TIGAR has a dual role in cancer cell survival through regulating apoptosis and autophagy

Running title: TIGAR inhibits apoptosis and autophagy

Jia-Ming Xie¹,* , Bin Li³,* , Hong-Pei Yu¹ , Quan-Geng Gao³ , Wei Li¹ , Hao-Rong Wu¹,# , Zheng-Hong Qin²,#

¹Department of General Surgery, the Second Affiliated Hospital of Soochow University, Suzhou 215006, China

²Department of Pharmacology and Laboratory of Nervous Diseases, Jiangsu Key Laboratory of Translational Research and Therapy for Neuro-Psycho-Diseases, Soochow University School of Pharmaceutical Science, Suzhou 215123, China

³Department of General Surgery, the first hospital of Wu Jiang, Suzhou 215200, China

*: These authors contribute to this work equally.

#: Corresponding authors:

Hao-Rong Wu, MD

Department of General Surgery

The Second Affiliated Hospital of Soochow University

1055 San Xiang Road, Suzhou, 215006, China

Phone: 86-512-67783308

Fax: 86-512-67783308
Email: wuhaorong@vip.sina.com

Zheng-Hong Qin, PhD

Department of Pharmacology and Laboratory of Aging and Nervous Diseases

Soochow University School of Pharmaceutical Sciences

199 Ren Ai Road, Suzhou 215123, China

Phone: 86-512-65882071

Fax: 86-512-65882071

Email: qinzhenhong@suda.edu.cn

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Abstract

The p53-induced glycolysis and apoptosis regulator (TIGAR) inhibits glycolysis, resulting in higher intracellular NADPH, lower reactive oxygen species (ROS) and autophagy activity. In this study, we investigated whether TIGAR might exert dual impacts on cancer cell survival based on its ability to inhibit both apoptosis and autophagy. In liver or lung cancer cells treated with the anticancer drug epirubicin, TIGAR levels increased in a dose and time dependent manner. TIGAR silencing enhanced epirubicin-induced elevations in ROS levels and apoptosis rates, in a manner that was blocked by ectopic addition of NADPH or N-acetyl cysteine. These findings were correlated with reduced tumorigenecity and increased chemosensitivity in mouse xenograft tumor assays. In parallel, TIGAR silencing also enhanced the epirubicin-induced activation of autophagy, in a manner that was also blocked by ectopic addition of NADPH. Notably, TIGAR silencing also licensed epirubicin-mediated inactivation of the mTOR pathway, suggesting TIGAR also exerted a negative impact on autophagy. However, genetic or pharmacological inhibition of autophagy increased epirubicin-induced apoptosis in TIGAR-silenced cells. Overall, our results revealed that TIGAR inhibits both apoptosis and autophagy, resulting in a dual impact on tumor cell survival in response to tumor chemotherapy.
Introduction

Tumor suppressor gene TP53 plays an important role in the regulation of cellular metabolism, specifically glycolysis and oxidative phosphorylation (OXPHOS) via transcriptional regulation of its downstream genes TP53-induced glycolysis regulator (TIGAR) and synthesis of cytochrome c oxidase (SCO2) (1;2). It has been known that cancer cells utilize glycolysis, which yields less ATP and can occur in hypoxic tissues which cannot obtain sufficient ATP through oxidation/ phosphorylation. A theory has been proposed to explain this phenomenon known as “the Warburg effect” (3). The role of TP53 in the regulation of energy metabolism through TIGAR and SCO2 provides new insights into the puzzles of cancer cell metabolism and strategies for cancer therapy (4).

TIGAR functions to lower fructose-2,6-bisphosphate (Fru-2,6-P$_2$) levels in cells, resulting in an inhibition of glycolysis (5). The TIGAR protein shows similarity to the bisphosphatase domain of PFK-2/FBPase-2 (6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase), an enzyme that has an essential function in the regulation of glycolysis (5;6). The concept of Warburg effect depict that cancer cells preferentially utilize the glycolytic pathway to produce ATP even in the presence of oxygen, thus the ability of TIGAR to inhibit cell glycolysis seems to be harmful for cancer cell survival. However, a number of recent studies have reported that
TIGAR expression was significantly elevated in human cancers such as glioblastoma (7), invasive breast cancers (8) and colorectal cancers (unpublished observations). The question which urgently needs to be addressed is why cancer cells need more TIGAR if their survival is dependent on glycolysis?

TIGAR also functions to decrease intracellular reactive oxygen species (ROS) levels through increasing NADPH generation. ROS-adaptive response may play a critical role in protecting cells against cytotoxic effects of anticancer agents (9;10) and high intracellular concentrations of GSH have been implicated in resistance to several chemotherapeutic agents (5). Bensaad et al reported that TIGAR protects cells from ROS-associated apoptosis (5). These may be the reason why the cancer cells need high levels of TIGAR for survival. On the other hand, TIGAR-mediated ROS reduction may limit autophagy activity. Considering autophagy can function to decrease ROS levels, inhibit apoptosis and support energy production in nutrient starvation or metabolic stress conditions (11), the impact of TIGAR-mediated autophagy inhibition in tumor cell survival remains to be investigated. In addition, whether TIGAR plays a role in autophagy regulation independent of ROS also needs to be defined. A more complete understanding the role of TIGAR in ROS and autophagy regulation will pave a new avenue for developing new chemotherapeutic drugs. The present study provides the
evidence showing that TIGAR has dual roles in chemo-sensitivity of tumor cells through inhibition of apoptosis and autophagy.

Materials and methods

Cell culture

Human lung cancer cell line A549 cells and human hepatocellular carcinoma derived HepG2 cells were obtained from the American Type Culture Collection and cultured with Dulbecco's modified Eagle medium (DMEM; GIBCO, 11965500) containing 10% fetal bovine serum (FBS; WISTEN INC, 086150008), 100 IU/ml penicillin and 100 IU/ml streptomycin in a humidified incubator at 37°C under 5% CO2 atmosphere, and passaged at pre-confluent densities by use of 0.25% trypsin solution every 2-3 days. Cells were stored and used within 3 months after resuscitation of frozen aliquots.

