Int J Clin Exp Pathol 2017;10(9):9649-9653
www.ijcep.com /ISSN:1936-2625/IJCEP0058950

Original Article

C-erbB-2 expression is related with pathological progression of gastric cancer: results of a non-radioactive in situ hybridization

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Received June 7, 2017; Accepted August 23, 2017; Epub September 1, 2017; Published September 15, 2017

Abstract: Objective: To study the relationship of c-erbB-2 oncogene expression with major pathological characteristics of gastric cancer (GC) progression. Methods: Eighty-one GC specimens were studied for c-erbB-2 oncogene amplification using non-radioactive in situ hybridization method. The c-erbB-2 overexpression status was correlated with tumor differentiation, tumor invasion and lymph node metastasis. Results: Among the 81 pathology confirmed GC patients, 41 (50.6%) were found to have c-erbB-2 overexpression in cancer tissues. The rate of c-erbB-2 overexpression was significantly higher in those with poor tumor differentiation (63.0%, 29/46) than in those with well differentiated tumor (34.3%, 12/35) (χ^2=6.576, P<0.001); significantly higher in those that invaded into deep muscle and beyond (55.7%, 39/70) than in those with tumors limited to the superficial muscle (18.2%, 2/11) (χ^2=5.357, P<0.025); and significantly higher in those with lymph node metastases (59.6%, 34/57) than in those without lymph node involvement (29.2%, 7/24) (χ^2=6.278, P<0.025). Conclusions: c-erbB-2 oncogene overexpression may indicate a more aggressive biological behavior of the tumor and could be used as a predictive marker for GC pathological progression.

Keywords: Gastric cancer, c-erbB-2 gene, in situ hybridization

Introduction

Proto-oncogene c-erbB-2 (also called neu of HER-2) located on human chromosome 17q21 belongs to a subfamily of type I receptor tyrosine protein kinase, which encodes a 185 kDa transmembrane growth factor glycoprotein (also called P185 protein) that contains an extracellular ligand-binding domain and intracellular tyrosine kinase activity. The extracellular region is structurally similar to that of epidermal-growth-factor receptor [1]. Like epidermal growth factor, c-erbB-2 expression reflects an increase in the proliferative activity of a tumor [2]. c-erbB-2 expression is observed only in low levels in epithelial cells of most organs in normal human tissues and at slightly higher levels in fetal tissues. Overexpression is observed in many different malignancies such as breast, ovarian, gastric, lung, prostate, colonic, and other cancers [3-6]. In this paper, we studied c-erbB-2 expression profile in GC tissues using in situ hybridization, and explored the significance of this gene in GC pathological progression.

Materials and methods

Clinical specimens

Eight-one pathology-confirmed gastric carcinoma specimens were collected from Jan 2016 to Jun 2017 from the Affiliated Hospital of Taishan Medical College and the Institute of Oncology, Medical College of Wuhan University. There were 53 males and 28 females aged between 37 to 65 years old (mean age 58.6 years). Among these specimens were 35 cases with carcinomas at gastric cardia, 17 cases at gastric body and 29 cases at antrum. According to

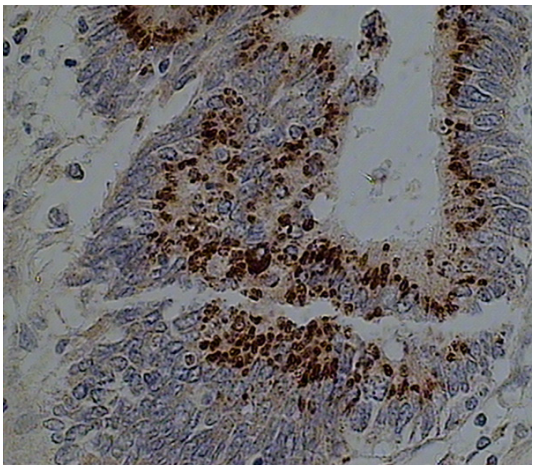

Figure 1. c-erbB-2 expression in gastric cancer tissues detected by non-radioactive labeled in situ hybridization. Frozen section gastric cancer slides were hybridized with digoxigenin-11-UTP labeled c-erbB-2 probe, developed by BCIP/NBT and counterstained by eosin. Positive stain was shown as dark brown particles in gastric cancer cell nests (400×).

