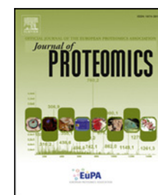
Journal of Proteomics 145 (2016) 127–136

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot



Proteomic analysis of human tooth pulp proteomes – Comparison of caries-resistant and caries-susceptible persons

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ARTICLE INFO

Article history:
Received 22 December 2015
Received in revised form 8 April 2016
Accepted 17 April 2016
Available online 22 April 2016

Keywords:
Human tooth pulp
DIGE
Proteome
Caries
Resistance

ABSTRACT

Most people in the world suffer from dental caries, >90% of adults experience caries on enamel and root surfaces during their life. However, the overall roles of all factors in the development of dental caries still remain unclear and are worthy of recent investigation. In this study we used a proteomic 2D-DIGE approach in connection with MS/MS to investigate the different protein abundances in the tooth pulp of human third molars obtained from caries-resistant and caries-susceptible people. Statistical analysis of the two protein maps obtained on large gel (17 cm length) and mini gel (7 cm length) followed by nLC-MS/MS analysis enabled the identification of 16 significantly changed spots with unique protein identifications corresponding to 12 non-redundant proteins. Seven proteins exhibited higher and four proteins exhibited lower expression in the caries-resistant samples compared to the caries-susceptible samples. Additionally, one protein (alpha-1-antitrypsin) exhibited both expressions (up and down). Most of the differentially expressed proteins were associated with protein metabolism, energy production, cytoskeletal organization and transport. These differentially expressed proteins are likely involved in the natural resistance or susceptibility of humans to the development of dental caries and suggest that the resistance mechanism is multifactorial.

Biological significance: Dental caries are not a serious and life-threatening disease, but their healing requires many remedies and takes up a lot of time. Moreover, neglecting the problem may lead to tooth loss, which can strongly reduce the quality of life. Therefore the identifying effective and safe oral medicine and determining the causes of caries-resistance were viewed as the main aims of this study. Our work aims to elucidate the mechanism of natural human resistance to the development of dental caries by studying the proteomes of tooth pulp isolated from patients who displayed different prevalences of tooth caries. This study is the first protein tooth pulp comparison of sound teeth obtained from caries-resistant versus caries-susceptible people.

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1. Introduction

Dental caries are currently a worldwide problem. Current research has shown that over 90% of all adults experience dental caries, only 10% of late adolescents and young adults in the United States are caries-free [1]. Dental caries continue throughout adulthood, with >95% of adults experiencing caries on enamel and root surfaces, and it leads to a complete loss of teeth in 25% of people. Permanent tooth caries belong to a group of human diseases with the highest prevalence among the world population [2]. Dental caries is a multifactorial disease that includes the participation of cariogenic bacteria, salivary components (proteins, enzymes) and fermentable carbohydrates, and other nutrients present in the dietary sources [1,3]. The overall roles of all these effects in the development of dental caries remain unclear and are worthy of further investigation.

Only a small proportion of the world's population (ca. 10%) is well protected against the prevalence of dental caries (http://www.who.int/oral_health/disease_burden/global/en; 27.10.2015). This anticaries resistance could be caused by the immune system, composition of saliva, nourishment, plaque composition, specific dental care and many other factors [4]. Last but not least, resistance could be caused by the presence of specific protein antibacterial agents inside the tooth tissues (pulp, dentin or enamel). Providing updated knowledge about biological principles determining the development and arrest of caries on the surface of teeth could make a significant contribution to planning and evaluating future preventive activities and oral health promotion. A recent study investigated tooth loss in adults living in the USA. It showed that a lower quality diet and reduced dietary intake of the most important nutrients (e.g. vitamins and minerals) may partly explain the risk of chronic disease in the population. After adjusting

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http://dx.doi.org/10.1016/j.jprot.2016.04.022
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for sociodemographic characteristics, physical activity and smoking status were positively associated with the number of natural teeth [5].

Immunological and microbiological characteristics in individuals also affect the incidence and prevalence of tooth caries. One of the major factors responsible for the prevalence of dental caries and ecological equilibrium in the mouth is saliva, which affects the colonization and growth of bacteria [6]. Saliva covers the oral hard and soft tissues with a conditioning film which governs the initial attachment of microorganisms. It furthermore contains proteins which can bind to bacteria, blocking their adherence to surfaces. These include lactoferrin, lactoperoxidase, lysozyme, and antimicrobial peptides. In this way saliva promotes the complexity of the oral microflora, which in itself protects against overgrowth of a few pathogenic species [7]. Only a few studies looking at the influence of saliva protein composition on the formation of dental caries have been performed. Some proteins (e.g. cystatins, lipocalin and acidic proline-rich proteins) were positively correlated with the caries-resistant group of subjects. Subjects with a higher number of dental caries had higher levels of amylase, immunoglobulin A and lactoferrin in the saliva [8]. Similarly, higher levels of cystatin S were present in the subjects with carious root, but a considerable variation in saliva protein composition was observed among the individuals [9]. The quantity of pathogenic species such as *Streptococcus mutans* is known to be significantly higher in the saliva of the caries-positive population, and is positively correlated with the number of dental caries. A correlation between the presence of salivary total immunoglobulin A and children with severe early childhood caries was also observed [10].

The pulp-dentin complex, which makes the tooth alive, demonstrates strong regenerative potential, which enables it to respond to disease and traumatic injury (for example dental pulp itself forms calcified tissue when transplanted subcutaneously) [11,12]. The pulp-dentin complex houses several biomarkers (e.g. bone morphogenic proteins, cathepsins), and they play important roles in the pathogenesis of dental caries and pulpal pathoses, because they are essential elements in the pathological process as reviewed in Emilia and Neelakantan [13]. The identification of the bioactive proteins present in the pulp has enabled their potential involvement in regenerative and other tissue responses to be better known. These proteins could potentially offer paths to novel clinical therapies [14]. The self-defence mechanism of the tooth against tooth caries remains unclear, but it is known that the tooth pulp reacts rapidly to the onset of caries on a non-perforated enamel surface. It was also noted that the cellular reactions along the pulp-dentin interface induced by rapid- and slow-progressing enamel lesions are different. These two type of lesions exhibit major differences in the tissue changes in the pulp-dentin complex [15,16]. These reactions of dental pulp cells could indicate the presence of a potential immune self-defence mechanism in tooth.

The proteome of human dental pulp is currently being extensively investigated [17–20]. The first complex proteomic study investigating human dental pulp proteins used two-dimensional gel electrophoresis followed by MS/MS identification of the proteins used, and in total 96 proteins were found. This study also compared the 2-DE gels of the pooled healthy and carious samples, but none of the 400 detectable protein spots were reproducibly changed. The cDNA microarray discovered several differentially expressed genes in carious tissues, but none of them were found in the 96 detected proteins. A comparison of the pulp tissues of healthy and mildly carious teeth revealed only slight expression changes, probably because the pulp of the carious teeth contained a high amount of healthy tissue [17]. The earlier proteomic study used difference gel electrophoresis to create a proteome reference map during the odontoblast-like differentiation of dental pulp cells in vitro. In total 23 proteins related to early odontogenetic differentiation were identified by MS [18]. Our group identified 342 proteins with high confidence in the human tooth pulp samples [19]. The identified tooth pulp proteins have a variety of functions: structural, catalytic, transporter, protease activity, immune response, and many others. In a comparison with dentin, 140 (pulp/dentin) shared proteins were identified, 37 of which were not observed in plasma. The most recent study used the terminal amine isotopic labelling of substrates technique for in-depth characterization of the human dental pulp proteome [20]. This study provided data for the Chromosome-centric Human Proteome Project (C-HPP). Using dental pulp as an unique connective tissue, they identified 4332 proteins. Furthermore, 17 missing protein candidates for the C-HPP project were discovered, highlighting the importance of using tooth pulp for proteomics [20]. The proteomics not only of the dental pulp but of the whole tooth was also recently reviewed [21].

