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ORIGINAL ARTICLE

Nrf2 knockdown by shRNA inhibits tumor growth and increases efficacy of chemotherapy in cervical cancer

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Abstract
Purpose NF-E2-related factor 2 (Nrf2) is a key transcription regulator for cellular response to oxidative stress in normal cells. In cancer cells, development of chemoresistance is associated with the constitutive activation of the Nrf2-mediated antioxidant defense system. Here, we investigated the role of Nrf2 in terms of cervical cancer cell proliferation and drug resistance.
Method To investigate whether cancer cells activate the Nrf2 system, we examined 40 surgical cervical cancer samples and 12 normal control tissues. Plasmids containing Nrf2-small hairpin RNA (shRNA) or non-targeting vector-control shRNA were transfected into CaSki cells. Using Western blots and RT-PCR assays, the expression levels of Nrf2 mediated-target genes were measured in CaSki cells stably expressing Nrf2-shRNA. To evaluate how the Nrf2 knockdown affected susceptibility to chemotherapeutic drugs, MTT and flow cytometry assays were done in vitro and confirmed by a mouse xenograft model in vivo.
Results The Nrf2-dependent defensive system was likely fully activated in cervical tumor tissues. Genetic knockdown of endogenous Nrf2 caused a global decrease in expression of Nrf2-regulated genes. This decrease in expression levels enhanced chemotherapeutic drug-induced apoptotic death in CaSki cells with a reduced cellular glutathione level. Additionally, the combination of cisplatin treatment and Nrf2 knockdown significantly suppressed tumor growth in vivo.
Conclusion Our findings provide evidence that the inhibition of Nrf2 activity by shRNA might be a promising therapeutic strategy to enhance the efficacy of anticancer drugs and thus can be applied further during the course of chemotherapy in the treatment of cervical cancer.

Keywords Cervical cancer · Chemotherapy · Susceptibility · Tumor growth · Nrf2 shRNA

Introduction

Globally, cervical cancer is the third most common malignancy among women, accounting for an estimated 529,800 total new cases and 275,100 total cancer deaths in 2008 [1]. Despite considerable improvements achieved through systemic therapy, the prognosis of cervical cancer patients with recurrent or metastatic disease remains unfavorable. The majority of cervix carcinomas are resistant to anticancer drugs and are generally non-responsive to initial chemotherapy [2]. Therefore, the control of such chemoresistance is required to develop effective treatments for cervical cancer.

Normally, nuclear factor erythroid-2-related factor 2 (Nrf2), a member of a cap "n" collar basic leucine-zipper transcription factor, originally regulates a transcriptional program involved in a variety of cellular defenses resistance to oxidative and electrophilic insults [3]. Under basal conditions, Kelch-like ECH-associated protein 1 (Keap1) negatively regulates Nrf2 activity by ubiquitin-mediated proteasomal degradation [4]. On exposure to oxidative stress, Nrf2 forms a heterodimer with one of the small Maf family proteins and translocates into the nucleus to induce

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constitutive expression of downstream antioxidant response proteins such as heme oxygenase (HO-1) [5] and phase II detoxifying enzymes especially for NAD(P)H:quinone oxidoreductase 1 (NQO1) [6–8]. Recent studies have shown that upregulation of HO-1 and NQO1 synergistically confer tumor cell survival and apoptosis inhibition by modulating cellular glutathione levels [9–11]. NQO1 acts to detoxify cancer drugs, whereas HO-1 contributes to cytoprotection by attenuating drug-induced oxidative stress and apoptosis [7, 12]. These findings suggest that constitutive activation of Nrf2 may facilitate cancer cell proliferation and prolong survival in oxidizing conditions that result from exposure to exogenous anticancer drugs. Thus, Nrf2 activation is likely linked to multidrug resistance.

A growing body of evidence shows that constitutive activation of Nrf2 may contribute to a malignant phenotype [13, 14]. Aberrant expression of Nrf2-induced self-defense genes has also been observed in specific tumors [13, 15–17]. Human-derived tumor cell lines were susceptible to Nrf2-mediated cytoprotection in vivo and in vitro [18]. The upregulation of the antioxidant defense system may increase cell proliferation by increasing the chemoresistance in cancer cells or by rapid removing anticancer agents from the cells and thereby enhancing cell tolerance to drug-induced apoptosis [16, 19, 20].

In the present study, we investigate how inhibiting Nrf2 affects cervical cancer cell proliferation and drug resistance. Stable inhibition in CaSki cells with an Nrf2-shRNA delivery system dramatically decreased the expression of Nrf2-regulated antioxidant defense genes, increased cell sensitivity to chemotherapeutic drugs, and ultimately suppressed tumor growth in a mouse xenograft model. These results suggest that shRNA-inhibition of Nrf2 activity might be a promising therapeutic strategy to increase the efficacy of anticancer drugs.

Materials and methods

Patient population

The study was reviewed and approved by the local ethics committee, and informed consent was obtained from all recruited participants. Cervical squamous carcinoma patients ($n = 40$) with a mean age of 43 (26–58) were randomly selected from the Department of Obstetrics and Gynecology, Xijing Hospital, Fourth Military Medical University, Xi'an, China. In addition, normal cervical tissues ($n = 12$) from patients with an average age of 37 (33–42) were collected as the control. All cases were clearly classified by experienced pathologists based on histology or cytology according to the criteria established by FIGO (International Federation of Gynecology and Oncology). None of the patients received radiotherapy or chemotherapy before recruitment. Patients with cervical cancer were divided into three groups as follows: 13 cases were of stage I, 18 of stage II, 9 of stage III–IV. Tumor tissue samples were removed and snap-frozen in liquid nitrogen for RNA and protein extraction.

