重新推荐自治区科技进步奖的项目需提交以下说明材料：

材料名称	上次推荐	本次推荐
推荐年度	2014	2018
项目名称	高尔基体蛋白 73 对肝癌早期诊断及其与肝癌上皮间质转化相关性研究	癌蛋白 GP73 在早期肝癌诊断及逆转侵袭转移的相关研究
创新内容（创新点）	1. 应用 GP73 这一新型肿瘤标志物在肝硬化的高危人群及 AFP 阴性的肝癌患者中进行筛查，提高了肝癌早诊的准确率。 2. 在肝癌组织中对 GP73 的表达与上皮间质转化之间的关系进行了探索性研究，为肝癌侵袭转移的机制提供了新的理论基础。	1. 在肝癌早诊方面：应用血清 GP73 与 AFP 二者联合检测优化了对早期肝癌的准确率；此外还在肝硬化人群及 AFP 阴性的肝癌患者中进行筛查，提高了早诊率，降低了漏诊率。 2. 对 GP73 这一关键分子靶点进行全面的功能学研究，发现 GP73 作为一个癌蛋白，具有促进肝癌增殖、侵袭、转移，抑制凋亡，影响细胞周期的重要调控功能。 3. GP73 通过以上恶性生物学功能影响对药物的敏感性：① GP73 降低肝癌细胞对化疗药物-顺铂的敏感性；② GP73 降低肝癌细胞对靶向药物-索拉非尼的敏感性导致治疗抵抗。 4. 通过分子生物学技术发现 GP73 通过上皮-间质转化促进肝癌的侵袭转移；在体外及小鼠转移瘤模型中均证实：靶向干预 GP73 后，逆转了上皮-间质转化，同时降低了肝癌的侵袭转移性。 5. 深入研究其分子机制发现：GP73 是通过介导 TGF-β1/ Smad2 信号通路调控上皮-间质转化，促侵袭、转移，并且还可能影响 PI3K/Akt 信号发挥作用。

经济效益（万元）	无	无
社会效益	1.申请：国家自然科学基金地区项目（编号：81560388）	1. 立项：自治区自然科学基金青年项目（编号：2014211C070） 2. 立项：新疆维吾尔自治区研究生科研创新项目（编号：XJGRI 2017082） 3.立项：新疆医科大学研究生创新创业项目（编号：CXCY2017021）
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		及 Smad2 的表达水平及其意义，中国全科医学，2016，19(26)：3191-3195.

重新推荐自治区科技进步奖的项目需提交以下说明材料

材料名称	上次推荐	本次推荐
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完成单位	新疆医科大学第一附属医院	新疆医科大学第一附属医院
完成人员	包永星、杨颖 、赵化荣 、张华 、肖蕾、古丽比也·沙比尔、毛睿、张瑞丽、张月芬、曹茜、马静	包永星、杨颖 、赵化荣 、张华 、肖蕾、古丽比也·沙比尔、毛睿、张瑞丽、张月芬、曹茜、马静
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		质转化，同时降低了肝癌的侵袭转移性。 5. 深入研究其分子机制发现：GP73 是通过介导 TGF-β1/Smad2 信号通路调控上皮-间质转化，促侵袭、转移，并且还可能影响 PI3K/Akt 信号发挥作用。
经济效益	无	无
社会效益	1.申请：国家自然科学基金地区项目（编号：81560388）	1. 立项：自治区自然科学基金青年项目（编号：2014211C070） 2. 立项：新疆维吾尔自治区研究生科研创新项目（编号：XJGRI2017082） 3.立项：新疆医科大学研究生创新创业项目（编号：CXCY2017021）
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Silencing of GP73 inhibits invasion and metastasis via suppression of epithelial-mesenchymal transition in hepatocellular carcinoma

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Abstract. Epithelial-mesenchymal transition (EMT) is associated with invasion and metastasis of cancer cells. Golgi protein 73 (GP73) is a serum biomarker for hepatocellular carcinoma (HCC) and our previous study demonstrated that the expression of GP73 correlated with aggressive behavior and EMT molecules in HCC. However, its role in metastatic mechanism of HCC is not clear. The aim of this study was to investigate the effect of GP73 on invasion and migration, and underlying mechanism of GP73 involved in EMT of HCC. The expression of GP73 was downregulated by small interfering RNA (siRNA). The metastatic and invasive abilities were analyzed using scratch assay and Transwell assay. Changes in EMT-related molecules were evaluated by western blot and qRT-PCR analyses, and epithelial-mesenchymal phenotype changes were also observed. Expression of GP73 was upregulated in the more metastatic HCC cell lines. Knockdown of GP73 by siRNA resulted in a significant decrease in migratory and invasive abilities in both MHCC97H and Bel-7404 cell lines. Importantly, EMT-related markers and morphological phenotypes significantly changed following by the inhibition of GP73. Silencing GP73 contributed to the reduction of invasion and metastasis via suppressing EMT in HCC. GP73 may serve as a novel molecular target against EMT in HCC metastasis therapy.

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Key words: Golgi protein 73, metastasis, epithelial-mesenchymal transition, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is the fifth-most common cancer worldwide and the third-leading cause of cancer death (1). Although great advances have been made in multiple therapeutic methods in recent years, the overall survival is poor, with a 5-year survival rate of ~5-6% (2). The dismal prognosis of HCC is mainly attributed to the aggressive metastasis and recurrence of HCC (3). Increasing number of studies have confirmed that the epithelial-mesenchymal transition (EMT) plays a vital role in promoting tumor metastasis, as the EMT process enables tumor cells to acquire migratory and invasive abilities (4-6). Therefore, exploring novel targeted molecular against EMT for HCC metastasis therapy is of great importance.

Golgi protein 73 (GP73, also termed GOLPH2 and GOLM1) is a 73-kDa type-II Golgi transmembrane glycoprotein that was originally cloned from a library derived from the liver tissue of a patient with adult giant cell hepatitis (7). It is reported that expressions of GP73 increased not only in viral infections (8-11) but also in certain cancer types, including HCC, prostate cancer, lung cancer, and gastric cancer (12-17), but more attention has been paid in the aberrant expressions of GP73 in liver diseases. It has been found that GP73 elevated moderately along with the progression of liver disease, from hepatitis to cirrhosis, and then it increased remarkably in HCC (18). We, and others, have proved that the serum GP73 is a promising and potential tumor marker for detecting HCC, for its sensitivity is superior to α-fetoprotein (AFP), especially in early HCC (19-22). However, the function and molecular mechanisms of GP73 remain obscure which seriously prevent it from clinical transformation as an HCC biomarker.

Our previous study demonstrated that GP73 was not only associated with poor prognosis in HCC patients, but also correlated with EMT representative molecules E-cadherin and Vimentin in HCC tissues by immunohistochemistry (23). Nevertheless, the above underlying mechanism of GP73 participating in the EMT progress remains unknown. In this study, we confirmed the critical role of GP73 in HCC invasion and metastasis. Moreover, we further verified that silencing GP73 contributed to the reduction of invasion and metastasis

via suppressing EMT. This may help to provide evidence of GP73 as a novel molecular target for HCC metastasis therapy.

Materials and methods

Cell lines and cultures. Human hepatocellular carcinoma cell lines MHCC97H, HCCLM3 and Bel-7404 and human normal liver cell line L-O2 were purchased from the Cell Bank of Chinese Academy of Science (Shanghai, China). All cells were supplemented with Dulbecco's modified Eagle's medium (DMEM, Hyclone, UT, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) at 37˚C in a humidified atmosphere with 5% CO_2 and maintained in RPMI-1640 medium (Hyclone).

Antibodies. Polyclonal rabbit antibodies against GP73, E-cadherin, N-cadherin, and Snail were purchased from Abcam (MA, USA); polyclonal rabbit antibodies against Vimentin and β-actin were purchased from Bioworld Technology (CA, USA). The appropriate peroxidas-econjugated goat anti-rabbit IgG and goat anti-mouse IgG secondary antibodies were obtained from Zhongshan Biotech (Beijing, China). The dilution of antibodies was used according to the manufacturer's instructions.

siRNA and transfection. Inhibition of *GP73* expression in MHCC97H and Bel-7404 cells was performed by small interfering RNA (siRNA). Both non-specific control siRNA and GP73 siRNA were designed, synthesized, and purified by GenePharma (Shanghai, China) and stored at -20˚C. When cells were grown to 60%, the GP73 siRNA or non-specific control siRNA was transfected using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). Six hours after transfection, the medium containing transfection reagents was removed. Forty-eight hours later, cells were harvested for western blot assay and subjected to the following assays. Non-specific siRNA was used as a negative control. Primers were GP73 siRNA sense, 5'-GUGGCUUAGAAUUUGAACATT-3' and antisense, 5'-UGUUCAAAUUCUAAGCCACTT-3'; and non-specific siRNA sense (negative control), 5'-UUCUCCGAACG UGUCACGUTT-3' and antisense, 3'-TTAAGAGGCUUGCA CAGUGCA-5'.

RNA isolation and quantitative real-time PCR. Total RNA was extracted using RNAiso Plus (Takara, Shiga, Japan) and transcribed into cDNA using a PrimeScript™ RT reagent kit (Takara) according to the manufacturer's protocols. GP73 mRNA expression was quantified by quantitative real-time PCR (qRT-PCR) using a 7500 Real-Time PCR system (Thermo Scientific, MA, USA). qRT-PCR was performed using SYBR Premix Ex Taq® (Takara) with the following GP73 primers: 5'-CAGCGCTGATTTTGAGATGAC-3' and 5'-ATGATCCGT GTCTGGAGGTC-3'. GP73 mRNA levels were normalized to β-actin with the following primers: 5'-TTCCAGCCTTCCTTC CTGGG-3' and 5'-TTGCGCTCAGGAGGAGCAAT-3'. PCR parameters consisted of an initial incubation of 60 sec at 95˚C, followed by 35 cycles at 95˚C for 20 sec each and 1 cycle each at 95˚C for 15 sec, 60˚C for 60 sec and 95˚C for 15 sec.

Western blot analysis. For western blot analysis, cells were harvested and washed twice with phosphate-buffered saline (Hyclone), the proteins were extracted using RIPA cell lysis buffer (Beyotime, Jiangsu, China), and the protein concentration was measured by enhanced bicinchoninic acid (BCA) Protein assay kit (Zhongshan Biotech). Equal amounts of protein from each group were loaded into an 8-10% SDS polyacrylamide gel electrophoresis (PAGE) (Zhongshan Biotech) and then electrotransferred to nitrocellulose filter membranes (Millipore, MA, USA) at 200 mA for 2 h. After being blocked for 2 h in 5% non-fat milk, the membranes were cut according the protein molecular weight and incubated with primary antibodies against GP73 (1:2,000; Abcam), anti-E-cadherin antibody (1:1,000; Abcam), anti-N-cadherin antibody (1:1,000; Abcam), anti-Snail antibody (1:1,000; Abcam), anti-vimentin antibody (1:1,000; Bioworld Technology), β-actin (1:5,000; Bioworld Technology) at 4˚C overnight. Washed thoroughly with washing TBST buffer containing Tween-20, the membranes were then incubated with corresponding secondary antibodies for 2 h at room temperature. Following several washes with washing buffer, the protein bands were visualized using an enhanced chemiluminescence (ECL) reagent (Thermo Scientific) and analyzed by ImageJ software. Experiments were performed in triplicate and normalized by the expression of β-actin.

Scratch assay. For the scratch assay (Haoran, Biotech, Shanghai, China), cells were grown to confluence in a 24-well plate, and a 'wounding' line was scratched into the cell monolayer with a sterile 200-μl pipette tip. The width of the wound was measured under a microscope at 0 and 48 h after the scratch to assess the migration ability of the cells.

Transwell assay. Transwell (6.5 mm) with 8.0-μm pore polycarbonate membrane coated inserts were purchased from Corning (NY, USA). Cells were seeded in 6-well plates ($2x10^5$ cells/well) and incubated for 24 h. After transfection, cells were cultured in complete medium for an additional 24 h. The cellular density was adjusted to $1x10^5$ cells/ml to account for non-adhered cells. For the invasion assay, $1x10^4$ cells in 100 μl serum-free DMEM were seeded in the upper chamber of the insert, with 15% Matrigel on the membrane of the upper chamber; 800 μl of DMEM containing 10% FBS was added to the lower chamber and incubated for 2 days. The medium and cells were then removed from the upper chamber using cotton swabs with 1X PBS. The cells were fixed with 800 μl methanol for 30 min, stained with a 0.5% crystal violet solution for 2 h, washed with 1X PBS and counted under a microscope.

Statistical analysis. Statistical analysis was carried out with SPSS software, version 16.0 (SPSS, Chicago, IL, USA). The results were expressed as the mean ± standard deviation (SD). The data among the groups were compared by one-way analysis of variance followed by Bonferroni correction. Each experiment was performed independently at least three times. Values of $P<0.05$ and $P<0.01$ were considered statistically significant.

Results

Expression of GP73 is upregulated in the more metastatic HCC cell lines. To determine the relationship between GP73

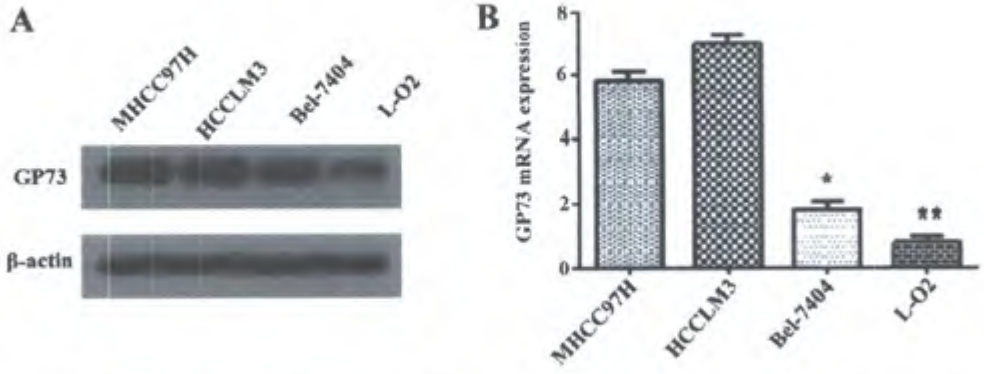

Figure 1. Expression of GP73 is upregulated in the more metastatic HCC cell lines. The relative expressions of GP73 in three human HCC cell lines MHCC97H, HCCLM3 and Bel-7404 and the normal human hepatocyte L-O2 cells were detected. (A) The protein level of GP73 was measured by western blot analysis. (B) The mRNA level of GP73 was performed by qRT-PCR. β-actin was detected as an internal control. Data are expressed as the mean ± SD of four independent experiments relative to β-actin. *P<0.05, **P<0.01 represents significant difference from MHCC97H cells.

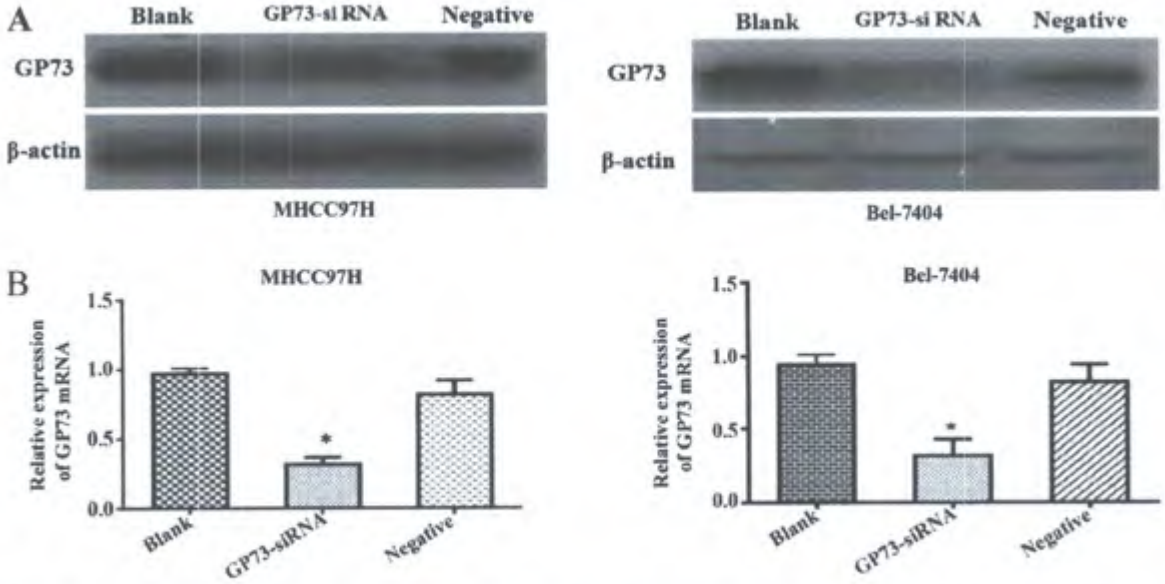

Figure 2. Effects of GP73 siRNA on GP73 expression in MHCC97H and Bel-7404 cells. Forty-eight hours after siRNA transfection, GP73 expression levels were significantly reduced by siRNA. GP73-siRNA group cells were transfected with GP73 siRNA whereas negative group cells were treated with non-specific control siRNA. (A) The protein level of GP73 was detected by western blot analysis. (B) The mRNA level of *GP73* was performed by qRT-PCR. β-actin was detected as an internal control. Data were expressed as the mean ± SD. *P<0.05 represents significant difference from blank control group.

expression and metastatic ability in cell lines, GP73 protein in three human HCC cell lines that had different metastatic potentials (MHCC97H, HCCLM3 and Bel-7404) and the normal human hepatocyte L-O2 cells were analyzed by western blotting (Fig. 1A). Higher GP73 expression was observed in the more metastatic cell lines, such as MHCC97H and HCCLM3, while relatively weak expressions was detected in Bel-7404 and L-O2. Similar results were obtained by qRT-PCR analysis for detecting GP73 (Fig. 1B). We therefore selected the higher GP73 expression cell line MHCC97H, and the lower Bel-7404, for further investigation.

Efficiency of the transient transfection of GP73 siRNA. Forty-eight hours after siRNA transfection, GP73 protein expression levels were significantly reduced by si-GP73 in both MHCC97H and Bel-7404 cells. To demonstrate the efficiency of the transfection of siRNA, western blot and qRT-PCR assays were conducted. Both protein and mRNA level of GP73 were clearly repressed in cells transfected with GP73 siRNA compared with that of cells transfected with negative control siRNA and blank control group cells ($P<0.05$; Fig. 2). These results demonstrated that the expression of GP73 in MHCC97H and Bel-7404 cells were effectively suppressed following transfection with specific GP73 siRNA.

