Twist1 correlates with poor differentiation and progression in gastric adenocarcinoma via elevation of FGFR2 expression

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Abstract

AIM: To explore the correlation between Twist-related protein (Twist)1, fibroblast growth factor receptor (FGFR)2 and gastric adenocarcinoma differentiation and progression.

METHODS: We evaluated Twist1 and FGFR2 in 52 gastric adenocarcinoma samples by immunohistochemistry and quantitative real time polymerase chain reaction, and analyzed the correlation between Twist1, FGFR2 and cancer differentiation. We also detected Twist1 and FGFR2 expression in gastric adenocarcinoma cell lines, and evaluated Twist1 influence on FGFR2 expression.

In addition, we studied the role of FGFR2 in Twist1-promoted cancer progression, including proliferation, invasion and epithelial-mesenchymal transition (EMT).

RESULTS: Twist1 and FGFR2 were detected in almost all the gastric adenocarcinoma samples. Twist1 (P = 0.0213) and FGFR2 (P = 0.0310) mRNA levels had a significant association with gastric adenocarcinoma differentiation. Moreover, Twist1 and FGFR2 expression in poorly differentiated cells (SNU-1 and SNU-16) was notably higher than in well-differentiated cells (MKN-7 and MKN-28). In poorly differentiated gastric adenocarcinomas, FGFR2 mRNA level was significantly positively correlated with Twist1 mRNA level (P = 0.004). Twist1 was proved to promote FGFR2 by regulating Twist1 expression by knockdown and overexpression. Additionally, Twist1 could induce proliferation, invasion and EMT in gastric cancer; of these, FGFR2 was required for invasion and EMT, rather than proliferation.

CONCLUSION: Twist1 and FGFR2 are highly associated with differentiation of gastric adenocarcinoma; Twist1 can facilitate invasion and EMT in gastric adenocarcinoma via promotion of FGFR2 expression.

Key words: Twist-related protein 1; Fibroblast growth factor receptor 2; Gastric adenocarcinoma; Cancer differentiation; Cancer progression

Core tip: The underlying mechanism of Twist1 and fibroblast growth factor receptor (FGFR)2 function in gastric cancer has not been fully elucidated. We assessed expression of Twist1 and FGFR2 in gastric adenocarcinoma tissues by immunohistochemistry and polymerase chain reaction, and demonstrated that Twist1 and FGFR2 expression was significantly associated with differentiation of gastric adenocarcinoma tissue. We found that Twist1 can function...
as a transcription factor to promote FGFR2 expression. Moreover, Twist1 can induce proliferation, invasion and epithelial-mesenchymal transition (EMT) of gastric cells, in which FGFR2 is required in the Twist1-induced cell invasion and EMT.

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INTRODUCTION

Gastric cancer is the fourth most common cancer and the second leading cause of cancer-related death worldwide[10]. Early clinical symptoms of gastric cancer are usually silent, and many patients are diagnosed at an advanced stage with lymph node invasion or metastasis, which leads to the fact that the 5-year survival rate is low, ranging from 20% to 30%[11]. Surgical resection is still the only curative method for gastric cancer, although many studies have demonstrated that perioperative and adjuvant chemotherapy, including chemoradiation, can improve the outcome of resectable gastric cancer. However, no effective targeted drugs except those targeting HER2 have been discovered, and more attention should be focused on finding other molecular targets for treatment of gastric cancer.

Twist-related protein (Twist)1, also known as class A basic helix-loop-helix protein (bHLHa)[12], has been proved to play an essential role in cell lineage determination and differentiation. Twist1 was first identified in Drosophila as one of the zygotic genes, and is essential for mesoderm specification and subdivision into different tissue types[13]. As a transcription factor, Twist1 is widely expressed in almost all tissues related to the mesoderm, and is involved in many biological processes such as organogenesis[14]. Despite that, ectopic Twist1 expression and function are found in many kinds of cancers, including breast, prostate, bladder and gastric cancer[15,16]. The distinguishing feature of Twist1 among other transcription factors is that it regulates gene expression differentially depending on dimer composition. The homodimers of Twist1 induce expression of fibroblast growth factor receptor (FGFR)2 and periostin (POSTN) while heterodimers repress FGFR2 and POSTN expression[17]. Another feature of Twist1 is that it induces epithelial-mesenchymal transition (EMT), which predicts that Twist1 may be a potential oncogenic protein in humans[18,19].

