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Analysis of the saliva proteome from patients with head and neck squamous cell carcinoma reveals differences in abundance levels of proteins associated with tumour progression and metastasis

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ABSTRACT

The objective of this study was to identify differentially expressed proteins in saliva from HNSCC patients compared to a control group. Saliva samples from eight individuals with non-malignant conditions of the head and neck region were employed as a control group and compared to saliva from eight patients with HNSCC using 2D DIGE analysis and subsequent mass spectrometry identification of candidate proteins. Beta fibrin (+2.77-fold), S100 calcium binding protein (+5.35-fold), transferrin (+3.37-fold), immunoglobulin heavy chain constant region gamma (+3.28) and cofilin-1 (+6.42) were all found to be significantly increased in the saliva from HNSCC samples compared to the control group whereas transthyretin (−2.92-fold) was significantly decreased. The increased abundance of one of the proteins identified (S100 calcium binding protein) was confirmed by immunoblot analysis. Many of these proteins are involved in tumour progression, metastasis and angiogenesis. The proximity of saliva to the developing tumour is undoubtedly a major factor in facilitating detection of these proteins and such a strategy may lead to the development of a panel of biomarkers useful for therapeutic monitoring and for early detection of HNSCC.

1. Introduction

The development of new proteomic diagnostics remains critical for the early detection and monitoring of head and neck squamous cell carcinoma. Ninety five percent of cancers arising from the mucosa of the upper aerodigestive tract, including the oral cavity, larynx, pharynx, nose and paranasal sinuses, are squamous cell carcinomas [1]. Head and neck squamous cell carcinoma (HNSCC) is an aggressive epithelial malignancy in which the early diagnosis of premalignant lesions is known to directly correlate with increased survival. Patients often present with advanced stage disease and despite combined therapy outcome remains poor. The major risk factors for head and neck cancer are chronic exposure of epithelia to tobacco smoke or alcohol, infection with the human papilloma virus types 16 and 18 (HPV) and genetic susceptibility [2].

Human tissue and biological fluids contain many proteins and small molecules which have the potential to be used as biomarkers [3]. Most research into cancer biomarkers has

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focused on blood components, such as plasma/serum and urine. Saliva, on the other hand, has been relatively poorly investigated as a source of biomarkers. The proximity to both premalignant and malignant head and neck neoplasms of saliva means it offers great potential for their detection [4]. A saliva-based diagnostic approach would be extremely advantageous because of its relatively non-invasive approach and with the developments in proteomic technology, identification of such diagnostic markers for early detection and prognostic markers for disease recurrence and treatment sensitivities employing saliva-based diagnostic tests are certainly attainable [5,6].

Analysis of the saliva proteome also presents many challenges. Similar to other biofluids such as serum, saliva displays a wide dynamic range with abundant proteins such as amylase and proline-rich proteins masking the presence of lower abundant proteins that are more likely to be of importance from a biomarker discovery point of view [6]. Specific saliva targeted immunodepletion columns regularly used in serum or plasma studies for the depletion of abundant proteins like albumin are not yet available for processing saliva [7]. The saliva proteome is a particularly hostile environment with proteins subjected to the affects of many host- and bacteria-derived enzymes, resulting in the generation of many modified proteins making analysis more complex [8]. However, recent developments in proteomics, especially in mass spectrometry and new labelling technologies will undoubtedly help in the successful analysis of the saliva proteome.

In order to examine differences in saliva protein levels associated with HNSCC, a proteomics based approach was initiated involving 2D DIGE analysis and subsequent mass spectrometry to generate a panel of biomarkers found to be differentially expressed between the cancerous and non-cancerous samples.

2. Materials and methods

2.1. Chemicals

Iodoacetamide (IAA), DTT, CHAPS, urea, thiourea, glycerol, 30% acrylamide-*N*,*N*,*N* bisacrylamide SDS, TEMED, ammonium persulfate, and glycine were acquired from Sigma-Aldrich (St. Louis, MO, USA). IPGs pH 3–11 NL, pharmalyte 3–11 NL, DIGE reagents and dry strip cover fluid were purchased from GE Healthcare (Uppsala, Sweden).

