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ORIGINAL ARTICLE

Human β-defensin 1 functions as a tumor suppressor *via* ER stress-triggered JNK pathway in hepatocellular carcinoma

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Summary

Purpose: *Human β-defensin 1 (DEFB1) belongs to defensins family that contribute to innate immune responses and was recently found to downregulate a variety of cancers, including renal, prostatic, and oral squamous cell carcinoma, and therefore is considered as a potential tumor suppressor. However, the role of DEFB1 in hepatocellular carcinoma (HCC) still needs to be elucidated.*

Methods: *Quantitative PCR and Western blot were used to measure the expression levels of interested proteins. CCK-8 and colony formation assays were performed to determine the ability of cell proliferation. Tumor formation experiments in nude mice were used to examine the tumor growth.*

Results: *The expression level of DEFB1 was dramatically downregulated in human HCC. Quantitative PCR and Western blot results also showed a pronounced decrease of DEFB1 expression in the liver cancer cell lines. Rescuing the expression of DEFB1 in Huh7 cells effectively suppressed cell proliferation and reduced the colony forming ability, probably by inducing cell apoptosis and cell cycle arrest. Moreover, tumor formation experiments in nude mice also showed inhibition of tumor growth by DEFB1 expression in vivo. Furthermore, induction of DEFB1 expression induced degraded protein increase and endoplasmic reticulum (ER) stress, which subsequently activated JNK pathway. Pharmacologic inhibition of ER stress by 4-phenylbutyrate, a compound to alleviate ER stress, effectively eliminated DEFB1-induction inhibition of cell proliferation and migration.*

Conclusion: *DEFB1 functions as a tumor suppressor in HCC through activating ER stress and JNK pathway, which may provide a potential strategy for HCC treatment.*

Key words: *DEFB1, defensins, hepatocellular carcinoma, tumor suppressor, ER stress*

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide and occurs most commonly in people with chronic liver diseases, especially cirrhosis caused by hepatitis B or hepatitis C infection [1,2]. According to the global cancer statistics in 2018, HCC is ranked the sixth most frequently diagnosed malignant tumor and the fourth leading cause of cancer-related mortality in the world [2]. The high mortality rate of HCC has been demonstrated to be a lack of biomarkers for early diagnosis and high rate of metastasis and recurrence after surgical excision [4]. Recently, despite considerable advances in the treatment of HCC, the overall survival rate of HCC remains unsatisfactory [5]. Therefore, it is urgent to understand the molecular mechanisms underlying the HCC genesis and identify new candidates for early detection and development of new therapeutic strategies.

Defensins are small, cysteine-rich cationic, antimicrobial peptides and play a critical role in the innate immune response [6,7]. Human β-defensin 1 (DEFB1) belongs to defensins family and mainly

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expresses and acts as an anti-microbial peptide against bacterial and fungal infections in various epithelial tissues, such as the skin, respiratory and urogenital tract [8-10]. Previous studies also found that DEFB1 prevents bacterial invasion for host defense in innate immunity [11]. In addition to the role of DEFB1 in antimicrobial and innate immune signaling activities, accumulating evidence demonstrated that DEFB1 was downregulated in a variety of cancers, including renal, prostatic, and oral squamous cell carcinoma [12-16], while rescuing DEFB1 expression effectively suppressed the proliferation of prostate, renal, and bladder cancer cells [17], suggesting DEFB1 may function as a potential tumor suppressor. Moreover, human DEFB1 has been found to inhibit tumor migration and invasion and may serve as an independent prognostic marker for oral squamous cell carcinoma [18].

DEFB1 was also detected in liver tissue and contributed to the biliary antimicrobial defense response [19-21]. A previous study has demonstrated a role of DEFB1 in HCV infection and liver cancer development [22]. In the present study, we systematically investigated the potential role of DEFB1 in gastric cancer and found that the expression level of DEFB1 was dramatically downregulated in human HCC. Rescuing the expression of DEFB1 dramatically inhibited cell proliferation and tumor growth *in vitro* and *in vivo*. This inhibitory effect of DEFB1 was mediated through ER stress and JNK signal pathway, which may provide a novel strategyfor the treatment of HCC.

