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SHORT COMMUNICATION

Salivary mucin MUC7 oligosaccharides in patients with recurrent aphthous stomatitis

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Abstract
Objectives The aetiology of recurrent aphthous stomatitis remains unknown. In this study, we investigate the composition of oligosaccharides from mucin MUC7 in recurrent aphthous stomatitis as these heavily *O*-glycosylated mucins confer many of saliva's protective properties such as defence against mucosal pathogens.
Materials and methods Unstimulated whole saliva samples were collected from six individuals, three with recurrent aphthous stomatitis and three corresponding sibling, without this condition. Oligosaccharides from salivary MUC7 were isolated and analysed by liquid chromatography-tandem mass spectrometry.
Results The types of oligosaccharides identified in the patients and control subjects were similar; however, statistical evaluation indicated semi-quantitative differences between specific oligosaccharide classes. These changes focused on a reduction in terminal glycan residues including fucosylation, sialylation and sulfation on galactose.
Conclusions This study was able to show differential MUC7 glycosylation in the patients suggesting functional changes to salivary mucins in this condition. The terminal glycans altered in disease have been shown to be important for a range of immunological and bacterial binding roles. Further investigation of these epitopes in a larger study may provide critical insights into the pathology of recurrent aphthous stomatitis.
Clinical relevance MUC7 glycosylation is altered in recurrent aphthous stomatitis. This may change the protective properties of this mucin against mucosal pathogens, which may effect this condition.

Keywords Oral mucosa · Mucin layer · Glycan · Saliva · Aphthous ulcers

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Introduction

Recurrent aphthous stomatitis (RAS) is an oral ulcerative condition of an idiopathic nature. The ulcers are painful and often debilitating for the patients given the recurrent nature of the disease. This chronic inflammatory condition typically commences in childhood or adolescence and decreases around the fourth decade of life. There are three main forms of RAS: minor, major and herpetiform, dependent on the size and the number of ulcers present. Factors suggested to trigger RAS include changes in salivary composition [1], bacteria [2] and genetic susceptibility [3]. RAS is a common disease with an estimated point prevalence of 1.5 %, annual incidence of 20 % and lifetime prevalence of 40 % in the USA [4]. The high prevalence and chronic nature of RAS make the identification of preventative treatment necessary.

Saliva is essential for oral health and is composed of electrolytes, nitrogenous products and proteins including mucins, immunoglobulins and enzymes. Mucins are large glycoproteins, with a high carbohydrate content, attached via the monosaccharide *N*-acetylgalactosamine (GalNAc) to a protein backbone through an oxygen atom on serine (Ser) or threonine

(Thr) residues (*O*-linked glycosylation). GalNAc residues are usually then further extended by a galactose (Gal) residue forming the core 1 family (Galβ1-3GalNAcα1-Ser/Thr (), which serves as a substrate for additional branching with *N*-acetylglucosamine (GlcNAc) to the core 2 family (Galβ1-3(GlcNAcβ1-4)GalNAcα1-Ser/Thr)() (symbols as seen in Fig. 1b). These two cores can then be extended further with additional Gal and GlcNAc residues and terminated with, for instance, fucose (Fuc), as is the case with the blood group ABO epitopes, and/or sialic acid (NeuAc). Mucins are produced by the major and minor salivary glands, with a range of roles including lubrication to aid mastication, speaking and swallowing, physical protection of the oral cavity and the aggregation and attachment of oral microorganisms [5, 6]. Many of these properties are directly provided by the glycans and are determined by the expression of specific glycosyl transferases located in the Golgi apparatus [7]. Due to polymorphisms in these enzymes, genetics play an important role in mucin glycosylation [8, 9].

Mucins, particularly those found in the saliva, have a high turnover rate and therefore respond quickly to altered conditions within the body. Changes in *O*-glycans have been observed in different disease states, particularly those involving an inflammatory response, where the changes in glycosylation can be observed in mucins also indirectly associated with the particular disease [10–12]. Given that mucins and their *O*-glycosylation affect a range of aspects of oral health and their changes in response to infection and inflammation, mucin glycosylation in RAS was investigated. The aim of this study was to identify changes of specific glycan types or families to understand more with regard to the disease mechanisms involved in RAS. MUC7 was selected rather than MUC5B as it is less affected by the blood group antigens which vary the *O*-glycan profile dramatically between patients [13]. Thus, MUC7 allows a clearer comparison between patient samples. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to compare the MUC7 *O*-linked oligosaccharides from patients with RAS and their healthy sibling. LC-MS/MS is a highly sensitive method that is not only able to define the composition of *O*-glycans but also their isomeric structures enabling a detailed analysis. This allows the identification of specific structural changes that may be essential to disease development and progression.

