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Identification of rearranged sequences of HPV16 DNA in precancerous and cervical cancer cases

D. Tsakogiannis [a], M. Bletsa [a], Z. Kyriakopoulou [a], T.G. Dimitriou [a], C. Kotsovassilis [b], E. Panotopoulou [c], P. Markoulatos [a, *]

[a] University of Thessaly, School of Health Sciences, Department of Biochemistry & Biotechnology, Microbiology-Virology Laboratory, Larissa, Greece
[b] General Hospital of Athens, Clinical Biochemistry Dpt, Athens, Greece
[c] Research Centre of Oncology and Experimental Surgery, Anticancer Oncology Hospital of Athens "St Savvas", Athens, Greece

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ABSTRACT

Integration of HPV16 DNA into the host chromosome is considered to be a crucial step towards genomic instability and cervical cancer development. Aim of the present study was to investigate the presence of HPV16 rearranged intra-viral sequences in HPV16-positive normal, precancerous and cervical cancer samples using the method of Restriction Site-PCR (RS-PCR). Sequence analysis of HPV16 integrants revealed for the first time in clinical samples two distinct rearranged intra-viral sequences, concerning the conjunction of E2 and L1 genes and the conjunction of E1 and L1 genes with inverted orientation. Furthermore mapping analysis of the E1 and E2 genes in cervical samples with rearranged intra-viral sequences of HPV16 genome was conducted in order to determine the integrity of viral genes. The identification of intra-viral rearrangements provides valuable information regarding the HPV16 DNA integration, and may be a significant biomarker for the presence of chromosomal instability and DNA damages in clinical samples.

1. Introduction

Human Papillomaviruses (HPVs) are non-enveloped, epitheliotropic, double stranded, circular DNA viruses that infect cutaneous and/or mucosal epithelia [1]. Over 150 different HPV types have been characterized, and approximately 40 of them are detected in cervical epithelia and classified as members of the genus Alphapapillomavirus [2,3]. Epidemiological studies revealed that persistent infection with high-risk HPV types is the main risk factor for the development of high-grade cervical intraepithelial neoplasia and cervical cancer with HPV16 and HPV18 types being the most frequently identified HPV types in invasive cervical cancer cases worldwide [4–6]. Cancer progression is related with high-risk HPV types infection and with the expression at increased levels of high-risk HPV16 E6/E7 oncogenes [7–10].

Persistent infection with high-risk HPV types is associated with an increasing risk of integration of viral episomes into the host chromosome and is a crucial event for the progression of precancerous cervical malignancies to cervical cancer development [11–14]. HPV integration usually disrupts E1 and/or E2 genes, converting the circular viral genome to linear DNA, in which the Long Control Region (LCR) and the E6, E7 oncogenes are always retained integrated into the host genome [15–17]. The HPV integration into host DNA leads in the increased expression and stability of transcripts encoding the viral oncogenes E6 and E7, which inactivate various cellular proteins such as p53 (E6) and pRB (E7) [16,18–21]. Two types of HPV DNA integration have been found in cervical dysplasia. Type I contains a single copy of the HPV genome integrated in the host DNA, while type II includes HPV integrants as head to tail concatamers of full length HPV genomes into a locus within the host chromosome [22].

HPV integration into the host chromosome is considered to occur randomly, and it has been reported that the most frequently detected integration sites involve transcribed regions (e.g. the locus of the myc oncogene) and common fragile site [23–25]. In addition, recent studies have supported the concept of non random integration concerning repetitive regions of the host genome [26] and homologous regions between HPV DNA and host cellular sequences [27]. HPV integration is associated with frequent genomic structural changes of the host chromosome at the viral insertion sites in

* Corresponding author. University of Thessaly, School of Health Sciences Department of Biochemistry & Biotechnology, Ploutonos 26 & Aiolou, 41221, Larissa, Greece.
E-mail address: markoulatos@bio.uth.gr (P. Markoulatos).

