Analysis of Protein Expression Profile of Oral Squamous Cell Carcinoma by MALDI-TOF-MS

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Abstract. *In this study, two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF-MS) technology was used to examine differentially expressed proteins in oral squamous cell carcinoma (OSCC) tissues from Norway (n=15) and the UK (n=45). Twenty-nine proteins were found to be significantly overexpressed in the OSCCs examined compared to the normal controls. Identified proteins included, family of annexin proteins that play important roles in signal transduction pathways and regulation of cellular growth, keratin-1, heat-shock proteins (HSP), squamous cell carcinoma antigen (SCC-Ag), cytoskeleton proteins, and proteins involved in mitochondrial and intracellular signalling pathways. The expression of four selected proteins (annexin II and V, HSP-27, and SCC-Ag) was verified using western blot analysis of 76 fresh tissue biopsy specimens in total, from Norway (n=53) and the UK (n=23). Proteomic analysis of OSCCs examined here demonstrated involvement of several proteins that might function as potential biomarkers and molecular targets for early cancer diagnostics, and may contribute to a novel approach to therapeutics and for predicting prognosis of OSCC.*

Oral squamous cell carcinoma (OSCC) represents one of the most common types of cancers of the head and neck region, and is a major health problem in Central, Eastern and Southeast Asia (1). This aggressive neoplasm afflicts about 500,000 new cases worldwide each year with approximately 62% of those occurring in developing countries, the disease is found more frequently in males than females (2). Worldwide, the frequency of the disease varies between 1% and 40% of all malignancies (2).

Although the incidence of OSCC seems to be higher in many developing countries compared with industrial ones, there has been a rising trend in incidence and mortality in several Western countries during the past decades (2, 3). Regular use of tobacco and excessive alcohol consumption together account for about 75% of all cases of OSCCs. Increasing use of these substances might be an important reason for the overall increase in the incidence rates that have been recently reported in some European countries (4). In Britain, OSCC accounts for approximately 3% of all new cancer cases, and is the 15th most common type of cancer, with an increase in incidence since the mid-1970s (5-7). In males, it is the 12th most common types of cancer in the UK (5), and among women, it is the 16th. In Norway, the relative frequencies of head and neck cancer (HNSCC), including OSCC, reported in the period from 1996-2001 was slightly higher than in the UK, affecting approximately 6.1% of male and 2.5% of female patients, and is linked to the social habits of tobacco smoking and alcohol consumption (8). Since the 5-year survival of patients diagnosed with oral cancer still remains low (maximum 50%), and the possibility of regional recurrence of the disease is high, there is a need for specific molecular biomarkers for early diagnosis and better therapeutic strategies. The most common anatomical sites for oral cancer are the tongue and the buccal mucosa. The life-styles associated with increased risk of oral cancer are now well-characterized (4, 9-12) for different populations of the globe.

Study of cancer genomics, as well as proteomics, has emerged as a powerful tool allowing for investigation of protein profiles and signatures for the purpose of screening, diagnosis, better understanding of OSCC pathogenesis, and possibly for improving therapeutics (13-20). Proteomics have

