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ORIGINAL ARTICLE

Inhibition of glutathione synthesis reverses Krüppel-like factor 4-mediated cisplatin resistance

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Abstract
Purpose To explore the protective effect of KLF4 against cytotoxicity induced by cisplatin and its possible mechanisms.
Methods The expression levels of KLF4 were detected by RT-PCR and western blot in cancer stem-like cells derived from hepatocarcinoma (T3A-A3) and the hepatocarcinoma cell line HepG2. KLF4 was knocked down in T3A-A3 by infection of pLVTHM-shKLF4 lentivirus and ectopic expressed in HepG2 by infection of pWPTS-KLF4 lentivirus. The MTT assay was carried out to determine the impact of KLF4 on cell survival in response to cisplatin. Cisplatin-induced DNA damage was measured by TUNEL staining. Glutathione content was measured by enzymatic assay. Buthionine sulfoximine was used to deplete the content of glutathione. The expression of γ-glutamylcysteine synthetase was analyzed by RT-PCR in HepG2 cells ectopic expressed KLF4.
Results With a higher level of KLF4, T3A-A3 cells were found to be more resistant to cisplatin than HepG2 cells. KLF4 knockdown was found to reduce cisplatin resistance in T3A-A3 cells. Ectopic expression of KLF4 in HepG2 cells was found to be associated with heightened resistance to DNA damage after exposure to cisplatin. Furthermore, the content of glutathione was found to be higher in T3A-A3 cells than in HepG2 cells. A nearly twofold increase in the cellular level of glutathione was identified in HepG2 cells with ectopic expression of KLF4. This was accompanied by heightened resistance to cisplatin. KLF4-mediated resistance to cisplatin in HepG2 cells was found to be completely abrogated by treatment with buthionine sulfoximine, an inhibitor of glutathione synthesis, which did not affect the expression of KLF4. Moreover, the mRNA expression of γ-glutamylcysteine synthetase, a rate-limiting enzyme of glutathione synthesis was up-regulated by KLF.
Conclusion We conclude that KLF4 regulates the cellular sensitivity to cisplatin in hepatocarcinoma stem-like cells and hepatocarcinoma cells by elevating intracellular glutathione levels.

Keywords Krüppel-like factor 4 · Cisplatin resistance · Glutathione · Cancer stem cell · Hepatocarcinoma

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Introduction

Chemotherapy is one of the most important therapies for cancer. However, the development of resistance to chemotherapeutic drugs in cancer cells significantly diminishes the intended effects of the therapy. Therefore, it is imperative to elucidate the mechanisms of chemoresistance in order to improve the effects of cancer therapeutics.

The mechanisms of chemotherapeutic resistance have been gleaned from various studies of cellular models of resistance, including changes in cellular drug accumulation [1],

drug detoxification [2, 3], inhibition of apoptosis [4], and repair of DNA adducts [5]. Recently, it was found that the content of total cellular glutathione (GSH) is an important determinant of chemoresistance [6]. The association between elevated cellular levels of GSH and chemoresistance has indicated that inactivation of cisplatin might occur through conjugation with GSH [7, 8]. The administration of GSH is also reported to have a protective effect on cisplatin toxicity in mice [9]. Intracellular GSH stores can be down-regulated by treatment with buthionine sulfoximine (BSO), which acts as an inhibitor of γ-glutamylcysteine synthetase (γ-GCS)—the rate-limiting enzyme for GSH synthesis. The suppression of intracellular GSH by the addition of BSO to cultured cancer lines was shown to heighten cisplatin sensitivity. Conversely, an elevation of GSH production by the addition of *N*-acetyl cysteine leads to increased cisplatin resistance [10].

It has been well accepted that cancer tissues are composed of heterogeneous cellular components with differing sensitivities to chemotherapy. Compared to bulk tumor cells, cancer stem cells have proven to be more resistant to standard chemotherapy [11]. It was presumed that cancer stem cells could evade chemotherapy, thereby resulting in tumor recurrence. It remains unclear how cancer stem cells develop chemoresistance. Genetic or epigenetic alterations have been shown to play a pivotal role in the regulation of chemotherapeutic resistance [12]. Krüppel-like factor 4 (KLF4) is an important sequence-specific DNA-binding protein capable of maintaining the self-renewing characteristics and pluripotency of stem cells. KLF4 regulates the transcription of genes involved in cell growth, proliferation, differentiation, and embryogenesis. In conjunction with three other transcription factors, KLF4 could transform murine fibroblasts into a state resembling that of embryonic stem cells [13]. KLF4 has a potent oncogenic role in mammary tumorigenesis, possibly by maintaining stem cell-like features and promoting cell migration and invasion [14]. Moreover, KLF4 has been reported to be involved in chemotherapeutic resistance. The overexpression of KLF4 in murine embryonic fibroblasts could lead to the development of cisplatin resistance [15]. It is worthwhile to study the role of KLF4 in chemoresistance of cancer stem cells or cancer cells.

In the present study, we investigated the role of KLF4 in chemoresistance, by comparing the chemotherapeutic sensitivity between hepatocarcinoma stem-like cells with high levels of KLF4 and HepG2 hepatocarcinoma cells with low levels of KLF4. Loss- and gain-of-function analyses in these cells were investigated to characterize the possible roles of KLF4 in regulation of chemotherapeutic sensitivity.

