Int J Clin Exp Med 2015;8(9):15556-15562
www.ijcem.com /ISSN:1940-5901/IJCEM0013346

Original Article

MiR-27a-3p promotes esophageal cancer cell proliferation via F-box and WD repeat domain-containing 7 (FBXW7) suppression

Xue-Zhi Wu[1], Kui-Peng Wang[2], Hong-Jie Song[3], Jian-Hua Xia[4], Yan Jiang[5], Yong-Lian Wang[6]

[1]Department of Cardiac Surgery, Zhumadian Center Hospital, Zhumadian 463000, Henan China; [2]The First Affiliated Hospital of Henan University of TCM, Zhengzhou 450000, Henan China; [3]Department of Gastroenterology, Zhumadian Center Hospital, Zhumadian 463000, Henan China; [4]Department of Neurology, Zhumadian Center Hospital, Zhumadian 463000, Henan China; [5]Department of Neurology, The Fifth Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan China; [6]Department of Thoracic Surgery, The First Affiliated Hospital of Xinxiang Medical University, Weihui 453100, China

Received July 24, 2015; Accepted September 10, 2015; Epub September 15, 2015; Published September 30, 2015

Abstract: Emerging evidence has suggested that dysregulation of microRNA-27a-3p (miR-27a-3p) may contribute to tumor development and progression in various types of cancers. However, its role in esophageal cancer is still unknown. In the present study, miR-27a-3p was significantly increased in esophageal squamous cell carcinoma (ESCC) tissues and cell lines. In esophageal cancer Eca109 cells, ectopic overexpression of miR-27a-3p promoted cell proliferation, meanwhile, cell proliferation was reduced by miR-27a-3p inhibition. Further studies showed that down-regulated miR-27a-3p expression could induced cell cycle arrest at the G1/S transition. In exploring mechanisms underlying the promotive role, our results revealed that miR-27a-3p markedly inhibited the expression of F-box and WD repeat domain-containing 7 (FBXW7). FBXW7, a tumor suppressor, exhibited significantly inhibitory effect on Eca109 cell proliferation. Thus our observations suggested that miR-27a-3p functioned as a tumor suppressor by targeting FBXW7. These findings indicated that miR-27a-3p could be considered as a potential therapeutic strategy for ESCC therapy.

Keywords: MiR-27a-3p, FBXW7, esophageal cancer, proliferation

Introduction

Esophageal cancer is one of the most common malignancies with high cancer-related morbidity and mortality worldwide [1]. Esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma are the two major histologic types of esophageal cancer [2]. Traditionally, surgery is considered the best treatment in early stage. In advanced esophageal cancer, a multidisciplinary approach that includes surgery, radiotherapy, and chemotherapy, alone or in combination has been developed [3]. However, the five-year overall survival of esophageal cancer is still not satisfactory [4]. Therefore, it is important to elucidate molecular mechanisms of esophageal cancer and to identify novel molecular targets for the treatment.

MicroRNAs (miRNAs) are a class of highly-conserved, non-coding RNAs (18-25 nucleotides) that function as negative regulators of gene expression at the post-transcription level, binding to the 3'-untranslated region (3'-UTR) of mRNAs transcripts and targeting them for degradation [5, 6]. Growing evidence showed that miRNAs are involved in pathogenesis of most cancers, where some can function as tumor suppressors or oncogenes [7, 8]. Aberrant regulation of miRNAs has been involved in various tumours, including esophageal cancers. For example, Meng et al reported that miRNA-330-3p functions as an oncogene in human esophageal cancer by targeting programmed cell death 4 [9]. Jiang et al suggested that miR-1179 could promote cell invasion through SLIT2/ROBO1 axis in esophageal squamous cell carcinoma [10]. Wang et al showed that

miRNA-218 inhibited the proliferation and metastasis of esophageal squamous cell carcinoma cells by targeting BMI1 [11]. Wang et al suggested that miR-145 could inhibit proliferation and invasion of esophageal squamous cell carcinoma by targeting c-Myc [12]. However, the expression and clinical significance of miR-27a-3p in esophageal cancer remains unknown.

