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ORIGINAL RESEARCH

LincRNA-ROR promotes metastasis and invasion of esophageal squamous cell carcinoma by regulating miR-145/FSCN1

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Background and objective: In an attempt to discover a new biomarker for early diagnosis and prognosis of esophageal squamous cell carcinoma (ESCC), the regulation mechanism of large intergenic non-coding RNA–regulator of reprogramming (lincRNA-ROR) as a microRNA (miRNA) sponge was studied.

Patients and methods: ROR expression in 91 pairs of ESCC tissue samples and matched adjacent tissues was quantified with real-time fluorescent quantitative polymerase chain reaction (qRT-PCR). The ROR–miRNA–mRNA regulatory network was built with 161 esophageal cancer (EC) tissues and 11 adjacent tumor tissues from The Cancer Genome Atlas (TCGA) database. A total of 96 cases of ESCC from TCGA database were collected for analysis on survival rates. The regulatory relationship between ROR, miR-145 and FSCN1 was verified in ESCC cells via qRT-PCR, dual luciferase reporter (DLR) assay, RNA immunoprecipitation (RIP) and Western blotting. The transwell method was used to detect cell migration and invasion.

Results: ROR expression in ESCC tumor tissues was significantly higher than in the adjacent tissues, $p<0.001$. The survival rate of ESCC patients with high ROR expression levels was lower than that of patients with low ROR expression levels ($p<0.001$). ROR overexpression could downregulate miR-145 by up to 50% was proven by RIP, DLR assay, and qRT-PCR. Two effective binding sites of ROR to miR-145 were verified by DLR assay. One of the sites has never been cited in the literature. The Western blotting results showed that FSCN1 was a downstream target of ROR/miR-145 ($p<0.05$). Transwell assays were used to show that overexpression of ROR enhanced migration and invasion behavior of ESCC and miR-145 hindered these effects.

Conclusion: ROR acted as a competitive endogenous RNA (ceRNA) of miR-145 in ESCC. A novel, effective miR-145 binding site of ROR was discovered. The ROR/miR-145/FSCN1 pathway was shown to take part in the metastasis of ESCC. ROR is likely an oncogene biomarker for ESCC early diagnosis and prognosis.

Keywords: ESCC, lncRNA, ceRNA, ROR, miR-145, FSCN1

Introduction

Esophageal cancer (EC) is the eighth most common cancer, the second most common cancer in China, and the sixth most common cause of cancer death worldwide.[1–3] Previous research indicated that the incidence of EC in China is ~23.9/100,000, with a 5-year survival rate of 20%–30%.[4–6] In China, >90% of EC are esophageal squamous cell carcinoma (ESCC).[3,7] ESCC has a high propensity for lymphatic spread via their rich network of submucosal lymphatic vessels, with a 5-year survival rate of 15%–34%.[4,7,8] Identifying biomarkers that could predict the prognosis of ESCC

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is needed. This would be instructive for therapeutic decision making as well as screening and early detection.

There are various long non-coding RNAs (lncRNAs) in ESCC that are proven to be dysregulated, such as HOTAIR, SPRY4-IT1, UCA1, and TUG1.[9–13] LncRNAs are RNA polymerase II transcripts that are longer than 200 nucleotides and lack an open reading frame. There are >10,000 types of lncRNAs that are thought to play crucial roles in development and differentiation of human disease, particularly tumor development.[14–21]

Among lncRNAs, the large intergenic non-coding RNAs (lincRNAs) are numerous and their functions are better understood. Several studies have proposed a model that suggests lincRNA function as a competitive endogenous RNA (ceRNA) when modulating concentration and biological functions of microRNAs (miRNA).[22–24]

The regulator of reprogramming (ROR, linc-ROR) was first identified in induced pluripotent stem cells (iPSCs).[25] ROR is ~2.6 kbp in length. It is also known as lincRNA-ST8SIA3, and it is located on chromosome 18 (hg19 chr18: 57, 054, 571–557, 072, 119) containing four exons. ROR is highly expressed in self-renewing human embryonic stem cells, iPSCs, and various cancer cells, including breast cancer, hepatocellular carcinoma, endometrial cancer, and pancreatic cancer.[26–30] There is a positive correlation between the expression level of ROR and the undifferentiation degree of the carcinoma tissues. ROR has been proven to have many functions in human cancers, including promoting the epithelial–mesenchymal transition, enhancing the hypoxia resistance of the tumor tissue, and reducing the sensitivity of the tumor to chemotherapy.[26–32] ROR is a known negative regulator of the p53 pathway.[33,34] ROR competitively inhibits cancer-suppressive miRNAs, such as miR-145, miR-205, and miR-124, in order to upregulate cancer-promoting transcription factors including Oct4, Sox2, and Nanog.[23,25,35–37]

The function of ROR in ESCC was investigated by building an ROR–miRNA–mRNA regulatory network based on The Cancer Genome Atlas (TCGA) database. Our previous study proved that the miR-145 was downregulated in ESCC and promoted lymph node metastases by regulating FSCN1. This corresponds with the aforementioned regulatory network.[38] It is proposed that ROR can accelerate the development of ESCC by sponging miR-145 and upregulating FSCN1. This study implemented an expression detection of ROR in both cell lines and tissues of ESCC, in order to study the relationship between ROR and ESCC incidence risk. The cox survival analysis of 96 cases collected from TCGA database was done to study the effects of ROR on ESCC prognosis. FSCN1 has been reported to be upregulated in many human carcinomas and positively correlated with the clinical aggressiveness of tumors, particularly invasion and metastasis,[39–41] indicating a poor prognosis. Transwell migration and invasion assays were performed to determine whether ROR could stimulate ESCC invasion and migration by regulating miR-145/FSCN1.

