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RESEARCH

Characterization of the Human Papillomavirus (HPV) Integration Sites into Genital Cancers

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Abstract Oncogenic HPVs have been found frequently integrated into human genome of invasive cancers and chromosomal localization has been extensively investigated in cervical carcinoma. Few studies have analyzed the HPV integration loci in other genital cancers. We have characterized the integration sites of HPV16 in invasive penile carcinoma by means of Alu-HPV-based PCR. Nucleotide sequence analysis of viral–human DNA junctions showed that HPV integration occurred in one case within the chromosome 8q21.3 region, in which the FAM92A1 gene is mapped, and in the second case inside the chromosome 16p13.3, within the intronic region of TRAP1 gene. These results confirm previous observations, summarized in a systematic review of the literature, on the HPV integration events in gene loci relevant to cancer pathogenesis.

Keywords Human Papillomavirus · HPV · Integration sites · Penile cancer · Vulvar Cancer

Abbreviations

HPV	Human Papillomavirus
PC	Penile Cancer

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Introduction

Infection of the anogenital mucosa with human papillomaviruses (HPVs) has been associated with premalignant and malignant lesions particularly at squamocolumnar junction of the uterine cervix [1]. Epidemiological studies showed that 12 high risk HPVs, with large predominance of the genotype 16, are associated with nearly all cases of preinvasive and invasive cervical carcinoma and with a significant number of vaginal, vulvar, penile, and anal cancers [2, 3].

In the general population HPV infection has a consistent higher prevalence in penile epithelium of asymptomatic men than in the cervix of cytological normal women. A systematic review of the literature showed that the prevalence of HPV infection in men ranged from 1.3 % to 72.9 % (most studies reporting ≥20 %), with HPV16 being the most common oncogenic viral type [4]. Conversely, in a recent meta-analysis assessing the burden of cervical HPV infection among 1 016 719 of women with normal cytology the estimated global HPV prevalence was 11.7 % (95 % confidence interval, 11.6–11.7 %), with HPV16 as the prominent type worldwide (3.2 %), [5]. The incidence of HPV-related high-grade penile intraepithelial neoplasia and invasive penile cancer, however, is much lower compared to that of high grade cervical intraepithelial neoplasia and invasive carcinoma suggesting that penile tissue is less prone to maintain a HPV persistent infection and to undergo neoplastic transformation [6]. Similarly, vulvar and vaginal cancers are rare, approximately one per 100 000 women per year, although HPV infection at these sites is common, suggesting a more benign course than cervical infections [7–9].

HPV-mediated transformation of human epithelial cells has been recognized as a multi-step process resulting from

integration of HPV DNA into human genome, deregulated transcription of the viral oncogenes E6 and E7, chromosomal instability, genetic or epigenetic oncogenic alterations and continuous selection of cells with a malignant phenotype [10]. The chromosomal mapping of more than 200 HPV-host junctions, mostly in cervical squamous cell carcinoma, showed that integration sites were unique, involving all chromosomes and fragile sites, translocation break points, and transcriptional active regions [11, 12]. Importantly, HPV genomes have been shown to be integrated into or close by cellular genes (VMP1, PVRL1, CHERP, CEACAM5, AHR, MRF-2) and within the common fragile sites of the human genome in a significant number (7.4 %) of high grade, but not low grade, cervical lesions indicating that this is a late and critical event in cancer progression [13].

Very few studies have investigated the HPV status and characterized the viral host-junctions in genital cancers arising at sites other than the transformation zone, particularly HPV-related penile, vaginal and vulvar carcinomas [14, 15]. The aim of the present study was to characterize the HPV–host junctions in penile carcinoma biopsies obtained from Ugandan patients using an HPV-Alu-based PCR strategy..

Materials and Methods

Samples Penile cancer biopsies histologically confirmed to be keratinizing squamous cell carcinomas were obtained from five Ugandan patients attending the New Mulago Hospital, Makerere University in Kampala (Uganda) and cryo-preserved in liquid nitrogen at −192°C until further molecular analysis. These samples were previously characterized in terms of histology, DNA quality, HPV genotypes, HPV16 variants and viral integration status [16].

