ELSEVIER

Cancer Genetics and Cytogenetics 175 (2007) 132–137

Cancer Genetics and Cytogenetics

Physical status of multiple human papillomavirus genotypes in flow-sorted cervical cancer cells

Christine F.W. Vermeulen[a,b], Ekaterina S. Jordanova[a,*], Károly Szuhai[c], Sandra Kolkman-Uljee[a], M. Albert Vrede[d], Alexander A.W. Peters[b], Ed Schuuring[a,e], Gert Jan Fleuren[a]

[a]*Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands*
[b]*Department of Gynecology, Leiden University Medical Center, P1-40, 2333 ZA Leiden, The Netherlands*
[c]*Laboratory for Cytochemistry and Cytometry, Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands*
[d]*Department of Pathology, Academic Hospital Paramaribo, Paramaribo, Suriname*
[e]*Department of Pathology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands*

Received 18 January 2006; received in revised form 19 February 2007; accepted 20 February 2007

Abstract Multiple human papilloma virus (HPV) infections have been detected in cervical cancer. To investigate the significance of multiple HPV infections, we studied their prevalence in cancer samples from a low-risk (Dutch) and a high-risk (Surinamese) population and the correlation of HPV infection with tumor cell aneuploidy. SPF_{10} LiPA was used for HPV detection in formalin-fixed cervical carcinoma samples from 96 Dutch and 95 Surinamese patients. Samples with HPV type 16 or 18 infections were sorted by flow cytometry, and fluorescence in situ hybridization was performed on the diploid and aneuploid subpopulations to detect HPV 16 and 18 genotypes simultaneously. Multiple HPV infections were present in 11 of 80 (13.8%) Dutch and 17 of 77 (22.1%) Surinamese carcinomas. Three cases had an HPV 16 and HPV 18 coinfection: in two cases, integrated HPV copies of HPV 16 or 18 were detected in the aneuploid fraction, and in one case both HPV 16 and 18 were present solely as episomes. Based on our findings, multiple HPV infections are present in cervical cancer samples from both high- and low-risk populations. Furthermore, multiple HPV types can be present in an episomal state in both diploid and aneuploid tumor cells, but integrated HPV genomes are detectable only in the aneuploid tumor cell subpopulations.

1. Introduction

Human papillomavirus (HPV) infection is a prerequisite for the development of cervical cancer [1]. Although HPV infections are common, the majority are transient and are cleared by the immune system [2]. When high-risk HPV is persistently present, low grade cervical intraepithelial lesions eventually progress to invasive cervical carcinoma [3]. HPV infects the basal epithelial cells and is frequently found in an episomal state in low- and high-grade cervical intraepithelial lesions. It is generally thought that viral integration into the human genome occurs during cervical carcinogenesis [2,4]. Upon integration, the viral E2 repressor is disrupted, leading to continued expression of the E6 and E7 oncoproteins. These oncoproteins inactivate the p53 and pRb tumor suppressor proteins, leading to uncontrolled cell proliferation and ultimately to cancer [5]. Viral integration frequency was shown to increase with disease severity [4,6,7].

The two most common high-risk HPV types found in cervical carcinoma are HPV 16 and HPV 18. Although both HPV 16 episomes and HPV 16 integrated copies are able to transform normal keratinocytes, HPV 18 has been reported to be present mainly in the integrated form [6,8].

Fluorescence in situ hybridization (FISH) has recently been used to investigate the physical state of HPV (episomal or integrated) in cells from cervical preinvasive or invasive lesions [9,10]. Several studies suggest that a diffuse nuclear signal is indicative of the presence of episomal HPV, whereas a punctate signal in the nucleus is characteristic of integrated HPV [11–13]. It was shown, however, that the diffuse signal can be excluded by harsh

* Corresponding author. Tel.: +31-71-5266596; fax: +31-71-5248158.
E-mail address: k.jordanova@lumc.nl (E.S. Jordanova).

0165-4608/07/$ – see front matter
doi:10.1016/j.cancergencyto.2007.02.009

pretreatment protocols, allowing the clear detection of integrated HPV copies [10].

In addition to the physical state of HPV, abnormal cellular DNA content or numerical chromosome aberrations have been suggested to be associated with the progression of cervical intraepithelial lesions to cervical carcinoma [14,15]. Despite investigations into whether HPV integration or DNA instability and aneuploidization is the first step in malignant transformation, the sequence of events is still under debate [16,17]. Data on malignant transformation and progression as a result of multiple HPV infections are even more limited. Such multiple HPV infections are frequent in premalignant stages and have recently been detected in invasive cervical cancer [18–20].

