Original Article

RBMX contributes to hepatocellular carcinoma progression and sorafenib resistance by specifically binding and stabilizing BLACAT1

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Abstract: Hepatocellular carcinoma (HCC) is one of the top five causes of cancer death. The interaction of RNA binding proteins and long non-coding RNA play vital role in malignant tumor progression, and even contribute to chemoresistance. RNA binding protein X (RBMX) plays a vital role in binding and stabilizing many proteins. In this study, we have identified RBMX significantly contributes to the tumorigenesis and sorafenib resistance of hepatocellular carcinoma (HCC). We observed that RBMX was highly expressed in both the HCC patient tissues and HCC cell lines. The HCC cell’s viability, proliferation, and sorafenib resistance ability were both increased when RBMX was overexpressed. Additional, RBMX also promotes HCC development and chemoresistance in vivo. Further, we found that the autophagy level was increased in HCC cells, which RBMX was up regulated, with sorafenib processing. Interestingly, our study found that long non-coding RNA bladder cancer associated transcript 1 (LncBLACAT1) was also raised in HCC. Mechanically, RIP, RNA pull-down and RNA Stability assay proved that RBMX could specially binds BLACAT1’s mRNA and matins its expression, which is high degree of consistency with catRAPI database prediction. This mechanism of action is beneficial for cancer cells proliferation, anti-apoptotic, and colony formation with sorafenib treatment. Further, the autophagy level and cancer cell stemness were also improved when RBMX/BLACAT1 upregulated. Our study indicated that hepatoma cells can improve their proliferation, colony ability and autophagy by RBMX stabilizing BLACAT1 expression then promote HCC development and drug resistance. Hence, RBMX could be considered as novel therapeutic target for HCC treatment strategies.

Keywords: Hepatocellular carcinoma, RNA binding protein X, sorafenib-resistance, bladder cancer associated transcript 1

Introduction

Hepatocellular carcinoma is one of the top five causes of cancer death among all ages in 2015 and till date remains as the most lethal with a 5-year survival rate of just 18% [1]. Treatment strategies for advanced stages of HCC are very limited due to the many underlying liver diseases which are associated with carcinoma [2]. Sorafenib (Nexaver, Bayer HealthCare Pharmaceuticals) is a drug that mechanistically inhibits the serine threonine kinase Raf and tyrosine kinase activity of vascular endothelial factor receptors, has been known to inhibit tumor cell proliferation and angiogenesis in different tumor models, including HCC. Sorafenib treatment has been considered as first option for patients with advanced stages of HCC, with the severity of the disease being high most complications arise from the carcinoma associated liver diseases [3-5]. Sorafenib in clinical studies such as the SHARP trial [6] and Asia-pacific trial [7] have been able to improve the survival rate of patients by 2.5 to 3 months in patients compared to the placebo group. HCC being a hugely heterogenetic disease, many patients seemed to show primary resistance to the sorafenib treatment [8]. The exact mechanism behind such resistance is still unclear. In the current study, we observed high expression of RBMX
gene (RNA binding motif) in HCC cell lines but additionally more increased expression in HCC-sorafenib resistant (SR) lines. Hence, we explore the potential role of RBMX in HCC progression and sorafenib resistance. Human proteome expression is incomplete without appropriate splicing of the nuclear mRNA and such splicing is carried out by many ribonucleoproteins (hnRNPs) which bind with the nascent mRNA and form complexes [9]. Among these, hnRNP G is coded by the RBMX gene and is relatively the least abundant but implicated in splicing of many vital mRNA [10]. Further, they play an important role in either addition or exemption of exons in the mRNA for many proteins. Recent studies have shown that hnRNP G associates itself with most units of RNA polymerase II hence being a basic component of general transcription process [11]. Another study had shown that RBMX binds with satellite I mRNA during mitotic phase, and lack of RBMX led to defective centromere cohesion and abnormal segregation, indicating its very crucial role during M phase [12].

A study by Heinrich et al., [13] had identified the hnRNP G’s RNA-recognition motif binds to a loose consensus sequence containing a CC (A/C) motif. In this study, we identified many similar CCAG consensus sequence by comprehensive mutational analysis of the RBMX-binding sites in the long non-coding RNA BLACAT1. Long non-coding RNAs (lncRNAs) have gained lot of recognition for their roles in various diseases [14, 15]. One such lncRNA known as HOTAIR (HOX transcript antisense RNA) has been identified to have a key role in promoting proliferation in pancreatic cancer, colorectal cancer and HCC. In many studies, HOTAIR has been specifically associated with HCC for its role in poor prognosis and survival rate [16-18]. Bladder cancer associated transcript-1 (BLACAT1) as the name suggests was first identified in bladder cancer [19], but later has been associated with colorectal cancer [20] and cervical cancer [21]. There are also studies implicating BLACAT1 to contribute to multidrug resistance [22]. As mentioned above there are some studies discussing RBMX and BLACAT1’s potential individual roles in tumorigenesis, but till data the roles of RBMX, and BLACAT1 in HCC’s progression and multidrug resistance is still unclear.

In this study, we identify RBMX contributes to the poor prognosis of HCC. We also observed its presence and overexpression in patient tumor tissues when compared with adjacent tissues. Further in vivo studies, we correlated RBMXs role through increased tumor size and also performed various silencing and overexpression studies to indicate its role in interaction with BLACAT1. We have finally observed that RBMX interacting and stabilizing BLACAT1 contributes to proliferation, anti-apoptosis, autophagy and multiple drug resistance (MDR) in HCC.

Materials and methods

Patient samples and tissue samples

Tumor samples from 60 tissues and adjacent normal tissues were obtained from patients who underwent liver resection at the Liver Transplantation Center in in Seventh People’s Hospital of Shanghai and simultaneously stored frozen in liquid nitrogen within 10 minutes. Patient’s character was presented in Table 1. Identification and confirmation of these tissues were performed using histology. All patients signed the informed consent and the study was approved by Institute Research Ethics Committee of the Seventh People’s Hospital of Shanghai University of TCM.

