Am J Cancer Res 2024;14(12):5665-5679
www.ajcr.us /ISSN:2156-6976/ajcr0148445

Original Article

miR-542-3p/PIK3R1 axis is involved in hsa_circ_0087104-mediated inhibition of esophageal squamous cell carcinoma metastasis

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Received December 12, 2022; Accepted June 20, 2023; Epub December 15, 2024; Published December 30, 2024

Abstract: Esophageal squamous cell carcinoma (ESCC), the most predominant subtype of esophageal cancer, is notorious for its high lymph node metastatic potential and poor prognosis. Growing evidence has demonstrated crucial function of circRNAs in human malignancies. However, the knowledge of circRNAs in lymph node metastasis of ESCC is still inadequate. In this study, a series of bioinformatic analyses and experimental validation were performed. By performing differential expression analysis and selection for GEO dataset GSE150476, a total of 8 circRNAs associated with lymph node metastasis of ESCC were identified. Expression analysis confirmed their low expression in ESCC tissues (relative to normal tissues) or metastatic sites (relative to primary sites). By combination of binding miRNAs from CSCD and starBase databases, six potential miRNAs (miR-532-5p, miR-2681-5p, miR-670-5p, miR-1252-5p, miR-382-3p and miR-542-3p) were predicted and a circRNA-miRNA regulatory network was constructed. Next, 695 target genes were predicted to bind to the 6 miRNAs. After conducting protein-protein interaction (PPI) network analysis, hub gene identification and expression analysis, a hub gene PIK3R1 was identified as the most potential downstream target gene of hsa_circ_0087104/miR-542-3p in ESCC. Hsa_circ_0087104 and PIK3R1 were decreased while miR-542-3p was increased in ESCC cells compared with normal esophageal epithelial cell line. Luciferase reporter and MS2-RIP assays confirmed the direct bind of miR-542-3p to hsa_circ_0087104 or PIK3R1. Hsa_circ_0087104 increased PIK3R1 expression but ectopic expression of miR-542-3p reversed hsa_circ_0087104-mediated PIK3R1 overexpression in ESCC. Overexpression of hsa_circ_0087104 suppressed *in vitro* migration and invasion of ESCC cells and this suppressive effect could be weakened by upregulation of miR-542-3p. Collectively, the current findings elucidated a potential hsa_circ_0087104/miR-542-3p/PIK3R1 axis that might be involved in suppression of lymph node metastasis of ESCC.

Keywords: Circular RNA (circRNA), hsa_circ_0087104, miR-542, esophageal squamous cell carcinoma (ESCC), metastasis

Introduction

Esophageal squamous cell carcinoma (ESCC), a malignant type originated from esophageal epithelial cells, ranks the seventh most frequent cancer and sixth leading causes of cancer-related deaths all over the world [1]. As the most common histological subtype, ESCC accounts for approximately 90% of all esophageal cancer cases [2]. Despite huge advancements regarding the diagnosis, therapy and prognosis have been achieved, the outcome of patients with ESCC is still dismal, with overall survival rate less than 20% [3]. Lymph node metastasis, a well-known poor prognostic factor of ESCC, may also contribute to treatment failures [4]. According to the statistics, the overall survival percentage of ESCC patients with lymph node metastasis decreases by 4% after five years [5]. Therefore, it is urgent need to explore the molecular mechanism of lymph node metastasis of ESCC and seek and develop effective therapeutic targets for treating lymph node metastasis of ESCC.

Circular RNAs (circRNAs) are a group of novel, endogenous and noncoding RNA transcripts characterized with covalently closed loops [6,

https://doi.org/10.62347/EFEO7205

7]. CircRNAs without 5'-cap and 3'-polyadenylated tail structure render them resistant to exonucleases and make them more stable than their linear counterparts [8]. Increasing studies have showed that circRNAs are critical regulators in multiple human malignancies, including breast cancer [9, 10], gastric cancer [11, 12] and hepatocellular carcinoma [13, 14]. However, only few circRNAs have been also reported to be closely associated with lymph node metastasis of ESCC. For example, Liu *et al*. showed that increased expression of serum exosomal hsa_circ_0026611 was associated with lymph node metastasis and poor prognosis of ESCC [15]; Zheng *et al*. suggested that hsa_circRNA_100873 overexpression linked to increased lymphatic metastasis of ESCC [16]. To date, the knowledge of circRNA's expression, function and mechanism in lymph node metastasis of ESCC is still inadequate and need to be further elucidated.

In this study, we first identified the candidate circRNAs associated with lymph node metastasis by performing circRNA differential expression analysis, intersection analysis and expression confirmation. Next, the downstream molecular mechanism of candidate circRNAs were predicted and explored. Finally, experimental validation for a hsa_circ_0087104/miR-542-3p/PIK3R1 axis in metastasis of ESCC was conducted. All the findings from this work might provide key clues for developing effective therapeutic targets for treating lymph node metastasis of ESCC in the future.

Materials and methods

Inclusion of datasets

In this study, we aimed to explore the possible role and mechanism of circRNAs involved in lymph node metastasis of ESCC by usage of NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo/) datasets. The included datasets should meet the following selection criteria: (1) the included datasets should study the circRNA expression profile; (2) only datasets regarding lymph node metastasis of ESCC would be included; (3) the datasets investigating cell lines or animals should be excluded. Finally, only one dataset GSE150476 met all the above criteria. GSE150476, based on the platform of GPL21825 074301 Array Human CircRNA microarray V2, contained three adjacent normal tissues, three ESCC tissues and three metastatic lymph node tissues.

GEO2R analysis

GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r/) is an online tool from the NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo/), which was used to perform data normalization and differential expression analysis in this study. Consequently, the differentially expressed circRNAs between normal and ESCC or between ESCC and metastatic tissues were obtained.

Intersection analysis

Intersection analysis was performed by an online tool, namely VENNY 2.1 (http://bioinfogp.cnb.csic.es/tools/venny). Using VENNY 2.1, the circRNAs that were commonly appeared in Cancer Vs. Normal, or Lymph Node Metastasis Vs. Cancer were acquired. Besides, VENNY 2.1 was also introduced to acquire the common binding miRNAs of circRNAs from CSCD and starBase databases.

circBase analysis

circBase (http://www.circbase.org/) [17] is an interactive database that can provide scripts to acquire known and novel circRNAs in sequencing data. In this study, circBase was utilized to obtain the genome location and the parental gene of circRNAs.

Cancer-specific circRNA database (CSCD) analysis

CSCD (http://gb.whu.edu.cn/CSCD) [18] is a cancer-specific circRNA database that can understand the functional effects of circRNAs by predicting their microRNA response element sites, RNA binding protein sites and open reading frames. This database was employed to draw the structural patterns of candidate circRNAs related to lymph node metastasis of ESCC.

miRNA prediction

The binding miRNAs of candidate circRNAs were predicted by two databases, consisting of CSCD (http://gb.whu.edu.cn/CSCD) [18] and starBase (http://starbase.sysu.edu.cn/) [19]. The binding miRNAs that were commonly

appeared in both the two databases were considered as candidate miRNAs.