Cell culture and reagents

Human cervical carcinoma CaSki cells were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 (FBS; HyClone Laboratories, Logan, UT) supplemented with 15% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA) at 37°C in a humidified incubator containing 5% CO_2. Primary antibodies against NQO1, HO-1, histone H3, and GAPDH were purchased from Cell Signaling Technology (Danvers, MA, USA). The Nrf2 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All chemicals including cisplatin, paclitaxel, doxorubicin and 5-fluorouracil were purchased from Sigma–Aldrich (Saint Louis, MO, USA). All transfections were carried out using Lipofectamine 2000 (Invitrogen), unless otherwise noted.

Generation of a cervical cancer cell line stably expressing Nrf2-shRNA

Sixth passage CaSki cells were seeded in 6-well plates (2×10^5 cells per well) and allowed to adhere for 24 h before transfection. Using Lipofectamine 2000 (Invitrogen), cells were transfected with plasmids containing shRNA directed against human Nrf2 (Nrf2-shRNA) (Santa Cruz Biotechnology), non-targeting vector-control shRNA (NC-shRNA) (Santa Cruz Biotechnology). In addition, copGFP control plasmid (Santa Cruz Biotechnology) was used to monitor and optimize transfection efficiency. At 48 h post-infection, puromycin (6.5 g/ml) was added to the culture medium for selection and further characterization. After 2 weeks, stable cell lines with reduced Nrf2 expression had been generated. Transfection efficiency of CaSki cells expressing Nrf2-shRNA was assessed by flow cytometry (Becton–Dickinson, Franklin Lakes, NJ, USA).

Preparation and quantification of protein sample

Snap-frozen tissue samples were homogenized in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol (v/v), 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1 mM dithiothreitol (DTT), and 100 mM PMSF protease inhibitor (Sigma–Aldrich)). After centrifugation of the homogenate at 13,000*g* for 1 min at 4°C, the supernatant containing the cytosolic extract was collected and stored at −80°C until use. Nuclear proteins were then extracted from the pellet.

Briefly, the pellet was resuspended in extraction buffer (2 M sucrose, 1 M HEPES, 2 M $MgCl_2$, 2 M KCl, 30% glycerol, 0.5 M EDTA, 1 M DTT, protease inhibitor cocktail, and 10% NP-40). After centrifugation at 12,000*g* for 15 min at 4°C, the supernatant containing the nuclear protein extract was collected and stored at −80°C for Western blotting.

Western blots of Nrf2, NQO1, and HO-1

Nuclear and cytosolic lysate from human cervical tissues and whole cell extracts (15–30 μg each) were subjected to 12% SDS–PAGE, subsequently blotted onto a PVDF membrane (Bio-Rad, Hercules, CA). The membrane was incubated with antibodies against Nrf2, NQO1, and HO-1, according to the manufacturer's instructions. For the cytosolic fraction, GAPDH was the loading control. For the nuclear fraction, β-actin and histone H3 were used as loading controls. Protein concentration was quantified using a Quant-iT protein assay kit (Invitrogen). Immunoreactive protein bands were quantified by densitometry using Quantity One (BioRad).

Total RNA extraction and real-time quantitative reverse transcription-PCR

Total RNA from tumor cells was extracted using the Trizol reagent (Invitrogen) and reverse transcribed for PCR using the QuantiTect SYBR-Green PCR Mix (Qiagen) according to the manufacturer's instructions. Target cDNA was amplified with the following cycling conditions: 50°C for 30 min, followed by an initial activation at 95°C for 15 min, and ending with 45 cycles of 94°C for 15 s, 55°C for 30 s, 72°C for 30 s. Primers used for quantitative PCR are as follows: NQO1: 5′-GGT TTG AGC GAG TGT TCA TAG G-3′(sense), 5′-GCA GAG AGT ACA TGG AGC CAC-3′(antisense); HO-1: 5′-CAG TGC CAC CAA GTT CAA GC-3′(sense), 5′-GTT GAG CAG GAA CGC AGT CTT-3′(antisense); GAPDH: 5′-ATG GGG AAG GTG AAG GTC G-3′(sense), 5′-GGG GTC ATT GAT GGC AAC AA-3′(antisense). The relative number of transcripts for each target gene was calculated using the $\Delta\Delta C_T$ method and normalized against GAPDH.

Cytotoxicity analysis

CaSki cells were seeded into a 96-well plate (3.0×10^4 cells/well) and transfected with NC-shRNA or Nrf2-shRNA as described previously [17]. After 24-h incubation, the cells were exposed to cisplatin, paclitaxel, doxorubicin, and 5-fluorouracil at various concentrations. To examine the cells' relative sensitivity to chemotherapeutic drugs, cell viability was measured after 48 h using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. Absorbance was measured at 560 nm with a microplate reader (Bio Tec Instruments Inc). All experiments were performed in triplicate.

Flow cytometry

The extent of apoptosis in CaSki cells induced by cisplatin, paclitaxel, doxorubicin, and 5-fluorouracil was assayed by annexin V/PI staining as described previously [21]. Following 48 h of drug exposure, an aliquot of cells were stained with annexin V-FITC and PI using an Apoptosis Detection Kit (Sigma–Aldrich) according to the manufacturer's instructions. Cells were counted with a FACSCalibur flow cytometer (Becton–Dickinson).

