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Original Article

Expression of Nrf2 and NQO1 in human gastric cancer and their clinical significance

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Abstract: Background: Accumulated evidence shows that constitutively high expression of Nuclear factor erythroid 2-related factor 2 (Nrf2) is associated with cancer growth and progression. NAD(P)H: quinone oxidoreductase 1 (NQO1), as a downstream gene of Nrf2, can also play an important role in occurrence and development of malignancy. In this study, we investigated expression of Nrf2 and NQO1 in gastric cancer and its clinical significance. Methods: Nrf2 and NQO1 protein expression were assessed using immunohistochemical (IHC) staining in 162 patients with gastric cancer. Their relationships and the correlation of Nrf2 and NQO1 expression with clinicopathological factors of gastric cancer were evaluated by Chi-square. Cumulative survival after tumor removal was calculated using the Kaplan-Meier method, and differences in survival curves were analyzed using the log-rank tests. Results: The positive rates of Nrf2 and NQO1 protein expression in gastric cancers were 59.26% and 54.32%, respectively. Both Nrf2 and NQO1 positivity were closely associated with tumor size, lymph node metastasis, TNM stage, tumor depth and the degree of histological differentiation ($P<0.05$ for all). Kaplan Meier analysis showed that patients with high Nrf2 or NQO1 expression had lower survival rates than those with low Nrf2 or NQO1 expression ($P<0.05$ for all). Both Nrf2 and NQO1 are significantly correlated with resistance to 5-fluorouracil combination chemotherapy in gastric cancer ($P = 0.002$ and 0.001, respectively). Conclusions: Nrf2 and NQO1 expression reflect aggressive behavior of gastric cancer. These findings indicate that Nrf2 and NQO1 might be useful as a poor prognostic biomarker of gastric cancer.

Keywords: Gastric cancer, Nrf2, NQO1, oxidative stress, chemoresistance

Introduction

Gastric cancer is one of the most common malignant tumors and the second leading cause of cancer death worldwide. A total of 989,600 new gastric cancer cases and 738,000 deaths are estimated to have occurred in 2008, accounting for 8% of the total cases and 10% of total deaths; over 70% of new cases and deaths occur in developing countries [1]. Although gastric cancer rates have decreased substantially in most parts of the world, it have limited efficacy because most gastric cancer patients are in the advanced stages when diagnosed; the five-year survival rate for patients with stage III/IV gastric cancer is around 10% [2]. In china, gastric cancer is still the second most frequently diagnosed cancer and the third leading cause of cancer death [3]. Gastric cancer results from a combination of genetics, lifestyle and environmental factors. Despite the advance in diagnosis and treatment, the prognosis for advanced gastric cancer is poor. Therefore, it is necessary to find novel cancer-related factors that can be used as markers for the diagnosis and treatment of gastric cancer.

Oxygen is essential for the survival of all aerobic organisms and Its metabolism results in partially reduced oxygen byproducts collectively known as reactive oxygen species (ROS) [4]. The balance between ROS production and elimination is crucial for the cell to remain viable and maintain its vital function [5]. Excess ROS produced under pathological conditions will cause oxidative damage to cellular DNA, lipids, and proteins; genetic changes and/or epigene-

Table 1. Correlation of clinicopathological factors with Nrf2 and NQO1 expression in gastric cancer

Clinical factors	No. of cases	Nrf2			NQO1		
		(+)	(-)	p	(+)	(-)	p
Age (years)							
<55	96	54	42	0.347	47	49	0.098
≥55	66	42	24		41	25	
Gender							
Male	103	59	44	0.498	52	51	0.195
Female	59	37	22		36	23	
Tumor size (cm)							
<5	89	43	46	0.002	39	50	0.003
≥5	73	53	20		49	24	
Tumor location							
Cardia, fundic, body	87	55	32	0.269	49	38	0.582
Antral	75	41	34		39	36	
TNM stage							
I, II	74	36	38	0.012	31	43	0.004
III, IV	88	60	28		57	31	
Tumor depth							
T1, T2	77	38	39	0.015	33	44	0.005
T3, T4	85	58	27		55	30	
Lymph node metastases							
Yes	91	62	29	0.009	56	35	0.037
No	71	34	37		32	39	
Differentiation							
Well	88	43	45	0.012	39	49	0.016
Moderate	43	30	13		27	16	
Poor	31	23	8		22	9	
Nrf2							
(+)	96				64	32	0.002
(-)	66				24	42	

