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ORIGINAL ARTICLE

Toll-interacting protein may affect doxorubicin resistance in hepatocellular carcinoma cell lines

Ayse Banu Demir[1] · Elif Baris[2] · Umay Bengi Kaner[3] · Hani Alotaibi[4,5] · Nese Atabey[5,6] · Ahmet Koc[7]

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Abstract

Background Liver cancer is the third leading cause of cancer-related deaths worldwide, and hepatocellular carcinoma (HCC) is the most common type of liver cancer. Transarterial interventions are among the chemotherapeutic approaches used in hardly operable regions prior to transplantation, and in electrochemotherapy, where doxorubicin is used. However, the efficacy of treatment is affected by resistance mechanisms. Previously, we showed that overexpression of the CUE5 gene results in doxorubicin resistance in Saccharomyces cerevisiae (S. cerevisiae). In this study, the effect of Toll-interacting protein (*TOLLIP*), the human ortholog of *CUE5*, on doxorubicin resistance was evaluated in HCC cells to identify its possible role in increasing the efficacy of transarterial interventions.

Methods and results The NIH Gene Expression Omnibus (GEO) and Oncomine datasets were analyzed for HCC cell lines with relatively low and high *TOLLIP* expression, and SNU449 and Hep3B cell lines were chosen, respectively. TOLLIP expression was increased by plasmid transfection and decreased by *TOLLIP*-siRNA in both cell lines and evaluated by RT-PCR and ELISA. Cell proliferation and viability were examined using xCELLigence and MTT assays after doxorubicin treatment, and growth inhibitory 50 (GI 50) concentrations were evaluated. Doxorubicin GI 50 concentrations decreased approximately 2-folds in both cell lines upon silencing *TOLLIP* after 48 h of drug treatment.

Conclusions Our results showed for the first time that silencing *TOLLIP* in hepatocellular carcinoma cells may help sensitize these cells to doxorubicin and increase the efficacy of chemotherapeutic regimens where doxorubicin is used.

Keywords Hepatocellular carcinoma · Toll-interacting protein · Doxorubicin · Cancer drug resistance

Introduction

Liver cancer is in sixth place among the most common cancer types, with an incidence rate of 4.7%, and is the third most common cause of cancer-related deaths, with a global mortality rate of 8.3% [1, 2]. The number of new liver cancer cases is predicted to increase by more than 50% by 2040; therefore, it is important for public health. Almost 80% of primary liver cancers are hepatocellular carcinomas (HCC) [3], which generally develop as a result of liver damage [1, 4]. The resulting liver damage causes inflammation, hepatocyte regeneration, liver matrix reorganization, fibrosis, and cirrhosis. Hepatitis B and Hepatitis C infections, excessive alcohol use, hemochromatosis, fatty liver, androgenic steroid use, and other metabolic disorders also pose a risk for HCC development [4]. In HCC, almost all carcinogenic pathways show some degree of impairment, and owing to disease heterogeneity, it is difficult to draw a clear molecular framework for the disease [4–11].

✉ Ayse Banu Demir
banu.demir@ieu.edu.tr

1 Faculty of Medicine, Department of Medical Biology, Izmir University of Economics, Sakarya Street, No:156, Balcova, Izmir 35330, Turkey

2 Faculty of Medicine, Department of Medical Pharmacology, Izmir University of Economics, Izmir, Turkey

3 Faculty of Medicine, Izmir University of Economics, Izmir, Turkey

4 Izmir International Biomedicine and Genome Institute, Dokuz Eylül University Health Campus, Izmir, Turkey

5 Izmir Biomedicine and Genome Center, Izmir, Turkey

6 Faculty of Medicine, Department of Medical Biology & Galen Research Center, Izmir Tinaztepe University, Izmir, Turkey

7 Faculty of Medicine, Department of Medical Genetics, Inonu University, Malatya, Turkey

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Liver transplantation and hepatic resection are generally used to treat HCC. Chemotherapy is also used for hardly operable patients or as adjuvant therapy after surgical resections [12–14]. Among chemotherapeutic approaches, systemic chemotherapy is not very effective in HCC owing to its low response rates and lack of survival advantage [4]. Due to their cytotoxic side effects, the development of molecular targeted therapeutic agents provides an opportunity for HCC clinical applications [15, 16].

Transarterial chemoembolization (TACE) is a therapeutic approach mainly used for the treatment of intermediate-stage HCC [17]. It is used as a pre-transplant adjuvant to reduce tumors in minimally invasive and hardly operable areas. Although there is no standardized therapeutic regimen for the treatment of TACE, the use of antineoplastic agents (e.g. doxorubicin, cisplatin, mitomycin, and their combinations) is the most optimized procedures [18]. Doxorubicin is one of the most commonly used agents for TACE [19]. Investigation of the effects of different genes that play a role in doxorubicin resistance in HCC may contribute to the efficacy of treatments, including TACE or combination therapies, as well as to the determination of new chemotherapeutic targets in HCC.

As a Toll-like receptor (TLR) inhibitor, Toll-interacting protein (TOLLIP) regulates the TLR signaling pathway in humans and has been shown to play a role in several diseases, such as tuberculosis [20]. TOLLIP has a wide range of functions, including inflammation, autophagy, vacuole trafficking, and nuclear interactions [21]. It acts as a critical adapter protein for the dynamic adaptation of the innate immune system to external factors and negatively regulates the NF-kB signaling pathway [22]. It also acts as an adapter protein in the initiation of chronic inflammation. As part of autophagy, it triggers autophagosome-lysosome fusion by combining ubiquitinated protein aggregates with LC3-coated autophagosomes [21]. A study showed that different chemotherapeutic drugs used in the treatment of HCC increased autophagy in HCC cells and increased cell viability by triggering drug resistance [23].

In our previous study, several genes, including *CUE5*, were found to play a role in high-dose doxorubicin resistance in the yeast strain *Saccharomyces cerevisiae* (*S. cerevisiae)* at the whole-genome level. In the literature, no direct data exists regarding the role of *TOLLIP*, which is the human ortholog of *CUE5*, in drug resistance. In yeast cells, it has been shown that increased cell sensitivity with Htt-96Q expression in the absence of *CUE5* can be compensated by the expression of *TOLLIP* [24]. This suggests that *TOLLIP* and *CUE5* may play similar roles in doxorubicin resistance. Therefore, in this study, we aimed to determine the function of *TOLLIP* gene in doxorubicin resistance and hypothesized that *TOLLIP* plays a role in doxorubicin resistance in HCC cells. We selected two HCC cell lines with relatively different *TOLLIP* expression levels and determined the role of *TOLLIP* in doxorubicin resistance via its overexpression and silencing in selected cell lines. Our results showed that *TOLLIP* may affect doxorubicin resistance, and its silencing in hepatocellular carcinoma cells may help sensitize these cells to doxorubicin treatment and increase drug efficacy.

Materials and methods

Cell culture

The HCC cell lines SNU449 and Hep3B were kindly provided by Prof. Dr. Nese Atabey. The cell lines were grown in appropriate cell media (for SNU449; RPMI 1640, Sigma Aldrich, R8758, for Hep3B; MEM, Sigma Aldrich M4655) with heat-inactive FBS (10%) and penicillin (100 U/mL) streptomycin (100 µg/mL) mL, Gibco, Carlsbad, CA) and incubated at 37 °C in the presence of 5% CO_2. Doxorubicin treatment was performed at least 24 h after cell seeding.