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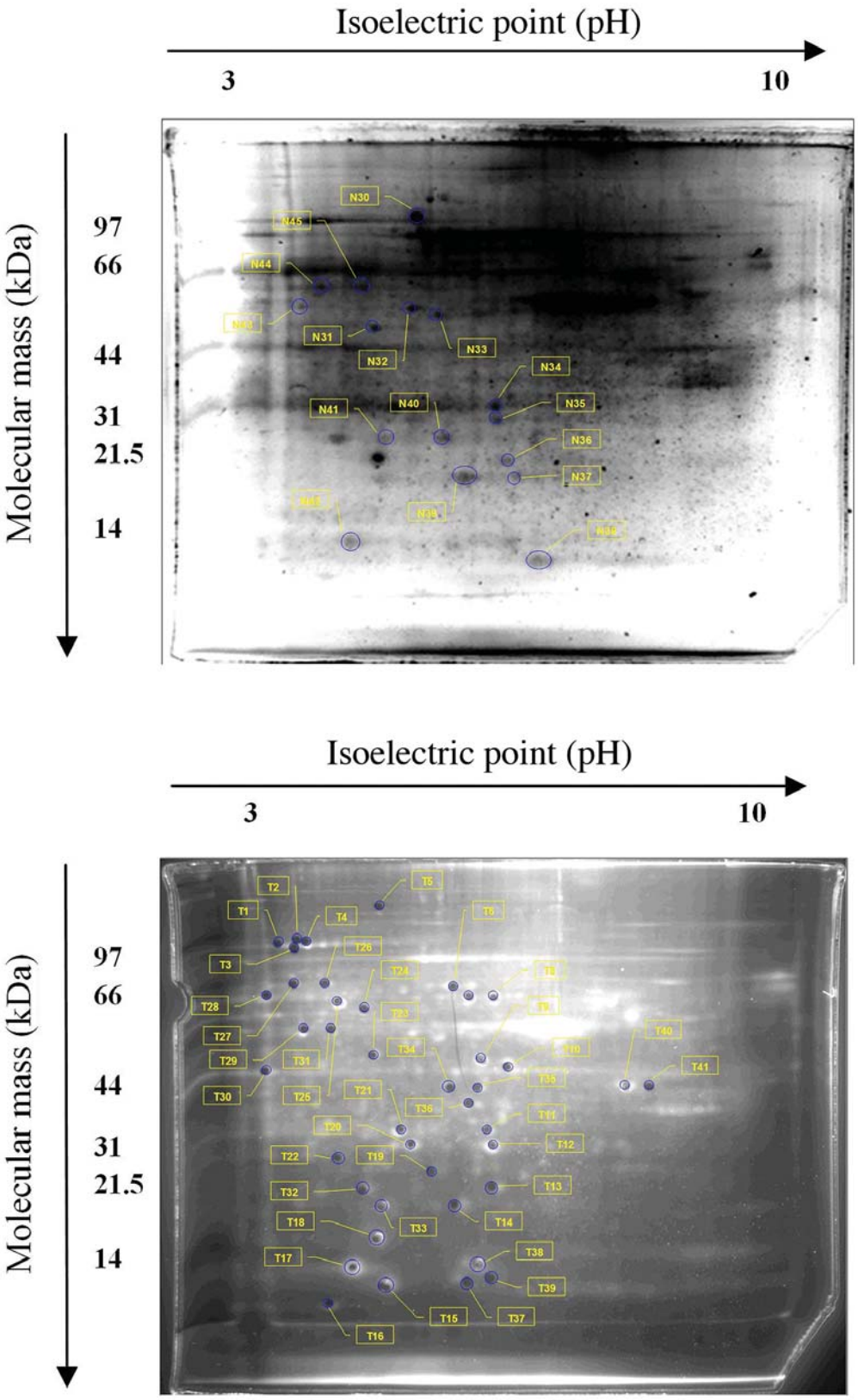

Figure 1. *Image of SYPRO-Ruby-stained 2-DE gel representing oral normal controls and tumour samples analyzed from patients from Norway. Identified protein spots were detected using peptide mass fingerprinting.*

previously been successfully used as a powerful method to investigate protein expression in malignancies of the breast, prostate, lung and several other cancer forms (21). In this study, we investigated OSCC tumour-associated proteins using two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF-MS) technologies. Western blotting was used to verify our findings for selected candidate proteins. The tumour-associated proteins found can be used as potential biomarkers for clinical diagnosis and as targeted proteins for pathogenic investigations. Cancer development involves a cascade of events leading to pathological changes that might alter functional proteins involved in the process of tumour development, and an early diagnosis is therefore of importance to fight the disease. The objective of the study was to undertake proteomic analysis of the OSCC samples to demonstrate the involvement of any overexpressed proteins that might lead to characterizing potential biomarkers and molecular targets for early cancer diagnostics.

