Int J Clin Exp Med 2017;10(1):1034-1042
www.ijcem.com /ISSN:1940-5901/IJCEM0039190

Original Article

Up-regulated Nrf2 in colorectal carcinoma and predicts poor prognosis

Pei Huang[1,2,4], Yan He[1,3,4], Jinming Cao[1,3,4], Aijing Dong[1,3,4], Wei Zhu[1,3,4], Xialin Chen[3,7], Han Cao[3], Hongying Qian[2,4], Cuicui Shen[2], Fengmei Cui[1,2], Jihua Nie[4,5,6], Jianping Cao[1,3,4]

[1]School of Radiation Medicine and Protection and Jiangsu Provincial Key Laboratory of Radiation Medicine and Protection, Medical College of Soochow University, Suzhou 215123, P. R. China; [2]Wuxi Second Hospital (Wuxi Municipal Second People's Hospital) Affiliated to Nanjing Medical University, Wuxi 214002, P. R. China; [3]Department of Radiation Genetics, School of Radiation Medicine and Protection, Medical College of Soochow University, Suzhou 215123, P. R. China; [4]Collaborative Innovation Center of Radiation Medicine of Jiangsu Higher Education Institutions and School for Radiological and Interdisciplinary Sciences (RAD-X), Soochow University, Suzhou 215123, P. R. China; [5]School of Public Health, Medical College of Soochow University, Suzhou 215123, P. R. China; [6]Jiangsu Key Laboratory of Preventive and Translational Medicine for Geriatric Diseases, Suzhou 215123, P. R. China; [7]The Department of Radiotherapy (Chen), People's Hospital of Shaoxing, Shaoxing, China

Received May 22, 2016; Accepted October 27, 2016; Epub January 15, 2017; Published January 30, 2017

Abstract: Colorectal cancer (CRC) is the third most common cancer worldwide. In the present study, the mechanism underlying Nrf2 activity in colorectal cancer was investigated. 149 CRC patients with available follow-up and clinical data were included for immunohistochemically studies. We found that the expressions of Nrf2, Prdx6 and SOD2 proteins were higher in CRC tissues than in the adjacent non-neoplastic tissues. Moreover, Nrf2 protein level was found to be significantly correlated with the lymph node status, TNM stage, differentiation state of CRC, as well as protein levels of Ki67, p53, Prdx6 and SOD2. Higher expression of Nrf2 protein also resulted in poorer 5-year overall survival (OS) outcomes, which could potentially be used to predict the prognosis of CRC patients. Our current study implicates Nrf2 in the progression and pathogenesis of CRC.

Keywords: Nrf2, colorectal carcinoma, prognosis

Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related deaths worldwide, only after lung and breast cancers [1]. An epidemiological study showed that the incidence rate of CRC has increased remarkably in China [2]. However, standard treatments, such as imaging, surgical techniques, neoadjuvant chemotherapy and radiotherapy, are ineffective in cases of advanced CRC [3]. It is therefore necessary to discover the molecular mechanisms that drive CRC tumorigenesis and to explore novel and more effective treatments for CRC.

Nuclear factor erythroid 2-related factor 2 (Nrf2), a basic leucine-zipper containing transcription factor that binds to the antioxidant responsive element (ARE), is a major regulator of many anti-oxidative and cytoprotective genes [4]. Anearlier study has shown that mice with downregulated Nrf2 were more prone to tumors when exposed to carcinogens [5]. In addition, uponexposure to different chemicals, such as-dextran sulfate sodium, azoxymethane, N-nitrosobutyl acid and 7, 12-xylene, the incidence of cancer was found to be increased in mice deficient in Nrf2 gene [6-9], which was also accompanied by increased levels of oxidation reactions and DNA damage in cells [10, 11]. Nrf2 also has an impact on carcinogenesis. Up-regulation of Nrf2 was observed in breast cancer, lung cancer, ovarian cancer, head and neck cancer, endometrial cancer, pancreatic cancer, hepatocellular cancer (HCC) and esophageal cancer [12-20], and was also reported to be associated with poor clinical outcomes and low patient survival in HCC, esophageal cancer, ovarian cancer and oral

Table 1. Clinical data for 149 CRC cases

Characteristics	N cases (%)
Sex	
Male	82 (55.0)
Female	67 (45.0)
Age	
≤ 60 years	44 (29.5)
>60 years	105 (70.5)
Tumor location	
Colon	98 (65.8)
Rectum	51 (34.2)
General type	
Uplift type	35 (23.5)
Infiltrating type	12 (8.0)
Ulcer type	102 (68.5)
Differentiation	
Well/Moderate	36 (24.2)
Poor	113 (75.8)
Invasion	
T1-2	30 (20.1)
T3-4	119 (79.9)
Lymphnode metastasis	
N0	95 (63.8)
N1-2	54 (36.2)
TNM stage	
I + II	95 (63.8)
III	54 (36.2)

squamous cell carcinoma [21-24]. These findings indicate that Nrf2 may be a potential target for antibody-based therapy. However, little study was conducted on the role of Nrf2 in CRC. Considering the wide reported functions of Nrf2 in carcinogenesis, it is necessary to further investigate its role in CRC.