Cell line and culture

The human normal hepatic cell LO2 and hepatocellular carcinoma cell lines (HepG2, Huh-7, SMMC7721, Hep3B, HCCLM3 and MHCC97H) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO2.

Establishment of sorafenib-resistant cells

To determine the half inhibitory concentration (IC50), cells were treated with sorafenib in 96 well plates and cell viability was determined for 3 days. Cells at a concentration of 1×10^4 cells/well were cultured in 6-well plates and incubated with sorafenib at a concentration just below their respective IC50. Further, the sorafenib concentration was gradually increased by 0.25
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Table 1. Association of clinicopathological characteristics with RBMX expression of the investigated HCC patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Low expression (n = 16)</th>
<th>High expression (n = 44)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of patients (%)</td>
<td>No. of patients (%)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>9 (56.3%)</td>
<td>26 (59.1%)</td>
</tr>
<tr>
<td>Female</td>
<td>7 (43.7%)</td>
<td>18 (40.9%)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21-40</td>
<td>4 (25.0%)</td>
<td>3 (6.8%)</td>
</tr>
<tr>
<td>41-60</td>
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<td>15 (34.1%)</td>
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<td>61-80</td>
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<td>22 (50.0%)</td>
</tr>
<tr>
<td>81-100</td>
<td>0 (0%)</td>
<td>4 (9.1%)</td>
</tr>
<tr>
<td>Liver cirrhosis history</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>10 (62.5%)</td>
<td>29 (65.9%)</td>
</tr>
<tr>
<td>No</td>
<td>6 (37.5%)</td>
<td>15 (34.1%)</td>
</tr>
<tr>
<td>Histological grade</td>
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<td></td>
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<tr>
<td>Low</td>
<td>5 (31.3%)</td>
<td>15 (34.1%)</td>
</tr>
<tr>
<td>Middle</td>
<td>8 (50.0%)</td>
<td>21 (47.7%)</td>
</tr>
<tr>
<td>High</td>
<td>3 (18.7%)</td>
<td>8 (18.2%)</td>
</tr>
<tr>
<td>TNM stages</td>
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<td></td>
</tr>
<tr>
<td>I</td>
<td>5 (31.3%)</td>
<td>1 (2.3%)</td>
</tr>
<tr>
<td>II</td>
<td>10 (62.5%)</td>
<td>18 (40.9%)</td>
</tr>
<tr>
<td>III</td>
<td>1 (16.2%)</td>
<td>22 (50.0%)</td>
</tr>
<tr>
<td>IV</td>
<td>0 (0%)</td>
<td>3 (6.8%)</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>3 (18.8%)</td>
<td>15 (34.1%)</td>
</tr>
<tr>
<td>Yes</td>
<td>13 (81.2%)</td>
<td>29 (65.9%)</td>
</tr>
</tbody>
</table>

RNA isolation was performed using Trizol reagent (Invitrogen) according to manufacturer’s instructions and SuperScript First-Strand Synthesis System were used for cDNA preparation (Invitrogen). PCR was performed according to the requirements. Internal control β-actin was also used. Gene relative expression was normalized to β-actin using 2⁻ΔΔCt. The experiments were performed in triplicate.

Western blot analysis

Western blotting analysis was performed using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The blots were incubated with primary antibodies overnight at 4°C. Following three washes, membranes were then incubated with secondary antibody overnight at 4°C. Signals were visualized with ECL. Antibodies were purchased as follows: ABCB1 from Santa Cruz Biotechnology (Santa Cruz, CA), LC3, RBMX, caspase3 and p62 from Cell Signaling Technology, β-actin from Sigma.

Immunohistochemistry assay

Immunohistochemistry analyses were performed as per [8]. Blocking of the samples was performed in normal goat serum with 5% BSA.
in TBS for 1 h at room temperature. Further, the sections were incubated with primary antibody at a dilution of 1:400 over-nights at 4°C and then washed with PBS three times. After incubation with secondary antibodies (16 h), the sections were subjected to a DAB reaction. The sections were photographed using a digitalized microscope camera (Nikon, Tokyo, Japan).

**In vivo tumor xenograft model**

The Huh7 cells were transfected with either scr or shRBMX; Hep3B cells were transfected with empty or RBMX overexpression vector. Matrigel (50%) were used to suspend the cells and the gels were subcutaneously implanted in the dorsal flanks of 6-week-old female *nu/nu* mice (1×10^6). Sorafenib was administrated when the tumor were up to 50 mm^3 in volume, at this point, the mice which transfected with same vector were randomized treat with DMSO or sorafenib (50 mg/kg/each 2 days, intraperitoneally). Further, monitoring of tumor growth was carried out using digital calipers, and the volume was calculated using the formula: 

\[
\text{tumor volume (mm}^3) = \text{[width (mm)] } \times \text{[length (mm)] } \times 0.5.
\]

Repeats were achieved by repeating experiments 2 individual times. Initial pilot experiments were performed to project the required number of mice per group. Tumors were collected for histopathological analysis, RNA and proteins extraction. Exclusion of animals were performed when humane end points were reached or in the event of death from procedure-related causes which did not cause differences in the numbers of experimental groups.

**TUNEL assay**

TUNEL staining was used to stain the tumor sections (Terminal deoxynucleotidyl transferase dUTP nick end labeling) (Roche, Shanghai, China). Cell positive for TUNEL were counted 20 fields with 200 × magnification. The apoptosis index was calculated according to the following formula: the number of apoptotic cells/total number of nucleated cells × 100%.

**Cells proliferation assay**

Cell proliferation was calculated using CCK8 assay according to the manufacturer’s instruction. After transfection and treatment, cells were incubated in well and add 10 ul cell count kit-8 solution. Co-incubation 2 h, 37°C, measurement of absorbance at 450 nm by spectrophotometer. Cell growth curve was further drawn with normalized values which were taken at OD 490.

**Colony-formation efficiency assay**

After transfection with RBMX and BLACAT1 gene expression vector, cells were seeded and incubated at 37°C in humidified incubator for 10 days. Cell colonies were stained with 0.5% Crystal Violet and the colonies were counted.

