MEDICAL SCIENCE MONITOR

LAB/IN VITRO RESEARCH

e-ISSN 1643-3750
© Med Sci Monit, 2018; 24: 7625-7632
DOI: 10.12659/MSM.909156

Received: 2018.01.25
Accepted: 2018.03.07
Published: 2018.10.25

Silence of Stomatin-Like Protein 2 Represses Migration and Invasion Ability of Human Liver Cancer Cells via Inhibiting the Nuclear Factor Kappa B (NF-κB) Pathway

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Source of support: Departmental sources

Background: Liver cancer is the third leading cause of tumor-related deaths worldwide. Stomatin-like protein 2 (STOML2) is obviously upregulated in various tumors. In this study, we explored the potential roles and mechanisms of si-STOML2 in the migration and invasion of human hepatoma LM3 cells.

Material/Methods: The expression levels of STOML2 in tissues and cells were separately analyzed with quantitative real-time PCR (qRT-PCR) and Western blotting. The viability, migration, and invasion of cells were assessed by cell counting kit-8 (CCK-8), wound healing, and transwell analysis, respectively. The mRNA and protein levels of various factors were separately measured using qRT-PCR and Western blotting. Correlation analysis between the expression of STOML2 and the clinicopathological features of liver cancer patients was evaluated using the chi-square test.

Results: Surprisingly, our results showed that STOML2 was upregulated in liver cancer tissue and cells, and this upregulation was linked to tumor size, histologic grade, and metastasis, but was not associated with sex, age, or TNM stage. The knockdown of STOML2 significantly repressed the viability, migration, and invasion of LM3 cells. We also observed that silencing STOML2 markedly downregulated the expression levels of matrix metalloproteinase-2 (MMP-2), MMP-9, metastatic tumor antigen 1 (MTA1), and nuclear factor kappa B (NF-κB), and upregulated levels of E-cadherin, tissue inhibitor of metalloproteinases 2 (TIMP2), and the inhibitor of kappa B (IκB).

Conclusions: STOML2 has a vital role in the progression of liver cancer. STOML2 silencing in LM3 cells obviously repressed the abilities of migration and invasion via suppressing the NF-κB pathway.

MeSH Keywords: Liver Neoplasms • Oncogene Protein v-cbl • Receptor Activator of Nuclear Factor-kappa B

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/909156

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Background

According to a recent report, liver cancer is the most common cause of tumor-related deaths worldwide [1]. Research has demonstrated that liver cancer can be characterized by obscure onset and difficult early diagnosis; therefore, liver cancer is generally diagnosed in advanced stage when it is prone to metastasis. Thus, the prognosis of liver cancer is extremely poor and is accompanied a high mortality rate [2]. The occurrence and development of tumors is a result of multi-gene participation and multi-step co-participation [3,4]. Therefore, to study the abnormal expression genes in liver cancer is of great medical significance, and by doing so we may be able to provide guidance for the diagnosis, treatment regimen, and prognostic evaluation of liver cancer.

The stomatin-like protein 2 (STOML2) gene was first discovered by and named in the Pathology Department of Yale University in the United States; and this gene is located in HSA chromosome 9p13.1 and consists of 10 exons and 9 introns with a length of about 3250bp [5]. STOML2 is also a new member of the stomatin gene superfamily, which is widely expressed in a variety of human tissues such as brain, lung, and kidneys [6]. In recent years, studies have proved that stomatin and its family members participate the development of malignant tumors [7–9]. In addition, a number of studies have shown that the expression of STOML2 was obviously upregulated in various malignant tumors, and STOML2 may play an important role in the incidence and development of tumors [10,11]. However, the relationship between STOML2 and liver tumors still remains unclear.

Nuclear factor kappa B (NF-κB) is an important nuclear transcription factor, which generally binds to inhibitor of kappa B (IκB) in an inactive state that exists broadly in the cytoplasm [12]. NF-κB is the convergent point of various signal transduction pathways, which regulates the expression of more than 500 genes involved in cell transformation, proliferation, and apoptosis [13]. It appears that the NF-κB pathway is closely related to the occurrence of diseases such as inflammation, cancer, and diabetes [14,15].

