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Research Article

Proteomic Analysis of Dacryoliths from Patients with or without Topical Rebamipide Treatment

Fukutaro Mano[a] Hiroki Takimoto[b] Masako Oe[c] Kuo-chung Chang[a]
Tomiya Mano[a] Yutaka Yoshida[b]

[a]Suita Tokushukai Hospital Eye Center, Suita, [b]Department of Structural Pathology, Kidney Research Center, Niigata University Graduate School of Medical and Dental Sciences, Niigata, [c]Tane Memorial Eye Hospital, Osaka, Japan

What Is It about?

This is the first report to clinically detect the different features of dacryoliths (lacrimal stones) related to long-term use of rebamipide eye drops. Each protein profile with or without topical rebamipide use was obtained by proteomic analysis and related to immune and inflammatory responses. Interestingly, bacterial infection had a stronger association with dacryoliths in the rebamipide (–) group compared to the rebamipide (+) group. It is possible that lacrimal protein alteration may be accelerated by topical rebamipide treatment, resulting in less bacterial infection. Our research contributes to the expansion of knowledge regarding proteomic analysis of dacryoliths.

Keywords

Dacryoliths · Dacryocystorhinostomy · Proteomic analysis · Rebamipide eye drops · Unipept

Abstract

Background: A dacryolith mainly contains organic material, but its specific protein content is unknown. We observed a unique dacryolith formation in patients with long-term use of rebamipide eye drops and tried to identify the differences in protein compositions of dacryoliths from patients with or without use of rebamipide eye drops using novel proteomic analysis. ***Methods:*** Dacryolith samples were obtained from 7 patients (4 samples were without rebamipide usage, 3 were with rebamipide usage) who underwent endo-dacryocystorhinostomy or lacrimal endoscopic surgery and were subjected to protein identification and metaproteomic analysis. ***Results:*** The proteomic analysis revealed that most core proteins of dac-

Fukutaro Mano and Hiroki Takimoto contributed equally to this manuscript.

Fukutaro Mano
21-1 Senriokanishi
Suita, Osaka 565-0814 (Japan)
E-Mail manofuku666@gmail.com

ryoliths are involved in immune and inflammatory responses and rebamipide-related proteins participated in several biological processes, including immune response, receptor-mediated endocytosis, and negative regulation of endopeptidase activity. Metaproteomic analysis of taxonomic diversity of dacryolith proteomes suggested less involvement of bacterial infections in dacryoliths from patients with long-term use of rebamipide. ***Conclusion:*** This is the first report to clinically detect the different features of dacryoliths related with long-term use of rebamipide eye drops with proteomic analysis. It is possible that lacrimal protein alteration may be accelerated by topical rebamipide treatment with less bacterial infection involvement, but this requires further study. Long-term rebamipide eye drop use may be restricted in patients with nasolacrimal duct obstruction.

Introduction

Lacrimal sac dacryoliths are often diagnosed during dacryocystorhinostomy (DCR), although their cause is unclear. Some authors have described the components of dacryoliths to reveal the mechanism of their formation. Several studies have described the etiology and gender and age-association of nasolacrimal duct obstruction (NLDO) and smoking; however, the results of each study differed in accordance with limited attendance [1, 2].

Reports have described the components of dacryoliths. Paulsen et al. [3] reported that trefoil factor (TFF) peptide and mucins are the major components of dacryoliths. Iliadelis et al. [4] performed a histochemical analysis of dacryoliths in a series of DCR procedures. The histological examination revealed acellular amorphous organic material and limited calcium salt depositions, while the chemical analysis showed mainly organic (over 90%) and minimal inorganic material as the components of dacryoliths. Orhan et al. [5] similarly described that dacryoliths comprised amorphous materials and organic materials. However, these previous studies failed to identify the type of organic material. Lew et al. [6] reported a change in the concentration of electrolytes and proteins in the tear samples of patients with NLDO, and detected low levels of lysozymes. However, no specific causative protein was detected immunologically in their study.

Dacryoliths are commonly characterized by yellowish hard stones accompanied with nasolacrimal discharge (Fig. 1). We have noticed unique dacryolith formation that was rather white and brittle in patients with long-term use (over 6 months) of rebamipide eye drops (Fig. 2). We hypothesized that there are different protein compositions between dacryoliths from patients who did not use rebamipide eye drops and dacryoliths from patients who had used rebamipide eye drops over a long period of time, and we tried to identify the major protein composition using novel proteomic analysis. The peptide sequences of the identified proteins were analyzed by Unipept, a recently developed open-source web application designed to explore biodiversity of complex metaproteome samples.

Materials and Methods

Patient and Tissues

A retrospective dacryolith evaluation using proteomic analysis was performed at a single center.

