Published in final edited form as:

Otolaryngol Head Neck Surg. 2009 November ; 141(5): 626–632. doi:10.1016/j.otohns.2009.08.011.

2D-DIGE proteomic characterization of head and neck squamous cell carcinoma

Mark A. Merkley, BS, Paul M. Weinberger, MD, Lana L. Jackson, MD, Robert H. Podolsky, PhD, Jeffrey R. Lee, MD, and William S. Dynan, PhD

Department of Otolaryngology, (Drs. Weinberger and Jackson), Institute for Molecular Medicine and Genetics, (Mr. Merkley, Dr. Dynan, Dr. Lee), and Center for Biotechnology and Genomic Medicine, Medical College of Georgia (Dr. Podolsky), Department of Pathology, Augusta VA Medical Center (Dr. Lee), Augusta, Georgia

Abstract

Objective—Identify proteins that are differentially expressed between head and neck squamous cell cancer and patient-matched normal adjacent tissue, and validate findings in a separate patient cohort.

Study Design—Cross-sectional study of surgical specimens.

Setting—Tertiary care academic medical center.

Subjects and Methods—Laser capture microdissection and two-dimensional difference gel electrophoresis were used previously to establish proteomic profiles for tumor and normal adjacent tissue from 14 patients. Here, significance analysis of microarray was used to rank candidate biomarkers. Spots meeting statistical and biological criteria of significance were analyzed by liquid chromatography and tandem mass spectrometry to obtain protein identifications. The expression pattern of the highest-ranked candidate biomarker (cornulin) was validated in a larger, independent patient cohort (n=68) by immunohistochemical staining of a tissue microarray.

Results—117/732 spots (15.9%) met criteria for significance. Identities were obtained for 40 spots, representing 19 different proteins. Four proteins were novel in the context of HNSCC: glutathione synthetase, which was upregulated, and cornulin (SEP53), guanylate binding protein 6, and GRP78, which were downregulated. Cornulin functions in the stress response in normal squamous epithelium, and reduced expression has been proposed as a marker of susceptibility to laryngopharyngeal reflux and other stressors. Loss of cornulin expression was confirmed in an independent HNSCC patient cohort ($P<.001$).

Conclusions—Downregulation of cornulin is a prominent feature of the molecular signature of HNSCC identified by comparative proteomics. Cornulin may represent a link between HNSCC and other pathologies arising in stratified squamous epithelium.

Please address correspondence to: William S. Dynan PhD, Institute for Molecular Medicine and Genetics, Medical College of Georgia, 1120 15th Street, Room CB-2803, Augusta Georgia 30912. Phone: 706.721.8756; FAX: 706.434.6440; wdyan@mccg.edu.

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Squamous cell carcinoma of the head and neck (HNSCC) is the sixth most common malignancy worldwide in adults, with nearly 50,000 new cases diagnosed in the United States each year.

¹ Risk factors include tobacco and alcohol use and infection with high-risk types of human papillomavirus. In contrast to most other cancer types, the overall survival rate (60%) for HNSCC has changed only slightly, if at all, in the last 30 years (epidemiology reviewed in²). These statistics are especially sobering in light of advances in imaging technology, surgical and non-surgical treatment methodologies, and peri-operative medical care for the head and neck cancer patient.

One promising avenue of research is the identification of biomarkers of disease state. These are available for other cancers, notably breast and prostate cancer, where they are beginning to be applied clinically. Biomarkers can be used in a number of ways, including early detection, establishment of prognosis, prediction of the response to specific therapies, analysis of surgical margins, and monitoring for disease recurrence. Biomarkers can also provide insights into pathobiology of specific disease states. A common starting point for identification of biomarkers is proteomic profiling to compare tissue or accessible body fluids from the HNSCC patient with corresponding uninvolved tissue or fluids from healthy subjects.