**In vitro apoptosis assay**

Approximately 1×10^5 cells were suspended in 100 μl buffer, 5 μl of Annexin V and 5 μl of propidium iodide were further added. The mix was incubated for 15 min at room temperature in dark, according to the manufacturer’s instruction (BD Biosciences, San Jose, CA). Flow cytomteric analysis was used to quantify the rate of apoptosis (3 repeats were performed) with the Beckman Coulter Epics Altra II cytometer (Beckman Coulter, California, USA).

**RNA immunoprecipitation**

Immunoprecipitation was performed with A/G-plus agarose beads in the cell lysates (Santa Cruz, Biotechnology, Inc. CA) incubated with appropriate antibodies for 4 h at 4°C. Further RNA was isolated, and RT-PCR was performed.

**RNA pull-down assay**

RNA pull-down assays were performed using the Pierce Magnetic RNA Protein Pull-down Kit (Thermo fisher). In brief, biotin-labeled BLACAT1 or antisense RNA was co-incubated with protein extracts and magnetic beads from Huh7 and Hep3B cells. The resulting magnetic bead-RNA-protein compounds were collected by low-speed centrifugation and then washed with a Handee spin column. Magnetic beads were boiled in sodium dodecyl sulfate buffer and subjected to Western blotting using GAPDH as a control.

**mRNA Stability measurement**

Huh7 or Hep3B cells transfected with scr or shRBMX-1 (shRBMX) expression vector and the stability of BLACAT1 mRNA variants were examined by treating the cells with actinomycin D.
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At different time points, (0, 15, 30, 60, and 120 min) RNA was isolated qRT-PCR were performed, as described above. Half-life (t1/2) of BLACAT1 mRNA variants were calculated as the time required for each mRNA variant to reduce to 50% after actinomycin D treatment from its initial abundance at time 0 min. Half-lives (t1/2) were determined by nonlinear regression analysis.

Bioinformatics analysis

In order to get a wider range of evidence, the deregulated gene expression of RBMX was further verified by The Cancer Genome Atlas (TCGA) database (n = 371); also the survival correlation (http://ualcan.path.uab.edu/analysis.html). CatRAPID program were used for interaction prediction (http://service.tartaglia-ab.com/page/catrapid_group).

Statistical analysis

The data are presented as the means ± standard deviations (SD). A two-tailed Student’s t-test and One-way analysis of variance (ANOVA) was used to evaluate the differences among more than three groups or six groups, in Graph Pad Prism 5 (Graph Pad Software, San Diego, CA, USA). Differences were considered significant for values of *P < 0.05, **P < 0.01, ***P < 0.001.

Results

RBMX is overexpressed in HCC tissues and HCC cell lines

To confirm the expression of RBMX in tumors, transcript levels were compared between the tumor and normal tissues. Based on the evidence from TCGA databases, RBMX transcript levels were observed to be highly upregulated when compared to the normal tissues (Figure 1A), as well as the association of clinicopathological characteristics of HCC patients with RBMX expression was showed in Table 1. And that, it was also observed the survivability of patients with high expression of RBMX had poor prognosis when compared to the patients with low or medium expression of RBMX (Figure 1B). In six individual patient tumor samples western blotting analysis was performed with comparisons to the normal tissue samples (Figure 1C). It was clear that there was significantly higher expression of RBMX in the patient samples, which was also observed in the mRNA level (Figure 1D). Immunohistochemistry analysis was performed in hepatocellular carcinoma (HCC) patient tissues and consistent results were evident that RBMX was highly expressed (Figure 1E). Commercially available hepatic cell lines such as LO2, HepG2, Huh-7, SMMC7721, Hep3B, HCCLM3 and MHCC97H were checked for RBMX expression (Figure 1F, 1G). Among these, Huh-7 displayed the highest expression of RBMX followed by HepG2. Further, the sensitivity of HCC cell lines to sorafenib was detected under increasing concentration (Figure 1H). The results indicated that there is some connection between expression of RBMX and HCC cells vitality in sorafenib. Then, Huh-7 or Hep3B cells were choose for further experiments.

Crucial role of RBMX in HCC cell’s proliferation and apoptosis

To understand the effect of RBMX in HCC progression, silence or overexpression experiments were performed in two different cell lines first. RBMX gene was silenced in Huh-7 cells, and silencing efficiency was confirmed using qRT-PCR analysis and western blotting analysis, and it was evident that the shRBMX-1 and shRBMX-2 had a much lower RBMX expression compared to the non-functional control shRNA (scr) (Figure 2A). RBMX overexpression assay was performed in Hep3B cells, and overexpressing efficiency was also verified in (Figure 2B). Then, proliferation assays were performed with these cells using cell counting kit. The results indicated that RBMX silenced cells have decreased proliferation (Figure 2C), on the contrary, RBMX overexpressed cells displayed a higher proliferation ability (Figure 2D). Further, colony formation capacity shows shRBMX Huh-7 cells had lesser colony forming units when compared to scr (Figure 2E), conversely, Hep3B cell’s colony units was significant increased when overexpressing RBMX (Figure 2F), this results showing a strong positive correlation between RBMX and proliferation. TUNEL assay were performed to evaluate the cells apoptosis in RBMX overexpressed and silenced cells. It was evident that the cells which RBMX were silenced had very high number of apoptotic cells when compared to the control (Figure 2G), contrarily, the dead cells
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**Figure 1.** RBMX is overexpressed in HCC tissues and HCC cell lines. (A) RBMX mRNA expression in tumor tissues and the corresponding adjacent tissues of HCC patients. (B) Patients with high RBMX (n = 89) expression presented a poor overall survival (OS) compared with the low/medium expression group (n = 276). RBMX expression is higher in tumor tissue samples of patients with poor prognosis. The results shown here (A and B) are in whole based upon data generated by the TCGA Research Network: http://cancergenome.nih.gov/. (C) The relative expression level of the RBMX protein in tumor tissues (T) and normal tissues (N) of HCC patients. (D) RBMX mRNA expression in tumor tissues and the normal tissues of HCC patients (n = 60). (E) The expression of RBMX was detected by IHC staining in 16 paraffin-embedded HCC and normal tissues specimens. (Scale bars = 100 μm). (F) Western blotting of RBMX protein and (G) RBMX mRNA expression in different human HCC cell lines. (H) Commercial HCC cell lines sensitivity to sorafenib were measured by CCK-8. Data are shown as the mean ± S.E.M., n = 6. *P < 0.05, **P < 0.01, ANOVA.