DNA Isolation Tissue biopsies were digested with Proteinase K (150 μg per ml at 37°C for 16 h) in 500 μl of lysis buffer (10 mM Tris–HCl pH 7.6, 5 mM EDTA, 150 mM NaCl, 1 % SDS), followed by DNA purification by phenol and phenol–chloroform–isoamyl alcohol (25:24:1) extraction and ethanol precipitation in 0.3 M sodium acetate (pH 4.6).

Amplification of Viral–Host Junctions Viral–host junctions were amplified by nested-PCR using four combinations of primers specific for human Alu repeats and HPV16 E2 region. PCR primer sequences are summarized in Table 1. Both outer and inner PCR reactions were performed in 50 μl reaction mixture (for all sets of primers) containing 500 ng of target DNA, 1X Hot Master buffer (2.5 mM $MgCl_2$), 20 pmol of each primer, 200 μM of dNTPs mix and 1.25 units of thermostable Taq DNA polymerase (5-Prime GmbH, Hamburg, DE). DNA was amplified in a Perkin-Elmer GenAmp PCR System 9700 thermal cycler with the following steps: an initial 2 min denaturation at 94°C, followed by 10 cycles of 52°C for 1 min, 68°C for 1 min, 94°C for 30s, and 30 cycles of 55°C for 1 min, 68°C for 1 min, 94°C for 30s and a final elongation at 68°C for 5 min. A reaction mixture without template DNA was included in each PCR run, as negative control.

Southern Blot Analysis PCR amplification products were electrophoretically separated on a 1 % agarose gel in 1X TAE buffer and transferred onto Hybond-N nylon membranes (GE Healthcare Biosciences, Pittsburgh, PA) with 0.4 N NaOH. The HPV-specific products were identified by hybridization at 68°C using a ^{32}P-labeled HPV16 E2 probe and visualized by autoradiography.

DNA Purification and Cloning PCR amplification products, positive by Southern blot analysis, were purified from 1.5 % agarose gel by GeneClean Turbo Protocols (MP Biomedicals, Solon, OH). The purified products were blunted by T4 DNA polymerase treatment, ligated into a pUC18 cloning vector, using Ready-To-Go pUC18 SmaI/BAP kit (GE Healthcare Biosciences, Pittsburgh, PA), and used to transform *E. coli* JM83 competent cells. Cells were plated on LB-Ampicillin (100 μg/mL) agar plates for overnight growth at 37°C. Colonies were transferred onto a nylon membrane and screened by colony hybridization using E2-3 primer as a ^{32}P-labeled HPV16 E2 probe. Plasmid DNAs from positive clones were isolated from minicultures (5 mL Luria broth) by Wizard Plus Minipreps DNA Purification System (Promega, Fitchburg, WI) and subjected to automated nucleotide sequence analysis at Primm Laboratories (Milan, IT) using the primer For specific for pUC18. Sequence homology search for human genome and HPV sequences present in the GenBank was performed using NCBI BLAST program (http//www.ncbi.nih.gov/BLAST/).

Review of the Literature

Published data on the HPV integration status and chromosomal location of the viral sequences in penile, vaginal and vulvar carcinoma were searched in Medline using the terms “HPV” AND “integration” AND (“vulva” OR “vulvar”) OR (“vagina” OR “vaginal”) OR (“penis” OR “penile”). Additional relevant references cited in retrieved articles were also evaluated. If data were published more than once, only the publication with more complete information was included. The search was updated on 20 October 2011.

Results

The aim of this study was to identify the chromosomal location of HPV integration sites in five penile carcinoma

