

## DETECTION OF HUMAN PAPILLOMAVIRUS AND CELLULAR REGULATORS p16<sup>INK4A</sup>, p53, AND NF-κB IN PENILE CANCER CASES IN KENYA

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**Summary.** – Human papillomavirus (HPV) E6 and E7 gene products play a central role in the induction of benign proliferation and malignant transformation by interacting with several cellular regulatory proteins such as p53, p16<sup>INK4a</sup>, and nuclear factor κB (NF-κB). In this study, HPV DNA was detected by *in situ* hybridization (ISH) and p53, p16<sup>INK4a</sup>, and NF-κB by immunochemistry in 22 penile cancer cases in Kenya. HPV DNA was found in 68.2% of the cases. There was no difference in the p53- and p16<sup>INK4a</sup>-positivities in HPV DNA-positive and HPV DNA-negative cases. In HPV DNA-positive cases, the NF-κB positivity in the nucleus, cytoplasm, and nucleus and/or cytoplasm amounted to 73.3%, 93.3%, and 100%, respectively, while in HPV DNA-negative cases, a 28.7% NF-κB positivity of in the nucleus and/or cytoplasm was observed. It is concluded that NF-κB in penile cancer is expressed more frequently in the presence of HPV infection than in its absence.

**Keywords:** Human papillomavirus; penile cancer; p16<sup>INK4a</sup>; p53; NF-κB; Kenya

### Introduction

HPV infection is associated with a broad spectrum of benign and malignant neoplastic epithelial changes. The association of HPVs with neoplastic transformation has been most extensively investigated in lesions of the uterine cervix and the role of HPVs in malignant transformation of the cervical epithelium has been well established. Although several epidemiological, clinical, and pathologic studies have indicated that this virus is sexually transmitted, the association of HPVs with penile cancer is not so much understood than that with a cervical cancer. The virus can affect the

squamous epithelium of the male genitalia in a similar way as the female genital tract (Gross and Pfister, 2004; Senba *et al.*, 2006; Tornesello *et al.*, 2008).

HPVs onco-proteins E6 and E7 are essential factors for HPVs oncogenesis. E7 and E6 react with the tumor suppressor gene products p53 and pRb proteins in host cell resulting in an induced cellular immortalization, transformation, and carcinogenesis due to their interference with cell cycle and apoptosis control (Munger *et al.*, 2004). The p16<sup>INK4a</sup> protein is a cyclin-dependent kinase inhibitor that decelerates the cell cycle by inactivating cyclin-dependent kinase inhibitors. The p16<sup>INK4a</sup> protein prevents the phosphorylation of pRb family members. Usually, overexpression of p16<sup>INK4a</sup> results in the inhibition of E2F-dependent transcription that inhibits cell cycle progression from the G1 to S phase (Zhang *et al.*, 1999). The nuclear and cytoplasmic overexpression of p16<sup>INK4a</sup> protein has been reported for some cancers (Sano *et*

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**Abbreviations:** HPV = Human papillomavirus; IκB = inhibitor κB; ISH = *in situ* hybridization; NF-κB = nuclear factor κB

*al.*, 1998; Klaes *et al.*, 2001; Humbey *et al.*, 2003; Ferreux *et al.*, 2003; Prowse *et al.*, 2008).

The causal relationship between chronic inflammation and cancer is widely accepted. Numerous investigations have identified NF- $\kappa$ B as an important modulator in driving chronic inflammation to cancer. This transcription factor is indispensable for the malignant progression of transformed cells associated with various inflammatory cells and a network of signaling molecules. The expression and the function of numerous cytokines, chemokines, growth factors, and survival factors are NF- $\kappa$ B-dependent. NF- $\kappa$ B activation has been implicated in a variety of processes related to the transformation and oncogenesis (Kiriakidis, *et al.*, 2003).