Materials and Methods

Patients and tissue specimens. Tissue samples of 60 tumours and 20 pairwise normal controls (NCs) were obtained from Norwegian (n=15: average age=64.4, range=31-89 years, SD=15.8 years) and UK (n=45: average age=57.8, range 22-92 years, SD=16.2 years) patients operated on at the Department of Surgical Sciences, Section of Otorhinolaryngology, Haukeland University Hospital in Norway and from a South London population in the UK. Among the UK samples, the sites of the primary carcinoma were the tongue (n=14), buccal mucosa (n=12), soft palate (n=3), floor of mouth (n=6), gingiva (n=4) and retromolar area (n=6). Among the Norwegian samples, the sites of primary tumour were the soft palate (n=6), tongue (n=5), floor of mouth (n=2) and gingiva (n=2). The samples, demographic anatomical sites and tissue preparation have been described in our earlier publication (22, 23).

For verification of the results from the 2-DE proteomic study, western blotting analysis was performed on 76 fresh frozen tissue biopsy specimens (3×30 μm each) of OSCCs from Norway (n=53: average age= 48.8, range= 15-93 years, SD=22 years) and from UK (n=23: average age= 58.5, range=22-92 years, SD=17.3 years).

Protein extraction. Samples selected for protein extraction were removed from the frozen RNA*later* and placed on a clean surface. Small pieces of approximately 25 mg of tissue specimens were cut out and washed 3-5 times for 30 min with phosphate-buffered saline (PBS), dried on tissue paper and homogenized in the presence of approximately 5 mg of alumina (Sigma-Aldrich, St. Louis, MO, USA). After resuspension in non-denaturing extraction and labelling buffer (Clontech, Mountain View, CA, USA), the insoluble tissue fragments were removed by centrifugation at 10,000 ×*g* for 30 min at 4˚C. Using bicinchoninic acid (BCA) Protein Assay Reagent kit (Pierce Biotechnology, Rockford, IL, USA), the protein concentration was assayed and the samples were stored at –20˚C in aliquots. Following protein extraction, a protease inhibitor for mammalian tissues (Sigma) was used to improve the yield of intact proteins. Due to difficulties of extracting sufficient amounts of protein from the tumour and normal controls, and thus making case by case study difficult, the samples were pooled in equal quantities resembling biological averaging after grouping of the samples according to the country of origin (OSCCs *vs*. NCs). For both populations, Norway and UK, four pools containing equal amounts of extracted total protein from OSCCs and NCs were prepared and used in further 2-DE-MALDI-TOF-MS analysis.

2-DE and MALDI-TOF-MS. The 2-DE and MALDI-TOF-MS methodology used was described in detail by Ibrahim *et al*. (24). In brief, 2-DE was carried out using four pools (two from each country) containing equal amounts of extracted total proteins. Following sample precipitation with cold 10% trichloroacetic acid (TCA), pellets were collected by centrifugation washed with cold 5% TCA, cold acetone and dried by air. Protein samples were solubilized in isoelectric focusing (IEF) buffer, containing 7 M urea, 2 M thiourea, 4% w/v 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.5% v/v Triton X-100, 20 mM dichlorodiphenyltrichloroethane (DTT), bromophenol blue and 0.5% v/v carrier ampholytes (Amersham-Pharmacia Biotech AB, Uppsala Sweden). Immobilized pH gradient (IPG) strips 18 cm, pH 3-10 non-linear; Amersham-Pharmacia Biotech AB) were rehydrated by protein samples in overnight incubation at room temperature in an Immobiline DryStrip Reswelling Tray (Amersham-Pharmacia Biotech). As described by the manufacturer, first dimension IEF was carried out on a Multiphor II system at 20˚C using Pharmacia EPS 3500 XL power supply (Amersham Pharmacia Biotech) in gradient mode. For second-dimension electrophoresis, IPG strips were equilibrated for 15 min with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 6 M urea, 30% v/v glycerol, 2% SDS, 16 mM (2.5 mg/ml) DTT in 50 mM Tris-HCl (pH 8.8), followed by an alkylation process carried out by 15 min incubation in the presence of 245 mM (45 mg/ml) iodoacetamide in sample buffer without DTT. Furthermore, second-dimensional SDS-PAGE was performed using Protean II XI (Bio Rad Laboratories, Hercules, CA, USA) apparatus at 20˚C with 12.5% polyacrylamide gel. For protein visualization, SYPROR Ruby protein staining kit (Bio-Rad) was used.

