A Cell Proteomic Approach for the Detection of Secretable Biomarkers of Invasiveness in Oral Squamous Cell Carcinoma

Alex M. Mlynarek, MD; Richard L. Balys, MD; Jie Su, MSc; Michael P. Hier, MD, FRCSC; Martin J. Black, MD, FRCSC; Moulay A. Alaoui-Jamali, PhD

**Objective:** To identify potential biomarkers of invasiveness in oral squamous cell carcinoma.

**Design:** A pilot proteomic study for the identification of secreted and cleaved proteins that can serve as potential biomarkers for head and neck carcinoma invasiveness.

**Subjects:** Two primary cell lines and their variants were established from 2 oral squamous cell carcinoma human tissue samples with distinct invasive phenotypes. The cell lines were confirmed to maintain the invasive capacity of the original cancer when implanted into the tongues of immunocompromised RAG-2/-c mice.

**Interventions:** Invasiveness was assessed by the capacity of cells to invade through a matrigel matrix using the Boyden chamber assay and correlated with the invasiveness seen clinically and histopathologically in patients. In parallel, cell lines were grown in serum-free conditioned medium, which was then used to identify secreted and/or cleaved proteins that emanate from cancer cells, using 2-dimensional gel electrophoresis and matrix-assisted laser desorption-ionization combined with tandem mass spectrometry.

**Results:** The invasion assays revealed a correlation between cell migration capacity through matrigel matrix and the aggressive phenotype seen in the clinical and histopathological assessments. More than 50 proteins were identified as being differentially secreted in media between the least and the more aggressive cell lines (P/H11021 0.05). These include proteins that regulate cell metabolism, cell structure, cell adhesion, and cell motility, as well as proteins with undefined function.

**Conclusions:** We report a sensitive and clinically relevant approach to screen for secreted biomarkers of oral squamous cell carcinoma invasiveness using proteomic technology. Both high- and low-abundant secreted proteins were identified and can represent potential biomarkers for oral cancer.

Arch Otolaryngol Head Neck Surg. 2007;133(9):910-918

**ORAL SQUAMOUS CELL CARCINOMA (OSCC), a variant of head and neck squamous cell carcinoma (HNSCC), is a relatively common and devastating disease, mainly among smokers. Presence of local, regional, and cervical lymph node metastasis remains the most significant prognostic factor of patient survival. Numerous genetic analyses of primary tumors, surgical margins, and saliva have been reported. However, no reliable genetic or protein marker has been proven to be useful for early diagnosis of HNSCC. This is likely owing to the complex biological process of HNSCC development and progression, both of which can be regulated, in addition to factors intrinsic to cancer cells, by a complex interaction between the tumor, its surrounding microenvironment, and the host. It is clear that a combination of genetic and proteomic profiling techniques in relevant models is needed to identify clinically useful biomarkers for HNSCC.**

Various biochemical and genomic approaches have been reported for studying the invasiveness of HNSCC. Most compared primary tumor cells with lymph node metastatic cells, and some have demonstrated gene differences between HNSCC cell lines with distinct invasion phenotype. In contrast to genomics, very few studies addressed protein changes that can predict invasiveness. As the DNA is transcribed into RNA and then translated into protein, a number of transcriptional, translational, and posttranslational modifications can occur, and therefore proteomic analysis holds great promise to accurately predict the function of marker proteins. One proteomic study published thus far on head and neck invasiveness used surface-enhanced laser desorption and ionization (SELDI) technology to com-
Table. Demographic Characteristics of the 2 Patients From Whom Tumor Tissue Samples Were Obtained.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patient 1</th>
<th>Patient 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>45</td>
<td>74</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>Male</td>
</tr>
<tr>
<td>Race</td>
<td>White</td>
<td>White</td>
</tr>
<tr>
<td>Cancer site</td>
<td>Oral tongue</td>
<td>Oral tongue</td>
</tr>
<tr>
<td>TNM stage</td>
<td>T4N2b</td>
<td>T3N1</td>
</tr>
<tr>
<td>Surgery</td>
<td>Total glossecotomy, laryngopharyngectomy, bilateral neck dissection</td>
<td>Hemiglossecotomy, bilateral neck dissection</td>
</tr>
<tr>
<td>Pathologic condition</td>
<td>Poorly differentiated SCC, with vascular, lymphatic, and perineural invasion</td>
<td>Moderately to poorly differentiated SCC, without vascular and perineural invasion</td>
</tr>
<tr>
<td>Previous treatments</td>
<td>Chemoradiotherapy</td>
<td>2 Surgical procedures</td>
</tr>
<tr>
<td>Medical history</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Medications</td>
<td>None</td>
<td>Oxycodone</td>
</tr>
<tr>
<td>Habits</td>
<td>Smoker, EtOH</td>
<td>Smoker, EtOH</td>
</tr>
<tr>
<td>Established cell line</td>
<td>OSCC-1</td>
<td>OSCC-2</td>
</tr>
</tbody>
</table>

