# Different Cellular Localization of NF-κB p65 Expression as an Indicator of Different Prognoses of Stage I–III Gastric Cancer Patients

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## Abstract

**Background:** Nuclear factor-κB p65 (NF-κB p65) may play a significant role as a biomarker in tumor progression and metastasis. However, the correlation between cellular localization of NF-κB p65 expression and the prognosis of gastric cancer (GC) patients has not been studied. The present study was designed to investigate the location of NF-κB p65 expression in GC, and evaluate its correlation with clinicopathological parameters of GC patients.
**Methods:** NF-κB p65 expressions in GC tissue and corresponding nonmalignant tissue from gastrectomy of 115 stage I–III GC patients were detected by immunohistochemistry. In addition, correlations between the staining results and the clinicopathologic features and survival of the GC patients were analyzed.
**Results:** The percentage of NF-κB p65 expression in GC tissue and the corresponding nonmalignant tissue was 73.9% and 46.80%, respectively. No significant correlation was found between NF-κB p65 expression and the clinicopathologic parameters. Cox univariate analysis indicated that both nuclear staining and cytoplasmic staining of NF-κB p65 expression correlated with the prognosis of GC patients (log-rank, $p = 0.0182$; $p = 0.0144$, respectively).
**Conclusion:** High nuclear expression of NF-κB p65 is an independent prognostic marker predicting a better survival, while high cytoplasmic staining indicates a worse prognosis of GC patients. Clin Trans Sci 2013; Volume 6: 381–385

**Keywords:** gastric cancer, NF-κB p65, cellular localization, prognosis

## Introduction

Although the incidence of gastric cancer (GC) has gradually declined worldwide,[1] GC remains one of the most common malignancies worldwide and a major cause of cancer mortality in Asia.[2] The clinical progression of GC is rapid and the prognosis is usually poor due to adjacent tissue invasion and distant organ metastasis at a very early stage.[3–5] Therefore, improving early diagnostic accuracy could increase the curative opportunity or prolong the survival of GC patients. However, the conventional serum markers for GC, such as carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen (CEA), lack sufficient sensitivity and specificity to facilitate early detection.[6] Thus, finding an indicator for early diagnosis of GC is of vital importance to improve the therapeutic effect and prolong survival of GC patients.

Nuclear factor-kappa B p65 (NF-κB p65) is a ubiquitously existing transcription factor that affects the proliferation, invasion, angiogenesis, and metastasis of cancer cells by regulating the expression of numerous genes.[7–9] In an inactive status, NF-κB p65 family members exist as dimmers with the predominance of p65/p50 heterodimers and are sequestered in the cytoplasm.[10] Activation of NF-κB p65 pathway by a series of stimuli would induce its translocation to the nucleus, thus activating the expression of downstream genes.[11,12] Constitutive activation of NF-κB p65 has been observed to positively correlate with tumor progression in various cancer types, including renal cancer, cervical cancer, and esophageal cancer.[13] Activated NF-κB p65 is therefore suggested as a potential therapeutic target for the treatment of these tumors.[14]

There is evidence that NF-κB level in GC cells is higher than that in adjacent normal epithelial cells, and activation of NF-κB in GC is related to lymphatic invasion.[15,16] It was reported that patients with high NF-κB activation in carcinoma tissue did not survive as long as those with low NF-κB activation, and p65, but not NF-κB, is a prognostic indicator of GC.[17] Although Kojima et al.[18] reported the expression level of NF-κB p65, its correlation with the clinicopathologic features (such as tumor progression) of GC patients, and its significance as a prognostic marker, the correlation between the location of NF-κB p65 expression and the prognosis of GC patients has not been reported. The present study was intended to investigate the potential correlation between the location of NF-κB p65 expression and the prognosis of GC patients by immunohistochemistry, in an attempt to further expound the role of NF-κB p65 expression in predicting the prognosis of GC patients, and see whether it could be used as an option for early clinical diagnosis and treatment of GC.

