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Temporal Characterization of Lymphatic Metastasis in an Orthotopic Mouse Model of Oral Cancer

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Abstract

Background—Overall mortality rate of head and neck squamous cell carcinoma (HNSCC) has not improved over the past 30 years; mostly due to high treatment failure rate among patients with regionally metastatic disease. To better understand the pathobiological processes leading to lymphatic metastasis development there is an urgent need for relevant animal models.

Methods—HNSCC cell lines were implanted into the tongues of athymic, nude mice. Histology, immunohistochemistry and ex vivo two-photon microscopy were used to evaluate tumor progress and spread.

Results—Orthotopic xenografts of different HNSCC cell lines produced distinct patterns of survival, tumor histology, disease progression rate, and lymph node metastasis development. Remarkably, all injected cell types reached the lymph nodes within 24 hours after injection, but not all developed metastasis.

Conclusions—This orthotopic xenograft model closely mimics several characteristics of human cancer and could be extremely valuable for translational studies focusing on lymphatic metastasis development and pathobiology.

Keywords

Orthotopic xenograft mouse model; head and neck squamous cell carcinoma; lymph node metastasis; physiologic characterization; oral cancer

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INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth most prevalent malignancy worldwide, with over 650,000 new cases a year, which result in over 350,000 deaths.⁽¹⁾ The incidence of HNSCC has been increasing over the past decades.^(2, 3) In 2012 HNSCC accounted for an estimated 52,610 new cases (3.4% of all new cancers), and 11,500 deaths in the United States.⁽⁴⁾ Despite significant recent advancement in therapy by surgery, radiation, and pharmacotherapy, costing an estimate of 3.2 billion dollars yearly in the US⁽⁵⁾, the overall mortality rate of HNSCC has not improved over the past 30 years and as a result, the 5-year patient survival remains among the lowest of major cancers.^(6, 7) The high treatment failure rate is mostly due to the fact that over 50% of HNSCC patients already have locoregional metastases at the time of presentation and many develop metastasis after initial diagnosis.⁽⁸⁻¹⁰⁾

The presence of cervical lymph node (CLN) metastases in HNSCC is the single most important predictor of poor outcome.^(11, 12) The primary route of spread in HNSCC is via the lymphatics to the CLNs and the 5-year survival of patients who present with CLN metastasis decreases by more than 50% compared to patients without regional metastasis - irrespective of therapy or the presence of distant metastases.^(8, 12, 13) Thus, to allow for more efficient therapies we need to better understand the pathobiological processes leading to lymphatic metastasis development in HNSCC as well as the relevant biology driving its association with mortality.

To address crucial, unanswered questions of HNSCC pathobiology there is an urgent need for relevant model systems, most importantly animal models that would allow us to study the complex biological processes leading to metastatic HNSCC in vivo. Although no current animal model is perfectly applicable to human cancer, recent technological advances produced an array of in vivo systems for HNSCC research.^(14, 15) Since the characterization of athymic nude mice^(16, 17), xenograft models have been frequently used for studies involving human tumor growth and spread as well as for developing and testing new antitumor drugs. Among all existing in vivo systems orthotopic xenograft models have been accepted to be the most clinically relevant for studying metastasis development and human tumor cell interactions with their microenvironment.^(14, 18) Although recently orthotopic models have been successfully used to address specific aspects of HNSCC pathobiology including metastasis development^(7, 19, 20), detailed characterization of these systems and how they recapitulate lymphatic metastasis in human HNSCC has not been thoroughly evaluated.

The objective of this study is to characterize an orthotopic nude mouse xenograft model of human HNSCC with emphasis on its ability to mimic the heterogeneity of human HNSCC with regard to host survival, primary tumor growth, histology, and, most importantly, metastatic potential with attention to cell physiologic mechanisms of progression.

