RESEARCH COMMUNICATION

Elevated Circulating CD19+ Lymphocytes Predict Survival Advantage in Patients with Gastric Cancer

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Abstract

Background: Circulating lymphocyte subsets reflect the immunological status and might therefore be a prognostic indicator in cancer patients. Our aim was to evaluate the clinical significance of circulating lymphocyte subset in gastric cancer (GC) cases. Methods: A retrospective study on a prevalent cohort of 846 GC patients hospitalized at Hospital from Aug 2006 to Jul 2010 was conducted. We calculated the patient's disease free survival (DFS) after first hospital admission, and hazard ratios (HR) from the Cox proportional hazards model. Results: Our findings indicated a significantly decreased percentage of CD3+, and CD8+ cells, a significantly increased proportion of CD4+, CD19+, CD44+, CD25+, NK cells, and an increased CD4+/CD8+ ratio in GC patients as compared with healthy controls (all P < 0.05). Alteration of lymphocyte subsets was positively correlated with sex, age, smoking, tumor stage and distant metastasis of GC patients (all P<0.05). Follow-up analysis indicated significantly higher DFS for patients with high circulating CD19+ lymphocytes compared to those with low CD19+ lymphocytes (P=0.037), with CD19+ showing an important cutoff of 7.91±2.98% Conclusion: Circulating lymphocyte subsets in GC patients are significantly changed, and elevated CD19+ cells may predict a favorable survival.

Keywords: Gastric carcinoma - flow cytometry - lymphocyte subsets - immune function - DFS

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Introduction

Gastric cancer (GC) is not only the second most common cancer, but also is the second most common cause of cancer-related death in the world, and it remains difficult to cure in developing countries, primarily because most patients present with advanced disease (Bertuccio et al., 2009; Babaei et al., 2010; Lee et al., 2011; Zhang et al., 2011). Even patients who present in the most favorable condition and who undergo curative surgical resection often die of recurrent disease (Duncavage et al., 2010; Dozois et al., 2011). Although preoperative tumor staging is useful to select the appropriate therapeutic strategy for GC patients, clinical tumor staging alone cannot predict patients' prognosis (Shimada et al., 2010; Cao et al., 2011; Feng et al., 2011). It has been reported that some immune-related biomarkers for diagnosis/prognosis and therapy monitoring of GC patients (Aburatani 2005; Tan et al., 2009). The host immune response to cancer cells is also associated with tumor progression (Hamaï et al., 2010). Once the tumor grow in vivo, the body's immune system may generate anti-tumor response involved in various ways to eliminate tumor cells or control tumor growth (Costache et al., 2010; Abès et al., 2011). Human anti-tumor immunologic mechanisms include cellular and humoral immune responses, with T lymphocyte immunity-based (Gross et al., 2008; Sabel et al., 2009; Sato et al., 2009). In recent studies, GC patients were often accompanied with low immune function and immune disorders (Saurer et al., 2009; Sagaert et al., 2010), but there were few reports with regard to the relationship between peripheral blood immunological parameters and prognosis. In this study, the parameters of cellular and humoral immunity were measured by flow cytometry (FCM) in 846 patients with GC at first hospital admission and 96 healthy persons. The significance of immune responses to gastric cancer cells and prognosis factors was evaluated.

Materials and Methods

GC patients

All procedures complied with the ethical guidelines for human specimens and use of laboratory of blood collection at Zhejiang Province Cancer Hospital, Zhejiang Cancer Center, China. All patients gave their written informed consent for this project. 846 patients (587 male, 259 female, age range 21-83, mean age 57.79) who were first diagnosed as GC and subsequently confirmed by histopathology using endoscopic biopsy between Aug 2006 to Jul 2010 in Zhejiang Province Cancer Hospital were enrolled in this study. All the patients did not receive

