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MicroRNA-124 inhibits proliferation and metastasis of esophageal cancer via negatively regulating NRP1

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Abstract. – OBJECTIVE: MicroRNAs are a kind of endogenous, non-coding RNAs, which exert a significant role in pathological processes. Previous studies have reported that microRNA-124 is a tumor suppressor. The specific effect of microRNA-124 on esophageal cancer, however, has not been fully elucidated. This stu[illegible] to explore the role of microRNA-124 in [illegible] geal cancer and its underlying mechanism[illegible]

PATIENTS AND METHODS: MicroRNA[illegible] expressions in 75 esophageal cancer tissu[illegible] paracancerous tissues, and esophageal ca[illegible] cer cell lines were detected b[illegible]CR (quan[illegible] titative Real-Time Polymeras[illegible]action). The relationship between p[illegible]RNA-[illegible]expression, clinical progressio[illegible]athologi[illegible]dica-tors, and prognosis of p[illegible] wit[illegible] cancer was analyzed. For [illegible]periments, we performed CCK-[illegible]ll cou[illegible]t-8), colony formation and tra[illegible]ll assay to [illegible] cell proliferation, migra[illegible]d invasion a[illegible]s after microRNA-124 [illegible]ression in TE-1 and EC-109 cells, res[illegible]tively. [illegible]rn blot was utilized to explore [illegible]e regulatory [illegible]f microRNA-124 in esoph[illegible]l cancer cells.

RESU[illegible]: MicroRNA-124 w[illegible] downregulated in [illegible]phage[illegible]ancer tissues than that of par[illegible]cerou[illegible]ssues. Patients with esophageal [illegible]er [illegible]o had [illegible]er expression level of micr[illegible]24 pre[illegible]ed higher tumor stage [illegible]meta[illegible]inci[illegible]ce, as well as lower sur-[illegible]te. *In [illegible]dies demonstrated a decrea[illegible] cell pr[illegible]ation and migration abilities after [illegible]icroRNA-124 overexpression. Western blo[illegible]ved upregulated PI3K and AKT, a[illegible]down[illegible]lated PTEN in esophageal cancer cells after overexpression of microRNA-124. [illegible]ermore, microRNA-124 was confirmed to n[illegible]ly regulate NRP1, so as to participate in the d[illegible]elopment of esophageal cancer.

CONCLUSIONS: MicroRNA-124 is downregulated in esophag[illegible] cancer tissues, which is re[illegible]kably correla[illegible] to the development, patho[illegible]l grade, [illegible] poor prognosis of esophageal [illegible]. Overexpressed microRNA-124 is capab[illegible]biting the malignant progression of esophageal cancer *via* negatively regulating [illegible]P1.

Key words:
MicroRNA-124; NRP1; Esophageal cancer; Proliferation; Metastasis.

Introduction

Esophageal cancer is one of the most common malignancies in the world with a high fatality rate. Globally, esophageal cancer leads to over 40,000 deaths each year[1,2]. Although the incidence of esophageal cancer in China has decreased, its mortality rate is still high[1]. At present, treatment methods of esophageal cancer include surgical treatment, radiotherapy, chemotherapy, and targeted therapy[3-5]. With the rapid development of molecular biology and genetic diagnosis technology, esophageal cancer is considered as the long-term interaction of genetic and environmental factors. The malignant transformation and irreversible genetic changes further result in the disordered cellular functions, including proliferation, apoptosis, and differentiation. Although great progresses have been made, the specific pathogenesis of esophageal cancer is still unclear[5-7]. More seriously,

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most patients with esophageal cancer are in the advanced stage when first diagnosed since the occult symptoms. About 40%-60% of these patients could not be operated because of the advanced stage and high surgical risk[5,8]. Searching for the key factors or new targets that participate in the development and metastasis of esophageal cancer could be beneficial to work out new treatment approaches[4,6]. Therefore, it is of great significance to elucidate the molecular mechanism of esophageal cancer for a better diagnosis and treatment.

MicroRNAs are a kind of single-stranded, non-coding RNAs with over 25 nucleotides in length. MicroRNAs could degrade or inhibit the translation of target mRNAs, so as to regulate gene expressions at the post-transcriptional level[9,10]. Functionally, microRNAs could not encode proteins, but exert their regulatory roles *via* complementary pairing of 3'UTR of target mRNAs[11,12]. A great number of studies have demonstrated that abnormally expressed microRNAs would lead to dysfunctions of multiple proteins[13]. MicroRNAs are also closely related to tumor development, which could be serve as markers in predicting malignant tumors[14,16]. Recent studies have found that microRNAs exert certain tissue specificities. It can promote proliferation, invasion, and metastasis of tumor cells through various pathways, and plays an essential regulatory role in the occurrence and development of tumors[17]. Differentially expressed microRNAs have been found in various tumor tissues, such as hepatocellular carcinoma, cancer, and non-small cell lung cancer[16,18-20]. Generally speaking, microRNAs exhibit biological functions in tumors through chromosome recombination, imprinting, epigenetic regulation, nucleocytoplasmic transfer, mRNA splicing, and translation[20,21].

