RESEARCH COMMUNICATION

Overexpression of Snail Accelerates Adriamycin Induction of Multidrug Resistance in Breast Cancer Cells

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Abstract

In addition to several molecular and morphologic changes, epithelial-mesenchymal transition (EMT) cells also show variation in sensitivity to chemotherapeutics agents. The aim of this study was to investigate whether overexpression of Snail in MCF-7 cells is associated with facilitated acquisition of P-gp mediated multidrug resistance (MDR). The results demonstrated that over-expression of Snail indeed resulted in slight enhancement of adriamycin-induced MDR in MCF-7/Snail cells without detectable increase of P-gp. However, in the longer term, MCF-7 cells overexpressing Snail were prone to be resistant to adriamycin, in this case with increased expression of P-gp. These results provide evidence that a strategy involving Snail inhibition may be a useful and promising therapeutic aspect in modulating MDR.

Key words: Breast cancer - Snail - multidrug resistance - P-gp - EMT cells

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Introduction

The epithelial-mesenchymal transition (EMT), characterized by long-lasting morphological and molecular changes in epithelial cells, refers to the trans-differentiation of epithelial cells to a more mesenchymal stateas (Hirohashi and Kanai, 2003; Lee et al., 2006; Spaderna et al., 2007). Snail belongs to a family of zinc finger-containing transcriptional repressors, and increasing evidence has indicated that Snail acts as a key regulator of EMT (Batlle et al., 2000; Cano et al., 2000; Carver et al., 2001). Snail triggers full EMT associated with the acquisition of invasive and tumorigenic properties (Marin et al., 2004; Peinado et al., 2004).

According to recent discoveries, EMT state malignant cells showed the close relationship with multigrug resistance (MDR). Epithelial tumor cells have been shown to be more sensitive to EGFR inhibitors than tumor cells, which have undergone an EMT-like transition and acquired mesenchymal characteristics (Frederick et al., 2007). Adriamycin- treated cells undergoing EMT displayed enhanced invasiveness and MDR. Depletion of Twist1, a member of the Snail family, could completely block the mesenchymal transformation, and partially reverse MDR (Li et al., 2009).

To observe whether over-expression of Snail induced EMT of breast cancer MCF-7 cells responded differentially to Adriamycin treatment, and elucidate the role of P-gp, the most important member of the protein superfamily of ABC transporters associated with MDR, in this study Snail gene was cloned and transfected into MCF-7 cells, and the expression of P-gp and the multidrug resistance index to adriamycin were detected after having been cultured with adriamycin.

Materials and Methods

Patients and paraffin-embedded tissue samples

A series of 48 infiltrating ductal carcinomas were analyzed (Weifang, China), obtained from patients with primary breast cancer having undergone surgery at the Affiliated Hospital of Weifang Medical University and collected at the institute of pathology. All the patients were female, and the average age was 38±8 years. Among them, 32 patients had small primary tumors (T1-2); extensive local disease was found in 16 (T3-4). About half were node-negative; in the remainder, metastasis was already present (N1-2). Obtained tissues were fixed in 4% formalin and embedded in paraffin.

Immunohistochemistry

Immunohistochemical staining was done on paraffin-embedded tissues. 5 μm thick sections of routine formalin-fixed and paraffin-embedded materials

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were analyzed. After microwave-based antigen retrieval with citric acid pretreatment, sections were incubated in 1% hydrogen peroxide for 10 min. Then the specimens were incubated with primary monoclonal antibodies for Snail and P-gp at room temperature for 1 h. Bound antibodies were detected using the avidin-biotin-complex (ABC) peroxidase method. Final staining was developed with DAB peroxidase, and hematoxylin was used for counterstaining. Non-tumorous epithelial cells served as positive control for E-cadherin staining while those in which the first antibody was omitted were used as negative control.