Patients and methods

Cell lines and culture conditions

EC cell lines, EC109 and EC9706, and immortalized esophageal epithelial cell line, H5E46, were provided by the Key Laboratory of Environmental Medicine Engineering of Southeast University, Ministry of Education, China. The cells were cultured in a humidified incubator at 37°C with 5% CO_2 in RPMI-1640 medium containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA).

Clinical tissue samples

Huaian is considered a high-risk region for EC in China. The 91 pairs of ESCC tissues and the matched tissues adjacent to carcinoma (>5 cm away from the cancer tissue) were all collected from the Department of Thoracic Surgery of Huaian First People's Hospital of Jiangsu Province, 2010. The patients whose tissue samples were used had provided written informed consent for their use in research. The inclusion criteria of the volunteers were as follows: 1) diagnosed with ESCC by pathological section or endoscope; 2) without radiotherapy or chemotherapy treatment; 3) >20 years of residence in Huaian; and 4) Han nationality. Ethical approval was provided by the institutional review board of the Southeast University, Affiliated Zhongda Hospital (Nanjing, China).

The extraction and purification of total RNA

The total RNA was extracted using the Trizol (Thermo Fisher Scientific) method. The RNA was then purified by DNase and a RNA centrifugal column purification kit (Tiangen, Beijing, China) in order to erase genome pollution. The purified RNA samples were stored at −80°C.

Real-time fluorescent quantitative polymerase chain reaction (qRT-PCR)

Reverse transcription (RT) of ROR was performed with a PrimeScript RT Master Mix Kit (Takara, Shiga, Japan). The RT reaction was performed in a gradient gene amplification instrument (Eppendorf, Hamburg, Germany). The fluorescent quantitative PCR was performed with a SYBR Premix Ex Taq II Kit (Takara), in accordance to the manufacturer's instructions. The reaction was performed in a StepOnePlus

System (Thermo Fisher Scientific). The ROR expression analysis was normalized with β-actin. The sequences of ROR and β-actin primers are listed in Table 1.

The RT reaction of miR-145 was implemented with deoxy-ribonucleoside triphosphate, moloney murine leukemia virus RT, 5× moloney murine leukemia virus buffer, RNase inhibitor (Thermo Fisher Scientific), and miRNA-specific Bulge-Loop RT Primer (RIBOBIO, Guangzhou, China). The reaction was performed in a gradient gene amplification instrument (Eppendorf).

MiR-145 was downregulated in ESCC, so prior to the fluorescent quantitative PCR preamplification of miR-145 for 12 cycles was added. The reaction system was a 20 μL mixture of cDNA 1 μL, Bulge-Loop Forward and Reverse Primers each 1.6 μL, 10× PCR buffer 2 μL, 2.5 mM deoxy-ribonucleoside triphosphate 1.6 μL, Taq DNA polymerase 0.2 μL (Takara), and RNase-free water 12 μL. The reaction condition was 94°C for 5 min, 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, 12 cycles, and 72°C for 5 min. The reaction kit and the quantitative PCR instrument were the same as ROR. The analysis of the miR-145 expression was normalized with U6. Quantitative PCR reaction of cell samples was repeated three times.

Establishment of stable ROR overexpressing ESCC cell line

EC109 was used to establish ROR high-level expression cell line, where it was transfected by the lentivirus expressing ROR and green fluorescent protein with puromycin resistance. The multiplicity of infection (MOI) was 20. The cells were cultured in a complete RPMI-1640 medium with puromycin (2 μg/mL) after the green fluorescence was observed with a fluorescence microscope. The stable expression cell line was established at the 8th passage. The transfection efficiency of the ROR was confirmed with qRT-PCR. The ROR expression in the positive group was 16,000 times higher than that in the negative control (NC) group.

ROR–miRNA–mRNA regulatory network

A total of 161 EC tumor samples and 11 adjacent tumor samples were collected from patients by TCGA database. The screening criteria were as follows: $p<0.05$, false discovery rate (FDR) <0.1, and fold change (FC) ≥ 2. A random variance model *t*-test was used to filter the differentially expressed genes in ESCC. The target miRNAs of ROR were predicted by miRanda. The target mRNAs of the miRNAs were predicted by miRanda and Targetscan.

RNA immunoprecipitation (RIP)

The RIP assay was performed in accordance to the manufacturer's instructions of the EZ-Magna RIP Kit (EMD Millipore, Billerica, MA, USA). The EC109 cells were divided into two groups: the ROR overexpressing group and the NC group. Each sample was immunoprecipitated by mouse IgG and Argonaute2 (AGO2) antibodies (EMD Millipore). The RIP procedure was subjected to qRT-PCR for detection of miR-145. The miR-145 expression was relatively low in ESCC cells. A 12-cycle pre-amplification was performed after RT and before quantitative PCR. The cycle threshold values of the miR-145 in the anti-AGO2 groups were normalized with the input groups.

DLR assay

The grouping of cells (EC109) is shown in Table 2. The cells were seeded in 48-well plates at a density of 2.5×10^4 cells per well, 24 h prior to transfection. The cells were cotransfected with a mixture of conditioned vector pmiR-GLO (Genewiz, Suzhou, China) and miRNA mimics. The three binding sites between miR-145 of the ROR are shown in Figure 1. They were mutated in this experiment.[26,29] Site 805-828 has not been reported in previous literature. The miR-145 and the NC mimics were obtained from TranSheepBio (Shanghai, China). The luciferase activity was measured with a DLR assay system (Promega Corporation, Madison, WI, USA) and a LB942 Modular Multimode Microplate Reader (Berthold, Bad Wildbad, Germany) after 48 h. The hLuc activity was normalized with the hRluc activity for comparison.

