miR-493 by regulating of c-Jun targets Wnt5a/PD-L1-inducing esophageal cancer cell development

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Abstract

Background: Esophageal cancer is one of the most common cancers across the globe; the 5-year survival of esophageal cancer patients is still low. MicroRNA (miRNA) dysregulation has been implicated in cancer development, and the miRNAs play a pivotal role in esophageal cancer pathogenesis. It is urgently needed to find out how miRNA dysregulation was involved in esophageal cancer (EC) development.

Methods: Through experiments in vivo and in vitro, we explored potential signaling pathways, miR-493/Wnt5A/c-JUN loop, in EC. Their mechanistic roles in EC cell proliferation, migration, and invasion were investigated through multiple validation steps in EC9706 and TE13 cell lines and EC specimens.

Results: Overexpression of miR-493 attenuates esophageal cancer cell proliferation, migration, and invasion in vivo and in vitro. Moreover, miR-493 downregulation is an unfavorable factor in EC and negatively correlated with Wnt5A. The existence of miR-493 is also an important attribute of metabolism. Based on mechanism analyses, we show that miR-493 inhibits the activity of c-JUN and p-PI3K/p-AKT with enhanced p21 and directly regulates Wnt5A expression and function, whereas c-JUN binds the promoter region of miR-493 and suppressed the expression of miR-493, forming a negative feedback loop.

Conclusions: The miR-493/Wnt5A/c-JUN loop is a molecular feedback loop that refers to the development of esophageal cancer cells and a potential target for the treatment of esophageal cancer.

KEYWORDS

c-JUN, esophageal cancer prognosis, miR-493, PD-L1, Wnt5A

INTRODUCTION

Esophageal cancer (EC) is the eighth most common cancer and the sixth leading cause of death from cancer across the globe.1 Among four of five cases, it happens in non-industrialized nations with the highest rates in Asia and Africa.2 The 5-year survival of EC patients is still low (around 15%–25%), although much progress has been made in treatment.3 Therefore, elucidation of the mechanisms involved in EC development is urgently needed.

MicroRNA (MiRNA) dysregulation has been implicated in cancer development.4 Accumulating evidence has shown that miRNAs play a pivotal role in EC pathogenesis, which provided a new era for the management of cancer and other diseases.5,6 MicroRNA (MiR)-493 as a tumor suppressor miRNA inhibits cell motility through downregulation of RhoC and FZD4 in bladder cancer.7 An increase of miR-493 expression during carcinogenesis prevents liver metastasis of colon cancer cells.8,9 MiR-493 also suppresses hepatocellular carcinoma cell proliferation and invasion.10–12 In lung cancer, miR-493 suppresses tumor growth, invasion and metastasis of lung cancer, and enforced expression of miR-493 in lung cancer cells promotes chemotherapy sensitivity to cisplatin.13–15 Moreover, miR-493 promotes proliferation,
invasion, and chemo-resistance in gastric cancer cells. However, the role of miR-493 in EC is still not investigated. Here, we studied the role of miR-493 and its underlying regulatory mechanisms in EC.

MATERIALS AND METHODS

Cell culture and transfection

Human EC EC9706 cells were cultured in Dulbecco’s modified Eagle’s medium and TE13 was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. For transfection, cells were cultured to 80% confluence and transfected with recombinant plasmids or small interfering RNA (siRNA) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendation. The detail was shown in Appendix S1 (Supplementary Materials and Methods).

Tissue specimens

The patients with esophageal squamous cell carcinoma were obtained from the Second Hospital of Hebei Medical University. Clinical protocols were approved by the Ethics Committees of Second Hospital of Hebei Medical University and patients gave informed written consent.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted from cell lines or tissues using Trizol (Invitrogen). RNA was then reverse-transcribed and complementary DNA (cDNA) was analyzed by regular or quantitative polymerase chain reaction (qPCR). The assays were performed in accordance with manufacturer’s instructions (Takara). The PCR reaction for each gene was repeated three times. Expression values (2^{-ΔΔCt}) were calculated. The detail was shown in Appendix S1 (Supplementary Materials and Methods).

Western blot

The tissues or cells were lysed with radio immunoprecipitation assay RIPA lysis buffer. The supernatant of the lysate was collected and quantified. Protein samples were submitted to 10% sodium dodecyl sulfate polyacrylamide gel and transferred to polyvinylidene difluoride membranes. The membranes were incubated with a primary antibody. c-JUN, p21, p-PI3K, PI3K, p-AKT, AKT, and PD-L1 were purchased from Cell Signaling Technology (Danvers, MA); Wnt5A and β-actin were purchased from Santa Cruz Biotechnology (Dallas, TX). After the membranes were subsequently incubated with secondary antibodies, protein bands were visualized using an ECL western blotting kit (Thermo Fisher Scientific). The details are shown in Appendix S1 (Supplementary Materials and Methods).