Methods

Plasmids and antibodies

Complementary DNA (cDNA) for humanDEFB1 were amplified from reverse-transcribed cDNA of HepG2 cells. Antibodies for AKT (4691), Phospho-AKT (4060), Erk1/2 (4695),and Phospho-Erk1/2 (4370) were purchased from Cell Signaling Technology (Danvers, MA, USA); antibodies for PERK (ab229912), ATF6 (ab227830), IRE1A (ab37073), JNK (ab179461), P-JNK (ab124956), DEFB1(ab170962) were purchased from Abcam (Cambridge, MA, USA). Anti-HA (H3663) tag and anti-β-actin (A5441) antibody was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cells and cell culture

LO-2, HepG2, Huh7 and HCCLM3 were all from National Infrastructure of Cell Line Resource (Peking Union Medical College, Beijing, China). All the cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% penicillin-streptomycin in a 37°C incubator with an atmosphere of 5% CO_2 and 95% air. Cells were passaged with Trypsin 2-3 times per week.

Evaluation of cell proliferation

Cell proliferation was assessed with Cell Counting Kit-8 (CCK-8; Beyotime, Shanghai, China, C0038) according to the manufacturer's instructions. Briefly, 6000 cells were suspended with 200 μL complete medium, and then were plated in 96-well plates; 20 μL of CCK8 reagent was added to each well and incubated for 60 min at 37°C. Molecular Device M2 was used tomeasure the absorbance at optical density (OD) 450.

Colony formation assay

Cells were harvested by trypsinization and resuspended to produce a single-cell suspension. 500 cells/well were plated into culture plates for triple experiments. Cell colonies were washed with phosphate buffered saline (PBS) and then were fixed and stained by a mixture of 6.0% glutaraldehyde and 0.5% crystal violet for 30 min. The glutaraldehyde crystal violet mixture was carefully removed and rinsed with tap water. The dishes or plates with colonies were left to dry in air at room temperature. Colonies were pictured and counted for analysis.

Tumor growth in nude mice

All procedures of animal experiment were approved by Animal Experimental Ethics Committee of Inner Mongolia Medical University. Female athymic BALB/c nude mice (aged 5-6 weeks) were purchased from the Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). To establish xenograft model, nude mice were randomized into two groups and 5×10^6 cells were respectively injected subcutaneously into the right flanks of mice of each group. Electronic caliper was used to measure the length and width of each tumor, and tumor volume was estimated by applying the following equation: volume = length×width2/2.

Cell migration assay

Cell migration was analyzed by Boyden chamber assay. Briefly, cells were harvested by trypsinization and then resuspended in serum-free medium, 8000 cells were plated into the upper chamber, and complete medium was added into the lower reservoir. Cells were cultured for 12 h before fixation by 70% ethanol, and then were stained with crystal violet for 30 min. The membranes were pictured under microscope. The cell migration ability was determined by the cell number in the membrane.

Quantitative real-time PCR

Total RNA was isolated from cells *via* TRIzol-based protocol (Invitrogen, Carlsbad, CA, USA), and complementary DNA (cDNA) was synthesized by a reverse transcriptase with oligo-(d)Ts. Real-time quantitative PCR analyses were performed using the ABI7500, Real-Time PCR System (Bio-Rad, Shanghai, China). The primer sequences were as follows: human glyceraldheyde 3-phosphate dehydrogenase (GAPDH): sense, 5´- GAA GGT GAA GGT CGG AGT C-3´; antisense, 5´-GAA GAT GGT GAT GGG ATT TC-3´; DEFB1: sense, 5´- CTC AGG TGG TAA CTT TCT CA-3´; antisense, 5´- TGT AAC AGG TGC CTT GAA T -3´.

Cell apoptosis assay

Cell apoptosis was analyzed by Annexin V/propidium iodide (PI) staining. Briefly, cells were collected by trypsinization and washed with PBS; cells were then resuspended in 1X binding buffer and then were stained with annexin V-FITC and PI at room temperature for 5 minutes in the dark. Stained cells were analyzed by flow cytometry.

Cell cycle assay

Cells were harvested with trypsinization and were fixed with ice-cold 70% ethanol at 4°C for 30 min, and then were centrifuged and washed with PBS twice at 850 g. RNase was added to remove the RNA and stained with PI. PI signals were collected by flow cytometry and the cell cycle profile was analyzed by Modfit.

Immunoblotting

Total cell lysate was prepared by radioimmunoprecipitation assay (RIPA) lysis buffer, and concentrations were determined by bicinchoninic acid (BCA) assay. 30μL of total protein were loaded and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, and then were transferred onto polyvinylidene fluoride (PVDF) membrane, which was blocked by 5% non-fat milk before incubation with primary antibody and secondary antibody. Chemiluminescence signal was collected by adding luminal reaction mix and pictured by Tanon-5200Multi.

