Immunohistochemical detection of KI polyomavirus in lung and spleen

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Article info

Little is known about the tissue tropism of KI polyomavirus (KIPyV), and there are no studies to date describing any specific cell types it infects. The limited knowledge of KIPyV tropism has hindered study of this virus and understanding of its potential pathogenesis in humans. We describe tissues from two immunocompromised patients that stained positive for KIPyV antigen using a newly developed immunohistochemical assay targeting the KIPyV VP1 (KVP1) capsid protein. In the first patient, a pediatric bone marrow transplant recipient, KVP1 was detected in lung tissue. Double immunohistochemical staining demonstrated that approximately 50% of the KVP1-positive cells were CD68-positive cells of the macrophage/monocyte lineage. In the second case, an HIV-positive patient, KVP1 was detected in spleen and lung tissues. These results provide the first identification of a specific cell type in which KVP1 can be detected and expand our understanding of basic properties and in vivo tropism of KIPyV.

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Introduction

KI polyomavirus (KIPyV) was discovered in 2007 in patients with respiratory tract infections (Allander et al., 2007). Subsequent studies have detected KIPyV in respiratory tract secretions, blood, stool and tonsil tissue (Babakir-Mina et al., 2011) and have suggested a seroprevalence of 55–70% (Nguyen et al., 2009; Kean et al., 2009), with infection occurring most frequently in childhood. KIPyV is not currently associated with any human disease(s). However, other polyomaviruses are known to be important human pathogens. BK polyomavirus (BKPyV) causes BK nephropathy and hemorrhagic cystitis, while JC polyomavirus (JCPyV) is the etiological agent of progressive multifocal leukoencephalopathy. For both BKPyV and JCPyV, disease only manifests when the host is immunocompromised (Knowles, 2006). As the more recently discovered Merkel cell carcinoma polyomavirus and Trichodysplasia spinulosa-associated polyomavirus are also thought to cause disease in the context of immunosuppression (Kazem et al., 2012; Gjoerup and Chang, 2010), a significant question is whether KIPyV follows this paradigm and causes disease in immunocompromised patients.

Since little is known about the in vivo tropism of KIPyV, it is not yet possible to accurately identify potential diseases with which it may be associated. The virus has most commonly been detected in respiratory secretions, but there have been only minimal efforts to explore additional specimen types. In addition, prior studies have relied exclusively on PCR approaches to detect viral genomes in bulk-extracted nucleic acid, making it impossible to define the specific cell type(s) that harbor KIPyV. There have also been no published reports describing the detection of KIPyV antigens in tissues. In order to define the tissue and cell tropism of KIPyV and as a step toward understanding the role of KIPyV in human disease, we developed an immunohistochemical (IHC) assay targeting KIPyV VP1 (KVP1), the viral capsid protein. We applied this assay to tissue specimens from patients positive for KIPyV by PCR described in the published literature (Siebrasse et al., 2012; Sharp et al., 2009). Tissues from two different patients were positive. KVP1 was detected in the lung tissue of a pediatric bone marrow transplant recipient, and a portion of the positive cells was identified as being CD68-positive using a double immunohistochemical stain. In addition, KVP1 was detected in the spleen and lung tissues of a deceased HIV-positive patient. These results provide the first insights into a specific cell type in which KIPyV can be detected.

Results

Establishment of a KVP1-specific immunohistochemistry assay

To study the cell and tissue tropism of KIPyV, we established an IHC assay using a newly developed monoclonal anti-KVP1
antibody. To validate the new IHC assay and evaluate its specificity, we developed a positive control cell pellet. Fig. 1a shows 293T cells transfected with the pDEST26-KVP1 construct, which expressed KVP1 from a CMV promoter, and stained with the KVP1 antibody. Several cells showed prominent dark brown staining (Fig. 1a and b), while cells from both a sequentially cut slide stained with the isotype control (Fig. 1c) and mock transfected 293T cells stained with the KVP1 antibody (Fig. 1d) did not.