Total GSH content

After 24 h of cell growth, CaSki cells (3.0×10^6 cells/well) stably transfected with Nrf2-shRNA were incubated with cisplatin, paclitaxel, doxorubicin, or 5-fluorouracil for 48 h. A non-specific vehicle-shRNA was used as control. The intracellular glutathione concentration (GSH) was measured using a CHEMICON Glutathione Detection kit (Millipore). Fluorescence was measured at 380 nm using a microplate reader. All experiments were conducted in triplicate.

Tumor xenografts study

CaSki cells (1.0×10^6 cells/mL) were injected subcutaneously into the flank of 6-week-old female BALB/c nude mice (Orient Bio Inc), and tumors (50–100 mm^3) developed before treatment. Mice were randomly assigned into six groups (cisplatin + Nrf2 shRNA, cisplatin + vector, cisplatin + PBS, saline + Nrf2 shRNA, saline + vector, saline + PBS) of eight individuals. Intratumoral administration 10 μg/kg/d of body weight of Nrf2-shRNA, NC-shRNA, or PBS followed intraperitoneal injections of cisplatin (5 mg/kg of body weight) for three of the six groups. The other three groups were treated with saline as controls. Either shRNA, cisplatin, saline, or PBS was injected every 3 days for 28 days. Animals were monitored for signs of tumor growth, and any tumors were measured with Vernier calipers. Tumor volumes were calculated according to the formula: [length (mm) × width (mm) × width (mm) × 0.52] [7, 22]. Upon termination, tumors were harvested, and tumor proteins were analyzed by Western blot. All animal experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC).

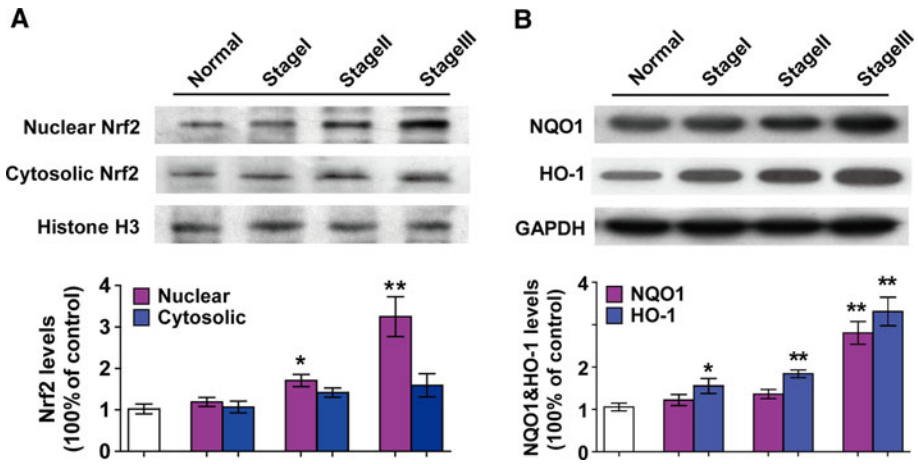

Fig. 1 Different expression of Nrf2-dependent target genes in normal and cervical cancer tissues. Protein expressions of **a** Nrf2 in the nucleus and **b** NQO1 and HO-1 in the cytosol were significantly up-regulated in cancerous tissues compared to normal cervical tissues. Cervical cancer tissues were separated into Stage I, Stage II, and Stage III. Nrf2 levels in stage II and III cervical cancer tissues were higher than those from stage I tissues. No significant difference in cytosolic protein expression of Nrf2 were observed between the tissues from different stages. Relative protein concentrations were determined by Western blot. Histone H3 and GAPDH were the protein loading controls. Columns, mean; *error bars*, standard deviation; $*P < 0.05$; $**P < 0.01$ versus normal cervical tissues alone

Statistical analysis

All values are expressed as mean ± Standard Error. Statistical significance was analyzed by Student's paired *t* test or one-way ANOVA followed by the Student–Newman–Keuls comparison method computed with SigmaStat (SPSS, Chicago, IL, USA). *P* values less than 0.05 were considered to be significant.

Results

Nrf2 concentration among the human cervical cancer tissues

To investigate the adaptive activation of the antioxidant defense system in cancer cells, we measured the endogenous expression levels of Nrf2 and its downstream targets including NQO1 and HO-1 in normal and cancerous (stages I, II, and III) cervical tissues. There were markedly elevated Nrf2 nuclear protein levels in cancerous tissues compared to normal cervical tissues (Fig. 1a). Similarly, Nrf2 levels in stage II and III cervical cancer tissues were higher than those from stage I tissues (Fig. 1a), suggesting a negative association Nrf2 expression and survival. Although the levels of the Nrf2-regulated antioxidant genes NQO1 and HO-1 were up-regulated in the cytosol of late-stage cervical cancer tissue (Fig. 1b), no significant difference in cytosolic protein expression of Nrf2 was observed between the tissues from different stages (Fig. 1a). These findings suggest that the Nrf2-dependent defensive system is fully activated in human cervical tumor tissues to acquire growth advantage.

Generation of cervical cancer cells that stably express Nrf2shRNA

Increasingly, evidence has shown that the development of chemoresistance is closely associated with the constitutive up-regulation of Nrf2-mediated antioxidant response element-driven genes. Along these lines, inhibiting Nrf2 function may increase sensitivity of CaSki cells to chemotherapeutic drugs. To evaluate this relationship, Nrf2-shRNA was stably transfected into CaSki cells. After puromycin selection, the transfection efficiency was calculated as 81.4% by flow cytometry (Fig. 2). According to real-time quantitative PCR and immunoblotting, both Nrf2 mRNA and protein levels in CaSki cells decreased significantly after the transfection of Nrf2-shRNA (Fig. 3). The relative mRNA of Nrf2 concentration in cells transfected with Nrf2-shRNA was 0.32 ± 0.03-fold lower than that in the NC-shRNA control ($P < 0.01$). The expression of Nrf2 was not different between control cells and untransfected cancer cells. These results reveal that the Nrf2-shRNA used in the present study successfully knocks down Nrf2 in CaSki cells.