Tissue specimens (dacryoliths in the lacrimal sac) were obtained from 7 patients (Table 1) who underwent endo-DCR or lacrimal endoscopic surgery for NLDO (1 male, 6 females, aged 69–82 years, mean age 75.6 years) at Tane Memorial Eye Hospital from April 2015 to

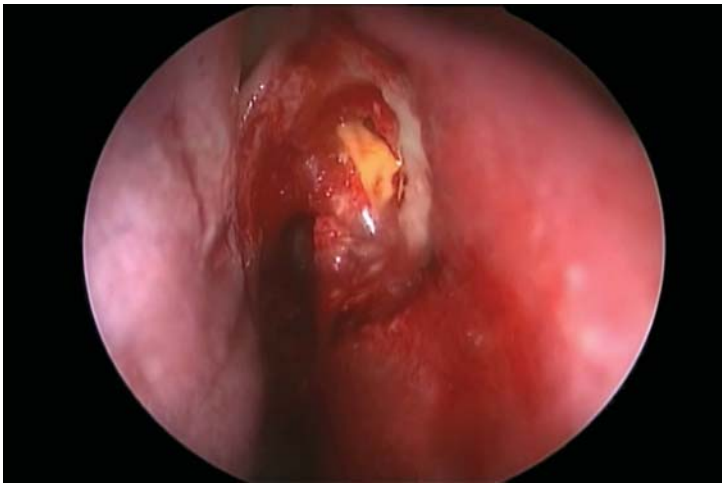

Fig. 1. A normal dacryolith characterized by yellowish hard stones.

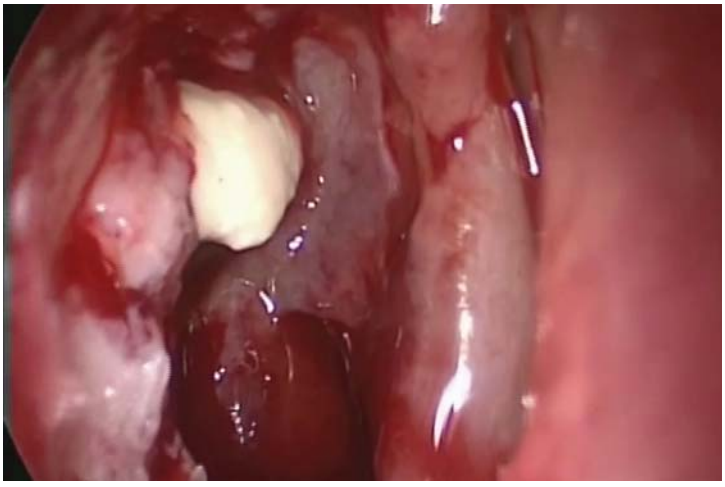

Fig. 2. A dacryolith from a patient who had used rebamipide eye drops over a long period of time was white and brittle.

Table 1. Dacryolith case information

Case	Gender	Age, years	Duration of epiphora, months	Diagnosis	Treatment	Rebamipide use	Duration of usage, months
1	female	75	30	NLDO	DCR	–	
2	female	75	6	canaliculitis + NLDO	DCR	–	
3	male	76	12	NLDO	endoscopic surgery	–	
4	female	78	20	NLDO	endoscopic surgery	–	
5	female	82	36	NLDO	DCR	+	6
6	female	74	18	NLDO	DCR	+	10
7	female	69	50	NLDO	DCR	+	40

NLDO, nasolacrimal duct obstruction; DCR, dacryocystorhinostomy; –, no rebamipide treatment; +, rebamipide treatment.

December 2016. Material from surgical procedures was obtained with the permission of the medical ethics commission and used in accordance with the Declaration of Helsinki. Patients were carefully reviewed for the duration of epiphora, history of acute dacryocystitis, general conditions (existence of cancer or Sjogren's syndrome, which may cause severe dry eye), usage of rebamipide eye drops, and duration of eye drop usage.

Dacryolith samples were analyzed at the Department of Structural Pathology, Kidney Research Center, Niigata University Graduate School of Medical and Dental Sciences, and used to perform protein identification and metaproteomic analysis.

Each patient had been diagnosed with NLDO (case 2 had canaliculitis, additionally), accompanied by refractory discharge, with a history of acute dacryocystitis, and underwent DCR or endoscopic surgery. The first four patients were never administered with topical rebamipide treatment in their medical history. Patients 5–7 patients had a history of topical rebamipide treatment.

In these three cases, rebamipide eye drops were administered over 6 months for severe dry eyes, and the dacryoliths with the use of rebamipide eye drops were larger and whiter than those without the use of rebamipide eye drops. The patients had no systemic symptoms, including Sjogren's syndrome and cancer.

