

Change in serum proteome during allogeneic hematopoietic stem cell transplantation and clinical significance of serum C-reactive protein and haptoglobin

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Abbreviations: AA, aplastic anemia; ALL, acute lymphocytic leukemia; APPs, acute phase proteins; CML, chronic myeloid leukemia; CRP, C-reactive protein; CsA, cyclosporin A; HLH, hemophagocytic lymphohistiocytosis; MDS, myelodysplastic syndrome; MMF, mycophenolate mofetil; MTX, methotrexate; NHL, non-Hodgkin lymphoma; SOS, sinusoidal obstruction syndrome

Abstract

Successful hematopoietic stem cell transplantation (HSCT) involves the restoration of hematopoietic function after engraftment, arising from the differentiation and proliferation of hematopoietic stem cells. Several factors could influence the course of allogeneic-HSCT (allo-HSCT). Therefore, knowledge of serum proteome changes during the allo-HSCT period might increase the efficacy of diagnosis and disease prevention efforts. This study conducted proteomic analyses to find proteins that were significantly altered in response

to allo-HSCT. Sera from five representative patients who underwent allo-HSCT were analyzed by 2-dimensional gel electrophoresis and liquid chromatography tandem mass spectrometry, and were measured on a weekly basis before and after allo-HSCT in additional 78 patients. Fourteen protein spots showing changes in expression were further examined, and most proteins were identified as acute phase proteins (APPs). Studies of 78 additional patients confirmed that C-reactive protein (CRP) and haptoglobin undergo expression changes during allo-HSCT and thus may have the potential to serve as representative markers of clinical events after allo-HSCT. Maximal CRP level affected the development of major transplant-related complications (MTCs) and other problems such as fever of unknown origin. Particularly, an increase in CRP level 21 days after allo-HSCT was found to be an independent risk factor for MTC. Maximal haptoglobin and haptoglobin level 14 days after allo-HSCT were predictive of relapses in underlying hematologic disease. Our results indicated that CRP and haptoglobin were significantly expressed during allo-HSCT, and suggest that their level can be monitored after allo-HSCT to assess the risks of early transplant-related complications and relapse.

Keywords: biological markers; C-reactive protein; haptoglobin; hematopoietic stem cell transplantation; proteomics; recurrence

Introduction

Successful HSCT or bone marrow transplantation involves the transplantation of new, functional immune systems after chemotherapy and radiotherapy. Allo-HSCT or bone marrow transplantation has been used successfully to treat hematopoietic malignancies, hematopoietic failure, autoimmune diseases, and genetic disorders (Copelan, 2006). The profiles of several serum proteins and peptides are altered during the processes of conditioning, stem cell infusion, and engraftment. Alterations in the serum proteome can reveal surrogate biomarkers, which can be assayed with relative ease to predict the outcomes and complications of allo-HSCT. These biomarkers, which include diffe-

Table 1. Clinical characteristics of 78 patients who underwent allo-HSCT.

Characteristics of the patients	Median (range)
Duration of follow-up, median range (months)	15.9 (1.0-70.9)
Patient age at transplantation, median range (years)	37.2 (16-62)
Time to ANC > 500/ μ l, median range (days)	13.0 (9-40)
Time to platelet > 20,000/ μ l, median range (days)	16.2 (7-89)
Time to platelet > 50,000/ μ l, median range (days)	22.7 (7-148)
	Number (%)
Sex (M/F)	40/38 (51.3/48.7)
Underlying disease	
AML/ALL/Other leukemia*	33/18/2 (42.3/23.1/2.6)
CML/MDS/AA/NHL/HLH	5/5/11/3/1 (6.4/6.4/14.1/3.8/1.3)
Disease status at HSCT	
Acute leukemia, CR1/CR2/NR	33/17/3 (62.3/32.1/5.6)
CML, CP/AP/BC	3/1/1 (60.0/20.0/20.0)
MDS, RA/RCMD/RAEB	1/2/2 (20.0/40.0/40.0)
AA, severe/very severe	8/3 (72.7/27.3)
NHL, s/p auto-PBSC/sensitive relapse	1/2 (33.3/66.7)
HLH	1 (100)
Graft type, sibling/untreated	54/24 (69.2/30.8)
Stem cell source, BM/PBSC	28/50 (35.9/64.1)
Sex compatibility, match/mismatch/data missing	49/28/1 (62.8/35.9/1.3)
ABO compatibility, match/mismatch/data missing	36/40/2 (46.2/51.3/2.6)
Conditioning regimen	
Fludarabine-based/CTX-TBI/BU-CTX/CTX-ATG	47/17/4/10 (60.3/21.8/5.1/12.8)
GVHD prophylaxis	
CsA-based/Tacrolimus-based/data missing	54/22/2 (69.2/28.2/2.6)
Engraftment, success/failure/data missing	73/4/1 (93.6/5.1/1.3)
Acute GVHD, yes/no	21/57 (26.9/73.1)
SOS, yes/no/data missing	4/73/1 (5.1/93.6/1.3)
MTC (aGVHD + SOS)	22/56 (28.2/71.8)
Acute other complication except MTC	35/43 (44.9/55.1)
Relapse after allo-HSCT during follow-up	24/54 (30.8/69.2)

ANC, absolute neutrophil count; ALL, acute lymphocytic leukemia; CML, chronic myelogenous leukemia; MDS, myelodysplastic syndrome; AA, aplastic anemia; NHL, non-Hodgkin's lymphoma; HLH, hemophagocytic lymphohistiocytosis; HSCT, hematopoietic stem cell transplantation; CR2, complete remission after induction chemotherapy in 1st relapsed patients; NR, non-remission; MDS, RA/RCMD/RAEB, myelodysplastic syndrome, refractory anemia/refractory cytopenia with multilineage dysplasia/refractory anemia with excess blast; BM, bone marrow; PBSC, peripheral blood stem cell; TBI, total body irradiation; BU-CTX, busulfan-cyclophosphamide; CTX-ATG, cyclophosphamide-antithymocyte globulin; CsA, cyclosporin A; GVHD, graft versus host disease; SOS, sinusoidal obstruction syndrome; MTC, major transplant-related complication

*Other leukemia' refers to biphenotypic leukemia and adult T-cell leukemia.

rentially expressed or excreted polypeptides and proteins, have the potential to improve the early and accurate diagnosis of allo-HSCT-related complications without the need for invasive procedures such as biopsy (Reddy and Ferrara, 2003; Schulenburg *et al.*, 2004). Recently, several studies have demonstrated the usefulness of proteomic approaches in the identification of marker peptide patterns for graft-versus-host disease (GVHD) and other allo-HSCT-related complications (Kaiser *et al.*, 2004; Srinivasan *et al.*, 2006; Weissinger *et al.*, 2006, 2007). The identification of specific patterns that are modulated during disease progression or therapy would

enhance current methods of diagnosis and follow-up care (Weissinger *et al.*, 2006).