MATERIALS AND METHODS

Cell Culture and Reagents

Head and neck cancer cell lines JHU-SCC-011, JHU-SCC-012, and JHU-SCC-019 were a gift from Dr. James Rocco (Boston, MA). OKF-TERT1 human, keratinocyte cells were a gift from Dr. Jim Rheinwald (Boston, MA). JHU-SCC cells were maintained in glutamine containing RPMI 1640 medium (Thermo Scientific HyClone, Logan, UT) supplemented with 10% fetal bovine serum (Invitrogen, Grand Island, NY) and 1X PenStrep solution (Invitrogen) at 37 °C in 5% CO₂. OKF-TERT1 cells were maintained in defined K-SFM medium (Invitrogen) at 37 °C in 5% CO₂.

Stable GFP-expressing Cell Lines

Green fluorescent protein (GFP)-expressing clones of the JHU-SCC-011, JHU-SCC-019, and OKF-TERT1 cell lines were generated using lentiviral particles containing MISSION TurboGFP Control Vector (Sigma, St. Louis, MO) following manufacturer's protocols. Briefly, each cell line was transduced at 50% confluence using ten-fold serial dilutions of the TurboGFP viral preparation in the presence of 8g/ml polybrene (Sigma). Stable, constitutively GFP-expressing clones were selected and propagated using puromycin (Sigma)-containing parent cell medium.

In Vitro Cell Growth Assays

Cell numbers were assessed using CellTiter 96 AQueousOne Solution Cell Proliferation Assay System (Promega, Madison, WI) following manufacturers protocol. Briefly, for each cell line, 5.0×10^2 cells/well were plated in 96-well plates in triplicates for each time point. Cells were incubated in RPMI 1640 under standard cell culture conditions and viable cell numbers at each time point were determined using colorimetric assay that generates an absorbance measurement for each well that is in linear correlation with the viable cell number. For each measurement the cells were incubated CellTiter 96 AQueousOne Reagent for 1 hour at 37 °C, in a humidified, 5% CO₂ atmosphere; then the absorbance of each well at 490nm was recorded using an ELx800 microplate reader (BioTek Instruments, Winooski, VT). Growth curves were plotted using Windows Excel 2003 (Microsoft, Redmont, WA).

Animals

Animal experiments were conducted in 4-6-week-old, athymic, BALB/c nu/nu mice (Charles River Laboratories, Wilmington, MA). Use of all animals was in accordance with the guidelines of the University of Texas Medical Branch Institutional Animal Care and Use Committee.

Orthotopic Mouse Model of Head and Neck Cancer

Preliminary tumor growth experiments established the appropriate cell inoculums of 1×10^5 - 1×10^6 range for all cancer cell lines used. For survival experiments all cell lines were used at the inoculum size of 1×10^6 cells suspended in 50µl phosphate buffered saline (PBS). For time course experiments 1×10^5 JHU-SCC-019 cells and 1×10^6 JHU-SCC-011, JHU-SCC-012, and OKF-TERT1 cells/mouse were used. Negative controls were inoculated with

50µl PBS containing no cells. After the mice were anesthetized, the cells were injected into the side of the tongue. After injection the mice were monitored daily until they were sacrificed based on weight loss (>20% of pre-injected weight), moribund appearance/behavior, or study timeline criteria. Time points used in the time course experiments were 7, 30, 60, and 90 days after injection. Tongues, cervical lymph nodes (CLNs) and lungs from all sacrificed animals were harvested and fixed in 4% paraformaldehyde. All CLNs were harvested from lateral to the submandibular salivary gland as one block from each side. These blocks contained 2-4 CLNs each and were sectioned as a single specimen. The total number of CLNs harvested from each mouse varied from 2 to 8.