Protein Extraction from Dacryolith

Each dacryolith sample was divided into small pieces using tweezers. Dacryoliths from patients treated with rebamipide eye drops were easier to cut than those from patients who had never received rebamipide. The dacryolith pieces were suspended in sodium dodecyl sulfate (SDS) sample buffer (2% SDS, 10% glycerol, 62.5 mM Tris-HCl pH 6.8, and 2% 2-mercaptoethanol) and homogenized using a Polytron homogenizer (Kinematica, Littau, Switzerland) and an ultrasonic disintegrator (Hielscher, Teltow, Germany). The homogenate was heated at 95°C for 10 min and centrifuged at 20,000 *g* for 20 min to obtain a supernatant as the protein extract.

Trypsin Digestion for Mass Spectrometric Analysis

The protein extract (30 µL containing 10 µg of protein) was transferred into a new microcentrifuge tube for "tube-gel digestion," a modified in-gel trypsin digestion procedure [7] to generate dithiothreitol-reduced, iodoacetamide-alkylated tryptic peptides for nano-flow liquid chromatography coupled with tandem mass spectrometer (LC-MS/MS). Briefly, the protein extract was mixed with 11.3 µL of acrylamide solution [acrylamide, 36% and *N*,*N*′-methylenebis (acrylamide), 4%], 0.4 µL each of 10% ammonium persulfate and *N*, *N*, *N*′, *N*′-tetramethylethylenediamine and polymerized for 60 min at room temperature. The polymerized tube gel was incubated for 30 min at room temperature in 50% methanol and 7% acetic acid and stored at 4°C in 5% acetic acid. The tube gels were subjected to in-gel digestion with trypsin (Sigma, Proteomics Sequencing Grade) using the protocol described by Katayama et al. [8].

Mass Spectrometry

The peptides generated from each dacryolith sample was finally dissolved in 15 µL of 0.3% formic acid and 5 µL of the sample was injected into nano-flow LC (Eksigent nanoLC 415 with ekspert cHiPLC, Sciex) coupled through a nano-electrospray ionization (ESI) ion source with a tandem mass spectrometer (TripleTOF5600+, Sciex). Analysis was conducted in duplicates for each sample under the trap and elute mode using a ChromeXP C18 Chip column (200 µm × 0.5 mm) as a trap column and a ChromeXP C18 Chip column (75 µm × 150 mm) as an analytical column. Mobile phases A and B were 0.1% formic acid and 0.1% formic acid in acetonitrile, respectively. Peptides were eluted by a 40-min gradient from 2% B to 32% B at 300 nL/min. MS spectrum (250 ms) followed by 10 MS/MS spectra (100 ms each) were acquired under a data-dependent mode. Dynamic exclusion time was set at 12 s. Autocali-

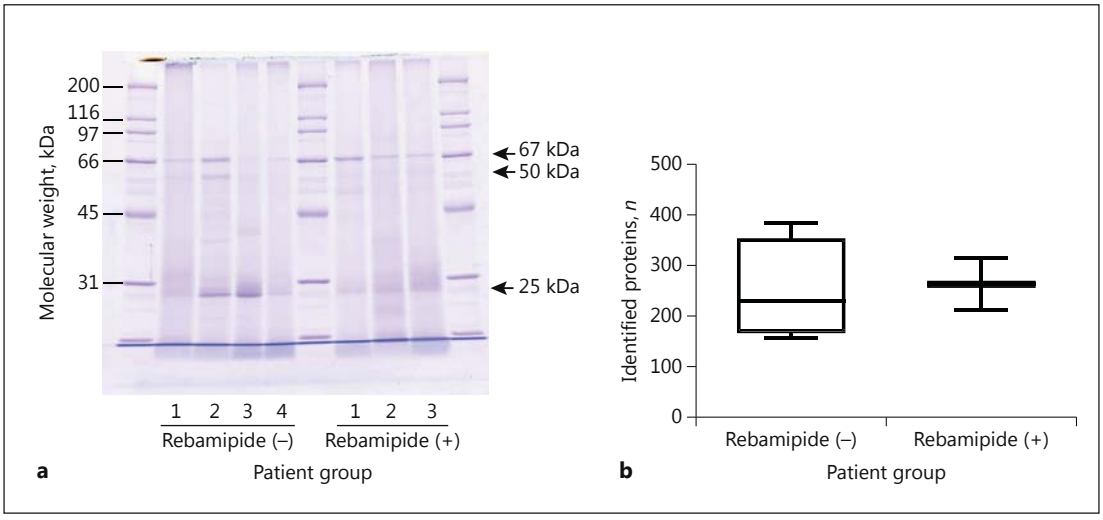

Fig. 3. a SDS-PAGE profiles of dacryolith proteins from patients with rebamipide (+) or without rebamipide (–) eye drop treatment. The protein extract (10 μg) from each sample was loaded and separated on 10% polyacrylamide gel, followed by staining of the gel with Coomassie Brilliant Blue R-250. **b** The number of proteins identified by mass spectrometry was compared between the two groups.

bration using 50-fmol tryptic peptides of bovine serum albumin (KYA Technology, Tokyo, Japan) was performed for every 5 or 6 samples.

