

## Papers

## CD40 upregulation is independent of HHV-8 in the pathogenesis of Kaposi's sarcoma

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Accepted for publication  
3 November 1998

**Abstract**

**Aims**—Human herpesvirus 8 (HHV-8) is now acknowledged as the infective cofactor in the pathogenesis of Kaposi's sarcoma. The mode by which HHV-8 causes Kaposi's sarcoma is unresolved and it is probable that it acts in conjunction with other factors including cytokines, anti-apoptosis proteins, and cell surface receptors. CD40, a cell membrane receptor belonging to the tumour necrosis factor receptor super family, promotes B cell survival and is expressed constitutively on endothelial cells. It is upregulated on cytokine treatment and has been documented recently in Kaposi's sarcoma. Because the HHV-8 genome contains cytokine homologues, this study investigated whether CD40 expression in Kaposi's sarcoma correlated with HHV-8 status, using a unique set of HHV-8 positive and negative specimens.

**Methods**—Twenty one paraffin wax embedded samples of Kaposi's sarcoma were selected, of which 18 were screened for the presence of HHV-8 using both conventional solution phase and TaqMan polymerase chain reaction (PCR). CD40 immunohistochemistry was assessed using a biotinylated amplification system. Staining was scored semiquantitatively.

**Results**—The results indicated that the expression of CD40 is independent of viral status, being present in both HHV-8 positive and negative specimens.

**Conclusions**—This suggests that HHV-8 promotes Kaposi's sarcoma cell survival following infection by mechanisms other than those involving CD40.

(J Clin Pathol: Mol Pathol 1999;52:32-36)

Keywords: human herpesvirus 8; Kaposi's sarcoma; CD40; immunohistochemistry; TaqMan polymerase chain reaction

Human herpesvirus 8 (HHV-8), a recently discovered DNA virus,<sup>1</sup> is now thought to be the infectious agent implicated in the pathogenesis of Kaposi's sarcoma. This is supported by its strong association with all subtypes of Kaposi's sarcoma<sup>2-5</sup> and convincing seroepidemiological

data.<sup>6-8</sup> HHV-8 has been categorised as a  $\gamma$ 2 herpesvirus, a group characterised by its lymphotropism, and is structurally homologous with herpesvirus saimiri (HVS) and Epstein Barr virus (EBV).<sup>9</sup> It has also been identified in multicentric Castleman's disease<sup>10</sup> and in a rare primary effusion lymphoma.<sup>11</sup> The mode by which HHV-8 causes Kaposi's sarcoma is unresolved and it is probable that it acts in conjunction with other factors including cytokines, cell surface receptors, and anti-apoptosis genes.<sup>12-15</sup>

CD40 is a cell surface receptor that belongs to the tumour necrosis factor/nerve growth factor (TNF/NGF) receptor superfamily.<sup>16</sup> It is expressed on a variety of cell types and promotes B cell survival and proliferation.<sup>17</sup> In addition, CD40 is expressed constitutively on endothelial cells.<sup>18</sup> It has also been shown that CD40 gene upregulation occurs on cytokine induction.<sup>18, 19</sup> In line with this, CD40 expression has been documented in both Kaposi's sarcoma samples and cell lines.<sup>14</sup>

Cytokines are implicated in the pathogenesis of Kaposi's sarcoma,<sup>12, 13</sup> and extensive analysis of the HHV-8 viral genome has revealed the presence of homologues of inflammatory mediators,<sup>20-23</sup> such as interleukin 6 (IL-6), which suggests that the virus might play a direct role in the inflammatory cascade. Because cytokines are known to upregulate the expression of the CD40 gene (and to be involved in the pathobiology of Kaposi's sarcoma), we decided to investigate whether there is an association between HHV-8 status and CD40 expression, using a unique set of both HHV-8 positive and HHV-8 negative Kaposi's sarcoma specimens.