Immunohistochemical staining of Snail, P- gp was defined as detectable immunoreaction in perinuclear and membrane, and was semiquantitatively estimated from 0 to 3: 0, corresponding to 0% positive cancer cells; 1, 1% to 25% positive cancer cells; 2, 26% to 50% positive cancer cells; and 3, >51% staining of cancer cells. Membranous P-gp expressions in the tumor sections were graded according to the proportions of positive cells and were classified into four groups: 0, <10% of the cancer cells stained or with complete absence of staining; 1, 10% to 49% positive expression; 2, 50% to 70% positive expression; and 3, >70% positive expression. Cancer cells that were immunostained at 0 or 1 were defined as having a reduced E-cadherin expression.

Cloning of Snail and construction of MCF-7/Snail

The total RNA from human white blood cells was extracted, and the sample was reversely transcripted into cDNA. 1μg cDNA worked as sample to amplify the whole length of Snail (GenBank accession number http://www.ncbi.nlm.nih.gov/mapview/maps.cgi) by PCR using pfu DNA polymerase. And the primers were as follows: forward primer, 5'-CCACTATGCCGCGCTCTTT-3'; reverse primer, 5'-TCAGCGGGGACATCCTGAGCA-3' (Sangon,China). After linker having been added, the products were ligated to T clone vector to generate pUCm-T-Snail constructs. The Snail gene were cut by Kpn = 1 * ROMAN I/Xba = 1 * ROMAN I from pUCm-T-Snail and ligated to pcDNA3.1 to construct pcDNA3.1-Snail. The expression vector pcDNA3.1-Snail was identified through digestion with Kpn = 1 * ROMAN I/Xba = 1 * ROMAN I, further identification was performed by sequencing.

Cell culture and transfection

MCF-7 cells were cultured in RPMI-1640 (HyClone) with 10% FBS (Gibico), penicillin, and streptomycin. The cells were incubated at 37°C in humidified air with 5% CO_2. The medium was replaced thrice weekly, and cells were maintained by serial passage after trypsinization with 0.1% trypsin. For the current experiment, MCF-7/pcDNA, MCF-7/Snail were induced by 50 μg/ml adriamycin for three months, and named MCF-7/pcDNA/A, MCF-7/Snail/A correspondingly.

Transfection of pcDNA3.1-Snail was carried out using LipofectamineTM2000 (Invitrogen) when the cells reached 80% cell fusion. 250 μl of DMEM medium without serum and control pcDNA3.1 or pcDNA3.1-Snail 4 μg per well were preincubated for 5-10 minutes at room temperature. 250μl of DMEM medium without serum was mixed with 10μl LipofectamineTM2000. And then, the mixtures were mixed and incubated for 20 minutes at room temperature for complex formation. After that, the cells were transfected according to manufacturer's protocol. The cells were then selected by G418 gradually for a month to establish MCF-7/pcDNA and MCF-7/Snail. Successfully transfected cells were selected with in culture medium 400 μg/ml G418 (Invitrogen) for 30 days and cultured at least 90 days, mixture of cells was used as stable cell.

Immunofluorescence and immunocytochemistry

Cells were seeded onto round gelatin-coated glass coverslips placed on 24-well plates, fixed with ice cold methanol for 10 min, permeabilized with 0.2% Triton for 3 min and blocked in 10% goat serum for 1 h at room temperature. E-cadherin and vimentin (Maxim, China) were probed with first antibody for 1 h at room temperature followed by detection with a rhodamine-conjugated goat anti-mouse secondary antibody for 1h at room temperature. Hoechst dye (1 μg/ml) was subsequently used to stain nuclei. Immunocytochemistry was used to detect the expressions of Snail and P-gp. The cells were incubated with primary monoclonal antibodies for Snail and P-gp at room temperature for 1 hour. Bound antibodies were detected using the avidin-biotin-complex (ABC) peroxidase method.

Cytotoxicity assay

For each assay, 1×104 cells in 100 μl were seeded in 96-well plates and allowed to attach for 24 h at 37°C in 5% CO2. The next day, culturing medium was removed and replaced with 100 μl of fresh complete culturing medium containing adriamycin at various concentrations (0.01, 0.1, 1, 10, 100 μg/ml), and such incubation was allowed to last for 72h. Control wells were replaced with fresh cell medium only. After incubation, 50μl of 1 mg/ml MTT was added to each well and the plates were incubated for 4h, medium was then removed from each well and replaced with 200 μl DMSO to dissolve the purple crystals. Absorbance was measured by a photometric microplate reader at 540 nm within 1 h. Results were recorded as mean absorbance ± standard deviation for each set of triplicates. To determine the IC50 values, the absorbance difference of control cells without drug was set at 1. A dose-response curve was plotted, and IC50 values were calculated based on multiple (at least three) independent experiments for each cell line.

