Negative regulation of TIMP1 is mediated by transcription factor TWIST1

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Received January 13, 2009; Accepted March 27, 2009

DOI: 10.3892/ijo_00000327

Abstract. TWIST1 is involved in tumor invasion and metastasis by promoting epithelial-to-mesenchymal transition. However, the molecular target of TWIST1 involving this mechanism is poorly understood. To identify the TWIST1-regulated genes, we established the Saos-2 cells stably expressing *TWIST1* gene by transfecting *TWIST1* cDNA into the cells and performed a differential display approach by using annealing control primers. Here, we report that one of the genes that were down-regulated in TWIST1 expressing cells is predicted to be *TIMP1*. TIMP1 has been reported as the naturally occurring specific inhibitor of matrix metalloproteinases (MMPs). Overexpression of *TWIST1* gene suppressed the expression of *TIMP1* mRNA but had no effect on *TIMP2* and *MMP-2* expression, as determined by semi-quantitative RT-PCR. We showed that TWIST1 was up-regulated in SCCBHY cells, which have a strong capacity of invasion into mandibular bone compared with SCCHN cells. Our present results suggest that TWIST1 is involved in tumor invasion by regulating the expression of *TIMP1*.

Introduction

TWIST1 belongs to a basic helix-loop helix family of transcription factors, which is expressed in mesodermal and cranial neural crest cells during embryogenesis (1-3). TWIST1 was identified as an important factor responsible for metastasis of breast cancer by promoting epithelial-to-mesenchymal transition (EMT) in an *in vivo* system (4). Down-regulation of Twist is able to suppress metastatic ability by repressing EMT (4,5). Exogenous expression of Twist1 promoted colony formation of mouse embryonic fibroblasts in soft agar and this process was mediated through suppression of the ARF/MDM2/p53 pathway (6). It was also reported that amplification of *Twist1* gene was associated with the development of acquired resistance to anticancer drugs and ectopic expression of *Twist1* gene leads to resistance to microtubule disrupting agents (7). TWIST1 is shown to affect AKT to mediate taxol resistance in nasopharyngeal carcinoma cells and up-regulation of AKT2 by TWIST1 promoted cell survival, migration and invasion in breast cancer cells (8,9). The increased expression of TWIST1 has been commonly used as molecular marker for EMT (10). These findings suggest that TWIST1 is a potent oncogene that induces tumorigenesis in non-malignant cells and promotes tumor progression in malignant cells. However, not much has been done to clarify TWIST1-targeted genes especially by using differential display procedures.

Several methods of subtractive assay have been used to identify differentially expressed genes (11). Differential display methods, which are based on PCR using short arbitrary primers, have been used in biological experiments due to their simplicity and fastness in spite of the higher rates of false positives. They are also biased toward detecting more abundant transcripts. A novel annealing control primer (ACP)-based differential display-RT-PCR technology has been used to identify differentially expressed genes in various cultured cells (12,13). The ACP-based PCR system facilitates the identification of the differentially expressed genes from samples displaying low mRNA levels without generating false positives (14).

Saos-2 cells express low level of *TWIST1* mRNA and have been used as a model to investigate the TWIST1 function (15). To identify the new targeted genes by TWIST1, we examined the mRNA profiles of Saos-2 cells transfected with the *TWIST1* gene inserted vector. Here, we describe a TWIST1-regulated gene which encodes tissue inhibitor of metalloproteinases-1 (TIMP-1).

