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MiR-874-3p is an independent prognostic factor and functions as an anti-oncomir in esophageal squamous cell carcinoma via targeting STAT3

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Abstract. – OBJECTIVE: Emerging evidence has suggested that dysregulation of miR-874-3p may be involved in tumor development and progression in various types of cancers. However, its expression and biological function in esophageal squamous cell carcinoma (ESCC) remains unclear. The aim of this study was to explore the roles of miR-874-3p in ESCC tumorigenesis and development.

PATIENTS AND METHODS: Quantitative Real Time-PCR was used to detect the expression of related mRNAs and miRNA in both ESCC tissues and cells. Then, statistical analysis was performed to determine the associations of miR-874-3p expression with the clinical features and the prognosis of ESCC. Cells proliferation and metastasis were assessed by cell viability assay and transwell assay. Luciferase reporter assays and Western blot were performed to analyze the regulation of putative target of miR-874-3p.

RESULTS: We found that the expressions of miR-874-3p in ESCC tissues and cell lines were much lower than that in normal control, respectively. Also, there is a statistically significance between miR-874-3p expression level and lymph nodes metastasis and clinical stage. Kaplan-Meier analysis showed that decreased miR-874-3p expression was associated with poor overall survival of patients. Multivariate Cox regression analysis showed that the expression of miR-874-3p was an independent prognostic factor for ESCC patients. After miR-874-3p mimics transfection, cell proliferation, migration, and invasion were significantly suppressed in the ESCC cells. Mechanistically, STAT3 was confirmed to be the downstream target of miR-874-3p in ESCC cells.

CONCLUSIONS: We indicate that miR-874-3p could be a new therapeutic target and prognostic marker of ESCC.

Key Words:
miR-874-3p, Esophageal squamous cell carcinoma, Prognosis, Proliferation, Migration, Invasion, STAT3.

Introduction

Esophageal cancer is a common digestive tract malignant tumor worldwide with a variable geographic distribution of incidence and histologic subtypes[1]. Esophageal squamous cell carcinoma (ESCC) and adenocarcinoma are the two main forms of esophageal cancer, and both have diverse pathologic characteristics[2,3]. Approximately, 70% of global ESCC cases occur in China[4]. Despite advancement in surgical techniques combined with chemotherapy and/or radiotherapy, the prognosis of ESCC still remains poor due to the highly invasive and metastatic nature of the disease[5,6]. To develop novel treatment strategies, a better understanding of the molecular mechanisms underlying the development of ESCC is critical to facilitate the prevention and treatment of advanced ESCC. MicroRNAs (miRNAs) are noncoding RNAs that are 18-23 nucleotides in length that inhibit post-transcriptional gene expression by binding to target mRNA at their 3'-untranslated region (UTR)[7]. Since one miRNA targets many mRNAs, miRNAs have been found to play crucial roles in various biological processes, such as inflammation, cell cycle regulation, differentiation, and cellular migration[8,9]. Furthermore, growing evidence has suggested that the deregulation of miRNAs plays a crucial role in cancer formation and metastasis, serving as either oncogenes or tumor suppressors[10-12]. In addition, several miRNAs have been identified in ESCC. For instance, miR-1290 was reported to promote cancer progression by targeting nuclear factor I/X in ESCC[13]. MiR-145 was shown to serve as a tumor suppressor because its overexpression could inhibit proliferation and invasion of ESCC in part by targeting c-Myc[14]. Also, in clinical practice, miRNAs may be used as biomarkers for dia-

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gnostic, prognostic, and monitoring purposes[15,16]. miR-874-3p, a tumor-related miRNA, is located on chromosome 5q31.2. Recently, miR-874-3p has been proved to act as either a suppressor in various types of human cancers, such as breast cancer[17], neck squamous cell carcinoma[18], and pancreatic ductal adenocarcinoma[19]. Many *in vitro* and *in vivo* researches indicated miR-874-3p as a regulator involved in development and progression of various tumors. However, to our best knowledge, the expression pattern of miR-874-3p and its biological function in ESCC remains largely unknown. In this study, we detected miR-874-3p expression in lung ESCC tissues and cell lines. Meanwhile, the association between miR-874-3p expression and clinicopathological factors as well as survival rates were analyzed. Finally, the biological function and potential molecular mechanism of miR-874-3p was investigated in ESCC cells.