Superoxide dismutase manganese (SOD2), an important component of the antioxidant cell protection system, can remove superoxide free radicals from aerobic organisms, and plays an important role in the balance between oxidation and antioxidant [25, 26]. When the organism is exposed to various kinds of harmful stimuli, SOD2 expression increases and results in reduced damage caused by oxidativestress [27, 28]. Peroxiredoxin 6 (Prdx6), a newly discovered antioxidant of theperoxiredoxins (Prdxs) family proteinsin the cytoplasm [29], was reported to reduce levels of the oxidant hydrogen peroxide and other reactive oxygen species [30].

We hypothesize that Nrf2 protein may be involved in the oncogenesis of CRC, and that the protein expression of Nrf2 can be a prognostic factor. To elucidate the prognostic value of Nrf2 protein in CRC, we analyzed the protein expression of Nrf2 with immunohistochemistry and assessed its associations with various clinicopathological parameters and 5-year overall survival (OS) rate outcomes in CRC patients. This study demonstrates a role of Nrf2 protein in CRC, and Nrf2 protein upregulationis correlated with reduced OS in CRC patients.

Materials and instruments

Patients and specimens

For this retrospective study, archival formalin fixed paraffin-embedded (FFPE) specimens from 149 CRC cases were obtained from the Second Hospital of Wuxi (Wuxi, China) from 2010 to 2012. As shown in **Table 1**, cases (82 male and 67 female individuals) with available follow-up and clinical data were included for immunohistochemically studies. Patients who had received chemotherapy or radiation therapy before surgery were excluded. Among the 149 CRC patients selected, 98 tumors were located in the colon, and 51 were in the rectum. Information regarding sex, age, disease stage, and histopathological parameters were retrieved from the patient medical records. The tumors were confirmed as malignant after surgery by pathologists from the Second Hospital of Wuxi. This study was approved by the Ethics Committees of the Second Hospital of Wuxi. Informed consent was provided by all patients. Of the 149 samples, 49 CRC samples had corresponding adjacent normal tissues. All of the tissue samples were routinely fixed in formalin and embedded in paraffin for immunohistochemically examination.

Immunohistochemistry

For immunohistochemistry, 4-μm-thick sections were cut from the FFPE tissue blocks and deparaffinized and rehydrated using xylene and a graded series of ethanol (absolute, 95%, 80%, 50%), followed by two 3-min washes in phosphate buffered saline with Tween-20 (PBST). Antigen (Nrf2, SOD2, Prdx6, P53, Ki67, C-erbB-2, nm23; Cell Signaling Tech., USA) retrieval was performed in 10 mmol sodium

