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ORIGINAL PAPER

Txr1: an important factor in oxaliplatin resistance in gastric cancer

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Abstract Oxaliplatin-based chemotherapy is the main treatment regimen for gastric cancer (GC), but can fail because of drug resistance. We investigated the role of a recently identified drug-resistance gene, taxol-resistant gene 1 (Txr1), in oxaliplatin resistance. A retrospective study based on banked tissue was carried out. We collected clinical data from 95 patients with stage II–III GC who were treated with radical D2 surgery and standardized first-line chemotherapy with oxaliplatin; paraffin blocks of their tumor specimens were prepared for a tissue microarray in which Txr1 expression was analyzed immunohistochemically and compared with their clinical data and their 3-year disease-free survival (DFS) rate. The human GC cell line, SGC7901, was developed into the oxaliplatin-resistant cell line, SGC7901/L-OHP, using slowly increased oxaliplatin concentrations over 6 months. The relationship between Txr1 expression and drug-resistance of oxaliplatin in GC was studied with drug intervention, gene silencing technology, real-time PCR and Western blot analysis. Of the 95 patients with GC, those with $TXR1^{-}$ GC had longer postoperative 3-year DFS (77.8 %) than those with $TXR1^{+}$ GC (52.9 %). In oxaliplatin-resistant SGC7901/L-OHP cells, the main expression location of Txr1 shifted from the nucleus to cytoplasm, and both the mRNA and protein expression of Txr1 were higher than that of the parental cells, whereas expression of thrombospondin-1 (TSP1) decreased. When the Txr1 gene was silenced, TSP1 expression increased and the oxaliplatin resistance was significantly reduced in SGC7901/L-OHP cells. Changed Txr1 expression in GC affects the efficacy of oxaliplatin-based chemotherapy. Increased Txr1 expression decreases TSP1 expression and inhibits apoptosis. Txr1 could be a target in reversing oxaliplatin resistance in GC.

Keywords Cancer · Oxaliplatin · Chemotherapy resistance · Txr1 · siRNA

Introduction

Gastric cancer (GC) is a common gastrointestinal cancer with occult incidence and high malignancy. For advanced gastric cancer, in addition to radical surgery, chemotherapy is a standard method in the comprehensive treatment for GC; oxaliplatin-based combination chemotherapy has become the mainstream chemotherapy for advanced GC. Oxaliplatin (L-OHP) is a third-generation platinum compound that significantly inhibits GC and many other tumors. However, L-OHP-treated GC often becomes drug resistant [1]. Reported possible mechanisms of oxaliplatin resistance are decreased drug accumulation; increased drug detoxification [2]; enhancement of DNA damage-repairing capacity [3]; increased platinum–DNA adducts; and changes in apoptosis-regulating genes [4, 5].

Recently, Cohen found a new drug-resistant gene that decreased secretion of thrombospondin-1 (TSP1) and caused resistance to taxol; therefore, it was called taxol-resistant gene 1 (Txr1) [6]. Others found that Txr1 had

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different levels of expression in breast cancer, GC, non-small cell lung cancer and nasopharyngeal carcinoma, which was mainly associated with taxol resistance and that Txr1 mRNA expression level in fresh tumor tissue was an independent prognostic factor [7–9]. Our previous study showed that Txr1 expression was related to the 5-year overall survival (OS) rate of patients with GC and could be used as an independent prognostic indicator [7]. Adjuvant chemotherapy for GC is mainly oxaliplatin-based, and relationship between Txr1 expression and chemotherapy is unknown. A retrospective study based on banked tissue was carried out to explore the possible relationship and mechanism between Txr1 expression in GC and oxaliplatin resistance.

Materials and methods

Patient information

From January 2006 to December 2008, we selected 95 patients with pathologically confirmed advanced GC at stages II and III, who had radical D2 surgery with post-operative oxaliplatin-based first-line chemotherapy (FOLFOX or XELOX) in Beijing Friendship Hospital. All cases had complete clinical information and tumor specimens. All patients received long-term outpatient and telephone follow-up to study the 3-year disease-free survival rate (DFS) as the primary end point. The study was approved by Beijing Friendship Hospital Ethics Committee.