In this study, we explored the expression levels of STOML2 in liver tumor tissues and cells. We also set out to determine the potential roles and mechanisms of si-STOML2 in the migration and invasion of human hepatoma LM3 cells.

Material and Methods

Tissue source

The 50 samples of liver cancer and adjacent normal tissues were acquired from liver cancer patients in the Affiliated Changzhou No. 2 People's Hospital, Nanjing Medical University, from 2011 to 2016. The patient's informed consent and the approval of the Ethics Committee were all obtained for the use of samples for later research.

Cell culture

Human normal hepatocyte line (LO2) and hepatoma cell line (Hep3B, MHCC97-L, and LM3) were purchased form Guangzhou Cellcook Biological Technology Co., Ltd. All cells were maintained in complete Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, USA) containing 10% fetal bovine serum (FBS; Gibco, Shanghai, China) and 1% penicillin-streptomycin (Yuanmu, Shanghai, China), and stored in a 95% humidified 5% CO_2 incubator (BC-J80S; Boxun, Shanghai, China) at 37°C.

RNA interference and transfection

For silencing of STOML2, human STOML2-target siRNA (5'-CGACAAUGUAACUCUGCAA-3') and unspecific scrambled siRNA (5'-ACGUGACACGUUCGGAGAATT-3') vectors were synthesized by BioVector (Beijing, China). Then, LM3 cells were transfected with vectors using Lipofectamine™ 2000 (Promega; Madison, Wisconsin, USA).

Cell counting Kit-8 (CCK-8) analysis

CCK-8 (MSK, Wuhan, China) was used to detect the viability of LM3 cells according to the manufacturer's instructions. Specifically, cells were inoculated in 96-well plates at the density of 1.5×10^3 cell/well and were cultured in an incubator for 24 h. After being cultured, cells were treated with PBS, unspecific scrambled siRNA vector, and human STOML2-target siRNA vector. The experiments were divided into control, negative control (NC), and si-STOML groups. After grouping, CCK-8 reagent was added to cells, and cells were seeded into an incubator for 4 h. Absorbance was analyzed at 450 nm by a microplate reader (FilterMax F3/F5; Molecular devices, California, USA).

Transwell analysis

Transwell assay was performed to measure the invasion of LM3 cells following a standardized method in which matrigel (Solarbio, Shanghai, China) was filled in the upper part of the transwell plate, and DMEM was added to the plate and maintained for 90 min. Then, the DMEM in plates was sucked out. The medium with FBS was dropped into the lower part of the transwell plate. Thus, cells suspension was filled in the upper part of the transwell plate and was then maintained for 24 h, and was fixed with paraformaldehyde (Thermo, Shanghai, China) for 10 min. Subsequently, cells were stained by hematoxylin for 18 min and washed by PBS 3 times. Finally, cells

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were observed and photographed using an inverted fluorescence microscope (M50C; Wanheng, Shanghai, China).

Wound healing assay

The migration ability of LM3 cells was assessed by wound healing assay under the protocols of the manufacturer. Briefly, after transfection, cells were scratched across the hole with a 1-mL pipette tip followed by washing in DMEM 2 times. Then, DMEM was added to the cells, which were then transferred into the incubator for 48 h. After being cultured, cells were rinsed in PBS 3 times and were fixed with paraformaldehyde for 25 min. Cells were stained with 0.1% crystal violet (Zhongxin Chemtech; Tianjin, China) for 30 min. Finally, cells were observed and photographed using an inverted fluorescence microscope.

Quantitative real-time PCR (qRT-PCR)

Total RNAs of the tissues and cells were harvested and collected by use of an RNA extraction kit (Promega, Beijing, China). We used 1 μg of RNA for synthesizing cDNA using the RevertAid™ cDNA Synthesis kit (Thermo, Shanghai, China). The reaction temperature and time were set as 85°C for 15 min and 4°C for 12 min. Then, cDNA was amplified by use of the SYBR Premix Taq™ II kit (Takara, Beijing, China). The reaction mixture (50 μL) contained 25 μL SYBR Green Mix, 19 μL distilled water, 4 μL cDNA, and 1 μL forward/reverse primer. The reaction temperature and time were set as: pre-degeneration at 96°C for 4 min, denaturation at 96°C for 20 s, annealing at 60°C for 30 s for 30 cycles, and extending at 72°C for 30 s. β-actin was used as the sample control. The sequences of primers are listed in Table 1. The formula $2^{-\Delta\Delta CT}$ was used to compare quantification.