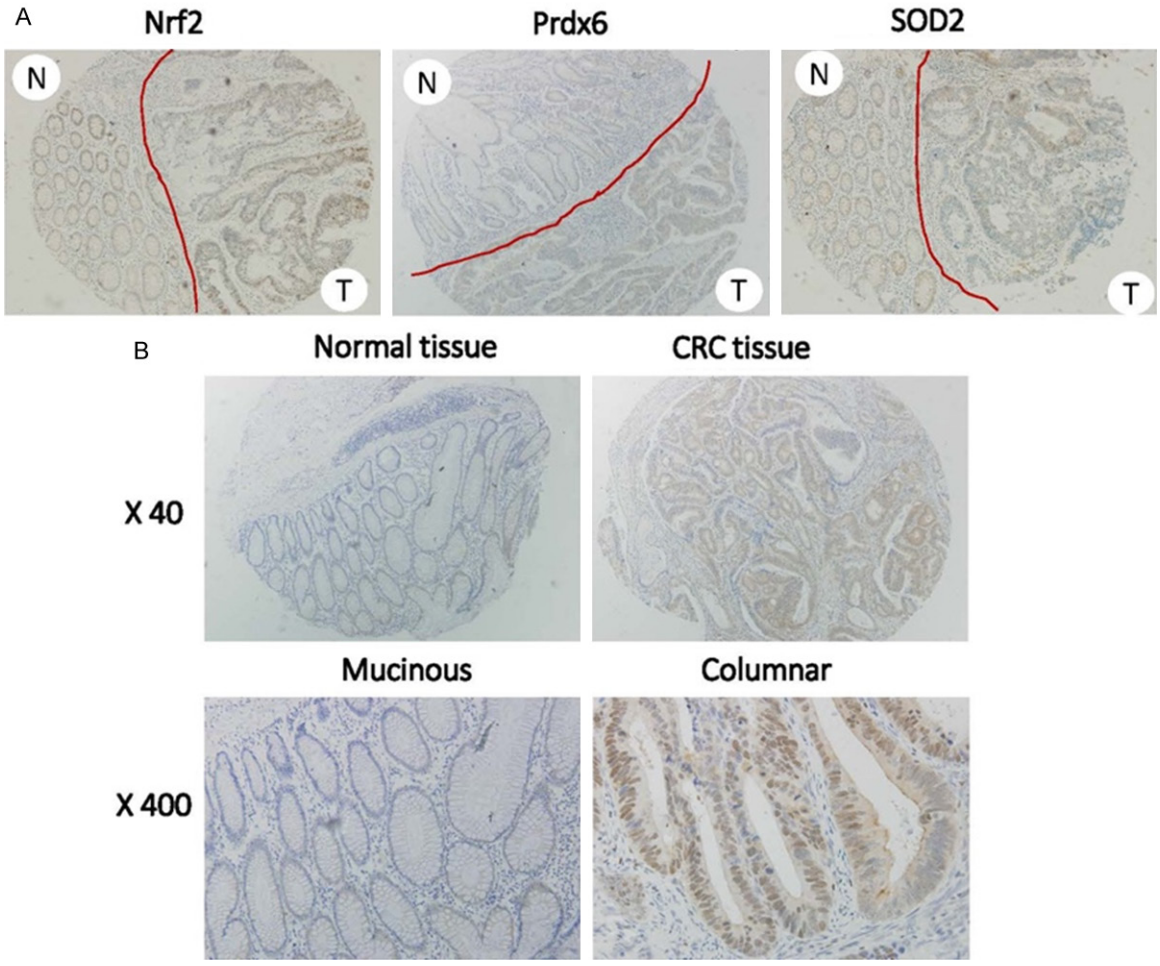

Figure 1. Increased expression of Nrf2, Prdx6 and SOD2 proteins in human colorectal cancer (CRC) and adjacent normal tissues. A. The red lines partitioned the sections into normal tissue region (N) and tumor tissue region (T). B. Cellular distribution of Nrf2 protein in CRC and adjacent normal tissues.

citrate buffer (pH 6.0), which was microwaved at 90-100°C for 20 min and then washed in PBST for 2 washes of 3 min each. The sections were then incubated for 30 min in 3% (v/v) hydrogen peroxide in methanol to block the endogenous peroxidase activity. They were then washed in PBST for 3 washes of 3 min each; blocked at room temperature for 30 min with 2% normal goat serum, 2% bovine serum albumin (BSA), and 0.1% Triton-X in phosphate buffered saline (PBS); and incubated overnight in a humidified chamber at 4°C with the primary antibodies. The sections were then washed in PBST 3 washes of 3 min and incubated for 1 h with secondary antibodies. After washing again with PBST 3 washes of 3 min, the sections were incubated with ready-to-use streptavidin peroxidase at room temperature for 10 min and then rinsed well with distilled water. The staining was developed with a DAB kit. The sections were then counterstained with hematoxylin, followed by dehydration and mounting. Negative controls were prepared by substituting the primary antibodies with PBS.

Immunoreactivity scoring

To evaluate the expression of Nrf2, P53, Ki67, C-erbB-2 and nm23 proteins, a reproducible semi-quantitative method that considers both staining intensity and area scores was adopted. The staining intensity was scored as follows: 1 (weak staining = light yellow), 2 (moderate staining = yellow brown), and 3 (strong staining = brown). The staining area was the percentage of positive tumor cells, which was scored as follows: 0 (no tumor cell stained), 1 (1-25% positive tumor cells), 2 (25-50% positive

Table 2. Protein expression levels of Nrf2, Prdx6 and SOD2 in 149 CRC samples

	Nrf2 expression	Prdx6 expression	SOD2 expression
CRC (149)	1.62 ± 0.86	1.75 ± 0.88	1.87 ± 0.73
Control (49)	0.77 ± 0.75	0.96 ± 0.89	0.94 ± 0.83
t	6.181	5.401	7.426
P	0.000*	0.000*	0.000*

*P<0.05, considered statistically significant.

Table 3. Correlation between Nrf2 protein expression level and clinicopathological parameters

Characteristics	Nrf2 expression		*P* Value
	Low	High	
N	50	99	
Sex			
Male	25	57	
Female	25	42	P = 0.38
Age			
≤60	12	32	
>60	38	67	P = 0.293
Tumor location			
Colon	32	66	
Rectum	18	33	P = 0.746
General type			
Uplift type	12	23	
Infiltrating type	6	6	
Ulcer type	32	70	P = 0.431
Differentiation			
Well/Moderate	6	30	
Poor	44	69	P = 0.014
Invasion			
T1-2	5	25	
T3-4	45	74	P = 0.028
Lymph node metastasis			
N0	40	55	
N1-2	10	44	P = 0.003
TNM stage			
I + II	21	74	
III	29	25	P = 0.000

There were no significant relationship between Nrf2 and the basic clinical parameters (age, sex, tumor and location). Positive correlations between Nrf2 expression and lymph node status, TNM stage, and differentiation were obvious.

tumor cells), 3 (50-75% positive tumor cells), and 4 (75-100% positive tumor cells). The final immunoreactivity score (IS) for each specimen was obtained as the product of the staining intensity and area scores. A score of 0-1 is considered as negative expression (-); 2-3 points, asweak expression, (+); 4-6 points, asmoderate expression, (++); and ≥6, as strong expression (+++). Based on Nrf2 expression the cases were divided into Nrf2 high (IS≥1) and Nrf2 low (IS<1) groups. Each section was assessed independently by two histopathologists, who were blinded to patient information. Positive samples were defined as those showing brown signals in the cytoplasm and/or the nuclei of cancer cells.