The main aim of our study was to use DIGE to investigate potential candidate proteins responsible for the resistance of the tooth to dental caries in humans. To the best of our knowledge, such a study comparing teeth samples had not been done before and could provide many new perspectives and benefits for human oral medicine.

2. Materials and methods

2.1. Sample preparation

Healthy and completely erupted permanent human third molars with closed apexes ($n = 12$) were extracted for various clinical reasons from twelve adults aged 24–40 years. Some of the teeth were extracted because of their anomalous position (mesial or distal inclination) or due to the difficulties associated with the process of their eruption. Another possible reason was a request from an orthodontist before the beginning of orthodontic treatment. All teeth were extracted in a dental clinic after acquiring the patient's informed consent regarding donation of tooth for research, and in accordance with The Code of Ethics of the World Medical Association for experiments involving humans.

The obtained teeth were divided into two independent groups according to the total number of dental caries (DMFT index) in the patient's oral cavity. One group ($n = 6$, 3 females and 3 males with ages ranging from 24 to 35 years, DMFT = 0) were teeth from the caries-resistant (R) group of people. These people never had any tooth caries in their mouth. The second group ($n = 6$, 4 females and 2 males with ages ranging from 27 to 40 years, DMFT ranging from 4 to 15) was formed of teeth from the caries-susceptible (S) group of people. These patients were cured for the tooth caries presence in the past and the number of the filled teeth in their mouth was ranging from 4 to 15 incidences. The teeth in each group were mixed and pooled.

The cementum was removed, and each tooth was horizontally cut (below the level of the enamel). The roots were then crushed in a jaw vice into smaller fragments, and the dental pulp was carefully removed and washed with 3×1.0 mL cell wash buffer (5 mM magnesium acetate, 10 mM Tris, pH 8.0). The pieces of pulp were lyophilized, frozen in liquid nitrogen, and stored for further processing (up to one year at −80 °C). The pulp sample taken from the roots of one tooth was about 2.5 mg dry weight.

For 2D-DIGE experiments, the pulp samples were pooled into two groups (R and S groups). In each group (ca. 15 mg of dry pulp) the pulp from in total six teeth was pooled. The pulp was then mixed and homogenized with a microhomogenizing drill in 0.8 mL of lysis buffer (30 mM Tris base, 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, and a mixture of protease inhibitors (Roche), pH was adjusted to 8.5). Lysed samples were kept at a temperature of 10–25 °C during homogenization and then were subjected to sonication (30 min, 5 °C) to improve the lysis process. Finally, the homogenate was filtered with centrifugal filter units (12,000g, Ultrafree 0.45 μm, Millipore) to remove insoluble material. Protein concentration was determined using a 2D Quant kit (GE Healthcare). The protein concentration in the extracts was adjusted to 1 mg/mL by adding the lysis buffer, and the samples were stored at −80 °C until further processing.

2.2. Two-dimensional difference gel electrophoresis (2D-DIGE)

The pH of the protein extracts was checked and eventually adjusted to 8.5 with 50 mM NaOH. Each 50 μg of protein sample was labelled with 400 pmol of Cy3, Cy5 or Cy2 according to the manufacturer's instructions for minimal labelling dyes (GE Healthcare). Briefly, 160 μL of protein sample from the caries-susceptible group was labelled with Cy3, and 160 μL of protein sample from the caries-resistant group was labelled with Cy5. Cy2 was used to label an equal mixture made of 80 μL of protein sample from the caries-susceptible group and 80 μL of protein sample from the caries-resistant group to form an internal standard. The samples were vortexed briefly and incubated on ice for 30 min in the dark. Reactions were quenched by the addition of 5.6 μL of lysine (10 mM) to each labelling dye for 10 min on ice in the dark. Labelled samples were combined in such a way that each mixture was comprised of protein samples from the caries-susceptible and caries-resistant samples and an aliquot of the internal standard (1:1:1, v/v/v). Finally, the mixture of labelled samples was mixed with an equal amount of sample buffer (8 M urea, 130 mM DTT, 4% (w/v) CHAPS, 2% (v/v) Bio-Lyte 3–10 buffer (Bio-Rad)) to form resulting samples suitable for application on IPG strips.

The samples (300 μL) were applied to broad-range 17 cm, pH 3–10 NL IPG strips (Bio-Rad). The strips were subjected to active rehydration at 15 °C for 12 h. Isoelectric focusing was carried out at 15 °C with a Protean IEF Cell system under mineral oil. Proteins were focused in four steps (250 V for 20 min, linear gradient; 10,000 V for 3 h, linear gradient; 10,000 V until complete 100,000 V h, rapid gradient). Prior to separation in the second dimension, the strips were equilibrated according to Görg et al. [22]. After the equilibration step, strips were rinsed in Tris-glycine buffer (pH 8.3), transferred to a homogeneous 12.5% (%T) SDS-polyacrylamide gel (with the same composition as in the previous section), and the gel strips were overlaid with 0.5% (w/v) agarose in SDS-PAGE running buffer containing bromophenol blue. Finally, 5 μL of Precision Plus Protein Standards was added at the top end of the gel. These large gels were run in the Protean II xi Cell system (Bio-Rad) for 30 min at 16 mA/gel followed by 40 mA/gel until bromophenol blue reached the end of the gel at 15 °C.

In the second stage of 2D-DIGE analysis, 130 μL of samples were applied to narrow-range 7 cm, pH 4–7 IPG strips (Bio-Rad). The strips were subjected to active rehydration at 15 °C for 12 h. Isoelectric focusing was carried out at 15 °C with a Protean IEF Cell system under mineral oil. Proteins were focused in four steps (250 V for 20 min, linear gradient; 500 V for 1 h, linear gradient; 1000 V for 1 h, linear gradient; 4000 V for 20,000 V h, rapid gradient). Equilibration of the strips before the second dimension was the same as for the 17 cm IPG strips. After the equilibration step, the strips were rinsed in Tris-glycine buffer (pH 8.3), transferred to a homogeneous 12.5% (%T) SDS-polyacrylamide gel and the gel strips were overlaid with 0.5% (w/v) agarose in SDS-PAGE running buffer containing bromophenol blue. These mini gels were run in the Mini-Protean Tetra Cell system (Bio-Rad) for 5 min at 50 V and 40 min at 200 V.

Two 2D-DIGE gels were run for each format (17 cm as well as 7 cm gel), giving two 2D-DIGE gels for each format as technical replicates. After electrophoresis, the gels were washed with ultrapure H_2O for 2×15 min before scanning.

