Abstract. Twist-related protein 1 (Twist1), is a class II basic helix-loop-helix transcription factor, which has been demonstrated to be a major regulator of epithelial-mesenchymal transition (EMT), and therefore is involved in promoting carcinoma metastasis. Previous studies have demonstrated that Twist1 expression is upregulated in cervical cancer cases with poor clinical outcomes. However, the mechanisms that mediate the role of Twist1 in cervical cancer metastasis are poorly understood. To the best of our knowledge, the present study provides the first evidence that the downregulation of Twist1 by short hairpin RNA lentivirus (LV-shRNA) resulted in the inhibition of invasion and migration of cervical cancer cells. Furthermore, the present study presents evidence that reducing Twist1 expression prevents cervical cancer cells from undergoing EMT. The expression of the epithelial cell marker, E-cadherin, was elevated; and the expression levels of mesenchymal cell markers [fibronectin, vimentin, matrix metalloproteinase-9 (MMP-9) and MMP-2] were reduced in the LV-sh-Twist1 group in cervical cells. Collectively, these findings indicate that Twist1-mediated modulation of EMT is important in the invasion and migration of cervical cells, and also indicates the potential therapeutic importance of strategies involving the inactivation of Twist1-mediated mesenchymal changes in cervical cancer.

Introduction

Cervical cancer is the second most common cause of cancer-associated mortality in women worldwide (1), and its global incidence increased at an annual rate of 0.6% between 1980 and 2010 (2). Invasion and metastasis are the primary causes of treatment failure and subsequent mortality in patients with cervical cancer (3). Recurrence and metastasis of cervical carcinoma to other sites, including the lymph nodes (4), bones (5), lungs (6) and liver (7) may also occur. Therefore, the inhibition of metastasis is an auxiliary strategy for curing patients of cancer. However, the molecular alterations that drive invasion and metastasis in cervical cancer are not well established. Identifying these molecular mechanisms may provide insights into potential targets for diagnosis and therapy.

Twist-related protein 1 (Twist1) is a class II member of the highly conserved family of basic helix-loop-helix transcription factors. Twist1 is overexpressed in a number of types of cancer, and correlates with low E-cadherin expression, high cancer aggressiveness and poor patient survival rates (8,9). The role of Twist1 in tumor invasion and metastasis has been attracting increasing interest. Previous studies have demonstrated that carcinoma invasion and metastasis are driven by a process termed epithelial-mesenchymal transition (EMT) (10), which is a process whereby epithelial cells acquire mesenchymal properties and override senescence (11). Twist1 overexpression promotes metastasis in vivo by inducing EMT (8). Suppression of Twist1 by small interfering RNA in prostate cancer cells induced the expression of epithelial components and specifically inhibited their capacity for invasion and metastasis (12,13). Furthermore, Twist1 expression also enhances cell migration and invasion in gastric cancer in vitro and in vivo (14). Therefore, targeting Twist1 may be a novel therapeutic approach for the treatment of cervical cancer.

The present study targeted Twist1 in human cervical cancer HeLa cells and reduced it's expression levels using a short hairpin (sh)RNA lentivirus (LV-sh-Twist1) and assessed the resulting effects on migration and invasion of the cells.

Materials and methods

Cell culture. The human cervical cancer cell line HeLa was maintained in RPMI-1640 medium (Gibco, Gaithersburg, MD, USA), supplemented with 10% fetal bovine serum (FBS) (MinHai Bio-Engineering, Lanzhou, China), 1%...

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penicillin-streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA) in a humidified incubator at 37°C, 5% CO₂ atmosphere and 95% air.

**Lentiviral shRNA production and stable knockdown of Twist1.** The GIPZ lentiviral shRNAmir-GFP system (Open Biosystems, Lafayette, CO, USA) was used to knockdown human Twist1 in HeLa cells. Lentiviruses (LVs) that co-express green fluorescent protein (GFP) and shRNAs targeting Twist1, and with expression of GFP and empty vector control were used to infect HeLa cells. The detailed method for making an shRNA-based stable knockdown of Twist1 cell lines is described in a previous study (15). The LVs produced were titered and stored according to the manufacturer's instructions.

