Immunohistochemistry

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Immunohistochemical methods were employed using monoclonal antibodies specific for HHV-6 viral proteins: early protein (p41), immediate-early protein (IE-2), late protein (gp116/64/54) and major capsid protein (gp82/105), which were supplied by the HHV-6 Foundation, Santa Barbara, CA. HSB2 cells (T cells) infected with the HHV-6 isolate GS were used as a positive control. Briefly, the cells were grown in 400mls of RPMI 1640 with 10% fetal bovine serum, penicillin and streptomycin (Sigma-Aldrich) and harvested when 50%-60% of the cells were positive by immunofluorescence for the late antigen gp116 core protein. The harvested cells were split evenly into two aliquots and pelleted (1000 rpm x 100g for 10 minutes). The supernatants were aspirated and the pellets were washed once in PBS (200ml per pellet). The cells were centrifuged again at 1000 rpm x 100g for 10 minutes and the PBS aspirated. The pellets were resuspended in 0.5ml of PBS each and frozen at -70°C. Lastly, the cell pellet was resuspended in liquid HistoGelTM (Perk Scientific) and embedded in paraffin.

Tissues from CD patients and controls, as well as the positive control (HSB2 infected cells) and negative control blocks (HSB2 uninfected cells) were cut at 5 µm and subjected to indirect immunostaining. The sections were placed on positively-charged glass slides and allowed to air-dry. Immunohistochemistry was performed on a Bond Max autostainer (Leica Microsystems, Bannockburn,

IL, USA) according to the manufacturer's protocol. Briefly, slides were dewaxed in Bond Dewax solution and re-hydrated in Bond Wash solution (Leica Microsystems). Antigen retrieval was performed at pH6 using Epitope Retrieval 1 solution (Leica Microsystems) for 20 min at 100°C. Slides were then incubated for 15 min at room temperature with the respective primary antibodies at the following dilutions 1:400, 1:400, 1:20,000 and 1:400, for early protein (p41), which reacts with both HHV-6 variant A and B infected cells, variant A specific IE-2, variant A and B specific late protein (gp116/64/54), and major capsid protein (gp82/105), which only reacts with variant A infected cells. Antibody detection was performed using the biotin-free Bond Polymer Alkaline Phosphatase Red and Refined systems (Leica Microsystems). Slides were then counterstained with hematoxylin. Antibodies against CD117 (C-Kit) (Dako, Carpinteria, CA) (for mast cells) and CD138 (Dako, Carpinteria, CA) (for plasma cells) were applied to the tissues of six patients and six controls to examine for non-specific immunoreactivity.

Table 1. Immunohistochemical analysis for HHV-6 viral antigens in CD and control patients

Monoclonal Antibody to	Dilution	Antigen Retrieval	Source	Immunoreactivity in CD patients	Immunoreactivity in controls
p^{41} (early antigen)	1:400	pH6	HHV-6 Foundation	-	-
IE-2 (immediate early antigen)	1:400	pH6	HHV-6 Foundation	+++	++
$gp^{116/64/54}$ (core antigen)	1:20,000	pH6	HHV-6 Foundation	+	+
$gp^{82/105}$ (capsid antigen)	1:400	pH6	HHV-6 Foundation	+++	++

+++ = 5-6 cells were immunoreactive per high power field, ++ = 2-3 cells were immunoreactive per high power field, + = 0-1 (rare) cells were immunoreactive per high power field and - = no immunoreactivity observed.