Silencing of GP73 inhibits migration and invasion of HCC cells. To explore the role of GP73 in HCC migration and invasion, *in vitro* motility assay were performed among the interfered cells, negative control siRNA and blank group cells. The scratch assay revealed that the GP73-siRNA cells resulted

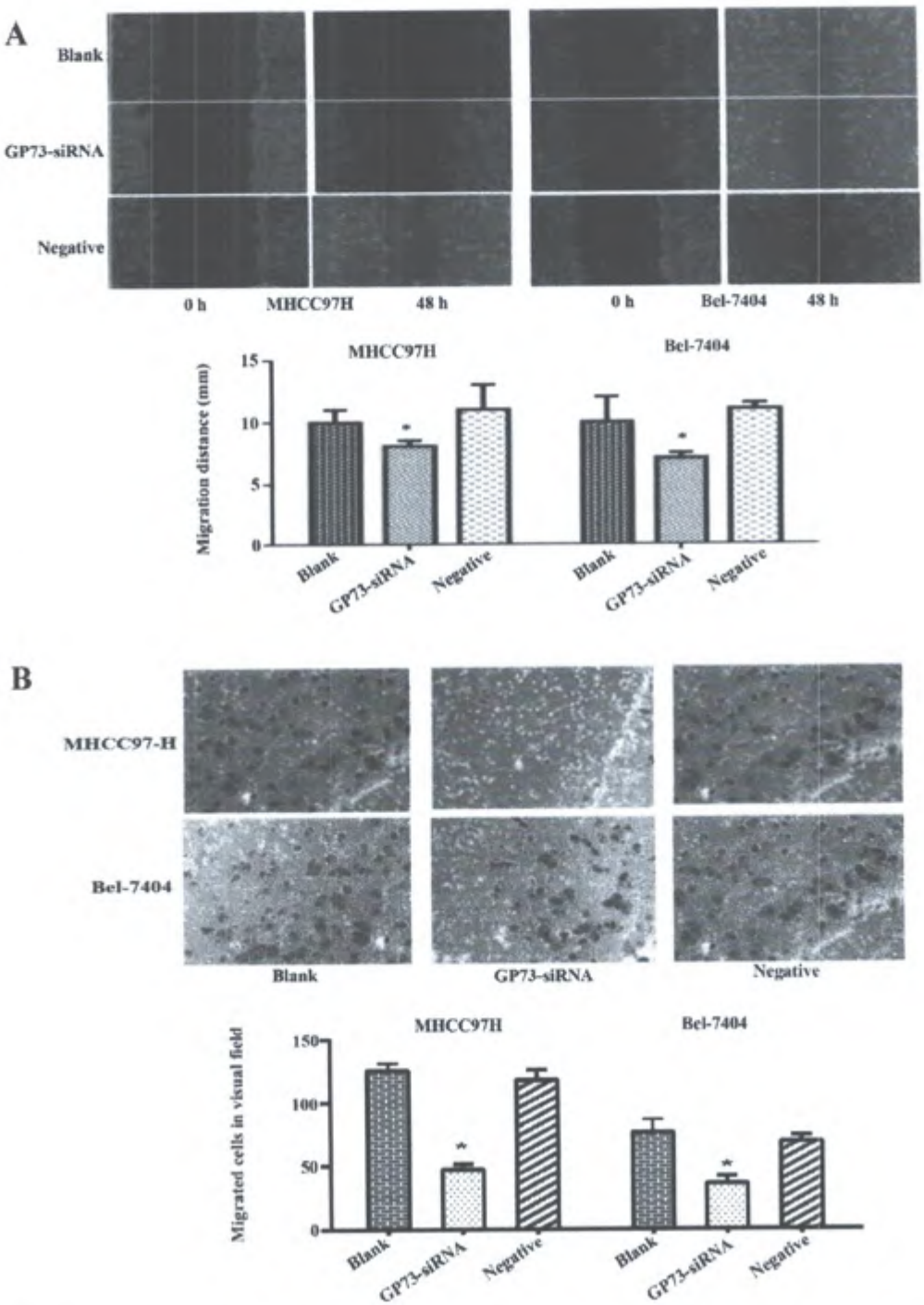

Figure 3. Silencing of GP73 inhibits migration and invasion of MHCC97H and Bel-7404 cells. GP73-siRNA group cells were transfected with GP73 siRNA whereas negative group cells were treated with non-specific control siRNA. (A) The ability of cell migration was assessed by scratch assay. (B) The ability of cell invasion was evaluated by Transwell assay. Results are expressed as mean ± SD. *P<0.05 represents significant difference from blank control group.

in a significant decrease in migratory ability both in MHCC97H and Bel-7404 cells (P<0.05; Fig. 3A). The Transwell assay showed that far fewer GP73-siRNA cells invaded through matrigel-coated chambers compared with blank and negative group cells in both MHCC97H and Bel-7404 cells (P<0.05; Fig. 3B). These findings provided evidence that knockdown of GP73 expression results in a significant decrease in migratory and invasive abilities in HCC cells.

Silencing of GP73 inhibits the process of EMT. Increasing numer of studies have proved that the EMT plays a vital role in promoting tumor metastasis, so we further studied the knockdown effect of GP73 on EMT in both MHCC97H and Bel-7404 cells. Western blotting results showed that the mesenchymal biomarkers N-cadherin and Vimentin, as well as the transcription factor Snail were markedly reduced, whereas the epithelial biomarker E-cadherin was overexpressed in

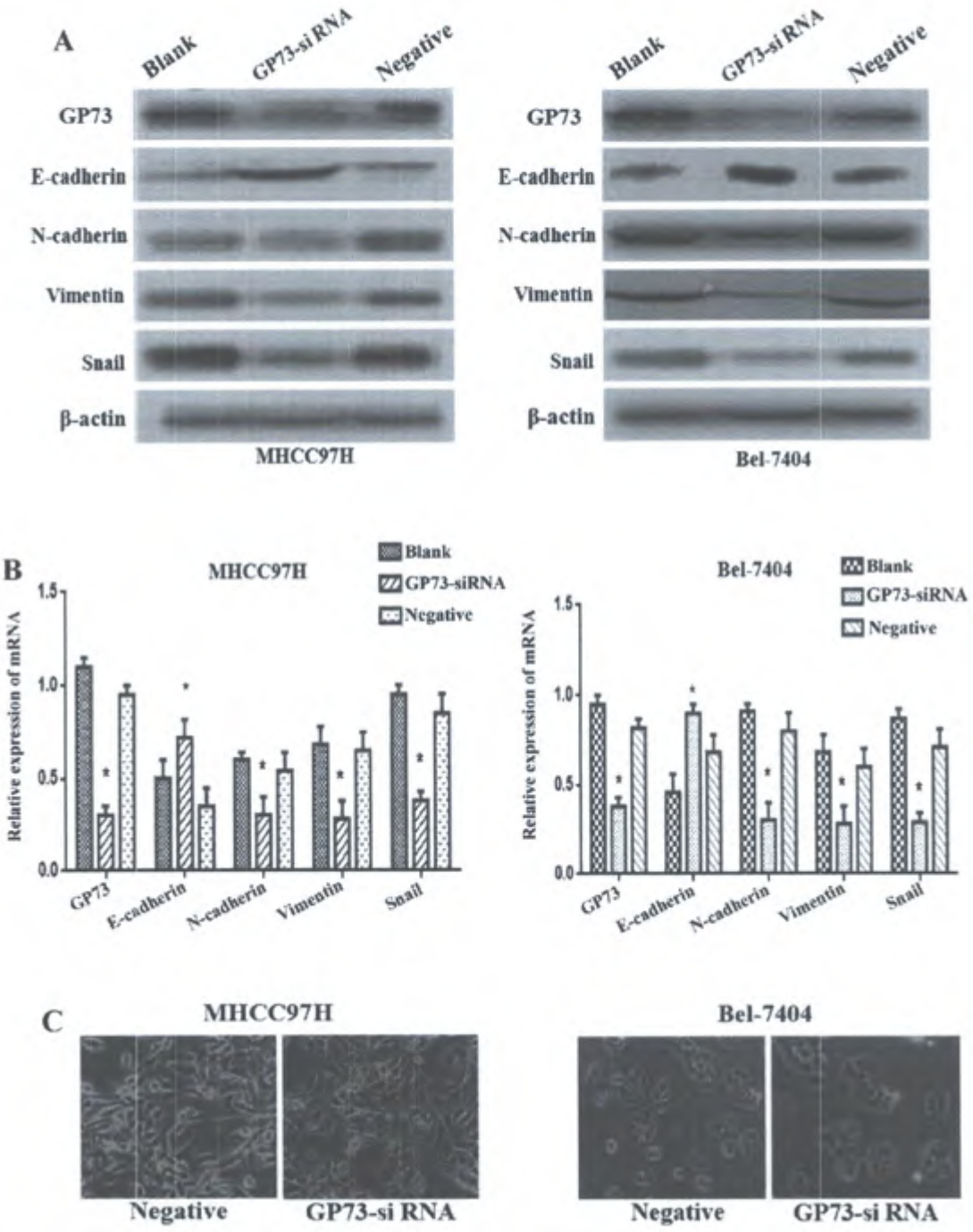

Figure 4. Silencing of GP73 inhibits the process of EMT. GP73-siRNA group cells were transfected with GP73 siRNA whereas negative group cells were treated with non-specific control siRNA. (A) Western blot analysis was performed to demonstrate the expression levels of EMT markers, including E-cadherin, N-cadherin, Vimentin and Snail. (B) The changes in cell morphology between GP73 knockdown cells and control group cells in the optical micrographs. Results are expressed as mean ± SD. *P<0.05 represents significant difference from blank control group.

GP73 siRNA-transfected cells compared with blank and negative group cells (Fig. 4A). Accordingly, similar results could be found in mRNA expression levels of these markers by qRT-PCR ($P<0.05$; Fig. 4B).

As shown in Fig. 4C, MHCC97H cells with reduced GP73 expression showed a major cell morphological change, from a spindle-shaped fibroblastic morphology to a cobblestone-shaped morphology. On the other hand, the Bel-7404 cells exhibited increasing cell-cell contact in GP73-siRNA group compared with negative group. The changes of morphological alterations were consistent with EMT markers. Thus, the above evidence indicated that the silencing of GP73 expression could attenuate the process of EMT in HCC cells.

Discussion

In this study, we found that GP73 was overexpressed in higher metastatic HCC cell lines. Also, downregulation of GP73

by siRNA could result in a significant decrease in migratory and invasive abilities in HCC cell lines. Importantly, both EMT-related markers and morphological phenotype significantly changed following the inhibition of GP73. These results suggest that silencing GP73 contributed to the reduction of invasion and metastasis via suppressing EMT in HCC. Our results highlight the possibility that GP73 could serve as a novel molecular target against EMT in HCC metastasis therapy.

GP73 is a highly phosphorylated protein and normally resides within the Golgi apparatus. It could be secreted into the extracellular space by cleavage at a proprotein convertase (PC) site, which results in the secretion of GP73 into the circulation (24,25). At present, increasing data indicate that serum GP73 levels are low in healthy controls, higher in cirrhosis and hepatitis, highest in HCC (18-22). Then, GP73 emerges as a potential serum tumor marker for detecting HCC. Currently, only few functional studies reported that overexpression of GP73 could promote proliferation and apoptosis (26). However, little is known about its molecular mechanisms in HCC progression which severely limits the GP73 clinical transformation as a promising biomarker.

Our previous study and other studies have showed that increased GP73 expression is strongly associated with poor prognosis and malignant biological behavior (such as tumor size, vein invasion, and metastasis) (23,27,28). To determine the relationship between GP73 and metastasis, we detected the protein and mRNA levels of GP73 in different metastatic ability cell lines. Higher GP73 were observed in more aggressive cells MHCC97H and HCCLM3, lower GP73 were detected in less aggressive cells Bel-7404 and non-aggressive cells L-O2 (Fig. 1). These results provided a clue that GP73 is likely related to metastasis of HCC.

To confirm the role of GP73 involved in invasion and metastasis of HCC, we silenced GP73 by specific siRNA (Fig. 2B). We initially demonstrated that siRNA could be successfully transfected into MHCC97H and Bel-7404 cell lines, resulting in significantly reduced GP73 expression. The scratch assay revealed that the GP73-siRNA cells resulted in a significant decrease in migration (Fig. 3A). In agreement with this, the Transwell assay showed that GP73-siRNA cells resulted in a decline in invasion (Fig. 3B). Similarly, the latest data also reported that depletion of GP73 could decrease the migration and metastasis of HCC cells (29,30). The above evidence suggests that GP73 may act as a key oncogene in regulating metastasis of HCC.

It is believed that the EMT plays an important role in cancer metastasis (5). During the metastatic cascade, carcinoma cells often initiate a key step known as EMT, a dynamic cellular process by promoting acquisition of invasive and migratory abilities. EMT is featured by loss of epithelial phenotype marker E-cadherin, and increased mesenchymal phenotype markers (Vimentin and N-cadherin), which contribute to the loss of cellular junction and polarity (5). Subsequently, epithelial cells obtained a fibroblastic phenotype, dissociate from the epithelium and migrate to distant organs. Consistent with these findings, our results revealed that silencing of GP73 increased the epithelial marker E-cadherin expression. At the same time, the mesenchymal markers N-cadherin and Vimentin, as well as the transcription factor Snail decreased in GP73-siRNA cells (Fig. 4A). Additionally, we found that knowndown of GP73 changed the morphology of the MHCC97H cells from the fibroblast-like shape to a cobblestone-like appearance. In Bel-7404 cells the cellular adherent junctions increased in GP73-siRNA cells compared with negative group cells (Fig. 4B). The results showed that knockdown of GP73 resulted in the suppression of EMT process in HCC. The next step is to validate these findings *in vivo* and explore whether GP73-siRNA has an effect on the activation of signaling pathways.

In conclusion, an important role of GP73 was found in the alteration of invasion and metastasis by regulating EMT in HCC cells. Our results highlight the possibility that GP73 may serve as a novel molecular target against EMT in HCC metastasis therapy.

Acknowledgements

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miR-29a suppresses growth and migration of hepatocellular carcinoma by regulating CLDN1

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ABSTRACT

CLDN1 (claudin1) is essential for intercellular junctions and has been reported to be involving in cell migration and metastasis, making it as an oncogene in various cancer types. However, the biological function roles and regulatory mechanisms of CLDN1 in hepatocellular carcinoma (HCC) are still not clarified. In this study, we found down-regulation of miR-29a and up-regulation of CLDN1 in HCC tissues and cell lines. Further found an inverse relation between the expressions of miR-29a and CLDN1 in HCC. Dual-luciferase reporter assay indicated that miR-29a regulated the expression of CLDN1 by binding to its 3' untranslated region (3'UTR). Knockdown of CLDN1 led to decrease in tumor cell growth and migration capacities *in vitro* and *in vivo*. While overexpression of miR-29a suppressed tumor growth and migration, these effects could be reversed by re-expressing CLDN1. Taken together, out data suggested that miR-29a may regulate tumor growth and migration by targeting CLDN1, providing promising therapeutic targets for HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the common cancers and ranks as the fifth malignant tumor worldwide [1,2]. Most suffers are diagnosed at advanced stages and the 5-year survival of HCC patients are still poor due to high degree of heterogeneity and migration into adjunct normal tissues [3]. Therefore, further insight of molecular mechanisms of impetus for tumor growth and migration should be excavated.

It has reported that the functions of miRNAs are different in various tissues [4]. MiRNAs which promote cancer development are known as oncogenic miRNAs, while the other miRNAs decreased tumor dissemination are considered to be suppressor miRNAs. MIR-29a has been demonstrated to be associated with physiological and pathological processes of tumor [5–7]. Recent studies have showed that miR-29a was a suppressor of tumor growth and migration in several solid tumors, such as liver cancer, breast cancer and ovarian cancer [8–10]. However, the biological roles of miR-29a in HCC remains unclear.

CLDN1, which encoding claudin1, mediates intercellular connection. Recent studies have shown that CLDN1 was expressed differently in various tumors. For instance, CLDN1 was up-regulated in cervical cancer, enhancing tumor invasion and migration though epithelial mesenchymal transition (EMT), while it was down-regulated in lung cancer, leading to shorter overall survival [11,12]. Previous studies have indicated that CLDN1 acted as oncogene in HCC, which correlated with EMT phenotype acquisition [13]. However, the roles of CLDN1 in tumor development and migration are largely unknown in HCC. Therefore, we speculated that if there was any miRNA that can interact with CLDN1 to modify tumor development in HCC.

In this study, we indicated that miR-29a was down-regulated in HCC tissues and cell lines, and negatively correlated with CLDN1 expression. Forced expression of miR-29a suppressed tumor growth and migration though decreasing the expression level of CLDN1. These data established a new axis consisting of miR-29a and CLDN1 in controlling tumor growth and migration in HCC.

2. Materials and methods

2.1. Tissues and cell culture

A total of 43 HCC tissues and 30 normal tissues were collected from First teaching hospital of Xinjiang medical university. Patients were proven to be HCC at initial diagnoses, and none of them had

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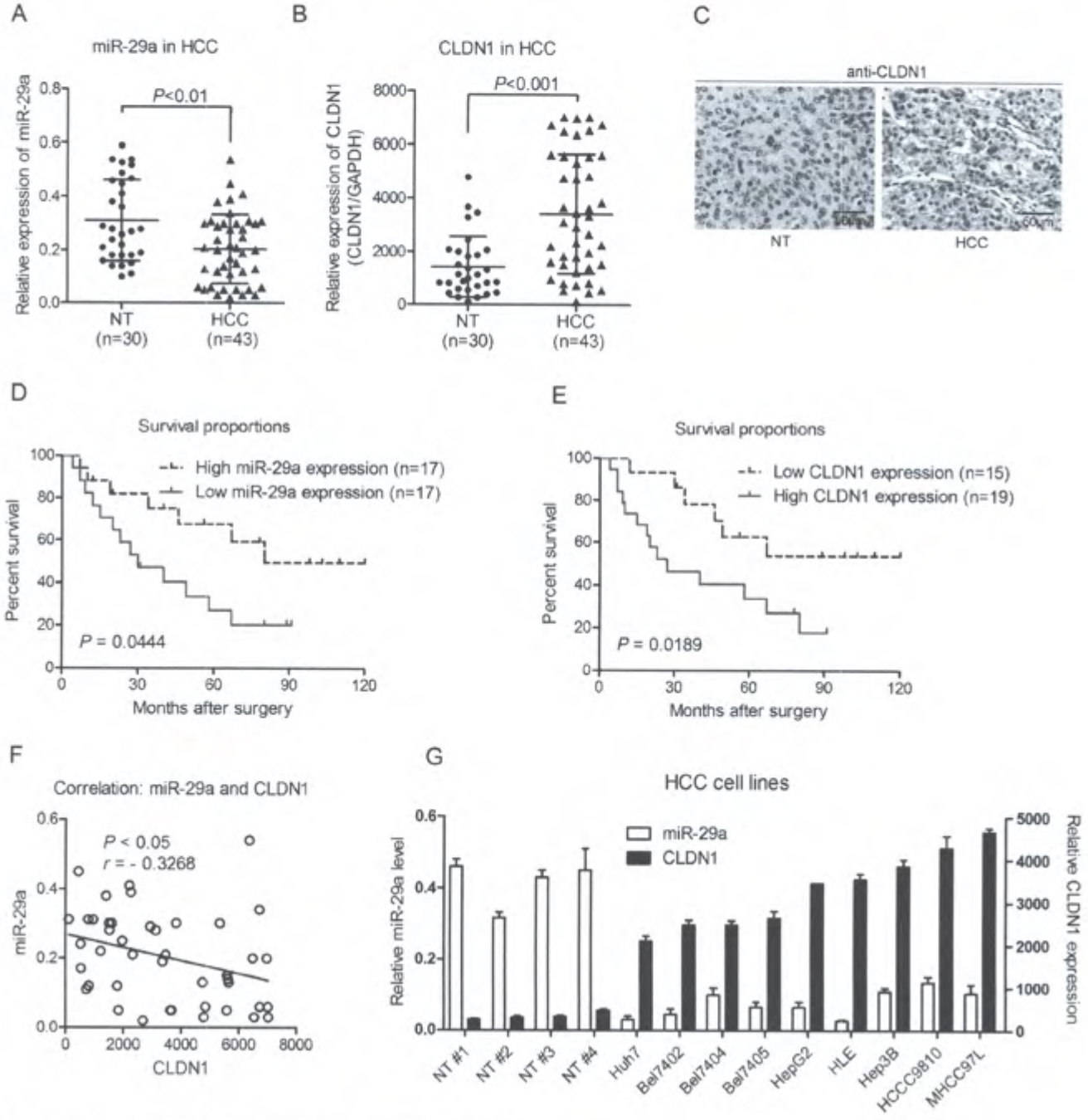

Fig. 1. miR-29a was down-regulated and correlated with CLDN1 expression in HCC.
(A) The expression of miR-29a in 46 HCC tissues and 30 normal tissues was detected bu qRT-PCR.
(B) qRT-PCR measured the expression of CLDN1 in HCC tissues and normal tissues.
(C) Immunohistochemical (IHC) staining analyzed the expression of the CLDN1. Scale bar, 50 μm.
(D) The survival of HCC suffers with high and low miR-29a levels were assessed by Mantel-Cox log-rank test.
(E) Mantel-Cox log-rank test was performed to examine the overall survival of HCC patients with different CLDN1 levels.
(F) The correlation between miR-29a and CLDN1 in HCC tissues were shown.
(G) Both the levels of miR-29a and CLDN1 were measured by qRT-PCR in a panel of HCC cell lines.

received chemotherapy or radiotherapy. Cell lines of HCC (HepG2, Hep3B, Huh7, Bel-7402, Bel-7404, Bel-7405, HLE, MHCC97L and HCCC9810) and 293T cells were obtained from the American Type Culture Collection (ATCC) and they were maintained in DMEM medium, which supplemented with 10% FBS, 100 μg/ml penicillin/streptomycin. Cells were maintained in a humidified atmosphere of 5% CO_2 at 37 C.