Abbreviations: EtOH, ethyl alcohol; OSCC, oral squamous carcinoma cell; SCC, squamous cell carcinoma.

pare proteins in primary tumors with proteins in metastatic lymph nodes. This study reported that proteins, such as enolase-α, annexin-I, and annexin-II, were up-regulated in invasive cases and suggested that they may predict invasiveness of HNSCC. In the present study, we report a sensitive proteomic approach for identifying secreted proteins from tongue squamous cell carcinoma cells isolated from matched patients with distinct disease aggressiveness and discuss candidate markers with potential to predict invasiveness of oral squamous cell carcinoma.

METHODS

CELL LINE ESTABLISHMENT

Tumor tissue samples from 2 human male patients with advanced OSCC of the tongue (Table) were used to establish matched cell lines. Briefly, the tissue was dissected under a stereomicroscope, sliced into small pieces, and maintained in culture as explants until the outgrowth of cancer cells was observed. Selected cells were maintained in culture in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% heat-inactivated fetal bovine serum (Mediatech Inc, Herndon, Virginia) and 50 U/mL of penicillin-streptomycin. From each patient’s cancer tissue, we isolated fibroblast-free primary OSCC population, which we referred to as first-generation (primary) OSCC-1.1 and OSCC-2.1 cells for patient 1 and patient 2, respectively. These cells maintained the same pathologic, tumorigenic, and invasive potential as their parental tissue when transplanted into the tongues of at least 5 RAG-2/γ(c) mice per cell type and when compared with the original human tissue (data not shown).

Further serial implantation of OSCC-1.1 and OSCC-2.1 cells in mouse tongues was used to create the second generation of cancer cells (Figure 1). In this case, OSCC-1.1 or OSCC-2.1 primary cells were implanted into tongues of at least 10 RAG-2/γ(c) mice. When the mice showed evidence of tumor growth and lost 20% of their weight, the tumor was excised and tissue explants were cultured to generate matched cell lines as described in the previous paragraph. These second-generation cells are referred to as OSCC-1.2 and OSCC-2.2 for patient 1 and patient 2, respectively. These second-generation cells were further transplanted into the tongues of at least 6 RAG-2/γ(c) mice per cell type to confirm their invasiveness in vivo.

INVASIVENESS ANALYSIS

Invasiveness, or the ability of the cancer to spread beyond its introduction site, was assessed clinically and quantified in vitro using the Boyden chamber assay. Clinical assessment of aggressiveness was determined in at least 8 mice per cell type by comparing the survival time after tissue implantation and the presence of lung metastasis at the time the mice were killed with the survival time of the human subjects after diagnosis. Histopathological grading of the cancer tissue induced by each cell line was performed by an experienced head and neck pathologist.

The Boyden chamber assay was performed using 8-µm porous chambers coated with matrigel (Becton Dickinson, Bedford, Massachusetts) according to the manufacturer’s recommendations. Briefly, serum-starved cells were placed into the
upper compartment and were allowed to invade through the matrigel matrix for 48 hours in the presence of the chemotactic growth factor EGF (epidermal growth factor) placed in the lower chamber. Invading cells on the underneath of the matrigel matrix were fixed and stained. Filters were viewed under bright-field microscopy (original magnification × 40), and cell counting was performed for at least 3 fields for each sample. Each experiment was performed at least 3 times, each in triplicate.

**PROTEOMIC TECHNIQUES**

Cell lines with confirmed differences in invasion activity were grown to subconfluence (60%-70% density) in complete medium and then washed twice in phosphate-buffered solution and grown in serum-free conditioned medium for 48 hours. Medium was then collected, clarified by centrifugation for 10 minutes at 1000g, and then concentrated by adding equal volume of 20% trichloro-acetic acid in acetone. After 2 hours at −20°C, protein pellet was collected by centrifugation at 15,000g for 15 minutes at 4°C, washed with ice-cold acetone, and then solubilized in a 50-µL solution of 8M urea, and 4% (weight/volume) CAHPS (3-[3-chloramidopropyl]dimethylamino]-1-propanesulfonate), 30mM Tris (pH 8.0), and protein concentration was determined using the Bradford assay.

