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Original Article

Overexpression of long non-coding RNA loc285194 suppresses cell proliferation and migration in hepatocellular carcinoma

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Abstract: The expression pattern of long non-coding RNA (lncRNA) has been reported to be a new player in various diseases, including cancer. Aberrant expression of these genes will affect tumorigenesis through either oncogenic or tumor suppressive pathways. Hepatocellular carcinoma (HCC), with the increasing incidence, has become the second deadly cancer in men and the sixth in women in the world. Given the high morbidity and mortality of hepatocellular carcinoma, this study aims at examining whether lncRNA loc285194 is a potential regulator of hepatocellular carcinoma and how can this lncRNA function in the tumor progression. The basic expression levels of lncRNA loc285194 were examined in HCC and adjacent noncancerous tissues and one cell line SMMC-7721 was selected. The overexpression of lncRNA loc285194 was detected by quantitative polymerase chain reaction (qPCR) in SMMC-7721 transducted with pcDNA3.1 carrying loc285194. In addition, compared with control groups, the ability of cell migration and invasion decreased in SMMC-7721 transducted with lncRNA loc285194. After transduction, the cell proliferation and the clone cell amount of SMMC-7721 cell line was obviously suppressed by lncRNA loc285194 overexpression, compared with control. The analysis of cell cycle via flow cytometry revealed that SMMC-7721 cells were arrested at G2/M stage with a great increasing of cell apoptosis. Finally, the result of western blot showed the overexpression of lncRNA loc285194 greatly changed the expression level of proteins controlling cell cycle and cell apoptosis to induce cell cycle arrest and cell apoptosis.

Keywords: Hepatocellular carcinoma, lncRNA loc285194, proliferation, migration, apoptosis

Introduction

It is reported that almost 70% of human genome is transcribed but only <2% of the total genome encodes proteins [1]. The great difference in the proportion of the genes that expressed indicates that human genome is not only the collection of protein-coding genes. There are "dark matters" within human genome functioning in the biological way to regulate cellular development and metabolism [2, 3]. RNA molecules without protein-coding function are such kind of "dark matters" and collectively named as non-coding RNAs (ncRNAs) [4]. Long non-coding RNA (lncRNA), one newly discovered group of ncRNAs, is founded to have cell developmental importance and its expression pattern may be new regulator in various diseases, including cancer. Aberrant expression of these "dark matters" will affect tumorigenesis through either oncogenic or tumor suppressive pathways [5, 6].

LncRNAs, ranging from 200 nt to 100 kilobases (kb), are transcripts like mRNAs without significant open reading frames and nearly do not have protein-coding ability [7]. LncRNA has been reported to function in a large number of human biological processes, including epigenetic modifications, to insure the normal cell functions. The expression level of lncRNA will be lower than the genes that encode proteins in general and a part of lncRNA appears to have special expression pattern like tissue specificity [8]. In addition, the fact that abnormal lncRNA expression can be found among various cancer types, indicates that the expression of lncRNA may be one regulator of tumorigenesis. The

increasing number of publications which focus on the relationship between lncRNA and cancer shows great attention in this area and many functional lncRNA have been found on the base of transcriptome analysis technologies, such as HOTAIR, MALAT1, HULC, H19 and so on [2].

The whole transcriptome sequencing has help to discover various lncRNA expression patterns and there are around 7000 to 23,000 unique lncRNAs found in human genome with unknown functions, which indicates they may play a significant role in the cell biology of both normal cells and cancer cells [7, 9]. The obvious different expression pattern of some particular lncRNA between cancer tissue and para-carcinoma tissue have obtained great suspicion from scientists, however, there is still no strong evidence to support this idea [10]. LncRNAs may have the ability to regulate the process of genomic imprinting and transcriptional regulation which is under the control of miRNA expression. The expression pattern of miRNA in cancer will be disrupted by epigenetic modifications, such as 'miRNA sponges', and two studies can be found to prove the function of lncRNA like 'miRNA sponges' to depress miRNA levels [11, 12].

One lncRNA loc285194 (GeneID: 285194), is also called LSAMP antisense RNA3, with the length of 2105 bases including four exons. There are studies revealing that loc285194 can be a suppressor of tumor genesis, such as osteosarcoma. The overexpression of this lncRNA is able to promote normal osteoblasts cell proliferation by regulating cell apoptotic and cell cycle transcripts. Despite the fact that decreased expressions of loc285194 can be found in several different kinds of cancers, the relationship between aberrant expression pattern of lncRNA loc285194 and hepatocellular carcinoma cell lines (HCC) remains unknown [13, 14].

