The Screening of Viral Risk Factors in Tongue and Pharyngolaryngeal Squamous Carcinoma

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Abstract. *Background: The oral cavity and pharyngolarynx is readily open to the environment, which provides a good atmosphere to viral infection and subsequently links to the local carcinogenesis. The aim of this study is to clarify the viral risk factors for tongue and pharyngolaryngeal squamous carcinomas and the oncogenic role of DNA viruses. Materials and Methods: Tongue, pharyngeal and laryngeal carcinomas, and corresponding non-neoplastic mucosa (NNM) were collected and subjected to microdissection and DNA extraction with integrity detected by beta-globin polymerase chain reaction(PCR). Additionally, we examined genomic DNA copies of Epstein-Barr virus (EBV), human papilloma virus (HPV) 16 and 18, and John Cunningham virus (JCV) by real-time PCR with a comparison of the clinicopathological features of the tumors. Results: All the extracted DNA samples showed good integrity. Compared with NNM, EBV and HPV16 copies were higher in the three kinds of head and neck carcinoma respectively ($p<0.05$). The same situation was also observed in tongue and pharyngeal carcinoma for HPV18, and pharyngeal carcinoma for JCV ($p<0.05$). There were fewer EBV copies in tongue than pharyngeal and laryngeal carcinoma ($p<0.05$). Pharyngeal carcinoma had a higher HPV16 copy number than tongue and laryngeal carcinoma ($p<0.05$). Moderately differentiated carcinoma of the head and neck had more EBV copies than well-differentiated ($p<0.05$). Conclusion: The viruses studied here might play an important role in the carcinogenesis of tongue and pharyngolaryngeal squamous carcinomas.*

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The oral cavity and pharyngolarynx is a door for the human body open to the environment. Tonsil is near to these anatomical sites, where lymph nodes are rich. Both provide a favorable atmosphere for viral infection, which is closely linked to the local carcinogenesis (1-3). Here, we targeted the viral risk factors of tongue and pharyngolaryngeal squamous carcinomas, including Epstein-Barr virus (EBV), human papilloma virus (HPV) and John Cunningham virus (JCV).

EBV is a ubiquitous human herpes virus infecting over 90% of the adult population worldwide and at least 95% of nasopharyngeal carcinomas (NPC) are EBV associated. The stable EBV infection of epithelial cells may require an undifferentiated phenotype. Although EBV receptor (CD21) that facilitates the infection of B-cells is not or is only a little expressed in epithelial cells, direct cell contact might allow EBV to infect CD21-negative cells, or the lytic infection of EBV in lymphocytes of the nasopharyngeal mucosa might also be a possible mechanism for EBV infection of epithelial cells. EBV has tumorigenic potential due to a unique set of latent genes such as latent membrane proteins (LMP) and EBV-determined nuclear antigens (EBNA). As a principal oncogene of NPC, LMP1 associates with the host membrane and can directly activate a number of signaling pathways including nuclear factor kappa-B, mitogen-activated protein kinases, and phosphoinositol-3-kinase, finally to prevent apoptosis and promote cancer development. LMP2 not only mediates tumor cell survival but also makes NPC cells migratory and invasive. EBNA1 is an unusual protein that binds the EBV genome to host chromosomes, and thus mediates equal partitioning of viral DNA into daughter cells during cell division. EBNA2 may be involved in the transactivation of LMP1. Evidence of EBV DNA in almost

all NPC cells that were studied supports the association of NPC with EBV. These findings indicate some role of EBV in carcinogenesis (4-6).

