RESEARCH

High ADAM8 Expression is Associated with Poor Prognosis in Patients with Hepatocellular Carcinoma

Yun Zhang · Yong-Fei Tan · Chao Jiang · Kai Zhang · Tian-Zhou Zha · Miao Zhang

Abstract In this study,we investigated the ADAM8 expression in hepatocellular carcinoma (HCC) and its correlation with clinicopathologic features,including the survival of patients with HCC. Furthermore,we examined the biological processes regulated by ADAM8 during the development of using HepG2 cell line as a model system. We used immunohistochemistry to compare ADAM8 protein expression in HCC and normal liver tissues and further analyze the ADAM8 protein expression in clinicopathologically characterized 105 HCC cases.We stably knocked down the endogenous expression level of ADAM8 in HepG2 cells with specific shRNA-expressing lentiviral vector. Following the successful establishment of stable cells,we examined in vitro cell growth by MTT assay,anchorage-independent growth by soft-agar colony formation assay and cell migration/invasion by transwell and boyden chamber assay. And in addition,we also investigated the in vivo tumor growth by xenograft transplantation of HepG2 cells into nude mice. Protein expression level of ADAM8 was markedly higher in HCC tissues than that in the normal liver tissues ($P=0.0058$).In addition,high expression of ADAM8 protein was positively correlated with serum AFP elevation,tumor size,histological differentiation,tumor recurrence,tumor metastasis,and tumor stage. Patients with higher ADAM8 expression showed a significantly shorter overall survival time than patients with low ADAM8 expression. Multivariate analysis suggested that ADAM8 expression might be an independent prognostic indicator ($p=0.016$) for the survival of patients with HCC. ADAM8-specific shRNA (shADAM8) successfully knocked down its endogenous expression in HepG2 cells. Compared to the parental and control shRNA-transfected (shCtrl) HepG2 cells,the shADAM8 cells exhibited significantly reduced in vitro cell growth,anchorage-independent growth,cell migration and invasion ($p<0.05$).In vivo,the xenograft transplants from shADAM8 cells gave rise to much smaller tumors as compared to those from shCtrl cells. High ADAM8 expression is associated with poor overall survival in patients with HCC. Down-regulation of ADAM8 inhibits the growth,anchorage-independent growth, migration and invasion of HepG2 cells. ADAM8 may be a potential target of antiangiogenic therapy for HCC.

Keywords ADAM8 · Hepatocellular carcinoma · Biomarker · Prognosis · Immunohistochemical analysis

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer deaths,with increasing incidence worldwide [1, 2]. It is more prevalent in Central Africa and parts of Asia, among which half are reported from China. Most patients with HCC have a very poor prognosis,with an annual number of newly diagnosed cases almost equal to the number of deaths [3]. This tragic situation stems from a lack of early diagnosis,the deteriorated condition of the cirrhotic liver from which most HCC cases develop,and the high resistance of HCC to chemotherapy [4]. Identification of novel molecular mechanisms involved in the development and progression of HCC may provide new strategies for the diagnosis and treatment of this life-threatening disease.

ADAM family members are implicated to be involved in the proteolytic processing of membrane-bound precursors, and they modulate cell-cell and cell-matrix interactions. ADAM8 encodes a protein of 824 amino acids with a COOH-terminal transmembrane domain and potential extracellular adhesion and protease domains [5, 6]. This

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molecule, localized to the plasma membrane,is processed by autocatalysis into two forms;one is derived by removal of a prodomain and the other is a remnant protein composed of the extracellular region with a disintegrin domain at the NH2 terminus [7]. ADAM8 behaves as an active metal-loprotease in vitro,hydrolyzing myelin basic protein and a variety of peptide substrates based on the cleavage sites of membrane-bound cytokines,growth factors,and receptors [8–11]. Other studies have demonstrated overexpression of some ADAM family proteins in a variety of human tumors [12, 13],but no report is available on the actual expression level of ADAM8 and the correlation between clinicopatho-logic features and prognosis of HCC patients.

In order to clarify the role of ADAM8 in the pathogenesis of HCC,we investigated the correlation of ADAM8 protein expression with clinicopathologic features with HCC in Chinese populations.We found that the expression level of ADAM8 protein was higher in HCC tissues than that in normal liver tissues. High expression of ADAM8 was asso-ciated with poor prognosis of HCC. Moreover,our results indicated that ADAM8 was involved in progression of HCC by promoting cell growth,invasion and migration of HCC cells.In our investigation,ADAM8 may act as an oncogene in the pathogenesis of HCC.

Materials and Methods

Sample Collecting

105 paraffin-embedded HCC samples and 50 noncancerous paraffin-embedded normal liver samples were obtained from Yixing people's hospital,Jiangsu Province,China.In 105 HCC cases,there were 55 male and 50 female ranging in age from 14 to 79 years (median,48 years). For the use of these clinical materials for research purposes,prior consents from the patients and approval from the Ethics Committees of Jiangsu Province were obtained and all the procedures have been performed in compliance with the Helsinki Declaration. All specimens had confirmed pathological diagnosis and were staged according to the 2002 hepatocellular carcinomas stag-ing system of the International Union Against Cancer(UICC).