2.3. 2D image analysis

Three different gel images were obtained from one gel at the appropriate wavelength. They are Cy2 (blue 488 nm laser and 520 nm band pass emission filter), Cy3 (green 532 nm laser and 580 nm band pass emission filter) and Cy5 (red 633 nm laser and 670 nm band pass emission filter) by using a Pharos FX™ scanner (Bio-Rad) at a resolution of 50 μm.

The analysis of DIGE gels was done using PDQuest™ software (Bio-Rad), version 8.0.1. The gel-to-gel spot matching and statistical analyses of the protein abundances among the samples were carried out according to the manufacturer's protocol. After automatic spot detection, spots were manually revised with edition tools for a correct detection. Gel groups were established according to the experimental design. Local regression model was chosen as a normalization method. Spot normalized volume was used to select statistically significant differential spots (paired Student's *t*-test). The significance level was set at $p \leq 0.05$. Protein spots that were differentially expressed in the caries-susceptible group compared with the caries-resistant group ($|\text{ratio}| > 1.5$, $p \leq 0.05$) were selected for further mass spectrometry identification.

Following fluorescence scanning, DIGE gels were also stained with colloidal Coomassie brilliant blue stain (Bio-Safe, Bio-Rad) according to the manufacturer's recommendations to enable the visual detection of the various abundances in the spots.

2.4. Protein identification

Spots with differential expression were excised from the Coomassie-stained gels, and then processed as described in Shevchenko et al. [23]. The resulting dried tryptic peptide extracts were stored at −80 °C before analysis.

An analysis of the tryptic digests with nano-liquid chromatography tandem mass spectrometry (maXis, quadrupole-time of flight, as mass spectrometer) was performed as in the previous study [19].

Proteins were identified by correlating tandem mass spectra to the International Protein Index (IPI, v. 3.87) and SwissProt (v. 2015_07, 24.6.2015) databases, using the MASCOT on-line search engine for protein identification using mass spectrometry data (http://www.matrixscience.com). The taxonomy was restricted to *Homo sapiens* to remove protein identification redundancy. Trypsin was chosen as the enzyme parameter. One missed cleavage was allowed, and an initial peptide mass tolerance of ±10.0 ppm was used for MS analysis and of ±0.05 Da for MS/MS analysis. Cysteines were assumed to be carbamidomethylated, proline and lysine to be hydroxylated, and serine, threonine and tyrosine to be phosphorylated; methionine was allowed to be oxidated. All these modifications were set to be variable. The monoisotopic peptide charge was set to 1+, 2+, and 3+. Only significant hits (MASCOT score ≥ 80 for proteins) were accepted. The Peptide Decoy option was selected during the data-search process to remove false-positive results.

2.5. Western blot analysis

Proteins were separated by standard SDS-PAGE or 2-DE using a homogeneous 12.5% (%T) polyacrylamide gel as described before [24]. The gels were blotted onto PVDF membrane (Immobilon P, Millipore) by tank transfer for 1 h at 100 V in Mini Trans-Blot cell (Bio-Rad). Membranes were then processed according to Nůsková et al. [25]. Briefly, the membranes were blocked in TBS-Tween buffer (0.15 M NaCl, 10 mM Tris-HCl, pH 7.5 with 0.1% (v/v) Tween-20), and incubated overnight at 4 °C in TBS-Tween buffer containing primary antibodies against either alpha-1-antitrypsin (Abcam, 1:200, rabbit polyclonal), ATP synthase subunit beta (Abcam, 1:1000, mouse monoclonal), or actin (Calbiochem, 1:6000, mouse monoclonal). After the incubation with primary antibodies, membranes were washed with TBS-Tween buffer, and incubated for 1 h at 25 °C with appropriate secondary antibody (Invitrogen, 1:3000, anti-rabbit or anti mouse) labelled with Alexa Fluor 680. The fluorescence signal was detected using Odyssey infrared imaging system (LI-COR) and quantified using Quantity One 4.6.8 software (Bio-Rad). The data were statistically analyzed paired Student's *t*-test using OriginPro v8.0 software. Results are represented as the average of 3 immunoblots ± S.E.M.

3. Results

3.1. Comparative proteomics between caries-resistant and caries-susceptible samples by 2D-DIGE

The principal aim of this study was to perform a comparative tooth pulp analysis of subjects with caries in their oral cavity and subjects without any caries to identify possible differing expression patterns of tooth pulp proteins. A comprehensive tooth pulp proteome analysis was performed on tooth pulp samples originating from sound molars of 12 human subjects (6 subjects with completely sound teeth and 6 subjects that have had at least 4 caries lesions in their oral cavity) using 2D-DIGE. Two formats of IPG strips were used. For detecting as many spots as possible, the large format gels with broad-range 3–10 pH were used. Secondly, to visualize the region at pH of ca. 4–7 and a molecular weight of ca. 30–80 kDa with many clustered spots, the appropriate narrow-range IPG strips were used. Protein spots in all the images were detected, analyzed, and compared using the software PDQuest™ (Bio-Rad). Following the detection of differences, protein identifications in selected spots were determined by mass spectrometry.

3.1.1. 2D-DIGE

Our previous study on human tooth pulp showed 2-DE analysis of human tooth pulp highly reproducible allowing the detection of >300 proteins in one 17 cm gel [19]. Also the representative 2-DE Coomassie gels from tooth pulp samples used in this study were analyzed and the profiles of protein maps were reproducible in terms of protein spot number, relative positions and intensities (data not shown). Taking all these data into account, in our 2D-DIGE analyses we decided to analyze each pool (R and S groups) twice in each format. Thus, a total of four 2D-DIGE gels were performed, two different images of each pool sample (R and S) were obtained in the large format (17 cm gel) and two images of each pool sample (R and S) were obtained in the small format (7 cm gel).

The samples (R and S) were labelled with Cy3 or Cy5 and were run in two large-format 2-DE gels along with a pooled standard, which was labelled with Cy2. The image analysis showed that the average number of spots detected by CyDye stains on this large gel was ca. 300. Protein spots exhibited migration positions from 15 to 250 kDa but were predominantly clustered between 20 and 150 kDa (Fig. 1). Two mini-format (7 cm) 2-DE gels were also analyzed. The samples were stained with CyDye in the same way as with in large gels. The average number of spots detected was ca. 60, less than in the large gel. Protein spots with p*I* ca. 4–7 were now much easier to resolve and analyze (Fig. 2). Small-format gels were sufficient in this case.

A differential analysis between caries-resistant and caries-susceptible samples on large-format gels (17 cm) or mini-format gels (7 cm) found 45 or 15 matched protein spots that satisfied the arbitrary parameters defined as the relative expression $ratio_{R/S} \geq 1.5$ for upregulated spots and ≤ -1.5 for downregulated spots, respectively. Only protein spots displaying significant changes ($p \leq 0.05$) were considered for further mass-spectrometry analysis.

In the large gel image analysis, 9 protein spots exhibited significant differences when comparing the caries-resistant and caries-susceptible samples. Out of the 9 spots, 5 exhibited greater and 4 showed lower expression of the caries-resistant samples compared to the caries-susceptible samples. The mini gel image analysis showed 7 protein spots with significant differences when comparing the caries-resistant and caries-susceptible samples. Six spots exhibited greater and one spot exhibited lower expression of the caries-resistant samples compared to the caries-susceptible samples (Fig. 2, Table 1). All these differential spots were cut from the gels, digested with trypsin and used for nLC-MS/MS analysis.

