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DNAJC6 promotes hepatocellular carcinoma progression through induction of epithelial–mesenchymal transition ☆

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ABSTRACT

Epithelial–mesenchymal transition (EMT) is a developmental program, which is associated with hepatocellular carcinoma (HCC) development and progression. DNAJC6 (DNA/HSP40 homolog subfamily C member 6) encodes auxilin, which is responsible for juvenile Parkinsonism with phenotypic variability. However, the role of DNAJC6 in HCC development and progression is limited. Here, we report that DNAJC6 is up-regulated in HCC tissues and up-regulation of DNAJC6 expression predicts poor outcome in patients with HCC. Furthermore, overexpression of DNAJC6 enhances the ability for acquisition of mesenchymal traits, enhanced cell proliferation and invasion. DNAJC6 positively regulated expression of EMT-related transcription factor, also activating transforming growth factor β (TGF-β) pathway to contribute to EMT. Our findings demonstrated an important function of DNAJC6 in the progression of HCC by induction of EMT, and they implicate DNAJC6 as a marker of poor outcome in HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent human malignancies which account for ~700,000 deaths annually and with an estimated 21,000 new diagnosed cases [1]. HCC has become a serious threat to human health due to its rising incidence and high metastatic recurrence and mortality rates [2]. The development and progression of HCC involves a multi-step, long-term process. The elucidation of the molecular mechanisms underlying the tumorigenicity, invasion and metastasis of HCC is critically important for the development of novel treatments for this disease.

DNAJC6 (DNA/HSP40 homolog subfamily C member 6) encodes auxilin, which was originally identified as a clathrin-associated protein that was neuro-specific and enriched in nerve terminals, suggesting that it may play a role in synaptic vesicle recycling [3]. Auxilin is the co-chaperone that recruits HSC70 to the clathrin coated vesicles (CCVs) for disassembly [4–7]. Defective auxilin causing Parkinsonism is in line with Parkinsonism often being an endosomal disorder commonly related to CCVs and synaptic vesicle recycling. Recent study indicated that DNAJC6 is responsible for juvenile Parkinsonism with phenotypic variability [8]. However, the role of DNAJC6 in carcinogenesis and progression is limited.

In this study, we evaluated the DNAJC6 expression levels in 30 tumor tissues of patients with HCC and found that DNAJC6 was significantly up-regulated in HCC. The Kaplan–Meier analysis indicated that the patients with high DNAJC6 expression had a poor outcome than the patients with low DNAJC6 expression. Based on functional analyses, we proved that DNAJC6 could promote HCC cell proliferation *in vitro*, whereas depletion of DNAJC6 inhibits HCC cell proliferation and invasion *in vitro* and tumor growth *in vivo*. Furthermore, DNAJC6 activates the transforming growth factor β (TGF-β) pathway to contribute to epithelial–mesenchymal transition (EMT). These findings suggest that DNAJC6 is a potential therapeutic target in HCC.

2. Materials and methods

2.1. Human tissue specimens

Tissue samples were obtained from 102 HCC patients who underwent surgery for tumor resection at the first hospital of Shijiazhuang city from February 2003 to December 2008. Thirty-four patients (33.3%) developed tissue-proven metastases, 10–60 months after resecting the primary HCC. Specimens from 102 patients with HCC

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and 30 cases of adjacent histologic normal tissues were immediately frozen in liquid nitrogen and stored at −80 °C until use. Follow-up data were obtained by reviewing the hospital records, direct communication with the patients after hepatic resection for all 102 patients. The DFS was defined as the length time after hepatectomy for HCC during which a patient survives with no sign of HCC. This study was approved by the Institutional Review Board of the first hospital of Shijiazhuang city and written consent was obtained from all participants.

2.2. Immunohistochemistry

Tissue samples embedded in paraffin were cut into 5 μm sections and stained according to standard immunohistochemistry protocols. After deparaffinized and rehydrated, tissue sections were performed antigen retrieval using citric acid (pH6.0) at 97 °C for 30 min and followed by treatment with 0.3% H_2O_2 for 10 min. Then, the slides were blocked with 10% BSA for 1 h at room temperature (RT). Primary antibody for DNAJC6 (Santa Cruz Biotechnology, CA, USA) was incubated overnight at °C and then the slides were incubated with peroxidase-conjugated secondary antibody at RT for 1 h. Next, the sections were treatment with diaminobenzidine and counterstained by haematoxylin.