To understand the role of miR-27a-3p in esophageal cancers, we investigated the expression of miR-27a-3p in esophageal cancer. Then, we introduced miR-27a-3p overexpression and down expression models to investigate the effect of miR-27a-3p on esophageal cancer cell proliferation. F-box and WD repeat domain-containing 7 (FBXW7), a tumor suppressor in a number of human cancers [13], has complementary binding site of miR-27a-3p at 3'-UTRs, which indicated that FBXW7 is putatively potential target of miR-27a-3p. Altogether, our study suggested a novel mechanism via which the tumor oncogenic function of miR-27a-3p depends on FBXW7, indicating the FBXW7-miR-27a-3p pathway as a potential therapeutic target in treatment of esophageal cancer.

Materials and methods

Clinical samples

Matched ESCC and corresponding adjacent non-tumor tissues were obtained from 28 patients who underwent esophagectomy for primary ESCC in the Department of Cardiac Surgery, Zhumadian Center Hospital. None of the patients had received preoperative chemotherapy or radiotherapy. All tissue samples were frozen in liquid nitrogen immediately after resection and stored at -80°C. The histologic diagnosis of these tissue samples was confirmed by a pathologist. This study was approved by the Ethics Committee of First Affiliated Hospital of Xinxiang Medical University, and written informed consent was obtained from all patients.

Cell culture

Human ESCC cell lines TE8, Eca109, EC9706, KYSE30 and normal esophageal cell line NEEC was purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Cells were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 U/ml penicillin and 100 μ g/ml streptomycin, within a humidified atmosphere containing 5% CO_2 at 37°C.

Cell transfection

A total of 1.5×10^5 cells were seeded into 6-well plates 24 h prior to transfection. Lipofectamine 2000 transfection reagent (Invitrogen) was used for the transient transfection of miR-27a-3p mimics, miR-27a-3p inhibitor and si-FBXW7 (Genepharma) according to the manufacturer's instructions. The final concentration of miR-27a-3p mimics, miR-27a-3p inhibitor or si-FBXW7 used in our study was 50 nM.

Luciferase assay

The FBXW7 3'-UTR was amplified from human genomic DNA and subcloned into the pGL3 basic luciferase reporter plasmid using MluI and XhoI sites. FBXW7 3'-UTR luciferase reporter plasmids was co-transfected with miR-27a-3p mimics using lipofectamine 2000 reagent. Transfected cells were serum shocked (20%) for 2 h and maintained in 2% RPMI1640 medium for 24 h before harvest. Dual-Luciferase Reporter Assay was performed according to the manufacturer's instructions. Data were presented as relative luciferase activity.

Cell proliferation assay

The transfected cells that were in the logarithmic growth phase were seeded into 96 well plates at a cell density of 1000 cells/well for Eca109. Cell counting kit-8 (CCK-8) solution (10 μL) was added to one well in each group every 24 h for four consecutive days, incubated at 37°C for 2 h, and the optical density was measured at 450 nm on a microplate reader (Thermo Fisher Scientific).

Cell cycle assay

A total of 1×10^4 transfected cells were seeded into 6 well plates and incubated in serum deprived medium for cell synchronization. Subsequently, cells were harvested at 24 h after incubating with complete medium. The cells were stained with PI and examined with a FACS flow cytometer. Data were analyzed with ModFit software (BD Biosciences).

