INTRODUCTION

More than 35 polyomaviruses have been discovered to date, and at least 13 of them are known to infect human cells (1,2). Among these viruses, BK polyomavirus (BKV), JC polyomavirus (JCV), Merkel cell polyomavirus (MCPyV), and trichodysplasia spinulosa (TS)-associated polyomavirus (TSPyV or human polyomavirus 8) are believed to cause diseases in humans. TSPyV was identified as the eighth human polyomavirus in 2010 from an immunocompromised patient with TS (3). TS is a rare skin disease characterized by development of follicular papules and keratin spines, especially on the face of immunocompromised patients (4). Serological studies revealed that TSPyV infection is relatively common in the general population worldwide (5–8). More than 60% of adults are positive for antibodies against TSPyV in the general Japanese population (9). TSPyV is a non-enveloped virus with a 5.2-kbp double-stranded DNA genome (3). The TSPyV genome can be divided into the early region encoding the large, middle, and small T antigens, and the late region encoding viral protein (VP)1, VP2, and VP3 (10). The large T antigen (LT) is a multifunctional protein that is necessary for viral replication (11). The activity of its helicase/ATPase domain is required for viral replication in infected cells. In oncogenic viruses such as SV40 and MCPyV, LT plays an important role in transformation (11). Almost all polyomavirus LTs have a retinoblastoma 1 (Rb1)-binding domain containing a conserved LXCXE motif (12–14). Many LTs can bind p53 via their helicase/ATPase domain, thereby suppressing p53-dependent apoptosis in virus-infected cells (15,16). Only MCPyV LT showed negligible or weak binding to p53 (14,17). Although TSPyV LT has similar functional domains to other polyomavirus LTs in its amino acid sequence, the functions of TSPyV LT have been reported rarely (10). In the present study, a full-length cDNA of TSPyV LT was cloned and its functions were investigated.

MATERIALS AND METHODS

Plasmids: To construct plasmids expressing LT, a DNA fragment encoding TSPyV LT was amplified from a full-length TSPyV genome (JPN-TMC strain) using primers TSV-LT-5034Kpn-F (5'-CACggtaccGGATAAGTTTTTAAGTAGAGA-3') and TSV-LT-2528XhoI-R (5'-CACctcgagTTATTGTGTTTGGAAACCAGA-3'), and the Platinum Taq DNA Polymerase system (Thermo Fisher Scientific, Waltham, MA, USA). TSPyV DNA was a non-enveloped virus with a 5.2-kbp double-stranded DNA genome (3). The TSPyV genome can be divided into the early region encoding the large, middle, and small T antigens, and the late region encoding viral protein (VP)1, VP2, and VP3 (10). The large T antigen (LT) is a multifunctional protein that is necessary for viral replication (11). The activity of its helicase/ATPase domain is required for viral replication in infected cells. In oncogenic viruses such as SV40 and MCPyV, LT plays an important role in transformation (11). Almost all polyomavirus LTs have a retinoblastoma 1 (Rb1)-binding domain containing a conserved LXCXE motif (12–14). Many LTs can bind p53 via their helicase/ATPase domain, thereby suppressing p53-dependent apoptosis in virus-infected cells (15,16). Only MCPyV LT showed negligible or weak binding to p53 (14,17). Although TSPyV LT has similar functional domains to other polyomavirus LTs in its amino acid sequence, the functions of TSPyV LT have been reported rarely (10). In the present study, a full-length cDNA of TSPyV LT was cloned and its functions were investigated.

