RESEARCH COMMUNICATION

Relationship Between Cytokine Levels and Clinical Classification of Gastric Cancer

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Abstract

Complex symptoms often make it difficult to choose optimized strategies suitable for gastric patients. Therefore, molecular markers are needed to assist doctor's diagnoses. In this study, to determine if the mRNA levels of Th1 and Th2 cytokines in peripheral blood mononuclear cells (PBMC) of patients with gastric cancer were correlated with their various stages, gastric patients, patients with benign gastric disease, and heathy people were recruited for detection of cytokine mRNA levels. Only the relative levels in comparison with levels of each patient's own β-actin were subjected to further statistical analyses. We found that there were significantly more positive detection of IL-4, IL-6, IL-10 mRNA expression in stage III and IV than those in patients with gastic cancer in stage I and II. It was also found that IL-4, IL-6, IL-10 mRNA expression in patients with low-level differentiations possessed significantly higher positive detection ratios than patients with moderate or high-level differentiation. These results suggest that positive detection of IL-4, IL-6 and IL-10 mRNA may be useful as a molecular marker approach for distinguishing the stage II and III of gastric cancer, as well as low-level and moderate cancer differentiation.

Key words: cytokine levels - clinical classification - gastric cancer

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Introduction

Gastric cancer is the most common malignant tumor in China, leading to a death of more than 260,000 each year. Currently, available strategies for treating gastric cancer include surgery, chemotherapy, radiotherapy and biological therapy (Frederick et al., 2002; Alberts et al, 2003; Falcone, 2003; Ang, 2010; Krejs, 2010; Li et al., 2010; Ahn et al., 2011). Doctors usually choose treatment strategies maily according to the diferent stages of gastric cancer. However, sometimes this is not accurate as patients have various disease symptoms, coocurring diseases, different ages, and so on. These make doctors difficult to choose optimized strartegies suitable for particulat patient. Therefore, molecular markers are needed to assist doctor's diagnosis.

It is known that cellular immunity of patients is important for retarding development of tumors. Studies show that levels of Th1 (T-helper cell type 1) cytokines (such as IFN-γ and IL-2) and Th2 type cytokines (such as IL-4, IL-6, and IL-10) varies in patients with gastric cancer in different stages (Liu et al., 1997; 1998; Servis et al., 2008). However, detailed relationship between mRNA levels of these cytokines and clinical stages of gastric cancer still needs to be investigated to determine if mRNA levels of certain cytokines could be used as molecular markers for clinical diagnosis of gastric cancer.

In this study, mRNA levels of Th1 and Th2 cytokines in peripheral blood mononuclear cells (PBMC) of heathy peple, patients with benign gastric disease, and gastric cancer in various stages were investigated. We found that there were significantly more positive detection of IL-4, IL-6, IL-10 mRNA expression in stage III and IV than those in patients with gastic cancer in stage I and II. It was also found that IL-4, IL-6, IL-10 mRNA expression in patients with low-level differentiations possessed significantly higher positive detection ratios than patients with moderate or high-level differentiation. These results suggest that positive detection of IL-4, IL-6 and IL-10 mRNA levels may be good for being used as molecular markers for distinguishing the stage II and III of garstric cancer, as well as distinguishing low-level and moderate cancer differentiation.

Materials and Methods

Patients

One hundred and twenty gastric patients, 60 patients

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with benign gastric disease, and 50 heathy people were recruited in this study, consisting of 3 groups (healthy control, benign gastric disease, and gastric cancer), respectively. Among the cancer group, numbers of the patients in stage I, II, III, and IV were 7, 25, 36, and 34, respectively. Numbers of patients with low-level, moderate, high-level cancer were 27, 60, and 15, respectively. Among the three groups, differences among sex and age were not significant.

RNA preparation

Peripheral blood mononuclear cells were isolated from blood samples of patients by centrifugation, washed with buffres twice, and stored at -800C for further analyses. Isolation of total RNAs wer performed using Guanidine isothiocyanate (GIT) methods as described previously (Wei et al., 2000). The purity of total RNAs was assessed by UV240 spectrophotometer (Daojin company, Japan).

