microRNA-1915-3p Promotes Cell Metastasis and Progression by Targeting Bcl2L11 in Hepatocellular Carcinoma

Wenjie Huang
Affiliated Liuzhou Municipal Liutie Central Hospital of Guangxi medical university
Sufen Li
Affiliated Liuzhou Municipal Liutie Central Hospital of Guangxi medical university
Xianhua Chen
Affiliated Liuzhou Municipal Liutie Central Hospital of Guangxi medical university
Lin Sun
Affiliated Liuzhou Municipal Liutie Central Hospital of university
Gangxi Pan
Affiliated Liuzhou Municipal Liutie Central Hospital of Guangxi medical university
Jianlin Chen
Affiliated Liuzhou Municipal Liutie Central Hospital of Guangxi medical university
Xiujuan Sun
Affiliated Liuzhou Municipal Liutie Central Hospital of Guangxi medical university
Yongqi Shen
Affiliated Liuzhou Municipal Liutie Central Hospital of Guangxi medical university
Yonggang Lu
Affiliated Liuzhou Municipal Liutie Central Hospital of Guangxi medical university
Yifan Sun (sunyifan@gxmu.edu.cn)
Guangxi Medical university https://orcid.org/0000-0003-3060-1539

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Abstract

Background

Increasing evidence suggests that miR-1915-3p plays vital regulatory roles in metastasis and progression of several types of cancer. However, the roles and underlying mechanism of miR-1915-3p in hepatocellular carcinoma (HCC) remains largely unclear.

Methods

We carried out a bioinformatic meta-analysis to investigate a possible role of miR-1915-3p as prognostic biomarkers. In vitro cellular models of HCC were used for functional studies exploring the role of miR-1915-3p in HCC development and progression. Finally, in vivo studies were performed to demonstrate that miR-1915-3p is a viable therapeutic target.

Results

This study showed that miR-1915-3p was significantly increased in HCC tissue samples and cell lines, and high miR-1915-3p expression was associated with a poor overall survival (OS) and disease-free survival (DFS) time of HCC patients. Overexpression or ablation of miR-1915-3p expression resulted in accelerated or inhibited cell proliferation, migration, and invasion respectively in HCC cells. In addition, miR-1915-3p induced downregulation of proapoptotic factors, including caspase3, caspase8, BAD, Bcl2L11, and P53. It also induced upregulation of antiapoptotic Bcl-2, protecting HCC cells from apoptosis. A biological analysis indicated that miR-1915-3p could be directly targeted to Bcl2L11 to regulate the proliferation, invasion, and migration of HCC cells. Furthermore, in vivo studies confirmed that treatment with miR-1915-3p retarded the growth of tumor in nude mice.

Conclusion

our study provided the evidence for the regulatory role of miR-1915-3p in HCC, which was causally linked to targeting of Bcl2L11. Medications that abrogate excessively expressed miR-1915-3p may offer novel targets for the management of HCC.

Highlights

1. miR-1915-3p was up-regulated in HCC tissues and cells.
2. Knockdown of miR-1915-3p inhibited HCC progression.
3. miR-1915-3p promoted HCC progression via linked to targeting of Bcl2L11.
4. Treatment with Bcl2L11 inhibited HCC growth in vivo.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers of the liver and is ranked as the third leading cause of cancer death and the sixth most common neoplasm (1). Unfortunately, HCC is often diagnosed at an advanced stage, which is no longer amenable to curative therapies due to a deficiency of biomarkers for early diagnosis. Thus, radiotherapy and chemotherapy remaining the only options (2), which have shown antitumor activity (3), but no definitive proof of survival benefit has been found (4). Presently, figuring out the molecular mechanisms regarding the progression and metastasis in HCCs and looking for the biomarkers and therapeutic targets for HCC are urgent necessities.

MicroRNAs (miRNAs) are a class of highly conserved small RNA molecules that act as critical regulators of gene expression in multicellular eukaryotes (5). Since their initial discovery, a growing body of evidence has documented that miRNAs play an important role in cellular homeostasis, and dysfunction of these molecules are linked to several human diseases (6). Now, there are lots of examples linking dysregulated expression of miRNAs to cancer, and miRNAs are increasingly viewed as potential therapeutic, diagnostic, and monitoring targets (7, 8). In HCC, miRNAs, including miR-101 (9), miR-221 (10), miR-21 (11) and miR-222 (12), can promote tumor development, whereas miR-203, miR-124 (13), miR-233 (14) and miR-122 (15) suppressed tumor growth and metastasis. These studies represent an important step toward the potential value of microRNA-based therapy for the treatment of HCC.

miR-1915-3p was another dysregulated miRNA in a variety of cancers, including gastric cancer (16), breast cancer (17), diffuse glioma (18) and lung cancer (19). Although upregulated expression of miR-1915-3p have been reported to be linked to intracellular oxidative stress status in HCC (20), its role for tumor metastasis and progression is still largely unclear. Therefore, we examined the expression of miR-1915-3p in HCC, matching adjacent liver tissues and several HCC cancer cell lines with different metastatic potentials. We found that miR-1915-3p promoted proliferation, invasion, and migration of HCC cells in vitro. By using bioinformatics analysis, we identified that Bcl2L11 was the direct target of miR-1915-3p in regulating the proliferation, invasion, and migration of HCC cells. Moreover, we confirmed our finding by the xenograft tumor model. Therefore, the increased miR-1915-3p suppressed Bcl2L11 translation for apoptosis inhibition in promoting the growth of HCC cells might prove to be a novel therapeutic candidate for HCC.

Materials And Methods

Patients and specimens

Tissue sections in immunohistochemistry were enrolled at Affiliated Liutie Central Hospital of Guangxi Medical University and provided informed consent for tissue to be used for research studies. All tissues were snap-frozen and stored in liquid nitrogen immediately after surgical resection. Tissue microarray slides were purchased from Shanghai Outdo Biotech Co., Shanghai, China. The clinicopathologic features of the patients are described in Table 1. Each patient was diagnosed based on World Health Organization criteria, and tumor differentiation grade was classified according to Edmondson and Steiner (21). Liver function was evaluated using the Child-Pugh scoring system. Tumor stages were classified based on the

International Union against Cancer TNM classification system. OS was defined as the time from surgery to death or surgery to last observation time. The OS data were obtained from the last follow-up for surviving HCC patients. Time to tumor recurrence was calculated from the surgical resection to the date of any relapse, including both extrahepatic metastasis and intrahepatic recurrence (22).

Cell lines and animals

Human HCC cell lines (SMC-7721, QGY-7701, QGY-7703, MHC-97H, HuH7, HepG2) and human normal liver cell lines LO2 were purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle medium (DMEM)(Gibco; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (Thermo Fisher Scientific) in 5% Co_2 at 37 °C.

Male Balb/c-nude mice (four-week-old) were purchased from the Shanghai Institute of Material Medicine and raised under specific pathogen-free conditions. The use of laboratory animals conformed to the NIH guide for Care and Use of Laboratory Animals, 8th ed.

Immunohistochemistry

Situ hybridization (ISH) was evaluated with miRCURY locked nucleic acid (LNA) detection probe for miR-1915-3p 5'-CCCCAGGGCGACGCGGCGGG-3' to detect the miR-1915-3p expression in HCC tissue arrays, as previously described with modifications(23). Briefly, digoxigenin double-labeled LNA-modified probe (500 nmol/L) was added to the hybridization solution and hybridized according to the manufacturer's protocol. The sample images were captured with 200X magnification after incubation with 4-nitroblue-tetrazolium and nuclear fast red. ISH signals for miR-1915-3p were scored by combining the proportion of positive cells and staining intensity. The intensity of staining cells was scored as: strong (3), intermediate (2), weak (1), and negative (0). The proportion of positive cells was scored as follows: 4 points: 75 to 100% of positive cells, 3 points: 50 to 74% of positive cells, 2 points: 25 to 49% of positive cells, 1 point: 1 to 24% of positive cells, 0 point: no positive cells. The staining index (SI) was calculated as staining intensity score times the proportion of positive tumor cells. High expression was explained by a $SI \geq 5$, while low expression was defined as an $SI < 5$.