2.3. Cell culture

Human HCC cell line SMMC-7721, Huh-7, HepG2 and Hep3B were purchased from the American Type Culture Collection (ATCC) and routinely maintained in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

2.4. Antibodies, plasmid and generation of stable cell line

Goat polyclonal anti-DNAJC6, rabbit polyclonal anti-β-actin, rabbit polyclonal anti-E-cadherin, rabbit polyclonal anti-Vimentin, rabbit polyclonal anti-ZO-1, rabbit polyclonal anti-N-cadherin, rabbit polyclonal anti-twist1, rabbit polyclonal anti-Smad2/3, rabbit polyclonal anti-pSmad2, mouse monoclonal anti-Slug and mouse monoclonal anti-ZEB1 antibodies were obtained from Santa Cruz Biotechnology. shRNA targeting DNAJC6 were also purchased from Santa Cruz Biotechnology. Following the manufacturer's instructions, an independent HepG2 stable transfectant was screened for depleted DNAJC6 and overexpressed DNAJC6 after 21 days selection in 800 μg/ml G418 (Invitrogen). Transfectant was routinely cultured under selection. The full-length coding region of DNAJC6 complementary DNA was PCR-amplified using following primers: 5′-GGAATTCATGAGCCTCCTCGGGAGCTAC-3′ and 5′-CCTCGAGATATAAGGGCTTTTGGCCTTG-3′, and then cloned into EcoR I and Xho I restriction sites of the mammalian expression vector pcDNA3.1-Neo (Invitrogen) (pcDNA3.1-DNAJC6).

2.5. Transfection

The cells were transfected with siRNA at a final concentration of 50 nM or 4 μg pcDNA3.1-DNAJC6 or control vectors using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instruction and the mRNA and protein levels in total cell lysates were analyzed by RT-qPCR and Western blot after 24 or 48 h.

2.6. Western blot

Protein lysates were separated by SDS–PAGE, and electrophoretically transferred to PVDF membrane (Millipore, Bedford, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with primary antibody at 4 °C overnight. Next day, the membranes were incubated with horseradish peroxidase conjugated-secondary antibody, and the bands were visualized using ECL detection reagents (Millipore). β-Actin served as the loading control.

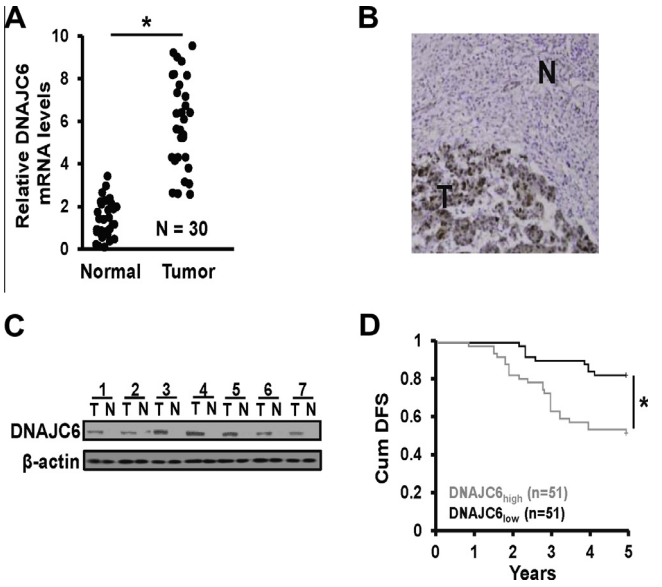

Fig. 1. DNAJC6 is up-regulated in HCC tissues and associated with shorter DFS. (A) The DNAJC6 mRNA expression in 30 cases of primary HCC tissues and the paired adjacent normal tissues. (B) and (C) The DNAJC6 protein levels in primary HCC tissue and the adjacent normal tissue by IHC (B) and Western blot (C). *T* = primary HCC tissue; *N* = the adjacent normal tissue. (D) The Kaplan–Meier plot of 5-year DFS curves stratified by DNAJC6 mRNA expression in 102 HCC patients. $^*P < 0.05$.