Histology and Immunohistochemistry

After fixation, harvested tissues were paraffin-embedded and serially sectioned at 4 µm thickness. Staining was performed using hematoxylin and eosin (both Sigma, St. Louis, MO) following standard technique. Findings were confirmed by immunohistochemistry with cytokeratin 5 antibody (Epitomics, Burlingame, CA) using Vecstatin Elite ABC kit (Vector Laboratories, Burlingame, CA) following manufacturer's recommendations. Briefly, all sections subject to immunohistochemistry were deparaffinized and rehydrated. Rehydrated sections were incubated in citrate-based antigen unmasking solution (Vector Laboratories, Burlingame, CA) at 90 °C for 20 minutes. After antigen retrieval, endogenous peroxidase was blocked using 3% hydrogen peroxide for 10 minutes. For serum blocking, incubation with primary and secondary antibodies and with ABC reagent, Vecstatin Elite ABC kit (Vector) was used following manufacturers recommendations; with the addition of monoclonal rabbit anti-cytokeratin 5 primary antibody (Epitomics) used at 1:100 dilution at room temperature for 1 hour. Negative controls were incubated with rabbit IgG (Vector) instead of primary antibody. ImmPact DAB (Vector) was used as chromogen and hematoxylin (Sigma) as counterstain. All stained sections were evaluated using a Leica DM LB microscope. Microphotographs were taken with a mounted Pixera PVC 100C camera.

H&E staining was performed for each serial section corresponding to every 50 µm depth interval of the tissue. Immunohistochemistry for cytokeratin 5 was performed on adjacent sections when the H&E staining was inconclusive. Analysis of each tissue block was continued until the first tumor was found in the tissue or until the whole tissue was found negative for tumor. A tissue was considered positive for tumor if any of its sections contained tumor tissue, defined as 2 or more adjacent tumor cells determined by H&E characteristics and/or positive cytokeratin 5 staining. (Individual cells showing positive cytokeratin 5 staining were not scored as tumor.) Scoring results were confirmed by a pathologist.

Two-photon microscopy

Freshly harvested lymph nodes were examined for the presence of GFP-positive cells by two-photon excitation fluorescence microscopy (TPM). Imaging was performed on a custom-built two-photon microscope based on a modified Zeiss 410 confocal laser scanning microscope having the capabilities for two-photon microscopy, second harmonic generation microscopy, and confocal microscopy; described in detail by Sun et al.⁽²¹⁾ GFP emission was isolated using a 525 ±25 nm bandpass filter. TPM images were obtained at several (3-4)

locations and depths on the lymph node, with images encompassing the capsule, subcapsular sinus, superficial cortex. Two lymph nodes per time point (1, 7, and 14 days) for each injected cell line were examined. For each imaged region, the number of GFP-positive cells was enumerated, averaged, and compared between time points and cell lines.

Statistics

The nonparametric Kaplan-Meier method was used for survival analysis. Differences between the survival curves were tested using pairwise log-rank (Mantel-Cox) tests. Relations between mean survival times of xenograft groups and GFP-positive cell numbers at different time points were analyzed using ANOVA and unpaired, two-tailed t-tests. P values <.05 were considered significant unless otherwise stated. All analyses were done using GraphPad Prism, Version 5.04 software (Graphpad Software, Inc., San Diego, CA).

RESULTS

Xenografts of Different HNSCC Cell Lines Result in Distinct Survival Patterns

Orthotopic xenograft modeling of human HNSCC has been optimized over the last 4 years in our laboratory. For survival studies we have selected three human HNSCC cell lines (JHU-SCC-011, JHU-SCC-012, and JHU-SCC-019) with different anatomical sites of origin (larynx, floor of mouth, tongue, respectively). For controls we used vehicle alone (saline) and OKF4/TERT-1 cells, a human, hTERT-immortalized, oral keratinocyte cell line⁽²²⁾ that has been found to be non-tumorigenic in our model in preliminary studies. Athymic, nude mice were injected with 1×10^6 cells in 50 μ l saline, monitored daily, and sacrificed based on weight loss criteria.

