Identification of prognosis-related proteins in gingival squamous cell carcinoma by twodimensional gel electrophoresis and mass spectrometry-based proteomics

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OBJECTIVES: *The aim of this study was to identify differentially expressed proteins in oral squamous carcinoma cells that could be potential prognosis-related cancer biomarkers.*
MATERIALS AND METHODS: *We compared protein expression patterns from gingival squamous cellc carcinoma (GSCC) tissues and adjacent non-cancerous matched tissues by proteomic analysis using two-dimensional gel electrophoresis coupled to mass spectrometry (2D-PAGE/MS).*
RESULTS: *Seventeen protein spots were found to be over-expressed and eight were under-expressed in cancerous tissue compared to the normal counterpart. Of these, annexin A2 and ezrin were validated by Western blot. We also demonstrated by immunohistochemistry that POSTN is highly expressed in the neoplastic tissues examined. Among the differentially expressed proteins, we focused our attention on Chloride intracellular channel 1 (CLIC1).*
CONCLUSION: *The 2D-PAGE/MS-based proteomics appears an efficient approach in detecting and identifying differentially expressed proteins that might function as potential biomarkers and/or molecular targets for early cancer diagnosis and prognosis and that might contribute to a innovative therapeutic strategies in GSCC. However, further validation and functional studies are needed to confirm and to support these promising, still preliminary data.*

KEY WORDS: Cancer biomarkers, Oral squamous cell carcinoma, Proteomics

Introduction

Oral squamous cell carcinoma (OSCC) is the most common cancer of head and neck and is generally regarded as the most widespread malignant neoplasm of the oral cavity [1-2]. OSCC is an aggressive cancer, which frequently leads to local recurrence and distant lymphatic metastasis, and is characterized by the accumulation of genetic and molecular alterations [3]. The pathogenesis of OSCC differs between foods, diet styles and countries and is frequently associated with environmental carcinogens [4]. Exposure to carcinogens, such as those present in tobacco smoke or derived from alcohol intake, is a remarkable element preceding the establishment of the disease in most cases [5]. For heavy smokers and drinkers, the consumption of both the substances shows a synergistic effect, increasing the risk of OSCC development by up to 35 times. Despite many efforts,the 5-year survival rate of OSCC patients is still approximately 50–60% [5].

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Squamous cell carcinoma of the gingiva has been reported to account for up to one quarter of the cases of OSCC, although estimates range from <10% to as high as 30% [6]. The lesion of the gingiva is very often confused with inflammatory conditions affecting the periodontium. Moreover, for anatomical reasons, squamous cell carcinoma of the gingiva tends to invade the underlying bone during the initial stage of the disease. These characteristics highlight the importance of an early diagnosis of the lesion to initiate treatment, prevent metastasis, and thereby improve the prognosis [7]. However, despite the advances in diagnosis and treatment of gingival OSCC, mortality rates have been not improved over the past decade [8]. Today, the prognosis of gingival OSCC patients is based exclusively on clinical staging and differentiation grade. The aim of this study was the identification of gingival OSCC tissue biomarkers. Therefore, we performed comparative proteomic analysis using a combination of 2-DE and mass spectrometry on OSCC lesions compared with the adjacent non-cancerous matched tissues (NCMTs). We identified quantitative differences in the expression pattern of about 25 proteins; among them, our attention was drawn by Ezrin, a protein involved in many cellular functions including regulation of actin cytoskeleton, control of cell shape, adhesion, mobility and modulation of signaling pathways, and by annexin2, that plays important roles in signal transduction pathways and regulation of cellular growth.

