

# RBMX promotes hepatocellular carcinoma progression and sorafenib resistance by stabilizing BLACAT1

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## Research

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# Abstract

**Background** Hepatocellular carcinoma 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.

**Method** RBMX and Lnc BLACAT1 were measured in HCC patient's tissue and commercial cell lines (Huh7 and Hep3B). And their relationship was verified by Co-IP assay and Overexpression experiment. Furthermore, the cell proliferation, colony formation and drug resistant assay were performed for effect evaluation of RBMX and BLACAT1 in HCC cell lines.

**Result** Both overexpression and positive correlation between RBMX and BLACAT1 were validated in HCC cell lines. Co-IP analysis also shows BLACAT1 could be specially bound by RBMX and increased the expression of BLACAT1. 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.

**Conclusion** 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.

## 1. Background

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%<sup>1</sup>. Treatment strategies for advanced stages of HCC are very limited due to the many underlying liver diseases which are associated with carcinoma<sup>2</sup>. 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<sup>3,4,5</sup>. Sorafenib in clinical studies such as the SHARP trial<sup>6</sup> and Asia-pacific trial<sup>7</sup> 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 heterogeneous disease, many patients seemed to show primary resistance to the sorafenib treatment<sup>8</sup>. 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<sup>9</sup>. Among these,

hnRNP G is coded by the RBMX gene and is relatively the least abundant but implicated in splicing of many vital mRNA<sup>10</sup>. 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<sup>11</sup>. 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<sup>12</sup>.

A study by Heinrich et al.,<sup>13</sup> 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) has gained lot of recognition for their roles in various diseases<sup>14,15</sup>. 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<sup>16,17,18</sup>. Bladder cancer associated transcript-1 (BLACAT1) as the name suggests was first identified in bladder cancer<sup>19</sup>, but later has been associated with colorectal cancer<sup>20</sup> and cervical cancer<sup>21</sup>. There are also studies implicating BLACAT1 to contribute to multidrug resistance<sup>22</sup>. As mentioned above there are some studies discussing RBMX and BLACAT1's potential individual roles in tumorigenesis, but till date 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 RBMX's 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.

## **2. Materials And Methods**

### **2.1 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.

### **2.2 Cell line and culture.**

The human hepatocellular carcinoma cell lines Huh7 and Hep3B cells 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% CO<sub>2</sub>.

## 2.3 Establishment of sorafenib-resistant cells

To determine the half inhibitory concentration (IC<sub>50</sub>), cells were treated with sorafenib in 96 well plates and cell viability was determined for 3 days. Cells at a concentration of 1 × 10<sup>4</sup> cells/well were cultured in 6-well plates and incubated with sorafenib at a concentration just below their respective IC<sub>50</sub>. Further, the sorafenib concentration was gradually increased by 0.25 μM every week, thus allowing selection of resistant cells. Sorafenib-resistant cell lines were obtained after 6 months, and were termed Hep3B-SR and Huh7-SR. These cells were maintained by continuously culturing in the presence of sorafenib.

## 2.4 RNA interference and plasmids

Two shRNA sequences targeting RBMX (shRBMX-1 and shRBMX-2) were synthesized by GenePharma (GenePharma Co, Ltd, Shanghai, China) to knockdown RBMX expression and the negative control shRNAs (scr) without sequence homology to human genes were provided by the same manufacturer. Cells were transfected with 100 nM shRNA duplexes using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) following manufacturer's protocols. The mock transfected cells were transfected with Lipofectamine 2000 without shRNA. pCMV6-RBMX (RBMX) and pCMV6-BLACAT1(BLACAT1) was synthesized and cloned into the cloning site of pCMV6 plasmid (Origene) respectively. pCMV6-Entry (vector) was as negative control.

## 2.5 Quantitative real-time polymerase chain reaction (qRT-PCR)

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<sup>-ΔΔCt</sup>. The experiments were performed in triplicate.

B-actin qRT-PCR forward primer: 5'-GCTACGAGCTGCCTGACG; reverse: 5'-GGCTGGAAGAGTGCCTCA.

RBMX qRT-PCR forward primer: 5'-CAGTTCGCAGTAGCAGTGGA; reverse: 5'-TCGAGGTGGACCTCCATAAC.

BLACAT1 qRT-PCR forward primer: 5'-CAAGAGGAGCCGGCTTAGCATCT; reverse: 5'-ACGGTTCCAGTCCTCAGTCAG.

## 2.6 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,  $\beta$ -actin from Sigma.

## 2.7 Immunohistochemical assay

Immunohistochemical analyses were performed as per (ref 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).

## 2.8 In vivo tumor xenograft model

The Huh7 cells were transfected with either scr or shRBMX – 1. 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. Further, monitoring of tumor growth was carried out using digital calipers, and the volume was calculated using the formula: tumor volume ( $\text{mm}^3$ ) = [width (mm)]<sup>2</sup>  $\times$  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 and flow cytometry. 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.

## 2.9 TUNEL assay

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

## 2.10 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 by spectrophotometer. Cell growth curve was further drawn with normalized values which were taken at OD 450.

## 2.11 Colony- formation efficiency assay

After transfection with RBMX knock down vector or BLACAT1 knock down 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.

## 2.12 In vitro apoptosis assay

Approximately  $1 \times 10^5$  cells were suspended in 100  $\mu$ l buffer, 5  $\mu$ l of Annexin V and 5  $\mu$ l 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 cytometric 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).

## 2.13 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.

## 2.14 mRNA Stability Assay

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 (Sigma). At different time points, (0, 15, 30, 60, and 120 min) RNA was isolated qRT-PCR were performed, as described above. Half-life ( $t^{1/2}$ ) 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 ( $t^{1/2}$ ) were determined by nonlinear regression analysis.

## 2.15 Database and software predictions

In order to get a wider range of evidence, the dysregulated 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.tartaglialab.com/page/catrapid\\_group](http://service.tartaglialab.com/page/catrapid_group)).

## 2.16 Statistical Analysis

The data are presented as the means  $\pm$  standard deviations (SD). Tukey's test or Student's t-test for the unpaired results was used to evaluate the differences among more than three groups or between two groups, respectively. Differences were considered significant for values of  $p < 0.05$ .

## 3. Results

## 3.1 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 (Fig. 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 (Fig. 1B). In six individual patient tumor samples western blotting analysis was performed with comparisons to the normal tissue samples (Fig. 1C). It was clear that there was significantly higher expression of RBMX in the patient samples, which was also observed in the mRNA level (Fig. 1D). Immunohistochemistry analysis was performed in hepatocellular carcinoma (HCC) patient tissues and consistent results were evident that RBMX was highly expressed (Fig. 1E). Commercially available hepatic cell lines such as LO2, HepG2, Huh-7, SMMC7721, Hep3B, HCCLM3 and MHCC97H were checked for RBMX expression (Fig. 1F, G). Among these, Huh-7 displayed the highest expression of RBMX followed by HepG2. And, Huh-7 or Hep3B cells were choose for further experiments.

## 3.2 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) (Fig. 2A). RBMX overexpression assay was performed in Hep3B cells, and overexpressing efficiency was also verified in (Fig. 2B). Then, proliferation assays were performed with these cells using cell counting kit. The results indicated that RBMX silenced cells have decreased proliferation (Fig. 2C), on the contrary, RBMX overexpressed cells displayed a higher proliferation ability (Fig. 2D). Further, colony formation capacity shows shRBMX Huh-7 cells had lesser colony forming units when compared to scr (Fig. 2E), adversely, Hep3B cell's colony units was significant increased when overexpressing RBMX (Fig. 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 (Fig. 2G), contrarily, the dead cells is minor in RBMX overexpressed Hep3B cells (Fig. 2H). Next, the data was further verified using flow cytometer analysis for RBMX silencing (Fig. 2I). Taken together, these date demonstrated that RBMX could facilitate HCC cells proliferation and inhibit cell apoptotic.