Statistics

The data were expressed as mean ± standard deviation. T-test and one-way analysis of variance (ANOVA) were used for the statistical analyses, using SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). P<0.05 showed statistical significance.

Results

DEFB1 is downregulated in hepatocellular carcinoma

DEFB1 was recently found to act as a tumor suppressor in a variety of cancers. To investigate the potential role of DEFB1 in HCC, the expression level of DEFB1 was first analyzed in human HCC samples from the clinically annotated genomic database, The Cancer Genome Atlas (TCGA). Compared with adjacent normal liver tissue, DEFB1 expression was significantly downregulated in HCC (Figure 1A). Moreover, compared with normal human livercell line LO2,the expression level of DEFB1 was also detected in different liver cancer cell lines, including HepG2, Huh7 and HCCLM3. Interestingly, quantitative PCR analysis showed that the mRNA level of DEFB1 was greatly diminished in all these cell lines (Figure 1B). Western blot results showed similar change in the protein level of DEFB1 (Figure 1C), suggesting that DEFB1 may play an important role in the progression of HCC.

Rescuing DEFB1 expression inhibits cell proliferation and tumor growth in vitro and in vivo

Next, the effect of DEFB1 in the development of liver cancer was investigated by overexpress-

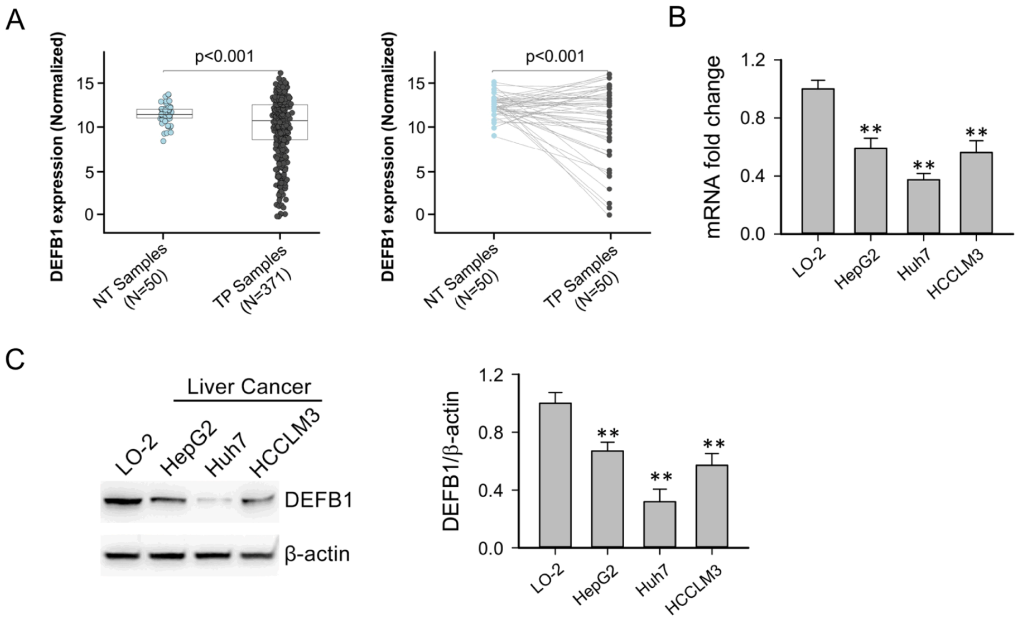

Figure 1. DEFB1 is downregulated in hepatocellular carcinoma. **A:** The expression level of DEFB1 in the clinical data from TCGA database. 50 normal and 371 patients in hepatocellular carcinoma. **B** and **C:** Quantitative PCR analysis and Western blot results showing the mRNA expression of DEFB1 in HepG2, Huh7 and HCCLM3 cancer cell lines. n=5. **p<0.01 versus normal Lo-2 group.

ing DEFB1 levels in Huh7 cell line. As shown in Figure 2A, DEFB1 was overexpressed after infection of lentivirus that contained HA-DEFB1 vector. Cell Count Kit 8 assay was performed to determine the cell proliferation ability. We found that DEFB1 overexpression caused a pronounced decrease in cell proliferation in a time-dependent manner (Figure 2B). Moreover, colony forming assay showed that the colony forming ability was also significantly impaired after DEFB1 overexpression (Figure 2C). To confirm this *in vivo*, Huh7 cells with DEFB1 overexpression were subcutaneously injected into nude mice. After 5 weeks, mice in the DEFB1 overexpression group developed a significantly larger tumor size that the control group (Figure 2D).

