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miRNA-331-3p directly targets E2F1 and induces growth arrest in human gastric cancer

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ABSTRACT

Deregulation of E2F1 activity is characteristic of gastric tumorigenesis, which involves in complex molecular mechanisms. microRNA is one of the post-transcriptional regulators for gene expression. Here, we report a member of miR-331 family, miR-331-3p, which was decreased in some kinds of malignancies. However, the biological function of miR-331-3p on gastric cancer is largely unknown. In this study, we screened the expressing levels of miR-331-3p and E2F1 in gastric cancer cell lines. We transfected precursor or inhibitor of miR-331-3p into gastric cancer cells. As results, miR-331-3p is down-regulated in all gastric cancer cell lines by real-time PCR. Over-expression of miR-331-3p blocked G1/S transition on SGC-7901 and AGS cell lines. Introduction of miR-331-3p dramatically suppressed the ability of colony formation and cell growth in vitro by interfering E2F1 activity. Our data highlight an important role of miR-331-3p in cell cycle control by targeting 3′-UTR of cell cycle-related molecule E2F1. We concluded that miR-331-3p is a potential tumor suppressor in gastric cancer. Restoring miR-331-3p in gastric cancer cells revealed potential application in gastric cancer therapy.

1. Introduction

Gastric cancer is the second leading cause of male cancer-related death and the third leading cause for female cancer-related death worldwide [1]. It is a major public health problem throughout the world [2]. In China, there are 400,000 new cases of gastric cancer and 300,000 deaths annually. Many cases who suffered from gastric cancer have lost curative chance with extremely poor outcome [3]. Therefore, developing novel and effective therapeutic methods is essential to reduce gastric cancer mortality. It has been known that the pathogenesis of gastric carcinomas is multifactorial, which includes genetic predisposition and environmental factors. There are a number of genetic alternations including tumor suppressor genes, oncogenes, cell adhesion molecules and growth factors [4]. Increased evidences suggested that E2F1 is an oncogenic factor during gastric carcinogenesis [5]. E2F1 is a downstream regulator of the Rb pathway and is a transcription factor that controls cell proliferation and cell cycle progression [6–9]. Recently, a cluster of miRNAs determining the regulation of E2F1 expression has been noticed [5].

miRNA is a class of small non-coding RNAs, has been discovered in animals and plants [10–12]. miRNA is 19–22 nt non-coding RNA, and able to bind complementary sequences in 3′-untranslated regions (3′-UTR) of target mRNA to induce their degradation or translational repression. They are encoded by genes that are presumably transcribed into single or clustered primary transcripts, which are processed and produced the mature miRNAs. They function as regulators of tumor initiation, progression and metastasis. The miRNAs involved in carcinogenesis are designated as oncogenic miRNAs (oncomiRs) [13–15]. miR-331-3p, located on 12q22, has been studied in prostate cancer and human lymphoblastoid cell lines. Their reports demonstrated significant correlation of miR-331 with the genes-related with cell cycle processes [16,17]. The miRNA expressing profile of gastric cancer cell lines suggested that miR-331-3p is one out of 146 down-regulated miRNAs in our laboratory. To elucidate and validate the potential function and expression pattern of miR-331-3p in gastric cancer, we constructed cell models of over-expressing miR-331-3p and down-expressing miR-331-3p in gastric cancer cells. We also screened expressing levels of miR-331-3p and E2F1 in a group of gastric cancer cells. By gain-of-function examination, E2F1 was identified as a significant target of miR-331-3p, contributing to the G1/S arrest.

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2. Materials and methods

2.1. Cell culture

Gastric cancer cell lines NCI-N87, SNU-16, AGS and KATO-III were obtained from American Type Culture Collection (Manassas, VA, USA). Gastric cancer cell lines MKN-45, MKN-28, SGC-7901, BGC-823, GES-1 immortalized gastric mucosa cells and human embryonic kidney cell line 293T (HEK 293T) were preserved in our institute. Cancer cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 with RPMI-1640 medium (Life Technologies, Grand Island, NY, USA) containing 10% fetal calf serum with 50 U/ml penicillin and 50 μg/ml streptomycin, HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma. St. Louis, MO) supplemented with 10% fetal calf serum (FCS). Exponentially growing cells were used for experiments.

