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Tumor-derived exosomal miR-103a-2-5p facilitates esophageal squamous cell carcinoma cell proliferation and migration

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Abstract. – OBJECTIVE: This study aimed to identify the different expression of microRNAs (miRNAs) in the plasma derived exosomes of patients with esophageal squamous cell carcinoma (ESCC).

PATIENTS AND METHODS: A total of 9 patients with ESCC and 9 patients with benign diseases were involved. miRNA sequencing was performed to screen differential expression of microRNAs in plasma exosomes between patients with ESCC and controls. The function of miRNA on proliferation and migration abilities was determined by CCK-8 analysis, wound scratch and transwell test. Predicted target genes were screened by databases and confirmed by RT-qPCR.

RESULTS: We identified a total of 10 miRNAs (7 upregulated and 3 downregulated) that were differentially expressed in plasma exosomes between patients with ESCC and control patients (fold change, FC ≥ 2.0 or ≤ -2.0, $p \leq 0.05$) by miRNA sequencing. Ten miRNAs were detected by qRT-PCR to verify the results of the miRNA sequencing. MiR-103a-2-5p demonstrated the most significant differential expression in both exosomes of ESCC cell lines and plasma of patients as compared with control patients and was therefore selected for subsequent functional experiments. Overexpression of miR-103a-2-5p promoted proliferation and migration in TE-1 cells, whereas inhibition of miR-103a-2-5p suppressed proliferation and migration in KYSE-150 cells. Exosomes extracted from the cells transfected with miR-103a-2-5p mimics significantly increased the proliferation and migration of two ESCC cell lines. Two genes, CDH11 and NR3C1 were identified as predicted targets of miR-103a-2-5p by the bioinformatics tools TargetScan, MiRanda, and mirDIP and RT-qPCR.

CONCLUSIONS: Our results shed light on how exosomal miR-103a-2-5p can promote proliferation and migration of ESCC cells and may represent a potential target for ESCC therapies.

Key Words:
Esophageal squamous cell carcinoma, Exosomes, MiR-103a-2-5p, Proliferation, Migration.

Introduction

Esophageal cancer (EC) ranked seventh cancer incidence and sixth in mortality worldwide in 2018. Globally, eastern Asia exhibits the highest incidence rates in men[1]. Esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC) are the two main histological subtypes of EC, and ESCC is prevalent in eastern Asia[2]. The prognosis for patients with ESCC remains grim despite improvements in diagnostic methods and multidisciplinary treatments[3]. Poor differentiation is known as an adverse predictor of patient outcomes[4].

MicroRNAs (miRNAs) are a class of small non-coding RNAs that regulate gene expression at post-transcriptional level; they function as crucial regulators of differentiation, proliferation, apoptosis, and metabolism in cancer cells[5,6]. It may be helpful to identify circulating miRNAs in the diagnosis and prognosis of EC[7] .

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Exosomes are small membranous vesicles derived from various cell types, including T cells, red blood cells, and tumor cells. They contain lipids, proteins, and nucleic acids (e.g., mRNA and miRNA). These RNAs can be transported to other cells and can function as a member of the new location[8]. Due to protection against endogenous RNase degradation, exosomal miRNAs are more stable than those not related to exosomes[9,10], thus, they have the potential to become specific biomarkers for diseases. Yang et al[11] showed that miRNAs transported by exosomes can promote the invasion of breast cancer cells.

While many tumor-specific miRNAs have been identified in tumor-derived exosomes in the plasma of patients with glioblastoma[12], thyroid cancer[13], hepatocellular carcinoma[14], lung cancer[15], breast cancer[16] and gastric cancer[17], few have been identified in the plasma of patients with EC. Therefore, we examined differential expression of miRNAs in the plasma of nine patients with ESCC and nine patients with benign diseases by miRNA sequencing. In the present study, the upregulated miR-103a-2-5p was selected for further analysis. Subsequently, we aimed to explore the influence of this miRNA on the biological function of EC cells when transmitted by exosomes.

Patients and Methods

Patients

Nine patients with newly diagnosed ESCC and nine patients with benign diseases were selected from January 2018 to November 2018 in the Xinqiao Hospital. All cases of ESCC were diagnosed by pathology. The management of the ESCC patients strictly follows the NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines) for Esophageal and Esophagogastric Junction Cancers. The eighth edition of the AJCC Cancer Staging Manual was used for cancer staging. Patients with acute inflammation and having other types of malignant tumors were excluded from this study. All patients with ESCC underwent an upper gastrointestinal fiberscope examination. Peripheral blood sampling was taken before surgery, radiotherapy, and chemotherapy. This investigation was approved by the Ethics Committee of Xinqiao Hospital. Signed written informed consents were obtained from all participants before the study. The blood samples were collected into blood collection tubes with EDTA and subsequently centrifuged at 2000 rpm for 10 minutes at 4°C, and then plasma was stored at -80°C until analysis.

Cell Culture

Normal human esophageal epithelial-1 cell line (Het-1A) was purchased from the American Type Culture Collection (ATCC), CRL-2692, Manassas, VA, USA); TE-1 (human ESCC well-differentiated cell lines, TCHu89) and KYSE-150 (human ESCC low-differentiated cell lines, TCHu236) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). These cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA), penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) and 10% exosomes-depleted fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA). All cells were cultured in an incubator (Series II 3111, Thermo Fisher Scientific, Waltham, MA, USA) that contained 5% CO_2 and was maintained at 37°C.

Isolation of Exosomes

Exosomes were extracted from 2 mL of plasma and 30 ml of cell culture supernatant using an exoEasy Maxi Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The method is described in the exoEasy instructions. Samples were filtered through 0.22 μm pore filters (EMD Millipore, Billerica, MA, USA) and then mixed 1:1 with Buffer XBP as sample (plasma or cell culture supernatant) and added to an exoEasy membrane affinity spin column. 10 mL Buffer XWP was be used to wash off the bound exosomes. Then, exosomes were eluted with 400 μL Buffer XE. A ZetaView® instrument (Particle Metrix, Meerbusch, Germany) was used to perform Nanoparticle Tracking Analysis (NTA). Bicinchoninic acid assay (BCA) Analysis Kit (Sangon Biotech, Shanghai, China) was used to detect the concentration of exosomal protein.

miRNA Sequencing

According the manufacturer's manual, total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and miRNeasy Micro Kit (Qiagen, Hilden, Germany). A 4200 TapeStation (Agilent, Santa Clara, CA, USA) was used to measure the RNA quality. RNA quantity was measured using a Qubit2.0 Fluorometer (Life Technologies, Gaithersburg, MD, USA). RNA samples were sent to Genminix Informatic Ltd. (Shanghai, China) for miRNA sequencing on an

Illumina Hiseq platform (Illumina, San Diego, CA, USA) with 10 M reads. The differentially expressed microRNAs were analyzed by Genminix Informatic Ltd. (Shanghai, China).

