Abstract. We previously showed that within primary tumors there exist subpopulations of cells expressing stem cell markers. Using immunofluorescence and western blotting, we examined the expression of stem cell markers tumor-rejection antigen 1-60 (TRA1-60) and octamer-binding transcription factor 3/4 (OCT3/4) to determine their relationship with cell invasiveness. Six human oral cancer cell lines were examined and a direct correlation was found between expression of these stem cell markers and invasion. Poor expression of E-cadherin and increased expression of N-cadherin was also found in TRA1-60- and OCT3/4-expressing cells. Phosphorylation of the major signaling molecule mitogen activated protein kinase (MAPK) was greatest in the TRA1-60- and OCT3/4-expressing cells. These results suggest that expression of specific stem cell markers in tumors may help guide a clinician’s choice of treatment.

Globally, oral cancer is the sixth most common cancer. Squamous cell carcinoma (SCC) accounts for more than 90% of all oral malignancies (1). The 5-year survival rate is only 50% and has not improved in over 50 years (1). At the time of diagnosis, 75% of patients present with regional metastasis (1). Stopping the invasive process is the key to controlling this disease.

Oral SCC consists of a heterogeneous population of cells ranging from an epithelial phenotype to a more fibroblast cell type. Within a single solid tumor, cells express a range of sensitivities to ionizing radiation and chemotherapy. Some cells may remain in a suspended period of dormancy until the appropriate signals induce a change in their behavior (2). Several transcription factors are known to be important regulators of pluripotency and self-renewal in embryonic stem cells. Octamer-binding transcription factor 3/4 (OCT3/4) is a transcription factor which induces rapid proliferation and tumorigenic properties of embryonic stem cells through activation of the UTF1 gene. Several studies suggest OCT3/4 sustains the self-renewal capacity of adult somatic stem cells (3). The tumor-rejection antigens (TRA) are embryonic stem cell markers that are highly expressed in embryonic stem cells but which vanish upon differentiation (4). We identify TRA1-60 and OCT3/4 as being markers of the invasive cell type. The identification of specific stem cell markers capable of predicting the invasive potential of a suspected lesion could be instrumental in the development of novel therapeutic approaches.

Materials and Methods

Cell culture. The SCC9 cell line (derived from a tongue lesion) was obtained from Dr. James Reinwald (Brigham and Woman’s Hospital, Harvard School of Medicine) and has been described elsewhere (5). SCC9β6 and SCC9β6D1 cells were generated in our laboratory through retroviral transduction of the SCC9 cells with the full-length β6 and β6D1 cDNA, which carries an 11 amino acid C-terminal deletion D1 (6, 7). Both β6 constructs were generous gifts of Dr. Dean Sheppard (UCSF) (8). The HSC series (HSC2, 3, and 4) was derived from a base of tongue lesion and was a generous gift of Dr. Randall Kramer (UCSF).

Growth conditions. Cells were routinely cultivated in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum at 37°C in 5% CO₂.

Reagents. Mouse monoclonal antibody to TRA1-60 (MAB 4360) was purchased from Millipore (Chemicon Division, Temecula, CA, USA). Rabbit monoclonal antibody to OCT3/4 (Cat.#: S0818) was purchased from Epitomics (Burlingame, CA, USA). Mouse monoclonal anti-E-cadherin and N-cadherin antibodies were from Zymed Laboratories (South San Francisco, CA, USA). Antibodies to β-actin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies to mitogen-activated protein kinase (MAPK) and pMAPK were from Cell Signaling Technology (Beverly, MA, USA).

Immunofluorescence microscopy. A total of 2x10⁵ cells/ml were plated onto uncoated glass coverslips (10 μg/ml) and cultured serum–free for 24 hours and then fixed with 2% paraformaldehyde and permeabilized with 0.1% Triton® X-100. The cells were incubated with monoclonal antibody for 1 hour and rinsed with phosphate- buffered saline (PBS) and then incubated with goat anti-
mouse or anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) for 30 minutes at room temperature. Coverslips were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). Cell reaction with the antibody was determined using a Nikon 80i immunofluorescence microscope.