We have previously reported proteomic profiling of HNSCC tissue and patient-matched normal adjacent tissue (NAT). We used laser capture microdissection (LCM) to obtain pure populations of cancer cells. Microdissection excludes stroma and necrotic tissue, potentially increasing specificity. We then used two-dimensional difference gel electrophoresis (2D-DIGE) to accurately measure abundances of 732 protein spots that were detected in >90% of the samples. We previously reported that there were no significant differences in the overall proteomic patterns of HNSCC from different anatomic subsites.³ Here we describe molecular identification of top-ranked biomarkers that differentiate HNSCC and NAT. The top-ranked biomarker identified in this study, cornulin, was validated in a larger cohort. Interestingly, cornulin has previously been implicated in resistance of normal tissue to several of the etiologic factors for HNSCC, including alcohol, chemical stressors, and pathogen infection.

Methods

Patient Selection

The cohort has been described,³ and included tissue samples obtained from patients with histologically confirmed HNSCC treated at the Medical College of Georgia (MCG) from 2004 to 2007 that enrolled in a voluntary tissue / tumor banking registry. Collection of tissue and subsequent analyses were approved by the MCG Institutional Review Board. All patients with available matching tumor and adjacent histologically normal frozen tissue at study inception were included. Biopsy specimens for the study were obtained pre- chemotherapy and/or radiotherapy. The cohort consisted primarily of patients with advanced TNM stage; three patients were TNM stage I/II, and eleven patients stage III/IV. Subsite of origin was oropharynx (3), larynx (4), and oral cavity (7).

LCM/2D-DIGE Analysis

The experimental procedure and data analysis were as described previously.³ Briefly, tumor and patient-matched NAT from 14 patients was stained with nuclear fast red and subjected to LCM using an Arcturus PixCell IIe microscope. Protein from captured cells was extracted and quantified, and an aliquot of each sample was labeled to saturation with Cy5 sulfhydryl-reactive dye. A mixed internal standard was prepared by combining an aliquot of protein lysate from each sample and labeled to saturation with Cy3 sulfhydryl-reactive dye. Each Cy5-labeled

sample was mixed with an aliquot of the Cy3-labeled internal standard and subjected to 2D gel electrophoresis prior to scanning and analysis.

Protein Identification

Proteins that met the statistical and biological criteria for significance, as explained in results, were considered candidates for molecular identification. LCM was used to obtain approximately 200 µg of protein from the patient samples. The protein was labeled with Cy5, mixed with 1 µg of the Cy3 labeled internal standard, and resolved by 2-dimensional electrophoresis. Spots were matched to the master analytical gel and picked by a robotic corer (Ettan Spot Picker, GE Healthcare Sciences). Proteins were digested with trypsin, and peptides were subjected to LC-MS/MS using an LTQ ion trap mass spectrometer (Thermo Scientific). Protein identities were determined from LC-MS/MS data using the Sequest algorithm as implemented by the BioWorks Browser v 3.2 (Thermo Scientific) and searching against the NCBI database. Identification was considered successful based on the Sf score ($>.5$), the P value ($<.05$), and consistency between experimental and predicted molecular weight and pI.

Immunohistochemistry

A commercial tissue microarray (TMA) (Biomaxx, Rockville, MD) was stained with anti-cornulin antibody, as described previously.⁴ Briefly, the slide was incubated with 1:100 anti-cornulin (Alexis, San Diego, CA) for 30 minutes. The slide was washed twice with PBS, then with HRP-conjugated goat anti-rabbit immunoglobulin (Envision+ HRP kit, Dako Corp. Carpinteria, CA.). The slide was rinsed twice with PBS and bound antibody was detected using diaminobenzidine. The slide was counterstained with hematoxylin. Scoring was determined by a board-certified pathologist (JRL) and reported on an ordinal scale as 0 (no expression) to 3 (strong expression).

Results

Proteomic profiling

Profiling was performed previously using 14 HNSCC and 14 patient-matched NAT samples.³ Patient demographics were also reported previously.³ Males and females were equally represented. Age at diagnosis ranged from 45 to 74 years. Patients were also classified by gender, primary subsite, tumor-node-metastasis (TNM) stage, histologic grade, tumor type (recurrent versus primary) and management. Samples were obtained from standard histologic sections by LCM. Proteins were extracted, fluorescently labeled, and analyzed by 2D-DIGE using an internal standard design. Spot maps were generated for 28 gels (14 HNSCC, 14 NAT). The maps were aligned with a master spot map, and relative abundance values were generated for each of 732 protein spots that were common to $>90\%$ of gels.