**Materials and methods**

HHV-8 POLYMERASE CHAIN REACTION (PCR)  
Representative paraffin wax embedded material from 18 patients (table 1) was dewaxed. Following a three day incubation in 0.1 mg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) digestion buffer (containing: 100 mM NaCl, 10 mM Tris, 25 mM ethylene diaminetetra acetic acid (EDTA), 0.5% sodium dodecyl sulphate (SDS), pH 8.4) the DNA was purified using a phenol/chloroform extraction method. The samples were placed in 3 M sodium acetate/ethanol overnight at

Table 1 Patient details, CD40 analysis, and HHV-8 status

Patient	HHV-8	HIV status	Sex	Site	CD40
1	Positive	Positive	M	Skin patch	++ sp, ++ endo
2	Positive	Positive	M	Skin nodular	+++ sp, +++ endo
3	Positive	Positive	F	Skin patch	+++ sp, +++ endo
4	Negative	Positive	F	Skin nodular	++++ sp, ++++ endo
5	Negative	Negative	M	Skin, stage?	++ sp, ++ endo
6	Positive	Negative	M	Skin patch	++ sp, ++ endo
7	Positive	Positive	M	Skin patch	+++ sp, +++ endo
8	Positive	Positive	M	Skin patch	+++ sp, - endo
9	Negative	Negative	M	Skin nodular	++ sp, + endo
10	Positive	Positive	M	Lymph node	+ sp, + endo
11	Positive	Positive	M	Lung	- sp, - endo
12	ND	Positive	M	Stomach	- sp, - endo
13	ND	Positive	M	Mouth	+ sp, - endo
14	Positive	Positive	M	Stomach	+ sp, - endo
15	Positive	?	?	?	++ sp, ++ endo
16	Negative	Positive	F	Skin nodular	+++ sp, +++ endo
17	Positive	Positive	M	Skin, stage?	++++ sp, ++++ endo
18	ND	Positive	M	Lung	+++ sp, +++ endo
19	Positive	Positive	M	Tongue	++++ sp, ++++ endo
20	Positive	Negative	M	Skin patch	- sp, - endo
21	Positive	Positive	M	Skin nodular	+++ sp, +++ endo

Endo, endothelial cell; sp, spindle cell; ND, not determined.

-20°C, with subsequent centrifugation and resuspension of DNA pellets in high performance liquid chromatography (HPLC) water. The amplifiability of the extracted DNA was assessed by  $\beta$ -globin amplification (yielding a 268 base pair (bp) product).

The presence of HHV-8 was detected using nested primers to the open reading frame (ORF)26 (VP23 capsid protein), which dictates the synthesis of a 233 bp fragment. Outer primer pair, KS4: 5'-AGCACTCGCAGGG-CAGTACG-3' and KS5: 5'-GACTCTTCG-CTGATGAAGTGG-3'. Inner primer pair, KS1: 5'-AGCCGAAAGGATTCCACCAT-3' and KS2: 5'-TCCGTGTTGTCTACGTCC-AG-3'.

All reactions were performed in a 480 DNA thermal cycler (Perkin Elmer, New Jersey, USA), with the following cycling parameters. Outer set: 94°C for 45 seconds, 60°C for 30 seconds, and 72°C for 45 seconds for 25 cycles; inner set: 94°C for 45 seconds, 55°C for 30 seconds, and 72°C for 45 seconds for 35 cycles, with a soak file of 4°C for both sets of reactions.

Reaction products were visualised on a 2% ethidium bromide stained agarose gel (Sigma, Poole, Dorset, UK) (fig 1). Positive and negative strand sequencing of PCR products was performed using the ABI PRISM 377 DNA sequencer.

