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ORIGINAL ARTICLE

Basic Study

MiR-451 inhibits proliferation of esophageal carcinoma cell line EC9706 by targeting CDKN2D and MAP3K1

Wen-Qiao Zang, Xuan Yang, Tao Wang, Yuan-Yuan Wang, Yu-Wen Du, Xiao-Nan Chen, Min Li, Guo-Qiang Zhao

Wen-Qiao Zang, Xuan Yang, Yuan-Yuan Wang, Yu-Wen Du, Xiao-Nan Chen, Min Li, Guo-Qiang Zhao, College of Basic Medical Sciences, Zhengzhou University, Zhengzhou 450001, Henan Province, China

Tao Wang, Department of Hemato-tumor, the First Affiliated Hospital of Henan University of TCM, Zhengzhou 450000, Henan Province, China

Author contributions: Zhao GQ and Zang WQ designed and guided the study; Zang WQ, Yang X, Wang YY, Wang T, Li M and Du YW performed and participated in analysis of laboratory experiment data; Zang WQ and Chen XN acquired and preserved the clinical samples; Zhao GQ and Zang WQ provided administrative support and funded experiments; all authors have contributed and approved the final manuscript.

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Correspondence to: Guo-Qiang Zhao, PhD, College of Basic Medical Sciences, Zhengzhou University, 100 Kexue Avenue, 450001 Zhengzhou, Henan Province, China. zhaogq@zzu.edu.cn
Telephone: +86-371-67781959

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Abstract

AIM: To investigate the underlying molecular mechanisms of miR-451 to inhibit proliferation of esophageal carcinoma cell line EC9706.

METHODS: Assays for cell growth, apoptosis and invasion were used to evaluate the effects of miR-451 expression on EC cells. Luciferase reporter and Western blot assays were used to test whether cyclin-dependent kinase inhibitor 2D (CDKN2D) and MAP3K1 act as major targets of miR-451.

RESULTS: The results showed that CDKN2D and MAP3K1 are direct targets of miR-451. CDKN2D and MAP3K1 overexpression reversed the effect of miR-451. MiR-451 inhibited the proliferation of EC9706 by targeting CDKN2D and MAP3K1.

CONCLUSION: These findings suggest that miR-451 might be a novel prognostic biomarker and a potential target for the treatment of esophageal squamous cell carcinoma in the future.

Key words: Esophageal squamous cell carcinoma; MiR-451; Cyclin-dependent kinase inhibitor 2D; MAP3K1; Proliferation

Core tip: Recently miR-451 has been reported to be tumor suppressor in human cancer cells. In the previous studies we have reported that miR-451

expression in esophageal squamous cell carcinoma (ESCC) tissues was significantly reduced, and that upregulated expression of miR-451 induced apoptosis and suppressed cell proliferation, invasion and metastasis in esophageal carcinoma. However, the underlying molecular mechanisms remain unclear. In this study, we supposed and showed that cyclin-dependent kinase inhibitor 2D (CDKN2D) and MAP3K1 are the targets of miR-451 by the bioinformatics algorithms (TargetScan and miRBase). Moreover, we found that CDKN2D and MAP3K1 contributed to ESCC malignancy.

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INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is one of the most lethal malignancies worldwide[1,2]. ESCC is the 8th most common cancer and the 6th leading cause of cancer-related death. The traditional treatment for ESCC includes chemotherapy and radiation therapy[3,4]. However, many patients who are treated with such traditional therapy still experience disease progression, which suggests that ESCC is resistant to traditional therapy. New treatment choices are critically required and the mechanism of tumorigenesis is to be further clarified.

MicroRNAs (miRNAs) are small, endogenous noncoding RNAs that have been identified as post-transcriptional regulators of gene expression. MiRNAs exert their functions through imperfect base-pairing with the 3'-untranslated region (3'-UTR) of target mRNAs[5-8]. In human cancer, miRNAs can act as oncogenes or tumour suppressor genes during tumourigenesis. Recently, miR-451 has been reported to be induced during zebrafish, mouse, and human erythroid maturation as a key factor involved in regulating erythrocyte differentiation[9-11]. It was also reported that miR-451 might function as a tumor suppressor and modulate MDR1/P-glycoprotein expression in human cancer cells[12]. In previous studies we have reported that miR-451 expression in ESCC tissues was significantly reduced, and that upregulated expression of miR-451 induced apoptosis and suppressed cell proliferation, invasion and metastasis in esophageal carcinoma[13,14]. However, the underlying molecular mechanisms remain unclear. In this study, we supposed and showed that cyclin-dependent kinase inhibitor 2D (CDKN2D) and MAP3K1 are the targets of miR-451 by the bioinformatics algorithms (TargetScan and miRBase). Moreover, we found that CDKN2D and MAP3K1 contributed to ESCC malignancy. Our data demonstrate that miR-451 has potential values as a prognostic marker and a therapeutic target for ESCC.

MATERIALS AND METHODS

Cell culture

EC9706 and KYSE150 cell lines were purchased from the Chinese Academy of Sciences Cell Bank. All cells were cultured in RPMI-1640 (Gibco, United States) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, United States) and grown in humidified 5% CO_2 at 37 ℃.

