

Original Article

Increase of miR-217 and decrease of β -catenin enhances sensitivity of esophageal cancer cells to cisplatin chemotherapy

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Abstract: Wnt/ β -catenin pathways can induce transcription of downstream cyclin D1 and are involved in the regulation of cell proliferation and apoptosis. Overexpression of β -catenin is correlated with esophageal squamous cell carcinoma (ESCC) onset and drug resistance. MicroRNA (miR)-217 expression is downregulated in ESCC tissue. This study aimed to investigate whether miR-217 regulates β -catenin expression and affects sensitivity of ESCC cells against cisplatin (DDP). ESCC patients were recruited for collection of tumor and adjacent tissues. Expression of miR-217, β -catenin, and cyclin D1 was measured. DDP-resistant cell line EC9706/DDP was established to compare expression of miR-217, β -catenin, and cyclin D1 with EC9706 cells, along with IC₅₀. Dual luciferase reporter gene assay was performed to investigate the targeted relationship between miR-217 and 3'-UTR of β -catenin. *In vitro* cultured EC9706/DDP cells were transfected with miR-217 mimic and/or si- β -catenin, followed by measurement of expression and cell proliferation by flow cytometry and CCK-8 assay. ESCC tissues had significantly higher expression of β -catenin and cyclin D1 plus lower miR-217 expression. EC9706/DDP cells had significantly higher β -catenin and cyclin D1 and lower miR-217 or IC₅₀ levels. miR-217 targeted 3'-UTR of β -catenin genes and inhibited expression. Transfection of miR-217 mimic and/or si- β -catenin suppressed β -catenin or cyclin D1 expression in EC9706/DDP cells. G0/G1 cycle was arrested, accompanied by reduced proliferation and increased DDP sensitivity. In conclusion, miR-217 targeted and inhibited β -catenin expression, suppressed cyclin D1, induced G0/G1 arrest, inhibited ESCC cell proliferation, and enhanced sensitivity towards DDP.

Keywords: MicroRNA-217, β -catenin, cyclin D1, DDP, drug resistance, esophageal carcinoma

Introduction

Esophageal cancer (EC) is a common malignant tumor, in the digestive tract, with complicated pathogenic mechanisms. These cause difficulties in prevention and early diagnosis, thus, causing high incidence, mortality, and worse prognosis [1]. Wnt/ β -catenin signal pathways are closely correlated with embryonic development and tissue/organ formation. They are involved in the regulation of various biological processes including cell proliferation, cycle, apoptosis, and differentiation [2]. Wnt/ β -catenin signal pathways can be correlated with tumor genesis, under abnormal activation. This has been demonstrated in lung cancer, gastric carcinoma [3], colorectal cancer [4], endometrium cancer [5], and pancreatic carcinoma [6]. A

positive regulator in this pathway, β -catenin can interact with multiple regulatory proteins to initiate expression of a series of Wnt-related target genes including c-myc, cyclin D1, and survivin, thus, modulating cell proliferation, cycle, and apoptosis [7]. Overexpression and abnormal accumulation of β -catenin significantly enhances positive regulation of Wnt/ β -catenin signal pathways and has been closely associated with occurrence, recurrence, and drug resistance of breast cancer [8], colorectal carcinoma [9], and gastric cancer [10]. Previous studies have shown that β -catenin overexpression participates in EC occurrence and affects patient survival and prognosis [11, 12]. Furthermore, abnormal activation of Wnt/ β -catenin signal pathways has been demonstrated to be associated with lower chemotherapy sensitivity

of various tumor cells including liver cancer [13], osteosarcoma [2], and lung cancer [14].

MicroRNAs (miRs) are a group of small non-coding RNA with 22~25 nucleotides length in eukaryotes. They can bind to 3'-untranslated region (3'-UTR) of target gene mRNA via complete or incomplete binding, thus regulating gene expression [15]. Studies have shown a possible association between miR-217 down-regulation and EC occurrence [16], indicating a potential role as a tumor suppressor gene. Bioinformatics analysis demonstrated complementary binding sites between miR-217 and 3'-UTR of β -catenin mRNA. This present study investigated whether miR-217 regulates β -catenin expression, affects EC cell sensitivity against chemotherapy reagent cisplatin, and participates in EC pathogenesis.

