RESEARCH ARTICLE

# Drug Resistance Effects of Ribosomal Protein L24 Overexpression in Hepatocellular Carcinoma HepG2 Cells

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## Abstract

**Background: The morbidity and mortality rate of liver cancer continues to rise in China and advanced cases respond poorly to chemotherapy. Ribosomal protein L24 has been reported to be a potential therapeutic target whose depletion or acetylation inhibits polysome assembly and cell growth of cancer. Materials and Methods: Total RNA of cultured amycin-resistant and susceptible HepG2 cells was isolated, and real time quantitative RT-PCR were used to indicate differences between amycin-resistant and susceptible strains of HepG2 cells. Viability assays were used to determine amycin resistance in RPL24 transfected and control vector and null-transfected HepG2 cell lines. Results: The ribosomal protein L24 transcription level was 7.7 times higher in the drug-resistant HepG2 cells as compared to susceptible cells on quantitative RT-PCR analysis. This was associated with enhanced drug resistance as determined by methyl tritiated thymidine (3H-TdR) incorporation. Conclusions: The ribosomal protein L24 gene may have effects on drug resistance mechanisms in hepatocellular carcinoma HepG2 cells.**

**Keywords:** Ribosomal protein L24 - drug resistance mechanism - liver cancer - HepG2 cells

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## Introduction

It had been reported by IARC that cancer has caused great threat to the people's health, regardless the state of economy. More than 8.2 million people died of cancer in 2012, there will be more than ten millions of people died of cancer in 2030 estimated according to the current development trend. 60% of world's total new annual cases occur in Africa, Asia and Central and South America. Hepatocellular cancer is the most common liver primary malignant tumor, the morbidity and mortality rate continues to rise in recent ten years worldwide, liver cancer has become one of the major diseases affecting human health (Ridge et al., 2013).

In terms of the treatment of liver cancer, surgery, ligation of hepatic artery, hepatic artery chemoembolization, radiofrequency, freezing, laser, microwave, chemotherapy, radiation therapy and other methods can be used, and individualized comprehensive treatment according to different stages was the key to improve the curative effect (Sawanyawisuth et al., 2012). The patients of metaphase and advanced phase of liver cancer were been used chemotherapy commonly, but the response is generally poor.

Multidrug resistance of tumor cells is the fundamental reason for the failure of cancer chemotherapy, and the influence of tumor metastasis and recurrence eventually lead to patient's death (Chen et al., 2012). Therefore, the approaches and reversal of hepatocellular carcinoma MDR has been the research hotspot in the field of tumor. The synthesis and overexpression of multidrug resistance gene is inseparable from the effect of a series of ribosomal protein (Wang et al., 2006; Kim et al., 2013; Liu et al., 2014). The abnormal expression of ribosomal protein will 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 hepatocellular carcinoma cell HepG2 adriamycin resistant strain has a high expression level of a series of ribosomal proteins, such as ribosomal protein L24 (RPL24).

RPL24 is a component of the 60S large ribosomal subunit (Chan et al., 1994), and it has been reported to be a potential therapeutic target whose depletion or acetylation inhibits polysome assembly and cell growth of breast cancer (Wilson-Edell et al., 2014). But the correlation between the function of RPL24 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 L24 and the result of real time RT-PCR showed RPL24 gene was highly transcribed in the amycin resistant strain than in the susceptible strain of HepG2 cells. Viability assay showed the enhanced amycin resistance in RPL24 transfected HepG2 cell line than control vector and null-transfected HepG2 cell line.

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## Materials and Methods

*Cell culture experiments*

Human adenocarcinoma cell line HepG2 (ATCC, CCL-185) was cultured in RPMI-1640 medium (DMEM, with 15% fetal bovine Serum), 100 U/ml streptomycin and 100 U/ml penicillin (Invitrogen) at 37°C in a humidified 5% $CO_2$ 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 hepatocellular carcinoma HepG2 cell line were selected from susceptible cell line, in each generation, the resistance level has been maintained by amycin treatment at $IC_{50}$ level. The $IC_{50}$ of drug-resistant human hepatocellular carcinoma HepG2 cell line is 2.76 μg/ml, 79-fold greater than that in the susceptible strain (0.035 μg/ml).

