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ORIGINAL ARTICLE

Negative Regulatory Role of TWIST1 on SNAIL Gene Expression

Mohammad Mahdi Forghanifard[1] · Sima Ardalan Khales[2] · Moein Farshchian[3] · Abolfazl Rad[1] · Masoud Homayouni-Tabrizi[4] · Mohammad Reza Abbaszadegan[2]

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Abstract Epithelial-mesenchymal transition (EMT) is crucial for specific morphogenetic movements during embryonic development as well as pathological processes of tumor cell invasion and metastasis. TWIST and SNAIL play vital roles in both developmental and pathological EMT. Our aim in this study was to investigate the functional correlation between TWIST1 and SNAIL in human ESCC cell line (KYSE-30). The packaging cell line GP293T was cotransfected with either control retroviral pruf-IRES-GFP plasmid or pruf-IRES-GFP-hTWIST1 and pGP plasmid. The KYSE-30 ESCC cells were transduced with produced viral particles and examined with inverted fluorescence microscope. DNA was extracted from transduced KYSE-30 cells and analyzed for copy number of integrated retroviral sequences in the target cell genome. The concentration of retroviral particles was determined by Real-time PCR. After RNA extraction and cDNA synthesis, the mRNA expression of TWIST1 and SNAIL was assessed by comparative real-time PCR amplification. Ectopic expression of TWIST1 in KYSE-30, dramatically reduces SNAIL expression. Retroviral transduction enforced TWIST1 overexpression in GFP-hTWIST1 nearly 9 folds in comparison with GFP control cells, and interestingly, this TWIST1 enforced expression caused a − 7 fold decrease of SNAIL mRNA expression in GFP-hTWIST1 compared to GFP control cells. Inverse correlation of TWIST1 and SNAIL mRNA levels may introduce novel molecular gene expression pathway controlling EMT process during ESCC aggressiveness and tumorigenesis. Consequently, these data extend the spectrum of biological activities of TWIST1 and propose that therapeutic repression of TWIST1 may be an effective strategy to inhibit cancer cell invasion and metastasis.

Keywords Epithelial-mesenchymal transition (EMT) · TWIST1 · SNAIL · ESCC cell line

Introduction

Epithelial-mesenchymal transition (EMT) is an extremely conserved developmental process leading loss of cell adhesion and polarity, dissociation and movement of epithelial cells to different sites throughout embryogenesis, as well as tumor cell invasion and metastasis [1, 2].

In cancer, several signaling pathways are involved in EMT including WNT, RAS, epidermal growth factor (EGF), transforming growth factor-β (TGF-β), Notch and PTEN/PI3K, which are induced by numerous transcription factors such as Zn-finger transcription factors SNAIL1 [3, 4], SNAIL2 (SLUG) [5], ZEB1 [6], ZEB2 [7] and TWIST1/2 [8]. The crosstalk between these signaling pathways through related transcription factors may provide the phenotypic changes in cancer cells during EMT.

Mohammad Mahdi Forghanifard and Sima Ardalan Khales are contributed equally in this study.

✉ Mohammad Reza Abbaszadegan
abbaszadeganmr@mums.ac.ir

[1] Cellular and Molecular Research Center, Sabzevar University of Medical Sciences, Sabzevar, Iran

[2] Division of Human Genetics, Immunology Research Center, Avicenna Research Institute, Mashhad University of Medical Sciences, Bu-Ali square, Mashhad 9196773117, Iran

[3] Molecular Medicine Research Department, ACECR-Khorasan Razavi Branch, Mashhad, Iran

[4] Department of Biochemistry and Biophysics, Mashhad Branch, Islamic Azad University, Mashhad, Iran

TWIST1, as a highly conserved member of the basic helix-loop-helix (bHLH) transcription factors, plays key roles in either embryogenesis or tumorigenesis. TWIST1 is a potentially metastatic oncogene which prevents different signaling pathways such as NF-kB [9], MYC- and p53-dependent apoptosis [10]. It can also promote tumor cells invasion and metastasis through induction of EMT, as well as tumor angiogenesis. TWIST1 function is regulated by spatial-temporal expression, phospho-regulation (phosphorylation of TWIST1 serine 68 by Ras-activated JNK, ERK and p38 MAPKs), and dimer choice [11–14]. While heterodimers of TWIST1 and other bHLH proteins usually repress transcription, TWIST1 homodimers generally activate transcription of genes of interest [15]. Such gene expressions are regulated through binding of dimers to the promoter E-box sequences (5′-CANNTG-3′) of genes of interest [16].