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radiotherapy and chemotherapy before blood tests. Among them, 617 patients received gastrectomy, of whom the pathological reports were available. 139 patients were treated with chemotherapy, while 55 patients did not receive any therapies due to the tumor progression or other reasons. The stages of tumor were determined according to the pathological diagnosis using endoscopic gastric mucosal biopsies in the patients without surgery. At the same time, age- and sex-matched 96 healthy volunteers were randomly selected in Hangzhou city, Zhejiang province, who is not merely the absence of disease and weakness, but also found no any disease including inflammation. Among these volunteers, 78 were male, 18 were female, the ages of the controls ranged from 29 to 74 years, and the median age was 48.5 years. All volunteers gave their informed consent. Follow-up deadline was in April 31, 2011, with lost less than 5%.

846 patients with GC were classified by different criterias. According to pathological types, there were 3 cases with mesenchymomas, 2 cases with squamous cell carcinomas, 541 cases with adenocaricinoma, 3 cases with adenosquamous carcinomas, 3 cases with neuroendocrine tumors, 1 case with mucinous carcinoma, 263 cases with signet ring cell carcinomas, 30 cases with unknown pathological types. While based on 7th UICC TNM staging system (Sobin et al., 2009), there were 143 patients with stage I (93 cases with IA and 50 cases with IB), 90 cases with stage II, 287 cases with III (149 cases with IIIA , 138 cases with IIIB), 184 cases with IV, 142 cases without stage data. Among all the patients, 95 cases were suffered from metastasis, while 622 cases were not. Metastasis in the remained 129 patients could not be determined. Grounded on the degree of differentiation, undifferentiated, poorly differentiated, low differentiated cases amounted to 581, moderately differentiated, highly differentiated cases amounted to 90, and 175 patients with unknown degree of differentiation.

Determination of lymphocyte subpopulations

Blood was collected into heparinized tubes 1 day at first hospital admission. One hundred microliters of the heparinized blood was mixed with 20 μL of each of the following fluorescent mouse anti-human monoclonal antibodies according to the reagent instructions: CD3, CD4, CD8, CD19, CD25, CD44 and CD56 (B-D Company, USA). The mixtures were incubated for 30 min in a dark room, washed with 3 mL of PBS, and centrifuged at 1500 rpm for 5 min. The supernatant was discarded, and the pellet was resuspended with 1 mL of PBS for flow cytometric analysis. The percentage of fluorescent-positive cells was determined by FACS Calibur flow cytometer (BD Biosciences, USA).

Statistical analysis

SPSS 17.0 (SPSS, Chicago, IL) software was used for statistical analysis by professional person. Initially, a normal distribution test was performed for measurement data. Normally distributed data were then analyzed using a t-test, whereas non-normally distributed data were analyzed using the Mann-Whitney U test. Survival time was calculated from the day of the initial flow cytometry examination. Survival rate was calculated using the Kaplan-Meier method and Cox regression models. All tests were two-sided, and a P value of less than 0.05 was considered significant.

Results

Comparison of T lymphocyte subsets and natural killer cells between GC patients and controls

On flow cytometry analysis, GC patients at first hospital admission had a significantly decreased percentage of $CD3^+$ and $CD8^+$ lymphocytes as compared with healthy controls ($P<0.05$). In contrast, the proportions of $CD4^+$, $CD19^+$, $CD25^+$, $CD44^+$ lymphocytes and NK cells, as well as the $CD4^+/CD8^+$ ratio, were significantly increased in GC patients as compared with healthy controls ($P<0.05$) (Table 1).

