Modification of STIM2 by m^{6}A RNA methylation inhibits metastasis of cholangiocarcinoma

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Background: N6-methyladenosine (m^{6}A) is the most frequent internal methylation of eukaryotic RNA (ribonucleic acid) transcripts and plays an important function in RNA processing. The current research aimed to investigate the role of m^{6}A-STIM2 axis in cholangiocarcinoma (CCA) progression.

Methods: The expression of STIM2 (Stromal Interaction Molecule 2) in CCA was measured using quantitative polymerase chain reaction (PCR) and immunohistochemistry (IHC). STIM2 was examined *in vivo* for its effects on the malignant phenotypes of CCA cells. The m^{6}A modification of STIM2 was assessed through MeRIP (methylated RNA Immunoprecipitation)-PCR.

Results: Based on the GEPIA (Gene Expression Profiling Interactive Analysis) 2 database findings, a low STIM2 mRNA (messenger RNA) level was related to a poor prognosis in individuals with CCA. Quantitative PCR and IHC assays indicated decreased protein satin in CCA tissues and were associated with extrahepatic metastasis. Vianude mice tail vein injection model indicated that increased STIM2 levels suppressed CCA cell metastasis *in vivo*, while KRT8 (keratin 8) was detected as the direct downstream target of STIM2-mediated CCA cell metastasis *in vivo*. Meanwhile, based on SRAMP database and MeRIP assays indicated that m^{6}A alteration resulted in abnormal STIM2 expression in CCA via METTL14 and YTHDC2.

Conclusions: Our findings revealed the epi-transcriptomic dysregulation in CCA and metastasis by proposing a complicated STIM2-KRT8 regulatory paradigm based on m^{6}A alteration.

Keywords: Intrahepatic cholangiocarcinoma; metastasis; STIM2; KRT8; N6-methyladenosine (m^{6}A)

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Introduction

Cholangiocarcinoma (CCA) is the second most aggressive form of primary liver cancer, with an increasing incidence worldwide (1,2). CCA has gradual signs, a high degree of malignancy, and a poor prognosis, as several parts of its clinical features are identical to those of hepatocellular carcinoma (HCCA) (3). Radiation therapy, chemotherapy, intervention, surgery, immune targeting, CCA ablation treatment, and other multidisciplinary approaches are all options for treating CCA (4-7). CCA is generally unresponsive to radiotherapy and chemotherapy, and consequently has inferior clinical outcomes.

CCA is a highly fatal malignant tumor originating from the bile duct epithelium, which is characterized by late clinical manifestations and lack of effective treatment. Chronic inflammation, including primary sclerosing cholangitis, Fasciola hepatica infection and hepatolithiasis, is listed as a risk factor, but the cause is unclear for most cases of CCA. The latest progress in molecular pathogenesis emphasizes the apparent legacy. The importance of genetic changes, including promoter hypermethylation and histone deacetylation, and genetic changes in cholangiocarcinoma (8). N6-methyladenosine (m^{6}A) is a common RNA (ribonucleic acid) alteration that predictably affects carcinogenesis and tumor growth (9). The alteration of m^{6}A RNA is a reversible process that is coordinated by methyltransferase (m^{6}A "writers" and "readers") and demethylase (m^{6}A "erasers") proteins (10-12). Recent research indicates that m^{6}A modification may play a critical role in various malignant cancers, including CCA (13-16). However, the particular chemical mechanism by which m^{6}A induces CCA development remains largely unknown.

Orai1 are essential primary proteins that are clustered and activated by STIM1 (Stromal Interaction Molecule 1) and STIM2 (Stromal Interaction Molecule 2). STIM1 is the direct activator of Orai1, and loss of the protein eliminates Orai1 activation, thereby regulating downstream Ca^{2+} (calcium)-dependent cell functions (17). STIM1 clustering of Orai1 in endoplasmic reticulum (ER)-plasma membrane (PM) junctions reinforces the coupling of Orai1-mediated Ca^{2+} entry with nuclear factor of activated T cells 1 (NFAT1) initiation (18). In contrast, the activation of diffusely localized channels in the membrane by the cytosolic STIM1 C terminus or SOAR (the STIM1 Orai activating region) fragments has been demonstrated in some research to cause nuclear translocation of NFAT1 and gene expression (17-20). STIM2 is also considered to be a relevant participant in pathological conditions associated with aging, Alzheimer's and Huntington's diseases, autoimmune diseases and cancer (21). STIM2 is considered to play a role in tumor development control; however, research on this is limited. However, the role of STIM2 in CCA regulation remains unclear. The purpose of this study was to investigate the clinical significance and biological roles of STIM2 in CCA.

