Decreased expression of the type III TGF-β receptor enhances metastasis and invasion in hepatocellular carcinoma progression

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Abstract. The transforming growth factor β (TGF-β) superfamily of cytokines is multifunctional and involved in the regulation of cell growth and differentiation. TGF-β can induce an epithelial-mesenchymal transition (EMT) of both epithelial and endothelial cells. This has consequences for cancer progression in regards to both migration and invasion abilities. The type III TGF-β receptor (TβRIII) is a ubiquitously expressed TGF-β co-receptor which regulates TGF-β signaling and the progression of various types of cancer. Previous studies have shown that TβRIII exhibits abnormal expression and plays an essential role in regulating cancer invasion and metastasis, while little is known in regards to its role in hepatocellular carcinoma (HCC) progression. In the present study, we designed the present research to study the role of TβRIII in the invasion and metastasis of HCC and the possible mechanisms involved. The results demonstrated decreased expression of TβRIII in HCC patient tissues and human HCC cell lines. TGF-β1 stimulation led to the increased migratory ability and reduced expression of TβRIII in HCC cells. In addition, knockdown of TβRIII by small interfering RNA (siRNA) promoted the migration and invasion of HCC cells and induced activation of the Smad2 and Akt pathways. All the results suggest that TβRIII is a novel suppressor of HCC progression.

Introduction

Hepatocellular carcinoma (HCC) represents the most common type of primary liver cancer (1). HCC is a highly prevalent and lethal neoplasia, and the management of HCC has significantly improved during the last few years (2). A better understanding of the pathogenesis and the molecular mechanisms facilitate a better prediction of prognosis and the most appropriate treatment approach. At present, curative therapies including resection and transplantation have increased the survival rate of HCC patients at an early stage, while those diagnosed at intermediate and advanced stages still suffered from a high risk of side-effects (3-5). Investigation of the cellular origins and the early molecular events resulting in HCC are urgently necessary for further understanding HCC disease progression.

The transforming growth factor β (TGF-β) superfamily of cytokines is composed of TGF-βs, activins, inhibins, bone morphogenetic proteins (BMPs) and anti-Müllerian hormone (AMH) and are expressed in all multicellular organisms (6). In mammals, the TGF-β family exerts regulatory functions on cell growth, differentiation, apoptosis, immunity, extracellular matrix (ECM) production, adhesion and even embryonic development. Alterations of the components of the TGF-β signaling pathway and abnormal production of TGF-βs are often observed in disease progression despite the involvement in the maintenance of tissue homeostasis under both normal and dynamic conditions (7,8). The TGF-β isoforms (TGF-β1-3) exert their biological effects by binding to three receptors: type I (TβRI), type II (TβRII) and type II (TβRIII). Once bound to TGF-β, the constitutively active TβRII recruits, binds and activates TβRI, thereby stimulating its protein kinase activity. The downstream substrates for TβRI are members of the receptor-activated Smads (R-Smads), namely Smad2 and Smad3. After inducing phosphorylation by receptors, R-Smads form complexes with common mediated Smad4 (Co-Smad), and then translocate to the nucleus where regulation of the transcription of certain genes occurs in cooperation with other transcription factors (9).

TβRIII is the most abundantly expressed and characterized TGF-β receptor, and is a proteoglycan comprised of 851 amino acids (10,11). As a TGF-β superfamily co-receptor, the role of TβRIII in regulating TGF-β signaling is context-dependent and complex (12,13). The significant and indispensable role of TβRIII in mediating signaling is exerted dependently through TβRII and TβRI as well as potential signaling independently through TβRII and TβI. Overexpression of TβRIII was found to restore TGF-β1 sensitivity in CAL-27 human oral squamous
cells (14). In MDA-MB-231 human breast cancer cells, TβRIII expression inhibited Smad2/3 signaling dependent on the TβRIII cytoplasmic domain, and the independent binding of TβRI/TβRII to TβRIII competed with TβRI/TβRII signaling complex formation, thus inhibiting TGF-β-mediated Smad signaling (15). Importantly, the alteration of TβRIII expression level and the mediating roles have been observed in the progression of several types of cancers including breast, prostate, lung and ovarian cancer (16-19). Furthermore, the anchorage-independent growth of human MCF-7 breast cancer cells was inhibited when TβRIII expression was recovered in vitro (20), and suppression of tumorigenicity occurred in MDA-MB231 breast cancer cells after increased TβRIII expression in athymic nude mice (21). The expression level of TβRIII and its regulatory role in HCC progression remain to be clarified.

