tumorigenesis promoter [10, 11]. The miR-543 cluster is in the DLK1-DIO3 region on human chromosome 14.

In ESCC, the expression levels of different miRNAs vary. Oncogenic miRNAs, including miR-92a, miR-31 and miR-21, are generally overexpressed in ESCC, while the expression levels of miRNAs that are often considered tumor suppressors, such as miR-145, miR-143 and miR-133a/b, are generally decreased [3]. It has been verified that miR-543 is markedly overexpressed in ESCC. However, the role of miR-543 in the progression of ESCC has not been systematically studied yet.

*PLA2G4A* is a possible target gene for miR-543. The enzyme that *PLA2G4A* encodes, as one member of the cytosolic phospholipase A2 group IV family, is related to the regulation of hemodynamics, inflammatory responses and other intracellular pathways. The enzyme catalyzes the hydrolysis of membrane phospholipids to release arachidonic acid [12-14]. Previous studies have already proved that *PLA2G4A* expression is significantly associated with invasion depth [15], and down-regulation of *PLA2G4A* might be related to poor patient survival in ESCC. However, the mechanism by which *PLA2G4A* expression is down-regulated has not yet been studied [16].

This study sought to clarify the biological function of miR-543 in ESCC cell mobility and invasiveness, to identify the exact relationship between miR-543 and *PLA2G4A*, and to determine the mechanism by which miR-543 and *PLA2G4A* jointly regulate the progression of ESCC.

## Materials and Methods

### Patients

Esophageal squamous cell carcinoma (ESCC) and adjacent normal pancreatic tissue samples (> 5 cm) were obtained from 47 patients hospitalized at the First Affiliated Hospital of Zhengzhou University. None of the patients received treatment before the radical surgical treatment. All sample tissues were frozen in liquid nitrogen and then stored at -80°C until use. Clinicopathologic data (Table 1) were collected, including age, sex, degree of differentiation, TNM stage and lymphatic metastasis (positive or negative). All data were obtained from the patients' medical records. The study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Zhengzhou University. Informed consent was obtained from each patient.

### Cell cultures

The ESCC cell lines KYSE30 and ECA-109, and the normal esophageal cell line Het-1A were acquired from BeNa Culture Collection (BNCC, Beijing, China). The ESCC cell lines TE-13 and TE-8 were purchased from Shanghai Enzyme Research Biotechnology Co, Ltd. (Shanghai, China). The ESCC cell line KYSE180 was purchased from KeLei Biological Technology Co, Ltd. (Shanghai, China). HEK-293T cells were purchased from the stem cell bank of the Chinese Academy of Sciences. KYSE30 cells were cultured in 90 % F-12 (Invitrogen) and 10 % FBS (Invitrogen). KYSE180, Het-1A and HEK-293T cells were cultured in 90 % high

glucose DMEM (Invitrogen) and 10 % FBS. ECA-109, TE-13 and TE-8 cells were cultured in 90 % RPMI-1640 and 10 % FBS.

#### Microarray analysis

The mRNA expression was analyzed by GSE67510 microarray from the GEO public database, where in two pairs of sublines (30-U/D and 180-U/D) with distinct motility capacities were established from two ESCC cell lines (KYSE30 and KYSE180). In addition, aberrantly expressed mRNA was screened by an R language analysis technique with fold change values more than 2 and *P* less than 0.05 as the screening conditions.

#### Cell transfection

MiR-543 mimics, miR-543 inhibitor, and pcDNA3.1-PLA2G4A were obtained from GenePharma (Shanghai, China). We chose KYSE30 and ECA-109 cell lines for the *in vitro* transfection experiment. Cells in the logarithmic growth phase were trypsin-digested after resuspension by adding the complete medium prior to transfection. The cells were inoculated into 6-well plates ( $1 \times 10^6$  cells) and incubated for 18-24 h in an incubator containing 5 % CO<sub>2</sub>. Cell transfection was carried out with the Lipofectamine 2000 (Life Technologies) system, according to the manufacturer's instructions. Then, all cells were incubated for 48 h in a 5 % CO<sub>2</sub> incubator at 37 °C.