Correlation between lymphocyte subpopulations and clinicopathological parameters

To assess the correlation between lymphocyte subpopulations and clinicopathological parameters, we performed a comparison of the immune cell subpopulations of PBLs in GC patients without therapy across the different

Table 1. Comparison of Immune Cell Subpopulations from the Peripheral Blood of GC Patients at First Hospital Admission and Controls ($\bar{x} \pm s$)

Immune cell subpopulations	Normal control (n=96)	Patients with GC (n=846)
CD3	73.32±7.65	67.51±10.97[a]
CD4	39.74±6.51	42.18±10.3[a]
CD8	28.68±4.83	18.40±8.13[a]
CD4/CD8	1.44±0.42	2.91±2.02[a]
CD44	45.81±13.50	73.21±16.00[a]
NK	17.10±8.00	20.54±11.02[a]
CD19	7.91±2.98	9.27±4.67[a]
CD25	17.10±5.21	28.67±8.79[a]

aindicate a significant difference in comparison with normal group ($P<0.05$)

Table 2. Correlation Between Lymphocyte Subpopulations and Clinicopathological Parameters

Groups	CD19	CD25	NK
Age			
<60	9.51±4.26	28.53±8.62	18.95±10.57*#
≥60	9.00±5.10	28.82±9.00	22.44±11.26*#
Gender			
Men	8.82±4.60*	29.28±9.23*	21.87±11.68*#
Female	10.29±4.68*	27.28±7.50*	17.48±8.61*#
Smoking			
+	8.81±4.65*#	30.25±9.42*	20.81±11.38
-	9.69±4.66*#	27.24±7.88*	20.30±10.70
Distant metastasis			
M0	9.41±4.66	28.86±8.79	19.87±10.71*#
M1	9.19±4.50	27.70±8.55	22.24±10.10*#
Clinical stages			
I/II	10.09±4.70*	28.82±8.00	20.00±10.05
III/IV	9.03±4.54*	28.67±8.94	20.34±10.85

*indicate a significant difference using a t-test ($P<0.05$); #indicate a significant difference using a binary logisitc regression analysis ($P<0.05$)

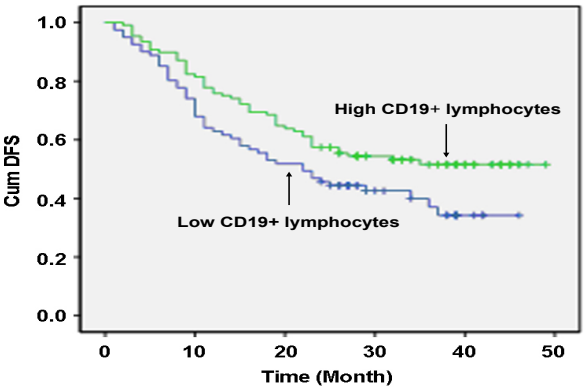

Figure 1. Kaplan-Meier Analysis Showed that CD19+ Lymphocyte was an Favorable Predictor for the Survival of GC Patients at First Hospital Admission, Which was Demonstrated by the Significantly Higher DFS of Patients with High CD19+ Lymphocytes Compared with Those with Low CD19+ Lymphocytes

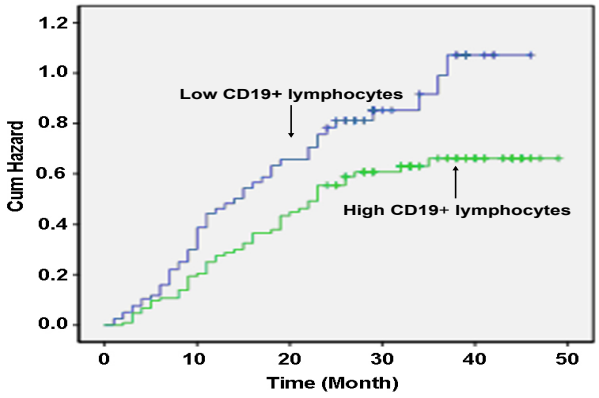

Figure 2. Cox Regression Models Indicated that Only CD19+ Lymphcyte is a Promising Independent Predictor of Survival in GC Patients (P = 0.037). The risk ratio, after adjustment for competing risk factors, sex, age, and stage of disease, was found to be 0.643 (95% confidence intervals, 0.431 to 0.958)