Statistical analysis

As the study endpoint, total survival time was considered as the time from surgery until the date of death or last follow-up (August 2015). To assess the correlations of demographic and clinical variables with Nrf2 protein expres-sion, the chi-square test and Fisher's exact test were used for categorical variables and the two-sample t-test was used for continuous variables. The chi-square test was also performed to compare the expression of Nrf2 protein between tumor tissues and adjacent benign tissues. Overall 5-year survival rates of all patientswere estimated by Kaplan-Meier analysis with a log-rank score for determining statistical significance. All *P* values were two-sided. P≤0.05 was considered as statistically significant. All statistical analyses were performed by SPSS 16.0 for Windows (Release 17.0, SPSS Inc.).

Results

Increased Nrf2 protein expression in CRC

Protein expression levels of Nrf2 in CRC were determined by immunohistochemistry (**Figure 1**). We compared the protein expression levels of Nrf2, Prdx6 and SOD2 in 149 CRC tissue samples. Nrf2 immunoreactivity was predominantly localized in the nucleus of the CRC tumor cells [14], although low levels of cytoplasmic-staining were also evident in some cancer cells as illustrated in **Figure 1**. Most adjacent non-neoplastic cells were not stained, and weak staining could be found in some normal counterparts in the non-dysplastic tissues. High and moderate expressions were found in the tumor cells. High expressions of Nrf2 protein was

Table 4. Correlation between Nrf2 protein expression and C-erbB-2, Ki-67, p53, and nm23 protein expressions

Characteristics	Nrf2 protein expression		*P* Value	R Value
	Low	High		
C-erbB-2 (-)	22	53		
C-erbB-2 (+)	28	46	P = 0.272	r = -0.90
Ki-67 (-)	18	18		
Ki-67 (+)	32	81	P = 0.016	r = 0.197
P53 (-)	39	38		
P53 (+)	11	61	P = 0.000	r = 0.374
nm23 (-)	44	94		
nm23 (+)	6	5	P = -0.126	r = 0.125

found in 66.4% (99/149) of CRC tissues. In contrast, Nrf2 protein expression was low in 75.5% (37/49) of the non-dysplastic tissues. The expression levels of Nrf2, Prdx6 and SOD2 proteins in 149 CRC samples were higher than those in the corresponding adjacent normal tissues (P = 0.000). There was a positive correlation between protein levels of Nrf2 and Prdx6 (r = 0.405, P = 0.000), as well as Nrf2 and SOD2 (r = 0.21, P = 0.01) in the CRC tissues (**Table 2**).

Association of protein expression levels of Nrf2 with the clinicopathological parameters of CRC

In order to explore the clinical role of Nrf2 protein in CRC, we assessed the correlations between Nrf2 protein expression levels and various clinicopathological parameters, including sex, age, location, invasion, tumor differentiation, lymphnode status and TNM stage of the CRC patients (**Table 3**). Among 149 CRC cases examined, high Nrf2 protein expression was detected in the carcinoma tissues of 99 cases. Statistical analysis showed that Nrf2 protein expression was not significantly correlated with clinical parameters such as age, sex and tumor location in CRC patients (P>0.05). However, significant correlations were found between Nrf2 protein expression and lymph node status (P = 0.003), TNM stage (P = 0.000) and tumor differentiation (P = 0.014) in CRC patients.

Correlation between Nrf2 protein expression and c-erb-2, Ki-67, P53 and nm23 proteins

As shown in **Table 4**, analyses for correlations between Nrf2 protein and c-erbB-2, Ki67, p53 and nm23 proteins showed positive correlation of Nrf2 protein expression withKi67 (P = 0.016) and p53 (P = 0.000) proteins; however, Nrf2 protein was not correlated with c-erbB-2or nm23 (P>0.05) proteins.

Protein expression level of Nrf2 and prognosis patients with CRC

Protein expression levels of Nrf2 were divided into two groups, Nrf2-low and Nrf2-high. The correlations were evaluated by Kaplan-Meier analysis for 5-year overall survival (OS). The results showed that there was a negative correlation between patient OS and Nrf2 protein expression level, and higher expression of Nrf2 protein resulted in poorer OS (**Figure 2A**). We compared the cases of 99 patients for which complete follow-up information was available. The mean survival time (MST) was 32.2 months and the 5-year survival rate (5-YSR) was 67.7% for the 65 patients with high Nrf2 protein expression; these values were significantly lower than those for the remaining 34 patients with low Nrf2 protein expression (MST>42.7 months, 5-YSR = 79.4%; P<0.001, **Figure 2A**). Furthermore, the results indicated that TNM stage (P = 0.027, P<0.05; **Figure 2C**) and Nrf2 protein (P = 0.029, P<0.05; **Figure 2A**) expression, as well as differentiation grade (P = 0.001, P<0.05; **Figure 2B**), could be used to predict the prognosis of CRC patients.

