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Analysis of salivary exosomal proteins in young adults with severe periodontitis

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Abstract

Objectives: Salivary exosomes harbour numerous constituents associated with oral and systemic diseases. However, no reports addressed components of salivary exosomes in patients with periodontitis. Our study aims to explore salivary exosomal proteins in young adults with severe periodontitis (SP) and to analyse the relationships between different proteins.

Materials and Methods: We collected saliva from 11 young adults with SP and 11 periodontally healthy subjects. After isolation of salivary exosomes, liquid chromatography–tandem mass spectrometry (LC-MS/MS) was used to analyse proteins. Gene ontology analysis was performed based on GeneCodis, and interaction network analysis for unique salivary exosomal proteins was performed by STRING.

Results: Twenty-six proteins were identified only in the SP group, and 58 proteins were identified only in the healthy group. Gene ontology analysis revealed that innate immune response, cytolysis and complement activation were highly enriched in the SP group. Interaction network analysis showed that the correlations among immune-related proteins (e.g. complement components and chemokine (C-C motif) ligand 28) were significant in the SP group. C6 proteins expressed only in the SP group were evaluated by Western blotting.

Conclusions: Salivary exosomes from periodontitis patients are enriched immune-related proteins that might participate in the immune response during the development of periodontitis.

KEYWORDS

exosomes, immune response, liquid chromatography–tandem mass spectrometry, saliva, severe periodontitis

1 | INTRODUCTION

Exosomes are small spherical vesicles measuring approximately 30–100 nm in diameter (Théry, Ostrowski, & Segura, 2009) and can be secreted by multiple cell types. Moreover, exosomes are found in various biological fluids, including serum, urine, saliva, breastmilk and amniotic fluid (Record, Subra, Silvente-Poirot, & Poirot, 2011). Because mRNAs, miRNAs, proteins and other

cellular constituents can be protected by exosomal lipid membranes (Balaj et al., 2011; Melo et al., 2015; Vlassov, Magdaleno, Setterquist, & Conrad, 2012), exosomes can carry different constituents to neighbouring or remote cells as a means of intercellular communication (Lotvall & Valadi, 2007). Salivary exosomes are mainly secreted by salivary glandular epithelial cells, oral mucosal epithelial cells and granulocytes (YáñezMó et al., 2015). Ogawa et al. indicated that salivary exosomes can participate in the local immune response in the oral cavity (Ogawa et al., 2011). Accumulating evidence has shown that the constituents of salivary exosomes are associated with systemic diseases. Zheng et al. identified PSMA7 as a salivary exosomal biomarker for inflammatory bowel disease, thus associating salivary exosomes with systemic inflammatory disease (Zheng et al., 2017). In addition, cancer-associated proteins have been detected in salivary exosomes from cancer patients (Lau & Wong, 2012; Sun et al., 2017). Moreover, the constituents of salivary exosomes are associated with oral diseases. Byun et al. identified miR-4484 in salivary exosomes as a diagnostic marker for oral lichen planus (OLP) (Byun, Hong, Choi, Jung, & Lee, 2015), while Aqrawi et al. explored LCN2, APMAP and CPNE1 as possible novel biomarkers in salivary exosomes for the diagnosis of Sjögren's syndrome (Aqrawi et al., 2017).

Periodontitis is an oral chronic inflammatory disease initiated by microbial plaque accumulation and exacerbated by the inflammatory immune response. Periodontitis can cause the destruction of alveolar bone and eventual tooth loss (Pihlstrom, Michalowicz, & Johnson, 2005). Periodontitis has a high global prevalence and has become a major public health problem (Dye, 2012). The condition leads to tooth loss and masticatory dysfunction and negatively affects aesthetics. During the development of periodontitis, the innate immune response plays an important role. Activation of the innate immune response can prevent microbial invasion but may destroy periodontal supporting tissue (Darveau, 2010). Increases in inflammatory factors and immune factors have been reported in the gingival crevicular fluid (GCF), saliva and serum of patients with periodontitis (Barros et al., 2016; Lira-Junior, Öztürk, Emingil, Bostanci, & Boström, 2017). However, there is no literature on protein profiling of salivary exosomes in patients with periodontitis.

In this study, our goal was to explore differences in the protein profiles of salivary exosomes between patients with severe periodontitis (SP) and healthy controls (HC). To this end, we collected the saliva of young adults with SP and young adults who were periodontally healthy and isolated exosomes from the saliva. Through mass spectrometry (MS) and gene ontology (GO) analysis, we compared the differences in salivary exosomal proteins between the SP group and the HC group and then further analysed the interactions between different proteins.