Table 1 Primers used for PCR amplification and as probe for Colony hybridization

Primer name	Primer sequence	Application
HPV16E2-1	5′ AGGACGAGGACAAGGAAAA	PCR
HPV16E2-2	5′ GGATGCAGTATCAAGATTTG	PCR
HPV16E2-3	5′ CACTGGCTGTATCAAAGAA	PCR and hybridization probe
HPV16E2-4	5′ATAGACATAAATCCAGTAGACAC	PCR
TC65+ Alu	5′ AAGTCGAATTCTTGCAGTGAGCCGAGAT	PCR
TC65- Alu	5′ CGA GAATTCATCTCGGCTCACTGCAA	PCR

samples all positive for HPV16 and all containing integrated viral genomes into the human chromosome. DNA samples were amplified using a HPV-Alu PCR strategy to identify viral–host junctions (Table1). PCR was employed using several combinations of primers specific to human Alu-repeats and HPV16 E2 gene in order to detect both ends of integrated HPV16 (Fig. 1). An HPV16 ^{32}P-labeled fragment of 2817 bp, spanning the E1 and E2 gene, was released from a recombinant plasmid containing the whole HPV16 genome by digestion with the restriction endonuclease *Pst* I, and was used to hybridize PCR products by alkaline Southern blot analysis. Positive bands were identified in samples PC8 and PC17 indicating that viral-host junctions were successfully amplified in these samples. The hybridization pattern showed one positive band of 850 bp in PC8 and two positive bands of 540 bp and 280 bp in PC17. The HPV16 E2-positive fragments were cut out from the agarose gel, purified and independently cloned into the SmaI/pUC-18