2.2. Databases searching

We searched following microRNA databases: http://microrna.sanger.ac.uk/; http://www.microrna.org/microrna/home.do; http://pictar.bio.nyu.edu/ and http://www.targetscan.org/vert_40/.

2.3. Total RNA preparation and reverse transcription

Total RNA from cell lines were extracted using miRNeasy Mini Kit (Qiagen, Hilden, Germany), which is suit for isolating small molecular weight nucleic acids. Concentration and purity of total RNA were measured by SmartSpec Plus spectrophotometer (Bio-Rad, CA, USA). The ratio of A260:A280 was used to indicate the purity of total RNA. cDNA was generated using the miScript Reverse Transcription Kit (Qiagen, Hilden, Germany). According to the manufacturer's instructions, 1 μg total RNA, 1 μl miScript Reverse Transcriptase Mix, 4 μl 5 × miScript RT buffer and appropriate volume RNase-free water were mixed well and incubated for 5 min at 95 °C to inactivate miScript Reverse Transcriptase Mix. All reverse transcriptions and no-template controls were run at the same time.

2.4. Real-time PCR analysis

A miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany) was used to measure the expression of mature miR-331-3p in gastric cancer cell lines after reverse transcription. Real-time polymerase chain reaction (PCR) was performed using on an Mx3005P QPCR System (Stratagene, USA), following the manufacturer's protocol. The 20 μl PCR mixture included 2 μl reverse transcription product, 10 μl 2 × QuantiTect SYBR Green PCR Master Mix, 2 μl 10 × miScript Universal Primer, 2 μl 10 × miScript Primer Assay (specific for miR-331-3p, purchased from Qiagen, Hilden, Germany), and 4 μl RNase-free water. The reaction mixtures were incubated at 95 °C for 15 min, followed by 40 amplification cycles of 94 °C for 15 s, 55 °C for 30 s, and 70 °C for 30 s. The threshold cycle (Ct) was defined as the fractional cycle number at which the fluorescence passed the fixed threshold. We also quantified transcripts of RNA, U6 small nuclear 2 using Hs_RNU6B_2 miScript Primer Assay (Qiagen, Hilden, Germany) for normalizing the levels of miR-331-3p. Hs_RNU6B_2 is widely used as endogenous reference RNA in miRNA quantification studies. The comparative ΔCt method was used for analysis [18].

2.5. Over-expression and down-expression of miR-331-3p

Stability-enhanced miR-331-3p precursor (GCCCCUGGGCCU AUCCUAGAA) and negative control RNA-oligonucleotides were purchased from Ambion Corporation (Austin, USA). To inhibit miR-331-3p function, a miRNA inhibitor (UUCUAGGAUAGGCCC-CAGGGGC) for miR-331-3p (AS-miR-331-3p) was used, along with the negative control (AS-miR-control). The day before transfection, SGC-7901 and AGS cells were seeded in antibiotic free medium. Transfection of miRNAs was carried out using Lipofectamine 2000 in accordance with the manufacturer's procedure (Invitrogen, CA, USA). miR-331-3p or negative control RNA-oligonucleotides were transfected at a final concentration of 50 nM unless otherwise indicated. The level of miR-331-3p expression in SGC-7901 and AGS cell lines was assayed by real-time RT-PCR 48 h after transfection as described above.

2.6. Cell growth activity assay

SGC-7901 and AGS cells (2×10^3 cells/well) were incubated with 100 μL culture medium in 96-multiwell plates and incubated for 1 day at 37 °C in a CO_2 incubator. The cells were transfected with miR-331-3p precursor or negative control. After incubation for different times (0, 24, 48 and 72 h), Cell growth activity was assessed using the Cell Counting Kit-8 (Dojindo, Japan) as following: CCK-8 (10 μL) was added to each well. After 1 h incubation at 37 °C, absorbance at 450 nm was measured with the ARVO MX plate reader (PerkinElmer, MA, USA). Background absorbance from empty wells was subtracted from sample wells. Cell growth activity was expressed as the absorbance at 450 nm. All experiments were performed in triplicate.

