Oral cavity tumors in younger patients show a poor prognosis and do not contain viral RNA


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SUMMARY

Background: Oral cavity and in particular oral tongue cancers occur with a rising incidence in younger patients often lacking the typical risk factors of tobacco use, alcohol use, and human papilloma virus (HPV) infection. Their prognosis when treated with chemoradiation has not been well studied and responsible risk factors remain elusive. A viral etiology (other than HPV) has been hypothesized.

Methods: First we analyzed outcomes from 748 head and neck cancer patients with locoregionally advanced stage tumors treated with curative-intent chemoradiation by anatomic site. Second, we analyzed seven oral tongue (OT) tumors from young, non-smokers/non-drinkers for the presence of viral mRNA using short-read massively-parallel sequencing (RNA-Seq) in combination with a newly-developed digital subtraction method followed by viral screening and discovery algorithms. For positive controls we used an HPV16-positive HNC cell line, a cervical cancer, and an EBV-LMP2A transgene lymphoma.

Results: Younger patients with oral cavity tumors had worse outcomes compared to non-oral cavity patients. Surprisingly none of the seven oral tongue cancers showed significant presence of viral transcripts. In positive controls the expected viral material was identified.

Conclusion: Oral cavity tumors in younger patients have a poor prognosis and do not appear to be caused by a transcriptionally active oncovirus.

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Introduction

Head and Neck Cancer (HNC) is the 6th most common cancer worldwide.1,2 Tobacco and alcohol use are the primary etiologic factors of HNC and account for the majority of head and neck tumors.2,3 Recently oropharyngeal cancer has been linked to infection with human papilloma virus (HPV). The incidence of these virus-associated cancers has been rising rapidly, suggesting increased HPV exposure and infection rates in the past two decades.3–5 HPV-associated HNC typically occur in non-smokers and non-drinkers, and are limited to the oropharynx.2,4,6

Unlike oropharyngeal cancers, tumors of the oral cavity, and in particular oral tongue tumors (which comprise a major subgroup of oral cavity tumors), rarely harbor HPV.7–10 even though p16 expression is frequently present.7,10 In addition, a significant proportion of oral tongue tumors occur in younger patients who lack classic HNC risk factors such as tobacco and/or alcohol use.11 These tumors tend to be clinically and histologically distinct from other head and neck cancers,12,13 and are reported to have an aggressive clinical phenotype often requiring intensive multimodality treatments.9,14 Nevertheless the clinical outcome of patients with oral
cavity tumors, in particular those occurring in younger patients, compared to other head and neck tumors is not well studied. An increasing overall incidence of oral tongue/oral cavity cancers, paralleling the trend for oropharyngeal tumors, has been noted. Furthermore oral cavity squamous cell carcinomas (OCSCC) frequently occur in transplant recipients. Due to these reasons it has been hypothesized that such tumors, located at a site of primary host-environment interaction with contact to foreign material (including bodily fluids) may be associated with an infectious agent such as a virus. Human papilloma virus-associated cancers are uncommon in the oral cavity and occur at a rate of 5.9%. Instead infectious agents such as Herpesviruses have been postulated as contributaries to oral tongue tumors, but this association remains unproven.

All known oncoviroses are associated with expression of viral proteins. The detection of viral transcripts or proteins is a primary mode of detection of oncoviroses in tumors and may also be used to distinguish an active viral influence on tumor maintenance from incidental viral infection. It is therefore essential to evaluate mRNA transcripts rather than just viral DNA.

Until recently, screening tissues for potential viruses required candidate approaches. With the advent of massively parallel sequencing technology, unbiased discovery of new, unknown viruses has become possible. To handle the large amounts of data, cloud computing-based methods have become available, but require large and expensive resources.

The primary goal of our study was to investigate differences in clinical outcome between tumors of the oral cavity compared to tumors in other sites of the head and neck region, and to discover a potential viral etiology of oral tongue tumors. To achieve this goal we screened seven oral tongue tumors for known and unknown viruses using short-read massively parallel sequencing. In addition, we also wanted to establish the bioinformatic tools necessary to detect unknown viral transcripts. By using tools readily available in a standard laboratory we obviate the need for cloud computing facilities or expensive multiprocessor workstations.

In this report we demonstrated significantly decreased survival rates for young patients (<45 years) with oral cavity tumors compared to patients with tumors at other locations in a cohort of 748 HNC patients. To our knowledge, this is the largest cohort of HNC patients on which a study like this has been done. In addition infectious agents such as Herpesviruses have been postulated as contributaries to oral tongue tumors, but this association remains unproven.