*RNA extraction*

Total RNA of cultured drug-resistant HepG2 cells and susceptible HepG2 cells was isolated using Trizol reagent.

*Real-time RT-PCR*

Real-time quantitative RT-PCR was done by LightCycler-RNA amplification Kit. By using of 3 purified RNA samples independently, the reaction was repeated; by using of LightCycler software (version 3.3), the threshold cycle number was determined. The experiment primers: RPL24 (forward: 5'-GGACTCACGGCGCTCATTAATTC-3', reverse: 5'-GACACGTGCGACACGACACC-3') and β-action (forward: 5'-ActccatcctgTgcctcgctt-3', reverse: 5'- GTTCTTCATCCAATTGCCTCCCT-3'). In one LightCycler run, the experiment was repeated three times in order to confirm the real-time RT-PCR accuracy and reproducibility. The ribosomal protein L24 quantitative RT-PCR result was normalized by β-actin gene. The overexpression fold was calculated (formula: 2 (Rt-Et)=2 (Rn-En), Rt: the threshold cycle number for the β-action gene in resistant strain; Et: RPL24 gene threshold cycle number in resistant strain; Rn: the threshold cycle number for the β-action gene in susceptible strain; En: RPL24 gene threshold cycle number in susceptible strain. Five-fold expression level were considered overexpression in experiment samples.

*Expression vector Construction*

After the specific primers were designed, the entire RPL24 coding region were PCR amplified.The reverse primer been used in this experiment was removed the original stop codon, in order to maintain the reading frame through the DNA encoding C-terminal peptide. Forward primer: 5'-TCGCTCTATGTCGGCCCACAATAA-3'; reverse primer: 5'-AATATCAGCTTTCTAGCTTGCGACGC-3'. The PCR conditions were: 94°C for 5 min, then 30 cycles of 94°C for 40 s, 57°C for 50 s, 72°C for 1 min, then 72°C for 10 min. The PCR product was electrophoresised and purified by quick Gel extraction kit of Qiagen. With T4 DNA ligase to the pcDNA3.1 (+) vector, the purified PCR product was ligated, and the reaction solution was transformed into HepG2 cells. By restriction analysis of recombinants with BamH I and Hind III, the positive clones were identified by PCR with specific primers and vector primers after selection. Then the RPL24/pcDNA3.1 (+) expression plasmid were been verified accuracy by further sequencing.

*Cell culture and stable transfection*

Prior to transfection, human adenocarcinoma cell line HepG2 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. When reached 50-60% confluence, the HepG2cells were transfected according to the manufacturer's instructions using Lipofectamine. Briefly, The day before transfection, trypsinize and count the cells. In each Plate well, 4 x 104 cells were isolated in 0.5 ml of complete growth medium. On the day of transfection, the cell density should reach 50~80% confluent. 0.5 μg DNA was diluted into 100 μl of Opti-MEM® I Reduced Serum Medium without serum for each well of transfected cells, and 1.5-2.5 μl of Lipofectamine® LTX was diluted into the above diluted DNA solution, gently mixed, then incubate to form DNA-Lipofectamine® LTX complexes at room temperature for 25 minutes.Remove growth medium from cells and replace with 0.5 ml of complete growth medium. 100 μl of DNA-Lipofectamine® LTX complexes were added directly into each wel, mix gently by rocking the plate back and forth. For the following transfection, the complexes do not have to be removed. Incubate the cells at 37°C, in $CO_2$ incubator for additional 48 h before being harvested for RT-PCR.

