

Expression of Cathepsin B and Its Relationship with Esophageal Squamous Carcinoma

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Abstract: Objective. To explore the relations between expression of Cathepsin B (CB) and development, invasion and metastasis of esophageal squamous cell carcinoma. **Methods.** The expression of CB protein and of CB mRNA were determined by immunohistochemistry and *in situ* hybridization. **Results.** CB protein expression and CB mRNA expression couldn't be detected in normal esophageal mucosa, while positive in metastatic group and non-metastatic group. CB protein expression and CB mRNA expression was significantly decreased. The out layer invasion group, rate of CB protein expression and CB mRNA expression in the tumor tissues and atypical hyperplasia in tumor-adjacent tissues was significantly elevated compared with low-muscle invasion group and deep-muscle invasion group. The positive rates was slightly higher in deep-muscle invasion than in low-muscle invasion without statistical significance. The positive rates of CB protein expression and of CB mRNA expression in the carcinoma tissues were significantly higher than atypical hyperplasia tissues in both the metastatic and non-metastatic group. **Conclusion.** CB protein expression and CB mRNA expression in human esophageal squamous cell carcinoma were increased, which indicated that CB is related to the development, invasion and metastasis of esophageal carcinoma [Life Science Journal. 2006;3(3):19-24] (ISSN: 1097-8135).

Keywords: esophageal squamous cell carcinoma; Cathepsin B; invasion; metastasis

Abbreviations: CB: Cathepsin B

1 Introduction

Invasion and metastasis of tumor is a complicated process, in which the relationship of extracellular matrix and metastasis of tumor is extremely close. A series of dynamic change develop between tumor cells and extracellular matrix. There are a lot of enzymes released that degrade the extracellular matrix and facilitate the invasion and metastasis of tumor cells^[1]. It is known that Cathepsin B (CB) is a lysosomal cysteine proteinase that can degrade laminin, fibronectin, IV type collagen and so on the extracellular matrix, and participating in the invasion and metastasis of tumor cells. In recent years, the research discovered that CB showed an obvious increase in varieties of malignant tumors such as prostate carcinoma, melanocytoma, carcinoma of colon and so on. It also participates in the invasion and metastasis of tumor. While both domestic and foreign scholars haven't carried out the research for CB and its relationship with invasion and metastasis of esophageal carcinoma. This research adopted the technique of immunohistoche-

mistry and *in situ* hybridization, to investigate CB expression and its relationship with the development, invasion and metastasis of esophageal carcinoma.

2 Materials and Methods

2.1 Materials

2.1.1 Reagents

CB immunohistochemical SP kit was provided by Wuhan Boster Biological Technology Company; SA-AP and BCIP/NBT were from American Promera corporation; CBcDNA probe with 5 terminal biotin marked (5'-GTTGACCAGCT-CATCCGACAGG-3') was synthesized by Beijing Oker Biological Technology Limited Corporation.

2.1.2 Specimens

Forty-nine fresh samples of human esophageal carcinoma were obtained from oncological hospital of Anyang and the First Affiliated Hospital of Zhengzhou University. These samples were all confirmed by pathology as squamous cell carcinoma. There were 49 cases, 25 males and 24 females, with median age 58.3 ± 17.8 years old (from 40.5 years old to 76.1 years old).

According to lymphatic metastasis: metastatic group (20 cases) and non-metastatic group (29 cases); according to the depth of invasion: low-muscle group (10 cases), deep-muscle group (15 cases) and out layer group (24 cases); according to development: esophageal carcinoma group (49 cases), atypical hyperplasia of tumor-adjacent group (30 cases) and normal group (49 cases).

2.2 Methods

2.2.1 Specimen preparation

The tissues of carcinoma, atypical hyperplasia of tumor-adjacent and normal tissues obtained were formalin-fixed and paraffin-embedded respectively for immunohistochemistry and *in situ* hybridization.

2.2.2 Immunohistochemistry

Paraffin sections were de-paraffined routinely, rinsed in PBS 3 times for 5 min each. Under the high pressure, samples were treated with 0.01 mol/L Citrate Tris-sodium for 15 min. Samples were rinsed 3 times with PBS for 5 min each, and blocked by serum for 30 min, to remove non-specific staining. Then drop wise 1:100 rabbit anti-human CB antibody was added and incubated at 4 °C overnight. The next day sections were taken for 30 min at room temperature, rinsed with PBS 3 times for 5 min each. So did the secondary antibody and the tertiary antibody. After the following DAB staining, hematoxylin staining, samples were dehydrated and mounted. PBS replaced the primary antibody in the negative control slices.

