Original Article
Up-regulated Nrf2 in colorectal carcinoma and predicts poor prognosis

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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]. A previous study has shown that mice with downregulated Nrf2 were more prone to tumors when exposed to carcinogens [5]. In addition, upon exposure 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...
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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 up-regulation is 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
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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 tumor cells), 3 (50-75% positive tumor cells), 4 (75-100% positive tumor cells).

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

<table>
<thead>
<tr>
<th></th>
<th>Nrf2 expression</th>
<th>Prdx6 expression</th>
<th>SOD2 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRC (149)</td>
<td>1.62 ± 0.86</td>
<td>1.75 ± 0.88</td>
<td>1.87 ± 0.73</td>
</tr>
<tr>
<td>Control (49)</td>
<td>0.77 ± 0.75</td>
<td>0.96 ± 0.89</td>
<td>0.94 ± 0.83</td>
</tr>
<tr>
<td>t</td>
<td>6.181</td>
<td>5.401</td>
<td>7.426</td>
</tr>
<tr>
<td>P</td>
<td>0.000*</td>
<td>0.000*</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

*P<0.05, considered statistically significant.

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

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Nrf2 expression</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>N</td>
<td>50</td>
<td>99</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>25</td>
<td>57</td>
</tr>
<tr>
<td>Female</td>
<td>25</td>
<td>42</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤60</td>
<td>12</td>
<td>32</td>
</tr>
<tr>
<td>&gt;60</td>
<td>38</td>
<td>67</td>
</tr>
<tr>
<td>Tumor location</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>32</td>
<td>66</td>
</tr>
<tr>
<td>Rectum</td>
<td>18</td>
<td>33</td>
</tr>
<tr>
<td>General type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uplift type</td>
<td>12</td>
<td>23</td>
</tr>
<tr>
<td>Infiltrating type</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Ulcer type</td>
<td>32</td>
<td>70</td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well/Moderate</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td>Poor</td>
<td>44</td>
<td>69</td>
</tr>
<tr>
<td>Invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1-2</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>T3-4</td>
<td>45</td>
<td>74</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>40</td>
<td>55</td>
</tr>
<tr>
<td>N1-2</td>
<td>10</td>
<td>44</td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I + II</td>
<td>21</td>
<td>74</td>
</tr>
<tr>
<td>III</td>
<td>29</td>
<td>25</td>
</tr>
</tbody>
</table>

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.

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 expression, 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 patients were 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...
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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), TMN stage (P = 0.000) and tumor differentiation (P = 0.014) in CRC patients.

Correlation between Nrf2 protein expression and c-erbB-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 with Ki67 (P = 0.016) and p53 (P = 0.000) proteins; however, Nrf2 protein was not correlated with c-erbB-2 or 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 Prdx6 and SOD2 proteins in CRC tissues. Recent study has revealed that Nrf2 function was regulated by Kelch-like ECH-associated protein 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.
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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...
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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.

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Disclosure of conflict of interest

None.

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