2.2. Clinical specimens

All 16 patients were treated at the Royal Victoria Eye and Ear Hospital, Dublin, between 2006 and 2007 and approval to conduct this study was granted by the Royal Victoria Eye and Ear Hospital Ethics Committee. The control group consisted of 8 patients with non-cancerous tumours/conditions of the head and neck region. The mean age of the group was 51.88 years with a standard deviation of ±13.91 years (Table 1). Using a control group with inflammatory non-malignant conditions of the head and neck allows for a greater chance that any protein identified with a difference in abundance levels between the cancer and control groups is a tumour specific protein and not associated with the inflammatory/immune response. The HNSCC group consisted of 8 patients. The mean age of the group was 64.13 years with a standard deviation of ±11.35 years (Table 1).

Table 1 – Clinical information for the control and HNSCC groups used in this study

Sex	Age	Diagnosis	Smoker
Control group			
F	66	MNG	No
M	47	Otitis externa	Yes
M	61	Sinusitis	Yes
M	39	Cholesteatoma	Yes
F	69	SNHL	No
M	25	Apthous mouth ulcers	No
M	49	Cholesteatoma	Yes
M	59	External exostosis EAC	Yes
HNSCC group			
F	48	Max Sinus SCC	Yes
M	71	Oesophageal SCC	Yes
M	66	Hypoparyngeal SCC	Yes
M	48	Pyriform Fossa SCC	Yes
M	56	Suprglottic SCC	No
M	81	VC SCC	Yes
M	71	Hypopharyngeal SCC	No
M	72	R vocal cord SCC	Yes

The control group consisted of 8 patients with non-cancerous tumours/conditions of the head and neck region. The mean age of the group was 51.88 years with a standard deviation of ±13.91 years. MNG: multinodular goiter, SNHL: sensorineural hearing loss, EAC: external auditory canal. The HNSCC group consisted of 8 patients. The mean age of the group was 64.13 years with a standard deviation of ±11.35 years.

2.3. Sample collection and preparation

Unstimulated whole saliva samples were collected in the morning. All the subjects were asked to be on an empty stomach, without having had any drink or eaten any kind of food since the night before. In order to minimize degradation of the proteins, the samples were processed immediately and kept on ice during the process. Between 1 and 4 mL of saliva was obtained from each subject with protease inhibitory cocktails (PICs) added immediately. To remove debris and cells, the saliva was centrifuged at 14,000 ×*g* for 15 min at 4 °C. Proteins from the resulting supernatants were concentrated using 5 kDa Amicons (Millipore) by centrifugation at 4000 ×*g* for 45 min at 4 °C. 200 μl of the resulting retenate was treated using the ReadyPrep 2-D cleanup kit (Bio-Rad) to precipitate the protein and remove agents that interfere with IEF. PICs was added to all buffers used in the ReadyPrep 2-D cleanup kit. The isolated saliva proteins were resuspended in buffer containing (4% (w/v) CHAPS, 7 M urea, 2 M Thiourea, 10 mM Tris–HCl, 5 mM Magnesium Acetate pH 8.5). Insoluble material was removed by centrifugation at 14,000 ×*g* for 10 min at 4 °C. The protein amount was estimated using an RC DC protein assay from Bio-Rad. BSA was used as a standard.

2.4. Labelling of samples

Saliva were labelled with *N*-hydroxy succinimidyl ester-derivatives of the cyanine dyes Cy2, Cy3, and Cy5 following the protocol described previously [9]. Typically, 50 μg of protein was minimally labelled with 200 pmol of either Cy3 or Cy5 for comparison on the same 2D gel. Labelling reactions were performed on ice in the dark for 30 min and then quenched with a 50-fold molar excess of free lysine to dye for 10 min on ice. A pool of all samples was also prepared and labelled with Cy2 to be used as a standard on all gels to aid image matching and cross-gel statistical analysis. The Cy3 and Cy5 labelling reactions (50 μg of each) from each lysate were mixed and run on the same gels with an equal amount (50 μg) of Cy2-labeled standard [10].

