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REVIEW

Biomarkers for acute GVHD: can we predict the unpredictable?

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Acute GVHD remains an important complication after allogeneic hematopoietic cell transplantation (HCT). Many efforts have been devoted to identifying potential noninvasive peripheral blood biomarkers to help improve the diagnosis or management of acute GVHD while avoiding invasive tissue biopsies. Early attempts to identify biomarkers focused on inflammatory cytokines, especially IL-2 or TNF-α, however, both of these and others were not specific for GVHD, often being elevated in the setting of generalized inflammation, accompanying other major complications of HCT as well. More recent efforts have focused on additional cytokines and other cell-surface molecules, which function in leukocyte trafficking and activation with the hope that these can also serve as targets for novel therapeutic approaches. Modern proteomic methods have allowed the screening of large numbers of patient samples and yielded several novel candidate biomarkers, including elafin and reg3α, which may not be directly involved in the immunological pathogenesis of GVHD, but may be unique biomarkers for end-organ injury. Combining these new molecules with traditionally identified cytokines to form an acute GVHD biomarker panel has recently shown the ability to predict outcomes in patients who develop acute GVHD. The ultimate goals of identifying a specific biomarker are to refine diagnosis, guide therapy and develop risk-adapted approaches in order to better treat patients and improve outcomes after allogeneic HCT. These approaches include differential treatment for patients who develop acute GVHD with a high-risk biomarker profile as well as pre-emptive therapy in patients after HCT prior to the development of symptoms. With the recent progress summarized below, these goals may soon be realized.

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INTRODUCTION

The diagnosis of acute GVHD after allogeneic hematopoietic cell transplantation (HCT) remains clinical, taking into account patient symptoms, laboratory values and affected tissue histology. Currently, there are no validated noninvasive biomarkers which are used in routine clinical applications for acute GVHD. Identifying specific biomarkers measured from peripheral blood samples would clearly be a valuable diagnostic and clinical tool to avoid invasive diagnostic procedures and assist in personalizing care after HCT. Traditionally, candidate biomarkers were investigated through hypothesis-driven approaches. These efforts were mostly single-center studies, limited by the relatively small number of patient samples analyzed, and lacked validation in larger cohorts. Candidate biomarkers were generally chosen because of a postulated role in the pathophysiology of GVHD, and thus, these biomarkers could ultimately serve as targets for therapy as well. Recently, modern proteomic technology has allowed the efficient analysis of large number of samples in an unbiased fashion, allowing the first identification of potential biomarkers, which are not involved in the pathophysiology of GVHD, but rather secreted as a result of end-organ damage.

To be of use, candidate biomarkers should possess some of the following characteristics (Table 1): (1) ease of testing, (2) widely available technique with good reproducibility, (3) relatively low cost, (4) adequate sensitivity with high specificity, (5) predictive value, (6) correlation with severity and (7) correlation with treatment response. If a biomarker was specific for diagnosis, delays in obtaining biopsies and pathological interpretation could be avoided. Furthermore, ambiguous cases could be clarified and managed more appropriately. If a biomarker was detectable prior to symptom onset, pre-emptive therapy could be given, akin to how CMV reactivation is currently managed after HCT. Furthermore, if a biomarker level correlates with the severity of disease and subsequent response (that is, similar to CA-125 or PSA), serial measurements could help to guide the withdrawal of immunosuppression. Ultimately, the utility of a biomarker is dependent upon the demonstration that clinical outcomes are altered as a result of having measured it.

INDIVIDUAL CANDIDATE BIOMARKERS

Cytokines are logical biomarkers for acute GVHD, given the accepted role of systemic inflammation in acute GVHD. The two most studied cytokines have been IL-2 and TNF-α.

IL-2

Given the role of IL-2 in T-cell activation and proliferation, IL-2 or its soluble receptor (sIL-2R) was an obvious choice to study. Miyamoto *et al.*[1] first suggested that sIL-2R levels at day +3 after HCT could predict acute GVHD and this was supported by a small German study, which demonstrated a clear correlation between sIL-2R levels and the severity of GVHD. However, investigators cautioned that CMV reactivation led to increased levels as well.[2] Foley *et al.*[3] conducted a study in 36 patients that measured weekly levels of sIL-2R after HCT and found that sIL-2R levels

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Table 1. Ideal characteristics of a noninvasive blood biomarker for acute GVHD

Characteristic	*Clinical Application*
Ease of testing	Can potentially avoid invasive biopsies
Widely available technique	
Good reproducibility	
Low cost	
Adequate sensitivity	
High specificity	More accurate diagnosis
Predictive value	Can guide pre-emptive therapy
Correlation with treatment response	Can guide immunosuppression withdrawal
Involved in pathophysiology	Can be targeted for novel therapy

started to increase 1–2 weeks prior to clinical onset of acute GVHD, however, they also showed a correlation with sepsis and hepatic veno-occlusive disease. Similarly, Mathias *et al.*[4] confirmed a correlation between serial sIL-2R levels and the development and severity of acute GVHD, but noted that critically ill patients without acute GVHD had comparable levels.

