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Effects of Targeting NRF2 on Inhibiting Gastric Cancer Growth and Tumor Development in Vitro and in Vivo

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Keywords:

NRF2; Gastric Cancer; ROS; GADD45α

Abbreviations:

NRF2: Nuclear factor related-E2 factor; ROS: Reactive Oxygen Species; ShRNA: Short hairpin RNA; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

1. Abstract

1.1. Background: Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2) is a transcription factor that regulates the expression of the cellular antioxidant response. Traditionally, Nrf2 is regarded as a tumor suppressor owing to its characteristic which prevents oxidative or electrophilic insult. However, there is increasing evidence that Nrf2 is activated in malignant cells. At present, little is known about the effect of targeting Nrf2 on gastric cancer.

1.2. Methods: Western blot analysis and qRT-PCR were utilized to determine the expression of Nrf2 in the gastric cell line. The effects of knockdown NRF2 were analyzed on cell proliferation, cell cycle, and ROS production in vitro. Mouse xenograft models were used to research the role of Nrf2 in vivo. More importantly, the expression level of Nrf2 was detected by immunohistochemistry in gastric cancer tissues and adjacent normal gastric tissues. Kaplan-Meier curve was employed to evaluate the correlation between the expression of Nrf2 and the clinical prognosis.

1.3. Results: Nrf2 expression was increased in GC lines, especially in AGS and HGC27 cells lines. Down-regulation of NRF2 is related to the inhibition of proliferation, resulting in overproduction of ROS in gastric cancer cells. Importantly, high expression of Nrf2 was detrimental to the clinical outcome. Furthermore, suppression of NRF2 could inhibit gastric tumor growth in xenograft tumor model.

1.4. Conclusion: Our findings demonstrate that Nrf2 could be a good biomarker for the prognosis as well as a novel therapeutic target of great potential in gastric cancer.

2. Introduction

Gastric cancer is a common lethal malignancy and causes great cancer-related mortality in the global. Even though uniform declines in incidence have been observed in many parts of the world for decades, gastric cancer remains the second leading cause of cancer-related mortality worldwide and the most prevalent cancer in Eastern Asia [1, 2]. These symptoms about gastric cancer are vague, non-specific, as a result, the disease is often advanced and incurable [3]. Complete surgical resection remains the only opportunity for cure in gastric cancer, otherwise, gastric cancers are routinely diagnosed in relatively advanced stage. Gastric cancer represents a significant burden on society, and improvements in the treatment of this disease are in need. Intratumoural, intrapatient, and interpatient heterogeneity in gastric cancer remains a crucial barrier to development new targeted therapies and therefore more research is needed to understand how these challenges can be overcome [4]. The prognosis of gastric cancer is

positively related to patient biological factors and clinical pathological conditions, necessitating the identification of novel bio-markers for diagnosis and treatment [5]. Therefore, it is necessary to find a reliable prognostic biomarker to help clinicians predict the characteristic of the malignancy and decrease the rate of unfavorable outcomes in a high -risk population.

Overproduction of Reactive Oxygen Species (ROS) in tumors has been shown to induce a variety of biological effects, including inhibitory cell proliferation, DNA damage and genetic instability, adaptability, cell damage and cell death, autophagy and resistance [6-9]. Nuclear factor E2-related factor 2(Nrf2) is a key transcription that controls the expression of a pool of antioxidant and cytoprotective genes regulating cellular response to oxidative and electrophilic stress [10]. Due to its cytoprotective function, Nrf2 has been traditionally studied in the field of chemoprevention and be considered a tumor suppressor, however, in the past years, there are much evidence that supports the idea that Nrf2 activation in malignant cells could be harmful to the evolution of the disease as well as to the outcomes of the treatment and findings of the mutation and aberrant signaling of the Nrf2 pathway in cancer reveal a new role for this factor beyond its function in chemoprevention [11]. But there are limited reports about the expression, significance, and function of Nrf2 in gastric cancer. In this study, we investigated the expression level of Nrf2 in gastric cancer cell lines in vitro and the effect of knockdown of NRF2 on the proliferation of gastric cancer cells. Besides, the correction of its expression and prognostic significance was also been researched.

