Published in final edited form as:

Otolaryngol Head Neck Surg. 2013 July ; 149(1): 97–104. doi:10.1177/0194599813482879.

***In vitro* evaluation of Sialyl Lewis X relationship with head and neck cancer stem cells**

Michael J. Czerwinski^{1,4}, Vincenzo Desiderio, MS^{1,3,4}, Omar Shkeir, BSE^{1,4}, Petros Papagerakis, DDS, PhD², Martian C. Lapadatescu, MD¹, John H. Owen, MS¹, Maria Athanassiou-Papaefthymiou, DSci^{1,2}, Li Zheng, DDS/PhD², Gianpaolo Papaccio, MD/PhD³, Mark E. Prince, MD¹, and Silvana Papagerakis, MD/PhD^{1,†}

¹Department of Otolaryngology, Head & Neck Surgery, University of Michigan (UM) Ann Arbor, MI

²Department of Otolaryngology, Pediatric Dentistry and Orthodontics, University of Michigan (UM) Ann Arbor, MI

³Department of Experimental Medicine, Second University, Naples, Italy

Abstract

Objective—To evaluate *in vitro* the potential links between sialyl Lewis X (sLeX) and cancer stem cells (CSC) in head and neck squamous cell carcinoma (HNSCC). HNSCC is an aggressive malignancy with high mortality mainly due to metastasis. CSC have emerged as important players in HNSCC metastasis. sLeX is a tetrasaccharide carbohydrate known to play a key-role in metastatic dissemination by promoting binding of the tumor cells to the endothelium.

Study Design—Experimental, *in vitro*.

Setting—Laboratory of Head and Neck Cancer Metastasis, University of Michigan.

Subjects and Methods—A panel of stage- and anatomic-site specific primary and metastatic HNSCC cell lines was assessed by flow cytometry to quantify sLeX relative expression levels. Serum-free conditioned media from the same HNSCC lines was collected over a time-course of 72 hours and assessed by Western-blot for secreted sLeX expression. Representative HNSCC cell lines were cultured as floating orospheres (condition that enhance CSC growth) or under normal adherent conditions and characterized by flow cytometry for CSC markers (CD44, aldehyde dehydrogenase [ALDH]) comparatively with sLeX expression.

Results—sLeX is predominantly expressed in carcinomas originating from the oral cavity. Secreted sLeX is also found to be high in oral carcinomas and increased over the analyzed time

[†]Corresponding Author: Silvana Papagerakis MD/PhD, Department of Otolaryngology – Head and Neck Surgery, Director – Head and Neck Cancer Metastasis Laboratory, 1150 W. Medical Center Drive, Room 5434 Med Sci I, Ann Arbor, MI 48109-5616, silvanap@umich.edu; Phone: (734) 615-7085; Fax: (734) 764-0014.

⁴These authors contributed equally to this work.

Request for Reprints: please contact corresponding author

This article was accepted for oral presentation at the 2012 AAO-HNSF Annual Meeting & OTO EXPO; September 9-12, 2012; Washington, DC.

Disclosures: Competing interests: None.

course. Floating orospheres were strongly positive for CD44 and ALDH confirming CSC enrichment of the orospheres. Tumor cells grown as orospheres are 95-100% positive for sLeX compared to 10-40% of adherent counterpart.

Conclusion—These studies provide the first evidence of sLeX relationship with CSC in HNSCC.

Keywords

head and neck squamous cell carcinomas; sLeX (sialyl Lewis X); CD44; stem cells; *in vitro* analysis

Introduction

The mortality associated with head and neck squamous cell carcinoma is still unacceptably high and it remains among the highest of the major solid cancers.¹ Despite improvements in the local control of HNSCC many patients continue to die from this disease due to the development of distant metastases.² The oral, head and neck cancer 5-year survival rate is a dismal 17% for patients with distant metastases, compared to over 80% for those with localized disease.² Novel prognostic tools and therapeutic approaches are greatly needed.