## 3.3 RBMX promote tumor growth in model

Next, the role of RBMX in tumor growth was studied. For this, shRBMX Huh-7 cells were injected into the dorsal flanks of *nu/nu* mice. The tumor volumes were subsequently measured every week using digital calipers. It was evident that the tumor size increased significantly from day 7 to day 35 in the scr group (Fig. 3A, B). But the development of tumors was significantly much slower in shRBMX group, when compared to the control group at the same time points. At day 35 the mice were sacrificed, and the tumor weight was measured. It was clear that the shRBMX group tumors were significantly smaller when compared to the scr group (Fig. 3A, C). Further, these samples were subjected to immunohistochemistry staining. Silencing efficiency was confirmed with RBMX immunohistochemistry staining, where in the shRBMX group tumor section displayed less to no positive cells for RBMX antibody (Fig. 3D right). Additionally, hematoxylin and eosin staining of the tumor sections from the control group showed distinct cells with clear darker nuclei typical of cancerous cells, but shRBMX group showed lesser nuclei (Fig. 3D left), indicating a decrease in tumorous cells. Ki67 staining was also performed which indicated more proliferative cells present by the darker nuclei in the scr group when compared to the shRBMX group. Finally, TUNEL assay of these sections displayed higher apoptotic cells in the shRBMX group compared to the scr (Fig. 3D middle). Taken together, these data indicated that RBMX promote tumor growth by improve cells proliferation and decrease apoptotic.

### **3.4 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 resistant evaluation. As show in Fig. 4A B, There have a huge 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 compare to Huh7/Hep3B native cell. More than that, the expression level of RBMX was further enhanced under sorafenib treatment (Fig. 4C, D). Further, cell viability assay was performed for evaluation of RBMX function in RBMX overexpressing or silenced cells. With increasing concentrations (0–20  $\mu$ M) of sorafenib, it was evident that the cell relative viability of Huh7/Huh7-SR cells which RBMX have been silenced significantly decreased (Fig. 4E). But RBMX overexpressing cells, Hep3B/Hep3B-SR, had higher cell viability than empty vector (Fig. 4G), thus indicating a important role of RBMX in resistance to sorafenib. Apoptosis level was also checked using flow cytometry. It was observed that the percentage of apoptotic cells in different RBMX expression level is consistent with cell viability (Fig. 4F, H). Simultaneously, to identify specific indicators of drug resistance and its association with RBMX, we checked the presence of ABCB1 (ATP Binding Cassette Subfamily B Member 1, a vital indicator of multidrug resistance) in HCCs (Fig. 4I). Western blotting data indicated that increased expression of RBMX did increase ABCB1 expression. And, ABCB1 expression decreased significantly in both Huh7/Huh7-SR and Hep3B/Hep3B-SR cell lines when RBMX was silenced. Simultaneously, to further understand the correlation of RBMX and apoptosis, caspase 3 expression was checked. From the data, it was clear that caspase-3 expression was reduced while RBMX was upregulated (Fig. 4I), but when RBMX was silenced caspase-3 expression was highly

upregulated, confirming our previous study of RBMX's role in cell proliferation. Based on these data we could declare that RBMX plays a key role in sorafenib resistance of HCCs.

### **3.5 Autophagy is a consequence of RBMX mediates sorafenib resistance**

Further experiments were also performed to understand the mechanism of how RBMX participate in sorafenib resistance. Interestingly, it was observed that the cells autophagic marker LC3 significantly increased in sorafenib resistant cells and RBMX up-regulated cells, there have an opposite expression levels in HCC normal cells and RBMX down-regulated cells (Fig. 5A-D). 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 opposed (Fig. 5E, F). Thus, the above-mentioned experiments specifically indicated the roles of RBMX mediate sorafenib resistance of hepatocellular carcinoma cells by activating autophagy.

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

Many studies have showed the chemotherapeutics resistant ability of BLACAT1 in multi-cancers, but the specific mechanism of action is unclear. For this concern, we talk about the relationship between RBMX and BLACAT1 in HCCs. First of all, BLACAT1 level was measured in HCC patient tissues and commercial HCC lines (Fig. 6A, B), and results show that there have a positive correlation between them (Fig. 6C). 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 lncBLACAT1.

We identified a total of 29 such sites in BLACAT1 sequence (Fig. 6D). More than that, *CatRAPID* online algorithm also performed for RNA-protein interaction prediction (Fig. 6E). Of interest, this program revealed a higher interaction DP value, reach up to 98%. Next, RNA immunoprecipitation assays were go ahead for interaction validation. RIP analysis of the samples showed the RBMX presences in both the Huh7 and Hep3B cells, which contain BLACAT1, thus indicating a strong interaction between BLACAT1 and RBMX (Fig. 6F, G). Additionally, RNA immunoprecipitation analysis followed by qRT-PCR also showed that sample precipitated with RBMX had a high fold expression of BLACAT1 (Fig. 6H, I). Experiments to understand the stability of BLACAT1 mRNA in the absence of RBMX were studied. Cells were either transfected with scr or shRBMX and then treated with actinomycin D to block de novo transcription. Further, 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 min h in both Huh7 and Hep3B cells when RBMX was silenced (Fig. 6H, I). This indicated that the stably expressing of BLACAT1 is potential dependent on RBMX exist. Overexpression studies were performed for further

verification (Fig. 6J, K). The expression level of BLACAT1 was distinctly increased with the overexpression of RBMX in all these cells, and when RBMX was silenced, BLACAT1 was downregulated. Taken together, these data indicating a strong interaction between RBMX and BLACAT1.

### **3.7 RBMX knockdown inhibited BLACAT1-induced cell proliferation**

Overexpression studies were further used to evaluate the effect of BLACAT1 on cell proliferation. The data shows, it's evident that BLACAT1 overexpression (Fig. 7A) in Huh7 cells increased the proliferation of HCC cells (Fig. 7B), also with an increase in colony forming capacity (Fig. 7C). The same trend of cells proliferation (Fig. 7D) and colony formation ability (Fig. 7E) also appeared in Hep3B cell line which transfected with BLACAT1 overexpressed vector (Fig. 7F). However, Huh7 cells and Hep3B cells which BLACAT1 gene overexpressed (Fig. 7G, J) lost their both proliferation (Fig. 7H, K) and colony forming capacity (Fig. 7I, L) when RBMX was silenced. 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. This indicates BLACAT1's proliferation capacity is dependent on RBMX.