Materials and methods

Materials. Dulbecco's, α and Opti modified Eagle's Minimal Essential Medium (D-MEM, α-MEM and Opti-MEM, respectively) were purchased from Gibco BRL (Gaithersburg, MD, USA). Fetal bovine serum (FBS) was obtained from JRH Biosciences (Lenexa, KS, USA). Plastic dishes were from Iwaki (Chiba, Japan). Anti-ß-actin and anti-TWIST1

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Key words: differential display, tumor invasion, matrix metalloproteinases, Saos-2, squamous carcinoma cell

monoclonal antibodies were purchased from Sigma (St. Louis, MO, USA) and Bio Matrix Research (Chiba, Japan), respectively. Anti-TIMP1 polyclonal antibody was from IBL (Takasaki, Japan). Full length human *TWIST1* cDNA (NM_ 000474) was purchased from GeneCopoeia (Germantown, MD, USA). It was subcloned to pDEST26 vector (Invitrogen, Carlsbad, CA, USA) by using Gateway cloning technology according to the manufacturer's direction. Other materials used were of the highest grade commercially available.

Cells and culture conditions. Human osteoblastic osteosarcoma cell line Saos-2 was obtained from the American Type Culture Collection (Rockville, MD, USA). Human oral squamous carcinoma cell lines SCCHN and SCCBHY were kindly provided by Dr K. Harada. The cells were cultured in plastic dishes containing α-MEM for Saos-2 cells or D-MEM for SCCHN and SCCBHY cells. Each medium contained 10% (v/v) FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were cultured at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air. Saos-2 cells were plated in 100-mm dishes and maintained in α-MEM containing 10% FBS until reaching 70-80% confluence. Before transfection, the cells were washed once with serum-free medium Opti-MEM. Transfection was performed with Lipofectamine reagent (Invitrogen). Twenty μg of expression vector (pDEST26) containing 6x histidine sequence-inserted *TWIST1* gene (pTWIST26) and 20 μl Lipofectamine in 1.5 ml Opti-MEM was mixed for 20 min. Serum-free medium (3 ml) was added to the mixture and the complexes were overlaid on to the cells. The cells were incubated with the complexes for 4 h at 37˚C. After incubation, culture medium containing complexes was replaced with the fresh one containing 10% FBS and the incubation continued. At 48 h after transfection, the cells were passaged at a 1:10 dilution into the medium containing geneticin (400 μg/ml, Sigma) for neomycin selection. After 2 weeks of culture in geneticin, the positive clones were selected and expanded for further characterization.

cDNA cloning and sequencing. GeneFishing kits (Seegene, Seoul, Korea) were used for the differential display methods following the manufacturer's instructions. The PCR products were cloned into TA cloning vector (Qiagen, Germantown, MD, USA) and used to transform competent DH5α *Escherichia coli* cells (Takara, Osaka, Japan). The colonies were grown for 16-18 h at 37˚C on Luria broth agar plates containing ampicillin, X-gal (5-bromo 4-chloro 3-indoyl-ß-D-galactopyranoside) and isopropyl-ß-D-thiogalactopyranoside for blue/white colony selection. The plasmids were extracted and the inserts were subjected to sequencing with T7 promoter primer. Sequence identity was confirmed by BLAST searches, accessed through the National Center for Biotechnology Information Homepage (http:www.ncbi.nlm.nih.gov/).

Semi-quantitative RT-PCR. Total RNA was isolated from TWIST- and empty vector-transfected Saos-2 cells by Isogen (Nippon Gene, Tokyo, Japan) followed by phenol extraction and ethanol precipitation. Purified RNA was further incubated with DNase I (Sigma) to digest the contaminated DNA. The mRNA samples were eluted in 30 μl double-distilled DEPC-treated water and reverse transcription was performed in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 6 mM $MgCl_2$, 2 mM DTT, 1 mM of each dNTP, 20 U of RNase inhibitor and 200 U superscript III (Invitrogen). The reaction mixture was incubated at 42˚C for 90 min and 94˚C for 2 min. The mRNAs of several genes were then detected by RT-PCR with the following specific primer pairs using reagents supplied with AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA). TWIST1, forward, 5'-GTCCGCAGTCTTACGAG GAG-3' TWIST1, reverse, 5'-CACGCCCTGTTTCTTTG AAT-3' TIMP1, forward, 5'-CTGTTGTTGCTGTGGCT GAT-3' TIMP1, reverse, 5'-TGCAGTTTTCCAGCAAT GAG-3' TIMP2, forward, 5'-AAAGCGGTCAGTGAGA AGGA-3' TIMP2, reverse, 5'-CTTCTTTCCTCCAACGT CCA-3' MMP2, forward, 5'-AGGCAAGTGGTCCGTGT GAA-3' MMP2, reverse, 5'-ACAGTGGACATGGCGGTCT CAG-3' GAPDH, forward, 5'-GACCCCTTCATTGACCTC AAC-3' GAPDH, reverse, 5'-CTTCTCCATGGTGGTGA AGA-3'. The PCR products were run on 1.2% agarose gels in 1X TBE buffer. The gels were stained with 1 μg/ml ethidium bromide and visualized under ultraviolet light.