2 | MATERIALS AND METHODS

2.1 | Saliva collection and preparation

Eleven young adults diagnosed with SP (Armitage 1999) and eleven young adults who were periodontally healthy were recruited at Peking University School and Hospital of Stomatology from 2016 to 2018. The inclusion criteria of the SP group were as follows: 18 ~ 35 years of age, maximum interdental clinical attachment level (CAL) ≥5 mm, maximum probing pocket depth (PPD) ≥6 mm and bone loss extending to the mid-third of the root and beyond. The individuals with PPD ≤ 3 mm and no obvious CAL were defined as HC. The exclusion criteria of all subjects were as follows: current smoking and former smoking within the past 5 years, periodontal treatment in the previous 12 months, antibiotics or other drugs affecting their periodontal status taken in the previous 3 months, oral diseases (e.g. progressive caries, oral ulcer, oral lichen planus or Sjogren's syndrome) and systemic diseases (e.g. cardiovascular diseases, diabetes mellitus, immunodeficiency diseases, tumours). This research was approved by the Ethical Committees of Peking University Hospital of Stomatology (PKUSSIRB-201631108), and written informed consent was obtained from all participants. The participants' information is provided in Table 1.

Salivary samples were collected from the recruited individuals. All individuals were asked not to eat, drink or brush their teeth starting at midnight on the day before sampling. After rinsing their mouths with purified water, individuals were required to harvest their saliva into a 50-ml sterile tube; the individuals were also asked not to speak during the collection procedure. This process lasted for 5–10 min, and almost 5 ml of saliva was collected from each participant. The unstimulated whole saliva samples were immediately maintained on ice and centrifuged at 12,000 *g* for 15 min at 4°C. Then, the supernatant of the saliva samples was distributed into several 1.5-ml tubes and immediately stored at − 80°C until further analysis.

TABLE 1 Full-mouth periodontal parameters of the two groups

Variables	Healthy controls (*n* = 11)	Severe periodontitis (*n* = 11)	*p*-value
Age (years)	27.36 (±2.42)	29.45 (±3.01)	.088
Bleeding Index	1.49 (±0.28)	3.84 (±0.39)	<.001
Probing Pocket Depth (mm)	2.55 (±0.16)	4.69 (±0.50)	<.001
Clinical Attachment Level (mm)	0.17 (±0.22)	2.57 (±0.12)	<.001

Note: The data are presented as the means (±*SD*). *p*-value: Independent-samples *t* test or chi-square test.