In general, all patients receive chemotherapy or irradiation prior to allo-HSCT, as a means to suppress immune reactions and help eradicate the disease prior to the infusion of hematopoietic stem cells (HSCs). However, this process may lead to undesirable tissue damage, triggering the release of inflammatory cytokines and promoting hemostatic alterations. Sinusoidal obstruction syndrome (SOS) is one of the most common early complications of HSCT, leading to significant morbidity and mortality. This disease appears to stem from endothelial damage to the sinusoids and small

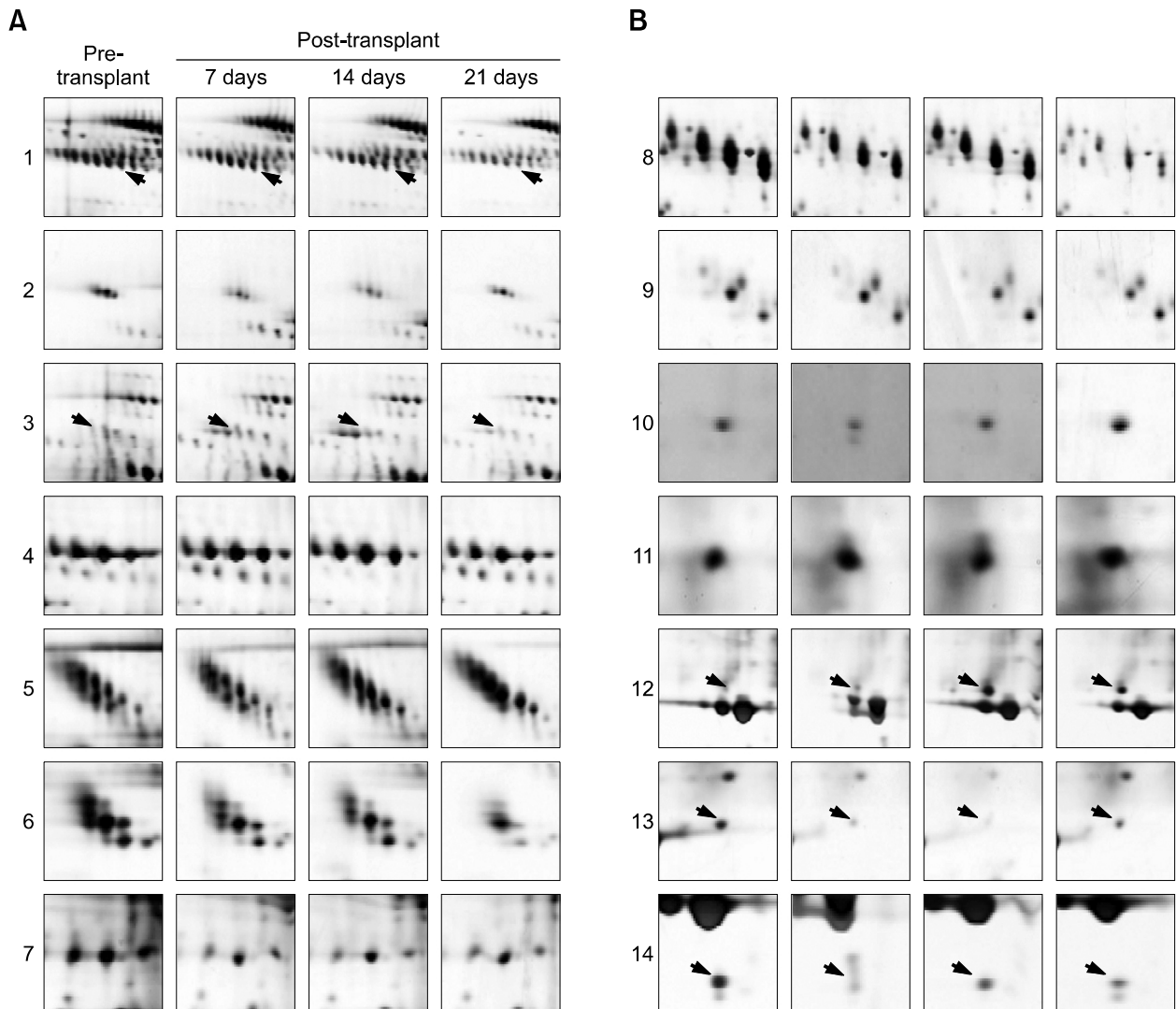


Figure 1. The analyses of human sera via 2-DE. Enlargement of the 2-DE gel spots identified by LC-MS/MS.

hepatic venules in response to conditioning regimens. Subclinical clotting and alterations in fibrinolytic profiles have occurred in patients receiving HSCT (Notoya *et al.*, 1998), and fibrinolytic and clotting parameters are predictive of SOS after HSCT (Tanikawa *et al.*, 2000; Villalon *et al.*, 2000; Sartori *et al.*, 2005). Acute GVHD also is one of the major early complications after HSCT, manifesting as SOS, wherein host tissues are perceived as antigenically foreign and are attacked by T cells in the graft. Proinflammatory cytokines are overproduced in response to total body irradiation (TBI) and/or high-dose chemotherapy, which directly or indirectly facilitates T-cell activation by upregulating the level of adhesion and costimulation molecules on antigen-presenting cells (Schultz and Arnold, 1990). Patient serum protein profiles undergo

changes during the development of acute GVHD after HSCT. Weissinger and colleagues reported the usefulness of proteomic screening in the early detection of acute GVHD occurring after allo-HSCT (Weissinger *et al.*, 2006, 2007). In addition, several proteins present in the body fluid of patients undergo changes in response to allo-HSCT. Severe infection and other complications of HSCT also influence protein profiles.

In this study, we conducted proteomic analyses to identify proteins that were differentially expressed in response to allo-HSCT. Among such proteins, the expression of C-reactive protein (CRP) and haptoglobin were significantly altered in response to transplantation.

Table 2. Fourteen proteins identified in five patients.

