·Basic Research·

Expression and clinical significance of c-Cbl, Cbl-b, and epidermal growth factor receptor in gastric carcinoma

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[**Abstract**] **Background and Objective:** c-Cbl and Cbl-b are two ubiquitous members of the Casitas B-lineage lymphoma (Cbl) family, which play important roles in the downregulation of epidermal growth factor receptor (EGFR) by acting as E3 ubiquitin ligases and multiadaptor proteins. This study investigated the expression of c-Cbl, Cbl-b, and EGFR in gastric carcinoma and its clinical significance. **Methods:** The expressions of c-Cbl, Cbl-b, and EGFR were detected by immunohistochemistry using tissue microarrays consisting of 124 specimens of gastric carcinoma and 16 specimens of normal gastric mucosa. The relationship between the expressions of c-Cbl, Cbl-b, and EGFR and clinicopathologic factors of gastric carcinoma were analyzed statistically. **Results:** The positive rates of c-Cbl, Cbl-b, and EGFR were higher in the gastric carcinoma group than in the normal group (71.0% vs. 18.0%, $P < 0.01$; 82.3% vs. 25.0%, $P < 0.01$; 56.5% vs. 12.5%, $P < 0.01$, respectively). The expression of c-Cbl was positively correlated with depth of invasion ($r = 0.219$, $P = 0.015$), and TNM staging ($r = 0.266$, $P = 0.003$). The expression of Cbl-b was positively correlated with lymph node metastasis ($r = 0.190$, $P < 0.034$) and TNM staging ($r = 0.298$, $P < 0.001$). The expression of EGFR was positively correlated with depth of invasion ($r = 0.286$, $P < 0.001$) and TNM staging ($r = 0.362$, $P = 0.000$). The expression of both c-Cbl and Cbl-b was positively correlated with EGFR ($r = 0.241$, $P = 0.007$; $r = 0.183$, $P = 0.042$, respectively). Synchronous strong-positive expressions of c-Cbl, Cbl-b, and EGFR were observed in 27 specimens of gastric carcinoma, most of which were at advanced stage. **Conclusions:** Overexpressions of c-Cbl, Cbl-b, and EGFR are closely related to the invasion and progression of gastric carcinoma. c-Cbl and Cbl-b may serve as novel molecular markers for gastric carcinoma.

Key words: c-Cbl proteins, Cbl-b proteins, epidermal growth factor receptor (EGFR), gastric carcinoma

Epidermal growth factor receptor (EGFR) is a member of the ErbB family and a membrane-receptor protein of 170 kDa with tyrosine kinase (TK) activity. When binding to the growth factor, it activates intracellular signal transduction and promotes cell proliferation, while the receptor per se is internalized and downregulated.[1] c-Cbl and Cbl-b are two important members of the Casitas B-lineage lymphoma (Cbl) family. Acting as bridge molecules and E3 ubiquitin ligases, they can target a series of receptor proteins with TK activity, representatively EGFR, and induce the degeneration of these receptors, and thus have important roles in maintaining homeostasis in the body. However, the coexpression of c-Cbl, Cbl-b, and EGFR in gastric cancer and its association are rarely reported in foreign and domestic literature. By creating microarrays with gastric cancer tissue and using the streptavidin-peroxidase (SP) immunohistochemical technique to measure the expression profile of these three proteins in gastric cancer, our study analyzed the correlation of the expression of c-Cbl, Cbl-b, and EGFR with the clinical and pathologic features of gastric cancer to investigate the clinical implications.

