Cellular localisation of HHV-8 in Castleman’s disease: is there a link with lymph node vascularity?


Abstract

Aims—Human herpesvirus 8 (HHV-8) has been identified in multicentric Castleman’s disease and in angioimmunoblastic lymphadenopathies. However, the presence of the virus does not necessarily indicate an aetiological role in these conditions. This study investigates the cell types infected by HHV-8 in Castleman’s disease and examines the correlation between HHV-8 and Castleman’s disease lymph node angiogenesis.

Methods—Sixteen formalin fixed, paraffin wax embedded samples from patients with Castleman’s disease (six multicentric, 10 solitary) were examined for the presence of HHV-8 using the polymerase chain reaction (PCR), non-isotopic in situ hybridisation, PCR in situ hybridisation (PCR-ISH), and real time quantitative TaqMan PCR to HHV-8 open reading frame 26 (ORF-26), and viral (v)-cyclin encoding regions. Vascularity was assessed using CD34, CD31, and factor VIII immunocytochemistry, and lymph nodes were scored as “low” or “high”.

Results—Five multicentric Castleman’s disease and two solitary Castleman’s disease biopsies were positive for HHV-8. HHV-8 was identified in approximately 10% of intranodal B lymphocytes, in endothelial cells, and in subcapsular spindle cell proliferations. The copy number of HHV-8 was low at 10–50 copies/1000 cells. The highest copy number was in subcapsular spindle cells. There was no correlation between vascularity score and HHV-8 status.

Conclusion—The preferential localisation of HHV-8 in subcapsular spindle cell proliferations (where early intranodal Kaposis’s sarcoma initiates) and endothelial cells in Castleman’s disease might finally explain the link between intranodal Kaposis’s sarcoma and Castleman’s disease.

Keywords: Castleman’s disease; human herpesvirus 8; Kaposis’s sarcoma

Recently, human herpesvirus 8 (HHV-8) sequences have been identified in cases of multicentric Castleman’s disease and in angioimmunoblastic lymphadenopathies, implicating a role for HHV-8 in the pathobiology of these conditions. Castleman’s disease is a morphologically distinct form of lymph node hyperplasia. Microscopically, there are two major categories described. The first is designated hyaline vascular type (angiofollicular), the second is the plasma cell variant. Clinically, Castleman’s disease is divided into a solitary and a multicentric type. The solitary form presents as a mass, commonly located in the mediastinum, but also found in the neck, lung, mesentery, axillary lymph nodes, peritoneum, soft tissues, and nasopharynx. Microscopically, over 90% of these cases are of the hyaline vascular type and the remainder are of the plasma cell type.

The multicentric or systemic form of Castleman’s disease is nearly always of the plasma cell type. This presents as a generalised lymphadenopathy, which might involve the spleen. This is an atypical lymphoproliferative disorder, showing a polygonal lymphoid proliferation with vascular hyperplasia. The prognosis of systemic Castleman’s disease is poor, with the disease tending to persist for months or years. Patients usually succumb to renal and pulmonary complications. Furthermore, some patients have coexistent Kapossi’s sarcoma (18–20% of patients). Others develop large cell lymphomas of the immunoblastic type. Evidence of clonal rearrangement of immunoglobulin and T cell receptor genes has been found in some patients with systemic Castleman’s disease, together with Epstein-Barr virus (EBV) genomes. However, no such features have been described in the solitary form of the disease. It is clear from these data that multicentric Castleman’s disease differs from the classic localised type, and might eventually evolve into clonal lymphoproliferation.