#### qRT-PCR

Total RNA isolation was performed by the TRIzol reagent (Invitrogen). The RNA purity and concentration were qualified. The extracted RNA was reverse transcribed using a first strand synthesis kit (Thermo Fisher Scientific) before qRT-PCR was performed. PCR was carried out following the Maxima SYBR Green qPCR Master Mix (2X) kit (Thermo) protocol. Step One Software v2.1 was applied to analyze the results, and the relative gene expression levels were measured by the 2<sup>-ΔΔCt</sup> method. The RNA primers used were produced by Sangon Biotech (BBI, Shanghai, China) and are displayed in Table 2.

**Table 1.** The relationship between PLA2G4A expression and clinicopathological characteristics in esophageal squamous cell carcinoma. \**P* < 0.05 was recognized as a significant difference

Item	Characteristic	Data (N=47)	PLA2G4A expression		P value
			low	high	
Gender	Male	36	24	12	0.477
	Female	11	6	5	
Age	<60	25	13	12	0.547
	≥60	22	12	10	
Tumor location	Upper	4	2	2	0.416
	Middle	32	24	8	
	Lower	11	7	4	
Tumor grade	G1	2	2	0	0.365
	G2	38	30	8	
	G3	7	4	3	
TNM stage	I-II	21	12	9	0.601
	III	26	15	11	
Lymphatic metastasis	Positive	37	31	6	0.039*
	Negative	10	6	4	
Distant metastasis	Yes	7	4	3	0.048*
	No	40	35	5	

**Table 2.** Primer sequences for qRT-PCR

	Forward (5'-3')	Reverse (5'-3')
PLA2G4A	GCTCGGACAGTGTGATGAAT	ACGATGAATCCAACCTTGCTTGA
miR-543	ATGAGAAGTTGCCCGTGTT	GCGAATGTTTCGTCACAAAT
GAPDH	CGGAGTCAACGGATTTGGTTCGTAT	AGCCTTCTCCATGGTGGTGAAGAC
U6	ATTGGAACGATACAGAGAAGATT	GGAACGCTTCACGAATTTG

### Western Blot

Total protein was dissolved by RIPA (radio immunoprecipitation assay) lysate. Then, a BCA protein assay kit was used to calculate the protein concentration. The proteins were subjected to SDS-PAGE and blotted on a PVDF membrane, followed by incubation in the primary antibodies anti-phospholipase A2 (ab58375, 1:500), anti-E-cadherin (ab15148, 1:500), and anti-vimentin (EPR3776, 1:5000) overnight at 4 °C. After being washed three times by TBS, the membranes were incubated with the HRP-labeled goat anti-rabbit IgG (ab6721, Abcam) antibody for 1.5 h. The chromogenic reaction of the membranes was tested by ECL Plus from Life Technology. GAPDH was used as the control.

### Nude mice allogeneic experiments

5-week-old nude mice were purchased from the Guangdong Medical Laboratory Animal Center (Guangdong, China) and were randomly divided into two groups. All mice were injected with KYSE30 cells with either p-*PLA2G4A* or p-NC. The serum-free cell suspension ( $1 \times 10^7$ /ml) of KYSE30 cells transfected with p-*PLA2G4A* or p-NC was injected subcutaneously into the back of each mouse (0.2 mL). When a tumor had grown to approximately 100-200 mm<sup>3</sup>, the tumor volume was calculated by the following formula:  $1/2 \times L^2 \times W$ , where L is the length (mm), and W is the width (mm) of the tumor. The average tumor volume and tumor weight were measured every 3 days for a total of 3 times. At the termination of the experiment (the 21st day), the mice were sacrificed, and the tumors were excised from each mouse to measure the average tumor volume and weight. The tumor tissues were used to perform qRT-PCR analysis to determine the expression of *PLA2G4A*.

### Luciferase activity assay

The possible microRNA target *PLA2G4A* was identified by TargetScan. The 3'-UTR segment of *PLA2G4A* was cloned into the plasmid pGL3 at the XbaI and FseI loci. Next, 293T cells were placed in 24-well plates, and transfection was carried out using the plasmid containing the *PLA2G4A* 3'-UTR wild-type sequence or the mutant sequence. Meanwhile, miR-543 mimics or control mimics were co-transfected by the Lipofectamine 3000 transfection reagent (Life Technology Corporation, Invitrogen™, USA). The Renilla luciferase vector pRL-SV50 (Promega) was used to equalize the transfection efficiency. The luciferase activity was determined using the dual-luciferase reporter assay system (Promega) 48 h after transfection.