Western blotting

Total proteins of the tissues and cells were harvested and lysed with an enhanced RIPA lysis buffer (Leagene, Beijing, China). Proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12%SDS-PAGE). Then, the protein was bound to the PVDF membrane (Hongda; Zhuzhou, Guangdong China). Subsequently, 5% skim milk was used to seal the membranes for 1.5 h, and membranes then were incubated with anti-STOML2 (Abcam, EP18708, 1: 1000), anti-E-cadherin (Abcam, ab15148, 1: 1200), anti-tissue inhibitor of metalloproteinases2 (TIMP2) (Abcam, ab180630, 1: 800), anti-metastatic tumor antigen 1 (MTA1) (Abcam, ab71153, 1: 1000), anti-matrix metalloproteinase-2 (MMP-2) (Invitrogen, MA1-772, 1: 1200), anti-MMP-9 (Invitrogen, PA5-13199, 1: 1000), anti-NF-κB (Abcam, E379, 1: 800), anti-IκB (CST, 9242S, 1: 1000), and anti-β-actin (R&D, MAB8969, 1: 2000) at 4°C in a refrigerator for 24 h. On the next day, the membranes were incubated with the secondary antibodies (Goat anti-mouse IgG, Abcam, ab7064, 1: 8000; Rabbit anti-mouse IgG, CST, #58802, 1: 7000; Mouse anti-rabbit IgG, CST, #3678, 1: 8000) at 37°C for 60 min. Proteins were analyzed by use of the iBright™ imaging system (A32749, Thermo, Shanghai, China).

Table 1. Sequences of the primers.

Primer name	Sequence (5'-3')	Product size (bp)
STOML2-forward	GCAGAAGGGAAGAAACAGGC	209
STOML2-reverse	GAGAACGCGCTGACATACTG	
MMP-2-forward	CAGCCCTGCAAGTTTCCATT	210
MMP-2-reverse	GTTGCCCAGGAAAGTGAAGG	
MMP-9-forward	GAGACTCTACACCCAGGACG	221
MMP-9-reverse	GAAAGTGAAGGGGAAGACGC	
MTA1-forward	CAGTCTGCTATAAGGCCGGA	246
MTA1-reverse	AGAAGAAATCCTCCCGCTCC	
E-cadherin-forward	TCACATCCTACACTGCCCAG	229
E-cadherin-reverse	AGTGTCCCTGTTCCAGTAGC	
TIMP2-forward	CTCTGTGACTTCATCGTGCC	217
TIMP2-reverse	CACTTCTCTTGATGCAGGCG	
β-actin-forward	GGGAAATCGTGCGTGACATT	219
β-actin-reverse	AGGTAGTTTCGTGGATGCCA	

Statistical analysis

All samples were assessed in triplicate, and the experiment was repeated at least 3 times. Experimental data are shown as mean ± standard deviation (SD) using Microsoft Excel 97-2003. One-way ANOVA was used to evaluate the differences among groups. Correlation analysis between the expression of STOML2 and the clinicopathological features of liver cancer patients was tested using the chi-square test. $P<0.05$ was considered as statistically significant.

