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Toward Biomarkers for Chronic Graft-versus-Host Disease: National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in Chronic Graft-versus-Host Disease: III. Biomarker Working Group Report

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ABSTRACT

Biology-based markers that can be used to confirm the diagnosis of chronic graft-versus-host disease (GVHD) or monitor progression of the disease could help in the evaluation of new therapies. Biomarkers have been defined as any characteristic that is objectively measured and evaluated as an indicator of a normal biologic or pathogenic process, a pharmacologic response to a therapeutic intervention, or a surrogate end point intended to substitute for a clinical end point. The following applications of biomarkers could be useful in chronic GVHD clinical trials or management: (1) predicting response to therapy; (2) measuring disease activity and distinguishing irreversible damage from continued disease activity; (3) predicting the risk of developing chronic GVHD; (4) diagnosing chronic GVHD: (5) predicting the prognosis of chronic GVHD; (6) evaluating the balance between GVHD and graft-versus-leukemia effects (graft-versus-leukemia or GVT); and (7) serving as a surrogate end point for therapeutic response. Such biomarkers can be identified by either hypothesis-driven testing or by high-throughput discovery-based methods. To date, no validated biomarkers have been established for chronic GVHD, although several candidate biomarkers have been identified from limited hypothesis-driven studies. Both approaches have

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merit and should be pursued. The consistent treatment and standardized documentation needed to support biomarker studies are most likely to be satisfied in prospective clinical trials.

KEY WORDS
Graft-versus-host disease • Biomarkers

BACKGROUND

The approach currently used to establish the diagnosis of chronic graft-versus-host disease (GVHD) depends almost exclusively on the clinical history, physical examination, and histopathologic confirmation. This approach is not fully informative in predicting the severity of the disease, response to therapy, or survival, and is not adequate to distinguish disease activity from irreversible tissue damage during treatment. These limitations may be best illustrated by the difficulty of designing appropriate inclusion criteria for clinical trials to treat chronic GVHD. Improvements could come from biologically based indicators or markers that could be used together with or in place of standard clinical and histologic criteria for diagnosing chronic GVHD, or for predicting or evaluating the response to therapy.

PURPOSE OF THIS DOCUMENT

The purpose of this document is to identify an approach for the identification, validation, and application of biomarkers for chronic GVHD. Toward this goal, this document provides: (1) definitions of biomarkers and their applications; (2) a proposed classification of biomarkers by pathophysiological process-specific pathways in chronic GVHD; (3) methodologic considerations in the identification and measurement of biomarkers for chronic GVHD; (4) current applications of biomarkers in chronic GVHD, and (5) recommendations for validation studies.

SUMMARY OF RECOMMENDATIONS

The Biomarkers Working Group made the following recommendations.

1. *A multidisciplinary, coordinated approach to the identification, validation, and application of biomarkers should be supported, particularly within the context of clinical therapeutic trials.*
2. *Both hypothesis-driven and discovery-based approaches for identification of chronic GVHD biomarkers should be pursued simultaneously.*
3. *Chronic GVHD clinical therapeutic trials should include correlative biologic studies focused on the identification, validation, and application of biomarkers whenever possible.*
4. *Samples from well-documented cases with and without chronic GVHD should be stored to create a resource for future biomarker studies.*
5. *Once chronic GVHD biomarkers have been validated, selected biomarkers should be tested for use as surrogate end points in clinical trials.*

DEFINITIONS OF BIOMARKERS AND THEIR APPLICATIONS

Biomarkers have been defined as characteristics that are objectively measured and evaluated as indicators of normal biologic or pathogenic processes, pharmacologic responses to a therapeutic intervention, or as a surrogate end point intended to substitute for a clinical end point. As a secondary goal, biomarkers could be used to elucidate the biologic mechanisms of a disease. For the purposes of this document, certain evaluations that are routinely performed to determine the diagnosis of chronic GVHD or to assess the severity of the disease were not considered as biomarkers. Examples of such evaluations include skin, liver, and intestinal biopsies, pulmonary functions tests, high-resolution computed tomography scans, performance scores, and Schirmer's tests.

Biomarkers could be used for a variety of purposes.