Western blotting

The cells were washed with phosphate-buffered saline and lysed in a radioimmunoprecipitation assay buffer (Thermo Fisher Scientific) that contained a protease inhibitor. The protein concentrations were quantified with a BCA Protein Assay Kit (Thermo Fisher Scientific). Proteins were separated with

Table 1 Primers of ROR and ACTB

Gene	Primers	
ROR	Forward	5′-CTTGATGGCATTGTCGCTAA-3′
	Reverse	5′-TCCAGTGGCTGTGCTAGATG-3′
ACTB	Forward	5′-CCACTGGCATCGTGATGGA-3′
	Reverse	5′-CGCTCGGTGAGGATCTTCAT-3′

Abbreviation: ROR, regulator of reprogramming.

Table 2 Grouping of DLR assay

Treatment	Group 1	Group 2	Group 3	Group 4	Group 5
Plasmid	ROR (WT)	ROR (WT)	MUT1	MUT2	MUT3
RNA mimic	NC	miR-145	miR-145	miR-145	miR-145

Abbreviations: DLR, dual luciferase reporter; NC, negative control; ROR, regulator of reprogramming.

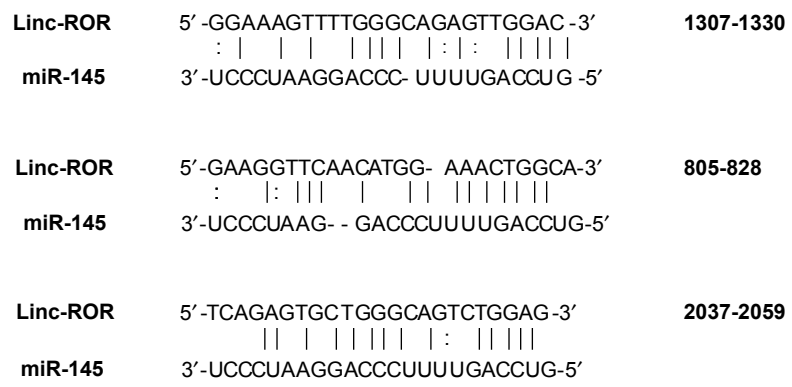


Figure 1 The three miR-145 binding elements in ROR in order (site 805-828 has not been previously reported).
Abbreviation: ROR, regulator of reprogramming.

a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to the polyvinylidene fluoride (PVDF) membranes. The membranes were incubated overnight at 4°C with rabbit monoclonal anti-human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1,000, Cell Signaling Technology) and rabbit polyclonal anti-human Fascin (1:10,000, Abcam, Cambridge, MA, USA). The anti-rabbit IgG and horseradish peroxidase-linked antibody (1:2,000; Cell Signaling Technology, Danvers, MA, USA) were washed and applied to the cells at room temperature for 1 h. The signals were detected with the SuperSignal West Femto Trial Kit (Thermo Fisher Scientific).

Transwell migration and invasion assay

The transwell assay was performed with a 6.5 mm in diameter and an 8-mm pore size chamber (Corning Incorporated, Corning, NY, USA). The invasion assay was performed 24 h after transfection, where 3×10^5 cells in the RPMI-1640 medium supplemented with 0.1% FBS were seeded onto the Matrigel-coated membrane matrix of the upper chamber. The lower chamber was filled with RPMI-1640 medium, supplemented with 10% FBS. The chamber was incubated for 24 h, and the cells that were invading the bottom of the membrane were fixed with methyl alcohol and stained with 0.1% crystal violet. The number of invaded cells was counted with a FSX100 bioimage navigator (Olympus Corporation, Tokyo, Japan).

The migration assay was performed 24 h after transfection, where 2×10^5 cells in RPMI-1640 medium were seeded in the upper chamber. The lower chamber was filled with a RPMI-1640 medium supplemented with 10% FBS. The fixation, staining, and counting methods of migrated cells were the same as the invasion assay.

Table 3 Relative expression of the ROR in the three cell lines

Cell lines	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$	*p*-value
H5E46	21.442±0.771			
EC109	18.007±0.217	−3.435±0.570	10.815	0.002
EC9706	16.560±0.463	−4.882±0.998	29.487	0.001

Abbreviation: ROR, regulator of reprogramming.

Statistical analysis

The quantitative variables were compared by the Student's *t*-test or the analysis of variance (ANOVA). The data were expressed as the mean value ± SD. $p<0.05$ was considered statistically significant. A cox regression analysis was used to detect the relationship between the risk of ESCC incidence and the ROR expression. The survival analysis was used to study the relation between the ROR expression and the prognosis of ESCC. The statistical analysis and the graph presentation were performed by SPSS 18.0 and GraphPad Prism 5 software.

Results

Highly upregulated ROR in ESCC cells and tissues

The ROR expression levels in ESCC cell lines and clinical sample tissues were determined by the qRT-PCR assay. Table 3 and Figure 2 show that the expression level of ROR

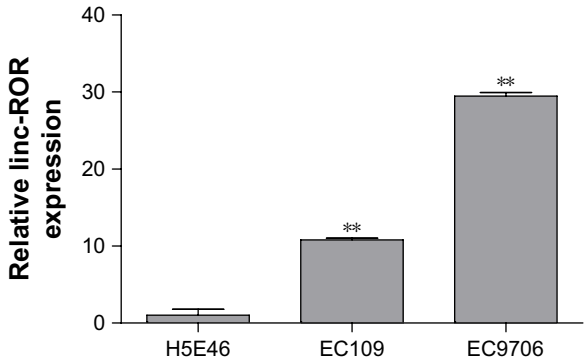


Figure 2 The ROR expression was higher in ESCC cell lines EC109 and EC9706 than the immortalized esophageal epithelial cell line H5E46 (**$p<0.01$).
Abbreviations: ESCC, esophageal squamous cell carcinoma; ROR, regulator of reprogramming.