We investigated the prevalence of multiple HPV infections in cervical cancer for a low-risk (Dutch) and a high-risk (Surinamese) population. The cases carrying a double HPV 16 and HPV 18 infection were analyzed with FISH on flow-sorted pure tumor cell subpopulations to determine the integration status of the multiple HPV types in relation to tumor cell aneuploidy.

2. Materials and methods

2.1. Patient samples

A total of 189 patients with invasive cervical carcinoma, FIGO stage 1B or 2A, were included in the present study. Patients were living in the Netherlands ($n = 98$), a region of low incidence for cervical cancer, or Suriname ($n = 99$), a region of high incidence. Patients were diagnosed with cervical carcinoma between 1989 and 1995. All Dutch patients were treated in the Leiden University Medical Center (LUMC; Leiden, the Netherlands), and the tumor tissue was stored in the archive of the Pathology Department. Of the Surinamese patients, 45 were treated in the LUMC and their tumor tissue was also kept in the LUMC laboratory. The other 49 patients were treated in Suriname, and tissue samples were stored in the laboratory of the Pathology Department, Academic Hospital, Paramaribo, Suriname.

2.2. HPV detection and genotyping

DNA was isolated from formalin-fixed, paraffin-embedded biopsy samples as previously described [21]. Care was taken to prevent cross-contamination during preparation of the sections from the paraffin blocks. Beta-globin polymerase chain reaction (PCR) was performed using primers RS40 and RS42 [21] to determine whether the isolated DNA was suitable for amplification. The DNA was subjected to a short PCR fragment assay using the SPF_{10} primer set, which amplifies a 65-bp fragment in the L1, according to the manufacturer's instructions (Innogenetics, Ghent, Belgium). Each experiment was performed with separate positive and several negative controls.

The presence of HPV was established using a microtiter plate–based hybridization assay, and SPF_{10}-PCR products from HPV DNA-positive cases were directly genotyped using a reverse hybridization line probe assay (LiPA; Innogenetics). With this assay, 25 individual HPV genotypes can be identified simultaneously: HPV 6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70, and 74.

On the HPV 16-positive (HPV 16^+) and HPV 18-positive (HPV 18^+) specimens, HPV type 16- and 18-specific PCRs were performed as described previously [22].

2.3. Flow cytometry and sorting

Flow cytometry sorting of formalin-fixed, paraffin-embedded samples positive for multiple HPV types was performed as described previously [23]. Briefly, paraffin-embedded 60-μm sections were treated with a combined mechanical–enzymatic method to obtain single cells. Cells were then stained with a mix of monoclonal antibodies directed against keratin and vimentin, containing clones MNF116 (anti-keratin; DAKO, Glostrup, Denmark), AE1/AE3 (anti-keratin; Chemicon International, Temecula, CA), and V9-2b (anti-vimentin; Department of Pathology, LUMC). A standard FACSCalibur (BD Biosciences, San Jose, CA) was used for flow cytometric analysis. Subsequently, flow sorting was performed using a FACSVantage flow-sorter (BD Biosciences). Tumor cells were sorted based on keratin and vimentin expression, combined with a gate on DNA content. Diploid and aneuploid tumor fractions and normal cell fractions were collected for DNA isolation; in addition, cell fractions were directly sorted separately onto glass slides for FISH processing.

2.4. Interphase FISH analysis

FISH was modified to detect multiple HPV genotypes simultaneously. Interphase FISH analysis was performed on flow cytometry–sorted cell fractions using an adapted protocol for FISH on formalin-fixed, paraffin-embedded tissue [24]. Approximately 400 cells were sorted by flow cytometry directly onto glass slides that had been cleaned with rinsing in 96% ethanol. After spotting, the slides were dried overnight at room temperature to ensure cell adhesion. If needed, the slides were incubated in a 0.1 mol/L solution of sodium borate ($Na_2B_4O_7$) to permit swelling of the nuclei. Afterwards, the slides were rinsed in phosphate-buffered saline and sterile water.

The HPV 16 probe was labeled with digoxigenin and the HPV 18 probe was labeled with biotin (both purchased from PanPath, Science Park Amsterdam, Amsterdam, the Netherlands). Hybridization and immunodetection were performed as previously described for nuclei isolated from paraffin-embedded material [24].

In each experiment, negative control slides, spotted with sorted vimentin-positive nuclei not infected with HPV, were