In some cases of Castleman’s disease of the hyaline vascular type, there might be an undue prominence of stromal elements. These types of lesions can occasionally acquire the features...
of an angiomatous hamartoma or a cellular spindle cell proliferation resembling Kaposi's sarcoma or haemangiopericytoma. Conceivably, some of these vascular proliferative lesions might result from the secretion of angiogenic factors by lymphoid elements. Interestingly, cutaneous and nodal forms of Kaposi's sarcoma have been reported to be associated with multicentric Castleman's disease. In light of previous findings of HHV-8 in multicentric Castleman's disease and angio-immunoblastic lymphadenopathy, we investigated the prevalence of HHV-8 in multicentric and solitary Castleman's disease, calculated viral copy numbers in such lesions, and identified the cell types infected by HHV-8. In addition, we examined any possible links between the degree of angiogenesis in Castleman's disease lymph nodes and the presence of HHV-8.

**Materials and methods**

Sixteen biopsies from lymph nodes of patients affected with Castleman's disease were examined for the presence of HHV-8. These biopsies were from three human immunodeficiency virus (HIV) positive and 13 HIV negative patients (table 1). Ten control reactive lymph nodes were also examined.

**HISTOLOGICAL AND IMMUNOCYTOCHEMISTRY ANALYSIS**

To investigate any possible link between the presence of HHV-8 and the type of Castleman's disease or the amount of angiogenesis, the blood vessels in all Castleman's disease biopsies were stained for factor VIII, CD34, and CD31, and vascularity was scored as "low" or "high". A biopsy score was attributed with non-overlapping primers derived from the major capsid protein of HHV-8 (ORF-25): outer primers, 5'-AGGCGAACGTCTATTGCCAATGG-3'.

For HHV-8 detection, KS4 primer (5'-AGACTGCGAGGCCAGTACG-3') and KS5 primer (5'-GACTCTTGCGCAGTGAACTCTGG-3'), derived from the putative minor capsid protein (homologue of open reading frame 26 (ORF-26) of herpesvirus saimiri) were used and 100 ng of starting DNA template was amplified for 25 cycles. To increase the sensitivity and verify the specificity of the amplicon obtained with these primers, 2 µl of amplified product was reamplified (for 25 minutes). Pretreatment was achieved using either a pressure cooking technique or protease treatment for 20 minutes.

After antigen unmasking, an anti-CD31 antibody (JC 70) diluted 1/30 in Tris buffered saline (TBS) was applied to the tissue section and allowed to interact for 30 minutes. Two washes in TBS for five minutes each were then performed. The secondary antibody (biotinylated rabbit antimouse) (Dako, Copenhagen, Denmark) diluted 1/400 in TBS (pH 7.6) was then incubated with the tissue section at room temperature. Immunocytochemical detection was achieved as described above.

**CD34**

No pretreatment was required for CD34 staining. The primary antibody dilution consisted of anti-CD34 (Q bend) diluted 1/100 in TBS (pH 7.6). Immunocytochemical detection was achieved as above.

**Factor VIII**

Pretreatment with either pressure cooking or trypsin digestion for 20 minutes was required for factor VIII staining. Neat antifactor VIII antibody (JC 70) was used as the primary antibody. Immunocytochemical detection was achieved as above.

**DETECTION OF HHV-8 BY SOLUTION PHASE POLYMERASE CHAIN REACTION (PCR)**

DNA was extracted from three or four 5 µm sections of paraffin wax embedded biopsies by treatment with xylene, digestion with proteinase K, extraction with phenol/chloroform, and alcohol precipitation. The quality of the extracted DNA was controlled by PCR amplification of β actin gene fragment using the following primers: forward primer, 5'-TCACCCACACTGTGCCCATCTACGA-3' and reverse primer, 5'-CAGCGGAACCGCTCATGTCCAATGG-3'.

For HHV-8 detection, KS4 primer (5'-AGACTGCGAGGCCAGTACG-3') and KS5 primer (5'-GACTCTTGCGCAGTGAACTCTGG-3'), derived from the putative minor capsid protein (homologue of open reading frame 26 (ORF-26) of herpesvirus saimiri) were used and 100 ng of starting DNA template was amplified for 25 cycles. To increase the sensitivity and verify the specificity of the amplicon obtained with these primers, 2 µl of amplified product was reamplified (for 25 cycles) using internal primers KS1 (5'-AGCGGAAAGGATTCCACCATG-3') and KS2 (5'TCCGTGTGTGTACTACGTC-3').