Vectors and cell transfections

miR-1915-3p ectopic expression level lentiviral vector (hU6-MCS-Ubiquitin-EGFP-IRES-puromycin-miR-501-3p) and anti-miR-1915-3p and its negative control were purchased from Genomeditech (Shanghai, China). Bcl2L11 expression vector (CMV-MCS-SV40-Neomycin-Bcl2L11) and shRNA Bcl2L11 vector, as well as its negative control, were also purchased from Genomeditech (Shanghai, China). The transfected clones were selected with puromycin and G418 (Sigma-Aldrich, USA), according to the manufacturer's protocol, and the transfected efficiency was verified by quantitative PCR (qPCR) and western blot assays. wt or mt 3'-UTR were sequenced from Bcl2L11 and were cloned into the pGL3-promoter vector (Promega, Madison, WI). All transfections were performed as previously described (24).

RNA extraction and quantitative real-time PCR (q RT-PCR)

Total RNA was extracted from cell lines and fresh-frozen HCC tissues with Trizol reagent (Thermo Fisher Scientific). A synthesis of complementary DNA (cDNA) was performed by PrimeScript RT Reagent Kit (Takara, Shiga, Japan) and detected at the ABI7500 Sequence Detection System (Applied Biosystem, Foster City, CA, USA), according the manufacturer's guidelines. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control for mRNA and U6 was used for microRNA. The gene expression relative to the endogenous control was analyzed by ΔC_t and $2^{-\Delta\Delta Ct}$. The following primers were used for qRT-PCR detection:

Western blot

Western blot was performed with a modified procedure as previously described (25). Briefly, total protein was extracted from HCC tissues and cells by using a RIPA Lysis Buffer (Thermo Scientific, USA) containing 1% phenylmethanesulfonyl fluoride on ice. The cell lysates (50 μg of protein) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a PVDF membrane that was blocked with Tri-buffered saline containing 0.1% Tween 20 and 5% bovine serum albumin (BSA) for two hours at room temperature. They were then incubated with the following antibodies at 4℃ overnight: anti-caspase3 (Abcam, ab4051, Cambridge, UK), anti-caspase8 (Abcam, ab108333, Cambridge, UK), anti-Bcl2 (Abcam, ab32124, Cambridge, UK), anti-BAD (Abcam, ab32445, Cambridge, UK), anti-Bcl2L11 (Abcam, ab32158, Cambridge, UK), anti-P53 (Abcam, ab131442, Cambridge, UK), and anti-GAPDH (Proteintech, 60004-1-Ig, USA). The membrane was incubated with the secondary antibodies (Proteintech, SA00001-1, USA) at room temperature for one hour. The immunoblots were detected by enhanced chemiluminescence assays and GAPDH was used as the loading control.

Wound-healing, cell proliferation and transwell invasion assay

The wound healing assay was conducted with a modified method as previously described (25). Briefly, hepatocarcinoma cells were grown to 90% confluence and then starved for 12 hours. A scraped line was created with a p200 pipette tip and washed twice with PBS. The cells were subsequently incubated for 48 hours with a starvation medium (DMEM without FBS). Cellular migration toward the scratch was observed and recorded after 48 hours, and the percentage of open area was assessed. The CCK8 assay was used to measure the proliferation of HCC cells. Briefly, cells (104/well) were inoculated into 96-well assay plates. Then, the cells were incubated with 10 μl CCK-8 solution (Dojindo, Tokyo, Japan) for two hours. Absorbance was acquired by using an automatic microplate reader at 450 nm (Thermo Fisher Scientific, Inc, Waltham, MA, USA). For the transwell invasion assays, the cells were plated in Transwell upper chambers (Corning, New York, USA) at a density of 2 × 104 in 200 μl of serum-free medium, while 800 μl of normal-containing culture medium was placed in the lower chambers. After 48 hours of incubation at 37℃, the cells that invaded the lower surface were fixed with 4% paraformaldehyde for 15 minutes and stained with 0.1% crystal violet dye (Ameresco, LIC, USA) for six minutes. They were photographed in an inverted microscope (×200 magnification) and counted by using Image J software.

Luciferase reporter assay

For the luciferase assay, cells were seeded in 96-well plates and cultured for 24 hours. In all, 10 ng of Bcl12L11 wild-type (WT) or mutant (MUT) 3'-UTR reporter vectors were transfected into HCC cells using the Lipofectamine 2000 (Invitrogen, USA), according to the manufacturer's instructions. After an incubation of 48 hours, cells were harvested and a luciferase reporter gene assay was conducted using the Dual Luciferase Reporter Assay Kit (Promega).

Animal experiments

Approximately 1×10^7 HCCSMMC-7721 cells, which stably transfected with either miR-1915-3p or the NC vector, were suspended in 100 μl Dulbecco's modified Eagle's medium and then cutaneously injected into the left flank of each BALB/c nude mouse. After injections for four weeks, the subcutaneous tumors were cut equally into small pieces and transplanted into the livers of nude mice. Transplanted mice were sacrificed six weeks later and tumor volume (V) was calculated with the following formula: (length × width^2)/2 (26). The paraffin-fixed livers were serial sectional and tunel assay was performed using a DeadEndTM Fluorometric TUNEL System (Promega), according to the manufacturer's instructions.

mRNA-seq

Total RNA was extracted from cells by Trizol reagent (Invitrogen, USA) and RNA samples were purified by magnetic oligo (dT). mRNA samples were then reverse transcribed into first-strand cDNA, followed by second strand synthesis. Fragmented DNA samples were blunt ended and adapters were ligated to construct a library. DNA was quantified with Qubit (Invitrogen, CA, USA). Sequencing was carried out using an Illumina HiSeq 3000 SBS instrument according to the manufacturer's recommendations. We quantified transcript expression levels with a fragment per kilobase of transcripts per million fragments mapped (FPKM), and analyzed the gene transcripts between different samples.

Statistical analysis

All quantified data are presented as mean ± the standard deviation (SD). To compare quantitative data between groups, students' t-tests were used. For categorical parameters, the chi-square test was used to analyze the data. Kaplan-Meier survival analysis was used to estimate OS. The correlation between groups was determined using *Pearson's correlation* tests. All statistical analyses in this study were performed using SPSS-24 (IBM, NY, USA) and Prism 6 (GraphPad). $P < 0.05$ was considered significant.

Results

miR-1915-3p is upregulated in HCC tissue samples and cell lines, and is associated with a poor prognosis for patients with HCC.

To characterize miR-1915-3p expression in human hepatic cancer, a tissue microarray containing 45 cases of normal, adjacent, and cancer tissue samples was immunohistochemically stained with a miR-

1915-3p antibody (Fig 1A). miR-1915-3p levels rose with a closing tumor, with the cancer tissue having significantly higher levels than normal or adjacent tissues. In addition, the level of miR-1915-3p was increased in most of the HCC cells lines (QGY-7701, QGY-7703, MHC-97H, HuH7, HepG2), whereas miR-1915-3p was decreased in SMMc-7721 cells in comparison with the normal hepatic cell line LO2 (Figure 1B). Next, the expression of miR-1915-3p was examined in HCC specimens (n = 45). We found that miR-1915-3p was associated with significantly increased cancer tissue compared to the adjacent cells (Figure 1C). The correlation between the clinicopathologic features of 45 HCC patients and the expression of miR-1915-3p is shown in Table 1. The results indicated that the increase of the miR-1915-3p level was significantly correlated with the parameters of tumor differentiation (p = 006) and TNM stage (p = 0.02). However, the miR-1915-3p level was not correlated with other clinical characteristics, including sex, hepatitis B surface antigen, cirrhosis, tumor number, preoperative alpha-fetoprotein, alanine, and gamma glutamyl transferase (Table 1). In addition, a Kaplan-Meier analysis showed the one-year, three-year, and five-year overall survival (OS) rates for HCC patients with high miR-1915-3p levels were significantly shorter than in patients with low miR-1915-3p levels. Moreover, HCC patients with high miR-1915-3p levels showed shorter one-year, three-year, and five-year disease-free survival rates than those with low miR-1915-3p levels (Figure 1D). These results indicated that upregulation of miR-1915-3p might play a potential role in promoting the malignant progression of HCCs.

miR-1915-3p promoted proliferation, invasion, and migration of HCC cells in vitro.