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Materials and Methods

Clinical data

Specimens of gastric cancer tissue were collected from 124 patients who underwent surgical resection at the First Hospital of China Medical University and Liaoning Cancer Hospital and Institute between October 2005 and August 2006 and who were radiotherapy-, chemotherapy- and immunotherapy-naïve before

surgery. Of these patients, 92 were men and 32 were women. They ranged in age from 24 years to 85 years, with a median age of 60 years. In all patients, pathology was adenocarcinoma. As for the level of differentiation, the disease was rated as highly differentiated in 11 patients, moderately differentiated in 29 patients, and poorly differentiated in 84 patients. Based on the pTNM staging system revised by the International Union Against Cancer (UICC) in 1997, the disease was rated as T1 in 3 patients, T2 in 73 patients, T3 in 40 patients, and T4 in 8 patients. Lymph node metastasis was seen in 100 cases and distal metastasis in 5 cases. The cancer was rated as stage I in 17 patients, stage II in 46 patients, stage III in 37 patients, and stage IV in 24 patients. In 16 of the 124 cases, normal tissue was obtained as controls from mucosa 5 cm away from the cancer lesion.

Methods and reagents

A total of 124 specimens of gastric cancer and 16 specimens of normal gastric tissue were prepared into tissue chips (a technique supported by Shanghai Outdo Biotech Co., Ltd). A brief description of the preparation follows. Paraffin-embedded tissue was first confirmed by a pathologic specialist. Tissue in the paraffin was then localized, and a tissue microarray apparatus was used for sampling (0.6 mm in diameter) from two points in each specimen, and a tissue array was thus prepared. Continuous sections of 4 μm in thickness were sliced. The sections were suspended, dried, and pathologically confirmed again to prepare several tissue chips for immunohistochemical staining (Figure 1). The SP technique was used for the staining, which was conducted as described in the protocol of the reagent kit. Known positive control sections were used as positive controls and phosphate buffered saline replaced the primary antibody as the negative control. The mouse anti-human c-Cbl monoclonal antibody was produced by BD Company; the mouse anti-human Cbl-b monoclonal antibody was produced by Santa Cruz; the mouse anti-human EGFR monoclonal antibody, the SP detection reagent kit, and the diaminobenzidin (DAB) solution were from Maxim Biotech Co., Ltd.

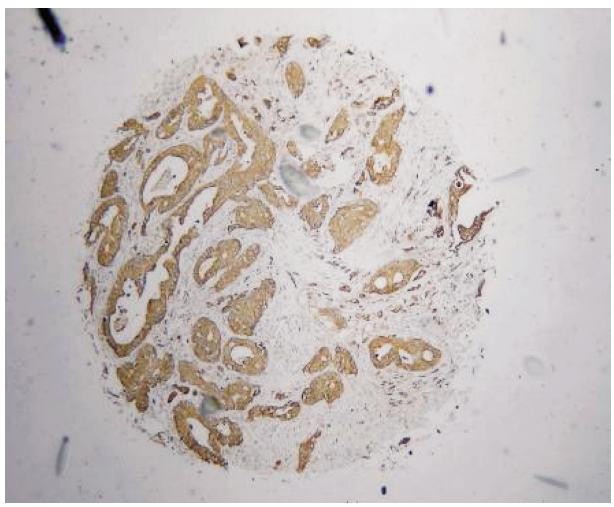

Figure 1 A stained spot of the tissue microarrays (SP ×40)

The evaluation of the immunohistochemical staining results

Positive staining of c-Cbl, Cbl-b, and EGFR was all indicated as yellowish brown granules in the cytomembrane, the cytoplasm, or both. Sections were observed through microscopic examination (×400) by staff in a blinded fashion. From each section, five visual fields were randomly selected and the score for each visual field depended on its percent of positive cells and their staining intensity. For the percent of positive cells, ≤5%, 6%–25%, 26%–50%, 51%–75% and ≥ 76% were recorded as 0, 1, 2, 3, and 4 points, respectively. For staining intensity, non-stained, light yellow, yellowish brown, and brown were recorded as 0, 1, 2, and 3 points, respectively. The arithmetic product of these two scores (percent score and intensity score) was regarded as the score for that visual field, and the average score of those five visual fields were regarded as the final score for that section. Finally, 0–1 point was recorded as (-), which indicated negative staining; 2–4 points were recorded as (+), which indicated weak-positive staining; 5–7 points were recorded as (++) and ≥ 8 points as (+++), which indicated strong-positive staining.

