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Research paper

Searching beyond the usual papillomavirus suspects in squamous carcinomas of the vulva, penis and head and neck

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ABSTRACT

Human Papillomaviruses (HPVs) are involved in the etiology of anogenital and head and neck cancers. The HPV DNA prevalence greatly differs by anatomical site. Indeed, the high rates of viral DNA prevalence in anal and cervical carcinomas contrast with the lower fraction of cancer cases attributable to HPVs in other anatomical sites, chiefly the vulva, the penis and head and neck. Here we analyzed 2635 Formalin Fixed Paraffin Embedded surgical samples that had previously tested negative for the presence of HPVs DNA using the SPF10/DEIA procedure, in order to identify the presence of other PVs not explicitly targeted by standard molecular epidemiologic approaches. All samples were reanalyzed using five broad-PV PCR primer sets (CP1/2, FAP6064/FAP64, SKF/SKR, MY9/MY11, MFI/MFII) targeting the main PV main clades. In head and neck carcinoma samples (n = 1141), we recovered DNA from two BetaHPVs, namely HPV20 and HPV21, and from three cutaneous AlphaPVs, namely HPV2, HPV57 and HPV61. In vulvar squamous cell carcinoma samples (n = 902), we found one of the samples containing DNA of one cutaneous HPV, namely HPV2, and 29 samples contained DNA from essentially mucosal HPVs. In penile squamous cell carcinoma samples (n = 592), we retrieved the DNA of HPV16 in 16 samples. Our results show first that the SPF10/DEIA is very sensitive, as we recovered only 2.1% (55/2635) false negative results; second, that although the DNA of cutaneous HPVs can be detected in cancer samples, their relative contribution remains anyway minor (0.23%; 6/2635) and may be neglected for screening and vaccination purposes; and third, their contribution to malignancy is not necessarily warranted and needs to be elucidated.

1. Introduction

Human Papillomaviruses (HPVs) are involved in the etiology of anogenital and oropharyngeal cancers (Forman et al., 2012). Within *Papillomaviridae*, HPVs belong into five different genera, namely Alpha-, Beta-, Gamma-, Mu- and NuPVs (Bernard et al., 2010). The large majority of HPVs, essentially Beta- and GammaPVs, cause asymptomatic infections and can be detected in healthy skin swabs and, for some GammaPVs, also in mucosal rinses (Bottalico et al., 2011; Gottschling et al., 2009). AlphaPVs is a very heterogeneous clade regarding tropism and clinical manifestation of the disease. Although most infections by human AlphaPVs are clinically asymptomatic some of them cause productive cutaneous warts; other cause productive mucocutaneous warts; finally a number of human AlphaPVs with mucosal tropism can induce malignant transformation after decades of persistent infection and are identified as carcinogenic or possibly/probably carcinogenic for humans (Doorbar et al., 2012; IARC, 2007).

Careful retrospective investigations have shown that infections by human AlphaPVs are the most likely etiologic cancer agent, accounting for nearly 100% of cervical cancer cases (de Sanjose et al., 2010), 88% of anal cancer cases in both males and females (Alemany et al., 2015) and for 74% of cancers of the vagina (Alemany et al., 2014). These high rates of viral DNA prevalence contrast with the lower fraction of cancer cases attributable to HPVs in other anogenital sites, chiefly the vulva and the penis. In vulvar cancers, infections by HPVs have been associated with less than 30% of the cancer cases (de Sanjose et al., 2013) while HPV DNA is found in around 30% of penile cancer (Alemany et al., 2016). Finally, in head and neck (HN) cancers, the most consistent findings relate to oropharyngeal cancers, where HPV DNA has been detected in 25% of cancer cases in contrast with the rest of the oral cavity, where HPV DNA is found in less than 10% of the cases (Castellsague et al., 2016; D'Souza et al., 2007).