Survival analysis

Seven hundred and forty eight patients with advanced head and neck squamous cell carcinoma (HNSCC) were identified from the University of Chicago Departments of Otolaryngology, Medical Oncology, and Radiation Oncology protocols spanning from 1990 to 2007. The presence of HNSCC was confirmed in all cases by review of histology records. Tumor stages were comparable among subgroups (Supplementary Fig. 1). Disease free and overall survival times were calculated as the difference between the date on which the patient joined the study and the last date of follow-up or date of death. Survival times were estimated using the method of Kaplan–Meier, and groups were compared using the log-rank test stratified by study protocol and treatment arm. The influence of covariates (anatomical location, tumor stage, tobacco/alcohol use, age) on survival was analyzed using Cox regression analyses.

Samples

Seven frozen oral tongue tumors were selected from the University of Chicago Head and Neck Cancer tissue bank (IRB approved protocol UCCC#8980) based on age, low or absent use of alcohol (social: < one glass of wine or equivalent per day) and non-use of tobacco (Table 1). Areas of tumor involvement were marked and a punch biopsy performed to achieve tumor content of ≥70%. Known HPV16(+), virus genome integrated UM-SCC47 cells were obtained from Dr. Thomas Carey (University of Michigan) and harvested at 70–80% confluence. A mouse lymphoma containing the EBV LMP2A transgene was used as a second control. A third control, publicly available RNA-Seq data of an HPV16-positive cervical carcinoma were downloaded from the NCBI Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/Traces/sra). All tumors were screened for HPV status using an established nested PCR method.

### Table 1

Sample overview and clinical characteristics of sequenced oral tongue cancers. CRT = concurrent chemoradiation, S = surgery, RT = Radiation, OT = Oral tongue tumor sample.

<table>
<thead>
<tr>
<th>Source</th>
<th>Patient</th>
<th>Patient</th>
<th>Patient</th>
<th>Patient</th>
<th>Patient</th>
<th>Patient</th>
<th>Patient</th>
<th>Cell line derived from patient</th>
<th>Cell line derived from patient</th>
<th>Cell line derived from patient</th>
<th>Literature derived</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Male</td>
<td>Female</td>
<td>Female</td>
<td>Male</td>
<td>Male</td>
<td>Male</td>
<td>Male</td>
<td>Male</td>
<td>Male</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Age</td>
<td>42</td>
<td>44</td>
<td>21</td>
<td>33</td>
<td>33</td>
<td>44</td>
<td>42</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>Alcohol</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>Tumor Stage T4N0</td>
<td>Initially T1N0 with several recurrences</td>
<td>Recurrence post surgery and CRT for T2N0 tumor</td>
<td>Recurrence post surgery for T1N0 tumor</td>
<td>Surgery, CRT, palliative chemotherapy</td>
<td>Surgery, CRT</td>
<td>Surgery, CRT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>CRT</td>
<td>Surgery, then CRT, then palliative chemotherapy</td>
<td>Surgery, RT, CRT</td>
<td>Surgery, RT</td>
<td>RT, CRT</td>
<td>CRT</td>
<td>CRT</td>
<td>surgery, CRT, palliative chemotherapy</td>
<td>CRT</td>
<td>CRT</td>
<td></td>
</tr>
<tr>
<td>Outcome</td>
<td>Cancer free</td>
<td>Undergoing treatment for metastatic disease</td>
<td>Deceased from tumor</td>
<td>Cancer free</td>
<td>Cancer free</td>
<td>Cancer free</td>
<td>Cancer free</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Figure 1 Survival analysis on the clinical data of 748 HNC patients estimated using Kaplan–Meier analysis. (A) Analysis for patients < 45 years of age. Patients with tumors of the oral cavity (OC+, dashed line) show a significantly decreased overall survival rate (Log-rank $p = 0.008$) when compared to younger patients with tumors at other locations of the HN region (OC-, solid line). This indicates the more aggressive phenotype of OC tumors in young patients. (B) No such difference can be observed for patients > 45 years of age (Log-rank $p = 0.66$). (C) A Cox proportional hazards model shows that significant risk factors influencing survival are tobacco/alcohol use and the interaction of young age and oral cavity tumors ($py =$ pack years, $OC =$ oral cavity).