Target gene prediction

miRNet (http://www.mirnet.ca) [20] is a network-based visual database for miRNA functional analysis and systems biology prediction, which was introduced to predict the possible target genes that could bind to candidate miRNAs. The miRNA-target gene regulatory network was also constructed using miRNet.

STRING analysis

STRING database (https://string-db.org/) [21] is a comprehensive database aiming to integrate all known and predicted associations between proteins, which was introduced to perform protein-protein interaction (PPI) network analysis for the target genes of candidate miRNAs. A PPI network was constructed by the database and could be directly downloaded from the database.

Hub gene screening

The hub genes among all target genes of candidate miRNAs were identified according to node degree by calculation of CytoHubba using Cytoscape software.

Expression analysis

The expression levels of the top 30 hub genes in esophageal carcinoma and normal controls were analyzed using starBase database (http://starbase.sysu.edu.cn/) which is a database for exploring miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-seq data [19].

Cell lines and cell culture

The normal esophageal epithelial cell line HEEC and ESCC cell lines TE-1, KYSE-150, KYSE-180 and ECA-109 were purchased from the Cell Bank of the Chinese Academy of Sciences and were cultured in RPMI-1640 medium (Gibco, Life Technologies, USA) supplemented with 10% FBS under a humidified atmosphere of 5% CO_2 at 37°C.

Cell transfection

The circRNA overexpressed plasmid, miRNA mimic and their corresponding negative controls were designed and purchased from RiboBio Co. Ltd. (Guangzhou, China). Cell transfection was conducted using Lipofectamine 3000 reagent (Invitrogen, Shanghai, China) according to the manufacturer's instructions as previously described [22].

Wound healing assay

The transfected cells were re-plated into six-well plates which were grown to 100% confluence in six-well plates, after which a micropipette tip was used to make a cross wound. Then, photographs were taken using a microscopy at 0 or 24 hours after wounding.

Transwell invasion analysis

Transwell invasion analysis was performed to assess cells' invaded abilities. 50,000 transfected cells were suspended into 0.2 ml serum-free medium and were added into Transwell inserts (Corning, USA) pre-coated with Matrigel (BD Bioscience, USA), after which the cells were cultured for 48 hours at 37°C. Subsequently, the cells on the super surfaces of the membrane were removed by a cotton swab and the cells on the lower surfaces of the membrane were fixed using 100% methanol and were stained using 0.1% crystal violet. Finally, the invaded cells were counted through a microscopy.

RNA isolation and qRT-PCR

The total RNAs from cells were extracted by usage of RNAiso plus Reagent (TaKaRa, Japan). Then, the RNAs were reversely transcribed into complementary DNA (cDNA) by the PrimeScript™ RT Reagent Kit (TaKaRa, Japan). Subsequently, PCR was performed in triplicates using a Roche LightCycler480 II Real-Time PCR Detection System by SYBR Premix Ex Taq (TaKaRa, Japan). Finally, the expression level was normalized to GAPDH or U6 and was calculated by the method of 2^{-ddCt}.

Statistical analysis

The bioinformatic statistical analyses in this study were directly performed using the above online databases or tools. The results from experiments in this study were shown as mean ± standard deviation (SD) and were analyzed by Students' *t*-test using GraphPad Prism software (Version 7). *P*-value <0.05 was considered as statistically significant.

Results

Identification of candidate circRNAs associated with lymph node metastasis of ESCC

Aiming to explore the underlying role and mechanism of circRNA in lymph node metastasis of ESCC, a GEO dataset GSE150476 was employed in this study. By usage of GEO2R online tool, the differentially expressed circRNAs (DECs) between ESCC and normal or metastatic tissues were obtained after performing data normalization (**Figure 1**). Next, the 12 downregulated circRNAs that were commonly appeared in Cancer Vs. Normal and Metastasis Vs. Cancer circRNA sets were selected as candidate circRNAs associated with lymph node metastasis of ESCC by performing intersection analysis (**Figure 2A**, **2B**). No upregulated circRNAs were commonly appeared in Cancer Vs. Normal and Metastasis Vs. Cancer circRNA sets. 8 of the 12 downregulated circRNAs were confirmed in circBase database. For improving the analytic accuracy, the expression data of the 8 circRNAs were downloaded from GEO database and re-calculated using GraphPad Prism software. As presented in **Figure 2C-J**, the expression levels of the 8 circRNAs were gradually decreased in order of normal, ESCC and metastatic ESCC. The genome location and parental genes of the 8 candidate circRNAs were listed in **Table 1**. Therefore, the 8 circRNAs might be potential circRNAs associated with lymph node metastasis of ESCC.

The structural and mechanistic patterns of candidate circRNAs

Previous studies have demonstrated that circRNAs exerts their function by mediating three possible mechanisms, consisting of miRNA sponge, RBP bind and protein or peptide encode. Thus, the structural patterns of the 8 circRNAs were explored using CSCD database. Consequently, the loop graphs of 5 of 8 circRNAs were found (**Figure 3**). As shown in **Figure 3**, all the five circRNAs (hsa_circ_0072389, hsa_circ_0005062, hsa_circ_0072391, hsa_circ_0072386 and hsa_circ_0087104) possessed miRNA response element site and RNA binding protein site. Among the 5 circRNAs, only hsa_circ_0005062 had a predicted open reading frame (**Figure 3B**).

Prediction of potential binding miRNAs of circRNAs related to lymph node metastasis of ESCC

Next, the binding miRNAs of the five candidate circRNAs related to lymph node metastasis of ESCC were predicted by two prediction programs, consisting of CSCD and starBase database. For improving analytic accuracy, only miRNAs that were commonly appeared in both the two databases were acquired by performing intersection analysis. As shown in **Figure 4A-E**, 1 (miR-532-5p), 2 (miR-2681-5p and miR-670-5p), 0, 3 (miR-1252-5p, miR-382-3p and miR-532-5p) and 1 (miR-542-3p) miRNAs were forecasted to potentially bind to hsa_circ_0072389, hsa_circ_0005062, hsa_circ_0072391, hsa_circ_0072386 and hsa_circ_0087104, respectively. Subsequently, a circRNA-miRNA regulatory network was constricted using Cytoscape software (**Figure 4F**).

