The trichodysplasia spinulosa-associated polyomavirus : discovery - prevalence - infection - expression

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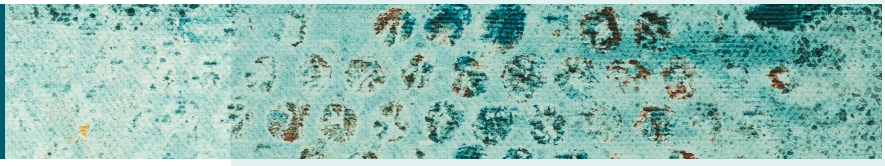

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With the identification of several new human polyomaviruses, in particular of the polyomavirus associated with trichodysplasia spinulosa (TSPyV), an exciting new field of polyomavirus research opened up. The general findings described in this thesis, especially regarding TSPyV, will be discussed in this concluding chapter together with leads for further study.

GENERAL DISCUSSION

Methods of polyomavirus discovery and antibody detection

Various methods were used for the identification of new human polyomaviruses (HPyVs) from different tissue samples, such as degenerate PCR, random (RT)-PCR, rolling-circle amplification (RCA) and next-generation sequencing (NGS). For small, circular DNA viruses such as the HPyVs, RCA has several advantages. It is a straightforward, rapid, cheap and easy method that preferentially amplifies small circular dsDNA molecules (HPyV genomes!) from complex biological, host genome-containing samples. RCA in combination with random primers will therefore remain a valuable tool for the identification of new PyVs, next to the extremely powerful but still expensive NGS techniques.

A limitation of amplification by RCA could be the detection limit, although the sensitivity of RCA can be increased by pre-treatment of the sample with exodeoxyribonuclease V in combination with a restriction enzyme unlikely to digest PyV DNA, in order to reduce the host genomic DNA content.[37] Directed RCA (dRCA), a modified RCA using specific primers, increases the RCA sensitivity significantly.[211] However dRCA will only be beneficial for PyV discovery while using degenerated PyV primer sets. In case of very low DNA concentrations, the somewhat more laborious screening of cDNA or DNA libraries by NGS might be a better tool for PyV discovery.

After TSPyV identification by RCA, we wanted to learn about the prevalence and epidemiology of TSPyV. For this purpose a TSPyV immunoassay was developed that measures seroreactivity against the immunogenic major capsid protein VP1. Nowadays, two types of immunoassays are used for PyV serology. One based on VP1 presented as virus-like particles (VLPs), the other on glutathione S-transferase (GST)-VP1 fusion protein. The latter can be displayed on a Luminex platform enabling simultaneous measurement of seroresponses against a multitude of viral antigens, for instance VP1 of different PyV species. We chose for the GST-VP1-based Luminex system for our studies, which in our hands turned out to be highly reproducible with very low cross-reactivity between VP1 antigens, even among closely related HPyVs.

One might doubt the presence of conformational epitopes in a GST-VP1 based immunoassay. However, similar TSPyV seroprevalences of ~75% in healthy adults were measured in our Dutch and Australian studies,[62,67] and in a Finish and a Japanese study using VP1-based VLPs.[66,202] VLPs are known to resemble native viral particles and thus retain conformational epitopes. A recent comparison between the two immunoassays using a set of 396 serum samples tested for BKPyV seroreactivity, again demonstrated a high correlation between both methods (r_s 0.823) (Wunderink et al., unpublished data). Based on these results, it is likely that GST-fusion proteins present the VP1 antigen in a comparable fashion to VP1-VLP and therefore can be used for PyV serological screening. Furthermore, since it has been described for MCPyV that VLP antibody levels are correlated to virus-neutralization,[247] it is anticipated that our TSPyV serological assay detects neutralizing antibodies.

The less immunogenic large T (LT) antigen might be useful in an immunoassay to discriminate between active and latent TSPyV infections. This has been described for MCPyV, where MCPyV LT seroreactivity reflected tumor burden in MCC patients.[185] Likewise, it would be interesting to test antibodies against the new T-antigens identified for TSPyV, middle T (MT) and alternative T (ALTO).

