Clinicopathologic Implications of Eukaryotic Initiation Factor 3f and Her-2/neu Expression in Gastric Cancer

Yu Cheng, Ph.D.^{1*}, Jin Zhou, Ph.D.^{2*}, and Honglun Li, B.M.³

Abstract

Background: To detect the expression of eIF3f and human epidermal growth factor receptor 2 (Her-2)/neu in gastric cancer (GC), correlation with their clinicopathological parameters and the relationship of eIF3f and Her-2/neu in the occurrence and development of GC.

Methods: A total of 195 gastrectomy specimens with stage I to III were examined for eIF3f expression by immunohistochemistry and for Her-2/neu expression by fluorescence *in situ* hybridization (FISH) with the median follow-up period of 38 months.

Results: The positive expression rate of eIF3f in GC and adjacent noncancerous tissue were 33.8% and 59.5%, respectively. eIF3f levels were linked to more advanced tumor stages and likelihood of recurrence (all $p < 0.05$). The Kaplan–Meier survival curves indicated that decreased expression of eIF3f could serve as a prognosis marker for poor outcome of GC patients ($p = 0.04$). 15.9% of GC specimens were positive for Her-2/neu, but whose expression was of no correlation with patients' survival. Patients who were positive for Her-2/neu also had high eIF3f expression levels ($p = 0.0295$).

Conclusion: Results suggest that eIF3f may play an important role in recurrence, thus representing a promising predictive marker for the prognosis of GC. But Her-2/neu has no relationship with the prognosis of GC. The clinical significance of eIF3f and Her-2/neu remains to be further investigated. Clin Trans Sci 2015; Volume 8: 320–325

Keywords: gastric cancer, eIF3f, Her-2/neu, biomarker, prognosis

Introduction

Gastric cancer (GC) is one of the most common malignant tumors worldwide. The WHO statistics shows that there are approximately one million new cases and 700,000 death all over the world in 2008.^{1–3} In the past several decades, intensive efforts have been made to identify tools to improve prognostication of GC. Such as miR-21, miRNA-106a, and miR-143, as well as miR-203 are considered to be promising novel biomarkers for GC.⁴ However, except for the cancer staging, which adjuvant treatment depends on, definite predictive factors of the prognosis of GC are relatively scarce.^{5–8} Thus, finding new prognostic tools, such as biomarkers, for accurate diagnosis of GC is of vital importance to improve the therapeutic effect and prolong the survival of patients. The eukaryotic initiation factor 3f (eIF3f) is the p47 subunit of the multisubunit eIF3 complex and plays an important role in translation initiation.^{9–11} Although the role of eIF3f in the eIF3 complex has not been defined,^{12–14} shutoff experiments in *Schizosaccharomyces pombe* show that in a long-term period, eIF3f is essential for viability.¹⁵ Translation system indicate that in mammalian cells eIF3f is a negative regulator of translation, which plays an important role in human cancer.^{12,16} Study of Roberta Marchione showed that the f subunit of eIF3 represents a promising candidate molecule to use for bio-therapeutic applications.¹⁷ Previous studies identified eIF3f as a protein involved in apoptotic signaling is a negative regulator of translation and we also demonstrated that eIF3f expression significantly decreased in many human cancers, which plays an important role in human cancer.^{11,16,18,19} Shi et al. report that eIF3f is downregulated in several human tumors, and that its overexpression inhibited cell proliferation in melanoma and pancreatic cancer cells.¹¹ Recent studies of Ana E. Higareda-Mendoza and Marco A. Pardo-Galván also show that eIF3f is essential for A549 cell proliferation and its absence induces the cell to enter apoptosis.²⁰

Human epidermal growth factor receptor 2 (Her-2) is a transmembrane tyrosine kinase receptor involved in development and progression of various solid tumor types, such as breast cancer, pulmonary adenocarcinoma, and colorectal and GC.^{21,22} Moreover, high expression of Her-2/neu has been recognized as one of the poor prognostic factors of breast cancer. Although a great deal of researches has been conducted, the significance of Her-2/neu overexpression in GC and its impact on survival is still controversial.