The identification of cancer stem cells (CSC), the highly tumorigenic subpopulation of cells in human cancers has created a new area of research with promising applications in the prognosis and therapeutics of cancer.³⁻¹² Characterizing CSC cancer subpopulations in HNSCC will lead to a better understanding of cancer recurrence, metastasis, resistance to treatment, and should pave the way for more effective therapies for HNSCC. Work by Prince *et al.* in 2007 was the first to identify CSC in HNSCC using the cellular marker CD44 and flow cytometry.³ These cells possessed the qualities of self-renewal, tumorigenesis, and the ability to recapitulate a heterogeneous tumor.³ We have previously designed both *in vitro* and *in vivo* models of HNSCC metastasis to study the behavior of this unique tumor cell subpopulation, the CSC, and our data has shown that CSC possesses a greater capacity for tumor growth, increased mobility, and invasive characteristics. Our *in vivo* experiments have confirmed the greater metastatic potential of CSC compared to non-CSC,¹³ suggesting that CSC may be responsible for the development of HNSCC metastasis. Clinically, CSC enrichment has been found enhanced in recurrent disease, treatment failure, and metastasis (reviewed in ¹⁴). However, it remains unclear how HNSCC CSC carry out the metastatic process.

Metastatic dissemination is directly related to the binding of cancer cells to endothelial cells (EC) through preferential expression of EC ligands.¹⁵⁻¹⁸ Our preliminary studies indicated that sLeX, a glycan which binds with strong affinity to the E-selectin, facilitates HNSCC binding to EC under conditions of flow.^{19,20} Expression of sLeX has been observed in several human malignancies, including HNSCC, and circulating levels of sLeX positive tumor cells were reported to be predictive for metastasis in GI carcinomas.^{15,18, 21-22} Therefore, we postulated that CSC preferentially express the endothelial adhesion molecules ligand, sLeX that may increase their ability to adhere to the lining of blood vessels and thereby to form metastasis. In the current study, we examine the level of expression of sLeX

in a panel of HNSCC cell lines established at the University of Michigan (UMSCC 10A, 10B, 11A, 11B, 22A, 22B, 12, 6, 103, 14A, 14B, 47;²³ See table 1 in publication by Carey²³); UMSCC 103 cell line was freshly isolated from a very aggressive HNSCC primary oral carcinoma resistant to treatment with poor clinical outcome (manuscript under review). We assessed the expression profile of sLeX both as membrane-bound and secreted soluble forms. Our goal was to investigate the potential links between sLeX and CSC in HNSCC and to determine its relationship with the CD44 and ALDH status. Our findings provide the first evidence of sLeX implications in CSC in head and neck carcinomas, and extend our previous studies on the molecular characterization of CSC in HNSCC.

Materials and Methods

Cell lines and Culture of Adherent Cells

All cell lines used were established at the University of Michigan under a protocol approved by the Institutional Review Board Office in accordance with the university's regulations;²³ their characteristics are illustrated in Table 1. They were cultured in Dulbecco's Modified Eagle Medium, 1% nonessential amino acids, 100× (Invitrogen), 1% Penicillin-Streptomycin, 100× (Invitrogen, Carlsbad, CA). The cell lines tested negative in periodic monitoring for mycoplasma. The cell lines were genotyped to rule out cross-contamination and to confirm their identity and their morphology was regularly examined. Human Normal Oral Keratinocytes (NOK) were used as control and were grown in basal keratinocyte growth media (KGM) supplemented with hydrocortisone, insulin (bovine), human epithelial growth factor (EGF) and bovine pituitary extract, in accordance with the manufacturer's instructions (Lonza, Walkersville, Maryland).

Supernatant Collection

We have previously found that the expression profile of various tumor-promoting cytokines secreted by HNSCC at varying time periods (4, 8, 24, 48 and 72 hours) are indicative of their metastatic potential.²⁴ Supernatants were collected in each of these time points as previously described²⁴ and analyzed for the sLeX expression by Western blot. Protease inhibitors (Complete Mini Kit with EDTA, Roche, San Francisco, California) were added to the supernatants, which were then processed in accordance with the manufacturer's instructions.

