·Basic Research·

Correlation of Fas/FasL expression to cell apoptosis in Epstein-Barr virus-associated gastric carcinoma

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[**Abstract**] **Background and Objective:** Epstein-Barr virus (EBV) has been detected in about 10% of gastric carcinomas. However, the pathogenetic role of EBV in gastric carcinoma is uncertain. This study was to explore the correlation of Fas/FasL expression to the apoptosis of tumor cells and tumor-infiltrating lymphocytes (TIL) in EBV-associated gastric carcinoma (EBVaGC). **Methods:** Fas/FasL expression in 49 specimens of EBVaGC, 20 specimens of EBV-negative gastric carcinoma (EBVnGC) and 12 specimens of normal gastric mucosa was detected by immunohistochemistry. The apoptotic index (AI) of cells was determined by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL). **Results:** The positive rates of Fas were 91.7% in normal gastric mucosa and 76.8% in gastric carcinoma ($P < 0.05$); those of FasL were 16.7% in normal gastric mucosa and 58% in gastric carcinoma ($P < 0.05$). The positive rate of Fas was lower in EBVaGC than in EBVnGC (71.4% vs. 90.0%, $P > 0.05$). The positive rate of FasL in EBVaGC was significantly higher than that in EBVnGC (63.2% vs. 45%, $P < 0.05$). The AI of EBVaGC cells was significantly lower than that of EBVnGC cells ($P = 0.002$). The number and AI of TIL in EBVaGC were higher than those in EBVnGC ($P < 0.05$). The AI of TIL was positively correlated with the level of FasL expression in tumor cells (r=0.237, $P = 0.028$). **Conclusion:** Up-regulation of FasL expression and decrease of TIL apoptosis in EBVaGC may facilitate the escape of tumor cells from the host immunosurveillance, and it might contribute to the development and progression of carcinoma.

Key words: Gastric neoplasm, Epstein-Barr virus, immunohistochemistry, TUNEL, Fas, FasL

Esptein-Barr virus (EBV) belongs to γ subfamily of herpes virus and EBV is the first known oncogenic virus. After 40 years of investigation, it has domstrated that EBV is closely related to the development of a variety of malignant tumors[1]. Since EBV was firstly found in gastric carcinoma cells in 1990, the relationship between EBV and gastric carcinoma has became a research hotspot[2]. As known currently, EBV-associated gastric carcinoma (EBVaGC) accounts for 7% –10% of all gastric carcinomas, and about 90 000 new cases were found yearly. EBVaGC develops from the proliferation of a single EBV-infected cell, indicating that EBV plays an important role in EBV positive gastric carcinoma development and progression, but the mechanism is unknown yet[3].

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Cell apoptosis is a genetically controlled initiative cell death. The imbalance between cell apoptosis and cell proliferation is the main reason for tumor development and progression. Fas/FasL is an important cell apoptosis signal pathway, which mediates cell apoptosis by interactions, also plays roles in immune surveillance. Tumor-infiltrating lymphocyte (TIL) is a key effector of anti-tumor immunity. Tumor cells can induce TIL production and escape from the immune damage. FasL of tumor cells shields the Fas-mediated cytotoxicity to tumor cells through Fas 'tumor counterattack' mechanism.

In this study, we used immunohistochemisty and TdT-mediated dUTP-biotin nick end labeling (TUNEL) to detect Fas/FasL expression, TIL, and cell apoptosis in 49 specimens of EBVaGC and 20 specimens of EBV-negative gastric carcinoma (EBVnGC), and explored the possible pathogenesis of EBVaGC.