2.2.3 *In situ* hybridization

Paraffin sections were deparaffined to water routinely, and performed in 3% hydrogen peroxide for 30 min at room temperature for blocking endogenous peroxidase activity. Sections were rinsed with H₂O 3 times for 5 min each time and digested for 20 min with pepsin to expose mRNA nuclear section, rinsed once with H₂O for 5 min, fixed with 1% formalin / 0.1 M PBS (pH 7.2 - 7.6, contain 0.1% DEPC) for 10 min at room temperature. Rinsed by H₂O 3 times for 5 min each time, and dropwised 20 μl /slice hybridization solution. The sections were set in 20% glycerine to prehybridize for 3 - 4 h at 42 °C dropwise 20 μl labeled probe added to specimen, then covered paraffin membrane to hybridize for 12 - 16 h at 42 - 43 °C. The next day, sections were dropwised with 0.1 × SSC for 4 times for 15 min each time to elute non-specific hybridization. Sections were processed with 1% acetylated BSA for 10 min at 20 °C. Sections were in Streptavidin Alkaline Phosphatase (SA-AP) for 20 min at 37 °C, Tris-HCl buffer I 3 times for

10 min each, and Tris-HCl buffer II 2 times for 1 min. Sections were dropwised BCIP/NBT (33 μl NBT and 16.5 μl BCIP added to 5 ml buffer II) substrate solution freshly, stained in the darkness for 10 - 120 min, nuclear fast red after stained for a few min, neutral gum mounted, prehybridization solution without probe hybridized and before hybridization specimens were processed as negative control by RNase.

The results were judged according to Naoki method^[3]. Positive cells account for 0% for (-), 0 - 20% for (±), 20 - 80% for (+), 80% for (++) , (-) and (±) were set as negative, (+) and (++) were positive.

2.2.4 Statistical analysis

All the data were analyzed by spss 10.0 system. Comparison between positive rates adopted chi-square test (χ^2 -test).

3 Results

3.1 CB protein expression and CB mRNA expression in esophageal carcinoma tissues, atypical hyperplasia tissues and normal tissues

The positive signals of CB protein were localized in cytoplasm or membrane of esophageal carcinoma or atypical hyperplasia cell and showed brown granules. Periphery matrix of carcinoma nest showed positive as well (Figure 1) but not in negative control. Positive signals of CB mRNA was localized in cytoplasm of esophageal carcinoma or atypical hyperplasia cells and showed blue granules (Figure 2). Normal esophageal mucosa cells couldn't be stained. No positive signals appeared in the negative control.

3.2 Relationships of CB and esophageal carcinoma metastasis

3.2.1 Relationship of CB protein and esophageal carcinoma metastasis

In the 20 cases with lymphatic metastasis, the positive rates of expression of CB protein in the tissues of esophageal carcinoma, atypical hyperplasia tissues and normal tissues were 100% (20/20), 60% (6/10) and 0% (0/20), respectively; while in the 29 cases without lymphatic metastasis, the positive rates CB protein expression in the esophageal carcinoma tissues, atypical hyperplasia tissues and normal tissues were 62.07% (18/29), 10% (2/20) and 0% (0/29), respectively. The difference of CB had statistical significance (carcinoma tissues group *vs.* normal tissues group, $P < 0.01$; atypical hyperplasia group *vs.* normal tissues group, $P < 0.01$). Table 1 showed the details.

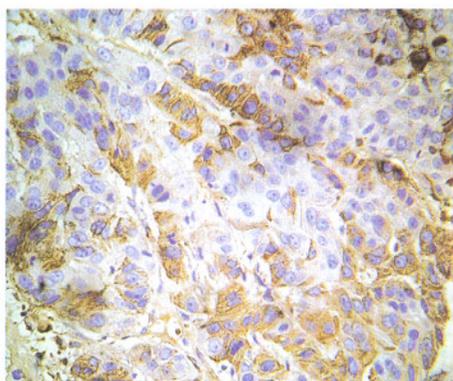


Figure 1. Immunohistochemical staining of esophageal squamous carcinoma (×400)

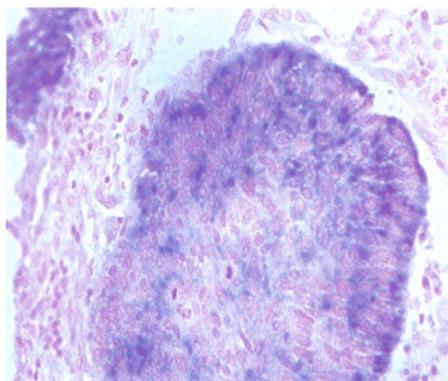


Figure 2. In situ hybridization staining of esophageal squamous carcinoma (×400)

