Human Papilloma Virus (HPV) Infection is an Etiologic Factor of Tonsillar Carcinoma

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INTRODUCTION

The prevalence of human papillomavirus (HPV) infection is high in the oropharyngeal mucosal regions, of which the tonsil is the most commonly affected. There may be a link between HPV and the pathogenesis of TC, because of common anatomical characteristics between cervical and tonsillar cancer (TC). We aimed to clarify whether HPV directly affects the oncogenesis and biologic behavior of TC by making a comparison between infection prevalence, physical status and viral loading numbers, and clinicopathologic prognostic factors.

MATERIALS AND METHODS

Selection of Tissue Samples; Samples from 52 patients were collected from archived, paraffin-embedded, tonsillar squamous cell carcinoma registry from the Yonsei University Medical School, Department of Pathology and head and neck oncology division of Otolaryngology during the period between January 1995 and May 2005. A total of 69 tonsillectomy samples for chronic follicular tonsillitis (CFT) were selected as control group. There were no significant differences in demographic data including age, sex between two groups.

HNA Extraction ; QiaAmp DNA minikit (Qiagen, USA, CA). The quality (ratio of 260/280 nm) and quantity (absorbance at 260 nm) of isolated DNA were determined by optical density measurement.

HPV genotyping ; HPV genotyping DNA chip (Biocore, Korea, Seoul) arrayed by multiple oligonucleotide probes of L1 sequence of 26 types of HPV.

Real-time PCR ; A TaqMan-based 5′-exonuclease quantitative real-time PCR assay based on DNA amplification of a 76 bp sequence of the E2 ORF and a 81 bp sequence of the E6 ORF in the presence of HPV-16 E2- and E6-specific hybridization probes.

Tissue Microarray ; Recipient blocks were made from purified agar in 3.8 x 2.2 x 0.5 cm frames. Consecutive 4-μm-thick sections were cut from the recipient blocks using an adhesive coated slide system (Instrumedics, Inc., New Jersey).

To compare HPV-related molecules between TC and CFT, p16, survivin, HIF-1a, skp-1, cyclin A, cyclin B1, c-myc and EGFR were investigated.

Imunohistochemistry ; Immunoperoxidase staining was performed using the streptavidin-biotin peroxidase complex method (LSAB universal kit, DAKO, Carpinteria, CA). For negative controls, the antibodies were replaced with the equivalent amounts of the subtype-matched normal mouse IgG. Immunostaining was graded and scored as follows: negative (0%), 1+ (weak and focal), 2+ (weak diffuse or strong and focal staining), 3+ (strong and diffuse).

Fluorescent In situ hybridization ; Two-color FISH was done on 3.5-μm consecutive sections from the same TMA paraffin blocks. Hybridization signals were enumerated by the ratio of orange signals (for c-myc) to green signals (for CEP8) in morphologically intact and non-overlapping nuclei. At least a 3-fold increase of the c-myc signals over CEP8 signals in the tumor cells was considered the criterion for gene amplification.

Statistical Analysis; Fisher's exact test with SAS software, version 9.1 (SAS Institute Inc., Cary, NC, USA).

RESULTS

HPV prevalence in TC and CFT ; HPV was detected in 38/52 (73.1 %) of the TC. Thirty-four (89.5 %) of the HPV positive tumors were HPV-16 positive, and the rest of four samples were infected by non-16 high risk types. Among the 69 CFT specimens, HPV were detected in 8 patients (11.6 %): Three was HPV-16, and the rest of them were infected by HPV-58 and low risk types of HPV-6, HPV-11 or HPV-84.

Viral physical status *Investigation;* HPV-16 integration was found in 94.1% of HPV-16 positive tonsillar cancers. These include 5 specimens (14.7%) without detectable E2 sequence, indicating complete integration if viral genes into host genome, and 27 specimens (79.4%) with E2/E6 ratio between 0 and 1, which were indicative of presence of mixed integrated and episomal forms. Pure episomal form was found in only 2 samples (5.9%) in 34 HPV-16 positive tonsillar cancers. Among 32 HPV-16 non-episomal status samples, 31 cases showed predominance of the viral integrated form, according to the value less than 1 of the ratio E2 to integrated E6. All three samples of HPV-16 positive CFT showed episomal forms.

Pathologic Correlation according to HPV infection ; For the verification of the speculation that HPV associated tonsillar SCC originates from the cryptal epithelium, whereas non-HPV related SCC emerge from the surface epithelium, we classified TC into as inverted tumors arising from the crypt and fungating or veruccoid tumors directly arising from the surface on macroscopic and microscopic reviews (Fig. 1). The crypt-originated TCs tend to grow centrifugally in a radiation manner (Fig. 2A), with multinodularity (Fig. 2B), and occasionally invading to the surface epithelium (Fig. 2C) in a pagetoid manner (Fig. 2D). Of the total of 52 TC specimens, 30 originated from the cryptal epithelium, 12 from the surface epithelium and 8 from both crypt and surface (mixed form). In the remaining 2 cases, it was not impossible to verify its origin. Of the 34 HPV-16 positive tumors, 27 cases originated from the cryptal epithelium, 1 cases from the surface epithelium and 6 cases from both crypt and surface (mixed form). The different phenotypes of TC according to HPV infection status was statistically significant ($p<0.0001$ by Fisher's exact test). Tumor grading was relatively poorer in case of HPV-positive group ($p= 0.0106$ by Fisher's exact test).

