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Twist Promotes the Migration of Hepatocellular Carcinoma Cells

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OBJECTIVE To study the effect of the Twist gene on the migration of human hepatocellular carcinoma cells and the possible mechanisms involved.
METHODS RT-PCR was used to detect expression of the Twist gene in primary (Hep11) and recurrent (Hep12) cell lines from the same HCC patient. Hep11 cells were stably transfected with Twist-cDNA, and Hep12 cells were transiently transfected with Twist RNAi plasmid. Cell migration assays were performed on Twist up-regulated Hep11 cells and Twist RNAi Hep12 cells. RT-PCR and Western blot were used to test the expression of EMT markers.
RESULTS Twist was expressed higher level and had increased migration capability in recurrent Hep12 cells than those in primary Hep11 cells. Cell models (Twist-Hep11) in which Twist protein was steadily and highly expressed were obtained. Compared with pcDNA3-Hep11 cells, migration of Twist-Hep11 cells was clearly increased. However, migration of Twist RNAi (Si-Twist-Hep12) Hep12 cells were reduced. Overexpression of Twist in Hep11 cells promoted expression of N-cad and vimentin.
CONCLUSION These results indicate that Twist promotes the migration of hepatocellular carcinoma cells in vitro and may play an important role in the upregulation of mesenchymal markers.

KEY WORDS: Twist, RNA interference, gene transfection, migration and invasion, HCC.

Introduction

Hepatocellular carcinoma (HCC) has been ranked the fifth most common cancer worldwide and every year, approximately 500,000 people die from the disease[1]. While chemotherapy and radiotherapy only show little benefit for HCC patients, surgery is considered the most effective treatment. However, even after surgery, HCC is inclined to recur[2]. Hence, improving the effect of chemotherapy and radiotherapy and preventing recurrence are important goals for the clinical therapy of HCC.

EMT (epithelial-mesenchymal transition) involves the conversion of a sheet of tightly attached epithelial cells into highly mobile mesenchymal or neural crest cells. Twist can contribute to tumor invasion and metastasis by promoting this latent developmental program of EMT[3]. However, the effect and mechanism of the Twist gene on the invasion and metastasis of hepatocellular carcinoma remain enigmatic. Therefore, in the present work, primary (Hep11) and recurrent (Hep12) cell lines were isolated from the same HCC patient in which the effect of the Twist gene on cell migration was investigated.

Materials and Methods

Cell culture

Two human liver carcinoma cell lines from primary (Hep11) and recurrent (Hep12) hepatocellular carcinoma from the same HCC patient were obtained by Dr. Xiaolan Xu in our laboratory. Cells were cultured in RPMI 1640 medium (Gibco Biocult, Paisley, UK) that contained 10% fetal bovine serum at 37°C in a humidified 5% CO_2.

Generation of stable Twist transfectants

The Twist expression vectors (pcDNA3-Twist) were kindly provided by Dr. Glackin (Division of Molecular Medicine, Beckman Research Institute of the City of Hope, CA). Cell transfection was carried out using Lipofectamine 2000 according to the manufacturer's instructions. Briefly, cells were grown to 80%-90% confluence, without antibiotics. Vectors that contained the different constructs (10 μg) were diluted in Opti-MEM (100 μl) and mixed with the transfection solution for 20 min. Cells were incubated with the transfection mixture at 37°C for 48 h, and then allowed to grow in fresh medium with antibiotics and 400 mg/ml G418 (Geniticin; Amresco, Solan, OH, USA) for 2 weeks. Single geneticin-resistant clones were selected and expanded. Cells transfected with the pcDNA3 vector were used as controls.

Generation of transient Si-Twist transfectants

The Twist-siRNA vector was generated using the pSuper RNAi System (OligoEngine) according to the manufacturer's instruction. Briefly, the primers containing the short hairpin RNA (shRNA) sequence targeting the Twist gene-coding region were annealed and cloned into the pSuper vector to generate the small interfering RNA (siRNA) expression vector. The sequences of the Si-Twist primers were Si-Twist-F: 5'-GAT CCG CTG AGC AAG ATT CAG ACC TTC AAG AGA GGT CTG AAT CTT GCT CAG CTT TTT TGG AAA-3', and Si-Twist-R: 5' -AGC TTT TCC AAA AAA GCT GAG CAA GAT TCA GAC CTC TCT TGA AGG TCT GAA TCT TGC TCA GCG-3'. The resulting vectors were then transfected into the Hep12 cells using Lipofectamine 2000. Cells transfected with the pSuper vector were used as controls.

