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Tg737 acts as a key driver of invasion and migration in liver cancer stem cells and correlates with poor prognosis in patients with hepatocellular carcinoma

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ABSTRACT

We previously demonstrated that the Tg737 gene plays a critical role in the carcinogenesis of hepatocellular carcinoma (HCC). However, few systematic investigations have focused on the biological function of Tg737 in the invasion and migration of liver cancer stem cells (LCSCs) and on its clinical significance. In this study, Tg737 overexpression was achieved via gene transfection in MHCC97-H side population (SP) cells, which are considered a model for LCSCs in scientific studies. Tg737 overexpression significantly inhibited the invasion and migration of SP cells in an extracellular signal-regulated kinase1/2 (ERK1/2)/matrix metalloproteinase-2 (MMP-2)-dependent manner. Furthermore, Tg737 expression was frequently decreased in HCC tissues relative to that in adjacent noncancerous liver tissues. This decreased expression was significantly associated with tumor differentiation, the American Joint Committee on Cancer (AJCC) stage, metastasis, tumor size, vascular invasion, alpha-fetoprotein (AFP) levels, and tumor number. Moreover, multivariate Cox regression analyses demonstrated that Tg737 expression was an independent factor for predicting the overall survival of HCC patients. Notably, Kaplan–Meier analysis further showed that overall survival was significantly worse among patients with low Tg737 expression. Collectively, our findings demonstrated that Tg737 is a poor prognostic marker in patients with HCC, which may be due to its ability to promote LCSCs invasion and migration. These results provide a basis for investigating of Tg737 as a novel prognostic biomarker and therapeutic target.

1. Introduction

Since the 1990s, hepatocellular carcinoma (HCC) has been the second leading cause of cancer-related death in China [1]. Surgical resection is considered the first-line treatment for patients with early-stage HCC, and the combination of resection with adjuvant therapy can significantly prolong survival [2,3]. However, local invasion and a high rate of intra-hepatic and distant migration remain common. Thus, invasion and migration are the key factors that impede the curative efficiency of current therapies and reduce long-term survival [4]. The difficulty in preventing invasion and migration in patients with HCC may be because conventional treatments target the bulk of HCC cells and leave behind liver cancer stem cells (LCSCs) [5].

Evidence suggests that in many malignancies, including HCC, residual cancer stem cells (CSCs) [6–8] are responsible for tumor recurrence and metastasis. CSCs initiate and sustain tumor growth and they translocate from the primary tumor to distant tissues where they give rise to new tumors [9–11]. Treatments that specifically target CSCs may be useful and necessary for effectively combating HCC invasion and migration highlighting the need for a better understanding of the molecular processes behind CSCs-related HCC invasion and migration. Furthermore, although additional molecular processes with high sensitivity and specificity for HCC have been proposed, the routine use of any of these processes has not been justified in clinical practice to date [12]. Hence, a better understanding of whether molecular processes related to LCSCs invasion and migration con-

Abbreviations: AFP, alpha-fetoprotein; AJCC, American Joint Committee on Cancer; CSCs, cancer stem cells; DMEM, Dulbecco's Modified Eagle Medium; ELISA, Enzyme-linked immunosorbent assay; ECM, extracellular matrix; ERK1/2, extracellular signal-regulated kinase1/2; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; H & E, hematoxylin and eosin; LCSCs, liver cancer stem cells; LOH, loss of heterozygosity; MMP-2, matrix metalloproteinase-2; MP, main population; PBS, phosphate-buffered saline; PI, propidium iodide; p-ERK1/2, phospho-ERK1/2; SP, side population; TGF-β, transforming growth factor-β; TMA, tissue microarray; TNM, tumor node metastasis; TPR, tetratricopeptide repeat

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tribute to the prognosis of patients with HCC is essential for developing more effective targeted therapies.

The Tg737 gene is a liver tumor suppressor gene of within the tetratricopeptide repeat (TPR) family. Our previous results showed that Tg737 may play an essential role in HCC metastasis [13,14], and a significant down-regulation of the Tg737 gene was observed in 59% of HCC tissues [15]. Based on these findings, we hypothesized that Tg737 plays an important role in hepatic carcinogenesis. However, the role of Tg737 in LCSCs invasion and migration and as a potential biomarker and therapeutic target in HCC is unclear. Systematic investigation focusing on its exact function in LCSCs invasion and migration, expression pattern, clinical relevance is needed.

One approach to obtain LCSCs is to isolate them from preparations of side population (SP) cells. SP cells are less abundance cells that coexist with (and may demonstrate significant functional differences from) the main population (MP) of cells and are considered a model for LCSCs in scientific studies [16,17]. In the present study, we wanted to evaluate the influence of Tg737 overexpression on SP cells invasion and migration and to examine the underlying molecular mechanisms involved. In addition, we sought to investigate the correlations between Tg737 expression and the clinicopathological characteristics and prognosis of patients with HCC. Our results indicate that Tg737-targeted therapies may represent a promising novel strategy to prevent disease metastasis and progression in patients with HCC.

