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

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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.

refrain from eating, drinking, or brushing teeth for at least 1 hour before collection. Immediately before collection, the subject rinsed his or her mouth with water. Whole saliva was collected over a 15-minute period directly into a sterile 50-mL vial that was kept on ice throughout. If a subject had difficulty producing adequate saliva, a funnel was inserted on top of the vial to facilitate collection.

After collection, saliva was centrifuged at 17,000 × *g* for 15 minutes at 4°C to remove bacteria and mucosal cells. The supernatant was removed and treated with Protease Inhibitor Cocktail (50 μL/1 mL of whole saliva; Sigma-Aldrich, St. Louis, MO) to minimize protein degradation. Saliva samples were divided into 0.5-mL aliquots and stored at −80°C.

Labeling of Saliva Samples

Proteins from 4 different pools of saliva, obtained from the oral cGVHD(+) and oral cGVHD(−) groups collected in phase I (Table 1), as well as from 2 groups of healthy adults (to allow identification of salivary proteins that showed altered expression as a result of normal physiological aging), were first trypsinized and labeled with isobaric Tags for Relative and Absolute Quantification (iTRAQ) chemical tags. Protein samples (240 g) from each pooled saliva sample were prepared in an 8 M urea buffer after trichloroacetic acid precipitation and then reduced by incubation with 5 mM trifluoroacetic acid for 1 hour at 37°C; cysteine residues were blocked by incubation for 10 minutes in 10 mM methyl methane at room temperature. Proteins were digested by the addition of 5 μL of sequencing-grade trypsin (Promega, Madison, WI) and incubation at 37°C for 2 hours, followed by a second incubation with an additional 5 μL of trypsin at 37°C for 16 hours. The trypsin-digested samples were labeled with iTRAQ reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol, as described previously [17]. Each pooled saliva sample was labeled with a different iTRAQ label, and then the 4 saliva samples were combined to create a single sample for MS analysis.

Glycoprotein Enrichment and Analysis

Glycoproteins/peptides were separately isolated from the iTRAQ-labeled saliva pools as described previously [18]. After trypsin digestion and iTRAQ labeling, the samples were desalted through a C18 column (Waters, Milford, MA) with 0.1% trifluoroacetic acid to remove uncoupled iTRAQ reagent. The samples were then oxidized with sodium periodate (final concentration 12.5 mM) by incubation at room temperature for 1 hour. After a further desalting step on a C18 column, the samples were dried, and the flow-through fraction was saved for tandem MS analysis. The freeze-dried samples were coupled to hydrazide resin (Bio-Rad, Hercules, CA) in 0.1 M sodium acetate and 0.15 M NaCl (pH 5.5). After the 24-hour binding step, the supernatant was collected, and the hydrazine resin was washed 3 times with 1.5 M NaCl, 3 times with 80% acetonitrile, 3 times with 100% methanol, 3 times with deionized water, and finally 3 times with 0.1 M NH_4HCO_3 (pH 8.3). The supernatants (nonglycopeptides) were saved for tandem MS analysis. The hydrazine resin was then treated with Peptide:N-Glycosidase F (PNGase F) (New England Biolabs, Beverly, MA) dissolved in 0.1 M NH_4HCO_3 (pH 8.3) for a total of 48 hours. The supernatant containing the released glycopeptides was collected, and the resin was rinsed 3 times in 0.05 M NH_4HCO_3 buffer and then twice with 80% acetonitrile, followed by centrifugation at 3000 × *g* for 5 minutes after each wash. The glycopeptide-containing supernatants were then processed for tandem MS as described below.

Tandem MS Analysis and Protein Identification

Quantitative tandem MS analysis was performed using the 4800 Proteomics Analyzer with TOF/TOF Optics (AB SCIEX, Framingham, MA). The MS reflector positive ion mode was used, with automated acquisition in the 800- to 4000-m/z range with 1000 shots per spectrum. A maximum of 15 peaks were selected per spot, with a minimum signal-to-noise ratio of 50 and a cluster area of 500. Approximately 48,872 precursors were subjected to tandem MS in the entire study. Proteins were identified using the MASCOT algorithm (Matrix Science, Boston, MA) and searched against a human International Protein Index (IPI) database (version 3.84) with ProteinPilot version 3.0 and the Paragon method [19]. The raw peptide identification results from the Paragon algorithm searches were further processed with the Pro Group algorithm within the ProteinPilot software before final display. Protein quantification was achieved by averaging iTRAQ ratios of all the peptides identified within each polypeptide. Normalization using a Gaussian distribution with median of 1 (when comparing all peptides between the control and experimental groups) was performed after iTRAQ ratios were calculated. A protein was considered significantly increased in expression if the iTRAQ ratio [eg, oral cGVHD(+)/oral cGVHD(−)] was >1.6 and significantly decreased if this ratio was <0.65 [20].