Preparation of tissue microarray and immunohistochemical staining

Of the 95 subjects, tumor specimens, which had been preserved in paraffin blocks, were collected to make into a tissue microarray for immunohistochemistry staining, according to the method we previously developed [7]. Primary antibody to Txr1 (LifeSpan BioSciences, Seattle, WA, USA) was diluted to 1:200. PV-9001 secondary antibody and horseradish peroxidase-labeled streptavidin–avidin were purchased from Zhongshan Biotechnology Company (Beijing, China). Results were judged by two blinded experienced pathologists after examining tissue sections. Slides were considered to be $Txr1^+$ by brown staining in nuclei and/or cytoplasm.

Generation of oxaliplatin-resistant GC cell line

The culture method was in the literature [7]. Briefly, oxaliplatin (Eloxatin, Sanofi-Aventis, France) concentration was gradually increased (from 1 to 5 μg/mL) over 6 months to induce oxaliplatin resistance in SGC7901 cells.

Cell experiments

Cells in logarithmic growth phase were passaged and cultured in 25-cm^2 flasks (Costar, USA) at 37 °C with 5 % CO_2. When the cells covered 70–80 % of the flask side wall, an inverted microscope (Nikon) was used to observe differences in cell morphology. Cell viability was measured with an MTT assay. We calculated the inhibition rate of the drug on cells and drew cell growth inhibition curves. We used a modified Karber method to calculate IC_{50} values.

Txr1 expression positioning in cells was tested using immunofluorescent staining. Single cell suspension at 1×10^6/mL was used to prepare cell wafers in sterile 6-well culture plates (Costar, USA). After being rinsed, fixed and permeabilized, they were closed at room temperature with 5 % fetal calf serum (Gibco, Carlsbad, CA, USA), the primary antibody to Txr1 (1:400) and fluorescent secondary antibody (DyLight488 goat antibody-labeled rabbit antibody 1:400, Beijing Zhongshan Golden Bridge Company, Beijing, China) were added to seal plates, which were excited under the fluorescent microscope (Nikon, Japan), and the photographic film was read. The procedure was from the kit protocol.

Western blot analysis

Cell protein extraction, quantification and Western blot analysis were as previously described [7]. Txr1 antibody was from LifeSpan BioSciences and mouse anti-human β-actin monoclonal antibody was from Sigma. Rabbit anti-human TSP1 antibody was from Boster Biological Engineering (Wuhan, China). Rabbit polyclonal anti-human survivin antibody, mouse anti-human GST-π monoclonal antibody, secondary goat anti-rabbit IgG and secondary goat anti-mouse IgG were all purchased from Zhongshan Biotechnology (Beijing, China).

Quantitative real-time PCR

The 7500 Real-Time PCR (Applied Biosystems, Foster City, CA, USA) was used for real-time quantitative PCR. We formulated 20 μL of total test sample, including 10 μL of SYBR-Green PCR MasterMix (Applied Biosystems Company), 300 ng of cDNA template (Sangon, Shanghai, China) and 500 nmol of primer. Three assays were done for each reaction tube. Results were analyzed with the $2^{-\Delta\Delta Ct}$ method. All primers were designed online, according to the report [7].

Cell cycle analysis

Cells were harvested (1×10^6), washed, fixed, stained according to manufacturer's protocol and detected on FACScan flow cytometry. Data were analyzed for the cell cycle distribution and apoptosis using Cellquest software (Becton–Dickinson, San Jose, CA, USA).