Immunohistochemical Analysis

All 105 tissue specimens were subjected to immunohisto-chemical analysis using the avidin-biotin-peroxidase meth-od. Sections were deparaffinized in xylene and dehydrated using a graded alcohol series before endogenous peroxidase activity was blocked with 0.5 % H_2O_2 in methanol for 10 min. Nonspecific binding was blocked by incubating sections with 10 % normal goat serum in phosphate-buffered saline(PBS) for 1 h at room temperature. Without

washing,sections were incubated with anti-ADAM8(1:100; Abnova,Taipei,Taiwan) in PBS at 4°Covernight in a moist box. Biotinylated goat anti-rabbit immunoglobulin G (IgG)(1:400; Sigma,St.Louis,MO,USA) was incubated with the sections for 1 h at room temperature and detected with a streptavidin-peroxidase complex. The brown color indica-tive of peroxidase activity was developed by incubating sections with 0.1 % 3,3-diaminobenzidine (Sigma) in PBS with 0.05 % H_2O_2 for 5 min at room temperature. The tissue specimens were viewed separately by two pathologists un-der double-blinded conditions,where they had no prior knowledge of the clinical or clinicopathological status of the specimens. Expression of ADAM8 in the HCC speci-mens was evaluated by scanning the entire tissue specimen under low magnification (×40),and then confirmed under high magnification(×200 and×400). The intensity of posi-tive staining was measured as described [14] and in the Supporting Information. The intensity of ADAM8 was clas-sified into high expression (mean area of positive staining as cutoff value, ADAM8^{high}, ≥50% of tumor section,and low expression ADAM8^{low},<50 %).

Cell Line

The hepatoma cell line HepG2 was acquired from the Cancer Institute,Central South University and cultured in RPMI1640 medium (HyClone Inc,USA) supplemented with 10 % fetal bovine serum (FBS) (PAA Laboratories,Inc,Austria) in a 37 °C, 5 % CO_2 incubator.

Extraction of Total RNA and Reverse Transcription Followed by PCR (RT-PCR)

Total RNA was extracted from approximately $1×10^6$ cells using Trizol reagent (Invitrogen,USA) following the manu-facturer's instructions.cDNA was then synthesized from 2 μmug total RNA using oligo(dT)15 as the primer along with the MMLV reverse transcriptase (Takara Inc,Japan).To de-termine the steady-state mRNA level of ADAM8 in HepG2 cells, PCR was performed with the following primers: AD-AM8 forward primer 5'-GAGGGTGACGGTGATAAGAA-3',reverse primer 5'-GAAACATTGGTGGCTACAGG-3', and the amplicon size is 377 bps. GAPDH (internal control) forward primer 5'-TTCGCTCTCTGCTCCTC-3',reverse primer 5'-GATGATCTTGAGGCTGTTGT-3',and the amplicon size is 520 bps. The PCR condition was one cycle of denaturation at 94 °C for 2 min,28 cycles of denaturation at 94 °C for 20 s (s),annealing at 55 °C for 30 s and extension at 72 °C for 30 s,followed by one cycle of final extension at 72 °C for 10 min. After the PCR reaction,the PCR products was loaded on 1 % agarose gel and visualized by ethidium bromide staining.

Construction of ADAM8-shRNA Lentiviral Vector

The BLOCK-iT RNAi lentiviral expression plasmid was purchased from Invitrogen Inc. The shRNA sequence targeting ADAM8 (shADAM8) and a control shRNA sequence (shCtrl) targeting no known human genes was designed using the BLOCK iT RNAi Designer http://www.invitrogen.com as follows: shADAM8, sense 5'-CACCGCCGTGAAATCAACAGCCAAAACGTTGGCTGTTGATTTCACGG-3';antisense 5'-AAAACCGTGAAATCAACAGCCAACTTTTGGCTGTTGATTTCACGGC-3. shCtrl, sense 5'-CACCGCCCTGAATTGAACAGCCAAAACGTTGGCTGTTGATTTCACGG-3'; 5'-AAAACCGTGAAATCAACAGCCAACTTTTGGCTGTTCAATTCAGGGC-3'. The shRNA primers were cloned into the lentiviral expression plasmid following the manufacturer's instruction and confirmed by sequencing.

Establishment of HepG2 Cell Line Stably Expressing shRNA

shRNA-expressed lentiviral plasmid (either ADAM8-specific or control) was transfected into HepG2 cells using Lipofectamin2000 (Invitrogen) according to the Valkovskaya N's instructions [15]. 48 h later,the cells were subjected to Blasticidin selection at a final concentration of 1.2 μmug/mL. Following two weeks of selection,stable pooled cells were further expanded for future experiments.

Real-Time PCR

Real-time PCR was performed to measure the knock down efficiency of ADAM8 mRNA expression using SYBR Premix Ex Taq (Takara,Japan) as described previously [16]. The sense primer: 5'-CAGCCAACAAATACCAAGTCT-3',Antisense primer: 5'-GTTCTCGATCTCCCACAGC-3'. GAPDH gene was used as an inner control. The sense primer:5'-GAAGGTCGGAGTCAACGG-3', Antisense primer: 5'-TGGAAGATGGTGATGGGATT-3'.

Western Blot Analysis

Approximately 5×10^6 cells were lysed in RIPA Buffer (50 mM Tris–HCl pH 8.0,1 mM EDTA pH 8.0,5 mM DTT,2 % SDS) and total protein concentration determined with BCA assay (Beyotime Inc,China).60 μmug of total protein were loaded onto 10 % SDS-PAGE gel. Antibodies used for Western blot analysis included:rabbit polyclonal anti-ADAM8 antibody (Proteintech Inc,1:200),anti-tubulin antibody (Santa Cruz,USA,1:400),and HRP-conjugated anti-rabbit secondary antibody (Amersham Pharmacia Biotech,1:50).