Transwell assay was also conducted to investigate the effect of DEFB1 on cell migration and invasion of Huh7 cells. The cell migration ability was determined by counting the cell numbers that migrated through the membranes after 24 h. As shown in Figure 2E, the migration ability was significantly decreased by DEFB1 overexpression. These results indicated an inhibitory effect of DEFB1 in cell proliferation, migration, and tumor growth in Huh7 cells.

DEFB1 overexpression induces cell apoptosis and cell cycle arrest

To understand how DEFB1 affected cell proliferation, the effect of DEFB1 overexpression on cell apoptosis was examined using PI staining combined and flow cytometry analysis. We found that induction of DEFB1 expression dramatically elevated the apoptotic rate in Huh7 cells (Figure 3A). Consistent with this result, the expression level of pro-apoptotic markers, such as Bax, caspase-3, was upregulated, while anti-apoptotic protein, Bcl-2, was downregulated (Figure 3B). Moreover, the cell cycle profile after DEFB1 overexpression was also evaluated by flow cytometry. As shown in Figure 3C, DEFB1 overexpression in Huh7 cells caused a pronounced increase in the number of S-phase cells, while it dramatically decreased G2/M-phase cells. These results indicated that DEFB1 inhibited cell proliferation by inducing cell apoptosis and cell cycle arrest.

DEFB1 overexpression triggers ER stress and activates JNK pathway in Huh7 cells

To understand the possible mechanisms underlying the DEFB1-mediated liver cancer cell growth and migration, we screened a variety of

