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***Basic Study***

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

Zang WQ *et al.* miR-451 inhibits proliferation of EC9706

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**Abstract**

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

**METHODS:** Assays for cell growth; apoptosis and transwell invasion were used to evaluate the effects of miR-451 expression on EC cells. Luciferase reporter and Western blotting assays were used to test whether CDKN2D and MAP3K1 act as major targets of miR-451.We found that CDKN2D and MAP3K1 were direct 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 novel prognostic biomarkers and potential targets for the treatment of esophageal squamous cell carcinoma in the future.

**Keywords:** Esophageal squamous cell carcinoma; miR-451; CDKN2D; MAP3K1; Proliferation

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**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-451expression 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 the esophageal carcinoma. However, the underlying molecular mechanisms remain unclear. In this study, we supposed and showed that CDKN2D and MAP3K1 were the targets of miR-451by the bioinformatics algorithms (TargetScan and miRBase), moreover, 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 of ESCC includes chemotherapyandradiation therapy[3,4]. However, many patients who are treated with such traditional therapy still experience progression of disease, 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 theirfunctions through imperfect base-pairing with the 3’- untranslatedregion (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 regulates erythrocyte differentiation[9-11]. It was also reported that miR-451 might function as tumor suppressor and modulate MDR1/P-glycoprotein expression in human cancer cells[12]. In the previous studies we have reported that miR-451expression in ESCC tissues were significantly reduced, and that upregulated expression of miR-451 induced apoptosis and suppressed cell proliferation, invasion and metastasis in the esophageal carcinoma[13,14]. However, the underlying molecular mechanisms remain unclear. In this study, we supposed and showed that CDKN2D and MAP3K1 were the targets of miR-451by the bioinformatics algorithms (TargetScan and miRBase), moreover, CDKN2D and MAP3K1 contributed to ESCC malignancy. Our data demonstrate that miR-451 has potential values as a prognostic marker and a therapeutic target of 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% CO2 at 37 ℃.

***Oligonucleotides and cell transfection***

The miR-451 mimics used in this study was synthesized by Shanghai GenePharma Co. Ltd. For transfection, 2 × 105 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 LipofectamineTM2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, United States). After 6h, the medium was changed to complete medium, and cells were cultured at 37 ℃ in 5% CO2.

***Cell growth assay***

The different experimental groups of EC9706 and KYSE150 cells were plated in 96-well plates at 1 × 104 cells per well and incubated for 48 h after transfection. The viability of cells was determined according to Cell Counting Kit-8 manufactures (CCK-8; Dojindo, Japan). Viable cell numbers were estimated by measurement of optical density (OD) at 450 nm. All experiments were performed in triplicates.

***Colony formation assays***

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 × 105 cells per dish. The plates were incubated for 2 wk at 37 °C in a 5% CO2 incubator, and the number of colonies was counted after staining with 0.1% crystal violet solution. All experiments were performed in triplicates.

***Cell invasion assay***

The experimental groups of EC9706 cells were adjusted at 2 × 105/mL in each group 48 h after transfection. The upper chamber of 24-well TranswellPermaeable 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% CO2 at 37 °C 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 triplicates.

***Apoptosis assay***

EC9706 cells were harvested 48h after transfection and adjusted cell concentration to 1 × 106 cells. Annexin V-FITC/PI Apoptosis Detetion Kit Ι(BestBio, Shanghai, China) was used to detect AnnexinⅤ. Results were obtained using FACScan Flow Cytometer(BD Biosciences, San Jose, CA, United States). Tests were repeated in triplicate. Dates were analyzed by Cell Quest software. All experiments were performed in triplicates.

***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 °C and incubated with RNase at 37 °C for 30 min. Nuclei were stained with propidium iodide for 30 min. A total of 104 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 cells in each group were lysed in lysisbuffer for total protein extration. Protein concentrations were measured using a 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 °C 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 bands density was determined with Kodak Digital ID Image Analysis Software and was normalization with the density of β-actin. All experiments were performed in triplicates.

***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 LipofectamineTM2000. Luciferase activities were measured using a Dual-Luciferase assay kit (Promega) according to manufacturer’s instructions at 48h post-transfection. All experiments were performed in triplicates.

