Original Article

Correlation between expression of NF-E2-related factor 2 and progression of gastric cancer

Jie Bao, Jiansheng Li, Dongying Li, Zhenjie Li

Department of Gastroenterology, The First Affiliated Hospital, Zhengzhou University, Zhengzhou 450052

Received May 23, 2015; Accepted July 11, 2015; Epub August 15, 2015; Published August 30, 2015

Abstract: Objective: Nuclear factor E2-related factor 2 (Nrf2) plays a part in antioxidant and phase II detoxification enzymes in cells by the up regulation of many antioxidant response elements (ARE) related gene transcription. Nrf2 not only protect the normal cells, but also can protect cancer cells from the effect of cell stress, which is helpful to the survival of cancer cell. Some studies show that the expression of Nrf2 has important clinical significance in cancer patients, but the analysis of gastrointestinal tumor Nrf2 comprehensive expression has not been reported. The aim of this study is to evaluate the expression of Nrf2 in gastric cancer by immunohistochemistry and analyze its related clinical significance. Methods: 180 cases of gastric cancer patients receive the gastrectomy and lymphade-nectomy, and the resection of tissue is expressesed in paraffin embedded sections by immunohistochemical analysis of Nrf2. And the difference between groups use χ² (chi-square criterion) test, and will be analyzed by Fisher’s exact test and Mann-Whitney U test. Use univariate and multivariate analysis, Kaplan-Meier curve and log-rank to test and evaluate the correlation between the expression of Nrf2 and the clinical pathological features. Results: The immune reaction of Nrf2 is mainly found in gastric cancer cell nucleus, which positive expression is closely related to the tumor size, depth of invasion, lymph node metastasis, lymphatic invasion and histological analysis (all \( P < 0.05 \)). The log-rank test shows that the survival rate of Nrf2 positive expression group is significantly lower than that of the negative expression group (\( P < 0.01 \)). The Nrf2 positive expression is closely related to the drug resistance of adjuvant chemotherapy on the basis of 5FU (\( P = 0.022 \)). Conclusion: There is a positive correlation between the expression of Nrf2 and the invasion of gastric cancer, which can be used as a potential indicator of patients’ poor prognosis.

Keywords: Nuclear factor E2-related factor 2 (Nrf2), gastric cancer, antioxidant

Introduction

Gastric cancer is the fourth most common gastrointestinal cancer, but its mortality is the second in all gastrointestinal tumors. Asian countries are the high incidence area of gastric cancer. Early diagnosis, radical operation and endoscopic treatment are the key to improve the prognosis of patients [1, 2]. However, some patients are even treated with radical resection, the late recovery is still not ideal, which has closely relationship with TNM factors [3, 4]. Besides clinical pathological factors, the prognosis of gastric cancer is also influenced by the biological variables, so it’s necessary to search for the new cancer related marker factors to the usage of the diagnosis and treatment of gastric cancer. The widely accepted understanding is that oxidative stress (OS) participates in the physiological and pathological process of degenerative disease. The production and increase of OS and the damage of DNA are all related to the damage and malignant transformation of gastric mucosa. Therefore, OS plays an important role in the cancerous process of gastric cancer [5, 6]. Nuclear factor E2-related factor 2 (Nrf2), is the basic of the redox sensitive bZIP transcription factor, which plays a part in antioxidant and phase II detoxification enzymes in cells by the up regulation of many antioxidant response elements (ARE) related gene transcription. Nrf2 binds to Kelch-like ECH-associated protein 1 (KEAP1). And the type cul3 ubiquitin ligase E3 regulates Nrf2 ubiquitin and protease dependent degradation [7-10]. When cells exist in oxidative stress or the environment of chemical substances, Nrf2 transfer to the nucleus, form heterologous dimerization of two and Maf partner, bind to ARE sequence position of DNA and activate the
Correlation between expression of Nrf2 and progression of gastric cancer

transcription of downstream gene such as antioxidant and phase II detoxifying enzyme [11-13]. The significance of Nrf2 is that it can play a protective role in many diseases and pathological changes in human, including cancer, neurodegenerative diseases, cardiovascular disease, inflammation, pulmonary fibrosis, acute non-injury and etc, and it is also associated with delaying senility [14-18]. So that Nrf2 is regarded as the “useful” transcription factors which can protect the body from oxidative stress injury [19]. But some studies also report that there exist abnormal activation of Nrf2 pathway in cancer cells and tumor tissues [20]. Nrf2 not only prevent normal cells turn into cancer cells, but also protect cancer cells from the effect of cell stress at the same time which can promote the survival of cancer cells, so it’s related to the growth and invasiveness of tumor. At present, there is no overall analysis on the expression of Nrf2 in gastric cancer cell nucleus, which has a correlation with the clinical prognosis in patients. Therefore, the aim of this study is to develop the preliminary exploration on it, and further elaborate the important significance of oxidative stress related Nrf2 expression on clinical practice.