Previously extracted and stained gel pieces were scanned using Fuji FLA-2000 phosphoimager (Fuji Photo Film Co. Ltd., Tokyo, Japan), and image acquisition was carried out by Image reader and Image Gauge software (Fuji Photo Film Co. Ltd., Tokyo, Japan). Detection, quantification and comparison of spot samples were performed using Image Master 2D Platinum Version 5.0 software as recommended by the manufacturer (Amersham Biosciences). For each sample, gel images obtained from OSCCs and NCs were compared to each other pairwise and normalized volume differences (% vol) were statistically calculated. Significantly different protein spots were selected and subjected to further analysis by MALDI-TOF-MS. SYPRO-Ruby stained 2-DE (Molecular Probes, Inc., Eugene, OR USA) protein spots were excised (as duplicate/or triplicate) from OSCC/NC 2-DE gels from Norwegian and UK cases under stringent conditions, transferred to 1.5 ml Eppendorf tubes and submitted to Proteomic Unit at University of Bergen (PROBE), Norway (http://www.probe.uib.no/), for peptide mass fingerprinting (PMF) using standard protocols. Spots were cut, washed twice with 50% acetonitrile in 25 mM ammonium bicarbonate for 15 min, followed by drying in a SpeedVac for 20 min. Dehydrated gels were rehydrated for 30 min on ice in 30 μl of 1.25 ng/μl trypsin (sequence

Table I. *Summary of the proteins found using Peptide-Mass Fingerprinting (PMF) search in the National Center of Biotechnology Information (NCBInr) database for the samples representing normal controls.*

Spot number	Protein name	Accession no.	Mass (kDa)/mass accuracy (ppm)	Matched peptides	Mascot score	Sequence coverage (%)/pI
NN29	Chain A, crystal structure of human serum albumin	gi 55669910	67/22	18	112	29/5.6
NN30	Squamous cell carcinoma antigen	gi 239552	44/23	16	153	36/6.4
NN34	Glutathione transferase	gi 20664359	23/9	8	65	57/5.1
NN35	Chain J, thioredoxin peroxidase B	gi 9955016	22/24	10	95	47/5.4
NN45	Chain A, crystal structure of human serum albumin	gi 55669910	67/22	34	290	63/5.6
NN46	Serum albumin	gi 23307793	71/26	20	141	31/6.1
NN50	Keratin 1	gi 7331218	66/24	10	71	27/8.2

NN: Norway normal.

Table II. *Summary of the proteins found using Peptide-Mass Fingerprinting (PMF) search in the National Center of Biotechnology Information (NCBInr) database for the samples representing oral squamous cell carcinoma from patients from Norway.*

Spot number	Protein name	Accession no.	Mass (kDa)/mass accuracy (ppm)	Matched peptides	Mascot score	Sequence coverage (%)/pI
NT3	PRO1400	gi 6650772	65/24	22	163	42/6.9
NT5	ER-60 protein	gi 2245365	57/18	19	178	44/5.9
NT6	Fibrinogen gamma	gi 223170	47/20	14	115	34/5.5
NT8	Beta actin variant	gi 62897625	42/21	11	75	34/5.4
NT9	Gamma-actin	gi 178045	26/14	9	94	56/5.7
NT10	Heat shock protein 27 kDa protein 1	gi 4504517	23/20	7	91	40/6.0
NT11	Chain B, glutathione transferase	gi 20664359	23/19	8	79	52/5.1
NT16	Fatty acid binding protein 5	gi 47125412	15/21	10	99	58/6.6
NT19	Chain B, glutathione transferase	gi 20664359	23/20	8	92	56/5.1
NT20	Heat-shock protein 27 kDa protein 1	gi 4504517	23/19	9	98	53/6.0
NT27	Keratin 1	gi 7331218	66/19	16	77	25/8.2
NT28	Squamous cell carcinoma antigen 1	gi 25005272	45/23	19	166	44/6.5
NT29	Annexin A2	gi 16306978	39/23	15	125	54/7.6
NT30	Squamous cell carcinoma antigen 1	gi 25005272	45/23	13	103	30/6.5
NT31	GDP-bound RAB2A GTPase	gi 73535756	20/25	8	88	42/6.5
NT33	Annexin A4	gi 1703319	36/24	15	94	43/5.8
NT34	Annexin A3	gi 12654115	36/18	16	148	40/5.6