TSPyV prevalence and infection

As TSPyV was discovered in a very rare skin disease affecting hair follicles called trichodysplasia spinulosa (TS), eyebrow hairs of asymptomatic kidney transplant patients and forehead skin swaps of healthy individuals were tested for TSPyV.[28,65] This revealed a TSPyV DNA prevalence of 4% and 2%, respectively, both with low DNA loads of <1 copy/cell. Despite the low TSPyV DNA prevalence in TS-asymptomatic healthy and immunosuppressed individuals, the overall TSPyV seroprevalence is high, 70% in healthy individuals and 89% in kidney transplant patients. These findings match with the general idea that HPyVs are ubiquitous viruses that infect their host without causing apparent primary disease. TSPyV IgG seroresponses remain detectable through life, although waning of TSPyV seroreactivity among seropositives is observed at older age.[61,62,202]
The large difference between TSPyV prevalence (based on viral DNA detection of skin) and seroprevalence (based on detection of virus-specific seroresponses) might be partly explained by the sensitivity of the TSPyV PCR. When only few skin cells are infected, local skin sampling might miss the sparsely TSPyV-positive skin cells. But the skin prevalence-seroprevalence discrepancy could also indicate that TSPyV is not a cutaneous HPyV *per se*. Of note, the low TSPyV DNA prevalence in skin samples is comparable to what is observed in other materials, such as nasopharyngeal, fecal, and urine samples.[32,248] Moreover, we showed that the serological profile of TSPyV does not resemble that of other HPyVs found on the skin, it rather resembles that of BKPyV.
The biological basis behind the different HPyV serological profiles is not known. They might reflect differences in the extent of virus exposure (localized infection or systemic), route of infection, cell tropism or antigenicity. The structure of the major capsid protein VP1 might influence all of these aspects. For instance HPyV6 and HPyV7 show a unique VP1 surface morphology with elongated VP1 surface loops which could be relevant for antigenicity but also for receptor binding, because as a consequence of these elongated loops non-sialylated receptors are utilized by these viruses.[249] In general for HPyV receptor-binding, VP1 is linked with terminal sialic acids (alpha2,3 or alpha2,6) of mainly gangliosides on the host cell membrane,[250] after which they are internalized using different entry pathways. Recent VP1 studies for Merkel cell polyomavirus (MCPyV) and HPyV9 show variations on this attachment mechanism.[251,252] For TSPyV it was recently shown that the location of

the VP1 binding site differed from any other identified PyV VP1 binding site. The data suggests that glycolipids rather than glycoproteins are important for TSPyV infection.[210] This knowledge is potentially relevant to identify the porte d'entrée and route of infection of TSPyV.

TSPyV and pathogenesis

The high TSPyV DNA loads detected in TS-affected skin, which argues against integration of viral DNA into the host genome, suggests differences in pathogenic mechanisms between TSPyV and MCPyV. In this aspect, TSPyV seems more related to the oncogenic raccoon polyomavirus, a highly replicating virus present in neuroglial brain tumors of raccoons and not found to be integrated.[253,254]

For JCPyV and BKPyV it was shown that pathogenicity is caused by reactivation of the persistent latent virus under immunosuppression. However, for TSPyV we showed that TS, at least in the one patient where this could be studied, represents a manifest primary TSPyV infection. At the moment we do not know whether this is true for all described TS cases, but recently we could confirm this pattern in another TS case. It is tempting, therefore, to propose that the rarity of TS is explained by the unlikely event of a primary TSPyV infection occurring after childhood in a severely immunocompromised host.

As immunosuppression is an important factor in TS development, lowering immunosuppressive medication without risking organ rejection and negatively influencing leukemia treatment, would benefit the outcome of TS. However, reducing the dose of immunosuppressive drugs is not always possible. Various antiviral treatments have been tried on TSPyV-infected TS patients, for instance topical cidofovir and oral valganciclovir. Cidofovir is a cytosine analogue and valganciclovir a guanosine analogue, both inhibiting polymerase activity and thereby likely influencing polyomavirus replication which is dependent on human polymerase. However, direct interference of cidofovir with the helicase/ATPase activity of large T (LT) or with the small T (ST) induced viral replication has also been postulated as modes of action.[255,256] The most effective therapy for TS so far seems the use of topical cidofovir, 1–3%. In most treated cases the external features of TS improved dramatically upon cidofovir cream treatment and the effect was already visible 2–4 weeks post-treatment. We were able to study the effect of cidofovir on the TSPyV DNA load in two TS patients by testing skin samples pre- and post-treatment. In both patients a rapid clinical response to treatment was observed. In one patient, however, only a 10 fold decrease in TSPyV DNA load was detected after treatment. Since TS patients are showing various responses upon cidofovir treatment, more studies are needed on the mechanism of action of cidofovir and on the identification of additional targets for antiviral drug treatment of polyomavirus-related disease.

TSPyV T-antigens and pathogenicity

We characterized the TSPyV T-antigens in detail, which is important for further studies on the pathogenic mechanisms of TSPyV. A unique T-mRNA transcription pattern was unraveled. Next to the commonly expressed HPyV small T (ST) and large T (LT) antigens, MT and ALTO expression was identified. Until recently, expression of the (oncogenic) MT protein was thought to be confined to rodent PyVs. Expression of ALTO, reminiscent of the C-terminal part of MT, was noted for MCPyV, but MT expression has not been described before for any HPyV. Whether ALTO is expressed by alternative splicing of the primary T-antigen mRNA or involves internal translation initiation driven by an IRES is unclear at the moment.