Protein Identification

The raw data generated by Analyst TF1.6 (Build 6211) were converted to mascot generic files by MS Converter (Sciex). Two mascot generic files generated from duplicate runs for one sample were merged and searched against the Swiss-Prot protein sequence database (2017-03 release) under the taxonomy of *Homo sapiens* parameter settings as follows: instrumental setting, ESI-QUAD-TOF; peptide tolerance, ±20 ppm; MS/MS tolerance, ±0.1 Da. Modification settings were as follows: fixed modification of carbamidomethylation on cysteine; variable modifications of deamidation on asparagine and/or glutamine (*N*-terminal glutamine to pyroglutamate and *N*-terminal glutamate to pyroglutamate) and oxidation on methionine. A maximum of two missed cleavages was allowed. The false discovery rate was targeted below 1%. For quantitative analysis, we used the normalized spectral abundance factor (NSAF), a label-free, spectral counting method that correlates with protein abundance (relative molar ratio) [9].

After completion of the identification of human proteins, unassigned queries (MS/MS spectra) were extracted and searched against all taxonomies (all entries of the Swiss-Prot database) to evaluate the convolution of bacterial proteins. Other parameters were the same as those for the identification of human proteins as described above.

Results

Proteomic Profiling of Dacryolith

Figure 3a shows the comparison of SDS polyacrylamide gel electrophoresis (PAGE) results of dacryolith proteins from patients with or without rebamipide eye drop treatment. Bands with molecular weights of approximately 67, 50, and 25 kDa, possibly representing

Table 2. Supposed functions of the top 20 abundant core proteins of dacryolith

Protein name	Gene name	Supposed function
S100-A9	S100A9	Regulation of inflammatory processes and immune response; predominantly found calprotectin (s100a8/a9)
S100-A8	S100A8	Regulation of inflammatory processes and immune response; predominantly found calprotectin (S100A8/A9)
Lysozyme C	LYZ	Bacteriolytic, associated with the monocyte-macrophage system
Neutrophil defensin 1	DEFA1	Antibacterial, antifungal, and antiviral activity
Cathepsin G	CTSG	Antibacterial activity; serine protease cleaves complement C3
Azurocidin	AZU1	Neutrophil granule-derived antibacterial and monocyte- and fibroblast-specific chemotactic proteins
Neutrophil elastase	ELANE	Modifies the functions of natural killer cells, monocytes, and granulocytes
Prolactin-inducible protein	PIP	Detection of chemical stimulus involved in sensory perception of bitter taste; negative regulation of T cell apoptotic process
Eosinophil cationic protein	RNASE3	Cytotoxin and helminthotoxin with low-efficiency RNase activity; exhibits antibacterial activity
Myeloperoxidase	MPO	Part of the host defense system of polymorphonuclear leukocytes; responsible for antimicrobial activity
Lactotransferrin	LTF	Antimicrobial activity and able to permeabilize different ions through liposomal membrane
Myeloblastin	PRTN3	Polymorphonuclear leukocyte serine protease that degrades elastin, fibronectin, laminin, vitronectin, and collagen types 1, II, and IV (in vitro)
Neutrophil gelatinase-associated lipocalin	LCN2	Iron-trafficking protein involved in multiple processes such as apoptosis, innate immunity, and renal development

The top 20 abundant core proteins of dacryoliths were selected from online supplementary Table 2S. Of these, plasma proteins (hemoglobin subunit alpha, hemoglobin subunit beta, hemoglobin subunit delta, serum albumin) and immunoglobulins (immunoglobulin kappa constant, immunoglobulin kappa light chain, immunoglobulin lambda constant 2) were excluded. Supposed functions were provided from neXtProt (release 2017-04-12, http://www.nextprot.org), a well curated human protein database.

albumin and heavy and light chains of immunoglobulins, respectively, were commonly observed in samples from both groups. The electrophoretic pattern was more similar in samples from patients with rebamipide treatment [rebamipide (+)] as compared with those from patients without rebamipide treatment [rebamipide (–)].

