Detection of Human Papillomavirus and p53 Protein in Oral Verrucous Carcinomas — Preliminary Report —

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ABSTRACT. Objective: To investigate coexpression of the human papillomavirus (HPV) and p53 protein in oral verrucous carcinomas (OVCs). Materials and Methods: Eight surgical specimens from five Japanese OVC patients who were treated in the Department of Oral Surgery of Kawasaki Medical School between 1975 and 1997 were studied using immunohistochemistry (IHC) and the consensus primer-mediated polymerase chain reaction (PCR). Results: None of OVCs was found to have p53-immunoreactivity by IHC, and none of the cases was HPV-positive by the PCR. Of interest was a case of HPV-negative and p53-negative oral papilloma which turned into an HPV-negative and p53-negative OVC at the same site. Conclusions: It seems possible that the oral cavity may not be susceptible to HPV-induced verrucous carcinomas with p53 protein overexpression. Furthermore, detection of p53 expression by IHC is thought to have great potential as a biomarker of the proliferating cells. Therefore, p53-negativity in OVCs as shown in this study may explain why OVC patients have significantly better survival than patients with oral squamous cell carcinomas, which often have a higher p53-positivity.

Key words: oral verrucous carcinoma — human papillomavirus — p53

High-risk human papillomavirus (HPV) type 16 is the most commonly found virus in oral squamous cell carcinomas, accounting for 2.6 to 35% of cases.[1-3] Studies on oral verrucous carcinomas (OVCs), a relatively rare variant of squamous cell carcimoma of low-grade malignancy, have found an HPV type 16-positivity of between 0[4,5] and 15%,[6] using the polymerase chain reaction (PCR) plus in situ DNA hybridization (ISH),[4] and ISH.[5,6] HPV type 18 was detected by PCR in one study.[7] Using immunohistochemistry (IHC), which is less sensitive than the PCR or ISH, 30% of laryngeal verrucous carcinomas in a small series of cases were found to be both HPV and p53-positive.[8] Binding to the p53 protein by the HPV-E6 open reading frame leads to absence of a checkpoint in the G1-phase of the cell cycle that has been shown to result in loss of its ability to function as a tumor suppressor.[9] To date, however, there has been no agreement on HPV-p53 correlation in OVCs. Further studies in this regard have been awaited, and we conducted this investigation.

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MATERIALS AND METHODS

Among 314 head and neck cancers treated in the Department of Oral Surgery of Kawasaki Medical School between 1975 and 1997, we found five

TABLE 1. Clinical data and evaluation of cases for detection of HPV and p53 protein

Case No.	Age/Gender	Smoking/Drinking	Primary site	TNM	Initial treatments	Outcomes	p53 stain	L1-PCR	Koliocyte
1	54/F	No/No	Buccal mucosa	T1N0M0	Excision	NED 113 mo	Ne	Ne	Yes
2	65/M	No/Yes	Tongue	T2N1M0	Excision UND RCIF	NED 77 mo	Ne	Ne	No
3	79/F	No/No	Tongue	T1N0M0	Excision	NED 40 mo	Ne	Ne	No
4	92/F	No/No	Palate-Buccal mucosa	T4N0M0	NT	EFO	Ne	Ne	No
5	77/F	No/No	Gingiva	T2N0M0	Excision	NED 99 mo	Ne	Ne	No

Stain : immunostaining, PCR : polymerase chain reaction, UND : upper neck dissection, RCIF : reconstruction with cervical island flap, NED : no evidence of disease, NT : not treated, EFO : excluded from outcome, Ne : negative

