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Asian Pacific Journal of Tropical Medicine

journal homepage: http://ees.elsevier.com/apjtm

Original research http://dx.doi.org/10.1016/j.apjtm.2015.10.001

Inhibition effect of miR-577 on hepatocellular carcinoma cell growth via targeting β-catenin

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ARTICLE INFO

Article history:
Received 15 Aug 2015
Received in revised form 20 Sep 2015
Accepted 30 Sep 2015
Available online 9 Oct 2015

Keywords:
mircoRNA-577
Hepatocellular carcinoma
β-catenin
Proliferation
Apoptosis

ABSTRACT

Objective: To investigate the expression and the regulation effect of cell growth of microRNA-577 in hepatocellular carcinoma (HCC).

Methods: qRT-PCR was applied to detect the relative expression of miR-577 in 70 paired HCC and matched tumor adjacent tissues collecting from resection between March 2011 and March 2014. Pearson chi-square test was used to analyze the relationship between the miR-577 expression and clinical features. The miR-577 mimics were transfected into HepG2 cells; cell cycles were detected by flow cytometry, cell proliferation was measured by MTT assay and BrdU incorporation assay, and cell apoptosis was determined by flow cytometry and caspase3/7 activity analysis. The expressions of β-catenin were measured by immunohistochemistry. Spearman correlation analysis was used to analyze the relationship between miR-577 and β-catenin. qRT-PCR and western-blot were used to detect the expression of β-catenin in transfected HepG2 cells.

Results: The relative expressions of miR-577 was significantly lower in HCC tissues compared to the matched normal tumor-adjacent tissues ($P < 0.05$). Low expression of miR-577 was significantly associated with large tumor size ($\geq$5 cm, $P < 0.05$) and advanced tumor node metastasis stage (III+IV, $P < 0.05$). Transfection of miR-577 mimics could inhibit repress cell proliferation, enhance cell apoptosis and block the cell cycles in G_0/G_1 phase ($P < 0.05$). miR-577 in HCC group had a significant negative correlation relationship with the expression of downstream target of β-catenin ($P < 0.05$). Both the mRNA and protein expression in HepG2 cells were down-regulated after transfection ($P < 0.05$).

Conclusions: Low expression of miR-577 is related to the malignant clinicopathological features in HCC tissues, and miR-577 may suppress HCC growth through down-regulating β-catenin.

1. Introduction

Hepatocellular carcinoma (HCC), short for hepatic carcinoma, is a kind of digestive system cancer with a high malignant degree [1]. In recent years, the morbidity of HCC has been constantly rising. Because of its insidious symptoms, rapid development and difficulties in early diagnosis, most patients are in the tumor advanced stage when they seek medical treatment. Molecular targeted drugs are mainly used in advanced liver cancer including interventional embolization treatment and oral administration of Sorafenib. However, the reaction rate is low in the patients and the 5-year survival rate is less than 20% [2]. Therefore, HCC has become one of the important factors to endanger people's health.

microRNA is a kind of single non-coding RNA with the length of about 19–25 nucleotides. Through the specificity of complete or incomplete complementary combination with 3′-untranslated region of mRNA target, mRNA degradation can be performed, and the role of the translation process can be inhibited [3]. Studies have shown that the abnormal expressions of a variety of miRNA exist in HCC tissues. For example, the expressions of miR-21 increase in HCC tissue and are closely related with HCC clinical stages as well as poor prognosis; also

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Peer review under responsibility of Hainan Medical College.
Foundation Project: Supported by Heilongjiang Province Science Foundation for Youths (QC2009C110).