Statistical analyses

SPSS version 13.0 was used for the statistical analyses. Numerical data were compared between groups using a χ^2 test for a four-fold table. Expression levels of c-Cbl, Cbl-b, and EGFR were shown as sequentially ranked data, which were compared between groups using the Mann-Whitney *U* test. Analysis on correlation was performed using the Spearman rank correlation. $P<0.05$ indicated statistical significance.

Results

High expression levels of c-Cbl, Cbl-b, and EGFR in gastric cancer tissue

Positive staining of c-Cbl, Cbl-b, and EGFR was seen in the cytoplasm, the cytomembrane, or both, as homogenous yellowish brown granules (Figure 2). In gastric cancer tissue, the positive rates of c-Cbl, Cbl-b, and EGFR were 71.0%, 82.3%, and 56.5%, respectively. Whereas in normal gastric tissue, the positive rates of these molecules were 18.0%, 25.0%, and 12.5%, respectively. The expression levels of all these molecules in gastric cancer tissue were significantly higher than those in normal tissue ($P \leq 0.001$).

Correlations between the expression of c-Cbl, Cbl-b, and EGFR and the clinical and pathologic features of gastric cancer (Table 1)

c-Cbl was positively expressed at various levels in 71.0% of all gastric cancer tissue. Its expression was positively correlated to the depth of infiltration and the pathologic staging of gastric cancer ($r = 0.219$, $P = 0.015$; $r = 0.266$, $P=0.003$), but not to sex, age, the level of differentiation, or lymph node metastasis.

Out of the 124 specimens of gastric cancer, 102 expressed the Cbl-b protein to various extents, and its expression was positively correlated to the presence of lymph node metastasis and the pathologic staging of gastric cancer ($r = 0.190$, $P =$

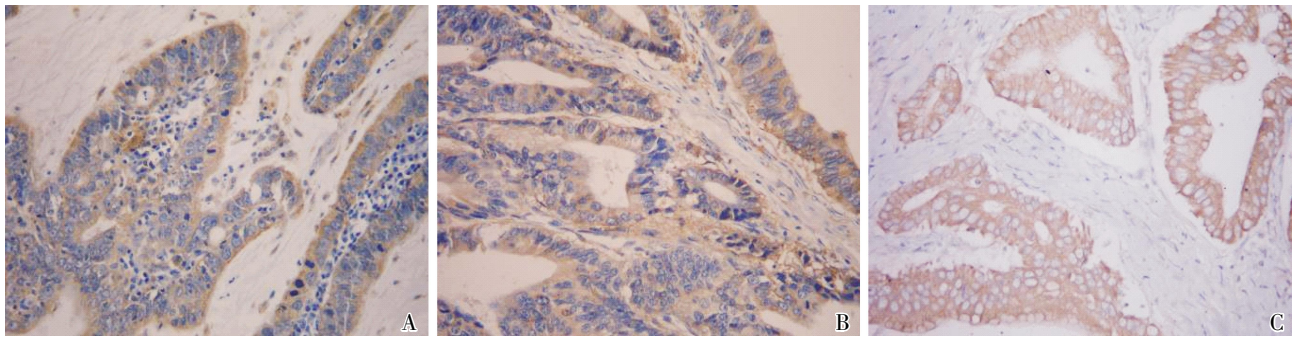

Figure 2 Expressions of c-Cbl, Cbl-b, and EGFR in gastric carcinoma (SP ×200)

A, strong-positive staining of c-Cbl in gastric carcinoma. Typically brown granules were diffusely distributed in the membrane or the cytoplasm of cancer cells.

B, strong-positive staining of Cbl-b in gastric carcinoma. Typically brown granules were diffusely distributed in the membrane or the cytoplasm of cancer cells.