Cell line transfections

For *TOLLIP* overexpression, 3 µg of sequence-validated plasmid DNA (pCMV-AC-GFP control (Origene, PS100010) or pCMV-AC-TOLLIP-GFP (Origene, RG200227)) was transferred to Hep3B and SNU449 cells in a 3:1 ratio with Fugene liposomal agent in a 6-well plate. All transfections were performed using 150,000 cells at passages four or five as described in the Fugene and Origene plasmid administration protocols. Briefly, cells to be transfected were seeded in cell medium with only FBS in a volume of 1.5ml. 3 µg of plasmid and transfection agent were incubated at room temperature for 5 min with 250 µl of FBS-free and antibiotic-free cell medium in separate tubes and subsequently incubated together for 15 min at room temperature. A total of 500 µl of FBS-free and antibiotic-free plasmid DNA and transfection agent were added to the seeded cells and incubated for 48 h at 37°C, 5% CO_2. After 48 h, the medium of the cells was replaced with fresh medium containing only FBS and further incubation was carried out for 24 h at 37°C, 5% CO_2. Transfection of cells was confirmed at RNA and protein levels prior to any further experiments.

TOLLIP gene silencing was performed as described in the siRNA and transfection agent protocols. Briefly, 10ul of Silencer® Select siRNA (Ambion, Cat #: 4,392,420, siRNA ID#: s29037) or control siRNA (Ambion, negative control siRNA No.1, Cat#:4,390,853) and 7.5 µl of the transfection solution Lipofectamine RNAimax were applied in 6-well plates using 150,000 cells. Cells for transfection were seeded in FBS-only cell medium in a volume of 1.5ml. 10µM

siRNA and lipofectamine transfection agent were incubated at room temperature for 5 min with 250 µl of FBS-free and antibiotic-free cell medium in separate tubes and then were combined and incubated together for 15 min at room temperature. A total of 500 µl of siRNA and transfection agent mix were added to the seeded cells and incubated at 37°C, 5% CO_2 for 48 h. After 48 h, the medium was replaced with fresh FBS-only medium, and the cells were incubated for 24 h. The silencing rates of cells were confirmed at the RNA and protein levels prior to any further protocols. All experiments were performed in four biological replicates, including three technical replicates.

Cell proliferation and viability assays

Cell proliferation was measured by electrical impedance measurement using E-Plate 16 plates on the xCELLigence instrument (xCELLigence RTCA DP 3×16, Acea Biosciences, San Diego, CA). Cell-free media was used as a negative control, and basal impedance measurements were made before doxorubicin application. Cells were cultured (10,000 cells/well) and incubated for 30 min at room temperature. After a 24-hour proliferation period in the instrument at 37°C, 5% CO_2, varying concentrations of doxorubicin were applied to the cells as described in the literature. The change in cell number was followed at 1-hour intervals for 48 h after the first 24 h of proliferation and drug treatment. By evaluating the change in cell number in the presence of doxorubicin, the Growth inhibitory 50 (GI 50) value, in which the growth of cells was inhibited by 50% [25], was determined by nonlinear regression analysis for 24 and 48 h after drug administration.

GI 50 values determined by xCELLigence were also confirmed by MTT assays (ABP Biosciences, Cell-QuantTM MTT Cell Proliferation Assay Kit, A015) as described by the manufacturer and are indicated as growth inhibition 50 (GI 50). Briefly, the cells were seeded in 96-well plates at a density of 5000 cells/well. After 24 h, the media were removed from the wells and a total of 100µL of fresh media was added to each well containing the corresponding doxorubicin concentrations. No drugs were administred to the controls. Media were removed from the wells at 24 and 48 h according to the time of drug administration, and 100 µl of fresh medium and 10 µl of MTT reagent were added to each well. The cells were incubated at 37°C for 4 h. Afterwards, the media was removed and 100 µl Detergent solution (or DMSO) was added to each well and colorimetric measurements were taken at 570 nm using a microplate reader (Thermo Scientific, Multiskan FC). GI 20, GI 50 and GI 80 calculations were confirmed by MTT and calculated as the percentage of the control. All experiments were performed with 4 biological replicates containing 3 technical replicas.

RNA isolation and quantitative real-time PCR

Total RNA was isolated from the cell lines (K0732, Thermo Scientific) and 1 µg of RNA was transcribed into copy DNA (cDNA) using a cDNA synthesis kit (K1622, Thermo Scientific). The cDNA samples were evaluated for expression profiles of the genes of interest (BioRad, CFX Connect) using the SYBR-Green method with the following forward and reverse primer pairs: GAPDH-Fwd:5'-ACCCACTCCTCCACCTTTGAC-3', GAPDH-Rev:5'-CATACCAGGAAATGAGCTTGACAA-3' [26]; TOLLIP-Fwd:5'-ATGGACGACCGCATTGC-3', TOLLIP-Rev:5'-ACTTGTCCTCCACCTGCCC-3'. GAPDH was used as a reference gene. PCR reaction conditions were performed as 10 minutes for polymerase activation/denaturation at 95°C; 40 cycles of (15 seconds at 95°C, 60 seconds at 50°C) for amplification, post-amplification melting curves at 65 °C' After 5 s, between 65 and 95 °C in 0.5 °C increments every 5 s. Relative mRNA expression values were calculated using the $2^{-\Delta\Delta Ct}$ method [27]. All experiments were performed with 4 biological replicates containing 3 technical replicas.

Protein assays

SNU449 and Hep3B cell lines were seeded 24 h prior to doxorubicin treatment, and TOLLIP protein levels in cell lysates were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Cusabio, CSB-E14976h) as described by the manufacturer. Briefly, the medium was removed from approximately 1 million/ml adherent cells and the cells were washed with cold PBS (pH7.2-7.4). The cells were scraped with a cell scraper in PBS and were transferred to 15 ml falcon tubes and washed once more with PBS and the cell lysates were stored at -20°C overnight. After two freeze-thaw cycles, cell lysates were centrifuged at 5000xg for 5 min at 4°C and the supernatants were collected. Solutions included in the kit (biotin antibody, HRP-avidin, wash solution and standards) were prepared as described in the kit. 100 µl of standard and sample were added to each well, covered with an adhesive tape and incubated at 37°C for 2 h. Then, the supernatant was removed from the wells and 100ul of Biotin antibody (1X) was added to each well, covered with an adhesive tape and incubated at 37°C for one hour. The samples were cooled to room temperature, the supernatant was removed and the wells were washed three times with 200 µl of wash solution (1X). The supernatant was completely removed and after incubation at room temperature for 2 min, 100 µl of HRP-avidin (1X) was added and covered with an adhesive tape and incubated at 37°C for one hour. The supernatant was removed and the samples were washed three times with washing solution

