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BRIEF ARTICLE

Expression of calreticulin is associated with infiltration of T-cells in stage ⅢB colon cancer

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Abstract

AIM: To investigate the correlation between expression of calreticulin and infiltration of lymphocytes in stage ⅢB colon cancer.

METHODS: Sixty-eight pathologically-confirmed specimens were obtained from stage ⅢB (T3N1M0) colon cancer patients who underwent radical resection between January 1999 and May 2002 at the Cancer Center of Sun Yat-Sen University, Guangzhou, China. Immunohistochemical analysis was performed to show infiltration of lymphocytes and expression of calreticulin in colon cancer. Association between calreticulin expression, infiltration of lymphocytes, and 5-year survival rate of patients was assessed.

RESULTS: The expression level of calreticulin was lower in cancer nest than in its adjacent normal epithelium since 61.8% (42/68) of the samples were stained with calreticulin in colon cancer. The expression of calreticulin in colon cancer was associated with the infiltration of CD45RO+ cells rather than with that of CD3+ cells. In addition, the stronger expression of calreticulin and the higher infiltration of CD3+ and CD45RO+ cells in colon cancer were associated with the higher 5-year survival rate of patients.

CONCLUSION: Expression of calreticulin is associated with infiltration of T-cells, which implies that a low expression level of molecular marker may represent a new mechanism underlying immune escape in colon cancer.

Key words: Calreticulin; Tumor-infiltrating lymphocyte; Colon cancer; Immune escape

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INTRODUCTION

Mounting evidence indicates that colorectal cancer is immunogenic[1]. For example, when immunologic effector cells, such as CD3+ T-cells, CD45RO+ T-cells and macrophages, infiltrate colorectal cancer tissue, tumor progression is decreased[2-7]. However, colorectal cancer cells have developed multiple immune escape mechanisms, such as reduced expression of HLA-I molecules, expression of immune inhibitors including TGF-β and indoleamine 2,3-dioxygenase (IDO), and induction of Treg cells[8-11]. More importantly, targeting the immune system can stimulate an immune response in colorectal cancer[12-16].

The mechanisms by which colorectal cancer cells induce an immunologic response remain unknown. Recently, evidence indicates that a subset of damage-associated molecular patterns (DAMPs) is involved in the induction of immune response. During the apoptosis of cancer cells, translocation of calreticulin (CRT) from endoplasmic reticulum (ER) to membrane (ecto-CRT exposure), expression of heat-shock proteins, release of HMGB1 from cells, and expression of NKG2D may serve as danger signals, thereby inducing an immune response[17,18]. CRT is a highly conserved 46 kDa Ca^{2+}-binding protein, which is mainly located in the lumen of ER and has various versatile functions, such as chaperone activity, regulation of Ca^{2+} homeostasis, adhesion signaling, involving gastrointestinal mucin synthesis at the stage of folding and oligomerization in ER, inhibiting angiogenesis and tumor growth[19-23]. Within ER, CRT interacts with various molecules like ERp57 and calnexin (CNX) to aid in proper folding of proteins[24]. Furthermore, CRT plays an important role both in the assembly of MHC class I molecule and in the loading of antigen peptides onto the MHC- I molecule within ER[25]. Besides, it has been shown that nuclear CRT can regulate nuclear protein transport and influence signaling *via* nuclear steroid receptors and integrins[26,27]. When cells are treated with anthracyclines, oxaliplatin, radiation and hypoxia, ecto-CRT exposure works as an "eat me" signal for dendritic cells and macrophages, initiating an immune response[28-30].

Altered expression of calreticulin has been detected in melanoma, and in liver, bladder, prostate, lung, pancreatic and breast cancers[31-37]. However, the clinical significance of CRT expression remains poorly understood and controversial. It has been reported that overexpression of CRT is related to the promotion of breast cancer and decreases the progression of prostate cancer[34,36]. Additionally, interaction between calnexin and calreticulin contributes to metastasis of melanoma[31]. Since the clinical evidence supporting the immune regulation of CRT is scant, this study examined the expression of CRT in stage ⅢB colon cancer patients to evaluate whether the expression of CRT is associated with the immunogenicity of colon cancer.