Patients and Methods

Patients and Samples

Samples of ESCC tissues and adjacent normal esophageal tissues were obtained from 121 ESCC patients who had undergone surgery at the Daqing Oil Field General Hospital between January 2011 and May 2013. There were 79 male and 42 female, and the average age was 53.4 (41 to 71) years. None of the patients had received preoperative chemotherapy or radiotherapy. The extent of the disease was determined by TNM staging based on the 7th IUCC/AJCC recommendations. Clinicopathological features of patients are summarized in Table I. Written, informed consent was obtained from each patient and approval was obtained from the Ethical Committee on Human Research of Daqing Oil Field General Hospital.

Cell Lines and MiR Transfection

All the cell lines, including human normal esophageal cell line (HEEC) and three human ESCC cell lines (ECA109, KYSE410, and TE-1), were purchased from Cell Bank of the Chinese Academy of Science (Xuhui, Shanghai, China). The cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL, Haidian, Beijing, China) supplemented with 10% fetal bovine serum (FBS), (Grand Island, NY, USA) as well as 100 U/ ml penicillin (Solulink, San Diego, CA, USA) and 100 μg/ml streptomycin (Cell Biologics, Chicago, IL, USA). Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

Table I. Association between miR-874-3p expression and different clinicopathological features of ESCC.

Clinicopathological features	No. of cases	miR-874-3p expression		
		High	Low	*p*-value
Gender				NS
Male	79	41	38	
Female	42	19	23	
Age (years)				NS
< 60	58	27	31	
≥ 60	63	33	30	
Tumor size (cm)				NS
< 4	66	37	29	
≥ 4	55	23	32	
Tumor location				NS
Upper/Lower	41	22	19	
Middle	80	38	42	
Histological grade				NS
G1	68	38	30	
G2+G3	53	22	31	
Tumor stage				NS
T1-T2	61	34	27	
T3-T4	60	26	34	
Lymph nodes metastasis				0.021
Absence	68	40	28	
Presence	53	20	33	
Clinical stage				0.011
I-II	75	44	31	
III-IV	46	16	30	

Table II. Primers used in this study.

Primers	Sequences (from 5′ to 3′)
miR-874-3p-F	GAACTCCACTGTAGCAGAGATGGT
miR-874-3p-R	CATTTTTTCCACTCCTCTTCTCTC
STAT3-F	GAAGAATCCAACAACGGC
STAT3-R	TCACAATCAGGGAAGCAT
GAPDH-F	GCACCGTCAAGGCTGAGAAC
GAPDH-R	TGGTGAAGACGCCAGTGGA

ECA109 and TE-1 cells (4 $\times 10^4$ cells per well) were seeded into 6-well culture plates and grown overnight. MiR-874-3p mimic and miR-negative control (miR-NC) were synthesized and purchased from RiboBio (Guangzhou, Guangdong, China). At 70% confluency, the cells were subjected to transfection using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

RNA Extraction, Reverse Transcription, and Quantitative RT-PCR

Total RNA was extracted from cells and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The extracted RNA was dissolved with RNAase-free water that was pretreated with diethyl pyrocarbonate. First-Strand cDNA Synthesis kit (Invitrogen, Carlsbad, CA, USA) was used to reversely transcribe from total RNA. Quantitative Real Time-PCR (qRT-PCR) was performed with the 7300 sequence detection system (Biosystems, Foster City, CA, USA) using SYBR Green Master Mix (Biosystems, Foster City, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous reference, and each sample was normalized to its GAPDH content. Relative expression levels of target genes were calculated by the $2^{-\Delta\Delta Ct}$ method. The primers were obtained from Tianlan Biotechnology (Xicheng, Beijing, China). Primer sequences are shown in Table II.

Cell Proliferation Assay

The cell proliferation was monitored by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 2×10^3 ECA109 and TE-1 cells were transfected with either miR-874-3p mimic or miR-NC and plated onto 96-well plates. After 0, 24, 48, 72, or 96 h, MTT reagent (Sigma-Aldrich, Xuhui, Shanghai, China) (20 μL) was added and cells were incubated for 4 h at 37°C. The absorbance of samples at 450 nm was measured using the Thermo Plate microplate reader (Rayto Life and Analytical Science Co. Ltd., Dusseldorf, Germany).