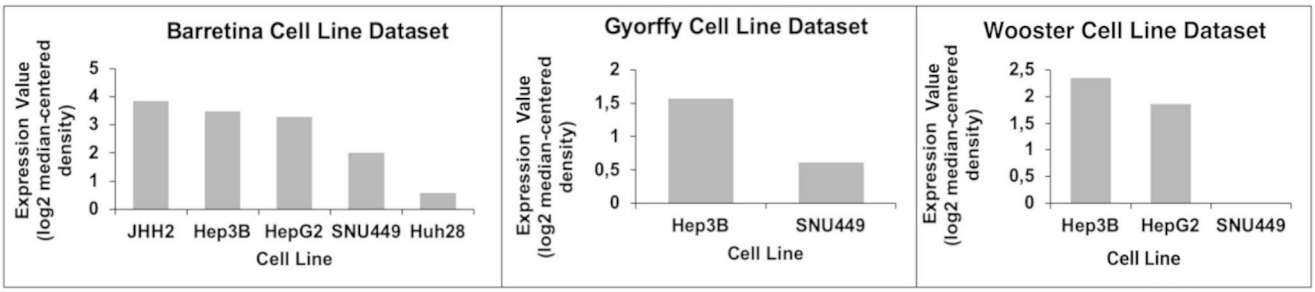

Fig. 1 Comparison of the *TOLLIP* expression values among different HCC cell lines in microarray data obtained from Oncomine Cell Line Datasets

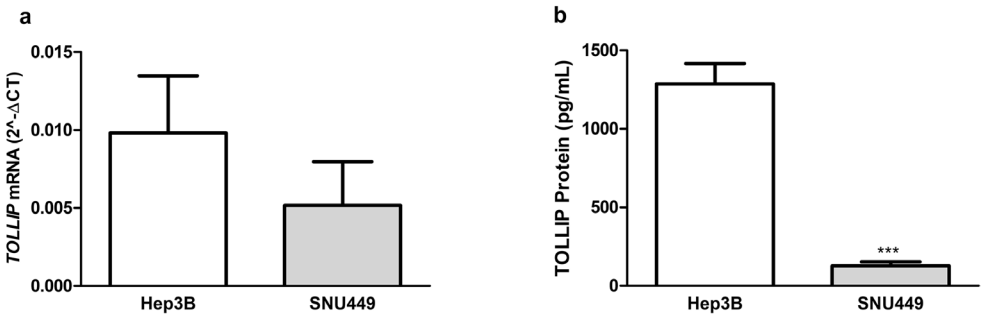

Fig. 2 Baseline **(a)** mRNA and **(b)** protein expression patterns of the *TOLLIP* gene in Hep3B and SNU449 cell lines. ***: $p < 0.001$ vs. Hep3B.

(1X) and completely removed from the supernatant. 90ul of TMB substrate was added to each well and incubated in the dark at 37°C for 30 min. 50 µl of reaction stopping solution was added and after mixing, the samples were read in a microplate reader at 450 nm wavelength within 5 min. All experiments were performed with 4 biological replicates containing 3 technical replicas.

Statistical analysis

Post-hoc Tukey-Kramer multiple comparison tests and one-way analysis of variance (ANOVA) were used to compare the mean values of proliferation experiments. Half growth inhibitor concentration (GI 50, defined as 50% inhibitory concentration) was calculated using nonlinear regression analysis (GraphPad Prism 5, La Jolla, CA). Comparisons between the two groups in Real-time PCR and MTT analyses were performed using paired t-test analysis. Data are shown as the mean ± standard error of the mean (S.E.M) and $p < 0.05$ was considered statistically significant.

Results

Toll-interacting protein (TOLLIP) is expressed relatively higher in Hep3B cells compared to SNU449 cells

Hepatocellular carcinoma (HCC) cell line microarray data from NIH GEO (Gene Expression Omnibus) database were scanned *for TOLLIP* expression patterns. A total of 3613 datasets of HCC cell lines were obtained and a total of 8 datasets, which contained the expression data of the *TOLLIP* gene in untreated HCC cell lines, were selected for analysis. Geo2R analysis was performed on these datasets by using the corresponding *TOLLIP* gene-specific probe IDs (GSE112788, probe ID: 1555865_at; GSE79232, probe ID: TOLLIP; GSE97172, probe ID: 7,945,620; GSE85274 and GSE132119, probe ID: 11722171_a_at; GSE88812, probe ID: 1555865_at; GSE73219, probe ID: TC11001258.hg.1; GSE36139, probe ID: 54472_at). Additionally, HCC cell line datasets from the Oncomine website were scanned and three HCC cell line datasets were compared in terms of *TOLLIP* expression (Fig. 1). Although not significant, the expression of *TOLLIP* gene was relatively higher in some cell lines than in others. Considering the opposing results in several datasets, the analyzed data revealed that the expression of *TOLLIP* gene was higher in Hep3B cells than in SNU449 cells. The *TOLLIP* expression patterns in these cell lines were confirmed at the RNA and protein levels. Similar to the microarray data analyzed, *TOLLIP* gene expression at the RNA level did not differ significantly between Hep3B and SNU449 cells, whereas at the protein level, Hep3B cells showed significantly higher TOLLIP levels than SNU449 (Fig. 2). Therefore, these two cell lines were selected for further experimentation.

Hep3B cells are more sensitive to doxorubicin compared to SNU449 cells

To determine the doxorubicin concentrations that suppressed cell growth by 50% (GI 50) at 24th and 48th hours, cellular proliferation was evaluated by real-time cell index (CI) analysis using xCELLigence. GI 50 concentrations for 24th and 48th hours were found to be 0.93 µM and 0.65 µM for Hep3B cells (Fig. 3a) and as 16.5 µM and 12 µM for SNU449 cells, respectively (Fig. 3b). These values were confirmed by the MTT assay (Fig. 3c and d). Doxorubicin concentrations that suppressed cell viability by 20% (GI 20), 50% (GI 50) and 80% (GI 80) for each cell line were identified for 24th and 48th hours. In Hep3B cells, GI 20 concentrations were found as 0.7 µM and 0.6 µM at 24th and 48th hours, respectively, while for SNU449 cells, these concentrations were 12 µM at 24th and 8 µM at 48th hours. GI 80 concentrations were found as 2 µM and 0.8 µM for Hep3B cells at 24th and 48th hours, respectively, whereas these concentrations were 40 µM at 24th hour and 30 µM at 48th hour (Fig. 3c and d).

TOLLIP expression levels vary in different doxorubicin treatment periods, although increasing doxorubicin concentrations seems not to affect *TOLLIP* expression levels significantly

To evaluate the time-dependent variation in *TOLLIP* expression upon doxorubicin treatment, *TOLLIP* expression levels were analyzed for 24 and 48 h of doxorubicin treatment at the RNA and protein levels. In Hep3B cells, *TOLLIP* mRNA expression was significantly increased at 48 h of doxorubicin treatment compared with that of 24 h. In SNU449 cells, no significant difference in *TOLLIP* expression was seen between 24-hours and 48-hours doxorubicin treatments (Fig. 4a). At the protein level, TOLLIP expression did not differ significantly compared to that of the control groups in Hep3B cells between 24 and 48 h of treatment, but significantly decreased in SNU449 cells after 48 h of doxorubicin treatment compared to their corresponding control groups (Fig. 4b).