SDS-PAGE and Western blot analysis. The cells were washed twice with PBS and then scraped into lysate buffer containing 1 mM DTT, 1 mM PMSF, 1 μg/ml leupeptin, 2 μg/ml aprotinin and 5 mM EGTA. The protein concentration was determined by using the protein assay reagent (Bio-Rad, Hercules, CA, USA) and diluted to a concentration of 1 mg/ml with lysate buffer. Twelve micrograms of each sample and pre-stained molecular weight markers were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Medford, MA, USA). The membranes were incubated for 2 h at ambient temperature in a blocking solution consisting of 5% non-fat skim milk in PBS containing 0.05% Tween-20 (PBS-Tween), washed briefly in PBS-Tween and then incubated overnight at 4˚C in PBS-Tween containing specific antibodies (diluted at 1:1000). For Western blot analysis, anti-ß-actin and anti-TWIST1 monoclonal antibodies and anti-TIMP1 polyclonal antibody were used. After the membranes had been washed 4 times within 30 min in PBS-Tween, they were incubated for 2 h at ambient temperature in PBS-Tween containing HRP-conjugated secondary antibodies (diluted at 1:5000, MP Biomedicals, Aurora, OH, USA). The membranes were washed again as described above and the proteins recognized by the antibodies were visualized with an ECL detection kit (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's directions.

Results

Identification and sequence analysis of the differentially expressed genes. To identify the specifically expressed genes in Saos-2 cells transfected with TWIST1-expressing vector, we compared the profiles of mRNA expression between TWIST1 (pTWIST26)- and empty vector (pDEST26)-transfected cells using a differential display method (Fig. 1A). DNA fragment of ~560-bp was amplified when cDNA representing the RNA was isolated from the cells transfected with empty vector (Fig. 1A, pDEST26) or the cells without transfection (data not shown). The corresponding band was

not detected in the cells overexpressing TWIST1 (Fig. 1A, pTWIST26). The 560-bp fragment was excised from the gel and subcloned. Analysis of nucleotide sequence of the isolated cDNA and comparison with the GenBank database indicated that the gene we isolated should be a part of *TIMP1* (NM_003254, Fig. 1B).

TIMP1 gene is down-regulated in TWIST1-overexpressing cells. To confirm the results of the ACP-RT-PCR analysis described above, mRNA was isolated from the untransfected, empty vector-transfected (pDEST26), or *TWIST1*-transfected (pTWIST26) Saos-2 cells. They were subjected to semi-quantitative RT-PCR using specific primers for *TIMP1* (Fig. 2). The expression of *TIMP1* decreased in the cells transfected with *TWIST1* gene when compared with that in the untransfected or empty vector-transfected cells. There were no significant alterations of mRNA in *TIMP2*, *MMP2* and *GAPDH* genes between the empty vector-transfected and *TWIST1* gene-transfected Saos-2 cells (Fig. 3). These results indicate that overexpression of TWIST1 in Saos-2 cells was specifically correlated with the expression of *TIMP1* gene,

