The trichodysplasia spinulosa-associated polyomavirus : infection, pathogenesis, evolution and adaptation

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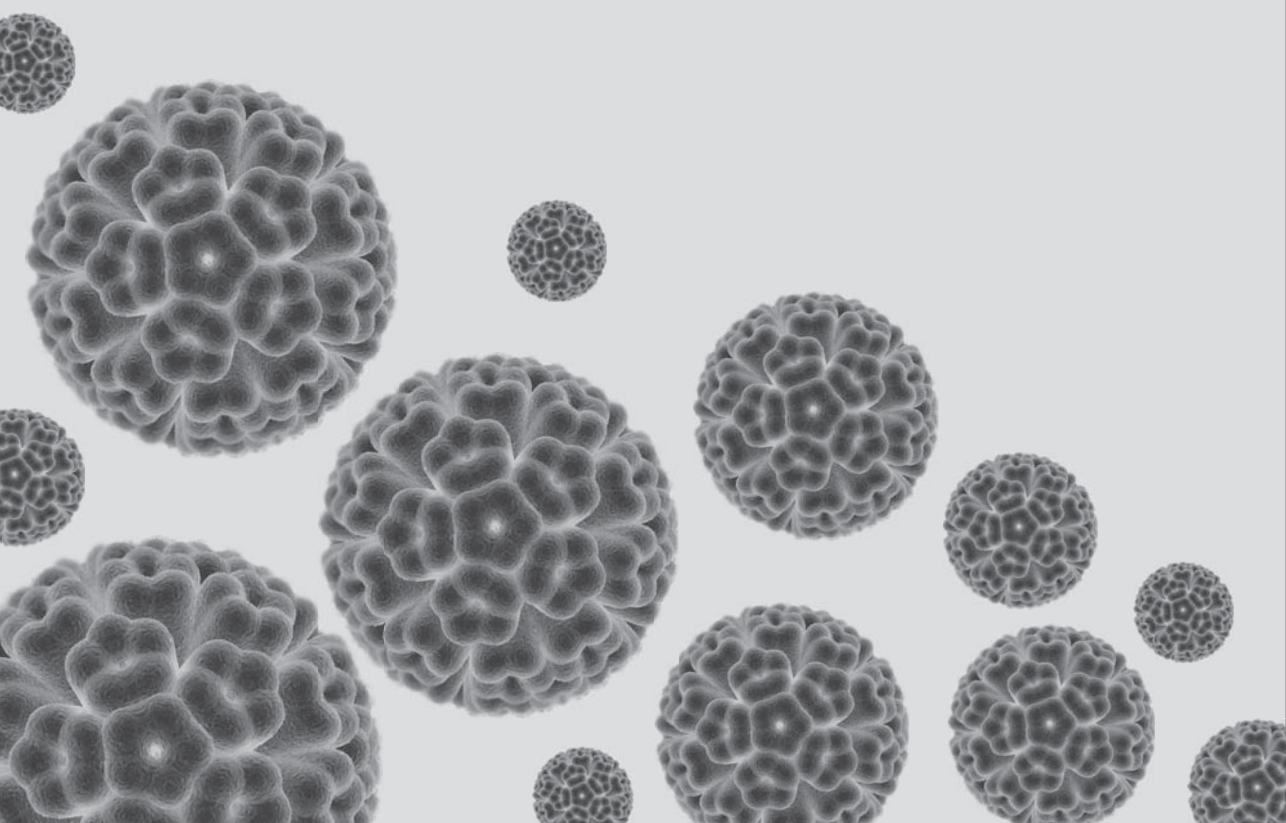

Chapter 3

Active TSPyV infection in trichodysplasia spinulosa

Adapted from:*

Trichodysplasia spinulosa is characterized by active polyomavirus infection

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Abstract

In this chapter, we aimed to corroborate the relationship between TSPyV infection and TS disease by analyzing the presence, load, and precise localization of TSPyV infection in TS patients and in controls. For this purpose, archived TS lesional and non-lesional skin samples were retrieved from TS patients through a PubMed search. Samples were analyzed for the presence and load of TSPyV DNA with quantitative PCR, and for expression and localization of viral protein with immunofluorescence. Findings obtained in TS patients (n = 11) were compared to those obtained in healthy controls (n = 249). TSPyV DNA detection was significantly associated with disease ($P < 0.001$), with 100% positivity of the lesional and 2% of the control samples. Quantification revealed high TSPyV DNA loads in the lesional samples (~10^6 copies/cell), and low viral loads in the occasionally TSPyV-positive non-lesional and control samples (<10^2 copies/cell). TSPyV VP1 protein expression was detected only in lesional TS samples, restricted to the nuclei of inner root sheath cells overexpressing trichohyalin. Altogether, the high prevalence and load of TSPyV DNA only in TS lesions, and the abundant expression of TSPyV protein in the affected hair follicle cells demonstrate a tight relationship between TSPyV infection and TS disease, and indicate involvement of active TSPyV infection in TS pathogenesis.

