Virology 525 (2018) 182–191

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/virology

High prevalence of *Gammapapillomaviruses* (*Gamma*-PVs) in pre-malignant cutaneous lesions of immunocompetent individuals using a new broad-spectrum primer system, and identification of HPV210, a novel *Gamma*-PV type

Elisa M. Bolatti[a,b,1], Lea Hošnjak[c,1], Diego Chouhy[a,b], Maria F. Re-Louhau[a], Pablo E. Casal[b], Hebe Bottai[d], Boštjan J. Kocjan[c], Emma J. Stella[a], Mario D. Gorosito[e], Adriana Sanchez[f], Ramón Fernandez Bussy[f], Mario Poljak[c,*], Adriana A. Giri[a,b,**]

[a] Grupo Virología Humana, Instituto de Biología Molecular y Celular de Rosario (CONICET), Suipacha 590, 2000 Rosario, Argentina
[b] Área Virología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, 2000 Rosario, Argentina
[c] Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Zaloška 4, 1000 Ljubljana, Slovenia
[d] Área Estadística y Procesamiento de Datos, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, 2000 Rosario, Argentina
[e] División de Anatomía Patológica, Facultad de Ciencias Médicas, Universidad Nacional de Rosario, Santa Fe 3100, 2000 Rosario, Argentina
[f] División de Dermatología, Facultad de Ciencias Médicas, Universidad Nacional de Rosario, Santa Fe 3100, 2000 Rosario, Argentina

ARTICLE INFO

Keywords:
Gammapapillomavirus
Gamma-PV PCR
CUT PCR assay
Actinic keratosis
HPV210
Genomic characterization

ABSTRACT

Genus *Gammapapillomavirus* (*Gamma*-PV) is the most diverse and largest clade within the *Papillomaviridae* family. A novel set of degenerate primers targeting the E1 gene was designed and further used in combination with the well-known CUT PCR assay to assess HPV prevalence and genus distribution in a variety of cutaneous samples from 448 immunocompetent individuals. General HPV, *Gamma*-PV and mixed infections prevalence were significantly higher in actinic keratosis with respect to benign and malignant neoplasms, respectively ($p = 0.0047$, $p = 0.0172$, $p = 0.00001$). *Gamma*-PVs were significantly more common in actinic keratosis biopsies than *Beta*- and *Alpha*-PVs ($p = 0.002$). The full-length genome sequence of a novel putative *Gamma*-PV type was amplified by 'hanging droplet' long-range PCR and cloned. The novel virus, designated HPV210, clustered within species *Gamma*-12. This study provides an additional tool enabling detection of HPV infections in skin and adds new insights about possible early roles of *Gamma*-PVs in the development of cutaneous malignant lesions.

1. Introduction

Papillomaviruses (PVs) are a highly diverse group of non-enveloped and double stranded DNA viruses that possibly infect mucosal and cutaneous epithelia of all vertebrates (de Villiers et al., 2004; Bravo et al., 2010). Classification of PVs is based on phylogenetic relationships of their complete L1 gene sequences (de Villiers et al., 2004). Currently, 324 human papillomavirus (HPV) types have been described: 221 officially recognized from the International Human Papillomavirus Reference Center at the Karolinska Institutet (Stockholm, Sweden; http://www.nordicehealth.se/hpvcenter/reference_clones) and 103 identified only by Next Generation Sequencing (NGS) techniques and available at the Papillomavirus Episteme (https://pave.niaid.nih.gov; Van Doorslaer et al., 2017). HPVs are grouped within five (*Alpha*, *Beta*, *Gamma*, *Mu* and *Nu*) out of more than 30 PV genera recognized to date, and further classified into mucosal/genital and cutaneous HPV types, based on the clinical manifestation of infections.