are fewer in RBMX overexpressed Hep3B cells (**Figure 2H**). Taken together, these data demonstrated that RBMX could facilitate HCC cells proliferation and inhibit cell apoptotic.
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Figure 2. Crucial role of RBMX in HCC cell’s proliferation and apoptosis. (A) Huh7 cells were transfected with shRBMX-1, shRBMX-2 or scr and the silencing efficiency was further detected by RT-PCR and WB. (B) Hep3B cells transfected with RBMX or empty vector (control) and the efficiency was detected by RT-PCR and WB. Cell proliferation of Huh7 cells with silenced RBMX gene (C) and Hep3B cells with overexpressed RBMX (D) was tested by using Cell Counting kit-8 assay. (E) Colony-formation assay revealed that RBMX knockdown decreased colony formation when compared with normal Huh7 cells. (F) Colony-formation assay revealed that RBMX overexpression increased colony number when compared with the normal Hep3B cells. TUNEL assay detected in transfected Huh7 cells (G) and Hep3B cells (H). (Scale bars = 50 μm). Data are shown as the mean ± S.E.M., n = 6. **P < 0.01, ANOVA.
RBMX contributes to HCC cell lines sorafenib resistance

To further understand the role of RBMX in chemoresistance, sorafenib resistance HCC lines were established as previous (Huh7-SR, Hep3B-SR), and CCK-8 assay was performed for drug resistance evaluation. As show in Figure 3A, 3B, resistant cells show great improvement of sorafenib resistance ability. Then, the expression of RBMX was measured in sorafenib resistance cell lines. We can observe that, the level of RBMX mRNA and protein significantly increased in Huh7-SR/Hep3B-SR cells compared to Huh7/Hep3B native cell (Figure 3C, 3D). To further clarify the role of the RBMX gene in cell resistance, following the processing as parental cells, Huh7-SR and Hep3B-SR cells were transfected with shRBMX or RBMX overexpression vectors, respectively. The transfection efficiency was validated by RT-PCR and western blots (Figure 3E, 3F). Next, CCK-8 assay was performed for evaluation of RBMX function in RBMX overexpressing and silenced cells. With increasing concentrations (0-20 M) of sorafenib, it was significantly decreased of cell relative viability of Huh7/Huh7-SR cells which RBMX silenced (Figure 3G, 3H). On the contrary, RBMX overexpressed cells, Hep3B/Hep3B-SR, had higher cell viability than empty vector group (Figure 3I, 3J), thus indicating an important sorafenib resistance role of RBMX in HCC cells. Consistent with cell viability assay, cells apoptosis level, measured by flow cytometry, was negative with RBMX expression (Figure 3K-P). For further clarification, chemoresistance and apoptotic maker genes were checked next. ABCB1 (ATP Binding Cassette Subfamily B Member 1, a vital indicator of multidrug resistance), specific indicators of drug resistance was measured with western blot in parental and SR cells. Results indicated that increased expression of RBMX did increase ABCB1 expression in Hep3B/Hep3B-SR cells (Figure 3Q). And, ABCB1 expression decreased significantly in both Huh7/Huh7-SR cells when RBMX was silenced. Simultaneously, caspase-3 was selected as apoptotic maker. From the data, it was clear that caspase-3 expression was reduced while RBMX was upregulated (Figure 3Q), but when RBMX was silenced caspase-3 expression was highly upregulated. Taken together, RBMX could positive regulate parental and SR cells proliferation and drug resistance under sorafenib processing, which indicated that RBMX plays a key role in sorafenib resistance of HCC cells.

RBMX promote tumor growth and sorafenib resistance in model

Next, the role of RBMX in tumor growth and drug resistance was studied. For this, shRBMX Huh-7 cells and RBMX up-regulated Hep3B cells were injected into the dorsal flanks of nu/nu mice with or without sorafenib treatment, respectively. The tumor volumes were subsequently measured every week using digital callipers. It was evident that the tumor size increased slowly from day 7 to day 35 in the shRBMX group of Huh7 cells injection, when compared to the scr control group at the same time points (Figure 4A). On the contrary, the tumor size significantly increased in RBMX group compared to empty vector group of Hep3B cells injection (Figure 4B). There also showed a positive effect of RBMX in HCC tumor growth under sorafenib treatment. The tumor volume increased dramatically when RBMX overexpression, even that with sorafenib treatment. But, RBMX knockdown aggrandized the therapeutic efficacy of sorafenib (Figure 4A, 4B). At day 35 the mice were sacrificed and the tumors were taken out for weight measurement and pathologic analysis. As show in Figure 4C, it was observably that no matter what kind of cells in tumorigenesis, tumor weight was heavier in the group with higher RBMX expression. More intuitively, tumors feature were showed in Figure 4D and 4E. Further, these samples were subjected to histopathological analysis (Figure 4F). Silencing and overexpression efficiency was confirmed with RBMX immunohistochemistry staining with anti-RBMX antibody, where in the shRBMX group tumor section displayed less to no positive cells, and RBMX overexpression group showed great proportion of RBMX staining. Additionally, hematoxylin and eosin staining of the tumor sections from the control group and RBMX up-regulation group showed distinct cells with clear darker nuclei typical of cancerous cells, but shRBMX group and sorafenib treatment groups showed lesser nuclei, indicating a decrease in tumorous cells. TUNEL assay of these sections displayed more apoptotic cells in the shRBMX group compared to the scr group; and the RBMX overexpression decreased cells death under sorafenib treat-
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RBMX promotes hepatocellular carcinoma development