1. Predict response to therapy. *For example, a biomarker could be developed to guide the choice of treatment.*
2. Measure disease activity and distinguish irreversible damage from continued disease activity. *For example, a biomarker could identify changes that may improve with therapy, as distinguished from those that will not.*
3. Predict risk of developing chronic GVHD. *For example, gene polymorphisms in either the donor or recipient may be associated with risk of chronic GVHD.*
4. Diagnose chronic GVHD. *For example, a biomarker could be used together with clinical criteria to determine eligibility for a clinical trial.*
5. Assess prognosis or establish staging of chronic GVHD. *For example, a biomarker could be used to determine the risk category or to guide decisions about the need for treatment.*
6. Evaluate GVHD versus graft-versus-leukemia (GVL) or graft-versus-tumor (GVT) effect. *Biomarkers could also be used to assess the GVL or GVT response in patients who have hematopoietic cell transplantation to treat malignancy.*

Biomarkers that could be used to predict response to treatment, measure disease activity, or distinguish

reversible disease activity from irreversible damage would have very high clinical use, because currently available clinical tools are not adequate for these purposes. On the other hand, biomarkers that could predict risk of developing chronic GVHD would likely have lower use, because interventions that could be used to change the risk of chronic GVHD are not currently available. Biomarkers that could be used to diagnose chronic GVHD would have lower use, because very good clinical tools have already been established for this purpose. Similarly, biomarkers that could assess the prognosis of chronic GVHD would have limited use, because clinical indicators of nonrelapse mortality have already been established. Lastly, biomarkers that could weigh GVHD versus GVL effects (GVL or GVT) would have limited use, because the relative clinical threats of chronic GVHD and recurrent malignancy can be reasonably well assessed through the use of currently available clinical indicators.

Clinical end points are of greatest relevance in establishing relevant measures of a therapeutic response. These end points assess how a patient feels, functions, or survives. A surrogate end point is expected to predict clinical benefit (or harm or lack of benefit) based on epidemiologic, therapeutic, pathophysiologic, or other scientific evidence [1]. Biomarkers can be used as surrogate end points in clinical trials only after extensive validation. After such validation, surrogate end points can be used to indicate therapeutic response before a clinical response has occurred and could be very useful for regulatory review [2].

PROPOSED CLASSIFICATION OF BIOMARKERS BY PATHOLOGIC PROCESS-SPECIFIC PATHWAYS IN CHRONIC GVHD

Numerous studies have been carried out to evaluate biomarkers for correlation with the presence or absence of chronic GVHD. These biomarkers can be categorized according to processes or mechanisms by which they are thought to contribute to the pathogenesis of chronic GVHD (Table 1), and in many cases, they correlate either qualitatively or quantitatively in one way or another with chronic GVHD activity.

Allogeneic Disparity

HLA antigen mismatching is associated with an increased risk of chronic GVHD [3]. Other biomarkers for allogeneic disparity include nonsynonymous single nucleotide polymorphisms in genes encoding minor histocompatibility antigens that differ between the donor and recipient. This hypothesis is supported by observations that the risk of chronic GVHD is increased when the donor and recipient have different racial or ethnic origins [4]. It is also possible that individual HLA antigen molecules reduce (or increase) the risk of chronic GVHD by preferentially presenting a selected repertoire of minor histocompatibility antigens [5]. Lastly, the association of chronic GVHD with increased numbers of activated B cells and anti-HLA antigen antibodies highlight the role of allogeneic disparity in the pathogenesis of chronic GVHD [6].

Direct Allogeneic Immune Responses Against Specific Antigens

Evaluations of direct allogeneic immune responses have not demonstrated strong correlation between the number of minor histocompatibility antigen-specific T cells and the presence of chronic GVHD [7,8]. In general, the antigens recognized by these donor-derived recipient-reactive T-cell populations are poorly characterized, and the response of donor T cells against recipient alloantigens appears to be highly heterogeneous and polyclonal. The best characterized minor histocompatibility antigens presented by major histocompatibility complex class I molecules are encoded on the Y chromosome. These H-Y antigens have been invoked to explain why the incidence of chronic GVHD is higher with the use of female donors for male recipients than with other donor and recipient sex combinations. Higher frequencies of T cells recognizing H-Y antigens presented by HLA-A2 and HLA-B7 molecules correlate with the presence of chronic GVHD, and the number of H-Y reactive T cells decreases after response to GVHD therapy [9]. HA-1 is another antigen that may be recognized in the development of chronic GVHD. Results of one study showed that HA-1–reactive T cells are present in patients with GVHD and that their frequencies decrease as GVHD improves during therapy [9]. Subsequent studies have shown a correlation of HA-1 mismatch with risk of acute GVHD but not with chronic GVHD [10]. The low frequency of HA-1 disparity makes it difficult to test associations with clinical outcome [11]. Results of another study showed that recipient mismatching for CD31 at all 3 codons 125, 563, and 670 was associated with an increased risk of acute GVHD and with a trend suggesting an increased risk of chronic GVHD [12]. Results of these studies need to be confirmed.

