

Down-regulated P53 by siRNA increases Smad4's activity in promoting cell apoptosis in MCF-7 cells

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Abstract. – **BACKGROUND:** Wildtype p53 and Smad4 are important suppressor genes in tumor genesis and development. The mutation in p53 gene is closely related to the early formation of breast cancer. In this research, we try to explore the status of Smad4 in breast cancer cell line MCF7 after knocking down p53.

MATERIALS AND METHODS: MCF-7 was bought from American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) in incubator containing 5% CO₂ at 37°C. In this study we apply to analytical methods such as growth ratio analysis *in vitro*, immunoblot analysis, flow cytometry for cell cycle analysis, RNA extraction and Real-Time PCR and data analysis.

RESULTS: The results indicated that specific down regulating of p53 can significantly increase the expression of Smad4 in cancer cell line MCF7 and promote cell apoptosis. In MCF7 cell line p53 gene is negatively related to Smad4 in great extent.

CONCLUSIONS: In our study, we detect the expression and function of Smad4 by suppressing p53 expression in MCF7, trying to figure out the relationship and signaling pathway between them. In this study, we try to found the affection of p53 on Smad4, so as to induce apoptosis in tumor cell, achieving the goal of targeted cancer prevention and treatment.

Key Words:

Wildtype p53, Breast cancer, Smad4, Cell apoptosis.

Introduction

Breast cancer is a genetic disease caused by multiple genes, including oncogenes and tumor suppressor genes. The occurrence of breast cancer is a process of multiple-factors and multiple-

steps, which is produced by variety of heredity and variation, resulted from the accumulation of many abnormal oncogenes and tumor suppressor genes. The activation of oncogenes and inactivation of tumor suppressor genes lead to host metabolic changes, cell signal transduction abnormalities and induced carcinomas, which is the leading cause of tumor occurrence^{1,2}. According to the studies of different indicators related to breast cancer, p53 oncogene protein is a better indicator of primary breast cancer³. Mutation in p53 gene is most common in human tumors, and p53 is also found so far to be the gene most closely related to human tumors. Linzer and Lane^{4,5} found p53 gene nearly at the same time in 1979. Besides, the mutation and expression of p53 oncogene in the early stage of breast cancer make it become to be one of the evaluations in judging the malignant tendency of precancerous lesions of breast cancer. As a member of sequence-conservative Smad family, Smad4 is mostly involved in Transforming Growth Factor β (TGF- β) superfamily's intracellular signaling transduction. TGF- β superfamily can inhibit the proliferation of epithelial cell. Smad4, being a potential tumor suppressor, play an essential role in TGF- β superfamily's signaling pathway. So it has a significant impact on tumor occurrence, development and metastasis.

Existing researches indicated that breast cancer is closely associated with genetic abnormality of p53, Smad4, etc. Besides, studies have also proved that low expression of Smad4 gene is significantly related to the high expression of p53 protein⁶. In this research we detected the migration and apoptosis in breast cancer cell line MCF7 after knocking down mutant p53 by RNAi. We also check down-regulated p53's impact on the expression of Smad4.

Materials and Methods

Breast Cancer Cell Line

MCF-7 was bought from American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) in incubator containing 5% CO₂ at 37°C. Transfection with silencing RNA (siRNA): 5*10⁴ cells were placed in 10 cm dish at the first day and change the medium into serum-free DMEM the next day when the cell confluence reached 70%-80%, incubating 30 min. Fluorescein-conjugated control siRNA was used to detect transfection efficiency. 18 µl siRNA stock solution of 10 µM and 10 µl Lipofectamine 2000 were diluted in 200 µl serum-free DMEM separately in expanded polyethylene (EP) tubes. After statically placed at room temperature, they were mixed together and placed statically for 20-30 min. The mixture was then gradually dripped into dishes containing 5 ml medium. Change the medium into DMEM with 10% FBS after culturing in the incubator for 4-6 hours.

Growth Ratio Analysis in vitro

Cells were plated in 96-well plate at the confluence of 2×10³ cells per well. Culture in incubator of 37°C, add 50 µl (1 mg/ml) MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) into the medium 24h, 48h, 72h and 96h after and incubate for 4 hours. Each treatment was repeated three times. Blue crystal was dissolved in 100 µl dimethylsulfoxide (DMSO) and vibrated at room temperature for 10 min. Absorbance were measured at 570 nm using microplate reader with 630 nm as a reference.