TWIST1 is overexpressed in a variety of human tumors and contributed in cancer aggressiveness specially in melanomas, neuroblastomas, as well as gastric, prostate, and breast cancers [8, 17]. Furthermore, its upregulation can enhance invasion and metastasis of esophageal squamous cell carcinoma (ESCC) [18, 19]. Since TWIST1 is involved in aggressiveness and metastasis behavior of different tumors, identification of its downstream target genes and their contribution in EMT is crucial to realize the related molecular mechanisms.

SNAIL family of zinc-finger transcription factors includes three members called SNAIL (SNAIL1), SLUG (SNAIL2) and SMUC (SNAIL3). SNAIL functions as a core regulator of both developmental and pathological EMT. The SNAIL promoter is consisting of several conserved regulatory cis-acting elements including AP1 and AP4 sites, LEF1 binding sites, SMAD-binding elements, as well as E-boxes [17]. SNAIL protein has two functional domains including a conserved C-terminal DNA-binding domain with the highest affinity for 5′-CACCTG-3′ sequence [20] and the N-terminal regulatory domain which is essential for transcriptional repression [21]. Several signaling pathways regulate SNAIL gene transcription including PI3 kinase (PI3K), ERK2 and GSK-3β/NF κB [22, 23]. Interestingly, the functional 5′-CACCTG-3′ E-box sequence in SNAIL promoter acts as a negative regulatory element through binding of SNAIL protein to this element, creating a negative feedback loop repressing its own gene expression [24].

TWIST1 and SNAIL, as inducers of EMT, are involved in invasiveness and aggressiveness of a variety of malignancies. Since ESCC is a highly aggressive tumor, our aim in this study was to investigate the functional correlation between TWIST1 and SNAIL in human ESCC cell line, KYSE-30. Our findings indicated negative regulatory role of TWIST1 on SNAIL gene expression.

Materials and Methods

In Silico Sequence Analysis

Related sequences for SNAIL mRNA, SNAIL gene, and SNAIL flanking sequence were obtained from Genbank (accession numbers NM 005985.3, NC 000020.11, and AF155233, respectively). Sequence analysis was performed using CLC Main Workbench version 5.6 (CLC bio, Aarhus, Denmark).

Cell Lines and Culture Condition

Packaging cell line GP293T and human ESCC cell line KYSE-30 were purchased from the Pasture Institute (Tehran, Iran), and cultured in DMEM and RPMI 1640 mediums (Gibco), respectively, supplemented with 10 % (*v*/v) fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 95 % humidified atmosphere and 5 % CO2.

Retroviral Transduction and Overexpression Study

The packaging cell line GP293T was transfected with 5 μg of either control retroviral pruf-IRES-GFP plasmid or pruf-IRES-GFP-hTWIST1 and 4 μg of pGP plasmid in 500 μl of DMEM media with no supplements (plasmids were kindly gifted by Dr. Stan Gronthos; Mesenchymal Stem Cell Group, Division of Haematology, Institute of Medical and Veterinary Science/ Hanson Institute/ CSCR, University of Adelaide, SA, Australia). The DNA mixture with 5 μl X-tremeGENE HP DNA Transfection Reagent (Roche Diagnostics GmbH, Germany) was added to the GP293T cells and then incubated for 48 h. Harvested supernatant containing infectious particles was filtered through 0.45 μm Nalgene filter (Nalgene Labware, Rochester, NY). Filtered particles were added to semiconfluent cultures of KYSE-30 ESCC cell line (1 x 10^5 cells/6-well in RPMI-60 + 10 % FBS) at cell passage 3 or 4 for 24 h. The Cells were examined with inverted fluorescence microscope (Olympus IX-70) and stably transducted high-expressing GFP (control) and GFP-hTWIST1 KYSE-30 (>95 % positive) were generated and used for functional study.