2.2. RNA isolation and qRT-PCR

Total RNAs extracted for HCC specimens and cell lines were isolated by TRIZOL reagent (Invitrogen, Carlsbad, CA). The GAPDH

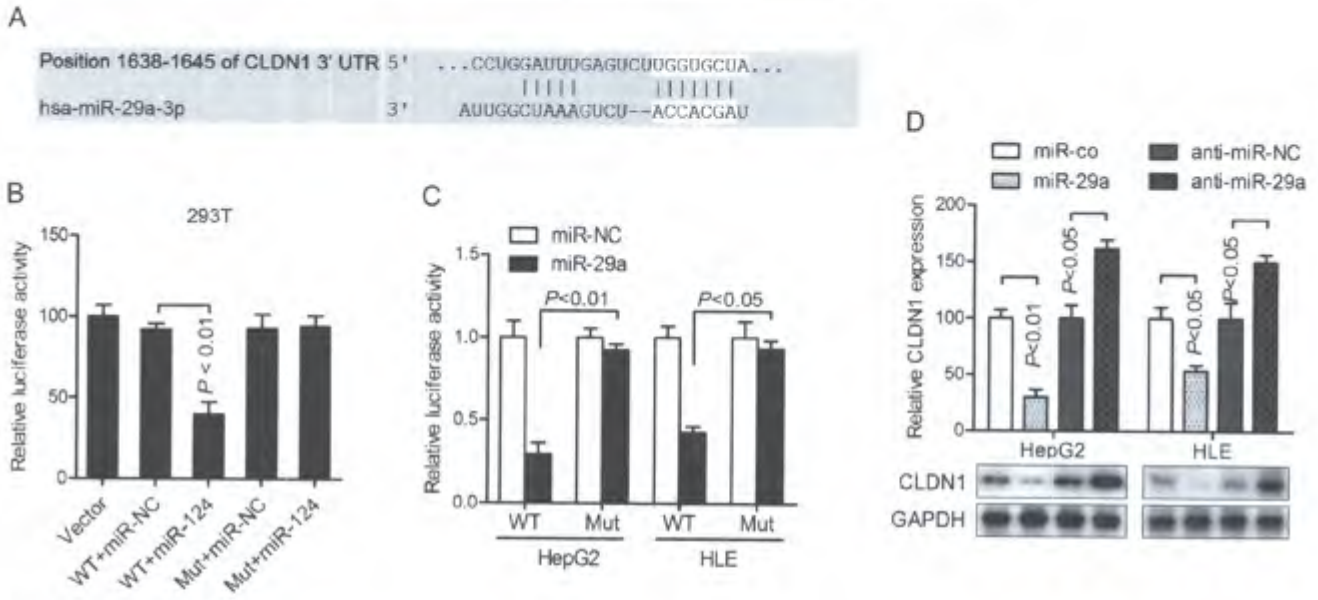

Fig. 2. CLDN1 was a target of miR-29a.
(A) The potential sequences of miR-29a binding site in 3'UTR of CLDN1were shown.
(B) Wide-type (WT) and mutant (Mut) of CLDN1 3'UTR, pre-miR-29a (miR-29a) and pre-miR-NC (miR-NC) were co-transfected into 293T cells. And the luciferase activities were measured 48 h after post-transfection.
(C) Luciferase reporter assay was also performed in HepG2 and HEL cells.
(D) After transfected with miR-29a, anti-miR-29a and their corresponding controls, the expression of CLDN1 was determined by qRT-PCR and Western blot.

and U6 were used as a control. PCR was performed using SYBR Green PCR kit (Invitrogen) on the Applied Biosystems. Primers of CLDN1 were: 5'-GCA TGA AGT GTA TGA AGT GCT TGG A-3'(forward), 5'-CGA TTC TAT TGC CAT ACC ATG CTG-3' (reverse); primers of GAPDH were: 5'-TTG GCA TCG TTG AGG GTC T-3' (forward) and 5'-CAG TGG GAA CAC GGA AAG C-3' (reverse). TaqMan MicroRNA Assay was applied to reverse translation of RNAs.

2.3. Western blot and immunohistochemistry (IHC)

For western blot assays, we firstly extracted proteins. The primary antibodies were: CLDN1 (Cell Signaling Technology, Beverly, MA, USA) and GAPDH (Abcam). The signals were then analyzed using the chemiluminescent detection system (PIERCE). For IHC assays, the specimens were cut into 4-μm sections. The samples were cultured with antibody of CLDN1, and then photographed with Olympus microscope.

2.4. Luciferase assay

The mutant and wide type sequences of CLDN1 3'UTR were amplified by PCR and clone into control vectors. 293T, HepG2 and HLE were transfected with wild type (WT) or mutant (Mut) of CLDN1-3'UTR, pre-miR-29a or pre-miR-control. Cells were obtained 48 h after transfected. The luciferase activities were performed according to the manufacturer's illustration and analyzed using Dual-Luciferase Reporter Assay System (Promega).

2.5. Cell proliferation assays

The tumor cells were treated with short hairpin RNA (shRNA) against CLDN1 and seeded into 96-well plates. The proliferation was examined by the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS, Promega). The OD value were evaluated at 562 nm after MTS solution added.

2.6. Cell migration assay

Cell migration assay was detected with transwell chamber. Cells were seeded in upper transwell of 24-well plates with serum-free medium. The chamber was fostered in a well 10% serum-containing medium for 24 h. The cells of upper side was wiped with cotton tips, while the cells on the bottom of the chamber were stained and dislodged. We then photographed and counted those cells under microscope.

2.7. In vivo assay

The 6-week-old female mice (Weitonglihua Biotechnology, Beijing, China) were inoculationed subcutaneously with 2×10^6 HepG2 cells. For some experiments, HepG2 cells were transfected with control vectors (sh-NC) or sh-CLDN1. For some experiments, HepG2 cells were transfected with control vectors (NC), CLDN1-overexpressing vectors (CLDN1), pre-miR-control or pre-miR-29a. Caliper measurements were performed every 4 days to evaluate tumor volume and the tumor volume was calculated by the formula π (length $\times$ width^2)/6.

2.8. Statistical analysis

A Student *t*-test and one-way ANOVA was performed to analysis the data of different groups. Data represents means ± SD at least 3 separate experiments. Statistical analyses were performed using the SPSS 16.0 software. $P < 0.05$ was considered statistically significant.

3. Results

3.1. miR-29a was down-regulated in HCC and negatively correlated with CLDN1

To determine the expression levels of miR-29a and CLDN1 in HCC, we firstly collected 46 HCC tissues and 30 normal tissues. As described in Fig. 1A, miR-29a were dramatically down-regulated in HCC tissues compared with the adjunct normal tissues ($P < 0.05$). In contrast, CLDN1 were highly expressed in HCC tissues than those in normal tissues ($P < 0.05$, Fig. 1B). The expression of CLDN1 was further confirmed by IHC staining (Fig. 1C). Moreover, the overall survival of patients were different according to the levels of miR-29a and CLDN1. The outcomes of suffers with low miR-29a expression were poorer than those with high expression (Fig. 1D), while the situations were opposite in CLDN1 (Fig. 1E). Interestingly, a strong negative relation between miR-29a and CLDN1 were observed in HCC tissues ($P < 0.05$, Log-rank (Mantel-Cox); Fig. 1F). Additionally, a panel of HCC cells were obtain to explore the expression levels of miR-29a and CLDN1, similar inverse relation were found in cell lines (Fig. 1G).

3.2. CLDN1 was a target of miR-29a in HCC

To further explore the relation between CLDN1 and miR-29a, we consulted Targetscan (http://www.targetscan.org). As speculated, CLDN1 was identified as a target of miR-29a (Fig. 2A). To ravel the correlation between miR-29a and CLDN1, we transfected putative sequences of wild-type (WT), mutant-type (Mut) of CLDN1, pre-miR-29a or pre-miR-control into 293T cells (Fig. 2B). Luciferase reports revealed that overexpression of miR-29a reduced the activity of wide-type CLDN1 though binding to the 3′UTR of CLDN1, whereas the effects of miR-29a were abolished in cell with mutant 3′UTR. Similar results were found using both HepG2 and HLE cells (Fig. 2C). Forced expression of miR-29a inhibited CLDN1 expression levels, while a strong enhancement of CLDN1 expression can be observed in miR-29a deletion cells (Fig. 2D). Western blot assays were performed to further confirm the expression levels of CLDN1 in miR-29a overexpressing cells and miR-29a restrained cells. Taken together, these results suggested that the expression of CLDN1 was modified by miR-29a.

3.3. Down-regulation of CLDN1 suppressed tumor growth and migration in HCC

To further examine the function of CLDN1 in HCC, we transfected HepG2 and HLE with short hairpin RNA (shRNA). As expected, the expression of CLDN1 was significantly silenced in CLDN1 specific shRNA (sh-CLDN1) transfected cells (Fig. 3A). Cell proliferation assays were performed to assessed the effects of

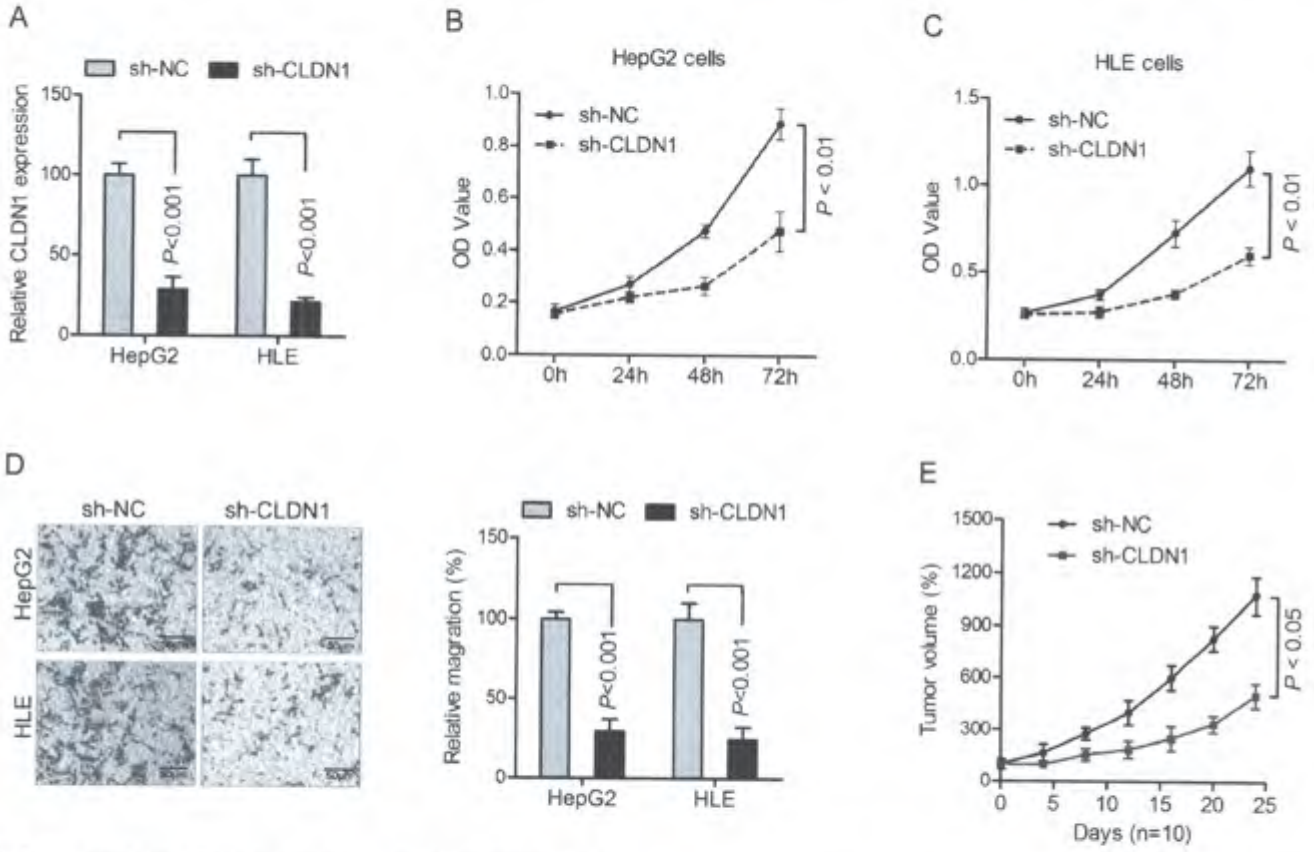

Fig. 3. Down-regulation of CLDN1 suppressed tumor growth and migration in HCC.
(A) Relative CLDN1 levels were measured in cells transfected with control (sh-NC) or CLDN1-specific shRNA(sh-CLDN1).
(B) After transfection, the effects of CLDN1 on cell growth were assessed in HepG2 cells.
(C) The OD value were analyzed in HLE cells to monitor cell proliferation.
(D) After treatment with specific shRNA against CLDN1 (sh-CLDN1), cell migration abilities were examined by transwell assays.
(E) Mice xenograft tumor models were established by injected CLDN1 silencing HepG2 cells (sh-CLDN1) or control cells (sh-NC). Tumor volume was measured every 4 days.

CLDN1 on cell growth, indicating that deletion of CLDN1 damped the proliferation abilities of HepG2 (Fig. 3B) and HLE cells (Fig. 3C). Transwell assays indicated that a smaller number of CLDN1-silencing cells were able to migrate though transwell chambers compared to the control cells (Fig. 3D). Furthermore, we established a nude mice xenograft tumor models to determine the effects of CLDN1 on HCC growth *in vivo*. As preconceived, there was a remarkable decrease of tumor volume in CLDN1 silencing group, compared to control group ($P < 0.05$, Fig. 3E).

3.4. MiR-29a suppressed tumor growth and migration by targeting CLDN1

Based on the findings above, we hypothesized that miR-29a may inhibit tumor growth and migration though regulating CLDN1. To further validate this hypothesis, we firstly transfected HepG2 and HLE cell with pre-miR-29a or control miRNA. The results demonstrated that the levels of miR-29a were increased after transfection, while the proliferation were declined (Fig. 4A). Transwell assays demonstrated that the migration abilities of miR-29a over-expressing cells were decreased (Fig. 4B), which was further confirmed by comparing the number of migrated cells (Fig. 4C). To ascertain the roles of CLDN1 on miR-29a induced cell migration suppression, CLDN1 was stably transfected into HepG2 and HLE cells and the mRNA and protein levels of CLDN1 were analyzed by qRT-PCR (Fig. 4D) and Western blot (Fig. 4E). Indeed, the expanded expression of CLDN1 prompted cell proliferation and migration (Fig. 4F and G). While miR-29a suppressed the abilities of cell

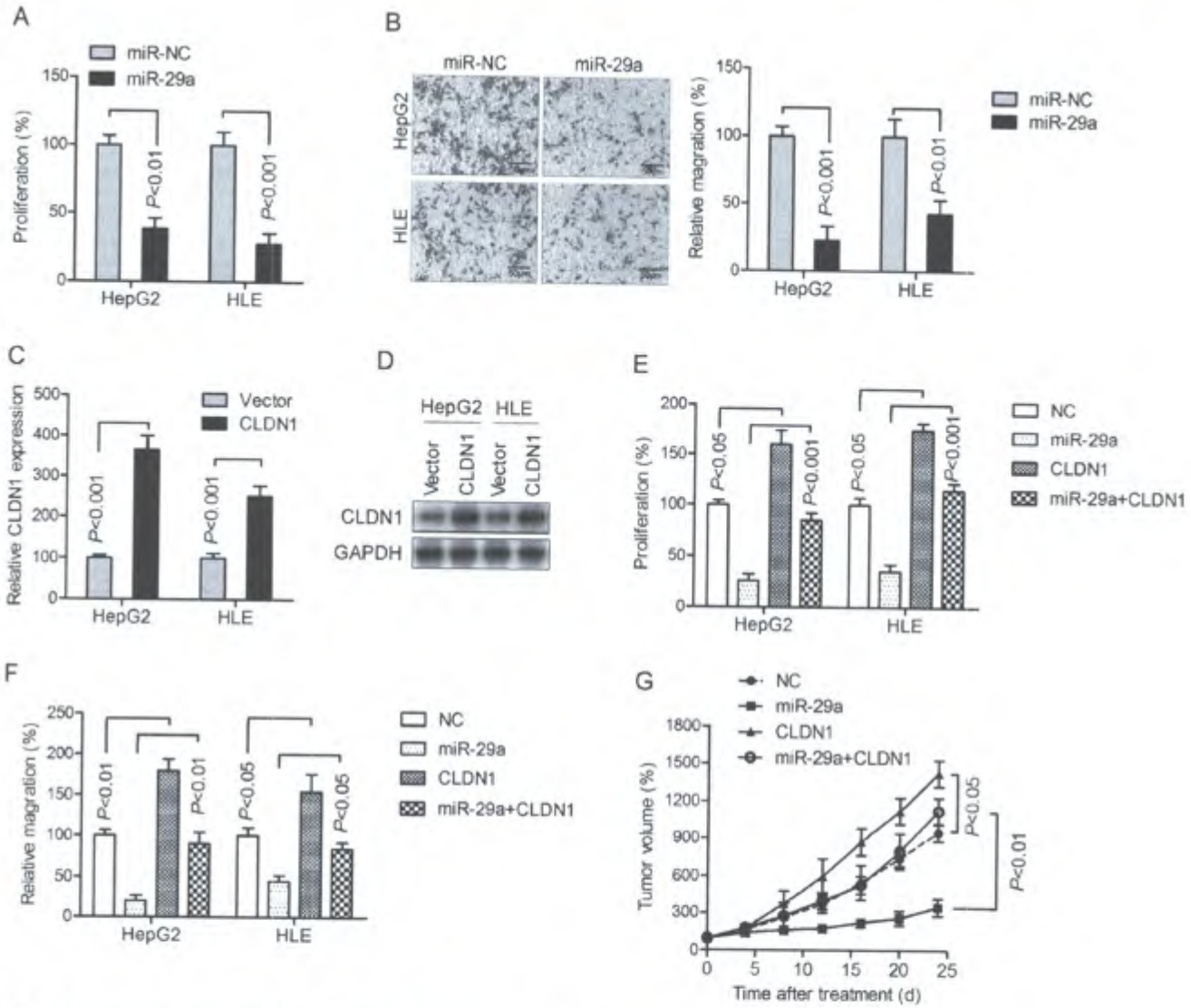

Fig. 4. MiR-29a suppressed tumor growth and migration by targeting CLDN1.
(A) HepG2 and HLE cells were transfected with vector containing miR-29a sequences, and cell proliferation were examined.
(B) Cells were transfected with pre-miR-29a and the migration capacities were measured.
(C) HepG2 and HLE were transfected with vector containing CLDN1, and the mRNA levels of CLDN1 were determined by qRT-PCR.
(D) The cells were treated above, Western blot was performed to analyze the protein levels of CLDN1.
(E) miR-29a and CLDN1 affected cell proliferation abilities.
(F) Tanswell assays were performed to evaluate the effects of miR-29a and CLDN1 on cell migration.
(G) The effects of miR-29a and CLDN1 on tumor growth were determined *in vivo*.

proliferation and migration, re-expression of CLDN1 significantly reversed the these abilities (Fig. 4F and G). CLDN1 also restored miR-29a mediated tumor growth suppression *in vivo*.