HCC is one of the common malignant tumors with the fifth morbidity and third fatality rate among different cancer types and this disease has become the second deadly cancer in men and the sixth in women in the world [15, 16]. Given that high-risk HCC does not have obvious pathognomonic molecular markers, this study aims to find whether another lncRNA, loc-285194, can regulate the tumorigenesis in HCC, like HULC. One lentivirus vector was used to overexpress lncRNA loc285194 into one human HCC cell line SMMC-7721. The overexpression of lncRNA loc285194 can suppress cell proliferation in HCC and cells were arrested at sub-G1 stage with obvious cell apoptosis. This result may provide useful evidence to find the pathognomonic molecular marker of HCC.

The basic expression levels of lncRNA loc-285194 were examined in HCC and adjacent noncancerous tissues and one normal human liver cell line SMMC-7721 was selected. The overexpression of lncRNA loc285194 was detected by quantitative polymerase chain reaction (qPCR) in SMMC-7721 transfected with pcDNA3.1 carrying loc285194. After transduction, MTT assay was performed to detect the cell proliferation of SMMC-7721 cell line. The results showed that the cell proliferation of SMMC-7721 cell line was obviously suppressed by lncRNA loc285194 overexpression, compared with control. In addition, compared with control groups the ability of cell migration and invasion decreased in SMMC-7721 transfected with lncRNA loc285194. The analysis of cell cycle via flow cytometry revealed that SMMC-7721 cells were arrested at G2/M with a great increasing of cell apoptosis. Finally, the result of western blot showed the overexpression of lncRNA loc285194 greatly changed the expression level of proteins controlling cell cycle and cell apoptosis. The expression of cyclin B1 and cdc2, which promote cells into correct cell cycle, as well as Cleaved-caspase 3 and PARP, which control cell apoptosis, were decreased while the expression of cleaved-PARP increased. In conclusion, the overexpression of lncRNA loc285194 can suppress cell proliferation in HCC and promote cell apoptosis. lncRNA loc285194 could be a candidate of tumorigenesis regulator in HCC.

Materials and methods

Patients and specimens

Clinical specimens were obtained from 15 patients with HCC, who underwent surgery at the First Affiliated Hospital of Zhengzhou University from 2014 to 2015. Adjacent non-tumoral tissues from these patients served as normal controls. Both tumor and non-tumor tissues were confirmed by two experienced pathologists. None of these patients received neo-adjuvant or adjuvant treatment before opera-

tion. The study was approved by the First Affiliated Hospital of Zhengzhou University. Informed consent was obtained from all of the patients for use of the clinical specimens.

Cell culture

In this study, human HCC cell lines (HepG2, SMMC-7721, Bel-7402, Hep 3B) and a normal human liver cell line HL-7702 were obtained from Cell Bank of Shanghai Institute of Biochemistry Cell Biology, Chinese Academy of Sciences. All the cells lines were cultured in DMEM (Hyclone, Cat no. SH30243.01B+), which is supplemented with 10% FBS (Biowest, Cat no. S1810) at 37°C with 5% CO_2.

Cell transfection

HCC SMMC-7721 cells were used to perform overexpression experiment. The overexpression plasmid vector of loc285194 (pcDNA3.1 carrying the sequence of loc285194) was synthesized in Life Technology (Invitrogen, USA). The empty vector pcDNA3.1 was used as a negative control. After transfection, total protein was extracted from 1×106 cells to test the effectiveness of transfection.

Reverse transcription PCR and quantitative PCR

Total RNA was isolated from the tissue samples or the transfected cells using TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. PCR products were analyzed on 1% agarose gels. The primers used in this study were synthesized by Invitrogen with the sequences as follows: for loc285194, 5'-TGTGCCTGTTTGACCTCTGA-3' (sense) and 5'-AGGAAGGATAAAAGACC GACCA-3' (antisense); for β-actin, 5'-GTGGACATC CGCAAAGAC-3' (forward), 5'-AAAGGGTGTAACGCAACTA-3' (reverse). The relative expressions of target genes were calculated using the $2^{-\Delta\Delta Ct}$ method.

Flow cytometric analysis of cell cycle and apoptosis

Cell cycle was performed with PI/RNase staining solution (Tian-jin Sungene Biotech Co., Ltd., China). 1×106 HCC SMMC-7721 cells without IL-3 were collected and then fixed in ethanolat (70%) at 4°C overnight. After washing with PBS, cells were permeabilized with 100 μl RNAase in PBS for 30 min at 37°C in the absence of light, and then cells were stained with 400 μL of propidium iodide (Sigma, US) for 30 min. The cell-cycle phases were analyzed by flow cytometry system (Beckman Coulter, Indianapolis, IN) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. The transfected cells were resuspended in binding buffer containing Annexin V-FITC and propidium iodide, and assessed by flow cytometer according to the manufacturer's instructions.