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted by using TRIzol as described above. Then, a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) was used to conduct reverse transcription with 1 μg of total RNA. U6 was used as the internal control for miRNA. qRT-PCR was conducted using upstream and downstream primers shown in Table I, SYBR Green PCR master mix (Toyobo, Osaka, Japan) on an ABI 7900HT StepOne Plus system (Applied Biosystems, Foster City, CA, USA).

Cell Transfection

MiR-103a-2-5p mimics, miR-103a-2-5p mimics-NC (negative control), miR-103a-2-5p inhibitors, and miR-103a-2-5p inhibitors-NC were purchased from Genecreate (Wuhan, China). Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was used to conduct transfection. The sequences used in this experiment are shown in Table II.

Exosome Uptake Assay

Exosomes from TE-1 cells transfected with miR-103a-2-5p mimics and miR-103a-2-5p mimics-NC were extracted as described above. According to the protocol for PKH26 dye kit (# MINI26)[18], exosomes were re-suspended in 1 mL diluents C, and 4 μL PKH26 dye was diluted in another 1 mL diluent C. Then, 1 mL of diluent C containing exosomes was mixed with the dye solution by gentle pipetting and incubated for 5 min at RT. 2 mL 10% exosome-depleted FBS in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) was used to react with excess dye. Then, 1 μg of PKH26-labelled exosomes were added into cell culture and co-cultured in 24-well plates for 24 h, and cells were then washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde at RT for 15 min, nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) for 5 min. After washing with PBS, the samples were analyzed and photographed using a fluorescence microscope (TS-100, Nikon, Tokyo, Japan).

Cell Proliferation Assay (CCK-8)

TE-1 and KYSE-150 were seeded in a 96-well plate with approximately 1×10^4 cells per well. TE-1 cells were transfected with miR-103a-2-5p mimics and mimics-NC for 24 h, and KYSE-150 cells were transfected with inhibitor and inhibitor-NC for 24 h. CCK-8 (Dojindo Molecular Technologies, Kumamoto, Japan) kit was used for cell proliferation detection at 0, 24, and 48 h. The absorbance value was measured by using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 450 nm wavelength. For functional analysis of exosomes, exosomes from cells transfected with miR-103a-2-5p mimics and miR-103a-2-5p mimics-NC were respectively extracted as described above. TE-1 and KYSE-150 cells were co-cultured with the two types of purified exosomes (20 μg/mL), respectively, for 24 h. CCK-8 was conducted in the same way as above.

Transwell and Wound Scratch Assay

Migration ability of ESCC cells was measured by using transwell chambers (Corning, Corning, NY, USA). The cells were suspended in serum-free DMEM and adjusted to a concentration of 5×10^4 cells/mL. Cell suspension (200 μL) was seeded into the upper chamber. The bottom chamber was filled with 600 μL of DMEM medium containing 10% exosome-depleted FBS. Following 36 h of incubation at 37°C, the migrated cells were stained with crystal violet (Beyotime, Shanghai, China) at RT for 20 min, then washed with PBS. Stained cells were observed using a microscope (CKX-41, Olympus, Tokyo, Japan) and counted. For functional analysis of exosomes, exosomes from cells transfected with miR-103a-2-5p mimics and miR-103a-2-5p mimics-NC were extracted as described above. TE-1 and KYSE-150 cells were co-cultured with the two types of purified exosomes (20 μg/mL), respectively, for 24 h. Approximately 200 μl of cell suspension (1×10^4 cells) in serum-free DMEM was added into the upper well of the chamber in triplicate wells. Transwell was conducted in the same way as above.

Cells were seeded in medium at 9×10^5 cells per well in a 6-well plate (Biofil, Guangzhou, China), incubated under standard conditions until 80-90% confluency, then wounded by a pipette tip. Floating cells were removed, and medium without FBS was added to each well. For functional analysis of exosomes, cells (TE-1 and KYSE-150) were seeded in medium at 9×10^5 cells per well in a 6-well plate (Biofil, Guangzhou, China), incubated under standard conditions until 80–90% confluency, then wounded by a pipette tip. Floating cells were removed and DMEM with exosomes (20 μg/mL)

Table I. The primer sequences used in qPCR.

miRNA and gene	Forward primer	Reversed primer
hsa-miR-103a-2-5p	5'-ACACTCCAGCTGGGAGCTTCTTTACAGTGCT-3'	5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCAAGGCAG-3'
hsa-let-7g-3p	5'-ACACTCCAGCTGGGCTGTACAGGCCACTG-3'	5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGCAAGGCA-3'
hsa-miR-301a-3p	5'-ACACTCCAGCTGGGCAGTGCAATAGTATTGT-3'	5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGCTTTGAC-3'
hsa-miR-130a-5p	5'-ACACTCCAGCTGGGGCTCTTTTCACATTGT-3'	5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGTAGCAC-3'
hsa-miR-33a-3p	5'-ACACTCCAGCTGGGCAATGTTTCCACAGTG-3'	5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGTGATGCA-3'
hsa-miR-2355-5p	5'-ACACTCCAGCTGGGATCCCCAGATACAAT-3'	5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTTGTCCAT-3'
hsa-miR-3192-5p	5'-ACACTCCAGCTGGGTCTGGGAGGTTGTAGCA-3'	5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTTCCACTG-3'
hsa-miR-429	5'-ACACTCCAGCTGGGTAATACTGTCTGGTAA-3'	5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACGGTTTT-3'
hsa-miR-125b-1-3p	5'-ACACTCCAGCTGGGACGGGTTAGGCTCTTG-3'	5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGCTCCCA-3'
hsa-miR-149-5p	5'-ACACTCCAGCTGGGTCTGGCTCCGTGTCTTC-3'	5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGGGAGTGA-3'
Human CDH11	5'-AGAGAGCCCAGTACACGTTGA-3'	5'-TTGGCATGATAGGTCTCGTGC-3'
Human NR3C1	5'-TGCCGCTATCGAAAATGTCTT-3'	5'-GGGTAGGGGTGAGTTGTGGT-3'
GAPDH	5'-GGACCTGACCTGCCGTCTAG-3'	5'-CCTGCTTCACCACCTTCTTGA-3'
U6	5'-TCGCTTCGGCAGCACATATAC-3'	5'-TATGGAACGCTTCACGAATTTG-3'

Table II. The sequences used for miR-103a-2-5p upregulation and downregulation.