MATERIALS AND METHODS

Materials

Sixty-eight pathologically-confirmed specimens were obtained from patients with stage ⅢB (T3N1M0; AJCC, 2002) colon cancer between January 1999 and May 2002 at the Cancer Center of Sun Yat-Sen University, Guangzhou, China (Table 1). All the patients underwent radical resection and 5-FU-based adjuvant chemotherapy after operation for 6 mo. The patients were evaluated every 3 mo during the first year, every 6 mo in the second year, and once every year thereafter for a total of 5 years. If a recurrence or a metastasis occurred, 5-FU-based chemotherapy was given according to the national comprehensive cancer network (NCCN) guidelines. No patients received preoperative blood transfusion or non-steroidal anti-inflammatory drugs. Overall survival was defined as the time from surgery to death. Data analysis was done on the last known day when the patient was alive.

Immunohistochemical assay and scoring systems

Formalin-fixed, paraffin-embedded tissue was cut into 4-μm thick sections. The size of each tissue section was about 1 cm × 1 cm. Then, the sections were dewaxed, rehydrated, and blocked with hydrogen peroxide. Antigens were retrieved in 10 mmol/L citrate buffer (pH 6.0) for 10 min and cooled to room temperature. After blocked with sheep serum, the sections were incubated overnight at 4℃ with either rabbit polyclonal antibody against human calreticulin at a dilution of 1:2000 (Abcam, Cambridge, MA, USA) or mouse monoclonal antibody against human CD3 and CD45RO (Zymed, San Diego, CA, USA), both of which were diluted to 1:100. Subsequently, biotinylated secondary antibodies and streptavidin-biotinylated horseradish peroxidase complexes were used. The sections were developed with diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin. Negative controls in which primary antibody was replaced with a phosphate buffered solution (PBS), were employed.

Infiltration of lymphocytes in the tumor was scored with Hussein's method[38] and expression of calreticulin in colon cancer was interpreted *via* immunoreactivity using the 0-4 semi-quantitative system derived from Remmele and Stegner for both the intensity of staining and the percentage of positive cells (labeling frequency percentage)[39]. The cells were counted in at least 10 different fields for each section, and the size of each high-power field (× 400) was about 300 μm × 300 μm. The cells were counted in tumor stroma. The highest infiltration areas of lymphocytes were chosen. Necrotic areas were avoided. Two observers counted the cells at the same time and in the same field under a multiple-lens microscope. The results were expressed as mean ± SE. Cytoplasm staining

Table 1 Parameters of patients (n = 68)

Parameters	n (%)
Age (yr)	
< 60	30 (44.1)
⩾ 60	38 (55.9)
Gender	
Male	38 (55.9)
Female	30 (44.1)
Tumor site	
Left hemicolon	45 (66.2)
Right hemicolon	23 (33.8)
Pathological grade	
G1	10 (14.7)
G2	51 (75.0)
G3	7 (10.3)
Survival time (mo)	
⩾ 60	52 (76.5)
< 60	16 (23.5)

was divided into no staining/background of negative controls (score = 0), weak staining above background (score = 1), moderate staining (score = 2), and intense staining (score = 3). The labeling frequency was scored as 0 (⩽ 1%), 1 (1%-24%), 2 (25%-49%), 3 (50%-74%), and 4 (⩾ 75%), respectively. The product index was obtained by multiplying the intensity and percentage and scored as (-), (+), (++), and (+++), which indicate the cross-indices of 0-2, 3-5, 6-8, and 9-12, respectively. (-) was defined as no or negative expression, and (+)-(+++) as positive expression. Each section was independently scored by two pathologists. If an inconsistency occurred, a third pathologist was consulted to achieve a consensus.