2.7. Flow cytometric analysis for cell cycle

One day before transfection, 1×10^6 cells of SGC-7901 and AGS cells were seeded into 6-well culture plates without antibiotics. The cells were transfected with miR-331-3p, miR-331-3p inhibitor or negative control. Forty-eight hours after transfection, cells were harvested and fixed in 70% ethanol at −20 °C overnight, and then stained with 250 μg/mL propidium iodide (Sigma–Aldrich), 5 μg/mL RNase A (Sigma–Aldrich) and 5 mmol/L EDTA in PBS (pH 7.4) for 30 min. The cell cycle analysis was done by FACScan (Beckman Instruments, Fullerton, CA, USA). Each transfection was performed in triplicate.

2.8. Soft agar colony formation assay

SGC-7901 or AGS cancer ells (3×10^3) were trypsinized to single-cell suspension and then plated in six-well plates, in complete culture medium containing 0.3% agar on top of 0.6% agar in the same medium. The plates were incubated at 37 °C in 5% CO_2 for 16 days; colonies were fixed with 70% ethanol, and stained with 0.2% crystal violet. The colonies containing at least 50 cells were counted. The data were presented as means ± SD by counting five fields randomly.

2.9. Western blot analysis

Protein from gastric cancer cell lines was extracted by mammalian protein extraction reagent (Pierce, USA) supplemented with protease inhibitors cocktail (Sigma, USA). HEK 293T cells were transfected with miR-331-3p, miR-331-3p inhibitor or negative control in six-well plates. After transfection, cells were cultured for 72 h, protein was extracted by mammalian protein extraction reagent (Pierce, USA) supplemented with protease inhibitors cocktail (Sigma, USA). Protein samples 50 μg were resolved by 10% SDS–PAGE and then transferred to PVDF membranes. The membranes were blocked by TBST buffer (TBS plus 0.1% Tween-20) containing 5% w/v non-fat milk and hybridized with primary antibody, followed by incubation with specific HRP-conjugated secondary

antibody. Protein bands were visualized by the ECL detecting system (Amersham Biosciences, Uppsala, Sweden). Mouse monoclonal anti-E2F1 (1:1000, Abcam, USA) was used. Monoclonal anti-GAPDH (1:5000, Abcam, USA) was used for loading control.

2.10. Luciferase activity assay

A 256-bp fragment of the wild-type (WT) E2F1–3′-UTR containing the putative miR-331-3p binding site was amplified by PCR, using the forward primer: 5′-CATACTAGTTGATACCCCAACTCCCTCTACC-3′ and the reverse primer: 5′-CTTAAGCTT CTGCCCACATCAGTGAAGGTCC-3′ After digestion of the PCR product by Spe I and Hind III, the E2F1–3′-UTR was cloned into the Spe I and Hind III sites of pMIR-Report (Ambion), yielding pMIR-Report-E2F1. A mutant 3′-UTR of E2F1 was introduced in potential miR-331-3p binding sites using the Quick-Change site-directed mutagenesis Kit (Stratagene). This construct, named E2F1/UTR or E2F1/UTR-Mut was used for transfection in HEK293T cell line in 24-well plates. In each well, 10 ng of renilla luciferase, phRL-TK vector (Promega, Madison, USA) was co-transfected for normalizing transfection efficiency. Reporter 500 ng, E2F1/UTR or E2F1/UTR-Mut together with 10 nM miR-331-3p, miR-331-3p inhibitor or negative control was co-transfected using Lipofectamine 2000 and Opti-MEM I reduced serum medium (Life Technologies, CA, USA). After 48 h, cells were harvested with 100 μL PLB reagent (Promega, Madison, USA) and 20 μL cell lysates prepared in reporter lysis buffer (Promega). Firefly luciferase activity was measured for each well using the dual luciferase assay Kit (Promega) with an analytical luminometer (TD-20/20, Turner Designs, Sunnyvale, USA) according to the manufacturer's instruction. Briefly, 10% volume of cell lysate (20 μL) was added to 100 μl of LAR II. Reaction was stopped with the addition of 100 μl Stop & Glo® reagent. Normalized relative luciferase activity (RLA) was calculated as the following formula: RLA = [firefly luciferase]/[renilla luciferase].