A B-cell response to H-Y minor histocompatibility antigens correlates with the development of chronic GVHD. Increased titers of antibodies reactive with donor and H-Y antigen have been shown to be present in patients with chronic GVHD [13,14]. Although the anti-H-Y antibody response might be promising as a marker of chronic GVHD, this response can occur only in male patients who have female donors.

Table 1. *Proposed Classification of Biomarkers Based on Process-Specific Mechanisms in Chronic Graft-versus-Host Disease*

Type of Response	Examples	Methods of Measurement	Comments
Allogeneic disparity [3-8]	HLA-A, B, DR	HLA typing, incompatibility SNPs	HLA typing well established as a prognostic marker for development of chronic GVHD
Direct allogeneic immune response measurements [7-17]	Immunity against minor histocompatibility antigens (MiHA) (e.g. H-Y, HA-1) nonspecific autoantibodies (ASMA, ANA)	MiHA-reactive antibodies (ELISA) MiHA reactive T cells and B cells (ELISPOT, proliferation assays, tetramer)	Only indirect evidence for H-Y antigen involvement No established correlation between chronic GVHD and any other HA
Inflammatory responses [18-36]	Th1/Tc1 and Th2/Tc2 DC1 and DC2 Eosinophils CpG response (TLR9) NOD2 polymorphism (TLR2)	Immunophenotyping, Stimulation assays with measurement of cytokine production, SNP for cytokine polymorphisms Stimulation assays, SNP, RT-PCR	Chronic GVHD may involve Th1- and Th2-driven pathology. Cytokine polymorphism may be predictive of development of chronic GVHD Early unpublished correlation of CpG ODN response and NOD2/CARD15 polymorphism.
Regulatory immune cell populations [37-54]	Regulatory T cells (Treg) Antigen-presenting cells (B cells)	Immunophenotyping, PCR for Foxp3, functional studies evaluating regulatory T cells	The role of Treg cells at present is controversial, as different studies have shown opposing results The response of chronic GVHD to rituximab therapy suggests a role for B cells outside antibody production.
Immune-modulatory consequences of chronic GVHD or therapy [31,55-59]	TREC and TCR Vβ repertoire analyses, and chimerism (T and B cells, DCs), Platelet counts, salivary IgA	Immunophenotyping chimerism studies	Single center studies at present
Nonimmune chronic GVHD biomarkers [28]	Von Willebrand factor and thrombomodulin	Standard laboratory	Only single center observation

HLA indicates human leukocyte antigens; GVHD, graft-versus-host disease; TREC, T cell restriction excision circles; TCR, T cell receptor; ELISA, enzyme-linked immunosorbent assay; MiHA, minor histocompatibility antigens; ELISpot, enzyme-linked immunospot; RT-PCR, reverse transcriptase polymerase chain reaction; CpG ODN, CpG oligodeoxynucleotide; Treg, regulatory T cells; DC1, type 1 dendritic cells.

Some groups have also evaluated T-cell clonality as a marker for chronic GVHD. Clonal T cells have been isolated from patients with myositis resulting from chronic GVHD [15]. Others have shown that clinical improvement during photopheresis occurred more frequently among patients with clonal T-cell receptor-γ rearrangements than among those who did not have such rearrangements [16]. The interpretation of these studies remains uncertain, because many patients without chronic GVHD have clonal expansions of T cells as part of the typical pattern of immune reconstitution after hematopoietic cell transplantation [17].