Table 4 ROR expression was higher in the ESCC tissues than in the adjacent tissues

Group	N	ΔCT	Fold change	OR (95% CI)
Tumor	91	11.147±2.993	1.717***	2.138 (1.376–3.323)**
Normal	91	12.057±3.277	1.000	

Note: **$p<0.01$ and ***$p<0.001$.
Abbreviations: ESCC, esophageal squamous cell carcinoma; OR, odds ratio; ROR, regulator of reprogramming.

in the two ESCC cell lines was 10.8 times and 29.5 times higher than the normal esophageal epithelial cell line.

The qRT-PCR results of the 91 pairs of ESCC tumor tissues and the adjacent tissues are shown in Table 4. The ROR expression in ESCC tissues was significantly higher than normal esophageal epithelial tissues. The high ROR level could increase the risk of ESCC. The relative ROR expression in tumor tissue and normal tissue is shown in Figure 3.

High ROR expression level could reduce the survival rate of ESCC patients

The 96 cases of ESCC provided by TCGA database were collected to perform the survival analysis of ROR expression, using age as the covariate. The SPSS 18.0 results are shown in Figure 4. This result showed that high ROR level reduced the survival rate of ESCC patients.

An ROR–miRNA–mRNA regulatory network was built with TCGA database

There were 82 miRNAs ($p<0.001$, FDR <0.1, and FC ≥2) and 1,398 mRNAs ($p<0.001$, FDR <0.05, and FC ≥2) after the differential screening of the TCGA EC data with the random variance model *t*-test. Out of the 82 miRNAs, 11 were predicted to bind to ROR by miRanda and were downregulated in the EC. The target miRNAs and the binding sites are shown in Table 5. Out of the 1,398 mRNAs, 45 were targets of the 11 miRNAs and were upregulated in EC. The ROR–miRNA–mRNA regulatory network is shown in Figure 5. Our previous work suggested that miR-145 decreased in

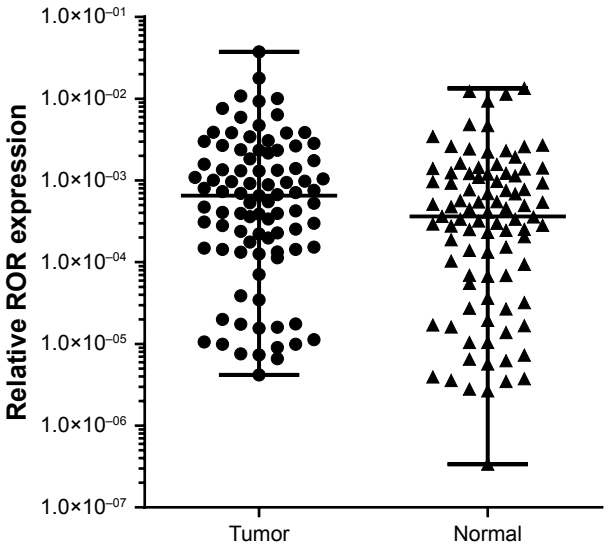


Figure 3 Relative ROR expression in tumor tissue and normal tissue ($p<0.001$).
Abbreviation: ROR, regulator of reprogramming.

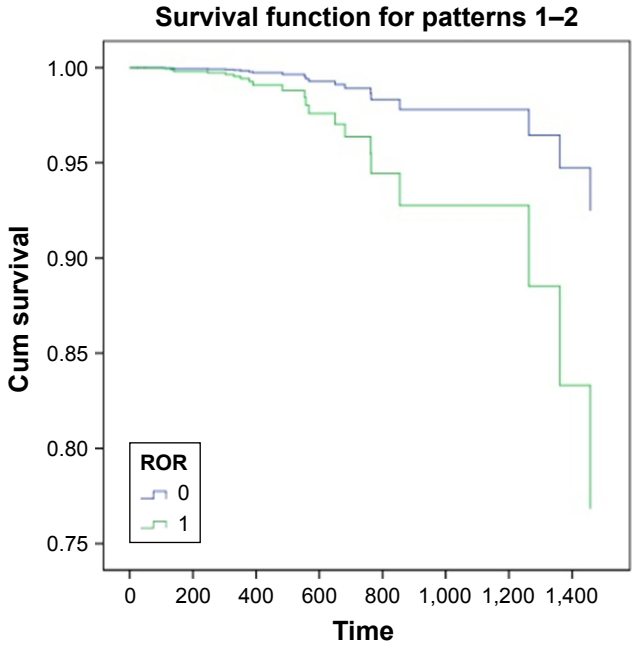


Figure 4 Survival curve of ESCC patients (0= low ROR expression, 1= high ROR expression, and time = day; $p<0.001$).
Abbreviations: ESCC, esophageal squamous cell carcinoma; Cum, Cumulative; ROR, regulator of reprogramming.