To independently evaluate the specificity of the KVP1 monoclonal antibody, a subset of the transfected cells were lysed to extract proteins for Western blot analysis (Fig. 1e). As negative controls, lysates of 293T cells from a mock transfection and from a transfection of an analogous plasmid expressing the VP1 protein of Wu polyomavirus (pDEST26-WVP1) were included. We first blotted with a primary antibody against Wu polyomavirus VP1 (WVP1), which is the most closely related virus to KIPyV and has also been detected in respiratory tract secretions. A single band was detected in the WVP1 lysate, while no band was seen in the KVP1 lysate or in the mock transfected cells (Fig. 1e). After the membrane was stripped, we blotted with the KVP1 monoclonal antibody. A single band corresponding to the predicted size of KVP1 was detected in the KVP1 lysate, while no band is seen in mock transfected cells or in the WVP1 lysate (Fig. 1e). The blot was stripped a second time and blotted for actin (Millipore #MAB1501, Billerica, MA) as a loading control. Given these data, we concluded the KVP1 antibody is specific for KI polyomavirus, and the KVP1 IHC assay can detect KI polyomavirus antigen in formalin-fixed paraffin-embedded cell pellets.

Detection of KVP1 in a pediatric transplant recipient

During the course of a recent study to evaluate the prevalence of human polyomaviruses in a prospective pediatric transplant cohort (Siebrasse et al., 2012), we identified one patient (#3001) whose nasopharyngeal aspirate sample (NPA) was strongly positive for KIPyV by real-time PCR. The patient’s clinical parameters have been described in detail (Siebrasse et al., 2012). In brief, the patient was a 17-month-old child who received a bone marrow transplant as treatment for Fanconi anemia in April 2009. The patient’s disease course was complicated by recurrent pulmonary hemorrhage, severe graft-versus-host disease (GVHD), and renal failure. The patient ultimately died of acute respiratory failure and extensive pulmonary hemorrhage several months later. The autopsy of the lung revealed evidence of chronic pulmonary hemorrhage with numerous hemosiderin-laden macrophages in the alveolar spaces. Diffuse alveolar hemorrhage leading to respiratory failure was the listed likely cause of death. Infection was considered less likely due to the negative results of routine microbiology testing (via culture and/or PCR). There was no significant inflammation or airway fibrosis to suggest GVHD in the lungs. A NPA sample collected 24 days prior to the death of the patient was strongly positive for KIPyV (1.3 × 10^9 genome copies per mL of transport media) (Siebrasse et al., 2012).

Tissue blocks obtained at autopsy from 17 different body sites were available for KVP1 IHC testing. These included skin, liver, lung, esophagus, stomach, small intestine, large intestine, pancreas, spleen, right kidney, left kidney, bladder, left ventricle, right ventricle and pituitary, right adrenal and left adrenal glands. Of these, only the lung was positive by IHC (Fig. 2). Two patterns of cellular staining were seen—strong, dark nuclear staining (arrows) and weaker, granular staining exclusively in the cytoplasm (arrow heads). Several controls were performed on serial sections to
analyze this staining pattern, including a corresponding IgG2b isotype control (Fig. 2a) and staining performed without the primary antibody or without both the primary and secondary antibodies (not shown). The weaker cytoplasmic staining was occasionally seen in controls and thus was potentially non-specific, but the strong nuclear staining was exclusively seen in the KVP1 stained tissue. Our subsequent analyses focused on these strong, nuclear staining cells. There were positive cells scattered throughout the section (similar to Fig. 2b and c) with a few localized regions containing a high density of positive staining cells.

**Detection of KVP1 in CD68-positive cells**

We used a double IHC (dIHC) staining approach to identify the cell type(s) that were KVP1-positive. Many of the positive cells were found within the alveolar spaces and were morphologically consistent with immune cells, so we began our analysis by establishing a dIHC assay using the KVP1 monoclonal antibody and an antibody against human CD45, which marks all cells of hematopoietic origin. Double IHC using the CD45/KVP1 assay showed clearly staining double positive cells throughout the tissue (Fig. 3b, c, and e). Of the 105 KVP1-positive cells counted in one tissue section, 51 (49%) were also CD45-positive. An isotype control (IgG2b for the KVP1 antibody) performed on a serial section (Fig. 3a and d) was negative. A hematoxylin and eosin stain showed dark staining clusters, morphologically consistent with phagocytic cells (Fig. 3f). Based on these data, we hypothesized that the CD45/KVP1-positive cells may be alveolar macrophages.

To test this hypothesis, we established a second dIHC assay using the KVP1 monoclonal antibody and an antibody against human CD68, which primarily marks macrophages and monocytes, and applied it to the lung tissue from patient 3001. Clear double staining was seen in ~48% of KVP1-positive cells (Fig. 4a–c), while no staining was seen in the isotype control (not shown). This demonstrated a subset of the KVP1-positive cells were likely to be alveolar macrophages. In addition, the morphology of a positive staining cell presented in Fig. 4c resembles that of a foamy macrophage, a specific morphotype of macrophage loaded with lipid droplets (Russell et al., 2009).