Materials and methods

Study population

Unstimulated whole saliva samples were collected for 5 min from three patients (P1 male, 19 years; P2 male, 19 years; P3 male, 28 years) with RAS and three corresponding siblings (C1 male, 17 years; C2 male, 21 years; C3 female, 25 years) without this condition. All the patients were selected from the referral population at the Clinic of Oral Medicine, Public Dental Health, Gothenburg, the Region Västra Götaland of Sweden during the year of 2013. The study subjects did not use antibiotics or antibacterial mouthwash 3 months prior to sampling, and were tobacco free with no to moderate alcohol consumption. Patients with RAS manifested with symptoms at least once per month during the previous year but did not receive any treatment for their condition at least 3 months prior to sampling. All patients suffered from a combination of minor and major RAS but did not suffer from any other oral mucosal disease. Overall, dental health was not examined in detail, although no patient or control suffered from any severe caries or periodontal disease. No eating, drinking or brushing of the teeth was allowed for 1 h pre-sampling. The Ethical Review Board in Gothenburg approved the study (Dnr 386-10) and written informed consent was obtained from all subjects and controls. The guidelines of the World Medical Association Declaration of Helsinki were followed.

Sample preparation and analysis

Saliva samples (0.3 to 5 mL) were stored at −80 °C. The procedure for sample preparation, isolation and characterisation of MUC7 oligosaccharides has been described previously [14]. Briefly, MUC7 from saliva was isolated by SDS-AgPAGE, blotted to polyvinylidene (PVDF) membranes and *O*-linked oligosaccharides released by reductive β-elimination before liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of released oligosaccharides. Oligosaccharides were assigned based on their parent ion mass and fragmentation spectra, which was compared to previously reported MUC7 structures [14]. Xcalibur™ (Rev.2.0.7, Thermo Fisher Scientific) software was used for semi-quantification of the abundance of each glycan structure as described previously [15]. Relative intensities (RI) were derived by comparison of each glycan or subset of glycans to the total glycan intensity and displayed as relative percentages.

Results

Characterisation of the MUC7 glycosylation in patients with RAS and their corresponding siblings

Mucins MUC5B and MUC7 were detected as two major and well-separated bands both in the unstimulated whole saliva samples from the three patients with RAS and their corresponding siblings (Fig. 1a). The migration and intensity of the two bands between patients and control subjects did not indicate any consistent trends. *O*-linked oligosaccharides were released from excised MUC7 bands by reductive β-elimination and analysed by LC-MS/MS. The glycan profiles