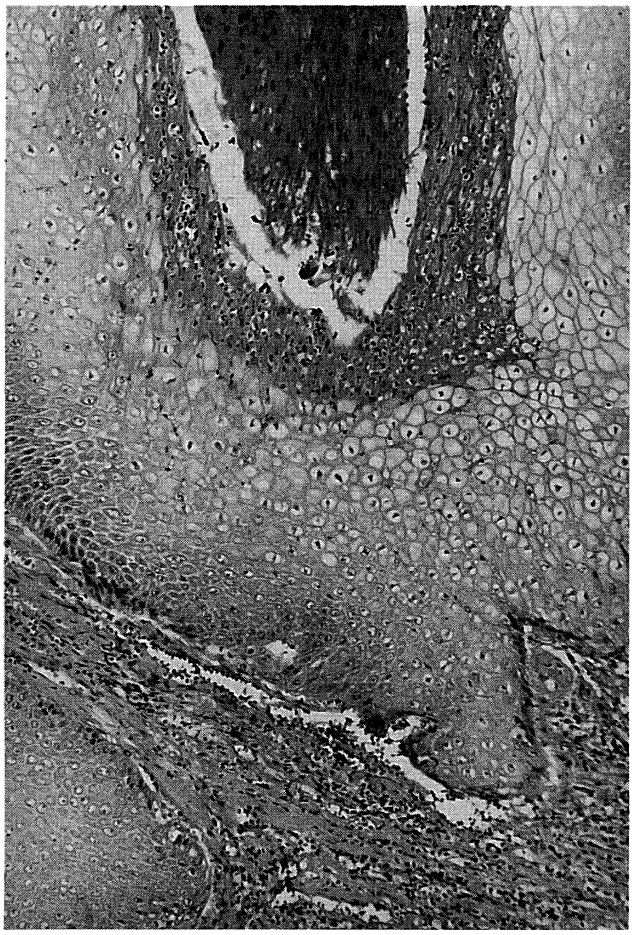

Fig 1. Histological findings of an oral verrucous carcinoma (case #5, H.E.×100).

patients with OVC, to which IHC and the consensus primer-mediated PCR were applied for the detection of HPV and p53 protein (Table 1). IHC for p53 protein is widely available and the consensus primer-mediated PCR for HPV is more sensitive than IHC and ISH. Interestingly enough, one of the patients (case #5) had had four lesions biopsied, which were histologically diagnosed as oral papilloma of the right lower gingiva, OVC of the right lower gingiva, leukoplakia of the tongue and verruca vulgaris of the left lower gingiva, respectively. Then the papilloma of the lower gingiva turned into an OVC at the same site after two years (Fig 1).

(1) immunohistochemical analysis

All tissue specimens were either biopsied or surgically removed, fixed in formalin, routinely processed, and embedded in paraffin. Then 3 μm thick sections were used for immunohistochemical staingig with the streptavidin-biotin complex method. Endogenous peroxidase activity was blocked with 0.1% H_2O_2 for five minutes. Citric acid (0.01 M) was used in conjunction with a microwave treatment (five times for five minutes at 600 W) for p53 antigen retrieval. The primary antibody against p53 was the monoclonal anti-p53 (DO-7, 1 : 40, Novocastra, U.K.), which recognizes both mutant and wild type p53 protein. As a positive control for p53, we used sections of colonic adenocarcinoma. As a negative control, the primary antibodies were replaced with buffer.

(2) Consensus primer-mediated PCR analysis

The PCR with consensus primers for the Ll region (Ll-PCR) has amplified at least nine HPV types; namely, 6, 11, 16, 18, 31, 33, 42, 52, 58, and the amplified HPV DNA could be typed by subsequent restriction mapping.[10] Briefly, total cellular DNA from these specimens was extracted according to the proteinase K procedure. The consensus primers for the Ll region were synthesized according to the previously described method of Yoshikawa *et al.*[10] The Ll-PCR employed in this study consisted of 40 cycles of denaturation (95°C, 1.5 min), annealing (48°C, 1.5 min) and extension (72°C, 2 min) on a DNA Thermal Cycler PJ-2000 (Perkin Elmer Cetus, U.S.A.). Each reaction mixture (100 μl) contained 50 mM KCl, 10 mM Tris-Cl pH 8.4, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 1 μM of each primer and four units of Taq DNA polymerase (Roche Diagnostic Systems, U.S.A.). After the reaction, one-tenth (10 μl) of the reaction mixture was electrophoresed through 3% NuSieve 3 : 1 agarose (FMC BioProducts, U.S.A.) gel containing 0.5 μg/ml ethidium bromide and was photographed after visualization by UV light. If amplified, HPV fragments could be typed on the basis of the restriction fragment length polymorphisms among HPVs. Rsa I, Dde I and Hae III (Takara Shuzo, Japan) were used to type the HPV fragments amplified by the Ll-PCR, as previously described.[10] Aliquots of genomic DNA isolated from the SiHa cell line were used as the positive control for HPV type 16.