Orospheres Culture

Tumor cells were plated (60,000 cells/well) in six-well ultra-low attachment plates (Corning Inc., Corning, NY) in DMEM/F12 medium with supplements (N2, B7, human EGF and beta-Fibroblast Growth Factor, FGF; 20ng/ml; Sigma) as we have previously described.^{25,26}

Aldehyde dehydrogenase (ALDH) Assay

We used the non-immunologic enzymatic ALDEFLUOR™ Kit (STEMCELL Technologies Inc., Vancouver, Canada) as previously reported.²⁷

Fluorescence Activated Cell Sorting (FACS)

FACS was employed to characterize sLeX and CD44 protein expression levels as previously described.^{13,25,26} The anti-CD44 (mouse anti-human, clone G44-26, BD Pharmingen, San Diego, California) and anti-sLeX (mouse anti-human, a gift from Dr. Lloyd Stoolman, Pathology Department, University of Michigan) were detected by APC633 (for CD44) and Alexa Fluor488 (for sLeX) anti-mouse antibodies (InVitrogen). Sorting experiments were performed on BD Biosciences FACS Vantage Cell Sorter at the University of Michigan Flow Cytometry Core. Gates were set as follows: cells incubated without antibody were used to account for autofluorescence; cells incubated with the isotype control antibodies (APC mouse IgG2b k (for CD44) and FITC mouse IgM (for sLeX) were used to control for nonspecific binding. We used a human lymphoma line U937 (gift from Dr. Lloyd Stoolman, University of Michigan), which was 100% sLeX+ as positive control.²⁸ Viable cells were assessed by DAPI exclusion and gated for forward and orthogonal light scatter with filters appropriate for the fluorescent antibody tag.

Immunofluorescence (ICF) and Immunocytochemistry (ICH)

ICF and ICH were performed as we have previously described.²⁹⁻³¹ Cells were grown in slide chambers (BD), then fixed with 4% formalin for 30min on ice. Image FX™ enhancer (InVitrogen) was used prior to blocking. Freshly prepared paraffin embedded sections from the corresponding primary tumors (HN-111) used for the derivation of the UMSCC103 were stained for sLeX. Images were obtained on a Nikon scope equipped with an Olympus camera and processed on Olympus DSP Image Manager.

Western-blot

Proteins from HNSCC supernatants or total cell lysates were extracted in RIPA buffer, 1% SDS and loaded on 4-12% Tris Novex (Life Technologies, Grant Island, NY) and run as previously described.²⁹⁻³¹ Western-blot was carried out with the same sLeX antibody (incubation in PBS-0.1% Tween with 5% milk, at 37°C for 1hr), then with the secondary (anti-mouse HRP) for 30min. Development was done with SuperSignal West Pico System (Thermo Scientific, Rockford, Illinois).

Statistical analysis

Values for repeats have been averaged and standard deviations have been calculated. We have used two-tailed T-Test to compute the exact probability (*P*) values; <.05 were considered significant.

Results

sLeX expression in HNSCC grown as adherent cells

Our FACS analysis (Figure 1) indicates that among the various head and neck sites, oral-derived HNSCC expressed highest sLeX, also showing an increasing tendency in cases that developed metastasis (UMSCC-103) or recurrences (UMSCC-14B). In contrast, NOK do not express sLeX (not shown). Figure 1 indicates the percentage of the cell population with sLeX expression as detected by flow cytometry. Secreted sLeX expression is also higher in

oral carcinomas and it increases over the analyzed 72-hour time course (Figure 2; Illustrated are UMSSC-103, -14A and -14B cell lines whose sLeX levels of expression were among the highest as detected by flow cytometry). Overall, these data reveals that sLeX expression is predominant in oral-derived HNSCC, with increasing tendency with metastasis and recurrence.