The genome of HPV is divided into an encoding region for viral DNA replication and cell transformation, an encoding region to produce the structural proteins of the virus particles, and a non-coding region of viral DNA replication and transcriptional regulatory elements. Although terminally differentiated epithelial cells generally cannot support DNA synthesis, HPVs can encode E6 and E7 proteins that create a state competent for DNA replication in these cells, especially in squamous epithelial lesions. A small fraction of people infected with high-risk HPVs will develop cancer, which usually arises many years after the initial infection. Therefore, the International Agency for Research on Cancer recognized that the high-risk HPV types 16 and 18 were carcinogenic in humans. Through wounds or abrasions, these viruses infect actively dividing basal epithelial cells and the viral DNA is maintained and undergoes DNA replication with cellular differentiation in the nuclei of infected epithelial cells. For example, HPV has also been detected in a variety of head and neck tumors including NPC, although strongly implicated in the genesis of human cervical carcinoma. Current data suggest that approximately 15~20% of head and neck squamous cell carcinomas (HNSCC) are linked to HPV infection (7-10).

John Cunningham virus (JCV) constitutes a family of polyoma viruses. In its genome, the early region is alternatively spliced to produce large T antigen and small t antigen. T antigen, a large nuclear phosphoprotein for viral DNA replication, binds to viral replication region to promote the unwinding of double helix and recruitment of cell proteins that are required for DNA synthesis. The late region encodes three capsid structural proteins due to alternative splicing and the small regulatory protein known as agnoprotein. Serological studies have indicated an asymptomatic JCV infection in about 90% of the adult population, but it may be activated under immunosuppressive conditions, leading to lethal demyelinating disease, progressive multifocal leukoencephalopathy. Evidence from transgenic and infectious animal models indicated that JCV can transform cells and cause various malignancies. In recent years, links have been suggested between JCV and various types of human cancer, including colorectal, prostate and esophageal cancer, brain tumors, bronchopulmonary carcinoma and B-cell lymphoma, pointing out its roles as an oncovirus. A body of evidence suggests that the respiratory tract might be a door for JCV infection, which provides its possible link to tongue and pharyngolaryngeal carcinomas (11-15).

In the present study, we examined the genomic DNA copies of EBV, HPV and JCV in tongue and pharyngolaryngeal squamous carcinomas and analyzed these data with regard to clinicopathological parameters of the tumors to clarify the viral risk factors for these types of the carcinoma and the oncogenic role of the DNA viruses.

Materials and Methods

Patients. Tongue (n=33), pharyngeal (n=24) and laryngeal (n=65) carcinomas were collected from surgical materials in Kouseiren Takaoka Hospital after obtaining permission from the patients or their relatives. The tongue (n=8), pharyngeal (n=8) and laryngeal (n=14) non-neoplastic mucosa (NNM) was also collected in the same way. All samples were fixed in 10% formalin and embedded in paraffin. The head and neck carcinoma cases were 100 men and 22 women (aged 27-91 years, mean=65.5 years). The histological diagnosis of the squamous carcinomas was based on the WHO criteria (1). Raji cells (EBV cultured lymphoma cell line) served as the standard curve for EBV copies. The plasmid PBR322-HPV16 and PBR322-HPV18 were purchased from JCRB GeneBank, Japan, and pBS-JCV Mad1 was kindly donated by Associate Professor H Sawa, Department of Neuropathology, Hokkaido University, Japan. The Ethics Committee of the Hospital gave approval for genetic experiments restricted to viruses.

DNA extraction and checking. Paraffin-embedded blocks were sectioned at 10 μm and cancer lesions on slides were microdissected with reference to hematoxylin and eosin (HE) staining of consecutive sections, and subject to deparaffinization. DNA was extracted from the cell line and deparaffinized samples by standard proteinase K digestion and phenol/chloroform extraction. All the DNA samples were amplified using β-globin primers, sense: 5'-ACACAACTGTGTTCAC TAGC-3'and anti-sense: 5'-GTCTCCTT AAACCTGTCTTG-3' (175 bp) by 30 cycles of denaturation at 95˚C for 30s, annealing at 55˚C for 30s, and extension at 72˚C for 30s to confirm their integrity.