In humans, the FGF family has 22 known members, and the FGFRs have four members, FGFR1-4[20,21]. Different FGFs bind different FGFRs to regulate many fundamental biological processes, including embryogenesis, tissue and stem cell maintenance, angiogenesis and wound healing[22-24]. The FGFR family is differentially expressed in various tissues and cancers. FGFRs are correlated with progression and prognosis in many cancers, including prostate, breast and bladder cancer, cholangiocarcinoma and gastric cancer[25,26]. FGFR2 is distinguished among the FGFRs because it has isoforms FGFR2(β)I/IIb and FGFR2(β)IIc. The FGFR2 IIb form is a high-affinity receptor of FGF1, FGF2, FGF10 and FGF7, whereas FGFR2 IIc binds both FGF1 and FGF2 but not FGF7[27]. The oncogenic role of FGFR2 in many cancers has been identified, including endometrial, ovarian, breast, lung and gastric cancers[28-30]. In gastric cancer, upregulation of FGFR2 was observed and associated with poor clinical outcomes. Moreover, anti-FGFR2 specific antibodies GAL-FR21 and GAL-FR22 have potential for the treatment of gastric tumors. However, the underlying mechanism by which FGFR2 leads to progression and poor prognosis of gastric cancer is not well elucidated and should attract more attention.

In our study, we assessed the expression of Twist1 and FGFR2 in gastric adenocarcinoma tissues by immunohistochemistry, and subsequently analyzed the correlation between Twist1 and FGFR2 expression with differentiation of gastric adenocarcinoma. Moreover, we detected Twist1 and FGFR2 expression in gastric adenocarcinoma cell lines with different differentiation. By regulating Twist1 and FGFR2 expression by knockdown or overexpression, we detected the correlation between Twist1 and FGFR2 expression, and explored the role of Twist1 and FGFR2 in gastric adenocarcinoma cell line progression.

MATERIALS AND METHODS

Cells and reagents

Well-differentiated adenocarcinoma MKN-7 and MKN-28 were purchased from RIKEN Bioresource Center (Koyadai, Japan). Moderately differentiated adenocarcinoma SGC-7901 was obtained from YRGENE Bioresource Center (Changsha, China). Poorly differentiated adenocarcinoma SNU-1 was from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and poorly differentiated adenocarcinoma SNU-16 was from the American Type Culture Collection (Manassas, VA, United States). All gastric adenocarcinoma cell lines were cultured in RPMI-1640 medium (HyClone, Logan, UT, United States) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin and 100 µg/mL streptomycin (HyClone) in 5% CO2 resuscitation. Anti-Twist1 antibody (Cat. No. ab50887) and anti-FGFR2 antibody (Cat. No. ab38201) were from Abcam (Cambridge, United Kingdom). EMT detection kit was from Cell Signaling Technology (Cat. No. 9782; Danvers, MA, United States). All reagents without special instruction were purchased from Sigma-Aldrich (St Louis, MO, United States).
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**Samples and immunohistochemistry**

All gastric adenocarcinoma samples were obtained from Shandong Cancer Hospital and Institute and Qilu Hospital of Shandong University, with prior patient consent and approval of the Institutional Clinical Ethics Review Board. The diagnosis was confirmed with routine pathology by two senior pathologists. A small amount of cancer tissue was immediately placed in liquid nitrogen to detect mRNA by quantitative polymerase chain reaction (QPCR), with no influence of normal pathological diagnosis. Pathologic tumor-node-metastasis (pTNM) staging was based on the 7th staging classification of the American Joint Committee on Cancer/Union for International Cancer Control (2009).