Materials and methods

Reagents and materials

ES cell line EC9706 was purchased from Cell Biology Institute, Chinese Academy of Science. Normal esophageal epithelial cell line HEEC was purchased from Hongshun Bio (China). RPMI1640 and DMEM culture medium were purchased from Gibco (US). Fetal bovine serum (FBS) was purchased from Hyclone (US). Penicillin-streptomycin was purchased from Lonza (US). TRIZOL and Lipofectamine 2000 were purchased from Invitrogen (US). PrimerScript RT reagent kit and SYBR Green Real-time PCR master Mix were purchased from Takara (Japan). miR-217 nucleotide fragments and PCR primers were synthesized by RuiBo Bio (China). Mouse anti-human β -catenin antibody was purchased from Cell Signaling Technology (US). Mouse anti-cyclin D1 antibody was purchased from Santa Cruz (US). HRP conjugated secondary antibody was purchased from Jackson Abonva (US). Cell Cycle Analysis Kit was purchased from Beyotime (China). CCK8 test kit was purchased from Toyobo (Japan). Luciferase gene reporter vector pLUC Luciferase vector was purchased from Ambion (US). Dual-luciferase reporter assay system was purchased from Promega (US). Chemotherapy reagent cisplatin (DDP) was purchased from Qilu Pharmacy (China).

Clinical information

A total of 49 ESCC patients receiving treatment in the First Affiliated Hospital of Soochow

University, from November 2015 to June 2016, were recruited. There were 26 males and 23 females, with an average age of 61.3 ± 10.7 years. Tumor tissues were collected from surgery, along with adjacent tissues which served as the control group. All patients signed informed consent and this study was approved by the hospital Ethics Committee.

Cell culture

EC cell line EC9706, EC9706/DDP, and normal esophageal epithelial cell line HEEC were seeded into RPMI 1640 medium containing 10% FBS, 100 U/mL penicillin, and 100 μ g/ml streptomycin. Cells were cultured under 37°C with 5% CO₂. Cells at log-growth phase with satisfactory status were used for further experiments.

Establishment of drug resistant cell line EC9706/DDP

DDP-resistant cell line was generated by intermittent treatment of a moderate concentration of DDP. Briefly, EC9706 cells at log-growth phase were treated with medium containing 0.1 μ g/mL DDP. After 48 hours, DDP-containing medium was discarded and fresh DDP-free medium was added for continuous culture. Cell passage was performed when they reached stable growth without death, followed by a second round of 25%~50% DDP treatment. Repeated drug treatment and passage regained cells that could maintain stable culture even under 1.0 μ g/mL DDP culture.

Drug resistant index

In vitro cultured EC9706 and EC9706/DDP cells were treated with various concentrations of DDP. MTT assay was used to test relative proliferation activity of all groups. Relative inhibitory rate (%) = 1 - relative proliferation activity (%). IC₅₀ refers to drug concentration under which 50% cell growth was inhibited. Resistance index (RI) = IC₅₀ (drug resistant cells)/IC₅₀ (original cells).

Construction of luciferase reporter gene construct and dual luciferase reporter gene assay

Using HEK293T genomic DNA as the template, full length fragments of wild type or mutant forms of 3'-UTR of β -catenin genes were amplified and cloned into pLUC plasmid. Recombinant plasmid was then used to transform DH5 α competent cells. Positive clones with

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correct sequences were confirmed by sequencing and named as pLUC- β -catenin-3'-UTR-wt and pLUC- β -catenin-3'-UTR-mut. Lipofectamine 2000 was used to co-transfect pLUC- β -catenin-3'-UTR-wt (or pLUC- β -catenin-3'-UTR-mut) and miR-217 mimic into HEK293T cells. After 48 hours of incubation, dual luciferase activity was measured by Dual Luciferase Reporter Assay System.