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in vivo experiment confirms that miR-21 can promote tumor antiapoptotic effect through down-regulating apoptosis related proteins PDCD4 and Fas-L [4,5]. miR-218 with a high biological activity can promote the proliferate ability of HCC cells through degrading CDK6 and BMI-1 [6]. miR-577 has regulating effect on the occurrence and development of many malignant tumors, which has down-regulated expression and even deficiency in a variety of human malignant tumors. At the same time miR-577 is closely related many biological actions including tumor proliferation and apoptosis [7]. Through bioinformatics search, it is found that β-catenin is one of the downstream potential targets for miR-577, which is the *CTNNB1* gene coding located in the chromosome 3p21. β-catenin is one of the key molecular in Wnt/β-catenin signaling pathway. Clinical trial results of II phrase show that imatinib mesylate can interfere Wnt/β-catenin signaling pathway and prevent tumor metastasis, which obtain better efficiency in the treatment of Philadelphia chromosome positive leukemia [8]. Other experimental targeted drugs that aimed at Wnt/β-catenin such as M475271 [9], AZD0530 [10], and Bosutinib [11] have also obtained certain results in decreasing tumor metastasis like prostatic cancer and breast cancer. Therefore, β-catenin has important research value in tumor biological treatment. Currently, clinical pathological significance and specific molecular mechanism of miR-577 in HCC is still unclear. The present study investigated the clinical significance and action mechanism of miR-577 in the occurrence and development of HCC through studying the expressions of miR-577 in HCC and matched tumor adjacent tissues, its biological function and its regulating effect of potential β-catenin target. The study can provide a new molecular target for the diagnosis and treatment of HCC.

2. Materials and methods

2.1. Clinical specimens

The present study was performed after the approval of the medical ethics committee of our study. A total of 70 cases of HCC as well as matched tumor adjacent tissues were collected from March 2011 to March 2014 that were performed surgical excision and confirmed with pathology. Among them, 49 were males and 21 were females aging 42–74 years [average age (55.7 ± 1.6) years]. All the specimens were *in vitro* materials stored in liquid nitrogen or neutral formalin solution.

2.2. qRT-PCR

microRNA and mRNA were extracted from the tissue or cell according to the method of the TRIZOL reagent instruction. cDNA was compounded through reverse transcription, and then 2 μL cDNA was taken for amplification detection by real-time PCR. *RNU6B* gene was regarded as the internal reference of microRNA detection, and *β-actin* gene was regarded as the internal reference of mRNA detection. $2^{-\Delta\Delta Ct}$ method was used to calculate the quantity of relative expression.

2.3. Cell culture

Immortalized human hepatocyte cell line LO2 and HCC including HepG2, Hep3B, SMMC-7721, Bel-7402 and Huh7 were stored in our laboratory. All the cells were cultured in suitable environment, and further experiment was performed after stable passage of 2–3 generations.

2.4. Cell transfection

HepG2 cells were cultured in 6-well plate to about 50% of fusion degrees and underwent intervention. Experimental group (per well): 100 pmoL miR-577 mimics & 5 μL Lipofectamine™ 2000; Control group (per well): 100 pmoL NC mimics & 5 μL Lipofectamine™ 2000. After incubation with serum-free medium for 6–8 h, the complete medium was changed for further culture.

2.5. MTT test

HepG2 cells transfected for 0, 24, 48, and 72 h respectively were collected and incubated in 96-well plate. After incubated to cell adherence in the incubator, MTT reagent was added and cultured for 4 h. The supernatant was discarded and DMSO was added to dissolve the crystals. ELIASA was used to detect the absorbance value at OD 490 nm.

2.6. BrdU analysis

The transfected miR-577 mimics of HepG2 were incubated in 96-well plate; then BrdU solution was added to incubate for 24 h and fixed by adding stationary liquid. Antigen-antibody method was used to detect the BrdU synthesis.

2.7. Cell cycle and cell apoptosis detection

HepG2 cells transfected for 72 h were taken to detect the cell cycle by PI simple staining and to detect cell apoptosis by Annexin V-FITC as well as PI simple staining.

2.8. Detection of HepG2 cell apoptosis by Caspase3/7 activity analysis

HepG2 cells transfected for 72 h were incubated in 96-well plate and three blank wells were set up. Caspase3/7 intracellular activity was detected by Apo-ONE® Caspase-3/7 Assay Kit, and Cell fluorescence intensity at 499 nm was measured by ELISA Tablet counter for quantitative assessment.