In vitro invasion assay

Invasion assays were done in a Boyden chamber with polyethylene terephthalate filter inserts for 24-well plates (Becton Dickinson Labware, NY, USA). Briefly, 200 μl of cells were seeded in the upper chamber at a final concentration of 1.0×10^5/ml in serum-free medium with 1% FBS. Three hundred microliters of medium conditioned with 10% FBS were placed in the lower compartment of the chamber. Twenty-four hours after incubation, the remaining tumor cells on the upper surface of the filters were removed by wiping with cotton swabs, and the invading cells on the lower surface were fixed with 2% formaldehyde in PBS followed by a staining procedure with 0.5% toluidine blue in 2% Na_2CO_3 and then observed under a microscope.

RT-PCR

Cells were lysed in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and total RNA was prepared according to the manufacturer's instructions. cDNA was synthesized by the M-MLV First Strand Synthesis System (Invitrogen). The cDNA was then amplified by PCR with specific primers, Twist (201 bp): sense 5′-GGA GTC CGC AGT CTT ACG AG-3′; antisense: 5′-TCT GGA GGA CCT GGT AGA GG-3′. E-cad (362 bp): sense 5'-TCC ATT TCT TGG TCT ACG CCT-3'; antisense 5'-CAC CTT CAG CCA TCC TGT TT-3'; N-cad (370 bp): sense 5' -GTG CCA TTA GCC AAG GGA ATT CAG C-3'; antisense 5'-GCG TTC CTG TTC CAC TCA TAG GAG G-3'; Vimentin (278 bp): sense 5'-CGC CAG ATG CGT GAA ATG G-3'; antisense 5'-ACC AGA GGG AGT GAA TCC AGA-3'. The cDNA of GAPDH (452 bp) was amplified as a control for the amount of cDNA present in each sample (sense: 5'-ACC ACA GTC CAT GCC ATC AC-3'; antisense 5'-TCC ACC ACC CTG TTG CTG TA-3'). PCR conditions: 95°C for 5 min, then 30 cycles at 94°C for 45 s, at 57°C for 45 s, at 72°C for 45 s, and then 72°C for 10 min.

Western blotting

Briefly, cell lysates were prepared by suspending the cell pellets in a modified radioimmunoprecipitation buffer (50 mmol/L Tris-HCl [pH 8.0], 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS), and the protein concentration was measured using the protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein (20 μg) were separated by electrophoresis on SDS-polyacrylamide gel and blotted onto the PVDF membrane (Amersham, Piscataway, NJ). After blocking with 5% nonfat dry milk for 1 h, the membrane was incubated with primary antibodies at room temperature for 1 h against Twist (1:250, prepared by our laboratory), N-cad (1:4000, Zymed), vimentin (1:3000, Boehringer-Mannhein). After washing with TBS-T, the membrane was incubated with secondary antibodies against rabbit IgG or mouse IgG, and the signals were visualized by enhanced chemiluminescence Western blotting system (Amersham). Expression of β-actin was also measured as an internal loading control using a specific antibody (1:10000, Boehringer-Mannheim).

Statistical analysis

Results were expressed as mean ± SD with triplicate measurements. Differences between groups were tested with Student's *t*-test, and the value of $P < 0.05$ was considered significant.