Materials and Methods

Clinical samples

A total of 49 cases which pathologically diagnosed as gastric adenocarcinoma and confirmed by EBV-encoded small RNA (EBER) in situ hybridization as EBVaGC were collected from the Third Affiliated Hospital of Sun Yat-sen University, the Second

Affiliated Hospital of Sun Yat-sen University and the First Hospital of Guangzhou between January 1, 2000 and December 31, 2006. These patients included 41 men and 8 women, with a median age of 53. Twenty cases of EBVnGC diagnosed by in situ hybridization were collected simultaneously. These patients included 15 men and 5 women, with a median age of 57.5. Twelve cases of normal gastric mucosa (excluding atrophy, intestinal metaplasia, and atypical hyperplasia, and only contained mild chronic inflammatory cell infiltration in lamina propria) were selected as control.

Reagents and methods

Reagents Anti-CD95/Fas polyclonal antibody (40022Ra) was purchased from Fuzhou Maixin Biotechnology Development Co., Ltd. Mouse anti-human FasL monoclonal antibody (M-0245) was purchased from Shanghai Changdao Biotechnology Co., Ltd. Mouse anti-human CD45RO monoclonal antibody (M0701) was purchase from Dako Co. TUNEL kit (QIA33) was from Calbiochem Co.

Immunohistochemistry EnVision two-step method was used for immunohistochemistry. According to instructions, the paraffin sections were dewaxed, hydrated and immersed in 0.01 mol/L citrate solution (pH=6.0), and incubated in microwave for antigen retrieval for 10 min. First antibody was replaced by PBS for negative control. The sections of liver cancer tissue, testis tissue and tonsil were served as positive controls for Fas, FasL and CD45RO, respectively.

Detection of cell apoptosis Apoptotic cells were detected by in situ TUNEL method according to instructions.

Estimation of immunohistochemistry

The cytoplasm and membrane of Fas- and FasL-positive cells were stained in brown. Fas and FasL staining was scored according to the proportion of positive cells: '-' stood for a proportion of less than 25%; '+' stood for 26%–50%; '++' stood for 51%–75%; '+++' stood for 76%–100%.

CD45RO was expressed in cytoplasm and on membrane in brown. According to the expression scope and intensity of CD45RO in TIL (lymphocytes in interstitial and around tumor nests), TIL infiltration was graded as mild, moderate and severe according to the Sydney visual analog scale system.[4]

Estimation of TUNEL

Positive cells showed dense distribution of dark brown yellow granules in cell nucleus. 1000 cells were randomly selected and observed under no less than five high-power microscope fields. Number of cells and positive cells were calculated. Apoptosis index = number of positive cells/number of total cells × 100%. Cell apoptosis index was divided into high and low groups by average bound.

Statistical analysis

Measurements were displayed by $x \pm s$. *t* test was used for comparison of two samples. Classification information was stood by n (%). χ^2 test was used. Spearman rank correlation analysis was used for correlation analysis. Differences between ranked samples were compared by Mann Whitey U test. α=0.05 was served as standard and all calculation was completed by SPSS13.0.

Results

Immunohistochemisty of Fas and FasL

The positive rates of Fas were 91.7% in 12 cases of normal gastric mucosa and 76.8% in 69 cases of gastric carcinoma. The expression intensity of the former was mainly '+++' and the later was '+' to '++'. The positive rates of FasL were 16.7% in normal gastric mucosa and 58% in gastric carcinoma respectively. The expression of Fas and FasL in gastric carcinoma had significant differences comparing to normal gastric mucosa ($P < 0.05$).

The expression of Fas, FasL, TIL and AI in EBVaGC and EBVnGC cells

The positive rates of Fas were 71.4% and 90.0% in EBVaGC and EBVnGC tissues respectively and there were no significant differences between two groups (Table 1). However, the former was mainly '+' and the latter was '++' (Figure 1). The positive rates of FasL were 63.2% and 45% in EBVaGC and EBVnGC tissues respectively (Figure 2). The distribution of TIL was unequal, the number of TIL was significantly larger in tumor margin and normal tissues, while fewer in tumor tissues and scattered. The number of TIL was higher in EBVaGC than in EBVnGC ($P = 0.005$), as shown in Figure 3. The AI of EBVaGC (0.47 ± 0.36) % was significantly lower than that in EBVnGC (1.04 ± 1.13) %, the differences had statistical significance ($P = 0.002$) as shown in Figure 4.