We next attempted to identify the remaining subset of KVP1-positive cells that were CD45-negative. Based on the observation that KVP1-positive cells lined the alveoli in another case described below, we established a dIHC assay using antibodies against KVP1 and cytokeratin, which labels epithelial cells. While cells staining single positive for either KVP1 or cytokeratin were evident, no double positive cells were seen (Supplementary Fig. 1), suggesting KIPyV was not present in epithelial cells in this lung specimen. One caveat to this interpretation is the absence of a gold standard positive control (i.e. epithelial cells known to express KVP1 in lung tissue) to validate the cytokeratin/KVP1 double stain in the context of lung tissue.

**Detection of KVP1 in an HIV-positive patient**

Sharp et al. (2009) previously screened 97 autopsy samples of lymphoid tissue by PCR and identified four samples positive for KIPyV, three from AIDS patients and one from an HIV-negative patient. A spleen sample from one patient had KVP1-positive staining cells scattered throughout the section with a few localized regions containing a high density of positive staining cells (Fig. 5a-c). This sample was derived from a 42-year-old HIV-positive male who had initially presented with peripheral neuropathy and atypical mycobacterial infection. He was re-admitted four months later for cytomegalovirus (CMV) retinitis, and his condition continued to deteriorate until death. Autopsy showed disseminated mycobacterial infection, CMV encephalitis, low grade CMV encephalitis and vacuolar myelopathy.

Most of the KVP1 positive cells were in areas of white pulp, which also showed poorly formed granulomas attributed to a mycobacterial infection, according to the autopsy report. One such example with positive cells surrounding a blood vessel consistent with splenic white pulp is shown in Fig. 5b and c. The isotype matched control antibody (J6.36) targeting the E2 glycoprotein of Hepatitis C virus (Sabo et al., 2011) yielded no staining, consistent with specific anti-KVP1 staining (Fig. 5a). Based on morphology and the detection of KVP1 in CD68-positive cells in the previously described case, we suspected hematopoietic cells may harbor KIPyV. Double staining of KVP1 with CD45 or CD68, however, yielded only single positive cells (Supplementary Fig. 2). Given these negative results, we hypothesized that the KVP1-positive cells may be endothelial cells, so we established a dIHC assay with the anti-KVP1 antibody and an antibody against CD31, which primarily marks endothelial cells. However, only single positive cells were seen (Supplementary Fig. 3). Therefore, the identity of the positive staining cells in the spleen is currently unknown.

We obtained sections of all other available tissues from this patient from the tissue bank for further analysis. These included the adrenal gland, carotid artery, colon, heart, ileum, kidney, liver, lung, pancreas, pituitary gland, prostate, testis, thyroid gland, tongue and a lymph node. Besides the spleen, only the lung specimen was positive by IHC for KVP1 with strong staining throughout the tissue as presented in Fig. 5d–f. Closer inspection of the lung showed KVP1 staining of some alveolar surfaces, raising the possibility that the infected cells might be pneumocytes. In addition, other cells (Fig. 5f, arrows) of unknown identity that did not border the alveolar spaces...
were also positive, suggesting that KIPyV infects multiple cell types present in the lung. Definitive identification of the specific cell types staining positive in this tissue was not possible due to the lack of available tissue. According to the autopsy report, the lungs showed bronchopneumonia, but no granulomas or acid fast bacilli were identified. The lung weights and pathological findings did not suggest an acute interstitial pneumonia.

Discussion

We detected KIPyV VP1 (KVP1) antigen in lung and splenic tissues for the first time using a newly developed IHC assay. In one patient, we identified a subset of the KVP1-positive lung cells as being CD68-positive. Together with morphologic data, these results suggest that alveolar macrophages harbor KVP1. The majority of prior KIPyV studies focused on respiratory secretions and relied on PCR of bulk-extracted DNA, making it impossible to determine specific cell types. Nonetheless, the simplest hypotheses regarding KIPyV tropism have focused on respiratory epithelial cells, the cell types infected by common respiratory viruses such as RSV (Hacking and Hull, 2002) and influenza (Kruiken and Taubenberger, 2008). While the IHC results alone do not prove that KIPyV can productively infect the CD68-positive cells, they do demonstrate expression of capsid protein from the late region of the genome, a step in the polyomavirus life cycle that is generally
thought to occur concomitantly with DNA replication (White et al., 2009). These results also suggest that efforts to develop in vitro culture systems for KIPyV should explore the possibility that macrophage and monocyte cell lines may be permissive for productive infection.