Proteins identified in the dacryolith samples are summarized in online supplementary Table 1S (for all online suppl. material, see www.karger.com/doi/10.1159/000487585) along with their NSAF values. No significant difference was observed in the number of dacryolith proteins identified from patients with and without rebamipide treatment (Fig. 3b). However, the variability in the number of identified proteins among samples was obvious in

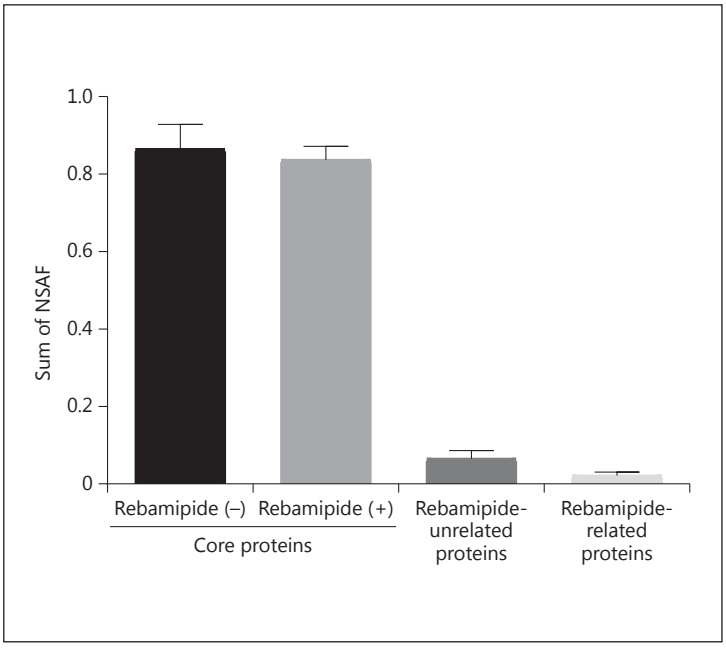

Fig. 4. Protein abundance, expressed as the normalized spectral abundance factor (NSAF), of core, rebamipide-unrelated, and rebamipide-related proteins of dacryoliths from patients with rebamipide (+) or without rebamipide (–) treatment. Core proteins (76 proteins) were identified from all dacryolith samples from both groups, while rebamipide-related proteins (57 proteins) were commonly identified in dacryolith samples from the rebamipide (+) group, but not the rebamipide (–) group. Rebamipide-unrelated proteins (16 proteins) were commonly identified in dacryolith samples from the rebamipide (–) group, but not the rebamipide (+) group.

the rebamipide (−) group in comparison to the rebamipide (+) group, possibly corresponding to the difference in SDS-PAGE patterns between the two groups (Fig. 3a). It is interesting that the number of proteins commonly identified in samples of the rebamipide (+) group (n = 133) was larger than the number of proteins commonly identified in samples of rebamipide (−) group (n = 92).

We compared the 133 and 92 proteins and found that 76 proteins were commonly identified in all the samples from both groups. We termed these proteins as "core proteins" of the dacryoliths. In addition, 57 proteins were commonly identified in dacryolith samples from the rebamipide (+) group, but not the rebamipide (−) group; these proteins were termed as "rebamipide-related proteins." Moreover, 16 proteins that were commonly identified in the dacryolith samples from the rebamipide (−) group, but not the rebamipide (+) group, were termed as "rebamipide-unrelated proteins." The sum of NSAF representing relative protein abundance was compared among core, rebamipide-related, and rebamipide-unrelated proteins. This comparison clearly indicated that core proteins are highly abundant, while the abundance of rebamipide-related and rebamipide-unrelated proteins was much less (Fig. 4; Table 2; online suppl. Table 1S).

Bioinformatics of Dacryolith Proteome

Gene ontology analysis based on biological process terms was adopted to characterize core proteins of the dacryoliths. David Bioinformatics Resources 6.8 (https://david.ncifcrf.gov) indicated innate immune response, proteolysis, receptor-mediated endocytosis, inflammatory response, defense response to bacterium, and immune response as the top six enriched biological processes (Fig. 5). This result suggests the involvement of immune and inflammatory responses in dacryolith generation, which was confirmed by the functions of top 20 abundant proteins (Table 2). Most of the abundant proteins were related to immune and inflammatory responses.

The relative abundance of rebamipide-related and unrelated proteins was very low as compared with that of core proteins (Fig. 4; online suppl. Table 2S). Gene ontology analysis