The spots appearing either in the large-format or in mini-format gels in the pH 4–7 and 30–80 kDa region were identified by mass

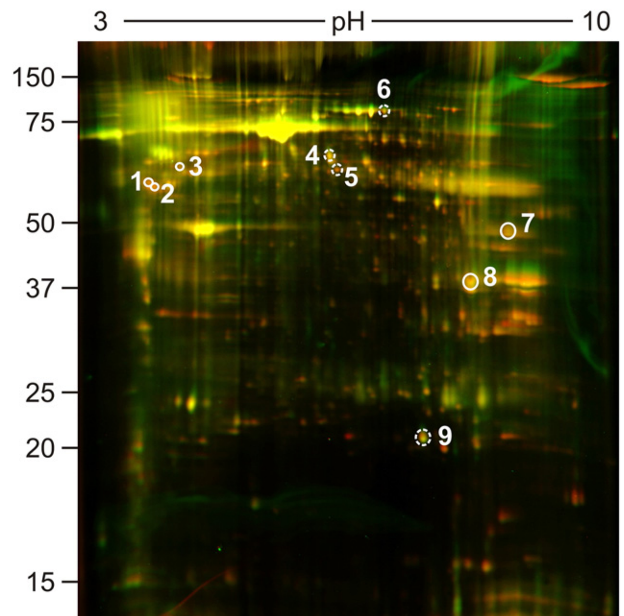

Fig. 1. 2D-DIGE comparative proteomics analysis between caries-resistant and caries-susceptible tooth pulp samples. The samples were separated on a 17 cm IPG strip with a non-linear pH gradient (3 − 10) followed by SDS-PAGE electrophoresis using a 12% homogeneous gel. A representative 2D-DIGE gel is shown as an overlay of Cy3 and Cy5 derived from a single gel, highlighting significant differentially expressed protein spots: a solid line for up-regulation and a dashed line for down-regulation in the caries-resistant samples. Molecular weight markers and their values in kDa are indicated. The highlighted spots were excised, digested with trypsin and identified by nLC-MS/MS. Information about the proteins corresponding to the spot numbers is listed in Table 1.

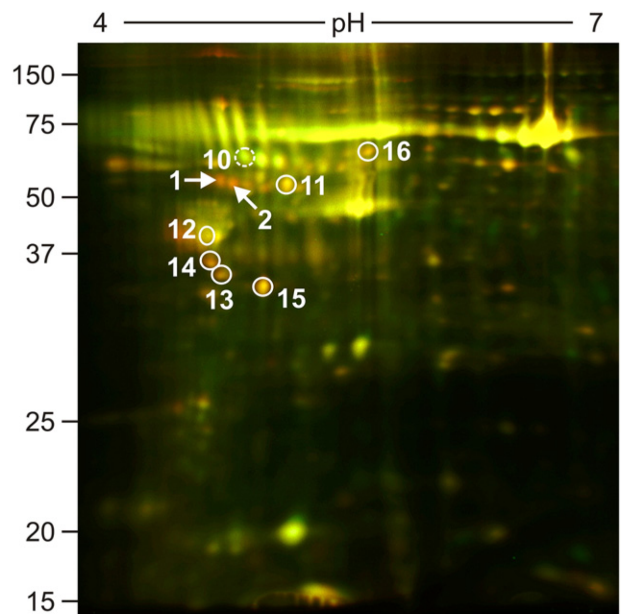

Fig. 2. The 2D-DIGE comparative proteomics analysis between caries-resistant and caries-susceptible tooth pulp samples. The samples were separated on a 7 cm IPG strip with a pH gradient (4–7) followed by SDS-PAGE electrophoresis using a 12% homogeneous gel. A representative 2D-DIGE gel as an overlay of Cy3 and Cy5 derived from a single gel, highlighting significant differentially expressed protein spots: a solid line for up-regulation and a dashed line for down-regulation in the caries-resistant samples. The spots that were statistically up or down-regulated on the 17 cm gel and their corresponding proteins that were identified as identical with spots in this gel are indicated with arrows. Molecular weight markers and their values in kDa are indicated. The highlighted spots were excised, digested with trypsin and identified by nLC-MS/MS. Information about the proteins corresponding to the spot numbers is listed in Table 1.

Table 1
List of significant differentially expressed proteins identified in human tooth pulp samples from caries-resistant and caries-susceptible subjects using 2D-DIGE.

Spot ID[a]	Accession SwissProt	Symbol (gene name)	Name of protein	Fold change (R/S)[b]	Uniq. peptides	Mascot score	Mr, teor.	pI, teor.
Upregulated protein								
Protein metabolism[c]								
1	A1AT_HUMAN	SERPINA1	Alpha-1-antitrypsin	6.15	11	539	46.7	5.3
2	A1AT_HUMAN	SERPINA1	Alpha-1-antitrypsin	4.32	14	727	46.7	5.3
16	CH60_HUMAN	HSPD1	Heat shock 60 kD protein 1, mitochondrial	2.26	34	1787	61.0	5.6
Metabolism; energy pathways[c]								
11	ATPB_HUMAN	ATP5B	ATP synthase subunit beta, mitochondrial	1.61	11	569	56.5	5.1
7	PGK1_HUMAN	PGK1	Phosphoglycerate kinase 1	2.25	18	1118	44.6	9.2
Cell growth and/or maintenance[c]								
13	TPM1_HUMAN	TPM1	Tropomyosin alpha-1 chain	1.85	41	2417	32.9	4.6
14	TPM1_HUMAN	TPM1	Tropomyosin alpha-1 chain	1.56	38	2199	32.9	4.6
3	VIME_HUMAN	VIM	Vimentin	2.25	30	1518	53.6	4.9
12	VIME_HUMAN	VIM	Vimentin	1.66	27	1279	53.6	4.9
Cell communication; signal transduction[c]								
8	ANXA2_HUMAN	ANXA2	Annexin A2	3.42	17	1069	38.6	8.5
15	ANXA5_HUMAN	ANXA5	Annexin A5	1.68	16	854	35.9	4.8
Downregulated protein								
Protein metabolism[c]								
10	A1AT_HUMAN	SERPINA1	Alpha-1-antitrypsin	0.58	18	959	46.7	5.3
Metabolism; energy pathways[c]								
9	PRDX1_HUMAN	PRDX1	Peroxiredoxin-1	0.66	9	440	22.1	9.2
5	SCOT1_HUMAN	OXCT1	Succinyl-CoA:3-ketoacid-coenzyme A transferase 1, mitochondrial	0.48	6	327	56.1	7.8
Transport[c]								
4	APOH_HUMAN	APOH	Beta-2-glycoprotein 1	0.62	16	887	38.3	9.5
6	TRFE_HUMAN	TF	Serotransferrin	0.43	26	1674	77.0	7.0

[a] Spot ID - The numbers correspond to the specific spots as indicated in Figs. 1 and 2.
[b] Average ratio DIGE (caries-resistant (R) and caries-susceptible (S) samples).
[c] Functional categories according to Human Protein Reference Database (www.hprd.org).

spectrometry. Comparison of the spot position in the gels with their relevant mass spectra enabled us to better identify the proteins in the spots.

