lncRNA MT1JP-overexpression abolishes the silencing of PTEN by miR-32 in hepatocellular carcinoma

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Abstract. Previous studies have shown that long non-coding RNA (lncRNA) MT1JP plays a role as a tumor suppressor in several types of cancer. The present study aimed to explore the role of MT1JP in hepatocellular carcinoma (HCC). Paired HCC and non-tumor tissues from 64 patients with HCC were subjected to RNA isolation and reverse transcription-quantitative PCR (RT-qPCR) to analyze the differential expression of MT1JP, microRNA (miR)-32 and phosphatase and tensin homolog (PTEN) in HCC. Cell transfections, followed by RT-qPCR and western blotting, were carried out to investigate the interactions among MT1JP, miR-32 and PTEN. The role of MT1JP, miR-32 and PTEN in regulating HCC cell proliferation was assessed using a Cell Counting Kit-8 assay. It was found that MT1JP was downregulated in HCC cancer tissues compared with that in non-cancer tissues. Survival analysis showed that patients with low MT1JP expression levels exhibited a significantly higher 5-year overall survival rate compared with patients with high MT1JP levels. The expression of MT1JP in HCC tissues was positively associated with PTEN and negatively associated with miR-32. Overexpression of MT1JP increased the expression levels of PTEN and decreased the expression levels of miR-32. Overexpression of miR-32 did not affect the expression of MT1JP but decreased the expression levels of PTEN and attenuated the effect of overexpression of MT1JP on the expression of PTEN. Overexpression of MT1JP and PTEN decreased the proliferation of HCC cells. Overexpression of miR-32 played an opposite role and attenuated the effects of overexpression of MT1JP. Therefore, MT1JP may upregulate PTEN by downregulating miR-32 to regulate HCC cell proliferation.

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Introduction

Hepatocellular carcinoma (HCC) is the most common subtype of liver cancer with a steady increase in incidence and remains the fifth most common cancer worldwide (1). Every year, >780,000 novel HCC cases are reported globally, causing ~745,000 mortalities (2,3). Patients at different stages of HCC have tried different treatment strategies, but the median overall survival of patients is only 6-20 months (3). Therefore, it is urgent to find an effective treatment.

Long non-coding RNAs (lncRNAs, >200 nucleotides in length) are RNA transcripts without protein-coding capacity but with the ability to regulate gene expression and participate in human diseases, including osteoarthritis (4), non-alcoholic fatty liver disease (5) and rheumatoid arthritis (6). lncRNAs play an important role in a variety of physiological and pathological processes such as cell proliferation and apoptosis, cell proliferation and senescence, immune activation and inactivation (7,8). Multiple lncRNAs have been confirmed by previous studies to play a vital role in HCC, such as TMPO-AS1 (9), MYLK-AS1 (10) and DLX6-AS1 (11). However, there are relatively few reports on the role of lncRNA MT1JP in HCC, and its ability to predict the prognosis of this disease is still limited.

MicroRNA (miRNA/miR) is a type of endogenous non-coding single-stranded small RNA, which is widely distributed in eukaryotes. It is composed of 19-25 nucleotides (12). A growing body of research over the past decades has demonstrated that miRNAs can act as tumor suppressors or promoters during the development of HCC (13,14). miR-32 is abnormally expressed in gastric cancer (15), glioma (16) and cervical cancer(17). However, the function and mechanism of miR-32 in HCC needs further exploration.

PTEN is a tumor suppressor gene with critical roles in diverse biological processes, such as cell cycle regulation and apoptosis (18). In cancer biology, PTEN mainly inhibits the PI3K/Akt signaling pathway, which is the main cell survival pathway, to induce cell death and suppress tumor growth (19).

It is known that PTEN in HCC can be targeted by oncogenic miRNAs, such as miR-32 (20). The current study investigated the expression levels of MT1JP and its prognostic value for patients with HCC, and explored whether the PTEN/miR-32 axis was involved in HCC progression via interaction with lncRNA MT1JP. The present study may identify a novel therapeutic target for HCC.