Materials and methods

Cell lines and drugs

The hepatocarcinoma cell line HepG2 and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, USA), supplemented with 10% heat-inactivated fetal bovine serum (Gibco, USA), 100 units/mL penicillin (Sigma, USA), and 100 μg/mL streptomycin (Sigma, USA), and maintained in the presence of 5% CO_2 at 37°C.

Derived from microvascular endothelial cells of human liver cancer [16], T3A cells contain a subpopulation of cells capable of self-renewal as spherical clones under anchorage-independent, serum-starved culture conditions. The NOD/SCID mice were subcutaneously inoculated. Monoclonal screening was performed; a single cell clone (T3A-A3) with rapid proliferation and high tumorigenicity was selected using an MTT assay and by examination of tumorigenesis in immunodeficient mice. T3A-A3 cells express stem cell markers and possess the characteristics of cancer stem cells. T3A-A3 cells were suspended in serum-free DMEM supplemented with B27 (1:50; Invitrogen, USA), 20 ng/mL human epidermal growth factor (hEGF; Invitrogen, USA), 20 ng/mL basic fibroblast growth factor (bFGF; R&D Systems, USA), 40 U/mL heparin (Sigma, USA), 2 mM glutamine (Sigma, USA), 100 units/mL penicillin (Sigma, USA), 100 μg/mL streptomycin (Sigma, USA), 5 μg/mL insulin (Sigma, USA), 0.5 μg/mL hydrocortisone (Sigma, USA) and plated into ultra-low attachment plates (Corning, USA).

The drugs employed included cisplatin (Sigma, USA), BSO (Sigma, USA), fluorouracil (Tianjin Jinyao Amino Acid Co, China), cyclophosphamide (Jiangsu Hengrui Medicine Co, China), doxorubicin (Sigma, USA), and vincristine (Shenzhen Main Luck Pharmaceuticals Inc, China).

Lentivirus production and transduction

Lentivirus production and tittering were carried out according to the following protocols from Trono Lab (http://tronolab.epfl.ch). In HEK293T cells, the pLVTHM vector containing KLF4-short hairpin RNA (shRNA) (GGA CGGCTGTGGATGGAAA) [15] or the shRNA-negative control (kindly provided by Prof. Kosik) [17] was co-transfected with the envelope plasmid pMD2.G and the packing vector psPAX2 via calcium phosphate precipitation. Virus-containing medium was collected 48 h after transfection, and the viruses were concentrated by ultracentrifugation at 28,000 rpm for 2 h. Concentrated viruses were reconstituted in phosphate-buffered saline (PBS). Full-length human KLF4 cDNA was amplified with a pair

of primers (forward: 5′-ATGGATCCCTGCTTCGGGC TGCCGAGGACCTTCTGGG-3′; reverse: 5′-TCGCAGTC GACGGCAGTGTGGGTCATATCCACTGTCTGGGA-3′). The PCR products were ligated, digested with *Bam*HI and *Sal*I, and subsequently cloned into the corresponding site of pWPTS vectors. The lentivirus pWPTS-KLF4 or pWPTS-GFP was produced as indicated previously.

Total RNA extraction and semiquantitative RT-PCR

Total RNA was extracted with Trizol (Invitrogen, China) according to the manufacturer's protocol. For mRNA analysis, 1 μg of total RNA was used for reverse transcription by Omniscript reverse transcriptase (Qiagen, China). The cDNA was amplified with primer pairs: KLF4 forward: 5′-CCCAATTACCCATCCTTCCT-3′; reverse: 5′-AGGTTTCTCACCTGTGTGGG-3′ (amplification product: 240 bp); γ-GCS forward: 5′-CAAGGACGTTCTCA AGTGGG-3′; reverse: 5′-CATACTCTGGTCTCCAAAG G-3′ (amplification product: 170 bp); β-actin forward: 5′-AACAAGATGAGATTGGCATGG-3′; reverse: 5′-AGT GGGGTGGCTTTTAGGAT-3′ (amplification product: 251 bp), β-actin mRNA levels were used as an internal normalization control. The PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide.

Western blot

To determine the levels of protein expression, the cells were harvested, lysed in RIPA lysis buffer (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.25% sodium deoxycholate, and 1 mM EDTA) with freshly added protease inhibitor cocktail (Roche, USA) for 30 min on ice, and subsequently centrifuged at 13,000 rpm for 10 min. The total protein concentration of whole-cell extracts was measured with Bradford reagent (Bio-Rad, Hercules, CA, USA). The proteins were resolved by SDS–PAGE (Bio-Rad, USA). After electrophoresis, the proteins were electrotransferred to polyvinylidene fluoride membranes (Millipore, USA), blocked with 5% skimmed milk, and probed with the following primary antibodies diluted in PBS/BSA: KLF4 (Santa Cruz, USA) followed by horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma, USA). Binding was detected by enhanced chemiluminescence (Millipore, USA).