Introduction

As indicated in the previous chapters, TS is a rare follicular skin disease observed only in immunocompromized solid organ transplant and lymphocytic leukemia patients. To our knowledge, approximately 30 cases of TS have been reported in the literature (**Chapter 2**) [1 - 3]. TS is characterized by follicular papules and keratotic protrusions (spicules), most commonly noticed on the face [4 - 6], sometimes accompanied by alopecia of the eyebrows and eyelashes [6]. Histopathology of the affected skin shows enlarged and dysmorphic hair follicles predominantly populated by trichohyalin-overproducing inner root sheath (IRS) cells. A viral cause of TS was suspected since the initial description of TS in 1999 by Haycox *et al*. [5], but attempts to identify the virus from plucked spicules of TS patients failed until the isolation of TSPyV genome by the use of rolling-circle DNA amplification technique [4].

To confirm the association between TSPyV infection and the development of TS, in this chapter we studied the presence and activity of TSPyV infection in a series of TS patients and in healthy controls, comparing presence and load of viral DNA, as well as expression and localization of viral protein.

Methods and Materials

Study populations and sample collection

Through a PubMed search on April 2010, we identified twelve articles reporting TS cases (search terms can be requested from the authors). Authors of eligible cases were contacted, asked to collaborate and to provide archival TS samples for additional analyses in our laboratory. This strategy resulted in the inclusion of six TS cases. The remaining six did not reply (n = 4), no longer possessed samples (n = 1) or declined collaboration (n = 1). The sample set was completed with an unpublished case brought forward by one of the contacted authors [7], three new cases sent to us for TSPyV analysis of which one was subsequently published [1], and the TS patient from which TSPyV was originally isolated [4].

From a total of 11 patients, formalin-fixed paraffin-embedded (FFPE) lesional biopsies or sections were obtained, numbered TS1 - TS11 according to the time of arrival at our laboratory (**Table 1**). From TS4 and TS5, we also received a non-lesional skin sample (NLS4 and NLS5). From TS1, also a forehead skin swab was obtained.

Forehead skin swabs were included from a control group of 249 individuals (age 4 - 79 years) participating in a clinical trial that monitored the effects of treatment against common skin warts (Clinical Trial Gov. registration number ISRCTN42730629) [8]. The samples were collected prior to treatment with a cotton-tip brushing uninvolved skin of the central forehead. The cotton-tip was placed in a sterile phosphate-buffered 0.9% saline solution.

Table 1: Overview of TS cases analyzed in this study

TS Case [Ref]	Sex	Age	Underlying Disease	TS onset	Description	Country
TS1 [4]	Male	15	Heart transplant	2009	TS	NLD
TS2 [9]	Male	8	ALL (T cell)	2003	TS	AUS
TS3 [10]	Male	19	ALL (pre-B cell)	2004	VATS	USA
TS4 This study	Male	5	Renal transplant	2009	TS	USA
TS5 This study	Female	63	Heart transplant	2010	TS	USA
TS6 This study	Male	62	Lung transplant	2002	TS	USA
TS7 [7]	Female	37	Heart transplant	1997	TOI	USA
TS8 [6]	Female	5	Heart transplant	2007	TS	CAN
TS9 [1]	Female	7	ALL (pre-B cell)	2010	TS	USA
TS10 [11]	Male	5	Heart transplant	2008	VATD	USA
TS11 [5]	Male	43	Renal transplant	1997	TS	USA

Abbreviations: ALL, acute lymphocytic leukemia; TS, trichodysplasia spinulosa; VATS, virus associated trichodysplasia spinulosa; TOI, trichodysplasia of immunosuppression; VATD, viral-associated trichodysplasia of immunosuppression; NLD, The Netherlands; AUS, Australia; USA, United states of America; CAN, Canada.