Real-time PCR. A real-time, fluorescence probe-based PCR method was used to quantify JCV copies with reference to a previous protocol (15). To quantify the viral copy number, HPV16 (PBR322-HPV16)-, HPV18 (PBR322-HPV18), and JCV (pBS-JCV Mad1)-containing plasmids were serially diluted and served as a standard reference. As for EBV, the copies per μg DNA extract from Raji cell lines was comparatively quantified using JCV real-time PCR standard and diluted for the standard reference. Amplicon development was monitored using a double-dye probe labeled with 6-carboxyfluorescein (FAM) at the 5' end and 6-carboxytetramethylrhodamine (TAMRA) at 3' end. Twenty-five microliter reaction mixtures contained 12.5 μl TaqMan® (*2) with 2.25 μl (10 μM) of each primer, 2.5 μl (2.5 μM) of double dye probe and 100 ng of template DNA. The protocol included the following parameters: an initial 10 min of incubation at 95ºC for TaqMan DNA polymerase activation followed by 60 cycles of denaturation at 95ºC for 30 s, annealing for 1min, and extension at 72ºC for 30 s. The primers, probes and annealing temperatures are shown in Table I.

Statistical analysis. Statistical evaluation was performed using Mann-Whitney *U*-test to differentiate the means. $P<0.05$ was considered as statistically significant. SPSS 10.0 software was employed to analyze all data.

Table I. *Nucleotide sequences of TaqMan primers and probes for targeted genes used in quantitative real-time PCR.*

Genes	Orientation	Nucleotide sequence (position)	Temperature (˚C)	Length (base)
EBV	Forward	5'CGGAAGAGGTTGCAAACAAAGG3'	55	22
(LMP)	Reverse	5'AAAGCAGCGTAGGAAGGTGTG 3'		21
	Probe	5'CACCGCCGCCACCGTCTGTCATC3'		23
HPV16	Forward	5'GCACCAAAAGAGAACTGCAATG 3'	55	22
	Reverse	5' GCAGCTCTGTGCATAACTGTG3'		21
	Probe	5'CTGAGCACCCTGTCCTTTGTGTGTCCG3'		27
HPV18	Forward	5'ACGACCTTCGAGCATTCCAG 3'	55	20
	Reverse	5' TTACTGCTGGGATGCACACC3'		20
	Probe	5'AGGACCCACAGGAGCGACCCAGAAAG3'		26
JCV	Forward	5' GCCACCCCAGCCATATATTG 3'	55	20
	Reverse	5' GTTGACAGTATCCATATGACCAGAGAA 3'		27
	Probe	5' T AAAACAGCATTGCCATGTGCCCCA 3'		25

Table II. *Relationship between viral copy number and clinicopathological parameters of tongue and pharyngolaryngeal squamous carcinoma.*

Clinicopathological parameter	n	EBV copies/μg (Mean±SD)	HPV16 copies/μg (Mean±SD)	HPV18 copies/μg (Mean±SD)	JCV copies/μg (Mean±SD)
Gender					
Male	100	9.07±20.56	352.29±430.78	1.07±3.55	332.61±204.46
Female	22	5.63±4.72	359.29±435.89	1.10±3.90	296.01±28.95
Age					
<65 years	51	9.48±24.63	351.89±387.05	1.18±3.20	311.43±46.94
≥65 years	71	7.71±13.16	354.75±466.11	1.00±3.88	336.48±240.58
Differentiation					
Well-differentiated	63	7.60±15.18*	326.16±389.99	1.06±3.04	339.96±249.12
Moderately differentiated	30	12.13±30.32	384.02±433.59	1.97±5.63	306.46±41.56
Poorly differentiated	29	6.49±60.06	381.55±555.06	0.18±0.97	315.92±95.35

SD: standard deviation. *Significantly different at $p<0.05$ compared with moderately differentiated subtype.

Results

Clear bands of *beta-globin* appeared for all DNA samples, indicating good integrity following PCR amplification (Figure 1). Among these viral examinations, HPV16 and JCV showed higher copy numbers than the other two (Figure 2). In fact, HPV18 was not detected in laryngeal carcinomas despite the 60 cycles employed in the present study. Only one case (4.2%) of pharyngeal carcinoma and 14 cases (42.4%) of tongue carcinoma showed HPV18 existence. In addition, EBV was not detected in 5 cases of tongue carcinoma, 2 pharyngeal carcinoma and 16 laryngeal carcinomas.