C, strong-positive staining of EGFR in gastric carcinoma. Typically brown granules were diffusely distributed in the membrane or the cytoplasm of cancer cells.

0.034; $r = 0.298$, $P = 0.001$). In addition, Cbl-b expression tended to increase with the depth of infiltration ($P = 0.057$). Cbl-b expression was not related to sex, age, or the level of differentiation.

EGFR expression was correlated with the depth of infiltration and the pathologic staging of gastric cancer, but not sex, age, the level of differentiation, or lymph node metastasis. In gastric cancer, Spearman correlation coefficients of EGFR expression to the depth of infiltration and pathologic staging were 0.286 ($P = 0.001$) and 0.362 ($P < 0.001$), respectively, which indicated positive correlations. However, EGFR expression was not significantly correlated to other clinical or pathologic factors, including the level of differentiation ($P > 0.05$).

Table 1 Relationship between levels of expression of c-Cbl, Cbl-b, and EGFR and clinicopathologic characteristics of gastric carcinoma

Clinicopathological characteristic	Patient No.	c-Cbl expression [patient No.(%)]			P	Cbl-b expression [patient No.(%)]			P	EGFR expression [patient No.(%)]			P
		(-)	(+)	(++) (+++)		(-)	(+)	(++) (+++)		(-)	(+)	(++) (+++)	
Total	124	36(29.0)	19(15.3)	69(55.6)		22(17.7)	13(10.5)	89(71.8)		54(43.5)	33(26.6)	37(29.8)	
Sex													
Men	92	23(25.0)	15(16.3)	54(58.7)	0.150	13(14.1)	10(10.9)	69(75.0)	0.130	35(38.0)	27(29.3)	30(32.6)	0.058
Women	32	13(40.6)	4(12.5)	15(46.9)		9(28.1)	3 (9.4)	20(62.5)		19(59.4)	6(18.8)	7(21.9)	
Age (years)													
≤60	65	18(27.7)	12(18.5)	35(53.8)	0.878	10(15.4)	5 (7.7)	50(76.9)	0.211	30(46.2)	16(24.6)	19(29.2)	0.639
>60	59	18(30.5)	7(11.9)	34(57.6)		12(20.3)	8(13.6)	39(66.1)		24(47.0)	17(28.8)	18(30.5)	
Differentiation													
Well and moderate	40	11(27.5)	4(10.0)	25(62.5)	0.411	4(10.0)	6(15.0)	30(75.0)	0.417	12(30.0)	16(40.0)	12(30.0)	0.173
Poor	84	25(29.8)	15(17.9)	44(52.4)		18(21.4)	7 (8.3)	59(70.2)		42(50.0)	17(20.2)	25(29.8)	
Depth of invasion													
T1,T2	76	29(38.2)	10(13.2)	37(48.7)	0.015	17(22.4)	9(11.8)	50(65.8)	0.057	41(53.9)	19(25.0)	16(21.1)	0.002
T3,T4	48	7(14.6)	9(18.8)	32(66.7)		5(10.4)	4 (8.3)	39(81.3)		13(27.1)	14(29.2)	21(43.8)	
Lymph-node metastasis													
No	24	9(37.5)	0 (0.0)	15(62.5)	0.907	7(29.2)	4(16.7)	13(54.2)	0.035	12(50.0)	3(12.5)	9(37.5)	0.984
Yes	100	27(27.0)	19(19.0)	54(54.0)		15(15.0)	9 (9.0)	76(76.0)		42(42.0)	30(30.0)	28(28.0)	
TNM staging													
Ⅰ,Ⅱ	63	31(49.2)	1 (1.6)	31(49.2)	0.003	20(31.7)	5 (7.9)	38(60.3)	0.001	40(63.5)	10(15.9)	13(20.6)	0.002
Ⅲ,Ⅳ	61	5 (8.2)	18(29.5)	38(62.3)		2(3.3)	8(13.1)	51(83.6)		14(23.0)	23(37.7)	24(39.3)	

*$P < 0.05$.