sLeX expression in HNSCC grown as orospheres

sLeX expression was also analyzed by FACS using HNSCC cell lines grown in standard adherent condition (“*adherent cells*”) and grown as floating spheres (“*orospheres*”) in ultralow attachment plates (a condition that favors CSC growth).^{25,26,32} Interestingly, HNSCC cell lines grown as orospheres were 93 to 100% positive for sLeX compared to 10-40% of the adherent counterparts, and their sLeX content increase with each passage (Figure 3). The differences between orospheres and adherent counterparts were statistically significant for all the cell lines analyzed (UMSSC-14A, -14B and 103: $p = .013$; $p = .001$; and $p = .002$, respectively); in contrast, the differences between -14B and -14A were not. After cell sorting, both sLeX+ and sLeX- cells were able to form orospheres (Figure 4). However, sLeX+ cells formed larger and more defined spheres. Of particular interest were the orospheres derived from sLeX- cells, which become 100% sLeX+ after 48 to 72 hours in culture, suggesting sLeX expression is a key characteristic of HNSCC CSC.

sLeX relationship with CD44 and ALDH, the traditional stem cells markers in HNSCC^{3,13}

We previously reported the CD44 expression levels as detected by flow cytometry in the UMSSC-6, -10A, -10B, -12, -14A, -14B, -47 and -103 (see Figure 2 in Davis et al¹³). The 10 to 15% of cells with the highest CD44 expression were collected as CD44^{high}, while the 10 to 15% with the lowest or no expression as CD44^{low} cells.¹³ We extended this analysis here by quantifying the cell populations with CD44 expression in the remaining lines from our panel: UMSSC-11A (9.59%) and -11B (83.16%). The U937 (100% sLeX+) expressed 48.65% CD44^{high}. We then assessed by flow the levels of co-expression for sLeX and CD44 cells, sorting the double positive cells, and found that metastatic and recurrent HNSCC, irrespective of primary site, exhibit higher percentage of sLeX/CD44 positivity. Interestingly, the highest % of double positive cell population is expressed by oral carcinoma cell lines, which either developed distant metastasis (UMSSC-103, a stage IV OSCC, patient survival time 10 months; 15.4%) or have recurred (UMSSC-14B, 7.5%) post-treatment failure. They are followed by UMSSC-11B, a stage IV larynx carcinoma refractory to chemotherapy that develop recurrence (patient survival time 14 months): 7.27%; the paired UMSSC-10: derived from a patient with a stage III larynx carcinoma which developed lymphnodes metastasis and had a survival time of 22 months (-10A: 7.08%; -10B: 6.57%); UMSSC-47, an oral carcinoma which initially presented with lymphnode involvement (T3N1M0): 4.82%; UMSSC-22B, a larynx lymphnode metastasis (patient survival time 22 months): 2.55% and its corresponding primary, UMSSC-22A: 1.17%. Our immunocytochemistry analysis identifies high sLeX levels in cells freshly cultured from a highly aggressive oral cancer resistant to standard treatment (UMSSC-103 and its corresponding primary, HN-111) (Figure 5A-B), as well as the sLeX/CD44 co-expression (Figure 5C). Taken together, these findings suggest that sLeX may contribute to

identifying the metastatic CSC cell subpopulations in conjunction with CD44 in HNSCC cell lines.

Next, we extended our analysis to another molecule identified by our group as a marker for the head and neck CSC, the ALDH.²⁵ Given that the highest % of sLeX+ or double positive sLeX+/CD44+ cells is expressed by oral-derived HNSCC, we focus our subsequent analysis on oral carcinomas cell lines, particularly one that developed distant metastasis (UMSCC-103, 8% ALDH+), one recurrence (UMSCC-14B, 6.3% ALDH+), and one that presented with locoregional (lymph nodes) metastasis (UMSCC-47, 31% ALDH+). Of interest, the percentage of ALDH+ cells significantly increases in sLeX+ sorted cells than in sLeX- or unsorted cell subpopulations (Figure 5D). These preliminary findings show promise for the potential of sLeX in identifying CSC, particularly the ones from more aggressive HNSCC prone to metastasize or recur; additional studies are underway to further confirm these findings.