DNase I treatment and reverse transcription

Five microliters of RNA extract was treated with 1 U of DNase I (Bao Biotech. Co., China) at 37 0C for 30 min. The reaction was stopped by adding 1 μl of EDTA (final concentration, 2.5 mM), followed by incubation for 10 min at 85°C to denature DNAs prior to reverse transcription. The treated RNA samples were stored on ice for further analyses. One microgram of DNase-treated sample was added to 20 μl of reaction system containing 1 μl (200 U) of MMLV (Promega, USA), deoxyribonucleoside triphosphates at 10 mM each (Promega, USA), 10 mM dithiothreitol, 50 pmol of backward primers (primers were synthesized by Shenggong Bio Co., Shanghai, China), 40 U of rRNasin RNase inhibitor (Promega, USA), and 4 μl of 5x first-strand buffer in 0.5 ml snap-seal apex tubes (Alphalabs, Eastleigh, Hampshire, United Kingdom) and incubated at 37°C for 60 min. Samples were heated to 75°C for 10 min prior to PCR.

PCR

PCR were performed at a PCR machine (PE-2400, Perkin Elmer Cetus, USA) in a volume of 100 μl, containging 10 μl of cDNA samples, 10 μl of PCR buffer, 2 U of Taq DNA polymerase (Promega, USA), 125 nM primer, 250 μM each deoxyribonucleoside triphosphate (Shenggong Bio Co., Shanghai, China), and 2.5 mM of $MgCl_2$. The primers are given in Table 1. The PCR products were separated on agarose gels. The density of product band were analyzed by a gel analysis system (Chemi Imager 5500, Kadak) using 1D Image Analysis Software (Kadak, USA).

Statistical analyses

Data analyses were perfermed with SPSS (version 11.5) statistical software. For patiens, the sex and age differences among groups were not statistically significant. Data wer analyzed using $\chi 2$ test or the exact fourfold table method. The data measurements were conducted using a paired t test or independent sample t test. Data with $P < 0.05$ for the difference was considered as statistically significant.

Table 1. Primers Used for RT-PCR

Primers	Sequences of the primers
β-actinF	5'-GTGGGGCGCCCCAGGCACCA-3'
β-actinR	5'-CTCCTTAATGTCACGCACGATTT-3'
IFNγF	5'ATGAAATATACAAGTTATATCTTGGCTTT3'
IFNγR	5'GATGCTCTTCGACCTCGAAACAGCAT3'
IL-2F	5'ATGTACAGGATGCAACTCCTGTCTT3'
IL-2R	5'GTTAGTGTTGAGATGATGCTTTGAC3'
IL-4F	5'ATGGGTCTCACCTCCCAACTGCT3'
IL-4R	5'CGAACACTTTGAATATTTCTCTCTCTCAT3'
IL-6F	5'ATTGACAAACAAATTCGG3'
IL-6R	5'TTACATTTGCCGAAGAG3'
IL-10F	5'ATGCCCCAAGCTGAGAACCAAGACCCA3'
IL-10R	5'GTTTCGTATCTTCATTGTCAT3'

Results

Detection of levels in gastric cancer patients

To detect the cytokine mRNA levels in gastric cancer patients, total RNAs were isolated from patients. The mRNA expression levels were determined using RT-PCR. The PCR products of cykine RNAs were separated on agarose gels and analyzed by Kodak Gel Analysis System. For each patient, the levels of cytokines were compared with level of their own β-actin. The relative levels were calculated by the following equation: RV (relative level) = (IFN-γ, IL-2, IL-4, IL-6, or IL -10) value / β-action value. Only the relative levels (RV) were subjected to further statistical analyses.