Therefore, in the present study, we investigated the expression of TβRIII at both the mRNA and protein levels in HCC patient tissues and cell lines. In vitro, we also studied the regulatory roles of TβRIII in metastasis and invasion of HCC and the related mechanisms.

Materials and methods

HCC patient tissues. Quantitative real-time PCR (qRT-PCR) and western blotting were utilized to examine the expression of TβRIII at the mRNA and protein level, respectively, in 10 HCC tissue specimens, which were collected at the First Affiliated Hospital of Anhui Medical University. All of the tissue specimens were pathologically confirmed as primary hepatic carcinoma and were promptly placed into sterile vials, and stored at -80°C until required. Tissue collection was approved by the Ethics Committee of Anhui Medical University. All procedures involving specimens obtained from human subjects were performed following informed consent from the patients.

Cell culture. Human HCC cell lines with stepwise metastatic potential (HepG2, SMMC-7721, MHCC97H, HCCLM3) and the normal immortalized liver cell line L02 were cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% (v/v) fetal bovine serum (FBS) (both from Gibco, Life Technologies, USA) and 100 U/ml of penicillin and 100 µg/ml of streptomycin. Normal human liver L02 cells, and human HCC cell lines HepG2 and SMMC-7721 were purchased from Shanghai HepG2 Cell Bank, Chinese Academy of Sciences (Shanghai, China). MHCC97H and HCCLM3 cells were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Shanghai, China). All of the cell lines were maintained at 37°C in a 5% CO2 atmosphere.

Quantitative real-time PCR (qRT-PCR). qRT-PCR was carried out to examine the mRNA expression of TβRIII. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as an internal control. Total RNA was extracted using TRIzol reagent (Invitrogen, Life Technologies, USA). Total RNA (2 µg) was reverse transcribed using the RevertAid First Strand cDNA Synthesis kit (Fermentas, Lithuania) according to the manufacturer’s instructions. The detection was carried out in a real-time PCR detection system (ABI 7500) using the SYBR-GreenER qPCR SuperMix Universal kit (Invitrogen, Life Technologies) according to the manufacturer’s instructions. The primer sequences for each gene were: GAPDH forward primer, 5'-AGGTCCGAGTCAACCGATT TG-3' and reverse primer, 5'-CCTGGAAGTGGTGATGGG AT-3'; TβRIII forward primer, 5'-ACCCCCAACTCTAACCC TG-3' and reverse primer, 5'-GGCAATACTGTGAGAAC AATAATTTC-3'. The cycle threshold value was defined as the PCR cycle number at which the reporter fluorescence crosses the threshold. The cycle threshold value of each product was determined and normalized against that of the internal control, GAPDH.

TβRIII small interfering RNA (siRNA) transfection. According to the TβRIII gene sequences, three siRNA duplexes targeting the TβRIII gene were designed (GenePharma, China): TβRIII-homo-2729 sense, 5’-GGGCCAUGAUGCAAAUATT-3’ and antisense, 5’-UUAAUCUGCAUAUGGCGCTT-3’; TβRIII-homo-2059 sense, 5’-GUGUGGUCUCAUAAC UTT-3’ and antisense, 5’-AGUUAUGAGACCACACCTT-3’; TβRIII-homo-759 sense, 5’-GGUCACACUUCACCUGAAT T-3’ and antisense, 5’-AUCAGGGAAGUGGAGCTT-3’. The transfection operation was performed under guidance of the Lipofectamine 3000 protocol (Invitrogen, Life Technologies). The transfection efficiency was confirmed by western blotting.