Ethical approval

The Institutional Review Board (IRB) of the Leiden University Medical Center (LUMC) stated that no medical ethical approval was needed to analyze the archival patient samples collected for TS diagnosis for the presence of the TS-associated pathogen.

Medical ethical approval to use skin swabs collected from the healthy controls for TSPyV analysis was obtained from the LUMC IRB. Informed consent (child as well as parental informed consent for participants less than 18 years of age) was obtained from each participant.

DNA extraction and PCR

FFPE tissue sections were received in sterile Eppendorf tubes (TS2, TS3, TS6, TS7 and TS11) or on glass slides (TS8 and TS10). TS4, TS5, NLS4 and NLS5 were received as paraffin blocks and 5-µm sections were cut using a clean blade for each tissue block. One section each was treated with Proteinase-K lysis buffer in a total volume of 100 µL. Upon processing, the supernatants were used for PCR analysis. Samples of TS1 and TS9 consisted of previously extracted DNA. The forehead skin swabs were used directly for PCR analysis.

To detect and quantify TSPyV DNA, three previously described real-time quantitative PCRs were performed with primers and probes located in VP1 and LT open reading frames, and in the non-coding control region (NCCR) [4]. In parallel, a pUC19 plasmid-series containing the TSPyV genome titrated in a background of 5 ng/µL human genomic DNA (Promega) was analyzed. To normalize for input DNA and calculate input cell numbers, a β-actin household gene PCR was conducted on the same samples [12]. For the skin swabs no normalization with β-actin household gene was performed, because of lack of sufficient human genomic DNA in the collected skin swabs. In these samples, TSPyV copies are indicated as copies per PCR reaction.

Histology, immunofluorescence and electron microscopy

Hematoxylin and eosin (H&E) staining for histology and immunofluorescence (IFA) to detect trichohyalin (1:250, AE15 (sc-80607), Santa Cruz) and TSPyV VP1 protein (1:1000), was performed on deparaffinized 4-µm sections. To detect VP1, a polyclonal rabbit antiserum was raised against a synthetic TSPyV VP1 peptide (TGNYRTDYSANDKL). As a TSPyV VP1-specificity control, serum from the same rabbit was used collected prior to VP1 peptide immunization. The sections were incubated in a dark chamber with a secondary Donkey-α-Rabbit-Cy3 antibody (1:1000, Invitrogen), a Goat-α-Mouse-Alexafluor488 antibody (1:300, Jackson) and Hoechst nuclear staining (1:100), and analyzed under a fluorescence microscope.

For electron microscopy (EM), a 50-µm FFPE section from lesional sample TS5 was treated as described [13], with slight modifications. In short, the section was deparaffinized, rehydrated and washed with cacodylate buffer. Upon O/N fixation in 2% glutaraldehyde plus 2% paraformaldehyde, it was post-fixated with 1% osmium tetroxide and transferred in an Epon capsule. One-hundred-nm sections were cut and placed on carbon/formvar-coated copper grids, poststained in 7% uranyl acetate/lead citrate, and analyzed with a Tecnai-12 electron microscope (Model Eagle, Fei Company, The Netherlands).

Results

Confirmation of trichodysplasia spinulosa in TS4, TS5 and TS6

TS-compatible clinical and histological findings were previously described for patients TS1 - TS3 and TS7 - TS11, including spicules on the face, distention of hair follicles and increase of trichohyalin-overexpressing IRS cells [1, 4 - 7, 9 - 11]. To verify TS in the unpublished cases (**Table 1**), histology was performed on TS4, TS5 and TS6. H&E staining of these samples showed enlarged hair follicles with numerous IRS cells containing large cytoplasmic eosinophilic granules (**Figure 1A**). The non-lesional samples did not display these histological features. In ultra-thin lesional sections of TS5, we confirmed the presence of intranuclear 38 - 42-nm virus particles in nuclei of IRS cells (**Figure 1B**) [10, 14]. These cells also contained electron-dense cytoplasmic protein accumulations, probably trichohyalin [5, 15, 16].

Presence of TSPyV DNA in TS patients and controls

The presence of TSPyV DNA was assessed by PCR in the lesional and non-lesional TS samples, and in the healthy control samples. TSPyV DNA was detected in all lesional samples (11 of 11, 100%), in one of two non-lesional samples, and in 6 out of 249 healthy controls (2%) (**Table 2**). This revealed a statistically significant association between the presence of TSPyV DNA and symptomatic TS ($P < 0.001$, Chi-square test).

