Expression of small leucine rich extracellular matrix proteoglycans biglycan and lumican reveals oral lichen planus malignant potential

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Abstract

Objectives

The aim of this study was to examine molecular alterations on the protein level in lesions of oral lichen planus (OLP), oral squamous cell carcinoma (OSCC) and healthy mucosa.

Materials and methods

Global protein profiling methods based on liquid chromatography coupled to mass spectrometry (LC-MS) were used, with a special emphasis on evaluation of deregulated extracellular matrix molecules expression, as well as on analyses of IG2F and IGFR2 expression in healthy mucosa, OLP and OSCC tissues by comparative semiquantitative immunohistochemistry.

Results

Mass spectrometry based proteomics profiling of healthy mucosa, OLP and OSCC tissues (and accompanied histologically unaltered tissues, respectively) identified 55 extracellular matrix proteins. Twenty among identified proteins were common to all groups of samples. Expression of small leucine rich extracellular matrix proteoglycans lumican and biglycan was found both in OSCC and OLP and they were validated by Western blot analysis as putative biomarkers.

A significant increase ($p<0.05$) of biglycan expression in OLP-AT group was determined in comparison with OLP-T group, while lumican showed significant up-regulation ($p<0.05$) in OLP-T and OSCC-T groups vs. adjacent and control tissue groups. Biglycan expression was only determined in OSCC-AT group. Immunohistochemical analysis of IGF2 and IG2FR expression revealed no significant difference among groups of samples.

Conclusion / Clinical relevance

Biglycan and lumican were identified as important pathogenesis biomarkers of OLP that point to its malignant potential.

Keywords: oral lichen planus, proteomics, biglycan, lumican, IGF2, IGF2R

1. INTRODUCTION

Oral lichen planus (OLP) is a chronic T cell mediated autoimmune inflammatory mucocutaneous disease that usually affects middle-aged females [1,2]. It is categorised as a potentially malignant disorder associated with an increased risk of oral cancer [3],with variable estimated rates ranging from 0-5.3 % [4]. The association between OLP and OSCC has been investigated at the molecular pathophysiological level with modern technologies [5-11]. It was suggested that chronic inflammation in OLP induces DNA damage, leading to increased cell proliferation and the development of oral cancer [10,11].

Mass spectrometry-based proteomics approach is a valuable tool for identification of novel cellular determinants and molecular mechanisms in many types of cancers, including head and neck cancers [12].In particular, novel potential oral cancer biomarkers might be exploited in individualised and improved therapeutic management of patients. Such cancer biomarkers might have a prognostic or therapeutic value in the clinical practice. Therefore, the rationale of presented study was to investigate in more details molecular alterations on the protein level in such lesions by use of global protein profiling methods based on liquid chromatography coupled to mass spectrometry (LC-MS) with a special emphasis on evaluation of deregulated extracellular matrix molecules expression, which is known to be of particular importance in pathogenesis of many diseases including tumours. In addition, malignant transformation in many types of cancers, including head and neck cancer, correlates with deregulation of the insulin-like growth factor receptor signalling pathways [13-16]. This signalization is important in initial steps of malignant transformation of many carcinomas and alternations in the Insulin growth factor receptor (IGFR) - mediated cellular signalling pathway are also associated with head and neck cancers [8-11, 17-21] In addition, expression of the IGF2R was found to be essential for cytotoxic T cell-mediated apoptosis of target cells both *in vitro* and *in vivo* conditions which might be important in malignant disease as well 22]Another factor associated with inflammation that contributes to malignant transformation and tumor growth is a disrupted extracellular matrix (ECM) dynamics and composition that favorizes a tumor- promoting microenvironment [23,24]. Such ECM changes positively impact extracellular-mediated intracellular signal transduction processes relevant for cell proliferation [25].

In this paper we therefore, present OLP and OSCC proteomics results, with a particular emphasis on expression analysis of ECM biglycan and lumican and corresponding IGF2R axis status.

2. MATERIALS AND METHODS

2.1. Tissue samples

A total of 62 biopsy samples were analysed by immunohistochemistry, including samples obtained from 24 patients diagnosed with oral lichen planus (collected during surgery on Department of Oral Medicine, School of Dental Medicine, University of Zagreb; OLP group), 20 samples obtained from untreated patients diagnosed with oral squamous cell carcinoma (collected during surgery on Department of Maxillofacial Surgery, Clinical Hospital Dubrava, Zagreb; OSCC group) and 18 samples of clinically healthy oral mucosa (archival samples of the Department of Pathology „Ljudevit Jurak“, Clinical Hospital „Sisters of Mercy“, Zagreb; CONTROL group). The main clinical and pathological characteristics of patients are shown in Table 1. Characteristic clinical findings of patients with OLP and OSCC are shown in Figures 1 and 2. In patients with OLP and OSCC diagnosis was established according to clinical and histopathological criteria [26-28]. Only patients with both clinically and histopathologically confirmed diagnosis of OLP and OSCC were included in the study Exclusion criteria were non-conformation of clinical and histopathological diagnosis or treatment with chemo- or radiotherapy in patients with OSCC before taking the tissue sample, to avoid the effect of therapy on results of tissue analysis. For immunohistochemical analysis, all biopsies were fixed in 10% phosphate buffer formaldehyde and embedded in paraffin blocks. In addition, tissue samples from 3 patients with both clinically and histopathologically confirmed diagnosis of oral lichen planus, 3 patients with both clinically and histopathologically confirmed diagnosis of OSCC and 3 patients with clinically healthy oral mucosa and without previous anamnestic data regarding oral diseases, which were selected after clinical examination, were collected for global proteomic profiling. From each patient with OLP and OSCC two tissue samples were taken- one from clinically changed mucosa (OLP-T, OLP tissue; OSCC-T, OSCC tissue) and the other from clinically healthy adjacent mucosa (OLP-AT, OLP adjacent tissue; OSCC-AT, OSCC adjacent tissue). Tissue samples from OLP patients were collected during surgery on Department of Oral Medicine, School of Dental Medicine, University of Zagreb., Tissue samples of OSCC patients were collected during surgery on Department of Maxillofacial Surgery, Clinical Hospital Dubrava, Zagreb. Tissue samples of healthy oral mucosa were collected during alveotomy on Department of Oral Surgery, School of Dental Medicine, University of Zagreb; CONTROL group). All aforementioned tissue samples (15 samples from 9 patients) were frozen in liquid nitrogen immediately after sampling. The main clinical and pathological characteristics of patients are shown in Table 2. All experiments were conducted with the approval of the Ethics committee of School of Dental Medicine, University of Zagreb; Clinical Hospital Dubrava and Clinical Hospital Centre „Sisters of Mercy“, Zagreb. Our research was registered at the U.S. National Institutes of Health (clinicaltrials.gov) (trial identifier: NCT03026361).The informed consent was signed by all research participants.

