

miR-1269a promotes tumor growth and progression of liver cancer via regulating Rbms3/c-Myc axis

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Abstract

Background: MicroRNAs play important roles in regulating liver cancer formation, progression and metastasis. miR-1269a, a cancer associated miRNA, its role in liver cancer is still unclear. **Methods:** The clinical, survival and miR-1269a expression of both liver cancer and normal tissues were analyzed from the TCGA database. The effects of miR-1269a on liver cancer cells were investigated through cell counting kit-8 assay, Ki67 immunofluorescence staining and animal models (Balb/c nude mice). The interaction between miR-1269a and Rbms3 was investigated via luciferase reporter assay. **Results:** Here, we showed that miR-1269a was highly expressed in primary patient tumor tissues using the TCGA database. miR-1269a inhibition through overexpressing its sponge in HepG2 and Hep3B cells significantly repressed cell proliferation, but had no effect on cell apoptosis and migration. Through directly targeting the 3' untranslated region, miR-1269a inhibits Rbms3 expression. Rbms3 inhibition promoted the expression of c-Myc, resulting in the increased proliferation of liver cancer cells. **Conclusions:** Our results reveal a novel miR-1269a/Rbms3/c-Myc axis, which plays a critical role in liver tumor growth and progression, and may serve as a promising biomarker for the early diagnosis of liver cancer, as well as a possible therapeutic target in liver cancer treatments.

Background

Liver cancer, including hepatocellular carcinoma and hepatoblastoma, is the third most prevalent cause of tumor related death and leads to 745,500 worldwide deaths each year [1, 2]. Among all cancers, the liver cancer has the second poorest survival rate and the 5-year relative survival rate is only 10.1% in China [3]. One key reason of the high mortality is the lack of diagnosis at the early stage of this disease [4, 5]. Early-stage diagnosis of liver cancer offers effective treatment options, including surgical resection or liver transplantation [6]. However, for patients with advanced disease, treatments are still unsatisfactory due to the poor prognosis and high frequency of tumor recurrence [7]. Thus, the critical challenge in liver cancer is to diagnose the disease as early as possible. Clarification of the underlying molecular mechanisms of liver cancer formation and progression, as well as the identification of novel biomarkers for early diagnosis and effective intervention, is urgently needed.

Similar to other cancers, liver cancer, a highly heterogeneous disease, is driven by the cumulation of genetic and epigenetic aberrations, including gene mutations, abnormal expression of various genes, signaling network disorders and abnormal epigenetic regulation [8-11]. Epigenetic regulation, such as histone modification and DNA methylation, often occur alongside genetic regulation to affect the development of malignancies. In addition, non-coding RNA, including MicroRNAs (miRNA) and lncRNA, is reported to take indispensable roles in tumor formation and progression [12, 13]. However, the exact functions and regulatory mechanisms of these epigenetic regulators in liver cancer remain poorly understood.

miRNAs, a family of highly conserved small noncoding RNAs, control target genes expression at the post-transcriptional level via directly binding to the mRNAs and inhibiting their translation, or causing direct mRNAs degradation [13]. Interestingly, miRNAs can promote gene expression through binding to promoter regions as well [14]. Previous studies demonstrate that approximately 50% of miRNA-coding genes are situated in tumor-associated genomic regions, suggesting a close relationship between miRNAs situated and the formation and progression of cancer [15,16]. Furthermore, the relationship between miRNAs and their target genes brings new perspectives on the biological mechanisms that contribute to human cancer [17]. Specific miRNAs, such as miR-34a, let-7 and the miR-200 family, function as either promoters or suppressors of cancer via a variety of mechanisms [18-21]. Although there are numerous miRNAs that are reported to be closely related with liver cancer, there remains a large number of miRNAs with unknown functions that are closely associated with liver cancer occurrence and progression [22]. Understanding the roles of these miRNAs could be crucial for the development of new and improved methods for diagnosis or therapeutic treatments in liver cancer.

miR-1269a, belonging to the miR-1269 family, is consistently highly expressed in late-stage colorectal cancer, and its expression in primary tumors is significantly associated with an increased risk of colorectal cancer relapse [23]. However, its role in liver cancer is still unknown. Here, we investigated the relationship between miR-1269a and liver cancer, and found that the expression of miR-1269a was conspicuously higher in liver cancer tissues compared with normal tissues. Further, we elucidated that miR-1269a suppressed liver cancer cell proliferation in vitro and in vivo. Additionally, we demonstrated that miR-1269a inhibiting Rbms3 expression by directly targeting the 3' untranslated region.

Methods

TCGA data processing

Differentially expressed miRNAs: The level3 miRNA sequencing data were downloaded from TCGA database (<https://cancergenome.nih.gov/>). Some data were acquired from a total of 49 patients, including miRNA sequencing data of both cancer tissues and matched normal tissues. The different expression of miRNAs between cancer and the match normal tissues were calculated using edgeR package. Afterwards, the fold changes (FCs) of individual miRNA expressions were analyzed, and miRNAs, those differentially expressed with $\log_2|FC| > 2.0$ and $P < 0.05$, were treated to be significant.

miR-1269a expression: The reads per million miRNA data of miR-1269a were log2 transformed and the expression of miR-1269a in 49 paired liver cancer cancer tissues and matched normal tissues were calculated.