	Sequence
miR-103a-2-5p mimics	5'-AGCUUCUUUACAGUGCUGCCUUG-3'
miR-103a-2-5p mimics-NC	5'-UUGUACUACACAAAAGUACUG-3'
miR-103a-2-5p inhibitor	5'-CAAGGCAGCACUGUAAAGAAGCU-3'
miR-103a-2-5p inhibitor-NC	5'-CAGUACUUUUGUGUAGUACAA-3'

from the cell culture supernatant of TE-1 mimics or TE-1 mimics-NC was added to each well. An inverted microscope (Olympus, Tokyo, Japan) was used to observe and photograph the cells. Photographs were taken at each indicated time. The area of wound closure was measured using ImageJ software (NIA, Bethesda, MD, USA).

Statistical Analysis

All experimental data were analyzed using GraphPad Prism Software 8.0 (GraphPad Software Inc, San Diego, CA, USA) and R Studio Version 1.1.463 (Boston, MA, USA). Differences were determined by the Chi-square test, unpaired Student's *t*-test, or ANOVA followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered statistically significant. All data are expressed as mean ± standard deviation (SD). All data were performed in triplicate.

Results

Levels of MiRNAs In Plasma Exosomes Differed Between the Patients With ESCC and the Control Patients

To investigate the differences in tumor-delivered exosomal microRNA in ESCC patients, the plasma exosomes from nine ESCC and nine control patients were extracted. Characteristics of patients are shown in Table III. Nanoparticle Tracking Analysis (NTA) was used to detect the particle size distribution of purified exosomes, and 90% of the particles were under 150 nm (Figure 1A). Then, miRNA sequencing was performed to explore the expression of miRNAs in exosomes. Differentially expressed miRNAs were considered those having a difference of more than twofold between their geometrical mean expression in the compared groups and a statistically significant *p*-value (< 0.05) by DEseq2 analysis. The expression levels of 61 miRNAs in plasma exosomes derived from ESCC patients were significantly different from those of control patients. Among those, 49 miRNAs were upregulated and 12 miRNAs were downregulated (Figure 1B). Volcano plots were used to represent the differential expression of miRNAs (Figure 1C). Based on the miRNA sequencing data, 10 miRNAs were identified as having a fourfold differential expression between groups (FC ≥ 2.0 or ≤ -2.0, $p < 0.05$, Table IV). Of these, 7 miRNAs were upregulated and 3 miRNAs were downregulated. Expression profiles of these 10 miRNAs were established and clustered using hierarchical cluster analysis (Figure 1D). Details of differential expression of microRNAs in plasma exosomes between patients with ESCC and controls were shown in Figure 2.

Table III. Characteristics of patients with esophageal squamous cell carcinoma and patients with benign diseases.

Characteristic	Control Group (n=9) (%)	ESCC Group (n=9) (%)	*p*-value
Gender			
Male	5 (55.6%)	6 (66.7%)	
Female	4 (44.4%)	3 (33.3%)	0.629
Age			
Mean± SD, y	54.6 ±7.1	60.0 ± 4.2	0.076
Reasons for admission			
ESCC		9 (100%)	
pulmonary bulla	5 (55.6%)		
achalasia of cardia	2 (22.2%)		
Other	2 (22.2%)		

