Gene Expression Profiling-Based Identification of CD28 and PI3K as New Biomarkers for Chronic Graft-Versus-Host Disease

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Chronic graft-versus-host disease (cGVHD) is a major cause of morbidity and mortality after allogeneic hematopoietic stem cell transplantation. Currently, no reliable biomarkers are available to predict the onset or progression of cGVHD. Therefore, in this study, we collected peripheral blood mononuclear cells from four patients with cGVHD and four ones with non-GVHD after hematopoietic stem cell transplantation and employed Affymetrix GeneChip Human U133 Plus 2.0 microarrays to screen the genes differentially expressed in cGVHD versus non-GVHD groups, with the aim to identify potential clinical biomarkers to predict cGVHD risk or progression. Microarray analysis demonstrated that the expression of 3180 genes changed significantly in cGVHD versus non-GVHD, with 879 genes upregulated and 2301 genes downregulated. Among them we chose *CD28* and *PI3K* as candidates for further verification. Flow cytometry and quantitative real-time polymerase chain reaction analysis confirmed the significant upregulation of *CD28* and *PI3K* in samples from patients with cGVHD compared with patients with non-GVHD, respectively. In conclusion, our study suggested that the upregulation of *CD28* and *PI3K* contributed to the onset and progression of cGVHD and provided evidence that *CD28* and *PI3K* may serve as promising biomarkers for cGVHD.

Introduction

CHRONIC GRAFT-VERSUS-HOST DISEASE (cGVHD) occurs in >50% of long-term survivors after allogeneic hematopoietic stem cell transplantation (HSCT) and is associated with significant morbidity and mortality (Flowers *et al.*, 2008). With greater availability and use of human leukocyte antigen (HLA)-matched unrelated donors, the incidence of cGVHD is rising rapidly (Seaton *et al.*, 2003). Several risk factors for cGVHD after HSCT have been proposed, including prior acute GVHD, older age of the patients, the use of donor lymphocyte infusions, and the use of unrelated or HLA-mismatched donors (Poloni *et al.*, 2011). However, none of them can indicate the onset and progression of cGVHD with high correlation before the disease is evident clinically. Therefore, it is urgent to search for novel clinical biomarkers to allow for accurate diagnosis and monitor of cGVHD.

Microarray analysis can provide a global view on hematologic diseases with its promise capacity for profiling expression patterns of thousands of genes in a single experiment. Several studies have investigated the gene expression profiles occurring in acute GVHD (Takahashi *et al.*,

2008) or acute myeloid leukemia (Wilson *et al.*, 2006; Bonadies *et al.*, 2011), whereas only few studies have addressed the global gene expression in cGVHD condition. Therefore, in this study, we performed gene expression profiling analysis by employing microarrays to screen the genes differentially expressed in patients with cGVHD versus non-GVHD, with the aim to identify potential clinical markers to predict cGVHD risk or progression. Our results demonstrated that *CD28* and *PI3K* were significantly upregulated in patients with cGVHD compared with patients with non-GVHD, suggesting that these two molecules may serve as good markers for evaluating cGVHD status.

Patients and Methods

Patients

Between April 2005 and December 2008, four patients with cGVHD and four patients with non-GVHD, all of whom received allogeneic HSCT at Guangdong General Hospital, were enrolled in this study (Table 1). The diagnosis and global assessment of cGVHD were based on the National Institutes of Health consensus criteria (Figuerola *et al.*, 2005). Four patients with non-GVHD, who did not suffer

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TABLE 1. PATIENT CHARACTERISTICS

Patient No.	Non-GVHD				cGVHD			
	1	2	3	4	5	6	7	8
Age (years)	36	34	31	44	22	33	27	50
Sex	F	M	M	M	F	M	M	M
Diagnosis	AML	AML	AML	AML	AML	AML	AML	AML
Donor	Sibling	Sibling	Sibling	Sibling	Sibling	Sibling	Sibling	Sibling
HLA disparity	0	0	0	0	0	0	0	0
Prophylaxis GVHD		...CSA/sMTX/MM F...				...CSA/sMTX/MM F...		
Grade of cGVHD	Severe	Severe	Severe	Severe	Severe	Severe	Severe	Severe

F, female; M, male; AML, acute myeloid leukemia; CSA, cyclosporine; sMTX, short-term MTX; MMF, mycophenolate mofetil; cGVHD, chronic graft-versus-host disease.

from cGVHD after allogeneic HSCT for 2 years follow-up, were designated as study controls. This study was approved by the Ethics Committee of Guangdong General Hospital and informed consent was obtained for all patients.