It is reported[22-25] that microRNA-124 is involved in the progression of gastric cancer, colorectal cancer, nasopharyngeal cancer, and liver cancer. The specific role of microRNA-124 in esophageal cancer, however, is not fully elucidated. In the present study, we detected microRNA-124 expression in esophageal cancer tissues and paracancerous tissues. Moreover, the effect of microRNA-124 on regulating biological functions of esophageal cancer cells were further detected. We aim to explore the role of microRNA-124 in the occurrence and development of esophageal cancer, so as to improve the treatment strategy.

Patients and Methods

Patients

75 esophageal cancer tissues and paracancerous tissues were collected by surgical resection. All enrolled patients were pathologically diagnosed as esophageal cancer according to TNM stage in the eighth edition of UICC/AJCC for International Cancer Control/American Committee on Cancer. Patients did not receive preoperative radiotherapy or chemotherapy. This investigation was approved by the Hospital Ethics Committee. All the patients signed the informed consent.

Cell Lines and Treatments

Four human esophageal cancer cell lines (OE19, OE21, TE-1 and EC-109) and one human normal esophageal epithelial cell line (HEEC) were purchased from ATCC (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% FBS (fetal bovine serum), 100 U/mL penicillin and 100 μg/mL streptomycin (Hyclone, South Logan, UT, USA), and incubated in 5% CO_2 incubator at 37°C.

Cell Transfection

MicroRNA-124 plasmids and negative control were constructed by Gene Pharma (Shanghai, China). Cells in good growth condition were selected and seeded in the 6-well plates. Cell transfection was performed when the confluence was up to 70% according to the instructions of Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA). After transfection for 48 h, cells were collected for the following *in vitro* experiments.

Cell Counting Kit-8 (CCK-8) Assay

Transfected cells were seeded into 96-well plates with 2×10^3 per well. 10 μL of the CCK-8 solution (Dojindo, Kumamoto, Japan) was added in each well after cell culture for 6, 24, 48, and 72 h, respectively. The absorbance at 490 nm of each sample was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

Colony Formation Assay

Transfected cells were seeded in the 6-well plates at a density of 200 cells per well. Culture medium was replaced once in the first week and twice in the second week. After culturing for 2 weeks, cells were fixed with methanol and stained

with 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) for 20 min, followed by the detection of colony formation.

Transwell Assay

The upper chamber of transwell chamber was previously coated with 100 µL of Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) and maintained in an incubator for 2 h. After cell density was adjusted to 2×10^5/mL, 200 µL of cell supernatant and 500 µL of culture medium containing 10% FBS were then added in the upper and lower chamber, respectively. Transwell chamber was removed after incubation for 24 h, and the non-migrated cells in the chamber were gently wiped off with a cotton swab. The chamber was fixed with 4% paraformaldehyde for 30 min, washed with phosphate-buffered saline (PBS) twice, and stained in 1% crystal violet for 30 min. Finally, 5 randomly selected fields were captured for cell count.

QRT-PCR (Quantitative Real-Time Polymerase Chain Reaction)

We used TRIzol (Invitrogen, Carlsbad, CA, USA) to extract total RNA for reverse transcription according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). The expression level of the target gene was calculated using the $2^{-\Delta\Delta CT}$ method. Primers used in this experiment were as the follows: MicroRNA-124, 5′-UAAGGCACGC[illegible]UGA[illegible]GCC-3′; U6, 5'-TGCGGGTGCT[illegible]TTCG[illegible]AGC-3'; NRP1, forward, 5'-CC[illegible]AAC[illegible]ACTCG-3', reverse, 5'-AG[illegible]ACCCAACATTCC-3'; β-actin, forward, [illegible]TGGCACCCAGCACAAT-3', reverse, 5'-TC[illegible]TAGGTGTCCCTTTG-3'.

Western Blot

Total protein was extracted from treated cells by radioimmunoprecipitation assay (RIPA) solution (Beyotime, Shanghai, China). The protein sample was separated by electrophoresis on 10% SDS-[illegible] (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and then transferred [illegible] PVDF (polyvinylidene difluoride) membrane (Millipore, Billerica, MA, USA). After membranes were blocked with skimmed milk, the m[illegible] incubated with primary antibodies (Cell Signaling Technology, Danvers, [illegible] USA) overnight at 4°C. The membranes were then washed with TBS-T (Tris-buffered Saline with Tween 20) and followed by the incubation of secondary antibody. The protein blot on the membrane was exposed by enh[illegible] chemiluminescence (ECL).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 statistical software (IBM, Armonk, [illegible] USA) was used for data analysis. Measurement data were expressed as mean ± standard deviation ($\bar{x}$ ± s) [illegible] compared using the t-test. The classified variable was compared using chi-square analysis or Fisher's exact test. Kaplan-Meier [illegible] produced [illegible] evaluating the overall survival of patients. Log-rank test was utilized for comparing differences between curves. [illegible] 0.05 considered the difference was statistically significant.