Spot No.	Accession No.	Identified proteins	Seq. Cov. (%)	Matched peptide	pI	MW (kDa)	PLGS score	Mascot score
1	IPI00017601	CP	6.8 (10)*	5 (8)*	5.4	122.9	630	319
2	IPI00017696	C1s	6.4 (5)	3 (3)	4.8	78.1	278	115
3	IPI00019568	Prothrombin	8.1 (13)	4 (7)	5.6	71.4	265	147
4	IPI00019591	CFB	10.6 (8)	7 (12)	6.8	143.1	517	140
5	IPI00550991	SERPINA3	29.9 (30)	13 (15)	5.4	50.7	1931	483
6	IPI00022431	AHSG	- (8)	- (3)	5.8	47.5	-	93
7	IPI00021891	FGG	15.4 (14)	7 (14)	5.3	52.1	762	241
8	IPI00431645	Haptoglobin	25.9 (42)	8 (23)	8.4	31.6	746	643
9	IPI00291262	APOJ	16.9 (15)	5 (5)	5.8	53.0	244	165
10	IPI00021841	APOE	28.3 (30)	7 (12)	5.6	36.2	631	434
11	IPI00022391	APCS	27.3 (34)	9 (14)	6.1	25.4	686	424
12	IPI00022381	CRP	13.8 (16)	3 (4)	5.4	25.1	201	74
13	IPI00021841	APOA1	62.1 (64)	16 (70)	5.5	30.7	1583	914
14	IPI00022423	PRBP	9.9 (23)	2 (5)	5.7	23.3	284	70

CP, Ceruloplasmin; C1s, Complement C1s subcomponent; CFB, Complement factor B; SERPINA3, α 1-antichymotrypsin; AHSG, α 2-HS-glycoprotein; FGG, Fibrinogen γ chain; APOJ, Apolipoprotein J; APOE, Apolipoprotein E; APCS, Serum amyloid P; CRP, C-reactive protein; APOA1, Apolipoprotein A1; PRBP, plasma retinol-binding protein

*Numbers in parentheses were sequenced and mapped data using the MASCOT DAEMON program.

Results

Clinical characteristics of patients after allo-HSCT

Venous blood samples were obtained from five representative patients who underwent allo-HSCT, and examined by proteome analysis. The five patients were diagnosed with AML (data not shown). The differentially expressed proteins identified in 2-DE analysis were validated using sera from 78 additional patients who underwent allo-HSCT at CNUH between June 2002 and April 2008. The majority of the patients ($n = 66$) received transplants for hematologic malignancy [*i.e.*, 33 patients with AML, 18 with acute lymphocytic leukemia (ALL), 5 with chronic myeloid leukemia (CML), 5 with myelodysplastic syndrome (MDS), 3 with non-Hodgkin lymphoma (NHL), 1 with acute biphenotypic leukemia, and 1 with adult T cell leukemia], whereas 12 patients received transplants because of hematopoietic failure syndrome [*i.e.*, 11 patients with aplastic anemia (AA) and 1 with hemophagocytic lymphohistiocytosis (HLH)]. The characteristics of these patients are summarized in Table 1.

Differentially expressed proteins in serum after allo-HSCT

To investigate proteins that were differentially expressed in patient serum during allo-HSCT, serum proteins were enriched via the depletion of albumin and IgG. Proteins from whole and

depleted sera separated by 2-DE (data not shown). Protein profiles between pre- and post-transplantation were compared using the Progenesis SameSpots program V2.0, and significant changes were visualized. 14 spots were examined using LC-MS/MS, and most proteins were identified as acute phase proteins (APPs) (Figure 1, Table 2). Comparison of the protein level before and after transplantation revealed three distinct patterns. Level of serum amyloid P, APOE, and CRP were higher 14 days or 21 days after allo-HSCT than before transplantation. However, concentrations of haptoglobin and α 2-HS-glycoprotein decreased after transplantation. Level of C1s, α 1-anti-chymotrypsin, prothrombin, complement factor B, fibrinogen γ chain, ceruloplasmin, PRBP, APOA1, and APOJ were modulated in irregular patterns. These results demonstrated that the level of some APPs was changed in response to transplantation and may therefore serve as potential markers for the diagnosis or evaluation of patients who receive allo-HSCT.

Validation of CRP and haptoglobin level in 78 patients after allo-HSCT

To further validate the differentially expressed proteins found in 5 patients, additional 78 patients who underwent allo-HSCT were analyzed. Changes of CRP and haptoglobin level within initial group of 5 patients showed more consistent pattern than other spots. Thus serum level of CRP and haptoglobin were measured on a weekly basis before

Table 3. Clinical characteristics of patients with or without MTC.

Characteristics	MTC (+) (n=22)	MTC (-) (n=56)	P value
Patient age, median range (years)	33.6 (17-51)	38.7 (16-62)	0.428 ^b
Patient age, < 40 or ≥ 40	16/6 (72.7/27.3)	26/30 (46.4/53.6)	0.036 ^d
Sex (M/F)	11/11	29/27	0.829 ^d
Time to ANC > 500/μl, median range (days)	14.0 (9-40)	8.3 (9-19)	0.757 ^c
Time to platelet > 20,000/μl, median range (days)	23.2 (8-89)	7.1 (7-89)	0.031 ^c
Time to platelet > 50,000/μl, median range (days)	32.4 (12-148)	19.47 (7-42)	0.087 ^c
Underlying disease			0.541 ^d
AML/ALL/Other leukemia	9/6/0	24/12/2	
CML/MDS/AA/NHL/HLH	3/2/2/0/0	2/3/9/3/1	
Disease status at HSCT ^a	17/5	48/8	0.500 ^c
Graft type, sibling/untreated	12/10	42/14	0.078 ^d
Stem cell source, BM/PBSC	7/15	21/35	0.638 ^d
Sex compatibility, match/mismatch/missing	13/9/-	39/19/1	0.600 ^d
ABO compatibility, match/mismatch/data missing	8/13/1	28/27/1	0.317 ^d
Conditioning regimen			0.276 ^d
Fludarabine-based	11	36	
CTX-TBI	8	9	
BU-CTX	1	3	
CTX-ATG	2	8	
GVHD prophylaxis			0.277 ^d
CsA-based	13	41	
Tacrolimus-based	8	14	
Data missing	1	1	

CTX-TBI, cyclophosphamide-total body irradiation; BU-CTX, busulfan-cyclophosphamide.

