Clinics and Research in Hepatology and Gastroenterology (2019) xxx, xxx–xxx

Available online at ScienceDirect www.sciencedirect.com

Elsevier Masson France EM|consulte www.em-consulte.com/en

ORIGINAL ARTICLE

Overexpression of CSN6 promotes the epithelial-mesenchymal transition and predicts poor prognosis in hepatocellular carcinoma

Ming Xu[a,1], **Lonbo Zhen**[c,1], **Liumei Lin**[b,1], **Ke Wu**[b], **Yifan Wang**[b], **Xiujun Cai**[b,*]

[a] *Department of Hepatobiliary Surgery, the First Affiliated Hospital of Bengbu Medical College, 233000 Bengbu, China.*
[b] *Department of General Surgery, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, 3 East Qingchun Road, 310016 Hangzhou, Zhejiang Province, China.*
[c] *Department of general surgery, the affiliated hospital of Qingdao University, Qingdao, China*

KEYWORDS
CSN6;
Hepatocellular carcinoma;
Prognosis;
Epithelial-mesenchymal transition

Summary
Background and aim: CSN6, as a critical subunit of the constitutive photomorphogenesis 9 (COP9) signalosome (CSN), has been previously reported to be increased in various cancers; however, its effect in hepatocellular carcinoma (HCC) remains unknown, which is the aim of present study, in terms of its explore the expression and role of CSN6 in HCC.
Methods: QRT-PCR, Western blot and immunohistochemistry (IHC) were used to examine the expression of CSN6. Kaplan-Meier survival analysis and univariate and multivariate Cox analyses were used to investigate the clinical and prognostic significance of CSN6 expression in HCC patients. Furthermore, the biological function of CSN6 on HCC cell proliferation and migration was investigated through CCK-8, transwell migration and invasion assays. Besides, the associations between CSN6 and epithelial-mesenchymal transition (EMT) were determined.
Results: CSN6 was increased in HCC tissues, and its overexpression was found to be associated with a poor prognoses for HCC patients. Overexpression of CSN6 promoted processes of HCC cell proliferation, migration, and invasion, while these processes were inhibited when CSN6 was silenced. Additionally, CSN6 was found to promote EMT by inhibiting E-cadherin, which were significantly mitigated via upregulation of Snail as a result of MEK/ERK pathway activation.

* Corresponding author.
E-mail address: srrsh_cxj@zju.edu.cn (X. Cai).
[1] These authors contributed equally to this article.

https://doi.org/10.1016/j.clinre.2019.07.012

Please cite this article in press as: Xu M, et al. Overexpression of CSN6 promotes the epithelial-mesenchymal transition and predicts poor prognosis in hepatocellular carcinoma. Clin Res Hepatol Gastroenterol (2019), https://doi.org/10.1016/j.clinre.2019.07.012

Conclusions: CSN6 up-regulation may play a contributory role in HCC metastasis and poor prognosis via activation of EMT, and may serve as an independent predictor for HCC prognosis.

Introduction

Hepatocellular carcinoma (HCC) is currently the sixth most commonly diagnosed cancer, and is the third most frequent cause of cancer related deaths [1,2]. Although perioperative management and surgical techniques have been improved, the prognosis of HCC patients remains unfavorable due to tumor metastasis and recurrence [3]. Therefore, it is of great significance to identify novel genes related to HCC invasion and metastasis.

CSN6 is one subunit of the COP9 signalosome (CSN), which is a multiprotein complex that consists of 8 subunits involved in protein degradation [4]. Recent studies have demonstrated that CSN6 is involved in tumorigenesis. For example, CSN6 is overexpressed in colorectal cancer and promotes proliferation and invasion of colorectal cancer cell by enhancing β-catenin signaling [5]. In glioblastoma, CSN6 decreases CHIP expression and increases EGFR expression, thereby driving the tumor progression [6]. Nevertheless, the role of CSN6 in HCC has not been studied.

In this study, it is found that CSN6 overexpression is closely associated with poor outcomes in HCC patients, and that CSN6 could enhance HCC cell invasion and metastasis both in vitro and in vivo. In addition, CSN6 was found to promote HCC carcinogenesis by regulating MEK/ERK pathway.

Materials and methods

Patients and tissue specimens

This study was approved by the research ethics committee of Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, China. Informed consent was obtained from all the patients. Paraffin-embedded pathological samples were obtained from 106 HCC patients undergoing initial hepatectomy from January 2006 to December 2010 in Sir Run Run Shaw Hospital (SRRSH), School of Medicine, Zhejiang University, China. Additionally, another cohort of 15 paired frozen fresh tumor tissues and non-tumor tissues were collected from September 2016 to December 2016 for analyzing the mRNA and protein level of CSN6. Clinicopathological information of the cohort was summarized in Table 1.

Table 1 Association between CSN6 expression and clinicopathology feature in patients with HCC.