Discussion

The physiological and pathological functions of Nrf2 protein are highly similar to an oncogene, due to its ability to upregulate the expressions of enzymes and antioxidant proteins, which in turn changes the redox state and maintains the balance of cells [31]. Results in our current study showed that Nrf2 protein expression exhibited a positive correlation with Prdx6and SOD2 proteins in CRC tissues. Recent study has revealed that Nrf2 function was regulated by Kelch-like ECH-associatedprotein I (Keap1) [32]. Under normal circumstances, the combination of Keap1 and Nrf2 play a negative regulatory role, resulting in the degradation of Nrf2 protein. In the case of oxidative stress and the presence of electrophilic reagents, Nrf2 cannot be degraded, because Keap1, which is rich in cysteine, cannot be ubiquitinated. Moreover, Nrf2 protein is enriched in the nucleus and binds to the antioxidant response element

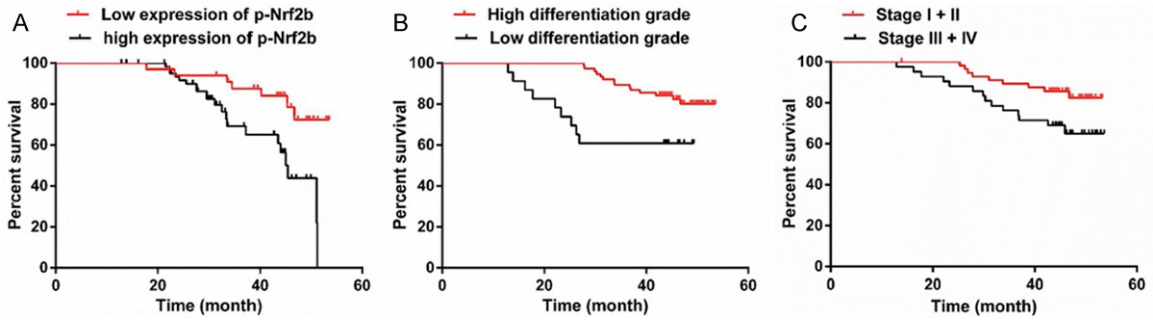

Figure 2. Survival curves of patients. A. 5-year overall survival curves of patients with CRC, subdivided according to Nrf2 expression. Group 1 = Nrf2 low expression, n = 34; Group 2 = Nrf2 high expression, n = 65; P = 0.029. B. 5-year overall survival curves of patients with CRC, subdivided according to differentiation. Group 1 = differentiation well/moderate, n = 68; Group 2 = differentiation poor, n = 31; P = 0.001). C. 5-year overall survival curves of patients with CRC, subdivided according to TNM stage. Group 1 = TNM I + II, n = 58; Group 2 = TNM III, n = 41; P = 0.027).

(ARE), which activates the expression of a series of antioxidant genes and two phase detoxification enzymes to protect the cell from DNA damage [33, 34].

The TNM stage and the lymph node status are two widely used prognostic indexes for CRC in the clinic. Poorly differentiated cancer cells of CRC are often aggressive and highly metastatic. A recent study has shown that the Nrf2 protein expression level was correlated with tumor stage and lymph node metastasis [35]. E-cadherin may limit the localization and transcriptional activity of Nrf2 protein in the nucleus, and in the absence of activated Nrf2 protein, tumor growth and metastasis increase [22]. Considering the association observed between Nrf2 protein expression and the clinicopathological characteristics in our study, Nrf2 protein could be used as a potential factor to predict tumor progression and poor prognosis in CRC.

Ki67 and p53 protein are two commonly used clinical indicators of cancer. Ki67, a non-histone protein, is a nuclear antigen related to cell proliferation, and is currently the most used positive marker of nuclear proliferation [36, 37]. Recent studies have shown that Ki67 was abnormally expressed in many tumors and precancerous lesions, and was positively associated with the degree of tumor malignancy [38-41]. The mutation and/or deletion of p53 are considered to be the major cause of many tumors. Mutant p53 not only promotes the growth of cancer cells, but also enhances the invasion capacity of tumor cells and metastasis [42]. In this study, we found that the expression of Nrf2 protein exhibited a positive correlation with Ki67 and p53 proteins, which suggested that Nrf2 protein may be involved in the origin and development of CRC tumor, but the specific mechanism needs to be further elucidated.

Although the Keap1-Nrf2 signaling pathway enhances the tolerance of tissue cells to carcinogenic factors, abnormalities in the activation of the Nrf2 signaling pathway may play a role in promoting the process of carcinogenesis. Homma et al. [43] confirmed that the tumor cell proliferation rate was significantly slowed when the expression of Nrf2 was repressed with interference techniques, and they also found that cells were arrested in G1 phase when the expression of Nrf2 was completely eliminated. Another study has discovered mutations in Keapl in tumor cells, which affected the negative regulation on Nrf2 and led to its overexpression and sustained activation [44]. In our study, the expression of Nrf2 protein in CRC was higher than that in the corresponding adjacent normal tissues (P = 0.001). Most adjacent non-neoplastic cells were not stained, although weak stainings could be found in some normal counterparts of non-dysplastic tissues. In contrast, high and moderate stainings of Nrf2 protein were observed in the tumor cells of CRC.

Furthermore, early report has demonstrated that Nrf2 expression correlated with the survival of patients with malignant diseases, indicating that Nrf2 could facilitate the proliferation of tumor cells and increase their resistance

to chemical drugs and radiation [45]. Mitsuishi et al. found that Nrf2 played an important role in the proliferation of A549 lung cancer cells [46]. Studies have shown that cancer cells overexpressing Nrf2 were less sensitive to common chemotherapeutic drugs, such as etoposide, cisplatin, 5-fluorouracil (FU) and adriamycin (ADM), than those with low Nrf2 expression [12-43], and Nrf2 overexpression may also protect the cancer cells against ionizing radiation [48]. Yang et al. [49] found that patients with high Nrf2 positive stainings (75%-100%) were less sensitive to chemotherapy than those with low Nrf2, indicating that high Nrf2 expression may predict poor prognosis.