^aEither acute leukemia in first remission or MDS; RA/RCMD or CML; CP, aplastic anemia or HLH; compared with acute leukemia in second remission/non-remission or MDS; RAEB or CML; AP/BC or non-Hodgkin's lymphoma, ^bStudent's T test, ^cMann-Whitney, ^dChi-square test, ^eFisher's exact test.

and after allo-HSCT. Based on a previously used definition (Glucksberg *et al.*, 1974; Shulman *et al.*, 1980; Przepiorka *et al.*, 1995), the observed transplant-related complications in an MTC-positive group (*i.e.*, ≥ grade II aGVHD, SOS) and an MTC-negative group (*i.e.*, FOU, ≥ grade II mucositis, hemorrhagic cystitis, grade I aGVHD) were further categorized. Of the 22 patients who developed MTC, 4 (18.1%) experienced fatal outcomes within 100 days after allo-HSCT. Relapse after allo-HSCT occurred in 24 patients (30.8%), with 13 (54.1%) experiencing a relapse within 100 days.

When MTC-positive and -negative groups were considered separately, the median overall CRP level were showed a characteristic monophasic pattern that varied with time and were significantly higher on days 14 and 21 in the MTC-positive group [*i.e.*, when these data are expressed as medians (25%, 75%), the values were 3.60 (1.70, 9.60) and 1.70 (0.65, 3.10), respectively, on day 14 ($P = 0.004$); 1.05 (0.40, 3.90) and 0.50 (0.20, 0.80), respectively, on day 21 ($P = 0.013$)] (data not shown). Mean haptoglobin level showed decreasing pattern during the period of observation by proteomic analysis. When remission and relapse groups were observed separately, the level were

significantly high on day 14 in the relapsed group than in the remission group (*i.e.*, mean ± SD values of 114.4 ± 78.9 and 65.6 ± 58.2 , respectively; $P = 0.027$), but were not correlated with MTC concentrations (data not shown).

To establish potential risk factors for the development of MTC, association of variables suspected to influence the development of complications after allo-HSCT were examined to the MTC-positive and -negative groups (Table 3). There were no significant differences in age, gender, underlying disease, transplant type, stem cell source, conditioning regimen, GVHD prophylaxis, ABO incompatibility, or time (days) from allo-HSCT to an absolute neutrophil count > 500/μl. However, the MTC-positive group required more time than did the MTC-negative group to achieve a platelet count greater than 20,000/μl after allo-HSCT ($P = 0.031$). Also, age differences (age < 40 yr versus ≥ 40 yr) were significantly increased the risk factor for the occurrence of MTC ($P = 0.036$). More patients younger than 40 yr were included in the MTC-positive group (72.7% in the MTC-positive group and 46.4% in the MTC-negative group). Time (days) from allo-HSCT to have a platelet count > 50,000/μl and patients group underwent

Table 4. Correlation between serum CRP and haptoglobin level and the development of complications or relapse after allo-HSCT.

		<i>P</i> value
Acute GVHD (\geq Grade II)	Maximal CRP level	$< 0.001^a$
	Maximal Haptoglobin level	0.810 ^b
	CRP level on day 14*	0.010 ^a
	CRP level on day 21*	0.030 ^a
Sinusoidal obstruction syndrome	Maximal CRP level	0.004 ^a
	Maximal Haptoglobin level	0.840 ^b
	CRP level on day 7*	0.019 ^a
	CRP level on day 14*	0.003 ^a
Other acute complications	CRP level on day 21*	0.018 ^a
	Maximal CRP level	0.002 ^a
	Maximal Haptoglobin level	0.602 ^b
	CRP level on pre-transplant	0.005 ^a
	CRP level on day 7	$< 0.001^a$
Relapse after HSCT	CRP level on day 14*	$< 0.001^a$
	CRP level on day 21*	0.001 ^a
	Haptoglobin level on pre-transplant	0.036 ^b
	Maximal CRP level	0.230 ^a
	Maximal Haptoglobin level	0.042 ^a
	Haptoglobin level on day 14*	0.027 ^b

^aMann-Whitney test, ^bStudent's T test.

*Once a week after transplant.

allo-HSCT from unrelated donor were related to the occurrence of MTC without statistical significance ($P = 0.087$, $P = 0.078$, respectively).

It also analyzed according to the correlation between representative APPs and complications after HSCT (Table 4). The maximal CRP level in each patients were showed significant effects to the development of complications during further courses of allo-HSCT, including acute GVHD ($P < 0.001$), SOS ($P = 0.004$) and other acute complications such as FUO ($P = 0.002$). And also, when these data were analyzed according to the median CRP level for each time points, the occurrence of MTC was significantly affected to the median CRP level on days 14 and 21. Overall, maximal haptoglobin level were not significantly associated with other acute complications ($P = 0.602$); however, maximal pre-transplant haptoglobin level were associated with the development of other acute complications ($P = 0.036$).

Consequently, univariate and multivariate analyses of factors that might potentially increase the risk of MTC were performed (Table 5). Our results showed that MTC was significantly associated with young age (*i.e.*, less than 40 yr old) and maximal CRP level ($P = 0.049$ and $P < 0.001$, respectively). CRP level on days 14 and 21 were also risk factors for MTC. When these data were analyzed according to maximal CRP level, the risk of MTC increased more than 6.9-fold in patients with CRP level ≥ 20 mg/dl than in patients with

CRP level < 10 mg/dl. Multivariate analysis of the same factors revealed that young age (*i.e.*, less than 40 yr old) and CRP level on day 21 were the strongest independent variables ($P = 0.017$ and 0.032 , respectively). Relapse is a major problem in patients undergoing allo-HSCT. When the level of CRP and haptoglobin in patients with relapse after allo-HSCT was compared, there were no significant differences with regards to pre-transplant variables and the timing of engraftment. However, maximal haptoglobin level were correlated to relapse after allo-HSCT ($P = 0.042$) and mean haptoglobin level on day 14 were significantly higher in relapsed patients ($P = 0.027$) (Table 4). This relationship was not found when the maximal and median CRP level at each time points were analyzed (data not shown). Univariate analysis of relapse risk after allo-HSCT revealed that maximal haptoglobin level, disease status at the time of transplantation, and mean haptoglobin level on day 14 were risk factors for relapse ($P = 0.019$, $P = 0.005$, and $P = 0.030$, respectively). When these data were analyzed according to maximal haptoglobin level, the risk of relapse increased more than 12.7-fold in patients with haptoglobin level ≥ 200 mg/dl compared to patients with haptoglobin level < 150 mg/dl. Multivariate analysis of the same factors revealed that disease status was the strongest independent variable ($P = 0.003$) (Table 5).

These results indicate that the expression

Table 5. Univariate and multivariate analyses of factors influencing the risk of MTC and relapse after allo-HSCT.