Comparative Real-Time PCR Analysis

Total RNA was prepared from approximately 3 × 10^3 either GFP control or GFP-hTWIST1 transduced cultured KYSE-30 cells using the TRIZOL reagent (Invitrogen). Isolated total RNA from each subpopulation was then used as template for cDNA synthesis, prepared with a first-strand cDNA synthesis kit (Invitrogen Life Technologies, Carlsbad, CA). The mRNA expression of TWIST1 and SNAIL was assessed with primer sets presented in Table 1 using comparative real-time PCR amplification, as described before [25].

Table 1 Primer sets sequences used in Real-time PCR

Gene	Forward primer sequence	Reverse primer sequence
TWIST1	GGAGTCCGCAGTCTTACGAG	TCTGGAGGACCTGGTAGAGG
SNAIL	TTCAACTGCAAATACTGCAACAACAAG	CGTGTGGCTTCGGATGTG

Scratch Assay (Wound Healing Assay)

To evaluate invasion capacity of GFP-hTWIST1 transduced KYSE-30 cells, scratch assay was done according to the introduced protocol by Chun-Chi Liang [26], scrapping KYSE-30 cells with p1000 pipet tip after being confluent in 10 cm plate. Spreading cells between parallel lines was measured by Inverted Microscope (Olympus, USA) and photographed after 0, 24 and 72 h.

Statistical Analysis

Data analyses were performed by SPSS 19.9 statistical package (SPSS, Chicago, IL, USA). The correlation between gene expressions was assessed using either the χ^2 or Fisher exact tests and Pearson's correlation. A P values less than 0.05 was considered statistically significant.

Results

Sequence Analysis of SNAIL Promoter

Having considered the CANNTG as E-box consensus sequence, we analyzed 2 kb upstream of transcription start site of SNAIL gene to find probable existing E-box sequences contributing in promoter region. Furthermore, we searched the transcription unit of SNAIL gene to explore downstream putative E-boxes which may potentially have role in SNAIL gene transcription. Based on the results, fifteen different E-box sequences were found in the 2 kb upstream region which two of them are located near to the transcription start site definitely in positions −137 and −384. Others are distributed from position −523 to −1779 (Fig. 1). In addition, 37 potentially E-box were found within the transcription unit of SNAIL gene. 23 of 37 are located in intronic sequences and 14 of 37 are located in exons 2 and 3 (Table 2).

Enforced Expression of TWIST1 down-Regulates SNAIL mRNA Expression in KYSE-30 Cells

After retroviral transduction, functional study was performed on stably transduced high-expressing GFP-hTWIST1 KYSE-30 cells (>95 % positive) to determine whether the ectopic expression of TWIST1 in examined cells induce TWIST1 upregulation, and what are the effects of induced TWIST1 overexpression on SNAIL mRNA expression. To answer these questions, comparative real-time PCR analysis was carried out on GFP control and GFP-hTWIST1 transduced cultured KYSE-30 cells. The results demonstrated that retroviral transduction enforced TWIST1 overexpression significantly in GFP-hTWIST1 nearly 9 folds in comparison with GFP control cells, and interestingly, this enforced expression of TWIST1 caused a significant −7 fold decrease in the level of transcripts for SNAIL in GFP-hTWIST1 compared to GFP control cells (Fig.2, $P < 0.01$).

Scratch Assay (Wound Healing Assay)

The migration of GFP-hTWIST1 KYSE30 cells was examined using scratch assay. As shown in Fig. 3, the migration of GFP-hTWIST1 KYSE30 cells was increased in comparison with GFP control KYSE30 cells, significantly. Forty eight hours after scratching, the scratch was closed in TWIST1 overexpressed cells completely, but not in control KYSE30 cells.

