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miR-518b is down-regulated, and involved in cell proliferation and invasion by targeting Rap1b in esophageal squamous cell carcinoma

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ABSTRACT

MicroRNAs (miRNAs) represent a class of small non-coding RNAs that regulate gene expression at the post-transcriptional levels. Recent studies show that miRNAs may function as oncogenes or tumor suppressor genes. In this study, we demonstrated that miR-518b was down-regulated in esophageal squamous cell carcinoma (ESCC) tissues and correlated with metastasis and survival. miR-518b suppressed the proliferation by inducing apoptosis and repressed the invasion in ESCC cells, but had no effect on the cell cycle. Furthermore, Rap1b was revealed to be directly regulated by miR-518b. These findings indicate that miR-518b may function as a tumor suppressor by targeting Rap1b in the development of ESCC and has important clinical and prognostic value.
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1. Introduction

Esophageal cancer is the sixth leading cause of cancer deaths, of which esophageal squamous cell carcinoma (ESCC) accounts for most cases. The incidence of ESCC appears to be broadly varied by geographic locations. The highest incidence rates were found in China, Iran, South America, and South Africa, and one-half of all ESCC cases in the world occur in China [1]. Despite a myriad of improvements in both diagnostic and therapeutic techniques over the past three decades, esophageal cancer continues to have a poor prognosis, with 5-year survival rates between 10% and 13% [2]. Research over the last 30 years has identified a number of genetic alterations relating to induction of ESCC. However, the molecular mechanisms responsible for the cellular deregulation in ESCC have not been fully understood [3].

MicroRNAs (miRNAs) represent a class of small non-coding RNAs that regulate gene expression at the post-transcriptional levels. MiRNAs play important roles in a series of biological processes and changes in miRNAs expression have been observed in a variety of human pathological conditions. Recent studies showed that miRNAs were aberrantly regulated in different oncogenic pathways and/or various types of cancers, indicating that some miRNAs may function as oncogenes or tumor suppressor genes [4]. For example, miR-21 is over-expressed in most tumor types, and acts as an oncogene by targeting many tumor suppressor genes related to proliferation, apoptosis, and invasion [5]. And the human let-7 family of miRNAs, containing 13 members located on nine different chromosomes, was deregulated in many human cancers and restoration of expression may be a useful therapeutic option in cancers [6]. These findings also point out the potential value of miRNA in cancer screening, diagnosis, treatment, and prognosis. Currently, only limited data is available on the miRNA signature of ESCC. In our previous study, we investigated the miRNAs expression profile of ESCC. The results show the deregulated expression of miRNAs in ESCC, and 11 miRNAs of which were considered significant statistical difference [7]. It is of great importance to further explore the clinical significance, biological functions, and target genes of miRNAs in ESCC.

In this study, we examined the expression level of miR-126, miR-518b, and miR-433 in human ESCC specimens, and explored their relationship with clinicopathologic features and prognosis.

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Further, we analyzed miR-518b functions and screened its potential target genes in ESCC cells. Our data suggest that miR-518b may exert its tumor suppressor function via targeting Rap1b.

2. Materials and methods

2.1. Patient samples and cell lines

Fresh cancer tissues and paired normal adjacent tissues (NAT) were obtained from patients with ESCC in First Affiliated Hospital of Medical School of Xi'an Jiaotong University from Oct-2008 to Oct-2010. The tissue samples were collected from patients undergoing surgery without chemo- and radiotherapy. Tissues were snap frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Tissue sections were stained with H&E for histological diagnosis and heterogeneity evaluation. Only those sections comprised of more than 70% tumor cells in total cell population were used in this study. All the samples were accrued with informed consent and the Institutional Ethics Committee approval for this project was obtained. The median follow-up time for disease progress was 14.05 months, ranging from 1 to 22 months.

The ESCC cell lines (Eca109, Ec9706, and TE-1) were from the Cell Bank of Shanghai (China) and cultured in RPMI 1640 medium supplemented with 10% FBS, 100 units/ml penicillin and 100 g/ml streptomycin at 37 °C in a 5% CO_2 incubator.

2.2. RNA extraction and quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and RNeasy mini kit (QIAGEN, Valencia, CA, USA) following the manufacturer's instruction. qRT-PCR was carried out using a BioRad iQ5 Real-Time PCR Detection System to confirm the expression levels of mRNAs and miRNAs as described before [7]. U6 snRNA and GAPDH mRNA levels were used for normalization. The expression levels in cancer tissues relative to NAT were calculated using the formula: $2^{-\Delta\Delta CT}$, $\Delta\Delta CT = (CT_{miRNA} - CT_{U6\ RNA})_{ESCC} - (CT_{miRNA} - CT_{U6\ RNA})_{NAT}$.

2.3. Plasmid construction

Anti-miR-518b inhibitor (anti-miR-518b) and anti-miR-Inhibitors-Negative Control (Control) were purchased from AngRang Inc. (Xi'an, China). The sequence of precursor miR-518b was synthesized and cloned into pcDNA™6.2-GW/EmGFP-miR expression vector (Invitrogen, Carlsbad, CA, USA). The Rap1b 3′-UTR target site sequence and sequence with mutation of three bases in the miR-518b target-site were synthesized and cloned into downstream of the luciferase gene in the pmirGLO luciferase vector (Promega, Madison, WI, USA). These vectors were named pcDNA-miR-518b, Rap1b-3′-UTR-WT and Rap1b-3′-UTR-MU, respectively. All constructs were sequenced.

2.4. Cell transfection

Cell transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Total RNA and protein were prepared 48 h after transfection and were used for qRT-PCR or Western blot analysis.

2.5. MTT assays

The cells were seeded at a density of 5×10^3 cells/well in 96-well plates at a final volume of 180 μl in incubation, at 37 °C, with 5% CO_2. After different time incubation, 20 μl of 5 mg/ml solution of MTT (Sigma, St. Louis, MO, USA) in PBS was added to each well. The plates were then incubated for 4 h at 37 °C. The reaction was then solubilized in 100% dimethylsulfoxide (Sigma, St. Louis, MO, USA), 20 μl/well, and shaken for 15 min. Absorbance of each well was measured on a multidetection microplate reader (BMG LABTECH, Durham, NC, USA) at a wavelength of 570 nm. pcDNA™6.2-GW/EmGFP-miR (mock) and anti-miR-Inhibitors-Negative Control (Control) were used as the control for pcDNA-miR-518b and anti-miR-518b inhibitor, respectively. All experiments were done in quadruplicate.

2.6. Cell cycle analysis

After harvested by trypsinization and washed with PBS, cells were fixed in ice-cold 70% ethanol, washed, and resuspended in 1 ml PBS containing final concentrations of 1 mg/ml RNase A for 30 min at room temperature, and finally, stained with 20 μg/ml propidium iodide (PI) for 30 min. The stained cells were analyzed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA) and DNA content was quantified using Coulter System software (Beckman Coulter Inc., Fullerton, CA). All of the samples were assayed in triplicate.

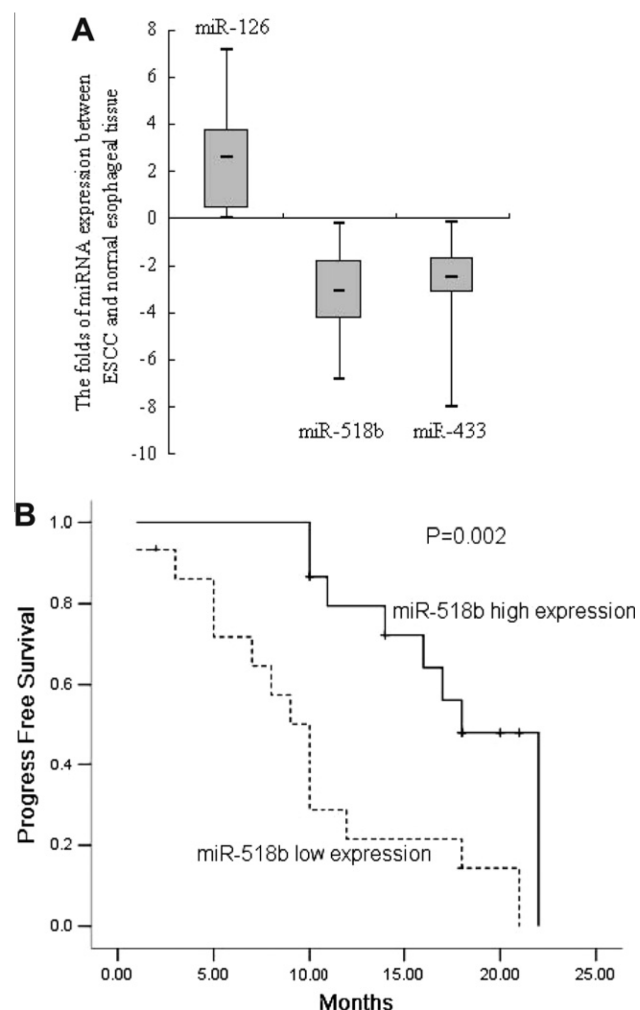


Fig. 1. The expression of miR-518b in esophageal squamous cell carcinoma and its association with survival. (A) The fold change in miR-126, miR-518b, and miR-433 expression in ESCC versus normal tissues. (B) Progression free survival curves for ESCC patients plotted on miR-518b expression.