Cytotoxicity assays

Cells were seeded in 96-well tissue culture plates at density of 5×10^3 cells/well and incubated for 24 h in a CO_2 incubator. The medium was refreshed with medium containing different drugs. After a 72-h incubation, the surviving cells were detected in the wells by using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-2H-tetrazolium-bromide) assays. MTT (5 mg/mL) was added into each well (50 μL/well). After a 4-h incubation at 37°C, the water-insoluble formazan dye was solubilized with DMSO (150 μL/well) and its optical density quantified using an ELISA reader (Multiskan MK3; Thermo, USA) at 492 nm. The cell viability was calculated as the ratio of absorbance in wells containing drugs and drugs plus modulators versus the control group. The IC_{50} values were calculated from a linear regression obtained from the dose-dependent curves of at least five points.

To detect the effect of GSH on KLF4-mediated cell survival responded to cisplatin, cells were plated in 96-well dishes at density of 5×10^3 cells/well in 50 μL of medium. After overnight incubation, 50 μL of medium containing 1 mM BSO was added to each well. The following day, 100 μL of medium containing different dose cisplatin was added to achieve concentrations ranging from 0 to 100 μM in a total volume of 200 μL. After continued incubation for 48 h, MTT assay was performed as described earlier.

TUNEL staining

Cells were plated in a 96-well plate and cultured in an incubator under 5% CO_2 for 24 h. After a 72-h incubation period with cisplatin, the cells were washed, fixed in 4% paraformaldehyde, and then permeabilized with 0.1% Triton X-100 in PBS/BSA solution. The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed with in situ cell death detection kits (Roche, USA).

Determination of intracellular glutathione

GSH concentrations were measured as described previously [10]. Briefly, cells grown to 80% confluence in T-75 flasks were harvested with trypsin, washed in cold PBS, and resuspended in 0.154 M NaCl. Protein extracts were prepared by multiple freeze/thaw cycles and by sonication. These extracts were normalized for total protein content using the modified Bradford assay (Bio-Rad). Total GSH was quantified with an enzymatic assay [18].

Statistical analysis

The results are expressed as mean ± SD, and two-tailed *t* tests were used to evaluate the intergroup differences with Prism 5.0 software (GraphPad, USA). The linear regression analysis for IC_{50} values and paired t test was performed using Excel and the SigmaStat Statistical Analysis System, version 1.01. The statistical significance was set at $P < 0.05$, and $P < 0.01$ indicates a highly significant difference.

Results

Correlation of KLF4 with cisplatin sensitivities in HepG2 and T3A-A3 cells

To determine whether KLF4 marks a phenotype that is highly resistant to cisplatin, we first compared the expression level of KLF4 in hepatocarcinoma-derived stem-like T3A-A3 cells and HepG2 cells. Then, the sensitivity to cisplatin was analyzed in these two cell types. As shown in Fig. 1a and b, T3A-A3 cells express high levels of KLF4, while HepG2 cells express very low levels of KLF4. The MTT assay was used to evaluate the cytotoxicity of cisplatin with respect to T3A-A3 and HepG2 cells. The results showed that viable cell numbers were 1.71-fold higher in the T3A-A3 cells than in HepG2 cells after incubation with 6.25 μM cisplatin for 72 h ($P < 0.01$). In T3A-A3 cells, cisplatin was found to have an IC_{50} of 38.48 μM, whereas in HepG2 cells, it was found to have an IC_{50} of 9.80 μM (Fig. 1c). These results suggest that the expression of KLF4 is positively correlated with resistance to cisplatin.

Lowering of cisplatin resistance by KLF4 shRNA in T3A-A3 cells

To address the relationship between the elevated expression of KLF4 and low sensitivity to cisplatin in T3A-A3 cells, we altered the functional levels of KLF4 in T3A-A3 cells. T3A-A3 cells were infected with a lentivirus harboring KLF4 shRNA or a negative control shRNA. The expression of the co-expressed fluorescent marker EGFP was monitored by fluorescence microscopy at 72 h after infection (Fig. 2a). As expected, the expression level of KLF4 was lowered significantly by shKLF4 lentivirus infection (Fig. 2b, c). Furthermore, the MTT assay was carried out to determine the impact of KLF4 knockdown on cell survival in response to cisplatin. As shown in Fig. 2d, T3A-A3 cells became sensitized to cisplatin after knockdown of KLF4. The IC_{50} for shKLF4-infected T3A-A3 cells was found to be 19.37 μM versus 39.57 μM in the control cells (Fig. 2d). Cisplatin is generally considered as a cytotoxic drug which kills cancer cells by damaging DNA and inhibiting DNA synthesis. How cells respond to cisplatin-induced DNA damage plays a critical role in deciding cisplatin sensitivity. One of the most widely used methods for detecting DNA damage in situ is TUNEL staining. Therefore, the cisplatin-induced DNA damage was measured by TUNEL staining to observe the effects of KLF4 on DNA damage. As shown in Fig. 2e, the cisplatin-induced DNA damage was significantly increased by the infection of shKLF4 in T3A-A3 cells relative to the control (shKLF4: 48.57 ± 13.74% vs. shNC: 13.64 ± 10.48%; $P < 0.01$).