Cell transfection

Cell transfection was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Serum-containing medium was replaced after 4–6 h. Cells were then cultured continuously. Transiently transfected cells were harvested after 48 h, whose mRNA and protein were extracted for real-time PCR and Western blot to confirm the efficiency of small interfering RNA (siRNA). The non-specific silencing sequence of synthetic Txr1 has a fluorescent marker (Invitrogen, USA) that shows green when excited under fluorescence microscopy and can be used to calculate transfection efficiency.

siRNA-mediated down-regulation of genes

Three pairs of siRNA duplex sequences against Txr1 were synthesized by Invitrogen [7]. An RNA silencing test was done using siRNA duplex 2 [7] as the silencing sequence and Lipofectamine 2000 as a carrier. Cells were harvested at 48 h since transfection and used for drug intervention trials, MTT test, cell cycle and apoptosis detection.

Statistical analysis

All experimental data were analyzed with the statistical analysis software SPSS13.0, and $P < 0.05$ was considered significant.

Results

Relationship between the Txr1 expression in GC tissues and 3-year DFS of patients with GC

We collected 96 consecutive patients with clinical GC at stages II and III in our study, consisting of 64 males and 31 females with a mean age of 62.06 years (range 32–86). The 3-year DFS of patients with $Txr1^+$ GC at stages II and III was significantly shorter than that of patients with $Txr1^-$ GC ($P < 0.05$; Fig. 1). There was no significant correlation between Txr1 expression and patient's gender, age, tumor type, depth of invasion or lymph node metastasis ($P > 0.05$; Table 1).

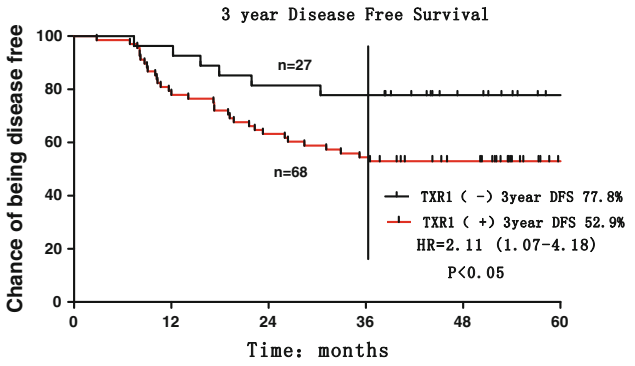

Fig. 1 Three-year disease-free survival rate and Txr1 expression for 95 gastric cancer patients

Txr1 expression was associated with oxaliplatin resistance in GC cells

As oxaliplatin concentration increased, a stably passaged oxaliplatin-resistant GC cell line, SGC7901/L-OHP, was successfully established. As shown in the growth curves of SGC7901/L-OHP and SGC7901 (Fig. 2a), cell doubling times did not significantly differ (28.4 h for SGC7901; 29.6 h for SGC7901/L-OHP). Immunofluorescence analysis showed that Txr1 expression was mainly located in the nucleus of SGC7901 cells, but in the cytoplasm of SGC7901/L-OHP cells (Fig. 2e).

Flow cytometry results initially showed that SGC7901/L-OHP and SGC7901 have no significant difference in distribution by cell cycle or apoptosis. However, after treatment with L-OHP, apoptosis in SGC7901 was significantly increased, whereas the apoptosis rate of SGC7901/L-OHP was not changed significantly (Table 2; Fig. 2b).

Real-time PCR and Western blot showed that both mRNA and protein expressions of Txr1 and survivin in SGC7901/L-OHP cells were higher than those in SGC7901 cells, while TSP1 expression was lower than that in SGC7901 cells (Fig. 2c, d).

Silenced Txr1 gene decreases oxaliplatin resistance in SGC7901/L-OHP

Under fluorescence microscopy, transfected cells were counted and transfection efficiency was calculated at about 40 %. Real-time PCR results suggested that cell transfection was valid (Fig. 3a). Using the MTT results, cell growth–inhibition curves were drawn for every experimental group; IC_{50} values for oxaliplatin in 48 h were calculated as 8.88, 26.69 and 27.1 (Fig. 3b) for the Txr1 silencing group, the uninterfered group and the negative control group, respectively.

