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Human papillomavirus infection induces NF-κB activation in cervical cancer: A comparison with penile cancer

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Abstract. This study aimed to determine the relationship between human papillomavirus (HPV) and nuclear factor-κB (NF-κB) in cervical cancer using 62 tissues of cervical cancer, and to compare the findings to penile cancer. HPV-DNA integration is a crucial factor for malignant transformation in cervical cancer and can be identified using *in situ* hybridization. Of the 62 cases, HPV infection was detected in 28 (45.2%). This frequency was lower than in penile cancer (68.2%) as shown by our previous study. The earliest age of onset of cervical and penile cancer was 18 and 35, respectively, whereas the mean age of the initial diagnosis of cervical and penile cancer was 50.1 and 59.6, respectively. The discrepancies of HPV prevalence, earliest ages of onset and mean ages between cervical and penile cancer patients may result from the gender-based synergistic action of HPV associated with multiple epidemiological co-factors. Of the 28 HPV-infected cases, NF-κB expression was observed in the nucleus in 18 (64.3%), in the cytoplasm in 19 (67.9%) and in the nucleus and/or cytoplasm in 27 cases (96.4%). The overexpression of NF-κB in cervical cancer cases suggests that NF-κB activation is a key modulator in driving chronic inflammation to cancer.

Introduction

Genital human papillomavirus (HPV) is the most common virus of sexually transmitted infections. In the 1970s, it was suggested that the possible role of HPV in cancer be investigated (1). It was recently established that HPV is crucial in human carcinogenesis. HPV causes infected epithelial cells in mucous membranes and skin to become abnormal. More than 40 HPV genotypes are able to infect the genital organs of females and males, including the vulva, vagina, cervix and penis (2,3). Although HPV-DNA is detected in numerous cervical cancer tissues, the presence of HPV may be insufficient by itself to establish full malignant transformation. Other factors should therefore be considered in the carcinogenic process. The synergistic action of HPV associated with poor hygienic condition may be required for malignant transformation. Epidemiological evidence suggests that numerous individuals are infected with HPV, although only a small percentage progress to being classified as malignant over a period of years, often decades (4,5).

The causal relationship between chronic inflammation and cancer is widely accepted. Numerous investigators have identified nuclear factor-κB (NF-κB) as a key modulator in driving chronic inflammation to neoplastic cells. This transcriptional factor is indispensable in the malignant progression of transforming cells within the various inflammatory conditions containing a network of signaling molecules. The NF-κB transcription factor family in mammals comprises the proteins, RelA (p65), RelB, c-Rel, p105/p50 (NF-κB1) and p100/p52 (NF-κB2). These proteins form homo- and heterodimeric complexes through their conserved prototypical Rel homology domain. NF-κB plays a critical role in the diverse cellular processes associated with proliferation, cell death and development. Experimental evidence showing specific mechanisms by which NF-κB affects cancer initiation, promotion and progression has been reported (6,7). The expression and function of numerous cytokines, chemokines, growth factors and survival factors are NF-κB-dependent. NF-κB activation plays a role in a variety of processes correlated to transformation and oncogenesis (8).

Initially, we attempted to detect HPV genotypes employing PCR and DNA sequences as in our previous studies (9-11), but the HPV-DNA could not be extracted from the

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Table I. Comparison between cervical and penile cancer in Western Kenya.

	Cervical cancer	Penile cancer
Earliest age of onset (years)	18.0	35.0
Mean age (years)	50.1	59.6
Rate of HPV infection	45.2%	68.2%
NF-κB expression in HPV infection case	96.4%	100.0%
NF-κB expression in HPV non-infection case	52.9%	28.6%

HPV, human papillomavirus; NF-κB, nuclear factor-κB.

paraffin-embedded tissues. Therefore, *in situ* hybridization (ISH) was used to confirm the presence of HPV in cervical cancer. Cervical cancer is the most common cancer found among females in sub-Saharan Africa. This study aimed to determine the relationship between HPV infection and NF-κB in cervical cancer in Western Kenya. This report also investigated HPV in penile cancer from the same study area (12). To the best of our knowledge, the relationship between cervical and penile cancer associated with HPV in the same area using tissue materials has yet to be reported.