Table 1 Fas and FasL expression, TIL infiltration and AI in Epstein-Barr virus (EBV)-positive and -negative gastric carcinomas

Group	Patient No.	Fas expression				FasL expression				TIL infiltration			AI(%)
		–	+	++	+++	–	+	++	+++	light	moderate	heavy	
EBVaGC	49	14	16	10	9	18	5	16	10	22	18	9	0.47 ± 0.36
EBVnGC	20	2	5	9	4	11	9	0	0	16	4	0	1.039 ± 1.13
P			>0.05				>0.004				>0.005		0.002

TIL, tumor-infiltrating lymphocytes; AI, apoptosis index; EBVaGC, EBV-associated gastric carcinoma; EBVnGC, EBV-negative gastric carcinoma. All values of AI are presented as mean ± SD.

Fas, FasL, CD45RO and TUNEL expression and the clinically pathological characteristics of EBVaGC

The frequency of FasL expression was significantly higher in diffuse type EBVaGC than in intestinal type EBVaGC, significant difference was shown ($P = 0.015$), while no obviously statistical significance was shown comparing with other clinically

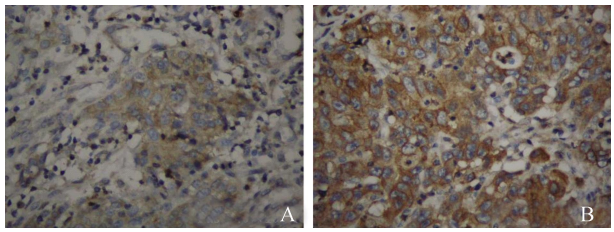

Figure 1 Fas expression in EBVaGC and EBVnGC tissues (EnVision ×400)

Fas is lowly expressed in EBVaGC tissues (A), while highly expressed in EBVnGC tissues (B).

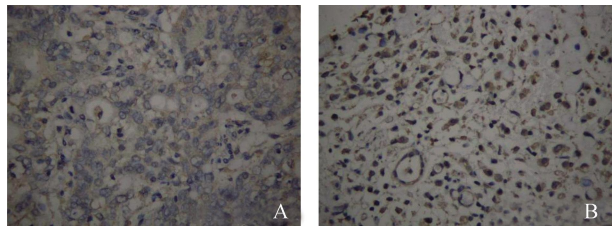

Figure 2 FasL expression in EBVaGC and EBVnGC tissues (EnVision ×400)

FasL is lowly expressed in EBVnGC tissues (A), while highly expressed in EBVaGC tissues (B).

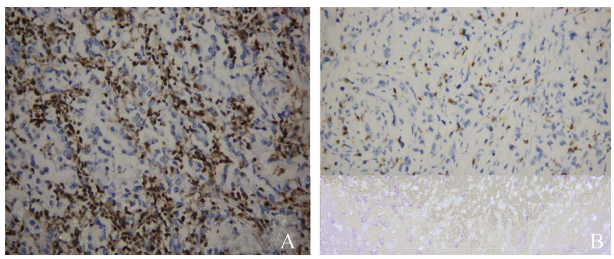

Figure 3 Tumor-infiltrating lymphocytes (TIL) in EBVaGC and EBVnGC tissues (Envision ×200)

A lot of TIL can be seen in EBVaGC tissues (A), but only a few in EBVnGC tissues (B).