Western blotting analysis

Cells were lysed in 200 μl lysis buffer (0.5 M Tris-HCl, pH 6.8, 2 mM EDTA, 10% glycerol, 2% SDS, and 5% β-mercaptoethanol). Protein extracts (20 μg) were electrophoresed on 10% SDS polyacrylamide gels (30% Acrylamide, 1.5 M Tris pH 8.8, 10% SDS, 10% Ammonium Persulfate, TEMED) and then transferred to polyvinylidene fluoride membranes (PVDF, Millipore, Billerica, MA, USA). Primary antibodies used in this study were at the following concentrations: Caspase-3 (Santa Cruz) 1:500, PARP (Santa Cruz) 1:1000, Cyclin B1 (Santa Cruz) 1:1000, β-actin (Santa Cruz) 1:2000. Blots were quantified by densitometry, and normalized by β-actin.

MTT assay

The proliferation assay of the HCC SMMC-7721 cells was performed using 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT, SigmaAldrich, USA). Cells were grown in a 96-well plate and the cell density was adjusted to 1×104/well. The plates were incubated for 24 h in a humidified atmosphere containing 5% CO_2 at 37°C. Then the cells were incubated in 0.1 mg/ml MTT for 4 h and lysed in dimethyl sulfoxide (DMSO) in a humidified atmosphere containing 5% CO_2 at 37°C for 10 min. The absorbance in each well was calculated at 490nm by using a microplate reader (Bio-Rad, CA). Each experiment was performed in triplicate wells and repeated three times.

Cell migration and invasion assay

The migration and invasion assays of the HCC SMMC-7721 cells were performed using transwell chambers. In migration assay, 5×10^4 cells were added into the upper chamber of transwells (BD Bioscience, USA). In the transwell

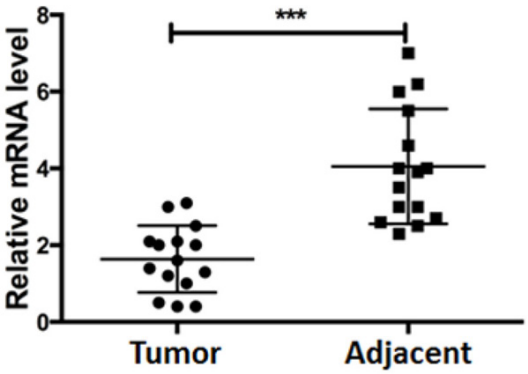

Figure 1. The expression of Loc285194 in HCC tissues. The expression of Loc285194 in 15 paired HCC tissues and adjacent non-tumor tissues from HCC patients were confirmed by qRT-PCR. The results indicated that Loc285194 is downregulated in HCC. ***P<0.001.

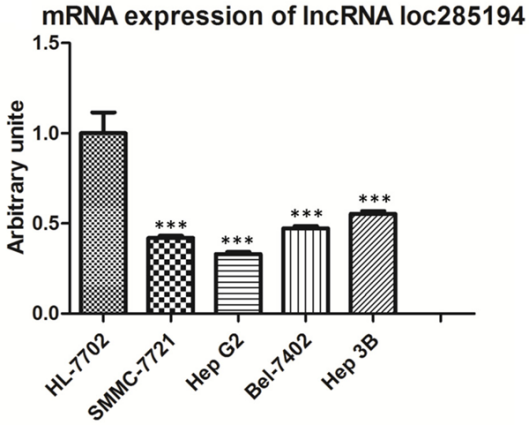

Figure 2. The expression of Loc285194 in HCC cell lines. The Loc285194 expression of human HCC cell lines and a normal human liver cell line HL-7702 was detected by qRT-PCR and the results showed that the Loc285194 expression of human HCC cell lines (HepG2, P<0.001; SMMC-7721 P<0.001; Bel-7402, P<0.001; Hep 3Bl, P<0.001) was decreased compared with the normal human liver cell line.

assay, 1×10^5 cells were added into the upper chamber precoated with matrigel (BD Bioscience, USA). Cells were maintained in medium without serum in the upper chamber, and medium containing 10% FBS was added to the lower chamber as chemoattractant in both assays. After 24 hours incubation, we wiped out the cells that did not migrate or invade through the membrane. Then the membranes were fixed and stained with 0.5% crystal violet. Three random fields were counted per chamber by using an inverted microscope (Olympus, Jap). Each experiment was performed in triplicate wells and repeated three times.