Immunohistochemistry was performed by the streptavidin peroxidase complex method. After deparaffinization with xylene and graded alcohol, slides were incubated in 3% hydrogen peroxide for 10 min, then placed in citrate buffer (pH 6.0), which was boiled by microwave oven heating. Phosphate-buffered saline (PBS) with 5% bovine serum albumin was used to block nonspecific binding. After blocking, slides were incubated in primary antibody at a dilution of 1:100 at 4 °C overnight. After washing the slides with PBS three times for 3 min each, the tissues were incubated with corresponding secondary antibody at 37 °C for 1-2 h. Following peroxidase complex reagent application, 3,3'-diaminobenzidine solution was used for incubation until satisfactory staining appeared. Results of every slide were finally evaluated by two senior pathologists unaware of the clinical information.

**Plasmid construction, siRNA and transfection**

A Twist1 open reading frame was amplified by PCR from the plasmid purchased from Origene (Rockville, MD, United States) and then subcloned into vector pFLAG-CMV2 with the double-digestion method of EcoRI and BamHI. Successful plasmid construction was finally confirmed by DNA sequencing. Twist1 (s14523) and control (4611) siRNAs were acquired from Ambion (Austin, TX, United States). FGFR2 siRNAs (sc-29218) were purchased from Santa Cruz Biotechnology (Dallas, TX, United States). Transfection was performed with lipofectamine 2000 (Carlsbad, CA, United States) according to the manufacturer’s instructions.

**RNA extraction and real-time analysis**

Total RNA was purified from cancer tissue with TRIzol reagent after tissue grinding and homogenization. Synthesis of cDNA and quantitative PCR was realized by the StepOnePlus real-time PCR system (Applied Biosystems; Carlsbad, CA, United States) in the SYBR Green method according to the manual of the manufacturer. GAPDH was applied as an internal control. The sequences of primers used for real-time PCR were designed as described previously[20]. Twist1-forward: TACATCGAATCCCTATTCCAGGTTC; Twist1-reverse: TAGTGGAACCGCGACATGGA; FGFR2-forward: CGCTGTTGAAGGATAACAACG; FGFR2-reverse: TGGAAGTTCATACTCGGAGACCC.

**Immunoblotting**

Measurement of expression of Twist1 or FGFR2 was carried out by immunoblotting. Total protein was first extracted according to the protocol of the Total Cellular Protein Extraction Kit (Millipore, United States). Cells were washed twice with PBS and lysed with lysis buffer containing a cocktail of protease inhibitors. The tissues or cells were homogenized for 20 s and then put on dry ice for 15 s. Following rotation of the homogenized tissues or cells at 4 °C for 20 min, the cells were centrifuged at 11000 rpm at 4 °C for 20 min, and the supernatant was collected. After detection of the protein concentration with the BCA detection kit (Beyotime Institute of Biotechnology, Shanghai, China), 10 μg protein was run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene fluoride membrane (PALL, United States) and incubated with primary antibody (1:1000) overnight at 4 °C. After washing three times in Tris-buffered saline, the membrane was incubated in secondary antibody labeled with horseradish peroxidase for 2 h at 37 °C and visualized by electrochemiluminescence (Millipore). Quantification of immunoblotting results was accomplished by Image J software (NIH, United States).

**Proliferation assay**

Proliferative ability was detected by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Detected cells were trypsinized and passed into 96-well plates at a density of 5000 cells/well and then starved in serum-free medium overnight. The medium was changed to normal medium containing 10% FBS, when time was set as 0 d. After termination by addition of 10 μL MTT at 10 mg/mL concentration, cells were cultured for 4-6 h. Medium was finally decanted carefully and crystals were dissolved by 100 μL dimethylsulfoxide and incubated for 15 min for complete solution. Absorbance at 490 nm was read by a microplate reader and all data were standardized by comparison with the control group. Data were analyzed by t test, and shown as mean ± SE, and confirmed by three independent experiments.