Stable expression cell lines were created once the cells were confirmed express the expected protein. The cell line sensitivity were been tested (0.032 μg/ml amycin, which can kill cells within one week). Forty-eight hours post-transfection, remove the transfection solution, add fresh medium without amycin, then the cells were splited (1:5 ratio), allowed to attach for 20 min, then the selective medium was added. The cells were incubated at 37°C after themedium was changed into medium containing 0.053 μg/ml amycin. Eevery 3-4 days, the selecting medium was replaced, until the clones were observed. Eight days later, the medium was replaced with medium containing 0.032 μg/ml amycin. By using dilution method in 96-well microtiter plate, the resistant cell lines were isolated, until only one colony was found, and the plate was incubated. The cells were harvested when the colony filled most area of the well, and the cells were transferred to a 24-well plate with 0.5 ml fresh medium (0.002 μg/ml amycin), the clone was expanded, by the sequence of 12- well plates, 6-well plates and T-25 flask finally. RT-PCR and Western blotting were used for the cell expression analysis.

*Total RNA Isolation and RPL24 transcript RT-PCR analysis*

The total RNA of RPL24 was isolated by using Trizol reagent from transfected cells. In each sample, 5mg isolated total RNA was used for first-strand cDNA synthesis as a template. The cDNA was synthesized at 70 °C for 5 min, and 0°C for 5 min, then 37°C1.5 h with a random primer using Avian myeloblastosis virus (AMV) reverse transcriptase (TaKaRa). At 99 °C for 5 min, the reverse

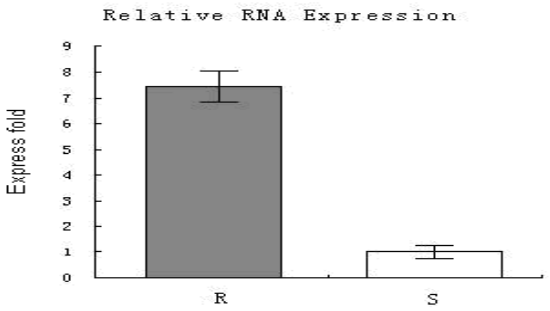


**Figure 1. Quantitative RT-PCR assay of RPL24 mRNA in Resistant and Susceptible Strains of HepG2 Cell Line.** Quantitative RTPCR was performed by using a Lightcycle-RNA amplification Kit SYBR Green and repeated using three independently purified RNA samples. The enhancement of fluorescence was found to be proportional to the initial concentration of template cDNA. RPL24 transcript copy numbers were normalized based on expression of the housekeeping β -actin from respective strains. R represents drug-resistant strain and S represents a susceptible strain. The data are presented as means±SD. n=3, $p$=0.03

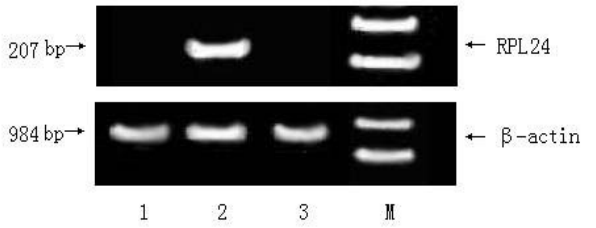


**Figure 2. RT-PCR Analysis of RPL24 mRNA in HepG2 Cells using Forward Primer of RPL24 and Reverse Primer of Vector (1: null cell; 2: RPL24 Transfected Cell; 3: Null Vector).** The production of the transcripts was detected in cells transfected with RPL24 (lane 2); No signal was detected either in normal cells (lane 3) or in cells transfected with vector (lane 1)

transcriptase was inactivated. The forward gene-specific primer (5'-ATGTCGGCCCACAAAACGTCTC-3') and the reverse vector primer (5'-TAGAGCGCACAGTCAAGGCTA-3') in RPL24 gene amplification were been used for transcriptional express confirmation. The primers used for β-actin PCR amplification was: forward: 5'-CTCCATCCAGGCCTCGTTGT-3', reverse: 5'- GGTTCATCTCCAATGCCTGCTT -3'. The RT reaction product (1mg) was used for routine PCR as the reaction template. The cycling parameters: 95°C for 5 min followed by 26 cycles of 94°C for 40 s, 57 °Cfor 50 s and 72°C for 1 min, followed by a final extension step of 72°C for 10 min.