Figure 3. RBMX contributes to HCC cell lines sorafenib resistance. (A) Sorafenib resistance cells verification by CCK-8 assay. (B) IC50 of Huh7/Huh7-SR/Hep3B/Hep3B-SR cells. (C) qRT-PCR and (D) Western blot were used to assay the expression of RBMX in parental and SR cells. Huh7-SR (E), Hep3B-SR (F) cells were either used as a silencing system or overexpression system for RBMX gene. Then, the parental and SR cells were both further treated with different concentrations (0, 2.5, 5, 10, 20 μM) of sorafenib. After 48 h the CCK8 assay was performed to detect cell sensitivity to sorafenib (G-J). (K-N) Flow cytometry analysis for apoptotic rate measurement in Huh7/Huh7-SR, Hep3B/Hep3B-SR cells post treat with sorafenib (20 μM) for 24 h, and apoptotic rate was quantized in histogram (O, P). (Q) Western blotting analysis of ABCB1 and caspase-3 from Huh7/Huh7-SR, Hep3B/Hep3B-SR cells with either RBMX overexpression or silencing, and sorafenib processing (20 μM) for 24 h before. Data are shown as the mean ± S.E.M., n = 6. **P < 0.01, ANOVA.
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Figure 4. RBMX promote tumor growth and sorafenib resistance in model. Huh7 cells transfected with shRBMX and Hep3B cells transfected with RBMX overexpression vector were injected into nude mice with or without sorafenib treatment (n = 3), respectively. The tumor volumes (mm$^3$) were calculated every week after injection (A, B). (C) Tumor weight (gram) and (D, E) the photography of xenograft tumors post 35 days of injection. The bars indicate S.D. Data were means of value from mice, mean ± S.E.M., n = 3. *P < 0.05, **P < 0.01, ANOVA. (F) Pathologic analysis. Hematoxylin-eosin (HE) staining, TUNEL, ki67 and RMBX immunostaining were performed in xenograft tumor samples. (Scale bars = 100 μm).

Finally, Ki67 staining was also performed which indicated more proliferative cells present by the darker nuclei coloration in higher RBMX expression group, this shows the RBMX expres-
sion rescued the HCC cells life from sorafenib toxicity. Taken together, these data indicated that RBMX promote tumor growth and sorafenib resistance by improve cells proliferation and decrease apoptotic.

**RBMX mediates sorafenib resistance by induction of autophagy**

Further experiments were also performed to understand the mechanism of how RBMX participate in sorafenib resistance. Interestingly, we found that autophagic flux was abundant in hepatoma carcinoma cells with sorafenib performance; but decrease rapidly when cells pretreatment with autophagy inhibitor, bafilomycin A1 (BAF) (Figure 5A, 5B). The protein detection of LC3 also indicated that autophagy was happened in hepatoma carcinoma cells responses to sorafenib stimulation. To further confirm whether RBMX regulates sorafenib-induced cellular autophagy, RBMX deletion assays were performed in HCC cells. It was observed that the cells autophagy maker LC3 significantly increased in cells which RBMX overexpressed, but reduced when RBMX deleted (Figure 5D, 5E). Western blotting analysis indicated that the LC3 II was significantly increased and p62 was downregulated in the presence of RBMX, but when RBMX was silenced then LC3 II and p62 were significantly opposite (Figure 5F, 5G), respectively. Thus, the above-mentioned experiments specifically indicated the roles of RBMX mediate sorafenib resistance of hepatocellular carcinoma cells by activating autophagy.

**RNA-binding protein RBMX binds and stabilizes BLACAT1**

Many studies have showed the chemotherapeutics resistant ability of BLACAT1 in multicancers, but the specific mechanism of action is unclear. For this concern, we study the relationship between RBMX and BLACAT1 in HCCs. First of all, BLACAT1 level was measured in HCC patient tissues (Figure 6A), also the HCC TNM stage and survival rate associate with BLACAT1 level were analyzed respectively (Figure 6B, 6C). As shown, BLACAT1 is significantly expressed and has a poor prognosis in HCC patients. Besides, BLACAT1 is overexpressed in commercial HCC lines (Figure 6D). Additionally, BLACAT1 expression is positively correlated with RBMX (Figure 6E). As mentioned previously, another study had identified the hnRNP G’s RNA-recognition motif binds to a loose consensus sequence containing a CC (A/C) motif (13). In this study, we identified many similar CCAG consensus sequence by comprehensive mutational analysis of the RBMX-binding sites in the IncBLACAT1. We identified a total of 29 such sites in BLACAT1 sequence (Figure 6F). More than that, CatRAPID online algorithm also performed for RNA-protein interaction prediction (Figure 6G). Of interest, this program revealed a higher interaction DP value, reach up to 98%. On the basis of this evidence, RNA immunoprecipitation assays were go ahead for interaction validation. RNA immunoprecipitation analysis followed by qRT-PCR showed that sample precipitated with RBMX had a high fold expression of BLACAT1 (Figure 6H, 6L). Also, the RNA pull-down shows BLACAT1 bound with RBMX (Figure 6I and 6M). Further experiments were performed to investigate the stability of BLACAT1 mRNA in the absence of RBMX. Cells were either transfected with scr or shRBMX and then treated with actinomycin D to block de novo transcription. Then, the levels of BLACAT1 were quantified using qRT-PCR and normalization was performed to 18S rRNA levels. It’s obvious that, BLACAT1 mRNA had decayed to almost 60% within 8 mins in both Huh7 and Hep3B cells when RBMX was silenced (Figure 6J, 6N). This indicated that the stably expressing of BLACAT1 is potential dependent on RBMX exist. As opposed to Figure 2A, 2B, to further verify the intracellular expression relationship between RBMX and BLACAT1, Huh7 and Hep3B cells were transfected with RBMX overexpression or knockdown vectors, respectively (Supplementary Figure 1). The expression level of BLACAT1 was distinctly increased with the over-expression of RBMX in all these cells, and when RBMX was silenced, BLACAT1 was reduced (Figure 6K, 6O). Taken together, these data indicate a strong positive interaction between RBMX and BLACAT1.