Taken together, expression of eIF3f or Her-2/neu both play important roles in many kinds of malignant tumors. In this study, we first focused on analyzing the relationship between eIF3f and Her-2/neu. Next, we investigated the expression levels of eIF3f and Her-2/neu by immunohistochemistry and FISH respectively in gastric carcinoma and their correlation with clinicopathological variables, aiming to find a new prognostic marker for GC and new diagnosis and treatment strategies.

Materials and Methods

Patient samples

A review of surgical specimens at the hospital between November 2007 and March 2009 yielded 195 cases of GC patients who had undergone curative surgery. The mean age of the patients was 62 years (range, 31–90 years). The median follow-up period was 38 months (range: 3–54 months). Of the 195 cases, 142 were male (72.8%) and 53 were female (27.2%). Histological type: adenocarcinoma: low differentiated (156 cases 80.0%) moderately differentiated (27 cases 13.8%) well differentiated (12 cases 6.2%). TNM stage: the stage of tumors was assessed according to the 7th ed. of TNM classification of GC.²³ Stage I: 52 patients (26.7%); II: 95 (48.7%); and III: 48 (24.6%). No preoperative chemotherapy or radiotherapy; primary sporadic tumors. The clinical characteristics of these cases were reviewed in Table 1.

¹Department of First Medical Oncology, Affiliated Yantai Yuhuangding Hospital of Qingdao University Medical, Yantai, Shandong, China; ²Department of Endocrinology, Affiliated Yantai Yuhuangding Hospital of Qingdao University Medical, Yantai, Shandong, China; ³Department of Radiology, Affiliated Yantai Yuhuangding Hospital of Qingdao University Medical, Yantai, Shandong, China.

*The authors contributed equally to this work.

Correspondence: Honglun Li (honglunli@126.com)

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Variable	Nucleophosmin/B23 staining		p Value	Her-2/neu staining		p Value
	High	Low		Positive31	Negative164	
Gender (n = 131)						
Male	49	93	0.87	19	123	0.32
Female	17	36		12	41	
Age (n = 131)						
<60	33	51	0.17	18	66	0.96
≥60	33	78		13	98	
Tumor differentiation (n = 131)						
Low	54	102	0.46	15	141	0.0049
Moderately	4	23		12	15	
Well	8	4		4	8	
TNM classification (n = 131)						
I-II	53	83	<0.02	5	131	0.0019
III	13	46		26	33	
Primary/recurrent (n = 131)						
Primary tumor	46	70	0.004	10	106	0.0063
Recurrent tumor	20	59		21	58	

Table 1. Correlation among nucleophosmin/B23, Her-2/neu staining, and clinical characteristics.

For immunohistochemical, the paraffin-embedded tissue sections were obtained from 195 cases of radical resection of GC. The fragments were fixed in neutral formol. All the cases were reviewed by a pathologist to confirm the malignancy. The ethics committee of the medical faculty agreed to these investigations. The written informed consents were obtained from all the patients.

Immunohistochemistry

The polyclonal antibody (ab64177) to eIF3f was bought from Abcom company. The paraffin-embedded tissue blocks were sectioned in 3–4 mm slices and placed on antislides. After dewaxing and hydration, the slides were rinsed in phosphate-buffered saline (PBS), and blocked for 10 minutes with 3% hydrogen peroxide to deprive the endogenous peroxidase activity. After antigen retrieval with the use of a microwave, the specimens were incubated with the anti eIF3f MAb (diluted 1:100 in PBS) at 37°C for 1.5 hours. The sections were then washed 3 × 3 minutes in PBS and incubated with 1 and 2 reagent of PV9000 mouse/rabbit hypersensitivity two steps immunohistochemical Kit (Beijing fir Jinqiao) for total of 60 minutes at 37°C in a humid chamber. The sections were washed 3 × 3 minutes with PBS, followed by the addition of diaminobenzidine (DAB) as a chromogen for 3–5 minutes, which was strictly controlled under microscope. 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 undergone 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 eIF3f-specific immunostaining within a tissue section was determined by the percentage of cells with cytoplasm staining. The immunostaining was read in a semiquantitative manner. Three visual field were examined randomly and the rate of positive cells were divided into 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 can be divided into three grades: no staining (0 score), slightly yellowish (1 score), brownish yellow (2 score), and dark brown (3 score). The multiplication of the two were graded as follows: 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.

