Am J Transl Res 2020;12(12):7870-7884
www.ajtr.org /ISSN:1943-8141/AJTR0121717

Original Article

SCAMP3 is regulated by miR-128-3p and promotes the metastasis of hepatocellular carcinoma cells through EGFR-MAPK p38 signaling pathway

Le Kang[1*], Ze-Hua Zhang[1,3*], Ying Zhao[2]

[1]First Department of Medicine, University Hospital Center, Friedrich-Alexander University, Erlangen-Nuremberg, Germany; [2]Department of Jiamusi College, Heilongjiang University of Chinese Medicine, Jiamusi 154000, Heilongjiang Province, China; [3]Department of Cancer Hospital, Harbin Medical University, Harbin 150000, Heilongjiang Province, China. [*]Equal contributors and co-first authors.

Received September 4, 2020; Accepted October 22, 2020; Epub December 15, 2020; Published December 30, 2020

Abstract: Purpose: To explore the regulatory mechanism of secretory carrier membrane protein 3 (SCAMP3) and miR-128-3p in hepatocellular carcinoma (HCC). Patients and methods: Cancer tissues and adjacent tissues of 52 HCC patients treated in our hospital were collected to explore the prognostic factors affecting their 3-year survival. HCC cells were purchased, the gene expression of Huh-7 and MHCC97 were adjusted by transfection, and the levels of SCAMP3, miR-128-3p, EGFR, p-EGFR, MAPK p38, p-MAPK p38, N-cadherin, vimentin, E-cadherin, cell proliferation, migration, invasion, apoptosis and epithelial-mesenchymal transition (EMT) were detected. A nude mouse model of HCC was constructed to verify the effects of transfection of mimics. Results: SCAMP3 was elevated in HCC patients and cancer tissues of HCC patients, while miR-128-3p showed opposite effects. High level SCAMP3 and low level miR-128-3p were related to poor prognosis of HCC. Both of them were correlated with excessive drinking history, N-stage, M-stage and pathological differentiation degree of HCC patients, as well as prognostic factors of HCC patients. SCAMP3 up-regulation or miR-128-3p down-regulation could promote HCC cell proliferation, migration, invasion, and transcription and protein levels of EGFR, p-EGFR, MAPK p38, p-MAPK p38, N-cadherin and vimentin, and inhibit HCC cell apoptosis and transcription and protein levels of E-cadherin. Dual luciferase reporter identified the targeting relationship between SCAMP3 and miR-128-3p. When both SCAMP3 and miR-128-3p were elevated or reduced, the biological manifestation of cells was not different from that of miR-NC transfected with unrelated sequences. Besides, miR-128-3p inhibited tumor growth in the HCC model in nude mice. Conclusion: SCAMP3 can be controlled by miR-128-3p and can mediate the EGFR-MAPK p38 signaling pathway to inhibit HCC cell metastasis, which is expected to become a promising therapeutic target for HCC.

Keywords: SCAMP3, miR-128-3p, EGFR-MAPK p38, hepatocellular carcinoma, metastasis

Introduction

Hepatocellular carcinoma (HCC) is a typical phenotype of primary hepatic carcinoma. Its etiology is related to chronic hepatitis B/C virus [1, 2]. According to the epidemiological data of HCC, there are 854,000 new cases and 810,000 deaths worldwide every year, and the morbidity and mortality have an upward trend [3]. Hepatitis B/C and alcohol abuse are risk factors of HCC, and the risk of alcohol abuse is five times higher than that of hepatitis C. Moderate alcohol consumption and hepatitis virus vaccination are helpful to prevent HCC [4]. HCC is also an invasive tumor with poor prognosis, and its early symptoms are insidious. Without prevention or screening, HCC is often diagnosed in the late stage with a low cure rate. However, the treatment options for advanced patients are very limited, and it is currently a popular therapeutic direction to find anticancer or carcinogenic targets at the molecular level [5, 6]. At present, HCC is diagnosed by biopsy or imaging analysis, and molecular markers with high sensitivity and specificity at the early stage of patients still need to be explored [7]. Therefore, finding highly correlated molecular markers of HCC and exploring its molecular mechanism are of great value for the treatment and prevention of HCC.

miRNA-mRNA molecular functional network is favored by researchers for its predictive screening of HCC highly correlated oncogenic or anti-cancer genes and helping people find potential therapeutic targets [8]. miRNA is a small RNA molecule with 20-25 nucleotides, which mediates the metastasis mechanism of cancer by specifically binding human cancer-related mRNA [9]. As a member of miRNA family, miR-128-3p is correlated with the sensitivity of sorafenib-induced apoptosis in HCC, and is abnormally down-regulated in HCC cell lines, which can improve the apoptosis sensitivity of HCC through physical binding with DJ-1, suggesting that it may have anti-cancer properties in HCC [10]. At present, there is little research on its metastasis mechanism in HCC, but it is found to be play a vital part in the metastasis of esophageal squamous cell carcinoma and EMT, and mainly plays a role by targeting ZEB1, the key medium of EMT [11]. We also found the potential binding sites between secretory carrier membrane protein 3 (SCAMP3) and it. We speculated that miR-128-3p-SCAMP3 molecular network mediated HCC metastasis. SCAMP3 is a transmembrane protein that plays a role in plasma membrane, endosome and trans Golgi network. It is over-expressed abnormally in HCC gene expression profile, which may have carcinogenic activity and is beneficial to HCC metastasis [12, 13]. EGFR-MAPK p38 signaling pathway is a functional molecular pathway closely related to tumor metastasis. EGFR can enhance the mobility of cells by adjusting the cell adhesion strength induced by adhesion plaque, while MAPK can change the stability of adhesion plaque [14]. Studies have shown that EGFR-MAPK p38 pathway mediates early liver inflammation and injury stress of fibrosis, and is also involved in the migration of HCC [15, 16].

Based on the above investigation, we conjectured that the miR-128-3p-SCAMP3-EGFR-MAPK p38 regulatory network mediates HCC metastasis, which is hereby verified.

Materials and methods

Tissue sample collection

From January 2014 to January 2017, fifty-two HCC patients admitted to the Affiliated Hospital of Heilongjiang University of Chinese Medicine and Cancer Hospital of Harbin Medical University were recruited, and their cancerous tissues and adjacent tissues were collected. Inclusion criteria: Patients whose HCC was confirmed by pathological diagnosis [17]. Patients received treatment at the first time. Patients did not have other liver diseases. Exclusion criteria: Patients had other malignant tumors or severe systemic dysfunction. Patients who could not accept the follow-up visit. This experiment was conducted with the approval of the ethics committee of the Affiliated Hospital of Heilongjiang University of Chinese Medicine and Cancer Hospital of Harbin Medical University, and was in accordance with Helsinki Declaration, all the participants have agreed to participate in the experiment and signed the informed consent.

Follow-up

HCC patients were followed up for 3 years, mainly by telephone, interview and pathological inquiry, to record their treatment until death or the last day of follow-up, as overall survival (OS).

Cell culture and transfection

Human HCC cells Huh-7, SK-HEP-1, MHCC97, SNU-354 and human normal hepatocytes L-02 (Guan Dao Bioengineering Co., Ltd., Shanghai, China, C0638, C4138, C7056, C1903, DA-C6286) were purchased, cultured in DMEM containing 10% PBS (Kanglang Biological Technology Co., Ltd., Shanghai, China, KL-P0032) in an environment of 37°C and 5% CO_2, and added with 0.25% pancreatin (Aiyan Biotechnology Co., Ltd., Shanghai, China, S-310) for digestion, followed by further culture to complete passage.