NT: Norway tumor.

Table III. *Summary of the proteins found using Peptide-Mass Fingerprinting (PMF) search in the National Center of Biotechnology Information (NCBInr) database for the samples representing oral squamous cell carcinoma from patients from the UK.*

Spot number	Protein name	Accession no. (NCBI database)	Mass (kDa)/mass accuracy (ppm)	Matched peptides	Mascot score	Sequence coverage (%)/pI
UKT6	Rho GDP dissociation inhibitor	gi 76780069	23/24	10	76	38/5.0
UKT7	Proapolipoprotein	gi 178775	29/24	16	130	48/5.5
UKT8	MYL1	gi 48145855	21/18	10	85	51/5.0
UKT9	Keratin 1	gi 7331218	66/21	18	111	37/8.1
UKT18	S100 calcium-binding protein A9	gi 4506773	13/19	6	64	40/5.7
UKT19	Histone 2	gi 55960991	14/20	6	71	38/10.3
UKT20	Structure Of Human Ferritin L Chain	gi 110591407	20/22	7	74	32/5.5
UKT23	ER-60 protease	gi 1208427	57/19	16	129	29/6.0
UKT24	Human mitochondrial aldehyde dehydrogenase	gi 6137684	54/11	16	137	33/5.7
UKT30	Peroxisomal enoyl-coenzyme A hydratase-like protein	gi 70995211	36/50	7	52	28/8.2
UKT31	ANXA4 protein	gi 39645467	34/18	11	84	32/5.6
UKT32	Heat-shock 27 kDa protein 1	gi 4504517	23/12	9	91	48/6.0

grade modified; Promega, Woods Hollow Road, Madison, WI, USA) dissolved in 50 mM ammonium bicarbonate and incubated at 37˚C overnight. Following centrifugation, acidification and supernatant collection, gel spots were extracted once for 20 min at room temperature with 60% acetonitrile containing 0.1% trifluoroacetic acid (TFA) and supernatants were pooled. After concentration of peptide mixture (10-20 μl) on a SpeedVac, 1 μl was mixed with an equal volume of α-Cyano-4-hydroxy-cinnamic acid (CHCA) matrix, 10 mg/ml in 60% TFA, 0.1% TFA acid, and 1.5 μl of mixture was applied to MALDI-TOF-MS sample targets. Samples were analysed on Autoflex (Bruker Daltonics, Germany), peptide mass spectra were recorded and a PMF search was carried out from NCBInr database protein matching using MASCOT (www.matrixscience.com) database search algorithms. Trypsin autolytic peptides (m/z=842.51, 1045.56 and 2211.10) were used to internally calibrate each spectrum and, when not detected (as in the cases with very little protein), an external standard was applied from a neighbouring sample on the target. Up to one missed tryptic cleavage was considered and a mass accuracy of 100 ppm was used for all tryptic-mass searches. Searches were performed without constraining protein molecular weight or isoelectric point (pI), and allowed for carbamidomethylation of cysteine and partial oxidation of methionine residues. Only proteins found significant ($p<0.05$) according to Molecular Weight Search (MOWSE) highest search score(s) and with 20 ppm or better were selected. Cases with lower mass accuracy and score matching were run in duplicate or triplicate to ensure an accurate analysis.

