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

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 tonsillar 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 typing: 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, surivin, HIF-1α, skp-1, cyclin A, cyclin B1, c-myc and EGFR were investigated.

Immunohistochemistry: 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 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 CEPI) in morphologically intact and non-overlapping nuclei. At least a 3-fold increase of the c-myc signals over CEPI signals in the tumor cells was considered the criterion for gene amplification.


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 was detected in 8 patients (11.6 %). Three were 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 91.4% of HPV-16 positive tonsillar cancers. These include 5 specimens (14.7 %) without detectable E2 sequence, indicating complete integration of 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 observed 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 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 verrucoid 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 (Fig. 3A and B). 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<0.001 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).

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.