Table 1
Clinicopathologic variables and the expression status of miR-126, miR-518b, and miR-433.

Variables	*N*	miR-126		*P*	miR-518b		*P*	miR-433		*P*
		Low	High		Low	High		Low	High	
Age				0.232			1.000			0.722
<60	12	4	8		6	6		7	5	
⩾60	18	10	8		9	9		9	9	
Gende0072				1.000			0.109			0.694
Male	21	10	11		13	8		12	9	
Female	9	4	5		2	7		4	5	
Smoking				0.315			0.100			1.000
Yes	26	11	15		11	15		14	12	
No	4	3	1		4	0		2	2	
Drinking				0.072			1.000			0.657
Yes	24	9	15		12	12		12	12	
No	6	5	1		3	3		4	2	
Differentiation				0.329			0.388			0.690
Well	5	1	4		3	2		2	3	
Moderate	16	7	9		6	10		8	8	
Poor	9	6	3		6	3		6	3	
TNM stage				0.732			0.715			0.299
I–II	16	7	9		9	7		7	9	
III–IV	14	7	7		6	8		9	5	
Lymph node				0.464			0.003			0.066
Metastasis	15	6	9		12	3		11	4	
No metastasis	15	8	7		3	12		5	10	

Table 2
Univariate analysis for progression free survival.

Variables	*N*	Progression free survival (months)		*P*
		Median ± SE	95% CI	
miR-126				0.125
Low	14	16.24 ± 1.85	12.62–19.87	
High	16	12.24 ± 1.63	9.03–15.42	
miR-518b				0.002
Low	15	10.05 ± 1.66	6.80–13.30	
High	15	17.82 ± 1.31	15.26–20.39	
miR-433				0.263
Low	16	12.41 ± 1.83	8.81–16.00	
High	14	15.92 ± 1.63	12.72–19.13	
Age				0.389
<60	12	14.95 ± 1.99	11.04–18.86	
⩾60	18	13.37 ± 1.68	10.08–16.67	
Gender				0.122
Male	21	12.67 ± 1.48	9.77–15.57	
Female	9	16.78 ± 2.09	12.68–20.88	
Smoking				0.241
Yes	26	13.21 ± 1.38	10.51–15.91	
No	4	18.5 ± 0.83	16.88–20.13	
Drinking				0.139
Yes	24	13.20 ± 1.37	10.51–15.88	
No	6	17.28 ± 3.08	11.23–23.32	
Differentiation				0.688
Well	5	14.80 ± 3.91	7.13–22.47	
Moderate	16	14.47 ± 1.59	11.35–17.59	
Poor	9	11.98 ± 1.97	8.13–15.84	
TNM stage				0.760
I–II	16	14.82 ± 1.67	11.54–18.10	
III–IV	14	13.31 ± 2.01	9.37–17.25	
Lymph node				0.018
Metastasis	15	10.97 ± 1.78	7.50–14.45	
No metastasis	15	17.33 ± 1.42	14.56–20.11	

2.7. Apoptosis assay

The cells were washed twice with cold 10 mM PBS and resuspended in 1× binding buffer (BD Biosciences, San Jose, CA, USA). Apoptosis in ESCC cells was quantified by staining with annexin V-Phycoerythrin (PE) and 7-amino-actinomycin D (7-AAD). The samples were analyzed using flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA).

Table 3
Multivariate Cox proportional hazards analysis for progression free survival.

Variables	Progression free survival		*P*
	HR	95% CI	
miR-126			
High vs Low	2.144	0.68–6.78	0.194
miR-518b			
Low vs High	6.88	1.03–45.84	0.046
miR-433			
Low vs High	1.96	0.48–7.99	0.349
Lymph node			
Metastasis vs No metastasis	1.66	0.183–15.06	0.652

2.8. Cell invasion assay

For invasion assay, the membrane invasion culture system (transwell membranes of 6.5 mm diameter and 8 μm pore size; Costar) was used according to the standard protocol. Briefly, Harvested cells (1×10^5) suspending in 100 μl of serum free RPMI 1640 were added into the upper compartment of the chamber. A total of 1000 μl conditioned RPMI 1640 medium with 20% (v/v) fetal bovine serum was used as a source of chemoattractant and placed in bottom compartment of chamber. After 48 h, the non-invasive cells on the upper surface of the membrane were removed with a cotton swab. The transformed cells that migrated through the Matrigel matrix and stuck to the lower surface of the membrane were fixed with 4% paraformaldehyde, stained with 1% crystal purple. The invasive cells were then counted (five high-power fields/chamber) using an inverted microscope. Each tests repeated in triplicate.