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cervical carcinoma. Usually, these alterations contain genomic amplifications and rearrangements [17,28]. Moreover, rearranged intra-viral genome structures have also been detected in HPV16 DNA integrants of the CaSki cervical cancer cell line [17,22].

The present study focused on the identification of rearranged intra-viral sequences in clinical samples. Clinical samples that were revealed to contain HPV16 rearranged intra-viral sequences underwent extensive mapping analysis of the E1 and E2 genes in order to look at their integrity.

2. Material and methods

2.1. Cervical samples

Thirty six HPV16-positive specimens of Greek women (sixteen paraffin-embedded cervical biopsies and twenty Thin Prep samples) were diagnosed as cervical intraepithelial neoplasias of stage I, II, III and cervical cancer. Of these samples, six were diagnosed as normal cervical specimens, thirteen samples as high-grade cervical intraepithelial neoplasia (CIN II, III), six samples as low-grade cervical intraepithelial neoplasia (CIN I) and eleven samples as cervical cancer cases (nine paraffin-embedded cervical biopsies and two Thin Prep samples). Moreover the cancer cell line CaSki was also studied in the present analysis.

2.2. Nucleic acid isolation of clinical samples

Genomic DNA from Thin Prep samples, as well as from the cell line Caski was extracted using the chaotropic agent guanidine thiocyanate (GuSCN) [29]. DNA from formalin-fixed, paraffin-embedded cervical tissues was extracted using the established proteinase K method [30]. A 498 bp fragment of human β-actin was amplified by PCR as a quality control in order to assay for the integrity of the extracted DNA and to screen for the presence of PCR inhibitors [13]. Identification of HPV16 genome was carried out through Multiplex PCR method as was recently described [31].

2.3. Identification of HPV16 rearranged intra-viral sequences

The detection of HPV16 rearranged intra-viral sequences was performed through RS-PCR (Restriction Site PCR) [32]. RS-PCR was performed, using HPV16 site-specific primers in conjunction with four RSO primers (Restriction site oligonucleotide primers [25] (Table 1).

RS-PCR was performed in a final volume of 25 μl. PCR mixture contained 2 pmol of each virus-site specific primer and 20 pmol of the RSO primer, 5 μl 5X KAPA2G Buffer A (KAPA), 1.5 mM $MgCl_2$, 1.2 mM dNTPs (Invitrogen, Life Technologies, Carlsbad, CA, USA) and 0.5U of thermostable DNA Polymerase (KAPA2G Robust DNA Polymerase, KAPA, Boston, USA). The cycling conditions were: 30 cycles of 30 s at 95 °C, 1 min at 45 °C and 2 minmin at 72 °C. The first cycle was proceeded by a 2 min denaturation step at 95 °C and the last cycle was followed by a 5 min elongation step at 72 °C.

Nested PCR was performed using as template 2 μl of the first-round RS-PCR. Nested PCR was carried out in a final volume of 25 μl. PCR mixture contained 2 pmol of each virus-site specific primer and 20 pmol of the RSO primer, 5 μl 5X KAPA2G Buffer A (KAPA), 1.5 mM $MgCl_2$, 1.2 mM dNTPs (Invitrogen, Life Technologies, Carlsbad, CA USA) and 0.5U of thermostable DNA Polymerase (KAPA2G Robust DNA Polymerase, KAPA, Boston, USA). The cycling conditions were: 35 cycles of 30 s at 95 °C, 1 min at 55 °C and 2 min at 72 °C. The first cycle was proceeded by a 2 min denaturation step at 95 °C and the last cycle was followed by a 5 min elongation step at 72 °C.

Table 1
RS-PCR primers used to identify the rearranged intra-viral sequences of HPV16 genome in clinical samples. The number following HPV16 is related to the position of viral sequence, while the letters D and U indicates the orientation of primer (D forward primer, U reverse primer). Four RSOs were used with the restriction enzymes *Bam*HI, *Eco*RI, XmaI and *Sau*3AI, respectively.

