Biomedicine & Pharmacotherapy 96 (2017) 545–550

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

# An analysis of suppressing migratory effect on human urinary bladder cancer cell line by silencing of snail-1

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ARTICLE INFO

*Keywords:*
Snail-1
Urinary bladder cancer
Epithelial to mesenchymal transition
E-cadherin
Vimentin
MMPs

ABSTRACT

*Background:* Snail-1 actively participates in tumor progression, invasion, and migration. Targeting snail-1 expression can suppress the EMT process in cancer. The aim of this study was to investigate the effect of *snail1* silencing on urinary bladder cancer.

*Methods:* Quantitative RT-PCR was used to detect snail-1 and other related metastatic genes expression following siRNA knockdown in urinary bladder cancer EJ-138 cells. The protein level of snail1 was assessed by Western blot. MTT and TUNEL assays were assessed to understand if snail-1 had survival effects on EJ-138 cells. Scratch wound healing assay measured cell motility effects after snail1 suppression.

*Results:* The significant silencing of snail-1 reached 60 pmol siRNA in a 48-h post-transfection. The result of scratch assay showed that snail-1 silencing significantly decreased Vimentin, MMPs, and *CXCR4* expression; however, expression of *E-cadherin* was induced. The cell death assay indicated that snail-1 played the crucial role in bladder cancer survival rate.

*Conclusion:* These results propose that snail-1 plays a major role in the progression and migration of urinary bladder cancer, and can be a potential therapeutic target for target therapy of invasive urinary bladder cancer.

## 1. Introduction

Urinary bladder cancer is a common cancer in the urinary tract worldwide. Studies have reported that incidence of urinary bladder cancer is approximately 380,000 new cases and 150,000 deaths annually [1]. Surgery, chemotherapy, radiation, and immunotherapy are the strategies for urinary bladder cancer treatment, but the above strategies had relatively low efficiency in patients with invasive or metastasis urinary bladder cancer [2]. Targeted therapy is a novel strategy for invasive cancer treatment blocking or interfering with the specific molecules or mechanisms involved in the growth, progression, and migration of cancer. One of the most common methods used in targeted therapy is gene silencing via RNAi molecules [3]. In this technology, siRNA interacts with mRNA and inhibits the protein translation and its performance, as well as the technology known as posttranscriptional gene silencing. Several genes have been implicated in cancer invasion, including *snail-1*, *HMGA2*, *Bach-1*, *MMPs*, *CXCR4*, *Vimentin* etc [4–7]. Snail-1 is a zinc finger protein belonging to transcription factors family. Snail-1 can regulate epithelial to mesenchymal transition (EMT) by repressing E-cadherin (adhesion molecule) during embryogenesis and cancer.

Numerous studies have been conducted in the field of targeted therapy on cancer invasiveness and metastasis by targeting snail-1. Most of them showed that snail-1 was the one of the most important makers for the treatment of invasive types of cancer. Deep et al. showed that snail-1 was crucial for invasiveness of PC3 prostate cancer cells, and also they suggested that E-cadherin might be controlled by snail-1 [8]. Our pervious study showed that snail-1 might play an essential role in cell viability and EMT development in bladder cancer by regulating miR-21 and miR29b expression [4]. In another study, we demonstrated that *snail-1* silencing via specific snail-1 siRNA could arrest the cell cycle and inhibit cell migration in MDA-MB-468 breast adenocarcinoma cells [9].

According to recent studies concerning snail-1, we found that it could interfere EMT by regulating several genes involved in this process and it might be the major regulator of the invasion and metastasis of cancer. It is not illustrated yet which genes are regulated by snail-1 and the mechanism of this protein in the aggressiveness of urinary bladder

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http://dx.doi.org/10.1016/j.biopha.2017.10.044
Received 15 July 2017; Received in revised form 5 October 2017; Accepted 9 October 2017

cancer is unclear. Additionally, previous studies suggested that targeting of snail-1 might be a strategy for invasive clinical cancer treatment.

In this study, we determined the transcriptional and translational expression level of snail-1 after gene silencing in the urinary bladder cancer (EJ-138) and then analyzed the cellular events related to growth inhibition, apoptosis, and migration. Finally, we evaluated the association of snail-1 as a regulatory protein during the EMT process with the expression of *E-cadherin*, *MMPs*, *Vimentin* and *CXCR4*.

## 2. Materials and methods

### 2.1. Materials and reagents

Cell culture reagents, including RPMI1640, FBS, penicillin, streptomycin, and trypsin–EDTA were purchased from Gibco (Gaithersburg, Md.). Snail-1 siRNA, FITC conjugate negative control siRNA, transfection reagent, transfection media, primary goat antibody, and RIPA protein extraction kit were purchased from Santacruz Biotechnology (California, USA). Secondary horseradish peroxidase-conjugated rabbit anti-goat antibody and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT Powder) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). In addition, SYBR Green real-time master mix was purchased from Ampliqon (Odense M, Denmark). Furthermore, enhanced chemiluminescence Kit, PVDF membrane and In Situ Cell Death Detection Kit were purchased from Roche Diagnostics (Mannheim, Germany). Moreover, cDNA synthesis kit was purchased from Thermo Scientific (WI, USA).

### 2.2. Cell culture

The experimental study was performed on the EJ-138 cell line belonging to the human urinary bladder cancer. EJ-138 cell line was purchased from the cell bank of Pasture Institute (Iran, Tehran), and maintained in RPMI-1640 medium supplemented with 10% FBS, 1% antibiotics (100 unit/ml penicillin and 100 µg/mL streptomycin). Cells were cultured at 37 °C in a 95% humidified atmosphere containing 5% $Co_2$. The cells were passaged every 3 days following detaching with 0.25% trypsin/EDTA and the experiment was performed during the logarithmic phase of cell growth.

