

RESEARCH ARTICLE

Effects of Ribosomal Protein L39-L on the Drug Resistance Mechanisms of Lung Cancer A549 Cells

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Abstract

Background: Cancer is a major threat to the public health whether in developed or in developing countries. As the most common primary malignant tumor, the morbidity and mortality rate of lung cancer continues to rise in recent ten years worldwide. Chemotherapy is one of the main methods in the treatment of lung cancer, but this is hampered by chemotherapy drug resistance, especially MDR. As a component of the 60S large ribosomal subunit, ribosomal protein L39-L gene was reported to be expressed specifically in the human testis and human cancer samples of various tissue origins. **Materials and Methods:** Total RNA of cultured drug-resistant and susceptible A549 cells was isolated, and real time quantitative RT-PCR were used to indicate the transcribe difference between amycin resistant and susceptible strain of A549 cells. Viability assay were used to show the amycin resistance difference in RPL39-L transfected A549 cell line than control vector and null-transfected A549 cell line. **Results:** The ribosomal protein L39-L transcription level was 8.2 times higher in drug-resistant human lung cancer A549 cell line than in susceptible A549 cell line by quantitative RT-PCR analysis. The ribosomal protein L39-L transfected cells showed enhanced drug resistance compared to plasmid vector-transfected or null-transfected cells as determined by methyl tritiated thymidine (3H-TdR) incorporation. **Conclusions and Implications for Practice:** The ribosomal protein L39-L gene may have effects on the drug resistance mechanism of lung cancer A549 cells.

Keywords: ribosomal protein L39-L - drug resistance mechanism - lung cancer - A549 cells

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Introduction

The international agency for research on cancer (IARC) released the world cancer report showed that cancer has a major threat to the public health whether in developed or in developing countries. There are 7.6 million people died of cancer in 2008, there will be more than 11 millions of people died of cancer in 2030 estimated according to the current development trend (Ridge et al., 2013). Lung cancer is the most common lung primary malignant tumor, the morbidity and mortality rate continues to rise in recent ten years worldwide, lung cancer has become one of the major diseases affecting human health (Villanueva, 2014). Methods in the treatment of lung cancer were surgical operation, chemotherapy, radiation therapy, traditional Chinese medicine therapy and molecular targeted therapy at present, surgical operation was the common treatment of early lung cancer (Tardon, 2014), but approximately 80% of patients had belong to advanced cancer at the time of diagnosis, and distant metastasis has occurred,

and the best treatment is chemotherapy, but about 80% of patients at the time of diagnosis would have been in the late fall (Shrivastava et al., 2013), cancer cells have distant metastasis, the best treatment is chemotherapy at this time (Onn et al., 2014), which is one of the main methods in the treatment of lung cancer, but lung cancer has always been bothered by chemotherapy drug resistant like other malignanthe chemotherapy drugly cross resistance (multiple drug resistance, MDR), which affected the chemotherapy efficacy of lung cancer greatly. There have been many research studies done on the lung cancer multidrug resistance mechanism all over the world in recent years. In this field, the studies were focused on two aspects: the multiple drug resistance mechanism of the MDR gene and its encoded 170 P protein (Baguley, 2010), and multiple drug resistance mechanism of the non-MDR gene (Colijn et al., 2011; Chen et al., 2012). The synthesis and overexpression of multidrug resistance gene is inseparable from the effect of a series of ribosomal protein. The abnormal expression of ribosomal protein will

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affect ribosome's function, which lead to various diseases, such as cancer, autoimmune diseases, metabolic diseases, etc. Previous studies of our research group revealed that the lung cancer cell A549 adriamycin resistant strain has a high expression level of a series of ribosomal proteins, such as RPL39.

Ribosomal protein L39 is a component of the 60S large ribosomal subunit (Lin et al., 1984; Otsuka et al., 1996). It has been reported the mutant cells lacking RPL39-L display decreased translation accuracy, and RPL39-homology is depressed in a variety of cancer cells (Nadano et al., 2002). But the correlation between the function of RPL39-L and cancer cell drug resistance has not been reported to date. In the present study, we utilized Real-time quantitative RT-PCR to clone a cDNA partial sequence of ribosomal protein L39-L, and real time quantitative RT-PCR indicated that this gene is transcribed to a greater extent in the amycin resistant strain than in the susceptible strain of A549 cells. Viability assay showed the enhanced amycin resistance in RPL39-L transfected A549 cell line than control vector and null-transfected A549 cell line.

Materials and Methods

Cell culture experiments

Human adenocarcinoma cell line A549 (ATCC, CCL-185) was cultured in 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 lung cancer A549 cell line has been selected from a susceptible A549 cell line, and the resistance has been maintained by treatment with amycin at LC₅₀ of each generation. The LC₅₀ of drug-resistant human lung cancer A549 cell line (abbreviated for DR1 strain) is 0.205 µg/ml, 82-fold greater than that in the susceptible strain (0.0025 µg/ml).