Measurement of Cell Viability

The short-term effects of epirubicin or TIGAR knockdown on tumor cell growth was assessed with 3- (4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, M5655) assay. Cells were cultured in 96-well plates (1000 cells per well) in complete DMEM. Cells were treated with 1.25-20 μg/ml epirubicin (Pfizer, 10 mg per bottle) or vehicle for 12-48 h and then subjected to MTT assay. Percentage of growth
inhibition was calculated as \( \frac{\text{OD}_{\text{vehicle}} - \text{OD}_{\text{treatment}}}{\text{OD}_{\text{vehicle}}} \times 100\% \), where OD was measured at 570 nm with a microplate reader (BieTek, Burleigh QLD, Australian). In each experiment, quintuplicate wells were used for each drug concentration, and assays were repeated in three independent experiments.

The long-term effects of TIGAR knockdown on tumor cell proliferation was analyzed with clony formation assay. Cells transfected with TIGAR siRNA after 12 h were plated in 6-well plates at a density of 200 cells per well and epirubicin was added 24 h later. Medium was changed every three days. After 14 days, the colonies were stained with Giemsa for 15 min and rinsed with deionized distilled water, photographed using a Nikon D800 digital camera (36.3 Mega-pixel; Nikon Corp., Tokyo, Japan) and scored using Image J software (W.S. Rasband, Image J, NIH, Bethesda, MD).

**Transfection and RNA interference**

To inhibit TIGAR expression, two small-interference RNAs (siRNA) matching region 115-133 in exon 3 (GCAGCAGCTGCTGGTATAT; TIGAR siRNA1) and region 565-583 in exon 6 (TTAGCAGCCAGTGTCTTAG; TIGAR siRNA2) of the human TIGAR cDNA sequence were synthesized by GenePharma (Shanghai, China), and a scramble sequence (TTACCGAGACCGTACGTAT) was synthesized as a negative control. To inhibit autophagy, Atg5 siRNAs (GCAACTCTGGATGGGATTG, Atg5
siRNA1; CATCTGAGCTACCCGGATA, Atg5 siRNA2) were also synthesized.

A method called reverse transit transfection was adapted for transient transfection using the Lipofectamine 2000 reagent (Invitrogen, 11668019). HepG2 or A549 cells were added directly to the Lipofectamine™ 2000-TIGAR siRNAs or Atg5 siRNAs complexes diluted in Opti-MEM Reduced Serum Medium (GIBCO, 31985070) and transfection occurred while cells were attaching to the wall of well. For A549 cells, cells were plated at a density of 5x10⁵ cells in 6-well plates and, were then transfected with TIGAR siRNAs using Lipofectamine 2000 reagent diluted in Opti-MEM Reduced Serum Medium 24 h later. The final concentration of TIGAR siRNA and Atg5 siRNA was 80 nM and 40 nM. Complete medium was added to each well 6 h after transfection. Cells were trypsinized and harvested for Western blot analysis or flow cytometry at the indicated times.

To assess the transfection efficiency, HepG2 cells were transfected with LV-TIGAR-shRNA(TIGAR#2:5’-GATTAGCAGCCAGTGTCTTAG-3’; TIGAR#3: 5’-GCTTACATGAGAAGTCTGTTT-3’) (Genechem, Shanghai, China) and 72 h after trasfection, the expression of green fluorescent protein (GFP) was detected with a fluorescence microscopy. The transfection efficiency was estimated about 90%. For establish stable TIGAR knockdown cells, HepG2 cells were transfected with
LV-TIGAR-shRNA (Genechem, Shanghai, China,) and were selected in cell culture medium containing 1µg/ml poromycin for one week. The efficiency of TIGAR knockdown was determined with Western blot analysis. Cells were then cultured in culture medium for in vivo xenograft experiments.

Flow cytometry detection of apoptosis and ROS

Cell apoptosis was quantified with double staining of fluorescein isothiocyanate (FITC) conjugated annexin-V and propidium iodide (PI) (Biouniquer, BU-AP0103). Ten thousands cells per sample were acquired with a FACScan flow cytometer (FACScan, Becton Dickinson, USA). Freshly trypsinized cells were pooled, washed twice with binding buffer and processed following the manufacturer's instructions. Cells fluorescence was analyzed with flow cytometry using the Cell Quest Pro software (Beckman Coulter, Kraemer Boulevard Brea, USA).

2',7'-dichloro-dihydrofluorescein diacetate (H2-DCFDA, Molecular Probes) was metabolized by non-specific esterases to the non-fluorescence product, 2',7'-dichloro-dihydrofluorescene, which was oxidized to the fluorescent product DCF by ROS. ROS levels were determined by incubating the cells with cell culture medium containing 10 μM H2-DCFDA for 30 min at 37 °C. Then, the cells were washed twice in PBS, trypsinized, resuspended in PBS and measured for ROS content with FACS.
(FACScan, Becton Dickinson). Assays were performed in triplicates and were repeated in three independent experiments.

**Western blot analysis**

Protein was extracted from cells using cell lysis solution supplemented with protease inhibitors (Roche, 04693159001) and phosphorylase inhibitors (Roche, 04906845001). Protein concentration was determined with a BCA protein assay kit (Pierce, 23227). Equal amounts of protein were fractionated on Tris-glycine SDS-polyacrylamide gels and subjected to electrophoresis and transferred to NC membranes. Membranes were blocked with TBS containing 5% (w/v) dry milk with 0.1% Tween 20, washed with TBS containing 0.1% Tween 20 (TBST), and then incubated overnight at 4 °C with specific antibodies against TP53 (1:1,000; Cell Signaling, #2524), TIGAR (1:1,000; Abcam, ab37910), LC3 (1:1,000; Abcam, ab62721), p62 (1:1,000; American Research Product, 12-1107), Atg5 (1:1,000; Cell Signaling, #2630), p-mTOR (1:1,000; Cell Signaling, #2971), S6(1:1000; Cell Signaling, #2217), p-S6(1:1000; Cell Signaling, #4856), 4EBP-1(1:1000; Cell Signaling, #9452) and p-4EBP-1(1:1000; Cell Signaling, #9451) in non-fat milk containing 0.1% NaN₃. After washing in TBST, membranes were incubated with fluorescence secondary antibodies (1:10,000; Jackson ImmunoResearch, anti-rabbit, 711-035-152, anti-mouse, 715-035-150) at room temperature for 1 h. Immunoreactivity
was detected using ODYSSEY INFRARED IMAGER (Li-COR Biosciences). The signal intensity of primary antibody binding was quantitatively analyzed with Image J software (W.S. Rasband, Image J, NIH, Bethesda, MD) and was normalized to a loading control β-actin (1:4,000; Sigma, A5441).