Alveolar macrophages are long-lived, terminally-differentiated cells that permanently reside in the lung. They are thought to be one of the first cell types to respond to pathogens (Schneberger et al., 2011). The identification of alveolar macrophages as a potential site of KIPyV infection greatly expands our understanding of KIPyV tropism and provides new insights into the biology of KIPyV. The infection of macrophages and monocytes by BK polyomavirus (Traavik et al., 1988), early hematopoietic progenitor cells by JC polyomavirus (Monaco et al., 1996) and detection of Merkel cell polyomavirus (Mertz et al., 2010) in monocytes have been reported. Alveolar macrophages display α-2,3- and α-2,6-linked sialic acid (Yu et al., 2011), both are known receptors for other polyomaviruses. BK polyomavirus and murine polyomavirus both bind to α-2,3-linked sialic acid, and JC polyomavirus binds to α-2,6-linked sialic acid (Neu et al., 2009). It is currently unknown what receptors are utilized by KIPyV, but it is possible that KIPyV might use similar receptors to BK and JC polyomaviruses.

The detection of KVP1 in CD68-positive cells could arise from one or more possible scenarios. First, alveolar macrophages may be susceptible to productive infection. Alternatively, it is also conceivable that circulating monocytes become infected in the bloodstream and remained infected after differentiation into alveolar macrophages in the lung. Third, other respiratory viruses, such as type A influenza virus, are known to abortively infect alveolar macrophages (Short et al., 2012), where infection and subsequent steps of the viral life cycle occur but no virions are produced. Finally, we cannot rule out the formal possibility that the presence of KVP1 merely reflects phagocytosis of other virus infected cells. However, given the observation that KVP1 staining is nuclear, this seems like an unlikely option. Bona fide infection versus abortive infection of macrophages by KIPyV would likely lead to different outcomes, as is seen during influenza A infection (Short et al., 2012). For example, an abortive infection would make the macrophage a dead end host and might allow the human host to mount a more effective immune response and delay viral spread. Conversely, a productive infection, whether initiated in the blood or lung, could incite a pro-inflammatory response from the infected macrophages, leading to greater immunopathology and increased viral spread.

In both of the cases presented here, the patients were immunocompromised and had lung tissues with detectable KVP1, supporting the hypothesis that the lung is an important site for KIPyV infection. However, the two cases presented with unique clinical features and disease pathologies. Patient #3001 was a pediatric patient who underwent a bone marrow transplant with multiple complications, including severe graft-versus-host disease, and died from acute respiratory failure and extensive pulmonary hemorrhage. In contrast, the other patient was an HIV-positive adult with multiple AIDS-defining illnesses at the time of death. The detection of KVP1 in the spleen of the adult HIV case and its absence in patient 3001 may reflect a distinct modality of KIPyV infection or possibly a dependence on HIV-mediated immunosuppression. Regardless, the IHC detection of KVP1 in the spleen of this patient corroborates the published PCR results (Sharp et al., 2009), and provides evidence that additional specific cell types harbor KIPyV. Collectively, these results demonstrate that KVP1 can be detected in multiple distinct cell types in more than one...
tissue type in the human body and suggest the possibility that additional parameters, such as host immune status or age, may be important factors affecting viral tropism. While the role of KIPyV in human disease remains to be determined, it is interesting to speculate about a potential role in respiratory illness given the high titers of KIPyV in patient 3001.

In conclusion, we have established an immunohistochemical assay to detect KIPyV antigen in human tissue. We also demonstrated KVP1-positive staining in two lungs and one spleen from two separate patient cases. Further analysis revealed a subset of the positive cells in one lung was likely to be alveolar macrophages. This discovery furthers our understanding of KIPyV biology. Moreover, the knowledge that KIPyV can be detected in CD68-positive cells will benefit future efforts to develop cell culture systems for propagation of this virus.

