

# A proteomics approach to characterizing human submandibular gland cell lines by fluorescent two-dimensional differential in-gel electrophoresis

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Received August 29, 2005; Accepted October 3, 2005

**Abstract.** The human salivary glands have a variety of histologic features such as intercalated duct cells, myoepithelial cells and acinar cells. A neoplastic human salivary intercalated duct cell line (HSG) and its derivatives, HSG with a myoepithelial cell phenotype (HSG-AZA1) and HSG with an acinar cell phenotype (HSG-AZA3) induced by 5-aza-2'-dC treatment of HSG cells, have been reported. To identify characterization of intercalated duct cells, myoepithelial cells and acinar cells in the salivary gland, we selected HSG, HSG-AZA1 and HSG-AZA3 cell lines to perform two-dimensional electrophoresis analysis. We used a fluorescent two-dimensional differential in-gel electrophoresis (2-D-DIGE) for comparative proteomics, which improved the reproducibility and reliability of differential protein expression analysis between the samples. Furthermore, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) peptide mass fingerprinting (PMF) was used to identify the proteins. These methods were combined to approach the protein profiles associated with characterization between HSG, HSG-AZA1 and HSG-AZA3 cells. Using these strategies, we identified seven HSG associated proteins, such as actin- $\beta$ , hydrocephalus inducing protein, L-plastin, KIAA0657 protein, septin 6 isoform A, lamin A/C isoform 2 and superoxide dismutase 2, three HSG-AZA1 associated proteins such as ubiquitin carboxyl-terminal esterase L1, myosin light chain 2 and muscle creatine kinase, and two HSG-AZA3 associated proteins, microtubule-associated

protein 6 and Annexin A3. These results suggest that the proteins are associated with characterization of the salivary gland.

## Introduction

The salivary glands have diverse cell types. Terminal end pieces, associated with salivary secretions, are composed of acinar cells and myoepithelial cells, and their central lumina communicate directly with the intercalated duct (1,2).

The human salivary cell line, HSG, which is derived from the submandibular gland, has features of the intercalated duct. When HSG cells were treated with 5-aza-2'-dC, the cells underwent DNA hypomethylation. Therefore, HSG-AZA1 cells (myoepithelial cell phenotype) and the HSG-AZA3 cells (acinar cell phenotype) were isolated from the HSG cells. Myosin was the specific marker of HSG-AZA1 cells, and amylase of the HSG-AZA3 cells (3). Previous studies reported that HSG, HSG-AZA1, and HSG-AZA3 cells produced adenocarcinoma, myoepithelioma, and acinar cell carcinoma, respectively, in nude mice that had undergone transplantation with those cells (3-5) suggesting that cytodifferentiation therapy has the potential to treat salivary gland tumors.

Two-dimensional (2-D) gel electrophoresis is the principal step of proteomics and widely used in comparative studies of protein expression levels of certain samples. 2-D gel electrophoresis is a well-established technique for the simultaneous separation and display of hundreds to thousands of proteins (6). The method separates proteins into two dimensions according to their isoelectric point and molecular size. Traditionally, identification of significant differences between two or more proteomes by 2-D gel electrophoresis required the running and analysis of many gels. However, the reproducibility of comparing proteomes by this method is hindered by technical variations in sample preparation and gel-running conditions. Fluorescent 2-D differential in-gel electrophoresis (2-D-DIGE), described recently by several research groups

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**Key words:** HSG, HSG-AZA1, HSG-AZA3, submandibular gland, fluorescent two-dimensional differential in-gel electrophoresis

(7-11), allows multiplex analysis of three sample proteomes on the same gel. The protein extracts being compared are covalently labeled with different fluorescent CyDyes, such as Cy2, Cy3, and Cy5. Fluorescent imaging of the gels at the wavelengths specific for each CyDye generates images that can be overlaid directly by the DeCyder software to identify any differentially expressed proteins among the samples.