2.2 Immunohistochemistry analysis of IGF2 and IGF2R expression

Oral squamous cell carcinoma (OSCC), oral lichen planus (OLP) and healthy oral mucosa tissue sections (total number = 62 tissue sections) were de-paraffined in xylene twice for 10 minutes and rehydrated in a descending set of ethanol. Endogenous peroxidase activity was inhibited by incubating the slides for 30 min in 3% hydrogen peroxide, followed by heat-mediated antigen retrieval using EnVision Flex Target Retrieval Solution (Dako, Denmark). Immunohistochemistry staining was performed by using immune-peroxidase avidin-biotin method. Briefly, tissue sections were incubated with primary polyclonal IGF2 (1:1000, Abcam, UK) and IGF2R (1:1000, Abcam, UK) for 30 min at room temperature. In addition, incubation with EnVision Rabbit Linker (Dako, Denmark) was done for IGF2R antibody. After incubation with primary antibody, slides were incubated with secondary biotinylated anti- goat antibody (Dako, Denmark) for 15 min at room temperature. Slides were reacted for 30 min with the EnVisionTM FLEX (K8000 Dako, Denmark) detection system. The antigen-antibody reaction was visualized by diaminobenzidine (DAB)-containing substrate solution (EnVision FLEX DAB+Chromogen, Dako, Denmark) diluted with hydrogen peroxide containing solution (EnVision FLEX Substrate Buffer, Dako, Denmark) for 15 minutes at room temperature and washed with distilled water. Slides were counterstained with haematoxylin (EnVision FLEX Hematoxylin, Dako, Denmark). Positive control was adult human heart valve tissue in which expression of IGF2 and IGF2R is positive. As a negative control slide, the same tissue incubated without primary antibody was used. All slides were analysed using Zeiss Axiophot light microscope (Zeiss, Germany) and evaluated independently by two surgical pathologists who were blinded to the clinicopathological characteristics of each patient**.** Immunohistochemical reactions were determined at the sites of strongest antibody activity in ten different representative areas of each section selected upon the whole section inspection (magnification 100x), where 100 cells were counted for assessment of IGF2 and IGF2R expression. Obtained immunohistochemistry results were evaluated semi-quantitatively by use of immunoreactivity scoring system (IRS) according to previously described criteria [13]. In short, IRS score of IGF2 and IGF2R (range 0-9) was determined in OSCC, OLP and histologically normal oral mucosa tissue sections (‘CONTROL tissue’) by combination of staining intensity (SI) determined by the intensity of positive control staining and percentage (%) of stained cells. IRS score values 0-1 were considered as negative, 2-3 as weak, 4-6 as moderate and ≥7 as a high immunoreactivity. These results are presented in the Supplementary material.

2.3 Preparation of tissue homogenates for mass spectrometry and Western blot analysis

A total of 15 tissue samples divided in 5 groups (OSCC-T and matched OSSC-AT group; OLP-T and matched OLP-AT; CONTROL group) were mechanically ground and homogenized in a mortar with liquid nitrogen. Homogenized tissue was dissolved in 1ml of a lysis buffer [7M urea/2M thiourea (Sigma-Aldrich, USA), 4% (w/v) CHAPS (Sigma-Aldrich, USA), 0.2% (w/v) mixture of ampholytes pH 3-10 (Bio-Rad, USA), 1% (w/v) dithiothreitol (DTT) (Sigma-Aldrich, USA)] and 1x protease inhibitor cocktail (Roche, Switzerland). Obtained lysates were subjected to sonication with 4mm probe, power 6W (MicrosonTM, PGC Scientifics, USA), four times for 10 seconds. After sonication, 10µl of nuclease mix (Amersham, USA) was added to the samples and incubated for 1 hour at room temperature with gentle agitation on a thermos-shaker (Eppendorf, Germany). Samples were then centrifuged for 45 minutes at 14000 rpm and 4°C (Eppendorf, Germany). The supernatants were collected separately and stored at -80°C. Protein concentrations were determined by use of Qubit™ fluorometric (Invitrogen, USA) quantitation platform. Before measurement of protein concentration the platform was calibrated with Qubit protein standards (Invitrogen, USA).

2.4 Sample preparation procedure for mass spectrometry analyses

For mass spectrometry analysis (LTQ Velos Orbitrap Velos mass spectrometer, Thermo Scientific, San Jose, CA, USA) 15 tissue samples divided in 5 groups (OSCC-T and matched OSSC-AT group; OLP-T and matched OLP-AT; CONTROL group) were prepared according to manufacturer's instructions by use of protein digestion kit RapiGest SF (Waters, USA). Briefly, 50 µg of total proteins were precipitated in four volumes of ice cold acetone at -20°C overnight for removal of ionic impurities and lipids. After precipitation of proteins, samples were centrifuged for 10 minutes at 4°C and 14000 rpm followed by acetone removal and drying out the protein precipitates in a vacuum concentrator (Eppendorf, Germany). To each sample, 50 µl 0.1% working RapiGest SF solution and DTT at 5mM (Sigma-Aldrich, USA) were added. After incubation of 30 minutes at 50°C, the samples were cooled to room temperature, and iodoacetamide at final concentration 15mM was added in a dark chamber for 30 minutes. Enzymatic digestion of proteins was performed by addition of 5µl of sequencing grade trypsin at final trypsin concentration of 20 µg/ml (Promega, Madison, WI, USA) to each sample and incubation for 4 hours at 37°C. RapiGest SF surfactant (Waters, USA) was hydrolyzed by adding TFA (Sigma-Aldrich, USA) to a final concentration of 0.5% and incubation at 37 °C for 35 minutes. Acid treated samples were then centrifuged for 10 minutes at 15°C and 14000 rpm and obtained solutions were transferred to new vials. Aliquots containing 6 µg of proteins (2.5% of the original amount) were desalted by using ZipTip C18 pipette tips (Merck Millipore, Billerica, MA, USA) according to manufacturer recommendations. Peptides were eluted with 10 µl of a 60%

acetonitrile containing solution (Sigma-Aldrich, USA) with 0.1% TFA and dried in a vacuum concentrator (Eppendorf, Germany). The samples were re-dissolved in 20 µl of 3% acetonitrile and 0.1% formic acid for the LC/MS analyses.

