

Human papillomavirus infection in oral verrucous carcinoma: genotyping analysis and inverse correlation with p53 expression

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Running title: HPV genotyping and p53 expression in oral verrucous carcinoma

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## **Key Words**

HPV · Genotype · Oral verrucous carcinoma · p53 · Tumorigenesis

## **Abstract**

**Objective:** Verrucous carcinoma (VC) is a rare subtype of squamous cell carcinoma, occurring mostly in oral mucosa. To clarify the role of human papillomavirus (HPV) in VC tumorigenesis, we investigated localization and genotypes of HPV, and p53 expression in oral VC. **Methods:** We studied paraffin-embedded specimens of 23 VCs and 10 control non-neoplastic lesions in oral mucosa. To investigate HPV infection, HPV genotypes, and p53 expression, we respectively employed *in situ* hybridization (ISH), sequence analysis following short PCR fragment (SPF)-PCR assay, and immunohistochemistry. **Results:** Of the 23 VC specimens, 11 (48%) had HPV-DNA (detectable by PCR), and 6 (26%) had intranuclear HPV in the upper portion of the squamous epithelium (detectable by ISH). Nine of the 11 PCR positive specimens showed multiple infections with low- and high-risk HPVs. No HPV-16 infection was detected. Although HPV-6 and -18 were frequently detected by PCR, no HPV could be found in control specimens by ISH. p53 expression was inversely correlated with HPV infection. **Conclusion:** Thus, multiple infections with low- and high-risk HPVs and their rapid replication during hyperkeratinization may participate in the histogenesis of oral VC. Oral VC tumorigenesis may involve the inactivation of p53, which is associated with HPV infection.

## **Introduction**

Human papillomavirus (HPV) is an oncogenic virus frequently associated with uterine cervical carcinoma worldwide. The prevalence of HPV infection in cervical carcinomas is 75–100% [1-6]. Nearly 100 different types of HPV have been described. They can be classified on the basis of whether they cause benign or malignant tumors into a high-risk group (HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 54, 56, 58, 59, 66, 68, and 69) and low-risk group (HPV-6, 11, 26, 30, 34, 40, 42-44, 53, 55, 57, 61, 62, 64, 67, 70, 71, 73, 74, 79, and 81-84) [7].

HPV infection is associated with a wide variety of oral lesions such as squamous cell papilloma (SCP), epithelial dysplasia, and squamous cell carcinoma (SCC). However, in contrast to the consistently high prevalence of HPV infections in uterine cervical lesions, their prevalence in oral lesions is highly variable, *e.g.*, 0–78% for oral SCC [8-23]. This variability may stem from differences in the methods used to detect HPV-DNA such as PCR, Southern blot hybridization, and *in situ* hybridization (ISH).

Up to 75% of all verrucous carcinomas (VCs), a rare variant of SCC, occur in the oral mucosa [24]. Oral VC is an exophytic, warty, slowly growing malignant tumor with pushing margins, and histologically it consists of thickened club-shaped papillae and blunt stromal invaginations of well-differentiated squamous epithelium with marked keratinization. Unlike conventional SCC, VC has squamous epithelium without histological malignant features and rare mitosis mostly in the basal layers [24]. Therefore, VC is histologically more difficult to distinguish from SCP than from SCC.

The possible involvement of HPV in the pathogenesis of VC is suggested by the

prevalence of HPV in VC, which varies widely (0–100%) [10, 11, 20, 25, 26]. In addition, there are few reports about HPV genotype analysis of VC. The aims of our study was 1) to elucidate the association of VC with HPV infection by determining HPV genotypes in retrieved, paraffin-embedded oral VC specimens and 2) to assess the importance of HPV in oral VC carcinogenesis by examining the correlation between HPV infection and immunohistochemical p53 expression.