Results

MicroRNA-124 Was Downregulated in Esophageal Cancer Tissues and Cells

We detected microRNA-124 expressions in 75 e[illegible] cancer tissues and paracancerous tissues by qRT-PCR. Downregulated microRNA-124 was found in esophageal cancer tissues [illegible] of paracancerous tissues (Figure 1A and 1B). Besides, downregulated microRNA-124 was also observed in esophageal cancer cell lines than that of normal esophageal epithelial cell line (Figure 1C). Among them, TE-1 and EC-109 cells expressed a higher level of microRNA-124, which were selected for the following experiments.

MicroRNA-124 Expression Was Correlated With Clinical Stage and Overall Survival of Patients With Esophageal Cancer

Patients with esophageal cancer were assigned to high expression and low expression group according to their expression levels of microRNA-124. Through chi-square analysis, we found that microRNA-124 expression was negatively correlated with clinical stage of esophageal cancer, whereas not correlated with age, gender, lymph node metastasis, and distant metastasis (Table I). Follow-up data of enrolled subjects were collected to analyze the relationship between microRNA-124 expression and prognosis of esophageal cancer. Kaplan-Meier showed that downregulated microRNA-124 was remarkably associated with poor prognosis of esophageal cancer (Figure 1D). These data suggested that microRNA-124 could be served as a biomarker in predicting the prognosis of esophageal cancer.

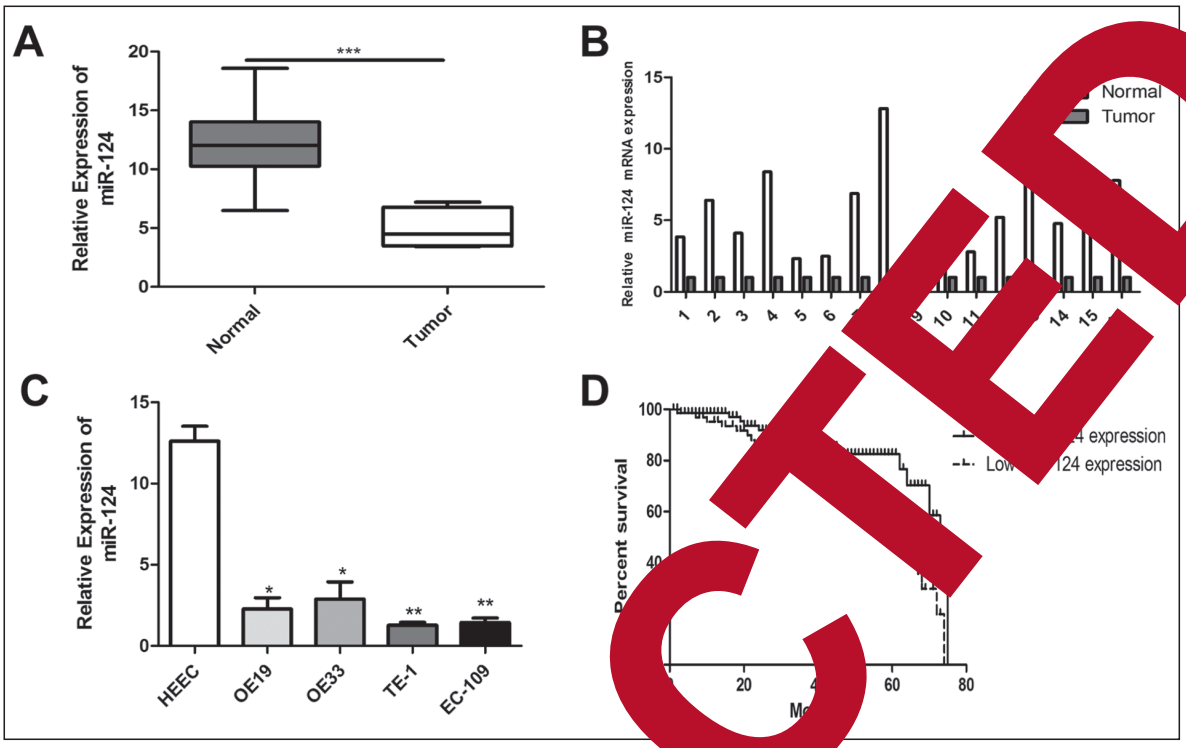


Figure 1. MicroRNA-124 was downregulated in esophageal cancer tissues [illegible] ***A***, ***B***, MicroRNA-124 was downregulated in esophageal cancer tissues than that of paracancer[illegible] ***C***, Expression levels of microRNA-124 in 4 esophageal cancer cell lines (OE19, OE33, TE-1 and EC-109) and n[illegible]ithelial cell (HEEC). ***D***, Kaplan-Meier survival curves of patients with esophageal cancer based on micr[illegible]-124 [illegible]ients in the high expression group had worse prognosis than those in the low expression group.