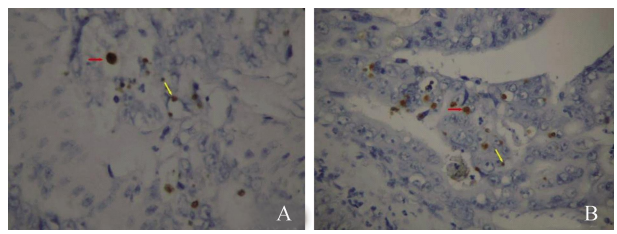

Figure 4 Apoptotic tumor cells and TIL in EBVaGC and EBVnGC tissues detected by TUNEL (×400)

A, In EBVaGC tissues, the apoptosis index (AI) of tumor cells is low, while that of TIL is high. B, in EBVnGC tissues, the AI of tumer cells in high, while that of TIL is low.

pathological characteristics. Fas, CD45RO and AI were not related to clinically pathological characteristics.

The relationship between Fas, FasL expression in TIL of EBVaGC and EBVnGC and TIL apoptosis

Apoptotic lymphocytes were scattered distribution in tumor stroma and the volume was smaller than tumor cells. AI values of TIL in EBVaGC and EBVnGC were 1.89 ± 1.20 and1.24 ± 1.09 respectively, and significant differences was shown (P = 0.036). However, the expression lever of Fas and FasL in TIL of EBVaGC and EBVnGC were not statistically significant (P > 0.05) (Table 3).

Table 2 Correlations of Fas and Fas expression, TIL infiltration, and AI to clinicopathologic features of EBV-associated gastric carcinoma (EBVaGC)

Parameter	Fas (patient No.)		FasL (patient No.)		TIL (patient No.)			AI (%)	
	+	−	+	−	light	moderate	heavy	High	Low
Total	35	14	31	18	22	18	9	16	33
Gender									
Female	7	1	5	3	4	2	2	4	4
Male	28	13	26	15	18	16	7	12	29
Age(years)									
< 40	10	1	7	4	4	5	2	3	8
40–60	17	6	15	8	9	10	4	5	18
> 60	8	7	9	6	9	3	3	8	7
Lauren classification									
Intestinal type	7	2	2	7	4	4	1	4	5
Diffuse type	28	12	29	11[a]	18	14	8	12	28
Depth of invasion									
T1,T2	2	2	1	3	0	4	0	1	3
T3,T4	33	12	30	15	22	14	9	15	30
Lymph node metastases									
+	30	10	26	14	20	13	2	12	28
−	5	4	5	4	2	5	2	4	5
Distant metastases									
+	0	0	0	0	0	0	0	0	0
−	35	14	31	18	22	18	4	16	33

[a] P = 0.015, vs. intestinal type.

Relationship between Fas, FasL expression in EBVaGC cells and AI of tumor cells

As shown in Spearman analysis, AI of EBVaGC cells was not related to Fas expression ($P > 0.05$). AI of TIL in EBVaGC was related to FasL expression in tumor cells (r=0.237, P=0.0284) (Table 3).

Table 3 Correlation between AI of tumor-infiltrating lymphocytes (TIL) and FasL expression in tumor cells

FasL	Cases	AI(%)
–	15	0.239 ± 0.089
+	18	0.303 ± 0.144
++	7	0.972 ± 0.783
+++	9	1.364 ± 0.328

r = 0.237, P = 0.028.

Discussion

Fas belongs to tumor necrosis factor/nerve growth factor receptor family and distributes widely in T and B lymphocytes, EBV transformed lymphocytes, some epithelial cells and tumor cells.[5] FasL is the natural legend of Fas, and the interaction of Fas and FasL is the main way to induce cell apoptosis. Fas bond with FasL, and transfered the signal to cytoplasm and leaded to the activation of Fas death domain, thus mediating the cell apoptosis in Fas expressed cells.[6] In tumor cells, through counteracting Fas mediated cell apoptosis and fighting back immune cells to cause T cell apoptosis were one of the most important mechanisms of tumor immune escape.

TIL were a group of heterogenous lymphocytes within tumor interstitial. TIL infiltration was a resistant phenomenon of immune system causing by tumor. The degree of infiltration was related to tumor invasion, metastasis and prognosis.