SDS-PAGE and western blot. Tissue samples (stored in RNA-Later stabilization solution) were extracted as described above. Supernatants were pooled according to their origin and cancer state: normal Norway, tumor Norway, normal UK, and tumor UK. Protein content was assayed with BCA. Samples were concentrated by ethanolic precipitation (90% ethanol, overnight, –20˚C), supernatants were removed by centrifugation and pellets were dried using SpeedVac concentrator. Dried samples were dissolved in SDS-PAGE sample buffer (×1) (25) to a final protein concentration of 1.5 mg/ml. Aliquots (40 μg/lane) were separated by SDS-PAGE using 12.5% gel (1.5 h 100 V) and transferred electrophoretically onto a nitrocellulose membrane (0.2 μm) (1 h at 100 V). Non-specific binding sites were blocked with non-fat dry milk powder (3%) in PBS (30 min normal temperature). Each membrane was further washed twice with PBS and was incubated overnight at 4˚C with the following anti-human antibodies (rabbit/anti-mouse); Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; β-actin mouse clone AC-15, 1:5000; Sigma-Aldrich, St. Louis, MO, USA; annexin II mouse, 1:2000 (Anni V); annexin V (FL-319); squamous cell carcinoma (SCCA1/2) (H390); heat shoch protein (HSP27) (H-77). All anti-human antibodies were used at 1:200 dilution as was recommended by the manufacturer.

Results

2-DE PAGE and MALDI-TOF-MS. Proteins (400 μg, TCA precipitates) used for the 2-DE were evenly-distributed in the 18-cm gel with pH in the range of 3-10 and with molecular masses of 14-97 kDa. Protein spots were detected in each gel by the Image Master software and some were present consecutive spots, indicating that modified proteins or isoforms were separated by 2-DE. In Figure 1, typical gel images for both tumours and normal controls for the Norwegian cases are shown. In Tables I-III, data for proteins (or the sum of the isoforms) that exhibited statistically significant differences are summarized with total normalized volumes [(% vol), using mean 100% and M.S.D. statistics] and fold differences found between tumour and NC samples. Approximately 17 and 16 proteins (or isoforms) were found to be up-regulated in the OSCCs from Norway and the UK, respectively.

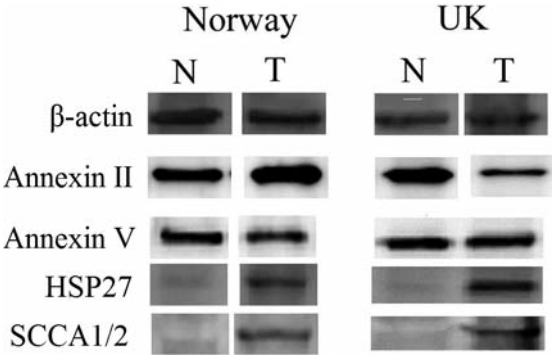

Figure 2. *Western blotting of β-actin, annexin II, V, heat-shock protein (HSP27) and squamous cell carcinoma (SCCA1/2) in tumour samples from Norway showing a significantly higher amount of annexin II in tumour than in normal control samples. Normal control samples from Norway showed higher expression of Annexin II than tumour samples from the UK. Tumour samples from Norway and UK showed higher expression of HSP27 and SCCA1/2 than did the normal controls.*

MALDI-TOF-MS and protein identification. From the SYPRO-Ruby-stained 2-DE, excised protein spots were subjected to in-gel tryptic digestion followed by MALDI-TOF-MS and database matching analysis. Several isoforms gave an identical primary structure in the protein matching and were classified as one protein. Proteins which displayed an apparent change in their expression level in tumour samples from the two countries included family of annexins (II and V), keratin-1, SCC1, HSP27 and actin, while those which displayed an apparent difference in their expression levels in normal controls compared to tumours included keratin-1 chain and crystal structure of human serum albumin (Tables I-III).

Western blot analysis (SDS-PAGE). As shown in Figure 2, confirmation of the results from MALID-TOF-MS was achieved by western blot analysis of the four proteins selected: β-actin, annexin II, annexin V, HSP27 and SCCA1/2 and as an internal control.