2.5. Protein separation by 2D gel electrophoresis and gel imaging

Immobilized 24 cm linear pH gradient (IPG) strips, pH 3–11, were rehydrated in rehydration buffer (7 M urea, 2 M Thiourea, 4% CHAPS, 0.5% IPG Buffer, 50 mM DTT) overnight, according to the manufacturer's guidelines. Isoelectric focusing was performed using an IPGphor apparatus (GE Healthcare) for a total of 40 kV/h at 20 °C, 50 mA. Strips were equilibrated for 20 min in 50 mM Tris–HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 1% (w/v) SDS containing 65 mM DTT and then for 20 min in the same buffer containing 240 mM iodoacetamide. Equilibrated IPG strips were transferred onto 18×20-cm 12.5% uniform polyacrylamide gels poured between low fluorescence glass plates. Strips were overlaid with 0.5% (w/v) low melting point agarose in running buffer containing bromphenol blue. Gels were run using the Ettan Dalt 12 apparatus (GE Healthcare) at 2.5 W/gel for 30 min then 100 W in total at 10 °C until the dye front had run off the bottom of the gels. All the images were collected on a Typhoon 9400 Variable Mode Imager (GE Healthcare). Statistics and quantitation of protein expression were carried out in Decyder software (GE Healthcare).

2.6. Spot digestion and LC–MS analysis

Preparative gels containing 300 μg of protein were fixed in 30% (v/v) methanol, 7.5% (v/v) acetic acid overnight and washed in water, and total protein was detected by post-staining with SyproRuby dye (Molecular Probes) for 3 h at room temperature or Colloidal Coomassie (Sigma) for 2 h at room temperature. SyproRuby stained gels were imaged using a Typhoon 9400 Variable Mode Imager (GE Healthcare) at the appropriate excitation and emission wavelengths for the stain. The subsequent gel image was imported into the BVA module of DeCyder software and was matched to images generated from DIGE analysis. Spots of interest were selected and confirmed using this software for subsequent picking using an Ettan Spot Picker. Individual gel pieces after dehydration were covered in digestion buffer (12.5 ng trypsin per μl of 10% Acetronitrile 40 mM Ammonium Bicarbonate) to cover the gel pieces. Exhaustive digestion was carried out overnight at 37 °C. After digestion, the samples were centrifuged at 12,000 ×*g* for 10 min using a bench top centrifuge. The supernatant was carefully removed from each sample and placed into clean and silconized plastic tubes. Samples were stored at −70 °C until analysed by MS.

Tryptic digested proteins were analysed by one-dimensional LC–MS using the Ettan MDLC system (GE Healthcare) in high-throughput configuration directly connected to a Finnegan LTQ (Thermo Electron). Samples were concentrated and desalted on RPC trap columns (Zorbax 300SB C18, 0.3 mm×5 mm, Agilent Technologies, and the peptides were separated on a nano-RPC column (Zorbax 300SB C18, 0.075 mm×100 mm, Agilent Technologies) using a linear acetonitrile gradient from 0% to 65% ACN (Sigma) over 60 min. All buffers used for nano LC separation contained 0.1% formic acid (Fluka) as the ion pairing reagent. Full scan mass spectra were recorded in profile mode and tandem mass spectra in centroid mode. The peptides were identified using the information in the tandem mass spectra by searching against the SWISS PROT database using SEQUEST.

2.7. 1-D gel and immunoblot analysis

10 ug of protein was loaded onto a 12% NuPAGE Bis–Tris Gels (Invitrogen) and electrophoretically separated using a MOS/SDS buffer according to the manufacturer's instructions. Electrophoretic transfer of proteins to Hybond-ECL nitrocellulose membranes (GE Healthcare) was carried out using a Bio-Rad Transblot SD cell (Bio-Rad). Proteins were transferred for 50 min at 0.34 mA. Efficiency of transfer was evaluated using Ponceau-S-Red staining of nitrocellulose membranes. Membranes were blocked for 1 h in 5% (w/v) fat-free milk powder in PBS containing 0.5% Tween-20. Membranes were then incubated overnight at 4 °C with the primary antibody, MRP14 [S100-Calcium binding protein A9] (Abcam ab24111) at a concentration of 1:1000. Nitrocellulose replicas were subsequently washed twice for 10 min in blocking solution and then incubated with corresponding peroxidase-conjugated secondary antibody (Abcam ab6728) for 1 h at room temperature at a concentration of 1:2000. Visualization of immuno-decorated 1D bands was carried out using an enhanced chemiluminescence kit (GE Healthcare). Statistical analysis was performed using ImageQuant TL (GE Healthcare).