Oligonucleotides and cell transfection

The miR-451 mimics used in this study was synthesized by Shanghai GenePharma Co. Ltd. For transfection, 2×10^5 cells were seeded into each well of six well plates and grown overnight until they were 50%-80% confluent. Cells were washed, placed in serum-free medium, and transfected using Lipofectamine™2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, United States). After 6 h, the medium was changed to complete medium, and cells were cultured at 37 ℃ in 5% CO_2.

Cell growth assay

The different experimental groups of EC9706 and KYSE150 cells were plated in 96-well plates at 1×10^4 cells per well and incubated for 48 h after transfection. The viability of cells was determined using Cell Counting Kit-8 (CCK-8; Dojindo, Japan) according to the manufacturer's instructions. Viable cell numbers were estimated by measurement of optical density (OD) at 450 nm. All experiments were performed in triplicate.

Colony formation assay

Cells were suspended in RPMI-1640 containing 0.35% low melting agarose, and plated onto solidified 0.6% agarose containing RPMI-1640 in six-well culture plates at a density of 1×10^5 cells per dish. The plates were incubated for 2 wk at 37 ℃ in a 5% CO_2 incubator, and the number of colonies was counted after staining with 0.1% crystal violet solution. All experiments were performed in triplicate.

Cell invasion assay

The experimental groups of EC9706 cells were adjusted at 2×10^5/mL in each group 48 h after transfection. The upper chamber of 24-well Transwell Permeable Supports with 8 μm pores (Corning Cat. No. 3422) was loaded with 200 μL of cell suspension, and the lower chamber was loaded with 500 μL of medium containing 10% serum for incubation in an atmosphere of 5% CO_2 at 37 ℃ for 48 h. Five wells were set for each group. The number of cells invading the matrigel

was counted from 5 randomly selected visual fields using an inverted microscope. All experiments were performed in triplicate.

Apoptosis assay

EC9706 cells were harvested 48 h after transfection and cell concentration was adjusted to 1 × 10^6 cells. Annexin V-FITC/PI Apoptosis Detetion Kit I (BestBio, Shanghai, China) was used to detect Annexin V. Results were obtained using FACScan Flow Cytometer (BD Biosciences, San Jose, CA, United States). Tests were repeated in triplicate. Data were analyzed with Cell Quest software. All experiments were performed in triplicate.

Cell cycle analysis

For cell cycle analysis by flow cytometry, cells in the logarithmic phase of growth were harvested by trypsinization, washed with PBS, fixed with 75% ethanol overnight at 4 ℃ and incubated with RNase at 37 ℃ for 30 min. Nuclei were stained with propidium iodide for 30 min. A total of 10^4 nuclei were examined in a FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA, United States). All experiments were performed in triplicate.

Western blot

The experimental groups of EC9706 and KYSE150 cells in each group were lysed in lysis buffer for total protein extration. Protein concentrations were measured using the BCA method (KeyGEN, China), and 30 μg of protein was separated by 12% SDS-PAGE and electroblotted onto a nitrocellulose membrane (Whatman, United States). The membrane was blotted overnight at 4 ℃ with primary antibodies (mouse anti- CDKN2D and anti-MAP3K1, 1:1000) in Tris-buffered saline with 5% non-fat milk. A secondary antibody (HRP-conjugated goat anti-mouse IgG) was incubated with the membrane for 1 h after three washes with TBST. The protein band density was determined with Kodak Digital ID Image Analysis Software and was normalized with the density of β-actin. All experiments were performed in triplicate.

Dual luciferase assay

The human CDKN2D and MAP3K1 fragments containing putative binding sites for miR-451 were amplified by PCR from human genomic DNA. The mutant CDKN2D and MAP3K1 3′-UTRs were obtained by overlap extension PCR. The fragments were cloned into a pmirGLO reporter vector (Promega), downstream of the luciferase gene, to generate the recombinant vectors pmirGLO-CDKN2D-wt, pmirGLO-CDKN2D-mut, pmirGLO-MAP3K1-wt and pmirGLO-MAP3K1-mut. For the luciferase reporter assay, cells were transiently co-transfected with miRNA (miR-451 mimics or scrambled-miR-451 negative control) and reporter vectors (wild-type reporter vectors or mutant-type reporter vectors), using Lipofectamine™2000. Luciferase activities were measured using a Dual-Luciferase assay kit (Promega) according to the manufacturer's instructions at 48 h post-transfection. All experiments were performed in triplicate.

Statistical analysis

Statistical testing was conducted with the assistance of SPSS 17.0 software. All data are expressed as mean ± SD. One-way analysis of variance (ANOVA) was used to analyze data. Results were considered significant when P values were < 0.05.

RESULTS

CDKN2D and MAP3K1 are direct targets of miR-451

We based on the following criteria to search for the direct target of miR-451: the target should have oncogenic property and regulate the cell migration and invasion. Among these targets of miR-451 predicted by the bioinformatics algorithms (TargetScan and miRBase), we selected CDKN2D and MAP3K1. The 3′-UTR of CDKN2D contains the seed regions for miR-451 at the position of base 240 nt - 246 nt (Figure 1A). Similarly, the 3′-UTR of MAP3K1 contains the seed regions for miR-451 at the position of base 6270 nt - 6278 nt (Figure 1B).