## Materials and Methods

### Specimen source

A total of 115 GC patients who underwent radical surgery at our hospital between November 2007 and March 2009 were evaluated retrospectively. They included 78 (67.8%) male patients and 37 (32.2%) female patients, with a mean age of 60.1 years (range 36–90 years) and a median follow-up period of 42 months (range 8–59 months). Histologically, they included 90 (78.3%) cases of poorly differentiated, 18 (15.7%) cases of moderately differentiated, and 7 (6%) cases of well differentiated adenocarcinoma. According to the seventh edition of TNM classification for GC as reported by Biondi et al.,[19] TNM stage of the 115 patients was assessed as follows: stage I in 31 patients (27%), II in 21 patients (18.3%), and III in 63 patients (54.7%). Her-2/neu overexpression was detected in 11 (10.5%) cases by postoperative fluorescence in situ hybridization (FISH). No patient received neoadjuvant treatment. No patient included in the study had evidence of distant metastasis at the time of diagnosis. Patients with a history of previous malignancy were excluded. The clinical characteristics of the patients are shown in *Table 1*.

| Characteristics | No. | NF-κB p65-high (n = 65) | NF-κB p65-low (n = 50) | p |
|---|---|---|---|---|
| Gender | | | | |
| Male | 78 | 45 | 33 | 0.713 |
| Female | 37 | 20 | 17 | |
| Age (y) | | | | |
| <60 | 55 | 31 | 24 | 0.974 |
| ≥60 | 60 | 34 | 26 | |
| Tumor differentiation | | | | |
| Well | 7 | 4 | 3 | 0.762 |
| Moderate | 18 | 11 | 7 | |
| Poor | 90 | 50 | 40 | |
| Tumor size (cm) | | | | |
| <4.5 | 59 | 32 | 27 | 0.067 |
| ≥4.5 | 56 | 33 | 13 | |
| TNM stage | | | | |
| I–II | 52 | 30 | 22 | 0.818 |
| III | 63 | 35 | 28 | |
| Her-2 status | | | | |
| Negative | 104 | 58 | 46 | 0.617 |
| Positive | 11 | 7 | 4 | |
| Primary/Recurrent | | | | |
| Primary tumor | 88 | 52 | 36 | 0.316 |
| Recurrent tumor | 27 | 13 | 14 | |

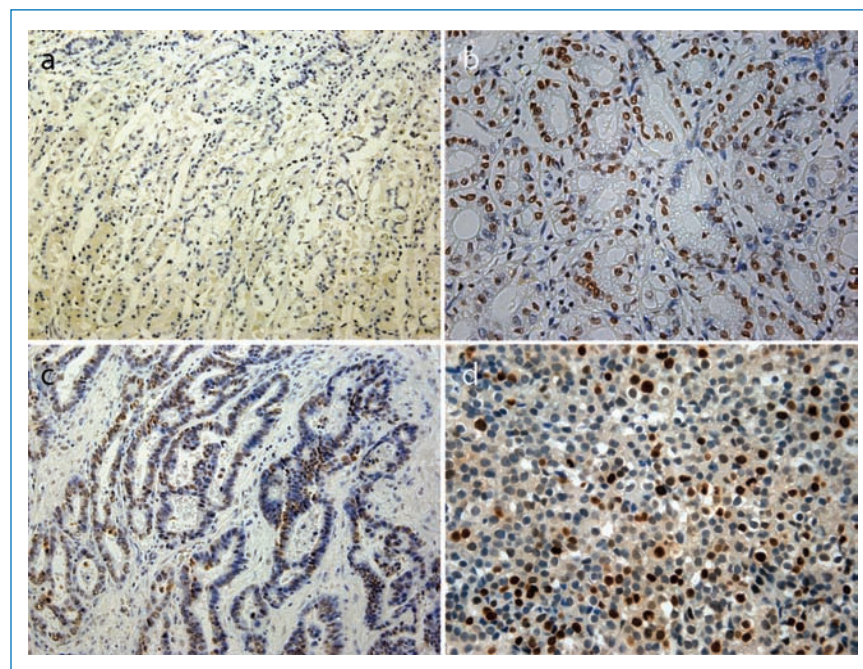**Table 1.** Correlation among NF-κB p65 staining and clinical characteristics.



**Figure 1.** No expression of NF-κB p65 in corresponding nonmalignant tissues (a) and low NF-κB p65 expression in corresponding nonmalignant tissues (b). Low NF-κB p65 expression in cancer tissues (c), high NF-κB p65 expression in cancer tissues (d). Original magnification of (a) and (c), ×200; original magnification of (b) and (d), ×400.

Specimens obtained from the 115 patients who underwent radical resection were fixed in neutral buffered formol saline and paraffin-embedded for immunohistochemical observation by a pathologist to confirm the malignancy. The study protocol was approved by the ethics committee of the medical faculty, and informed consent was obtained from all patients.