SUMMARY: Trichodysplasia spinulosa-associated polyomavirus (TSPyV or human polyomavirus 8) was identified from patients with trichodysplasia spinulosa, a rare skin disease affecting the faces of immunocompromised patients. Like other polyomaviruses, the TSPyV genome encodes a large T antigen (LT). However, the expression and functions of TSPyV LT in infected cells remain largely unknown. In the present study, we cloned a full-length TSPyV LT cDNA from cells transfected with the full-length of TSPyV LT DNA. Transfection study using green fluorescence protein-tagged LT expression plasmids showed that TSPyV LT was expressed in the nucleus of transfected cells. Analysis of deletion mutants identified a nuclear localization signal in TSPyV LT. Recombinant TSPyV LT exhibited an ATPase activity. TSPyV LT has a chitinase-like domain; however, no chitinase activity was detected. Immunoprecipitation assays revealed that TSPyV LT bound to retinoblastoma 1, but not to p53 in transfected cells. Expression of TSPyV LT in NIH3T3 cells induced colony formation in soft agar, suggesting its transformation activity. These data indicate that TSPyV LT may be associated with the pathogenesis of trichodysplasia spinulosa, which is a hyperplasia of keratinocytes in inner hair follicles.
Functions of TSPyV LT

(2094 bp, Fig. 1A) (10) was gel-purified using the QIAquick Gel Extraction Kit (Qiagen) and then cloned between the KpnI and XhoI sites of pcDNA4HisMaxA (pcDNA4HisMaxA-TSPyV-LT). Mutagenesis was performed with KOD plus mutagenesis kit (Toyobo, Osaka, Japan) to match the amino acid sequence to a reference strain of TSPyV (GenBank NC_014361).

SV40-LT cDNA was also cloned into the BamHI/XhoI sites of pcDNA4HisMaxA using primers SV40-LT-5163BamHI-F (5’-CACggtaccTGGATAAAGT TTAA ACAGAG-3’) and SV40-LT-2691XhoI-R (5’-CACctegag TATGTITCCAAGGTTCAGGGG-3’) (pcDNA4HisMaxA-SV40-LT).

To generate deletion mutants of TSPyV LT, cDNA was amplified using the following primers and the resulting amplicons were subcloned between the KpnI/BamHI sites of pEGFP-C3: TSV-LT-del1 (1320 bp); TSV-LT-del2, TSV-LT-5034KpnI-F/TSV-LT-3300BamHI-R (5’-CACggatccTGGATAAAGTTTTA ACAGAG-3’) (1320 bp); TSV-LT-del2, TSV-LT-5034KpnI-F/TSV-LT-4100BamHI-R (5’-CACggtaccCCCCCAAGCAAAAGCAAAATA-3’) (520 bp); TSV-LT-del4, TSV-LT-4069KpnI-F/TSV-LT-2528BamHI-R (5’-CACggatccTTATGTTGTGTGGAA ACCAGA-3’) (1541 bp); TSV-ST, TSV-LT-5034KpnI-F/TSV-ST-4438BamHI-R (5’-CACggtaccTTACTTACCATGCCG TTTTATGTTTCAGGTTCAGGGG-3’) (239 bp).

Recombinant large T antigens: cDNA encoding TSPyV LT was amplified from pcDNA4HisMaxA-TSPyV-LT using primers TSV-LT-5034BamHI-F2 (5’-CACggtaccTGGATAAAGTTTTA ACAGAG-3’) (1541 bp); TSV-ST, TSV-LT-5034KpnI-F/TSV-LT-4795BamHI-R (5’-CACggtaccCTGGCTACTGAAGCTAGTTG-3’) (239 bp).

Western blotting: Cells were suspended in mammalian protein extraction reagent (M-PER) (Thermo Fisher Scientific). Subsequently, 5–15 µg of total protein per lane were separated on 4–12% Bolt Bis-Tris Plus SDS-PAGE Gels (Thermo Fisher Scientific) and blotted on an Immobilon-FL transfer membrane (Merck Millipore, Burlington, MA, USA). For western blotting, the membrane was incubated with primary antibodies for 1 h and secondary antibodies (horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG) for 30 min. The membrane was incubated with SuperSignal West Dura Substrate (Thermo Fisher Scientific). Chemiluminescent signals were measured using an LAS3000 instrument (Fuji-film, Tokyo, Japan).