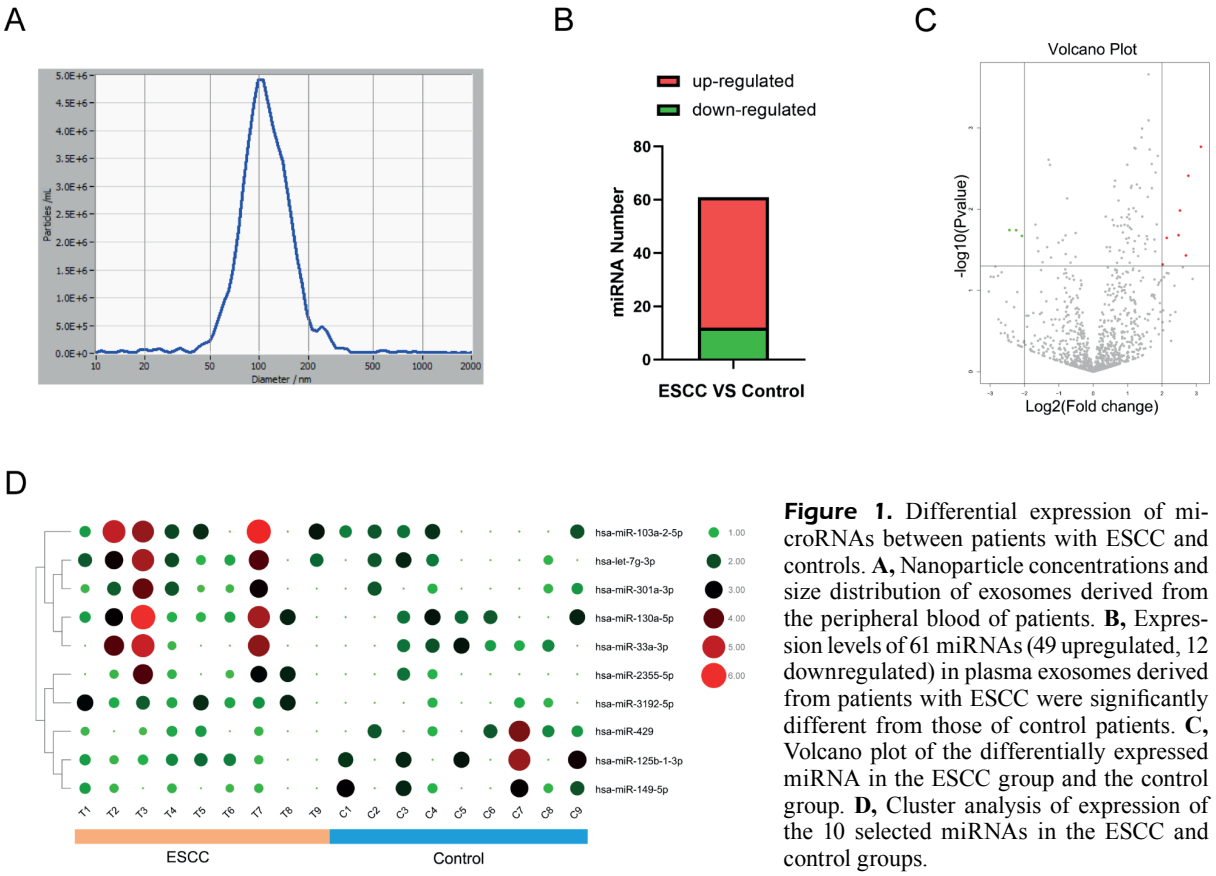

Figure 1. Differential expression of microRNAs between patients with ESCC and controls. **A,** Nanoparticle concentrations and size distribution of exosomes derived from the peripheral blood of patients. **B,** Expression levels of 61 miRNAs (49 upregulated, 12 downregulated) in plasma exosomes derived from patients with ESCC were significantly different from those of control patients. **C,** Volcano plot of the differentially expressed miRNA in the ESCC group and the control group. **D,** Cluster analysis of expression of the 10 selected miRNAs in the ESCC and control groups.

MiR-103a-2-5p Was Highly Expressed In Both Plasma-Delivered Exosomes and ESCC Cells

Het-1A (normal human esophageal epithelial-1 cell), TE-1 (high differentiation), and KYSE-150 (low differentiation) esophageal carcinoma cell lines were used to verify and explore the functions of the 10 selected miRNAs. As shown in Figure 3A, qRT-PCR results revealed that three miRNAs, including miR-103a-2-5p, miR-301a-3p, and miR-149-5p were overexpressed in KYSE-150 compared with Het-1A and TE-1. This result indicated that upregulation of these three microRNAs might be related to the malignant degree of ESCC. Furthermore, two microRNAs in KYSE-150 and TE-1, miR-33a-3p and miR-429, were significantly downregulated compared with Het-1A. Conjoint analysis of miRNA sequencing results from plasma exosomes showed that the expression trends of two overexpressed microRNAs

Table IV. Differential expression of 10 candidate miRNAs in plasma of ESCC Group vs. Control Group.

miRNA	Chrom	Length	ESCC Group (n=9)	Control Group (n=9)	*p*-value
hsa-miR-125b-1-3p	chr11	21	1.06±0.78	4.92±7.48	0.021
hsa-miR-429	chr1	21	0.49±053	3.13±5.97	0.018
hsa-miR-103a-2-5p	chr20	22	12.53±16.69	1.48±1.50	0.002
hsa-miR-301a-3p	chr17	22	3.49±5.36	0.66±0.95	0.020
hsa-let-7g-3p	chr3	20	6.37±8.30	1.08±1.55	0.004
hsa-miR-2355-5p	chr2	20	2.79±4.43	0.34±0.73	0.037
hsa-miR-130a-5p	chr11	21	10.37±17.46	1.79±1.89	0.010
hsa-miR-3192-5p	chr20	22	2.35±1.80	0.32±0.49	0.023
hsa-miR-149-5p	chr2	22	0.47±0.49	2.66±3.12	0.018
hsa-miR-33a-3p	chr22	21	7.63±12.3	1.52±1.55	0.049

(miR-103a-2-5p, miR-301a-3p) and two microRNAs with lower expression (miR-125b-1-3p, miR-429) were consistent. However, the expression level of miR-103a-2-5p in the ESCC patients was 8.76 times higher than in the control group, and its *p*-value is the lowest (p=1.70e-03). Furthermore, the expression of miR-103a-2-5p was confirmed to be significantly higher in the exosomes of KYSE-150 (Figure 3B). Clinical data from the OncomiR database (https://www.oncomine.org) demonstrate that miR-103a-2-5p was upregulated approximately 2.5-fold in ESCC tumor tissues compared with controls (p=1.76e-03) (Figure 3C). Compared with patients with lower miR-103a-2-5p level, the survival rate of those with higher level was significantly reduced (p=0.015). Additionally, similar result was observed in TCGA database (Figure 3D, p=0.014). These results suggested that elevated expression of miR-103a-2-5p in tumor tissues and exosomes may play a critical role in the progression of ESCC. Consequently, miR-103a-2-5p was chosen for the subsequent functional experiments.

MiR-103a-2-5p Promoted Proliferation and Migration In ESCC Cells

Experimental analysis (qRT-PCR) has confirmed that the expression of miR-103a-2-5p in TE-1 cells was lower than that in KYSE-150 cells. To determine the role of miR-103a-2-5p in the proliferation and migration of ESCC cells, TE-1 cells were transfected with miR-103a-2-5p