Subsequent Western blot analysis indeed showed that CDKN2D and MAP3K1 expression was down-regulated in EC9706 and KYSE150 cells following transfection with the miR-451 mimics (Figure 1C and D). In order to test the specific regulation through the seed region, we constructed a reporter vector which consists of the luciferase coding sequence followed by the 3′-UTR of CDKN2D and MAP3K1. Wild type (pmirGLO-CDKN2D-3′-UTR, pmirGLO-MAP3K1-3′-UTR) or mutated sequences (pmirGLO-CDKN2D-mut 3′-UTR, pmirGLO-MAP3K1-mut 3′-UTR) within the seed region sites were cloned into the pmirGLO reporter vector. We used a Dual-Luciferase reporter system containing either wild-type or mutant 3′-UTRs of CDKN2D and MAP3K1, respectively. Co-transfection experiments showed that miR-451 significantly decreased the luciferase activity of wild type in EC9706 and KYSE150 cells ($P < 0.05$; Figure 1E and F), but this was not observed in mutant type ($P > 0.05$; Figure 1E and F). These data indicate that miR-451 negatively regulates CDKN2D and MAP3K1 expression by directly binding to putative binding sites in the 3′-UTR. Our results thus demonstrated that CDKN2D and MAP3K1 are direct targets of miR-451.

CDKN2D and MAP3K1 overexpression reverses the effect of miR-451

To explore the function of CDKN2D and MAP3K1 in EC9706 cells, we constructed pcDNA3.1-CDKN2D and pcDNA3.1-MAP3K1 lacking the 3′-UTR, and then they were transfected into EC9706 cells. Western blot assay showed that transfection of miR-451 mimics inhibited