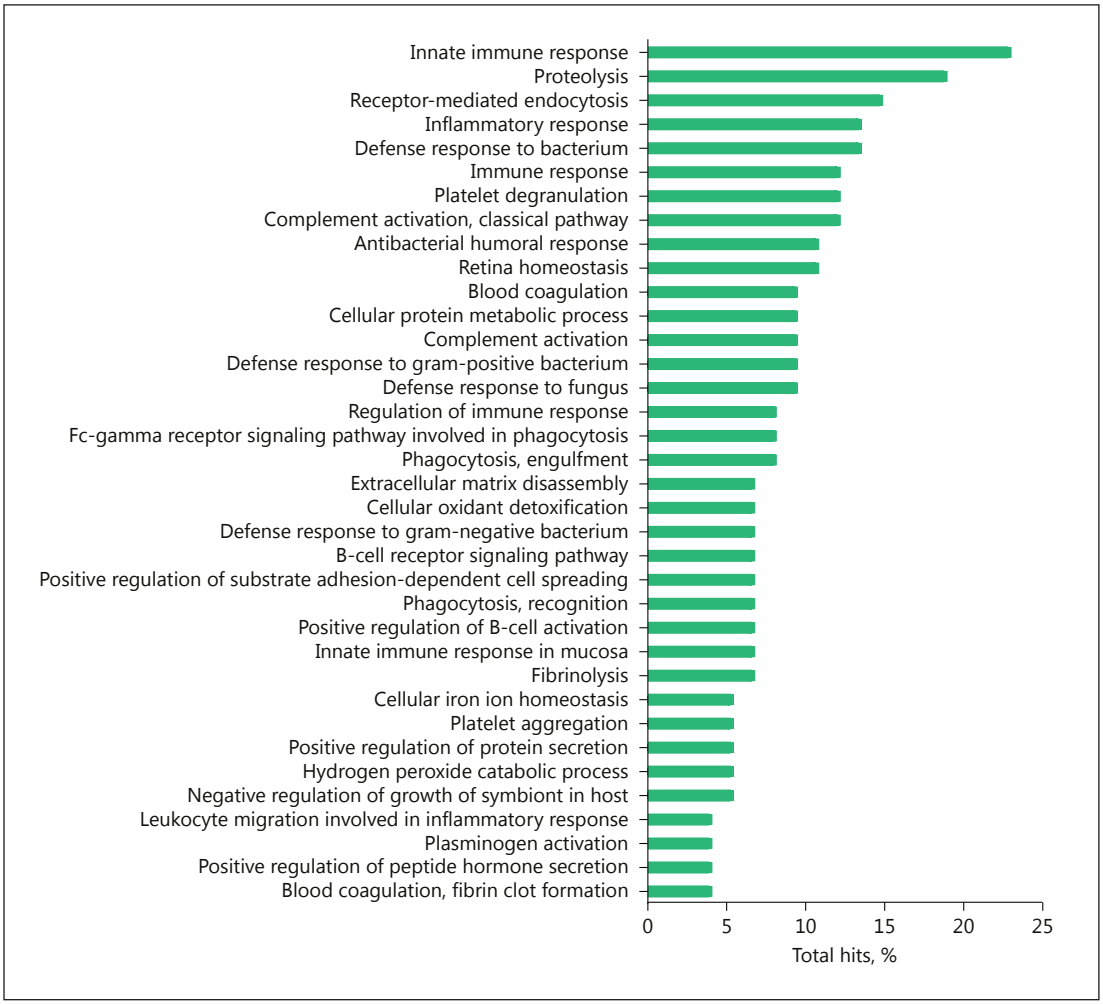

Fig. 5. Gene ontology analysis of core proteins of the dacryolith proteome using biological process terms. Core proteins, defined as proteins commonly identified in all dacryolith samples, were analyzed based on the Gene Ontology (GO) Biological Process using David Bioinformatics Resources 6.8 (https://david.ncifcrf.gov). Only overexpressed or enriched GO terms statistically significant from those with all the genes of *Homo sapiens* are shown ($p < 0.0001$ by modified Fisher's exact test).

using biological process terms showed that biological processes are significantly enriched in rebamipide-related proteins (Fig. 6). The same analysis on rebamipide-unrelated proteins, on the other hand, failed to give any significantly enriched biological processes.

Metaproteomic Analysis of Dacryoliths

Proteomic analysis of the core proteins of the dacryoliths indicated the predominance of proteins that were functional in immune and/or inflammatory responses. This result suggests that proteins with a microorganism origin may be components of dacryoliths. To explore this possibility, we re-searched unassigned spectra (MS/MS spectra excluded from searching against the *Homo sapiens* protein database) against Swiss-Prot under all entries (all taxa). This approach resulted in the identification of additional proteins as summarized in online supplementary Table 3S. Although many spectra cross-matched to proteins of other mammals, a significant number of spectra were well matched to proteins with a microorganism origin