4. Discussion

MiRNAs have been proved to be function as regulators of specific gene expression and they impact tumor development, migration, metastasis [14]. Previous researches indicated that miR-29a acted as suppressor in various types of cancer [15–17]. In this study, we found that miR-29a was down-regulated in HCC tissues and associated with poor outcome (Fig. 1A and D), suggesting that miR-29a may contribute to the development of HCC.

Claudin1, encoding by CLDN1 gene, belongs to traneransmembrane proteins and it is important for tight junctions [18]. Encouraging evidence have shown that abnormal expression of CLDN1 is associated with tumor development. Here, we found that CLDN1 was up-regulated in HCC tissues compared to normal tissues (Fig. 1B), similar results were found in a panel of HCC cell lines (Fig. 1G). It has been demonstrated that loss of CLDN1 means loss of intercellular adhesion and this would be an initial step for tumor metastasis [19]. As an oncogene, CLDN1 promoted tumor invasion and migration in colon and liver cancer [20]. However, the roles of CLDN1 on tumor growth and migration together with the upstream regulatory mechanism in HCC still need further investigation.

Interestingly, there existed a negative correlation between the expressions of CLDN1 and miR-29a (Fig. 1F), indicating that there would be interaction between miR-29a and CLDN1 in HCC. Indeed, the results from luciferase assays showed that miR-29a could down-regulated CLDN1 expression by directly binding to the 3′UTR of CLDN1 (Fig. 2), indicating that CLDN1 was a target of miR-29a.

Then, we explored the biological roles of CLDN1 in HCC by transfecting with shRNA targeting CLDN1. *In vitro* and *in vivo* studies indicated that recession of CLDN1 led to inhibition of tumor growth and migration (Fig. 3). Previous studies have demonstrated that CLDN1 contributed to EMT phenotype formation in HCC [13] Thus, CLDN1 may be a potential therapeutic target for HCC. Moreover, functional assays revealed that miR-29a could suppress tumor growth and migration *in vitro* and *in vivo* (Fig. 4). Notably, forced expression of CLDN1 could reversed the miR-29-mediated suppression events, indicating that miR-29a regulated tumor growth and migration by targeting CLDN1.

In conclusion, our data proposed an interesting correlation between miR-29a and CLDN1 in HCC. Down-regulation of miR-29a and up-regulation of CLDN1 were observed in HCC tissues and cell lines. MiR-29a suppressed cell proliferation and migration partly by reducing CLDN1 though binding to its 3′UTR. Thus, we highlighted the interaction between miR-29a and CLDN1 in tumor development, which provided novel targets for exploring new therapeutic strategies.

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Transparency document

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TMPRSS4 promotes cancer stem cell traits by regulating CLDN1 in hepatocellular carcinoma

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ABSTRACT

Encouraging advances in the treatment of hepatocellular carcinoma(HCC) have been achieved; however, a considerable part of patients still relapse or metastasize after therapy, and the underlying mechanisms have not been clarified yet. Here, we found that CLDN1 was markedly up-regulated in HCC tissues, and correlated with poor prognosis. Overexpression of CLDN1 dramatically promoted the capability of tumorsphere formation and cancer stem cell (CSC) traits. Furthermore, we found that TMPRSS4 was up-regulated in HCC tissues and there was a positive correlation between TMPRSS4 and CLDN1. In addition, the expression of CLDN1 was regulated by TMPRSS4. Moreover, TMPRSS4 mediated CSC properties and up-regulated CLDN1 by activating ERK1/2 signaling pathway. Taken together, our results revealed that CLDN1 contributed to CSC features of HCC, which was altered by TMPRSS4 expression via ERK1/2 signaling pathway, providing promising targets for novel specific therapies.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers, accounting for second cancer leading deaths worldwide [1,2]. Most patients with advanced HCC fail to rehabilitation after curative resection due to distance metastasis or tumor recurrence [3]. Although many researches have been carried out, the investigation of underlying mechanisms and consequent design of reasonable treatment strategies remain a large unmet need.

Emerging evidence indicates that the spreading cancer cells should have the self-renew ability to develop in a new place, and act as founders [4]. Recently, some researchers have speculated that these cells experiencing epithelial-mesenchymal transition (EMT) may share the traits of cancer stem cell (CSC) phenotype, exhibiting the ability of self-renew and chemoradiaion resistance [5,6]. This suggests that eliminating CSC population may be a promising strategy for cancer therapy.

Claudin-1, encoding by CLDN1 gene, is central for intercellular junctions, involving in cell growth and differentiation [7]. Recent studies have indicated that CLDN1 was abnormally highly expressed in various types of human cancers [8]. Newly emerging evidence suggested that CLDN1 was critical for the induction of EMT process in HCC [9]. Although CLDN1 is responsible for tumor progression, the roles and interaction genes of it on tumor development are largely unknown in HCC.

Type II transmembrane serine protease (TTSP), a protein belongs to cell surface-associated protease. As a novel member of TTSPs, transmembrane protease serine 4 (TMPRSS4) has been found to be overexpressed in pancreatic, colon, thyroid and gastric cancer tissues [10,11], contributing to tumor development and poor prognosis. Previous studies have elucidated that TMPRSS4 promoted a variety of biological functions, including tumor growth, cell invasion and tumor metastasis. Moreover, TMPRSS4 has been demonstrated to be a mediator during the epithelial mesenchymal transition (EMT) and an inducer of CSC phenotype in multiple tumor [12,13]. However, the biological roles of TMPRSS4 in HCC and its downstream signaling events were not clearly elucidated.

In this study, we examined the expression of CLDN1 in HCC and explored its roles in regulation of the upstream signaling events. Remarkable up-regulation of TMPRSS4 and CLDN1 were observed in HCC tissues. The ability of tumor sphere formation was further

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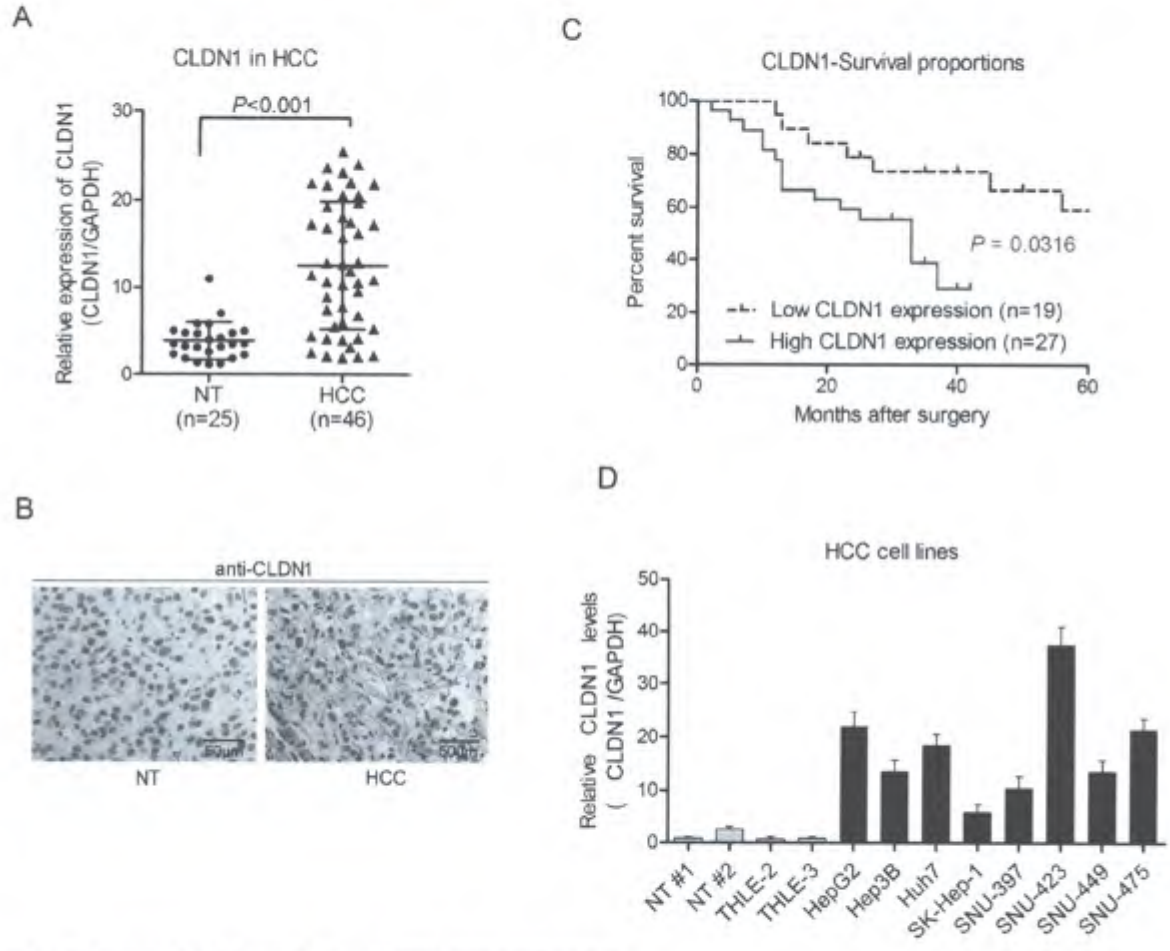

Fig. 1. CLDN1 was up-regulated in HCC tissues and correlated with poor overall survival
(A) The expression of CLDN1 was measured in 46 HCC and 25 adjacent normal tissues (NT) by qRT-PCR.
(B) Immunohistochemical (IHC) staining were performed to value the expression of CLDN1 in normal tissues and HCC tissues. The representative graphs were shown. Scale bar, 50 μm.
(C) The overall survival (OS) of patients with HCC was evaluated by Mantel-Cox log-rank test, containing of 19 cases of low CLDN1 expression and 27 suffers with high CLDN1 levels.
(D) Expression levels of CLDN1 were measured by qRT-PCR in a panel of HCC cell lines.

evaluated, and the expression of genes associated with EMT and CSC were detected. While overexpression of CLDN1 induced an EMT and CSC behaviors, which was regulated by TMPRSS4. For the first time, our results suggested that CLDN1 may facilitate EMT and CSC properties, which was regulated by TMPRSS4 expression via ERK1/2 signaling pathway in HCC.

2. Materials and methods

2.1. Clinical specimens and cell lines

Forty-six HCC tissues and twenty-five normal tissue were collected from First teaching hospital of Xinjiang medical university. Patients were proven to be HCC at initial diagnosis, and none of them had received chemotherapy or radiotherapy. 8 human HCC cell lines (HepG2, Hep3B, Huh7, SK-Hep-1, SNU-397, SNU-423, SNU-449 and SNU-475) and 2 non-tumorigenic human liver cell lines (THLE-2 and THLE-3) were obtained from ATCC. All cell lines were cultured in DMEM medium, containing 10% FBS, 100 μg/ml penicillin/streptomycin. Cells were maintained in humidified air at 37 °C with 5% CO_2.

2.2. RNA isolation and quantitative reverse transcription-PCR (qRT-PCR)

TRIZOL reagent (Invitrogen, Carlsbad, CA) was used to extract total RNAs, and cDNA was synthesized using SuperScript first strand synthesis system (Invitrogen). The qRT-PCR assay was performed using SYBR Green PCR kit (Invitrogen) on the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, USA). Primers of TMPRSS4 were: 5′-TCC AAG GAC CGA TCC ACA CT-3′ (forward), and 5′-AAG TTG TCG AAA CAG GCA GA G-3′ (reverse); primers of CLDN1 were: 5′-GCA TGA AGT GTA TGA AGT GCT TGG A-3′(forward), 5′-CGA TTC TAT TGC CAT ACC ATG CTG-3′ (reverse), primers of GAPDH were: 5′-TTG GCA TCG TTG AGG GTC T-3′ (forward) and 5′-CAG TGG GAA CAC GGA AAG C-3′ (reverse). The mRNA level of GAPDH was used for normalization. Other primers for genes were received from PrimerBank (http://pga.mgh.harvard.edu/primerbank/).

2.3. Western blot and immunohistochemistry (IHC)

Western blot assays were preformed to confirm the

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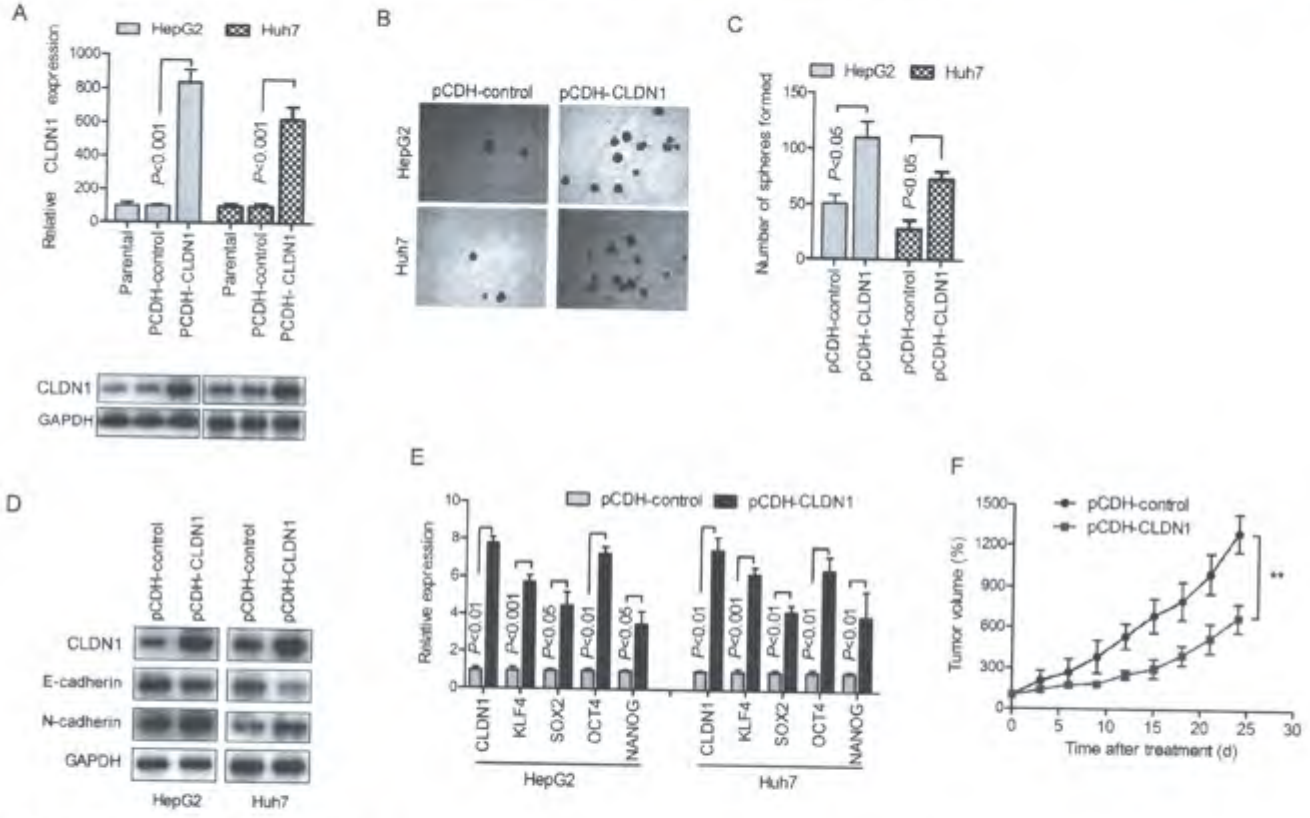

Fig. 2. Overexpression of CLDN1 promoted CSC traits

(A) The CLDN1 gene were stably transfected into HCC cell lines. qRT-PCR and Western blot were performed to measure the expression levels of CLDN1.
(B) Tumor sphere formation assay was carried out to evaluate the stemness of parental and stably transformed cells.
(C) And the cell number was counted.
(D) The protein levels of claudin-1 (CLDN1), E-cadherin and N-cadherin were measured.
(E) qRT-PCR was performed to confirm the expression of CLDN1 and CSC associated transcription factors, including KLF4, SOX2, OCT4 and NANOG.
(F) The volumes of xenograft tumor models were assessed every 4days after injected with CLDN1 overexpressing cells or control cells.

modification of protein expression of genes. Primary antibodies were: TMPRSS4, CD133(1:1000, Santa Cruz Biotechnology, USA), ERK1/2, phosphorylated ERK1/2 (p-ERK1/2), phosphorylated AKT (pAKT), AKT, Claudin-1 (CLDN1) and Claudin-7 (CLDN7) (1:1000, Cell Signaling Technology, USA) and GAPDH (Abcam). The signals were then analysed using the chemiluminescent detection system (PIERCE). For IHC staining of TMPRSS4 and CLDN1, 3 μm-thick sections were made and suffered from incubation with antibodies of TMPRSS4 and CLDN1 (1:200, Abcam).