All positive cases were confirmed by amplification with non-overlapping primers derived from the major capsid protein of HHV-8 (ORF-25): outer primers, 5'-AGGCGAACGTCTATTGCCAATGG-3'.
CAGATGTGAC-3' and 5'-GAAATTACCC ACAGGATGCG-3'; inner primers, 5'-CATG GGAGTACATTGCTACGCACC-3' and 5'-GAAATTATCTCGCAGGGTGCCC-3'.

MEASUREMENT OF HHV-8 VIRAL LOAD IN CASTLEMAN'S DISEASE

HHV-8 viral load in Castleman's disease biopsies was assessed by real time TaqMan PCR using the 7700 sequence detector (PE Biosystems, Warrington, Cheshire, UK) to amplify segments within the ORF-26 and the viral (v)-cyclin encoding region of the HHV-8 genome. This newly described technique uses the 5'→3' endonucleolytic activity of AmpliTaq DNA polymerase, and AmpErase UNG (exo-III, which destroys any unincorporated dNTPs) to anneal, extend, and destroy the DNA template. The TaqMan probe (5'→3') detects the expected product, when the probe is intact and only if the target region is present. The FAM reporter dye, which is attached to the 5' end of the probe, and the TAMRA (tetramethyl-rhodamine) which is attached by a linker arm modified nucleotide to the 3' end of the probe. The probe is chemically phosphorylated at its 3' end, which prevents probe extension during PCRs. The 5' end of the primer anneals to the target sequence, which is subsequently overlaid by an APES coated slide specifically designed for PCR-ISH. The tissue was first deparaffinized in xylene and cleaned with alcohol, followed by digestion in proteinase K (0.2–0.5 mg/ml) for 10–15 minutes at 37°C. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide solution (0.1% sodium azide) for 10 minutes. Slides were then washed in high performance liquid chromatography (HPLC) water and air dried.

PCR reagents used were as follows: 1 μM each primer, 200 μM dNTPs, 4.5 mM MgCl₂, 1× PCR buffer II (PE Biosystems) and 10 U AmpliTaq Gold in a reaction volume of 25–50 μl. HHV-8 inner primers as above were used. PCR reactions were performed in the GeneAmp PCR system 1000 (PE Biosystems). Slides were assembled on a dedicated assembly unit held at 70°C. A "hot start" modification was performed by heating the reaction solution to 70°C before the addition of AmpliTaq Gold. The PCR reaction mix was placed over the tissue section, which was subsequently overlaid by an Ampli Cover disc (silicon rubber) and clip.

PCR in situ hybridisation (PCR-ISH) IDENTIFICATION OF HHV-8 IN CASTLEMAN'S DISEASE

PCR-ISH was used to identify the precise cell types infected in Castleman's disease. All PCR-ISH assays were performed in triplicate. An EBV positive post-transplant lymphoproliferative disorder specimen was also included to exclude crossreactivity with EBV. Representative 5 μm sections from solution phase and TaqMan positive cases were cut on to APES coated slides specifically designed for PCR-ISH (PE Biosystems). Tonsil tissue was used as a negative control. The tissue was deparaffinized in xylene and cleaned with alcohol, followed by digestion in proteinase K (0.2–0.5 mg/ml) for 10–15 minutes at 37°C. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide solution (0.1% sodium azide). Slides were then washed in high performance liquid chromatography (HPLC) water and air dried.
Two negative controls were performed on each slide—one without PCR reagents and one with omission of AmpliTaq IS and/or primers. The following cycling conditions were used: 94°C for one minute, 94°C for 50 seconds, and 55°C for 55 seconds, for 40 cycles, followed by soaking to 4°C.