Cell Growth Analysis

Cell growth was determined by MTT assay (Sigma,USA). Briefly,1×10^3 cells were seeded into 96-well plate with quadruplicate for each condition.72 h later,MTT reagent was added to each well at 5 mg/mL in 20 μmuL and incubated for another 4 h. The formazan crystals formed by viable cells were then solubilized in DMSO and measured at 490 nm for the absorbance (A) values.

Soft Agar Colony Formation Assay

The anchorage-independent growth of hepatocytes was monitored by the soft agar colony formation assay.In brief, cells (100/well) resuspended in 1.5-ml mixture of 1.2 % low-melt agarose and 2×RPMI 1640 (v:v=1:1) were loaded in triplicate on the top of the solidified bottom agar comprising equal-volume mixture of 0.7 % lowmelt agarose and RPMI 1640 in 12-well plates. The cells were incubated at 37 °C,5 % CO_2 for two weeks. The colonies composed of more than 50 Cells were counted.

In Vitro Migration and Invasion Assay

Cells growing in the log phase were treated with trypsin and resuspended as single-cell solution. Then the cells were counted and 1×10^5 cells in 1 mL of serum-free RPMI 1640 medium were split into the upper chamber of transwell and boyden chamber (8-μmum pore size,BD Biosciences,USA) where the transwell membrane was coated either with (for invasion) or without (for migration) matrigel. Serum-free medium was also added to the lower chamber. The migration assay proceeded in 37 °C,5%CO_2 tissue culture incubator for 12 h and the invasion assay,18 h. The non-migrated/invaded cells in the upper chamber were removed using cotton swap,and the migrated/invaded cells fixed in methanol and stained with eosin for migrated cells or Gimsa for invaded cells. The cells trapped in or attached to the reverse side of the porous membrane were photographed through×200 microscope objective,the numbers of migrated cells in at least 5 random fields counted under phase contrast microscope,and the average calculated.

In Vivo Xenograft Tumor Growth in Nude Mice

All animal protocols were approved by the Animal Care and Use Committee of Jiangsu University. Nude mice between 4 to 6 weeks were purchased from the Animal Center,Central South University (N=5).1×10^6 cells growing in log phase were resuspended in serum-free RPMI 1640 medium and injected subcutaneously into the 4–6 week-old male BALB/c nu/nu mice.To minimize individual difference,shCtrl cells and shADAM8 cells were injected symmetrically to the leftright flank of the same mouse. The mice were maintained

in a barrier facility on HEPA-filtered racks. The animals were fed with an autoclaved laboratory rodent diet. All animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health (NIH) Guide for the Care and Use of Animals under assurance number A3873-1.15 days later,the mice were sacrificed with tumors isolated and their sizes and weights measured. Finally, the ADAM8 expression was examined again by quantitative real-time PCR in implanted nude mice of shRNAADAM8 and control cell groups. GAPDH gene was used as a normalizing control. The designed paired primers were as follow, ADAM8 forward,5'-CAGCCAACAAATACCAAGTCT-3' reverse:5'-GTTCTCGATCTCCCACAGC-3'. GAPDH forward,5' GAAGGTCGGAGTCAACGG 3' reverse, 5'-TGGAAGATGGTGATGGGATT-3'. The PCR reaction was carried out in a volume of 25 µl using SYBR Green Mix (Tiangen Inc,China) on MXP3000 Instrument (Stratagene Laboratory). Experiments were repeated three times.

Statistical Analysis

All data were analyzed by SPSS 13.0 software and presented as mean±SD. The χ2 test was applied to analyze the relationship between ADAM8 expression and clinicopathologic characteristics. Survival curves were plotted by the Kaplan-Meier method and compared by the logrank test. The significance of various variables for survival was analyzed by the Cox proportional hazards model in the multivariate analysis. One-way ANOVA was applied to test the differences between groups for all in vitro analyses. ANOVA test was used for the in vivo xenograft experiment.A P value of less than 0.05 was considered statistically significant.
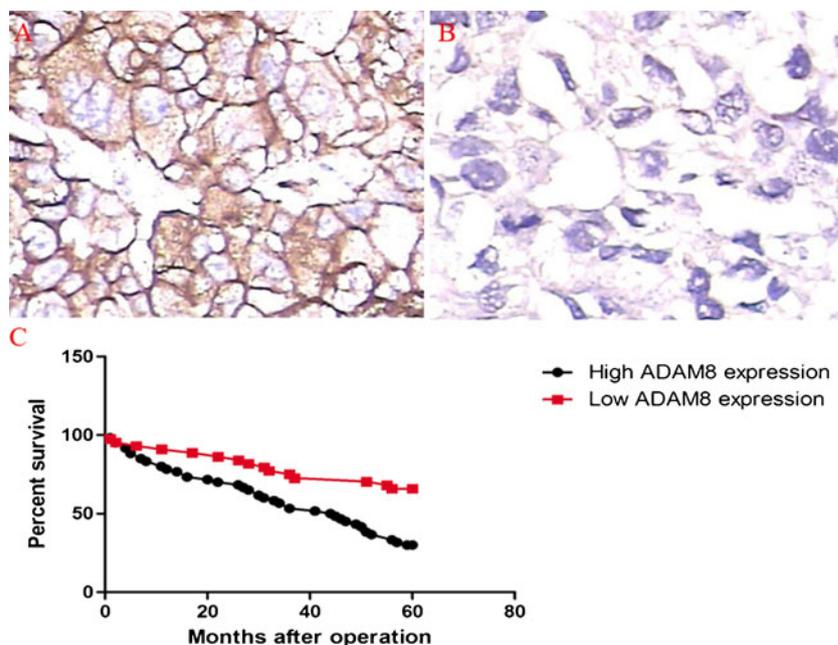
Results