Kaplan-Meier analysis of the survival data of a representative experiment (Figure 1A) yielded significant differences between treatment groups ($P < .0001$). Pairwise log-rank (Mantel-Cox) tests showed that the three different tumor cell line-injected groups had statistically distinct survival patterns ($P = .0019$ for JHU-SCC-011 vs. JHU-SCC-012 cells; $P = .0017$ for JHU-SCC-011 vs. JHU-SCC-019 cells; and $P = .0173$ for JHU-SCC-012 vs. JHU-SCC-019 cells.). Interestingly, mice injected with JHU-SCC-011 cells (011-injected mice) did not live significantly shorter than the controls, while JHU-SCC-012 cell-injected mice (012-injected mice) and JHU-SCC-019 cell-injected mice (019-injected mice) had significantly lower survival rates ($P < .01$). As expected, OKF4/TERT-1 cell-injected mice (OKT-injected mice) and normal saline injected control mice (NSC-injected mice) showed no significant difference in their survival rates.

Similarly, when we compared the mean survival times from all experiments, the pooled data showed distinct survival characteristics for the three different cancer cell-injected groups (Figure 1B). The mean survival times for 011-, 012-, and 019-injected mice were 155.2, 10.3, and 53.9 days, respectively with $P < .0001$ for 011- vs. 012-injected mice, $P = .0086$ for 011- vs. 019-injected mice, and $P = .0358$ for 012- vs. 019-injected mice. Most importantly, while 012- and 019-injected mice died significantly earlier than controls ($P < .0001$ and $P < .01$, respectively), 011-injected mice did not.

Primary Tumor Growth and Cervical Lymph Node Metastasis Development Depends on the Injected Cell Line

Temporal monitoring of these HNSCC cell lines showed characteristic tumor development patterns in our in vivo model (Table I), with 011-injected mice being primary tumor positive and lymph node metastasis negative, 012-injected mice being positive for both, and 019-injected mice showing a time dependent profile. In this time course experiment, some animals had to be sacrificed based on weight loss criteria outside the experimental time points. For data analysis they were grouped with the mice at the nearest time point.

For the studies described in Table I and Figures 2-4 the number of lymph nodes analyzed by histology and immunohistochemistry for each individual mouse depended on when the first tumor in each tissue block was found, as described in the Materials and Methods section. The total number of lymph nodes analyzed for 011-, 012-, 019-, OKT- and NSC-injected mice was 129, 22, 87, 27, and 23, respectively.

Histological analysis showed that all 011-injected mice had primary tumors in the tongue at 7, 30, 60, and 90 days after injection. Interestingly, at the 120-day time point we found the tongue of one animal tumor free. None of the 011-injected mice developed cervical lymph node metastasis.

All 012-injected mice had to be sacrificed within 12 days after injection, based on weight loss criteria. All of these animals had large tongue tumors and 83% of them already had lymph node metastases.

All 019-injected mice at the 7- and 30-day time points presented with tongue tumors. None of these mice had lymph node metastasis on day 7, but 40% had developed them by day 30. Six 019-injected mice needed to be sacrificed between the 30- and 60- day time points based on weight loss criteria; all of them had large tongue tumors and lymph node metastases as well. Four out of the 5 mice sacrificed 60 days after injection were found positive for both tongue and lymph node tumors, while 1 animal was negative for both. Surprisingly, only 1 out of the 4 remaining mice had tongue tumor 90 days after injection, but 2 of them were lymph node metastasis positive.

No control animals (OKT- and NSC-injected mice) developed tongue tumors or lymph node metastases (data not shown).

Different HNSCC Cell Line Xenografts Form Tumors of Different Histological Subtypes

Histological and immunohistochemical analysis of the tongues and cervical lymph nodes from the sacrificed animals revealed that the three HNSCC cell lines formed characteristic tumors of different HNSCC subtypes. Hematoxylin-eosin staining results were confirmed using immunohistochemical labeling for cytokeratin 5 that all cell types used in this study express in tissue culture (data not shown) and in vivo (Figures 2-4).