Materials and methods

Study patients. This study included 64 patients with HCC (36 males, 28 females; age range, 33-67 years old; mean age, 51.8±6.0 years old) selected from the 136 patients with HCC admitted to the Union Hospital Affiliated to Tongji Medical College of Huazhong University of Science and Technology (Wuhan, China) between April 2010 and April 2014. All patients were treated with surgical resection of the primary tumors and/or targeted therapies, radiotherapies and chemotherapies according to their conditions. The study was approved by the review board of the Ethics Committee of the aforementioned hospital. Inclusion criteria included: i) patients had not received radiofrequency ablation preoperatively; ii) newly diagnosed HCC case and iii) completed treatment and a 5-year follow-up in Union Hospital Affiliated to Tongji Medical College of Huazhong University of Science and Technology. Exclusion criteria included: i) Recurrent patients with HCC, ii) previous history of cancer, iii) family history of cancer and iv) other clinical disorders. All 64 patients with HCC provided written informed consent.

Tissue samples, treatment and follow-up. Before initiation of therapies, liver biopsy was performed under the guidance of MRI to collect both HCC and non-tumor (within 3 cm of the tumor) tissues. All tissues were snap-frozen and then transferred into a -80˚C freezer before use. HCC tissue samples were sectioned at 5-μm. The clinicopathological features of the patient specimens and the histological diagnosis of all HCC and normal tissues were independently reviewed and approved by two experienced pathologists. Pathologists performed a blinded assessment of the specimens. The TNM stage was evaluated based on the American Cancer Joint Commission Cancer Staging Manual (21). From the day of admission, all patients were followed-up for 5 years in a monthly manner through telephone to record their survival. The overall survival was defined as the day of diagnosis to the day of death or last follow-up. Patients who died of other causes or were lost during the follow-up were excluded. The 5-year overall survival rate was calculated as the proportion of the survivors among all patients with HCC at 5 years after hepatectomy.

HCC cells and transfections. Human HCC cell line SNU-398 (American Type Culture Collection) was used. A mixture of 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 90% RPMI 1640 medium was used to cultivate cells. Cells were cultivated under the conditions of 37˚C, 95% humidity and 5% CO_2. Cells were harvested at the confluence of 70-80% to perform cell transfections. The overexpression pcDNA3.1-MT1JP (MT1JP), pcDNA3.1-PTEN (PTEN) and control pcDNA3.1 recombinant plasmids were constructed by Shanghai GenePharma Co., Ltd. miRNA-NC and miR-32 mimic were obtained from Guangzhou RiboBio Co., Ltd. Lipofectamine 2000® (Sangon Biotech Co., Ltd.) was used to transfect 35 nM miRNA (miRNA-NC or miR-32) or 2 μg plasmid for 24 h in six-well plates. The control (C) cells for all transfections were untransfected cells. At 24 h post-transfection at the room temperature, cells were harvested to perform subsequent experiments. The sequences were as follows: miR-32 Mimic, 5'-AGUAUACGUCCUGCAGUC AGCU-3') and miRNA-NC, 5'-UGCUCCGGACGUGUG AGGUGT-3'.

Reverse transcription-quantitative (RT-qPCR). Total RNA from tissues and cells was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Total RNA was reverse transcribed into cDNA using oligo (dT)15, random primers (GenScript), M-MLV reverse transcriptase, dNTPs, 5X buffer and RNase inhibitor (all Tiangen Biotech Co., Ltd.) at 25˚C for 10 min, 42˚C for 50 min and 80˚C for 10 min. QuantiTect SYBR® Green PCR kit (Qiagen, Inc.) was used to prepare qPCR mixtures for measuring the expression levels of MT1JP and PTEN. GAPDH was used as the endogenous control of MT1JP and PTEN. To measure the expression levels of miR-32, all steps were performed using All-in-One™ miRNA qRT-PCR Detection kit (GeneCopoeia, Inc.). U6 was used as the endogenous control of miR-32. The following thermocycling conditions were used for the qPCR: Initial denaturation at 94˚C for 5 min; followed by 40 cycles at 94˚C for 10 sec, 60˚C for 20 sec and 72˚C for 30 sec; and final extension at 72˚C for 150 sec. The following primer pairs were used for the qPCR: MT1JP forward: 5'-GCAAGGGACGTCGGAGA-3' and reverse: 5'-TCCAGGTTGCAGTTGTT-3'; PTEN forward: 5'-TGGCTT CGACTTAG-3' and reverse: 5'-GGTTGGTTATGGTCTTTA AAAGG-3'; miR-32 forward: 5'-GCGGCGTATTGCACA TTACT-3' and reverse: 5'-TCGTATCCAGTGCAGGGTC-3'; GAPDH forward: 5'-AGATCATCAGCAATGCCTCCT-3' and reverse: 5'-TGAGTCCTTCCACGATACCAA-3' and U6 forward: 5'-CTCGCTTCGGCAGCACA-3' and reverse: 5'-AACGCTTCACGAATTTGCGT-3'. The $2^{-\Delta\Delta Cq}$ method (22) was used for data analysis and all experiments were performed in three replicates.