Discussion

Each year, over 400,000 new cases of HNSCC are diagnosed and more than 200,000 deaths are reported worldwide.¹ The main determinant of the HNSCC poor clinical outcome is the development of distant metastases.² Among the various sites of HNSCC, the oral-derived carcinomas remain the most aggressive and metastatic, with the poorest outcome.¹⁻² Cancer stem cells are defined as the small subpopulation of tumor cells that have distinctive biologic markers, exhibit self renewal and demonstrate the ability to generate new primary tumors and recreate the original tumor heterogeneity in immune deficient mice.³³⁻³⁷ The highly tumorigenic properties of the CSC suggest that they may be the critical population of cancer cells in the development of metastasis, just as they have been proven to be in primary tumor development and growth.²² Clinically, CSC enrichment is directly linked to tumor recurrence, treatment failure, and metastases in HNSCC (reviewed in ¹⁴). The role of CSC in the development of metastasis is not yet well understood.

Recently, Prince and colleagues provided the first evidence of CSC in HNSCC based on the overexpression of the cellular marker CD44 expression.³ We have since evaluated the metastatic potential of HNSCC CD44+ CSC using a panel of well characterized HNSCC cell lines generated at the University of Michigan, and determined that CD44+ expression by CSC correlates with the HNSCC *in vitro* invasive propensity and their ability to generate metastasis *in vivo*.¹³ Data from other types of carcinomas has indicated that the tumor cells' ability to metastasize may depend on their expression levels of sLeX, a glycan that promotes the attachment of the tumor cells to the endothelium, a critical initial step that leads to metastatic dissemination.^{15,38-40} Based on our previous work indicating that sLeX+ HNSCC cells can bind to endothelium under conditions of flow,¹⁹⁻²⁰ we hypothesized that sLeX expression by HNSCC facilitates cancer cell invasion and metastasis.

Emerging recent studies at the University of Michigan have identified CD44 and ALDH as a CSC markers in HNSCC.^{13,25,41,42} Our current data indicate that tumor cells grown as orospheres (a condition that favors CSC growth) were highly positive for sLeX compared to their adherent counterpart; even the sLeX- orospheres became 100% sLeX+ within few

days. Floating orospheres were strongly positive for CD44 and ALDH confirming sLeX preferential expression in CSC. Furthermore, our analysis of sLeX/CD44 co-expression has shown important expression overlap in our head and neck tumor cell lines, particularly in the metastatic ones. Of interest, sLeX+ tumor cells exhibit higher abilities in binding to the endothelium, in support of the sLeX involvement in critical steps of the metastatic process. Furthermore, our *in vitro* evaluation of the sLeX expression in HNSCC has indicated that among the various head and neck sites, sLeX expression was higher in oral-derived HNSCC, especially in those that developed metastasis or recurrences or have relapsed after failing standard treatment. These data are consistent with data generated in a parallel study in patient biopsies, where we found that sLeX expression was higher in oral and oropharynx carcinomas and associated with poor patient outcome (unpublished data). We are currently extending this analysis in our collections of tumor microarrays generated from primary and metastatic oral-derived carcinomas to further explore the clinical significance of sLeX as a reliable biomarker for metastatic potential in HNSCC. Our preliminary findings have indicated that sLeX expression correlates with lymph nodes metastasis (unpublished data); larger population-based studies are currently under investigation. Its potential predictive value for the clinical outcome and patient survival will be subsequently tested in our patient population.

The current study was designed to evaluate the expression profile of sLeX in relation with the CD44 status in CSC in HNSCC. Given the limitations of the present *in vitro* study, future studies focused on better understanding the clinical significance and the biological functions of sLeX in CSC as it relates to HNSCC are needed. In addition, further purification of the stemlike cell population in HNSCC is necessary to clarify what metastatic characteristics are indeed unique to these cells; such evidence would allow clinicians to exploit this particular set of attributes to target cancer cells that keep a tumor growing and allow it to spread. The long-term aim of this series of experiments is to better characterize CSC in HNSCC as a first step in designing novel modalities to prevent HNSCC metastasis and thus, to improve patient outcome.