tic alterations can lead to the dysregulation of oncogenes and tumor suppressor genes, ultimately contributing to the pathogenesis of cancer [4, 5]. Oxidative stress as a result of elevated levels of ROS has been observed in almost all cancers, including gastric cancer [6, 7]. To counteract the oxidative or electrophilic stress in cells, human bodies organize an antioxidant response through signaling mechanisms, such as the Kelch-like eCh-associated protein 1 (Keap1)-nuclear factor-e2-related factor 2 (nrf2)-antioxidant response element (ARE) pathway (Keap1-nrf2-ARE pathway) [8].

Nuclear factor erythroid 2-related factor 2 (Nrf2), one of the most important transcriptional activators in the pathway, plays a central role in regulation a battery of genes involved in xenobiotic metabolism and antioxidants [9, 10]. Nrf2 detoxifies ROS mainly through the transactivation of a series of genes including NAD(P)H: quinone oxidoreductase 1 (NQO1) and heme oxygenase-1 (HO1 (HMOX1)), which maintains cell homeostasis [5, 8]. Accumulated evidence shows that constitutively high expression of Nrf2 not only promotes cancer development, but also contributes to chemoresistance, enhances cancer cell survival and is associated with cancer growth and progression [11, 12]. In this study, we investigated the correlation of clinicopathological factors of gastric cancers with Nrf2 and NQO1 expression. We also assessed the prognostic value of high Nrf2 and NQO1 expression, and evaluated the effects of Nrf2 and NQO1 expression on chemosensitivity in patients with gastric cancer.

Materials and methods

Patients and tumor specimens

All tissue specimens from 162 cases of gastric cancer were collected from Gansu Provincial Hospital from Sep 2008 to Feb 2010. All patients underwent curative gastrectomy with lymph node dissection. Tissue samples for diagnostic purposes were obtained with the consent of each patient. None of these patients received radiotherapy, chemotherapy or other anti-cancer therapy before surgery, and 124 patients with stage II or III cancer received 5FU-based adjuvant chemotherapy for 6 to 8 cycles. All specimens were pathologically confirmed. The clinicopathological characteristics of these patients are summarized in **Table 1**. Histological diagnosis was based on the 2010