Table 5 The 11 target miRNAs of ROR and their binding sites

miRNA name	Align score	Energy	miRNA start	miRNA end	ROR start	ROR end
has-miR-29b-2-5p	147	−16.17	2	21	630	655
has-miR-129-5p	146	−15.05	2	19	1,650	1,670
has-miR-148a-3p	160	−15.42	2	21	2,507	2,526
has-miR-30c-2-3p	156	−16.07	2	19	1,767	1,792
has-miR-30c-2-3p	153	−15.67	2	19	1,138	1,160
has-miR-30c-2-3p	142	−15.85	2	21	1,952	1,975
has-miR-204-5p	141	−16.09	2	10	539	560
has-miR-153-3p	141	−15.03	2	10	1,496	1,517
has-miR-125a-5p	165	−24.98	2	23	928	952
has-miR-29c-3p	147	−15.74	3	21	419	441
has-miR-29c-3p	144	−14.31	3	17	2,529	2,550
has-miR-378a-5p	150	−18.41	2	21	1,689	1,712
has-miR-20b-5p	143	−16.96	3	22	1,321	1,342
has-miR-145-5p	134	−25.71	2	20	1,307	1,330
has-miR-145-5p	133	−18.48	3	21	805	828
has-miR-145-5p	128	−19.96	2	21	2,037	2,059

(*Continued*)

Table 5 (*Continued*)

miRNA name	Align score	Energy	miRNA start	miRNA end	ROR start	ROR end
has-miR-145-5p	127	−11.07	2	20	1,291	1,310
has-miR-145-5p	120	−11.35	2	8	207	229
has-miR-145-5p	120	−13.08	2	8	679	701
has-miR-145-5p	120	−17.6	2	21	1,144	1,166
hsa-miR-145-5p	119	−18.87	4	22	774	798
hsa-miR-145-5p	118	−10.76	2	11	2,282	2,304
hsa-miR-145-5p	118	−9.54	2	11	2,294	2,316
hsa-miR-145-5p	117	−16.64	2	15	1,521	1,546
hsa-miR-145-5p	110	−18.93	3	22	2,012	2,032
hsa-miR-145-5p	110	−13.77	4	20	2,427	2,450
hsa-miR-145-5p	108	−16.94	2	13	1,406	1,428
hsa-miR-145-5p	105	−14.76	3	22	1,553	1,575
hsa-miR-145-5p	104	−9.94	2	8	254	276
hsa-miR-145-5p	104	−13.5	4	22	315	339
hsa-miR-145-5p	104	−10.03	3	21	1,367	1,390
hsa-miR-145-5p	103	−10.4	2	12	478	500
hsa-miR-145-5p	103	−11.56	2	12	2,245	2,268
hsa-miR-145-5p	102	−12.31	3	20	2,521	2,547
hsa-miR-145-5p	100	−14.49	2	7	945	967

Abbreviations: miRNA, microRNA; ROR, regulator of reprogramming.

ESCC and could regulate FSCN1.[38] These findings were in accordance with this network, so the research of the ROR/miR-145/FSCN1 pathway in ESCC commenced.

ROR could competitively bind to miR-145 as an miRNA sponge in ESCC

ROR could downregulate miR-145 expression

The regulative relations of ROR and miR-145 were studied by implementing qRT-PCR of miR-145 in the ROR overexpressing group and the NC group cells. The results showed that the overexpression of ROR downregulated the expression of miR-145 in EC109 (Table 6 and Figure 6).

ROR could bind to miR-145

The binding relation of the ROR and the miR-145 was verified by performing an anti-AGO2 RIP assay to explore the effects of ROR overexpression in miR-145. MiR-145 of the ROR overexpressing group immunoprecipitated by AGO2 antibodies was significantly higher than the NC group (Figure 7). The cycle threshold values were normalized with