2.7. RNA isolation and reverse transcription-quantitative PCR (RT-qPCR)

Total RNA from HCC cells was extracted using Trizol Reagent (Invitrogen) according to the protocol provided by the manufacturer. Total RNA was reversely transcribed with the PrimeScript

Table 1
Relationship between clinicopathological factors and DNAJC6 expression in 102 HCC patients.

Clinicopathological factors	$DNAJC6_{low}$	$DNAJC6_{high}$	*P*
Age			
<55	31	35	0.575
⩾55	19	17	
Size (cm)			
⩽2	30	18	0.017*
>2	21	33	
Stage			
I–II	42	33	0.043*
III	9	18	
Grade			
I–II	43	32	0.014*
III	8	19	
Portal vein tumor thrombus			
Negative	30	28	0.776
Positive	10	8	
Missing	11	15	
HBV infection			
Negative	20	22	0.528
Positive	25	21	
Missing	6	8	

* $P < 0.05$.

RT reagent Kit (TaKaRa, Japan). RT-qPCR for the expression of mRNA analysis was performed using SYBR Green qRT-PCR master mix (TaKaRa) as described using GAPDH for normalization. The triplicate CT values of detectable gene were averaged, and the CT value of GAPDH was subtracted to obtain ΔCT. All samples were normalized to internal controls and fold changes were calculated through relative quantification ($2^{-\Delta CT}$).

2.8. Invasion assay

The invasion of cells *in vitro* was measured by the invasion of cells through Matrigel-coated transwell inserts (8 μm pore size, BD Biosciences, San Jose, CA, USA). 1×10^5 cells were seeded into the upper chamber with serum-free medium. DMWM with 10% FBS were put into lower compartment as chemo-attractant. Cells were allowed to migrate for 16 h. Remaining cells in the upper chamber were scraped out by cotton swap. The cells that had migrated to the lower surface of the membrane were fixed with 100% methanol and stained with crystal violet, followed by counting in five random optical fields.

2.9. Proliferation assay

Cell proliferation was analyzed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay as previously reported [9]. Briefly, cells were seeded in 96-well plates at a density of 1500 cells/well for 0, 24, 48 and 72 h. 20 μL of MTT (5 mg/ml) (Sigma, St. Louis, MO, USA) was added to each well and incubated at 37 °C for 3 h. Subsequently, the MTT solution was removed, DMSO was added and the absorbance value (OD) of each well was measured at 570 nm.

2.10. Xenografts assays in nude mice

Cells stably expressing negative control siRNA (shControl), cells stably expressing DNAJC6 siRNA (shDNAJC6) or cells stably expressing negative vector (Control), cells stably expressing