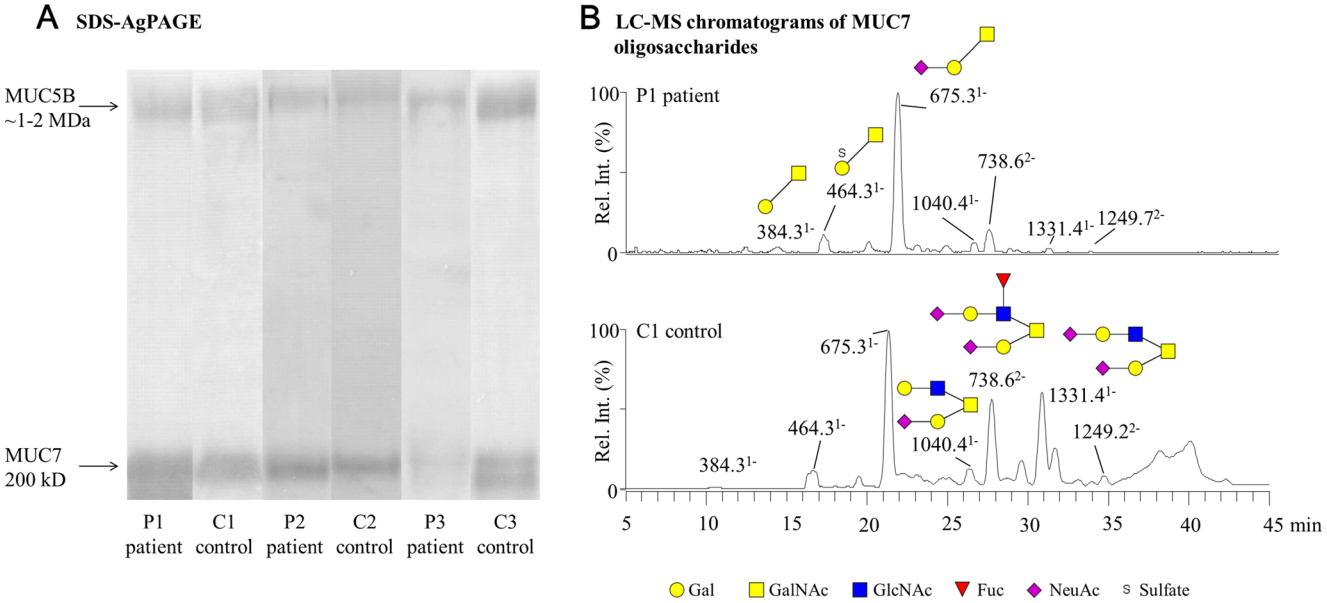

Fig. 1 Salivary mucins and MUC7 glycosylation in recurrent aphthous stomatitis (RAS). **a** Alcian blue-stained mucins from unstimulated whole saliva samples from patients with recurrent aphthous stomatitis (RAS) and their corresponding healthy sibling separated by sodium dodecyl sulfate-agarose/polyacrylamide composite gel electrophoresis (SDS-AgPAGE) and blotted to polyvinylidene fluoride (PVDF) membrane. **b** A base peak liquid chromatography mass spectrometry (LC-MS) chromatogram of oligosaccharides present on MUC7 of patient P1 and control C1. The detected negative ions of each peak are depicted together with structures assigned after MS/MS

of a patient and paired sibling are seen in Fig. 1b. The type of glycans identified in patients and control subjects showed no major differences and were consistent with those previously reported for MUC7 [14]. Glycans of the core 1 family (○—□) and the core 2 ( ) family were identified (symbols in Fig. 1b). Core 1 glycans were identified with the addition of the terminating glycan residues sulfate or sialic acid. A much larger range of core 2 structures of the controls were identified as shown in Fig. 1b including extended and branched glycans with the addition of fucose and the terminating glycan residue, sialic acid, in some cases making up the important sialyl-Lewis x epitope, NeuAcα2-3Galβ1-3(Fucα1-4)GlcNAcβ1-(-).

Investigation of terminal residues sulfate and sialic acid in patients with RAS

Given that the types of glycan structures identified in the patients with RAS and control subjects were similar, further semi-quantitative analysis was undertaken to identify differences in this small sample set. Quantitative differences were found between the glycan profiles both between patients and controls and between individuals (Fig. 1b). The intensity of each individual glycan structure was recorded. This analysis allowed the calculation of the average composition of each oligosaccharide found in individual samples, allowing the comparison of controls and patients (Fig. 2a), as previously described [16]. After performing this exercise, *O*-glycomic analysis was able to identify glycan changes in RAS.

The combined *N*-acetylhexosamine (HexNAc) residues *N*-acetylglucosamine (GlcNAc) and *N*-acetylgalactosamine (GalNAc) as well as the hexose (Hex) residue galactose (Gal) showed no differences between the patients and control subjects. These residues define the level of extension, showing that the oligosaccharide backbone was the same between the two groups. However, a trend is apparent for the decoration of this backbone by fucose, sialic acid and sulfation, the terminating monosaccharides. Overall, there is a reduction of these monosaccharides in the patients, especially prominent for sialic acid, where a large reduction was observed. This reduction of sialic acid, in combination with a less significant fucose reduction, suggests a reduced ability to form the sialyl-Lewis x epitope. Specific analysis comparing sialic acid and the sialyl-Lewis epitope showed a clear trend of the reduction of sialylation in general and the sialyl-Lewis x epitope in particular, in patients with RAS (Fig. 2b).