**Invasion assay.** The upper surface of an 8 μm pore-size Transwell® filter was coated with a thin layer of a reconstituted basement membrane (Matrigel) (BD Biosciences, San Jose, CA, USA) for 1 h at 37˚C to polymerize. Cells (2x10^5) were plated onto the upper surface, while the lower compartment contained 300 μl of fibroblast-conditioned medium. The cultures were placed at 37˚C for 16h in the presence of 5% CO2. To terminate the assay, the filters were fixed, and the upper surface wiped clean and stained with crystal violet. The filters were air dried and the number of cells that crossed the membrane was counted. Five random fields for each filter were evaluated.

**Western blotting.** Cells were serum starved for 24 hours, detached from tissue culture plates and reseeded back onto the plate for an additional 24 h. The cells were then lysed in Nonidet P-40 lysis buffer [1.5% Nonidet P-40, 150 mM NaCl, 0.2% sodium dodecyl sulfate (SDS), 1 mM ethylenediaminetetraacetic acid (EDTA), 20 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Na3VO4, 50 mM NaF] and the protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA). The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to a nitrocellulose membrane (Micron Separation Inc., Westborough, MA, USA) using a semi-dry blotting apparatus (Bio-Rad, Hercules, CA, USA) (11). The membranes were developed using the ECL chemiluminescence kit (AmershamPiscataway NJ, USA) and bands were detected by X-ray film. β-actin was used as a loading control. The blots were assigned relative value units (rvu) using the NIH Image program (http://rsb.info.nih.gov/nih-image).

**Results**

**Invasive potential of oral SCC cell lines.** The SCC9 and HSC series were compared separately. The SCC9β6 cells were 4- and 2-fold more invasive than SCC9 and SCC9β6D1 cell lines, respectively (Figure 1A). The HSC3 cells were 2.5- and 5- times more invasive than HSC2 and HSC4 cells, respectively (Figure 1B). The invasive potential of the cells was SCC9β6>SCC9β6D1>SCC9 and HSC3>>HSC2>HSC4. These results established the invasive potential of the cells for the subsequent experiments.

**Detection of TRA1-60 and OCT3/4 using immunofluorescence microscopy.** The SCC9 and HSC series were evaluated by immunofluorescent microscopy for expression of TRA1-60 and OCT3/4 antibodies. Expression of TRA1-60 and OCT3/4 was greatest in the SCC9β6 cells while little to no expression was detected in the SCC9 or SCC9β6D1 cells (Figure 2). TRA1-60 and OCT3/4 were detected in the HSC3 and not in HSC2 and HSC4 cell lines (Figure 2). TRA1-60 and OCT3/4 expression were detectable in the most invasive cell lines (SCC9β6 and HSC3).

**Expression of E-cadherin and N-cadherin in oral SCC.** Using western blotting, the correlation between pluripotency and expression of E-cadherin (an epithelial marker) and N-cadherin (a mesenchymal marker) was evaluated. E-cadherin expression was 5-fold and 4-fold greater in SCC9 and SCC9β6D1 cells in comparison to SCC9β6 (Figure 3). Additionally, E-cadherin expression was 4 fold greater in HSC2 and HSC4 compared to HSC3 (Figure 3). Conversely, N-cadherin expression was greatest in SCC9β6 and HSC3 cell lines (Figure 3). SCC9β6 and HSC3, the most invasive cells lines that expressed the greatest amount of TRA1-60 and OCT3/4, also expressed the greatest amount of N-cadherin.

**Activation of MAPK as a marker of invasive potential.** MAPK is a serine/threonine-specific kinase which responds to external stimuli and regulates a variety of actions, such as gene expression and tumor cell invasion. When evaluated by western blot, all six cell lines expressed approximately equivalent levels of total MAPK. However, SCC9β6 and HSC3, the cells expressing the highest quantities of TRA1-60 and OCT3/4, also expressed the largest quantity of phosphorylated MAPK (Figure 4).