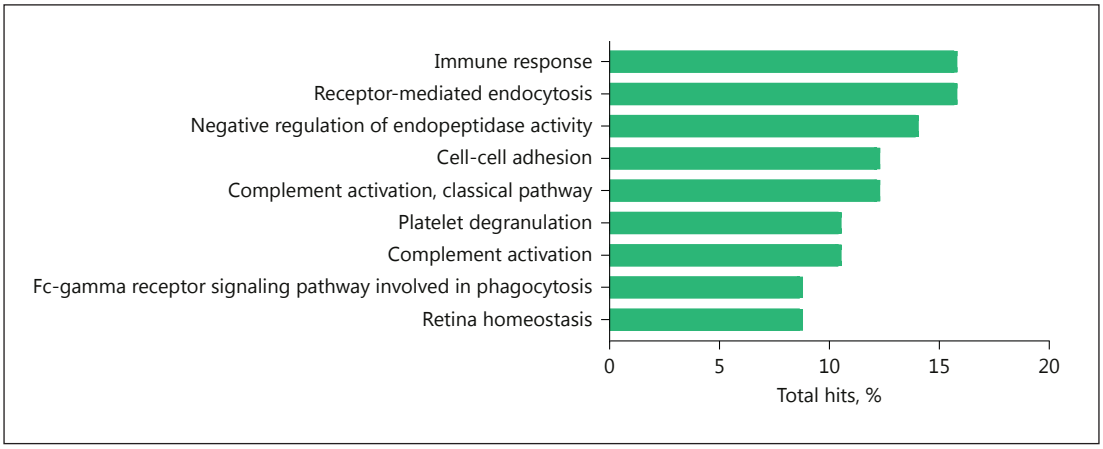

Fig. 6. Gene ontology analysis of rebamipide-related proteins of dacryolith proteome using biological process terms. Rebamipide-related proteins, defined as proteins uniquely identified in all dacryolith samples from the rebamipide (+) group, were analyzed based on the Gene Ontology (GO) Biological Process using David Bioinformatics Resources 6.8 (https://david.ncifcrf.gov). Only overexpressed or enriched GO terms statistically significant from those with all the genes of *Homo sapiens* are shown ($p < 0.0001$ by modified Fisher's exact test).

(online suppl. Table 3S). The bacterial elongation factor Tu was detected in all dacryolith samples from the rebamipide (−) group, while it was undetected in samples from the rebamipide (+) group.

The peptide sequences of the additionally identified proteins were further analyzed by Unipept 3.1 (http://unipept.ugent.be). Unipept is a recently developed open-source web application designed to explore biodiversity of complex metaproteome samples with excellent data visualization, and evaluate tryptic peptides that are unique to a particular genus, species, or any other taxon. Analysis using Unipept was performed without filtering out duplicate peptides and the results were visualized in tree-view images (Fig. 7a, b). In the tree-view images, the size of a node corresponded to the number of peptides associated with the node or any of its children, while the width of each branch corresponded to the size of the destination node [10, 11]. The bacterial-specific peptide sequence was shown in blue code, while the sequence nonspecific to bacteria peptides was shown in a green or orange code.

In 2 out of 3 dacryolith samples from the rebamipide (+) group (Fig. 7a), bacterial specific peptide sequences were undetected. In contrast, bacterial specific peptide sequences were found in all four samples from the rebamipide (−) group. In addition, the bacterial node and its branches appeared to be wide and large in 3 of 4 dacryolith samples from the rebamipide (−) group.

Discussion

Core proteins of dacryoliths were characterized by the immune and inflammatory responses which were confirmed by the top 20 abundant proteins.

Gene ontology analysis showed that biological processes are significantly enriched in rebamipide-related proteins and not so rich in rebamipide-unrelated proteins.

The metaproteomic analysis suggests that proteins of bacterial origin were less frequent in the rebamipide (+) group.