To determine if the expression of the *TOLLIP* gene changes with increasing doxorubicin concentrations, GI 20, GI 50 and GI 80 concentrations were applied to both cell lines for 24 and 48 h and *TOLLIP* expression patterns

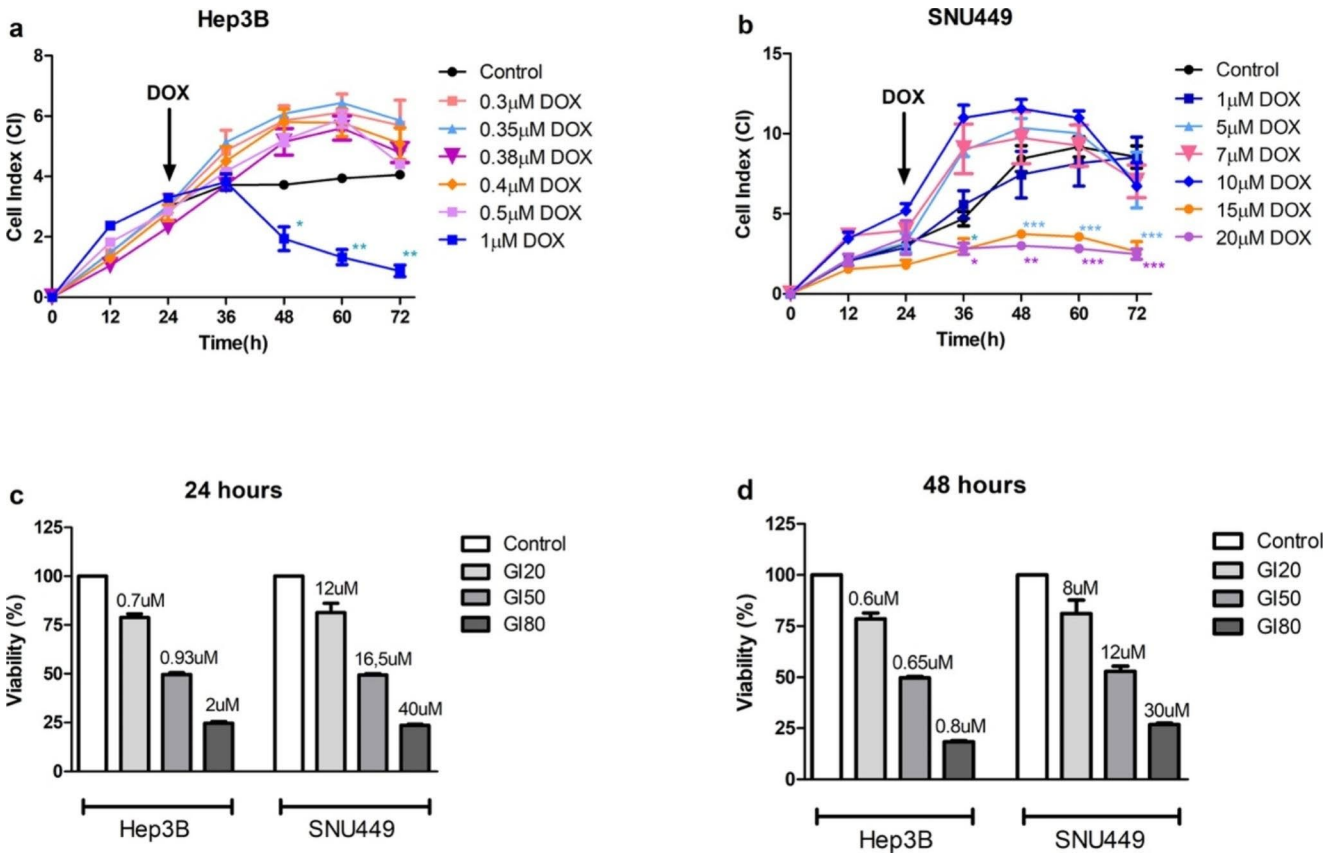

Fig. 3 Real Time Cell Index (CI) of **(a)** Hep3B and **(b)** SNU449 cells. Cell viability analysis for Hep3B and SNU449 cells at GI 20 and GI 80 concentrations of doxorubicin upon **(c)** 24 h and **(d)** 48 h of doxorubicin treatment. Cell growth inhibition concentration 20 (GI 20), growth inhibition concentration 50 (GI 50), growth inhibition concentration 80 (GI 80). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ vs. control

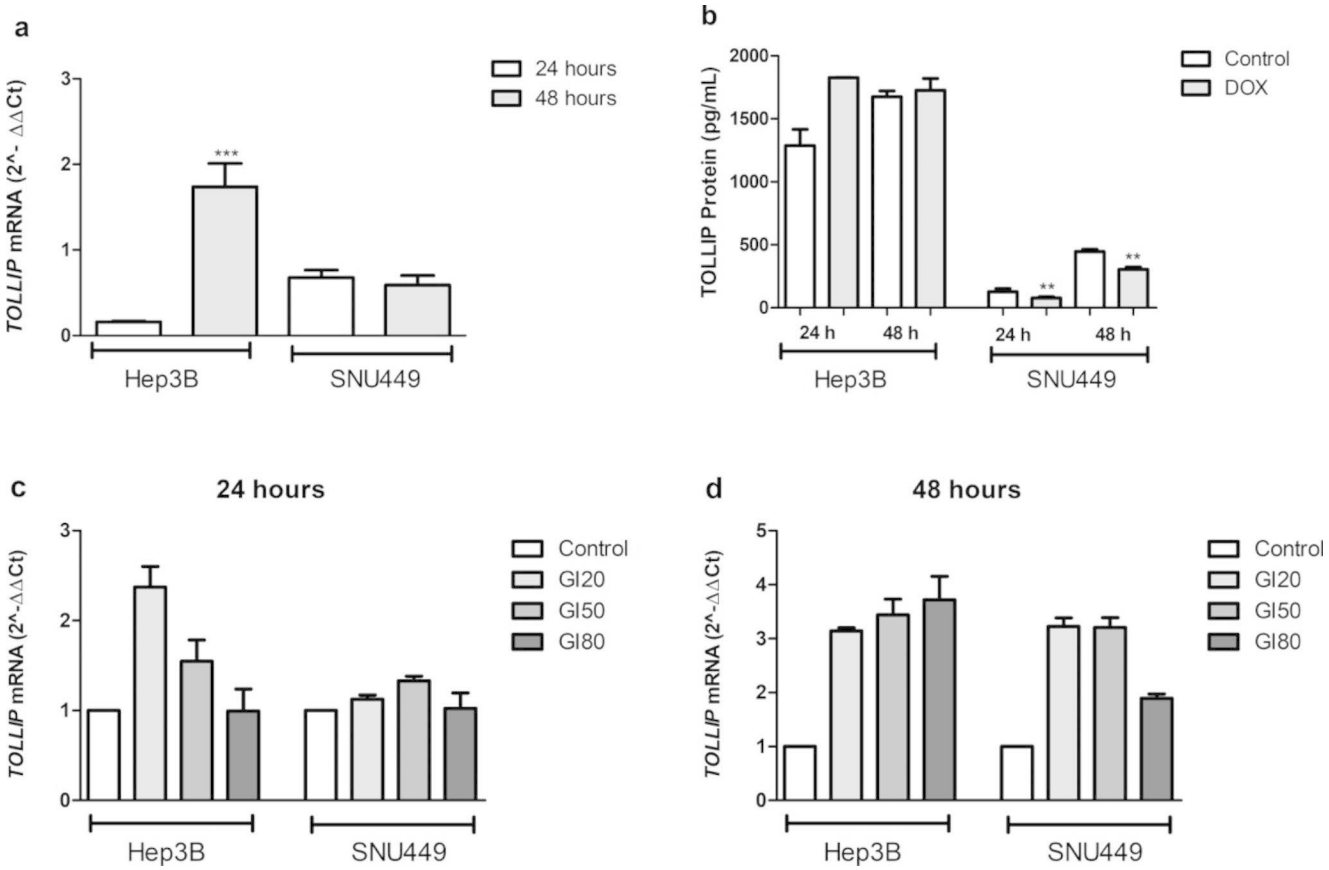

Fig. 4 *TOLLIP* expression in Hep3B and SNU449 cell lines at 24 and 48 h doxorubicin treatment (GI 50 concentration) at **(a)** mRNA and **(b)** protein levels and evaluation of the effects of increasing doxorubicin concentrations for **(c)** 24 h and **(d)** 48 h on *TOLLIP* expression in Hep3B and SNU449 cell lines at mRNA level. Cell growth inhibition concentration 20 (GI 20), growth inhibition concentration 50 (GI 50), growth inhibition concentration 80 (GI 80) values performed by MTT. **: $p < 0.01$, ***: $p < 0.001$ vs. control