## **Materials and methods**

### *Tissue specimens*

For the ISH, HPV genotyping, and immunohistochemistry, we retrieved 23 oral VC specimens from the files of the Division of Oral Pathology and Bone Metabolism, Unit of Basic Medical Sciences, Course of Medical and Dental Sciences, Nagasaki University Graduate School of Biomedical Sciences. The male-to-female ratio was 15:8, and mean age at the first visit was 71.3 years. The primary sites of the tumor were buccal mucosa (7 cases), gingiva (6), tongue (4), lips (2), oral floor (2), and palate (2). All tumor samples were removed when they were in clinical tumor stage I. Histological structure of an oral VC specimen stained with hematoxylin and eosin is shown in Fig. 1. Control non-neoplastic specimens included fibroepithelial polyp (3 cases), mucous extravasation phenomenon (3), inflammatory polyp (1), epulis fibrosa (1), epulis granulomatosa (1), and acute inflammation (1) from 10 patients (male: female=3:7; mean age at first visit: 46.7). All control lesions were covered with mucosal squamous epithelium. Excisions in all cases were performed at Nagasaki University Hospital, and specimens were fixed in neutral

buffered 10% formalin and embedded in paraffin.

#### *In situ hybridization (ISH)*

For the morphological detection of HPV-DNA, 3  $\mu\text{m}$ -thick paraffin sections were mounted on organosilane-coated glass slides. HPV screening was performed using a detection kit (Kreatech Diagnostics, Amsterdam, Netherlands). The pan-HPV probe labeled with digoxigenin used in our study was composed of a mixture of HPV types 6, 11, 16, 18, 31, and 33. The deparaffinized, washed, and air-dried VC and control sections were digested with pepsin work solution for 30 min at 37°C. The washed and air-dried slides were exposed to the pan-HPV probe and covered with coverslips, placed on a 95°C hotplate for 5 min, immediately transferred into a moist environment, and incubated overnight at 37°C. The sections rinsed with TBS buffer were incubated with alkaline phosphatase conjugated anti-digoxigenin antibody. For visualization of HPV-DNA, NBT/BCIP substrate was added to each specimen for 15 min at 37°C. The sections were counterstained with nuclear fast red, and mounted in an aqueous medium.

#### *DNA isolation from paraffin-embedded tissue*

To isolate the DNA from the paraffin blocks, three to five 10- $\mu\text{m}$ -thick paraffin sections were collected in a 1.5-ml tube. The microtome blade was changed after cutting each specimen under the cleanest possible conditions. We used the DNA isolator PS kit (Wako Pure Chemicals Industries, Ltd., Osaka, Japan). Briefly, the sections were deparaffinized in xylene, washed with 70% ethanol, and digested with protease. The DNA

was precipitated in isopropanol and washed with 70% ethanol. The dried DNA was dissolved in 20 µl of TE buffer.

#### *General primer-mediated HPV-PCR and sequencing*

General primer-mediated PCR and subsequent sequencing was performed for the detection and typing of HPV-DNA. All samples were subjected to PCR using PuReTaq Ready-to-Go PCR Beads (Amersham Biosciences Corp., Piscataway, NJ, USA) with 5 µM SPF primers located in the L1 open reading frame of the HPV genome (Table 1). The SPF system allowed detection of at least 43 different HPV genotypes, and had high sensitivity even in the paraffin-embedded samples [27, 28]. The PCR amplifications were carried out in a thermal cycler (Bio-Rad, Hercules, CA, USA) under the following conditions: 94°C for 9 min; 45 cycles of 30 sec at 94°C, 45 sec at 45°C, and 45 sec at 72°C; and a final extension of 5 min at 72°C. Amplified products were separated on a 3% Agarose 21 gel (Wako) and detected with ethidium bromide. The 65-bp short PCR fragments (SPF) were purified using the QIAEX II Gel Extraction Kit (Qiagen, Inc. Valencia, CA, USA) according to the manufacturer's instructions, cloned in the pGEM-T Easy vector (Promega Corp., Madison, WI, USA), and transformed into *Escherichia* DH5 alpha competent cells (Promega). Plasmids were isolated from several independent colonies using the Gene Elute Plasmid Miniprep Kit (Sigma-Aldrich Japan, Tokyo, Japan) and their inserts were sequenced using an Auto-Read Sequencing kit (Amersham Pharmacia Biotech, Inc. Piscataway, NJ, USA) and an ALF DNA Sequencer II (Amersham Pharmacia Biotech, Inc.). The sequences of the 22-bp interprimer region in the PCR products were compared

with GenBank database sequences using the BLAST program.