Overexpressed microRNA[illegible]ibited Cell Proliferation

To further explore t[illegible]ffect [illegible]icroRNA-124 on the prolifer[illegible]apac[illegible]ageal cancer cells, we first constructed corresponding transfection plasmids of microRNA-124 (Figure 2A and 2B). The CCK-8 assay showed decreased proliferative rate after

Table I. Association[illegible]R-124 expressio[illegible]linicopathologic characteristics of esophageal cancer.

Param[illegible]rs	[illegible]er of cases	miR-124 expression		*p*-value
		Low (%)	High (%)	
Age (y[illegible]				0.435
<[illegible]	32	20	12	
[illegible]	43	23	20	
G[illegible]				0.294
Ma[illegible]	27	22	15	
Female	38	18	20	
[illegible]e				0.021
[illegible]2	42	29	13	
T[illegible]4	33	14	19	
Ly[illegible]tasis				0.116
	45	30	15	
[illegible]es	30	14	15	
[illegible]tance metastasis				0.101
	60	38	22	
	15	6	9	

microRNA-124 overexpression in esophageal cancer cells (Figure 2C and 2D). Similar results were obtained in the colony formation assay (Figure 2E and 2F).

Overexpressed microRNA-124 Inhibited Cell Migration and Invasion

Transwell assay was performed to detect migration and invasion of esophageal cancer cells after altering microRNA-124 expression. The amount of transmembrane cells was remarkably reduced after overexpression of microRNA-124 in TE-1 cells, indicating the inhibited migration and invasion abilities (Figure 3A and 3B). Similar results were obtained in the EC-109 cells (Figure 3C and 3D).

Overexpressed microRNA-124 Activated PI3K/AKT Pathway

We next explored the mechanism of microRNA-124 in promoting proliferation and migration of esophageal cancer cells. PI3K/AKT pathway-related genes were detected by Western blot. The data illustrated downregulated PTEN and upregulated PI3K and AKT after microRNA-124 overexpression (Figure 4).

MicroRNA-124 Inhibited Development of Esophageal Cancer Via NRP1

To further investigate how microRNA-124 promoted the malignant progression of esophageal cancer, we screened out the target gene of microRNA-124 through bioinformatics analysis. Here, NRP1 expression was detected by qRT-PCR and Western blot. The data showed that NRP1 was remarkably upregulated in esophageal cancer tissues than that of normal cancer tissues (Figure 5A). NRP1 was also expressed in esophageal cancer cells compared with that of HEEC cells (Figure 5B). Furthermore, we selected 16 pairs of esophageal cancer tissues and corresponding paracancerous tissues to detect the expression levels of microRNA-124 and NRP1. MicroRNA-124 was found to negatively regulate NRP1 in TE-1 and EC-109 cells (Figure 5C). Small interfere sequence of NRP1 was constructed and its transfection efficacy was verified by qRT-PCR (Figure 6A and 6B). Rescue experiments indicated that microRNA-124 decreased proliferation and invasion abilities by microRNA-124 overexpression were reversed by NRP1 overexpression in TE-1 cells, indicating that microRNA-124 promoted malignant progression of esophageal cancer by negatively regulating NRP1 (Figure 6C and 6D).