Table 1. Relationship of CB expression and lymphatic metastasis

Group	Tissues	Cases(n)	Positive cases(n)	Positive rate (%)
Metastatic group	Tissues of carcinoma	20	20	100
	Tissues of tumor-adjacent	10	6	60
Non-metastatic group	Tissues of carcinoma	29	18	62.07
	Tissues of tumor-adjacent	20	2	10

Note: comparison of CB mRNA expression in carcinoma tissues between two groups: $\chi^2 = 9.782, P = 0.000$; comparison of CB mRNA expression in atypical hyperplasia tissues between two groups: $\chi^2 = 8.523, P = 0.004$

3.2.2 Relationship of CB mRNA expression and esophageal carcinoma metastasis

In the 20 cases with lymphatic metastasis, the positive rates of CB mRNA expression in esophageal carcinoma tissues, atypical hyperplasia tissues and normal tissues were 95% (19/20), 50% (5/10) and 0% (0/20), respectively; in the 29 cases without lymphatic metastasis, the positive

rates of CB mRNA expression in the tissues of esophageal carcinoma, atypical hyperplasia and normal tissues were 55.2% (16/29), 10% (2/20) and 0% (0/29), respectively. The positive rates of CB mRNA in the esophageal carcinoma tissues and atypical hyperplasia between two groups were statistically significant. Table 2 showed the details.

Table 2. Relationship of CB mRNA and esophageal carcinoma metastasis

Group	Tissues	Cases(n)	Positive cases(n)	Positive rate (%)
Metastatic group	Tissues of carcinoma	20	19	95
	Tissues of tumor-adjacent	10	5	50
Non-metastatic group	Tissues of carcinoma	29	16	55.2
	Tissues of tumor-adjacent	20	2	10

Note: comparison of CB mRNA expression in carcinoma tissues between two groups: $\chi^2 = 9.2, P = 0.002$; comparison of CB mRNA expression in atypical hyperplasia tissues between two groups: $\chi^2 = 5.963, P = 0.015$

3.3 CB protein, CB mRNA and esophageal carcinoma invasion

3.3.1 Relationship of CB protein expression and esophageal carcinoma invasion

In tissues of esophageal squamous cell carcinoma with low-muscle invasion, deep-muscle invasion and out layer invasion, the positive rates of CB protein were 50% (5/10), 60% (9/15) and 100% (24/24), respectively; in atypical hyperplasia tissues in different invasion depths, the positive rates of CB protein were 0% (0/8), 10% (1/10) and

58.33% (7/12). In the tissues of esophageal carcinoma and atypical hyperplasia of tumor-adjacent with out layer invasion, the positive rates of CB protein was obviously higher than the other two with statistical significance. While in the tissues of esophageal carcinoma and atypical hyperplasia with deep-muscle invasion, the difference of the positive rates of expression of CB protein in comparison with low-muscle invaded wasn't statistically significant ($P > 0.05$). Table 3 showed the details.

Table 3. CB protein Expression and esophageal carcinoma invasion

Depth of invasion	Tissues of carcinoma			Tissues of atypical hyperplasia		
	Positive cases(n)	Negative cases(n)	Positive rates(%)	Positive cases(n)	Negative cases(n)	Positive rates(%)
Low-muscle layer ^a	5	5	50	0	8	0
Deep-muscle layer ^b	9	6	60	1	9	10
Out layer ^c	24	0	100	7	5	58.33

Note: tissues of carcinoma: a vs. b: $\chi^2=0.244, P=0.612$; a vs. c: $\chi^2=14.069, P=0$; b vs. c: $\chi^2=11.345, P=0.001$; tissues of tumor-adjacent: a vs. b: $\chi^2=0.847, P=0.357$; a vs. c: $\chi^2=7.179, P=0.007$; b vs. c: $\chi^2=5.507, P=0.019$

3.3.2 Relationship of CB mRNA and esophageal carcinoma invasion

In tissues of esophageal squamous cell carcinoma with low-muscle invasion, deep-muscle invasion and out layer invasion, the positive rates of CB mRNA were 30% (3/10), 53.33% (8/15) and 95.83% (23/24), respectively; in atypical hyperplasia tissues in different invasion depths, the positive rates of CB mRNA were 0% (0/8), 10% (1/10) and 50% (6/12), respectively. In tissues of

esophageal carcinoma and atypical hyperplasia with deep-muscle invasion, the CB mRNA expression was higher compared with low-muscle invasion, but the difference wasn't of statistical significance ($P > 0.05$). While CB mRNA expression in the tissues of esophageal carcinoma and atypical hyperplasia with out layer invasion in comparison with the former both, the difference of the positive rates was statistically significant. Table 4 showed the details.

