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A pilot study of Merkel cell polyomavirus in squamous cell carcinoma of the tongue

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Introduction

The oral tongue and the floor of the mouth are the most common sites of squamous cell carcinoma (SCC) within the oral cavity, accounting for more than 50% of cases [1,2]. The increasing incidence of oral tongue SCC in young patients (ages 20–44 years) who have a lower incidence and duration of tobacco and alcohol exposure suggests that genetic, environmental or viral factors may contribute to the pathogenesis of oral tongue SCC [3–6]. The tongue has a significantly different histologic makeup with a rich lymphatic supply compared to the other areas of the oral cavity. As such, tumors of the tongue are more likely to invade and metastasize [7].

Viral associations with head and neck cancers are well-documented for sites such as the nasopharynx and oropharynx. Epstein-Barr virus (EBV) is associated with 99% of undifferentiated nasopharyngeal carcinomas and a small percentage of oral squamous carcinomas [8]. Human papillomavirus (HPV) has been detected in 25% of all head and neck cancers and 50–70% of oropharyngeal carcinomas [9]. A few studies have found an association of HPV with SCC of the oral tongue [10]. However, there has been no conclusive evidence that viral carcinogenesis has a role in SCC of the oral cavity, especially the oral tongue [11–13].

Clonal integration of a polyomavirus in human Merkel Cell Carcinoma (MCC) was first described in 2008 [14]. Interestingly the risk of other cancers such as chronic lymphocytic leukemia, basal cell carcinoma and SCC of skin, have been reported to occur with higher frequency in patients with MCC [15]. However, none of these cancers have been conclusively linked to MCPyV infection [16–22]. The Merkel Cell Polyomavirus (MCPyV) large T-antigen is responsible for the transformative properties of the virus, which can occur in both immunocompetent and immunocompromised patients. MCPyV is a component of the normal skin flora, and it rarely leads to malignant transformation [23]. Primary MCPyV infection in adults is generally asymptomatic, as MCPyV seropositivity has previously been noted among asymptomatic patients, and MCPyV DNA has been detected in normal skin samples [23–27]. In this study, we analyzed oral tongue SCC for the presence of MCPyV in a group of patients without the traditional risk factors for oral tongue SCC. We analyzed normal base of tongue (BOT) samples obtained during sleep apnea surgery as controls since normal oral tongue samples were unavailable.

Materials and methods

Samples

IRB approval was obtained, and charts were retrospectively reviewed for patients diagnosed with oral tongue SCC, who were < 45 yrs of age, regardless of risk factors of tobacco and alcohol exposure, or

Abbreviations: HPV, human papillomavirus; MCPyV, merkel cell polyomavirus; SCC, squamous cell carcinoma; PP2A, protein phosphatase 2A; BOT, base of tongue

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patients > 45 years of age with no known risk factors. These patients were chosen because they were either not exposed to known risk factors, or the time of exposure to risk factors was not congruent with the typical age group of patients who develop tobacco-associated malignancies. In addition, adequate tissue had to be available to be included in the study. Twenty-one patients with oral tongue SCC and samples of normal BOT from 7 patients undergoing surgery for sleep apnea were identified for the study. Both formalin-fixed paraffin embedded (FFPE) tissue and flash frozen (depending on availability) specimens were analyzed for the presence of MCPyV. DNA was extracted from the specimens, and polymerase chain reaction (PCR) was performed to amplify the regulatory and large T-antigen regions using primers specific to MCPyV. DNA sequencing of PCR products was performed by Lone Star Labs, Inc. (Houston, TX). The Basic Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov) was used to confirm detection of MCPyV sequences.

Specimen processing

Initially, 5 μm sections were cut from each paraffin block using a single blade. A second set of 10 μm sections was cut from the paraffin blocks using a new blade for each block to rule out potential cross contamination. The samples were deparaffinized with xylene washes and rehydrated with ethanol. DNeasy (Qiagen) reagents were used in accordance with the manufacturer's instructions to extract DNA from each sample.

Detection of Merkel cell polyomavirus (MCPyV) by polymerase chain reaction (PCR)

Samples were assessed for DNA integrity using the AG1/AG2 primers (human A-gamma globin gene). In addition, samples were analyzed for the presence of MCPyV, using primers specific for DNA sequences in the regulatory and the large T-antigen regions (Table 1) [28]. DNA samples and PCR reaction components (1 μM for each primer, 0.1 U/μl Taq polymerase, 20 μM dNTPs, reaction buffer containing 1.5 mM MgCl2) were assembled to a final volume of 50 μl. PCR amplification conditions for the AG1/AG2 primers were: 40 cycles of denaturation at 94 °C for 30 sec, annealing at 61 °C, and extension at 72 °C for 30 sec. PCR amplification conditions for the MCPyV primer sets were: 45 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C, and extension at 72 °C for 30 sec. A plasmid clone of MCPyV (generously provided by Patrick Moore, University of Pittsburgh Cancer Institute) was used as a positive control for PCR reactions. Amplicons were visualized on ethidium bromide-stained 2% agarose gels after electrophoresis. DNA sequence analysis was performed to confirm MCPyV.

