miR-1297 promotes cell proliferation by inhibiting RB1 in liver cancer

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Abstract. Liver cancer is the one of the most common causes of cancer-associated mortality worldwide. MicroRNAs (miRNAs or miRs) are important in various types of cancer, including liver cancer. In the present study, with miRNA expression profile data obtained from the Gene Expression Omnibus database, three independent methods were used to investigate the miRNAs that are involved in liver carcinogenesis, including Fisher's exact test, *t*-test and Wilcoxon test. Five differentially expressed miRNAs were identified. Among them, miR-1297 drew specific attention. Target gene analysis and Ingenuity Pathway Analysis revealed its potential impact on cell death and cell cycle. Cell Counting Kit-8 proliferation assay indicated that the HepG2 cell proliferation was promoted by miR-1297, while miR-1297 inhibitor could significantly inhibit the proliferation of HepG2 cells. Luciferase assays confirmed that miR-1297 directly bound to the 3'-untranslated region of retinoblastoma (RB)1, and western blotting demonstrated that miR-1297 suppressed the expression of RB1 at the protein level. RB1 is involved in the regulation of the human cell cycle pathway. It is possible that miR-1297 contributes to the carcinogenesis of liver cancer via downregulation of the tumor-suppressor gene *RB1*. Our results suggest that miR-1297 may serve as a potential therapeutic target of liver cancer.

Introduction

Liver cancer is one of the most common causes of cancer-associated mortality worldwide (1). Its five-year survival rate is significantly lower than that of other types of cancer (2), mainly because the majority of liver cancer patients are at late stage when they are diagnosed (3). Therefore, it is necessary to identify new biomarkers for early diagnosis of liver cancer (4).

MicroRNAs (miRNAs or miRs) are short non-coding RNAs with an average length of 20-25 nt (5). By binding to the 3' untranslated region (UTR) of their target messenger (m)RNA molecules, miRNAs inhibit the translation of their target mRNA molecules (6). However, in limited cases, the binding sites are located in the 5' UTR or in the coding region of the target mRNA (6). miRNAs are important in regulating various biological processes, including cell development, cell proliferation, cell differentiation and cell apoptosis. Several previous studies have reported that miRNAs participate in the initiation and carcinogenesis of cancer (7,8). The carcinogenesis of liver cancer and its associated miRNAs have become a research hotspot area (9). The results of previous studies have revealed that miRNAs could be candidate prognostic and diagnostic biomarkers for liver cancer (9).

The present study, with miRNA expression microarray data obtained from the Gene Expression Omnibus (GEO) database, aimed to identify the key miRNAs involved in the carcinogenesis of liver cancer. In order to obtain confident results, differentially expressed miRNAs in liver cancer were identified by combining the results of three independent methods: Fisher's exact test, *t*-test and Wilcoxon test. Target genes of the selected miRNAs were predicted also by three independent methods: DIANA (10), miRanda (11) and TargetScan (12). Ingenuity Pathway Analysis (IPA) was conducted using the targets of the identified miRNAs to explore the underlying mechanisms of carcinogenesis of liver cancer.

Materials and methods

Microarray data of miRNA. From the GEO database (https://www.ncbi.nlm.nih.gov/geo/), the microarray data of miRNA GSE6857 and GSE30297, which represent miRNA expression profile data from 496 liver cancer patients and 35 normal controls, respectively, were obtained. The data sets were based on the platforms GPL4700 and GPL8786, respectively. All raw data, including the original CEL, GPR and SOFT files, were obtained for further analysis.

Detection of differentially expressed miRNAs in liver cancer. Normalization of the raw miRNA data was performed in

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R platform (version 3.1.3; https://www.r-project.org/) using the Robust Multi-array Analysis (RMA) method (13). The final log_2-transformed RMA expression values were then stored for further analysis. Three independent tests, including Fisher's exact test, *t*-test and Wilcoxon test, were used to identify significantly differentially expressed miRNAs between liver cancer and normal control samples. The miRNAs that were supported by the above three tests were considered to be confidently involved in the carcinogenesis of liver cancer.

Prediction of miRNAs target genes. Prediction of target genes for the differentially expressed miRNAs was performed using current available methods, including DIANA (10), miRanda (11) and TargetScan (12). In order to reduce the false-positive rate of target prediction, the predicted targets supported by at least two independent methods were selected as reliable target genes of miRNAs. In addition, miRNAs target genes with wet experimental support in the TarBase 6.0 database (14) were also included in the final pathway analysis.