### *Immunohistochemistry*

To evaluate the correlation of HPV infection with the expression of tumor-suppressor p53 protein, we examined immunohistochemical expression of p53 in paraffin sections of oral VC and control lesions. After the retrieval of p53 antigen in 0.01M citrate buffer (pH: 6.0) by heating (121°C, 10 min) in an autoclave, immunostaining was carried out using p53 monoclonal antibody (DakoCytomation Co Ltd., Kyoto, Japan) and EnVision + system (DakoCytomation). The color reaction was developed with 3,3'-diaminobenzidine (Sigma-Aldrich Japan). The sections were counterstained with hematoxylin, dehydrated, and mounted in a synthetic mounting medium.

## **Results**

### *ISH for detecting HPV*

ISH detected HPV-DNA in 6 of 23 oral VC specimens (26%) (table 2). HPV-DNA was localized in the nuclei of neoplastic epithelial cells in the upper part of the spinous layer and keratinized layer. The signals for HPV-DNA were intense, particularly in the invaginated neoplastic epithelium of the VC. Signals were moderately intense in the keratinized layer of the papillary cell columns (Fig. 2). On the other hand, no HPV-DNA was detected in the non-neoplastic squamous epithelium in all 10 control specimens (table 2).

### *HPV genotyping*

Eleven of the 23 oral VC specimens (48%) were screened for HPV-DNA using the SPF-PCR method. From the DNA sequences of purified PCR products, HPV genotypes were identified in these 11 cases (Fig. 3 and Table 2). The results of HPV genotyping are summarized in Table 3. SPF-PCR detected high-risk HPV-18 in 10 of 23 VC specimens (43%), followed by HPV-6 (39%), HPV-74 (9%), and HPV-11 and -33 (4%). Many specimens (39%) were multiply infected; six specimens were infected with HPV-6 and -18, one with HPV-6, -18, and -33, one with HPV-6, -18, and -74, and one with HPV-11, -18, and -74. A single infection was detected in only two cases: one with HPV-6 and one with HPV-18. The multiple infections contained high-risk HPV: HPV-18 and -33.

In control specimens, HPV-DNA was detectable and HPV genotypes identifiable in 7 cases (70%) (table 2). Six specimens were multiply infected (HPV-6 and -18, 5 cases; HPV-11 and -18, 1 case), and 1 specimen was monoinfected (HPV-18) (table 3). Infection with both HPV-6 and HPV-18 was more frequent.

The Fisher's exact probability test revealed no significant difference in HPV prevalence (based on SPF-PCR data) between the oral VC and control non-neoplastic lesions ( $p > 0.05$ ).

### *Immunohistochemistry for p53*

p53 was immunohistochemically detected in 16 cases of oral VC (70%) (table 2). Many specimens showed nuclear expression focally or diffusely localized in the basal and/or suprabasal cells, and some tumors exhibited p53 expression in the differentiation



sequence from basal cells to prickly cells. The specimens with extensive diffuse expression of p53 had no detectable HPV-DNA (Fig. 4). The Fisher's exact probability test indicated that p53 expression was correlated inversely with HPV infection detected by PCR ( $p < 0.01$ ) and ISH ( $p < 0.01$ ). In three control specimens, p53 (30%) was expressed in focal basal cells and/or suprabasal cells. In control specimens, there was no significant correlation between p53 expression and HPV infection based on PCR ( $p > 0.05$ ) (Table 4).