Results

High expression of STOML2 in liver cancer tissue and hepatoma cells

To explore the expression levels of STOML2 in tumor and normal tissues/cells, the mRNA and protein expression levels of STOML2 were analyzed by qRT-PCR and Western blotting, separately. qRT-PCR assay showed that STOML mRNA was expressed higher in tumor tissue than in normal tissue, and that

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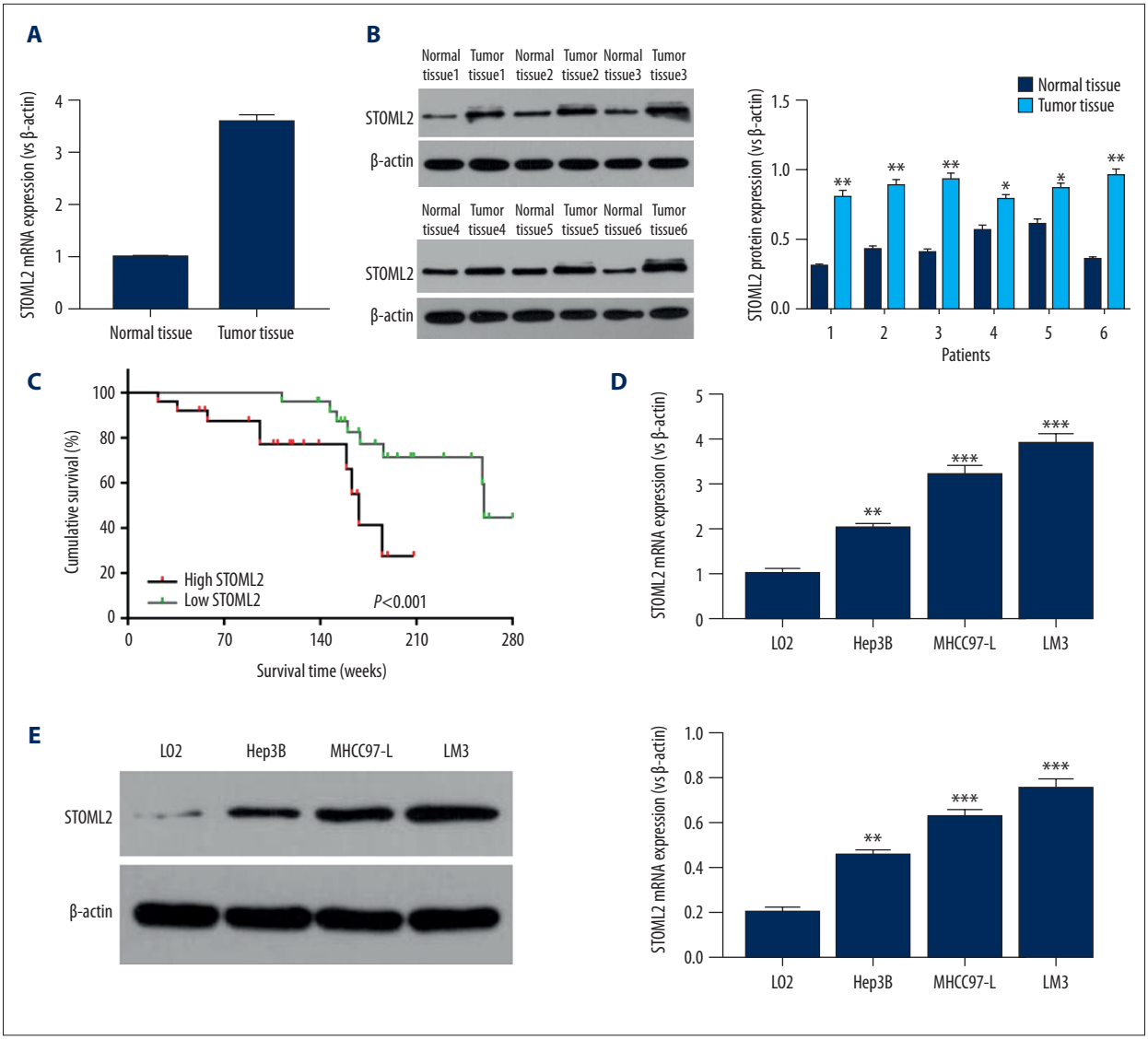

Figure 1. High expression of STOML2 in liver cancer tissue and hepatoma cells and correlated with tumor progression. (**A**) The expression level of STOML2 mRNA in liver cancer and adjacent normal tissues was tested by qTR-PCR. (**B**) The expression level of STOML2 protein in liver cancer and adjacent normal tissues was detected by Western blotting. (**C**) The correlation between STOML2 expression and the survival rate of the patients was quantified by GraphPad prism 7 software. (**D**) The mRNA levels of STOML2 in LO2, Hep3B, MHCC97-L, and LM3 cells were analyzed by qTR-PCR. (**E**) The protein level of STOML2 in cells was assessed by Western blotting. β-actin served as an internal control. Gray value was detected and counted by use of Quality One software. * $P<0.05$; ** $P<0.01$; *** $P<0.001$, compared to NC.