2.9. Immunohistochemistry

The tissue sample was produced routinely to 4 μm paraffin section. Rabbit anti-human β-catenin anti-body (1:100) was used to detect β-catenin protein expression of the issue. Each section was read and graded independently by the two high qualification pathologists in the case of double blind [12].

2.10. Western blot

Total protein of HepG2 cells transfected for 72 h was extracted and the concentration was detected. About 50 μg protein sample was added in each well and isolated with 10% SDS-PAGE gel electrophoresis. BIO-RAD Trans-Blot SD was used for transmembrane, primary antibody of β-catenin (1:1 000) and primary antibody of β-actin was used to detect the protein expression.

2.11. Statistical analysis

Data were analyzed by SPSS13.0 software. Pearson *Chi*-square test was used to analyze the enumeration data, and mean ± sd was used to express the measurement data. *t*-test or ANOVA was used for the comparison among groups. Pearson correlation test was used to analyze the expression correlations between miR-577 and β-catenin. $P < 0.05$ was considered as statistical significant difference.

3. Results

3.1. Relative expression of miR-577 in HCC and matched tumor adjacent tissues

Through the detection of qRT-PCR technique, the relative expression of miR-577 of HCC tissues in 70 cases was 2.402 ± 0.143 while that of matched tumor adjacent tissues was 5.868 ± 0.302. Among HCC tissues of 55 (84.29%) cases, the relative expression of miR-577 was lower than that of the matched tumor adjacent tissues. The statistical test showed that the difference was statistical significant ($P < 0.001$) (Figure 1).

3.2. Correlations between expression level of miR-577 and clinicopathologic feature of HCC patients

In order to investigate the correlations between expression level of miR-577 and clinicopathologic feature, 70 cases of HCC patients were classified with clinicopathologic features including age, sex, HBV infection or not, liver cirrhosis or not, serum AFP level, tumor volume, tumor number, blood vessel infiltration or not, histopathological grading, tumor node metastasis (TNM) periodization, *etc*. Through student's *t*-test, it was found that the expression of miR-577 in HCC tissues with larger volume (≥5 cm) and higher TNM periodization decreased significantly ($P < 0.01$) (Table 1).

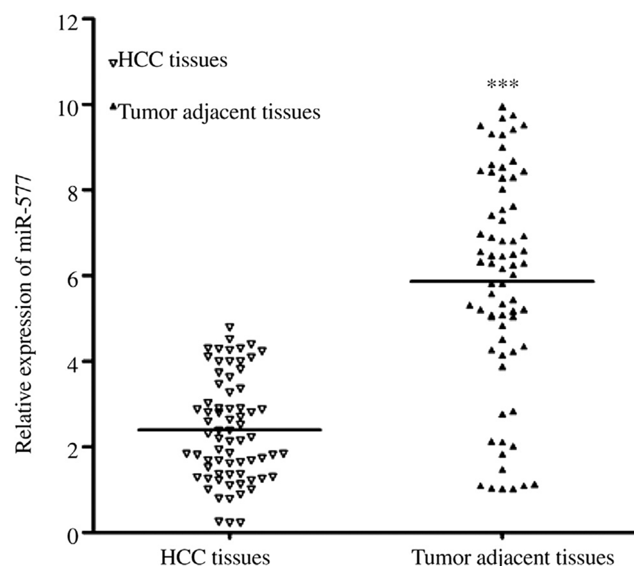

Figure 1. Expression of miR-577 in HCC and matched tumor adjacent tissues.

Table 1

Relationship between miR-577 expression and clinicopathologic feature of HCC patients (n = 70).

Clinicopathologic features	Classification	miR-577 expression	*P*
Age	>50 years of age	2.609 ± 0.101	0.098
	≤50 years of age	2.281 ± 0.134	
Sex	Male	2.434 ± 0.191	0.133
	Female	2.229 ± 0.107	
HBsAg	Positive	2.629 ± 0.131	0.714
	Negative	2.332 ± 0.135	
Liver cirrhosis	Yes	2.507 ± 0.132	0.081
	No	2.213 ± 0.084	
AFP	≥400 ng/mL	2.483 ± 0.011	0.873
	<400 ng/mL	2.403 ± 0.097	
Tumor volume	<5 cm	4.791 ± 0.141	0.003**
	≥5 cm	1.137 ± 0.119	
Tumor number	>1	2.284 ± 0.132	0.121
	1	2.461 ± 0.117	
Blood vessel infiltration	No	2.531 ± 0.112	0.076
	Yes	2.294 ± 0.170	
Edmondson grading	I+II	2.496 ± 0.092	0.595
	III+IV	2.151 ± 0.101	
TNM periodization	I+II	3.546 ± 0.111	0.006**
	III+IV	1.049 ± 0.136	