Real-time PCR

To observe whether the expression change of P-gp

in MCF-7/pcDNA and MCF-7/Snail with or without treatment of adriamycin is responsible for drug resistance, Quantitative RT-PCR used to observe the change of P-gp mRNA.

The mRNA levels of P-gp were measured by real-time RT-PCR. The mRNA levels of internal control gene and PBGD were measured and used to normalize the mRNA levels of the drug resistance genes. The forward and reverse primers for P-gp were 5'-GCCCTTGGAATTATTTCTTT-3' and 5'-TGGGTGAAGGAAAA TGTAAT-3'. The forward and reverse primers for PBGD were 5'-ACGATCCCGAGACTCTGCTTC-3' and 5'-GCACGGCTACTGGCACA CT-3'.

The constituents of each PCR (25 μl) were 5 μl of template RNA(200 ng/reaction) or dH2O, 2.5 μl MgCl2 (6 mM), 2× (forward and reverse) 1 μl of primer, 12.5 μl of 2×QuantiTect SYBR Green PCR, and 3 μl of dH2O. To compare the expression levels among different tumor samples, the relative expression levels of the resistance genes were calculated using the comparative CT method and compared with a calibrator.

Western blot assays

Cells were cultured in growth medium on 35-mm tissue-culturing plates to approximately 80 % confluence. After being briefly rinsed with PBS, the cells were lysed in ice cold lytic buffer containing 1% NP-40, 50 mmol/L Tris, 150 mmol/L NaCl, 0.1% SDS, 0.5% deoxycholate, 200 μg/ml PMSF and 50 μg/ml aprotinin. The cell lysates were for 5 min at 100°C. After SDS-PAGE, the proteins were transferred onto nitrocellulose membranes, which were blocked with 5% nonfat dry milk in TBS for 2 h and then incubated with the primary antibodies, afterwards, with horseradish peroxidase-conjugated rabbit antimouse IgG. As control for equivalent protein loading, filters were simultaneously incubated with a mouse MAb directed against GAPDH (glyceraldehyde-3-phosphatedehydrogenase). The protein-antibody complexes were visualized by ECL chemoluminescence.

Statistical analysis

Analysis of variance was conducted, followed by independent-samples T test. The $\chi 2$ test was used to compare immunohistochemical data. P value less than 0.05 was considered statistically significant.

Results

Expression of Snail and P-gp in human breast cancer

Snail in primary breast cancer tissues was identified in the cytoplasm as well as in the nucleus of cancer cells (Figure 1 A, C). P-gp expression in cancer cells was characterized by patterns with variable degrees of membrane staining (Figure 1B, D). Immunohistochemistry of 48 breast cancer tissues showed a moderate to strong Snail expression in 34 cases (71%), mainly in the invasive tumor front. More patients with high snail expression displayed high P-gp expression (71%), whereas high P-gp expression was found in 83% of specimens with high snail expression. This was statistically significant (P = 0.025, $\chi 2$ test).

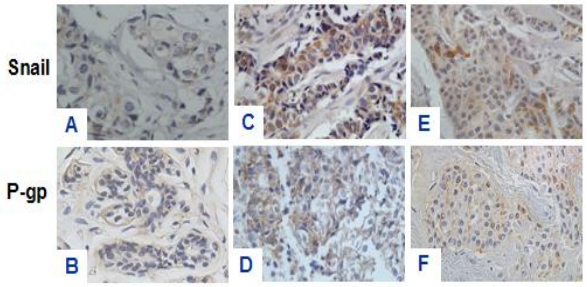

Figure 1. Immunohistochemical Findings. Staining of Snail was found in the perinucleus as well as in the nucleus of tumor cells (C, F). P-gp expression was identified in the cell membrane and less intensive in the cytoplasm (D, F). The expression of Snail and P-gp expressed higher than benign lesions (A, B) Tissue with high expression of Snail always accompanied with high expression of P-gp (C, D)