Western blotting. SNU-398 cells were counted, and total proteins in $3x10^5$ cells were extracted using RIPA solution (Sangon Biotech Co., Ltd.). Protein samples were incubated in boiling water for 10 min to denature proteins, followed by protein lysates (30 μg/lane) of each group were separated using 10% SDS-PAGE. Proteins were then transferred to PVDF membranes and 5% non-fat milk was used to block membranes at room temperature for 80 min. Rabbit polyclonal PTEN (1:1,400; cat. no. ab31392; Abcam) and GAPDH (1:1,400; cat. no. ab37168; Abcam) primary antibodies were incubated with membranes at 4˚C for 18 h, followed by incubation with goat anti-rabbit IgG H+L (1:1,000; cat. no. ab6721; Abcam) at room temperature for 2 h. After that, membranes were incubated with RapidStep™ ECL detection reagent (EMD Millipore) at room temperature for 10 min to develop signals. Data was processed using ImageJ v1.46 software (National Institutes of Health).

Table I. Association between MT1JP expression and clinicopathological features of patients with hepatocellular carcinoma.

Clinicopathological variables	Value, n	MT1JP expression		P-value
		Low, n=32	High, n=32	
Sex				0.325
Male	36	23	13	
Female	28	9	19	
Age, years				0.269
<50	32	13	19	
≥55	32	19	13	
HBsAg				0.221
Negative	28	15	13	
Positive	36	17	19	
Tumor size, cm				0.026
<5	35	9	26	
≥5	29	23	6	
TNM stage				0.025
I/II	37	15	22	
III/IV	27	17	10	

TNM, Tumor-Node-Metastasis; HBsAg, hepatitis B surface antigen.

Cell proliferation assay. SNU-398 cells were counted, and $3x10^4$ cells per well were mixed with 1 ml mixture of 10% FBS and 90% RPMI 1640 medium to prepare single-cell suspensions. Cells were cultivated in a 96-well plate (0.1 ml per well) under conditions of 37˚C, 95% humidity and 5% CO_2. Cell Counting Kit-8 solution (Sigma-Aldrich; Merck KGaA) was added into each well (10 μl per well) at 4 h before the end of cell culture. After cell culture, 10 μl DMSO was added and optical density values were measured at 450 nm wavelength using a microplate reader (Thermo Fisher Scientific, Inc.).

Statistical analysis. Statistical analyses were performed using SPSS version 23 (IBM Corp.). Data were expressed as mean values ± standard deviation of three biological replicates. Associations were analyzed by linear regression. Differences between two types of tissue and among different cell groups were analyzed using paired t-tests and ANOVA (one-way) in combination with the Tukey's test, respectively. Survival analyses were performed by dividing the 64 patients with HCC into high and low MT1JP level groups (n=32) according to the median of MT1JP expression (2.34) in HCC tissues. Kaplan-Meier plotter and log-rank tests were used to plot and compare survival curves. P<0.05 was considered to indicate a statistically significant difference.