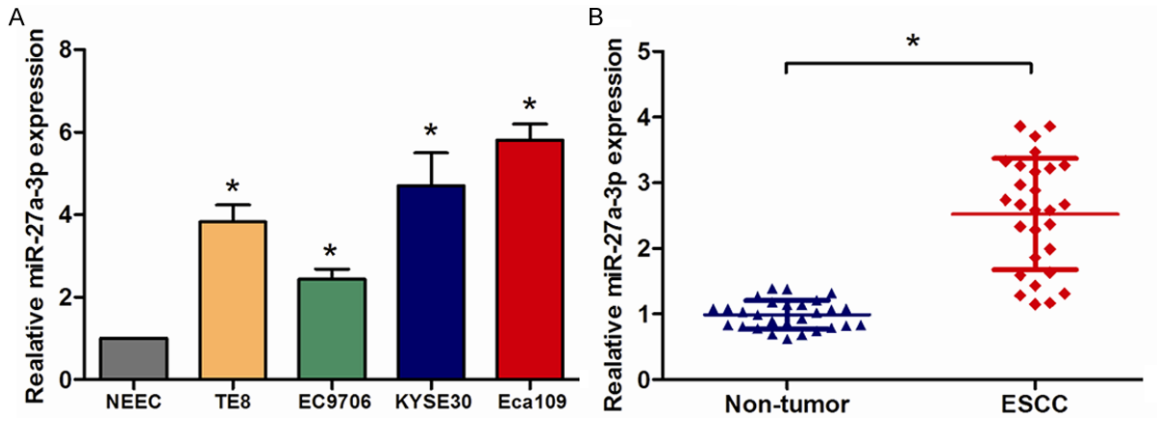

Figure 1. miR-27a-3p was up-regulated in human ESCC cell lines and tissues. A. Relative expression of miR-27a-3p in four ESCC cell lines and one normal esophageal cell line NEEC was determined by qRT-PCR. B. Relative expression of miR-27a-3p in primary ESCC tissues compared to non-tumor tissues. *P < 0.05.