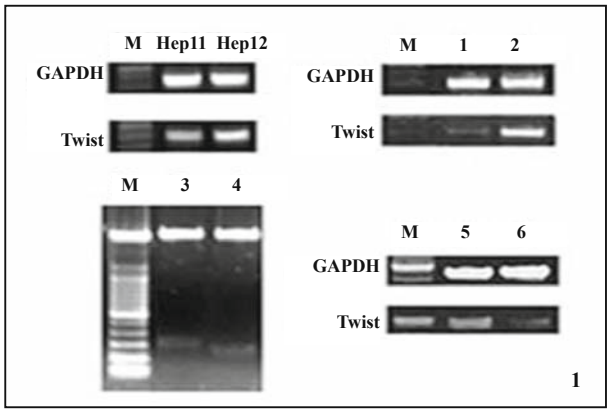
Results

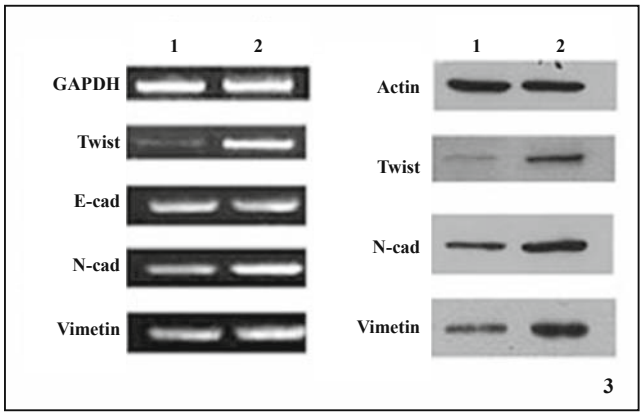
Up-regulation of Twist in Hep12 cells with increased migration ability

RT-PCR indicates that Twist expression is increased in recurrent Hep12 cells compared with that in primary Hep11 cells (Fig.1). A value representing cell migration was determined through the invasion assay, which was performed in vitro, and then the cell number was calculated. The cell number of Hep12 cells (203.67 ± 13.82) was obviously increased compared with that of Hep11cells (14.5 ± 3.86) ($P < 0.01$) (Fig.2). These results suggested that Twist overexpression is positively correlated with cell migration.

Overexpression of Twist leads to the promotion of migration ability

Hep11 cell clones transfected with pcDNA3 or pcDNA3-Twist were obtained after gene transfection. Expression of Twist protein was increased in the positive cell clones that were transfected with pcDNA3-Twist (Fig.1 and Fig.3). The cell number of Twist-Hep11 cells (205.75 ± 11.11) was increased significantly ($P < 0.01$) (Fig.2) compared with that of pcDNA3-Hep11 cells (49.00 ± 10.98). These results suggested that overexpression of Twist leads to the promotion of the migration ability in these cells.





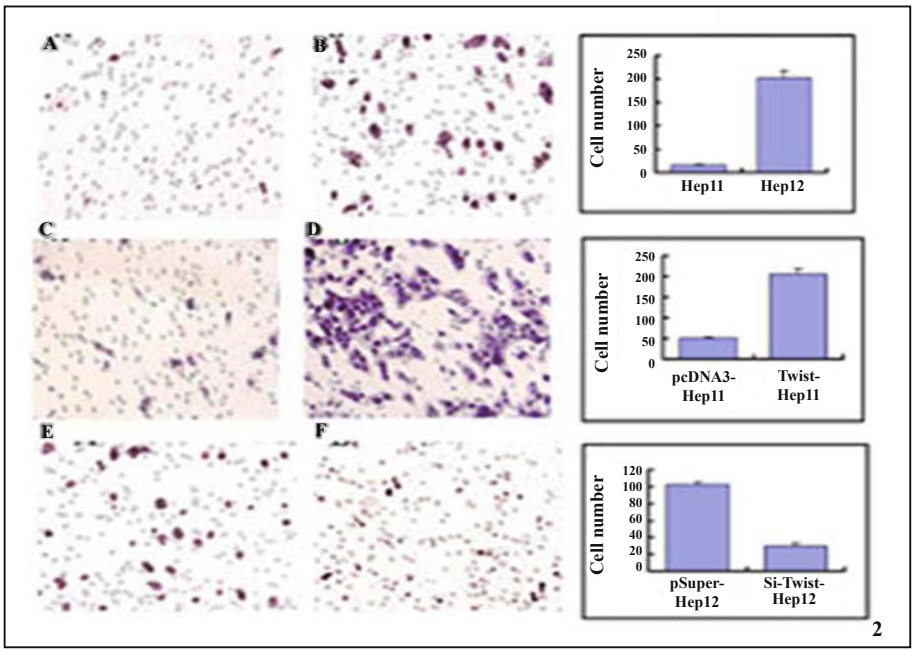


Fig.1. Analysis of Twist expression in Hep11, Hep12, Twist-Hep11 and Si-Twist-Hep12 cells. M, DNA Marker (100bp ladder); 1, pcDNA3- Hep11 cells; 2, Twist- Hep11 cells; 3 & 4, pSuper-Si-Twist recombinant and pSuper vector digested by EcoRI/HindIII and released 300bp and 240bp fragment respectively; 5, pSuper-Hep12 cells; 6, Si-Twist-Hep12 cells.