To further investigate the role of miR-1915-3p in HCCs, both loss and gain-of-function experiments were performed in HCCHepG2 and SMMC-7721 cell lines. As detected by qRT-PCR, we confirmed that the miR-1915-3p was successfully overexpressed by stable infection of miR-1915-3p lentiviral vectors in SMMC-7721, while the miR-1915-3p in HepG2 cells was downregulated by stable infection of anti-miR-1915-3p lentiviral vectors (Figure 2A). The CCK-8 assay showed that upregulation of miR-1915-3p in HCCSMMC-7721 cells increased cell proliferation, whereas dilution of miR-1915-3p in HepG2 cells inhibited cell proliferation (Figure 2B). Moreover, a wound-healing and transwell assay showed that the HCCSMMC-7721- miR-1915-3p cells had a higher wound-closure speed and cell invasion capacity than the SMMC-7721 cell, whereas the HepG2-anti-miR-1915-3p cells showed a lower migratory and invasive capacity than HepG2 cells (Figure 2C and D). Collectively, these results indicate that miR-1915-3p functions as a tumor promoter and accelerates cell proliferation, migration, and invasion in HCC cells.

miR-1915-3p inhibits apoptosis via downregulation of proapoptotic factors and upregulation of antiapoptotic Bcl-2.

The Bcl-2 family is categorized into anti-apoptotic and proapoptotic molecules, and the cell life and death are modulated by the balance between these two subsets. As shown in Figures 3A and B, the expression of the antiapoptotic molecule Bcl-2 was increased after miR-1915-3p upregulation in HCCSMMC-7721 cells, whereas the levels of proapoptotic molecules, including caspase3, caspase8, BAD, Bcl2L11, and P53, were all decreased. In contrast, inhibition of miR-1915-3p results in the downregulation of Bcl-2 and

the upregulation of proapoptotic molecules in HepG2 cells. Therefore, these data demonstrate that miR-1915-3p plays a critical role in HCC cell survival by increasing Bcl-2 expression.

Bcl2L11 was found as one of the targets for miR-1915-3p.

In order to investigate the underlying mechanisms for how miR-1915-3p exerts its functional impacts on HCC cells, TargetScan (http://www.targetscan.org) was used to identify potential miR-1915-3p target genes (Figure 4A). Among the identified potential targets, Bcl2L11 was first due to its crucial role in the apoptosis in HCC cells(27). Bcl2L11 has been previously reported to be a target of several miRNAs in HCC (28) and other cancers (29, 30). We found the complementary sequence of miR-1915-3p in the 3'-UTR of Bcl2L11 mRNA (Figure 4B). To further confirm Bcl2L11 was a direct target of miR-1915-3p, we performed a luciferase reporter gene assay. The results demonstrated that miR-1915-3p overexpression resulted in a decrease luciferase activity in wild-type (wt) construct of Bcl2L11 3'UTR, but there was no significant change of luciferase activity observed in mutant (mt) (Figure 4B). The relationship between miR-1915-3p and Bcl2L11 was analyzed in qRT-PCR. The results indicated that miR-1915-3p overexpression significantly decreased the Bcl2L11 level in SMMC-7721 cells, whereas a miR-1915-3p knockdown increased the level of Bcl2L11 in HepG2 (Figure 4C). Then, to further elucidate whether miR-1915-3p executed its function by suppressing Bcl2L11, we transfected SMMC7721-miR-1915-3p or HepG2-anti-miR-1915-3p cells with Bcl2L11 overexpression or Bcl2L11 silence, respectively. The results showed that Bcl2L11 expression was significantly decreased in SMMC7721-miR-1915-3p cells, even though Bcl2L11 overexpression existed. Moreover, Bcl2L11 expression was significantly increased despite the Bcl2L11 silencing in HepG2-anti-miR-1915-3p cells (Figure 4D). Therefore, these data suggested that Bcl2L11 could be a direct downstream target of miR-1915-3p in HCCs.

Bcl2L11 mediates the functional effects of miR-1915-3p on HCC cells.

Then, rescue experiments were performed to ascertain whether miR-1915-3p executed its functional effects by suppressing its target genes. A restoration of Bcl2L11 blocked the miR-1915-3p-mediated promotive effects on proliferation (Figure 5A), migration (Figure 5B), and invasion (Figure 5C) in SMMC-7721 cells. In addition, silencing of Bcl2L11 results in a reverse effect, which abrogates the inhibitory function of miR-1915-3p loss on HepG2 cells (Figure 5A-C). Taken together, our results revealed that Bcl2L11 acted as a functional downstream target of miR-1915-3p in HCC cells.

Bcl2L11 mediated the effects of miR-1915-3p in vivo.

The roles of miR-1915-3p-Bcl2L11 signaling on modulating tumor metastasis and tumor growth were examined in vivo. Restoration of Bcl2L11 expression (low-dose, medium-dose, and high-dose Bcl2L11) in SMMC7721-miR-1915-3p cells resulted in a significant decrease in tumor weight and volume compared to controls (Figure 6A-C). In addition, different Bcl2L11 concentration treatments induced significant apoptosis in a dose-dependent manner compared with the group of mice treated with saline (Figure 6D).

Discussion

Recurrence after resection or ablation and progression after effective chemoembolization are major drawbacks in the management of hepatocellular carcinomas. Thus, to understand the underlying mechanisms of HCC recurrence and to target the progression in order to provide more effective treatments of HCCs are urgent necessities. Since the initial application of miRNA in HCC, significant effort has been devoted to the development of therapeutics that utilize this pathway (31). Numerous microRNAs, including microRNA-195 (32), microRNA-494 (33) and microRNA-657(34), significantly suppressed or promoted HCC metastasis and progression, indicating the modulation of miRNA activity represented an attractive strategy for recurrence and progression of HCCs. Unfortunately, the underlying molecular mechanisms by these miRNAs in HCCs have not been fully revealed. In our study, the expression of miR-1915-3p, including HCC cell lines and tissue samples from humans, was a significant upregulation, which was consistent with previous reports (20). However, previous reports revealed only miR-1915-3p as an oxidative stress-responsive microRNA, but revealed its biological effect in HCC. This prompted us to speculate that miR-1915-3p may be important, but frequently neglected when correlated to metastasis or recurrence of HCC.

Similar with other findings, the serum level of miR-1915-3p was upregulated in breast cancer (17) and diffuse glioma patients (18) when compared with healthy volunteers. Our study has, for the first time to our knowledge, shown that the level of miR-1915-3p was higher in human liver cancer samples versus adjacent and normal samples. In addition, we also found miR-1915-3p was upregulated in HCC cell lines and associated with a poor prognosis for patients with HCC. In light of the expression level of miR-1915-3p that was decreased in the gastric cancer (16) and breast cancer tissues (35) and their cell lines, we postulated that miR-1915-3p may play different roles in various cancers, although further investigation is needed.

The aim of treatment of HCC is to increase survival while maintaining the highest quality of life. Survival benefits have been proven by surgical resection, ablation (36), lenvatinib (37), and regorafenib (38) treatments in HCC. However, tumor recurrence, including true recurrence due to de novo tumors with the oncogenic liver, complicates 70% of case at five years out (39). Our results showed that upregulation of miR-1915-3p promoted HCC progression by accelerating proliferation, migration, and invasion in vitro, whereas miR-1915-3p knockdown inhibited these activities. A recent study demonstrated that miR-1915-3p promotes the migration and proliferation of certain human breast cancer cells (17), which supports the oncogene role of miR-1915-3p in HCC. It should be noted that high miR-1915-3p expression levels also resulted in the reduction of proliferation and migration in breast cancer cell MCF-7 (35), and showed longer overall survival time than those with low miR-1915-3p expression levels via activation of Bcl-2 in gastric cancer patients (16). In our study, however, high miR-1915-3p expression predicted a reduced OS and DFS in HCC patients. The findings that both high and low miR-1915-3p expressions could augment the biological behavior of cancer cells seems contradictory. Nevertheless, previous studies have demonstrated that ERK1/2 could be activated by high miR-1915-3p expression (17). Moreover, ERK1/2 could act as a positive-regulator of Bcl-2(40, 41). Thus, we have postulated that the biological behavior of cancer cells could be promoted through an indirect activation of Bcl-2 via ERK1/2 signaling at a high miR-

1915-3p expression in breast cancer, and through direct activation of Bcl-2 via miR-1915-3p-binding mechanism at a low miR-1915-3p expression in gastric cancer.

The scientific interests in exploring the mechanisms responsible for cell apoptosis in HCC have blossomed for decades. Previous studies have demonstrated that the caspase and Bcl-2 family play an important role during the initiation of apoptosis in HCC invasion and metastasis (42-44). In our in vitro study, miR-1915-3p increased antiapoptotic molecule expression (Bcl-2) and decreased the expression of proapoptotic molecules (caspase3, caspase8, BAD, Bcl2L11, and P53) in HCC cells. The evidence indicated that miR-1915-3p possibly contributes to the development and progression of HCC by regulating the caspase and Bcl-2 family. Our findings may provide possible research directions for pathological mechanisms of HCC recurrence or progression, which better early detection and isolation.