2.5 Nano-LC-MS/MS analysis

Tryptic peptides were fractionated on a 75 µm × 12 cm column containing 3 µm Monitor C18 resin (Orochem Technologies, Inc., Lombard, IL, USA) and having an integrated 10 µm ESI emitter tip ("Self-Pack" PicoFrit column; New Objective, Woburn, MA, USA). Solvent A was 0.1 M acetic acid in water and solvent B was 0.1 M acetic acid in acetonitrile. Peptides were eluted with a linear acetonitrile gradient (0-70% solvent B over 60 min), operated at 200 nL/min using an Agilent 1200 HPLC (Agilent Technologies, Santa Clara, CA, USA). The column eluate was introduced directly into the LTQ Velos Orbitrap Velos mass spectrometer (Thermo Scientific, San Jose, CA, USA) with a 1.8 kV ESI voltage. Full MS scans in the m/z range 300-1700 at a nominal resolution of 60000 were collected in the Orbitrap, followed by data-dependent acquisition of MS/MS spectra for the ten most abundant ions in the LTQ ion trap. Only ions having a charge state ≥ 2 were considered for collision-induced dissociation. Repeated fragmentation of the same ion was minimized by employing a 30-second dynamic exclusion time.

2.6 Protein identification

MS/MS spectra were searched against the human Uniprot protein database (downloaded on 04/12/2014 from Uniprot) using the Mascot algorithm v.2.3.2 provided by Matrix Science. Mascot searches were performed with the following parameters: trypsin enzyme specificity, 2 possible missed cleavages, 20 ppm (LTQ OrbiTrap Velos mass spectrometer (Thermo Scientific, San Jose, CA, USA)) mass tolerance for precursor ions, 0.5 Da mass tolerances for fragment ions. Search parameters specified a differential modification of oxidation on methionine and a static modification of carbamidomethylation (+57.0215 Da) on cysteine. For high confidence on peptide sequence assignment and protein identification, data were filtered with following stringent criteria: Mowse score > 64 for all charge states, at least 3 peptides per protein, 1% peptide false discovery rate (FDR) and 1% protein FDR. Determination of subcellular localisation of identified proteins was done by detailed Uniprot database (http://uniprot.org) search. Additionally, biological functions of identified extracellular matrix proteins were individually analysed in Uniprot database.

2.7 Western blot analysis

For the Western blot procedure a total of 50 μg proteins from aforementioned 15 tissue samples divided in 5 groups (OSCC-T and matched OSSC-AT group; OLP-T and matched OLP-AT; CONTROL group) were resolved on 10 % SDS polyacrylamide gels using the Mini-protean cell (Bio-Rad, USA). The PVDF membranes (BioRad, USA) were incubated with primary antibodies raised against human lumican (1:1000, rabbit mAb, Abcam, UK) and biglycan (1:1000, rabbit mAb, Abcam, UK) at 4°C overnight respectively. Secondary antibody linked to anti-rabbit (1:1000, Dako, USA) was used. The signal was visualized by Western Lightning Chemiluminescence Reagent Plus Kit (Perkin Elmer, USA) on the ImageQuant LAS500 (GE Healthcare, USA). As the loading controls (1:1000, α-tubulin, mAb, SigmaAldrich,USA; 1:1000, GAPDH,mAb, GeneTex,USA) showed deregulated expression between different groups of samples, the signal intensities of particular bands were normalized with the total protein stain [29,30], a robust and reliable approach for semiquantitative Western blot analysis of protein expression in tissue samples and compared in Quantity One software (Bio-Rad, USA).

3. Statistical analysis

For determination of differences in lumican relative expression status between CONTROL and both OLP (OLP-T and OLP-AT) and OSCC (OSCC-T and OSCC-AT) groups parametric ANOVA in Microsoft Excel was performed. As biglycan expression was detected only both in OLP-T and OLP-T groups of samples and solely in OSCC-AT group of samples, parametric two-tailed paired t-test (Microsoft Excel) was used for analysis of biglycan relative expression within OLP group of samples. P- values lower than < 0.05 were considered statistically significant. If the signal was observed only in one condition, the relative expression value was set as 1. Differences in final immunoreactivity of IGF2 and IGF2R scores between different groups of samples (CONTROL, OLP and OSCC groups) were analysed by use non-paramteric statistics (Kruskal-Wallis ANOVA ($p<0.05$) test). For determination of differences between IGF2 and IGF2R final immunoreactivity scores within particular group of samples, non-parametric Wilcoxon rank-sum test was used. Statistical analyses of immunohistochemistry results were performed in Statistica (v.12.0) (These results are presented in the Supplementary material).

4. Results

4.1 Proteomics profiling

Global proteomics profiling was performed for 15 samples divided in 5 groups (OSCC-T and matched OSSC-AT group; OLP-T and matched OLP-AT; control group). Obtained data was analysed in Uniprot database (http://uniprot.org) with the emphasis on extracellular matrix proteins. In total, 55 extracellular matrix proteins were found to be expressed in analysed samples (Table 3) with very high