**MTT assay.** HeLa cells were cultured in 96-well plates at a density of 1x10⁴ cells per well overnight and infected with LV-sh-Twist1. After 0, 24, 48 and 72 h, 50 ml MTT (1 mg/ml) from Sigma-Aldrich (St Louis, MO, USA) was added to the cell media. After 4 h, the MTT was discarded and 150 ml dimethyl sulfoxide was loaded into each well. The spectrophotometric absorbance of the samples was measured using a microplate reader (Model 680; Bio-Rad Laboratories, Inc., Richmond, CA, USA) at 570 nm with a reference wavelength of 655 nm. The percentage of cell survival was calculated using the following formula: Cell viability = (absorbance value of infected cells / absorbance value of uninfected control cells) x 100. Six duplicate wells were measured at each concentration, and each experiment was performed at least three times.

**Apoptosis assay.** At 48 h after transfection, the HeLa cells were collected and washed twice with cold phosphate-buffered saline, resuspended in 400 µl Annexin V-fluorescein isothiocyanate (FITC) binding buffer at a density of 1x10⁶ cells/ml. The cells were stained with 5 µl Annexin V-FITC and 10 µl propidium iodide from an Apoptosis Detection kit (Jingmei Biotech, Shanghai, China) following the manufacturer's instructions. The cells were then subjected to flow cytometry (BD Biosciences, San Jose, CA, USA) to detect cell apoptosis. This experiment was conducted 3 times.

**Cell migration and invasion Transwell assays.** In vitro cell invasion assays were carried out in Matrigel-based Transwell plates with slight modifications. HeLa cells (500 µl of 1x10⁶ cells/ml in 0.1% FBS + RPMI-1640) were seeded into the Matrigel™-coated upper chambers of an 8 µm pore-sized polycarbonate membrane (Corning Costar, Cambridge, MA, USA). The lower compartments were filled with medium supplemented with 20% FBS. After 24 h, the cells that were present on the other side were stained with crystal violet dye and counted under a light microscope (DP70; Olympus, Melville, NY, USA). The number of cells were determined in eight random fields. The experiment was repeated 3 times for each group. Cell migration assays were carried out in a similar way but without Matrigel™. Migration or invasion of the cells through the chamber to the underside of the filter was assessed as described previously (16).

**Reverse transcription quantitative polymerase chain reaction (RT-qPCR) assay.** Total RNAs were prepared from the treated cells using TRIzol® reagent (Invitrogen Life Technologies). qPCR was carried out using the ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA) and performed by SYBR® Green dye according to the manufacturer's instructions. Oligonucleotide primers were synthesized by Sangon Biotech Co., Ltd (Shanghai, China). Sequences of primers used in the qPCR analysis are presented in Table I. The qPCR results were calculated using the 2-ΔΔCT method as described previously (17).

**Statistical analysis.** Statistical analysis was conducted using SPSS software (version 16.0; SPSS, Inc., Chicago, IL, USA). Data from at least three separate experiments are presented as the mean ± standard error of the mean. Paired Student's t-tests were used to determine any significant differences. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**LV-sh-Twist1 inhibits cervical cancer cell viability and induces cell apoptosis.** To determine the function of Twist1 in cervical cancer cells, Twist1 was knocked down in HeLa cells using LV-sh-Twist1, which was selected from three candidates by qPCR (data not shown). The effects of LV-sh-Twist1 on Twist1 expression levels were examined by western blotting in cervical cancer HeLa cells. The results demonstrated that transfection of LV-sh-Twist1 resulted in the downregulation of Twist1 (Fig. 1A and B). The expression level of Twist1 was reduced in HeLa cells at 24, 48 and 72 h after LV-sh-Twist1 transfection and that the level of inhibition increased in a time-dependent manner (Fig. 1A). The expression level of Twist1 was decreased at 48 h after LV-sh-Twist1 transfection compared with LV-GFP (Fig. 1B).

MTT assay was used to determine the effect of LV-sh-Twist1 on HeLa cell proliferation. The present results revealed that the knockdown of Twist1 evoked a marked inhibition effect...
on cell proliferation after 24, 48 and 72 h of LV-sh-Twist1 transfection in HeLa cells (73.2±5.3%, P<0.05; 57.4±6.1%, P<0.01; and 32±6.7%, P<0.01, respectively; Fig. 1C), whereas the cell viability in the LV-sh-Twist1 transfection group was reduced significantly compared with the LV-GFP group at 48 h (63.5±6.6%, P<0.01; Fig. 1D).