Bcl2L11, a member of the Bcl-2 family, reportedly conferred dual regulatory cell growth and apoptosis in gastric cancer (29), non-small cell lung cancer (45) and ovarian cancer (46). Based on the gene expression profiles and bioinformatics analysis, we found Bcl2L11 was a direct target gene of miR-1915-3p. On the one hand, Bcl2L11 overexpression suppressed promotion of the miR-1915-3p-mediated proliferation, migration, and invasion in SMMC-7721 cells. On the other hand, the silencing of Bcl2L11 resulted in a reverse effect, which abrogated the inhibitory function of miR-1915-3p loss on HepG2 cells. In addition, Bcl2L11 protected against miR-1915-3p-induced increase of tumor weight via promotion of apoptosis of the cells. Most importantly, our study has, for the first time, offered miR-1915-3p/Bcl2L11, a novel therapeutic target for metastasis and progression in HCC.

Conclusions

Collectively, to our knowledge, we presented the first evidence that miR-1915-3P was highly expressed in metastasis HCC tissue samples and cell lines. In addition, its upregulation was related to the poor prognosis of HCC patients, as well as the idea that miR-1915-3p promoted proliferation, invasion, and metastasis of HCC cells by suppression of Bcl2L11. While there clearly remains significant work to be done in HCC therapies, our findings highlight the roles of miR-1915-3p involved in HCC metastasis and progression, which will enable us to use it as a potential therapeutic target.

Declarations

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Authors' contributions

WH and SL designed the study and wrote the manuscript. WH, XC and SL mainly performed the experiments. LS and XC performed bioinformatics analysis and analysed the data. XS and JC performed molecular modelling and virtual screening procedure. YS supervised the study and was responsible for

funding acquisition. YS and YL conceived, designed and supervised the study, wrote and revised the manuscript. The author(s) read and approved the final manuscript.

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Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Liuzhou Municipal Liutie Central Hospital.

Consent for publication

Not applicable.

Competing interests

The author declare no competing financial interests.

Author details

[1] Department of Clinical Laboratory, Affiliated Liutie Central Hospital of Guangxi medical university, No.14 Fei-e Road, Liuzhou, 545007 Guangxi China.

[2] Department of Pathology, Affiliated Liutie Central Hospital of Guangxi medical university, No.14 Fei-e Road, Liuzhou, 545007 Guangxi China

[3] Department of Oncology, Affiliated Liutie Central Hospital of Guangxi medical university, No.14 Fei-e Road, Liuzhou, 545007 Guangxi China.

[4] Department of Hepatobiliary Surgery, Affiliated Liutie Central Hospital of Guangxi medical university, No.14 Fei-e Road, Liuzhou, 545007 Guangxi China.

References

1. Forner A, Reig M, Bruix J. Hepatocellular carcinoma. Lancet (London, England). 2018;391(10127):1301-14.
2. Su J, Li J, Yu Q, Wang J, Li X, Yang J, et al. Exosomal miRNAs as potential biomarkers for acute myocardial infarction. IUBMB life. 2020;72(3):384-400.
3. Salem R, Gordon AC, Mouli S, Hickey R, Kallini J, Gabr A, et al. Y90 Radioembolization Significantly Prolongs Time to Progression Compared With Chemoembolization in Patients With Hepatocellular Carcinoma. Gastroenterology. 2016;151(6):1155-63.e2.
4. Vilgrain V, Pereira H, Assenat E, Guiu B, Ilonca AD, Pageaux GP, et al. Efficacy and safety of selective internal radiotherapy with yttrium-90 resin microspheres compared with sorafenib in locally advanced and inoperable hepatocellular carcinoma (SARAH): an open-label randomised controlled phase 3 trial. The Lancet Oncology. 2017;18(12):1624-36.
5. Kato M, Slack FJ. microRNAs: small molecules with big roles - C. elegans to human cancer. Biology of the cell. 2008;100(2):71-81.
6. Kloosterman WP, Plasterk RH. The diverse functions of microRNAs in animal development and disease. Developmental cell. 2006;11(4):441-50.
7. Iorio MV, Croce CM. MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. EMBO molecular medicine. 2012;4(3):143-59.
8. Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. Nature reviews Drug discovery. 2017;16(3):203-22.
9. Su H, Yang JR, Xu T, Huang J, Xu L, Yuan Y, et al. MicroRNA-101, down-regulated in hepatocellular carcinoma, promotes apoptosis and suppresses tumorigenicity. Cancer research. 2009;69(3):1135-42.
10. Pineau P, Volinia S, McJunkin K, Marchio A, Battiston C, Terris B, et al. miR-221 overexpression contributes to liver tumorigenesis. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(1):264-9.
11. Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. Gastroenterology. 2007;133(2):647-58.
12. Wong QW, Ching AK, Chan AW, Choy KW, To KF, Lai PB, et al. MiR-222 overexpression confers cell migratory advantages in hepatocellular carcinoma through enhancing AKT signaling. Clinical cancer research : an official journal of the American Association for Cancer Research. 2010;16(3):867-75.
13. Furuta M, Kozaki KI, Tanaka S, Arii S, Imoto I, Inazawa J. miR-124 and miR-203 are epigenetically silenced tumor-suppressive microRNAs in hepatocellular carcinoma. Carcinogenesis. 2010;31(5):766-76.
14. Wong QW, Lung RW, Law PT, Lai PB, Chan KY, To KF, et al. MicroRNA-223 is commonly repressed in hepatocellular carcinoma and potentiates expression of Stathmin1. Gastroenterology. 2008;135(1):257-69.

15. Coulouarn C, Factor VM, Andersen JB, Durkin ME, Thorgeirsson SS. Loss of miR-122 expression in liver cancer correlates with suppression of the hepatic phenotype and gain of metastatic properties. Oncogene. 2009;28(40):3526-36.
16. Cui HW, Han WY, Hou LN, Yang L, Li X, Su XL. miR-1915-3p inhibits Bcl-2 expression in the development of gastric cancer. Bioscience reports. 2019;39(5).
17. Guo J, Liu C, Wang W, Liu Y, He H, Chen C, et al. Identification of serum miR-1915-3p and miR-455-3p as biomarkers for breast cancer. PloS one. 2018;13(7):e0200716.
18. Ohno M, Matsuzaki J, Kawauchi J, Aoki Y, Miura J, Takizawa S, et al. Assessment of the Diagnostic Utility of Serum MicroRNA Classification in Patients With Diffuse Glioma. JAMA network open. 2019;2(12):e1916953.
19. Xu C, Li H, Zhang L, Jia T, Duan L, Lu C. MicroRNA-1915-3p prevents the apoptosis of lung cancer cells by downregulating DRG2 and PBX2. Molecular medicine reports. 2016;13(1):505-12.
20. Wan Y, Cui R, Gu J, Zhang X, Xiang X, Liu C, et al. Identification of Four Oxidative Stress-Responsive MicroRNAs, miR-34a-5p, miR-1915-3p, miR-638, and miR-150-3p, in Hepatocellular Carcinoma. Oxidative medicine and cellular longevity. 2017;2017:5189138.
21. Wittekind C. [Pitfalls in the classification of liver tumors]. Der Pathologe. 2006;27(4):289-93.
22. Llovet JM, Di Bisceglie AM, Bruix J, Kramer BS, Lencioni R, Zhu AX, et al. Design and endpoints of clinical trials in hepatocellular carcinoma. Journal of the National Cancer Institute. 2008;100(10):698-711.
23. Sun Y, Long H, Sun L, Sun X, Pang L, Chen J, et al. PGM5 is a promising biomarker and may predict the prognosis of colorectal cancer patients. Cancer cell international. 2019;19:253.
24. Luo C, Yin D, Zhan H, Borjigin U, Li C, Zhou Z, et al. microRNA-501-3p suppresses metastasis and progression of hepatocellular carcinoma through targeting LIN7A. Cell death & disease. 2018;9(5):535.
25. Huang W, Tan M, Wang Y, Liu L, Pan Y, Li J, et al. Increased intracellular Cl(-) concentration improves airway epithelial migration by activating the RhoA/ROCK Pathway. Theranostics. 2020;10(19):8528-40.
26. Wang L, Tang ZY, Qin LX, Wu XF, Sun HC, Xue Q, et al. High-dose and long-term therapy with interferon-alfa inhibits tumor growth and recurrence in nude mice bearing human hepatocellular carcinoma xenografts with high metastatic potential. Hepatology (Baltimore, Md). 2000;32(1):43-8.
27. Seitz SJ, Schleithoff ES, Koch A, Schuster A, Teufel A, Staib F, et al. Chemotherapy-induced apoptosis in hepatocellular carcinoma involves the p53 family and is mediated via the extrinsic and the intrinsic pathway. International journal of cancer. 2010;126(9):2049-66.
28. Urtasun R, Elizalde M, Azkona M, Latasa MU, García-Irigoyen O, Uriarte I, et al. Splicing regulator SLU7 preserves survival of hepatocellular carcinoma cells and other solid tumors via oncogenic miR-17-92 cluster expression. Oncogene. 2016;35(36):4719-29.
29. Zhang H, Duan J, Qu Y, Deng T, Liu R, Zhang L, et al. Onco-miR-24 regulates cell growth and apoptosis by targeting BCL2L11 in gastric cancer. Protein & Cell. 2016;7(2):141-51.