were evaluated at the RNA level. 24th hour data of Hep3B cells showed that *TOLLIP* expression increased approximately 2-folds compared to that of the control group when GI 20 was administered, and the expression of the gene decreased proportionally with increasing doses of doxorubicin (Fig. 4c). When we looked at the 48th hour data for these cells, *TOLLIP* expression increased 3-folds when the GI 20 dose was administered, and there was a slight dose-dependent increase in the GI 50 and GI 80 doses (Fig. 4d). Considering the 24th hour data of SNU449 cells, *TOLLIP* expression almost did not change upon doxorubicin treatment, while it increased 3-folds when the GI 20 dose was applied for 48 h. This increase was similar to the GI 50 value but decreased when the GI 80 dose was administered (Fig. 4c and d). However, none of these changes were statistically significant. Although not significant, a proportional increase in *TOLLIP* expression with increasing doxorubicin concentration was only observed upon 48 h doxorubicin treatment in Hep3B cells.

Overexpression of Toll-interacting protein rendered SNU449 cells more resistant to doxorubicin, whereas silencing rendered both Hep3B and SNU449 cells more sensitive to doxorubicin

The possible role of *TOLLIP* in doxorubicin resistance was evaluated via its overexpression and silencing in both cell lines. *TOLLIP* gene expression increased approximately 76-folds in Hep3B cells (Fig. 5a) at the mRNA level and 1.6-folds at the protein level (Fig. 5b). Real-time cell index analysis showed that *TOLLIP* overexpression in Hep3B cells at 24 h of doxorubicin treatment increased the GI 50 concentration by approximately 1.3-folds (1.2 µM). Interestingly, in 48 h of doxorubicin treatment, this value decreased 2-folds (0.3 µM) compared to that of the control group (Fig. 5c and d). These GI 50 values were also confirmed by the MTT assay (Fig. 5e).

In SNU449 cells, *TOLLIP* expression increased by an average of 30-folds at the mRNA level while approximately 2.8-folds at the protein level (Fig. 6a and b). *TOLLIP* overexpression in these cells increased the GI 50 value

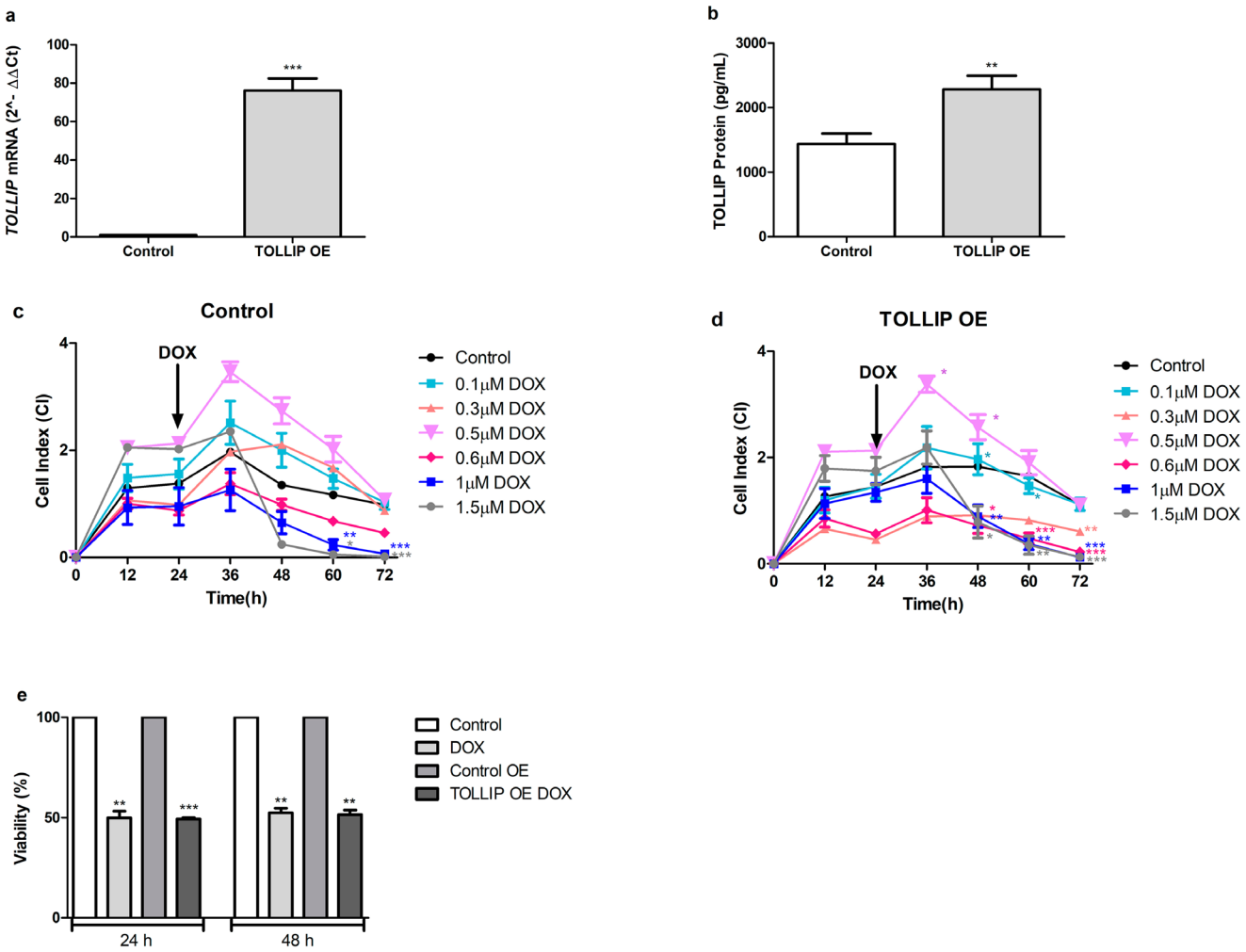

Fig. 5 **(a)** mRNA and **(b)** protein expression levels of *TOLLIP* in Hep3B cells after plasmid transfection. Cell proliferation patterns for **(c)** Hep3B control and **(d)** Hep3B *TOLLIP* OE conditions. **(e)** Cell viability confirmations for the GI 50 values by MTT assay. For 24 h; cell viability for DOX control (49,96% ± 3,2% S.E.M) and *TOLLIP* OE DOX (49,26% ± 0,82% S.E.M). For 48 h; cell viability for DOX control (52,48% ± 2,3% S.E.M) and *TOLLIP* OE DOX (51,44% ± 2,3% S.E.M). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ vs. control

by approximately 1.9-folds (34 μM) at 24 h doxorubicin treatment. At 48 h of doxorubicin treatment, this value was found to be 1.3 times higher (17.3 μM) compared to the control group (Fig. 6c and d). The MTT assay analysis also confirmed these values (Fig. 6e).