Tumors in the tongues of 011-injected mice showed the histopathology of well differentiated keratinizing squamous cell carcinoma with peripheral lymphovascular invasion (Figure 2). The lymph nodes from these animals were negative for metastatic cancer (data not shown).

Tongue tumors of 012-injected mice were moderately differentiated (Figure 3A). The tumor cells showed prominent nucleoli with brisk mitosis and apoptosis with focal tumoral necrosis. Lymphovascular invasion was also identified. In the lymph nodes of 012-injected mice subcapsular metastases were identified (Figure 3B). These metastases formed cohesive tumor clusters with pushing borders rather than infiltrative growth patterns. The tumor cells in the lymph nodes showed eosinophilic cytoplasm and active mitosis.

In contrast, tongue tumors of 019-injected mice were poorly differentiated with no obvious keratinization or differentiation (Figure 4A). These tumors showed lymphovascular invasion by individual cells and infiltrative growth pattern at the periphery. The tumor cells were highly mitotically active and discohesive with prominent nucleoli. Focally, tumor cells were spindle-shaped with some sarcomatoid features. In the lymph nodes of 019-injected mice metastatic cancer was identified with extensive subcapsular and intraparenchymal deposits (Figure 4B). These tumor cells showed markedly enlarged nuclei with prominent and multiple nucleoli. In the parenchyma of the lymph nodes, tumor cells were loosely infiltrating with several individual cancer cells.

No tumors were found in the tongues and lymph nodes of control animals (data not shown).

Injected Cells Reach the Cervical Lymph Nodes within 24 Hours after Injection Regardless of Cell Type

To determine how much time it takes for the tumor cells of different cell lines to reach the cervical lymph nodes, we used two-photon microscopy in time course experiments. We transfected JHU-SCC-011 and JHU-SCC-019 tumor cells as well as OKF4/TERT-1 control cells with green fluorescent protein (GFP) and selected for stable GFP-expressing (GFP+) clones for each (011G, 019G, and OKTG). Preliminary studies showed that the GFP+ clones were comparable to their parent cell lines in their in vitro growth patterns, although both of them showed a somewhat slower growth rate than their parent cell lines (Figure 5A-B). We injected 1×10^6 cells of each GFP+ cell line into the tongues of nude mice and analyzed their cervical lymph nodes by ex vivo, two-photon microscopy (Figure 5C-E), 1, 7, and 14 days after injection. For each cell line two lymph nodes (from different animals) per time point were analyzed (18 lymph nodes total).

Surprisingly, we found GFP+ cells in the lymph nodes of all injected animals at all time points analyzed, even as early as 24 hours after injection. When we compared the overall number of GFP+ cells found in the lymph nodes (subcapsular area and cortex together), there was no statistical difference among 011G-, 019G-, and OKTG-mice at the 24-hour time point. During the time course this number remained relatively steady for 011G-mice, while we observed an increase in 019G-mice and a decrease in OKTG-mice ($P < .05$).

DISCUSSION

Frequent treatment failures in HNSCC and the lack of improvement in survival are due to our limited understanding of the pathomechanism causing the poor outcome. Animal models could provide much needed insight, but to obtain relevant results it is important to understand the advantages and limitations of the available systems.⁽²³⁾ Currently available

systems include carcinogenic induction of tumors in the oral cavity by direct chemical exposure, transgenic induction of tumors, and orthotopic implantation of animal or human tumor cells.