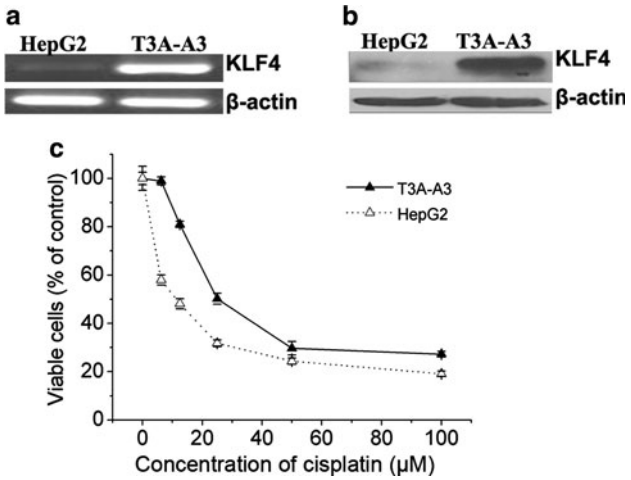

Fig. 1 Correlation of KLF4 with cisplatin sensitivities in HepG2 and T3A-A3 cells. The expression levels of KLF4 were compared in HepG2 and T3A-A3 cells, as detected by **a** RT-PCR and **b** western blot; β-actin was regarded as an endogenous control. **c** Comparison of cisplatin cytotoxicity in HepG2 and T3A-A3 cells by an MTT-based cytotoxicity assay

Ectopic expression of KLF4 in HepG2 cells induces enhanced resistance to cisplatin

To further characterize the effects of KLF4 on cisplatin sensitivity, we infected HepG2 cells with pWPTS-KLF4 to up-regulate KLF4. RT-PCR and western blot analyses showed that KLF4 was ectopically expressed in the pWPTS-KLF4 infected HepG2 cells (Fig. 3a, b). Then, the cytotoxicity of cisplatin was evaluated. The results showed that HepG2 cells infected with pWPTS-KLF4 had markedly increased resistance to cisplatin versus the control ($P < 0.01$) (Fig. 3c). The IC_{50} for HepG2 cells infected with pWPTS-KLF4 was 54.62 μM versus 11.08 μM for control cells. The survival rates of pWPTS-KLF4-infected HepG2 cells treated with 6.25 μM cisplatin were significantly higher than those of the control (Lenti-KLF4: 100.31 ± 4.58% vs. Lenti-GFP: 60.95 ± 2.17%, $P < 0.01$). As shown in Fig. 3d, the cisplatin-induced DNA damage was significantly reduced by the infection of pWPTS-KLF4 in HepG2 cells relative to the control (Lenti-KLF4: 18.03 ± 6.24% vs. Lenti-GFP: 59.01 ± 9.93%, $P < 0.01$).

Regulation of cisplatin sensitivity by KLF4 through an elevated intracellular level of glutathione

Although many mechanisms of cisplatin resistance have been proposed over the last decade, the inactivation of intracellular cisplatin by GSH has been regarded as an important mechanism of cisplatin resistance [19]. Therefore, we compared the intracellular levels of GSH in T3A-A3 and HepG2 cells. T3A-A3 cells were found to have a