In addition to its overexpression, *TOLLIP* was silenced using siRNA in Hep3B and SNU449 cell lines. In Hep3B cells, *TOLLIP* expression was inhibited by 80% at the mRNA level (Fig. 7a), which was reflected as a 1.6-folds decrease at the protein level (Fig. 7b). When *TOLLIP* gene was silenced in Hep3B cells, 1.2-fold (0.6 μM) and 2.2-folds (0.1 μM) decrease in GI 50 values were observed at 24-hours and 48-hours of doxorubicin treatment, respectively (Fig. 7c and d) and cell viabilities were confirmed for these concentrations by MTT assay (Fig. 7e).

TOLLIP was silenced by 91% at the mRNA level in SNU449 cells (Fig. 8a) and this silencing was reflected as a 1.5-fold reduction at the protein level (Fig. 8b). Upon silencing *TOLLIP*, no significant change in GI 50 concentrations was observed in 24-hours of doxorubicin treatment, while GI 50 concentrations was decreased 1.7-folds (3.9 μM) at 48 h of doxorubicin treatment (Fig. 8c and d), which was confirmed by the MTT assay (Fig. 8e).

Discussion

As in most cancer types, changes in drug influx and efflux, drug metabolism, DNA repair, tumor microenvironment, phenotypic transition and epigenetic changes are among the drug-resistance mechanisms in HCC [28, 29]. Although targeted therapies and direct chemotherapeutic approaches such as TACE overcome the side effects of therapies, drug resistance remains an obstacle for effective chemotherapeutic

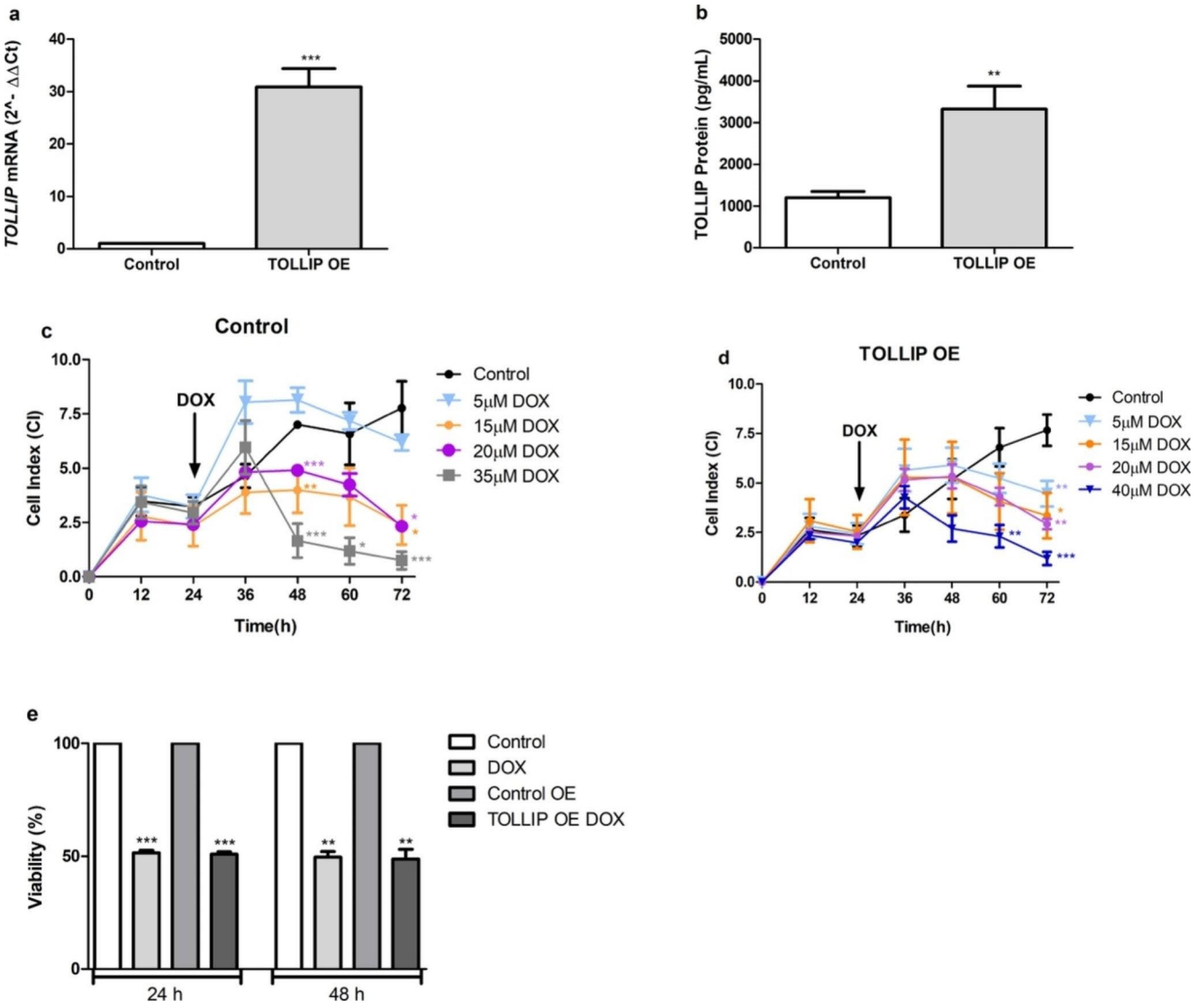

Fig. 6 **(a)** mRNA and **(b)** protein expression levels of *TOLLIP* in SNU449 cells after plasmid transfection. Cell proliferation patterns for **(c)** SNU449 control and **(d)** SNU449 *TOLLIP* OE conditions. **(e)** Cell viability confirmations for the GI 50 values by MTT assay. For 24 h; cell viability for DOX control (51,55% ± 1,02% S.E.M) and *TOLLIP* OE DOX (50,86% ± 1,06% S.E.M). For 48 h; cell viability for DOX control (49,61% ± 2,4% S.E.M) and *TOLLIP* OE DOX (48,68% ± 4,3% S.E.M). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ vs. control

regimes [30]. Direct involvement of *TOLLIP* in cancer drug resistance has not been shown before, but some of the proteins that forms a complex have been shown to be increased in HCC [31] and TOLLIP overexpression has been shown to be related to unfavorable outcomes in renal cell carcinoma [32]. Similarly, *TOLLIP* overexpression increased doxorubicin resistance in SNU449 cells. However, the effect of TOLLIP overexpression may depend on the HCC cell type, as we did not observe a similar increase in drug resistance in Hep3B cells. Overexpression of *TOLLIP* in SNU449 cells increased doxorubicin resistance by approximately 1.5-folds in the first 48 h in Hep3B cells, and its overexpression increased GI 50 concentrations by only 1.3-folds at 24 h and decreased by 2-folds at 48 h.