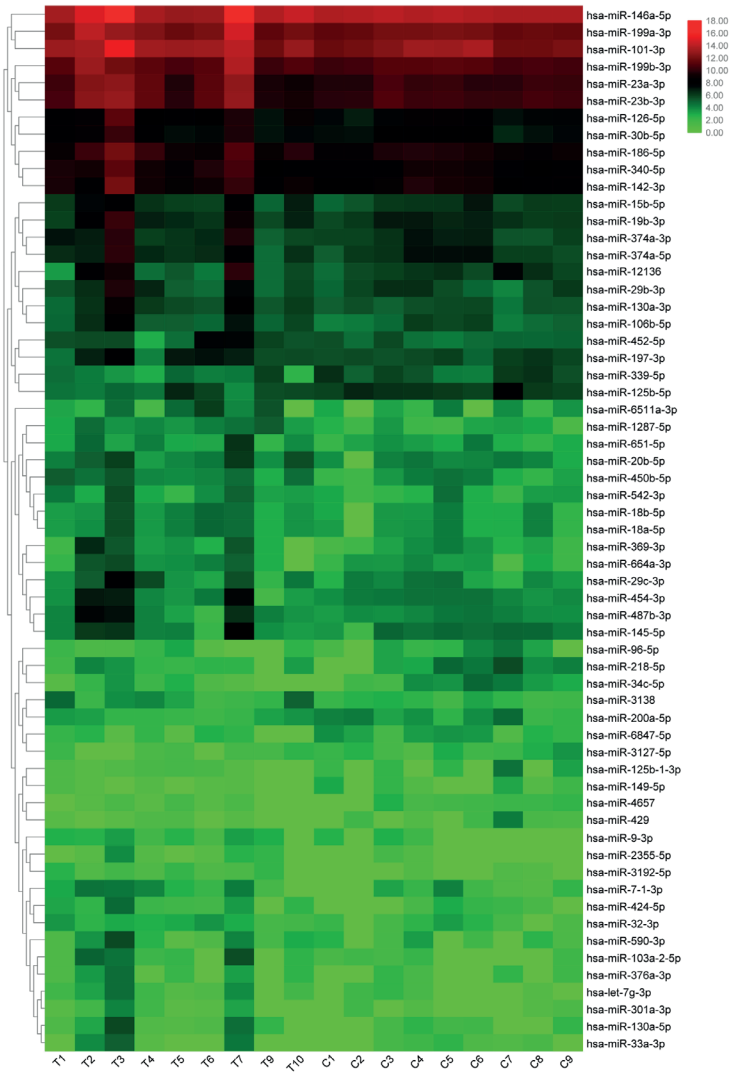

Figure 2. Heatmap of differential expression of microRNAs in plasma exosomes between patients with ESCC and controls.

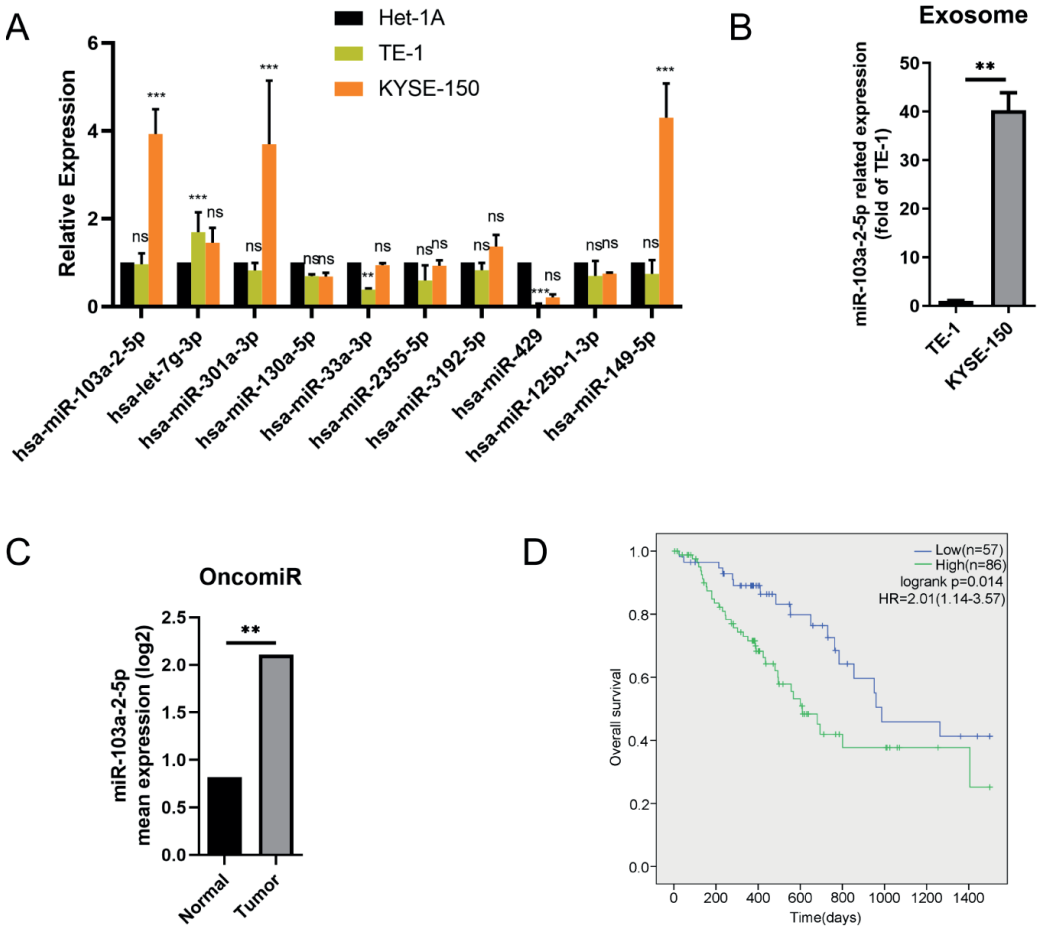

Figure 3. miR-103a-2-5p was highly expressed in both ESCC plasma exosomes and cells. **A,** qRT-PCR analysis of expression of the 10 selected miRNAs in Het-1A and ESCC cell lines (TE-1 and KYSE-150). ns, not significant. **B,** qRT-PCR analysis of miR-103a-2-5p expression in exosomes extracted from supernatant of TE-1 and KYSE-150 cell cultures. **C,** OncomiR data showing that miR-103a-2-5p was upregulated in ESCC tumor tissue compared with controls. **D,** Patients with highly expressed mir-103a-2-5p have a relatively higher risk of mortality (p=0.014) from TCGA databases. ns, not significant, **p<0.01, ***p<0.001.

mimics or mimics-NC; similarly, KYSE-150 cells were transfected with miR-103a-2-5p inhibitor or inhibitor-NC. Using qPCR analysis, the expression of miR-103a-2-5p in TE-1 cells transfected with mimics was significantly higher than that in mimics-NC after 48 h post-transfection (Figure 4A), Furthermore, CCK-8 assays were conducted to detect the function of miR-103a-2-5p in proliferation. Overexpression of miR-103a-2-5p promoted proliferation in TE-1 cells (p<0.01, Figure 4B). Wound scratch assays and transwell assays were conducted to verify the role of miR-103a-2-5p in migration. Results from these assays demonstrated that high-dose miR-103a-2-5p was more effective in promoting cell migration (Figure 4C and 4D). There is a significant decrease in expression shown in the KYSE-150 cells transfected with inhibitor compared with those transfected with inhibitor-NC (p<0.001, Figure 4E). Cell viability was suppressed in the KYSE-150 inhibitor group compared with the inhibitor-NC group (p<0.01, Figure 4F). In addition, consistent with the above results, a significant decrease in the number of migrated cells was shown in the miR-103a-2-5p inhibitor group compared with those in the NC group (Figure 4G and 4H). These findings illustrated that miR-103a-2-5p can promote the proliferation and migration capability of ESCC cell lines.