After amplification, the Ampli Cover discs and clips were removed and slides fixed in 100% ethanol for five minutes. Hybridisation was carried out with a 5'-end biotin labelled oligonucleotide probe (5'-TGTTGGTGTA CCACATCTACTCCAAAATAT-3'). The probe was applied at a concentration of 5 pmol/100 µl hybridisation buffer, composed of 5% dextran sulphate, 2× saline sodium citrate (SSC), and 10% formamide. Slides were denatured at 94°C for 15 minutes and then hybridised for four to 12 hours at 42°C. Coverslips were removed and sections washed using different stringencies as follows: 4× SSC at 22°C for 15 minutes, 2× SSC at 30°C for 15 minutes, 2× SSC at 42°C for 15 minutes, 2× SSC at 55°C for 15 minutes, 1× SSC at 55°C for 15 minutes, or 0.1× SSC at 55°C for 15 minutes, followed by a final wash in TBT (100 mM NaCl, 40 mM Tris, pH 7.2, 3% bovine serum albumin (BSA), and 0.05% Triton X-100) for 10 minutes. The intensity of the signal was dependent on the stringency of the post-hybridisation wash used.

A three step detection technique was used. Monoclonal antibiotin (Dako; diluted 1/40) was followed by biotinylated rabbit antimouse F(ab')2 (Dako; diluted 1/200), and finally avidin/peroxidase (Dako; diluted 1/50 in 5% dried skimmed milk solution). The product was detected by AEC chromagen (Histostain-SP kit; Zymed Lab Inc, San Francisco, USA) with careful monitoring of signal development (five to 15 minutes). Simultaneous immunohistochemistry was not performed because vascular spaces were easily identified morphologically.

Control amplifications and hybridisations were carried out using a pair of β globin primers (5'-ACACAACTGTGTTCACTAGC-3' and 5'-CAACTTCATCCACGTTCACC-3') and a biotinylated β globin oligonucleotide probe (5'-GACTCCTGTAGAGAAGTCT GCCGTACTGC-3'), as well as a biotinylated human papilloma virus 16 (HPV-16) 30 mer oligonucleotide probe (5'-TATTAAC TGTCAAAAGGCCACTGTGCTCTGA-3').

Labelled primer driven in situ amplification was performed on selected cases. The KS-1 primer was a 5'-end labelled 30 mer oligonucleotide (sequence as for the PCR-ISH oligonucleotide probe) and we used the same reaction con-

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Table 2  Angiogenesis and HHV-8 in Castleman’s disease (CD)

<table>
<thead>
<tr>
<th>CD subtype</th>
<th>Vessel count</th>
<th>HHV-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyaline vascular</td>
<td>Low</td>
<td>1/3</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>4/7</td>
</tr>
<tr>
<td>Plasma cell</td>
<td>Low</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>2/4</td>
</tr>
</tbody>
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HHV-8, human herpesvirus 8.

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Figure 1  (A) Haematoxylin and eosin stained section of a plasma cell variant Castleman’s disease node in a human immunodeficiency virus (HIV) positive patient with a high vascular score and early intranodal Kaposi’s sarcoma. (B) Human herpesvirus 8 (HHV-8) amplicons in lymphoid cells in the same patient identified using polymerase chain reaction in situ hybridisation (PCR-ISH). In addition, HHV-8 was identified in the early Kaposi’s sarcoma lesion. (C) Hyaline vascular type Castleman’s disease in an HIV negative patient with a high vessel count also negative for HHV-8 on solution phase PCR and negative here on PCR-ISH. (D) Haematoxylin and eosin section illustrating the high vessel count in this sample.

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ditions as for PCR-ISH. After amplification the slides were held at 55°C. Stringency washes and detection steps were as for PCR-ISH.

Positive control material for in cell PCR reactions included BC-1 and BC-3 cells (lymphoblastoid cell lines containing HHV-8) and Kaposi’s sarcoma biopsies. Negative tissues and cell line controls included myocardium and SiHa cells.

