Int J Clin Exp Med 2017;10(9):14083-14092
www.ijcem.com /ISSN:1940-5901/IJCEM0046052

Original Article

Impact of TILs on the prognosis of IIIB colon cancer associated with c-FLIP$_L$ expression

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Received December 7, 2016; Accepted May 15, 2017; Epub September 15, 2017; Published September 30, 2017

Abstract: Background: To find out useful tools to evaluate the prognosis of colon cancer, this study investigated how c-FLIP$_L$ and tumor-infiltrating lymphocytes (TILs) are correlated with the prognosis of patients with stage IIIB colon cancer. Methods: 180 cases of pathologically proven specimens with stage IIIB colon cancer (T3N1M0, AJCC, 7th edition) were collected at Sun Yat-sen University Cancer Center. The expression of c-FLIP$_L$ and the density of TILs in tumor tissues were examined through immunohistochemical analysis. The correlation of c-FLIP$_L$ and TILs, with the prognosis of patients was analyzed. Results: C-FLIP$_L$ was correlated with an unfavorable overall survival (OS). The densities of CD8+ and CD45RO+ T cells were associated with a favorable OS, whereas Foxp3+ Treg cells were related to an unfavorable OS. However, the relationship between TILs and prognosis varied for two groups of patients that were divided according to c-FLIP$_L$ expression. In the group with high c-FLIP$_L$ expression, a high density of CD8+ and CD45RO+ T cells or a low density of Foxp3+ Treg cells was correlated with a short OS; In the group with low c-FLIP$_L$ expression, a high density of CD8+ and CD45RO+ T cells or a low density of Foxp3+ Treg cells was correlated with a long OS. Conclusion: This study revealed that the relationship between the density of TILs and the prognosis of colon cancer depended on the expression of c-FLIP$_L$, indicating that the clinical outcome of immune response depended on not only the subtypes of TILs but also the genetic background of cancer cells.

Keywords: Colon cancer, prognosis, c-FLIP$_L$, tumor-infiltrating lymphocytes

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer deaths in China, accounting for the death of approximately 500,000 people every year [1]. Traditional therapies for colon cancer include chemotherapy drugs, such as irinotecan and oxaliplatin, and biological agents, such as bevacizumab and cetuximab [1-3]. However, the outcome of overall survival (OS) is still poor [2]. Therefore, efficient strategies for inhibiting disease progression and improve clinical outcomes should be urgently developed.

The density of tumor-infiltrating lymphocytes (TILs) is associated with favorable prognosis [4-6]. TILs can be divided into functional subtypes, including effector CD8+ T cells, memory CD45RO+ T cells, and regulator T cells (Tregs; Forkhead box protein P3, Foxp3) [7, 8]. Previous studies revealed that the absence of early signs of metastasis is correlated with the presence of a high density of intratumoral effector T cells [8-10]. Thus, this study proposed that the immune contexture may affect the clinical outcomes of patients.

Apoptosis is vital for cell and tissue growth, and it plays a major role in cancer treatments. This cellular suicide program is regulated by many different signals from both intracellular and extracellular stimuli [11, 12]. The cellular FADD-like IL-1β-converting enzyme inhibitory protein (c-FLIP) is a key inhibitor of death receptor signaling pathways [13, 14]. Having a similar structure to the caspase-8, the c-FLIP can efficiently block caspase-8 cleavage. Alternative splicing generates two types of c-FLIP: the long form (c-FLIP$_L$), which contains a caspase-like domain; and the short form (c-FLIPS and c-FLIPR), which lacks the caspase-like domain [15, 16].

Table 1. Characteristics of patients (n=180)

Characteristics	No. of patients (%)
Age (years)	
≥60	105 (58.3)
<60	75 (41.7)
Gender	
Male	102 (56.7)
Female	78 (43.3)
Tumor sites	
Left hemicolon	135 (75.0)
Right hemicolon	45 (25.0)
Pathological grade	
G1	19 (10.5)
G2	149 (82.8)
G3	12 (6.7)
Survival time (months)	
≥60	132 (73.3)
<60	48 (26.7)

Previous studies have shown that increased c-FLIP$_L$ expression can be observed in various cancers, including ovarian carcinoma, non-small cell lung, and colorectal cancers, and that increased c-FLIP$_L$ expression is correlated with poor prognosis [17, 18]. In addition, C-FLIP$_L$ plays an important role in promoting motility, epithelial-mesenchymal transition (EMT), and immune evasion [19, 20]. However, no study has explored the correlation among c-FLIP$_L$, TILs, and the prognosis of colon cancer.