To exclude salivary proteins that might be markers of normal physiological aging, all proteins showing a significantly increased iTRAQ ratio in both the allo-HSCT patient population [ie, oral cGVHD(+)/oral cGVHD(−)] and during normal aging (ie, elderly adults/young adults) were removed from the final MS dataset. The same exclusion criteria were applied to proteins that showed a significantly decreased iTRAQ ratio in both groups.

Protein Function and Pathway Analysis

Gene Ontology analysis was performed using the online program Database for Annotation, Visualization, and Integrated Discovery (DAVID), version 6.7 [21]. Proteins up-regulated and down-regulated in the oral cGVHD(+) group relative to the cGVHD(−) group were classified according to biological process, cellular component, molecular function, and cellular pathway.

ELISA Assays

Proteins were chosen for validation as potential oral cGVHD biomarkers based on a significant iTRAQ ratio (iTRAQ ratio <0.65 or >1.6) [20] and the availability of a high-quality ELISA assay. IL-1 receptor antagonist (IL-1ra) and cystatin B (CSTB, or Stefin B) were measured using ELISA assay kits (R&D Systems, Minneapolis, MN). Dilutions of saliva typically used in immunoassays were 1:1000 for IL-1Ra and 1:400 for CSTB. ELISA data were analyzed using parametric 4-PL curve fitting software (ReaderFit; Hitachi Solutions America, San Bruno, CA) to determine concentrations of samples based on a standard curve. The R^2 values for our ELISA standard curves were typically in the range of 0.98 to 0.995. Protein assays were carried out using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL), and ELISA immunoassay data were normalized to protein concentration, per milligram of salivary protein.

Statistical Analysis

Protein levels determined using ELISA immunoassays were compared between groups using the Mann-Whitney *U* test, and the correlation between marker protein levels was described by the Spearman rank correlation. Protein expression data were analyzed using Sigmaplot (Systat Software, San Jose, CA) or SPSS version 19 (IBM, Armonk, NY). A receiver operating characteristic (ROC) curve analysis was used to assess the diagnostic potential of selected proteins, including ROC curves for single and combined protein markers, using Sigmaplot or SPSS. Differences were considered statistically significant at $P < .05$. Patient characteristics were compared using the Student *t* test and exact chi-square test.

RESULTS

Patient Characteristics

Table 1 summarizes the clinical characteristics of the allo-HSCT recipients. The patients in the oral cGVHD(+) and oral cGVHD(−) groups with saliva samples collected in phase I were used for the MS studies, whereas patients with samples collected in both phase I and phase II were used for the validation studies using immunoassays. The 2 patient groups in each phase of the study were generally well matched in terms of age, sex, original disease, and type of transplant received. A history of acute GVHD was seen in 63% of the oral cGVHD(+) group, compared with 75% of the oral cGVHD(−) group (Table 1). In the oral cGVHD(+) group, the National Institutes of Health global severity score ranged from 1 to 10 (mean, 3.5; n = 42 patients), and the number of involved tissues varied from 1 to 4. After the oral mucosa (100% affected), skin was the most commonly involved site (52%; n = 22), followed by the eye (45%; n = 19). The vast majority (83%) of patients in the oral cGVHD(+) group had disease involvement at 2 or more sites.

The mean whole saliva flow rate was slightly lower in the cGVHD(+) group compared with the cGVHD(−) group and healthy adult controls, but the difference was not statistically significant (Table 2). Overall, patients in the cGVHD(+) group were at an average of 36 months post-HSCT at the time of saliva collection, compared with an average of 31.7 months for the oral cGVHD(−) group ($P = .65$).