Cell Migration and Invasion Assays

For migration assay, 2×10^3 cells were seeded in FBS-free DMEM in the upper chamber of a 24-well transwell (Corning Incorporated, Corning, NY, USA). For invasion assay, 4×10^3 cells were seeded in FBS-free DMEM in the upper chamber of a 24-well Matrigel transwell invasion insert (BD Biosciences, San Jose, CA, USA). Then, the DMEM medium combined with 10% FBS which acted as chemoattractant was supplemented to the lower chamber. The cells were incubated for 48 h at 37°C, and then, the cells on the top surface of the membrane were removed through wiping with a cotton swab. Cells were incubated for 24 h and the cells that did not migrate or invade through the pores were removed by a cotton swab. Migrated and invaded cells were counted under a light microscope (Olympus, Tokyo, Japan).

Luciferase Reporter Assays

The 3'-UTR sequence of STAT3 predicted to interact with miR-874-3p or the mutated sequen-

Table III. Univariate and multivariate analysis of overall survival in ESCC patients.

Clinicopathological features	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	*p*	Hazard ratio	95% CI	*p*
Gender	0.977	0.546-1.934	0.355	-	-	-
Age	1.233	0.748-2.331	0.289	-	-	-
Tumor size	1.566	0.559-2.452	0.113	-	-	-
Tumor location	0.832	0.477-1.675	0.169	-	-	-
Histological grade	0.844	0.573-2.213	0.137	-	-	-
Tumor stage	1.348	0.854-2.567	0.094	-	-	-
Lymph nodes metastasis	3.137	1.437-5.328	0.008	2.669	1.216-4.378	0.024
Clinical stage	3.457	1.568-6.339	0.005	2.833	1.138-5.046	0.015
miR-874-3p expression	3.119	1.439-6.449	0.001	2.779	1.318-5.673	0.008

ce within the predicted target sites was synthesized and inserted into the pGL3 control vector (Promega, Xuhui, Shanghai, China). Cells (4×10^4) were seeded in triplicates in 48-well plates and allowed to settle for 24 h. Then, TE-1 cells were transfected with 50 nM miR-874-3p mimic or miR-NC and then co-transfected with the wild-type or mutant 3'-UTR of the STAT3 gene with 0.2 mg/ml vector (Ambion, Austin, TX, USA). Luciferase and control signals were measured at 48 h after transfection using the Dual-Luciferase Reporter Assay Kit (Promega, Madison, WI, USA), according to manufacturer's instruction. All assays were performed in triplicate.

Western Blot

Thirty-six hours after transfection, cells were harvested and lysed using RIPA Lysis Buffer (Pierce Biotechnology, Rockford, IL, USA). Protein was extracted, separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% nonfat milk in Tris-buffered saline (TBS) containing 0.1% Tween 20, and then, they were incubated with corresponding primary antibody. Primary antibodies against STAT3 (Wanleibio, Shenyang, Liaoning, China) were used at a dilution of 1:1500, against GAPDH (Wanleibio, Shenyang, Liaoning, China) which was used at a dilution of 1:2000. The membranes were washed and then incubated for 60 min with specific secondary antibodies. The blot was developed using enhanced chemiluminescence (ECL) solution (Beyotime, Shanghai, China) and photographed using the FluorChem imaging system (Alpha Innotech Corp, San Antonio, TX, USA).

Statistical Analysis

All the statistical analyses were performed using SPSS13.0 for Windows (SPSS Inc., Chicago, IL, USA). Data are shown as the mean ± standard deviation (SD). The differences between the two groups were analyzed using the Student's *t*-test. The x^2-test was applied to determine the association between the miR-874-3p expression and the clinicopathological parameter of ESCC. Multi-group comparisons of the means were carried out by one-way analysis of variance (ANOVA) test with post-hoc contrasts by Student-Newman-Keuls test. Patient survival and their differences were determined by Kaplan-Meier method and log-rank test. A Cox proportional hazards regression analysis was used for univariate and multivariate analyses of prognostic values. A *p*-value less than 0.05 was considered statistically significant.

Results

MiR-874-3p Expression Was Down-Regulated in Both ESCC Tissues and Cell Lines

To determine the biological function of miR-874-3p, we first determined its expression in ESCC patients. The results of qRT-PCR assay showed that the expression of miR-874-3p was significantly decreased in ESCC tissues compared to that in adjacent non-tumor tissues ($p < 0.01$, Figure 1A). Then, the expression level of miR-874-3p was examined in three human ESCC cell lines (ECA109, KYSE410 and TE-) and one human normal esophageal cell line (HEEC). As shown in Figure 1B, we found that miR-874-3p expression levels were lower in all three ESCC cell lines than in HEEC ($p < 0.01$). Subsequently, to explore whether miR-874-3p expression was correlated with clinicopathological features in OS patients, statistical analysis was performed. As shown in Table I, we found that low expressions of miR-874-3p were significantly associated with lymph nodes metastasis ($p = 0.021$), and advanced clinical stage ($p = 0.011$). But no significant association with other clinical factors. Taken together, our findings revealed that miR-874-3p expression was down-regulated in ESCC and its low levels were significantly associated with clinical progression of ESCC patients.