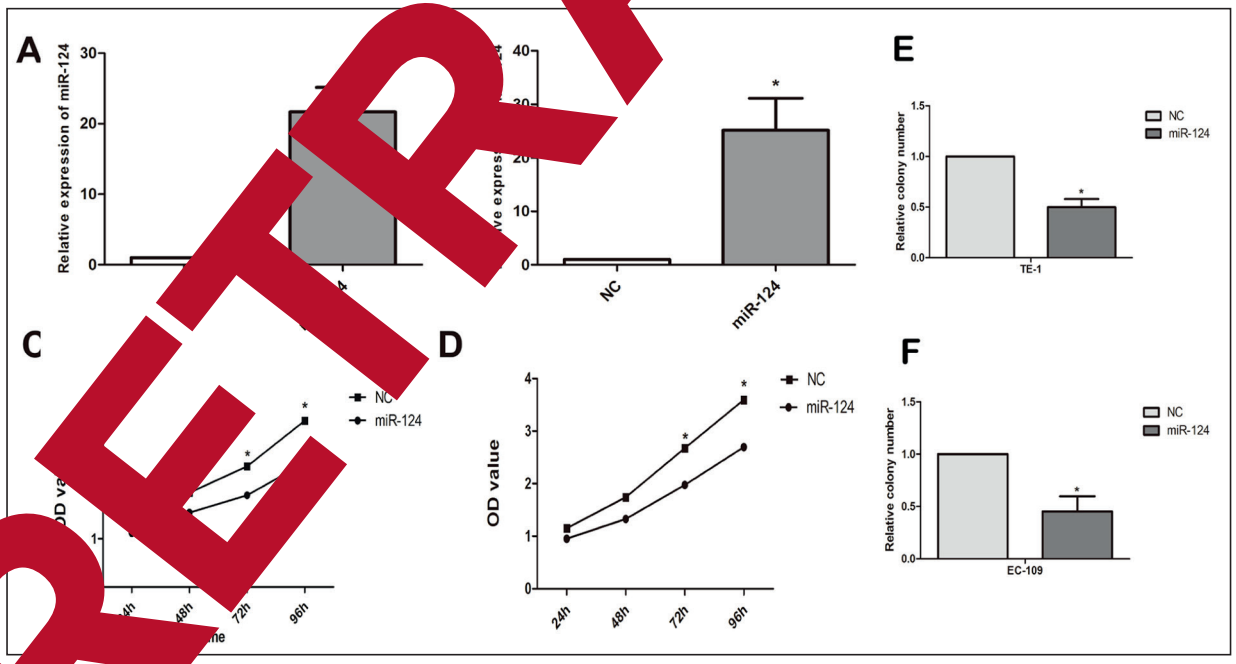


Figure 2. Overexpressed microRNA-124 inhibited cell proliferation. ***A, B,*** QRT-PCR was used to verify the transfection efficacy of microRNA-124 overexpression plasmid in TE-1 and EC-109 cells. ***C, D,*** Growth curve analysis showed the cell growth of TE-1 and EC-109 cells after microRNA-124 overexpression. ***E, F,*** Cell colony formation ability in TE-1 and EC-109 cells after microRNA-124 overexpression.

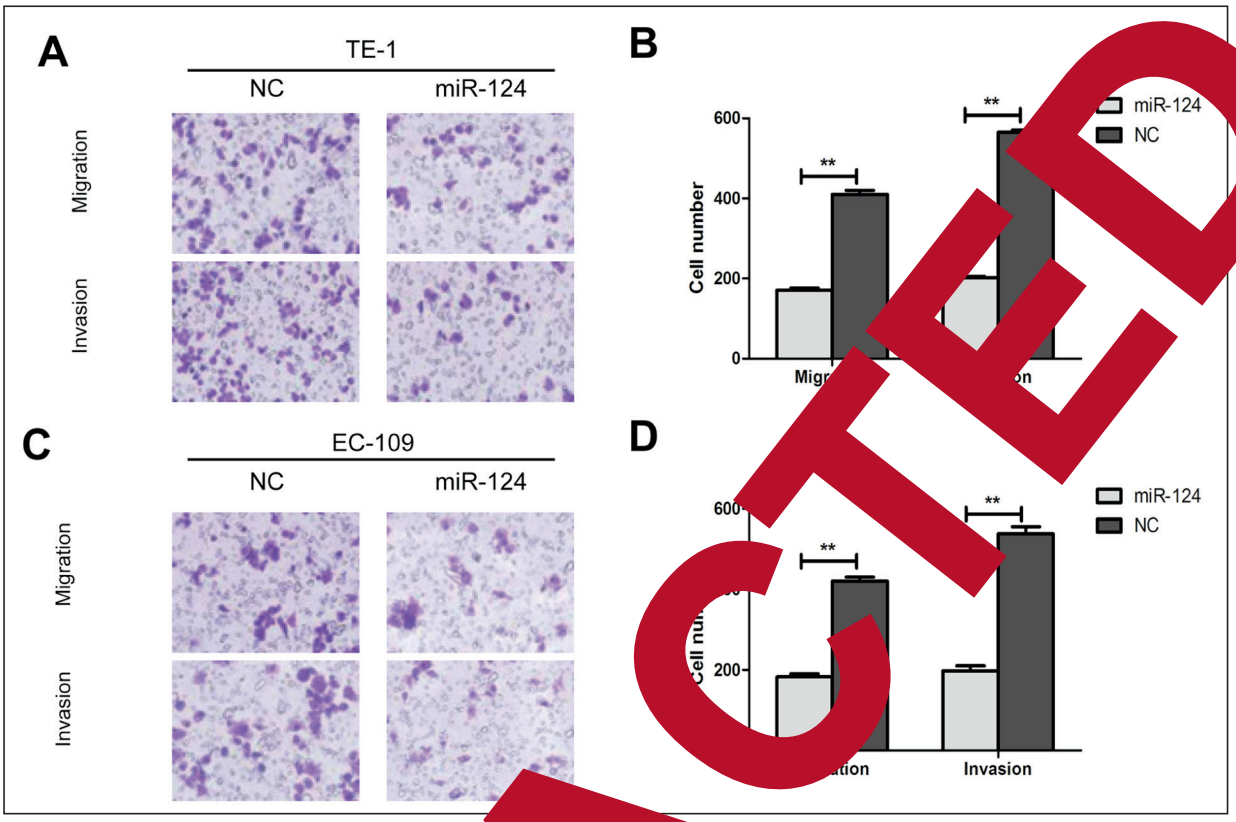
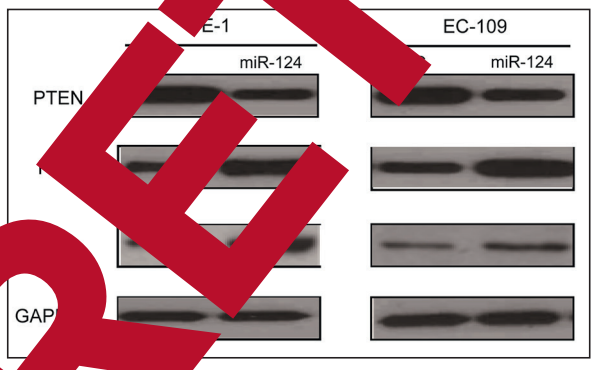