### **3.8 RBMX knockdown inhibited BLACAT1-induced autophagy and sorafenib resistance of HCC cancer cells *in vitro*.**

To further understand the role of BLACAT1 in RBMX mediated sorafenib resistance, Huh7 and Huh7-SR cells with overexpression of BLACAT1 and treatment with sorafenib were performed. Initially, we found that the Huh7 cell which BLACAT1 overexpressed remained higher cell viability in concentration gradually increased sorafenib treatment, than the empty vector, indicating that BLACAT1 itself could contribute for the resistance to sorafenib (Fig. 8A). But, the cells viability decreased significantly with sorafenib treatment when RBMX was efficiently silenced, even at as low as 2.5  $\mu$ M concentrations, making the cells highly susceptible to sorafenib (Fig. 8B). Further, the multidrug resistant marker (ABCB1) and apoptotic marker (caspase 3) were checked in the BLACAT1 over expressed and RBMX silenced cells. The data showed that ABCB1 expression raised and caspase3 declined clearly respectively, but the silence of RBMX reversed the expression levels of both (Fig. 8C), which indicated BLACAT1 similar to RBMX seemed to contribute to drug resistance in HCC. Similarly, the same experiments were performed in Huh7-SR cells which have uniform treatment as Huh7 cells. As we expected, there showed consistent consequence in cells viability, ABCB1 and caspase3 expression in RBMX silenced or not groups when BLACAT1 was overexpressed (Fig. 8D-F). Thus, overexpression of BLACAT1 increases cell's (both Huh7 and Huh7-SR cell lines) viability but the consequence were reversed as RBMX knock down. This indicated that RBMX binding to BLACAT1 is vital for the sorafenib resistance. Additionally, cells autophagy was detected. We observed that BLACAT1 overexpression seems to promote LC3 expression (Fig. 8G, H), but LC3 expression seems to be declined when RBMX is silenced even in the presence of BLACAT1. Specifically, although LC3 II expression seems to be upregulated in the BLACAT1 overexpression system, however it is

similar to vector control when RBMX is silenced, but LC3 I expression remains the same throughout (Fig. 8I). Also P62, another autophagy marker, seems to be downregulated when BLACAT1 is upregulated, but when RBMX is silenced they seem to be upregulated (Fig. 8H). Hence, silencing of RBMX significantly affects the drug resistance ability by upregulating apoptosis and downregulating autophagy. Taken together, these data indicate the vital role of RBMX in regulating BLACAT1 and sorafenib resistance in HCC cells.

## 4. Discussion

RBMX/hnRNP G has been recently acknowledged for its function in alternative splicing processes for many mRNA<sup>23</sup>. Interestingly, these functions vary between activation or inhibition depending on the mRNA. A study by Nasim et al.,<sup>10</sup> had shown that RBMX interacts with a splicing activator protein called hTra2 $\beta$ , and they together antagonistically are involved in inclusion or exclusion of exons in many mRNA. This particular procedure allows certain genes to be either suppressed or expressed in a particular cell type. Another study identified estrogen receptor alpha (ERa)<sup>24</sup> 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 ERa exon 7 by HTRA2-BETA1. Surprisingly, this inclusion and higher expression of hnRNP G aided in improved prognosis and survival in endometrial cancer. In contrast, in our study we observed increased expression of RBMX led to poor prognosis in HCC patients (Fig. 1B). Additionally, we observed overexpression of RBMX led to an increased proliferation and colony formation capacity in HCC cell lines (Fig. 2). This alternative role could be well explained, because hnRNP G has varying roles depending on the target mRNA<sup>10</sup>. 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<sup>10,25</sup>.

In our study, we identified one such mRNA which seems to be regulated by RBMX, which is BLACAT1 (Fig. 6). BLACAT1 which is also known as linc-UBC1 is a long intergenic non-coding RNA, they are among the recently identified class of ncRNA and have been neglected for years as just non-essential background noise<sup>19</sup>. In recent years many of such lncRNA are being brought into limelight for their role in cancer. One such lncRNA 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<sup>15,26</sup>. Previously, HOTAIR has been identified to contribute to poor prognosis, increased proliferation and metastasis in many cancers such as breast cancer<sup>15</sup>, pancreatic cancer<sup>16</sup>, colorectal cancer<sup>17</sup> and HCC<sup>18</sup>. In our study, we were very interested to identify the relationship between RBMX and BLACAT1. Previously, a study identified the RNA recognition motif of RBMX binds to a consensus sequence of CC(A/C) motif<sup>13</sup>, and in this study we identified BLACAT1 has total 29 such sites in BLACAT1 sequence (Fig. 6D). We also identified that BLACAT1 binds and interacts with RBMX through immunoprecipitation studies (Fig. 6F, G). It was also clear that RBMX not only binds to BLACAT1 (Fig. 6H, I) but positively upregulates the expression of BLACAT1 (Fig. 6J, K). Previous studies have shown BLACAT1 to be upregulated in bladder cancer<sup>19</sup>, gastric cancer<sup>22</sup> and colorectal cancer<sup>20</sup>.

From our evidence, RBMX also seems to bind and stabilize BLACAT1, as we observed post silencing of RBMX, BLACAT1 decayed quickly (Fig. 6J, K). This indicates the strong interactive role between RBMX and BLACAT1. It was also evident that lack of RBMX and in turn BLACAT1 seems to upregulate apoptosis, decrease proliferation, and also decrease autophagy in HCC cell lines (Fig. 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<sup>27</sup>. 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<sup>19</sup>. 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<sup>20</sup>. Hence, BLACAT1 could potentially be the chief contributing factor for tumorigenesis but it was evident that RBMX 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 RBMX and treated with sorafenib, the cell viability decreased significantly in HCC and HCC-SR cells (Fig. 8).

Another interesting observation from our data was that RBMX 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<sup>6,7</sup>. In our study, we observed sorafenib resistant (SR) cell lines also had very high expression of RBMX (Fig. 4C). Treatment of sorafenib had very less effect on decreasing the cell viability (Fig. 4E, G) of HCC-SR cell lines. But silencing of RBMX (Fig. 4E) aided in improving the sensitivity of sorafenib significantly, thereby indicating a strong role of RBMX in sorafenib resistance. Additionally, we observed in both the HCC and HCC-SR lines, sorafenib dependent apoptotic activity increased significantly after silencing of RBMX (Fig. 4E). There are various studies which have indicated that sorafenib can activate protective autophagic response in HCCs, 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<sup>28,29,30,31,32,33</sup>. 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<sup>34,35,36</sup>. Other genes such as PSMD10<sup>37</sup>, ADBR2<sup>38</sup> 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 (Fig. 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 ncRNA have been associated to contribute to such resistance. Specifically, the study by Zhou et al., which had proven that HOTAIR, a lncRNA, plays a vital role in cisplatin resistance to hepatocellular carcinoma<sup>15</sup>. 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<sup>39</sup>. 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<sup>40</sup>, miR-361<sup>22</sup> causes 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 (Fig. 8D).

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.

## 5. Conclusion

Overall, the results in this study indicated that under the mechanism of RBMX stabilize and promote BLACAT1 expression, hepatoma cells can increase their proliferation and colony ability then promote HCC development. Furthermore, the autophagy is also improved when co-incubate with sorafenib, contribute to chemotherapy resistance. Hence, RBMX could be considered as novel therapeutic target for HCC treatment strategies.

## Abbreviations

HCC (Hepatocellular Carcinoma); RBMX (RNA binding protein); BLACAT1 (bladder cancer associated transcript 1); SR (sorafenib-resistance); ABCB1 (ATP Binding Cassette Subfamily B Member 1); lncRNAs (Long non-coding RNAs); MDR (multiple drug resistance)

## Declarations

### Ethics approval and consent to participate

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.

### Consent for publication

Not applicable

### Availability of data and materials

Not applicable

### Competing interests

The authors declare that they have no conflict of interest.

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## Authors' contributions

All the authors contribute to design and discussion of this study. Furthermore, Y S and S H contribute to article writing; X M and M Z contribute to experiments performance; J Z and G W contribute to data analysis and figure construction; Y Y and W X provide guidance and assistance throughout the process.