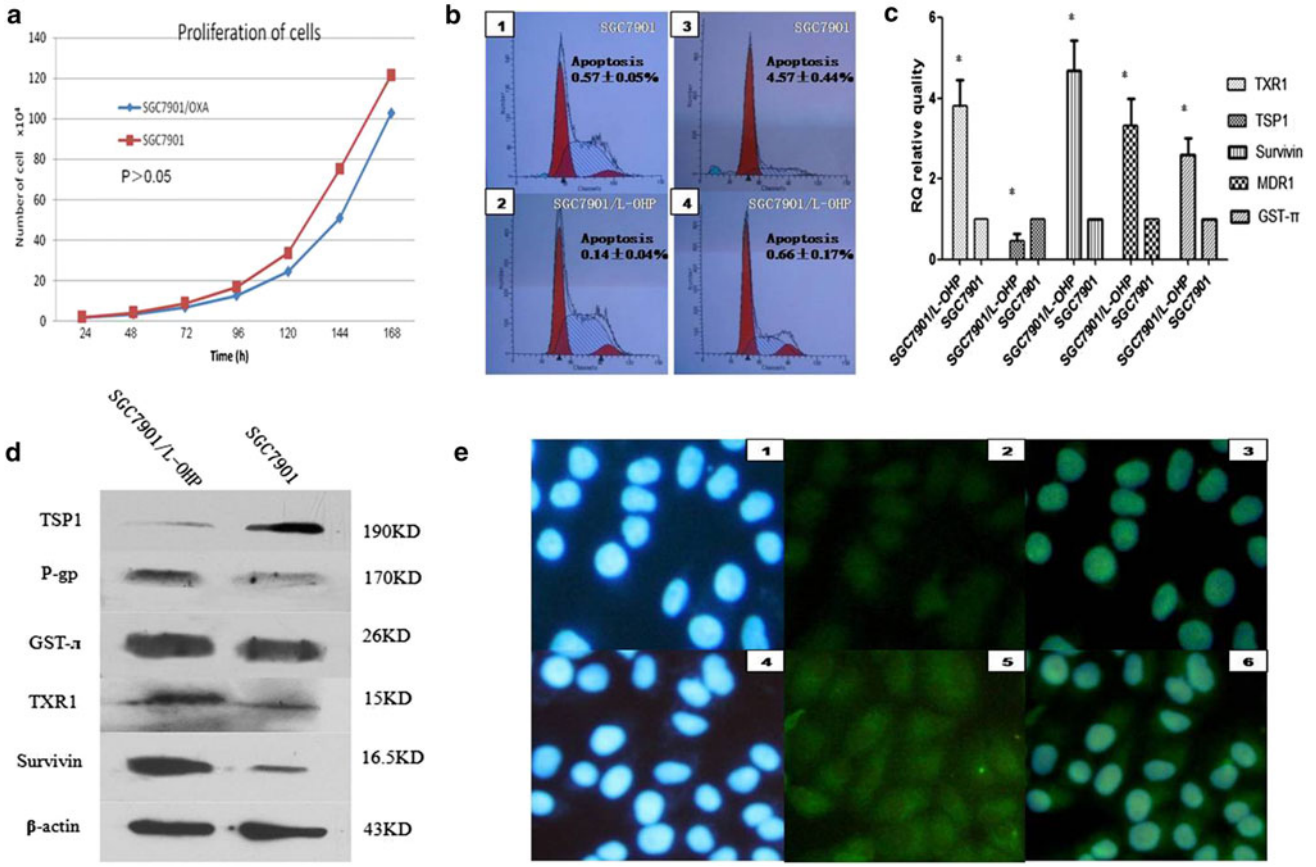

Fig. 2 **a** Growth curves for gastric cancer cells SGC7901/L-OHP and SGC7901. **b** Changes in apoptosis rate through FACS detection. **b1**, **b2**: After 24 h of normal culture. **b3**, **b4**: After 24 h of 14 μg/mL oxaliplatin treatment, apoptosis was significantly increased in SGC7901 cells ($P < 0.05$), but not in SGC7901/L-OHP cells ($P > 0.05$). **c**, **d** Real-time PCR and Western blot analysis of drug-resistance-related factors in gastric cancer cells: mRNA and protein expressions of Txr1, MDR1 and survivin in SGC7901/L-OHP cells were higher than that in SGC7901 cells, TSP1 expression was lower than that in SGC7901 cells ($P < 0.05$) while GST-π expression did not significantly differ in the two cell lines. **e** Immunofluorescence assay of Txr1 expression localization in cells (×200): **e1**, **e4** show DAPI-stained blue nuclei. **e2**, **e5** were stained with secondary antibody labels; Txr1 expression is shown as green fluorescence. **e3** shows **e1** merged with **e2**. **e6** shows **e4** merged with **e5**. **e1**–**e3** show Txr1 in SGC7901 cells is mainly expressed in the nucleus; **e4**–**e6** shows Txr1 in SGC7901/L-OHP cells is mainly expressed in the cytoplasm

Silencing of Txr1 gene affects SGC7901/L-OHP cell cycle and apoptosis

After the gene silencing of Txr1, 20 μg/mL of oxaliplatin was used for 24 h. Cell cycle and apoptosis in each group were detected on flow cytometry. Results showed that silencing of Txr1 led to a significant increase in apoptosis (Table 3 and Fig. 3c).

Effects of silencing Txr1 gene on expression of resistance-related factors in gastric SGC7901/L-OHP cells

After the gene silencing of Txr1, cells were incubated with 20 μg/mL of oxaliplatin for 24 h. Test results showed that mRNA and protein expression of Txr1 and survivin in the silenced group was significantly decreased, while that of TSP1 was significantly increased. Protein and mRNA expression of other resistance-associated factors were not significantly different (Fig. 3d, e).