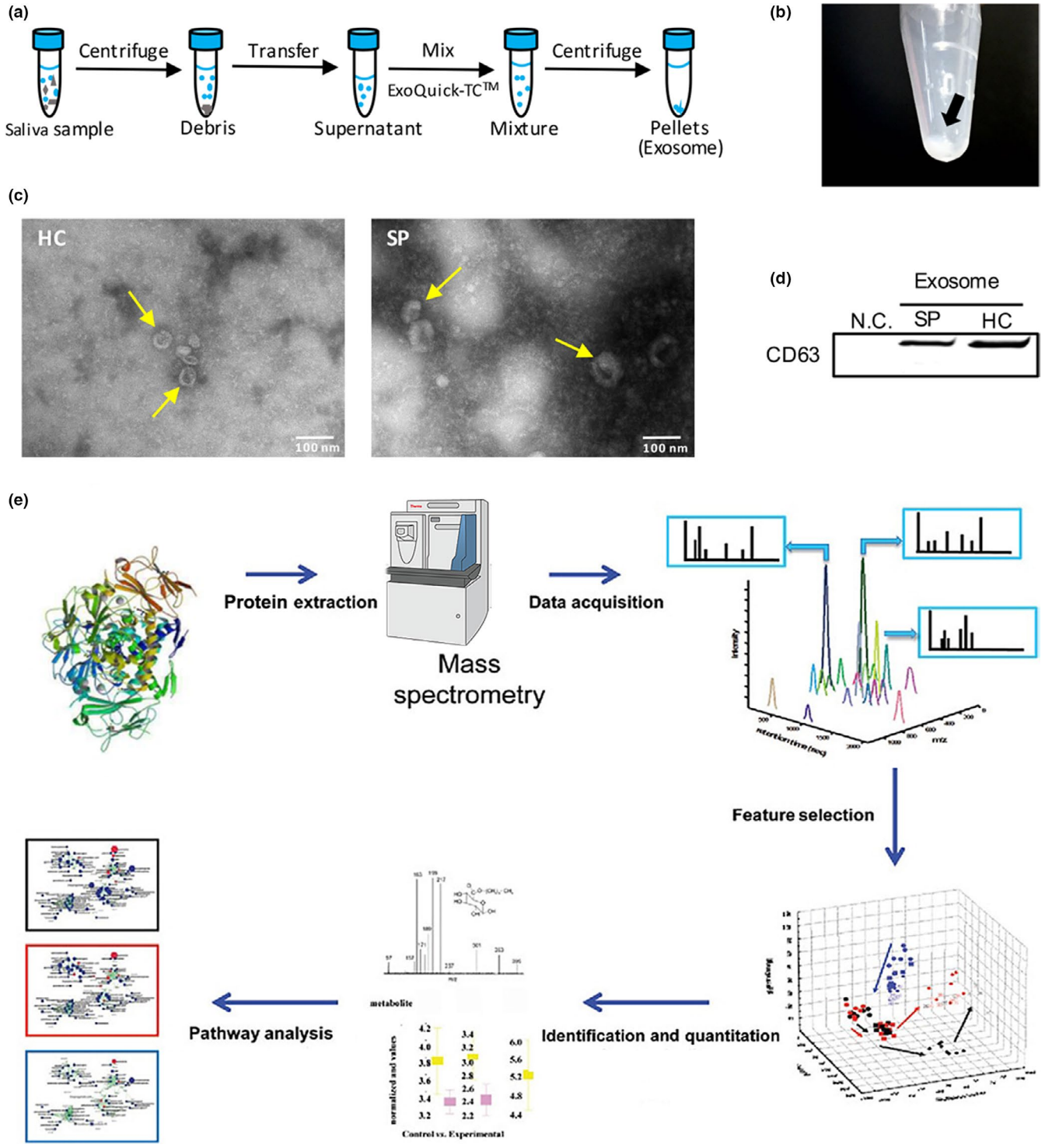

FIGURE 1 Brief illustration of the extraction and identification of salivary exosomes and the LC-MS/MS analysis. (a) Schematic diagram of exosome isolation from human saliva. After centrifuging the saliva and removing the debris, the supernatant was mixed with reagent and then centrifuged to obtain pellets. (b) The pellets collected in the bottom of the 1.5-ml tubes were present as very thin, barely visible white films. (c) Transmission electron microscopy images of salivary exosomes from HC and SP patients. The scale bar is 100 nm, and the exosomes are 30–100 nm in size and spherical in shape. (d) Western blotting of exosome-specific CD63. NC represents the saliva sample lacking isolated exosomes, while SP and HC represent exosome samples isolated from saliva. (e) Workflow of the LC-MS/MS analysis shows the general procedures, including protein extraction, data acquisition, feature selection, identification and quantitation, and pathway analysis

2.2 | Exosome isolation from saliva

Exosome isolation was performed using an ExoQuick-TC™ kit (System Biosciences Inc.) according to the manufacturer's protocol, with some modifications to adjust the kit for use with saliva (Figure 1a). In brief, saliva volumes of 0.5 ml were admixed with ExoQuick precipitation solution at a 2:1 ratio. The mixtures were incubated overnight at 4°C and centrifuged at 1,500 *g* for 30 min at 4°C. After aspirating the supernatant, the residual solution underwent a second round of centrifugation at 1,500 *g* for 5 min at 4°C, and the supernatant was again removed. The pellets were processed for further analysis (Figure 1b) (Zlotogorski-Hurvitz et al., 2015).

2.3 | Transmission electron microscopy (TEM)

The pellets in each tube were resuspended in 25 μl of phosphate-buffered saline (PBS). Drops of resuspended pellets were deposited on Formvar/carbon-coated electron microscopic grids and held at room temperature for 10 min. Then, the grids were transferred to 3% glutaraldehyde for 10 min. After washing the grids with triple-distilled water three times, negative staining was performed with freshly prepared 2% aqueous uranyl acetate for 10 min. The grids were dried at room temperature for 10 min, and exosomes were observed and imaged using a JEM-1400 PLUS transmission electron microscope (JEOL, Japan) at 120 kV (Théry, Amigorena, Raposo, & Clayton, 2006).