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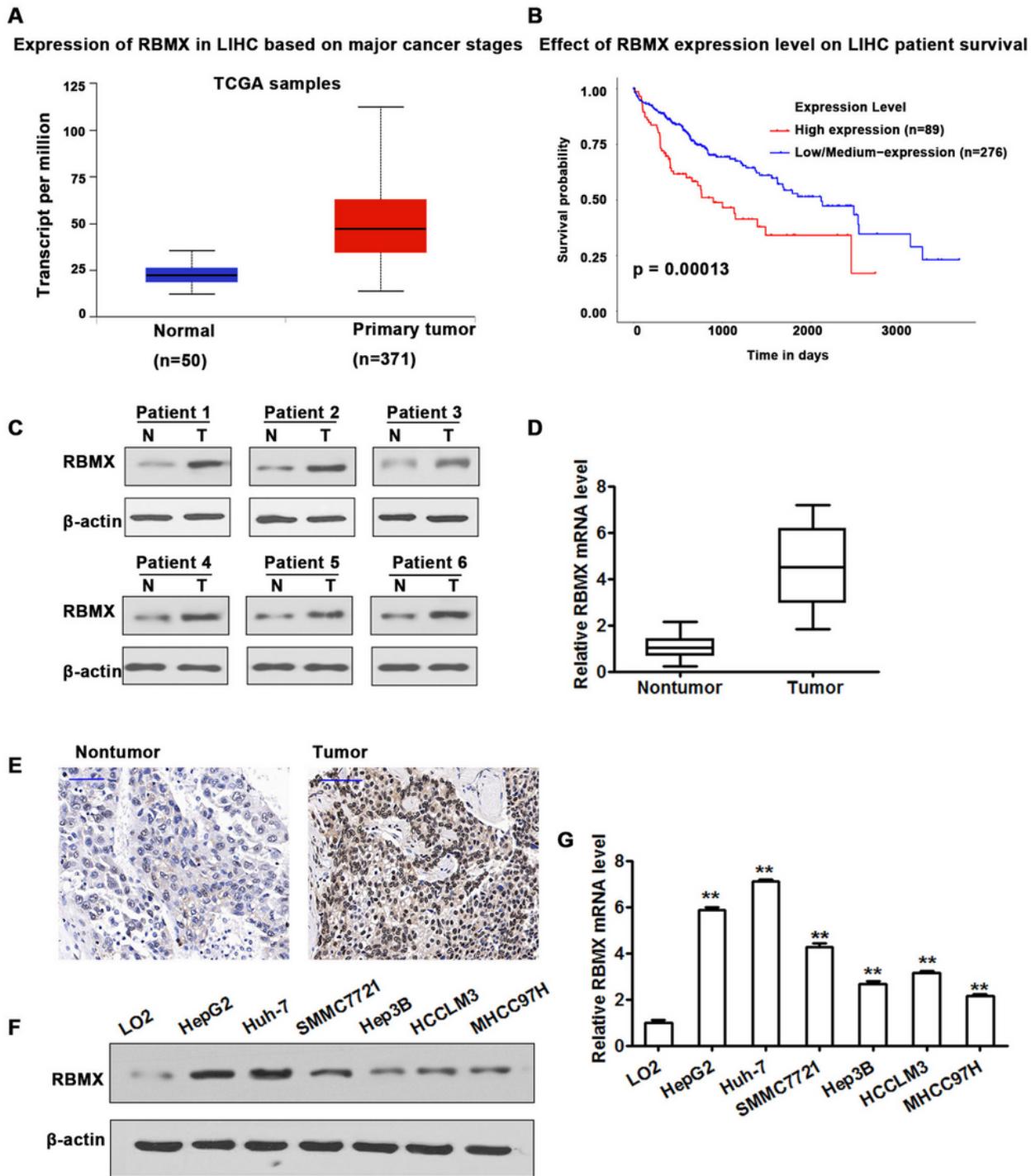
## Table 1

**Table 1**

Association of clinicopathological characteristics of the investigated HCC patients with RBMX expression. (n=60)

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

## Figures



**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 =70). (E) The expression of RBMX was detected by IHC staining in 16 paraffin-embedded HCC and normal tissues specimens. The original magnification was 200X. (F) Western blotting of RBMX protein and (G) RBMX mRNA expression in different human HCC cell lines. \*\*P<0.01