3. Material and Methods

3.1. Antibodies and Reagents

2,7-Dichlorofluorescein diacetate (DCF-DA, D6883) was purchased from Sigma-Aldrich (St Louis, MO, USA). Anti-NRF2 (ab31163) antibody was purchased from abcam. (Santa, Cruz, CA). Anti-cyclinB1 (4138), cdc2 (9116), gadd45α antibodies, and Horseradish peroxidase – conjugated anti-mouse and anti-rabbit antibodies were obtained from Cell Signaling Technology. Anti-β-actin (A5441) antibody was purchased from Sigma.

3.2. Cell Culture

Human gastric cell lines (GES, AGS, HGC27, MKN45, N87, KATO-III) were obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China). These cells were cultivated in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100mg/ml streptomycin) and maintained in air humidified 5% CO2 at 37^0C in a cell incubator.

3.3. Western Blot Analysis

Total protein concentration was determined with Bio-Rad Protein Assay after cells were lysed. Thirty to sixty ug protein lysates were electrophoresed on 10% SDS-PAGE gels and transferred to PVDF membranes. TBST containing with 5% nonfat milk or bovine serum albumin was used to block nonspecific binding for 2 h at room temperature. Then membranes were incubated with primary antibodies according to the instructions overnight at 4 ^{0}C with primary antibodies followed by Corresponding HRP-conjugated secondary antibodies (1:5000 dilutions) at room temperature for 2 h. Immune reactive bands were then detected using an ECL chemiluminescence system.

3.4. Cell Viability Assay

The Cell Counting Kit-8 (CCK-8)assay was used to measure cell viability. AGS and HGC27 cells were plated in 0.5 × 104 cells per well in 96-well plates. After a day (24h) of cultivation, according to the manufacturer's instructions (CK04, Dojindo, Japan), the CCK-8 reagent was added to the medium, and cells were incubated for a further 2h.The absorbance of samples (450 nm) was determined by using a scanning multi-well spectrophotometer every 24h.

3.5. Short Hairpin RNA (shRNA) Transfection

The pGP U6-shRNA plasmids were constructed by cloning the respective shRNAs into the pGPU6/GFP/Neo vector (Gene Pharma, Shanghai, China). The new plasmid was renamed as shRNA-Nrf2. And a blank vector (shNC) was used as a negative control. AGS and HGC27 cells were seeded in a 24-well plate at a concentration of 1×105 cells per well. According to the manufacturer's protocol, lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was used for transfection Fresh culture medium was replaced 6h after transfection, and the cells were harvested 48 h after transfection for analysis. The shNC was used as a negative control. Stable transfected cells were validated by qRT-PCR and Western blot analysis compared with the negative control cells.

3.6. RNA Extraction and Quantitative Real-Time PCR

Teal-time PCR was conducted as previously described[12].Briefly, Total RNA from AGS and HGC27 cells was extracted using Tirol reagent, and reverse transcription was carried out with 500ng RNA in a total 20 μL reaction volume using Prime Script™ RT Master Mix according to the manufacturer's instructions. Quantitative real-time PCR experiments were done with the 7500 Real-time PCR System (Applied Bio systems) using SYBR Premix Ex Taq reagents. Primers were designed and validated by Invitrogen Biotechnology Co. Ltd. All data were normalized to the human β-actin. Primers sequences are: β-actin, F, 5'-CTGG CACCACACCTTCTACAATG-3',R, 5'-AATGTCACGCACGATTTCCCGC-3';NRF2,F, GCCCATTGATGTTTCTGAT

, R, TTAGTGAAATGCCGGAGTCA.

3.7. Cell Cycle Analysis

To verify the effects on cell cycle distribution by using Cycle TEST DNA Reagent Kit (340242, BD Biosciences) according to the manufacturer's introductions. Harvested were seeded into 6-well plates, for cell cycle analysis, and fixed in 70% ice-cold ethanol in PBS after washing in ice-cold PBS. The cells were then pelleted in a cooled centrifuge and suspended in cold PBS. Cells were incubated at 37°C for 30 min followed by the addition of 100 ul bovine pancreatic RNAase

(Sigma) for 20 min at room temperature in the dark. At last, data were analyzed by BD FACS Canto II flow cytometer (BD Biosciences, CA).