Gene expression was altered via constructing recombinant plasmids. Low expression plasmid pSilencer-SCAMP3 of SCAMP3 (si-SCAMP3), high expression plasmid pEGFP-SCAMP3 of SCAMP3 (SCAMP3), negative control RNA (si-NC), miR-128-3p up-regulated sequence (mimics), miR-128-3p inhibition sequence (inhibitor), miR negative control (miR-NC) were transfected with the help of Lipofectamine™ 2000 kit (Woosen Biotechnology Co., Ltd., Hangzhou, China, 11668019).

Real-time quantitative PCR

Extraction of total RNA in cells was carried out by the aid of Trizol reagent (Yiji Industries Co., Ltd., Shanghai, China, YJ58182). Then, total

RNA (5 μg) was taken for reverse transcription of cDNA using a reverse transcription kit (Qiming Biotechnology Co., Ltd., Shanghai, China, OX02700). Synthesized cDNA (1 μL) was taken for amplification. β-Actin and U6 were regarded as internal reference for SCAMP3 and miR-128-3p, respectively, and $2^{-\Delta\Delta ct}$ was applied to analyze the data.

MTT detection of cell viability

Transfected cells were inoculated on a 96-well plate (4×10^3 cells/well) 24 h after transfection and incubated at 37°C. Twenty μL MTT solution (5 μmg/mL, Yiyan Biological Technology Co., Ltd., Shanghai, China, SL7133-10ml) was added at each time point, cultured for 4 hours at 37°C. Two hundred μL dimethyl sulfoxide was put into each well. Measurement of OD value was performed at 490 nm wavelength by Nabi spectrophotometer (Bunsen Health Technology Co., Ltd., Tianjin, China).

Transwell detection of cell migration and invasion abilities

Cells were collected and inoculated on a 24-well plate (3×10^4 cells/well) 24 hours after transfection. They were digested by trypsin and transferred to the upper compartment (containing 50 mg/L Matrigel, Hengfei Biotechnology Co., Ltd., Shanghai, China, M8370). And then, 200 μL RPMI1640 nutrient solution (Fuze Trading Co., Ltd., Shanghai, China, LS115-001) and 600 mL RPMI1640 containing 10% FBS were added to the upper compartment and the lower compartment, respectively, culturing at 37°C for 48 h. The upper compartment was rinsed with PBS (Rongbai biological technology Co., Ltd., Shanghai, China, MB13103) and stained with 0.5% crystal violet, and its invasiveness was observed with a microscope. For the detection of migration, all the other steps were the same as before except for Matrigel.

Flow cytometry detection of cell apoptosis

Transfected cells were digested by 0.25% trypsin, washed twice with PBS, and mixed with binding buffer (100 μL) to prepare as suspension (1×10^6/mL). Next, Annexinv-FITC and PI were successively put into it. Cell incubation was conducted at indoor temperature for 5 min away from light. And flow cytometry NovoCyte (Biocytocare Biotechnology Co., Ltd., Guangzhou, China, D2060R) was used for detection. In addition, Q2 and Q3 in flow cytometry represent the proportion of early and late apoptosis, respectively, so the sum of the two was the level of apoptosis.

Western Blot detection

RIPA lysis was applied for extraction of total protein of HCC cells, and the protein concentration was determined with BCA kit (Rongbai Biotechnology Co., Ltd., Shanghai, China, LCB-004). The dilution ratio of SCAMP3 and other primary antibodies was 1:1000, and all the antibodies were purchased from Shanghai Kemin Biotechnology Co., Ltd. Each membrane was washed with PBS and incubated with horseradish peroxidase labeled goat anti-rabbit secondary antibody. Finally, after the steps of ECL luminescence and development, the gray value was analyzed.

Dual luciferase report assay

Targetscan7.2 predicted miR-128-3p downstream target genes. SCAMP3 3'UTR-Wt, SCAMP3 3'UTR-Mut, mimics and miR-NC were transferred into HCC cells using Lipofectamine™ 2000 kit, and determination of luciferase activity was conducted using dual luciferase reporter gene kit (Qunji Biotech Co., Ltd., Shanghai, China, KA3784).

Establishment of HCC models in vivo

Female BALB/c nude mice aged 4-6 weeks (VitalRiver Laboratory Animal Technology Co., Ltd., Beijing, China, 401) were raised in a sterile environment. HCC cells (5×10^6) containing mimics and miR-NC were injected into the left abdomen of nude mice subcutaneously. There were five mice in each group, and tumor growth was measured every 7 days. Mice were killed by dislocation 28 days after being injected, and measurement of size and mass of tumors were conducted. All animal experiments involved in the research had been approved by the ethics committee of animal experiments in our hospital, and were conducted in the light of the guidance of the ethics committee of animal experiments, which was in line with the institutional ethical guidelines for animal experiments.

Statistical methods

GraphPad 6 was applied for data analysis and image rendering. Independent sample t test,

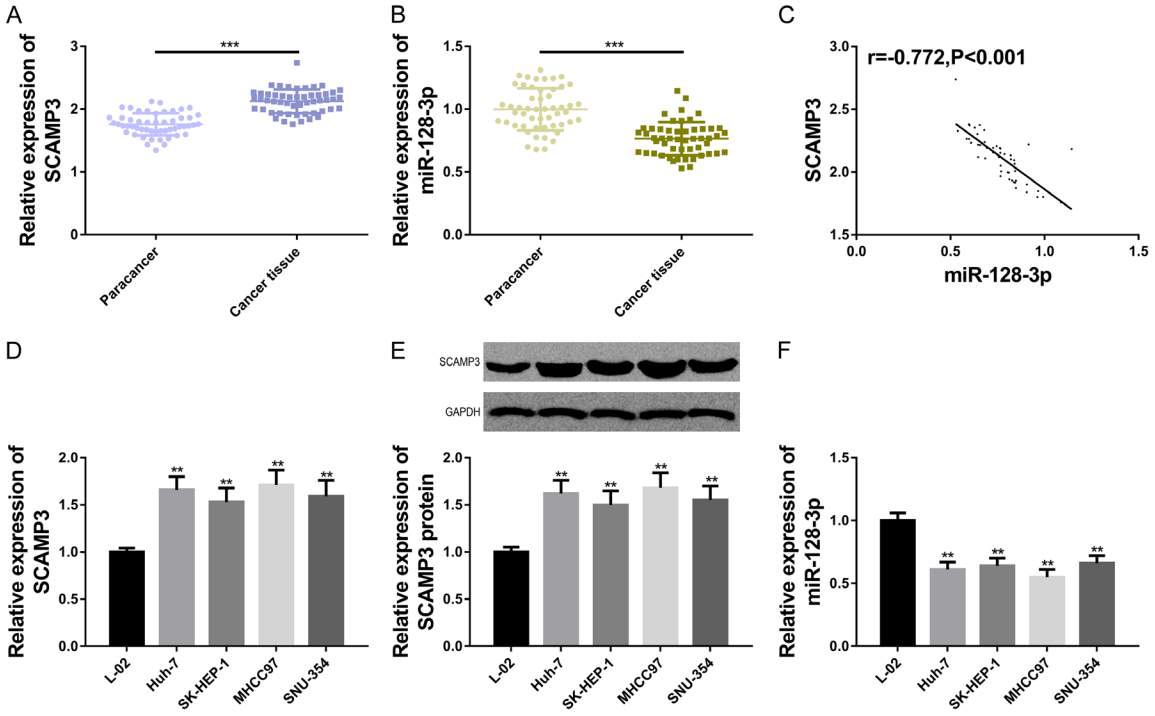


Figure 1. SCAMP3 is up-regulated in cancer tissues of HCC patients and is negatively correlated with miR-128-3p. A, B. The expression level of SCAMP3 is higher in HCC patients' cancer tissues, while the expression level of miR-128-3p is lower. C. There was a negative correlation between SCAMP3 and miR-128-3p (r=-0.772, P<0.001). D-F. The expression and protein levels of SCAMP3 in HCC cell lines were high, while the expression level of miR-128-3p was low, as well as the corresponding protein diagram. Notes: Comparison between the two groups or comparison with L-02 cells, **P<0.01, ***P<0.001. Abbreviation: SCAMP3, secretory carrier membrane protein 3; miR, microRNA; HCC, hepatocellular carcinoma.