Inflammatory Responses

Chronic GVHD has been associated with alloreactive helper and cytotoxic T cells, nonspecific suppressor cells, tumor necrosis factor-α–secreting macrophages, and autoreactive T cells [18,19]. In human beings, chronic GVHD may involve an alteration in the balance of helper T cell type 1/cytotoxic T cell type 1 (Th1/Tc1) and helper T cell type 2/cytotoxic T cell type 2 (Th2/Tc2) populations, with a predominant Th1/Tc1 response characterized by an aberrant pattern of interferon (IFN)-γ production without interleukin (IL)-2 production. Chronic GVHD has been associated with increased proportions of $CD8^+$ cells lacking CD28 [20] and a decreased proportion of natural killer ($CD3^-/CD16^+/CD56^+$) cells in the blood [20]. Cytokines also play a role in certain disease manifestations. Sclerosis has been associated with high concentrations of transforming growth factor-β, whereas fatigue and wasting have been associated with high concentrations tumor necrosis factor-α, and im-

munodeficiency has been associated with high concentrations of IL-10 and transforming growth factor-β [21].

Reduced expression of IL-10 and increased IFN-γ production has been described in patients with chronic GVHD as compared with healthy individuals and patients without GVHD, suggesting a more prominent role for Th1 cytokines in human chronic GVHD than in murine chronic GVHD [22]. These findings are further supported by the increased expression of IFN-γ detected in skin biopsy specimens from patients with cutaneous chronic GVHD [23]. Transcription of the Th2 cytokines IL-4 and IL-5 was not detected in these skin samples, and IL-10 expression was not significantly altered as compared with control subjects. In a separate study, however, Con A-stimulated T-cell production of IFN-γ was decreased in patients with chronic GVHD [24]. The consistent presence of autoantibodies against cytoskeletal proteins (tubulin, actin, myosin) indirectly supports a role for donor Th2 cells in human chronic GVHD [25]. Overall, it appears that chronic GVHD can present with either Th1/Tc1 or Th2/Tc2 predominance, and it is likely that predominance of one or the other may produce different clinical manifestations.

$CD4^+$ T cells can be divided into 3 classes: (1) naive ($CD45RA^+/CCR7^+/CD62L^{hi}$); (2) central memory ($CD45RA^-/CCR7^+/CD62L^{hi}$); and (3) effector memory ($CD45RA^-/CCR7^-/CD62L^{low}$). Whereas central memory $CD4^+$ T cells can have either Th1 or Th2 profiles, effector memory $CD4^+$ T cells produce high levels of IFN-γ, IL-4, and IL-5, and they produce moderate levels of IL-2 [26]. Severe chronic GVHD is associated with a preponderance of $CD4^+$ effector memory cells as opposed to central memory cells [27].

$CD8^+$ T cells appear to be important effectors in chronic GVHD. $CD8^+$ T cells infiltrates correlate with microvessel loss in the skin [28], and increased cytolytic $CD8^+$ T cells are found in intestinal biopsy specimens from patients with chronic GVHD [29]. Clinical improvement during extracorporeal photopheresis has been associated with decreased numbers of $CD8^+$ T cells and increased numbers of $CD4^+$ T cells in patients with chronic GVHD, but the changes were not striking [30]. The onset of chronic GVHD has been associated with T-cell activation, as evidenced by OX40 expression on $CD4^+$ and $CD8^+$ T cells, and clinical response to therapy has been associated with decreased T-cell expression of OX40 [31].

Polymorphisms of donor and recipient IL-1 and IL-6 genes have been associated with the incidence and severity of chronic GVHD [32]. The donor IL-6-174GG-homozygous genotype causes increased IL-6 production and has been associated with increased incidence and severity of chronic GVHD. The donor tumor necrosis factor receptor type II 169RR-homozygous genotype has been associated with an increased risk of chronic GVHD [33]. An increased risk of chronic GVHD has also been associated with the recipient IL-10 GG-homozygous genotype and with the recipient IL-1Ra polymorphism, IL1RN*2 [34,35].

Regulatory Immune Populations in Chronic GVHD

Both high and low levels of regulatory T-cell populations have been correlated with the presence of chronic GVHD [36-41]. Conflicting results might be resolved through studies of Foxp3 expression, a transcription factor that governs the development and function of regulatory T cells.

The role of plasmacytoid (IFN-α–producing) dendritic cells (DCs) in chronic GVHD remains unclear. Increased numbers of donor- or host-derived plasmacytoid DCs in the blood have been associated with the presence of chronic GVHD in human beings [4,42]. Other studies, however, have shown that grafts containing higher numbers of plasmacytoid DCs were associated with a lower incidence of chronic GVHD and a higher risk of recurrent leukemia [43]. The increased incidence of chronic GVHD observed after granulocyte colony-stimulating factor–stimulated blood cell transplantation could be caused by a paucity of plasmacytoid DCs [44].