Statistical analysis

Each experiment was repeated at least three times. All data were summarized and represented as mean ± standard deviation (SD). Comparison between different groups were determined using Student's t-tests and one-way analysis of variance (ANOVA) followed by Bonferroni t-tests. Statistical analyses were performed by SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). P<0.05 was considered as statistically significant. *indicates statistical difference with P<0.05, **indicates statistical difference with P<0.01 and *** indicates statistical difference with P<0.001.

Results

Loc285194 expression was downregulated in HCC

The expression of loc285194 in 15 paired HCC tissues and adjacent non-tumor tissues from HCC patients were confirmed by qRT-PCR. As shown in **Figure 1**, a significant downregulated expression of PCAT-1 was observed in HCC patients compared with the level of adjacent non-tumor tissues (**Figure 1A**, P<0.001). Furthermore, the loc285194 expression of human HCC cell lines (HepG2, SMMC-7721, Bel-7402, Hep 3B) was decreased compared with the normal human liver cell line HL-7702 (**Figure 2**, HepG2 P<0.001, SMMC-7721 P< 0.001, Bel-7402 P<0.001, Hep 3B P<0.001). These results indicated that Loc285194 could suppress neoplasia in HCC.

Loc285194 suppressed the proliferation of HCC cells

To investigate the role of loc285194 in HCC proliferation, we chose SMMC-7721 cells to perform experiment and MTT assay. Infection was observed to be efficient at 96 h, and over 80% SMMC-7721 cells were presented to be GFP positive in both pcDNA3.1-loc285194 and empty vector groups (**Figure 3A**). The results of overexpression of loc285194 in the SMMC-7721 cells were confirmed by qRT-PCR. As shown in **Figure 3B**, the expression of loc-285194 was significantly high in pcDNA3.1-loc285194 group compared to the empty vec-

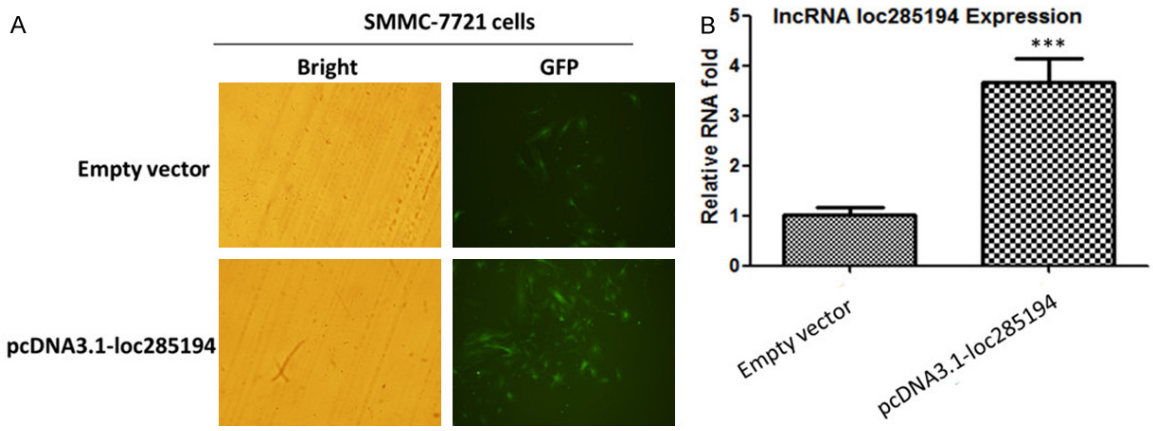

Figure 3. Overexpression of Loc285194 in SMMC-7721 cells by pcDNA3.1- Loc285194. A: Fluorescence microscopy examination of lentivirus infection efficiency in SMMC-7721 cells (Scale bar: 100 μm). B: RT-qPCR analysis of Loc285194 expression level in SMMC-7721 cells after pcDNA3.1-EDAG-1 infection. ***P<0.001.