### Transwell invasion assay

Cells were harvested after trypsin digestion, resuspended into serum-free medium and inoculated into the upper chamber of the Transwell system at  $5 \times 10^3$  per well in 200 μL of serum-free medium, and 500 μL of DMEM containing 10 % FBS was added to the lower chamber. After a 24-h incubation, the chambers were rinsed twice in PBS, and the cells were fixed using 4 % paraformaldehyde and subsequently stained with 0.1 % crystal violet. The upper chamber membrane was gently wiped with a cotton swab, and the image was recorded under a microscope.

### Transwell migration assay

After being digested by pancreatic enzymes and washed twice in PBS,  $5 \times 10^3$  cells were cultured in 1 % FBS medium and then placed into the Transwell chamber. After 24 h, the cells were soaked twice in calcium-free PBS, fixed using methyl alcohol and stained with 0.1 % crystal violet. The cells in the upper chamber were gently wiped out with a cotton swab. Finally, we observed the cells under a microscope and took pictures.

### Statistical analysis

All experiments were performed in triplicate and were independently conducted three times. GraphPad Prism 6.0 (Graphpad Software, USA) was used for the statistical analyses. The differences between the two groups were assessed by Student's t-test, while the differences among multiple groups were compared by one-way ANOVA. A p-value < 0.05 was considered significant.

## Results

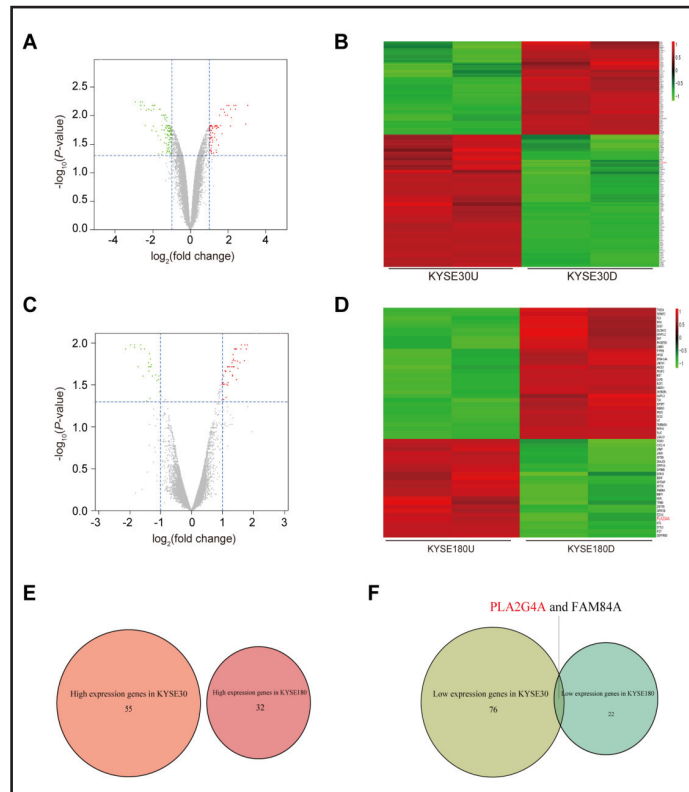
### Identification of mRNA expression patterns in ESCC cell lines by mRNA chip

By using information from the GPL570 platform, eight samples of the GSE67510 chip were analyzed (4 KYSE30 cell lines and 4 KYSE180 cell lines). The mRNA expression levels in

the KYSE30 cell lines (Fig. 1A-1B) and the KYSE180 cell lines (Fig. 1C-1D) were screened by the criteria of a fold-change value greater than 2 and  $P < 0.05$ . We identified no intersecting highly expressed genes of the KYSE30 and KYSE180 cell lines (Fig. 1E). We identified two common genes, *PLA2G4A* and *FAM84A*, in the KYSE30 and KYSE180 cell lines that had low expression levels (Fig. 1F). *PLA2G4A* had been researched in other cancers, but it was less studied in esophageal squamous cell carcinoma (ESCC), so we chose *PLA2G4A* as our research object.