Risk factor	Univariate analysis		Multivariate analysis	
	RR (95%, CI)	P value ^a	RR (95%, CI)	P value ^a
Age group (< 40 or ≥ 40)	2.557 (1.006, 6.601)	0.049	3.651 (1.256, 10.626)	0.017
Maximal CRP level	1.099 (1.051, 1.149)	< 0.001		
CRP level on day 14	1.080 (1.031, 1.131)	0.001		
CRP level on day 21	1.091 (1.012, 1.177)	0.023		0.032
Category of maximal CRP		0.001		
< 10	1			
≥ 10 and < 20	3.175 (1.170, 8.618)	0.023		
≥ 20	6.867 (2.328, 20.254)	< 0.001		
Disease status ^b	3.591 (1.466, 8.794)	0.005	4.168 (1.626, 10.684)	0.003
Maximal haptoglobin level	1.006 (1.001, 1.011)	0.019		
haptoglobin level on day 14	1.006 (1.001, 1.011)	0.030		
Category of maximal haptoglobin		0.006		
< 150	1			
≥ 150 and < 200	1.696 (0.693, 4.152)	0.247		
≥ 200	12.675 (2.657, 60.467)	0.001		

RR, relative risk; CI, confidence interval.

^aCox proportional regression analysis, ^bEither acute leukemia in first remission or MDS; RA/RCMD or CML; CP, aplastic anemia or HLH; compared with acute leukemia in second remission/non-remission or MDS; RAEB or CML; AP/BC or non-Hodgkin's lymphoma.

patterns of CRP and haptoglobin are significantly associated with the development of MTC and the risk of relapse after allo-HSCT, and suggest that monitoring of changes in CRP and haptoglobin level after allo-HSCT would allow for the identification of patients at risk for early transplant-related complications and relapse.

Discussion

When patients experience bone marrow failure or hematological malignancy, treatment generally involves chemotherapy or radiotherapy followed by the transplantation of a new and functional immune system. Although allo-HSCT has dramatically improved patient survival rate, the broader applications of this therapy remain limited because of the life-threatening complications that often occur after therapy, such as severe infection and acute GVHD (Horowitz *et al.*, 1990; Gratwohl *et al.*, 2002). Early diagnosis and better control of these complications would increase the safety of allo-HSCT, particularly with respect to the broad applications of the procedure. Because of the complexity of most problems that occur after allo-HSCT, knowledge of the behavior of single molecules and potential posttranslational modifications may not be sufficient to gain useful insight into the development of these conditions. However the generation of specific proteome patterns that are modulated during disease progression or

therapy would likely prove useful in the diagnosis and follow-up of patients who experience complications after allo-HSCT. Proteomic analyses have emerged as a technology capable of deciphering biological processes and discovering biomarkers present in tissues and bodily fluids. Previous studies strongly suggest that proteomic analyses of body fluids will become increasingly important in the clinical diagnosis of various diseases (Osin *et al.*, 1998; Voss *et al.*, 2001). Recent proteomic data have focused on hematologic malignancies such as AML (Cho *et al.*, 2004; Kwak *et al.*, 2004) and the follow-up care of patients after allo-HSCT (Kaiser *et al.*, 2004; Srinivasan *et al.*, 2006; Weissinger *et al.*, 2006, 2007).

In this study, 14 proteins, including CRP and haptoglobin, identified during allo-HSCT by 2-DE and LC-MS/MS are classified as APPs, a group of proteins whose plasma concentrations increase (*i.e.*, positive acute phase proteins) or decrease (*i.e.*, negative acute phase proteins) in response to inflammation. Generally, haptoglobin, CRP, C1s, prothrombin, fibrinogen γ chain, α 1-anti-chymotrypsin, complement factor B, serum amyloid P, and ceruloplasmin are positive APPs, whereas PRBP is considered a negative APP. Apart from the listed proteins, changes in APOE, APOJ, and APOA1 level were observed. Differentially expressed proteins, including APPs were involved in fibrinolytic processes and clotting parameters and changes in the expression level of these proteins may thus also be expected during the period

following allo-HSCT.

In the further validation of CRP and haptoglobin level in 78 patients after allo-HSCT, maximal CRP level influenced the development of acute complications, including acute GVHD, SOS, and FUO, after further courses of allo-HSCT. In particular, CRP level on days 14 and 21 was associated with the occurrence of MTC. The CRP level at each time points was closely associated with occurrence of other acute complications. In addition, the CRP level on day 21 was found to be an independent risk factor for MTC. Although young age (*i.e.*, less than 40 yr old) was the strongest predictive factor, our results may have been influenced by the fact that more young subjects were included in the MTC-positive group than in the MTC-negative group. CRP is an acute phase protein that is produced by the liver and is a reliable marker of systemic inflammation. The protein is induced in liver cells by IL-6 (Schultz and Arnold, 1990), which is produced by many types of cells such as monocytes, macrophages, fibroblasts, and endothelial cells. These cells are likely to survive the conditioning regimen and respond to the tissue damage caused by high-dose cytotoxic agents and infections. CRP and its role in the development of MTC after allo-HSCT were reported in the previous studies (Schots *et al.*, 1998, 2002, 2003). It is said that early increase in serum level of CRP is independent risk factor for the occurrence of MTC. As these studies, our results suggest that not only the severity and multiplicity of precipitating causes, but also individual inflammatory response level, determine outcome during the 3 weeks after allo-HSCT. In addition, a correlation between CRP and leukemic relapse was found, wherein mean CRP level throughout the early post-SCT episode were significantly lower in relapsing patients than in others (Min *et al.*, 2006). Also, GVHD was significantly associated with relapse. These studies have suggested that the procurement of inflammatory status, as reflected by higher serum CRP level, allows antigen-activated donor T cells to maintain their anti-leukemic effects, whereas the suppression of proinflammatory cytokines might interfere with the graft-versus-leukemia (GVL) phenomenon. However, we found no correlation between CRP level and leukemic relapse. Mean haptoglobin level during allo-HSCT exhibited a monophasic pattern, as did median CRP concentrations. However, maximal haptoglobin level and haptoglobin level at each time points were not associated with the development of MTC. Only haptoglobin concentrations prior to transplantation were correlated with other acute complications

such as FUO. Also, maximal haptoglobin level and haptoglobin level at day 14 emerged as risk factors for relapse after allo-HSCT in patients with disease at the time of transplantation. Haptoglobin is a positive acute-phase protein produced by liver cells (Arredouani *et al.*, 2003). Hepatic expression of haptoglobin is strongly induced by inflammatory mediators such as IL-6-type cytokines (Wang *et al.*, 2001). In addition to a role as a hemoglobin scavenger, haptoglobin has been shown to behave as an angiogenic (Cid *et al.*, 1993), antioxidant (Arredouani *et al.*, 2003), and anti-inflammatory factor (Langlois and Delanghe, 1996; Arredouani *et al.*, 2003). Haptoglobin and its variants are potent immunosuppressors of lymphocyte function (Arredouani *et al.*, 2003; Huntoon *et al.*, 2008), and also several studies have indicated that the protein may be useful as a cancer biomarker (Ahmed *et al.*, 2004; Bharti *et al.*, 2004; Ang *et al.*, 2006; Okuyama *et al.*, 2006; Sandoval *et al.*, 2007). Despite extensive research on haptoglobin, no studies have yet examined the correlation between haptoglobin and complications after allo-HSCT.