**RBMX and BLACAT1 interact to co-regulate HCC cells proliferation**

Overexpression and knockdown studies were further used to evaluate the effect of RBMX and BLACAT1 on cell proliferation. For this purpose, Huh7 cells of RBMX knock-down and Hep3B cells of RBMX overexpressing were transfected with BLACAT1 overexpressing and
Figure 5. RBMX mediates sorafenib resistance by induction of autophagy. (A) Immunofluorescent staining for autophagic flux in Huh7 and Hep3B cells co-incubated with sorafenib (20 μM), which cells were pretreated with or without BFA A1 (100 nM). Scale bars = 20 μm. (B) Fluorescence intensity and (C) protein level of autophagy maker LC3 were showed. Data were mean ± S.E.M., n = 3. **P < 0.01, ANOVA. (D) Huh7/Huh7-SR cells (E) Hep3B/Hep3B-SR cells were incubated with sorafenib 10 μM for 48 h, then transfected with shRBMX or RBMX overexpression vector, scr or empty vector as control. Then, green fluorescence for LC3 and DAPI for nuclear were stained. Scale bars = 20 μm. Quantification of LC3-positive puncta per cell. Data are shown as the mean ± S.E.M., n = 6. **P < 0.01, ANOVA. (F, G) Western blotting with anti-LC3 and anti-P62 antibodies for the protein samples from the cell lines.
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Figure A: Relative BLACAT1 mRNA level in non-tumor and tumor tissues.

Figure B: Relative BLACAT1 level in normal, III, and III/IV stages.

Figure C: Percent survival of BLACAT1 low expression vs. high expression.

Figure D: Relative BLACAT1 mRNA level in different cell lines.

Figure E: BLACAT1 mRNA expression vs. RBMX mRNA expression.

Figure F: Heatmap showing interaction propensity and discriminative power.

Figure H: Fold enrichment of BLACAT1 and GAPDH in HuH7 cells.

Figure I: Western blot analysis of RBMX antisense and GAPDH in HuH7 cells.

Figure J: BLACAT1 mRNA remaining with different treatments.

Figure K: Relative BLACAT1 level with different treatments.
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Figure 6. RNA-binding protein RBMX binds and stabilizes BLACAT1. BLACAT1 expression levels in patient’s tumor tissues (A), the TNM stage (B) and survival Kaplan-Meier curve (C) correlation with BLACAT1 level. BLACAT1 expression in commercial cell lines (D) Expression relevance analysis between RBMX and BLACAT1 (E). (F) RBMX consensus binding site sequence and BLACAT1 sequence alignment. The RBMX binding sequence is shown. (G) CatRIPAD online interaction prediction shows a higher DP value. (H, L) RIP assay shows BLACAT1 was identified in RBMX complex, and relative enrichment was measured by RT-PCR. Data are shown as the mean ± S.E.M., n = 6. *P < 0.05, **P < 0.01, ANOVA. (I, M) RNA pull-down assay, RBMX and GAPDH protein in immunoprecipitation with BLACAT1 RNA were evaluated by western blots. (J, N) Measurement of BLACAT1 mRNA stability. Cells transfected with scr or shRBXM were treated with actinomycin D to block de novo transcription and the levels of BLACAT1 were assessed by RT-qPCR analysis and normalized to 18S rRNA levels, also quantified by RT-qPCR analysis. (K, O) The expression of BLACAT1 was determined after overexpression of RBMX or knockdown of RBMX by qRT-PCR assays. Data are shown as the mean ± S.E.M., n = 6. *P < 0.05, **P < 0.01, ANOVA.
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Figure 7. RBMX and BLACAT1 interact to co-regulate HCC cells proliferation. (A, D) Re-transfection of BLACAT1 overexpressing or shBLACAT1 vectors in Huh7 and Hep3B cells, which RBMX gene already knockdown or overexpressed in these cells, respectively. The expression level of BLACAT1 was detected by qRT-PCR assays. (B, E) Cell Counting kit-8 assay and (C, F) Colony-formation assay were performed in A, D processed cells. Huh7-SR and Hep3B-SR cells are operated under the same steps with Huh7 and Hep3B cells in (A, D), then (G, J) the expression level of BLACAT1 was detected by qRT-PCR assays. (H, K) Cell Counting kit-8 assay and (I, L) Colony-formation assay were also performed. Data are shown as the mean ± S.E.M., n = 6. *P < 0.05, **P < 0.01, ANOVA.
shBLACAT1 vectors, respectively. The transfection efficiency, which BLACAT1 overexpression in Huh7 cells and BLACAT1 knockdown in Hep3B cells, were proved by QRT-PCR, MTT and colony formation assay (Supplementary Figure 2). Then, RT-PCR for BLACAT1, cell viability and colony formation assay were performed. The data shows, it’s evident that BLACAT1 is decreased (Figure 7A) in Huh7 cells when RBMX was silenced even BLACAT1 was overexpressed. As BLACAT1’s expression, the proliferation and colony formation ability of Huh7 cells also declined (Figure 7B, 7C), similarly. However, in Hep3B cells which BLAC1 was silenced while RBMX was up-regulated, the BLACAT1’s expression (Figure 7D), cell viability (Figure 7E) and colony formation density (Figure 7F) have not been rescued by RBMX overexpression compare to Hep3B cells of BLACAT1 was silenced only. These tests also performed in sorafenib resistance cells (Huh7-SR and Hep3B-SR) with same processing. Consistent with the above results, the BLACAT1 expression level (Figure 7G) and cells proliferation ability (Figure 7H, 7I) in Huh7-SR both similarly declined. Additional, Hep3B-SR cells appear identical tendency of these indicators (Figure 7J-L). In previous experiments, we had shown that RBMX increases cell viability; here we show that BLACAT1 can also increase cell viability but only in the presence of RBMX. Hence, indicates HCC cells proliferation capacity is dependent on RBMX and BLACAT1 co-regulation.