LSD-T test, repeated measurement ANOVA, Bonferroni were used for comparison between two groups, comparison among multiple groups, pair-wise comparison afterwards, and expression at multiple time points, respectively. Independent risk factors affecting patient prognosis were analyzed by multivariate Cox regression analysis. Kaplan-meier was used to draw the survival curve, and Log-rank was applied to estimate the difference of survival time between groups. When P<0.05, statistical difference was indicated.

Results

SCAMP3 is up-regulated in HCC and negatively correlated with miR-128-3p

SCAMP3 expression was higher in HCC tissues than that in adjacent tissues, while miR-128-3p expression was different from the former one. There was a negative correlation between the two (r=-0.772, P<0.001), and their in HCC cell lines was also detected, the former showed a higher level, while the latter showed a lower level. Since the expression of the two were most significant in Huh-7 and MHCC97 of HCC cells, Huh-7 and MHCC97 were chosen for further analysis. Moreover, we also found that the two were bound up with the history of excessive drinking history, N stage, M stage and pathological differentiation of HCC patients. All the above results were statistically significant (P<0.05). As shown in **Figure 1** and **Table 1**.

High level SCAMP3 or low level miR-128-3p is associated with poor prognosis of HCC patients

We have successfully completed a 3-year follow-up of 52 HCC patients, of which 14 patients survived were enrolled in good prognosis group, and 38 patients died were enrolled in poor prognosis group. The 3-year OS in this study was 26.92% (14/52). We found that high level SCAMP3 and low level miR-128-3p existed in the poor prognosis group. Then, we plotted the survival curve with the median of the two expression as the dividing point of the high and low expression. The results showed that high

Table 1. Correlation of SCAMP3 and miR-128-3p with the pathological data of HCC patients [n (%), mean ± SD]

Factors	n=52	SCAMP3	t value	*P* value	miR-128-3p	t value	*P* value
Gender			1.096	0.278		1.273	0.209
Male	28	1.94±0.14			0.90±0.15		
Female	24	1.90±0.12			0.95±0.13		
Age (years)			1.792	0.079		1.434	0.158
<60	25	1.89±0.13			0.94±0.14		
≥60	27	1.96±0.15			0.88±0.16		
Smoking history			1.479	0.146		1.503	0.139
No	37	1.88±0.16			0.92±0.16		
Yes	15	1.95±0.14			0.85±0.13		
Excessive drinking history			7.517	<0.001		3.304	0.002
No	32	1.79±0.18			0.95±0.17		
Yes	20	2.16±0.16			0.80±0.14		
T stage			1.967	0.055		1.418	0.162
T2/T3	23	1.85±0.14			0.90±0.14		
T4	29	1.93±0.1.5			0.84±0.16		
N stage			6.583	<0.001		3.687	<0.001
N0	34	1.70±0.19			1.00±0.18		
N1	18	2.03±0.13			0.82±0.14		
M stage			5.605	<0.001		4.119	<0.001
M0	36	1.77±0.2.0			1.05±0.17		
M1	16	2.08±0.1.4			0.85±0.14		
Degree of pathological differentiation			2.120	0.039		2.729	0.009
Poor differentiation	19	2.00±0.13			0.86±0.12		
Middle + high differentiation	33	1.90±0.18			0.97±0.15		

Abbreviation: SCAMP3, secretory carrier membrane protein 3; miR, microRNA; HCC, hepatocellular carcinoma; T, tumor infiltration; N, lymph node metastasis; M, distant metastasis.

level SCAMP3 or low level miR-128-3p was associated with lower 3-year OS of HCC patients. Cox regression analysis revealed that N stage, M stage, miR-128-3p, SCAMP3 were independent prognostic factors for HCC patients. All the above results were statistically significant (P<0.05). As shown in **Figure 2** and **Table 2**.

Up-regulation of SCAMP3 promotes metastasis of HCC cells

We down-regulated, up-regulated, and negatively controlled the SCAMP3 expression in Huh-7 cell and MHCC97 cell by transfection them with si-SCAMP3, sh-SCAMP3, and si-NC, respectively. Cell function analysis indicated that HCC cells had increased abilities of proliferation, migration and invasion after SCAMP3 up-regulation, and the cells were free from apoptosis. The protein levels of p-EGFR, EGFR, p-MAPK p38, MAPK p38, N-cadherin and vimentin elevated, while E-cadherin decreased. The results of downward adjustment of SCAMP3 was different from the above. All the above results were statistically significant (P<0.05). As shown in **Figure 3**.

Target-controlled relationship exists between SCAMP3 and miR-128-3p

Targeted binding sites between SCAMP3 and miR-128-3p were found via Targetscan7.2, and the results of dual luciferase activity indicated that after up-regulating miR-128-3p, only SCAMP3 3'UTR-Wt luciferase activity (instead of SCAMP3 3'UTR-mut) was decreased, and SCAMP3 expression and protein level in HCC cells were inhibited. All the above results were statistically significant (P<0.05). As shown in **Figure 4**.

Down-regulation of miR-128-3p promotes metastasis of HCC cells

We also up-regulated, down-regulated and negatively controlled Huh-7 and MHCC97 cells by

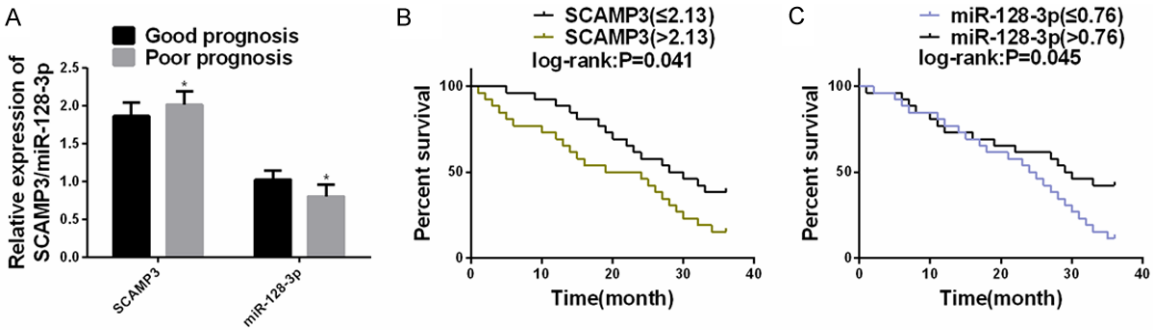


Figure 2. High level SCAMP3 or low level miR-128-3p is associated with poor prognosis of HCC patients. A. The expression level of SCAMP3 in serum of patients with poor prognosis is higher, while miR-128-3p is opposite. B, C. High level SCAMP3 or low level miR-128-3p is associated with lower 3-year OS in HCC patients. Note: Comparison with good prognosis, *P<0.05. Abbreviation: SCAMP3, secretory carrier membrane protein 3; miR, microRNA; HCC, hepatocellular carcinoma; OS, overall survival.

