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 seperated, 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

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Introduction

The international agency for research on cancer (IARC) reported that tumor had threatened the public's health in countires 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 sensitizer 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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protein L39-L.

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 seperated, 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, Invitrogene, 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'- GACTTTCACCATTAAGCGATTC-3', reverse: 5'-GACGAACCGACCACGAGA -3') and β-action (forward: 5'-TCCATCCTGGCCTCGCTGT-3', reverse: 5'- GGTTCTTCTCCAATGCCTCC -3'). To confirm the accuracy and reproducibility of real-time quantitative RT-PCR, the experiment was determined in three repeats within one LightCycler run. The results for ribosomal protein L39-L was normalized to housekeeping β-actin gene. Overexpression fold was calculated according to the formula 2 (Rt-Et)=2 (Rn-En), where Rt is the threshold cycle number for the reference gene in the ACC1 strain, Et is the threshold cycle number for the experimental gene in the ACC1 strain, Rn is the threshold cycle number for the reference gene in the susceptible strain and En is the threshold cycle number for the experimental gene in the susceptible strain. Sample that had expression lever five-fold was considered overexpressed.The date were analyzed t-test.

Construction of the expression vector

The entire coding region of RPL39-L was amplified by PCR using the specific primers designed. The reverse primer was designed to remove the original stop codon and maintained the reading frame through the DNA encoding the C-terminal peptide. The forward primer used was 5'-TCAAGCTTATGTCGGCCCACAAAACGT-3', and the reverse primer was 5'-TGAATTCAGCTTTAGCTTGGTGCGAC-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% CO_2 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 CO_2 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

instructions. Briefly, a kill curve was performed to test the cell line for sensitivity to 0.004 μg/ml amycin, which can kill cells within one week. Forty-eight hours post-transfection, the transfection solution was removed and fresh medium without amycin was added. The cells were split on a 1:5 ratio and allowed to attach for 20 min before the selective medium was added. The medium was removed and replaced with medium containing 0.003 μg/ml amycin, and the cells were incubated at 37°C. The selecting medium was replaced every 3-4 days until clones were observed. Eight days later, the medium was replaced with medium containing 0.002 μg/ml amycin. The resistant cell lines were isolated using a dilution method until only one colony was found in each well of a 96-well microtiter plate, after which the plate was incubated until the colony filled most area of the well. The cells were harvested and transferred to a 24-well plate with 0.5 ml fresh medium containing 0.002 μg/ml amycin, and the clone was expanded in 12- and 6-well plates, and finally a T-25 flask. The cells were analyzed for expression using RT-PCR and Western blotting.

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'- GTTCTTCTCCAATGCCTCCTT -3'. One microliter of the RT reaction product was used as the template for routine PCR. The following cycling parameters were used: 95°C for 5 min followed by 25 cycles of 95°C for 40 s, 58°C for 45 s and 72°C for 1 min, followed by a final extension step of 72°C for 10 min.

Western blot analysis

Cells transfected with the RPL39-L construct were washed with phosphate buffered saline (PBS) and lysed in extraction buffer containing 50 mM Tris, pH 7.8, 150 mM NaCl and 1% Nonidet P-40 (Sigma, St. Louis, USA). The cell lysate was centrifuged at 10,000g for 10 min, and the supernatant was collected and stored at 80°C until use. Protein concentration was confirmed by using the Bradford assay. Twenty micrograms of protein per lane was used for SDS-PAGE electrophoresis. Sample treatment buffer (0.125M Tris-HCl, pH 6.8, 10% glycerol, 2% b-mercaptoethanol, 2% SDS and 0.1% bromophenol blue) was added to the sample and heated at 95°C for 5min. The gels were run for 100 min at 100 V, the proteins were transferred to a PVDF membrane at 350 mA for 100 min. The membrane was washed in PBST (PBS containing 0.05% Tween 20) for 5min and blocked in PBST and 5% non-fat dry milk for 2h, washed in PBST twice for 5min, and then incubated at 4°C with mouse anti-His antibody (1:200 dilution; BD, USA) overnight. The membrane was washed twice for 5min with PBS-T, incubated for 1.5h with a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:2000; BD, USA) at room temperature, and then washed three times as described above. Detection was done with the diaminobenzidine (DAB) chromogenic kit (Boster BioTech Company, Wuhan, China) according to the manufacturer's instructions. The membrane was exposed in visible light for 10 min, then washed with ddH2O to terminate the reaction. Molecular weight was determined by comparison with molecular weight markers (Bio-Rad).

