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Original Research Article

MFI2-AS1 enhances the survival of esophageal cancer cell via regulation of miR-331-3p/SOX4

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Abstract

***Purpose:** To investigate the specific role of melanotransferrin antisense RNA (MFI2-AS1) in esophageal cancer (EC) progression.*
***Methods:** The differential expression of MFI2-AS1 in EC tissues and cells was determined using quantitative reverse transcription–polymerase chain reaction (qRT-PCR). Silencing MFI2-AS1 was performed by transfection with specific short hairpin RNAs targeting MFI2-AS1. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) and flow cytometry (FC) were used to assess cell viability and apoptosis of EC cells, respectively. The sponging microRNA (miRNA) of MFI2-AS1 was validated using luciferase activity and RNA immunoprecipitation assays while the downstream target gene of the sponging miRNA was evaluated by luciferase activity assay.*
***Results:** MFI2-AS1 was significantly enhanced in EC tissues ($p < 0.01$) and indicated a poor prognosis in EC patients. Knockdown of MFI2-AS1 in EC cells decreased cell viability and promoted cell apoptosis of EC cells. Functionally, MFI2-AS1 targeted miR-331-3p, and sex-determining region on Y-chromosome-related high-mobility-group box4 (SOX4) was identified as a target gene of miR-331-3p. Ectopic expression of SOX4 counteracted the suppressive effect of MFI2-AS1 knockdown on EC cell viability and stimulative effect on EC cell apoptosis.*
***Conclusion:** The pro-oncogenic effect of MFI2-AS1 on EC progression occurs via the regulation of the miR-331-3p/SOX4 axis, providing a new potential therapeutic target for EC.*

***Keywords:** MFI2-AS1, MiR-331-3p, SOX4, Esophageal cancer, Caner progression, Pro-oncogenic*

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INTRODUCTION

As a malignant tumor, esophageal cancer (EC) is one of the most common cancers and the leading cause of cancer-related deaths worldwide [1]. Therefore, to establish new treatment for EC, a better understanding of the molecular mechanism involved in EC progression is needed. Long non-coding RNAs (lncRNAs) have been reported to be critical regulators during tumor progression through epigenetic modification of target genes or sponging microRNAs (miRNAs) to mediate target mRNA stability [2]. The abnormal expression of lncRNAs regulates the occurrence and metastasis of various human tumors, including EC [3]. Melanotransferrin antisense RNA (MFI2-

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AS1), a pro-oncogenic lncRNA in hepatocellular carcinoma [4], has been reported to be dysregulated in EC [5]. However, its specific role in EC progression has not been reported until now. Functional networks formed by lncRNAs-miRNAs-mRNAs have been reported to be implicated in EC initiation and development [6]. MFI2-AS1 functions as a competing endogenous RNA of miR-134 to promote hepatocellular carcinoma progression [4]. The miRNA target of MFI2-AS1 in EC remains unclear and needs further investigation. miR-331-3p was reported to inhibit ovarian cancer metastasis [7] to suppress tumor progression. Recently, miR-331-3p has been reported to be a tumor recurrence predictor in esophageal adenocarcinoma [8], suggesting the involvement of miR-331-3p in MFI2-AS1-mediated EC progression.

The present study investigated the oncogenic role of MFI2-AS1 in EC progression, and then determined the potential miRNA-mRNA network involved in MFI2-AS1-mediated EC tumor progression.

EXPERIMENTAL

Collection of tumor tissues

Sixty-five patients who were diagnosed with EC by histological confirmation were enrolled in this study at the Affiliated Hospital of Guilin Medical University. All the patients provided written informed consent. The tumor tissues and adjacent normal tissues from the 65 patients were excised and stored in liquid nitrogen for subsequent experiments. The study received approval from the Ethics Committee of Affiliated Hospital of Guilin Medical University (approval no. 2019113). It was performed according to the 1964 Helsinki Declaration and its later amendments for ethical research involving human subjects [9].

Cell culture

The EC cell lines (KYSE-150, Eca-109, and KYSE-30) and normal esophageal epithelial cell line (HEEC) were purchased from the Chinese Academy of Sciences (Shanghai, China) and grown in fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) containing Dulbecco's modified Eagle's medium (DMEM; Transgene, Beijing, China) in a 37°C humidified atmosphere incubator containing 5% CO_2.