Statistical Analysis

SAM was used to evaluate the paired relative abundance values for each protein spot, and a false discovery rate (FDR) was estimated using permuted data sets. A false discovery rate of 0 was chosen as a statistical cutoff. Of 117 spots that met this criterion, 35 increased in HNSCC versus NAT and 82 decreased. We applied a second filter of 2-fold change, reasoning that proteins that had less than 2-fold change were unlikely to be robust biomarkers. Of 75 spots that passed both filters, 23 increased in HNSCC versus NAT, and 52 decreased.

Protein Identification

SAM analysis provides a statistically ranked list, which aids in prioritization of spots for identification. We attempted the identification of 51 spots by LC-MS/MS using an ion trap LTQ mass spectrometer. Of the 40 (78%) that were identified, seven were present in two or

more spots, leaving 19 unique identifications. Identified proteins are listed in Table 1 and are sorted by direction and magnitude of fold change. They included structural proteins, markers of cell proliferation, and stress proteins.

Marker Characteristics

To evaluate the ability of the 40 identified spots to collectively discriminate between HNSCC and NAT, we performed an unsupervised clustering analysis and represented the results in the form of a heat map (Fig 1A). HNSCC samples clustered separately from NAT, and the difference in expression pattern is readily apparent by inspection. We also performed a principal component analysis using the same group of identified spots (Fig 1B). The normal samples and cancer samples again clustered, indicating that the 40 identified spots robustly discriminate between groups.

As in a previous study of cervical cancer using a similar methodology,⁴ more spots are differentially absent in cancer (green on right half of figure) than are differentially present (red on right half of figure.) The overall “proteomic signature” thus defined includes both abnormally low and abnormally high values for individual proteins.

Cornulin

The statistically top-ranked marker was cornulin (also known as c1orf10 and squamous epithelial-induced stress protein of 53 kDa (SEP53)), a member of the calcium-binding S-100 protein family. Indeed, isoforms of cornulin were identified six times within the set of 40 identified spots. Relative expression values for cornulin in HNSCC and patient-matched NAT samples are graphed in Fig 2A. Cornulin levels decreased in all 14 patients, and there was no overlap between cornulin abundance values in HNSCC and NAT. Thus, the area under the receiver operating characteristic curve (ROC) was 1.0 (not shown), which is outstanding for a single marker.

Because of its statistical ranking and the prior evidence for involvement in a variety of squamous tissue pathologies (see Discussion), we sought to validate the cornulin findings in a larger cohort. We analyzed expression using anti-cornulin antibody to stain a tissue microarray containing normal oral squamous epithelium (14), well differentiated (16), moderately differentiated (26), and poorly differentiated (12) tumors (representative images, Fig. 2B). The mean graded staining intensity for normal tissue was 2.5 on a 0-3 scale. The mean graded intensities for the well, moderately, and poorly differentiated tumors were 0.0, 0.08, and 0.2, respectively ($\chi^2=59.8$, $P<.001$, Fig 2C). In 8 of 68 cores, strong focal expression of cornulin could be seen among nonstained tumor cells. These areas may correspond to maturing squamous morules and were not included in the grading scheme. An example of this focal staining is also shown (Fig 2b). The cores with intense focal staining were seen in well-differentiated HNSCC (4/16) and moderately differentiated HNSCC (4/26) but not in poorly differentiated cancer (0/12).

Discussion

Here we report a set of 19 identified proteins that strongly discriminated between HNSCC and patient-matched NAT. The use of patient-matched tissue was important for defining this data set. With cornulin, for example, abundance values in normal tissue varied, but the fold-decrease in HNSCC was similar in most cases (Fig 2). Similarly the use of LCM to enrich for pure populations of tumor cells may have been helpful in reducing confounding factors.