2.11. Statistical analysis

Statistical analysis was performed with SPSS15.0 software (SPSS Inc., USA). Data are expressed as the mean ± standard deviation from at least three separate experiments. The differences between groups were analyzed using Student *t* test, when only two groups, or assessed by one-way ANOVA when more than two groups. A value of $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Functions of miR-331-3p on gastric cancer cells

At first, we screened miR-331-3p and E2F1 expression in several gastric cancer cell lines by real-time PCR and Western blot. Different miR-331-3p and E2F1 expressing levels were found in eight cell lines (Fig. 1A). We chose SGC-7901 and AGS cancer cells for miR-331-3p precursor or miR-331-3p inhibitor transfection study, respectively. After transfection, we examined expression of miR-331-3p on cancer cells by real-time PCR. The expressing level of miR-331-3p was up-regulated 71.32-fold ($p < 0.01$) in SGC-7901 cells with miR-331-3p precursor transfection. The expressing level of miR-331-3p was down-regulated 46.43-fold ($p < 0.01$) in AGS cells with miR-331-3p inhibitor transfection, compared to negative control (Fig. 1B).

As shown in Fig. 1C, cell growth activity of SGC-7901 was significantly suppressed by restoring miR-331-3p expression, compared to miR-control group ($p < 0.05$). We evaluated colony formation ability on soft agar by miR-331-3p transfection. Colony formation rates were 2.20 ± 0.83%, 7.60 ± 0.60% and 8.2 ± 0.80% in miR-331-3p group, miR-control group and parental SGC-7901 group, respectively ($p < 0.05$, Fig. 1D). The colony formation rate of ASG cells showed same tendency in miR-331-3p group, miR-control group and parental group.

We examined cell cycle after transfecting miR-331-3p precursor by flow cytometry. miR-331-3p-transfected SGC-7901 and AGS showed higher percentage of G0/G1 phase cells and lower percentage of S phase or G2/M phase cells ($p < 0.05$, Fig. 2A and B). The interfering efficacy to cell cycle disappeared after transfecting miR-331-3p inhibitor into AGS cells. It means that over-expression of miR-331-3p blocks G1/S transition.

3.2. miR-331-3p targets 3′-UTR of E2F1 gene

Based on databases search, the association of miR-331-3p and E2F1 was hinted. We evaluated the effect of miR-331-3p on E2F1, a crucial regulatory gene for cell cycle (Fig. 3A). We examined protein levels of E2F1 in HEK 293T cells after transfecting miR-331-3p precursor, miR-331-3p inhibitor or control into cells. As shown in Fig. 3B, transfection of miR-331-3p precursor led to a significant down-regulation of E2F1 protein. Alternatively, transfection of miR-331 inhibitor did not alter E2F1 protein levels compared to control-transfected cells.

To verify a direct interaction between miR-331-3p and the 3′-UTR of E2F1, we cloned the 3′-UTR region that is predicted to interact with miR-331-3p into a luciferase reporter vector. We assessed luciferase activity by co-transfecting the luciferase reporter vector bearing the 3′-UTR of E2F1 with the miR-331-3p precursor, miR-331-3p inhibitor or control plasmids. Luciferase activity of reporter plasmid with wild-type 3′-UTR of E2F1 was markedly decreased in cells transfected with miR-331-3p precursor, compared to luciferase activity of reporter plasmid with mutant 3′-UTR (Fig. 3C, mutated CGG to TCA, $p < 0.05$). Conversely, the luciferase activity of reporter plasmid was not interfered after transfection with miR-331-3p inhibitor. Taken together, these data imply that miR-331-3p may attenuate the expression of E2F1 by directly targeting the 3′-UTR of E2F1.