Immunohistochemical Analysis of ADAM8 Protein Expression in HCC and Normal Liver Tissues

We measured the expression level and subcellular localization of ADAM8 protein in 105 archived paraffinembedded HCC samples and 50 noncancerous samples using immunohistochemical staining expression. Specific ADAM8 staining was mainly founded in the plasma membrane and cytoplasm of noncancerous and malignant epithelial cells. Overall,ADAM8 was positively and negatively expressed in 82(78.1 %) and 23 (21.9 %) of the 105 HCC patients, respectively. ADAM8 was highly and lowly expressed in 60 (57.1 %) and 45 (42.9 %) of the 105 HCC patients, respectively(Fig. 1a-b).In comparison,ADAM8 was positively and negatively expressed in 26 (52 %) and 24 (48 %) of 50 noncancerous samples,respectively. ADAM8 was highly and lowly expressed in 15 (30 %) and 35 (70 %) of 50 noncancerous samples,respectively. Protein expression level of ADAM8 was markedly higher in HCC tissues than that in the normal liver tissues ($P=0.0058$).

Correlation Between ADAM8 Expression and Clinicopathologic Characteristics in Chinese HCC

ADAM8 expression level in HCC was associated with serum AFP elevation($P=0.031$) but not with age,gender, chronic HBV infection,and liver cirrhosis (Table 1). Histologically,high ADAM8 expression occurred more often in larger tumors ($P=0.017$) and in late-stage (Stage III-IV) HCC ($P=0.025$).In addition,ADAM8 expression level was

Fig. 1 Detection of ADAM8 protein in HCC and Kaplan-Meier plots of overall survival duration in patients with HCC. **a** High ADAM8 expression in HCC samples. **b** Low ADAM8 expression in HCC samples (original magnification×200). **c** Negative expression of ADAM8 in HCC samples;D. Kaplan-Meier survival analysis of overall survival duration in 105 HCC patients according to ADAM8 protein expression. The log-rank test was used to calculate p values

associated closely with histological differentiation ($p=$ 0.029),tumor Recurrence ($p=0.016$),and tumor metastasis ($p=0.014$) (Table 1).

Univariate and Multivariate Analyses of Prognostic Variables in HCC Patients

The prognostic effect of ADAM8 on the overall survival rate of HCC patients with a high or low ADAM8 protein expression level was compared using Kaplan-Meier survival curves and the log-rank test respectively,showing that high ADAM8 expression was a significant prognostic factor for poor overall survival rate of HCC patients. The 5-year survival rate of HCC patients with a high or a low ADAM8 protein expression level was 30 % and 66.7 %, respectively. A

Table 1 Relation between ADAM8 expression in HCC and clinicopathological features of hepatocellular carcinoma patients

Clinicopathological variable(n)	Low ADAM8 expression $n=45$	High ADAM8 expression $n=60$	P value
Age(yr)			
<60(59)	25	35	0.843
≥60(46)	20	25	
Gender			
Male(55)	20	28	0.846
Female(50)	25	32	
Tumor size(cm)			
≤5(25)	18	11	0.017
>5(80)	27	49	
Histological differentiation			
Well/moderate(54)	29	25	0.029
Poor(51)	16	35	
Liver cirrhosis			
With(62)	28	36	0.843
Without(43)	17	24	
Recurrence			
Yes(33)	7	23	0.016
No(72)	38	37	
Metastasis			
With(23)	4	18	0.014
Without(82)	41	42	
HBsAg			
Positive(89)	35	51	0.444
Negative(16)	10	9	
AFP(ng/ml)			
≤400(17)	3	14	0.031
>400(88)	42	46	
Tumor stage			
I-II(32)	23	17	0.025
III-IV(73)	22	43	

significant difference was observed on the Kaplan-Meier survival curves for HCC patients with a high or a low expression level of ADAM8 ($P<0.001$,log-rank test, Fig. 1c). Univariate Cox regression analysis also identified that clinical variables including serum AFP,tumor size,histological differentiation, tumor recurrence,tumor metastasis,and tumor stage and ADAM8 expression were significantly associated with overall survival (Table 2). Furthermore,to evaluate the potential of ADAM8 expression as an independent predictor for overall survival of HCC,multivariate Cox regression analyses were performed. While the others failed to demonstrate independence,serum AFP,tumor size,histological differentiation,tumor recurrence,tumor metastasis,tumor stage andADAM8 expression may play a role in predicting the overall survival in HCC (Table 2).

ADAM8 Was Highly Expressed in HepG2 Cells

To examine the biological functions of ADAM8,we first measured the expression level of endogenous ADAM8 in HepG2 cells by RT-PCR.As shown in Fig. 2a, when the PCR cycle number was controlled (28 cycles) so that both the ADAM8 and GAPDH products were in the linear range, ADAM8 showed an expression level comparable to that of the housekeeping gene GAPDH,suggesting ADAM8 is highly expressed in HepG2 cells,which also indicates that HepG2 is a good model system for studying the functions of endogenous ADAM8 by loss-of-function approach.

