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Longitudinal investigation of salivary proteomic profiles in the development of early childhood caries

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ABSTRACT

Objectives: To investigate differentially expressed salivary peptides in the development of early childhood caries (ECC) in 3–4 year-old children.

Materials and methods: Eighty-two caries-free children at baseline were followed-up for 1 year, during which period 15 of them had developed ECC (Group C), whilst another 15 cases out of the 31 individuals who remained healthy were marked as Group H. Stimulated whole saliva samples were collected at 0, 6 and 12 months, and analyzed using weak cation exchange magnetic beads combined with matrix assisted laser desorption/ionization time-of-flight mass spectrometry. Corresponding peptide mass fingerprints were obtained to develop a discriminating model for ECC development. Q-Exactive mass spectrometry was then performed to identify the possible proteins where these peptides might derive from.

Results: Nine peptide peaks were found to be significantly different in Group C among the three sampling time points and might correlate with development of caries. Levels of three of them increased over time, whilst that of the other six decreased gradually. We chose three peptides (1346.6, 2603.5 and 3192.8 Da) which exhibited the best capability of classification, to establish a model for children at high risk of caries. One peptide (1346.6 Da) was identified to be salivary histatin-rich peptide.

Conclusions: Our results indicate that peptidomic methods can be applied to help identify new candidate biomarkers for the occurrence and development of ECC.

Clinical significance: The change of salivary peptides may be an indicator of ECC, facilitating more effective measures to be taken in prevention of this disease.

1. Introduction

Early childhood caries (ECC) is one of the most common oral infectious diseases in children, affecting 66% of 5-year-olds in China [1]. Deciduous teeth are more vulnerable to caries, making the disease progresses rapidly. The incidence of ECC in 3–4 year-old children was 56.2% in Alabama [2] and 43.6% in some cities of China [3]. ECC has a negative effect on children's oral and general growth [4] and has a great influence on the oral health-related quality of life of 2–5 year-old children, as well as their parents [5]. Even after treatment, the incidence of ECC was significantly higher in children with a previous history of this disease [2]. Thus, there is an urgent need for diagnosis at an early stage to be helpful for taking appropriate preventive measures before apparent clinical lesions occur.

Saliva is the peripheral environment of teeth and contains a lot of molecular biological information. Recent advances adopting high-throughput technologies have allowed for better understanding of the complexity of many diseases at the protein level. So far, almost 3390 proteins have been identified in the human oral cavity [6]. Saliva, as a common body liquid, can be collected more easily and non-invasively than serum, and has been extensively used in the early diagnosis of oral diseases (such as dental caries and periodontal disease), cancer, diabetes and other systemic disorders [7–9].

Caries-associated proteins in saliva were studied by a number of

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researchers. In these studies, different levels of expression of proteins were found between caries-active group and healthy control. Previous studies mainly used two-dimensional electrophoresis (2-DE), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and liquid chromatography-mass spectrometry (LC–MS) to separate proteins by molecular weight first, followed by mass spectrometric analysis. Some phosphorylated peptides correlated with caries-free subjects, whereas the expression of amylase, immunoglobulin A and lactoferrin appeared to be higher in individuals with caries [10–12]. Conventional methods, however, are time-consuming, costly and technically demanding, and may miss some low-expressed but essential peptides. Improvement in high-throughput proteomic and peptidomic approaches facilitates new ways to elaborate and illustrate the etiology and pathogenesis of disease. Hart et al. found that the combination of analysis of salivary proteins and plaque microbiota led to better predictive models for caries-active and caries-free patients [13]. Our previous cross-sectional study detected eleven protein peaks that were significantly different between 3-year-olds with severe early childhood caries (s-ECC) and healthy children, using weak cation exchange magnetic beads (WCX) combined with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) [14]. Another study by our research group aimed to identify any differences after s-ECC children been completely treated. In that study, ten children with s-ECC were sampled before, 1 and 4 weeks after dental treatment. Seven peptide peaks were differentially expressed when comparing among the three time points, and two of them were identified to be segments of histatin-1 [15]. However, few studies have investigated the longitudinal variation of salivary peptides associated with the development of pediatric dental caries.

The aim of the present study was to explore the salivary protein profile differences associated with the development of caries in different follow-up periods in preschool children. We assumed that certain salivary peptide biomarkers would exist to be a manifestation of dental caries status.