RNA isolation, amplification, and hybridization

For each of the four patients with cGVHD and four patients with non-GVHD, 4 mL of venous blood was collected. Total RNA from nucleated blood cells was isolated following the lysis of erythrocytes and removal of cell debris. In brief, immediately after sample collection, leukocytes were concentrated by centrifugation, and Buffer EL (Qiagen) was added to lyse erythrocytes. Cells were washed in Buffer EL to eliminate the abundance of globin species from mRNA pool. RNA was then purified with RNeasy Mini kit (Qiagen) (Buzzeo *et al.*, 2008). The quality and quantity of purified total RNA were assessed by Agilent 2100 Bioanalyzer RNA 6000 NanoChip (Agilent). Only samples with an A_{260}/A_{280} between 1.7 and 2.2 were used for following experiments. About 5.0 µg total RNA was used to generate cDNA, which was then hybridized onto Affymetrix U133 Plus 2.0 GeneChip oligonucleotide arrays (Affymetrix) according to the manufacturer's instructions.

Microarray statistical analysis

The microarray data were normalized by using robust multiarray average (Irizarry *et al.*, 2003). Using the resulting normalized intensities, an empirical Bayes method was applied (Kendzierski *et al.*, 2003). The lognormal-normal model was employed to estimate the expression of each gene. Genes were ranked according to this value and the results were further filtered according to the magnitude of change in the expression; only genes that were at least twofold up- or down-regulated were considered. To evaluate the differences in the

variability of gene expression, an *F* test for equal variances of the cGVHD and non-GVHD control groups was performed for each gene. To account for multiple testing issues, *q*-values were calculated (Storey and Tibshirani, 2003) to provide a local expected false discovery rate for a list of genes with the most significant difference in the variance. All computations were performed using R language and environment.

Flow cytometry

Blood cells isolated from 20 patients with cGVHD and 20 patients with non-GVHD were subjected to flow cytometry analysis for the detection of CD28 in CD4+ and CD8+ T cells subpopulation on a FACScan (Becton Dickinson) equipped with a 15 mW air-cooled argon laser tuned at 488 nm. The cells were prepared for the measurement according to the protocols provided with the assays or antibodies; data acquisition and analysis were performed by the CellQuest (Becton Dickinson) software.

Quantitative real-time polymerase chain reaction

To validate the results of the microarray experiment, quantitative real-time polymerase chain reaction (qRT-PCR) was performed on 60 individual patient samples that had not been used in the microarray experiment. Total RNA was extracted with Trizol kit (Invitrogen) and 5.0 µg total RNA was used in the first-strand cDNA synthesis with random hexamers and Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Quantitative detection of PI3K expression in cDNA from peripheral blood mononuclear cells (PBMCs) was performed using TaqMan real-time PCR. To precisely determine the copy number of *PI3K*, a duplex vector including a fragment of the *PI3K* and *ABL* genes was constructed and used as a reference. Based on the DNA concentration, measured by spectrophotometry and confirmed by

