Long non-coding RNA RBMY2FP promotes proliferation of male hepatocellular carcinoma by directing DNA methylation and activating RBMY1A1 via DNMT1

Feng Liu[1,2,*], Fu Yang[1,*], Xia Wu[3,*], Jin-Feng Huang[1,*], Ji-Hang Yuan[1], Qi-fei Tao[4], Gui-hua Jiang[3], Jia-sheng Zheng[5], Shu-Han Sun[1]

[1]Department of Medical Genetics, Second Military Medical University, Shanghai, 200433, China

[2]Reproductive Medical Center, Obstetrics and Gynecology of Navy PLA General Hospital, Beijing 100048, China

[3]Department of Infectious Diseases, The Second Affiliated Hospital of Harbin Medical University, Harbin 150001, China

[4]The Third Department of Hepatic Surgery, Eastern Hepatobiliary Hospital, Second Military Medical University, Shanghai, 200433, China

[5]Center of Intervention Oncology, Beijing Youan Hospital, Capital Medical University, Beijing 100069, China

[*]These authors have contributed equally to this work

Correspondence to: *Shu-Han Sun,* ***email:*** *shsun@vip.sina.com*
Jia-sheng Zheng, ***email:*** *zhengjiasheng6@163.com*

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ABSTRACT

Sexual dimorphism is a major issue in hepatocellular carcinoma (HCC), with significantly higher incidence in males. We screened male specific Y chromosome transcripts through RNA sequence and discovered a long non-coding RNA RBMY2FP (RNA binding motif protein, Y-linked, family 2, member F pseudogene, lnc-RBMY2FP) that is specifically expressed in about 1/3 male HCC tissues, with no expression in adjacent livers. Positive expression of lnc-RBMY2FP in male HCC is related to poor patient survival. Lnc-RBMY2FP enhances HCC cell growth, proliferation and tumor stemness both *in vitro* and *in vivo*. Mechanistically, lnc-RBMY2FP interacts with DNMT1 and hampers its binding onto promoters of RBMY gene family. Thus, maintenance of promoter methylation status is disturbed because of inhibition of DNMT1 activity, leading to increased expression of RBMY1A1 protein and enhanced hepatocarcinogenesis. The finding of lnc-RBMY2FP may partially explain the male preference of HCC and potentially contribute to HCC treatment.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most prevalent cancers worldwide and the second cause of cancer related death in men and sixth in women [1]. Hepatitis B or C viral infection is one of the most common causes of HCC [2]. As known to all, males are more susceptible to HCC than females, with average male to female ratios between 2:1 and 4:1 [1, 3]. Sex hormones together with corresponding receptors and cytokines are considered to be responsible for the sexual dimorphism of HCC, such as androgen and androgen receptor (AR), estrogen and estrogen receptor (ER), and IL-6 [4–6]. After binding to its ligand, AR activates its transcriptional targets such as cell cycle–related kinases and drives hepatocarcinogenesis through corresponding signal pathways [7]. AR could regulate transcription of HBV RNA thus promoting hepatitis B virus-induced hepatocarcinogenesis [8], serving as a potential therapeutic target for the treatment of HCC [9]. Estrogen and ER play inverse functions, attenuating tumor progression in HCC [5, 10]. Other factors, such as Foxa1/2 factors and their targets are reported to play important roles in the sexual dimorphism of HCC [11]. However, the reasons still remain elusive.

In the past decade, long non-coding RNAs are emerging as important regulators in kinds of malignant tumors as well as in HCC. Yuan et al. reported that

the lncRNA-activated by TGF-β(lncRNA-TAB) was upregulated in HCC metastasis and promoted HCC invasion-metastasis cascade through binding miR-200 family and IL-11 mRNA [12]. LncRNA highly up-regulated in liver cancer (HULC) was demonstrated to promote tumor angiogenesis in liver cancer through miR-107/E2F1/SPHK1 signaling [13]. LncRNAs also play roles in HBV related hepatocarcinogenesis. In Huang JL's research, HBx-related long non-coding RNA DBH-AS1 promoted HCC cell proliferation and survival through activation of MAPK signaling pathway [14]. Tumor repressing lncRNAs were reported in HCC too. Wang TH and colleagues proved that long non-coding RNA AOC4P suppressed HCC metastasis via enhancing vimentin degradation and suppressing the EMT [15].

The biggest genetic difference between male and female is their respective sex chromosome composition, that is, XY as male and XX as female. The Y chromosome exists exclusively in male and harbors male-specific genes because of its male-specific region [16, 17]. We investigated whether male-specific male genes play important roles in the HCC gender disparity. A long non-coding RNA RBMY2FP (lnc-RBMY2FP) was discovered as the only significantly upregulated Y chromosome transcript in tumors by RNA sequence of tumor and liver tissues from 3 HCC patients. Furthermore, lnc-RBMY2FP is demonstrated to express specially in about 1/3 male HCC and its positive expression predicts poorer patient survival.

RBMY2FP gene locates in the RBMY (RNA biding motif on the Y chromosome) gene family locus. The RBMY gene family containing RNA recognition motifs at the N terminus is specifically expressed in male germ cell and conserved in mammalians [18, 19]. At least six subfamilies of RBMY have been identified because of multiple copies on the Y chromosome [20], among which RBMY1 is the largest and the only subfamily that is expressed. RBMY is primarily reported to function as a splicing regulator through regulating the activity of constitutive splicing factors in male germ cell and to be responsible for spermatogenesis [21–24]. Recently, activation of RBMY1 proteins have been reported specifically in male HCC and may relate with male predominance of HCC [25, 26]. RBMY1A1 is the predominant protein specially activated in 1/3 male HCC and functions as inhibitor of GSK3β, thereby hindering the GSK3β–dependent degradation of β-catenin and inducing the nuclear entry of β-catenin for the transcription of downstream oncogenes, eventually increasing tumor cell self-renewal, chemoresistance, and proliferation and resulting in poor survival of HCC patients [27]. Lnc-RBMY2FP (RNA binding motif protein, Y-linked, family 2, member F pseudogene) is a long non-coding RNA encoded by a pseudogene of the RBMY family and is found to promote HCC cell growth and proliferation both *in vitro* and *in vivo*. We demonstrate that lnc-RBMY2FP is actively expressed due to hepomethylation of the promoter region and combines with DNMT1 and inhibits DNA methylation maintenance of both its own promoters and the RBMY1A1 promoters in feedback. Enhancing expression of RBMY1A1 by lnc-RBMY2FP further promotes the carcinogenesis of HCC.