2. Materials and methods

2.1. Ethics statement

This study was ethically approved by Peking University Biomedical Ethics Committee (issuing number: PKUSSIRB-2013060) and followed the principles of the Declaration of Helsinki. Parents of the pediatric participants have all signed the informed consent form before the start of the study.

2.2. Subjects

According to previous studies, at least 10 subjects in each group (ECC and control) were needed. From the epidemiological literatures in China, we have found that the incidence of primary caries in 3–4 year-old children was about 43.6% (60% in a two-year study [16]). As we set the lost rate to 20% for each time point, at least 72 individuals were required to meet our need as a rough estimate. Hence, eighty-two preschool children aged 3 years old in one kindergarten in Beijing, China, who were found to be caries-free on examination (total number of decayed, missing and filled tooth surfaces = 0), were initially recruited at baseline in May 2014 and September 2014. Then, they were followed up every 6 months approximately and this would last for 1 year. The oral health status of each individual was determined by one professional dentist. The diagnostic criteria were according to the WHO Oral Health Surveys Basic Methods (5th edition, 2013). 36 children were excluded at the other two time points (details shown in S Table 1). Finally, 15 children who both meet diagnosis of ECC and the inclusion criteria at the 6- or 12-month time points were classified into the experimental group (Group C). The other 31 remained caries-free, and 15 of them (who were age- and sex-matched) were selected to be the healthy group (Group H). All study subjects were systemically healthy. Those with influenza or an upper respiratory tract infection and those who had received antibiotic therapy within 1 month of any sampling time were excluded from the study. Those who were absent at any time point were excluded. S Table 2 shows detailed information and caries status of the subjects.

2.3. Saliva collection and processing

All individuals were instructed to rinse orally with water after breakfast and then rest for 10 min before saliva collection at 8:30 a.m. Stimulated whole saliva samples were collected for 5 min. The 1.5 mL saliva samples were immediately placed on ice. Insoluble material, cells and debris were removed by centrifugation at 10,000 × *g* for 10 min at 4 °C. The supernatants were collected, and 1 mM ethylene diamine tetraacetic acid (Sigma, St. Louis, MO, USA) and 1 mM phenylmethyl sulfonyl fluoride (Sigma) were added to inhibit protease activity. Protein concentrations were measured by the Lowry method and the ELx808 Protein Assay (BioTek, Hercules, CA, USA). Supernatants were kept at −80 °C separately until further analysis.

2.4. WCX fractionation and MALDI-TOF MS

All saliva samples were fractionated using WCX magnetic beads (MB) (Bioyong Tech). Samples were purified and isolated by the following steps: (1) The beads were turned upside down to mix them well before use; (2) 20 μL of beads, 150 μL of MB-WCX binding solution, and 10 μL of salivary sample were gently mixed and incubated for 5 min at room temperature; (3) the tubes were placed on the MB separation device (Bioyong Tech) for 1 min, and the beads adsorbed onto the tube wall; (4) the supernatant was then removed, and the beads were washed by mixing thoroughly with 150 μL of MB washing solution. Two minutes later, the tubes were placed on the separation device for 1 min; (5) step 4 was repeated and the supernatants removed; (6) 10 μL of MB elution solution were added and mixed thoroughly with the beads, then the tubes were put on the separation device for 2 min; (7) the clear supernatant was transferred into a fresh tube, and the peptides were analyzed directly on a ClinTOF instrument (Bioyong Tech) or stored at −20 °C and analyzed within 24 h.

The matrix solution was 8 mg/mL CHCA in 50% acetonitrile/0.1% TFA/49.9% deionized water. First, 1 μL of purified peptide solution was spotted onto a MALDI-TOF MS target from ClinTOF. After drying at room temperature, 1 μL of matrix solution was spotted to cover the sample, which was dried again before analysis. MALDI-TOF MS was performed using a ClinTOF instrument. Calibration of the MALDI-TOF MS was done by a three-peptide mixture (monoisotopic molecular weights of 1533.8582, 2465.1989, and 5730.6087 Da; Sigma product numbers P2613, A8346, and I6279, respectively) before analysis. Profile spectra were acquired from an average of 400 laser shots per sample. The mass range 1000–10,000 Da was collected. Each sample of saliva was analyzed three times, and the mean values of the intensities and masses of each peak were used for analysis.