Target gene prediction and hub gene identification

The target genes of potential miRNAs were predicted using a comprehensive target gene prediction database, namely miRNet. Consequently, a total of 695 target genes of these miRNAs were found. For better visualization, a miRNA-target gene regulatory network was established as shown in **Figure 5**. PPI network analysis demonstrated that these target genes had close connections. As presented in **Figure 6A**, a PPI regulatory network among these target genes was constructed by STRING database. After calculating by CytoHubba, the top 30 hub genes were identified based on node degree and a sub-PPI network was re-constructed (**Figure 6B**, **6C**). Subsequently, expression analysis for the top 30 hub genes in esophageal carcinoma was performed. As presented in **Figure 6D**, among these genes, only PI3KR1 was significantly downregulated in esophageal carcinoma when compared with normal controls. By matching the circRNA-miRNA or miRNA-target gene pairs, PIK3R1 was the downstream target gene of hsa_circ_0087104/miR-542-3p axis. Taken together, hsa_circ_0087104/miR-542-3p/PIK3R1 might be a

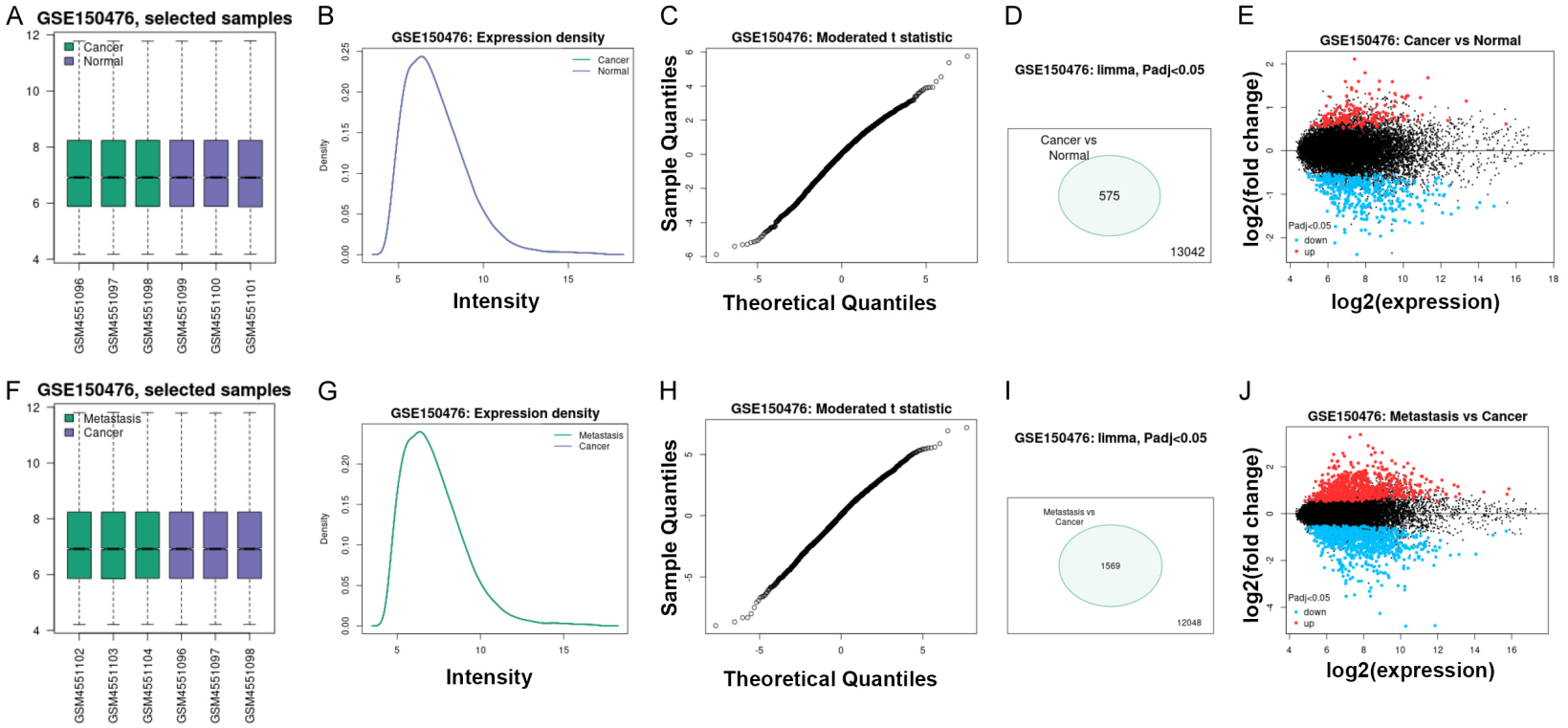


Figure 1. Screening of differentially expressed circRNAs (DECs) associated with lymph node metastasis of ESCC. A. The normalization of the tissue samples (3 normal and 3 ESCC samples) from GSE150476. B. The expression density of circRNAs from 3 normal samples and 3 ESCC samples in GSE150476. C. The moderated t statistic of each circRNA from 3 normal samples and 3 ESCC samples in GSE150476. D. The significant DECs between normal and ESCC samples in GSE150476. E. The volcano plot of the DECs between normal and ESCC samples in GSE150476. F. The normalization of the tissue samples (3 ESCC and metastatic samples) from GSE150476. G. The expression density of circRNAs from 3 ESCC and metastatic samples in GSE150476. H. The moderated t statistic of each circRNA from 3 ESCC and metastatic samples in GSE150476. I. The significant DECs between ESCC and metastatic samples in GSE150476. J. The volcano plot of the DECs between ESCC and metastatic samples in GSE150476. The red dots and blue dots represent the significant upregulated and downregulated DECs in metastatic sites (relative to primary sites) or ESCC tissues (relative to normal tissues), respectively. The black dots represent circRNAs that are not differentially expressed between primary ESCC and metastatic ESCC or normal controls in GSE150476.