Cell transfection

Short hairpin RNAs (shRNAs) targeting MFI2-AS1 (shRNA-1 and shRNA-2) and the scrambled oligonucleotide (Control), miR-331-3p mimics, inhibitor and negative control (NC mimic and NC inh) were purchased from GenePharma (Shanghai, China). The full-length cDNA of sex-determining region on the Y-chromosome-related high-mobility-group box4 (SOX4) was inserted into the pcDNA4.1 vector (Invitrogen, Carlsbad, CA, USA). Next, KYSE-150 or KYSE-30 cells were transfected with pcDNA-SOX4, pcDNA vector (Vector), miR-331-3p mimics, inhibitor, NC mimic, NC inh, shRNA-1, shRNA-2 or control with Lipofectamine 2000 (Invitrogen).

Cell viability

KYSE-150 or KYSE-30 cells (5×10^3 cells/well) were seeded at the indicated time (0, 24, 48, 72 and 96 h) before incubation with 20 μL of 5 mg/mL of MTT (Sigma-Aldrich, St. Louis, MO, USA). After 4 hours of incubation, the medium was removed and of the cells was added, and the absorbance value at 490 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA).

Cell apoptosis

KYSE-150 or KYSE-30 cells (1×10^6 cells) were trypsinized and resuspended in binding buffer (KeyGEN BioTech, Jiangning, Nanjing, China). Next, the cells were double stained with 5 μL propidium iodide and 5 μL Annexin V-fluorescein isothiocyanate by Annexin V-FITC kit (Biosea Biotechnology, Beijing, China). Apoptotic cells were analyzed using flow cytometry (FACSCalibur, Becton-Dickinson, San Jose, CA, USA).

Dual-luciferase reporter assay

MFI2-AS1 and the 3′-untranslated region of SOX4 containing miR-331-3p binding sites were cloned into pmirGLO Vector (Promega, Madison, WI, USA) and named MFI2-AS1-wt or SOX4-wt. Similarly, MFI2-AS1 and SOX4 3′-untranslated region carrying mutated sequences were also inserted into the pmirGLO vector and were named MFI2-AS1-mut or SOX4-mut, respectively. Human embryonic kidney-293 cells were plated and co-transfected with the luciferase vectors and miR-331-3p mimics or NC mimic. The luciferase activity was measured 48 h later.

RIP assay

KYSE-150 or KYSE-30 cells were lysed using NP40 lysis buffer (Sigma-Aldrich). After shearing by ultrasound on ice to harvest chromatin, co-precipitated RNA bound to primary IgG or AGO2

antibodies were isolated and then performed using quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis.

RNA isolation and qRT-PCR

Total RNA from EC tissues or cells was extracted using Trizol (Invitrogen). After the determination of the concentration of RNAs using ultraviolet spectrometer, 20 ng RNAs were reverse-transcribed into cDNAs. Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) was then performed under the following conditions: 55°C for 1 min, followed by 35 cycles of 94°C for 30 s, 58°C for 45 s and 72°C for 35 s using SYBR Green Master (Roche, Mannheim, Germany). The gene expression levels were normalized to those of GAPDH or U6 via the comparative threshold cycle CT ($2^{-\Delta\Delta CT}$) method. The primer sequences are shown in Table 1.

Western blotting

KYSE-150 cells were lysed with radioimmunoprecipitation buffer (Sangon Biotech, Shanghai, China), and the protein concentration was measured using the BCA protein assay kit (Pierce, Rockford, IL, USA). Proteins (30 μg) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then the bands were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). After blocking in 5% non-fat milk, the membranes were probed with the SOX4 (1:2000) or β-actin (1:2500) primary antibody (Abcam, Cambridge, UK) overnight at 4°C. Next, the membranes were incubated with the goat anti-human secondary antibody conjugated with horseradish peroxidase (1:5000; Abcam), and then the immunoreactive signals were visualized using enhanced chemiluminescence.

Statistical analysis

All the experiments were performed at least three times, and the data were presented as means, ± standard deviation. Statistical significance was evaluated by independent sample *t*-test followed by Tukey's post-hoc test. Survival curve analysis was performed using the Kaplan–Meier method and the log-rank test. $P < 0.05$ was considered as statistically significant.

RESULTS

MFI2-AS1 is highly expressed in EC

The expression level of MFI2-AS1 was first analyzed in EC tissues or cells. EC tissues contained a relatively high level of MFI2-AS1 compared with that in adjacent normal tissues (Figure 1 A). In addition, highly expressed MFI2-AS1 was correlated with a poor prognosis of EC patients, indicating that patients with high MFI2-AS1 expression showed a shorter overall survival than those with low MFI2-AS1 expression (Figure 1 B). Similarly, MFI2-AS1 expression in EC cells was also higher than in the normal esophageal epithelial cell line (HEEC) (Figure 1 C), suggesting a close relationship between MFI2-AS1 and EC.