The function of EBV in gastric carcinoma development and progression has not been discovered. However, it has been proved that the mechanism of EBV associated gastric carcinoma and EBV negative gastric carcinoma was different according to many years studies.[7,8] The relationship between EBV and cell apoptosis and proliferation associated genes is the hotspot for EBV research. It has been proved that the cell apoptosis index in EBV positive gastric carcinoma was lower than EBV negative carcinoma, and related to EBV existence, but the reasons has not been discovered.[9,10] Fas and FasL are more precise cell apoptosis regulated genes, but their roles in EBVaGC has not been found.

In the present study, the expression of Fas and FasL in gastric carcinoma had significant differences comparing with normal tissues, indicating that gastric carcinoma development was related to abnormal expression of Fas and FasL. The apoptosis index in EBVaGC was significant lower than in EBVnGC, and the results were confirmed to Chang *et al.*[11] reported. The expression of Fas in EBVaGC and EBVnGC did not have any significant difference. However, the intensity of Fas expression in EBVaGC was lower than in EBVnGC. The Fas expression in cell surface reached to the critical value was the chief condition for the sensitivity of cell apoptosis induced by Fas system, which may be the main reason for the apoptosis index in EBVaGC was lower than in EBVnGC.

Resent studies shown that FasL was overexpressed in many tumor cells, and tumor cells caused T cell apoptosis through Fas/FasL mediated pathway. It had been found that after co-culturing human lung cancer line with Jukat T lymphocytes, the expression of Fas increased in Jurkat cells, while expression of FasL in lung cancer cells may produce anti-apoptosis effects, making the growth of T cells inhibited and inducing cell apoptosis. That was Yoong *et al*[12,13] referred counterattack model. In this study, we found that the expression of FasL was higher in EBVaGC than in EBVnGC, and upregulation of FasL expression could kill immune active cells and escape the immune response. The total apoptosis index of TIL in EBVaGC was higher than in EBVnGC, which may be related to the higher expression of FasL in EBVaGC comparing to EBVnGC. Overexpression of FasL made tumor cells establishing an effective counterattack model to resist immune attack, which meant through Fas/FasL pathway to induce apoptosis by increasing expression of Fas in TIL. In addition, we observed that the number of TIL was larger in EBVaGC than in EBVnGC, that may be associated with the chemotactic effects in lymphocytes induced by some cytokines secreted from EBV-positive tumor cells. EBERs could induced IL-10 expression in Burkitt lymphocytes,[14] and the IL-10 expression was also detected in nasopharyngeal carcinoma and NK/T lymphoma. IL-10 played important roles in upregulating FasL expression in nasopharyngeal carcinoma.[15] Therefore, we speculated that upregulation of FasL was related to IL-10 expression induced by EBV.

Mitsuhiko *et al.*[16] explored that FasL expression was associated with the differentiation of gastric carcinoma. Our study showed that FasL expression in EBVaGC was related to histological type, and the expression in diffuse type was higher than in intestinal type (P = 0.015). Based on the above results, we speculated that the intensity of Fas expression decreased in EBVaGC, which contributed to the escape of tumor cells from immune attack and reduced the cell apoptosis index. The FasL expression was higher in EBVaGC than in EBVnGC, which promoted the apoptosis of TIL, thus the body escaped the immune surveillance and further reduced cell apoptosis in tumor cells. FasL expression was relevant to tumor differentiation and histological, which could reflect the special nature of tumor and the degree of malignancy.

The mechanism of EBV leaded gastric carcinoma was not clear, however, the final way was by inhibiting cell apoptosis and enhancing cell proliferation to cause tumorigenesis. Fas/FasL was not only an important system in regulating cell apoptosis, but also related to tumor development and progression. Our study demonstrated that upregulation of FasL expression and decreased apoptosis of TIL were favorable for tumor cells escape the immune surveillance and supplied conditions for tumor development and progression.

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