*3H-TdR incorporation*

HepG2 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, the HepG2 cells were washed with 0.05 M PBS (pH 7.4) three times, and detached from the microtiter wells by trypsinization. Detached HepG2 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 HepG2 (cell associated 3H-TdR) was determined using a Wallac 1414 (WALLAC, Finland) liquid scintillation counter according to the manufacturer's instructions. The relative viability was calculated in treated cells by contrast of control cells (HepG2 cells transfected with vector and null-transfected HepG2 cells) as the ratio of 3H-TdR reduction. 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= (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 RT-PCR*

In order to analysis the amplification fold of RPL24 gene, real-time quantitative RT-PCR was used in drug-resistant human hepatocellular carcinoma HepG2 cell line and in susceptible HepG2 cell line. In order to determine the relative copy numbers, the cycle number of RPL24 was normalized against β-actin cycle number by which the amplification reached the threshold between drug-resistant human hepatocellular carcinoma HepG2 cell line and susceptible HepG2 cell line. The RPL24 exhibited 7.7-fold higher level of transcription in drug-resistant human hepatocellular carcinoma HepG2 cell line than in susceptible HepG2 cell line. The results suggested that RPL24 expression was up-regulated in drug-resistant human hepatocellular carcinoma HepG2 cell line (Figure 1).

*Transcription and expression of RPL24 in HepG2 cells*

After stable transfection cell line were gained, total RNA was isolated from control cells and RPL24-transfected cells, and RT-PCR was performed using forward RPL24-specific primer and the reverse vector-specific primer. A pair of β-actin primers of HepG2 cell line was used to ensure the validity of the reaction system. A 207 bp product, was observed in RPL24 gene transfected cells only (Figure 2), confirming that RPL24 had been transcribed in the transfected cells.

*Viability assay*

To investigate the RPL24 expression in relation to amycin resistance, HepG2 cell line stably transfected with RPL24 or control vector and null-transfected HepG2 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. HepG2 cell line that is transfected with either RPL24 or control vector gene or

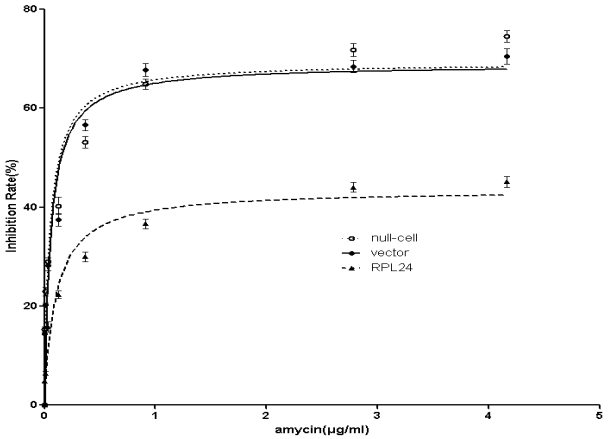


**Figure 3. Inhibitive Effect of Amycin on Cell Viability.** The cultured cells were treated with different concentration of amycin and incubated for 72 h. The inhibiting effect (E) of the amycin on the cell viability was described by the equation: E= (Emax*C) / ($EC_{50}$+C)

null-transfected HepG2 cells were treated with various concentrations of amycin (0, 0.003, 0.012, 0.057, 0.196, 0.425, 2.187, 3.215, 4.374 μg/ml) for 72 h and cell survival was analyzed according to 3H-TdR incorporation in order to observe the cell viability. The $EC_{50}$ and 95% confidence intervals of null-transfected cells are 0.053 (0.032-0.074), the $EC_{50}$ and 95% confidence intervals of vector-transfected cells are 0.058 (0.038-0.078), and the $EC_{50}$ and 95% confidence intervals of RPL24 transfected cells are 0.103 (0.073-0.134) (Figure 7). Obvious cell viability augmentation was observed in the RPL24 transfected HepG2 cells compared to null-transfected or vector-transfected cells (Figure 3).