## **Discussion**

HPV is an oncogenic virus and its oncogenicity has been well documented for squamous cell carcinoma of the uterine cervix. HPV promotes the development of cervical cancer *in vivo* and can immortalize cervical epithelial cells *in vitro* [29, 30]. HPV-infected epithelial cells produce E6 and E7 oncoproteins, which can inactivate the tumor-suppressor functions of p53 and RB genes, respectively [31, 32]. Interactions of the HPV oncoproteins with the cell cycle proteins, such as cyclin D, cyclin E, p16, p21, and p27 are involved in the activation or repression of cell cycle progression in cervical carcinogenesis [33]. Cells infected with high-risk HPVs are more capable of performing these oncogenic functions than cells infected with low-risk HPVs [34].

In this study, HPV-DNA was detected in 26 and 48% of VC specimens, using ISH and PCR, respectively. This difference was evidently due to differences in the ISH and PCR methods. We also detected a high prevalence of multiple HPV infections by DNA sequence analysis, *i.e.*, 82% of HPV-positive VC and 86% of HPV-positive control specimens were multiply infected, especially with both low-risk HPV-6 and high-risk

HPV-18. We previously found a similarly high proportion of multiple HPV infections in penile cancer specimens [28]. Thus, SPF-PCR with HPV- DNA sequencing appears to be a superior method for the detection of multiple infections.

Interestingly, HPV-16 was not identified in our study. The HPV-16 genotype is an important predictor of transformation of infected cells into malignancy, and is associated with oral SCC [2, 9-12, 14, 17-19, 35-37]. Kingsley *et al.*, who studied an oral SCC cell line transfected with HPV-16, found that HPV-16 can measurably increase proliferative potential and adhesion to fibronectin. Their report suggests that HPV-16 is the inducer of cell proliferation and infiltration into the surrounding stroma [38]. Surprisingly, Shroyer *et al.* used PCR followed by DNA slot-blot hybridization to demonstrate the participation of low-risk HPV-6 and -11 [26], and Mitsuishi *et al.* used PCR with sequence analysis and restriction fragment polymorphism analysis to show the participation of high-risk HPVs other than HPV-16 [25]. Tracking an oral VC patient longitudinally, Lubbe *et al.* identified HPV-11 (a low-risk virus) in an early-stage biopsy and HPV-16 in a late-stage biopsy specimen [39]. Taken together, the results of our study and previous investigations suggest that HPV-16 is not initially related to oncogenesis of oral VC but rather to progression of the tumor, which may therefore require activation after secondary infection with HPV-16. The absence of HPV-16 in our study might be due to the fact that all our biopsy or excision specimens were obtained during the early stage of VC development.

The correlation between histological characteristics and low-risk HPV infection in oral VC is not clear. Nevertheless, we speculate that low-risk HPV infection may induce exophytic or warty proliferation histologically in oral VC, as it does in SCP.

The successful detection of HPV by ISH is thought to be dependent on the amount of HPV-DNA. In SCP, immediately after HPV infection in the basal and suprabasal cells, early HPV proteins are synthesized from uncoated viral DNA, and steady-state viral DNA replication occurs in these cells. Later, rapid DNA replication, capsid protein production, and assembly of the virion particles take place in spinous, granular, and keratinized cells, and virion particles are released from the surface keratinized cells. For replication of the virus DNA and assembly of the virus particles, differentiation of squamous epithelial cells, *i.e.*, keratinization, must occur [40]. In VC, nuclear localization of HPV-DNA occurs in the upper spinous layer and keratinized layer, but not in the basal layer of the squamous epithelium. Presumably, the amount of HPV-DNA in the basal layer and lower spinous layer was too low to be detected by ISH in our study. However, the SPF-PCR method was sensitive enough to detect HPV-DNA in 70% of control non-neoplastic oral tissues and 48% of oral VC tissues. We conclude that the HPV in HPV-infected epithelial cells of control tissues does not enter into a rapid DNA replication cycle. The squamous epithelia lining the control lesions, unlike VC lesions, showed only mild keratinization. The lack of rapid HPV-DNA replication in the non-neoplastic lesions may be related to poor or absent keratinization. Conversely, it was reported that HPV E7 protein induces hyperplasia of the squamous epithelium [41]. Therefore, our results suggest that HPV in the infected basal cells and/or suprabasal cells of the control group is inactive.