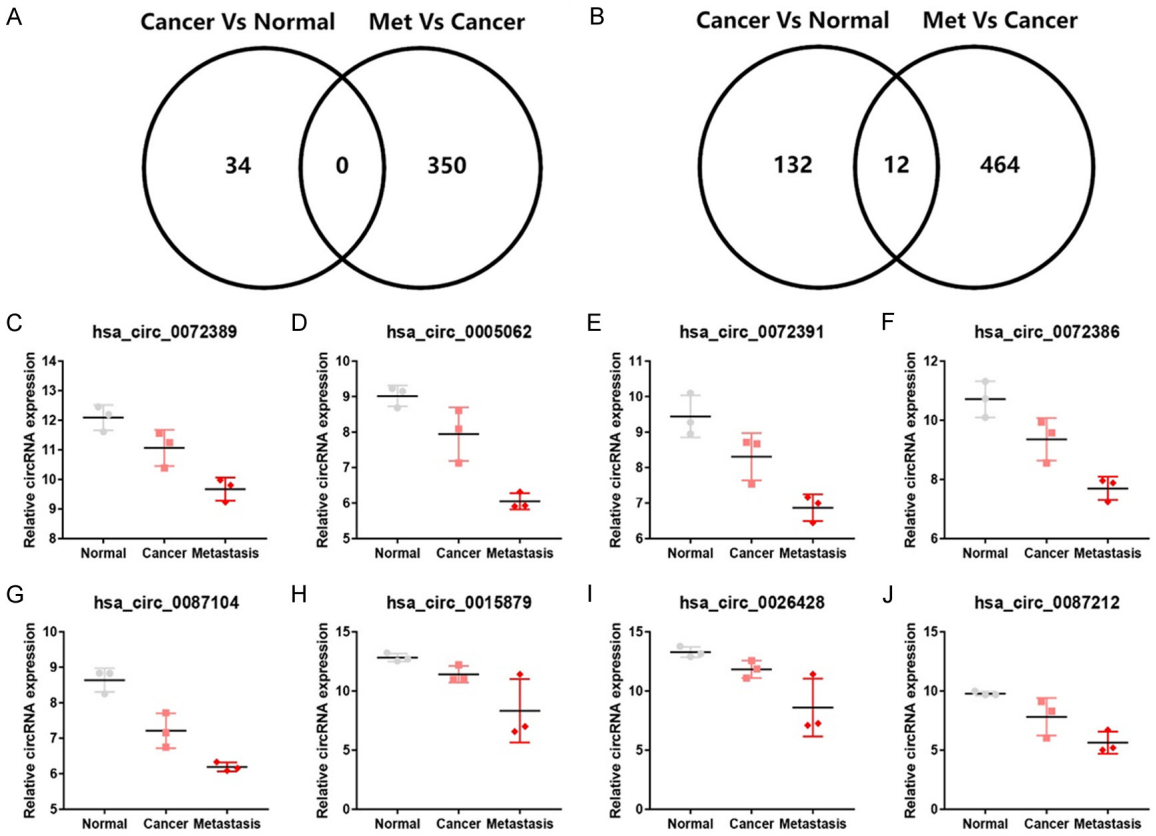


Figure 2. Identification of potential circRNAs associated with lymph node metastasis of ESCC. Intersection analysis for the circRNAs that were commonly upregulated (A) or downregulated (B) in Cancer Vs. Normal and Metastasis Vs. Cancer circRNA sets. The expression levels of hsa_circ_0072389 (C), hsa_circ_0005062 (D), hsa_circ_0072391 (E), hsa_circ_0072386 (F), hsa_circ_0087104 (G), hsa_circ_0015879 (H), hsa_circ_0026428 (I) and hsa_circ_0087212 (J) in normal, ESCC and metastatic tissues.

Table 1. The genome location and parental genes of candidate circRNAs associated with malignant progression of esophageal squamous cell carcinoma

circRNA ID	circBase ID	Location	Parental gene
hsa_circRNA_103830	hsa_circ_0072389	chr5:43294157-43299077	HMGCS1
hsa_circRNA_005062	hsa_circ_0005062	chr9:710803-713464	KANK1
hsa_circRNA_103831	hsa_circ_0072391	chr5:43295853-43297268	HMGCS1
hsa_circRNA_103828	hsa_circ_0072386	chr5:43292575-43299077	HMGCS1
hsa_circRNA_104785	hsa_circ_0087104	chr9:39165929-39178357	CNTNAP3
hsa_circRNA_015879	hsa_circ_0015879	chr1:201286699-201297996	PKP1
hsa_circRNA_026428	hsa_circ_0026428	chr12:52882111-52884517	KRT6A
hsa_circRNA_087212	hsa_circ_0087212	chr9:75775718-75780125	ANXA1

potential pathway involved in lymph node metastasis of ESCC.

Expression, bind and regulation validation of hsa_circ_0087104/miR-542-3p/PIK3R1 axis in ESCC

By usage of a series of bioinformatic analyses as mentioned above, a potential circRNA/miRNA/mRNA axis (hsa_circ_0087104/miR-542-3p/PIK3R1) that might be involved in lymph node metastasis of ESCC was identified. Expression analysis showed that hsa_circ_0087104 and PIK3R1 were lower while miR-542-3p was higher in ESCC cell lines than that in normal esophageal epithelial cell line (**Figure 7A-C**). Two assays, consisting of dual-luciferase reporter and MS2-RIP, were used to confirm the

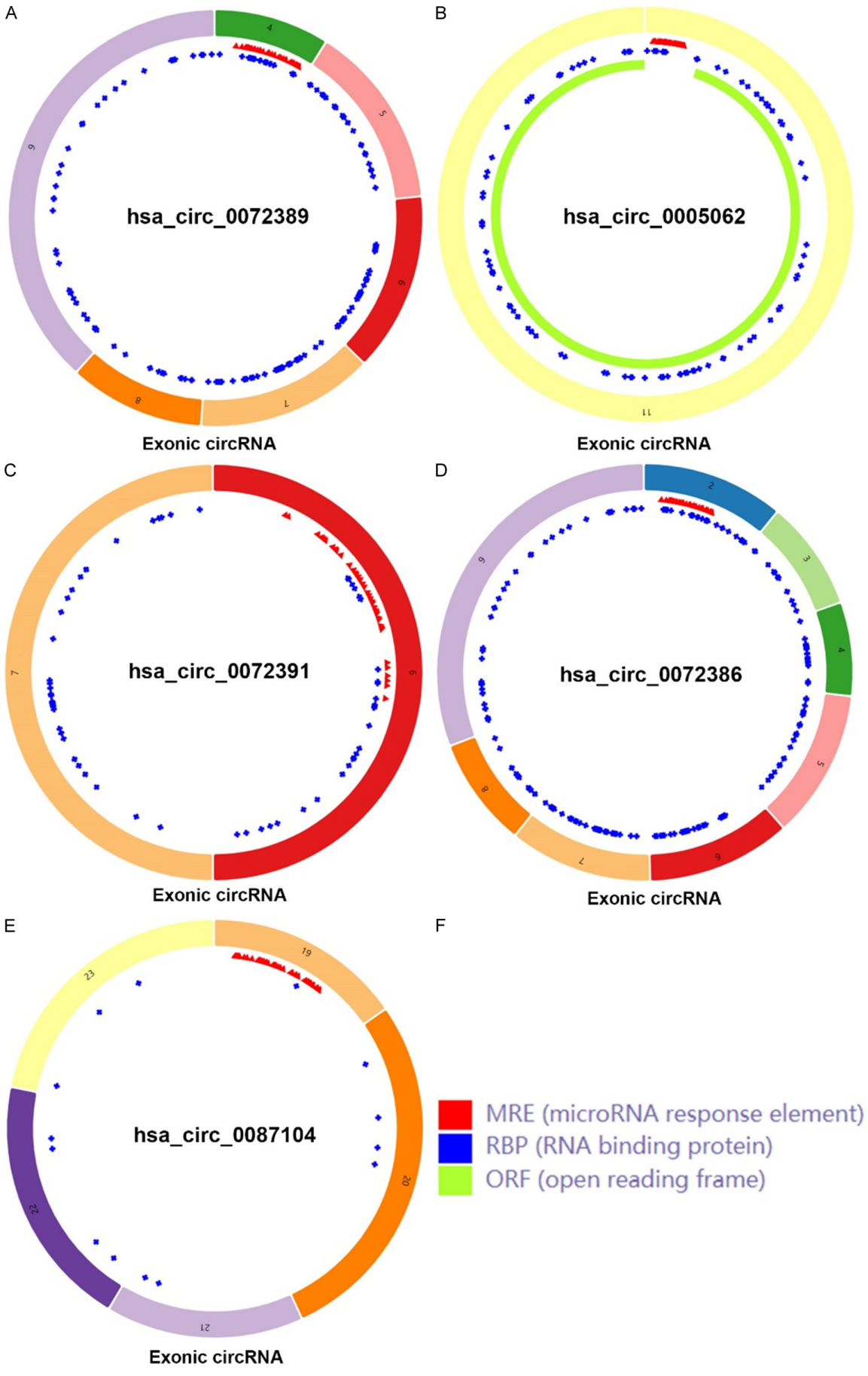

A
hsa_circ_0072389
Exonic circRNA
B
hsa_circ_0005062
Exonic circRNA
C
hsa_circ_0072391
Exonic circRNA
D
hsa_circ_0072386
Exonic circRNA
E
hsa_circ_0087104
Exonic circRNA
F
MRE (microRNA response element)
RBP (RNA binding protein)
ORF (open reading frame)