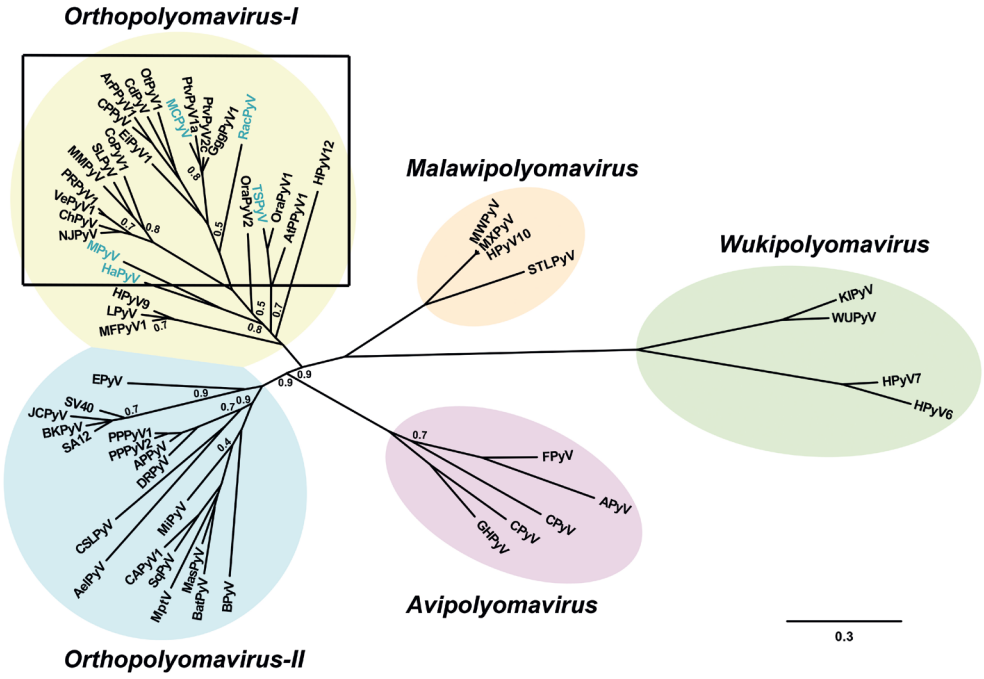

FIGURE 1. PHYLOGENETIC TREE OF POLYOMAVIRUSES.
The box within the *Orthopolyomavirus-I* clade represents the ORF5-positive polyomaviruses putatively expressing MT and/or ALTO. Polyomaviruses known to be involved in transformation or proliferation are depicted in blue.

In another recent study by our group not included in this thesis, we showed that the MT/ALTO-encoding ORF5 is largely conserved within a monophyletic cluster of mammalian PyVs, the *Orthopolyomavirus-I* lineage (**FIGURE 1, box**), and being subject of strong evolutionary selection.[105,204] This selection involves an unique, binary codon-constrained valine-alanine toggling imposed by conservation of the overlapping CxLxE pRB-binding motif in the LT-encoding ORF2. The strong evolutionary pressure on ORF5 makes it very likely that MT/ALTO expression is important for the evolution of this cluster of PyVs that contains all the PyVs thus far associated with proliferation and oncogenic transformation (**FIGURE 1, in blue**). Despite inducing hyperproliferation, no indication has been obtained so far of involvement of TSPyV in oncogenesis.

