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iTRAQ-based quantitative analysis of alveolar bone resorption in rats with experimental periodontitis

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ABSTRACT

Objective: Periapical periodontitis results in alveolar bone resorption around the root apex. During the progression of inflammation, host cells release various inflammatory mediators and pro-inflammatory cytokines through immune responses. However, the pathological mechanisms associated with periapical bone destruction remain unclear. This study was objected to identify differentially regulated proteins in periapical periodontitis via a quantitative proteomics approach using isobaric tags for relative and absolute quantification (iTRAQ) labelling of peptides.

Methods: A model of periapical periodontitis by sealing LPS into the pulp chambers of rats was established. iTRAQ was employed to screen differentially expressed proteins in alveolar bone between periapical lesions and healthy controls. These proteins were further analysed by bioinformatics. And four proteins were validated by western bolt.

Results: We identified 4398 proteins, of which 7 were up-regulated and 151 were down-regulated in periapical periodontitis compared to normal tissue. Using bioinformatics tools such as GO and KEGG pathway analysis, we found that our proteomics strategy could identify and quantify differentially expressed proteins that were not described in previous studies examining periapical periodontitis; these proteins included hexokinase, legumain and members of the keratin family.

Conclusion: In summary, our results represent potential biomarkers for the detection of periapical periodontitis and demonstrate that quantitative proteomics is a robust discovery tool for the identification of differentially regulated proteins in periapical periodontitis.

1. Introduction

Periapical periodontitis (otherwise known as apical periodontitis and endodontic infection) is a chronic inflammation of periapical tissue, which is known caused by microbial infection within the root canal system of the affected tooth. The inflammatory process involving mast cells, angiogenesis and cytokines, ultimately results in alveolar bone resorption around the root apex, which is a prominent pathological and clinical feature of periapical periodontitis (Aspriello et al., 2009; Kawashima & Stashenko, 1999; Lucarini et al., 2009; Zizzi et al., 2013; Zizzi, Aspriello, Rubini, & Goteri, 2011). Periapical periodontitis is common, and lifetime prevalence estimates can be as high as 20–100% per individual (Iqbal & Kim, 2008).

Periapical lesions are characterized by periradicular periodontal ligament and bone resorption due to bacterial infection from the root canal system (Graunaite, Lodiene, & Maciulskiene, 2012). Diverse inflammatory mediators have been associated with periradicular lesions. During the inflammatory disorder, host cells release various inflammatory mediators and pro-inflammatory cytokines through innate and adaptive immune responses (Marton & Kiss, 2014). Studies have demonstrated that adipokine leptin is expressed in human periapical periodontitis (Martin-Gonzalez et al., 2015). Researchers have shown that there is a positive correlation between the expression levels of bone-resorptive cytokines and periapical soft tissue lesions in periapical cysts and granulomas with significant clinical symptoms (Menezes et al., 2006). However, currently, despite advances in our knowledge of the pathogenesis of periradicular lesions, the specific bone regulatory cytokines and the key pathological mechanism associated with the regulation of bone resorption remain unclear. Therefore, there is a growing need to understand the role that cytokines play in the pathogenesis of apical periodontitis

Proteomics is the study focusing on constitution, distribution and interaction of proteins in a whole cell or organisms. Recently, proteomics has been widely used to obtain substantial information about

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individual proteins in various oral diseases, including periapical periodontitis (Bantscheff, Lemeer, Savitski, & Kuster, 2012; Khurshid et al., 2016). Isobaric tagging for relative and absolute protein quantification (iTRAQ) has recently been demonstrated to be a sensitive quantitative proteomic method for high-throughput protein identification and quantification (Wiese, Reidegeld, Meyer, & Warscheid, 2007). The iTRAQ method involves a set of amine-specific isobaric tags that are used for multiplexed relative quantification of proteins by mass spectrometry. This method has been employed in many cancer biomarker studies, including analyses of saliva in breast cancer (Mohan, Letchoumy, Hara, & Nagini, 2008), human prostate cancer cell lines (Glen et al., 2008), and chronic myeloid leukaemia (Griffiths et al., 2007).

In this study, we used iTRAQ to quantitatively compare the proteomes of periapical periodontitis cases and healthy controls. The differentially expressed proteins identified in this study may serve important roles in the progression of periapical periodontitis. Additionally, our findings may contribute to a better understanding of the molecular events underlying this disease and could be used to predict the development of periapical periodontitis.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Ethics Committee of the Institute and Hospital of Stomatology, Nanjing University Medical School. All surgeries were performed under anaesthesia and all efforts were made to minimize animal suffering.