**Evaluation of LC3 fluorescent puncta**

HepG2 cells were seeded onto cover glass (Fisher Scientific, #032910-9) in 24-well plate and were transfected with scramble siRNA, TIGAR siRNA1 or TIGAR siRNA2 24 h later. Cells were treated with or without the treatment of 5 μg/ml epirubicin for 12 h. After 72 h of trasfection, cells were washed with PBS (pH 7.4) and fixed with 4% paraformaldehyde and then blocked in PBS containing 1% normal bovine serum albumin and 0.1% Triton X-100 for 1 h at room temperature. Cells were then incubated with rabbit polyclonal anti-LC3 antibody (Abcam, ab62721) followed by incubation with FITC-conjugated anti-rabbit secondary antibodies (1:200, Boster, BA1105). After 1 h incubation and several rinses, cells were incubated with 0.5 μg/ml 4,6-diamidino-2-phenylindole (DAPI) for 10 min. Sections were then washed in PBS and cover slipped with fluoromount Aqueous Mounting Medium (Sigma, F4680). The slides were analyzed with Nikon C1 plus laser scanning confocal microscopy (Nikon D-Eclipse C1 plus, Tokyo, Japan). Six fields were analyzed for each of the samples stained with a
given antibody, and the number of LC3 puncta/cell was evaluated as the total number of dots divided by the number of nuclei in each microscopic field and at least 150 cells were examined in each treatment. Duplicates of three independent experiments were analyzed for each group.

**Transmission electron microscopy**

HepG2 cells were seeded into six-well plates and transfected with TIGAR siTIGAR2 or Scramble siRNA for 72 h, epirubicin was applied 12 before the end of transfection. Cells were then fixed for 2 h with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), post-fixed in 1% OsO₄ dehydrated in graded ethanol and then embedded in epoxy resin. Blocks were cut on an ultramicrotome into ultrathin sections which were poststained with uranyl acetate and lead citrate, and viewed under a Hitachi 7100 electron microscopy.

**Redox-State Analysis**

For GSH/GSSG measurement, a GSH and GSSG Assay Kit (Beyotime, China) was used according to the manufacturer's protocol. NADPH levels of cells were detected with enzychromTM NADP+/NADPH assay kit (ECNP-100) (BioAssay Systems, USA) according to the manufacturer's protocol.

**In vivo tumor growth analysis**
HepG2 cells and stable TIGAR knockdown HepG2 cells (1*10^6) were subcutaneously inoculated into the right oxter of 6-week-old female athymic nude mice (Shanghai SLAC Laboratory Animal Co. Ltd). Two weeks after tumor formation, 2 mg/kg epirubicin was intraperitoneally administrated every three days for another two weeks, and then mice were anesthetized and photographed. After the mice were sacrificed, the tumors were removed and photographed and weighted. Tumor proteins were extracted for Western blot analysis. All animal procedures were approved and monitored by the local Animal Care and Use Committee in Soochow University (License NO. Syxk (Su-0062)).

**Statistical analysis**

All data were presented as means±SEM. Data were subjected to one-way ANOVA using the GraphPad Prism software statistical package (GraphPad Software, San Diego, CA, USA). When a significant group effect was found, post hoc comparisons were performed using the Newman–Keuls t-test to examine special group differences. Independent group t-tests were used for comparing two groups. Significant differences at p<0.05, 0.01 and 0.001 are indicated by *, **, ***, respectively. All calculations were performed using the 14.0 SPSS software package (SPSS Inc.).
Results

Epirubicin induced TIGAR expression

Epirubicin, a DNA damaging anticancer agent induced growth inhibition in both HepG2 cells (Fig.1A) and A549 cells (Supplemental Fig. S1A) in a concentration- and time-dependent manner. To detect TIGAR expression in response to epirubicin treatment, HepG2 cells were seeded onto 6-well plates for 24 h and were then treated with different concentrations of epirubicin (1.25-20 μg/ml) for 12 h or 5.0 μg/ml epirubicin for different length of time (12-48 h). An increase in TIGAR protein levels was observed at the low concentration of epirubicin (1.25-5μg/ml), but returned to that of control levels at 10 μg/ml (Fig. 1C). A time-course study showed that TIGAR expression was elevated at 12-24 h, but quickly declined and fell down to that of control at 36 h (Fig. 1E). As TIGAR was known as a TP53-inducible gene, the expression of TP53 in HepG2 cells treated with different concentrations of epirubicin or 5.0 μg/ml epirubicin for different length of time was detected with immunoblotting. The results showed that the elevated expression of TIGAR correlated with the expression of TP53 as seen in Fig. 1B and 1D. Similar results were obtained in A549 cells (Supplemental Fig. S1B-E). To further investigate whether the elevation of TIGAR was correlated with the increased expression of TP53, PFT-α, a TP53 inhibitor was used. Western blot analysis showed that expression
of Bax and TIGAR were decreased in a time- and dose-dependent manner (Supplemental Fig. S2), which indicated that TIGAR induction was dependent on TP53 in HepG2 cells.

Based on these observations, we treated HepG2 and A549 cells with 5 µg/ml epirubicin for 12 h in rest of experiments, as this treatment protocol induced a minor inhibition of cell viability but a significant increase in TIGAR protein levels.