## Discussion

The recurrence of hepatocellular carcinoma metastasis is an important factor affecting the long-term survival of human, tumor recurrence or distant metastasis will occurr in more than 70% of clinical curative surgical resection or liver transplantation patients with hepatocellular carcinoma (Llovet, 2014). Above 90% of death factor were related to tumor metastasis and recurrence. The clinical correlation study has revealed the tumor size, number, vascular invasion and disseminated lesions were important prognostic factors in hepatocellular carcinoma (Huang et al., 2013; Xu et al., 2014), but clinical practice suggests that the clinical and pathological features of tumor, such as size and number, cannot predict the metastatic potential of tumor recurrence accurately. Biological characters of tumors may be the real key of recurrence and metastasis. Previous studies have gained large number of molecular markers of hepatocellular carcinoma recurrence and metastasis (Enooku et al., 2012; Hou et al., 2013). But because of the complexity of the recurrence and metastasis of molecular regulatory networks, single hepatocellular carcinoma molecular markers have limited value of interpretation and intervention of recurrence and metastasis mechanism. A lot of molecular information can be obtained by the use of high off chip technology, some molecular labels has been found which can represent for the different biological behavior of liver cancer, some key genes and proteins was the key of later research. From the tiny RNA (microRNA) study, miRNA spectra of hepatocellular carcinoma recurrence and metastasis has been found recently (Wang et al., 2014), which also tells us that all the stages were involved in the regulation of hepatocellular carcinoma biological characteristics, such as gene transcription, post transcription regulation and protein expression.

Chemotherapy was the most common used method for the liver cancer treatment despite surgical operation (Kataoka et al., 2014) but the multiple drug resistance has become the biggest obstacle in hepatocellular carcinoma treatment. Research in the gene expression changes of liver cancer cell resistance to chemotherapy drugs mainly focused on multidrug resistance mechanism, include MDR genes and non MDR genes (Oishi et al., 2014). In this study, the results showed that the ribosomal protein L24 transcription level was 7.7 times higher in drug-resistant human hepatocellular carcinoma HepG2 cell line than in susceptible HepG2 cell line. Expression of RPL24 also increases the amycin resistance in susceptible HepG2 cell. Although we do not have enough information to pinpoint the exact amycin resistance role of RPL24, our results suggested that RPL24 was a good candidate for further research of amycin resistance.

As a ribosomal protein, RPL24 can influence the synthesis of a subset of proteins (Barkic et al., 2009). The altered translational profile may enhance the overall fitness of the cells and increase the tolerance of the cells, then and the organisms can against harmful chemicals, such as amycin. It has been reported the expression of ribosomal proteins was important for mitochondrial biogenesis (Haque et al., 2010). 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 RPL24 mediates amycin resistance. RPL24 may also help to increase the expression of other proteins to help to degrade amycin. Proteomic study to characterize those target proteins was needed to elucidate the mechanism of RPL24-dependent amycin resistance.

Since RPL24 is likely to be 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 RPL24 can confer resistance against a broad range of antibiotics. In addition, the highly conserved nature of RPL24 also suggests that the antibiotic resistance associated RPL24 is present in a variety of species. The study of RPL24 functions has a broad application.

In summary, we have studied the primary function of RPL24 in drug resistance field. 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 RPL24 may confer some amycin resistance in human hepatocellular carcinoma HepG2 cell line. Research results had provided a basis for further studies on the gene function of antibiotic resistance, which will improve our understanding of the molecular basis of ribosomal protein L24 mediated resistance in human carcinoma cell line.

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