Cell viability assays

Cell viability was analyzed using methyl thiazolyl tetrazolium (MTT) assay as described previously. Each experiment was performed in triplicate, and results are presented as the mean ± SD. The details are shown in Appendix S1 (Supplementary Materials and Methods).

Cell cycle

To evaluate cell-cycle phases (G1, S, and G2 phase), cells were fixed with 70% cold ethanol (700 μL) at 4 °C overnight and then stained with propidium iodide (PI) at 37 °C for 30 minutes. Cells were introduced to a fluorescence-activated cell sorting (FACS) flow cytometer (BD Biosciences). The cells in different cell-cycle phases were counted. Each experiment was done in triplicate. The details are shown in Appendix S1 (Supplementary Materials and Methods).

Wound healing

Cells were seeded in 6-well plates at 2 × 10^5 cells per well. When the cells reached 80%-90% confluency, the cells were scratched with 200 μL pipette tips. The width of the scratch was observed at the 0- and 48-hour time points, and the relative wound closure was calculated by comparing with the original width. Each experiment was repeated in triplicate.

All methods were carried out in accordance with relevant guidelines and regulations.

RESULTS

miR-493 attenuates esophageal cancer cell proliferation, migration, and invasion in vivo and in vitro

To understand the biological role of miR-493 in EC development, we overexpressed miR-493 in EC cells (EC9706 and TE13) by transfecting with mimics. More than 5-fold increase in miR-493 expression was observed in EC9706 and TE13 cells treated with miR-493 mimics compared with the negative control (NC) group by real-time quantitative reverse transcription (qRT)-PCR (Figure S1). Subsequently, cell proliferation was examined in EC9706 and TE13 cells. We found that miR-493 overexpression inhibited cell growth and G1 to S transit by MTT (Figure 1(a)), colony formation (Figure 1(b)) and cell-cycle analysis (Figure 1(c)). To investigate the effect of miR-493 on migration and invasion in EC cells, a wound healing and transwell assay was
performed. Results showed that miR-493 overexpression significantly reduced the ability of EC cell migration and invasion (Figure 1(d)–(f)). Levels of c-JUN, p-Pi3K, and p-AKT were significantly reduced whereas p21 was enhanced after overexpression of miR-493 in EC9706 and TE13 cells (Figure 1(g)). Next, in vivo experiment was performed by subcutaneously injecting EC9706/TE13-miR-493 or control cells into nude mouse. The size of xenograft tumor was measured. We found that tumor developed from miR-493 overexpressed cells were significantly smaller than control tumor (Figure 1(h)). To evaluate the effects of miR-493 on tumor metastasis in vivo, nude mice were injected intravenously into the tail vein with EC9706/TE13-miR-493 or control cells, respectively, and then assessed lung

**Figure 1** MiR-493 attenuates esophageal cancer cell proliferation, migration, and invasion in vivo and in vitro. The effect of miR-493 on the growth of EC9706 and TE13 cells was examined by a MTT (a), colony formation (b) and cell-cycle analysis (c) mean ± SD (n = 3) The effect of miR-493 on EC9706 and TE13 cell migration and invasion was measured by wound healing (d) and transwell assay with (e) or without pre-coating with Matrigel (f). (g) Protein levels of c-JUN, P21, p-Pi3K, and p-AKT were detected by western blot in EC9706 and TE13 cells transfected with NC or miR-493. (h) The volume of xenograft tumors was periodically measured for each mouse and the growth curves of tumor were plotted (i) Representative image of metastatic tumor nodules in the lung of nude mouse intravenously injected with EC9706/TE13-miR-493 or control cells.
metastasis by bioluminescence imaging. MiR-493 over-expressed cells exhibited a decreasing number of lung nodules compared with control cells (Figure 1(i)). Together, these data indicate that miR-493 plays a pivotal role in esophageal cancer in vitro and in vivo.

miR-493 directly regulates Wnt5A in esophageal cancer cell

Using TargetScan bioinformatics algorithms for target gene prediction, we found that Wnt5A was predicted to be a direct target of miR-493 (Figure 2(a)). Overexpression of miR-493 downregulated the protein levels of Wnt5A in EC9706 and TE13 cells (Figure 2(b)). Next, the 3’UTR of human Wnt5A gene was cloned into the pGL3-luciferase reporter vector to test whether miR-493 directly target Wnt5A. Consistent with binding data, there was a decrease in relative luciferase activity when the Wnt5A 3’UTR was co-transfected in EC9706 and TE13 cells with the miR-493, however, there was no difference in relative luciferase activity for mutant Wnt5A 3’UTR transfection (Figure 2(c)).