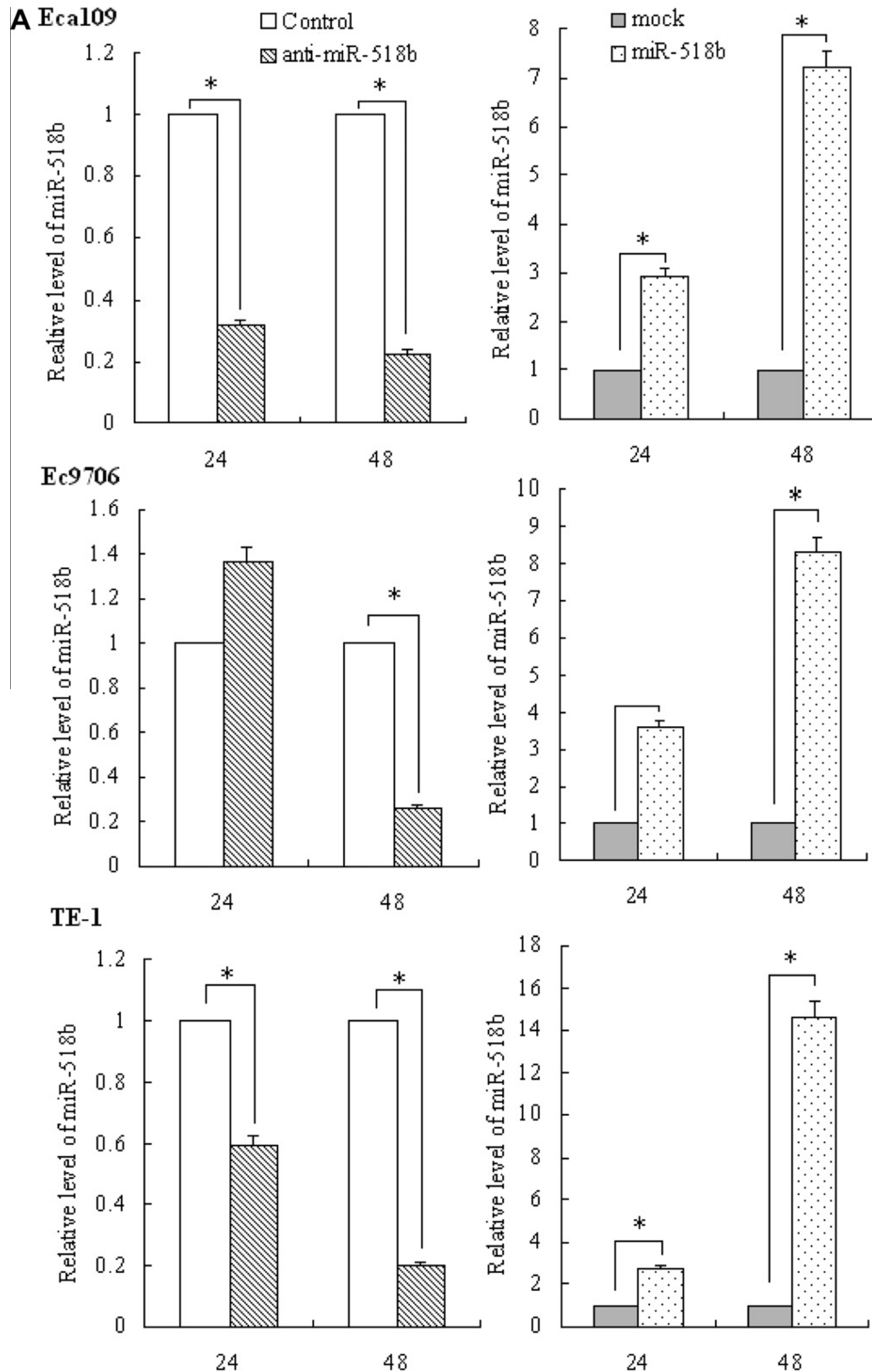
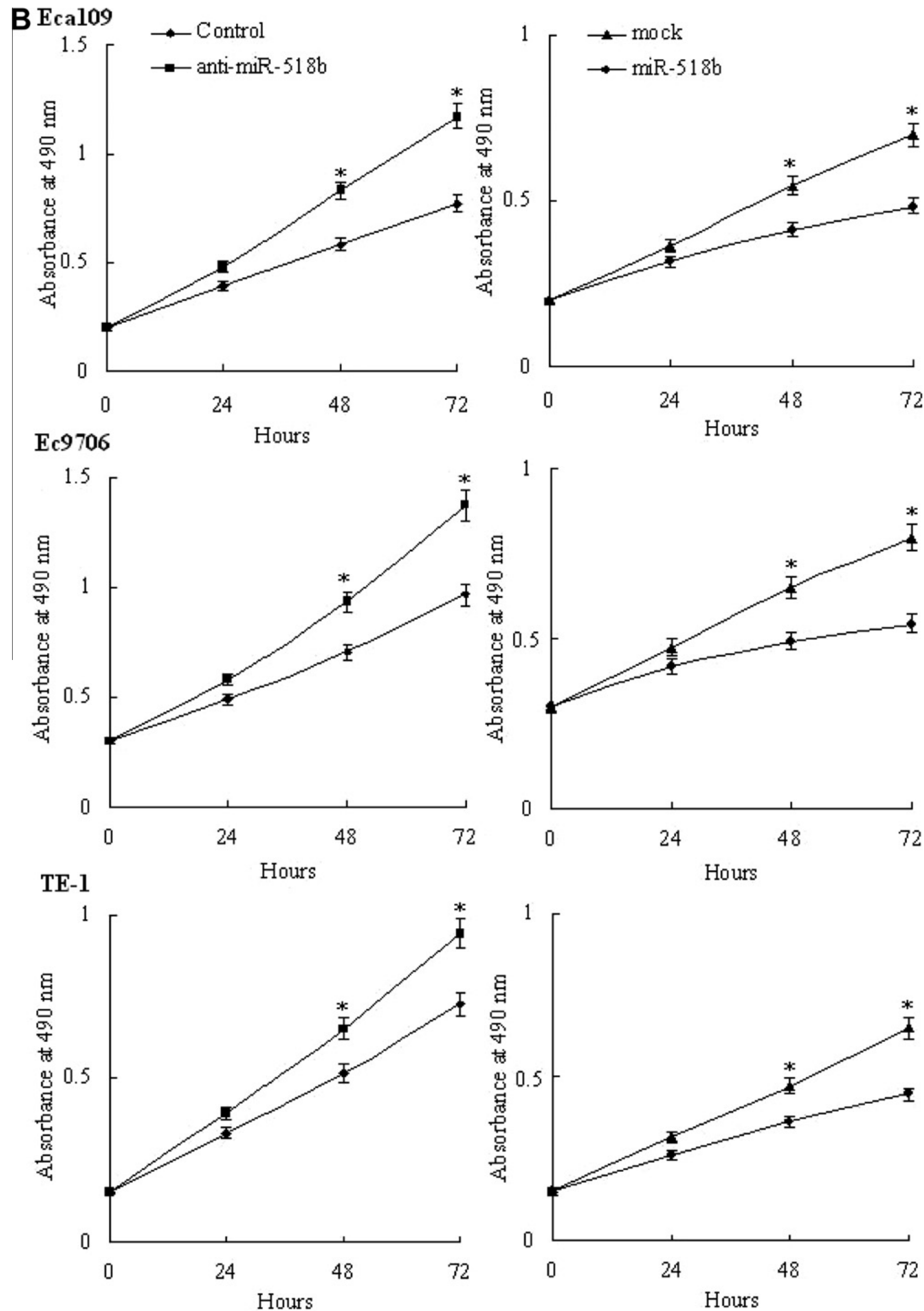


Fig. 2. Effect of miR-518b on cell proliferation, cell cycle and apoptosis. (A) Anti-miR-518b inhibitor (anti-miR-518b)/pcDNA™6.2-GW/EmGFP-miR-518b (miR-518b) could significantly change the expression of miR-518b at 48 h after transfection compared with anti-miR-Inhibitors-Negative Control (Control)/pcDNA™6.2-GW/EmGFP-miR (Mock). (B) After miR-518b or anti-miR-518b transduction, the cell growth of ESCC cells was analyzed at each different time point (24, 48, and 72 h, respectively) compared with the controls by the MTT assay. (C) Flow cytometric analysis of the effect of miR-518b on the cell cycle of Eca109 cells. (D) Effect of alteration of miR-518b expression on cell cycle detected by flow cytometric analysis in total three ESCC cells. (E) Flow cytometric analysis of the effect of miR-518b on apoptosis of Eca109 cells. (F) Effect of alteration of miR-518b expression on cell apoptosis detected by flow cytometric analysis in total three ESCC cells. $^{*}P < 0.05$ compared with respective control.

2.9. Western blot analysis

Anti-Rap1b and anti-β-actin antibodies were obtained from Santa Cruz Biotech. For Western blot analyses, 20 μg of total protein were electrophoresed on a 10% SDS–PAGE gel, transferred onto to PVDF membrane, blocked, and then incubated with primary antibody as indicated above. Corresponding horseradish peroxidase (HRP)-conjugated secondary antibody was then used on