Proteins that increase in HNSCC

There were seven unique proteins that were upregulated in HNSCC versus NAT. One of these, glutathione synthetase, is novel in the context of HNSCC. Elevation could reflect oxidative stress in the tumor microenvironment. Oxidative stress could also be responsible for the elevation of manganese superoxide dismutase, a finding that is consistent with many prior reports.⁵⁻⁷ Manganese superoxide dismutase expression has also been suggested to correlate with metastatic potential in cell lines.⁸ Elevation of keratin-17 is consistent with previous reports that expression increases in HNSCC, is correlated with metastatic potential, and is seen in positive lymph nodes.^{7,9,10} Elevated proliferating cell nuclear antigen is consistent with the role of this protein in DNA replication and with a previous report that this is an adverse prognostic marker for HNSCC.¹¹

Proteins that decrease in HNSCC

There were 11 unique proteins that were downregulated in HNSCC versus NAT. The most highly ranked protein was cornulin, which is a novel biomarker in the context of HNSCC. Prior studies have shown that cornulin is expressed in normal stratified squamous epithelium. Strikingly, its normal function is to promote resistance to some of the etiologic factors for HNSCC, including alcohol and other chemical insults.¹² It also bears the evolutionary signature of a pathogen resistance gene,¹³ which is of interest because the association of a subset of HNSCC with viral infection. Loss of cornulin has been implicated as a predisposing factor in symptomatic laryngopharyngeal reflux disease.¹⁴ Loss of expression has also been reported previously in esophageal cancer and to some extent in Barrett's esophagus, the precursor of esophageal adenocarcinoma.^{15,16} Similarly it is lost in cervical cancer and its precursor, high-grade squamous intraepithelial lesions.⁴ Indeed, in this recent study, cornulin was the only biomarker that quantitatively discriminated normal cervix, high grade squamous intraepithelial lesions (HSIL), and invasive cancer.⁴ The similar down-regulation of cornulin in HNSCC, esophageal, and cervical cancers may reflect their common origin in squamous epithelium.

Guanylate binding protein 6 is also a novel biomarker in the context of HNSCC. Interestingly, this member of the 65 kDa GTPase family is normally up-regulated in response to interferon- γ .¹⁷ Decreased expression in cancer could reflect lower expression of IFN- γ in the tumor-microenvironment or loss of ability to respond to this inflammatory cytokine.

Heat shock 70 kDa protein 5, also known as glucose-regulated protein 78 (GRP78) was a third novel biomarker in the context of HNSCC. The two-fold decrease was unexpected, as this stress protein has been shown to be up-regulated in a number of other cancers, including breast, prostate, liver, colon, and gastric cancers.¹⁸ There have been no prior reports of either increased or decreased expression in HSNCC. Further investigation of this potential marker is warranted.

The present survey also identified many proteins that have been reported before as HNSCC biomarkers. Expression of cytokeratins 4, 8, and 13 decreased in HNSCC relative to NAT, consistent with several reports.^{19,20} Keratin-4 was one of only two markers where there was no overlap in abundance values between HNSCC and NAT (cornulin was the other). Annexin A1 decreased in HNSCC relative to NAT in all 14 patients. Expression of Transglutaminase 3, which cross-links proteins in cornified epithelium, was decreased in HNSCC consistent with prior reports.^{9,21} Cystatin-B, a cathepsin-B inhibitor was decreased, although in a prior study it was unchanged.²² There was a decrease in the leukocyte elastase inhibitor (Serpin B1), which is consistent with our previous findings in cervical cancer.⁴ We saw a decrease in Annexin A, consistent with three previous studies, although other studies have reported an increase; this discrepancy is unexplained.^{5,6,23-25}

Conclusion

Comparative proteomics identified 19 unique proteins that distinguish HNSCC from NAT, four of which are novel in the context of HNSCC. Loss of one of these proteins, cornulin, is a molecular feature linking various pathologies affecting stratified squamous epithelium and with sensitivity to etiologic factors for HNSCC.