2.2. Animals and treatment

Sixty male Wistar rats that weighed between 200–220 g were obtained from the Experimental Animal Center of Nanjing Province. Periapical lesions were induced using a previously described method (Yang & Peng, 2008). All rats were anesthetized with intraperitoneal ketamine (90 mg/kg). The pulps of the right mandibular first molars was exposed with a #1/4 round bur until the bur head sank into the pulp chamber. Small cotton swabs soaked in 1 mg of lipopolysaccharide (LPS) from Escherichia coli serotype 055: B5 (Sigma-Aldrich, USA) per 1 mL of 1x Tris buffer were placed into the pulp chamber to induce periapical periodontitis. The exposed teeth were left open to the oral environment to induce the formation of periapical lesions. The left mandibular first molars were left untreated and served as controls. The animals were sacrificed 2 weeks after lesion induction. Some tissue specimens of rat mandibles were immediately frozen in liquid nitrogen and stored at −80 °C for iTRAQ.

2.3. iTRAQ labelling and SCX fractionation

The right and left mandibles from the first molars of rats were used for the iTRAQ labelling experiment. Protein samples (200 μg each) were reduced using 10 mM TCEP (tris (2-carboxyethyl) phosphine) for 30 min at room temperature and alkylated with iodoacetamide (55 mM) for 60 min in the dark at room temperature. After that, samples were diluted to 1.5 M urea using TEAB (500 mM), digested for 16 h at 37 °C with trypsin (10 μg). The tryptic peptides were then labelled with iTRAQ Reagents 4-plex Kit (Applied Biosystems) according to the manufacturer's protocol. The samples were labelled as 116 (15 DPA), 118 (20 DPA), 119 (25 DPA), and 121 (30 DPA), respectively. After labelling, the iTRAQ-labelled peptide mixtures were pooled, fractionated by strong cationic exchange (SCX) chromatography (AB Sciex, No. 4352160), desalted by Sep-Pak C18 cartridges (waters), re-constituted in 100 μL of 5% ACN and 0.05% formic acid, and subjected to mass spectrometry.

2.4. LC–MS/MS separation and data analysis

iTRAQ-labelled samples were analysed using a Q-Exactive mass spectrometer (Thermo Fisher Scientific) coupled to an UltiMate 3000 HPLC system (Dionex LC Packings). Five-microliter samples were first loaded onto a trap column (C18 PepMap100, 300 μm × 1 mm, 5 μm, 100 Å; Thermo Scientific Dionex, Sunnyvale, CA) and separated by a C18 column (Acclaim PepMap C18, 15 cm × 75 μm × 3 μm, 100 Å; Thermo Scientific Dionex) at a flow rate of 0.26 μL/min. Peptides were detected in the Orbitrap at a resolution of 60,000 at 400 *m/z*. Peptides were applied to a 90 min linear gradient ranging from 2% to 40% B (mobile phase A, 0.1% formic acid in water; mobile phase B, 0.1% formic acid in ACN). The selection for MS/MS was conducted using a high-energy collision dissociation (HCD) operating mode with a normalized collision energy setting of 40%, and the 10 most intense signals in survey scan were fragmented. Overall, 100,000 ions were set as the automatic gain control for generation of HCD spectra. Dynamic exclusion was performed with a repeat count of 2 and an exclusion duration of 120 s. Proteins were identified using the Triticum-aestivum database IWGSC1.0 + popseq.28.pep 5.6.3 (Ensembl) by Proteome Discoverer 1.4 software coupled with Mascot. Unique proteins with at least two unique peptides with a false discovery rate < 0.01 were qualified for further data analysis.

2.5. Bioinformatics analysis

Functional classifications and enrichment analyses of the GO of differentially expressed proteins were carried out using the DAVID database (http://david.abcc.ncifcrf.gov/). Proteins were classified according to the GO category (http://www.geneontology.org), including "biological process," "cell component," and "molecular function," respectively. The following procedure were followed: p-value for each GO term was calculated, and only terms with p-value less than 0.05 were shown.

2.6. Western blotting

The right and left mandibles from first molars of rats were lysed in ice-cold lysis buffer containing a protease inhibitor and phosphatase inhibitor cocktail. Western blot analyses were performed as previously described (Qian et al., 2015). The following antibodies were used: hexokinase (1:1000, ab150423, Abcam), legumain (1:1000, ab125286, Abcam), cytokeratin5 (1:1000, ab53121, Abcam), cytokeratin17 (1:1000, ab53707, Abcam) and GAPDH (1:5000, 2118, Cell Signaling). Proteins were visualized with an ECL kit. Densitometric analyses of immunoblot bands were performed using Image J software and were normalized to GAPDH (http://rsb.info.nih.gov/ij/). Normalized data were represented as a percentage of or fold change compared to the corresponding control, which was set to 1 or 100.

3. Results

3.1. Proteome analysis

Technical replicates were prepared by labelling peptides derived from normal tissue with 116 and 118 iTRAQ labels and peptides derived from periapical periodontitis tissue with 119 and 121 iTRAQ labels. Quantitative analysis and the identification of LC–MS/MS spectra comparing normal tissue and periapical periodontitis mandibles yielded 4398 proteins comprising 23,936 unique peptides (data available on request).

The fold changes were calculated from the ratio of intensity of the iTRAQ reporter ions obtained for the peptides from the periapical periodontitis tissue to peptides derived from normal tissue. Using cut-off values of 1.5-fold for over-expression and 0.67-fold for under-expression of a protein, 158 differentially expressed proteins were