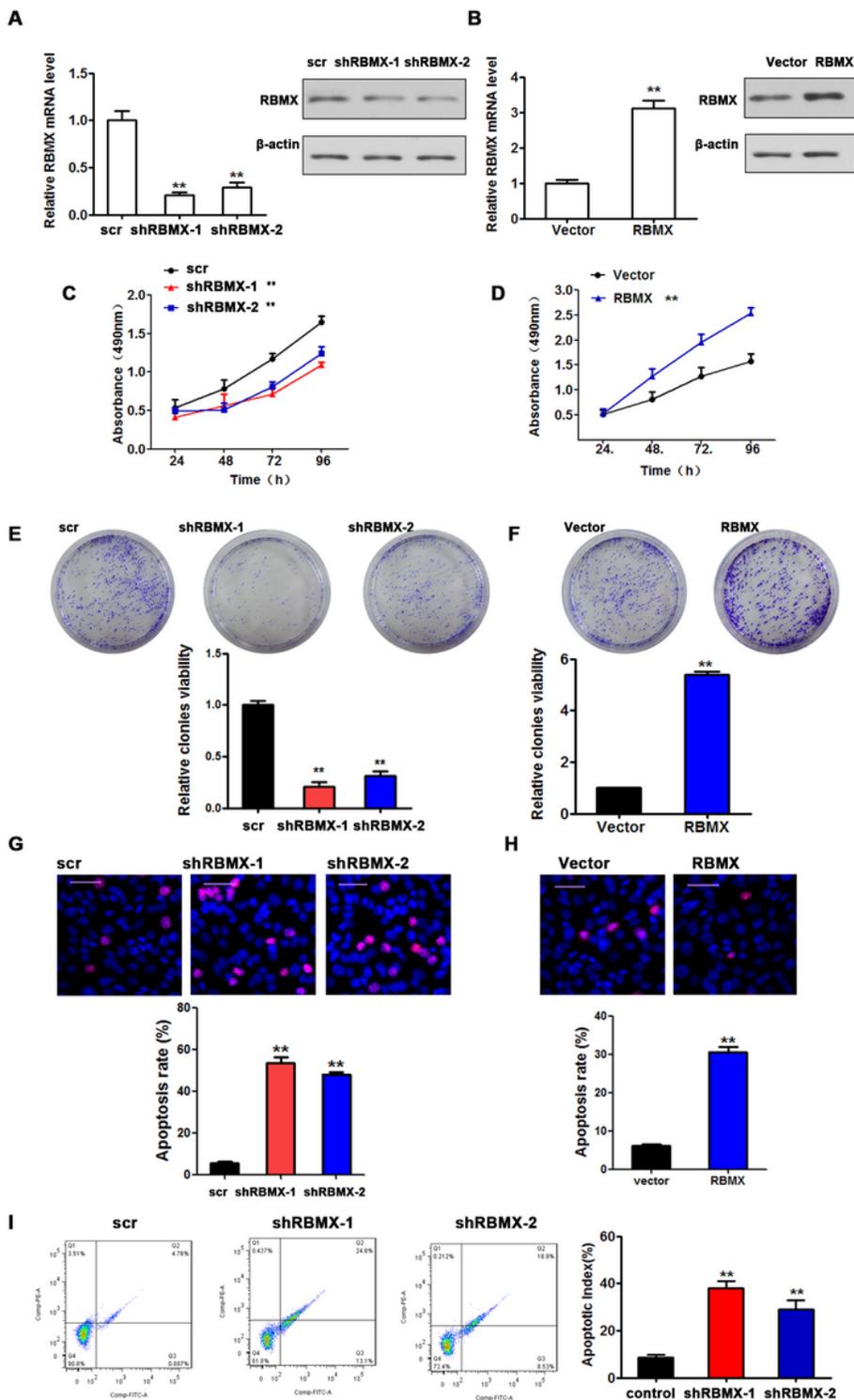
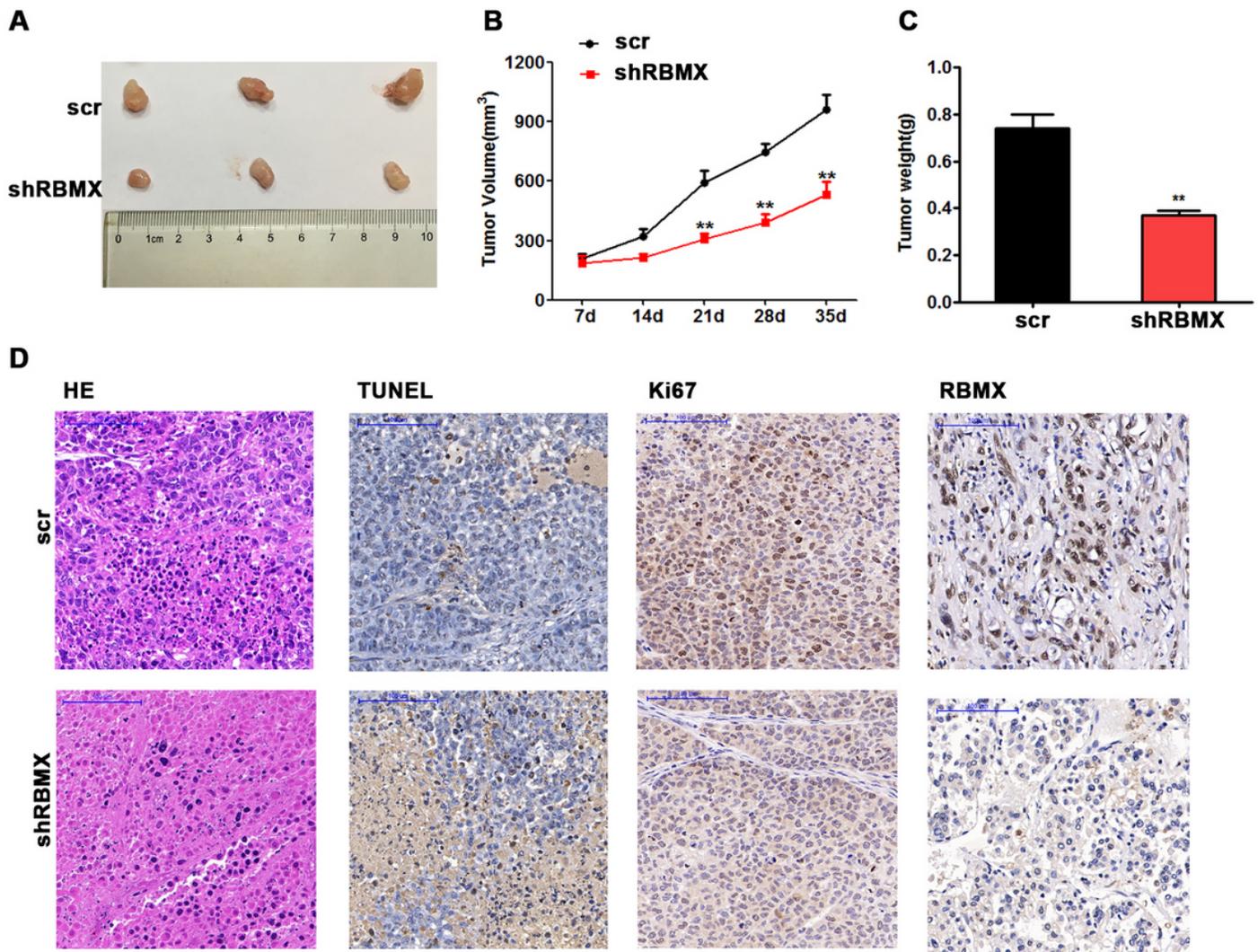


Figure 2

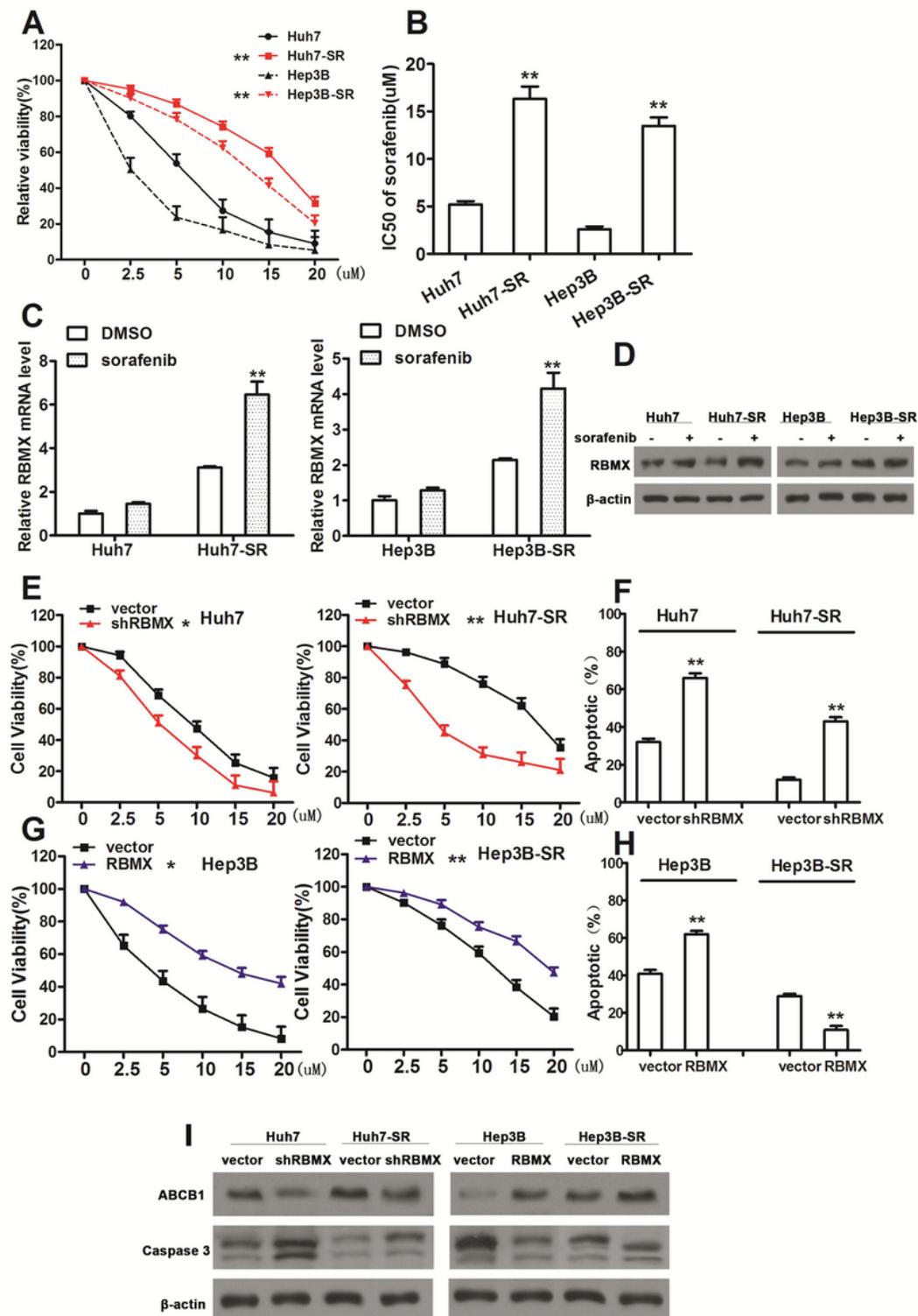
Crucial role of RBMX in HCC cell's proliferation and apoptosis. (A) Huh7 cells were transfected with shRBMX-1, shRBMX-2 or scr and the silencing efficiency was further detected by RT-PCR and WB. (B) Hep3B cells transfected with RBMX or empty vector (control) and the efficiency was detected by RT-PCR 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). (I) Flow cytometry showed that knockdown of RBMX could obviously induce Huh7 cell apoptosis, with a graph to indicate percentage index. \*\*P<0.01