Decreasing Nrf2 expression in CaSki cells causes decreased in expression of self-defense genes

To investigate whether decreased levels of Nrf2 also downregulated the expression levels of genes in the Nrf2-dependent pathway, we measured the expression of Nrf2 downstream target genes. Western blots showed that protein expression levels for NQO1 and HO-1 decreased significantly in cells expressing Nrf2-shRNA (Fig. 3A). Transcript levels of NQO1

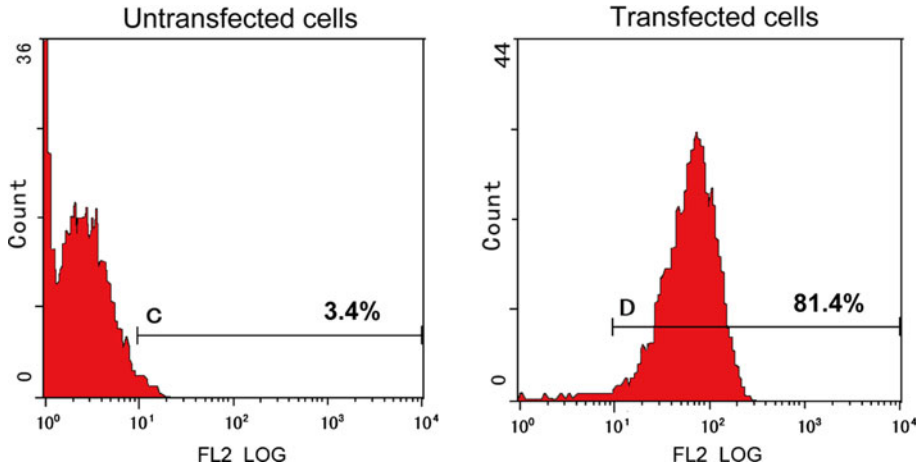

Fig. 2 CaSki cells were transfected with plasmids containing Nrf2-shRNA. After selection with puromycin, the transfection efficiency in the stably expressing cells was 81.4% (*left*) compared to 3.4% in untransfected cells (*right*) by flow cytometry. The fluorescence signal intensity was measured at 488 nm by FL2 fluorescence channel

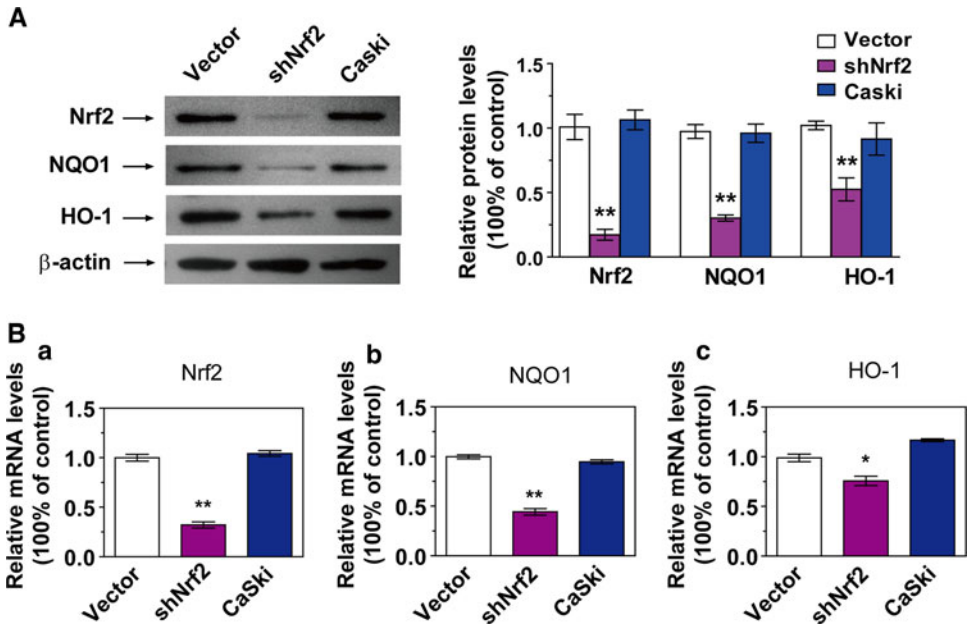

Fig. 3 Inhibition of Nrf2 expression by Nrf2-shRNA causes down-regulation expression of Nrf2-mediated self-defense genes. **A** Immunoblot of whole protein extracts from stable cells expressing Nrf2-shRNA (*middle*), control cells transfected with NC-shRNA (*left*), or untransfected CaSki cells (*right*). **B** mRNA levels of *a* Nrf2, *b* NQO1, and *c* HO-1 quantified using real-time RT-PCR. *β*-actin and GAPDH were internal control, respectively. Columns, mean; *error bars*, standard deviation; $*P < 0.05$, $**P < 0.01$ versus NC-shRNA-treated control cells

and HO-1 also declined in cells expressing Nrf2-shRNA but were not significantly different between control cells expressing NC-shRNA and untransfected cells (Fig. 3B, b–c).