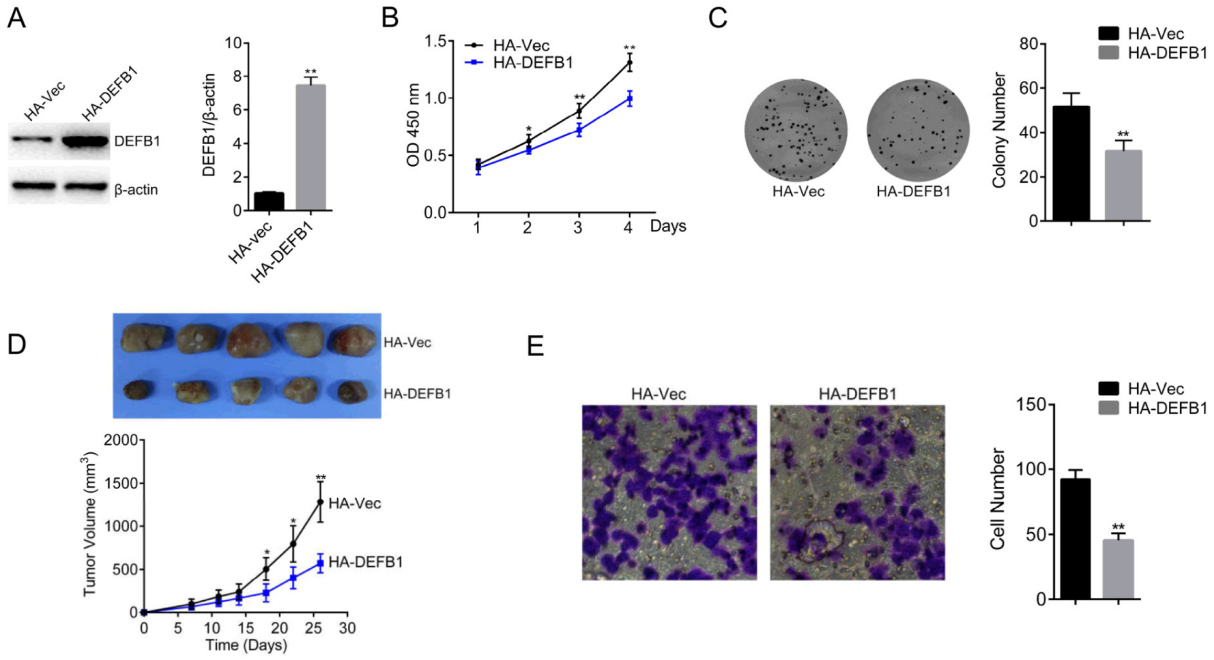

Figure 2. DEFB1 overexpression inhibits cell proliferation and tumor growth *in vitro* and *in vivo*. **A:** DEFB1 was overexpressed in Huh7 cells assayed by Western blot. **B:** CCK8 assay showing the cell proliferation after DEFB1 overexpression. **C:** Left: representative images showing that DEFB1 overexpression caused a pronounced decrease in colony forming ability of Huh7 cells. Right: Collective data of the number colonies. **D:** Left: typical image showing the size of subcutaneous tumors in nude mice five weeks after inoculation. Right: The size of subcutaneous tumors was weighed to measure the rate of cell growth. **E:** Transwell migration assay showing the capacity of cell migration after DEFB1 overexpression. Left: representative images; Right: the number of cells (blue in the image) was counted that migrated through the membrane. **p<0.01 versus HA-vec group.

signaling pathways after DEFB1 overexpression in Huh7 cells. Interestingly, we observed that rescuing DEFB1 expression in Huh7 cells significantly elevated the expression levels of ER sensors, such as PERK, ATF6 and IRE1A (Figure 4A), demonstrating an ER stress caused by DEFB1 overexpression. Moreover, c-Jun N-terminal kinase (JNK) signal pathway that involves multiple important cellular processes, including cell growth, invasion, and apoptosis, was dramatically activated by elevating its phosphorylation upon DEFB1 overexpression (Figure 4B). However, activation of ERK or AKT signals were observed in Huh7 cells after DEFB1 overexpression (Figure 4B), suggesting that JNK signal pathway may mediate the inhibitory effect of DEFB1 in liver cancer cell proliferation.

Pharmacologic inhibition of ER stress and JNK pathway eliminate the inhibitory effect of DEFB1 in cell proliferation and migration

To further investigate the roles of ER stress and JNK pathway in the inhibitory effect of DEFB1 in cell proliferation, we first used 4-phenylbutyrate (4-PBA), a compound that can interact with unfolded or misfolded proteins, to alleviate ER stress. Furthermore, DEFB1-induced ER stress by upregulation of PERK, ATF6 and IRE1A was effectively eliminated upon 2 μM 4-PBA treatment. Importantly, the inhibition of DEFB1 overexpression in the cell proliferation and migration was also impaired by administration of 4-PBA (Figure 4C and 4D).

In addition, SP600125, a common JNK inhibitor, was used to block JNK pathway by inhibiting the phosphorylation of JNK. Expectedly, 20 μM SP600125 suppressed the DEFB1-mediated JNK phosphorylation but did not changed DEFB1-mediated upregulation of PERK, ATF6 and IRE1A, indicating that JNK pathway is the downstream of ER stress. Meanwhile, SP600125 treatment significantly abolished DEFB1-mediated inhibition of cell proliferation and migration. These results indicated that ER stress-triggered JNK pathway activation mediated the inhibitory effect of DEFB1 in cell proliferation and migration of liver cancer cells.

Discussion

Hepatocellular carcinoma has become one of the most common malignancies with high morbidity and mortality worldwide [1-3], which may be attributed to a lack of biomarkers for early diagnosis and high rate of metastasis and recurrence after surgical excision [4,5]. In this study, we demonstrated an inhibitory effect of DEFB1 in