We present the following article in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-21-6485/rc).

Methods

Samples and cell lines from human origin

The Eastern Hepatobiliary Surgery Hospital (EHBH) used CCA tissues and neighboring normal tissues from 10 CCA patients undergoing curative surgical resections. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by Ethics Review Committee of Eastern Hepatobiliary Surgery Hospital and all patients provided written informed consent. Human CCA cell lines HuCCAT1 (ATCC, Manassas, USA)were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium with 100 g/mL streptomycin, 100 U/mL penicillin, and 10% fetal calf serum (GE Healthcare, Life Sciences, USA). The third-party biology services used short tandem repeat (STR) analysis to characterize all cell lines (Feiouer Biology Co., Ltd., Chengdu, China).

RT and qPCR (reverse transcription-reaction and quantitative polymerase chain reaction)

The PrimeScript RT reagent kit (Takara Bio Inc., China) produced the first-strand cDNA (complementary DNA). To synthesize microRNA (miRNA), the first-strand cDNA Poly-A Tailing Kit (Sangon Bio Inc., Shanghai, China) was used to reverse-transcribe miRNAs. In a quantitative RT-PCR, SYBR Premix Ex TaqII (Takara Bio Inc., Dalian, China) was utilized (qRT-PCR). The endogenous controls for Messenger RNA (mRNA) and miRNAs were β-actin and U6, respectively. The relative fold-change of target expression was calculated using the comparative $2^{-\Delta\Delta Ct}$ technique. Sangon designed and manufactured all of the primers (Shanghai, China).

Immunohistochemical (IHC) staining

IHC was performed according to the previously described method (22). The evaluation fields were set at 200× magnification power for H-score calculations (23). In malignant cells, staining concentration was sorted as 0, 1, 2, or 3, referring to the existence of negative, weak, intermediary, or solid brown staining, respectively. All of the cells were counted and stained at different intensities to obtain an overall cell count. To calculate the average percentage of positive cells, we used the formula shown below.

H-score = (% of cells stained at intensity level 1×1) + (% of cells stained at intensity level 2×2) + (% of cells stained at intensity level 3×3). H-scores of 0–3 were obtained, with 0 indicating that all cells stained negatively and 3 indicating that all cells stained positively.

Establishment of stable cell lines overexpressing STIM2 and Keratin 8 (KRT8)

Hanbio provided the lentivirus vectors expressing STIM2 or KRT8 (LV-STIM2 or LV-KRT8) and its control vector (LV-NC) (Shanghai, China). CCA cells were infected with the lentivirus vector and then treated with puromycin (at a 2 μg/mL concentration) for 1 week.

In vivo experimentation through metastasis assays

A protocol was prepared before the study without registration. Animal experiments were approved by the Second Military Medical University's Institutional Animal Care and Use Committee (Shanghai, China), in compliance with the Second Military Medical University guidelines for the care and use of animals. Four-week-old male BALB/c mice were purchased from the Second Military Medical University Animal Center and maintained in pathogen-free conditions with a regular pellet diet and water. Metastatic lung and liver models were used as previously described (23).

Methylated RNA immunoprecipitation (MeRIP) PCR

MeRIP-PCR analysis was performed according to a previously published technique (24).

Statistical analysis

The mean± standard error of the mean (SEM) were used to present the data. All data were obtained from three independent experiments. GraphPad Prism v.8 (San Diego, USA) and SPSS v.25.0 (Chicago, USA) software were used for all statistical analyses. Survival curves were calculated using the Kaplan-Meier method, and the differences were determined using the log-rank test. Univariate and multivariate Cox proportional hazards regression models were used to examine the independent components. P values less than *0.05, **0.01, and ***0.001 were deemed statistically significant.

Results

STIM2 deficiency in CCA tissues is related to a poor prognosis and extrahepatic metastases

According to the GEPIA(Gene Expression Profiling Interactive Analysis) 2 database, patients with CCA who had lower levels of STIM2 had a worse overall prognosis and were more likely to die from the condition (*Figure 1A*). STIM2 mRNA expression was significantly lower in 10 matched CCA tissues compared to adjacent normal tissues by quantitative PCR (qPCR). Notably, the CCA that metastasized extra hepatically had the lowest STIM2 mRNA expression (*Figure 1B*). Meanwhile, IHC revealed that STIM2 protein levels were commensurate with the trend in mRNA levels (*Figure 1C*,*1D*). Collectively, these findings suggested that STIM2 may be involved in the evolution of CCA and may serve as a possible prognostic indicator for patients with CCA.