3.2. Identification of differentially expressed proteins

Sixteen protein spots were successfully identified after the nLC-MS/MS analysis of spots of interest on both 2D-DIGE gels (these spots had statistically significant changes at the $p \le 0.05$ level) with high confidence after database searches (these spots are marked in the gels in Fig. 1 and Fig. 2). The combined spectra were searched against the IPI-human database using the Mascot engine. The protein name, accession number, theoretical molecular weight, and p*I* values, Mascot score, spot number, number of the gel, and where the spot was identified are shown in Table 1. In total, 16 spots remained with unique protein identifications corresponding to 12 non-redundant proteins.

The identified proteins were categorized according to the classification system used in the public database available at http://www.hprd.org. They have a variety of functions in biological processes (Table 1). The categories include energetic metabolism (33.3%), protein metabolism (16.7%), transport (16.7%), cell growth (16.7%), and cell communication (16.7%). Among the sixteen differentially expressed spots with identified proteins, seven proteins exhibited an increased expression level, while the remaining four proteins were downregulated in the caries-resistant group. One protein detected among the statistically different spots exhibited either up- or downregulation in this group. Some of these spots were highly upregulated; i.e., spot 1 corresponding to alpha-1-antitrypsin was upregulated by a factor of 6.2. Similarly another spot of alpha-1-antitrypsin was upregulated by a factor of 4.3. Some spots displayed downregulation, i.e. spot 6 corresponding to serotransferrin was downregulated by a factor of 0.43 (Fig. 1).

In some cases, MS analysis revealed the same identity for multiple protein spots appearing as separated entities in 2-DE gels, indicating the possibility of the presence of various isoforms of those proteins. The presence of several isoforms is due to many complex combinations of post-translational modifications, leading to multiple distinct posttranslationally modified protein spots for one protein [26]. In our gels, some distinct proteins were identified in several spots due to possible postranslational modifications or due to the cleavage or degradation of proteins generating different proteolytic fragments distinguished by 2-DE electrophoresis. Interestingly, the majority of the different isoforms of any particular protein exhibited an identical shift in expression (either up- or down-regulation) in the caries-resistant group (e.g. annexin A2, serotransferrin, tropomyosin alpha-1 chain, and vimentin), with one exception: alpha-1-antitrypsin, where up-regulation as well as down-regulation shifts on the spots were observed. Some of these discussed isoform spots satisfied the arbitrary parameters defined as the relative expression $ratio_{R/S} \ge 1.5$ for upregulated spots and ≤ -1.5 for downregulated spots, respectively, but did not satisfy the $p \le 0.05$ criterium for Student's *t*-test. Thus only the spots with a statistically significant fold change value are reported in Table 1 and are marked in Fig. 1.

All of the results of protein identification in the selected spots along with a comparison of caries-resistant protein spot relative abundances relative to the caries-susceptible group are summarized in Table 1.

3.3. Confirmation of protein expression by Western blot

To evaluate the performance of the 2D-DIGE quantitative proteomic approach used in this study, the expression level of tooth pulp proteins alpha-1-antitrypsin and ATP synthase subunit beta were further examined by Western blot on SDS-PAGE and/or 2-DE gels (Figs. 3 and 4). ATP synthase subunit beta was significantly upregulated in the caries-resistant group as compared to the caries-susceptible group (Fig. 3). One intensive spot of alpha-1-antitrypsin (spot 10) was slightly down-regulated in the caries-resistant group, whereas two other less intensive alpha-1-antitrypsin spots (spots 1 and 2) were strongly upregulated in the caries-resistant group as compared to the caries-susceptible group (Figs. 3 and 4). The agreement between the results, obtained by 2D-

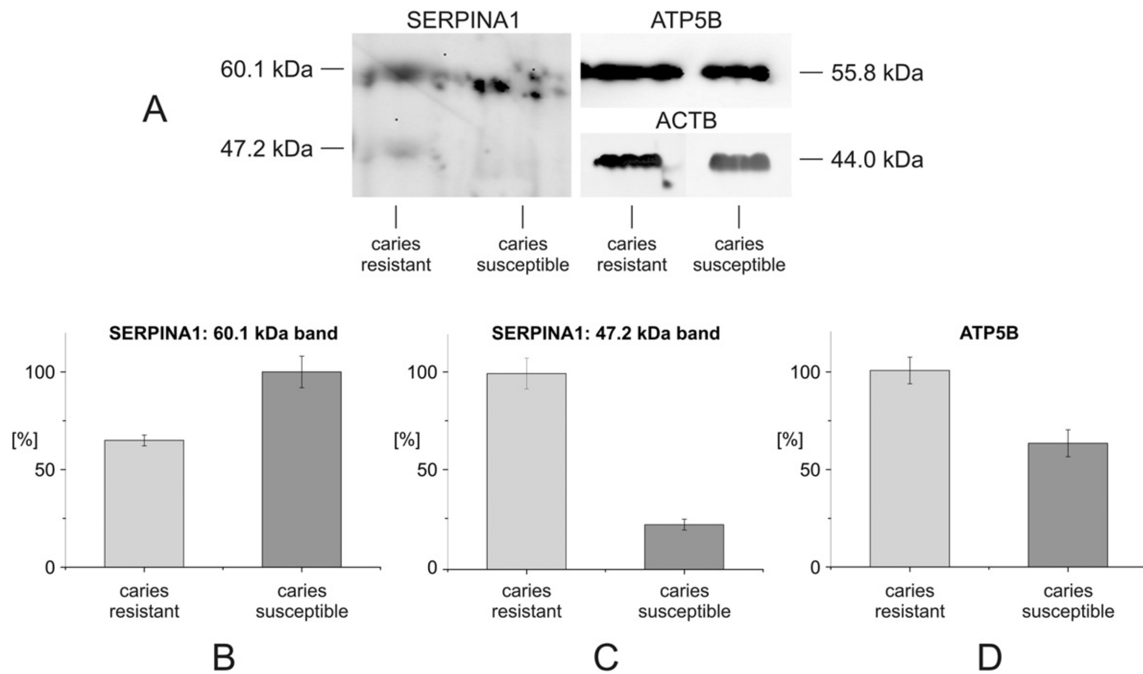

Fig. 3. Validation of protein expression by Western blot analysis. (A) SDS-PAGE using a 12% homogeneous gel for analysis of tooth pulp samples obtained from caries-resistant and caries-susceptible groups. Antibodies against SERPINA1 (alpha-1-antitrypsin), ATP5B (ATP synthase subunit beta), and ACTB (actin) were used for the samples as indicated. ACTB was used as an internal control. Image analysis showing the expression of SERPINA1 60.1 kDa band (B), SERPINA1 47.2 kDa band (C), and ATP5B (D). Bars represent the average of 3 immunoblots ± S.E.M. The expression pattern of SERPINA1 and ATP5B was consistent with the 2D-DIGE results.

DIGE and Western blot respectively, signifies the validity of the proteomic approach.