#### TaqMan PCR

Samples were amplified further using the technique of TaqMan PCR, which we have

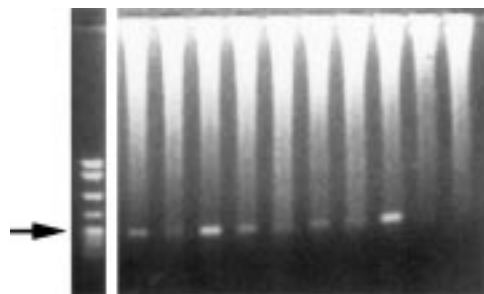


Figure 1 Sample 2% agarose gel exhibiting the characteristic 233 bp amplicons (ORF26).

described previously.<sup>24 25</sup> TaqMan PCR refers to the addition of a fluorescent labelled oligonucleotide probe to the amplification reaction, which also contains conventional primer pairs. The fluorescent reporter label is quenched by a quencher dye when the probe is intact. When amplification occurs, the reporter is cleaved (because of the exonuclease activity of Taq DNA polymerase), resulting in increased sample fluorescence, which can be detected using a luminescence spectrometer. Cleavage only occurs during specific amplification of the target template, thus obviating the need to perform southern blot analysis.

Extracted DNA was amplified for HHV-8 (ORF26) using the following protocol: 1× PCR buffer II (Perkin Elmer), AmpErase UNG (Perkin Elmer) 0.01 U/ $\mu$ l, 4 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTPs, 300 nM forward and reverse primers (KS1: 5'-AGCCGAAAGGATTCC ACC- AT-3' and KS2: 5'-TCCGTGTTG TCTACGTCC- A- G-3', respectively), and 200 nM TaqMan probe (5'- F-CGCTATTC TGCAGCAGCT- GTTGGTGTACCA-T- 3', where F = FAM and T = TAMRA).

#### CD40 IMMUNOCYTOCHEMISTRY

Representative 5  $\mu$ m paraffin wax embedded material from 21 patients with Kaposi's sarcoma was selected, cut on to slides, and dewaxed using xylene (table 1). The sections were hydrated and then stained with anti-CD40 antibodies (clone EA-5, code no. MCA 1213; Serotec, Kidlington, Oxfordshire, UK) using the Dako catalysed signal amplification (CSA) system, HRP kit (gift of Professor K Gatter) (Dako, Cambridge, UK). This procedure uses a mouse primary antibody, which is detected by a biotinylated secondary antibody. The secondary antibody is then incubated with a streptavidin-biotin-peroxidase complex, in which peroxidase catalyses the precipitation on to the specimen of a biotinylated phenolic compound. This results in an increase in the number of biotin molecules available for further binding to the streptavidin-peroxidase reagent. The target is finally visualised by diaminobenzidine (DAB)/hydrogen peroxide. The procedure results in pronounced amplification of the signal, allowing the detection of small quantities of target antigen.

Sections were incubated for five minutes in 3% hydrogen peroxide, after which a protein block was applied for a further five minutes (serum free protein in phosphate buffered saline with 0.015 M sodium azide). The primary antibody was applied (1/100 antibody dilution) for 15 minutes. After washing, the slides were incubated sequentially for 15 minute periods with biotinylated link antibody, streptavidin-biotin-peroxidase complex, amplification reagent (biotinylated tyramide and hydrogen peroxide), and streptavidin-peroxidase. The amplified target antigen was visualised after a five minute incubation in DAB/hydrogen peroxide.

Tonsil tissue was used as a positive control. Staining was assessed semiquantitatively on a