Exosome-Mediated Transfer of MiR-103a-2-5p Promoted Proliferation and Migration In ESCC Cells

The previous studies showed that miRNAs can be transferred between cells by exosomes and

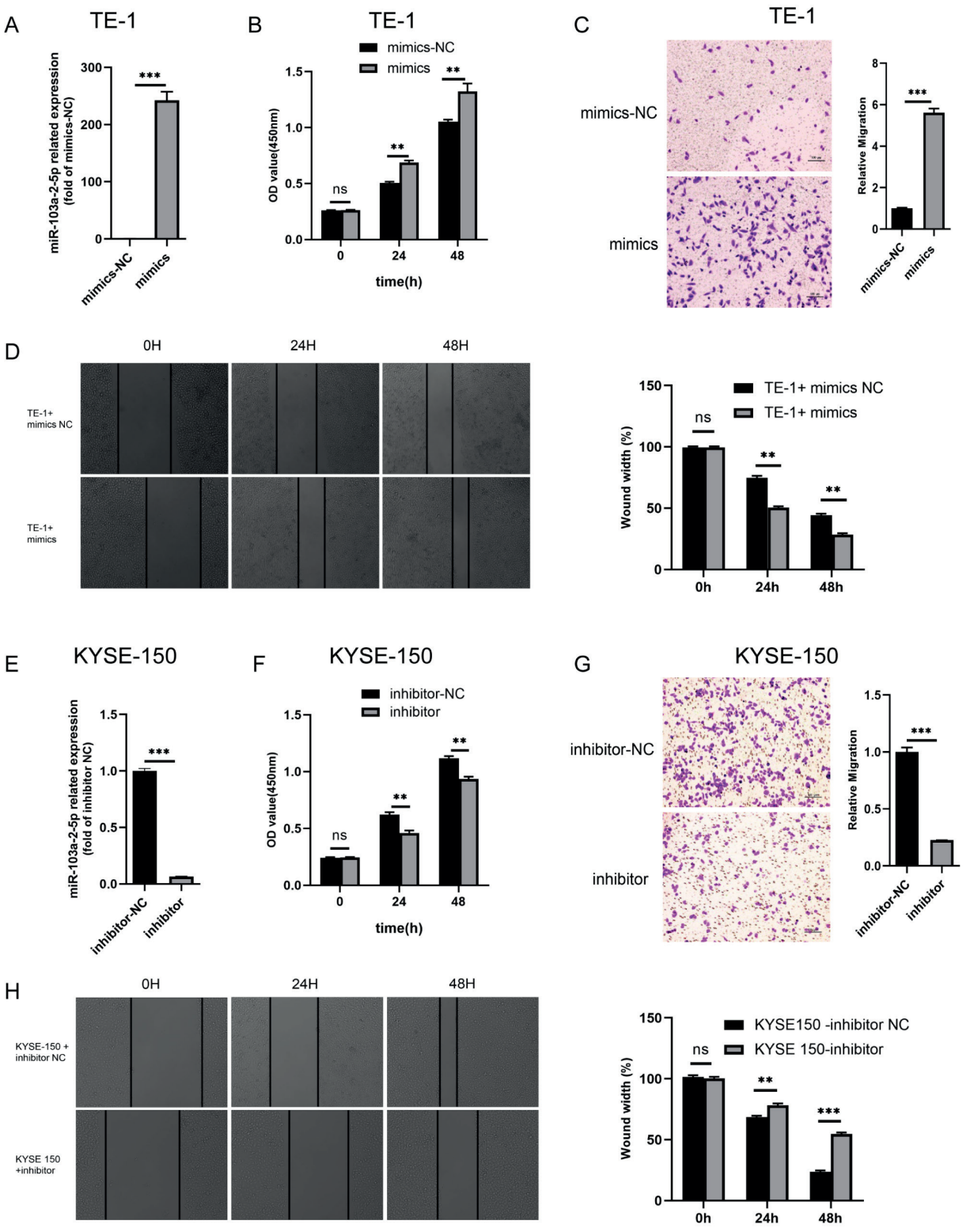

Figure 4. MiR-103a-2-5p expression in cytoplasm was associated with the proliferation and migration ability of ESCC cells. **A,** Expression of miR-103a-2-5p in TE-1 cells transfected with mimic or mimic-NC. **B,** Proliferation rates according to CCK-8 assays of TE-1 mimic-NC and mimic groups. **C-D,** Transwell and wound scratch assays determining the migration ability of TE-1 mimic-NC and mimic groups. (magnification: 40×) **E,** Expression of miR-103a-2-5p in KYSE-150 cells transfected with miRNA inhibitor-NC or inhibitor. **F,** Proliferation rates according to CCK-8 assays of KYSE-150 inhibitor-NC and inhibitor groups. **G-H,** Transwell wound scratch assays determining the migration ability of KYSE-150 inhibitor-NC and inhibitor groups. ns, not significant, (magnification: 40×) $^{**}p<0.01$, $^{***}p<0.001$.