Cell transfection

In vitro cultured EC9706 cells were divided into five groups: miR-NC transfection group, miR-217 mimic transfection group, si-NC transfection group, si- β -catenin group, and miR-217 mimic + si- β -catenin group. Cells were collected for assay, 72 hours after transfection. Nucleotide fragments used were: si- β -catenin sense: 5'-AUUACAUCGGUUGUGAACGUCCC-3'; si- β -catenin anti-sense: 5'-GGGACGUUCAACCGGAUUGUAAU-3'; si-NC sense: 5'-UUCUC GAAC GUGUC ACGUU UdTdT-3'; si-NC anti-sense, 5'-UUAAG AGGCU UGCAC AGUGC AdTdT-3'.

qRT-PCR for gene expression

Cultured cells were lysed for RNA extraction by TRIzol method. PimerScript RT reagent kit was used to synthesize cDNA from RNA through reverse transcription. Using cDNA as the template, PCR amplification was performed under the direction of TaqDNA polymerase. Primer sequences were: miR-217_F: 5'-TACTC AACTC ACTAC TGCAT CAGGA-3'; miR-217_R: 5'-TATGG TTGTT CTGCT CTCTG TGTC-3'; U6_F: 5'-ATTGG AACGA TACAG AGAAG ATT-3'; U6_R: 5'-GGAAC GCTTC ACGAA TTTG-3'; β -catenin_F: 5'-AGGAC CACCG CATCT CTACA T-3'; β -catenin_R: 5'-GCAGT TTTGT CAGTT CAGGG A-3'; CyclinD1_F: 5'-GCTGC GAAGT GGAAA CCATC-3'; CyclinD1_R: 5'-CCTCC TTCTG CACAC ATTTG AA-3'; β -actin_F: 5'-GAACC CTAAG GCCAA C-3'; and β -actin_R: 5'-TGTC ACGAC GATTT CC-3'. In a 10 μ L PCR system, one added 5.0 μ L 2XSYBR Green Mixture, 0.5 μ L forward and reverse primers (2.5 μ M/L), and 1 μ L ddH₂O. PCR conditions were as follows: 95°C for 15 seconds, followed by 60°C for 30 seconds, and 74°C for 30 seconds. Forty cycles were performed on ABI 7500 fluorescent PCR cyler.

Western blot

Cells were collected for protein extraction. After washing with PBS twice, 1X SDS lysis buffer

was added followed by 5 minutes of boiling. Afterward, 40 μ g supernatant protein was separated in 8% DS-PAGE for 3 hours under 80 V, and was then transferred to PVDF membrane at 250 mA for 3 hours. Membrane was blocked in 5% defatted milk powder for 60 minutes at room temperature. Primary antibody (β -catenin at 1:300, cyclin D1 at 1:200, β -actin at 1:600) was added at 4°C for overnight incubation. After PBST washing three times, HRP conjugated secondary antibody (1:10 000 dilutions) was added for 60 minutes at room temperature for incubation. After PBST washing three times, ECL reagent was added for development for 1~3 minutes at room temperature. The film was exposed and scanned for data collection. Gray value was analyzed by Image J (NIH, Bethesda, MD, USA).

Flow cytometry for cell cycle

Cells were digested in trypsin and centrifuged at 1000 g for 5 minutes. Cells were rinsed in pre-cold PBS, once, and fixed in pre-cold 70% ethanol at 4°C for 24 hours. PBS was added for removing ethanol by centrifugation. PI staining buffer, including PI and RNase A, was added for 37°C dark incubation for 30 minutes. DNA content was measured by flow cytometry.