Cutaneous HPVs are distributed over all five HPV genera and represent approximately 75% of all HPV types described to date (de Villiers, 2013). In contrast to mucosal high-risk HPV types, clustering to the *Alpha*-PV genus, which have been recognized as oncogenic by WHO (WHO/ICO, 2010), the association of specific cutaneous HPV types with skin carcinogenesis remains to be demonstrated (Quint et al., 2015). Nevertheless, the relationship between *Beta*-PV type infections and

* Corresponding autor at: Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Zaloška 4, 1000 Ljubljana, Slovenia.
** Corresponding author at: Grupo Virología Humana, Instituto de Biología Molecular y Celular de Rosario (CONICET), Suipacha 590, 2000 Rosario, Argentina.
E-mail addresses: mario.poljak@mf.uni-lj.si (M. Poljak), giri@ibr-conicet.gov.ar (A.A. Giri).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.virol.2018.09.006
Received 25 June 2018; Received in revised form 5 September 2018; Accepted 7 September 2018

devolvement of non-melanoma skin cancer (NMSC; basal and squamous cell carcinomas) has been established in patients with epidermodysplasia verruciformis (Nindl et al., 2007; Lazarczyk et al., 2009). Moreover, *Beta*-PV DNA has been detected in a high proportion of cutaneous squamous cell carcinomas and their precursor lesions, actinic keratosis, in both organ-transplant recipients (Feltkamp et al., 2008; Proby et al., 2011) and immunocompetent individuals (Chahoud et al., 2016). Epidemiological data have shown that one in 100–1000 skin tumor cells harbor *Beta*-PV genomes, with higher prevalence and viral loads in actinic keratosis than in squamous cell carcinomas (Pfister et al., 2003; Weissenborn et al., 2005). These observations, together with molecular and histopathological data (Quint et al., 2015), support the current model of *Beta*-PV types as co-factors, together with UV-radiation, in the early pathogenesis of skin cancer (Pfister, 2003; Akgül et al., 2006; Nindl et al., 2007). However, the etiological role of cutaneous HPV infection and the mechanism(s) involved in skin carcinogenesis remain to be further elucidated (Howley and Pfister, 2015; McLaughlin-Drubin, 2015; Quint et al., 2015).

Gamma-PV genus is the most diverse and largest clade within the family *Papillomaviridae*, with 185 completely sequenced HPV types, surpassing *Alpha*- and *Beta*-PV genera, with 66 and 65 HPV types, respectively (Van Doorslaer et al., 2017). Improved molecular methods used for detection of cutaneous HPVs have led to the identification of many novel *Gamma*-PV types in common warts and other skin tumors (Bzhalava et al., 2013; Ekström et al., 2013; Hošnjak et al., 2015). Recent data have shown that HPV197 (species *Gamma*-24) is the most commonly detected HPV type in squamous cell carcinomas (Arroyo Muhr et al., 2015). Moreover, it has been demonstrated that six out of nine HPV types/putative types (HPV158, FA9, GC05, KC45, SE126 and SE253), belonging to the *Gamma*-PV genus, are by far more abundant (> 10-fold) in pooled samples of squamous cell carcinomas and actinic keratosis, in comparison to keratoachantomas (Bzhalava et al., 2014). Nevertheless, so far there is no evidence that *Gamma*-PVs contribute to the development of NMSC in immunosuppressed or immunocompetent individuals.

Since *Gamma*-PV genus is much more diverse than previously known (Van Doorslaer et al., 2017) and among 193 distinct commercial HPV tests currently available for detection of HPV infection, all but two target *Alpha*-PV types only (Poljak et al., 2016), novel detection methods are needed to establish tissue tropism and potential clinical relevance of novel and previously known *Gamma*-PVs. In the present study we describe the design of a novel primer system which enables the amplification of HPV types clustering to the *Gamma*-PV genus (*Gamma*-PV PCR). *Gamma*-PV PCR was further used in a combination with the well-known CUT PCR assay (Chouhy et al., 2010; Brancaccio and Robitaille et al., 2018) in order to assess the HPV prevalence and genus distribution in a variety of cutaneous samples from immunocompetent individuals. Additionally, we report the identification of 15 novel putative HPV types and the complete molecular and phylogenetic characterization of HPV210, a novel HPV type clustering to the species *Gamma*-12.

2. Materials and methods

2.1. Patients' data, sample collection and processing

Overall, 653 samples from cutaneous epithelia, obtained from 448 immunocompetent individuals [227 males and 221 females, median age: 55 years (range, 16–92 years)] were analyzed in this study (Table 1).

All subjects provided written informed consents for participation in the study and were interviewed about their demographic characteristics (age, gender, place of birth), history of skin pathologies and family history of skin cancer. All enrolled patients were examined by a dermatologist and all lesions were additionally subjected to histopathological evaluation at the División de Anatomía Patológica, Facultad de Ciencias Médicas, Universidad Nacional de Rosario, Argentina. The study was conducted according to the Declaration of Helsinki and was approved by the Institutional Review Board of the Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina (reference number 6060/134).