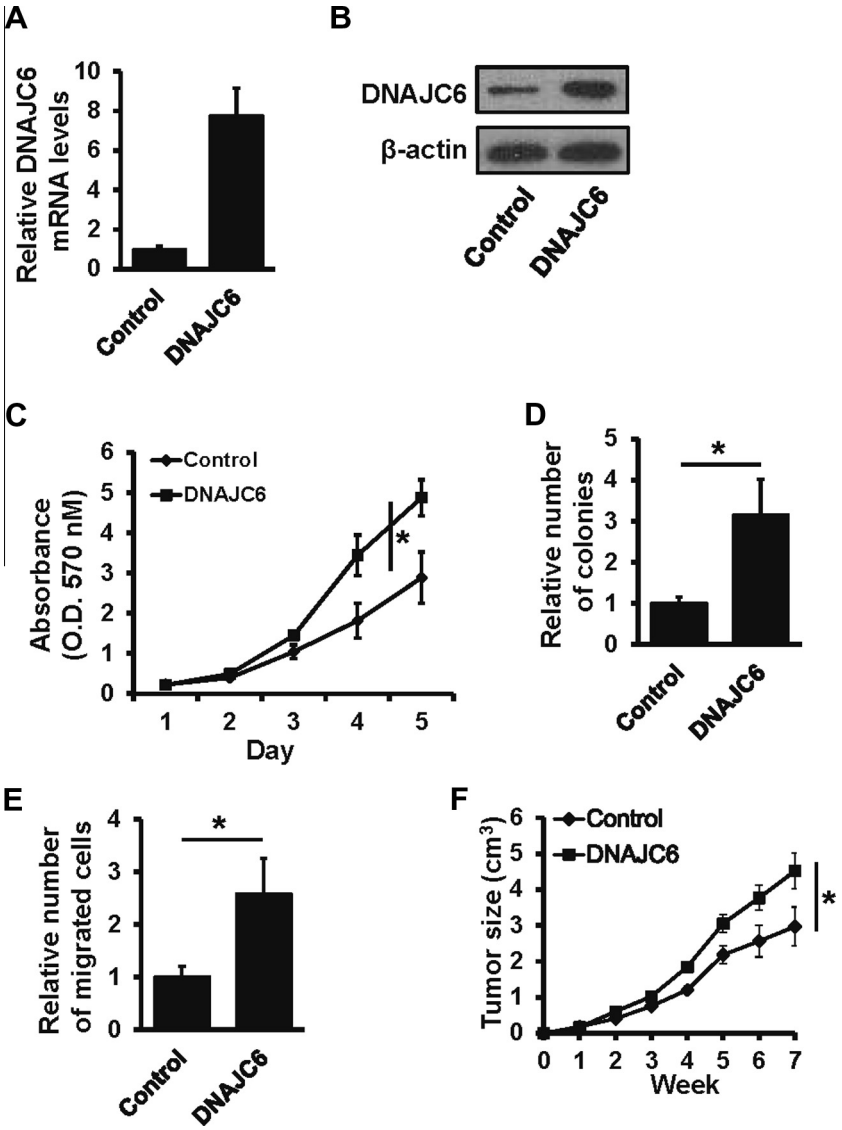

Fig. 2. Overexpression of DNAJC6 promotes HCC cell proliferation and migration *in vitro*. (A) and (B) DNAJC6 expression in DNAJC6-transfected and control HepG2 cells detected by RT-qPCR (A) and Western blot (B). (C) and (D) Cell proliferation ability of DNAJC6-overexpressed and control HepG2 cells by MTT (C) and colony formation (D) assays, respectively. (E) Cell migration ability of DNAJC6-overexpressed and control HepG2 cells by transwell assay. (F) 1×10^7 cells as in (A) were inoculated into the right subaxillary region of athymic nude mice and tumor growth was recorded with a caliper-like instrument. $^*P < 0.05$.