Western blotting. Liver samples and human HCC cell lines were collected and cultured respectively, according to the methods described above. Liver tissue was homogenized in RIPA lysis buffer and phenylmethylsulfonyl fluoride (PMSF) at a ratio of 99:1 (v/v). The cells were washed twice with ice-cold phosphate-buffered saline (PBS) after the corresponding treatment and lysed in cell lysis buffer. The protein concentration was determined with the BCA protein assay kit (Thermo Fisher Scientific, USA). A protein sample was mixed with 5X sample buffer (4:1) (Bio-Rad, Hercules, CA, USA) and heated in boiling water for 10 min. The proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA), and incubated with blocking buffer (0.05% Tween-20 PBS with 5% non-fat milk) for 2 h. Immunoblots were incubated with the indicated primary antibodies overnight at 4°C, followed by the appropriate horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature, and immunodetection was visualized by enhanced chemiluminescence (Pierce, Rockford, IL, USA) using hydrogen peroxide and luminol as substrates. Autoradiographs were scanned using ImageQuant LAS 4000 mini (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The density of the specific bands was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Wound healing assay. SMMC-7721 cells were seeded in a 6-well plate at 70-90% confluency and the cell monolayer was scraped in a straight line to create a ‘scratch’ with a P200 pipette tip. The debris was removed and the edge of the scratch was smoothed by washing the cells twice with 1 ml PBS. Then, 2 ml serum-free DMEM was added with different
concentrations of TGF-β1 (0.2, 1, 5 and 25 ng/ml) and images were captured using a computer-based microscopic imaging system at the 0 h time point at a magnification of x200. After 24 h, images of the wound were captured again under magnification of x200.

Changes in the migratory ability of the human SMMC-7721 cells after transfection with TβRIII siRNA were also assessed by wound healing assay. SMMC-7721 cells were seeded to 70-90% confluency and starved for 12 h. TβRIII siRNA was transfected into the SMMC-7721 cells under the guidance of the Lipofectamine 3000 protocol. After 12 h, the wound was created using the same method as described above. Images at 0 and 24 h were captured by a computer-based microscopy imaging system under a magnification of x200, respectively. Cell motility was evaluated according to the following formula: Cell motility ratio = (distance 24 h - distance 0 h)/distance 0 h.

Transwell assay. SMMC-7721 cells plated in 6-well plates previously transfected with the TβRIII siRNA for 12 h were harvested by trypsinization. The cells were washed once with PBS to remove the influence of FBS and then the cell pellet was resuspended in serum-free DMEM at 1x10^6 cells/ml. The cells (25,000-50,000) were seeded in the upper chamber of a Transwell filter, coated with Matrigel (BD Biosciences) in order to determine the cell invasive ability. The cells were cultured for 48 h at 37°C in a 5% CO₂ incubator through Matrigel toward the lower chamber, containing medium with 10% FBS as the chemoattractant. After incubation, the medium in the upper chamber was discarded and then the upper chamber was removed gently from the 24-well plates. The filter was fixed in 90% alcohol for 30 min and hexamethyl pararosaniline staining for 5 min. Images were captured of representative fields of each filter, and the number of cells was quantified.

Statistical analysis. All data are expressed as means ± SD. Statistical analysis was performed using one-way ANOVA. Statistical calculations were conducted using SPSS 10.0 software. The results were considered statistically significant for P-value <0.05.

Results

Decreased expression of TβRIII in tumor and non-tumor tissues of HCC patients. To investigate whether TβRIII expression is altered in HCC patient specimens, we initially analyzed the mRNA expression level in 10 human HCC tissue samples and compared the level with the matched non-tumor tissue samples as normal controls by qRT-PCR. From all the specimens, we observed a significant decrease in TβRIII mRNA expression in tumor tissues compared with the matched non-tumor tissue samples (Fig. 1A). To confirm decreased expression of TβRIII in HCC patient tissues, we examined TβRIII expression at the protein level by western blot analysis. The result showed that the protein expression of TβRIII was significantly decreased in the HCC tumor tissues compared with the level in the matched non-tumor tissue samples (Fig. 1B). The results revealed the impaired expression of TβRIII at both the mRNA and protein level.