confidence, Mowse score > 64 for all charge states, at least 3 identified peptides per protein, false discovery rate on protein level and peptide estimated at 1%. Comparative analysis of identified extracellular matrix proteins between different groups showed that twenty among identified proteins were common to all groups of samples (Table 3). Six identified proteins, namely ladinin 1, eukaryotic initiation factor 4A-I, cathepsin D, cofilin-1, laminin subunit beta-3 and protein S100-A9 were unique to OSCC-T group and three identified proteins to OSSC-AT group, namely mimecan, suprabasin and cystatin-A (Table 3). In addition, two identified extracellular matrix proteins were unique to OLP-T group, namely tenascin-X and Ras-related C3 botulinum toxin substrate 1, two proteins were unique to OLP-AT group, namely X isoform of 40S ribosomal protein S4 and mitochondrial stress-70 protein and four proteins were unique to CONTROL, namely collagen alpha-1(XII) chain, versican core protein, fibulin-1 and asporin (Table 3). Furthermore, Uniprot database (http://uniprot.org) search of unique extracellular matrix proteins identified in OSCC-T group showed their functional involvement in a number of cellular processes that contribute to disease progression, such as for example reorganization of the actin cytoskeleton (*i.e.* cofilin-1), remodelling of extracellular matrix molecules (*i.e.* laminin subunit beta-3 and cathepsin D), chemokine production (*i.e.* protein S100-A9) and processing of ribonucleic acids and proteins (Eukaryotic initiation factor 4A-I). On the other hand, only two unique extracellular matrix proteins were identified in the OLP-T group, namely tenascin X and Ras-related C3 botulinum toxin substrate 1, which were both associated with enhanced cellular adhesion on extracellular matrix. Also, mass spectrometry analysis revealed a number of small leucine rich extracellular matrix proteoglycans, *i.e.* mimecan, decorin, biglycan and lumican and among them only biglycan was common to OLP-T, OSCC-T and their matched, adjacent tissue samples. Lumican was, on the other side, identified in all analysed groups of samples. Aforementioned small leucine rich proteoglycans were further subjected to semiquantitative Western blot analysis.

4.3 Western blot results

Expression of small leucine rich extracellular matrix proteoglycans lumican and biglycan was found both in OSCC and OLP and were thus, additionally semiquantitatively validated by Western blot analysis as putative biomarkers. Chemiluminescent signals for biglycan were detected in OLP-AT, OLP-T and OSCC-T groups while lumican expression was detected in all analysed groups of samples (Fig 3A), which was in accordance with results of global proteomics profiling. Also, significant increase ($p<0.05$) of biglycan expression in OLP-AT group was determined in comparison with OLP-T group (Fig 3B), while lumican showed significant up-regulation ($p<0.05$) in OLP-T and OSCC-T groups *vs.* adjacent and control tissue groups (Fig 3B). Biglycan expression was only determined in OSCC-AT group (Fig 3B).

5. DISCUSSION

New analytical methods are continuously underpinning development of research in the field of malignant disorders. In particular, identification of novel biomarkers, *i.e.* diagnostic markers would help clinicians in the diagnostic process, pre-symptomatic interventions or prediction of treatment response. One interesting discipline for analysis of novel, putative biomarkers is proteomics based on mass spectrometry. In this study therefore, proteomics was used to analyse in more details extracellular matrix (ECM) proteins and proteins related to ECM signalization and that have a well-established role in malignant transformation and invasion of tumor cells. The ECM is the non-cellular component present within all tissues and organs. It is composed by a complex mixture of glycoproteins, proteins, proteoglycans and polysaccharides [25]. Besides its general role in being physical scaffold for the cellular constituents, the proper dynamics of ECM macromolecules is crucial for correct tissue morphogenesis [31]. There are numerous evidence indicating that deregulated composition and architecture of ECM macromolecules are involved in cellular malignant transformation and cancer progression [32]. For that purpose, premalignant oral lichen planus (OLP) lesions were studied in comparison with oral squamous cell carcinoma (OSCC) and healthy oral mucosa. OLP is a chronic mucocutaneous autoimmune disease with already recognised potential for developing in oral squamous cell carcinoma (OSCC) [3, 33], the most common form of head and neck cancer [34]. Although numerous studies have investigated pathology of OLP, the aetiology of its progression to OSCC is still unknown. To investigate a potential link between OLP and OSCC pathologies and contribution of deregulated ECM molecules to OLP malignant transformation, a mass spectrometry based proteomics profiling of normal mucosa, OLP and OSCC tissues (and accompanied clinically unaltered tissues, respectively) was performed. With this approach 55 extracellular matrix proteins were identified in analysed samples (Table 3). Among them 6 identified proteins were unique to OSCC including ladinin 1, eukaryotic initiation factor 4A-I, cathepsin D, cofilin-1, laminin subunit beta-3 and protein S100-A9 and 3 were unique to adjacent, histologically unaltered tissues including mimecan, suprabasin and cystatin-A. These findings are in line with previous studies which showed increased expression of the protease cathepsin D [32], eukaryotic initiation factor 4A-I [35], cofilin -1 [36] and S100A9 protein [37] in OSCC which was correlated with progression and invasion of tumor cells. In addition, decreased expression of small leucine rich proteoglycan mimecan was previously reported in the oesophageal squamous cell carcinoma [38]. In OLP tissues, proteomics profiling revealed only two unique proteins, tenascin X and Ras-related C3 botulinum toxin substrate 1. Tenascin X was recently suggested as a novel diagnostic marker for ovarian cancer [39] and malignant mesothelioma [40], so its identification only in OLP tissues implies its role in a malignant transformation of oral lichen planus as well. Similarly, Ras-related C3 botulinum toxin substrate 1 can be associated with rapid epithelial differentiation and invasive properties of human cholesteatoma [41] and increased head and neck squamous cell carcinoma (HNSCC) cells invasion potential [42]. This

also points to a potentially malignant nature of OLP. Interestingly, in normal mucosa tissues, ECM proteins fibulin-1 and asporin were identified (Table 3). This result is in line with literature data as diminished fibulin-1 expression has been previously reported in various malignant diseases [43] while asporin bears a tumor suppressive role in breast carcinoma [44]. Comparative analysis of identified extracellular matrix proteins between different groups of samples showed that 20 identified proteins were common to all groups of samples (Table 3), for example the small leucine rich proteoglycan lumican. Validation of mass spectrometry results for lumican by use of Western blot showed its expression in all analysed groups and significantly increased lumican expression was assessed for OSCC-T and OLP-T in comparison with their matched adjacent tissue and with normal mucosa tissues, respectively (Figure 3). Still, mass spectrometry analysis did not reveal presence lumican in all analysed samples as shown by Western blot (Table 3 and Figure 3). This might be a consequence of heavy glycosylation of proteoglycans lumican and biglycan [45] that is known to alter physico-chemical characteristics of peptides and which strongly influences ionization of glycoconjugates during the mass spectrometry analysis [46]. Lumican is constitutive protein expressed in many tissues [47, 48], and has a role in maintenance of normal tissue integrity [49]. Moreover, recent evidence shows that elevated lumican expression has a negative impact on proper spatial organization of collagen fibres which facilitates local invasion in breast and pancreas cancer cell lines [50, 51]. Also, overexpression of lumican was associated with enhanced migration of colon cancer cell lines [52] and lumican overexpression correlated with cisplatin – resistance in OSCC cell line and clinical HNSCC tissue specimens [53]. According to these results, it may be assumed that lumican expression assessed in OLP and OSCC tissues by Western blot analysis might reflect similar pathologies of OLP and OSCC and potentially contribute to malignant transformation of oral lichen planus lesions.