Colony formation assay

Next, 1.2% and 0.7% agarose with low melting point was prepared in distilled water. The 1.2% agarose and 2 \times RPMI 1640 medium (20% FBS) was mixed at a proportion of 1:1, which was cooled and solidified, as bottom agar was kept in 37°C 5% CO₂ incubator. Then, 0.7% agarose and 2 \times 1640 medium (20% FBS) was mixed at a proportion of 1:1, followed by the addition of 0.2 mL cell for sufficient mixing. The mixture was put on the 1.2% agarose-1640 plate, forming a double layer agar. The agar was cooled, solidified, and kept in 37°C 5% CO₂ incubator. Every three days, 0.2mL 1640 medium (20% FBS) was supplemented. After 14 days of culturing, the number of colonies was observed and counted under an inverted microscope (Olympus, Shinjuku, Tokyo, Japan). The upper culture medium was carefully removed and 0.4 mL of 0.1% gentian violet dye was added into each hole. After dyeing for 15 minutes, cells were observed and photographed.

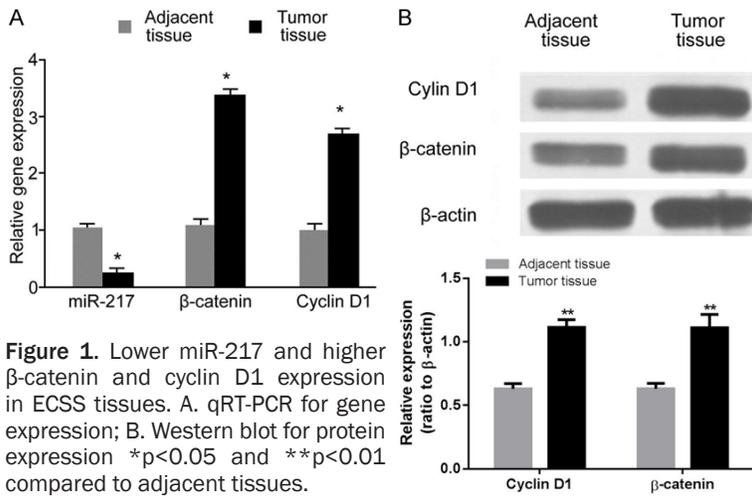


Figure 1. Lower miR-217 and higher β-catenin and cyclin D1 expression in ECSS tissues. A. qRT-PCR for gene expression; B. Western blot for protein expression *p<0.05 and **p<0.01 compared to adjacent tissues.

Statistical analysis

SPSS 18.0 was used for data analysis. Measurement data are presented as mean ± standard deviation (SD). Comparison of measurement data between groups was performed by student's t-test. One-way ANOVA was performed to assess statistical significance among multiple treatment groups, followed by LSD as post-hoc test. Statistical significance was defined as p<0.05.

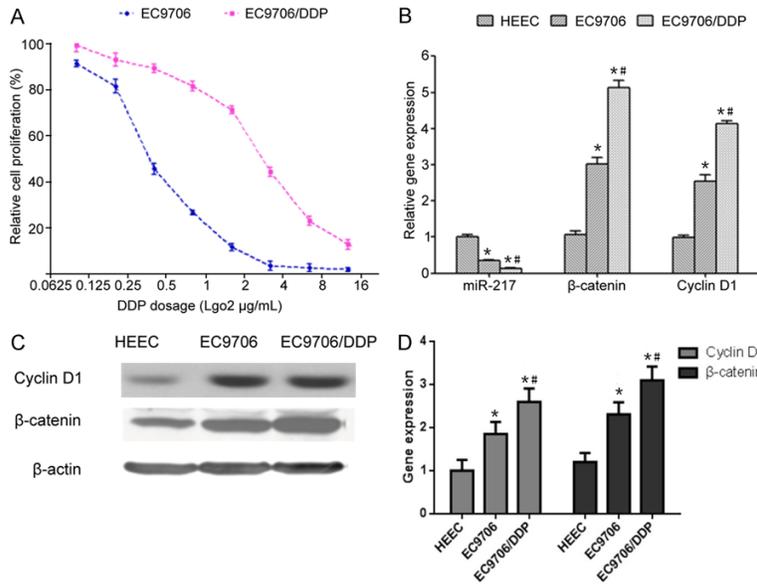


Figure 2. Correlation between miR-217 downregulation and β-catenin and cyclin D1 upregulation with drug resistance. A. CCK-8 for cell proliferation activity; B. qRT-PCR for gene expression; C. Western blot for protein expression. D. gray value analysis for Western blotting data. *, p<0.05 compared to HEEC cells. #, p<0.05 compared to EC9706 cells.

