High Expression of NLRC5 is associated with prognosis of gastric cancer

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Abstract: Objective: To explore the relationships of NLRC5 with clinicopathological characteristics and prognosis of gastric cancer patients.

Methods: A total of 97 gastric cancer patients undergoing radical gastrectomy were enrolled. All patients were diagnosed by immunohistochemical staining. The relationship between NLRC5 expression and clinicopathological characteristics of gastric cancer was analyzed via univariate and multivariate Cox regressions.

Results: NLRC5 expression was positive in 70 cases (72.2%) and negative in 27 cases (27.8%). No significant differences in age, sex, or tumor size or differentiation were found between the negative and positive groups. NLRC5 expression was related to tumor site, and in the positive group, it was high in the fundus and low in the pylorus ($\chi^2=7.359$, $P=0.125$). NLRC5 expression was significantly related to lymph nodes and tumor node metastasis (TNM) staging ($\chi^2=6.295$; $\chi^2=6.268$). Multivariate Cox regression indicated positive NLRC5 expression was independently and significantly associated with prognosis of gastric cancer patients (HR=2.92, 95%CI: 1.51-5.63).

Conclusions: NLRC5 is closely related to TNM staging and lymph node metastasis of gastric cancer and is an independent risk factor for the prognosis of gastric cancer patients.

Keywords: Gastric cancer; NLRC5; Clinicopathological characteristics; Prognosis

1 Introduction

As a malignant epithelial tumor and major health concern, gastric cancer (GC) is the fourth most common cancer and the second leading cause of cancer-related death worldwide [1]. Early detection and radical resection of GC significantly contribute to improving the 5-year overall survival rates, up to 90%. Despite the recent progress in chemotherapy, radiotherapy and surgical techniques for GC treatment, the outcomes of GC patients remain dismal and the 5-year overall survival rates are less than 25% [2]. One main reason for the low overall survival is the lack of appropriate identifiable molecular biomarkers, which means most GC patients are diagnosed at advanced stages and miss the best opportunity for curative surgery. Therefore, in-depth research should be conducted to explore mechanisms and novel molecules for early diagnosis and treatment, which are hotspots of basic GC research. GC development consists of multiple stages and can be promoted by many factors, including activation of oncogenes and inactivation of tumor suppressors [3]. Finding key molecules and their mutual relationships in gastric carcinogenesis is of great significance for the diagnosis and treatment of GC.

Mutations of several nucleotide-binding oligomerization domain-like receptors (NOD)-like receptors (NLRs), including NOD1, NOD2, NLRP1, NLRP3, NLRP7 and NLRP12, have been implicated in tumorigenesis [4, 5]. NLRC5 (NLR family CARD domain containing 5), one member of the innate immune system receptor (or NLR) family, can regulate immune responses and is associated with chronic inflammatory diseases [6, 7]. NLRC5 is involved in suppressing a type of very important compound in the nuclear factor (NF)-κB signaling pathway in innate immune cells, which can effectively regulate the activity of immune cells, preventing the body from damage caused by sustained inflammation [8]. NLRC5 can also negatively regulate the secretion of proinflammatory factors such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α in RAW264.7 cells [9]. A previous study reported that NLRC5 is expressed in renal carcinoma, cervical cancer, prostate cancer, hepatocellular carcinoma and
rectal cancer. It was also reported that NLRC5 is highly expressed in lung cancer and associated with prognosis in non-small cell lung cancer [10]. Furthermore, overexpression of NLRC5 promoted the proliferation, migration and invasion of HCC cells in vitro. Up-regulation of NLRC5 not only positively correlates with the increase of beta-catenin but also coordinates the activation of the downstream Wnt/beta-catenin signaling pathway [11]. However, the biological function of NLRC5 in GC has not been well demonstrated yet. Therefore, we aim to explore NLRC5 expression and its effect on prognosis in GC patients for the first time.

2 Materials and methods

This study was approved by Institutional Review Board of the Second Affiliated Hospital of Zhengzhou University (2017-10-0038).

2.1 Tissue samples

Tissue samples were collected from patients who received surgical operation between June 2011 and December 2012 in the Second Affiliated Hospital of Zhengzhou University. No patient received radiotherapy or chemotherapy before surgical operation. All patients were confirmed by pathological examination after specimen fixation, paraffin embedding, and staining.