IPA. The most significantly differentially expressed miRNAs identified in the previous steps were selected for IPA. The Ingenuity Knowledge database (http://www.ingenuity.com/products/ipa) and IPA tools were used to identify the enriched roles of miRNAs and their target genes in cellular functions, pathways and diseases.

Cell culture and transfection. The human liver cancer cell line HepG2 was obtained from the Chinese Center for Type Culture Collection (Beijing, China). The HepG2 cell line was first cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.). Cells were maintained in a humidified atmosphere with 5% CO_2 at 37˚C. HepG2 cells were seeded in 24-well plates at $6x10^5$ cells/well and incubated overnight. Transfection of the miR-1297 mimics, anti-miR-1297, inactive control cel-mir-67 or pMIR-REPORT vector (all Thermo Fisher Scientific, Inc.) was performed using Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) with 300 nmol miRNA or 1 μg/ml DNA plasmid, respectively. Total proteins of HepG2 cells were isolated at 48 h post-transfection using M-PER Reagent (Thermo Fisher Scientific, Inc.).

Western blotting. Proteins were separated by 12% SDS-PAGE and then transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Membranes were blocked with 5% non-fat milk and incubated with anti-retinoblastoma (RB)1 antibody (cat. no. ab181616; Abcam, Shanghai, China) or anti-β-actin antibody (cat. no. ab8227; Abcam) at 4˚C overnight. Following extensive washes with TBST, a secondary antibody (cat. no. ab150077; Abcam) was added to the system. Finally, enhanced chemiluminescence (Abcam) was used to detect the immunoreactive protein bands.

Cell proliferation assay. Cell proliferation assay was performed with Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). HepG2 liver cancer cells were plated at $6x10^5$ cells/well in 24-well plates. Then, cells were incubated in 10% CCK-8 at 37˚C for color conversion. Proliferation rates were detected at 24, 48 and 72 h post-transfection.

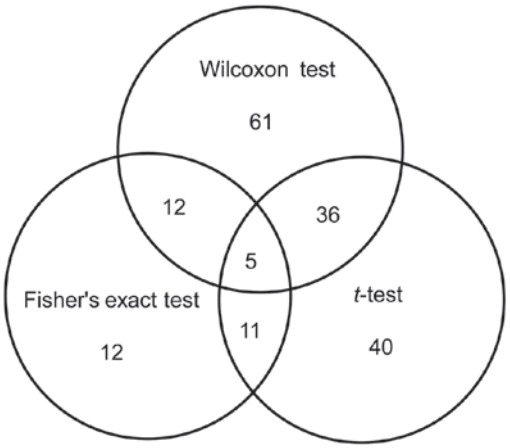

Figure 1. Venn diagram of the differentially expressed microRNAs identified by three independent tests. The overlap of the results from the three tests was selected for further analysis.

Luciferase assay. HepG2 cells were seeded at $6x10^5$ cells/well in 24-well plates and incubated for 24 h. Subsequently, the cells were co-transfected with 0.8 μg pGL3-RB1-3'UTR or pGL3-RB1-3'UTR Mut plasmid or with 0.08 ng phRL-SV40 control vector (all Promega Corporation, Madison, WI, USA), and with 100 nM miR-1297 or inactive control RNA, using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The *Renilla* luciferase and firefly luciferase activities were detected using a dual luciferase assay (Promega Corporation) at 24 h post-transfection.

Statistical analysis. Statistical analyses were performed using R (version 3.1.3; https://www.r-project.org/). Values were expressed as means ± standard deviation. Differences between groups were estimated with the Student's *t*-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Differentially expressed miRNAs in liver cancer. Based on the combined results of the three independent tests, five miRNAs were identified to be significantly differentially expressed in liver cancer (Fig. 1 and Table I), including three upregulated and two downregulated miRNAs. Among these miRNAs, miR-1297 had the most significant deregulation.