Materials and methods

Western blot test the expression of Nrf2 in gastric cancer cell lines

Gastric cancer cell lines which include MKN74, MNK45, KATOIII and NUGC4 are purchased by the Chinese academy of sciences cell institute. Cell incubation liquid which contains RPMI 1640, 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin, which is incubated in 37°C environment, and then cells obtained by centrifugation. The total proteins are extracted by lysates after the phosphate buffer solution (PBS) has been washed. Prepare the nuclear extracts and cytoplasmic groups of Nrf2 Western blotting analysis by using the nuclear/cytoplasmic component kit (K266-25, BioVison, California, USA). Denaturation of the protein from the nucleus or cytoplasm transfers to the hybrid membrane after being separated on SDS polyacrylamide gel, and then close overnight in 5% skim milk Tris buffer solution (TBS). The membrane is incubated overnight (sc-365949, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) by anti Nrf2 mouse monoclonal antibody which is in the concentration of 1:500. TBS-Tween 20 (TBST) conjugates horseradish peroxidase (HAF007, RD Syestems, Minneapolis, USA) by anti mouse IgG when the membrane has been rinsed, and then incubate for 15 minutes. Use ECL-Plus detection reagent to show the immunoreactive bands in X ray film, and distinguish nuclear and cytoplasmic components at the same time. When the membranes has been washed by WB solution for 15 minutes, the cell nucleus and cytoplasmic component will be marked and detected again by anti Lamin B1 antibody (ab16048, Abcam, Cambridge, USA, 1:1000) and anti α tubulin antibody (CP06, Calbiochem, MA, USA, 1:1000).

Patient and specimen

The study includes 180 cases of gastric adenocarcinoma patients in total, and the tumors of them have invaded the submucosa tissues over. All of patients have received the subtotal gastrectomy and lymphadenectomy from January 2006 to December 2012. There are 59 cases of patients have received the distal gastrectomy, 17 cases of patients have received proximal gastrectomy, 99 cases of patients have received total gastrectomy and 5 cases of patients have received partial gastrectomy in 180 cases of patients. The male to female ratio is 121:59, and the average age is 65 years old (33-82 years old). The cases of I-III staging of gastric cancer are 50 cases, 36 cases and 94
Correlation between expression of Nrf2 and progression of gastric cancer

Histopathologic classification is based on tumor lymph node metastasis classification of seventh edition that 75 cases are differentiated type (papillary, high differentiation and moderately differentiated tubular adenocarcinoma) and other 105 cases are undifferentiated type (low differentiated adenocarcinoma, mucinous adenocarcinoma and signet ring cell carcinoma). All of patients have not received chemotherapy before the operation, at the same time the related contents of the research have been informed consent by the patients and their family and approved by our hospital ethics committee.

**Immunohistochemical analysis of Nrf2 in gastric cancer**

Tumor specimen will be embedded in paraffin sections when it has been fixed in PBS with 10% formalin (the thickness is 4 um), which is used for immunohistochemical analysis by fixed on the glass slides. Use xylene to make formalin chemical treatment for sections, at the same time the sections are dewatered by a series of concentration gradient ethanol. Endogenous peroxidase activity of sections is destroyed when dipping in 3% catalase-methanol solution at room temperature for 10 minutes. After being washed three times by PBS (5 minutes each time), sections are processed by 1% bovine serum at room temperature for 30 minutes to block the nonspecific reaction. The sections which are stained by anti Nrf2 antibody should be preprocessed by using citrate buffer solution in advance to get antigenicity at 121°C for 10 minutes. The sections are closed for 10 minutes at room temperature with 3% skim milk powder PBS kit after washed three times by PBS (5 minutes each time). Then sections are incubated overnight at 4°C in the PBS containing anti Nrf2 antibody (sc-365949, Santa Cruz Biotechnology, Inc., 1:200). Use anti biotin-peroxidase kit with streptavidin (Nichirei, Tokyo, Japan) to stain the sections next. The incubation of sections and diaminobenzidine tetrahydrochloride will achieve visualization of immune complexes when the sections have been washed three times by PBS. And then, after washing sections, use hematoxylin to double stain and fix the sections. Normal placental tissue regard as a positive control of Nrf2. The expression of Nrf2 is evaluated by the number of the nuclei of the tumor cells which are marked by anti Nrf2 antibody, and all sections are evaluated by two independent observers (who don’t know the clinical data and prognosis). Choose 10 representative regions in the tumor and evaluate the expressions of 100 cells by high power microscope (×400). We will choose the undifferentiated region to evaluate if a section exist differentiation and undifferentiated regions at the same time even the area is smaller than the differentiation area. The average labeling index of Nrf2 is evaluated by the number of positive cells within each region. The expression of Nrf2 is based on the ratio of positive cells and the staining intensity, and the latter distinguishes it by three grades: 0, 1+, 2+, which is evaluated by the degree of reaction according to Solis and other method in the form of a percentage. The score of immunohistochemistry is obtained by the product of the staining intensity and the degree of reaction (0-200). Because the median value of Nrf2 expression in gastric cancer is 110 in this study, the cut-off value is 100.