2.2 Data collection

With a standardized excel spread sheet, clinical and pathological data were collected from each patient including sex, age, tumor site, tumor size, differentiation, lymph nodes number, and tumor node metastasis (TNM) stage. The follow-up deadline was June 30, 2016. The primary outcome was death.

2.3 Immunohistochemistry

Anti-NLRC5 antibody (Abcam Co. Ltd, USA, ab105411), beta-catenin antibody (Cell Signaling Co. Ltd, USA), goat antimouse IgG, and goat polyclonal secondary antibody to rabbit IgG (Beijing Zhonghua Jinqiao Biotechnology Co., Ltd) were used. NLRC5 was detected by an immunohistochemical method. Typically, 3-μm tissue specimens were deparaffinized in xylene and microwave-treated for 10 min at moderate power in a 0.01 M citrate buffer (pH=6.0). After cooling for 30 min, the specimens were washed in a phosphate buffer saline (PBS) cushion fluid, and endogenous peroxidase was blocked with 3% hydrogen peroxide for 30 min, followed by incubation with PBS containing 10% normal goat serum for 30 min. The specimens were incubated overnight at 4°C with anti-NLRC5 antibody at a dilution of 1:100. The specimens were immunostained using a Chemmate kit (Dako, Glostrup, Denmark) with 3,3-diaminobenzidine as the chromogenic substance.

The specimens were observed under low-power magnification and confirmed under high-power magnification. NLRC5 expression was judged as follows: each view was scored 0-4 according to the staining intensity (0: ≤10%, 1: 11%-30%, 2: >30%, 3: 30%-60%, 4: >60%). The staining scores of 0-1 and 2-4 were considered as tumors with negative and positive expressions, respectively.

2.4 Statistical analyses

All statistical analyses were finished on Statistical Product and Service Solutions (SPSS) 20.0, with the significant level at \( P<0.05 \). NLRC5 expressions were compared according to sex (female vs. male), age (<60 vs. ≥60), tumor site (fundus, pylorus, antral), tumor size (<4cm vs. ≥4cm, cutoff value determined using the receiver operator characteristic curve), differentiation (low, low-middle, middle), number of lymph nodes, and TNM stage (I II III) through Chi-square test because these were qualitative data. The effect of NLRC5 on the prognosis of GC patients was assessed via univariate and multivariate Cox regression because this is survival data. Hazard risk (HR) and relative 95% confidence interval (CI) were calculated. The overall survival rates between low- and high-expression groups were compared via the log-rank test.

3 Results

3.1 Clinical and histopathological characteristics

Table 1 presents the clinical and histopathological characteristics of the 97 cases, including 72 males and 25 females. The patients were aged 38 to 83 years old (median = 64), and 62 of them were above age 60. There were 27, 65 and 5 cases with distal, proximal and total gastrectomy, respectively. According to the 7th edition of the Ameri-
can Joint Committee on Cancer (AJCC) Staging System for gastric cancer, the patients were restaged to stage I (n=20), II (n=23) and III (n=54). Fifty-three patients (54.6%) had lymph node metastasis. The tumor sites included fundus (n=49), pylorus (n=32) and antral (n=16).

### 3.2 NLRC5 expression and clinicopathological parameters

NLRC5 expression in GC was stronger than the negative group (Figure 1). NLRC5 expression was positive in 70 of the 97 cases (72.2%) and negative in 27 cases (27.8%). No significant difference in age or sex was found between the negative and positive groups ($\chi^2=0.676, P=0.411$; $\chi^2=2.349, P=0.125$). NLRC5 expression was related to tumor site, and in the positive group, it tended to be high in fundus and low in pylorus ($\chi^2=7.359, P=0.025$). No significant differences were observed in tumor size or differentiation between the positive and negative groups ($\chi^2=1.327, P=0.249$; $\chi^2=2.968, P=0.227$). NLRC5 expression was significantly related to both lymph node number and TNM stage ($\chi^2=6.782, P=0.035$; $\chi^2=6.268, P=0.045$).