Target genes prediction. Since miRNAs serve their functions by targeting mRNAs, the predicted target genes of the differentially expressed miRNAs were retrieved. The most significant deregulated miRNA, miR-1297, attracted our attention because one of its target genes supported by multiple evidences is the tumor-suppressor gene *RB1* (Fig. 2), which is involved in the regulation of the cell cycle and in human

Table I. Differentially expressed miRNAs identified from three tests.

miRNA	*t*-test	Wilcoxon test	Fisher's exact test	Log (fold-change)
hsa-miR-1297	$1.30x10^{-4}$	$4.49x10^{-3}$	$2.21x10^{-3}$	1.69
hsa-miR-18a*	$7.15x10^{-4}$	$1.23x10^{-3}$	$1.46x10^{-2}$	1.40
hsa-miR-183	$1.43x10^{-3}$	$1.70x10^{-3}$	$2.48x10^{-2}$	1.28
hsa-let-7e	$1.32x10^{-2}$	$1.54x10^{-2}$	$3.73x10^{-2}$	-1.17
hsa-miR-126	$4.78x10^{-2}$	$2.75x10^{-2}$	$4.28x10^{-2}$	-1.20

miRNA/miR, microRNA; hsa, *Homo sapiens*.

Table II. Top networks associated with microRNA-1297 target genes.

Identity	Associated network functions	Score
1	Cell death and survival, behavior, nervous system development and function	43
2	Carbohydrate metabolism, small molecule biochemistry, skeletal and muscular disorders	32
3	Cell cycle, cell morphology, cellular function and maintenance	30
4	Cancer, gastrointestinal disease, auditory disease	27
5	Cell cycle, cell death and survival, tumor morphology	24

Table III. Diseases and functions associated with microRNA-1297 target genes.

A, Diseases and disorders

Name	P-value, range	Molecules, n
Neurological disease	$3.66x10^{-4}$-$3.62x10^{-2}$	18
Organismal injury and abnormalities	$3.66x10^{-4}$-$4.79x10^{-2}$	13
Cancer	$6.07x10^{-4}$-$4.88x10^{-2}$	74
Gastrointestinal disease	$6.07x10^{-4}$-$2.93x10^{-2}$	44
Respiratory disease	$5.38x10^{-3}$-$3.34x10^{-2}$	11

B, Molecular and cellular functions

Name	P-value, range	Molecules, n
Cell cycle	$4.70x10^{-5}$-$4.79x10^{-2}$	14
Cellular development	$1.37x10^{-3}$-$4.79x10^{-2}$	17
RNA post-transcriptional modification	$4.21x10^{-3}$-$3.62x10^{-2}$	5
Molecular transport	$5.91x10^{-3}$-$4.46x10^{-2}$	8
Carbohydrate metabolism	$6.12x10^{-3}$-$2.43x10^{-2}$	1

cancer pathways (hsa04110 and hsa05200 Kyoto Encyclopedia of Genes and Genomes pathways) (15-17).

IPA. The predicted target genes of miR-1297 were collected and imported into the IPA system to investigate their biological functions in liver cancer. Table II contains the top five most significant networks identified by IPA. Among these networks, cell death and survival was the most frequent function, with a significant score of 43 (Table II). IPA also indicated that miR-1297 target genes were involved in various biological functions, including cell cycle and cellular development (Table III). Cell death and survival as well as glutamate receptor signaling were the most significant pathways enriched in target genes of miR-1297 (Fig. 3).

miR-1297 promotes liver cancer cell proliferation. The potential impact of miR-1297 on liver cancer cell proliferation was assessed in the HepG2 cell line. HepG2 cells were transfected with miR-1297 mimics or miR-1297 inhibitor, or with the inactive control cel-mir-67. CCK-8 proliferation assay