Although increased sIL-2R levels do correlate with the development and severity of acute GVHD, sIL-2R levels also rise in the setting of other inflammatory conditions after HCT. Hence, sIL-2R levels likely serve as a marker for generalized activation of the immune system, and not a specific biomarker for acute GVHD. However, identification of the importance of IL-2 in the pathophysiology of acute GVHD did contribute to the rationale for targeted therapy in the form of specific monoclonal antibodies such as daclizumab[5] and drug conjugates such as denileukin diftitox.[6]

TNF-α

In 1990, Holler *et al.*[7] first reported that increased serum levels of TNF-α preceded acute GVHD. However, such increases were also observed in patients who had interstitial pneumonitis and veno-occlusive disease. Similarly, a study by Or *et al.*[8] showed that serum levels of soluble TNF receptors (TNFRs) were also associated with major transplant-related complications and not just acute GVHD. More recently, Choi *et al.*[9] measured soluble TNFR1 levels before HCT and at day +7 in 438 patients undergoing myeloablative HCT and showed that increases in TNFR1 >2.5 times the baseline level correlated with the development of grade II–IV acute GVHD and increased TRM. Similar to the experience with IL-2R, these associations between TNF and acute GVHD were clearly not specific, but were present for any significant complication, such as infection, veno-occlusive disease and pulmonary toxicity. However, insight into the role of TNF-α in complications after HCT led to the justification for targeted therapy for acute GVHD.[10]

Other recent hypothesis-driven biomarkers

Table 2 summarizes several recent studies concerning hypothesis-driven acute GVHD biomarkers. As significant inflammation was thought to be a major part of the initial trigger of acute GVHD, early studies focused on acute-phase reactants, such as IL-1 and IL-6, in addition to TNF-α.[11–14] A Japanese study measured C-reactive protein in a heterogeneous group of over 200 patients with analysis showing that although higher C-reactive protein levels could predict for acute GVHD, other factors including conditioning regimen intensity, infection and unrelated donors were also associated with higher levels.[15]

Acute GVHD has traditionally thought to be a Th1 response, and thus, cytokines including IL-12 and IFN-γ have been shown to have correlations with acute GVHD.[12,14,16] IL-18, which induces the production of Th1 cytokines, has been reported by two studies to correlate with acute GVHD, with evidence that IL-18 levels decrease with successful treatment.[17,18] Paradoxically, some studies have shown that cytokines with anti-inflammatory effects are associated with acute GVHD. IL-10, which is thought to downregulate the synthesis of certain cytokines, such as IL-2, TNF-α and IFN-γ, has been shown to be elevated in acute GVHD with the theory that it is produced in response to the significant inflammation present.[12,13] Similarly, transforming growth factor β, which has roles in regulatory T-cell development and inhibition of lymphocyte activation, has also been studied.[12]

IL-7 and IL-15 are thought to function in the homeostatic peripheral expansion of donor T cells after the lymphodepletion induced by conditioning, and both have been studied. Thiant *et al.*[19] showed that day +14 levels of IL-7 could predict for grades II–IV acute GVHD. IL-15 also peaked at day +14 but lost its association in multivariate analysis and is thought to be more a marker of generalized inflammation. In addition, IL-8, a cytokine that functions in neutrophil chemotaxis and degranulation, was shown to remain elevated in one study in patients with β-thalassemia who developed GVHD and decreased in patients who did not develop GVHD.[20]