RESULTS

Lnc-RBMY2FP is specifically expressed in 1/3 male HCC and predicts poor prognosis

First, we compared Y chromosome transcripts between male HCC tissues and corresponding adjacent livers from 3 male HCC patients by RNA sequence. Lnc-RBMY2FP was the only transcript significantly upregulated in tumors (Table S2). Next, we detected lnc-RBMY2FP in HCC tumor tissues and adjacent non-tumor tissues in HCC cohort 1 of 20 HCC patients (15 males and 5 females). We found that lnc-RBMY2FP was specifically expressed in 5 male patients' tumor tissues, while lnc-RBMY2FP was not detectable in compared adjacent non-tumor tissues, nor in the other 10 male patients or female patients (Figure 1A, Supplementary Figure S1). Protein RBMY1A1 was found to be activated in those lnc-RBMY2FP positive tumor tissues by western blot (Supplementary Figure S2A). Lnc-RBMY2FP was not found in other kinds of tumor tissues, including colon cancer, esophageal cancer, pancreatic cancer, and lung cancer, regardless of gender (data not shown).

We further investigated roles of lnc-RBMY2FP in male HCC in consideration of its specific expression in male HCC. In HCC cohort 2 containing 68 male HCC patients, lnc-RBMY2FP was verified to actively express in 23 patients (Figure 1B). Furthermore, survival analysis showed that patients with positive lnc-RBMY2FP expression in HCC had significantly worse prognosis than those without lnc-RBMY2FP expression, with lower overall survival rate (log rank: $P = 0.0138$) (Figure 1C). RBMY1A1 was found to be activated together with lnc-RBMY2FP ($p<0.001$) (Supplementary Figure S2B). These findings suggested that lnc-RBMY2FP might play important roles in male HCC and be a tumor-promoting gene in male HCC, so we conducted a series of experiments to explore roles of lnc-RBMY2FP in male HCC.

Over-expression of lnc-RBMY2FP promotes HCC cell proliferation *in vitro*

We found no expression of lnc-RBMY2FP in HCC cell lines in our lab, so we mainly conducted over-expression experiments. First, we cloned lnc-RBMY2FP from positive HCC tissues following RACE assay (Supplementary Figure S3A). Ectopic Over-expression of lnc-RBMY2FP was confirmed by PCR in SMMC-7721 and HepG2 cell lines (Supplementary Figure S3B).

In CCK8 assays, lnc-RBMY2FP apparently promoted HCC cell growth both in SMMC7721 and hepG2 cell lines, indicating that cell proliferation was enhanced by the overexpression of lnc-RBMY2FP (Figure 2A). In clone formation assay, number of clones formed by lnc-RBMY2FP ectopically over-expressed cells was much bigger than corresponding control cells (Figure 2B). Then we performed EdU (5-ethynyl-2'-deoxyuridine) immunofluorescence staining in the lnc-RBMY2FP-overexpressed SMMC 7721 cells. As shown, number of EdU-positive nuclei was increased by lnc-RBMY2FP-overexpression in SMMC 7721 cells (Figure 2C). Cell cycle distribution showed an increase of S-phase population and a reduction of G0/G1 population after over-expression of lnc-RBMY2FP in cell cycle analysis assay by flow cytometry (Figure 2D). In nonadherent spheroid culture assay, overexpression of lnc-RBMY2FP resulted in more spheres formed at day 5, demonstrating that lnc-RBMY2FP enhanced stemness-like activity of HCC cells(Figure 2E). Altogether, these results indicate that lnc-RBMY2FP promotes HCC cell growth and proliferation.

Lnc-RBMY2FP also promotes HCC growth *in vivo*

To further verify the effects of lnc-RBMY2FP, we conducted a series of *in vivo* experiments. First, lnc-RBMY2FP overexpressed and controlled HepG2 cells were injected subcutaneously into the armpit of nude mice. Growth speed of tumors was accelerated by lnc-RBMY2FP compared with that of tumors formed from control xenografts (Figure 3A). Moreover, tumor weight and volume were bigger in lnc-RBMY2FP overexpression group than the control at the end point (Figure 3B-3C).

To monitor the growth of lnc-RBMY2FP in liver, we labeled the lnc-RBMY2FP overexpressed SMMC 7721 cells and correlated control cells with firefly luciferase and inoculated cells intrasplenically into nude mice. HCC cells colonized and proliferated in liver after intrasplenic injection. Overexpression of lnc-RBMY2FP led to more colonized foci and faster growth speed. As a result, there were more and bigger tumors formed in the lnc-RBMY2FP overexpressed group (Figure 3D-