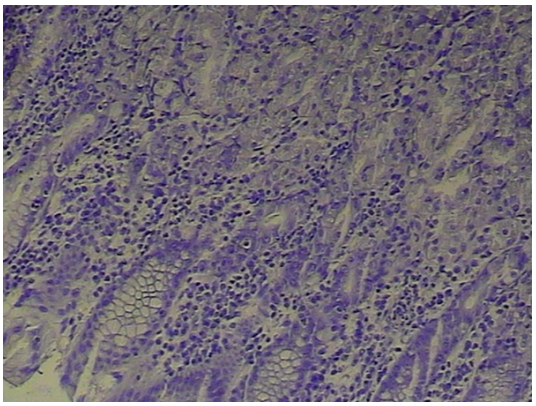

Figure 2. (Negative control) non radioactive labeling in situ hybridization to detect the expression of c-erbB-2 in normal gastric mucosa. The gastric mucosa biopsy dig -11-UTP labeling c-erbB-2 probe hybridization in frozen sections, stained by BCIP/NBT and eosin. Negative staining showed that the developed gastric cell nests deep blue granules (200×).

Lauren classification, there were 33 cases with intestinal type carcinoma and 48 cases with diffused type carcinoma. Thirty-five cases were with well-differentiated carcinoma and 46 were poorly differentiated. In 11 cases cancer invaded superficial muscle layer and in the rest cancer invaded beyond deep muscle. Fifty-seven of these cases had lymph node metastases and 24 had no lymph node metastases. After surgical removal, the specimens were snap frozen in liquid nitrogen and cryo-sectioned at -260C, with thickness between 6 to 10 μm, which were mounted on aminopropyltriethoxysilane (APES, Sigma, St Louis, Ohio, USA) treated slides.

In situ hybridization

c-erbB-2 cDNA probe was a kind gift of Professor Yamamoto from Tokyo University, Japan. It is the product of c-erbB-2 cDNA degradation by endonucleases Dra Iand Sma I, with the length of 461 base pairs (bp). Non-radioactive digoxigenin-11-UTP labeling was performed using random priming method, with reagent kit from Boehringer mannheims (purchased from Sino-American Biotechnology Company, No. 007, Sanshan Road, National Hi-Tech Industry Development Zone, Luoyang, Henan Province, China). The probe concentration was 15 μg/ml and sensitivity was 0.1 pg DNA. After treatment with 0.1 N HCl, RNase A, protease K and 0.1% Glycin, the slides were pre-hybridized for one hour at room temperature in pre-hybridization solution (4×SSC, 50% formamide, 1× Denhardt solution, 0.5% PEG and 0.5 mg/ml ssDNA). After washing the hybridization solution (probe concentration 0.2 μg/ml) was added, the slides were sealed with cover glass and treated in formamide chamber at 95°C for 10-15 min. Then the slides were hybridized overnight at 42°C in a humid chamber, after which the cover glass was removed in 2×SSC solutions, and alkaline phosphatase labeled anti-DIG antibody (1:500 dilution) was added and incubated for 2 h. After washing, 5-bromo-4-chloro-3-indoxyl phosphate disodium (BCIP)/nitroblu-tetrazolium (NBT) color development substrate was added and kept in the dark for color reaction, which was terminated by TE buffer washing when color reaction was sufficient. The slides were counter-stained with eosin, and sealed. Normal gastric tissues from the same specimens were treated in the same fashion as negative controls.

Slides interpretation

When the slides were viewed under microscope (400×), dark brown-blue granules and particles could be found in positively stained cells, and the background was stained red by eosin. Positive and negative stained specimens were recorded respectively. Data on conventional pathology of the specimens were collected from routine pathological report based on gross pathology and hematoxylin and eosin (HE) stained tissue slides.

Table 1. c-erbB-2 expression and the pathological characteristics of the GC studied

Pathological characteristics	Number	c-erbB-2 positive			*P* value*
		n	Percent of the group	Percent of the total (%)	
Lauren classification					
Intestinal type	33	17	51.5	21.0	NS
Diffuse type	48	24	50.0	29.6	
Differentiation					
Well differentiated	35	12	34.3	14.8	χ^2=6.576
Poorly differentiated	46	29	63.0	35.8	P<0.001
Depth of invasion					
Superficial muscle	11	2	18.2	2.5	χ^2=5.357
Deep muscle	70	39	55.7	48.1	P<0.025
Lymph node metastases					
Yes	57	34	59.6	42.0	χ^2=6.278
No	24	7	29.6	8.6	P<0.025

NS: not significant. *Chi-square test.