3H-TdR incorporation

Adenoid cystic carcinoma cells were kept in the presence of various concentrations of amycin for 72 h before 3H-TdR incorporation. Eighteen hours before harvesting, 1 lCi of 3H-TdR was added to the medium of each well. To harvest the cells, DMEM medium was discarded, and cells were washed three times with 0.05 M PBS (pH 7.4), and detached from the microtiter wells by trypsinization. Detached adenoid cystic carcinoma cells were harvested onto glass fiber filter paper using a mini-MASH II microharvesting device (Whittaker MA Bioproducts, Walkersville, MD) and 3H-TdR incorporated into adenoid cystic carcinoma cell (cell associated 3H-TdR) was determined using a Wallac 1414 (WALLAC, Finland) liquid scintillation counter according to the manufacturer's instructions. Relative viability (%) was calculated as the ratio of 3H-TdR reduction in treated cells to that of control cells (adenoid cystic carcinoma cells transfected with vector and null-transfected adenoid cystic carcinoma cells). Each condition was performed in triplicate, and the date were analyzed t-test. The inhibiting effect (E) of the amycin on the cell viability was described by the equation: $E = (Emax*C) / (EC_{50}+C)$. 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 EC_{50} (50% effective dose [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

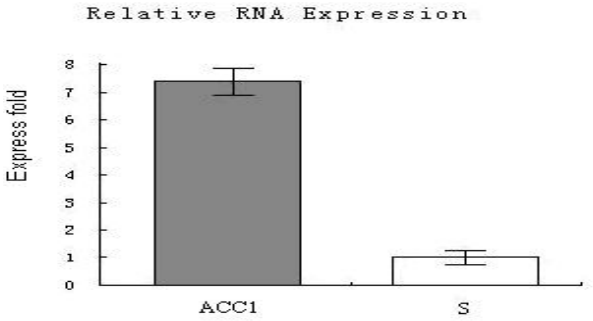

Figure 1. Quantitative RT-PCR Assay of RPL39-L mRNA in Resistant and Susceptible Strains of ACC cell line. Quantitative RTPCR was performed by using a Lightcycle-RNA amplification Kit SYBR Green and repeated using three independently purified RNA samples. The enhancement of fluorescence was found to be proportional to the initial concentration of template cDNA. RPL39-L transcript copy numbers were normalized based on expression of the housekeeping β-actin from respective strains. ACC1 represents drug-resistant ACC cell line, and S represents a susceptible strain. The data are presented as means±SD. T-test: n =3, p= 0.03

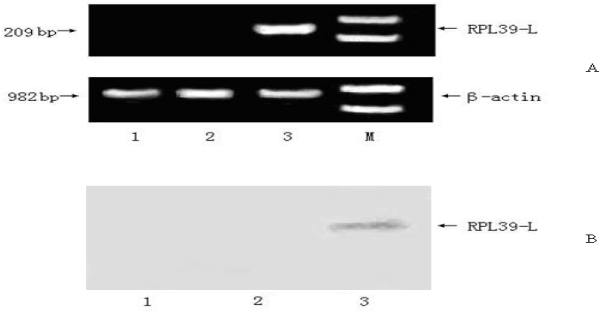

Figure 2. RPL39-L Expression in Adenoid Cystic Carcinoma(ACC) Cells Figure 2. A) RT-PCR analysis of RPL39-L mRNA in adenoid cystic carcinoma cells (1: null cell; 2:null vector; 3: RPL39-L transfected cell) .The production of the transcripts was detected in cells transfected with RPL39-L (lane3); No signal was detected either in normal cells (lane 1) or in cells transfected with vector (lane 2). Figure 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 (lane3); No signal was detected either in normal cells (lane 1) or in cells transfected with vector (lane 2)

