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Brief Communication

Establishment of an *in vitro* equine papillomavirus type 2 (EcPV2) neutralization assay and a VLP-based vaccine for protection of equids against EcPV2-associated genital tumors

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ABSTRACT

The consistent and specific presence of *Equus caballus* papillomavirus type 2 (EcPV2) DNA and mRNA in equine genital squamous cell carcinoma (gSCC) is suggestive of an etiological role in tumor development.

To further validate this concept, EcPV2-neutralizing serum antibody titers were determined by an EcPV2 pseudovirion (PsV) neutralization assay. Furthermore, an EcPV2 L1 virus-like particle (VLP)-based vaccine was generated and its prophylactic efficacy evaluated *in vivo*.

All 6/6 gSCC-affected, but only 3/20 tumor-free age-matched animals revealed EcPV2-neutralizing serum antibody titers by PsV assay. Vaccination of NZW rabbits and BalbC mice with EcPV2 L1 VLP using Freund's or alum respectively as adjuvant induced high-titer neutralizing serum antibodies (1600–12,800). Passive transfer with rabbit EcPV2–VLP immune sera completely protected mice from experimental vaginal EcPV2 PsV infection.

These findings support the impact of EcPV2 in equine gSCC development and recommend EcPV2 L1 VLP as prophylactic vaccine against EcPV2 infection and associated disease in equids.

Introduction

Papillomaviruses (PV) are a large group of epitheliotropic DNA viruses that ubiquitously infect vertebrates, causing mostly benign papillomas (warts), and rarely malignant intraepithelial neoplasia and invasive cancer. More than 60 animal PV and 160 human PV (HPV) genotypes have been completely characterized thus far (Bernard et al., 2010). In humans, 15 to 20 oncogenic (high-risk) HPV types are causally associated with development of squamous cell carcinomas (SCC) of the anogenitalia – most notably the uterine cervix – and the oropharynx, which together contribute 5% to the human cancer cases worldwide (de Martel et al., 2012; Parkin, 2006; zur Hausen, 2009).

SCC represents the most common type of horse cancer (~20% of all equine malignant tumors), which can affect any body site, yet predominate at sites with muco-cutaneous transition such as the external genitalia (~57%), the head and ocular region (~43%) (Knowles et al., 2015; Strafuss, 1976). Equine SCC usually emerges from non-invasive precursor lesions including plaques, papillomas and *in situ* carcinomas. There is growing evidence for *Equus caballus* papillomavirus type 2 (EcPV2) being causally involved in the pathogenesis of equine genital SCC (gSCC) and its precursor lesions (Knight et al., 2011; Scase et al., 2010; Sykora et al., 2012). Viral DNA and transcripts were consistently found in lesions, but not in normal adjacent tissue or non-genital SCC (Scase et al., 2010), and only sporadically in genital swabs or smegma of tumor-free horses (Bogaert et al., 2012; Fischer et al., 2014; Sykora et al., 2012). In previous studies EcPV2 DNA was detected in 45–90% of all penile SCC (Bogaert et al., 2012; Knight et al., 2011). Little is known about the natural route of EcPV2 transmission, although anecdotal reports indicate that virus can be transmitted via direct contact (Sykora et al., 2012). Moreover, the assumed EcPV2 infection-related mechanisms underlying tumor pathogenesis remain to be elucidated, and information on the seroprevalence of EcPV2 in gSCC-affected horses is still lacking. Addressing these issues is of importance, as similar pathological features, the

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presumed direct route of transmission, and external genitalia as preferential sites of infection could establish EcPV2-positive gSCC as a correlative naturally occurring PV-associated genital tumor.

For humans three multivalent HPV vaccines have been introduced that target mucosal PV infections. These subunit vaccines comprise virus-like particles (VLPs) self-assembled from the L1 major capsid proteins of high-risk HPV16/18 (bivalent), HPV6/11/16/18 (quadrivalent), or HPV6/11/16/18/31/33/45/52/58 (nonvavalent) adsorbed to adjuvant. VLPs are empty capsids that morphologically and immunologically mimic authentic virions and induce high-titers of type-restricted neutralizing antibodies. Animal vaccination trials with VLP-based immunogens have been conducted with CRPV (cottontail rabbit PV) in rabbits (Breitburd et al., 1995), COPV (canine oral PV) in dogs (Suzich et al., 1995), BPV4 in cattle (Kirnbauer et al., 1996), and, more recently, BPV1/2 in horses (Hainisch et al., 2012). A murine challenge model based on vaginal infection with PV pseudovirions (PsV), i.e. PV L1/L2 capsids harboring a reporter plasmid, mimics the natural situation *in vivo*. Following infection of mucosal keratinocytes and expression of the reporter protein (Luciferase), the latter can be detected *in vivo* by bioluminescence imaging (Roberts et al., 2007). This model has proven more sensitive in the detection of protective antibodies induced by PV vaccines compared to *in vitro* PsV neutralization assays (Longet et al., 2011).

In a serological pilot study, we have developed an EcPV2 neutralization assay and evaluated the immune response of horses to natural exposure with EcPV2. Furthermore, given the severity of equine gSCC disease and current limitations of therapy, we have generated a prophylactic EcPV2 L1 VLP vaccine and addressed its protective efficacy in a murine experimental EcPV2 PsV challenge model.