2.4. Gene overexpression and silencing

The TMPRSS4 cDNA sequences were constructed into the lentiviral expression vector pCDH-EF1-MCS-T2A-Puro at EcoRI and BamHI sites. The lentivirus was obtained from 293T cells by co-transfection with pCDH-TMPRSS4 and the lentiviral packaging plasmids pMD2.G and pspAX2. After infected with lentivirus, TMPRSS4 positive HCC cells were selected using 5 μg puromycin. The CLDN1 expressing cells were transfected with pcDNA3.1-CLDN1, with empty vector as control. Transfection was performed with LipofectamineTM 2000 (Invitrogen) according to the manufacturer's protocol. For gene silencing, HCC cell lines were transfected with short-hairpin RNA (shRNA) to silencing TMPRSS4 expression. Cells were seeded in 6-well culture plates at a density of 3×10^5 cells/well and transfected with shRNA against TMPRSS4 (sh-TMPRSS4) and negative controls (sh-control) were performed 24 h later using lipofectamineTM 2000 (Invitrogen).

2.5. Sphere formation assay

In tumor sphere formation assay, cells were incubated in anchorage-independent conditions. 1×10^5 cells were seeded into 6-well plates, and maintained in medium without serum. Fresh epidermal growth factor (EGF; 20 ng/ml; R&D Systems) and basic fibroblast growth factor (bFGF; 10 ng/ml; R&D Systems) were added every other day. After 2 weeks, the radius of each tumor spheroid and the number of tumor spheres were was measured using NIS-Elements Microscope Imaging Software (Nikon, Tokyo, Japan).

2.6. In vivo assay

2×10^6 HepG2 cells or CLDN1 silencing HepG2 cells were injected into the 6-week-old female mice (Weitonglihua Biotechnology, Beijing, China). For some studies, mice injected with CLDN1-overexpressing HepG2 cells and control cells. Tumor volume was calculated by the formula π (length × width2)/6.

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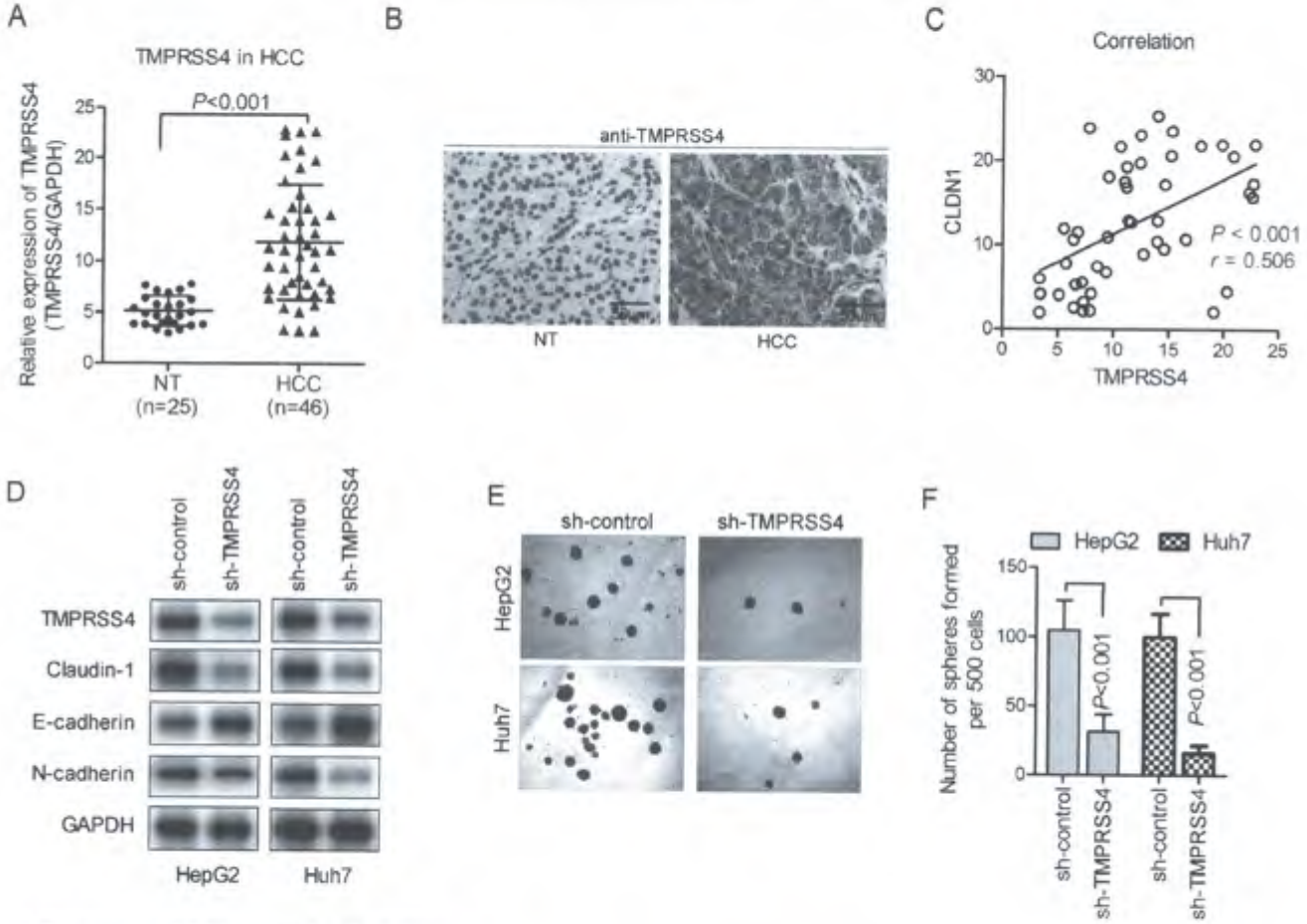

Fig. 3. The expression of CLDN1 was regulated by TMPRSS4

(A) The expression levels of TMPRSS4 in HCC tissues and normal tissues.

(B) The expression levels of TMPRSS4 was confirmed by IHC staining. Representative graphs were shown. Scale bar, 50 μm

(C) The relationship between the levels of TMPRSS4 and CLDN1 in HCC tissues were shown ($r = 0.506$, $P < 0.001$).

(D) HepG2 and Huh7 cells were transfected with shRNA against TMPRSS4, and the expressions of TMPRSS4, p-ERK, ERK, p-AKT, AKT, claudin-1 (CLDN1) were examined by Western blot.

(E) The effects of TMPRSS4 on tumor sphere formation was assessed.

(F) And the cell number was counted.

2.7. Statistical analysis

Student *t*-test was used to analyze differences between two groups and one-way ANOVA was employed in case of data consisted of more than two groups. Data represents means ± SD at least 3 separate experiments, Statistical analyses were performed using the Graphpad prism 5 software. A two-tailed value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. CLDN1 was up-regulated in HCC tissues and correlated with poor overall survival

Here, we firstly collected 46 HCC and 25 adjacent normal tissues (NT). As described in Fig. 1A, the expression of CLDN1 were significantly higher in HCC samples compared with adjacent normal tissues ($P < 0.001$). The levels of CLDN1 were further confirmed by IHC staining (Fig. 1B). In addition, we found that the patients had different overall survival according to the expression of CLDN1. Patients with high CLDN1 levels had a poorer prognosis than those with low levels ($P = 0.0316$, Log-rank (Mantel-Cox); Fig. 1C). Furthermore, a panel of HCC cell lines together with normal cells were selected to determine the expression of CLDN1, and also found overexpression of CLDN1 in HCC cells. Collectively, these data indicated that CLDN1 may be involved in tumor development of HCC.

3.2. Overexpression of CLDN1 promoted EMT and CSC traits

To further investigate the function of CLDN1 in HCC, HepG2 and Huh7 cells were stably transfected with lentiviral vector expressing CLDN1 (pCDH-CLDN1) or its empty control (pCDH-control). The expression of CLDN1 in parental and transfected cells were further confirmed by qRT-PCR and Western blot (Fig. 2A). Notably, a remarkable tumor sphere formation ability was found in cells with stable CLDN1 expression (Fig. 2B), and the number of tumor spheres were counted (Fig. 2C), indicating that CLDN1 may regulate the cancer stem cell (CSC) properties of HCC. Since epithelial-mesenchymal transition (EMT) is thought to be associated with CSC properties, forced CLDN1 expression led to decrease of E-cadherin and increase of N-cadherin in our case (Fig. 2D). Additionally,

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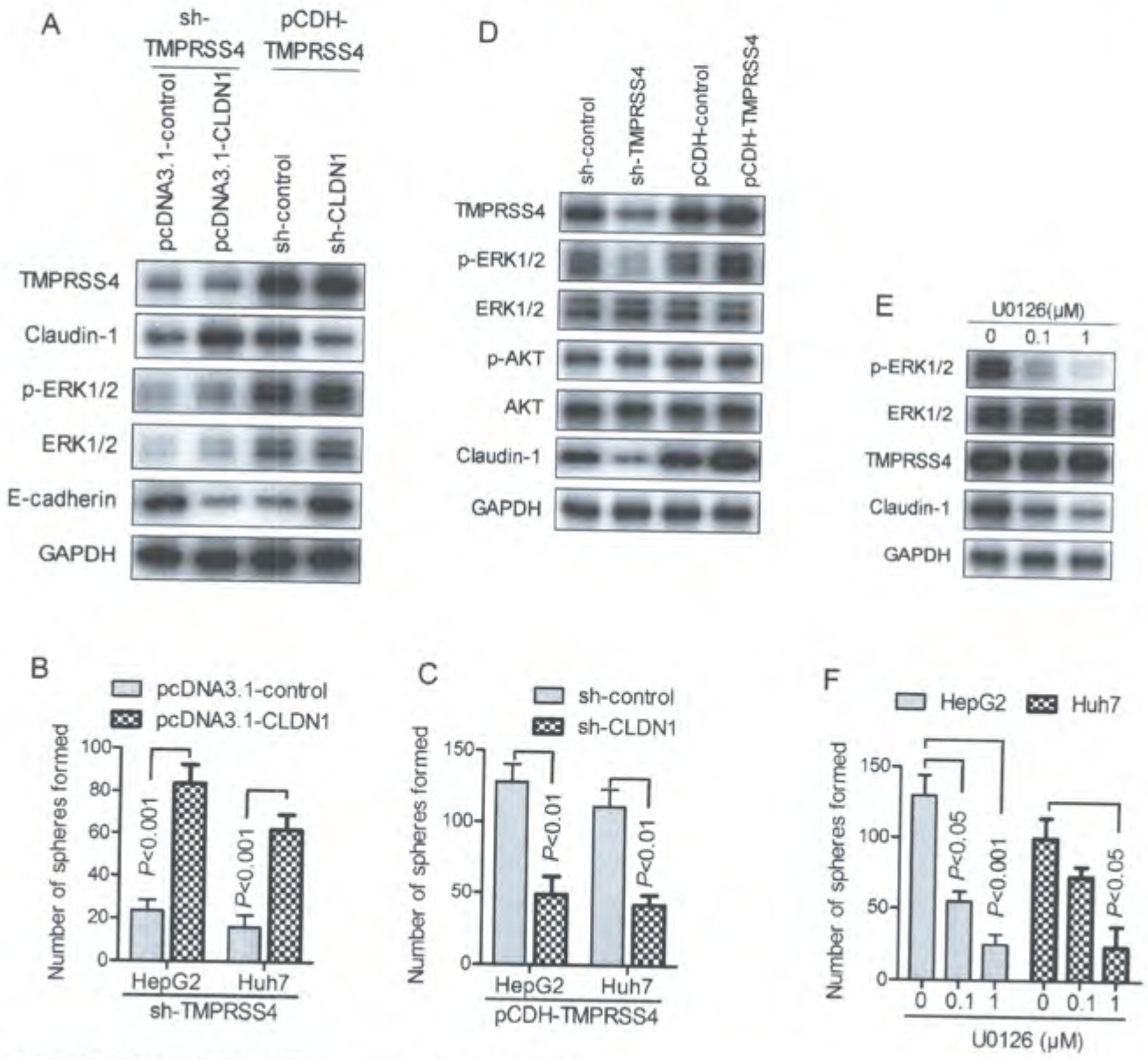

Fig. 4. TMPRSS4 modified CSC traits and CLDN1 expression though ERK1/2 pathway

(A)The CLDN1 overexpressing vector (pcDNA3.1-CLDN1) or empty control (pcDNA3.1-control) were transfected into sh-TMPRSS4 HepG2 cells. And CLDN1 specific shRNA (sh-CLDN1) or control (sh-control) was transfected into pCDH-TMPRSS4 HepG2 cells. The levels of TMPRSS4, claudin-1 (CLDN1), p-ERK, ERK and E-cadherin in these cells were detected by Western blot.

(B) After transfection with CLDN1 overexpressing vector (pcDNA3.1-CLDN1) or empty control (pcDNA3.1-control), number of spheres in sh-TMPRSS4 HepG2 cells was counted

(C) After transfection with CLDN1 specific shRNA (sh-CLDN1) or control (sh-control), number of spheres in pCDH-TMPRSS4 HepG2 cells was counted.

(D) After transformed with TMPRSS4 expressing vector or shRNA against TMPRSS4, the levels of p-ERK, ERK, TMPRSS4 and claudin-1 (CLDN1) were measured.

(E) After treated with ERK1/2 inhibitor U0126 (0 μM, 0.1 μM and 1 μM), the levels of p-ERK, ERK, TMPRSS4, claudin-1(CLDN1) were valued again.

(F) Number of sphere formation were measured after treated with ERK1/2 inhibitor in HepG2 and Huh7 cells.

we also found a high expression of the CSC associated transcription genes, KLF4, SOX2, OCT4 and NANOG, in CLDN1-expressing cells (Fig. 2E), providing another link between CLDN1 and stemness in HCC.

To further confirm our data, we established xenograft tumor models. CLDN1 overexpressing cells and control cells were injected into the models, and tumor volumes were assessed every 4 days. The experiments were performed to examine whether forced CLDN1 expression can enhance tumor growth. The results showed that cells with high CLDN1 levels grew faster than the controls (Fig. 2F), suggesting that CLDN1 expression levels were associated with tumor malignancy and tumorigenicity.

3.3. The expression of CLDN1 was regulated by TMPRSS4

To further determine the relation between CLDN1 and TMPRSS4, we examined the expression of TMPRSS4 in HCC tissues and adjacent normal tissues. As shown in Fig. 3A, the levels of TMPRSS4 were up-regulated in HCC tissues ($P < 0.05$). IHC staining confirmed the results (Fig. 3B). Interestingly, there was an obvious positive correlation between the expression of TMPRSS4 and CLDN1 ($r = 0.506$, $P < 0.001$; Fig. 3C). To further determine the regulatory

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mechanism between TMPRSS4 and CLDN1, we stably down-regulated TMPRSS4 expression in HepG2 and Huh7 cells by shRNA-mediated RNA interference. The gene-silencing efficiency of TMPRSS4 were confirmed by Western blot. As shown in Fig. 3D, the expression of CLDN1 were suppressed in TMPRSS4 silencing cells, whereas the cells experienced an EMT-like phenotypic switch in TMPRSS4 lacking cell. Moreover, we found a decreased sphere forming efficiency in both HepG2 and Huh7 cells after silencing of TMPRSS4 (Fig. 3E). Furthermore, number of tumor sphere were decreased in TMPRSS4 depleted cells (Fig. 3F). Taken together, these findings revealed that TMPRSS4 may promote tumorsphere formation and regulate CLDN1 expression in HCC.

3.4. TMPRSS4 modified CSC traits and CLDN1 expression though ERK1/2 pathway

To ascertain whether TMPRSS4 can mediate stem like properties though regulating CLDN1, we augmented CLDN1 levels in TMPRSS4 silenced cells, on the contrary, shRNA targeting CLDN1 were transfected into TMPRSS4 overexpressed cells. As shown in Fig. 4A, enhanced CLDN1 expression led to decrease of E-cadherin in TMPRSS4 lacking cells, while CLDN1 depletion up-regulated E-cadherin levels of TMPRSS4 overexpressing cells. However, no obvious differences in p-ERK1/2 and TMPRSS4 levels were detected, indicating that CLDN1 could be a downstream molecule of TMPRSS4/ERK1/2 pathway and regulate EMT progress in HCC. Furthermore, accompanied by enhanced CLDN1 expression, the ability of tumor sphere forming was restored in TMPRSS4-lacking cells (Fig. 4B). And the number of tumor spheres were decreased after CLDN1 silencing in TMPRSS4-overexpressed cells (Fig. 4C). These data suggested that rehabilitation of CLDN1 expression in HCC cells could promote CSC phenotype formation in a TMPRSS4-independent manner.

Previous studies have shown that activation of PI3K/AKT and ERK1/2 signaling pathways contributes to the tumor accelerating effect of TMPRSS4. In this study, the activity of ERK1/2 was decreased in TMPRSS4 knockdown cells, whereas it was elevated in TMPRSS4 overexpressing cells (Fig. 4D). However, there was no change in the phosphorylation of AKT. Based on these findings, we hypothesized that TMPRSS4 may regulate CLDN1 expression and CSC formation though activating ERK1/2 pathway. To further investigate this hypothesis, an inhibitor of ERK1/2, U0126, was used. Accompanied by decreased ERK1/2 activity, application of U0126 reduced the expression of CLDN1 and tumorsphere-forming activity of TMPRSS4-expressing cells in a dose-dependent manner (Fig. 4E and F). Thus, these data manifested that TMPRSS4 may induce CSC phenotype and up-regulate CLDN1 though ERK1/2 pathway.

4. Discussion

CLDN1, a central protein for intercellular junctions, plays crucial role in cell growth and differentiation [14]. Recently, some studies have reported that CLDNs abnormally expressed in various cancer types, including hepatocellular carcinomas [15]. Furthermore, accumulating evidences have elucidated that overexpression of CLDN1 (Claudin-1) was associated with lymph node metastasis, advanced pathological grade and poor prognosis, making it as an oncogene [16]. Previous studies suggested that forced expression of CLDN1 resulted in expression of the snail and slug and thereby mediated EMT [7]. In this study, we found CLDN1 was up-regulated in HCC tissues and cell lines, correlating with patients overall survival, suggesting that CLDN1 seemed to be regulating factors of tumor development and progression, which may help to predict outcome of patients with HCC.

It has been widely believed that cancers are driven by cancer stem cells (CSCs), which have capacities of differentiation, migration and radiation resistance [6]. Present researchers have demonstrated that CLDN1 could induce epithelial-mesenchymal transition (EMT) in HCC [15]. Since EMT played a vital role in the acquisition of CSC traits, we presumed CLDN1 could generate HCC cells with CSC features. CD133, CD44, KLF4 and SOX2 are common cancer stem cell markers used in HCC research [17]. As expected, forced CLDN1 expression resulted in the formation of CSC and changes of EMT and embryonic stem cell (ESC) related genes (Fig. 2).

TMPRSS4, a member of TTSP family, was found highly up-regulated in several cancer types, including pancreatic, colon, thyroid and gastric cancer tissues [18–20]. In addition, TMPRSS4 was correlated with self-renew ability in colon and lung cancer, and knockdown of TMPRSS4 levels resulted in stem cell markers decline, which accompanied with PI3KAKT and/or ERK1/2 phosphorylation [21]. It was reported earlier that TMPRSS4 was involved in tumor angiogenesis and metastasis in HCC cells [22]. In this study, we found overexpression of TMPRSS4 in HCC tissues, which were associated with CLDN1 expression (Fig. 3). Western blot analysis demonstrated that activation of ERK1/2, but not AKT, was found in TMPRSS4 overexpressing cells. By using of an ERK1/2 inhibitor, we further convinced that ERK1/2 signaling pathway was involved in TMPRSS4-mediated CSC formation in HCC. Moreover, we found that CLDN1 could be activated via ERK1/2 in TMPRSS4-overexpressing cells. We further found that CLDN1 played a crucial role in acquisition of CSC phenotype in HCC as downstream effector of the TMPRSS4/ERK1/2 signaling pathway.

