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*Original Article*

# Long non-coding RNA TUG1 regulates the development of multidrug resistance in hepatocellular carcinoma via P-gp and MDR1

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**Abstract:** Multidrug resistance (MDR) is a significant impediment to the treatment of hepatocellular carcinoma (HCC), which leds to increasing resistance to different drugs. Taurine up-regulated gene 1 (TUG1), a 7.1-kb long non-coding RNA (lncRNA), was found to be up-regulated in HCC and promoted cell growth and apoptosis. However, its clinical significance and potential role in MDR in HCC remain unclear. In the present study, we identified the mRNA levels of TUG1 in HCC tissues of adriamycin-resistant patients and two kinds of adriamycin-resistant cells SMMC-7721/ADM and HepG2/ADM by qRT-PCR. After knockdown or overexpression of TUG1, the anticancer drug resistance was assessed by testing the cytotoxicity of adriamycin and apoptosis rate utilized by CCK-8 assay and flow cytometry, respectively. The expression level of several genes related to apoptosis, PARP and Caspase-3, were also detected by western blotting. Furthermore, we investigated the expression levels of P-gp and MDR1 by qRT-PCR and western blotting. TUG1 was upregulated in HCC tissues of adriamycin-resistant patients and adriamycin-resistant cells. Moreover, SMMC-7721/ADM and HepG2/ADM cells transfected with TUG1 siRNA and treated with adriamycinled to the lower survival rate and the higher tumor cells apoptosis. While, TUG1 overexpression led to the resistance to apoptosis in SMMC-7721 and HepG2 cells transfected with pcDNA-TUG1 and treated with adriamycin. Furthermore, we found that TUG1 down-regulation inhibited the expression of P-gp and MDR1. Taken together, knockdown of lncRNA TUG1 in HCC can effectively suppress the development of MDR by targeting P-gp and MDR1, suggesting a promising therapeutic target for reversing MDR in HCC therapy.

**Keywords:** Long non-coding RNA (lncRNA), TUG1, multidrug resistance (MDR), hepatocellular carcinoma (HCC), P-gp, MDR1

## Introduction

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related deaths worldwide [1]. With improvements of medical standard, chemotherapy, radiotherapy and surgical techniques have been applied for HCC over the past few decades and death rate of HCC was low now. Chemotherapy is the most frequently used primary medical treatment for HCC [2]. However, the prospect for HCC patients was not really optimistic, which is caused by the development of resistance to multiple chemotherapeutic agents after continuous single chemotherapy. It was reported that several drug resistance common mechanisms involves changes in drug export, metabolism, apoptosis, DNA repair, cell survival and other cell functions [3-7]. In addition, several studies have shown that some factors such as fucosyltransferase 4 (FUT-4) can mediate the multidrug resistance of human HCC [8]. Still, the chemo-resistance of HCC remains largely unexplored.

Long non-coding RNA (lncRNA), which are defined as being longer than 200 nucleotides lack of significant open reading frames, play an important role in tumorigenesis [9], cancer cells growth [10], apoptosis [11] and metastasis [12]. Taurine up-regulated gene 1 (TUG1), a 7.1-kb lncRNA, was firstly detected in a genomic screen for genes up-regulated in response to

taurine treatment of developing mouse retinal cells [13]. Inhibition of TUG1 results in accelerating apoptosis. Several researchers have observed that TUG1 can stimulate the cell proliferation of osteosarcoma [14] and urothelial carcinoma of the bladder [15]. TUG1 also has been reported to be down-regulated in non-small cell lung carcinoma (NSCLC) [16]. In addition, TUG1 was found to be up-regulated in HCC and promoted cell growth and apoptosis [17]. However, the role of TUG1 in multidrug resistant HCC remains largely unexplored.

In this study, we aim to explore the role of lncRNA TUG1 in adriamycin resistance HCC and further investigate the effects of TUG1 on the expression of genes associated with multidrug resistant, including P-gp and MDR1.

## Materials and methods

This study was approved by the ethical committees of the School of Medicine Zhejiang University and all animal experiments were conducted under the guidelines on humane use and care of laboratory animals for biomedical research pblished by Mational Institutes if Health (No. 85-23, revised 1996).