2.5. Data processing and statistical analysis

All the spectra obtained from the saliva samples were analyzed using BioExplorer (Bioyong Tech) to obtain the mean relative peak intensities; then baselines were subtracted, spectra normalized using total ion current, and peak *m/z* values and intensities determined in the mass range 1000–10,000 Da. A signal-to-noise (S/N) ratio > 6 was required. To align the spectra, a mass shift of no more than 0.1% was determined. The peak area was used for quantitative standardization. The k-nearest neighbor (KNN) algorithm in this software suite was used to establish the best pattern of differentiation model for the development of ECC. Analysis of variance (ANOVA) or Kruskal-Wallis test was used to identify differences in protein levels of saliva samples in the two

groups among the three time points. The data were analyzed using the BioExplorer statistical package. $p < 0.05$ were considered to indicate statistical significance.

2.6. Identification of potential peptide biomarkers by nano-LC/ESI–MS/MS

First, the supernatants were processed by WCX as described above, and then the impurities like beads were removed by centrifugation at 10,000 × *g* for 20 min at 4 °C. Before LC–MS/MS, the supernatants were purified by a 0.22 μm-level filter. Each sample (20 μL) was separated by nano-HPLC system EASY-nLC1000 (Proxeon Biosystems, now Thermo Fisher Scientific). The flow rate of the equilibrated BEH nano ACquity column 100 × 100 mm was 400 nL/min.

Tandem mass spectrometry (MS/MS) was then performed using Q-Exactive mass spectrometer (Thermo Fisher Scientific). The analyzing time was set as 120 min, and the voltage of ion source was 3.5 kV. Fragmentation of peptides was performed using higher-energy collision dissociation (HCD). Mass-to-charge ratio (*m/z*) of the peptide fragments was collected by gathering 20 fragmented fingerprint after each single full scan. Survey scans were acquired at a resolution of 70,000 at *m/z* 200. Resolution for HCD spectra was set to 17,500 at *m/z* 200. After the MS/MS figure was input to the PD software (Proteome Discoverer 1.4, Thermo Fisher Scientific), an initial screening was carried out. The parameters used were shown below: range of molecular weight of parent ion was 350–6000 Da, the minimum number of peaks in MS/MS figures was set as 10, and the thresholds of S/N ratio was 1.5. After initial screening, the mass spectrum was searched by Mascot software (version: 2.3.2) based on fixed modification (Cysteine carbamidomethylation) and variable modification (Oxidation of methionine and N-acetylation of protein). Then we used database NCBInr 20120419 (17893860 sequences; 6141683785 residues) and Percolator method (false discovery rate [FDR] ≤ 1%; accuracy of peptide tol: 20 ppm) for quantitative analysis of the spectrum.

3. Results

3.1. Sample information

Finally, thirty children (mean age 43.9 ± 5.5 months at baseline) were selected in our peptidomic analysis, of whom 15 developed caries (Group C), whilst the other 15 remained healthy (Group H). In the Group C, nine children developed caries after 6 months, whereas the disease came up in other six participants at the time point of 12 months. However, two children from the Group C were completely treated (without active caries) at the final stage, so we removed their 12-month samples from the analysis. Hence, there were 88 samples in total. Detailed subject information and caries status were shown in S Table 2.

3.2. Peptide profiles

Salivary peptidome fingerprint peaks from each patient were characterized based on the maximum intensity within a particular *m/z* range.

An average of 117 peptide mass peaks was detected in the Group C. When compared at 0, 6 and 12 months, the peak intensities differed significantly for 35 peptides ($p < 0.05$). Twenty-six of them were excluded because almost the same results for these peaks were found in Group H. Thus, we observed nine peaks that were potentially related to the development of caries: 1066.9, 1346.6, 2140.6, 2225.3, 2234.6, 2603.5, 3049.6, 3058.3 and 3192.8 Da (Figs. 1 and 2). Three of them gradually increased over time (1346.6, 2225.3 and 2234.6 Da; Fig. 2c), whereas the mass peaks for the peptides at 1066.9, 2140.6, 2603.5, 3049.6, 3058.3 and 3192.8 Da were highest at baseline, and then turned to lower intensity at the later time points (Fig. 2a and b). Moreover, almost all of the samples in Group C showed a time-dependent decrease in the 3192.8 Da peptide (Fig. 2e; please note that subjects on the left side of the red dotted line turned to caries-active at the 12-month time point, whilst those on the right side fell in caries status by the 6-month time point). Apart from the 3049.6 Da peptide, the other eight peaks were not detected in healthy controls. In the Group H, little difference was observed in the abundance of the 3049.6 Da peptide; it stayed at a relatively high level at 0 and 6 months, with a slight decrease at the latest time point (Fig. 2d).