RESULTS

Immunohistochemical analysis showed none of the OVCs to be p53-positive, despite the positive control (Fig 2A, B). No HPV-positive cases were found by the PCR (Fig 3). In addition, both the oral papilloma and the OVC which developed at the same location in case #5 were negative for HPV and

p53, as were the leukoplakia and verruca vulgaris found in different locations in the same patient.

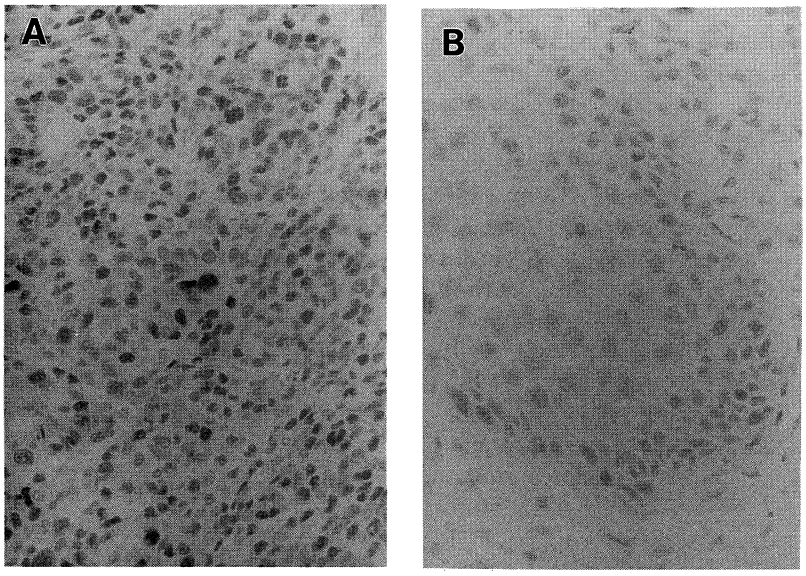

Fig 2. Immunohistochemical analysis for p53 protein (×200)
(A) Positive control (B) None of the OVCs was p53-positive.