Endogenous ADAM8 was Successfully Knocked Down by shRNA-Expressing Lentiviral Vector

To stably knock down the endogenous expression of ADAM8,we applied a lentiviral vector expressing specific shRNA sequence targeting ADAM8 (shADAM8).As a control,we stably transfected the HepG2 cells with the same lentiviral vector expressing a control shRNA sequence (shCtrl) not targeting any known human genes.By mRNA and protein expression analysis,we found that the shCtrl cells have similar ADAM8 level as the parental HepG2 cells,which were significantly higher than that in the shADAM8 cells (Fig. 2b,c).

ADAM8 Knockdown Inhibited the Growth of HepG2 Cells

After successfully knocking down the endogenous expression of ADAM8,we first examined its effect on cell growth. As shown in Fig. 3,the parental HepG2 cells had a similar growth rate as the shCtrl cells over a seven-day period,while starting from day 4 the growth of shADAM8 cells were significantly slower than the former two cells ($P<0.05$), suggesting ADAM8 promotes the growth of HepG2 cells.

Table 2 Univariate analysis and multivariate analysis showing the overall survival rate for hepatocellular carcinoma patients

| Variables	Univariate analysis		P value	Multivariate analysis		P value
	HR	95%CI		HR	95%CI	
ADAM8	1.763	1.112–3.542	0.012	1.723	1.143–3.621	0.016
Age	0.912	0.245–1.681	0.472			
Gender	0.724	0.316–1.196	0.575			
Tumor size	1.278	0.582–2.145	0.007	1.123	0.679–2.376	0.006
Histologic grade	1.523	0.584–2.421	0.012	1.296	0.751–1.891	0.021
Liver cirrhosis	1.256	0.832–2.148	0.568			
Recurrence	1.218	0.773–2.316	0.016	1.513	1.001–2.214	0.013
Metastasis	1.324	1.001–1.923	0.019	1.433	0.517–1.721	0.012
HBsAg	1.782	1.163–2.455	0.633			
AFP(ng/ml)	1.221	1.012–1.814	0.033	1.621	1.121–2.257	0.023
Tumor stage	1.267	0.768–1.912	0.017	1.215	0.723–1.812	0.012

ADAM8 Knockdown Inhibited Cellular Transformation

We next explored the effect of ADAM8 on cellular transformation. Since anchorage-independent growth is a hallmark for transformed cells,we measured the growth of different cells on soft agar. Both the parental HepG2 cells and the shCtrl cells formed similar number of colonies on soft agar over a two-week period [(37.2±4.6) vs. (36.4±4.1)].In contrast, knocking down endogenous ADAM8 dramatically reduced the number of colonies (10.1±2.0)($p<0.05$),implying an essential role of ADAM8 in regulating cellular transformation(Fig. 4).

ADAM8 Knockdown Reduced Cell Migration and Invasion

Cell migration and invasion are integral steps for the process of tumor development and metastasis. When testing the abilities of HepG2 cells to migrate/invade through 8-μmum pores on the polycarbonate membrane either without or with pre-coated matrigel,we found the knocking down endogenous ADAM8 significantly reduced the potentials of HepG2 cells to both migrate and invade ($p<0.05$),as compared to the parental or shCtrl cells (Fig. 5).

Fig. 2 ADAM8 was highly expressed in HepG2 cells and this level was successfully knocked down by shRNA. **a** Total RNA was extracted from HepG2 cells and RT-PCR was applied to examine the steady-state mRNA levels of ADAM8 and GAPDH. **b** and **c**. Parental HepG2 cells and those stably transfected with either control (shCtrl) or ADAM8-specific (shADAM8) shRNA were examined for ADAM8 expression by quantitative real-time PCR and Western-blot. GAPDH and α-tubulin were used as internal control,respectively
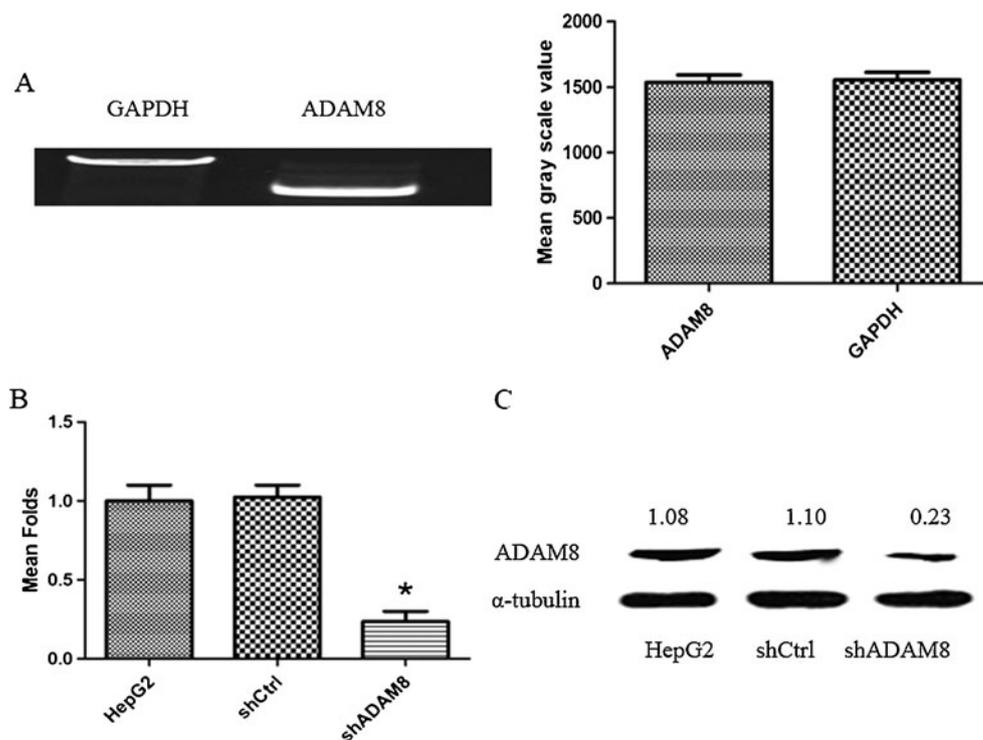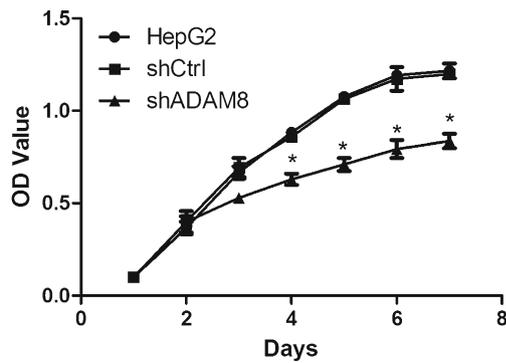
Fig. 3 Down-regulation of ADAM8 inhibited cell growth. The cell growth of parental HepG2 cells and their stable derivatives,shCtrl and shADAM8, were examined by MTT assay over a seven-day period.*P <0.05,as compared to parental HepG2 cells and shCtrl cells