B cells may facilitate the development of chronic GVHD through their ability to present alloantigens and produce autoantibodies. The appearance of TLR9-positive activated B cells has been associated with the development of chronic GVHD [45] and with increased concentrations of IgG in the blood [46,47]. Contrasting data have shown that decreased B-cell lymphopoiesis and decreased numbers of B-cell precursors in the blood of patients correlate with established chronic GVHD [48] and with a decrease in the numbers of IgA producing plasma cells in the marrow [49]. Anti-CD20 monoclonal antibody has also been reported to be effective for treatment of chronic GVHD [50,51]. Chronic GVHD in human beings is associated with autoantibody-mediated diseases such as myasthenia gravis, but the mechanisms that account for autoantibody production remain obscure. Autoantibodies associated with chronic GVHD can have a variety of specificities, including antinuclear antibody, rheumatoid factor, antimitochondrial antibody, Coombs antibody, antismooth muscle antibody, anticardiolipin antibody, platelet antibodies, and antineutrophil antibodies [46,47,52,53].

Immune-Modulatory Consequences of Chronic GVHD

T cells from patients with chronic GVHD are poorly responsive to mitogen and alloantigen stimu-

lation, suggesting the presence of defects in T-cell activation pathways [54,55]. Some investigators have suggested that the effector cells stimulated by recipient alloantigens can be identified by their constitutive expression of "activation" markers, including CD69, IL-2R, Fas receptor (CD95), and Fas ligand [31,55-57]. Autoimmune mechanisms are thought to account for at least some of the clinical manifestations of chronic GVHD. Autoimmunity could reflect impaired deletion of autoreactive T cells in the thymus and altered presentation of cryptic antigens caused by increased production of IL-6 [58].

Nonimmune Chronic GVHD Biomarkers

Localized nonimmune biomarkers of chronic GVHD such as Von Willebrand factor and thrombomodulin have also been identified as correlating with endothelial damage [28].

METHODOLOGIC CONSIDERATIONS IN THE IDENTIFICATION AND MEASUREMENT OF BIOMARKERS FOR CHRONIC GVHD

Prospective collection of samples for evaluation of either an existing set of hypothesis or discovery-based studies or as a resource for future studies is strongly encouraged. In the following section, we discuss the types of tissues that may be collected, potential confounding factors, and recommendations around sample acquisition.

Tissue Location of Markers

Although blood-based biomarkers have received the most attention, tissue-based biomarkers should not be overlooked. For example, cytokine expression in skin biopsy specimens [24] and increased numbers of eosinophils in gut biopsy specimens [59] have been associated with chronic GVHD. Tissue eosinophilia has been associated with increased severity of chronic GVHD and with eosinophilia in the blood [60]. Mast cells may help to direct T-cell trafficking and activation in the microvasculature of patients with chronic GVHD [61].

GVHD causes inflammation of salivary gland ducts and increased epithelial permeability in salivary glands. These changes increase the concentrations of albumin, IgG, lactoferrin, and electrolytes in saliva samples from patients with chronic GVHD [62-65]. One study found 74% sensitivity and a 91% predictive value for associations between salivary Na^+ concentrations and histopathologic findings of chronic GVHD in salivary glands [62]. Such salivary changes are also reversible with decreased inflammation [64], suggesting that these measures could be useful in monitoring the response to therapy. Oral manifestations of chronic GVHD have been correlated with the presence of GVHD manifestations affecting the eye, liver, and skin, suggesting that saliva-based tests could be used as a general marker of disease activity [65].

Potential Confounding Factors

A variety of confounding factors limit the ability to interpret results of many previous biomarker studies. These include the onset time of GVHD after transplantation, the age of the recipient, the type of graft (peripheral blood, bone marrow, or umbilical cord blood), treatment of the donor with granulocyte colony-stimulating factor, the type and intensity of immune suppressive treatment (steroids, calcineurin inhibitor, mammalian target of rapamycin (mTOR) inhibitor), the presence of infections, and lingering effects of acute GVHD. The interpretation of immune-related biomarkers must also account for time from transplantation, because immune reconstitution occurs gradually, even in the absence of chronic GVHD.