4. Discussion

microRNA is a novel class of regulatory molecules with the ability to control gene expression at the post-transcriptional level. It appears to decrease protein production by blocking translation of mRNA into protein. Therefore, identification of cancer-specific microRNAs is critical for understanding their role on tumorigenesis and may be important for finding novel therapeutic targets [19,20]. To date, over 150 microRNAs are reported to concern with carcinogenesis (miRbase: http://microrna.sanger.ac.uk/). Half of them have been reported in gastric cancer [5,19–27]. miR-331-3p belongs to cancer-associated microRNA. Epis et al. found that miR-331-3p regulated ERBB-2 protein expression and blocked downstream PI3K/AKT signaling in prostate cancer [16]. Wang et al. found that miR-331-3p was significantly correlated with the genes in cell cycle-related biological processes, which is consistent with important role of miRNAs in cell cycle regulation [17]. Up to date, there is no report of miR-331-3p on gastric cancer. miR-331-3p belongs to miR-331 family, which is located on chromosome 12 from 57845502 to 57845595 loci. In this study, we firstly revealed that miR-331-3p expression is significantly decreased in human gastric cancer cells.

Inhibiting oncogenes or restoring the expression of silenced tumor suppressor genes are basic strategy for cancer gene therapy. Saito et al. revealed that many miRNAs are under epigenetic control and could be therapeutic targets of cancer [28]. From this study, restoring miR-331-3p expression in human gastric cancer

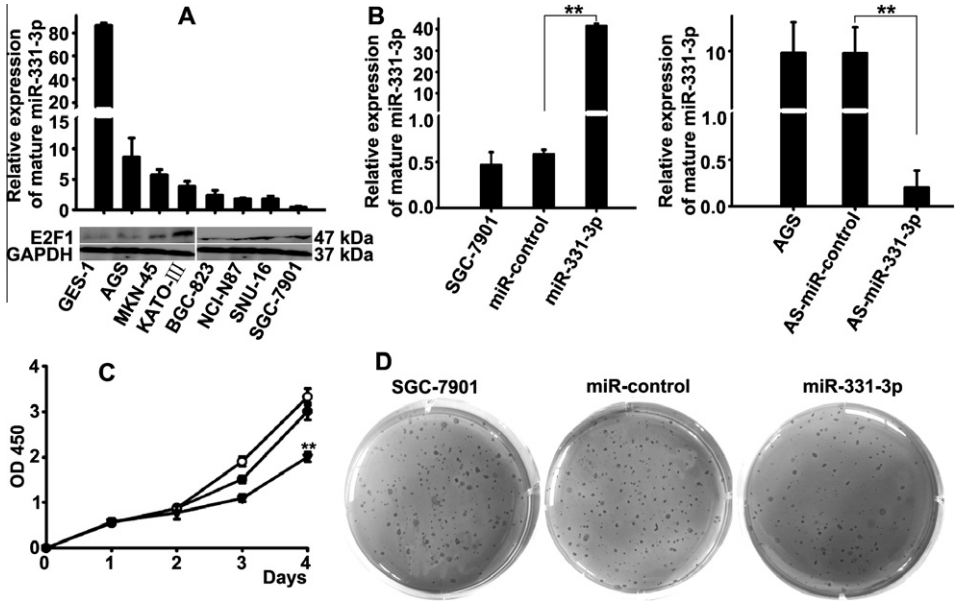

Fig. 1. Functions of miR-331-3p on gastric cancer cells. (A) The different expressing levels of miR-331-3p and E2F1 on seven gastric cells lines and one immortalized gastric mucosa cells. (B) Expression of miR-331-3p was up-regulated in SGC-7901 cells by miR-331-3p precursor transfection, and down-regulated in AGS cells by miR-331-3p inhibitor transfection, compared to controls ($p < 0.01$). (C) Over-expression of miR-331-3p precursor leads to cell growth arrest by CCK-8 assay ($p < 0.01$). (D) Colony formation rates were significantly different between miR-331-3p precursor transfection group and controls in SGC-7901.