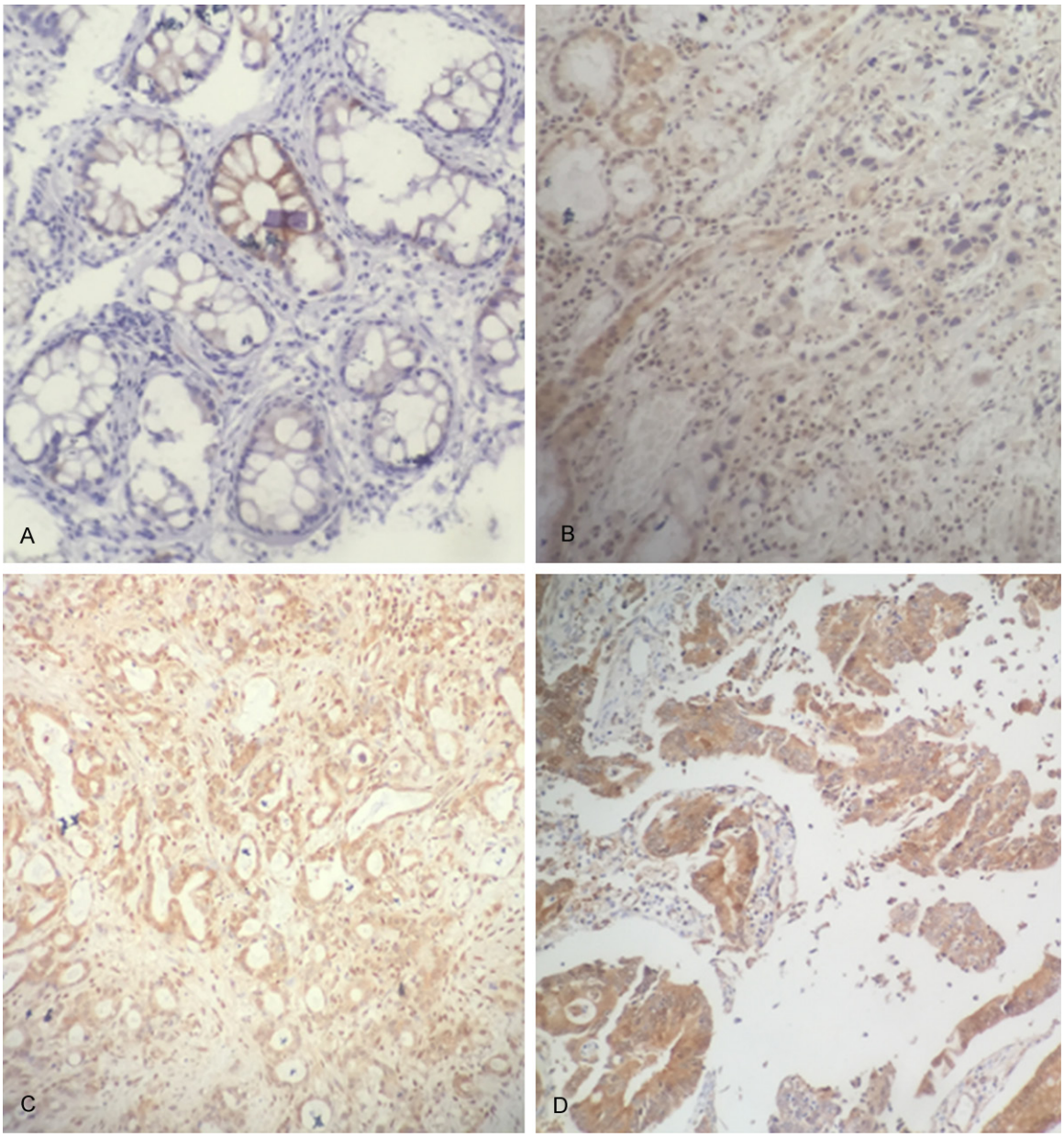

Figure 1. Immunostaining of Nrf2 in clinical gastric cancer samples. Representative immunostaining of Nrf2 in (A) normal stomach tissue; (B-D) Gastric cancer tissues; (B) well-differentiated gastric carcinoma; (C) middle-differentiated gastric carcinoma; (D) poor-differentiated gastric carcinoma.

edition of the World Health Organization digestive system cancer guidelines. The staging of gastric cancer was according to the 7th edition of tumor node metastasis classification. "5FU resistance" was defined as gastric cancer that recurred after adjuvant chemotherapy [7]. This study was approved by the Ethics Committee of Gansu Province People's Hospital (syll2015009), and all patients gave their informed consent prior to inclusion in the study.

Immunohistochemistry (IHC)

Tumor samples were fixed with 10% formaldehyde in PBS, embedded in paraffin, sectioned into 4-μm thick sections and mounted on glass slides for immunohistochemical analysis. The procedure was in accordance with the immunohistochemical SP detection kit instructions. The diluted density of mouse anti-human Nrf2 antibody was 1:200 and NQO1 antibody was