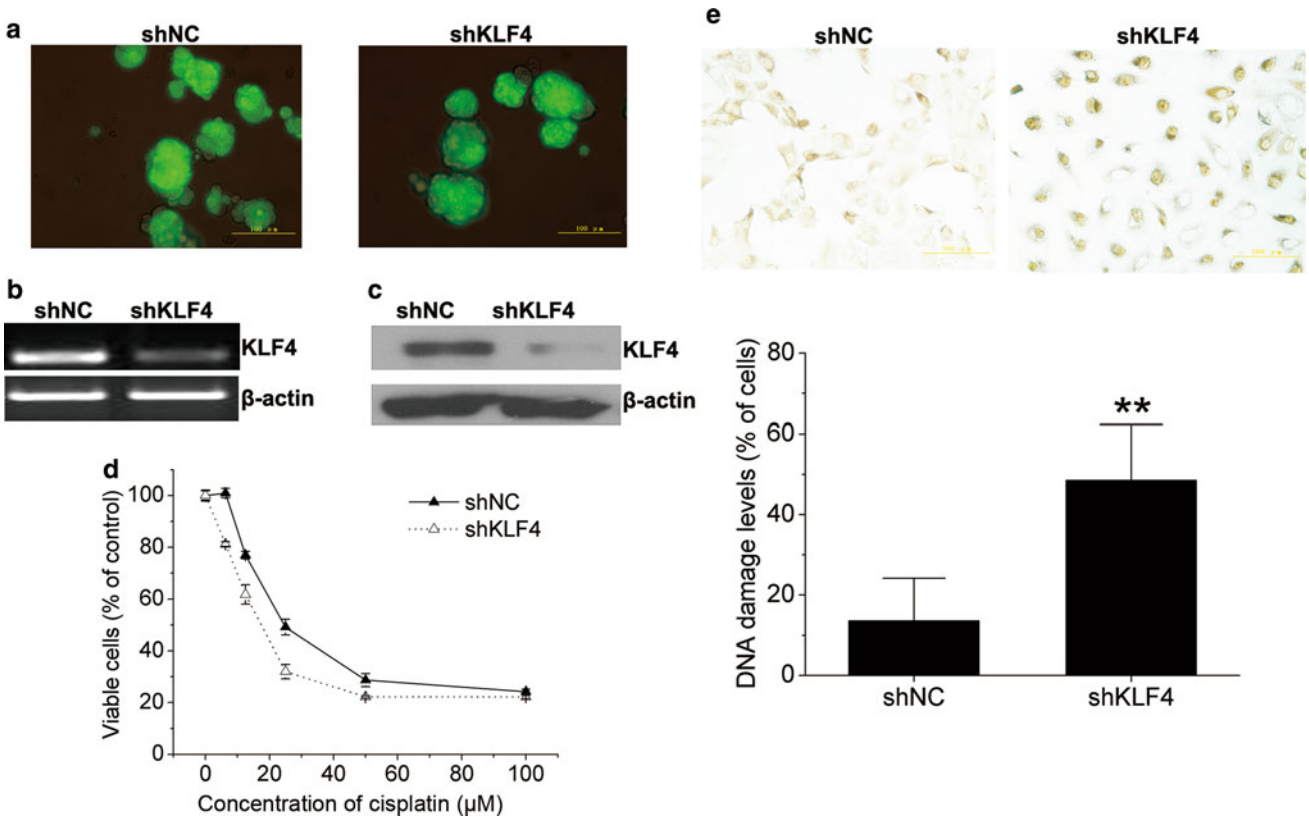

Fig. 2 Lowering of cisplatin resistance by KLF4 shRNA in T3A-A3 cells. **a** Detection of infection efficiency. EGFP, a co-expressed fluorescent marker of the vector, was monitored in T3A-A3 cells by fluorescence microscopy at 72 h after infection. **b** The expression of KLF4 was detected by RT-PCR. **c** KLF4 expression was detected by western blot and normalized by β-actin (shKLF4, T3A-A3 cells infected with KLF4 shRNA lentivirus; shNC, T3A-A3 cells infected with negative control lentivirus vector). **d** Effect of shKLF4 on cisplatin resistance in T3A-A3 cells. The MTT-based cytotoxicity assay was performed at 72 h after infection. **e** Effect of KLF4 on cisplatin-induced DNA damage in T3A-A3 cells. T3A-A3 cells infected with shKLF4 or shNC were cultured in 12.5 μM cisplatin for 72 h. DNA damage was detected by TUNEL staining. The percentage of TUNEL-positive cells was analyzed by IPP imager software. $^{**}P < 0.01$ shKLF4 versus shNC

higher content of GSH than HepG2 cells (Fig. 4a). Thus, the expression of KLF4 might be positively correlated with the intracellular level of GSH. Furthermore, GSH might contribute to KLF4-mediated cisplatin resistance. To test this hypothesis, the GSH content was measured in HepG2 cells infected with lentivirus harboring pWPTS-KLF4 or pWPTS-GFP. As shown in Fig. 4b, a significant difference in the intracellular level of GSH was observed between pWPTS-KLF4 and pWPTS-GFP (Lenti-KLF4: 111.73 ± 18.33 nmol/mg protein vs. Lenti-GFP: 58.08 ± 9.93 nmol/mg protein; $P < 0.01$). To confirm the relationship between GSH and increased survival mediated by KLF4 after cisplatin exposure, the survival rate of HepG2 ectopic expressed KLF4 exposed to cisplatin was evaluated with or without pre-treated with BSO. BSO was found to deplete the stores of GSH in both the pWPTS-GFP and pWPTS-KLF4 transductions to a similar basal level (Fig. 4c). Next, the cell viability was determined under exposed to different concentrations of cisplatin in the presence or absence of BSO. It was demonstrated that pre-incubation with BSO restored cellular sensitivity to cisplatin in KLF-4 ectopic expressed HepG2 cells (Fig. 4d). These results suggested that the resistance to cisplatin in HepG2 cells with ectopic expression of KLF4 was GSH depended. An alternative explanation would be that BSO exposure causes suppressed expression of KLF4 in the KLF4 transductions. However, western blotting in the presence or absence of BSO revealed no significant decrease in the expression of KLF4 in the GFP or KLF4 ectopic expressed HepG2 cells (Fig. 4e).

Expression ofγ-GCS was up-regulated by ectopic expression of KLF4 in HepG2 cells

To investigate the mechanism of the up-regulation of glutathione in KLF4-ectopic expressing HepG2 cells, the expression of γ-GCS was quantitated by RT-PCR. In the KLF4 ectopic expressed HepG2 cells, there was a further 50% increase in the γ-GCS mRNA level than in GFP ectopic expressed HepG2 cells (Fig. 5). The γ-GCS