### *Tissue samples from HCC patients*

A total of 80 patients with primary HCC were enrolled in the study. The HCC diagnosis was histopathologically confirmed. In addition, none of the patients received any preoperative therapy. HCC clinical stage was determined according to the BCLC staging classification. After resection and stored at -80°C, fresh samples were snap-frozen in liquid nitrogen in no time. Non-tumor specimens were obtained from the resected tumor specimen as farthest as better. The tumor tissue samples were collected from the above patients. This study received approval from the ethics committee of *** Hospital, and all patients provided written informed consent.

### *Cell lines and culture*

Human HCC cell lines SMMC-7721 and HepG2 purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) were cultured in RPMI 1640 medium(Hyclone, USA) supplemented with 10% of fetal bovine serum (Hyclone, USA), 100 U/ml of penicillin and 100 mg/ml of streptomycin. The cells were grown at 37°C in a humidified incubator with 5% $CO_2$. Adriamycin was obtained from Shanghai Shenggong Biological Engineering Co., Ltd. (Shanghai, China). The adriamycin-resistant SMMC-7721/ADM cells were developed from the parental SMMC-7721 cells that were subjected to persistent exposure to adriamyc in with a stepwise increasing concentration from 0.05 to 2 μg/ml over several months, until the cells could survive and be subcultured in the culture medium containing 1 μg/ml. The adriamycin-resistant HepG2/ADM cells were obtained by the same way. Prior to each experiment, SMMC-7721/ADM and HepG2/ADM cells were cultured in drug-free RPMI 1640 medium for 1 week.

### *Plasmids and transfection*

TUG1-overexpression lentiviral vector (pcDNA-TUG1) and control lentiviral vector (pcDNA-3.1) purchased from Shanghai Cancer Institute, China. Three different siRNAs against TUG1 gene were designed to specifically knock down TUG1 expression to avoid off-target effects and ensure the efficiency of interference. TUG1 siRNAs were as follows: siTUG1-1: sense 5'-CUACAACUAUCUUCCUUUACCACCG-3' and antisense 5'-CGGUGGUAAAGGAAGAUAGUUGUAGCA3'; siTUG1-2: sense 5'-CACGACCAUGGUUGUCAUCCATT-3' and antisense 5'-UGGAUGACAACCAUGGUCGUGTT-3'; siTUG1-3: sense 5'-CAGCUGUUACCAUUCAACUUCUUAA-3' and antisense 5'-UUAAGAAGUUGAAUGGUAACAGCUG-3', which were synthesized (Ribobio, Guangzhou, China). SMMC-7721 and HepG2 cells (or SMMC-7721/ADM and HepG2/ADM cells) were seeded in six-well plates with antibiotic-free medium and transfected with specific siRNA oligonucleotides by using Lipofectamine RNAi MAX, according to the manufacturer's protocol (Invitrogen, USA). After 24 h transfection, the cells were purified by ultracentrifugation (4000× g) at 4°C for 10 min and then filtered through 0.45 μm filter.

### *CCK-8 assay*

Cell Counting Kit-8 (CCK-8) assay was performed to detect the cytotoxicity of gene transfection. After transfected si-TUG1 or pcDNA-TUG1 into SMMC-7721/ADM and HepG2/ADM, cells were seeded in 96-well plates, each well of which contained 100 ml PRMI-1640 medium

supplemented with 10% FBS ($5\times10^4$ cells/well). And then, cells were treated with 1 μg/ml adriamycin. After culturing for 24 h, 36 h and 48 h, 10 μl of CCK-8 reagent was added and incubated at 37°C for 1 h in humid atmosphere containing 5% $CO_2$. The optical density (OD) was read at 450 nm using Microplate Reader (BioRad, USA).

*Flow cytometry for cell apoptosis analysis*

Cells transfected with si-TUG1, pcDNA-TUG1 or respective control were harvested 48 h and collected. Then, cell pellet were incubated in a solution containing 5 μl FITC-Annexin V and 1 μl of Propodium iodide (PI, 50 μg/ml). Flow cytometric evaluation was conducted within 5 min. The cells were examined by flow cytometry using an EPICS XL-MCL FACS can (Bectone-Dickinson, USA). The experimental data was analyzed with the MultiCycle Software for Windows (Phoenix Flow Systems, USA). Plots in the lower left quadrant present the viable cells and in the right quadrant present the apoptotic cells on the dual parameter fluorescent dot.