***Statistical analysis***

Statistical testing was conducted with the assistance of SPSS 17.0 software. All data are expressed as means ± standard deviation (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 selectCDKN2D and MAP3K1. The 3' untranslated region (3'UTR) of CDKN2D contains the seed regions for miR-451 at the position of base 240nt-246nt (Figure 1A). Similarly, the 3' untranslated region (3'UTR) of MAP3K1 contains the seed regions for miR-451 at the position of base 6270nt-6278nt (Figure 1B).

Subsequent western blot analysis indeed showed that CDKN2D and MAP3K1 expression were down-regulated in EC9706and KYSE150cells following transfection with the miR-451 mimics (Figure1C 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 sequence (pmirGLO-CDKN2D -mut 3’UTR, pmirGLO-MAP3K1 -mut 3’UTR) within the seed region sites were cloned into thepmirGLO 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 KYSE150cells (*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-451negatively regulates CDKN2D and MAP3K1 expression by directly binding to putative binding sites in the 3' UTR. Our results thus demonstrated that CDKN2D and MAP3K1were direct targets of miR-451.

***CDKN2D and MAP3K1 overexpression reversed 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 intoEC9706 cells. Western blot assay showed that transfection of miR-451 mimics inhibited 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 (Figure2A). Similarly, co-transfection of pcDNA3.1-MAP3K1and 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 recombinantCDKN2D 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 (Figure2B 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 reversetheanti-proliferation of miR-451 (Figure 2B and C). From these results we conclude that expression of CDKN2D and MAP3K1could 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 recombinantCDKN2D 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 didn't show significantly decreased levels of apoptosis (Figure 2D). But cells transfected with pcDNA3.1-MAP3K1 alone showed significantly decreased levels of apoptosis compared to the Blank control (Figure2E), 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 (Figure2E).

In the transwell assays we found that exogenous expression of miR-451 decreased cell invasiveness (Figure2F and G). Subsequently, we exogenously expressed recombinantCDKN2D 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 invasiveness (Figure 2F and G). 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 (Figure2F).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 (Figure2G).

Cell-cycle analysis showed that administration of miR-451 mimics 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 reverseG1 arrest of miR-451 (Figure2H). 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 reverseG1 arrestof miR-451 (Figure 2I).

***MiR-451 inhibits the proliferationof EC9706cells by targeting CDKN2D, 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(Figure3A).

CCK8 array showed that CDKN2D, MAP3K1 silencing and miR-451 overexpression inhibited the proliferation (Figure3B and C). For EC9706 cells transfected withsi-CDKN2D, the inhibition is more obvious than cellstransfected with si-MAP3K1 (Figure 3B and 3C). Compared to 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, theinhibitioneffectsare similar to cells transfected withthe overexpression of miR-451(Figure3D).Furthermore, Colony formation assay obtained the similar results that CDKN2D, MAP3K1 silencing and miR-451 over-expression inhibited EC9706cellscolonynumbers (Figure3E).

Invasion arrays showed that knockdown of MAP3K1 and the overexpression of miR-451 repressed the invasion capacities of EC9706 cells (Figure 3G). For cells transfected withsi-MAP3K1, the numbers of invasive cellsareless than cellstransfected 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 cells apoptosis significantly compared to the Blank control. For EC9706 cells transfected withsi-MAP3K1, the apoptosis cellsaremore than cellstransfected with si-CDKN2D. Co-transfected cells with si-CDKN2D and si-MAP3K1, theapoptosiseffectsare similar to cells transfected withthe overexpression of 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 (Figure3H).Altogether, these results confirm that miR-451 inhibits the proliferation of EC9706 by targeting CDKN2D and MAP3K1.

**DISCUSSION**

Cyclin-dependent kinase inhibitor 2D (CDKN2D) (p19INK4d),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 beenshown to form a stable complex with CDK4 or CDK6, and prevent the activation of the CDK kinases, thus function 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 involved in this protein was shown to participate in repressing neuronal proliferation, as well as permatogenesis[15-20]. Little is known of its role in cancer development and prognosis.CDKN2Dexpression 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 mediatorsof evolutionarily conserved signaling networks that playan 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 identifiedas the mammalian homolog of the yeast MAP3Ks Ste11and Byr2 that function in pheromone responsive signaling. Studies have demonstrated that MAP3K1 functions in cell survival, apoptosis, and cell motility/migration in multiple normaland tumor cell types[24].