3.8. Measurement of Reactive Oxygen Species (ROS)

Treated Cell were collected and examined as previously described [13].Intracellular reactive oxygen species (ROS) generation was measured by flow cytometry using oxidation sensitive fluorescent probe (DCFH-DA) according to the manufacturer's protocols. The cells were harvested and then stained with 10μM DCFH-DA probe at 37 °C for 20 min. Cells were washed three times with PBS, and then the fluorescence intensity examined by flow cytometry, with excitation and emission settings of 488 nm and 530 nm, respectively. In all experiments, 10,000 viable cells were analyzed, and performed three times.

3.9. Immunohistochemically (IHC) Assay

A gastric cancer tissue microarray was purchased from Shanghai Outdo Biotech (Shanghai, China), which contained 90 carcinoma tissues and paired para-carcinoma tissues. All patients had been pathologically diagnosed with gastric adenocarcinoma. IHC of NRF2 was performed using anti-NRF2 the IHC analysis was performed as follows. In brief, the tissue sections were dewaxed and endogenous peroxidase was blocked by 1% hydrogen peroxide. After incubated with primary antibody against NRF2 overnight at 4°C and being washed, tissue sections were treated with biotinylated secondary antibody for 1 h at room temperature. Finally, tissue sections were reacted with 3,3-diaminobenzidine and counterstained with hematoxylin. The total score (values 0-12) of protein expression was calculated by multiplying the percentage of positive areas (0-25% = 1, 26-50% = 2, 51-75% = 3, >75% = 4) and immunostaining intensity (negative = 0, weak = 1, moderate = 2, or strong = 3). The score ≥ 6 defined high expression, while score < 6 defined low expression.

3.10. Immunohistochemistry Staining

The tissue sections were cut from human gastric tissue blocks and xenograft tumor nodes. The harvested tissues were fixed in 10% formalin at room temperature, processed, and embedded in paraffin. The tissue sections were dewaxed and endogenous peroxidase was blocked by 1% hydrogen peroxide. For Ki67 and Nrf2 immunohistochemistry, sections were incubated with primary antibodies overnight at 4°C and being washed followed by the secondary antibody for 1 h at room temperature. The signal was detected by biotinylated secondary antibodies. Finally, slides were developed by the DAB reagent.

3.11. Xenograft Experiment

The mice were randomly divided into two groups with five mice in each group. Each mouse nude was injected on the mammary pads with vector-transfected HGC27 cells (2×10^6) on the right side. Measure the length and width of the tumor with a Vernier caliper every 3 days and their volumes were calculated using the equation (width2 * length)/2. Mice were sacrificed after 25 days, and the xenograft tumors were isolated and weighted. Values are means ± S.D. of each group. Then these tumor tissues were stained with immunohistochemistry.

3.12. TUNEL Assay

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP Nick end labeling (TUNEL) was used to detect apoptotic cells that undergo extensive DNA degradation. The apoptotic reaction of tumor tissue was detected by apoptosis kit (C1086, Beyotime) and observed under the fluorescence microscope.

3.13. Statistical Analysis

Three independent experiments were carried out for each test. Data were analyzed using SPSS software version 19.0 or GraphPad Prism v6.0. All data were presented as mean ± standard derivation (SD). Statistical comparison in each group was performed using unpaired Student's t-test. $\chi 2$ test was used to determine the correlation between Nrf2 expression and clinicopathologic variables. Survival curves were estimated using the Kaplan-Meier method. P-values < 0.05 were considered to be statistically significant.

4. Results

4.1. Overexpression of Nrf2 in Gastric Cancer Cell Lines

We first investigate the protein expression of Nrf2 in gastric cancer cell line by western blotting, and then the mRNA expression of Nrf2 was analyzed by qRT-PCR. The result shows that all gastric cancer cell lines (AGS, HGC27, MKN45, N87, and KATO-III) had an overexpression of Nrf2 compared to normal gastric cell line (GES) (Figure 1A). AGS and HGC27, with higher expression levels of Nrf2, were therefore chosen for further experiments.