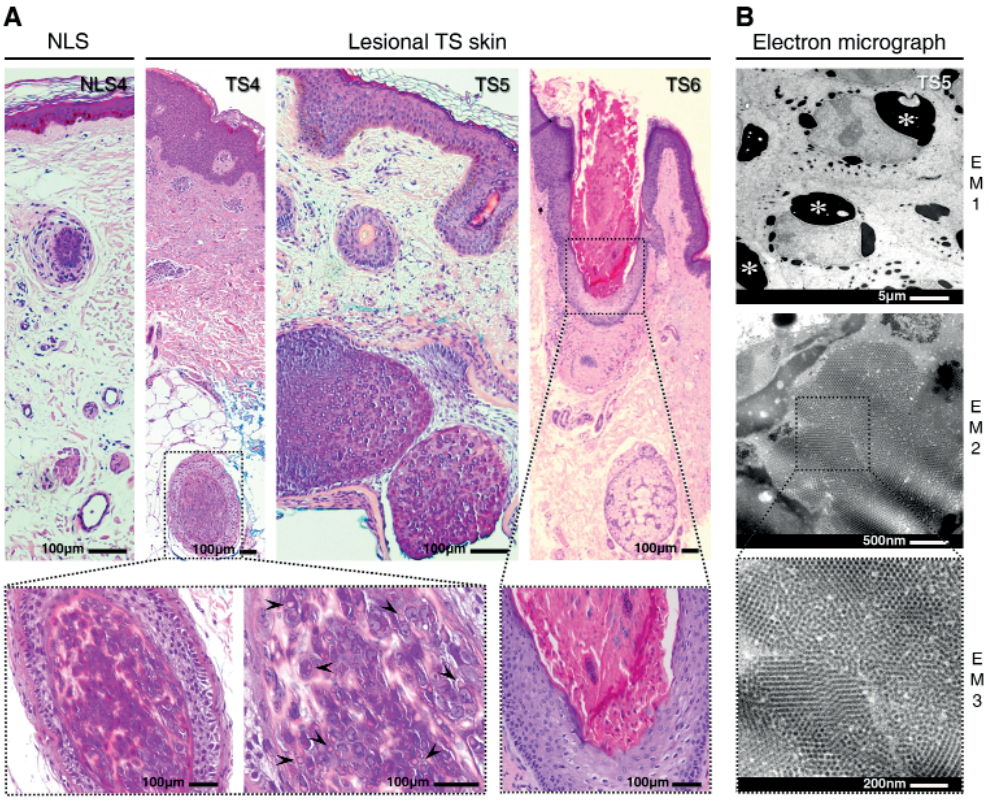

Figure 1. Histology and electron microscopy of non-lesional and lesional TS tissues. Panel **A** shows the H&E staining of non-lesional (NLS) and lesional (TS) skin from patients TS4, TS5 and TS6. The non-lesional sample NLS4 appears normal and shows absence of enlarged hair follicles. The lesional tissues contain distended and dysmorphic hair (follicles) with increased numbers of IRS cells. The insets show magnifications of IRS cells in TS4 with eosinophilic granules in their cytoplasm (arrowheads). The inset of TS6 shows abrupt cornification and protrusion of an eosinophilic spicule. Panel **B** shows electron micrographs of TS5. Asterisks indicate electron-dens protein accumulation within IRS cells (EM1). EM2 and EM3 show intranuclear crystalloid-structured arrangements of viral particles within an IRS cell.

TSPyV DNA load in TS patients and controls

As a measure of viral replication, we calculated TSPyV DNA load in TSPyV DNA-positive samples. High viral loads above 10^4 copies/cell were measured in all, except one, lesional TS samples (91%), with an average of ~10^6 (**Figure 2A, Table 2**). The non-lesional TSPyV-positive sample NLS5 contained less than 5 copies/cell, a 1,000,000-fold reduction compared to the lesional sample from the same patient (TS5, **Figure 2A**). In the six TSPyV DNA-positive healthy control skin swabs, the average amount of detectable TSPyV DNA copies was 33, being 10,000–100,000-fold lower compared to the forehead skin swab of TS1 (**Figure 2B**).