RNA extraction

Total RNA of cultured drug-resistant A549 cells and susceptible A549 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'-AGACTTTCACCATTAAGCGATTCC -3', reverse: 5'-TGACGAACCGACCACGAGAC -3') and β-action (forward: 5'-CTCCATCTGGCCTCGCTGT-3', reverse: 5'-GGTCTTCTCCAATGCCTCCTT -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 $2^{-(Rt-Et)/2} = 2^{-(Rn-En)}$, where Rt is the threshold cycle number for the reference gene in the DR1 strain, Et is the threshold cycle number for the experimental gene in the DR1 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.

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'-TCAAGCTTATGTTCGGCCACAAAACGTTCC-3', and the reverse primer was 5'-CTGAATTCAGCTTTAGCTTGGTGGCAGCGC-3'. The PCR conditions were: 95°C for 5 min, then 30 cycles of 95°C for 40 s, 58°C for 40 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 A549 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 adenocarcinoma cell line A549 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 4×10⁴ 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 48h 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 1.5h 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'-ATGTCGGCCACAAAACGTTCC-3') and the reverse vector primer (5'-TAGAACGCACAGTCGAGGCTA-3') for confirmation of transcriptional expression. The primers used for β-actin PCR amplification was: forward: 5'-CTCCATCCTGGCCTCGCTGT-3', reverse: 5'-GGTCTTCTCCAATGCCTCCTT-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 40 s and 72°C for 1 min, followed by a final extension step of 72°C for 10 min.

3H-TdR incorporation

A549 cells were kept in the presence of various concentrations of amycin for 72h before 3H-TdR incorporation. Eighteen hours before harvesting, 1 ICI 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 A549 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 A549 (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 (A549 cells transfected with vector and null-transfected A549 cells). Each condition was performed in triplicate.

Statistical analysis

The inhibiting effect (E) of the amycin on the cell viability was described by the equation: $E = (E_{max} * C) / (EC_{50} + C)$. The horizontal axis is the concentration of amycin, vertical axis is the inhibition rate; E_{max} is the concentration at which maximum effect is reached, while EC_{50} (50% effective dose [ED_{50}]) 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 lung cancer A549 cell line and in susceptible A549 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 lung cancer A549 cell line and susceptible A549 cell line. The RPL39-L exhibited 8.2-fold higher level of transcription in drug-resistant human lung cancer A549 cell line than in susceptible A549 cell line. The results suggested that RPL39-L expression was up-regulated in drug-resistant human lung cancer A549 cell line (Figure 1).

Transcription and expression of RPL39-L in A549 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

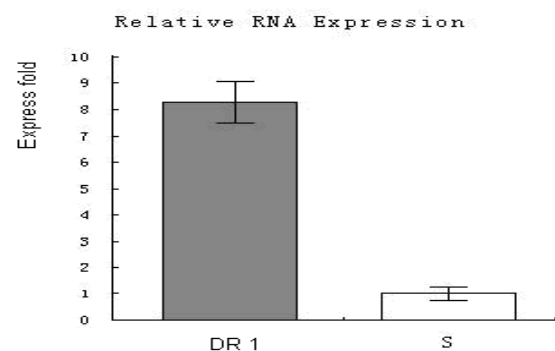


Figure 1. Quantitative RT-PCR Assay of RPL39-L mRNA in DR1 and Susceptible Strains of A549 Cell Line. Quantitative RTPCR was performed by using a Lightcycle-RNA amplification Kit SYBR Green and repeated using three independently purified RNA samples. The enhancement of fluorescence was found to be proportional 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. DR1 represents drug-resistant strain and S represents a susceptible strain. The data are presented as means±SD. n=3, p=0.03.

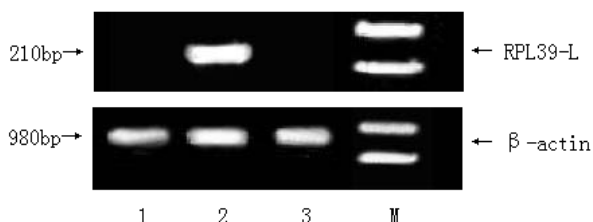


Figure 2. RT-PCR Analysis of RPL39-L mRNA in A549 Cells using Forward Primer of RPL39-L and Reverse Primer of Vector (1: null cell; 2: RPL39-L Transfected Cell; 3: Null Vector). The production of the transcripts was detected in cells transfected with RPL39-L (lane 2); No signal was detected either in normal cells (lane 3) or in cells transfected with vector (lane 1)