In the previous studies we have reported that miR-451expression in ESCC tissues were significantly reduced, and that upregulated expression of miR-451 induced apoptosis and suppressed cell proliferation, invasion and metastasis in the 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, 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, 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, andalteration 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.EachmiRNA has the potential to target hundreds of genes that harbor in their 3' UTRs sequences complementary to the seed region of the miRNA[26-29]. In the study,forco-transfected cells with si-CDKN2D and si-MAP3K1, theinhibitioneffectsare similar to cells transfected withthe overexpression of miR-451.

However, miRNAs may function according to a combinatorialcircuits model, in which a single miRNA may target multiplemRNAs, and several coexpressedmiRNAs may targeta single mRNA. Recent studies have suggested that the biologicalconcept of “one hit–multiple targets” could be used in clinicaltherapeutics[30]. If the primary molecular defect of a disease isin the expression of a miRNA, the expression of several criticalprotein targets could be deregulated. In that case, one mightrecover the normal phenotype of the cells by normalizing themiRNA expression. Although individual targets responsible for observed phenotypes have been proposed for many miRNAs, itis likely that a specific miRNA may function through cooperativedown-regulation of multiple targets. Thus, other target genes ofmiR-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-451might be novel prognostic biomarkers and potential targets 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 treatment of ESCC includes chemotherapyand radiation therapy. However, many patients who are treated with such traditional therapy still experience progression of disease, 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 regulates erythrocyte differentiation. It was also reported that miR-451 might function as tumor suppressor and modulate MDR1/P-glycoprotein expression in human cancer cells.

***Innovations and breakthroughs***

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

***Applications***

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

***Terminology***

miRNAs are small, endogenous noncoding RNAs that have been identified as post-transcriptional regulators of gene expression. MiRNAs exert theirfunctions through imperfect base-pairing with the 3’- untranslated region (3’-UTR) of target mRNAs.

***Peer-review***

The manuscript is basically good. The authors have done designed and guided the study. This is an in vitro study that addressed the mechanism of tumor suppressive functions of a microRNA, 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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**Figure 1 CDKN2D, MAP3K1 are direct targets of miR-451 in EC9706 cells**. A: The putative miR-451 binding sequences for theCDKN2D 3' UTRs. The 3' untranslated region (3'UTR) of CDKN2D contains a seed region for miR-451. B: The putative miR-451 binding sequences for theMAP3K1 3' UTRs. The 3' untranslated region (3'UTR) of MAP3K1 contains a seed region for miR-451. C:Western blotanalysedCDKN2D expression in transfected cells. Transfection of miR-451 mimics resulted in significant reduction of CDKN2D protein expression by western blot in EC9706 and KYSE150 cells. β-actin was used as a reference. D:Western blotanalysedMAP3K1 expression in transfected cells. Transfection of miR-451 mimics resulted in significant reduction of MAP3K1 protein expression by western blot 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. F: miR-451 significantly decreased the luciferase activity of MAP3K1 3’UTR-Wt in EC9706 and KYSE150(a*P* < 0.05 *vs* control group).



**Figure 2CDKN2D and MAP3K1 overexpression reversed the effect of miR-451.** A: CDKN2D and MAP3K1 protein level was 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 CDKN2Dor MAP3K1expression. 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 was performed. a*P* < 0.05 *vs* control group. C: The expression of MAP3K1could partially reverse the anti-proliferation function of miR-451.Colony formation assays was performed. a*P* < 0.05 *vs* control group. D: The expression of CDKN2D didn’t 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 were 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 were 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 cellinvasionwas assessed usingtranswell 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 cellinvasionwas assessed usingtranswell 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 MAP3K1didn’t reverseG1 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).



**Figure 3 MiR-451 inhibits the proliferation of EC9706 cells by targeting CDKN2D, MAP3K1.** A: CDKN2D and MAP3K1protein level was 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 proliferation. CCK8 array was used to assess EC9706 proliferation. C: MAP3K1 silencing and miR-451 overexpression inhibited the 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 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 were assessed using flow cytometry assay. G: CDKN2D, MAP3K1 silencing and miR-451 overexpression suppressed EC9706 cell invasiveness. The cellinvasionwas assessed usingtranswell 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 (a*P* < 0.05 *vs* control group). The cell-cycle was assessed using flow cytometry assay.