**Down-regulation of TIGAR enhanced epirubicin-induced apoptosis.**

To investigate the effects of TIGAR on the cell growth of HepG2 and A549 cells, two different TIGAR siRNA molecules (TIGAR siRNA1, TIGAR siRNA2) were designed to silence TIGAR expression. HepG2 cells were transfected with scramble siRNA, TIGAR siRNA1 or TIGAR siRNA2 at the concentration of 80 nM for 72 h. The transfection efficiency was ~90% as detected with a fluorescence microscopy (Supplemental Fig. S3A) Western blot analysis of TIGAR protein levels showed 89% and 91% of silencing efficiency with TIGAR siRNA1 and TIGAR siRNA2, respectively (Fig. 2A). A 65.6% (TIGAR siRAN1) and 71.2% (TIGAR siRNA2) of silencing efficiency was also detected in A549 cells (Supplemental Fig. S3B). The short-term effect of TIGAR knockdown on cell growth was examined with an MTT assay in both A549 and HepG2 cells. Knockdown of TIGAR had no significant effect on the cell proliferation under normal condition. However, TIGAR knockdown significantly enhanced the
inhibitory effects of epirubicin on growth of HepG2 cells, suggesting a protective effect of TIGAR on cancer cell survival (Fig. 2B). Similar effects were obtained in A549 cells with combination of TIGAR knockdown and epirubicin treatment (Supplemental Fig. S3C). To evaluate the long-term effect of TIGAR knockdown on cell proliferation, clony formation assay was used. The formation of clones of HepG2 cells after TIGAR knockdown was only slightly reduced under normal condition. The epirubicin-induced inhibition of the clony formation was greatly enhanced by knockdown of TIGAR (Fig. 2C and D).

To further investigate whether down-regulation of TIGAR enhances the chemo-sensitivity of HepG2 and A549 cells, we analyzed apoptosis of HepG2 and A549 cells in response to epirubicin after knockdown of TIGAR. TIGAR interference enhanced the apoptotic index when HepG2 cells treated with 5.0 μg/ml epirubicin compared to those treated with epirubicin alone (Fig. 2E and F). Similar effects of TIGAR knockdown on epirubicin-induced apoptosis were observed in A549 cells (Supplemental Fig. S3D). Furthermore, the present study showed that knockdown of TIGAR promoted apoptosis partly through the activation of conventional caspase-dependent apoptotic pathway with a significant enhancement in the activation of caspase 3 (Supplemental Fig. S4A). The apoptosis was inhibited with the pan-caspase inhibitor Z-VAD-FMK (Supplemental Fig.
These results suggest that TIGAR knockdown enhances the chemo-sensitivity of tumor cells to epirubicin.

Inhibition of TIGAR expression reduced tumorogenecity and enhanced chemo-sensitivity to epirubicin in vivo.

To evaluate the effects of TIGAR on tumor growth and chemo-sensitivity of hepatocellular carcinoma to epirubicin, a xenograft nude mouse model was used. HepG2 cells with stable TIGAR knockdown (Fig. 3A and B) were inoculated into the right oxter of 6-week-old female athymic nude mice. A mild inhibition of tumor growth in vivo was observed in stable TIGAR knockdown cells as compared to vector-transfected cells. Epirubicin treatment (2 mg/kg) reduced tumor size and the effect was substantially enhanced in stable TIGAR knockdown group (Fig. 3C-E). The reduced expression of TIGAR was confirmed with GFP-fluorescence (Fig. 3F) and Western blot analysis (Fig. 3G). These in vivo results revealed that inhibition of TIGAR expression can reduce tumorogenecity and enhance the chemo-sensitivity of hepatocellular carcinoma to epirubicin.

TIGAR knockdown enhanced epirubicin-induced elevation of ROS levels.

TIGAR is reported to inhibit glycolysis and decrease intracellular ROS levels, thus protects cells from ROS-associated apoptosis. To detect whether this increased
Chemo-sensitivity induced by TIGAR knockdown was associated with increased ROS levels, we detected the ROS levels in the cells transfected with TIGAR siRNAs and treated with epirubicin. The results showed that ROS levels were slightly increased in HepG2 cells after TIGAR knockdown. The treatment with epirubicin after TIGAR knockdown produced greater increase in ROS levels compared with control cells and cells treated with TIGAR scramble siRNA (Fig. 4A and B). To confirm that the enhancement of epirubicin-induced apoptosis after TIGAR knockdown was attributable to the increased cellular ROS levels, NADPH was supplemented to HepG2 cells. Cells were transfected with TIGAR siRNA2 and were treated with 10 μM NADPH 1 h before epirubicin treatment. Addition of NADPH significantly reduced ROS levels in cells transfected with TIGAR siRNA2 and treated with epirubicin (Fig. 4A and B). Similar results were seen with the ROS scavenger NAC, (Supplemental Fig. S5A and B). GSH/GSSG and NADPH levels after TIGAR knockdown with or without treatment of epirubicin were also detected and results showed a significant decrease in GSH/GSSG and NADPH levels after TIGAR knockdown HepG2 cells treated with 5 μg/ml epirubicin (Fig. 4C and D). These effects were partly blocked with supplementation of 10 mM NAC 1 h before epirubicin treatment (Supplemental Fig. S5C and D). The clony formation assay also showed a protective effect of NAC on tumor cell growth (Supplemental Fig.
Apoptosis of HepG2 cells after TIGAR knockdown with or without treatment of 5 μg/ml epirubicin was significantly reduced after applying of NADPH (Fig. 4E), however, compared to control cells, the apoptotic rate of HepG2 cells treated with 5.0 μg/ml epirubicin after TIGAR knockdown with addition of NADPH was still higher than that of epirubicin alone, suggesting that enhancement of chemo-sensitivity by knockdown of TIGAR is partially mediated by elevation of ROS levels.