At the beginning of experiments, we tried to detect HPV genotypes, but the HPV DNA could not be extracted from paraffin embedded cancer tissues. Therefore, ISH was used to confirm HPV presence and to determine a localization of HPV within the penile cancer tissues. The purpose of this study was to determine the prevalence of HPV in penile cancer and the subsequent overexpression of p16<sup>INK4a</sup>, p53, and NF- $\kappa$ B.

## Materials and Methods

**Tissue specimens.** We studied biopsy materials of 22 cases of penile cancer among specimens that were submitted for pathologic diagnosis to the Department of Histopathology, Rift Valley Provincial General Hospital from various hospitals in Kenya (Western, Nyanza, and Rift Valley provinces, years 1983–1998). This investigation was authorized by the Government of Kenya (research permit No. OP.13/001/8C224/36). The specimens were fixed in 10% formalin and embedded in paraffin for histology, immunohistochemical analyses, and ISH. Histological analysis was performed using 3.5  $\mu$ m sections of tissue stained with hematoxylin and eosin. Penile cancer was classified as keratinizing squamous cell carcinoma or non-keratinizing squamous cell carcinoma. Parallel sections were prepared also for ISH.

**HPV DNA detection by ISH.** Paraffin-embedded tissue specimens were cut into 3.5  $\mu$ m sections and collected on silane-coated glass slides. For the detection of HPV DNA, a HPV screening detection kit (Kreatech Diagnostics) was used. Pan HPV DNA probe,

ISH-positive control probe, ISH-negative control probe, and HPV-positive control slides (supplied with the kit) were examined. The detection kit used a digoxigenin-labeled pan HPV-encoded DNA probe made of a mixture of HPV 6, 11, 16, 18, 31, and 33. ISH-negative control was a digoxigenin-labeled DNA probe derived from plasmid DNA (pSP) that did not contain any sequences of human or viral origin. After hybridization with the probes, the alkaline phosphatase conjugated antibody against digoxigenin was applied to the sections. The localization of HPV DNA was detected using NBT/BCIP substrate and observed under a light microscope.

**Immunohistochemical detection of p16, p53, and NF- $\kappa$ B.** Sections of 3.5  $\mu$ m in thickness were placed on silane-coated glass slides that were deparaffinized and dehydrated. Immunohistochemical analysis of p16<sup>INK4a</sup> was performed using a p16<sup>INK4a</sup> research kit containing monoclonal antibody E6H4 (MTM Laboratories) following manufacturer's instructions. For p53, the slides were then boiled in 0.01 mol/l citrate buffer pH 7.0 for 5 mins for antigen retrieval and cooled at room temperature for 30 mins. The slides were then rinsed in 0.01 mol/l PBS pH 7.4, and the endogenous peroxidase activity was blocked with 3% H<sub>2</sub>O<sub>2</sub> and absolute methanol for 10 mins. The tissue sections were covered with mouse monoclonal antibodies anti-human p53 (Dako) and anti-NF- $\kappa$ B (Cell Signaling Technology) diluted 1:50 or with a control serum at 37°C for 3 hrs. After washing with PBS, the sections were covered with EnVision (Dako) at 37°C for 40 mins and rinsed again with PBS. Antigenic sites on sections were demonstrated by the reaction with mixture of 0.05% 3, 3'-diaminobenzidine tetrahydrochloride in 0.05 mol/l Tris-HCl buffer and 0.01% hydrogen peroxide for 10 mins. The sections were then counterstained with methyl green for 10 mins, dehydrated in ethanol, cleared in xylene, and mounted. Results are evaluated using a light microscope.