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR</th>
<th>95% CI</th>
<th>P value$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSCC status (OC+ vs OC-)</td>
<td>0.92</td>
<td>(0.64, 1.32)</td>
<td>0.65</td>
</tr>
<tr>
<td>Age (≤45 years vs. &gt;45 years)</td>
<td>0.86</td>
<td>(0.64, 1.23)</td>
<td>0.42</td>
</tr>
<tr>
<td>Alcohol Use (none, low, moderate, heavy)</td>
<td>1.12</td>
<td>(1.01, 1.24)</td>
<td>0.037</td>
</tr>
<tr>
<td>Tobacco Use (Never, &lt;20py, 20–40py, &gt;40py)</td>
<td>1.32</td>
<td>(1.19, 1.45)</td>
<td>8.8×10⁻⁸</td>
</tr>
<tr>
<td>Interaction: Young patient * OC+</td>
<td>2.47</td>
<td>(1.19, 5.10)</td>
<td>0.015</td>
</tr>
<tr>
<td>Tumor stage (I, II, III, IV)</td>
<td>1.31</td>
<td>(0.89, 1.94)</td>
<td>0.17</td>
</tr>
</tbody>
</table>

HR=Hazard Ratio, CI=Confidence Interval

$^1$ P values from Wald test of multivariate Cox regression model after adjusting for covariates.
RNA-Seq library preparation

RNA was extracted using Qiagen RNeasy columns (Qiagen, CA) and polyA purified (Ambion Micro Poly(A) purist), using between 1.5 and 10 μg RNA. cDNA libraries were prepared as described in the Supplementary methods.

Sequencing

Short read sequences were collected from an Illumina Genome Analyzer II (Illumina, CA) with a standard protocol for 36 bp or 56 bp read-lengths on one lane per sample in the GAII flowcell (Illumina, CA) at Argonne National Laboratories (Bolingbrook, IL). Expression of CDKN2A-transcripts was quantified as described in the Supplementary methods.

Virus algorithms

An overview of both algorithms is provided in Fig. 2A. A detailed explanation of the algorithms, the databases used, and the criteria to accept positive viral calls can be found in the Supplementary methods and Supplementary Tables 2 and 3. All analyses were run on a desktop computer (Apple iMAC, 2.4 Ghz, 8 Gb RAM, Apple, CA).

In-silico simulation

To evaluate the ability to detect unknown viruses, HPV16 and EBV sequences were deleted from all databases, and both virus algorithms were repeated using the UM-SCC47/EBV-LMP2A-transgene lymphoma short read sequences.

Results

Survival analysis

Survival of 748 HNC patients treated at the University of Chicago with chemoradiotherapy was analyzed. A lower rate of survival was evident in younger patients with oral cavity tumors compared to those with non-oral cavity tumors: among the young patients (Age < 45 years, n = 135), patients with primary neoplasms of the oral cavity (n = 24) had 5-year overall survival of 42%, compared to 65% 5-year survival in 111 patients with nonoral cavity neoplasms [p = 0.008, HR = 3.25, (95%CI: 1.50–7.05.)] (Fig. 1A). Progression free survival was also different with 35.7% of oral cavity patients versus 64% in non-oral cavity patients progression-free at 5 years [p = 0.002; HR = 2.96, (95%CI: 1.42–6.17)]. This difference is by and large not driven by good prognosis oropharynx tumors (majority HPV(+) based on prior data31–33), with young oral cavity tumor patients showing worse overall survival than both oropharynx, and non-oropharynx/non-oral cavity tumors (p = 0.023, log-rank test comparing three groups; Supplementary Fig. 2). Within the cohort of young patients with oral cavity tumors there was no difference in clinical outcome between oral tongue (50% of young oral cavity patients, Supplementary Fig. 3) and all other oral cavity primary sites (p = 0.70, Supplementary Fig. 4). In the age group of patients >45 years there was no significant difference in overall survival for oral cavity vs.

Figure 2 (A) Flowchart visualizing the different steps during the rapid and the discovery algorithm. (B) The amount of data is efficiently reduced by computational subtraction of host and non-viral microbial reads. (C) A further reduction is achieved by assembling the remaining reads into longer sequences (contigs) of which ~90% are of host origin. Only a minor fraction of contigs matches to a virus or to both a viral and a host sequence and will be evaluated subsequently. (Height of the bars correspond to the mean of all samples, error bars indicate standard deviation.)
Seven oral tongue cancers from young patients (average age 37 years) with no or minimal tobacco and alcohol use were selected from the University of Chicago tissue bank (Table 1). Patients were treated depending on clinical stage with surgery, chemoradiotherapy or combined modality approaches. Five patients were disease free at the time of manuscript preparation, one patient was deceased, and one patient was undergoing palliative treatment for metastatic disease. Of note, three patients experienced recurrences after initial treatment despite adequate therapy results (43%) and one patient progressed on induction chemotherapy (14%). All tumors were confirmed to be HPV-negative using an established PCR method.