In our study, Kaplan-Meier analysis showed that patient survival time was negatively correlated with the Nrf2 protein expression level, and higher Nrf2 protein expression was correlated with shorter survival time in CRC patients. The observed correlation between high Nrf2 protein expression and poor prognosis in CRC patients in our current study is consistent with the abovementioned studies. Thus, Nrf2 protein expression may be a clinically relevant prognostic marker in various malignancies, including CRC.

Acknowledgements

This research was supported by key scientific development program of China (2016YFC09-04702), the National Science Foundation of China (81472917, 81573080, and 81402626), and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), Jiangsu Key Laboratory of Preventive and Translation Medicine for Genetic Diseases, China Postdoctoral Science Foundation Grant (2015M571811), Jiangsu Province Postdoctoral Science Foundation Grant (1402175C).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jianping Cao, School of Radiation Medicine, Medical College of Soochow University, Renai Road 199, Suzhou 215123, PR China. Tel: +086-512-65880037; Fax: +086-512-65884830; E-mail: jpcao@suda.edu.cn; Dr. Jihua Nie, School of Public Health, Medical College of Soochow University, Renai Road 199, Suzhou 215123, PR China. Tel: +086-512-65880073; Fax: +086-512-65880036; E-mail: niejihua@suda.edu.cn

References

[1] Gansler T, Ganz PA, Grant M, Greene FL, Johnstone P, Mahoney M, Newman LA, Oh WK, Thomas CR Jr, Thun MJ, Vickers AJ, Wender RC, Brawley OW. Sixty years of CA: a cancer journal for clinicians. CA Cancer J Clin 2010; 60: 345-350.

[2] Li M, Ma Y, Huang P, Du A, Yang X, Zhang S, Xing C, Liu F and Cao J. Lentiviral DDX46 knockdown inhibits growth and induces apoptosis in human colorectal cancer cells. Gene 2015; 560: 237-244.

[3] Yu JL, May L, Lhotak V, Shahrzad S, Shirasawa S, Weitz JI, Coomber BL, Mackman N and Rak JW. Oncogenic events regulate tissue factor expression in colorectal cancer cells: implications for tumor progression and angiogenesis. Blood 2005; 105: 1734-1741.

[4] Hudson JI, Hiripi E, Pope HG Jr, Kessler RC. The prevalence and correlates of eating disorders in the National Comorbidity Survey Replication. Biol Psychiatry 2007; 61: 348-358.

[5] Ramos-Gomez M, Kwak MK, Dolan PM, Itoh K, Yamamoto M, Talalay P and Kensler TW. Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. Proc Natl Acad Sci U S A 2001; 98: 3410-3415.

[6] Osburn WO, Karim B, Dolan PM, Liu G, Yamamoto M, Huso DL and Kensler TW. Increased colonic inflammatory injury and formation of aberrant crypt foci in Nrf2-deficient mice upon dextran sulfate treatment. Int J Cancer 2007; 121: 1883-1891.

[7] Khor TO, Huang MT, Prawan A, Liu Y, Hao X, Yu S, Cheung WK, Chan JY, Reddy BS, Yang CS and Kong AN. Increased susceptibility of Nrf2 knockout mice to colitis-associated colorectal cancer. Cancer Prev Res (Phila) 2008; 1: 187-191.

[8] Fahey JW, Haristoy X, Dolan PM, Kensler TW, Scholtus I, Stephenson KK, Talalay P and Lozniewski A. Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of Helicobacter pylori and prevents benzo-[a]pyrene-induced stomach tumors. Proc Natl Acad Sci U S A 2002; 99: 7610-7615.

[9] Xu C, Huang MT, Shen G, Yuan X, Lin W, Khor TO, Conney AH and Kong AN. Inhibition of 7,12-dimethylbenz(a)anthracene-induced skin tumorigenesis in C57BL/6 mice by sulforaphane is mediated by nuclear factor E2-related factor 2. Cancer Res 2006; 66: 8293-8296.

[10] Hirayama A, Yoh K, Nagase S, Ueda A, Itoh K, Morito N, Hirayama K, Takahashi S, Yamamoto

M and Koyama A. EPR imaging of reducing activity in Nrf2 transcriptional factor-deficient mice. Free Radic Biol Med 2003; 34: 1236-1242.

[11] Morito N, Yoh K, Itoh K, Hirayama A, Koyama A, Yamamoto M and Takahashi S. Nrf2 regulates the sensitivity of death receptor signals by affecting intracellular glutathione levels. Oncogene 2003; 22: 9275-9281.

[12] Lister A, Nedjadi T, Kitteringham NR, Campbell F, Costello E, Lloyd B, Copple IM, Williams S, Owen A, Neoptolemos JP, Goldring CE and Park BK. Nrf2 is overexpressed in pancreatic cancer: implications for cell proliferation and therapy. Mol Cancer 2011; 10: 37.