2.8. Statistical analysis

Two-sided, Student's *t*-tests were used to analyze differences in protein levels between saliva samples from the control group and saliva from patients with head and neck cancer. A *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. DIGE analysis of saliva

50 μg of protein from each sample was labelled with Cy2, Cy3, or Cy5. In total 16 gels were run comprised of 8 gels (8 HNSCC samples v 8 Control samples) run in duplicate. All sixteen samples were labelled with Cy3 or Cy5 incorporating dye-swapping (i.e. reciprocal labelling of Cy3 and Cy5) to eliminate any dye bias. All 16 samples analysed in the experiment were used in the Cy2 labelled internal pooled standard. Samples

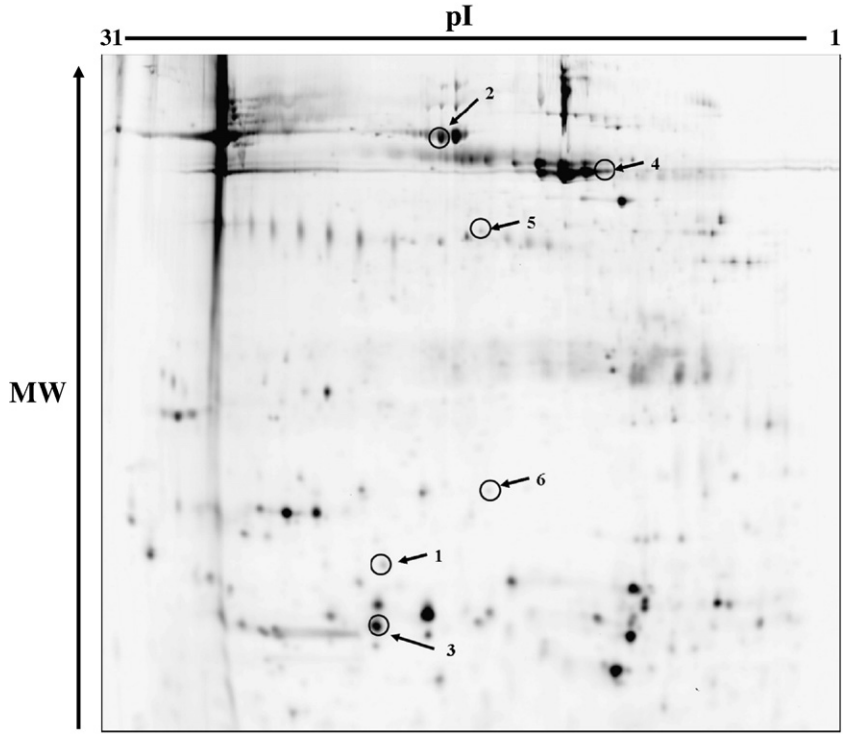

Fig. 1 – Image of saliva proteins analyzed using two-dimensional polyacrylamide gel electrophoresis (2D DIGE). Proteins were separated based on their isoelectric point, (3–11 range) and their molecular weight to generate a protein expression map (PEM). Highlighted are 6 proteins that were found to have significant differences in abundance levels between the samples.

were combined and separated by 2D gel electrophoresis. For DeCyder image analysis, the differential in-gel analysis mode of DeCyder was first used to merge the Cy2, Cy3, and Cy5 images for each gel and to detect spot boundaries for the calculation of normalized spot volumes/protein abundance. At this stage, features resulting from non-protein sources, namely dust particles and scratches, were filtered out. The analysis was used to rapidly calculate abundance differences between samples run on the same gel. The biological variation analysis mode of DeCyder was then used to match all pairwise image comparisons from difference in-gel analysis for a comparative cross-gel statistical analysis. Operator intervention was required at this point to set landmarks on gels for more accurate cross-gel image superimposition. Comparison of normalized Cy3 and Cy5 spot volumes with the corresponding Cy2 standard spot volumes within each gel gave a standardized abundance. A 2D DIGE combined image of Cy2, Cy3 and Cy5 labelled saliva proteins from both the control and HNSCC groups is shown in Fig. 1. Highlighted are 6 proteins that were found to have significant differences in abundance levels between the samples. The 6 differentially expressed proteins included in Table 2 all had a p-value of ≤ 0.05. Proteins found to have higher abundance levels in HNSCC saliva compared to the control group included beta fibrin, S100 calcium binding protein, transferrin, cofilin-1 and immunoglobulin heavy chain constant region gamma 2. Transthyretin was found to have a lower abundance level in HNSCC saliva compared to the control group.