Our PCR procedure showed high rate of the HPV infection (70%) in non-neoplastic control lesions. Six SPF primers used in our PCR method could detect at least 43 different HPV genotypes, and this SPF system had high sensitivity even in the

paraffin-embedded samples [27, 28]. Actually, HPV infection in the normal oral mucosa has been described [12, 14, 20-22, 25, 36, 42]. The prevalence rate of HPV including high-risk HPV such as HPV-16, -18 in the normal squamous epithelium was 0–67%. Thus, the high prevalence rate of HPV in our control group was not a unique finding. We hypothesize that the HPV-DNA in non-neoplastic oral epithelium is in a steady-state and inactive, *i.e.*, unable to synthesize the oncoproteins that induce epithelial hyperplasia and keratinization.

Inactivation of tumor-suppressor p53 protein plays an important role in cervical carcinogenesis [31, 32]. Our examination demonstrated the inverse correlation between HPV infection and p53 expression, and inactivation of p53 was suggested as crucial in the tumorigenesis of oral VC. Similarly, Cheng *et al.* immunohistochemically showed inverse correlation of HPV infection with p53 expression in lung cancer specimens [43].

In conclusion, histogenesis of oral VC appears to involve multiple infections with high- and low-risk HPVs, especially high-risk HPV-18 and low-risk HPV-6. The undetectability of HPV-16, a virus with an effective transformation function, in oral VC specimens suggests to us that it is not directly involved in the oncogenesis of oral VC. Development of oral VC may require rapid HPV replication and activation during hyperkeratinization of the oral epithelium as well as inactivation of p53 tumor-suppressor protein involved in HPV infection. Moreover, using DNA extraction from paraffin-embedded tissue specimens, the highly sensitive SPF-PCR procedure and sequence analysis can be applied retrospectively in the study of HPV genotypes in surgical and biopsy materials.

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Table 1. Short PCR fragment (SPF) primers for HPV.

SPF primers	Sequence 5'→3'	Position
SPF1A	GCiCAGGGiCACAATAATGG	6582-6601
SPF1B	GCiCAGGGiCATAACAATGG	6582-6601
SPF1C	GCiCAGGGiCATAATAATGG	6582-6601
SPF1D	GCiCAAGGiCATAATAATGG	6582-6601
SPF2B	GTiGTATCiACAACAGTAACAAA	6624-6646
SPF2D	GTiGTATCiACTACAGTAACAAA	6624-6646

i: inosine

Assay from Kleter *et al.*, 1998. [27]

Table 2. Results of ISH, HPV genotyping, and p53 expression of oral VC and control lesion specimens.

No.	Age/sex	ISH	HPV genotype	p53 expression*	No.	Age/sex	ISH	HPV genotype	p53 expression*
Oral VC									
1	71/F	+	6	-	13	81/M	-		++
2	85/F	-	11, <u>18</u> , 74	-	14	68/M	-		++
3	83/M	-	6, <u>18</u>	+	15	79/M	-		+
4	37/M	+	6, <u>18</u>	+	16	71/F	-		++
5	77/F	+	6, <u>18</u>	-	17	84/M	-		+++
6	75/M	-		++	18	75/M	-		+
7	39/M	-		++	19	54/M	-		+
8	65/F	-		+	20	88/M	-	6, <u>18</u> , <u>33</u>	+
9	59/M	-	6, <u>18</u>	-	21	81/M	+	6, <u>18</u>	-
10	75/M	-		+++	22	76/F	-	<u>18</u>	+
11	78/M	-		++	23	75/F	+	6, <u>18</u> , 74	-
12	65/M	+	6, <u>18</u>	-					
Control non-neoplastic lesion									
1	10/F	-	6, <u>18</u>	-	6	69/F	-	6, <u>18</u>	+
2	25/F	-	6, <u>18</u>	-	7	69/M	-		+
3	62/M	-		-	8	20/F	-	6, <u>18</u>	-
4	8/F	-	11, <u>18</u>	-	9	65/M	-		+
5	74/F	-	6, <u>18</u>	-	10	65/F	-	<u>18</u>	-