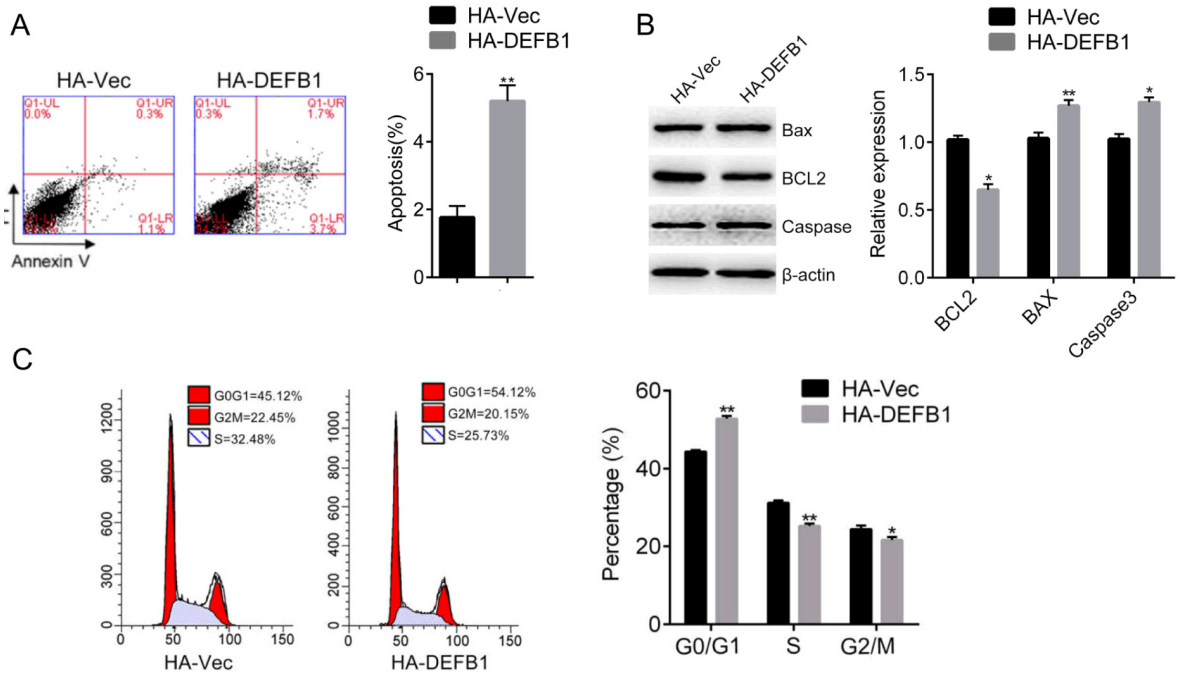

Figure 3. DEFB1 overexpression induces cell apoptosis and cell cycle arrest. **A:** Flow cytometry analysis showing the cell apoptosis was significantly increased after DEFB1 expression in Huh7 cells. **B:** Western blot results showing the expression levels of Bax, caspase-3 and Bcl-2. **C:** Left: representative images of propidium iodide staining and flow cytometry analysis showing cells in different cell cycle stages, including S-phase, G0/G1-phase and G2/M-phase. Right: Comparison of the percentage of S-phase, G0/G1-phase and G2/M-phase cells. *p<0.05, **p<0.01 versus HA-vec group.