4.2. Knock Down of NRF2 Inhibits Proliferation of Human Gastric Cancer Cells

To knockdown the endogenous expression of Nrf2 in AGS and HGC27 cells, we applied a plasmid vector expressing specific shRNA sequence targeting Nrf2 (shRNA-Nrf2). We stably transfected the AGS and HGC27 cells with specific plasmid vector and use a control shRNA sequence (shNC) that did not target any known human gene. As expected, qRT-PCR and western blotting of AGS and HGC27 confirmed that NRF2 expression was apparently decreased in shRNA-NRF2 group compared to shRNA-Control group (Figure 1B). Then the viability of the cells was determined by cell proliferation assay. Our study demonstrated that downregulating Nrf2 significantly reduced the viability of AGS and HGC27 (Figure 1C).

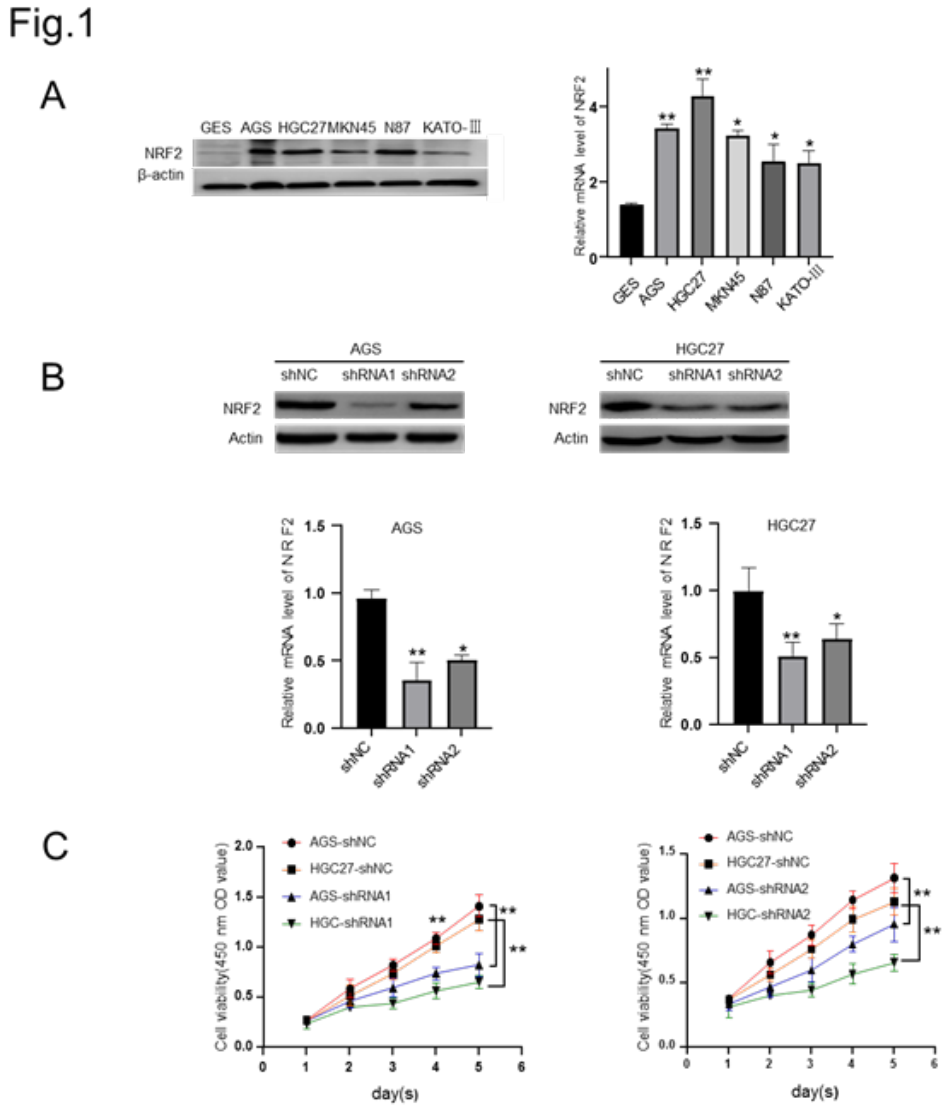

Figure 1: The proliferation-suppressive effect of NRF2 knockdown on GC cells. (A) NRF2 protein expression and mRNA expression was detected in various GC cell lines (GES, AGS, HGC-27, MKN45, N87 and KATO-3) by Western blotting. The protein expression of NRF2 was significantly higher in AGS, HGC27 and N87 compared with other three GC cells, and Human Gastric Mucosal Epithelial Cell Line GES1. (B).Transduced with pGU6/GFP/Neo/shRNANRF2 vector. NRF2 expression significant suppressed at both protein and mRNA level after knockdown NRF2 (**p <0.01,**P <0.01). (C) CCK8 