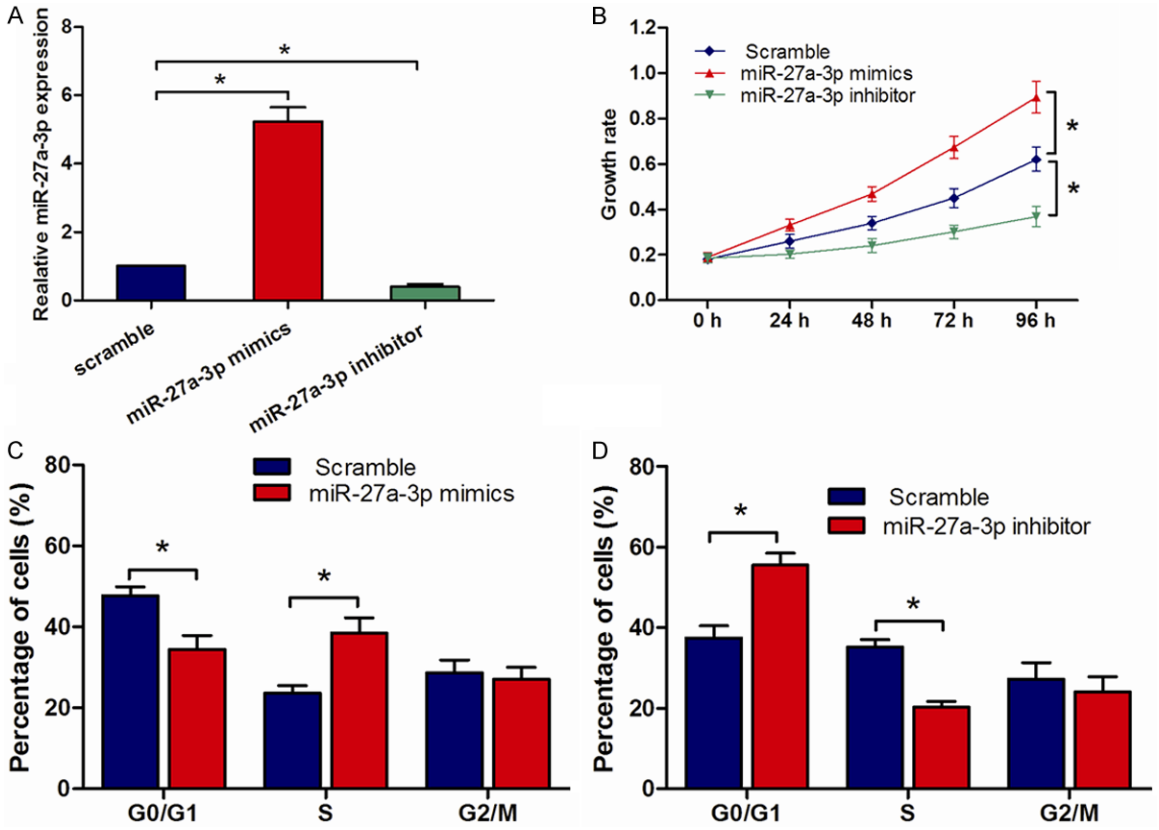

Figure 2. Overexpression of miR-27a-3p promoted ESCC cell proliferation. (A) Expression of miR-27a-3p were determined by qRT-PCR after transfection of miR-27a-3p mimics, inhibitors or scramble. (B) Eca109 cells transfected with miR-27a-3p mimics, inhibitors or scramble, were examined by CCK8 assay after different time periods. (C, D) The cell cycle phase of Eca109 cells transfected with miR-27a-3p mimics (C) or miR-27a-3p inhibitors (D) were analyzed by flow cytometry. *P < 0.05.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from human tissues or cells using Trizol reagent, and purified using a miRNA easy column (Qiagen). The RNA was reversely transcribed into cDNA using a reverse transcript reagent kit. MiR-27a-3p expression was determined using TagMan microRNA

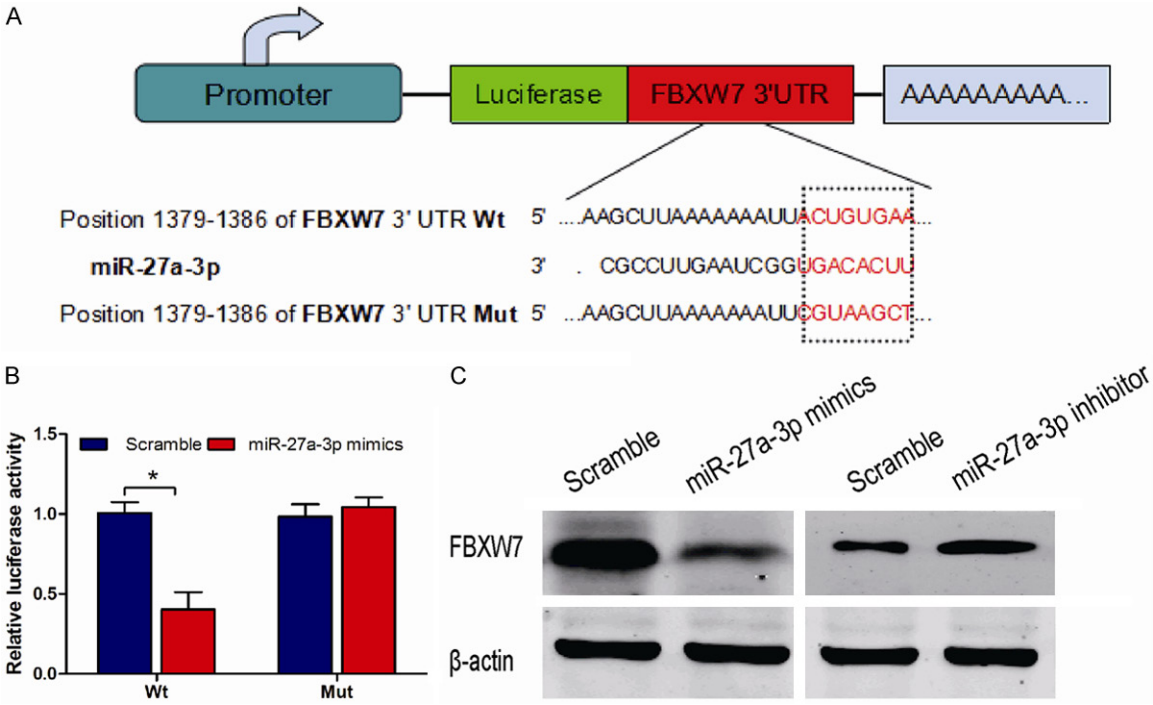

Figure 3. FBXW7 was a target of miR-27a-3p in ESCC cells. A. The potential miR-27a-3p binding sequence of FBXW7 3'-UTR and the Mut. B. Eca109 cells were co-transfected with miR-27a-3p mimics with Wt or Mut FBXW7 3'-UTR. Luciferase activity was assayed. C. Protein level in Eca109 cells transfected with miR-27a-3p mimics or miR-27a-3p inhibitor was detected by Western blot. *P < 0.05.

assays (Applied Biosystems). U6, a small nuclear RNA, was used as an internal control. Specific TaqMan primers, targeting mature miR-27a-3p or U6 were obtained from Applied Biosystems. Quantitative real-time PCR (qRT-PCR) was performed in an ABI 7500 real-time PCR system. Results were presented as the relative changes of miR-27a-3p following normalization to U6.