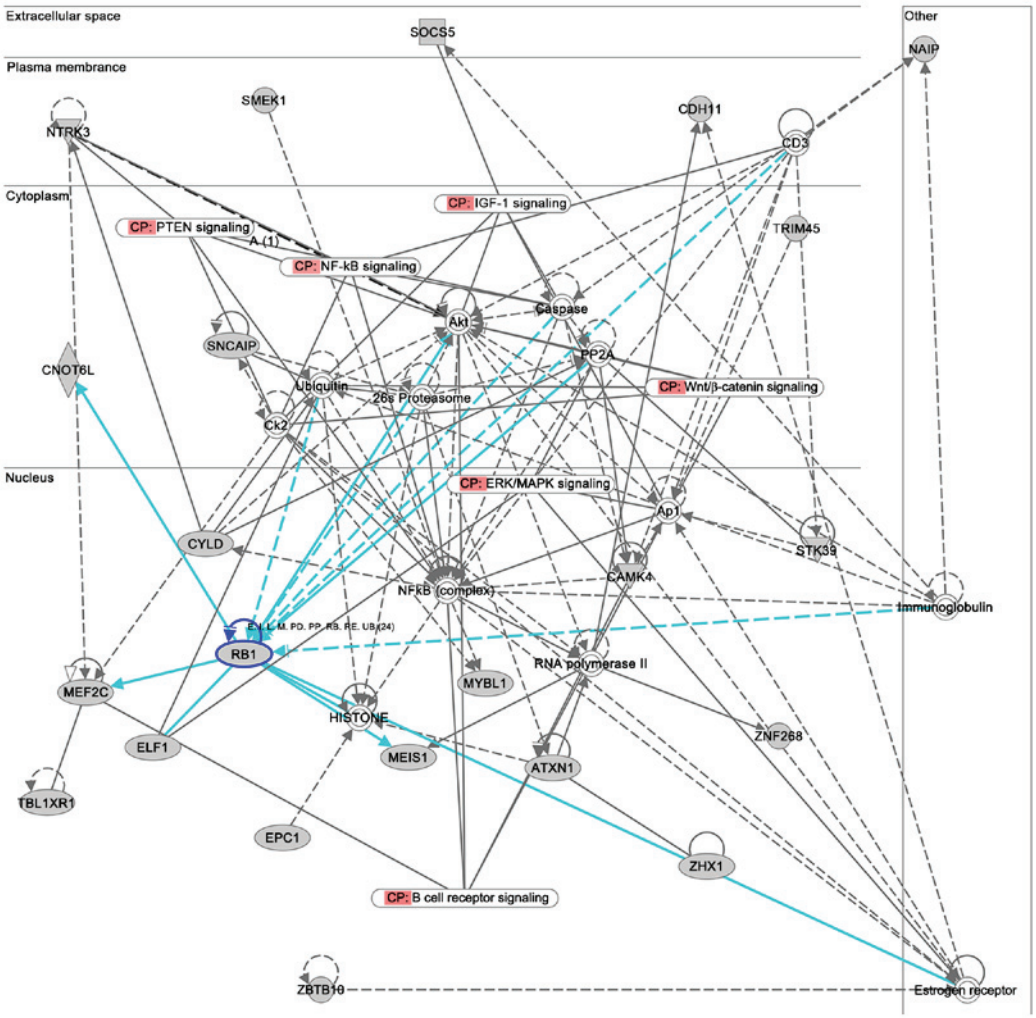

Figure 2. The most highly-rated network of microRNA-1297 target genes in Ingenuity Pathway Analysis. A solid line indicates a direct interaction between the two proteins, while a dotted line reveals an indirect interaction.