Materials and methods

Clinico-Pathological Characteristic of the Patients and Sample Collection

Samples were collected between September 2011 and January 2012 at the Oral and Maxillofacial Surgery, Magna Graecia University, Medical School, Catanzaro, Italy, and officially registered. All patients were asked to give informed written consent approved by the ethical committee of the Medical School. Tissues were obtained from 3 patients (2 men, 1 women) affected by gingival squamous cell carcinoma. The tumor stage was confirmed by immunohistochemistry analysis. After surgical resection, the tissues were immediately frozen at -80 °C. The protein extracts were prepared by the OSCC and adjacent non cancerous marched tissues (NCMTs) in lysis buffer [8M urea, 4% (w/v) CHAPS], 1x protease inhibitor (Halt TM protease inhibitor cocktail- Thermo Scientific), 1x phosphatase inhibitor (Halt TM phosphatase inhibitor cocktail-Thermo Scientific), 70mM DTE and 1mM EDTA. The mixture was homogenized (Ultra Turrax, IKA) at 11,000 pm-2s - 12 on ice and then left at 4° for 1 h. The homogenate was centrifuged (TLA 100.4 Beckan) at 40,000 rpm for 1h at 4°C. The supernatant was transferred to a fresh tube and the protein concentration was determined with the Bradford assay method using BSA as the standard. For each patient, 300 mg of protein extract from normal and cancerous tissues, were precipitated over-night in ice-cold acetone, centrifuged at 15000xg for 30 min and respectively pooled to create two groups (normal and cancer), in the attempt to minimize intra-class sample variability. Resulting pools were subjected to high resolution 2D electrophoresis.

Two-Dimensional Page Separation

Samples were diluted into Isoelectrofocusing (IEF) sample buffer containing 8 M urea, 4% CHAPS, 0.1 M DTT, 0.8% pH 3–10 non linear (NL) carrier ampholyte buffer. IEF was carried out on non-linear immobilized pH gradients (pH 3–10 NL; 24-cm-long IPG strips; GE Healthcare). The first dimension IPG strips were run on an GE Healthcare IPGphor unit, until a total of 70 000 Vh was reached. Prior to SDS-PAGE, IPG strips were equilibrated in a dithiothreitol (10 mg/mL) SDS equilibration solution followed by a treatment with iodoacetamide (25 mg/mL) SDS equilibration solution as described in the GE Healthcare Ettan DIGE protocol. The second dimension separation was run on 10% SDS-polyacrylamide gels, (2W/gel; 25°C) until the bromophenol blue dye front reached the end of the gels. Gels were stained with MS-compatible silver staining [9,10]. Gel image analysis was carried out using the Image Master 2D Platinum software,version 6.0 (GE Healthcare BioSciences). The spot auto-detect function was used for all group comparisons using identical parameters. Groups were matched automatically and corrected manually if necessary. Differences in protein expression were identified using the relative volume (%Vol) option of the software. This option allows the data to be independent of experimental variations between gels caused by differences in loading or staining [11]. Changes in average volume larger than ±50 % of the average spot volume and the significance level of $P < 0.05$ (normal vs. cancer group) was the criterion used for excision. Spots, were manually excised, destained, and acetonitrile-dehydrated. They were then rehydrated in trypsin solution, and in-gel protein digestion was performed by overnight incubation at 37 °C. The resulting tryptic peptides were purified through Pierce® C18 Spin Columns (Thermo Fisher Scientific Inc.) according to the manufacturer's procedure, eluted with 40μL of 70% acetonitrile and dehydrated in a vacuum evaporator [12]. Each purified tryptic peptide was analized through Nanoscale LC-MS/MS.

Nano Lc-Ms/Ms and Database Search

Chromatography was performed on an Easy LC 1000 nanoscale liquid chromatography (nanoLC) system

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(Thermo Fisher Scientific, Odense, Denmark). The analytical nanoLC column was a pulled fused silica capillary, 75 μm i.d., in-house packed to a length of 10 cm with 3 μm C18 silica particles from Dr. Maisch (Entringen, Germany) [5]. uL of the peptide mixtures were loaded at 500 nL/min directly onto the analytical column. A binary gradient was used for peptide elution. Mobile phase A was 0.1% formic acid, 2% acetonitrile, whereas mobile phase B was 0.1% formic acid, 80% acetonitrile. Gradient elution was achieved at 350 nL/min flow rate, and ramped from 0% B to 30% B in 15 minutes, and from 30% B to 100% B in additional 5 minutes; after 5 minutes at 100% B, the column was re-equilibrated at 0% B for 10 minutes before the following injection. MS detection was performed on a quadrupole-orbitrap mass spectrometer Q-Exactive (Thermo Fisher Scientific, Bremen, Germany) operating in positive ion mode, with nanoelectrospray (nESI) potential at 1800 V applied on the column front-end via a tee piece. Data-dependent acquisition was performed by using a top-5 method with resolution (FWHM), AGC target and maximum injection time (ms) for full MS and MS/MS of, respectively, 70,000/17,500, 1e6/5e5, 50/400. Mass window for precursor ion isolation was 2.0 m/z, whereas normalized collision energy was 30. Ion threshold for triggering MS/MS events was 2e4. Dynamic exclusion was 15 s. Data were processed using Proteome Discoverer 1.3 (Thermo Fisher Scientific, Bremen, Germany), using Sequest as search engine, and the HUMAN-refprot-isoforms.fasta as sequence database. The following search parameters were used: MS tolerance 15 ppm; MS/MS tolerance 0.02 Da; fixed modifications: carbamidomethylation of cysteine; variable modification: oxidation of methionine, phosphorylation of serine, threonine and tyrosine; enzyme trypsin; max missed cleavages 2; taxonomy Human.
Protein hits based on two successful peptide identifications (Xcorr> 2.0 for doubly charged peptides, >2.5 for triply charged peptides, and >3.0 for peptides having a charge state >3) were considered valid.