**Immunohistochemistry**
Streptavidin-peroxidase immunohistochemical technique was used to determine the level of NF-κB p65. The monoclonal antibody (E379, ab32536, Abcam Inc.) to NF-κB p65 was from Abcam (Cambridge, United Kingdom). Paraffin-embedded tissue blocks were sliced into 3–4 mm sections and placed on antislides. The sections were dewaxed, hydrated, rinsed in phosphate-buffered saline (PBS), blocked with 3% hydrogen peroxide for 10 minutes to deprive the endogenous peroxidase activity. After antigen retrieval with the use of a microwave, the sections were incubated with the antinucleolin MAb (diluted 1:100 in PBS) at 37°C for 70 minutes, washed $3 \times 3$ minutes in PBS, and incubated with 1 and 2 Reagent of PV9000 Mouse/Rabbit hypersensitivity two-step immunohistochemical Kit (Beijing fir Jinqiao, China) for 50 minutes at 37°C in a humid chamber. The sections were washed again $3 \times 3$ minutes with PBS, followed by the addition of diaminobenzidine (DAB) as a chromogen. Antibodies were optimized using positive control tissue according to manufacturer's instructions. In negative controls, the primary antibody was replaced with PBS. The rest procedures were undertaken in parallel with other specimens. Each slide was scored in a blinded fashion by two pathologists according to the manufacturer's recommended criteria at ×100 and ×200 magnification. The overall percentage of cells on an immunostained section was determined according to the pattern of intracellular localization. The extent and pattern of the NF-κB p65-specific immunostaining within a tissue section was determined by the percentage of cells with a nucleus and cytoplasm staining. The immunostaining was read in a semiquantitative manner. Three visual fields were examined randomly and the rate of positive cells was classified as less than 5% (0 score), 6–25% (1 score), 26–50% (2 score), 51–75% (3 score), and more than 75% (4 score). The staining intensity was classified as four grades: no staining (0 score), slightly yellowish (1 score), brownish yellow (2 score), and dark brown (3 score). The multiplication of the two

| | No. | HR (95% CI) | p |
|---|---|---|---|
| Univariate analysis | | | |
| Gender | | | |
| Male | 78 | | 0.9164 |
| Female | 37 | | |
| Age (y) | | | |
| <60 | 55 | | 0.4858 |
| ≥60 | 60 | | |
| Tumor differentiation | | | |
| Well | 7 | | 0.2724 |
| Moderate | 18 | | |
| Poor | 90 | | |
| Tumor size (cm) | | | |
| <4.5 | 59 | | 0.9465 |
| ≥4.5 | 56 | | |
| TNM stage | | | |
| I–II | 52 | | 0.4460 |
| III | 63 | | |
| Her-2 status | | | |
| Negative | 104 | | 0.2585 |
| Positive | 11 | | |
| Nucleolar staining of nucleolin | | | |
| Low | 50 | | 0.0182 |
| High | 65 | | |
| Cytoplasmic staining of nucleolin | | | |
| Low | 96 | | 0.0144 |
| High | 19 | | |
| Multivariate analysis | | | |
| Nucleolar staining of nucleolin | | | |
| Low | 50 | 0.4 | 0.0232 |
| High | 65 | 0.181–0.882 | |
| Cytoplasmic staining of nucleolin | | | |
| Low | 96 | 2.714 | 0.0193 |
| High | 19 | 1.176–6.266 | |

**Table 2.** Univariate and multivariate analysis of overall survival in patients with GC.

was graded as 0 (0 score), 1+ (1–4 score), 2+ (5–8 score), and 3+ (9–12 score). Intensity scores of 0 or 1+ were designated as low expression, and 2+, 3+ were designated as high expression.

## Statistical analysis
Statistical analysis was performed using SAS 9.2 software system (SAS, Cary, NC, USA). Chi-square statistics and Fisher's extract test were applied to assess correlations between NF-κB p65 and clinicopathological parameters. Kaplan-Meier survival analysis was performed for univariate analysis of the survival rate, and Cox regression analysis was performed for multivariate analysis. Univariate analysis was also performed to assess the prognostic value of NF-kappa B p65 expression and other clinicopathological features. A p-value less than 0.05 was considered statistically significant.

## Results

### NF-κB p65 expression and clinicopathological characteristics
All the 78 male and 37 female patients in this series received radical resection for GC, including 88 primary and 27 recurrent GC patients. Pathologically, all the 115 cases were adenocarcinoma, of which 7 cases were well differentiated, 18 were moderately differentiated, and 90 cases were poorly differentiated (including 3 cases of signet-ring cell carcinoma). For all specimens, 11 cases were confirmed as Her-2/neu-positive.

### Immunohistochemical analysis
NF-κB p65 immunohistochemical staining showed a nuclear staining pattern in both nonmalignant tissue (*Figure 1*, panel b) and GC tissue (*Figure 1*, panel a–b). The nuclear expression of NF-κB p65 in GC tissue was significantly higher than that in nonmalignant tissue. Cytoplasmic staining was detected in 16.5% (19/115) cases of cancer tissue (*Figure 1*, panel d). High expression of NF-κB p65 was detected in 60.87% (65/115) cases of cancer tissue, while no high expression of NF-κB p65 was detected in the adjacent benign gastric tissue.