In the present study, to investigate specific markers of intercalated duct cells, myoepithelial cells, and acinar cells, we selected three cell lines (HSG, HSG-AZA1, and HSG-AZA3), and performed proteomic analysis using 2-D-DIGE and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

## Materials and methods

**Sample preparation.** The three cell types (HSG, HSG-AZA1 and HSG-AZA3) were transplanted into nude mice to produce tumor tissue (3). The expression of specific markers, such as carcinoembryonic antigen (CEA), myosin, and amylase, of these tumor tissues were confirmed as described previously (3,12). We then selected the tumor tissues to be used in this study. The tumor tissues were washed in cold phosphate-buffered saline (PBS). Samples were crushed by liquid nitrogen and resuspended in lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 10 mM Tris pH 8.0). Insoluble materials were removed by centrifugation at 13,000 rpm for 20 min at 10°C. The protein concentration was determined using a commercial Bradford reagent (Bio-Rad, Richmond, CA). The model proteins were dissolved in lysis buffer to provide stock solutions with final concentrations of 1 mg/ml that were used to prepare the experimental samples.

**Sample labeling.** The pooled standard sample was prepared by pooling protein from each of the samples before labeling. A 1-mM stock of each dye was diluted 1:5 with anhydrous dimethyl formamide just before the labeling reaction. Approximately 50 µg of each protein extract (in 4 µl) was mixed with 1 µl of CyDye Cy2, Cy3, or Cy5 (0.2 mM) (Amersham Biosciences, Buckinghamshire, UK), vortexed, and incubated on ice for 30 min in the dark as described by Skynner *et al* (11). The reaction was quenched by the addition of 10 mM lysine.

**2-D gel electrophoresis.** Before isoelectric focusing (IEF), pooled Cy2-labeled extracts were combined with the Cy3-labeled sample extracts and Cy5-labeled extracts according to the experimental design (Table I). The combined labeled samples were added to an equal volume of 2X the sample buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 2% v/v Pharmalytes pH 3-10), vortexed, and incubated on ice for another 10 min in the dark. In addition, the volume of the combined labeled samples was adjusted to 450 µl with a standard rehydration buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 1% v/v Pharmalytes pH 3-10). Immobiline DryStrips (pH 3-10 NL, 24 cm) (Amersham Biosciences) were rehydrated overnight.

IEF was carried out at 500 V for 500 V/h, 1,000 V for 1,000 V/h, and 8,000 V for 64,000 V/h at 20°C and a maximum current setting of 50 µA/strip in an IPGphor II IEF unit

Table I. Experimental design for 2-D-DIGE.

Gel	Cy2	Cy3	Cy5
1	Pooled standard	HSG	HSG-AZA1
2	Pooled standard	HSG-AZA1	HSG
3	Pooled standard	HSG	HSG-AZA3
4	Pooled standard	HSG-AZA1	HSG-AZA3
5	Pooled standard	HSG-AZA3	HSG

Approximately 50 mg of each extract and the pooled standard were labeled with the indicated CyDye and mixed. Electrophoresis analysis was then performed on 2-D gel as indicated.

(Amersham Biosciences). Before sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), each strip was equilibrated with 10 ml equilibration buffer A (6 M urea, 100 mM Tris-HCl pH 8.0, 30% v/v glycerol, 2% w/v SDS, 0.5% w/v DTT) for 15 min, followed by 10 ml equilibration buffer B (6 M urea, 100 mM Tris-HCl pH 8.0, 30% v/v glycerol, 2% w/v SDS, 4.5% w/v iodoacetamide) for another 15 min.

The strips then were loaded and run on 12% acrylamide isocratic Laemmli gels (13) using the Ettan DALT six apparatus (Amersham Biosciences). Gels were run at 2.5 W/gel for 30 min and then increased to 100 W after the proteins migrated into the resolving gel. The gel electrophoresis run was terminated once the bromophenol blue dye (Amersham Biosciences) migrated off the bottom of the gel.

**Image acquisition and analysis.** Labeled proteins were visualized using the Typhoon 9400 Imager (Amersham Biosciences). The Cy2 images were scanned using a 488-nm laser and a 520-nm emission filter, band pass (BP) 40. Cy3 images were scanned using a 532-nm laser and a 580-nm emission filter BP30. Cy5 images were scanned using a 633-nm laser and a 670-nm emission filter BP30.