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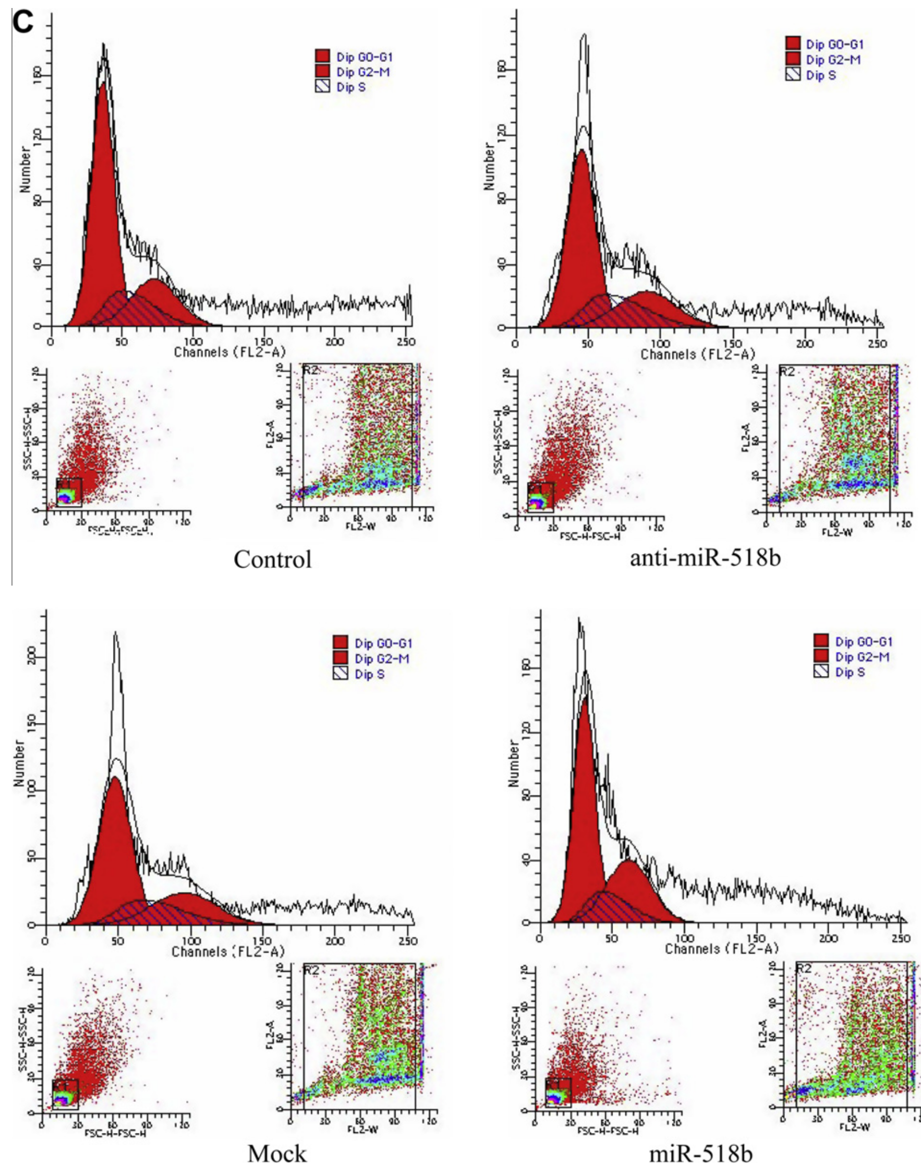
them at room temperature for 2 h. After chemiluminescence reaction with enhanced ECL detection reagents (Amersham, Little Chalfont, Buckinghamshire, England) according to the manufacturer's instructions, the membranes were visualized by exposure to X-ray film in dark. Densitometric analysis was performed using Scion Image software.

2.10. Luciferase assay

Forty-eight hours after transfection, cells were assayed for both firefly and renilla luciferase activity using the Dual-GLO® Luciferase Assay System (Promega, Madison, WI, USA). Briefly, cells were lysed and the firefly luciferase substrate was added (75 μl/well Dual-GLO® Substrate/Buffer). After 15 min, luciferase activity was measured using a luminometer (BMG LABTECH, Durham, NC, USA). Next the renilla luciferase substrate was added (75 μl/well Stop & GLO® Substrate/Buffer) and the luminescence measured after further 10 min incubation. The renilla/firefly luciferase ratio was calculated from the mean luminescence values of triplicate wells, after blanking against values from untransfected cells. The percentage knockdown using test miR-518b was calculated compared with the control and data are shown as the mean of three experimental replicates.

2.11. Immunohistochemical staining and analysis

The Streptavidin-Peroxidase technique (Golden Bridge International: SP-9000) was used according the manufacturer's



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instruction. A sheep polyclonal antibody against Rap1b (Santa Cruz Biotechnology, Santa Cruz, CA: sc-1481) was used at dilutions of 1:400. An irrelevant sheep antiserum served as a negative control. Two observers who were blinded to clinical features evaluated staining results independently and co-observed for a consensus when they were divergent with the method as described [8].

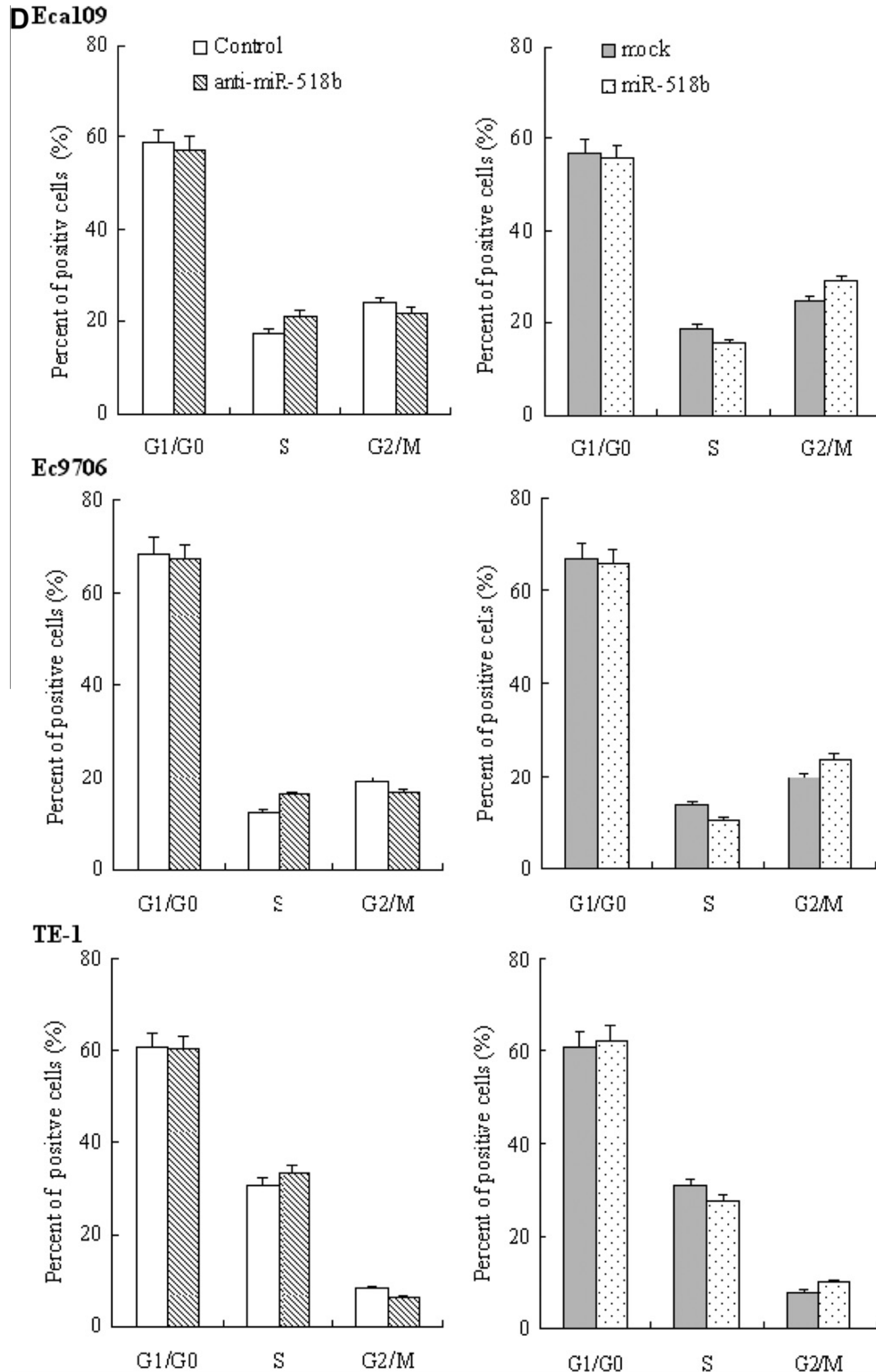
2.12. Statistical analysis

To investigate the association with clinicopathologic features and survival, miRNA expression values were dichotomized into low and high groups using the median expression value within cohort as a cutoff. A Fisher's exact text was used to analyze the relationship between miRNAs expression level and various clinicopathologic characteristics. The survival curves were built by the Kaplan–Meier method, and the resulting curves were compared using the log-rank test. The joint effect of covariables was examined using the Cox proportional hazard regression model. For other analysis, data are expressed as the mean ± standard deviation from at least 3 separate experiments performed in triplicate. Differences between groups were assessed by unpaired, two-tailed Student's t test, $P < 0.05$ was considered significant.