Results
Seven of 16 Castleman’s disease (five multicentre and two solitary) cases yielded HHV-8 amplicons. Tables 1 and 2 give results for HHV-8 and Castleman’s disease and relate the vascularity count to HHV-8 positivity. HHV-8 was found by PCR and TaqMan PCR in Castleman’s disease of all types (but predominantly multicentric Castleman’s disease) and in HIV positive and negative individuals. Three HIV associated Castleman’s disease cases were positive for HHV-8, with two of these patients having coexistent multicentric Castleman’s disease and cutaneous Kaposi’s sarcoma. In one of these biopsies, there was evidence of microscopic Kaposi’s sarcoma within the lymph node affected by Castleman’s disease. Four of 13 HIV negative patients with Castleman’s disease were positive for HHV-8, and one of these patients had underlying Kaposi’s sarcoma. In total, two of six plasma cell variant Castleman’s disease...
nodes, and five of 10 hyaline vascular Castle-
man’s disease nodes were positive for HHV-8.
When scored for angiogenesis, six of 11 high,
and one of five low nodes were positive for
HHV-8 (table 2). None of the reactive lymph
nodes contained HHV-8 by either solution
phase PCR or TaqMan PCR.

Using PCR-ISH under high and low strin-
gency post-hybridisation washing conditions,
HHV-8 amplicons were detected predomi-
nantly in lymphoid cells and occasionally in
endothelial cells (in two cases) within germinal
centres (figs 1–4). PCR-ISH analysis of a solu-
tion phase PCR HHV-8 negative lymph node
did not reveal any amplicons (fig 1).

HHV-8 amplicons were detected in lym-
phoid cells in B cell rich areas of lymph nodes
(predominantly germinal centres) and in a sin-
gle lymphoid cell in an afferent subcapsular
lymphatic (fig 3). In any one node, the number
of positive lymphoid cells was less than
10–15% of the nodal lymphoid population.
The HHV-8 copy number in each 1000 cells in
the lymph node as assessed by TaqMan PCR
was 10–50. Corrected for by PCR-ISH results,
this represents one to three copies of HHV-8
DNA for each infected endothelial and lym-
phoid cell. The efficiency of TaqMan PCR can
be assessed by the slope of the standard curve
(generated by positive plasmid controls con-
taining the gene of interest; fig 5). The slope
here is −4.016, with a correlation coefficient of
0.997. This represents 100% efficient PCR
(given the slope of the curve is 1/log(1 + E),
where E is the efficiency of the PCR).

In some cases (three), HHV-8 amplicons
were found preferentially in focal areas of spin-
dle cell proliferations beneath the capsule of
the node (fig 4). To confirm the precise cell
infected by HHV-8 in these areas, we microdis-
sected out lymphoid cells. Figure 4E and F
shows the localisation of HHV-8 amplicons in
spindle like cells in these areas. This occurred
in all of the three cases, which incidentally were
scored as high vascularity. This result was
reproducible on repeated investigation. One
case in particular showed very weak in situ
signals when the probe alone was applied, but

Figure 4 (A) Haematoxylin and eosin stained section showing a hyaline vascular type Castleman’s disease specimen with
a high vessel count, as demonstrated by CD34 staining (B). (C) β-globin gene polymerase chain reaction in situ
hybridisation (PCR-ISH) on the same specimen and (D) a parallel negative control. (E) And (F) human herpes virus 8
(HHV-8) amplicons (indicated by arrows) in spindle cells in a subcapsular location in a Castleman’s disease lymph node,
after microdissection of lymphoid cells. Note the suboptimal morphology after proteolytic digestion.
HHV-8 v-cyclin gene. The slope of the curve is 

\[
-4.016, \text{ with a correlation coefficient of 0.997, illustrating high reproducibility of the assay.}
\]

Figure 5 Standard curve for viral (v)-cyclin TaqMan PCR using plasmid standards containing the human herpes virus 8 (HHV-8) v-cyclin gene. The slope of the curve is 

\[
-4.016, \text{ with a correlation coefficient of 0.997, illustrating high reproducibility of the assay.}
\]