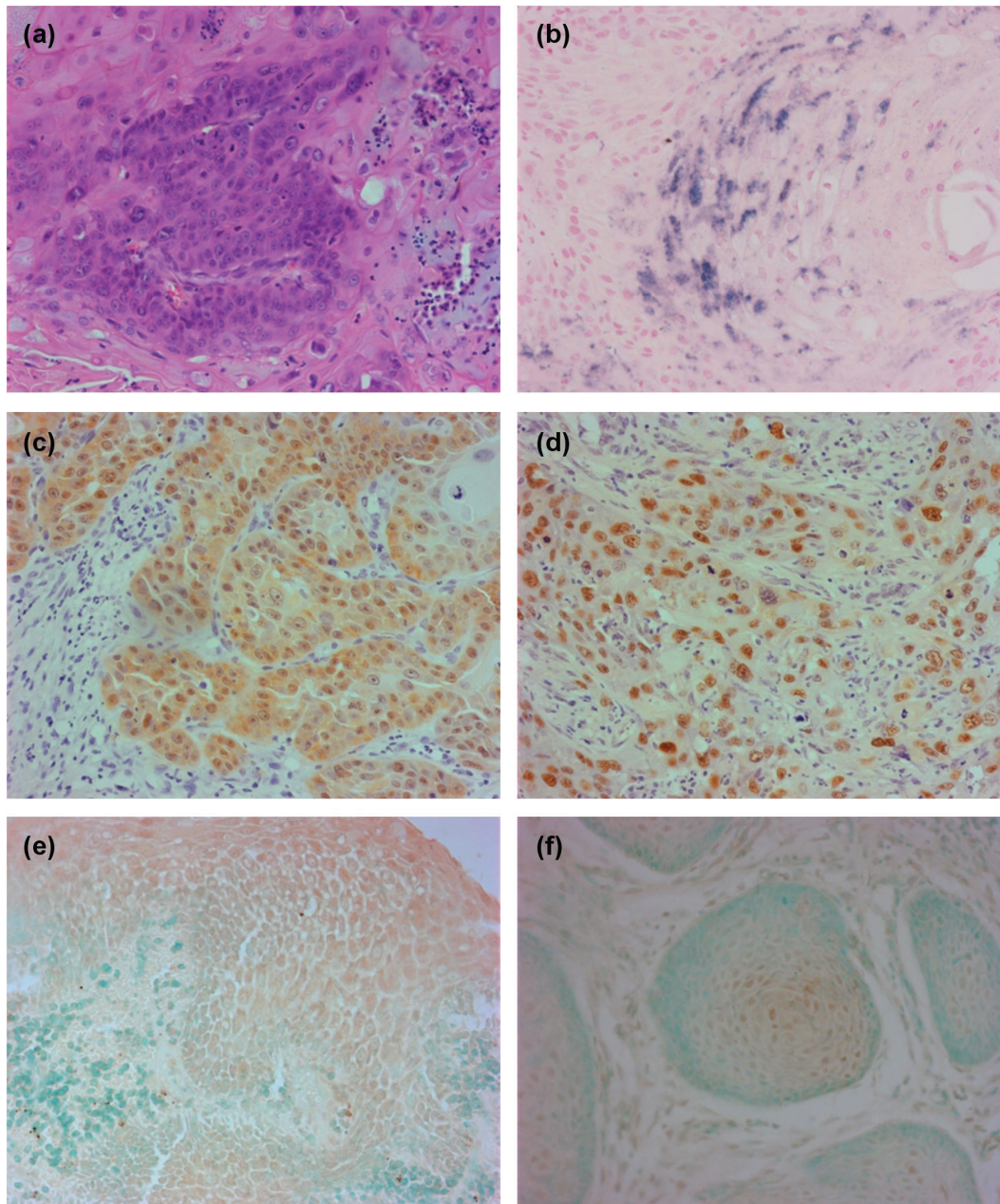
## Results

### Clinicopathologic findings

The age of penile cancer patients ranged from 35 to 82 years with an average of 59.6 years. Twenty-two cases of penile cancer were evaluated and 19 cases were classified as keratinizing squamous cell carcinoma and 3 cases as non-keratinizing squamous cell carcinoma (Fig. 1a).