2. Materials and methods

2.1. Cell culture and isolation of SP cells

We chose MHCC97-H cells for SP cells isolation. All related experiments were performed using these SP cells because MHCC97-H cells contain a high percentage of SP cells as described by our group [16]. MHCC97-H cells (maintained in our laboratory, originally obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 IU/ml penicillin, 400 IU/L trypsin, and 100 μg/ml streptomycin. SP cells were obtained via procedures as previously reported [16,18]. After sorting, SP cells were cultured in Williams'E medium supplemented with 10% FBS, 100 IU/ml penicillin, 400 IU/l trypsin and 100 μg/ml streptomycin. The cells were plated in 75-cm^2 flasks and cultured at 37 °C with 5% CO_2 and 95% humidified air. The medium was replaced every 2 days.

2.2. Analysis of Tg737 gene expression in SP and MP cells

SP and MP cells were processed for protein extraction, and western blot analysis was performed as previously described [19]. The primary antibodies used were as follows: anti-Tg737 (diluted 1:600, mouse polyclonal, Abnova, Taipei, Taiwan) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; diluted 1:400, mouse monoclonal C-2; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.3. Construction of the targeting vector and transient transfection

The pcDNA3.1-Tg737 plasmid was constructed and transiently transfected into SP cells as described previously [14]. Either pcDNA3.1-Tg737 or pcDNA3.1 (-) were transiently transfected into SP cells for 6 h using LipofectamineTM 2000 (simply described as SP cells transfected with pcDNA3.1-Tg737 or SP cells transfected with pcDNA3.1 (-), respectively in below). Under the same transient transfection experimental conditions, SP cells were also incubated with LipofectamineTM 2000 alone for 6 h (simply described as SP cells incubated with LipofectamineTM 2000 alone in below). Meanwhile, SP cells were treated under the same transient transfection experimental conditions for 6 h in the absence of pcDNA3.1-Tg737, pcDNA3.1 (-) and LipofectamineTM 2000 treatment (simply described as blank SP cells in below). After these treatments, the different interfered cells were used in subsequent related experiments unless otherwise noted.

2.4. Analysis of Tg737 gene overexpression

The different interfered cells (described in Construction of the targeting vector and transient transfection) were grown in medium supplemented with 1% FBS for 12 h. The cells from the different groups were harvested and processed for protein extraction, and western blot, a widely accepted analytical technique that is used to detect specific proteins in a given sample, was performed as previously described [19]. The primary antibodies used included anti-Tg737 (diluted 1:500) and anti-GAPDH (diluted 1:400).

2.5. Annexin V/propidium iodide (PI) assay

To exclude the possibility of cell viability-related effects in subsequent experiments, Annexin V/PI assay was performed using an apoptosis detection kit (Annexin V-fluorescein isothiocyanate (FITC)/PI Staining Kit; Immunotech Co., Marseille, France) according to the manufacturer's instructions. Briefly, the different interfered cells (described in Construction of the targeting vector and transient transfection) were grown in medium supplemented with 1% FBS for 12 h. Cells from the different groups were then collected, washed in cold phosphate-buffered saline (PBS), incubated for 15 min with fluorescein-conjugated Annexin V and PI and analyzed via flow cytometry. Before transfection, the viability of the SP cells (incubated with medium supplemented with 10% FBS) was also analyzed.

2.6. Cell invasion and migration assays

The different interfered cells (described in Construction of the targeting vector and transient transfection) were subjected to invasion and migration assays. Cell migration was measured using transwells with 8-μm pore filters (Costar, MA, USA). The lower chamber was filled with DMEM supplemented with 10% FBS and 5 μg/ml fibronectin (Sigma, St. Louis, MO, USA), and 2×10^4 cells in 0.5 ml of media supplemented with 1% FBS were loaded into the upper chamber. Cells that did not migrate after 12 h were removed from the upper face of the membrane by scrubbing with a cotton swab; after that, the membrane was fixed with 4% formaldehyde and stained with 0.5% crystal violet dye. Finally, migratory cells were counted at 100× magnification from ten different fields for per transwell. For the Matrigel invasion assay, the procedures were the same as described above, except that the transwell membrane was coated with 500 ng/μl Matrigel (BD, CA, USA).

2.7. Scratch assay

The different interfered cells (described in Construction of the targeting vector and transient transfection) were subjected to the scratch assay. Cells in the different groups were manually scratched with a 200 μl pipette tip, washed with PBS three times and incubated with 1% FBS for 12 h. Wound healing was observed at the same view at 0 and 12 h after scratching to observe the process of wound healing.

2.8. Cell morphological characteristics

The different interfered cells (described in Construction of the targeting vector and transient transfection) were grown in medium supplemented with 1% FBS for 12 h. Cells in the different groups were then fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.1% Triton-X100 in PBS for 4 min, incubated with FITC-phalloidin in PBS for 30 min and rinsed three times. Images of the cells stained with FITC-labeled phalloidin were acquired using a fluorescence microscope.