Table 2 – Table of proteins found to have increased or decreased abundance levels in HNSCC saliva compared to the control group

Position on gel	Protein ID	Protein AC	Average ratio	Identification method	No. of matched peptides
1	Transthyretin	gi|339685|	−2.92	LC–MS	4
2	Transferrin	gi|37747855|	3.37	LC–MS	16
3	S100 calcium binding protein A9 (calgranulin B)	gi|56205191|	5.35	LC–MS	4
4	Immunoglobulin heavy chain constant region gamma 2	gi|12054074|	3.28	LC–MS	8
5	Fibrin beta	gi|223002|	2.77	LC–MS	11
6	Cofilin 1 (non-muscle)	gi|30582531|	6.42	LC–MS	6

Listed are the protein identities obtained by LC–MS analysis, gene index number, average ratio and matched number of peptides.

3.2. *Statistical analysis and mass spectrometry*

Protein identities were achieved using the Ettan MDLC system (GE Healthcare) in high-throughput configuration directly connected to a Finnegan LTQ (Thermo Electron). Fig. 2 shows a base peak chromatogram (full ms) generated by LC–MS–MS analysis of the S100 calcium binding protein digest using the Finnegan LTQ mass spectrometer (Fig. 2A). Also shown is a continuous *b* or *y*-ion series of at least six residues for peptide DLQNFLK from S100 calcium binding protein A9 (Fig. 2B). Protein identification was performed using the Turbo-SEQUEST algorithm in the BioWorks 3.1 software package (Thermo Electron) and the Swiss-Prot human database (Swiss Institute of Bioinformatics, Geneva, Switzerland). The identified peptides were further evaluated using charge state versus cross-correlation number (XCorr). The criteria for positive identification of peptides was XCorr >1.5 for singly charged ions, XCorr >2.0 for doubly charged ions, and XCorr >2.5 for triply charged ions.

3.3. *Western blot analysis*

Western blot and statistical analysis of saliva from control and HNSCC samples was performed using an antibody to MRP14 (S100 calcium binding protein). Western blot analysis was performed on all 16 samples with the representative figure. (Fig. 3) showing results for 8 of the individual samples (4 HNSCC samples v's 4 Control samples). The results confirm the increase in abundance levels of S100 calcium binding protein in HNSCC samples compared to the control group.