Sulfation is a terminal residue of low abundance on MUC7. However, given the interesting results of the other terminal glycans, the amount and nature of sulfation was addressed using the LC-MS and LC-MS/MS data. Sulfation was found either attached to the 3 position of Gal or the 6 position of GlcNAc among the identified structures (Fig. 2c). A statistically significant decrease of structures with sulfation at the 3 position of Gal was found, but the sulfate linked to the 6 position of GlcNAc did not show a significant difference, rather trending towards an increase in the patient samples. The most prominent structure carrying this Gal 3-sulfation was the core 1 glycan. This structure was decreased

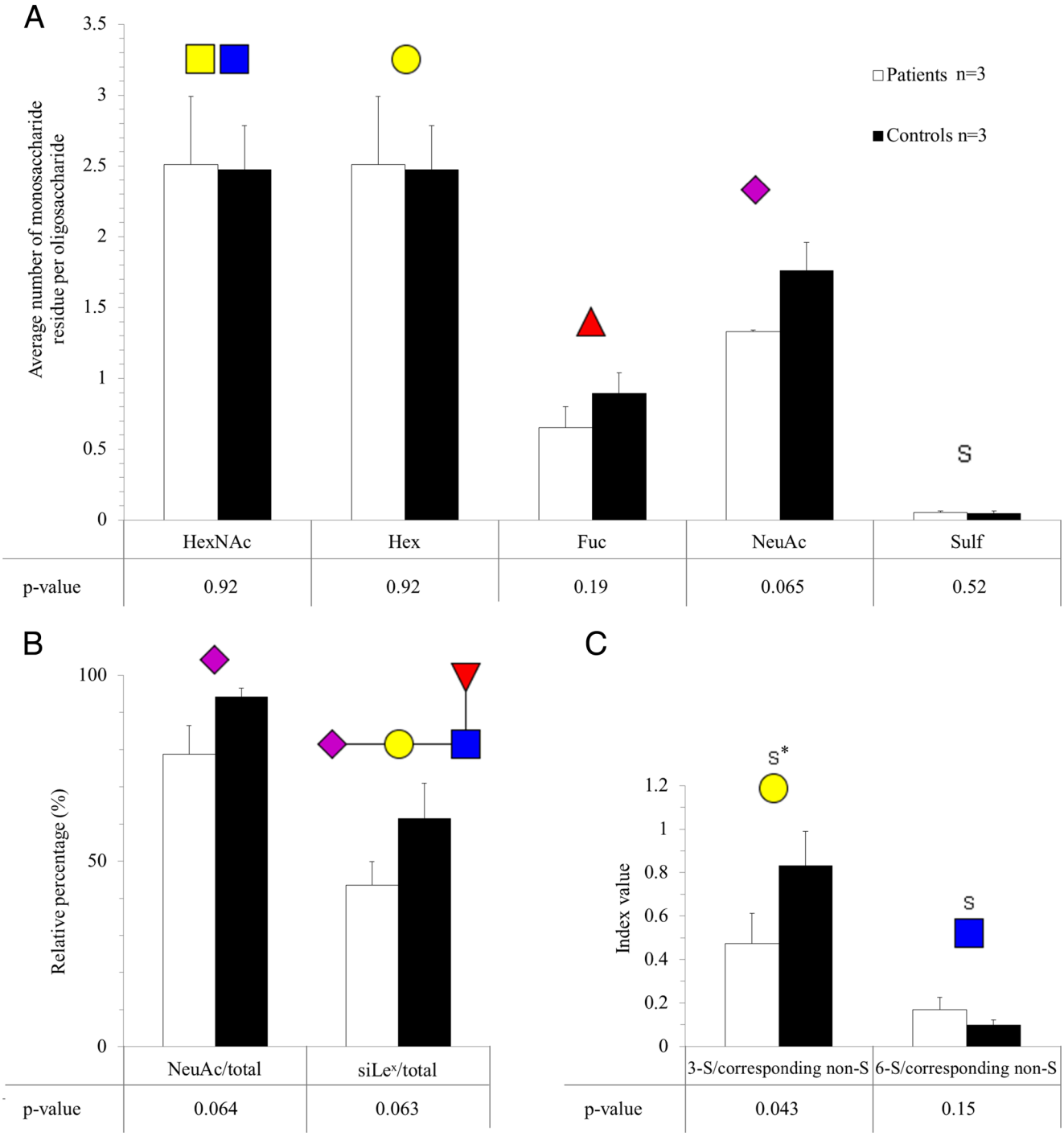

Fig. 2 Comparison of the mass spectrometric intensities of detected oligosaccharides. **a** The average number of monosaccharide residues per oligosaccharide between pooled patients and pooled controls, based upon relative mass spectrometer intensities (RMSI) of oligosaccharide peaks in liquid chromatography mass spectrometry (LC-MS) chromatograms. HexNAc, *N*-acetylhexosamine (terminal *N*-acetylgalactosaminitol) in the reduced *O*-linked oligosaccharides is also included. *Hex* hexose, *Fuc* fucose, *NeuAc N*-acetylneuraminic acid (sialic acid), *Sulf*, sulfate. **b** The relative percentage of sialylation and sialyl-Lewis x of patients and controls, based upon RMSI. *Si-Le*^{x} sialyl-Lewis x, *NeuAc* sialic acid-containing structures, *total* sum of RMSI of all structures. **c** Sulfation in patients and controls. *3-S* sulfated on hexose, *6-S* sulfated on non-reducing end *N*-acetylhexosamine, *corresponding non-S* corresponding non-sulfated oligosaccharide structure. Index value = RMSI of oligosaccharide structure divided by RMSI of its non sulfated precursor. *Statistically significant difference between patients and controls (P = 0.04). The statistical dispersion used is standard deviation

in patients and was the only intact oligosaccharide that showed a statistically significant difference (P = 0.04). This analysis shows how essential the structurally specific LC-MS/MS analysis was to identifying the differences between these

samples and just how important this in-depth analysis is to understanding disease pathology.

Overall, this study was able to show that there is a decrease in terminal epitopes on MUC7 in patients with RAS. This trend is especially clear in sialic acid, the sialyl-Lewis x epitope and sulfate linked to the 3 position of Gal.

Discussion

This study was able to show that the terminal oligosaccharides attached to MUC7 were altered in patients with RAS. Sialic acid was one of these altered residues with a reduction observed in patients with RAS. This monosaccharide is part of a range of oligosaccharide structures including sialyl-Lewis x, which showed a trend towards a reduction in RAS (Fig. 2b; $P = 0.06$). The sialyl-Lewis x epitope is thought to be of importance in the homing of leukocytes [17, 18]. More specifically, it has been suggested to