*Protein extraction and Western blotting analysis*

Cells were washed twice with ice-cold PBS and lysed in 2× SDS Sample Buffer which contained 100 mM Tris-HCl (pH 6.8), 10 Mm EDTA, 4% SDS and 10% Glycine. Equal amounts (15 μg) of protein were loaded onto a 10% SDS-PAGE and electrophoresis at 80 V for 30 min and 150 V for 1 h. The gel was transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked by 1% bovine serum albumin (BSA) in TBST at room temperature for 1 h and then probed with primary antibodies overnight. Primary antibodies used were as below: anti-PARP (Abcam, England); anti-Caspase-3 (Abcam, England); anti-P-gp (Abcam, England); anti-β-actin (Sigma, USA). After washed by TBST, membranes were incubated with horseradish peroxidase conjugated secondary antibodies for 2 h at room temperature. The membrane was washed by TBST and signals were detected by enhanced chemiluminescence (ECL).

*Quantitative real-time PCR*

Total RNAs was extracted from human HCC cell lines using Trizol reagent (Invitrogen, CA, USA). Then, single strand cDNA was obtained using M-MLV reverse transcriptase kit (Promega, USA) for quantitative real-time PCR (qRT-PCR) analysis to examine protein expression levels. The TUG1 primers were as follows: forward 5'-CAAGAAACAGCAACACCAGAAG-3' and reverse 5'-TAAGGTCCCCATTCAAGTCAGT-3'. The MDR1 primers were as follows: forward 5'-ACCAAGCCGCTCCGATACA-3', and reverse 5'-TCATTGGCGAGCCTGGTAGTC-3'. β-actin was used as internal control and primers were as follows: forward 5'-GTGGACATCCGCAAAGAC-3', and reverse 5'-AAAGGGTGTAACGCAACTA-3'. PCR was carried out by PCR reaction mixture containing 10 μl of 2× SYBR premix EX Taq, 0.8 μl of forward and reverse primers (2.5 μM), 5 μl of cDNA (2 ng), 4.2 μl of $ddH_2O$. Cycling conditions were a denaturation at 95°C for 1 min, 40 cycles of annealing at 60°C for 20 s and extension at 72°C for 10 min using BioRad-connet Real-time PCR platform. The expression levels were calculated using $2^{-\Delta\Delta CT}$ formula.

*Statistical analysis*

All data were expressed as mean ± SD of at least three independent experiments. Statistical comparisons were assessed using the Student's t-test on SPSS 18.0 software. Values of $P < 0.01$ were considered as statistically significant difference.

## Results

*TUG1 involves the development of adriamycin resistance*

To investigate whether TUG1 is associated with the development of adriamycin resistance in HCC tissues, we detected the mRNA levels of TUG1 in the HCC tissues of adriamycin-sensitive patients (n=20) and adriamycin-resistant patients (n=20). The results of the sample for the test have shown that significantly increased mRNA level of TUG1 was observed in the HCC tissues of adriamycin-resistant patients comparing to adriamycin-sensitive patients ($P < 0.01$) (**Figure 1A**). To verify this differential expression of TUG1, we detected the TUG1 expression in two kinds of adriamycin-resistant cells SMMC-7721/ADM and HepG2/ADM, which were developed from the parental SMMC-7721 cells and HepG2 cells, respectively. Consistent with the results in HCC tissues, as shown in **Figure 1B**, high levels of TUG1 was