ADAM8 Contributes to in Vivo Xenograft Tumor Growth

In addition to examining the biological functions of ADAM8 in vitro,we also assessed the in vivo function of ADAM8 using a xenograft transplantation model.By subcutaneously transplanting the shCtrl or shADAM8 cells into nude mice,we monitored the tumor growth over a 15-day period.As shown in Fig. 6,by measuring the tumor weights,we found that shADAM8 cells gave rise to significantly smaller tumors than shCtrl cells ($p<$ 0.05). Real-time qPCR showed that ADAM8 expression was obviously reduced in implanted nude mice of shADAM8 cell groups compared with control cell groups (Fig. 6c).

Discussion

Despite many advances in diagnostic imaging of tumors, combination chemotherapy,and radiation therapy,little improvement has been achieved within the last decade in terms of prognosis and quality of life for patients with hepatocellular carcinoma. Given the frequent failure of conventional treatment strategies,many cancer-related molecules have been characterized toward the goal of developing novel anticancer therapies such as molecular-targeted drugs and antibodies or cancer vaccines [17, 18]. Molecular-targeted therapies are expected to be highly specific to malignant cells,with minimal adverse effects due to their well-defined mechanisms of action. Equally desirable in prospect are minimally invasive,highly sensitive,and specific new diagnostic methods that would adapt readily to clinical settings. From these points of view,tumor-specific transmembrane/ secretory proteins should have significant advantages because they are presented either on the cell surface or within the extracellular space and/or in serum,making them easily accessible as molecular markers and therapeutic targets.