4. Discussion

4.1. Tooth samples and 2D-DIGE analysis

Human teeth (third sound molars) were extracted for clinical reasons from human adult patients in a dental clinic. Ca. 20% of the whole population undergoes extraction of the third molars during their lifetime. Additionally, ca. 10% of young people at the age of ca. 20–35 and even several percent of older people do not have any carious teeth in their oral cavity. These caries-free people were suitable donors of teeth for the caries-resistant group in our study. The second group of donors is the much larger group of caries-susceptible people suffering from at least four cured carious lesions in their set of teeth. The donors were randomly selected according to their health and social status. All members of both groups did not have periodontitis.

It is known that periodontitis is a complex immune-inflammatory disease, and it also plays an important role in the health status of the oral cavity and teeth. Periodontitis results from a pre-established infection in gingiva, mainly due to Gram-negative bacteria that colonize deeper in the gingival sulcus. Host inflammatory and immune responses have both protective and destructive roles. Although cytokines, prostaglandins, and proteases fight against the microbial burden, these molecules promote connective tissue loss and alveolar bone desorption, leading to several histopathological changes, which can converge to cause tooth loss. There is no available biomarker for periodontitis diagnosis, prognosis and treatment evaluation. Some genes, transcripts or proteins have already exhibited a different expression in healthy subjects and in patients [27].

Changes in dentin permeability caused by carious lesions, including tertiary dentin formation following a stepwise excavation procedure, represents a secondary biological reaction that is not only produced to protect the pulp, but also as a consequence of changes in the cariogenic environment to help stop the progress of the lesions [16]. We can assume that these reactions in the dentine/pulp complex are a defensive reaction of the dental pulp, and this cascade continues throughout the pulp-dentin complex and dentin tubules to create the anti-caries protective reaction which can stop and destroy the lesion in its initial

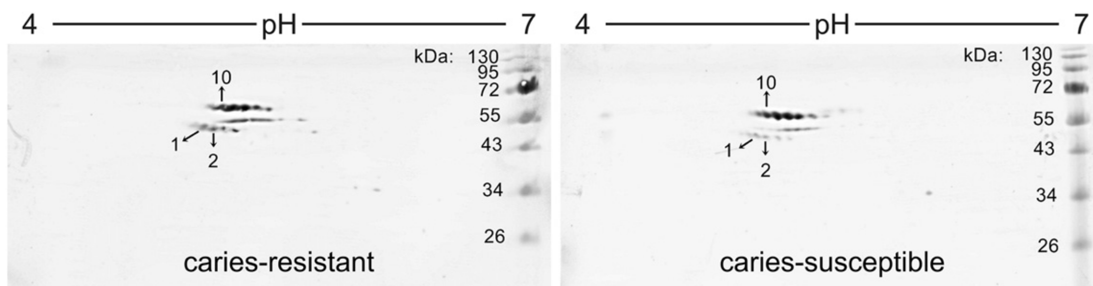

Fig. 4. Validation of protein expression by Western blot analysis. Two-dimensional resolution of alpha-1-antitrypsin content in samples obtained from caries-resistant group (left) or caries-susceptible group (right). The samples were separated on a 7 cm IPG strip (Bio-Rad) with a pH gradient (4–7) followed by SDS-PAGE using a 12% homogeneous gel and detected by an antibody against alpha-1-antitrypsin. The numbered spots represent statistically significant up or down-regulation. Molecular weight markers and their values in kDa are indicated. Information about the proteins corresponding to the spot numbers is listed in Table 1. The expression pattern of SERPINA1 spots was consistent with the 2D-DIGE results.

stage. We are working under the assumption that the dental pulp of caries-resistant people can be more active in its defensive reactions to non-cavitated caries and is better at keeping the tooth sound than the caries-susceptible group. Comparing the dental pulp proteomes of both studied groups could determine the proteins that take part in these processes. In this study we did not detect any upregulated proteins with antibacterial function in the tooth pulp of caries-resistant group, but our previous study revealed that these proteins are present in the tooth pulp [19]. The possibility that these antibacterial protein biomarkers are upregulated in pulp-dentin complex of caries-resistant people in a case of starting caries lesion is worth of future investigation.

Only a few studies have compared the protein composition of the oral cavity between caries-susceptible and caries-resistant people so far. These measurements have only been made on saliva samples. In this way, a significantly higher level of lipids and proteins was observed in the caries-susceptible group [28]. In a project comparing two groups of old people with and without root caries and one group of young people without root caries, 14 protein markers were observed [9]. Another study, focusing on enamel root caries, compared two groups of young people, and detected 44 differences in the saliva protein content. This study also compared the proteins of the in vitro created pellicle, and also found significant differences in the proteomes of the studied groups [8]. Finally, the biomarkers were very different in the "saliva" studies, probably due to using different sources and/or detection techniques [29]. A comparison of all these "saliva" studies with our results identified uniquely identical to what we found in our experiments, the study of Preza et al. also reported the specific upregulation of serotransferrin in the caries-susceptible [9]. Considering the fact that the proteomes of saliva and dental pulp are different, such coincidental detection of at least one biomarker protein is significant and promising. However, the specific role of serotransferrin in the caries-supporting process still remains unknown.

A 2D-DIGE technique was used to compare the tooth proteomes of different population groups with comparable age and having a varying number of tooth caries. It is a sensitive, precise and reproducible technique, capable of reliably detecting as little as 0.5 fmol of protein, and this detection system is linear over a 10,000-fold concentration range [30]. It can visualize hundreds to thousands of protein species, giving important information about changes in p*I* and Mr. Currently, due to the undeniable benefits of the method, 2D-DIGE still remains the most comprehensive top-down method to study changes in the abundance of intact proteins [31].

In this study, soluble proteomes of caries-resistant and caries-susceptible tooth pulp samples were separated with 2D-DIGE on 17 cm IPG strips with a mean number of ca. 300 spots. When the 2D-DIGE on broad-range IPG strips (17 cm, pH 3–10, non-linear gradient) was performed, the protein alpha-1-antitrypsin, ATP synthase subunit beta, and vimentin proteins (spots 1–5) were slightly clustered around a region of molecular mass ca. 45–55 and pH 4.9–5.3 (Fig. 1). The narrow-range IPG strips (7 cm, pH 4–7) were successfully used to improve the resolution of these protein spots, as is shown in Fig. 2.

In this study, twelve proteins were found to be differentially expressed between the caries-resistant and caries-susceptible samples and they are summarized in Table 1. Almost all of these proteins with significantly altered expression (except heat shock 60 kD protein 1) were also detected in the human dental pulp in our previous study [19]. Six of these proteins (alpha-1-antitrypsin, phosphoglycerate kinase 1, peroxiredoxin 1, serotransferrin, tropomyosin alpha-1 chain, and vimentin) were observed by Pääkkönen et al. in the human tooth pulp tissue [17]. Additionally, vimentin was detected in human dental pulp cells [18]. Surprisingly, none of them are in accordance with a comprehensive list of proteins published in one of the more recent studies by Eckhard et al. [20]. Though the reason behind the discrepancy between their results and the results presented in this study remains unclear, it could be attributed to the completely different analytical approach used in their study.

4.2. Proteins related to metabolism and energy production

The functional categories with the highest number of differentially expressed spots of proteins were involved in the energetic and protein metabolism (Table 1).