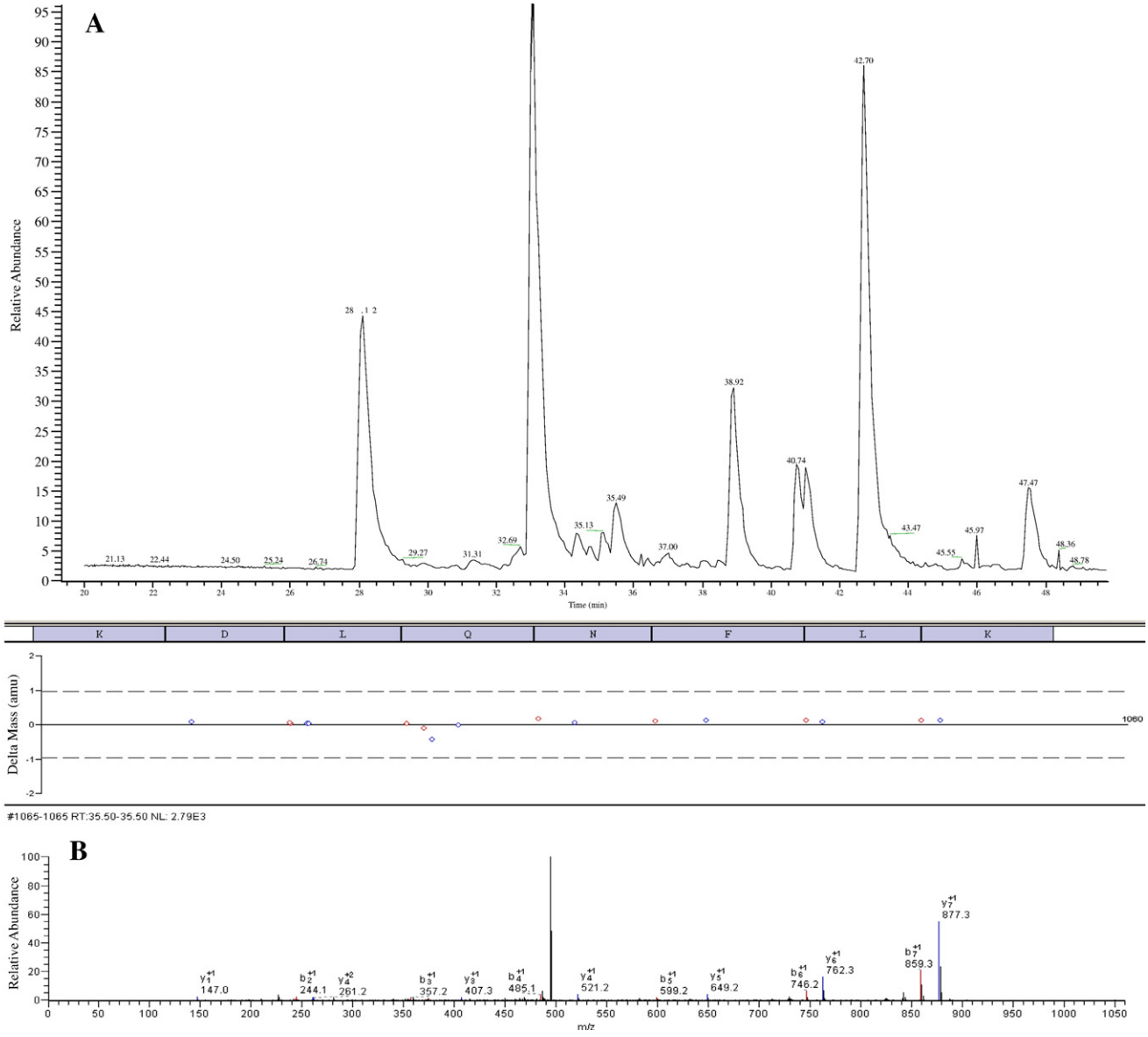

Fig. 2 – (A) Base peak chromatogram (full ms) generated by LC–MS–MS analysis of the S100 calcium binding protein digest using the Finnegan LTQ mass spectrometer coupled to the GE Healthcare MDLC. (B) continuous *b* or y-ion series of at least six residues for peptide KDLQNFLK from S100 calcium binding protein. The criteria for positive identification of peptides were XCorr >1.5 for singly charged ions, XCorr >2.0 for doubly charged ions, and XCorr >2.5 for triply charged ions.

Western blot analysis showed a 4.6-fold increase in abundance levels for S100 calcium binding protein which compares favourably to the 5.35-fold increase generated using 2D DIGE. To show visually alterations in corresponding spot intensity proportions, a 3-D image of S100 calcium binding protein A9 is shown (Fig. 3). This figure demonstrated the increase in abundance levels for this protein in saliva samples from patients with HNSCC compared to the control group. Images were generated using the Biological variation analysis (BVA) module of DeCyder software. Due to limited sample amounts, validation of the 2D DIGE results was performed with only one protein, S100 calcium binding protein. This supports the results seen in the 2D DIGE analysis. Future work will investigate the expression of the other candidate proteins by western blot analysis in larger sample sets.