### 3.3 NLRC5 and survival

The relationship between NLRC5 and prognosis in GC patients was evaluated via univariate and multivariate Cox regression. The univariate regression indicated lymph node number (HR=3.26, 95%CI: 1.83-5.80 for 1-7, $P<0.001$; HR=2.34, 95%CI: 1.43-3.84, $P=0.001$), TNM stage (stage III: HR=2.84, 95%CI: 1.43-5.67, $P=0.003$) and positive NLRC5 expression (HR=2.92, 95%CI: 1.51-5.63, $P=0.001$, Figure 2)
Table 2: Univariate Cox regression analysis of relationship between clinicopathological characteristics and prognosis in patients with gastric cancer

| Parameters         | Beta | SE   | Wald χ² | P    | HR (95% CI) |
|--------------------|------|------|---------|------|-------------|
| Age (year)         |      |      |         |      |             |
| <60                |      |      |         |      |             |
| ≥60                | 0.031| 0.221| 0.019   | 0.889| 1.03 (0.67-1.59) |
| Sex                |      |      |         |      |             |
| Male               |      |      |         | 1.00 |             |
| Female             | -0.166| 0.335| 0.247   | 0.619| 0.85 (0.44-1.63) |
| Tumor site         |      |      |         |      |             |
| Fundus             |      |      |         | 1.00 |             |
| Pylorus            | 0.019| 0.334| 0.003   | 0.955| 0.98 (0.51-1.89) |
| Antral             | 0.367| 0.339| 1.170   | 0.279| 1.44 (0.74-2.80) |
| Tumor size         |      |      |         |      |             |
| <4cm               |      |      |         | 1.00 |             |
| ≥4cm               | -0.032| 0.243| 0.017   | 0.895| 1.00 (0.60-1.56) |
| Differentiation    |      |      |         |      |             |
| Low                |      |      |         | 1.00 |             |
| Low-middle         | 0.082| 0.518| 0.025   | 0.874| 1.09 (0.39-3.00) |
| Middle             | 0.318| 0.527| 0.364   | 0.546| 1.37 (0.49-3.86) |
| Lymph nodes        |      |      |         |      |             |
| 0                  |      |      |         | 1.00 |             |
| 1-7                | 1.160| 0.294| 15.755  | <0.001| 3.18 (1.64-5.74) |
| ≥7                 | 0.781| 0.246| 11.407  | 0.002| 2.18 (1.44-3.26) |
| TNM stage          |      |      |         |      |             |
| I                  |      |      |         | 1.00 |             |
| II                 | 0.584| 0.335| 3.040   | 0.081| 1.79 (0.93-3.46) |
| III                | 1.045| 0.352| 8.844   | 0.003| 2.84 (1.43-5.67) |
| NLRC5              |      |      |         |      |             |
| -                  |      |      |         | 1.00 |             |
| +                  | 1.070| 0.336| 10.153  | 0.001| 2.92 (1.51-5.63) |

Figure 1: Expression of NLRC5 in GC specimens (A: negative; B weakly positive; C strongly positive; SP*400)

Figure 2: Relationship between NLRC5 expression and prognosis of GC

were all associated to survival status of GC patients. The multivariate regression (Table 3) indicated positive NLRC5 expression significantly increased the risk of adverse outcomes by 69% (HR=1.69, 95%CI: 1.17-2.45, P=0.005). Lymph node metastasis significantly enhanced the risk of death in GC patients (HR=1.77, 95%CI: 1.23-2.55, P=0.002 for 1-7;
Discussion

Recent research suggests the development and progression of GC are caused by multiple environmental and genetic factors. An epidemiological study confirms that chronic inflammation increases the risk of malignancy [12]. Under long-term infection stimulation, the human organism will enter a state of chronic inflammation, which subtly induces mutations of normal cells to malignant cells and further promotes the development, invasion and metastasis of cancer cells [13]. Inflammatory factors such as ILs, TNFs, cyclooxygenase-2 and interferon-γ play important roles in the development and occurrence of GC [14-17]. These anti- or pro-inflammatory factors have been extensively concerned, as they can activate the natural immune system and build the first-line defense barrier against the invasion of pathogens. They also can detect pathogen-associated molecular patterns through specific pattern- recognition receptors [18]. After the invading pathogens are identified, the body activates a series of signaling pathways and defense reactions and generates immune and inflammatory responses [19].

The NLRC5 gene, located at the 16q13 locus of human genome, has a full-length of 6822 bp mRNA encoded by 49 exons, resulting in 1866 amino acids [20]. NLRC5 is composed of N-terminal recruitment domains (CARDs), centrally located nucleotide binding domains (NBDs) and C-terminal leucine-repeat sequences (LRRs). NLRC5 is the largest NLR because of the 204 kDa LRR. NLRC5 mainly regulates the immune response in the body. As reported previously, NLRC5 is involved in regulating the expressions of the MHC class I gene and MHC class I antigen-presenting related genes. The specific knockout of NLRC5 can promote NF-κB signal transduction and induce the expressions of downstream inflammatory cytokines, such as TNF-α and IL-6 [8, 21]. NLRC5 can also promote the type I IFN signaling pathway and antiviral immune responses [22].