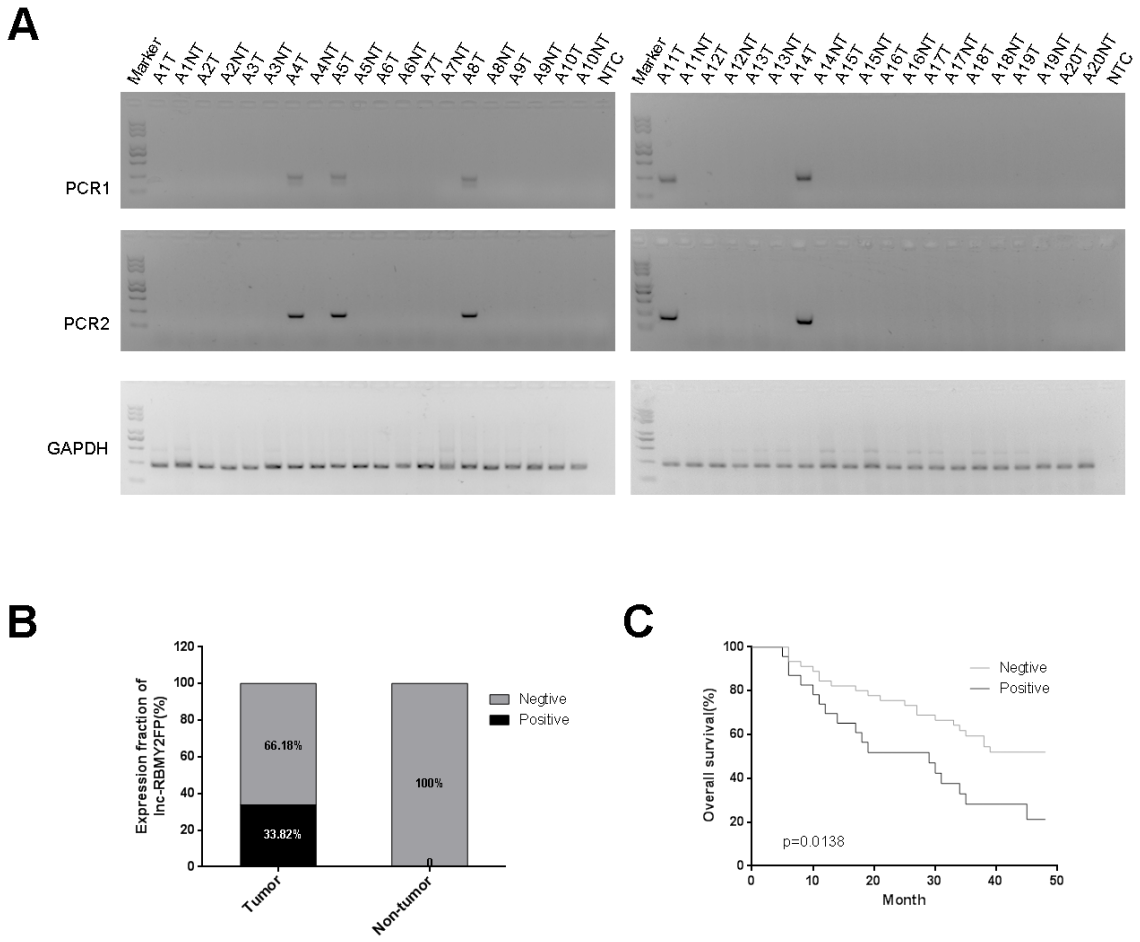

Figure 1: Expression of lnc-RBMY2FP in clinical HCC tissues. A. Expression of lnc-RBMY2FP in cohort 1 detected by PCR. (Male: A1-A15; female: A16-20) **B.** Analysis of lnc-RBMY2FP expression in HCC cohort 2 using Taqman real-time PCR. **C.** Kaplan–Meier curves depicting overall survival of patients in cohort 2 according to lnc-RBMY2FP expression.

3E). Tumors were confirmed by histological section and HE staining(Figure 3F). At the same time, proliferation markers were detected in the tumors formed in nude mice liver by IHC, including Ki-67 and PCNA. Positive rates of Ki-67 and PCNA were higher in tumors formed by lnc-RBMY2FP overexpressed cells than those by controlled cells(Figure 3G).

Effects of lnc-RBMY2FP on colonization and proliferation *in vivo* were further investigated in tail vein injection modeling system. HCC cells were directly

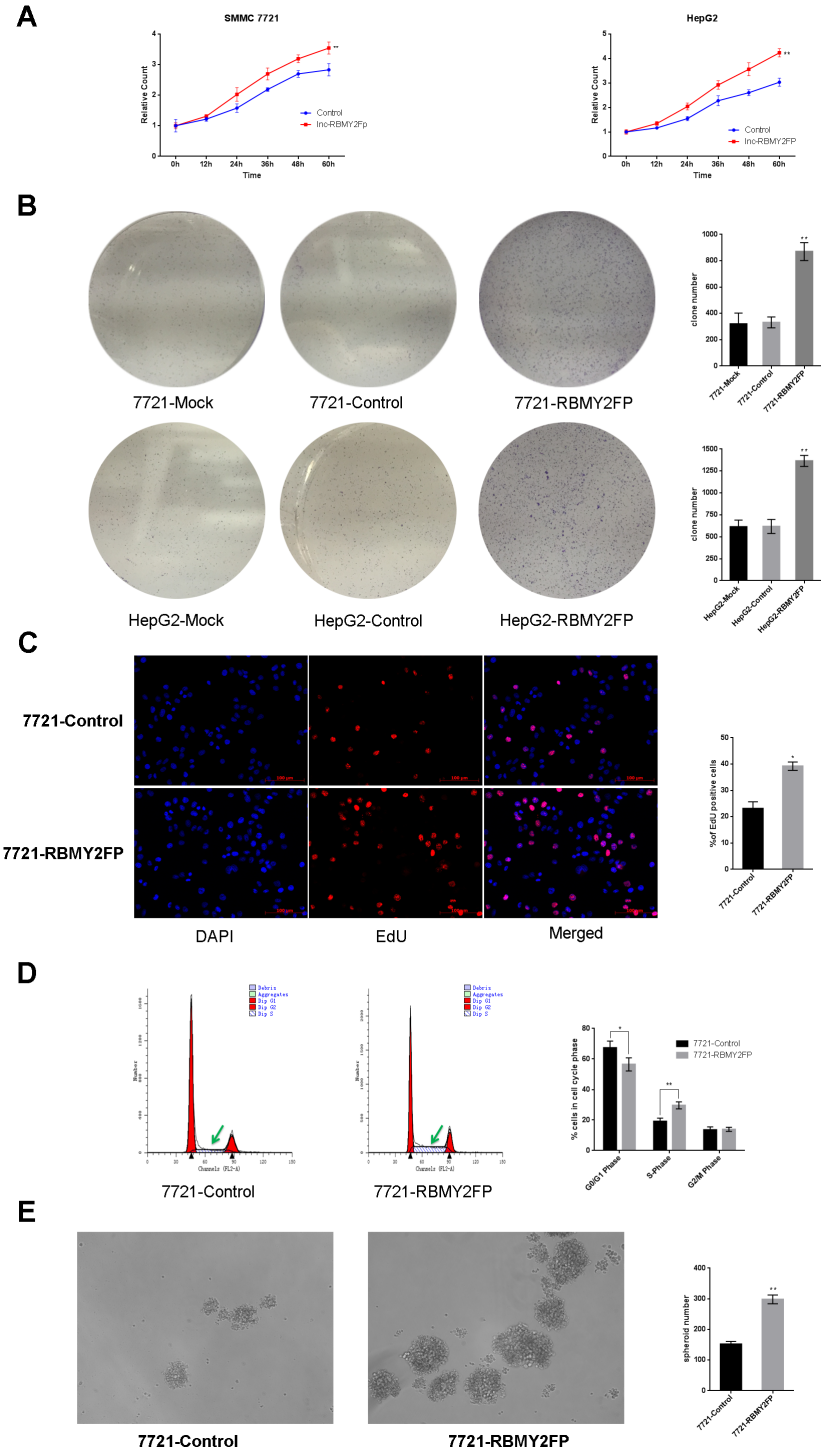

Figure 2: lnc-RBMY2FP enhances HCC cell proliferation *in vitro*. A. Proliferation of HCC cells assessed by CCK8 assay. **B.** Clone formation assay of HCC cells. **C.** EdU immunofluorescence staining of differently treated HCC cells. **D.** Cell cycle analysis of SMMC 7721 cells by flow cytometry. **E.** Nonadherent spheroid culture assay of SMMC 7721 cells.

injected in tail veins of nude mice and colonized in lungs. We also observed a significant increase in the lung colonization generated by lnc-RBMY2FP overexpressed SMMC-7721 cell, displaying more tumor foci and bigger tumors than controlled SMMC 7721 cells (Figure 4A-4B). Higher positive rates of Ki-67 and PCNA were found in lnc-RBMY2FP overexpression group too (Figure 4C). Taken together, these data demonstrated that lnc-RBMY2FP promotes HCC cell growth and proliferation *in vivo*.