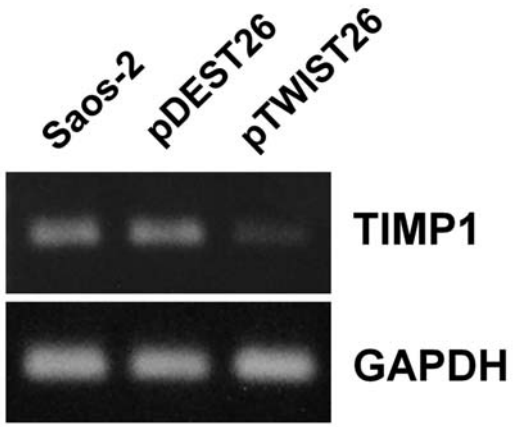
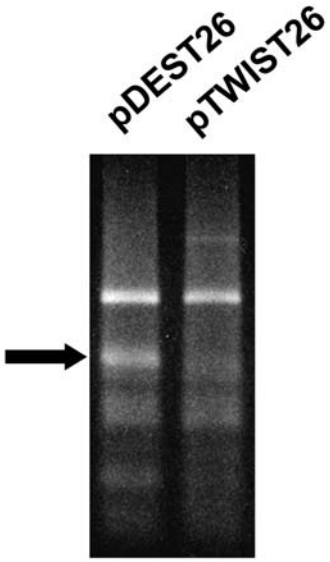


Figure 2. Identification of *TIMP1* mRNA in Saos-2 cells expressing pDEST26 or pTWIST26. Amplified DNA products were separated on a 2% agarose gel and stained with ethidium bromide. The *GAPDH* transcript was used as an internal standard to confirm the integrity of the mRNA samples.

but not other matrix-related genes *TIMP2* and *MMP2*. We further analyzed the expression pattern of TIMP1 protein by using Western blot analysis. The expression of TIMP1 protein in TWIST1 overexpressing cells (pTWIST26, Fig. 4) was significantly lower than that in the cells transfected with the control vector (pDEST26, Fig. 4). ß-actin was used as an internal standard.

TWIST1 is up-regulated in SCCBHY cells. It was reported that SCCBHY squamous carcinoma cells expressed lower level of TIMP1 protein when compared with SCCHN cells (16). Next, we used these cell lines to examine whether TWIST1 could negatively regulate the expression of TIMP1 protein by Western blot analysis. Fig. 5 shows the reaction between the antibodies indicated and the proteins extracted from the SCCHN and SCCBHY cells. SCCHN cells abundantly expressed TIMP1 protein, whereas SCCBHY cells expressed TIMP1 weakly. The expression of TWIST1 protein was higher in the extract prepared from SCCBHY cells compared with

A



B

TCGGTACCACGCATGCTGCAGACGCGTTACGTATCGGATCCAGAATTCGTGAT
gtctaccaggcattcgcttcatgggggatgccgctgacatccggttcgtctacacccccgccatggagagtgtctgcggatactt
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gtggctccctggaacagcctgagcttagctcagcgccggggcttcaccaagacctacactgttggctgtgaggaatgcacagtgt
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acccaaaaaaaaaaaaaaaaaaaaccccccatcgtagtcgcagcattcacaga
ATCTGAATTCGTCGACAAGCTTCTCGAGCCTAGGCTAGCTCT

Figure 1. Identification of differentially expressed genes in Saos-2 cells by ACP-based PCR. (A) Messenger RNA from the cells transfected with empty vector (pDEST26) or TWIST1 expressing vector (pTWIST26) was employed for the synthesis for first-strand cDNA using dT-ACP1. Using combination of dT-ACP2 (reverse primer) and arbitrary ACP (ACP9, forward primer), second-strand cDNA sequences were amplified during second-stage PCR. The differentially expressed genes were separated on a 2% agarose gel and stained with ethidium bromide for visualization. A band indicated by arrow was excised from the gel for further cloning and sequencing. A representative diagram of the ACP system from 3 experiments is shown. (B) The inserts in TA-cloning vector were subjected to sequencing with T7 promoter primer. Nucleotide sequence of the isolated cDNA was analyzed by sequencing and is represented with underlined lower letters.