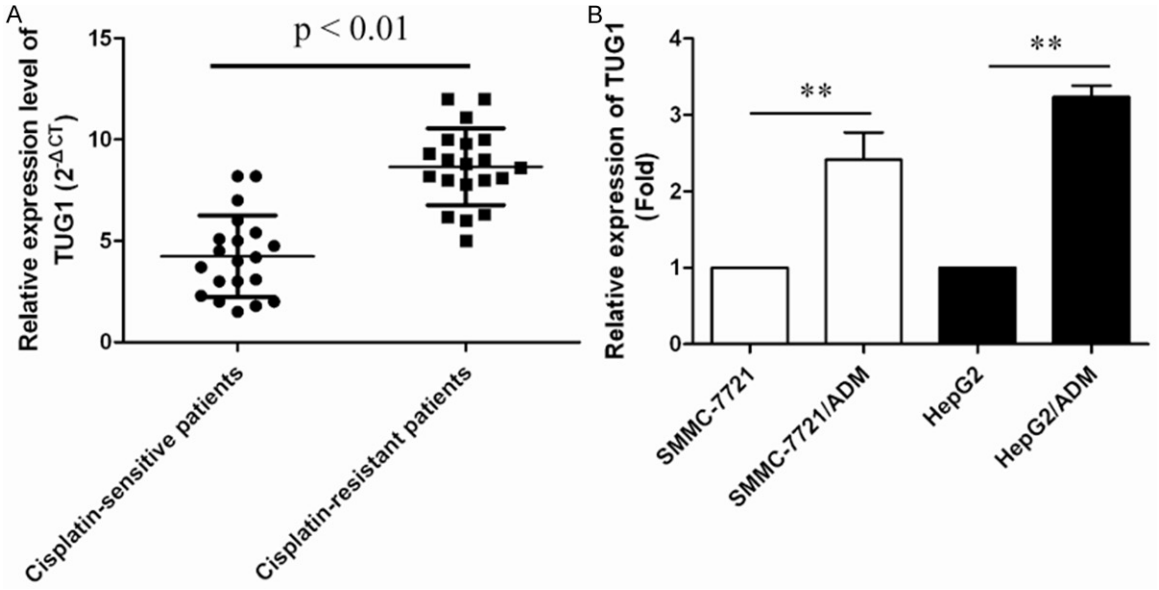


**Figure 1.** TUG1 is associated with the adriamycin-resistance. A. The mRNA levels of TUG1 in gastric cancer tissue of adriamycin-sensitive patients and adriamycin-resistant patients. B. The mRNA levels of TUG1 in the adriamycin-resistant SMMC-7721 and HepG2 cells. All values are mean ± SD. ** vs. control, P < 0.01.

observed in adriamycin-resistant cells SMMC-7721/ADM and HepG2/ADM (P < 0.01). Taken together, these results demonstrated that TUG1 may be involvethe development of adriamycin resistance in HCC.

*Knockdown of TUG1 reverses the adriamycin resistance in adriamycin-resistant HCC cell lines*

Based on the above results, we tested the effect of TUG1 knockdown on adriamycin-induced cytotoxicity and apoptosis in SMMC-7721/ADM and HepG2/ADM cells. As shown in **Figure 2A**, si-TUG1-1, si-TUG1-2 and si-TUG1-3 significantly decreased the expression of TUG1 in SMMC-7721/ADM and HepG2/ADM cells and si-TUG1-1 group had more obvious influence. After transfected siTUG1, SMMC-7721/ADM and HepG2/ADM cells were treated with adriamycin for 24 h, 36 h and 48 h, and CCK-8 assay was utlized to examine the cellular activity. As shown in **Figure 2B**, after transfected siTUG1, cells had a remarkedly lower cell viability than that in si-Scramble group, and cellular survival rate clearly reduced with time of adriamycin treatment (P < 0.01). Moreover, we utilized flow cytometry assay to examine the percentage of apoptotic tumor cells. After stained with Annexin-V and PI, SMMC-7721/ADM and HepG2/ADM cells transfected siTUG1 and treated with adriamycin were analyzed. The results show that increased apoptotic cells were detected in siTUG1 group than that in si-Scramble group (**Figure 2C**). Furthermore, western blotting analysis was utilized to detect the protein level of several genes related to apoptosis. As shown in **Figure 2D**, the expression level of PARP and caspase-3 was notably enhanced in the si-TUG1 infected HCC cells (P < 0.01). These results indicate that siTUG1 reverses the adriamycin resistance in adriamycin-resistant HCC cell lines.