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The development of PCR methods using general primers for amplification of a broad-range of HPVs had a major impact on the molecular epidemiology of viral-related infections. Many different primer sets targeting the *L1* gene have been designed and used for the detection of a broad-range of HPVs, as this gene is the most conserved one at the nucleotide level (Mengual-Chulia et al., 2016). Given their overwhelming contribution to cancer, most of these consensus primers were designed to target oncogenic AlphaPVs. Among these are the single pair of consensus primers GP5/6 (Snijders et al., 1990) and its extended version GP5+/6+ (de Roda Husman et al., 1995); and the MY09/11 (Manos et al., 1989) and its extended version PGMY09/11 pair of degenerate primers (Gravitt et al., 2000). PCR methods using PGMY09/11 primers have been extensively used in epidemiologic studies of HPVs (Giuliano et al., 2001; Richardson et al., 2005; Richardson et al., 2003; Schiffman et al., 2005; Tabrizi et al., 2005). The MY-based method generates an amplicon of 450 bp and targets a wide spectrum of HPVs, including all known possibly/probably/oncogenic AlphaPVs. The GP5+/6+ primer set has also been extensively used in many epidemiologic HPVs studies, either directly (Frisch et al., 1997; Hampl et al., 2006; Madsen et al., 2008; Skapa et al., 2007) or nested, after the MY primer amplification (Fox et al., 2005; Hampl et al., 2007; Piketty et al., 2003). This GP-based PCR generates an amplicon of 150 bp and can amplify at least 20 mucosal AlphaPVs (de Roda Husman et al., 1995). The SPF10 primer set has been extensively used in epidemiological studies due to its high sensitivity and specificity. This method is able to amplify 69 known AlphaPVs generating a small fragment (65 bp) of the *L1* gene (Kleter et al., 1998). Tests that rely on shorter fragments of the viral genome are considered to be more sensitive and usable for less preserved specimens. None of these widely used methods of HPVs detection are generally able to detect Beta- or GammaPVs. Another system widely used is the FAP primer set, which is very useful in identifying new PVs (Forslund et al., 2002; Forslund et al., 1999). This system is able to detect a broad-spectrum of PVs from both human and animal species (Antonsson et al., 2000; Antonsson and Hansson, 2002) and is usually the choice for detecting the presence of unknown, largely divergent PVs (Antonsson and McMillan, 2006; Bzhalava et al., 2014; Garcia-Perez et al., 2014).

The aim of the present study was to reanalyze samples from squamous cell carcinomas of the HN, penis and vulva that had previously tested negative for the presence of HPVs DNA using the SPF10/DEIA procedure in order to identify the presence of other PVs not targeted by standard epidemiologic approaches, covering mucosal as well as cutaneous HPVs.

2. Materials and methods

2.1. Sample collection

Samples were obtained from a Formalin Fixed Paraffin Embedded (FFPE) repository from a retrospective cross-sectional study coordinated by the Catalan Institute of Oncology (ICO), Barcelona, Spain, designed and constructed for the assessment of the HPV contribution to a number of anogenital and HN human tumours (Alemany et al., 2016; Alemany et al., 2015; Alemany et al., 2014; Castellsague et al., 2016; de Sanjose et al., 2013; de Sanjose et al., 2010). All specimens were tested for the presence of tumour tissue as well as for the presence of HPV DNA using a two-step SPF10/DEIA/LiPA25 protocol (Kleter et al., 1999). Amplification products testing positive for the presence of HPVs DNA but that resulted negative for LiPA25 genotyping were Sanger-sequenced to identify the nature of the viral DNA amplified. The detailed protocols and results for cervical (de Sanjose et al., 2010), anal (Alemany et al., 2015), vaginal (Alemany et al., 2014), vulvar (de Sanjose et al., 2013), penile (Alemany et al., 2016) and HN cancers (Castellsague et al., 2016) are described elsewhere. For the purpose of this study, only squamous cell carcinoma samples from the vulva, penis and HN cancer that had tested negative for the presence of HPV DNA using the SPF10/DEIA protocol were included in the analyses. As positive controls, for each anatomical location we included further 5–10% samples of the same study that had tested positive for the presence DNA from a single HPV. The final dataset for the present work consisted of 1141 cases randomly chosen among all HPV DNA negative squamous head-and-neck cancers (380 cases from the larynx, 380 cases from the oral cavity and 381 cases from the pharynx) and 59 controls for squamous head-and-neck cancers, 902 cases and 83 controls for squamous vulvar cancers and 592 cases and 57 controls for squamous penile cancers.