Figure 3. The structural patterns of candidate circRNAs. The loop graphs of hsa_circ_0072389 (A), hsa_circ_0005062 (B), hsa_circ_0072391 (C), hsa_circ_0072386 (D) and hsa_circ_0087104 (E) obtained from CSCD database. (F) The representation of microRNA response element, RNA binding protein and open reading frame.

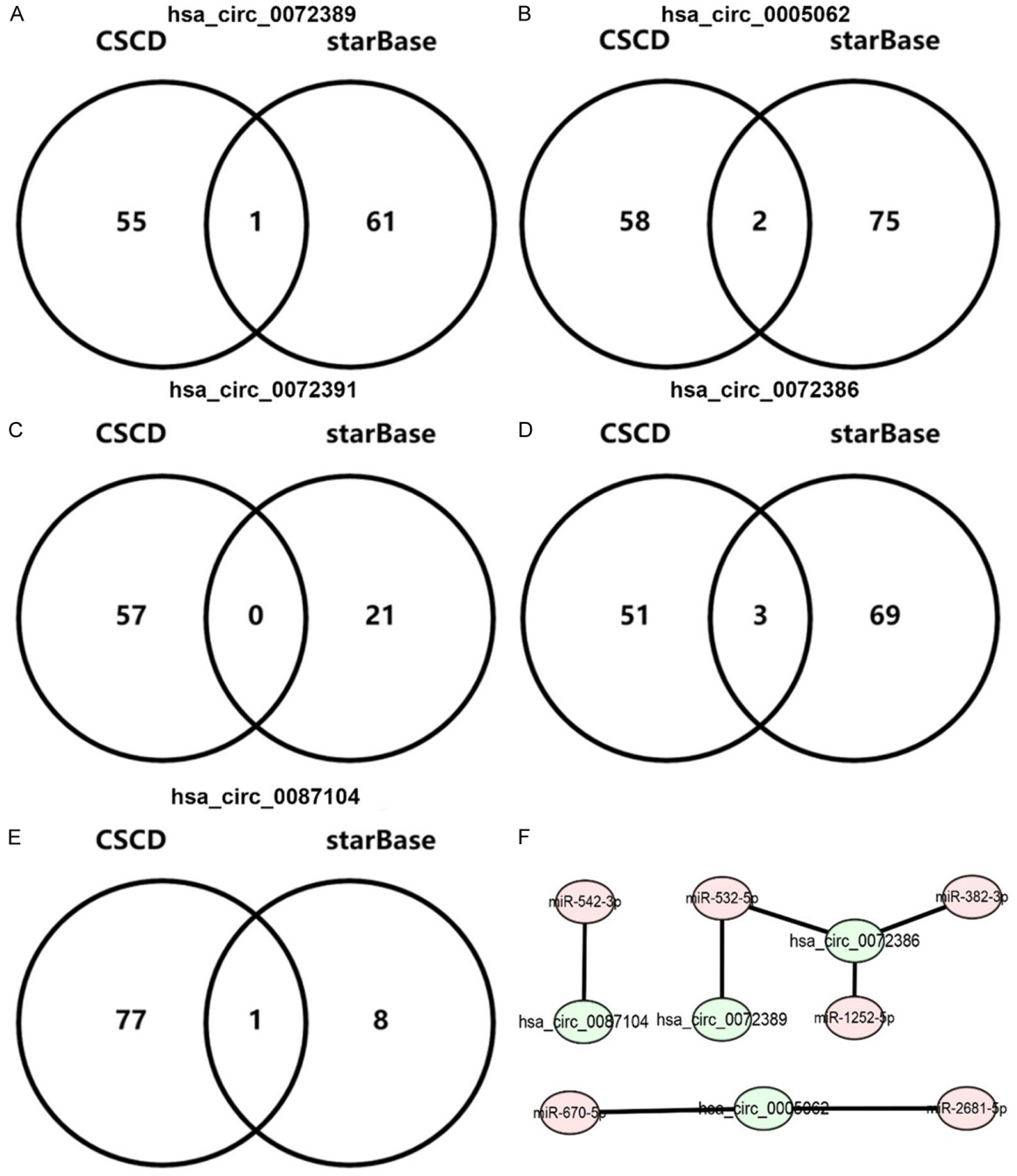


Figure 4. Construction of a potential circRNA-miRNA regulatory network associated with lymph node metastasis of ESCC. Intersection analysis for the binding miRNAs of hsa_circ_0072389 (A), hsa_circ_0005062 (B), hsa_circ_0072391 (C), hsa_circ_0072386 (D) and hsa_circ_0087104 (E) from CSCD and starBase databases. (F) The potential circRNA-miRNA network established by Cytoscape software.