Five mass peaks (the 1066.9, 1346.6, 2603.5, 3058.3 and 3192.8 Da) in Group C were selected for clustering analysis. The relative intensities of the five peaks in the samples were arranged by unsupervised, Ward-linkage hierarchical clustering using standard correlation as a distance metric between 0 and 6 months (Fig. 3a), and 0 and 12 months (Fig. 3b). In Fig. 3a and b, rows represented samples, whilst columns showed *m/z* peaks by average molecular weight. Considering the peptides which could only be detected in the caries group, the 1346.6, 2603.5 and 3192.8 Da peptides exhibited the best separation of the three time points (Fig. 4). Figs. 3 and 4 showed good separation of the samples in Group C from the three time points, indicating that the fitting results were satisfactory. Thus, the peaks at 1346.6, 2603.5 and 3192.8 Da were selected as candidate biomarkers for ECC.

With Q-Exactive mass spectrometry, two of the nine differentially expressed peptides (experimental *m/z* values: 1346.6 and 2225.3 Da) were successfully identified to be salivary His-rich peptide and PITSLRE protein kinase beta SV1 isoform, respectively (S Table 3).

4. Discussion

Caries-susceptible children may have a greater possibility of getting infected in both the primary and the permanent dentition. Even though, dental caries is reversible in early stages, so prompt detection and prevention is of great importance. Previous studies have indicated that salivary flow rate, characteristics and components are related to the status of caries [17]. With significant advances in proteomic and peptidomic techniques, salivary peptides have been widely investigated to explore their relationship with dental decay, but without confirmative conclusions [18–20]. MALDI-TOF MS is a rapid, high-throughput, inexpensive, and reliable technique with high sensitivity and resolution, and it has been used to analyze peptides in many oral and systemic diseases, such as Sjögren's syndrome [21,22], ovarian cancer [23], gastric cancer [24] and lung adenocarcinoma [25], as well as orthodontic treatment duration [26] and dental caries [14,15]. In the present study, magnetic beads were used in the process of sample preparation and purification to remove high-abundance proteins (such as amylase, albumins and IgGs), for better observation of the remaining low-abundance proteins and their detection [27,28].

The present study was performed in a group of young children who were an at-risk population for natural caries development. In this study, three of the nine peaks gradually increased during the process of caries occurrence and development (1346.6, 2225.3 and 2234.6 Da; Fig. 2c). We speculated that the increase might be under the influence of the cariogenic microorganisms. For example, Vitorino et al. found a high number of peptide fragments in their caries-susceptible group, suggesting a high proteolytic activity [11]. Smith et al. showed that glucosyltransferase B significantly related to the severity of s-ECC [29]. With the development of caries, the balance of the natural oral microbiota becomes disturbed and cariogenic microbiota become predominant, and this condition gradually changes as the damage progresses. As a result, proteins produced by bacterial metabolism may change simultaneously.

By contrast, the peptides of 1066.9, 2140.6, 2603.5, 3049.6, 3058.3 and 3192.8 Da were at a relatively high level at baseline, and then decreased at the other two time points (Fig. 2a and b). The intensity of the 3192.8 Da peptide tended to decrease with time in all the 15 subjects of the caries group, albeit with some individual variation (Fig. 2e). These peptides might originate from host secretions that