Associations of miR-1269a expression level and patients' survival: The level3 miRNAs sequencing data and the relative clinical prognosis information were obtained from TCGA database. The inclusion criterion was set as follows: cancer tissue samples containing both miR-1269a sequencing data and prognosis

1×10⁴ liver cancer cells were incubated in 24-well plates each well. After 48 h, the cells were fixed with 4% paraformaldehyde for 20 min. Afterwards, the cells were washed by PBS and permeabilized with 0.25% Triton X-100 for 5 min, then blocked using PBS (10% FBS) for 1 h. Next, cells were incubated with primary antibody anti Ki67 (abcam, Cambridge, England) in PBS (10% FBS) overnight at 4°C. After rinsing with PBS, the cells were treated with secondary antibody in PBS (10% FBS) for 2 h at room temperature. At last, the cells were incubated with Hoechst 33342 for 5 min, then washed with PBS and imaged using fluorescence microscopy (Nikon).

Reverse Transcription and Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated with Trizol reagent (TaKaRa, Dalian, China) according to the manufacturer's manual and guideline. For each sample, 500 ng RNA was reverse transcribed to cDNA with Prime-Script RT reagent kit (TaKaRa). The resulting cDNA was amplified using Takara Ex Taq PCR kit (TaKaRa). qRT-PCR was performed with the Stratagene Mx3000 QPCR system (Stratagene, Foster City, CA) and analyzed via the $\Delta\Delta$ CT method. The qRT-PCR sequences used in this study were shown as follows: Gapdh-PF: GGAGCGAGATCCCTCCAAAAT, Gapdh-PR: GGCTGTTGTCATACTTCTCATGG; Rbms3-PF: TGGACCATCCCATGTCAATGC, Rbms3-PR: CCAACGAAAGGTGATTATCTGC; c-Myc-PF: GTCAGAGGCGAACACACAAC, c-Myc-PR: TTGGACGGACAGGATGTATGC.

Luciferase reporter assay

To make the Rbms3 3'UTR vector, PCR was carried out with the lucRbms3-F: cgctcgcagGTAGGACAAGCTGCATTTTCTG and lucRbms3-R: cgcaagcttACGTTAGCCCCCACTACAAA primers (the lower-case letters represent restriction enzyme sites for XhoI and HindIII). The resulting PCR fragment was ligated to pGL-3 luciferase reporter vector opened with XhoI and HindIII. 293T cells grown in 24-well plates were transfected with 50 nM Control miRNA, miR-1269a mimics and miR-1269a inhibitor (Ribobio Corporation, Guangzhou, China), 0.5 ug pGL-3 luciferase reporter vector that contain the Rbms3 3'UTR, and 0.02 ug a control Renilla luciferase vector (pRL-TK; Promega) in the presence of Fugene HD (Roche). The activities of Firefly and Renilla luciferase in the cell lysates were detected with a Dual-Luciferase Reporter Assay System (Promega) at 24 hours after transfection.

Animal experiments

10 Male Balb/c nude mice (4 weeks old, SLAC Laboratory Animal Company, Shanghai, China) were bred according to National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) in Animal Center of Shanghai University of Medicine & Health Sciences. The Institutional Animal Care and Use Committee of Shanghai University of Medicine & Health Sciences approved all the protocols. The mice were maintained on a 12 h light/12 h dark schedule (lights on from 7:00 to 19:00). The food and water were allowed ad libitum in the SPF house. 5×10⁶ Hep3B cells from control and miR-1269a sponge groups were injected subcutaneously into the right forelimb axillary of mice. Then the mice were euthanised using pentobarbital sodium intraperitoneally (120 mg/kg) 1 h after the treatment. Tumors were excised and the weight was evaluated.

Statistical Analysis

All statistical analyses were analyzed using SPSS 17.0 software. Data were analyzed using t-test and one-way ANOVA. Error bars represented the SEM of three independent experiments. All the data were represented as mean ± SEM. *, ** and *** were indicative of p<0.05, p<0.01, and p<0.001, respectively.

Results

miR-1269a is closely associated with liver cancer

To uncover the relationship between miR-1269a and liver cancer, we calculated the expression levels of various miRNAs in both liver cancer and normal tissues from the TCGA database [25]. The top ten most significantly up-regulated miRNAs in cancer tissues were analyzed and are shown in Fig. 1a. We then examined the effect of these miRNAs on cancer survival curves and found that high expression of miR-1269a significantly reduced the survival time of cancer patients (Fig. 1b). Next, we compared the expression of miR-1269a in cancer tissues and normal tissues. The results demonstrated that miR-1269a expression was significantly increased in cancer tissues (Fig. 1c). To further verify this result, we collected carcinoma tissues from liver cancer patients and detected the expression of miR-1269a. Real-time PCR analysis revealed that the expression of miR-1269a was significantly increased in the carcinoma tissues of liver cancer patients compared to the corresponding normal tissues (Fig. 1d). These data suggest that miR-1269a might be closely associated with the degree of tumor malignancy.