Statistical analysis

Correlation between calreticulin expression, or infiltration of lymphocytes and parameters of patients was analyzed by χ^2 test or Fisher's exact test. Factors, including gender and age of the patients, pathologic grade, tumor site, infiltration of CD3+ cells and CD45RO+ cells, and calreticulin expression level in colon cancer, were assessed by univariate and multivariate analysis to determine their influence on the overall survival rate of patients. Kaplan-Meier curve and log-rank test were used to estimate the distribution of variables in relation to survival. Cox regression model was used to correlate the assigned variables with the overall survival rate. All statistical analyses were carried out using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Expression of CRT and infiltration of lymphocytes in stage ⅢB colon cancer

CRT was stained in cytoplasm rather than in nuclei of cancer cells and normal epithelium. The expression level of CRT was lower in colon cancer than in its adjacent normal epithelium. Of the stained samples, 61.8% (42/68) were positive for CRT in cancer nest (Figure 1A and B,

Table 2 Relation between expression of calreticulin, infiltration of lymphocytes and parameters of patients (n = 68)

Parameters	Calreticulin expression		P
	(-)	(+)-(+++)	
Gender			0.813
Male	15	23	
Female	11	19	
Age (yr)			0.204
⩾ 60	12	26	
< 60	14	16	
Tumor site			0.245
Left hemicolon	15	30	
Right hemicolon	11	12	
Pathological grade			0.643
G1	5	5	
G2	19	32	
G3	2	5	
Infiltration of CD3+ cells per high-power field			0.387
> 18	18	33	
⩽ 18	8	9	
Infiltration of CD45RO+ cells per high-power field			0.010
> 26	15	36	
⩽ 26	11	6	

Table 2). CD3+ and CD45RO+ cells were observed in all samples from tumor stroma and its adjacent normal mucosa. Positively stained antigens were found on cell membrane (Figure 1C-F).

Relation between expression of CRT and infiltration of CD3+ or CD45RO+ cells

The cut-off value for infiltration of lymphocytes in colon cancer was 75%. Infiltration of 18 CD3+ cells and 26 CD45RO+ cells was observed in per high-power field, and was thus recorded as high and low infiltration. Log-rank test was used to analyze the relation between expression of CRT and infiltration of CD3+ and CD45RO+ cells in colon cancer, showing that positive expression of CRT (+-+++) was associated with high infiltration of CD45RO+ cells (P = 0.010, Table 2). No correlation was observed between expression of CRT and infiltration of CD3+cells or other parameters of the patients, such as age, gender, tumor site.

Univariate and multivariate survival analysis

By the end of a 5-year follow-up period, 52 patients were alive with a 5-year survival rate of 76.5%. Kaplan-Meier survival analysis indicated that positive expression of CRT was associated with a higher 5-year survival rate. The 5-year survival rate of patients with positive and negative expression of CRT was 85.5% (36/42) and 61.5% (16/26), respectively (P = 0.022, Table 3). However, the survival curves for patients with positive and negative expression of CRT were crossed at 18 mo (Figure 2). In addition, high infiltration of CD3+ and CD45RO+ cells in colon cancer was associated with a higher 5-year survival rate (P = 0.000, Figure 3).