Materials and methods

Tissue specimens. The biopsy materials studied were obtained from 62 cervical cancer cases from specimens that had been submitted for pathologic diagnosis to the Department of Histopathology, Rift Valley Provincial General Hospital from various hospitals in the western part of Kenya (Western, Nyanza and Rift Valley provinces, 1983-2000). This was a retrospective study and the specimens used were archival. This investigation was authorized by the Government of Kenya (research permit No. OP.13/001/8C224/36). The specimens were fixed in 10% formalin, embedded in paraffin and examined histologically and by ISH. Histological analysis was carried out using 3.5 μm sections of tissue stained with H&E. Parallel sections were prepared for ISH. Findings from the cervical cancer were compared to those of the penile cancer in a previous study (12).

In situ hybridization. The paraffin-embedded tissue specimens were cut into 3.5 μm sections and collected on silane-coated glass slides. For the detection of HPV-DNA, an HPV screening detection kit (Kreatech Diagnostics Co., Amsterdam, The Netherlands) 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, which is composed of a mixture of HPV 6, 11, 16, 18, 31 and 33. ISH-negative control is a digoxigenin-labeled DNA probe, derived from plasmid DNA (pSP) and does not contain any sequences of human or viral origin. The steps involved in the ISH procedure using the HPV screening detection kit are: following hybridization with the probes, alkaline phosphatase-conjugated antibody against digoxigenin was applied to the sections. The localization of HPV-DNA was detected using a NBT/BCIP substrate and observed under a light microscope.

Immunohistochemistry of NF-κB. Sections of 3.5 μm were placed on silane-coated glass slides. The sections were deparaffinized to remove the embedded medium and dehydrated. The slides were then boiled in 0.01 mol/l citrate buffer, pH 7.0, at 98˚C for 40 min for antigen retrieval and cooled at room temperature for 30 min. After the slides had been rinsed in 0.01 M phosphate-buffered saline (PBS), pH 7.4, the endogenous peroxidase activity was blocked with 3% H_2O_2 and absolute methanol for 10 min. The tissue sections were covered with 1:50 dilution of mouse monoclonal anti-human NF-κB antibody (Cell Signaling Technology Inc., Beverly, MA, USA) or control serum at 37˚C for 3 h. After being washed with PBS, the sections were covered with the peroxidase-labeled dextran polymer (Dako, Carpinteria, CA, USA) at 37˚C for 40 min and rinsed in PBS. Target antigenic sites on the sections were demonstrated by reacting with a chromogen of 0.05% 3,3'-diaminobenzidine tetrahydrochloride in 0.05 M Tris-HCl buffer and 0.01% hydrogen peroxide for 10 min. The sections were then counterstained with methyl green for 10 min, dehydrated in ethanol, cleared in xylene and mounted.

Statistical analysis. Statistical analysis was performed for possible relationships between the HPV infection and nuclear and/or cytoplasmic expression of NF-κB.

Results

Clinicopathological findings. The results of the comparison between cervical and penile cancer are shown in Table I. Variations were found in the clinical data between the two types of cancer. The age at the initial diagnosis of the 62 cervical cancer patients ranged from 18 to 73 years (mean 50.1). The earliest age of onset and mean age of cervical cancer were 18 and 50.1, respectively. The cervical carcinomas were investigated histologically, and divided into 54 keratinizing and 8 non-keratinizing squamous cell carcinomas.