However, regardless of the cell line type, silencing of the *TOLLIP* gene led to doxorubicin sensitivity, both in Hep3B cells that represent earlier stages of HCC and SNU449 cells that represent the later stages of the disease. Interestingly, even when *TOLLIP* gene expression was increased or decreased, Hep3B cells became approximately 2-folds more sensitive to doxorubicin at 48 h of treatment. It appears that changing the *TOLLIP* expression balance in these cells leads to doxorubicin sensitivity. Hep3B cells were more sensitive to doxorubicin than SNU449 cells, and at basal conditions, express *TOLLIP* more than SNU449 cells. This is consistent with the results obtained from *TOLLIP* overexpression in these cells. However, this *TOLLIP*-imbalance-dependent sensitivity seems to be specific for Hep3B cells. In addition,

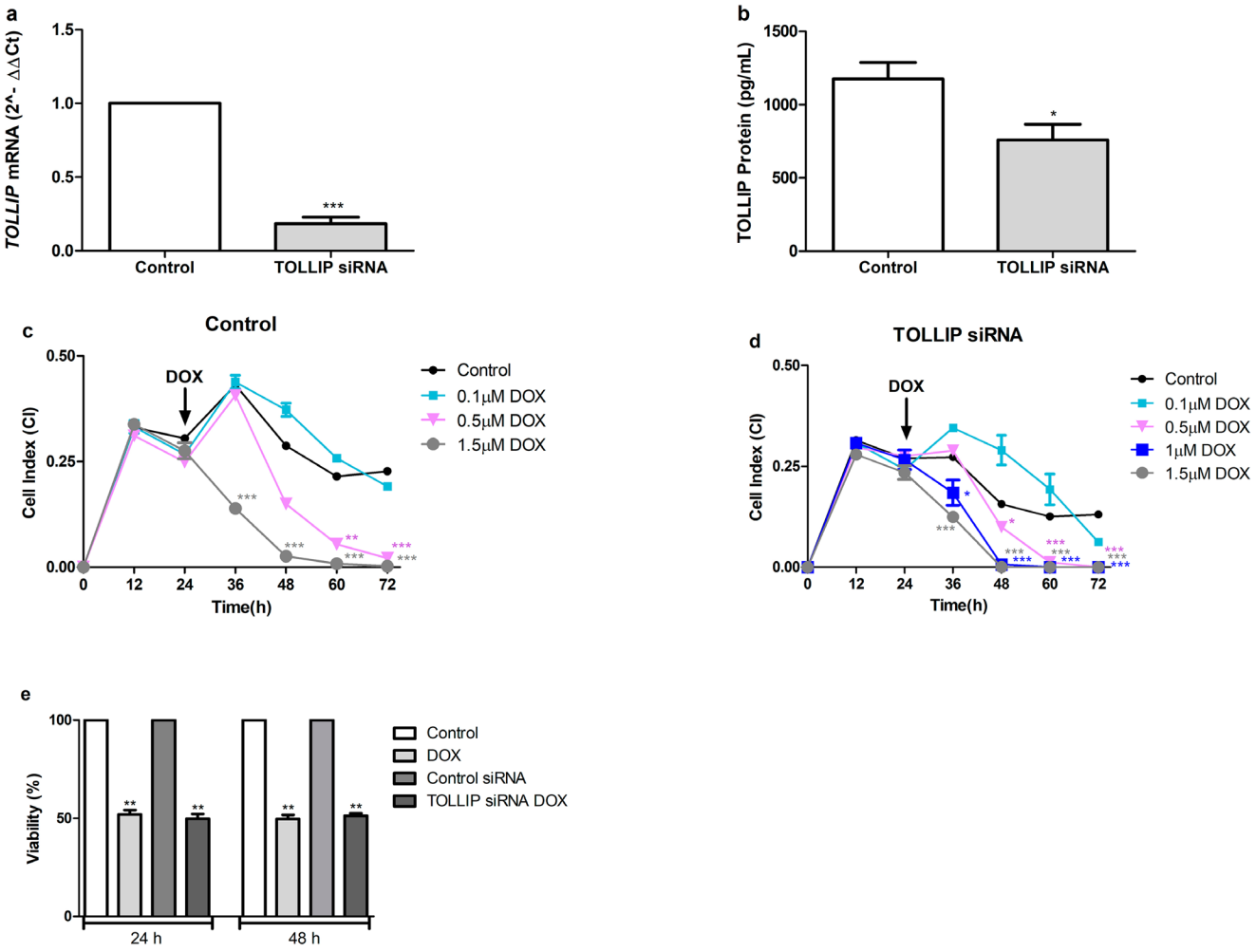

Fig. 7 **(a)** mRNA and **(b)** protein expression levels of *TOLLIP* in Hep3B cells after siRNA transfection. Cell proliferation patterns for **(c)** Hep3B control and **(d)** Hep3B *TOLLIP* siRNA conditions. **(e)** Cell viability confirmations for the GI 50 values by MTT assay. For 24 h; cell viability for DOX control (52% ± 2,1% S.E.M) and *TOLLIP* siRNA DOX (49,74% ± 2,3% S.E.M). For 48 h; cell viability for DOX control (49,64% ± 2,1% S.E.M) and *TOLLIP* siRNA DOX (51,25% ± 1,3% S.E.M). **: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ vs. control

we cannot exclude the possibility that this sensitivity may be due to other regulatory responses. Basal *TOLLIP* expression in Hep3B cells increased at 48 h of doxorubicin treatment. Increased expression through genetic manipulation may lead to the activation of *TOLLIP*-independent pathways to protect the cell, which may result in drug sensitivity. However, this hypothesis requires further investigation.

Toll-interacting protein, which acts as an intracellular ubiquitin adapter and was first known for its inhibitory protein function in the TLR2/4-mediated MYD88 signaling pathway, is a multifunctional protein involved in many signaling pathways in the cell due to three different domains in its structure [21, 33, 34]. The cellular functions of *TOLLIP* are important in the regulation of autophagy [24, 35, 36]. Its absence abolishes endosomal-lysosomal fusion [37] and is involved in lysosomal recycling events, such as carrying interleukin-1 receptor (IL-1R) and transforming growth factor beta-1 receptor (TGFβ-1R) [38–40]. Autophagy is among the mechanisms that affect drug resistance [30, 41]. It has been shown that cytotoxic protein aggregates are cleared by autophagy as a result of *TOLLIP* gene overexpression [24]. Therefore, reduction of the autophagic functions, along with lysosomal recycling events in the absence of the TOLLIP protein, can be one of the possible mechanisms that lead to drug sensitivity.

Another important role of TOLLIP is in the regulation of inflammatory pathways. It may either trigger pro- or anti-inflammatory responses [21]. It is well known for its anti-inflammatory function as an inhibitor of IL-1β and TLR2/4 dependent NF-κB signaling pathways [42–44]. However, it can promote inflammation under low-dose endotoxin treatment by translocating to the mitochondria to induce reactive oxygen species [37]. Inflammation is an important parameter in cancer progression and toll-like receptors play an