Fig.2. Overexpression of Twist promoted migration in Hep11 cells and downregulation of Twist inhibited migration in Hep12 cells. A, Hep11 cells; B, Hep12 cells; C, pcDNA3-Hep11 cells; D, Twist-Hep11 cells; E, pSuper-Hep12 cells; F, Si-Twist-Hep12 cells.

Fig.3. Overexpression of Twist promoted expression of N-cad and vimentin. 1, pcDNA3-Hep11 cells; 2, Twist-Hep11 cells.

Down-regulation of Twist leads to suppression of migration ability

Consequently, a Twist siRNA vector was generated, and we successfully suppressed Twist expression by using RNA interference in Hep12 cells (Fig.1). Compared with that of pSuper-Hep12 cells (102.67 ± 2.52), the cell number of Si-Twist-Hep12 cells (30.00 ± 3.83) was obviously suppressed ($P < 0.01$) (Fig.2). These results suggested that the downregulation of Twist leads to the inhibition of the migration ability in these cells.

Overexpression of Twist promoted expression of N-cad and vimentin

We investigated whether overexpression of Twist affects the expression of E-cad, N-cad and vimentin. Results showed that N-cad and vimentin RNA and protein levels were markedly higher in Twist-Hep11 cells than in pcDNA3 control cells (Fig.3). However, no significant difference in expressions levels of E-cad RNA between the two groups was detected.

Discussion

Twist, a member of the bHLH transcription factor family, has shown importance in its role in controlling cell type determination and differentiation. Twist has also been found to have oncogenic properties. For example, overexpression of Twist in rhabdomyosarcoma inhibits myc-induced apoptosis[4] and upregulation of Twist is associated with malignant transformation in T-cell lymphoma[5]. The role of Twist in cancer metastasis was first reported in a breast cancer model, which suggested that Twist induced EMT, resulting in the promotion of tumor invasion[6]. However, the verdict regarding EMT-mediated tumor metastasis is controversial. Some studies have shown that Twist overexpression correlated with EMT-mediated metastasis in prostate and breast cancers[6,7]. In contrast, other studies reported that Twist had no correlation with EMT-mediated metastasis in gastric and colon cancers[8,9]. Regarding HCC, a recent report has shown that overexpression of Twist gene positively correlated with HCC metastasis[10]. However, no definitive results have indicated that Twist promotes the migration of HCC. In this study, we first reported that endogenous Twist was expressed abundantly in recurrent Hep12 cells, but expressed much lower in primary Hep11 cells. Therefore, we transfected Hep11 cells with the Twist-cDNA plasmid. Our findings suggested that the migration ability of Twist-Hep11 cells was promoted through the overexpression of the Twist gene. However, after we transfected Hep12 cells with the Si-Twist vector, the results indicated that the suppression of Twist inhibited cell migration. Therefore, we think that Twist may play an important role in the migration of HCC.

Normally, we think that Twist binds DNA using similar E-box sequence motifs responsible for repressing E-cadherin[11]. However, as shown in Fig.3, we found that N-cad and vimentin RNA and protein levels were markedly upregulated in Twist-Hep11 cells, but no obvious changes in E-cad RNA levels were detected. Taken together, the results of our study suggested that Twist might correlate with HCC cell migration because Twist may promote the expressions of N-cad and vimentin.

In conclusion, Twist may contribute to the migration of HCC cells by promoting N-cad and vimentin mainly through EMT. As a novel player in the tumor metastasis and with multidrug-resistant properties[12], Twist is drawing much attention. Our findings of the presence of a functional link between Twist and cell migration suggest that targeting Twist may be a novel therapeutic modality for recurrent HCC intervention in the future.

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