Table 2: Overview of TSPyV DNA-PCR results

Group	TSPyV DNA-positive n (%)	TSPyV DNA-load * average copy number (range)
Lesional TS samples (n=11)	11 (100)	1226845 (78.0 - 4479917.9)
Non-lesional TS samples (n=2)	1	5
Healthy control samples (n=249)	6 (2)	33 (13.6 - 67.3)

* Viral copy numbers were calculated per cell for the lesional and non-lesional TS samples, and per PCR reaction for the healthy control samples

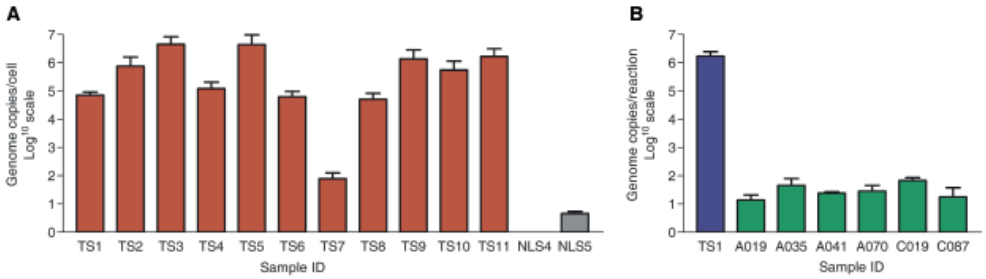

Figure 2. Quantification of TSPyV DNA in TS patients and healthy controls. Bars in graph **A** illustrate the amount of TSPyV genome copies per cell measured in the lesional (TS1 - TS11) and non-lesional (NLS4 and NLS5) samples. The bars in graph **B** show the amount of TSPyV genome copies per PCR-reaction measured in skin swabs from TS patient 1 (TS1) and from the six TSPyV DNA-positive healthy individuals (A019-C087). The graphs are shown in a Log10 scale. The error bars represent the standard deviation of the mean viral load calculated with three different quantitative PCRs (NCCR, VP1 and LT).

Expression and localization of TSPyV protein

To elaborate on the activity of TSPyV infection in TS patients, expression and localization of the TSPyV major capsid protein VP1 was assessed in lesional and non-lesional TS samples. In all of the lesional samples available for this purpose (TS3 - TS5, TS8, TS10 and TS11), TSPyV VP1 was easily detectable within the distended hair follicles with dystrophic morphology (**Figure 3**, and data not shown). In every case, VP1 was observed almost exclusively within the nuclei of IRS cells that overexpressed trichohyalin. In the non-lesional TS samples, no TSPyV VP1 was detected and trichohyalin expression was not increased.