*Overexpression of TUG1 inhibits apoptosis in HCC cell lines*

To have further elucidation the role of TUG1, we further examined the effect of TUG1 overexpression on cell apoptosis in HCC cell lines. As shown in **Figure 3A**, transfection of pcDNA-TUG1 into parental SMMC-7721 and HepG2 cells led to marked enhancement of TUG1 mRNA. These results confirmed that SMMC-7721 and HepG2 cells transfected with pcDNA-TUG1 showed the upregulation of TUG1 mRNA expression. After transfected pcDNA-TUG1, cells had a remarkedly higher cell viability than that in pcDNA-3.1 group (P < 0.01) (**Figure 3B**). Next, we examined the effect of pcDNA-TUG1

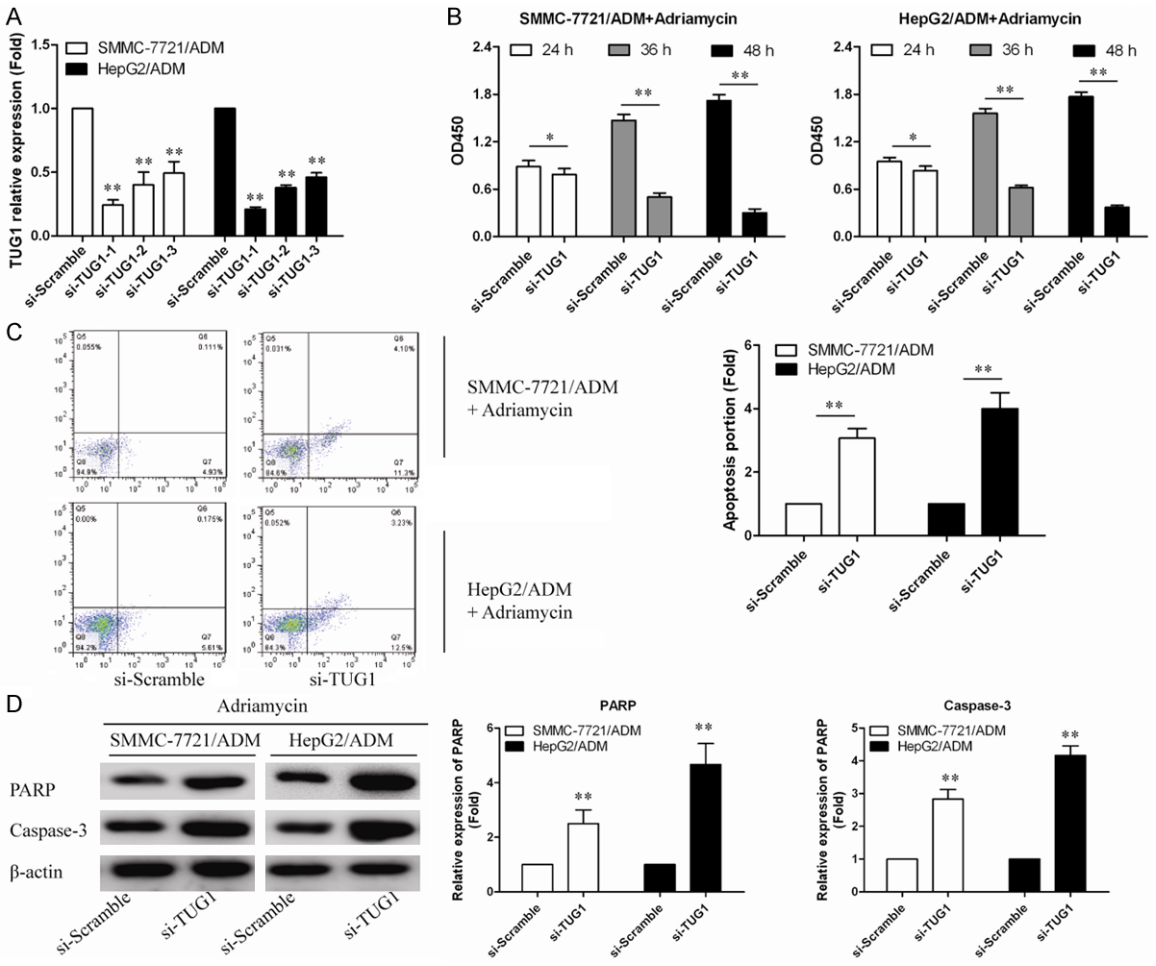


**Figure 2.** TUG1 knockdown reverses the adriamycin-resistance in adriamycin-resistant gastric cancer cells. A. The efficiency of TUG1 small RNA on the mRNA level of TUG1. B. The effect of TUG1 knockdown on cell viability of SMMC-7721/ADM and HepG2/ADM cells transfected with siTUG1 and treated with adriamycin. C. Percentage of apoptotic cells was analyzed by flow cytometry. D. The expression level of PARP and caspase-3 after si-TUG1 infected HCC cells. All values are mean ± SD. ** vs. control, $P < 0.01$.