**Invasion assay**

Invasive activity of gastric cells was evaluated by Transwell assay. Pre-Matrigel-coated Transwell 24-well plates were purchased from BD Company (New Jersey, United States). Cells were trypsinized and passed into the Transwell upper chamber and cultured for 6 h to allow adhesion. Serum-free medium was changed to starve cells for 6 h. After that, medium in the lower chamber was changed to 10% FBS-containing medium and the cells were cultured for 12 h. Finally, cells were fixed in methanol and stained with Giemsa stain. Cells on the upper filter surface were removed using a cotton swab and invasive cells were observed and counted at × 200 magnification in at least five random visual fields. Cell numbers in the control group were set as baseline and the
Twist1 and FGFR2 expression in gastric adenocarcinoma

Twist1 and FGFR2 expression were analyzed in well-, moderately and poorly differentiated gastric adenocarcinomas. Twist1 was mostly observed in the nucleus and cytoplasm in gastric adenocarcinoma (Figure 1A and B), while FGFR2 was seen in both the cytoplasm and cell membrane (Figure 1C and D). Twist1 and FGFR2 were detectable in most cases of gastric adenocarcinoma (86.5% and 88.5%, respectively). With immunohistochemical detection, Twist1 and FGFR2 seemed to have stronger staining in poorly differentiated cancer tissue, suggesting they may be associated with differentiation in gastric adenocarcinoma.

Twist1 and FGFR2 expression correlated with gastric adenocarcinoma differentiation

To confirm the immunohistochemical results, we performed QPCR to detect and quantify the mRNA level of Twist1 and FGFR2 in gastric adenocarcinoma with different degrees of differentiation. As expected, Twist1 and FGFR2 had higher expression in poorly differentiated gastric adenocarcinoma compared with well-differentiated cancer ($P = 0.0213$ and $0.0310$, respectively), which implies that high Twist1 and FGFR2 expression may promote poorly differentiated gastric adenocarcinoma (Figure 2A and B). To confirm this result in cell lines, we collected gastric adenocarcinoma cell lines of different differentiation status and detected the expression of Twist1 and FGFR2. In all these cells, levels of Twist1 and FGFR2 were detected by immunoblotting and QPCR (Figure 2C-E). Immunoblotting (Figure 2C) and QPCR (Figure 2D and E) demonstrated that poorly differentiated SNU-1 cells had significantly higher Twist1 expression than well-differentiated MKN28 cells (Figure 2D), whereas poorly differentiated SNU-1 and SNU-16 cells had markedly higher FGFR2 expression compared with that in well-differentiated MKN7 and MKN28 cells (Figure 2E).

Twist1 promotes FGFR2 transcription and expression

Twist1 and FGFR2 expression can be elevated by Twist1 homodimers,

So we firstly verified whether FGFR2 expression was associated with Twist1 in gastric adenocarcinoma. We separated gastric adenocarcinoma samples into well-, moderately and poorly differentiated groups, which were further divided into high or low Twist1 expression numbers in the other groups were standardized by ratio to baseline. Analyzed data were from three independent experiments.

**Statistical analysis**

All data without special note were analyzed by Student's $t$ test using SPSS version 17.0. Column and spot graphs values were displayed with ± SE. $P < 0.05$ was considered statistically significant.

**RESULTS**

Twist1 and FGFR2 expression in gastric adenocarcinoma

Consistent with previous studies, Twist1 was mostly observed in the nucleus and cytoplasm in gastric adenocarcinoma (Figure 1A and B), while FGFR2 was seen in both the cytoplasm and cell membrane (Figure 1C and D). Twist1 and FGFR2 were detectable in most cases of gastric adenocarcinoma (86.5% and 88.5%, respectively). With immunohistochemical detection, Twist1 and FGFR2 seemed to have stronger staining in poorly differentiated cancer tissue, suggesting they may be associated with differentiation in gastric adenocarcinoma.