Inhibition of tumor metastasis by STIM2 in vivo

The mRNA level of STIM2 was then studied in three CCA cell lines, with RBE exhibiting relatively lower STIM2 expression, while HUCCAT1 and Huh28 exhibited markedly higher STIM2 expression (*Figure 2A*). To investigate the role of STIM2 in preventing CCA cell metastasis, RBE cells were infected with LV-STIM2 or LV-NC (*Figure 2B*), and the effects of STIM2 on tumor metastasis were examined *in vivo*. To create the liver orthotopic-implanted models, cells were infected with LV-STIM2 or LV-NC and transplanted into the livers of nude mice. Six weeks after transplantation, the fluorescence signal intensities of metastatic liver nodules in the LV-STIM2 group were significantly lower than those in the LV-NC group (*Figure 2C*). There were significantly fewer metastatic foci found in liver tissue slices from patients who had been treated with LV-STIM2 (*Figure 2D*,*2E*), indicating

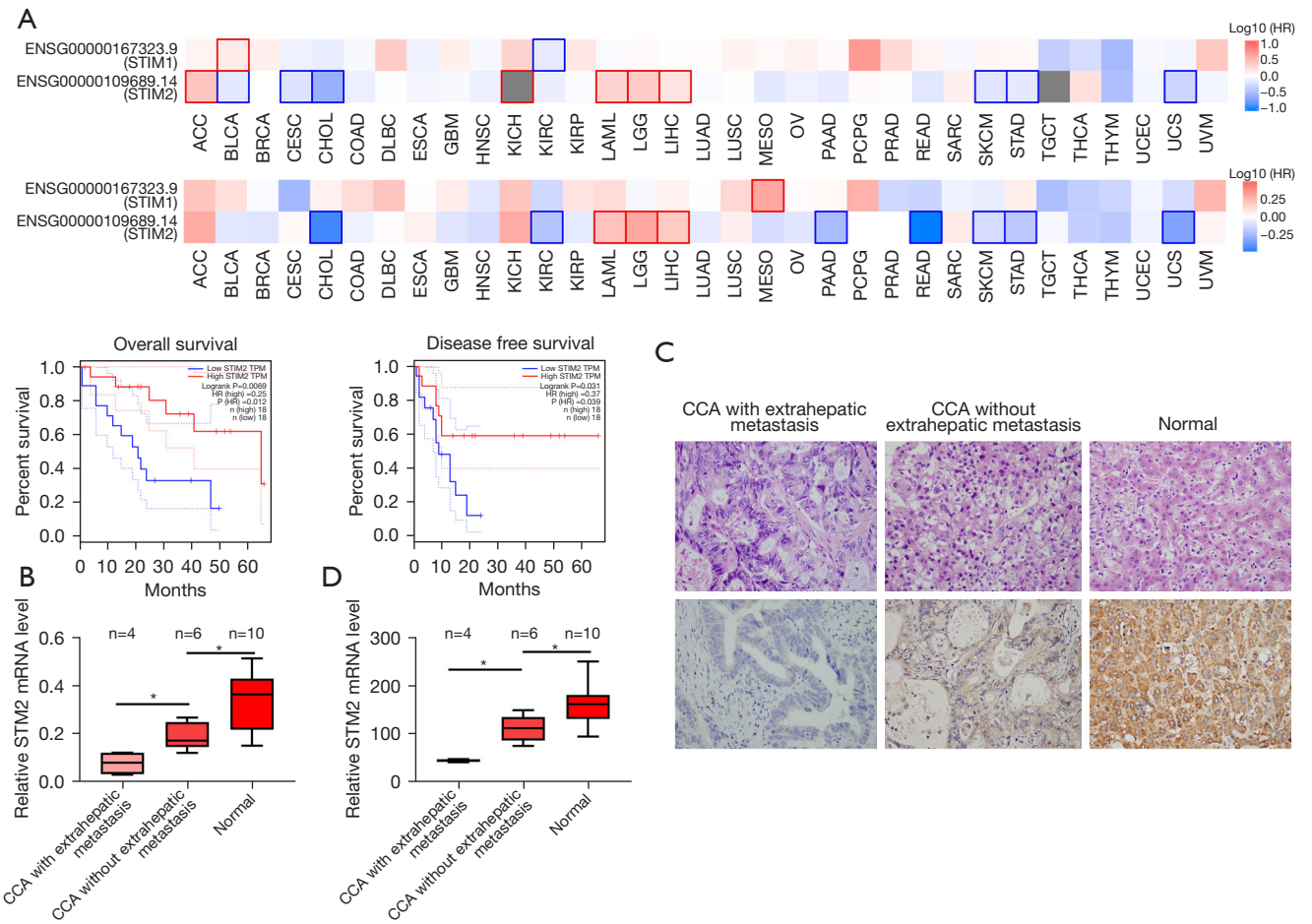

Figure 1 Decreased STIM2 in CCA tissues is associated with poor prognosis and extrahepatic metastasis. (A) *STIM2* expression and overall survival or disease-free survival in CCA patients were compared using Kaplan-Meier survival analysis. A median expression level from the GEPIA 2 database served as the cutoff point. (B) The mRNA expression levels of STIM2 were examined in 10 paired CCAs (with or without extrahepatic metastases) and