** $P < 0.01$.

3.3. Transfection of miR-577 mimics into HepG2

The results showed that expression of miR-577 could be detected in all the cell lines, among which LO2 expression was the highest, and that of HepG2 was the lowest ($P < 0.01$) (Figure 2A). Then, HepG2 was selected to be the intervention cell. miR-577 mimics were transfected into HepG2 transiently using liposomes method. The results showed that the transfected miR-577 mimics could increase the intracellular expression level of miR-577 of HepG2 significantly ($P < 0.001$) (Figure 2B).

3.4. Inhibition of overexpression of miR-577 on HepG2 cell proliferation

Figure 3A showed that compared with normal control group, the transfected miR-577 mimics could decrease the HepG2 cell viability significantly ($P < 0.01$). Then, BrdU was mixed into transfected cells, and through cellular immunofluorescence staining and ELIASA analysis detection, it was found that DNA synthesis ability of HepG2 was significantly reduced after the miR-577 mimics transfection ($P < 0.01$) (Figure 3B). The result indicated that cell proliferation was inhibited.

3.5. Effect of overexpression of miR-577 on the G_0/G_1 phase of HepG2

Figure 4 showed that compared with normal control group, percentage of HepG2 after miR-577 mimics transfection in G_0/G_1 phase was significantly increased ($P < 0.05$), while the cell percentage in S period was significantly decreased ($P < 0.05$), which indicated that miR-577 would block tumor cells at DNA presynthesis stage.

3.6. Effect of miR-577 on HepG2 cell apoptosis

The results confirmed that after transfection of miR-577 mimics for 72 h, apoptosis percentage of miR-577 group was

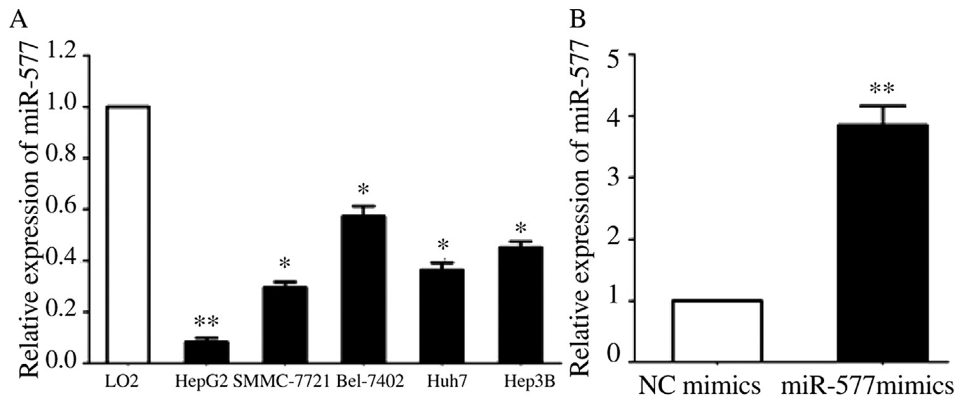

Figure 2. Transfection of miR-577 mimics into HepG2.
A: Expression of miR-577 in different cell lines ($^{**}P < 0.01$, $^{*}P < 0.05$); B: After miR-577 mimics transfected into HepG2, expression level of miR-577 was significantly increased ($^{**}P < 0.01$).