Discussion

In 1995, Soulier and colleagues found HHV-8 DNA sequences in 14 of 14 cases of HIV associated multicentric Castleman's disease (which included five cases without detectable Kaposi's sarcoma). In addition, HHV-8 was detected in seven of 17 cases of multicentric Castleman's disease in HIV negative patients, including one patient with cutaneous Kaposi's sarcoma. HHV-8 DNA sequences were also found in lymph nodes and peripheral blood samples from these patients. In Soulier's control cohort of 34 non-multicentric Castleman's disease reactive lymph nodes (follicular and/or interfollicular hyperplasia) in HIV negative patients, HHV-8 was detected in only one case. These results suggest that HHV-8 might also play a role in the pathogenesis of multicentric Castleman's disease, especially in HIV infected patients. Alternatively, the findings might represent viral passenger positivity. These results have subsequently been confirmed by other investigators. In addition, HHV-8 DNA sequences have been identified in a related condition, angioimmunoblastic lymphadenopathy.

In our study, HHV-8 was identified in seven of 16 cases of Castleman's disease. Interestingly, five of six multicentric cases were positive for HHV-8. The presence of HHV-8 in the Castleman's disease samples tested is not linked to a histological subtype of Castleman's disease and neither is it always seen in highly vascular cases (\(\chi^2 = 0.55873; p < 0.50\)), although it is seen more often in multicentric Castleman's disease. Although one of the HIV negative, HHV-8 positive patients was considered not to have an increased risk of developing Kaposi's sarcoma (that is, did not have multicentric Castleman's disease), there is in fact an increased incidence of Kaposi's sarcoma in individuals with any type of Castleman's disease. The presence of HHV-8 in only a proportion of patients with Castleman's disease (43%), as described here, might suggest a reactivation of latent HHV-8 infection in such patients. The cytokine rich microenvironment of these lesions, as well as of other angiogenic lymphoproliferations, could be particularly favourable to HHV-8 replication, or to the host cells which harbour this virus. However, it is also possible that HHV-8 can help to induce angiogenesis in Castleman's disease—for example, through the production of HHV-8 viral interleukin 6 (vIL-6). Preliminary data from our own group (using peptide nucleic acid (PNA-ISH) vIL-6 probes) suggests that this might be an important mechanistic action of HHV-8 in patients with Castleman's disease. Indeed, using TaqMan PCR, HHV-8 vIL-6 transcripts are relatively abundant in Castleman's disease (J O'Leary et al, 1999, unpublished data).

PCR-ISH conveniently localises the cells infected by HHV-8 in Castleman's disease. Of importance, is the finding of HHV-8 in lymphoid cells, which is as predicted from peripheral blood assays. However, the localisation of HHV-8 amplicons to endothelial cells (fig 2) and to subcapsular spindle cell areas (fig 4), where a high HHV-8 copy number is seen, raises important pathobiological questions.

In lymph nodes, early Kaposi's sarcoma is usually seen in the subcapsular and trabecular sinuses, with advanced Kaposi's sarcoma effacing the lymph node architecture. If this is the case, then the localisation of HHV-8 amplicons to this site might not occur by chance alone, and might lead to the following sequence of events. Circulating infected HHV-8 B cells enter the lymph node through theafferent lymphatics (fig 3). Here, viral mesenchymal cells and/or endothelial cells are infected and assume a spindle cell morphology (akin to Kaposi's sarcoma development). With permissive infection, spindle cell proliferation proceeds and small foci of spindle cells appear in the subcapsular space of the node. Two events can then follow: HHV-8 is disseminated throughout the node, thereby contributing to increased vascularity (via v-IL-6 production) and Castleman's disease, or the virus may remain localised and support focal subcapsular spindle cell proliferation, leading to the development of Kaposi's sarcoma.

The questions that now remain are: (1) are Castleman's disease and Kaposi's sarcoma inextricably linked by a viral factor, that factor being HHV-8; and (2) are some forms of Castleman's disease early intranodal Kaposi's sarcoma, as has been suggested by some clinical studies?


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