RBMX stabilizes BLACAT1 facilitate HCC cancer cells sorafenib resistance by autophagy

To further determine whether BLACAT1 is involved in sorafenib resistance in hepatocellular carcinoma cells via RBMX, toxicity experiments were performed in Huh7 and Hep3B cells with different expression of RBMX and BLACAT1. Initially, we found that the Huh7 cells which BLACAT1 overexpressed remained higher cell viability with concentration gradually increased sorafenib processing (Figure 8A), than which the empty vector group. In opposite, the Hep3B cells viability, which BLACAT1 was silenced, was decreased quickly under sorafenib challenge compare to scr group (Figure 8D). This indicating that BLACAT1 itself could contribute to sorafenib resistance of HCC cells. Then, the effect of RBMX in BLACAT1 mediated HCC cells resistance was studied next. As the results showed (Figure 8B), contrast with BLACAT1 overexpressed cells, the Huh7 cells viability decreased significantly with sorafenib treatment when RBMX was efficiently silenced, which making the cells highly susceptible to sorafenib, even if BLACAT1 was overexpressed. But, what interesting is, RBMX up regulation cannot rescue Hep3B cells vitality when BLACAT1 was silenced (Figure 8E). This explored that RBMX is indispensable in BLACAT1 mediated HCC cells resistance. Further, the multidrug resistant marker (ABCB1) and apoptotic marker (caspase-3) were checked in these two cells by western blot. The data showed that ABCB1 expression raised and caspase3 declined clearly respectively, but the silence of RBMX reversed the expression levels of maker genes (Figure 8C). As expected, ABCB1 and caspase-3 expressions just the reverse in Hep3B cells compare to Huh 7 (Figure 8F). Additionally, cells autophagy was detected for further verification. Consistent with cell viability, we observed that BLACAT1 overexpression seems to promote LC3 expression in Huh7 cells with sorafenib processing (Figure 8G), but LC3 expression seems to be declined when RBMX was silenced even in the presence of BLACAT1. Specifically, the LC3 expression deduced observably in Hep3B cells when BLACAT1 was silenced under sorafenib treatment, however, this expression level not recovered while RBMX overexpressed. And, the intensity of LC3 was quantified in Figure 8H. This indicated that the autophagy induced by BLACAT1 for sorafenib resistance regulated via RBMX. Furthermore, western blot were performed next. The proteins extract from Huh7 and Hep3B cells, which with sorafenib processing, are measured. Coincident with foregoing data, LC3 protein level also improved when BLACAT1 up regulated in Huh7 cells, but decreased when shRBMX present (Figure 8I). The LC3 of Hep3B always downregulated when BLACAT1 silenced, even though RBMX present (Figure 8J). Also P62, another autophagy marker, seems to be downregulated when BLACAT1 is upregulated in Huh7 cells, but when RBMX is silenced they seem to be upregulated (Figure 8I). And, this is different in Hep3B cells (Figure 8J). Hence, silencing of RBMX significantly affect the drug resistance ability by upregulating apoptosis and downregulating autophagy. Taken together, these data indicating the RBMX is essential in BLACAT1 mediated sorafenib resistance in HCC cells.
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Figure 8. RBMX stabilizes BLACAT1 facilitate HCC cancer cells sorafenib resistance by autophagy. Huh7 cells were transfected with empty vector, BLACAT1 overexpression vector, BLACAT1 overexpression vector + scr, BLACAT1 overexpression vector + shRBMX, respectively. Hep3B cells were transfected with scr, shBLACAT1, empty vector + shBLACAT1, RBMX overexpression vector + shBLACAT1, respectively. (A, B, D and E) CCK8 assay was used to detect cells sensitivity to sorafenib. Western blotting with anti-ABCB1 and anti-caspase3 on protein extracted from Huh7 cells (C) and Hep3B cells (F). (G) Immunofluorescence of autophagy (LC3-RFP) in Huh7 and Hep3B cells. Scale bars = 20 μm. (H) Quantification of LC3-positive puncta per cell. (I, J) Western blots with anti-LC3 antibody and anti-P62 antibody in Huh 7 and Hep3B cells. Data are shown as the mean ± S.E.M., n = 6. *P < 0.05, **P < 0.01, ANOVA.

Discussion

RBMX/hnRNP G has been recently acknowledged for its function in alternative splicing processes for many mRNA [23]. Interestingly, these functions vary between activation or inhibition depending on the mRNA. A study by Nasim et al., [10] had shown that RBMX interacts with a splicing activator protein called hTr-a2b, and they together antagonistically are involved in inclusion or exclusion of exons in many mRNA. This particular procedure allows...
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certain genes to be either suppressed or expressed in a particular cell type. Another study identified estrogen receptor alpha (ERα) [24] expression is regulated by hnRNP G and a splicing regulator/opponent HTRA2-BETA1. In this study, hnRNP G was required for the inhibition of the splicing of ERα exon 7 by HTRA2-BETA1. Surprisingly, this inclusion and higher expression of hnRNP G aided in improved prognosis and survival in endometrial cancer. In contrary, in our study we observed increased expression of RBXM led to poor prognosis in HCC patients (Figure 1B). Additionally, we observed overexpression of RBXM led to an increased proliferation and colony formation capacity in HCC cell lines (Figure 2). This alternative role could be well explained, because hnRNP G has varying roles depending on the target mRNA [10]. There are many such studies showing hnRNP G to have multiple splicing roles in mRNA of proteins such as survival motor neuron SMN2, Tau and dystrophin proteins [10, 25].

In our study, we identified one such mRNA which seems to be regulated by RBXM, which is BLACAT1 (Figure 6). BLACAT1 which is also known linc-UBC1 is a long intergenic non-coding RNA, they are among the recently identified class of IncRNA and have been neglected for years as just non-essential background noise [19]. In recent years many of such IncRNA are being brought into limelight for their role in cancer. One such IncRNA is HOX transcript antisense RNA (HOTAIR) which seems to be interacting with polycomb repressive complex and in turn upregulate the trimethylation of histones, which subsequently lead to downregulation of many genes [15, 26]. Previously, HOTAIR has been identified to contribute to poor prognosis, increased proliferation and metastasis in many cancers such as breast cancer [15], pancreatic cancer [16], colorectal cancer [17] and HCC [18]. In our study, we were very interested to identify the relationship between RBXM and BLACAT1. Previously, a study identified the RNA recognition motif of RBXM binds to a consensus sequence of CC (A/C) motif [13], and in this study we identified BLACAT1 has total 29 such sites in BLACAT1 sequence (Figure 6F). We also identified that BLACAT1 binds and interacts with RBXM through immuno-precipitation studies (Figure 6H, 6L, 6I and 6M). It was also clear that RBXM not only binds to BLACAT1 but positively upregulates the expression of BLACAT1 (Figure 6K, 6O). Previous studies have shown BLACAT1 to be upregulated in bladder cancer [19], gastric cancer [22] and colorectal cancer [20].