**Figure 3**

RBMX promote tumor growth in model. Huh7 cells transfected with shRBMX were injected into nude mice (n = 6). The tumor volumes were calculated every week after injection. (A) The photography of xenograft tumors post 35 days of injection. (B) Tumor volumes. (C) Tumors weight (gram). The bars indicate S.D.

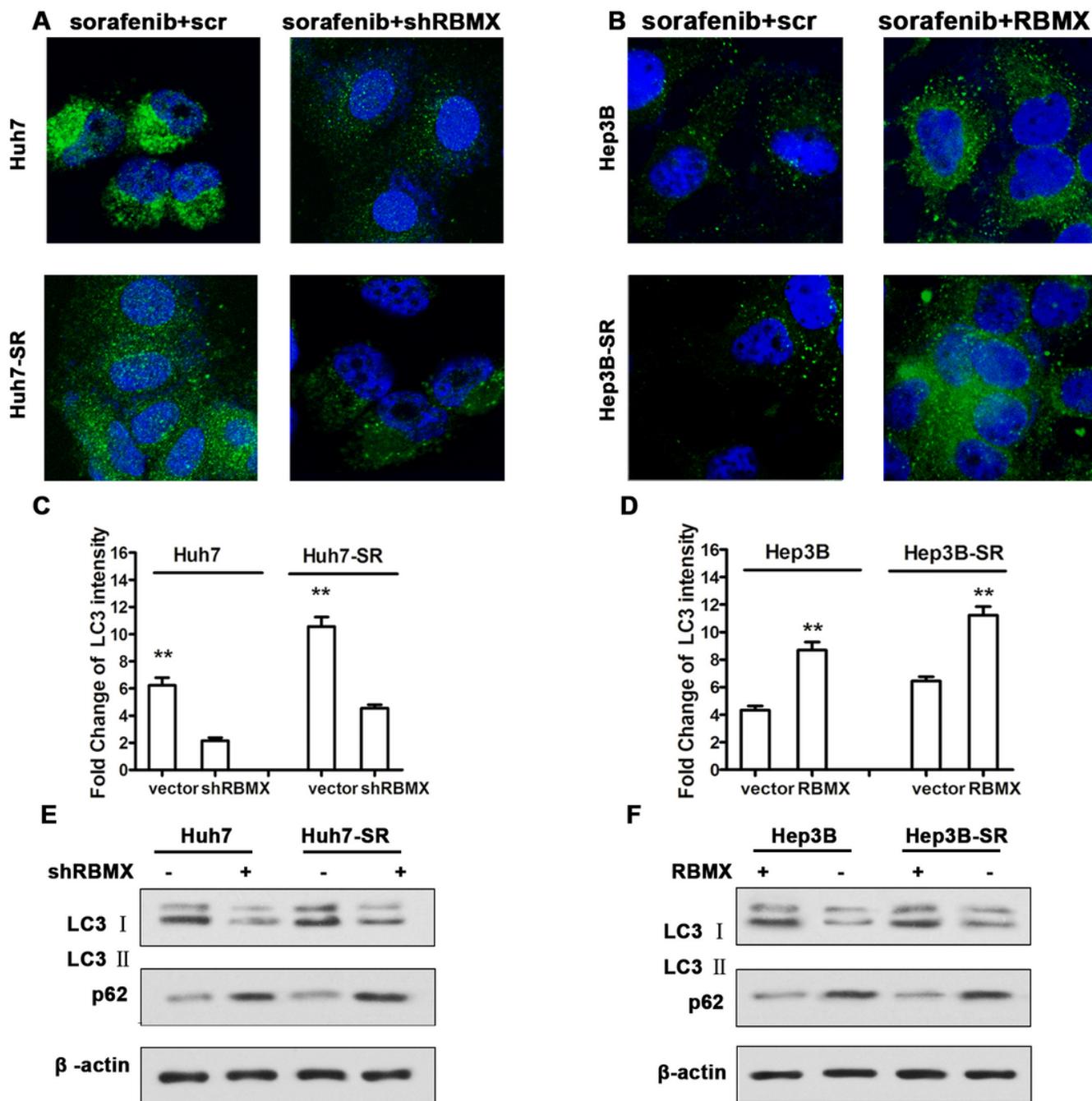
Data were means of value from mice, mean  $\pm$  S.E.M., n = 6. \*, p<0.05; \*\*, p<0.01. (D) Hematoxylin-eosin (HE) staining of xenograft tumors, TUNEL staining, anti-ki67 and anti-RBMX immunostaining in xenograft tumor samples. (Scale bars = 100 $\mu$ m)