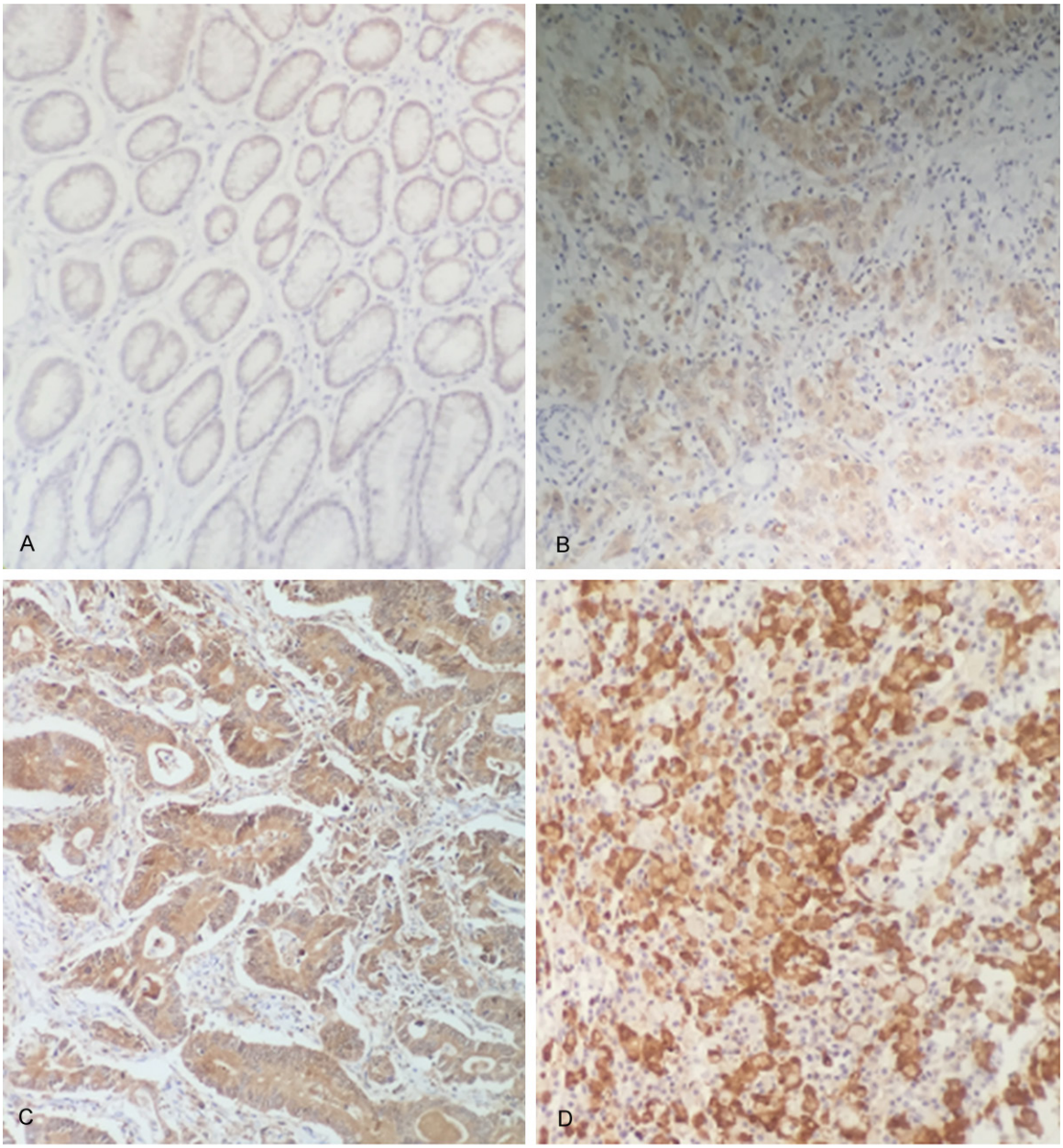

Figure 2. Immunostaining of NQO1 in clinical gastric cancer samples. Representative immunostaining of Nrf2 in (A) normal stomach tissue; (B-D) Gastric cancer tissues; (B) well-differentiated gastric carcinoma; (C) middle-differentiated gastric carcinoma; (D) poor-differentiated gastric carcinoma.

1:400. Nrf2 immunoreactivity was detected in the nucleus. NQO1 protein positive products were mainly localized in the cytoplasm, and it was also weakly detected in the nucleus.

Evaluation of IHC staining

Positive results were judged by both the proportion of positive cells and the intensity of staining. The staining intensity score was 0 (negative), 1 (weak), 2 (medium), and 3 (strong). The integral of the rate of positive cells was 0 (≤5%), 1 (6-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%). Immunohistochemical scores, which ranged from 0 to 12, were calculated by multiplying the values of the intensity and the proportional score. A score ≥4 was considered to be a positive expression.