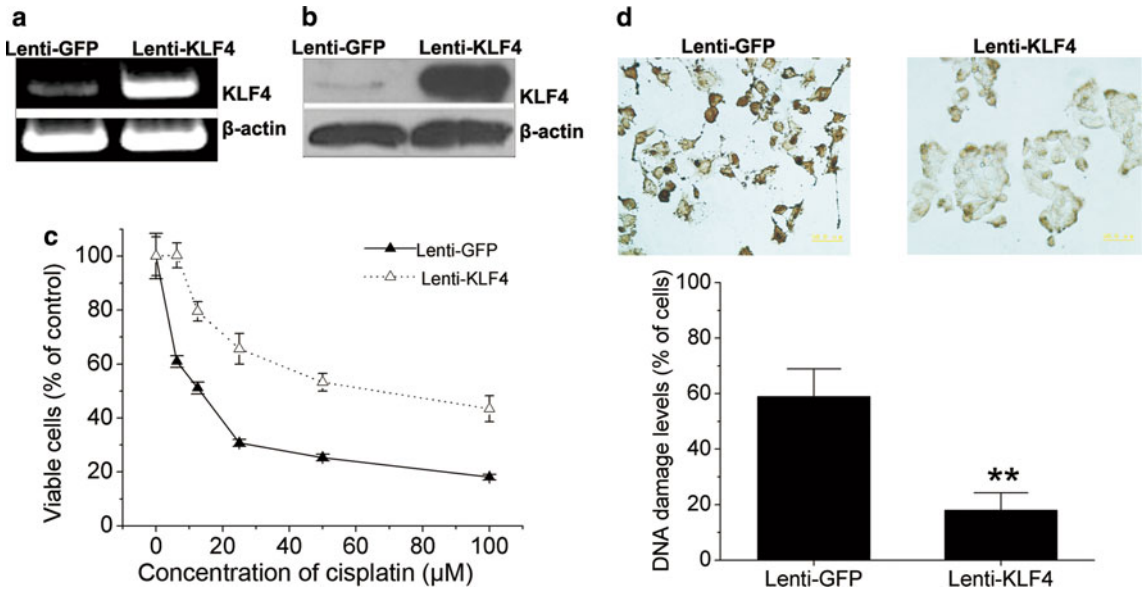

Fig. 3 Enhancement of cisplatin resistance by ectopic expression of KLF4 in HepG2 cells. The KLF4 expression in HepG2 cells infected with pWPTS-KLF4 or pWPTS-GFP lentivirus for 72 h was detected by **a** RT-PCR and **b** western blot (Lenti-KLF4, HepG2 infected with pWPTS-KLF4 lentivirus; Lenti-GFP, HepG2 infected with pWPTS-GFP lentivirus). **c** Comparison of cisplatin cytotoxicity in HepG2 cells infected with pWPTS-KLF4 or pWPTS-GFP by the MTT assay. **d** Effect of KLF4 on cisplatin-induced DNA damage in HepG2 cells. HepG2 cells infected with pWPTS-KLF4 or pWPTS-GFP were cultured in 25 μM cisplatin for 72 h. DNA damage was detected by TUNEL staining. The percentage of TUNEL-positive cells was analyzed by IPP imager software. $^{**}P < 0.01$ for Lenti-KLF4 versus Lenti-GFP