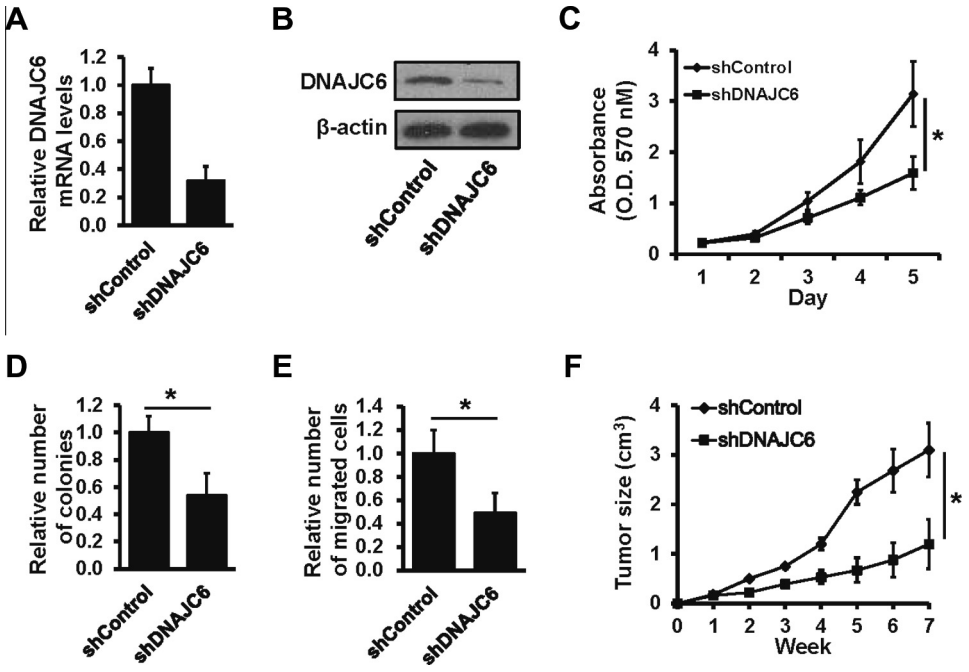

Fig. 3. Depletion of DNAJC6 expression suppresses CRC cell proliferation and migration *in vitro* and tumor growth *in vivo*. (A) and (B) The DNAJC6 expression of HepG2 cells stable expressing DNAJC6 siRNA (shDNAJC6) or non-sense siRNA (shControl) RT-qPCR (A) and Western blot (B). (C) and (D) Cell proliferation ability of DNAJC6-depleted and control HepG2 cells by MTT (C) and colony formation (D) assays, respectively. (E) Cell migration ability of DNAJC6-depleted and control HepG2 cells by transwell assay. (F) 1×10^7 cells as in (A) were inoculated into the right subaxillary region of athymic nude mice and tumor growth was recorded with a caliper-like instrument. $^*P < 0.05$.

DNAJC6 (DNAJC6) were inoculated into the right subaxillary region of athymic nude mice ($n = 5$, per group). Tumor growth was recorded once a week with a caliper-like instrument. Tumor volume was calculated according to the formula volume = (width2 × length)/2. Seven weeks after inoculation, mice were killed, and the final volume and weight of tumor tissues were determined.

2.11. Statistical analysis

One-way analysis of variance (ANOVA) was used to analyze differences among clinicopathological variables by DNAJC6 mRNA expression. A Student's *t*-test was used to analyze differences between 2 experimental groups. DNAJC6 expression was dichotomized at the median, which is the most conservative cutoff point for categorizing a continuous variable [10]. Survival analysis was carried out according to the methods of Kaplan and Meier. All calculations were performed with the SPSS for Windows statistical software package (SPSS Inc., Chicago, IL, USA). A P value < 0.05 was considered statistically significant.

3. Results

3.1. The DNAJC6 expression in HCC tissues

To evaluate the expression of status of DNAJC6 in HCC, we first performed reverse transcription-quantitative PCR (RT-qPCR) for 30 paired of HCC and corresponding adjacent normal liver tissues. The results showed that the DNAJC6 expression level was significantly higher in HCC tissues than that in the adjacent normal liver tissues (Fig. 1A). Subsequently, we validated our results by IHC and Western blot for 7 matched pairs of HCC and normal tissues. The results showed that the protein expression level of DNAJC6 was also elevated in HCC tissues (Fig. 1B and C). These results shows that DNAJC6 expression is elevated in HCC tissues.

3.2. The relationship between DNAJC6 expression and clinicopathological factors

To further investigate the clinical significance of DNAJC6 in HCC, we examined DNAJC6 expression in 102 HCC samples by RT-qPCR. When dichotomized at the median expression level, down-regulation of DNAJC6 was associated with significantly decreased DFS in a Kaplan–Meier analysis (Fig. 1D).

To further analyze the association between DNAJC6 mRNA levels and clinicopathological factors, we compared the distribution of DNAJC6 mRNA levels with different clinicopathological factors, including patient age, tumor size, clinical stage, grade portal vein tumor thrombus and HBV infection. As shown in Table 1, the DNAJC6 expression levels were correlated with tumor size, clinical stage and grade. The $DNAJC6_{high}$ group had larger tumor size ($P = 0.017$), advanced clinical stage ($P = 0.043$) and high grade ($P = 0.014$) compared to the $DNAJC6_{low}$ group (Table 1).

Together, these results indicate that up-regulation of DNAJC6 expression predicts poor outcome.