On individual gels, the Cy2-labeled images (pooled standard) were compared with either the Cy3 or the Cy5 images from the individual HSG, HSG-AZA1, and HSG-AZA3 cell lines using the DeCyder-differential in-gel analysis software (Amersham Biosciences). The estimated number of spots for each co-detection procedure was set to 3,000. Gel-to-gel matching of the standard spot maps from each gel, followed by statistical analysis of protein abundance changes between samples, was then performed using the DeCyder-biological variation analysis software module (Amersham Biosciences). The statistical significance of each expression level was calculated using the Student's t-test on the logged ratios. This analysis is equivalent to doing a paired t-test and has increased power because it takes into account the pairing of samples within gels. Protein spot expression levels, which showed a statistically significant ( $P < 0.001$ ) increase or decrease on every gel used in the study, were accepted as being differentially expressed between the extracts under comparison. The 2-D-DIGE workflow is described in Fig. 1.

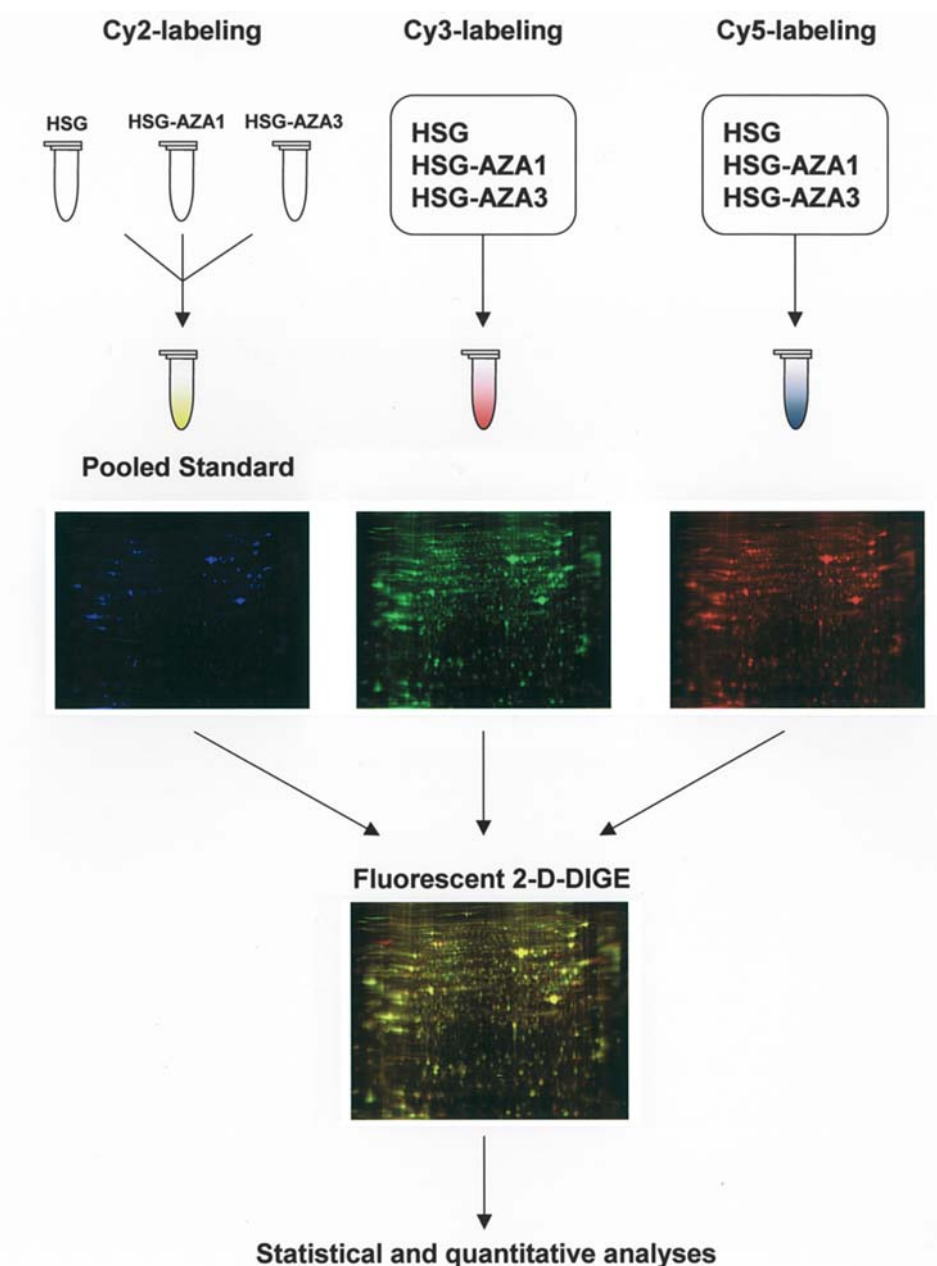


Figure 1. 2-D-DIGE workflow. The pooled standard was labeled with Cy2. Experimental proteins, HSG, HSG-AZA1, and HSG-AZA3, were labeled with Cy3 and Cy5, respectively, according to Table I. The pooled standard and experimental proteins were then mixed for electrophoresis on the same 2-D-DIGE gel. Statistical and quantitative analyses were performed using DeCyder software.