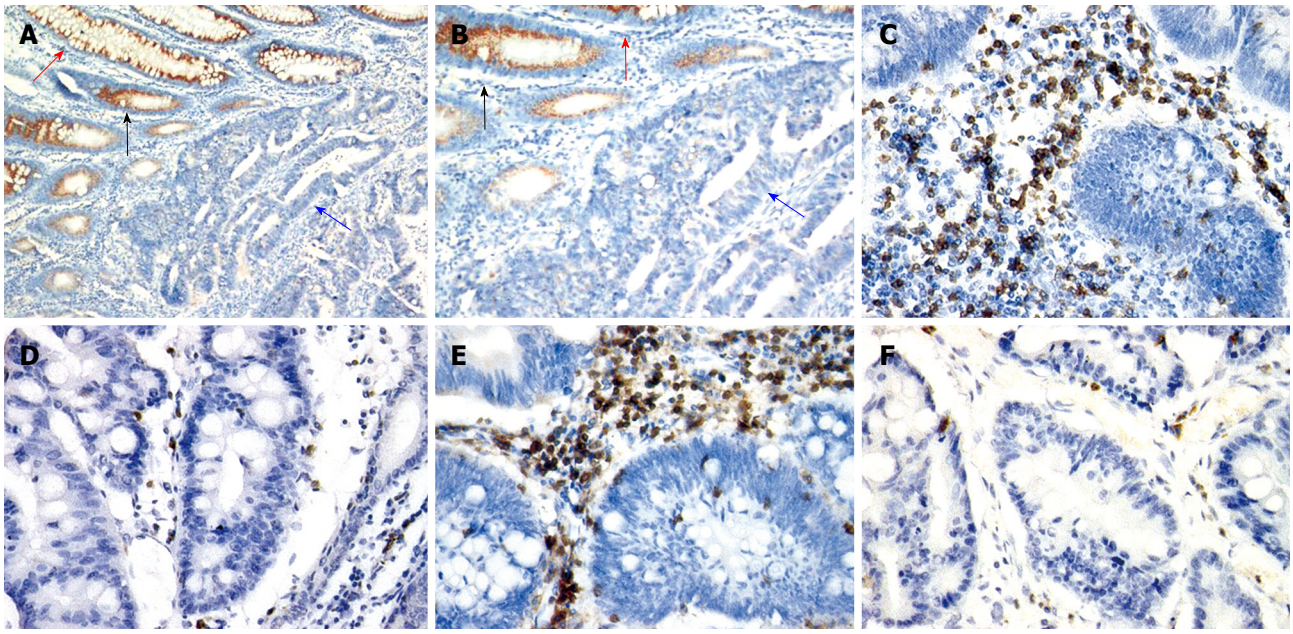

Figure 1 Expression level of CRT in cancer nest (A) and in its adjacent normal epithelium (B) [× 100 in A, × 200 in B, normal epithelium (red arrow), atypical hyperplasia (black arrow) and tumor tissue (blue arrow), respectively], high infiltration of CD3+ cells (C) and CD45RO+ cells (E) in colon cancer stroma (× 400), low infiltration of CD3+ cells (D) and CD45RO+ cells (F) in colon cancer stroma (× 400).

Table 3 Univariate survival analysis (n = 68)

Parameters	Survival time (mo)		*P*
	< 60	⩾ 60	
Gender			0.542
Male	10	28	
Female	6	24	
Age (yr)			0.973
⩾ 60	9	29	
< 60	7	23	
Tumor sites			0.722
Left hemicolon	10	35	
Right hemicolon	6	17	
Pathological grade			0.919
G1	2	8	
G2	12	39	
G3	2	5	
Infiltration of CD3+ cells per high-power field			0.000
> 18	6	45	
⩽ 18	10	7	
Infiltration of CD45RO+ cells per high-power field			0.000
> 26	5	46	
⩽ 26	11	6	
Calreticulin expression			
(-)	10	16	
(+)-(+++)	6	36	0.022

Cox regression model revealed that neither expression of CRT nor infiltration of CD3+ cells or CD45RO+ cells in colon cancer had an independent prognostic value (Table 4).

DISCUSSION

In this study, the expression level of CRT was lower in colon cancer than in its adjacent normal epithelium, indicating that expression of CRT is associated with infiltration of CD45RO+ cells rather than with CD3+ cells and may serve as a mechanism underlying immune escape in colon cancer, although it is not an independent prognostic indicator.