Variable	CSN6 expression		*P* value
	Low (%)	High (%)	
Age			
< 60 years	28 (44.4)	21 (48.8)	0.656
≥ 60 years	35 (55.6)	22 (51.2)	
Gender			
Male	52 (82.5)	36 (83.7)	0.874
Female	11 (17.5)	7 (16.3)	
HBsAg			
Negative	12 (19.0)	4 (9.3)	0.169
Positive	51 (81.0)	39 (90.7)	
AFP (ng/mL)			
< 400	47 (74.6)	19 (44.2)	**0.002**
≥ 400	16 (25.4)	24 (55.8)	
Liver cirrhosis			
No	32 (50.8)	17 (39.5)	0.254
Yes	31 (49.2)	26 (60.5)	
Tumor size			
< 5 cm	43 (68.3)	23 (53.5)	0.124
≥5 cm	20 (31.7)	20 (46.5)	
Tumor number			
Single	56 (88.9)	38 (88.4)	1.000
Multiple	7 (11.1)	5 (11.6)	
Tumor differentiation			
Well/moderately	35 (55.6)	18 (41.9)	0.166
Poorly	28 (44.4)	25 (58.1)	
Tumor thrombi			
No	58 (92.1)	34 (79.1)	0.052
Yes	5 (7.9)	9 (20.9)	
TNM stage			
I+II	61 (96.8)	35 (81.4)	**0.014**
III+IV	2 (3.2)	8 (18.6)	
BCLC stage			
A	62 (98.4)	34 (79.1)	**0.001**
B	1 (1.6)	9 (20.9)	

HCC: hepatocellular carcinoma; HBsAg: hepatitis B surface antigen; AFP: α-fetoprotein; TNM: tumor node metastasis; BCLC: Barcelona clinic liver cancer.

Cell lines and culture

In this study, two human HCC cell lines (HepG2 and LM3) were obtained from Chinese Academy of Sciences (Shanghai, China). Cells were routinely cultured in minimum essential medium (MEM) or Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL, Rockville, MD) with 10% fetal bovine serum supplemented (Gibco, USA) at 37 °C in a humidified 5% CO2 atmosphere. For drug inhibition experiments, cells were cultured in medium with 20 uM PD98059 (Selleck, USA) and harvested 24 hours later for western blot analysis.

Stable cell lines and short interference RNA

Stable cell lines expressing CSN6 or shCSN6 were generated by transfection of pCMV-CSN6 or GV493-shCSN6 which were obtained from Genechem (Shanghai, China) into LM3 and HepG2 cells and cultured for 20 days with 400 μg/mL G418 or 2 μg/mL puromycin 72 h after infection. Positive clones were then selected, and transfection was verified

Please cite this article in press as: Xu M, et al. Overexpression of CSN6 promotes the epithelial-mesenchymal transition and predicts poor prognosis in hepatocellular carcinoma. Clin Res Hepatol Gastroenterol (2019), https://doi.org/10.1016/j.clinre.2019.07.012

by quantativereal-time PCR (qPCR) and western blot. Plasmid and negative controls were transient transfected into HCC cells at a working concentration of 50 nM using Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's protocol. Gene silencing effect was confirmed by qPCR and western blot at 48—72 h post transfection.

Quantitative real-time PCR

Total RNAs from primary tumor and adjacent non-tumor tissue samples were extracted using TRIzol reagent (Ambion, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 μg of RNA from each sample using iScript™ cDNA Synthesis Kit (BIO-RAD, USA). Quantitative real-time PCR was performed using a 7500 Real-time PCR system (Applied Biosystems, Inc., USA). Primer sequences used for CSN6 detection were as follows: forward 5′-TCATCGAGAGCCCCCTCTTT-3′ and reverse 5′- CCAATGCGTTCCGCTTCCT-3′. The $2-\Delta Ct$ method was used to quantify the relative CSN6 expression levels and normalized using the β-actin (forward 5′-CATGTACGTTGCTATCCAGGC-3′, reverse5′-CTCCTTAATGTCACGCACGAT-3′) expression. Each sample was tested at least three independent replicates.

Western blot analysis

Total proteins from frozen HCC tissues or cells were extracted with RIPA lysis buffer containing protease inhibitors (Beyotime, China) and quantified using the Piece BCA Protein Assay Kit (Thermo Scientific, USA). About 30 ug of total protein was separated by 10% SDS-PAGE. Samples were transferred to PVDF membranes (Millipore, USA) and incubated overnight at 4 °C with the primary antibodies. The antigen—antibody complex on the membrane was detected with Clarity™ Western ECL Substrate (BIO-RAD, USA). Sources of antibodies and concentrations used were as follows: CSN6 (1:1000, Santa Cruz), E-cadherin (1:1000, CST), Snail (1:1000, CST), p-MEK1/2 (1:1000, CST), p-ERK1/2 (1:1000, CST), p-AKT (1:1000, Beyotime), AKT (1:1000, Beyotime) and β-actin (1:1000; Sigma).

Immunohistochemistry analysis

Paraffin-embedded specimens were cut to 4 μm, deparaffinized with xylene, and dehydrated in a graded series of alcohols. Antigen retrieval was performed using 0.01M citrate buffer for a 3-min boil. Hydrogen peroxide was applied to block peroxidase, and then the slides were incubated with normal goat serum. The primary antibody for CSN6 (1:300, Santa Cruz) and E-cadherin (1:150, CST) was incubated overnight at 4 °C. Normal goat serum was used as a negative control. Immunohistochemistry (IHC) staining for tissues was detected by the Mo&Rb GTVision III Detection System (Gene Tech, China). DAB visualization was then performed, and the slides were countersigned with hematoxylin. CSN6 protein expression in cancer cells was scored according to the intensity of cytoplasmic staining by a 4-point system: 0, negative; 1, weakly positive; 2, positive; and 3, strongly positive. To examine the association of CSN6 expression level with clinicopathological features, patients were divided into two groups: low CSN6 expression (0 and 1) or high CSN6 expression (2 and 3).