Acknowledgments

This work was in part supported by NIH Grant 5R33CA095941-04. We thank Eric Miller (BS) and Wenbo Zhi (PhD) of the Medical College of Georgia proteomics core for data collection.

References

1. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2007. *CA Cancer J Clin* 2007;57:43–66. [PubMed: 17237035]
2. Argiris A, Karamouzis MV, Raben D, et al. Head and neck cancer. *Lancet* 2008;371:1695–709. [PubMed: 18486742]
3. Weinberger PM, Merkley MA, Lee JR, et al. Combination proteomic analysis demonstrates molecular similarity of head and neck squamous cell carcinoma arising from different subsites. *Arch Otolaryngol Head Neck Surg*. In press.
4. Arnouk H, Merkley MA, Podolsky RH, et al. Characterization of Molecular Markers Indicative of Cervical Cancer Progression. *Proteomics Clin Appl*. In press.
5. Ralhan R, Desouza LV, Matta A, et al. Discovery and verification of head-and-neck cancer biomarkers by differential protein expression analysis using iTRAQ labeling, multidimensional liquid chromatography, and tandem mass spectrometry. *Mol Cell Proteomics* 2008;7:1162–73. [PubMed: 18339795]
6. Wang Z, Jiang L, Huang C, et al. Comparative proteomics approach to screening of potential diagnostic and therapeutic targets for oral squamous cell carcinoma. *Mol Cell Proteomics* 2008;7:1639–50. [PubMed: 18458027]
7. Patel V, Hood BL, Molinolo AA, et al. Proteomic analysis of laser-captured paraffin-embedded tissues: a molecular portrait of head and neck cancer progression. *Clin Cancer Res* 2008;14:1002–14. [PubMed: 18281532]
8. Ye H, Wang A, Lee BS, et al. Proteomic based identification of manganese superoxide dismutase 2 (SOD2) as a metastasis marker for oral squamous cell carcinoma. *Cancer Genomics Proteomics* 2008;5:85–94. [PubMed: 18460737]
9. Ohkura S, Kondoh N, Hada A, et al. Differential expression of the keratin-4, -13, -14, -17 and transglutaminase 3 genes during the development of oral squamous cell carcinoma from leukoplakia. *Oral Oncol* 2005;41:607–13. [PubMed: 15975524]
10. Toyoshima T, Vairaktaris E, Nkenke E, et al. Cytokeratin 17 mRNA expression has potential for diagnostic marker of oral squamous cell carcinoma. *J Cancer Res Clin Oncol* 2008;134:515–21. [PubMed: 17786476]
11. Pich A, Chiusa L, Navone R. Prognostic relevance of cell proliferation in head and neck tumors. *Ann Oncol* 2004;15:1319–29. [PubMed: 15319236]
12. Darragh J, Hunter M, Pohler E, et al. The calcium-binding domain of the stress protein SEP53 is required for survival in response to deoxycholic acid-mediated injury. *FEBS J* 2006;273:1930–47. [PubMed: 16640557]
13. Little TJ, Nelson L, Hupp T. Adaptive evolution of a stress response protein. *PLoS One* 2007;2:e1003. [PubMed: 17925851]
14. Johnston N, Dettmar PW, Lively MO, et al. Effect of pepsin on laryngeal stress protein (Sep70, Sep53, and Hsp70) response: role in laryngopharyngeal reflux disease. *Ann Otol Rhinol Laryngol* 2006;115:47–58. [PubMed: 16466100]