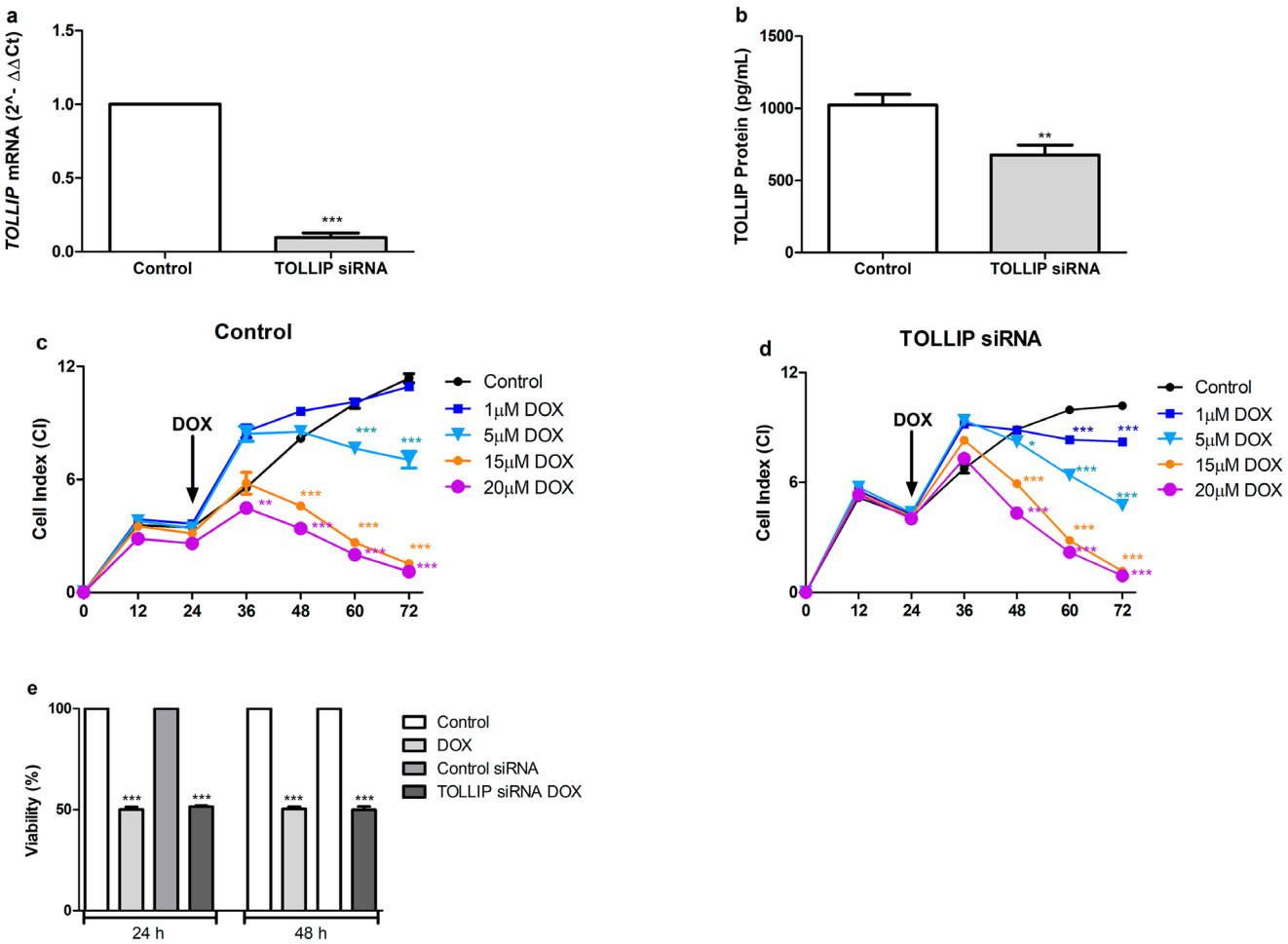

Fig. 8 **(a)** mRNA and **(b)** protein expression levels of *TOLLIP* in SNU449 cells after siRNA transfection. Cell proliferation patterns for **(c)** SNU449 control and **(d)** SNU449 *TOLLIP* siRNA conditions. **d)** Cell viability confirmations for the GI 50 values by MTT assay. For 24 h; cell viability for DOX control (49,96% ± 1,4% S.E.M) and *TOLLIP* siRNA DOX (51,44% ± 0,4% S.E.M). For 48 h; cell viability for DOX control (50,31% ± 1,2% S.E.M) and *TOLLIP* siRNA DOX (49,85% ± 1,6% S.E.M). **: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ vs. control

important role [33]. Since TOLLIP is an inhibitory protein of TLR2/4 dependent NF-κB signaling, it is expected that inhibition of TOLLIP would lead to increased activation of NF-κB and further increase in its target genes involved in inflammation, cell growth, survival and proliferation [45, 46]. Our results, however, showed that the proliferation status of the cells was not affected, even though proliferation was slightly decreased in Hep3B cells upon silencing *TOLLIP*. This may indicate that the effect of *TOLLIP* on doxorubicin resistance is not due to changes in cell proliferation but probably due to several other events that it plays a role in. Inflammation is an important regulator of drug-metabolizing enzymes and transporters (DMETs) [47]. Some of these enzymes and transporters are important in the doxorubicin response [48]. Therefore, another possible mechanism for the effect of *TOLLIP* on doxorubicin resistance can be the differential expression of some DMETs.

TOLLIP negatively affects the TGF-β signaling pathway [40] and it has been shown that TOLLIP plays a role in HCC progression via the PI3K/AKT pathway [49]. Silencing *TOLLIP* gene may have rendered cells more sensitive to the drug in means of its involvement in several signaling pathways. The response given by signaling pathways is highly dependent on the cellular background due to cross-talk between several signaling pathways. Further investigations of the signaling pathways that change upon silencing or overexpression of TOLLIP may help explain its effects on drug resistance status.

Doxorubicin intercalates into DNA molecules, inhibits topoisomerase enzyme and induces DNA breaks. It also damages the cell and cell membrane by triggering the formation of free radicals and inhibiting the topoisomerase-II-mediated DNA repair mechanism [50]. Several regulatory and adaptor protein functions of TOLLIP may directly or indirectly affect drug resistance. Although the role of

TOLLIP gene expression in doxorubicin resistance may not be a natural response of the cell, in this study, we showed that it may partially affect resistance through external interventions in gene expression.

The effects of the *TOLLIP* gene on doxorubicin resistance are generally in line with our previous findings [51], although shows differences on cellular basis. In addition, even significant increases and decreases in doxorubicin resistance have been observed with temporary manipulation of the *TOLLIP* gene; these changes are not very large. This may be because of the silencing and overexpression processes at the mRNA level reflected as smaller changes at the protein level. This inconsistency among mRNA and protein levels and among cell lines might be due to the complex regulation of gene expression at both the post-transcriptional and post-translational levels. mRNA stability or miRNA-mediated events might have affected the translation efficiency and protein levels, leading to differences in mRNA and protein expression levels. Post-translational modifications can also affect protein stability, although the mRNA levels do not change. In addition, the mRNA and protein levels may not always be in sync due to differences in the spatiotemporal regulation of gene expression. In this manner, temporary manipulations of the *TOLLIP* gene may be considered as a limitation of this study and permanently changing *TOLLIP* gene expression in the nuclear genome in these cell lines may lead to more effective results. In addition, further analysis of the post-transcriptional and post-translational changes in TOLLIP in HCC cells upon doxorubicin treatment would help to better understand the regulation of TOLLIP gene expression.

Conclusion

Overall, in this study, we have shown for the first time that the *TOLLIP* gene may affect doxorubicin resistance in hepatocellular carcinoma cells. Although further investigations are needed to determine the exact molecular mechanisms, reduction of *TOLLIP* gene expression may render HCC cells more sensitive to doxorubicin, regardless of the HCC grade and have the potential to contribute to the efficacy of chemotherapeutic treatments, including TACE.

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Author contributions Study conception and design was performed by Ayse Banu Demir. Material preparation, data collection and analysis were performed by Ayse Banu Demir, Elif Baris, Umay Bengi Kaner, Hani Alotaibi, Nese Atabey, Ahmet Koc. The first draft of the manuscript was written by Ayse Banu Demir and Elif Baris. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Competing interests *The authors have no relevant financial or non-financial interests to disclose.*

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