Relationship Between MiR-874-3p Expression and ESCC Patients' Survival

To further verify the potential clinical utility of miR-874-3p high expression, we investigated the association between miR-874-3p expression and survival of ESCC patients by Kaplan-Meier analysis and log-rank test. As shown in Figure 2, we found that patients with low miR-874-3p expression had a poor overall survival compared to patients with high miR-874-3p expression ($p = 0.016$), indicating that down-regulated miR-874-3p was closely associated with poor prognosis of ESCC patients. In addition, univariate and multivariate analyses were utilized to evaluate whether the miR-874-3p expression level and various clinicopathological features were independent prognostic parameters of patient outcomes. As shown in Table III, univariate analysis revealed that the

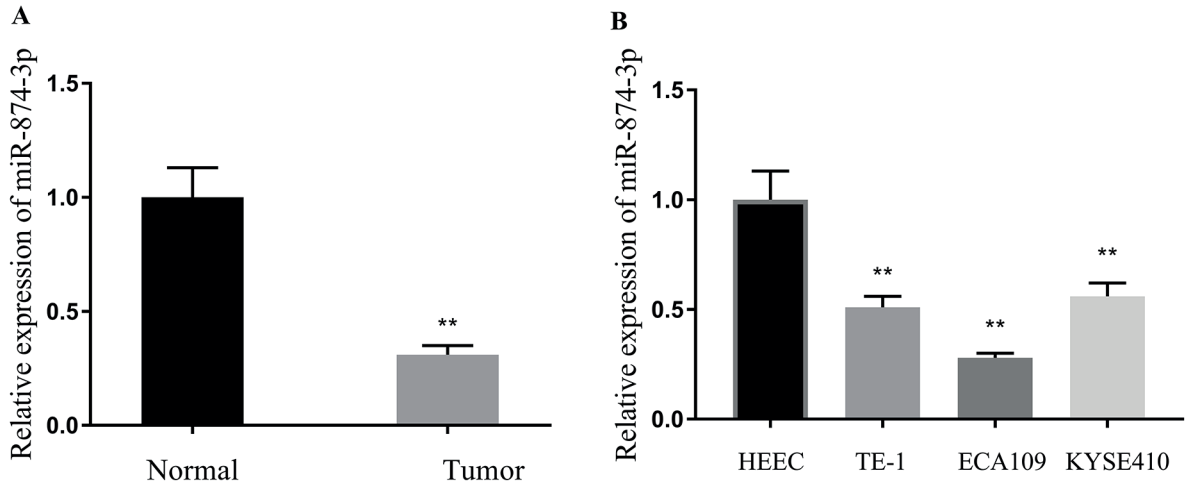

Figure 1. miR-874-3p was down-regulated in ESCC tissues and cell lines. ***(A)*** The expression of miR-874-3p in ESCC tissues and adjacent non-tumor tissues were examined by qRT-PCR. ***(B)*** The expression of miR-874-3p in the normal esophageal epithelial cells and ESCC cell lines (ECA10, KYSE410, and TE-1). $^{**}p < 0.01$.

relative level of miR-874-3p ($p = 0.001$), lymph nodes metastasis ($p = 0.008$), and clinical stage ($p = 0.005$) were significantly associated with overall survival of ESCC. Of note, multivariate analysis revealed that miR-874-3p expression ($p = 0.008$), lymph nodes metastasis ($p = 0.024$), and clinical stage ($p = 0.015$) were independent prognostic markers for ESCC.