To identify quantitative differences in proteins secreted by cell lines of different invasive potentials, the 2-dimensional gel electrophoresis tagged with silver staining was performed. Consistent candidate proteins that reliably showed increased expression across samples were cut from the gels, in-gel trypsin digested, and analyzed by matrix-assisted laser desorption-ionization and tandem mass spectroscopy for identification. Proteins with the greatest quantitative differences were analyzed sequentially by searching the Mascot database of mass spectra.

**DATA ANALYSIS**

Statistical analysis was carried out using the 2-tailed t-test. *P* < .05 was considered statistically significant.

**RESULTS**

**INVASIVENESS RESULTS**

To investigate markers for oral cancer invasiveness, we adopted a tissue cell–based strategy wherein clinical specimens and matched cell lines were implanted orthotopically to immunocompromized mice and the phenotype was investigated in vivo and in vitro (Figure 1). The clinical assessment of invasiveness showed that mice implanted with the first-generation OSCC-1.1 and OSCC-2.1 cell lines survived 2 times longer than mice implanted with the second-generation cell lines OSCC-1.2 and OSCC-2.2 (mean ± SD, 1.3 ± 0.11 vs 0.5 ± 0.08 months for OSCC-1.1 and OSCC-1.2, respectively, and 4.9 ± 0.28 vs 2.1 ± 0.23 months for OSCC-2.1 and OSCC-2.2, respectively). These differences in survival time between OSCC-1 and OSCC-2 and between the first- and second-generation OSCC-1 and OSCC-2 cells were statistically significant (*P* ≤ .01).

**2-DIMENSIONAL GEL ELECTROPHORESIS RESULTS**

Two-dimensional gel electrophoresis revealed more than 50 protein spots in the media with both OSCC-1 and OSCC-2 cell lines. The majority of identified spots were distinct and easy to cut out. However, there were some large spots on the gel, most likely representing protein aggregates.

The spots, which were found to be differentially expressed (found in different quantities) by 5-fold or more, were excised, trypsin digested, and analyzed using QTRAP matrix-assisted laser desorption-ionization and tandem mass spectroscopy (Applied Biosystems, Foster City, California). Analysis of the gels from the OSCC-1 cell line revealed 32 protein spots as being differentially expressed between the more invasive and the less invasive phenotypes. Twenty-seven of these protein spots were found in higher abundance (up-regulated), and 5 in lower abundance (down-regulated) in the more aggressive phenotype. Analysis of the OSCC-2 cell line revealed 30 protein spots to be differentially expressed: 25 were up-regulated and 5 were down-regulated (Figure 3). Of importance, the serum-free medium alone did not contain any proteins when it underwent the same analysis (data not shown). Therefore, any proteins that were identified in this study originated from the cancerous cells.

**MASCOT RESULTS**

Sixty proteins were found to be differentially expressed between the different invasive variants of the OSCC-1 cell line and were found to be significant (*P* < .05). Of these proteins, 56 were up-regulated and 4 were down-regulated in the more aggressive phenotype (*P* < .05). An analysis of the OSCC-2 cell line revealed 69 differentially expressed proteins, with 53 up-regulated and 16
down-regulated in the variant with the more aggressive phenotype.

In the 2 cell lines, 27 of these proteins were found to be in common. Twenty-three proteins were up-regulated in the media with the more aggressive phenotypes. These included actin, annexin, cofilin, migration-inducing gene 10 protein (also called phosphoglycerate kinase 1), mitochondrial malate dehydrogenase, non-metastatic 23, p64, peptidylprolyl isomerase A, phosphoglycerate mutase 1, pigment epithelial-differentiating factor, RNA-binding protein regulatory subunit, transgelin 2, tropomyosin, and ubiquitin-conjugating enzyme. Of all the proteins expressed differentially in the media with both cell lines, the migration-inducing gene 10 protein, which corresponds to the phosphoglycerate kinase 1, was the most significant pro-
tein (ie, had the highest Mascot score). The mass spectrum of the migration-inducing gene 10 protein is represented in Figure 4. Only 1 protein, proapolipoprotein, was down-regulated in the medium with the more aggressive phenotypes of the 2 cell lines. Three proteins were found in both cell line media but at different expression levels: 14-3-3 protein Tau, glutathione S-transferase, and profilin. All the proteins found to be differentially expressed in the serum-free media from both cell lines are represented as Venn diagrams in Figure 5 (details of the complete proteomic data are available at: http://www.medicine.mcgill.ca/pharma/alaouijamalilab).