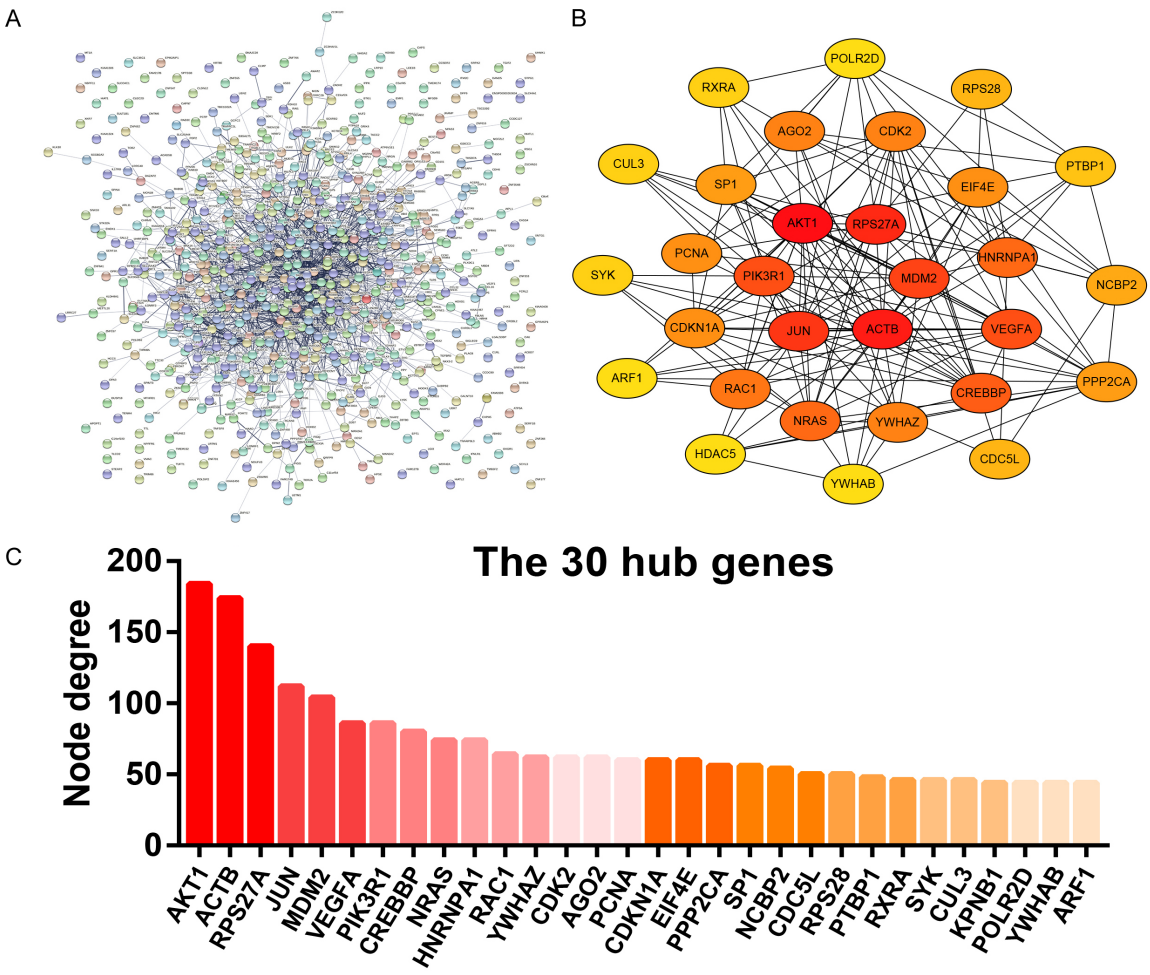
bind relationship among hsa_circ_0087104, miR-542-3p and PIK3R1 as we previously described [23]. **Figure 7D** suggested luciferase activity of the reporter with wild-type-hsa_circ_0087104 or wild-type-PIK3R1 was significantly decreased while luciferase activity of

Figure 5. The miRNA-target gene regulatory network constructed by miRNet database.

the reporter with mutant-hsa_circ_0087104 or mutant-PIK3R1 presented no statistical change when transfected with miR-542-3p mimic. MS2-RIP assay also indicated that miR-542-3p could directly bind to hsa_circ_0087104 and PIK3R1 in ESCC cells (**Figure 7E**). Furthermore, expression regulation research revealed that hsa_circ_0087104 could positively modulate PI3KR1 expression and miR-542-3p reversed hsa_circ_0087104-caused upregulation of PI3KR1 in ESCC cells (**Figure 7F**, **7G**).

Has_circ_0087104 suppressed in vitro metastasis of ESCC by regulating miR-542-3p/PIK3R1 axis

To confirm the role of hsa_circ_0087104/miR-542-3p/PIK3R1 axis in metastasis of ESCC, further functional experimental validation was performed. Firstly, wound healing assay was conducted. Considering the low expression of hsa_circ_0087104 in ESCC (relative to normal tissue) or metastatic site (relative to primary site), hsa_circ_0087104 overexpression assay was conducted. As presented in **Figure 8A-D**, high expression of hsa_circ_0087104 significantly suppressed *in vitro* migration of ESCC cells but hsa_circ_0087104-meidated suppression of migration of ESCC cells could be markedly reversed after overexpression of miR-542-3p. Moreover, transwell invasion assay demonstrated that increased hsa_circ_0087104 inhibited *in vitro* invasion of ESCC cells while miR-542-3p overexpression could weaken hsa_circ_0087104' effect in invasion of ESCC cells (**Figure 8E-G**). All these findings suggested that hsa_circ_0087104/miR-542-



D

Gene symbol	AKT1	ACTB	RPS27A	JUN	MDM2	VEGFA	PIK3R1	CREBBP	NRAS	HNRNPA1
FC	1.13	1.68	1.26	1.08	1.73	2.21	0.66	1.05	2.25	1.64
P-value	4.10E-01	7.00E-10	4.20E-02	8.70E-01	1.30E-01	1.20E-02	2.50E-02	4.20E-01	5.00E-04	4.80E-07
Gene symbol	RAC1	YWHAZ	CDK2	AGO2	PCNA	CDKN1A	EIF4E	PPP2CA	SP1	NCBP2
FC	1.75	2.21	2.83	1.47	3.61	1.27	1.27	1.44	1.41	2.18
P-value	1.20E-09	1.30E-09	1.60E-13	3.70E-03	3.10E-15	1.80E-01	1.20E-02	4.50E-05	1.40E-04	5.90E-08
Gene symbol	CDC5L	RPS28	PTBP1	RXRA	SYK	CUL3	KPNB1	POLR2D	YWHAB	ARF1
FC	1.77	1.17	1.88	1.03	1.59	1.13	1.81	1.61	1.61	1.5
P-value	3.10E-04	3.80E-01	9.30E-17	7.70E-01	1.30E-02	2.90E-01	1.50E-10	1.30E-05	3.90E-08	8.90E-07

Figure 6. Analysis for the target genes of candidate miRNAs. A. The protein-protein interaction (PPI) network established by STRING database. B. The sub-PPI network of the top 30 hub genes constructed by Cytoscape software. C. The presentation of the top 30 hub genes. D. The expression landscape of the top 30 hub genes in ESCC and normal controls. "Red" represents "High expression"; "Green" represents "Low expression"; "Grey" represents "No statistical difference".

3p/PIK3R1 axis might play a suppressive role in lymph node metastasis of ESCC as vividly depicted in **Figure 9**.

Discussion

ESCC is the most frequent histological subtype of esophageal cancer and is malignant tumor with the features of high metastatic potential and poor prognosis [1, 5]. Therefore, it makes sense to put efforts to study the molecular mechanism of lymph node metastasis of ESCC.

It has been widely acknowledged that circRNAs play key roles in initiation and progression of human malignancies, including esophageal cancer [24]. However, only few circRNAs have been reported to link to lymph node metastasis of ESCC, including hsa_circ_0026611 [15] and hsa_circRNA_100873 [16].