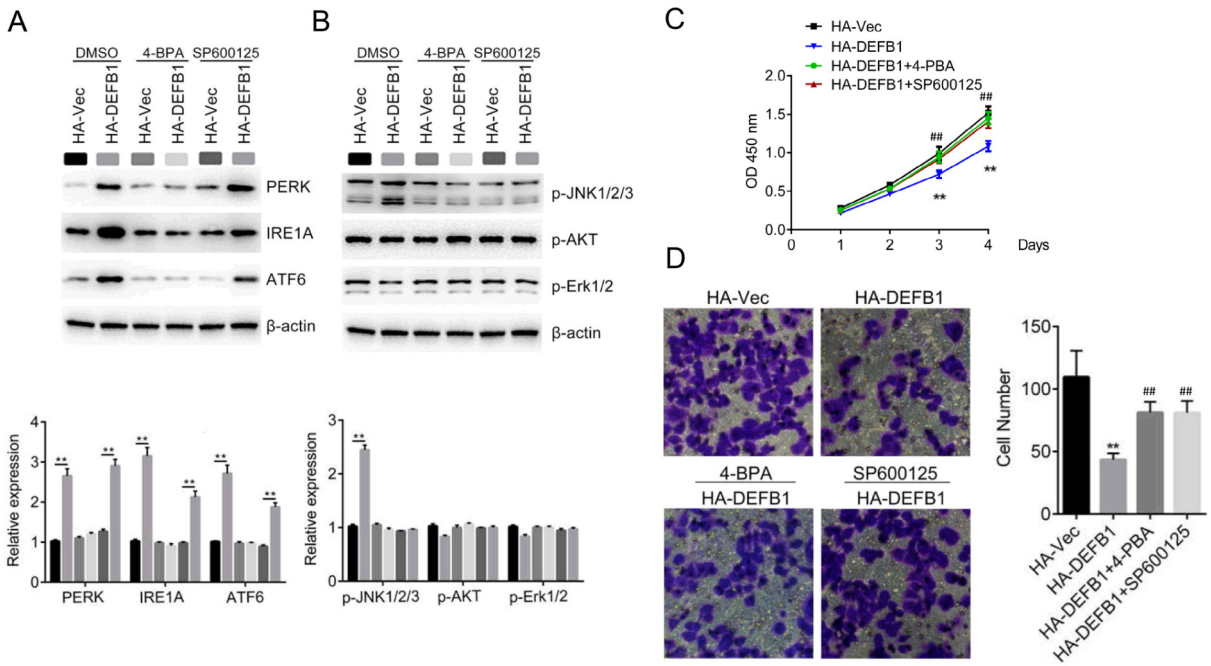

Figure 4. DEFB1 overexpression triggers ER stress and activates JNK pathway in Huh7 cells. **A:** Western blot results showing the expression levels of ER sensors, such as PERK, ATF6 and IRE1A. **B:** Comparison of phosphorylation level of JNK, ERK and AKT upon DEFB1 overexpression. **p<0.01 versus HA-vec group. **C:** CCK assay showing the cell proliferation after 2 μM 4-PBA or 20 μM SP600125 treatment. **D:** Transwell migration assay showing the capacity of cell migration after 2 μM 4-PBA or 20 μM SP600125 treatment. **p<0.01 versus HA-vec group, ##p<0.01 versus HA-DEFB1 group.

cell proliferation and invasion through ER stress-induced activation of JNK signal pathway in HCC. Interestingly, the expression level of DEFB1 was significantly downregulated both in HCC human samples from TCGA database and three liver cancer cell lines, which may allow DEFB1 to serve as a biomarker in HCC.

DEFB1 is an antimicrobial peptide that is mainly expressed in various epithelial tissues, such as the skin, respiratory and urogenital tract, and function as defender against bacteria and fungi [8-10]. Previous investigations of DEFB1 function mainly focus on the host defense in innate immunity [11]. However, in addition to the role of DEFB1 in antimicrobial and innate immune signaling activities, accumulating evidence demonstrated that DEFB1 plays roles in cell growth and tumorigenesis [12]. The downregulation of DEFB1 expression was observed in various cancers, including renal, prostatic, and oral squamous cell carcinoma [12-16]. Importantly, DEFB1 showed an inhibitory effect in the cell proliferation and tumor migration and invasion in prostate, renal, bladder cancer cells and oral squamous cell carcinoma [17,18], suggesting DEFB1 may function as a potential tumor suppressor. In addition, DEFB1 was also detected in liver tissue and contributed to the biliary antimicrobial defense response [19-21]. A role of DEFB1 was found in HCV infection and liver cancer development [22]. In the present study, we reported that DEFB1 attenuated tumor progression in HCC, which provided a new insight about the role of DEFB1 in HCC.

The mechanism of how DEFB1 suppressed the proliferation, migration and invasion of HCC cells was also investigated. The signaling pathways of IRE1, ATF6, and PERK, three ER stress sensors are involved in the unfolded protein response and regulated the functions of endoplasmic reticulum [23]. In our study, the expression of IRE1, ATF6, and PERK was significantly increased after DEFB1 overexpression, suggesting DEFB1 overexpression induced an ER stress in Huh7 cells. Previous reports have demonstrated that ER stress was essential for cancer cell initiation, proliferation, invasion and apoptosis [24,25]. However, the role of ER stress in cancer cell growth is still controversial [25]. Clearly, DEFB1 overexpression triggered ER stress, suppressed cell proliferation and induced cell apoptosis in HCC. It is well established that in response to ER stress, IRE1 activated and recruited TRAF2, which subsequently activated ASK1 and led to JNK activation [26]. Consistently, the phosphorylation level of JNK was dramatically increased during ER stress after overexpression DEFB1, while no activation was observed in the PI3K-AKT

signaling pathway. Pharmacologic inhibition of ER stress and JNK pathway effectively eliminated the inhibitory effect of DEFB1 in cell proliferation and migration, indicating that ER stress induced JNK pathway activation mediated by the inhibitory effect of DEFB1 in HCC. However, how DEFB1 overexpression triggered ER stress still needs to be further investigated.

Conclusions

In summary, we found that the expression level of DEFB1 was dramatically downregulated in human HCC and the liver cancer cell lines. DEFB1 exerts an inhibitory effect in cell proliferation in tumor growth through ER stress induced JNK pathway activation in HCC. Our findings support the idea that DEFB1 function as a tumor suppressor in different cancers.

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Conflict of interests

The authors declare no conflict of interests.

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