Flowcytometry For cell Cycle Analysis

Cells were placed in 10 cm dish at the confluence of 10⁶ and were used for flowcytometry analysis 24 hours after transfection. Wash cells with phosphate buffered saline (PBS) twice and treat them with RNAase (200 µg/ml) at 37°C for 30 min. Then add 50 µg/ml propidium iodide and treat on ice for 30 min in darkness. The value of fluorescence was measured at 585 nm using Flow Cytometer^a (BD FCS Calibur flow cytometer. St Josè, CA, USA). Each treatment was repeated three times.

Immunoblot Analysis

Cells were washed by pre-cooling PBS twice and collected. Cold lysis buffer (20 mM HEPES

[4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid] pH 7.4, 1% NP-40, 2 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonide fluoride (PMSF), 1 mM tosylphenylalanylchloromethyl ketone (TPCK), 2 mM Na₃VO₄, and proteasome inhibitor) were added into cells at the concentration of 100 µl per 10⁶ cells. The total amount of protein was measured and sodium dodecyl sulphate (SDS) loading buffer was added to protein for 10 min at 95°C. Proteins were separated on 10% SDS-PAGE gels (polyacrylamide gels) with 50 µg protein loaded on each lane. Size separated proteins were then transferred onto polyvinylidene fluoride (PVDF) membrane of 0.22 µm. Membranes were probed with corresponding primary antibody and horse radish peroxidase (HRP)-conjugated second antibody for 1 hour respectively. Enhanced chemiluminescence (ECL) was added onto the membrane and films were developed in dark room.

RNA Extraction and Real-Time PCR

Total cellular RNA, from treated and untreated cells, was extracted using TRIzol™ reagent (Invitrogen, Rockville, MD, USA), according to the manufacturer's instructions. Five micrograms of total RNA and oligo (dT) 12-18 primer or random hexamers was taken in diethyl pyrocarbonate (DEPC)-treated water. cDNA synthesis was initiated using 200 units of Moloney-murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen, Rockville, MD, USA), under conditions recommended by manufacturer and the reaction was allowed to proceed at 37°C for 50 min. Reaction was terminated by heating at 70°C for 15 min. Each real time-polymerase chain reaction (RT-PCR) contained 10% of cDNA, 20 pM of each primer in 20 mM Tris-HCl (pH 8.4) containing 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTP (deossi-nucleotide-tri-phosphate) mix, and 1 unit of platinum Taq DNA polymerase (Invitrogen, Rockville, MD, USA) in a final volume of 20 µl. After an initial denaturation for 2 min at 95°C, 30 cycles of denaturation (94°C for 1 min), annealing (for 1 min), and extension (72°C for 1 min) were performed on a DNA thermal cycler (Techne, Cambridge, UK) with a final extension for 10 min at 72°C. The primer pairs used were as follows: p53 5'-CTGAGGTTGGCTCTGACT-G T A C C A C C A T C C - 3' (F) 5' - CTCATT CAGCTCTCGGAACATCTGGAAGCG-3' (R), and β-actin, 5'-ATCTGGCACCACAC-CTTCTACAATGAGCTGCG-3' (F) 5' -

CGTCATACTCCCTGCTTGCTGATCCACATCTGC-3'(R). The melting temperature of P53, Smad4 and β -actin are 55°C, 58°C and 56°C.

Statistical Analysis

Data are expressed as the mean of three independent results. Statistical comparisons are made using Student's *t* test and *p* value < 0.05 was considered as significant.

Results

Inhibition on p53's Expression by Different Concentration of p53 siRNA

MCF7 cells were transfected with p53 siRNA of different final concentration (0 μ M, 0.03 μ M, 0.06 μ M, 0.12 μ M, 0.24 μ M, 0.48 μ M, 1.0 μ M, 2.0 μ M, 10 μ M). Detect mRNA level of p53 quantitatively 24 hours after transfection using Real-Time PCR and calculated that p53 siRNA IC₅₀ = 0.21 μ M (Figure 1).

48 hours after siRNA transfection, cells were collected for Western Blot (WB) to check the expression level of p53 and Smad4. Results indicated that the expression of p53 was significantly depressed with the increasing concentration of siRNA. When the concentration of siRNA reached 0.4 μ M, p53's expression cannot be detected anymore. And protein level of Smad4 significantly increased (Figure 2).