Results

Lower miR-217 and higher β-catenin and cyclin D1 expression in ECSS tissues

qRT-PCR results showed that, compared with adjacent tissues, ESCC tumor tissues had statistically lower miR-217 expression ($p = 8.99 \times 10^{-5}$) and significantly higher β-catenin ($p = 6.36 \times 10^{-5}$) and cyclin D1 mRNA levels ($p = 1.84 \times 10^{-4}$) (Figure 1A). Western blot results showed remarkably higher β-catenin ($p = 0.003$) and cyclin D1 ($p = 0.002$) protein levels in ESCC tumor tissues, compared with adjacent tissues (Figure 1B).

Decreased miR-217 and increased β-catenin and cyclin D1 expression correlated with drug resistance

CCK-8 for cell proliferation activity

All groups of cells were seeded into 96-well plate at 1×10^4 per well density. After attachment, cells were cultured for 48 hours. Then, 10 μL CCK-8 solution was added into each well. After 4 hours of incubation, absorbance values at 450 nm (A450) were measured by a microplate reader. Relative proliferation activity = (A450 of treatment wells - A450 of blank wells) / (A450 of control wells - A450 of blank wells) × 100%.

CCK-8 assay showed obviously lower DDP sensitivity in EC9706/DDP cells than in EC9706 cells after treatment with different doses of DDP ($p = 0.021$) (Figure 2A). IC₅₀ calculation results showed significantly higher IC₅₀ of DDP in EC9706/DDP cells than that of EC9706 cells, as EC9706/DDP cells had a drug resistance index of 8.51 (Table 1). qRT-PCR results showed significantly lower miR-217 expression in EC9706/DDP cells than in EC9706 cells ($p = 1.30 \times 10^{-3}$), while expression of β-catenin and cyclin D1 mRNA levels were higher. EC9706

Table 1. IC₅₀ values between EC9706 and EC9706/DDP cell

Drug	IC ₅₀ (μg/mL)		P value	RI
	EC9706	EC9706/DDP		
DDP	0.37 ± 0.02	3.15 ± 0.13	<0.001	8.51

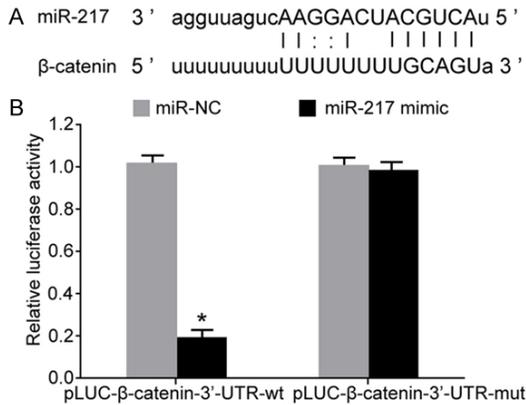


Figure 3. β-catenin as the target gene of miR-217. A. Functional sites between miR-217 and 3'-UTR of β-catenin mRNA; B. Dual luciferase reporter gene assay. *, p<0.05 comparing between miR-217 mimic and miR-NC.

cells had lower miR-217 levels than HEEC cells and higher β-catenin ($p = 5.42 \times 10^{-4}$) and cyclin D1 mRNA levels ($p = 1.10 \times 10^{-3}$) (Figure 2B). Western blot showed consistent results, as EC9706/DDP cells had higher β-catenin and cyclin D1 protein levels than EC9706 cells ($p = 0.0359, 0.0323$), with further higher level than HEEC cells ($p = 0.0022, 0.0010$) (Figure 2C, 2D). These results reveal that decreased miR-217 and elevated β-catenin and cyclin D1 expression may be related to EC onset and DDP drug resistance.