In addition, significantly elevated expression of the small leucine rich proteoglycan biglycan was assessed in adjacent tissues of both OSCC-T and OLP-T groups (Figure 3). This result might be explained in the context of biglycan role in inflammatory processes which was well documented previously [54]. Indeed, in normal physiological conditions biglycan has a role in maintenance of the extracellular matrix integrity. After tissue damage, biglycan is released by proteolytic cleavage from extracellular matrix and acts as an endogenous ligand for Toll-like receptors 2 and 4 on a surface of innate immune cells, *i.e.* macrophages [55]. Activation of Toll-like receptors induces strong immune response mediated by NfkB signalling pathway and production of proinflammatory cytokines occurs. This process promotes further infiltration of immune cells on a site of inflammation. It is well known that cytokines and growth factors released from the host immune, stromal and endothelial cells promote tumor cells growth and disease progression [56]. Tumor-associated inflammation is indeed, a common event in HNSCC carcinogenesis as well [57] and it may be assumed that elevated biglycan expression in OLP and OSCC adjacent tissues contributes to enhanced infiltration of immune cells on

a site of oral lichen planus and oral squamous cell carcinoma tissues and promotes malignant progression of both diseases.

The second aim of presented study was evaluation of the Insulin-like growth factor receptor 2 (IGF2R) and IGF2 role in OLP. The Insulin-like growth factor receptor 2 (IGF2R) is a developmentally regulated multifunctional transmembrane glycoprotein that lacks intracellular tyrosine kinase domain [58]. It is primarily a transport protein for M6P-bearing glycoproteins, but an increasing body of evidence implies its role in suppression of various malignant diseases [59, 60]. This may be due to IG2FR ability to inhibit cellular growth through negative regulation of IGF2 mitogenic activity [61]. Comparative semi-quantitative immunohistochemical analysis of IGF2 and IG2FR expression in samples of healthy oral mucosa, OLP and OSCC showed variable IGF2 immunoreactivity in all analysed samples, mainly moderate or weak intracellular immunostaining (Supplementary material). No statistical difference in IGF2 expression between analysed groups of samples was assessed. These results are in line with the study of Zhi et al. [57 62], where no statistically significant differences in IGF2 expression was observed in various forms of head and neck carcinoma (including squamous tumors of the larynx, oral cavity and pharynx) in comparison with adjacent tumor unaffected tissues. The authors suggested to analyse circulation levels of IGF1 and IGF2 growth factors in tumor tissues and in serum samples in future studies, which might provide more precise data in the IGF-2 role in malignant transformation. Similarly, IG2FR immunostaining in all analysed groups of samples was strong to weak and was observed mainly intracellularly. No significant differences in IG2FR expression was observed between analysed groups of samples, although an increased expression trend was observed in the control group *vs.* OLP and OSCC (Supplementary material). Similar observation was reported in the study of Mountzios et al. [13], where association between IG2FR expression and clinico-pathological parameters of patients that suffer from operable laryngeal squamous cell carcinoma was not observed. In addition, observed increased expression for IG2FR in normal healthy mucosa can be explained by literature data since IGF2R was found to be active in healthy mucosa where it acts as a tumor-supressor. Its mutation is directly linked with increased risk of HNSCC [63] so herein presented result adds evidence on the precancerous potential of OLP and suggests that IGF2R axis might be included in the carcinogenesis of these potentially malignant oral lesions. Still, IGF2R expression should be evaluated in more details and on a larger cohort within prospective studies. Interestingly, a statistically significant difference between final IRS scores between IGF2 and IG2FR expression was determined for all analysed groups of samples (Supplementary material). This might point to the IG2FR role in regulation of extracellular IGF2 levels which can be considered as the indirect mechanism that influences cellular behaviour [61]. It is known that IGF-s including IGF2 are powerful mitogens whose expression is associated with various malignant diseases, and that observed expression level differences between IG2FR and IGF2 expression might be a consequence of

the IG2FR antitumor role in OLP and OSCC where IG2FR acts as a negative regulator of IGF2 activity.

Conclusions

Proteomic profiling of OLP and OSCC by use of nano-LC-MS/MS revealed a number of altered ECM proteins and proteins connected with ECM signalization. In particular, small leucine rich extracellular matrix proteoglycans biglycan and lumican were identified as important pathogenesis biomarkers of OLP that point to its malignant potential. Biglycan was indeed, identified in tumor and adjacent tissue samples of patients diagnosed both with OLP and OSCCT. Significantly elevated expression of biglycan in adjacent tissues of OSCC and OLP is probably due to enhanced infiltration of immune cells on a site of oral lichen planus and oral squamous cell carcinoma tissues where they promote malignant progression of both diseases. Lumican was identified in all analysed groups of samples but its significantly increased expression was found only in OSCC and OLP lesions in comparison with their matched, adjacent tissue and with normal mucosa tissues which points to its role as a potential contributor to malignant transformation of oral lichen planus lesions. At last, comparative semi-quantitative immunohistochemical analysis of IGF2 and IG2FR expression in samples of healthy oral mucosa, OLP and OSCC showed variable immunoreactivity in all analysed samples and no statistical differences in IGF2 and IGF2R expression between analysed groups of samples. However, a statistically significant difference between final IGF2 and IG2FR IRS scores was determined for all analysed groups of samples in favour to IGFR2. This result may further corroborate the IG2FR antitumor role in OLP and OSCC where it acts as a negative regulator of IGF2 activity.

Compliance with Ethical Standards

Conflict of Interest:

The authors have no conflict of interests to declare.