3.3. Overexpression of DNAJC6 promotes HCC proliferation and invasion in vitro and tumor growth in vivo

For an in-depth understanding the role of DNAJC6 in HCC, we transfected pcDNA3.1-DNAJC6 into HepG2 cells and constructed the stably cell clones. As shown in Fig. 2A and B, the DNAJC6 expression was significantly up-regulated in DNAJC6-transfected cells. MTT analysis showed that HepG2-DNAJC6 cells grew much faster than control cells (Fig. 2C). Colony formation assay also revealed that HepG2-DNAJC6 cells formed more colonies than control cells (Fig. 2D). These results indicated that overexpression of DNAJC6 significantly promotes cell growth in HepG2 cells. To test whether overexpression of DNAJC6 affect the cancer cell invasion, we performed transwell assay. As shown in Fig. 2E, the number of migrated cells was much higher in DNAJC6-transfected cells than

control cells, suggesting that DNAJC6 promotes cell invasion in HCC. Together, these results indicated that overexpression of DNAJC6 promotes cell proliferation and invasion in HCC *in vitro*.

We next performed xenograft assays in nude mice to determine if overexpression of DNAJC6 in HepG2 cells was able to affect tumor growth *in vivo*. DNAJC6 and control cells (1×107) were injected into the right subaxillary region of nude mice ($n = 5$ per group). Tumor formation and volume of the tumor in each mouse were examined, measured and recorded for seven weeks and tumor growth curves were determined. As shown in Fig. 2F, tumor