Candidate biomarkers, which are not cytokines, have also been studied recently and these include hepatocyte growth factor (HGF), cytokeratin 18 fragments, syndecan-1, granzymes A and B and CD30. HGF is a circulating molecule with many functions, including stimulating hematopoiesis and tissue repair after injury, and thus, is likely produced as a response to the inflammation present in GVHD.[21] Cytokeratin 18 is an intermediate filament present in epithelial and parenchymal cells, which is cleaved by caspases when apoptosis is induced, resulting in the release of cytokeratin 18 fragments. Luft *et al.*[22] showed that serum cytokeratin 18 fragment levels correlated with the severity of hepatic and intestinal acute GVHD and decreased in response to successful treatment. Syndecan-1 is a transmembrane heparin sulfate proteoglycan present on the surface of most epithelial cells, including skin, liver and intestine, and serum levels were shown to be significantly elevated in acute GVHD with a correlation with the severity of disease.[23] Granzymes A and B are products of cytotoxic T cells and natural killer cells, and serum levels appeared to parallel the incidence and severity of acute GVHD, although there was an association with CMV infection as well.[24] CD30, a member of the TNFR superfamily, is expressed on the surface of certain activated T cells. Recent studies have shown that CD30 expression appears to be higher on memory T cells in patients with acute GVHD,[25] and soluble CD30 levels have been shown to be increased in the setting of GVHD,[26] possibly revealing a novel target for therapeutic intervention.

Lymphocyte-trafficking molecules

The circulation of lymphocytes in the body is controlled by a multitude of specific cell-surface molecules, which participate in the process of lymphocyte trafficking.[27,28] Acute GVHD appears to be no different from any other adaptive immunological response and, thus, should possess similar requirements of lymphocyte trafficking.[29,30] α4β7 integrin is a surface molecule, which specifically traffics lymphocytes to intestinal lymphoid tissue.[31] Recently, a retrospective analysis showed that increased expression of α4β7 integrin on memory T-cell subsets was associated with the subsequent development of intestinal GVHD.[32] A later study, using samples at presentation of symptoms of GVHD and prior to any treatment, showed that memory $CD8^+$ T cells from patients with intestinal acute GVHD had higher levels of α4β7 expression.[33] A large study collecting serial samples at the standard time points after HCT is ongoing in attempts to validate the predictive value and study if expression of α4β7 integrin correlates with severity of disease and prognosis.

Table 2. Recent hypothesis-driven candidate biomarkers

Category	*Biomarker*	*Function*	*Reference*
Acute-phase reactants	IL-6	Acute-phase response of inflammation, production of neutrophils	Schots *et al.*[11]
	CRP	and maturation of B cells	Imamura *et al.*[14]
		Acute-phase reactant, which activates complement and innate immunity	Fuji *et al.*[15]
Th1 cytokines	IL-12	T-cell growth factor, stimulates production of TNF-α and IFN-γ	Nakamura *et al.*[16]
	IL-18	Activated NK cells, induces IFN-γ production	Fujimori *et al.*[17]
			Shaiegan *et al.*[18]
			Mohty *et al.*[52]
T-cell homeostasis	IL-7	Peripheral expansion of donor T cells after lymphodepletion from conditioning	Thiant *et al.*[19]
Anti-inflammatory cytokines	IL-10	Downregulates expression of Th1 cytokines	Visentainer *et al.*[12]
	TGF-β	Inhibits Ag presentation	Visentainer *et al.*[12]
		Blocks activation of lymphocytes	Liem *et al.*[13]
		Aids in differentiation of regulatory T cells	
Other circulating molecules	IL-8	Neutrophil chemotaxis and degranulation	Uguccioni *et al.*[20]
	HGF	Regulator of cell growth, motility, morphogenesis and tissue	Okamoto *et al.*[21]
	Cytokeratin-18 fragments	repair after injury	Luft *et al.*[22]
		Intermediate filament that is cleaved by caspases when	Seidel *et al.*[23]
	Syndecan-1	apoptosis is induced	Kircher *et al.*[24]
	Granzymes A and B	Transmembrane proteoglycan on epithelial cells released upon injury	Hubel *et al.*[26]
	CD30 and sCD30	Cytoplasmic enzymes released by effector T and NK cells	Chen *et al.*[25]
		Expressed on activated lymphocytes, unclear function	
Lymphocyte-trafficking molecules	α4β7 integrin	Mediator of lymphocyte trafficking to the intestine and associated lymphoid tisse	Chen *et al.*[32]
			Chen *et al.*[33]
	CXCL10	Ligand for CXCR3, stimulated monocytes, NK cells and T-cell migration	Piper etl al.[34]
	CCL8	Chemokine attracting leukocytes to sites of inflammation	Hori *et al.*[35]

Abbreviations: CRP = C-reactive protein; CCL8 = C-C motif ligand 8; CXCL10 = C-X-C motif chemokine 10; HGF = hepatocyte growth factor; NK = natural killer; sCD30 = soluble CD30; TGF = transforming growth factor.