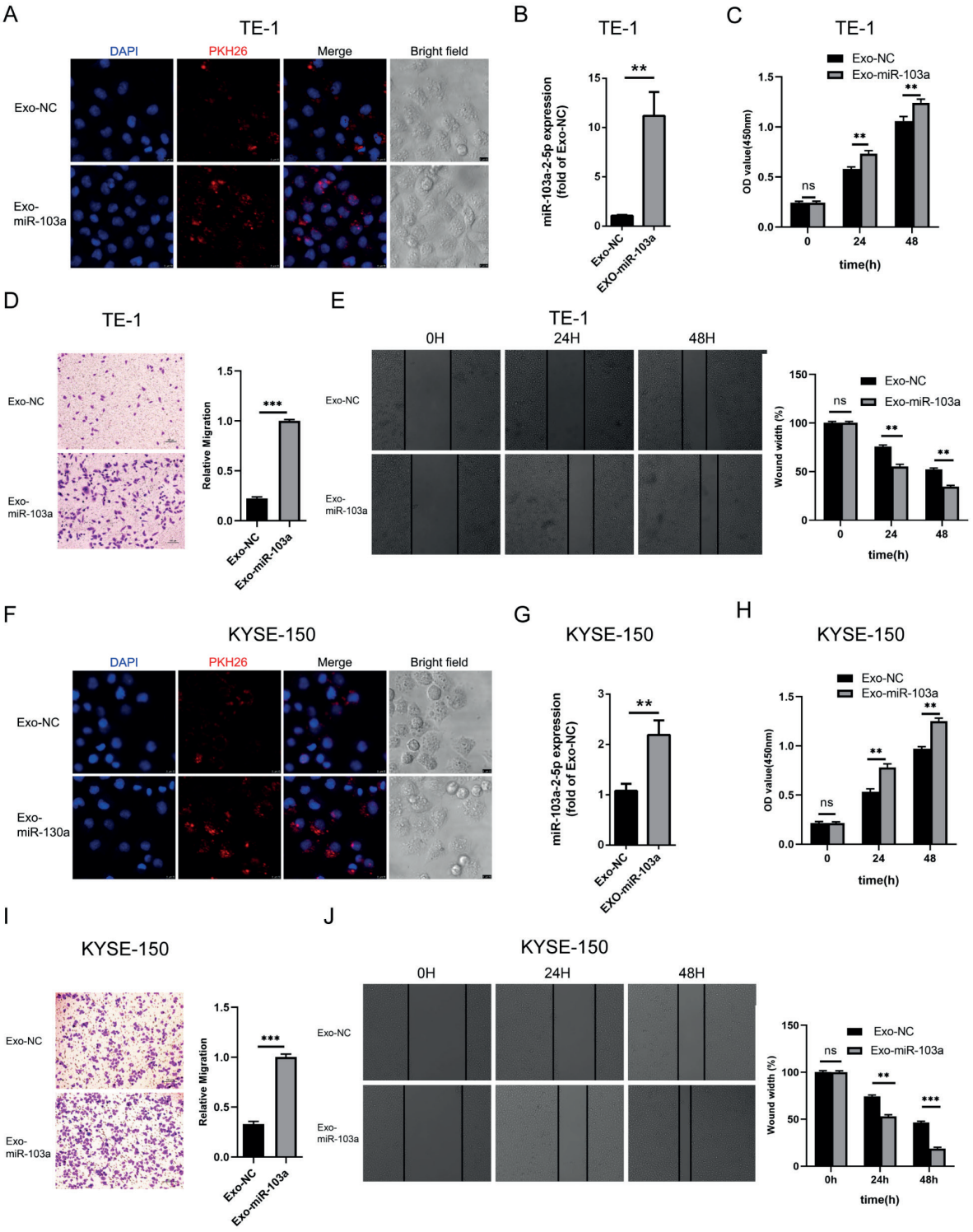

Figure 5. Effects of miR-103a-2-5p transferred by exosomes on the biological function of recipient TE-1 and KYSE-150 cells. Fluorescence microscopy of exosome uptake by TE-1 (**A**) and KYSE-150 (**F**) cells incubated with PKH26-labeled Exo-NC or Exo-miR-103a (magnification: 100×). Scale bar = 10 **μ**m. **B-G,** qPCR was performed to detect the level of miR-130a-2-5p in the cytoplasm of ESCC cells after exosome transfer. Proliferation rates according to CCK-8 assay of TE-1 (**C**) and KYSE-150 (**H**) cells incubated with Exo-NC or Exo- miR-103a. **D-E,** Transwell and wound scratch assays determining the migration ability of TE-1 cells incubated with Exo-NC or Exo- miR-103a. (magnification: 40×) **I-J,** Transwell and wound scratch assays determining the migration ability of KYSE-150 cells incubated with Exo-NC or Exo- miR-103a. ns, not significant, (magnification: 40×) **p<0.01, ***p<0.001.

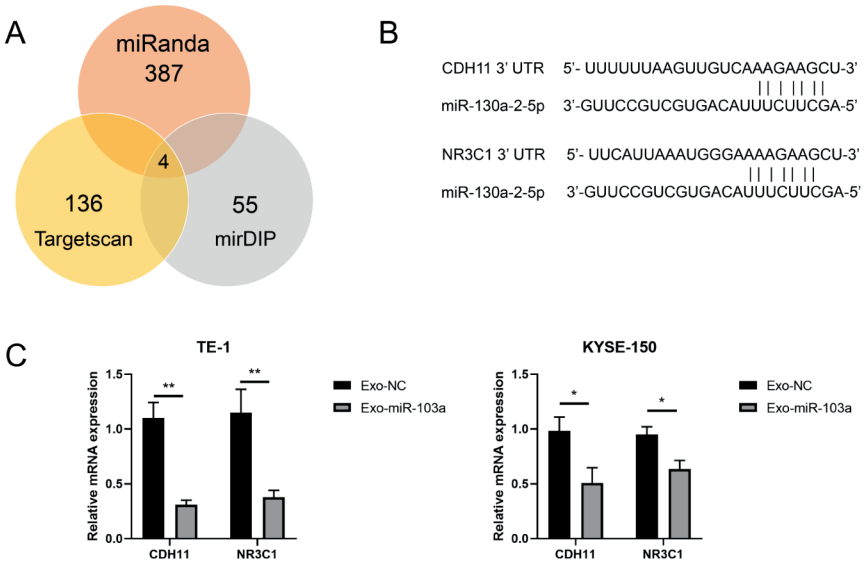

Figure 6. Potential targets analysis of miR-103a-2-5p. **A,** Venn plot for the potential target genes of miR-103a-2-5p. **B,** Alignment of potential *CDH11* and *NR3C1* base pairing with miR-103a-2-5p as identified by TargetScan. **C,** Downregulation of *CDH11* and *NR3C1* was confirmed by RT-PCR. **p*<0.05, ***p*<0.01.

play a key role in increasing proliferation and migration of tumor cells[11]. To confirm the regulatory effects of miR-103a-2-5p transferred by exosomes on the ESCC cells, exosomes were extracted from TE-1 cells transfected with miR-103a-2-5p mimics and mimics-NC. Then, these exosomes were labeled with PKH26 and incubated with TE-1 and KYSE-150 cells. Confocal images indicated that the PKH26-labeled exosomes (red) could be taken up by both TE-1 and KEYS-150 cells. Moreover, the uptake exosomes were mainly located in the cytoplasm. These demonstrated that circulating exosomes were internalized by TE-1 cells and KYSE-150 (Figure 5A and 5F). As shown in Figure 4B and 4G, qRT-PCR results confirmed that miR-103a-2-5p was transferred into recipient ESCC cells.