(-) negative; (+) weak positive; (++) (+++) strong positive.

Correlations between the expression of c-Cbl/Cbl-b and EGFR and their coexpression

Expression levels of both c-Cbl and Cbl-b were positively related to the expression level of EGFR in gastric cancer (r = 0.241, P = 0.007; r = 0.183, P = 0.042) (Table 2).

Out of 124 cases of gastric cancers, 27 showed strong-positive expression of all three proteins, mostly in patients with poorly differentiated cancer, profound tumor infiltration, numerous metastatic lymph nodes, and later-stage disease (Table 3).

Table 2 Correlation between the expression levels of c-Cbl, Cbl-b, and EGFR

EGFR expression	Patient No.	c-Cbl expression [patient No.(%)]			r	P	Cbl-b expression [patient No.(%)]			r	P
		(-)	(+)	(++) (+++)			(-)	(+)	(++) (+++)		
(-)	54	23(42.6)	4 (7.4)	27(50.0)	0.241	0.007	15(27.8)	4 (7.4)	35(64.8)	0.183	0.042
(+)	33	10(30.3)	8(24.2)	15(45.5)			5(15.2)	4(12.1)	24(72.7)		
(++)(+++)	37	3 (8.1)	7(18.9)	27(73.0)			2 (5.4)	5(13.5)	30(81.1)		

(-) negative; (+) weak positive; (++) (+++) strong positive. $P < 0.05$.

Table3 Clinicopathologic characteristics of the 27 cases with simultaneously strong-positive expressions of c-Cbl, Cbl-b, and EGFR

Pathological characteristic	Number	%
Differentiation		
Well and moderate	10	37.0
Poor	17	63.0
Depth of invasion		
T1	1	3.7
T2	10	37.0
T3–T4	16	59.3
Lymph-node metastasis		
N0	7	25.9
N1	10	37.0
N2–N3	10	37.0
Stage		
Ⅰ	3	11.1
Ⅱ	7	25.9
Ⅲ	7	25.9
Ⅳ	10	37.0

Discussion

The binding of EGFR to its ligand promotes the formation of dimers and the phosphorylation of EGFR and thus activates EGFR. Activated EGFR can transduce signals into the cells. On the other hand, activated EGFR recruits c-Cbl/Cbl-b, which, in turn, induces the ubiquitination of EGFR. Thereby ubiquitinated EGFR combines to CIN85 (Cbl-interacting protein, relative molecular mass 85000) and endophilin (endophilin changes the phosphorylation status of the cytomembrane by its acyltransferase activity and thereby promotes curvature changes and invagination of the cytomembrane in the early stage of internalization) to create a complex, which is then internalized into the multivesicular bodies and finally degraded in the lysosomes.[2]

In this study, we detected high expression levels of c-Cbl, Cbl-b, and EGFR in gastric cancers. In addition, the expression of c-Cbl was related to the depth of infiltration and pathologic staging of gastric cancer, the expression of Cbl-b related to lymph node metastasis and pathologic staging, and EGFR expression related to the depth of infiltration and pathologic staging. Currently domestic and foreign studies regarding the coexpression of c-Cbl, Cbl-b, and EGFR and their correlation in gastric cancer are rare. In the only relevant report, Japanese scientists Ito et al measured the coexpression of c-Cbl and EGFR in gastric cancer.[3] Their study revealed that c-Cbl expression was positive in 67% of the cases and was correlated to the depth of infiltration of the tumor and the presence of lymph node metastasis. Moreover, it was positively correlated to EGFR expression, and patients with coexpressed c-Cbl and EGFR had poor prognosis. Our study reached similar conclusions. The multivariate analysis by Ito *et al*[β] suggested that the most significant factor influencing prognosis was tumor staging, rather than the expression of the c-Cbl protein.[3] Therefore, we cannot completely exclude the possibility that high expression levels of c-Cbl and Cbl-b in gastric cancer may also be an accompanying event in tumor occurrence. The exact role of c-Cbl and Cbl-b in gastric cancer needs further investigation.