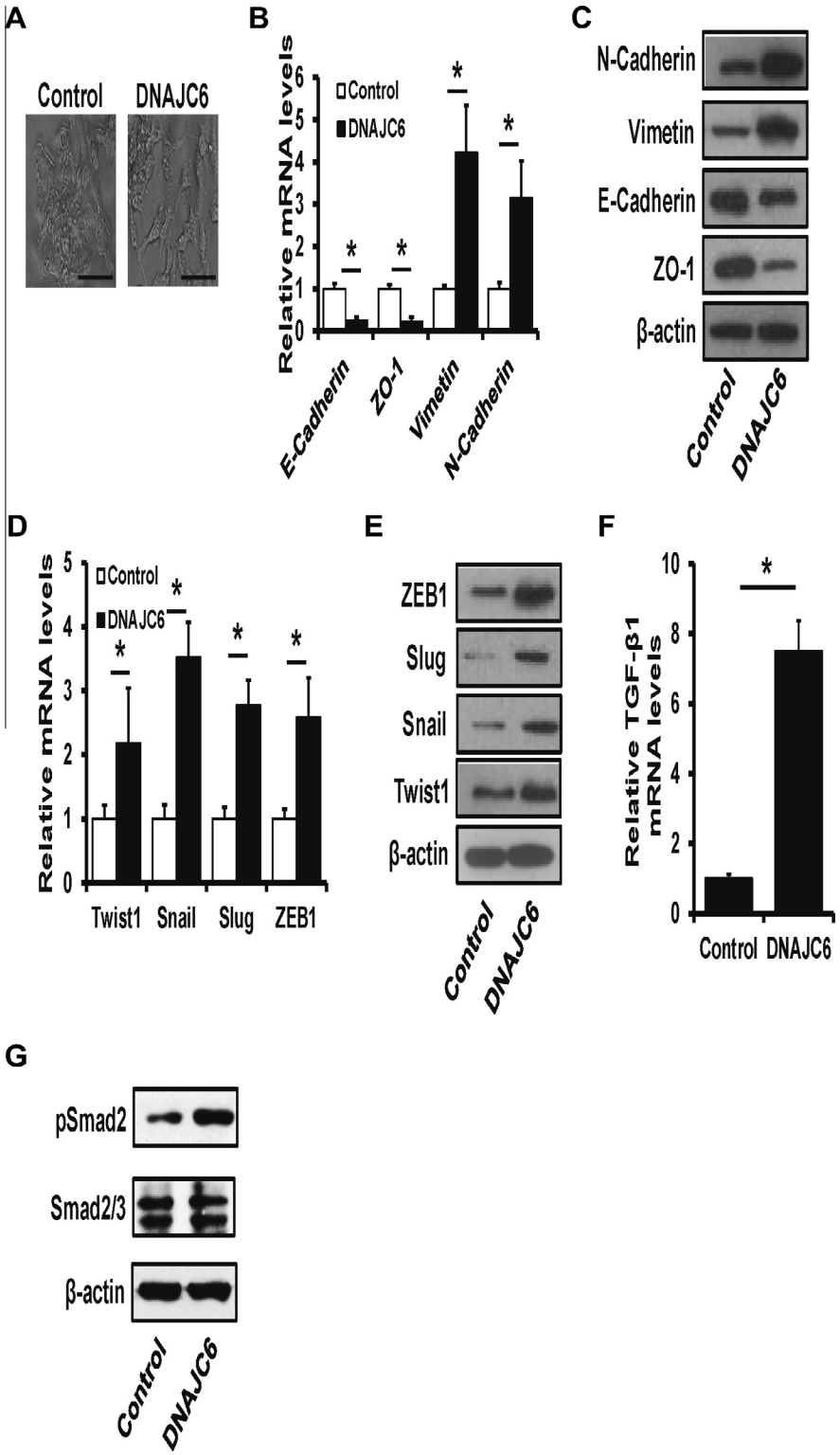

Fig. 4. Overexpression of DNAJC6 induces an EMT-like phenotype. (A) Morphologic change of DNAJC6-overexpressed and control HepG2 cells. (B) and (C) RT-qPCR (B) and Western blot (C) analyses for the indicated EMT markers in DNAJC6-overexpressed and control HepG2 cells. (D) and (E) RT-qPCR (D) and Western blot (E) analyzed for some EMT-related transcription factors in the DNAJC6-overexpressed and control HepG2 cells. (F) RT-qPCR analyzed for TGF-β1 expression. (G) Western blot of pSmad2 and total Smad2/3 protein. Scale bar, 200 μm, $^{*}P < 0.05$.

growth of DNAJC6 cells was significantly increased in nude mice when compared with those of control cells, suggesting that overexpression of DNAJC6 promotes tumor growth *in vivo*.

Thus, these results indicate that overexpression of DNAJC6 promotes HCC proliferation and invasion *in vitro* and tumor growth *in vivo*.

3.4. Depletion of DNAJC6 suppresses HCC proliferation and invasion in vitro and tumor growth in vivo

To evaluate the effect of depletion of DNAJC6 on HCC proliferation and invasion, we selected DNAJC6 RNAi HepG2 clone (shDNAJC6) as well as its control shRNA clone (shControl) for further analyses. RT-qPCR and Western blot analysis indicated that expression levels of DNAJC6 mRNA and protein were down-regulated in shDNAJC6 cells compared with those in shControl cells (Fig. 3A and B). Cell proliferation analyses showed that depletion of DNAJC6 in HepG2 significantly inhibited cell growth. ShDNAJC6 cells grew much slower than that of shControl cells in MTT assay (Fig. 3C). Colony formation assay also revealed that shDNAJC6 cells formed fewer colonies than shControl cells (Fig. 3D). As shown in Fig. 3E, shDNAJC6 cells displayed much lower transwell migratory rates than shControl cells. These results indicated that depletion of DNAJC6 suppresses HCC proliferation and invasion *in vitro*.

We next performed xenograft assays in nude mice to determine if depletion of DNAJC6 in HepG2 cells was able to affect tumor growth *in vivo*. shDNAJC6 and shControl cells (1×107) were injected into the right subaxillary region of nude mice ($n = 5$ per group). Tumor formation and volume of the tumor in each mouse were examined, measured and recorded for seven weeks and tumor growth curves were determined. As shown in Fig. 3F, tumor growth of shDNAJC6 cells was significantly reduced in nude mice when compared with those of shControl cells, suggesting that depletion of DNAJC6 inhibits tumor growth *in vivo*.

Thus, these results indicate that depletion of DNAJC6 expression suppresses HCC proliferation and invasion *in vitro* and tumor growth *in vivo* thereby preventing tumor development.