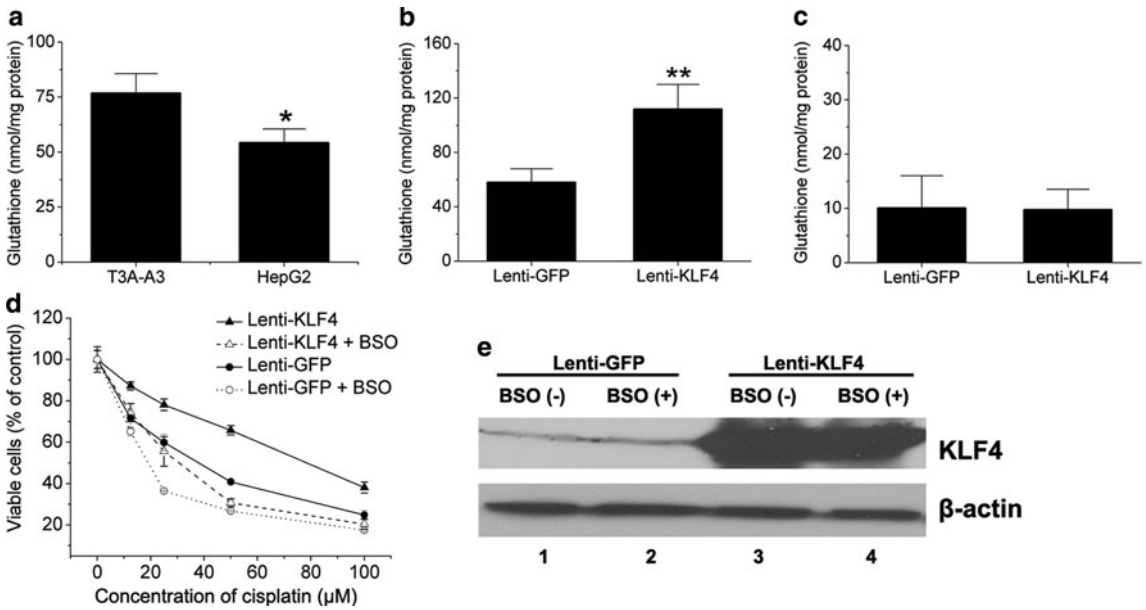

Fig. 4 : Regulation of cisplatin sensitivity by KLF4 through an elevated intracellular level of glutathione. **a** Total cellular glutathione in T3A-A3 and HepG2 cells. $^{*}P < 0.05$ for HepG2 versus T3A-A3. **b** Total cellular glutathione in HepG2 cells infected with pWPTS-KLF4 or pWPTS-GFP lentivirus for 72 h. $^{**}P < 0.01$ for Lenti-KLF4 versus Lenti-GFP. **c** The effect of BSO on glutathione content of HepG2 cells infected with pWPTS-KLF4 or pWPTS-GFP. The cells were incubated for 24 h with 0.5 mM BSO, followed by measurements of the glutathione content. **d** The depletion of glutathione inhibits KLF4-mediated cisplatin resistance in HepG2 cells. HepG2 cells infected with pWPTS-KLF4 or pWPTS-GFP were cultured with various concentrations of cisplatin in the presence or absence of 0.5 mM BSO, and the viable cells were detected by the MTT assay after 48 h. **e** The expression of KLF4 was analyzed by western blotting in HepG2 cells infected with pWPTS-KLF4 or pWPTS-GFP. *Lane 1*, *3* extracts from untreated cells; *Lanes 2*, *4* extracts from cells after a 24-h incubation period in the presence of 0.5 mM BSO

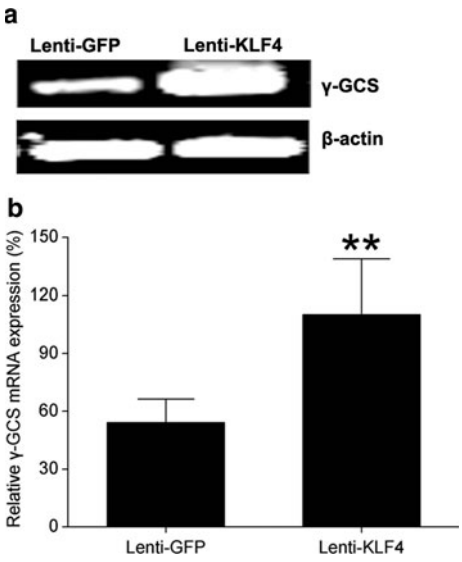

Fig. 5 Expression of γ-GCS was up-regulated by ectopic expression of KLF4 in HepG2 cells. **a** The γ-GCS mRNA expression was detected by RT-PCR. HepG2 cells were infected with pWPTS-KLF4 or pWPTS-GFP lentivirus for 72 h, and β-actin was regarded as an endogenous control. **b** Quantitative analysis. Bands were quantitated by densitometric analysis and normalized by β-actin. $^{**}P < 0.01$ for Lenti-KLF4 versus Lenti-GFP

enzyme controls a reaction that is typically rate-limiting for glutathione synthesis [20]. This suggested the possibility that glutathione synthesis was regulated by KLF4.

The effects of ectopic expression of KLF4 on cellular sensitivity to various chemotherapy drugs

Since GSH and its associated enzymes play a critical role in the cell susceptibility to the cytotoxic effect of chemotherapy drugs, the cellular sensitivity to chemotherapy drugs beside cisplatin was also detected in KLF4-ectopic expressed HepG2. The results showed that ectopic expression of KLF4 increased cellular resistance to fluorouracil (Fig. 6a) and cyclophosphamide (Fig. 6b), but no significant effects on cellular sensitivity to doxorubicin (Fig. 6c) and vincristine (Fig. 6d).

Discussion

In recent years, cancer stem cells have been isolated from human tumors or tumor endothelial cells [21]. Such cells have been found to be significantly more resistant to chemotherapeutic drugs than common tumor cells [11, 22]. In the present study, we explored the mechanisms of chemoresistance by comparing cancer stem cells and cancer cells and GSH levels, which are influenced by the regulation of KLF4.

Firstly, it was found that KLF4 expression is significantly higher in hepatocarcinoma stem-like cells T3A-A3 than in the hepatocarcinoma cell line HepG2. With exposure to cisplatin, T3A-A3 cancer stem-like cells exhibit more resistance to cisplatin than HepG2 cells do. These results suggest that high expression of KLF4 in hepatocarcinoma cells is a characteristic of a more cisplatin-resistant phenotype. KLF4 has been implicated in tumorigenesis and the regulation of stem cells. Wong et al. [23] reported a surprising link between KLF4 and the regulation of telomerase. This has provided important insights into the contribution of KLF4 to tumorigenesis and the regulation of stem cells. In addition, KLF4 protein levels are inversely correlated with the extent of genotoxic stress. This indicates that KLF4 might play a role in controlling the switch in the p53 response. In response to cytostatic repairable DNA damage, KLF4 is activated by p53 to promote the transactivation of proarrest p53 target gene p21 and causes cell cycle arrest [24]. The complicated functions of KLF4 in cell growth, proliferation, and survival indicate that it might be involved in drug resistance of cancer stem cells.

Under various DNA-damaging stimuli, profound changes of gene expression affect the cellular outcome of survival or nonlethal injury. To analyze the relationship of KLF4 with chemoresistance, we analyzed cisplatin sensitivity after KLF4 knockdown in T3A-A3 cells and the ectopic expression of KLF4 in HepG2 cells. T3A-A3 cells became sensitized to cisplatin after knockdown of KLF4, while HepG2 cells became resistant to cisplatin after ectopic expression of KLF4. These results suggest that KLF4 is negatively correlated with the sensitivity of cancer cells to cisplatin.