Chemically induced HNSCC models most frequently use 4-nitroquinoline-1 oxide (4-NQO) in rodents to mimic the effects of tobacco carcinogens.⁽²⁴⁻²⁶⁾ The benefit of this model lies in the fact that it is an immunocompetent model and as such is well suited for the study of immune effects. The model also generally produces multiple lesions at different stages thus providing an opportunity to study all stages of tumorigenesis including dysplasia, invasive tumor at the primary site, and tumor at regional lymphatic sites. The limitations of the model include a long timeline (at least 48 weeks) for tumor development, unpredictable tumors in size and number, and, most importantly, the fact that lymphatic metastasis occurs at a low rate and generally require an even longer timeline to develop.⁽²⁴⁾

Genetically engineered mouse (GEM) models have been used to pinpoint the role of selected molecules in cancer development and progression by overexpressing/activating certain target genes (Ras, TGF β) and eliminating/inactivating others (Smad4).⁽²⁷⁻³⁰⁾ This approach produces oral tumors that metastasize to regional lymph nodes in a manner that mimics human disease histologically; however, this model is predicated on genetic alterations that differ from those characterized as essential in human oral carcinogenesis, thus, raising questions relating to the specific molecular pathways and their relevance to human disease. Additionally, GEM models also develop regional lymphatic metastases at low rates (12-35% at 80 weeks).^(15, 30)

The use of orthotopic implantation has several strong advantages. The timeline to tumor development can be manipulated by varying the number of cells implanted. This approach has been shown to faithfully recapitulate metastatic site distribution in a number of tumors of different type.⁽³¹⁾ In the case of oral cancer, one can use established mouse oral cavity tumor cell lines to reintroduce into a syngeneic mice.^(32, 33) This model allows for the study of disease in wild type immunocompetent mice and allows the ability to modify tumor cells as desired prior to implantation. Its limitation relates to the fact that molecular changes in mouse oral carcinogenesis have been found to be different from the ones driving disease in humans in general. Alternatively, human tumor cells can be introduced into either SCID mice or nude mice with the productive generation of primary tumors and regional lymphatic metastases.^(31, 34) This approach retains the histological characteristics of disease while maintaining the relevant tumor promoting genetic changes and allowing for the stable introduction of experimental changes. The limitation here relates to the use of immunodeficient mice. In the case of SCID mice, the immunodeficiency is profound, inactivating multiple arms of the immune system that include T-cells, B-cells, and NK cells.⁽³⁵⁾ Nude mice, by contrast, have a specific defect which only affects $\alpha\beta$ T cells. They still retain normal B cells, NK cells, macrophages, antigen presenting cells, and $\gamma\delta$ T cells, and show some capacity for immune function, albeit functionally compromised in light of a lacking helper T cell function.^(36, 37)

Based on the profound impact of CLN metastasis formation on the outcome of human HNSCC and the considerations discussed above, in this study we evaluated an orthotopic

nude mouse xenograft model of human HNSCC for its potential relevance to human cancer. Our main goal was to develop a model that allows us to study the temporal progress of HNSCC towards metastasis formation and the effects of this progress on survival.

We showed here that different HNSCC cell line xenografts result in distinct survival patterns as well as exhibit characteristically different ability for CLN metastasis formation in our model. Most importantly, we found a strong correlation between these two important features of cancer, closely mimicking the observed and to date unexplained correlation between cervical lymph node metastasis and survival in human HNSCC.^(3, 38, 39) 012-injected mice only survived for approximately 10 days on average and we found metastatic cancer in 83% of them at the time of death. In contrast, despite developing large tongue tumors, 011-injected mice had a mean survival time of about 150 days, not significantly less than controls. Interestingly, none of the 011-injected mice had CLN metastasis. 019-injected mice presented a third, distinct phenotype. They survived for approximately 50 days on average, and in time course experiments they showed a slower progression towards metastatic cancer than 012-injected mice. This progress seemed to be gradual from no metastasis at day 7 to 91% of the animals having metastatic cancer by day 60. More importantly, all 019-injected mice that had to be sacrificed based on weight loss criteria had CLN metastasis at the time of their death. Intriguingly, we observed a decline in the ratio of 019-injected mice with metastasis among the surviving animals on day 90. Moreover, 75% of these surviving animals were primary tumor free. These unexpected results suggest that this model could be used not only for tumor progression studies and preclinical investigations of new cancer therapeutics, but may contribute insight towards mechanisms of tumor clearance.