From our evidence, RBXM also seems to bind and stabilize BLACAT1, as we observed post silencing of RBXM, BLACAT1 decayed quickly (Figure 6J, 6N). This indicates the strong interactive role between RBXM and BLACAT1. It was also evident that lack of RBXM and in turn BLACAT1 seems to upregulate apoptosis, decrease proliferation, and also decrease autophagy in HCC cell lines (Figure 8). There have been other studies where in binding of certain proteins are necessary to stabilize them, one study had shown the RNA binding protein known as “quaking” was necessary to stabilize SIRT2 mRNA for it to perform its functions [27]. Understanding the potential functions of BLACAT1 leads to one of the earliest studies which identified its role in bladder cancer due to its interaction with polycomb repressive complex 1 and has been associated with lymph node metastasis and poor overall survival [19]. BLACAT1 has previously also been associated to increased cell proliferation due to its ability to bind with EZH2 and suppresses p15, thereby disrupting the cell cycle in colorectal cancer [20]. Hence, BLACAT1 could potentially be the chief contributing factor for tumorigenesis but it was evident that RBXM was regulating the BLACAT1 in HCC. When cells were overexpressed for BLACAT1 and treated with sorafenib, the proliferation of these cells were higher than the empty vector. But when these same cells were silenced for RBXM and treated with sorafenib, the cell viability decreased significantly in HCC and HCC-SR cells (Figure 8).

Another interesting observation from our data was that RBXM also contributed to multidrug resistance in HCC. In this study, we considered one of the important treatment strategy sorafenib that has been used to treat advanced stages of HCC [6, 7]. In our study, we observed sorafenib resistant (SR) cell lines also had higher expression of RBXM (Figure 3C, 3D). Treatment of sorafenib had very less effect on sorafenib resistant (SR) cell lines also had higher expression of RBXM (Figure 3C, 3D). Treatment of sorafenib had very less effect on cell viability (Figure 3A) of HCC-SR cell lines. But silencing of RBXM (Figure 3G and 3H) aided in improving the sensitivity of sorafenib significantly, thereby indicating a strong role of RBXM in sorafenib resistance. Additionally, we
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observed in both the HCC and HCC-SR cell lines, sorafenib dependent apoptotic rate increased significantly after silencing of RBMX (Figure 3K, 3L). There are various studies which have indicated that sorafenib can activate protective autophagic response in HCC cells, and detailed experiments where in various pharmacological inhibitors such as bafilomycin 1, chloroquine or knockdown of various autophagic genes such as beclin or atg5 have been indicated to improve the cytotoxic effect of sorafenib [28-33]. Another study had shown CD24 to be highly expressed in HCC, which in turn activates protein phosphatase 2, this additionally seems to activate mTOR/AKT pathway which contributes to autophagy [34-36]. Other genes such as PSMD10 [37], ADBR2 [38] also have shown to promote HCC progression through their regulation of autophagy. In our studies too, we observed autophagy to be highly upregulated post treatment with sorafenib in the resistant cell lines (Figure 5A). But when RBMX was silenced, autophagy was highly downregulated with decrease in proliferation, thus overcoming sorafenib associated protective mechanism in HCC-SR.

In recent years, different cancers have been observed to develop multidrug resistance, specifically IncRNA have been associated to contribute to such resistance. Specifically, the study by Zhou et al., which had proven that HOTAIR, an IncRNA, plays a vital role in cisplatin resistance to hepatocellular carcinoma [15]. They observed that HOTAIR induced MDR through STAT3 (signal transducer activated transcription), which in turn phosphorylated ABCB1. One type of MDR is actually due to ABCB1 (ATP-binding cassette sub-family B member) which pushes out the anticancer drugs out of the cells thereby reducing the intracellular drug [39]. Recently, many studies have also shed light on the role of another non-coding RNA microRNA (miR). Studies have indicated miRs such as miR-129-5p [40], miR-361 [22] cause’s chemo-resistance in gastric cancer through activation of ABCB1. One type of MDR is actually due to ABCB1 (ATP-binding cassette sub-family B member) which pushes out the anticancer drugs out of the cells thereby reducing the intracellular drug [39]. Recently, many studies have also shed light on the role of another non-coding RNA microRNA (miR). Studies have indicated miRs such as miR-129-5p [40], miR-361 [22] cause’s chemo-resistance in gastric cancer through activation of ABCB1. In our study too, we observed ABCB1 to be upregulated in sorafenib resistant cells, specifically after treatment with sorafenib. But once these cells were silenced for RBMX, ABCB1 expression decreased significantly (Figure 8C).

Based on our studies and multiple evidences from literatures, we could conclude that RBMX through BLACAT1 induces tumorigenesis, which was observed by increasing proliferation and decreasing apoptosis. Further it also regulates MDR through ABCB1 and autophagy (LC3). Our results could contribute in development of therapeutic strategies for advanced stages of HCC and other cancers whose treatment is a limited to specific type of therapeutic strategy.

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Disclosure of conflict of interest

None.

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References

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Supplementary Figure 1. Huh7 and Hep3B cells transfection efficiency validation. Huh7 cells were transfected with RBMX overexpression vector, empty vector as control; on the contrary, right panel, Hep3B cells were transfected with shRBMX, shscr as control. After 24 h, RBMX level was measured by qRT-PCR and western blot. Data were shown as the mean ± S.E.M., n = 3. *p < 0.05, **p < 0.01, ***p < 0.001, ANOVA.
Supplementary Figure 2. Huh7 and Hep3B cells transfection efficiency validation. Huh7 cells were transfected with BLACAT1 overexpression vector, empty vector as control; below, Hep3B cells were transfected with shBLACAT1, shscr as control. After 24 h, RBMX level was measured by qRT-PCR and transfection efficiency was verified by MTT and colony formation assay. Data were shown as the mean ± S.E.M., n = 3. *p < 0.05, **p < 0.01, ANOVA.