Knockdown of endogenous Nrf2 sensitizes cervical cancer cells to chemotherapeutic drugs

To determine how Nrf2 knockdown affects chemosensitivity, we exposed cells stably expressing Nrf2-shRNA to cisplatin. The Nrf2 knockdown increased the cytotoxicity of cisplatin, resulting in increased cell death in a dose-dependent manner compared to control cells expressing NC-shRNA (Fig. 4a). The IC_{50} of cisplatin was significantly lower in cells stably expressing Nrf2-shRNA (10.62 ± 1.06 μg/mL) compared to vehicle-treated control cells (33.15 ± 3.22 μg/mL; Table 1). Additionally, we investigated whether there was an increased sensitivity to paclitaxel, doxorubicin, and 5-fluorouracil. For all three drugs, cell viability was reduced in cells expressing Nrf2-shRNA compared to the vector-control cells (Fig. 4b–d). CaSki cells expressing Nrf2-shRNA were most susceptible to paclitaxel. After 48 h of drug exposure, the IC_{50} of paclitaxel was 3.04 ± 0.66 (μg/ml) with a dramatic inhibition rate of 71.93% (Table 1). The inhibition rate of 5-FU was relatively slighter (56.70%). Sensitivity to the anticancer agents was similar between the control cells transfected with NC-shRNA and untransfected CaSki cells. These results suggest that Nrf2 activity is negatively associated with susceptibility to chemotherapeutic agents in CaSki cells.

Nrf2 knockdown increased drug-induced apoptosis and decreased GSH in CaSki cells

Previous results suggested that a decrease in Nrf2 expression might be directly related to an increased susceptibility to chemotherapeutic drugs. Using flow cytometry analysis to investigate anticancer drug-induced apoptosis, we

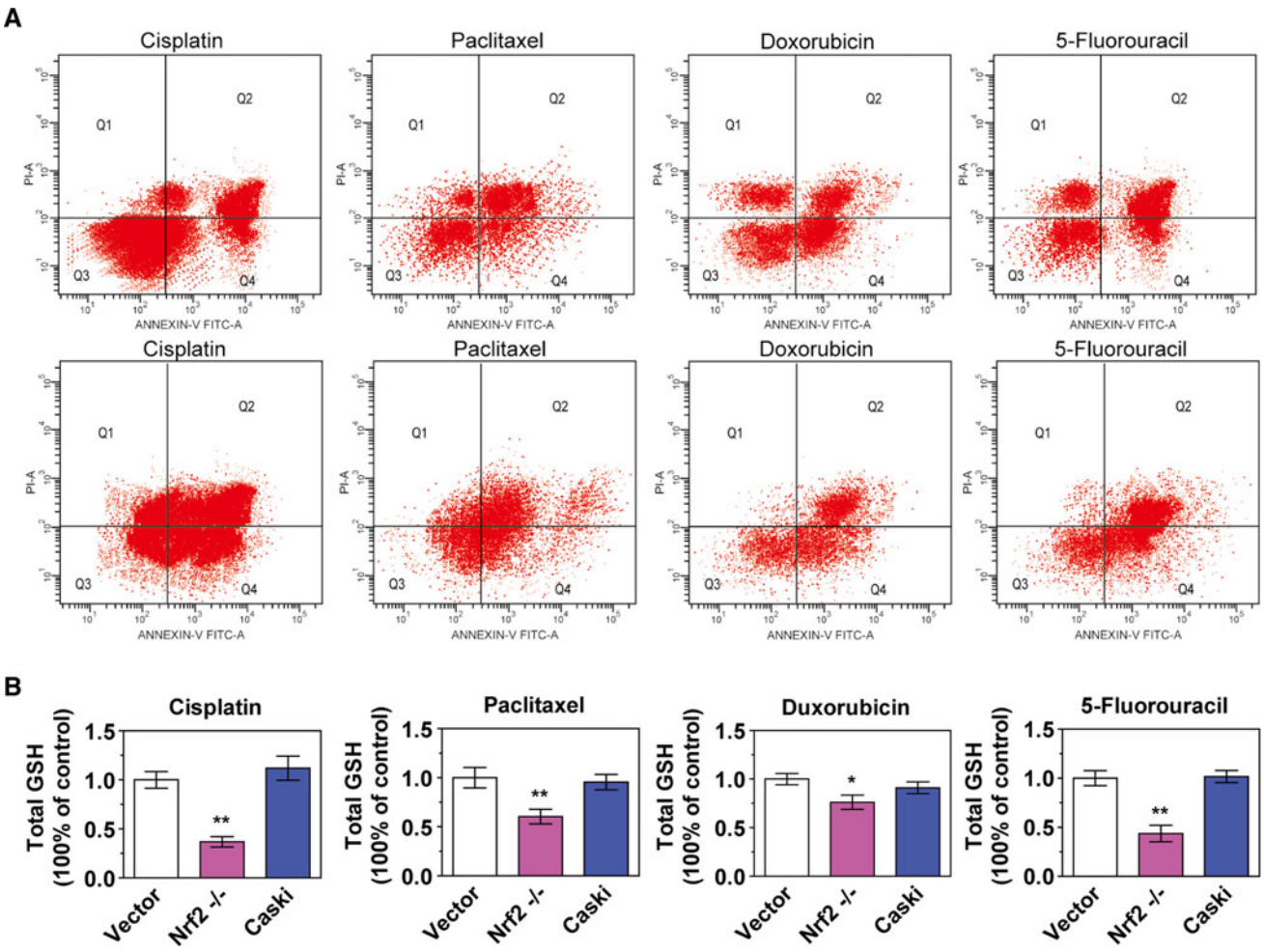

Fig. 4 Genetic knockdown of Nrf2 results in increased sensitivity to cytotoxicity induced by chemotherapeutic drugs. CaSki cells transfected with Nrf2 shRNA (*open square*) or NC shRNA (*open triangle*) were exposed to **a** cisplatin, **b** paclitaxel, **c** doxorubicin, and **d** 5-fluorouracil for 48 h, respectively. Cell viability was monitored by MTT assay. Cancer cells expressing NC-shRNA were used as the control. Mean cytotoxicity from three independent experiments. Representative experiments are shown