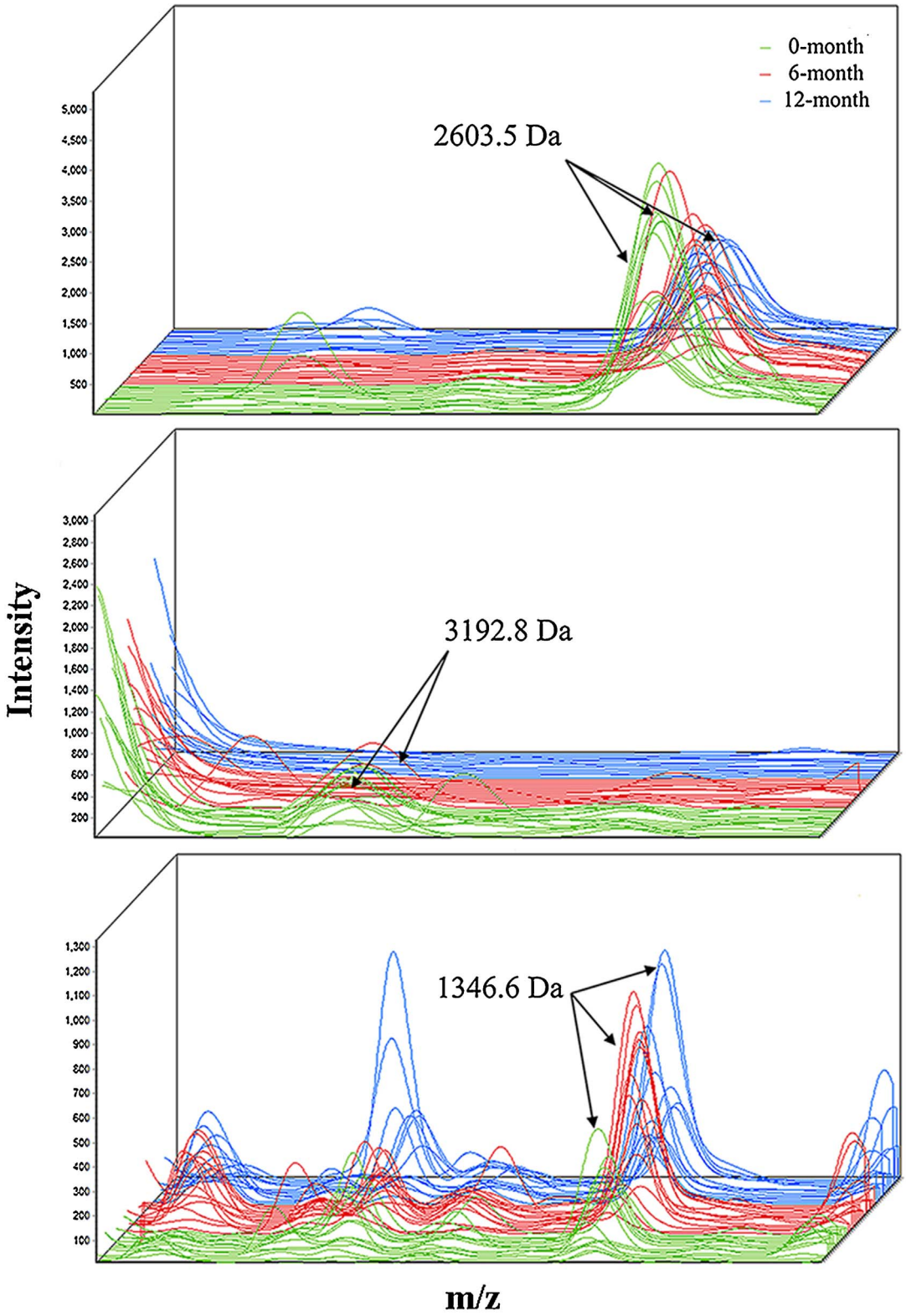

Fig. 1. Three-dimensional *m*/*z*-ratio-intensity maps showing intensities of peptides at 2603.5, 3192.8 and 1346.6 Da, which had significantly different expression. Green represents the baseline; red, 6 months; blue, 12 months. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

protect hard tissues of the teeth. For instance, some phosphorylated proteins that participate in the formation of acquired pellicles are considered to be capable of maintaining tooth integrity [10–12,30]. As mentioned above, the changes could also result from alternation of the predominant microbiota.

The 3049.6 Da peak was observed in the Group H at a relatively stable level, much higher than that in the Group C at 6 and 12 months (Fig. 2d). This variation would fit the hypothesis that this peak arises from a protective peptide. A slight decrease was observed at the 12-month point of the Group H, indicating some of the members in that group might be at risk of future development of caries. However, the other eight peaks were not observed in Group H, implying that these