STOML protein was aberrantly upregulated in tumor tissue. Meanwhile, we found that the mRNA and protein expression levels of STOML2 was expressed at a higher level in Hep3B, MHCC97-L, and LM3 cells than in LO2cells, and that STOML2 expression in LM3 cells was the highest. Thus, LM3 cells were selected for later research (Figure 1A, 1B, 1D, 1E).

High expression of STOML2 was correlated with tumor progression

In order to demonstrate the prognostic significance that STOML-upregulated in liver cancer patients, our study examined the correlation among the expression of STOML2, the clinicopathological features, and cumulative survival rate of liver cancer patients. We observed that the cumulative survival rate of liver cancer patients with high STOML2 expression was lower than in those with STOML2 low expression, and that survival time

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Table 2. Correlation analysis between the expression of STOML2 and the clinicopathological features of liver cancer patients.

Groups	Number of patients	Low STOML2 expression	High STOML2 expression	*P* value
Gender				
Male	35	17	18	0.32
Female	15	5	10	
Age(years)				
<60	18	4	14	0.054
≥60	32	16	16	
Tumor size (cm)				
<3	20	12	8	0.018*
≥3	30	8	22	
TNM stage				
I/II	23	13	10	0.945
III/IV	27	15	12	
Histologic grade				
G1	8	3	5	0.041*
G2	25	8	17	
G3	17	7	10	
Metastasis				
No	30	19	11	0.021*
Yes	20	6	14	

* $P<0.05$, Chi-square test.

of patients with high STOML2 expression was shorter than in those with low STOML2 expression (Figure1C). Chi-square test results showed that expression levels of STOML2 were clearly related to tumor size, histologic grade, and metastasis, but was not associated with sex, age, or TNM stage (Table 2).

Silencing STOML2 inhibited the viability, migration, and invasion of LM3 cells

The transfection efficiency was tested by qRT-PCR and Western blotting. Our results indicated that STOML2 obviously had a low expression in si-STOML2. In comparison with NC, expression levels of STOML2 were about 50% that in si-STOML2 (Figure 2A, 2B). In addition, we explored the effects of si-STOML2 in terms of viability, migration, and invasion of LM3 cells by using CCK-8, wound healing, and transwell assays. As CCK-8 results show, when cells were transfected with si-STOML2, in comparison to NC, the viability of cells markedly decreased in a time-independent manner and the rates of migration and invasion in si-STOML2 cells were reduced by 63% and 50%, respectively, compared to those in NC (Figures 2C, 3).

Silencing STOML2 regulated the expression of metastasis-related factors in LM3 cells

To investigate the effect of si-STOML2 on metastasis-related factors in LM3 cells, qRT-PCR and Western blotting were performed. We found that mRNA levels of E-cadherin and TIMP2 were went up significantly, whereas the levels of MMP-2, MMP-9, and MTA1 were noticeably attenuated in si-STOML compared with NC. Moreover, Western blotting results showed that the protein expression trend of the above factors was consistent with the expression trend of mRNA (Figure 4).

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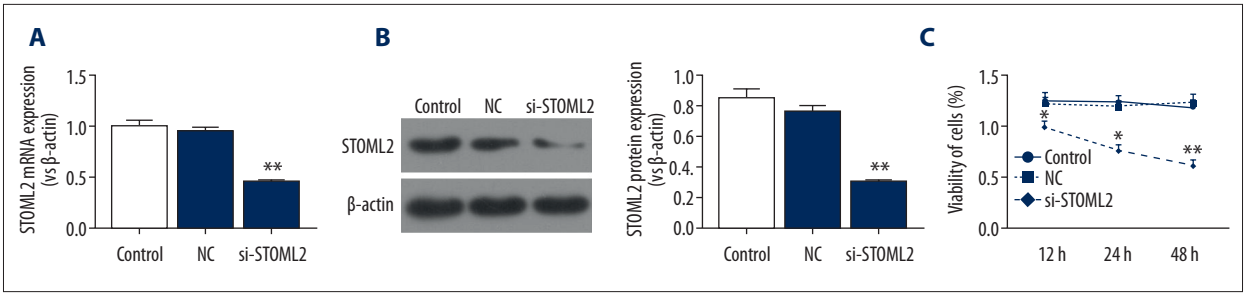