neighboring normal tissues. (C) IHC pictures showing *STIM2* staining in the CCA tumor or surrounding tissues (magnification ×200). (D) H-score from (C). ± SD. *, P<0.05 indicated statistical significance. CCA, cholangiocarcinoma; STIM2, Stromal Interaction Molecule 2; GEPIA 2, Gene Expression Profiling Interactive Analysis; mRNA, messenger RNA; IHC, immunohistochemical; SD, standard deviation.

that STIM2 inhibited the ability of CCA cells to spread intra-hepatically. Using nude mice as a model for lung metastasis, we injected cells labeled with firefly luciferase into their tail veins. Compared to the LV-NC group, the LV-STIM2 group had significantly lower bioluminescence signal intensities in mice, substantially lower fluorescence signal intensities of metastatic lung nodules (*Figure 2F*), and fewer metastatic foci in lung tissue sections (*Figure 2G,2H*), indicating that STIM2 can inhibit hepatoma cells' metastatic lung potential.

STIM2 inhibits tumor metastasis by decreasing KRT8 levels in vivo

Next, we explored the targeted gene of STIM2 inhibition of tumor metastasis *in vivo*. The UALCAN database (https://ualcan.path.uab.edu/) showed that the top five genes (*KRT18*, *KRT8*, *MPST*, *MRPS17*, and *C20orf24*) were negatively correlated with STIM2 in CCA (*Figure 3A*). We observed that LV-STIM2 lowered KRT8 mRNA expression in CCA cells, indicating that STIM2 may regulate KRT8 expression