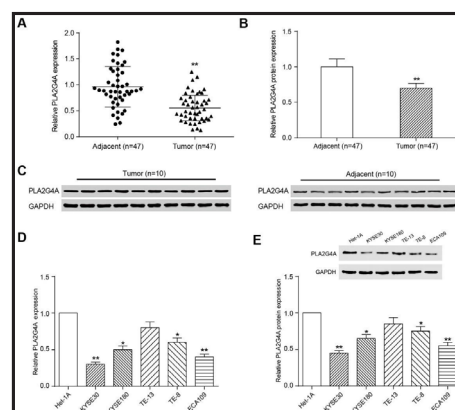
### Expression of *PLA2G4A* in ESCC

The qRT-PCR assay showed that the expression level of *PLA2G4A* in ESCC tissues was considerably lower compared with the expression level in adjacent normal tissues ( $P < 0.05$ , Fig. 2A). The expression level of the *PLA2G4A* protein in ESCC tissues was observably lower than that in adjacent normal tissues, as examined by Western blot assay ( $P < 0.05$ , Fig. 2B-C). The relationships between *PLA2G4A* expression and clinicopathological characteristics in ESCC are shown in Table 1. There was no significant correlation between the expression level of *PLA2G4A* and clinical features of ESCC patients such as age, gender, and tumor location. However, the expression level of *PLA2G4A* was significantly correlated with the lymphatic metastasis and distant metastasis of ESCC patients, which indicated that *PLA2G4A* may affect the



**Fig. 1.** Identification of mRNA expression patterns in ESCC cell line by mRNA chip(A) Volcano plot of mRNAs in KYSE30. (B) Heatmap of mRNAs screened by fold-change value greater than 2 and  $P < 0.05$  in KYSE30. (C) Volcano plot of mRNAs in KYSE180. (D) Heatmap of mRNAs screened by fold-change value greater than 2 and  $P < 0.05$  in KYSE180. (E) Venn diagram of highly expressed genes in both KYSE30 and KYSE180. (F) Venn diagram of genes with low expression levels in both KYS30 and KYSE180.

**Fig. 2.** Low expression of *PLA2G4A* in ESCC(A) qRT-PCR results of *PLA2G4A* levels in ESCC tissue and adjacent normal tissue. \*\*  $P < 0.01$ , compared with adjacent group. (B-C) Western blot results of *PLA2G4A* levels in ESCC and adjacent normal tissue. \*\*  $P < 0.01$ , compared with adjacent group. (D) Expression level of *PLA2G4A* mRNA in the ESCC cell lines KYSE30, KYSE180, ECA109, TE-13 and TE-8 and the normal esophageal squamous epithelium Het-1A cell line. \*  $P < 0.05$ , \*\*  $P < 0.01$ , compared with Het-1A. (E) Gray-scale value of protein expression of *PLA2G4A* in different cell lines. \*  $P < 0.05$ , \*\*  $P < 0.01$ , compared with Het-1A.



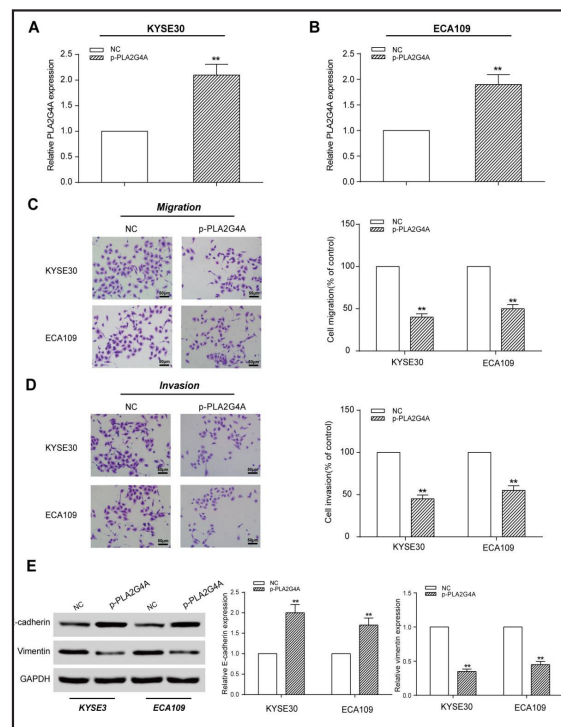
metastasis of ESCC. The mRNA expression levels of *PLA2G4A* in different ESCC cell lines were analyzed by qRT-PCR. The results showed that the ESCC cell lines KYSE30, KYSE180, ECA109, TE-13 and TE-8 all presented relatively lower expression levels of *PLA2G4A* mRNA compared with those of normal esophageal squamous epithelium Het-1A cell lines (Fig. 2D). Western blot found that the protein expression of PLA2G4A in ESCC cell lines was also lower than that in normal esophageal squamous cells (Fig. 2E), and the KYSE30 and ECA109 cell lines demonstrated the lowest PLA2G4A protein expression. Therefore, the KYSE30 and ECA109 cell lines, as well as the normal human esophageal squamous epithelium Het-1A cell line were used in the subsequent experiments.