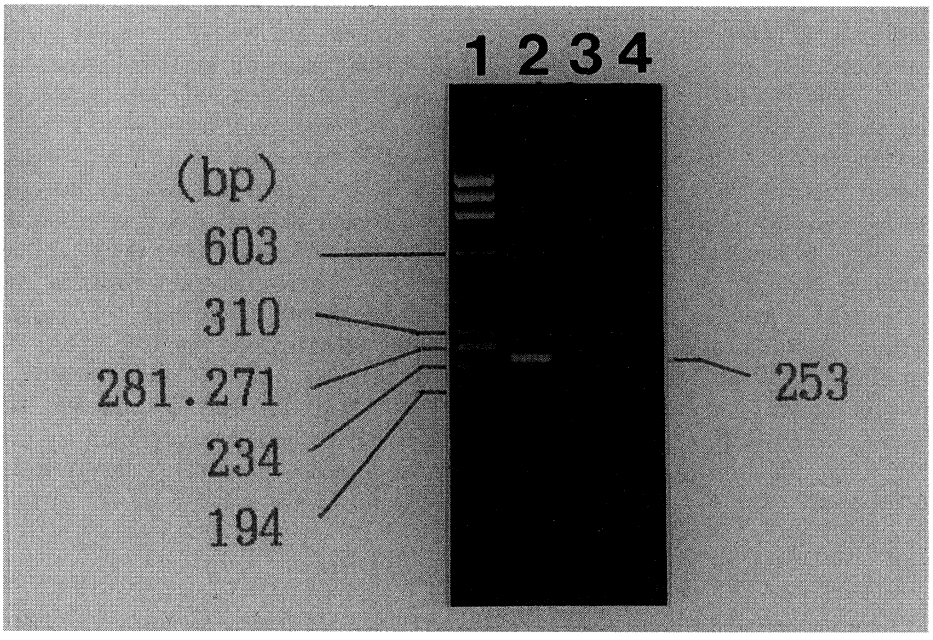

Fig 3. Ll-PCR analyiis for HPV.
lane 1, size markers (ϕ×174 digested with HaeIII); lane 2, positive control (SiHa cell line); lane 3, negative control (buffer); lane 4, patient sample. None of the HPV fragments was amplified by Ll-PCR.

DISCUSSION

HPV DNA has been detected in oral premalignant lesions and oral squamous cell carcinomas, although no biopsied normal epithelia from healthy oral mucosas have been found to be positive for HPV by PCR analyses.[2] These findings may suggest that HPV is not just a passenger in the carcinogenesis of oral squamous cell carcinomas. Another study has found that oral squamous cancer cell lines that transcribe very low levels of p53 transcripts but contain no p53 protein do not contain HPV type 16/18 DNA and that mutations such as insertions and deletions of the p53 gene in these cell lines may be linked to the absence of p53 protein.[11] In this context, it may be suggested that HPV is not always a critical factor in the carcinogenesis of p53-mutated oral squamous cell carcinomas. Mutations of the p53 gene were not examined in this study, but such mutations of the p53 gene may be responsible for both the HPV and p53-negative OVCs in this study.

Another reason for the absence of p53 protein in IHC may be methodological differences. We used a standard immunohistochemical method for p53 protein using 10% formalin-fixed tissue with a microwave treatment for p53 antigen retrieval. In a recent report,[12] absolute ethanol fixation without microwave enhancement was found to maintain and usually increase the strength of positive staining for p53 protein. Regarding the L1-PCR for HPV, it can detect 0.01 pg of DNA of HPV types 6, 11, 16, 18, 31, 33 and 52,[10] and has equal sensitivity to Southern blot hybridization, the 'gold standard' which can detect an average of 0.1 - 0.2 copies of HPV DNA per cell.[13] Therefore, results from the L1-PCR are reliable.

Geographical and ethnical differences in the HPV positivity of oral squamous cell carcinomas have been pointed out.[1] The HPV positivity of OVCs in Western population was between 0[4,5] and 30[8]%. The HPV positivity of OVCs in this study was 0%. The study of HPV positivity of OVCs in Japan will be needed.

As to differences in the primary sites of head and neck, 30% of laryngeal verrucous carcinomas had both p53 protein and HPV expression using immunohistochemistry. It seems possible that the larynx cound be susceptible to HPV-induced verrucous carcinomas with p53 protein overexpression,[8] while the oral cavity may not be. Furthermore, detection of p53 expression by IHC is thought to have great potential as a biomarker of the proliferating cells.[14] Therefore, p53 - negativity in OVCs as shown in this study may explain why OVC patients have significantly better survival than patients with oral squamous cell carcinomas, which often have a higher p53 - positivity.[14] However, our results cannot exclude the possibility that HPV can act as an initiator in the carcinogenesis of OVCs and then disappear (hit-and-run theory).[15] Of course, we know that the number of cases we studied is too small to conclude this. We stress that further studies including mutational analysis of the p53 gene with a large number of cases should be undertaken for better understanding.

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