**Figure 4**

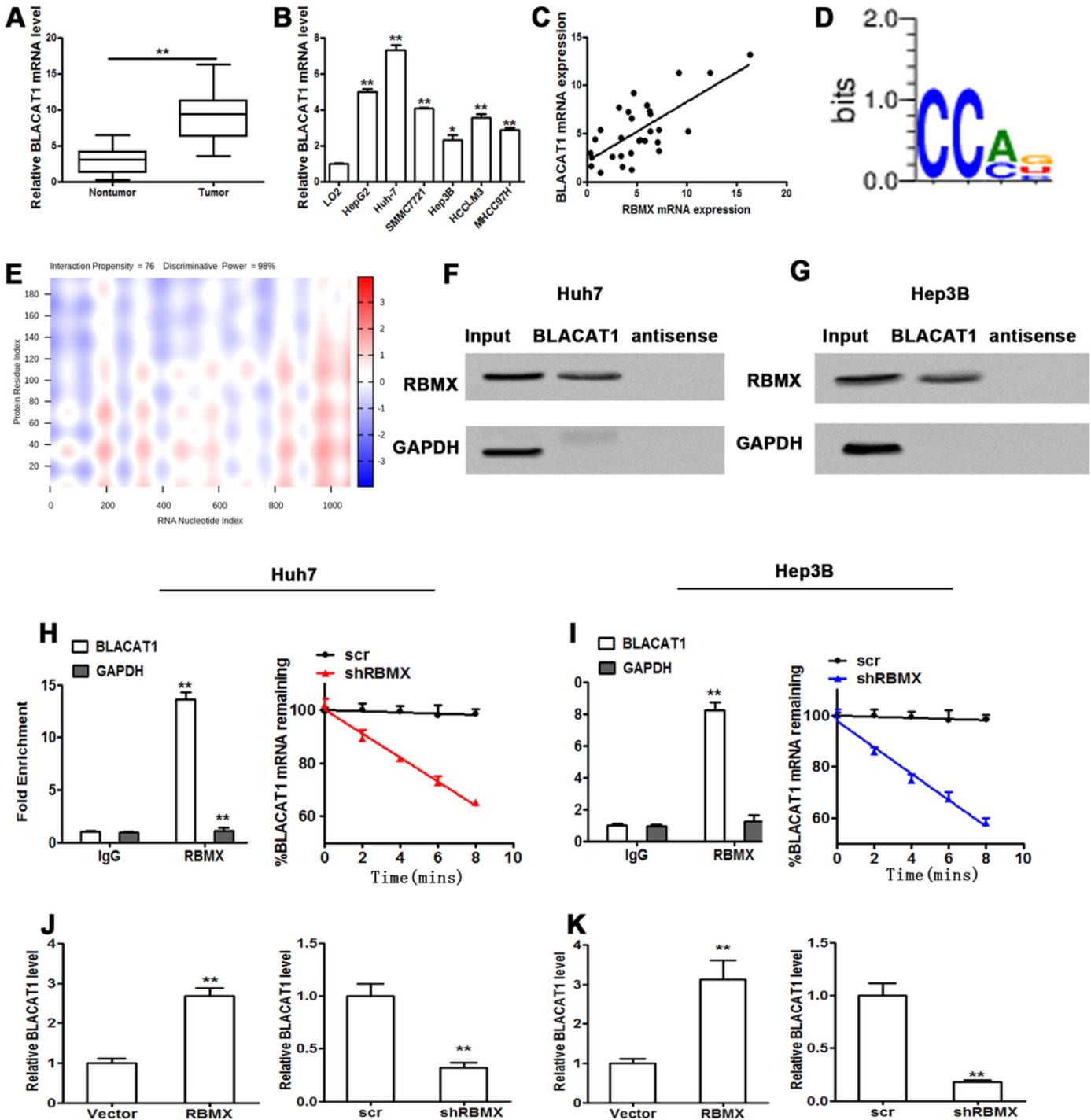
RBMX contributes to HCC cell lines sorafenib resistance. (A) Sorafenib resistance cells verification by CCK-8 assay. (B) IC50 of Huh7/ Huh7 -SR/ Hep3B/ Hep3B-SR cells. (C)qRT-PCR and (D) Western blot were

used to assay the expression of RBMX after cell were incubated with sorafenib (20  $\mu$ M) for 24h in Huh7 and Hep3B cells which was compared with sorafenib treatment in Huh7-SR and Hep3B-SR cells. Huh7/Huh7-SR (E), Hep3B/Hep3B-SR (G) cells were either used as an overexpression system or silencing system for RBMX gene and the cells were further treated with different concentrations (0, 2.5, 5, 10, 20  $\mu$ M) of sorafenib. After 48 h the CCK8 assay was performed to detect sorafenib sensitivity. (F, H) Flow cytometry analysis for apoptotic rate measurement in Huh7/Huh7-SR, Hep3B/Hep3B-SR cells post treatment with sorafenib (20  $\mu$ M) for 24h. (I) Western blotting analysis of ABCB1 and caspase 3 from Huh7/Huh7-SR, Hep3B/Hep3B-SR cells with either RBMX overexpression or silencing. \*\*P<0.01



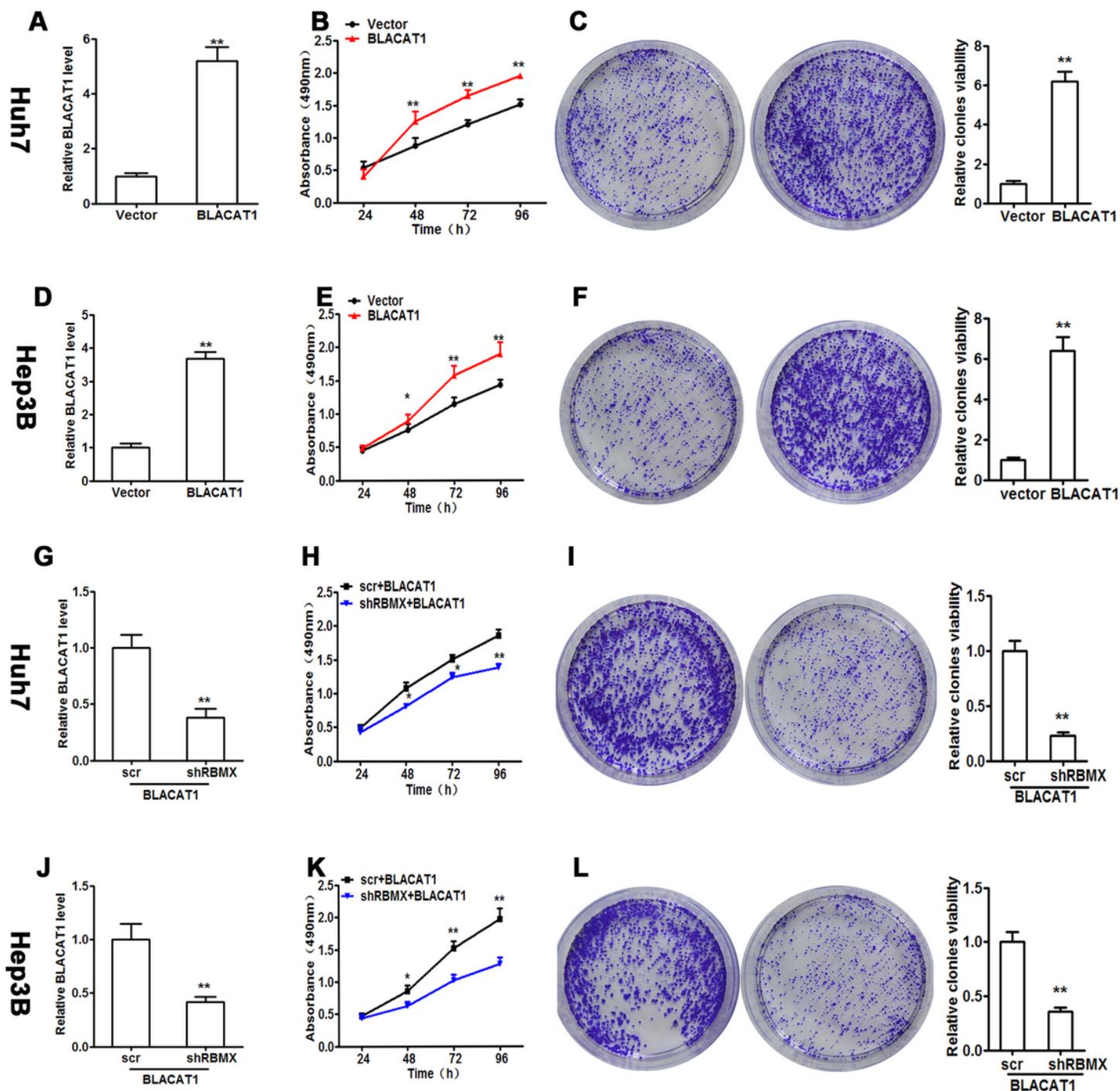
## Figure 5

Autophagy is a consequence of RBMX mediates sorafenib resistance. (A) Huh7/Huh7-SR cells (B) Hep3B/Hep3B-SR cells were incubated with sorafenib 10  $\mu$ M for 48 h, then transfected with scr or shRBMX. The cells were stained for LC3 and DAPI for nuclear staining. (C, D) Quantification of LC3-positive puncta per cell. Data are shown as the mean  $\pm$  S.E.M., n=6. \*\*P<0.01, \*\*\*P<0.001, ANOVA. (E, F) Western blotting with anti-LC3 and anti-P62 antibodies for the protein samples from the cell lines. \*\*P<0.01