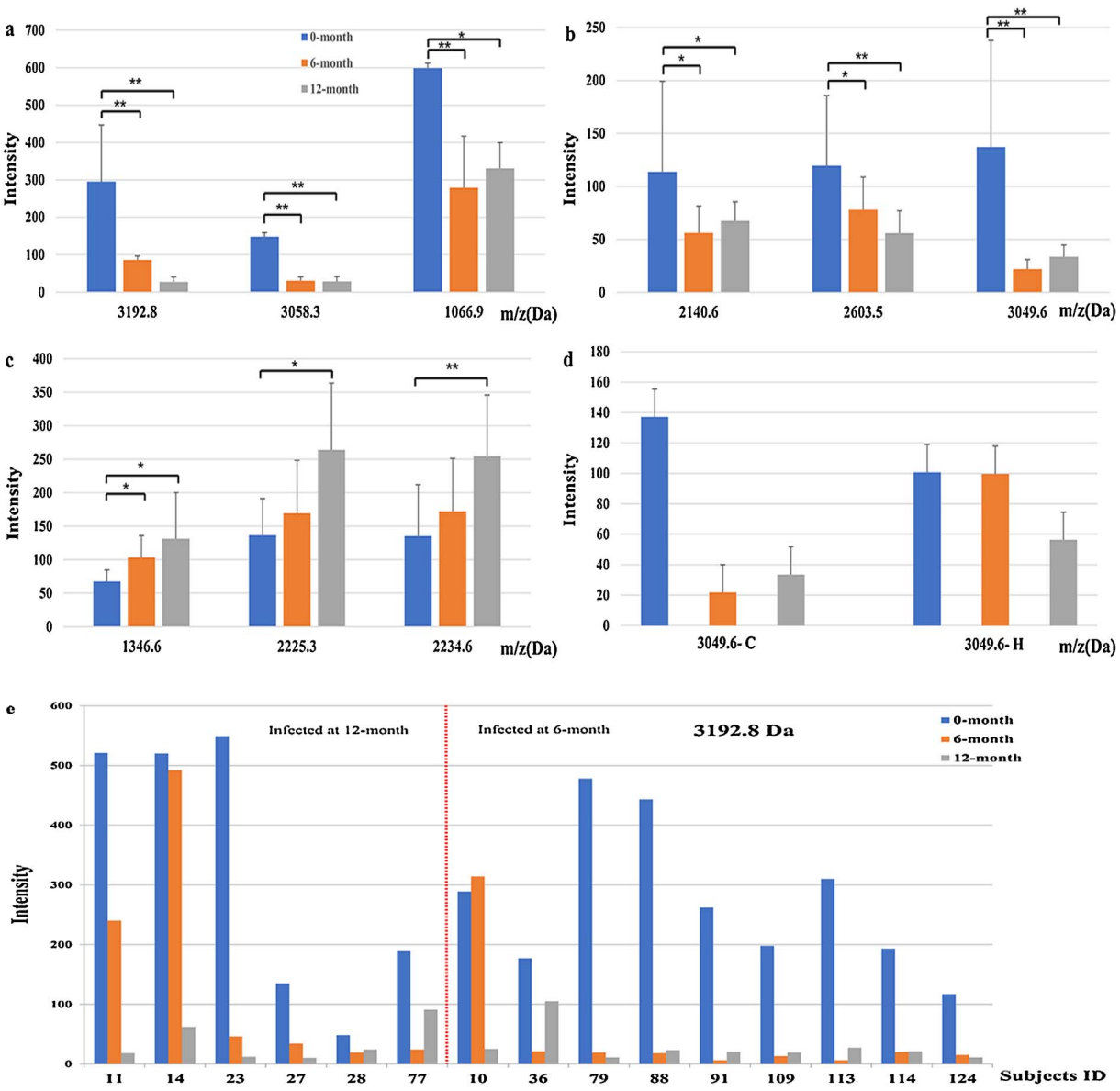

Fig. 2. Column view of mass spectral intensities in Group C (the group with caries). **(a–c)** Time-dependent increase of peptides of 1346.6, 2225.3 and 2234.6 Da, and decreasing intensities of peptides of 1066.9, 2140.6, 2603.5, 3049.6, 3058.3 and 3192.8 Da (* $p < 0.05$; ** $p < 0.01$). **(d)** The intensity of the 3049.6 Da peptide in Group C and H, showing no significant difference at the three time points in Group H. **(e)** Histogram of the mass spectral peak intensity of the 3192.8 Da peptide. Almost all the patients from Group C showed a decreased level of this peptide over time, which appeared to be related to the development of caries.

peptides may be specifically related to dental caries.

Two previous studies by our group identified different peptide peaks between caries-susceptible and caries-free children. However, the experimental designs were different from each other and also not the same as the present study. One was cross-sectional [14], and the other [15] investigated a group of children going from caries status to non-active caries status after treatment. It is also implied that the different stages of the disease resulted in the varied expression levels of salivary peptides. Furthermore, the participants came from different kindergartens, in which the dietary structure and oral hygiene practices were distinct to some extent. Proteins are prone to be affected by such circumstances, adding to the difficulty in achieving consistency in caries-related research.