Results

MT1JP is downregulated in HCC and predicts poor survival. To illustrate the role of MT1JP in the progression of HCC, the expression of MT1JP and the basic information, such as clinical pathological features of patients with HCC, are displayed in Table I. The expression levels of MT1JP in non-tumor and HCC tissues were measured by qPCR and compared using a paired t-test. Compared with non-tumor tissues, significantly lower expression levels of MT1JP were observed in HCC tissues (P<0.05; Fig. 1A). Compared with patients in the high MT1JP level group, a significantly lower overall 5-year survival rate was observed in the low MT1JP level group (P=0.0078; Fig. 1B). It is worth noting that the expression levels of MT1JP were not significantly affected by HBC and HCV infections as well as clinical stages (data not shown).

Expression of MT1JP is associated with the expression of PTEN and miR-32 in HCC. The expression levels of PTEN and miR-32 were also measured by qPCR and compared using paired t-tests between non-tumor and HCC tissues. Compared with non-tumor tissues, significantly lower expression levels of PTEN (Fig. 2A) and significantly higher expression levels of miR-32 (Fig. 2B) were observed in HCC tissues (both P<0.05). Regression analysis showed that the expression of MT1JP in HCC tissues was positively associated with the expression of PTEN (Fig. 2C) and negatively associated with the expression of miR-32 (Fig. 2D).

MT1JP upregulates PTEN through downregulation of miR-32 in HCC cells. MT1JP and PTEN expression vectors, as well as miR-32 mimic, were transfected into SNU-398 cells and their expression levels were measured by qPCR at 24 h post-transfection. Compared with C and NC, the expression levels of MT1JP, miR-32 and PTEN were significantly increased (all P<0.05; Fig. 3A). Moreover, overexpression of MT1JP decreased the expression levels of miR-32 (P<0.05), whilst overexpression of miR-32 did not affect the expression of MT1JP (Fig. 3B). In addition, overexpression of miR-32 inhibited the mRNA and protein levels of PTEN (P<0.05),

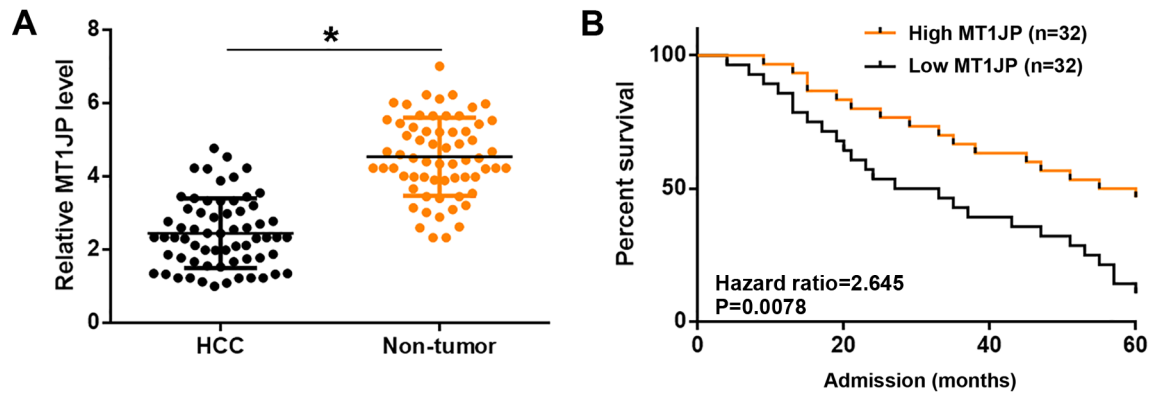

Figure 1. Downregulation of MT1JP in HCC tissues is associated with the poor survival of patients with HCC. (A) Expression levels of MT1JP in two types of tissues (non-tumor and HCC) were measured by quantitative PCR and compared by a paired t-test. (B) Kaplan-Meier and log-rank tests were used to plot and compare survival curves. Mean values of three biological replicates were presented. *P<0.05. HCC, hepatocellular carcinoma.