Eyebrow hair follicles, healthy forehead skin swabs, lesion swabs and lesion biopsies were collected, processed, and stored at − 80 °C until further analysis, as described previously (Kocjan et al., 2005; Chouhy et al., 2010; Bolatti et al., 2017). The adequacy of swab and biopsy samples for downstream analyses was determined by PCR amplification of the human beta-globin gene (Saiki et al., 1986).

2.2. Gamma-PV PCR primer design

Nucleotide sequences of the E1 open reading frame (ORF) of 64 completely characterized *Gamma*-PV types, available at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), were aligned using the ClustalW algorithm, provided within the MEGA6 software package (Tamura et al., 2013). Based on the alignment, several primer candidates, with a conserved sequence of 8–10 nucleotides at the 3′ region and a degenerate 5′ region, were found and subsequently analyzed for primer-dimer and primer-hairpin formations. Finally, two primers showing the lowest number of mismatches at the 3′ region were selected: FFPE1-Fw (5′-twiywghiytaaaacgaaagt-3′; i = inosine) and FFPE3-Rv (5′-sawwagwatytkcagyttcat-3′). The alignment of the 64 *Gamma*-PV types used to design the *Gamma*-PV primer system and the positions of the primer binding regions is shown in Supp. Table 1. The positions of the forward and reverse primer corresponded to nucleotides 1033-1053 and 1191-1171, respectively, of the HPV4 complete genome sequence (GenBank accession No. X70827), yielding an amplicon of 158 bp.

2.3. Determination of HPV infection in clinical samples

The presence of HPV infection was determined using the newly designed *Gamma*-PV PCR and an improved version of the original CUT PCR assay targeting L1 ORF, using modified primers. Briefly, CUT primers were originally designed using an L1 ORF nucleotide sequence alignment derived from 88 completely characterized cutaneotropic and mucosotropic HPV types from *Alpha*-, *Beta*-, *Gamma*-, *Mu*- and *Nu*-PV genera (Chouhy et al., 2010). Subsequently, the CUT PCR primer system was improved with the design of 3 novel primers [CUT1EFw (5′-trccigaycciaatagatttg-3′; i = inosine), CUT1CRv (5′-tcicacatrtciccrtcytg-3′; i = inosine) and CUT1DRv (5′-tcisccatrtciccrtcytg-3′; i = inosine)] based on the alignment of L1 ORF sequences of 244 additional HPV types/putative types. Furthermore, the original primers CUT1AFw, CUT1BFw and CUT1CFw (Chouhy et al., 2010) were excluded from the improved CUT PCR, based on the specificity analysis, as in some conditions these primers enabled the amplification of human genomic DNA. Current CUT primer-binding regions within the alignment of L1 ORFs of 64 Gamma-PVs are shown in Suppl. Table 1.

All PCR reactions were performed using the thermocycler Mastercycler Personal (Eppendorf, Hamburg, Germany).

Gamma-PV PCR was performed in a reaction mixture with a final volume of 25 µl, containing 3 µl of sample DNA. Reaction mixtures contained 0.8 µM of each primer, 150 µM of each dNTP (Thermo Scientific, Waltham, USA), 4 mM of $MgCl_2$, 0.2% BSA, 1 X PCR buffer with $(NH_4)_2SO_4$ and 2.5 U of Taq DNA polymerase (Thermo Scientific). Reaction mixtures were first heated for 2 min at 94 °C, followed by 5 cycles of 20 s at 94 °C, 20 s at 50 °C and 10 s at 66 °C, 10 cycles of 20 s at 94 °C, 20 s at 49 °C and 10 s at 66 °C, 10 cycles of 20 s at 94 °C, 20 s at 48 °C and 10 s at 66 °C, 20 cycles of 20 s at 94 °C, 20 s at 47 °C and 10 s at 66 °C, followed by 2 min at 66 °C.

CUT PCR was performed in a reaction mixture with a final volume of 25 µl, containing 5 µl of sample DNA. Reaction mixtures contained 0.4 µM of primer CUT1Fw, 0.4 µM of primer CUT1EFw, 0.266 µM of