expression on the apoptosis of HCC cells. The results of flow cytometry revealed that parental SMMC-7721 and HepG2 cells transfected pcDNA-TUG1 exhibited a significantly decreased apoptosis index compared to the pcDNA-3.1 groups ($P < 0.01$) (**Figure 3C**). As shown in **Figure 2D**, the expression level of PARP and caspase-3 was significantly reduced in the pcDNA-TUG1 infected HCC cells ($P < 0.01$). Together, these data indicate that overexpression of TUG1 inhibits adriamycin-induced apoptosis in parental SMMC-7721 and HepG2 cells.

*TUG1 influenced the expression of P-gp and MDR1*

To investigate the mechanism by which TUG1 regulate the adriamycin resistance of HCC cells, we examined the expression levels of several MDR-related proteins (P-gp and MDR1) by western blotting and qRT-PCR. The expression level of P-gp and MDR1 in SMMC-7721/ADM and HepG2/ADM cells transfected si-TUG1 were significantly lower than that in si-Scramble (**Figure 4A**, **4B**). On the contrary, as shown in **Figure 4C**, **4D**, the expression levels of P-gp and MDR1 both increased in parental SMMC-7721 and HepG2 cells transfected pcDNA-TUG1 than that in pcDNA-3.1. These results indicate that down-regulation of TUG1 inhibited the development of multidrug resistant in HCC via regulating the expression of multidrug resistant related gene P-gp and MDR1.

## Discussion

HCC is one of the major leading causes of cancer-associated mortality worldwide [18]. There

