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Re-induction of cell differentiation and ^{131}I uptake in dedifferentiated FTC-133 cell line by *TSHR* gene transfection ☆

Fang Feng [1], Hui Wang *, Shasha Hou [1], Hongliang Fu

Department of Nuclear Medicine, Xin Hua Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China

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ABSTRACT

Introduction: Radioiodine therapy is commonly used to treat differentiated thyroid cancer (DTC), but a major challenge is dedifferentiation of DTC with the loss of radioiodine uptake. TSHR is a key molecule regulating thyrocyte proliferation and function. This study aimed to test the therapeutic potential of TSHR in dedifferentiated DTC by gene transfection in order to restore cell differentiation and radioiodine uptake.
Methods: Dedifferentiated FTC-133 (*dFTC-133*) cells were obtained by monoclonal culture of FTC-133 cell line after ^{131}I radiation. Recombinant plasmid pcDNA3.1-hTSHR was transfected into *dFTC-133* cells by using Lipofectamine 2000 reagent. Immunofluorescence analysis was carried out to confirm TSHR expression and its location. Radioiodine uptake assay was thereafter investigated. mRNAs and proteins of TSHR and other thyroid differentiated markers were detected by real-time PCR and western blot respectively.
Results: Among the thyroid specific genes in *dFTC-133* cells with stable low radioiodine uptake, TSHR was down-regulated most significantly compared with FTC-133. Then, after *TSHR* gene transfection, augmented expression of TSHR was observed in *dFTC-133* cell surface and cytoplasm by immunofluorescence analysis. It was found that ^{125}I uptake was 2.9 times higher ($t=28.63$, $P<.01$) in cells with *TSHR* transfection than control. The mRNAs of TSHR, NIS, TPO and Tg were also significantly increased by 1.7 times ($t=13.8$, $P<.05$), 4 times ($t=28.52$, $P<.05$), 1.5 times ($t=14.43$, $P<.05$) and 2.2 times ($t=19.83$, $P<.05$) respectively compared with control group.
Conclusion: Decreased TSHR expression correlated with FTC-133 ongoing dedifferentiation. *TSHR* transfection contributed to the re-differentiation of dedifferentiated thyroid follicular carcinoma cells.

1. Introduction

Differentiated thyroid carcinomas (DTCs), including papillary or follicular types, are often curable with surgical resection and ^{131}I ablation. ^{131}I can effectively treat tumor foci exhibiting high ^{131}I uptake [1]. By contrast, the loss or absence of radioiodine uptake observed in some tumors with a less differentiated phenotype is an unfavorable prognostic factor and the molecular mechanisms remain still unclear [2].

Differentiated thyroid cells express a number of final differentiation markers such as thyroglobulin (Tg), thyroperoxidase (TPO) and the Na^+/I^- symporter (NIS), which are responsible for iodine uptake, synthesis of thyroid hormones and the differentiated thyroid phenotype. All of these molecules are regulated by thyrotropin (TSH) binding to its receptor. Previous studies suggested that expression anomalies of these thyroid specific proteins could occur in thyroid cancers, whereas TSHR expression was little reported [3].

TSHR molecules in the membrane are quite stable and signaling in the thyrocyte will be controlled mainly through circulating TSH levels. T4 withdrawal or TSH administration to DTC patients with functional TSHR ensures uptake of ^{131}I and the removal of malignant cells. However, dedifferentiated metastases may have no radioiodine uptake even if circulating TSH is high. So, we hypothesized that TSHR expression anomalies may play a key role in dedifferentiation of DTC. And in our preliminary studies [4], we proved that FTC-133 cells underwent dedifferentiation during long-term culture in vitro, and ^{131}I may promote this process with decrease in TSHR expression.