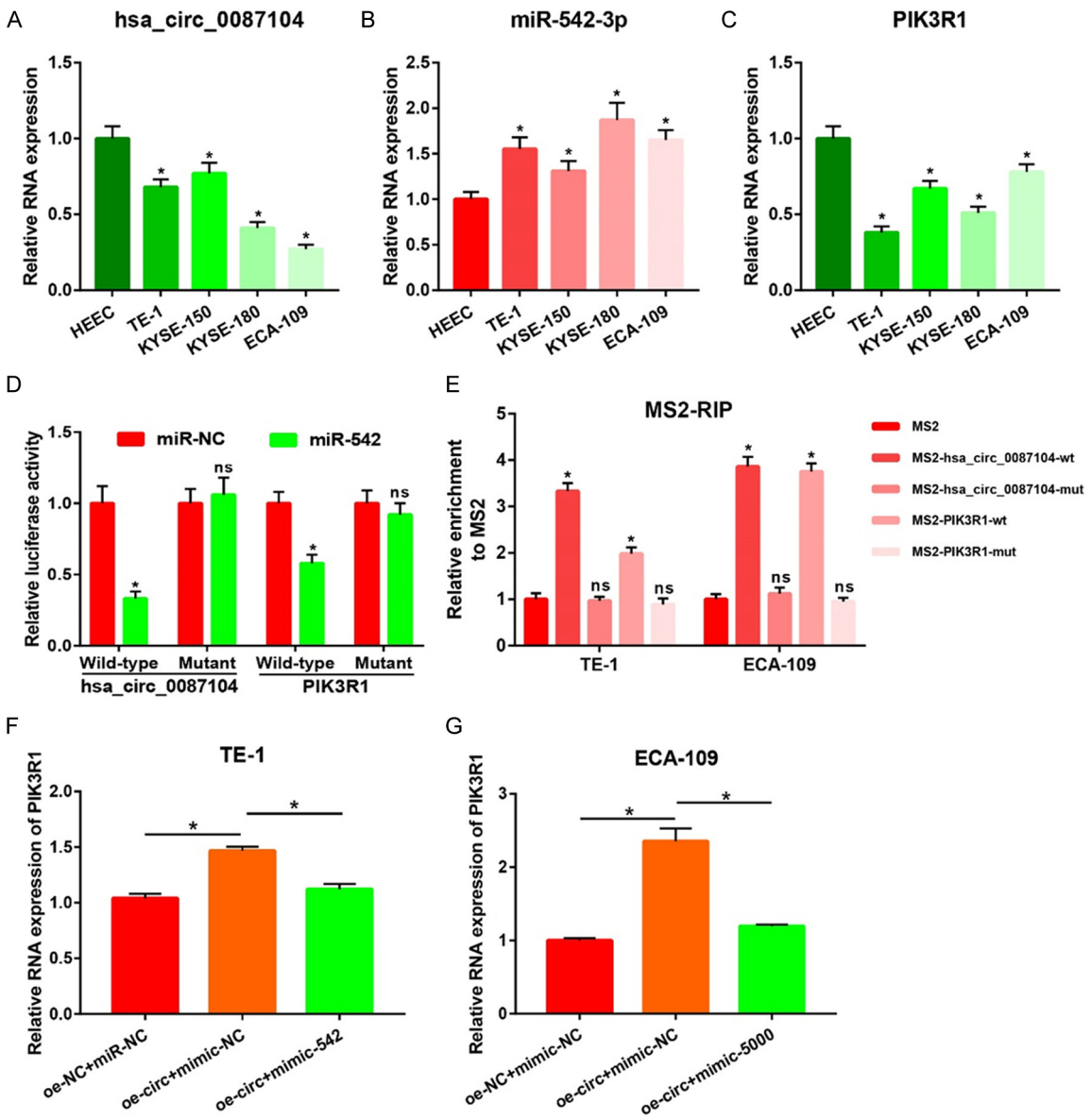


Figure 7. Expression, bind and regulation validation of hsa_circ_0087104/miR-542-3p/PIK3R1 axis in ESCC. A-C. The expression of hsa_circ_0087104, miR-542-3p and PIK3R1 in ESCC cell lines when compared with normal esophageal epithelial cell line. D, E. Dual-luciferase reporter and MS2-RIP assays confirmed that miR-542-3p directly bound to hsa_circ_0087104 and PIK3R1 in ESCC cells. F, G. High expression of hsa_circ_0087104 increased PIK3R1 expression but this effect could be weakened by upregulation of miR-542-5p in ESCC cells. $^{ns}P>0.05$; $^{*}P<0.05$.

In this study, a total of five candidate circRNAs that might be associated with lymph node metastasis of ESCC were identified by using a GEO dataset GSE150476, consisting of hsa_circ_0072389, hsa_circ_0005062, hsa_circ_0072391, hsa_circ_0072386 and hsa_circ_0087104. Some of these circRNAs have been found to participate in cancer development. For example, Liang *et al.* demonstrated that hsa_circ_0072389, hsa_circ_0072391 and hsa_circ_0072386 could aggravate glioma [25]; Zhen *et al.* indicated that hsa_circ_0072391 enhanced cell proliferation of hepatoblastoma [26].

MiRNAs have been widely reported to participate in regulation of circRNAs' expression and function [8, 27, 28]. Moreover, the bioinformatic analysis revealed that all the five candidate circRNAs related to lymph node metastasis of