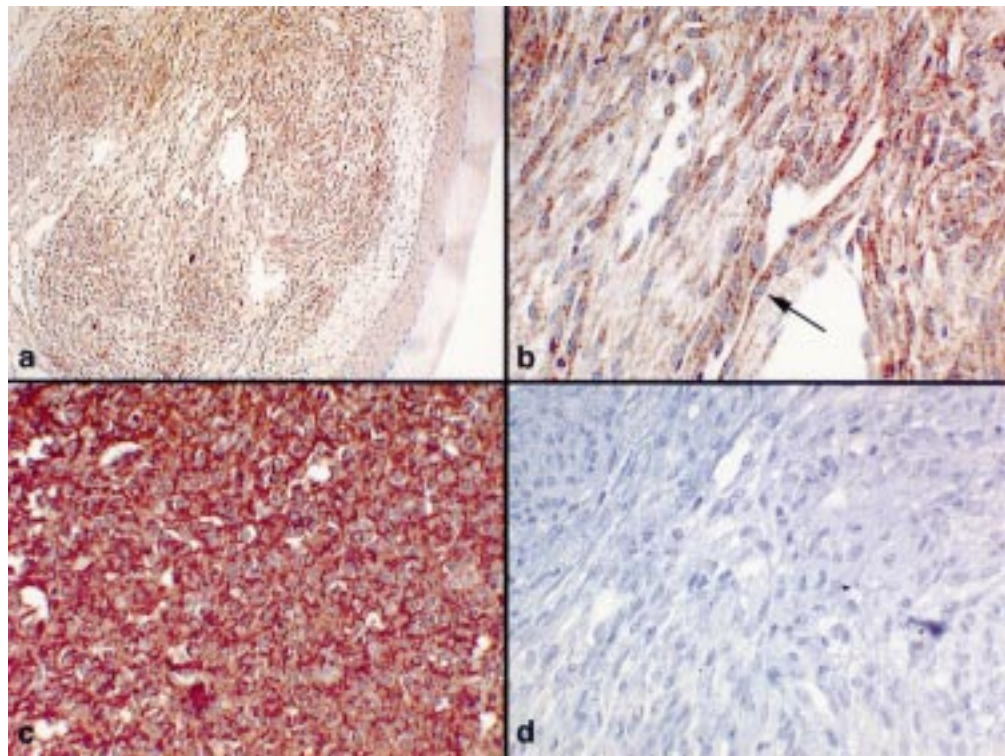


Figure 2 (a) CD 40 staining of nodular Kaposi's sarcoma, (b) cytoplasmic staining of both spindle and endothelial cells (arrow); (c) positive control (tonsil); (d) negative control (without primary antibody).

scale from + (weak) to ++++ (strong). Spindle cells and endothelial cells were assessed independently.

Staining was attempted with the anti-CD40 clone B-B20 (Serotec) but results were less satisfactory. Initial staining was also attempted using a conventional three step detection system but this yielded weak/equivocal signals.

### Results

Ten specimens exhibited strong staining (+++/++++) of endothelial and/or spindle cells (fig 2 and table 1). Three specimens were CD40 negative, two of which were positive for HHV-8. Overall, 14 specimens contained amplifiable HHV-8 sequences (table 1), which were screened using two different PCR protocols. There was no obvious relation with disease stage and/or anatomical site.

### Discussion

Kaposi's sarcoma has remained a tumour of uncertain histogenesis. It is unresolved as to whether or not it is a true neoplasm.<sup>26</sup> The recent development of a neoplastic cell line<sup>27</sup> and the demonstration that multifocal tumours show similar X-linked gene methylation patterns<sup>28</sup> suggest that in some instances Kaposi's sarcoma is a clonal neoplasm. In addition, epidemiological data have supported the theory that an infectious agent may be central to the pathogenesis of Kaposi's sarcoma, stimulating an inflammatory response mediated by cytokines.<sup>12 13</sup> To date, HHV-8 has emerged as the most likely candidate agent—

this is supported by its presence in most Kaposi's sarcoma lesions and compelling sero-epidemiological data.<sup>1-8</sup>

CD40, a 45–50 kDa glycoprotein, is a member of the TNF receptor superfamily, which includes CD30 and Fas. It is expressed on a variety of cells, including B lymphocytes, and activation of CD40 induces B cell activation, proliferation, and differentiation.<sup>16 17</sup> Although some members of the TNF receptor superfamily induce apoptosis (for example, Fas),<sup>16 17</sup> CD40 mediated signal transduction promotes cell survival, which might be mediated by Bcl-x.<sup>29</sup> In addition, CD40 is expressed constitutively on cultured endothelial cells and its expression is upregulated by cytokines, including TNF and  $\gamma$  interferon.<sup>18</sup> CD40 activation also promotes the expression of cell adhesion molecules<sup>18</sup> and thus is likely to play a central role in the inflammatory cascade.