Taken together, our results revealed a novel mechanism underlying the induction of cancer stem like cells (CSCs) in HCC fostered by TMPRSS4 mediated CLDN1 expression via ERK1/2 signaling pathway. Our findings also confirmed the link between TMPRSS4 and CLDN1 that contributed to the formation of CSCs, providing new targets for the treatment of HCC. Furthermore, up-regulation of TMPRSS4 and CLDN1 might be insensitive biomarkers for predicting poor prognosis for HCC patients.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2017.06.085.

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肝细胞癌组织中高尔基体蛋白73和转化生长因子β_1及Smad2的表达水平及其意义

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摘 要:目的 探讨高尔基体蛋白73(GP73)及转化生长因子β(TGF-β)/Smads信号通路与肝细胞癌(肝癌)的关系。方法 选取2010年1月—2014年12月新疆医科大学第一附属医院病理科收集的80例肝癌患者的肝癌组织及癌旁组织蜡块标本,并从本院病案室调阅患者的临床病历资料,包括性别、年龄、乙型肝炎表面抗原(HBs Ag)、血清甲胎蛋白(AFP)水平、有无肝硬化、有无门静脉癌栓、分化程度、TNM分期、有无血管侵犯。采用免疫组织化学En Vision二步法检测肝癌组织和癌旁组织GP73、TGF-β1和Smad2的阳性表达情况和积分光密度(IOD)。结果GP73定位于胞质,呈棕黄色颗粒。肝癌组织GP73阳性表达率为73.8%(59/80),高于癌旁组织的5.0%(4/80)(χ2=76.50,P<0.05)。TGF-β1和Smad2主要表达于胞质,偶见于胞核,为棕黄色深染颗粒。肝癌组织TGF-β1阳性表达率为81.2%(65/80),高于癌旁组织的6.2%(5/80)(χ2=65.67,P<0.05);肝癌组织Smad2阳性表达率为66.2%(53/80),高于癌旁组织的10.0%(8/80)(χ2=47.62,P<0.05)。Spearman相关分析结果显示,GP73与TGF-β1、Smad2呈正相关(rs=0.841、0.405,P<0.05)。不同性别患者肝癌组织GP73、Smad2表达水平比较,差异均无统计学意义(P>0.05),TGF-β1表达水平比较,差异有统计学意义(P<0.05);不同年龄、HBs Ag、AFP、有无肝硬化及门静脉癌栓患者肝癌组织GP73、TGF-β1和Smad2表达水平比较,差异均无统计学意义(P>0.05);不同分化程度、TNM分期、有无血管侵犯患者肝癌组织GP73、TGF-β1和Smad2表达水平比较,差异均有统计学意义(P<0.05)。结论 GP73在肝癌组织中多呈阳性表达,其可能通过调控TGF-β/Smads信号通路参与肝癌的发生与发展。

关键词:癌;肝细胞;转化生长因子β1;Smad2蛋白质;高尔基体蛋白73;

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Expression Level and Significance of GP73,TGF-β_1 and Smad2 in Hepatocellular Carcinoma Tissues

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Abstract: Objective To investigate the relationship between Golgi protein 73(GP73),TGF-β/Smads signaling pathway and hepatocellular carcinoma. Methods 80 cases of paraffin- embedded samples of hepatocellular carcinoma tissues and para- carcinoma tissues collected by Pathology Department of the First Affiliated Hospital of Xinjiang Medical University were selected from January 2010 to December 2014. And clinical data of these patients were consulted from the Medical Records Room of our hospital,including gender,age,hepatitis B surface antigen(HBs Ag),alpha fetoprotein(AFP) level,with or without liver cirrhosis,with or without portal vein tumor thrombus, differentiation degree, TNM staging, with or without vascular invasion. Immunohistochemistry En Vision system was adopted to detect positive expression and IOD of GP73 and TGF-β1,Smad2 in hepatocellular carcinoma tissues and para- carcinoma tissues. Results GP73, claybank particles, were located in cytoplasm. The positive expression rate of GP73 in hepatocellular carcinoma tissues was 73. 8%(59 /80),higher than 5. 0%(4 /80)(χ2= 76. 50,P < 0. 05) of para- carcinoma tissues. TGF-β1,Smad2 mainly expressing in cytoplasm,occasionally in karyon,were claybank dense granule. TGF-β1positive expression rate in hepatocellular carcinoma tissues was 81. 2%(65 /80),higher than 6. 2%(5 /80)(χ2= 65. 67, P < 0. 05) of para- carcinoma tissues. Smad2 positive expression rate in hepatocellular carcinoma tissues was 66. 2%(53 /80),higher than 10. 0%(8 /80)(χ2= 47. 62,P < 0. 05) of para-carcinoma tissues. Spearman relevant analysis results showed that GP73 correlated positively with TGF-β1,Smad2(rs= 0. 841,0. 405,P < 0. 05). The comparison of the expression levels of GP73, Smad2 in patients with different gender were not statistically significant(P > 0. 05), the comparison of the expression levels of TGF-β1in patients with different gender was statistically significant(P < 0. 05); the comparison of the expression levels of GP73, TGF-β1and Smad2 in patients with different age,HBs Ag,AFP,with or without liver cirrhosis,with or without portal

vein tumor thrombus were not statistically significant(P > 0. 05); the comparison of the expression levels of GP73, TGF-β1and Smad2 in patients with different differentiation degree,TNM staging,with or without vascular invasion were all statistically significant(P < 0. 05). Conclusion GP73,mainly expressing positively in hepatocellular carcinoma tissues,may be involved in the occurrence and development of hepatocellular carcinoma through the regulation of TGF-β / Smads signaling pathway.

Keyword : Carcinoma, hepatocellular; Transforming growth factor beta1; Smad2 protein; Golgi protein 73

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肝细胞癌(肝癌)是人类最常见的恶性肿瘤之一,根据全球最新数据统计,肝癌发病率在全身恶性肿瘤中位居第6位,病死率位居第2位,在我国形势更加严峻,发病和死亡人数占全球50%以上,堪称肝癌大国[1]。高尔基体蛋白73(Golgi protein 73,GP73)是一种新颖的Ⅱ型高尔基体跨膜糖蛋白,是潜在的肝癌血清肿瘤标志物,然而其在肝癌发生、发展中的功能及调节机制尚罕见报道。转化生长因子β_1(transforming growth factorβ_1,TGF-β_1)是一种多功能的细胞因子,其介导的TGF-β/Smads信号通路与多种肿瘤的发生及侵袭、转移密切相关。本研究采用免疫组织化学染色的方法,对肝癌组织及癌旁组织中GP73、TGF-β_1和Smad2的表达水平进行检测,从组织学水平上初步探讨GP73及TGF-β/Smads信号通路与肝癌发生/发展的可能机制,为进一步在分子学水平上探讨肝癌发病机制提供理论依据。

1 材料与方法

1.1 组织标本

经新疆医科大学第一附属医院伦理委员会批准,选取2010年1月—2014年12月新疆医科大学第一附属医院病理科收集的80例肝癌患者的肝癌组织及癌旁组织蜡块标本,并从本院病案室调阅该80例患者的临床病历资料,包括性别、年龄、乙型肝炎表面抗原(HBs Ag)、血清甲胎蛋白(AFP)水平、有无肝硬化、有无门静脉癌栓、分化程度、TNM分期、有无血管侵犯。80例患者病历资料完整,术前均未行放疗、化疗、射频消融及生物治疗等内科治疗,术后病理学确诊为肝癌,组织蜡块均经病理科两名资深医生进行HE染色,形态符合肝癌确诊依据[2]。

1.2 方法

1.2.1 主要试剂与仪器

兔抗人GP73多克隆抗体购自美国Proteintech Group公司,兔抗人TGF-β_1多克隆抗体和兔抗人Smad2多克隆抗体购自武汉博士德生物工程有限公司,免疫组织化学检测试剂盒、磷酸盐缓冲液(PBS)、枸橼酸抗原修复液购自北京中杉金桥生物技术有限公司。

1.2.2 GP73、TGF-β_1和Smad2检测

采用免疫组织化学En Vision二步法检测肝癌组织和癌旁组织GP73、TGF-β_1和Smad2的表达情况。组织标本常规石蜡包埋,5μm厚连续切片。石蜡切片经烤箱脱蜡后水化,3%H_2O_2阻断内源性过氧化物酶,微波炉枸橼酸抗原修复液修复,冷却后滴加一抗4℃冰箱过夜。每批实验均设阳性和阴性对照,其中阳性对照为兔抗人GP73多克隆抗体、兔抗人TGF-β_1多克隆抗体和兔抗人Smad2多克隆抗体,阴性对照为不加抗体的PBS;一抗工作液体浓度均为1∶50。次日滴加辣根过氧化物酶标记的二抗,37℃温箱孵育30 min,光镜下观察二氨基联苯胺(DAB)显色,苏木素复染,脱水,透明,中性树胶封固,镜下观察。

1.2.3 结果判定

(1)由两名病理科医生根据染色强度独立进行结果判定,以胞质显色为棕黄色至棕褐色颗粒为阳性细胞;按照着色强度评分:无色为0分,淡黄色为1分,棕黄色为2分,棕褐色为3分。使用Image J2x图像分析软件计算每个视野下阳性部位面积的百分比,每张切片随机选择5个视野,取其平均值为该张切片的阳性表达面积;按照阳性表达面积百分比评分:无阳性表达面积为0分,1%~20%为1分,21%~50%为2分,51%~80%为3分,>80%为4分。两项得分相乘,0~1分为"-",2~4分为"+",5~7分为"++",8~12分为"+++"。将"-~+"定义为蛋白低表达即阴性表达,将"++~+++"定义为蛋白高表达即阳性表达。(2)应用CMIAS真彩色医学图像分析系统,每张切片随机选择5个高倍(×200)视野,分别对各组5个视野中的免疫组织化学结果进行积分光密度(integral optical density,IOD)测定,然后计算平均IOD。

1.3 统计学方法

采用SPSS 17.0软件进行统计学分析,计量资料以($\bar{x}\pm s$)表示,两组间比较采用独立样本t检验;计数资料的分析采用χ^2检验;相关性分析采用Spearman相关分析。以P<0.05为差异有统计学意义。

2 结果

2.1 肝癌组织和癌旁组织GP73、TGF-β_1和Smad2阳性表达率比较

GP73定位于胞质,呈棕黄色颗粒(见图1,本文彩图详见本刊官网www.chinagp.net电子期刊相应文章附件)。肝癌组织GP73阳性表达率为73.8%(59/80),癌旁组织GP73阳性表达率为5.0%(4/80),两者GP73阳性表达率比较,差异有统计学意义(χ^2=76.50,P<0.05)。TGF-β_1和Smad2主要表达于胞质,偶见于胞核,为棕黄色深染颗粒(见图2、3)。肝癌组织TGF-β_1阳性表达率为81.2%(65/80),癌旁组织TGF-β_1阳性表达率为6.2%(5/80),两者TGF-β_1阳性表达率比较,差异有统计学意义(χ^2=65.67,P<0.05);肝癌组织Smad2阳性表达率为66.2%(53/80),癌旁组织Smad2阳性表达率为10.0%(8/80),两者Smad2阳性表达率比较,差异有统计学意义(χ^2=47.62,P<0.05)。

2.2 肝癌组织GP73与TGF-β_1、Smad2的相关性

Spearman相关分析结果显示,GP73与TGF-β_1、Smad2呈正相关(r_s=0.841、0.405,P<0.05)。

2.3 不同临床特征患者肝癌组织GP73、TGF-β_1和Smad2表达水平比较

不同性别患者肝癌组织GP73、Smad2表达水平比较,差异均无统计学意义(P>0.05),TGF-β_1表达水平比较,差异有统计学意义(P<0.05);不同年龄、HBs Ag、AFP、有无肝硬化及门静脉癌栓患者肝癌组织GP73、TGF-β_1和Smad2表达水平比较,差异均无统计学意义(P>0.05);不同分化程度、TNM分期、有无

血管侵犯患者肝癌组织GP73、TGF-β_1和Smad2表达水平比较,差异均有统计学意义(P<0.05,见表1)。

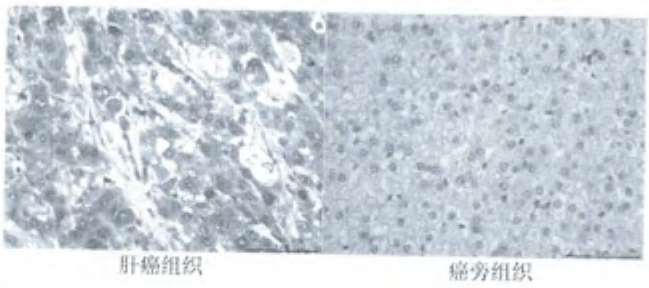

图1 GP73在肝癌组织及癌旁组织中的表达(免疫组织化学DAB显色,×400)Figure 1 Expression of GP73 in hepatocellular carcinoma tissues and para-carcinoma tissues

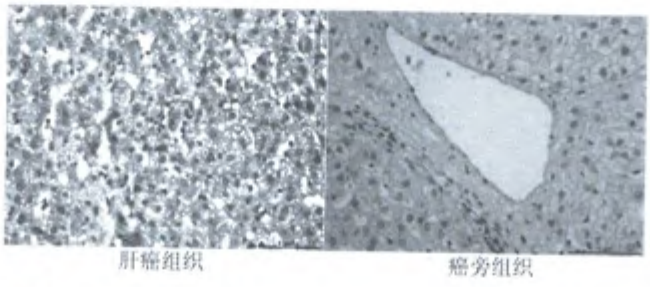

图2 TGF-β1在肝癌组织及癌旁组织中的表达(免疫组织化学DAB显色,×400)Figure 2 Expression of TGF-β1in hepatocellular carcinoma tissues and para-carcinoma tissues

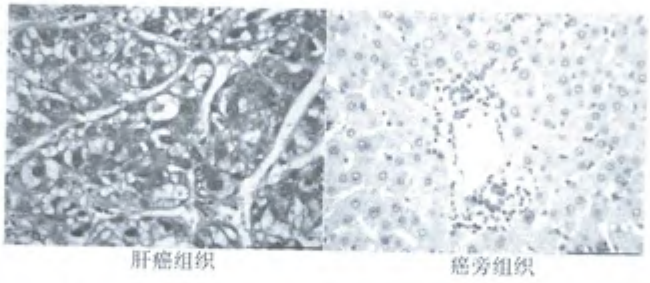

图3 Smad2在肝癌组织及癌旁组织中的表达(免疫组织化学DAB显色,×400)Figure 3 Expression of Smad2 in hepatocellular carcinoma tissues and para-carcinoma tissues

3 讨论

GP73是一种新近发现的Ⅱ型高尔基体跨膜糖蛋白,因其在十二烷基硫酸钠(SDS)-聚丙烯酰胺凝胶(PAGE)电泳中显示相对分子量为7.3×10^4而得名。2000年,KLADNEY等[3] 在构建成人巨大细胞性肝炎(giant cell hepatitis,GCH)时首次发现GP73,进一步研究发现,在病毒性肝炎、自身免疫性肝炎、酒精性肝硬化患者肝组织中GP73的表达明显高于正常组[4] 。2004年,MAITRA等[5] 研究发现GP73可以在血清中检测到,并认为GP73可以作为肝脏疾病的标志物。在亚洲人群的研究中,毛一雷等[6] 初步确立了我国正常人GP73参考范围,并说明GP73不但可以作为诊断肝癌的敏感性指标,而且对预测预后有一定的价值。同时,本课题组前期研究也证实血清GP73水平在肝癌患者中升高最为明显,临床上认为其可能成为肝癌早期诊断的理想标志物[7-9] 。

表1 不同临床特征患者肝癌组织GP73、TGF-β_1和Smad2表达水平比较($\bar{x}\pm s$)Table 1 Comparison of expression levels of GP73,TGF-$\beta_1$and Smad2 in different clinical features 下载原表

临床特征	例数	IOD 值		
		GP73	TGF-β_1	Smad2
性别				
男	49	17.1±3.2	15.3±1.8	13.5±4.4
女	31	16.4±4.1	14.5±1.4	13.1±5.1
t 值		0.854	2.103	0.372
P 值		0.396	0.039	0.711
年龄(岁)				
>60	35	18.2±2.8	15.6±3.1	14.0±2.2
≤60	45	17.5±4.4	16.2±3.1	13.3±3.3
t 值		0.820	0.859	1.390
P 值		0.415	0.393	0.168
HBsAg				
阳性	30	17.2±3.5	15.2±3.0	14.3±3.9
阴性	50	17.9±3.0	14.9±3.5	14.6±3.9
t 值		0.949	0.391	0.328
P 值		0.346	0.697	0.744
AFP(μg/L)				
>400	14	18.3±3.1	15.6±2.9	14.5±4.2
≤400	66	16.6±3.7	15.3±3.6	13.9±5.0
t 值		1.602	0.291	0.418
P 值		0.113	0.772	0.677
肝硬化				
有	32	17.5±2.9	14.9±3.2	14.4±3.3
无	48	18.3±3.1	16.0±2.9	14.9±2.9
t 值		1.168	1.422	0.702
P 值		0.246	0.159	0.485
门静脉癌栓				
有	30	16.9±3.6	16.5±2.9	13.9±4.7
无	50	17.5±3.1	15.4±3.2	14.3±3.9
t 值		0.657	1.540	0.405
P 值		0.513	0.128	0.686
分化程度				
高中分化	37	14.4±2.9	10.9±2.1	8.9±3.5
低分化	43	20.1±3.6	16.1±3.5	15.9±4.1
t 值		7.714	7.893	8.230
P 值		<0.001	<0.001	<0.001
TNM 分期(期)				
Ⅰ~Ⅱ	39	13.3±3.2	9.9±2.8	10.2±2.2
Ⅲ~Ⅳ	41	21.5±3.6	17.3±3.3	16.9±3.3
t 值		10.748	10.642	10.628
P 值		<0.001	<0.001	<0.001
血管侵犯				
有	42	22.5±3.8	16.9±3.4	17.6±3.5
无	38	14.2±2.8	10.0±2.8	10.3±2.9
t 值		11.024	9.847	9.964
P 值		<0.001	<0.001	<0.001

2011年肿瘤权威教授CHAFFER等[10]指出肿瘤细胞只有发生上皮-间质转化(epithelial-mesenchymal transition,EMT)具备了运动侵袭能力,才能进行局部浸润和远处转移。因此研究肝癌发生EMT的关键分子及信号通路对阻断肝癌细胞侵袭、转移的发生和发展有非常重要的意义。2013年本课题组就GP73在肝癌发生、发展中的调节机制进行初步研究发现,GP73与EMT关系密切,发生EMT的主要分子特征是上皮细胞标志物如E-钙黏蛋白(E-cadherin)表达下调和间质细胞标志物如波形蛋白(Vimentin)表达上调[11-12]。2014年ZHANG等[13]首先报道在肝癌细胞株Hep G2及BEL-7402中应用siRNA对GP73进行干扰后亦提示GP73在肝癌发生、发展的机制中起到重要作用。目前尽管国内外学者对GP73作为肝癌相关性蛋白的研究已取得一定进展,但是,绝大多数研究仅停留在GP73作为肝癌血清肿瘤标志物的方向,其在肝癌发生、发展中的功能及调节机制未明确阐明,严重阻碍其临床价值的转化。