Chemokines, which are small molecules that bind to a family of heterotrimeric G proteins and participate in lymphocyte trafficking, are also being studied. Piper *et al.*[34] performed a prospective analysis in 34 patients after HCT and showed a two-fold increase in serum levels of the chemokine CXCL10 in patients with acute GVHD relative to control patients. Hori *et al.*[35] used a proteomics approach in murine models of HCT to identify serum levels of the chemokine CCL8. Looking at human samples, there was preliminary evidence that serum CCL8 levels were increased in the setting of acute GVHD, although only 14 human patients were evaluated. Targeting mediators of lymphocyte trafficking may soon become a clinically applicable approach for acute GVHD, as such therapies are already being used for multiple sclerosis[36] and inflammatory bowel disease.[37]

Proteomic screening for biomarkers of acute GVHD. Rather than focussing on one or a few hypothesized proteins, which participate in the pathophysiology of acute GVHD, some investigators have attempted to use a large-scale proteomics approach to identify candidate biomarkers (see Table 3). Investigators from the University of Michigan first performed a discovery study, comparing 21 patients with severe acute GVHD with samples from 21 matched control patients. They found 35 candidate biomarkers, using a large Ab-microarray analysis, consisting of 120 various acute-phase reactants, cytokines, angiogenic factors, tumor markers, leukocyte-adhesion molecules and metalloproteinases or their inhibitors. From these 35 proteins, the eight most significantly different proteins (IL-2Rα, C-reactive protein, IL-8, ICAM-1, TIMP-1, TNFR1, HGF and CA19.9) were chosen for ELISA-based analysis in a training set of 282 patients. Logistic regression determined that a linear combination of values for a four-marker panel comprised of IL-2Rα, TNFR1, HGF and IL-8 levels produced the best model and had impressive accuracy when applied to a validation set of 142 additional patients. In addition to being able to confirm the diagnosis of GVHD, this panel was also able to predict survival independent of disease severity.[38] Most recently, a three-biomarker panel, consisting of IL-2Rα, TNFR1 and elafin (see below), was tested prospectively at pre-conditioning, day +7 and day +14 after allogeneic HCT in separate training ($n = 342$) and validation ($n = 171$) sets. Analysis showed reasonably good specificity (75%) and fair sensitivity (57%) for the development of acute GVHD.[39]

Other investigators have used mass spectrometry-based approaches. The advantage of using mass spectrometry is that the identification of candidate proteins is not biased by the availability of antibodies, as is the case for microarray or ELISA-based techniques. The disadvantages include the labor and time necessary and the lack of identification of specific proteins and limited sensitivity for proteins at low levels.[40] Kaiser *et al.*[41] collected urine from transplant recipients and showed that a panel of 16 polypeptides could reliably differentiate patients with GVHD from those without. After refining their sample preparation, the same group analyzed urine samples, using the same methods on a training set of 63 samples, and identified an acute GVHD-specific model of 31 polypetides. When applied to a large validation set, this panel had a sensitivity of 83.1% and specificity of 75.6%.[42] Paczesny *et al.*,[43] who identified the four-marker panel mentioned above, have used such an approach on pooled plasma from patients with and without skin GVHD to discover the protein elafin, an elastate inhibitor overexpressed in inflamed epidermis, as a potential organ-specific biomarker. In a validation set of >400 samples, the level of elafin remained specific for skin GVHD and was also able to predict TRM and OS. Most recently, using the same approach, this group also has identified reg3α as a potential organ-specific biomarker for intestinal GVHD.[44]

MOVING TOWARD RISK-ADAPTED APPROACHES

Risk-adapted approaches, either in pre-emptive intervention or differential treatment after the diagnosis of acute GVHD, are one goal of the larger efforts to personalize care after allogeneic HCT. Recently, investigators from the University of Michigan spearheaded an effort to evaluate the prognostic value of a panel of biomarkers comprised of TNFR1, IL-2Rα, IL-8, HGF, elafin and reg3α. Samples