The CCK8 results demonstrated that exosomes extracted from TE-1 cells transfected with miR-103a-2-5p mimics can significantly increase the proliferation of two ESCC cells (Figure 5C and 5H). As expected, results of transwell (Figure 5D and 5I) and scratch (Figure 5E and 5J) experiments revealed that the migration ability of ESCC cells was also enhanced by co-cultured with exosomes with high level miR-103a-2-5p. All these results indicate that exosomes-mediated transfer of miR-103a-2-5p can promote proliferation and migration in ESCC cells.

Potential Targets Analysis of MiR-103a-2-5p

Three databases were used to identify potential targets of miR-103a-2-5p. A total of 387 candidate genes from miRanda, 136 candidate genes from TargetScan Human 7.2, and 55 candidate genes from mirDIP were found. Finally, four candidate target genes, cadherin-11 (*CDH11*), mannosidase alpha class 2A member 1 (*MAN2A1*), N-terminal glucocorticoid receptor isoform C1 (*NR3C1*), and transportin 1 (*TNPO1*) were screened out from the results of merging the three datasets (Figure 6A). Previous studies reported that low expression of *CDH11*[19] and *NR3C1*[20] is involved in promoting cancer cell proliferation and migration. Bioinformatics analysis with TargetScan was performed and it was revealed that human miR-103a-2-5p was a potential binding candidate of *CDH11* and *NR3C1* (Figure 6B). Furthermore, mRNA levels of CDH11 and NR3C1 were found to be downregulated in TE-1 and KYSE-150 cells after treated with exosomes extracted from miR-103a-2-5p transfected TE-1 cells (Figure 6C). These results indicated that miR-103a-2-5p might facilitate the proliferation and migration abilities of ESCC cells, perhaps by downregulating expression of these two genes.

Discussion

In 2005, esophageal cancer ranked 4th in mortality among both men and women in China[21]. A prior study showed that oncogenic miRNAs delivered by exosomes could regulate the invasive-

ness of co-cultured breast cancer cells[11]. The results of this study indicate that the invasiveness of cancer cells may be regulated by miRNA which transported into recipient cells via exosomes after donor cell transfection. Thus, miRNAs delivered by exosomes may represent key treatment targets for anticancer therapy. However, the specific miRNAs in plasma exosomes from patients with cancer are still unclear. Therefore, the mechanisms underlying tumor cell-exosomal miRNAs interactions require further investigation, especially in the ESCC model.

Liu et al[22] have demonstrated that the expression of miR-103a in esophageal cancer cells is significantly higher than in normal esophageal cells, which is consistent with our findings. miR-103a has been identified as an onco-miRNA in colorectal cancer[23] and breast cancer[24]. Nevertheless, the role of miR-103a-2-5p in ESCC remained unknown. In this study, we found that miR-103a-2-5p was highly expressed in plasma exosomes from patients with ESCC and showed that miR-103a-2-5p facilitates ESCC cell proliferation and migration. Furthermore, we investigated exosomes may be taken up by ECSS cells and indicated that miR-103a-2-5p mimics could be transferred between ESCC cells by exosomes. These results demonstrate that miR-103a-2-5p is a key regulator of ESCC, and this is the first report of a functional analysis of miR-103a-2-5p in ESCC.

Prior studies demonstrated that microRNA-21 transferred by exosomes promotes cell migration and invasion in ESCC[25] and that miR-93-5p transferred by exosomes promotes proliferation in ESCC[26]. These studies utilized miRNA microarrays to profile microRNA expression in patients with esophageal cancer versus controls. The limitations of miRNA microarrays mainly display in following several aspects: (1) reduced sensitivity for miRNAs expressed at low levels and (2) lack of ability to explore new miRNAs and structural sequence changes. In contrast, miRNA sequencing can overcome these limitations. To our knowledge, this is the first study reporting a miRNA sequencing analysis characterizing exosomal miRNAs in ESSC.

Exosomal miR-103a-2-5p has a great impact on ESCC cells relative to biological function. As a result, we want to explore the possibility of exosomal miR-103a-2-5p serving as diagnostic or prognostic biomarkers for ESCC. This will require a huge amount of efforts to detect exosomal miR-103a-2-5p in patient plasma and analyses the relationship of exosomal miR-103a-2-5p with prognosis, survival rate, etc. Based on the miRNA sequencing data, hsa-miR-301a-3p and hsa-miR-149-5p also significantly upregulated. Four candidate genes were found based on three databases, several molecular studies have confirmed that CDH11[19], NR3C1[20] are regarded as tumor suppressors in many types of cancers. However, another two target genes, MAN2A1 and TNPO1, were reported to have an overexpression and promote tumor progression in tumors[27-29]. Further investigations should be performed to confirm the function of these four target genes. A shortcoming of our results is the small number of samples, due to the high cost of miRNAs sequencing and isolation of plasma exosomes, and they should be confirmed by detecting a large number of confirmed ESCC and control patients.

Conclusions

Summarily, we have established that exosomal miR-103a-2-5p is differentially expressed in patients with ESCC *vs.* healthy controls. As circulating miRNAs in exosomes are more stable than those in other forms, these findings suggest that circulating exosomal miR-103a-2-5p could serve as a novel, noninvasive diagnostic and prognostic biomarker in the early diagnosis of ESCC. Moreover, our findings suggest that miR-103a-2-5p may promote receptor ESCC cell proliferation and migration.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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