30. Cai Y, Wang W, Guo H, Li H, Xiao Y, Zhang Y. miR-9-5p, miR-124-3p, and miR-132-3p regulate BCL2L11 in tuberous sclerosis complex angiomyolipoma. Laboratory investigation; a journal of technical methods and pathology. 2018;98(7):856-70.
31. Rossi JJ. New hope for a microRNA therapy for liver cancer. Cell. 2009;137(6):990-2.
32. Wang R, Zhao N, Li S, Fang JH, Chen MX, Yang J, et al. MicroRNA-195 suppresses angiogenesis and metastasis of hepatocellular carcinoma by inhibiting the expression of VEGF, VAV2, and CDC42. Hepatology (Baltimore, Md). 2013;58(2):642-53.
33. Chuang KH, Whitney-Miller CL, Chu CY, Zhou Z, Dokus MK, Schmit S, et al. MicroRNA-494 is a master epigenetic regulator of multiple invasion-suppressor microRNAs by targeting ten eleven translocation 1 in invasive human hepatocellular carcinoma tumors. Hepatology (Baltimore, Md). 2015;62(2):466-80.
34. Zhang L, Yang L, Liu X, Chen W, Chang L, Chen L, et al. MicroRNA-657 promotes tumorigenesis in hepatocellular carcinoma by targeting transducin-like enhancer protein 1 through nuclear factor kappa B pathways. Hepatology (Baltimore, Md). 2013;57(5):1919-30.
35. Jin ML, Kim YW, Jin HL, Kang H, Lee EK, Stallcup MR, et al. Aberrant expression of SETD1A promotes survival and migration of estrogen receptor α-positive breast cancer cells. International journal of cancer. 2018;143(11):2871-83.
36. Llovet JM, Real MI, Montaña X, Planas R, Coll S, Aponte J, et al. Arterial embolisation or chemoembolisation versus symptomatic treatment in patients with unresectable hepatocellular carcinoma: a randomised controlled trial. Lancet (London, England). 2002;359(9319):1734-9.
37. Kudo M, Finn RS, Qin S, Han KH, Ikeda K, Piscaglia F, et al. Lenvatinib versus sorafenib in first-line treatment of patients with unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial. Lancet (London, England). 2018;391(10126):1163-73.
38. Bruix J, Qin S, Merle P, Granito A, Huang YH, Bodoky G, et al. Regorafenib for patients with hepatocellular carcinoma who progressed on sorafenib treatment (RESORCE): a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet (London, England). 2017;389(10064):56-66.
39. Hasegawa K, Kokudo N, Makuuchi M, Izumi N, Ichida T, Kudo M, et al. Comparison of resection and ablation for hepatocellular carcinoma: a cohort study based on a Japanese nationwide survey. Journal of hepatology. 2013;58(4):724-9.
40. Joo MC, Jang CH, Park JT, Choi SW, Ro S, Kim MS, et al. Effect of electrical stimulation on neural regeneration via the p38-RhoA and ERK1/2-Bcl-2 pathways in spinal cord-injured rats. Neural regeneration research. 2018;13(2):340-6.
41. Chen G, Li P, Liu Z, Zeng R, Ma X, Chen Y, et al. Enrichment of miR-126 enhances the effects of endothelial progenitor cell-derived microvesicles on modulating MC3T3-E1 cell function via Erk1/2-Bcl-2 signalling pathway. Prion. 2019;13(1):106-15.
42. Zhong P, Yang H, Lin S, Peng J, Lin J. A Traditional Chinese Medicine Herb Mixture Qingjie Fuzheng Granules Inhibits Hepatocellular Carcinoma Cells Growth by Inducing Apoptosis. Journal of evidence-based integrative medicine. 2018;23:2515690x18789632.

43. Hong NR, Park HS, Ahn TS, Jung MH, Kim BJ. Association of a Methanol Extract of Rheum undulatum L. Mediated Cell Death in AGS Cells with an Intrinsic Apoptotic Pathway. Journal of pharmacopuncture. 2015;18(2):26-32.
44. Cillessen SA, Meijer CJ, Notoya M, Ossenkoppele GJ, Oudejans JJ. Molecular targeted therapies for diffuse large B-cell lymphoma based on apoptosis profiles. The Journal of pathology. 2010;220(5):509-20.
45. Baumgartner U, Berger F, Hashemi Gheinani A, Burgener SS, Monastyrskaya K, Vassella E. miR-19b enhances proliferation and apoptosis resistance via the EGFR signaling pathway by targeting PP2A and BIM in non-small cell lung cancer. Molecular cancer. 2018;17(1):44.
46. Chen J, Lin Y, Jia Y, Xu T, Wu F, Jin Y. LncRNA HAND2-AS1 exerts anti-oncogenic effects on ovarian cancer via restoration of BCL2L11 as a sponge of microRNA-340-5p. Journal of cellular physiology. 2019;234(12):23421-36.

Tables

Table1 Correlation between the clinicopathologic characteristics and miR-1915-3p expression in HCCs

Clinical parameters		miR-1915-3p		P value
		Low (n=45)	High (n=45)	
Age (years)				0.138
	≤50	24	17	
	>50	21	28	
Gender				0.270
	Male	39	35	
	Female	6	10	
HBsAg				0.764
	Positive	38	39	
	Negative	7	6	
AFP (ng/ml)				0.274
	≤400	31	26	
	>400	14	19	
ALT (U/L)				0.398
	≤40	19	23	
	>40	26	22	
GGT (U/L)				0.288
	≤54	17	22	
	>54	28	23	
Cirrhosis				0.535
	No	7	5	
	Yes	38	40	
Tumor size (cm)				0.398
	≤5	26	22	
	>5	19	23	
Tumor number				0.270
	Solitary	39	35	
	Multiple	6	10	

Tumor differentiation				**0.006***
	□/□	31	18	
	□/□	14	27	
TNM stage				**0.020***
	□/□	42	34	
	□/□	3	11	

Chi-square test was used in all analysis

HBsAg hepatitis B surface antigen, AFP alpha-fetoprotein, ALT alanine aminotransferase□GGT gamma glutamyl transferase, TNM tumor–node–metastasis