ATPase assay: ATPase activity was examined using a High Throughput Colorimetric ATPase Assay Kit (Innova Biosciences, Cambridge, UK). Michaelis–Menten curve, Km, and Vmax were calculated with GraphPad Prism (GraphPad Software, San Diego, CA, USA). In addition, an ATPase activity assay using radioisotopes was performed as described previously (18).

Chitinase activity: Chitinase activity was measured using a Chitinase Assay kit (Sigma-Aldrich, St. Louis, MO, USA).

Immunoprecipitation: Immunoprecipitation was performed as described previously (19). Anti-p53 (clone DO-7, Dako, Copenhagen, Denmark), anti-Rb1 (ab24, Abcam, Cambridge, UK), and anti-His (Sigma-Aldrich) antibodies were used for immunoprecipitation.

Colony formation assay: The colony formation assay was performed using the CytoSelect 96-Well Cell Transformation Assay (Cell Bioslabs, San Diego, CA, USA).

Genome informatics: Multiple alignments and phylogenetic analyses were performed using Genetyx software (ver. 14, Genetyx, Tokyo, Japan). TSPyV sequences used in the present study were as follows: AB873001 (JPN-TMC) and NC_014361 (reference sequence). Protein domain searching was performed in the Conserved Domains site of NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). For prediction of nuclear localization signals (NLS), cNLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) was used (20).

RESULTS

Cloning and structure of a full-length TSPyV LT: To obtain a full-length cDNA of TSPyV LT, RT-PCR was performed on RNA extracted from 293 cells transfected with a full DNA genome of TSPyV-LT. Three bands were obtained and sequenced (Figs. 1A and 1B). Sequence analysis revealed that a band of 2094 bp size corresponded to a full-length TSPyV-LT (80 kDa) as reported previously (10), whereas other two bands corresponded to short forms of 21 and 27 kDa of TSPyV LTs. Multiple alignment of TSPyV LT and SV40-LT amino acid sequences showed putative functional domains in TSPyV LT including a DNA junction domain (DnaJ), an LXCXE motif (Rb-binding domain), an origin binding domain (OBD), a zinc finger, and a
helicase/ATPase domain (Figs. 1C and 1D). Unlike other polyomaviruses, domain searching identified a chitinase-like domain in TSPyV LT.

**Localization of TSPyV LT and identification of NLS:** Transfection study showed nuclear localization of TSPyV LT in HeLa cells transfected with a full-length TSPyV-LT genome. Sizes of three major bands are indicated. (B) Sequence analysis of TSPyV LT cDNA. Three forms were identified. Nucleotide positions in the reference sequence of TSPyV (GenBank NC_014361) are indicated in the top. (C) Structure and putative functional domains of TSPyV LT. LXCXE: Rb1-binding motif, NLS: nuclear localization signal, OBD: origin-binding domain. (D) Amino acid alignment of TSPyV LT and SV40-LT.

**Fig. 1.** (Color online) Cloning a full-length of TSPyV LT cDNA. (A) Electrophoresis of a RT-PCR product for TSPyV LT cDNA from 293 cells transfected with a full-length TSPyV-LT genome. Sizes of three major bands are indicated. (B) Sequence analysis of TSPyV LT cDNA. Three forms were identified. Nucleotide positions in the reference sequence of TSPyV (GenBank NC_014361) are indicated in the top. (C) Structure and putative functional domains of TSPyV LT. LXCXE: Rb1-binding motif, NLS: nuclear localization signal, OBD: origin-binding domain. (D) Amino acid alignment of TSPyV LT and SV40-LT.

To evaluate the function of LT, a full-length 80 kDa recombinant TSPyV LT protein was produced using a baculovirus-
Functions of TSPyV LT

An ATPase assay demonstrated that recombinant TSPyV LT induced release of free phosphate from ATP (Fig. 3C). According to Michaelis–Menten curve-fitting, $V_{\text{max}}$ of SV40 and TSPyV LTs were 2.549 (standard error (se) 0.059) and 2.075 (se 0.315) $\mu$M/min, respectively, and $K_m$ were 56.91 (se 7.00) and 585.3 (se 178.3) $\mu$M, respectively. In addition, free radiolabeled phosphate was detected by chromatography after adding TSPyV LT to radiolabeled ATP (Fig. 3D). These data suggested that TSPyV LT possess ATPase activity, similar to that of SV40-LT.