Immunofluorescence analysis

Cells were seeded on cover slips with 5×10^5 cells into 6-well plates. After 48 h, cells were washed with cold PBS and then fixed with 4% formaldehyde in PBS (pH 7.4) for 15 min at room temperature and then probed with primary antibodies against E-cadherin (1:200, CST). The cover slips were then incubated with fluorescein isothiocyanate (FITC)-conjugated goat against rabbit IgG (1:500, Thermo Fisher Scientific) and counterstaining with 4′, 6-diamidino-2-phenylindole (DAPI, Beyotime).

Cell proliferation assay

Cell proliferation was assessed using cell counting kit 8 (CCK-8, Bimake). Cells were plated into 96-well plates at 1000 cells per well, and cell viability (absorbance 450 nm) was measured every 24 h for 6 days. At each time point, 10 ul of CCK-8 substrate solution for each 100 uL culture medium per well was added and the mixture was incubated at 37 °C for 2 h. The absorbance was measured at 450 nm using Varioskan™ Flash multimode reader (Thermo Fisher Scientific).

Migration and invasion assays

For the migration assay, about 1×10^5 cells suspended in serum-free media were seeded in the upper chamber. For the invasion assay, the membrane was coated with Matrigel (BD Biosciences, USA) to form a matrix barrier, and then 2×10^5 cells were placed in the upper chamber. In each lower chamber, 600 mL of DMEM medium with 10% fetal bovine serum were added. After 48 h of incubation at 37 °C, the cells that had migrated through the pore were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Then, the cells were counted in five randomly chosen fields (100×) under a microscope.

In vivo metastasis assays

As described previously [7], nude mice (4 weeks old, male) were anesthetized with pentobarbital and a transverse incision was made in the left flank through the skin and peritoneum. The spleen was carefully exposed and 5×10^6 viable HepG2 cells carrying control shRNA or shCSN6 were injected under the spleen capsule via a 27-gauge needle. Six weeks after the injection, the mice were sacrificed under anesthesia to research liver metastases.

Statistical analysis

Statistical analyses and graphical representations were performed using SPSS PASW Statistics 18.0 software (SPSS, Inc., Chicago, IL) and GraphPad Prism 5. OS and DFS curves were obtained by the Kaplan—Meier method with log-rank test. Univariate analysis and multivariate analysis were analyzed

Please cite this article in press as: Xu M, et al. Overexpression of CSN6 promotes the epithelial-mesenchymal transition and predicts poor prognosis in hepatocellular carcinoma. Clin Res Hepatol Gastroenterol (2019), https://doi.org/10.1016/j.clinre.2019.07.012

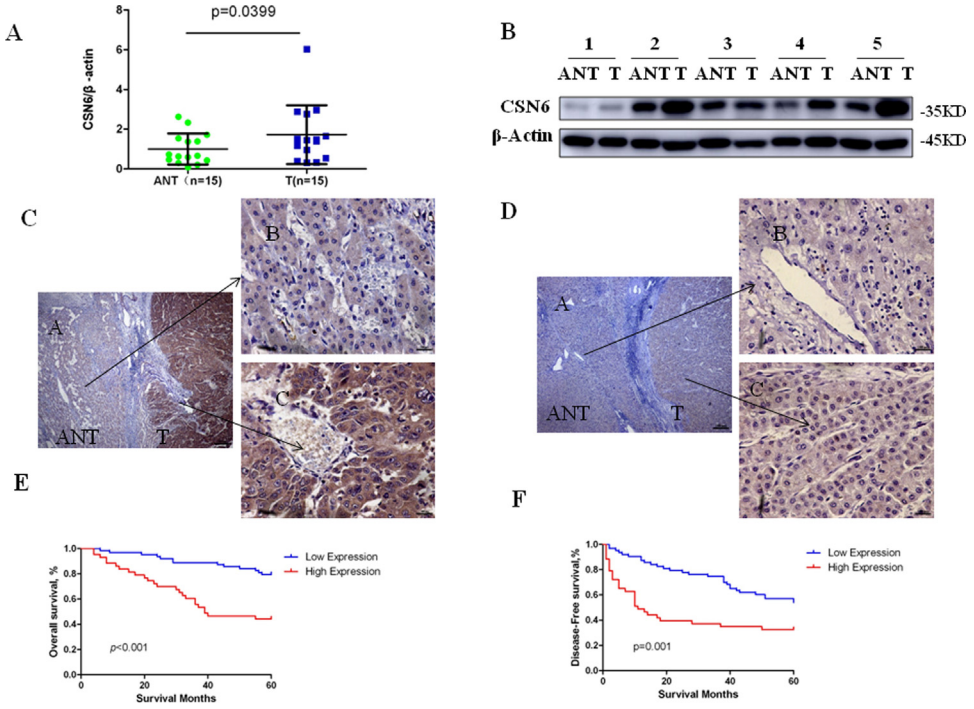

Figure 1 Overexpression of CSN6 indicated poor prognosis of HCC patients. A. qRT-PCR analysis of CSN6 expression in 15 pairs of HCC tissues and adjacent non-tumor tissues (ANT). B. CSN6 expression is upregulated in HCC tissues analyzed by western blot. C and D. Representative images of CSN6 staining in HCC tissues and paired non-tumor tissues (magnification ×40, scale bar: 100 μm). The region was further enlarged (magnification ×400, scale bar: 100 μm). E. Kaplan-Meier analysis of the correlation between CSN6 expression and the overall survival of HCC patients. F. Kaplan-Meier analysis of the correlation between CSN6 expression and the disease free survival of HCC patients.

with Cox proportional hazard regression model to estimate their independent prognostic value on cancer. A two tailed P value of < 0.05 was considered as statistical significance. The analysis of qPCR was performed by paired Student's t test. A two-sided P value < 0.05 was considered statistically significant.