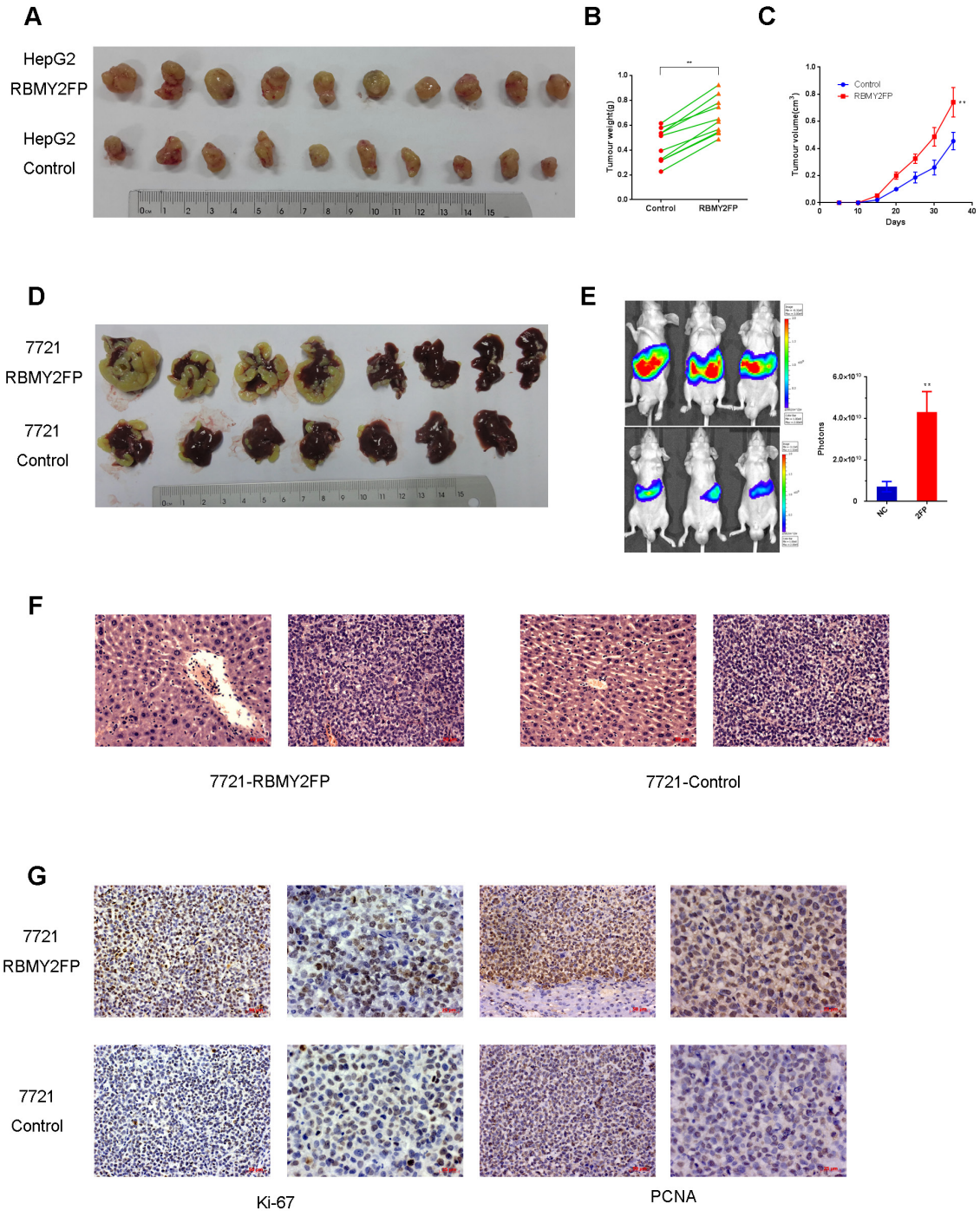

Figure 3: lnc-RBMY2FP promotes HCC cell growth *in vivo*. A. Photos of tumors formed subcutaneously. **B.** Weight of tumors dissected from armpit of mice. **C.** Tumor growth curves over time after subcutaneous injection. **D.** Photo of livers dissected from mice 8 weeks after intrasplenic injection. **E.** Representative photograph of mice by imaging with the IVIS Imaging System. **F.** Histological confirmation of tumors formed in liver by HE staining. **G.** IHC of tumors to detect proliferation markers Ki-67 and PCNA.

Activation of lnc-RBMY2FP is related to aberrant DNA methylation

There exists a CpG island in the promoter region of RBMY2FP gene locus. Then we detected the DNA methylation state of the CpG island. A hypermethylation state was found in non-tumor liver tissues (Figure 5A). However, there was a dramatic decrease of the methylation rates in the promoter in lnc-RBMY2FP positive tumor tissues. On the contrary, promoter hypomethylation was not seen in lnc-RBMY2FP negative tumors (Figure 5A). Hypomethylation state was also found in RBMY1A1 promoter region in positive tumor tissues (Figure 5B). We could speculate that aberrant methylation state of promoter regions might result in activation of RBMY2FP in a portion of male patients.

Lnc-RBMY2FP combines with DNMT1 as a DNMT1-interacting RNA

Di Ruscio A et al. reported that there existed some active transcriptions regulating chromatin methylation states [32]. In their study, DNMT1-interacting RNA ecCEBPA arising from CEBPA locus was demonstrated to combine with DNMT1 and prevent CEBPA gene locus methylation. Then we investigated whether lnc-RBMY2FP could combine with DNMT1 like ecCEBPA. First we performed RNA pulldown using biotin labelled lnc-RBMY2FP synthesized *in vitro*. DNMT1 was enriched by sense RNA, however not by antisense RNA control or beads only without RNA control (Figure 5C). On the other hand, RNA immunoprecipitation (RIP) validated a specific association between DNMT1 and lnc-RBMY2FP as expected. Lnc-RBMY2FP was significantly enriched by