Compared with NNM, EBV and HPV16 copies were higher in the three kinds of head and neck carcinoma ($p<0.05$). The same situation was also observed in tongue pharyngeal carcinoma for HPV18, and pharyngeal carcinoma for JCV ($p<0.05$). There were fewer EBV copies in tongue than pharyngeal and laryngeal carcinoma ($p<0.05$). Pharyngeal carcinoma had a higher HPV16 copy number than tongue and laryngeal carcinoma ($p<0.05$). Moderately differentiated squamous carcinoma of tongue and pharynolarynx had a higher EBV copy numbers than well-differentiated carcinoma ($p<0.05$).

Discussion

EBV DNA was detected in 72% of oral carcinimas in Okinawa, south western islands about 2,000 km far from mainland Japan (16), but the incidence decreased to about 25% in the western district of Japan (17). The virus initially enters the body through the oropharyngeal mucosa and infects B lymphocytes through the CD21 receptor (18). The EBV-mediated disruption of cell growth checkpoints relies on a direct modulation of cytokine receptor signaling mechanisms and alterations in the expression levels of various cytokines. LMP1 and other latent EBV proteins exert pleiotropic effects when expressed in the cells, resulting in the up-regulation of interleukin (IL)-6 and (IL-8), and growth-activating factors. It was documented that EBV was associated with aggressive

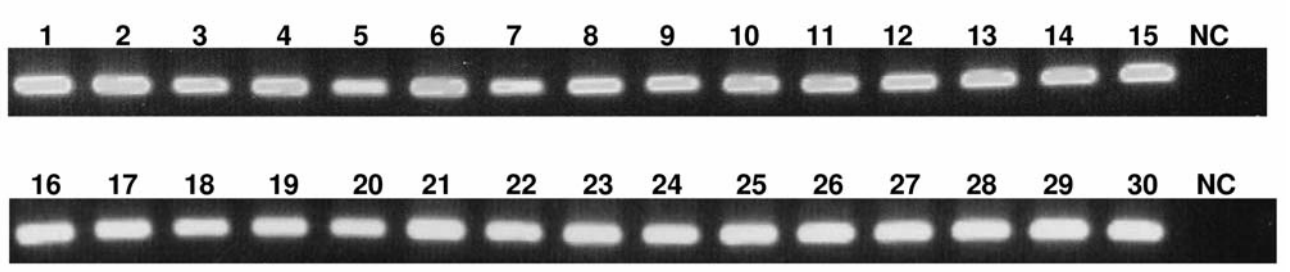

Figure 1. *Detection of β-globin in tongue and pharyngolaryngeal squamous carcinomas. β-Globin detection was positive in all cases of head and neck squamous carcinoma.*

types of oral tumors, especially in immunosuppressed patients and most EBV-related oral tumors involving the tongue (19). Primary EBV infection probably occurs in the polarized mucosal epithelium of the oropharynx, the virus then spreads to B lymphocytes and establishes latent infection. Here, we found that EBV copy numbers were higher in these three kinds of head and neck carcinoma than in the corresponding NNM, although EBV was not detectable in all cases. In contrast, EBV replication was seen in 1.3% of tongue mucosal samples, but not in tongue carcinomas. According to our data, EBV infection might be a risk factor for the carcinogenesis of tongue and pharyngolaryngeal squamous carcinomas. Additionally, there were clearly fewer EBV copies in tongue than in pharyngeal and laryngeal carcinoma, indicating the differential potential for this virus to live or integrate in the tongue and pharyngolaryngeal carcinoma cells. Moderately differentiated squamous carcinoma of tongue and pharynolarynx had higher EBV copy numbers than well-differentiated ones, indicating that EBV infection might be associated with the differentiation of tongue and pharyngolaryngeal carcinoma.