The Th1 and Th2 cytokine levels were set up according to classification criteria as previously reported (Haiming et al., 2000). Briefly, if PCR amplification bands with expected right sizes were not observed or observed but the levels were lower than that of β-actin, it was considered as negative (RV was set as <0.1 in following statistical processing). If a band with right size was stronger than that of β-actin, it was considered as positive (the RV was set as ≥ 0.1 in following statistical processing). One of these PCR experimental results was given in Figure 1.

Comparison of levels of cytokine mRNA among healthy persons, patients with benign gastric diseases or gastric cancer

The mRNA levels of five types of cytokines, along with of β-actin mRNA, in healthy persons, patients with benign gastric diseases or gastric cancer were detyermined by RT-PCR and then subjected to statistical analyses using the methods described in the Method section. As shown in Table 2, between healthy and patients with benign gastric diseases, there were no significant difference in the IFN-γ, IL-2, IL-4, IL-6, and IL-10 mRNA expression levels in peripheral blood PBMC (P values were 0.648, 0.868, 0.835, 0.780, and

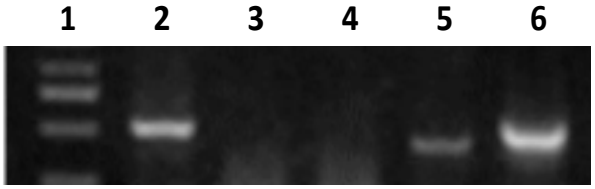

Figure 1. Electrophoresis of PCR Products

0.949, respectively). When compared with healthy group and the benign disease group, the gastric cancer patient group has a lower positive IFN-γ and IL-2 ratios (P values were 0.026 and 0.007; 0.002 and 0.003, respectively). When IL-4, IL-6, IL-10 mRNA levels were compared with those of healthy control group and benign disease group, it was found that the gastric cancer patient group has higher positive IL-4, IL-6, IL-10 ratios (P values <0.001). These results suggest that patients with gastric cancer have changed mRNA levels and that the mRNA levels of these cytokines may be used as molecular markers for clinical diagnosis.

Relationship between Th1 and Th2 cytokine levels in PBMC with different clinical gastric cancer stages

Since mRNA levels of thse cytokines were changed in gastric cancer patient group, we contimue to investigate if the cytokine mRNA levels are related to the stage of cancer. Among patients with gastric cancer in stage I, II, III, and IV, only a few were detected for positive IFN-γ and IL-2 mRNA transcription, therefore, IFN-γ and IL-2 mRNA levels are not good for use as markers in diagnosis (Table 3). However, for expression of IL-4, IL-6, and IL-10 mRNA, it was found that there were significantly more positive detection in stage III and IV than those in patients with gastic cancer in stage I and II (P values were 0.003,0.000,0.000; 0.000,0.000,0.000, respectively). Between stage III and IV, or stage I and II, there were no significant difference in positive detections of IL-4, IL-6 and IL-10 mRNA levels (P values were 0.124, 0.155, and 0.454, respectively). These results suggest that positve detection of IL-4, IL-6 and IL-10 mRNA levels may be used as molecular markers for distinguishing the stage II and III of garstric cancer.

Table 2. Comparison of mRNA Levels of Cytokines with those of Actin among Patients (healthy, with benign tumors or gastric cancer)

groups	Case #	Ratio (%) with potivitive cytokine mRNA detections				
		IFN-γ	IL-2	IL-4	IL-6	IL-10
healthy	50	12.0	22.0	12.0	10.0	8.0
benign tumors	60	15.0	23.3	13.3	11.6	8.3
gastric cancer	102	2.9	6.8	67.6	62.7	58.8

Table 3. Comparison of mRNA Levels of Cytokines with those of Actin among Patients with Gastric Cancer at Different Clinical Classification Stage

stages	Case #	Ratio (%) with positive cytokine mRNA detection				
		IFN-γ	IL-2	IL-4	IL-6	IL-10
Stage I	7	0.0	0.0	57.1	42.8	42.8
Stage II	25	0.0	4.0	40.0	28.0	20.0
Stge III	36	2.7	5.5	77.7	75.0	75.0
Stage IV	34	5.8	7.4	91.1	88.2	82.3