**MALDI-TOF peptide mass fingerprinting (PMF) analysis and database search.** Separate preparative gels electrophoresed with individual HSG, HSG-AZA1, and HSG-AZA3 cell lines were fixed and then stained with SYPRO Ruby (Molecular Probes, Inc., Eugene, OR). The resulting fluorescent images were matched to CyDye images using DeCyder software. A spot-picking list was generated from DeCyder and exported to an automated spot-handling system (Ettan Spot Picker, Amersham Bioscience). The gel spots were excised as circular plugs 2 mm in diameter and delivered into 96-well microtiter plates.

In-gel digestion was performed with an enzyme solution containing 50 mM  $\text{NH}_4\text{HCO}_3$ , 5 mM  $\text{CaCl}_2$ , and 12.5 ng/ $\mu\text{l}$  trypsin. Aliquots of the purified samples were spotted on

matrix crystals of  $\alpha$ -cyano-4-hydroxyl-cinnamic acid on a stainless-steel target and air dried. Mass determinations were performed on the AXIMA-CFR Mass Spectrometer (Shimadzu Co. Ltd., Kyoto, Japan). The proteins were identified by the PMF method, using Mascot Search on the Web (<http://www.matrixscience.com/cgi/index.pl?page=../home.html>) (Matrix Science, Ltd., London, UK).

**Immunohistochemistry.** To confirm the results of PMF analysis, immunohistochemistry was carried out. Briefly, the 4  $\mu\text{m}$  paraffin-embedded specimen of normal submandibular glands were deparaffinized and hydrated. The slides were treated with endogenous peroxidase in 0.3%  $\text{H}_2\text{O}_2$  for 30 min, and the sections were blocked for 2 h at room temperature

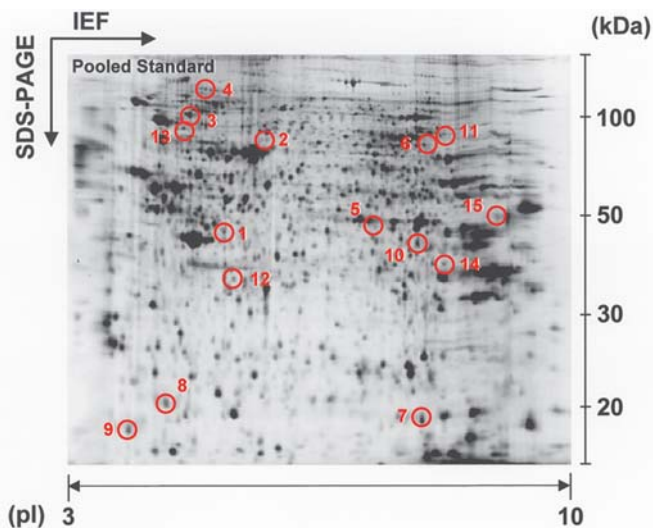


Figure 2. Typical 2-D-DIGE gel images of the pooled standard. The protein lysates were labeled with either Cy3 or Cy5 and subjected to 2-D-DIGE using pH 3-10 Immobiline DryStrips. HSG-associated proteins are represented by spots 1-7; HSG-AZA1-associated proteins are represented by spots 8-10; HSG-AZA3-associated proteins are represented by spots 11 and 12.