Results

CSN6 is overexpressed and associated with poor prognosis in HCC

To identify the clinical significance of CSN6 in HCC, the expression levels of CSN6 in 15 paired HCC tumor and adjacent normal tissues were investigated. The results show that CSN6 expression was significantly increased in terms of mRNA ($P = 0.0399$, Fig. 1A) and protein (Fig. 1B) levels (5 paired specimens) in HCC tissues compared with adjacent non-cancerous tissues. The clinical significance of CSN6 was further investigated by immunohistochemistry using 106 HCC specimens. Representative immunohistochemical staining results are shown in Fig. 1C and 1D. Correlations between CSN6 protein expression and the clinicopathological parameters of HCC patients are summarized in Table 1. In details, in HCC, CSN6 protein expression was significantly correlated with AFP ($P = 0.007$), tumor size ($P = 0.014$), TNM stage ($P = 0.002$) and BCLC stage ($P = 0.018$).

To determine the association between CSN6 expression and prognosis of HCC patients, all patients were followed-up for overall survival and disease free survival after surgery. As a result, Kaplan-Meier survival scores indicate that patients in the CSN6 high expression group have a poorer 5-year overall survival (OS) ($P = 0.002$, Fig. 1E) and a shorter disease-free survival (DFS) time ($P = 0.004$, Fig. 1F) than those in the CSN6 low expression group.

To further study whether CSN6 expression was an independent risk factor for HCC patients, univariate and multivariate analyses were performed. The univariate results indicate that CSN6 overexpression is significantly correlated with OS and DFS in HCC patients (Table 2). Multivariate analysis revealed that CSN6 was an independent risk factor for HCC (OS: hazard ratio (HR) = 3.30, 95% confidence interval (95%CI): 1.63—6.71, $P < 0.001$; DFS: HR = 1.91, 95%CI: 1.11—3.30, $P = 0.020$) (Table 2). These findings suggest that CSN6 was upregulated in HCC and might be a new prognostic biomarker for HCC.

CSN6 is required for proliferation of HCC cells

To check whether CSN6 promoted HCC cells proliferation, a CSN6 loss function study in HepG2 cells was performed, which showed a relatively higher level of CSN6. The knockdown efficiency of CSN6 was confirmed by qPCR and Western blot (Fig. 2A), and the result of CCK-8 assay shows that CSN6 silencing significantly reduced cell viability as reflected by the growth curve (Fig. 2C). On the contrary, overexpression

Please cite this article in press as: Xu M, et al. Overexpression of CSN6 promotes the epithelial-mesenchymal transition and predicts poor prognosis in hepatocellular carcinoma. Clin Res Hepatol Gastroenterol (2019), https://doi.org/10.1016/j.clinre.2019.07.012

Table 2 Univariate and multivariate analyses of prognostic factors for overall survival and disease-free survival of HCC patients in the study cohort.

Variable	OS				DFS			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95%CI)	*P*	HR (95%CI)	*P*	HR (95%CI)	*P*	HR (95%CI)	*P*
Age								
≥60	0.91 (0.48–1.74)	0.779			0.70 (0.42–1.18)	0.179		
Gender								
Male	0.83 (0.36–1.89)	0.654			1.25 (0.59–2.64)	0.556		
HBsAg								
Yes	0.76 (0.33–1.73)	0.512			1.18 (0.56–2.49)	0.667		
AFP								
≥400	1.48 (0.77–2.83)	0.239			1.13 (0.66–1.93)	0.660		
Liver cirrhosis								
Yes	1.20 (0.62–2.29)	0.587			1.44 (0.85–2.43)	0.176		
Tumor size								
≥5 cm	2.46 (1.29–4.70)	**0.006**	2.14 (1.06–4.32)	**0.035**	2.25 (1.34–3.78)	**0.002**	2.08 (1.18–3.66)	**0.011**
Tumor number								
Multiple	1.04 (0.37–2.93)	0.943			1.70 (0.81–3.59)	0.164		
Tumor differentiation								
Poorly	1.24 (0.65–2.36)	0.52			0.88 (0.53–1.48)	0.629		
Tumor thrombi								
Yes	2.31 (1.05–5.05)	**0.037**	1.35 (0.54–3.41)	0.52	3.76 (1.97–7.17)	**<0.001**	2.99 (1.47–6.08)	**0.003**
TNM stage								
III+IV	3.48 (1.52–7.99)	**0.003**	1.26 (0.44–3.59)	0.663	3.18 (1.50–6.74)	**0.003**	1.17 (0.48–2.83)	0.730
Group								
High	3.71 (1.88–7.30)	**<0.001**	3.30 (1.63–6.71)	**<0.001**	2.29 (1.36–3.84)	**0.002**	1.91 (1.11–3.30)	**0.020**

HCC: hepatocellular carcinoma; OS: overall survival; DFS: *disease-free survival*; HBsAg: hepatitis B surface antigen; AFP: α-fetoprotein; TNM: tumor node metastasis; HR: hazard risk ratio; CI: confidence interval.

of CSN6 in HCC-LM3 cells promoted the cell proliferation (Fig. 2B and D), suggesting that CSN6 is important for proliferation of HCC cells.