assay were used to assess the effect of NRF2 on cell proliferation. Data are presented as mean ± SD from three independent experiments with each running in triplicate. Unpaired student's t-test was used for the comparison between the two groups (**p < 0.01, **p < 0.01).

4.3. Silencing the Expression Nrf2 Triggers G2/M Cell Cycle Arrest in GC Cells

The cell cycle is a process that participates in the growth and proliferation of cells. The prolonged cell-cycle block disrupts the balance of cell proliferation and cell death. Within this G2/M gate, almost all of the cells were in G2 after transfected with shRNA sequence targeting Nrf2, with very few cells entering into mitosis, indicating activation of the G2/M cell cycle checkpoint. Moreover, to confirm the G2/M phase arrest in GC cells by silencing the expression Nrf2 induction of cdc2, CyclinB1, and GADD45α expressions, cell cycle signaling cdc2, CyclinB1 were measured by Western blot, and apoptosis signaling proteins such as GADD45α were detected by Western blotting. To sum up, our data support that knocking down the expression of Nrf2 was involved in G2/M phase arrest which inhibited the proliferation of GC cells by alterations in cdc2/CyclinB1/ GADD45α proteins (Figure 2A, 2B). The phenomenon that cells with cell cycle arrest had higher levels of reactive oxygen species was observed (Figure 2C).