Table 2. Univariate and multivariate Cox regression analysis

Index	Univariate		Multivariate	
	HR (95% CI)	P	HR (95% CI)	P
Gender	1.146 (0.480-2.703)	0.669		
Age (years)	1.054 (0.424-3.108)	0.764		
Smoking history	1.099 (0.892-1.536)	0.098		
Excessive drinking history	0.748 (0.455-1.263)	0.274		
T stage	0.354 (0.125-0.999)	0.056		
N stage	5.108 (2.206-20.740)	0.028	2.217 (1.120-3.775)	0.009
M stage	1.736 (1.003-3.001)	0.001	1.832 (1.080-3.202)	0.024
Degree of pathological differentiation	0.553 (0.748-1.416)	0.102		
SCAMP3	3.870 (1.492-3.513)	<0.001	3.370 (2.324-8.529)	0.001
miR-128-3p	2.221 (1.213-3.781)	0.010	2.534 (0.820-6.469)	0.001

Abbreviation: SCAMP3, secretory carrier membrane protein 3; miR, microRNA; T, tumor infiltration; N, lymph node metastasis; M, distant metastasis.

transfecting them with mimics, inhibitor and miR-NC, respectively. When miR-128-3p decreased, HCC cells would have enhanced abilities of proliferation, migration and invasion, and suppressed apoptosis ability. The protein levels of p-EGFR, EGFR, p-MAPK p38, MAPK p38, N-cadherin and vimentin elevated, while E-cadherin decreased. The above results were reversed after miR-128-3p up-regulation. All the above results were statistically significant (P<0.05). As shown in **Figure 5**.

Simultaneous up-regulation or down-regulation of SCAMP3 and miR-128-3p can offset the above transfer promotion effect

When SCAMP3 and miR-128-3p were both up-regulated or down-regulated, no remarkable difference could be found in biological functions and molecular performance of HCC cells compared with miR-NC (P>0.05). However, compared with HCC cells with up-regulated miR-128-3p, the proliferation, migration and invasion abilities, as well as the protein levels of p-EGFR, EGFR, p-MAPK p38, MAPK p38, N-cadherin and vimentin, were notably increased, while the cell apoptosis ability and E-cadherin protein level were suppressed. Compared with cells with down-regulated miR-128-3p, the above results were all opposite, and the differences are statistically significant (P<0.05). As shown in **Figure 6**.

Up-regulation of miR-128-3p can inhibit tumor growth in nude mice

We further conducted in vivo study by establishing a nude mouse model. The results showed that the HCC model in vivo transfected with mimics successfully realized the over-

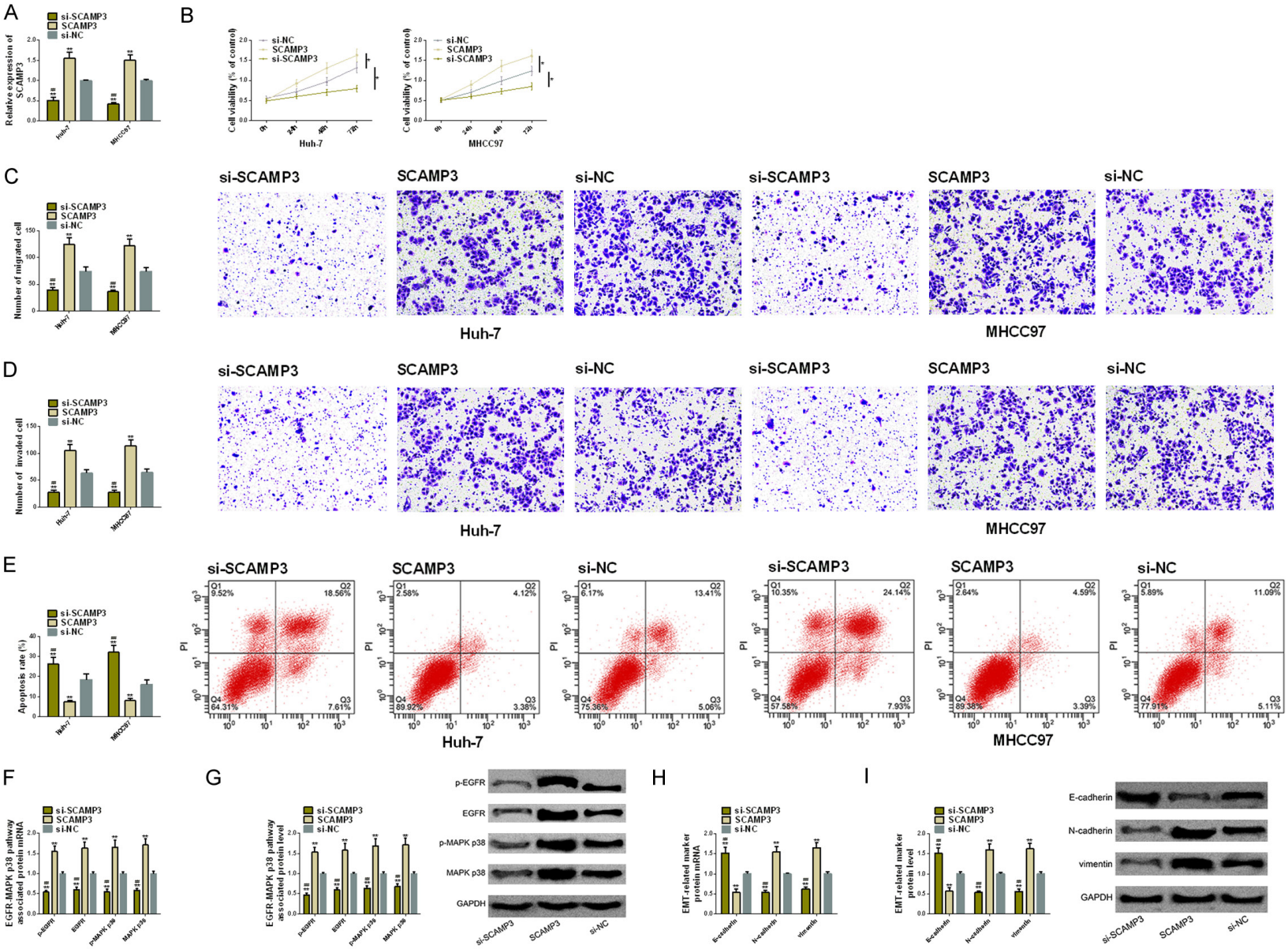


Figure 3. Up-regulation of SCAMP3 promotes metastasis of HCC cells. A. Transfection efficiency of SCAMP3. B-D. The proliferation, migration and invasion of HCC cells increases after SCAMP3 is up-regulated. E. After up-regulating SCAMP3, the apoptosis rate of HCC cells decreases, as well as the corresponding cell flow

diagram. F, G, After up-regulating SCAMP3, expression and protein levels of p-EGFR, EGFR, p-MAPK p38, MAPK p38 increase, as well as the corresponding protein diagram. H, I, After regulating SCAMP3, expression and protein levels of N-cadherin and vimentin increase, while the expression and protein level of E-cadherin decrease, as well as the corresponding protein diagram. Notes: Comparison with si-NC or between two groups, *P<0.05, **P<0.01. Comparison with sh-SCAMP3, ##P<0.01. Abbreviation: SCAMP3, secretory carrier membrane protein 3; NC, negative control; HCC, hepatocellular carcinoma; p-EGFR, phospho-epithelial growth factor receptor; EGFR, epithelial growth factor receptor; p-MAPK p38, phospho-p38 mitogen-activated protein kinase; MAPK p38, p38 mitogen-activated protein kinase; si, short interfering; PI, propidium iodide; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; EMT, epithelial-mesenchymal transition.