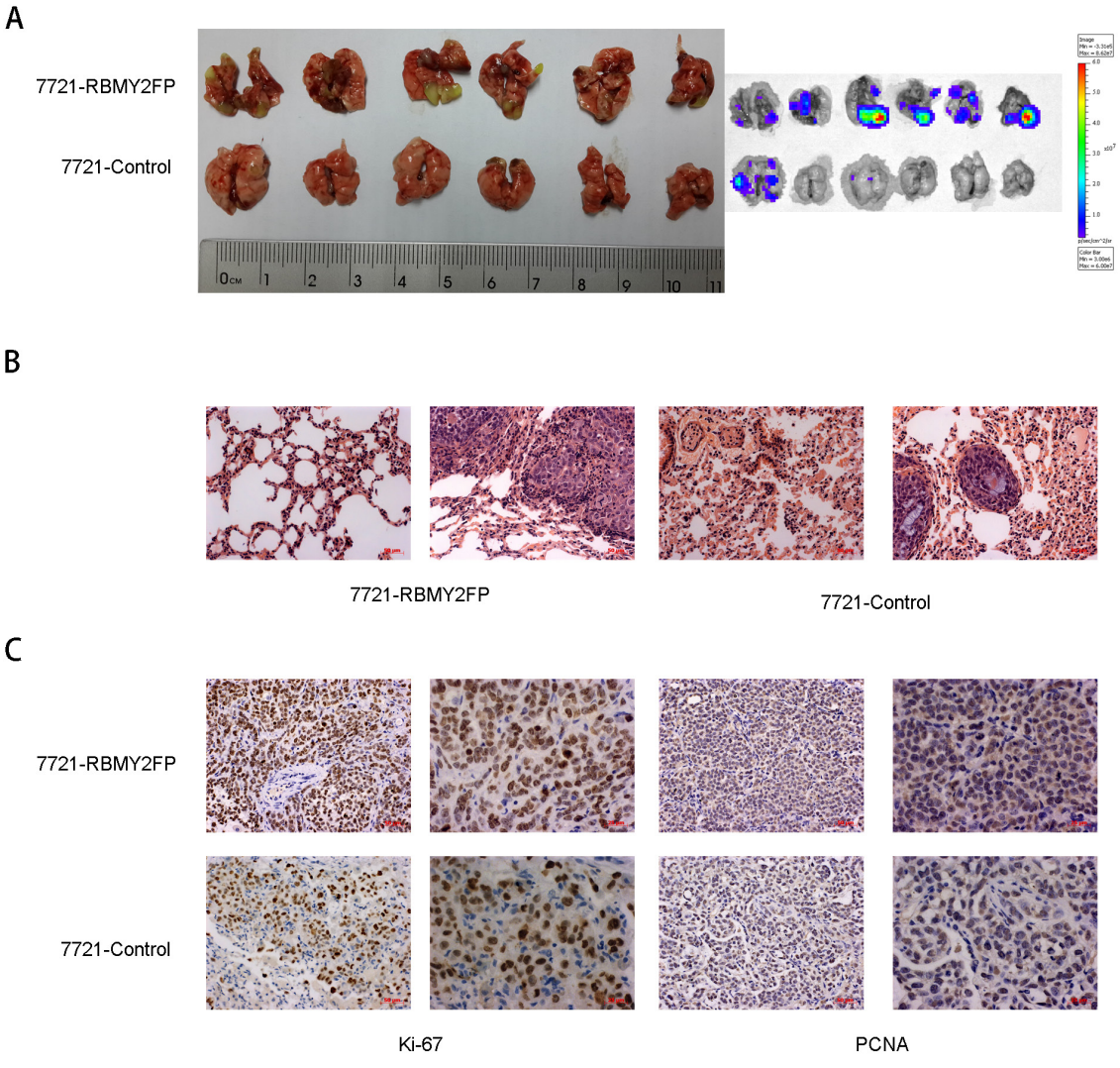

Figure 4: lnc-RBMY2FP enhances lung colonization in tail vein injection model. A. Pictures of lungs resected at the end point and luciferase activity assed by the IVIS Imaging System. **B.** Histological confirmation of tumors colonized in lung by HE staining. **C.** IHC of tumors to detect proliferation markers Ki-67 and PCNA.

the DNMT1 antibody compared with the nonspecific IgG control antibody (Figure 5D). These results indicate the association between lnc-RBMY2FP and DNMT1.

Lnc-RBMY2FP blocks methylation maintenance of RBMY gene locus and activates RBMY1A1

Then we investigated roles of lnc-RBMY2FP on DNMT1 function. In non-tumor liver and lnc-RBMY2FP negative HCC tissues, DNMT1 bound promoter region of RBMY2FP gene locus (Figure 6A) and maintained methylation state. However, in lnc-RBMY2FP positively expressed HCC tissues, DNMT1 was repressed by lnc-RBMY2FP and enrichment of DNMT1 on promoter region was reduced compared with that in non-tumor livers tissues (Figure 6A). DNMT1 was found to bind promoter of RBMY1A1 gene locus in non-tumor liver and HCC tissues as well (Figure 6B). Just as RBMY2FP gene, promoter of RBMY1A1 gene bound by DNMT1 was reduced in lnc-RBMY2FP positively expressed HCC tumors (Figure 6B). We concluded that the aberrant methylation state of RBMY2FP gene promoter activated the expression of lnc-RBMY2FP and lnc-RBMY2FP disturbed maintenance of DNA methylation of RBMY region through blocking DNMT1 in turn. In HepG2 cells, RBMY1A1 was elevated by over-expression of lnc-RBMY2FP, suggesting activation of RBMY1A1 by aberrant methylation (Figure 6C). RBMY1A1 was reported to increase HCC resistance to sorafenib [27]. We also found an increase in resistance to sorafenib in HepG2 cells overexpressing lnc-RBMY2FP (Figure 6D).

To verify the inhibiting roles of lnc-RBMY2FP on DNMT1, we conducted DNMT1 stable overexpressing experiments on the basis of lnc-RBMY2FP overexpression. Ectopic expression of DNMT1 in lnc-RBMY2FP overexpressed HepG2 cells decreased