Algorithm results

An overview of the two-algorithm approach and its performance is provided in Fig. 2 and Supplementary Table 2. After applying positive selection criteria (Supplementary Table 3), the rapid algorithm clearly identified HPV16 and EBV in the positive control samples (Fig. 3A and Supplementary Fig. 5A).

In contrast, none of the oral tongue tumors showed significant matches to known viruses. To further investigate the reliability of the virus call criteria, and to foster the distinction of false positive calls by the rapid algorithm, the proportion of reads determined as being of viral origin divided by all reads obtained per sample was calculated (Fig. 4A). Even though some reads aligned to viral genomes for the oral tongue samples, the number of reads aligning to a virus normalized for all input reads is significantly higher for the positive controls (Kruskal-Wallis p = 0.017). Moreover, in the control samples a mean of 95.7% (95%CI of the true expected mean 86.4%-100%) of all viral reads aligned to a single best virus (HPV16/EBV, Fig. 4B). This shows, that true viral reads dominate the total virus count over false positive hits. Similarly high values cannot be observed for the OT samples. The amounts of viral reads in the OT samples rather resemble the amount and distribution of viral hits identified in UM-SCC47 that do not match to HPV16, and that can serve as a baseline of expected false positive hits (Figs. 3A and 4B).

Sequences that were aligned to viruses by the rapid algorithm, but did not fulfill the positive call criteria, were subjected to a manual BLAST search. For the majority of those sequences, either sequence homology to human genes or low complexity/repetitive reads were reported, thus supporting the notion of being false positives. The large number of viral hits that also match human sequence may relate to the homology of human and viral ORFs. Up to 8% of the human genome are reported to have originated from endogenous retroviruses

Furthermore, using the discovery algorithm, HPV16 and EBV (HHV4) were also unequivocally identified in all positive control samples (Fig. 3B and Supplementary Fig. 5B).

Despite the presence of only one single transgenic viral protein (LMP2A) in one of the control samples, both algorithms identified the presence of EBV, suggesting a high degree of sensitivity.

To simulate the detection of yet unknown viruses related to known viruses, HPV16 and EBV sequences were deleted from the databases and the algorithm was rerun for UM-SCC47 and the EBV-LMP2A transgene lymphoma. The rapid algorithm produced identical results with the singular change of HPV16 and EBV matches missing (not shown). The discovery algorithm identified E6 transcripts in UM-SCC47 derived sequences (Fig. 3C). In addition, a multitude of HPV viruses from different HPV types and species (that by definition share 60–70% sequence similarity)

![Figure 3](image-url)
are identified (Fig. 3D), demonstrating a high degree of sensitivity for related viruses.

For the EBV-LMP2A transgene lymphoma the discovery algorithm identified a single candidate hit (Herpesvirus papio latent membrane protein 2 (Supplementary Fig. 5C), an alpha-herpesvirus occurring in baboons\(^3\)). Since there is only one single EBV protein in the LMP2A transgenic lymphoma, as expected fewer herpesvirus hits are identified. Other hits e.g. Kaposi’s sarcoma
virus (HHV8), and Herpes simplex virus 2 (HHV2) are also found, but did not meet the candidate criteria (Supplementary Fig. 5D).

In three of the seven oral tongue tumors, possible viral candidates meeting the positive virus call criteria were reported, but verification of the respective contigs by a manual web-based BLAST query readily identified them as most likely being false positive hits (Supplementary Table 4); detailed output for each sample is available in the Supplementary materials). None of the seven oral tongue tissue samples showed convincing evidence of viral transcripts in the rapid or the discovery algorithm underlining the absence of viral transcripts belonging to either a known virus or a novel virus related to a known virus.

p16/CDKN2A expression

p16 is commonly used as a surrogate marker for HPV in oropharyngeal tumors. Interestingly CDKN2A mRNA (p16) was significantly expressed in three of the seven samples \( (p = 0.037, \text{one-sided Welch } t\text{-test}) \); Fig. 5). This is consistent with recent reports suggesting that p16 is expressed in OCSCC in the absence of HPV, making it an unreliable HPV surrogate marker when evaluating oral cavity tumors.7,10