Effects of MiR-874-3p on the Proliferation, Migration, and Invasion of ESCC Cells

After confirming the oncogenic role of miR-874-3p in ESCC, the functional effects of miR-874-3p on ESCC cells were explored. MiR-874-3p mimic/miR-NC was transfected into ESCC cell lines (ECA109 and TE-1). As shown in Figure 3A, the results of qRT-PCR showed that miR-874-3p mimic could significantly increase miR-miR-874-3p levels compared to negative control groups ($p < 0.01$). Then, MTT assay revealed that forced miR-874-3p expression significantly decreased ECA109 and TE-1 cells proliferation ability compared to that in the control group (Figure 3B and 3C). To measure the effect of miR-874-3p on tumor cell migration, the transwell apparatus assay was used. As shown in Figure 3D, we found that up-regulation of miR-874-3p significantly decreased migration of ECA109 and TE-1 cells. Also, overexpression of miR-874-3p inhibited the invasion ability of ECA109 and TE-1 cells when compared with control cells (Figure 3E). Therefore, our results demonstrated that overexpression of miR-874-3p expression can inhibit proliferation, migration, and invasion of ESCC cells.

STAT3 is the Target Gene of MiR-874-3p

To reveal the molecular mechanism underlying the inhibition of the proliferation and metastasis

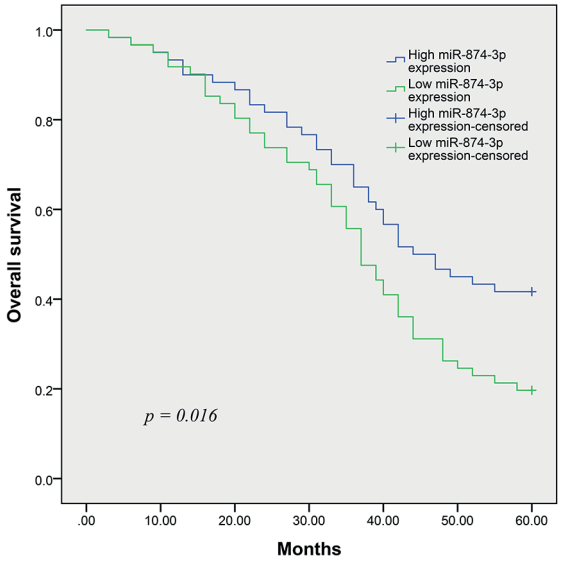

Figure 2. Kaplan-Meier survival curves of patients with ESCC based on miR-874-3p expression status. Patients in the low miR-874-3p expression group had significantly poorer prognoses than those in the high miR-874-3p expression group ($p = 0.016$, log-rank test).