HPV, particularly the tumorigenic 16 subtype, plays a causative role in the development of most oropharyngeal carcinomas, but in only a small percentage of head and neck carcinomas arising in other sites, such as the tongue (20). It has been thought that HPV type 16 and 18 are two of the causative agents of head and neck cancer (20-22). However, oral cavity and oropharynx including tonsil and tongue should be distinctly divided since the detection rate of HPV-DNA is clearly different between them. In a recent large-scale investigation, 43 to 50% of oropharyngeal cancer cases showed HPV-DNA positivity, while oral cancer cases demonstrated positivity in only 2.9 to 14% (21-23). Moreover, Ha *et al*. (23) concluded that HPV-16 infection and integration is seldom found in oral premalignant lesions and invasive carcinoma, and therefore rarely contributes to malignant progression in the oral cavity. Miller and White noted that there was a significantly higher rate of HPV positivity reported by those studies using PCR techniques for either consensus or type-specific HPV sequences (24). Here, HPV16 copy numbers were higher in three kinds of head and neck carcinoma in comparison with NNM, with higher copy numbers of HPV16 in pharyngeal carcinoma than the others, while HPV18 was only detectable in one case (4.2%) of pharyngeal carcinoma and 14 cases (42.4%) of tongue carcinoma. Therefore, it was suggested that HPV16 might be more involved in the carcinogenesis of tongue and pharyngolaryngeal carcinoma, especially in pharyngeal. Reportedly, the E6 protein of the high-risk HPV binds and induces the degradation of the p53 tumor suppressor protein *via* a ubiquitin-mediated process, while the HPVE7 protein binds and destabilizes the retinoblastoma (Rb) tumor suppressor protein and related proteins (25, 26). HPV16 possibly contributed to the tumorigenesis of these squamous carcinomas.

Although the precise mechanisms responsible for JCV-induced cellular transformation and tumor development are not completely understood, it is believed that T-antigen plays a critical role in malignant transformation by interacting with several cell regulatory proteins, including p53 and pRb, and also by modulating several critical growth signaling pathways, such as the insulin growth factor-I receptor and Wnt signaling pathways. It has been indicated that integration of JCV T-antigen into the cellular genome might promote the proliferative ability of cells, possibly through inactivation of p53 or pRb pathways. Another target of T-antigen is β-catenin, whose translocation into the nucleus or down-regulation of the membranous type may contribute to tumorigenesis. It was found that JCV T-antigen increased the stability of β-catenin, leading to the overexpression of nuclear β-catenin, but not the membranous type (11-15).

In this study, we examined JCV targeting T-antigen and found it to be present at higher copy numbers only in pharyngeal carcinoma, compared with corresponding mucosa, in line with our previous reports (12-14), indicating its possible oncogenic role in pharyngeal carcinogenesis. Previous work demonstrated the presence of replicating JCV DNA in B lymphocytes from peripheral blood, tonsils, and spleen, and it was hypothesized that lymphocytes might be