The present study investigates c-FLIP$_L$ expression and TILs in colon cancer tissues, and evaluates the prognostic significance in predicting the survival outcomes of patients with stage IIIB colon cancer. Furthermore, the correlation between TILs and OS for different levels of c-FLIP$_L$ is analyzed.

Materials and methods

Patients

A total of 180 cases of pathologically proven specimens with stage IIIB colon cancer (T3N-1M0, AJCC, 7th edition) were collected between January 1999 and December 2007. All of the patients underwent radical resection at the Cancer Center of Sun Yat-sen University in Guangzhou, China, and all of the samples were pathologically confirmed (**Table 1**). All of the patients received 5-FU-based adjuvant chemotherapy after their operation. Patients were evaluated every 3 months during the 1st year, every 6 months during the 2nd year, and then annually for the next 5 years. All of the patients were contacted via telephone or e-mail. If recurrence or metastasis occurred, 5-FU-based chemotherapy was administered in accordance with the National Comprehensive Cancer Network (NCCN) guidelines. OS time was defined as the period from the time surgery was performed to the patient's death. This study was conducted in accordance with Helsinki Declaration and approved by the Research Ethics Committee of Sun Yat-sen University.

Immunohistochemical assay and scoring systems

Paraffin-embedded tissues were cut into 4 μm thick sections and heated for 1 h at 65°C. The sections were then de-paraffinized, rehydrated, and blocked with hydrogen peroxide. The antigens were retrieved by pressure cooker treatment in ethylenediaminetetraacetic acid (EDTA) (pH 8.0) for 4 min and cooled to room temperature. Then, the endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide. The sections were incubated overnight at 4°C with primary antibodies: mouse anti-FLIP antibody (Abcam, Cambridge, MA, USA), mouse monoclonal antibody against human CD8, CD45RO (Zymed, San Diego, CA, USA), and mouse monoclonal anti-Foxp3 monoclonal antibody (eBioscience, San Diego, CA, USA); all of which were diluted 1:200. Afterward, the tissue sections were washed with phosphate-buffered saline (PBS) and treated with an anti-mouse secondary antibody, which reacts with the streptavidin horseradish peroxidase (HRP) complex, for 15 min. The samples were developed with diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin. As a negative control, PBS was used instead of the primary antibody.

Both the intensity of staining and the extent of immunoreactivity were evaluated and scored to quantify c-FLIP$_L$ expression. The intensity of cytoplasmic staining was scored as follows: negative = 0, weak = 1, moderate = 2, or strong = 3. The percentage of positive cells was scored as follows: 0 (≤10%), 1 (10%-24%), 2 (25%-49%), 3 (50%-74%), and 4 (≥75%). The product index was obtained by multiplying the two scores. To quantify expression of the TILs (CD45RO, CD8,