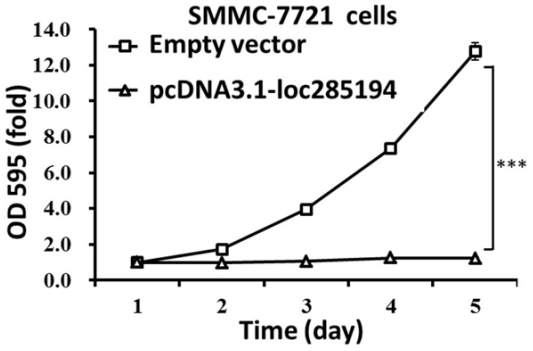

Figure 4. Overexpression of Loc285194 promoted the viability and proliferation of SMMC-7721 cells. Growth curves of SMMC-7721 cells in two groups (Empty vectors and pcDNA3.1-Loc285194) measured by MTT assay. ***P<0.001.

tor group (P<0.001). Then we explored the effect of Loc285194 on HCC cell proliferation by MTT assay. According to our experimental results, the expression of loc285194 and the proliferation rate of HCC cells transfected with pcDNA3. 1-loc285194 was significantly decreased compared with the empty vector group (**Figure 4**, P<0.001) These results indicated that loc285194 could inhibit HCC cell proliferation.

Loc285194 inhibited HCC cells migration and invasion

To investigate the role of loc285194 in HCC cell migration and invasion, we chose SMMC-7721 cells to perform Transwell migration assay and Transwell invasion assay. According to our experimental results, overexpression of loc285194 inhibited breast cancer cell migration and invasion compared with negative control (**Figure 5**, migration P<0.001; invasion P< 0.001). These findings made it clear that loc285194 could inhibit the development and progression of breast cancer.

Overexpression of loc285194 induced the HCC cell cycle arrest

We further investigated the cell cycle progression for the mechanisms underlying the inhibition of cell growth by loc285194 overexpression. The cell cycle distribution of SMMC-7721 cells after loc285194 overexpression was analyzed through FACS assay (**Figure 6A**). As shown in **Figure 7B**, 34.24%±0.28% pcDNA3.1-loc285194 cells were in G0/G1 phase compared to 56.84±0.52% of control group (P< 0.001), whereas 41.45±0.53% of pcDNA3. 1-loc285194 cells in G2/M phase compared to 19.78±0.21% of control cells (P<0.001), indicating that cell cycle was blocked in G2/M phase. These results suggested overexpression of loc285194 inhibited HCC cell growth by inducing cell cycle arrest and apoptosis.

Overexpression of loc285194 promoted the apoptosis of HCC cells

The discrimination of apoptosis changes was measured to detect the role of loc285194 in regulating SMMC-7721 cells apoptosis by 7-AAD and annexin V-APC (**Figure 7A**). As a result, the apoptotic percentage of the pc-