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

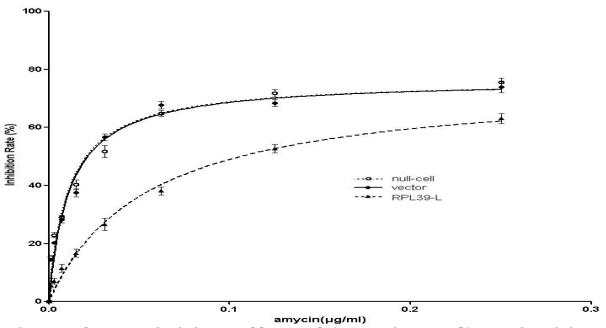

Figure 3. . Inhibitive Effect of Amycin on Cell Viability. The cultured cells were treated with different concentration of amycin and incubated for 72 h. The inhibiting effect (E) of the amycin on the cell viability was described by the equation: E= (Emax*C) / (EC50 + C) . T-test: n =3, p= 0.04.null-cell: nothing transfected in ACC cell line; vector: ACC cell line transfected with vector; RPL39-L: ACC cell line transfected with RPL39-L

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),

rarely used for guiding clinical therapy at present(Iqbal et al., 2014). Current views of clinical study for effect of ACC chemotherapy is not consistent, the choice of chemotherapy plan is difference. This may be related to the biological characteristics, histological type, degree of differentiation, cell dynamics of the tumor and patient's individual difference of the chemotherapeutic sensitivity and tolerance. In clinical practice, different patients often been used the same kind of chemotherapy in the treatment of the same type of cancer, even for patients with different tumor,this is obviously not scientific. Selection of chemotherapy drugs according to different patients and drug sensitivity test, then formulate individualized chemotherapy plan (Saijo et al., 2012; Fishman et al., 2013), was the trend of chemotherapy.

Study on the gene expression changes of cancer cell resistance to chemotherapy drugs mainly focused on MDR genes and non MDR genes of multidrug resistance mechanism. In this study, we found that the ribosomal protein L39-L transcription level was 6.5 times higher in drug-resistant human adenoid cystic carcinoma cell line than in susceptible adenoid cystic carcinoma cell line. Expression of RPL39-L in susceptible adenoid cystic carcinoma cell line also increases resistance against amycin. Although we do not have enough information to pinpoint the exact role of RPL39-L in amycin resistance, our results suggest that RPL39-L is a good candidate for future studies of amycin resistance.

As a ribosomal protein, RPL39-L can influence the synthesis of a subset of proteins (Petrov et al., 2004; Zhao et al., 2013). The altered translational profile may enhance the overall fitness of the cells and increase the tolerance of the cells and the organisms against harmful chemicals such as amycin (Lippert et al., 2014; Maeda et al., 2014; Hong et al., 2014). It has been reported that RPL39-L expression is important for mitochondrial biogenesis (Nadano et al., 2002; Tan et al., 2007). Since mitochondria are the organelles responsible for the oxidation reaction, their increase can accelerate the rate to detoxify amycin. This may be a possible way that RPL39-L mediates amycin resistance. RPL39-L may also help to increase the expression of other proteins to help to degrade amycin. Proteomic study to characterize those target proteins is needed to elucidate the mechanism of RPL39-L-dependent amycin resistance.

Since RPL39-L is likely an upstream factor of amycin resistance, its increase may cause a more significant increase in downstream factors, which can be more beneficial than other resistance-associated markers. The upstream role also makes it likely that RPL39-L can confer resistance against a broad range of antibiotics. In addition, the highly conserved nature of RPL39-L also suggests that the antibiotic resistance associated RPL39-L is present in a variety of species. The study of RPL39-L functions has a broad application.

In summary, we have studied the primary function of RPL39-L gene. Based on the characteristics of the gene, it is a member of the ribosomal protein family. The convergence of data in the present study suggests that RPL39-L may confer some amycin resistance in human adenoid cystic carcinoma cell line. Research carried out to date has provided a basis for further studies on the gene function associated with antibiotic resistance, which will improve our understanding of the molecular basis of ribosomal protein L39-L mediated resistance in human cancer cell line.

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