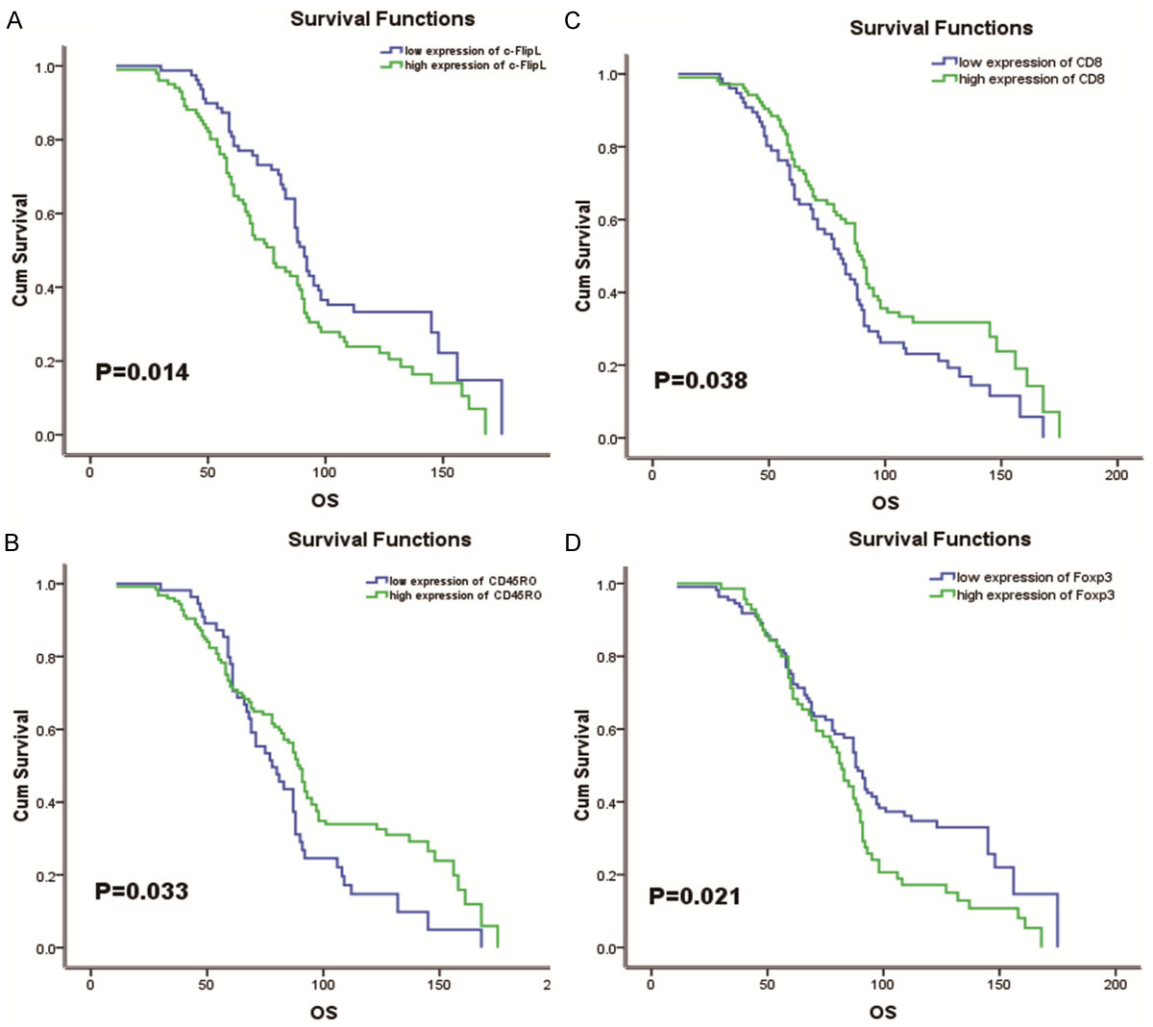

Figure 1. Kaplan-Meier analysis of overall survival of c-Flip$_L$, CD8, CD45RO and Foxp3.

and Foxp3), the number and size of the cells were counted in at least 10 different fields of each section. The size of each high-powered field (400×) was approximately 300 um × 300 um, and the cells were counted in the intratumoral compartment. The areas with the highest densities were selected. Two observers simultaneously counted the cells in the same field with the use of a multiple-lens-microscope. The results were expressed as the ± standard error of the mean.

Statistical analysis

All statistical analyses were performed with a SPSS19.0 statistical software package. The median value was used to differentiate the high and low expression for each maker. A chi-square test was performed to analyze the correlation among c-FLIP$_L$, density of TILs, patient characteristics, and OS. The TILs of the different levels of c-FLIP$_L$ were assessed with both univariate and multivariate analyses to determine their influence on OS. Kaplan-Meier curves were utilized to estimate the distribution of variables in relation to survival. The Cox regression model was employed to correlate assigned variables with OS. OS was defined as death from any cause. Statistical significance was assumed for a two-sided P<0.05.

Results

Patient characteristics

The 180 patients consisted of 102 men and 78 women. Their age ranged from 19 to 85 years old, with a median age of 61. All of the patients were pathologically proven with IIIB (T3N1M0) colon cancer (**Table 1**).