Discussion

The EMT process is an important preliminary event in local invasion of different human epithelial cancers. Although, morphological and cellular changes of epithelial cells during the later stages of EMT and metastasis (dissemination) are remained unclear, recent cell-based EMT studies elucidated an alternative model for the simultaneous function of multiple EMT inducers and their cooperation at various spatial

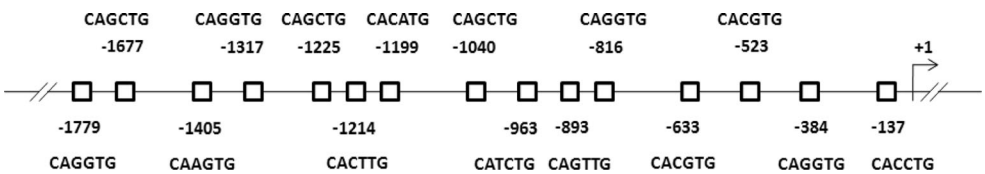

Fig. 1 Schematic view of the position and sequence of fifteen E-box hexanucleotide (consensus sequence, CANNGT) within 2 Kb upstearm of SNAIL transcription start site

Table 2 The number and positions of E-box hexanucleotide consensus sequence (CANNGT) in SNAIL transcription unit (GenBank accession number: AL121712.27). Asterisk indicates positions in exon 2 and 3

	Sequence	Number	Position(s) (23 + 14)
1	CACCTG	5	231–6, 880–5*,1896–01, 2762–7, 5637–42*
2	CAAGTG	4	311–6, 1166–71*, 4523–8, 5709–14*
3	CATCTG	1	943–8*
4	CAGGTG	5	164–9,1664–9,2736–41, 4661–6, 5766–71*
5	CACATG	1	3202–7
6	CAGATG	3	1041–6*, 1557–62, 4978–83*
7	CACTTG	2	2881–6, 5694–9*
8	CATGTG	1	4031–6
9	CAATTG	2	978–83*, 2468–73
10	CAGTTG	2	2293–8, 2551–6
11	CAGCTG	4	1178–83*, 2777–82, 5311–16*, 5387–92*
12	CAACTG	3	645–50, 1228–33*, 2498–03
13	CAAATG	1	1608–13,
14	CATATG	2	1885–90, 2026–31
15	CATTTG	1	1948–53

(primary tumor vs. metastases), and temporal (initiation vs. maintenance) EMT stages [27]. It has been recently demonstrated that a reciprocal expression pattern may be existed between TWIST1 and SNAIL in MCF10A cells; however, the exact mechanism was not described [28]. This study presents evidences for significant association of TWIST1 and SNAIL genes expression in invasive ESCC cell line KYSE-30. Interestingly, we revealed significant down-regulation of SNAIL following ectopic expression of TWIST1 in the cells. Since KYSE-30 is an invasive cell line and TWIST1 and SNAIL are two prevalent transcription factors of EMT

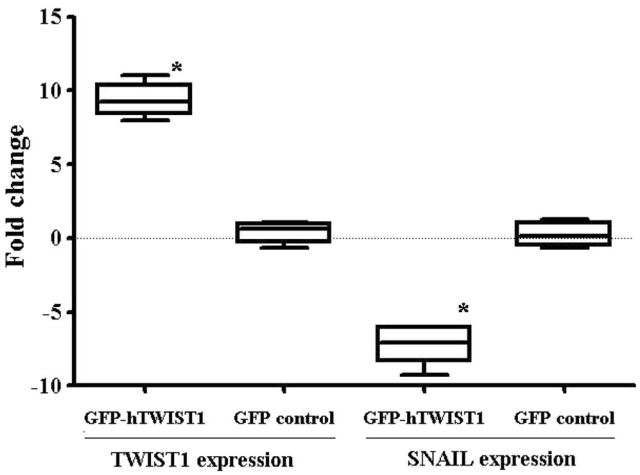

Fig. 2 Enforced expression of TWIST1 down-regulates SNAIL mRNA expression in KYSE-30 cells. Retroviral transduction enforced significant TWIST1 overexpression in GFP-hTWIST-1 nearly 9 folds compared to GFP control cells causing a − 7 fold decrease in SNAIL mRNA expression, significantly. Asterisk indicates statistically significance

promotion, this inverse correlation may play critical role in regulation of KYSE-30 invasiveness.