Western Blotting Analysis

OSCC and NCMTs tissue proteins were boiled for 10 minutes in SDS sample buffer, separated by 10% SDS-PAGE and the proteins were transferred to a nitrocellulose membrane by electroblotting. Non-specific reactivity was blocked in nonfat dry milk in TPBS [5% (w/v) Milk in PBS (pH 7.4) and 0.005% Tween20] for 2 hours at room temperature. The membrane was treated with rabbit anti-ezirin antibody (1:1000, 3145 Cell Signalling) and anti-annexinA2 antibody (1:1000 8235 Cell Signalling) for 2 hours at room temperature, followed by incubation with antirabbit IgG horseradish peroxidase-linked secondary antibody (1;10000; 7074S Cell Signalling). The membrane was developed by ECL-Western blot detection reagents according to the manufacturer's instructions (Santa Cruz Biotechnology). γ-tubulin was used as a loading control. IMMUNO

Histochemical analysis

Archival formalin-fixed and paraffin-embedded tissues were used for these study. Seriated deparaffinated sections (4μm-thick) were used for staining procedures, haematoxylin and eosin and immunohistochemistry. All procedures were carried out at room temperature. All cases were stained by a rabbit anti-human periostin antibody (Abcam); All samples were also tested without primary antibody as control. The best results were obtained with a dilution of 1:400 and with MW antigen retrieval using an automated immunostainer. Intensity of immunostaining was evaluated by two independent observer with the following score: score 0, no staining; score 1, weak staining; score 2, moderate staining; score 3, strong staining.

Results

2-De Analysis of Gingival Squamous Cell Carcinoma[8]

To identify proteins differentially expressed in gingival squamous cell carcinoma versus normal tissues, we investigated the two-dimensional electrophoresis (2DE) protein profiles of cancerous tissues and healthy matched tissues. Fig. 1 shows a representative 2D gel. 25 protein spots with a fold-change greater than 1.8 were excised from the silver stained gels, trypsin digested and subjected to LC-MS/MS analysis. The list of the 25 proteins, 17 of which are up-regulated and 8 are down-regulated, is shown in Table I, together with their gene name, MW, pI, number of identified peptides, Identification Score (IS) and relative fold-change. Among the 25 proteins, 17 were up regulated and 8 were down regulated in cancerous tissues compared to the normal counterpart. It appears that the majority of these products correspond to molecules that bind to actin filaments in muscle and non-muscle cells, to molecules that regulate membrane-membrane and membrane-cytoskeletal interactions and to molecules involved in the cell shape control, adhesion and mobility.

Validation of 2DE findings by Western Blot Analysis

To strengthen the findings of 2DE analysis, Western blots were performed on protein extract of cancerous and healthy tissues, using antibodies against annexinA2 and ezrin. Both proteins are consistently overexpressed in neoplastic tissues (Fig. 2) thus confirming the proteomic data. Even though not identified by 2DE, the expres-

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TABLE I - *List of up-regulated and down-regulated proteins.*