**Correlation between the expression of Nrf2 and chemical resistance in gastric cancer**

In order to evaluate whether the expression of Nrf2 has influence on chemical resistance in gastric cancer, we compare the levels of Nrf2 expression of the patients with gastric cancer who have received 5-fluorouracil (5-FU) chemotherapy. “5-FU resistance” is defined that patients’ tumor recurrence after they received adjuvant chemotherapy, at the same time only the patients who are in II and III staging of gastric cancer receive chemotherapy, and the patients who can’t receive chemotherapy because of side reaction are ruled out, so final-
Correlation between expression of Nrf2 and progression of gastric cancer

Data analysis

All data record and analysis are performed by using SPSS 16 software, and use χ² (chi-square criterion) test to examine the differences between groups, and will be analyzed by Fisher’s exact test and Mann-Whitney U test. Use univariate and multivariate analysis, Kaplan-Meier curve and log-rank to test and evaluate the correlation between the expression of Nrf2 and the clinical and pathological features. If P<0.05, the differences have statistical significance.

Results

Expression of Nrf2 in gastric cancer cell lines

Western blotting analysis was performed in order to detect the protein level of Nrf2. The result showed that Nrf2 is mainly expressed in the nucleus, and only a small amount of expression in the cytoplasm (Figure 1).

Correlation between the immunohistochemical expression of Nrf2 and the clinical pathological features

Use immunohistochemistry to determine the expression of Nrf2 for 180 cases of paraffin sections of gastric cancer. Table 1 shows the
Correlation between expression of Nrf2 and progression of gastric cancer

Table 2. Correlation between the expression of Nrf2 and clinical pathological factors

<table>
<thead>
<tr>
<th>Clinical factors</th>
<th>Expression of Nrf2</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative group</td>
<td>Positive group</td>
</tr>
<tr>
<td>Age</td>
<td>(n=69, 38.3%)</td>
<td>(n=111, 61.7%)</td>
</tr>
<tr>
<td>&lt;65 ages</td>
<td>24</td>
<td>47</td>
</tr>
<tr>
<td>≥65 ages</td>
<td>45</td>
<td>64</td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td>53</td>
</tr>
<tr>
<td>Female</td>
<td>16</td>
<td>46</td>
</tr>
<tr>
<td>Tumor volume</td>
<td>&lt;5 cm</td>
<td>36</td>
</tr>
<tr>
<td>≥5 cm</td>
<td>33</td>
<td>84</td>
</tr>
<tr>
<td>Tumor invasion depth</td>
<td>T1a, T1b, T2</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>T3, T4a, T4b</td>
<td>37</td>
</tr>
<tr>
<td>Lymphatic metastasis</td>
<td>Yes</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>34</td>
</tr>
<tr>
<td>Staging</td>
<td>IA, IB</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>IIA, IIB</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>IIIA, IIB, IIIC</td>
<td>27</td>
</tr>
<tr>
<td>Lymphatic invasion</td>
<td>Yes</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>18</td>
</tr>
<tr>
<td>Differentiation</td>
<td>Differentiated type</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Undifferentiated type</td>
<td>32</td>
</tr>
</tbody>
</table>

In 180 cases of patients, we classified 111 cases (61.7%) as Nrf2 positive group which Nrf2 immunohistochemical scores are more than 100, the remaining 69 cases (38.3%) are negative group. Table 2 showed the correlation between the expression of Nrf2 and the clinical pathological features. The positive expression of Nrf2 was significantly related to the gender, volume of tumor, depth of invasion, lymph node metastasis, lymphatic invasion and analysis of tissue clinical stages.

Correlation between the immunohistochemical expression of Nrf2 and 5FU drug resistance

In 77 cases, 61 cases are Nrf2 positive who received 5FU adjuvant chemotherapy, 16 cases were negative. In 61 cases of positive patients, 44 cases (72.9%) showed 5FU drug resistance. While in 16 cases of negative patients, only 5 cases (38.5%) showed drug resistance. The difference has statistical significance (P=0.022).

Analysis of survival rate

Kaplan Meier analysis shows that the overall survival rate of Nrf2 positive group was significantly lower than that of Nrf2 negative group (62% vs. 83%, P<0.01) (Figure 3). At the same time, univariate analysis showed that the tumor depth of invasion, volume of tumor, lymph node metastasis, histological type and lymphatic invasion were also significantly related the factors which significantly influenced the prognosis of patients. Multivariate analysis of the above factors showed that lymph node metastasis was the related indicator of independent prognosis (Table 3).