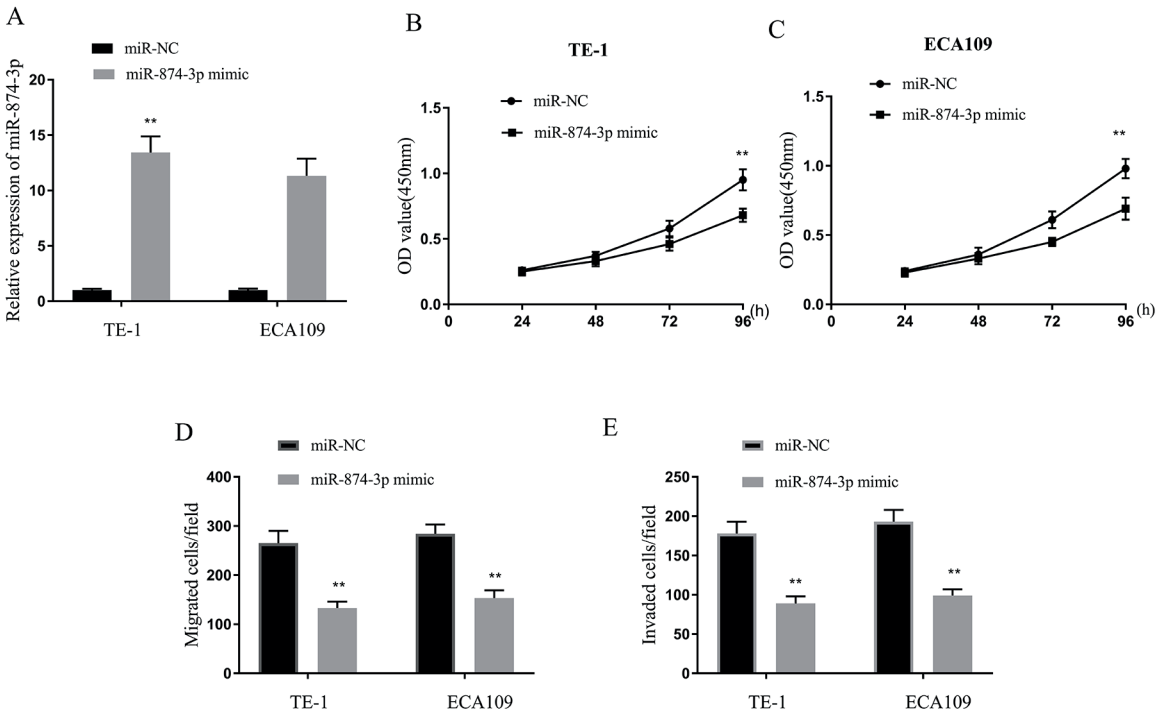

Figure 3. Effect of miR-874-3p on ESCC cell proliferation, migration, and invasion. ***(A)*** The expression of miR-874-3p in the ECA10 and TE-1 cell after transfected with miR-874-3p mimic or miR-NC using qRT-PCR. ***(B,C)*** MTT assay was performed to determine cell proliferation ability in ECA10 and TE-1 transfected with miR-874-3p mimic or NC. The cells invasion (D) and migration (E) were examined in ECA10 and TE-1 cells transfected with miR-874-3p mimic or NC. $^{**}p < 0.01$.

of ESCC cells by miR-874-3p, by using the miRNA database TargetScan, we identified a putative miR-874-3p binding site located in the 3'-UTR of STAT3 mRNA (Figure 4A). To further investigate whether STAT3 was a direct target of miR-874-3p, STAT3 3'-UTR was cloned into a luciferase reporter vector, and the putative miR-874-3 binding site in the miR-874-3p 3'-UTR was mutated. As shown in Figure 4B, the results of the luciferase assay showed that miR-874-3p suppressed STAT3 luciferase activities in TE-1 cells and this suppression of activity was abrogated by mutations in the miR-874-3p binding sites. To further study the association between miR-874-3p and STAT3, RT-PCR and Western blot were performed. We found that miR-874-3p overexpression could decrease STAT3 expression on mRNA level (Figure 4C) and protein level (Figure 4D). Taken together, our results suggest that there is a functional target site of miR-874-3p in the STAT3 3'UTR.

Discussion

The role of miRNAs in the development of ESCC remains ambiguous, and the discovery of new specific therapeutic targets may provide effective management of disease[20]. In this study, we first determined the expression levels of miR-874-3p in ESCC tissues and cells and found that miR-874-3p was significantly down-regulated in both ESCC tissues and cells, indicating that it may act as a negative regulator in the progression of ESCC. Clinically, low expression levels of miR-874-3p correlated with lymph nodes metastasis and advanced clinical stage, suggesting that miR-874-3p may be involved in the metastasis of ESCC cells. Kaplan-Meier analysis indicated that patients with high miR-874-3p expression had poorer overall survival compared with low miR-874-3p, which might show that miR-874-3p was an influential factor for the prognosis of ESCC. Notably, the results of univariate and multivariate analyses confirmed that expression of miR-874-3p could be an independent factor for predicting the prognosis of ESCC patients. Our findings firstly provided evidence that miR-874-3p may be a potential prognostic biomarker. Previous research has demonstrated the tumor-suppressive function of miR-874-3p in several human malignancies. For instance, Zhang et al[21] reported that miR-874 serves as a tumor suppressor by inhibiting an-

giogenesis through STAT3/VEGF-A pathway in gastric cancer. Dong et al[22] showed that miR-874 expression was significantly down-regulated in osteosarcoma and its forced expression could suppress cells proliferation and metastasis by targeting E2F3. Jiang et al[23] found that overexpression of miR-874 suppressed hepatocellular carcinoma cell epithelial-mesenchymal transition and further disrupted migration and invasion. Leong et al[24] revealed that miR-874-3p was lowly expressed in hepatocellular carcinoma and associated with poor prognosis of hepatocellular carcinoma patients. Further gain-function assay indicated that forced miR-874-3p expression suppressed cell proliferation and colony formation via down-regulation of PIN1 expression. Consistent with these finding, we demonstrated that miR-874-3p acted as a suppressive role in ESCC growth and metastasis. These data suggested that miR-874-3p may be a therapeutic target in ESCC. Signal transducer and activator of transcription 3 (STAT3) is a transcription factor which in humans is encoded by the STAT3 gene[25]. As a transcription activator and an oncogene, STAT3 is frequently detected with persistent activation in most human cancer cell lines and tumor tissues[26]. Increasing evidence[27,28] shows that STAT3 plays a critical role in many physiologic processes, including cell differentiation, proliferation, and apoptosis. Previously, several miRNAs have been shown to exert their tumor-suppressive role by targeting STAT3 in various tumors, such as miR-124[29], MiR-1181[30], and miR-506[31]. In this work, STAT3 was identified as a potential target gene of miR-874-3p using bioinformatics analysis. Zhao et al[32] reported that miR-874 inhibits cell growth and induces apoptosis by targeting STAT3 in human colorectal cancer cells. However, whether miR-874-3p exerts its anticancer role in ESCC by targeting STAT3 remains unknown. To validate the STAT3 gene as the target gene of miR-874-3p, luciferase reporter was employed, and the results confirmed STAT3 as a direct target gene of miR-874-3p. We also found that overexpression of miR-874-3p could suppress the expression of STAT3 on both mRNA and proteins levels. Taken together, our finding indicated that miR-874-3p suppressed ESSC cells proliferation, migration, and invasion by targeting STAT3.