**TIGAR knockdown enhanced epirubicin-induced autophagy activation.**

To investigate the effects of epirubicin on autophagy, HepG2 cells were seeded onto 6-well plates for 24 h and were then treated with different concentrations of epirubicin (1.25-20 μg/ml) for 12 h or 5 μg/ml epirubicin for different length of time (12-48 h). The data showed that autophagy was activated if treated with higher concentration (10-20 μg/ml) of epirubicin for longer time (36-48 h) with increases in LC3-II/LC3-I ratio and decreases in p62 levels (Fig. 5A-D). It was reported that TIGAR can inhibit autophagy through down-regulating ROS levels. To determine whether autophagy was activated by knockdown of TIGAR, HepG2 cells were left untreated or treated with 5 μg/ml epirubicin with or without TIGAR knockdown and the LC3 levels were detected with Western blot analysis. The results showed that knockdown of TIGAR alone did not significantly increase the LC3-I/LC3-II ratio. Treatment of HepG2 cells with epirubicin
(5 μg/ml) did not significantly induce activation of autophagy at the time examined. However, knockdown of TIGAR resulted in significant activation of autophagy after treatment of epirubicin (Fig. 5E). Meanwhile, the greater increase in the LC3 fluorescence intensity and the number of LC3 patches were seen in HepG2 cells after treatment with epirubicin and TIGAR knockdown (Fig. 5F). The greater decrease in p62 was also found to in response to epirubicin treatment after TIGAR-knockdown (Fig. 5G).

To further confirm the enhancement of autophagy activity by knockdown of TIGAR, HepG2 cells were transfected with scramble siRNA or TIGAR siRNA2 with or without treatment of epirubicin and electron microscopy was used to detect the formation of autophagosomes. Results showed that compared with control, the formation of autophagosomes was detected in HepG2 cells after TIGAR knockdown or treated with 5.0 μg/ml epirubicin, however, the increased number of autophagosomes, secondary lysosomes and vacuolated mitochondria were seen in HepG2 cells treated with combined epirubicin and TIGAR knockdown (Fig.6). These results suggest an enhancement in the autophagy activation by TIGAR knockdown in response to epirubicin treatment.

To further investigate whether this enhanced autophagy activity after TIGAR knockdown was caused by the elevation of ROS levels, HepG2 cells transfected with TIGAR siRNA2 were treated with NADPH 2 h before the treatment of 5.0 μg/ml
epirubicin. Results showed that the increment of epirubicin-induced autophagy activity by TIGAR knockdown was partially inhibited with supplementation of NADPH (Fig.5H). This suggests that in addition to ROS, other mechanisms may be involved in TIGAR-mediated autophagy regulation (11). As mTOR is a major regulator of autophagy, the inhibition of which has been shown to induce activation of autophagy in response to nutrient starvation (12), we then detected the phosphorylation of mTOR and its downstream protein S6 and 4EBP1 in response to epirubicin after TIGAR knockdown. Results showed that silencing TIGAR expression alone had no significant effect on phosphorylation of mTOR under normal condition. Treatment of HepG2 cells with epirubicin caused a robust increase in the levels of phosphorylated mTOR but not its downstream S6 and 4EBP1. However, knockdown of TIGAR almost completely blocked the phosphorylation of mTOR induced by epirubicin (Fig. 7A). Moreover, knockdown of TIGAR reduced phosphorylated S6 and 4EBP1 below the control levels after combined epirubicin treatment (Fig.7B and C). These results indicate that TIGAR may have a direct regulatory role on mTOR signaling pathway.

**Inhibition of autophagy enhanced TIGAR knockdown-induced increases in apoptosis.**

To investigate if the activation of autophagy induced by TIGAR knockdown affects...
chemo-sensitivity of epirubicin, 3-MA, an autophagy inhibitor that has been reported to inhibit the activity of PI3 kinase and block the formation of autophagosomes, was applied 12 h before the end of the treatment. After applying 3-MA, the rate of apoptosis of HepG2 cells treated with 5 μg/ml epirubicin and TIGAR knockdown was significantly increased (Fig. 7D and E). Clony formation assay also showed a decrease in clones after autophagy was inhibited by 3-MA. It has reported that 3-MA has dual role in modulation of autophagy via different temporal patterns of inhibition on class I and class III PI3K (13). To further investigate the effects of autophagy induced by TIGAR knockdown on cell apoptosis and chemo-sensitivity, HepG2 cells were co-transfected with Atg5 siRNA and TIGAR siRNA for 48 h. The co-transfection efficiency was 62.33% for Atg5 and was 72.05% for TIGAR (Fig. 7F). HepG2 cells co-transfected with Atg5 siRNA and TIGAR siRNA were then treated with 5 μg/ml epirubicin for 12 h and the cell viability was assessed with MTT assay. The results showed that inhibition of autophagy by knockdown of Atg5 in TIGAR-silenced HepG2 cells, cell survival was greatly reduced after the treatment with epirubicin (Fig. 7G). These results suggested that enhanced autophagy activity induced by TIGAR knockdown reduces the cytotoxicity of epirubicin.

Discussion
Alteration of energy metabolism is one of the hallmarks of cancer cells and has recently inspired particular interest in investigating regulation of metabolic pathways in cancer chemotherapy (14). The increased dependency of cancer cells on glycolytic pathway for ATP generation provides a biochemical basis for the design of therapeutic strategies to preferentially kill cancer cells by pharmacological inhibition of glycolysis. Thus development of novel glycolytic inhibitors as a new class of anticancer agents is likely to have broad therapeutic applications (15). Several glycolysis inhibitors are now in preclinical and clinical development, such as lactate dehydrogenase A inhibitor FX11 (16) or hexokinase inhibitor 2-deoxy-glucose (15;17) and lonidamine (18). The function of TIGAR as a kind of glycolytic inhibitor seems to be unfavorable to cancer cell survival from the view of glycolysis. However, recent studies showed that TIGAR was over-expressed in several types of cancer cells and its expression was favorable to tumor cell survival (5). The role and mechanisms by which TIGAR affect cancer cell survival need to be further studied.