3. Results

3.1. miR-518b is down-regulated in ESCC and associated with shorter progression free survival

We examined the expression of miR-126, miR-518b, and miR-433 in a set of 30 paired samples using qRT-PCR. The results showed that the three miRNAs are significantly altered in ESCC tissues when compared to the normal controls ($P < 0.05$, Fig. 1A). miR-126 was up-expressed while miR-518b and miR-433 was down-expressed in ESCC. Next, the correlation of miRNAs expression with the clinicopathologic factors was examined. There was a correlation between expression status of miR-518b and metastasis of lymph node (Table 1). Low expression of miR-518b and metastasis of lymph nodes were relevant to decreased progress free survival (PFS) through Kaplan–Meier survival curve analysis with a log rank comparison, whereas other parameters were not



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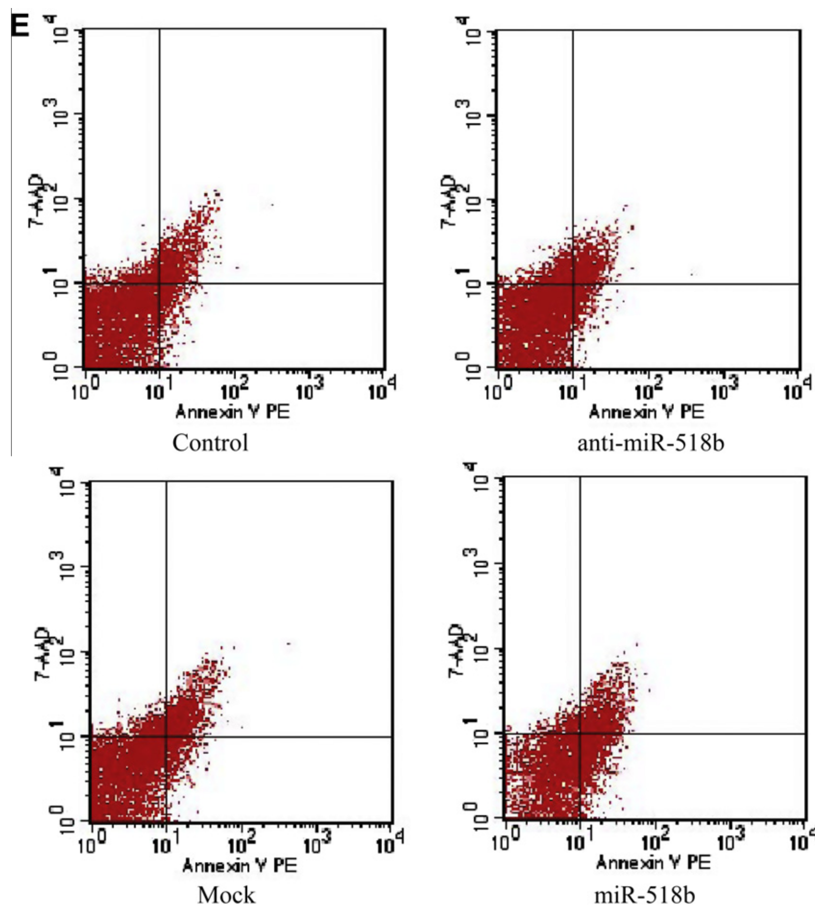
significant (Table 2, Fig. 1B). Decreasing expression of miR-518b were independent prognostic factors for PFS (Table 3).

3.2. miR-518b represses cell proliferation by inducing apoptosis in ESCC cells

Since miR-518b had important relationship with clinicopathologic factors and survival, we further investigated the function of miR-518b in ESCC. To determine the role of miR-518b in tumor cell proliferation, the plasmid pcDNA™6.2-GW/EmGFP-miR-518b (miR-518b) and an anti-miR-518b inhibitor (anti-miR-518b) were used to over-express and block miR-518b expression in ESCC cells, respectively. The results showed that the expression of miR-518b was significantly affected by the plasmids at 48 h after transfection, so total RNA and protein were prepared 48 h after transfection and were used for qRT-PCR or Western blot analysis



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(Fig. 2A). Using the MTT assay, the over-expression of miR-518b repressed the cell viability, while down-expression of miR-518b promoted the proliferation (Fig. 2B). Next, effect of miR-518b on cell cycle and apoptosis was analyzed by flow cytometry. The alteration of miR-518b expression was ineffective in cell cycle distribution in ESCC cells (Fig. 2C and D). ESCC cells transfected with miR-518b showed enhanced apoptosis rate, including the early apoptosis and late apoptosis, compared to mock group, while anti-miR-518b group got the opposite results (Fig. 2E and F). These results indicated that miR-518b suppressed the ability of ESCC cells to proliferate by inducing apoptosis in ESCC cells.

3.3. miR-518b regulates cell invasion in vitro

We further analyzed the effects of miR-518b on the invasive behavior of ESCC cell lines. After incubation, the cells migrated to the basal side of the membrane were counted and captured (Fig. 3A). The numbers of miR-518b-transfected ESCC cells passing through the matrigel were significantly lower than the numbers of cells transfected with pcDNA™6.2-GW/EmGFP-miR (Mock). On the contrary, the numbers of anti-miR-518b-transfected ESCC cells passing through the matrigel were significantly higher than the numbers of cells transfected with anti-miR-Inhibitors-Negative Control (Fig. 3B). These findings suggest that miR-518b expression seems to be closely associated with the invasion of ESCC cell lines.