2.2. PCR and sequencing

DNA was released from FFPE material by incubation of four 5 μm slices with 250 μL of Proteinase K solution (10 mg/mL proteinase K, 50 mM Tris-HCl, pH 8.0) overnight at 56 °C. Samples were subsequently incubated at 95 °C for 8 min to inactivate proteinase K and stored at −80 °C until use. To serve as a control for the presence of input DNA, the human tubulin gene was targeted to generate an amplicon of 65 bp, the same length as the one generated by the SPF10 primers on the HPVs genomes (Alemany et al., 2015). For vulvar and penile cancer samples, the DNA solutions obtained after proteinase K treatment had been stored for several months at −80 °C. For samples from these locations, to facilitate the release of DNA adsorbed to the plastic walls, tubes were heated at 60 °C during 48 h prior to aliquot withdrawing for PCR.

Samples were analyzed using different sets of previously described primers, listed in Table S1, designed to detect a broad range of mucosal and cutaneous PVs: i) CPI/CPII (Tieben et al., 1993); ii) FAP6085/FAP64 (Li et al., 2013); iii) MY9/MY11 (Manos et al., 1989); iv) SKF/SKR (Sasagawa and Mitsuishi, 2012). Additionally, we designed a new set of broad-HPV primers by using CODEHOP (Rose et al., 2003), (MFI/II) specifically targeting the *E1* gene of cutaneous AlphaPVs. The MFI/II primer set was designed in order to complement the HPV detection of the SK primer set, as not all cutaneous AlphaPVs can be detected by using the SK primer set.

All PCRs reaction mixtures contained: 0.05 U/μL DNA Polymerase (Biotools), 1.0× PCR reaction buffer (10×), 1.5 mM MgCl2, 0.4 mM dNTPs (Invitrogen), 0.2 μM of each primer (Biolegio) and 100 ng of DNA. Each PCR mixture underwent 40 amplification cycles with different annealing temperatures for each primer set: 45 °C for SKF/R; 50 °C for CPI/II, FAP6084/64, MFI/II; and 47 °C for MY09/11. Finally, PCR products from those samples with an amplicon were sequenced at the Genoscreen facilities (Lille, France) in both strands, using the same primers used for amplification.

For all samples previously classified as SPF10/DEIA-negative in which we identified by Sanger-sequencing the presence of DNA from an HPV included in the 69 AlphaPVs detected by DEIA, we additionally performed a fresh SPF10 PCR followed by the LiPA25 genotyping assay (Kleter et al., 1999).

3. Results

We have tested a total of 2635 FFPE surgical samples from squamous carcinomas of the HN, penis and vulva that had previously tested negative for the presence of HPV DNA using the SPF10/DEIA procedure.

For squamous cancers of the HN, we were able to properly identify and genotype the HPV-DNA present in 31 out of 59 controls (53%) (Table 1). In all cases, the HPV hereby identified matched the one previously genotyped by LiPA25. We tested 1141 SPF10/DEIA-negative HN cancer samples, and we retrieved sequences specific for a one particular HPV in 15 out of these 1141 (1.3%) samples (Table 1, Table S2). In ten out of these 15 samples (66.7%), we detected DNA from mucosal HPVs, namely HPV16 (n = 8), HPV51 (n = 1) and HPV74 (n = 1). Finally, we recovered DNA from two BetaHPVs, namely HPV20 and HPV21, and from three cutaneous AlphaPVs, namely HPV2, HPV57 and HPV61. Although ten from these 15 samples were expected to have tested positive for the initial SPF10/DEIA screening (i.e. those containing either