Western blot

After transfection, cells were lysed using the mammalian protein extraction reagent RIPA (Beyotime) supplemented with a protease inhibitor cocktail (Roche) and phenylmethylsulfonyl fluoride (Roche). Equal amount proteins were separated by 8% SDS-PAGE gel and transferred to PVDF membranes (Bio-Rad). Membranes were probed with primary antibodies at 4°C overnight followed by incubation with HRP conjugated secondary antibodies. β-actin (abcam) was used as a loading control. Protein was detected with Image Acquisition using ImageQuant™ LAS 4000 (GE Healthcare Life Sciences).

Statistical analysis

Statistical analyses were performed using SPSS version 18.0. Data are presented as mean ± SD. Statistical analysis was performed by using one-way analysis of variance, and followed by Dunnett's test for multiple comparisons and by student's *t*-test for comparisons between two groups. *P* values lower than 0.05 was considered statistically significant.

Results

miR-27a-3p is overexpressed in ESCC cell lines and tissues

To determine the effect of miR-27a-3p on the progression of ESCC, We first explored the miR-27a-3p expression in ESCC cell lines using qRT-PCR. The results showed that miR-27a-3p expression was significantly up-regulated in ESCC cell lines compared to normal esophageal cell line NEEC (P < 0.05, **Figure 1A**). We further tested the miR-27a-3p expression in human ESCC tissues. We found that the expression of miR-27a-3p in ESCC tissues was signifi-