Gene ID	Name	Sequest score	N. peptide	MW	PI	Spot ID	Fold change Cancer vs normal
P38606	V-type proton ATPase catalytic subunit A	7,15	3	68,26	5,52	12	-4,35
P08670	Vimentin	15,19	6	53,62	5,12	11	-2,20
P19971	Thymidine phosphorylase	27,27	9	45,17	8,85	10	1,62
P24752	Acetyl-CoA acetyltransferase, mitochondrial	13,27	6	39,40	8,09	18a	-1,02
P04075	Fructose-bisphosphate aldolase A	21,70	10	39,40	8,09	18	1,23
P11142	Heat shock cognate 71 kDa protein	13,61	5	70,85	5,52	7	1,44
P07951	Tropomyosin beta chain	21,33	8	32,83	4,70	8	1,30
P07355	Annexin A2	21,47	9	38,58	7,75	16b	1,68
P04083	Annexin Al	14,58	7	38,69	7,02	16a	1,15
P04406	Glyceraldehyde-3-phosphate dehydrogenase	6,49	2	36,03	8,46	16c	-1,60
P07355	Annexin A2	5,49	4	38,58	7,75	14a	1,81
P40926	Malate dehydrogenase, mitochondrial	9,22	5	35,48	8,68	14b	-1,21
P00338	L-Iactate dehydrogenase A chain	6,80	3	36,67	8,27	17	-l,56
P22626	Heterogeneous nuclear ribonucleoproteins A2/B1	8,30	3	37,41	8,95	14c	2,31
Q16836	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	1,89	2	34,27	8,85	15b	1,08
095398	Rap guanine nucleotide exchange factor 3	1,41	2	103,69	7,56	15a	l,58
P05976	Myosin light chain 1/3, skeletal muscle isoform	10,59	5	21,13	5,03	1	-1,21
P15311	Ezrin	2,02	2	69,37	6,27	4e	l,54
Q15691	Microtubule-associated protein RP/EB family member 1	1,70	3	29,98	5,14	3	1,68
O00299	Chloride intracellular channel protein 1	4,61	4	26,91	5,17	3a	10,00
Q9UPR5	Sodium/calcium exchanger 2	1,66	2	100,30	5,15	17a	-1,75
P00558	Phosphoglycerate kinase 1	Il,50	5	44,59	8,10	18b	3,06
P01857	Ig gamma-1 chain C region	6,76	3	36,08	8,19	18d	1,19
P22626	Heterogeneous nuclear ribonucleoproteins A2/B1	8,27	3	37,41	8,95	19	2,06
Q96C19	EF-hand domain-containing protein D2	6,45	4	26,68	5,20	4a	1,98

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sion levels of Periostin (POSTN), a secreted protein that has been suggested to promote invasion and angiogenesis in OSCC were analysed by immunohistochemistry. Interestingly, POSTN resulted to be highly expressed in all the neoplastic tissues examined (Fig. 3).

CLASSIFICATION OF PATHWAYS AMONG DIFFERENTIALLY EXPRESSED PROTEINS

The molecular pathways potentially altered in OSCC were investigated by importing into the Ingenuity Pathway Analysis Tool (IPA) the list of the 25 differentially expressed proteins. This analysis revealed two significant networks, with an IPA score higher than 15: 1) Cancer, Gastrointestinal Disease, Hepatic System Disease (with 15 focus molecules and IPA score 39), shown in Fig. 3; 2) Cellular Assembly and Organization, Cellular Function and Maintenance, Cell Cycle (with 8 focus molecules and IPA score 18), shown in supplementary Fig. S1. It must be noted that, in the IPA analysis, a score of 3 indicates a 1/1000 chance that the focus genes are in a network not due to random chance.