**COMMENT**

Attempts to establish oral cancer cell lines have been of limited success. One of the reasons for the low success rate of establishing cell lines from primary tumors is considered to be bacterial contamination when the tumor tissue is resected and the low proliferative activity of primary tumors compared with that of the metastatic tumors. In the present study, we have successfully created 2 cell lines of human tongue squamous cell carcinoma (OSCC-1 and OSCC-2) and their variants with distinct invasive phenotype.

The isolated cells induced aggressive (or less well-differentiated) phenotype when serially implanted into the tongues of immunocompromised RAG-2/γ(c) mice. This approach has been reported earlier for HNSCC. Cell lines derived from recurrent tumors or metastasis tend to be less differentiated and demonstrate a morphological divergence displaying fewer desmosomes and tonofilaments than cells from primary tumors. The in vivo reimplanted cell lines we isolated were more aggressive in the clinical assessment, as well as in the objective Boyden chamber invasion assay. We attribute this enhanced invasive potential to selection or enrichment of a cell subpopulation with more invasive capacity from the more heterogeneous primary oral cancer cells. Nevertheless, OSCC-2 variants were always less invasive than OSCC-1 variants, which correlate with the clinical observation on invasiveness.
Proteins secreted into conditioned medium are tumor specific and can represent potential biomarkers that may circulate in the bloodstream.18 Two recent literature reviews on the invasiveness of HNSCC have been published.19,20 They distinguished 4 categories of molecules associated with the process of invasion and metastasis: (1) proteases that mediate invasion of the extracellular matrix, (2) proteins responsible for growth factor signaling and angiogenesis, (3) adhesion proteins responsible for cell migration, and (4) tumor suppressor genes. Using the PANTHER (Protein Analysis Through Evolutionary Relationships) classification system (version 6.0; Applied Biosystems),21 we were able to classify proteins secreted by OSCC-1 and OSCC-2 cells as being involved in metabolic adaptation, extracellular matrix remodeling, cell cytoskeleton structure, cell adhesion, and angiogenesis.

Proteases or proteolytic enzymes, which primarily catalyze the hydrolytic breakdown of proteins into peptides or amino acids, were the most abundant in cancer cell-conditioned medium. For example, we identified several dehydrogenases to be significantly hypersecreted by cells with the more aggressive phenotype. These enzymes include glyceraldehyde-3-phosphate dehydrogenase,22 lactate dehydrogenase,23,24 malate dehydrogenase,25 and isocitrate dehydrogenase.26 Other enzymes we identified to be significantly up-regulated in the medium with the more aggressive phenotypes of OSCC cell lines have been previously reported to play major roles in oncogenesis. They include CuZn superoxide dismutase,27 fumarylacetoacetate,28 ubiquitin-conjugating enzyme,29 prolyl isomerase,30 triosephosphate isomerase,25 phosphoglycerate mutase,26 and transelin 2.31,32 The latter enzyme is an actin-binding protein that has been reported to act as a regulator of matrix metalloproteinase expression,33 and it is thought to be involved in carcinogenesis of esophageal squamous cell carcinoma.32 Pigment epithelial differentiating factor is a glycoprotein that can be classified into the angiogenesis group, since it is regulated by vascular endothelial growth factor and has been shown to have antiangiogenic properties in OSCC.33 Pigment epithelial differentiating factor is found to be significantly up-regulated in the medium from the more aggressive OSCC cell lines.