## Figure 6

RNA-binding protein RBMX binds and stabilizes BLACAT1. BLACAT1 expression levels in patient's tumor (A) and commercial cell lines (B). (C) The correlation of RBMX and BLACAT1. (D) RBMX consensus binding site sequence and BLACAT1 sequence alignment. The RBMX binding sequence is shown. (E) CatRIPAD online interaction prediction shows higher DP value. (F, G) Immunoprecipitation studies of BLACAT1 and western blot analysis of RBMX. (H, I) RNA immunoprecipitation of RBMX, control using IgG followed by quantification of BLACAT1 in Huh7 or Hep3B cells using RT-qPCR analysis. Cells transfected with scr or shRBMX 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. (J, K) The expression of BLACAT1 was determined after overexpression of RBMX or knockdown of RBMX by qRT-PCR assays. \*\*P<0.01

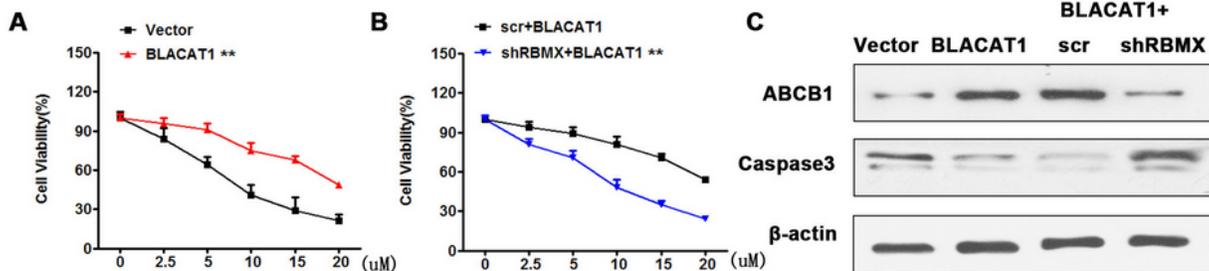


**Figure 7**

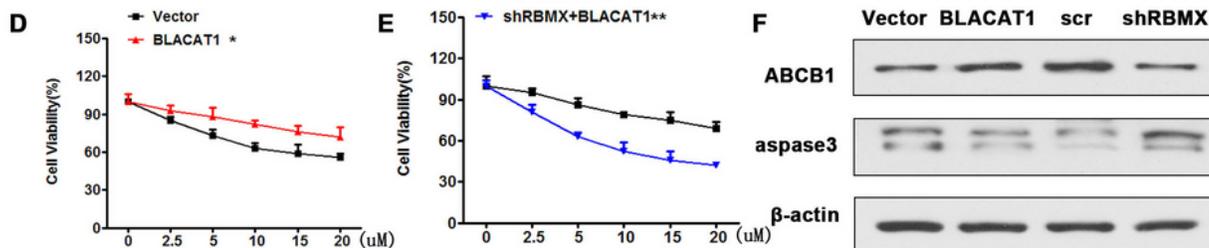
RBMX has an impact on BLACAT1 and regulates HCC cell proliferation in vitro. (A, D) Huh7 and Hep3B cells transfected with vector or BLACAT1 and the expression of BLACAT1 was detected by qRT-PCR assays. (B, E) Cell Counting kit-8 assay and (C, F) Colony-formation assay were performed in A, D treatment cells. (G, J) Huh7 and Hep3B cells co-transfected with BLACAT1 and scr or BLACAT1 and

shRBMX were used to detect BLACAT1's dependence on RBMX, then, (H, K) Cell Counting kit-8 assay and (I, L) Colony-formation assay were performed. \*\*P<0.01.

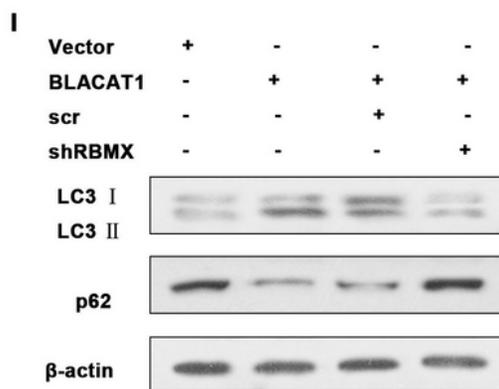
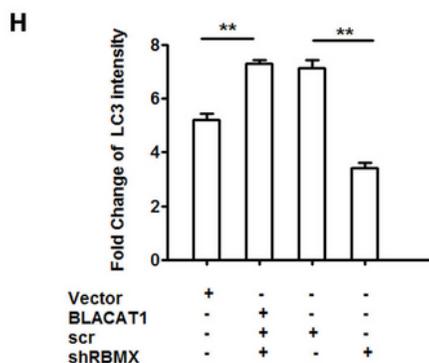
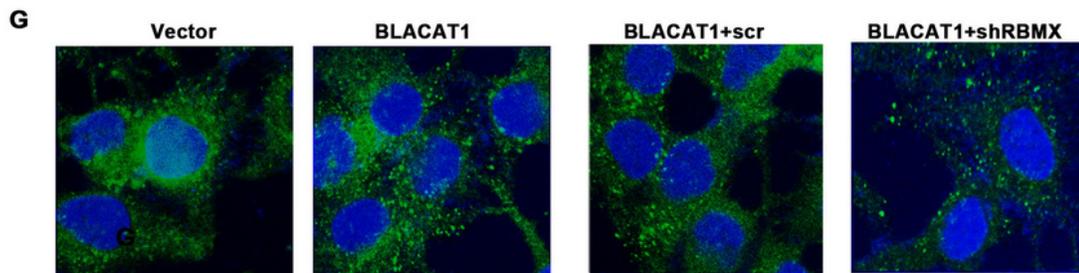
### Huh7+sorafenib



### Huh7-SR+sorafenib



### Huh7-SR+sorafenib



**Figure 8**

RBMX knockdown inhibited BLACAT1-induced autophagy and sorafenib resistance of HCC cancer cells in vitro. CCK8 assay was used to detect the impact of BLACAT1 on cell proliferation. Huh7 cells with over expression of BLACAT1 (A), Huh7 cells with over expression of BLACAT1 but silenced for RBMX (B). (C)

Western blotting with anti-ABCB1 and anti-caspase3 on protein extracted from cell line post over expression of BLACAT1, over expression of BLACAT1 but silenced for RBMX. CCK8 assay was used to detect the impact of BLACAT1 on Huh7-SR cell proliferation. Huh7-SR cells with over expression of BLACAT1 (D), Huh7-SR cells with over expression of BLACAT1 but silenced for RBMX (E), all the above mentioned groups were treated with different concentrations (0, 2.5, 5,10, 20  $\mu$ M) of sorafenib for 24h. (F)Western blotting with anti-ABCB1 and anti-caspase3 on protein extracted from Huh7-SR cell. (G) Observation of autophagy (LC3-RFP) in cell line with either BLACAT1 over expression or BLACAT1 over expression but silenced for RBMX. (H) Quantification of LC3-positive puncta per cell. (I) Western blotting analysis with anti-LC3 antibody and anti-P62 antibody in cell line with either BLACAT1 over expression or BLACAT1 over expression but silenced for RBMX. \*\*P<0.01.