### 2.3. Transfection of siRNA

For transfection, we used a Pooled of three different siRNA duplexes sequences targeting Snail-1 mRNA (Table 1). Firstly, we needed to optimize the time and dosage affected by the siRNA in the cell line. Briefly, Ej-139 cells were cultured at a density of $2 \times 10^5$/well of 6-well plates in the condition of serum and antibiotic free medium and transfected at 60–80% confluence with various times (24–72 h.) and concentrations of transfection reagent (4–8 µl) according to the Santa Cruz siRNA transfection instructions. Six hours after transfection, 1 ml of RPMI-1640 medium containing 20% FBS medium was added to each well. Cells were cultured for another 48 h. Snail-1 silencing was measured using qRT-PCR, and western blot in both mRNA and protein levels, respectively. The transfection efficiency was measured by FITC conjugated negative control siRNA (Control siRNA). To insure siRNA delivery into cells and specify of our siRNA against SNAIL1, the cells were imaged by live cell imaging system (citation 5, Biotek).

**Table 1**
Sequences of snail-1 siRNA.

| Snail-1 siRNA | Sense | Antisense |
|---|---|---|
| A | GGACUUUGAUGAAGACCAUtt | AUGGUCUUCAUCAAAGUCCtt |
| B | CACGAGGUGUGACUAACUAtt | UAGUUAGUCACACCUCGUGtt |
| C | GCGAGCUGCAGGACUCUAAtt | UUAGAGUCCUGCAGCUCGCtt |

### 2.4. Quantitative real time PCR (qRT-PCR)

All pair of primers sequences were designed by primer design tool and blasted using the primer-blast software on the NCBI website (http://www.nchi.nlm.nih.govhttp://www.nchi.nlm.nih.gov) prior to the experiment (Table 2).

RiboEx reagent (GeneAll Biotechnology, Seoul, Korea) was used to isolate total RNA from the cells according to the manufacturer's protocol. cDNA was synthesized with 1 µl total RNA (1 µg) using 1 µl random hexamer primer, 1 µl MMLV (Moloney Murine Leukemia Virus), 4 µl 5× reaction buffer and 2 µl of 10 mM dNTP and added DEPC buffer to the final volume of 20 µl. The expression of snail-1 and other genes was performed by qRT-PCR using SYBR master mix and light cycler 96 system (Roche Life Science, Germany). The thermos-cycling conditions were 95 °C for 10 min followed by 40 cycles at 95 °C for 10 s, 59 °C for 30 s and 72 °C for 20 s. Relative snail-1 mRNA expression was calculated with Livak method ($2^{-\Delta\Delta CT}$). The data were normalized with an internal *β-actin* control.

### 2.5. Protein expression analysis by western blotting

In order to isolate total protein, the cell lysate was obtained by using the RIPA protein isolation kit containing lysis buffer, PMSF, protease inhibitor, and phosphatase inhibitor. The lysate was then centrifuged at 13000 rpm for 10 min at 4 °C and Bradford method was used to determine the concentration of protein. Subsequently, 25 µg of protein samples was mixed with protein sample buffer (10% Glycerol, 50 Mm Tris pH 8.6, 2% SDS, 1% Bromophenol blue, and 100 mM DTT), after 5 min incubation in 95 °C dry bath, then the proteins were separated by 12.5% SDS-PAGE and blotted to the methanol activated PVDF membrane. The membrane was blocked with blocking buffer (0.5% Tween 20) overnight at 4 °C. The membrane was incubated with primary goat polyclonal antibody against snail-1 (in a dilution of 1:1000) and mouse monoclonal antibody against beta-actin (in a dilution of 1:3000) as an internal control for 1 h. The anti-goat antibody conjugated with horseradish peroxidase was used as a secondary antibody (in a dilution of 1:5000) for 1 h. The signal was detected by ECL kit, and recorded by western blotting system (Sabz Company, Iran). Density of bands was evaluated by ImageJ software (National Institutes of Health, Bethesda, Maryland, USA), and signals of the sample bands were normalized to β-actin signals.

### 2.6. Cell viability assay

Cytotoxicity of siRNA and involved reagents was assessed using the MTT assay. Briefly, the cells were plated at a density of $15 \times 10^3$ cells/well in 96-well culture plates. After the cell treatment, 50 µl of MTT (2 mg/mL) was added to each well and then incubated for further 4 h. The formazan crystals were solved by adding 200 ml of DMSO + Sorensen buffer to each well. After 30-min incubation, absorbance of the solubilized formazan was measured at a wavelength of 570 nm using a microplate reader (Tecan, Austria) and all experiments were performed in triplicate.

### 2.7. Apoptosis detection assay (TUNEL)

To determine whether apoptosis was induced after *snail-1* silences or not, DNA strand breaks were performed by the terminal deoxynucleotidyl transferase nick end labeling (TUNEL) assay. Ej-138 cells were cultured at a density of $15 \times 10^3$ cell/well in 96-well cell culture plates and then transfected with optimal specific siRNAs concentration (60 pmol). After incubation in an optimal time for *snail-1* silencing, the cells were fixed with 4% paraformaldehyde (pH: 7.4). Then, the wells were incubated with blocking solution (3% H2O2 in methanol) after the