Table 3. Proteomic approaches to identifying biomarkers

Reference	*Biomarker panel*	*Reported accuracy*	*Limitations*
Paczesny *et al.*[38]	IL-2α TNFR-1 IL-8 HGF	AUC 0.91 in training set AUC 0.86 in validation set	Excluded patients with VOD, IPS and sepsis
Paczesny *et al.*[39]	IL-2α TNFR-1 Elafin	Sensitivity 57% Specificity 75% Accuracy 65%	Excluded patients with VOD, IPS and sepsis
Kaiser *et al.*[41]	16 polypeptides from urine found by CE-MS	Sensitivity 100% Specificity 82% (training set only)	Small number of HCT patients ($n = 35$) No validation set
Weissenger *et al.*[42]	31 polypeptides from urine found by CE-MS	Sensitivity 83.1% Specifivity 75.6%	Difficult to identify individual peptides Complicated technique
Srinivasan *et al.*[53]	8 ions with distinct *M/Z* ratios	Sensitivity 100% Specificity 100%	Small sample size Excluded patients with other complications

Abbreviations: CE-MS = capillary electropheresis mass spectrometry; HGF = hepatocyte growth factor; TNFR = TNF receptor; VOD = veno-occlusive disease.

were prospectively collected from patients who enrolled on BMT CTN 0302, a multicenter, randomized, four-arm phase II clinical trial studying initial therapy for acute GVHD.[45] Samples were collected at treatment initiation (day 0 of the trial), day 14 and 28 of treatment. Logistic regression analysis was then used to create biomarker panels for each time point, and results showed that measurements at the time of GVHD onset could predict for both death by day 180 and treatment failure at day 28.[46] In addition, investigators from France recently reported in a smaller study on the prognostic value of measuring fecal concentrations of calprotectin and α1-antitrypsin at diagnosis of intestinal GVHD.[47] Although not being able to distinguish between other GI maladies, these widely available stool protein assays can potentially assist in stratifying risk. Further validation in different populations is required, but progress is certainly being made towards a risk-adapted clinical trial for the initial therapy of acute GVHD (see Figure 1a).

Developing a strategy for pre-emptive intervention against acute GVHD is more difficult to achieve, given the inherent treatment of some patients who would not have developed disease. In 1999, Bacigalupo *et al.*[48] used a clinical scoring system, measured at day +7 after HCT, to predict risk of both acute GVHD and TRM. A pilot study in 18 patients followed, which treated high-risk patients with pre-emptive anti-thymocyte globulin,[49] and this clinical scoring system was then refined to include day +7 serum levels of BUN, cholinesterase, total proteins, gamma glutamyl transferase, along with donor type and cell dose.[50] Subsequently, a large prospective trial was conducted, randomizing intermediate and high-risk patients to either no treatment or pre-emptive anti-thymocyte globulin therapy. Results showed that pre-emptive treatment with anti-thymocyte globulin did appear to lower the risk of acute GVHD in high-risk patients, but did not have a significant effect on transplant-related mortality or overall survival.[51] A similarly designed trial could be conducted using biomarkers measured at a certain time point after HCT (see Figure 1b). If the panel of biomarkers used includes biomarkers that are important in the pathophysiology of acute GVHD, such pre-emptive therapy can potentially be more specifically targeted.

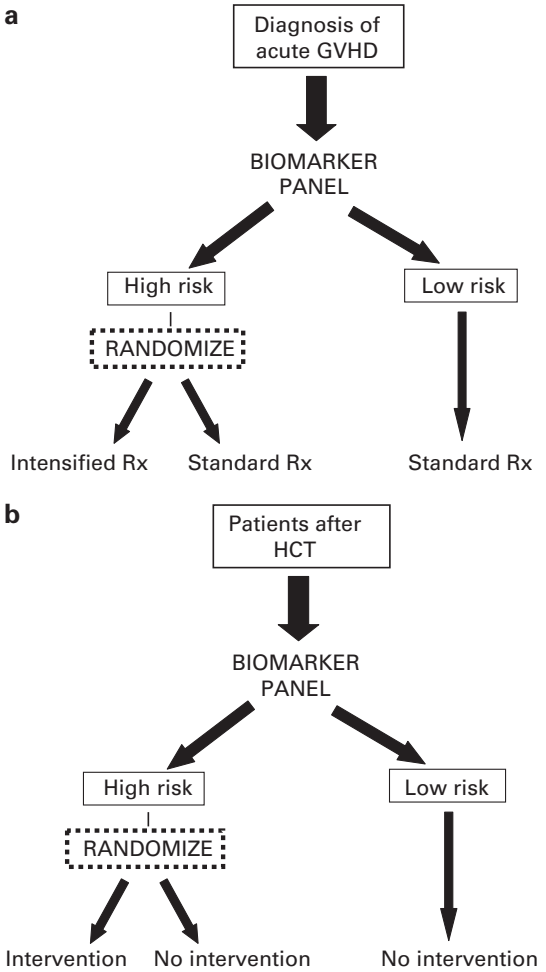

Figure 1. Suggested future risk-adapted clinical trial designs. (**a**) Risk-stratified approach for patients with newly diagnosed acute GVHD. (**b**) Risk-stratified approach for the pre-emptive treatment to prevent acute GVHD.