4.2.1. Caries-resistant proteins related to metabolism and energy production

Statistically significant changes were detected in three spots of alpha-1-antitrypsin. Two less intensive spots were upregulated (>4-fold) and one more intensive spot with a slightly higher molecular weight and higher p*I* value was downregulated (ca. two-fold) in the caries-resistant group (Table 1, Figs. 1, 2 and 4). Incidentally, alpha-1-antitrypsin was also detected in other large and relatively intensive spots that were statistically insignificant. According to the reference map in SWISS-PROT database, alpha-1-antitrypsin has multiple, naturally occurring MW and p*I* isoforms (http://world-2dpage.expasy.org/swiss-2dpage/viewer&map5CSF_HUMAN&ac5P01009). Therefore, the appearance of the multiple alpha-1-antitrypsin isoforms occurring as distinct spots with different MW and p*I* values in our human tooth pulp samples is not surprising. These isoforms can undergo differential posttranslational modification (e.g. glycosylation) or get truncated [32]. The alteration in the levels of alpha-1-antitrypsin variants can also be used as markers of various diseases [33]. Therefore, our observation of the different alpha-1-antitrypsin spots (spot 1, 2, or 10) in the human tooth pulp samples needs further in-depth investigation.

Alpha-1-antitrypsin is a major circulating and tissue inhibitor of serine proteinases implicated in the regulation of inflammation and host defence. It may also exhibit anti-inflammatory activities independent of its protease inhibitor function. Alpha-1-antitrypsin at physiologic and inflammatory concentrations positively modulates the proliferation and motility of stromal cells from human exfoliated deciduous teeth in vitro. Thus Aldonyte et al. demonstrated the importance of this protein in the maintenance and regulation of tissue progenitor cells in vivo [34]. Recent studies in animal models have revealed oxidative stress and oxidative damage in the pathogenesis of alpha-1-antitrypsin deficiency. Escribano et al. studied the oxidative stress status and antioxidant enzyme activity in children with alpha-1-antitrypsin deficiency. It was revealed that increased oxidative stress, together with reduced antioxidant defence is involved in the pathophysiology of the early stages of alpha-1-antitrypsin deficiency [35]. Alpha-1-antitrypsin also inhibits *Moraxella* (a genus of Gram negative bacteria) IgD binding protein-induced B cell activation in vitro that is unrelated to its protease inhibitory activity and is not dependent on the *Moraxella* IgD binding protein binding to the cell surface [36]. To summarize the previous discussion on alpha-1-antitrypsin effects in the tooth pulp, we can hypothesize that the increased level of alpha-1-antitrypsin could support inhibition of the activity of serine proteinases in tooth tissues, leading to an elevated resistance of teeth to the development of tooth caries. It could also act as anti-inflammatory in the patients without dental caries in their "sound" oral cavity.

ATP synthase subunit beta and phosphoglycerate kinase 1, other up-regulated proteins in caries-resistant samples in present study, are important enzymes that provide energy for the cell to use. ATP synthase (up-regulated 1.6-fold, Table 1) catalyzes the final coupling step of oxidative phosphorylation to supply energy in the form of ATP [37].

Phosphoglycerate kinase 1 (up-regulated 2.3-fold) catalyzes an important ATP-generating step in glycolysis. Honda et al. studied the functional role of phosphoglycerate kinase 1 in the developing tooth germ of the mouse lower first molar. The strong expression of phosphoglycerate kinase 1 mRNA was seen in the odontogenic epithelial cells and surrounding mesenchymal cells of the tooth germ from embryonic day 10.5 to E18.0. It was shown that phosphoglycerate kinase 1 forms an 84-kDa protein complex in these embryonic organs. Their overall results suggested that phosphoglycerate kinase 1 plays some functional roles in the development of tooth germ and other embryonic organs by forming

a protein complex with glyceraldehyde-3-phosphate dehydrogenase [38]. The function of the phosphoglycerate kinase 1 in connection to the prevalence of dental caries remains unclear.

It is interesting that both of the above enzymes are upregulated in the caries-resistant patients, which could indicate an enhanced cell turnover and production of ATP in these teeth. Liu et al. provided evidence for the existence of the functional machinery required for ATP release and degradation in human dental pulp, and that pannexin channels are involved in external dentin stimulation-induced ATP release. These findings support a plausible role for ATP signaling in dentin hypersensitivity and dental pain [39]. Zhang et al. studied the mechanisms that lead to the regulation of NOD-like receptor family, pyrin domain-containing 3 (NLRP3) inflammasome expression and activation in human dental fibroblasts. The NLRP3 inflammasome plays an important role in the cellular defence against invading pathogens, and is reported to be expressed in human dental pulp fibroblasts. They declared that ATP promoted the generation of reactive oxygen species and activated the NLRP3 inflammasome in a reactive oxygen species-dependent manner [40].

The other up-regulated protein in the caries-resistant samples is heat shock 60 kD protein 1, up-regulated by ca. 2.3-fold. Heat shock protein 60 kD (also known as chaperonin) is a protein that is responsible for the transportation and refolding of proteins from the cytoplasm into the mitochondrial matrix. The protein has been implicated in atherosclerosis and it contributes to T-cell activation [41]. Heat shock protein 60-based immuno-regulation is being exploited clinically for the treatment of autoimmune disorders; the administration of a peptide from this human chaperonin has been experimentally reported to halt Sjögren's syndrome (a chronic autoimmune disease in which the body's white blood cells specifically destroy the salivary and lacrimal glands) [42].

4.2.2. Caries-susceptible proteins related to metabolism and energy production

In addition to the above proteins which exhibited increased expression levels, some other identified proteins exhibited reduced levels in the caries-resistant samples, such as peroxiredoxin-1 (0.66-fold), and succinyl-CoA:3-ketoacid-coenzyme A transferase 1 (0.48-fold).

Peroxiredoxin-1 is an antioxidant enzyme that catalyzes oxidation/reduction processes and is able to serve as redox biomarker in various human diseases, and is also the key regulator controlling the redox state of functional proteins. Redox regulators with antioxidant properties related to active mediators, cellular organelles, and the surrounding environments are all connected within a network and are involved in diseases related to redox imbalance including cancer, ischemia/reperfusion injury, neurodegenerative diseases, as well as normal aging [43]. It is known that the salivary concentrations of thiobarbituric acid-reacting substances (markers of lipid peroxidation) are associated with periodontal status assessed as the papillary bleeding index. Celecová et al. analyzed the salivary markers of oxidative stress in dental patients in relation to age, gender and oral health. They revealed that the caries index negatively affected the salivary concentrations of advanced oxidation protein products and positively affected the salivary concentrations of thiobarbituric acid-reacting substances. These phenomena indicate that dental caries have an influence on reactive oxygen species – an increased production of peroxiredoxin-1 could be the response to this situation [44].

Succinyl-CoA:3-ketoacid-coenzyme A transferase 1 plays a crucial role in ketone-body metabolism. Its deficiency is one of the main defects in patient ketolysis [45]. Any supporting information for this enzyme's function in the development of or resistance to dental pulp caries is still unknown.

4.3. Structural proteins involved in cytoskeletal organization

Two spots corresponding to annexins were detected in this study. Annexin A2 was detected as 3.4-fold upregulated in caries-resistant samples (spot 8, Fig. 1) and annexin A5 was also found to be ca. 1.7-fold upregulated in caries-resistant samples (spot 15, Fig. 2). Annexins are soluble proteins that belong to a family of calcium-dependent phospholipid membrane-binding proteins. Annexins seem to play a role in various cellular activities such as vesicle trafficking, calcium signaling, cell division, cell growth regulation, and apoptosis [46].