15. Xu Z, Wang MR, Zin X, et al. Novel Human Esophagus-Specific Gene C1orf10: cDNA Cloning, Gene Structure, and Frequent Loss of Expression in Esophageal Cancer. *Genomics* 2000;69:322–30. [PubMed: 11056050]
16. Yagui-Beltran A, Craig AL, Lawrie L, et al. The human oesophageal squamous epithelium exhibits a novel type of heat shock protein response. *Eur J Biochem* 2001;268:5343–55. [PubMed: 11606197]
17. Degrandi D, Konermann C, Beuter-Gunia C, et al. Extensive characterization of IFN-induced GTPases mGBP1 to mGBP10 involved in host defense. *J Immunol* 2007;179:7729–40. [PubMed: 18025219]
18. Lee AS. GRP78 induction in cancer: therapeutic and prognostic implications. *Cancer Res* 2007;67:3496–9. [PubMed: 17440054]
19. Kainuma K, Katsuno S, Hashimoto S, et al. Differences in the expression of genes between normal tissue and squamous cell carcinomas of head and neck using cancer-related gene cDNA microarray. *Acta Oto-Laryngologica* 2006;126:967–74. [PubMed: 16864496]
20. Matthias C, Mack B, Berghaus A, et al. Keratin 8 expression in head and neck epithelia. *BMC Cancer* 2008;8:267. [PubMed: 18803884]
21. Choi P, Jordan CD, Mendez E, et al. Examination of oral cancer biomarkers by tissue microarray analysis. *Arch Otolaryngol Head Neck Surg* 2008;134:539–46. [PubMed: 18490578]
22. Strojjan P, Budihna M, Smid L, et al. Prognostic significance of cysteine proteinases cathepsins B and L and their endogenous inhibitors stefins A and B in patients with squamous cell carcinoma of the head and neck. *Clin Cancer Res* 2000;6:1052–62. [PubMed: 10741734]
23. Chen J, He QY, Yuen AP, et al. Proteomics of buccal squamous cell carcinoma: the involvement of multiple pathways in tumorigenesis. *Proteomics* 2004;4:2465–75. [PubMed: 15274141]
24. Wu W, Tang X, Hu W, et al. Identification and validation of metastasis-associated proteins in head and neck cancer cell lines by two-dimensional electrophoresis and mass spectrometry. *Clin Exp Metastasis* 2002;19:319–26. [PubMed: 12090472]
25. Turhani D, Krapfenbauer K, Thurnher D, et al. Identification of differentially expressed, tumor-associated proteins in oral squamous cell carcinoma by proteomic analysis. *Electrophoresis* 2006;27:1417–23. [PubMed: 16568407]

A. Heat Map

B. Principal Component Analysis

Figure 1. Statistical Analysis

A. Results of unsupervised cluster analysis of 40 spots containing identified candidate biomarkers, presented in the form of a heat map. **B.** Principal component analysis derived from the expression levels of the same set of 40 spots containing identified proteins.

A. Cornulin Abundance: 2D-DIGE

B. Immunohistochemical Staining

Normal Adjacent

Well Differentiated

Moderately Differentiated

Poorly Differentiated

Focal Expression

C. Cornulin Abundance: Immunohistochemistry

Figure 2. Validation of cornulin as a candidate biomarker

A. Graph of the normalized log₂ abundance values for cornulin. Lines connect abundance values for patient-matched samples. Results are for spot 464, one of six spot corresponding to cornulin isoforms. All showed very similar expression trends. **B.** Immunohistochemistry. A tissue microarray containing 80 cores (70 cancer, 10 normal) was stained with anti-cornulin. Representative images from normal adjacent tissue, and well differentiated, moderately differentiated and poorly differentiated tumors are shown. An example of focal staining is also shown. Brown staining indicates the presence of the protein. **C.** Average graded for the intensity of staining for each group.

Table 1

Identified Proteins

Table is sorted by direction and magnitude of fold change. The proteins at the top of the table (no shade) are the proteins, which were elevated in normal versus cancer. The proteins at the bottom of the table (shaded gray) are proteins, which were elevated in cancer versus normal. 31 out of 40 submitted proteins were identified. P (pro) is the p-value for the identified protein. Scores less than 11 were not significant. PC (peptide coverage) indicates the amount of the identified proteins that the peptides covered. Accession numbers are from UniProt and NCBI.