Underlined HPV genotypes are categorized as high-risk types.

\* -: negative.

+: expression localized in basal cells and/or suprabasal cells.

++: expression extending from basal cells to lower half of the epithelium.

+++ : expression extending from basal cells to more than the lower half of the epithelium.

Table 3. Infection rate of HPV in oral VC and control lesions.

HPV genotype	Number of positive cases (infection rate)
Oral VC	
HPV-6	9 (39%)
HPV-11	1 (4%)
HPV- <u>18</u>	10 (43%)
HPV- <u>33</u>	1 (4%)
HPV-74	2 (9%)
HPV-6 and - <u>18</u>	8 (35%)
Control non-neoplastic lesion	
HPV-6	5 (50%)
HPV-11	1 (10%)
HPV- <u>18</u>	7 (70%)
HPV-6 and - <u>18</u>	5 (50%)

Underlined HPV genotypes are categorized as high-risk types.

Table 4. Correlation of p53 expression with HPV infection in oral VC and control lesions.

Parameters	p53 expression		<i>P</i>
Oral VC			
	Negative (n=7)	Positive (n=16)	
HPV-DNA detected by PCR			
Negative (n=12)	0	12	
Positive (n=11)	7	4	0.0014
HPV-DNA detected by ISH			
Negative (n=12)	2	15	
Positive (n=11)	5	1	0.0034
Control non-neoplastic lesions			
	Negative (n=7)	Positive (n=3)	
HPV-DNA detected by PCR			
Negative (n=3)	1	2	
Positive (n=7)	6	1	0.3662

## Figure legends

Fig. 1. Histological features of VC. (a) The lesion shows exophytic papillary proliferation of markedly keratinized squamous epithelium. Broad blunt and well-differentiated rete ridges extend into the submucosa. (b) The rete ridges have pushing margins, and the basement membrane remains intact. Chronic inflammatory cells infiltrate in the underlying connective tissue.

Fig. 2. ISH for pan-HPV in oral VC (Case #21). HPV-DNA is predominantly in the upper portion of the neoplastic squamous epithelium (a) and within the nuclei (b).

Fig. 3. Sequence analysis of HPV-DNA. Arrows indicate the inserted HPV-DNA. (a), (b), and (c) are part of the sequences of HPV-6, -11, and -18, respectively.

Fig. 4. Immunohistochemistry for p53. (a) Intense expression is observed in the nuclei from the basal cells to near the surface of the squamous epithelium. HPV-DNA is not detected in this case (Case #17). (b) The epithelium shows no p53 expression. This figure shows a serial section of the Fig. 2 specimen (Case #21), which is infected with HPV.