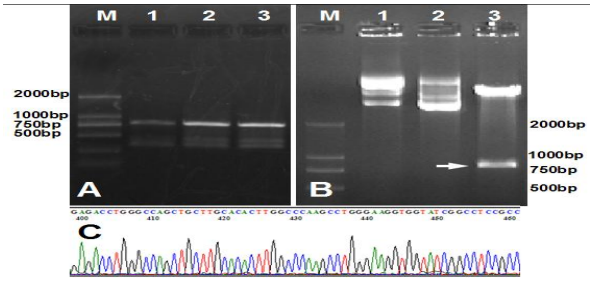

Figure 2. pCDNA-Snail. (A) Identification of constructed pCDNA-Snail plasmid. Amplified the whole length of Snail gene by RT-PCR, and the fragment is about 800bp. Lane 1, 2 and 3 represent the product of RT-PCR, M for maker. B. Identification of pCDNA-Snail by Hind III/Bgl II digestion. Lane 1, 2 represent the clones, lane 3 represent the positive clones, M for marker. (C) Part sequencing diagram

RT-PCR and pcDNA3.1-Snail

The RT-PCR amplified products were identified by electrophoresis, with the whole length of 799 bp (Figure 2A). After being ligated to pCDNA3.1 to generate pCDNA-Snail which was indentified by electrophoresis after being digested by Hind III/Bgl II, positive clone had a about 800bp segment (Figure 2B). And further identification was performed by sequencing (Figure 2 C).

Downregulation of E-cadherin and up-regulation of vimentin of MCF-7/Snail

EMT process involves losing epithelial phenotype and obtaining some specific characteristics of mesenchymal cells. As observed, the expression of E-cadherin was down-regulated with reduced membrane localization in the MCF-7/Snail cells, while that of vimentin was up-regulated, whereas vimentin expression was markedly short in the MCF-7 cells. These hallmark shifts at molecular levels indicated an EMT transformation of

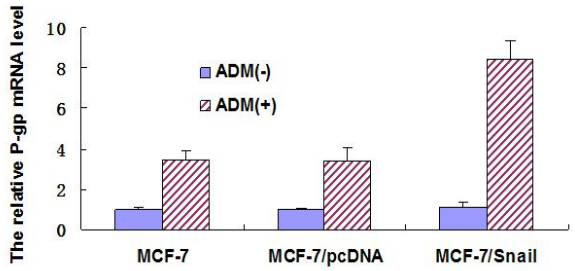

Figure 3. Expression Changes of E-cadherin and Vmentin of MCF-7 Cells after Transfection with Snail gene with or without Adriamycin

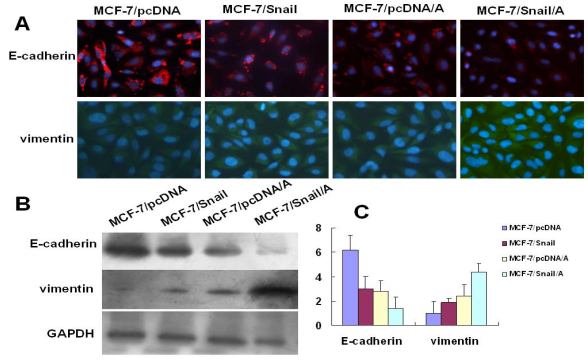

Figure 4. P-gp mRNA Examined by Real time RT-PCR. GQuantification and statistical analyses were done for three independent experiments. MCF-7/Snail cells treated with adriamycin expressed more P-gp mRNA than MCF-7/pcDNA cells treated with adriamycin did (P<0.05)