Further characterization revealed other advantages of our model, as well as some of its limitations. Histological analysis showed that the three HNSCC cell lines formed characteristic tumors of different subtypes *in vivo*, mimicking human HNSCC.^(40, 41) 011-injected mice presented with well differentiated squamous cell carcinoma (SCC), while 012- and 019-injected mice developed moderately and poorly differentiated SCC, respectively. Furthermore, metastatic tumors showed the same histological subtype as the corresponding primary tumor.

Finally, our attempts to monitor early stages of metastasis development revealed an unexpected feature of this model that, to our knowledge, has never been reported. Two-photon microscopy analysis of GFP-expressing clones showed that injected cells reached the CLNs within 24 hours after injection regardless of cell type. One explanation could be that the injection of the xenograft into the tongue produces enough hydrostatic pressure to push the injected cells into the naturally fenestrated lymphatic vessels driving them into the cervical lymph nodes within hours after injection.

These two-photon microscopy results seemingly contradict our findings with histological and immunohistochemical analysis, since we found GFP-positive cells in the CLNs of 011G-, and OKTG-injected animals 7 days after injection, but we found the CLNs of 011-, and OKT-injected mice negative for tumor at the same time point by histology. However, in some of those negative CLNs we did find several individual cells that stained positive for

cytokeratin-5, but we did not score them as tumors based on the fact that their morphology was not consistent with the characteristic tumor-cell morphology found in larger tumors in this study. We hypothesize, that these individual cells were indeed injected tumor cells, that found their way to the CLNs, but could not establish a growing tumor there. The number and localization of these cells are similar to those of the GFP-positive cells found in CLNs of 011G-, and OKTG-injected mice by two-photon analysis. According to this theory, after arriving to the CLNs, while 012- and 019-cells would be able to grow and develop into metastasis, 011-, and OKT-cells would not.

Clearly, further studies will be necessary to test this hypothesis, but once corroborated by additional experiments, it could also explain the shift within 14 days after injection, in the location of GFP-positive 019G cells from cortical to subcapsular areas of CLNs, found by two-photon microscopy. Initially, 019G cells would arrive to both locations, but preferential growth in the subcapsular microenvironment would result in a shift in cell numbers towards this location.

Since even the injected OKF-TERT1 cells - that never formed tumors in vivo – reached the CLNs, we concluded that this model may not be suitable to study the early stages of the multistep process that has been suggested to lead to metastasis formation.⁽⁴²⁻⁴⁴⁾ On the other hand, it could be exceptionally well-suited for investigating the late stages of the metastatic process. Intriguingly, not all tumor cell lines formed tumors in the CLNs. None of the 011-injected mice in survival or time course experiments presented with CLN tumors (Table I) despite the presence of individual JHU-SCC-011 cells in the CLNs (Figure 5). This result could be explained by the recently recognized importance of tumor microenvironment in metastasis formation and that different tumor cell types may or may not be able to successfully interact with the cellular and non-cellular factors within the lymph node.⁽⁴⁵⁻⁴⁷⁾ In this regard, the orthotopic xenograft model could be especially valuable for studies focusing on these poorly understood aspects of tumor metastasis development.

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Figure 1.

Orthotopic xenografts of different HNSCC cell lines produce distinct survival patterns in nude mouse model. 1×10^6 cells were implanted per animal. Mice were sacrificed once weight loss exceeded 20% body weight. (A) Kaplan-Meier survival curve for nude mice implanted with cancer cells. Data shown are from one representative experiment out of four independent experiments. (n=5 for 011-, 012-, and 019-cell injected mice; n=4 for OKT- and NSC-injected mice - 23 mice total) (B) Mean survival times of nude mice implanted with cancer cells. Pooled data from four independent experiments. (n=5, 12, 10, 4, and 4 for 011-,