Statistical analysis

Statistical analyses were performed using SPSS 17.0 software. Statistical analysis of group differences was performed using the χ^2

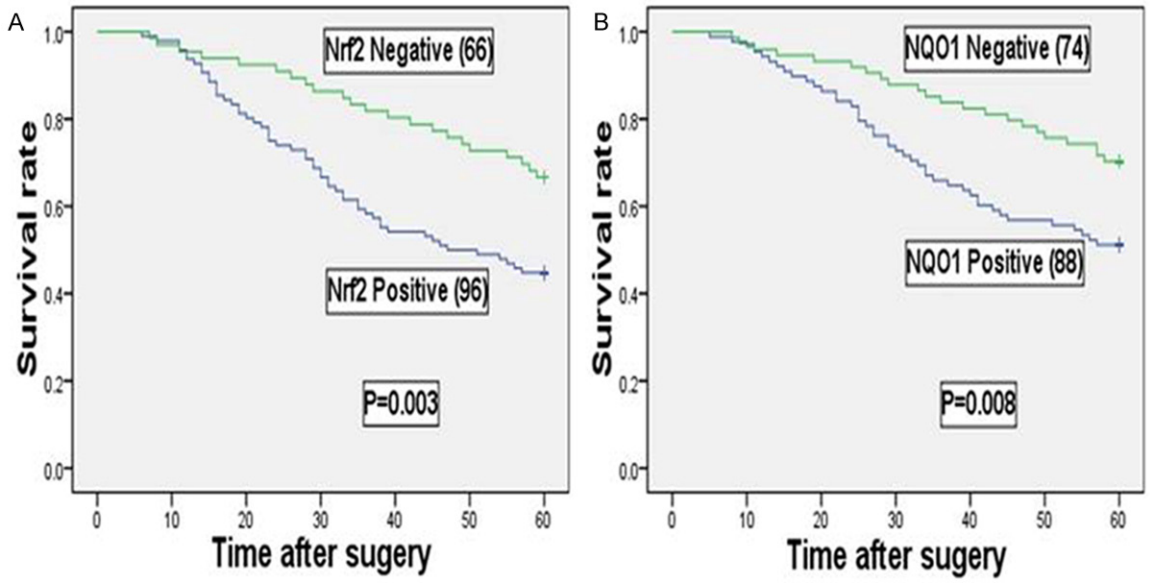

Figure 3. Kaplan-Meier survival curves of patients with gastric cancer according to Nrf2 (A) and NQO1 (B) protein expression. Correlation between overall survival of the patients and Nrf2 expression was found to be statistically significant (log rank P = 0.003) as well as that between survival and NQO1 expression (log rank P = 0.008).

Table 2. Correlation of Nrf2 and NQO1 expression with 5FU resistance

5FU sensitivity	Nrf2			NQO1		
	(+)	(-)	p	(+)	(-)	p
Sensitive	24	27	0.002	16	31	0.001
Resistant	54	19		53	24	

test and Fisher's exact tests. Cumulative survival after tumor removal was calculated using the Kaplan-Meier method, and differences in survival curves were analyzed using the log-rank tests. P<0.05 was considered statistically significant.

Results

Nrf2 and NQO1 immunohistochemical expression in gastric cancer tissues and adjacent normal tissue

Immunohistochemical results showed that Nrf2 protein is mainly located in the nucleus of gastric cancer cells (**Figure 1**). The positive rate of Nrf2 protein expression was 59.26% (96/162) in the 162 tumor tissues, which was significantly higher than that in adjacent non-tumor tissues (19.14%, 31/162) (P<0.001). NQO1 immunoreactivity was detected mainly in the cytoplasm, and it was also weakly detected in the nucleus (**Figure 2**). The positive rate of NQO1 in the 162 tumor tissues was 54.32% (88/162). It was significantly higher than in the corresponding normal tissues (13.58%, 22/162) and the difference was statistically significant (P<0.001).