#### Effect of silencing PLA2G4A on ESCC cell mobility and invasiveness

The experimental group (p-*PLA2G4A* group) of KYSE30 and ECA109 cells was transfected with pcDNA3.1-*PLA2G4A*, and the control group (NC group) of KYSE30 and ECA109 cells was transfected with the empty plasmid. The *PLA2G4A* expression in KYSE30 and ECA109 cells was significantly increased in the p-HOTAIRM1 group when compared with that in the NC group ( $P < 0.01$ , Fig. 3A-3B). The Transwell results showed that the mobility and invasiveness ability of the KYSE30 and ECA109 cells in the p-*PLA2G4A* group were notably lower than those of the cells in the NC group ( $P < 0.01$ , Fig. 3C-3D). E-cadherin is a calcium-dependent transmembrane protein that participates in cell-to-cell adhesion and plays a critical part in tumor metastasis. Vimentin is present in mesenchymal cells and is closely related to tumor occurrence and metastasis. Western blot analysis revealed that the protein expression level of E-cadherin in the p-*PLA2G4A* group was markedly higher than that in the NC group, whereas the protein expression level of vimentin was lower in p-*PLA2G4A* group than in the NC group ( $P < 0.01$ , Fig. 3E).

#### PLA2G4A suppressed ESCC growth in vivo

KYSE30 cells transfected with p-*PLA2G4A* or the empty plasmid were injected subcutaneously into the backs of nude mice. The results of the nude mice allogeneic experiments revealed that the average tumor size in the p-*PLA2G4A* group was smaller than that of the NC group (Fig. 4A). The average tumor volume was measured every 3 days. The average tumor volume of the p-*PLA2G4A* group was significantly smaller than that of the NC group (Fig. 4B). At the termination of the experiment (the 21st day), the mice were sacrificed, and the tumors were excised from each mouse to measure the average weight. The average



**Fig. 3.** PLA2G4A reduced invasion and migration of ESCC cells (A-B) PLA2G4A expression levels were detected in KYSE30 (A) and ECA109 (B) cells after transfection with pcDNA3.1-PLA2G4A (p-PLA2G4A) and the empty plasmid (NC) by qRT-PCR analysis. (C) Transwell migration assay results showed that the p-PLA2G4A group down-regulated the migration of KYSE30 and ECA109 cells compared with the NC group. (D) Transwell invasion assay results in KYSE30 and ECA109 cells. (E) Western blot showed that p-PLA2G4A increased the protein expression level of E-cadherin and decreased the protein expression level of vimentin in KYSE30 and ECA109 cells. \*\*  $P < 0.01$ , compared with the control group. \*\*  $P < 0.01$ , compared with the NC group.

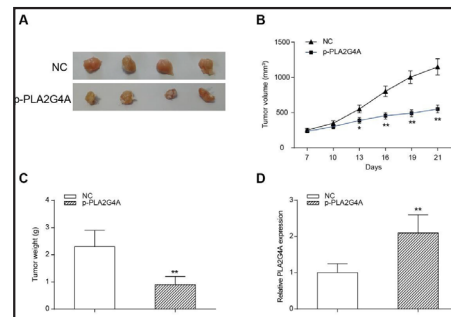
tumor weight of the p-*PLA2G4A* group was also significantly less than that of the NC group ( $P < 0.01$ , Fig. 4C). The expression of *PLA2G4A* was up-regulated approximately 110 % in the p-*PLA2G4A* group compared with the NC group, as revealed by qRT-PCR ( $P < 0.01$ , Fig. 4D).