Inhibition of miR-1269a can significantly suppress the proliferation of liver cancer cells

Next, we examined the function of miR-1269a in liver cancer at the cellular level. Since miR-1269a is highly expressed in liver cancer, we overexpressed a miR-1269a sponge sequence in liver cancer cell lines HepG2 and Hep3B in order to suppress its expression. Overexpression of the miR-1269a sponge resulted in a

significant downregulation of miR-1269a expression, demonstrating that the sponge sequence was effective at inhibiting miR-1269a expression (Fig. 2a). Furthermore, we examined the effects of miR-1269a on cancer cell proliferation. Overexpression of the miR-1269a sponge significantly reduced the levels of CCK8 in HepG2 and Hep3B liver cancer cells, indicating that the miR-1269a sponge significantly inhibited the proliferation of liver cancer cell lines (Fig. 2b). Moreover, an immunofluorescence staining assay showed significantly decreased levels of Ki67 following overexpression of the miR-1269a sponge, suggesting a significant reduction in the number of cells in the mitosis stage (Fig. 2c). Meanwhile, the colony formation assay showed that overexpression of the miR-1269a sponge resulted in a significant decrease in the number of clones (Fig. 2d and 2e). Together, these results indicate that when miR-1269a is inhibited, the proliferation of liver cancer cell lines is also significantly inhibited.

miR-1269a directly targets the 3' untranslated region of Rbms3 and inhibits Rbms3/c-Myc axis

We investigated the molecular mechanism of action of miR-1269a in liver cancer cells. Rbms3 is a tumor suppressor gene closely associated with tumor cell proliferation. We predicted that miR-1269a may directly target Rbms3 using Targetscan (Fig. 3a). In addition, the mRNA level of Rbms3 was significantly increased following inhibition of miR-1269a (Fig. 3b). Moreover, upregulation of Rbms3 resulted in a significant decrease in the expression of c-Myc following inhibition of miR-1269a (Fig. 3b). These results suggest that miR-1269a likely regulates the proliferation of tumor cells by regulating Rbms3 expression. We then used a luciferase reporter assay to verify whether miR-1269a could directly regulate Rbms3, and found that miR-1269a regulated Rbms3 by binding to its 3'UTR region (Fig. 3c). Notably, mutation of the 3'UTR sequence resulted in the failure of miR-1269a to bind to the 3'UTR region, indicating that miR-1269a specifically binds to the WT 3'UTR sequence of Rbms3 (Fig. 3c). To further confirm the relationship between miR-1269a and its target Rbms3, qRT-PCR analyses proved that the expression of Rbms3 was significantly downregulated in the carcinoma tissue of liver cancer patients compared to the corresponding normal tissue (Fig. 3d). The patients were divided into high- and low-level expression groups based on the level of miR-1269a expression, and we then detected the relative expression of Rbms3 and c-Myc. We found that patients in the high miR-1269a expression group had significantly downregulated expression of Rbms3, but significantly upregulated expression of c-Myc (Fig. 3e). These data indicate that miR-1269a can promote the expression of c-Myc via direct inhibition of Rbms3, and ultimately affect the proliferation of liver cancer cells.

Inhibition of miR-1269a can significantly depress tumor growth of liver cancer cells in vivo

To further explore the function of miR-1269a in liver cancer, we detect whether miR-1269a inhibit the development of liver cancer in vivo. Hep3B cells from the control group and miR-1269a sponge overexpression group, were injected subcutaneously into five Balb/c nude mice. 28 days after injection, the mice were euthanised and the solid tumors were obtained, and the volume and weight of each tumor was detected (Fig. 4a). Interestingly, the weight of tumors from miR-1269a sponge overexpression group was markedly reduced (Fig. 4b). Meanwhile, the tumor volume from miR-1269a sponge overexpression group was significantly were significantly decreased (Fig. 4c). These data reply that miR-1269a inhibition inhibit liver cancer development in vivo.

Discussion

Liver cancer is a complex disease that is associated with many pathogenic factors, including gene mutations, gene expression variation and epigenetic changes. However, the specific roles that miRNAs play in this process remain unclear. In this study, we demonstrated that miR-1269a is one of the most significantly up-regulated miRNAs in carcinoma tissues in patients with liver cancer. Survival curve analysis showed that the expression of miR-1269a was significantly associated with the survival time of cancer patients. Further study into the mechanisms of miR-1269a revealed that it binds to the cancer cell proliferation-related gene Rbms3 and inhibits its expression. Thus, our study reveals that miR-1269a promotes tumor proliferation and liver cancer by inhibiting Rbms3.