The EBV latent membrane protein 1 (LMP1) has been shown in vitro to promote infected B cell survival by upregulating the bcl-2 gene,<sup>30</sup> as well as inducing upregulation of the CD40 gene, which may partly mediate its anti-apoptosis function.<sup>31 32</sup> Extensive HHV-8 viral genome analysis has revealed a homologue of Bcl-2 that can inhibit Bax mediated apoptosis.<sup>33</sup> However, our group has been unable to correlate bcl-2 gene expression with viral status.<sup>34</sup> An additional development has been the documentation of cytokine gene homologues within the HHV-8 genome,<sup>20 21 23</sup> including IL-6, which has been demonstrated to inhibit apoptosis in a murine plasmacytoma cell line.<sup>21</sup> Interestingly, CD40 positivity has been documented in IL-6 dependent myeloma

cell lines,<sup>17</sup> which raises the possibility that CD40 gene expression might be upregulated directly by HHV-8 viral encoded genes. However, the expression of these may be limited to the lytic phase and it has been demonstrated that most Kaposi's sarcoma cells are latently infected with HHV-8.<sup>35</sup> An alternative scenario is that cytokines released by bystander inflammatory cells upregulate CD40 gene expression in a paracrine manner.

In their study, Pammer and colleagues<sup>14</sup> demonstrated that CD40 was expressed on both Kaposi's sarcoma tissue and cell lines and that CD40 gene expression was upregulated by cytokine treatment. Similarly, CD40 expression was noted on endothelial cells in areas adjacent to tumours and in granulation tissue with faint/negative staining in normal skin. Interestingly, HHV-8 was not identified in cell lines on PCR screening.<sup>14</sup> This prompted us to determine whether a relation existed between HHV-8 status and CD40 expression. Our results suggest that CD40 expression does not correlate with the presence of HHV-8, being found in both HHV-8 positive and negative specimens. Conversely, two CD40 negative cases contained amplifiable HHV-8 sequences. It also appears that CD40 expression is independent of the disease stage. In addition, CD40 expression varied from lesion to lesion with six specimens exhibiting weak/negative staining. This is somewhat at variance with the findings of Pammer *et al*, who reported strong staining in all patients.<sup>14</sup> However, they did report that only one of four cell lines expressed CD40.

We have also demonstrated the use of the EA5 clone, which yielded satisfactory results when used in conjunction with the CSA system. The latter enables the target signal to be greatly increased by exploiting a biotin deposition amplification system.<sup>36</sup> This enhances the visualisation of low copy target antigens and thus permits the use of higher antibody dilutions than are used in conventional immunocytochemical detection systems. In addition, an antigen retrieval step is eliminated, with the obvious advantage of improved tissue morphology. In contrast to other investigators,<sup>14</sup> less satisfactory results were obtained with the anti-CD40 B-B20 clone.

In summary, we have shown that CD40 is expressed in both HHV-8 positive and HHV-8 negative Kaposi's sarcoma specimens. We are confident that our HHV-8 negative specimens were truly negative because we used TaqMan PCR, which has a sensitivity of one viral genome in 10<sup>6</sup> DNA sequences (JJ O'Leary *et al*, unpublished, 1998). Even though our numbers are small, we have also shown that some HHV-8 positive specimens did not exhibit CD40 immunohistochemical staining. This suggests that HHV-8 might promote cell survival following infection by anti-apoptosis mechanisms not involving CD40. An additional consideration is that CD40 expression is dependent on the inflammatory milieu, including the presence of certain cytokines that may or may not promote CD40 gene up-

regulation (for example,  $\gamma$  interferon).<sup>18, 19</sup> The presence of these probably vary at different stages of tumour development, even though we were unable to demonstrate a relation to disease stage. It is likely that the pathogenesis of Kaposi's sarcoma is a complex multifactorial process that involves viral homologues of cell cycle genes,<sup>37</sup> apoptosis genes, and inflammatory mediators operating in both a paracrine and autocrine manner.

The authors thank Serotec, Kidlington, Oxford, UK for providing the antibodies used in this study.

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*Mol Path* 1999 52: 32-36  
doi: 10.1136/mp.52.1.32

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