Some studies on restoring iodide uptake of dedifferentiated thyroid carcinoma had been done targeting NIS with agents such as retinoic acids and NIS gene transfection, but the results have not been encouraging [5]. Most recently, the enhanced radioiodine uptake and retention, combining NIS and TPO gene transfection, in a model of a nonsmall cell lung cancer cell line were observed [6]. Therefore, we hypothesized that TSHR gene transfection, which can enhance both NIS and other thyroid specific molecular expression, may enhance iodine uptake obviously in dedifferentiated DTC.

The aim of the present study was to investigate the correlation of THSR expression anomalies and dedifferentiation of FTC-133, and the

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* Corresponding author. Tel.: +86 21 25076989; fax: +86 21 25076989.
E-mail address: wanghuishanghai@hotmail.com (H. Wang).

[1] Fang Feng and Shasha Hou to be joint first authors.

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role of *TSHR* gene transfection in redifferentiation of dedifferentiated thyroid carcinoma.

2. Materials and methods

2.1. Cell culture

The human DTC cell line FTC-133 was obtained from the Department of Nuclear Medicine, Ruijin Hospital, Shanghai, China. The cells were cultured in Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture (DMEM/F12; Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum containing 1% penicillin/streptomycin (Invitrogen Life Technologies) and 10 mU/ml the human TSH (Sigma-Aldrich) and grown at 37 °C and 5% CO_2.

2.2. dFTC-133 cell line establishment

In our early studies, we found that dedifferentiation of FTC-133 cells was enhanced by ^{131}I radiation [4]. So, dedifferentiated FTC-133 (*dFTC-133*) cell line was established by limiting dilution analysis after radiation with ^{131}I. Briefly, FTC-133 cells were seeded in 6-well plates incubated with 15 μCi $Na^{131}I$ for 3 days and then cultured in activity-free medium for 3 months. Then the cells were suspended, and by graded dilutions, the suspension of cells ultimately contained 10 specific cells in 1 ml of culture medium. Then, 100 μl of suspension was plated into 96-well plates for an appropriate culture period, and some wells would be found with one cell clone. These wells were selected and cultured. Cells with stable lowest radioiodine uptake were set as *dFTC-133* and their iodide-metabolizing molecules were detected.

2.3. Plasmid amplification, extraction, purification, and transfection

Recombinant eukaryotic expression plasmid *pcDNA3.1-hTSHR* was a gift from the Department of Nuclear Medicine, Ruijin Hospital, Shanghai Jiaotong University School of Medicine and empty vector *pcDNA3.1 (+)* was a gift from professor Xu Rang, Xinhua Hospital, Shanghai Jiaotong University School of Medicine. The plasmid *pcDNA3.1-hTSHR* or the empty vector *pcDNA3.1(+)* was transformed into E. coli DH5α (Tiangen Biotech, Beijing, China) respectively. A single bacterial colony was selected and inoculated with 150–200 ml of LB containing the appropriate ampicillin (100 μg/ml). Plasmid extraction was available for isolation of plasmid DNA. An EndoFree plasmid kit (Tiangen Biotech) was used to purify the plasmid DNA with an affinity column.

The *pcDNA3.1-hTSHR* and empty *pcDNA3.1(+)* were transfected into *dFTC-133* cells by using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Briefly, cells were seeded in 24 well plates for one day in order to achieve 90%–95% confluency per well at the time of transfection. 0.80 to 4 μg of *pcDNA3.1-hTSHR* or *pcDNA3.1 (+)* cDNA was mixed with Opti-MEM respectively. 2 to 10 μl of Lipofectamine in Opti-MEM was added to diluted DNA and incubated for 20 min at room temperature. Then, The DNA and Lipofectamine mixture was added in the cells overlaid with serum free DMEM. After five hours, the complexes were removed and the cells were cultured with normal medium. Blank group of cells were set with non transfection.