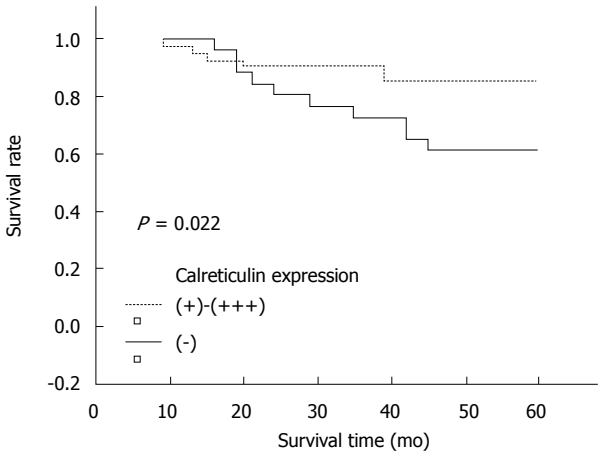

Figure 2 Relation between CRT expression and 5-year survival rate.

A few studies have examined the role of CRT in the progression of colon cancer[40,41]. Toquet *et al*[40] reported that the expression of CRT is decreased in non-mucinous colonic adenocarcinoma while Vougas reported that the expression of CRT is increased in poorly-differentiated colon cancer and advanced tumor[41]. In this study, however, neither the pathological classification nor the differentiation grade was associated with the expression level of CRT.

It is still unknown whether CRT is involved in the mechanism underlying immune escape in colon cancer. Colorectal cancer with microsatellite instability (MSI-H) is immunogenic due to infiltration of a large number of lym-

Table 4 Multivariate survival analysis (n = 68)

Parameters	B	SE	Wald	df	Sig.	Exp(B)	95% CI for Exp (B)	
							Lower	Upper
Pathological grade	0.172	0.261	0.435	1	0.510	1.188	0.712	1.979
Gender	-0.027	0.247	0.012	1	0.912	0.973	0.599	1.580
Age	0.079	0.255	0.095	1	0.758	1.082	0.656	1.784
Tumor site	0.085	0.276	0.094	1	0.759	0.919	0.535	1.579
Infiltration of CD3+ cells	-0.057	0.511	0.013	1	0.911	0.944	0.347	2.537
Infiltration of CD45RO+ cells	-0.743	0.552	1.809	1	0.179	0.476	0.161	1.404
Expression level of calreticulin	0.133	0.279	0.226	1	0.634	0.876	0.507	1.512

B: Regression coefficient; SE: Standard error; df: degree of freedom; Sig.: Significant; Exp (B): Odds ratio; CI: Confidence interval.