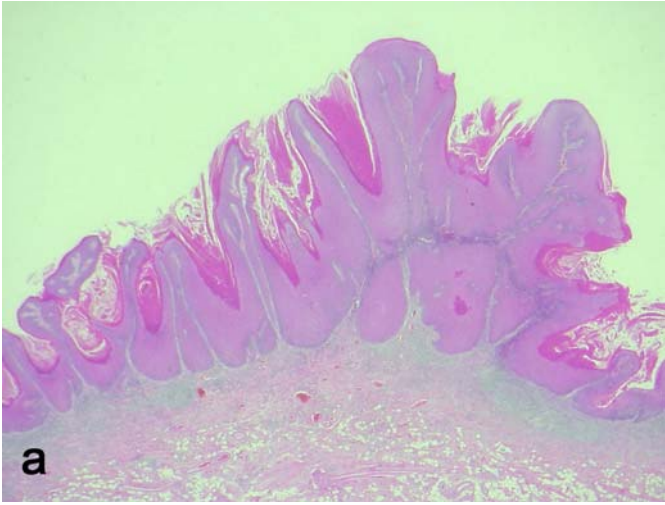


Fig. 1a

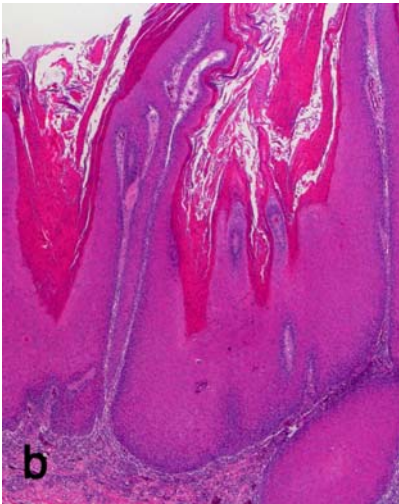


Fig. 1b

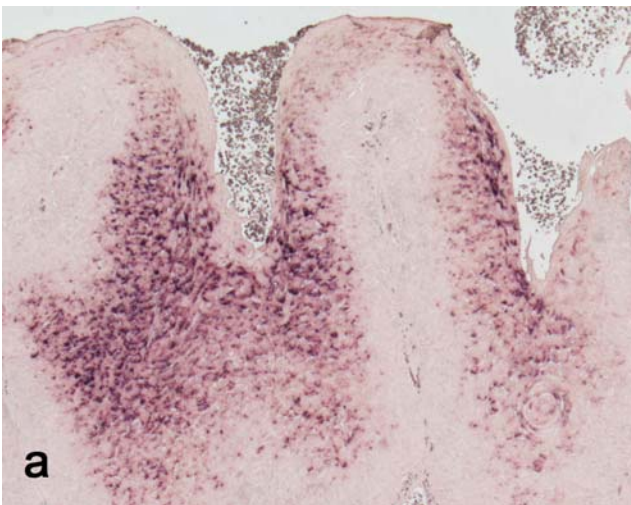


Fig. 2a



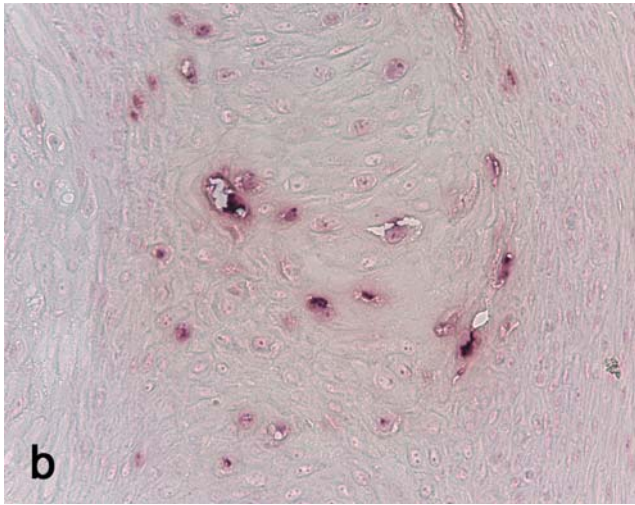


