RESEARCH ARTICLE

Influence of Ribosomal Protein L39-L in the Drug Resistance Mechanisms of Lacrimal Gland Adenoid Cystic Carcinoma Cells

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Abstract

Background: Cancer constitutes a key pressure on public health regardless of the economy state in different countries. As a kind of highly malignant epithelial tumor, lacrimal gland adenoid cystic carcinoma can occur in any part of the body, such as salivary gland, submandibular gland, trachea, lung, breast, skin and lacrimal gland. Chemotherapy is one of the key treatment techniques, but drug resistance, especially MDR, seriously blunts its effects. As an element of the 60S large ribosomal subunit, the ribosomal protein L39-L gene appears to be documented specifically in the human testis and many human cancer samples of different origins. Materials and Methods: Total RNA of cultured drug-resistant and susceptible lacrimal gland adenoid cystic carcinoma cells was separated, and real time quantitative RT-PCR were used to reveal transcription differences between amycin resistant and susceptible strains of lacrimal gland adenoid cystic carcinoma cells. Viability assays were used to present the amycin resistance difference in a RPL39-L transfected lacrimal gland adenoid cystic carcinoma cell line as compared to control vector and null-transfected lacrimal gland adenoid cystic carcinoma cell lines. Results: The ribosomal protein L39-L transcription level was 6.5-fold higher in the drug-resistant human lacrimal gland adenoid cystic carcinoma cell line than in the susceptible cell line by quantitative RT-PCR analysis. The ribosomal protein L39-L transfected cells revealed enhanced drug resistance compared to plasmid vector-transfected or null-transfected cells as determined by methyl tritiated thymidine (3H-TdR) incorporation. Conclusions: The ribosomal protein L39-L gene could possibly have influence on the drug resistance mechanism of lacrimal gland adenoid cystic carcinoma cells.

Keywords: Ribosomal protein L39-L - drug resistance mechanism - lacrimal gland adenoid cystic carcinoma cells

Introduction

The international agency for research on cancer (IARC) reported that tumor had threatened the public’s health in countries all over the world, whether rich or poor (McCormack et al., 2012). 7.6 million people were died of cancer in 2008, and the number were estimated to rise to 11 millions in the year of 2030 according to the present progress pattern (Bray et al., 2012). As a kind of highly malignant epithelial tumor, lacrimal gland adenoid cystic carcinoma can occur in any part of the body, such as salivary gland (Kondo et al., 2014), trachea (Chang et al., 2011), lung (Wang et al., 2013), breast (Kim et al., 2014), skin (Rocas et al., 2013) and lacrimal gland (Bradley et al., 2013). At present the treatment of adenoid cystic carcinoma (ACC) were combined therapy, including operation, chemotherapy, radiotherapy, radiotherapy sensitizing and palliative chemotherapy, etc (Martinez et al., 2011; Jensen et al., 2011; Shen et al., 2012; Kaur et al., 2013). Chemotherapy is one of the key treatment techniques, but drug resistant, especially MDR, had seriously affected its effect. Multidrug resistance is one of the important reasons leading to tumor drug resistance and chemotherapy failure.

There were several research experiments been executed to study the multidrug resistance mechanism across the world lately. Within this field, the studies were concentrated on two aspects: the multiple drug resistance(MDR) (Koshkin et al., 2012) mechanism gene, which encoded 170 P protein, and non- multiple drug resistance gene (Tay et al., 2014). The synthesis and overexpression of multidrug resistance gene is inseparable from the effect of a series of ribosomal protein. The ribosome’s function can be affected by abnormal expression of ribosomal protein, which can cause various diseases, such as tumor, metabolic diseases, autoimmune diseases, etc. Earlier experiments of our research group collected the results to prove that the adenoid cystic carcinoma (ACC) adriamycin resistant strain has a high ribosomal proteins expression level, such as ribosomal

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Ribosomal protein L39(RPL39) is one protein of that 60S large ribosomal subunit. As a homolog of ribosomal protein L39, ribosomal protein L39-L(RPL39-L) is normally expressed in the human testis and derepressed in multiple cancer cells (Nadano et al., 2002; Sugihara et al., 2010). Even so, the relationship between the function of RPL39-L and cancer cell drug resistance has not been confirmed now. In this study, Total RNA of cultured drug-resistant and susceptible lacrimal gland adenoid cystic carcinoma cells was separated, and real time quantitative RT-PCR revealed the RPL39-L gene is transcribed to a greater extent in the amycin resistant strain than in the susceptible strain of ACC cells. Viability assay demonstrated that the amycin resistance in RPL39-L transfected ACC cell line were higher compared to control vector and null-transfected ACC cell line.