Discussion

In this work, 2-DE-MALDI-TOF-MS technology was used to identify proteomic changes in OSCCs from Norway and UK. Differential expression of 29 proteins was found at significant levels in the OSCC samples examined. Among

the Norwegian samples, overexpression of transferrin, family of actins, HSP27, family of fibrins, keratin-1, family of annexins (II, III, IV, V), SCCA1, endoplasmic reticulum (ER-60) protein and transferrin protein (PRO1400) were found. Interestingly, similar findings were observed among the UK tumour samples showing a similar pattern of overexpressed proteins. Moreover, the UK samples also showed overexpression of the S100 calcium-binding protein A9, histone-2, human ferritin L chain, myosin light chain 3 (MYL1) and Rho GDP dissociation inhibitor.

An increased level of salivary transferrin has been previously reported as being strongly correlated with the size and stage of the tumour and that it might function as a biomarker for the early detection of oral cancer (26). The family of actins belongs to relatively highly-conserved contractile proteins involved in cell motility, blood coagulation, adherence junction organization and axon guidance, and may play an important role in tumourigenesis. It has been suggested that the presence of γ-actin in saliva may contribute to cancer diagnosis and treatment follow-up (27). γ-Actin has been reported as being up-regulated in human small cell lung cancer and as a biomarker for leiomyosarcoma (28, 29). Our study showed a significant elevation of fibrin which is involved in signal transduction. The presence of fibrin is also a feature of blood coagulation and platelet activation, and is a histological feature of wound-healing and is found in many pathological and inflammatory processes. However, its role in cancer genesis is under investigation and remains poorly understood (30, 31). Previous investigations demonstrated involvement of fibrin in the progression of a variety of tumour types and its association with tumourigenesis resulting from extravasation and subsequent clotting of fibrinogen from plasma. Extravagated from blood vessels, fibrinogen becomes clotted and cross-linked within minutes, resulting in formation of a three-dimensional gel that often is found in presence of individual tumour cells or clumps of tumour cells (30). However, its direct function in the process of tumour genesis in the oral cavity needs further investigation. We also found an overexpression of HSP27 that interacts with actin and intermediate filaments, contributes in the process of cell differentiation and is involved in activation of the proteasome. It has been suggested that dysregulation and overexpression of HSP27 may be a frequent event during the progression of oral tongue SCC, and it might function as an independent marker for this disease (32). Another *in vitro* study of HSP27 silencing showed decreasing metastatic behaviour in head and neck SCC (33). Overexpression of HSP27 has also been associated with good prognosis for patients diagnosed with OSCC (34). We also found an elevation of the keratin-1 (KRT1) protein, which is involved in the development of epidermis, fibrinolysis, regulation of angiogenesis and in response to oxidative stress. KRT1 was previously reported as being expressed in mesenchymal tumours including schwannomas, epithelioid sarcomas and synovial sarcomas (35). An experimental mouse model using the carcinogen 4-nitroquinoline 1-oxide (4-NQO) in drinking water showed that 4-NQO was able to alter the expression patterns of the intermediate filament proteins of KRT1 that were expressed in the epithelial supra-basal layers, in addition to the basal layer, in tongues from carcinogen-treated animals (36).