*MiR-543 was highly expressed in ESCC*

Bioinformatics predicted the binding sites of miR-543 and *PLA2G4A* (Fig. 5A). In addition, the luciferase activity of cells co-transfected with *PLA2G4A* wild-type 3'-UTR and miR-543 mimics was decreased in comparison with that of the control group. In contrast, co-transfection with *PLA2G4A* mutant 3'-UTR and miR-543 mimics had little effect on the luciferase activity ( $P < 0.01$ , Fig. 5A). qRT-PCR analysis demonstrated that the expression level of miR-543 in ESCC tissues was conspicuously higher than in the adjacent normal tissues ( $P < 0.01$ , Fig. 5B). The expression levels of miR-543 in different ESCC cell lines (KYSE30, KYSE180, ECA109, TE-8 and TE-13) were higher than that in the normal esophageal squamous epithelium Het-1A cell line, as revealed by qRT-PCR. In addition, the expression level of miR-543 was the highest in the KYSE30 and ECA109 cells (Fig. 5C). The protein expression level of *PLA2G4A* in the KYSE30 and ECA109 cells was verified by Western blotting. MiR-543 mimics notably inhibited *PLA2G4A* protein expression, whereas the miR-543 inhibitor markedly increased *PLA2G4A* protein expression ( $P < 0.01$ , Fig. 5D).

*MiR-543 enhanced the invasion-metastasis cascade of ESCC by inhibiting the expression of PLA2G4A*

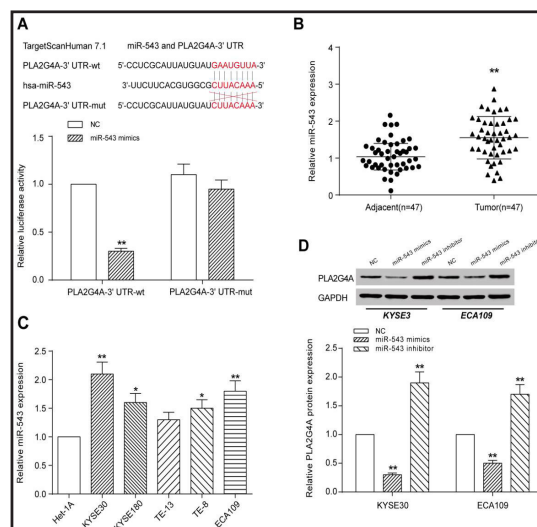
The expression levels of miR-543 and *PLA2G4A* were determined in the groups with NC, miR-148a mimics, the miR-148a inhibitor or p-HOTAIRM1 and miR-148a mimics in KYSE30 and ECA109 cells, and the results indicated that the transfection efficiency was satisfactory ( $P < 0.01$ , Fig. 6A-6B).



**Fig. 4.** HOTAIRM1 inhibited tumor formation in nude mice (A) Overexpression of *PLA2G4A* dramatically suppressed ESCC tumor growth in comparison to that of the NC group. KYSE30 cells were transfected with NC or p-*PLA2G4A* and inoculated into nude mice, after which the tumor size was monitored. (B) Tumor volumes were measured every 3 days. (C) Tumor weight were measured the 21st day after the injection. (D) The expression level of HOTAIRM1 was significantly higher in ESCC mouse tumor tissue compared with the NC group. \*\*  $P < 0.01$ , compared with NC group.