It has been shown that ectopic expression of TWIST1 in epithelial cancer cells not only can cause loss of E-cadherin-mediated cell-cell adhesion and trigger cell motility and metastatic ability in vivo, but can also induce EMT, angiogenesis, cell survival, and chemoresistance in vitro [8]. Moreover, TWIST1 ectopic expression in mouse embryonic fibroblasts induced soft agar colony formation displaying its important role in malignant transformation [10].

Since the metastasis is a multistep process, specific factors are required to help cancer cells for local invasion, intravasation, survival in circulation, extravasation and finally to generate macrometastases at distant organs [29].

TWIST1 can elevate the escape of tumor cells from their primary sites (intravasation), penetration of tumor cells via the endothelium of lymphatic or blood vessels entering the systemic circulation, and exiting of tumor cells from the capillaries and enter organs (extravasation).

Gene expression profiling of three different primary mammary carcinoma cell lines with ability of intravasation (4 T1, 4TO7,168FARN) revealed TWIST1 as the second most highly upregulated gene in significant correlation with invasiveness and aggressiveness behavior of the cell lines [8]. On the other hand, it has been shown that down regulation of TWIST1 in highly metastatic carcinoma cells dramatically declined the metastasis efficiency [8, 30].

SNAIL expression is controlled at either transcriptional or post-transcriptional levels through complex signaling networks [31]. SNAIL is direct transcriptional suppressor of E-cadherin in epithelial cells inducing EMT [32]; however, other factors than SNAIL may be also involved in the loss of E-cadherin expression in ESCC such as direct interaction of TWIST1 with specific E-boxes in the proximal E-cadherin promoter during tumor progression.

Human E-cadherin promoter contains three E-boxes (A, B and C) which are located in the proximal promoter regions (A and B) and in exon 1 (C). These E-boxes are essential for transcriptional suppression in cancer cell lines [33]. It has been shown that EMT-inducing regulators including bHLH TWIST1 and E47, and the zinc finger factors ZEB1, ZEB2, SLUG and SNAIL can interact with E-boxes of E-cadherin promoter to suppress its expression during EMT [6, 22, 34].

Nuclear factor kappa B (NF-kB) signaling is an essential mediator between inflammation and cancer [35]. It has been indicated that NF-kB is an inducer of SNAIL expression [22, 36]. In addition, TWIST1 is a downstream target gene of NF-kB pathway [37, 38]. TWIST1 overexpression in response of hyperactive NF-kB is indispensable for EMT and metastasis in progressive breast cancer cells. Importantly, Hector L et al. showed negative feedback loop between cytokines activating

