High JC virus load in tongue carcinomas may be a risk factor for tongue tumorigenesis

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Abstract The John Cunningham virus (JCV) asymptotically infects a large proportion (~90%) of the population worldwide but may be activated in immunodeficient patients, resulting in progressive multifocal leukoencephalopathy. Recent reports demonstrated its oncogenic role in malignancies. In this paper, the presence of JCV-targeting T antigen was investigated in tongue carcinoma (TC, n=39), dysplastic tongue epithelium (DTE, n=15) and glossitis (n=15) using real-time polymerase chain reaction (PCR) and in situ PCR and immunohistochemistry, and JCV copies were analyzed with the clinicopathological parameters of TCs. The results demonstrated that glossitis and DTEs had significantly lower copies of JCV (410.5±44.3 and 658.3±53.3 copies/μg DNA respectively) than TCs (981.5±14.0, p<0.05). When they were divided into three groups with 0–200 copies/μg DNA (low), 201–1,000 (moderate) and more than 1,001 (high), TCs showed 3 (7.6%) in the low group, 21 (53.8%) in the moderate group and 15 (38.4%) in the high group and glossitis showed 11 (73.3%) in the low group, 0 (0%) in the moderate group and 4 (26.6%) in the high group. The DTEs occupied an intermediate position between them (p<0.001). In situ PCR demonstrated that the nuclei of TC and DTE cells are sporadically T-antigen positive but not in nasal turbinate epithelial cells. Immunohistochemistry for T-antigen protein revealed four positive cases only in TCs. The existence of JCV T-antigen DNA was not associated with the clinicopathological variables of TCs. In conclusion, the presence of JCV may be a risk factor of tongue carcinogenesis.

Keywords Tongue cancer · JC virus · Carcinogenesis

Introduction

John Cunningham virus (JCV), firstly isolated from the brain of patients with progressive multifocal leukoencephalopathy [12], is a member of the polyomavirus group together with simian virus 40 (SV40) and BK virus. These share major characteristics, such as non-enveloped virions, double-stranded circular DNA genomes, and oncogenic potential under the appropriate conditions [7]. It has been generally thought that JCV has neurotropic oncogenicity both in rodents and humans [14]. The JCV infects a large proportion of the population worldwide, and 80–90% of all adults are seropositive for the virus. Primary infection occurs during childhood and remains asymptomatic with the virus persisting quiescent in the kidneys and lymphoid tissues. However, JCV may be activated when the immune system is impaired [18]. JCV can be detected in tonsillar tissue [10], and the respiratory and digestive tracts are thought to be the primary sites for JCV to enter human body. A recent report described that lung tissue expresses
abundant levels of JCV receptor-type sialic acids which mediate JCV infection [5].

JCV has been detected in human brain tumors [2, 3] and also in several epithelial malignancies, such as colon, prostate, and esophageal carcinomas [4, 6, 19]. A transgenic mouse strain expressing the JCV T antigen is reported to exhibit putitary tumors in approximately 50% of the animals by 1 year of age, some progressing to malignant peripheral nerve-sheath tumors [14]. T antigen is considered to play an important role in JCV oncogenesis as it interferes with two tumor suppressor proteins that regulate cell-cycle progression, including pRb and p53. T antigen binds to pRb and displaces E2F, thereby promoting cell-cycle progression and the inappropriate cell proliferation of ontogenetically transformed cells [9]. The release of E2F from Rb normally activates p14ARF expression which leads to stabilization of p53. However, T antigen binds to and inactivates p53 to prevent inhibition of the cell cycle, providing an optimal cell environment for viral replication and packaging during the JCV lytic infection and then facilitating the transformation in non-permissive cells [17]. T antigen can increase the stability of β-catenin and cause its accumulation by directly binding so that the protein then translocates to the nucleus where it enhances the expression of such genes as c-myc and cyclin D1, which are typical of the deregulation in the Wnt signaling pathway [8]. In this paper, we examined JCV-targeting T antigen in combination with real-time polymerase chain reaction (PCR), in situ PCR, and immunohistochemistry to clarify the oncogenic role of JCV in tongue carcinogenesis.

Materials and methods

Cases

Formalin-fixed and paraffin-embedded samples were selected from the pathological archive of the University of Toyama Hospital from 1982 to 1999 with informed consent of the patients [39 cases of tongue squamous cell carcinoma (TC), 58.5±15.2 years (mean±SD), male/female=23:16; 15 cases of dysplastic tongue epithelium (DTE), 60.2±10.4 years, 11:4; 15 cases of glossitis; 67.7±9.8 years, 9:6]. Among the 39 TCs, 14 cases had lymph node metastasis, and the average longitudinal tumor size was 3.1±0.95 cm (mean±SD). The staging for each tongue carcinoma was evaluated according to the Union Internationale Contre le Cancer system showing stage I in 7 cases, stage II in 15, stage III in 11, and stage IV in 6. Twenty-seven patients were followed-up for more than 5 years and are still alive, but 12 patients died due to tumor progression within 5 years. The Ethics Committee of the Hospital gave approval for genetic experiments restricted to JCV.