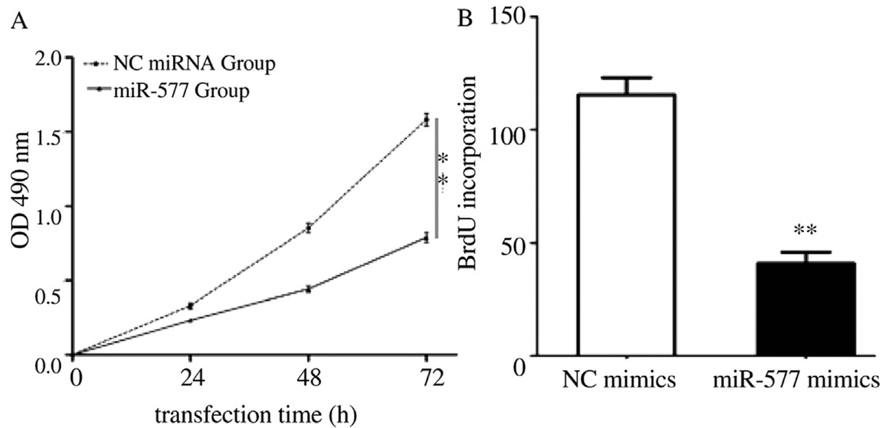

Figure 3. Effect of miR-577 on cell proliferation capacity of HepG2.
A: HepG2 cell viability ($^{**}P < 0.01$); B: DNA synthesis of HepG2 ($^{**}P < 0.01$).

significantly increased compared with normal control group ($P < 0.01$) (Figure 5A and B). Caspase3/7 activity analysis also showed that related enzyme activity of HepG2 cell apoptosis was statistically increased after miR-577 transfection ($P < 0.01$) (Figure 5C). The results showed that miR-577 *in vitro* could inhibit HepG2 cell proliferation and promote its apoptosis.

3.7. Correlation of protein expressions between miR-577 and β-catenin in HCC tissues

Immunohistochemical results showed that the protein expression intensity of β-catenin in miR-577 low expression group was significantly higher than that in miR-577 high expression group ($P < 0.05$) (Figure 6A). Pearson correlation analysis showed that there was a significantly negative correlation between miR-577 and β-catenin expression ($P < 0.001$) (Figure 6B).

3.8. Effect of overexpression of miR-577 on expression of β-catenin in HepG2

It was found that compared with control group, overexpression of miR-577 down-regulated the expression levels of *β-catenin* mRNA (Figure 7A) and protein (Figure 7B) significantly in HepG2 ($P < 0.01$).

4. Discussion

HCC is common in the world and is one of the digestive system malignant tumors with a high lethality rate. It has a morbidity of 622 000 people per year and a mortality rate of 600 000 people per year [13]. The appearance of targeted drug Sorafenib prolongs the survival time of HCC patients at advanced stage effectively [14] and is also proved that molecular targeting treatment plays an important role in improving the prognosis of HCC patients. Therefore, searching for new molecular targeted antitumor and exploring its antitumor mechanism have been one of the key methods to improve the therapeutic effect of patients as well as ameliorate the prognosis of HCC patients.

HCC tissues exist a large number of microRNA abnormal expressions and play an important effect in the occurrence and development of HCC. For example, expressions of miR-218 in HCC tissues and HCC cells reduced significantly and *in vitro* overexpression of miR-218 could inhibit proliferate ability of HCC cells and induce apoptosis significantly through down-regulating the expression CDK6 and BMI-1 [15]. While the expression of miR-27a in HCC tissues increased significantly compared with normal liver tissue and was closely related to patients with poor prognosis; and overexpression of miR-27a in HepG2 and Huh7 promoted the ability of cell proliferation,