Materials and Methods

Cell culture experiments

Human adenoid cystic carcinoma cell was cultured within RPMI-1640 medium (DMEM, Invitrogen, Carlsbad, U.S.A) with 15% fetal bovine Serum (PAA, Pasching, Austria), 100 U/ml streptomycin and 100 U/ml penicillin (Invitrogen) at 37°C in a humidified 5% CO2 atmosphere. Cultures of cells were harvested at 80% confluence 24 hours before stimulation, counted and seeded in six well plates at a density of 30,000 cells per ml. The drug-resistant human adenoid cystic carcinoma cell cell line has been selected from a susceptible adenoid cystic carcinoma cell line, and the resistance has been maintained by treatment with amycin at LC50 of each generation. The LC50 of drug-resistant human adenoid cystic carcinoma cell line (abbreviated for ACC1 strain) is 0.186 μg/ml, 80.1-fold greater than that in the susceptible strain (0.0023 μg/ml).

RNA extraction

Total RNA of cultured drug-resistant adenoid cystic carcinoma cells and susceptible adenoid cystic carcinoma cells was isolated using Trizol reagent (Gibco BRL, Grand Island, NY, USA) according to the manufacturer’s protocol.

Real-time quantitative RT-PCR analysis

Real-time quantitative RT-PCR was done using a LightCycler-RNA amplification Kit SYBR Green I (Roche, Germany). The reaction was repeated using three independently purified RNA samples, and the threshold cycle number was determined using LightCycler software version 3.3. Two pairs of primers were designed for this experiment: RPL39-L (forward: 5'-TCAAGCTTATGTCGGCCCACAAAACGT-3', and the reverse primer was 5'-TGAATTCAGCTTATGTCGGCCCACAAAACGT-3'). The PCR conditions were: 95°C for 5 min, then 30 cycles of 95°C for 50 s, 59°C for 50 s, 72°C for 1 min, then 72°C for 10 min. The PCR product was purified from the gel following electrophoresis using a quick Gel extraction kit (Qiagen). The purified PCR product was ligated with T4 DNA ligase to the pcDNA3.1 (+) vector (Invitrogen, Carlsbad, USA), and the ligation reaction solution was transformed into adenoid cystic carcinoma cells (Invitrogen). Positive clones were identified by restriction analysis of recombinants with HindIII and EcoR I, and by PCR with specific primers and vector primers. The accuracy of the expression plasmid RPL39-L-pcDNA3.1 (+) was further verified by sequencing.

Cell culture and stable transfection

Human adenoid cystic carcinoma cell line was cultured in DMEM with 10% FBS, 100 mg/ml streptomycin and 100 units/ml penicillin (Invitrogen) at 37°C in a humidified 5% CO2 atmosphere prior to transfection. When the cells were at 50-60% confluence, they were transfected using Lipofectamine according to the manufacturer's instructions. Briefly, The day before transfection, trypsinize and count the cells. Plate 4x10^4 cells per well in 0.5 ml of complete growth medium. Cell density should be 50–80% confluent on the day of transfection. For each well of cells to be transfected, dilute 0.5 μg of DNA into 100 μl of Opti-MEM® I Reduced Serum Medium without serum. For each well of cells, dilute 1.5-2.5 μl of Lipofectamine® LTX into the above diluted DNA solution, mix gently and incubate for 25 minutes at room temperature to form DNA-Lipofectamine® LTX complexes. Remove growth medium from cells and replace with 0.5 ml of complete growth medium. Add 100 μl of the DNA-Lipofectamine® LTX complexes directly to each well containing cells and mix gently by rocking the plate back and forth. Complexes do not have to be removed following transfection. Incubate the cells at 37°C in a CO2 incubator for additional 48 h before being harvested for RT-PCR.

Once it had been confirmed that the cells were expressing the expected protein, stable expression cell lines were created according to the manufacturer’s
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Isolation of total RNA and RT-PCR analysis of the specific RPL39-L transcript

Total RNA was isolated from transfected cells by using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Five micrograms of isolated total RNA from each sample was used as a template for first-strand cDNA synthesis. The cDNA was synthesized at 70°C for 5 min, and 0°C for 5 min, then 37°C 1.5 h with a random primer using Avian myeloblastosis virus (AMV) reverse transcriptase (TaKaRa). The reverse transcriptase was then inactivated at 99°C for 5 min. PCR amplification of the RPL39-L gene was performed with the forward gene-specific primer (5'-ATGTCGGCCCACAAAACGTTC-3') and the reverse vector primer (5'-AGAACGCACAGTCGAGGCTA-3') for confirmation of transcriptional expression. The primers used for β-actin PCR amplification was: forward: 5'-CTCCATCCTGGCCTCGCTG-3', reverse: 5'-ATGTCGGCCCACAAAACGTTC-3'. The horizontal axis is the concentration of amycin, vertical axis is the inhibition rate; Emax is the concentration at which maximum effect is reached, while EC50 is the concentration at which 50% of the maximum effect is reached, while ED50 is the concentration at which 50% of the maximum effect is reached. The 95% confidence intervals were used to determine significant differences among different cells.