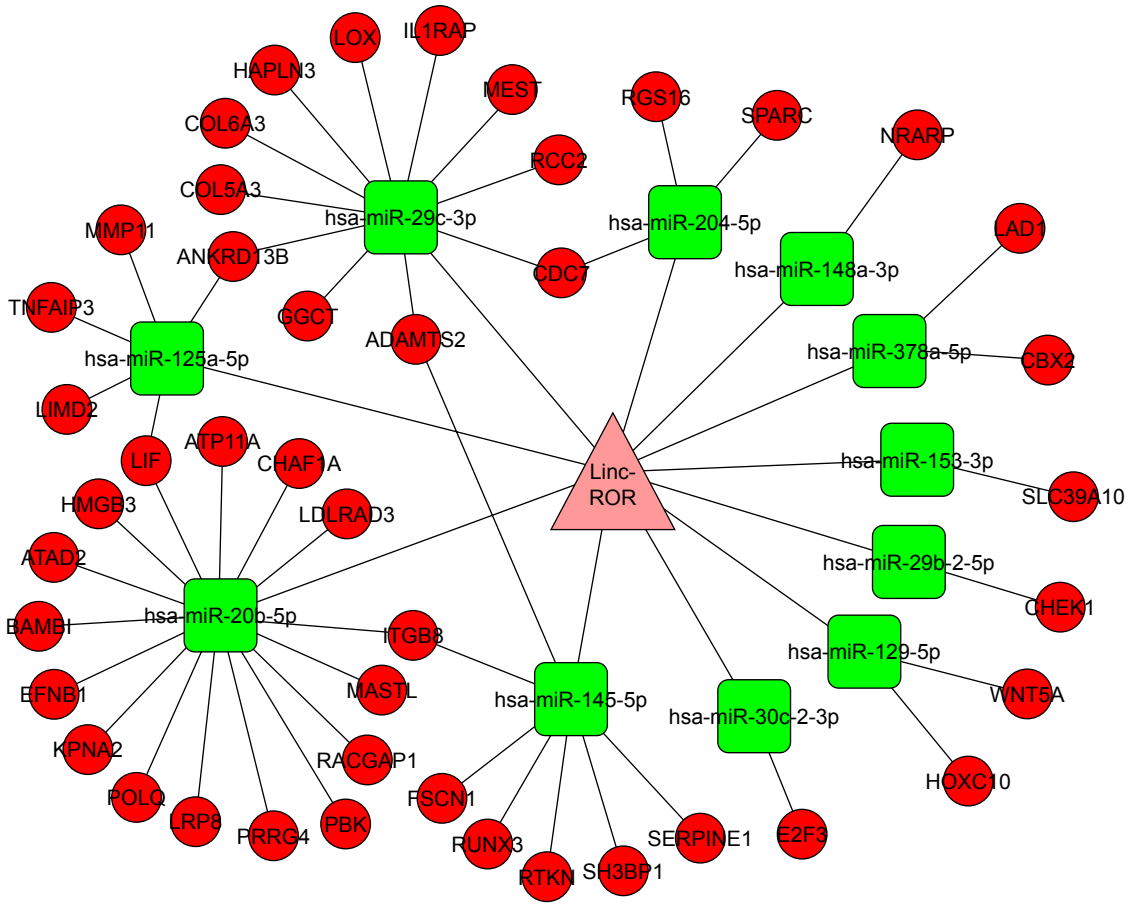


Figure 5 The ROR–miRNA–mRNA regulatory network.
Abbreviations: miRNA, microRNA; ROR, regulator of reprogramming.

Table 6 The downregulation of miR-145 induced by ROR overexpression

Group	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$	*F*	*p*-value
ROR	5.991±0.229				
NC	4.976±0.250	−1.015±0.250	2.021	0.027	0.007

Abbreviations: NC, negative control; ROR, regulator of reprogramming.

the input. It can be suggested that ROR could bind to miR-145 and was recruited to the AGO2-related RNA-induced silencing complexes (RISCs).

Two predicted binding sites of ROR to miR-145 were proved effective

A DLR assay was implemented to verify the predicted binding sites of ROR and miR-145. The vector utilized was pmiR-GLO, and hLuc/hRluc was for the fluorescence signal intensity. Figure 8 and Table 7 show that group 2 (WT+miR-145) signal was significantly lower than group 1 (WT+nc mimic), group 3 (MUT1+miR-145), and group 4 (MUT2+miR-145). This meant that ROR could bind to miR-145 via at least two binding elements. Site 1307-1330 and the novel 805-828 site were effective miR-145 binding sites that are likely the targets of ESCC molecular therapy.

An ROR/miR-145/FSCN1 axis was proved to be working in ESCC

The TargetScan algorithm found FSCN1 to be among the highest scoring predicted mRNA targets of miR-145. Previous literature suggested that miR-145 could suppress the metastasis of ESCC by targeting the FSCN1.[38] The regulative relations of ROR, miR-145, and FSCN1 were further explored by Western blotting.

The Western blotting results showed that miR-145 downregulated FSCN1 (Figure 9). ROR indirectly upregulated FSCN1. The ROR/miR-145/FSCN1 pathway was proven and played a role in ESCC progress on the protein level.

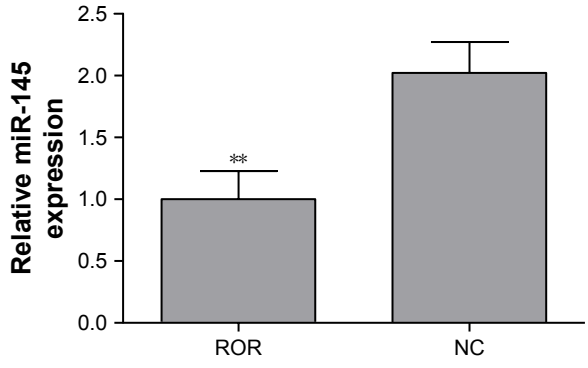


Figure 6 The overexpression of ROR downregulated miR-145 (**$p<0.01$).
Abbreviations: NC, negative control; ROR, regulator of reprogramming.

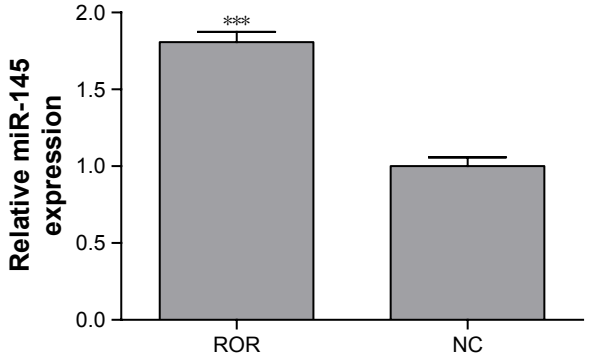


Figure 7 The anti-AGO2 RIP followed by qRT-PCR confirmed that ROR could bind to miR-145 (***$p<0.001$).
Abbreviations: NC, negative control; qRT-PCR, real-time fluorescent quantitative polymerase chain reaction; RIP, RNA immunoprecipitation; ROR, regulator of reprogramming.

ROR could promote migration and invasion of ESCC cells

Previous research has shown that FSCN1 promotes tumor metastasis in various cancers.[39–41] ROR functions in migration and invasion of ESCC were explored, in order to verify its regulation of miR-145/FSCN1. The EC109 cells transfected with lentivirus (that were overexpressing ROR or only the vector) were chosen as the treatment (ROR) group or the NC group. The regulating relationship between ROR and miR-145/FSCN1 was supplemented with miR-145 mimics or NC mimics as a treatment of the following Transwell migration and invasion assays. The grouping of the EC109 cells is shown in Table 8.