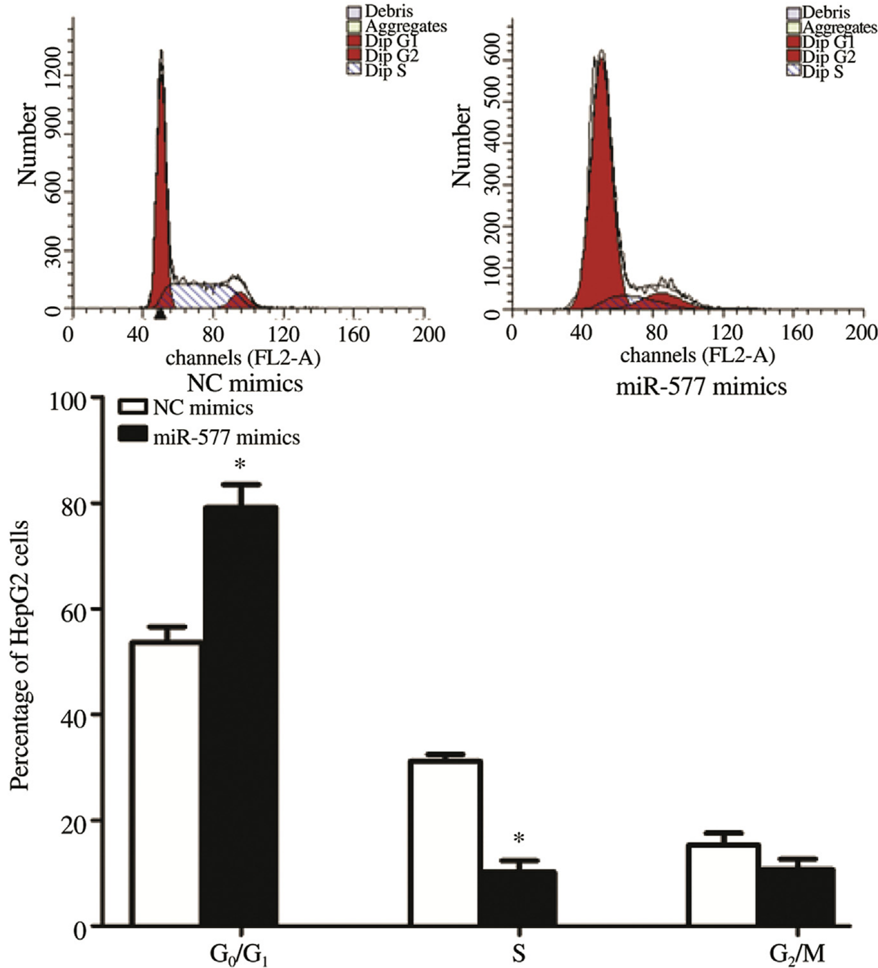

Figure 4. Effect of miR-577 on HepG2 cell cycle.
$^{*}P < 0.05$.

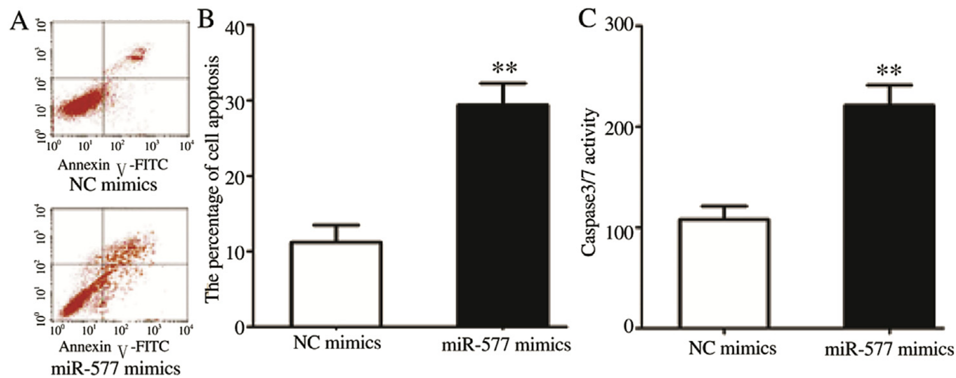

Figure 5. Effect of miR-577 on cell proliferation and apoptosis ability of HepG2.
A & B: Apoptosis percentage of HepG2 significantly ($^{**}P < 0.01$); C: Related enzyme activity of HepG2 cell apoptosis significantly ($^{**}P < 0.01$).

invasion and metastasis significantly [16]. The present study has confirmed that the expression level of miR-577 in HCC tissues was significantly lower than that in matched tumor adjacent tissues, and its low expression was closely related to malignant clinicopathologic features such as the enlargement of tumor volume and high TNM periodization. It is showed that miR-577 has important regulating effect in HCC progress.