Results

Real-time quantitative RT-PCR analysis

Real-time quantitative RT-PCR was used to analysis the amplification fold of RPL39-L in drug-resistant human adenoid cystic carcinoma cell line and in susceptible adenoid cystic carcinoma cell line. The cycle number of RPL39-L at which the amplification reached the threshold was normalized against β-actin cycle number to determine the relative copy numbers between drug-resistant human adenoid cystic carcinoma cell line and susceptible adenoid cystic carcinoma cell line. The RPL39-L exhibited...
6.5-folds higher level of transcription in drug-resistant human adenoid cystic carcinoma cell line than in susceptible adenoid cystic carcinoma cell line. The results suggested that RPL39-L expression was up-regulated in drug-resistant human adenoid cystic carcinoma cell line (Figure 1).

**Transcription and expression of RPL39-L in adenoid cystic carcinoma cells**

After stable transfection cell line were gained, total RNA was isolated from control cells and RPL39-L-transfected cells, and RT-PCR was performed using forward RPL39-L-specific primer and the reverse vector-specific primer. A pair of β-actin primers of adenoid cystic carcinoma cell line was used to ensure the validity of the reaction system. A PCR product of the expected size, about 209 bp, was observed only in cells that were transfected with the RPL39-L gene (Figure 2A), Western blot analysis using anti-RPL39-L antibodies identified a protein in cells transfected with RPL39-L gene (Figure 2B), which confirming that RPL39-L had been transcribed in the transfected cells successfully.

**Viability assay**

To investigate the RPL39-L expression in relation to amycin resistance, adenoid cystic carcinoma cell line stably transfected with RPL39-L or control vector and null-transfected adenoid cystic carcinoma cells were used for this assay. The time course and dose-response of cell viability over a wide range of concentrations of amycin was measured based on the 3H-TdR incorporation assay. Adenoid cystic carcinoma cell line that is transfected with either RPL39-L or control vector gene or null-transfected adenoid cystic carcinoma cells were treated with various concentrations of amycin (0.001, 0.003, 0.007, 0.015, 0.031, 0.062, 0.125, 0.25μg/ml) for 72 h and cell survival was analyzed according to 3H-TdR incorporation in order to observe the cell viability. Expression of the RPL39-L gene improved the viability of amycin-treated adenoid cystic carcinoma cells, the maximal protective effect was observed at 0.125μg/ml amycin (Figure 7). The EC$_{50}$ and 95% confidence intervals of null-transfected cells are 0.0019 (0.0014-0.0022), the EC$_{50}$ and 95% confidence intervals of vector-transfected cells are 0.0021 (0.0017-0.0024), and the EC$_{50}$ and 95% confidence intervals of RPL39-L transfected cells are 0.197 (0.182-0.205). Obvious cell viability augmentation was observed in the RPL39-L transfected adenoid cystic carcinoma cells compared to null-transfected or vector-transfected cells (Figure 3).

**Discussion**

The research of drug sensitivity of malignant tumor has been a major topic in tumor treatment. quick and accurate tumor chemosensitivity test has great significance to guide the selection of clinical chemotherapy drugs and the improvement of curative effect (Takebayashi et al., 2013; Cree et al., 2013; Canadas et al., 2014). But most research mainly concentrated in the laboratory research (Tadbir et al., 2012; Ashkavandi et al., 2013; Jaafari et al., 2013),

![Figure 1. Quantitative RT-PCR Assay of RPL39-L mRNA in Resistant and Susceptible Strains of ACC cell line.](image1)

![Figure 2. RPL39-L Expression in Adenoid Cystic Carcinoma (ACC) Cells Figure 2. A) RT-PCR analysis of RPL39-L mRNA in adenoid cystic carcinoma cells (1: null cell; 2: null vector; 3: RPL39-L transfected cell). The production of the transcripts was detected in cells transfected with RPL39-L (lane 3); No signal was detected either in normal cells (lane 1) or in cells transfected with vector (lane 2). B) Western blot analysis of RPL39-L in adenoid cystic carcinoma cells (1: null cell; 2: null vector; 3: RPL39-L transfected cell). The RPL39-L production was detected in cells transfected with RPL39-L (lane 3); No signal was detected either in normal cells (lane 1) or in cells transfected with vector (lane 2).](image2)

![Figure 3. Inhibitive Effect of Amycin on Cell Viability.](image3)
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