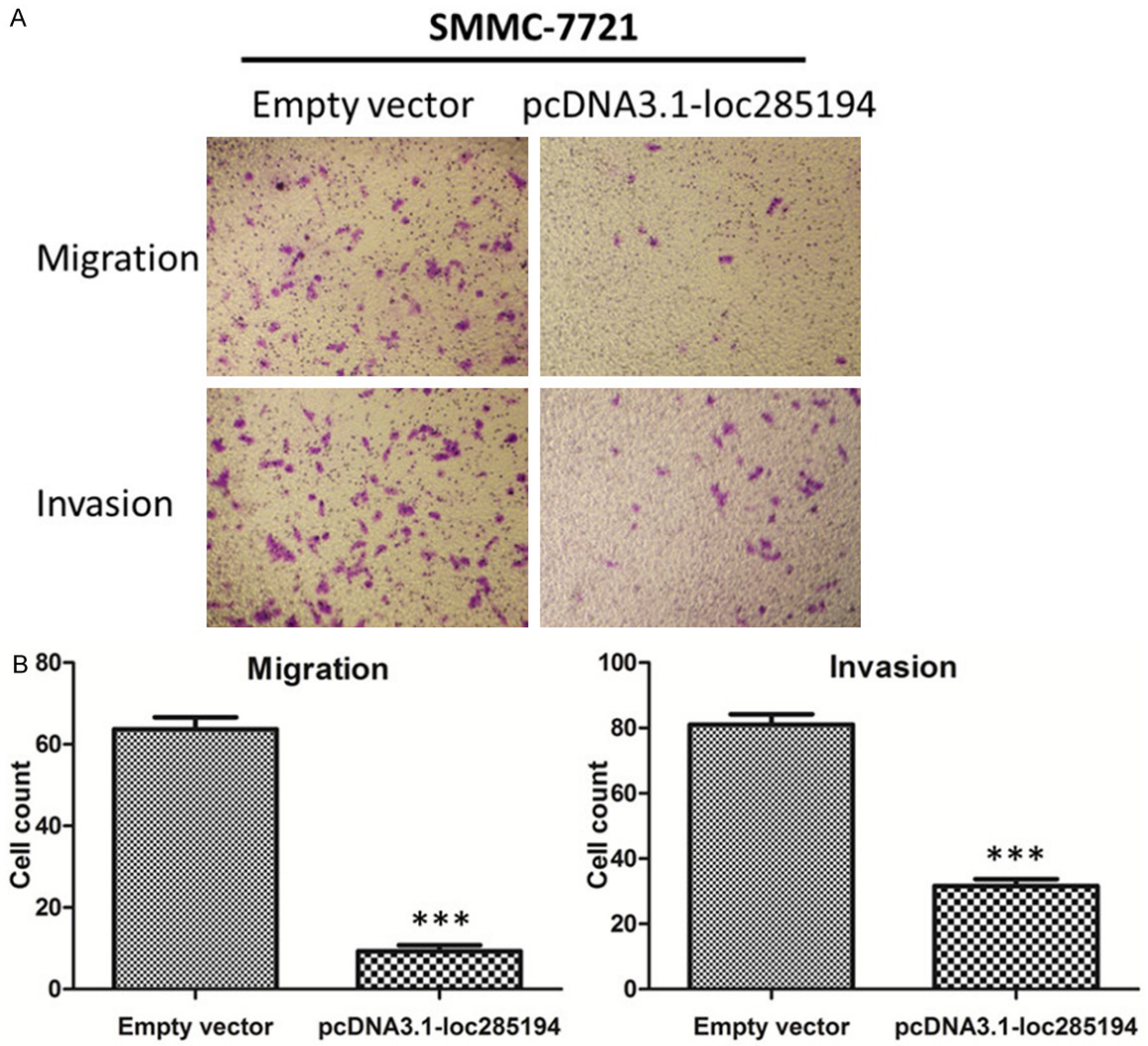

Figure 5. Loc285194 inhibited SMMC-7721 cells migration and invasion. The migration and invasion assays of the HCC SMMC-7721 cells were performed using transwell chambers. The results showed that overexpressing of Loc285194 inhibited the migration of SMMC-7721 cells. Data are presented as means ± SD from three independent experiments. ***P<0.001.

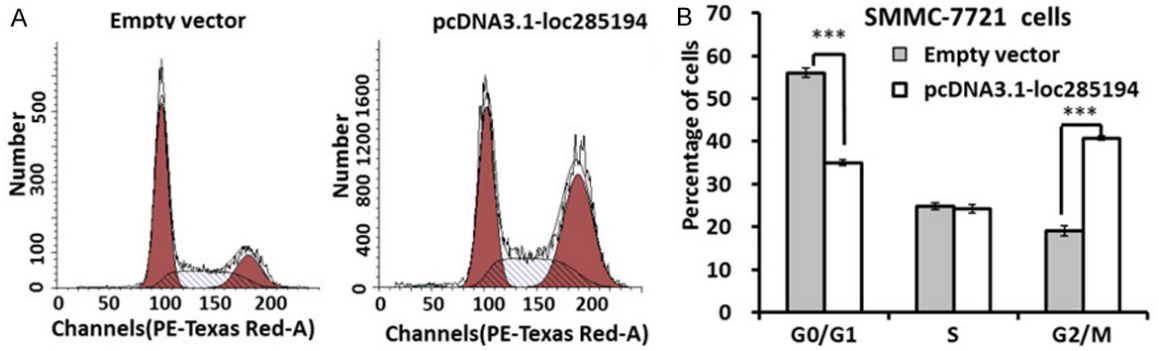

Figure 6. Overexpression of loc285194 blocked the cell cycle progression of SMMC-7721 cells. A: FACS analysis of cell cycle distribution of SMMC-7721 cells in two groups (Empty vector, pcDNA3.1-Loc285194). B: Overexpression of loc285194 in SMMC-7721 cells led to an increase of cells at G2/M phase and concomitantly a decrease of cells at G0/G1 phase. Data represent means ± SD from three independent experiments. ***P<0.001.