with 1.5% blocking serum (Santa Cruz Biotechnology, Santa Cruz, CA) in PBS before reacting with anti-human myosin light chain 2 (MLC2) polyclonal antibody (Santa Cruz Biotechnology) at a dilution of 1:100. They then were incubated with primary antibody and non-immune control antibody for 3 h at room temperature in a moist chamber. Thereafter, samples were incubated with biotinylated anti-goat

IgG and peroxidase-conjugated streptavidin. The peroxidase reaction was performed using a 3,3'-diaminobenzidine tetrahydrochloride (Dako Japan Inc., Kyoto, Japan). Finally, the slides were counterstained with hematoxylin, dehydrated in graded ethanol, cleaned in xylene, and mounted.

## Results

**Proteomic analysis of differentially expressed proteins among HSG, HSG-AZA1, and HSG-AZA3 cells by 2-D-DIGE.** To identify the different factors among the HSG, HSG-AZA1, and HSG-AZA3 cells, we used the 2-D-DIGE technology to monitor changes in the abundance of proteins. The samples were run in triplicate to obtain statistical significance for protein differences. An average of 2,500 spots was detected across all five gels by the DeCyder software (Fig. 2). We found seven protein spots in the HSG cells that were up-regulated >3-fold, and three protein spots that were down-regulated >3-fold compared with the HSG-AZA1 cells (Tables II and III) ( $P < 0.001$ ). We also identified seven spots as up-regulated and five spots as down-regulated proteins compared with the HSG-AZA3 cells (Tables II and III) ( $P < 0.001$ ). Of the up-regulated proteins in the HSG cells, we identified seven proteins that were expressed in common with the HSG-AZA1 and HSG-AZA3 cells. Moreover, 5 spots were differentially expressed >3-fold between the HSG-AZA1 and HSG-AZA3 cells (Table IV) ( $P < 0.001$ ).

**MALDI-TOF PMF analysis.** Based on the comparative analysis of protein expression patterns among the HSG, HSG-AZA1, and HSG-AZA3 cells, the 15 differentially

Table II. Up-regulated proteins in HSG compared with HSG-AZA1 or HSG-AZA3.

Accession no.	Protein	Theoretical pI/Mw (kDa)	t-test	Average ratio
Compared with HSG-AZA1				
Hs. 426930	ACTB <sup>a</sup>	5.6/40.5	0.00036	-12.68
Hs. 47115	HYDIN protein <sup>a</sup>	5.7/80.7	7.50e-07	-7.1
Hs. 381099	L-plastin <sup>a</sup>	5.2/70.8	2.30e-08	-5.89
Hs. 6654	KIAA0657 protein <sup>a</sup>	5.3/138.2	6.60e-06	-4.67
Hs. 90998	Septin 6 isoform A <sup>a</sup>	6.4/49.2	0.00011	-3.16
Hs. 436441	Lamin A/C isoform 2 <sup>a</sup>	6.4/65.2	2.70e-06	-3.12
Hs. 384944	SOD2 <sup>a</sup>	6.9/22.2	9.50e-06	-3.04
Compared with HSG-AZA3				
Hs. 426930	ACTB <sup>a</sup>	5.6/40.5	0.0008	-8.33
Hs. 381099	L-plastin <sup>a</sup>	5.2/70.8	3.00e-07	-5.37
Hs. 6654	KIAA0657 protein <sup>a</sup>	5.3/138.2	0.0001	-3.78
Hs. 436441	Lamin A/C isoform 2 <sup>a</sup>	6.4/65.2	6.60e-06	-3.61
Hs. 90998	Septin 6 isoform A <sup>a</sup>	6.4/49.2	0.00011	-3.32
Hs. 47115	HYDIN protein <sup>a</sup>	5.7/80.7	4.00e-07	-3.26
Hs. 384944	SOD2 <sup>a</sup>	6.9/22.2	9.60e-08	-3.02

<sup>a</sup>HSG-associated protein.



Table III. Down-regulated proteins in HSG compared with HSG-AZA1 or HSG-AZA3.