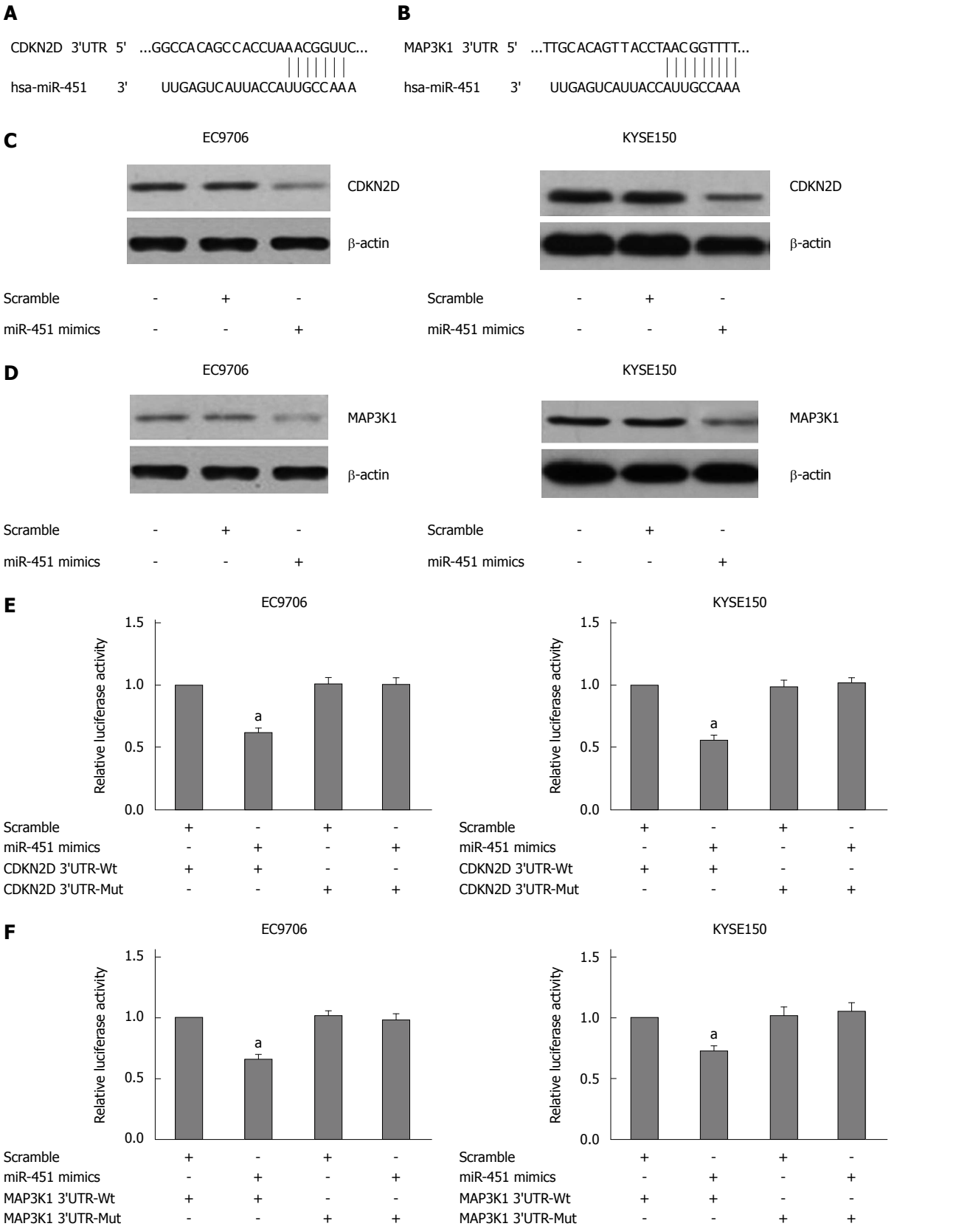


Figure 1 CDKN2D and MAP3K1 are direct targets of miR-451 in EC9706 and KYSE150 cells. A: The putative miR-451 binding sequence for the CDKN2D 3'-untranslated region (3'-UTR). The 3'-UTR of CDKN2D contains a seed region for miR-451; B: The putative miR-451 binding sequence for the MAP3K1 3'-UTR. The 3'-UTR of MAP3K1 contains a seed region for miR-451; C: Western blot analysis of CDKN2D expression in transfected cells. Transfection of miR-451 mimics resulted in a significant reduction of CDKN2D protein expression in EC9706 and KYSE150 cells. β-actin was used as a reference; D: Western blot analysis of MAP3K1 expression in transfected cells. Transfection of miR-451 mimics resulted in a significant reduction of MAP3K1 protein expression in EC9706 and KYSE150 cells. β-actin was used as a reference; E: MiR-451 significantly decreased the luciferase activity of CDKN2D 3'-UTR-Wt in EC9706 and KYSE150 cells; F: MiR-451 significantly decreased the luciferase activity of MAP3K1 3'-UTR-Wt in EC9706 and KYSE150 cells ($^aP < 0.05$ *vs* control group).

the expression of CDKN2D and MAP3K1, respectively (Figure 2A). Co-transfection of pcDNA3.1-CDKN2D and miR-451 abrogated the effects of miR-451 on CDKN2D expression (Figure 2A). Similarly, co-transfection of pcDNA3.1-MAP3K1 and miR-451 abrogated the effects of miR-451 on MAP3K1 expression (Figure 2A).

In the colony formation assays we found that exogenous expression of miR-451 decreased cell colony formation numbers (Figure 2B and C). Subsequently, we exogenously expressed recombinant CDKN2D lacking the 3'-UTR sequence (pcDNA3.1-CDKN2D) or MAP3K1 lacking the 3'-UTR sequence (pcDNA3.1-MAP3K1) in EC9706 cells. Cells transfected with pcDNA3.1-CDKN2D or pcDNA3.1-MAP3K1 alone showed significantly increased cell colony formation numbers (Figure 2B and C). When we, however, co-transfected cells with pcDNA3.1-CDKN2D or pcDNA3.1-MAP3K1 and miR-451, the expression of CDKN2D and pcDNA3.1-MAP3K1 lacking the 3'-UTR sequence were found to reverse the anti-proliferation of miR-451 (Figure 2B and C). From these results we conclude that expression of CDKN2D and MAP3K1 could partially reverse the anti-proliferation function of miR-451.

Our apoptosis assay indicated that exogenous expression of miR-451 increased cell apoptosis induced by serum starvation (Figure 2D and E). Subsequently, we exogenously expressed recombinant CDKN2D lacking the 3'-UTR sequence (pcDNA3.1-CDKN2D) or MAP3K1 lacking the 3'-UTR sequence (pcDNA3.1-MAP3K1) in EC9706 cells. Cells transfected with pcDNA3.1-CDKN2D alone did not show significantly decreased levels of apoptosis (Figure 2D). However, cells transfected with pcDNA3.1-MAP3K1 alone showed significantly decreased levels of apoptosis compared to the blank control (Figure 2E), and that when we co-transfected cells with pcDNA3.1-MAP3K1 and miR-451, the expression of MAP3K1 lacking the 3'-UTR sequence was found to reverse the pro-apoptotic functions of miR-451 (Figure 2E).

In the transwell assays we found that exogenous expression of miR-451 decreased cell invasiveness (Figure 2F and G). Subsequently, we exogenously expressed recombinant CDKN2D lacking the 3'-UTR sequence (pcDNA3.1-CDKN2D) or MAP3K1 lacking the 3'-UTR sequence (pcDNA3.1-MAP3K1) in EC9706 cells. Cells transfected with pcDNA3.1-MAP3K1 alone showed significantly increased cell invasiveness (Figure 2G). When we, however, co-transfected cells with pcDNA3.1-CDKN2D and miR-451, the expression of CDKN2D lacking the 3'-UTR sequence was found to reverse the anti-migration functions of miR-451 (Figure 2F). Similarly, when we co-transfected cells with pcDNA3.1-MAP3K1 and miR-451, the expression of MAP3K1 lacking the 3'-UTR sequence was found to reverse the anti- migration functions of miR-451 (Figure 2G).

Cell cycle analysis showed that administration of miR-451 mimic oligonucleotides significantly increased the percentage of cells in the G1 phase and decreased the percentage of cells in the S phase (Figure 2H and I). When we co-transfected cells with pcDNA3.1-CDKN2D and miR-451, the expression of CDKN2D lacking the 3'-UTR sequence was found to reverse G1 arrest of miR-451 (Figure 2H). When we co-transfected cells with pcDNA3.1-MAP3K1 and miR-451, the expression of MAP3K1 lacking the 3'-UTR sequence was not found to reverse G1 arrest of miR-451 (Figure 2I).

