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Proteomic Analysis of Saliva from Patients with Oral Chronic Graft-Versus-Host Disease

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A B S T R A C T

Chronic graft-versus-host disease (cGVHD) is an immune-mediated disorder and is the major long-term complication of allogeneic hematopoietic stem cell transplantation (allo-HSCT). The oral mucosa, including the salivary glands, is affected in the majority of patients with cGVHD; however, at present there is only a limited understanding of disease pathobiology. In this study, we performed a quantitative proteomic analysis of saliva pooled from patients with and without oral cGVHD—cGVHD(+) and cGVHD(−), respectively—using isobaric tags for relative and absolute quantification labeling, followed by tandem mass spectrometry. Among 249 salivary proteins identified by tandem mass spectrometry, 82 exhibited altered expression in the oral cGVHD(+) group compared with the cGVHD(−) group. Many of the identified proteins function in innate or acquired immunity, or are associated with tissue maintenance functions, such as proteolysis or the cytoskeleton. Using ELISA immunoassays, we further confirmed that 2 of these proteins, IL-1 receptor antagonist and cystatin B, showed decreased expression in patients with active oral cGVHD ($P < .003$). Receiver operating curve characteristic analysis revealed that these 2 markers were able to distinguish oral cGVHD with a sensitivity of 85% and specificity of 60%, and showed slightly better discrimination in newly diagnosed patients evaluated within 12 months of allo-HSCT (sensitivity, 92%; specificity 73%). In addition to identifying novel potential salivary cGVHD biomarkers, our study demonstrates that there is coordinated regulation of protein families involved in inflammation, antimicrobial defense, and tissue protection in oral cGVHD that also may reflect changes in salivary gland function and damage to the oral mucosa.

INTRODUCTION

Chronic graft-versus-host disease (cGVHD) is a life-threatening immunologic condition that occurs following allogeneic hematopoietic stem cell transplantation (allo-HSCT), affecting 30% to 70% of patients who survive more than 3 months [1-3]. Its presentation may be progressive, arising from acute GVHD that merges into cGVHD, or de novo, with no previous acute GVHD. cGVHD has been characterized as both an alloimmune and autoimmune disease, affecting multiple tissues of the transplant recipient, including the skin, oral mucosa, liver, and eyes [1]. It involves different, predominantly T cell–mediated immunologic mechanisms, including donor-derived alloreactive T lymphocytes, autoreactive T lymphocytes, and dysregulated expression of inflammatory mediators [4]. Although T lymphocytes are the primary mediators of cGVHD, a role for B lymphocytes in cGVHD is suggested by the presence of serum autoantibodies and B cell markers, such as B cell activating factor, as well as recent promising results obtained using drugs that target B cell surface antigens [5].

The oral mucosa is affected in the majority (51% to 63%) of cGVHD patients at the initial diagnosis and is the second most commonly involved tissue after the skin [2]. Common manifestations of oral cGVHD include tissue atrophy, erythema, edema, lichenoid changes, and mucoceles [6,7]. Damage to salivary glands frequently leads to xerostomia, which, together with reduced salivary immunoglobulin production, increases the risk of oral infections [8]. In more severely affected patients, significant pain associated with oral lesions and sclerodermatous changes can lead to fibrosis, which causes trismus (limited mouth opening) and compromised oral function. In addition to the significant morbidity and mortality associated with cGVHD, the disease can mimic other autoimmune or inflammatory conditions, such as scleroderma and lichen planus [1,9]. Thus, biomarkers that can distinguish cGVHD from other clinically similar immune conditions would be very useful diagnostic tools.

Given that the oral mucosa and salivary glands are major target organs of numerous human diseases, salivary proteomics is an appropriate methodology for the molecular profiling of oral-associated diseases, including cGVHD, Sjögren's syndrome, periodontitis, and oral cancer (reviewed in [10]). Saliva represents an ideal starting point for identifying potential cGVHD biomarkers, because changes in the salivary proteome should be directly associated with the localized

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oral pathology. Saliva also is less proteomically complex than serum, which in principle reduces the time and cost required for the analysis of mass spectrometry (MS) data [11]. Whole saliva is composed of fluid including proteins produced by major and minor salivary glands, as well as both secreted and nonsecreted proteins produced by mucosal, periodontal, and immune cells that reside in the mouth. Several previous studies have used MS and immunoassay-based approaches to identify potential oral GVHD markers [7,12,13]; however, to date there is little consensus as to whether any of the identified salivary proteins might be useful for diagnosis or predicting patient outcomes.

In an effort to obtain a global profile of proteomic alterations occurring in oral cGVHD, we used a quantitative MS approach to identify salivary proteins displaying altered expression in patients with active oral cGVHD. We identified 82 salivary proteins that showed quantitative changes in expression in oral cGVHD, and further validated 2 of these proteins using ELISA immunoassays.