Conclusion

Taken together, our findings provide the first evidence of sLeX relationship with the CD44/ALDH status in CSC from head and neck carcinomas, and extend our previous studies on the molecular characterization of CSC in HNSCC. The aim of these studies is to lay a foundation for the design of novel diagnostic and therapeutic approaches, resulting in improvement of HNSCC management and patient outcome.

Acknowledgments

We thank the Flow Cytometry Core and the Department of Pathology (Dr. Jonathan McHugh) at the University of Michigan for their help in acquiring this data.

Funding source: This work was made possible by funding from the University of Michigan Comprehensive Cancer Center (grant G008872, Drs. S. Papagerakis and Prince), the American Academy of Otolaryngology Percy Memorial Award (N011395, Drs. S. Papagerakis and Prince), the NCI/NIDCR P50 CA 97248 (University of Michigan Head and Neck Cancer Specialized Program of Excellence in Research, SPOR, Career Development Award – Dr. S. Papagerakis), and the University of Michigan Undergraduate Research Opportunity Program

(UROP). None of the funding sources had any role in the design, conduct, or interpretation of the above-mentioned experiments.

References

1. Jemal A, Bray F, Center MM, et al. Global cancer statistics. *CA Cancer J Clin.* 2011; 61:10–30.
2. Shah, J.; Johnson, NW.; Batsakis, JG. *Oral Cancer.* London, United Kingdom: Martin Dunitz Press; 2003.
3. Prince ME, Sivanandan R, Kaczorowski A, et al. Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. *Proc Nat Acad Sci USA.* 2007; 104:973–978. [PubMed: 17210912]
4. Al-Hajj M, Wicha MS, Benito-Hernandez A, et al. Prospective identification of tumorigenic breast cancer cells. *Proc Nat Acad Sci USA.* 2003; 100:3983–3988. [PubMed: 12629218]
5. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med.* 1997; 3:730–737. [PubMed: 9212098]
6. Chiba T, Kita K, Zheng YW, et al. Side population purified from hepatocellular carcinoma cells harbors cancer stem cell-like properties. *Hepatology.* 2006; 44:240–251.
7. Collins A, Berry PA, Hyde C, et al. Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res.* 2005; 65:10946–10951. [PubMed: 16322242]
8. Dalerba P, Dylla SJ, Park IK, et al. Phenotypic characterization of human colorectal cancer stem cells. *Proc Nat Acad Sci USA.* 2007; 104:10158–10163. [PubMed: 17548814]
9. Li C, Heidt DG, Dalerba P, et al. Identification of pancreatic cancer stem cells. *Cancer Res.* 2007; 67:1030–1037. [PubMed: 17283135]
10. O'Brien C, Pollett A, Gallinger S, et al. A human colon cancer cell capable of initiating tumor growth in immunodeficient mice. *Nature.* 2007; 445:106–110. [PubMed: 17122772]
11. Singh S, Clarke ID, Terasaki M, et al. Identification of a cancer stem cell in human brain tumors. *Cancer Res.* 2003; 63:5821–5828. [PubMed: 14522905]
12. Singh S, Hawkins C, Clarke ID, et al. Identification of human brain tumour initiating cells. *Nature.* 2004; 432:396–401. [PubMed: 15549107]
13. Davis SJ, Divi V, Owen JH, et al. Metastatic potential of cancer stem cells in head and neck squamous cell cancer. *Arch HN Surg.* 2010; 136:1260–1266.
14. Chinn SB, Owen AD, Peters RD, et al. The role of head and neck squamous cell carcinomas in tumorigenesis, metastasis, and treatment failure. *Front Cancer Endocrinol.* 2012; 90:1–6.
15. Papagerakis S, Teknos T, Islam M, et al. Functional evaluation of oral cancer invasion. *Oral Oncol.* 2007; 5:105–105.
16. Papagerakis S, Thornhill M. Therapeutic targets in oral cancer. *Toxicol Pathol.* 2006; 34(7):1009–1012.
17. Fujii Y, Yoshida M, Chien LJ, et al. Significance of carbohydrate antigen sialyl-Lewis X, sialyl-Lewis A, and possible unknown ligands to adhesion of human urothelial cancer cells to activated endothelium. *Urol Int.* 2000; 64(3):129–133. [PubMed: 10859542]
18. Dohi T, et al. Fucosyltransferase-producing sialyl Le(a) and sialyl Le(x) carbohydrate antigen in benign and malignant gastrointestinal mucosa. *Cancer.* 1994; 73:1552–1561. [PubMed: 7908856]
19. Irimura T, Nakamori S, Matsushita Y, et al. Colorectal cancer metastasis determined by carbohydrate-mediated cell adhesion: role of sialyl-LeX antigens. *Semin Cancer Biol.* 1993; 4:319–324. [PubMed: 7903054]
20. Nakamori S, Kameyama M, Imaoka S, et al. Increased expression of sialyl Lewis X antigen correlates with poor survival in patients with colorectal carcinoma: clinicopathological and immunohistochemical study. *Cancer Res.* 1993; 53:3632–3637. [PubMed: 8101764]
21. Nakagoe T, Sawai T, Tsuji T, et al. Difference in prognostic value between sialyl Lewis(a) and sialyl Lewis(x) antigen levels in the preoperative serum of gastric cancer patients. *J Clin Gastroenterol.* 2002; 34:408–415. [PubMed: 11907351]