β-catenin as the target gene of miR-217

Bioinformatic analysis showed a targeted relationship between miR-217 and 3'UTR of β-catenin mRNA (Figure 3A). Dual luciferase reporter gene assay showed that transfection of miR-217 mimic significantly decreased the relative luciferase activity in HEK293T cells transfected with β-catenin-3'-UTR-wt plasmid ($p = 1.01 \times 10^{-4}$) (Figure 3B). No significant changes occurred in those cells transfected with β-catenin-3'-UTR-mut plasmid, indicating that miR-217 targeted 3'-UTR of β-catenin mRNA and inhibited its expression.

Elevated miR-217 and decreased β-catenin enhanced DDP sensitivity of EC cells

To stabilize proliferation activity of EC9706/DDP cells above 80% ($= IC_{20}$), DDP concentration of 0.75 μg/mL was used to observe DDP sensitivity of all transfected cells. Results showed that transfection of miR-217 mimic, β-catenin siRNA, and miR-217 mimic + β-catenin siRNA, respectively, significantly decreased β-catenin ($p = 2.61 \times 10^{-5}, 1.74 \times 10^{-5}, 7.71 \times 10^{-6}$) and cyclin D1 expression in EC9706/DDP cells ($p = 2.35 \times 10^{-5}, 1.22 \times 10^{-5}, 8.75 \times 10^{-6}$) (Figure 4A and 4B), increased G0/G1 ratio, and decreased S phase percentage (Figure 4C). Cell proliferation activity under 0.75 μg/mL DDP was also statistically decreased after being treated with miR-217 mimic, β-catenin siRNA, and miR-217 mimic + β-catenin siRNA, respectively ($p = 0.0014, 0.0018, 0.0002$). Proliferation of cells was minimized when levels of miR-217 was increased and expression of β-catenin reduced (Figure 4D, 4E). Further assays showed different DDP sensitivities in all groups of transfected cells, as shown by significantly lower IC₅₀ in miR-217 mimic or β-catenin siRNA transfection groups and minimal IC₅₀ levels in co-transfection group (Table 2).

Discussion

EC is a common malignant tumor, ranked as eighth most popular and sixth deadliest cancer, worldwide [17]. Early diagnosis of EC is difficult. The current approach is mainly surgical resection, in combination with chemo- and radio-therapy. Although chemotherapy plans for EC are continuously changing, long-term usage of drugs often leads to resistance, an important factor causing post-op recurrence and metastasis compromising clinical efficiency. Identification of target molecules, therefore, is of importance in helping diagnosis, decreasing resistance, improving efficiency, and improving prognosis.

Canonical Wnt/β-catenin pathways are correlated with regulation of embryonic development, cell proliferation, differentiation, and migration [18]. Previous studies have shown that overexpression of β-catenin might be involved in EC pathogenesis [11, 12]. Abnormal activation of Wnt/β-catenin signal pathways has been shown to be related with acquirement of