**Table 1. Detection of HPV DNA p16<sup>INK4a</sup>, p53, and NF- $\kappa$ B in penile carcinoma**

Cases	HPV DNA		p16 <sup>INK4a</sup>		p53		NF- $\kappa$ B in nucleus		NF- $\kappa$ B in cytoplasm		NF- $\kappa$ B in nucleus and/or cytoplasm	
	No.	%	n	%	n	%	n	%	n	%	n	%
HPV-positive cases	15	68.18	7	46.67	9	60.00	11	73.33	14	93.33	15	100.00
Keratinizing squamous cell carcinoma	14	63.64	7	50.00	8	57.14	10	71.43	13	92.86	14	100.00
Non-keratinizing squamous cell carcinoma	1	4.55	0	0	1	100.00	1	100.00	1	100.00	1	100.00
HPV-negative cases	7	31.18	3	42.86	4	57.14	2	28.57	2	28.57	2	28.57
Keratinizing squamous cell carcinoma	5	22.73	2	33.33	3	60.00	2	33.33	2	33.33	2	33.33
Non-keratinizing squamous cell carcinoma	2	9.09	1	100.00	1	50.00	0	0	0	0	0	0
Total	22	10.00	10	45.45	13	59.09	13	59.09	16	72.73	17	77.27

**Fig. 1**

Detection of HPV DNA (b), p16<sup>INK4a</sup> (c), p53 (d), and NF-κB in the cytoplasm (e) and nucleus (f) in penile carcinoma cells  
Hematoxylin and eosin staining of keratinizing squamous cell carcinoma (a).

#### *Detection of HPV DNA, p16<sup>INK4a</sup>, p53, and NF-κB*

Of the 22 cases of penile cancer, 15 cases (68.2%) were HPV DNA positive as determined by ISH (Fig. 1b). In the group of keratinizing squamous cell carcinoma, 14 cases of 19 (73.7%) were HPV DNA-positive. In the group of non-

keratinizing squamous cell carcinoma type, only 1 case of 3 (33.3%) was HPV DNA-positive.

Immunohistochemical analyses for p16<sup>INK4a</sup>, p53, and NF-κB were performed for all specimens. The results in relation to HPVs are summarized in Table 1. Of the 22 cases, 15 cases (68.2%) were HPV-positive and 7 cases (31.2%) were



HPV-negative. Of the 15 HPV-positive cases, 7 (46.7%) were p16<sup>INK4a</sup>-positive (Fig. 1c) and 9 (60.0%) were p53-positive (Fig. 1d). Of the 7 HPV-negative cases, 3 (42.9%) were p16<sup>INK4a</sup>-positive, and 4 (57.1%) were p53-positive. Of the 15 HPV-positive cases, 14 cases (93.3%) were NF-κB-positive in cytoplasm (Fig. 1e) and 11 cases (73.3%) in the nucleus (Fig. 1f). NF-κB was detected in all 15 cases of HPV-positive specimens of penile cancer in the nucleus and/or cytoplasm. Of the 7 HPV-negative cases, 2 (28.6%) were NF-κB-positive in both nucleus and cytoplasm.

### Discussion

HPV is a large family of DNA viruses with more than 100 different HPVs, which can infect genital mucosa after a sexual transmission. The prevalence of HPV DNA is significantly greater in cancer of the genital organs than in other organs. HPVs-related disease is well-documented in lesions of the female genital organs using a wide range of epidemiological, clinical, and molecular techniques. The prevalence of HPVs in tumor tissue has been reported to vary considerably. The frequency of HPV-positive penile cancer (68.2%) reported in this study is similar to the frequency of 71% and 66% reported by Picconi *et al.* (2000) and Cupp *et al.* (1995), respectively. Higher rates 79%, 82%, and 83% were reported by Senba *et al.* (2006), Sarkar *et al.* (1992), and Tornesello *et al.* (1992), respectively. Lower rates 46%, 42%, and 31% were reported by Tornesello *et al.* (2008), Rubin *et al.* (2001), and Cubilla *et al.* (1998), respectively.