Table 1
Table of PCR primers. Names are based on nucleotide position in MCP Reference sequence NC_010277. F (forward) and R (reverse) designate whether the primer is sense and antisense.

Primer name	Sequence
Human A-gamma globin gene	
AG1(2)	CTCAGACGTTCCAGAAGCGAGTGT
AG2(2)	AAACGGCTGACAAAAGAAGTCCT
MCP Large T antigen	
M2(3) (MCP 3694F)	TTGCAGTAATTTGTAAGGGGACT
M1(3) (MCP 3872R)	GGCATGCCTGTGAATTAGGA
LT3(3) (MCP 4704F)	ATATAGGGGCCTCGTCAACC
LT3(3) (MCP 5012R)	TTGTCTCGCCAGCATTGTAG
MCP 2307F	AAAGCCCTCTGTTGCCCC
MCP 2366F	AGGCGCCACTGAAAGGACC
MCP 2701F	ATCAGGCAAGCACCAAATAAGAC
MCP 3016F	GCTATTTTGCCCTTTCACATCCTC
MCP 3347R	AGAAGCACCTAGAGAGATTAAGAGATTT
MCP 3612R	GCTTGTGAATATGAGCTAGACGACC
MCP 3812R	TTGCTATTAAGAATTTTTGCTCTACC
MCP 4228R	CCCGTGTTCCTCTGCCGAG
MCP 4295R	CTGAGCCTCCCTCGTCCTC
MCP 4347R	AGAACGGATGGCACCTGGGAG
MCP regulatory region	
MCP 5341F	AGCTTGCAGAGAGCCTCTCT
MCP 247R	ACCACAACTTGGCTGCCTAG
MCP 5375F	GGATAAATCCATCTTGTCTATATGCAG
MCP 212R	CAAGTTGGCAGAGGCTTG

Analysis and statistical considerations

The Fisher exact test was used to determine significant associations between presence of MCPyV with gender, stage, smoking, alcohol use, recurrence and perineural invasion. The Wilcoxon rank sum test was used to compare the age and length of follow-up in patients with and without MCPvY.

Results

Demographic and baseline characteristics of cancer patients are shown in Table 2. The median age of tongue cancer patients was 40 years (range, 25–85 years). The median follow-up time was 39 months (0–178 months). 10 of the 21 patients (52.4%) were male. The T staging was as follows: T1 = 3 patients (14.3%), T2 = 11 patients (47.3%), T3 = 2 patients (9.5%), T4 = 3 patients (14.3%). The nodal staging was as follows: N0 = 13 (57.1%), N1 = 3 (14.2%), N2a = 1 (4.7%), N2b = 1 (4.7%), N2c = 1 (4.7%). Tumor samples from 6 of 21 (28.6%) patients tested positive for MCPyV DNA sequences. All of the 7 normal BOT samples were found to be negative for MCPyV DNA. The mean age of these 7 patients was 52.7 years, and the male to female ratio was 4:3. These patients either underwent BOT reduction surgeries for sleep apnea or had biopsies of normal BOT for identification of a primary site for unknown primary malignancies. However, all were negative for cancer. The time of the last follow-up was not available for 4 patients. The other 17 patients were still alive at their last follow-up with a mean follow-up time of 3.5 years (S.D. 4.9) after diagnosis. Table 3 details the comparison of demographic and clinical factors for patients with MCPyV-positive and MCPyV-negative oral tongue SCC. None of the 6 patients whose tumors were positive for MCPyV had a recurrence while 9 of the 15 (75%) MCPyV-negative patients had recurrences ($p = 0.011$). Older patients (greater than 45 years of age) were more likely to be MCPyV-negative than the younger patients, but this difference was not statistically significant. Overall survival times were similar between patients who were positive and negative for MCPyV. There was no significant association between presence of MCPyV and perineural invasion ($p > 0.99$).

Discussion

MCPyV is a non-enveloped double-stranded DNA virus and one of

Table 2
Demographics of patient characteristics (n = 21).

Characteristic/Outcome	Number (%) or Mean ± SD, median, range
Merkel cell virus +	6 (28.6)
Perineural invasion	6 (28.6)
Male	11 (52.4)
Stage: 1, 2	12 (63.2) Missing # 2
3, 4	7 (36.8)
Smoking history	10 (52.6) Missing # 2
Alcohol use	9 (47.4) Missing # 2
Recurrence	9 (56.2) Missing # 5
Alive at last follow up	17 (100.0)
Age (years)	41.5 ± 14.7, 39.0, 25–85
Follow up (years)	3.5 ± 4.9, 0.9, 0–14.9