Decreased expression of TβRIII in human HCC cell lines along with increased metastatic potential. To further confirm the alteration in the expression in TβRIII in HCC progression, human liver L02 cells and four HCC cell lines with stepwise metastatic potential (HepG2, SMMC-7721, MHCC97H and HCCLM3) were selected to observe the expression change of TβRIII in vitro by western blotting. Western blot results demonstrated the highest expression level in human liver L02 cells, while the expression of TβRIII was gradually decreased with increasing metastatic potential in the HCC cell lines. Moreover, the expression of TβRIII in the HCC cell lines MHCC97H and HCCLM3 which possess high metastatic potential was markedly lower than that in the HepG2 cells, an HCC cell line with low metastatic potential (Fig. 2). These results showed that a reduction in the expression of TβRIII occurred simultaneously with the increasing metastatic potential of the HCC cells.
TGF-β1 treatment enhances the migratory ability of human HCC cell lines. TGF-β1 is overexpressed and involved in the regulation of the progression of various types of cancer, and we investigated the effect of TGF-β1 on the migratory ability
of human HCC cells. Human SMMC-7721 cells were seeded in a 6-well plate (5x10^5 cells/well) and starved for 12 h on the following day. Then, SMMC-7721 cells were exposed to TGF-β1 for 24 h at concentrations of 0.2, 1, 5 and 25 ng/ml. The wound healing assay showed that TGF-β1 significantly elevated the migratory ability of the SMMC-7721 cells in a concentration-dependent manner, particularly in the 5 and 25 ng/ml treatment groups (Fig. 3). The results indicated that TGF-β1 promoted the metastasis of the HCC cells.

**TGF-β1 stimulation reduces the expression of TβRIII in human HCC cells.** To further characterize the effect of TGF-β1 on the expression of TβRIII, we treated human SMMC-7721 cells with TGF-β1 at stepwise concentrations (0.2, 1, 5 and 25 ng/ml) and assessed the protein level of TβRIII using western blotting. Treatment with TGF-β1 (5 and 25 ng/ml) resulted in a significantly decreased expression level of the TβRIII protein (Fig. 4). To assess whether the effects of TGF-β1 on TβRIII were specific, we analyzed the effect of TGF-β1 on TβRII expression. Western blot results showed no significant changes in the level of TβRII expression upon TGF-β1 treatment at various concentrations (Fig. 4). The results above indicated that it was TβRIII instead of TβRII that was downregulated by TGF-β1 in the human SMMC-7721 cells.

**Decreased expression of TβRIII elevates the migratory and invasive abilities of human HCC cells.** We detected the gradually decreased expression of TβRIII in human HCC cell lines along with increasing metastatic potential. To investigate the roles of TβRIII in regulating the migratory and invasive abilities of the HCC cells, we silenced the expression of TβRIII by transfection of siRNA targeting the TβRIII gene. The wound healing and Transwell assays were performed to detect the migratory and invasive abilities of the SMMC-7721 cells transfected with the siRNA targeting TβRIII, respectively. The wound healing assay results revealed that the migratory ability of the SMMC-7721 cells in the siRNA transfection groups was significantly increased compared with the corresponding control groups (Fig. 5). The Transwell assay showed a significantly increased invasive ability of the SMMC-7721 cells in the siRNA transfection groups compared with the corresponding control groups (Fig. 6). Collectively, these results confirmed that the downregulation of TβRIII expression promoted HCC cell migration and invasion in vitro.

**Silencing of TβRIII expression by siRNA activates the Smad2 and Akt pathways in the SMMC-7721 cells.** TGF-β1 was found to promote the migration of HCC cells and reduced the expression of TβRIII, and furthermore, silencing of the expression of TβRIII elevated the migration and invasion abilities of the HCC cells. Thus, we hypothesized that the inhibitory effects of TβRIII on migration and invasion of HCC cells may be through regulation of the TGF-β downstream signaling pathway. Therefore, to further confirm this hypothesis, we examined Smad2 and Akt activation by western blotting after transfection of TβRIII siRNA. The results revealed that silenced expression of TβRIII in the siRNA transfection groups increased the expression levels of p-Smad2 and p-Akt compared with the corresponding control groups (Fig. 7).