As it has been demonstrated that cell apoptosis serves a considerable role in the progression and development of tumors (18), the present study further explored whether the cell proliferation inhibition observed was due to the induction of apoptosis. Flow cytometry was used to determine the level of cell apoptosis at 48 h after transfection with LV-sh-Twist1 or LV-GFP. (C) HeLa cells were treated with LV-sh-Twist1. MTT assay was performed to determine cell viability rates at 0, 24, 48 and 72 h, respectively. (D) At 48 h after transfection with LV-sh-Twist1 or LV-GFP, the viability of HeLa cells was assessed using MTT assay. Data are presented as the mean ± standard deviation from three independent experiments. *P<0.05 and **P<0.01 compared with control. Twist1, Twist-related protein 1; sh, short hairpin; LV, lentivirus; GFP, green fluorescent protein.

Table I. List of reverse transcription-polymerase chain reaction primers used in the present study.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Sequence Product length (bp)</th>
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<tbody>
<tr>
<td>β-actin</td>
<td>F: 5’TGACGTGGACATCCGCAAAG3’ 205</td>
</tr>
<tr>
<td></td>
<td>R: 5’CTGGAAGGTGACAGCGAGG3’</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>F: 5’TGCCGCCCATCGCTTACAC3’ 179</td>
</tr>
<tr>
<td></td>
<td>R: 5’TGCTTAACCCCTACCTTTGA3’</td>
</tr>
<tr>
<td>Vimentin</td>
<td>F: 5’AATGGCTGTCATCTTACC3’ 186</td>
</tr>
<tr>
<td></td>
<td>R: 5’GGGTATCAACCCAGGGAGGTG3’</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>F: 5’TGCAACCTTTACAGACTATTCC3’ 122</td>
</tr>
<tr>
<td></td>
<td>R: 5’GAAATGTGAGATGCTGGTG3’</td>
</tr>
</tbody>
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F, forward; R, reverse.

Figure 1. Inhibitory effect of LV-sh-Twist1 on the survival rate of HeLa cells. (A) The expression levels of Twist1 by LV-sh-Twist1 in HeLa cells at 0, 24, 48 and 72 h, respectively. (B) The expression levels of Twist1 at 48 h after transfection with LV-sh-Twist1 or LV-GFP. (C) HeLa cells were treated with LV-sh-Twist1. MTT assay was performed to determine cell viability rates at 0, 24, 48 and 72 h, respectively. (D) At 48 h after transfection with LV-sh-Twist1 or LV-GFP, the viability of HeLa cells was assessed using MTT assay. Data are presented as the mean ± standard deviation from three independent experiments. *P<0.05 and **P<0.01 compared with control. Twist1, Twist-related protein 1; sh, short hairpin; LV, lentivirus; GFP, green fluorescent protein.

Altered expression of Twist1 influences migration and invasion in cervical cancer cells. The present study further examined whether the migration and invasion of cervical cancer cells is attenuated by the reduction of Twist1 expression via LV-mediated shRNA. Transwell® assays were employed to investigate whether the migration and invasion of cervical cells were affected by LV-sh-Twist1. Notably, when Twist1 was downregulated by LV-sh-Twist1 transfection, the migration of HeLa cells was reduced by ~53% compared with the control group (Fig. 3A; P<0.05). Similar results were obtained from invasion assays, which showed a reduction of ~5% compared with the control group (Fig. 3B; P<0.05).

LV-sh-Twist1 inhibits EMT of cervical cells. As numerous studies have revealed that EMT is closely associated with the migration and invasion of cancer cells (19,20), the present study investigated the effect of LV-sh-Twist1 transfection on EMT processes in cervical cells by examining the changes in
E-cadherin, fibronectin, MMP-9, MMP-2 and vimentin expression. RT-qPCR results demonstrated that the expression of the epithelial cell marker, E-cadherin, was significantly elevated in HeLa cells 48 h after LV-sh-Twist1 transfection (P<0.05). As hypothesized, the expression levels of mesenchymal cell markers (fibronectin and vimentin) were significantly reduced in the LV-sh-Twist1 transfection group (P<0.05; Fig. 4). In addition, similar results were observed from western blot analysis; the expression levels of fibronectin, vimentin, MMP-9 and MMP-2 were downregulated, whereas E-cadherin was upregulated in the LV-sh-Twist1 transfection group (Fig. 4). These results provide evidence for a potential role of EMT...
during the effects of Twist1 downregulation, which reduces the migration and invasion of cells in cervical cancer.