Spot #	Identification	Uniprot Accession	Fold Change	P value	Score	PC (%)	MW (kd)
464	Cornulin	Q9UBG3	5.28	1.00E-30	108.3	35	53502
455	Cornulin	Q9UBG3	4.33	2.66E-12	30.26	13	53502
2089	Keratin, type II cytoskeletal 4	P19013	4.62	1.17E-09	186.2	33	57250
543	Keratin, type II cytoskeletal 4	P19013	4.10	4.05E-10	154.2	31	57250
524	Keratin, type II cytoskeletal 4	P19013	3.94	8.54E-10	144.2	29	57250
449	Cornulin	Q9UBG3	3.58	1.00E-30	70.29	31	53502
563	Keratin, type II cytoskeletal 4	P19013	3.58	4.67E-12	126.2	26	57250
469	Cornulin	Q9UBG3	3.37	1.11E-15	80.3	31	53502
517	Keratin, type II cytoskeletal 4	P19013	2.91	4.52E-08	90.18	22	57250
546	Keratin, type II cytoskeletal 4	P19013	3.16	2.66E-11	132.2	28	57250
1979	Cystatin-B	P04080	3.07	1.73E-13	50.17	61	11133
528	Keratin, type II cytoskeletal 4	P19013	2.81	1.80E-09	138.2	25	57250
557	Keratin, type II cytoskeletal 4	P19013	3.25	3.88E-09	164.2	30	57250
725	Keratin, type I cytoskeletal 13	P13646	2.95	1.92E-05	124.1	25	45837
939	Leukocyte elastase inhibitor	P30740	2.68	5.77E-15	120.2	29	42715
457	Cornulin	Q9UBG3	3.09	5.55E-15	80.29	31	53502
553	Keratin, type II cytoskeletal 4	P19013	3.14	4.67E-09	146.2	31	57250
1977	Cystatin-B	P04080	2.73	3.83E-13	26.17	7	11133
269	Serum Albumin	P02768	2.64	9.46E-10	130.2	18	65993
1110	Annexin A1	P04083	2.53	3.61E-11	126.2	39	38690
2099	Annexin A1	P04083	2.45	8.81E-12	120.2	40	38690
663	Keratin, type II cytoskeletal 8	P05787	2.51	1.31E-09	28.15	15	30759
361	Guanylate-binding protein 6	Q6ZN66	2.62	1.99E-13	70.14	12	72399
719	Cornulin	Q9UBG3	2.36	1.67E-14	80.29	31	53502
1148	Annexin A1	P04083	2.34	1.71E-10	96.19	35	38690

Spot #	Identification	Uniprot Accession	Fold Change	P value	Score	PC (%)	MW (kd)
357	Guanylate-binding protein 6	Q6ZN66	2.33	0.0002	30.12	8	72399
272	Protein-glutamine gamma-glutamyltransferase E	Q08188	2.26	1.81E-06	64.13	11	76584
1109	Annexin A1	P04083	2.223	1.72E-09	108.2	35	38690
564	Keratin, type II cytoskeletal 4	P19013	2.13	1.15E-07	166.2	21	62423
1114	Annexin A1	P04083	2.14	1.97E-09	100.2	35	38690
532	Heat shock 70kDa protein 5 (GRP78)	B0QZ61	2.01	2.31E-14	320.2	44	72289
285	Keratin, type II cytoskeletal 5	P13647	2.12	1.08E-08	112.2	16	62423
1705	Ferritin light chain	P02792	2.49	3.07E-13	36.19	19	20007
935	Macronuclear actin	P10922*	2.31	1.41E-12	42.14	17	41720
744	Keratin, type I cytoskeletal 17	Q04695	3.26	1.13E-12	136.2	30	48076
726	Glutathione synthetase	P48637	3.19	0.00017	38.12	14	52352
1674	Superoxide dismutase [Mn]	P04179	2.51	4.88E-08	10.12	7	24695
1188	Proliferating cell nuclear antigen	P12004	2.63	4.11E-07	40.18	20	28750
1677	Superoxide dismutase [Mn]	P04179	2.74	6.09E-11	14.15	11	24695
739	Keratin, type I cytoskeletal 17	Q04695	4.57	3.19E-11	174.2	44	48076