012-, 019-, OKT- and NSC-injected mice, respectively – 35 mice total) 011, JHU-SCC-011 cells; 012, JHU-SCC-012 cells; 019, JHU-SCC-019 cells; OKT, OKF4/TERT-1 cells; NSC, normal saline control. *P<.05; **P<.01; ***P<.0001

Figure 2. JHU-SCC-011 cell xenografts form well differentiated keratinizing squamous cell carcinomas in the tongue. Representative histological sections of tongue xenografts in nude mice (n=24) were stained by hematoxylin/eosin (H&E) and immunohistochemistry for cytokeratin 5 confirming epithelial origin of labeled cells. Black square denotes the area visualized by higher magnification.

Figure 3.

JHU-SCC-012 cell xenografts produce moderately differentiated squamous cell carcinomas in the tongue and cervical lymph nodes. Representative histological sections of (A) tongue xenografts and (B) metastatic cancer cells in the cervical lymph nodes of nude mice (n=6) are shown. Tissue sections were stained by hematoxylin/eosin (H&E) and immunohistochemistry for cytokeratin 5 confirming epithelial origin of labeled cells. Black square denotes the area visualized by higher magnification.

Figure 4. JHU-SCC-019 cell xenografts produce poorly differentiated squamous cell carcinomas in the tongue and cervical lymph nodes. Representative histological sections of (A) tongue xenografts and (B) metastatic cancer cells in the cervical lymph nodes of nude mice (n=25) are shown. Tissue sections were stained by hematoxylin/eosin (H&E) and immunohistochemistry for cytokeratin 5 confirming epithelial origin of labeled cells. Black square denotes the area visualized by higher magnification.

Figure 5.

Orthotopically implanted cells reach the cervical lymph nodes within 24 hours. In vitro growth curves of GFP-expressing (A) 011G and (B) 019G cells compared to their parent cell lines. Absorbance measurements are in linear correlation with cell numbers. Error bars represent SD (n=3). Representative microscopy images of lymph nodes from mice after tongue injection with GFP-expressing (C) JHU-SCC-011 and (D) JHU-SCC-019 cells. Grayscale image at low magnification (10× objective, 1200 × 1200 μm field-of-view) of a lymph node demonstrating the distribution of GFP signal. Insets are specific regions-of-

interest imaged by two-photon microscopy using 40× objective, showing individual cells within the subcapsular and cortical space. (E) GFP-positive cell counts obtained from two-photon micrographs in regions-of-interest (ROI) in the lymph nodes at day 1, 7, and 14 post-injection. White and grey bars represent cells in the subcapsular and cortical area, respectively. For each cell line two animals per time point were analyzed (18 animals total). Error bars represent SD for ROIs (n = 5 for all time points). *P<.05, 011G/019G/OKTG, stably GFP-transfected JHU-SCC-011/JHU-SCC-019/ OKF4/TERT-1 cells, respectively. JHU-011, JHU-SCC-011 cells; JHU-019, JHU-SCC-019 cells.

Table 1

Primary Tumor Growth and Presence of Cervical Lymph Node Metastasis In Vivo

Days	No. of Mice	Tongue		Lymph Nodes
		Tumor (%)	Size* (mm)	Tumor (%)
JHU-011 cells				
7	4	100	1.4±0.2	0
30	5	100	1.2±0.2	0
60	5	100	1.2±0.3	0
90	5	100	2.4±0.9	0
120	5	80	1.4±0.8	0
JHU-012 cells				
4-12	6	100	5.0±1.2	83
JHU-019 cells				
7	5	100	1.0±0.2	0
30	5	100	2.0±0.6	40
43-60**	11	91	4.0±1.3	91
90	4	25	4.8±0.0	50

Abbreviations: No., number; %, percent of mice with tumor;

* *average tumor size ± standard deviation;*

** 6 mice were sacrificed between days 43-51 and 5 mice were sacrificed on day 60