2.4 | Western blotting and validation of immune-related proteins

Purified exosomal pellets were treated with 1 × RIPA buffer and protease inhibitors (CWBio). Protein concentrations were determined using a BCA protein assay kit (CWBio). Samples were combined with 4 × SDS-PAGE loading buffer and boiled at 95°C for 5 min. Approximately 50 μg of total protein was loaded onto 12% SDS–polyacrylamide gels and transferred to PVDF membranes. The proteins were probed with a primary rabbit anti-CD63 antibody (Cat# EXOAB-CD63A-1; System Biosciences Inc.) at a dilution of 1:1,000. After the membranes were incubated with a secondary anti-IgG fluorescently labelled antibody, an Odyssey® LI-COR Imaging System (LI-COR Biotechnology) was used to visualize the proteins (Zlotogorski-Hurvitz et al., 2015). In addition, in the validation groups of 8 samples, CD63 was probed as described above. Then, the membranes were incubated with a primary rabbit anti-C6 antibody (1:500; Cat# 17239–1-AP; Proteintech).

2.5 | Protein extraction and in-gel digestion

Purified exosomal pellets were treated with 1 × RIPA buffer and protease inhibitors (CWBio). The total protein concentrations were determined by the BCA assay. They were then separated by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). Gels were fixed and stained with Coomassie Brilliant Blue. The in-gel proteins were reduced by incubation with dithiothreitol followed by alkylation with iodoacetamide. After washing and dehydration, the proteins were digested with sequencing grade trypsin at 37°C overnight (sequencing grade-modified; Promega, Madison, WI) (Olsen, Ong, & Mann, 2004). The peptides were extracted from the gel pieces with 0.1% formic acid and 80% acetonitrile, and the extracts were dried in a vacuum centrifuge.

2.6 | Liquid chromatography–tandem mass spectrometry (LC-MS/MS)

After being resuspended in 10 μl of 0.1% FA, peptides were analysed with an Easy-nLC1200 nanoflow UHPLC (Thermo Fisher Scientific Inc.) coupled to an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific Inc.). Five microlitres of sample was loaded at a speed of 0.3 μl/min in 0.1% FA onto a trap column (C18, Acclaim PepMap TM 100 75 μm × 2 cm nanoViper Thermo) and eluted across a fritless analytical resolving column (C18, Acclaim PepMap TM 75 μm × 15 cm nanoViper RSLC Thermo). Gradient elution was performed from 4% to 30% LC-MS buffer B (LC-MS buffer A includes 0.1% formic acid; LC-MS buffer B includes 0.1% formic acid and 80% ACN) at 300 nl/min. High-energy collision dissociation (HCD) was used to fragment the peptide precursor ions. Peptides were injected into a mass spectrometer via a nanoelectrospray ion source with an electrospray voltage of 2.2 kV. All data were acquired in profile mode using positive polarity. A full MS scan was conducted from 300 to 1,500 *m/z* at a resolution of 120 K, and an AGC target set to 1e6. MS/MS spectral data were acquired in the Orbitrap, with a resolution of 30 K and an intensity threshold of 1.6 K. Maximum fill times were 50 ms for MS. The respective Q3 software versions used were Tune 2.6.0 and Xcalibur 2.1 (Thermo Fisher Scientific Inc.).

2.7 | Database search

Raw files were loaded into the PD software package (version 2.2) and searched against the UniProt protein database downloaded from the UniProt human protein sequence database. All searches were performed with tryptic specificity, allowing up to two missed cleavages. For protein identification, the FDR of 1% was applied at both the peptide and the protein level. The protein modifications were carbamidomethylation (C) (fixed), oxidation (M) (variable) and protein N-terminal acetylation (variable), and the maximum missed cleavages were set to 2. Data were searched with a 10-ppm precursor tolerance and a 0.02-Da tolerance for fragment ions.

2.8 | Statistical analysis

Data analysis was carried out using SPSS software (version 20; IBM). An independent-samples *t* test was used to compare the differences in ages between the SP and HC groups, while a chi-squared test was used to compare the differences in the bleeding index, PPD and CAL between these two groups. To obtain the biological process

(BP), cellular component (CC), molecular function (MF) and pathway information of the proteins involved, GO analysis was performed based on GeneCodis (Tabas-Madrid, Nogales-Cadenas, & Pascual-Montano, 2012). To identify potential protein interactions, interaction network analysis was performed using STRING (Szklarczyk et al., 2017).