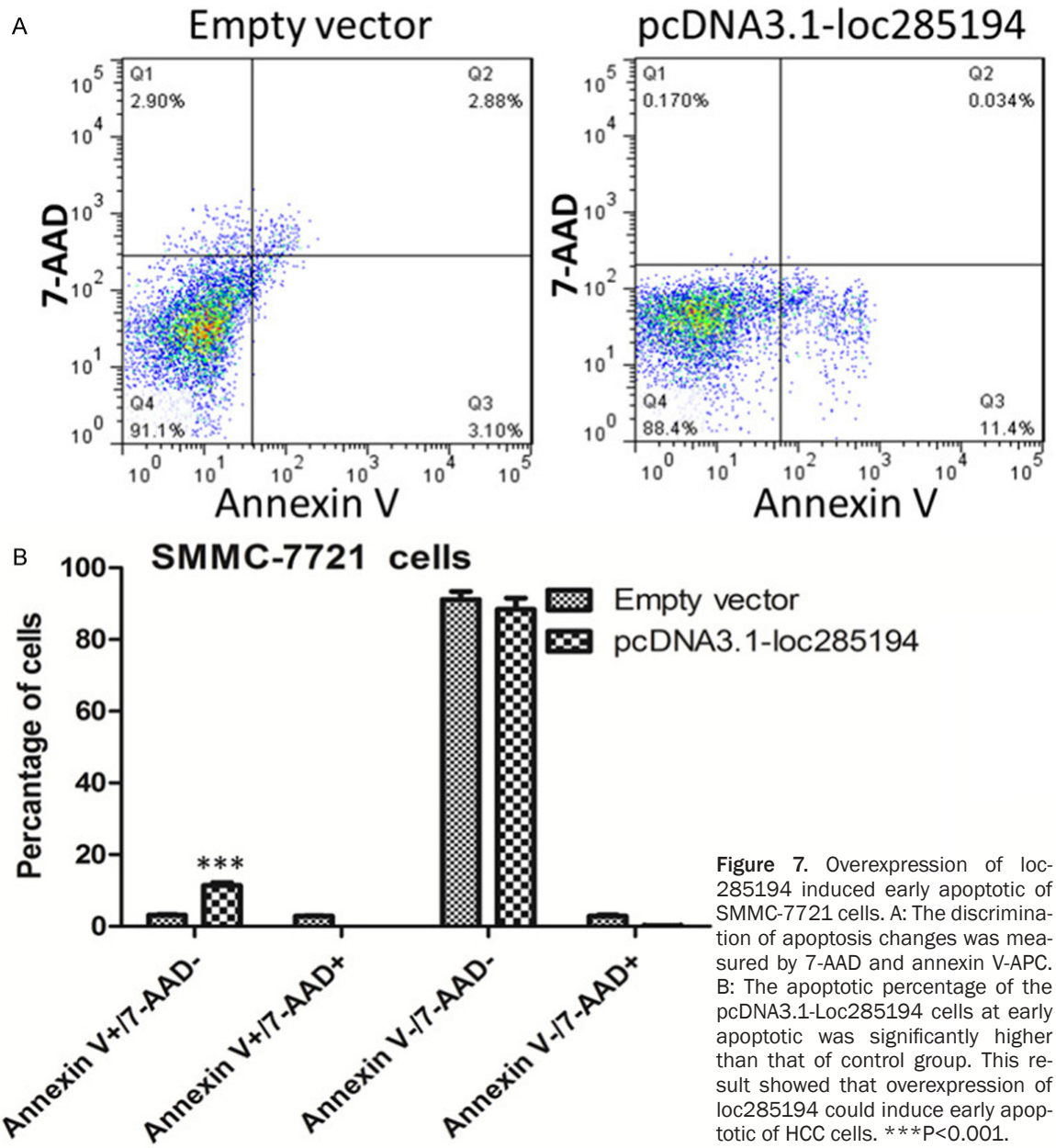

Figure 7. Overexpression of loc-285194 induced early apoptotic of SMMC-7721 cells. A: The discrimination of apoptosis changes was measured by 7-AAD and annexin V-APC. B: The apoptotic percentage of the pcDNA3.1-Loc285194 cells at early apoptotic was significantly higher than that of control group. This result showed that overexpression of loc285194 could induce early apoptotic of HCC cells. ***P<0.001.

DNA3.1-loc285194 cells at early apoptotic was significantly higher than that of control group (**Figure 7B**). This result showed that overexpression of loc285194 could induce early apoptotic of HCC cells. To further explore how overexpression of loc285194 inhibited human breast cancer cells growth, the alterations of Caspase 3, PARP, Cleaved-caspase 3, cyclin B1 and cdc2 were detected and analysed. As shown in **Figure 8**, the expression of cyclin B1 and cdc2, which promoted cells into correct cell cycle, as well as Cleaved-caspase 3 and PARP, which controlled cell apoptosis, were decreased while the expression of cleaved-PARP increased. Combined with former results, we suggested that overexpression of loc285194 might induce cell cycle arrest by downregulating the expression level of cyclin B1 and cdc2 and induce cell apoptosis through cleavage of PARP inducted by activation of Caspase 3.