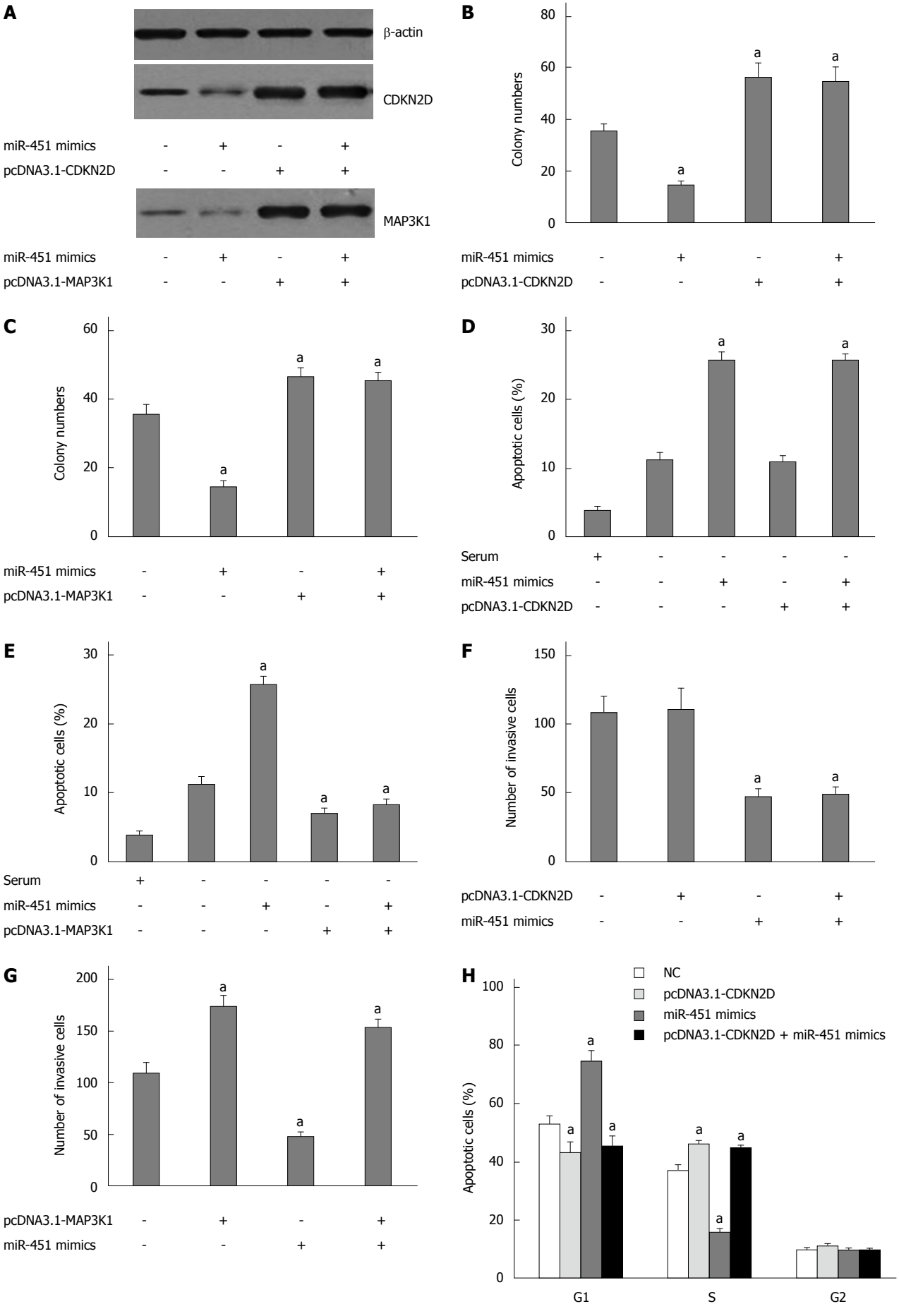
MiR-451 inhibits the proliferation of EC9706 cells by targeting CDKN2D and MAP3K1

To further explore the biological significance of CDKN2D**,** MAP3K1 and miR-451 in EC9706 cells, CDKN2D-siRNAs, MAP3K1-siRNAs and miR-451 mimics were transfected into EC9706 cells. Western blot assay showed that transfection of CDKN2D-siRNAs, MAP3K1-siRNAs and miR-451 mimics inhibited the expression of CDKN2D and MAP3K1, respectively (Figure 3A).

CCK8 assay showed that CDKN2D, MAP3K1 silencing and miR-451 overexpression inhibited the cell proliferation (Figure 3B and C). For EC9706 cells transfected with si-CDKN2D, the inhibition was more obvious than cells transfected with si-MAP3K1 (Figure 3B and C). Compared to the NC group, co-transfection of si-CDKN2D and si-MAP3K1 also significantly inhibited the proliferation of EC9706 cells (Figure 3D). In addition, for co-transfected cells with si-CDKN2D and si-MAP3K1, the inhibitory effects were similar to those for cells overexpressing miR-451 (Figure 3D). Furthermore, colony formation assay obtained the similar results that CDKN2D, MAP3K1 silencing and miR-451 overexpression reduced EC9706 cell colony numbers (Figure 3E).

Invasion assay showed that knockdown of MAP3K1 and the overexpression of miR-451 repressed the invasion capacities of EC9706 cells (Figure 3G). For cells transfected with si-MAP3K1, the numbers of invasive cells were less than cells transfected with si-CDKN2D (Figure 3G). To investigate the effect of si-CDKN2D, si-MAP3K1 and miR-451 on apoptosis, we performed apoptosis assay. As showed in Figure 3F, CDKN2D, MAP3K1 silencing and the overexpression of miR-451 induced EC9706 cell apoptosis significantly compared to the blank control. For EC9706 cells transfected with si-MAP3K1, the apoptotic cells were more than those for cells transfected with si-CDKN2D. For co-transfected cells with si-CDKN2D and si-MAP3K1, the apoptosis effects were similar to those for cells overexpressing miR-451.