3 | RESULTS

3.1 | Verification of salivary exosomes

Exosomes are 30–100 nm in size and spherical in shape, with an observable bilayer lipid membrane (Figure 1c). To further verify the extraction of exosomes, Western blotting was used to detect the exosomal marker CD63 in samples with or without isolation of exosomes (Figure 1d). Two samples for which exosomes were isolated exhibited bands specific for this protein, whereas this marker was not detected in a sample for which exosomes were not isolated. Thus, these results confirm the successful isolation of exosomes from saliva samples.

3.2 | Profiling of salivary exosomal proteins

To explore the differences in salivary exosomal proteins between the SP group and the HC group, we randomly selected four samples from these two groups and pooled two samples in the same group together. LC-MS/MS was used to identify salivary exosomal proteins in these two groups; the procedure is briefly illustrated in Figure 1e. In total, 921 and 999 salivary exosomal proteins were identified for the SP group and HC group, respectively (Tables S1, S2, S3 and S4). As shown in Figure 2, 439 salivary exosomal proteins were shared by both groups. In addition, 58 proteins were identified specifically in the HC group, and 26 proteins were unique to the SP group, as listed in Table S5.

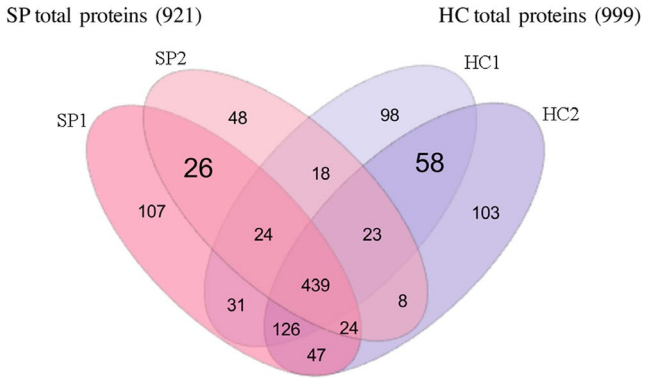

FIGURE 2 Venn diagram of the identified salivary exosomal proteins. We randomly selected four samples from the severe periodontitis (SP) group and the healthy control (HC) group and pooled two samples from the same group to generate samples SP1, SP2, HC1 and HC2. SP1: a pooled sample from the SP group; SP2: another pooled sample from the SP group; HC1: a pooled sample from the HC group; HC2: another pooled sample from the HC group [Colour figure can be viewed at wileyonlinelibrary.com]

3.3 | Gene ontology analysis of salivary exosomal proteins

To analyse the functions of the salivary exosomal proteome, GO analysis was carried out based on the unique proteins, and statistical tests were undertaken to identify the enriched function categories with $p < .001$. As shown in Figure 3, GO biological process (GOBP), molecular function (GOMF), cellular component (GOCC) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were classified. In GOBP, the SP group and the HC group possessed completely different enrichment items, except blood coagulation (Figure 3a). Innate immune response, cytolysis and complement activation were significantly enriched only in the SP group. Complement-related items also displayed the most obvious enrichment in the KEGG section unique for the SP group (Figure 3d). According to Figure 3b, "extracellular space/region" showed high enrichment in both groups. For GOMF, compared with the HC group, the SP group only showed enrichment in "flavin reductase activity" (Figure 3c).

3.4 | Protein–protein interaction network analysis of salivary exosomal proteins

The results of the protein–protein interaction network analysis for unique salivary exosomal proteins from the SP group and the HC group are displayed in Figure 4. One of the identified protein networks in the SP group consists of nucleosome components, such as histone H1B, H1D, H2A and H2B (Figure 4a). In addition, the other protein cluster-containing complement components (C6, C8A and C8B) were shown in the SP group. The interaction between chemokine (C-C motif) ligand 28 (CCL28) and serum amyloid A1 (SAA1) was also proposed in the SP group. For the HC group, the interaction map revealed a more diverse network (Figure 4b). The core interactions in the network of the HC group were related to nuclear metabolism (heterogeneous nuclear ribonucleoprotein K (HNRNPK) and heterogeneous nuclear ribonucleoprotein R (HNRNPR)). In addition, stress response and redox proteins (e.g. COP9 signalosome complex subunit 8 (COPS8) and superoxide dismutase 1 (SOD1)) were found only in the HC group.