The Impact of Different Transfection Time of p53 siRNA on p53 and Smad4's Expression

12, 24 and 48 hours after 0.1 μ M p53 siRNA transfection, cells were collected respectively for WB to check the expression level of p53 and

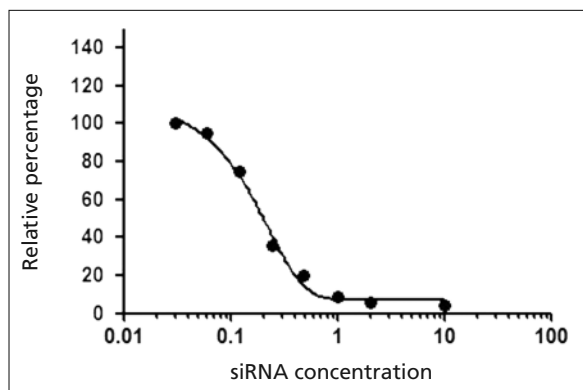


Figure 1. Inhibition on p53 mRNA by different concentration of miRNA. Impact of decreased p53 on Smad4.

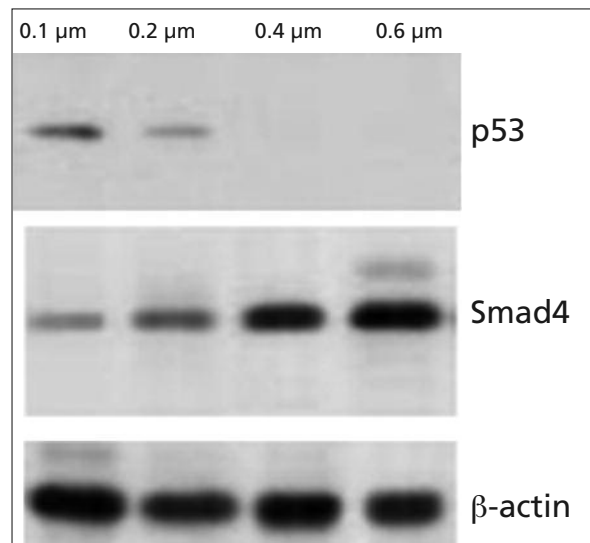


Figure 2. The impact of different concentration siRNA on p53 and Smad4's expression.

Smad4. Results indicated that the expression of p53 was significantly depressed with the increasing time of transfection. 48 hours after transfection, p53 protein level decreased to 1/10 of that in control group. And protein level of Smad4 significantly increased (Figure 3).

The Impact of p53 siRNA Transfection on cell Growth and Apoptosis

After p53 siRNA transfection, the growth ratio of MCF-7 cell was 50% lower than that of normal cell (Figure 4). The ratio of cell apoptosis was accelerated as well, with apoptotic ratio

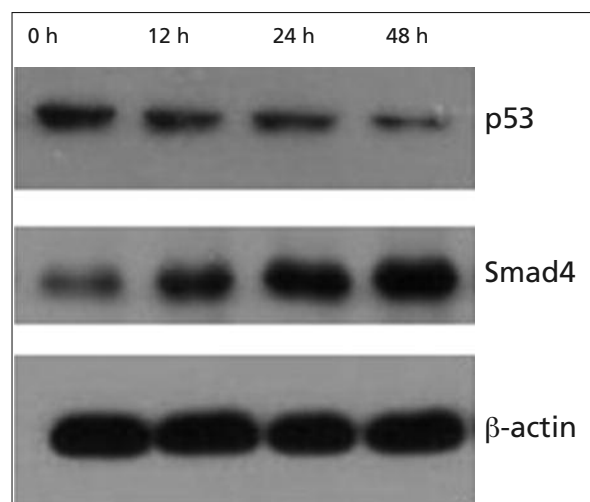


Figure 3. The impact of different transfection time of p53 siRNA on p53 and Smad4's expression.

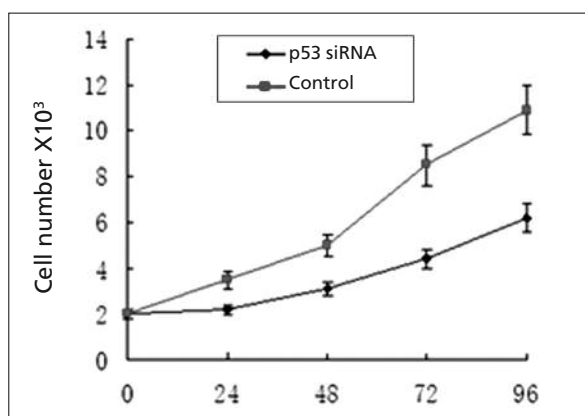


Figure 4. The impact of siRNA transfection on cell growth.

reaching to 58% 3 days after transfection, significantly higher than that of normal cell (Figure 5).

The Impact of p53 siRNA Transfection on cell Cycle

Cell cycle was detected by Flow Cytometer and results showed that siRNA interference reduced the ratio of G1 phase while increased the ratio of S and G2 phase in cell cycle. In overall, cell cycle became shorter and cell apoptosis was promoted (Figure 6).