The early stage of liver cancer exhibit few symptoms, and therefore the vast majority of liver cancers are diagnosed when it has already progressed to an advanced stage. Even with recent advances in cancer medication and intervention, successful treatment for patients with liver cancer in the advanced stages remains poor [26]. Therefore, early detection and diagnosis is crucial for the effective treatment of liver cancer. Numerous miRNAs that exist in the blood for a prolonged period play important roles in the formation of liver cancer [27]. Therefore, there is great potential for the utilization of miRNAs in the blood as valuable indicators for the detection of early stage liver cancer. For example, previous studies have found that miR-216b can be used as an early indicator of liver cancer, due to its significantly lowered expression in the plasma of liver cancer patients, as well as its ability to inhibit cancer cell proliferation, migration and invasion via HBx/miR-216b/IGF2BP2 signaling [28]. In this study, we found that the expression of miR-1269a in liver cancer was abnormally upregulated. Furthermore, its expression was closely related to patient's age, carcinoma stage and survival time. Our study revealed for the first time that miR-1269a may be an important oncogene in the formation and development of liver cancer. Similarly, previous studies have found that miR-1269a is abnormally elevated in colorectal cancer and promotes tumor formation [23]. Interestingly, the TCGA dataset revealed that high expression of miR-1269a was also significantly associated with a poor survival rate in patients with kidney renal papillary cell carcinoma. Therefore, it is of interest to investigate the function of miR-1269a in other cancers such as kidney renal papillary cell carcinoma. Besides, sustaining proliferative signaling, resisting cell death and activating metastasis are hallmarks of tumorigenesis, progression and recurrence [29]. Proliferation is very important for tumor initiation. Therefore, miR-1269a may play a key role in tumorigenesis and should be a potential biomarker for the early diagnosis of liver cancer.

Rbms3, an RNA-binding protein, is expressed in adult organisms widely. Recent studies have shown that Rbms3 may act as a tumor suppressor gene in nasopharyngeal carcinoma, esophageal squamous cell carcinoma and lung squamous cell carcinoma [30-32]. Rbms3 can directly bind to the promoter region of the oncogene c-Myc and inhibit its expression. Decreased expression of Rbms3 is associated with an increased risk of tumor malignancy and advanced tumor stage. Here, we demonstrated that Rbms3 is strongly associated with carcinoma formation, and furthermore, its upstream regulator miR-1269a robustly promotes cancer formation by binding to the 3'UTR and causing mRNA degradation. Interestingly, several other miRNAs, including miR-19-3p, have been predicted to directly target the 3'UTR of Rbms3 and inhibit its expression. It may be very valuable to uncover the function of miR-19-3p in liver cancer. Thus, we reveal that the miR-1269a/Rbms3 pathway is critical in liver cancer, and their functions can possibly be extended to numerous other cancers.

c-Myc is well known as an oncogene, whose activation is a hallmark of various cancers due to the promotion of cancer cell growth and genomic instability [33-35]. Recently, it is reported that the innate role of MYC-mediated cell competition in development is conserved in human cancers [36]. In this study, we revealed miR-1269a/Rbms3 to be a novel upstream regulator of c-Myc, and explored the regulatory network of c-Myc. Thus, co-detection of the expression levels of miR-1269a, Rbms3 and c-Myc may be a promising method for the assessment of liver cancer prognosis.

There are several limitations in our study. Though the cell proliferation, cell apoptosis and migration have been detected in this work, other important features of advanced cancer, such as cell invasion, multidrug resistance, immune escape and cell metabolism, still need further study. Besides, Foxo1, a tumor suppressor that is closely associated with cell metabolism and DNA damage repair, is a potential target gene of miR-1269 [37, 38]. It is possible that miR-1269a control liver cancer cell metabolism via regulating Foxo1. Additional research should be conducted to reveal more function of miR-1269a in liver cancer in vitro and in vivo.

Conclusions

In summary, the present study demonstrated that miR-1269a is highly expressed in liver cancer, and can promote liver cancer tumor growth and development by targeting the 3'UTR of Rbms3 (Fig. 4d). We not only explored the role of a new miRNA that is closely related to liver cancer, but also revealed the role of a novel miR-1269a/Rbms3 pathway in the regulation of the oncogene c-Myc. Thus, miR-1269a is a fascinating molecule that is intrinsically involved in liver cancer pathogenesis and progression, and may serve as a potential biomarker for the early diagnosis of liver cancer as well as a prognostic indicator following liver resection.

Abbreviations

miRNAs: microRNAs; TCGA: the cancer genome atlas; Rbms3: RNA binding motif single stranded interacting protein 3; c-Myc: MYC proto-oncogene; UTR: untranslated regions.

Declarations

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interpretation of the data, or preparation of the manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

J.S., Z.D. played critical roles in experimental design and revised the paper. Y.L., Y.Z., Y.Y. performed all the experiments and wrote the paper. L.M., Y.M. analyzed the data. J.S., Z.D. wrote and revised the paper. All authors have read the manuscript (including the data) and have approved it for publication.