Overall, these results demonstrate that if CRP level are elevated during the third week after transplantation, clinicians should recognize the probability of MTC and seek clinical evidence suggestive of this condition. Although any correlation between relapse and CRP level did not show, maximal level of haptoglobin was associated with relapse after allo-HSCT. Haptoglobin is a positive APP like CRP; however, the proteins differ in clinical significance. The underlying pathophysiological mechanism remains unclear, and also our studies have some limitations as these data come from heterogeneous patient group, conditioned with different regimens and transplanted with differing cell sources, but the identification of a new biomarker able to predict relapse after allo-HSCT may be of considerable interest. Finally, we suggest that monitoring the expression of CRP and haptoglobin after allo-HSCT will allow for identification of patients who are at risk of early transplant-related complications and relapse.

Methods

Patients

Venous blood samples were obtained prior to allo-HSCT and weekly after allo-HSCT. The serum was separated and stored at -80°C until further use. The study protocol was approved by the Ethics Committee of Chonnam National University Hwasun Hospital (CNUH). Subjects were fully informed and written consent was obtained from each participant.

Conditioning and transplantation

Forty-seven patients were treated with a fludarabine-containing regimen. Of these, 32 patients received fludarabine (30 mg/m²/day for 6 days) and busulfan (0.8 mg/kg every 6 h for 4 days). In addition, Campath[®] (alemtuzumab) was added to the fludarabine and busulfan regimens of 15 patients who underwent unrelated allo-HSCT. Standard-intensity protocols were used to treat 21 patients. Of these, 17 patients received TBI (6 × 2 Gy over 3 days) and cyclophosphamide (60 mg/kg for 2 days), and 4 patients received busulfan (4 mg/kg for 4 days) followed by cyclophosphamide (120 mg/kg for an additional 2 days). Ten patients received cyclophosphamide (50 mg/kg for 4 days) and ATG (Thymoglobuline) (2.5 mg/kg for 3 days).

A total of 54 patients received stem cells from HLA-identical family donors, and 24 patients received transplants from unrelated donors [i.e., 18 patients received transplants from a matched unrelated donor (MUD) and 6 patients received transplants from a mismatched donor).

Stem cells were derived from peripheral blood stem cells in 50 patients and bone marrow in 28 patients. As prophylactic treatment against GVHD, 54 patients were treated with methotrexate (MTX) or mycophenolate mofetil (MMF) and cyclosporin A (CsA), and 22 patients were treated with Prograf[®] (tacrolimus, FK506) with or without methotrexate (MTX). The remaining two patients were treated with cyclosporin A (CsA) alone. All patients received standard supportive care, including empirical broad-spectrum antibiotic therapy in case of fever, low-dose continuous infusions of heparin (100 U/kg daily) to prevent SOS, and high-dose immunoglobulins and acyclovir to prevent herpes simplex/CMV. Platelet transfusions were given when platelet counts decreased to less than 20 × 10⁹ platelets/L. Granulocyte colony stimulating factor was administered to all patients until the achievement of stable neutrophil recovery, defined by an absolute neutrophil count (ANC) > 1.0 × 10⁹/L.

Definition of engraftment and major complications

Engraftment was estimated by the time required to obtain an ANC of 500 or more and the maintenance of a platelet count of 20,000 to 50,000 for 3 days, without the need for a platelet transfusion. Bone marrow aspiration and section biopsies were performed on day 21 after stem cell infusion. In addition, we checked for the presence of a short tandem repeat (STR) after HSCT and compared patients with donor STR, and performed FISH analysis of sex chromosomes in sex-mismatched transplant patients. Acute GVHD was graded on a four-point scale [i.e., ranging from mild disease (I) to severe disease (IV)] (Glucksberg *et al.*, 1974; Przepiorka *et al.*, 1995) and chronic GVHD was classified as either limited or extensive, as previously described (Shulman *et al.*, 1980; Atkinson *et al.*, 1989). Fever of unknown origin (FUO) was defined by a body temperature ≥ 38.3°C for at least 2 consecutive days. SOS was diagnosed according to the presence of hyperbilirubinemia (> 3.0 mg/dl), weight gain (> 2.5% of initial weight), and right upper quadrant pain, with or without the presence of hepatomegaly (McDonald *et al.*, 1984). Hematological and non-hematological toxicity were

measured and graded according to the criteria described by the National Cancer Institute-Common Toxicity Criteria (NCI-CTC) version 3.0.

Depleted serum sample preparation

The two major protein components of human serum (i.e., albumin and IgG) were depleted via immunoaffinity with albumin and IgG depletion kit according to the manufacturer's protocol (Sigma).

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis (2-DE) was performed using isoelectric focusing (IEF) and SDS-PAGE analysis. Dry-strips were rehydrated with 200 µg of protein in solubilization solution (9 M urea; 2% CHAPS; 4 M thiourea; 2% IPG buffer, pH 4 to 7; 18 mM DTT and a trace of bromophenol blue) and loaded onto Immobiline Drystrips. Rehydration proceeded for 12 h at room temperature and IEF was conducted at 20°C in gradient mode for 1 minute at 200 V, 90 min at 3,500 V and 10 h at 3,500 V. Following IEF separation, the dry-strips were equilibrated for 30 minutes in an equilibration buffer (50 mM Tris-HCl pH 6.8, 6 M urea, 30% glycerol, 2% SDS and a trace of bromophenol blue). We then added 0.25% DTT to the first equilibration buffer, and replaced DTT with 4.5% iodoacetamide in the second equilibration buffer. The second dimension of the separation was carried out on a SDS polyacrylamide vertical slab gel. After 2-DE, proteins were visualized by silver staining according to the manufacturer's protocol (GE Healthcare).

Image analysis

The 2-DE images were analyzed using the Progenesis SameSpots program, V2.0. (Nonlinear Dynamics, Nonlinear, Inc) (Mattacks *et al.*, 2003).