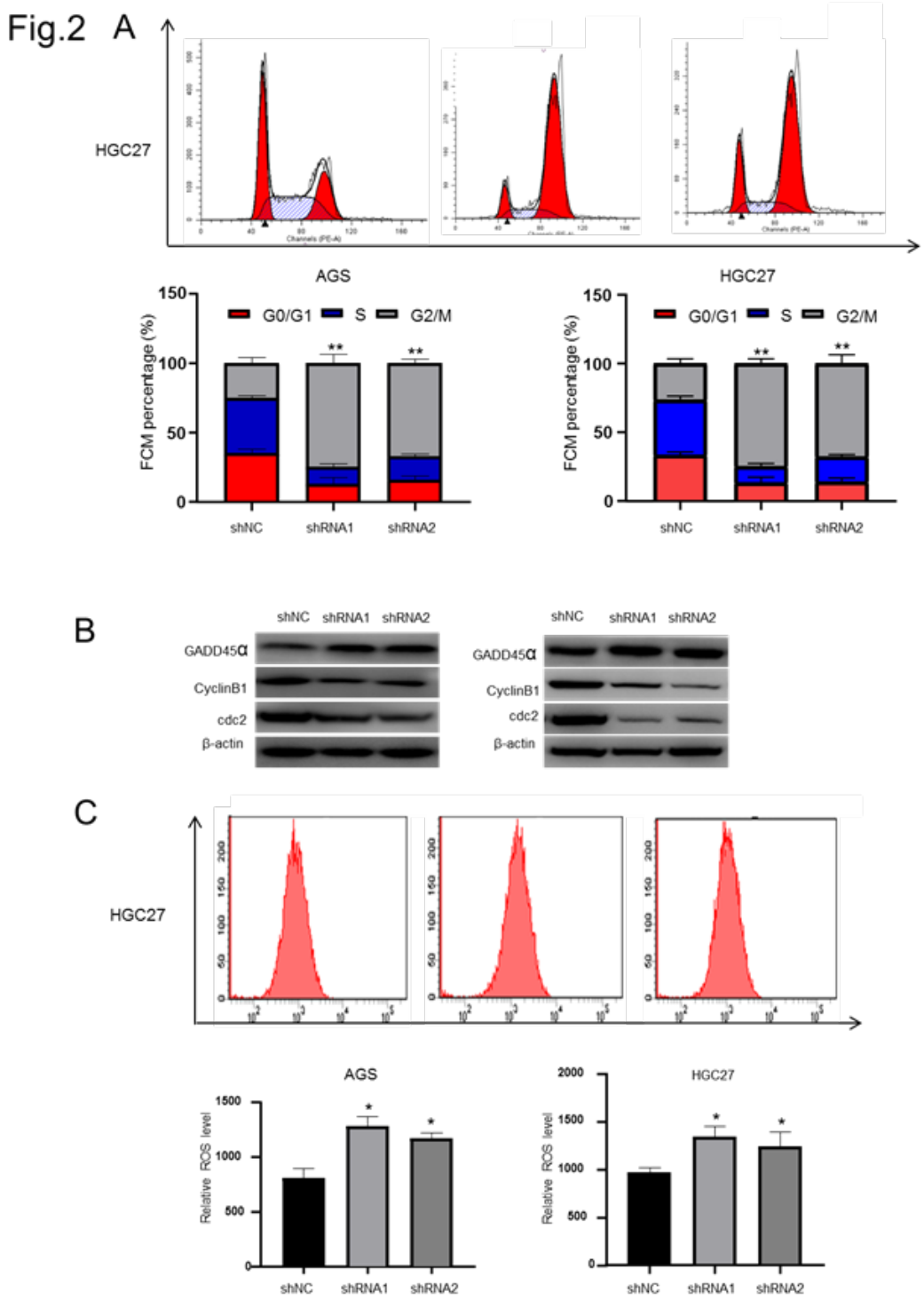

Figure 2: NRF2 knockdown induced G2/M phase arrest in human gastric cancer cells. (A)Cell-cycle analysis was conducted by using Flow cytometry. Suppressed NRF2 significantly induced cell cycle arrest G2M phase in AGS and HGC27 cells. (B)Western blot to use to examine the expression of GADD45α,cyclinB1,cdc2 after knockdown of NRF2 in AGS and HGC27 cells,β-actin expression was used as a loading control.(C) ROS production was measured using DCFH-DA by flow cytometry. Data presented as means ± S.D. are representative of three independent experiments when compared with the vehicle control. (*$p < 0.05$,**$p < 0.01$).

4.4. Nrf2 Promotes Tumor Growth in Mouse Xenograft Models

Subsequently, shNC cells and shNrf2 cells were subcutaneously inoculated into the nude mice, which were employed to further probe the tumor-promoting effect of Nrf2 in vivo. The volume of mice was measured once 5 days for 25 days. The tumor size of the shNC group was significantly larger than that of the shNrf2 group. The tumor mass of shNrf2 treated mice was significantly less than that of the control group (Figure 3A,3B). The immunohistochemistry staining of xenograft tissues revealed a lower expression of ki67 in shNrf2 treated tumors, whereas suggested higher apoptosis in shNrf2 treated tumors by TUNEL (Figure 3C). To summarize, knockdown NRF2 inhibited gastric cancer growth in vivo.

4.5. NRF2 Overexpression Correlated with Poor Prognosis of Gastric Cancer

We then performed IHC staining of gastric adenocarcinoma tissue arrays to illustrate the protein level of NRF2 in 90 pairs of gastric adenocarcinomas tissue and adjacent normal gastric tissue. As shown in (Figure 3A), Nrf2 was significantly overexpressed in 65.5% (59/90) GC cases. In contrast, 12.2%(11/90)of adjacent noncancerous tissues showed Nrf2 overexpression (Figure 4A,4B). Moreover, Kaplan Meier analysis shows that the overall survival rate of the high-expression of Nrf2 group was significantly lower than that of the Nrf2 low-expression group (P<0.01) (Figure 4C). Therefore, NRF2 overexpression has a potential role in gastric cancer development and correlates with poor outcome for gastric cancer patients.