**Fig. 5.** MiR-543 was highly expressed in ESCC (A)

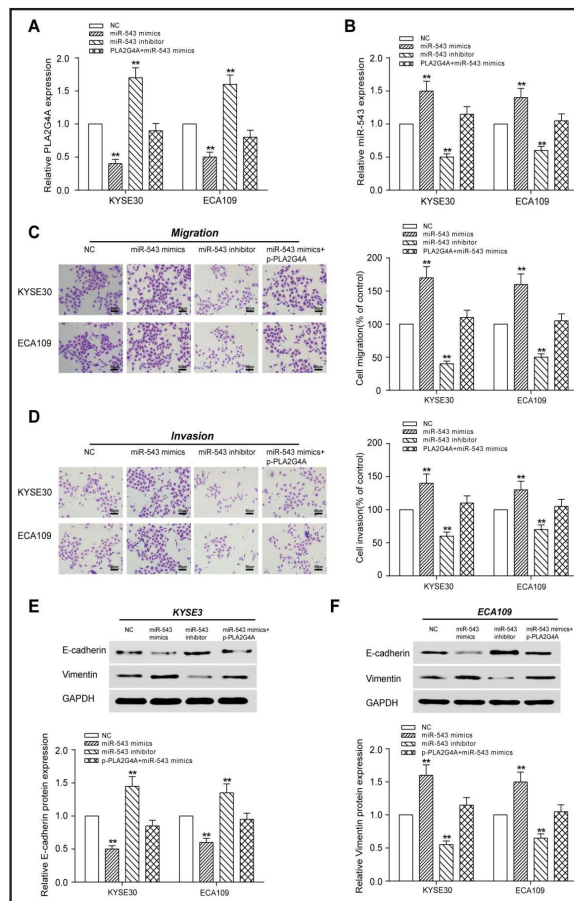
The binding sites of miR-543 and *PLA2G4A* were predicted by bioinformatics. Then, luciferase activity analysis indicated that transfection with miR-543 mimics could significantly inhibit the fluorescence activity of the *PLA2G4A* wild-type luciferase gene. \*\*  $P < 0.01$ , compared with the NC group. (B) The qRT-PCR results showed high expression levels of miR-543 in ESCC tissue. \*\*  $P < 0.01$ , compared with the adjacent tissue. (C) Expression levels of miR-543 in ESCC cell lines KYSE30, KYSE180, ECA109, TE-13 and TE-8 and in the normal esophageal squamous epithelium Het-1A cell line. \*  $P < 0.05$ , \*\*  $P < 0.01$ , compared with the Het-1A cell line. (D) Western blot analysis demonstrated the *PLA2G4A* protein expression levels in KYSE30 and ECA109 cells after transfection with miR-543 mimics and inhibitor. \*\*  $P < 0.01$ , compared with the NC group.



The transwell invasion and migration experiments demonstrated that the two cell lines transfected with miR-543 mimics exhibited significantly increased metastasis ability. Moreover, the number of migratory or invasive cells in the miR-543 inhibitor group was drastically lower. When *PLA2G4A* and miR-543 were overexpressed at the same time, the ability of ESCC cells to migrate and invade was returned to the control level ( $P < 0.01$ , Fig. 6C-6D). Western blot indicated that the protein expression level of E-cadherin in the miR-543 mimics group was relatively lower than in the NC group, whereas it was much higher in the miR-543 inhibitor group compared with NC group. The presence of miR-543 mimics in both cell lines could relieve the inhibitory effect of *PLA2G4A* and allow E-cadherin levels to recover ( $P < 0.01$ , Fig. 6E). The protein expression level of vimentin was higher in the miR-543 mimics group than in the NC group, while it was lower in the miR-543 inhibitor group in comparison with the NC group. Similarly, miR-543 mimics could relieve *PLA2G4A* inhibition and restore the vimentin levels ( $P < 0.01$ , Fig. 6F).

## Discussion

Increasing numbers of studies have indicated that miRNAs are involved in various biological processes, such as cell reproduction, differentiation, apoptosis, and metabolism, as well as tumorigenesis [17]. Recently, it has been revealed that altered expression of miRNA contributes to the invasion and metastasis of many cancers [4, 6, 18]. Increasing numbers of studies have indicated that among numerous other miRNAs, miR-543 is also involved in the processes of initiation and maintenance of many kinds of cancers, including ESCC [17]. It has also been reported that miR-543 is down-regulated in breast cancer [19] and endometrial cancer [20] but functions as an oncogene in hepatocellular carcinoma [10, 21]. These different conclusions may be caused by differences in the etiology and pathogenesis of these cancers. Increasing numbers of studies have indicated that a poor survival rate in ESCC patients is highly associated with frequent local invasion and distant metastasis [17]. Furthermore, abnormal