Discussion

Specific tumor cell line is important research resource in the study of tumor cell biology. There are no sufficient researches on the occurrence and development of breast cancer cell and new developed strategies for treatment, which have greatly

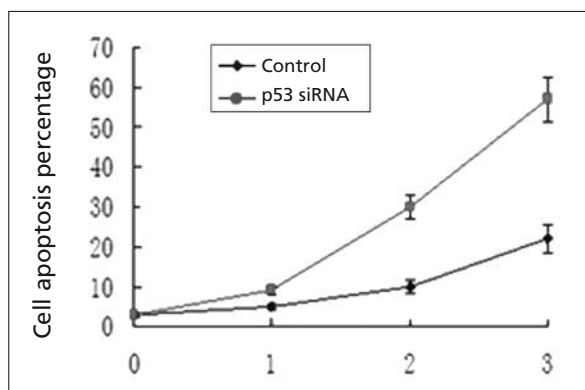


Figure 5. The impact of siRNA transfection on cell apoptosis.

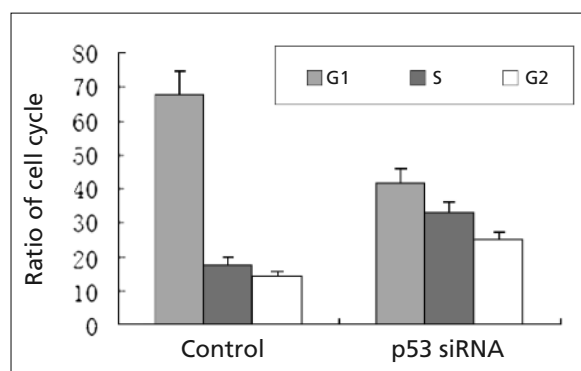


Figure 6. The impact of p53 siRNA transfection on cell cycle.

hindered the progress of breast cancer research⁶. MCF-7 is a good breast cancer model with wild-type p53 expression. However, p53 in different stages of breast cancer cell has not been fully reported⁷. Besides, different cell line, experimental procedure, growth status and genetic background will lead to different results. Compared with other genetically similar cell lines, MCF-7 is different in p53's expression, which is very important in the research of p53's function⁸⁻¹⁰. Since discovered, Smad4 gene has always been a hotspot in research. Smad4 regulates cell cycle and many signaling pathway, affects invasion and metastasis of cancer cell. It also plays important role in cell growth, development, apoptosis and many other biological process. However, the mutation of Smad4 gene doesn't happen in MCF-7, while its mRNA level decreased, indicating that there may be some regulation mechanisms in the level of translation and post-translation. So further studies of these genes' mutation, especially the relationship between their mRNA/protein level and tumor genesis/prognosis, will help us understand the mechanism of tumor occurrence more deeply, and provide new evidence for tumor diagnosis, gene therapy and prognostic judgment. Researches on the p53 and Smad4's function in tumor can help to understand the mechanism of cell signal transduction and open up a new way for gene therapy¹¹⁻¹⁵.

Some researches¹⁶⁻¹⁸ have proved that in malignant tumor of mammary gland which is a classic target organ regulated by estrogen p53 have higher positive reaction rate. And the rate is unrelated to tumor size, pathological category, histological differentiation and lymph node metastasis but related to Estrogen Receptor (ER). These researched have found that there is close relation

between p53 mutation and ER. Nowadays it is generally recognized that the lower lifespan of ER negative patients is an indicator for prognosis of breast cancer patients, which indicates that mutant p53 lose the supersession of malignant cellular phenotype leading to worse biologically malignant behavior of breast cancer cell. In breast cancer, gene expression of p53 is negatively related to ER status.

Cell homeostasis is maintained by the interaction between innumerable growth regulatory factors (such as peptide and lipophilic hormone), among which the genetic-level interaction of Smad4 and estrogen receptor signaling pathway plays an important role in maintaining the balance of reproductive system. Although the exact molecular mechanisms have not been elucidated, more and more studies have shown that Smad4 can modulate the signal transduction of ER which regulates the expression of growth factors. Estrogen is very important in many physiological processes. Many experiments have confirmed that many estrogenic physiological and pathological effects are mediated by ER. ER is group of proteins that can be phosphorylated and their biological functions are regulated by their status of phosphorylation. Smad4 signal transduction can change the phosphorylation status of ER and ER-dependent signaling network and biological activity no matter ER ligand is present or not.