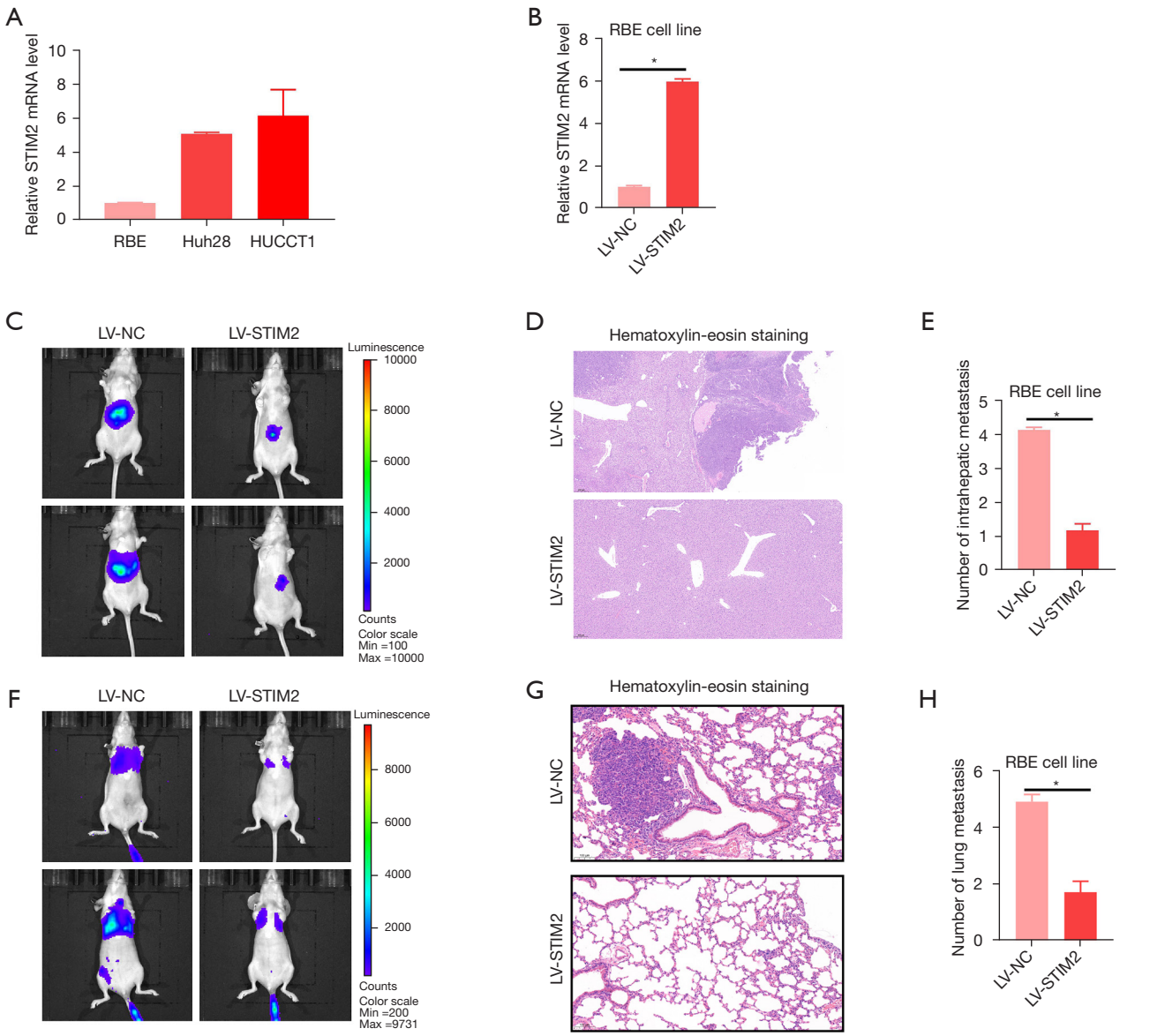

Figure 2 STIM2 inhibits tumor metastasis *in vivo*. (A) qRT-PCR was used to verify the mRNA levels of *STIM2* in three CCA cells. (B) RBE cells overexpressed *STIM2*. qRT-PCR was used to verify the efficacy of the overexpression. (C) Photographs of the intrahepatic metastases taken in bioluminescence. (D) Sample microscopic pictures of metastatic lung foci stained with HE to show the indicated CCA cells in liver tissue from which to extrapolate, magnification ×50. (E) Quantitative findings from (D). (F) The IVIS@ Lumina II imaging system (Caliper Life Sciences, Hopkinton, MA, USA) captured bioluminescence images of the mouse tail vein and injection metastasis lung models at specific periods. (G) A microscopic picture of pulmonary metastatic foci identified CCA cells in pulmonary tissue sections stained with HE, magnification ×50. (H) Quantitative statistics from (G). *, P<0.05. qRT-PCR, quantitative reverse transcription-polymerase chain reaction; CCA, cholangiocarcinoma; STIM2, Stromal Interaction Molecule 2; HE, hematoxylin-eosin.

in CCA cells (*Figure 3B*). As a result, we are curious as to whether STIM2 inhibits tumor metastasis via decreased KRT8 levels. SK-Hep1 cells transfected with LV-STIM2 or LV-NC were infected *in vitro* with LV-KRT8 or LV-N, respectively (*Figure 3C*). LV-STIM2 decreased the capacity of CCA to metastasize in orthotopic implanted and lung metastasis models. Simultaneously, LV-KRT8 restored the inhibitory effect of STIM2 on CCA cell metastasis