Figure 3. Overexpressed microRNA-124 inhib[ited] cell mi[gration] [and] invasion. ***A, B,*** TE-1 cells transfected with microRNA-124 overexpression plasmid displayed sig[nifica]ntly [lo]wer mi[gra]tion and invasion capacities. ***C, D,*** EC-109 cells transfected with microRNA-124 overexpression plasmi[d] [exhibited] significantly lower migration and invasion capacities.

Discussi[on]

Esophageal cancer i[s one] of [the] malignancies of the [upper] tract. Exploration of the pa[thogenesis] [and] development mechanisms is of [great] significan[ce] [to] improv-



[Fig]ure 4. Overexpressed microRNA-124 activated PI3K/[AKT] pathway. Overexpressed microRNA-124 significantly [de]creased expressions of P13K/AKT pathway-related genes, including PTEN, P13K and AKT.

[ing] the diagnosis and prognosis of patients with esophageal cancer[2,6]. Molecular genetic changes in esophageal cancer cells, such as alterations in gene copy numbers and coding sequences, could remarkably affect phenotypes of tumor cells[5]. In recent years, the incidence and mortality of esophageal cancer in China have gradually increased. The early diagnostic rate of esophageal cancer is extremely low, and most of these patients are in the advanced stage when first diagnosed[5,7,8]. Genetics, diet, unhealthy lifestyles, and precancerous lesions are all closely related to the occurrence of esophageal cancer. Clinically, over 50% of patients with esophageal cancer experienced micrometastases before radical surgery[5,26]. Therefore, early diagnosis, effective treatment, and postoperative adjuvant therapy of esophageal cancer have been well studied.

Current studies have confirmed that microRNAs possess significant biological functions in tumors, which provides new directions for better tumor treatment[14]. MicroRNAs are capable of regulating proliferation, apoptosis, and migration