Detection of HPV-DNA and NF-κB. ISH showed that 28 cases (45.2%) were HPV-DNA-positive (Fig. 1A and B). This frequency was lower than in penile cancer (68.2%) according to a previous study (12). Of the 54 cases of keratinizing and 8 of non-keratinizing squamous cell carcinomas, 24 (44.4%) and 4 (50%) were infected with HPV, respectively.

Immunohistochemical analysis for NF-κB was performed on all 62 specimens. Of the 28 HPV-positive cases, 18 (64.3%)

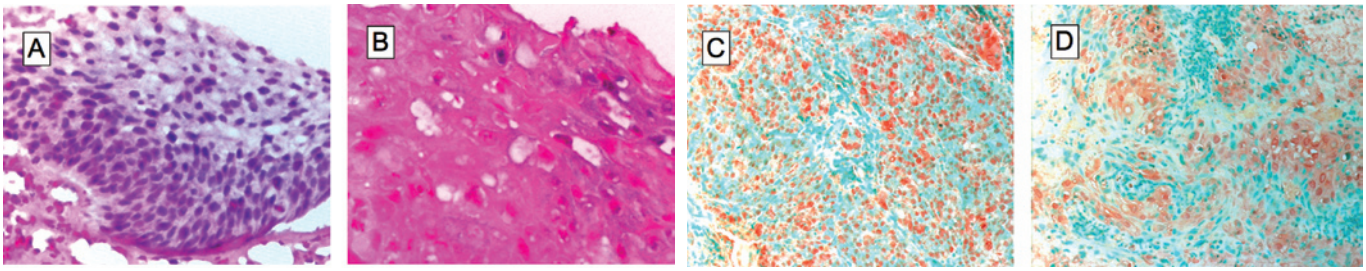

Figure 1. (A) *In situ* hybridization for human papillomavirus signal exhibited on a section of cervical cancer. (B) *In situ* hybridization for human papillomavirus signal exhibited on a section of cervical cancer with a condylomatus lesion. (C) Immunohistochemical expression of NF-κB in the nucleus in cervical cancer. (D) Immunohistochemical expression of NF-κB in the cytoplasm in cervical cancer.

Table II. Correlation of NF-κB expression with HPV infection in cervical cancer.

NF-κB expression	HPV-DNA		p-value
	Positive (n=28)	Negative (n=34)	
Positive in nucleus (n=32)	18	14	
Negative in nucleus (n=30)	10	20	0.0700
Positive in cytoplasm (n=33)	19	14	
Negative in cytoplasm (n=29)	9	20	0.0653
Positive in N and/or C (n=45)	27	18	
Negative in N and/or C (n=17)	1	16	0.0001

HPV, human papillomavirus; NF-κB, nuclear factor-κB; N and/or C, nucleus and/or cytoplasm.

were NF-κB-positive in the nucleus (Fig. 1C) and 19 (67.9%) were NF-κB-positive in the cytoplasm (Fig. 1D). Additionally, NF-κB was detected in the nucleus and/or the cytoplasm in 27 cases (96.4%). Of the 34 HPV-negative cases, NF-κB localization in the nucleus, cytoplasm and nucleus and/or cytoplasm was observed in 14 (41.2%), 14 (41.2%) and 18 cases (52.9%), respectively (Table II).

Statistical analysis. The Fisher's exact probability test showed that the nuclear and/or cytoplasmic expression of NF-κB was correlated with HPV infection ($p<0.01$) (Table II).

Discussion

Between 2000 and 2004, cervical cancer was the thirteenth most common cancer in the United States, with an incidence rate of 808 per 100,000 individuals (13). Cervical cancer is the second most common cancer among females worldwide, with an estimated 493,000 new cases and 274,000 deaths in 2002 (14). Globally, 83% of the cases of this cancer occur in developing areas, including sub-Saharan Africa, Melanesia, Latin America and the Caribbean, South-Central Asia and Southeast Asia (14). While the causal relationship between HPV infection and squamous cell carcinoma of the genital tract is well established (15), the role of HPV in the development of the malignancy has yet to be elucidated. However, mounting evidence shows the involvement of HPV types in the process of oncogenesis. Genital HPV infection is the cause of this most common sexually transmitted disease, thus, the risk factor for HPV infection is increased by sexual behaviors. The highest prevalence of HPV infection is noted in sexually active adolescent and young adults. HPV infection is a key factor in cervical oncogenesis. However, high-risk HPV is a necessary but insufficient cause of cervical cancer, which develops in a multi-step manner from precursor lesions.