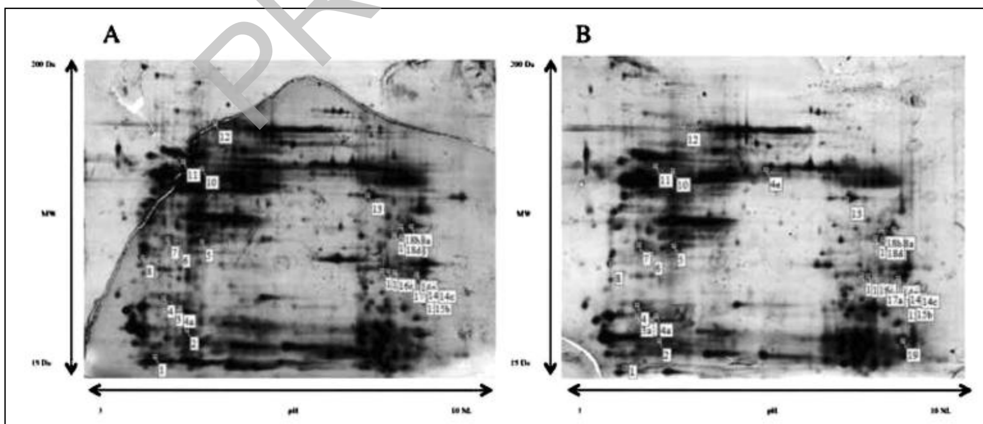

Fig. 1: Representative 2D gels of healthy matched tissues (A) and cancerous tissues (B).

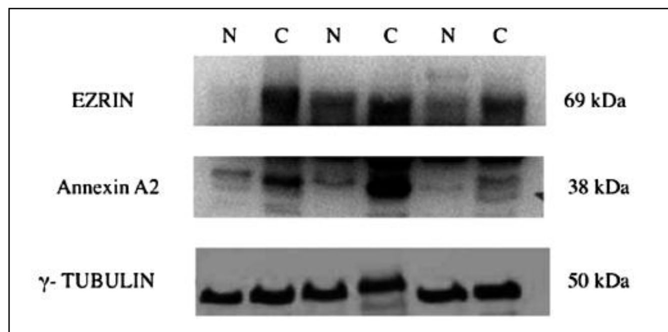

Fig. 2: Western blot analysis on protein extract of cancerous (C) and healthy tissues (N) for anti Ezrin, Annexin A2 and γ-Tubulin antibodies.

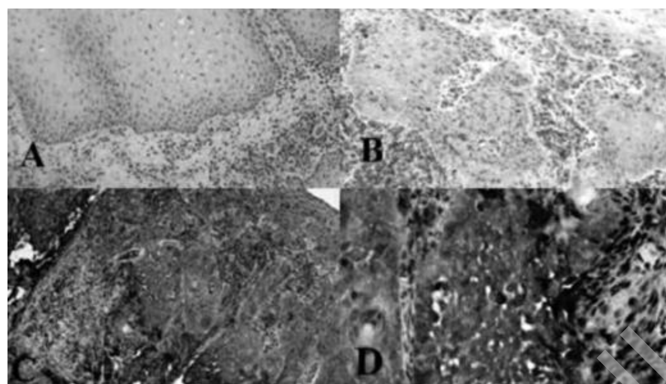

Fig. 3: A. Achantotic epithelium in oral leucoplakia negative for periostin (20x); B. Moderate staining of SCC (10X); C and D Intense staining in cancer cells and extracellular matrix in a deeply invasive SCC (10x and 40x).

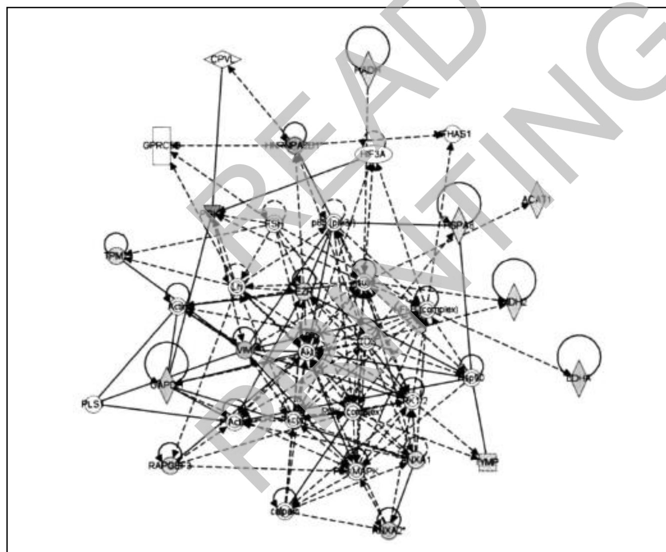

Fig. 4: Network analysis of the proteins expression was performed using the Ingenuity software. Each network displays the proteins products as nodes (different shapes representing the functional classes of proteins) and the biological relationships between the nodes as lines. The length of each line reflects the amount of literature evidence supporting this node-to-node relationship. The color intensity of each node indicates the degree of upregulation (red) or downregulation (green) of the respective proteins.