CSN6 promotes invasion and metastasis of HCC cells in vitro and in vivo

To investigate whether CSN6 is involved in motility of HCC cells, transwell migration and matrigel invasion assays were performed in CSN6 knockdown or overexpression HCC cells. The results indicate that CSN6 silencing significantly inhibited the migration and invasion ability of HepG2 cells (Fig. 2E), whereas overexpression of CSN6 promoted the migration and invasion ability of LM3 cells (Fig. 2F). To further evaluate the impact of CSN6 upon cell invasion and metastasis in vivo, shCSN6- and control shRNA-infected HepG2 cells injected into the spleen of nude mice. After 6 weeks, liver metastases were significantly reduced in the mice injected with HepG2-shCSN6 cells compared with the mice injected with HepG2-scramble cells (Fig. S1). Collectively, our data suggested that CSN6 is capable of promoting the invasion and metastasis potential of HCC cells both in vitro and in vivo.

CSN6 inversely regulates E-cadherin in HCC cells in vitro and in vivo

Since EMT occurs during cancer metastasis and is a well reported mechanism for tumor cell migration and invasion, CSN6 is hypothesized to regulate EMT of HCC cells. As shown in Fig. 3A, CSN6 silencing promoted the level of epithelial marker (E-cadherin), and decreased the expression levels of mesenchymal markers (N-cadherin). Meanwhile, overexpression of CSN6 promoted EMT. Analysis of a clinical patient database (TCGA), by GEPIA (http://http://gepia.cancer-pku.cn/), demonstrated a weak negative correlation between CSN6 and E-cadherin (Fig. 3B). Immunofluorescence analysis further showed

Please cite this article in press as: Xu M, et al. Overexpression of CSN6 promotes the epithelial-mesenchymal transition and predicts poor prognosis in hepatocellular carcinoma. Clin Res Hepatol Gastroenterol (2019), https://doi.org/10.1016/j.clinre.2019.07.012

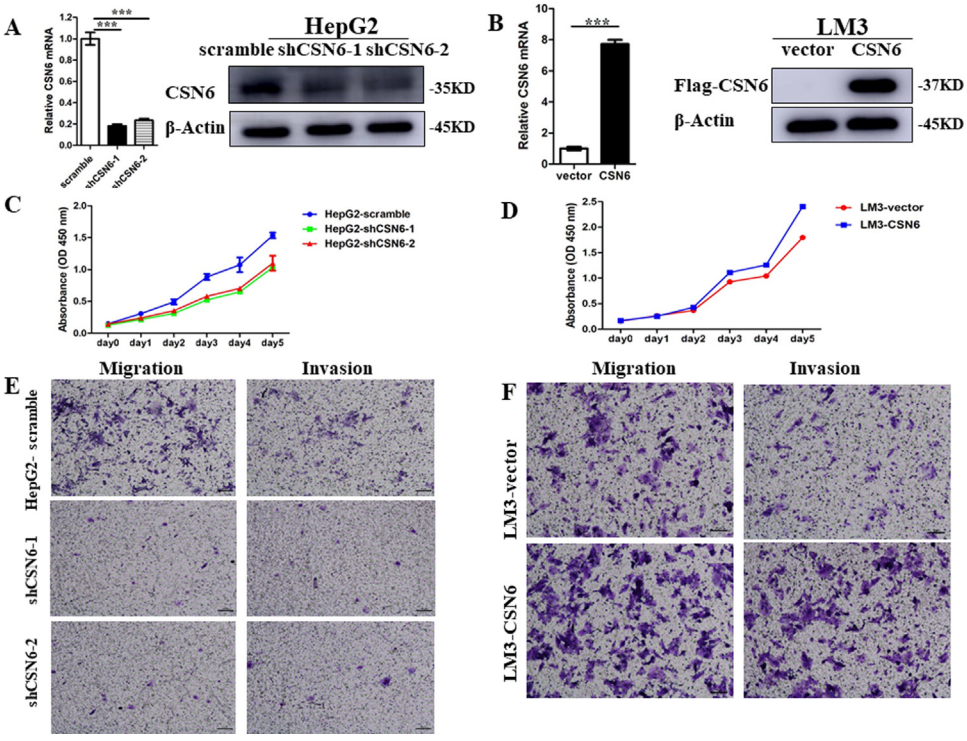

Figure 2 Effect of CSN6 expression on proliferation, invasion and metastasis of HCC cells. A. qRT-PCR (left) and western blot (right) analysis of CSN6 expression in HepG2 cells transfected with indicated shRNA. β-Actin was detected as an internal control. B. qRT-PCR (left) and westernblot (right) analysis of CSN6 expression in LM3 cells transfected with empty vector or CSN6. β-Actin was detected as an internalcontrol. C. silencing endogenous CSN6 inhibited cell growth by CCK8 assay. D. CSN6 overexpression promoted cell growth. E and F. effects of CSN6 depletion or CSN6 overexpression on the migration and invasion of HCC cells were assessed by transwell migration assay and matrigel invasion assay, respectively. Representative results are shown in the upper panel. (Magnification: 100×, scale bar: 100 um).

that overexpression of CSN6 reduced expression of the E-cadherin, whereas knockdown CSN6 increased E-cadherin expression (Fig. 3C). Then, the expression levels of CSN6 and E-cadherin proteins in the same HCC specimens were assessed by immunohistochemistry (Fig. 3D), and the results showed that specimens with low expression of CSN 6 had high E-cadherin expression. These data signifies that the negative regulation of CSN6 on E-cadherin occurs both in vitro and in vivo.