The cell migration and the invasion assay results are shown in Figure 10. ROR enhanced the migratory and the invasive capacities of EC109. It seems that miR-145 had the opposite function, where it restrained the effects of ROR to some extent. The mechanisms could be regulating tumor invasion and metastasis-related gene FSCN1 by sponging miR-145.

Discussion

ROR is a typical lincRNA that plays important regulatory roles in the pathogenesis and progression of tumors.[23,35,36] Previous studies suggested that ROR could promote cell reprogramming and enhance the malignancy of multiple tumors, including breast cancer and pancreatic cancer.[23,25,27,30] ROR could even enhance the therapeutic resistance of tumors.[28,31,37] But its clinical significance and prognostic value in ESCC remain largely unknown. In this study,

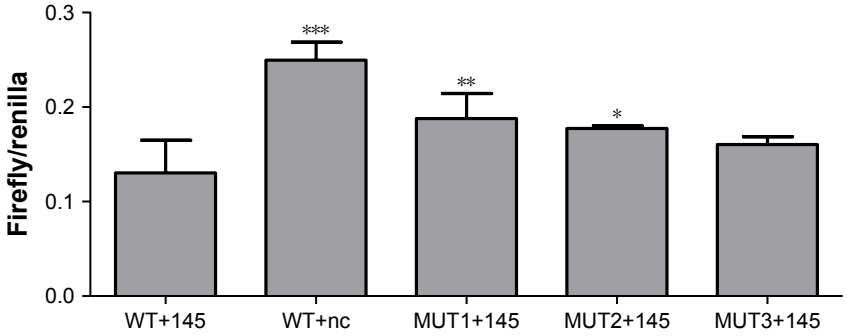


Figure 8 The DLR signal intensity of the five groups (compare with WT+145 group; *$p<0.05$, **$p<0.01$, and ***$p<0.001$).
Abbreviation: DLR, dual luciferase reporter.

ROR expression in both ESCC cells and tissues was tested. The qRT-PCR results showed that ROR was significantly upregulated in ESCC cells and tissues than the normal. The results showed that the ROR expression was positively correlated with the ESCC incidence risk. The cox survival analysis of 96 ESCC patients, recorded in TCGA database, showed that the survival time of patients with a higher ROR expression was significantly shorter than the patients with a lower ROR expression. This suggested that the high ROR expression level also predicted a poor prognosis of ESCC. ROR is likely a biomarker for early screening and poor prognosis of ESCC.

Recent literature has suggested that ROR could act as a molecular sponge in many tumors, where it absorbed and inhibited the activity of miRNAs. MiR-145 seems to suppress the development of various tumors by promoting apoptosis or inhibiting proliferation and invasion.[23,25–27,29,33–36] ROR is predicted by miRanda to have multiple binding sites with miR-145. It was speculated that ROR could enhance the malignance of ESCC by competitively binding to miR-145.

Previous literature showed that miR-145 was downregulated in ESCC. The reduced expression of miR-145 showed a trend of lymph node metastasis by regulating FSCN1.[38] FSCN1 was higher in ESCC tissues than normal epithelium. Its overexpression was associated with poor prognosis or lymph node metastasis in several human epithelial cancers, including colonic, gastric, breast, skin, urothelial carcinomas, and ESCC.[42] In this study, it was suggested that ROR could promote metastasis and invasion of ESCC by regulating miR-145 and FSCN1. An ROR–miRNA–mRNA regulatory network was built in ESCC that contained 11 downregulated miRNAs and 95 upregulated mRNAs based on TCGA database. The results were in agreement with our hypothesis that the ROR/miR-145/FSCN1 pathway existed in ESCC.

The qRT-PCR showed that ROR overexpression downregulated miR-145. The RIP results confirmed that ROR bound to miR-145 in ESCC cells. The binding relations of ROR and miR-145 were further studied by predicting binding sites with miRanda. The top three sites obtained high scores, but only two of the sites had been previously reported. The DLR assay was used to prove that the novel site 805-828 effectively bound to miR-145, meaning that this site could be a new target for ESCC molecular therapy.

Table 7 The DLR fluorescent signal intensity

Group	Signal intensity	*p*-value
WT+145	0.1305±0.0343	
WT+nc	0.2496±0.0190	<0.001
MUT1+145	0.1878±0.0265	0.008
MUT2+145	0.1773±0.0028	0.024
MUT3+145	0.1605±0.0081	0.118

Abbreviation: DLR, dual luciferase reporter.

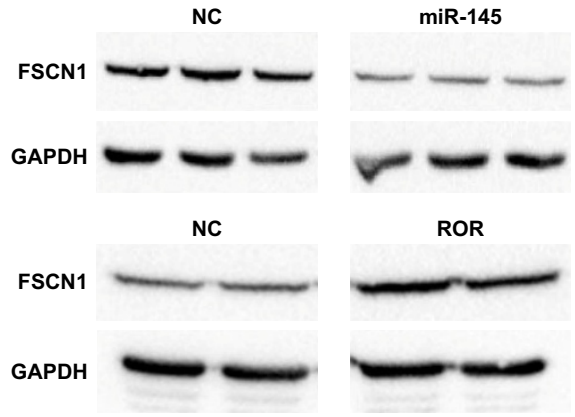


Figure 9 Overexpression of miR-145 downregulated FSCN1 ($p<0.01$) and ROR upregulated FSCN1 ($p<0.05$).
Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NC, negative control; ROR, regulator of reprogramming.