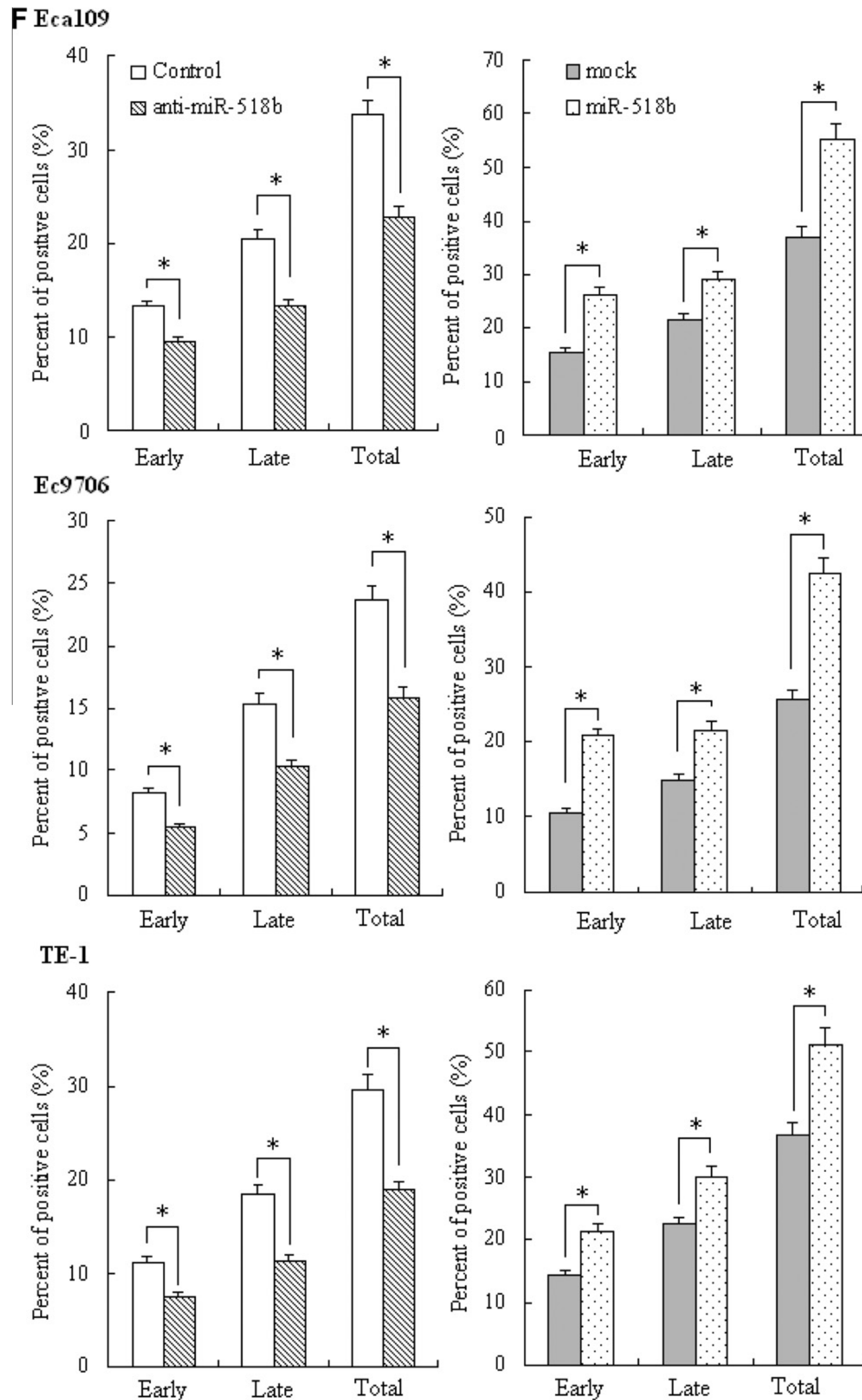
3.4. Rap1b is a target of miR-518b

Based on the correlation of miR-518b with metastasis of lymph node and miR-518b-induced suppression of the invasion of ESCC cell lines, we hypothesized that miR-518b inhibited the malignancy of ESCC cells by regulating oncogenes and/or genes involved in cell invasion. We used the TargetScan (http://www.targetscan.org/l) and MicroCosm (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) for prediction of targets of miR-518b. It was showed that Rap1b, EGR1 (early growth response 1), TFAP2A (transcription factor AP-2 alpha), HOXC8 (homeobox C8) and TSN (translin) were some of the predicted targets of miR-518b. Since Rap1b is known to play a role in the progression of angiogenesis and migration, so we elected to validate the role of miR-518b on the regulation of Rap1b [9].

First, we detected the expression of miR-518b and Rap1b in ESCC cells by qRT-PCR or Western blots, respectively, using the normal esophageal tissues as control. It was found that ESCC cells had a lower level of miR-518b expression (Fig. 4A), a similar level of Rap1b mRNA expression (Fig. 4B), and a higher level of Rap1b protein expression (Fig. 4C). We then evaluated expression levels of Rap1b in tissues by immunohistochemical analysis. It was demonstrated that Rap1b up-regulated in tumor tissues compared with NAT (Fig. 4D).

Further, we used Western blots to determine the effect of miR-518b on levels of endogenous Rap1b protein in ESCC cells. We showed that Rap1b protein expression was significantly down-regulated in extracts from miR-518b-transfected ESCC cells when compared with miR-518b control-transfected ESCC cells (Fig. 4E). But qRT-PCR analysis showed that miR-518b had no effect on the mRNA expression of Rap1b (Fig. 4F).

Last, we constructed luciferase reporter vectors to determine whether miR-518b was capable of targeting Rap1b (Fig. 4G). After co-transfection of miR-518b with Rap1b-wild type or mutated 3′-UTR luciferase reporter vector into ESCC cells, we found that miR-518b reduced Rap1b 3′-UTR luciferase activity compared with



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the control. Furthermore, miR-518b had little observable effects on the mutated Rap1b 3′-UTR luciferase reporter (Fig. 4H, I, and J), indicating that miR-518b indeed was able to silence Rap1b gene expression.

4. Discussion

In this study, we showed that miR-518b was significantly down-regulated in ESCC tissues when compared with adjacent normal tissue. Furthermore, the expression of miR-518b had a significant relationship with lymph node metastasis and survival. Importantly, we showed that Rap1b expression was negatively regulated by miR-518b in ESCC cells, suggesting a role for miR-518b dysregulation in the pathogenesis of ESCC.

Esophageal carcinogenesis is a multi-stage process, involving a variety of changes in gene expression and physiological structure change. MiRNAs are a class of small non-coding endogenous RNA molecules, regulating gene expression at the post-transcriptional

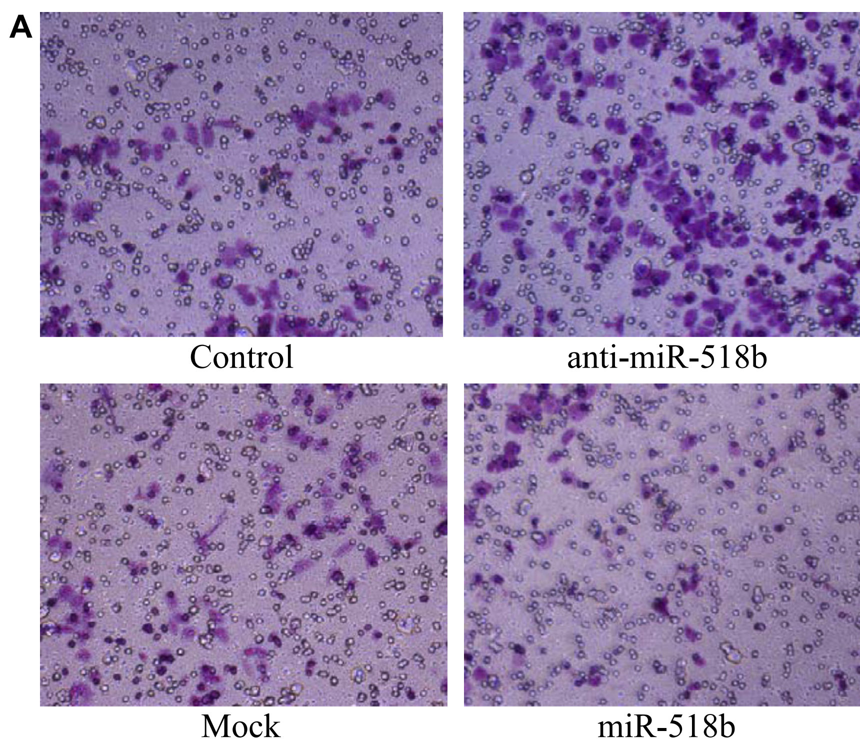

Fig. 3. Effect of miR-518b on cell invasion in vitro. (A) Cell invasion assay. Cells transfected with anti-miR-518b inhibitor (anti-miR-518b), anti-miR-Inhibitors-Negative Control (Control), pcDNA™6.2-GW/EmGFP-miR-518b (miR-518b) and pcDNA™6.2-GW/EmGFP-miR (Mock) were subjected to transwell invasion assays, as described. After incubation, cells that invaded through the pores to the under surface of the membrane were fixed, stained and counted. (B) Invading cell numbers are the average count of five random microscopic fields. Each bar represents the mean ± S.D. of the counts from a single representative experiment. $^{*}P < 0.05$ compared with respective control.

level, and play important roles in a series of biological processes including cell differentiation, proliferation, and apoptosis. Frequent dysregulation of miRNAs in malignancy highlights the study of molecular factors upstream of gene expression to reveal the important role of miRNAs in carcinogenesis [10–12].

Reports found that the expression of miRNAs was aberrant in ESCC, suggesting that miRNAs played an important role in the progression of ESCC [12–15]. Lots of previous studies have demonstrated the role of miRNAs, notably miR-21, in the carcinogenesis of ESCC [16–22]. miR-21 was shown to act as an oncogene in ESCC through targeting programmed cell death 4(PDCD4) and PTEN [16–18]. miR-223, miR-205, and miR-375 were down-regulated in ESCC and had an inhibitory effect on cell invasion [18–20]. A recent study looking at serum samples taken from 290 ESCC patients and 140 age- and sex-matched controls showed that a profile of 7 serum miRNAs (miR-10a, miR-22, miR-100, miR-148b, miR-223, miR-133a, and miR-127-3p) as ESCC biomarkers. More importantly, this panel of 7 miRNAs clearly distinguished stage I/II ESCC patients from controls [22].

Our previously study revealed that miR-126 up-regulated whilst miR-518b and miR-433 down-regulated in ESCC. In this study, we examined the expression levels of these three miRNAs in 30 paired specimens and found that miR-518b was significantly down-regulated in ESCC tissues. These results were consistent with previous studies that miR-518b only expressed in non-tumor tissues compared with hepatocellular carcinoma tissues and miR-518b was down-regulated in highly invasive pancreatic cancer cell lines but not in highly metastatic cell lines [23,24]. In addition, the levels of miR-518b in tumor tissue of patients with lymph node metastasis were significantly lower than in those without lymph node metastasis. Further, low expression of miR-518b was an independent factor indicating a poor prognosis of ESCC patients. These results suggest that down-regulation of miR-518b in tumor cells may play roles in the development of ESCC and have prognostic values.