Fluorescence *in situ* hybridization (FISH)

FISH analysis was performed using the PathVysion Her-2/neu probe kit (Vysis, Abbott Park, IL, USA) according to the manufacture's protocols. Briefly, the neutral formol fixed and paraffin-embedded sections (3–4 μm) were placed on Anti slides and then were kept in oven overnight at 56°C. After deparaffinized and dehydrated at room temperature, the slides were treated with pretreatment solution (sodium thiocyanite) and protease solution for 15 minutes and were dehydrated with 70%, 80%, and 100% alcohol for 5 minutes each and air dried. The probe was denatured at 80°C for 5 minutes and was added to each slide and sealed under a small glass cover slip before overnight hybridization at 42°C. The procedure was followed by washing in 0.4 × sodium saline citrate (SSC)/0.3% Nonidet (NP40) and 2 × SSC. After hybridization, the nuclei counterstaining was achieved with 4',6-diamidino-2-phenylindole (DAPI). Slides were scored for hybridization signals on a cell-by-cell basis using a fluorescence microscope. Results were expressed as the ratio of Her-2/neu signal (orange) to centromere 17 signal (green)

Figure 1. Immunohistochemical findings of the degree of expressions of eIF3f in GC tissue (A–D) and ANCT (E–H) and Her-2/neu expression in GC tissue by FISH (I–J). eIF3f was localized in the cytoplasm of different levels and percentages. A and E (–); B and F (+); C and G (++) ; D and H (+++). Negative expression of Her-2/neu (I); positive expression of Her-2/neu (J) (magnification of A, D, E, and H $\times 100$); (magnification of F and G $\times 200$); (magnification of B and C $\times 400$).

and the readings were read as follows: the expected ratio 1–1.8 indicates no gene amplification (negative), a ratio of >2.2 as Her-2/neu gene amplification (positive), and a ratio between 1.8 and 2.2 as equivocal cases. The polysomy 17 was also recorded in the cells as four spec green signals as moderate polysomy and >4 spec green signals as high polysomy.

Statistical analysis

Differences between groups were analyzed using a Student's *t*-test for continuous variables and a chi-square test or Fisher's exact test for proportions. The overall survival rate was estimated by the Kaplan–Meier method. Univariate analyses (Cox proportional hazard regression models) were also performed to assess the prognostic value of nucleolin expression and other clinicopathological features. We utilized SAS 9.2 software system for statistical analysis.

Results

clinicopathological characteristics

A total of 142 men and 53 women all accepted radical operation of GC, including 116 primary and 79 recurrent GC patients. Pathologically, all of the 195 cases were adenocarcinoma, which covered 12 well differentiated, 27 moderate differentiated, 156 low differentiated, and 4 signet-ring cell carcinoma.

eIF3f expression in GC and adjacent noncancerous tissue (ANCT)

Immunohistochemical stain for eIF3f showed cytoplasm staining pattern in both GC tissues (Figure 1A–D) and ANCT (Figure 1E–H). Immunoscoring was calculated when the presence of eIF3f deposits in tumor glands and cytoplasmic staining were positive.

The rate of high eIF3f expression in GC tissues was 33.8% (66/195) whereas 59.5% (116/195) in ANCT.

Correlation between eIF3f expression and clinicopathological features

The association of eIF3f levels with clinicopathological features in GC was also assessed in Table 1. Low eIF3f expression in GC was significantly associated with more advanced tumor stages ($p = 0.02$) and likelihood of recurrence ($p = 0.04$). However, eIF3f expression was not significantly correlated with sex ($p = 0.87$), age ($p = 0.17$), and tumor differentiation ($p = 0.46$). A total of 53 (40.0%) of 136 stage I and II cases had high eIF3f staining densitometry values higher than cases of stage III ($p = 0.02$). Furthermore, the overall densitometric analysis of the immunostaining revealed that the down regulated eIF3f protein expression varied significantly between primary and recurrent tumors ($p = 0.04$).