Fig. 2b

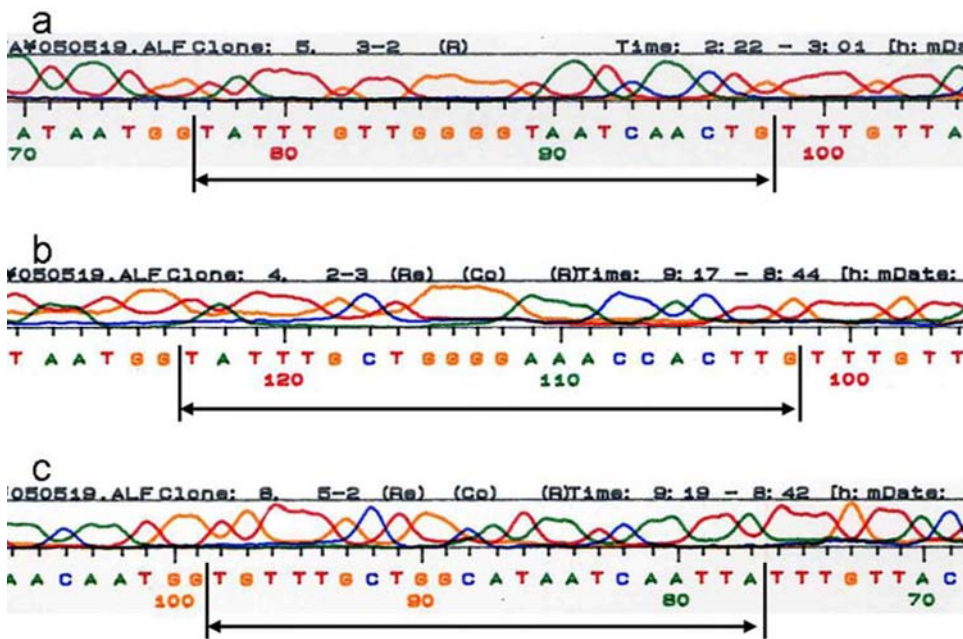


Fig. 3

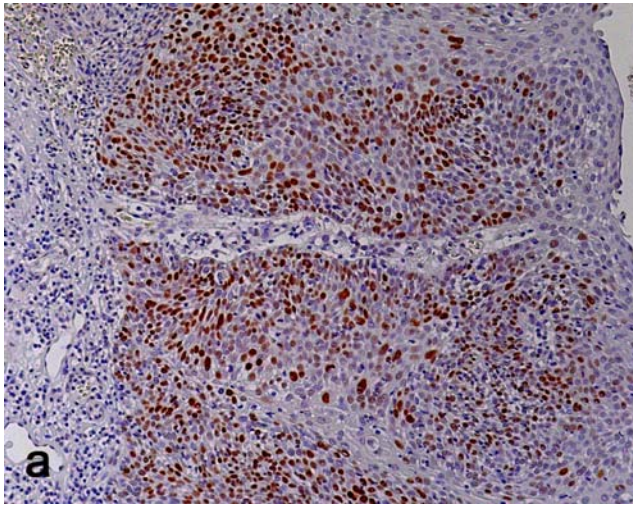


Fig. 4a

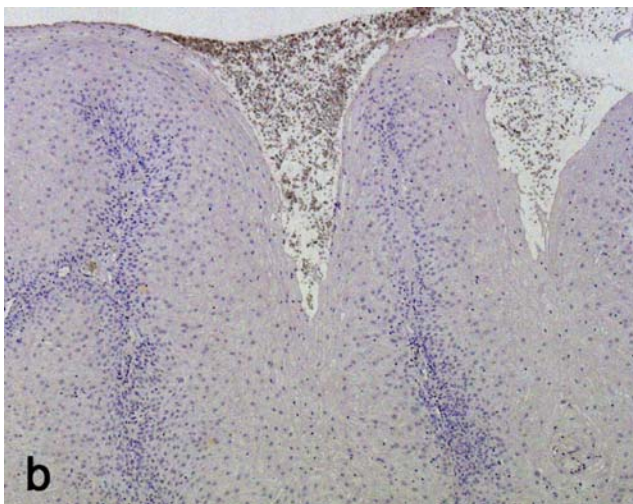


Fig. 4b