TABLE 2. SEQUENCES OF PRIMERS AND PROBES FOR QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

Primers/probes	Sequence	Purpose
PI3K-f	5'-CGGCTTTTTC AACCTTTTAA	Forward primer
PI3K-b	5'-CATGCCGATAGCAAAACCAAT	Reverse primer
PI3K-p	5'-FAM-CAGTAGGCAACCGTGAAGAAAAGATCCTCA-TAMRA	Probe
ABL-f	5'-GATGTAGTTGCTTGGGACCCA	Forward primer
ABL-b	5'-TGGAGATAACACTCTAAGCATAACTAAAGGT	Reverse primer
ABL-p	5'-FAM-CCATTTTGGTTTGGGCTTCACACCATT-TAMRA	Probe

quantitative gel electrophoresis, standard dilutions of the vector from 10^7 to 10^1 copies were prepared. Briefly, PCR was performed in a 25 μ L total volume containing 2 μ L cDNA, 25 pmol of each primer (PI3K-f and PI3K-b for *PI3K* gene amplification; ABL-f and ABL-b for *ABL* gene amplification), 10 nmol dNTPs, 1.5 U AmpliTaq Gold (Applied Biosystems), 5 pmol 6FAM-TAMRA probe, and PCR buffer containing 4.5 mM $MgCl_2$. After an initial denaturation at 95°C for 5 min, 40 cycles consisting of 95°C for 15 s and 64°C for 1 min were performed. Primers and probes for *PI3K* and *ABL* gene amplification were synthesized by Invitrogen and are listed in Table 2.

Results

Differential gene expression patterns in cGVHD versus non-GVHD control

Microarray analysis demonstrated that 3180 genes showed significant changes in expression between the cGVHD and non-GVHD groups, with 879 genes upregulated and 2301 genes downregulated (Fig. 1). A representative list of these genes was compiled, with focus on the immune-related genes which show the greatest magnitude fold change in expression (Table 3).

Upregulation of CD28 in T-cell subpopulation from patients with cGVHD

As *CD28* showed more than fivefold increase of expression between cGVHD and non-GVHD (Table 3), we performed flow cytometry to detect *CD28* expression level in both *CD4+* and *CD8+* T-cell subpopulation. The portion of *CD8+**CD28+*

T cells was higher in cGVHD than in non-GVHD patients ($46.71\% \pm 24.61\%$ vs. $35.61\% \pm 12.67\%$, $p=0.03$; Fig. 2), but no significant difference in the portion of *CD4+**CD28+* T cells was found between cGVHD and non-GVHD patients ($44.07\% \pm 12.07\%$ vs. $40.85\% \pm 11.05\%$, $p=0.568$; Fig. 2).

Upregulation of *PI3K* in PBMCs from patients with cGVHD

PI3K showed about threefold increase of expression in patients with cGVHD (Table 3). To confirm the upregulation of *PI3K* in cGVHD, we performed qRT-PCR to examine the level of *PI3K* mRNA in the PBMCs derived from 40 patients with cGVHD, including 20 low-grade cGVHD (grade I or II) and 20 high-grade cGVHD (grade III or IV), and 20 non-GVHD controls. The results demonstrated that *PI3K* mRNA level was significantly higher in samples from patients with cGVHD than from those with non-GVHD. Further, we found a statistically significant difference in *PI3K* mRNA level between low-grade cGVHD and high-grade cGVHD samples. Data were analyzed using a one-way analysis of variance ($F_{2,57}=18.70$, $p=0.00$) followed by the Dunnett test. *PI3K* mRNA level of low-grade cGVHD samples showed significant difference from that of non-GVHD ($p=0.01$) and also from that of high-grade cGVHD ($p=0.01$) (Fig. 3).

Discussion

Recently, genome-wide expression profiling analysis has been applied to the studies of many diseases, leading to significant advances in our understanding of their pathogenesis

FIG. 1. Heat map of the genes differentially expressed in cGVHD versus non-GVHD patients. The red, green, black, and gray colors represent the upregulation, downregulation, no change, and no expression, respectively. cGVHD, chronic graft-versus-host disease. Color images available online at www.liebertonline.com/dna

TABLE 3. REPRESENTATIVE GENES DIFFERENTIALLY EXPRESSED IN CHRONIC GRAFT-VERSUS-HOST DISEASE VERSUS NONCHRONIC GRAFT-VERSUS-HOST DISEASE