Ethics approval and consent to participate

All methods in this study were approved by the Research Medical Ethics Committee of Shanghai Pudong Hospital. All experimental protocols were performed in accordance with the Institutional Ethics Committee of Shanghai University of Medicine & Health Sciences. This article adheres to the ARRIVE guidelines for the reporting of animal experiments.

Consent for publication

Not applicable.

Competing interests

All authors have read the manuscript and approved to submit to your journal. No conflict of interest exists in the submission of this manuscript.

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Figure Legends

Fig. 1. miR-1269a is upregulated in liver cancer. (a) The top 10 upregulated miRNAs were ranked by $\log_2|FC|$. (b) Overall survival time in liver cancer patients, stratified into high-level and low-level groups according to median miR-1269a expression using a Kaplan-Meier curve and a Log-rank test. (c) Expression of miR-1269a in liver cancer tissues and matched normal tissues. (d) qRT-PCR analysis of miR-1269a expression in liver cancer tissue samples and matched normal tissue samples. For c and d, *** represents $p < 0.001$.

Fig. 2. miR-1269a sponge significantly suppresses the proliferation of liver cancer cells. (a) qRT-PCR analysis of miR-1269a expression in control and miR-1269a sponge-overexpressing HepG2 and Hep3B cells. (b) CCK8 analysis of control and miR-1269a sponge-overexpressing HepG2 and Hep3B cells. (c) Ki67 immunofluorescence staining analysis of the control and miR-1269a sponge-overexpressing HepG2 and Hep3B cells. (d and e) Colony formation assay analysis of control and miR-1269a sponge-overexpressing HepG2 and Hep3B cells. For b and e, *, ** and *** represent $p < 0.05$, $p < 0.01$ and $p < 0.001$.

Fig. 3. miR-1269a binds the 3' untranslated region of Rbms3 and regulates its expression. (a) Putative binding of miR-1269a and Rbms3 predicted by Targetscan software. (b) qRT-PCR analysis of Rbms3 and c-Myc expression in control and miR-1269a sponge-overexpressing HepG2 and Hep3B cells. (c) Left: dual luciferase assay analysis showing the interaction of miR-1269a and the Rbms3 3'UTR. 293T cells were co-transduced with retroviruses containing control siRNA + WT UTR, miR-1269a siRNA + WT UTR or miR-1269a inhibitor + WT UTR; right: dual luciferase assay analysis showing the interaction of miR-1269a and the Rbms3 3'UTR. 293T cells were co-transduced with miR-1269a siRNA + control UTR, miR-1269a siRNA + WT UTR or miR-1269a siRNA + MT UTR. (d) qRT-PCR analysis of Rbms3 expression in liver cancer tissue samples and matched normal tissue samples. (e) qRT-PCR analysis of Rbms3 and c-Myc expression in liver cancer tissue samples stratified into high- and low-level expression groups according to median miR-1269a expression. *, ** and *** represent $p < 0.05$, $p < 0.01$ and $p < 0.001$.

Fig. 4. miR-1269a inhibition depress liver cancer in vivo. (a) The shape of tumors from the control and miR-1269a sponge-overexpressing Hep3B cells. (b) The weights of tumors from the control and miR-1269a sponge-overexpressing Hep3B cells. (c) The tumor volume in the development of mice after control and miR-1269a sponge-overexpressing Hep3B cells injection. The tumor volumes were evaluated with the follow formula: $\text{Volume} = \text{length} \times \text{width} \times \text{width} / 2$. The evaluations were taken at the indicated time points. (d) The possible mechanism of miR-1269a in the regulation of liver cancer proliferation.