22. Shah MH, Sainger RN, Telang SD, et al. E-cadherin truncation and sialyl Lewis-X overexpression in oral squamous cell carcinomas and oral precancerous conditions. *Neoplasma*. 2009; 56:40–47. [PubMed: 19152244]
23. Carey, TE. Head and neck cancers. In: Masters, JRW.; Palsson, B., editors. *Human Cell Culture*. Vol. II. Kluwer Academic Publishers; 1999. p. 185-255.
24. Shkeir O, Atahassiou-Papaefthymiou M, Lapadatescu MC, et al. In vitro cytokines release profile: Predictive for metastatic potential in head and neck squamous cell carcinomas. *Head Neck*. 2012 in press.
25. Tirino V, Desiderio V, Paino F, et al. Cancer stem cells in solid tumors: an overview and new approaches for their isolation and characterization. *FASEB J*. 2012 Sep 28.
26. Tirino V, Desiderio V, Paino F, et al. Methods for cancer stem cell detection and isolation. *Methods Mol Biol*. 2012; 879:513–529. [PubMed: 22610581]
27. Clay MR, Tabor M, Owen JH, et al. Single-marker identification of head and neck squamous cell carcinomas stem cells with aldehyde dehydrogenase. *Head Neck*. 2010; 32:1195–1201. [PubMed: 20073073]
28. Harris P, Ralph P. Human leukaemic models of myelomonocytic development: a review of the HL-60 and U937 cell lines. *Oncogenes*. 1999; 18:6496–6504.
29. Papagerakis S, Shabana AH, Depondt J, et al. Altered plakoglobin expression at mRNA and protein levels correlates with clinical outcome in patients with oropharynx squamous carcinomas. *Hum Pathol*. 2003; 45:565–572. [PubMed: 12827610]
30. Papagerakis S, Shabana AH, Depondt J, et al. Altered desmoplakin expression at the transcriptional and protein level provides prognostic information in human oropharyngeal cancer. *Hum Pathol*. 2009; 33:594–603.
31. Papagerakis P, Pannone G, Shabana AH, et al. Aberrant beta-catenin and LEF1 expression may predict the clinical outcome for patients with oropharyngeal cancer. *Int J Immunopathol Pharmacol*. 2012; 25:135–146.
32. Krishnamurthy S, Nor JE. Orospheres assay: a method for propagation of head and neck cancer stem cells. *Head Neck*. 2012 Jul 13.
33. Al-Hajj M, Clarke MF. Self-renewal and solid tumor stem cells. *Oncogene*. 2004; 23:7274–7282. [PubMed: 15378087]
34. Cho RW, Clarke MF. Recent advances in cancer stem cells. *Curr Opin Genet Dev*. 2008; 18:48–53. [PubMed: 18356041]
35. Lobo NA, Shimono Y, Qian D, et al. The biology of cancer stem cells. *Annu Rev Cell Dev Biol*. 2007; 23:675–699. [PubMed: 17645413]
36. Reya T, Morrison SJ, Clarke MF, et al. Stem cells, cancer, and cancer stem cells. *Nature*. 2001; 414:105–111. [PubMed: 11689955]
37. Wicha MS, Dontu G. Cancer stem cells: an old idea--a paradigm shift. *Cancer Res*. 2006; 66:1883–1890. [PubMed: 16488983]
38. Chambers AF, Naumov GN, Varghese HJ, et al. Critical steps in hematogenous metastasis: an overview. *Surg Oncol Clin N Am*. 2001; 10:243–255. [PubMed: 11382585]
39. Haier J, Nicolson GL. The role of tumor cell adhesion as an important factor in formation of distant colorectal metastasis. *Dis Colon Rectum*. 2001; 44:876–884. [PubMed: 11391152]
40. Pantel K, Brakenhoff RH. Dissecting the metastatic cascade. *Nat Rev Cancer*. 2004; 4:448–456. [PubMed: 15170447]
41. Krishnamurthy S, Dong Z, Vodopyanov D, et al. Endothelial cell-initiated signaling promotes the survival and self-renewal of cancer stem cells. *Cancer Res*. 2010; 70:9969–9978. [PubMed: 21098716]
42. Campos MS, Neiva KG, Meyers KA, et al. Endothelial derived factors inhibit anoikis of head and neck cancer stem cells. *Oral Oncol*. 2012; 48:26–32. [PubMed: 22014666]