CSN6 regulated E-cadherin expression through MEK/ERK pathway

To determine the underlying mechanism by which CSN6 regulates cell migration, invasion and EMT in HCC, the phosphorylation status of AKT and ERK were analyzed. It is found that CSN6 affected activation of ERK but not AKT signaling. The activation status of ERK and the upstream gene MEK1/2 in cells with stable CSN6 knockdown and overexpression were analyzed and t data showed that CSN6 increased just the levels of phosphorylated MEK1/2 (p-MEK1/2) and ERK1/2 (p-ERK1/2), rather than the total MEK and ERK (Fig. 4A). Snail which was reported as a downstream effector of MEK/ERK pathway could repress E-cadherin transcription by binding to E-box of E-cadherin promoter [8] was also influenced by CSN6.

To further confirm the function of MEK/ERK pathway in CSN6-induced EMT phenotype, the MEK activity was inhibited by MEK inhibitor of PD98059. The phospho-ERK1/2 was downregulated by PD98059, which inhibited the expression of Snail, thereby increasing E-cadherin protein expression so as to rescue the E-cadherin expression suppressed by CSN6 overexpression in LM3 cells (Fig. 4B). Meanwhile, transwell assays showed that the PD98059 reduced the migration of LM3-CSN6 (Fig. 4C). In contrast, the converse E-cadherin expression pattern was observed when HGF was stimulated in CSN6-knockdown cells (Fig. S2). Taken together, these data suggests that CSN6 regulated EMT progression, at least in part, via the upregulation of MEK/ERK signaling pathways.

Discussion

As HCC is one of the most common and lethal malignancies in the world [9], and the dismal outcome of HCC patients are contributed by invasion and metastasis, the identification of new predictive biomarkers of HCC invasion and prognosis is of great significance [10]. Recent researches have revealed that CSN6, a critical subunit of the constitutive photomorphogenesis 9 (COP9) signalosome which is an evolutionarily conserved multi-protein complex found in plants and animals, is upregulated in numerous human cancers [5,6,11—15]. However, the expression status and the role of CSN6 in HCC are far away from being well understood.

Please cite this article in press as: Xu M, et al. Overexpression of CSN6 promotes the epithelial-mesenchymal transition and predicts poor prognosis in hepatocellular carcinoma. Clin Res Hepatol Gastroenterol (2019), https://doi.org/10.1016/j.clinre.2019.07.012

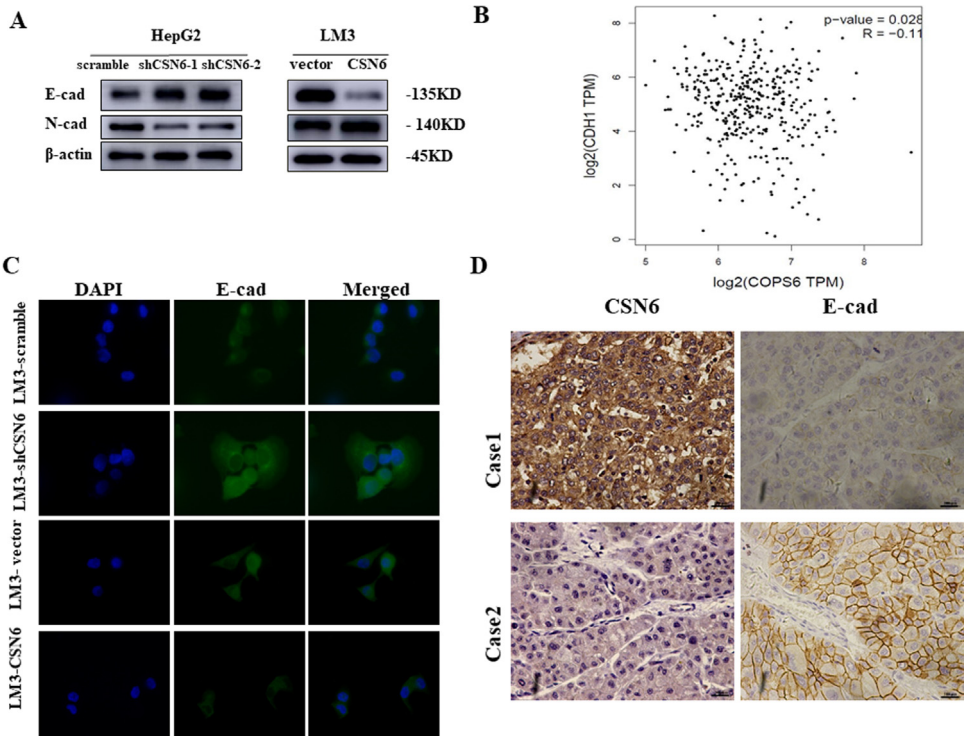

Figure 3 CSN6 inversely regulates E-cadherin in HCC cells in vitro and in vivo. A. CSN6 regulated the E-cadherin (E-cad) and N-cadherin (N-cad) expression in HepG2 and LM3 cells as confirmed by western blot. B. Correlation analysis of CSN6 level and CDH1 mRNA in TCGA dataset of HCC patients ($r = -0.11$, $P = 0.028$). C. The expression of E-Cadherin was examined by immunofluorescence. D. Representative CSN6 and E-cadherin (E-cad) immunohistochemical images of HCC specimens from two patients (Magnification: 400×, scale bar: 100 um).

In the very study, the clinical significance of CSN6 expression in HCC samples was investigated, and found that CSN6 expression was also upregulated in HCC tissues compared with ANTs, and that high-level CSN6 expression was an independent prognostic marker for HCC patients. Further clinical analysis suggested that HCC patients with higher CSN6 expression had shorter 5-year OS and DFS. Gain-of-function and loss-of-function experiments revealed that silencing of CSN6 expression inhibited the migration, invasion, and proliferation of HCC cells, while upregulated expression of CSN6 yielded the opposite results. All these evidences indicate that CSN6 might be a novel marker associated with a poor prognosis and a high risk of tumor metastasis in HCC.