Table 1 Relative sensitivity of CaSki cells to chemotherapeutic drugs

Drugs	IC_{50} value (μg/ml)		
	Vector	shNrf2	CaSki
Cisplatin	33.15 ± 3.22	10.62 ± 1.06*	28.70 ± 0.51
Paclitaxel	10.83 ± 0.94	3.04 ± 0.66*	11.66 ± 0.33
Duxorubicin	1.43 ± 0.62	0.44 ± 0.19*	1.68 ± 0.60
5-Fluorouracil	157.90 ± 8.37	68.37 ± 5.93*	186.71 ± 12.40

Relative sensitivity of CaSki cells to chemotherapeutic drugs. Data are represented as percentage of viable cells relative to the vehicle-treated control. There was no significant difference in cellular sensitivity to all anticancer agents between the control cells transfected with NC-shRNA and untransfected CaSki cells

* $P < 0.05$ versus control cells transfected with NC-shRNA

observed that compared to NC-shRNA transfected cells, both early and late apoptotic cells were significantly more common in cells transfected with Nrf2-shRNA (Fig. 5A).

An increase in intracellular GSH has been correlated with a reduced sensitivity to chemotherapeutic drugs. Intracellular GSH concentration was significantly decreased in cells transfected with Nrf2-shRNA and treated with cisplatin, paclitaxel, doxorubicin, or 5-fluorouracil (Fig. 5B), confirming an increase in apoptosis in these cells. There was no significant difference in GSH concentration between cells transfected with NC-shRNA and untransfected CaSki cells. Collectively, these data provide support to the hypothesis that the inhibition of Nrf2 increases drug-induced apoptosis in cancer cells, presumably through a decrease in GSH concentration.

Therapeutic efficacy of the combination of cisplatin and Nrf2 inhibition on tumor growth in vivo

To elucidate whether Nrf2-shRNA and cisplatin can act synergistically in vivo, we transplanted CaSki cells into mice bearing subcutaneous tumors. In conjunction,