DNA extraction

Paraffin-embedded specimens were cut into consecutive 10 μm sections for microdissection with reference to hematoxylin-and-eosin staining. Then tissues were deparaffinized in xylene followed by incubation with proteinase K and sodium dodecyl sulfate at 55°C overnight. DNA was extracted using phenol–chloroform–isoamyl alcohol (GAPDH) primers (sense, 5'-ACATCAAAGAAGGT GGTGAGC-3' and anti-sense, 5’-CTTGGTCCTGAGCC AAATCGT-3'; 199 bp) by 35 cycles of denaturation at 95°C for 60 s, annealing 53°C for 60 s, and extension at 72°C for 60 s followed by an additional 7 min at 72°C to confirm the integrity of the DNA. All reactions were modulated using the Gene Amp 9700 PCR System™ (Applied Biosystem, Forster City, USA). Ten-microliter aliquots of each PCR product were examined by electrophoresis on 8% polyacrylamide gels. Finally, the gels were stained with ethidium bromide and photographed under ultraviolet light. The cases examined in the present study were limited to the ones that showed a clear single band of GAPDH.

Quantitation of JCV DNA by real-time PCR

A real-time, fluorescence probe-based PCR method was employed for quantitative assessment of JCV in samples using the Mx3000P™ Real-Time PCR system (Stratagene, La Jolla, CA, USA). For establishment of the quantitative technique, a JCV-containing plasmid (pBS-JCVMad1, kindly provided by Associate Professor H Sawa, Department of Neuropathology, Hokkaido University, Graduate School of Medicine) was serially diluted and served as a standard reference. This standard and DNA samples of TCs, DTEs, and nasal turbinate epithelial cells (NTEs) were subjected to PCR amplification of the 128-bp sequence of the T-antigen using RT-JCV1, nucleotides 3492–3511, 5'-GCCACCCCAGCCTATTG-3' and RT-JCV-2, nucleotides 3619–3595, 5'-GTTGACATATCCATGCCAC AGAA-3' as the forward and reverse primers, respectively. The monitoring of the amplicon development was performed using a double-dye probe, RT-JCV-3, nucleotides 3515–3539, 5’-GAGCCACGGCATATGG-3'. The reactions were performed using the 2x TaqMan® Universal PCR Master Mix (Applied Biosystems). Reaction mixtures (25 μl) contained 12.5 μl TaqMan® Universal PCR master mix 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
95°C for TaqMan® DNA polymerase activation followed by 60 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 30 s.

In situ PCR

Sections (10 μm thick) were digested with proteinase K (10 μg/ml) for 15 min at 37°C, then topoisomerase I was applied for 30 min at room temperature. After rinsing with phosphate-buffered saline (PBS), the tissue was fixed in 4% neutralized paraformaldehyde and subjected to washing with 2× saline sodium citrate (SSC). Then a 125-μl aliquot of the PCR solution (1 μM primers of JCT-1A, nucleotides 3052–3069, 5′-CGTAAAGTTCTAGGCA-3′ and JCT-1AS, nucleotides 3225–3207, 5′-AGTCAAGGGATTACCTTC-3′, 200 μM DIG-11-dUTP, 4.5 mM MgCl₂, PCR buffer, 2 U Taq polymerase) was placed on the tissue under membrane sealing, and then PCR was performed on the slide griddle of a programmable thermal controller: 94°C for 3 min, followed by 15 cycles of 92°C for 15 s, 55°C for 15 s, 72°C for 30 s, and finally 72°C for 5 min. These primers result in the amplification of a 174-bp fragment of JCV. In the next step, the tissue was washed with 2× SSC and incubated with blocking solution, 100 μg/ml Salmon Testis DNA (Sigma, Germany), 10 μg/ml yeast tRNA (Sigma), and 5% bovine serum albumin in PBS, for 1 h. Finally, the sections were incubated with an anti-digoxigenin antibody coupled to alkaline phosphatase overnight, followed by DAKO® Fuchsin + Substrate-Chromogen System (DakoCytomation) and then were counterstained with methyl green. JCV infected neuroblastoma (JCI) cells (kindly provided by Dr. Kazuo Nagashima, Hokkaido University, Sapporo, Japan) were used as positive controls in this experiment.