Fig. 4 Down-regulation of ADAM8 inhibited cell transformation. The anchorage-independent growth of parental HepG2 cells and their stable derivatives,shCtrl and shADAM8,were examined by soft agar colony formation assay. **a** Colonies were photographed. **b** Bar graph showed the differences of colony formation among the three groups. Data were presented as mean± SD for three independent experiments.*P<0.05,as compared to parental HepG2 cells and shCtrl cells
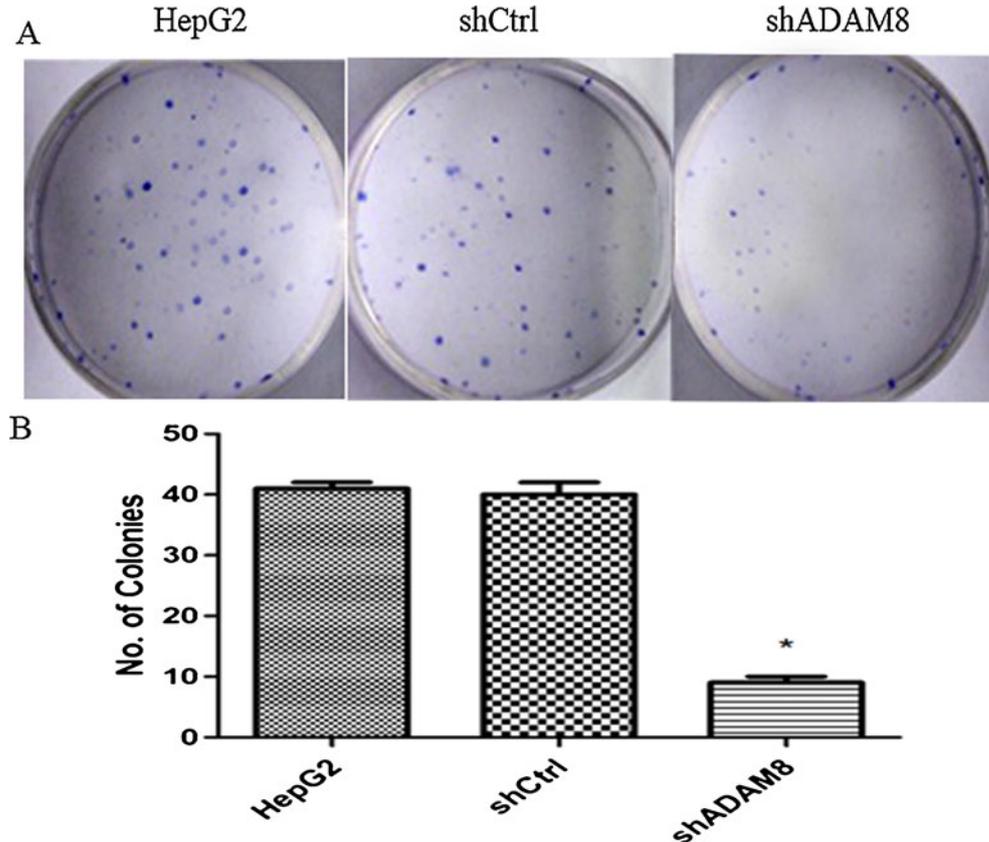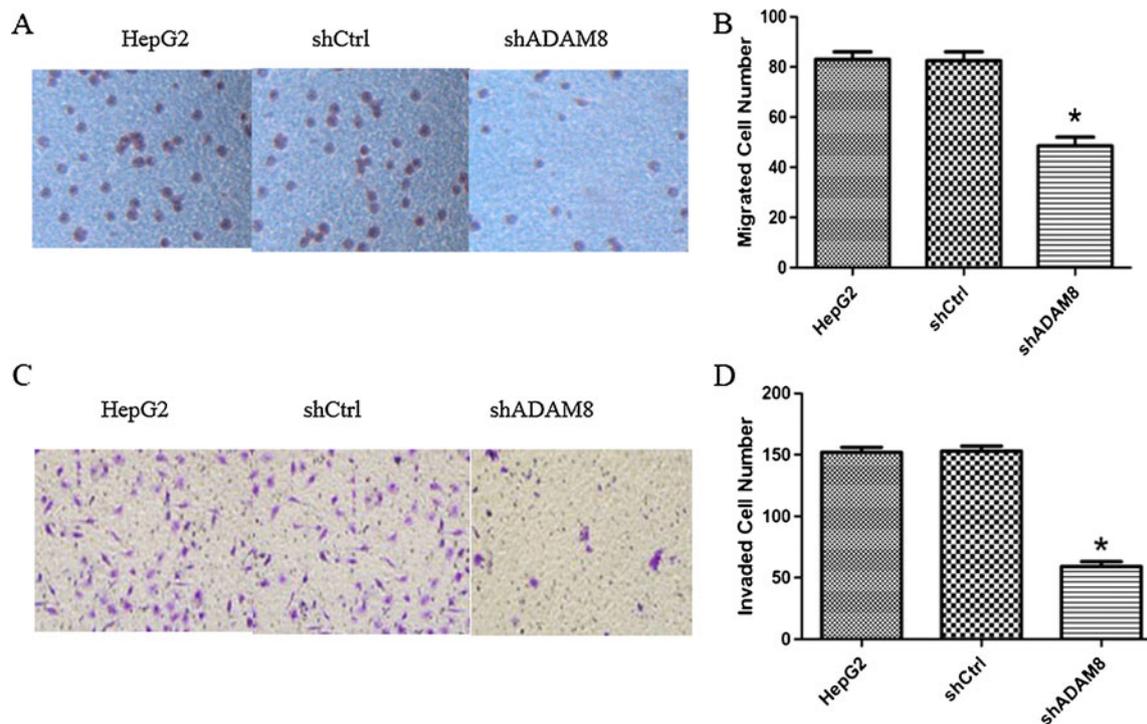
Fig. 5 Down-regulation of ADAM8 reduced cell migration and invasion. The migrating **a** and **b** and invading **c** and **d** capabilities of parental HepG2 cells and their stable derivatives,shCtrl and shADAM8,were examined by transwell and boyden chamber assay. **a** and **c** Migrated or invaded cells were photographed under the microscope (200×). **b** and **d** Quantifications of migration and invasion were presented as mean±SD for three independent experiments.*$P<0.05$,as compared to parental HepG2 cells and shCtrl cells

Some tumors pecific markers already available,such as CYFRA or Pro-GRP,are transmembrane/secretory proteins [19, 20]; the example of rituximab (Rituxan),a humanized monoclonal antibody against CD20-positive lymphomas, provides proof that targeting specific cell surface proteins

can result in significant clinical benefits [21–24]. As an approach to identifying novel cancer-specific cell surface or secretory proteins,we have been exploiting the power of genome-wide expression analysis to select genes that are overexpressed in cancer cells. Analysis of candidate