Figure 2. Silencing STOML2 inhibited the viability of LM3 cells. (**A**) LM3 cells were administrated with PBS (control), human STOML2-target siRNA (si-STOML2), and unspecific scrambled siRNA (NC) vectors, respectively. The mRNA level of STOML2 in LM3 cells was explored by qTR-PCR. (**B**) The protein level of STOML2 was determined by Western blotting and normalized to the levels of β-actin. The gray value was measured and calculated by use of Quality One software. (**C**) CCK-8 was performed to detect cell viability. * $P<0.05$; ** $P<0.01$, compared to NC.

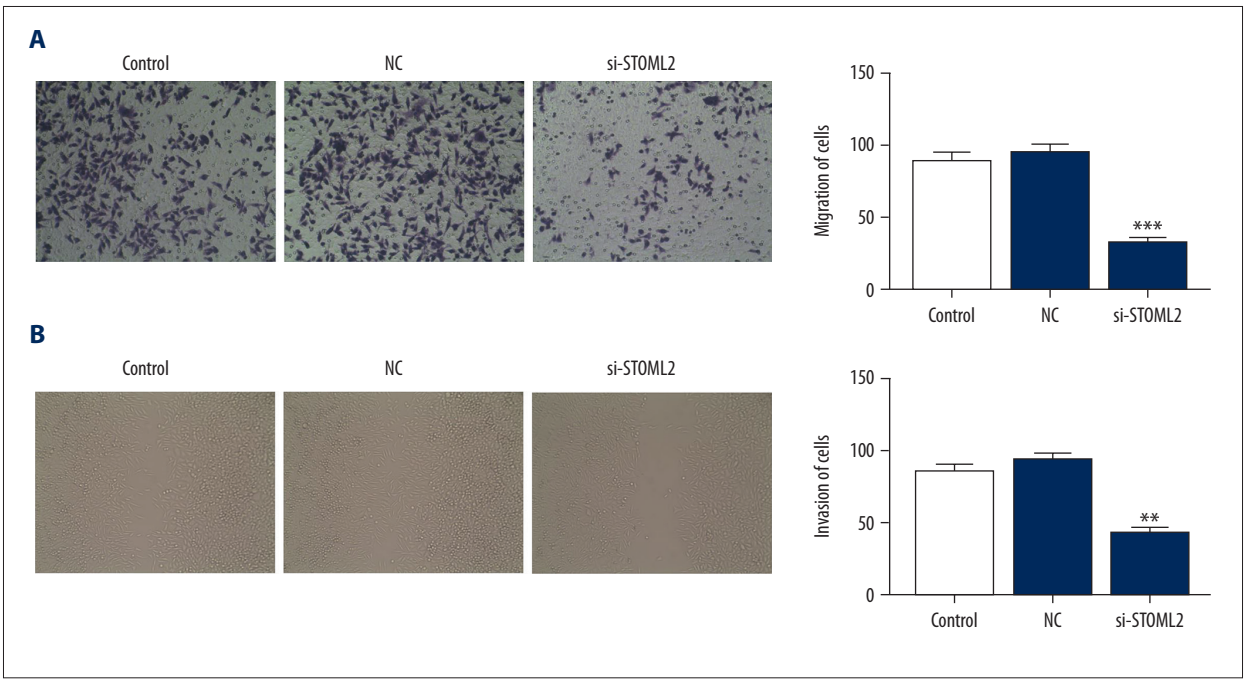

Figure 3. Silencing STOML2 repressed the migration and invasion ability of LM3 cells. (**A**) The ability of migration was assessed using wound healing assay. (**B**) The ability of invasion was assessed with transwell assay. * $P<0.05$; ** $P<0.01$, *** $P<0.001$, compared to NC.