Probe ID	Gene symbol	Gene name	FC ^a
221658_s_at	IL21R	Interleukin 21 receptor	7.13
209823_x_at	HLA-DQB1	Major histocompatibility complex, class II, DQ beta 1	6.34
206295_at	IL18	Interleukin 18 (interferon-gamma-inducing factor)	5.88
211861_x_at	CD28	CD28 molecule	5.07
211410_x_at	KIR2DL5A	Killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 5A	4.53
205125_at	PLCD1	Phospholipase C, delta 1	3.93
216876_s_at	IL17A	Interleukin 17A	3.87
205945_at	IL6R	Interleukin 6 receptor	3.7
216482_x_at	ZNF79	Zinc finger protein 79	3.64
209929_s_at	IKBKG	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma	3.33
209828_s_at	IL16	Interleukin 16 (lymphocyte chemoattractant factor)	3.27
208375_at	IFNA1	Interferon, alpha 1	3.26
216133_at	TRD@	T cell receptor delta locus	3.25
208991_at	STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)	3.22
200799_at	HSPA1A	Heat shock 70 kDa protein 1A	3.22
204891_s_at	LCK	Lymphocyte-specific protein tyrosine kinase	3.08
203879_at	PIK3CD	Phosphoinositide-3-kinase, catalytic, delta polypeptide	2.99
204683_at	ICAM2	Intercellular adhesion molecule 2	2.87
211372_s_at	IL1R2	Interleukin 1 receptor, type II	2.87
201648_at	JAK1	Janus kinase 1	2.79
216355_at	PCDHB17	Protocadherin beta 17 pseudogene	2.60
211799_x_at	HLA-C	Major histocompatibility complex, class I, C	2.58
201208_s_at	TNFAIP1	Tumor necrosis factor, alpha-induced protein 1 (endothelial)	2.45
204777_s_at	MAL	mal, T-cell differentiation protein	2.23
205456_at	CD3E	CD3e molecule, epsilon (CD3-TCR complex)	2.09
217478_s_at	HLA-DMA	Major histocompatibility complex, class II, DM alpha	2.06
210031_at	CD247	CD247 molecule	2.05
203373_at	SOCS2	Suppressor of cytokine signaling 2	-2.08
210354_at	IFNG	Interferon, gamma	-2.38
203953_s_at	CLDN3	Claudin 3	-2.50
208474_at	CLDN6	Claudin 6	-2.63
208375_at	IFNA1	Interferon, alpha 1	-3.23
207533_at	CCL1	Chemokine (C-C motif) ligand 1	-3.23
220034_at	IRAK3	Interleukin-1 receptor-associated kinase 3	-3.23
207901_at	IL12B	Interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2,	-3.33
220813_at	CYSLTR2	Cysteinyl leukotriene receptor 2	-3.57
204932_at	TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b	-3.95
221319_at	PCDHB8	Protocadherin beta 8	-4.35
221399_at	EDA2R	Ectodysplasin A2 receptor	-4.76
211876_x_at	PCDHGA12	Protocadherin gamma subfamily A, 12	-9.09

^aLog₂-fold change in signal intensity calculated as [2^{log₂(avg cGVHD_avg non-GVHD)}]. FC > 0, upregulated; FC < 0, downregulated. FC, fold change; TCR, T-cell receptor.

(Wagner *et al.*, 2004; Borovecki *et al.*, 2005; Takata *et al.*, 2005). In this study, we screened genes differentially expressed in PBMCs between patients with cGVHD and with non-GVHD by employing Affymetrix GeneChip Human U133 Plus 2.0 microarrays, which could reveal more accurate and comprehensive gene expression patterns than long cDNA microarrays could (Ljubimova *et al.*, 2001). The comparison of gene expression profiles between cGVHD and non-GVHD samples demonstrated that interleukin 21 receptor (*IL21R*), major histocompatibility complex class II DQ alpha 1 (*HLA-DQB1*), killer cell immunoglobulin-like receptor two domains long cytoplasmic tail 5A (*KIR2DL5A*), and interleukin 6 receptor (*IL6R*) were significantly upregulated, whereas protocadherin gamma subfamily A 12 (*PCDHGA12*), ectodysplasin A2 receptor (*EDA2R*), and interleukin 12B were downregulated. In accordance with the known pathological features of cGVHD,

these differentially expressed genes included a large number of inflammatory cytokines such as IL-6, IL-17, and TNF- α , which have been proposed to play important roles in the development of cGVHD (Tanaka *et al.*, 1995).