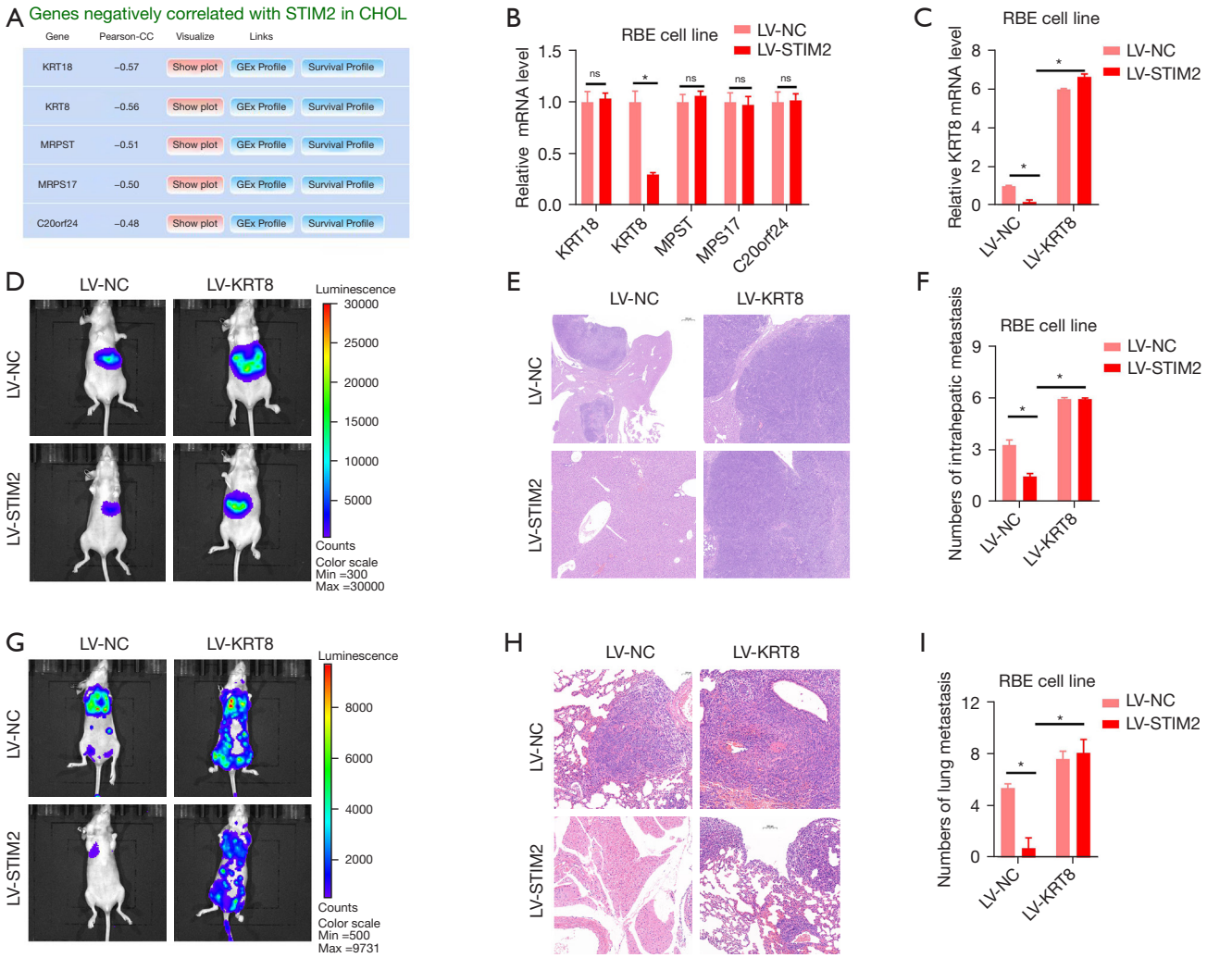

Figure 3 *STIM2* inhibits tumor metastasis by decreasing *KRT8* levels *in vivo*. (A) Five top genes (*KRT18*, *KRT8*, *MPST*, *MRPS17*, and *C20orf24*) negatively correlated with *STIM2* in CCA based on the UALCAN database (https://ualcan.path.uab.edu/). (B) The mRNA level of *KRT8* in CCA with LV-NC or LC-*STIM2* was validated using qRT-PCR. (C) *KRT8* was overexpressed in CCA cells with LV-NC or LV-*STIM2*. The overexpression efficiency was validated using qRT-PCR. (D) Representative bioluminescence photographs of the intrahepatic metastases. (E) Microscopic representations of pulmonary metastatic foci in liver tissue slices stained with HE identified CCA cells, magnification ×50. (F) Statistics derived from (E). (G) The IVIS@ Lumina II imaging system captured bioluminescence images of the mouse tail vein and injection lung metastasis models at specific times. (H) Representative microscopic pictures of pulmonary metastatic foci from indicated CCA cells were obtained through HE staining, magnification ×50. (I) Quantitative statistics from (H). *, P<0.05. CCA, cholangiocarcinoma; STIM2, Stromal Interaction Molecule 2.

in vivo (*Figure 3D-3I*), demonstrating that STIM2 suppresses tumor metastasis *in vivo* by lowering KRT8 levels.

Abnormal STIM2 levels in CCA are responsible for m^{6}A methylation modification

Through GEPIA 2 analysis, we observed the relationship between STIM2 and the expression of methylase METTL3, METTL14, WTAP, and KIAA1429, as well as demethylase ALKBH5 and FTO. In the CCA, we detected that STIM2 was related toMETTL14 and YTHDC2 (*Figure 4A*). Meanwhile, *Figure 4B* shows that STIM2 mRNA had three very confident m^{6}A sites, indicating that m^{6}A modification may be related to aberrant STIM2