To determine the response and role of TIGAR in cancer cells with relation to chemotherapy, this study investigated the regulation of chemo-sensitivity by TIGAR in HepG2 and A549 cells. Consistent with the previous findings (5;19;20), TIGAR expression in A549 and HepG2 cells was elevated following treatment with epirubicin in
face of mild or transient stress. TIGAR knockdown mildly decreased the cell viability, partially due to the increased rate of apoptosis. Furthermore, the epirubicin-induced decline in cell viability and the increase in apoptosis were greatly enhanced when TIGAR was knocked down. These studies suggest a protective effect of TIGAR on cancer cell survival under basal and stress conditions. The in vivo studies further showed a reduction in tumorigenicity and an increase in chemo-sensitivity to epirubicin after TIGAR knockdown, suggesting TIGAR may be a therapeutic target for cancer therapy.

Spitz et al reported that cancer cells produced greater amount of superoxide and hydrogen peroxide than normal cells (21;22). To reduce the hydroperoxide toxification, the pentose pathway was upregulated to produce more NADPH. TIGAR can inhibit glycolysis, however, this effect may be masked by other regulatory mechanisms in tumor cells. In agreement with Spitz et al (21;22), we observed the lactate production was increased in colon cancer tissues compared to corresponding normal tissues. We also observed a high expression of G6PD in colon cancer tissues alone with TIGAR over expression (unpublished observations), thus TIGAR can increase the generation of NADPH by promoting flow of pentose phosphate pathway (PPP). To investigate if TIGAR knockdown-induced enhancement in chemo-sensitivity is caused by the increases in ROS, total cellular ROS levels, GSH/GSSG ratio and NADPH levels were detected.
The increases in ROS levels and decreases in GSH/GSSG ratio or NADPH levels were found, which confirmed the role of TIGAR in suppressing ROS production. To further confirm that the enhanced apoptosis to epirubicin after TIGAR knockdown was mediated by elevation of cellular ROS, NADPH and NAC were applied. The results demonstrated that cell apoptosis was apparently reduced if the NADPH or NAC was added to cells. However, the apoptotic rate of cancer cells treated with NADPH after TIGAR knockdown was still higher than that of the control cells, indicating other mechanisms are also involved in TIGAR-regulated chemo-sensitivity. Caspase 3, an important death protease (23) was found to be activated in cells transfected with TIGAR siRNA or treated with epirubicin, this effect was also amplified with combination of TIGAR knockdown and epirubicin. Cell apoptosis induced by TIGAR knockdown, epirubicin or combination of both was partially rescued after supplementation of Z-VAD-FMK, a pan-caspase inhibitor. It suggests that the promotion of chemo-sensitivity by TIGAR knockdown is more or less due to the activation of caspase-dependent apoptotic pathway.

Recent reports showed that TIGAR expression was correlated with the ex vivo sensitivity of chronic lymphocytic leukemia cells to fludarabine (24). Lui et al demonstrated that TIGAR/NADPH cascade was involved in antitumor effects of a new c-Met tyrosine kinase inhibitor, suggesting that inhibition of TIGAR may have an
antitumor potentiality (25). ECyd, an RNA-directed nucleoside anti-metabolite, elicits its antitumor effect via TIGAR down-regulation (26). Wanka et al, demonstrated that TIGAR was elevated shortly after irradiation, and silencing TIGAR expression resulted in cell viability inhibition and the damnification of DNA repair in glioblastoma cell lines, with the possibility to enhance radio-sensitization (20). In consistent with these findings, the present study suggests that TIGAR may be a valuable target for chemotherapy.

Bensaad et al, demonstrated that TIGAR can modulate ROS in response to nutrient starvation or metabolic stress, and functions to inhibit autophagy (11). Ling Ye et al, reported that knockdown of TIGAR induced apoptosis and autophagy in HepG2 hepatocellular carcinoma cells (27). How does TIGAR influence autophagy activity and its consequence on cell viability, however, is not clearly defined. Autophagy is an evolutionarily conserved mechanism for degradation of intracellular substances, responsible for the recycle of metabolic substances and the maintenance of intracellular stability (28). Whether autophagy kills or protects cancer cells is complex (29). In most chemotherapeutic strategies, elevation of autophagy activity in varying degrees after treatment could be observed (30). This can be considered as a protective strategy for tumor cells to avoid being entirely killed by drugs. The pro-survival ability of autophagy renders tumor cells resistant to anticancer drugs, which greatly compromises curative
efficacy of chemotherapy. A complex interplay between apoptosis and autophagy has also been reported (31). In many cases, autophagy can inhibit apoptosis by removing damaged mitochondria, preventing the release of apoptogenic factors such as cytochrome c from the mitochondria and the activation of the apoptotic cascade in cancer cells (32;33). In agreement with other investigators (11;27), knockdown of TIGAR alone only slightly increased autophagy activity, but significantly enhanced epirubicin-induced autophagy activation. To explore the effects of activated autophagy on cell viability after TIGAR knockdown, 3-MA or Atg5 siRNA was implied. Inhibition of autophagy with 3-MA or Atg5 knockdown further increased the epirubicin-induced apoptosis when TIGAR was knocked down, which suggests a protective effect of autophagy on cancer cell survival.

It has been proposed that TIGAR affects autophagy as a result of reduction in ROS. In the present study, NADPH partially blocked TIGAR knockdown-induced enhancement of autophagy activity, suggesting additional mechanisms may be involved. This study found that knockdown of TIGAR alone had no significant effect on mTOR but robustly decreased the epirubicin-induced the phosphorylation of mTOR and its downstream protein S6 and 4EBP1. This suggests that TIGAR may influence autophagy activity through regulating ROS levels and a direct inhibitory effect on mTOR pathway.