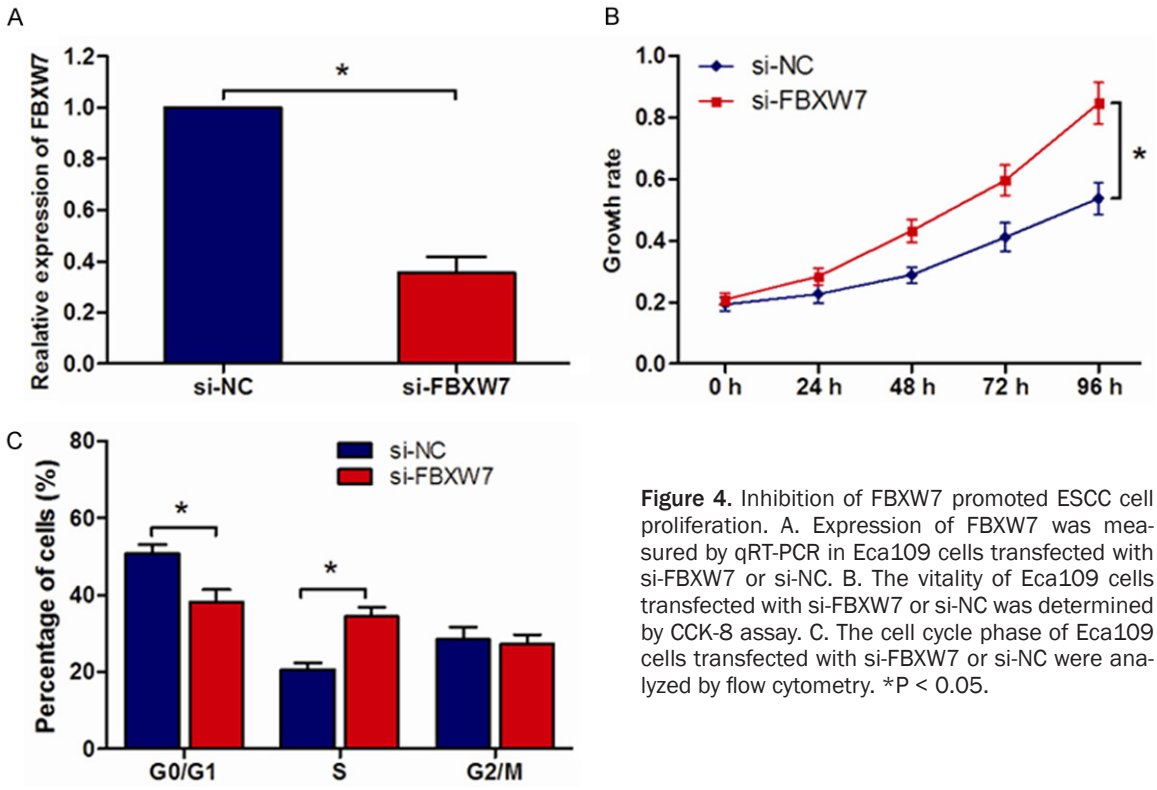

Figure 4. Inhibition of FBXW7 promoted ESCC cell proliferation. A. Expression of FBXW7 was measured by qRT-PCR in Eca109 cells transfected with si-FBXW7 or si-NC. B. The vitality of Eca109 cells transfected with si-FBXW7 or si-NC was determined by CCK-8 assay. C. The cell cycle phase of Eca109 cells transfected with si-FBXW7 or si-NC were analyzed by flow cytometry. *P < 0.05.

cantly higher than that in the adjacent non-tumor tissues (P < 0.05, **Figure 1A**). These data indicated that miR-27a-3p was associated with the progression of ESCC.

miR-27a-3p promotes ESCC cell proliferation

To explore the role of miR-27a-3p in esophageal cancer, ESCC cell line Eca109 was transfected with miR-27a-3p mimics or miR-27a-3p inhibitor. The transfection efficiency was confirmed by qRT-PCR (**Figure 2A**). CCK8 assay showed cell proliferation was significantly higher in miR-27a-3p mimics transfected Eca109 cells compared to scrambled control cells. Conversely, miR-27a-3p inhibitor significantly decreased proliferation of Eca109 cells (**Figure 2B**). In addition, we analyzed the cell cycle by flow cytometry, our results showed that ectopic overexpression of miR-27a-3p induced a significant decrease in the percentage of cells in G1/G0 phase and an increase in the percentage of cells in S phase (**Figure 2C**), In contrast. miR-27a-3p inhibition showed a significant increase in the percentage of cells in G1/G0 phase and a decrease in the percentage of cells in S phase (**Figure 2D**). All these data suggested that miR-27a-3p could promote the proliferation of Eca109 cells.

miR-27a-3p targets FBXW7 in ESCC cells

The 3'-UTRs of FBXW7 has binding site of miR-27a-3p, which indicated FBXW7 is putatively potential target of miR-27a-3p (**Figure 3A**). To examine the effect of miR-27a-3p on FBXW7 transcription activity, a 3'-UTR of FBXW7-containing luciferase reporter plasmid was co-transfected with miR-27a-3p mimics in Eca109 cells. In the Dual-Luciferase assay, our data revealed that miR-27a-3p overexpression significantly decreased wild type (Wt) but not the mutant (Mut) 3'-UTR luciferase activity (**Figure 3B**). Furthermore, in the FBXW7 protein analysis from Western blot, miR-27a-3p overexpression decreased protein level, but miR-27a-3p inhibition increased FBXW7 protein level (**Figure 3C**). Thus, these findings indicated that miR-27a-3p could inhibited the FBXW7 transcription activity.

FBXW7 knockdown increases Eca109 cell proliferation

FBXW7 acted as a tumor suppressor protein in a number of human cancers [13]. However, it is less involved the role of FBXW7 in esophageal cancer. To study the effect of FBXW7 on ESCC cell proliferation, Eca109 cells were transfect-

ed with specific FBXW7 siRNA (si-FBXW7) or negative control siRNA (si-NC). The effect of si-FBXW7 was detected by qRT-PCR (**Figure 4A**). CCK-8 assay showed that si-FBXW7 significantly increased ESCC cell proliferation ability compared to si-NC group (**Figure 4B**). Furthermore, our data revealed that si-FBXW7 decrease the percentage of cells in G1/G0 phase and increase the percentage of cells in S phase (**Figure 4C**). These data showed that inhibition of FBXW7 have a similar effect with miR-27a-3p overexpression.

Discussion

The present study was set to explore the role of miR-27a-3p in the regulation of proliferation of esophageal cancer cells and possible mechanisms. In the present study, our results showed that miR-27a-3p was significantly increased in ESCC cell lines and tissues. Functional studies revealed that miR-27a-3p significantly promoted ESCC cell proliferation, suggesting miR-27a-3p could act as a tumor oncogene in the progression of ESCC. In addition, we identified that FBXW7 was a direct target of miR-27a-3p in ESCC cells. Thus, our results identify a new role of miR-27a-3p in esophageal cancer.