*$P < 0.05$□Bold values signify P-value <0.05

Figures

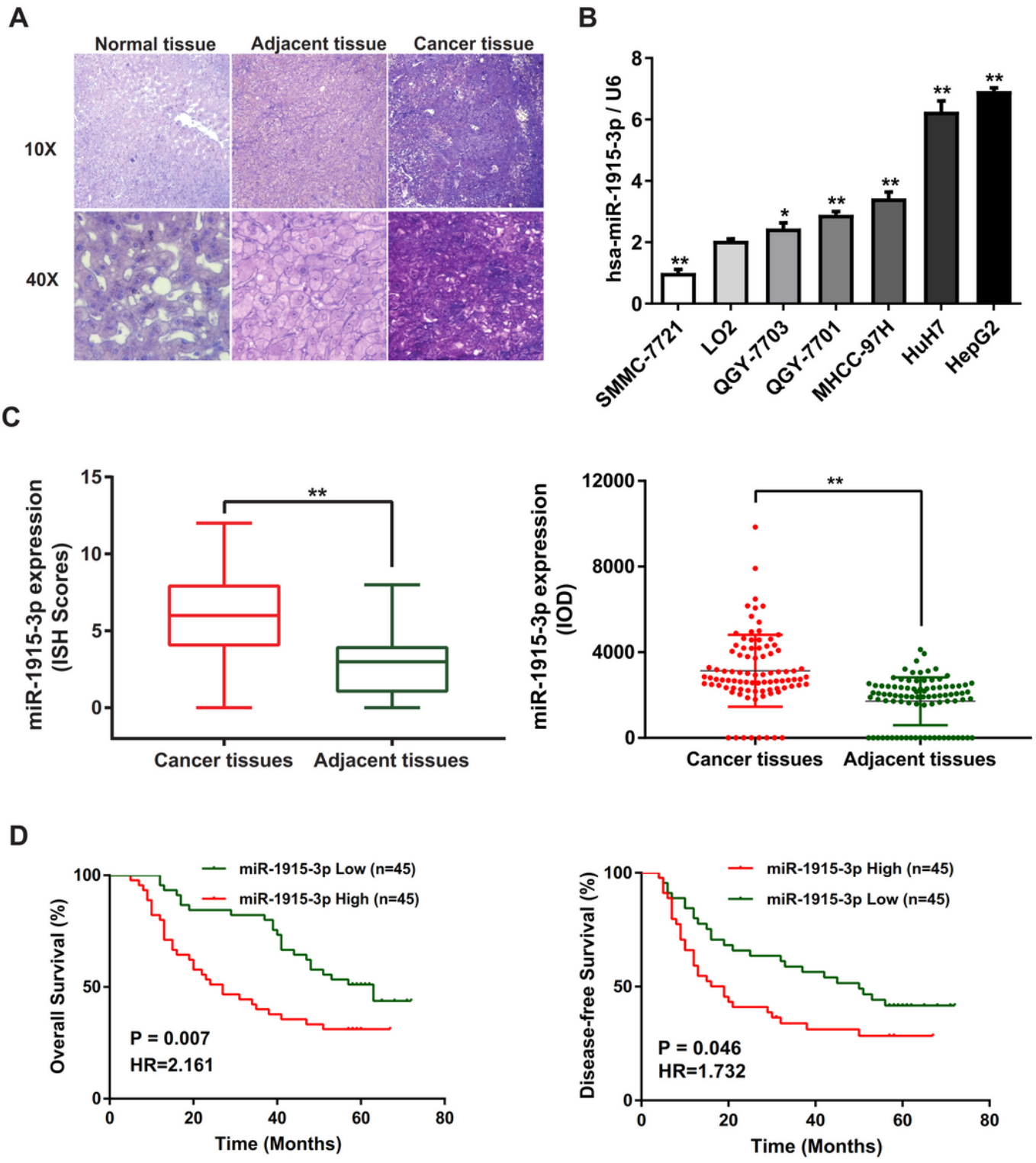

Figure 1

miR-1915-3p expression level is increased in HCC tissue samples and cell lines and associated with a poor prognosis for patients with HCC. A. miR-1915-3p immunohistochemistry-stained sections of normal and adjacent, cancer tissues of HCC in human (×400 magnification). B. The expression levels of miR-1915-3p in six HCC cell lines (SMC-7721, QGY-7701, QGY-7703, MHC-97H, HuH7, HepG2), and human liver cell lines LO2 (n = 3 independent experiments, *P < 0.05, **P < 0.01 versus LO2 cells). C. ISH and RT-

qPCR was used to detect the expression of miR-1915-3p in 45 pairs of HCC tissues and corresponding adjacent tissues. **P < 0.01 versus the corresponding adjacent tissues. D. Kaplan-Meier's analysis showed the association of miR-1915-3p expression and overall survival (OS), disease free survival (DFS) in patients with HCC (n = 45 individuals for low or high expression of miR-1915-3p patients). Data are presented as mean ± standard deviation.

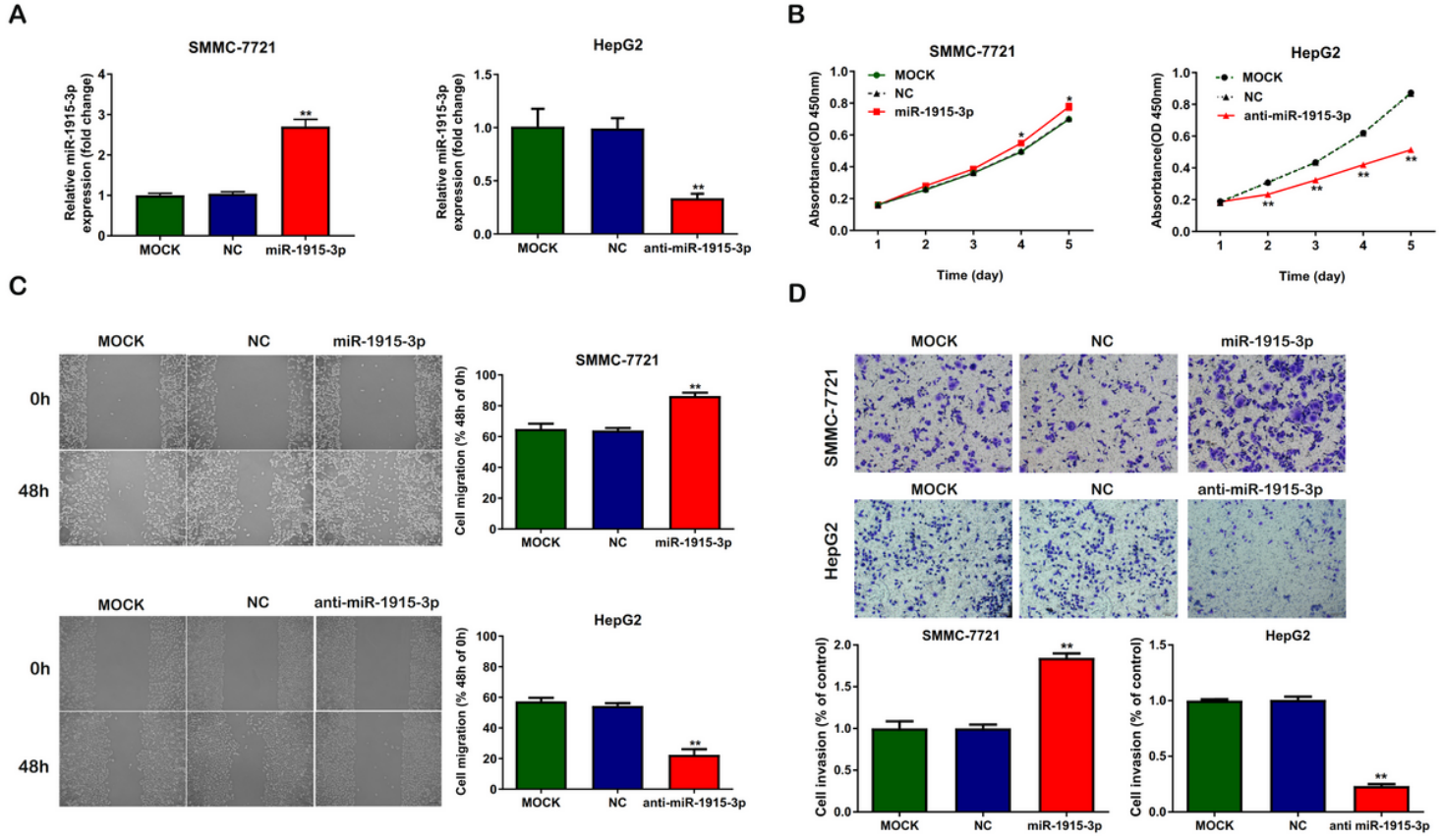

Figure 2

miR-1915-3p promotes the proliferation, migration and invasion of HCC cells in vitro. A. Expression of miR-1915-3p (n = 3 independent experiments, *P < 0.05, **P < 0,01) in SMMC-7721 and HepG2 cells that were transfected with miR-1915-3p overexpression or miR-1915-3p knockdown vectors. B. The effect of miR-1915-3p on cell proliferative ability was determined by CCK8 assay for 1, 2, 3, 4 and 5 day (n = 3 independent experiments, *P < 0.05, **P < 0,01). C. Wound healing as assessed by scratch assay and the quantification of the percentage of wound area were shown (n = 3 independent experiments, Scal bar: 50 μm, **P < 0,01). D. Cell invasion was examined using transwell invasion assays after knockdown or overexpression of miR-1915-3p in HCC cells (n = 3 independent experiments, Scal bar: 50 μm, **P < 0,01). Data are presented as mean ± standard deviation.