In summery, TIGAR was induced by epirubicin. Knockdown of TIGAR expression
by siRNA enhanced the antitumor effects of epirubicin with increased cellular ROS levels, active caspase 3 and the rate of apoptosis. The effects can be partially blocked by supplementing NADPH or NAC. Knockdown of TIGAR slightly increased activation of autophagy but significantly enhanced epirubicin-induced autophagy activation. The TIGAR knockdown-induced autophagy activation involved in elevation of ROS and inhibition of mTOR. Inhibition of autophagy activity enhanced epirubicin-induced apoptosis. Therefore, TIGAR exerts dual effects on survival of tumor cells, with one favorable effect mediated by reducing ROS and an unfavorable effect due to reducing autophagy activity. However, based on current information, the predominant role of TIGAR on cancer cells is pro-survival. This study also suggests that if TIGAR is targeted for chemotherapy, inhibiting autophagy would provide additional benefits.

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**Figure legends**

**Fig. 1 Epirubicin inhibited cell growth and induced TIGAR expression.** (A) The dose-response and the time-cause of the effect of epirubicin on cell viability. HepG2 cells were treated with different concentrations of epirubicin (1.25-20 μg/ml) for 12 h, 24 h, 36 h and 48 h. Cell viability was detected with MTT assay. (B and D) The effects of epirubicin on protein levels of TP53. HepG2 cells were treated with different concentrations of epirubicin (0, 1.25, 2.5, 5, 10, 20 μg/ml) for 12 h or 5 μg/ml epirubicin for different length of time (12, 24, 36 and 48 h). The protein levels of TP53 were detected with Western blotting. β-actin protein was used as a loading control. Quantitative analysis was performed with Image J. (C and E) The effects of epirubicin on protein levels of TIGAR. The HepG2 cells were treated as described above. Values are means ± SD from 3 independent experiments. * p< 0.05, ** p< 0.01, *** p<0.001, ns p>0.05 versus control group.
Fig. 2 Down-regulation of TIGAR inhibited cell survival and enhanced epirubicin-induced apoptosis. (A) Knockdown efficiency of TIGAR in HepG2 cells. Transient transfection of siRNAs was applied to knockdown TIGAR expression for 72 h and epirubicin was added 12 h before the end of experiment. The protein levels of TIGAR were detected with Western blotting. β-actin protein was used as a loading control. Quantitative analysis was performed with Image J. (B) Short-term effects of epirubicin on cell growth after TIGAR knockdown. HepG2 cells were treated as described above and cell growth was assessed with MTT assay. (C) Long-term effects of epirubicin on cell growth after TIGAR knockdown. HepG2 cells were treated as described above and cell growth was assessed by clony formation assay. (D) Quantitative analysis of clone numbers of HepG2 cells after TIGAR knockdown with or without epirubicin treatment. (E) The effects of epirubicin on apoptosis after TIGAR knockdown. HepG2 cells were treated as described above and apoptosis was detected with flow cytometry detection. (F) Quantitative analysis of percentage of apoptotic HepG2 cells after TIGAR knockdown with or without treatment of epirubicin. Values are means ± SD from 3 independent experiments. * p< 0.05, ** p< 0.01, *** p<0.001, ns p>0.05 versus corresponding group.
Fig. 3 Inhibition of TIGAR expression enhanced chemo-sensitivity to epirubicin in vivo. (A) Transfection efficiency of lentivirus in HepG2 cells. Cells were transfected with LV-TIGAR-shRNA at a MOI value of 10. GFP-green fluorescence was detected with a fluorescence microscopy after 72 h. (B) Western blot analysis of TIGAR protein expression after HepG2 cells transfected with LV-shTIGAR and selected with 1 μg/ml poromycin for one week. GAPDH was used as a loading control. (C) HepG2 cells and stable TIGAR knockdown HepG2 cells (1*10^6) were subcutaneously inoculated into the right oxter of 6-week-old female athymic nude mice. Two weeks after tumor formation, 2 mg/kg epirubicin was intraperitoneally administrated every three days for another two weeks. Mice were anesthetized and photographed. (D) The visual comparison of tumor size from six mice in each group. (E) Tumor weights from six mice in each group were measured. Values are means ± SD. * p< 0.05 versus control group, ns p>0.05 # p<0.05 versus corresponding group. (F) GFP-green fluorescence was detected with fluorescence microscope in xenograft tumor tissues (frozen section, 10 μm thickness), DAPI-blue fluorescence-field was photographed at the same vision. (G) Western blot analysis of TIGAR protein expression in xenograft tumor tissues. GAPDH was used as a loading control.
Fig. 4 TIGAR knockdown enhanced epirubicin-induced elevation of ROS levels. (A) ROS levels after TIGAR knockdown and epirubicin treatment in the presence and absence of NADPH. The HepG2 cells were transfected with scramble siRNA or TIGAR siRNA2 for 60 h and then were left untreated or treated with 5 μg/ml epirubicin for 12 h in the presence or absence of NADPH. The ROS levels and cell apoptosis were measured with flow cytometry after treatment of epirubicin for 12 h. (B) Quantitative analysis of ROS levels after TIGAR knockdown and epirubicin treatment in the presence and absence of NADPH. (C) GSH/GSSG ratio in HepG2 cells after TIGAR knockdown with or without treatment of 5 μg/ml epirubicin. (D) NADPH levels in HepG2 cells after TIGAR knockdown with or without treatment of 5 μg/ml epirubicin. (E) Cell apoptosis after TIGAR knockdown and epirubicin treatment in the presence and absence of NADPH. The HepG2 cells were treated as described above and apoptosis was detected with flow cytometry. Values are means ± SD from 3 independent experiments. * p< 0.05, ** p< 0.01, *** p<0.001, ns p>0.05 versus corresponding group.