Accession no.	Protein	Theoretical pI/Mw (kDa)	t-test	Average ratio
Compared with HSG-AZA1				
Hs. 76118	UCHL1 <sup>a</sup>	5.3/25.2	0.00035	12.06
Hs. 50889	MLC2 <sup>a</sup>	4.9/19.1	0.00049	11.23
Hs. 334347	MCK <sup>a</sup>	6.8/43.3	6.90e-05	5.98
Compared with HSG-AZA3				
Hs. 239444	MAP6 <sup>b</sup>	9.3/77.1	8.00e-7	6.67
Hs. 442733	Annexin A3 <sup>b</sup>	5.6/36.5	0.0001	3.29
Hs. 1742	IQGAP1	5.6/108.0	6.40e-05	3.22
Hs. 286049	PSAT1	7.6/40.8	3.40e-05	3.18
Hs. 58324	ADAMTS5	9.2/58.8	0.00056	3.03

IQGAP1, IQ motif containing GTPase activating protein 1; PSAT1, phosphoserine aminotransferase isoform 1; ADAMTS5, a disintegrin-like and metalloprotease (repolyisin type) with thrombospondin type 1 motif, 5 (aggrecanase-2). <sup>a</sup>HSG-AZA1-associated protein. <sup>b</sup>HSG-AZA3-associated protein.

Table IV. Proteins differentially expressed in HSG-AZA1 and HSG-AZA3.

Accession no.	Protein	Theoretical pI/Mw (kDa)	t-test	Average ratio
Highly expressed in HSG-AZA1				
Hs. 76118	UCHL1 <sup>a</sup>	5.3/25.2	0.00026	11.57
Hs. 50889	MLC2 <sup>a</sup>	4.9/19.1	0.00032	8.5
Hs. 334347	MCK <sup>a</sup>	6.8/43.3	0.00024	6.55
Highly expressed in HSG-AZA3				
Hs. 239444	MAP6 <sup>b</sup>	9.3/77.1	1.20e-05	-7.19
Hs. 442733	Annexin A3 <sup>b</sup>	5.6/36.5	0.0001	-6.25

<sup>a</sup>HSG-AZA1-associated protein. <sup>b</sup>HSG-AZA3-associated protein.

expressed proteins were selected for further analysis. These spots were robotically excised from the gels for MALDI-TOF PMF analysis. All were specifically digested by trypsin and got peptide masses maps from the PMF analysis. Fifteen known proteins were extracted from the analysis. Fig. 3 shows representative results of PMF analysis.

**Protein identification by MALDI-TOF PMF analysis.** We identified seven HSG-associated proteins, i.e., actin-β (ACTB), hydrocephalus-inducing protein (HYDIN protein), L-plastin, KIAA0657 protein, septin 6 isoform A, lamin A/C isoform 2, and superoxide dismutase 2 (SOD2); three HSG-AZA1 associated proteins, i.e., ubiquitin carboxyl-terminal esterase L1 (UCHL1), myosin light chain 2 (MLC2), and muscle creatine kinase (MCK); and two HSG-AZA3-associated proteins, microtubule-associated protein 6 (MAP6) and Annexin A3 (Tables II-V). Of these proteins, the functions are known for eight and unknown for three. In addition, these proteins were functionally categorized, based on the known

or inferred biologic functions, into structural protein, structural protein activity, actin-binding protein, guanosine triphosphate-binding protein, SOD activity, hydase activity, and creatine kinase activity.

**3-D images and immunohistochemistry of identified protein.** Typical results from the DeCyder software for MLC2 protein spots are shown in Fig. 4A. In the 2-D and 3-D images of the HSG-AZA1 cells, we detected overexpression of this protein. We did not detect these protein spots in the HSG and HSG-AZA3 cells. In this study, the differences in protein expression were detected more easily by the DeCyder 3-D simulations of the spots.

To investigate the distribution of MLC2 protein expression *in vivo*, we performed immunohistochemical staining of the protein in the submandibular gland. Fig. 4B shows representative staining results of MLC2. Myoepithelial cells lining the acini periphery were positive for MLC2 in the normal submandibular gland.

