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In vitro evaluation of the therapeutic potential of nevirapine in treatment of human thyroid anaplastic carcinoma

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ABSTRACT

Anaplastic thyroid carcinoma (ATC) is a severe thyroid malignancy with poor prognosis, due to its early metastasis and unresponsiveness to both radiation and chemotherapy. Nevirapine, a non-nucleoside reverse transcriptase inhibitor, has been used as a re-differentiation agent to treat cancers in several human cancer models. So far, the effects of nevirapine on human thyroid anaplastic carcinoma cells have not been documented. The aim of this study was to evaluate the therapeutic potential of nevirapine in treatment of human thyroid anaplastic carcinoma. Cell proliferation was determined by methly thiazolyl tetrazolium (MTT) assay. Cell apoptosis was analyzed by Hoechst 33258 staining. The mRNA expression of NIS and TSHR was determined by real-time quantitative reverse transcription-polymerase chain reaction (real time RT-PCR). Iodine uptake was determined by ^{125}I radioactivity assay. At all doses (100, 200, 350, 500 μmol/L) tested, nevirapine significantly inhibited cell proliferation after 48 h treatment. At high dose (500 μmol/L), nevirapine significantly increased the percentage of apoptotic cells compared with control ($P < 0.01$). At lower doses (200 μmol/L and 350 μmol/L), nevirapine did not induce cell apoptosis, but up-regulated NIS and THSR mRNA expression in a dose-dependent manner. In FRO cells pre-treated with nevirapine, the increase in NIS expression had no obvious effect on iodine uptake. These findings indicate that nevirapine has an anti-proliferative effect on FRO cells, which correlates with an induction of cell differentiation.

1. Introduction

Thyroid malignancies of follicular cell origin have been classified as either well-differentiated thyroid carcinoma (WDTC) or anaplastic/undifferentiated thyroid carcinoma (ATC). ATC is estimated to account for 1–2% of all thyroid malignancies (Smauridge, 2009). Due to the early metastases that characterize ATC (Kurukahvecioglu et al., 2007), complete surgical resection is usually not possible. The effectiveness of other therapeutic modalities including radiation and chemotherapy also remains unsatisfactory. Thus, patients with ATC commonly have poor prognosis, with a median survival of 7.2 months from the time of diagnosis (Are and Shaha, 2006).

The unique fundamental function of the thyroid follicular epithelial cells is to utilize iodide to synthesize thyroid hormones, a process that involves several thyroid-specific iodide-handing genes (Nillson, 2001). A key step is the uptake of circulating iodide into the thyroid cell, which is mediated by the sodium-iodide symportor (NIS). The NIS expression is under the control of thyroid-stimulating hormone (TSH), which acts by binding to the TSH receptor (TSHR) on the cell surface. The expression of NIS gene is often lost in ATC leading to the failure of radioiodine concentrating in ATC cells, which partly accounts for the unresponsiveness of ATC cells to radioiodine treatment (O'Neill et al., 2010).

Reverse transcriptase (RT) coding gene has been demonstrated to plays important roles in regulation of cell proliferation and differentiation (Landriscina et al., 2007; Spadafora, 2004). Nevirapine, a non-nucleoside reverse transcriptase inhibitor, has been shown to inhibit endogenous RT activity in various transformed mammalian cells (Mangiacasale et al., 2003). More recently, nevirapine has been used as a re-differentiation agent to treat cancers in several human cancer models (Landriscina et al., 2007). So far, the effects of nevirapine on human thyroid anaplastic carcinoma cells have not been documented. In the present study, we aimed to quantitate the effects of nevirapine on FRO cell proliferation, survival and differentiation-related genes expression, and evaluate the therapeutic potential of this agent in treatment of human thyroid anaplastic carcinoma.

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2. Materials and methods

2.1. Human thyroid anaplastic carcinoma cell line

FRO cell line was obtained from the First Affiliated Hospital, Chinese Medical University (Shenyang, P.R. China).

2.2. Cell cultures

FRO cells were cultured in Dulbecco's Modified Eagle Medium containing 10% (v/v) fetal bovine serum (Gibco Life Technology, Grand Island, NY, USA), 1 μM L-glutamine, and 100 IU/mL penicillin and 100 μg/mL streptomycin solution at 37 °C in a humidified atmosphere of 5% CO_2 in air. Human recombinant TSH (rhTSH) (Sigma–Aldrich, St. Louis, USA) was added at a concentration of 2 mU/mL. Nevirapine standard substance was dissolved in dimethylsulfoxide (DMSO) (Sigma–Aldrich, St. Louis, USA). Nevirapine or the same volume of DMSO (0.1%, control) was added to the cultures. Nevirapine-containing medium was refreshed in every 48 h.

2.3. Cell proliferation assay

FRO cells were seeded into 96-well culture plates at 10,000 cells/well. Cells were treated with different doses of nevirapine (0, 100, 200, 350 and 500 μmol/L) for 48 h. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye (5 mg/mL) was added to each well for additional 4 h, and the reaction was then stopped by the addition of DMSO. Optical density was measured at 490 nm on a multi-well plate reader. Background absorbance of the medium was subtracted. All samples were assayed in triplicate, and mean for each experiment was calculated. Data were expressed as fold increases over control.