Oral cGVHD Proteome

For MS studies (phase 1), the 4 pooled saliva samples collected from 40 subjects, split into 2 groups of allo-HSCT

Table 2
Salivary Flow Rates of Allo-HSCT Recipients and Healthy Adult Subjects

Study Group	No. of Subjects	Salivary Flow Rate, mL/min ± 1 SD	*P* Value*
Oral cGVHD(+)	37	0.37 mL/min ± 0.23	—
Oral cGVHD(−)	15	0.415 mL/min ± 0.16	.49
Elderly adults	10	0.415 mL/min ± 0.05	.53
Young adults	10	0.45 mL/min ± 0.1	.30

* Comparing the salivary flow rate of each group of subjects to the oral cGVHD(+) group, using a 2-tailed Student *t* test.

recipients and 2 groups of healthy adults, were each labeled with a different iTRAQ label and then combined and subjected to tandem MS simultaneously. Out of a total of 249 proteins identified by tandem MS, 82 proteins were significantly changed in expression as a result of oral cGVHD, based on the iTRAQ data comparing saliva samples from the cGVHD(+) and cGVHD(−) groups. Forty-four of those 82 proteins were significantly up-regulated in the oral cGVHD(+) group (Table 3), whereas the other 38 were down-regulated (Table 4). Of the 82 salivary proteins altered in the oral cGVHD(+) group, 13 were identified as glycoproteins by hydrazine-affinity chromatography and tandem MS (Tables 3 and 4). Proteins involved in innate and acquired immunity and inflammation, as well as oral (tooth) protection and various housekeeping functions, were prominently represented in the MS dataset (see below).

Additional analysis of the iTRAQ dataset obtained from the 2 healthy adult groups revealed that 29 proteins (35%) identified as part of the oral cGVHD proteome showed the opposite trend in expression, based on iTRAQ data, during normal physiological aging (Tables 3 and 4). Specifically, 15

Table 3
Salivary Proteins Up-Regulated in Patients with Oral cGVHD

Accession	Protein/Gene Name	Peptides, n (% Coverage)	iTRAQ Ratio [GVHD(+)/GVHD(−)]	iTRAQ Ratio (Adult Control)*
IPI00790899	55-kDa protein	10 (19.7)	1.58	0.94
IPI00300786	Alpha-amylase 1/AMY1A	889 (83.4)	4.88	0.65
IPI00847635	Alpha-1-antichymotrypsin isoform 1/SERPINA3	8 (16.3)	2.38	1.23
IPI00552432	Basic salivary proline-rich protein 2/PRB2	12 (29.7)	12.3	1.3
IPI00019482	Basic salivary proline-rich protein 4/PRB4	12 (29.7)	10.72	1.14