Recommendations for Sample Acquisition

Samples should be banked for future studies with a link to patient data regarding diagnosis of chronic GVHD, GVHD histology, and response to GVHD therapy as outlined in other working group reports in a manner that complies with regulations for disclosure of protected health information. We recommend that donor and patient samples be obtained before transplantation. Additional patient samples should be obtained at the onset of chronic GVHD and whenever the patient is assessed for response after initiation of treatment. Because the immune environment changes with posttransplantation immune reconstitution, time-matched samples should also be obtained from patients who do not have GVHD. In the absence of chronic GVHD, samples should be obtained at 3, 6, 9, 12, and 24 months after transplantation. Ideally, DNA, RNA, cells, plasma, and serum from blood and other sources such as urine, saliva, bronchoalveolar lavage fluid, and material from tissue biopsy specimens (e.g., skin, oral mucosa, liver, lung) would be collected. More realistically, minimum requirements would focus on collection of samples at 3, 6, and 12 months and liquid nitrogen storage of viable peripheral blood cells and serum or plasma collected from the same sample.

Handling of specimens for centralized testing and differences in local processing procedures can confound the assessment of biomarkers, because many biologic assays may be affected by processing, transport and storage conditions, and time delay. Standard procedures for the collection, processing, storage, and transport of samples should be agreed upon widely among chronic GVHD centers. Studies to document assay sensitivity to these variables should be per-

formed before centralized testing begins. Finally, permission to use samples in future studies and to exchange materials with other institutions should be embedded in approved consent documents, thereby allowing such studies to be conducted in the future without the need for explicit re-consenting of patients.

CURRENT APPLICATIONS OF BIOMARKERS IN CHRONIC GVHD

A limited number of biomarkers have been evaluated in hypothesis-driven testing for specific clinical applications (Table 2). The data have come primarily from a single center or from a small number of centers, and in most cases, the findings have not been tested as part of large multicenter trials.

1. Predict response to therapy. *Although some biomarkers have shown correlations with response during treatment of chronic GVHD [66] no marker has been identified as a predictor of response.*
2. Measure disease activity and distinguish irreversible damage from continued disease activity. *As discussed above, a variety of markers correlate in one way or another with disease activity, but they have not been used to distinguish irreversible damage from reversible disease activity. Specific tissue markers may be of particular value for this application.*
3. Predict risk for developing chronic GVHD. *Preliminary results suggest that polymorphisms of the IL-10 promoter and NOD2/CARD15 [67] could have use for predicting risk of chronic GVHD.*
4. Diagnose chronic GVHD. *Several markers have the potential to assist in the diagnosis of chronic GVHD, but the sensitivity and specificity of these biomarkers in the diagnosis of chronic GVHD has received only limited attention. These include: (a) platelet, absolute lymphocyte, and eosinophil counts; (b) serum bilirubin, creatinine kinase, and aldolase (myositis) concentrations; (c) serum immunoglobulin and salivary IgA concentrations; (d) autoantibodies including antinuclear, anticardiolipin, antimitochondrial, antismooth muscle, and anti-acetylcholine receptor. Other markers that still require confirmation in larger studies include: (a) assessment of H-Y reactive antibodies and B cells; (b) B-cell response to CpG ODN; and (c) the presence or absence of regulatory cells.*
5. Assess prognosis of chronic GVHD. *The only established biomarker for prognosis of chronic GVHD is platelet count.*
6. Evaluate GVHD versus GVL effect (GVL or GVT). *Currently the only marker consistently associated with an increased risk of recurrent malignancy is the persistence of recipient immune cell populations (T cell, B cell, and DC) after the transplantation.*
7. Act as surrogate end points for therapeutic response. *No biomarkers have received sufficient study and validation to justify their use as surrogate end points.*

Table 2. *Biomarker Candidates for Application in Chronic Graft-versus-Host Disease*

Marker Application	Predict Response to Therapy	Distinguish Reversible versus Irreversible	Predicts Risk of Developing cGVHD	Diagnosis	Prognosis or Staging	Evaluate for Graft-versus-Leukemia	Surrogate End point
Potential Biomarker	H-Y antibody	None known	a. Allogeneic disparity b. Polymorphism for 1. NOD2/CARD15 2. IL-10	a. Platelet count eosinophil count. b. Bili, CPK, aldolase c. Serum Igs, salivary IgA d. Autoantibody (ANA, ACA, AMA, ASMA, Anti-acetyl cholinesterase antibody e. H-Y antibodies f. CpG response B cells	Platelet count	None known	None established