Cell cycle analysis showed that knockdown of CDKN2D and the overexpression of miR-451 significantly increased the percentage of cells in the G1 phase and decreased the percentage of cells in the S phase (Figure 3H). Altogether, these results confirm that miR-451 inhibits the proliferation of EC9706 by targeting CDKN2D and MAP3K1.


A
β-actin
CDKN2D
miR-451 mimics - + - +
pcDNA3.1-CDKN2D - - + +
MAP3K1
miR-451 mimics - + - +
pcDNA3.1-MAP3K1 - - + +
B
Colony numbers
80
60
40
20
0
a
a
a
a
miR-451 mimics - + - +
pcDNA3.1-CDKN2D - - + +
C
Colony numbers
60
40
20
0
a
a
a
a
miR-451 mimics - + - +
pcDNA3.1-MAP3K1 - - + +
D
Apoptotic cells (%)
30
20
10
0
a
a
a
a
Serum + - - - -
miR-451 mimics - - + - +
pcDNA3.1-CDKN2D - - - + +
E
Apoptotic cells (%)
30
20
10
0
a
a
a
a
Serum + - - - -
miR-451 mimics - - + - +
pcDNA3.1-MAP3K1 - - - + +
F
Number of invasive cells
150
100
50
0
a
a
a
a
pcDNA3.1-CDKN2D - + - +
miR-451 mimics - - + +
G
Number of invasive cells
200
150
100
50
0
a
a
a
a
pcDNA3.1-MAP3K1 - + - +
miR-451 mimics - - + +
H
NC
pcDNA3.1-CDKN2D
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pcDNA3.1-CDKN2D + miR-451 mimics
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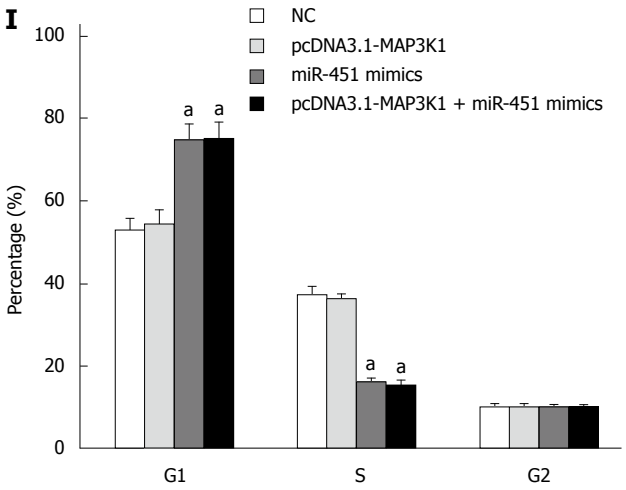

Figure 2 CDKN2D and MAP3K1 overexpression reverses the effect of miR-451. A: CDKN2D and MAP3K1 protein levels were detected by Western blot assay. Western blot assay showed that transfection of miR-451 mimics inhibited the expression of CDKN2D or MAP3K1. Co-transfection of pcDNA3.1-CDKN2D or pcDNA3.1-MAP3K1and miR-451 abrogated the effects of miR-451 on CDKN2D or MAP3K1 expression. β-actin was used as a reference; B: The expression of CDKN2D could partially reverse the anti-proliferation function of miR-451. Colony formation assays were performed. $^{a}P < 0.05$ *vs* control group; C: The expression of MAP3K1 could partially reverse the anti-proliferation function of miR-451. Colony formation assays were performed. $^{a}P < 0.05$ *vs* control group; D: The expression of CDKN2D did not reverse the pro-apoptotic function of miR-451. Cells were transfected with pcDNA3.1-CDKN2D (not including 3'-UTR) or (and) miR-451. The cell apoptosis was assessed using flow cytometry assay; E: The expression of MAP3K1 reversed the pro-apoptotic function of miR-451. Cells were transfected with pcDNA3.1-MAP3K1 (not including 3'-UTR) or (and) miR-451. The cell apoptosis was assessed using flow cytometry assay; F: The expression of CDKN2D reversed the anti-migration function of miR-451. Cells were transfected with pcDNA3.1-CDKN2D (not including 3'-UTR) or (and) miR-451. The cell invasion was assessed using transwell assay; G: The expression of MAP3K1 reversed the anti-migration function of miR-451. Cells were transfected with pcDNA3.1-MAP3K1 (not including 3'-UTR) or (and) miR-451. The cell invasion was assessed using transwell assay; H: The expression of CDKN2D reversed G1 arrest of miR-451. Cells were transfected with pcDNA3.1-CDKN2D (not including 3'-UTR) or (and) miR-451. The cell cycle was assessed using flow cytometry assay; I: The expression of MAP3K1 did not reverse G1 arrest of miR-451. Cells were transfected with pcDNA3.1-MAP3K1 (not including 3'-UTR) or (and) miR-451. The cell cycle was assessed using flow cytometry assay ($^{a}P < 0.05$ *vs* control group).