Destaining and in-gel trypsin digestion of protein and extraction

Protein spots separated by 2-DE were excised manually from 2-DE gels and destained using oxidation buffer (15 mM potassium ferricyanide and 50 mM sodium thiosulphate). Destained gels were re-swelled and dehydrated using 100 µl of 100 mM ammonium bicarbonate in 50% acetonitrile. The gels were re-swollen and dehydrated for an additional two to three times, then dried. The dried gels were digested by 50 mM ammonium bicarbonate, pH 8, containing 0.05 µg/µl trypsin for 16 hours at 37°C. The tryptic peptides were extracted three times to recover all of the peptides from the gel particles. The recovered peptides were concentrated by drying the final volume of the extracts in a vacuum centrifuge. The concentrated peptides were then mixed with 20 µl of 0.1% formic acid in 3% acetonitrile in preparation for liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis.

LC-MS/MS analysis and database searches

Nano LC of tryptic peptides was performed using a Waters

Nano LC system equipped with a Waters C18 nano column. Samples were loaded onto the column. Peptides were eluted from the column with a gradient ranging from 2% to 40% binary solvent B1 for 30 min at 0.4 μ l/min. Mass spectrometry analysis of tryptic peptides was performed using a Waters SynaptTM HDMS. The mass spectrometer was operated in V-mode for all measurements. All analyses were performed using a positive mode Nano ESI with a NanoSpray source. Accurate mass LCMS data were collected via an alternating [i.e., low energy (MS) and elevated energy (MS^E)] mode or Data Dependent Acquisition (DDA) mode of acquisition. Continuum LC-MS/MS data were processed and used in database searches using the Protein Lynx Global Server (PLGS), version 2.3 (Waters). Ion detection, clustering and normalization were performed using PLGS. Processed data were used to search the IPI human database (Liu *et al.*, 2004; Wang *et al.*, 2005). Processed ions were sequenced and mapped against the IPI human database using the PLGS and MASCOT DAEMON programs (<http://www.matrixscience.com>).

Measurement of CRP and haptoglobin level

Level of CRP were measured using an Hitachi 7600-110 automated biochemical analyzer (Hitachi High Technologies., Tokyo, Japan) and turbidimetric immunoassays (TIA), with a reference range of 0 mg/dl to 0.5 mg/dl. Haptoglobin level were determined via immunoturbidometry using a commercial HAPT kit (Roche, Switzerland) and the COBAS Integra 800 biochemical analyzer (Roche, Switzerland), with a reference range of approximately 30 mg/dl to 200 mg/dl.

Statistical analysis

Student's *t* tests were performed to test for differences in the means of continuous measurements and these results were confirmed using the Mann-Whitney test. The association between categorical variables was investigated using the Chi square or Fisher's exact tests. Factors of interest were compared between patient groups. Preliminary univariate analyses were performed to identify factors that could be used to build a Cox proportional hazard regression model. This model was then used to determine the probability of major transplant-related complications and relapse.

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References

Ahmed N, Barker G, Oliva KT, Hoffmann P, Riley C, Reeve S, Smith AI, Kemp BE, Quinn MA, Rice GE. Proteomic-based

identification of haptoglobin-1 precursor as a novel circulating biomarker of ovarian cancer. *Br J Cancer* 2004; 91:129-40

Ang IL, Poon TC, Lai PB, Chan AT, Ngai SM, Hui AY, Johnson PJ, Sung JJ. Study of serum haptoglobin and its glycoforms in the diagnosis of hepatocellular carcinoma: a glyco-proteomic approach. *J Proteome Res* 2006;5:2691-700

Arredouani M, Matthijs P, Van Hoeyveld E, Kasran A, Baumann H, Ceuppens JL, Stevens E. Haptoglobin directly affects T cells and suppresses T helper cell type 2 cytokine release. *Immunology* 2003;108:144-51

Atkinson K, Horowitz MM, Gale RP, Lee MB, Rimm AA, Bortin MM. Consensus among bone marrow transplanters for diagnosis, grading and treatment of chronic graft-versus-host disease. Committee of the International Bone Marrow Transplant Registry. *Bone Marrow Transplant* 1989;4: 247-54

Bharti A, Ma PC, Maulik G, Singh R, Khan E, Skarin AT, Salgia R. Haptoglobin alpha-subunit and hepatocyte growth factor can potentially serve as serum tumor biomarkers in small cell lung cancer. *Anticancer Res* 2004;24:1031-8

Cho JW, Kim JJ, Park SG, Lee DH, Lee SC, Kim HJ, Park BC, Cho S. Identification of B-cell translocation gene 1 as a biomarker for monitoring the remission of acute myeloid leukemia. *Proteomics* 2004;4:3456-63

Cid MC, Grant DS, Hoffman GS, Auerbach R, Fauci AS, Kleinman HK. Identification of haptoglobin as an angiogenic factor in sera from patients with systemic vasculitis. *J Clin Invest* 1993;91:977-85

Copelan EA. Hematopoietic stem-cell transplantation. *N Engl J Med* 2006;354:1813-26

Glucksberg H, Storb R, Fefer A, Buckner CD, Neiman PE, Clift RA, Lerner KG, Thomas ED. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors. *Transplantation* 1974; 18:295-304

Gratwohl A, Brand R, Apperley J, Biezen Av A, Bandini G, Devergie A, Schattenberg A, Frassoni F, Guglielmi C, Iacobelli S, Michallet M, Kolb HJ, Ruutu T, Niederwieser D. Graft-versus-host disease and outcome in HLA-identical sibling transplantations for chronic myeloid leukemia. *Blood* 2002;100:3877-86

Horowitz MM, Gale RP, Sondel PM, Goldman JM, Kersey J, Kolb HJ, Rimm AA, Ringden O, Rozman C, Speck B, *et al.* Graft-versus-leukemia reactions after bone marrow transplantation. *Blood* 1990;75:555-62

Huntoon KM, Wang Y, Eppolito CA, Barbour KW, Berger FG, Shrikant PA, Baumann H. The acute phase protein haptoglobin regulates host immunity. *J Leukoc Biol* 2008; 84:170-81

Kaiser T, Kamal H, Rank A, Kolb HJ, Holler E, Ganser A, Hertenstein B, Mischak H, Weissinger EM. Proteomics applied to the clinical follow-up of patients after allogeneic hematopoietic stem cell transplantation. *Blood* 2004;104: 340-9