Biomarkers can be identified both through hypothesis-driven and discovery-based methods. To date, virtually all studies of chronic GVHD have used a hypothesis-driven approach with a small number of candidate biomarkers. Discovery-based methods with the use of high-throughput methods have not yet been used to identify biomarkers for chronic GVHD. High-throughput methods using microarray-based or proteinomic-based assays could be used to discover new DNA, RNA, or protein biomarkers for chronic GVHD. Such studies could use both structured and unstructured statistical evaluations for correlation with the intended clinical application. The choice among DNA, RNA, or protein biomarkers depends on the specific application under development. For example, DNA-based assays should be perfectly adequate for correlations with risk of GVHD, but they would not be useful as measures of disease activity. The use of RNA-based assays depends on the availability of specimens that contain cells expressing the biomarker of interest. For instance, RNA from blood cells could provide informative immunologic biomarkers, to the extent that blood cells participate in the pathogenesis of chronic GVHD. Similar considerations apply for protein-based assays.

RECOMMENDATIONS FOR VALIDATION STUDIES

Most of the listed biomarkers are derived from single center or single laboratory evaluation and have not been validated. Validation of chronic GVHD markers requires comparison of the biomarker with an accepted gold standard for a specific application. Several reasons account for the lack of validation of results from previous studies. Testing may require relatively difficult laboratory-specific techniques or reflect the specific interests of investigators. Techniques for testing may differ among centers, because there is no forum for development of standardized biomarkers assays. For example, the lack of standardized assays has made it very difficult to interpret the results of assays that measure T-regulatory cells. The recent experience of the National Institutes of Health–funded Immune Tolerance Network exemplifies a successful approach toward standardization of assays in collaborative biomarker studies.

Validation studies require correlations between biomarker assessment and standardized clinical data documenting chronic GVHD diagnosis and response to treatment. Four types of studies will be needed to develop biomarkers in chronic GVHD. The initial discovery step involves immune reconstitution, proteomics, and microarray studies to generate hypotheses. These initial studies are followed by more limited validation studies designed to test specific candidates from the discovery results with material from a limited number of centers. Technical validation is then needed to demonstrate that testing of validated markers can be carried out reliably in a larger number of centers and to confirm the earlier results. The final step involves clinical application and validation in large clinical studies. At this step the biomarker could be used to identify subtypes of chronic GVHD for classification purposes, prognostication, and response to therapy.

It is likely that only large, well-conducted clinical trials will provide the consistency of treatment and standardized documentation needed to support validation studies correlating biomarkers with response to therapy. Single or limited institution observational studies in which standardized diagnostic criteria are used and in which samples are obtained from patients with and without chronic GVHD may be sufficient for initial studies correlating biomarkers with the diagnosis of chronic GVHD. Subsequent validation would require the application of similar diagnostic criteria and testing of the marker in patient populations across several centers. In the absence of a clinical trial, the only gold standard for the diagnosis and response to therapy of chronic GVHD suitable for validation of chronic GVHD biomarkers is a retrospective evaluation of clinical and histologic data for each case, perhaps performed by an expert review group.

Although the ideal biomarker will have both high specificity and high sensitivity with low false-positive and low false-negative rates, some biomarkers may be selected for either high sensitivity or high specificity. As an example in diagnosis, high specificity may be more important than high sensitivity, whereas high sensitivity may be more important as a response biomarker once the diagnosis of chronic GVHD has been established. Multivariate analyses should be considered to identify potential combinations of biomarkers and clinical characteristics that may increase specificity. Expert statistical design and analysis is essential for this type of study. Suggested statistical criteria are included in Appendix 1.

In conclusion, much work will be required both to validate candidate biomarkers from previous studies and to implement high-throughput methods with appropriately collected specimens for future discovery-bases approaches. Close coordination between multispecialty clinical and laboratory-based groups will be needed to pursue such studies. Identification and validation of biomarkers will greatly assist in the evaluation of new approaches for treatment of chronic GVHD.

NATIONAL INSTITUTES OF HEALTH CONSENSUS DEVELOPMENT PROJECT ON CRITERIA FOR CLINICAL TRIALS IN CHRONIC GVHD STEERING COMMITTEE

Steven Pavletic and Georgia Vogelsang (project chairs); LeeAnn Jensen (planning committee chair);

Lisa Filipovich (diagnosis and staging); Howard Shulman (histopathology); Kirk Schultz (biomarkers); Dan Couriel (ancillary and supportive care); Stephanie Lee (design of clinical trials); James Ferrara, Mary Flowers, Jean Henslee-Downey, Paul Martin, Barbara Mittleman, Shiv Prasad, Donna Przepiorka, Douglas Rizzo, Daniel Weisdorf, and Roy Wu (members). The project group also recognizes contributions of numerous colleagues in the field of blood and marrow transplantation, medical specialists and consultants, the pharmaceutical industry, National Institutes of Health, and US Food and Drug Administration professional staff for their intellectual input, dedication, and enthusiasm on the road to completion of these documents.