Ectopic expression of Wnt5A mitigates miR-493 suppression of EC proliferation, migration, and invasion

To determine whether Wnt5A of miR-493 target affect the function of miR-493, we constructed the Wnt5A expression plasmid. The Wnt5A was transfected into miR-493-overexpressing EC cells and cell proliferation was detected by MTT. The ectopic expression of Wnt5A increased cell proliferation ability (Figure 3(a)). Ectopic expression of Wnt5A also rescued miR-493-mediated effects on cell cycle, migration, and invasion in EC9706 and TE13 cells (Figure 3(b)–(d)). These indicated that the effects of miR-493 on EC cells specifically depend on Wnt5A.

c-JUN binds the promoter region of miR-493

To test the transcriptional regulatory mechanisms of miR-493 expression, bioinformatics software (UCSC and PROMO) was used to analyze the promoter region of miR-493. Three c-JUN-binding motifs were identified at miR-493 promoter region and mutant was also shown (Figure 4(a)). To illustrate the effect of c-JUN on the miR-493, c-JUN was knocked down in EC9706 and TE13 cells by RNA interference. c-JUN suppression greatly increased the expression of miR-493 (Figure 4(b)). Subsequently, chromatin immunoprecipitation assay (ChIP) was used to determine whether endogenous c-JUN binds to the miR-493 promoter in EC cells. We found that there was a significant enrichment of the binding region from c-JUN antibody compared to NC immunoglobulin G (IgG) pulldown (Figure 4(c)). Next, dual-luciferase reporter assay indicated c-JUN reduced the luciferase activities of the wild-type compared with the mutant miR-493 promoter after co-transfection with c-JUN in EC9706 and TE13 cells (Figure 4(d)). These data indicate that c-JUN binds to the promoter of miR-493 and inhibits its transcription.

Increased miR-493 induced the sensitivity of EC cells to cis-dichlorodiammine platinum

Next, we examined the effect of miR-493 on the sensitivity of EC cells to cis-dichlorodiammine platinum (DDP). Inhibition rates at 48 hours after treatment at different
FIGURE 3  Ectopic expression of Wnt5A mitigates miR-493 suppression of EC proliferation, migration, and invasion. (a) MTT assays for EC9706 and TE13 cells were performed after transfection with Wnt5A and/or miR-374a. (b) Cell cycle (c) migration and (d) invasion assays were also detected as indicated. Mean ± SD (n = 3).

FIGURE 4  C-JUN binds the promoter region of miR-493. (a) Schematic diagram of putative c-JUN binding sites including wild-type and mutant (Mut) in the promoter regions of miR-493. (b) miR-493 expression was detected after knockdown of c-JUN in EC9706 and TE13 cells. (c) ChIP assay was performed in EC9706 and TE13 cells by using antibodies against c-JUN. (d) Dual-luciferase reporter assay indicated the luciferase activities of the wild type, mutant miR-493 promoter after co-transfection with c-JUN in EC9706 and TE13 cells. Mean ± SD (n = 3).
concentrations of DDP were calculated for cells transfected with control or miR-493. The results showed that EC cell lines stably overexpressing miR-493 significantly enhanced the sensitivity to DDP (Figure 5(a)). Furthermore, the miR-493-overexpressing cells were transfected with Wnt5A and we found that overexpression of Wnt5A decreased sensitivity of miR-493-overexpressing EC cells to DDP (Figure 5(b)).

**Metabolic analysis on modulation of miR-493 expression**

The energy requirements of cancer cell growth rely on the profound alterations of cellular metabolism. Subsequently, we evaluated the impact of modulation of miR-49 on EC cell metabolism. Overexpression of miR-49 decreased glucose uptake and adenosine 5'-triphosphate (ATP) generation in both EC9706 and TE13 cell lines (Figure 6(a),(b)). Next, the transfection of Wnt5A into the miR-493-overexpressing cells ameliorated the effect of miR-49 decreased glucose uptake and ATP generation (Figure 6(c),(d)). Therefore, the existence of miR-493 is an important attribute of metabolism.

**Clinical association between miR-493 and Wnt5A expression in EC tissues**

To investigate the relationship between the expression levels of miR-493 and Wnt5A in clinical specimens, we collected EC specimens. Real time PCR showed that the expression of miR-493 was low in clinical samples of EC patients (Figure 7(a)). In addition, a Kaplan–Meier survival analysis was used to analyze the significance of miR-493. The overall survival (OS) results illustrated that EC patients with low expression of miR-493 have a poorer prognosis compared with the patients with high expression of miR-493 (Figure 7(b)). The expression of Wnt5A was also examined in clinical specimens and we found that Wnt5A was leveled up in EC samples (Figure 7(c)). Notably, a reverse correlation was observed between relative miR-493 and Wnt5A expressions (Figure 7(d)).