[13] Wang XJ, Sun Z, Villeneuve NF, Zhang S, Zhao F, Li Y, Chen W, Yi X, Zheng W, Wondrak GT, Wong PK and Zhang DD. Nrf2 enhances resistance of cancer cells to chemotherapeutic drugs, the dark side of Nrf2. Carcinogenesis 2008; 29: 1235-1243.

[14] Singh A, Misra V, Thimmulappa RK, Lee H, Ames S, Hoque MO, Herman JG, Baylin SB, Sidransky D, Gabrielson E, Brock MV and Biswal S. Dysfunctional KEAP1–NRF2 Interaction in Non-Small-Cell Lung Cancer. PLoS Med 2006; 3: e420.

[15] Shibata T, Ohta T, Tong KI, Kokubu A, Odogawa R, Tsuta K, Asamura H, Yamamoto M and Hirohashi S. Cancer related mutations in NRF2 impair its recognition by Keap1-Cul3 E3 ligase and promote malignancy. Proc Natl Acad Sci U S A 2008; 105: 13568-13573.

[16] Jiang T, Chen N, Zhao F, Wang XJ, Kong B, Zheng W and Zhang DD. High levels of Nrf2 determine chemoresistance in type II endometrial cancer. Cancer Res 2010; 70: 5486-5496.

[17] Zhang P, Singh A, Yegnasubramanian S, Esopi D, Kombairaju P, Bodas M, Wu H, Bova SG and Biswal S. Loss of Kelch-like ECH-associated protein 1 function in prostate cancer cells causes chemoresistance and radioresistance and promotes tumor growth. Mol Cancer Ther 2010; 9: 336-346.

[18] Shibata T, Kokubu A, Saito S, Narisawa-Saito M, Sasaki H, Aoyagi K, Yoshimatsu Y, Tachimori Y, Kushima R, Kiyono T and Yamamoto M. NRF2 mutation confers malignant potential and resistance to chemoradiation therapy in advanced esophageal squamous cancer. Neoplasia 2011; 13: 864-873.

[19] Shibata T, Kokubu A, Gotoh M, Ojima H, Ohta T, Yamamoto M and Hirohashi S. Genetic alteration of Keap1 confers constitutive Nrf2 activation and resistance to chemotherapy in gallbladder cancer. Gastroenterology 2008; 135: 1358-1368, 1368, e1351-1354.

[20] Solis LM, Behrens C, Dong W, Suraokar M, Ozburn NC, Moran CA, Corvalan AH, Biswal S, Swisher SG, Bekele BN, Minna JD, Stewart DJ and Wistuba II. Nrf2 and Keap1 abnormalities in non-small cell lung carcinoma and association with clinicopathologic features. Clin Cancer Res 2010; 16: 3743-3753.

[21] Sasaki H, Suzuki A, Shitara M, Hikosaka Y, Okuda K, Moriyama S, Yano M and Fujii Y. Genotype analysis of the NRF2 gene mutation in lung cancer. Int J Mol Med 2013; 31: 1135-1138.

[22] Nioi P, Nguyen T. A mutation of Keap1 found in breast cancer impairs its ability to repress Nrf2 activity. Biochem Biophys Res Commun 2007; 362: 816-821.

[23] Frohlich DA, McCabe MT, Arnold RS, Day ML. The role of Nrf2 in increased reactive oxygen species and DNA damage in prostate tumorigenesis. Oncogene 2008; 27: 4353-4362.

[24] Zhou S, Ye W, Zhang M, Liang J. The effects of nrf2 on tumor angiogenesis: a review of the possible mechanisms of action. Crit Rev Eukaryot Gene Expr 2012; 22: 149-160.

[25] Wallace DC, Fan W. The pathophysiology of mitochondrial disease as modeled in the mouse. Genes Dev 2009; 23: 1714-1736.

[26] Miao L, St Clair DK. Regulation of superoxide dismutase genes: implications in disease. Free Radic Biol Med 2009; 47: 344-356.

[27] Ramachandran A, Lebofsky M, Weinman SA, Jaeschke H. The impact of partial manganese superoxide dismutase (SOD2)-deficiency on mitochondrial oxidant stress, DNA fragmentation and liver injury during acetaminophen hepatotoxicity. Toxicol Appl Pharmacol 2011; 251: 226-233.

[28] Jang YC, Van Remmen H. The mitochondrial theory of aging: insight from transgenic and knockout mouse models. Exp Gerontol 2009; 44: 256-260.

[29] McCarron RM, Shohami E, Panikashvili D, Chen Y, Golech S, Strasser A, Mechoulam R and Spatz M. Antioxidant properties of the vasoactive endocannabinoid, 2-arachidonoyl glycerol (2-AG). Acta Neurochir Suppl 2003; 86: 271-275.

[30] Zaccagnino P, D'Oria S, Romano LL, Di Venere A, Sardanelli AM, Lorusso M. The endocannabinoid 2-arachidonoylglicerol decreases calcium induced cytochrome c release from liver mitochondria. J Bioenerg Biomembr 2012; 44: 273-280.

[31] Lau A, Villeneuve NF, Sun Z, Wong PK, Zhang DD. Dual roles of Nrf2 in cancer. Pharmacol Res 2008; 58: 262-270.