3.5 | Validation of immune-related proteins by Western blotting

Four samples from the SP group and four samples from the HC group were randomly selected for validation. Western blotting was performed to verify C6 expression in the different groups (Figure 5). The Western blotting results showed that all four samples in the SP group but none of the four samples in the HC group exhibited C6 expression.

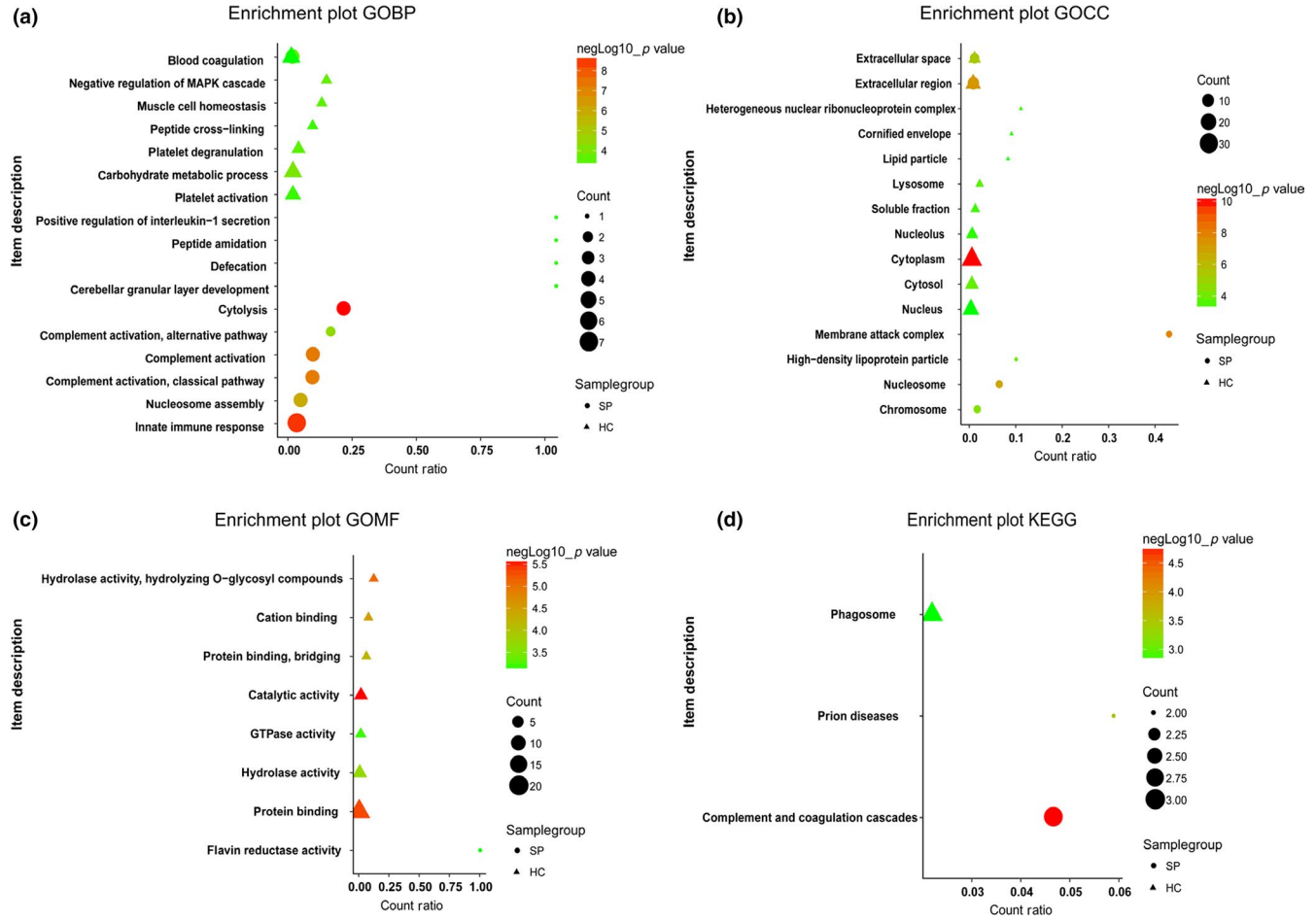

FIGURE 3 Gene ontology analysis of the unique salivary exosomal proteins from the SP group and the HC group. (a) Gene ontology biological process (GOBP); (b) gene ontology cellular component (GOCC); (c) gene ontology molecular function (GOMF); (d) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. HC: the group containing proteins found only in healthy controls; SP: the group containing proteins found only in patients with severe periodontitis. The x-axis indicates the ratio of the number of proteins enriched in each function category (Count) to the whole number of annotated proteins in the category. The colour indicates the degree of enrichment after statistical analysis