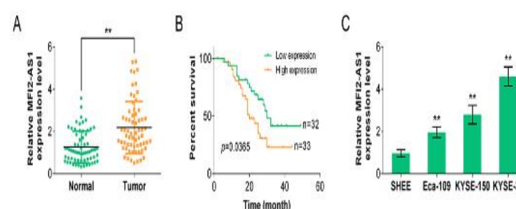

Figure 1: MFI2-AS1 is highly expressed in EC. Expression of MFI2-AS1 in EC tissues and adjacent non-cancer tissues, as determined by qRT-PCR (n = 65). Overall survival analysis of EC patients with high MFI2-AS1 expression and low levels of MFI2-AS1. (A) Expression of MFI2-AS1 in EC cell lines and SHEE, as determined by qRT-PCR; ***p* < 0.01

MFI2-AS1 regulates EC proliferation and apoptosis

Loss-of-function experiments were performed to evaluate the effect of MFI2-AS1 on EC tumor progression.

Table 1: Primer sequences used in the study

Name	Primer	Sequence (5′–3′)
MFI2-AS2	Forward	TACATACAGTGACCCAAAGAGCA
	Reverse	CAGTGCTTCTGAACGCCTCTT
miR-331-3p	Forward	GAGCTGAAAGCACTCCCAA
	Reverse	CACACTCTTGATGTTCCAGGA
U6	Forward	CTCGCTTCGGCAGCACATA
	Reverse	AACGATTCACGAATTTGCGT
GAPDH	Forward	ACCACAGTCCATGCCATCAC
	Reverse	TCCACCACCCTGTTGCTGTA

The expression of MFI2-AS1 was effectively silenced in KYSE-150 or KYSE-30 cells by transfection with shRNA-1 and shRNA2 (Figure 2 A). The MTT assay data revealed that the knockdown of MFI2-AS1 decreased the cell viabilities of KYSE-150 and KYSE-30 cells (Figure 2 B). Cell apoptosis was significantly promoted using shRNA-1 transfection in KYSE-150 and KYSE-30 cells (Figure 2C). Thus, MFI2-AS1 may contribute to EC tumor progression.

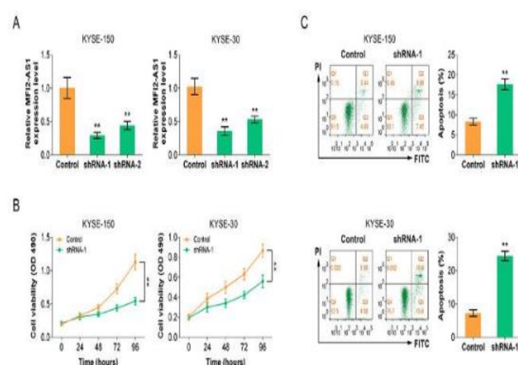

Figure 2: MFI2-AS1 regulates EC proliferation and apoptosis. Transfection efficiency of shRNA-1 and shRNA-2 in KYSE-150 and KYSE-30 cells as detected by qRT-PCR. (A) Effect of MFI2-AS1 on the cell viability of KYSE-150 and KYSE-30 cells as detected by the MTT assay. Effect of MFI2-AS1 on the cell apoptosis of KYSE-150 and KYSE-30 cells, as detected by flow cytometry; ***p* < 0.01

MFI2-AS1 directly targets miR-331-3p

To further unravel the regulatory effect of MFI2-AS1 in EC tumor progression, dual-luciferase and RNA immunoprecipitation (RIP) assays were performed to verify the specific binding target of MFI2-AS1. The sponging miRNA of MFI2-AS1 was predicted to be miR-331-3p (Figure 3 A). The luciferase activity of the MFI2-AS1-wt luciferase reporter vector was substantially reduced in HEK-293T cells transfected with miR-331-3p mimics (Figure 3 B). However, the luciferase activity of MFI2-AS1-mut showed no obvious changes in HEK-293T cells transfected with either miR-331-3p mimics or NC mimic (Figure 3 B). The RIP assay results showed that the MFI2-AS1 and miR-331-3p expression levels were enriched via immunoprecipitation with AGO2 compared with those in the anti-IgG group (Figure 3 C), suggesting the specific interaction between MFI2-AS1 and miR-331-3p. Moreover, the expression of miR-130a-5p was decreased by shRNA-1 transfection in KYSE-150 and KYSE-30 cells (Figure 3 D). Thus, MFI2-AS1 directly targets miR-331-3p to suppress its expression in EC.