The other class of proteins we identified are adhesion molecules, which mediate cell-to-cell and cell-extracellular matrix adhesions and have a function in cell migration. In our study, we found several interesting proteins (all up-regulated), which can be included into this group of adhesion molecules including actin, tropomyosin, and translation elongation factor 1. Actin is a protein important for maintaining cell shape, adhesion, and motility. Tropomyosins are actin-binding proteins that have been reported to be deregulated in OSCC cell lines34 and have been proposed as therapeutic targets.35 Translation elongation factor 1 plays a major role in oncogenic transformation.36 Other important proteins involved in cell-to-cell adhesions and cell motility that we...
found to be up-regulated include annexin,37 cofilin,26 fatty acid–binding protein,26,38 and RNA-binding regulatory subunit.39 Interestingly, migration-inducing gene 10 protein, also called phosphoglycerate kinase 1, was the most significant protein (the highest Mascot score) identified. This protein has been reported to be secreted in serum from patients with lung adenocarcinoma and can predict patient survival.40 Phosphoglycerate kinase 1 is involved in glycolysis pathway, and according to the PANTHER classification system it is involved in other functions such as macrophage-mediated immunity, signal transduction, neurogenesis, and cell structure. To our knowledge, this is the first report of this protein being secreted by an OSCC cell line.

![Venn diagram](https://via.placeholder.com/150)

**Figure 5.** A Venn diagram representing the differentially expressed proteins in the oral squamous carcinoma cell (OSCC)-1 and OSCC-2 cell lines (P<.05).

Up-regulated and down-regulated signifies proteins found at higher and lower levels in the second generation of cells compared with the first generation of cells, respectively. The overlapping Venn diagram represents proteins found to be secreted in both OSCC-1 and OSCC-2 cells. GDP indicates guanine nucleotide diphosphate.
Finally, mutations in tumor suppressor genes, such as p53, have been found to be the most common genetic alterations involved in head and neck cancer. Interestingly we have identified 2 tumor suppressor gene proteins, p64 and nonmetastatic 23, as being upregulated in the media containing the more invasive cell line forms. Nonmetastatic 23 is described as a metastatic suppressor gene. Reduced nonmetastatic 23 expression has been correlated with increased cellular motility and is thought to play an important role in oral cancer tumorigenesis.

CONCLUSIONS

We report a reliable and sensitive in vitro–in vivo approach to screen for clinically relevant models of OSCC invasiveness using proteomic technology. Both high- and low-abundant candidate proteins were identified as being secreted by the cancer cells into the culture medium. Several candidate proteins were identified as being selectively associated with oral cancer invasiveness and were found to be significant (P < .05). Further validation is ongoing in our laboratory to determine the preclinical and clinical utility of these proteins as biomarkers for HNSCC. The combined use of proteomic technology and clinically relevant cancer models is a promising approach for the identification of low-molecular-weight protein markers for head and neck cancer.

Submitted for Publication: July 27, 2006; final revision received May 23, 2007; accepted May 25, 2007.

Correspondence: Moulay A. Alaoui-Jamali, PhD, Lady Davis Institute for Medical Research, Segal Comprehensive Cancer Center, 3755 Chemin Côte Ste-Catherine, Room E534, Montréal, QC H3T 1E2, Canada (moulay .alaoui-jamali@mcgill.ca).

Author Contributions: Dr Alaoui-Jamali (principal investigator and laboratory director) had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Mlynarek, Balys, Su, Hier, Black, and Alaoui-Jamali. Acquisition of data: Mlynarek, Balys, Su, Black, and Alaoui-Jamali. Analysis and interpretation of data: Mlynarek, Balys, and Alaoui-Jamali. Drafting of the manuscript: Mlynarek, Balys, Su, Hier, Black, and Alaoui-Jamali. Critical revision of the manuscript for important intellectual content: Mlynarek, Hier, Black, and Alaoui-Jamali. Obtained funding: Mlynarek, Hier, Black, and Alaoui-Jamali. Administrative, technical, and material support: Mlynarek, Balys, Su, Black, and Alaoui-Jamali. Study supervision: Mlynarek, Hier, Black, and Alaoui-Jamali.

Financial Disclosure: None reported.

Funding/Support: This work was supported by McGill University Head and Neck Cancer Fund, and in part by the Canadian Breast Cancer Research Alliance and Canadian Institutes for Health Research. Dr Alaoui-Jamali is an FRQS (Fonds de la recherche en santé du Québec) Scholar and a recipient of Dundi and Lyon Sachs Distinguished Scientist Award.

Additional Contributions: Susan James, PhD, Marcos Di-Falco, PhD, and Leonid Kriazhev, PhD (Genome Quebec Innovation Centre: Proteomics Platform) provided technical support and valuable advice on the proteomic analysis.

REFERENCES