Statistical analysis

Comparisons between c-erbB-2 positive rates in different pathological subgroups were analyzed using Chi-square test, with P<0.05 as statistical significance.

Results

c-erbB-2 expression in GC tissues

Of the 81 GC specimens, 41 (50.6%) were found to have c-erbB-2 overexpression, which showed dark-brown granules in cancer tissue (**Figure 1**), negative results are shown in **Figure 2**. The relationship of c-erbB-2 overexpression and the pathologic characteristics of tumors were summarized in **Table 1**.

Correlation between c-erbB-2 overexpression and major pathological characteristics pertaining to cancer progression

Three major pathological parameters related to cancer progression-tumor differentiation, depth of tissue invasion and lymph node metastasis-were particularly analyzed in this study to explore their correlation with c-erbB-2 expression.

In terms of tumor differentiation, there were 35 (43.2%, 35/81) cases with well differentiated tumors and 46 (56.8%. 46/81) poorly differentiated tumors. Among the 41 c-erbB-2 positive cases, 12 were well differentiated tumor, accounting for 14.8% (12/81) of the total specimens studied and 34.3% (12/35) of the well differentiated group, and 29 were poorly differentiated, accounting for 35.8% (29/81) of the total and 63.0% (29/46) of the poorly differentiated group (χ^2=6.576, P<0.001) (**Figure 3**).

In terms of tumor invasion, there were 11 cases (13.6%, 11/81) with tumors invasion limited to the superficial muscle layer and 70 cases (86.4%, 70/81) with tumors invading beyond the deep muscle layer. In cases with superficial muscle layer invasion, c-erbB-2 overexpression was found in 2 cases, accounting for 18.2% (2/11) of the group or 2.5% (2/81) of the total specimens studied. On the other hand, in cases with deep muscle invasion, c-erbB-2 overexpression was observed in 39 cases, accounting for 55.7% (39/70) of the group or 48.1% (39/81) of the total specimens studied. The difference in c-erbB-2 expression between these two groups were statistically very significant (χ^2=5.357, P<0.005) (**Figure 3**).

In terms of lymph node metastases, there were 57 cases (70.4%, 57/81) with lymph node metastases and 24 cases (29.6%, 24/81) without lymph node metastases. Of the lymph node positive group, c-erbB-2 overexpression was found in 34 cases, which was 59.6% (34/57) of the group or 42.0% (34/81) of the total specimens studied. In contrast, in the lymph node negative group, c-erbB-2 overexpression was found in 7 cases, accounting for 21.2% (7/24) of the group or 8.6% (7/81) of the total. The differences between these two groups reached statistically significance (χ^2=6.278, P<0.001) (**Figure 3**).

Discussion

GC is one of the most common malignant tumors worldwide and continues to be a highly aggressive malignancy with poor prognosis and low survival rates. The survival of patients with