Results

EcPV2 seroprevalence in gSCC-affected and clinically tumor-free horses

EcPV2 pseudovirions (PsV), i.e. viral capsids harboring the alkaline phosphatase-expressing reporter plasmid pYSEAP, were generated as virion surrogate for the development of an EcPV2 neutralization assay. Codon-modified synthetic genes encoding EcPV2 L1 major and L2 minor capsid proteins (Scase et al., 2010) were synthesized and transferred into 293TT cells, PsV were purified on Optiprep gradients and a PsV-based neutralization assay (PBNA) was established according to Buck et al. (2004). Using this assay, sera of six horses with histologically confirmed, EcPV2 DNA-positive gSCCs (Sykora et al., 2012), and of 29 apparently tumor-free control horses were assessed for the presence of EcPV2-neutralizing antibodies (Table 1).

PBNA revealed EcPV2-neutralizing antisera in 6/6 (100%) horses diagnosed with penile or vulvar SCC confirmedly harboring EcPV2 DNA, with titers ranging from 50 to 3200. From 20 age-matched control animals, three (15%; #12, 14 and 21) were seropositive, with titers ranging between 50 and 400. Interestingly, genital swabs/smegma obtained from these three horses had also scored positive for EcPV2 DNA. Furthermore, two of the three seropositive control horses (#12 and #14) had been co-stabled for many years with animals bearing gSCC (an Icelandic gelding not included in this study and mare #6). No information on the stabling history was available for #21. In contrast to EcPV1-induced juvenile papillomas, gSCC mainly affect older animals (Junge et al., 1984) thus it is noteworthy that the small number of younger horses enrolled in this study ($n=9$; medium age 3.8 years; maximum age 8 years) tested seronegative for EcPV2. This finding indicates that either infection occurs later in life (by increasing cumulative number of close or sexual contacts), or that seroconversion is rare or short-lived in transiently infected animals but may increase with persistent infection.

Generation of EcPV2 L1 virus-like particles (VLP)

To develop a prophylactic vaccine for preventing infection and ensuing disease, EcPV2 L1 VLP was generated. The EcPV2 L1 ORF was PCR amplified from DNA isolated from an equine penile carcinoma, sub-cloned into baculovirus transfer vector pEVmod (Kirnbauer et al., 1992), and verified by bidirectional sequencing. Two clones were isolated, one harboring a wild-type (wt) L1 sequence (Scase et al., 2010), and one containing a mutated (mut) form of L1. The latter comprised an unintentionally introduced point mutation (A1110G) predicted to change the highly conserved amino acid (aa) 370 Glutamic acid to Glycine (E370G). Two recombinant baculoviruses were isolated and L1 proteins expressed in Sf9 insect cells (Schellenbacher et al., 2009). SDS-PAGE and Coomassie-staining of crude cell lysates and gradient-purified protein preparations (Shafti-Keramat et al., 2003) revealed EcPV2 wt L1 VLP and mut L1 migrating as ~50 KD proteins (Fig. 1A, *). Negatively stained preparations were visualized by transmission electron microscopy (TEM), demonstrating assembly into spherical particles with the expected diameter of ~50–55 nm for EcPV2 wt L1, whereas EcPV2 mut L1 aggregated into irregular complexes (Fig. 1B).

Immunization with EcPV2 wt L1 VLP and EcPV2 mut L1 protein

To analyze immunogenicity of EcPV2 wt L1 VLP in comparison to non-assembled mut L1 protein, one New Zealand White (NZW) rabbit each was immunized on days 0, 21 and 42 with 50 μg of the respective protein using complete or incomplete Freund's adjuvant (CFA/IFA). In addition, five Balb/C mice were subcutaneously immunized on days 0, 14 and 28 with 10 μg of EcPV2 wt L1 VLP using clinically relevant (less reactogenic) aluminum hydroxide adjuvant. All sera were drawn two weeks after the final boost. Rabbit antisera were tested in serial log dilutions (10^{-2} to 10^{-7}) for L1-specific antibody responses by ELISA (Fig. 2A), using either wt L1 VLP or mutated L1 protein as ELISA antigens. Under native ELISA conditions, post-immune but not pre-immune sera revealed antibody titers of 100,000 against L1 VLP, and of 1,000,000 against mut L1 protein. Similar titers were observed when using denatured preparations of L1 VLP (10,000) or mut L1 protein (1,000,000) as antigens (not shown).

Vaccination with EcPV2 wt L1 VLP, but not mut L1 protein, induced high-titer neutralizing antisera in rabbits and mice

Neutralizing antisera raised to PV virions are directed to conformation-dependent epitopes present on assembled VLP (or pentamer subunits) and correlate with vaccine efficacy (Harro et al., 2001). Therefore rabbit immune sera were tested in PBNA using EcPV2 PsV consisting of assembled wt EcPV2 L1 and L2, enclosing the reporter plasmid pYSEAP (Fig. 2B). Rabbit antiserum to L1 VLP was neutralizing at high titers (6400), whereas antiserum to mut L1 protein or pre-immune serum did not neutralize EcPV2 PsV at the highest concentration tested (dilution of 1:50). Each of 5 mouse antisera against EcPV2 wt L1 VLP also neutralized PsV with titers ranging between 1600 and 12,800 (Fig. 2B).

Passive transfer of neutralizing antiserum raised against VLP protected mice against vaginal infection with EcPV2 PsV

To evaluate efficacy of prophylactic vaccination with EcPV2 L1 VLP, we used a well-established murine genital challenge model with EcPV2 PsV (Roberts et al., 2007). Mice were passively transferred (i.v.) with 20 μl of rabbit pre-immune or immune serum to EcPV2 wt L1 VLP, EcPV2 mut L1 protein, or BPV1 L1 VLP and challenged with luciferase-encoding EcPV2 PsV. Mice (5 per group) that had received EcPV2 VLP immune serum were completely protected against vaginal