In HPVs infection, the interaction of HPV proteins with cellular pRb and p53 proteins is significant. Remarkably, pRb and p53 proteins, which are important molecules in the cell cycle and apoptosis control, are mutated in many human cancers. Both E7 and E6 HPVs oncogenes interact with pRb and p53 proteins, which inhibit activities of these tumor suppressors. The cell cycle in S phase would normally lead to the apoptosis by the action of pRb. However, in HPVs infected cells, this process is counteracted by the viral E6 protein, which targets p53 for proteolytic degradation (Munger *et al.*, 2004). The loss of p53 function is implicated in the pathogenesis of the tumor as well as in the prognosis of many neoplasms. The mutant protein accumulates in the nuclei of tumor cells and can be identified by immunohistochemical reaction. Nuclear accumulation of p53 has been previously reported in 41–89% of penile squamous cell carcinomas (Lam and Chan 1999; Lopes *et al.*, 2002; Humbey *et al.*, 2003).

There is a close association between p16<sup>INK4a</sup> overexpression and a high-risk of HPVs infection. Therefore, the overexpression of p16<sup>INK4a</sup> is suggested to be a useful marker for evaluating HPVs activity in cancer lesions (Sano *et al.*, 1998; Ferreux *et al.*, 2003). The frequency of the overexpression

of p16<sup>INK4a</sup> has been reported in 55–97% cases of cervical squamous cell carcinoma (Sano *et al.*, 1998; Klaes *et al.*, 2001; Humbey *et al.*, 2003) and in 48–80% of cervical adenocarcinoma (Milde-Langosch *et al.*, 2001; Zielinski *et al.*, 2003). Previously, the frequency of overexpression of p16<sup>INK4a</sup> has been reported in 29–50% cases of penile squamous cell carcinoma (Ferreux *et al.*, 2003; Prowse *et al.*, 2008). Klaes and co-workers (2001) reported that p16<sup>INK4a</sup> was a specific biomarker for identification of the dysplastic cervical epithelia in cervical biopsy samples or cervical smears. Overexpression of the p16<sup>INK4a</sup> protein product was observed only in 10 cases (45.5%) of 22 specimens of penile cancer and accordingly has a much lower correlation with HPV-positive penile cancers. However, higher levels of p16<sup>INK4a</sup> were reported in cervical cancer cases (Kalof *et al.*, 2005; Dehn *et al.*, 2007). In our experiments, we did not find any significant association between abnormal expression of p16<sup>INK4a</sup> and p53 and the presence of HPV DNA in penile cancer.

Increased NF-κB activity is associated with many cancers including cancers associated with a viral infection. NF-κB activity is modulated for many different viruses, such as Human immunodeficiency virus 1, Human T-lymphotropic virus 1, Epstein-Barr virus, Hepatitis B virus, adenoviruses, and HPVs (Nees *et al.*, 2001; Spitkovsky *et al.*, 2002; Havard *et al.*, 2002, 2005; Mishra *et al.*, 2006; James *et al.*, 2006). NF-κB dependent proliferation of cells and protection from an apoptosis are likely to have significant effects on the oncogenesis associated with of HPVs infection. To the best of our knowledge, the immunohistochemical localization of NF-κB associated with HPV infection in penile cancer has not been reported yet. In the HPV-positive cases, NF-κB was detected in nucleus and/or cytoplasm in 100% of samples. On the other hand, NF-κB was detected in nucleus and/or cytoplasm only in 28.6% of the HPV-negative cases. HPVs E6- and E7-positive cells have shown IL-1b-induced NF-κB activation and exhibit elevated levels of NF-κB components (Havard *et al.*, 2002). The expression of E7 rather than E6 was found to be associated with the nuclear location of these components. Viral E6 and E7 oncoproteins are important regulatory proteins inside the host cells, which are associated with the transcriptional activity of NF-κB. A fraction of the E7 protein is found in association with the inhibitor κB (IκB) kinase complex and attenuates the induced activity of IκB kinase α, what results in an impaired phosphorylation and degradation of the kinase. While E7 obviates inhibitor of IκB kinase activation in the cytoplasm, the E6 protein reduces NF-κB p65-dependent transcriptional activity within the nucleus. It is suggested that HPVs oncogene-mediated suppression of NF-κB activity contributes to HPVs escape from the immune system (Spitkovsky *et al.*, 2002).

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