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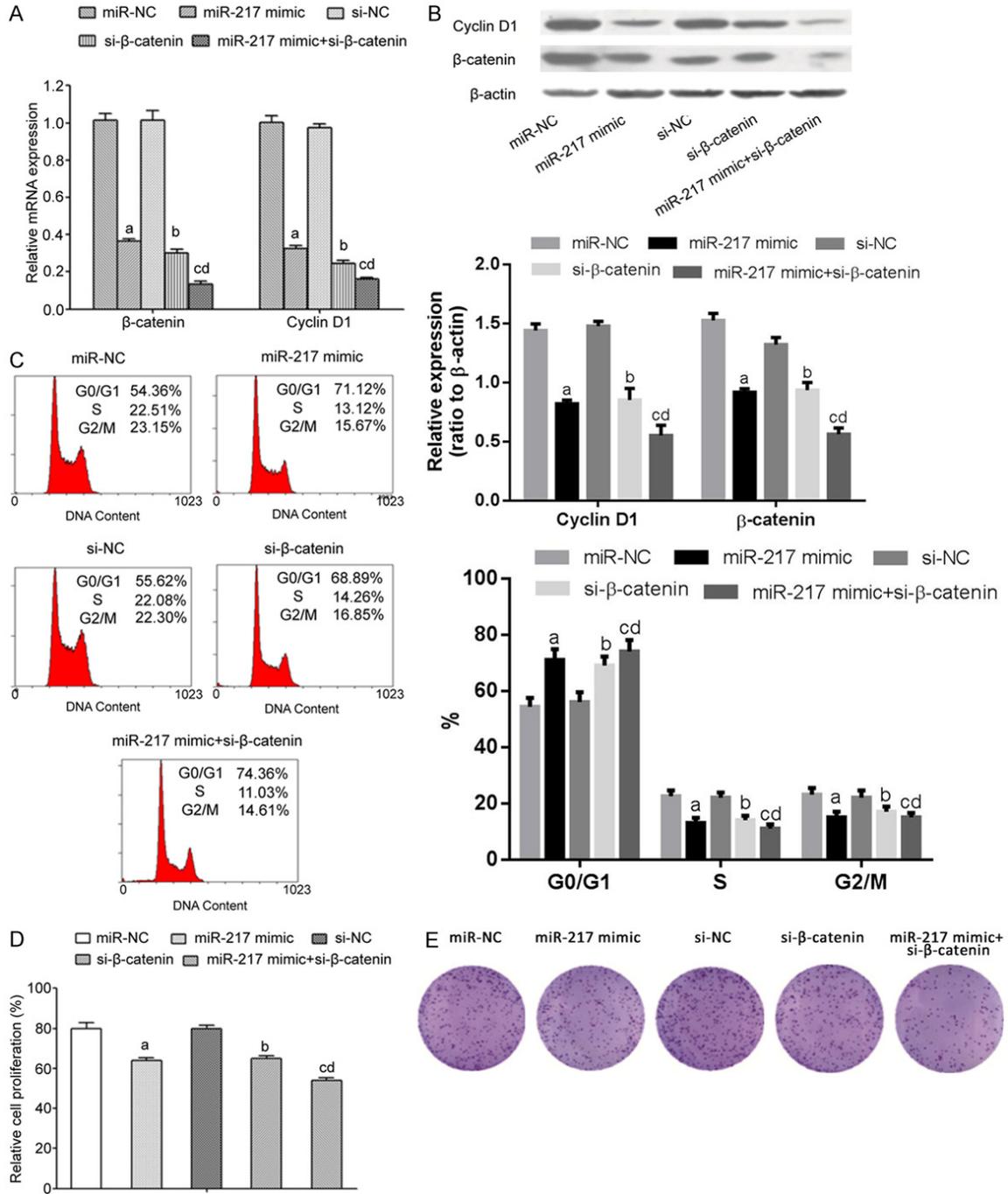


Figure 4. Enhanced DDP sensitivity in EC cells by miR-217 upregulation and β -catenin downregulation. A. qRT-PCR for gene expression; B. Western blot for protein expression; C. PI staining for cell cycle; D. CCK-8 for cell proliferation activity. E. Colony formation assay. a, $p < 0.05$ comparing between miR-217 mimic and miR-NC groups; b, $p < 0.05$ comparing between si- β -catenin and si-NC groups; c, $p < 0.05$ comparing between miR-217 mimic + si- β -catenin and miR-217 mimic groups; d, $p < 0.05$ comparing between miR-217 mimic + si- β -catenin and si- β -catenin groups.

chemotherapy drug resistance in various tumor cells such as liver cancer [13], osteosarcoma [2], and pulmonary cancer [14]. miR-217 downregulation may also be related with EC pathogenesis [16], in which miR-217 may work as a

tumor suppressor gene. Bioinformatics analysis showed complementary binding sites between miR-217 and 3'-UTR of β -catenin mRNA. This present study, thus, investigated whether miR-217 plays a role in regulating β -catenin