Discussion

The mechanism of drug resistance to taxol was recently found to be mediated by Txr1, which down-regulates TSP1 in tumors [7–9]. In our previous study, we found that the 5-year OS rate for patients whose GC specimens expressed high Txr1 was shorter than that with low Txr1 expression, which could be used as an independent prognostic indicator [7]. However, the relationship between Txr1 expression and oxaliplatin resistance in GC is unclear. In this study, patients with stage II–III GC received D2 radical surgery, and oxaliplatin-based adjuvant chemotherapy were subjected to

Table 1 Clinicopathological features and TXR1 expression in the tissue of GC patients

	No. of cases	Txr1		X^2	P
		Negative	Positive		
Gender				4.131	0.052
Male	64	14	50		
Female	31	13	18		
Age (years)				0.893	0.345
≤60	42	14	28		
>60	53	13	40		
Lauren classification				1.976	0.227
Intestinal type	79	21	59		
Mixed or diffuse type	16	7	9		
Differentiation					
Poor or undifferentiated	60	16	44	0.246	0.620
Moderate or well	35	11	24		
Depth of wall invasion				2.521	0.472
T1	2	0	2		
T2	11	2	9		
T3	75	24	51		
T4	7	1	6		
Lymph node metastasis				0.481	0.488
Yes	78	21	57		
No	17	6	11		
pTNM stage				0.326	0.568
II	25	6	19		
III	70	21	49		
Disease-free survival (DFS)					
≥36 months	61	25	36		
<36 months	34	2	32		
3-year DFS		92.3 % (25/27)	52.9 % (36/68)	13.221	0.0003

inclusion criteria that made the chemotherapy results more clearly comparable and conducive to analysis of influencing factors. Mechanisms and relationships between Txr1 expression and oxaliplatin resistance of GC cells were explored preliminarily in vitro experiment, which complemented and helped develop further experiments.