To reveal the exact role of miR-518b in ESCC, we then tested the effect of miR-518b on proliferation, cell cycle, apoptosis, and invasion by up-and down-regulating the expression level of miR-518b. The results showed that increased miR-518b inhibited the proliferation of ESCC cells, while decreased miR-518b promoted proliferation, suggesting that it could play a role in ESCC tumourgenesis. miR-518b had no influence on cell cycle but promoted the apoptosis rate, indicating that miR-518b suppressed the ability of ESCC cells to proliferate by inducing apoptosis in ESCC cells. Since miR-518b had a relationship with metastasis, we then examined the implication of miR-518b in ESCC cells invasion, and data showed that miR-518b had a negative effect on invasion. These suggest that down-regulation of miR-518b in tumor cells may play roles in the development of ESCC through enhancing cell proliferation, inhibiting apoptosis, and promoting cell invasion. However, these should be confirmed with further in vivo experiments.

Considering the value of miR-518b in metastasis and invasion, it will be interesting to explore mechanisms mediating miR-518b directly or indirectly affects cell progression in ESCC. In order to understand the pathways involved in miR-518b-mediated inhibition of cell invasion, we investigated potential target genes of miR-518b. Bioinformatics analysis revealed Rap1b, EGR1, TFAP2A, HOXC8 and TSN as potential targets. Some of these target genes have been previously confirmed to involve in tumor progression. Rap1b is an isoform of Rap1, a small GTPase regulating several basic cellular functions including adhesion, migration, polarity, differentiation, and growth [25]. Studies provided evidence that Rap1b was required for angiogenesis and had mitogenic properties [9,26]. We then chose Rap1b for further experiments based on the

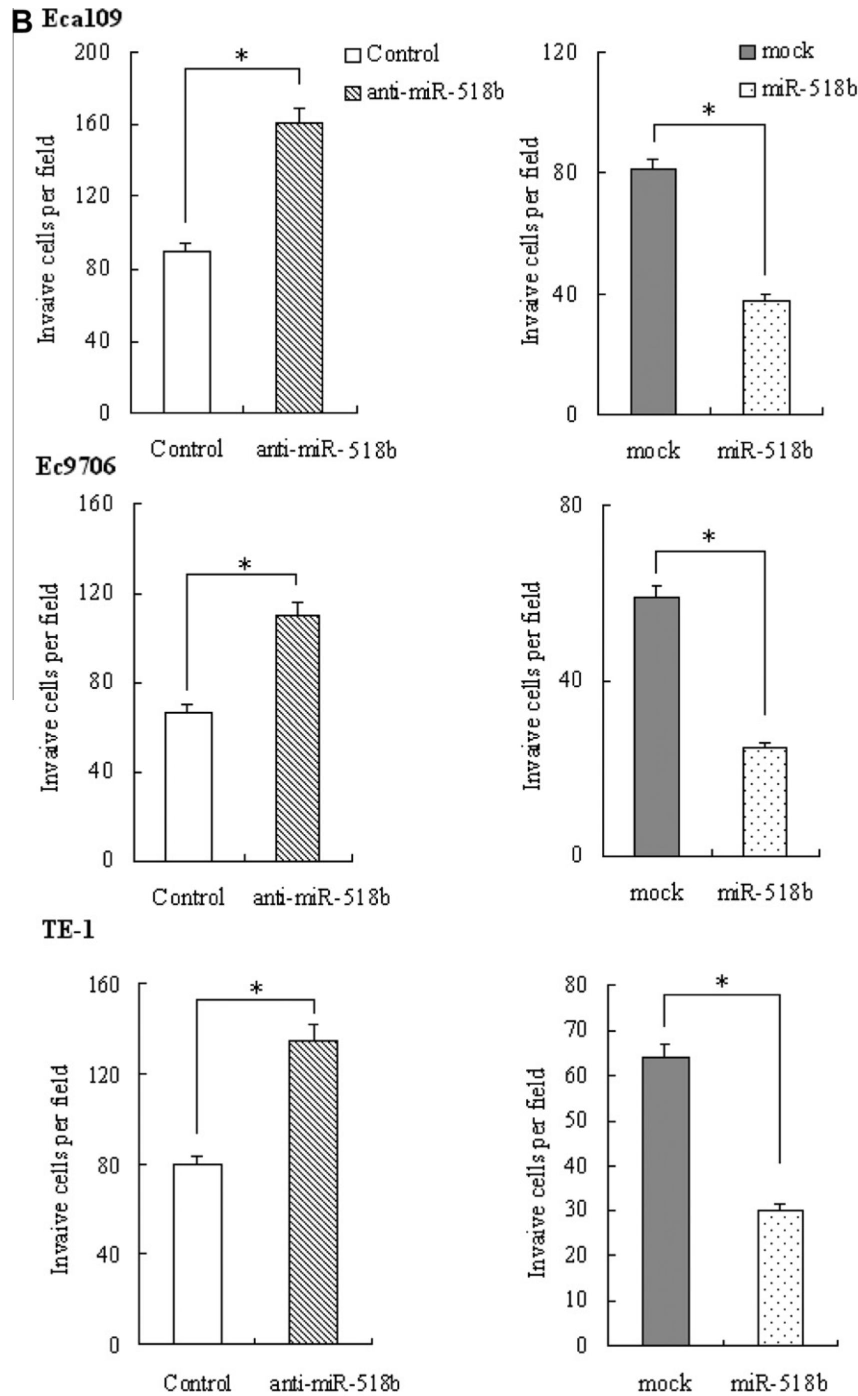

Fig. 3 (*continued*)

correlation of miR-518b with metastasis of lymph node and suppression of the invasion in ESCC. There were increase expression of Rap1b in both cells and tissues. Following results showed that miR-518b could significantly down-regulate Rap1b protein expression, but had no effect on the mRNA expression. So we drew the conclusion that miR-518b post-transcriptionally regulated expression of Rap1b. Using a miRNA reporter system, we then showed that miR-518b specifically down-regulated endogenous Rap1b expression at the post-transcriptional level. These results provided insight into regulation mechanism of miR-518b in ESCC. Rap1b might be the target via which miR-518b mediates its effects on cell growth and invasion. On the other hand, there are opposite observations in the expression of miR-518b in other cancers. For example, miR-518b up-regulated in HCC compared to non-cancerous tissue and in extranodal marginal zone lymphomas (MALT lymphoma) compared to gastritis [27,28]. As known to all, each miRNA can influence the expression of several hundred different target genes containing oncogenes and tumor suppressor genes. In other words, the action for the anti-tumor or pro-tumor roles of a miRNA depends on the competition among its target genes in specific types of cancer. Thus, we speculate that miR-518b function as a tumor suppressor gene in ESCC was due to the inhibition of its target