Figures

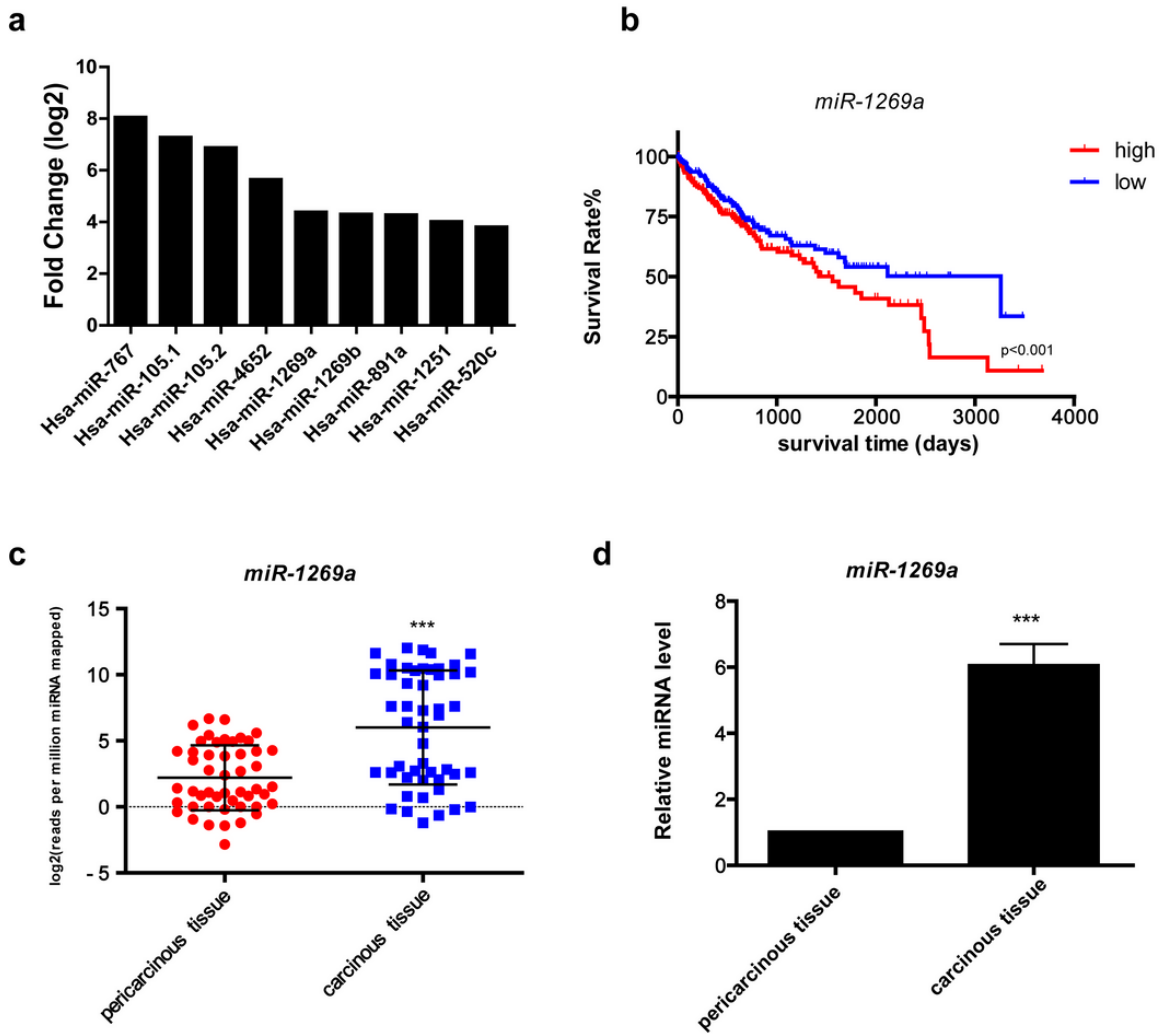


Figure 1

miR-1269a is upregulated in liver cancer. (a) The top 10 upregulated miRNAs were ranked by log₂|FC|. (b) Overall survival time in liver cancer patients, stratified into high-level and low-level groups according to median miR-1269a expression using a Kaplan-Meier curve and a Log-rank test. (c) Expression of miR-1269a in liver cancer tissues and matched normal tissues. (d) qRT-PCR analysis of miR-1269a expression in liver cancer tissue samples and matched normal tissue samples. For c and d, *** represents p < 0.001.