Molecular comparison according to HPV infection ; On analysis of p16 immunohistochemistry, p16 expression was variably positive predominantly in nuclei (Fig. 3). In this study, 71.2% of TC exhibited strong and diffuse nuclear staining for p16, whereas there were no p16 expressing cells in CFT. The p16 overexpression was significantly associated with HPV infection ($p<0.0001$ by Fisher's exact test) and histologic grading ($p=0.0426$). On analysis of c-myc fluorescent in situ hybridization, c-myc amplification was seen in 25% (Fig. 4). The c-myc amplification was significantly associated with HPV-16 integration status ($p=0.034$) and histologic grading ($p=0.036$).

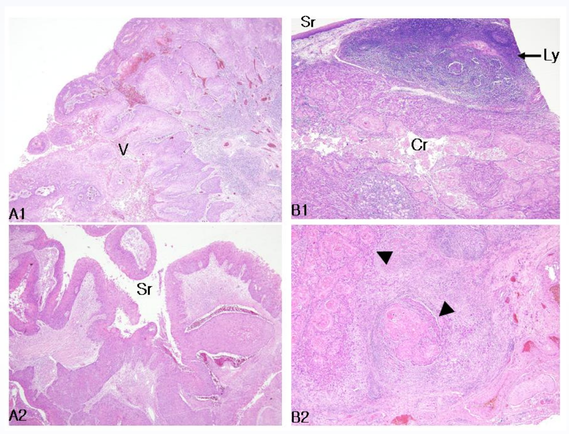

Fig. 1. Microscopical difference between surface-originated and crypt-originated tonsillar cancer. A1: Veruccous or papillary changes are restricted to the surface (labeled as V). A2: Carcinomatous changes are totally involving the whole tonsillar surface epithelium (Sr). B1: Papillary changes are restricted to the adjacent to the crypt (labeled as Cr) densely packed lymphoid cells (labeled as Ly). Notice a normal-looking surface epithelium (Sr) in contrast to A1. B2: Multilobular pattern (arrowheads) of growth is characteristic of TC arising from the crypt.

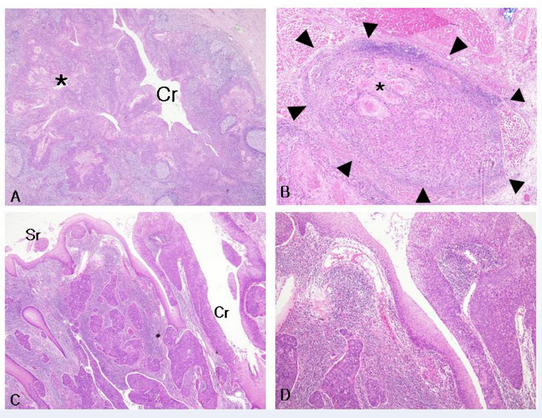

Fig. 2. Microscopic findings to suggest the crypt origin in tonsillar cancer. A: Early carcinoma (labeled as asterisk) develops from the squamous epithelium lining the crypt [labeled as Cr]. B: Lobular arrangement (arrowheads) is another evidence of cryptal origin. Notice well-developed keratinizing pearls (asterisk). C: Underlying nested cancer tissues invade the tonsillar surface (labeled as Sr). Carcinoma in situ was recognized predominantly in the crypt epithelium (labeled as Cr), but spared in the surface epithelium (Sr). D: Higher magnification of carcinoma in situ along the cryptal epithelium

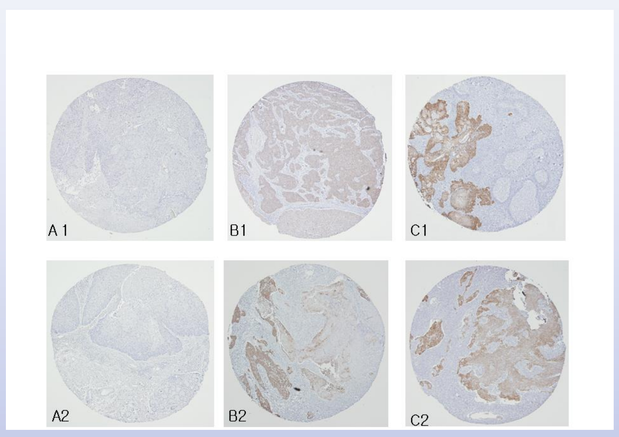

Fig. 3. Immunohistochemical results of p16 from tissue microarray. A1 and A2: negative staining for p16, B1, B2: grade 2 staining (diffuse weak or focal strong) for p16, C1 and C2: grade 3 staining (diffuse strong) for p16.

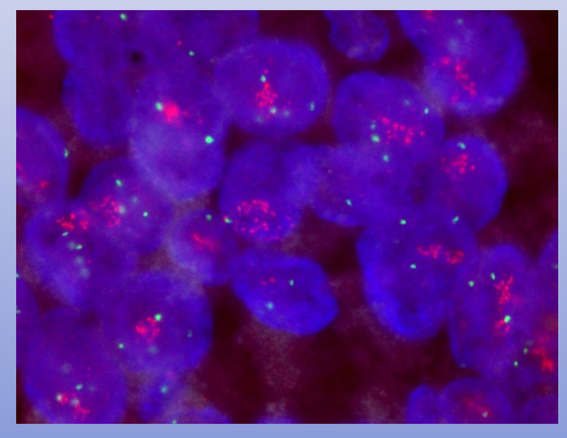

Fig. 4. Fluorescent in situ hybridization (FISH). FISH shows increased c-myc gene copy numbers in tonsillar cancer (orange, c-myc; green, CEP8 control).

CONCLUSION

HPV-16 integration could be directly related to tonsillar carcinogenesis initially in tonsillar crypts followed by cell cycle aberration, such as p16 overexpression related to the G1-S phase and amplification of c-myc oncogene.