Table 4. CB mRNA and esophageal carcinoma invasion

Depth of invasion	Tissues of carcinoma			Tissues of tumor-adjacent		
	Positive cases(n)	Negative cases(n)	Positive rates(%)	Positive cases(n)	Negative cases(n)	Positive rates(%)
Low-muscle layer ^a	4	6	40	0	8	0
Deep-muscle layer ^b	8	7	53.33	1	9	10
Outer layer ^c	23	1	95.83	6	6	50

Note: tissues of carcinoma: a vs. b: $\chi^2=0.427, P=0.513$; a vs. c: $\chi^2=13.459, P=0$; b vs. c: $\chi^2=10.226, P=0.001$; tissues of tumor-adjacent: a vs. b: $\chi^2=0.847, P=0.357$; a vs. c: $\chi^2=5.714, P=0.017$; b vs. c: $\chi^2=4.023, P=0.045$

3.4 Relationship of CB protein, CB mRNA and esophageal carcinoma development

Above-mentioned results showed, in the tissues of normal esophageal mucosa, CB protein expression and CB mRNA expression were seldom detected. In the carcinoma tissues of metastatic group, the positive rates of CB protein expression and CB mRNA expression were 100% (20/20), 95% (19/20), respectively. In the matched tissues of tumor-adjacent atypical hyperplasia, the positive rates of CB protein expression and CB mRNA expression were 60% (6/10), 50% (5/10),

respectively; in the carcinoma tissues of non-metastatic group, the positive rates of CB protein expression and CB mRNA expression were 62.07% (18/29), 55.2% (16/29) respectively. In the matched tissues of tumor-adjacent atypical hyperplasia, both of the positive rates of CB protein expression and CB mRNA expression were 10% (2/20). The positive rates of CB protein expression and CB mRNA expression were compared to those in the matched tumor-adjacent atypical hyperplasia, with the statistically significant difference ($P < 0.01$). Table 5 showed the details.

Table 5. Correlations of CB protein expression, CB mRNA expression and esophageal carcinoma development

Group	Tissues of carcinoma [#]		Tissues of tumor-adjacent [*]	
	Expression of protein(%)	Expression of mRNA(%)	Expression of protein(%)	Expression of mRNA(%)
Metastatic group ^a	100.00	95.00	60.00	50.00
Non-metastatic group ^b	62.07	55.20	10	10

Note: # : correlation of a and CB mRNA expression, $r=20.328, P=0.000$; * : correlation of b and CB mRNA expression, $r=3.649, P=0.01$

3.5 Correlations of CB protein and CB mRNA

In the metastatic group and non-metastatic group, the expression of CB protein and mRNA in the tissues of carcinoma and atypical hyperplasia of

tumor-adjacent had positively relative tendency, the difference had statistical significance (carcinoma tissues: $P < 0.01$; atypical hyperplasia tissues: $P < 0.01$). Table 6 showed the details.

Table 6. Correlations between CB protein expression and of CB mRNA expression

Group	Tissues	Cases (n)	Expression of protein		Expression of mRNA	
			Positive cases(n)	Positive rates	Positive cases(n)	Positive rates
Metastatic group	Tissues of carcinoma	20	20	100.00	19	95.00
	Tissues of tumor-adjacent	10	6	60.00	5	50.00
Non-metastatic group	Tissues of carcinoma	29	18	62.07	16	55.2
	Tissues of tumor-adjacent	20	2	10	2	10

Note: correlation of carcinoma tissues and CB mRNA expression: $r = 20.328, P < 0.01$; correlation of atypical hyperplasia tissues and CB mRNA expression: $r = 3.649, P < 0.01$

4 Discussion

The mechanism of tumor is unclear, but it's known that metastasis would lead to worse prognosis. Therefore index, which is relevant to metastasis of tumor has been research hot-spot domestically and abroad. Recent years, CB was found to participated in extracellular matrix and facilitated the metastasis of tumor. Miyake *et al*^[5] discovered that in the serum of prostate carcinoma with lymphatic metastasis, the level of CB protein was obviously increased compared to those without lymphatic metastasis; Yu *et al*^[6] discovered that the positive rates of expression of CB protein in the tissues of colorectal carcinoma with lymphatic metastasis was also elevated. Xu *et al*^[7] determined expression of CB protein and mRNA of hepatoma tissues and found that CB participated in the metastasis of hepatoma.