Fig. 6 Down-regulation of ADAM8 inhibited in vivo xenograft tumor growth. **a** hCtrl and shADAM8 cells were injected subcutaneously into nude mice (*N*=5 for each group) and the tumors were isolated two weeks later. **b** Tumor weights were quantified from 5 mice and presented as mean±SD.*$P<0.05$,as compared to shCtrl cells
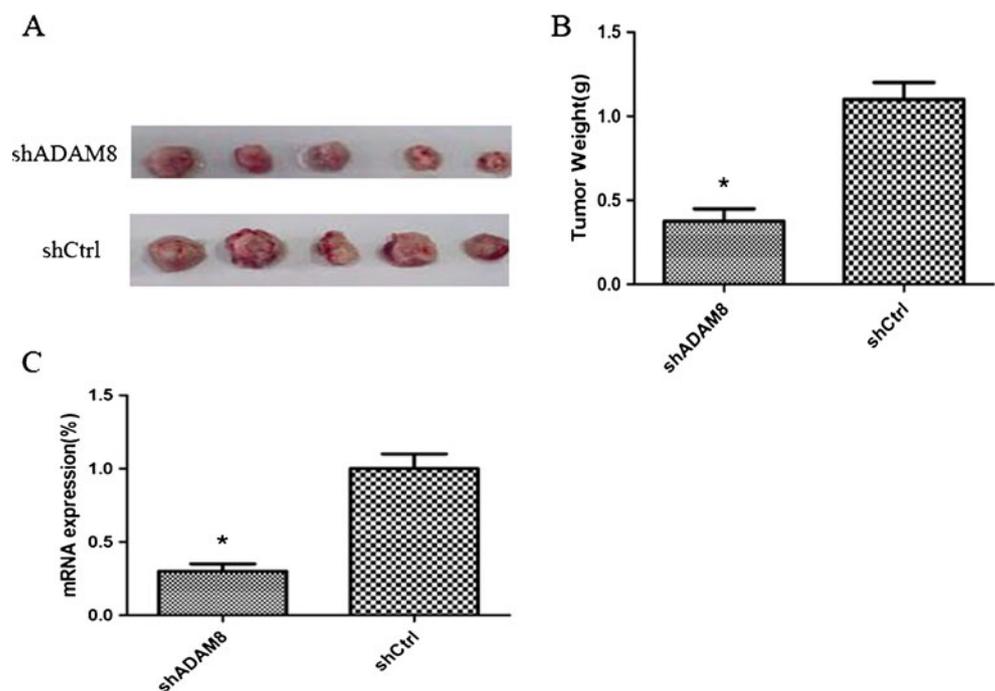
molecules revealed ADAM8 as a potential target for development of novel tools for diagnosis and treatment of hepatocellular carcinoma.

ADAM8 protein is homologous to a snake disintegrin, Reprolysin (M12B),a zinc metalloprotease [6]. Members of the ADAM family are cell surface proteins with a unique structure combining potential adhesion and protease domains.A published report has suggested that the ADAM8 ectodomain is cleaved by ADAM8 itself [7]. Because various matrix metalloproteinases and ADAM family proteins had been described as being overexpressed in human cancers [21],ADAM8 seemed likely to have a potential role in tumor development or progression.In this sutdy,we evaluated the association between ADAM8 tissue expression and patients' prognosis in hepatocellular carcinoma.In our tumour material,ADAM8 was positively expressed in 78.1 % of HCC patients and highly expressed in 57.1 % of HCC patients.

The ADAM8 expression level was closely correlated with serum AFP elevation and tumor stage,confirming the association of poorly differentiated HCC with AFP elevation [22]. In addition,high ADAM8 expression occurred more often in larger tumors ($P=0.017$) and in late-stage (Stage III-IV) HCC ($P=0.025$). Late-stage HCC exhibited portal vein invasion and was correlated closely with poorer prognosis [23]. Our findings therefore support the suggestion that ADAM8 expression enhances metastatic potential of transformed cells,as well as human cancers,including HCC [24, 25]. Our results suggested that ADAM8 expression was not related to HBV infection and liver cirrhosis, indicating that ADAM8 may be not associated with the initiation of HCC [26]. In this study,the prognosis of HCC patients with high ADAM8 expression was poor,and Cox regression analysis indicated that high expression level of ADAM8 was a significant prognostic factor for a poor overall survival rate of HCC patients,suggesting that ADAM8 may become a novel prognostic marker for HCC.

Further,we analyzed the function of ADAM8 in HepG2 cells.By transfecting the cells with shRNA-expressing lentiviral vector followed by selection with Blasticidin,we successfully established stable cells expressing either control or ADAM8-specific shRNA,with the latter showing dramatically reduced ADAM8 level as compared to the former. The subsequent functional studies demonstrated that knocking down the endogenous expression of ADAM8 led to significant reduced in vitro cell growth transformation,migration, invasion,as well as in vivo xenograft tumor formation. Compared with the previous gain-of-function studies,this loss-of-function study offers more insights on the functions of endogenous ADAM8,minimizing the confounding factors that might be introduced by overexpressed ADAM8 at a physiologically irrelevant high level. Our study also revealed a novel function of ADAM8 in HCC,that is,to promote the cell migration and invasion,suggesting its potential involvement in cancer metastasis. This is consistent with the finding by Valkovskaya N etal.that downregulation of ADAM8 inhibits the invasion of pancreatic cancer cells [15] which further indicates that the biological functions of ADAM8 are not unique to a specific cancer,but common to multiple cancers. The observations that ADAM8 regulates multiple cellular processes such as cell growth,cell transformation,migration,invasion,and is a prognostic factor for multiple cancers implies its importance as a therapeutic target for treating multiple human cancers,including HCC.

In conclusion,our results provide a basis for the concept that high ADAM8 expression in hepatocellular carcinoma may be important in the tumor progression and serves as an independent biomarker for poor survival. Thus,high ADAM8 expression identifies patients at high risk and is a potential novel therapeutic target for hepatocellular carcinoma.

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