MATERIALS AND METHODS

Study Population

Clinical details and demographics of the allo-HSCT study population are presented in Table 1. Patients who had undergone allo-HSCT procedure at the Fred Hutchinson Cancer Research Center/Seattle Cancer Care Alliance were recruited for this study through the Long-Term Follow-Up Program, either at the anniversary visit or during a later appointment required for ongoing treatment of cGVHD [14]. At these appointments, a comprehensive assessment of patients was completed by an attending oncologist, including a computed tomography scan, complete physical examination including evaluation of cGVHD status, and profiling of serum proteins and electrolytes as needed. Global cGVHD severity was scored according to the National Institutes of Health global severity scale [15] on a scale of 0 to 4 for each of 7 organs (mouth, skin, eye, gastrointestinal tract, liver, lung, and joints/fascia; with asymptomatic involvement scored as 0, to severe as 4). All allo-HSCT recipients were in remission at the time of saliva collection.

Oral examinations to assess oral cGVHD were performed at the Oral Medicine clinic at Seattle Cancer Care Alliance. Diagnosis of oral cGVHD was based on mucosal changes, including atrophy of the mucosal surfaces, with loss of normal surface keratinization of the gingiva and dorsal tongue; erythema, especially vascular inflammation; hyperkeratinization, including lichenoid and plaque-like changes; mucoceles, especially on the soft palate and lower labial mucosa; ulcers; and erythema of the parotid duct [6]. The protocol was approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center, and all participants provided informed consent.

For phase 1 of the study, an additional 20 healthy adults were recruited for salivary proteomic analysis through the University of Washington School of Dentistry. These individuals were divided into 2 groups, middle-aged to elderly adults (n = 10), with an average age of 58 years (range, 50 to 68 years), and young adults (n = 10), with an average age of 27 years (range, 21 to 34 years). Each group contained an equal number of males and females. The protocol was approved by the University of Washington's Institutional Review Board.

Saliva Collection and Processing

Unstimulated whole saliva was collected from each consented human subject essentially as described previously [16]. The subject was asked to

Table 1
Clinical Characteristics of Allo-HSCT Recipients Used in Proteomic Studies

Characteristic	Phase 1			Phase 2		
	Oral cGVHD(+) (n = 10)	Oral cGVHD(−) (n = 10)	*P* Value*	Oral cGVHD(+) (n = 36)	Oral cGVHD(−) (n = 10)	*P* Value*
Age, yr, median (range)	55 (38-67)	56.5 (21-63)	.91	42 (25-76)	38.5 (31-68)	.51
Sex, n (%)						
Male	5 (50)	5 (50)	1.0	23 (64)	3 (30)	.077
Female	5 (50)	5 (50)		13 (36)	7 (70)	
Original disease, n (%)						
AML	5 (50)	3 (30)	1.0	9 (25)	2 (20)	.77
ALL	0	2 (20)		4 (11)	2 (20)	
CML	0	2 (20)		2 (5.5)	1 (10)	
Myelofibrosis	2 (20)	0		3 (8.3)	0	
NHL	1 (10)	2 (20)		3 (8.3)	1 (10)	
Other	2 (20)	1 (10)		15 (41.6)	4 (40)	
Conditioning regimen, n (%)						
Radiotherapy/chemotherapy	6 (60)	6 (60)	1.0	23 (64)	3 (30)	.77
Chemotherapy only	4 (40)	4 (40)		13 (36)	7 (70)	
Type of donor, n (%)						
Related	4 (40)	4 (40)	1.0	16 (44.4)	5 (50)	1.0
Unrelated	6 (60)	5 (50)		20 (55.6)	5 (50)	
Haploidentical	0	1 (10)		0	0	
Cell source, n (%)						
Bone marrow	0	6 (60)	.003	8 (22.2)	1 (10)	.13
PBSCs	10 (100)	3 (30)		27 (75)	7 (70)	
Cord Blood	0	1 (10)		1 (2.8)	2 (20)	
Type of transplant, n (%)						.67
Myeloablative	5 (50)	4 (40)	1.0	29 (80.6)	7 (70)	
Nonmyeloablative	5 (50)	6 (60)		7 (19.4)	3 (30)	
GVHD prophylaxis, n (%)						
MTX + tacrolimus	3 (30)	4 (40)	.76	18 (50)	4 (40)	.62
MMF + tacrolimus	2 (20)	1 (10)		1 (2.8)	0	
CSP + MMF	3 (30)	5 (50)		5 (13.9)	1 (10)	
CSP + MTX	1 (10)	0		3 (8.4)	0	
Other	1 (10)	0		9 (25)	5 (50)	
Acute GVHD, n (%)†	7 (70)	9 (90)	.58	20 (55.5)	6 (60)	1.0
Months of post-transplantation saliva collection, mean (range)	16.1 (6.7-34.5)	23.3 (12-62.7)	.26	40.8 (10-121.6)	24.4 (12-80.9)	.16

ALL indicates acute lymphoblastic leukemia; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; NHL, non-Hodgkin lymphoma; CSP, cyclosporin; MMF, mycophenolate plus Cellcept and Myfortic; MTX, methotrexate.

* Age and saliva collection data were compared between oral cGVHD(+) and oral cGVHD(−) patient groups using a 2-tailed Student *t* test. The other patient characteristics were compared using a 2-tailed exact chi-square test.

† Indicates number of patients in whom aGVHD was seen in 1 or more tissues.