Discussion

OSCC is characterized by a high degree of local invasiveness and metastasis potential, which leads to a high incidence of mortality [13]. A delayed diagnosis, usually carried out in the advanced stages of the disease, is the main contributing factor to its poor 5-year survival rate; therefore, the identification of reliable biomarkers holds great potential for the early detection of OSCC, as well as for monitoring cancer progression [13]. The gingival form of squamous cell carcinoma (GSCC) is relatively rare, representing less than 10% of OSCC [14]. Because of its proximity to the teeth and periodontium, the tumor can mimic the advanced periodontal disease, sharing many symptoms, such as swelling, bleeding, tooth mobility, deep periodontal pockets and bone destruction [14]. For this reason, the discovery of molecules might be used as biomarkers in GSCC is even more significant and desirable. Recently, high-throughput genomic and proteomic techniques have identified transcripts and proteins with an altered expression in OSCC samples, but their use as diagnostic or prognostic markers is still under investigation [15]. Most of these studies have been initially performed on cell lines, and the identified proteins have been subsequently tested on human cancer specimens; not surprisingly, significant discrepancies in the fold change of relative mRNA or protein abundance have emerged between the in vivo and in vitro analysis [15]. In this study, 2DE and mass spectrometry technologies were used to evaluate the whole proteome of GSCC tissue samples. The analysis led to the identification of 25 proteins with an altered expression in the neoplastic tissues compared to the normal counterpart. Gene ontology analysis indicates that the vast majority of these proteins is involved in metabolic processes related to the establishment of a neoplastic phenotype and in cell cycle control mechanisms. Among the differentially expressed molecules, the annexins ANXA1 and ANXA2 were identified. Annexins are a family of calcium-binding proteins, implicated in membrane trafficking, calcium signalling as well as in cell motility, differentiation and proliferation [16]. Our findings, which demonstrate an upregulation of ANXA2 expression in cancer samples, are in agreement with those reported in the current literature, thus confirming its role as potential biomarker in OSCC [17]. On the other hand, the ANXA1 protein, which has been reported to be consistently down-regulated in OSCC cell lines [18], appears significantly overexpressed, both by 2D-PAGE and Western blotting, in our study set. This discrepancy, that may possibly reflect a different behavior of cultured tumor cells compared to surgery-derived cancer tissue specimens, requires further analysis and larger sample size to be explained. On this basis, we wish to suggest a cautious approach on the use of ANXA1 as marker for OSCC patients. Among the up-regulated proteins, the Chloride intracellular channel 1 (CLIC1) was the most notable in the tumor samples

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and it is novel in the contest of OSCC. CLIC1 is expressed in many human tissues and it has been reported to be involved in the regulation of cell cycle, cell proliferation, and differentiation. Recent studies have found that CLIC1, upregulated in a variety of cancers, might function as a sensor and an effector during oxidative stress [19], thus playing a major role in tumor invasion and metastasis. Therefore, we propose that CLIC1 might be considered a potential diagnostic/prognostic marker for GSCC. In this work, we have also performed immunohistochemical analysis of POSTN expression in the GSCC samples. POSTN is a cell adhesion protein that regulates bone and tooth formation as well as cardiac development [20]. The role of POSTN in tumorigenesis is supported by a growing number of evidence showing that this protein is frequently over-expressed in many cancer types, such as colon [21], lung [22], head and neck [23], breast [24], ovarian [25] and prostate cancer, although with contradictory data concerning the identity of periostin expressing cells (i.e. stroma, tumor cells or both) [26]. POSTN expression is a hallmark of epithelial-to-mesenchymal transition, a switch of paramount importance in OSCC [27]. A survey of the literature in this specific cancer demonstrates, in most cases, a predominant stromal localization of POSTN. Interestingly, in our study, POSTN expression was evenly increased both in the cellular and in the ECM component of tumor tissue, and parallels with the depth of invasion. The results described in the present work shed novel insight into the whole cell proteome of OSCC, with a specific focus on the gingival form of this tumor. The differentially expressed molecules discovered in our study bring to light a number of biological processes and reveal potentially important information in understanding the molecular basis and the progression of OSCC.

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Appendix A. Supplementary material

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