T and N stages of the tumors and across the different clinical stages of patients. The results indicated that the proportions of CD3+, CD4+, CD8+, CD44+, CD19+, or CD25+ lymphocytes and NK cells, as well as CD4+/CD8+ ratio, were not associated with lymph node metastasis, histological type and histological differentiation (P>0.05), but the proportion of NK cells was positively associated with age, gender and distant metastasis, respectively (P<0.05); the proportion of CD19+ lymphocytes was positively associated with gender, smoking and clinical stage of tumor, respectively (P<0.05); the proportion of CD25+ lymphocytes was positively with gender and smoking, respectively (P<0.05) (Table 2). The binary logisitc regression analysis of the proportions of NK+, CD19+ and CD25+ cells in peripheral blood showed that there was a positive correlation of NK+ cells levels in the GC patients with age, gender and distant metastasis; the proportion of CD19+ lymphocytes in GC patients was closely correlated with smoking (P <0.001) (Table 2).

CD19+ lymphocytes and survival in GC patients

Kaplan-Meier analysis showed that CD19+ lymphocytes was an favorable predictor for the survival of GC patients at first hospital admission, which was demonstrated by the significantly higher disease free survival (DFS) of patients with high CD19+ lymphocytes as compared with those with low CD19+ lymphocytes (Figure 1). In contrast, CD4+, CD19+, CD3+, CD25+, CD25+, and NK cells, and the CD4+/CD8+ ratio, had no prognostic significance for DFS. Retrospective analysis using Cox regression models indicated that only CD19+ lymphocytes is a promising independent predictor of DFS in GC patients (P=0.037). The risk ratio after adjustment for competing risk factors, sex, age, and stage of disease was found to be 0.643 (95% confidence intervals, 0.431 to 0.958) (Figure 2). The results showed that increases in CD19+ lymphocytes had a greater impact on the prognosis than tumor clinical stage.

Discussion

Tumor immunity is really a complex process and the immune function in patients with malignant tumors is often abnormal (Block et al., 2009). Cellular constituents in immune system mainly include T, B cells, NK cells, macrophages and dendritic cells (Jeong et al., 2011). The immune system can not only identify and eliminate the invasive pathogenic microorganisms, but also can recognize and kill mutant tumor cells, aging cells and other adverse components (Block et al., 2009; Jeong et al., 2011; Schreiber et al., 2011). In this study, using flow cytometry (FCM) to measure the expression levels of CD3+, CD4+, CD8+, CD4+/CD8+, CD19+, CD25+, CD44+ and NK cells in peripheral blood from 846 GC patients and 96 control donors, we found that as compared with those in control group, the expression of CD3+ and CD8+ lymphocytes in GC patients were obviously lower, while the expression of CD4+, CD19+, CD25+, CD4+/CD8+, CD44+, NK+ were significantly higher (P<0.05).

Furthermore, the results by the logistic regression analysis showed that there were significant differences in the immune function of GC patients grouped based on sex, age, tumor stage and distant metastases. Many previous studies showed that smoking was positively correlated with GC (Lindblad et al., 2005; Huang et al., 2011; Tramacere et al., 2011). In our study, the ratio of male and female smoker was 64.67:1. Tobacco contained carcinogens, affecting the immune function, but the specific regulatory mechanisms remained unknown. It was found a correlation between smoking and GC in these data, while we observed the correlation between smoking and the expression of CD19+, CD25+ lymphocytes in the GC patients (P < 0.01). In the GC patients group based on age (≥60 or <60), we found the age was negatively correlated with immune function. For each additional one-year-old age, the risk rate of GC increased by 0.972%. In elder patients, due to slow gastric motility, delayed gastric emptying, and decreased gastric mucosal defense function making the retention of food in the stomach, carcinogenic nitroso compounds were synthesized in the stomach and repeatedly stimulated the gastric mucosa in a long term, finally induced GC (Pisanu et al., 2007; Saif et al., 2010). On the other hand, elder patients often have important organ dysfunction, associated with more complications,