The overall prevalence of any type of HPV in the general populations of sub-Saharan Africa for females with normal cytology is 21.8% (16). Yamada *et al* (17) reported that the overall prevalence of HPV infection in the uterine cervix associated with abnormalities was 27% in Nairobi, Kenya. The prevalence of HPV-16 and/or HPV-18 among invasive cervical cancer case ranges from 43.7% in Senegal to 90.2% in Ethiopia (16). The overall prevalence average of HPV16/18 among the invasive cervical cancer cases is 69.2% in sub-Saharan Africa (16). The overall prevalence of HPV-DNA in cervical cancer in this study using ISH was 45.2%, which is relatively similar to the 43.7% in Senegal and 44.4% in Guinea (16). This variability may depend not only on the different methods of HPV detection used, but also on the geographic variation in HPV distribution. Our studies showed that both cervical and penile cancers were associated with HPV infection. Notably, the prevalence of cervical cancer with HPV (45.2%) was lower than penile cancer with HPV (68.2%) in surgical specimens in Kenya (12). The earliest age of onset for cervical cancer cases was 18 years, while for penile cancer cases it was 35 years (12). The mean age for cervical cancer cases was 50.1 and for penile cancer cases 59.1 years (12). Variations in the synergistic action of HPV between genders may occur, associated

with multiple epidemiological co-factors, such as life environment, genital hygiene, sexual habits and cultural practices. To clarify these discrepancies, further research is required.

NF-κB comprises of a family of transcription factors that modulate signaling pathways to inhibit apoptosis, growth factors and cell cycle regulatory proteins. NF-κB activation promotes cell survival and growth, and therefore plays a critical role in inflammation-based and cancer progression. This transcription factor is indispensable for the malignant progression of transforming cells in the environment, including network signaling molecules mediated by various inflammatory cells. The expression of numerous cytokines, chemokines, growth factors and survival factors is NF-κB-dependent. NF-κB activation is modulated for HPV (18-23). In this study, NF-κB was detected in the nucleus and/or cytoplasm in 96.4% of the HPV-positive cases. On the other hand, NF-κB was detected in the nucleus and/or cytoplasm in only 52.9% of the HPV-negative cases. We demonstrated the correlation between HPV infection and nuclear and/or cytoplasmic NF-κB expression using the Fisher's exact probability test. HPV infection was considered to activate NF-κB resulting in cell transformation (11,12,24).

The integration of HPV-DNA in the host cell DNA involves cancer formation and development. The HPV viral oncogenes E7 and E6 are the main contributors to the development of HPV-induced cancers, probably due to integration of the viral genes in the host cell genome. E7- and E6-induced genetic instability leads to the activation of oncogenes and the inactivation of tumor suppressor genes. Inactivation of tumor suppressors p53 and pRb is a common event in the carcinogenesis of human cells. Notably, p53 and pRb genes are mutated in various types of human cancer. Overexpression of viral E6 and E7 oncogenes reacts with the tumor suppressor gene products p53 and pRb proteins in the host cells, resulting in an induced cell immortalization, transformation and oncogenesis due to their interference with the cell cycle and apoptosis control (25). HPV E6 and E7 oncogenes are key regulatory proteins inside host cells and are associated with the transcriptional activity of NF-κB (19). Therefore, activation of NF-κB by viral oncogenes may be the mechanism of tumor formation in cervical cancer.

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