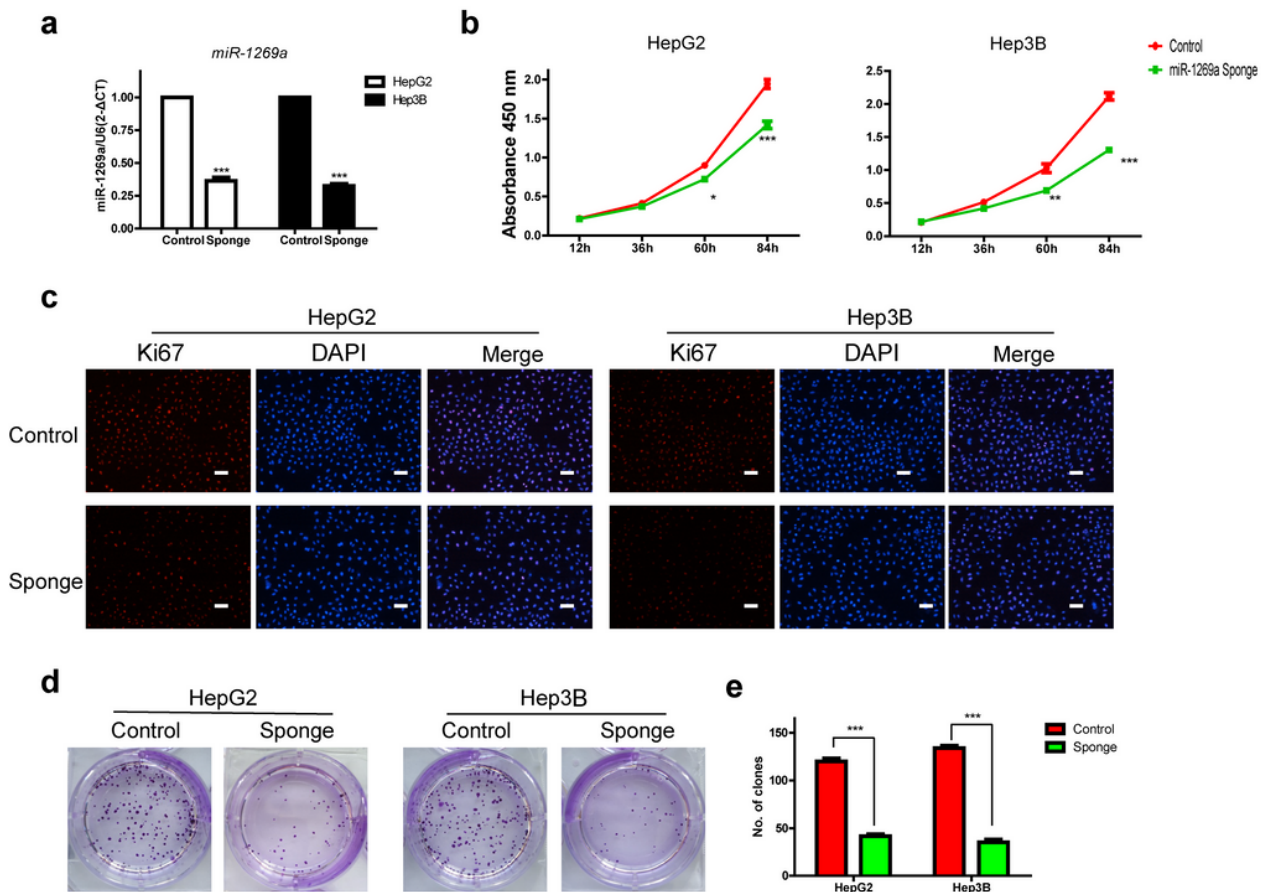


Figure 2
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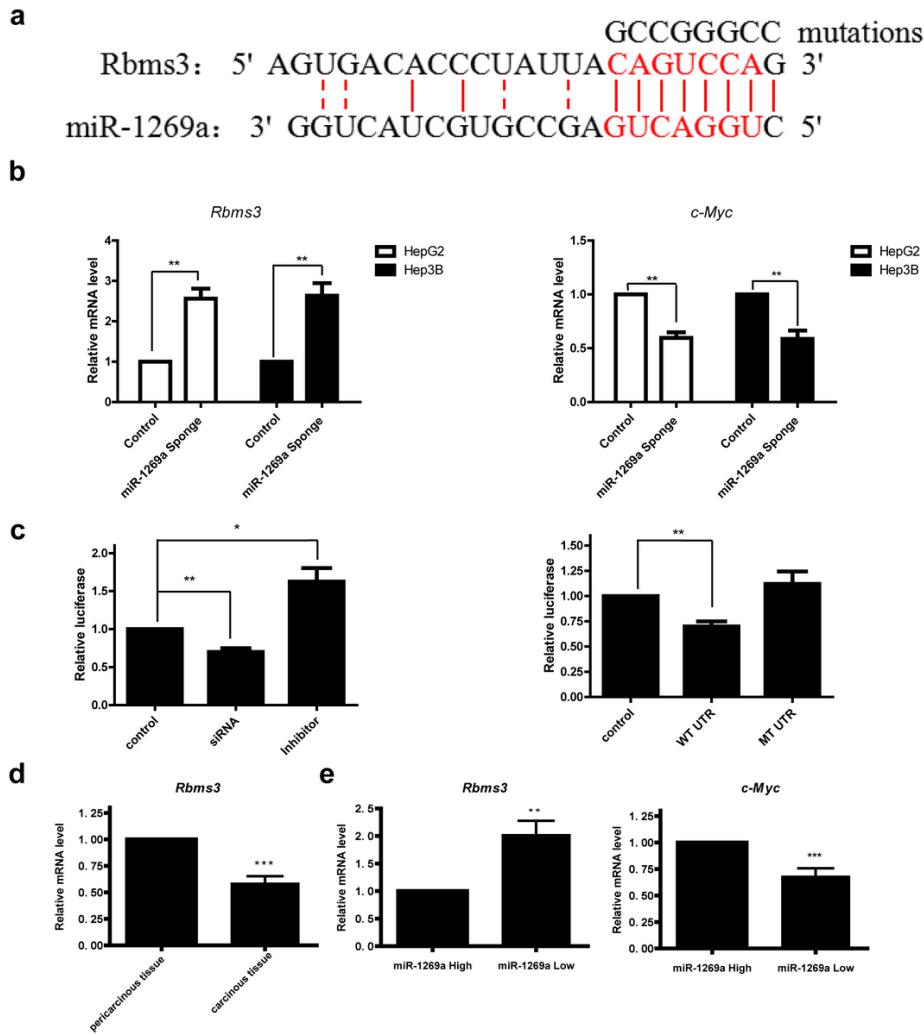