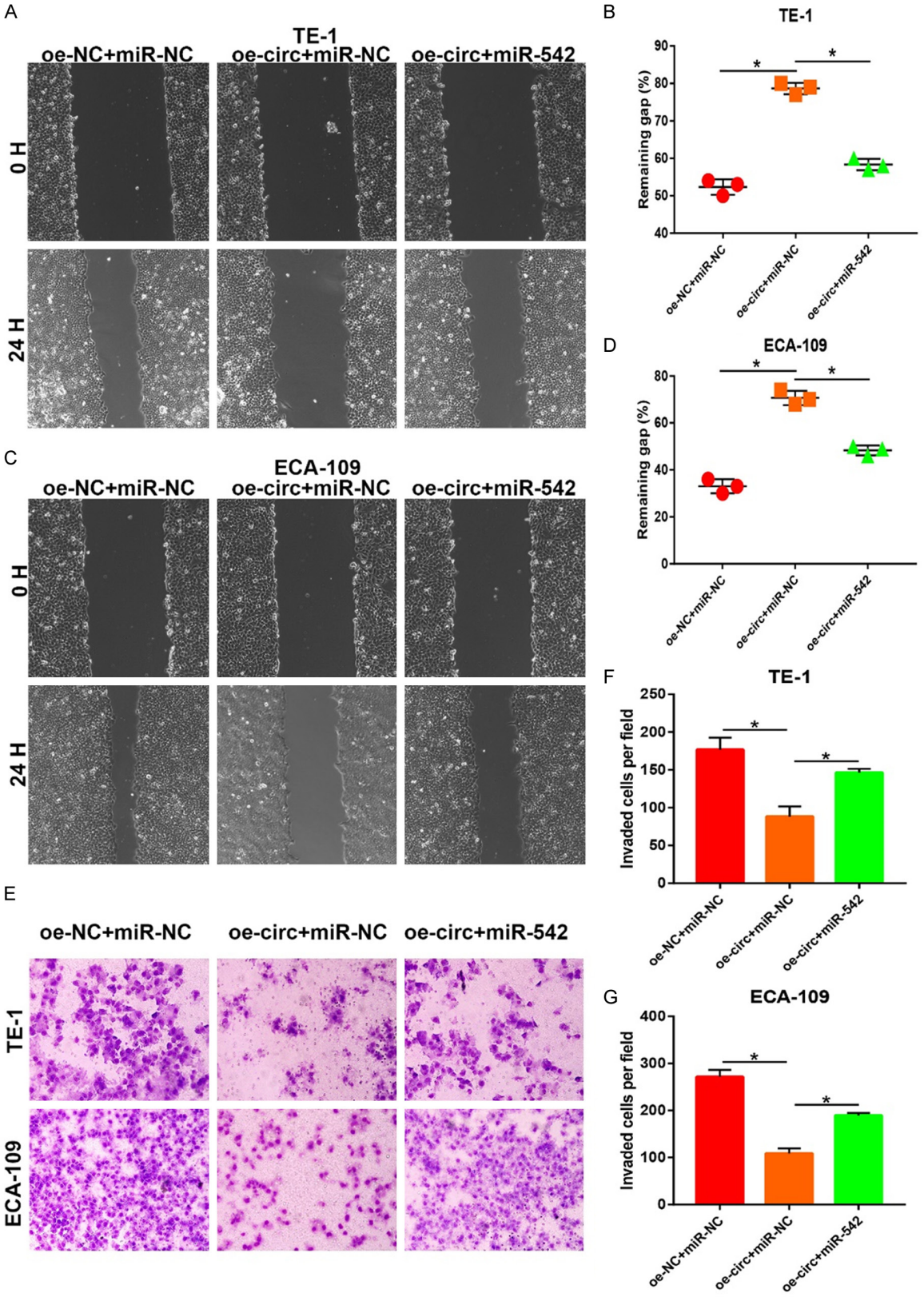


Figure 8. Has_circ_0087104 suppressed *in vitro* metastasis of ESCC by regulating miR-542-3p/PIK3R1 axis. A, B. High expression of hsa_circ_0087104 inhibited migration of TE-1 but this effect could be reversed by upregulation of miR-542-5p. C, D. High expression of hsa_circ_0087104 inhibited migration of ECA-109 but this effect could be reversed by upregulation of miR-542-5p. E-G. High expression of hsa_circ_0087104 inhibited invasion of ESCC cells but this effect could be reversed by upregulation of miR-542-5p. *P<0.05.

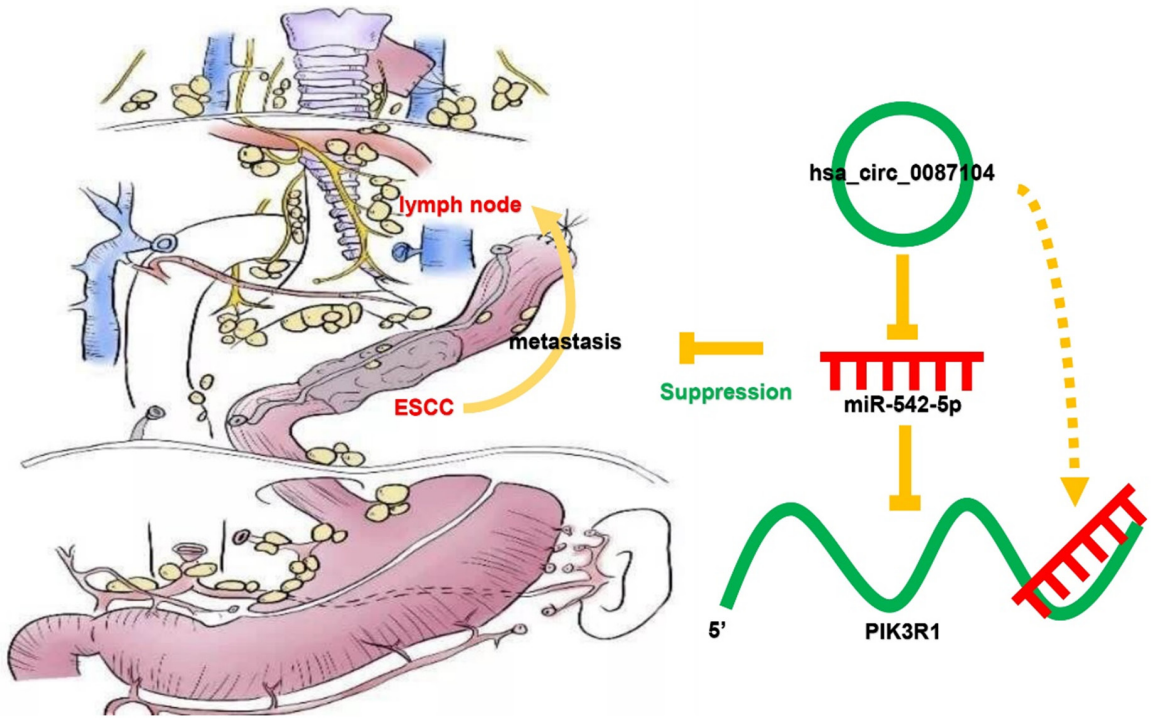


Figure 9. The molecular action mechanism of hsa_circ_0087104/miR-542-5p/PIK3R1 axis in suppression of lymph node metastasis of ESCC.

ESCC possessed miRNA response element sites. After performing binding miRNA prediction from CSCD [18] and starBase [19], a total of 6 binding miRNAs the 5 candidate circRNAs related to lymph node metastasis of ESCC were identified, including miR-532-5p, miR-2681-5p, miR-670-5p, miR-1252-5p, miR-382-3p and miR-542-3p. Previous studies have suggested that some of these miRNAs are involved in carcinogenesis of ESCC. For instance, Song *et al*. confirmed that miR-670-5p participated in hsa_circ_0000337-mediated progression of ESCC [29]; miR-382-3p functioned as a tumor suppressor in ESCC and its downregulation was linked to poor prognosis of patients with ESCC [30, 31].

It has been widely acknowledged that miRNAs exert their biological roles by mainly negatively regulating downstream target gene expression and function [32]. Consequently, a total of 695 target genes were predicted for the six miRNAs. After performing PPI network analysis, hub gene identification and expression analysis, PIK3R1 was identified as the most potential target gene of hsa_circ_0087104/miR-542-3p in ESCC. As previously reported, PIK3R1 was believed to function as a tumor suppressor in several types of human cancer, including lung adenocarcinoma [33], osteosarcoma [34] and cervical cancer [35]. Moreover, our experimental validation revealed that miR-542-3p could directly bind to hsa_circ_0087104 and PIK3R1 in ESCC cells, and overexpression of hsa_circ_0087104 suppressed ESCC cell migration and invasion by regulating miR-542-3p/PIK3R1 axis *in vitro*.

Taken together, the current findings suggested a tumor suppressive hsa_circ_0087104 in lymph node metastasis of ESCC and elucidated its potential downstream miR-542-3p/PIK3R1 molecular mechanism. The identified hsa_circ_0087104/miR-542-3p/PIK3R1 axis might be involved in suppression of lymph node metastasis of ESCC, which should be further confirmed by much more basic cell and animal experiments and large clinical trials in the future.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (NSFC-82203239).

Disclosure of conflict of interest

None.

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