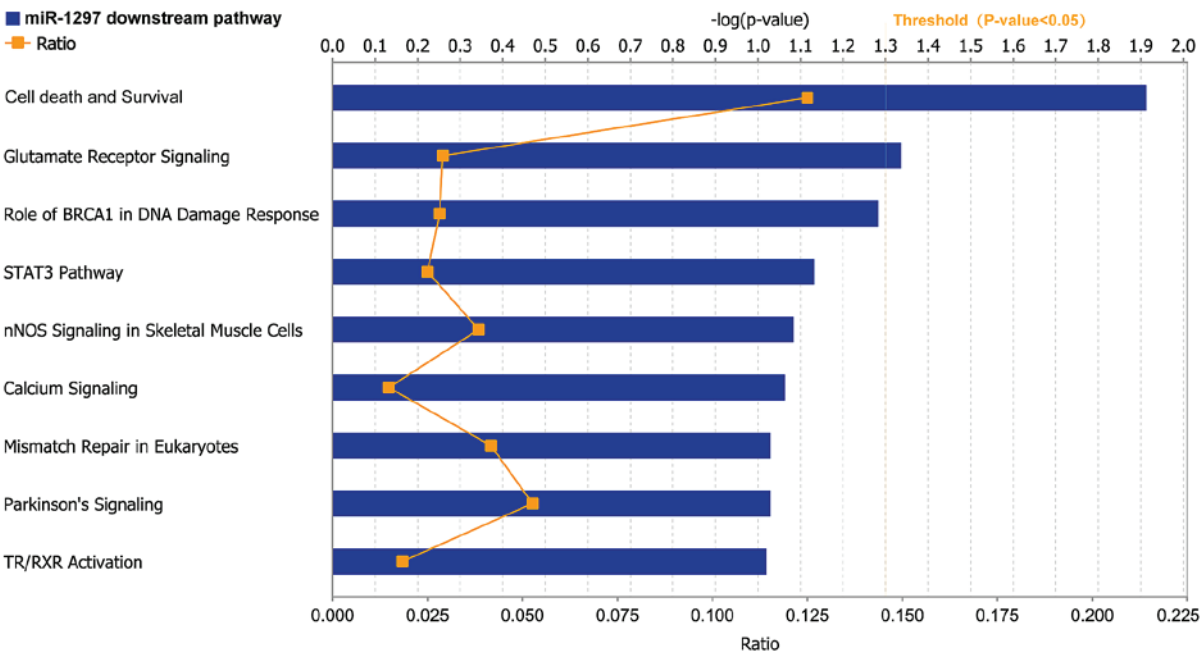

Figure 3. Enriched pathways of miR-1297 target genes. The y-axis reveals the top-rated pathways, as calculated by Ingenuity Pathway Analysis, while the x-axis indicates the ratio between the number of target genes that map to the pathway and the number of all known genes in the pathway. miR, microRNA; BRCA, breast cancer; STAT3, signal transducer and activator of transcription; nNOS, neuronal nitric oxide synthase; TR, thyroid hormone receptor; RXR, retinoid X receptor.

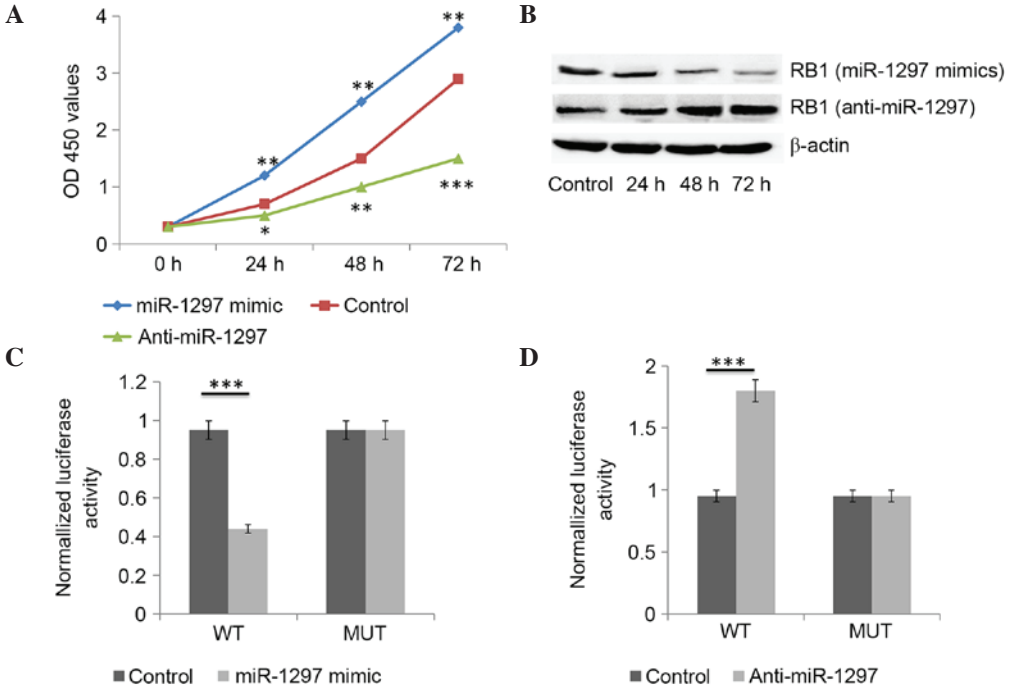

Figure 4. miR-1297 targets RB1 and promotes cell proliferation in liver cancer cells. (A) miR-1297 regulates cell proliferation in the HepG2 cell line. (B) miR-1297 negatively regulates RB1 expression in HepG2 cells. (C) Analysis of the relative luciferase activities of RB1-WT and RB1-MUT in HepG2 cells upon transfection with miR-1297 mimics. (D) Analysis of the relative luciferase activities of RB1-WT and RB1-MUT in HepG2 cells following transfection with miR-1297 inhibitors. *P<0.05; **P<0.01; ***P<0.001. OD, optical density; miR, microRNA; RB, retinoblastoma; WT, wild type; MUT, mutant.