**miR-493 regulates the expression of PD-L1**

The blocking of programmed death receptor 1 (PD1) and/or its ligand (PD-L1) as an immune checkpoint has been successfully used for treatment of some malignancies. We analyzed the associations between miR-493 and PD-L1 expression. Results showed that miR-493 overexpression downregulated the expression of PD-L1 in EC (Figure 8(a)). And then overexpression of c-Jun decreased the effect of miR-493 on PD-L1 (Figure 8(b)). Next, PD-L1 was knocked down in EC cells (Figure 8(c)). We found that the inhibition of PD-L1 enhanced the sensitivity of EC cells to DDP (Figure 8(d)). These findings suggest miR-493 is a critical regulator of PD-L1 expression by c-JUN and a potential therapeutic target to enhance antitumor activity.

**DISCUSSION**

MiR-493 dysregulation has been linked with several types of cancer. However, the roles and molecular mechanisms of miR-493 in EC have not been reported. In this study, we found that miR-493 attenuates EC cell proliferation, migration, and invasion in vivo and in vitro. These results suggest that miR-493 functions as a potential tumor suppressor in EC. Our results are consistent with the previous reports of miR-493 inhibiting tumor development.

After profoundly studying the underlying mechanisms, we show that miR-493 inhibited the activity of c-JUN and p-PI3K/p-AKT with enhanced p21 and directly regulates Wnt5A expression and function, whereas c-JUN binds the promoter region of miR-493 and suppressed the expression of miR-493, forming a negative feedback loop. This miR-493/Wnt5A/c-JUN loop is essential for the development of EC cells.

Cancer is characterized by uncontrolled proliferation resulting from cell-cycle progression. In this study, miR-493 inhibited G1 to S transit in EC cells. The inhibiting effect of miR-493 on EC growth might be because of the arrest of G1 to S transit by miR-493. Cell-cycle arrest can also induce the chemotherapy sensitivity in tumor. Furthermore, we also observed that increased miR-493 induced the sensitivity of EC cells to DDP, P21, as a cyclin-dependent kinase inhibitor, promotes cell-cycle arrest in response to many stimuli and functions as both a sensor and an effector of multiple anti-proliferative signals.
The Wnt family members play a crucial role in regulating cellular and organ function. Wnt5a, a member of the non-canonical WNT signaling pathways, has been reported to function in cell proliferation, cell cycle, and invasion. Recent studies show that Wnt5a is involved in regulating cancer cell invasion, metastasis, metabolism, and inflammation. In this study, ectopic expression of Wnt5A as the target of miR-493 mitigates miR-493 suppression of EC proliferation, migration, and invasion. These results demonstrate that miR-493 directly targets Wnt5A to suppress EC proliferation, migration, and invasion.

The transcriptional regulation of miRNAs also influences the development of various diseases. c-JUN as a proto-oncogene is required for tumor development.
We observed that c-JUN bound to the promoter region of miR-493 and negatively modulate miR-493 expression. Together, these results indicate that miR-493 can induce its own expression through a miR-493/Wnt5A/c-JUN loop in EC pathogenesis.

Cancer cells exhibit a higher rate of glucose metabolism than normal cells, which provide the energy and metabolites demands of tumor progression. Metabolic reprogramming contributes to esophageal tumorigenesis and a better understanding of how EC cells retain glucose metabolism is essential for treating EC. Overexpression of miR-49 decreased glucose uptake and ATP generation in EC cells. However, Wnt5A ameliorated the effect of miR-49 on glucose uptake and ATP generation.

Consistent with their roles in vitro and in vivo, we found that the low expression of miR-493 was negatively correlated with the high expression of Wnt5A in clinical samples of EC patients. In addition, the overall survival illustrated that miR-493 might be a prognosis factor for EC patients.

Immune checkpoint inhibitors are one of the promising treatments for cancer, especially the drugs targeting either the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) or the PD1, yet the mechanisms are still not clear. Here, we examined the effect of miR-493 on PD-L1 and found that miR-493 regulates the expression of PD-L1 by c-JUN and then the sensitivity of EC cells to DDP.

Together, our results elucidate a molecular feedback loop that involves miR-493, Wnt5A, c-JUN, and PD-L1 in EC. In future, these mechanistic findings may provide a useful therapeutic option for the treatment of EC.

**CONCLUSIONS**

Our results elucidate a molecular feedback loop that involves miR-493, Wnt5A, and c-JUN in EC. In the future, these mechanistic findings may provide a useful therapeutic option for the treatment of EC.
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SUPPORTING INFORMATION
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