Discussion

In this study, we analyzed differences in survival and clinical outcome of a prospectively treated cohort of 748 HNC patients (Supplementary Table 1). We found a significantly inferior outcome for young patients (<45 years) with cancer of the oral cavity (OCSCC) compared to young patients with HNC in other anatomic locations. To our knowledge, this is the largest cohort on which a study of this kind has been done. Our study highlights the influence of tumors at different anatomical locations on the overall survival rate of young HNC patients. Our data show that there are prognostic differences between younger and older oral cavity patients, in contrast to the majority of recent reports claiming a comparable stage by stage prognosis for younger and older HNSCC patients.38–41 To date no known risk factor could be shown to convey the decreased prognosis in young OCSCC patients. In fact young OCSCC patients have a lower exposure to known risk factors: never-smokers represent 20.7% of the young non-oral cavity patients, but 37.5% of the young oral cavity patients in our study cohort. In addition in our dataset a non-significant trend towards lower stages was present for young OCSCC patients.

The worse outcome in young OCSCC patients led us to investigate the hypothesis that tumors of the mobile tongue, which are a majority of oral cavity cancers, may have a viral etiology9 specifically implying a so far unknown virus. We therefore developed a novel viral screening algorithm using massively parallel short-read sequencing and were able to demonstrate the absence of viral transcripts in our collection of oral tongue cancers.

Our method focuses on mRNA transcripts, an approach that has proven successful for viral detection in other tumors,42,43 as viral protein expression is essential for viral oncogenesis in all known human oncoviruses. While we believe that our approach provides a high level of sensitivity for detection of all active oncoviruses, there are possible scenarios which would lead the algorithms to be falsely negative: (1) a virus that does not produce mRNA transcripts or does not polyadenylate them.44 It has been hypothesized that viruses cause cancer by a “hit-and-run” mechanism45–47 or by viral oncomodulation.48,49 Nevertheless, there are currently no known examples of viral oncogenesis of this type in humans. (2) Oncogenesis by an unknown virus without sufficient homology to any known viruses. Such lack of homology would lead to the inability of BLAST to detect a new virus. Given the marked partial homology between distinct viral families, we think this is a remote possibility. We estimate that the discovery algorithm (based on BLAST identification of distant HPV-species, Fig. 3C/D) will identify viruses down to 60–70% sequence homology. Furthermore homol-
ogy within a small part of the viral genome would be sufficient for detection. (3) A virus may use sequences that closely resemble human sequences leading the automated algorithm to discard such “human” information. This again seems unlikely since all known oncorviruses have distinct viral proteins, that are essential to proper viral function, and are therefore reliably identifiable. (4) Involvement of non-viral microbial factors (e.g. bacteria/fungi): Because there is a substantial overlap in genetic material between viruses and bacteria and the presence of bacterial/fungal DNA contamination of samples from the oral cavity subtraction of non-viral microbial sequences was necessary. (5) Finally, our small sample set of seven tumors may have led us to randomly only select non-virus associated tumors. We specifically selected representative, phenotypically ideal cases (i.e. young age, non-smokers, non-drinkers). We believe that our cohort is optimal at representing the clinical phenotype, but larger sample sets, that will undoubtedly become available in the future, can further substantiate our results.

In oropharyngeal cancer, measurement of p16 protein expression is a reliable surrogate marker for HPV infection and is used widely. In oral cavity tumors however, two recent reports demonstrate a high false positive rate when evaluating p16 for OCSCC. In our study a high abundance of CDKN2A/p16 mRNA-transcripts in 3 out of 7 (43%) HPV-negative OT samples is observed. Bearing in mind, that mRNA-expression levels do not perfectly reflect protein levels, this finding nonetheless validates previous reports questioning p16 as a single HPV-surrogate marker in OCSCC.

In summary, our algorithm successfully identifies viruses in three positive control tumors, even in the presence of just a single viral gene, and when simulating the discovery of a distantly related but potentially unknown virus. No viruses were found in the oral tongue cancer samples. We thereby show for the first time that oral tongue tumors in non-smokers/non-drinkers do not contain transcriptionally active oncoviruses, and are therefore unlikely to be virus-associated. As such we propose refocusing the etiological investigations of OCSCC on non-viral environmental and genetic factors. Our report also has broader implications for the field of virus discovery, making it feasible for smaller laboratories to perform such analyses using a rapid and sensitive dual algorithm for known and unknown viruses.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.oraloncology.2013.02.003.

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