Annexin A2 is an abundant protein which plays multiple roles in signal transduction, cell proliferation, apoptosis, endocytosis, exocytosis, and inflammation [47]. In addition to an intracellular role, annexin A2 has been reported to participate in processes localized to the cell surface, including extracellular protease regulation and cell-cell interactions. Differential expression of annexin A2 was observed between normal and malignant tissues [48]. Recent results from Li et al. indicate that annexin A2 regulates autophagy, thereby contributing to host immunity against bacteria through the Akt1-mTOR-ULK signaling pathway [49].

Annexin A5 is the smallest member of the annexin family and plays a central role in the machinery of membrane repair via the formation of a protective two-dimensional bandage at a membrane damage site. Current knowledge on cell membrane repair and findings on the role of annexin A5 in membrane resealing were recently reviewed [50]. Liu et al. proposed that annexin A5 inhibits the inflammatory effects of phospholipids, and decreases vascular inflammation. Annexin A5 promotes the induction of regulatory T cells and thus is potentially interesting as a therapeutic agent [41].

Both of these annexins were also detected in human dentin [24] and annexin A2 was also found in tooth cementum [51]. This provides evidence of the presence of these proteins in almost the whole tooth. These annexins (A2 and A5) upregulated in the caries-resistant group could reinforce oral immunity. Some of their roles in defence mechanisms were proved in recent studies [41,49].

Vimentin was the other protein found in several spots in this study. Two of them were statistically significantly upregulated (spots 3 and 12, Figs. 1 and 2 in the caries-resistant group). One of the upregulated spots (spot 12) was relatively more intensive and proved to only include the peptides from the "middle" part of the whole protein amino acid chain (amino acids from the 101 to 410 position – without the protein "head" and "tail"). In the less intensive vimentin spot (spot 3), we could detect peptides covering almost the whole protein chain (from 14 to 440 amino acid position). Its longer amino acid chain is in accordance with its higher observed molecular weight in the gel. Moreover, the presence of vimentin in multiple spatially relatively distant spots (due to its modifications) is not surprising. This was also shown in the previous tooth pulp studies [17,19]. Vimentin belongs to a large family of intermediate filament proteins that play important structural and functional roles in the formation and regulation of the cytoskeleton. The regulation of vimentin is highly complex and is driven by posttranslational modifications such as phosphorylation and cleavage by intracellular proteases [52]. An immunolocalization vimentin study performed in human incisors and canine tooth germs demonstrated spatio-temporal expression, indicating the participation of vimentin in cell proliferation and migration, and the differentiation of preodontoblasts and preameloblasts [53].

Tropomyosin alpha-1 chain was also an upregulated protein found in the caries-resistant samples (Fig. 2). It was found in two spots (13 and 14) exhibiting a (1.85- and 1.6-fold increase). Both tropomyosin spots were characterized by MS analysis as two isoforms of this protein with molecular weights of 32.9 kDa and 32.7 kDa. Tropomyosin naturally exists in multiple isoforms in all mammalian cells and tissues. In humans tropomyosins play important roles in actin cytoskeleton functions, such as intracellular vesicle movement, cell migration, cytokinesis, cell proliferation and apoptosis. In vitro biochemical studies and in vivo localization studies suggest that different tropomyosin isoforms have differences in their actin-binding properties and their effects on other actin-binding protein functions [54].

The function of vimentin and tropomyosin alpha-1 chain in dental pulp in relation to the anti-caries mechanism remains unknown.

4.4. Other proteins

Among the five down-regulated proteins in the caries-resistant samples detected in this study, two of them belong to proteins with transport functions: beta-2-glycoprotein 1 and serotransferrin.

Beta-2-glycoprotein 1 (also known as apolipoprotein H) was found to be downregulated by a factor of 0.6 in this study. It is a plasma glycoprotein protein that in vivo interacts with the lipid layer of membranes. It is also associated with low-density lipoprotein thus having an influence on the cholesterol levels in blood [55].

Serotransferrin was also found to be downregulated by a factor of 0.4 in this study. The serotransferrin was detected as isoforms producing five main spots with variable intensity at the same molecular weight and different p*I* (Fig. 1). Serotransferrin was shown to be quantitatively downregulated in almost all of the spots, but the difference was only found to be statistically significant in one spot (8). This significant spot of serotransferrin probably consist one of the modified forms, however it is very difficult to ascertain the type. Serotransferrin is known to be the main iron transport protein in the blood, but it can also bind and transport other alternative metal ions [56]. The cause of elevated serotransferrin and beta-2-glycoprotein 1 levels observed in the caries-susceptible tooth pulp samples remains unknown. However, our observation is in accord with the large comparative study of saliva conducted by Preza et al. who also found elevated levels of serotransferin in saliva samples of the caries-susceptible group [9].

4.5. Applications of the results

We tried to find the tooth protective factors (antimicrobial proteins or peptides) in our samples. These factors could be employed in various practical products and applications. Our findings of the proteins possibly responsible for the resistance of humans to dental caries might be built upon in several ways. If the anti-caries function of the presented proteins is confirmed, they could be tested in tooth pastes, as nutritional additives or as novel drug treatments. One modern approach how for improving human oral cavity health is using bioactive or biologically similar synthesized compounds: Li et al. successfully applied a novel peptide based on the enamel protein amelogenin to promote a remineralization of initial enamel caries lesions. This peptide also inhibited the progress of enamel caries [57].

5. Conclusion

We present for the first time a proteomic 2D-DIGE analysis to elucidate the changes in expression between the third molar tooth pulp of caries-resistant and caries-susceptible people. This study found overexpressed proteins in caries-resistant samples: annexin A2, annexin A5, ATP synthase, phosphoglycerate kinase 1, heat shock protein 60, tropomyosin alpha-1, isoforms of alpha-1-antitrypsin and vimentin. These proteins play roles in protein metabolism, cytoskeletal protein binding, calcium ion binding or are structural constituents of the cytoskeleton. We assume that these proteins are likely involved in the natural resistance of teeth to the development of dental caries. On the other hand, some proteins were overexpressed in caries-susceptible samples (peroxiredoxin-1, succinyl-CoA:3-ketoacid coenzyme A transferase, beta-2-glycoprotein, and isoforms of serotransferrin and serpin B3). Our findings also suggest that resistance to the development of dental caries is a multifactorial phenomenon. We believe that this study improves our current understanding and provides information that will help to achieve better stomatology prevention and treatment. It is not possible to declare that we have found proteins that will prevent people from developing dental caries, but surely the first step in comparative proteomics of human teeth in connection to dental caries has been taken. Further studies in this direction would be necessary to better understand these defensive, anti-caries mechanisms.

Conflict of interest

All authors declare no conflicts of interest.

Acknowledgments

Dr. Marek Vrbacký is acknowledged for his kind help with performing Western blot analysis. This work was supported by the Ministry of Health Departmental Program for Research and Development (NT14324-3/2013), and with support for long-term conceptual development of research organization RVO:67985823.

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