**Discussion**

The three highly homologous isoforms of TGF-β in humans (TGF-β1, TGF-β2 and TGF-β3) share a receptor complex and signal in similar ways while their expression levels vary depending on the tissue (22). TGF-β can induce an epithelial-mesenchymal transition (EMT) of both epithelial and endothelial cells, which contributes to disease progression in both cancer and fibrosis (23). EMT promotes cellular migratory and invasion properties, as cellular migration requires the loss of cell-cell contacts and acquisition of fibroblastic characteristics. Several studies have demonstrated the increased expression of TGF-β1 in various types of cancers and the close association with tumor metastasis, invasion and the poor prognosis of patients (24,25). Although the increased expression of TGF-β1 has been reported to be closely associated with a poor prognosis and tumor angiogenesis in HCC (26-28), the mechanism concerning the role of the promotion of tumorigenesis remains unclear. The research based on the MDA-MB-231 breast cancer and Ovca420 ovarian cancer cells in vitro revealed...
Figure 6. Effects of TβRIII siRNA on the invasive ability of SMMC-7721 cells. (A) The invasive ability was assessed by Transwell invasion assay. (B) Histogram shows the effects of transfection on invasion ability. The data from three independent experiments are shown as the mean ± SD. NC, negative control; siTβRIII, TβRIII siRNA. **P<0.01 compared with the control group; ##P<0.01 compared with the TGF-β1 group.

Figure 5. Effects of TβRIII siRNA on the migration of SMMC-7721 cells. (A) The migratory ability was assessed by wound healing assay. (B) Histogram shows the effects of transfection on migratory ability. Cell motility was evaluated according to the following formula: Cell migration ratio = (distance 24 h - distance 0 h)/distance 0 h. The data from three independent experiments are shown as the mean ± SD. NC, negative control; siTβRIII, TβRIII siRNA. **P<0.01 compared with the control group; ##P<0.01 compared with the TGF-β1 group.

Figure 6. Effects of TβRIII siRNA on the invasive ability of SMMC-7721 cells. (A) The invasive ability was assessed by Transwell invasion assay. (B) Histogram shows the effects of transfection on invasion ability. The data from three independent experiments are shown as the mean ± SD. NC, negative control; siTβRIII, TβRIII siRNA. **P<0.01 compared with the control group; ##P<0.01 compared with the TGF-β1 group.
that TGF-β1 reduced the expression of TβRIII at the mRNA and protein levels in a dose- and time-dependent manner (29). In our previous study, we established an HCC animal model by intraperitoneally injecting diethylinitrosamine (DEN) in 14-day-old C57BL/6J mice. The results revealed significantly increased expression of TGF-β1 and induced activation of Smad2 signaling in a time-dependent manner after the injection (30). In the present study, we observed that TGF-β1 promoted the migratory ability and induced decreased expression of TβRIII in HCC cells. These results suggest that the TGF-β1 expression level may be a diagnostic predictor of HCC progression, and its promotive effect on the migration of HCC cells may be associated with the low expression of TβRIII.

The negative regulation of TβRIII expression by TGF-β1 may provide insight into the tumor-promoting effects of elevated TGF-β1 and the role of decreased TβRIII expression in HCC as well. The expression level of TGF-β1 was found to be elevated and correlated with the migration and invasion in a spectrum of human cancers (24,25). Nevertheless, the mechanism by which the high level of TGF-β1 exerts tumor-promoting effects remains unclear. A recent study found that TβRIII inhibited TGF-β-mediated signaling through the independent binding to TβRI and TβRII and competition with TβRI/TβRII complex formation (15). We found decreased expression of TβRIII concomitant with the elevated level of TGF-β1, as well as the role of TGF-β1 in promoting metastasis in the HCC cell lines. Thus, the present study confirmed decreased TβRIII expression as a possible mechanism for the tumor-promoting role of TGF-β1.