Figure 1. Flow cytometry analysis of siayl Lewis X (sLeX) expression in head and neck squamous cell carcinomas (HNSCC)

The threshold for expression was defined by staining with an isotype control antibody. Our data indicate that sLeX is predominantly expressed in oral-derived carcinomas, showing an increasing tendency in cases that developed metastasis or recurrences.

Figure 3. Sialyl Lewis X (sLeX) expression in orospheres versus adherent oral carcinoma cells Orospheres express higher sLeX than adherent cells (blue; corresponding isotype control, red); its expression increases with each passage (1st: 93.1%; green; 2nd : 97.2%; orange).

Figure 4. Orospheres formation with sLeX- and sLeX+ sorted oral carcinoma cells
 Both sLeX+ and sLeX- sorted cells are able to form orospheres (20×). Orospheres derived from sLeX-cells become 100% sLeX+ after 48-72 hours of culture.

D.

Figure 5. Sialyl Lewis X (sLeX) and cancer stem cells (CSC) markers

sLeX in metastatic line UMSCC-103 (**A**, 40 $\times$) and corresponding primary (HN111, **B**, 20 $\times$). sLeX/CD44 co-localization illustrates that some of the sLeX+ cells also stained positive for CD44 with areas of co-expression in UMSCC-103 (immunocytofluorescence, nuclei are stained blue with DAPI, **C**, 60 $\times$. ALDH+ cells = subpopulation of sLeX+ cells (**D**).

Table 1

Characteristics of the head and neck squamous cell carcinoma cell lines.^a

Cell line	Site	Stage	Type
UMSCC-14A	Floor of mouth	I	Primary
UMSCC-14B	Floor of mouth	I	Recurrence
UMSCC-6	Base of tongue	II	Primary
UMSCC-47	Lateral tongue	III	Primary
UMSCC-103	Lateral tongue	IV	Primary
UMSCC-10A	True vocal cord	III	Primary
UMSCC-10B	Lymph node	III	Metastasis
UMSCC-11A	Epiglottis	IV	Primary
UMSCC-11B	Supraglottis	IV	Recurrence
UMSCC-22A	Hypopharynx	III	Primary
UMSCC-22B	Lymph node	III	Metastasis
UMSCC-12	Larynx	III	Primary

^aTable indicates the characteristics of the HNSCC cell lines originated from various head and neck anatomic sites including the stage of the cancer at the time of cell line derivation (Stage), whether the biopsy was of primary, recurrent or metastatic lesion (Type of lesion), what the primary site of occurrence had been (Primary Site).