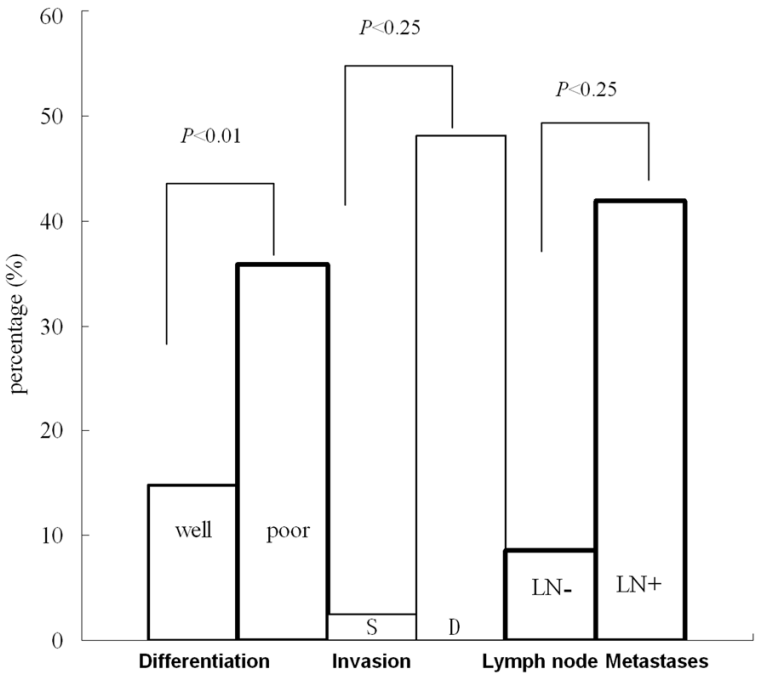

Figure 3. Relationship between c-erbB-2 overexpression and pathological characteristics in gastric cancer. Well = well differentiated, poor = poorly differentiated; S = tumor invading superficial muscle layer, D = tumor invading deep muscle layer; LN- = lymph node metastasis negative, LN+ = lymph node metastasis positive. The percentage of c-erbB-2 overexpression was much higher in poorly differentiated tumors (35.8%) than in well differentiated tumors (14.8%) (P<0.001), much higher in tumors invading deep muscle (48.1%) than those invading superficial muscle (2.5%) (P<0.001), and much higher in tumors with lymph node metastases (42.0%) than in those without lymph node metastases (8.6%) (Chi-square test).

GC depends mainly on the stage of the disease, with early GC having a 5 year survival of 90-100% and advanced tumors a 5 year survival of 15-25%. The role of other prognostic factors in these tumors is still under investigation [7]. Conventional pathology demonstrated that certain types of GC usually showed much more aggressive biological behavior than other types, and such pathological features include tumor differentiation, depth of tumor invasion and lymph node metastasis [8]. With the development of molecular biology, it has been found that there are many genes involved in GC development and pathological progression, including the plasminogen activator (uPA) and its inhibitor PAI-1 (plasminogen activator inhibitor type 1), the cell-cycle regulator cyclin E, epidermal growth factor (EGF), growth factor receptors c-erbB-2, the apoptosis inhibitor bcl-2, the cell adhesion molecule E-cadherin, and the multifunctional protein beta-catenin [9]. The relationship between these molecular markers and the conventional pathological parameters is currently under intensive investigation, as elucidation of these molecular profiles may provide more information on biological behavior of GC.

Using highly sensitive in situ hybridization method, we found 50.6% (41/81) of the gastric tumors had c-erbB-2 overexpression in the present work. Previous studies of c-erbB-2 in GC have shown that the frequency of its overexpression varies from 9 to 38% [10-13]. Two major reasons may account for the fact that c-erbB-2 overexpression was higher in our series than those reported previously. First, most of our patients were late stage GC. Among the 81 tumors, 57 (70.3%) had lymph node metastasis and 70 (86.4%) had tumors spreading beyond the deep muscle layer, as examined by routine pathology. These figures are much higher than those reported by other investigators. Secondly, we used in situ hybridization method to detect c-erbB-2 overexpression at mRNA level on freshly obtained GC tissues snap frozen in liquid nitrogen immediately after resection. This method can preserve the gene product to the maximum. On the other hand, most previous study used immunohistochemical method to study c-erbB-2 protein expression on paraffin embedded tissue blocks fixed in various fixatives. This method has limited sensitivity as demonstrated by a study that confirmed fixation and paraffin wax embedding affect the results of immunohistochemical demonstration of c-erbB-2 in GC [14].

Our results suggested that c-erbB-2 overexpression was related with GC pathological progression. The percentage of c-erbB-2 overexpression was higher in poorly differentiated GC (35.8%) than in well differentiated GC

(14.8%), higher in tumors invading beyond deep muscle 48.1% than in those limited to superficial muscle (2.5%), and higher in tumors with lymph node metastases (42.0%) than those without lymph node metastases. These results are in consistency with previous findings suggesting that c-erbB-2 overexpression may denote a group of highly aggressive GC with poor prognosis [7, 9, 13]. As c-erbB-2 may play an important role to predict more aggressive biological behavior of gastric cancer, this molecule could be used as a valuable marker for GC prognosis.

Acknowledgements

Shandong science and technology development plan (No. 2013YD21013); Shandong medical and health science and technology development plan (No. 2011HW083).

Disclosure of conflict of interest

None.

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