DISCUSSION

CDKN2D (p19^{INK4d}), a negative regulator of the cell cycle, is located on chromosome 19p13. The protein encoded by this gene is a member of the INK4 family of cyclin-dependent kinase inhibitors. This protein has been shown to form a stable complex with CDK4 or CDK6, and prevent the activation of the CDK kinases, thus functioning as a cell growth regulator that controls cell cycle G1 progression. The abundance of the transcript of this gene was found to oscillate in a cell-cycle dependent manner with the lowest expression at mid G1 and a maximal expression during S phase. The negative regulation of the cell cycle involving this protein was shown to participate in repressing neuronal proliferation, as well as spermatogenesis[15-20]. Little is known of its role in cancer development and prognosis. CDKN2D expression in cancers has been examined in only a few studies and, to date, it has not been linked to cancer development.

Mitogen-activated protein kinases (MAPKs) are key mediators of evolutionarily conserved signaling networks that play an essential role in multiple aspects of cell physiology[21,22]. MAP3K1 or MEKK1 (MEK kinase 1) is a 196-kDa serine-threonine kinase that belongs to the MAP3K family and the STE superfamily[22,23]. MAP3K1 was originally identified as the mammalian homolog of the yeast MAP3Ks Ste11 and Byr2 that function in pheromone responsive signaling. Studies have demonstrated that MAP3K1 functions in cell survival, apoptosis, and cell motility/migration in multiple normal and tumor cell types[24,25].

In previous studies we have reported that miR-451 expression in ESCC tissues were significantly reduced, and that upregulated expression of miR-451 induced apoptosis and suppressed cell proliferation, invasion and metastasis in esophageal carcinoma[13,14]. In this study, we identified CDKN2D and MAP3K1 as the direct and functional targets of miR-451, which facilitated our understanding of the mechanisms underlying ESCC progression. Additionally, a further study indicated that CDKN2D and MAP3K1 overexpression reversed the effect of miR-451, and that miR-451 inhibited the proliferation of EC9706 by targeting CDKN2D and MAP3K1. The study demonstrates that miR-451 prefers to act as a potential target for the treatment of ESCC in the future.

MiRNAs have been shown to be important in the development and maintenance of normal cellular function, and an alteration in expression of miRNAs can result in human cancer initiation and tumor progression. MiRNAs can regulate target genes by increasing mRNA decay or by repressing translation. Each miRNA has the potential to target hundreds of genes that harbor in their 3'-UTR sequences complementary to the seed region of the miRNA[26-29]. In the study, for co-transfected cells with si-CDKN2D and si-MAP3K1, the inhibitory effects are similar to