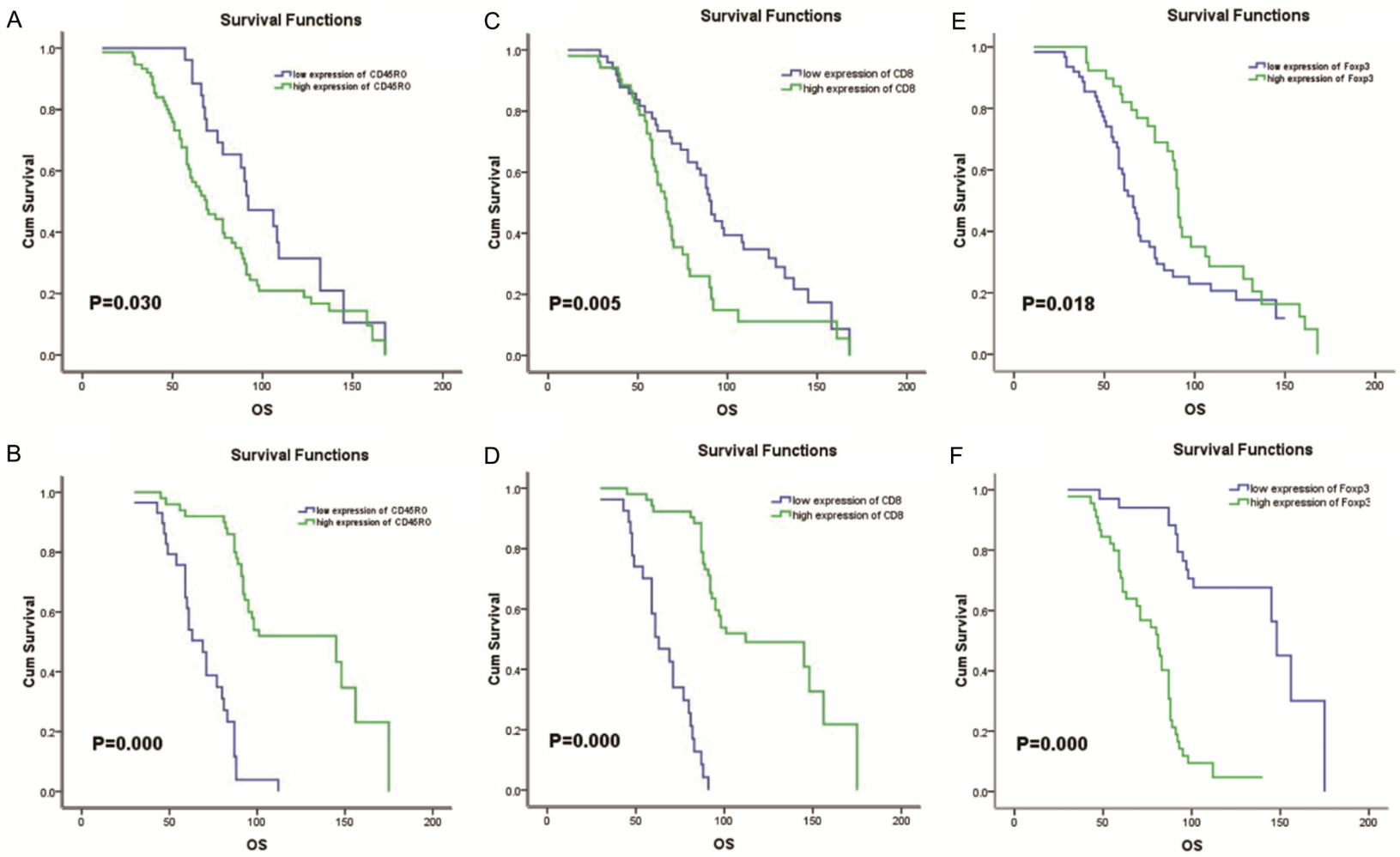

Figure 2. Kaplan-Meier analysis of OS for TILs in high and low expression of c-Flip$_L$ groups. A, C, E: Showed OS for TILs in high expression of c-Flip$_L$; and B, D, F: Showed OS for TILs in low expression of c-Flip$_L$.