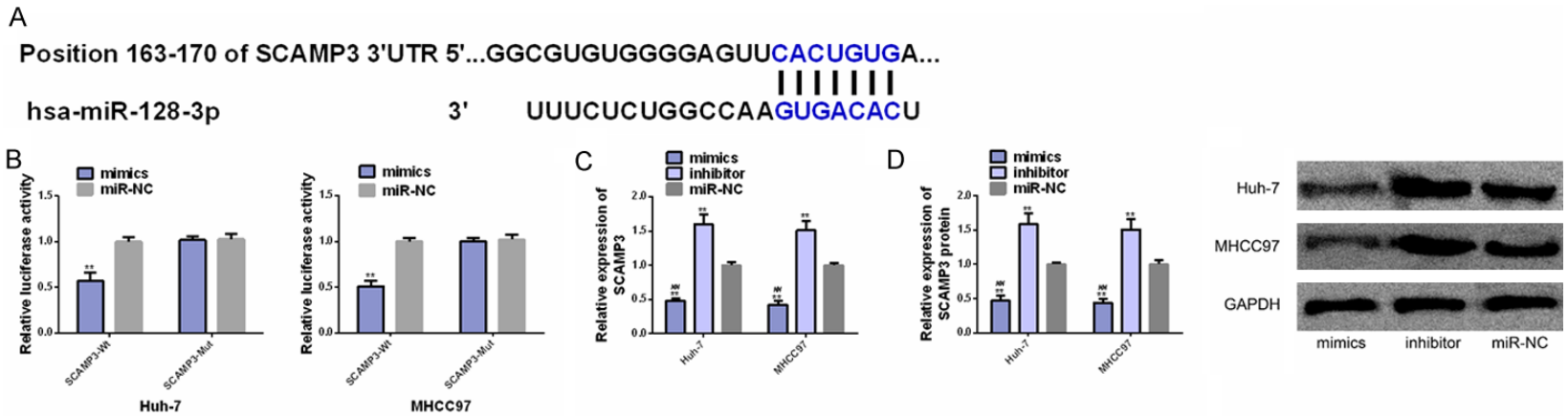


Figure 4. Target-controlled relationship exists between miR-128-3p and SCAMP3. A. Potential targeting sites exist between miR-128-3p and SCAMP3. B. Relative luciferase activity-dual luciferase reporter. C. Expression level of SCAMP3 in HCC cells after transfection. D. Protein level and protein diagram of SCAMP3 in HCC cells after transfection. Note: Comparison with miR-NC, **P<0.01. Comparison with inhibitor, ##P<0.01. Abbreviation: SCAMP3, secretory carrier membrane protein 3; miR, microRNA; NC, negative control; Wt, wild type; Mut, mutant.

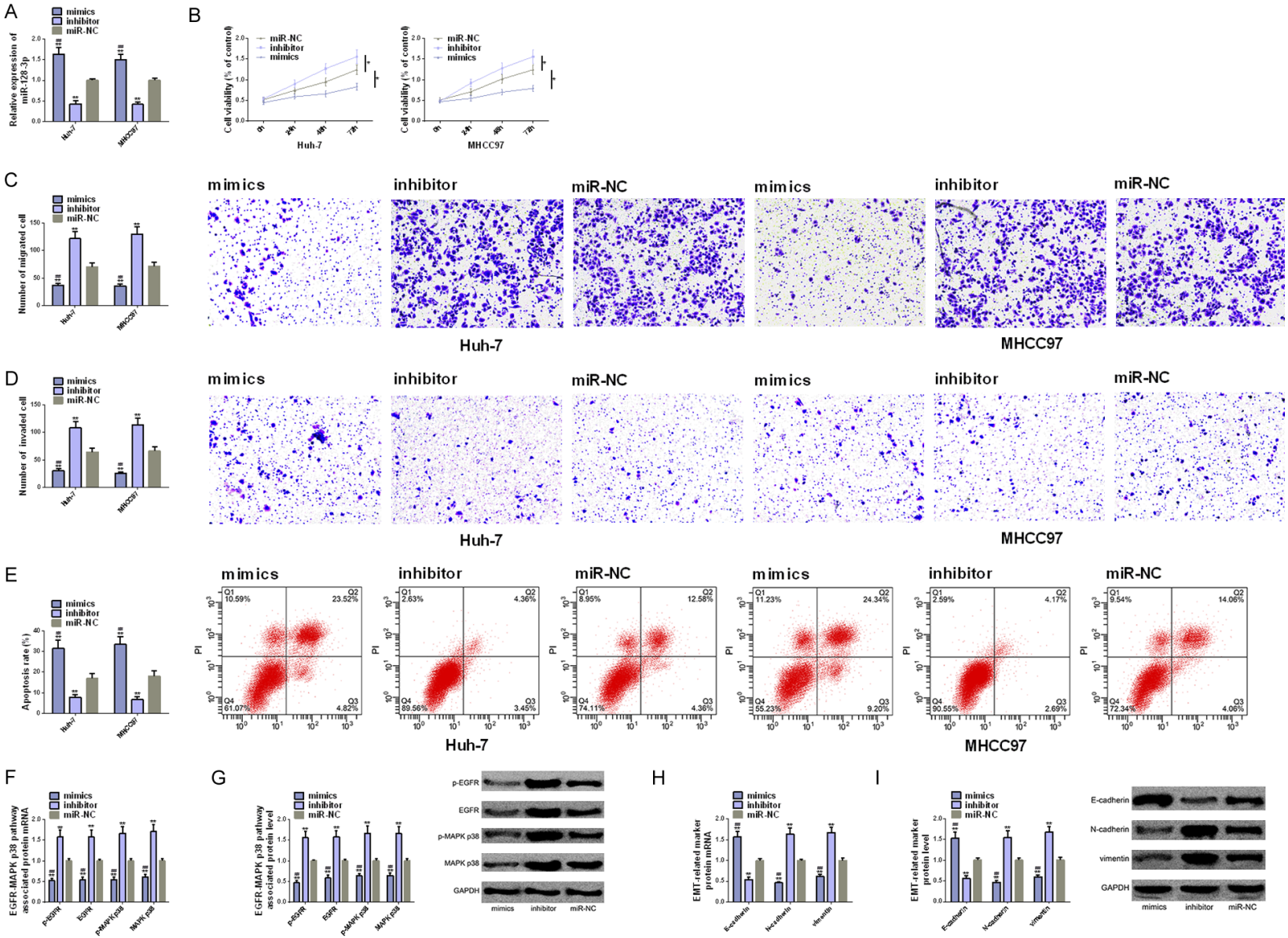
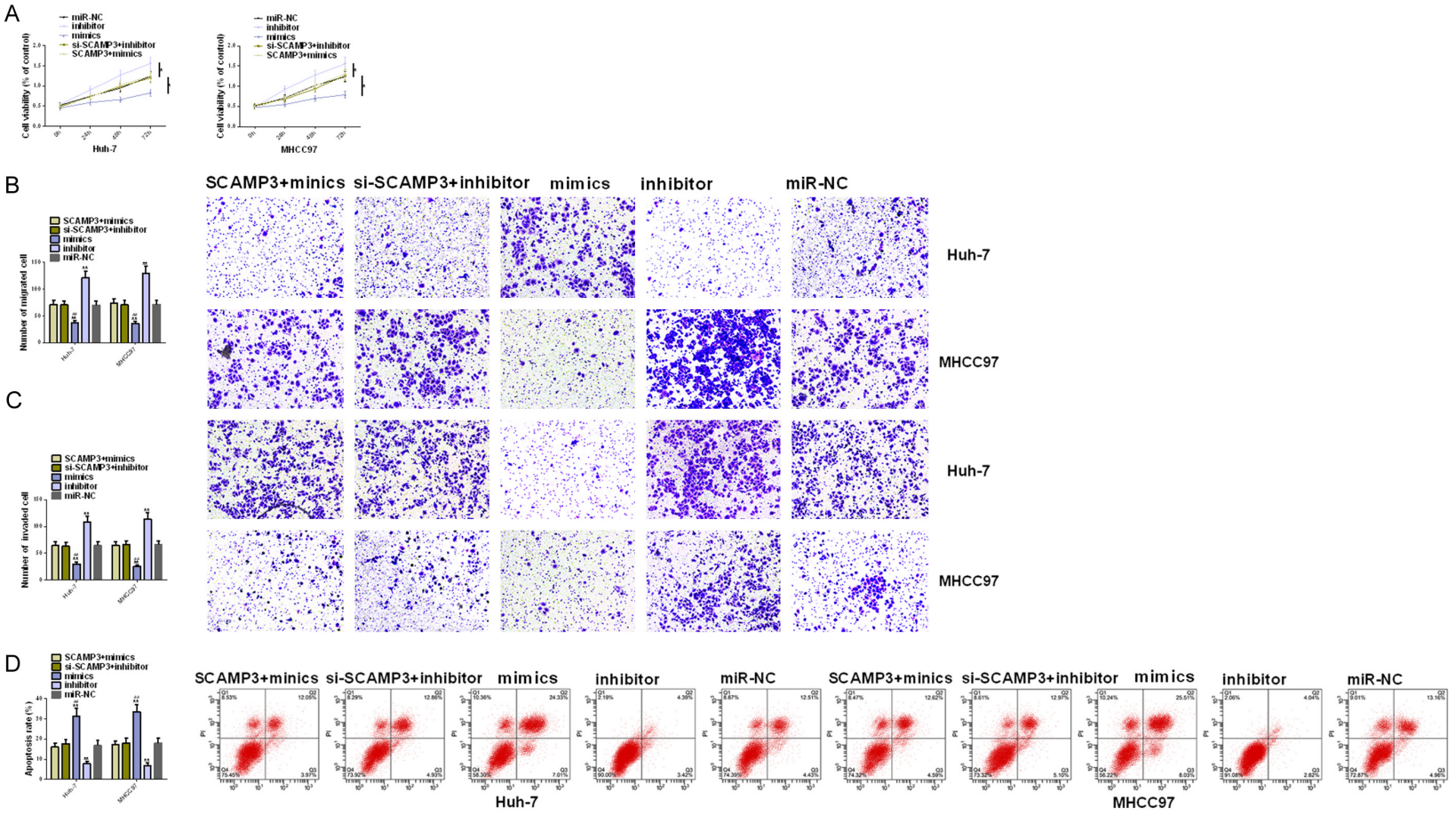