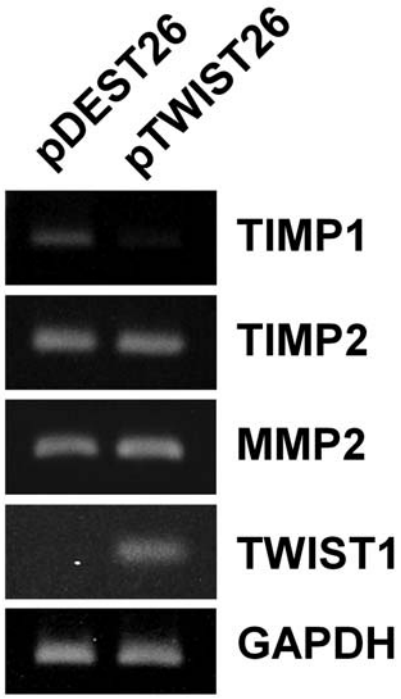

Figure 3. RT-PCR analysis of the genes (*TIMP1*, *TIMP2*, *MMP2* and *TWIST1*) in the cells expressing pDEST26 or pTWIST26. Amplified DNA products were separated on a 1.5% agarose gel and stained with ethidium bromide. The *GAPDH* transcript was used as an internal standard to confirm the integrity of the mRNA samples.

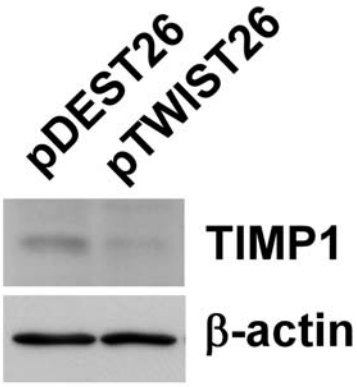

Figure 4. Expression of TIMP1 and TWIST1 proteins in the cells expressing pDEST26 or pTWIST26. Proteins from Saos-2 cells were subjected to Western blot analysis using TIMP1 antibody. The staining intensity of ß-actin in both preparations revealed that equal amounts of the protein was loaded.

that in SCCHN cells. These results correlated with the ACPs-based PCR in which the level of TIMP1 protein expression was lower in the cells expressing TWIST1. Equal loading of the samples was determined by using ß-actin antibody as an internal control (Fig. 5).

Discussion

To identify new TWIST1-regulated genes, we transfected Saos-2 cells with TWIST1-expressing vector, followed by performing a new ACP-based differential display PCR method. With this technique, we identified several genes that were predominantly up-regulated or down-regulated in the TWIST1-overexpressing cells. We identified one of these genes to be *TIMP1* and assessed its expression in more detail by using semi-quantitative PCR method. We found that expression of TIMP1 at mRNA and protein levels decreased in the TWIST1-overexpressing cells. These results indicate that TWIST1 is involved in the negative regulation of TIMP1 at both mRNA and protein levels.

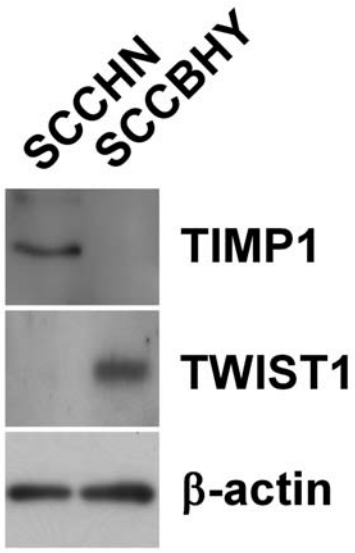

Figure 5. Identification of TIMP1 and TWIST1 proteins in SCCHN and SCCBHY cells. Proteins prepared from the cultured cells were analyzed by Western blotting using the antibodies indicated.