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Ethical approval:

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All experiments were conducted with the approval of the Ethics committee of School of Dental Medicine, University of Zagreb; Clinical Hospital Dubrava and Clinical Hospital Centre „Sisters of Mercy“, Zagreb (Croatia). This trial was registered at the U.S. National Institutes of Health (clinicaltrials.gov) (trial identifier: NCT03026361).

Informed consent:

The informed consent was obtained from all individual participants included in the study.

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Tables

Table 1. Main clinical and pathological characteristics of patients whose biopsy specimens were used for immunohistochemical analyses. Histological grading of OSCC was determined according to Broder et al.(28) (G1=well-differentiated tumor, G2=moderately good differentiated tumor; G3=poorly differentiated tumor).

Diagnosis	**Samples (No.)**	**Mean age (years)**	**Gender**		**Site of biopsy**	**Clinical type of OLP/ Histological grading of OSCC**
			Female	**Male**		
Oral lichen planus (OLP)	24	53,5	18	6	Buccal mucosa	Reticular type of OLP (N=15) Erosive type of OLP (N=9)
Oral squamous cell carcinoma (OSCC)	20	54,5	2	18	Tongue (N=8) Tonsillae (N=7) Retromolar mucosa (N=3) Gingiva (N=2)	G1 (N=3), G2 (N=2), G3 (N=3) G1 (N=2), G2 (N=1), G3 (N=4) G1 (N=1), G3 (N=2) G1 (N=2)
Healthy mucosa	18	41	9	9	Gingiva (N=18)	-

Table 2. Main clinical and pathological characteristics of patients whose biopsy specimens were used for global proteomic profiling (G2= moderately good differentiated tumor, G3=poorly differentiated tumor (28).

Diagnosis	Patients (No.)	Mean age (years)	Gender		Site of biopsy	Clinical type of OLP/ Histological grading of OSCC
			Female	Male		
Oral lichen planus (OLP)	3	68	3	0	Buccal mucosa	Erosive OLP (N=3)
Oral squamous cell carcinoma (OSCC)	3	64	0	3	Tongue	G3 (N=3)
Healthy mucosa	3	30	3	0	Retromolar mucosa (N=3)	-

Table 3. List of identified extracellular matrix proteins and associated biological processes (determined by detailed Uniprot database search /http://uniprot.org/ considering subcellular localization of proteins and biological processes they are involved in: in the healthy oral mucosa tissue samples (CONTROL group), in the oral lichen planus and corresponding histologically unaltered adjacent tissue samples (OLP-T and OLP- AT groups) and in the tumor and corresponding tumor-free adjacent tissue samples (OSCC-T and OSCC-AT groups). Abbreviations: CONTROL - normal mucosa tissue samples; OLP-AT- oral lichen planus corresponding histologically unaltered adjacent tissue samples; OLP-T- oral lichen planus tissue samples; OSCC-AT –oral squamous cell carcinoma corresponding tumor-free adjacent tissue samples; OSCC -T- oral squamous cell carcinoma tissue samples; (+) -positive identification of extracellular matrix protein in one sample within the analysed group; (++) - positive identification of extracellular matrix protein in two samples within the analysed group; (+++) - positive identification of extracellular matrix protein in all samples within the analysed group; (-)- extracellular matrix protein was not identified within the analysed group.

Uniprot ID	**Protein name**	**Biological process**	**CONTROL**	**OLP-AT**	**OLP-T**	**OSSC-AT**	**OSCC-T**
P12111	Collagen alpha-3(VI) chain	cell adhesion	+++	+++	+++	++	+++
P35555	Fibrillin-1	extracellular matrix organization	++	++	++	+++	+++
P35579	Myosin-9	actin cytoskeleton reorganization	+++	+++	+++	+++	++
P12109	Collagen alpha-1(VI) chain	cell adhesion	++	+++	+++	+++	+
Q6UYC3	Prelamin-A/C	structural molecule activity	+++	++	+++	+++	++
P11021	78 kDa glucose-regulated protein	ATPase activity	++	-	+	-	++
Q15149	Plectin	hemidesmosome assembly	++	++	+++	++	++
P08670	Vimentin	movement of cell or subcellular component	+++	+++	+++	+++	+++
Q05707	Collagen alpha-1(XIV) chain	extracellular matrix organization	++	-	+++	++	-

P07900	Heat shock protein HSP 90-alpha	chaperone-mediated protein complex assembly	+++	++	+++	++	++
D3DTX7	Collagen, type I, alpha 1, isoform CRA_a	extracellular matrix structural constituent	+++	+	++	++	-
P15924	Desmoplakin	desmosome organization	+++	+++	+++	+++	+++
P11142	Heat shock cognate 71 kDa protein	ATP metabolic process	++	+	++	++	++
P02751	Fibronectin	cell adhesion	++	-	-	+	++
P51884	Lumican	collagen fibril organization	++	++	+++	++	++
P12110	Collagen alpha-2(VI) chain	cell adhesion	++	+++	++	+++	+
P07585	Decorin	glycosaminoglycan metabolic process	++	++	+++	+++	-
P01009	Alpha-1-antitrypsin	glycoprotein binding	++	++	+++	+++	++
P10809	60 kDa heat shock protein, mitochondrial	'de novo' protein folding	+	+	-	-	+
P98160	Basement membrane-specific heparan sulphate proteoglycan core protein	glycosaminoglycan biosynthetic process	+	+	+	+	-
P25705	ATP synthase subunit alpha, mitochondrial	ATP biosynthetic process	+	-	+	-	++
P13639	Elongation factor 2	positive regulation of translation	+++	++	+++	+++	++
P14618	Pyruvate kinase isozymes M1/M2	ATP biosynthetic process	++	+	+++	+++	++

P09382	Galectin-1	apoptotic process	++	+	++	++	+
P07237	Protein disulphide-isomerase	cell redox homeostasis	++	++	+	++	++
P04792	Heat shock protein beta-1	intracellular signal transduction	++	++	++	++	++
Q06830	Peroxiredoxin-1	hydrogen peroxide catabolic process	++	+	++	++	+
Q99715	Collagen alpha-1(XII) chain	collagen fibril organization	+	-	-	-	-
P08123	Collagen alpha-2(I) chain	extracellular matrix organization	+	+	++	++	+
P13611	Versican core protein	cell adhesion	+	-	-	-	-
P06576	ATP synthase subunit beta, mitochondrial	ATP biosynthetic process	++	++	+	++	++
B1AHL2	Fibulin-1	extracellular matrix organization	+	-	-	-	-
P51888	Prolargin	keratan sulphate biosynthetic process	+	-	-	+	-
Q9BXN1	Asporin	biomineralization	+	-	-	-	-
P02452	Collagen alpha-1(I) chain	extracellular matrix organization	+	-	-	+	-
O75369	Filamin-B	myogenesis	++	++	++	+	-
P24821	Tenascin	cell adhesion	-	+	+	-	++
Q00610	Clathrin heavy chain 1	intracellular protein transport	-	+		++	++
P78371	T-complex protein 1 subunit beta	protein folding	-	+	+	-	+
P14625	Endoplasmin	response to stress	-	+	++	++	++