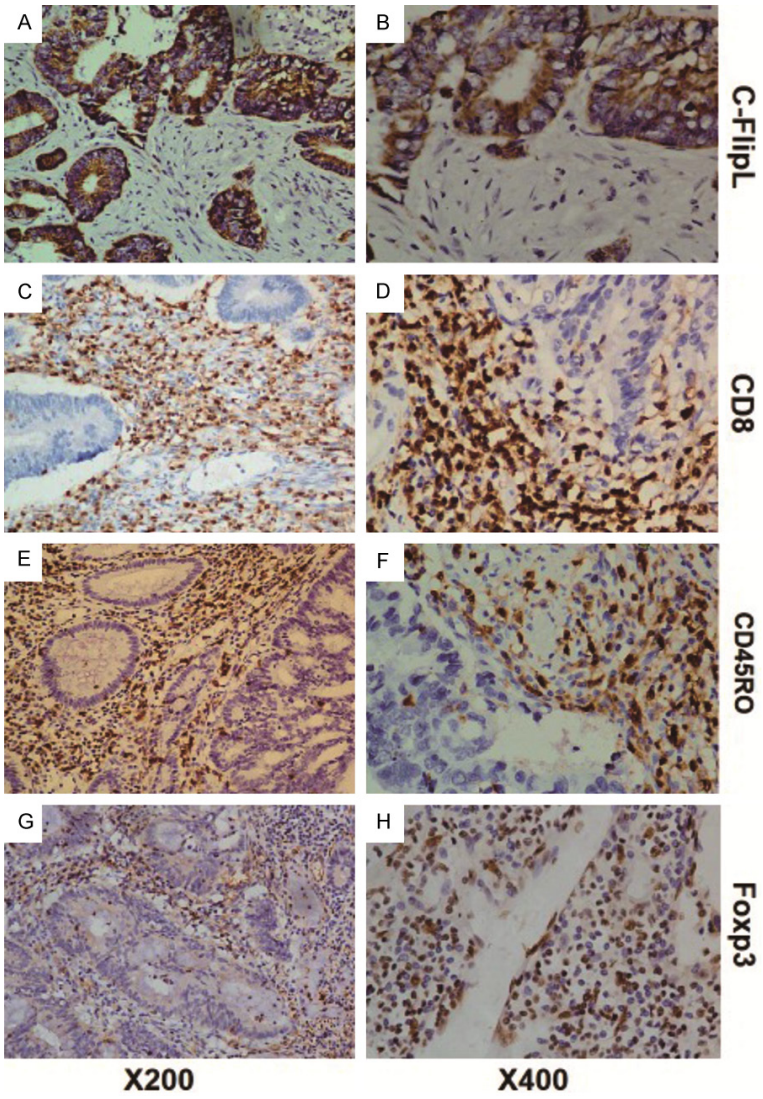

Figure 3. Immunohistochemical staining for c-Flip$_L$, CD8, CD45RO and Foxp3 (200× and 400×). A, B: c-Flip$_L$ in colon cancer tissue. C, D: CD8+ T lymphocytes in colon cancer tissue. E, F: CD45RO+ T lymphocytes in colon cancer tissue. G, H: Foxp3+ T lymphocytes in colon cancer tissue.

Immunohistochemical analysis and clinicopathological parameters

An immunohistochemical analysis was performed on 180 colon cancer samples. The TILs in colon cancer were defined as CD8 (effector T cells), CD45RO (memory T cells), and Foxp3 (regulating T cells). The results revealed that the TILs (i.e., CD8, CD45RO, and Foxp3) were mainly located in the tumor stroma. CD8 and CD45RO stained the cell membrane, whereas Foxp3 stained the cell nucleus. C-FLIP$_L$ was mainly located in the cytoplasm of the cells (**Figures 3** and **4**). According to the immunohistochemical scores, 101 samples (56.1%) showed high levels of c-FLIP$_L$ expression, whereas the remaining 79 samples (43.9%) exhibited low or no c-FLIP$_L$ expression; 125 samples (69.4%) demonstrated high levels of CD45RO expression, whereas the remaining 55 samples (30.6%) presented low or no CD45RO expression; 104 samples (57.8%) displayed high levels of CD8 expression, whereas the remaining 76 samples (42.2%) indicated low or no CD8 expression; 73 samples (40.6%) showed high levels of Foxp3 expression, whereas the remaining 107 samples (59.4%) exhibited low or no Foxp3 expression. The infiltrating lymphoid cells were counted.

Relationship among c-FLIP$_L$ expression, TIL density, and patient characteristics

The markers were divided into two groups on the basis of the median value. The cutoff value for the score of c-FLIP$_L$ was 5, whereas the cutoff value for the density of CD8+, CD45RO+, and Foxp3+ T cells were 34, 36, and 10 cells, respectively. The scores of the markers were analyzed and associated with the clinical pathologic characteristics of colon cancer. c-FLIP$_L$ was divided into two groups, namely, high and low expression, as shown in **Tables 2**-**4**. The densities of CD8+, CD45RO+, and Foxp3+ T cells were correlated with different OS times for various levels of c-FLIP$_L$ ($P<0.05$). However, the density of TILs was not significantly associated with age, gender, tumor sites, or pathological grade for both levels of c-FLIP$_L$.