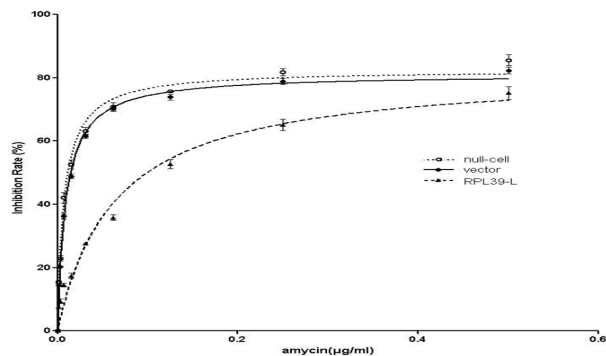


Figure 3. Inhibitive Effect of Amycyn on Cell Viability. The cultured cells were treated with different concentration of amycyn and incubated for 72 h. The inhibiting effect (E) of the amycyn on the cell viability was described by the equation: $E = (E_{max} * C) / (EC_{50} + C)$.

forward RPL39-L-specific primer and the reverse vector-specific primer. A pair of β -actin primers of A549 cell line was used to ensure the validity of the reaction system. A PCR product of the expected size, about 210 bp, was observed only in cells that were transfected with the RPL39-L gene (Figure 2), confirming that RPL39-L had been transcribed in the transfected cells.

Viability assay

To investigate the RPL39-L expression in relation to amycyn resistance, A549 cell line stably transfected with RPL39-L or control vector and null-transfected A549 cells were used for this assay. The time course and dose-response of cell viability over a wide range of concentrations of amycyn was measured based on the 3H-TdR incorporation assay. A549 cell line that is transfected with either RPL39-L or control vector gene or null-transfected A549 cells were treated with various concentrations of amycyn (0.001, 0.003, 0.007, 0.015, 0.031, 0.062, 0.125, 0.25, 0.5 μ g/ml) for 72h 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 amycyn-treated A549 cells, the maximal protective effect was observed at 0.125 μ g/ml amycyn (Figure 7). The EC_{50} and 95% confidence intervals of null-transfected cells are 0.0021 (0.0018-0.0024), the EC_{50} and 95% confidence intervals of vector-transfected cells are 0.0023 (0.0019-0.0026), and the EC_{50} and 95% confidence intervals of RPL39-L transfected cells are 0.203 (0.199-0.210). Obvious cell

viability augmentation was observed in the RPL39-L transfected A549 cells compared to null-transfected or vector-transfected cells (Figure 3).

Discussion

Due to the continuous rise of morbidity and mortality rate, lung cancer focused the attention of clinical treatment (Maurice, 2013). Despite surgical operation, chemotherapy is the most common used methods for the lung cancer treatment (Saha et al., 2013). Scientists have made unremitting efforts to genetic studies on drug resistance in lung cancer cells. Genetic variants in the PI3K, PTEN, AKT or mTOR pathway were been confirmed can be used for the prediction of platinum-based chemotherapy response of advanced non-small cell lung cancers (Xu et al., 2012); the overexpression of hypoxia-inducible factor 1 promoter-induced JAB1 were been reported can enhance the chemotherapeutic sensitivity of lung cancer cell line A549 in an anoxic environment (Hu et al., 2012). Among all the drug resistance mechanism studies of lung cancer treatment, the multiple drug resistance has become the biggest obstacle in treatment of lung cancer (Pendharkar et al., 2013). Study on the gene expression changes of lung cancer cell resistance to chemotherapy drugs mainly focused on MDR genes and non MDR genes of multidrug resistance mechanism (Brewer et al., 2011; Chauhan et al., 2012). While decreasing the expression of P-glycoprotein (P-gp), multi-drug resistance-associated protein (MRP1) and glutathione-S-transferase II (GST-II), vinorelbine can increase the drug sensitivity to cisplatin and intracellular accumulation of rhodamine-123 in A549/DDP cells (Zhou et al., 2013); P-glycoprotein (P-gp), lung resistance-related protein (LRP) and glutathione-S-transferase- π (GST- π) were all involved in the MDR mechanism of breast cancer (Chen et al., 2013). In this study, we found that the ribosomal protein L39-L transcription level was 8.2 times higher in drug-resistant human lung cancer A549 cell line than in susceptible A549 cell line. Expression of RPL39-L in susceptible A549 cell line also increases resistance against amycyn. Although we do not have enough information to pinpoint the exact role of RPL39-L in amycyn resistance, our results suggest that RPL39-L is a good candidate for future studies of amycyn resistance.

As a ribosomal protein, RPL39-L can influence the synthesis of a subset of proteins (Tan et al., 2007). 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 amycyn (Edrington et al., 2014). It has been reported that RPL39-L expression is important for mitochondrial biogenesis. Since mitochondria are the organelles responsible for the oxidation reaction, their increase can accelerate the rate to detoxify amycyn. This may be a possible way that RPL39-L mediates amycyn resistance. RPL39-L may also help to increase the expression of other proteins to help to degrade amycyn. Proteomic study to characterize those target proteins is needed to elucidate the mechanism of RPL39-L-dependent amycyn resistance.

Since RPL39-L is likely an upstream factor of amycyn 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 lung cancer A549 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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