Kwak JY, Ma TZ, Yoo MJ, Choi BH, Kim HG, Kim SR, Yim CY,

- Kwak YG. The comparative analysis of serum proteomes for the discovery of biomarkers for acute myeloid leukemia. *Exp Hematol* 2004;32:836-42
- Langlois MR, Delanghe JR. Biological and clinical significance of haptoglobin polymorphism in humans. *Clin Chem* 1996;42:1589-600
- Mattacks CA, Sadler D, Pond CM. The cellular structure and lipid/protein composition of adipose tissue surrounding chronically stimulated lymph nodes in rats. *J Anat* 2003;202:551-61
- McDonald GB, Sharma P, Matthews DE, Shulman HM, Thomas ED. Venocclusive disease of the liver after bone marrow transplantation: diagnosis, incidence, and predisposing factors. *Hepatology* 1984;4:116-22
- Min CK, Kim SY, Eom KS, Kim YJ, Kim HJ, Lee S, Kim DW, Lee JW, Min WS, Kim CC. Patterns of C-reactive protein release following allogeneic stem cell transplantation are correlated with leukemic relapse. *Bone Marrow Transplant* 2006;37:493-8
- Notoya A, Sawada K, Ieko M, Tarumi T, Koizumi K, Fukada Y, Sato N, Yasukouchi T, Koike T. Subclinical alterations in coagulation and fibrinolysis in patients undergoing autologous peripheral blood stem cell transplantation. *Leuk Lymphoma* 1998;28:405-13
- Okuyama N, Ide Y, Nakano M, Nakagawa T, Yamanaka K, Moriwaki K, Murata K, Ohigashi H, Yokoyama S, Eguchi H, Ishikawa O, Ito T, Kato M, Kasahara A, Kawano S, Gu J, Taniguchi N, Miyoshi E. Fucosylated haptoglobin is a novel marker for pancreatic cancer: a detailed analysis of the oligosaccharide structure and a possible mechanism for fucosylation. *Int J Cancer* 2006;118:2803-8
- Osin P, Shipley J, Lu YJ, Crook T, Gusterson BA. Experimental pathology and breast cancer genetics: new technologies. *Recent Results Cancer Res* 1998;152:35-48
- Przepiorka D, Weisdorf D, Martin P, Klingemann HG, Beatty P, Hows J, Thomas ED. 1994 Consensus Conference on Acute GVHD Grading. *Bone Marrow Transplant* 1995;15:825-8
- Reddy P, Ferrara JL. Immunobiology of acute graft-versus-host disease. *Blood Rev* 2003;17:187-94
- Sandoval JA, Turner KE, Hoelz DJ, Rescorla FJ, Hickey RJ, Malkas LH. Serum protein profiling to identify high-risk neuroblastoma: preclinical relevance of blood-based biomarkers. *J Surg Res* 2007;142:268-74
- Sartori MT, Spiezia L, Cesaro S, Messina C, Paris M, Pillon M, Saggiorato G, Pagnan A, Girolami A, Zanesco L, Cella G. Role of fibrinolytic and clotting parameters in the diagnosis of liver veno-occlusive disease after hematopoietic stem cell transplantation in a pediatric population. *Thromb Haemost* 2005;93:682-9
- Schots R, Kaufman L, Van Riet I, Lacor P, Trullemans F, De Waele M, Van Camp B. Monitoring of C-reactive protein after allogeneic bone marrow transplantation identifies patients at risk of severe transplant-related complications and mortality. *Bone Marrow Transplant* 1998;22:79-85
- Schots R, Van Riet I, Othman TB, Trullemans F, De Waele M, Van Camp BM, Kaufman L. An early increase in serum levels of C-reactive protein is an independent risk factor for the occurrence of major complications and 100-day transplant-related mortality after allogeneic bone marrow transplantation. *Bone Marrow Transplant* 2002;30:441-6
- Schots R, Kaufman L, Van Riet I, Ben Othman T, De Waele M, Van Camp B, Demanet C. Proinflammatory cytokines and their role in the development of major transplant-related complications in the early phase after allogeneic bone marrow transplantation. *Leukemia* 2003;17:1150-6
- Schulenburg A, Turetschek K, Wrba F, Vogelsang H, Greinix HT, Keil F, Mitterbauer M, Kalhs P. Early and late gastrointestinal complications after myeloablative and nonmyeloablative allogeneic stem cell transplantation. *Ann Hematol* 2004;83:101-6
- Schultz DR, Arnold PI. Properties of four acute phase proteins: C-reactive protein, serum amyloid A protein, alpha 1-acid glycoprotein, and fibrinogen. *Semin Arthritis Rheum* 1990;20:129-47
- Shulman HM, Sullivan KM, Weiden PL, McDonald GB, Striker GE, Sale GE, Hackman R, Tsoi MS, Storb R, Thomas ED. Chronic graft-versus-host syndrome in man. A long-term clinicopathologic study of 20 Seattle patients. *Am J Med* 1980;69:204-17
- Srinivasan R, Daniels J, Fusaro V, Lundqvist A, Killian JK, Geho D, Quezado M, Kleiner D, Rucker S, Espina V, Whiteley G, Liotta L, Petricoin E, Pittaluga S, Hitt B, Barrett AJ, Rosenblatt K, Childs RW. Accurate diagnosis of acute graft-versus-host disease using serum proteomic pattern analysis. *Exp Hematol* 2006;34:796-801
- Tanikawa S, Mori S, Ohhashi K, Akiyama H, Sasaki T, Kaku H, Hiruma K, Matsunaga T, Morita T, Sakamaki H. Predictive markers for hepatic veno-occlusive disease after hematopoietic stem cell transplantation in adults: a prospective single center study. *Bone Marrow Transplant* 2000;26:881-6
- Villalon L, Avello AG, Cesar J, Odriozola J, Lopez J, Oteyza JP, Larana JG, Cantalapiedra A, Navarro JL. Is veno-occlusive disease a specific syndrome or the exacerbation of physiopathologic hemostatic changes in hematopoietic stem cell transplantation (HSCT)? *Thromb Res* 2000;99:439-46
- Voss T, Ahorn H, Haberl P, Dohner H, Wilgenbus K. Correlation of clinical data with proteomics profiles in 24 patients with B-cell chronic lymphocytic leukemia. *Int J Cancer* 2001;91:180-6
- Wang Y, Kinzie E, Berger FG, Lim SK, Baumann H. Haptoglobin, an inflammation-inducible plasma protein. *Redox Rep* 2001;6:379-85
- Weissinger EM, Mischak H, Ganser A, Hertenstein B. Value of proteomics applied to the follow-up in stem cell transplantation. *Ann Hematol* 2006;85:205-11
- Weissinger EM, Schiffer E, Hertenstein B, Ferrara JL, Holler E, Stadler M, Kolb HJ, Zander A, Zurbig P, Kellmann M, Ganser A. Proteomic patterns predict acute graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. *Blood* 2007;109:5511-9