Although many studies show NLRC5 is highly expressed in various human solid malignancies and involved in the occurrence, invasion and metastasis of malignant tumors, the findings about the role of NLRC5 in malignant tumors remain controversial. A retrospective study presented high NLRC5 expression in stage III non-small cell lung cancer (NSCLC) and found that positive NLRC5 expression may be one predictor of poor prognosis in stage III NSCLC patients [23]. Moreover, NLRC5 was highly expressed in hepatocellular carcinoma and hepatocellular carcinoma cell lines, and promoted the proliferation, migration and invasion of hepatocellular carcinoma by targeting the Wnt/β-catenin signaling pathway [24]. However, Rodriguez found Bcl-2 cells stably expressing NLRC5 (B16-5 cell line), compared with T cell costimulatory molecule CD80 (B16-CD80 cell line) or their combination (B16-5/80 cells Strain), highly expressed the major histocompatibility complex-1, low molecular mass polypeptide (LMP)-2, LMP7 and transporter associated with antigen processing-1 genes of antigen processing molecules [25]. B16-5 cells effectively transmitted the melanoma antigen peptide gp10025-33 to effector cytotoxic gp100-petide T cell

| Parameters | Beta | SE  | Waldχ² | P    | HR(95%CI) |
|------------|------|-----|--------|------|-----------|
| Lymph nodes |      |     |        |      |           |
| 0          |      |     |        | 1.0  |           |
| 1-7        | 0.570| 0.187| 9.253  | 0.002| 1.77(1.23-2.55) |
| ≥7         | 0.450| 0.141| 10.112 | 0.001| 1.57(1.19-2.07) |
| TNM stage  |      |     |        |      |           |
| I          |      |     |        | 1.0  |           |
| II         | 0.231| 0.218| 1.130  | 0.288| 1.26(0.82-1.93) |
| III        | 1.131| 0.155| 53.353 | 0.000| 3.10(2.29-4.20) |
| NLRC5      |      |     |        |      |           |
| -          |      |     |        | 1.00 |           |
| +          | 0.526| 0.188| 7.840  | 0.005| 1.69(1.17-2.45) |
receptor (Pmel-1 TCR) transgenic CD8 (+) T cells, inducing their proliferation, and stimulated Pmel-1 cells in the presence of CD80, even without gp100 peptide, suggesting NLRC5 promotes the processing and presentation of the primary tumor antigen [25]. B16-5 cells also significantly inhibited tumor growth in C57BL/6 hosts after subcutaneous implantation, but not in immune-deficient hosts, suggesting NLRC5-tumor cells elicit anti-tumor immunity [25]. The researchers believed NLRC5 can be used to restore tumor immunogenicity, which in turn stimulates the activation of protective anti-tumor immunity, thereby exerting the ability to inhibit tumor invasion and metastasis. Therefore, NLRC5 expression and its relationship with clinicopathological characteristics in GC patients are worthy of investigation. In the present study, we mainly explored NLRC5 expression and its relationships with the clinicopathological characteristics and prognosis of GC patients. Results showed NLRC5 was highly expressed in GC, with a positive rate of 72.2%. The survival rates of two groups with different NLRC5 expressions were analyzed by Kaplan-Meier method. It was found the prognosis of the low expression group was better than that of the high expression group. Multivariate analysis showed the positive NLRC5 expression was an independent risk factor of prognosis in GC patients, suggesting high NLRC5 expression is one molecular marker of poor prognosis in GC patients. NLRC5 may be involved in the invasion and metastasis of GC. Interestingly, our study showed NLRC5 expression levels differed significantly among different tumor sites. We speculate that the possible reason was that difference of GC microenvironment affected the NLRC5 expression. We did not collect the information about post-operative treatment, which requires further research and may have little effect because patients usually are treated according to the guidelines.

In conclusion, NLRC5 is widely expressed in gastric cancer tissues, and its expression is closely related to TNM staging and lymph node metastasis of gastric cancer. Multivariate Cox regression shows NLRC5 overexpression is an independent risk factor for the prognosis of gastric cancer patients.

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