Silencing STOML2 suppressed the migration and invasion of LM3 cells via inhibiting NF-κB pathway

To further identify the potential mechanism of si-STOML2 inhibition of cell migration and invasion, the proteins expression of NF-κB and IκB were carried out using Western blotting. Data showed that when cells were administrated with si-STOML2, the protein level of NF-κB was markedly downregulated and was reduced by about 43.75% compared to NC. Furthermore, in contrast to NC, si-STOML2 significantly upregulated IκB expression, and IκB protein level was increased by about 2.32-fold.

Discussion

A number of studies have shown that STOML2 is widely expressed in human tissues; however, the expression of STOML2 is obviously upregulated in malignant tumors [10,16,17]. The Chinese Academy of Medical Sciences has, for the first time, found that the expression of STOML2 in esophageal squamous carcinoma tissue was upregulated by more than 6-fod compared to that in normal esophageal mucosa [17]. Nevertheless, the role of STOML2 in liver cancer and its related mechanisms are not yet known. Therefore, we set out to study whether STOML2 was highly expressed in liver tumor tissue and cells, to determine the correlation between the expression of STOML2 and

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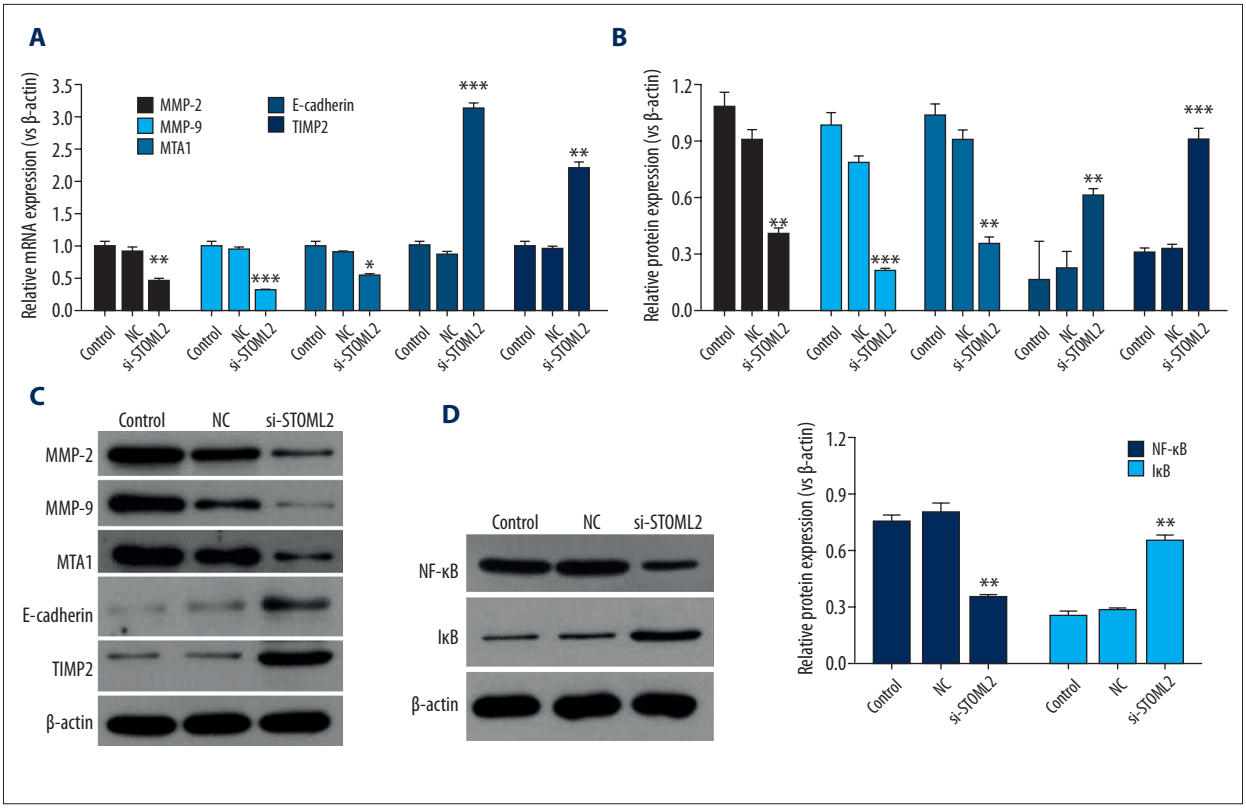