4 | DISCUSSION

In this study, we extracted salivary exosomes from young SP patients and periodontally healthy subjects and compared the differences in the protein compositions of salivary exosomes between these two groups by LC-MS/MS. GO and protein–protein interaction network analyses were then conducted to identify proteins related to periodontitis.

To confirm the extraction of exosomes, we used TEM to characterize exosome morphology and Western blotting to detect the representative exosomal protein marker (CD63). Exosomes are spherical bilayer membrane vesicles with a diameter of 30–100 nm (Lötvall et al., 2014). Through TEM, we observed the structures of the exosomes in saliva samples from the SP group and the HC group (Figure 1c). Zlotogorski-Hurvitz et al. demonstrated that ExoQuick-TC^{TM}, a chemical precipitation reagent for exosome isolation, is suitable and efficient for saliva, especially small volumes of saliva (Zlotogorski-Hurvitz et al., 2015), and is similar to the classical method of ultracentrifugation. Several specific membrane proteins, such as TSG101, CD63, CD9 and CD81, participate in exosome formation (Simons & Raposo, 2009). Some of these proteins are commonly considered exosomal markers. In this study, CD63 was detected in the exosomes isolated from saliva samples by Western blotting (Figure 1d). In addition, in GOCC (Figure 3b), "extracellular space/region" showed high enrichment in both the SP group and the HC group, which also indicated that the isolation of exosomes was successful.

Salivary exosomes are one of the important components in saliva with nanoscale size and the ability to carry proteins, RNA and miRNA to cells as a means of intercellular communication (Lotvall & Valadi, 2007). Recently, the constituents of salivary exosomes have been reported to be associated with oral and systemic diseases, especially in some diseases related to inflammation and immune reactions, such as OLP, Sjögren's syndrome, breast cancer and lung cancer (Aqrawi et al., 2017; Byun et al., 2015; Lau & Wong, 2012; Sun et al., 2017). Periodontitis is a common oral

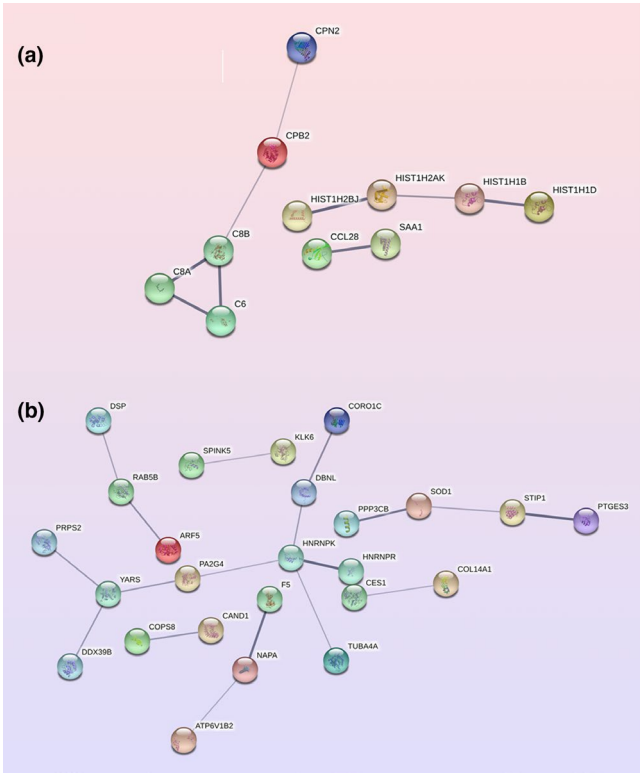

FIGURE 4 Interaction network analysis for unique salivary exosomal proteins in the SP group (a) and the HC group (b). The names used in the molecular network are gene names