In the present study, we performed microarray analysis to demonstrate that cGVHD has some unique molecular signatures and identifies networks of genes or pathways that may be of significance in the pathogenesis of cGVHD. Interestingly, the microarray results indicated that the expression of *CD28* and *PI3K* was increased in patients with cGVHD. Although gene microarray results are generally accurate and reproducible, many authors agree that these data must be verified by subsequent mRNA analysis (Cao *et al.*, 2002). On the basis of a recent report that treatment of refractory cGVHD with mesenchymal stem cells led to the downregulation of *CD28* (Weng *et al.*, 2010), we assumed

FIG. 2. Percentages of CD8+CD28+ and CD4+CD28+ T cells in cGVHD and non-GVHD patients detected by flow cytometry. NS, not significant.

that CD28 and its downstream effector PI3K play important roles in the pathogenesis of cGVHD. Therefore, we further validated the upregulation of CD28 and PI3K in cGVHD by flow cytometry and qRT-PCR analysis.

CD28, a costimulatory molecule, is present on the cell membrane and can be detected highly sensitively by flow cytometry. Based on flow cytometric analysis, the proportion

of CD28+ T cells was higher in patients with cGVHD than non-GVHD, which is consistent with the microarray data. Moreover, we validated by flow cytometric analysis that CD28 on the CD8+ T cells, but not the CD4+ T cell subpopulation, increased with extended course of cGVHD. This is partly due to the fact that T-lymphocyte subsets of CD8+ cells serve as active cytolytic T lymphocytes (Parkman, 1993) and can participate in transplantation immunity and rejection reaction (Gilliam *et al.*, 1996; Rojas *et al.*, 2005). CD28 expressed on CD8+ T cells in patients with cGVHD thus regulates the differentiation after antigen stimulation into long-term survival memory T cells, implicating that these cells participate in cGVHD.

The previous studies reported that cGVHD starts with the expansion of mature donor T cells that recognize minor or major histocompatibility antigens of the recipient (Perez-Simon *et al.*, 2006), and efficient T-cell activation requires antigen recognition and costimulation (Wulfing *et al.*, 2002). CD28 is one of the best-understood costimulatory molecules and is expressed predominantly on T cells. It can deliver a costimulatory signal, which in conjunction with T cell receptor signal leads to T cell activation. CD28 propagates intracellular signals to promote T cell proliferation and enhance the expression of cytokine/chemokine, thus playing an important role in the pathogenesis of multiple myeloma (Bahlis *et al.*, 2007). In accordance with these reports, our results show that the proportion of CD8+CD28+ T cells was higher in patients with cGVHD, indicating that the increased proliferation of CD8+ T cells accelerates cGVHD, which may be initiated by interaction between CD28 and its ligands (Yu *et al.*, 1998). These results suggest that inhibiting CD28 costimulation by blocking CD28 ligands or using monovalent anti-CD28 reagents before transplantation could inhibit the expansion of CD8+ T cells and decrease the incidence of cGVHD.

More significantly, we found that the expression of *PI3K* was correlated with the occurrence and grade of cGVHD. We confirmed by qRT-PCR that *PI3K* mRNA level was increased in patients with cGVHD. In this study, a high level of *PI3K* mRNA was detected in the PBMCs but not in T cells, because the study was retrospective and the blood samples of enrolled patients were from our specimen bank in which single T cells had not been separated. Thus, we need further select venous blood from patients with cGVHD to confirm that the expression of *PI3K* is upregulated in specific T cells from these patients.