Figure 4. Silencing STOML2 regulated metastasis-related factors and NF-κB pathway in LM3 cells. (**A**) qRT-PCR was used to evaluate the mRNA levels of MMP-2, MMP-9, MTA1, E-cadherin, and TIMP2. (**B, C**) Western blotting was performed to investigate the protein levels of MMP-2, MMP-9, MTA1, E-cadherin, and TIMP2. (**D**) The protein levels of NF-κB and IκB were evaluated by Western blotting. β-actin was used as internal control. The gray value was evaluated and calculated by use of Quality O software. * $P<0.05$; ** $P<0.01$, *** $P<0.001$, compared to NC.

the clinicopathological features of patients diagnosed with liver cancer and the survival analysis of liver cancer patients. Similar to previous studies, our data show that STOML2 expression in liver cancer tissue and cells was higher than that in adjacent normal tissue and cells. Meanwhile, augmented expression of STOML2 was significantly related to the clinicopathological features (tumor size, histologic grade, and metastasis), which are associated with tumor progression. Moreover, patients with high expression of STOML2 had a poor 5-year survival rate. These results suggest that upregulation of STOML2 is associated with the poor prognosis of liver cancer.

Reports provided evidence that metastasis is a typical hallmark of hepatocellular carcinoma development, which is one of the important factors of poor prognosis [18,19]. Research found that STOML2 over-expression facilitates the migration and invasion of gastric cancer [20]. A previous study indicated that STOML2 silencing can reduce the growth and motility of tumor cells [21]. According to these findings, in our experiment, the STOML2 gene was silenced in LM3 cells and we investigated the effects of si-STOML2 on viability, migration, and invasion of cells. As expected, si-STOML2 decreased the viability, migration, and invasion of cells. Hence, STOML2 depletion might possibly reduce the spread of liver cancer by repressing the migration and invasion of cancer cells.

In order to further test the inhibition effect of STOML2 silencing on LM3 cell migration and invasion capacities, we assessed the expression levels of metastasis-related factors. Researchers showed that matrix metalloproteinase 2 and 9 (MMP 2 and 9) promote the metastasis of malignant tumors [22,23], and another study proved that TIMP2-overexpressed can reduce the invasion of pancreatic tumor cells [24]. MTA1 is a metastasis-associated gene that is overexpressed in various tumors [25]. A previous study substantiated that the deletion of adhesion protein E-cadherin accelerates metastasis of cancer cells [26]. From the data obtained in the present study, we found that si-STOML2 clearly reduced the levels of MMP2, MMP9, and MTA1, but it increased E-cadherin and TIMP2 levels. These results suggest that silencing of STOML2 attenuates the capacities of migration and invasion via regulating metastasis-related factors.

NF-κB signaling generally is seen as a cancer promoter in the development of tumors [27]. In recent years, increasing evidence

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indicates that suppression of the NF-κB pathway significantly reduces the progression of cancer [28,29]. Furthermore, a study reported that glabridin represses the migration and invasion of hepatocellular carcinoma cells via downregulating the NF-κB pathway [30]. Consistent with previous studies, we found that silencing of STOML2 depressed abilities of migration and invasion via suppressing the NF-κB pathway. Hence, STOML2 may be a target for the treatment of liver cancer, but our results need to be verified *in vivo*.

Conclusions

To conclude, the present study shows that expression levels of STOML2 are closely related to the incidence of human liver cancer. High expression of STOML2 was markedly correlated with the 5-year cumulative survival rate. STOML2 silencing in LM3 cells significantly suppressed the abilities of migration and invasion via repressing the NF-κB pathway.

Conflict of interest

None.

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