Figure 5. Down-regulation of miR-128-3p promotes HCC cell metastasis. A. Transfection efficiency of miR-128-3p. B-D. The proliferation, migration and invasion of HCC cells increases after miR-128-3p is down-regulated. E. The apoptosis rate of HCC cells decreases after miR-128-3p is down-regulated, as well as the corre-

sponding cell flow diagram. F, G. Expression and protein levels of p-EGFR, EGFR, p-MAPK p38, MAPK p38 in HCC cells increase after miR-128-3p is down-regulated, as well as the corresponding protein diagram. H, I. The expression and protein levels of N-cadherin and vimentin in HCC cells increases after miR-128-3p is down-regulated, while the expression and protein level of E-cadherin decreases, as well as the corresponding protein diagram. Note: Comparison with miR-NC or between two groups, *P<0.05, **P<0.01. Comparison with inhibitor, ##P<0.01. Abbreviation: miR, microRNA; NC, negative control; HCC, hepatocellular carcinoma; p-EGFR, phospho-epithelial growth factor receptor; EGFR, epithelial g-MAPK p38, phospho-p38 mitogen-activated rowth factor receptor; p protein kinase; MAPK p38, p38 mitogen-activated protein kinase; PI, propidium iodide; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; EMT, epithelial-mesenchymal transition.



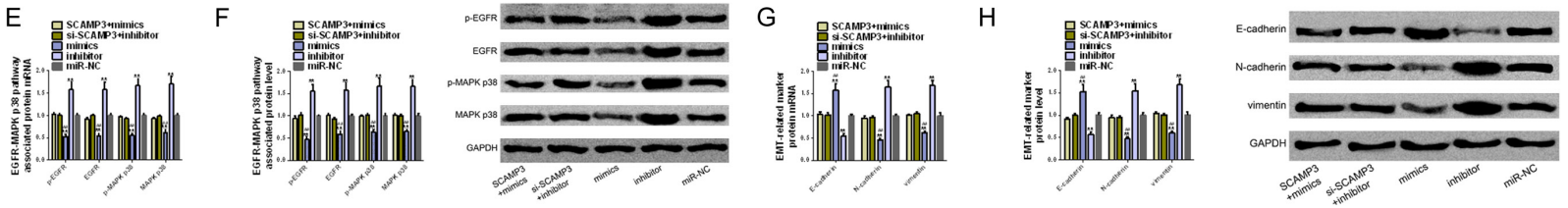


Figure 6. Simultaneous up-regulation or down-regulation of SCAMP3 and miR-128-3p can offset the above transfer promotion effect. A-C. Simultaneous up-regulation or down-regulation of SCAMP3 and miR-128-3p could counteract the promotion of proliferation, migration and invasion of HCC cells. D. Up-regulating or down-regulating SCAMP3 and miR-128-3p simultaneously can offset the apoptosis inhibition effect of HCC cells, as wells as the corresponding cell flow diagram. E, F. Up-regulating or down-regulating SCAMP3 and miR-128-3p simultaneously can offset the increase in the levels of p-EGFR, EGFR, p-MAPK p38 and MAPK p38 proteins in HCC cells, as well as the corresponding protein diagram. G, H. Simultaneous up-regulation or down-regulation of SCAMP3 and miR-128-3p can offset the increase of N-cadherin and vimentin protein level and the inhibition of E-cadherin protein level in HCC cells, as well as the corresponding protein diagram. Note: Comparison with miR-NC or between two groups, *P<0.05, **P<0.01. Comparison with inhibitor, ##P<0.01. Abbreviation: SCAMP3, secretory carrier membrane protein 3; miR, microRNA; NC, negative control; HCC, hepatocellular carcinoma; p-EGFR, phospho-epithelial growth factor receptor; EGFR, epithelial growth factor receptor; p-MAPK p38, phospho-p38 mitogen-activated protein kinase; MAPK p38, p38 mitogen-activated protein kinase; si, short interfering; PI, propidium iodide; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; EMT, epithelial-mesenchymal transition.

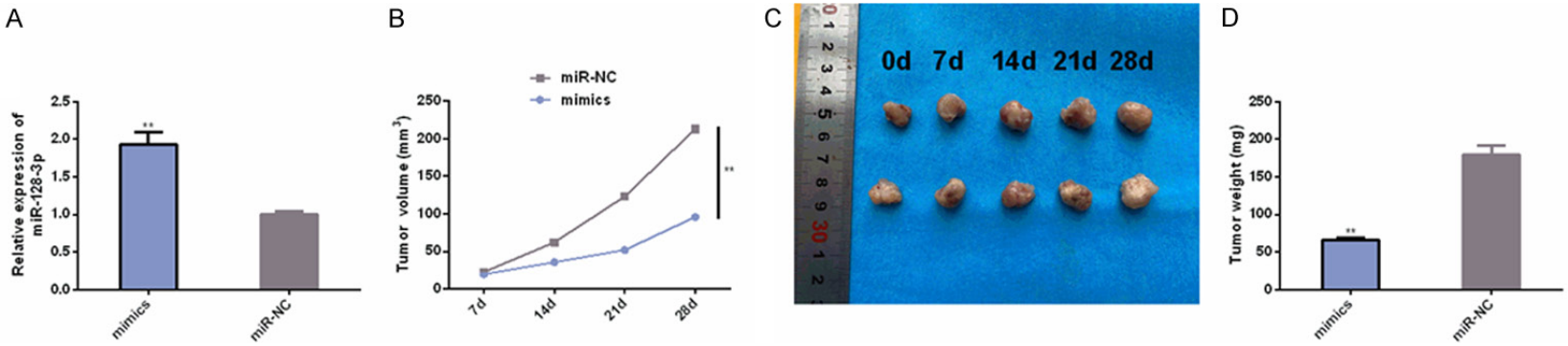


Figure 7. Up-regulation of miR-128-3p can inhibit tumor growth in nude mice. A. miR-128-3p transfection efficiency of HCC models in vivo. B-D. Up-regulation of miR-128-3p significantly inhibited tumor growth rate, size and mass of HCC models in vivo. Note: Comparison with miR-NC or between the two groups, **P<0.01. Abbreviation: miR, microRNA; NC, negative control; HCC, hepatocellular carcinoma.

expression of miR-138-5p, and the tumor growth rate was notably inhibited. Besides, its tumor size and mass were significantly lower than that of the HCC model in vivo transfected with miR-NC. The differences were statistically significant ($P<0.05$). As shown in **Figure 7**.