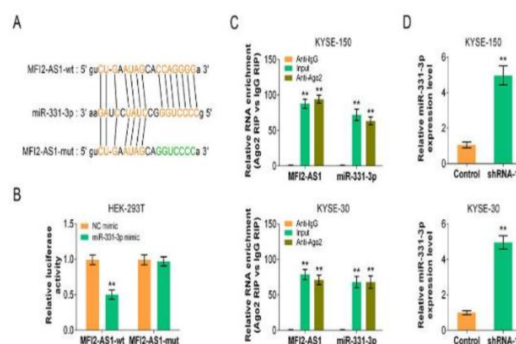

Figure 3: MFI2-AS1 directly targets miR-331-3p. The potential binding target of MFI2-AS1 was predicted to be miR-331-3p. Effect of miR-331-3p on luciferase activity on MFI2-AS1-wt or MFI2-AS1-mut. Binding affinity between MFI2-AS1 and miR-331-3p, as detected by RIP. Effect of MFI2-AS1 on miR-331-3p expression in KYSE-150 and KYSE-30 as determined by qRT-PCR; ***p* < 0.01

miR-331-3p directly targets SOX4

To further investigate the regulatory effect of the MFI2-AS1/miR-331-3p axis in EC, dual-luciferase and RIP assays were performed to verify the specific binding target of miR-331-3p. The target gene of miR-331-3p was predicted to be SOX4 (Figure 4 A), and miR-331-3p mimics decreased the luciferase activity of the SOX-wt luciferase reporter vector. However, the luciferase activity of SOX4-mut was not affected by miR-331-3p mimics (Figure 4 B). Moreover, SOX4 protein expression was decreased by miR-331-3p mimics transfection in KYSE-150 cells (Figure 4 C). To verify the MFI2-AS1/miR-331-3p/SOX4 axis in EC, KYSE-150 cells were cotransfected with shRNA-1 and miR-331-3p inhibitor. The decreased protein expression of SOX4 due to shRNA-1 transfection was reversed by additional transfection with miR-331-3p inhibitor (Figure 4 D), suggesting that MFI2-AS1 directly targets miR-331-3p to increase SOX4 expression in EC.

MFI2-AS1 regulates EC proliferation and apoptosis through miR-331-3p-mediated SOX4

To determine the effect of the MFI2-AS1/miR-331-3p/SOX4 axis on EC progression, KYSE-150 cells were cotransfected with shRNA-1 and pcDNA-SOX4. Transfection with pcDNA-SOX4 attenuated the MFI2-AS1 silence-induced decrease in SOX4 (Figure 5 A). However, over-expression of SOX4 reversed the suppressive effect of shRNA-1 on the cell viability of KYSE-150 (Figure 5 B). Rescue experiments indicated that the over-expression of SOX4 counteracted the promoting effect of shRNA-1 on the cell apoptosis of KYSE-150 (Figure 5 C). Taken

together, the data show that MFI2-AS1 regulates EC proliferation and apoptosis through miR-331-3p-mediated SOX4.

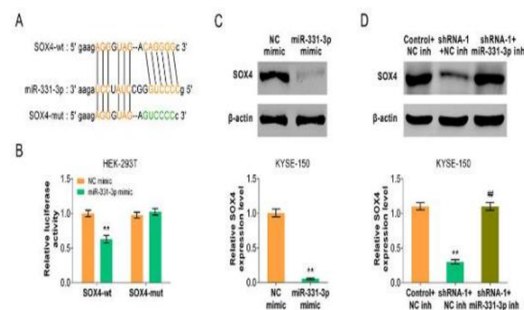

Figure 4: miR-331-3p directly targets SOX4. The potential miR-331-3p binding target was predicted to be SOX4 via Targetscan (http://www.targetscan.org/vert_71/). Effect of miR-331-3p on the luciferase activities of SOX4-wt and SOX4-mut. Effect of miR-331-3p on the protein expression of SOX4 in KYSE-150 cells. Effect of miR-331-3p inhibitor and shRNA-1 on the protein expression of SOX4 in KYSE-150 cells; ** or ##*p* < 0.01