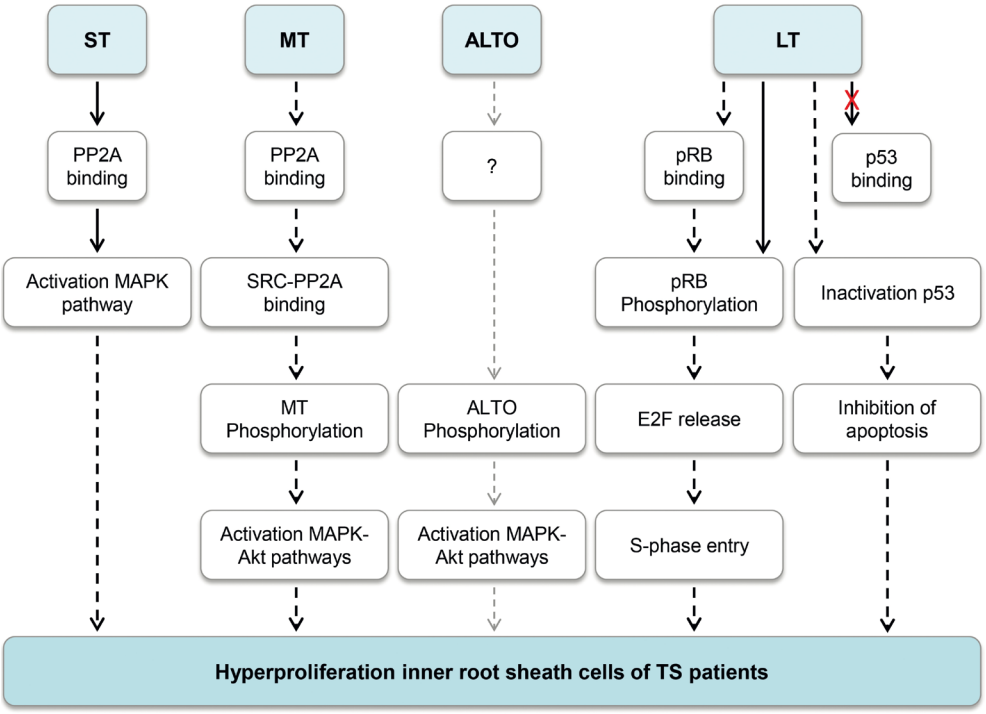

FIGURE 2. MODEL BY WHICH TSPyV T-ANTIGENS MIGHT INFLUENCE CELLULAR PATHWAYS AND THEREBY AFFECTING PROLIFERATION OF IRS CELLS IN TS PATIENTS.

Most of the pathways were described for SV40 and MPyV (dashed arrows). For TSPyV binding of ST to PP2A A and C subunits and MAPK-pathway involvement, an association between LT with hyperphosphorylated pRB, and no interaction between LT and p53 (red cross) were experimentally proven (solid arrows).[31,218,219,257] Polyomavirus ALTO interactions with host factors are not described yet (grey dashed arrows).

The role of T-antigens in inner root sheath (IRS) hyperproliferation was studied by immunohistochemistry (IHC) on TS-affected skin of patients. In the hyperproliferating IRS cells we showed LT co-expression with the tumor suppressor gene pRB, which was hyperphosphorylated and therefor most likely promoting cell cycle entry. Direct interaction between TSPyV LT and pRB was not studied yet, but this could be assessed in the future by *in vitro* GST pull-down assays or by (co-)immunoprecipition experiments like the ones performed for TSPyV LT-p53.[257] Due to insufficient TS lesional sections, we were unable to perform p53 IHC, but in a recent study no direct interaction between the tumor suppressor gene p53 and TSPyV LT was found.[257] This does not consequently mean that the p53-pathway is not influenced by TSPyV. For instance, for MCPyV an indirect LT-p53 interaction was described accompanied by p53 transcription inhibition.[258]
Next to interference with the cellular pRB pathway, there is an increasing body of evidence showing that TSPyV disrupts cellular protein kinase pathways. For instance the studies by Tyring and co-workers showed that TSPyV ST interacts with subunits of PP2A,[218] and as a consequence activates the MAPK pathway.[219] Whether next to ST, also MT and ALTO interact with cellular phosphokinase pathways is subject for further studies. Both MT and ALTO harbor a C-terminal transmembrane-binding domain which is known to be important for the transforming character of MPyV MT. ALTO, however, lacks the PP2A-binding domain encoded by the first exon of MT, and therefore must use other strategies than MT to influence phosphokinase pathways. In the flowchart in **FIGURE 2**, an overview is given of the cellular pathways described above, through which the early genes of TSPyV might induce proliferation of the follicular IRS cells.

Concluding remarks

With the expansion of the number of human polyomaviruses since 2007, polyomavirus research has boomed. In this thesis the first steps were made to learn about TSPyV infection and pathogenesis. We found evidence, despite the rarity of TS, that TSPyV is widely circulating in the general population, like what is observed for most other HPyVs. Although TSPyV is causing a skin disease, from the serological profile, DNA-prevalence data and case study one can conclude that TSPyV infection is not restricted to the skin and most likely involves other organs as well. Aspects such as transmission, route of infection, cell tropism and site of latent infection are not yet understood for TSPyV. Further research on these topics is essential to understand these infections better, and to develop strategies for treatment and prevention.
The T-antigens are the usual suspects when it comes to understanding the pathogenic mechanisms employed by TSPyV. Now that the TSPyV T-antigenome has been unravelled, future steps in TSPyV research can be planned. These studies should focus on the role(s)

of MT and ALTO in virus replication and cell proliferation, and possibly transformation. The importance of these studies is indicated by the pivotal role that ORF5 (encoding MT and ALTO) seems to play in the evolution of the *Orthopolyomavirus-I* clade as a whole. Moreover, TSPyV is so far the only PyV species in which intra-species COCO-VA toggling is observed and the role of these two variants can be studied. It is expected that the findings of these studies will further increase our understanding of HPyV infection in general, and of pathways involved in cell growth and replication as well, contributing to new treatment modalities against viral disease and cancer.