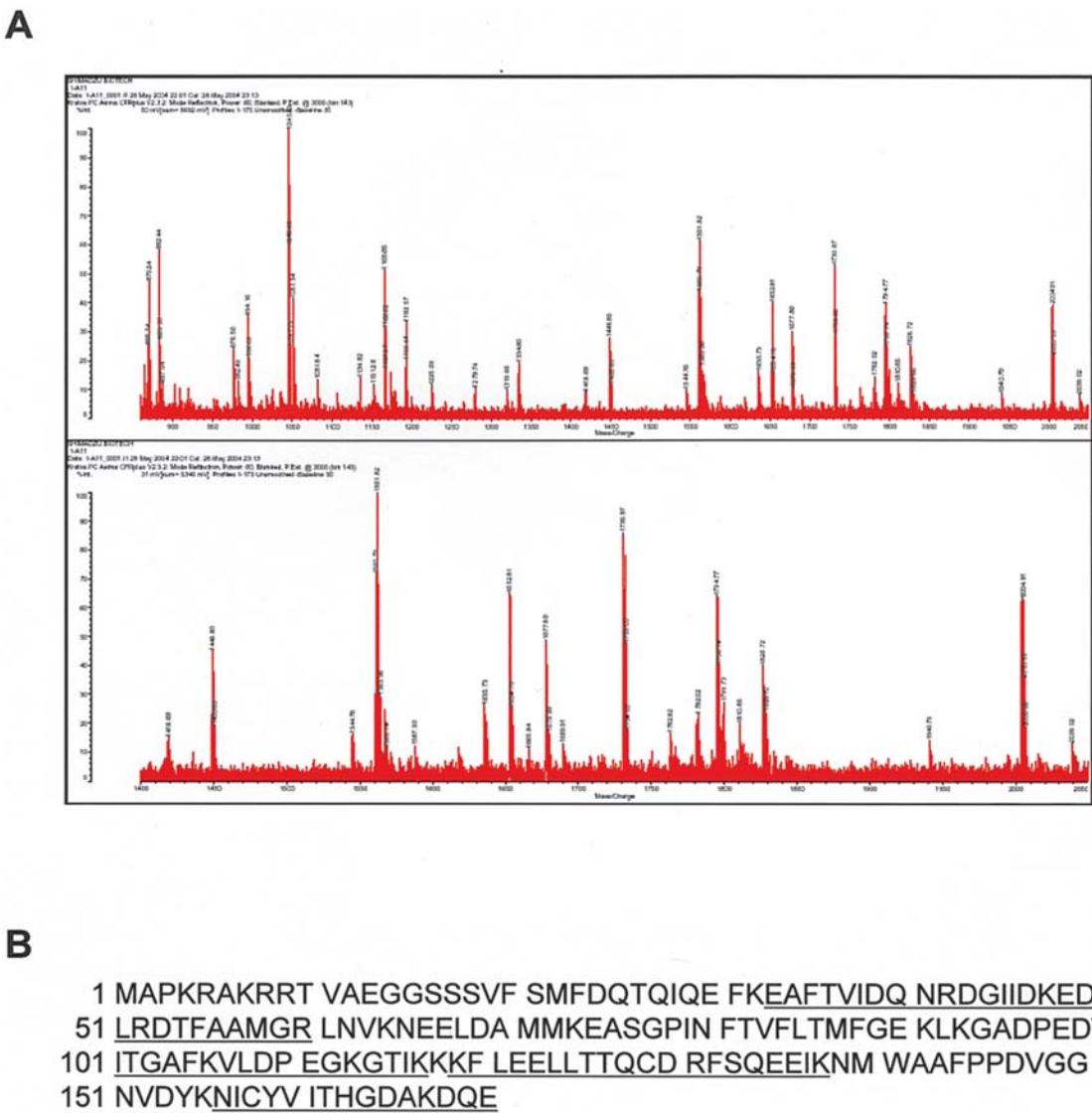


Table V. Associated proteins in HSG, HSG-AZA1, and HSG-AZA3 cell lines.