No uniform international evaluation system for adjuvant chemotherapy currently exists. 3-year DFS is mainly used as a reference. The latest CLASSIC III clinical study [10] results showed XELOX-adjuvant chemotherapy after D2-type radical gastrectomy to be superior to observation alone, which meant the chemotherapy was effective. However, GC patients with same stage treated with the same surgery and adjuvant regimens show varying responses. Therefore, developing resistance to chemotherapy drugs accounts for much of this variation. According to the experimental methods and conclusions in this study, the 3-year DFS of patients with $Txr1^+$ GC was significantly lower than that with $Txr1^-$ GC, which suggests that Txr1 is associated with efficacy differences and chemotherapy drug resistance. Therefore, Txr1 expression could be used as prognostic indicator. Souglakos et al. [8] suggested that mRNA expression of Txr1 could indicate resistance of non-small cell lung cancer to taxol and help evaluate prognoses, which is similar to results in this study.

In vitro experiments showed Txr1 expression in SGC7901 cells was mainly focused in the nucleus, whereas that in drug-resistant cell line SGC7901/L-OHP was primarily in the cytoplasm, while nuclear Txr1 was significantly reduced. When treated with the same oxaliplatin concentration, the apoptosis rate of SGC7901/L-OHP was significantly lower than that of SGC7901, which suggests that cytoplasmic Txr1 predominated and might inhibit apoptosis—thus making cells less sensitive to chemotherapeutic drugs. Knauer et al. [11] in a study of prognosis of patients with colorectal cancer and the mechanism of survivin nucleo-cytoplasmic transport found a similar phenomenon in which survivin expression in the nucleus was dominant and enhanced chemotherapy sensitivity. Related reports in the literature suggested that change of survivin expression and localization affected efficacy of chemotherapy, tumor metastasis and invasiveness, as well as prognoses of cancer patients, which agreed with this study [12]. However, the specific molecular mechanism is still unknown and needs further study.

TSP-1 is an effective apoptosis-inducing protein, whose role in different cells varies by the presence of cell surface receptors and the degree of TSP-1 expression. Mateo reported that, in leukemia cells, TSP1 induced a non-caspase-dependent apoptosis through CD47 [14] and believed that this might be a common phenomenon in tumor cells [15]. Studies from Lih et al. [6] suggested that taxane cytotoxicity

Table 2 Differences of cell cycle distribution and apoptosis rate of SGC7901/L-OHP and SGC7901

Groups	Treated with L-OHP (24 h)	G_0/G_1 %	S %	G_2/M %	Apoptosis %
SGC7901	0	42.33 ± 1.8	51.48 ± 0.83	6.44 ± 0.72	0.57 ± 0.05
SGC7901	14 μg/mL	78.35 ± 1.77	19.93 ± 1.7*	1.73 ± 0.07*	4.57 ± 0.44*
SGC7901/L-OHP	0	41.31 ± 2.41	52.21 ± 2.2	6.48 ± 0.21	0.14 ± 0.04
SGC7901/L-OHP	14 μg/mL	63.18 ± 6.15	29.92 ± 2.95*	6.91 ± 3.2	0.66 ± 0.17