Relationship between c-FLIP$_L$, TILs, and patients' prognosis

The median observation period was 82.0 months (ranging from 11 to 175 months); the

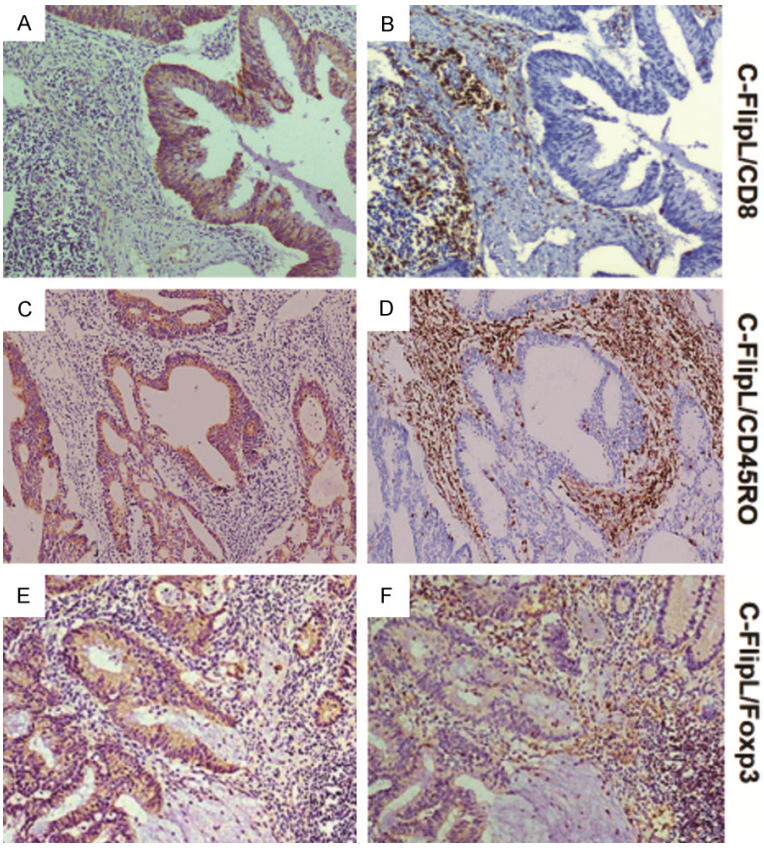

Figure 4. Co-expression of c-Flip$_L$ and TILs.

5-year survival rate of the 180 IIIB colon cancer patients was 73.3%. Kaplan-Meier survival analysis indicated that the OS was significantly shorter for patients with high c-FLIP$_L$ expression. The high expression of CD8+ and CD45RO+ T cells is correlated with a long OS time, whereas that of Foxp3 is associated with a short OS time (P<0.05, **Figure 1**). Afterward, the association between the density of TILs and OS time were analyzed for both the high and low c-FLIP$_L$ expression groups. Patients with high densities of CD8+ and CD45RO+ T cells presented a significantly shorter OS time under the condition of high c-FLIP$_L$ expression (P<0.05). By contrast, patients with high densities of CD8+ and CD45RO+ T cells exhibited a significantly longer OS time under the condition of low c-FLIP$_L$ expression (P<0.01). In addition, a high density of Foxp3 was correlated with a significantly longer OS time under the condition of high c-FLIP$_L$ expression (P<0.05), whereas a high density of Foxp3 was associated with a significantly shorter OS time under the condition of low c-FLIP$_L$ expression (P<0.01, **Figure 2**).

Univariate analysis of factors associated with OS

Univariate analysis demonstrated that CD8+, CD45RO+, and Foxp3+ T cells and age were significantly correlated with OS for both levels of c-FLIP$_L$. Under the condition of high c-FLIP$_L$ expression, a high density of CD8+ and CD45RO+ T cells presented a significantly shorter OS time, whereas a high density of Foxp3+ T cells correlated with a significantly longer OS time. Under the condition of low c-FLIP$_L$ expression, a high density of CD8+ and CD45RO+ T cells exhibited a significantly longer OS time, whereas a high density of Foxp3+ T cells had a significantly shorter OS time. However, no significant correlations were found between OS and gender, tumor sites, and pathological grade (**Table 5**).

Multivariate analysis of factors associated with OS

A multivariate analysis was performed on gender, age, tumor sites, pathological grade, CD8+, CD45RO+, and Foxp3+ T cells for different levels of c-FLIP$_L$. Among the 180 stage IIIB colon cancer patients, multivariate analysis revealed that CD8+, CD45RO+, and Foxp3+ T cells had a significant correlation with OS for both levels of c-FLIP$_L$. Age was correlated with OS for a low expression of c-FLIP$_L$. However, no significant correlations were found between OS and gender, tumor sites, and pathological grade (**Table 6**).