Table 2. IC₅₀ of all transfected cells

Group	IC ₅₀ (μg/mL)
miR-NC	3.29 ± 0.21
miR-217 mimic	1.85 ± 0.09 ^a
si-NC	3.45 ± 0.19
si-β-catenin	1.69 ± 0.07 ^b
miR-217 mimic + si-β-catenin	1.14 ± 0.05 ^{c,d}

Note: a, p<0.05 comparing between miR-217 mimic and miR-NC groups; b, p<0.05 comparing between si-β-catenin and si-NC groups; c, p<0.05 comparing between miR-217 mimic + si-β-catenin and miR-217 mimic groups; d, p<0.05 comparing between miR-217 mimic + si-β-catenin and si-β-catenin groups.

expression, mediating DPP sensitivity of EC cells, and EC pathogenesis.

This study's results showed significantly lower miR-217 expression in ECSS tumor tissues, with remarkably higher β-catenin expression, indicating that miR-217 downregulation might cause elevation of β-catenin expression, leading to facilitation of ESCC occurrence. Want et al. found lower miR-217 expression in ESCC tumor tissues [16], supporting the tumor suppressor role of miR-217 in EC onset. Xi et al. also showed lower miR-217 in EC tissues compared with normal esophageal tissues [19]. This present study found miR-217 downregulation in ESCC tumor tissues, consistent with Want et al. [16] and Xi et al. [19].

Cyclin D1 is coded by human CCND1 genes and plays important roles in cell cycle regulation. Activation of Wnt/β-catenin pathways can facilitate cyclin D1 gene transcription and expression, thus, playing a role in facilitating cell proliferation and tumor occurrence. In this study, cyclin D1 expression was significantly elevated in ESCC tumor tissues, probably due to abnormal expression of β-catenin. Wang et al. [20] showed remarkably higher cyclin D1 expression in ESCC tumor tissues, consistent with the present study. Deng et al. showed higher β-catenin positive rates in ESCC tumor tissues and its correlation with tumor size, cell differentiation grade, clinical stage, and lymph node metastasis [21]. Abnormal elevation of β-catenin can work as a predictive index for tumor progression and unfavorable prognosis. Xu et al. also demonstrated higher β-catenin expression levels in ESCC tumor tissues than in normal esophageal mucosa, with significant correlation between expression levels and pathology

grade as well as tumor infiltration depth [22]. This study found abnormally elevated β-catenin expression in ESCC tumor tissues, consistent with Deng et al. [21], Xu et al. [11], and Tang et al. [22].

Intermittent DDP rushing generated drug resistant cell lines, in which this study found significantly lower miR-217 expression than its parental cell line EC9706, with lower expression of β-catenin and cyclin D1. In addition, this study reveals that depressed miR-217 expression and elevated β-catenin and cyclin D1 expression might be correlated with suppressed DDP sensitivity in EC9706/DDP cells. Moreover, this study found significantly lower miR-217 expression in EC cells than in normal esophageal epithelial cells, consistent with Xi et al. [19], who showed remarkably lower miR-217 in EC cell lines than in immortalized esophageal epithelia (IEE) cells. Further analysis showed that transfection of miR-217 mimic and/or β-catenin siRNA remarkably decreased β-catenin and cyclin D1 expression in EC9706/DDP cells and decreased G0/G1 phase ratio, with increased S phase ratio. Analysis also revealed reduced proliferation potency of cells under certain DDP concentrations. These results suggest that elevation of miR-217 and downregulation of β-catenin decreases cyclin D1 expression, suppresses DDP resistance of cells, and suppresses proliferation potency.

In conclusion, microRNA-217 can induce G0/G1 phase arrest and inhibit proliferation ability of EC cells via targeted inhibition of β-catenin expression and subsequent decrease of downstream cyclin D1 expression, eventually enhancing DDP drug sensitivity.

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Disclosure of conflict of interest

None.

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