As shown in Fig. 1, TSPyV LT has a chitinase-like domain, a hydrolase which breaks the glycosidic bonds present in chitin. Phylogenetic analysis of chitinase proteins revealed that the TSPyV-encoded chitinase-like domain was similar to that of Clostridium species, but divergent from that of humans (Fig. 4A). Chitinase activity of TSPyV LT was assessed using a Chitinase Assay Kit (Sigma-Aldrich), but neither TSPyV LT nor SV40-LT exhibited chitinase activity (Fig. 4B).

Interaction of TSPyV LT with p53 and Rb1, and colony formation assay: LT encoded by oncogenic polyomavirus such as SV40 binds to Rb1 and p53, indicating an oncogenic role in infected cells.
Fig. 3. Production of recombinant LTs and ATPase assay. (A) Coomassie brilliant blue (CBB) stained gel. Recombinant LTs produced with a baculovirus-expression system were electrophoresed. Elution fractions 1–3 of TSPyV LT are shown. SV40-LT represents a control. (B) Western blotting of recombinant LTs with an anti-His antibody. Samples were the same as in (A). (C) Michaelis-Menten curve of ATPase assay for recombinant TSPyV and SV40-LTs using an ATPase analysis kit. The horizontal and vertical axes indicate substrate concentration (µM) and reaction rate (µM/min), respectively. (D) Chromatography of the ATPase assay output with radioisotope-labeled ATP. Percentages of free radiolabeled phosphate are shown in the bottom of panel.

Fig. 4. Chitinase-like domain in TSPyV LT. (A) Phylogenetic analysis of chitinase domains from various species. Amino acid sequences used here are follows; Chit1-human (GenBank CHIT1_HUMAN), Chit3-human (NP_001267), Arthrobacter subterraneus (WP_090584994), Asticcacaulis tainhuensis (WP_090645045), Bacillus oceanisediminis (AND39947), Clostridium (EDP18750), Eubacterium oxidoreducens (WP_090170714), Herbiconix gingens (WP_092551006), Lachnospiraceae bacterium C7 (WP_090076020), Leifsonia sp (WP_089912933), Merdimonas faecis (WP_094078741), Oryza sativa Japonica (EUB09005), Paenibacillus peoriae (ALA43820), Paraburkholderia phenazium (WP_090068325), Pseudomonas panipatensis (WP_090263822), and TSPyV LT (272-339aa in YP_003800007). (B) Chitinase activity analysis. 4-Nitrophenyl N-acetyl-β-D-glucosaminidase, 4-Nitrophenyl N, N'-diacetyl-β-D-chitobioside, and 4-Nitrophenyl β-D-N, N'-triacetylchitotriose were used as substrates. Glutathione S transferase (GST) was used as a negative control. The results were averages of duplicate experiments. Error bar indicates standard error.
Functions of TSPyV LT

Subsequently, we investigated the oncogenic roles of TSPyV LT. To investigate its binding to p53 and Rb1, His-tagged LT was transfected to 293 or 293T cells and immunoprecipitated with anti-p53 or Rb1 antibody. Immunoprecipitation assays revealed that TSPyV LT did not bind to or only weakly bound cellular p53. In contrast, SV40-LT did bind p53 (Figs. 5A and 5B). However, immunoprecipitation assays showed an interaction between Rb1 and LT in 293 cells and 293T cells (Figs. 5C and 5D). These results indicated that TSPyV LT and SV40-LT interacted with Rb1. Finally, a colony formation assay in soft agar was performed to investigate transformation activity of TSPyV LT. Colonies of TSPyV LT and SV40-LT-transfected NIH3T3 cells were observed significantly more frequently than those of cells transfected with empty vector (Fig. 6). Significant differences between TSPyV LT and vector-transfected cells were observed in 0.32–0.40% agarose gel (Fig. 6B), but not in lower concentrations (0.09–0.18%, data not shown). Colonies of TSPyV LT-transfected cells were detected more frequently than those of SV40-LT-transfected cells, whereas the expression level of TSPyV LT was similar to that of SV40-LT in western blotting (Fig. 6C). These data suggest the transformation activity of TSPyV LT in mammalian cells.