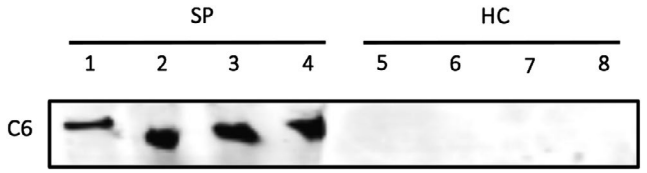

FIGURE 5 Verification of immune-related protein (C6) expression in salivary exosomes by Western blotting. The results indicate that C6 was expressed in all four samples from the SP group but in none of the four samples from the HC group

inflammatory disease; however, currently, there is no research on salivary exosomes associated with periodontitis. Thus, in our study, we compared salivary exosomal proteins in SP patients and periodontally healthy subjects. We identified 26 proteins exclusive to the SP group and 58 proteins exclusive to the HC group. After we performed GO analysis on these proteins, the results of GOBP and KEGG analysis showed that the most remarkable function in the SP group involved immunity, especially innate immune response and complement activation. Then, the interactions between these unique proteins were further analysed. We found that in the SP group, the main interaction is the interaction between the complement components (C6, C8A and C8B) (Figure 4a). The complement system is a cascade system closely related to immunity and inflammation. Activation of the complement system can mediate microbial phagocytosis, recruit and activate inflammatory cells, and cause direct lysis of microorganisms (Ricklin, Hajishengallis, Yang, & Lambris, 2010). Among the complement system components, C6 is a member of the membrane attack complex (MAC), which binds to C5b at the initiation of MAC formation and then participates in complement activation (Sarma & Ward, 2011). Furthermore, CCL28 is a chemokine that can be selectively expressed in certain mucosal tissues, such as the exocrine glands, colon and trachea, and plays a significant participatory role in the immune process and calcium mobilization (Hieshima et al., 2003). These immune-related proteins have been reported to be elevated in the GCF of patients with periodontitis (Ertugrul, Sahin, Dikilitas, Alpaslan, & Bozoglan, 2013). In our studies, we further verified by Western blotting that C6 was expressed only in salivary exosomes from the SP group.

It is recognized that periodontitis is not only a chronic inflammatory disease in the oral cavity but also may affect systemic diseases; it can even become a risk factor for certain systemic diseases (Cullinan & Seymour, 2013). Many epidemiological and clinical studies have shown correlations between periodontitis and systemic diseases, including diabetes mellitus, and cardiovascular disease (Preshaw et al., 2007; Kjellström, Rydén, Klinge, & Norhammar, 2016). Recent reports have indicated that salivary exosomes may be associated with systemic diseases. For example, serum exosomes from lung cancer patients contained specific markers for lung cancer. In addition, lung cancer-related proteins were also detected in salivary exosomes (Sun et al., 2017). By analysing salivary exosomal proteins in SP patients, we learned that salivary exosomes from SP patients contained complement components (C6, C8A and C8B) and CCL28, which were related to the immune response. The immune response plays an important role in diabetes mellitus and cardiovascular disease (Ouyang, Rutz, Crellin, Valdez, & Hymowitz, 2011; Wang et al., 2014). In patients with diabetes mellitus, periodontitis can lead to an increase in immune-inflammatory factors (e.g. C-reactive protein, plasminogen activator inhibitor-1 and other cytokines), which may promote insulin resistance and induce diabetic complications (King, 2008; Lalla & Papapanou, 2011). Beck et al. elucidated that pro-inflammatory cytokines induced by periodontitis (e.g. interleukin-1β (IL-1β), IL-6 and tumour necrosis factor (TNF)) may enter the systemic circulation and induce acute-phase reactions in the liver, thereby promoting atherosclerosis (Beck et al., 2005). Considering that exosomes can enter the circulatory system, we suggest that salivary exosomes containing immune-related proteins may participate in the local immune defence during the process of periodontitis and be vehicles linking periodontitis with systemic diseases.

5 | CONCLUSION

In summary, we compared the salivary exosomal proteins between young adults with SP and HCs. The results of GO analysis revealed that the most notable function in the SP group was related to immunity. These findings indicated that salivary exosomes may be involved in the immune response in periodontitis and link periodontitis

with systemic diseases. However, the underlying mechanism requires further research.

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CONFLICTS OF INTEREST

All authors have declared no conflicts of interest.

AUTHOR CONTRIBUTIONS

H. X. recruited subjects, collected samples, conducted the experiments and drafted the manuscript. H. X. participated in recruiting subjects and collecting samples. Z. M. analysed the data and revised the manuscript. Z. Q. conceived and designed the study, and made a critical revision of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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