Our study revealed overexpression of calcium-dependent phospholipid-binding protein annexin-A2, a member of the annexin family, involved in several cell processes including signal transduction and regulation of cellular growth. Comparative proteomic analysis of the annexin-A2 protein demonstrated its significant up-regulation in OSCC cell lines accompanying cellular transformation (37). Annexin A2 seems to interact with carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) which is found to be down-regulated in colon, prostate, breast and liver cancer (38). Interaction of annexin-A2 was also found in connection with the *S100A10* gene, also known as *p11* (39). Unlike other S100 proteins, the second helix-loop-helix domain (EF-hand) of protein p11 is incapable of binding calcium due to a series of mutations caused by deletions and substitutions. Attracted to negatively-charged phospholipids, annexin-A2 binds to p11 at the Ca^{2+}-binding site (13). Moreover, it has been suggested that annexin-A2 is involved in membrane cytoskeleton interactions and contributes to regulation of ion currents and substances across the membrane (40). S100A10 and annexin A2 seem to form a heterotetrameric protein complex that is able to resemble the structure and function of S100 proteins activated by the binding of calcium. The structure of that complex is more stable than the p11 dimer, and therefore overexpression of the annexin II gene results in higher levels of p11 protein (40, 41). In a previous study of OSCC from Sudan and Norway by genomic microarrays, we detected amplification of 1q21 harbouring S100A1–A14 in 72% of Sudanese and 46% of Norwegian samples, which is consistent with previous studies regarding potential involvement of S100A10 in OSCC (23). Our proteomic and western blot analyses showed increased expression of another Ca^{2+}-binding protein, annexin-V, which is involved in cell membrane organization and dynamics, and functions as an anti-coagulant, indirectly inhibiting the thromboplastin-specific complex involved in the blood coagulation cascade. It has been suggested that annexin-V has a potential influence on cell proliferation, and is involved in both invasive capacity and main tumour characteristics of oral carcinoma (42). In another study investigating the proteomics of cancer stem cells in prostate cancer, annexin-V was found to be positively- correlated with *CD44* gene expression. In that study, CD44 + cells seemed to possess stem cell characteristics and highly

expressed genes known to be important in stem cell maintenance, which may be valuable in regard to the development of new cancer diagnostic tools (43).

SCCA1, found in our study, has been reported as being associated with oral cancer, and used as a positive immunohistochemical marker of pathologic lymph node metastasis is associated with advanced tumour stage, and a higher rate of distant metastasis (44). The preoperative serum SCCA level has been reported as a potential prognostic indicator in disease-free survival and overall survival (45). However studies with a longer observation period are necessary to confirm these results. In another study, serum concentrations of SCCA were reported to be significantly higher than in healthy controls, suggesting that SCCA may function as a useful biomarker in OSCC (46). Proteomic study of human tongue cancer cell line Tca8113 subcutaneously inoculated into nude mice and compared with stem cell treated control nude mice showed overexpression of SCCA only in tumour-bearing mice (47). In that particular study, SCCA was also found to be up-regulated in clinical patients with tongue cancer using real-time polymerase chain reaction (RT-PCR) and western blotting. This may strongly suggest that SCCA may be of great potential as a biomarker of not only for tongue cancer but probably other SCCs of the head and neck region, and may function as a therapeutic target for gene therapy.

Although the number of samples, especially from Norway, needed to be higher in this study, our results seem to be consistent with previous findings (24). One of the major problems working with protein profiles derived from cancer tissues is the heterogeneous nature of the tumour samples used. Some possible sources influencing heterogeneity may include the differences between the proportions of cancer cells in relation to non-cancer cells, the degree of tissue damage, necrosis and cell apoptosis (48). In our study, only samples histologically-confirmed as containing more than 70% of tumour cells were used. Since the primary tumour samples may contain several other cell types in addition to tumour cells, it may be difficult to exactly define the major cellular changes occurring during the cell conversion from normal to malignant stage.

To conclude, the overexpression of annexin family proteins, keratin-1, HSP27 and SCCA reported in this study using proteomic technology and simultaneously confirmed by western blotting might provide valuable information towards their serving as potential protein biomarkers of OSCC. Findings of this study point towards global protein changes that seem to be specific for the two populations investigated. Further development of early diagnostic methods, therapeutical improvements, prediction of tumour invasion and prediction of survival of the patients affected by OSCC may depend on the precise identification of functional proteins that are specific for OSCC. Although several different specific proteins were found to be overexpressed in the samples from Norway and the UK, lack of concordance of data may reflect the differences in tobacco usage patterns and variation in alcohol consumption in the two populations. Our study of oral cancers seem to be in an agreement with several previous studies of the OSCC marker proteins. Our study provides new information on candidate proteins and their further testing as diagnostic, prognostic biomarkers and/or targets for therapy. Larger studies involving cases of OSCCs from other populations are warranted. In particular, the family of annexins, actins and S100, and moreover SCCA, keratin 1 and HSP27 may require more attention and further investigation in oral cancer.

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