* $P < 0.05$

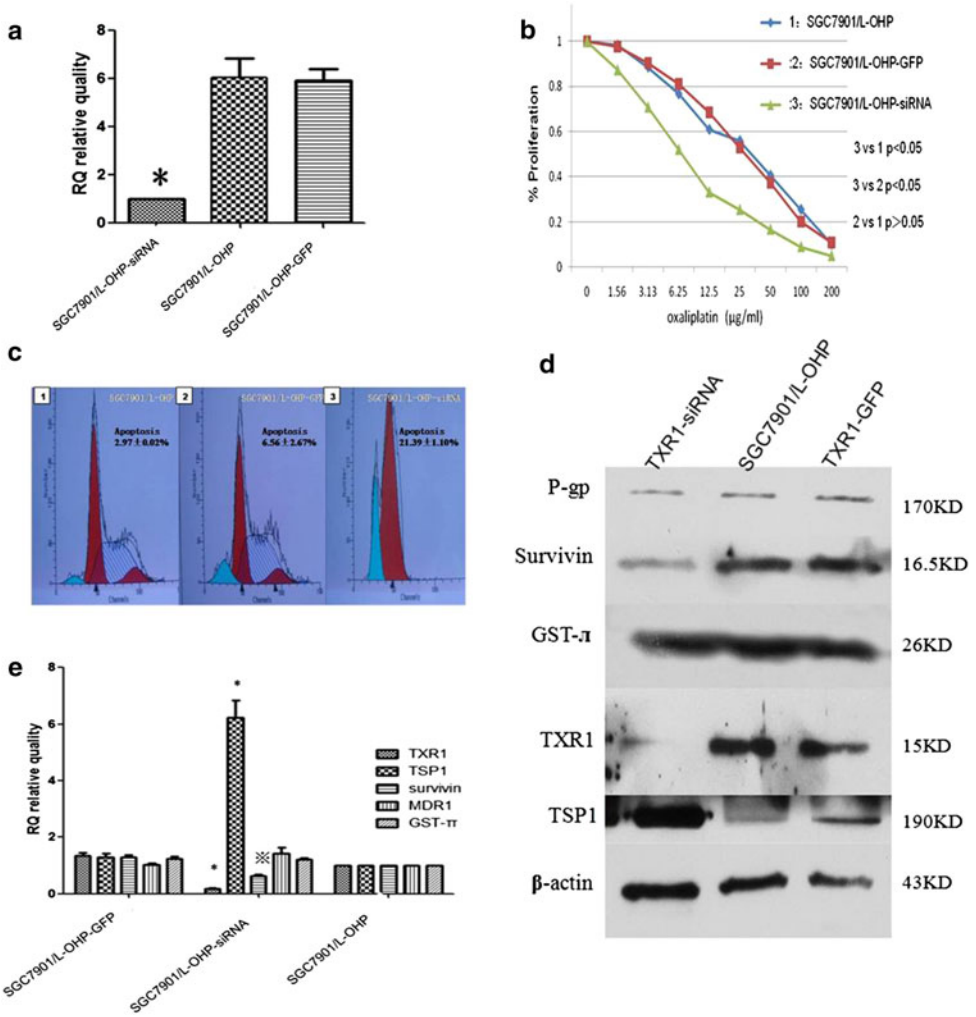

Fig. 3 **a** RNA silencing efficiency test results: After transfection, Txr1 mRNA in the silenced group was significantly less than that in the non-silenced group and negative control group ($P < 0.05$); Txr1 mRNA in the silenced group and negative control group did not significantly differ. **b** Cell growth–inhibition curve within 48 h after Txr1-siRNA was transfected in each group; **c** effect of Txr1 gene silencing on the apoptosis of gastric cancer SGC7901/L-OHP: Apoptosis rate in the silenced group was significantly lower than that in non-silenced group and negative control group ($P < 0.05$); apoptosis rates of the non-silenced group and negative control group did not significantly differ. **d**, **e** After gene silencing, effect of Txr1 on the drug-resistance-associated factor mRNA and protein expression of SGC7901/L-OHP cells: mRNA and protein expressions of Txr1 in the silenced group (TXR1-siRNA) were significantly decreased ($P < 0.05$); the negative control group (TXR1-GFP) and non-silenced group (SGC7901/L-OHP) had no significant change; mRNA and protein expressions of TSP1 in the silenced group were greater than those in the negative control and non-silenced groups ($P < 0.05$); mRNA and protein expressions of survivin in the silenced group were less than those in the control and non-silenced groups ($P < 0.05$)

was regulated by TSP1 through activation of CD47-integrin-associated signaling pathways, whereas Txr1's inhibitory effect on apoptosis occured at the transcriptional level, which affected chemoresistance of tumor cells by decreasing TSP1 expression. In this study, it was found that, under action of oxaliplatin with the same concentration, the apoptosis rate of SGC7901/L-OHP was significantly lower than that of SGC7901. Protein and mRNA expressions of Txr1 were higher than those of SGC7901, and their expression increased in a time- and concentration-dependent manner, which indicated that the increased Txr1 expression was induced by the drug. On the other hand, its TSP1 expression was lower than that of SGC7901, which was inverse to Txr1 expression and consistent with that in literature [2, 4, 5, 13]. After the Txr1 gene silencing, Txr1 protein expression in SGC7901/L-OHP was decreased as TSP1 expression increased, while apoptosis rate and sensitivity to oxaliplatin were increased. This result further indicated that increased Txr1 expression decreased TSP1 expression, resulting in inhibited apoptosis, which might be a mechanism of GC oxaliplatin resistance.