Among the nine peaks we identified here which may be indicative of caries formation, five peaks (the 1066.9, 1346.6, 2603.5, 3058.3 and 3192.8 Da) showed good clustering before and after development of caries (Fig. 3), suggesting these peptides might be relevant to caries. Among them, the 1346.6 Da, 2603.5 Da and 3192.8 Da peptides showed the best classifying capability (Fig. 4). As a consequence, we chose the 1346.6 Da, 2603.5 Da and 3192.8 Da peptide peaks as candidate biomarkers for ECC development. The peptides of 2603.5 Da and 3192.8 Da showed a high expression level at baseline and then decreased at the other two time points, while the 1346.6 Da revealed the opposite trend. However, all the three peptides were not detected in the control group, which might indicate that those children were less sensitive to the disease. The detection of these peptides and the showing up of corresponding trends could be a signal of danger.

With Q-Exactive mass spectrometry, we identified two peptides, which experimental *m/z* values were 1346.6 and 2225.3 Da. The former one was predicted to be salivary His-rich peptide, whilst the latter was considered as PITSLRE protein kinase beta SV1 isoform. His-rich peptides, namely histatins, are a family proved to have antimicrobial and antifungal activities. They are secreted by parotid and submandibular glands. The most common isoforms in saliva are Histatin-1, −3 and −5 (HTN-1, 3, 5), taking up 85% of the overall content of histatins. Moreover, they are the essential components of acquired enamel

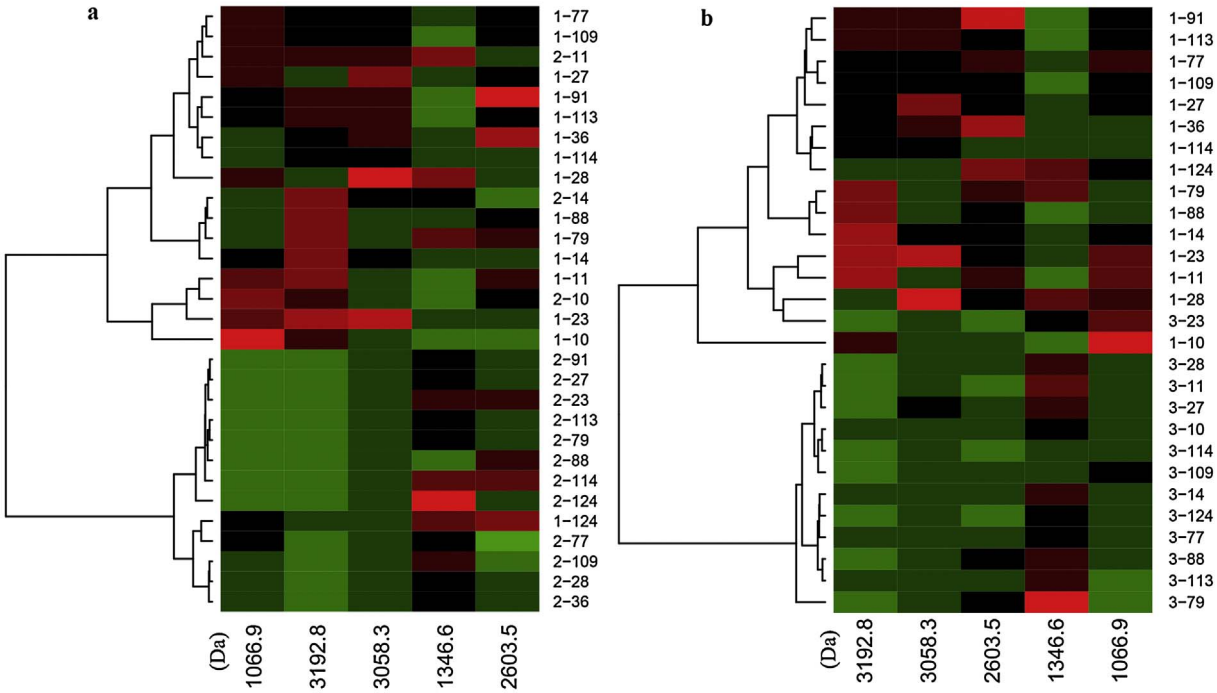

Fig. 3. The clustering analysis of five peaks (the 1066.9, 1346.6, 2603.5, 3058.3 and 3192.8 Da) in their distribution between subjects in Group C at **(a)** 0 and 6 months, and **(b)** at 0 and 12 months. In the column labels, "1" represents a sample from baseline; "2", a sample from 6 months; "3", a sample from 12 months. The numbers behind "-" represents the ID of the subjects.