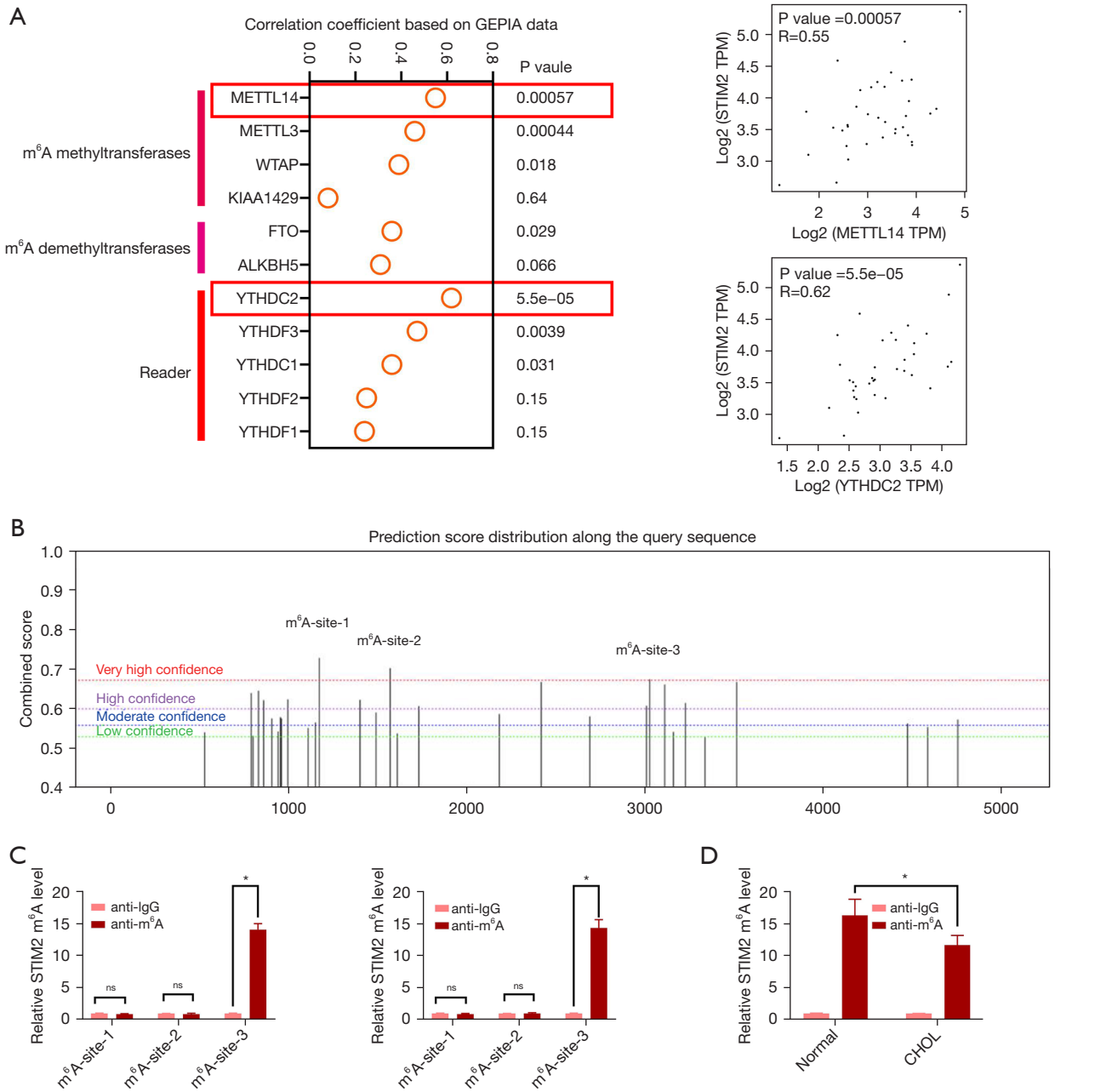

Figure 4 Abnormal *STIM2* levels in CCA are responsible for m^{6}A methylation modification. (A) Expression correlation with methylase (*METTL3*, *METTL14*, *WTAP,* and *KIAA1429*), demethylase (*ALKBH5* and *FTO*), and readers (*YTHDC1-2* and *YTHDF1-3*) in CCA based on the GEPIA database. (B) The m^{6}A site of STIM2 mRNA was predicted by the SRAMP database. (C) m^{6}A-site level *STIM2* in CCA cells were obtained by MeRIP-qPCR. (D) m^{6}A *STIM2* levels in 10 paired primary CCA were obtained by MeRIP-qPCR. *, P<0.05. CCA, cholangiocarcinoma; STIM2, Stromal Interaction Molecule 2.

levels in CCA, as demonstrated by the SRAMP (sequence-based RNA adenosine methylation site predictor) database (https://www.cuilab.cn/sramp). Further research using a RIP (RNA immunoprecipitation) assay confirmed the presence of the m^{6}A-3-site in STIM2 mRNA (*Figure 4C*). In the clinic, MeRIP-qPCR results showed that in 10 paired CCA patients, the m^{6}A CPEB1 level was lower in the tumors than the surrounding normal tissues (*Figure 4D*).