TIMP1 is a prototypic and original ancestral member of the mammalian TIMP families. The mammalian TIMP family consists of TIMP1, TIMP2, TIMP3 and TIMP4 proteinase inhibitors that suppress extracellular matrix (ECM) degradation by forming an inhibitory complex with MMPs (17). The maintenance of normal tissue structure in part depends on the balance between the expression and activity of ECM-degrading MMPs and the expression of TIMPs. Blockade of MMP-1 activity inhibited migration and invasion of prostate cancer cells (18). Treatment of mice with an MMP-1-specific inhibitor significantly decreased prostate tumor growth and the incidence of lung metastasis (18). It was also reported that the differential expression of MMPs and TIMPs of ovarian tumors was observed in serous or mucinous tumors (19). These findings suggest that the changes in the ratio of the expression/activity of TIMPs and MMPs associated with tumor initiation and growth through loss of cell adhesion and evasion of apoptosis (2,20).

In this study, we also used two kinds of oral squamous carcinoma cell lines to further demonstrate the relationships between TWIST1 and TIMP1. SCCBHY cells secrete TIMP2 but only trace level of TIMP1, whereas SCCHN cells secrete TIMP1 but only trace level of TIMP2 (16). We examined whether TWIST1 expression could be correlated with TIMP1 expression in these carcinoma cells. The level of TWIST1 expression was higher in the SCCBHY cells, whereas the

expression of TIMP1 was weak in these cells. It was reported that SCCBHY cells, but not SCCHN cells invaded the mandibular bone when they were inoculated in masseter muscle of the nude mouse (16). These findings suggest that down-regulation of TIMP1 by TWIST1 correlates with the higher potent invasion of SCCBHY cells.

It was suggested that TWIST1 is a new positive factor in the development and progression of cancer and plays important roles in invasion and metastasis (21). In addition, TWIST1 was shown to enhance the intravasation step of metastasis, although it did not have any significant effect on the survival, extravasation, or growth rate of the tumor cells (4). Inactivation of TWIST1 suppressed migration and invasion abilities, which was correlated with the induction of E-cadherin expression as well as morphologic and molecular changes associated with MET (22). In the present study, we demonstrated that TWIST1 did not affect *TIMP2* and *MMP2* expression. Among the matrix-related proteins, TWIST1 might down-regulate TIMP1 expression, which in turn should break the balance between matrix-degrading enzymes and their inhibitors. The above evidence in combination with our present results suggest that TIMP1 is one of the target genes that were negatively regulated by TWIST1. The present results also suggest that down-regulation of TIMP1 by TWIST1 promotes tumor invasion.

The bHLH transcription factors including TWIST1 bind to the conserved E-box region (CANNTG) on the promoter of genes and then activate or inhibit their transcription (23). Although E-box region is not located within a 1-kb fragment upstream of the translation start site of *TIMP1*, the UTE-1 motif (5'-TGTGGTTTCCG-3') is located at -62 to -52, which is 10-bp downstream of the SRE. The UTE-1 element is required for high-level promoter activity in a wide variety of cell types. Runx-1 and -2 regulate transcription of *TIMP1* via this UTE-1 site (24). It was also reported that C-terminal 'Twist domain' located in Twist1 gene binds to Runx proteins and inhibits Runx transcriptional activity (25). From these findings, TWIST1 might regulate the TIMP1 expression through the interaction of other transcription factors that bind directly to the promoter region. However, further studies are needed to clarify the mechanisms in transcriptional regulation of *TIMP1* by TWIST1.

In conclusion, overexpression of *TWIST1* down-regulates TIMP1 expression through affecting the transcriptional activity on the *TIMP1* gene. The insight in our present study that TWIST1 might regulate the transcription of the *TIMP1* gene is attractive, because both genes are closely related with tumor invasion. The effect of TWIST1 on *TIMP1* gene expression may result from the disruption of balance between proteolytic enzymes and their inhibitors, by which TWIST1 might be involved in tumor invasion.

Acknowledgements

We thank Dr K. Harada for the kind gift of SCCHN and SCCBHY cells. This study was supported in part by grants from the Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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