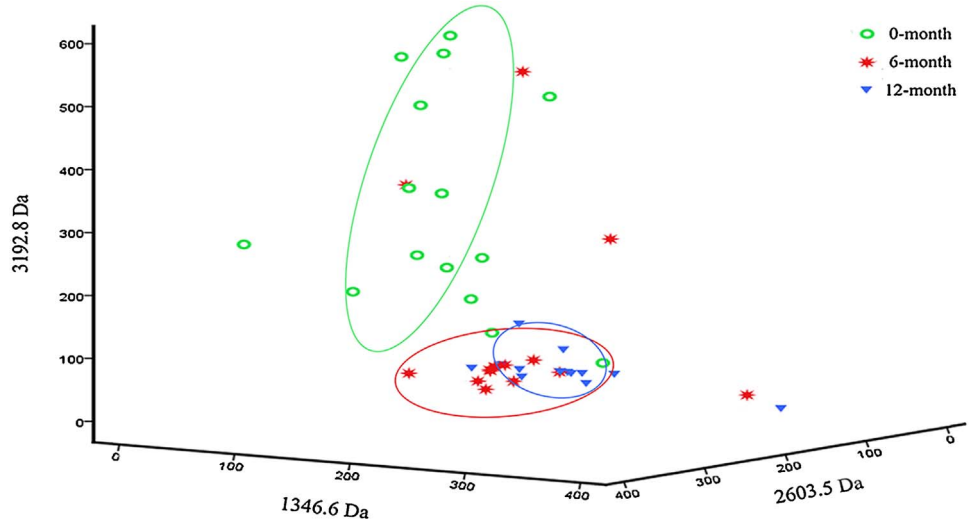

Fig. 4. Scatter plots of the subjects in Group C at 0, 6 and 12 months, established by combining the peptides at 1346.6, 2063.5 and 3192.8 Da.

pellicle. In this study, the tendency of the 1346.6 Da peptide was consistent with many previous studies. Jurczak et al. [31] found that the concentration of HTN-5 significantly increased in ECC group compared to caries-free children, and correlated with the progression of the disease. Gornowicz et al. [32] demonstrated that adolescents with high severity of caries (DMF > 11) had significantly increased level of HTN-5 compared to those with lower intensity of caries. Colombo et al. [33] reported positive correlation between HTN-5 and Streptococci mutans counts. HTN-5 has also been reported to be most active in inhibition and elimination of candida organisms, which can damage DNA and membranes of cell organelles, leading to the fungal and bacterial cell death [34]. The 1346.6 Da peptide has the same 10-amino-acid sequence as a part of HTN-5, suggesting that this peptide has the possibility to be derived from HTN-5. In addition, we found PITSLRE protein kinase beta SV1 isoform might be candidate biomarker as well. PITSLRE, also known as cyclin-dependent kinase 11 (CDK11), is a serine/threonine protein kinase which seems to play numerous roles in transcription, RNA processing, regulating cell-cycle progression, cytokinesis, and apoptosis [35]. Unfortunately, no relevant literatures have reported the effect of PITSLRE in oral diseases.

Some limitations of the present study need to be addressed. First, stimulation of saliva tends to modulate pH value of the fluid and dilutes the concentration of proteins of interest [36] as well as some other analytes [37]. Second, the different sources of the peptides, the dynamic transformations after been secreted, and the protein databases that are far from completeness, will all make the identification of all the peptides much challenging. Third, mass spectrometry methods may have some impacts on the stability of the results. In further studies, a larger sample size is needed to confirm the relationship between dental caries and salivary proteins. In addition, further validation of the

candidate peptides can provide stronger evidence. It sounds that saliva-based protein profile research is inherently a bit complex, however, with the development of proteomic and peptidomic technologies and the enrichment of protein databases, we expect to be able to carry out more convincing studies on the intrinsic mechanisms of dental caries and the contributions of salivary proteins to this disease. Functional analysis of the identified biomarkers is also helpful for us to better understand ECC and thus take more effective measures on early diagnosis and prevention strategy.

5. Conclusions

Our results indicate that peptidomic methods can be applied to help identify new candidate biomarkers for ECC. Three peptides of our discriminating model, namely 1346.6, 2603.5 and 3192.8 Da, might be indicators of developing ECC. Among them, the 1346.6 Da peptide was identified to be His-rich peptide. In the future, we hope that children at risk of dental caries can be screened out by detecting some specific salivary peptides (e.g. in form of a testing strip), and this disease can be controlled by more effective prophylactic measures.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jdent.2017.04.006.

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