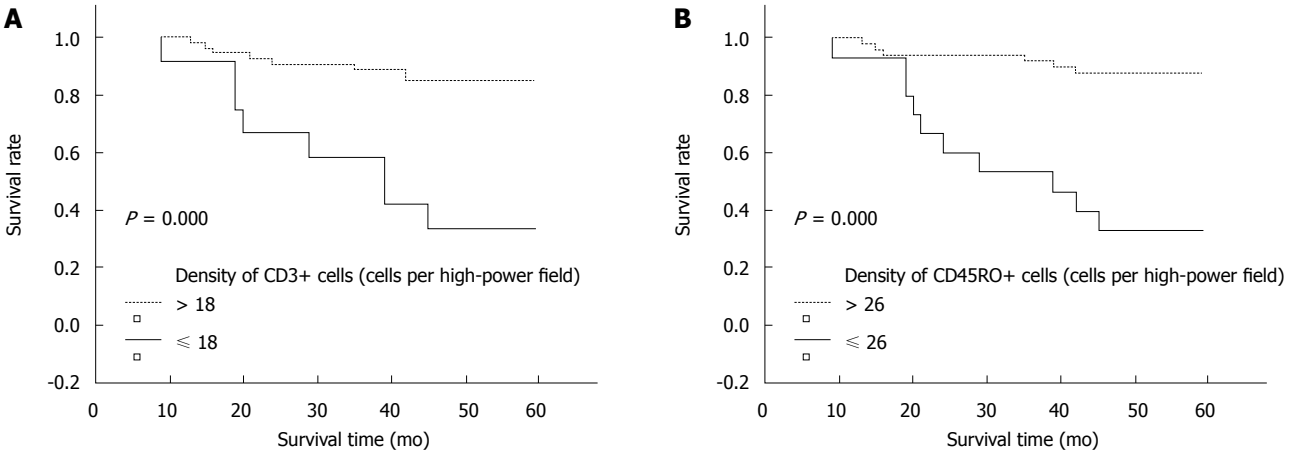

Figure 3 Correlation between high infiltration of CD3+ cells (A) and CD45RO+ cells (B) in colon cancer and 5-year survival rate of patients.

phocytes, resulting in a favorable prognosis[42,43]. Microarray analysis showed that CRT expression was up-regulated in 27 cases of colorectal cancer with MSI-H, while quantitative RT-PCR analysis failed to confirm it in another 26 cases of colorectal cancer with MSI-H[44]. In this study, the relation between expression level of CRT and infiltration of lymphocytes was assessed, which indicates that expression of CRT is related with infiltration of CD45RO+ cells in colon cancer. Since CD45RO+ cells contribute to a favorable prognosis of colon cancer patients, it is reasonable to infer that CRT expression is involved in immune response that occurs in colon cancer. However, this study failed to show that either CRT expression or infiltration of CD45RO+ cells was associated with the 5-year survival rate in a Cox statistical model. Therefore, further study is needed to confirm the role of CRT expression in the progression of colon cancer.

In conclusion, a low expression level of endogenous "danger signals", such as CRT, may represent a new mechanism underlying immune escape in colon cancer.

COMMENTS

Background

There is evidence that colorectal cancer is immunogenic. The mechanisms by which colorectal cancer cells induce an immunologic response remain unknown. A subset of damage-associated molecular patterns (DAMPs) has been recently found to be involved in the induction of immune response. Calreticulin is one of the most important DAMPs. Whether calreticulin is associated with the immunogenicity of colon cancer remains controversial.

Research frontiers

Calreticulin is a multifunctional chaperone protein which mainly locates in the lumen of endoplasmic reticulum (ER). When exposed on the cell surface, calreticulin works as an "eat me" signal for dendritic cells and macrophages, initiating an immune response. Whether the expression of calreticulin in colon cancer is involved in induction of immune response is a hot topic on the role of calreticulin in pathogenesis of colon cancer.

Innovations and breakthroughs

Studies indicate that the expression of calreticulin is decreased in non-mucinous colonic adenocarcinoma while is increased in poorly -differentiated colon cancer and advanced tumor. Other studies have shown that one of the calreticulin fragments inhibits angiogenesis and growth of colon cancer cells. However, the correlation between expression of calreticulin and survival of colon cancer patients remains unknown. In this study, the expression of calreticulin in stage ⅢB colon cancer was associated with the lower infiltration of CD45RO+ lymphocytes which was associated with a shorter 5-year survival time, suggesting that reduced expression of CRT may serve as a mechanism underlying immune escape in colon cancer.

Applications

In this study, reduced expression of endogenous "danger signals", such as calreticulin, was found to be a new mechanism underlying immune escape in colon cancer, which is important for a better understanding of the immunogenicity of colon cancer, thus providing a new immunotherapy for colon cancer.

Terminology

Calreticulin: A highly conserved 46 kDa Ca^{2+}-binding protein, which is mainly located in the lumen of ER and has various versatile functions, like chaperone activity, regulation of Ca^{2+} homeostasis, and adhesion signaling. When cells were treated with anthracyclines, oxaliplatin, radiation and hypoxia, calreticulin exposure works as an "eat me" signal for dendritic cells and macrophages, initiating an immune response.

Peer review

This is a well-conducted study on the correlation between calreticulin expression and infiltration of lymphocytes in colon cancer and their impact on the 5-year survival time of patients with stage ⅢB colonic cancer.

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