IPI00296654	BPI fold-containing family B member 2/BPIFB2	17 (22.7)	2.36	0.50†
IPI00032293	Cystatin C/CST3	11 (45.9)	2.1	0.9
IPI00002851	Cystatin D/CST5	23 (43)	2.85	1.14
IPI00032294	Cystatin S/CST4‡	ND	2.94	0.72
		127 (67.4)	3.76	0.86
IPI00305477	Cystatin SN/CST1‡	ND	1.92	1.03
		136 (66.7)	37.5	1.13
IPI00220327	Cytokeratin-1/KTR1	25 (34.9)	3.26	1.44
IPI00021304	Cytokeratin-2e/KRT2	10 (14.9)	2.25	1.19
IPI00300725	Cytokeratin-6A/KRT6A	5 (8.7)	10.73	0.80
IPI00019359	Cytokeratin-9/KRT9	5 (8)	1.87	0.9
IPI00009865	Cytokeratin-10/KRT10	23 (29.5)	2.26	1.48
IPI00873806	FAM3B isoform A/FAM3B‡	ND	3.79	0.89
IPI00784293	Golgi membrane protein 1/GOLM1	7 (17)	2.03	0.64†
IPI00853068	Hemoglobin alpha-2/HBA2	10 (53.5)	11.0	0.23†
IPI00654755	Hemoglobin subunit beta/HBB	13 (55.1)	15.0	0.35†
IPI00012024	Histatin-1/HTN1	18 (36.8)	6.82	0.33†
IPI00012026	Histatin-3/HTN3	0.56 (27.5)	5.53	0.395†
IPI00387120	Ig kappa chain V-IV region Len	ND	2.29	1.02
IPI00854743	IgG heavy chain variable region/ENSP00000375034	2 (13.5)	2.10	0.99
IPI00304808	Kallikrein-1/KLK1	0.93 (23.3)	1.67	1.23
IPI00383717	Kallikrein, intron-containing (fragment)	6 (22.2)	1.53	1.09
IPI00025023	Lactoperoxidase/LPO	20 (19.5)	3.05	0.47†
IPI00848342	Lactotransferrin precursor/LTF‡	ND	1.53	0.96
		61 (48.3)	3.16	0.39†
IPI00019038	Lysozyme C/LYZ	16 (37.8)	7.85	0.14†
IPI00009123	Nucleobindin-2/NUCB2	12 (23.3)	1.94	0.87
IPI00646304	Peptidyl-prolyl cis-trans isomerase B/PPIB	5 (20.4)	1.67	0.70
IPI00004573	Polymeric immunoglobulin receptor/PIGR	87 (43.9)	1.91	0.26†
IPI00748533	PRB3 protein/PRB3	18 (28.5)	2.20	0.94
IPI00022974	Prolactin-inducible protein/PIP	29 (65.8)	13.0	0.41†
IPI00377025	Proline-rich protein HaeIII subfamily 1/PRH1; PRH2	190 (79.5)	12.76	0.43†
IPI00856018	Proline-rich protein 4/PRR4	7 (26.1)	5.40	0.74
IPI00023011	Proline-rich protein 3/SMR3B	72 (67.1)	10.37	0.84
IPI00008580	Secretory leukocyte protease inhibitor/SLPI	1 (9.1)	3.1	0.48†
IPI00642739	TIMP1 metallopeptidase inhibitor 1/TIMP1	2 (14.7)	2.06	0.64†
IPI00299729	Transcobalamin-1/TCN1	5 (10.6)	9.91	0.82
IPI00414884	Uncharacterized protein C4orf40/C4orf40	5 (23.3)	2.29	0.96
IPI00374315	UPF0762 protein C6orf58/C6orf58	11 (24.6)	1.65	0.45†
IPI00006705	Uteroglobin/SCGB1A1	1.1 (11)	1.61	1.08
IPI00784119	V-ATPase subunit S1/ATP6AP1	ND	2.04	0.76
IPI00166729	Zinc-alpha-2-glycoprotein/AZGP1	61 (52)	21.57	0.69