[32] Kaspar JW, Niture SK, Jaiswal AK. Nrf2:INrf2 (Keap1) signaling in oxidative stress. Free Radic Biol Med 2009; 47: 1304-1309.

[33] Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD and Yamamoto M. Keap1 re-

presses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev 1999; 13: 76-86.
[34] Kobayashi A, Kang MI, Okawa H, Ohtsuji M, Zenke Y, Chiba T, Igarashi K and Yamamoto M. Oxidative Stress Sensor Keap1 Functions as an Adaptor for Cul3-Based E3 Ligase To Regulate Proteasomal Degradation of Nrf2. Mol Cell Biol 2004; 24: 7130-7139.
[35] Bao J, Li J, Li D, Li Z. Correlation between expression of NF-E2-related factor 2 and progression of gastric cancer. Int J Clin Exp Med 2015; 8: 13235-13242.
[36] Hall PA, Levison DA, Woods AL, Yu CC, Kellock DB, Watkins JA, Barnes DM, Gillett CE, Camplejohn R, Dover R, et al. Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: An index of cell proliferation with evidence of deregulated expression in some, neoplasms. J Pathol 1990; 162: 285-294.
[37] Fujimori Y, Fujimori T, Imura J, Sugai T, Yao T, Wada R, Ajioka Y and Ohkura Y. An assessment of the diagnostic criteria for sessile serrated adenoma/polyps: SSA/Ps using image processing software analysis for Ki67 immunohistochemistry. Diagn Pathol 2012; 7: 807-816.
[38] Kobayashi T, Iwaya K, Moriya T, Yamasaki T, Tsuda H, Yamamoto J and Matsubara O. A simple immunohistochemical panel comprising 2 conventional markers, Ki67 and p53, is a powerful tool for predicting patient outcome in luminal-type breast cancer. BMC Clin Pathol 2013; 13: 5.
[39] Ikenaga M, Takano Y, Ohtani Y, Tsukamoto H, Hiki Y, Kakita A and Okayasu I. Low levels of apoptosis and proliferative activity in colorectal villous tumors: comparison with tubular tumors. Pathol Int 1998; 48: 453-459.
[40] Saleh HA, Jackson H, Banerjee M. Immunohistochemical expression of bcl-2 and p53 oncoproteins: correlation with Ki67 proliferation index and prognostic histopathologic parameters in colorectal neoplasia. Appl Immunohistochem Mol Morphol 2000; 8: 175-182.
[41] Triest BV, Pinedo HM, Blaauwgeers JL, Diest PJ, Schoenmakers PS, Voorn DA, Smid K, Hoekman K, Hoitsma HF and Peters GJ. Prognostic Role of Thymidylate Synthase, Thymidine Phosphorylase/Platelet-derived Endothelial Cell Growth Factor, and Proliferation Markers in Colorectal Cancer. Clin Cancer Res 2000; 6: 1063-1072.
[42] Montero S, Guzman C, Vargas C, Ballestin C, Cortes-Funes H, Colomer R. Prognostic value of cytosolic p53 protein in breast cancer. Tumour Biol 2001; 22: 337-344.
[43] Homma S, Ishii Y, Morishima Y, Yamadori T, Matsuno Y, Haraguchi N, Kikuchi N, Satoh H, Sakamoto T, Hizawa N, Itoh K and Yamamoto M. Nrf2 enhances cell proliferation and resistance to anticancer drugs in human lung cancer. Clin Cancer Res 2009; 15: 3423-3432.
[44] Komatsu M, Kurokawa H, Waguri S, Taguchi K, Kobayashi A, Ichimura Y, Sou YS, Ueno I, Sakamoto A, Tong KI, Kim M, Nishito Y, Iemura S, Natsume T, Ueno T, Kominami E, Motohashi H, Tanaka K and Yamamoto M. The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. Nat Cell Biol 2010; 12: 213-223.
[45] Kim WD, Kim YW, Cho IJ, Lee CH, Kim SG. E-cadherin inhibits nuclear accumulation of Nrf2: implications for chemoresistance of cancer cells. J Cell Sci 2012; 125: 1284-1295.
[46] Mitsuishi Y, Motohashi H, Yamamoto M. The Keap1-Nrf2 system in cancers: stress response and anabolic metabolism. Front Oncol 2012; 2: 200.
[47] Ohta T, Iijima K, Miyamoto M, Nakahara I, Tanaka H, Ohtsuji M, Suzuki T, Kobayashi A, Yokota J, Sakiyama T, Shibata T, Yamamoto M and Hirohashi S. Loss of Keap1 function activates Nrf2 and provides advantages for lung cancer cell growth. Cancer Res 2008; 68: 1303-1309.
[48] Singh A, Bodas M, Wakabayashi N, Bunz F, Biswal S. Gain of Nrf2 function in non-small-cell lung cancer cells confers radioresistance. Antioxid Redox Signal 2010; 13: 1627-1637.
[49] Yang H, Wang W, Zhang Y, Zhao J, Lin E, Gao J and He J. The role of NF-E2-related factor 2 in predicting chemoresistance and prognosis in advanced non-small-cell lung cancer. Clin Lung Cancer 2011; 12: 166-171.