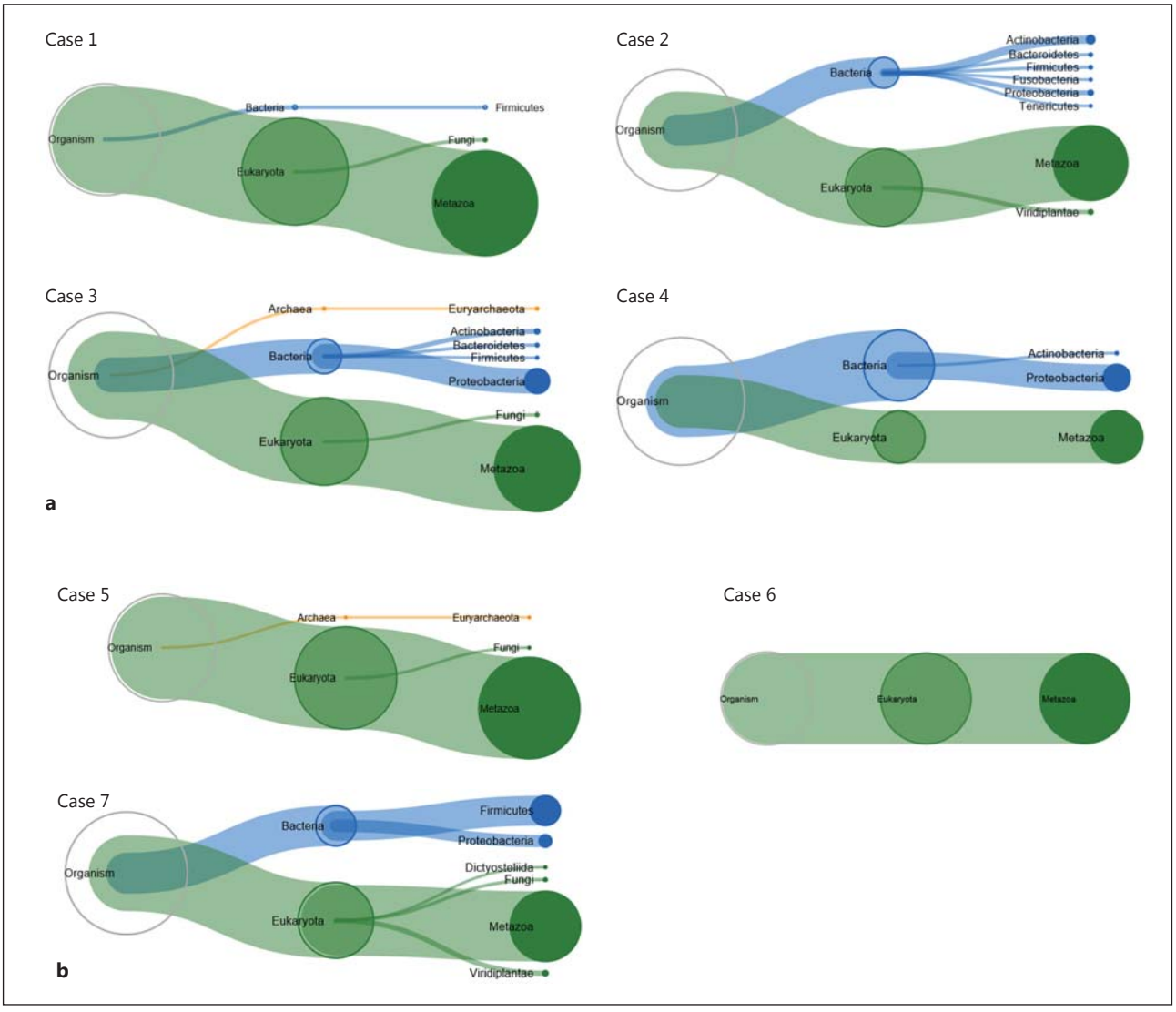

Fig. 7. Metaproteomic analysis of taxonomic diversity of the dacryolith proteome. After completion of the identification of human proteins using the Mascot search engine with the Swiss-Prot protein sequence database under taxonomy *Homo sapiens*, unassigned queries (MS/MS spectra) were extracted and searched against all entries in the Swiss-Prot database (see Materials and Methods for more details). Identified peptides (online suppl. Table 3S) were analyzed by Unipept 3.1, an open-source web application (http://unipept.ugent.be) without filtering duplicate peptides. Results were visualized in tree-view images. **a** Results of dacryolith samples from the rebamipide (–) group (cases 1–4). **b** Results from the rebamipide (+) group (cases 5–7).

This is a first report about proteomic analysis of dacryoliths. It contributes a lot to further study because the previous study could not identify the proteins of dacryoliths. Many proteins are related to immune and inflammatory responses and have been proven to be associated with bacterial infection. This suggests bacterial infection is one of the processes involved in the creation of dacryoliths. In addition, proteomic analysis revealed that the TFF peptide, considered as the predominant component in previous report [3], was not a major component of the dacryoliths in our study. However, the possibility of the TFF peptide may not be excluded because no histological examination was performed in our study.

The rebamipide-related proteins were characterized with less involvement of bacterial infections and highly enriched biological process. This evidence indicates that the original category of dacryoliths related to the long-term use of rebamipide eye drops may be clarified. It is possible that lacrimal protein alteration may be accelerated by topical rebamipide treatment with less involvement of bacterial infection; however, this requires further study. Whether these results of the proteomic analysis directly represent the features in stiffness and colors of dacryoliths is still unclear. Other factors of inorganic materials may also determine the characters of dacryoliths.

Clinically, we should note the different characters of dacryolith formation with long-term use of rebamipide eye drops. How often dacryoliths occur in patients with long-term use of rebamipide eye drops is questionable; however, rebamipide use may be withheld in patients with NLDO because it may create dacryoliths.

There are several limitations of this study which should be acknowledged. For one, our small sample size did not have the power to determine whether or not a statistical significance existed between the two groups. Also, the samples were obtained only from patients who had dacryoliths; therefore, the results in this study are subject to selective bias from unobserved differences. Secondly, proteomic analysis is basically quantitative. Histopathological assessment is much more qualitative but was not performed correspondingly. Accordingly, a prospective study with larger samples based on rebamipide use needs to be performed using well-matched controls to confirm our findings.

Disclosure Statement

None of the authors has any financial/conflicting interests to disclose.

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