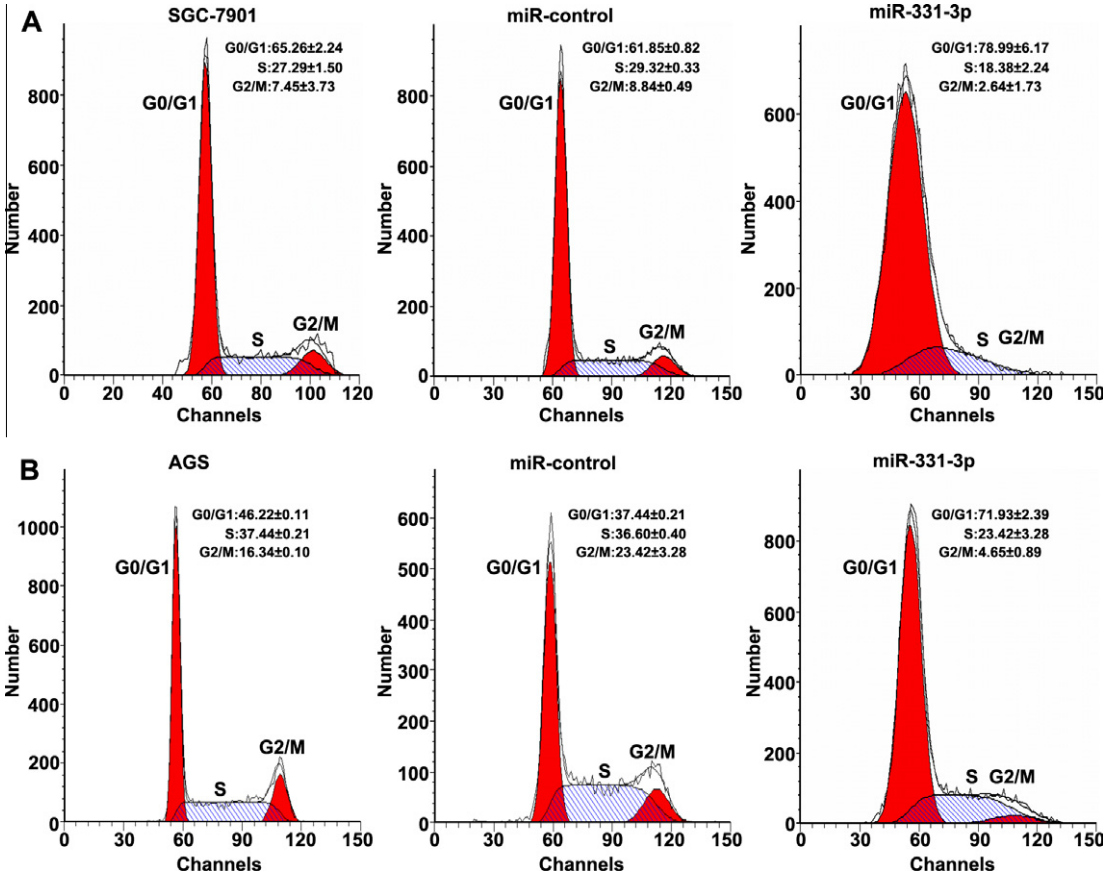

Fig. 2. Effect of miR-331-3p transfection on cell cycle. (A) miR-331-3p-transfected SGC-7901 showed increased percentage of G0/G1 phase cells and decreased percentage of S and G2/M phase cells ($p < 0.05$). (B) miR-331-3p-transfected AGS showed increased percentage of G0/G1 phase cells and decreased percentage of S and G2/M phase cells ($p < 0.05$).

cells could significantly increase the percentage of G0/G1 phase cells and decrease the percentage of G2/M phase cells, and then result in growth arrest of gastric cancer cells. These findings provided evidence that miR-331-3p functions as key regulator in cell