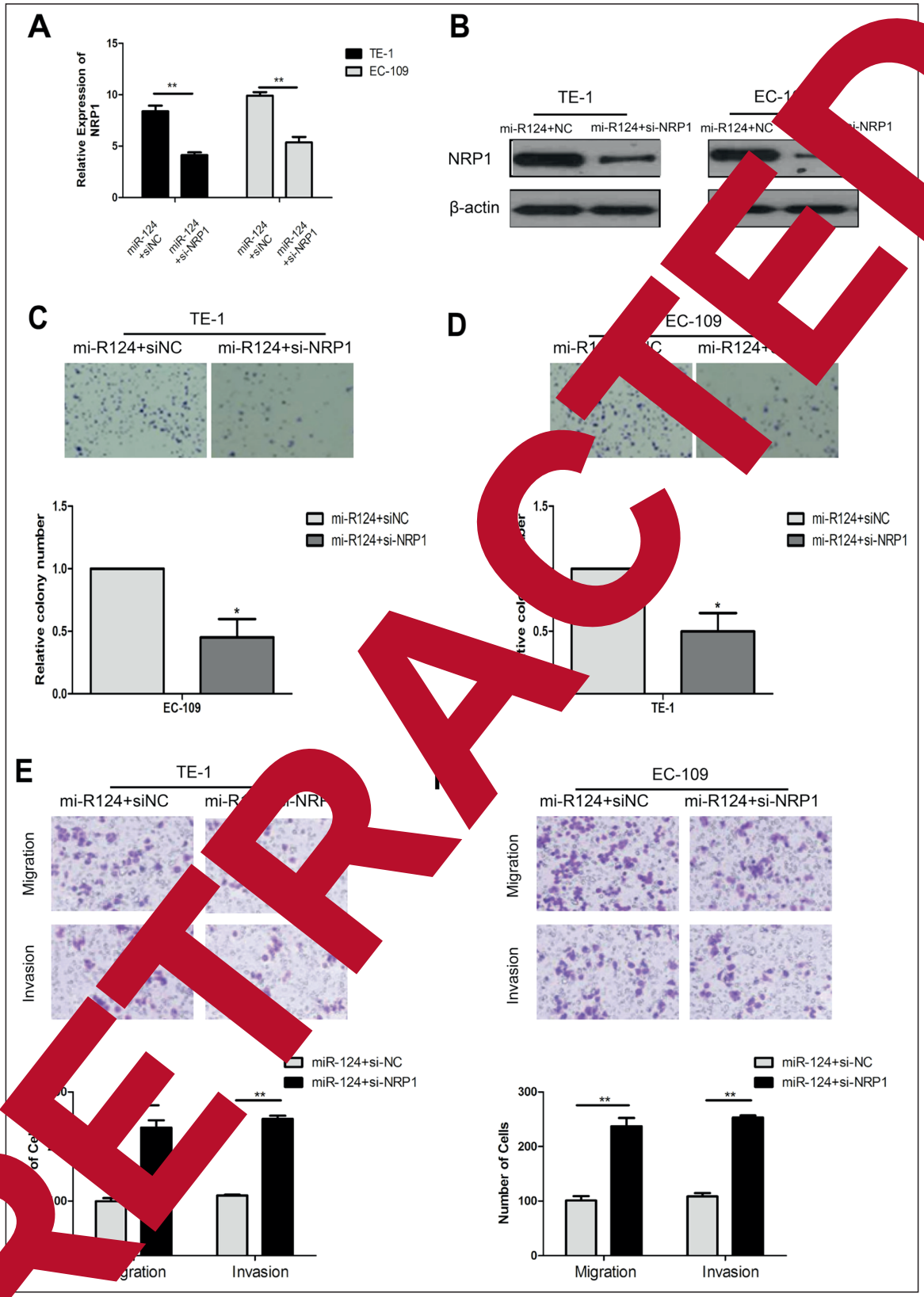


Figure 5. MicroRNA-124 inhibited development of esophageal cancer *via* NRP1. ***A, B,*** The mRNA expression level of NRP1 relative to GAPDH in human esophageal cancer tissues, paracancerous tissues, and cell lines were detected using qRT-PCR. ***C,*** A negative correlation was found between microRNA-124 and NRP1 in esophageal cancer samples.