Primers	Sequence (5′-3′)
RS-PCR	
HPV16-768-24D	ACAAAGCACACACGTAGACATTCG
HPV16-1545-26D	AGTAATAAATCAACGTGTTGCGATTG
HPV16-2386-25D	TTTGTTTACAACCATTAGCAGATGC
HPV16-2929-24D	GTGCCAACACTGGCTGTATCAAAG
HPV16-5156-27U	TACCAATTCTACTGTACCTAATGCCAG
HPV16-5883-27U	ACTTATTGGGGTCAGGTAAATGTATTC
HPV16-6686-25U	AGTAGATATGGCAGCACATAATGAC
HPV16-7591-22U	GTTGGCAAGCAGTGCAGGTCAG
Nested RS_PCR	
HPV16-790-25D	CGTACTTTGGAAGACCTGTTAATGG
HPV16-1587-26D	GGACTTACACCCAGTATAGCTGACAG
HPV16-2414-26D	AATAGGTATGTTAGATGATGCTACAG
HPV16-2964-25D	ACAAGCAATTGAACTGCAACTAACG
HPV16-5121-25U	GAGGTTAATGCTGGCCTATGTAAAG
HPV16-5850-28U	CCCTGTATTGTAATCCTGATACTTTAGG
HPV16-6651-25U	TGCGTGTAGTATCAACAACAGTAAC
HPV16-7524-26U	TTAAACCATAGTTGCTGACATAGAAC
RSO primers	
RSO-Bam	TAATACGACTCACTATAGGGAGANNNNNNNNNNGGATCC
RSO-Eco	TAATACGACTCACTATAGGGAGANNNNNNNNNNGAATTC
RSO-Xma	TAATACGACTCACTATAGGGAGANNNNNNNNNNCCCGGG
RSO-Sau	TAATACGACTCACTATAGGGAGANNNNNNNNNNGATC

2.4. Sequence analysis

Each individual amplicon of the second-round nested PCR was subjected to cloning using the pGEM-T Easy Vector system (Promega, Madison, WI, USA). The recombinant plasmid DNA was purified using the Nucleospin plasmid kit (Macherey Nagel, Duren, Germany) and the plasmids were subjected to sequencing at Macrogen Europe, Amsterdam, the Netherlands. The cloned sequences were characterized by database alignments using National Centre for Biotechnology Information (NCBI) using the Mega BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The numbering of nucleotide positions were determined through sequence alignment among the prototype strain of HPV16 genome and the identified rearranged HPV16 nucleotide sequences using the MUSCLE algorithm, in MEGA v.5 software [33,34]. The HPV16 reference sequence is documented at the HPV sequence database PaVE (http://pave.niaid.nih.gov) and is available from the National Center for Biotechnology Information (NCBI GI no. 333031) at http://www.ncbi.nlm.nih.gov.

2.5. Rearrangement confirmation

The HPV16 rearranged intra-viral sequences were confirmed using primer sets that amplify from both sides the locus of rearrangement, using the appropriate cervical sample as template. The primer sets were designed according to the nucleotide sequences of the identified HPV16 DNA rearrangement forms. The primers were constructed through the program Primer3 (Whitehead Institute; http://bioinfo.ut.ee/primer3-0.4.0). In particular, two distinct primer sets were designed for the rearrangement form E7, E1/L1 (RSE1F1-RSE1R1 and RSE1F1-RSE1R2), while one primer set was designed in order to confirm the rearrangement form E2/L1 (RSE2F1-RSE2R2) (Table 2).

PCR amplifications were performed in a final volume of 50 μl. Each PCR mixture contained 25 pmol of each primer set, 10× Taq buffer (GeneON, Ludwigshafen am Rhein, Germany), 1.5 mM $MgCl_2$, 1 mM dNTPs (Invitrogen, Life Technologies, Carlsbad, CA USA) and

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