EMT的发生涉及多个环节,其分子通路十分复杂,但在诱导EMT发生的众多细胞因子和转录因子中,TGF-β被证实是诱导EMT的关键因子,TGF-β共有3种,即TGF-β_1、TGF-β_2、TGF-β_3,其中TGF-β_1在肿瘤中主要参与刺激细胞生长、诱导细胞分化和调亡等精细调节活动,近年来诸多研究报道TGF-β能够诱导体内、体外多种肿瘤细胞发生EMT,认为肿瘤细胞系中转入外源性TGF-β后获得EMT特征,侵袭、转移能力增加;反之抑制TGF-β信号通路后能逆转EMT的发生,抑制肿瘤细胞的侵袭和转移[14-15]。TGF-β在EMT中起着中心作用,即肿瘤转移的关键分子事件。TGF-β介导EMT主要涉及Smad依赖[16]和Smad非依赖[17]两条信号通路。在Smad依赖信号通路中,TGF-β与细胞膜上Ⅱ型受体结合,Ⅱ型受体激酶诱导Ⅰ型受体磷酸化,并与Smad2/3形成复合物。Smad2/3磷酸

化后,与Smad4形成异三聚体或四聚体,使E-cadherin表达下调和Vimentin表达上调,介导EMT发生。因此本研究采用免疫组织化学染色的方法在组织学水平初步探讨GP73与TGF-β/Smads信号通路的关系。本研究结果显示,80例组织蜡块中GP73和TGF-β_1、Smad2在肝癌组织中阳性表达率高于癌旁组织,并且Spearman相关分析显示,在肝癌组织中GP73与TGF-β_1、Smad2均呈正相关,GP73、TGF-β_1和Smad2表达水平与肝癌的分化程度、TNM分期及血管侵犯关系密切。

综上所述,本研究结果表明,GP73的表达与TGF-β/Smads信号通路关系密切,但其具体作用机制及是否还有其他信号通路的参与,进而调节肝癌的发生与发展,有待进一步的分子水平实验研究加以证实。

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本文无利益冲突。

本研究背景:

肝细胞癌(肝癌)作为一种常见的恶性肿瘤,预后极差,导致肝癌预后差的主要原因是高侵袭、转移性。因此,研究肝癌侵袭、转移的机制,寻找有效的分子干预靶点对改善肝癌预后具有重要意义。本课题组前期研究发现:高尔基体蛋白73(GP73)与上皮-间质转化(EMT)具有相关性。推测GP73参与调控肝癌发生EMT从而获得侵袭、转移特征。为证实这一假说,本课题组拟利用分子生物学技术方法和原理,在组织学水平上初步探讨GP73是否作为调控肝癌EMT发生的关键因子及其传导通路,有助于阐明肝癌发生侵袭、转移的分子机制,为寻求肝癌有效的干预靶点提供新的理论依据。

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· 短篇论著 ·

靶向干扰高尔基体蛋白73基因对肝癌细胞侵袭转移能力的影响

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【关键词】 肝肿瘤； RNA，小分子干扰； 肿瘤侵润

Effect of targeted interference of Golgi protein 73 gene on invasion and metastasis of liver cancer cells

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【Key words】 Liver neoplasms; RNA, small interfering; Neoplasm invasiveness

肝细胞癌（HCC）是临床常见恶性肿瘤之一，对人类的健康危害极大，5年生存率约为45%[1]。研究结果显示HCC的侵袭、转移是多因素相互作用、相互影响的一个复杂过程，其分子机制尚未明确。因此，阐明HCC侵袭、转移的分子机制，有利于为治疗肝癌提供新的靶点提供参考，对指导临床提高肝癌疗效有重要意义。

高尔基体糖蛋白73（golgi glycoprotein 73，GP73）又称Ⅱ型高尔基体膜蛋白（golgi phosphoprotein2，GOLPH2），由美国学者Kladney等2000年发现[2-3]。GP73是一种肝癌相关性蛋白，在肝癌的诊断和鉴别诊断中具有重要的应用价值[4-8]。

我们在前期工作中应用免疫组织化学技术，发现GP73蛋白在肝细胞癌组织中表达明显高于癌旁组织[9-12]，其高表达与肿瘤血管浸润、病理学分期密切相关[13]，由此推测GP73可能与HCC的侵袭、转移等恶性生物学特性相关。本研究采用脂质体介导的RNA靶向干扰（RNA interference，RNAi）技术，沉默GP73基因表达，探讨GP73在肝癌发生、发展机制中的作用，尤其是在侵袭、转移方面，探讨GP73基因能否作为一个抑制肝癌侵袭、转移的治疗靶点。

一、材料与方法

1. 细胞系与试剂：人肝癌细胞SMMC-7721、Bel-7404、人正常肝细胞株Chang均购自美国Sigma公司；DMEM高糖培养基及胎牛血清均购自美国Hyclone公司，总RNA提取试剂盒及逆转录试剂盒均购自上海莱工公司；RT-PCR引物由上海生物工程有限公司合成；lipofectamineTM2000购自美国Invitrogen公司；Western blot试剂盒购自瑞士Roche公司；Transwell小室购美国BD公司。

2. 细胞培养：上述细胞株均接种于含有10%胎牛血清的高糖DMEM（不含抗生素），并放置在37℃、5% CO_2孵箱内培养，取对数生长期的细胞用于实验。

3. 定量PCR法：细胞总RNA使用Trizol试剂（购自美国Invitrogen公司）从两株肝癌细胞系中分离，并使用分光光度计定量。提取的RNA使用逆转录试剂盒（美国Thermo，Madison，WI）进行逆转录，分析是根据定量PCR试剂盒说明书(SYBR绿色，购自瑞士Roche公司)。β-肌动蛋白(actin)作为参考，引物序列：上游5'-TGTGTCCGTCGTGGATC-3'；下游5'-CCTGCTTCACCACCTTCTTGA-3'；GP73引物序列为：上游5'-CAGCGCTGATTTTGAGATGA-3'，下游5'-ATGATCCGTGTCTGGAGGTC-3'。以β-肌动蛋白作为一个比较CT方法确定基因的表达水平参考。实验进行3次，定量分析是由$2^{-\Delta\Delta CT}$值进行比较。

4. 质粒构建和转染：小干扰RNA(siRNA)由吉玛公司合成并提供，分为3组：(1) 实验组：上游5'-GUGCUUGGUAACAGCAAGUTT-3'，下游5'-ACUUGUUACCAAGCACTT-3'；(2)阴性对照组：上游5'-UUCUCCGAACGUGUCACGUTT-3'，下游5'-ACGUGACACGUUCGGAGAATT-3'；(3) 空白对照组；按lipofectamine TM2000说明转染SM77-21及Bel-7404细胞株，48 h后使用RT-PCR和Western blot法来确定所述目标站点与干扰效率。

5. 检测细胞侵袭和运动能力：采用Transwell小室，取200 μl无血清培养基至小室上室置37℃培养箱内30 min，取转染后的培养48 h的SMMC-7721及Bel-7404细胞制成1×10^6个/L单细胞悬液，取200 μl移入上室内，下室内加入500 μl含10%胎牛血清的DMEM培养液，37℃培养24 h后取出。以棉签小心刮除滤膜上室面的细胞，侵袭到下室面的细胞以甲醛固定，结晶紫染色20 min，用磷酸盐缓冲液洗涤1次，在200倍光镜下任取5个视野计算细胞数，以其均数表示侵袭细胞数。

6. 划痕实验：SMMC-7721和Bel-7404细胞接种于24孔板培养，转染后用10 μl枪头垂直划伤，然后磷酸盐缓冲液洗涤1次，加入含10%胎牛血清的DMEM培养液，分别0 h、48 h后在倒置显微镜下观察细胞迁移，迁移的距离由迁移细

胞前沿与划痕伤边缘进行比较。迁移率＝（干预组细胞迁移距离／未给予干预组细胞迁移距离）×100%。实验分别至少进行4次。

7. 统计学方法：数据使用SPSS17.0统计软件进行数据分析。数据以均数±标准差（$\bar{x}\pm s$）表示。两样本均数间比较t检验，多样本均数间比较行方差分析，方差不齐时先进行变量转换，$P<0.05$为差异有统计学意义。

二、结果

1. GP73基因沉默效果：通过RNA干扰，48 h后提取细胞RNA，经RT-PCR法检测mRNA水平，肝癌SMMC-7721细胞实验组GP73 mRNA的相对表达量（0.42±0.05）显著低于空白对照组（0.97±0.04），差异有统计学意义（$P<0.05$）；肝癌SMMC-7721细胞实验组GP73 mRNA的相对表达量（0.42±0.05）显著低于阴性对照组（0.69±0.17），差异有统计学意义（$P<0.05$）；肝癌Bel-7404细胞实验组GP73 mRNA的相对表达量（0.32±0.11）显著低于空白对照组（0.96±0.07），差异有统计学意义（$P<0.05$）；肝癌Bel-7404细胞实验组GP73 mRNA的相对表达量（0.32±0.11）显著低于阴性对照组（0.71±0.18），差异有统计学意义（$P<0.05$）；以上结果显示，通过脂质体介导的siRNA转染使GP73 mRNA表达均下降（图1）。

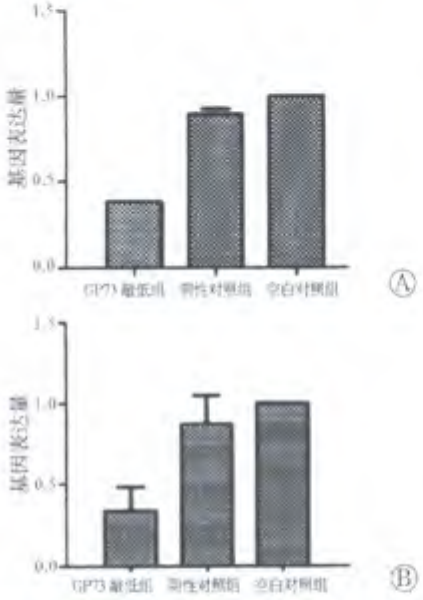
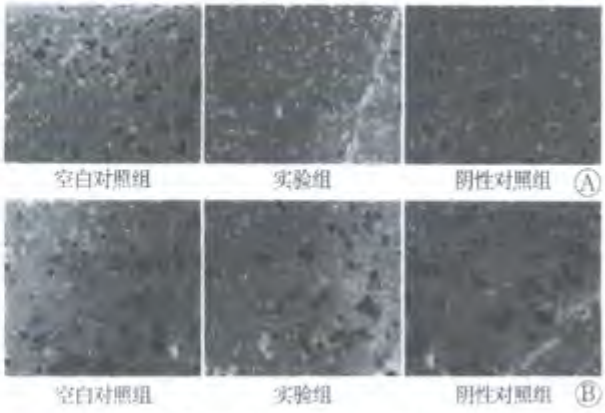


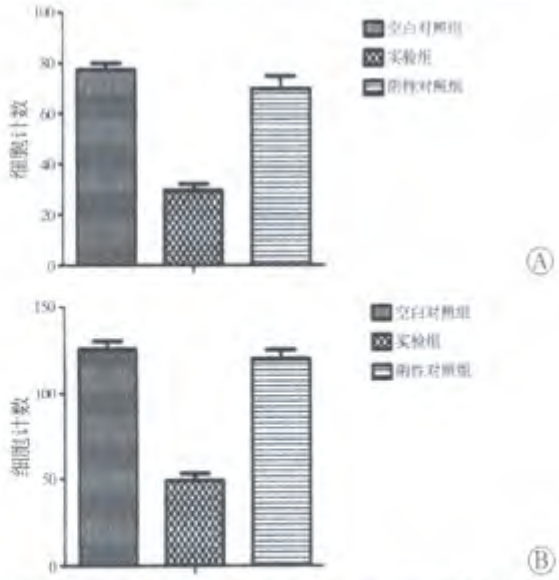
图1 RT-PCR检测肝癌细胞SMMC-7721与Bel-7404中GP73基因的表达情况

2. 沉默GP73基因对肝癌细胞侵袭能力影响：用Transwell小室法沉默SMMC-7721及Bel-7404细胞内GP73基因对肝癌细胞侵袭能力的影响。实验组SMMC-7721细胞穿过Transwell小室底部纤维膜数量（47±4.16，$P<0.001$）显著低于空白对照组（125.33±5.64，$P<0.001$）和阴性对照组（117.54±8.44）；实验组Bel-7404细胞穿过Transwell小室底部纤维膜数量（25.41±5.82，$P<0.001$）显著低于空白对照组（75.75±10.36，$P<0.001$）和阴性对照组（68.32±4.95），见图3。



注：侵袭实验紫红色为穿过小室底部的细胞

图2 SMMC-7721细胞及Bel-7404细胞Transwell小室试验结果



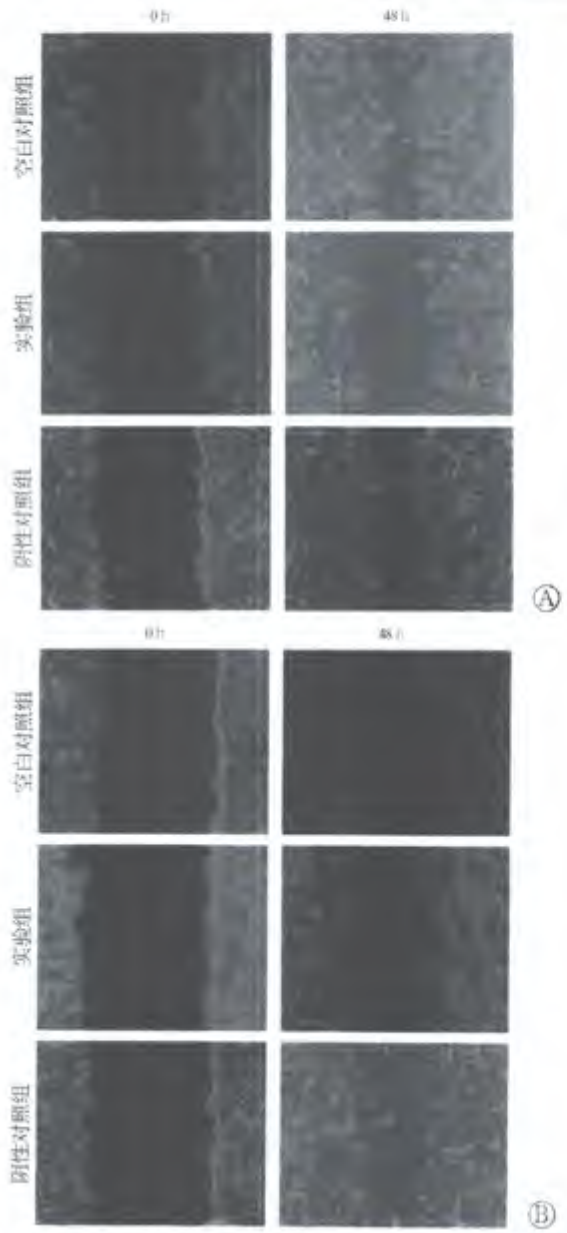
A：SMMC-7721细胞穿过小室底部数量；B：Bel-7404细胞穿过小室底部数量，结晶紫染过细胞的计数，实验组与空白对照组相比，$P<0.05$，阴性对照组与空白对照组相比，$P>0.05$

图3 Transwell小室试中穿过小室底部的细的计数柱状图

3. 沉默GP73基因对肝癌细胞转移能力影响：转染GP73 siRNA后，细胞划痕试验观察对细胞迁移能力的影响，0 h SMMC-7721细胞空白对照组，实验组与阴性对照组对比，划痕愈合距离比之间差异无统计学意义（$P>0.05$）。转染48 h后SMMC-7721细胞空白对照组，实验组与阴性对照组迁移率分别约为41%，20%和38%（$P<0.001$，图4A）；0 h Bel-7404细胞空白对照组，实验组与阴性对照组对比，划痕愈合距离比之间差异无统计学意义（$P>0.05$）。转染48 h后Bel-7404细胞空白对照组，实验组与阴性对照组迁移率分别约为37%，17%和32%。（$P<0.001$，图4B）。

三、讨论

HCC是一种常见的恶性肿瘤，其死亡率不断上升。在全球每年60万人的因HCC死亡[14]。由于它高异质性和肝癌致癌具体机制的不确定性，高侵袭转移性，发现时多为晚期，对治疗造成了障碍，故寻求抑制肝癌侵袭、转移治疗靶点，对指导临床提高肝癌治疗疗效有重要意义。GP73



A：下调 GP73 表达的 SMCC-7721；B：Bel-7404 细胞对的细胞迁移的影响（×100）

图 4　SMMC-7721 细胞及 Bel-7404 细胞划痕试验结果

是 II 型高尔基跨膜糖蛋白，它最初是从成年巨细胞性肝炎患者中得到的[3]。在正常的肝脏中 GP73 主要是在汇管区的胆管上皮细胞中表达，且定位在管腔侧的细胞核和细胞膜之间，大部分的正常肝细胞是不表达 GP73 的[15-16]，其在肝炎，肝癌患者的血清中逐渐升高，在肝癌中升高最明显[12-13]。

Mao 等[9]报道在国际组的大样本，即 GP73 在肝癌的敏感度和特异度均比甲胎蛋白更高，这表明 GP73 是一种新型的肝癌的早期诊断的肿瘤标志物[9]，然而目前绝大多数研究仅仅停留在 GP73 作为肝癌血清肿瘤标志物的方向，而其在肝癌的转移侵袭中的功能及调节机制却没有阐明。

在本研究中我们首先针对 GP73 基因设定了 siRNA 靶点作为实验组，同时设定了一条阴性对照 siRNA。通过 PCR 初步检测发现，两组细胞在转染 48 h 后与对照组靶基因 GP73 相比，实验组 GP73 mRNA 表达量显著低于阴性对照组及空白对照组，差异有统计学意义。本研究中选择 SMMC-7721 及 Bel-7404 细胞检测 GP73 在人类肝癌细胞中的活性。划痕实验和 Transwell 小室法是用于评估肿瘤细胞运动经典方法。试验结果显示：在转染 48 h 后，SMMC-7721 细胞和 Bel-7404 细胞实验组细穿过 Transwell 小室底部纤维膜数量显著低于空白对照组和阴性对照组；而划痕试验结果显示：转染 48h 后两组细胞实验组与空白对照组和阴性对照组相比迁移率低并有统计学意义，故根据实验结果可得知 GP73 对肝癌细胞的侵袭转移能力是有明显的影响，同时此结果与前期发现的肝癌细胞组织中 GP73 蛋白的表达明显高于癌旁组织，同时其高表达与肿瘤血管浸润、病理分期密切相关等结果相符合，推测 GP73 可能与 HCC 的侵袭、转移等恶性生物学特性相关，从而推断出 GP73 可参与肝癌细胞的迁移、侵袭过程，与肝癌病情进展关系密切。可能成为肝癌基因治疗的新靶点。