Correlation of clinicopathological factors with Nrf2 and NQO1 expression

To evaluate the role of Nrf2 and NQO1 protein in gastric cancer progression, the correlation between Nrf2 and NQO1 expression and clinicopathological features of patients was analyzed. As summarized in **Table 1**, there were no significant correlations between the expression level of Nrf2 protein and patient age, gender, location in patients with gastric cancer. However, there was a close relationship between the positive rate of Nrf2 protein and tumor size (P = 0.002), lymph node metastasis (P = 0.009), TNM stage (P = 0.012), tumor depth (P = 0.015) and the degree of histological differentiation (P = 0.012). Expression of NQO1 protein in the gastric cancer tissue was correlated with tumor size (P = 0.003), lymph node metastasis (P = 0.037), TNM stage (P = 0.004), tumor depth (P = 0.005) and the degree of histological differentiation (P = 0.016), but not with age, gender, location (P>0.05).

Association between Nrf2 and NQO1 expression and prognosis of gastric cancer patients

NQO1 protein was closely related to expression of Nrf2 in gastric cancer tissues (P = 0.002)

(**Table 1**). Kaplan Meier analysis indicated that patients with high Nrf2 expression had lower survival rates than those with low Nrf2 expression (P = 0.003) (**Figure 3A**). Similarly, the survival rates of the NQO1 positive group was significantly poorer than that of the NQO1 negative group (P = 0.008) (**Figure 3B**).

Correlation of Nrf2 and NQO1 expression with 5FU resistance

Table 2 shows correlation of Nrf2 and NQO1 expression with 5FU resistance. The patients of 5FU resistance were 54 (69.23%) in the 78 Nrf2 positive patients, whereas which were only 19 (41.30%) in the 46 Nrf2 negative patients. Similarly, the patients of 5FU resistance in the 69 NQO1 positive and the 55 NQO1 negative patients were 53 (76.81%), 24 (43.64%), respectively. Both Nrf2 and NQO1 are significantly correlated with resistance to 5FU-based adjuvant chemotherapy in gastric cancer. *P* values were 0.002, 0.001, respectively and the difference is statistically significant.

Discussion

Nrf2 belongs to the cnc ("cap 'n' collar") subfamily of the basic region leucine zipper transcription factors [13, 14]. Furthermore, several studies have confirmed that activation of Nrf2 and its downstream gene is caused by defects in Keap1 function [13]. Previous studies have demonstrated that Nrf2 immunoreactivity is frequently detected in various human malignancies, such as breast, lung, gastric, pancreatic, intrahepatic cholangiocellular, gallbladder, endometrial, colorectal, and ovarian carcinomas [15, 16]. At the same time, the overexpression of its downstream genes (such as NQO1) also detected by using IHC in many types of human cancer, including human breast, ovarian, pancreatic, thyroid, adrenal, colon, corneal and gastric tumors [17, 18]. Therefore, it can be theoretically proposed that defects of Nrf2/NQO1 pathway play an important role in occurrence and development of malignancy. Our paper is the first report to reveal that an association between the Nrf2/NQO1 pathway and clinicopathological features of gastric cancer patients.

In the present study, Nrf2 immunoreactivity was detected predominantly in the nucleus, and its rate of immunopositivity was 59.26% (96/162) in the 162 tumor tissues, which was significantly higher than that in adjacent non-tumor gastric tissues. Our results are similar to previous researches. Previous studies have reported that the rate of Nrf2 immunopositivity in gastric cancer was 55.9% [19] and 61.7% [7]. However, Hu et al. reported that Nrf2 immunoreactivity was detected predominantly in the cytoplasm [19]. Kawasaki et al. thought that the different result may be due to using different antibodies in the course of the experiment [7]. Meanwhile, NQO1 immunoreactivity was detected mainly in the cytoplasm, and the positive rate of NQO1 in the 162 tumor tissues was 54.32% (88/162), which was significantly higher than that in the corresponding normal tissues. Previous studies have reported that NQO1 protein and mRNA expression levels are abnormally elevated within many solid tumors [18]. And some scholars believed that NQO1 may be a useful diagnostic indicator for some tumors [18, 20, 21]. In addition, according to the current data, we found that there was a positive correlation between expression of Nrf2 and NQO1. Many relative researches have demonstrated that the overexpression of Nrf2 could up-regulated NQO1 expression [10, 22]. We therefore considered that Nrf2 protein expression is consistent with NQO1 expression in gastric cancer specimens. These results suggest that Nrf2 and NQO1 may act as a new parameter for diagnosis of gastric cancer.