Discussion

HCC is the second major cause of cancer death in human beings, which often begins with the deterioration of chronic liver diseases [18]. miRNA-mRNA regulatory network is a regulatory pathway bound up with the pathogenesis of HCC. Exploring its molecular mechanism is helpful for us to formulate therapeutic strategies for HCC [19]. Here, we focused on the promising regulatory function of miR-128-3p-SCAMP3 in HCC metastasis mechanism, hoping to provide new options in treating HCC patients.

More and more researchers have focused their research enthusiasm on miR-128-3p and SCAMP3, and published numerous research results to fill the gaps in the application of the two in HCC. For example, in the research of team of Yu [20], miR-128-3p participates in the regulation mechanism of HCC through targeted inhibition of CYP2C9. Another example is the study of Wei et al. [21], SCAMP3 is proved to be a prognostic indicator of HCC. It is overexpressed in HCC tissues and is bound up with vascular invasion and tumor staging. Downregulation of its expression is also helpful to inhibit HCC cell proliferation and block cell cycle. Here, we found that miR-128-3p in HCC cancer tissues and cell lines was relatively low, while SCAMP3 was contrary, indicating that both the two may participate in the occurrence and progression of HCC. Further exploration of the value of the two in differentiating HCC cancer tissues indicated that the AUC of the two in differentiating HCC cancer tissues from adjacent healthy tissues was as high as 0.850, suggesting that both of them have the potential to assist in the screening of HCC. The results of correlation analysis revealed that there was a negative correlation between the two, indicating that the two may have a negative correlation in the pathological regulation of HCC. Analysis of pathological parameters showed that the two were also closely related to the excessive drinking history, N stage, M stage, and degree of pathological differentiation of HCC patients, suggesting that the two have certain predictability for the pathological conditions of patients. We also analyzed the prognosis of patients. The results exhibited that high level SCAMP3 or low level miR-128-3p were strongly associated with patient poor prognosis, indicating that both may be prognostic indicators of HCC. Cox analysis revealed that miR-128-3p, SCAMP3, N stage and M stage are independent prognostic indicators for HCC patients. In the research of the team of Huang [22], miR-128-3p has been proved to be a prognostic indicator for HCC patients. Its low level is significantly related to short disease-free survival, which is similar to our research results. In addition, the 3-year OS of HCC patients in our study was 26.92%, which was similar to the study of Wang et al. [23].

We have focused on the regulatory mechanisms of the two in HCC cell lines, and mainly studied the role of the two in HCC tumor cell metastasis mechanisms. Studies show that main manifestations of EMT process include low level E-cadherin and high levels of N-cadherin and vimentin, and the main contribution of EMT to tumor metastasis comes from its enhancement of tumor invasiveness [24, 25]. In addition, we also studied the effects of the two on EGFR-MAPK p38 signaling pathway. Studies have shown that there is a differential protein SCAMP3 in the subset of CD81-related proteins in HCC cells, which may mediate the activation of EGFR-MAPK p38 signaling pathway and thus participate in the regulation of hepatitis c virus on host transmembrane protein assembly. We suspect that this is also a potential connection between SCAMP3 and the pathway in HCC metastasis mechanism [26]. Two cell lines with the most significant differential expression were chosen for analysis, namely Huh-7 and MHCC97. According to our study, SCAMP3 up-regulation or miR-128-3p down-regulation is beneficial to malignant metastasis of HCC cells, which is manifested by enhanced proliferation, migration, invasion, EMT, decreased apoptosis level and activation of EGFR-MAPK p38 signaling pathway. The reverse treatment of the two genes showed the opposite effects, suggesting that the development of SCAMP3 inhibitors or miR-128-3p promoters may be a new treatment option for HCC patients, which is beneficial to inhibit tumor

metastasis of HCC. Similar to our results, a study suggests that miR-128-3p is also notably down-regulated in HCC cells, and can inhibit HCC progression by regulating phosphoinositide-3-kinase regulatory subunit p85α (PIK3R1), while its up-regulation can induce cell stagnation in G1 phase and inhibit proliferation and migration [22]. We further verified the relationship between SCAMP3 and miR-128-3p through dual luciferase reporter. The results exhibited that miR-128-3p over-expression only notably reduced the luciferase activity of SCAMP33' UTR-wt, and the transcription and protein level of SCAMP3 were significantly reduced. While its down-regulation showed opposite results, indicating that there was indeed a targeted regulatory relationship between the two. In addition, we further verified through co-transfection experiments. When the two were up-regulated or down-regulated at the same time, the biological function changes of HCC cells had no significant difference in comparison with miR-NC. Compared with up-regulated miR-128-3p, however, the cell proliferation, migration, invasion, EMT were enhanced, EGFR-MAPK p38 pathway were activated, and cell apoptosis level were decreased. Meanwhile, miR-128-3p down-regulation led to opposite results, which again confirms the targeted regulatory relationship between the two. SCAMP3 has been reported to have targeted binding relationships with a variety of miRNAs. For example, it is regulated by miR-584-3p in melanoma cells, mediating the inhibition of metformin on tumor cell growth and movement. Another example is that it is negatively regulated by miR-27a/b-3p and forms a feedback loop during lipogenesis to regulate metabolic processes [27, 28]. Although the targeted relationship between SCAMP3 and miR-128-3p has not been reported, the regulatory role of the latter in HCC has been confirmed by numerous studies [10, 20, 22], indicating that miR-128-3p is highly associated with HCC, and further confirming that the axis of miR-128-3p-SCAMP3 is likely to be a key regulatory axis for HCC metastasis. Moreover, we further investigated the effects of miR-128-3p on HCC model in nude mice, and found that its up-regulation inhibited tumor growth rate, size, other aspects of tumor of nude mice model to different degrees, indicating that its elevated gene expression has a certain therapeutic effect on tumors in vivo, indicating that miR-128-3p expression promoters may have a certain positive effect on the treatment of HCC patients.

Although this paper confirmed that miR-128-3p can regulate the tumor metastasis mechanism of HCC through targeted negative regulation of SCAMP3 expression and mediation of EGFR-MAPK p38 signal transduction pathway, however, there is still room for improvement. First of all, whether miR-128-3P-SCAMP3 has influence on HCC chemical resistance can be analyzed, and its promising regulatory mechanism on HCC chemical sensitivity can be clarified. Secondly, we can supplement whether there are upstream regulatory role molecules of miR-128-3p and explore potential target control mechanisms.

Conclusion

We proposed for the first time the tumor mechanism of miR-128-3p-SCAMP3-EGFR-MAPK p38 axis regulatory network regulating HCC, and clarified the potential regulatory mechanism of miR-128-3p as a tumor suppressor in HCC progression. The miR-128-3p-SCAMP3-EGFR-MAPK p38 axis is crucial in the pathological development of HCC and may provide a new therapeutic direction for HCC patients.

Disclosure of conflict of interest

None.