Table 4. Comparison of mRNA Levels of Cytokines with those of Actin among Patients with Gastric Cancer at Different Tumor Differentiation Degrees

Tumor differentiation	Case #	Ratio (%) with potivitive cytokine mRNA detections				
		IFN-γ	IL-2	IL-4	IL-6	IL-10
high	15	0.0	0.0	46.6	33.3	33.3
moderate	60	3.3	38.3	53.3	40.0	45.0
low	27	3.7	7.4	88.8	85.2	81.4

Relationship between Th1 and Th2 cytokine levels in PBMC with different cancer differentiation degrees

The relationship between mRNA levels of cytokines and cancer differentiation degrees were also investigated (Table 4). Because there were not enough positive detection for IFN-γ and IL-2, IFN-γ and IL-2 are not suitable for use as molecular markers. As shown in Table 4, IL-4, IL-6, IL-10 mRNA expression in patients with low-level differentiations possessed significantly higher positive detection ratios than patients with moderate or high-level differentiation (P values were 0.003,0.001,0.002; 0.015,0.020,0.018, respectively). Howevere, between patients with moderate or high-level differentiation, there were no significant difference in positive detection numbers (P values were 0.239, 0.064, 0.133, respectively) in IL-4, IL-6, and IL-10 mRNA expression levels. These results suggested that positve detection of IL-4, IL-6 and IL-10 mRNA levels may be also used as molecular markers for distinguishing low-level and moderate cancer differentiation.

Discussion

When compared with healthy people, the cytokine levels are usually changed due to the growth of tumors and tumor-induced immune responses. It was reported that Th1 and Th2 cytokines were expressed with various levels in patients with solid tumors (Chau et al., 2000; De Vita et al., 2000; Hattori et al., 2003; Servis et al., 2008). Most of these reports focus on the roles of cytokines in the immune response in patients with tumors (Botella-Estrada et al., 2005; Dillman, 2011; Kanazawa 2005; Musha et al., 2005), including patients with pancreatic cancer (Bellone et al., 1999), colorectal cancer (Kawamura et al., 2002; Ren et al., 2001), urinary system cancer (Filella et al., 2000; Onishi et al., 2001; Tahir et al., 2001), malignant melanoma (Lauerova et al., 2002; Grivennikov and Karin, 2011), Hodgkin lymphoma (Skinnider and Mak, 2002), chronic lymphocyte leukemia (Podhorecka et al., 2002), and glioblastoma (Hao et al., 2002). However, it is not reported that if there is a relationship between

these cytokines and the clinical stage of cancers. If so, certain cytokines could be used as molecular markers for clinical diagnosis, which will be very helpful for doctors and patients to choose the best treatment strategies.

In this paper, we detected several cytokine levels in healthy people, patients with benign tumors and patients with gastric cancers in various stages. We found that there were significantly more positive detection of IL-4, IL-6, IL-10 mRNA expression in patients with stage III and IV gastric cancers than those with gastric cancers in stage I and II. It was also found that IL-4, IL-6, IL-10 mRNA expression in patients with low-level differentiations possessed significantly higher positive detection ratios than patients with moderate or high-level differentiation. These results suggest that positive detection of IL-4, IL-6 and IL-10 mRNA levels may be good for being used as molecular markers for distinguishing the stage II and III of garstric cancer, as well as distinguishing low-level and moderate cancer differentiation.

In this study, one hundred and twenty gastric patients, 60 patients with benign gastric disease, and 50 heathy people were recruited. For each patient, the levels of cytokines were compared with level of their own β-actin. Only the relative levels in compariosn with levels of each patien's own β-actin were subjected to further statistical analyses. Our results will provide a basis for the possible use of positive detection (in comparison with detection of β-actin) of IL-4, IL-6 and IL-10 mRNA as molecular markers in the clinical diagnosis.

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