Current studies have suggested that the EGF receptor/ligand system was involved in the proliferation of gastric mucosa and the occurrence and development of gastric cancer, and that overexpression or increased activation of EGFR was closely related to the occurrence and prognosis of gastric cancer.[4] Both single-variate and multivariate analyses demonstrate that a high expression level of EGFR was an independent prognostic factor for poor prognosis in patients with gastric cancer.[5,6] Arao et al used the EGFR TK-inhibitor ZD6474 in animal models laden with undifferentiated gastric cancer and revealed significant inhibition to tumor growth.[7] Our results showed that the hyperexpression of EGFR was positively correlated to profound infiltration and the later stages of gastric cancer. This was consistent with published literature.[3-8] Therefore, EGFR is considered a valuable marker in determining the malignant level and invasiveness of gastric

cancer.

Studies have shown that, as inhibitors of receptor (including EGFR) tyrosine kinase (RTKs), c-Cbl and Cbl-b downregulated RTKs signals.[9] However, some studies also suggested that the c-Cbl and Cbl-b proteins were involved in promoting the infiltration of tumor cells.10 C-Cbl and Cbl-b proteins bind to the SH3 domain of CIN85, but the binding is largely dependent on the tyrosine phosphorylation of c-Cbl and Cbl-b as induced by EGF. Nam *et al.*[10] reported that in the breast cancer cell line MDA-MB-231, the interaction between endogenous AMAP1 (an effector of GTP-Arf6 involved in the metastasis of breast cancer[11,12]) and c-Cbl and Cbl-b was dependent on EGF stimulation. Their study showed that CIN85 could also bind to AMAP1 and thus promote the interaction between c-Cbl, Cbl-b, and AMAP1. The study used small interfering ribonucleic acid (siRNA) to inhibit the effects of c-Cbl and Cbl-b and revealed that the infiltration activity in MDA-MB-231 cells was significantly inhibited. Knockdown of the c-Cbl and Cbl-b genes also inhibited matrix degradation, and cells transfected with the mutated Cbl-b gene lost its matrix degradation activity. These findings suggest that c-Cbl and Cbl-b were involved in the infiltration of breast cancer. Our study revealed high expression levels of c-Cbl and Cbl-b in gastric cancer and a significantly positive correlation between high expression levels of c-Cbl and the depth of infiltration of gastric cancer. The levels of expression of Cbl-b tended to increase with infiltration depth (P = 0.057), with a significantly positive correlation to the presence of lymph node metastasis in gastric cancer. These findings indicate that c-Cbl and Cbl-b might be involved in the infiltration of the tumor.

The study by Nam *et al.*[10] showed that the interaction between c-Cbl, Cbl-b, and CIN85/AMAP1 was initially dependent on EGF stimulation in MDA-MB-231 cells, indicating that c-Cbl, Cbl-b, and CIN85 were involved in promoting EGF-induced increased infiltration activity in breast cancer cells. Our study demonstrated a significantly positive correlation between the levels of expression of c-Cbl, Cbl-b, and EGFR in gastric cancer, suggesting that c-Cbl and Cbl-b might also be involved in promoting EGF-induced increased infiltration activity in gastric cancer cells.

Our study showed that c-Cbl, Cbl-b, and EGFR were highly expressed in gastric cancer tissue and that their expression levels were related to the invasion and development of gastric cancer. Codetection of c-Cbl and Cbl-b and EGFR was significant in determining the malignant level of gastric cancer. Both c-Cbl and Cbl-b were positively correlated with EGFR, indicating their possible synergy in the proliferation, infiltration, and metastasis in gastric cancer. Codetection of c-Cbl and Cbl-b and the EGFR system might be somewhat valuable in the further exploration into the biologic behavior of gastric cancer. c-Cbl and Cbl-b might become new molecular markers for gastric cancer.

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