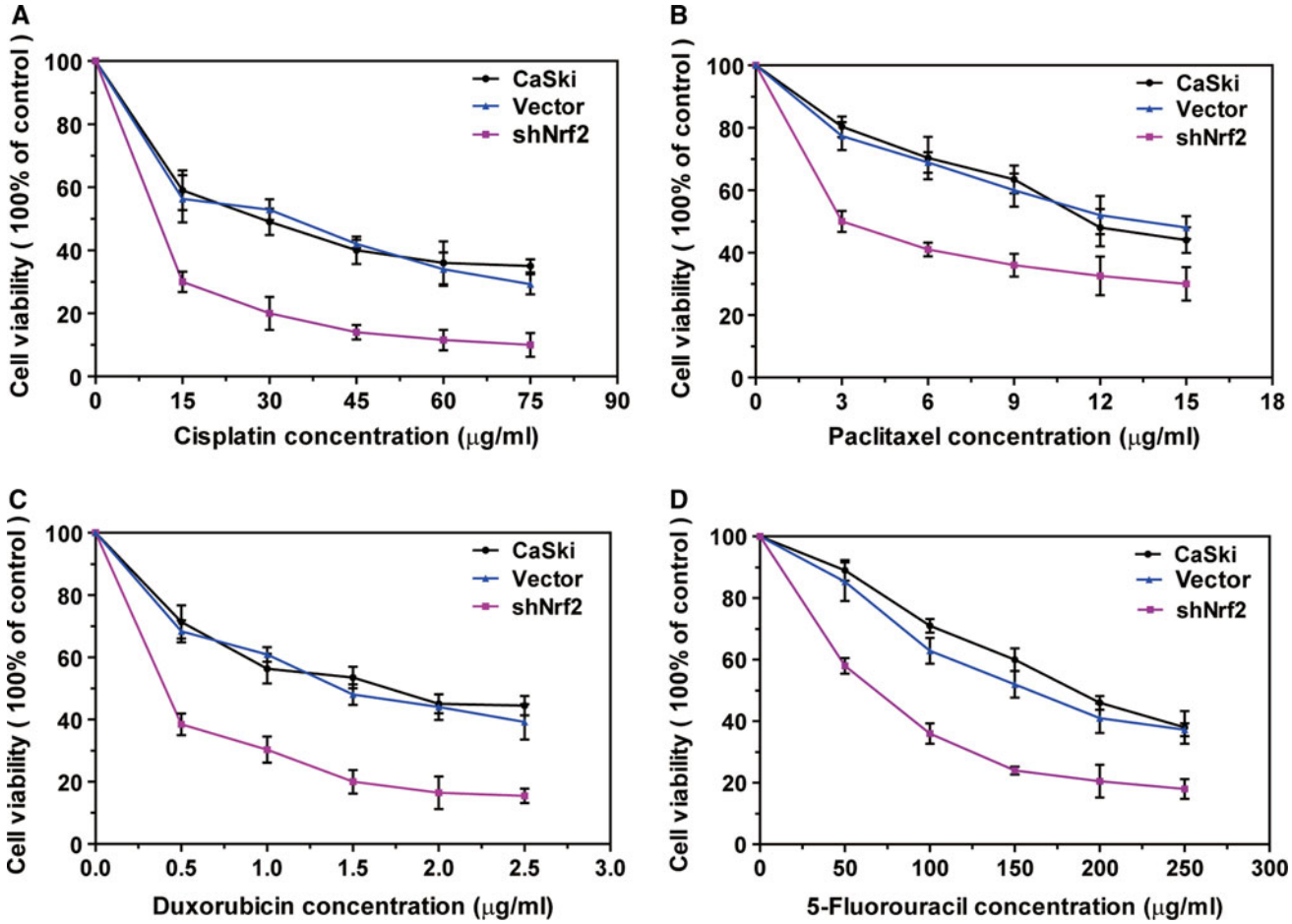

Fig. 5 Nrf2 knockdown increased drug-induced apoptosis and reduced cellular GSH content in CaSki cells. The proportion of cisplatin-, paclitaxel-, doxorubicin-, and 5-fluorouracil-induced apoptosis in CaSki cells (*upper* of **a**) and CaSki cells stably expressing Nrf2-shRNA (*lower* of **a**) using Annexin V and propidium iodide staining. **b** Total GSH levels in cells stably transfected with Nrf2-shRNA and control cells transfected with NC-shRNA or untransfected CaSki cells. Columns, mean; *error bars*, standard deviation; $^{*}P < 0.05$, $^{**}P < 0.01$ versus NC-shRNA-treated control cells

some mice were treated with cisplatin. Tumor growth was significantly inhibited in mice injected with cisplatin (Fig. 6a). When cisplatin treatment was combined with Nrf2-shRNA expression, tumor growth was inhibited significantly more compared to treatments with cisplatin treatment alone (71.61% vs. 49.64%, $P < 0.05$) (Table 2). Interestingly, injections of the Nrf2-shRNA plasmid alone also suppressed tumor growth (27.36%) (Table 2) without affecting body weight. Tumor weight did not differ significantly between controls treated with NC-shRNA and control mice injected with PBS. Gene expression analysis of tumors showed remarkable decrease in Nrf2 (Fig. 6b). Our findings support the hypothesis that the combination of cisplatin and Nrf2-shRNA can significantly suppress cervical cancer tumor growth in vivo. The observed synergistic effects were achieved through the knockdown Nrf2 with a shRNA delivery system.

Discussion

Recent studies have showed that therapeutic resistance in cancer cells occurred as a result of genetic mutations that conferred tumorigenic potential and survival advantage against chemotherapy [23–26]. Here, we found that human cervical cancer cells might activate the antioxidant defense system. The Nrf2 protein levels were markedly elevated in cervical cancer cells. The Nrf2 mediated-defense system closely correlated with advanced staging, suggesting that an increase nuclear Nrf2 concentration might result in poor prognosis of cervical cancer. Using RNAi to suppress Nrf2 expression, we found that the suppression of Nrf2 and its downstream genes inhibited tumor growth and enhanced cytotoxic effect of the anticancer drugs in vitro. Therefore, the shRNA-mediated knockdown of Nrf2 during chemotherapy is a promising approach for the treatment of cervical cancer.

Table 2 The weight and inhibition rate of tumor 28 days after CaSki cells implantation

Group (n = 6–8)	Tumor weight (g)	Inhibition rate
Saline		
PBS	4.13 ± 0.38	–
Vector	3.96 ± 0.36	4.12%
shNrf2	3.00 ± 0.11[a]	27.36%
Cisplatin		
PBS	2.08 ± 0.24[a]	49.64%
Vector	1.92 ± 0.15[a]	53.74%
shNrf2	1.17 ± 0.23[a,b]	71.61%

The weight and inhibition rate of tumor 28 days after CaSki cells implantation

* $P < 0.05$

[a] Compared with mice administrated with saline + PBS, [b] relative to mice treated with cisplatin + PBS

Nrf2, a basic redox-sensitive bZIP transcription factor, activates cytoprotective pathways against oxidative injury, inflammation, and apoptosis through transcriptional induction of a large number of self-defense genes involved in phase II detoxification enzymes and antioxidant stress enzymes [27]. Keap1 negatively regulates Nrf2 activity through ubiquitin-mediated proteasomal degradation, indicating that complete loss of Keap1 activity leads to constitutive activation of Nrf2 [20, 28, 29]. High levels of Nrf2 determined chemoresistance in type II endometrial cancer [24]. In contrast, downregulation of the Nrf2-dependent antioxidant response by overexpressing Keap1 or by transient-transfection of Nrf2-siRNA in lung carcinoma, breast adenocarcinoma, and neuroblastoma resulted in greater susceptibility to cisplatin, etoposide, and doxorubicin [28, 30–32]. These observations support the idea that a decrease in the Nrf2-dependent protective response may increase efficacy of chemotherapeutic agents.