A growing body of data demonstrates that increased resistance to chemotherapy depends upon the cellular production of GSH. Increased detoxification of cisplatin by GSH-related reactions is an important biochemical mechanism of cisplatin resistance. It was reported that intracellular levels of GSH appear to be the rate-limiting parameter in the detoxification of cisplatin in human liver tumor cells [25]. Currently, three principal mechanisms have been proposed for the role of GSH in the regulation of cisplatin resistance in cancer chemotherapy [19]. The first mechanism involves the effects of GSH on ATP-binding cassette (ABC) transporter-mediated cisplatin transport. The second mechanism is the redox-regulating capacity of GSH in detoxifying cisplatin toxicity. The third mechanism includes the regulation of the intracellular copper pool, which affects the uptake of cisplatin. It was also reported that co-treatment with a GSH-depleting agent also potentiates apoptosis provoked by cisplatin [26]. CD44 is an adhesion molecule expressed in cancer stem-like cells. Ishimoto et al. [27] reported that human gastrointestinal cancer cells with high levels of CD44 expression have an

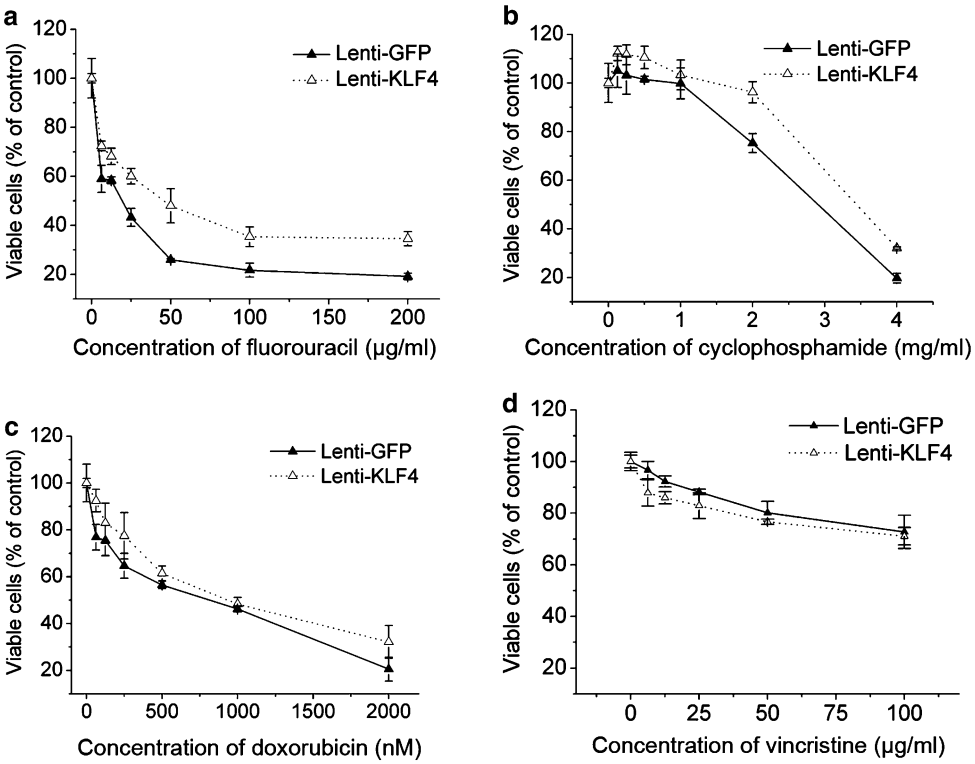

Fig. 6 Evaluation of cellular resistance to various chemotherapy drugs in HepG2 cells with ectopic expression of KLF4. Cell survival was evaluated by MTT assay in HepG2 cells after 72 h infected with pWPTS-KLF4 or pWPTS-GFP lentivirus by the MTT assay. **a** Fluorouracil. **b** Cyclophosphamide. **c** Doxorubicin. **d** Vincristine

enhanced capacity for GSH synthesis. Our present data show that the levels of GSH in cancer stem-like cells are higher than in HepG2 cells. In addition, GSH levels increase when KLF4 is ectopically expressed in HepG2 cells. Pre-incubation of HepG2 cells with ectopically expressed KLF4 with the GSH-depleting agent BSO regenerates sensitivity to cisplatin. However, BSO did not affect the expression of KLF4. These findings suggest that KLF4-mediated cisplatin resistance is associated with up-regulated levels of GSH.

The control of GSH levels and the redox state involves a complex interplay between biosynthesis, utilization, degradation, oxidation/reduction, and transport [28]. All of these factors must be considered in order to evaluate the significance of GSH as a signaling component in cisplatin resistance. Several important factors control the rate of de novo GSH synthesis. One factor is the activity of γ-GCS, an enzyme catalyzing the first and rate-limiting reaction in de novo GSH synthesis. In this respect, it is interesting that the mRNA expression of γ-GCS was found to be up-regulated in HepG2 cells by ectopic expressing KLF4. However, further studies are still warranted to assess the capacity of KLF4 for altering GSH synthesis and degradation as a mechanism of chemoresistance.

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Conflict of interest None.

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