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Up-regulation of gastric cancer cell invasion by *Twist* is accompanied by N-cadherin and fibronectin expression ☆

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Abstract

Twist, a newly found EMT-inducer, has been reported to be up-regulated in those of diffuse-type gastric carcinomas with high N-cadherin level. We show here MKN45, a cell line derived from undifferentiated carcinomas cells, expresses high levels of *Twist*. Down-regulation of *Twist*, using an antisense *Twist* vector in MKN45 cells, inhibits cell migration and invasion, companied with a morphologic changes associated with MET. Suppression of *Twist* also decreases the expressions of N-cadherin and fibronectin, but not of E-cadherin in MKN45. In contrast, overexpression of *Twist* in MKN28, a cell line derived from moderate differentiated carcinomas, results in up-regulation of N-cadherin and fibronectin, companied with down-regulation of E-cadherin. Taken together, our results suggest that *Twist* regulates cell motility and invasion in gastric cancer cell lines, probably through the N-cadherin and fibronectin production.

Keywords: *Twist*; EMT; MET; Gastric cancer; Invasion; Migration

Epithelial–mesenchymal transition (EMT), characterized by a gain of mesenchymal cell markers (fibronectin, vimentin, smooth muscle actin, and N-cadherin) and a loss of epithelial markers (E-cadherin and catenins) [1,2], is a process whereby cells acquire molecular alterations that facilitate cell motility and invasion [3]. *Twist*, a basic-helix–loop–helix (bHLH) transcription factor known to be involved in EMT process, has been recently identified capable of mediating carcinoma metastasis [4,5]. Indeed, Yang et al. found that *Twist* stood out as one of the most strongly up-regulated genes responsible for invasiveness and/or intravasation of mouse mammary tumor. Inhibition of *Twist* expression through small interfering RNA was sufficient to greatly impair the metastatic ability of the most fully metastatic tumor cell lines, 4T1 [6]. Moreover, endogenous *Twist* expression was found to be correlated with metastatic potential in a panel of human epithelial tumor cell lines such as breast and prostate cancers [6–8]. Further studies implicate that *Twist* regulate tumor metastasis, probably through EMT [4,6,9]. The best evidence is that *Twist* has been shown to down-regulate the activity of the E-cadherin promoter by directly binding to, and inhibiting, the activity of a positive regulator of E-cadherin transcription [6]. Studies in prostatic cancers have demonstrated that up-regulation of nuclear *Twist* led to the recruitment of E-cadherin, which was correlated with the development of distal metastatic disease in primary prostatic cancer specimens [10]. Down-regulation of *Twist* in

Abbreviations: EMT, epithelial–mesenchymal transitions; MET, mesenchymal to epithelial transition; FN, fibronectin; DAPI, 4′,6-diamidino-2-phenylindole.

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androgen-independent prostate cancer cell lines, resulted in suppression of the invasion ability of prostate cancer cells correlated with induction of E-cadherin expression [7].

Despite these increasing studies, little information is available concerning the role of *Twist* in gastric cancer. Recently, Rosivatz et al. reported that overexpression of *Twist* gene was more frequently found in those of diffuse-type gastric carcinoma tissues with high N-cadherin gene [11]. A more recent study of prostate carcinoma further implicated that *Twist*1 was necessary for N-cadherin transcriptional activation [12]. However, no direct evidence has yet been reported concerning the role of *Twist* in gastric cancer invasiveness. Therefore, in present work, two gastric cancer cell lines with different differentiation were transfected with sense or antisense *Twist* vector. Our data present evidence that *Twist* promotes gastric cancer cells invasion, probably through N-cadherin and fibronectin production.

Materials and methods

Cells, plasmids, and reagents. Human gastric carcinoma cell line MKN28 (JCRB0253) and MKN45 (JCRB0254) were obtained from the Japanese Collection of Research Bioresources (JCRB, Japan). NIH-3T3 cell line was obtained from the American Type Culture Collection (ATCC, USA). The *Twist* sense and antisense expression vector (pcDNA3/*Twist*S, pcDNA3/*Twist*AS) were kindly provided by Dr. C. Glackin [13]. The identity of *Twist* PCR product was confirmed by nucleotide sequencing. Primary antibodies (anti-*Twist*, anti-E-cadherin, anti-FN, anti-N-cadherin, and anti-β-actin) were from Santa Cruz Biotechnology (Santa Cruz, CA) and secondary antibodies (HRP-linked IgG and FITC-conjugated IgG) were from Cell Signaling Technology (Beverly, MA).

Confocal microscopy. The subcellular localization of *Twist* in gastric cell lines was determined using Confocal Laser Scanning Microscope (CLSM, Leica Microsystems GmbH, Germany). Briefly, MKN45 cells were seeded on glass coverslips in a six-well plate for 48 h in RPMI-1640 medium (Hyclone) containing 10% fetal bovine serum (FBS). After fixation, the cells were incubated with antibody against *Twist* (1:200) overnight at 4 °C. Cells were then incubated with FITC-conjugated Ab (1:100) for 1 h at room temperature before observed by CLSM.