ND indicates not determined.

* iTRAQ ratios of healthy elderly adult/young adult saliva sample.

† Proteins (n = 15) in the adult control saliva dataset that show the opposite trend in relative expression compared with the oral cGVHD(+)/oral cGVHD(−) dataset.

‡ Proteins obtained from the glycoprotein dataset. In some cases, the same protein was identified in both the glycoprotein (‡) and nonglycoprotein samples.

Table 4
Salivary Proteins Down-Regulated in Patients with Oral cGVHD

Accession No.	Protein/Gene Name	Peptides, n (% Coverage)	iTRAQ Ratio [GVHD(+)/GVHD(−)]	iTRAQ Ratio (Adult Control)*
IPI00021263	14-3-3 protein zeta-delta/YWHAZ	1.43 (28.6)	0.50	2.0†
IPI00747533	56-kDa protein/PGD	8 (16.2)	0.52	1.23
IPI00021439	Actin, cytoplasmic 1/ACTB	ND (60.8)	0.44	2.09†
IPI00465248	Alpha-enolase/ENO1	19 (35.3)	0.60	1.5†
IPI00419215	Alpha-2-macroglobulin-like protein 1/A2ML1‡	ND	0.56	1.46
		ND (7.7)	0.66	0.95
IPI00654709	Aldehyde dehydrogenase/ALDH3A1	3 (5.7)	0.59	1.30
IPI00304557	BPI fold-containing family A member 2 (SPLUNC2)/BPIFA2‡	42 (63.5)	0.47	1.42
			0.60	0.69
IPI00027486	Carcinoembryonic antigen/CEACAM5‡	ND	0.47	1.78†
IPI00292532	Cathelicidin antimicrobial peptide/CAMP	1 (5.2)	0.58	2.08†
IPI00021828	Cystatin-B/CST6	31 (79.6)	0.45	1.34
IPI00021885	Fibrinogen alpha chain/FGA	7 (6.4)	0.45	1.02
IPI00298497	Fibrinogen beta chain/FGB	9 (20)	0.37	1.37
IPI00219757	Glutathione S-transferase P/GSTP1	7 (43.3)	0.63	1.90†
IPI00478493	Haptoglobin-related protein/HRP‡	ND	0.49	1.66†
IPI00641737	Haptoglobin/HP‡	ND	0.49	1.47
IPI00003111	Ig kappa chain V-I region AU/LOC652694	0.97 (28.8)	0.49	0.86
IPI00430847	IGKC protein/IGKC	0.88 (55.1)	0.44	0.86
IPI00829626	IGL@ protein	23 (36.5)	0.37	0.70
IPI00479708	Ig mu chain C region/IGHM‡	ND (9)	0.56	2.06
		24 (27.7)	0.19	1.74†
IPI00175024	IL-1 receptor antagonist protein/IL1RN	9 (49.7)	0.40	1.24
IPI00219077	Leukotriene A-4 hydrolase, isoform 1/LTA4H	1.10 (5.2)	0.57	1.54†
IPI00004310	Ly6/PLAUR domain-containing 3/LYPD3‡	ND	0.56	1.57†
IPI00027409	Myeloblastin/PRTN3	6 (25.8)	0.64	1.81†
IPI00236556	Myeloperoxidase, proteinase 3/MPO‡	ND	0.54	0.96
		8 (7.3)	0.55	0.72
IPI00005721	Neutrophil defensin 1/DEFA1	1.34 (19.2)	0.57	1.71†
IPI00027769	Neutrophil elastase/ELANE	2 (2.6)	0.61	1.405
IPI00169383	Phosphoglycerate kinase 1/PGK1	8 (23.7)	0.61	1.48
IPI00010796	Protein disulfide-isomerase/P4HB	15 (30.3)	0.45	1.45
IPI00479186	Pyruvate kinase isozymes M1, M2/PKM2	9 (16)	0.60	1.41
IPI00816799	Rheumatoid factor D5 light chain (fragment)	7 (35.6)	0.41	1.04
IPI00027462	S100A9/MRP14	45 (80.7)	0.48	3.07†
IPI00216298	Thioredoxin/TXN	11 (51.4)	0.57	1.085
IPI00300376	Transglutaminase-3/TGM3	8 (11.1)	0.48	1.41
IPI00788802	Transkelotase variant (fragment)/TKT	1.07 (8.8)	0.62	1.13
IPI00426051	Uncharacterized protein DKFZp686C15213	11 (20)	0.55	1.02
IPI00423462	Uncharacterized protein DKFZp686K18196	88 (34.7)	0.50	1.07
IPI00877792	Fibrinogen gamma chain/FGG‡	ND	0.38	1.64†
		1.04 (11)	0.32	1.08
IPI00887678	Uncharacterized protein/LOC654188	6 (21.5)	0.60	1.27

* iTRAQ ratios of healthy elderly adult/young adult saliva sample.
† Proteins (n = 14) in the adult control saliva dataset that show the opposite trend in relative expression compared with the oral cGVHD(+)/oral cGVHD(−) dataset.
‡ Proteins obtained from the glycoprotein dataset. In some cases, the same protein was identified in both the glycoprotein (‡) and nonglycoprotein samples.

proteins that were up-regulated in oral cGVHD(+) saliva samples were down-regulated during normal aging (comparing young adults and middle-aged adults), and 14 proteins that were down-regulated in oral cGVHD(+) saliva samples were up-regulated with normal physiological aging.

Functional Classification and Pathway Analysis

To functionally classify the salivary proteins that displayed altered expression in the oral cGVHD(+) group, we performed Gene Ontology analysis using the DAVID bioinformatics knowledge base [21]. The majority (33 of 59; 56%) of the cGVHD-altered proteins identified by this database were either secreted or known to have an extracellular function (Supplementary Figure 1A). Many of these proteins also had a protein-binding activity, either as proteinase inhibitors or as enzymes, such as proteases and hydrolases.

To identify specific trends in the expression data, we grouped proteins belonging to the same gene family or that were functionally similar, and determined a mean iTRAQ value (Supplementary Figure 1B). Among the proteins up-regulated in the oral cGVHD(+) group were numerous proteins that function in innate immunity or oral tissue protection, including salivary cystatins, histatins, and proline-rich proteins (Supplementary Figure 1B). Cytokeratins were also abundant in oral cGVHD(+) saliva. Among the down-regulated proteins were several proteinases, including neutrophil elastase and myeloperoxidase, as well as several glycolytic enzymes and antiapoptotic factors (Supplementary Figure 1B). The results show that proteins within a family or that have a common function, such as proteolysis or antimicrobial activity, were frequently coregulated in the oral cGVHD(+) group.