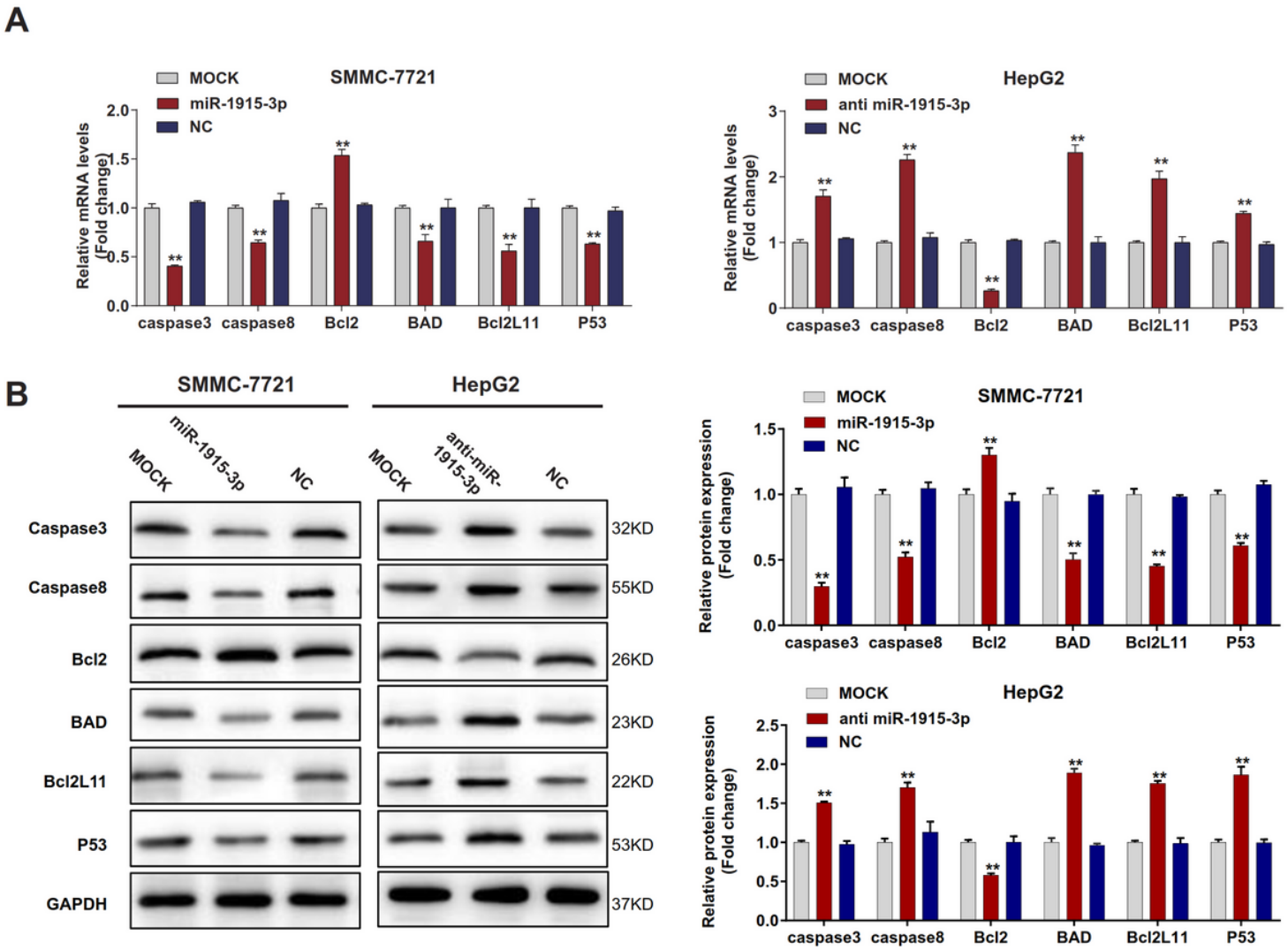

Figure 3

miR-1915-3p inhibits apoptosis via downregulation of proapoptotic factors and upregulation of antiapoptotic Bcl2. qRT-PCR (A) and western blot analysis (B) show changes in proapoptotic factors (caspase3, caspase8, BAD, Bcl2L11 and P53) and antiapoptotic Bcl2 following stable downregulation or upregulation of miR-1915-3p expression (n = 3 independent experiments, **P < 0,01). Data are presented as mean ± standard deviation.

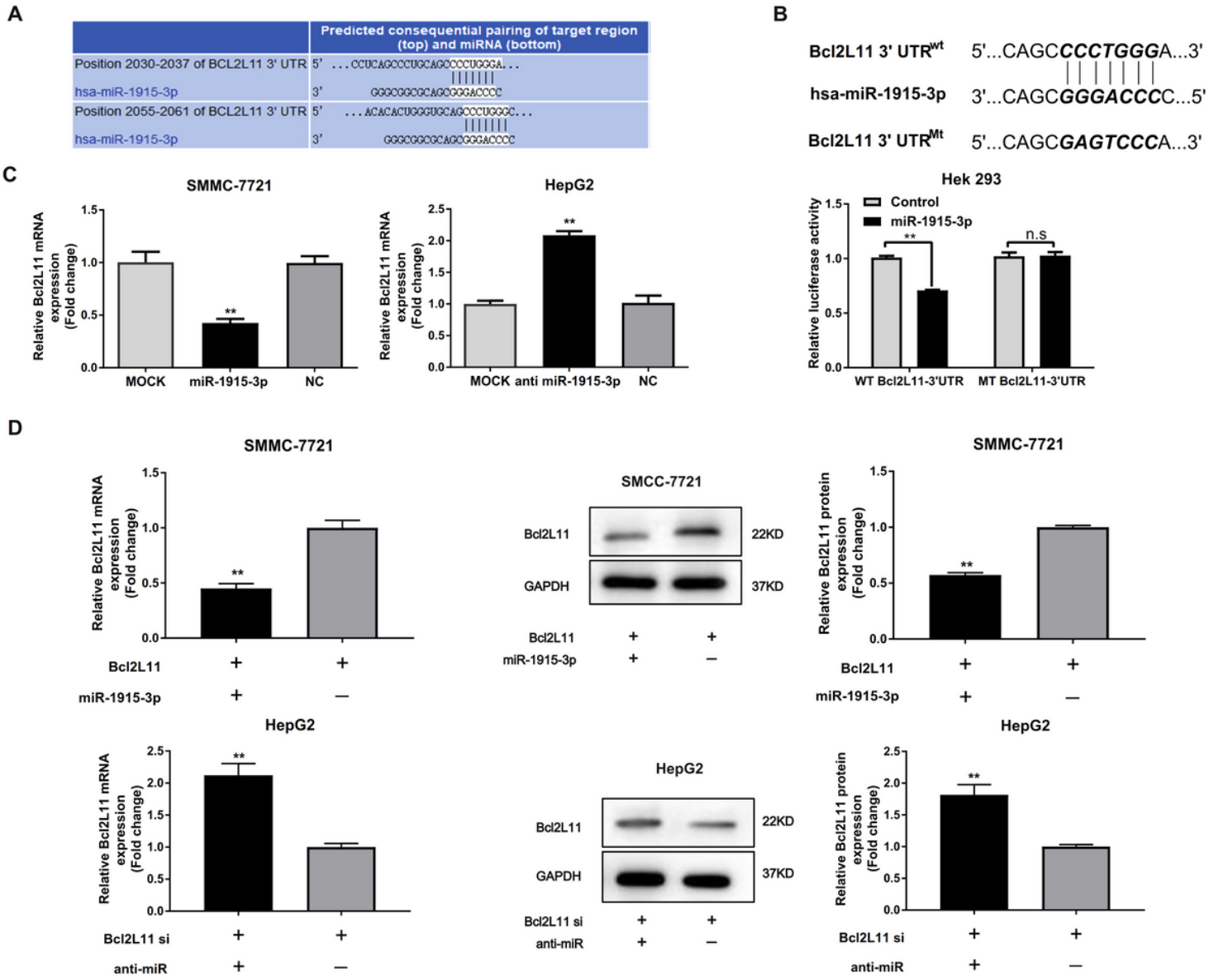

Figure 4

miR-1915-3p inhibits apoptosis via downregulation of proapoptotic factors and upregulation of antiapoptotic Bcl2. qRT-PCR (A) and western blot analysis (B) show changes in proapoptotic factors (caspase3, caspase8, BAD, Bcl2L11 and P53) and antiapoptotic Bcl2 following stable downregulation or upregulation of miR-1915-3p expression (n = 3 independent experiments, **P < 0,01). Data are presented as mean ± standard deviation.