FUTURE CONSIDERATIONS

In looking to the future, one significant obstacle will be continued advances in allogeneic HCT, including the use of alternative donor stem cell sources, novel conditioning regimens, newer combinations for GVHD prophylaxis and improvements in supportive care. Clearly, the complex immunological environment after allogeneic HCT can be influenced by all of these factors, and it is unclear if biomarkers identified in one setting will accurately translate to another. Indeed, a report by Mohty *et al.*[52] on 113 patients undergoing reduced intensity HCT analyzed a panel of cytokines and found an association with acute GVHD only with serum IL-12p70 levels, suggesting that much of the cytokine elevations reported in past studies could be at least partly attributed to myeloablative conditioning.

Clearly, large collaborative studies with many patients from multiple centers will be needed to validate any biomarker or more

likely, a panel of biomarkers. As stated above, the ideal biomarker panel would not only be specific for diagnosis but also predictive and responsive to therapy. Studying these biomarkers in other inflammatory settings, such as cases of autologous or syngeneic GVHD, will also be interesting to see if they are truly 'allo-specific'. Once such a panel is agreed upon, risk-adapted clinical trials can be designed for both prophylaxis and treatment, with the hope of being able to prevent or modify acute GVHD in patients destined to experience significant morbidity. Furthermore, with a therapy-responsive set of biomarkers, a clinical trial designed to manage immunosuppression with guidance from biomarkers can potentially be conducted as well. Lastly, active investigation should continue in the search for new candidate biomarkers. Current technology has allowed us to rapidly screen large numbers of patient samples, and many centers have had the foresight to collect and store these samples, giving valuable material to be used for future analysis.

Unfortunately, even with the significant progress made in the last few years, no noninvasive biomarker is ready for clinical use. Although some candidate biomarker assays are available commercially, these tests have not been definitively validated in clinical trials and have not yet been proven to be useful in guiding clinical decisions. In the near future, strong efforts should be made to collaborate in multicenter efforts to agree on a consensus panel of biomarkers and conduct the clinical trial strategies suggested in Figures 1a and b.

CONCLUSION

In conclusion, many groups have thus far attempted to identify candidate biomarkers for acute GVHD. As shown above, many single-center small retrospective series have proposed multiple potential circulating or cell-surface biomarkers, but most of these candidate biomarkers are limited by inadequate specificity for GVHD relative to other significant complications after HCT. These biomarkers fall into three general categories: (1) markers of generalized inflammation (such as IL-2 and TNF-α), (2) lymphocyte surface molecules (such as CD30, $\alpha4\beta7$ integin) and (3) products secreted when end organs are damaged (such as elafin, reg3α and cytokeratin 18 fragments). The historical efforts, including those studying IL-2 and TNF-α, yielded valuable insights into understanding the pathophysiology of acute GVHD and led to the development of several novel therapies. Such discovery continues in the form of clinical trials targeting new molecules, such as $\alpha4\beta7$ integrin and CD30, and others. Newer methods such as modern proteomic techniques have allowed the identification of several organ-specific candidate biomarkers, including elafin for skin GVHD and reg3α for intestinal GVHD. Neither of these molecules appears to be involved in the pathophysiology of GVHD, but, rather, are molecules secreted as a result of end-organ damage. Incorporation of elafin and reg3α with traditional cytokine markers into a biomarker panel has recently been shown to correlate with prognosis. The ultimate goals of identifying specific biomarkers for acute GVHD are to refine diagnosis and guide therapy in order to improve outcomes for our patients undergoing allogeneic HCT. With the progress we have made thus far, and the continued efforts of ongoing investigation, these goals are not far from being realized.

CONFLICT OF INTEREST

Dr Y-B Chen has received funding for clinical trials from Millennium Pharmaceuticals, Inc. and has served as a consultant for and received funding for clinical trials from Seattle Genetics.

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