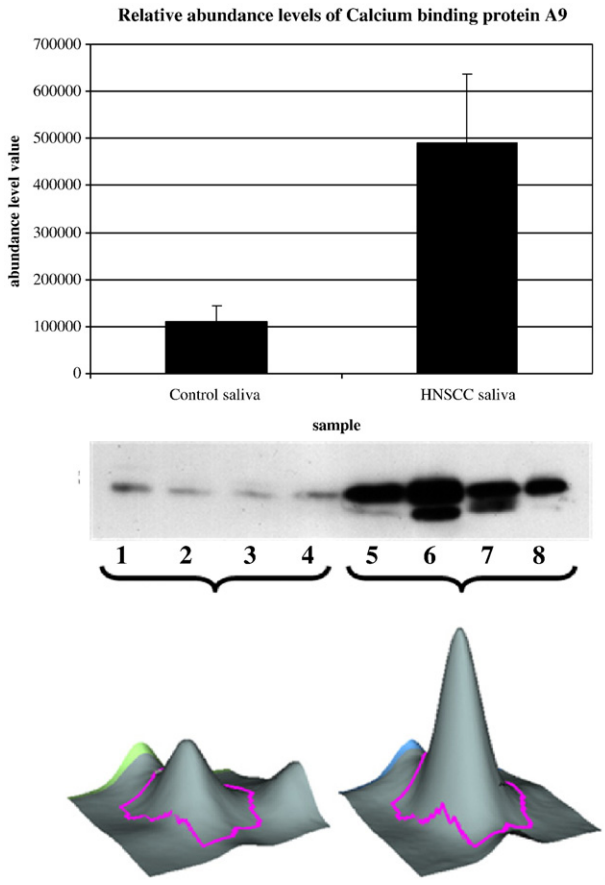

Fig. 3 – Western blot and statistical analysis of saliva from control and HNSCC samples using an antibody to MRP14 (S100 calcium binding protein). The results confirm the increase in abundance levels of S100 calcium binding protein in HNSCC samples compared to the control group. Western blot analysis showed a 4.6-fold increase in abundance levels for S100 calcium binding protein which compares favourably to the 5.35-fold increase generated using 2D DIGE. This is a representative blot for analysis on all samples performed in duplicate. Also displayed is a 3-D image of S100 calcium binding protein A9. This figure demonstrated the increase in abundance levels for this protein in saliva samples from patients with HNSCC compared to the control group. Images were generated using the BVA module of DeCyder software. Control saliva: lanes 1–4, HNSCC saliva: lanes 5–8.

4. Discussion

In this study, we quantified the protein levels of saliva proteins in HNSCC compared to a control group with the purpose of detecting differences in the levels of proteins which could serve as markers for the disease. HNSCC is very difficult to diagnose and often early small lesions can be missed. As a result, this cancer is usually diagnosed at an advanced stage. A panel of biomarkers detectable in the saliva of patients with HNSCC may allow for the development diagnostics assays for early detection and prognostic markers for disease recurrence and treatment sensitivities. Many papers have now been published on analysis of the saliva proteome investigating disease specific proteins. The p53 antibody can be detected in the saliva of patients diagnosed with oral squamous cell carcinoma [11] and increased levels of human alpha-defensin 1 (HNP-1) was found to be indicative of the presence of oral SCC in a study by Mizukawa et al. [12]. Recently an examination into salivary soluble CD44 (solCD44) expression in HNSCC patients and normal controls showed that solCD44 ELISA seems to effectively detect HNSCC at all stages [13]. Interleukin 8 was detected at higher concentrations in saliva of patients with OSCC (oral cavity and oropharyngeal squamous cell carcinoma) as measured by real-time polymerase chain reaction analysis and enzyme-linked immunosorbent assay [14]. Salivary biomarkers for other cancers have also been identified. The tumour marker c-erbB-2 (erb) was found in the saliva of women diagnosed with breast carcinoma, as compared with patients with benign lesions and healthy controls [15], and elevated salivary levels of CA 125 were detected in patients with malignant ovarian tumours [16].

The six proteins listed in Table 2 were detected in all 16 samples (8 HNSCC samples and 8 Control samples). Five were found to have significantly increased abundance levels in the saliva from the HNSCC samples compared to the control group, namely beta fibrin (+2.77-fold), S100 calcium binding protein (+5.35-fold), transferrin (+3.37-fold), immunoglobulin heavy chain constant region gamma (+3.28) and cofilin-1 (+6.42). Transthyretin (−2.92-fold) was the only proteins found to be significantly decreased.

The S100 protein family consists of 20 known members and are likely involved in many of the aspects of tumourigenesis and metastasis. Of the 20 known members of the S100 protein family, only serum levels of S100B is used clinically in melanoma patients at all stages of the tumour. S100A9 which forms a heterodimeric complex with S100A8, is found with increased abundance levels in gastric and colorectal cancer [17,18]. Yong and Moon recently demonstrated that by employing small-interfering RNA (siRNA) targeting S100A8 or S100A9, the invasive and migratory phenotypes of SNU484 cells were significantly inhibited [17].

Of increased relevance to our study, the recent paper by Sewell and co-workers [19], identified potential biomarkers of laryngeal cancer tissue specimens using two-dimensional differential in-gel electrophoresis and mass spectrometry. The results of their analysis showed that the expression of a

number of proteins was significantly altered in the tumour specimens when compared to match normal controls, including S100 calcium binding protein A9. Melle and co-workers demonstrated that calgranulin A and calgranulin B were detectable in protein lysates from positive areas and were absent in the negative areas of pharyngeal squamous tumour epithelial cells, using ProteinChip arrays [20]. Driemel and colleagues showed that S100A8 and S100A9 can differentiate between normal mucosa, inflammatory and hyperproliferative lesions, and oral squamous cell carcinoma with a sensitivity of 100% and specificity of 91% using SELDI-ToF [21].