**Discussion**

Invasive oral SCC exhibits a significant degree of plasticity. This plasticity is a characteristic of disease progression and is reflected during epithelial – mesenchymal transition.
(EMT). During EMT, specific markers of the mesenchymal phenotype are up-regulated and the expression of some markers associated with the epithelial phenotype decreases (9). Identifying markers indicative of invasiveness could define a novel set of therapeutic targets.

The process of invasion is a complex version of cell migration in which the cell must remodel its surrounding extracellular matrix either by degradation, deposition, or mechanical rearrangement of the existing environment. Our data clearly suggest that within oral SCC, a subpopulation of cells that have different degrees of invasive potential exists. We defined SCCβ6 and HSC3 as being the most invasive oral SCC cell lines within their respective groups (SCC9 and HSC series). The SCCβ6 and HSC3 cell lines also have the greatest expression of TRA1-60 and OCT3/4 and exhibit markedly higher phosphorylation of MAPK.

Cancer cells are inherently unstable and this instability has made the use of cancer vaccines unsuccessful. A prime example of this instability concerns the intercellular adhesion molecule E-cadherin, whose targeted disruption during tumor

---

**Figure 2.** TRA1-60 and OCT3/4 expression. The SCC9 (Panel A: A and B), SCC9β6 (Panel A: C and D), SCC9β6D1 (Panel A: E and F), HSC2 (Panel B: A and B), HSC3 (Panel B: C and D) and HSC4 (Panel B: E and F) cell lines (2 x 10⁵ cells/ml) were grown on glass coverslips for 24h under serum free conditions at 37°C and then evaluated by immunofluorescence microscopy using anti-TRA1-60 and -OCT3/4 antibodies. Note the membranous expression of TRA1-60 and the nuclear localization of OCT3/4 in SCC9β6 (Panel A: C and D) and HSC3 cells (Panel B: C and D).

**Figure 3.** Expression of E-cadherin and N-cadherin in oral Squamous cell carcinoma. Lysate from all cell lines was evaluated for the expression of E-cadherin (A) and N-cadherin (B). Cell lysate was separated by SDS-PAGE and subjected to western blotting using monoclonal antibodies to E-cadherin (A) and N-cadherin (B). Antibodies to β-actin were used as a loading control. Note the expression of N-cadherin correlates with the expression of TRA1-60 and OCT3/4. Relative value units (rvu) were assigned using dosimetry.
progression is one of the most common alterations in cancer (10). Other changes include the expression of different integrin receptors. For example, our group showed that αvβ6 is highly expressed in invasive oral cancer cells and not in the poorly invasive variety (7). We previously reported that integrin αvβ6 ligand binding results in activation of the src-family kinase member Fyn and of focal adhesion kinase (FAK); which ultimately activates the MAPK pathway. The MAPK pathway responds to external stimuli and regulates a variety of actions, such as gene expression and tumor cell invasion. SCC9β6 and HSC3 cells are both β6-positive and SCC9β6D1 expresses a β6 cytoplasmic truncation. Our current results show that the activation of MAPK was significantly elevated in the highly invasive variety (7). We previously reported that integrin αvβ6-Fyn signaling promotes fibroblast-like differentiation of human mesenchymal stem cells. Lysate from all cell lines was evaluated for phosphorylation of MAPK. The lysate was separated by SDS-PAGE followed by western blotting with antibodies to the phosphorylated form of MAPK (pERK1/2). Total MAPK (ERK1/2) was used as a loading control. The cells lines (SCC9β6 and HSC3) with high expression of OCT3/4 and TRA1-60 have the greatest level of pMAPK. Relative value units (rvu) were assigned using dosimetry.

Acknowledgements

This work was supported by a grant from the University of California Cancer Research Coordinating Committee and additional funds provided by the Department of Orofacial Sciences University of California San Francisco.

References


Received February 2, 2012
Revised February 27, 2012
Accepted February 28, 2012