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Twist1 promotes FGFR2 transcription and expression

Previous studies have demonstrated that FGFR2 expression can be elevated by Twist1 homodimers, so we firstly verified whether FGFR2 expression was associated with Twist1 in gastric adenocarcinoma. We separated gastric adenocarcinoma samples into well-, moderately and poorly differentiated groups, which were further divided into high or low Twist1 expression...
groups. In poorly differentiated cancer, the higher Twist1 group had significantly higher FGFR2 expression than the lower Twist1 group ($P = 0.004$), indicating that high Twist1 expression can promote FGFR2 expression (Figure 3A). SNU-1 and MKN28 cells were selected as models for Twist1 knockdown and overexpression,
respectively, because SNU-1 cells had higher native Twist1 expression and MKN28 cells had lower native Twist1 expression. We transfected SNU-1 cells with Twist1 siRNA to accomplish Twist1 knockdown, and transfected MKN28 cells with Twist1 plasmid, and then detected FGFR2 mRNA and protein levels by QPCR and immunoblotting. QPCR and immunoblotting both showed that FGFR2 was downregulated after Twist1 knockdown, and FGFR2 expression was elevated after Twist1 overexpression in MKN28 cells. Forty-four hours after transfection with pFLAG-FGFR2 or control vector, MKN28 cells were lysed and FGFR2 expression was detected; D: FGFR2 mRNA level was regulated by Twist1 overexpression or knockdown. Total mRNA was extracted in SNU-1 cells 48 h after transient transfection of Twist1 siRNA or Twist1 vector. FGFR2 mRNA level was evaluated with QPCR. FGFR2 expression decreased when Twist1 was knocked down and increased when Twist1 was overexpressed. \( \text{a} \) P < 0.05, Twist1 siRNA vs scrambled RNA; \( \text{c} \) P < 0.05, Twist1 overexpression vs control vector.

**FGFR2 is required in Twist1-induced invasion and EMT in gastric cell lines**

In previous studies, Twist1 has been identified to promote proliferation in gastric cancer cell lines [10,31], but the target molecule of Twist1 is still controversial. In the experiments above, we suspected that FGFR2 may be one of the candidate targets of Twist1 and investigated its role in Twist1-induced gastric adenocarcinoma progression. SNU-1 cells were transfected with Twist1 plasmid and/or FGFR2 siRNA to achieve knockdown, and a series of functional assays, including proliferation, invasion and EMT, was performed. Twist1 overexpression notably accelerated proliferation, while FGFR2 knockdown had no significant influence on this effect (Figure 4A), suggesting that FGFR2 was not involved in Twist1-induced proliferation. Invasive ability was evaluated by Transwell assay with 10% FBS as the chemotactic factor. The invasion increased markedly when Twist1 was overexpressed, indicating that Twist1 played an essential role in SNU-1 cell invasion. When FGFR2 was knocked down, this Twist1-induced invasion...
Figure 4 Fibroblast growth factor receptor 2 is required in Twist1-induced invasion and epithelial-mesenchymal transition rather than proliferation. A: Fibroblast growth factor receptor (FGFR) 2 knockdown did not block Twist1-induced proliferation in MKN28 cells. Proliferative ability was assessed by MTT assay in MKN28 48 h after transfection with Twist1 vector and FGFR2 siRNA. Twist1 overexpression promoted proliferation, and FGFR2 knockdown had little influence on that. Data are displayed as mean ± SE from three independent experiments. a P < 0.05 Twist1 overexpression vs control group. B-E: Representative Transwell figures of MKN28 without transfection (B), and MKN28 transfected with control pFLAG-CMV2 vector (C), pFLAG-Twist1 vector (D), and pFLAG-Twist1 + siFGFR2 (E); F: Invasive cell number was counted and analyzed by Student’s t-test. Invasive ability was evaluated by Transwell assay 48 h after transfection of Twist1 vector and FGFR2 siRNA. The invasive ability was markedly elevated by Twist1 overexpression and this tendency decreased significantly when FGFR2 was knocked down by siRNA, indicating that FGFR2 played an essential part in Twist1-induced invasion in MKN28 cells. Data were acquired from three independent experiments and displayed as mean ± SE; a P < 0.05 Twist1 overexpression vs control; c P < 0.05 Twist1 + siFGFR2 vs Twist1 overexpression; G: FGFR2 was partly required in Twist1-induced EMT. Twist1 overexpression significantly promoted E-cadherin decrease, and N-cadherin and Snail-2 increase, demonstrating that Twist1 promoted EMT in MKN28 cells. When FGFR2 was knocked down and Twist1 was overexpressed, the change in E-cadherin and N-cadherin was impaired, whereas there was little change in Snai12, indicating that FGFR2 played an essential role in Twist1-induced EMT. (H) Signals from (G) were quantified by Image J software. Data were from 5 independent experiments, analyzed by Student’s t-test and displayed by ± SE. *P < 0.05 vs corresponding control group respectively; **P < 0.01 vs control group regarding to FGFR2 expression.
was reduced significantly, which demonstrated that FGFR2 was required in Twist1-involved invasion (Figure 4B-F). Previous studies have shown that Twist1 is an important protein in gastric adenocarcinoma EMT. After transfection, we detected the change in EMT-related factor expression by immunoblotting (Figure 4G). Decreased E-cadherin and increased N-cadherin induced by Twist1 overexpression were impaired by FGFR2 knockdown, while Snail2 expression had no change, which indicates that FGFR2 was required in Twist1-promoted EMT changes in E-cadherin and N-cadherin.