Materials and methods

Development of anti-KVP1 monoclonal antibodies

To develop anti-KVP1 monoclonal antibodies, the KVP1 Gateway pENTR/SD/D-TOPO construct previously described (Nguyen et al., 2009) was transferred into the Gateway pDEST17 plasmid (Life Technologies, Carlsbad, CA) and expressed in Escherichia coli. The resulting recombinant His-tagged KVP1 protein was purified via an affinity Ni-NTA column (Pierce Biotechnology, Rockford, IL). A BALB/c mouse was immunized with three consecutive doses of the purified antigen. Its spleen was harvested for hybridoma fusion with the murine myeloma line P3 × 63Ag8.653 (Sigma-Aldrich, St. Louis, MO). We screened for clones producing anti-KVP1 antibody by ELISA and Western blot using purified GST-tagged KVP1 as the target antigen. Positive clones were then screened by ELISA using GST-tagged WVP1 (Nguyen et al., 2009), and those demonstrating cross-reactivity to WVP1 were excluded. Two rounds of limiting dilutions were performed to achieve clonality. The anti-KVP1 monoclonal antibody used in the following experiments, NN-Ab03, was isotype IgG2b. The anti-WVP1 monoclonal antibody (NN-Ab06) used shown in Fig. 1e is developed in an analogous manner and is also isotype IgG2b.

Generation of a KVP1 positive control

We generated a positive control for the immunohistochemistry assay by transfecting 293T cells with a plasmid (pDEST26, Life Technologies, Carlsbad, CA) encoding KVP1. Cells were harvested three days after transfection, and a portion was fixed in 10% neutral buffered formalin for 24 h, embedded in paraffin, and sections of the cell block were transferred to glass slides.

Immunohistochemistry (IHC)

Tissue sections were deparaffinized in three changes of xylene and then rehydrated in a series of graded ethanol solutions. Antigen retrieval was accomplished in citrate buffer pH 6.0 (10 mM citric acid, 0.05% Tween 20) at 95 °C in a water bath for 35 min. Slides were then stained using the Histostain™–Plus 3rd Generation IHC Detection kit (Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions, with Superblock T20 (Thermo #37516, Rockford, IL) used as the blocking agent. The aforementioned KVP1 monoclonal antibody or an isotype control (mouse IgG2b, BD Biosciences #557351, San Jose, CA) was used as the primary antibody. After completion of the Histostain protocol, the slides were counterstained with hematoxylin and dehydrated in a series of graded ethanol solutions and xylene.

Slight modifications to this assay were used for staining performed on the pediatric transplant recipient’s tissue. Tissue sections were deparaffinized in xylene for 15 min and then rehydrated in a series of graded ethanol solutions. Endogenous peroxidases were quenched in 3% hydrogen peroxide for 15 min. Antigen retrieval was accomplished in citrate buffer in a pre-warmed pressure cooker (Nesco PC6–25, Two Rivers, WI) for 3 min on the high setting. After blocking in 1.5% normal horse serum (Vector Labs #S-200, Burlingame, CA), the tissues were incubated first in primary antibody and then in secondary antibody (biotinylated anti-mouse IgG, Vector BA-2000, Burlingame, CA). The staining was developed using the Vectastain standard ABC kit (Vector Labs #PK-6100, Burlingame, CA) and DAB (Vector Labs #SK-4100, Burlingame, CA), counterstained with hematoxylin and dehydrated in a series of graded ethanol solutions and xylene.

Double immunohistochemistry (diHIC)

Double staining of the tissues utilized a protocol similar to that mentioned above for the transplant recipient’s tissues with the addition of several steps. Following chromagen development of the first antibody using either DAB or ImmPACT SG (Vector Labs #SK-4705, Burlingame, CA), tissues were blocked with avidin and biotin (Vector Labs #SP-2001, Burlingame, CA) and a second time with 1.5% normal horse serum. They were then incubated with the second primary and secondary antibodies and then developed as per the above IHC protocol. Other primary monoclonal antibodies used were against CD45 (BD Biosciences #555480, San Jose, CA), CD68 (Dako #M081401, Glostrup, Denmark) and CD31 (Dako #M0823, Glostrup, Denmark). Citrate buffer was used for antigen retrieval in the double IHC assay with CD45 and CD68, while Tris-EDTA buffer pH 9.0 (10 mM Tris, 1 mM EDTA, 0.05% Tween 20) was utilized for the CD31 double IHC assay.

Image manipulation

Images were cropped to squares. The resolution was changed to 500 dpi in Photoshop with constrained proportions and no resampling. No other image manipulation was conducted.

Human studies

This study was approved by the Human Research Protection Office of Washington University in St. Louis under IRB 201108413.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.08.005.

References