Discussion

This study observed that the prognostic role of TILs was associated with the c-FLIP$_L$ expression in colon cancer cells. To exclude the influence of clinical stages to the prognosis, 180 cases of stage IIIB colon cancer patients were chosen in this study. For a high expression of c-FLIP$_L$ in cancer cells, a high density of CD8+ T cells or a low density of Treg cells was associated with shorter survival against colon cancer, which is in contrast to the conventional notion that CD8+ T cells infiltrate into tumor tissues.

Table 2. Correlation between c-Flip$_L$ and CD8

CD8	c-Flip$_L$					
	High (n=101)			Low (n=79)		
	H (n=52)	L (n=49)	P	H (n=52)	L (n=27)	P
Age						
≥60	32	28	0.653	27	18	0.209
<60	20	21		25	9	
Gender						
Male	28	32	0.241	27	15	0.759
Female	24	17		25	12	
Tumor site						
Left	39	35	0.685	40	21	0.932
Right	13	14		12	6	
Pathology grade						
G1	5	5	0.862	8	1	0.265
G2	45	41		39	24	
G3	2	3		5	2	
Survival time (mo)						
≥60	30	38	0.005	49	15	0.000
<60	22	11		3	12	

Table 3. Correlation between c-Flip$_L$ and CD45RO

CD45RO	c-Flip$_L$					
	High (n=101)			Low (n=79)		
	H (n=75)	L (n=26)	P	H (n=50)	L (n=29)	P
Age						
≥60	47	13	0.257	26	19	0.242
<60	28	13		24	10	
Gender						
Male	43	17	0.471	26	16	0.785
Female	32	9		24	13	
Tumor site						
Left	58	16	0.117	39	22	0.827
Right	17	10		11	7	
Pathology grade						
G1	9	1	0.171	8	1	0.146
G2	61	25		39	24	
G3	5	0		3	4	
Survival time (mo)						
≥60	43	25	0.030	46	18	0.000
<60	32	1		4	11	

In general, the densities of CD8+ T cells, CD45RO+ T cells, and Treg cells were associated with good prognosis in colorectal cancer [21-25]. For example, Galon et al explored 454 cases of human colon cancer specimens and found that patients with a high density of CD45RO+ T cells had a significantly prolonged disease-free survival (DFS), and that the type, density, and location of immune cells were better predictors of patient survival [26]. In this study, 180 cases of colon cancer specimens were examined, and the infiltration of CD8+ and CD45-RO+ T cells was found to correlate with a favorable OS time. Our finding is consistent with previous studies. However, since a part of the follow-up data has been lost, we did not analyze the correlation between the TILs and DFS.

Cancer cells develop multiple mechanisms to escape immune pressure. Previous studies focus on how cancer cells escape from immunity killing by restraining tumor antigen presentation, impeding lymphocytes, infiltrating tumor tissues, and forming immunosuppression microenvironments [27-31]. Limited attention has been devoted to whether immune pressure would promote the progress. Hughes R et al found that (M2) subpopulation of tumor-associated macrophages (TAM) accumulated around blood vessels in tumors after chemotherapy, where they promote tumor revascularization and relapse. However, if the patients were given a kind of immunosuppression drug to impede the function of immunity, the tumor may not relapse [32]. C-FLIP$_L$ is a multiple functional protein that not only inhibits apoptosis, autophagy, and necroptosis, but also promotes EMT and transfers the fas-associated apoptosis signal to the survival signal by activating JNK, AKT and NFκ-B pathways [33-37]. Kim Y et al investigated that cancer associated gene (CAGE) regulates expression of epithelial-mesenchymal transition (EMT)-related protein. Snail, an EMT-related protein, mediates the effect of CAGE on the induction

Table 4. Correlation between c-Flip$_L$ and Foxp3

Foxp3	c-Flip$_L$					
	High (n=101)			Low (n=79)		
	H (n=39)	L (n=62)	P	H (n=34)	L (n=45)	P
Age						
≥60	22	38	0.134	29	16	0.001
<60	17	24		5	29	
Gender						
Male	21	39	0.121	22	20	0.820
Female	18	23		12	25	
Tumor site						
Left	28	46	0.882	25	25	0.894
Right	11	16		9	20	
Pathology grade						
G1	3	7	0.533	6	3	0.298
G2	32	54		26	37	
G3	4	1		2	5	
Survival time (mo)						
≥60	33	35	0.018	32	32	0.000
<60	6	27		2	13	