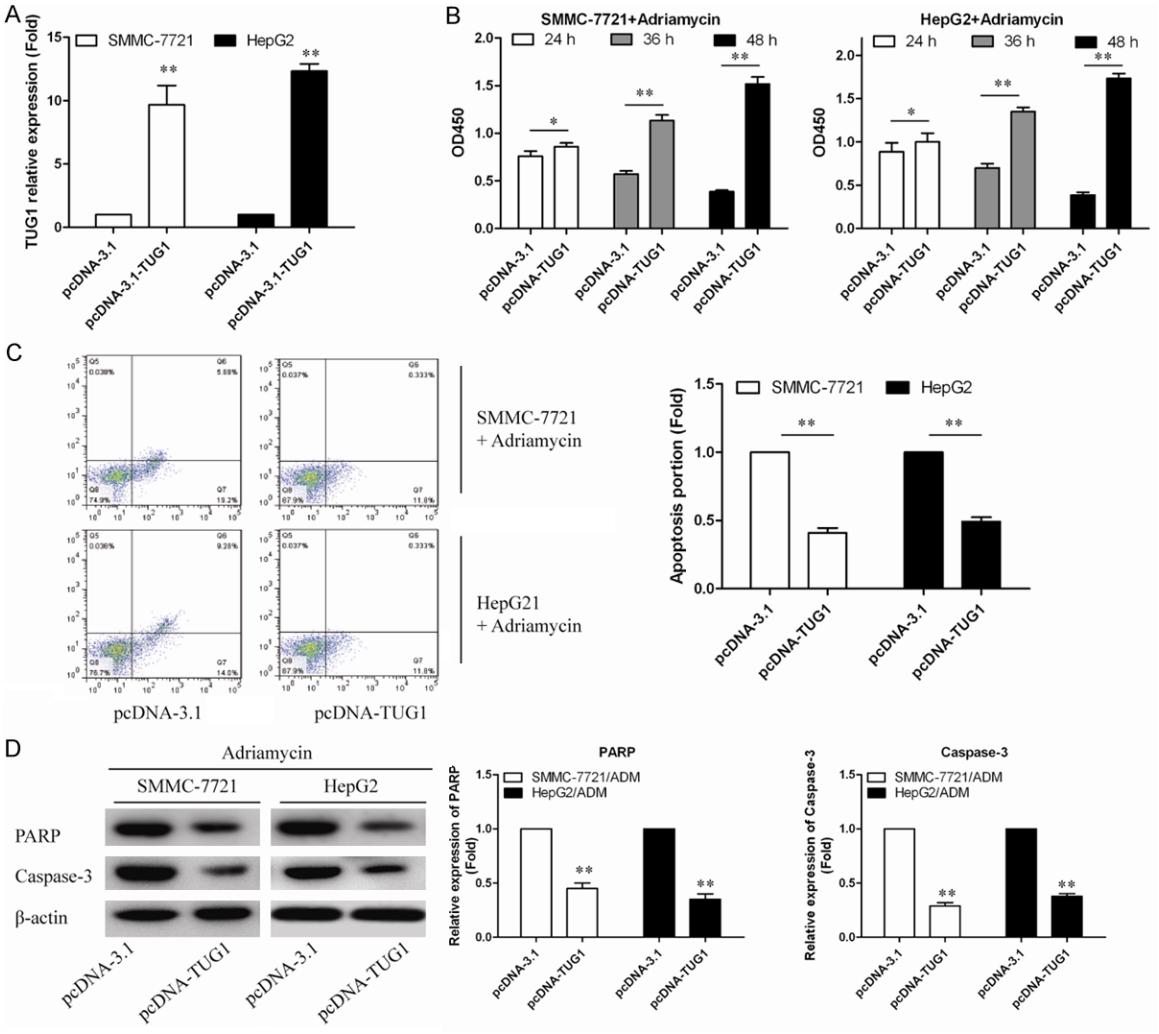


**Figure 3.** Overexpression TUG1 inhibits apoptosis in the adriamycin-resistant HCC cells. A. Cells transfected pcDNA-TUG1 plasmid overexpressed TUG1. B. The effect of TUG1 overexpression on cell viability of SMMC-7721 and HepG2 cells transfected with pcDNA-TUG1 and treated with adriamycin. C. Percentage of apoptotic cells was analyzed by flow cytometry. D. The expression level of PARP and caspase-3 after pcDNA-TUG1 infected HCC cells. All values are mean ± SD. ** vs. control, $P < 0.01$.

have been significant advances in clinical diagnosis and bringthe HCC under control and lots of therapeutic strategies including chemotherapy, radiotherapy and surgical techniques have been improved [19]. The treatment prospects of HCC is not optimistic, however, the 5-year overall survival rate remains very poor. MDR is a significant impediment to the treatment of HCC, which leads to increasing resistance to diffenent drugs [20]. Adriamycin, as an important drug used in HCC chemotherapy, could trigger apoptosis by inducing DNA damage [21]. However, adriamycin could lose their potency over time due to the development of multiple drug resistance in cancer cells. The mechanism for drug resistance is associated with a vary of pathological and physiological changes in the body. Nevertheless, the specific mechanism of MDR is acontinuous need to find out.

An increasing number of researches have concentrated on the lncRNAs which plays an important role in tumorigenesis, cancer cells growth, apoptosis and metastasis in human cancers, such as gastric cancer [22], breast cancer [12], prostate cancer [23] and HCC [24]. LncRNAs exert their effects through regulating gene expression at the level of chromatin modification, transcription and post-transcriptional processing. It was found that the dysregulation of