Numerous miRNAs have been identified as oncogenes or tumor suppressor in various human cancers [14]. For example, Wang et al reported that miR-27a-3p was significantly upregulated in peripheral blood mononuclear cells of pancreatic cancer patients [15]. Wataru et al found that miR-27a-3p was markedly upregulated in primary tumor samples from patients and associated with poor progression-free survival of clear cell renal cell carcinoma, furthermore, they showed that miR-27a-3p could promote renal cancer cell proliferation, migration and invasion [16]. Xu et al revealed that miR-27a-3p could promote cell proliferation in glioma cells via regulation of MXI1 expression [17]. Our data expanded the role of miR-27a-3p in ESCC. Forced expression of miR-27a-3p could promote ESCC cell proliferation.

F-box/D repeat-containing protein 7 (FBXW7) belonged to F box protein family, which is the first member of the family [18]. As one of ubiquitin ligase E3 complex, FBXW7 is a key factor of specifically recognizing substrates, regulating its expression by p53 at the transcriptional level [19]. Accumulating evidences suggested that FBXW7 play an important role in the progression of tumorigenesis. For example, Fu et al showed that FBXW7 overexpression could inhibit renal cancer cell proliferation and induced apoptosis [20]. Wang et al found that FBXW7 could inhibit hepatocellular carcinoma migration and invasion via Notch1 signaling pathway [21]. Yang et al suggested that FBXW7 could suppress epithelial-mesenchymal transition, stemness and metastatic potential of cholangiocarcinoma cells [22]. Gong et al reported that miRNA-25 promoted gastric cancer proliferation, invasion, and migration by directly targeting FBXW7 [23]. In the present study, we found that inhibition of FBXW7 remarkably promoted the proliferation of ESCC cells, which expanded the function of FBXW7 in ESCC.

In conclusion, our data showed that miR-27a-3p promoted ESCC cell proliferation through FBXW7 repression. This newly identified miR-27a-3p/FBXW7 link provided a novel therapeutic strategy for the treatment of ESCC.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yan Jiang, Department of Neurology, The Fifth Affiliated Hospital of Zhengzhou University, No. 3, Kangfuqian Street Zhengzhou 450052, Henan China. E-mail: zhongminwang65@163.com

References

[1] Jemal A, Bray F, Center MM, erlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011; 61: 69-90.

[2] Enzinger PC, Mayer RJ. Esophageal cancer. N Engl J Med 2003; 349: 2241-2252.

[3] Hofstetter W, Swisher SG, Correa AM, Hess K, Putnam JB Jr, Ajani JA, Dolormente M, Francisco R, Komaki RR, Lara A, Martin F, Rice DC, Sarabia AJ, Smythe WR, Vaporciyan AA, Walsh GL, Roth JA. Treatment outcomes of resected esophageal cancer. Ann Surg 2002; 236: 376.

[4] Wouters MW, Gooiker GA, van Sandick JW, Tollenaar RA. The volume-outcome relation in the surgical treatment of esophageal cancer. Cancer 2012; 118: 1754-1763.

[5] He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. Nat Revie Genet 2004; 5: 522-531.