利益冲突　无

作者贡献声明　迫力热吧·博力东：试验的操作，资料分析与解释，文章撰写；杨颖：论文选题，对文章内容审阅；黄凤玲：文献收集；刘攀，张瑞丽，张宋安，肖蕾：支持性贡献；包水星：指导及支持性贡献

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·读者·作者·编者·

本刊对来稿中统计学处理的有关要求

1. 统计研究设计：应交代统计研究设计的名称和主要做法。如调查设计（分为前瞻性、回顾性或横断面调查研究）；实验设计（应交代具体的设计类型，如自身配对设计、成组设计、交叉设计、析因设计、正交设计等）；临床试验设计（应交代属于第几期临床试验，采用了何种盲法措施等）。主要做法应围绕4个基本原则（随机、对照、重复、均衡）概要说明，尤其要交代如何控制重要非试验因素的干扰和影响。

2. 资料的表达与描述：用 $\bar{x} \pm s$ 表达近似服从正态分布的定量资料，用M（QR）表达呈偏态分布的定量资料；用统计表时，要合理安排纵横标目，并将数据的含义表达清楚；用统计图时，所用统计图的类型应与资料性质相匹配，并使数轴上刻度值的标法符合数学原则；用相对数时，分母不宜小于20，要注意区分百分率与百分比。

3. 统计分析方法的选择：对于定量资料，应根据所采用的设计类型、资料所具备的条件和分析目的，选用合适的统计分析方法，不应盲目套用 t 检验和单因素方差分析；对于定性资料，应根据所采用的设计类型、定性变量的性质和频数所具备的条件以及分析目的，选用合适的统计分析方法，不应盲目套用 χ^2 检验。对于回归分析，应结合专业知识和散布图，选用合适的回归类型，不应盲目套用简单直线回归分析，对具有重复实验数据的回归分析资料，不应简单化处理；对于多因素、多指标资料，要在一元分析的基础上，尽可能运用多元统计分析方法，以便对因素之间的交互作用和多指标之间的内在联系进行全面、合理的解释和评价。

4. 统计结果的解释和表达：当 $P<0.05$（或 $P<0.01$）时，应说明对比组之间的差异有统计学意义，而不应说对比组之间具有显著性（或非常显著性）的差别；应写明所用统计分析方法的具体名称（如：成组设计资料的 t 检验、两因素析因设计资料的方差分析、多个均数之间两两比较的 q 检验等），统计量的具体值（如 $t=3.45$，$\chi^2=4.68$，$F=6.79$ 等），应尽可能给出具体的 P 值（如 $P=0.0238$）；当涉及到总体参数（如总体均数、总体率等）时，在给出显著性检验结果的同时，再给出95%可信区间。

项目编号	XJGRI2017082
研究类别	科研项目
申报类型	临床医学

新疆研究生科研创新项目

协 议 书

项目名称：	GP73 调控 PI3K/AKT 信号通路对肝癌细胞索拉非尼耐药的影响及其机制研究
项目管理方：	新疆医科大学研究生学院
项目依托方：	新疆医科大学第一临床学院
研究生姓名：	杨颖
学 号：	107601160027
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联系电话：	15022944632
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批准金额：	1.8 万元
执行时间：	2017.10-2018.10

新 疆 医 科 大 学 研 究 生 学 院 制 表

填写说明

1、项目立项有关信息参照《2017年度新疆研究生科研创新项目立项名单及经费表》进行填写。

2、项目研究内容应与《新疆研究生科研创新项目申请书》一致。

3、本协议一式三份，用A4纸打印，于左侧装订成册。

为做好新疆医科大学“新疆研究生科研创新项目”执行工作，新疆医科大学（以下简称甲方）与项目申请人 杨颖 （以下简称乙方）及项目负责人所在学院（以下简称丙方）就新疆医科大学“新疆研究生科研创新项目”签定执行协议书，确定相关条款，以保证此项目的顺利进行：

一、本合同一经签订，甲、乙、丙及导师各方均应认真履行协议条款的义务。履行协议过程中，如需变动或修改，各方应友好协商解决或签定相应的补充协议，补充协议与本协议具有等同的法律效力。

二、甲方负责建立项目经费本进行资助经费的管理，经费分两次下拨，立项后拨付资助经费的75%，结题合格后给予拨付剩余的20%，资助经费的5%作为项目管理费使用。经费只用于申请项目的研究，不能挪作它用。经费的使用必须经过导师及研究生学院签字确认，方可报销。

三、项目必须进行中期检查，由甲方根据实际情况确定中期检查时间和要求，乙方向甲方提交《中期检查报告》，对于中期检查不合格的项目，将冻结剩余经费直到项目验收通过后。

四、应按研究计划完成项目研究内容并按时结题。除了结题报告外，结题要求不得低于项目申请书的预期研究成果要求。

五、资助项目发表的论文、专著、学位论文、设计、调研报告等成果，均应标注“新疆维吾尔自治区研究生科研创新项目资助”及项目批准号，未标注的不得作为结题验收材料，项目研究成果属新疆医科大学所有，个人不得私自转让。

六、乙方应在毕业前，按时按要求结题，并将本项目成果交付甲方，否则甲方可终止协议并追回资助经费。

七、丙方及导师协助甲方管理本项目，负责监督，保证项目实施，并负责组织、协调项目实施过程中出现的问题。

八、本协议自各方签字盖章后生效。一式3份，甲、乙、丙方各执一份。

九、本协议的解释权归甲方所有。

一、项目目标和主要研究内容(800 字内)

1.1 研究目标

在细胞水平研究 GP73 调控 PI3K/Akt 信号通路→促进 EMT，增强干性→索拉非尼抵抗→获得耐药

①证明耐药株对索拉非尼治疗抵抗，比较耐药株和亲本株对增殖、凋亡、活性、侵袭、转移的不同，共聚焦显微镜观察形态改变；

②证明耐药株中 GP73 表达上调及 PI3K/Akt 信号通路激活，EMT 及肿瘤干性相关指标表达均增强。

1.2 研究内容

1）耐药株和亲本株的建立及功能鉴定 ：

①索拉非尼耐药细胞株和亲本细胞株的建立：hepG2-SR 与 hepG2 及 Huh7-SR 与 Huh7 细胞。采用浓度梯度递增法建立肝癌对索拉非尼获得性耐药胞株。

②耐药株和亲本株功能鉴定：证明耐药株对索拉非尼治疗抵抗，亲本株对其治疗敏感，应用克隆实验、流式细胞仪、CCK-8 法、Transwell 小室、划痕实验对细胞增殖、凋亡、活性、侵袭、转移能力的不同，共聚焦显微镜观察耐药株和亲本株形态区别。

2）证明 GP73 与 PI3K/Akt 通路诱导肝癌发生 EMT 及增强干性的调控作用：

①耐药株中 GP73 表达升高及 PI3K/Akt 信号通路激活 ：检测耐药株和亲本株中 GP73、PI3K、t-Akt、p-Akt、m-TOR 基因和蛋白。

②耐药株发生 EMT：在耐药株和亲本株中，qRT-PCR 检测 EMT 相关分子：E-cadherin、N-cadherin、Vimentin、Western blot 方法检测上述指标蛋白。

③耐药株肿瘤干性表型增强：Western blot 方法检测耐药株和亲本株中干性相关基因 Oct-4,CD44,SOX-2 的 mRNA 蛋白表达。

二、项目实施方案(600 字内)

1）**耐药株和亲本株的建立及功能鉴定** ：

①索拉非尼耐药细胞株和亲本细胞株的建立：hepG2-SR 与 hepG2 及 Huh7-SR 与 Huh7 细胞。采用浓度梯度递增法建立肝癌对索拉非尼获得性耐药胞株。将肝癌细胞在含 0.5umol/L 索拉非尼培养液中培养，待细胞能稳定生长，逐渐提高索拉非尼的浓度，每次提高 0.25umol/L，直至细胞能在相当于临床最高耐受浓度(含 5umol/L 索拉非尼)的培养液中稳定生长。MTT 方法检测耐药株和亲本株的 50%抑制浓度(IC50, half maximal inhibitory concentration)被测量的拮抗剂的半抑制浓度，耐药株 IC50 是亲本株的 3 倍以上，符合耐药标准。

②耐药株和亲本株功能鉴定：证明耐药株对索拉非尼治疗抵抗，亲本株对其治疗敏感，应用克隆实验、流式细胞仪、CCK-8 法、Transwell 小室、划痕实验分别检测耐药株和亲本株对细胞增殖、凋亡、活性、侵袭、转移能力的不同，共聚焦显微镜观察耐药株和亲本株形态区别。

2）**证明 GP73 与 PI3K/Akt 通路诱导肝癌发生 EMT 及增强干性的调控作用**：

①耐药株中 GP73 表达升高及 PI3K/Akt 信号通路激活 ：在耐药株和亲本株中，采用 Western blot 方法分别检测耐药株和亲本株中 GP73、PI3K、t-Akt、p-Akt、m-TOR 基因和

②耐药株发生 EMT ：在耐药株和亲本株中，采用 qRT-PCR 检测 EMT 相关分子：E-cadherin、N-cadherin、Vimentin，Western blot 方法检测上述指标蛋白。免疫荧光染色后，激光共聚焦扫描显微镜下观察上皮-间质表型转化的细胞形态改变，上皮标志物 E-cadherin、间质标志物 N-cadherin 和 Vimentin 的定位及表达变化。

③耐药株肿瘤干性表型增强：在耐药株和亲本株中，采用 Western blot 方法检测肿瘤干性相关基因 Oct-4, CD44, SOX-2 的 mRNA 蛋白表达。

三、项目的年度计划及时间安排

时间	研究计划
2017.10-2017.03	细胞水平研究耐药株中 GP73 调控 PI3K/Akt 信号通路→促进 EMT，增强肿瘤干性→索拉非尼抵抗
2018.04-2018.10	总结，补遗，上报结题。发表 1-2 篇 SCI 收录论文。顺利结题。

四、预期研究成果

1.阐明 GP73 调控 PI3K/Akt 通路促进肝癌发生 EMT，增强干细胞表型，导致索拉非尼耐药的分子机制。

2.发表研究论文 1-2 篇。

五、项目经费管理　　计划资助总额: ____ (万元)

序号	经费开支科目	经费预算金额（万元）
1	试剂费	1.75
2	复印费	0.05
经费合计：	（万元）	

六、协议签署

甲方：

主管领导签章：

年　月　日

乙方：

项目申请人签字：

年　月　日

丙方：

主管领导签章：

年　月　日

项目编号	CXCY2017021
研究类别	自然科学
申报类型	创新项目

新疆医科大学研究生创新创业项目

协 议 书

项 目 名 称： GP73 调控 PI3K/AKT 信号通路对肝癌细胞索拉非尼耐药的影响及其机制研究

项目管理方： 新疆医科大学研究生学院

项目依托方： 新疆医科大学　　学院

研究生姓名： 杨颖

学　　号： 107601160027

培 养 层 次： √博士研究生　□硕士研究生

专　　业： 肿瘤学

导 师 姓 名： 包永星

联 系 电 话： 15022944632

电 子 邮 箱： 297215484@qq.com

批 准 金 额： 1 万元

执 行 时 间： 2017.6-2018.6

新 疆 医 科 大 学 研 究 生 学 院 制 表

填写说明

1、项目立项有关信息参照《2017 年新疆医科大学研究生创新创业项目立项名单》进行填写。

2、项目研究内容应与《新疆医科大学研究生创新创业项目申请书》一致。

3、参与人不得超过 5 人（含负责人），同一人主持和参加的项目不得超过 2 项，否则课题将予以取消。

4、本协议一式三份，用 A4 纸双面打印，于左侧装订成册。

为做好“新疆医科大学研究生创新创业项目”执行工作，新疆医科大学（以下简称甲方）与项目申请人_____（以下简称乙方）及项目负责人所在学院（以下简称丙方）就新疆医科大学“新疆医科大学研究生创新创业项目”签定执行协议书，确定相关条款，以保证此项目的顺利进行：

一、本合同一经签订，甲、乙、丙及导师各方均应认真履行协议条款的义务。履行协议过程中，如需变动或修改，各方应友好协商解决或签定相应的补充协议，补充协议与本协议具有等同的法律效力。

二、甲方负责建立项目经费本进行资助经费的管理。经费只用于申请项目的研究，不能挪作它用。经费的使用必须经过导师及研究生学院签字确认，方可报销。

三、项目必须进行中期检查，由甲方根据实际情况确定中期检查时间和要求，乙方向甲方提交《中期检查报告》，对于中期检查不合格的项目，将冻结剩余经费直到项目验收通过后。

四、应按研究计划完成项目研究内容并按时结题。除了结题报告外，结题要求不得低于项目申请书的预期研究成果要求。

五、资助项目发表的论文、专著、学位论文、设计、调研报告等成果，均应标注“新疆医科大学研究生创新创业项目资助”及项目批准号，未标注的不得作为结题验收材料，项目研究成果属新疆医科大学所有，个人不得私自转让。

六、乙方应在毕业前，按时按要求结题，并将本项目成果交付甲方，否则甲方可终止协议并追回资助经费，其导师三年内不得指导研究生申报研究生课题。

七、丙方及导师协助甲方管理本项目，负责监督，保证项目实施，并负责组织、协调项目实施过程中出现的问题。

八、本协议自各方签字盖章后生效。一式 3 份，甲、乙、丙方各执一份。

九、本协议的解释权归甲方所有。

一、项目目标和主要研究内容(800 字内)

1.项目目标

索拉非尼是晚期肝癌一线治疗的首选方案，但耐药是治疗失败的主要原因，因此，阐明耐药机制对克服其治疗抵抗具有重要意义。目前认为上皮间质转化（EMT）与干细胞在肿瘤耐药中发挥重要作用。我们前期研究发现上调高尔基体蛋白 73（GP73）促进肝癌 EMT，靶向沉默 GP73 逆转 EMT；进一步通过基因芯片数据发现：索拉非尼耐药细胞株中 GP73 表达上调， EMT 和肿瘤干性标志分子均表达升高，据此推测 GP73 促进肝癌 EMT，诱导干性是索拉非尼耐药的可能机制。结合预实验结果：GP73 调控 PI3K/Akt 通路介导肿瘤干性表型，因此提出科学假说 **“GP73 调控 PI3K/Akt 通路促进肝癌 EMT，增强肿瘤干性表型导致索拉非尼耐药”**，本研究拟通过体内、外实验比较耐药和亲本模型中 GP73 及 PI3K/Akt 通路表达变化，应用靶向沉默 GP73 和 Akt 阻断技术，着重探讨拮抗肝癌 EMT，减弱干性表型后对索拉非尼耐药的逆转作用。本课题有助于阐明索拉非尼的耐药分子机制，有助于克服其治疗抵抗，为开发新的治疗靶点奠定临床研究基础。

2.研究内容

1）在细胞水平研究 GP73 调控 PI3K/Akt 信号通路→促进 EMT，增强干性→索拉非尼抵抗→获得耐药

①证明耐药株对索拉非尼治疗抵抗，比较耐药株和亲本株对增殖、凋亡、活性、侵袭、转移的不同，共聚焦显微镜观察形态改变（已部分完成）；

②证明耐药株中 GP73 表达上调及 PI3K/Akt 信号通路激活，EMT 及肿瘤干性相关指标表达均增强。采用 qRT-PCR，Western blot，流式细胞术分别检测耐药株和亲本株中 GP73 及 PI3K/Akt 通路关键分子，EMT 及肿瘤干性相关指标。

二、项目实施方案(600 字内)

1）**耐药株和亲本株的建立及功能鉴定 ：**

①索拉非尼耐药细胞株和亲本细胞株的建立：hepG2-SR 与 hepG2 及 sk-hep-1-SR 与 sk-hep-1 细胞。采用浓度梯度递增法建立肝癌对索拉非尼获得性耐药胞株。将肝癌细胞在含 0.5umol/L 索拉非尼培养液中培养，待细胞能稳定生长，逐渐提高索拉非尼的浓度，每次提高 0.25umol/L，直至细胞能在相当于临床最高耐受浓度(含 5umol/L 索拉非尼)的培养液中稳定生长。MTT 方法检测耐药株和亲本株的 50%抑制浓度(IC50, half maximal inhibitory concentration)被测量的拮抗剂的半抑制浓度，耐药株 IC50 是亲本株的 3 倍以上，符合耐药标准（已完成）。

②耐药株和亲本株功能鉴定：证明耐药株对索拉非尼治疗抵抗，亲本株对其治疗敏感，应用克隆实验、流式细胞仪、CCK-8 法、Transwell 小室、划痕实验分别检测耐药株和亲本株对细胞增殖、凋亡、活性、侵袭、转移能力的不同，共聚焦显微镜观察耐药株和亲本株形态区别（已部分完成）。

2）**证明 GP73 与 PI3K/Akt 通路诱导肝癌发生 EMT 及增强干性的调控作用：**

①耐药株中 GP73 表达升高及 PI3K/Akt 信号通路激活 ：在耐药株和亲本株中，采用 Western blot 方法分别检测耐药株和亲本株中 GP73、PI3K、t-Akt、p-Akt、m-TOR 基因和

②耐药株发生 EMT ：在耐药株和亲本株中，采用 qRT-PCR 检测 EMT 相关分子：E-cadherin、N-cadherin、Vimentin、Slug，Western blot 方法检测上述指标蛋白。免疫荧光染色后，激光共聚焦扫描显微镜下观察上皮-间质表型转化的细胞形态改变，上皮标志物 E-cadherin、间质标志物 N-cadherin 和 Vimentin 的定位及表达变化。

③耐药株肿瘤干性表型增强：在耐药株和亲本株中，采用 Western blot 方法检测肿瘤干性相关基因 Oct-4,CD44,SOX-2 的 mRNA 蛋白表达。

三、项目的年度计划及时间安排

2017.06-2017.12：细胞水平研究耐药株中 GP73 调控 PI3K/Akt 信号通路→促进 EMT，增强肿瘤干性→索拉非尼抵抗

2018.01-2018.06：总结，补遗，上报结题。发表 1 篇收录中文核心论文。顺利结题。

四、预期研究成果

阐明 GP73 调控 PI3K/Akt 通路促进肝癌发生 EMT，增强干细胞表型，导致索拉非尼耐药的分子机制。发表研究中文核心论文 1 篇。

五、项目经费管理　　计划资助总额：1（万元）

序号	经费开支科目	经费预算金额（万元）
	试剂	0.64
	劳务费	0.36

经费合计：　　（万元）

六、项目组主要成员

姓名	学号	所在学院	联系电话	所在学科	项目负责内容
杨颖	107601160027	一附院	15022944632	肿瘤学	Western blot 检测
李志鹏	107602158859	一附院	15894622995	肿瘤学	细胞培养
沙娅·玛哈提	107601150802	一附院	18690828013	肿瘤学	PCR 检测
贾春丽	201006100301432	一附院	15099189144	肿瘤学	Western blot 检测
杨志芳	107602160473	一附院	15099606440	肿瘤学	数据处理

七、协议签署

甲方：

主管领导签章：

年　月　日

乙方：

项目申请人签字：

年　月　日

丙方：

主管领导签章：

年　月　日