Examination of IL-1ra and CSTB as Potential Oral cGVHD Biomarkers

Figure 1 summarizes the salivary expression of IL-1ra and CSTB in the oral cGVHD(+) and oral cGVHD(−) groups, normalized to salivary protein concentration. Levels of IL-1ra and CSTB were both reduced by ~2.3-fold in the oral cGVHD(+) group compared to the oral cGVHD(−) group

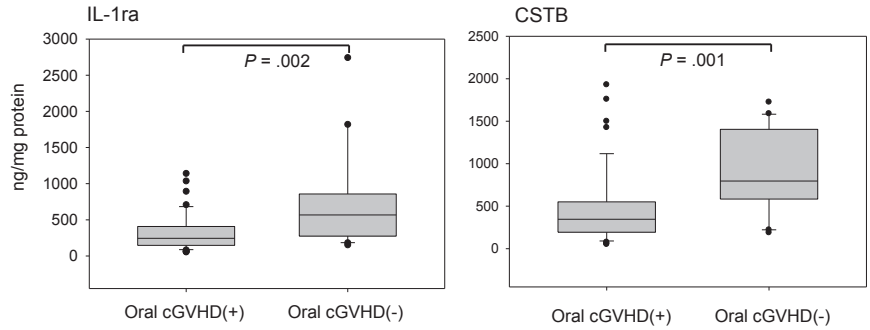

Figure 1. IL-1ra and CSTB are down-regulated in oral cGVHD. The levels of salivary IL-1Ra and CSTB were measured in individual patients in the oral cGVHD(+) and oral cGVHD(−) groups by ELISA, and the results displayed as boxplots. The top and bottom of each box indicate the 75th percentile and 25th percentile, respectively. The line inside each box indicates the median concentration. The top and the bottom ends of the whiskers represent 90th and 10th percentiles. Outliers are shown as dots. Significance levels were calculated using the Mann-Whitney *U* test. The median values (from left to right) are as follows: IL-1ra: 244.7 ng/mL for oral cGVHD(+) and 567.5 ng/mL for oral cGVHD(−); CSTB: 345.8 ng/mL for oral cGVHD(+) and 796.5 ng/mL for oral cGVHD(−).

($P < .003$, Mann-Whitney *U* test) (Figure 1). The Spearman rank correlation between the 2 proteins was 0.69 ($P < .001$).

We performed ROC curve analysis to further assess marker performance. The area under the curve for the oral cGVHD(+) group was 0.76 for the 2 markers, with a sensitivity of 85% and specificity of 60% (Table 5). Patients with oral cGVHD who were examined within 12 months of allo-HSCT exhibited slightly better sensitivity (92%) and specificity (73%) with the 2-marker panel, although the number of patients was relatively small (n = 24) (Table 5).

To date, all proteins measured by immunoassay, including IL-1ra and CSTB, have shown the same upward or downward trend in expression as predicted from the iTRAQ analysis of pooled saliva samples. These findings support the use of the iTRAQ/tandem MS approach for proteomic discovery [17,20].

DISCUSSION

cGVHD remains the primary long-term complication of allo-HSCT and is a major hurdle to the more widespread use of allo-HSCT worldwide [2,22]. Chronic inflammation, tissue damage, and apoptosis are central aspects of cGVHD pathobiology; this tissue damage can be mediated directly by infiltrating T lymphocytes and indirectly by proinflammatory cytokines, including IL-1, TNF-α, and IL-17 released by various types of immune cells [4,8,23]. In accordance with this disease model, we identified numerous proteins associated with oral cGVHD that function in inflammation and/or innate immunity [24], as well as proteins involved in normal cellular maintenance and survival (Tables 3 and 4). The presence of elevated cytokeratins in oral cGVHD(+) saliva samples (Supplementary Figure 1B) is likely a biochemical marker of mucosal tissue damage and cell death [9]. Alterations in innate and acquired immunity is closely associated with the pathobiology of cGVHD in different target organs [13,25,26] and is clinically relevant to cGVHD-associated mortality, which in most cases results from serious bacterial and/or fungal infections [1].

Saliva is largely a product of the parotid and submandibular salivary glands, and its protein and nonprotein composition is a reflection of both local and systemic (serum-derived) sources. In cGVHD, damage to salivary glands is rapid and often severe, affecting both overall saliva production and composition [27-29]. Patients typically have higher concentrations of metal ions such as sodium and magnesium, as well as albumin, IgG, and total protein [30,31]. Salivary flow rates are generally reduced [27], although some recent studies have found no difference in flow rates between patients with and those without oral cGVHD ([13] and this study). Damage to salivary glands likely varies depending on the intensity of conditioning (including whether or not radiotherapy is used) and accompanying levels of T lymphocytes and proinflammatory cytokines.