The relationship between the expression level of eIF3f and prognosis

The prognostic effect of eIF3f on the survival rate of GC patients was investigated by comparing the survival rate of patients with high or low levels of eIF3f protein expression in tumors using Kaplan–Meier survival curves and log-rank test. Univariate Cox regression analysis showed that clinical variables, including tumor recurrence (log-rank, $p < 0.01$; Figure 2A) and eIF3f expression (log-rank, $p = 0.04$; Figure 2B) both significantly associated with overall survival (Table 2). Data indicate that high expression of eIF3f protein was a significant prognostic factor for better survival of GC patients. Furthermore, multivariate Cox regression analyses showed that eIF3f expression was not an independent predictor for the overall survival of patients with GC.

Correlation between Her-2/neu expression and tumor grade, TNM classification, and tumor recurrence

We detected the expression of Her-2/neu by FISH and the overexpression was detected in 31 (15.9%) tumors (Figure 1I–J). The possible association of Her-2/neu levels with clinical and pathological factors in gastric carcinoma was also assessed. The chi-square test did not detect significant difference between the expression level of Her-2/neu and the patients' sex, age ($p > 0.05$), but correlated with the differentiation degree, TNM classification, tumor recurrence, or distant metastasis. Compared with the eIF3f, the expression of Her-2/neu was found higher in 15 (7.7%) of 141 poorly differentiated adenocarcinoma than 4 (2.1%) of 8 well-differentiated cases ($p = 0.0049$; Table 1). Simultaneously, 26 (13.3%) of 33 stage III cases had Her-2/neu staining densitometry values higher than 5 (2.6%) of 131 cases of stage I and II ($p = 0.0019$; Table 1). When determined the possible relationship between Her-2/neu values and tumor recurrence or progression, we found that positive Her-2/neu expression was observed in 10.8% of recurrent or metastasis tumors compared with 5.1% of primary tumors ($p = 0.0063$; Table 1).

Figure 2. (A) Kaplan-Meier overall survival curves for primary or recurrent GC patients. Recurrent GC patients had significantly shorter overall survival ($p < 0.01$) than primary GC patients. (B) A comparison of the overall survival curve between patients with low and high eIF3f expression. The median PFS was 42.5 months in patients with high eIF3f expression, compared to 33 months in patients with low eIF3f expression. (C) A comparison of the overall survival curve between patients with negative and positive Her-2/neu expression ($p = 0.12$).

The relationship between the expression level of Her-2/neu and prognosis

From the above results, it was concluded that Her-2/neu expression is closely related to invasion and cancer metastasis and can be used to evaluate the biological behavior. Some authors documented that Her-2/neu appears to be an important

prognostic factor in GC. However, univariate analysis revealed Her-2/neu level was not a prognostic factor for patients' overall survival in our study group ($p = 0.12$; Figure 2C).

The relationship between eIF3f and Her-2/neu

Our present study reveals that Her-2/neu is overexpressed in GC and with no expression in ANCT. This is not in accordance with those of eIF3f. We also found that GC tissues with a high proportion of eIF3f overexpressing, especially those defined as 3+, also present a high level of Her-2/neu immunostaining ($p = 0.0295$; Table 3). The results showed that eIF3f and Her-2/neu expressions were both closely related to later TNM stage and recurrence or metastasis of GC. However, unlike eIF3f, which is identified as a significant and independent prognostic factor of GC, Her-2/neu expression in FISH has no prognostic significance in this study. Meanwhile, this research does not support value of Her-2/neu expression as a prognostic tool in GC.

Conclusion

Despite the incidence and mortality rate of GC have fallen over past several decades, GC is still the fourth most common cancer and the second leading cause of cancer-related death in the world.^{24,25} Disappointingly, in terms of prognosis and quality of life for GC patients, little improvement has been achieved within the past decade although many advances in tumor diagnosis and treatment.^{26,27} Given the high failure rate of conventional treatment strategies, many cancer-related molecules have been characterized with the goal of developing novel anticancer strategy.

eIF3f is another eIF3 subunit whose function is not well known.^{18,28} In this study, we demonstrated that overexpression of eIF3f inhibits cell proliferation and induces apoptosis in melanoma and pancreatic cancer cells, suggesting that down-regulation of eIF3f is involved in tumorigenesis for many types of cancer.^{11,16,19} However, there is no large sample report about the expression and clinical significance of eIF3f in human GC progression and prognosis. In this study, we investigated the expression of eIF3f in

GC as well as its correlation with the clinicopathological features and prognosis.