As an important suppressor molecule, miR-577 can play a role of antitumor through down-regulating the expression levels of multiple oncogenes in tumor. Zhang *et al* found that miR-577 can down-regulate the expression of lipoprotein receptor related protein 6, which is the key molecule of Wnt signaling pathway to inhibit the growth of malignant brain glioma [17]. The study of Yuan *et al* also showed that miR-577 down-regulates TSGA10 to achieve the goals of proliferation inhibition, apoptosis induction and cell cycle arrest of esophageal squamous cells [18]. In the present study, miR-577 mimics were used for the overexpression of miR-577 in HepG2, and it has been proved by MTT and BrdU experiment that miR-577 can inhibit cell proliferation through restraining HepG2 cell viability and DNA synthesis. Cell cycle analysis showed that after the overexpression of miR-577, cell counts in DNA replicative phase (S

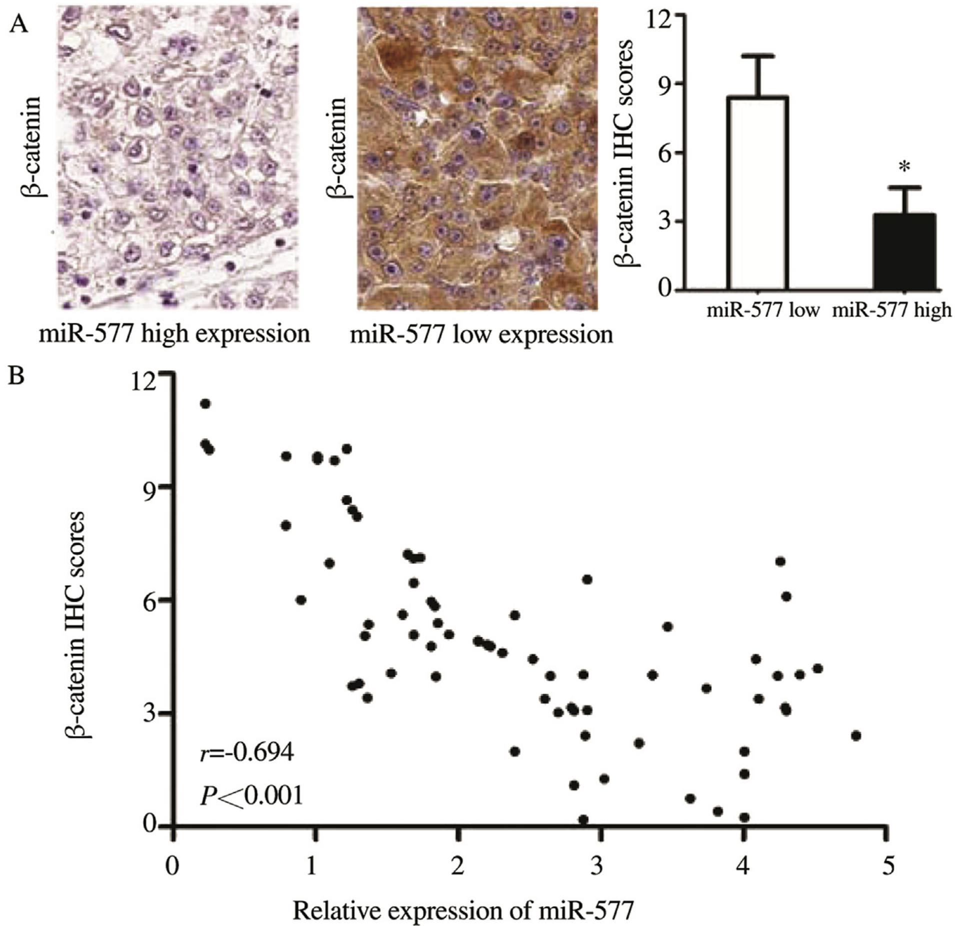

Figure 6. Expression of β-catenin protein in HCC tissues and the correlation with miR-577.
A: Expressions of β-catenin protein in different miR-577 expression levels of HCC tissues ($^{*}P < 0.05$); B: Correlation between miR-577 and β-catenin expression.