DNA direct sequencing for PCR products

PCR amplicons were further confirmed by direct sequencing because there is some homology between polyomaviruses SV40, BK virus, and JCV [20]. After 35 cycles of PCR under the same conditions as real-time or in situ PCR, DNA was purified on MicroSpin Columns (Amersham Biosciences) before sequencing according to the manufacturer’s instructions using a BigDye® Terminator v3.1 cycle sequencing kit and an ABI PRISM® 3100 genetic analyzer (Applied Biosystems). All sequences were analyzed for homology using the nucleotide–nucleotide “BLAST” search feature located on the National Center for Biotechnology Information Web site.

Immunohistochemistry

For immunohistochemistry, 4 μm thick sections of formalin-fixed and paraffin-embedded TCs, DTEs, NTEs, and JCI cells were deparaffinized with xylene, dehydrated through an alcohol gradient, immersed in heated target-retrieval buffered solution (DAKO) with intermittent microwave irradiation for 15 min. A methanol solution with H₂O₂ was applied for 5 min to block endogenous peroxidase. The primary antibody used was a mouse monoclonal anti-simian virus T antigen that cross-reacts with JCV T antigen (1:100 dilution; clone Pab 101; Santa Cruz Biotecology, Santa Cruz, CA, USA). The tissue was incubated with the primary antibody overnight, followed by exposure to DAKO EnVision™ Labeled Polymer (DAKO) for 30 min. Staining was developed by a reaction with diaminobenzidine chromogen, and counterstaining for 1 min with hematoxylin was then performed.

Statistical analysis

A statistical evaluation was performed using the chi-square test to compare the positive rate and Mann-Whitney U test to differentiate the non-parametric means. SPSS 10.0 software was employed to analyze all data, and p<0.05 was considered to be statistically significant.

Results

Real-time PCR for T antigen was successful with all cases examined with a quantitative standard using plasmid pBS-JCVMad1. The PCR products for the T-antigen sequence amplified by real-time PCR were verified to be JCV (not the BK or SV40 species of polyomavirus) by DNA sequencing. The viral DNA loads were 981.6±14.0 copies/μg DNA (mean±SE; standard error range 37.8–2,338.4) in TCs, 658.3±53.3 copies/μg DNA (3.5–2,883) in DTEs and 410.5±44.3 copies/μg DNA (0–2,070.0) in glossitis. There were significant differences between TCs and DTEs and glossitis (p<0.05, Fig. 1a). When the JCV copy numbers in the TCs, DTEs and glossitis were divided into three groups, low (0–200 copies/μg DNA), moderate (201–1000), and high (≥1001), there were significant differences among them (p<0.01, Table 1). Figure 1b,c, and d show the average copy numbers in each group. The value for the control of JCI cells was 4.39×10⁶ copies/μg DNA. In the TCs, the correlation between JCV copy number (low, 0–1000; high, ≥1001) and such clinicopathological variables as tumor size, stage, differentiation, and lymph node metastasis was examined, but no correlation was observed (Table 2).

In situ PCR amplification and immunohistochemical staining was accompanied by a positive control for JCV using the genomic DNA from JCI cells, which persistently is infected with JCV. In situ PCR demonstrated clear positive staining in 20–30% of the JCI cells (Fig. 2a). Some
positive cells were also noted in DTEs (Fig. 2b). In some cases, positive cells were sporadically found in areas of the TCs (Fig. 2c). In the glossitis, no signals were observed in any of the cases. However, each of these PCR amplicons was confirmed further through DNA sequencing; and in each instance, the presence of JCV was validated. In this study, we observed JCV T-Ag expression occasionally in TC samples (4 of 39, 10.3%). Figure 3 shows the immunohistochemical data obtained from JCI cell line and TC. Positive nuclear staining was observed in the positive control JCI cells (Fig. 3a). The nuclear expression pattern of T antigen also appeared in TC (Fig. 3b), and no detection of T antigen was noted in glossitis. Direct cycle sequencing indicated no variation in the DNA sequences of PCR amplicons (data not shown).

Discussion

In the present study, the results of real-time PCR for determining JCV T-antigen load revealed significantly higher levels in TCs compared with DTEs and glossitis.