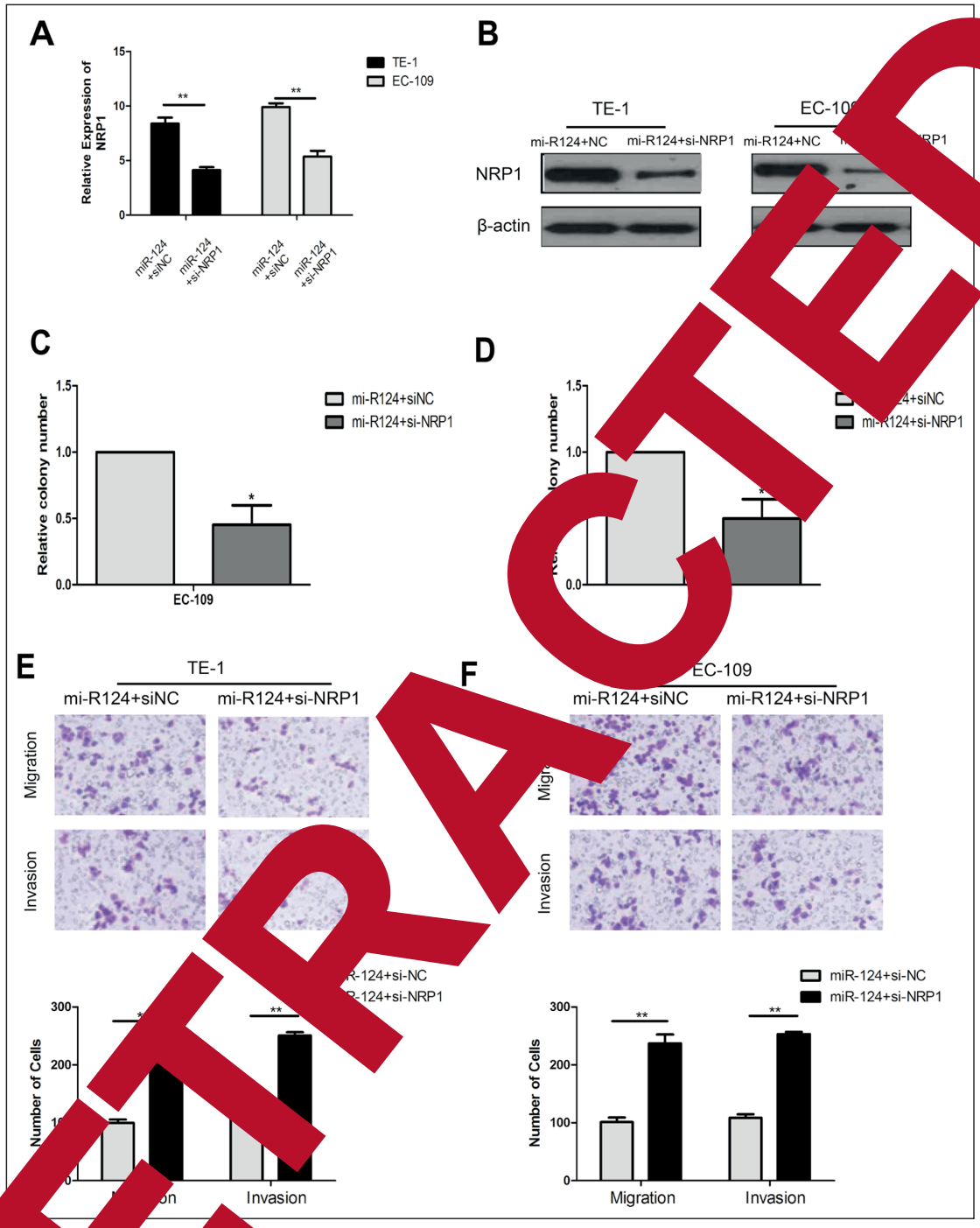


Figure RNA-12 ulated NRP1 expression. ***A,*** NRP1 expression was verified by qRT-PCR in co-transfected ll lines. rn bl s used to verify NRP1 expression. ***C, D,*** The roles of microRNA-124 and NRP1 in regulating n and ophageal cancer cells were examined by transwell assay. A representative data set was displayed as SD valu 0.05, $^{**}p < 0.01$).

of o as to affect the chemotherapy radiotherapy sensitivities[10,17]. It is reported there are many differentially expressed miRNAs in esophageal cancer tissues, which may fluence the diagnosis and prognosis of these patients. In the present study, we found that microRNA-124 was downregulated in esophageal cancer tissues than that of paracancerous tissues, which was negatively correlated with the pathological grade and prognosis of esophageal cancer.

We, therefore, speculated that microRNA-124 could inhibit the malignant progression of esophageal cancer. For *in vitro* experiments, overexpressed microRNA-124 remarkably inhibited proliferation, invasion, and migration of esophageal cancer cells.

PI3K/AKT pathway is considered to be involved in tumorigenesis[27]. The activity of PI3K/AKT pathway is negatively regulated by the lipid phosphatases PTEN and SHIP[27,28]. So far, no specific phosphatases have been found to be able to downregulate AKT activity. On the contrary, phosphatase inhibitors could increase the phosphorylation and activity of AKT. Recent studies showed that AKT can be inactivated by a c-terminal regulatory protein (cTMP) that binds to AKT and blocks downstream signaling by inhibiting AKT phosphorylation. Overexpression of cTMP prevents AKT from being inactivated by the dephosphorylation of PP2A phosphatase, thus protecting AKT activity[28,29]. PI3K/AKT pathway plays a pivotal role in the proliferation and metastasis of multiple cancers, such as breast cancer, colon cancer, lung cancer, prostate cancer, liver cancer, and pancreatic cancer[29-32]. In this [illegible] Western blot results showed that PTEN, protein in PI3K/AKT pathway, was downregulated after microRNA-124 overexpression, while PI3K and AKT were remarkably upregulated, indicating that microRNA-124 inhibits proliferation and metastasis of esophageal [illegible] *via* PI3K/AKT pathway.

Neuropilin 1 (NRP1) is a type I transmembrane glycoprotein expressed on [illegible] face, which belongs to the [illegible] family. NRP1 is a multifunctional receptor that exerts a crucial role in the nervous system, vascular system, and tumor. Traditionally, NRP1 not only participates in the regulation of neuronal guidance and axon growth, but also regulates activation, proliferation, and migration of endothelial cells[34]. Studies have shown that NRP1 is differentially expressed in various types of tumors such as bladder cancer and breast cancer, and is closely related to tumor cell growth and tumor angiogenesis. It has been reported that NRP1 expression is positively correlated with gliomas malignancy. Additionally, overexpressed NRP1 is also associated with lower overall survival of patients with prostate cancer. Cellular experiments have demonstrated that NRP1 could lead to metastasis of tumor cells *via* stimulating cell proliferation and increasing expressions of adhesion factors in epithelial cells[36]. In the present study, we found the interaction between microRNA-124 and NRP1 by the rescue experiment, which further provides a theoretical basis for diagnosing and treating esophageal cancer.

Conclusions

We showed that microRNA-124 was downregulated in esophageal cancer tissues, which was remarkably correlated with the development pathological grade, and poor prognosis of esophageal cancer. Overexpressed microRNA-124 was capable of inhibiting the malignant progression of esophageal cancer by negatively regulating NRP1.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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