Table 8 Grouping type

Treatment	Group 1	Group 2	Group 3	Group 4
Cell type	ROR	ROR	NC	NC
RNA mimics	nc	miR-145	nc	miR-145

Abbreviations: NC, negative control; ROR, regulator of reprogramming.

Western blotting was used to verify the regulation of miR-145 and ROR on FSCN1 expression. The results proved that miR-145 decreased FSCN1 and the overexpression of ROR upregulated FSCN1. This verified the ROR/miR-145/FSCN1 axis in ESCC. Previous research[38,39] led us to perform transwell migration and invasion assays, which proved that ROR enhanced metastasis and invasion of ESCC. miR-145 counteracted the effects of ROR to some extent.

Conclusion

In this study, it is supposed that ROR is an oncogene of ESCC, which positively correlates with ESCC incidence and results in poor prognosis. ROR can promote metastasis and invasion of ESCC by regulating miR-145/FSCN1. ROR is likely a biomarker that can be used for early screening and will suggest poor prognosis for ESCC patients. ROR can probably be an effective target of miR-145 in molecular therapy for ESCC.

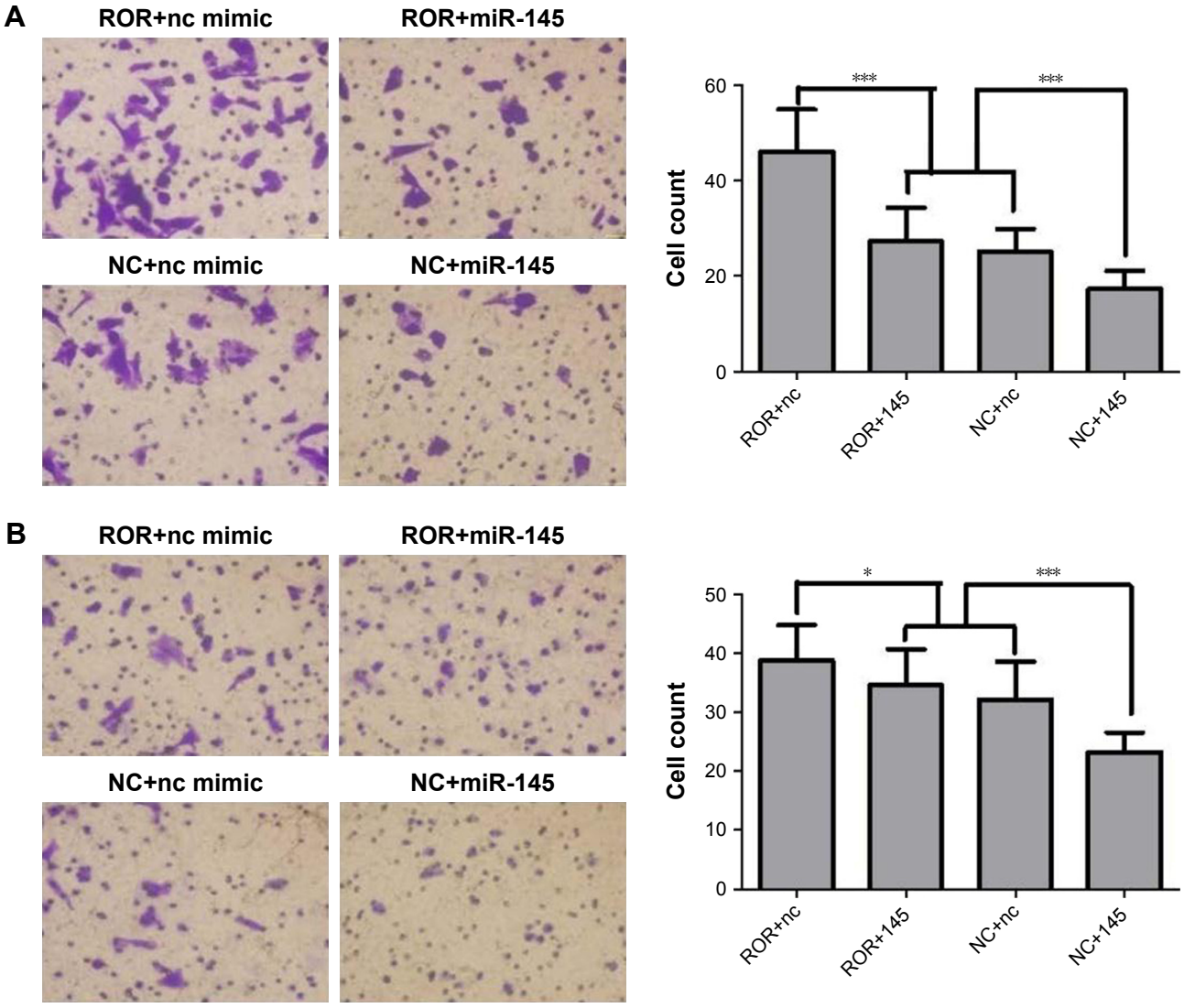


Figure 10 ROR promoted migration and invasion of ESCC cells (*$p<0.05$ and ***$p<0.001$).

Notes: (**A**) Transwell migration assay demonstrated that the cell count of the ROR overexpressing group was significantly higher than the NC group. The miR-145 mimics reversed these effects. (**B**) Transwell invasion assay demonstrated that the cell count of the ROR overexpressing group was significantly higher than the NC group. The miR-145 mimics reversed these effects.

Abbreviations: ESCC, esophageal squamous cell carcinoma; NC, negative control; ROR, regulator of reprogramming.

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Disclosure

The authors report no conflicts of interest in this work.

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