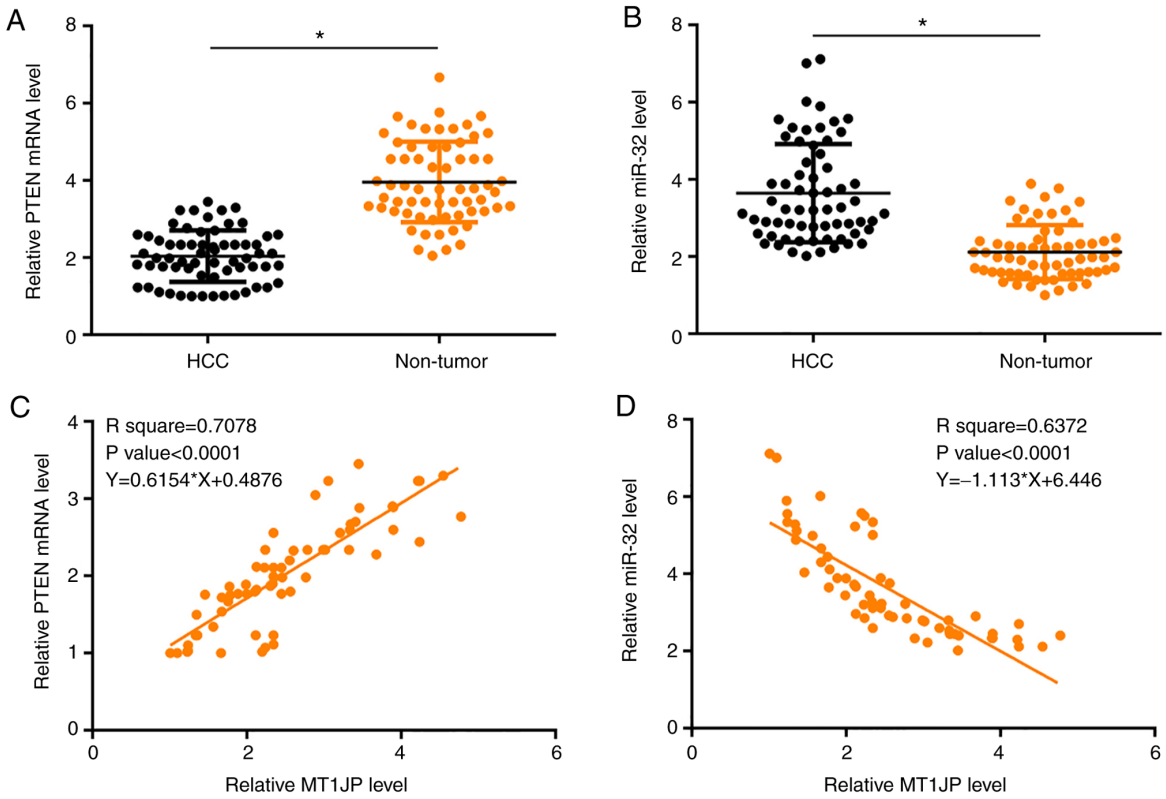

Figure 2. Association between MT1JP and either PTEN or miR-32 in HCC. Expression levels of (A) PTEN mRNA and (B) miR-32 were measured by quantitative PCR and compared by paired t-test between two types of tissues (non-tumor and HCC). Association between (C) MT1JP and PTEN mRNA and (D) MT1JP and miR-32 were analyzed by linear regression. Mean values of three biological replicates were presented. *P<0.05 vs. non-tumor. HCC, hepatocellular carcinoma; PTEN, phosphatase and tensin homolog; miR, microRNA.

which could be reversed by simultaneous upregulation of MT1JP (Fig. 3C).

MT1JP inhibits HCC cell proliferation through PTEN and miR-32. The effects of overexpression of MT1JP, miR-32 and PTEN on the proliferation of SNU-398 cells were analyzed using a cell proliferation assay. Compared with NC and C, overexpression of MT1JP and PTEN resulted in significantly inhibited proliferation of HCC cells (P<0.05). miR-32 promoted cell proliferation. Moreover, the suppressive effect of MT1JP on cell proliferation was reversed by miR-32 (P<0.05; Fig. 4).