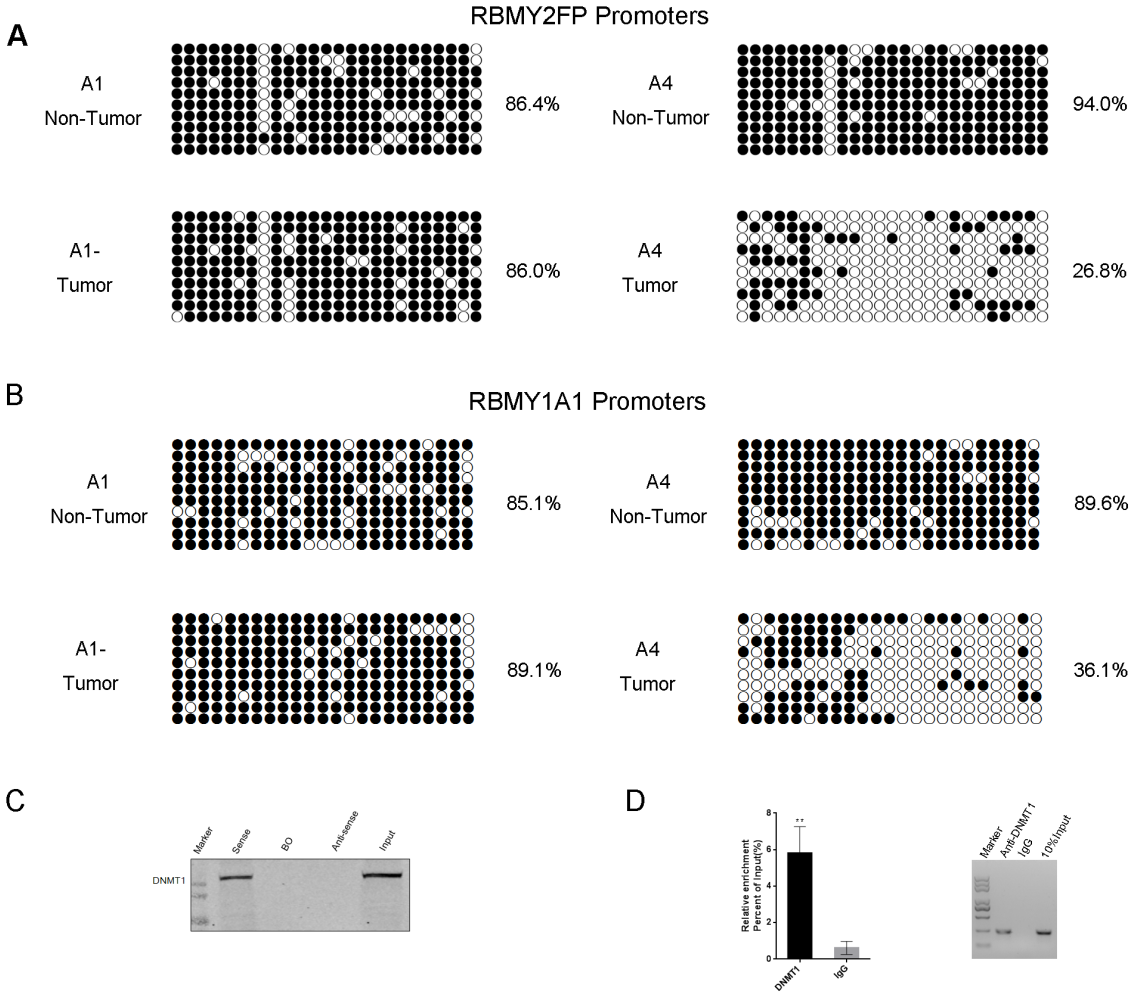

Figure 5: Methylation of RBMY2FP promoters. A. Bisulfite DNA sequencing (bisulfite sequencing PCR) of RBMY2FP promoters in lnc-RBMY2FP negatively expressed Patient A1 and lnc-RBMY2FP positively expressed Patient A4. ($P < 0.01$, Wilcoxon rank-sum test). **B.** Bisulfite DNA sequencing of RBMY1A1 promoters. ($P < 0.01$, Wilcoxon rank-sum test). **C.** Western bot of DNMT1 in RNA pulldown by lnc-RBMY2FP in HepG2 cells. BO: beads only. **D.** Enrichment of lnc-RBMY2FP in DNMT1 RIP and agarose gel electrophoresis of lnc-RBMY2FP after DNMT1 RIP in HepG2-RBMY2FP cells. Lnc-RBMY2FP primer pair1 was used.

RBMY1A1 levels (Figure 7A). Enhanced cell growth and proliferation by lnc-RBMY2FP was repressed by DNMT1(Figure 7B-7D). Increased resistance to sorafenib was also reversed by DNMT1 as a result of RBMY1A1 inhibition. These suggested that DNMT1 mediates the activating roles of lnc-RBMY2FP on RBMY1A1.

DISCUSSION

In this study, we found a male specific tumor-promoting long noncoding RNA RBMY2FP in HCC, which may explain male preference of HCC. Lnc-RBMY2FP is exclusively actively expressed in 1/3 male HCC tissues

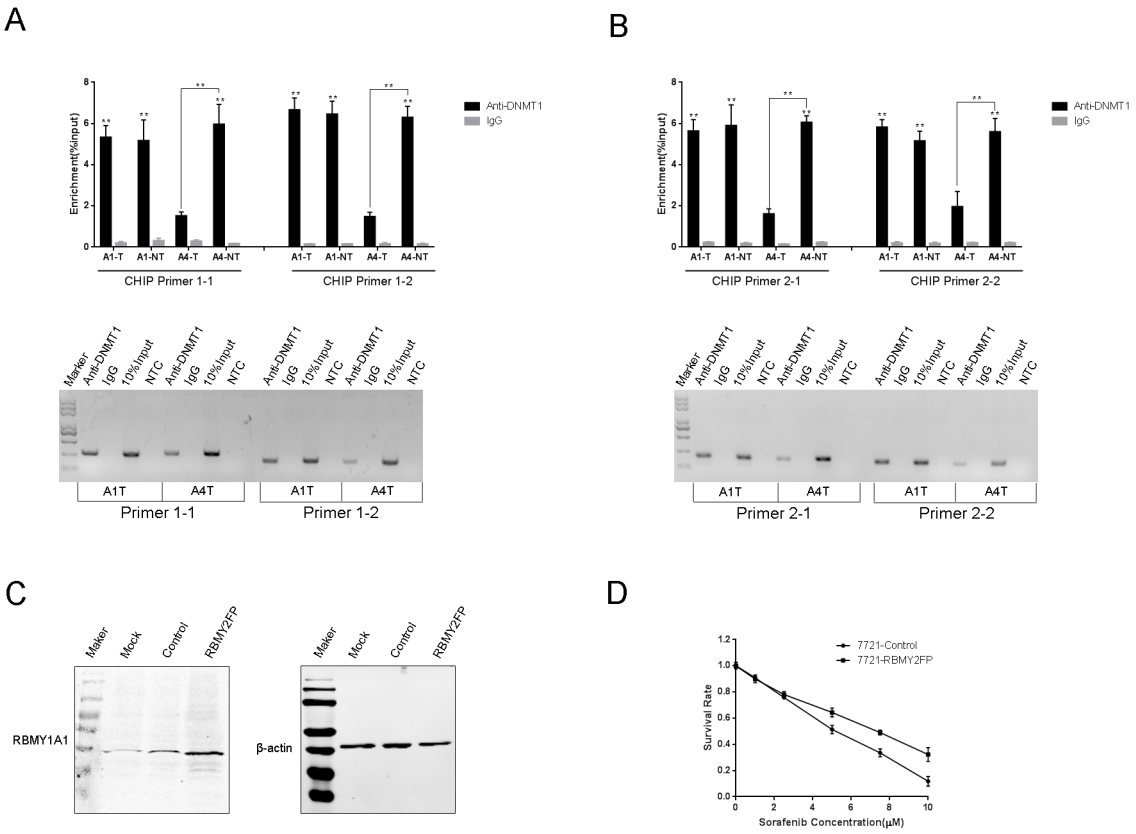

Figure 6: lnc-RBMY2FP inhibits function of DNMT1 and activates RBMY1A1. A. Binding of DNMT1 on RBMY2FP promoters assessed by CHIP assay in patient tissues. **B.** Binding of DNMT1 on RBMY1A1 promoters assessed by CHIP assay. **C.** Western bot of RBMY1A1 in hepG2 cells (normalized to β-actin). **D.** Survival of cells after treatment of sorafenib for 2 days assessed by CCK8.