An earlier report described that colon cancers and adenomas similarly demonstrated a higher JCV T-antigen load calculated by real-time PCR than background tissue or normal colonic mucosa [15]. From the available data, the viral DNA loads in formalin-fixed and paraffin-embedded samples in colon [15], gastric [11], and lung [20] cancers appear to be similar to our finding. According to the results of the real-time PCR, the JCV copy number was calculated to be around one copy per ten cells in TCs on the basis of the DNA extraction procedure from oral squamous cell carcinoma cells (about 100,000 cells/μg DNA, data not shown). If the presence of JCV is associated with the development of cancer, then one might expect to find one virus genome per human genome in cancer tissue but not in normal tissue. In a previous study of gastric cancers using fresh frozen samples, the JCV DNA loads were found to be about 4,800 copies/μg DNA in gastric cancers, about 5,400

Table 1  JCV viral DNA loads divided into low, moderate and high groups in TCs, DTEs, and glossitis

| Groups | 0–200 copies/μg DNA | 201–1,000 copies/μg DNA | ≥1001 copies/μg DNA |
|--------|---------------------|------------------------|---------------------|
| TC     | 3                   | 21                     | 15                  |
| DTE    | 5                   | 7                      | 3                   |
| NTE    | 11                  | 0                      | 4                   |

Table 2  Correlation between the clinicopathological variables and JCV viral DNA loads

| Groups | 0–1000 copies/μg DNA | 1001– copies/μg DNA | p value |
|--------|----------------------|--------------------|---------|
| Size   |                      |                    | >0.05   |
| Stage  | 0–II                 | 14                 | 8       |
|       | III, IV              | 10                 | 7       |
| Differentiation | Well | 16 | 8 | >0.05 |
|       | Mod, poor            | 8                  | 7       |
| Metastasis | −    | 17 | 8 | >0.05 |
|       | +                    | 7                  | 7       |
in paired adjacent noncancerous tissues, and 540 in normal gastric tissues. When the corresponding samples were processed by formalin fixation and paraffin embedding, the JCV DNA load was reduced to one sixth in gastric cancers, 1/20 in adjacent non-cancerous tissues, and one fifth in normal gastric mucosa [11]. The value of 981.5 copies/μg DNA in TCs in the present study may thus correspond to 4,900 (×5) to 19,630 (×20) in fresh frozen samples. Those numerical values of JCV copies may indicate its oncogenic role in the malignancies, including TCs.

When the viral DNA loads were divided into three groups, low, moderate and high, the TCs included 15 high cases (38.4%), 21 moderate (53.8%), and 3 low (7.7%), on the contrary, glossitis had only 4 high cases (26.6%) and almost none of JCV DNA in the other 11 cases. DTEs occupied an intermediate position between them. It is possible that a JCV infection of the glossitis is a major risk factor for tongue carcinomas, and some of them develop into dysplasia and finally carcinoma. In fact, Epstein–Barr virus was not detected by in situ RNA hybridization in our cases. It was the same for human papilloma virus type 16 and 18 by PCR (data not shown). The presence of high viral DNA loads was not correlated with any clinicopathological variables that may support the hypothesis that JCV infection is not directly linked to cancer progression. However, it is still possible that JCV may play some role in the oncogenesis of tongue carcinoma.

While Zambrano et al. [19] used in situ hybridization (ISH) to reveal the sporadic presence of JCV in normal prostatic glandular epithelium, data on the actual infection levels were not available. No signals were detected by ISH in the current study except in the positive controls (data not shown), possibly due to the small copy number. In contrast, in situ PCR can detect even a few viral copies per cell [16], and sporadic nests of positive cells were detected in TCs and DTEs but not in glossitis, which is consistent with the viral DNA loads observed. T-antigen protein expression was observed by immunohistochemistry in only four cases of TCs and DTE as opposed to the observations of Shin et al. [13]. Although we employed the antibody against SV40 T antigen, our data should be reliable enough to confirm the JCV localization because we evaluated the immunolabeling positivity of JCV T antigen taken together with the data of real-time PCR. In these experiments, it was difficult to prove the clear positivity of T-antigen protein, but the antibody used in the present study had limited success. It is thought that the half-life of T-antigen protein might be very short, and it is not easy to detect the T-antigen positivity...
when formalin-fixed and paraffin-embedded tissues are used.

An earlier study demonstrated the presence of replicating JCV DNA in B lymphocytes from peripheral blood, tonsil, and spleen, and it has been hypothesized that lymphocytes may thus be one site of JCV persistence [10]. The detection of viral gene products in renal tubules and the excretion of JC virions in the urine indicate JCV persistence in the kidney [1]. In the present study, JCV DNA contamination from JCV persistent B lymphocytes in the oral mucosa, which contain B lymphocytes, could not be ruled out. However, the majority of JCV-infected cells observed by in situ PCR were tonsil stromal cells rather than B lymphocytes with using tonsil tissues (data not shown), which is consistent with the findings of Monaco et al. [10].

In conclusion, the presence of JCV in normal tongue tissue may be a risk factor for tumorigenesis of tongue carcinoma, and the possibility that JCV may play some oncogenetic role can thus not be ruled out. Further investigations to explore this possibility therefore appear to be warranted.

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