indicated that cell proliferation was significantly promoted in miR-1297-mimics-transfected HepG2 cells compared with that in inactive control cel-mir-67-transfected cells (Fig. 4A). Conversely, miR-1297 inhibitor significantly inhibited the proliferation of HepG2 cells (Fig. 4A).

miR-1297 targets and negatively regulates RB1 in liver cancer cells. miR-1297 mimics significantly reduced the protein levels of RB1 in liver cancer cells (Fig. 4B). Conversely, miR-1297 inhibitor significantly increased the protein levels of RB1 in liver cancer cells (Fig. 4B). As predicted by bioinformatics analysis, there was complementarity between hsa-miR-1297 and the 3'UTR of RB1. The effect of miR-1297 on the translation of RB1 mRNA into protein was then determined using a luciferase reporter assay. miR-1297 mimics significantly reduced the luciferase activity of the reporter gene with the wild-type construct but not with the mutant RB1 3'UTR construct (Fig. 4C). The inhibitor of miR-1297 significantly enhanced the luciferase activity of the reporter gene with the wild-type construct but not with the mutant RB1 3'UTR construct (Fig. 4D). These evidences indicate that miR-1297 directly binds to the 3'UTR region of RB1. In general, miR-1297 targets and negatively regulates RB1 in liver cancer cells.

Discussion

In the present study, using three independent tests (Fisher's exact test, *t*-test and Wilcoxon test), five differentially expressed miRNAs were identified, which may play crucial roles in the carcinogenesis of liver cancer (Table I). Upon retrieving and analyzing the target genes of these five miRNAs, the most significantly deregulated miRNA, miR-1297, attracted our attention. One of its target genes with various supporting evidences is the tumor-suppressor gene *RB1*. The *RB1* gene encodes a negative regulator of the cell cycle, and was known to be a tumor suppressor of multiple types of cancer (15-17), including liver cancer (18). In addition, RB1 is involved in the human cancer pathway (http://www.kegg.jp/kegg/pathway.html, hsa04110 and hsa05200). This leads to the hypothesis that miR-1297 may be important in liver cancer.

Therefore, IPA was conducted to analyze the biological function of the target genes of miR-1297. IPA is based on the Ingenuity Knowledge Base, which derives known biological functions and interactions of genes from published studies. IPA allows the identification of biological networks, functions and pathways that are associated with the target genes of miR-1297. The results indicated that cell death and survival was the highest-rated miR-1297 downstream biological network, with a significance score of 43. The cell cycle was the most enriched cellular function of miR-1297 target genes, as shown in Table II. These results revealed that miR-1297 may participate in cancer through regulating cell death or the cell cycle. Generally, miR-1297 may be important in the carcinogenesis of liver cancer through regulation of its target genes, particularly the tumor-suppressor gene *RB1*. To date, no other studies have reported an association between miR-1297 and liver cancer. However, miR-1297 has been reported to regulate the carcinogenesis of colorectal cancer (19), lung adenocarcinoma (20) and laryngeal squamous cell carcinoma (21). In addition, its predicted target gene, *RB1*, is a negative regulator of the cell cycle and a tumor-suppressor gene (22). Therefore, the roles of miR-1297 in liver cancer were validated by wet experiments.

CCK-8 proliferation assay indicated that cell proliferation was promoted by miR-1297 in the HepG2 cell line, while miR-1297 inhibitor could significantly inhibit the proliferation of this cell line. Western blotting revealed that miR-1297 suppressed the expression of RB1 at the protein level in HepG2 cells. Furthermore, luciferase assays confirmed that miR-1297 directly bound to the 3'UTR of RB1 and suppressed its expression. In conclusion, these results indicated that miR-1297 promotes cell proliferation in liver cancer by negatively regulating the cell cycle-inhibitory gene *RB1*. Therefore, miR-1297 may be a potential therapeutic target for liver cancer in the future.

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