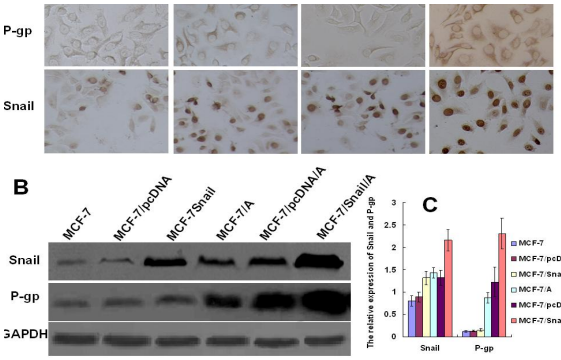

Figure 5. Expression Changes of P-gp and Snail Protein by Immunochemistry and Western Blotting. Over-expression of Snail could lead to the increase of multidrug resistance index, but did not result in obvious increased expression of P-gp directly. However, when the MCF-7/pcDNA and MCF-7/Snail cells were treated with adriamycin, MCF-7/Snail cells were more resistant to adriamycin and expressed more P-gp protein than MCF-7/pcDNA did (P<0.01). A. P-gp and Snail protein were examined by Immunochemistry. B. P-gp and Snail protein were examined by western blotting. C. Analyses of P-gp and

MCF-7/Snail cells (Figure 3). Flow cytometry analysis was performed eliminate the influence of apoptosis on the expression of E-cadherin and vimentin. Flow cytometry analysis for the expression of E-cadherin and vimentin showed that MCF-7/Snail/A cells had an average of 43 ± 3.7% (mean ± SE, n = 6, P <0.001) lower expression of E-cadherin and 64 ± 6.8% higher expression of vimentin (mean ± SE, n = 5, P < 0.001) compared with control cells.

Table 1. The IC_{50} Value to Adriamycin, RR for Resistance Index, Ratio of IC_{50}

Cell lines	IC_{50}(μg/ml)	RR
MCF-7	1.96±0.22	1
MCF-7/ pCDNA	1.88±0.231	0.96
MCF-7/Snail	4.74±0.63	4.42
MCF-7/A	24.05±3.98	12.26
MCF-7/ pCDNA/A	21.13±4.02	10.78
MCF-7/Snail/A	45.61±6.85	23.27*

*P < 0.05 versus MCF-7/ pCDNA/A group; †P < 0.01 versus MCF-7/ pCDNA/A group; #P < 0.01 versus MCF-7/Snail/A group

Expressions of MCF-7/Snail induced by adriamycin

P-gp mRNA expression was estimated by real-time RT-PCR. The P-gp mRNA relative levels of MCF-7/Snail and MCF-7/Snail/A were 1.02 and 8.21, respectively. After being induced by 50μg/ml adriamycin for three months, the P-gp mRNA expression of MCF-7/Snail/A cells was increased compared to those of cells without adriamycin induction (Figure 4). Western blot demonstrated that cells were induced by 50μg/ml adriamycin for three month, the the P-gp protein expression of MCF-7/Snail/A was also increased compared to MCF-7/Snail cells without induction of adriamycin. Over-expression of Snail could lead to the increase of multidrug resistance index, but did not result in obviously increased expression of P-gp directly. However, when the MCF-7/pcDNA and MCF-7/Snail cells were treated with adriamycin, MCF-7/Snail cells were more resistant to adriamycin and expressed more P-gp protein than MCF-7/pcDNA did (P<0.01) (Figure 5).

Drug resistance phenotype after overexpression of Snail

Cytotoxicity experiment was performed to detect whether overexpression of Snail in MCF-7/Snail could enhance the multidrug-resistant phenotype. Multidrug-resistant phenotype was assessed by comparison with IC50 values determined by cell proliferation assay. The degree of adriamycin resistance of parent MCF-7 was regarded as 1, the adriamycin resistance index of MCF-7/Snail increased obviously to 4.42, and the adriamycin resistance index of MCF-7/Snail/A greatly increased to 23.27 compared with 10.78 of MCF-7/pCDNA/A (P<0.01). The various degrees of adriamycin resistance are summarized in Table 1.