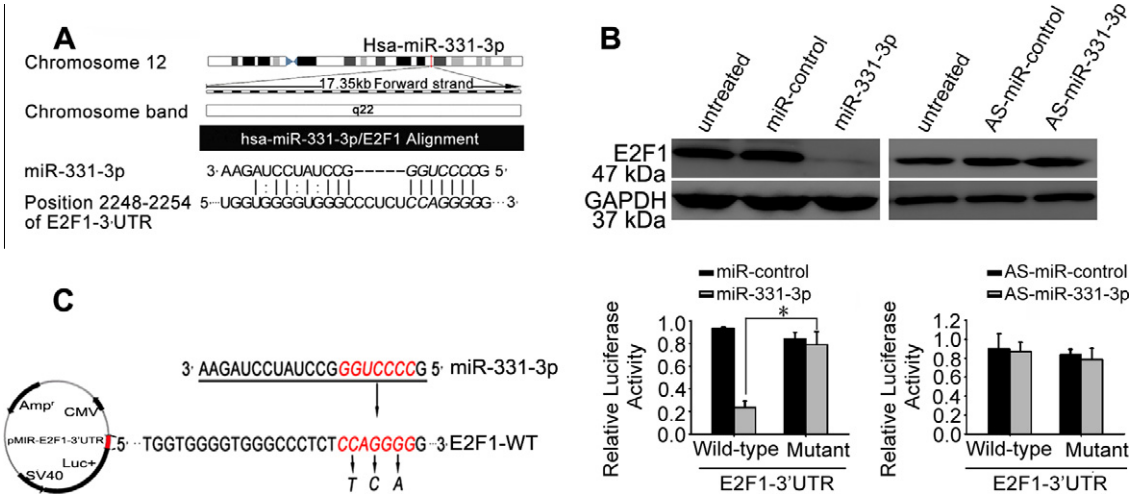

Fig. 3. miR-331-3p targets 3′-UTR of E2F1 gene and attenuates the E2F1 expression. (A) Schematic graph of chromosome location of miR-331-3p. Representative nucleotide sequence matches between possible target sequences and miRNA. Only matched nucleotides with miRNA seed sequences are indicated with vertical lines, the red vertical line indicates the location of miR-331-3p in chromosome 12. The miR-331-3p seed sequence (GGUCCCC) is shown in italic nucleotides. (B) Forty-eight hours after miR-331-3p precursor or inhibitor transfection on 293T cells, E2F1 protein level was significantly reduced in miR-331-3p transfected cells by Western blot. By contrast, miR-331-3p inhibitor transfection did not influence protein expression of E2F1. (C) Left: schematic graph of 3′-UTR binding site for miR-331-3p. E2F1–3′-UTR-WT or E2F1–UTR-Mut was inserted into downstream of luciferase of pMIR-reporter vector. Right: luciferase activity of cells was measured on HEK293T cells. A total of 1×10^5 HEK 293T cells were transfected with 500 ng of wild-type-UTR-reporter or mutant-UTR-reporter constructs together with 10 nM of miR-331-3p precursor or miR-331-3p inhibitor. Each bar represents mean values ± SD from three independent experiments (asterisk means, $p < 0.05$).

growth and may be a promising target for gastric cancer treatment. It is well known that miRNAs regulate a variety of cellular activities through regulating expression of multiple target genes [29,30]. In our study, E2F1 was verified as a promising target gene, which is related to G1/S transition. Our analysis revealed that restoring miR-331-3p expression attenuated protein level of E2F1 by post-transcription regulation, and therefore inhibited cell cycle progression in gastric cancer. So, the miR-331-3p could be considered as tumor suppressor genes. Tumor suppressor miRNAs usually prevent tumor development by negatively regulating oncogenes or genes that control cell cycle or apoptosis. Currently, several miRNAs are reported as tumor suppressor genes. For instance, miRNA let-7 was taken as tumor suppressor miRNA. RAS oncogene is a direct target of miRNA let-7. Let-7 negatively regulates RAS expression by pairing at the 3′-UTR of RAS mRNA for translational repression [31,32]. Because miRNAs function as oncogenes or tumor suppressors, it might be possible to regulate miRNA expression in order to inhibit cancer growth, similar to the use of antisense mRNAs or RNAi, which are widely used as tools for studying gene functions and in some case of gene therapy [33].

In summary, we have found that miR-331-3p is markedly down-regulated in human gastric carcinoma. Over-expression of miR-331-3p leads to cell cycle arrest and inhibits cell growth. E2F1 is identified as direct target gene of miR-331-3p, which is responsible for cell cycle arrest and cell growth inhibition. It implied that miRNAs cannot be overlooked as a class of molecules on regulating biological functions and on cancer therapy. Our data enhance understanding the functions of miRNAs on gastric carcinogenesis.

Competing interests statement

The authors declare that they have no competing financial interests.

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