Spot no.	Accession no.	Protein
HSG-associated proteins		
1	Hs. 426930	ACTB
2	Hs. 47115	HYDIN protein
3	Hs. 381099	L-plastin
4	Hs. 6654	KIAA0657 protein
5	Hs. 90998	Septin 6 isoform A
6	Hs. 436441	Lamin A/C isoform 2
7	Hs. 384944	SOD2
HSG-AZA1-associated proteins		
8	Hs. 76118	UCHL1
9	Hs. 50889	MLC2
10	Hs. 334347	MCK
HSG-AZA3-associated proteins		
11	Hs. 239444	MAP6
12	Hs. 442733	Annexin A3

## Discussion

Because the functional molecules in the cells are protein, proteome analysis based on 2-D gel electrophoresis has several advantages over cDNA/oligonucleotide microarray systems for clinical use. First, the system measures primarily high-abundance proteins, which are ideal specific biomarkers because they can be measured and targeted easily. mRNA levels are not directly associated with the amounts of functional proteins (14). Second, using proteomics, we can analyze the post-translational modifications that are essential for protein formation and function. For many proteins, post-translational modifications are important for their functions, and they can be analyzed only by the methods used in proteomics (15,16).

The present study showed that 2-D-DIGE is a robust method for identifying statistically significant changes in protein abundance among three samples. The technique provides reliable identification of proteomic differences among the samples, because multiple samples can be analyzed on a single gel and an internal standard can be included for accurate matching across gels (17). The use of an internal standard helps to minimize false positives and negatives because it provides a separate control for each individual protein spot on all gels in the analysis (17). It is possible that the CyDyes may show some artifactual preferential labeling of some proteins, although none was detected in this study.

To investigate the proteins associated with cytodifferentiation of myoepithelial cells or acinar cells from parental cells (intercalated duct cells), we performed 2-D-DIGE

analysis. We identified seven HSG-associated proteins, three HSG-AZA1-associated proteins, and two HSG-AZA3-associated proteins.

Because HSG-AZA1 and HSG-AZA3 cells were affected by 5-aza-2'-dC, the forms differed from the HSG cells. Therefore, expressions of structural proteins recognized the differences among the HSG, HSG-AZA1, and HSG-AZA3 cells. Of the seven HSG-associated proteins, we found three structural proteins, such as ACTB, L-plastin, and lamin A/C. ACTB is a structural constituent of cytoskeleton (18); L-plastin is a member of a family of actin-binding proteins (19); lamin A/C is related to structural molecular activity 20. The expression of lamin A/C, a nuclear membrane protein, lacks several types of undifferentiated cells (20). Since HSG cells can differentiate into myoepithelial and acinar cells, it was thought that lamin A/C was overexpressed in HSG cells compared with HSG-AZA1 and HSG-AZA3 cells.

Of the HSG-AZA1-associated proteins, we found MLC2 and MCK. These proteins are related to muscle differentiation (21) and were reported to be specific markers of muscle (21). The marker of the HSG-AZA1 cells, myosin, was reported previously (3,22). In the present study, we identified overexpression of MLC2 and MCK in the HSG-AZA1 cells. Therefore, these results suggested that MLC2 and MCK are specific markers of both the muscle cells and the myoepithelial cells.

Of the HSG-AZA3-associated proteins, MAP6 is a calmodulin-binding and calmodulin-regulated protein that is involved in microtubule stabilization (23). The MAP family was reported to be associated with pancreas acinar cells (24). This observation together with our results suggests that MAP6 may play a role in the acinar cells of the salivary gland.

Several studies have shown that CEA, myosin, and amylase were expressed in salivary gland-derived cell lines such as HSG, HSG-AZA1, and HSG-AZA3 (3,12,25-27). On the other hand, we could not detect spots of the specific markers for HSG and HSG-AZA3 cell lines. The proteins showing up- or down-regulation at >3-fold were selected for PMF analysis. One possible explanation for this discrepancy is that expression level of specific markers was <3-fold up-regulation.

In conclusion, our 2-D-DIGE technology combined with MALDI-TOF PMF analysis identified candidate specific markers among the myoepithelial phenotype (HSG-AZA1), the acinar cell phenotype (HSG-AZA3), and their parental cells derived from the intercalated duct (HSG). Although salivary gland tumors consist of various types of cells, the proteins identified in this study could be useful biomarkers of salivary gland diseases.

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