[6] Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009; 136: 215-233.
[7] Berkhout B, Jeang KT. RISCy business: MicroRNAs, pathogenesis, and viruses. J Biol Chem 2007; 282: 26641-26645.
[8] Zhang B, Pan X, Cobb GP, Anderson TA. microRNAs as oncogenes and tumor suppressors. Dev Biol 2007; 302: 1-12.
[9] Meng H, Wang K, Chen X, Guan X, Hu L, Xiong G, Li J, Bai Y. MicroRNA-330-3p functions as an oncogene in human esophageal cancer by targeting programmed cell death 4. Am J Cancer Res 2015; 5: 1062-75
[10] Jiang L, Wang Y, Rong Y, Xu L, Chu Y, Zhang Y, Yao Y. miR-1179 promotes cell invasion through SLIT2/ROBO1 axis in esophageal squamous cell carcinoma. Int J Clin Exp Pathol 2015; 8: 319.
[11] Wang T, Chen T, Niu H, Li C, Xu C, Li Y, Huang R, Zhao J, Wu S. MicroRNA-218 inhibits the proliferation and metastasis of esophageal squamous cell carcinoma cells by targeting BMI1. Int J Mol Med 2015; 36: 93-102.
[12] Wang F, Xia J, Wang N, Zong H. miR-145 inhibits proliferation and invasion of esophageal squamous cell carcinoma in part by targeting c-Myc. Onkologie 2013; 36: 754-758.
[13] Akhoondi S, Sun D, von der Lehr N, Apostolidou S, Klotz K, Maljukova A, Cepeda D, Fiegl H, Dafou D, Marth C, Mueller-Holzner E, Corcoran M, Dagnell M,Nejad SZ, Nayer BN, Zali MR, Hansson J, Egyhazi S, Petersson F, Sangfelt P, Nordgren H, Grander D, Reed SI, Widschwendter M, Sangfelt O, Spruck C. FBXW7/hCDC4 is a general tumor suppressor in human cancer. Cancer Res 2007; 67: 9006-9012.
[14] Rufino-Palomares EE, Reyes-Zurita FJ, Lupiáñez JA, et al. MicroRNAs as oncogenes and tumor Suppressors. MicroRNAs in Medicine 2014; 223-243.
[15] Wang WS, Liu LX, Li GP, Chen Y, Li CY, Jin DY, Wang XL. Combined serum CA19-9 and miR-27a-3p in peripheral blood mononuclear cells to diagnose pancreatic cancer. Cancer Pre Res 2013; 6: 331-338.
[16] Nakata W, Uemura M, Sato M, Fujita K, Jingushi K, Ueda Y, Kitae K, Tsujikawa K, Nonomura N. Expression of miR-27a-3p is an independent predictive factor for recurrence in clear cell renal cell carcinoma. Oncotarget 2015; 6: 21645-54.
[17] Xu W, Liu M, Peng X, Zhou P, Zhou J, Xu K, Xu H, Jiang S. miR-24-3p and miR-27a-3p promote cell proliferation in glioma cells via cooperative regulation of MXI1. Int J Oncol 2013; 42: 757-766.
[18] Fujii Y, Yada M, Nishiyama M, Kamura T, Takahashi H, Tsunematsu R, Susaki E, Nakagawa T, Matsumoto A, Nakayama KI. Fbxw7 contributes to tumor suppression by targeting multiple proteins for ubiquitin-dependent degradation. Cancer Sci 2006; 97: 729-736.
[19] Mao JH, Perez-losada J, Wu D, Delrosario R, Tsunematsu R, Nakayama KI, Brown K, Bryson S, Balmain A. Fbxw7/Cdc4 is a p53-dependent, haploinsufficient tumour suppressor gene. Nature 2004; 432: 775-779.
[20] Fu Y, Lin Y, Yang Z, Yang G, Li G, Liu Y, Tan X, Huang Y, Wu X, Wang Y, Xiong H, Zhang M, Fang L, Ge Y, Zeng J, Cai Y, Bai J, Wu S. FBXW7 overexpression suppresses renal cancer cell proliferation and induces apoptosis. Med Oncol 2015; 32: 656-656.
[21] Wang X, Zhang J, Zhou L, Sun W, Zheng ZG, Lu P, Gao Y, Yang XS, Zhang ZC, Tao KS, Dou KF. Fbxw7 regulates hepatocellular carcinoma migration and invasion via Notch1 signaling pathway. Int J Oncol 2015; 47: 231-243.
[22] Yang H, Lu X, Liu Z, Chen L, Xu Y, Wang Y, Wei G, Chen Y. FBXW7 suppresses epithelial-mesenchymal transition, stemness and metastatic potential of cholangiocarcinoma cells. Oncotarget 2015; 6: 6310-6325.
[23] Gong J, Cui Z, Li L, Ma Q, Wang Q, Gao Y, Sun H. MicroRNA-25 promotes gastric cancer proliferation, invasion, and migration by directly targeting F-box and WD-40 Domain Protein 7, FBXW7. Tumor Biol 2015; 36: 7831-40.