TGF- β signaling pathway is mediated by two membrane receptors (T β RI and T β RRI) located on cell membrane in serine/threonine kinase system (a signaling pathway different from tyrosine kinase receptor signaling pathway). And the signal on cell membrane is then transmitted to nucleus by smads (Sma and Mad-related protein) which is an intracellular signal conduct protein and is composed of many members. 9 members have been found in current studies, including Smad1, Smad2, Smad3, Smad4, Smad5, Smad6, Smad7, Smad8 and Smad9, each of which plays a different role in cell signal transduction. Smad2 and Smad3, two receptor-regulated signal conduct protein, transmit TGF- β signal. T β RRI is stimulated after combining with homologous ligand, then phosphorylates and activates T β RRI. Activated T β RRI serine/threonine kinase phosphorylates Smad2 and Smad3. Phosphorylated Smad2 and Smad3 combine with general signal conduct protein Smad4 and form stable heterogeneous complex, which transfers to nucleus and activates transcription. However, inhibitory Smad7 can interact with activated T β RRI to suppress TGF- β

signal transduction. Researches in recent years showed that many intracellular proteins affect TGF- β signal transduction through interaction with effect molecule Smads¹⁹.

With the further study of signaling pathway, it has been found that Smad4 as a general regulatory factor of Smad family has an effect of ER- α related suppressor in RE and TGF- β signaling pathway. From the gene mapping analysis of interaction region, we get the conclusion that ER- α and AF-1 region are indispensable in Smad4's function. However, MHI region and Hinge region are also needed when ER interacts with Smad4. Corresponding to previous results, Wu et al²⁰ also proved that Smad3 can enhance the ER-mediated transcription activity. But interestingly, Smad4 can reverse this activity. Smad3 and Smad4 can interact with ER individually. Smad3 alone can activate transcription while TGF- β and Smad3-Smad4 complex can suppress ER-mediated transcription activity. Intracellular TGF- β signal is transmitted through phosphorylated Smad3. The Smad3-Smad4 complex was then located into nucleus to regulate the transcription. So, Wu et al²⁰ pointed out that TGF- β suppresses ER-mediated transcription activity with existence of Smad4. But when Smad4 is mutated or removed, TGF- β may activate gene transcription instead of suppress it.

Nowadays, there has been certain understanding of each component of p53 Smad4 signaling pathway and their function, which has provided theoretical foundation for further studied on the interaction between each step and the induction of physiological effects in cell. However, the role of p53-Smad4 interaction in tumorigenesis has not been fully elucidated, which still need further studies.

In our study, we detect the expression and function of Smad4 by suppressing p53 expression in MCF7, trying to figure out the relationship and signaling pathway between them. Experiments have proved that activity of Smad4 increased and cell apoptosis was promoted after p53 was downregulated by siRNA. Expression of Smad4 is significantly positively related to p53 siRNA. The more p53 siRNA was transfected, the expression of p53 was lower, mRNA/protein level of Smad4 was higher and apoptosis in MCF7 was more obvious. 6 hours after transfection of p53 siRNA p53 mRNA level dropped significantly. 12 hours after transfection p53 protein level was suppressed. 24 hours after transfection Smad4 mRNA level increased ob-

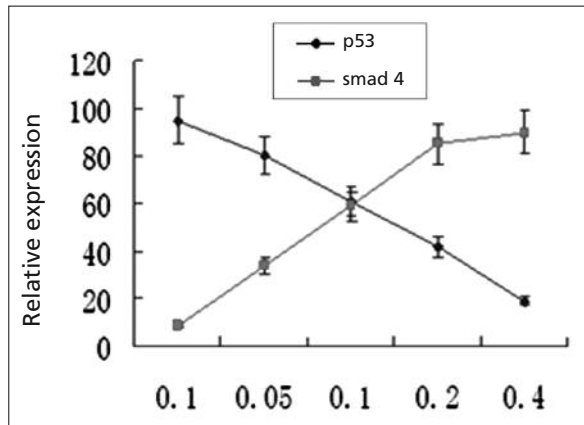


Figure 7. The impact of p53 siRNA transfection on cell cycle.

viously. 48 hours after transfection cell apoptosis increased and cell cycle became shorter. With the inhibition of p53, Smad4 mRNA was activated (Figure 7) and cell apoptosis was promoted. Wild type p53 and Smad4 belonged to tumor suppressor gene, which negatively regulated tumor cell growth. They are negatively related in the same breast cancer cell. In this study we try to found the affection of p53 on Smad4, so as to induce apoptosis in tumor cell, achieving the goal of targeted cancer prevention and treatment.

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