Discussion

As a newly discovered class of non-coding genes, lncRNAs have been recently found to be pervasively transcribed in the genome [17, 18].

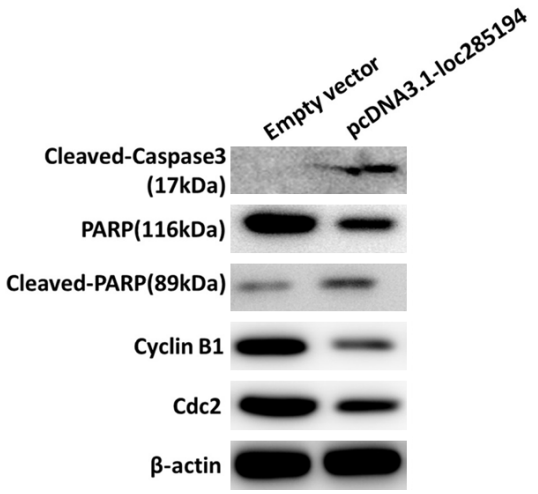

Figure 8. Overexpression of loc285194 decreased the expression of cyclin B1 and cdc2, as well as Cleaved-caspase 3 and PARP, but increased the expression of cleaved-PARP in SMMC-7721 cells. The alterations of Caspase 3, PARP, Cleaved-caspase 3, cyclin B1 and cdc2 in SMMC-7721 cells were detected and analysed by Western blotting. Blots were quantified by densitometry, and normalized by β-actin. The expression of cyclin B1 and cdc2, as well as Cleaved-caspase 3 and PARP was decreased, while the expression of cleaved-PARP was increased.

The expression levels of lncRNAs as well as their cognate RNA-binding proteins are often associated with human diseases, in particular cancer [19-21]. HCC is one of the most frequently occurring cancers with poor prognosis and dysregulation of many HCC-related lncRNAs such as HULC, HOTAIR, MALAT1, and H19 have been identified [22].

Loc285194 is a novel lncRNA located in chr3q13.31, consisting of four exons [13]. Recent studies have shown loc285194 is commonly downregulated in a lot of tumors. Tong et al [14] found that loc285194 expression was significantly down-regulated in esophageal squamous cell carcinoma (ESCC) and low expression of loc285194 was associated with larger tumor size. Qi et al [23]. showed that loc285194 expression was significantly decreased in colorectal cancer tissues and cell lines and low expression of loc285194 was associated with poor prognosis. However, the role of loc285194 in HCC is still unknown.

In this study, we explored the role of lncRNA loc285194 in HCC progression. According to our results, the expression of loc285194 was significantly decreased in HCC tissues compared to matched normal tissues. Furthermore, the expression of loc285194 was also downregulated in HCC cell lines (HepG2, SMMC-7721, Bel-7402, Hep 3B) compared with the normal liver cell line HL-7702. In addition, overexpressing loc285194 would inhibit the proliferation, migration and invasion of SMMC-7721 cells. Aberrance of cell cycle progression might lead to tumor-specific phenotype, such as unlimited cell proliferation, abnormal cell division, and chromosomal instability [24]. So we analysisd the cell cycle via flow cytometry, revealing that high level of loc285194 could arrest SMMC-7721 cell cycle at G2/M with a great increasing of cell apoptosis. 7-AAD and annexin V-APC were performed to measure the discrimination of apoptosis changes. According to our results, overexpression of loc285194 could induce early apoptosis of SMMC-7721 cells. Western blotting was performed to detect the level of cell cycle-related molecular cyclin B1 and cdc2 as well as apoptosis factor Cleaved-caspase 3 and PARP in SMMC-7721 cells after transfection with pcDNA3.1- loc285194. Our results showed that the expression of cyclin B1, cdc2, Cleaved-caspase 3 and PARP was downregulated while the expression of cleaved-PARP was upregulated. Overexpression of loc285194 might induce cell cycle arrest by downregulating the expression level of cyclin B1 and cdc2 and induce cell apoptosis through cleavage of PARP inducted by activation of Caspase 3.

In conclusion, the Overexpression of lncRNA loc285194 can suppress cell proliferation in HCC and promote cell apoptosis. LncRNA loc285194 could be a new therapeutic target for the treatment in HCC. More work is needed to confirm the molecular mechanisms of loc285194 as a candidate biomarker for HCC in the clinic.

Acknowledgements

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Disclosure of conflict of interest

None.

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