Fig. 5 TIGAR knockdown enhanced epirubicin-induced autophagy activation. (A) The dose response of epirubicin on protein levels of p62. HepG2 cells were treated with different concentrations of epirubicin for 12 h. Cells were lysed and the protein levels of
p62 were detected with Western blotting. β-actin was used as a loading control. Quantitative analysis was performed with Image J. (B) The dose response of epirubicin on protein levels of LC3. HepG2 cells were treated as described above. (C) The time-course of epirubicin on protein levels of p62; HepG2 cells were treated with 5 μg/ml of epirubicin for 12, 24, 36 and 48 h. Cells were lysed and the protein levels of p62 were detected with Western blotting. β-actin was used as a loading control. Quantitative analysis was performed with Image J. (D) The time-course of epirubicin on protein levels of LC3; HepG2 cells were treated as described above. (E) The effects of TIGAR knockdown on LC3 in the presence and absence of epirubicin. HepG2 cells were transfected with scramble siRNA or TIGAR siRNA1 and TIGAR siRNA2 for 60 h and then were left untreated or treated with 5 μg/ml epirubicin for 12 h. The protein levels of LC3 were detected with Western blotting. β-actin was used as a loading control. Quantitative analysis was performed with Image J. (F) Distribution of LC3 puncta in TIGAR knockdown HepG2 cells. HepG2 cells were planted onto 24-well plate and were treated as described above and then fixed with 4% paraformaldehyde and processed for immunofluorescence. Cells were analyzed with a confocal microscopy. LC3 was stained green and DAPI was stained blue. Scale bar = 5 μm. The number of LC3 dots/cell was quantitatively analyzed in analyzed. (G) Effects of TIGAR knockdown on p62 in the
presence and absence of epirubicin. HepG2 cells were treated as described above. (H) The effects of NADPH on autophagy induced by TIGAR knockdown. HepG2 cells were transfected with TIGAR siRNA2 for 60 h and then were treated with 5 μg/ml epirubicin for 12 h, NADPH was added 2 h before the treatment of epirubicin. The levels of LC3 were detected by Western blot analysis. β-actin was used as a loading control. Values are means ± SD from 3 independent experiments. * p< 0.05, ** p< 0.01, *** p<0.001, ns p>0.05 versus control group.

**Fig. 6 TIGAR knockdown increased epirubicin-induced autophagosome formation.**

Electron micrographs showed the ultrastructure of control HepG2 cells or HepG2 cells transfected with scramble siRNA or TIGAR siRNA2 with or without treatment of 5.0 μg/ml epirubicin. (A and B) HepG2 control cells or cells transfected with scramble siRNA appeared to be normal ultrastructure with healthy looking mitochondria, lysosomes and the nuclei. (C) HepG2 cells transfected with TIGAR siRNA2 showed the autophagosomes and secondary lysosomes. Mitochondria vacuolation was found. (D) HepG2 cells treated with 5.0 μg/ml also appeared secondary lysosomes and mitochondria vacuolation, as well as autophagosomes formation. (E) HepG2 cells transfected with TIGAR siRNA2 combined with treatment of 5.0 μg/ml epirubicin increased the number
of vacuoles and the autophagosomes in the cytoplasm. The increases in secondary lysosomes and mitochondrial vacuolation, condensation of chromatin were also observed. Scale bars: 1 μm. Arrows indicate autophagosomes, # indicates lysosomes, *indicates mitochondria.

**Fig. 7** TIGAR knockdown enhanced epirubicin-induced autophagy by inhibiting mTOR pathway and inhibition of autophagy enhanced TIGAR knockdown-induced increases in apoptosis. (A) Effects of TIGAR knockdown on phosphorylation of mTOR in the presence and absence of epirubicin. HepG2 cells were treated as described above, the protein levels of phosphorylated mTOR were detected with Western blot analysis. β-actin was used as a loading control. Quantitative analysis was performed with Image J. (B and C) Phosphorylation of mTOR downstream protein S6 and 4EBP1 after TIGAR knockdown on HepG2 cells in the presence and absence of epirubicin, β-actin was used as a loading control. (D and E) The effects of 3-MA on TIGAR knockdown-induced increases in apoptosis. Sixty h following transfection of scramble siRNA or TIGAR siRNA2, HepG2 cells were left untreated or treated with 5 μg/ml epirubicin for 12 h in the presence and absence of 10 mM 3-MA. The apoptotic rate was measured with flow cytometry of annexin V-FITC and PI double stained cells. (F) Efficiency of
co-knockdown of Atg5 and TIGAR. HepG2 cells were co-transfected with Atg5 siRNA and TIGAR siRNA for 48 h and the protein levels of Atg5 and TIGAR were determined with Western blot analysis. β-actin protein was used as a loading control. Quantitative analysis was performed with Image J. (G) Effects of Atg5 knockdown on TIGAR knockdown-induced increases in apoptosis. HepG2 cells were co-transfected with Atg5 siRNA and TIGAR siRNA for 48 h. Cell viability was detected with MTT assay after TIGAR and Atg5 knockdown with or without the treatment of epirubicin. Values are means ± SD from 3 independent experiments. * p< 0.05, ** p< 0.01, *** p<0.001, ns p>0.05 versus corresponding group.
Fig. 1
Fig. 2

A

Scramble siRNA

TIGAR siRNA1

TIGAR siRNA2

TIGAR

β-Actin

-30 KD

-42 KD

Optical Density

(TIGAR/Actin) × 100

Scramble siRNA

TIGAR siRNA1

TIGAR siRNA2


B

Cell survival

(OD value) (% control)

Scramble siRNA

TIGAR siRNA1

TIGAR siRNA2

Epi 5 μg/ml


C

Scramble siRNA

TIGAR siRNA1

TIGAR siRNA2

Epi

TIGAR siRNA1 + Epi

TIGAR siRNA2 + Epi


D

Number of colonies

Scramble siRNA

TIGAR siRNA1

TIGAR siRNA2

Epi 5 μg/ml


E

Scramble siRNA

TIGAR siRNA1

TIGAR siRNA2

Epi

TIGAR siRNA1 + Epi

TIGAR siRNA2 + Epi


F

Apoptosis (%)

Scramble siRNA

TIGAR siRNA1

TIGAR siRNA2

Epi 5 μg/ml


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Figure 5
Fig. 6
Fig. 7
TIGAR has a dual role in cancer cell survival through regulating apoptosis and autophagy

Jia-Ming Xie, Bin Li, Hong-Pei Yu, et al.

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