2.4. Measurement of apoptosis by Hoechst 33258 staining and cell viability assay

Apoptosis of the treated FRO cells were determined according to the morphological changes in cell nuclei using Hoechst 33258 staining (Sigma–Aldrich, St. Louis, USA). Cells, after washing with cold phosphate-buffered saline (PBS), were fixed with paraformaldehyde for 30 min. Hoechst 33258 (10 μg/mL) was added and incubated for 20 min before being detected under fluorescence microscopy.

Cell viability was determined by fluorescence microscopy after staining with 0.4% trypan blue for 5 min. Necrotic cells were stained blue, normal cells could not be stained. Cell viability (%) was expressed as normal cells/(normal cells + necrotic cells) × 100.

2.5. RNA extraction and real-time RT-PCR analysis

Total RNA was extracted using the Trizol Reagent according to the manufacturer's procedures (TaKaRa Biotechnology, Dalian, P.R. China). For the first strand synthesis of cDNA, 5 μg of RNA was used in a 20 μL reaction mixture using a cDNA Superscript III (Invitrogen, NY, USA) following the supplier's instructions. For real-time PCR, 2 μL cDNA sample was amplified utilizing the Platinum SYBR Green qPCR Supermix DUG (TaKaRa Biotechnology, Dalian, P.R. China) in a real time detection system (Hoffmann-La Roche, Basel, Switzerland). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. The following primers were used: NIS, forward 5′-CCATCCTGGATGACAACTTGG-3′, reverse 5′-AAAAACAGACGATCCTCATTG-3′ (PCR product 99 bp); TSHR, forward 5′-CCATCAGGAGGAGGACTTCA-3′, reverse 5′-ATTGGGCAGATTAGAAAATG-3′ (PCR product 138 bp); GAPDH, forward 5′-CAAGGCTGAGAACGGGAA-3′, reverse 5′-GCATCGCCCCACTTGATTTT-3′ (PCR product 89 bp). Primers were designed to be intron spanning. Reaction conditions were 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C, 30 s at 58 °C (both NIS and TSHR were 58 °C), 30 s at 72 °C and 72 °C for 5 min. Results were expressed as fold increases over control and normalized to GAPDH.

2.6. Western blotting

Equal amounts of protein from cell lysates were loaded in each well of a 12% sodium dodecyl sulfate–polyacrylamide gel. After electrophoresis, proteins were transferred to PVDF membranes, blocked for 1 h with 5% fat-free milk at room temperature, and blotted with the indicated primary antibodies (monoclonal rabbit anti-phospho-p44/42 ERK1/2 antibody and polyclonal rabbit anti-pan-ERK(c-14) antibody, 1:1000; Cell signaling Technology) overnight at 4 °C with gentle agitation. The membranes were incubated with HRP-conjugated secondary antibody for 1 h at room temperature. The immune complexes were detected using ECL plus kit (Hyclone UK Ltd., United States), and visualized on a Storm 860 Gel and Blot Imaging System.

2.7. In vitro iodine uptake assay

FRO cells were incubated for 48 h in the presence of 350 μmol/L nevirapine, and then the medium was removed and washed with 1 mL HBSS containing10 μmol/L $Na^{125}I$. After 30 min at 37 °C in a humid atmosphere, cells were washed with ice-cold HBSS. Radioactivity was counted in a γ-counter (Packard, USA). Cell numbers were also determined and iodide uptake was expressed as counts per minute per 10^6 cells.

2.8. Statistical analysis

All data was expressed as mean ± SEM. Differences between groups were examined by Student's *t* test or ANOVA test using SPSS17.0 software. Value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Nevirapine inhibited cell proliferation

To evaluate the effect of nevirapine on cell proliferation, FRO cells were treated with nevirapine (0, 100, 200, 350, 500 μmol/L) for 48 h. Cell proliferation was then measured by MTT assay. Nevirapine inhibited FRO cells proliferation in a dose-dependent manner (Fig. 1). Cell viability was evaluated by trypan blue staining. The results showed that nevirapine doses of 200 or 350 μmol/L had no obvious effect on cell viability; while 500 μmol/L nevirapine decreased the cell viability by 5.3% ± 0.77% compared to control ($P < 0.05$)(Fig. 2).

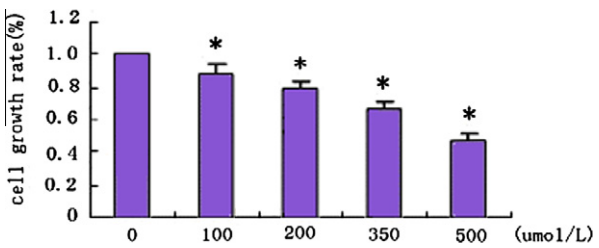

Fig. 1. Inhibition of FRO cells proliferation by nevirapine. Cells were treated with increasing doses of nevirapine (0, 100, 200, 350, 500 μmol/L) for 48 h. Cell proliferation was determined by MMT assay. Data (means ± SEM, $n = 3$) are expressed as fold increase over control. *$P < 0.05$ vs. control.