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APPENDIX 1: STATISTICAL CONSIDERATIONS FOR VALIDATION OF BIOMARKERS FOR CHRONIC GVHD

In validating a biomarker, we specify the conditions of measurement. These include the time of day the sample is taken (unless known to be unimportant), the observer/person doing the measurement, conditions known to affect the measurement, type of automated instrument, and characteristics of the subject being measured.

Reliability refers to:

- measurements are measuring what we think they are measuring (face validity).
- the measurement is repeatable over time (when time is short enough that we do not expect a change in the measurement).
- the measurement is repeatable over evaluations (whether an instrument or a person—and the technique of collection, storage, transport may be important).
- variability of the measurement is small enough for useful inference to be made.

The variation in a measurement consists of variation caused by the subject (V_s), variation caused by instrument, evaluator, age, sex, etc. We denote the sum of the measurement errors as V_e. We define reliability as:

$$\frac{V_s}{V_s + V_e}.$$

This is related to the intraclass correlation coefficient (ICC). We estimate these quantities from the expected values of the sums of squares from an analysis of variance table. Other quantities that are used in reliability evaluation are the coefficient of variation (ratio of the SD to the mean expressed as a percent). Typically, we want reliability to be "high"–0.8 is excellent, 0.6 to 0.8 is substantial, 0.4 to 0.6 is moderate, 0.2 to 0.4 is fair. Our recommendations are that biomarkers for chronic GVHD should be selected with an ICC of greater than 0.6.

We determine *face validity* by noting how well the measure agrees with an attribute as determined by experienced individuals. In many situations, the attribute determined by experienced individuals is categorical. In this case, we measure the validity by the agreement between the categories predicted by the measurement and those determined by consensus. This could be a χ^2 statistic or a measure of association (gamma, Spearman, or Kendall correlation).

Content validity is defined as how the biomarker measures a variety of aspects of the disease. Because many biomarkers are quite specific in their measurement aim, this concept may be less useful for biomarkers. However, if one is attempting to obtain a global measure of disease activity or improvement, we can use a combination of biomarkers and then this concept will be helpful.

Construct validity measures the association of one biomarker with other measures most likely based on clinical and histologic criteria. We measure this by a nonparametric correlation coefficient. Criterion validity measures the agreement with a gold standard.

We expect a biomarker to address a biologic process. To address face validity the physician or physicians provide an attribute that the biomarker will address. In some cases, this may be difficult or determined by the biomarker itself. These may be (ordered) categorical or continuous attributes. The biomarker need not be in the same scale as the attribute. That is, the biomarker may be continuous, whereas the attribute may be categorical. The agreement can be assessed by various statistical techniques. For example,

if both attribute and biomarker are continuous, regression (possibly multiple to adjust for age, sex, etc.) may be used. If the attribute is an ordered categorical variable and the biomarker is continuous, we often use an ordered logistic regression.

To address the reliability of a measure, the ICC is widely used. In this case, we conduct an analysis of variance and estimate sources of variation from subject, instrument, evaluator, time, etc. Then, we compute the ICC. Note that one can compute a number of ICCs depending on whether we are concerned with subject, instrument, evaluator, or time.

As these studies progress, there will be need to develop prognostic indicators, diagnostic indicators, etc. These will often combine biomarkers and other variables to predict outcomes, diagnoses, etc. Thus, it will be important to determine the key set of biomarkers and to have the full set of key biomarkers for patients and control subjects. Many statistical methods are available to combine variables (including biomarkers) for these purposes. Multiple linear regression is useful to predict continuous outcomes; logistic regression is the widely accepted method for predicting dichotomous outcomes. However, these methods rely on linear relationships between predictor and response. In some cases, there will be nonlinear responses. A common one is a threshold response: there is no response until a variable reaches a certain level. Models such as classification and regression trees have been very useful in this context. These are exploratory analyses that are tailored to the specific problem addressed.

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