Fig.3

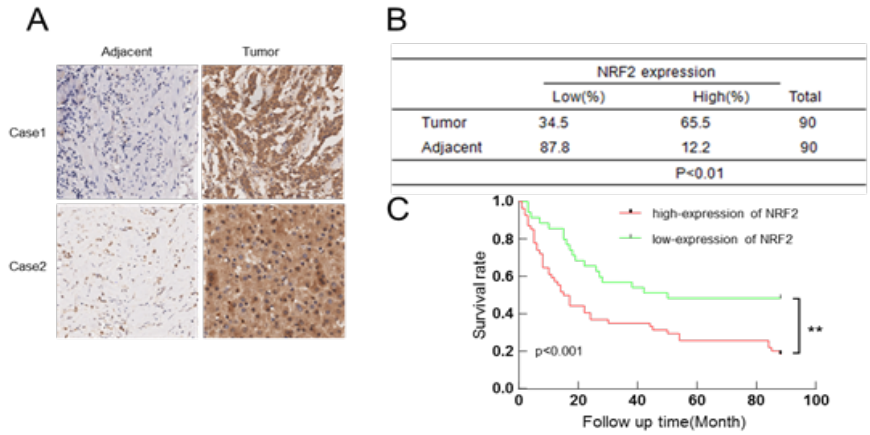

Figure 3: (A). Representative immunohistochemical staining images of NRF2 expression (high or low) in tissue microarrays constructed from gastric cancers and paired adjacent gastric tissues. Scale bar, 100μm. (B).A summary of immunohistochemical staining results of NRF2 expression in tissue microarrays. (C). Kaplan-Meier analysis of overall survival of gastric cancer patients stratified by nrf2 expression.

Fig.4

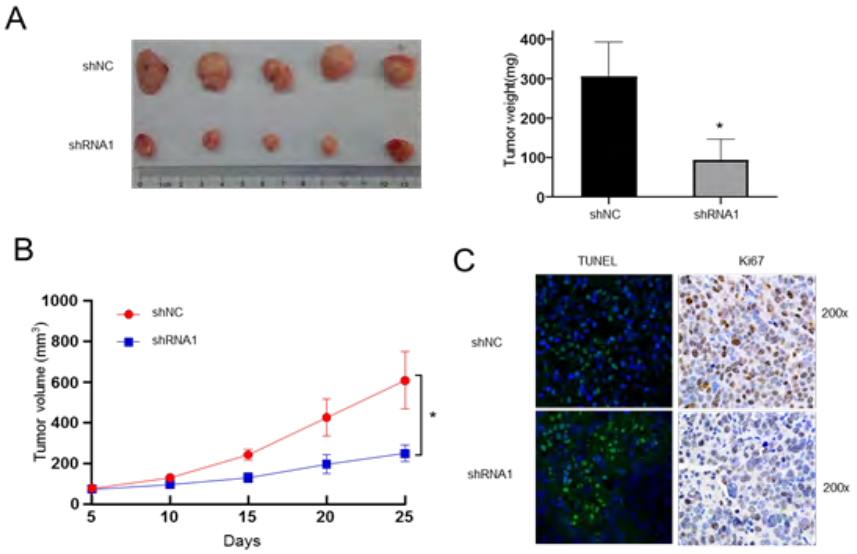

Figure 4: NRF2 knockdown suppressed tumor growth in vivo. (A) Tumors (shRNA-Control, n=5; shRNA-NRF2, n=5) were harvested imaged and measured after 25 days, Statistical analyses demonstrated that the tumor weight (B), tumor volume (C) of treated and untreated mouse. *P<0.05, **P<0.01 vs. control group (n=5). (C) Immunohistochemistry analysis of ki67 expression of cancer tissue of the two groups; TUNEL to analyze the apoptosis cells. (Magnification ×400) Scale bars, 100μm.