leading to immune function disorder, particularly T lymphocytes and immune surveillance depression, further increased risk of gastric cancer (Kalnina et al., 2010; Ferguson et al., 2011; Schreiber et al., 2011). Whalen et al showed that the early immune response of cancer was usually local-based and the suppression of immune function in the body gradually obvious along with the tumor development and metastasis (Keystone et al., 2010; Jochems et al., 2011; Qualls et al., 2011). As the disease progression in the patients with gastric cancer, the immune function between stage I/II and stage III/IV groups was significantly different, suggesting that the immune status of patients with gastric cancer was negatively correlated with TNM staging. There was a vicious circle between immune injury and distant metastasis. Our results were consistent with the previous studies (Nguyen et al., 2008; Pawelek et al., 2008; Powell et al., 2011).

In recent years, many researchers focused on the correlation between the immune function and prognosis of the patients with cancer (Domschke et al., 2009; Dai et al., 2010; Sweetenham et al., 2010). It was found that the expression level of $CD3^+$, $CD4^+/CD8^+$ and NK^+ cells in preoperative patients with colorectal cancer was positively correlated with the prognosis (Wang et al., 1999). The studies indicated that $CD4^+/CD8^+$ ratio was an objective indicator to evaluate the prognosis of GC patients, a lower ratio meaning a higher risk of local recurrence and distant metastasis, resulting poor prognosis (Rey-Ferro et al., 1997). A few studies have shown that $CD4^+$, $CD8^+$ expression levels in patients with esophageal cancer was significantly positively correlated with survival (Cho et al., 2003; Nozoe et al., 2005). In addition, some studies have shown a humoral immunity was important to assess the prognosis of patients with node- negative breast cancer (Schmidt et al., 2008). In present study, the expression of $CD19^+$ cells in peripheral blood of the GC patients was significantly higher than control group and positively correlated with prognosis ($P = 0.04$), but the remaining seven markers were not statistically significant. $CD19^+$ is a 95KD I-type transmembrane glycoprotein and an antigen specific CD molecules that widely distributed on B cell surface (Mahmoud et al., 1999; Ishikawa et al., 2002). $CD19^+$ cells can regulate B cell activation and proliferation, the expression of which starting from the B progenitor cells to mature B cells through the whole process of B cell differentiation. Therefore, the expression levels of $CD19^+$ lymphocytes in peripheral blood can reflect the differentiation of B lymphocytes (Schmidt et al., 2008). $CD19^+$ B cell activating factor (BAFF) can specifically bind to B lymphocytes and induce its proliferation, differentiation and secretion of immunoglobulin, playing an important role in humoral immunity. The receptors of BAFF contains BCMA B cell maturation antigen, transmembrane protein complex TACI and B cell activating factor receptor BAFF-R (Smyth et al., 2004; Ben-Baruch 2006; Kim et al., 2006). BAFF deficiency can result in humoral immune function disorder. Humoral immunity to tumor usually indicates B cell and antibody-dependent cytotoxicity. The patients with cancer can produce antibodies for tumor-associated antigen that can inhibit tumor growth; On the other hand, B cell surface immunoglobulin can bind to tumor-associated antigen, then process and present antigens to induce immune response of T cells, or interact with macrophages and complement system to kill tumor cells (Mahmoud et al., 1999; Ishikawa et al., 2002; Smyth et al., 2004; Ben-Baruch 2006; Kim et al., 2006; Schmidt et al., 2008). In our study, the increased expression of CD19+ cells enhanced humoral immunity, thus modifying the immune status and prognosis, and further improving the DFS of GC patients.

Cellular and humoral immune both play an important role in anti-tumor immunity (Wu et al., 2011). For different types of tumors, or the same tumor at different periods, different body's immune status is accompanied by different expression of CD molecules (Nakajima et al., 2009). By detecting changes of lymphocyte subsets in peripheral blood from patients to monitor the immune status, evaluate the immune function and predict the prognosis, that may be helpful to instruct clinical therapy.

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