Conclusions

We indicated that miR-874-3p was down-regulated in ESCC and that down-regulated miR-874-3p was associated with worse prognosis. The

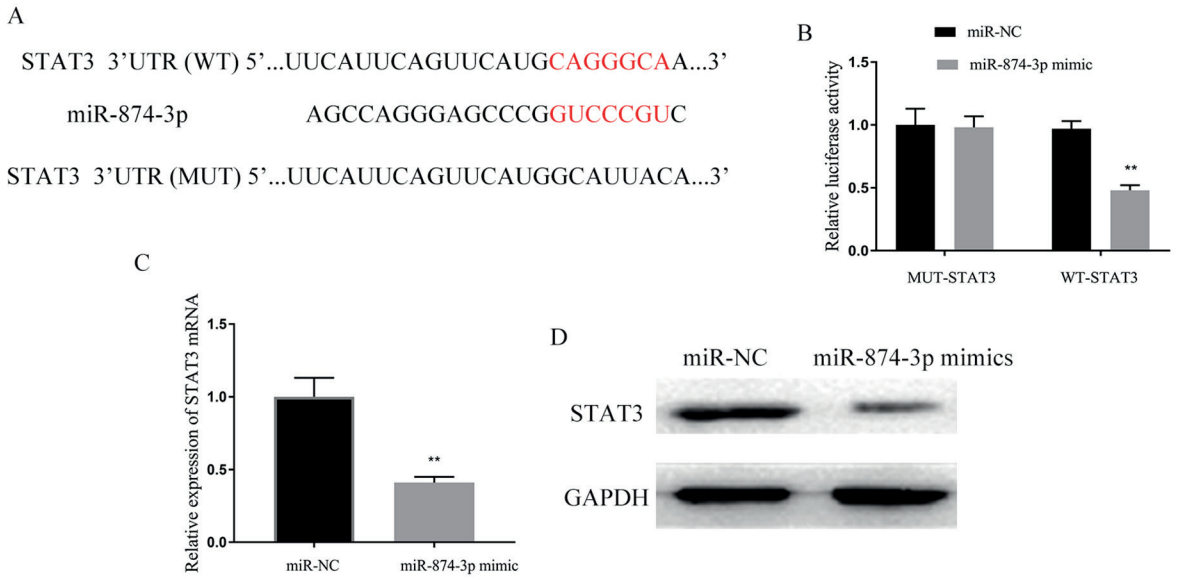

Figure 4. miR-874-3p targets and regulates STAT3. ***(A)*** Diagram of the predicted miR-874-3p targeting site in the 3'-UTR of STAT3. ***(B)*** Dual-luciferase reporter assay proved that miR-874-3p directly targeted STAT3 by binding its 3'UTR in the TE-1 cells. ***(C)*** STAT3 mRNA expression levels were detected by qRT-PCR in TE-1 cells transfected with the miR-874-3p mimics or miR-NC. ***(D)*** STAT3 proteins expression was measured by Western blot in TE-1 cells transfected with the miR-874-3p mimics or miR-NC. $^{**}p < 0.01$.

ectopic expression of miR-874-3p inhibited ESCC cells proliferation, migration, and invasion *in vitro*. Further assay revealed that STAT3 was a direct and functional target of miR-874-3p in ESCC cells. These findings demonstrated that miR-874-3p could serve as a potential biomarker and therapeutic target for ESCC.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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