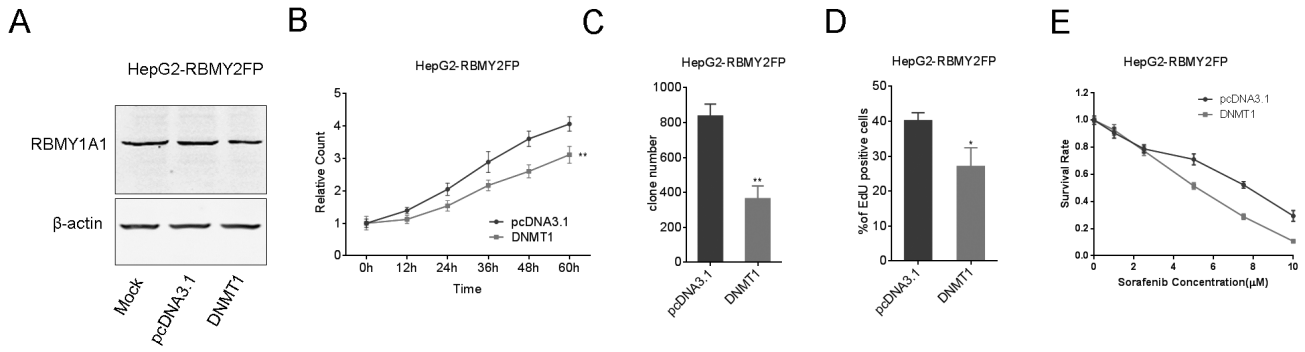

Figure 7: DNMT1 reversed roles of lnc-RBMY2FP. A. Western bot of RBMY1A1 in hepG2-RBMY2FP cells (normalized to β-actin). **B.** Proliferation of HCC cells assessed by CCK8 assay. **C.** Clone formation assay of HCC cells. **D.** EdU immunofluorescence staining of differently treated HCC cells. **E.** Survival of cells after treatment of sorafenib for 2 days assessed by CCK8.

and related to worse prognosis of HCC patients. Ectopic overexpression of lnc-RBMY2FP enhances HCC cell growth and proliferation both *in vitro* and *in vivo*.

DNA methylation at the 5 positions of cytosine is one of the important epigenetic modifications and is associated with multiple biological processes and diseases such as cancers [33, 34]. Aberrant DNA methylation state changes are frequently found in HCC [35]. *De novo* DNA methylation depends on DNMT3A and DNMT3B, and maintenance of methylation state relies on DNMT1 [36, 37]. Recently, evidence is emerging that lncRNAs are involved in regulation of DNA methylation. Di Ruscio A demostrated that lncRNA could change genomic methylation patterns by interacting with DNMT1 [32]. LncRNA TARID (TCF21 antisense RNA inducing demethylation) was reported to activate TCF21 expression by interacting with both the TCF21 promoter and GADD45A (growth arrest and DNA-damage-inducible, alpha a regulator of DNA demethylation) and inducing demethylation of TCF21 promoter [38]. LncRNA Dum (developmental pluripotency-associated 2 (Dppa2) Upstream binding Muscle lncRNA) in skeletal myoblast cells silenced its neighboring gene, Dppa2, in cis through recruiting Dnmt1, Dnmt3a and Dnmt3b, thus promoting myoblast differentiation and damage-induced muscle regeneration [39]. In our research, lnc-RBMY2FP was verified to combine with DNMT1 and inhibit activity of DNMT1 in maintaining DNA methylation of RBMY promoters, herein resulting in promoter hypomethylation of RBMY family. Hypomethylation of promoters activated expression of lnc-RBMY2FP and RBMY1A1, forming a positive feed-back loop. Activation of RBMY1A1 together with lnc-RBMY2FP enhances HCC tumorigenesis and chemoresistance.

Taken together, we discover an important factor explaining male preference of HCC and adding to the evidence for lncRNA and DNA methylation crosstalk. Enhancing chemoresistance to sorafenib by lnc-RBMY2FP prompts other therapies for lnc-RBMY2FP positively expressed patients in clinical practice.

MATERIALS AND METHODS

Patients and specimens

Frozen HCC tumor and paired non-tumor liver tissues were randomly obtained from the Eastern Hepatobiliary Hospital (Shanghai, China). Fresh tissue samples were collected and processed within 10 minutes. Each sample was snap-frozen in liquid nitrogen and then stored at -80°C. The paired adjacent normal tissues were isolated from at least 3 cm away from tumor boarder and with no microscopic tumor cells. The data do not contain any information that could identify the patients. All patients provided written informed consent and the ethical consent was granted from the Committees for Ethical Review of Research involving Human Subjects of Second Military Medical University (Shanghai, China).

Cell lines

The cell lines SMMC-7721 and HepG2 were obtained from the Chinese Academy of Sciences Cell Bank and cultured at 37°C in an atmosphere containing 5% CO2 and in Dulbecc's modified Eagl's medium supplemented with 10% fetal bovine serum.

Real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen CA). First-strand cDNA was generated using the M-MLV Reverse Transcriptase (Invitrogen) and random primers. Real-time PCR was performed in the StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, USA) using Taq PCR Mastermix (TianGen Biotech, Beijing, China), gene specific primers and probes or SYBR® Green (Takara, Dalian, China) and gene specific primers (Table S1). GAPDH mRNA was employed as an endogenous control. The relative expression of RNAs was calculated using the comparative CT method.

5' and 3' rapid amplification of cDNA ends (RACE) analysis

5'-RACE and 3'-RACE analyses were performed with 5 μg of total RNA using the SMARTer™ RACE cDNA Amplification Kit (Clonetech) according to the manufacturer's instructions. The gene-specific primers used for PCR were 5'GGGA CCATGTTTCGCAAATACTGC3'(5'RACE) and 5'TTGTCTCCATTGCTTTTGAGGGTG 3'(3'RACE).

CCK8 assays

HCC cells were seeded at a density of 1×10^3 cells per well in 96-well plates, and the viability of the cells was assessed from 3 replicates in 3 independent experiments by the Cell Counting kit-8 (Dojindo Laboratories, Japan) every 12 hours.

Clone formation assays

Cells were seeded at a density of 1000 cells per well in a 6-well culture plate and cultured for 2 weeks; the colonies were stained with 1% crystal violet and counted.

EdU immunofluorescence staining

EdU immunofluorescence staining was performed with an EdU kit (Roche). Cells were grown on cover slips until they reached approximately 50% confluence. Cell proliferation was assessed using EdU immunofluorescence staining according to the manufacturer's protocol.