Discussion

The present study investigated the function of MT1JP in HCC. It was found that MT1JP was downregulated in HCC and was associated with the survival of patients with HCC. In HCC, MT1JP may have upregulated PTEN through miR-32 to inhibit the proliferation of HCC cells.

MT1JP is shown to be downregulated in tissues of different types of cancer (23), such as gastric cancer (24), glioma (25) and bladder cancer (26). A recent study has demonstrated the inhibitory effect of MT1JP on the progression of HCC (27).

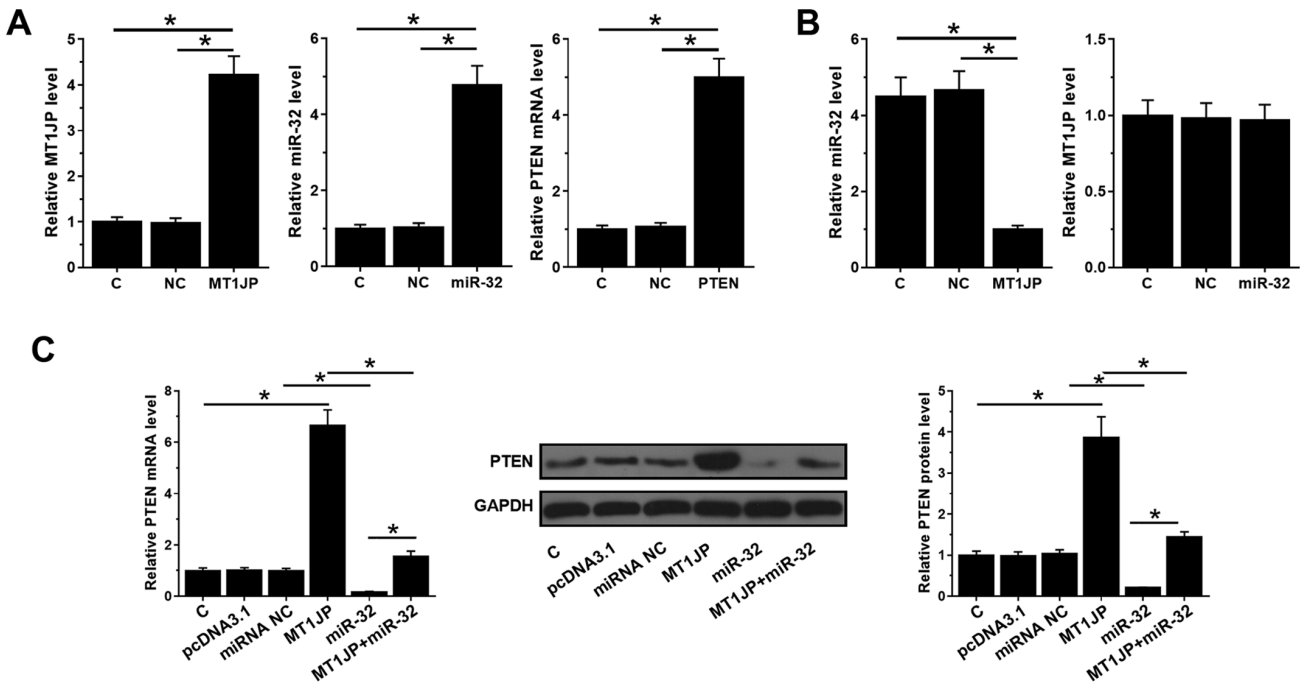

Figure 3. Overexpression of MT1JP increases the expression levels of PTEN by downregulating miR-32 in HCC cells. (A) MT1JP and PTEN expression vectors, as well as miR-32 mimic, were transfected into SNU-398 cells and overexpression was confirmed by qPCR at 24 h post-transfection. (B) Interaction between MT1JP and miR-32 was analyzed by qPCR. (C) Effects of overexpression of MT1JP and miR-32 on the expression of PTEN were analyzed by qPCR and western blotting. Mean values of three biological replicates were presented. *P<0.05 vs. C, NC or MT1JP. PTEN, phosphatase and tensin homolog; qPCR, quantitative PCR; C, control; NC, negative control.