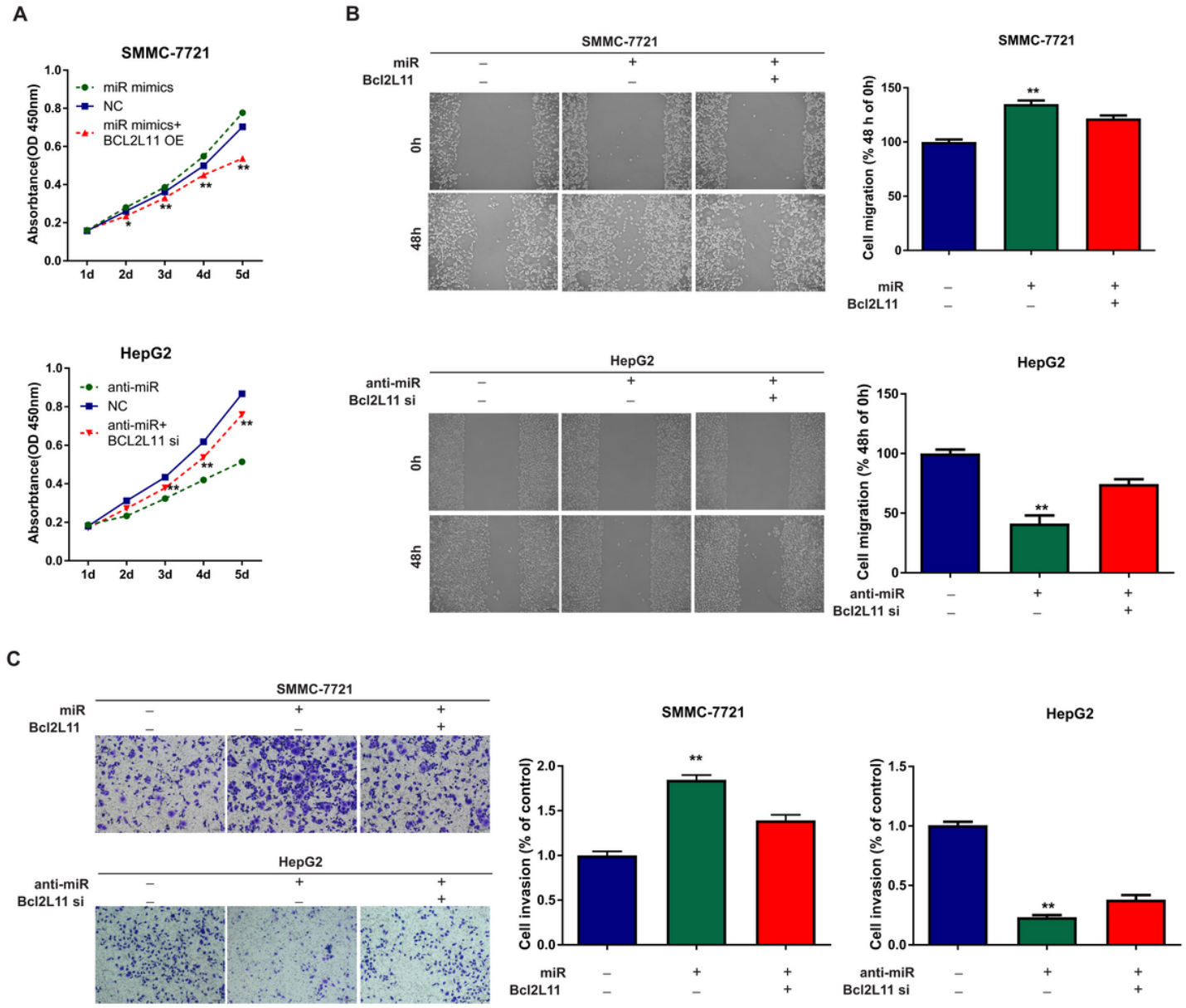

Figure 5

Modulation of Bcl2L11 expression inhibited miR-1915-3p-mediated cellular activities in vitro. A. CCK8 assay showed the proliferation of SMMC-7721-miR1915-3p that were transfected with Bcl2L11 vector and HepG2-miR1915-3p cells that were transfected with Bcl2L11 vector and their corresponding control (n = 3 independent experiments, **P < 0,01). The efects of Bcl2L11 on the migration and invasion of HCC cells was determined by B wound healing assay and C transwell assay (n = 3 independent experiments, scal bar: 50 μm, **P < 0,01). Data are presented as mean ± standard deviation.

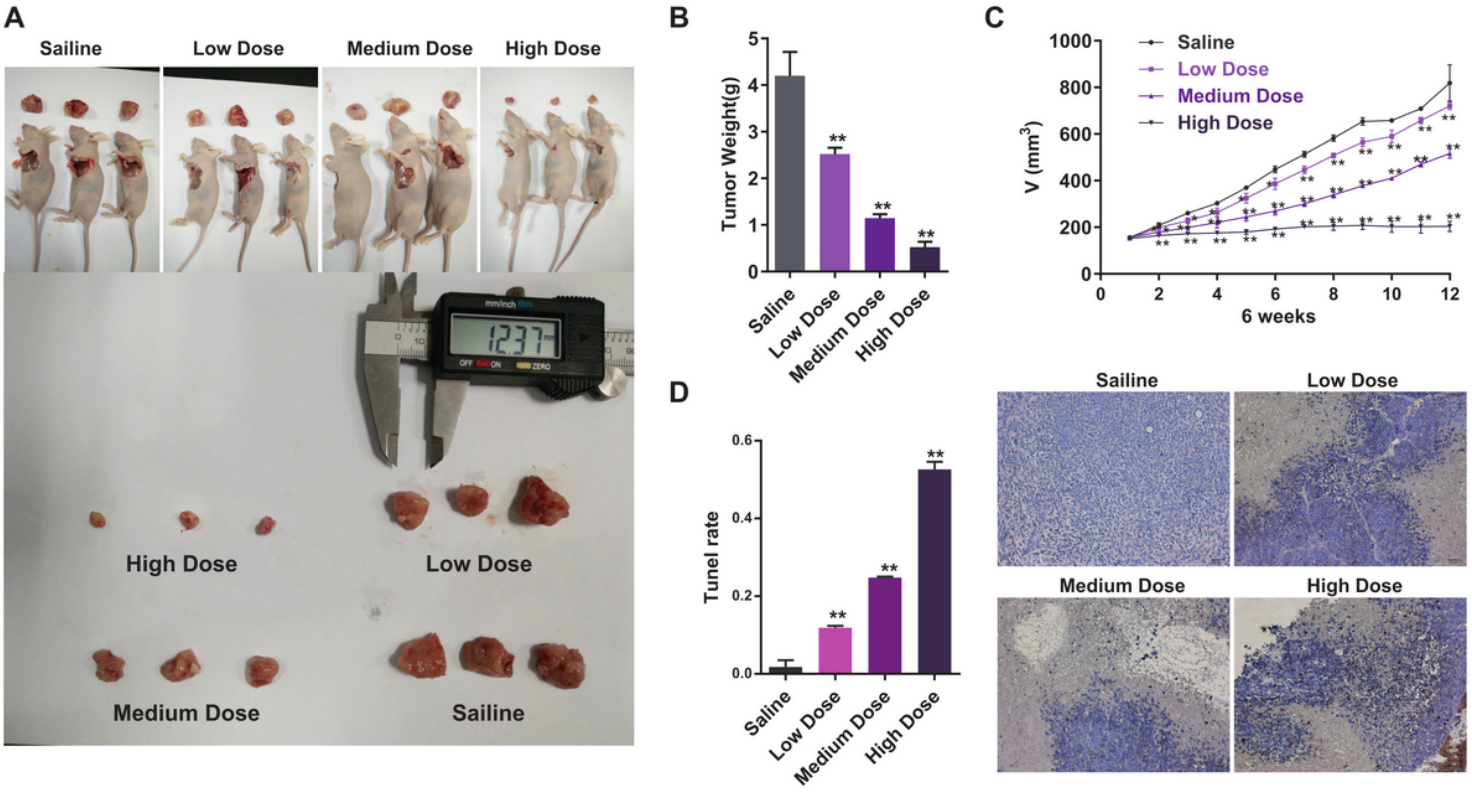

Figure 6

The effects of Bcl2L11 on miR-1915-3p-mediated functions in vivo. A. Macrograph of tumors after treatment with different Bcl2L11 concentration. B. Tumor weights were calculated(n = 3 independent experiments, **P < 0,01). C. Measurement of tumor volumes at the indicated time points(n = 3 independent experiments, **P < 0,01). D. A TUNEL assay for detecting apoptotic cells from tumor tissues after treatment with different Bcl2L11 concentration (n = 3 independent experiments, scal bar: 20 μm, **P < 0,01).