be involved in lymphocyte recirculation in the high endothelial venules (HEV) of lymph nodes [19]. A reduced content of sialic acid and the sialyl-Lewis x epitope on MUC7 may indicate an overall reduction in these structures in the oral cavity including the HEV. This may result in the ineffective clearance of oral bacteria as fewer leukocytes attach to mucosa-associated lymphoid tissue in the oral cavity. In fact, MUC7 has been shown to adhere oral streptococci and this adherence is lost when sialic acid residues are removed from MUC7 oligosaccharides [20]. Thus, our finding further strengthens the hypothesis that patients with RAS have a reduced ability to clear oral pathogens.

Our data suggest that patients with RAS have a less complex type of glycans compared to control subjects. This provides further hypotheses and insight into understanding the disease. Given that it has been suggested that bacteria may be ineffectively cleared in patients with RAS, it is possible that there is an abundance of bacterial oligosaccharide degrading enzymes reducing glycan complexity. Alternatively, the changes in glycosylation may result from an inflammatory response in the mucus layer altering the expression of the oligosaccharides. In a recent study, a method was developed that was able to specifically identify glycans from salivary MUC7 that were degraded, most likely by bacterial enzymes [21]. The use of this method on RAS samples would help to differentiate between bacterial degradation and inflammatory mediated glycosylation alterations.

Sulfation was addressed in detail to ascertain if this terminal residue is also altered in RAS. Although a difference was not observed for 6-linked sulfate, a significant reduction in the sulfate 3-linked to Gal was apparent in RAS samples, particularly when this epitope was part of the sulfated core 1 structure. Differential changes in sulfation are possible as two different enzymes are responsible for sulfate attachment to these two residues [22]. The core 1-sulfated glycan has been shown to be upregulated on MUC7 in patients with the chronic systemic inflammatory disease rheumatoid arthritis, indicating that this glycan is reactive to the immune environment [12]. Here, the results show that patients with RAS also have changes in this epitope. However, as a reduction was seen, a disease mechanism other than chronic inflammation is suggested.

Overall, this investigation showed that terminal *O*-glycan epitopes are altered in RAS. Further specific investigation of these important epitopes in a clinical setting will provide a greater insight into the pathogenesis of this disease and ultimately lead to the development of much needed preventatives. The recent improvements in sensitive quantitative large-scale-targeted *O*-glycomics make this type of large-scale study now a realistic possibility [12].

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Conflict of interest The authors declare that they have no competing interests.

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