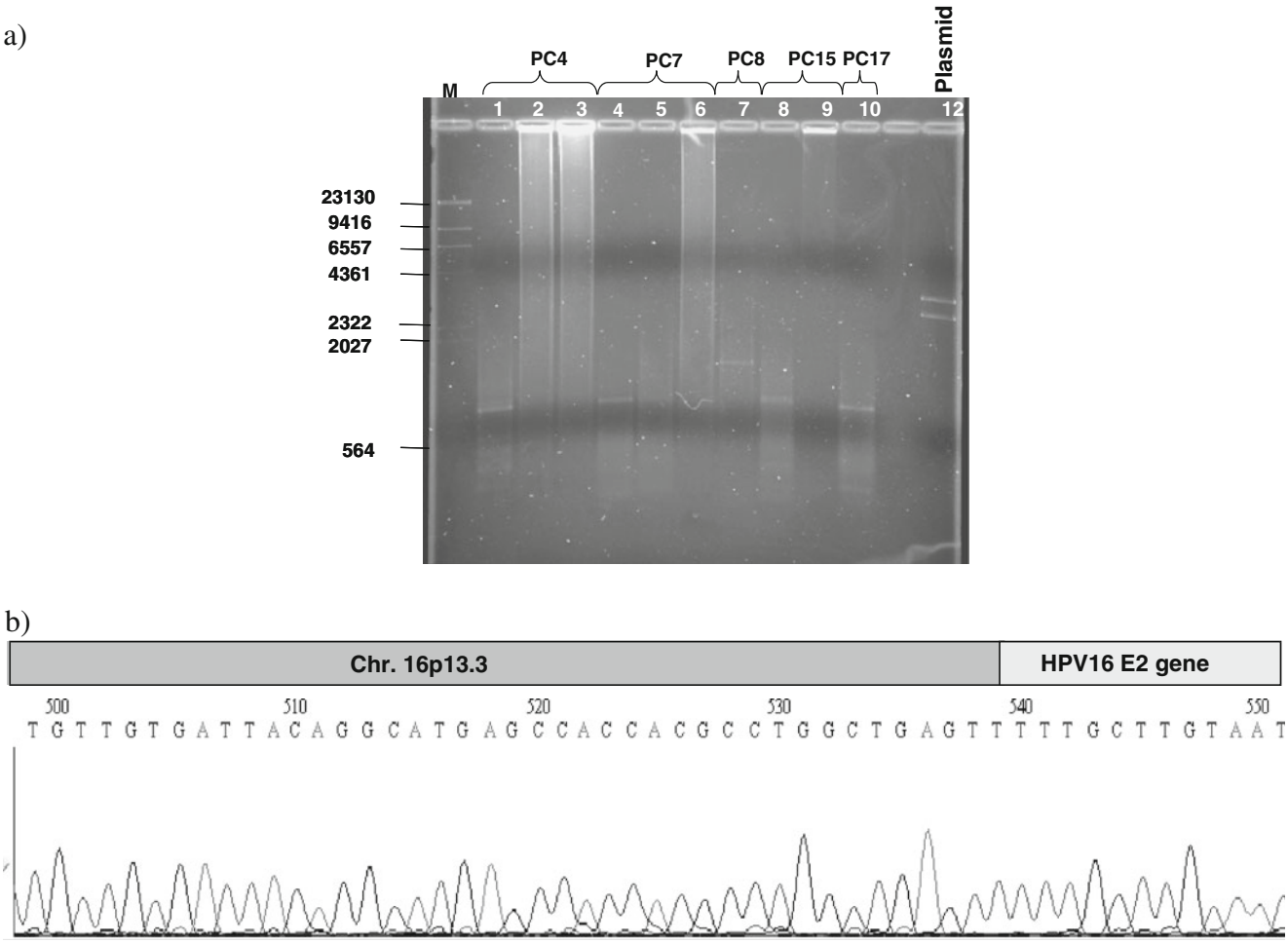

Fig. 1 **a** 1.0 % agarose gel electrophoresis of HPV-Alu PCR product. Lanes 1,4,8 and 10: PCR with HPV16E2-3 and TC65+ primer set. Lane2: PCR with HPV16E2-3 and TC65- primer set. Lanes 3,6,7 and 9: PCR with HPV16E2-4 and TC65- primer set. Lane 5: PCR with HPV16E2-4 and TC65+ primer set. M: Lambda DNA/Hind III marker. Lane 12: HPV16 E2 positive control. **b** Nucleotide sequence of viral–host junction isolated from clone number 1 of PC17

plasmid. The colony hybridization allowed the identification of four HPV-positive clones. Positive recombinant plasmids were subjected to automated DNA sequencing analysis.

A database homology search of human and HPV sequences in Genbank revealed the chromosomal locations of HPV–host junctions in two out of four clones. One viral sequence from PC8 sample was found integrated within the chromosome region 8q21.3, in which the FAM92A1 gene is mapped. The second viral sequence from PC17 was found integrated in the human chromosome 16, inside an intronic region of TRAP1 gene, coding for a heat shock protein of 75 kDa (HSP75). Two recombinant clones contained DNA fragments consisting of unidentified short DNA sequences adjacent to a HPV primer, probably originating from non-specific PCR products.

Discussion

Persistent infection with high risk HPVs has been associated with development of carcinomas arising from mucosal squamous epithelial cells of anogenital and oropharyngeal areas. In the low grade cervical intraepithelial neoplasia, the viral genome usually persists and replicates as an episomal molecule, whereas in high grade cervical neoplasia and in invasive cancer the HPV genomes have been found frequently integrated into the chromosomal DNA of the host cell, suggesting that this is a crucial event in cancer progression [17, 18]. To date a large number of virus-host integration sites have been mapped in chromosomes of cervical cancer cells and, although found randomly distributed into the human genome, they often have been found to alter host gene expression and to interrupt gene loci relevant for cell proliferation [19].

Limited information is available on the chromosomal integration loci of HPV-related cancers other than cervical neoplasia. In this study the characterization of viral–host junctions in two penile carcinoma samples showed that in one case the HPV16 integration occurred within the chromosome region 8q21.3, in which the FAM92A1 gene is mapped, and in the second case the viral DNA was integrated within the human chromosome 16p13.3 inside an intronic region of TRAP1 gene, TNF receptor-associated protein 1, [20]. The FAM92A1 gene encodes for thirteen different protein isoforms and has been suggested to be a new tumor-related gene with oncogenic potential in renal carcinogenesis based on its increased expression in renal cancer biopsies as well as on cell growth promotion by

Table 2 All characterized HPV integration sites in penile, vaginal and vulvar neoplasia