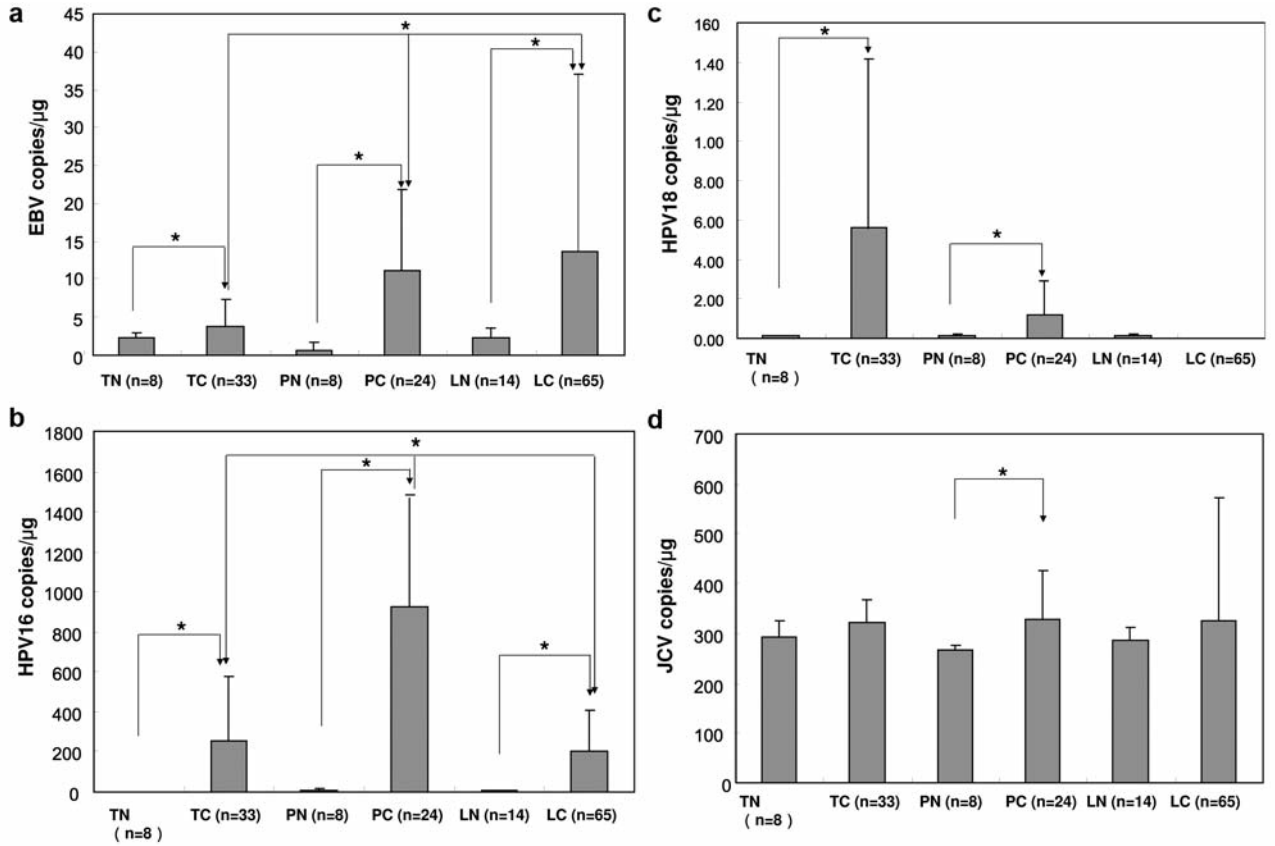

Figure 2. *Real-time PCR amplicons of EBV, HPV and JCV in tongue and pharyngolaryngeal squamous carcinomas. Comparison of EBV (a), HPV16 (b), HPV18 (c) and JCV (d) copy numbers was performed between the carcinoma and normal mucosal tissue of the tongue, pharynx and larynx. *Significantly different, p<0.05. TN: Tongue normal mucosa; TC: tongue carcinoma; PN: pharyngeal normal mucosa; PC: pharyngeal carcinoma; LN: laryngeal normal mucosa; LC: laryngeal carcinoma.*

one site of JCV persistence (27). Detection of viral gene products in renal tubules and excretion of JC virions in the urine suggested JCV persistence in the kidney (28). Monaco *et al*. (29) also reported that the majority of JCV infected cells assessed by in situ PCR were tonsil stromal cells rather than B lymphocytes with using tonsil tissue, leading to the hypothesis of JCV persistence in a quiescent state in lymphoid tissue during latency and infection of other cells upon immune suppression.

In conclusion, EBV and HPV16 and JCV might play an oncogenic role in the squamous carcinogenesis of tongue and pharynolarynx and be risk factors for these carcinomas. Further investigations to explore the molecular mechanisms of viral oncogenesis appear to be warranted.

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