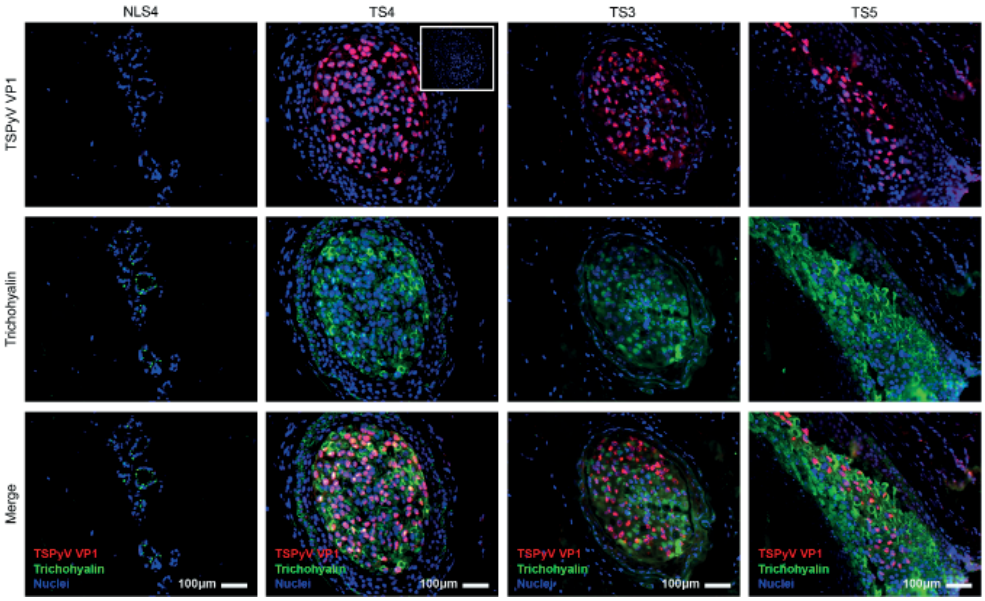

Figure 3. TSPyV VP1 protein expression in lesional and non-lesional TS samples. TS non-lesional (NLS4) and lesional (TS3 - TS5) skin sections were co-stained for TSPyV VP1 (red), trichohyalin (green) and chromatin (blue). In the non-lesional sample, no TSPyV VP1 was detected, whereas the lesional samples showed abundant intranuclear VP1 staining of the affected hair follicles (upper row). Staining with a pre-immune rabbit serum collected prior to TSPyV VP1 immunization (inset in TS4), revealed no staining of the lesional samples underscoring the specificity of TSPyV VP1 antiserum. Overexpression of trichohyalin was observed in IRS cells in the lesional samples but not in the non-lesional sample (middle row). Co-localization of both signals was frequently observed in the lesional samples (lower row).

Discussion

In previously published reports, different names have been used to describe TS, such as trichodysplasia or pilomatrix dysplasia of immunosuppression [7, 15, 17] cyclosporine-induced folliculodystrophy [18] or viral/virus-associated trichodysplasia [1, 5, 9 - 11, 14, 19]. However, despite terminology differences, all cases represented immunocompromized patients that had follicular papules and spiny lesions predominantly on the face histologically characterized by enlarged, dysmorphic hair follicles consisting of IRS cells packed with trichohyalin. By assembling the first representative series of TS samples from almost half of all previously described cases grouped under different names, we provided evidence that these patients indeed suffered from the same disease with TSPyV infection as a shared feature.

When one reasons about the involvement of a certain pathogen in the pathogenesis of a certain disease, the prevalence of the putative pathogen should be considerably higher in the patient group than in healthy controls [20]. As polyomaviruses cause lifelong

latent infections with shedding of small amounts of detectable virus, here the present amount of virus should also be taken into account. TSPyV was detected in all of the lesional TS samples, in one of two non-lesional TS samples and in a minority of controls, resulting in a highly significant statistical association between TSPyV infection and TS. High TSPyV DNA loads were measured exclusively in lesional TS samples indicating that active TSPyV infection with a high rate of replication is only seen in TS-affected skin, suggesting its involvement in the etiology of the disease. Preliminary sequence analysis revealed subtle TSPyV nucleotide substitutions in each of the lesional samples ruling out the possibility of TSPyV plasmid or sample contamination as a source of the positive findings. The observed TSPyV prevalence of 2% in the control swabs is comparable to the 4% prevalence that we measured in plucked eyebrows from a group of asymptomatic immunosuppressed renal transplant patients [4]. This probably reflects latent TSPyV infections, as indicated by serological studies [21, 22]. Unfortunately, we were unable to test the TSPyV DNA status of healthy skin biopsies. However, because comparable load measurements were obtained with the healthy skin swabs and the non-lesional biopsies, and a high TSPyV load was measured in the skin swab of TS1, we feel that skin swabs provide an appropriate sample for TSPyV analysis. Whether TSPyV load measurements in non-invasive skin swabs can be used as a TS diagnostic tool needs further investigation.

In every positive sample, the presence of TSPyV DNA was detected with all three PCRs, each located in a different region of the TSPyV genome (LT, VP1 and NCCR). With all of them comparable viral loads were measured indicating that, if detectable, the whole TSPyV genome was present. Host genomic integration of the viral DNA, as has been shown for MCPyV [23], was not investigated in this study but seems unlikely considering the high TSPyV loads that were measured.

Another indication that active TSPyV infection is involved in the development of TS was found in the presence of VP1 in all lesional TS samples tested, especially because the staining was restricted to the trichohyalin-overexpressing IRS cells that are known to be hyperproliferative in TS and are held responsible for the growth of the characteristic spines [4]. Taken together, with the TSPyV DNA prevalence and load data, despite the limited study size, these findings provide strong evidence that active TSPyV infection is associated with TS and probably involved in the pathogenesis of TS. Further *in vitro* studies are needed to confirm this hypothesis and to reveal the pathogenic mechanisms involved. Furthermore, the use of antiviral treatment, for example with topical cidofovir 1% cream that proved successful in treating some TS patients in the past [4, 10, 18], deserves serious consideration.

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