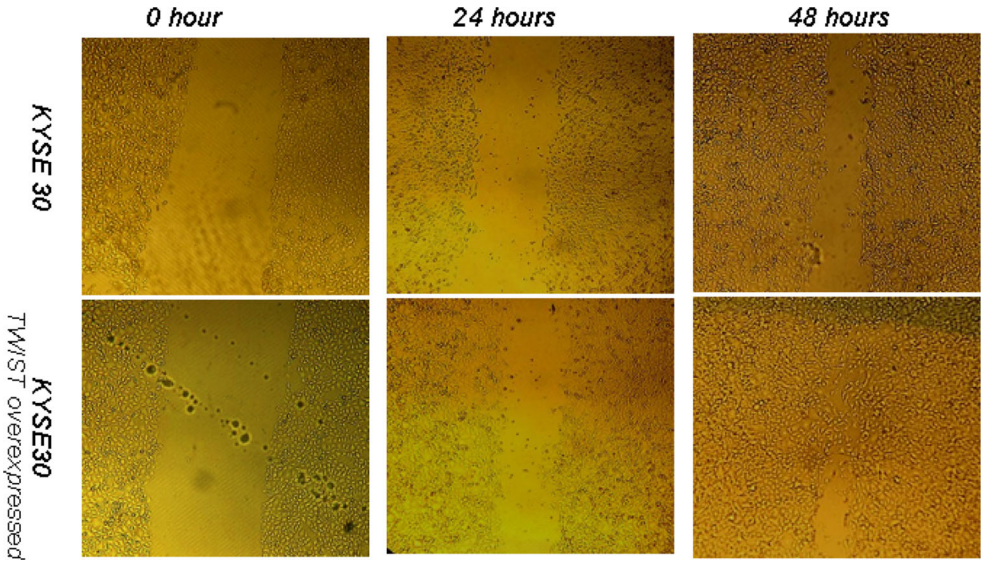

Fig. 3 Scratch assay. The migration of GFP-hTWIST1 KYSE30 cells was compared to GFP control KYSE30 cells. As can be clearly seen, the scratch was closed in TWIST1 overexpressed cells after 48 h

NF-kB and TWIST1 which resulted in prevention of NF-kB transactivation. Since the overexpression of TWIST1 prevents the NF-KB activation, down-regulation of SNAIL can be speculated subsequently, leading maintenance of EMT in cancer [9]. Our findings may confirm this hypothesis.

Chromatin immunoprecipitation (ChIP) assays revealed recruitment of the SNAIL1/2 to the E-boxes sequences through interaction between C-terminal WR (Trp-Arg) domain of TWIST1 and C-terminal zinc fingers of SNAIL1/2 proteins [39]. Therefore, TWIST1 may be hetrodimerized with SNAIL and negatively control gene expression. As mentioned before, SNAIL promoter has several E-box sequences which can act as negative regulatory elements (Fig.1). SNAIL can bind to these E-boxes, creating a negative feedback loop to repress its own gene expression in both PWP-1 and SW-480 cells [24]. Accordingly and based on our results, we may hypothesize that TWIST1-SNAIL heterodimers can probably bind to the E-box sequences and repress the SNAIL gene expression in KYSE-30 cells, although it should be cleared in details. So it may be extrapolated that TWIST1 is required as a molecular switch to activate or suppress target genes via utilizing numerous direct and indirect mechanisms for invasion and metastasis of tumor cells.

It has been shown recently that EMT is a multistep process from initiation to maintenance. During this event, the EMT-inducing transcription factors are generated transiently in the cells and function locally through both development and cancer [27]. The spatial and temporal expression of TWIST1 and SNAIL in human breast cancer revealed that SNAIL is needed to initiate EMT at the invasive edge of the primary tumor, while TWIST1 generally operates EMT maintenance during later stages of metastatic dissemination and probably in dormant micrometastases. Interestingly, the inverse correlation between TWIST1 and SNAIL expression is observed in breast cancer cell line, MCF10A, during EMT process [28]. In line with these reports and based on our results, we can propose such transient expression of EMT inducing transcription factor in ESCC cell line KYSE-30, where the activation of TWIST1 may leads to the maintenance of the late EMT through down-regulation of SNAIL, providing supports for their roles in ESCC aggressiveness and metastasis.

In conclusion, this study investigated the correlation between the EMT-inducing factors TWIST1 and SNAIL in ESCC KYSE-30 cells. Having confirmed inverse correlation of TWIST1 and SNAIL functionally, we may propose TWIST1 as possibly remarkable EMT maintenance gene in ESCC through suppression of SNAIL to induce malignance and metastasis potentials. To the best of our knowledge, this is the first report mainly describing the functional suppressive role of TWIST1 on SNAIL gene expression in ESCC cell line KYSE-30, and may introduce novel molecular gene expression pathway controlling EMT process during ESCC aggressiveness and tumorigenesis. These evidences extend the spectrum of biological activities of TWIST1 and suggest that therapeutic repression of TWIST1 may be an effective strategy to inhibit cancer cell invasion and metastasis.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interests.

Human and Animal Rights This article does not contain any studies with human participants or animals performed by any of the authors.

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