TGF-β binds to the constitutively active type II TGF-β receptor (TβRII) along with the consequent activation of the type I TGF-β receptor (TβRI), subsequently forming heteromeric complexes to induce downstream signaling transduction (9,12). Although lacking intrinsic enzymatic activity, TβRIII is the most abundantly expressed TGF-β superfamily receptor and has a complex- and context-dependent role in regulating TGF-β superfamily signaling and cancer progression with mechanisms yet to be defined. Recently, the decreased and even lost expression of TβRIII have been reported in several types of cancers including breast, prostate, lung and ovarian cancer (16-19). Several studies have also demonstrated the aberrant expression of TβRIII in HCC (27,31). However, the specific expression condition and roles of TβRIII in HCC development and progression require further research. The results of the present study showed that the expression of TβRIII in human HCC tissues was significantly decreased compared with the matched normal liver tissues, at the mRNA and protein levels. In our previous study of an animal model, we also found that the expression of TβRIII was decreased in DEN-induced HCC mice compared with normal control mice along with increasing time after DEN injection (30). These results suggest that the decreased expression of TβRIII has a negative regulatory role in HCC progression.

Notably, the regulatory roles of TβRIII in inhibiting tumor invasion, angiogenesis, and metastasis have also been defined. The restoration of TβRIII expression in murine mammary cancer cells and prostate cancer cells inhibited
tumor invasion, angiogenesis, and metastasis both basally and in response to TGF-β treatment in vivo and in vitro, respectively (16,17,21,32), and expression of TβRIII inhibited the motility and invasion of NSCLC cells and knockdown of TβRIII expression increased its invasion (18). To further investigate the roles of TβRIII in the regulation of HCC migration and invasion, we performed research using human HCC cell lines in vitro. Consistently, the results showed that gradually decreased expression of TβRIII was observed in HCC cell lines along with increasing metastatic potential. Human normal liver L02 cells showed the highest expression level of TβRIII protein, while the MHCC97H and HCCLM3 cell lines with high metastatic potential exhibited a markedly lower expression level than that in HepG2, an HCC cell line with low metastatic potential. Based on the findings of decreased TβRIII expression in HCC cell lines with increasing metastatic potential, we hypothesized that TβRIII may be a negative regulator of the migration and invasion of HCC. In addition, the results of siRNA targeting of the TβRIII gene revealed that knockdown of TβRIII led to the enhanced motility and invasiveness of human SMC-7721 cells both basically and under TGF-β stimulation. Collectively, based on the results in HCC patient tissues and in a previous HCC mouse model (30), we propose that low expression of TβRIII is involved in the malignant progression of HCC, and TβRIII plays a suppressive role in regulating the metastasis and invasion of HCC.

TβRIII has been previously found to exert inhibitory effects on the migration and invasion of HCC and on various types of cancers; however, the related mechanisms remain to be defined. TβRIII has been shown to have effects on both the Smad and non-Smad signaling pathways in a ligand-dependent or -independent manner, including the Smad, NF-κB and p38 signaling pathways (33-35). In mouse lung fibroblasts, the expression of TβRIII was found to reduce the phosphorylation of Smad2, Smad3 and Akt (36). Moreover, it has been reported that restoration of TβRIII in breast cancer inhibited the expression level of Smad2 or Smad3 in vivo and in vitro (16). In our previous study in an HCC mouse model, we detected that the expression of p-Smad2 was elevated in a time-dependent manner, while the TGF-β1 level was increased (30). In the present study, we detected the expression of p-Smad2 and p-Akt in SMMC-7721 cells after transfection with TβRIII siRNA. The results revealed that the expression levels of p-Smad2 and p-Akt were elevated in the siRNA groups compared with the corresponding control groups. These results suggest that TβRIII suppressed the progression of HCC by inhibiting the activation of Smad2 and Akt pathways, and subsequently decreasing the migration and invasion of HCC.

In conclusion, TβRIII displayed decreased expression with HCC progression in vivo and in vitro, which may be associated with the higher level of TGF-β1. An elevated level of TGF-β1 and decreased expression of TβRIII may lead to the activation of Smad2, and the mechanism between them is yet to be defined. Furthermore, TβRIII acts as a suppressive factor in regulating the migration and invasion of HCC, by inhibiting the Smad2 and Akt pathways. The present study confirmed that TβRIII may be a candidate molecular target in HCC diagnosis and treatment.

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