2.5. Overexpression of DNAJC6 induces EMT in HCC cells

We observed that HepG2 cells transfected with vector retained their cobblestone-like morphology with tight cell–cell adhesion, whereas DNAJC6-transfected cells displayed an elongated fibroblast-like morphology with scattered distribution in culture (Fig. 1A). To further investigate the effects of DNAJC6 on the EMT process of HepG2 cells, the relative mRNA and protein expression of mesenchymal phenotype cell biomarkers (N-cadherin and Vimentin), and epithelial phenotype cell biomarker (E-cadherin and ZO-1) in DNAJC6-transfected cells and corresponding control cells were measured by RT-qPCR (Fig. 4B) and Western blot (Fig. 4C). Our results indicated that forced expression of DNAJC6 in HepG2 cells led to a reduction of E-cadherin and ZO-1 expression and an increased expression of N-cadherin and Vimentin (Fig. 4B and C). Furthermore, the EMT-related transcription factors, Twist1, Snail, Slug, and ZEB1 were significantly up-regulated in DNAJC6-overexpressed HepG2 cells compared to control cells by RT-qPCR (Fig. 4D) and Western blot assays (Fig. 4E). Several lines of evidence have implicated the involvement of TGF-β in EMT process in HCC progression [11]. We next intended to identify whether TGF-β signaling is activated in DNAJC6-induced EMT. As shown in Fig. 4F, the TGF-β1 mRNA level was increased in DNAJC6-transfected HepG2 cells. In addition, the level of phosphorylated Smad2 protein, a downstream effector of TGF-β pathway, was significantly increased in DNAJC6-transfected HepG2 cells (Fig. 4G). Collectively, these results indicated that DNAJC6 promotes HCC progression through induction of EMT.

4. Discussion

DNAJC6 belongs to the evolutionarily conserved DNAJ/HSP40 family of proteins, which regulate molecular chaperone activity by stimulating ATPase activity. DNAJ proteins may have up to 3 distinct domains: a conserved 70-amino acid J domain, usually at the N terminus, a glycine/phenylalanine (G/F)-rich region, and a cysteine-rich domain containing 4 motifs resembling a zinc finger domain [12]. Diseases associated with DNAJC6 include Parkinson's disease and mental retardation epilepsy [8,13]. However, the role of DNAJC6 in carcinogenesis and progression is limited. In the current study, we demonstrated that DNAJC6 is up-regulated in HCC tissues and up-regulation of DNAJC6 correlated to advanced stage, high grade and large tumor size. Furthermore, our results revealed that overexpression of DNAJC6 expression in human HCC cells promotes cell proliferation and invasion *in vitro* and tumor growth *in vivo*. Moreover, we showed that depletion of DNAJC6 inhibits human HCC cells proliferation and invasion *in vitro* and tumor growth *in vivo*. These results indicated that DNAJC6 as a potential oncogene in HCC development and progression.

EMT occurs frequently during normal development in processes such as mesoderm and neural crest cell formation. During cancer progression, EMT is also crucial for loss of cell polarity of epithelial cells, thus facilitating migratory and invasive behavior [14,15]. Our results indicated that DNAJC6 induces EMT programs of human HCC cells, including up-regulation of mesenchymal markers (N-cadherin and Vimentin) and down-regulation of epithelial markers (E-cadherin and ZO-1). Moreover, we have shown in this study that DNAJC6 up-regulates the expression of several EMT-related transcription factors, notably the Twist1, Snail, Slug, and ZEB1 and an increase in cell and invasion *in vitro*. These findings demonstrated that DNAJC6 is not only linked to cell proliferation but promotes EMT by activation of essential effectors of the process such as Twist1, Snail, Slug, and ZEB1.

EMT can be induced or regulated by various growth and differentiation factors, including TGF-β, growth factors that act through receptor tyrosine kinases, such as fibroblast growth factor, and Wnt and Notch proteins [16,17]. Among these, TGF-β has received much attention as a major inducer of EMT during embryogenesis, cancer progression and fibrosis. Our results showed that DNAJC6 can activate TGF-β signaling thereby inducing EMT, resulting in HCC development and progression.

In summary, we demonstrated that DNAJC6 is up-regulated in human HCC, and DNAJC6 exhibits oncogene activity that affects the HCC cell growth, invasion, and metastasis both *in vitro* and *in vivo* through induction of EMT. This study suggests that DNAJC6 may be a potential target for HCC therapy.

Conflict of interest

The authors have no financial interests in or financial conflict with the subject matter discussed in this manuscript.

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