Address correspondence to: Ying Zhao, Department of Jiamusi College, Heilongjiang University of Chinese Medicine, No. 53 Guanghua Street, Qianjin District, Jiamusi 154000, Heilongjiang Province, China. Tel: +86-0454-6103996; E-mail: zhaoying66688@163.com

References

[1] European Association for the Study of the Liver (EASL); European Association for the Study of Diabetes (EASD); European Association for the Study of Obesity (EASO). EASL Clinical Practice Guidelines: management of hepatocellular carcinoma. J Hepatol 2018; 69: 182-236.

[2] Zamor PJ, deLemos AS and Russo MW. Viral hepatitis and hepatocellular carcinoma: etiology and management. J Gastrointest Oncol 2017; 8: 229-242.

[3] Fernandes ESM, Rodrigues PD, Alvares-da-Silva MR, Scaffaro LA, Farenzena M, Teixeira UF and Waechter FL. Treatment strategies for lo-

cally advanced hepatocellular carcinoma. Transl Gastroenterol Hepatol 2019; 4: 12.
[4] Tang A, Hallouch O, Chernyak V, Kamaya A and Sirlin CB. Epidemiology of hepatocellular carcinoma: target population for surveillance and diagnosis. Abdom Radiol (NY) 2018; 43: 13-25.
[5] Balogh J, Victor D, 3rd, Asham EH, Burroughs SG, Boktour M, Saharia A, Li X, Ghobrial RM and Monsour HP Jr. Hepatocellular carcinoma: a review. J Hepatocell Carcinoma 2016; 3: 41-53.
[6] Vilchez V, Turcios L, Marti F and Gedaly R. Targeting Wnt/beta-catenin pathway in hepatocellular carcinoma treatment. World J Gastroenterol 2016; 22: 823-832.
[7] Bruix J, Reig M and Sherman M. Evidence-based diagnosis, staging, and treatment of patients with hepatocellular carcinoma. Gastroenterology 2016; 150: 835-853.
[8] Zhang CJ and Du HJ. Screening key miRNAs for human hepatocellular carcinoma based on miRNA-mRNA functional synergistic network. Neoplasma 2017; 64: 816-823.
[9] Ma R, Wang C, Wang J, Wang D and Xu J. miRNA-mRNA interaction network in non-small cell lung cancer. Interdiscip Sci 2016; 8: 209-219.
[10] Guo XL, Wang HB, Yong JK, Zhong J and Li QH. MiR-128-3p overexpression sensitizes hepatocellular carcinoma cells to sorafenib induced apoptosis through regulating DJ-1. Eur Rev Med Pharmacol Sci 2018; 22: 6667-6677.
[11] Zhao L, Li R, Xu S, Li Y, Zhao P, Dong W, Liu Z, Zhao Q and Tan B. Tumor suppressor miR-128-3p inhibits metastasis and epithelial-mesenchymal transition by targeting ZEB1 in esophageal squamous-cell cancer. Acta Biochim Biophys Sin (Shanghai) 2018; 50: 171-180.
[12] Thomas P, Wohlford D and Aoh QL. SCAMP 3 is a novel regulator of endosomal morphology and composition. Biochem Biophys Res Commun 2016; 478: 1028-1034.
[13] Skawran B, Steinemann D, Weigmann A, Flemming P, Becker T, Flik J, Kreipe H, Schlegelberger B and Wilkens L. Gene expression profiling in hepatocellular carcinoma: upregulation of genes in amplified chromosome regions. Mod Pathol 2008; 21: 505-516.
[14] Dehghani M, Brobey RK, Wang Y, Souza G, Amato RJ and Rosenblatt KP. Klotho inhibits EGF-induced cell migration in Caki-1 cells through inactivation of EGFR and p38 MAPK signaling pathways. Oncotarget 2018; 9: 26737-26750.
[15] Berasain C and Avila MA. The EGFR signalling system in the liver: from hepatoprotection to hepatocarcinogenesis. J Gastroenterol 2014; 49: 9-23.
[16] Song MK, Kim YJ, Song M, Choi HS, Park YK and Ryu JC. Polycyclic aromatic hydrocarbons induce migration in human hepatocellular carcinoma cells (HepG2) through reactive oxygen species-mediated p38 MAPK signal transduction. Cancer Sci 2011; 102: 1636-1644.
[17] Ayuso C, Rimola J, Vilana R, Burrel M, Darnell A, Garcia-Criado A, Bianchi L, Belmonte E, Caparroz C, Barrufet M, Bruix J and Bru C. Diagnosis and staging of hepatocellular carcinoma (HCC): current guidelines. Eur J Radiol 2018; 101: 72-81.
[18] Wei Y, Ye W and Zhao W. Serum iron levels decreased in patients with HBV-related hepatocellular carcinoma, as a risk factor for the prognosis of HBV-related HCC. Front Physiol 2018; 9: 66.
[19] Lou W, Liu J, Ding B, Chen D, Xu L, Ding J, Jiang D, Zhou L, Zheng S and Fan W. Identification of potential miRNA-mRNA regulatory network contributing to pathogenesis of HBV-related HCC. J Transl Med 2019; 17: 7.
[20] Yu D, Green B, Marrone A, Guo Y, Kadlubar S, Lin D, Fuscoe J, Pogribny I and Ning B. Suppression of CYP2C9 by microRNA hsa-miR-128-3p in human liver cells and association with hepatocellular carcinoma. Sci Rep 2015; 5: 8534.
[21] Zhang X, Sheng J, Zhang Y, Tian Y, Zhu J, Luo N, Xiao C and Li R. Overexpression of SCAMP3 is an indicator of poor prognosis in hepatocellular carcinoma. Oncotarget 2017; 8: 109247-109257.
[22] Huang CY, Huang XP, Zhu JY, Chen ZG, Li XJ, Zhang XH, Huang S, He JB, Lian F, Zhao YN and Wu GB. miR-128-3p suppresses hepatocellular carcinoma proliferation by regulating PIK3R1 and is correlated with the prognosis of HCC patients. Oncol Rep 2015; 33: 2889-2898.
[23] Wang CY and Li S. Clinical characteristics and prognosis of 2887 patients with hepatocellular carcinoma: a single center 14 years experience from China. Medicine (Baltimore) 2019; 98: e14070.
[24] Li Y, Zhang T, Qin S, Wang R, Li Y, Zhou Z, Chen Y, Wu Q and Su F. Effects of UPF1 expression on EMT process by targeting Ecadherin, Ncadherin, Vimentin and Twist in a hepatocellular carcinoma cell line. Mol Med Rep 2019; 19: 2137-2143.
[25] Dituri F, Mancarella S, Cigliano A, Chieti A and Giannelli G. TGF-beta as multifaceted orchestrator in HCC progression: signaling, EMT, immune microenvironment, and novel therapeutic perspectives. Semin Liver Dis 2019; 39: 53-69.
[26] Zona L, Lupberger J, Sidahmed-Adrar N, Thumann C, Harris HJ, Barnes A, Florentin J, Tawar

RG, Xiao F, Turek M, Durand SC, Duong FH, Heim MH, Cosset FL, Hirsch I, Samuel D, Brino L, Zeisel MB, Le Naour F, McKeating JA and Baumert TF. HRas signal transduction promotes hepatitis C virus cell entry by triggering assembly of the host tetraspanin receptor complex. Cell Host Microbe 2013; 13: 302-313.

[27] Tseng HW, Li SC and Tsai KW. Metformin treatment suppresses melanoma cell growth and motility through modulation of microRNA expression. Cancers (Basel) 2019; 11: 209.

[28] Kulyte A, Kwok KHM, de Hoon M, Carninci P, Hayashizaki Y, Arner P and Arner E. MicroRNA-27a/b-3p and PPARG regulate SCAMP3 through a feed-forward loop during adipogenesis. Sci Rep 2019; 9: 13891.