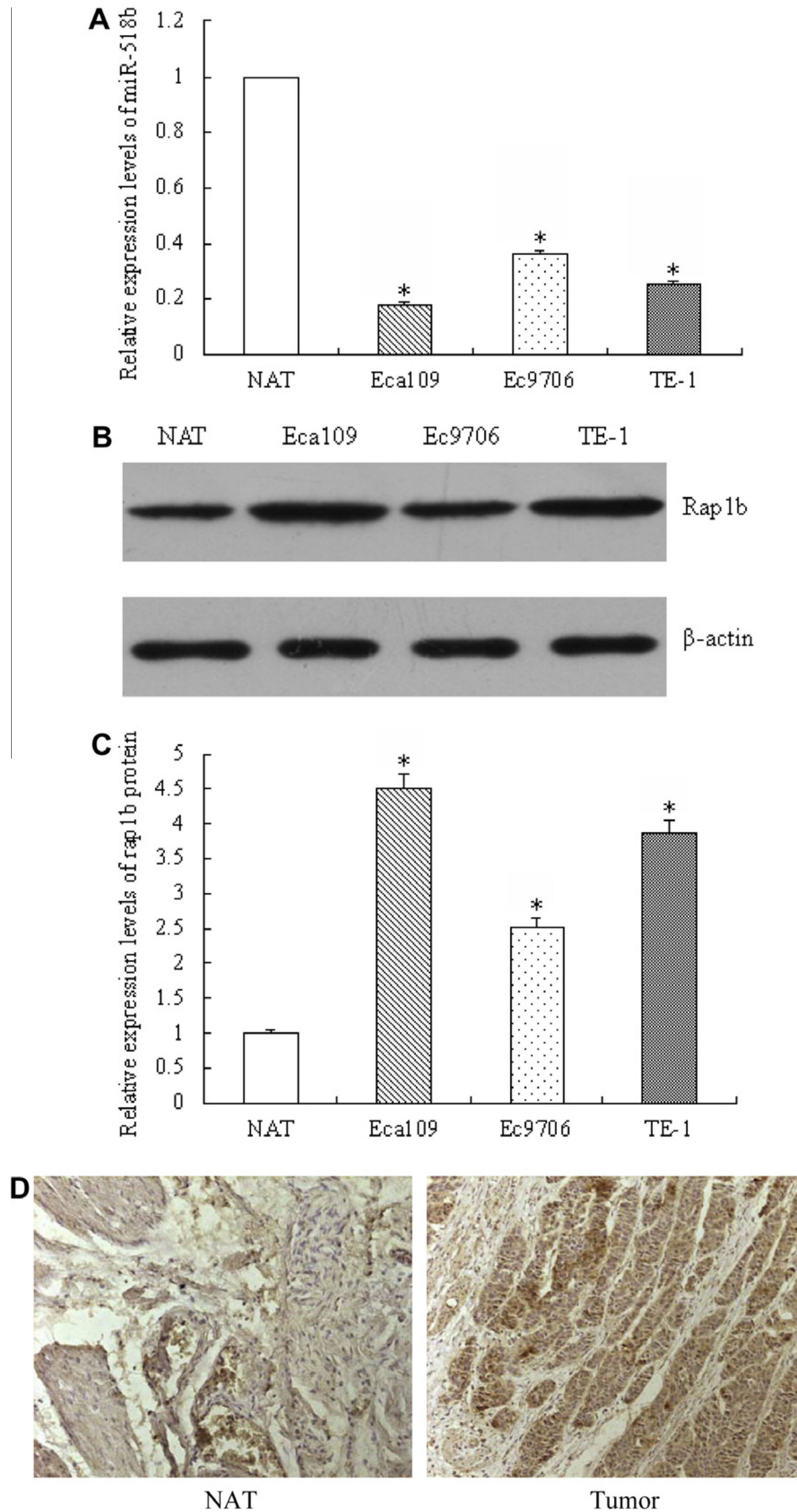

Fig. 4. Rap1b was a target of miR-518b. (A–C) The expression of miR-518b and Rap1b in ESCC cells were detected by qRT-PCR or Western blots, respectively, using normal adjacent tissues (NAT) as control. (D) Immunohistochemical analysis detected the expression of Rap1b protein in tumor tissues compared with NAT (magnification, ×200). (E and F) Effects of miR-518b on expression of Rap1b in mRNA and protein levels were detected by qRT-PCR or Western blots, respectively. NE represents for normal ESCC cells, miR-518b represents cells transfected with "pcDNA™6.2-GW/EmGFP-miR-518b", mock represents for cells transfected with "pcDNA™6.2-GW/EmGFP-miR". (G) Schematic graph of the putative binding sites of miR-518b in the Rap1b 3′-UTR (514–520). Mutant of Rap1b indicates the Rap1b 3′-UTR with mutation in miR-518b-binding sites. (H–J) Luciferase assay in Eca109 (H), Ec9706 (I), and TE-1 (J). miR-518b represents cells transfected with "pcDNA™6.2-GW/EmGFP-miR-518b", NE represents for normal ESCC cells, mock represents for cells transfected with "pcDNA™6.2-GW/EmGFP-miR", pmirGLO represents for pmirGLO luciferase vector, rap1b-m and rap1b-w represents for mutant and wide type luciferase vector. $^{*}P < 0.05$ compared with respective control.

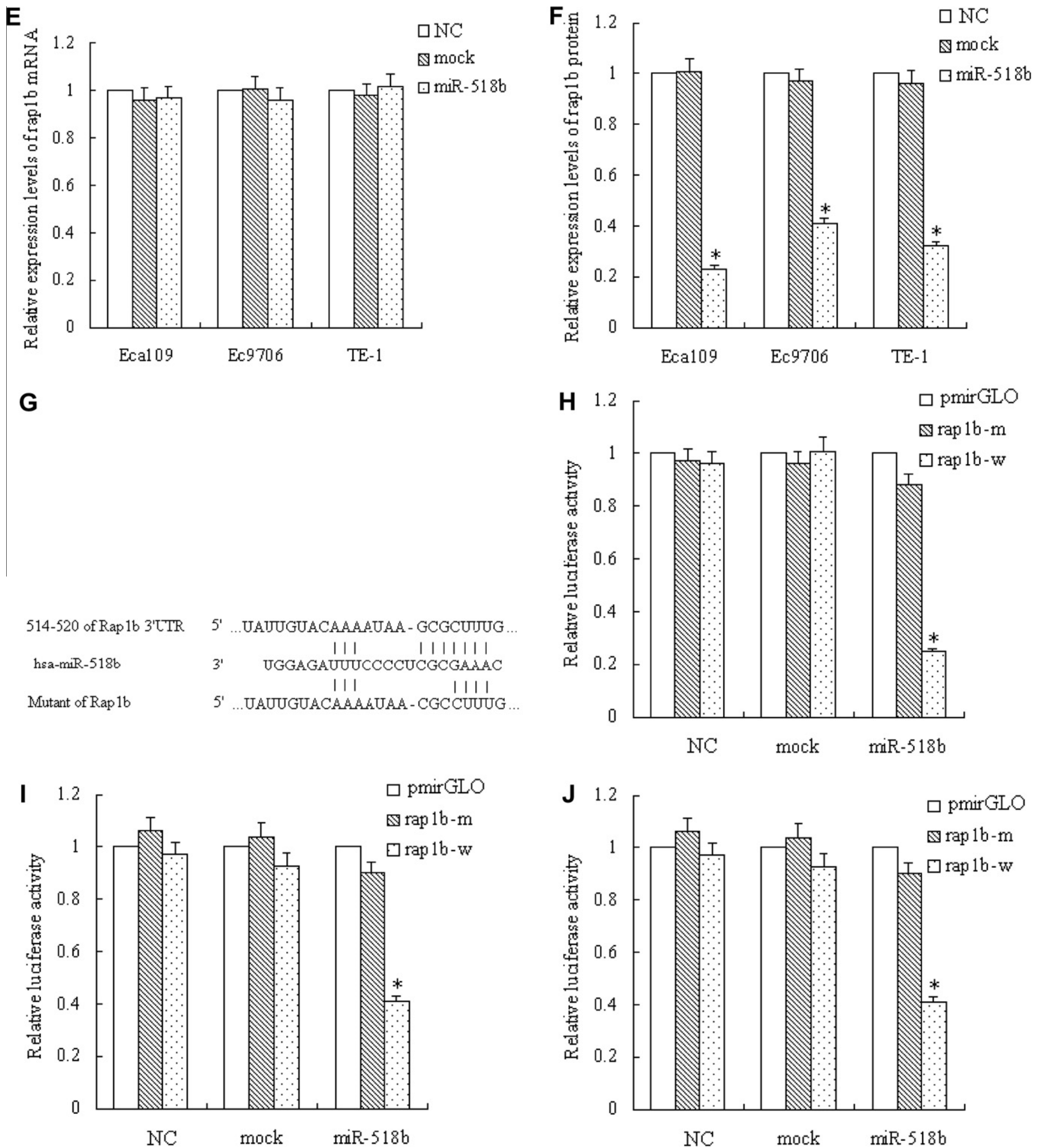

Fig. 4 (*continued*)

genes, which were mostly oncogenes in ESCC. So it is very important to validate targets of miR-518b by further functional assays.

One important limitation of our study is that only 30 patients were enrolled in the analysis of the clinicopathologic characteristics and the follow up data was only for progress free survival. We would like to confirm the findings in a larger patient population and analyze the relationship between miR-518b expression and overall survival.

In conclusion, this study provides new insights into the role of miR-518b in ESCC. Here we show that miR-518b is down-regulated in ESCC tissues and correlates with metastasis and survival, and is able to inhibit cell proliferation and invasion of ESCC cells in vitro. We used functional assays to confirm that miR-518b down-regulated Rap1b expression in ESCC cells. These results suggest that miR-518b is a tumor suppressor in ESCC, and might serve as a therapeutic target.

Conflict of interest statement

None declared.

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