Figure 3

miR-1269a binds the 3' untranslated region of Rbms3 and regulates its expression. (a) Putative binding of miR-1269a and Rbms3 predicted by Targetscan software. (b) qRT-PCR analysis of Rbms3 and c-Myc expression in control and miR-1269a sponge-overexpressing HepG2 and Hep3B cells. (c) Left: dual luciferase assay analysis showing the interaction of miR-1269a and the Rbms3 3'UTR. 293T cells were co-transduced with retroviruses containing control siRNA + WT UTR, miR-1269a siRNA + WT UTR or miR-1269a inhibitor + WT UTR; right: dual luciferase assay analysis showing the interaction of miR-1269a and the Rbms3 3'UTR. 293T cells were co-transduced with miR-1269a siRNA + control UTR, miR-1269a siRNA + WT UTR or miR-1269a siRNA + MT UTR. (d) qRT-PCR analysis of Rbms3 expression in liver cancer tissue samples and matched normal tissue samples. (e) qRT-PCR analysis of Rbms3 and c-Myc expression in liver cancer tissue samples stratified into high- and low-level expression groups according to median miR-1269a expression. *, ** and *** represent $p < 0.05$, $p < 0.01$ and $p < 0.001$.

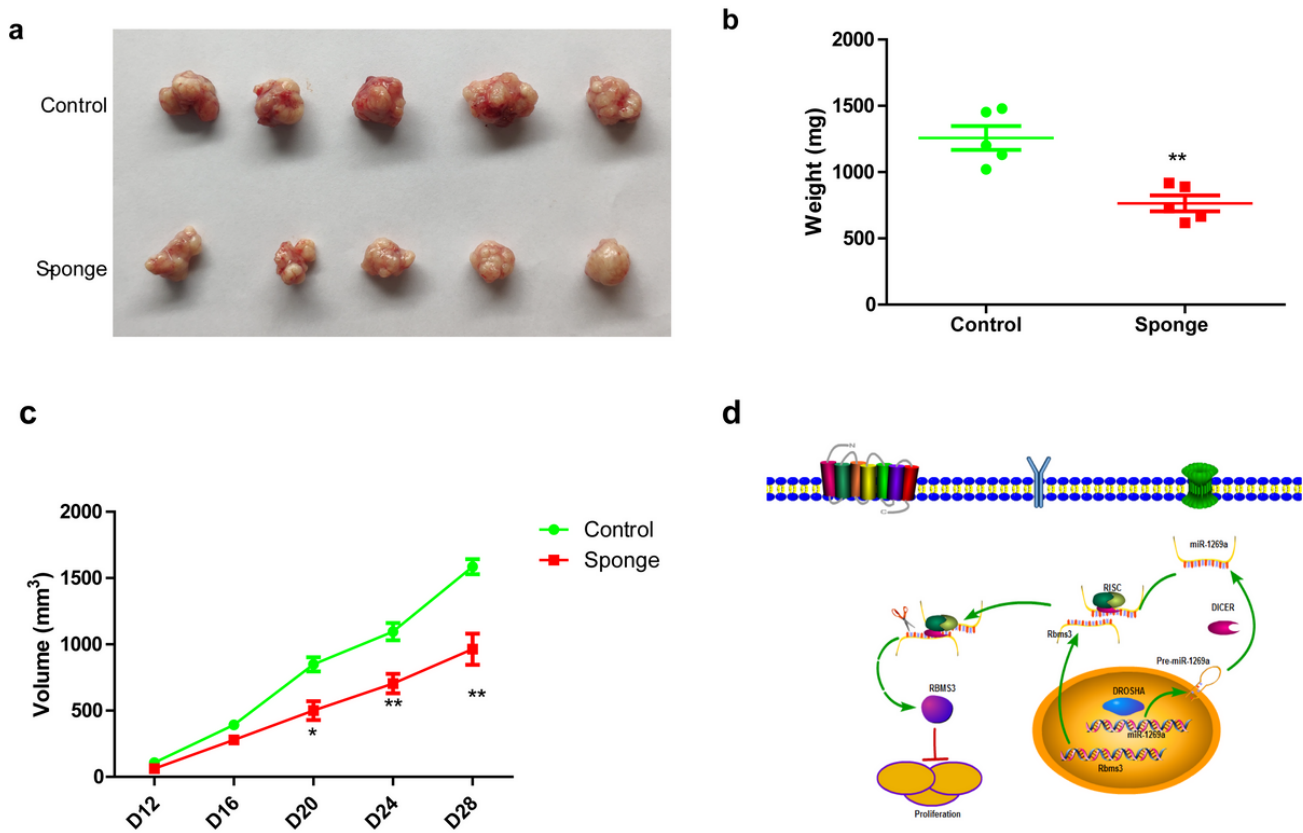


Figure 4

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Supplementary Files

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