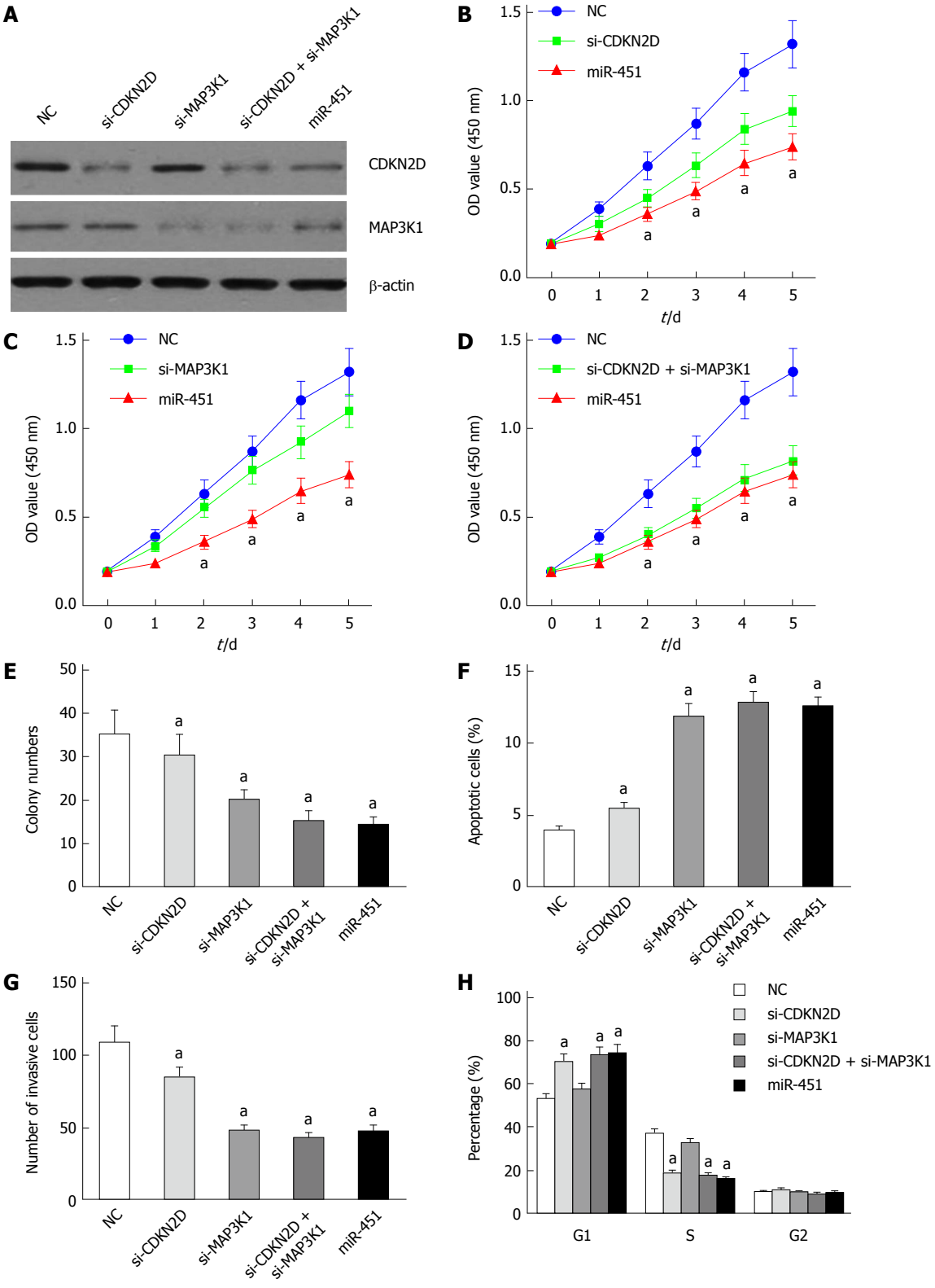

Figure 3 MiR-451 inhibits the proliferation of EC9706 cells by targeting CDKN2D and MAP3K1. A: CDKN2D and MAP3K1 protein levels were detected by Western blot assay. Western blot assay showed that transfection of CDKN2D-siRNAs, MAP3K1-siRNAs and miR-451 mimics inhibited the expression of CDKN2D and MAP3K1, respectively; B: CDKN2D silencing and miR-451 overexpression inhibited the cell proliferation. CCK8 array was used to assess EC9706 proliferation; C: MAP3K1 silencing and miR-451 overexpression inhibited the cell proliferation. CCK8 array was used to assess EC9706 proliferation; D: Co-transfection of si-CDKN2D and si-MAP3K1 inhibited the proliferation of EC9706 cells. CCK8 array was used to assess EC9706 proliferation; E: CDKN2D, MAP3K1 silencing and miR-451 overexpression reduced the growth of colonies of EC9706 cells. Colony formation assay was used; F: CDKN2D, MAP3K1 silencing and miR-451 overexpression induced EC9706 cell apoptosis. The cell apoptosis was assessed using flow cytometry assay; G: CDKN2D, MAP3K1 silencing and miR-451 overexpression suppressed EC9706 cell invasiveness. The cell invasion was assessed using transwell assay; H: CDKN2D silencing and the overexpression of miR-451 significantly increased the percentage of cells in the G1 phase and decreased the percentage of cells in the S phase ($^aP < 0.05$ *vs* control group). The cell cycle was assessed using flow cytometry assay.

those for cells overexpressing miR-451.

However, miRNAs may function according to a combinatorial circuits model, in which a single miRNA may target multiple mRNAs, and several coexpressed miRNAs may target a single mRNA. Recent studies have suggested that the biological concept of "one hit-multiple targets" could be used in clinical therapeutics[30]. If the primary molecular defect of a disease is in the expression of a miRNA, the expression of several critical protein targets could be deregulated. In that case, one might recover the normal phenotype of the cells by normalizing the miRNA expression. Although individual targets responsible for observed phenotypes have been proposed for many miRNAs, it is likely that a specific miRNA may function through cooperative down-regulation of multiple targets. Thus, other target genes of miR-451 may also contribute to tumorigenesis.

In conclusion, we have identified that miR-451 inhibited the proliferation, invasion and induced the apoptosis of ESCC cells *in vitro* and *in vivo* by directly targeting CDKN2D and MAP3K1. MiR-451 might be a novel prognostic biomarker and a potential target for the treatment of ESCC in the future.

COMMENTS

Background

Esophageal squamous cell carcinoma (ESCC) is one of the most lethal malignancies worldwide. ESCC is the 8th most common cancer and the 6th leading cause of cancer-related death. The traditional treatments for ESCC include chemotherapy and radiation therapy. However, many patients who are treated with such traditional therapy still experience disease progression, which suggests that ESCC is resistant to traditional therapy. In human cancer, microRNAs (miRNAs) can act as oncogenes or tumour suppressor genes during tumourigenesis.

Research frontiers

Recently miR-451 has been reported to be induced during zebrafish, mouse, and human erythroid maturation as a key factor involved in regulating erythrocyte differentiation. It was also reported that miR-451 might function as a tumor suppressor and modulate MDR1/P-glycoprotein expression in human cancer cells.

Innovations and breakthroughs

In previous studies the authors have reported that miR-451 expression in ESCC tissues were significantly reduced, and that upregulated expression of miR-451 induced apoptosis and suppressed cell proliferation, invasion and metastasis in esophageal carcinoma. However, the underlying molecular mechanisms remain unclear. In this study, the authors supposed and showed that CDKN2D and MAP3K1 are the targets of miR-451 by the bioinformatics algorithms (TargetScan and miRBase). Moreover, they found that CDKN2D and MAP3K1 contributed to ESCC malignancy.

Applications

The data suggest that miR-451 has potential values as a prognostic marker and a therapeutic target for ESCC.

Terminology

MiRNAs are small, endogenous noncoding RNAs that have been identified as post-transcriptional regulators of gene expression. MiRNAs exert their functions through imperfect base-pairing with the 3'-untranslated region of target mRNAs.

Peer-review

The manuscript is basically good. This is an *in vitro* study that addressed the mechanism of tumor suppressive functions of a miRNA, miR-451. The authors authentically conducted the required experiments using esophageal cancer cells and revealed that miR-451 targeted CDKN2D and MAP3K1 and worked tumor-suppressively through the inhibition of the two kinases. The findings are expected to contribute to the development of molecularly targeted therapy for esophageal cancer.

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