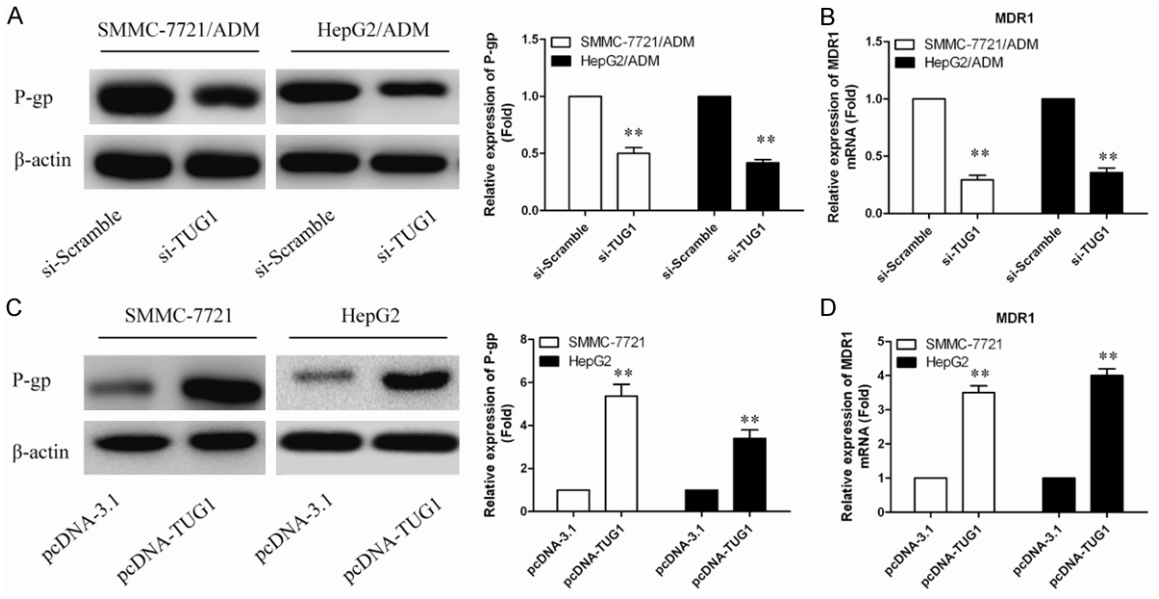


**Figure 4.** TUG1 influenced the expression of genes associated with apoptosis and multidrug resistant. A. The protein level of P-gp after TUG1 knockdown. B. The mRNA level of MDR1 after TUG1 knockdown. C. The protein level of P-gp after TUG1 overexpression. D. The mRNA level of MDR1 after TUG1 overexpression. All values are mean ± SD. ** vs. control, $P < 0.01$.

lncRNAs exercises a great influence in the clinicopathological symptoms and cancer prognosis [25]. Recent researches have shown that lncRNA MRUL is a potential target to reverse the MDR phenotype of MDR-HCC cell sublines, which could effectively promote the development of MDR in HCC cancers [26]. In the present study, we also found that lncRNA TUG1 involves the development of adriamycin resistance in HCC cell lines.

Here, we found the overexpression of TUG1 in HCC tissues of adriamycin-resistant patients and two kinds of adriamycin resistant cells SMMC-7721 and HepG2. Moreover, CCK-8 assay and flow cytometry analysis revealed that TUG1 knockdown remarkedly lowered the cellular survival rate and increased the percentage of apoptotic tumor cells in adriamycin-resistant cells transfected siTUG1 and treated with adriamycin, which indicated that TUG1 knockdown reverses the adriamycin resistance of adriamycin-resistant cells. To have further elucidation the role of TUG1, we further examined the effect of TUG1 overexpression on cell apoptosis in HCC cell lines. Overexpression of TUG1 exhibit the antiapoptotic property, which inhibited the apoptosis of parental SMMC-7721 and HepG2 cells treated with adriamycin. Furthermore, we detected the expression level of representative genes involved in multidrug resistant, including P-gp and MDR1. The results showed that P-gp and MDR1 expression were both increased after TUG1 overexpression.

It is noteworthy that cell apoptosis played a critical role in the development of MDR [27]. Against apoptosis-associated biological response might be the major influence factors for the failure in cancer treatment. Some research work has already showed the correlation between TUG1 and antiapoptotic activity [28]. However, this current study is limited and we could not tease out a specific molecular mechanisms of TUG1 in apoptosis unambiguously and further studies is absolutely essential. Several reports suggested that P-gpisone of the most criticalfactors being involved in MDR, which acts as an ATP-dependent efflux pump to convey anti-cancer drugs out of the cells [29, 30]. P-gp was encoded by the MDR1 gene [31] and MDR1 is a well characterized form of drug resistance. Up-regulation of P-gpcould contribute to a compromised chemotherapy response and prevent the intracellular accumulation of anticancer drugs, which is necessary for cytotoxic activity. Taken together, these results indicated that TUG1 might promote the development of MDRvia regulation of P-gp and MDR1.

In conclusion, these results reveal that TUG1 knockdown reversed the adriamycin resistance in adriamycin-resistant HCC cells and down-regulation of TUG1 inhibits the development of multidrug resistant in HCC via increasing the expression of multidrug resistant related gene (p-gp and MDR1). Further in-depth studies are recommended to fully understand the molecular mechanisms of TUG1 and MDR in HCC. Such breaking-through discovery offers a platform for researchers to widen therapeutic method in HCC treatments.

## Disclosure of conflict of interest

None.

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