Discussion

Cervical cancer remains the only major gynecological malignancy that is clinically staged. At present, no accurate, efficient techniques for indicating prognosis and diagnosing parametrium invasion and lymph node metastasis are available for the selection of the most suitable treatment (21). The identification and functional characterization of molecules critically involved in prognosis, parametrium invasion and lymph node metastasis may reveal targets for diagnostic and therapeutic applications.

The majority of cancer-related mortality events occur as a result of metastasis rather than the original tumor; therefore, inhibiting cancer cell metastasis is a crucial aspect of cancer prevention. Previous studies observed that Twist1 expression promoted the migration and invasion capabilities of human breast cancer and hepatocellular carcinoma cells (22,23), which are essential for tumor metastasis (8). Furthermore, Twist1 expression in cervical cancer is associated with poor disease outcome (24). These results indicate that Twist1 is closely correlated with the invasion of cervical cancer cells. The objective of the present study was to investigate whether the migration and invasion of cervical cancer cells was regulated by Twist1, and if this was the case, which molecular mechanisms and signaling pathways were involved. To investigate the therapeutic relevance of inhibiting Twist1 in cervical cancer, Twist1 expression was knocked down using shRNA and the effects on cell invasion and migration were assessed. The results of the present study indicated that specific inhibition of Twist1 expression resulted in marked reductions in cervical cancer cell invasion in vitro.

These findings are consistent with the pro-invasive functions of Twist1 in cervical cancer and support the therapeutic potential of inhibiting Twist1 or Twist1-mediated EMT to inhibit cervical cancer cell invasion and migration. The present data demonstrated that the inhibition of Twist1 expression resulted in a notable reduction in cervical cancer cell growth and an increased cell apoptosis rate. These results indicate that the inhibition of Twist1 may have therapeutic potential, resulting in the targeting of cervical cancer cell invasiveness that contributes to tumor growth, progression and treatment resistance. To further address this potential, ongoing and future studies should address the effects of Twist1 inhibition in cervical cancer cells on tumor growth, invasion and response to therapy in vivo. The present study revealed that the downregulation of Twist1 by LV-sh-Twist1 transfection attenuates the cell migration and invasion abilities of cervical cancer cells through the reversal of EMT or suppression of MMP expression.

EMT is characterized by increased migratory features, reduced epithelial cell adhesion, loss of cytoskeleton components and acquisition of mesenchymal components (25), which is also critical for cancer metastasis. Induction of EMT may
result in cancer cells invading the surrounding stroma, and to intravasation, dissemination and colonization of distant sites. Thus, reversal of EMT is considered to be an effective strategy against cancer metastasis (26). In the present study, downregulation of Twist1 by LV-sh-Twist1 was demonstrated to be capable of reversing the process of EMT by reducing the expression of mesenchymal markers vimentin and fibronectin, and increasing the expression of the epithelial marker E-cadherin. These findings indicate that the inhibition of invasion by LV-sh-Twist1 in HeLa cells may be partially attributable to the reversal of EMT.

MMPs serve a critical role in cancer invasion, migration, metastasis and tumorigenesis. Blocking tumor cell expression of MMPs significantly reduces tumor invasion and metastasis (27). MMP-2 and -9 are major components of the extracellular matrix and basement membrane. A number of human tumors have been reported to be associated with increased expression of MMP-2 and -9 (28), and tumor aggressiveness has been found to significantly correlate with increased levels of MMP-2 and -9 in prostate (29) and breast (30) cancer. Cytokines and inhibitors regulate MMP-2 and -9 expression in cerebral and ovarian cancerous cells (31). In the present study, knockdown of Twist1 reduced expression of MMP-2 and -9 in the cervical cancer cell line.

In conclusion, the present study provides experimental evidence that knockdown of Twist1 by LV-sh-Twist1 suppresses cell migration and invasion in cervical cancer, which in turn drives EMT. Therefore, the results demonstrate an effect of Twist1 on cervical cancer cell invasion and metastasis, which may lead to the identification of novel diagnostic markers and therapeutic targets, and thus aid the understanding of the mechanisms behind cervical cancer metastasis.

Acknowledgements

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References