**Fig. 6.** MiR-543 enhanced invasion-metastasis of ESCC by inhibiting the expression of PLA2G4A(A-B) The expression levels of miR-543 and PLA2G4A in the groups with the NC, miR-148a mimics, miR-148a inhibitor or p-PLA2G4A and miR-148a mimics in KYSE30 and ECA109 cells were determined by qRT-PCR. (C) Transwell migration assay results in KYSE30 and ECA109 cells of different transfection groups. (D) Transwell invasion assay results in KYSE30 and ECA109 cells of different transfection groups. (E) Western blot results showed that miR-543 mimics decreased the protein expression levels of E-cadherin, while the miR-543 inhibitor increased the protein expression levels of E-cadherin in KYSE30 and ECA109 cells. (F) Western blot results showed that miR-543 mimics increased the protein expression levels of vimentin, while the miR-543 inhibitor decreased the protein expression level of vimentin in KYSE30 and ECA109 cells. \*\*  $P < 0.01$ , compared with the NC group.



expression of miRNAs has been implicated in the metastasis and progression of cancers by the acquisition of metastatic potential [17]. However, there have been no reports on the expression of miR-543 in ESCC cells or on the role of miR-543 in the invasion and migration of ESCC cells. In this study, the expression level of miR-543 in several ESCC cell lines was determined by qRT-PCR assay, and it was revealed that miR-543 is up-regulated in multiple ESCC cell lines and that it enhances the metastasis of ESCC cells.

Many studies have confirmed that miRNAs can regulate the stability and translation of their target mRNAs by attaching to the 3'UTR of the target [6]. In addition, it has been proven that these miRNAs, including miR-21, miR-138, miR-223 and miR-92a, regulate ESCC development through the regulation of the expression of their target genes, such as *PTEN* (phosphatase and tensin homolog), *FBXW7* (F-box and WD repeat domain containing 7) and *PDCD4* (programmed cell death 4) [8]. In this study, we used a luciferase reporter assay to demonstrate that miR-543 was capable of directly targeting the 3'-UTR of *PLA2G4A*, and the results showed that *PLA2G4A* is a target of miR-543.

*PLA2G4A* encodes a calcium-dependent cytosolic phospholipase called cytosolic phospholipase A2 $\alpha$  (cPLA2 $\alpha$ ), which is expressed in a variety of tissues [13]. It is an enzyme that hydrolyzes membrane glycerophospholipids to release arachidonic acid and lysophospholipids that are implicated in cancer cell reproduction [22]. The enzyme that *PLA2G4A* encodes is also associated with the regulation of hemodynamics, inflammatory responses, and other intracellular pathways [13]. In addition, *PLA2G4A* is a gene correlated with invasion depth [15]. Some studies have suggested that down-regulation of *PLA2G4A* expression may be a risk factor for advanced clinic pathological classification and poor patient survival. These findings suggest that it may be possible to use *PLA2G4A* as an efficient marker for the prediction of outcomes in ESCC patients [16]. Our study showed that *PLA2G4A* up-regulated the protein expression of E-cadherin and down-regulated the protein expression of vimentin, which inhibited ESCC cell mobility and invasiveness. We also used a Transwell assay to confirm our conclusion.

Nevertheless, there were some limitations of this research. For instance, the specific mechanism by which *PLA2G4A* regulates the invasion and migration of ESCC cells is worthy of further study, which would help us better understand the process of invasion and migration of ESCC cells. In addition, we conducted our Transwell invasion and migration experiments in KYSE30 and ECA109 cell lines, and experiments in other ESCC cell lines such as KYSE180 and TE-8 should be conducted.

In our study, we confirmed that the expression of *PLA2G4A* was dramatically inhibited by the overexpression of miR-543, and then we used a Transwell assay to demonstrate that the metastatic ability of ESCC cells was enhanced by miR-543.

## Conclusion

Overall, the present study first confirmed the roles of miR-543 and *PLA2G4A* in ESCC cell mobility and invasiveness and then demonstrated the targeted relationship between miR-543 and *PLA2G4A*. The findings of our study could give a better understanding of the mechanism by which the invasion and migration of ESCC cells are regulated by miR-543 and could also indicate a potential target for therapy in the future.

## Disclosure Statement

No conflict of interests exists.

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