The data from our study showed that the levels of eIF3f protein were significantly lower in GC tissue compared with paired ANCT. Additionally, the decreased expression of eIF3f protein was significantly related to more advanced tumor stages ($p = 0.02$)

Variable	No.	Hazard ratio HR (95% CI)	p Value
Univariate analysis			
Gender			
Male	142		0.40
Female	53		
Age (years)			
<60	84		0.12
≥60	111		
Tumor differentiation			
Well	12		0.74
Moderate	27		
Low	156		
TNM classification			
I-II	136		0.88
III	59		
Her-2 expression			
Negative	164		0.12
Positive	31		
Primary/recurrent			
Primary tumor	116		<0.01
Recurrent tumor	79		
eIF3f expression			
Low	129		0.04
High	66		
Multivariate analysis			
Primary/recurrent			
Primary tumor	116	3.77	<0.01
Recurrent tumor	79	1.79–7.94	
eIF3f expression			
Low	129	2.27	0.04
High	66	1.05–4.92	

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

eIF3f expression	Her-2/neu expression		Total
	Positive	Negative	
1+	1	19	20
2+	14	17	31
3+	16	16	32
Total	31	52	83

Table 3. Her-2/neu expression in different degree of eIF3f immunostaining in GC.

and likelihood of recurrence ($p = 0.04$). No statistically significant relationship was found between the eIF3f levels and sex ($p = 0.87$), age ($p = 0.17$), and tumor differentiation ($p = 0.46$). The Kaplan–Meier survival curves showed that the patients with low eIF3f

expression had significantly poor survival compared with those patients with high eIF3f expression. Furthermore, multivariate Cox regression analyses showed that recurrence ($p < 0.01$) and eIF3f expression ($p = 0.04$) were both prognostic predictor to human GC after resection. Based on these results, we propose a bold hypothesis that a preoperative determination of eIF3f expression may be useful in predicting the therapeutic effect and postoperative survival of human GC.

Extensive studies of Her-2/neu have been conducted among a variety of tumor types. Researches show that overexpression of Her-2/neu oncogene is a frequent molecular event in multiple human cancers, such as breast cancer, pulmonary adenocarcinoma, and colorectal and GC.^{29,30} In previous studies, Her-2/neu overexpression in 10%–34% of breast cancer cases has been documented to be associated with a poor prognosis.³¹ Favorable clinical results with anti-Her-2/neu therapy in breast cancer have led to the analysis of its expression in other solid tumors, but the clinical significance of its expression is not fully clear yet. The rate of the expression of Her-2/neu is up to 15.9% in our study group. No relationship was found between Her-2/neu overexpression and patients' age, sex. Patients with distant metastasis, late stage, and poorly differentiated status have a significantly higher Her-2/neu expression rate than the remaining cases, which suggests that Her-2/neu may be activated during the process of infiltration and participate in the process of the distant metastasis.

This study of Her-2/neu reveals that the significance of Her-2/neu overexpression in GC and its impact on survival is controversial. Survival analysis in our study did not confirm the prognostic significance of Her-2/neu overexpression in GC patients, which is consistent with Halon's³² recent report. Similar results were observed by Tateishi et al.,³³ who found prognosis was independent of Her-2/neu expression.

We also found that high expression of eIF3f was in conjunction with an over accumulation for Her-2/neu in GC tissue sections. It is perhaps telling that there is a correlation between eIF3f and Her-2/neu in GC. If so, it will provide new insights into the carcinogenesis and progression of GC, thereby finding a novel diagnosis

or treatment and servings to clinic. However, it is all just speculation at this point and the correlation between the two needs to be further investigated at the molecular and cellular levels.

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