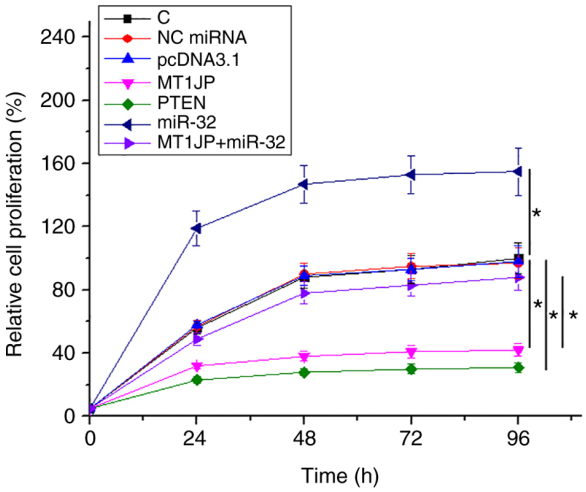

Figure 4. MT1JP regulates PTEN and miR-32 to inhibit hepatocellular carcinoma cell proliferation. Effect of MT1JP, miR-32 and PTEN overexpression on the proliferation of SNU-398 cells were analyzed by cell proliferation assay against controls. Mean values of three biological replicates were presented. *P<0.05. PTEN, phosphatase and tensin homolog; miR, microRNA.

This study found that MT1JP promotes apoptosis and inhibits cell proliferation and migration by inhibiting the expression of miR-24-3p. However, the study did not thoroughly explore the function of miR-24-3p target genes and the interaction of MT1JP, miR-24-3p and miR-24-3p target genes. Based on this, the present study explored another miRNA (miR-32) regulated by MT1JP and revealed that MT1JP promoted the expression of the miR-32 target gene PTEN. This was investigated by downregulating the expression of miR-32, thereby exerting an antitumor effect, which which inhibited the proliferation of HCC cells.

PTEN is a dual phosphatase with both protein and lipid phosphatase activities (28). Gao *et al* (29) revealed that PTEN is concomitantly downregulated in breast cancer tissues and cell lines, and overexpression of PTEN inhibits the progression of breast cancer. Wang *et al* (30) reported that PTEN inhibits the development of gastric cancer. The present study observed reduced expression levels of PTEN after the overexpression of miR-32, which further confirmed the targeting of PTEN by miR-32. The miR-32/PTEN axis can be modulated by certain lncRNAs, such as GAS5, to regulate cancer cell malignant behaviors (31). The present research showed that MT1JP can upregulate PTEN by inhibiting the expression of miR-32, thereby inhibiting cell proliferation. Moreover, previous studies have found that the PTEN promoter contains a p53-binding element. Thus, p53-mediated cell death requires coordinated repression of the cellular survival machinery via direct activation of PTEN transcription by p53 (32). Another study has shown that MT1JP enhances p53 translation by binding to the RNA binding protein TIAR, thereby regulating p53-related pathways such as cell cycle, apoptosis and proliferation (23). Therefore, MTIJP may also inhibit tumor growth through P53/PTEN signaling.

There are certain limitations to the current study. First, the effects of MT1JP/miR-32/PTEN on several signaling pathways remains to be elucidate. MT1JP could target multiple miRNAs to regulate its function in HCC, therefore other potential target miRNAs should be investigated. This study lacks *in vivo* trials, and our conclusions need to be validated *in vivo* in the future.

In conclusion, MT1JP played a tumor-suppressive role in HCC by upregulating PTEN through downregulation of miR-32 to suppress HCC cell proliferation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SZ and JX developed the project, carried out the experiments and clinical studies, collected the data and wrote the manuscript. QC and FZ collected the data, carried out the experiments and clinical studies, analyzed the data and edited the manuscript. HW and HG analyzed the data and carried out the literature research. SZ and JX confirm the authenticity of all raw data. All contributing authors have read and agreed to the final version of the manuscript.

Ethics approval and consent to participate

The Ethics Committee of Union Hospital Affiliated to Tongji Medical College of Huazhong University of Science and Technology approved the study (Wuhan, China). Written informed consent was obtained from all individual participants included in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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