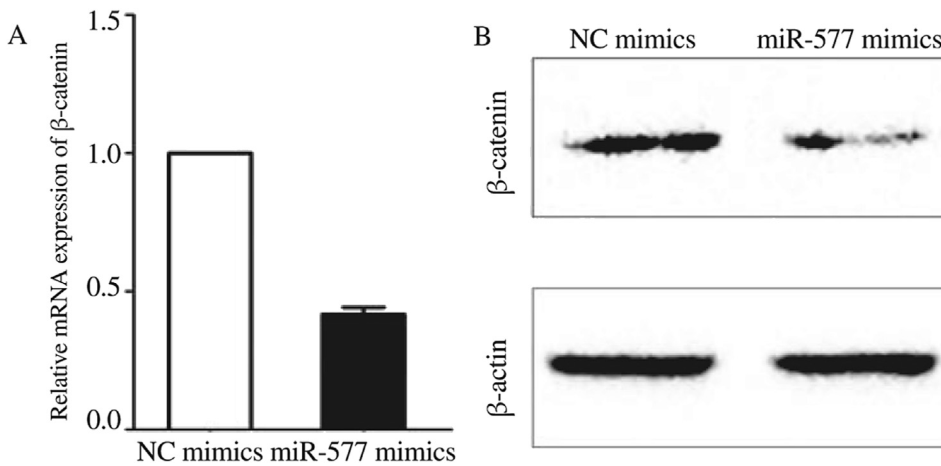

Figure 7. Overexpression of miR-577 down-regulating the expression of β-catenin in HepG2.
A: Expression of *β-catenin* mRNA of HepG2 ($^{**}P < 0.01$); B: Expression of β-catenin protein of HepG2.

period) decreased significantly, and most cells were blocked in prophase of DNA synthesis namely G_0/G_1 phrase. It was found in further study that the expression of miR-577 increased the related enzyme activities of cell apoptosis significantly. The result of flow cytometry analysis showed that the percentage of HepG2 apoptosis increased significantly. The above results showed consistently that miR-577 can inhibit cell proliferation of HepG2 significantly and promote the apoptosis.

Wnt signaling pathway plays a key role in the occurrence and development of HCC. Wnt signaling pathway activation leads to the accumulation of downstream molecule β-catenin in cytoplasm and migration to cell nucleus [19]. It complexes with LEF/TCF to form transcription regulation and promotes the transcription of cyclinD1, c-myc and MMP7 that related to multiple downstream tumor proliferation as well as invasion, and eventually led to tumor growth and metastasis [20].

It was found in Bioinformatics retrieval that β-catenin is one of the downstream potential biological targets of miR-577. To study the regulating effect of β-catenin in miR-577 of HCC, we firstly detected the protein expression of downstream in the potential target of β-catenin at histological level. The results showed that there was a negative correlation between miR-577 and β-catenin protein expression. We then overexpressed miR-577 in HepG2 successfully by *in vitro* transfection method. Through qRT-PCR and Western-Blot detection, it was found that after overexpression of miR-577, expression levels of *β-catenin* mRNA and protein in HepG2 down-regulated significantly. Molecular targeted drug used in clinical practice, which is mainly based on Sorafenib, plays an important role in the treatment of HCC. Recent researches have confirmed that the overexpression and intranuclear metastasis of β-catenin in HCC cells is one of the important reasons to promote HCC cell proliferation and Sorafenib treatment resistance [21]. Therefore, the negative regulation of miR-577 on β-catenin expression, which is confirmed by the present study, may have the important clinical value to ameliorate the treatment and prognosis of Sorafenib resistant patients.

In conclusion, expression of miR-577 in HCC issues down-regulated significantly, and low expression of miR-577 was closely related to malignant clinicopathological features of HCC. miR-577 may inhibit the HCC cell growth through down-regulating the expression of β-catenin. Hence, miR-577 has a certain development potential in the diagnosis and biological target therapy of HCC.

Conflict of interest statement

We declare that we have no conflict of interest.

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