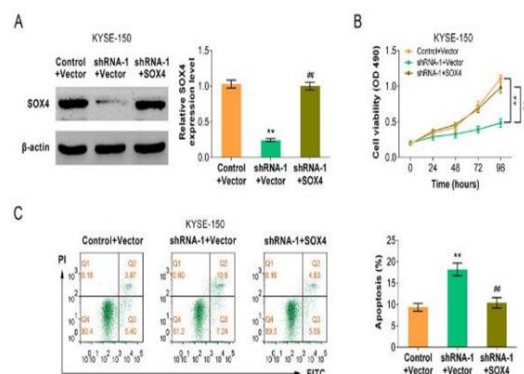

Figure 5: MFI2-AS1 regulates EC proliferation and apoptosis through miR-331-3p-mediated SOX4. (A) Effect of shRNA-1 and pcDNA-SOX4 on the protein expression of SOX4 in KYSE-150 cells. (B) Effect of shRNA-1 and pcDNA-SOX4 on the cell viability of KYSE-150. (C) Effect of shRNA-1 and pcDNA-SOX4 on the cell apoptosis of KYSE-150; ** or ##*p* < 0.01

DISCUSSION

The pivotal role of lncRNAs in tumorigenesis has been established recently, and lncRNA-based therapies represent novel approaches to suppress tumor progression [10]. Because MFI2-AS1 was shown to be dysregulated in EC [5], the role and mechanism of MFI2-AS1 in EC tumor progression were then evaluated in this study. The early diagnosis of EC is particularly important for patients because a delayed diagnosis could lead to metastasis and recurrence of EC [11]. Differentially expressed lncRNAs in EC suggest the potential clinical application of lncRNAs as either diagnostic or prognostic biomarkers [11]. In this study, the up-regulation of MFI2-AS1 in EC tissues demonstrated a poor prognosis in the patients, suggesting its prognostic role in EC. However, to further verify its diagnostic role in EC, the relationship between MFI2-AS1 and the clinicopathological parameters of EC patients should be investigated in a future study.

MFI2-AS1 was reported to be a common pro-oncogenic lncRNA in various tumors. Loss-of-function experiments in this study revealed that the knockdown of MFI2-AS1 decreased the cell viability and promoted the cell apoptosis of EC. However, the effect of MFI2-AS1 on EC migration, invasion and the epithelial–mesenchymal transition remains to be investigated. It has been reported that lncRNAs either directly alter protein activity and localization, modify chromatin remodeling and splicing patterns for transcriptional interference or sponge miRNAs in the pathogenesis of EC [3]. For example, study has shown that lncRNA deleted in lymphocytic leukemia 2 function as competing endogenous RNA during EC progression through binding to miR-30e-5p [12]. Data from luciferase activity and RIP assays showed that miR-331-3p is a direct target miRNA of MFI2-AS1 in EC and that MFI2-AS1 inhibits miR-331-3p expression. miR-331-3p serves as a tumor recurrence predictor in esophageal adenocarcinoma [8]. The role of miR-331-3p in EC progression has been poorly studied and warrants further investigation.

LncRNAs sponge miRNAs to modulate the expression of mRNAs, thus affecting tumorigenesis [3], and miR-331-3p functions as either a tumor suppressor or oncogenic miRNA in various tumors through different target genes. The target gene of miR-331-3p in EC was validated as SOX4 in this study. SOX4, a transcriptional factor, is involved in embryonic development and tumorigenesis via the activation of cancer-associated pathways, including the Wnt/β-catenin and TGF-β/SMAD pathways [13]. Previous studies have shown that SOX4 drives the epithelial–mesenchymal transition to facilitate tumor metastasis [14]. The clinicopathological significance of SOX4 in esophageal squamous cell carcinoma has been recently reported [15], and SOX4 promoted EC proliferation and invasion by silencing miR-31 [16]. Moreover, miR-133a-mediated inhibition of SOX4 suppressed EC migration and invasion [17]. In line with a previous study in which SOX4 was involved in urothelial carcinoma-associated 1/miR-204-mediated EC tumor progression [18],

this study demonstrated that the over-expression of SOX4 counteracted the regulatory role of MFI2-AS1 in EC proliferation and apoptosis, suggesting that lncRNA MFI2-AS1 modulates EC tumor progression in a competing endogenous RNA pattern via the regulation of the miR-331-3p/SOX4 axis.

CONCLUSION

The findings of this study provide evidence that MFI2-AS1, which is up-regulated in EC tissues and cells, plays a pro-oncogenic role in EC growth via miR-331-3p-modulated SOX4. Therefore, MFI2-AS1 may be a potential target for EC therapy.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was performed by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Feng Lin and Angui Li designed the study, supervised the data collection, and analyzed the data. Xiaomin Tang and Kai Hu interpreted the data and prepared the manuscript for publication. Jianfei Song and Haiyong Wang supervised the data collection, analyzed the data and reviewed a draft of the manuscript. All the authors have read and approved the manuscript for publication.

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