5. Discussion

Transient activation of Nrf2 can protect normal cells from aerobic stimulation, while structural activation is related to tumor development and drug resistance. This is the dual effect of Nrf2 in the occurrence and development of tumor [14]. Sporn MB confirmed that NRF2 activation prevents initiation of chemically induced lung cancer, but promotes progression of pre-existing tumors regardless of chemical or genetic etiology. Once tumors are initiated, NRF2 inhibition is effective against the progression of chemically and spontaneously induced tumors [15]. Nrf2 nuclear expression in patients with esophageal squamous cell carcinoma was related to worse survival and poorer therapy outcomes [16]. In this study, we report the correlation between the expression of Nrf2 in gastric cancer in vivo and in vitro and the clinical-pathological features.

Studies have shown that an increasing concentration of ROS is commonly associated with activation of p53, increased oxidative stress, DNA damage, and eventually, apoptotic cell death [17]. Some compounds reveal that ROS accumulation can induce Inhibitory Effects in numbers of cancer cells lines [18, 19]. Our study shows that Nrf2 in gastric cancer cell lines was highly expressed in both protein and mRNA levels, downregulation of NRF2 induce inhibitory of cell growth, with accumulation ROS. We consider that gastric cancer cell lines can cause the continuous production of antioxidants, which makes the cancer cells a higher ability of anti-ROS. In fact, numbers of reports point out that NRF2 maintains low ROS levels in cancer cells, which promotes quiescence renewal, anchorage-independent growth, and protects them from chemotherapy [9, 20, 21]. These evidence further suggests that Nrf2 serves as an oncogene to promote the growth and proliferation of tumor cells, possibly via an increase in the tumor resistance to oxidative stress.

Furthermore, to reveal the exact role of Nrf2 in gastric cancer, we explored the effect of Nrf2 on proliferation, apoptosis by modulating the expression level of Nrf2 using Nrf2-shRNA. Then, we investigated the potential mechanisms of Nrf2 in regulating proliferation, apoptosis. Nrf2 promotes cell proliferation by upregulate of cdc2-cyclin B complex, a kind of gene that expresses cell cycle-dependent or directly involved in cell cycle regulation [22]. The main function of GADD45α, an important gene related to DNA damage repair and plays an important role in apoptosis, in the cell cycle is to arrest G2/M transition through disrupting Cdk1/Cyclin B1 interaction [23, 24]. In the present study, we demonstrate that downregulation Nrf2 inhibits the growth of GC cells by inducing cell cycle arrest, which may triggered by ROS. Nevertheless, detailed mechanisms remain unclear and merit further investigation.

Previous reports have implicated Nrf2 in the pathophysiology of small cell lung, human breast, hepatocellular cancer, and glioblastoma and showed multiple human cancers frequently exhibit overexpression levels of Nrf2 [25-28]. When the primary tumor of renal cell carcinoma showed Nrf2 mutation or increased expression of Nrf2, the metastatic tumor had a poor response to targeted therapy of vascular endothelial growth factor [29]. The expression of mRNA and protein of NQO1, HO-1, and GST decreased significantly after Nrf2 knockout in renal cancer cells, thus increasing the sensitivity to Sunitinib [30]. Nrf2 is frequently deregulated in NSCLC through somatic mutations that disrupt the Nrf2-Keap1 interaction to constitutively activate Nrf2 [15]. Cancer cells acquire malignant properties by activating the Keap1-Nrf2 system, Nrf2 positive patients are more likely to develop drug resistance and the prognosis is very poor [31]. Our dates show Nrf2 activity strongly associate with poor patient prognosis. These results have important implications for NRF2-targeted cancer prevention and intervention strategies.

In summary, we have demonstrated that Nrf2 is highly expressed in gastric cancer cells, and knockdown of NRF2 shows anti-tumor effect in vitro and in vivo. Overexpression of NRF2 serves as a poor prognosis marker in human gastric cancer samples, implying that targeting NRF2 is a promising therapeutic strategy for gastric cancer treatment in future. These investigations may help with the development of personalized treatment for patients who have abnormal levels of Nrf2.

6. Ethics Approval and Consent to Participate

All animal study procedures were performed in accordance with protocols approved by Suzhou Hospital Affiliated to Nanjing Medical University Ethics Review Committee.

7. Declaration of Conflicting Interest

The authors declare that there is no conflict of interest.

8. Funding

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