As results of the study, it is found that there is an inverse correlation between CSN6 and E-cadherin in HCC, and that CSN6 reduced the expression of epithelial-mesenchymal transition marker of E-cadherin while upregulated N-cadherin. Epithelial-to-mesenchymal transition (EMT) activation is pivotal during cancer invasion and metastasis. Downregulation of E-cadherin is considered as a hallmark of Epithelial-mesenchymal transition (EMT)[16,17] leading to loss of epithelial differentiation and acquisition of mesenchymal-like cellular competence of tumor cells, which are the fundamental features of EMT [18]. To obtain a better understanding of the function of CSN6 on HCC cells, the effects of CSN6 on aberrant signaling of the MEK/ERK pathway were studied by Western blot. Existing studies have shown that activation of the MEK/ERK signaling pathway contributes to proliferation, invasion, and EMT [19—21], and may play critical roles in HCC [22—24]. Therefore whether CSN6 could regulate MEK/ERK pathways is determined, and observed a strong activation of MEK/ERK pathway and upregulation of Snail which reported as a critical downstream effector of MEK/ERK pathway [8,25] in CSN6-overexpressed HCC cells. However, PD98059, an MEK inhibitor, significantly inhibited the CSN6-induced downregulation of E-cadherin, indicating that CSN6-induced EMT was regulated by Snail as a result of MEK/ERK pathway activation.

In conclusion, this study revealed at the first ever that CSN6 is a prognostic indicator in HCC patients, and, CSN6 contributes to metastasis and EMT in HCC possibly by regulation of MEK/ERK pathway.

Disclosure of interest

The authors declare that they have no competing interest.

Acknowledgements

This work was supported by Major Scientific and Technological Project of Zhejiang Province, China, No. 2015C03026.

Please cite this article in press as: Xu M, et al. Overexpression of CSN6 promotes the epithelial-mesenchymal transition and predicts poor prognosis in hepatocellular carcinoma. Clin Res Hepatol Gastroenterol (2019), https://doi.org/10.1016/j.clinre.2019.07.012

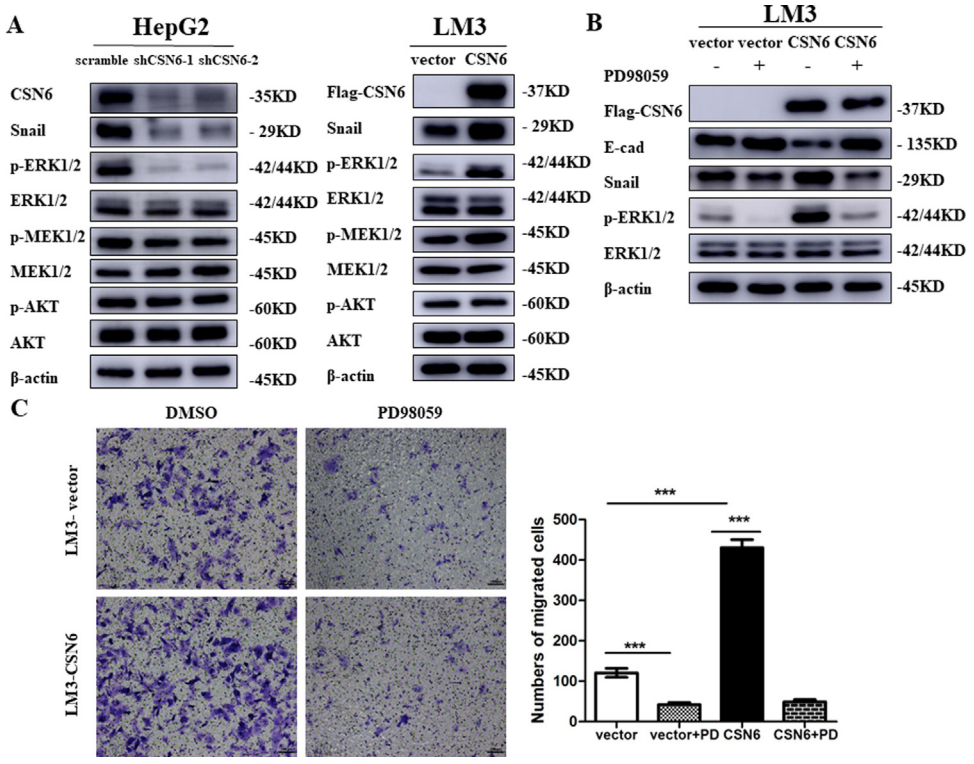

Figure 4 The effect of Snail and MEK/ERK pathway of CSN6 induced EMT. A. Protein expression levels of CSN6, Snail, AKT, p-AKT, MEK, p-MEK, ERK and p-ERK were analyzed by western blot in HepG2 cells transfected with shCSN6 and CSN6-overexpressed LM3cells B. Enhanced activity of the MEK/ERK signaling pathway significantly inhibited by PD98059, the inhibitor of MEK, and reversed the CSN6 induced EMT by down-regulating Snail expression. C. PD98059 treatment significantly suppressed the migration ability of LM3-CSN6 cells. The data were analysed by an unpaired two-tailed Student's t-test. ***indicates $P < 0.01$.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.clinre.2019.07.012.