In our study we found S100A9 to have a 5.35-fold increase in abundance levels in the saliva from the HNSCC group compared to the control group using 2D DIGE. This result was confirmed using Western blot analysis, showing a 4.6-fold increase in abundance levels for S100A9. S100A8 was also detected in our study and was found to be of increased abundance in the saliva from the HNSCC group but was excluded because the *p*-value for this protein was outside the selected value for this study.

This study and previous studies have demonstrated the importance of S100A9 in HNSCC as a potential biomarker and with further insight into its function within the tumour micro-environment it may be a valid target for new therapies. Ohshiro and co-workers recently described work on saliva samples from healthy individuals and HNSCC patients. Their analysis identified two proteins, α-1-B-glycoprotein and complement factor B proteins to be present in saliva samples from HNSCC patients but not in normal specimens. Cystatin S, parotid secretory factor, and poly-4-hydrolase ß-subunit proteins were detected in most normal saliva but not in patient specimens. Interestingly they identified S100 calcium binding protein A9 in both HNSCC patient saliva and in normal samples as described in this paper [22].

The presence of fibrin in and around tumours may play an important role in tumour growth [23]. Fibrin could form an adhesive support for cell anchorage during local expansion and emigration. Fibrin may also promote angiogenesis. The fibrin clot and the associated coagulation factors provide a highly pro-angiogenic environment [24] Previous studies documented the presence of fibrin and fibrinogen in situ in small cell carcinoma of the lung, renal cell carcinoma, malignant melanoma, and ovarian carcinoma [25–31]. In this study it was found that beta fibrin was 2.77 fold increased in abundance levels in the saliva from the patients with HNSCC compared to the control group. Beta fibrin has important functions in tumour progression and the detection of this protein in the saliva is significant. The proximity of the saliva to the developing tumour is fundamental to the detection of such proteins.

Transferrin, cofilin-1, immunoglobulin heavy chain constant region gamma 2 and transthyretin were also found to have significant differences in abundance level in HNSCC saliva compared to the control group. Transthyretin is a traditional biomarker for nutritional and inflammatory status. In this study it was found that the levels of transthyretin were significantly decreased in the saliva from the HNSCC group. Recently a truncated from of transthyretin was included in a panel of three proteins in a study on serum biomarker discovery for ovarian cancer in women [32]. Turhani et al., recently demonstrated that cofilin was 2.2 fold increased in abundance levels in fractions enriched in cytosolic and mitochondrial proteins from oral squamous cell carcinoma whole tumour samples compared to surrounding mucosa [33].

One of the most important aspects of investigations into expression-based clinical proteomics is the employment of a proper control group. Many proteins identified in biomarkers discovery studies are often found to be acute phase proteins, and are associated with the inflammatory state resulting from the disease [34]. In this study we were able to use controls with inflammatory non-malignant conditions of the head and neck therefore allowing a greater chance that any protein identified with a difference in abundance levels between the cancer and control groups is a tumour specific protein.

Human saliva is attractive for disease diagnostics because its collection is less invasive than that of blood for serum/plasma analyses. Markers found in saliva are likely to be relatively selective for HNSCC. Many of today's biomarkers are found associated with multiple cancers making precise diagnosis difficult. Many proteins that make up the saliva proteome have now been identified and catalogued, but many more are still to be discovered. Immunodepletion columns are now widely available for serum/plasma processing but a specific column for saliva is not yet available. Ohshiro and co-workers recently used an immunodepletion column commonly used to deplete serum, plasma or CSF of major proteins in their study on saliva proteins [22]. The results were very interesting with the removal of abundant proteins like albumin allowing analysis of lower level proteins. Immunodepletion of as many major saliva proteins such as mucins, amylases and immunoglobulins would allow researchers to mine deeper into the saliva proteome and analyse protein fragments and associated peptide that are disease specific. It is also interesting to note that abundant proteins such as transferrin and immunoglobulin heavy chain constant region gamma were found to be of particular interest in this study. The use of depletion columns would remove these proteins but it seems reasonable that analysis of the abundant protein fraction might also uncover some valuable information.

Recent developments in proteomic technologies like mass spectrometry, liquid chromatography and protein/peptide labelling technologies will undoubtedly help researchers detect low abundance molecules in the saliva proteome. The ultimate goal of a quick cost effective, minimally invasive test for groups at high risk of developing HNSCC and for the monitoring of response to specific therapeutic regimes are certainly achievable.

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