We examined the expression of 2 proteins selected from the MS dataset and confirmed in an expanded patient population that IL-1ra and CSTB had significantly altered expression in association with oral cGVHD (Figure 1). IL-1ra is a protein that binds to the IL-1 receptor and blocks IL-1 signaling. It is produced in both secreted (soluble) and intracellular forms by immune cells, keratinocytes, and several other cell types [32]. Altered expression of IL-1Ra is associated with numerous chronic inflammatory diseases, including rheumatoid arthritis. Donor and recipient IL-1Ra genotypes have been shown to affect the incidence and/or severity of acute GVHD and cGVHD, respectively [33,34]. An altered balance between IL-1 and IL-1ra has been proposed as a factor governing the severity of several inflammatory conditions, including rheumatoid arthritis and Crohn disease [35]. Salivary IL-1Ra levels were markedly lower in the oral cGVHD(+) group compared with the oral cGVHD(−) group (Figure 1), which might favor excessive IL-1 signaling in the oral mucosa [7]. CSTB, a protease inhibitor and member of the cystatin family, functions as an inhibitor of cathepsins L, H, and B and may regulate cathepsin-mediated apoptosis in neuronal cells [36]. Along with its action as a protease inhibitor, CSTB has been proposed to have other functions, including the regulation of glycolysis and inhibition of the INF-β pathway in association with HIV infection [37]. One of

Table 5
Receiver Operating Curve Characteristics for Oral cGVHD Biomarkers

Marker	AUC	*P* Value*	Sensitivity, %	Specificity, %
All allo-HSCT recipients†				
L-1ra	0.74	.002	87	60
CSTB	0.765	.001	78	80
IL-1ra + CSTB	0.76	.001	85	60
Newly diagnosed patients‡				
IL-1ra	0.76	.034	85	64
CSTB	0.78	.019	92	64
IL-1ra + CSTB	0.8	.014	92	73

AUC indicates area under the curve.
* Tests whether the AUC value is significantly different from 0.5.
† Comparison was between 46 patients with oral cGVHD(+) and 20 with oral cGVHD(−).
‡ Comparison was between 13 patients with oral cGVHD(+) and 11 with oral cGVHD(−) studied at ≤12 months post-transplantation.

the proteins with which CSTB interacts is pyruvate kinase M2, an enzyme that is down-regulated in oral cGVHD(+) saliva (Table 4). Thus, the markedly diminished expression of CSTB in oral cGVHD could affect multiple cellular functions, including cathepsin protease activity and INF signaling. CSTB is present in human saliva primarily in several S-modified forms; how these modifications might influence function is unclear, however [38].

An important focus of current GVHD research is to develop biomarker panels that eventually might serve as diagnostic tools and to inform patient prognosis and treatment strategies [39]. To date, a number of candidate cGVHD biomarkers have been reported, including serum B cell activating factor [25], serum MHC class I–related chain A and its antibodies [40], serum IL-15 [41], serum CXCL9 [42], and salivary lactotransferrin and lactoperoxidase [13]. Those studies differ in terms of the biofluid examined (serum or saliva), type of population studied (pediatric or adult), and proteomic analysis methodology. While there is considerable overlap between the serum and salivary proteome [11], it is likely that serum and salivary biomarkers for cGVHD will be distinct and will measure disease activity in different tissues. In the case of IL-1ra and CSTB, additional studies with both independent sets of cGVHD patients, as well as longitudinal studies, are needed to determine their potential utility as oral biomarkers of cGVHD. Another recent report indicated that salivary IL-1ra levels were reduced in oral cGVHD patients, suggesting that this protein indeed may have utility as an oral cGVHD biomarker [43].

In summary, we have used a proteomic approach to describe the alterations in salivary protein expression that occur in patients with oral cGVHD, and have identified 2 potential biomarkers for this disease. This study provides new insight into the inflammatory and anti-inflammatory pathways that may be involved in the persistence of this chronic inflammatory disease, and adds new proteins that might serve as research or diagnostic tools in the study of oral cGVHD.

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.bbmt.2014.03.031.

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