2.4. Immunofluorescence test

Cells were divided into three groups as described above in plasmid DNA transfection. Cells were seeded in 24 well plates with a cover glass per well and cultured for 24 h. Then, cells were fixed in PBS with 4% formaldehyde for one hour and permeabilized with 0.3% Triton X-100 for 30 min. After washing with PBS for three times, cells were incubated with PBS-3% bovine serum albumin (BSA) at room temperature for three hours. Then, cells were incubated with the monoclonal mouse anti-TSHR (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C, and then incubated with a fluorescein-conjugated secondary antibody (1:200; Santa Cruz Biotechnology) for two hours. After washing with PBS for three hours, slides were mounted and observed with a fluorescence microscope.

2.5. RAIU assay

Radioiodine uptake was measured as previously described [7]. Briefly, cells in five wells were washed (×3) with 1 ml of HBSS and then incubated at 37 °C with 500 μl buffered HBSS containing 0.1 μCi carrier-free $Na^{125}I$ and 10 μM sodium iodide for 5, 10, 15, 30 and 60 min respectively. Then, radioactive medium was aspirated and cells were washed (×3) with 1 ml of ice-cold HBSS for 1 min. Cells were harvested using trypsin and counted with a hemacytometer. One milliliter of 95% ethanol was added to each well for 20 min and then transferred into vials for counting with a gamma counter. The radioactivity was normalized to the number of cells present at the time of the assay as cpm every 10^6 cells.

2.6. Real-time polymerase chain reaction

Total RNA were extracted from cells using Trizol reagent (Invitrogen Life Technologies) followed by the manufacture's instructions. Five micrograms of each RNA sample was reverse transcribed with SuperScript III First-Strand Synthesis System (Invitrogen Life Technologies) in the presence of random hexamers to generate cDNA. Quantitative real-time polymerase chain reaction (PCR) was done using the SYBR Green I real-time PCR (Toyobo Life Science, Shanghai, China) with an ABI PRISM 7500 Real-time PCR System (Applied biosystems, Foster, California, USA). Primer pairs and annealing temperatures are described in Table 1. All amplification reactions were done in triplicate. The threshold cycle (Ct) values were used for calculating the relative expression ratios between control and treated cells. The relative gene expression was calculated with the following formulas: $\Delta Ct = Ct_{target} - Ct_{\beta\text{-actin}}$, $\Delta\Delta Ct = Ct_{experiment/control} - Ct_{blank}$, $2^{-\Delta\Delta Ct}$ = relative expression, and directly proportional to the gene expression.

2.7. Western-blotting

Cells were washed with PBS and harvested by scraping with RIPA lysis buffer. Lysates were quantified spectrophotometrically using the bicinchoninic-acid-based (BCA) method (Pierce Chemical, Rockford, IL, USA). Twenty-five micrograms of each sample was separated by gradient polyacrylamide gels and transferred onto polyvinyldifluoride membranes (Pierce Chemical). Membranes were incubated with one of the following primary antibodies—affinity purified mouse monoclonal anti-human NIS antibody (1:1500; Chemicon International, Hofheim, Germany), monoclonal mouse anti-TSHR (1:1000; Santa Cruz Biotechnology), TPO antibody (1:500; Thermo Fisher Scientific Inc., Fremont, CA, USA) or mouse anti-human β-tubulin antibody (1/2000; Santa Cruz Biotechnology)—overnight at 4 °C in TTBS/milk. The

Table 1
Primer sequences of TSHR, NIS, TPO and Tg.

Gene	Primer forward	Primer reverse	Product
GAPDH	ATTCCACCCATGGCAAATTC	GCATCGCCCCACTTGATT	121 bp
TSHR	TCATTTGACATAGCAGAAAC	TAATAGTGACCAAGTTCTGA	113 bp
NIS	GACAAACCTCTGAGGACAGGG	ATACTGGGGACGGTTGAAGC	223 bp
TPO	ACACAGGCAAATCGGAAATC	GCAATGTTTACAAGAAAAGGCC	137 bp
Tg	GGGCATGTTACTGCATGTC	TTTGAACACAGGTCTGCCA	273 bp