**DISCUSSION**

To the best of our knowledge, we demonstrated for the first time that Twist1 and FGFR2 expression was significantly associated with differentiation of gastric adenocarcinoma tissue. With experiments in vitro, we found that Twist1 functioned as a transcription factor to promote FGFR2 expression. Moreover, Twist1 induced proliferation, invasion and EMT of gastric cells, and FGFR2 was required for Twist1-induced cell invasion and EMT.

The extracellular domain of FGFR contains two or three immunoglobulin-like loops where the FGF binding site is encoded. It is generally considered that FGFR IIIb is mostly expressed in epithelial cells, while FGFR IIIc is mostly expressed in mesenchymal cells[22,23]. One of the most intriguing features of FGFR2 is its isoforms with different functions. FGFR2 IIIb has high affinity for FGF7 and FGF10, whereas FGFR2 IIIc has high affinity for FGF2 and FGF9. The diverse distributions and functions of FGFR2 isoforms may determine the different outcome of cytokine or other cell signals. This ligand specificity of FGFR2 isoforms was not our focus and could not be verified in our experiments. However, we hope our study triggers more in-depth research on FGFR2 isoform functions in gastric cancer.

In humans, pathologic Twist1 expression is mostly focused in cancer initiation, progression and metastasis, including breast cancer[34], hepatocellular carcinoma[35,36], prostate cancer[37], gastric cancer[38,39], esophageal squamous cell carcinoma[36,38], bladder cancer[40] and pancreatic cancer[41]. Many recent studies have highlighted the role of Twist1 in promoting cancer cell EMT and metastasis. EMT is demonstrated to generate poorly differentiated cells[41], which lose their polarity and become more invasive. One of our intriguing findings is that Twist1 and FGFR2 can lead to easier EMT of gastric adenocarcinoma cells, which may be the reason that Twist1 and FGFR2 were correlated with gastric cancer differentiation.

Specific monoclonal antibodies of FGFR2 have been proved to suppress gastric tumor growth by blocking FGFR2 signaling[16,24]. However, no drugs targeting FGFR2 are in current clinical use. We believe that FGFR2 and Twist1 could become new research hotspots in gastric cancer targeted therapy. We hope that our findings can bring new insight into drug development in gastric cancer, and help find a new therapy that targets Twist1.

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