Cell cycle analysis

Cell cycle analysis was performed by flow cytometry. Briefly, cells were harvested and fixed in ethanol (70%). After fixing in -20°C for 24h, samples were stained by propidium iodide (0.05mg/ml) at 37°C for 30min. Finally, samples were analyzed by flow cytometry in a FACScalibur cytometer.

Spheroid formation

The HCC cells were plated in 6-well ultra-low attachment culture dishes at 1×10^6 cells per well and cultured in DMEM without FBS for ten days [28]. The number of spheroids was counted and representative views were shown.

Western blot analysis

Cells were treated as indicated in figure legends, and the harvested cells were lysed for protein collection. Identical quantities of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose filter membranes. Antibody dilutions of 1:1000 were used for DNMT1 (Cell Signaling Technology, Boston, USA), RBMY1A1 (abcam) and 1:5000 for β-actin (Sigma-Aldrich). The secondary antibodies applied IRdye800-conjugated goat anti-rabbit IgG (Li-Cor Biosciences Inc., Lincoln, NE) and IRdye700-conjugated goat anti-mouse IgG (Li-Cor Biosciences Inc.) and were detected using an Odyssey infrared scanner (Li-Cor Biosciences Inc).

Vectors construction

The cDNA encoding lnc-RBMY2FP was PCR-amplified by the Prime STAR DNA Polymerase (Takara, Dalian, China) from positive HCC tissues and subcloned into the BamH I and Sal I sites of pSPT19 vector, named pSPT19-FTX. DNMT1 overexpressing vector was constructed using pcDNA3.1 vector in the former study [29].

Construction of lentivirus and stable cell lines

Construction of lentivirus containing the lnc-RBMY2FP gene was conducted as previously reported [30]. Empty vectors containing the green fluorescent protein wre used as a negative control. SMMC 7721 and HepG2 cells were infected with concentrated virus at a multiplicity of infection of 100 in the presence of 8 μg/ml polybrene (Sigma-Aldrich). The supernatant was replaced with complete culture media after 24 hr. The expression of lnc-RBMY2FP in the infected cells was confirmed by PCR 96 hr after infection. Transfected cells were selected with puromycin (1 μg/ml) for four weeks to obtain stable overexpressed cell lines.

To overexpress DNMT1 in lnc-RBMY2FP overexpressing HCC cells, pcDNA3.1-DNMT1 vectors were transfected as described before [31]. Transfected cells were selected with G418 (800 μg/ml) for four weeks to obtain stable overexpressed cell lines. Empty pcDNA3.1 vector was used as control.

RNA pull-down

Lnc-RBMY2FP was *in vitro* transcribed from vector pSPT19-RBMY2FP and biotin-labeled with the Biotin RNA Labeling Mix (Roche) and Sp6 RNA polymerase (Roche), treated with RNase-free DNase I (Roche), and purified with an RNeasy Mini Kit (Qiagen, Valencia, CA). One milligram of whole-cell lysates from hepG2 cells were incubated with 3ug of purified biotinylated transcripts for 1 hr at 25°C; complexes were isolated with streptavidin agarose beads (Invitrogen). The associated proteins were resolved by gel electrophoresis and detected by western blot.

RNA immunoprecipitation (RIP)

RNA immunoprecipitation (RIP) experiments were performed using the Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA) according to the manufacturer's instructions. The co-precipitated RNAs were detected by reverse transcription PCR. Total RNA (input controls) and corresponding species IgG controls were performed simultaneously to demonstrate that the detected signals were from the RNA that was specifically bound (n=3 for each experiment).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using an EZ ChIP Chromatin Immunoprecipitation Kit (Millipore, Bedford, MA) according to the manufacturer's instructions. Briefly, cross-linked chromatin was sonicated into 200-bp to 1000-bp fragments. The chromatin was immunoprecipitated using anti-DNMT1 (Abcam, Chicago, USA) antibody. Normal mouse immunoglobulin G (IgG) was used as a negative control. Quantitative polymerase chain reaction (PCR) was conducted using SYBR Green Mix (Takara Bio, Otsu, Japan). The primer sequences are listed in Table S1.

Bisulfite genomic sequencing

DNA obtained from tissues were bisulfite-treated. Modified genomic DNA was then amplified with primers specific to the RBMY2FP gene promoters by PCR. The primers used for detecting the RBMY2FP promoter were 5'- ATTTAGGATGTTAGTTAGGGTGTAGGTT-3' (forward) and 5'-CGCTTTTTAATAAAACAAA AAACCTAAAATT-3' (reverse). The primers used for detecting the RBMY1A1 promoter were 5'-

TATGTTTATAATTGTAAAGGGTTTATAG-3' (forward) and 5'- AAAAACAACTAAAACTTCCCTAAAAA-3' (reverse). Bisulfite genomic sequencing PCR products were gel-extracted, subcloned into pMD-18T Vectors (Takara, Dalian, China), and transformed into Escherichia coli. Candidate plasmid clones were sequenced by Majorbio Company (Shanghai, China).

Animal studies

The animal studies were approved by the Institutional Animal Care and Use Committee of the Second Military Medical University, Shanghai, China. Male athymic BALB/c nude mice (4-5 weeks old) were used for animal studies. Subcutaneous tumor growth assays were performed as previously described [12]. 1×10^6 cells were injected subcutaneously into the armpit and mice were sacrificed 8 weeks after injection. For intrasplenic injection model, a left-abdominal incision was made after anesthesia, and HCC cells were injected into the spleen. Spleen was resected after compression for 3 minutes allowing cells completely flow into liver. Finally the abdominal wall was closed carefully. For lung colonization model, cells with corresponding treatment were injected into the tail veins of nude mice. Lungs were analyzed at 8 weeks to quantify lung colonization *in vivo* by histology examination. The mice were kept in pathogen-free conditions. The growth of subcutaneous tumors was monitored using the IVIS Lumina II system (Caliper Life Sciences, Hopkinton, MA) 10 min after intraperitoneal injection of 4.0 mg of luciferin (Gold Biotech) in 50μl of saline.

Statistical analysis

Data are expressed as means ± SD of three independent experiments. All statistical analyses were performed using SPSS version 17.0 software (Abbott Laboratories, Chicago, IL). For comparisons, Student's t test (two-tailed), Fisher's exact test, Wilcoxon signed-rank test, Log-rank test, and nonparametric Mann-Whitney U test were performed as appropriate. A p value < 0.05 was considered significant. One star (*) in graphs represented $p < 0.05$, and two stars (**) represented $p < 0.01$.

Abbreviations

AR, androgen receptor; ER, estrogen receptor; EdU, 5-ethynyl-2'-deoxyuridine; HCC, hepatocellular carcinoma; lnc-RBMY2FP, long noncoding RNA RBMY2FP; lncRNA, long non-coding RNA, RBMY, RNA-binding motif on the Y chromosome.

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CONFLICTS OF INTEREST

The authors declare that none of them have any conflicts of interest.

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