Table 5. Univariate analysis of factors associated with OS in different levels of c-Flip$_L$

c-Flip$_L$	OS			
	High (n=101)		Low (n=79)	
	Median (95% CI)	P	Median (95% CI)	P
CD45RO				
High (n=125)	69 (56.4-81.6)	0.030	135 (81.7-188.3)	0.000
Low (n=55)	92 (70.1-114.0)		69 (59.9-78.1)	
CD8				
High (n=104)	66 (58.4-73.6)	0.005	112 (72.2-152.8)	0.000
Low (n=76)	91 (83.5-98.5)		63 (51.0-152.8)	
Foxp3				
High (n=73)	91 (88.9-93.1)	0.018	81 (67.2-95.0)	0.000
Low (n=107)	66 (58.9-73.1)		148 (136.5-160.0)	
Age				
≥60 (n=105)	70 (63.1-76.9)	0.134	87 (81.5-92.5)	0.001
<60 (n=75)	91 (74.2-107.8)		145 (64.5-225.5)	
Gender				
Male (n=102)	69 (56.6-81.4)	0.121	89 (82.9-95.1)	0.820
Female (n=78)	89 (71.8-106.2)		92 (84.9-99.1)	
Tumor site				
Left (n=135)	69 (58.1-79.9)	0.882	91 (84.3-97.7)	0.894
Right (n=45)	88 (68.2-107.8)		88 (81.1-94.9)	
Pathology grade				
G1 (n=19)	67 (40.2-93.8)	0.553	101 (92.2-109.8)	0.298
G2 (n=149)	78 (63.3-92.7)		89 (84.8-93.2)	
G3 (n=12)	68 (40.1-95.9)		87 (68.3-105.7)	

of matrix metalloproteinase-2 (MMP-2) and cancer cell motility. Meanwhile, c-FLIP$_L$ mediates the effect of CAGE on the induction of MMP-2 and cell motility by the induction of Snail, which might explain these phenomena [38]. The clinical impact of c-FLIP$_L$ on TILs has not been evaluated. Colon cancer cells generate adaptive changes when encountering immune pressure. In this paper, the expression of c-FLIP$_L$ and the infiltration of TILs are tested in local advanced colon cancer. Data showed that more than 50% colon cancer tissue express high c-FLIP$_L$. If the effect of c-FLIP$_L$ is znot considered, patients with high infiltration of effector T cells would appear to have favorable outcomes. However, if the effect of c-FLIP$_L$ is taken into account, as well as the high infiltration of CD8+ and CD45RO+ T cells or the low infiltration of Treg cells, patients with a high expression of c-FLIP$_L$ would present shorter OS time. By contrast, patients with a low expression of c-FLIP$_L$ under the same scenario would have longer OS time. These findings suggest that under specific conditions, cancer cells will adapt to the immune microenvironment and may transform from “immune surveillance” into “immune driving” [39]. However, since our time and cases were limited, whether this phenomena is specific to c-FLIP$_L$ or other inhibitors of apoptosis such as BCL-2, is still unknown, which needs further investigation.

Conclusion

This study revealed that the relationship between the den-

Table 6. Multivariate analysis of factors associated with OS in different levels of c-Flip$_L$

c-Flip$_L$	OS					
	High (n=101)			Low (n=79)		
	HR	(95% CI)	P	HR	(95% CI)	P
CD45RO	1.774	1.041-3.025	0.035	0.410	0.178-0.944	0.036
CD8	1.951	1.190-3.199	0.008	0.183	0.080-0.417	0.000
Foxp3	0.540	0.326-0.894	0.017	3.665	1.577-8.470	0.003
Age	1.294	0.789-2.124	0.307	2.090	1.112-3.930	0.022
Gender	0.602	0.362-1.003	0.051	0.996	0.544-1.825	0.991
Tumor site	0.837	0.490-1.432	0.516	0.662	0.301-1.376	0.269
Pathology grade	1.239	0.769-1.996	0.379	0.873	0.525-1.452	0.601

sity of TIL and the prognosis of colon cancer depends on the expression of c-FLIP$_L$ in cancer cells. The clinical outcome of immune response not only relied on the subtypes of TILs but also, more importantly, on the genetic background of cancer cells.

Acknowledgements

This study received support from the National Natur-al Science Founda-tion of China (Grant No. 81272341).

Disclosure of conflict of interest

None.

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