Survivin is a member of apoptosis-inhibiting protein family playing a large role in the development of GC. In this study, expressions of Txr1 and survivin were found to

Table 3 Effects of siRNA silencing on cell cycle distribution and apoptosis in gastric cancer

Groups	G0/G1 %	S %	G2/M %	Apoptosis %
SGC7901/L-OHP	47.12 ± 0.99	44.69 ± 0.48	8.14 ± 0.51	2.97 ± 0.02
SGC7901/L-OHP-GFP	42.66 ± 0.87	47.60 ± 0.96	8.29 ± 0.32	6.56 ± 2.67
SGC7901/L-OHP-siRNA	97.00 ± 2.00*	0.58 ± 0.10*	2.42 ± 1.90*	21.39 ± 1.10*

* $P < 0.05$; statistically significant compared with changes in the corresponding cell cycle and apoptosis in the undisturbed group and negative control group

Each group was tested after 24 h of the action of 20 μg/mL of L-OHP

be significantly greater in SGC7901/L-OHP than in SGC7901. When treated with the same concentrations of oxaliplatin, apoptosis in SGC7901 significantly increased, but did not significantly change in SGC7901/L-OHP cells. However, when the Txr1 gene was silenced, survivin expression declined, SGC7901/L-OHP sensitivity to oxaliplatin recovered and its apoptosis rate increased. All suggested that expressions of both Txr1 and survivin were closely related to apoptosis, and their increased expression led to oxaplatin resistance. Some studies had shown that treatment with a caspase inhibitor only partially reduced anti-apoptotic ability of survivin, suggesting that survivin mediates both the caspase-dependent and caspase-independent apoptotic pathway [16, 17]. Analysis of experimental results implied that silencing Txr1 decreased expression of survivin through some signal regulation, which in turn inhibited the apoptosis signaling pathways. On the other hand, silencing Txr1 led to increased TSP1 expression, promoted apoptosis of tumor cells by activating CD47-integrin-associated signaling pathways and decreased oxaliplatin resistance. Txr1, survivin and TSP1 were linked through a complex mechanism to affect oxaliplatin resistance in GC.

In this study, siRNA was used to specifically inhibit the Txr1 expression in SGC7901/L-OHP, resulting in more cells in the G_1 phase, fewer cells in S-phase, increased apoptosis, significantly decreased oxaliplatin resistance index, and significantly increased mRNA and protein expression of TSP1 and survivin. However, there was no significant change in mRNA and protein of MDR1, GST-π and other drug-related factors, which suggested that siRNA could specifically inhibit Txr1 gene transcription, regulate cell cycle and apoptosis, and reverse resistance of SGC7901/L-OHP to oxaliplatin, which implied that Txr1 could be a new therapeutic target, particularly in oxaliplatin-resistant GC.

Development of resistance to cancer drugs is multifactorial process that often involves the generation of many drug-resistance-related genes and multiple mechanisms. Here, we found that expressions of Txr1, MDR1, GST-π, survivin and other drug-resistance-related factors in drug-resistant cell SGC7901/L-OHP were increased, all of which might be involved in resistance to oxaliplatin, possibly interacting with each other to form a complex network of regulatory mechanisms. These results preliminarily confirmed that Txr1 is closely related to prognosis of gastric cancer and oxaliplatin resistance in chemotherapy. The Txr1 gene might affect apoptosis and cell division. Its natural function and its role in chemotherapy resistance are extremely complex, which merits further study.

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Conflict of interest We declare no conflict of commercial or associative interest in connection with the work.

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