Experiments may suggest that the highly expressed CB have relationship with the metastasis of prostate cancer, colorectal cancer, and liver cancer, but its relationship with the metastasis of esophageal carcinoma hasn't been reported. This experiment measured the CB protein expression and CB mRNA in the esophageal carcinoma tissues, tumor-adjacent atypical hyperplasia tissues and normal esophageal tissues and explored relationship of CB expression with esophageal carcinoma. Immunohistochemical results demonstrated in 20 cases with the lymphatic metastasis, the positive rates of CB protein expression in the tissues of esophageal carcinoma, tumor-adjacent atypical hyperplasia and normal tissues were 100%, 60% and 0%, respectively; in the 29 cases without lymphatic metastasis, the positive rates of expression of CB protein in the tissues of esophageal carcinoma, tumor-adjacent atypical hyperplasia and normal tissues were

62.07%, 10% and 0%, respectively. CB protein expression in metastasis group was significantly increased in tissues of carcinoma and tumor-adjacent atypical hyperplasia tissues compared with the non-metastasis group. *In situ* hybridization results demonstrated in 20 cases with lymphatic metastasis, the positive rates of CB mRNA expression were 95%, 50% and 0%, respectively; while in non-lymphatic metastasis group, the positive rates of expression of CB mRNA were 55.2%, 6% and 0%, respectively. The difference of the positive rates of CB mRNA expression in the carcinoma tissues and tumor-adjacent atypical hyperplasia tissues between the lymphatic group and non-lymphatic group was statistically significant. Above-mentioned results demonstrated that both CB protein and mRNA have close relationship with the metastasis of esophageal carcinoma.

CB is a lysosomal cysteine proteinase that can degrade the extracellular matrix and facilitate to go through barrier of basement membrane and extracellular matrix, which results in the invasion and metastasis of tumor cells. Accordingly, attentions were paid to the relationship between CB and the invasion of tumor, as well. Ejjan *et al*^[8] analyzed the tissues of transitional bladder carcinoma by Western blot, and the results demonstrated that the expression of CB protein was higher in invasive type than that in superficial type, which suggested that CB protein expression was related to the invasion of bladder carcinoma; Dohchin *et al*^[9] discovered that in the gastric carcinoma tissues with muscle layer invasion, CB protein expression was higher than tissues with the mucosa layer invasion, and relevant to the invasion of gastric carcinoma. This research results demonstrated: in the carcinoma tissues and tumor-adjacent atypical hyperplasia tissues with low-muscle invasion, deep-muscle invasion

and out layer invasion, the positive rates of CB protein expression and CB mRNA expression were both elevated. In the tissues of carcinoma and tumor-adjacent atypical hyperplasia of the out layer invasion, the positive rates of CB protein and CB mRNA were significantly highly expressed significantly compared with the low-muscle invasion and deep-muscle invasion both. So CB protein expression and CB mRNA expression was related not only to the metastasis but also to the invasion of esophageal carcinoma. Therefore CB may be a marker of invasion and metastasis of tumor cells and at the same time, provided important guidance for clinical therapy to prevent the invasion and metastasis.

Many abroad and domestic studies carried out were on CB and its relationship with the cancer invasion and metastasis of tumor, but seldom on relationship of CB and cancer development. This research observed also explored CB and its relationship with esophageal carcinoma development. Results demonstrated: CB protein and CB mRNA couldn't be detected in normal tissues, while in the lymphatic and non-lymphatic group, the positive rates of expression of CB protein and CB mRNA in the esophageal carcinoma tissues were higher than those in the tumor-adjacent atypical hyperplasia with statistical significance, which demonstrated that CB participated in the development of esophageal carcinoma.

Furthermore, this experiment results suggested, and non-metastatic group, the positive rates of CB protein expression and CB mRNA expression in carcinoma tissues and tumor-adjacent atypical hyperplasia tissues in metastasis group was significantly higher than that in non-metastasis group. This results further showed that except non-metastasis group, the positive rates of CB protein expression was consistent with CB mRNA expression, in carcinoma tissues and in the atypical hyperplasia tissues, but the positive rates of CB mRNA was lower than CB protein. To some extent, investigation of CB by immunohistochemistry was more sensitive than that by *in situ* hybridization.

It was noteworthy of the different location of CB protein positive staining. Positive staining in the tumor-adjacent mucosa and normal mucosa was only in the cytoplasm, while positive staining in the esophageal carcinoma was scattered in the cytoplasm, cellular membrane and peripheral cells. Weiss *et al* injected invasion tumor cell and non-invasion tumor cell to athmic mice respectively, and got the similar results, suggesting that tumor cells

secreted CB protein to peripheral matrix, which could degrade the matrix component and facilitated the invasion and metastasis of tumor.

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