Discussion

Figure 3. Result of survival prognosis after operation of 180 cases of patients on the basis of Nrf2 expression.

Nrf2 is regarded as antioxidant which can protect normal cells from oxidative stress damage. This is one of the protection mechanisms of...
Correlation between expression of Nrf2 and progression of gastric cancer

Table 3. Univariate and multivariate analysis of prognostic factors of gastric cancer

<table>
<thead>
<tr>
<th>Clinical factors</th>
<th>Single argument</th>
<th>Multiple variables</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P value</td>
<td>P value</td>
</tr>
<tr>
<td>Age</td>
<td>0.43</td>
<td>-</td>
</tr>
<tr>
<td>Gender</td>
<td>0.52</td>
<td>-</td>
</tr>
<tr>
<td>Depth of invasion</td>
<td>&lt;0.01</td>
<td>0.26</td>
</tr>
<tr>
<td>Tumor volume</td>
<td>&lt;0.01</td>
<td>0.33</td>
</tr>
<tr>
<td>Lymphatic metastasis</td>
<td>&lt;0.01</td>
<td>0.04</td>
</tr>
<tr>
<td>Lymphatic invasion</td>
<td>&lt;0.05</td>
<td>0.28</td>
</tr>
<tr>
<td>Tissue typing</td>
<td>&lt;0.05</td>
<td>0.19</td>
</tr>
<tr>
<td>Nrf2</td>
<td>&lt;0.05</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Wang and others, they find the expression of Nrf2 in gallbladder cancer is related to the tumor differentiation, staging and lymph node metastasis [23]. In the recently report of Hu and others, the significant effect on prognosis by the expression of Nrf2 in gastric cancer is also consistent with the results of this study. But they only detect the immunoreactivity of Nrf2 in the cytoplasm and find nothing in the nucleus, and may because of using different antibodies [26]. Many studies have confirmed that Nrf2 exerts its antioxidant activity must be completed from the cytoplasm to the nucleus [27]. The immunoblot analysis of this study shows that Nrf2 in gastric cancer cell lines mainly exists in the nucleus. The continuous and over expression of Nrf2 in cancer cell nucleus may be regarded as antioxidants to protect cancer cells from ROS damage. At the same time, the expression is also the performance of tumor invasive enhancement. Based on this theory, it is necessary to test and evaluate Nrf2 in the nucleus of gastric cancer tissue specimen, rather than the cytoplasm.

In this study, we evaluate the prognostic value of the expression of Nrf2 by univariate analysis. Although Nrf2 positive is not regarded as an evaluated factor of independent prognosis to be added to the multivariable evaluation, the result shows lymph node involvement as a marker for poor prognostic factor which can significantly affect the positive rate of Nrf2. Solis and Wang et al all report that there is a certain correlation between the expression of Nrf2 in small cell lung cancer and in gallbladder cancer and poor prognosis of patients, and they all support the view that the expression of Nrf2 can be regarded as the factor of evaluated prognosis to evaluate the survival time of patients after operation.

At present, some studies show that Nrf2 expression increases the survival rate of cancer cell by increasing the resistance of cancer cells to chemotherapy and radiotherapy [28-33]. This study shows the expression of Nrf2 is significantly related to the adjuvant chemotherapy on the basis of 5FU. So by evaluating the expression level of Nrf2 in gastric cancer nucleus of patients can assist decision-making.
Correlation between expression of Nrf2 and progression of gastric cancer

the chemotherapy program. Nrf2 gene or inhibition can lead the antioxidant of cell which is regulated by Nrf2 to dysfunction, including glutathione, thioredoxin, non-protein thiols etc. Finally, cause cancer cells to restore the sensitivity to anticancer drugs and radiation. Cho and others find the inhibition of Nrf2 can increase the sensitivity of cancer cells to alkylating anticancer drugs [31]. Ma et al report the in vivo can significantly inhibit the growth of tumor by knocking out the Nrf2 and using in combination with cisplatin at the same time [22]. Therefore, the new chemotherapy program which is involved in Nrf2 regulation of antioxidants will provide better curative effect for the clinic diagnosis and treatment of tumor, and improve the survival prognosis of patients.

In summary, there is a positive correlation between the expression of Nrf2 and the invasion of gastric cancer, which can be used as a potential indicator of patients’ poor prognosis.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jiansheng Li, Department of Gastroenterology, The First Affiliated Hospital, Zhengzhou University, Zhengzhou 450052. E-mail: jiansheng_li11@126.com

References

Correlation between expression of Nrf2 and progression of gastric cancer


[27] Li W, Kong AN. Molecular mechanisms of Nrf2-mediated antioxidant response. Mol Carcinog 2009; 48: 91-104.