Transient transfection and generation of stable transfectants. 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 containing the different constructs (10 μg) were diluted in DMEM (100 μl) and then mixed with the transfection solution for 15 min. After washing, the cells were incubated with the transfection mixture at 37 °C for 6 h and then were allowed to grow in fresh media. For a transient expression of MKN28 cells, the transfected cells were incubated at 37 °C for 24–48 h and used for analysis. Stable MKN45 transfectants were isolated by selection with 500 mg/ml of G418 (AMRESCO) for two weeks, and the total pool of transfectants (nine colonies) was used. MKN45 cells transfected with the pcDNA3 vectors were used as controls.

In vitro invasion and migration assay. Cell invasion assays were performed using Transwell™ chambers (Costar). Briefly, after coating the filter with 80 μg of Matrigel (BD) overnight at 4 °C, cells were seeded at densities of 10^5 cells per well in serum-free medium with 0.1% BSA (Sigma). Six hundred microliters of invasion buffer [containing 200 μl NIH-3T3 conditioned medium, 200 μl standard growth medium (the medium used to grow the cells in) and 200 μl basic medium] was added to the lower chamber as a chemoattractant. After 24 h, cells that had penetrated through the membrane were randomly counted in six different fields under a light microscope at 200× magnification. Each cell line was assayed in duplicate per experiment and repeated in triplicate. To assess migration, *in vitro* migration assays were done under the same conditions as the Transwell™ invasion assays, but in non-Matrigel-coated Transwell™ chambers.

Wound-healing assay. Cells were allowed to grow to 90% confluency before cell monolayer was wounded with a plastic pipette tip to generating a wound. Migration of cells was judged at indicated time points. Each assay was set up in triplicate.

RNA preparation, cDNA synthesis, and quantitative real-time PCR. Total RNA was extracted with Trizol (Invitrogen, CA). One microliter of total RNA was reverse transcripted to cDNA using the RevertAid First Strand cDNA synthesis kit (MBI Fermentas). PCR was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems, 4367649). The final reaction contained 10 μl SYBR green/enzyme reaction mix, 0.5 μM primer and 1 μl of cDNA in a total volume of 20 μl. PCR conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The primers of each gene are as follows: GAPDH-f, 5′-CCTCAAGATCATCAGCAAT-3′; GAPDH-r, 5′-CCATCCACAGTCTTCTGGGT-3′; *Twist*-f, 5′-ACAAGCTGAGCAAGATTCAGACC-3′; *Twist*-r, 5′-TCCAGACCGAGAAGGCGTAG-3′; E-cadherin-f, 5′-ACACCATCCTCAGCCAAGA-3′; E-cadherin-r, 5′-CGTAGGGAAACTCTCTCGGT-3′; FN-f, 5′-CCATCGCAAACCGCTGCCAT-3′; FN-r, 5′-AACACTTCTCAGCTATGGGCTT-3′. N-cadherin-f, 5′-ATCCTACTGGACGGTTCG-3′; N-cadherin-r, 5′-TTGGCTAATGGCACTTGA-3′. All assays were evaluated using the FTC2000 fluorescent quantitative PCR detection system (Funglyn, Canada), and relative expression was calculated by normalizing the C_t (threshold cycle) of the target gene to the C_t of the GAPDH housekeeping gene in the same sample. The mean normalized expression and statistical significance of differences in mRNA expression for the examined factors was analyzed using the REST-XL-version 2 software [14].

Protein extracts and Western blot analysis. Cytoplasmic and nuclear protein fractions were prepared as previously described [15]. For whole cell protein extraction, cells were lysed in 1 ml of RIPA lysis buffer. A total of 20 μg protein were separated by 10% SDS–PAGE gel and subsequently transferred onto polyvinylidene fluoride membranes for Western blotting. Following antibodies were used: anti-E-cadherin (1:500), anti-*Twist* (1:1000), anti-FN (1:500), anti-N-cadherin (1:500) and HRP-conjugated secondary antibodies (1:5000).

Statistical analysis. Statistical significant differences were determined by one way ANOVA and independent-sample *t* test. $P < 0.05$ was regarded as significant.

Results

Subcellular localization of Twist in non-treated gastric cells by CLSM

Nuclear *Twist*, activated by extracellular factors through PI3K/AKT, Wnt1 and NF-κB pathway in invasive carcinomas [4,6,16–18], functions as a repressor of downstream gene transcription such as E-cadherin [19]. Based on these studies, the subcellular localization of *Twist* in MKN45 cells (a gastric cancer cell lines with high potential for peritoneal dissemination [20]) was examined. *Twist* was observed located in both cytoplasm and nucleus, predominately in the cytoplasm in perinuclear area (Fig. 1A). Further demonstration was performed by Western Blot, an expected band with 28 kDa size in both cytoplasmic and nuclear extracts from MKN45 cells were detected, respectively (Fig. 1B).

Down-regulation of Twist inhibited cells motility and invasion in a human gastric cell line MKN45

To observe the invasion-promoting effect of *Twist* in gastric cancer cells, a pcDNA3/*Twist*AS vector was trans-