Discussion

The epithelial-mesenchymal transition (EMT) is a process by which cells of epithelial origin lose cell-cell adhesion and polarity, and acquire a mesenchymal phenotype, with increased cell migratory behavior, enhanced metastasis and recurrence (Moody et al., 2005; Gupta and Massagué, 2006; Moustakas and Heldin,

2007; Mani et al., 2008; Sarrió et al., 2008; Kalluri and Weinberg, 2009). EMT is often associated with a poor prognosis in women with breast cancer (Fuchs et al., 2002; Kokkinos et al., 2007; Li et al., 2009). Snail has a central role in morphogenesis (Carver et al., 2001). Expression of Snail represses expression of E-cadherin and induces EMT breast cancer cells (Batlle et al., 2000; Zhang et al., 2007), indicating that Snail plays a fundamental role in EMT, and over expression of Snail increases the risks for recurrence and for poor survival in some carcinoma patients (Cho et al., 2007; Hosono et al., 2007; Leroy and Mostov, 2007). Results of the experiment showed that overexpression of Snail in MCF-7 could induce transcriptional downregulation of E-cadherin, upregulating vimentin and resulting in EMT-like phenotype.

EMT is not only involved in morphologic change, but also can lead to carcinoma cell insensitivity to chemotherapeutics. Gain of N-cadherin expression is a typical example of the EMT signatures and it has been linked with drug resistance (Zhang et al., 2007). Epithelial tumor cells have been shown to be significantly more sensitive to EGFR inhibitors than tumor cells which have undergone an EMT-like transition and acquired mesenchymal characteristics (Grandis and Sok, 2004; Fuchs et al., 2008). These observations were later extended to other tumor types and EGFR antagonists (Buck et al., 2007; Shrader et al., 2007; Barr et al., 2008; Haddad et al., 2009). It has been shown that most basal-like breast cancers in EMT condition show unfavorable prognosis and resistance to chemotherapy (Azizun-Nisa et al., 2008; Brouckaert et al., 2009). And on the other way round, the resistance to some drugs can also lead to EMT of malignant cells (Peinado et al., 2004; Kokkinos et al., 2007).

P-gp is the most common member ABC transporters which are associated with MDR of human cancers. Although many previous experiments had demonstrated that EMT of malignant cells lead to insensitivity to chemotherapeutics, but seldom attentions were paid to malignant cells in EMT resisting to chemotherapeutic drugs through P-gp mediated MDR. In order to detect whether Snail could result in P-gp mediated MDR, Snail gene was cloned and transfected into MCF-7 cells, Cell viability in our study was measured via the colorimetric MTT method assessing the cell's mitochondrial activity after being transfected with Snail. Our results showed that the multidrug resistance of MCF-7/Snail to adriamycin increased slightly compared with MCF-7/pcDNA cells; nevertheless, there had no increased expression of both P-gp mRNA and protein correspondingly. So the increased resistance to adriamycin might not results from high expression of P-gp but other mechanisms. We previously detected the expression of Glut-1 by western blot and immunofluorescence, a transporter located on the membrane of tumor cells. When the MCF-7 cell was transfected with Snail, the expression of Glut-1 was increased. The over-expressed Glut-1 may involve in slightly increased resistance to adriamycin of MCF-7/Snail (Bentley et al., 1997).

However, with longer periods of exposure to adriamycin, the resistant drug indexes of MCF-7/pcDNA/A and MCF-7/Snail/A to adriamycin were 12.26 and 23.27, respectively. Adriamycin efflux assay was performed, after an accumulation and efflux period, and adriamycin fluorescence was quantitated by flow cytometric analysis. Compared with MCF-7/pcDNA/A group, the fluorescence intensity of adriamycin in MCF-7/Snail/A cells increased markedly. Beyond that, both expression of P-gp mRNA and protein were also increased obviously. The results indicated that breast cancer cells with over-expression of Snail were more likely to be resistant to adriamycin through the expressions of P-gp at both mRNA and protein levels. Clinically, breast cancer cells in EMT condition might be easier to produce multidrug resistance.

All of these findings suggest that overexpression of Snail resulting in EMT enhances MDR slightly in MCF-7/Snail cells without increase of P-gp; however, the cells having undergone EMT due to overexpression of Snail are prone to be resistant to adriamycin through the increased expression of P-gp.

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