Subsequent study found that there was a close relationship between the positive protein expression of Nrf2 and NQO1 and tumor size, lymph node metastasis, TNM stage, tumor depth and the degree of histological differentiation (**Table 1**). Our findings are in accordance with most of the previous reports. Such as, Kawasaki et al. reported that expression of Nrf2 was significantly associated with tumor size, tumor depth, lymphatic invasion, lymph node metastases, and tumor histology [7]. Yang et al. reported that the NQO1 expression level was markedly associated with histological grade, LN metastasis and Her2 expression levels [17]. Moreover, high expression of Nrf2 in colorectal cancer [10], Cholangiocarcinoma [11], Gallbladder Cancer [13], pancreatic adenocarcinoma [14], breast cancer [15] was associated with lower survival rates compared with patients with low-level Nrf2 expression. Cui et

al. and Buranrat et al. reported that NQO1 expression was associated with poor overall survival in ovarian carcinoma [20] and in small cell lung cancer patients [23], respectively. In our paper, we showed by Kaplan Meier analysis that patients with high Nrf2 or NQO1 expression had lower survival rates than those with low Nrf2 or NQO1 expression. We therefore thought that Nrf2 and NQO1 upregulation may promote the invasion and/or metastasis of gastric cancer cells, which may reflect aggressive behavior of gastric cancer. These findings indicate that Nrf2 and NQO1 might be useful as a poor prognostic biomarker of gastric cancer.

A growing body of evidence suggests that Nrf2 can not only mediate cancer chemoprevention in normal cells, but also promotes cancer cell survival [7, 8]. Constitutively expressed Nrf2 can promote cancer cell proliferation and is a profound protection from anticancer drugs or radiotherapy-induced cell death [24-26]. Therefore, Nrf2 is a potent determinant of chemo- and/or radioresistance of cancer [24]. Several literatures reported that Nrf2 expression is significantly correlated with increased proliferation and treatment resistance to radiation, cisplatin, gemcitabine, doxorubicin, 5-fluorouracil (5-FU), mitoxantrone and topotecan, seemingly through the induction of antioxidant genes [24, 27]. Zeekpudsa et al. believed that NQO1 may protect cancer cells by removing free radicals and making cells more resistant to anticancer agents, particularly to oxidative stress inducers [28]. Geng et al. reported that NQO1 expression reduced the effect of 5-fluorouracil combination chemotherapy in gastric cancer [29]. Inhibition of NQO1 by a pharmacological inhibitor, dicoumarol, suppressed urogenital and pancreatic cancer cell growth and also potentiated cytotoxicity of cisplatin and doxorubicin [28]. Our results also showed that both Nrf2 and NQO1 are significantly correlated with resistance to 5-fluorouracil combination chemotherapy in gastric cancer. We therefore thought that Nrf2/NQO1 pathway plays a crucial role in chemoresistance of cancer. Furthermore, Nrf2 and NQO1 may be potential molecular targets for elevating response of cancer cells to chemotherapeutic drugs by inhibiting the Nrf2/NQO1 pathway [7, 19, 26-32]. Based on the above evidence, we can predict the efficacy of 5FU-based adjuvant chemotherapy in gastric cancer by evaluation of Nrf2 or NQO1 expression. Of course, for the patients that they have resistance to chemotherapy and Nrf2 or NQO1 expression is positive, it may be possible to achieve sufficient efficacy of chemotherapy by concomitant inhibition of Nrf2 or NQO1.

In conclusion, there was a positive correlation between expression of Nrf2 and NQO1. Both of them correlated with tumor size, lymph node metastasis, TNM stage, tumor depth, the degree of histological differentiation and resistance to 5-fluorouracil combination chemotherapy in gastric cancer. These findings suggest that Nrf2 and NQO1 expression reflect aggressive behavior of gastric cancer. Nrf2 and NQO1 might be useful as a poor prognostic biomarker of gastric cancer. It may be possible to achieve sufficient efficacy of chemotherapy by concomitant inhibition of Nrf2 or NQO1 for the treatment of Nrf2 or NQO1 positive gastric cancer patients.

Disclosure of conflict of interest

None.

Authors' contribution

All authors read and approved the final manuscript.

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