According to the above findings, aberrant STIM2 levels in CCA are caused by m^{6}A alteration.

Discussion

CCA, which develops from epithelial cells confronting the biliary tree lumen, is the second most common primary hepatic tumor worldwide after HCCA (25). As a result of the lack of early recognized clinical symptoms and the unavailability of precise tumor biomarkers, CCA is typically detected at an advanced, incurable stage (26). Despite recent advances in the development of molecularly targeted medicines, the outlook for this lethal malignancy remains bleak. As a result, there is a high priority in unraveling the molecular mechanisms and pathways underlying this disease to improve the clinical outcomes of CCA patients. STIM2 is the primary protein involved in Orai1 clustering and activation (27).

There is little known regarding the involvement of STIM2 in the advancement of CCA. Based on the GEPIA 2 database, this study discovered that low STIM2 mRNA levels were related to poor survival in CCA patients. Quantitative PCR and IHC studies revealed a decrease in the protein expression in CCA tissues was linked to extrahepatic metastasis. Increased *STIM2* levels inhibited CCA cell metastasis *in vivo*, confirming the inhibitory effect of STIM2 on the tumorigenesis and metastasis of CCA.

Our mechanistic investigation revealed that the *STIM2-KRT8* axis is critical in CCA metastasis. *KRT8* (keratin 8) is an epithelial marker and one of the most important keratin proteins (28). *KRT8* and its filament partner, *KRT18*, have been found to control the cellular response to stress stimuli and contribute to cell resistance to apoptosis, in addition to maintaining cell mechanical integrity (29). Recent investigations showed that *KRT8* levels were higher in the aqueous humor of patients with age-related macular degeneration (AMD) (30). *In vitro* studies have also indicated that high *KRT8* levels could protect RPE cells from deterioration under oxidative stress (31). *KRT8* has been demonstrated to be important in a variety of malignant tumors (32-34). However, the molecular mechanism of *KRT8* in the pathogenesis of CCA remains unknown. In this study, the GEPIA database and qRT-PCR were used to determine the changed expression of the *KRT8* mRNA levels controlled by *STIM2*. According to functional studies, *STIM2* suppresses tumor metastasis *in vivo* by reducing *KRT8* levels. In future research, we will explore the unique regulatory mechanism of m^{6}A-induced STIM2 abnormalities in CCA.

A recent study revealed that m^{6}A mRNA modification is critical for RNA fate, including splicing, localization, translation, transport, and mRNA stability (35). A multicomponent complex composed of *METTL3*, *METTL14*, and *WTAP* mediates adenosine N6 methylation in mammals (36). To date, *FTO* and *ALKBH5* have been shown to influence m^{6}A demethylation (37). m^{6}A has been shown to interact with the targeted mRNA or miRNA and contribute to the progression of various types of cancer (9). In recent years, emerging studies have suggested that m^{6}A-related genes have crucial roles in the initiation and progression of cancers, including acute myeloid leukaemia, renal cell carcinoma, and hepatocellular carcinoma etc. (24). Several m^{6}A regulator abnormal expressed is closely related to the poor prognosis of tumor patients including CCA (38). Recently study identified PD-L1 mRNA as a target of ALKBH5 and reveals a role for ALKBH5 in regulating the tumor immune microenvironment and immunotherapy efficacy in CCA (39). However, research on the m^{6}A alteration of STIM2 in CCA is lacking. In the present study, the SRAMP database and MeRIP-seq (sequencing) were used to determine the altered expression of *STIM2* mRNA transcripts controlled by m^{6}A alteration. In this study, we found that the m^{6}A/IGF2BP2 axis functioned in enhancing CCA metastasis, suggesting that it might be a new target for CCA diagnosis and treatment. Finally, as previously stated, this was the first time that STIM2 was identified as a direct target of m^{6}A-mediated transformation. The particular regulatory mechanism through which m^{6}A induces *STIM2* abnormality in CCA remains an open question for further research.

Conclusions

Finally, our findings have provided new insight into the critical involvement of the m^{6}A-STIM2 axis in the progression of CCA metastasis. The current results highlighted the importance of the molecular process of m^{6}A epi-transcriptomic alteration in cancer research, which was previously undiscovered.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-21-6485/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by Ethics Review Committee of Eastern Hepatobiliary Surgery Hospital and all patients provided written informed consent. Animal experiments were approved by the Second Military Medical University's Institutional Animal Care and Use Committee (Shanghai, China), in compliance with the Second Military Medical University guidelines for the care and use of animals.

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