References

[1] Zhu RX, Seto WK, Lai CL, Yuen MF. Epidemiology of Hepatocellular Carcinoma in the Asia-Pacific Region. Gut Liver 2016;10:332—9.
[2] Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990—2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet 2015;385:117—71.
[3] Villanueva A, Hoshida Y, Battiston C, Tovar V, Sia D, Alsinet C, et al. Combining clinical, pathology, and gene expression data to predict recurrence of hepatocellular carcinoma. Gastroenterology 2011;140 [1501—1512 e1502].
[4] Wei N, Deng XW. The COP9 signalosome. Annu Rev Cell Dev Biol 2003;19:261—86.
[5] Fang L, Lu W, Choi HH, Yeung SC, Tung JY, Hsiao CD, et al. ERK2-dependent Phosphorylation of CSN6 is critical in colorectal cancer development. Cancer Cell 2015;28:183—97.
[6] Hou J, Deng Q, Zhou J, Zou J, Zhang Y, Tan P, et al. CSN6 controls the proliferation and metastasis of glioblastoma by CHIP-mediated degradation of EGFR. Oncogene 2017;36:1134—44.
[7] Zhou S, Xu M, Shen J, Liu X, Chen M, Cai X. Overexpression of NEDD9 promotes cell invasion and metastasis in hepatocellular carcinoma. Clin Res Hepatol Gastroenterol 2017;41:677—86.
[8] Lin Y, Dong C, Zhou BP. Epigenetic regulation of EMT: the Snail story. Curr Pharm Des 2014;20:1698—705.
[9] Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. CA Cancer J Clin 2018;68:7—30.
[10] Giannelli G, Koudelkova P, Dituri F, Mikulits W. Role of epithelial to mesenchymal transition in hepatocellular carcinoma. J Hepatol 2016;65:798—808.
[11] Chen J, Shin JH, Zhao R, Phan L, Wang H, Xue Y, et al. CSN6 drives carcinogenesis by positively regulating Myc stability. Nat Commun 2014;5:5384.
[12] Wang W, Tang M, Zhang L, Xu X, Qi X, Yang Y, et al. Clinical implications of CSN6 protein expression and correlation with mutant-type P53 protein in breast cancer. Jpn J Clin Oncol 2013;43:1170—6.
[13] Wen D, Liao T, Ma B, Qu N, Shi RL, Lu ZW, et al. Downregulation of CSN6 attenuates papillary thyroid carcinoma progression by reducing Wnt/beta-catenin signaling and sensitizes cancer cells to FH535 therapy. Cancer Med 2018;7:285—96.
[14] Yang L, Liu Y, Wang M, Qian Y, Dong X, Gu H, et al. Quercetin-induced apoptosis of HT-29 colon cancer cells via inhibition of the Akt-CSN6-Myc signaling axis. Mol Med Rep 2016;14:4559—66.
[15] Zhang SN, Pei DS, Zheng JN. The COP9 signalosome subunit 6 (CSN6): a potential oncogene. Cell Div 2013;8:14.
[16] Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest 2009;119:1420—8.

Please cite this article in press as: Xu M, et al. Overexpression of CSN6 promotes the epithelial-mesenchymal transition and predicts poor prognosis in hepatocellular carcinoma. Clin Res Hepatol Gastroenterol (2019), https://doi.org/10.1016/j.clinre.2019.07.012

[17] Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. Cell 2009;139:871—90.
[18] Mittal V. Epithelial mesenchymal transition in tumor metastasis. Annu Rev Pathol 2018;13:395—412.
[19] Elsum IA, Martin C, Humbert PO. Scribble regulates an EMT polarity pathway through modulation of MAPK-ERK signaling to mediate junction formation. J Cell Sci 2013;126:3990—9.
[20] Hu X, Zhai Y, Kong P, Cui H, Yan T, Yang J, et al. FAT1 prevents epithelial mesenchymal transition (EMT) via MAPK/ERK signaling pathway in esophageal squamous cell cancer. Cancer Lett 2017;397:83—93.
[21] Sheng W, Chen C, Dong M, Wang G, Zhou J, Song H, et al. Calreticulin promotes EGF-induced EMT in pancreatic cancer cells via Integrin/EGFR-ERK/MAPK signaling pathway. Cell Death Dis 2017;8:e3147.
[22] Liu M, Hu Q, Tu M, Wang X, Yang Z, Yang G, et al. MCM6 promotes metastasis of hepatocellular carcinoma via MEK/ERK pathway and serves as a novel serum biomarker for early recurrence. J Exp Clin Cancer Res 2018;37:10.
[23] Pan JX, Chen G, Li JJ, Zhu QD, Chen ZJ, Yu ZP, et al. Isocorydine suppresses doxorubicin-induced epithelial-mesenchymal transition via inhibition of ERK signaling pathways in hepatocellular carcinoma. Am J Cancer Res 2018;8:154—64.
[24] Zhao F, Gong X, Liu A, Lv X, Hu B, Zhang H. Downregulation of Nedd4L predicts poor prognosis, promotes tumor growth and inhibits MAPK/ERK signal pathway in hepatocellular carcinoma. Biochem Biophys Res Commun 2018;495:1136—43.
[25] Wang S, Cheng Y, Zheng Y, He Z, Chen W, Zhou W, et al. PRKAR1A is a functional tumor suppressor inhibiting ERK/Snail/E-cadherin pathway in lung adenocarcinoma. Sci Rep 2016;6:39630.

Please cite this article in press as: Xu M, et al. Overexpression of CSN6 promotes the epithelial-mesenchymal transition and predicts poor prognosis in hepatocellular carcinoma. Clin Res Hepatol Gastroenterol (2019), https://doi.org/10.1016/j.clinre.2019.07.012