DISCUSSION

In this study, we showed that TSPyV LT was expressed in the nuclei of TSPyV-transfected cells, and an NLS was identified in the LT protein sequence. TSPyV LT bound to Rb1, but not to p53. Transformation activity of TSPyV LT was suggested by the results of the colony formation assay. Transformation activity of TSPyV LT was suggested by both the similarity of the structure of its LT protein to other polyomaviruses and the results of the colony formation assay. The results of colony formation assay suggest a higher transformation activity of TSPyV LT than that of SV40-LT. However, negligible or low binding to p53 by TSPyV LT was observed by immunoprecipitation assays. Among human polyomaviruses, negligible or low binding to p53 has been demonstrated only for MCPyV-encoded LT, which is the closest LT to TSPyV genetically (14,17). Binding activity to Rb1 was shown for TSPyV LT here and elsewhere (21), and this interaction may be responsible for the transformation activities of TSPyV and MCPyV LTs (17,22). On the other hand, high transformation activity of TSPyV LT in colony formation assay suggests the presence of an unknown factor, apart from
Rb1, associated with transformation activity. In Merkel cell carcinoma cases, integration of MCPyV into the host genome and continuous expression of LT has been demonstrated (23,24). MCPyV-positive Merkel cell carcinoma cells do not express VP1, indicating no replication of the virus in tumor cells. In contrast, expression of VP1 was demonstrated in the inner root sheath cells of hair follicles in TS lesions (25), indicating that serial and productive infection occur in TS, which is a hyperplasia of epithelial cells and not a cancer. Although there are no reports of LT expression in TS, transformation activity of TSPyV LT may be associated with hyperplastic changes of the inner root sheath cells of hair follicles. Short term or low expression of LT may be insufficient for full transformation of cells in TS.

Based on the structure of TSPyV LT and the results of functional analyses, we presumed that TSPyV LT has similar roles of DNA binding, viral replication, and transformation activity as for other polyomaviruses. Interestingly, TSPyV LT has a chitinase-like domain, unlike other human polyomaviruses. Chitinase-like domains are also encoded by other mammalian polyomaviruses, such as Bornean orangutan polyomavirus and Ateles paniscus polyomavirus 1. The chitinase-like domain of TSPyV LT shares 96% amino acid homology with that of the Bornean orangutan polyomavirus (GenBank YP_003264534.1). Chitinase is a hydrolytic enzyme required to digest chitin, which is a component of the cell wall structure of fungi and animals (26). The human genome also encodes some
chitinases. Human chitinases can be target molecules in allergy, and are associated with the pathogenesis of some allergic diseases (27,28). Since the chitinase-like domain of TSPyV LT was similar to bacteria-encoded chitinases and divergent from mammalian chitinases in phylogenetic analyses (Fig. 4A), this domain in TSPyV LT might have been genetically transferred from other microbes. Although TSPyV LT did not show any chitinase activity in our assay, the domain might play a role in the pathogenesis of TS and/or infection of TSPyV. Further studies are required to reveal the function and immunogenicity of the TSPyV LT chitinase-like domain.

Although several functions of TSPyV LT were revealed in this study, its pathogenic functions, especially those related to hyperkeratosis and hyperplasia of keratinocytes in TS lesions, remain largely unknown. Animal models of infection will be necessary to observe the pathogenic functions of LT in vivo. In addition, an in vitro replication system of TSPyV will be required to reveal the whole role of TSPyV LT in virus replication and the natural lifecycle of TSPyV.

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Conflict of interest None to declare.

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