P62701	40S ribosomal protein S4, X isoform	positive regulation of cell proliferation	-	+	-	-	-
P38646	Stress-70 protein, mitochondrial	negative regulation of apoptotic process	-	+	-	-	-
A6NLG9	Biglycan	chondroitin sulphate biosynthetic process	-	++	+	+	-
P08311	Cathepsin G	cellular protein metabolic process	-	-	++	++	+
P22105	Tenascin-X	cell-matrix adhesion	-	-	+	-	-
P63000	Ras-related C3 botulinum toxin substrate 1	cell-matrix adhesion	-	-	+	-	-
P20774	Mimecan	keratan sulphate biosynthetic process	-	-	-	+++	-
Q6UWP8	Suprabasin		-	-	-	+	-
P01040	Cystatin-A	negative regulation of proteolysis	-	-	-	+	-
Q6IPJ9	Ladinin 1		-	-	-	-	+
P60842	Eukaryotic initiation factor 4A-I	regulation of translational initiation	-	-	-	-	++
P07339	Cathepsin D	collagen catabolic process	-	-	-	-	+
P23528	Cofilin-1	regulation of cell morphogenesis	-	-	-	-	++
Q13751	Laminin subunit beta-3	extracellular matrix organization	-	-	-	-	+
P06702	Protein S100-A9	chemokine production	-	-	-	+	+

