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Nrf2 overexpression is associated with *P*-glycoprotein upregulation in gastric cancer

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ABSTRACT

The efficacy of chemotherapeutic agents remains very poor in gastric cancer (GC) patients due to the development of multidrug resistance (MDR) phenotype. The nuclear factor erythroid 2-related factor 2 (Nrf2), is a pivotal transcriptional factor that regulates phase II detoxifying enzymes, antioxidants and efflux transporters including *P*-glycoprotein (*P*-gp). The aim of this study was to investigate the association of Nrf2 and *P*-gp and their correlations with clinicopathological criteria in GC patients.Nrf2 and MDR1/*P*-gp expressions in both mRNA and protein levels were examined by real-time PCR and immunohistochemical staining (IHC) respectively, in endoscopic biopsy samples from60 GC patients compared with those expressions in non-GC individuals. Our results from IHC examinations revealed that Nrf2 expression in GC patients (46.7%) is markedly higher than that in non-GC individuals (11.7%) ($p < 0.001$, Mann–Whitney test) which was confirmed by real-time PCR in mRNA levels. Induction of *P*-gp as a drug efflux pump, was associated with Nrf2 overexpression in these samples ($r = 0.55$, $p < 0.001$). There was also a strong correlation between Nrf2 overexpression and tumor size, histological grade, lymph node and distant metastasis while *P*-gp upregulation was shown to be associated only with the histological grade and tumor size (Chi-square, all $p < 0.05$). Our results suggest that therapeutic inhibition of Nrf2 expression can improve the efficacy of chemotherapeutic agents for GC patients by down regulation of *P*-gp expression.

1. Introduction

Gastric cancer (GC) is the third leading cause of cancer mortality and the fifth most common cancer globally [1]. Despite recent improvements in surgical approaches combined with adjuvant and neoadjuvant perioperative techniques including chemo- and radiotherapy, the prognosis and treatment of patients with advanced GC still remain poor [1,2]. Therefore, there is an urgent need to discover novel cancer related key molecules that are clinically applicable as potential markers for both early detection and targeted therapy in GC patients.

Nuclear factor E2-related factor 2(Nrf2), which is a member of the cap n collar (CNC) subfamily transcription factors, was initially known as a master regulator of phase II detoxifying and antioxidant genes through binding to antioxidant response elements (ARE) [3,4]. Under physiological condition, most of the cytoplasmic Nrf2 binds to Kelch-like ECH-associated protein-1(Keap1), a redox-regulated substrate adaptor protein for the cul3-based E3 ubiquitin ligase complex that regulates ubiquitin-dependent proteasomal degradation of Nrf2 [5]. In response to oxidative stress, degradation of Nrf2 is diminished, followed by releasing Nrf2 into the nucleus where increases the expression of downstream cytoprotective effectors [6–8]. Nrf2 was also suggested to be a tumor suppressor protein involved in the suppression of cancer initiation or progression [9,10]. However, up-regulation of Nrf2 is associated with treatment resistance and poor prognosis in some cancers [11–14]. Thus, Nrf2 is involved not only in cellular defense against oxidative stress-related damage or carcinogen-dependent destruction, but also in malignancy progression and chemoresistance.

The major obstacle to the ultimate success of chemotherapy is

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multidrug resistance (MDR). Mechanistically, the function of MDR is affected by a variety of factors including defective apoptosis pathway, altered targets, increased detoxification and reduced drug accumulation via the upregulation of membrane transporters [15,16]. *P*-glycoprotein (*P*-gp), an ATP-binding cassette (ABC) efflux pump and the main product of the ABCB1 (or MDR1) gene, is an important chemoresistance-related membrane transporter for efflux a wide range of structurally unrelated anticancer drugs [17–19]. Therefore, it is crucial to better understand the molecular mechanisms underlying MDR function to improve the efficacy of chemotherapeutics. A number of genes including ABC transporter gene family, as key modulators of MDR, are regulated by Nrf2-dependent signaling [9,20]. In the blood–brain barrier, upregulation of Nrf2 with sulforaphane, as a Nrf2 activator, can increase protein expression and efflux activity of *P*-glycoprotein in vivo or in vitro [21]. However, the importance of Nrf2-MDR axis in tumor progression and chemoresistance has still to be uncovered in cancer patients. In this study, we investigated the expression levels of Nrf2 and MDR1/*P*-gp in biopsy samples of GC patients in both mRNA and protein levels by real time PCR and immunohistochemistry (IHC), respectively. The association between these two molecules and also clinicopathological characteristics were investigated in these patients. Our results support the hypothesis that inhibition of Nrf2 signaling in GC patients with high Nrf2 expression can improve the effectiveness of chemotherapeutic agents by downregulation of MDR1/*P*-gp.