### Correlations between NF-κB p65 expression and clinicopathologic parameters
Data representing the correlations between NF-κB p65 levels and the clinicopathologic features of the 115 GC cases are summarized in *Table 1*. No significant correlation was found between NF-κB p65 expression and the clinicopathologic parameters.

### Correlations between the expression level of NF-κB p65 and prognosis
Correlation between the high expression of nuclear staining or cytoplasmic staining of NF-κB p65 and patient survival was assessed by Cox survival analysis. The result showed that NF-κB p65 overexpression was identified as the only independent prognostic factor for better survival of GC patients after adjusting patient age, gender, tumor size, differentiation, TNM classification, and Her-2/neu status (*Table 2*).

Patients with overexpression of nuclear staining had a longer survival time (log-rank, $p = 0.0182$; *Figure 2*, panel a). Inversely, high expression of cytoplasmic staining was found to be associated with a significantly high recurrence rate and a shorter survival rate (log-rank, $p = 0.0144$; *Figure 2*, panel b). Univariate survival analysis showed that high nuclear staining was correlated with a better prognosis, while high cytoplasmic staining was closely associated with a poor prognosis. In terms of prognosis of patients, high nuclear expression with low cytoplasmic expression indicated the best prognosis while low nuclear expression with high cytoplasmic expression indicated the poorest prognosis. Low nuclear expression with low cytoplasmic expression and high
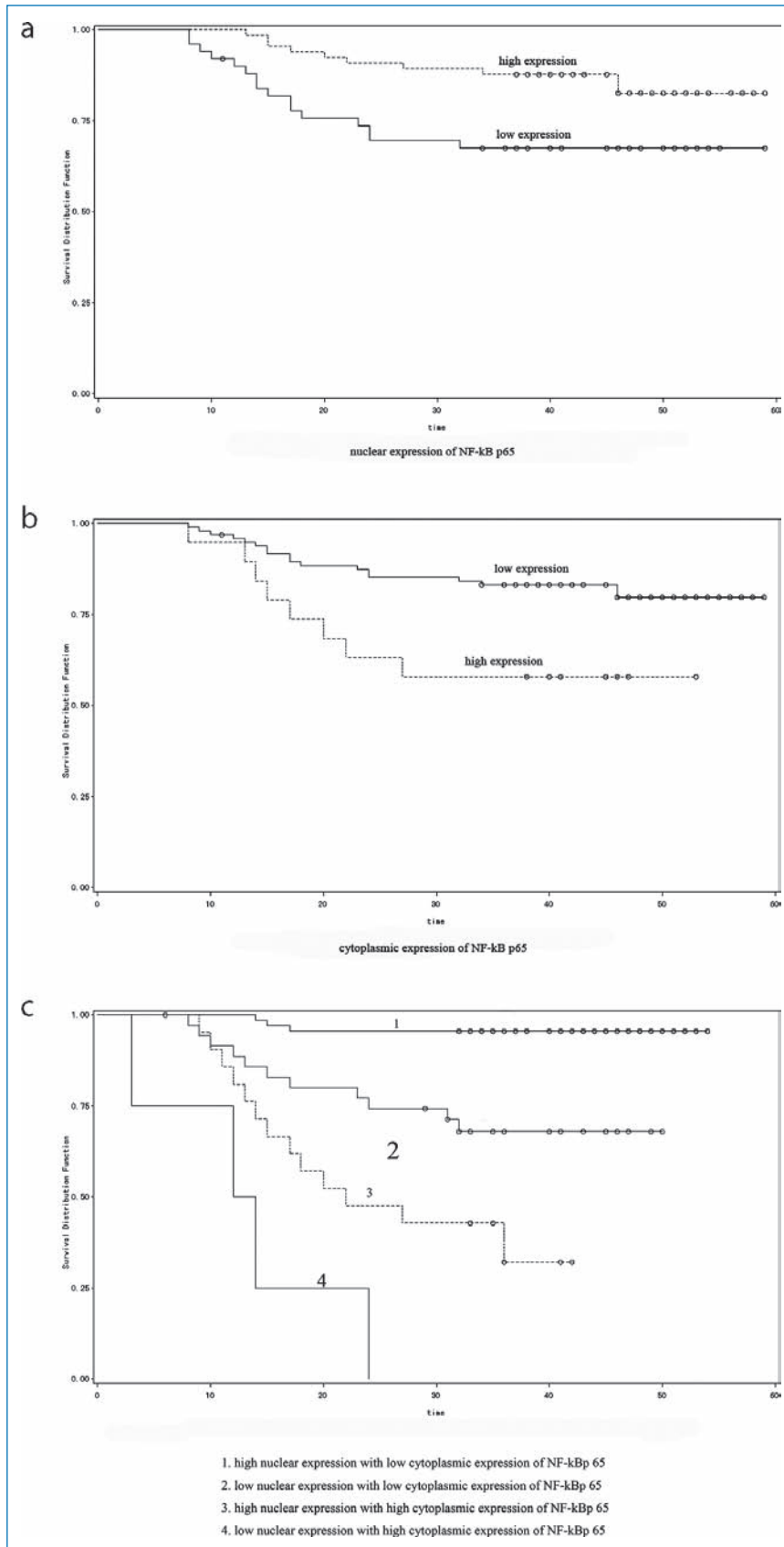
**Figure 2.** Survival analysis of NF-κB p65 expression in GC patients. Overexpression of nucleolar staining showed longer survival time (a), high expression of cytoplasmic staining associated with shortened survival rate (b) and different expression sites and levels of NF-κB p65 showed different prognosis (c).