Figures

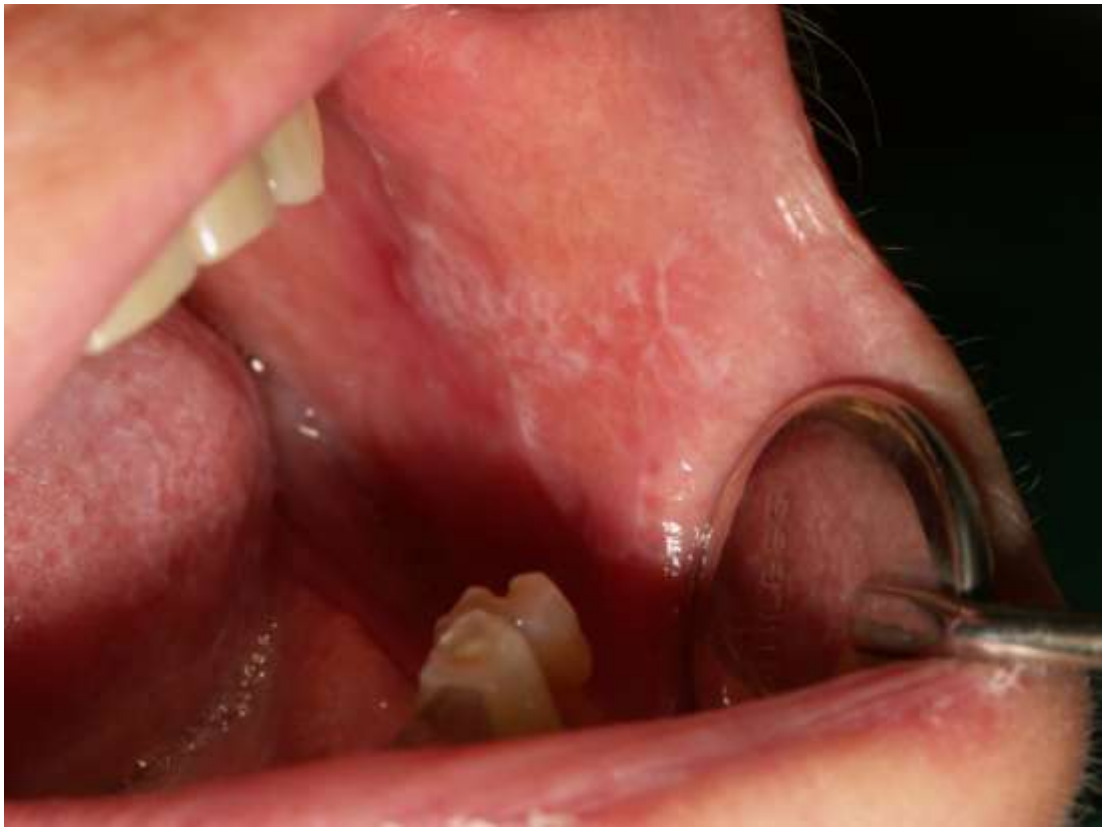

Figure 1. Characteristic clinical finding of patient with OLP

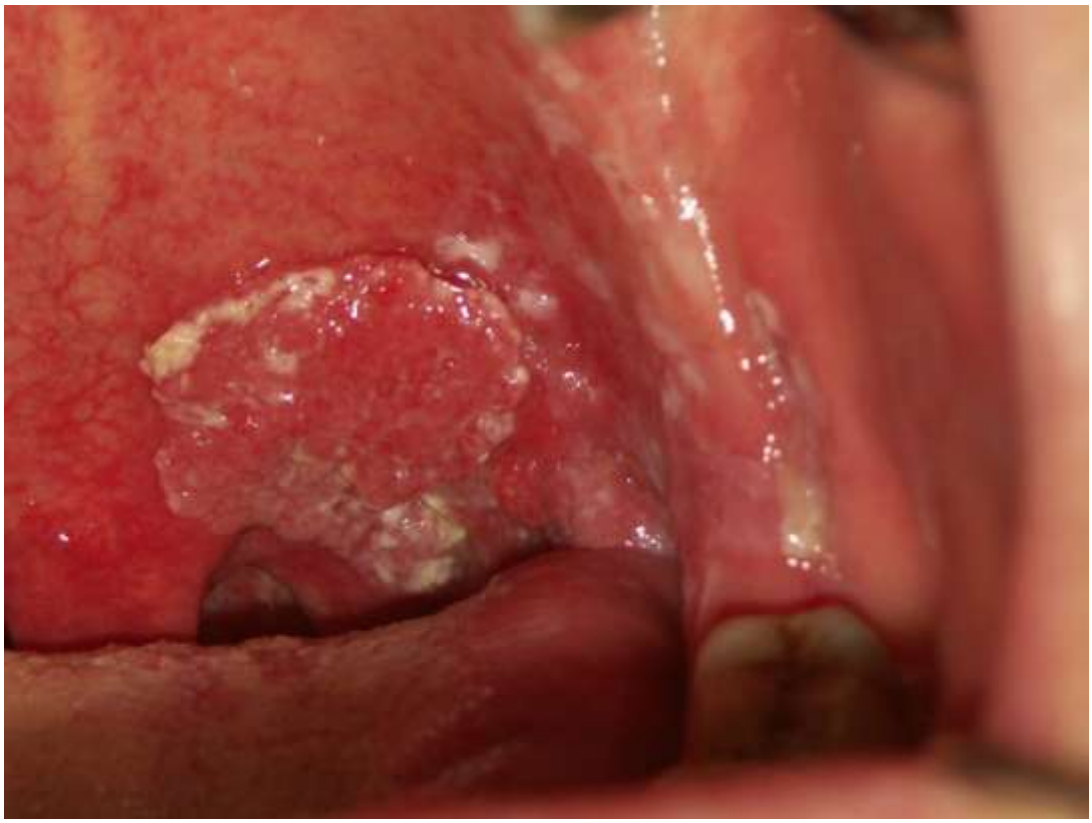

Figure 2. Characteristic clinical finding of patient with OSCC

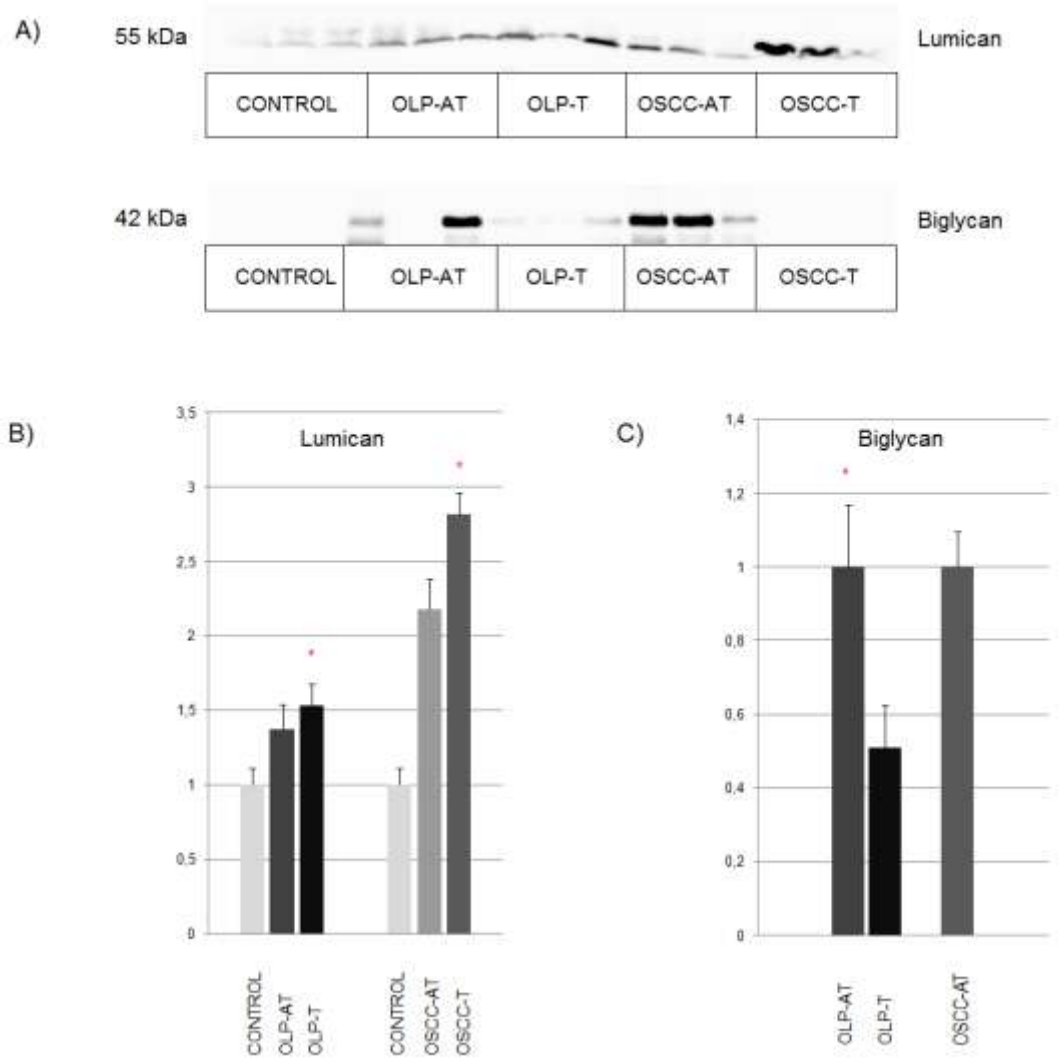

Figure 3. Representative blots (A) and graphical representations of lumican and biglycan relative expression (B, C). Graphical results are presented as mean relative expression of three replicate experiments + SD in the healthy oral mucosa tissue samples (CONTROL), oral lichen planus and corresponding histologically unaltered adjacent tissue samples (OLP-T and OLP- AT) as well as in the tumor and corresponding tumor-free, adjacent tissue samples (OSCC-T and OSCC-AT). Statistically significant changes (Student's t-test, $p<0.05$) are marked with an asterisk (*). Abbreviations: CONTROL - normal mucosa tissue (1-3); OLP-AT- oral lichen planus corresponding histologically unaltered adjacent tissue (4-6); OLP-T- oral lichen planus tissue (7-9); OSCC-AT –oral squamous cell carcinoma corresponding tumor-free adjacent tissue (10-12); OSCC -T - oral squamous cell carcinoma tissue (13-15).