2. Materials and methods

2.1. Patients and specimens

Participants (n = 120) including sixty patients with gastric adenocarcinoma and sixty non-GC individuals were recruited from Imam Reza and Madani Hospitals, Tabriz, Iran between April 2015 and February 2017. The study was approved by the Ethics Committee of Tabriz University of Medical Sciences (Ethical code: TBZmed.REC.1394.618). All participants received enough explanation about the study and provided written informed consent regarding the use of endoscopic gastric biopsies. Demographic and clinicopathological information of the participants are shown in Table 1. The fresh biopsy samples were collected in liquid nitrogen, then stored at −70 °C until RNA extraction, or fixed in 4% paraformaldehyde for subsequent dehydrating and paraffin embedding. The histological assessment was made for all the cases by two different pathologists, blinded to the clinical data, during their routine diagnostic work.

2.2. Immunohistochemical staining

IHC process was performed to determine protein expression levels of Nrf2 and *P*-gp in formalin-fixed paraffin-embedded (FFPE) blocks of tissues. Briefly, 4 μm sections were cut, mounted on glass slides, dried overnight at 60 °C, de-paraffinized in xylene and rehydrated through graded ethanol series (100%, 95%, 70%, and 50%). Endogenous peroxidase activity was quenched with 3% H2O2 in methanol for 10 min at room temperature. The tissue sections were subjected to antigen retrieval by heating in sodium citrate buffer, pH 6.0, in a microwave oven for 30 min. After washing three times with phosphate-buffered saline (PBS, pH 7.4) for 7 min each time, the tissue slides were incubated with rabbit polyclonal anti-Nrf2 IgG (diluted 1/100; sc-722, Santa Cruz Biotechnology, Inc.) or mouse monoclonal anti *P*-gp antibody (diluted 1/100; sc-390883, Santa Cruz Biotechnology, Inc.) for 45 min in a humidity chamber at room temperature, followed by 45 min incubation with anti-mouse/rabbit secondary antibody conjugated with horseradish peroxidase (HRP) (Dako, EnVision system, Denmark). Following twice washing with PBS solution, 3,3′-diaminobenzidine (DAB) tetrahydrochloride substrate (liquid DAB + substrate Chromogen, Dako, Denmark) was applied for about 5 min to develop the staining color. Sections were then immersed in running tap water, and 0.1% hematoxylin was used for counterstaining. Finally, stained slides were coverslipped with rapid mounting medium (Entellan, Merck, Germany) and analyzed under a light microscope. Negative controls for all immunostainings were obtained by exclusion of the primary antibodies.

2.3. Immunohistochemical analysis

All IHC results were evaluated by two independent investigators, who were unaware of any clinical information from participants. The intensity of staining was graded into 4 categories: no staining = 0, weak = 1, moderate = 2 and strong = 3. In addition, according to the percentage of stained cells, the expression was scored as 1 (< 25% of

Table 1
Association between clinicopathological criteria and protein expression of Nrf2 or *P*-gp in gastric cancer.

Clinical pathological criteria	No. of patients (%)	Nrf2		*P value*	*P*-gp		*P value*
		Positive (%) n = 28	Negative (%) n = 32		Positive (%) n = 18	Negative (%) n = 42	
Age				0.537			0.564
< 65 years	20 (33.3)	9 (45.0)	11 (55.0)		7 (35.0)	13 (65.0)	
≥65 years	40 (66.7)	19 (47.5)	21 (52.5)		11 (27.5)	29 (72.5)	
Gender				0.274			0.370
Male	40 (66.7)	21 (52.5)	19 (47.5)		14 (35.0)	26 (65.0)	
Female	20 (33.3)	7 (35.0)	13 (65.0)		4 (20.0)	16 (80.0)	
Tumor size				**0.001**			**0.004**
< 5 cm	37 (61.7)	11 (29.7)	26 (70.3)		6 (16.2)	31 (83.8)	
≥5 cm	23 (38.3)	17 (73.9)	6 (26.1)		12 (52.2)	11 (47.8)	
Histological grade				**0.011**			**0.049**
Well differentiated	14 (23.3)	5 (35.7)	9 (64.3)		2 (14.3)	12 (85.7)	
Moderately differentiated	36 (60)	14 (38.9)	22 (61.1)		10 (27.8)	26 (72.2)	
Poor differentiated	10 (16.7)	9 (90.0)	1 (10.0)		6 (60.0)	4 (40.0)	
Lymph node metastasis				**0.011**			0.089
Positive	32 (53.3)	20 (62.5)	12 (37.5)		13 (40.6)	19 (59.4)	
Negative	28 (46.7)	8 (28.6)	20 (71.4)		5 (17.9)	23 (82.1)	
Distant metastasis				**0.017**			0.071
Positive	11 (18.3)	9 (88.8)	2 (18.2)		6 (54.5)	5 (45.5)	
Negative	49 (81.7)	19 (38.8)	30 (61.2)		12 (24.5)	37 (75.5)	
Smoking history				0.755			0.181
Current/Former	13 (21.7)	7 (53.8)	6 (46.2)		6 (42.6)	7 (53.8)	
Never	47 (78.3)	21 (44.7)	26 (55.3)		12 (25.5)	35 (74.5)	

Nrf2: Nuclear factor erythroid 2-related factor 2, *P*-gp: *P*-glycoprotein.The bold characters denote that the *P* value is less than 0.05, which has the statistic significance.