nuclear expression with high cytoplasmic expression ranked the second and third (log-rank, $p < 0.01$; *Figure 2*, panel c).

## Discussion

Although a large number of investigations strongly suggest that NF-κB p65 is activated in gastric and other carcinomas and is an important transcriptional factor related to biological malignant characteristics such as anti-apoptosis and invasiveness of carcinoma,[20–22] the biological significance of different cellular localization of NF-κB p65 in carcinoma tissues remains unclear. In the present study, we focused on the prognostic value of different expression sites of NF-κB p65 in GC tissue. It was found that nuclear expression of NF-κB p65 was significantly higher in 72.1% GC tissue specimens versus 16.5% for cytoplasmic staining. Cox univariate analysis indicated that both cytoplasmic and nuclear staining of NF-κB p65 expression significantly correlated with the prognosis of GC patients.

Knowing that NF-κB p65 activation is a new prognostic parameter in GC as reported by previous studies,[23] the present study was intended to see whether NF-κB p65 was an independent prognostic indicator. At present, the most important factor in predicting the prognosis of GC patients is TNM stage, which is determined by the primary tumor size, degree of metastasis to regional lymph nodes, and distant metastasis.[24] These traditional clinicopathological parameters seem to be the most important risk factors for predicting overall survival of GC patients. However, no significant correlation was observed between TNM stage and NF-κB p65 (*Table 1*), suggesting that NF-κB p65 may be a prognostic indicator independent of TNM stage. In fact, our multivariate analysis showed that only NF-κB p65 was a prognostic parameter and that NF-κB p65 was independent of the traditional pathological parameters, including TNM stage.

In the past few years, several studies have shown the function of NF-κB p65 as a prognostic biomarker in various types of cancer.[23,25] Unlike many other transcription factors involved in cancer biology, NF-κB p65 plays a two-side role in the process of carcinogenesis.[26,27] The tumor-promoting role of NF-κB p65 has been studied exhaustively. Although previous studies reported that NF-κB p65 could induce G1 cell cycle arrest in human epithelial cells,[28] the mechanism underlying the tumor-suppressor role of NF-κB p65 remains poorly understood. Unexpectedly, this study has, for the first time, shown that the clinical outcomes are

different in GC cases with different cellular localizations of NF-κB p65, which is consistent with the previous studies. It was found in our study that patients who showed high cytoplasmic staining of NF-κB p65 in carcinoma tissue did not survive as long as those with high nuclear staining, indicating that a high level of nuclear expression of NF-κB p65 is a prognostic marker for better survival, while high cytoplasmic staining was closely associated with a worse prognosis for GC patients, which may suggest that different cellular localizations of NF-κB p65 may play a role in cancer progression.

In conclusion, our immunohistochemical results showed that nuclear expression of NF-κB p65 was markedly increased in GC tissue as compared with that in nonmalignant tissue. A high level of nuclear expression of NF-κB p65 was associated with a better prognosis and was an independent prognostic indicator for GC when overall survival was used as the endpoint. In addition, high cytoplasm staining indicates a worse prognosis, suggesting that the combination of cytoplasmic and nuclear detection could better predict the prognosis of GC. High cytoplasm expression with low nuclear expression may indicate a shorter survival, while low cytoplasm expression with high nuclear expression may indicate a better prognosis. Further investigation is needed to clarify the mechanism of NF-κB as a new prognostic biomarker in GC.

## Conflict of interest statement
We have no conflicts of interest to report.

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