Name	Pathology[a]	HPV	Chromosomal Integration site	Gene locus	Reference
PC-17	Penile Ca	16	16p13.3	TRAP1	This work
PC-8	Penile Ca	16	8q21.3	FAM92A1	This work
IC2	Penile Ca	16	8q24	c-myc	(Couturier et al. 1991)
P11	Penile Ca	16	8q24.2	POU5F1B/c-myc	(Kalantari et al. 2008)
P1	Penile Ca	16	20p12	JAG1	(Kalantari et al. 2008)
P24	Penile Ca	16	20p12	JAG1	(Kalantari et al. 2008)
P5	Penile Ca	16	3q28	TP63	(Kalantari et al. 2008)
P7	Penile Ca	16	19q13.4	ZSCAN-5A/ZSCAN -5B	(Kalantari et al. 2008)
P21	Penile Ca	16	9p23	PTPRD/TYRP	(Kalantari et al. 2008)
T27	Vaginal Ca	16	Xp22	HSX11910	(Ziegert et al. 2003)
T28	Vaginal Ca	16	4q21	BIKE	(Ziegert et al. 2003)
INT20	VaIN2	16	21q22		(Wentzensen et al. 2002)
INT14	VaIN3	18	15q12	HS150715	(Wentzensen et al. 2002)
INT4	VINX	16	3q28	FRA3C TP63	(Wentzensen et al. 2002)
Case 1	BD3	16	8q28		(Nakanishi et al. 2009)
Case 1	BD4	16	11q12 & 3q25		(Nakanishi et al. 2009)
Case 1	Vulvar Ca	16	8q24	c-myc	(Nakanishi et al. 2009)
SK-v- CL	VIN	16	12q14-q15	INT1/GLI	(Sastre-Garau et al. 1989)
INT18	VIN2	16	4q31	FRA4C	(Wentzensen et al. 2002)
INT21	VIN3	16	6q25	TCP1	(Wentzensen et al. 2002)
INT32	VIN3	16	3q28	FRA3C	(Wentzensen et al. 2002)
INT50	VIN3	16	13q14	KPNA3	(Wentzensen et al. 2002)
V1	VIN3	16	10p15		(Luft et al. 2001)

[a]BD, Bowen's disease; *v-CL* vulvar cell line; *VIN* vulvar intraepithelial neoplasia; *VaIN* vaginal intraepithelial neoplasia; *Ca* carcinoma

colony formation in vitro and by mouse xenograft assay in vivo [21]. The TRAP1 gene encodes a TRAP1/HSP75 protein which is a highly conserved molecular chaperone playing a key role in signal transduction, protein folding, protein degradation, and morphologic evolution [22]. Moreover TRAP1/HSP75 protein has been shown to regulate genes involved in cell cycle and metastases through the TNF pathway [23].

A systematic review of genomic integration sites of HPV DNA in penile, vaginal and vulvar neoplasia allowed to identify integration events in twelve invasive cancers and ten intraepithelial neoplasia. As shown in Table 2, the HPV genome integrants localized at different chromosome loci. The integration occurred on chromosomes 6, 9, 10, 11, 13, 15, 16, 19, 21 and X in 4.3 % of cases, on chromosomes 4 and 20 in 8.7 %, on chromosome 3 in 17.4 % and on chromosome 8 in 21.7 % of the cases. In all carcinoma samples, however, the integration took place within or nearby critical cellular genes, such as TRAP1, FAM92A1, POU5F1B, c-myc, JAG1, TP63, TYRP and PTPRD. This observation supports the hypothesis that HPV integrants identified in anogenital carcinomas represent the end-point of a clonal selection of cells with altered functional genes which overgrow and can give rise to invasive carcinomas. Of note, [24] analyzed the HPV16 integration sites in four lesions of multifocal vulvar Bowen's disease and in an invasive squamous vulvar carcinoma occurring synchronously in the same patient. Two out of four Bowen's disease lesions contained only episomal viral DNA, one the integrated virus at locus 4q28 and one integrated virus at loci 11q12 and 3q25. The squamous cell carcinoma contained integrated HPV16 at loci 8q24, where the c-myc is mapped, suggesting that cancer progression occurred when HPV integration affected a gene important for cell transformation.

Moreover, [25] using the W12 cell model showed that integrants can occur at anytime during episome maintenance and the integration sites of a significant number of single nonclonal population of W12 preferably occurs within chromosome bands containing a common fragile sites or containing cancer-associated coding gene or microRNA.

More studies are needed to identify the function of the affected host genes in order to elucidate the mechanisms leading to HPV-related carcinogenesis.

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Declaration of Interest The authors report no declarations of interest.

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