In the present study, the expression of Nrf2/ARE-dependent detoxification enzymes and glutathione-related enzymes like HO-1 and NQO1 was remarkably decreased in CaSki cells transfected with Nrf2-shRNA. HO-1 plays a critical role in adaptive and innate defense responses to oxidative stress in normal cells [11, 33]. Increased expression and activity of HO-1 have been found in tumors, such as renal cell carcinoma, prostate tumors, and hepatoma, which confer tumor cell growth and proliferation through its antiapoptotic effects [34–36]. The suppression of Nrf2-driven HO-1 increased the chemosensitivity of lung cancer A549 cells to cisplatin [37]. Similar to this study, changing the endogenous NQO1 levels revealed key regulatory mechanisms of β-lapachone for pancreatic cancer therapy [38]. The development of indolequinone mechanism-based inhibitors of NQO1 decreased human pancreatic MIA PaCa-2 cancer cells growth [39]. 5-Methoxy-1,2-dimethyl-3-[(4-nitrophenoxy) methyl]indole-4,7-dione (quinone oxidoreductase 1) inhibited the activity of human pancreatic cancer cells both in vitro and in vivo [40]. While a wide variety of detoxifying and antioxidant genes are involved in tumor development and progression, downregulation of cytoprotective genes may enhance antitumorigenic effects of cisplatin-based chemotherapy in patients with cervical cancer, presumably by inhibiting the transcriptional activation of Nrf2.

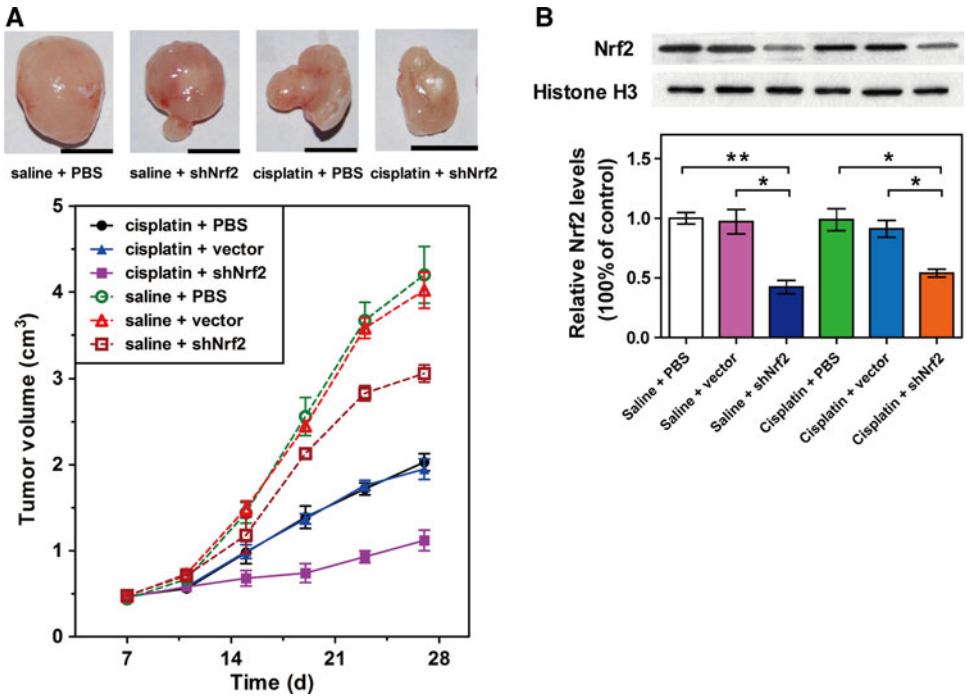

Fig. 6 Therapeutic efficacy of cisplatin in combination with Nrf2-shRNA on tumor growth in vivo. **A**-*a*, Four representative subcutaneous tumors from different tumor-cell injection therapies. Each group contained 8 animals. **A**-*b*, Inhibited tumor volume in each treatment. **B** Mean expression levels from randomly selected tumors from each treatment group showed a remarkable decrease in Nrf2 in the nucleus in both cisplatin + Nrf2-shRNA and saline + Nrf2-shRNA. *$P < 0.05$ compared with vehicle control. **$P < 0.01$ relative to control treated with PBS

Cisplatin is one of the most potent anticancer agents widely used for the treatment of metastatic cervical cancer [41]. Cisplatin kills cancer cells by generation of reactive oxygen species (ROS) that may exhaust the antioxidant capacity of cancer cells and thereby causing drug-induced apoptosis [42–44]. However, development of resistance to cisplatin limits its therapeutic use for treatment of cervical cancer. The transition of chemotherapy-responsive cancer cells to chemotherapy-resistant cancer cells may be involved in several different mechanisms, such as increased cellular detoxification and antioxidant systems [16, 24, 45].

GSH is a major endogenous antioxidant that contributes to rescuing cells from apoptosis. Previous studies have suggested that an elevated cellular GSH level caused decreased responses to chemotherapeutic agents [25, 46]. Bracht et al. observed a significant inverse correlation between intracellular GSH concentrations and sensitivity to doxorubicin in a panel of 14 human cancer cell lines [18]. Inhibition of Nrf2 by siRNA sensitized Nrf2 in over-expressing SKOV3 cells to cisplatin treatment by depleting total cellular GSH pool [23]. In human lung cancer, the siRNA-mediated repression of self-defense genes and of cellular GSH pool inhibited cell growth and sensitized to anticancer agents [47]. When the cells were treated singly, tumor growth decreased but not to the extent observed in the combination treatment. Changes in growth and drug sensitivity might be the result of the decreased GSH levels through inactivation of Nrf2 using the shRNA approach.

In summary, our findings fully support the hypothesis that inhibited tumor growth and increased efficacy of chemotherapy in cervical cancer depend on the specific downregulation of Nrf2 activity. Upregulation of Nrf2 in cancer cells may increase cellular antioxidant and detoxification abilities by inducing a wide variety of self-defense genes. Therefore, inhibition of Nrf2 activity, in combination with antineoplastic agents, might be a promising therapeutic strategy to control chemoresistance and thus augment apoptosis death in cervical cancer.

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Conflict of interest None.

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