Statistical analysis revealed different expression levels of *PI3K* between grade I or II and grade III or IV cGVHD patients. We could not compare all the grades of patients with cGVHD, because blood from patients in all grades was not available. Moreover, the grade is a subjective judgment of cGVHD severity (Sullivan *et al.*, 1991). Therefore, we only compared low grade (grade I or II) and high grade (grade III or IV) to evaluate dynamic expression of *PI3K* in different courses of cGVHD. Accumulating evidence has highlighted the critical role of the PI3K signaling pathway in the development, activation, and homeostasis of T cells (Koyasu, 2003; Okkenhaug and Vanhaesebroeck, 2003). In response to a variety of stimuli including alloantigens or autoantigens, which can differ between donor and recipient, PI3K signaling modulates the expression of survival and mitogenic factors in T cells (Harriague and Bismuth, 2002). The expansion

FIG. 3. Validation of the upregulation of *PI3K* mRNA expression in peripheral blood mononuclear cells from cGVHD patients by quantitative real-time polymerase chain reaction. The bar graph indicated the quantitative real-time polymerase chain reaction analysis of *PI3K* transcript level in peripheral blood mononuclear cells from non-GVHD, low-grade cGVHD, and high-grade cGVHD.

of these activated T cells at the early phase of cGVHD can subsequently induce damage to target organs directly or indirectly (Perez-Simon *et al.*, 2006). In addition, LY294002, a PI3K inhibitor, inhibited the activation of primary T cells (Crooks *et al.*, 1995). Based on these data, we propose that PI3K, as a switch of T cell activation, plays an important role in cGVHD. By investigating multiple-grade points throughout cGVHD development, rather than comparing PI3K expression in patients who suffered from cGVHD to that in non-GVHD controls, we can begin to establish a functional correlation between PI3K expression and prospective development of cGVHD. In this aspect, PI3K may serve as a novel biomarker to predict the incidence and development of cGVHD.

As the T cell responses induced by CD28-mediated co-signaling overlap the reported functions of PI3K in lymphocyte activation (Appleman *et al.*, 2002), it is conceivable that CD28-PI3K may be the critical signaling molecule in T cell costimulation in cGVHD (Harada *et al.*, 2001). CD28 can directly bind to PI3K by a well-characterized YNMN binding motif in its cytoplasmic domain (Cai *et al.*, 1995; Okkenhaug *et al.*, 2001), and PI3K signaling pathway is considered to be important for CD28-mediated costimulation (Pages *et al.*, 1994). Previous studies reported that dual inhibition of CD28 and PI3K may achieve more potent alleviation of pathological immune responses than that achieved with either inhibitor alone, suggesting that CD28 stimulation through the PI3K pathway allows efficient T cell activation (Garcon *et al.*, 2008). Although the relevant PI3K catalytic isoforms and the contribution of CD28 to PI3K activity in T cells have not been determined unequivocally, some reports indicated that CD28 and PI3K isoform (p110) act in parallel and complementary pathways to activate T cells (Okkenhaug *et al.*, 2007; Garcon *et al.*, 2008), whereas other studies suggested that it is only through p85 that PI3K binds to CD28, contributing to T-cell activation, survival, and division (Alcazar *et al.*, 2009). We will distinguish the subtypes of PI3K in activated T cells of cGVHD in a further study.

In summary, given the rising incidence of cGVHD and poor response of many patients to conventional immunosuppressive treatments, it is extremely urgent to develop sensitive biomarkers for the early diagnosis and therapy of cGVHD (Deeg *et al.*, 1998). Our data establish a correlation between CD28/PI3K expression and the incidence and severity of cGVHD. These findings suggest that anti-CD28 reagents or PI3K inhibitors could potentially reduce the risk of cGVHD. Moreover, increased CD8+CD28+ T cells and PI3K expression may predict the incidence and severity of cGVHD, which will facilitate the treatment of cGVHD. Further studies are necessary to elucidate the detailed mechanism by which specific CD8+CD28+ T cell subpopulations and PI3K pathway contribute to cGVHD following allogeneic HSCT. Longitudinal follow-up of transplantation patients will be required to establish whether CD28/PI3K downregulation is correlated with the alleviation of cGVHD and a better clinical outcome.

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Disclosure Statement

No competing financial interests exist.

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