

Human herpes virus 8 (HHV-8) in Kaposi's sarcoma: lack of association with Bcl-2 and p53 protein expression

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Abstract

Aims—Human herpes virus 8 (HHV-8) is the infectious agent implicated in the pathogenesis of Kaposi's sarcoma, although its mode of action is unclear. Recent work indicates that the HHV-8 genome encodes a viral Bcl-2 homologue (v-Bcl-2). The aim of this study was to explore Bcl-2 expression in Kaposi's sarcoma using a unique set of HHV-8 positive and negative cases, and to determine whether there is a relation with p53 expression.

Methods—Up to 18 specimens from 17 patients were selected. HHV-8 status was determined using nested polymerase chain reaction (PCR) to the open reading frame (ORF) 26, with further confirmation by TaqMan PCR. In addition, Bcl-2 and p53 immunohistochemistry were performed using standard protocols.

Results—The results suggest that Bcl-2 and p53 expression is independent of HHV-8 status. In addition, there does not appear to be a direct correlation with disease stage.

Conclusions—HHV8 histopathogenesis is likely to be a multifactorial complex process, which may be mediated in part by viral genes and apoptosis regulating homologues.

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Keywords: human herpes virus 8; Bcl-2; p53; immunohistochemistry; TaqMan PCR

Kaposi's sarcoma is a vasoformative tumour of uncertain histogenesis that might have an infectious aetiology.¹ The newly described human herpes virus 8 (HHV-8) or Kaposi's sarcoma associated herpes virus (KSHV)² is the most likely candidate agent, based on its strong association with all Kaposi's sarcoma subtypes.²⁻⁶ This association is confirmed by seroepidemiological studies, which show convincingly that the seroprevalence of HHV-8 parallels that of high risk groups, including homosexual/bisexual males and high risk endemic areas.⁷⁻¹¹ HHV-8 has also been implicated in the pathogenesis of the rare B cell primary effusion lymphoma¹² and Castleman's disease.¹³ More recent evidence has shown that bone marrow dendritic cells might harbour the virus, suggesting that HHV-8 may play a role in both multiple myeloma and monoclonal gammopathy of uncertain significance.¹⁴ Viral genetic analysis indicates that HHV-8 has sequence

homology with two other lymphotropic viruses, namely: Epstein-Barr virus (EBV) and herpes virus saimiri (HVS).¹⁵ In addition, the viral genome contains homologues of cellular genes including those encoding interleukin 6 (IL-6), a D-type cyclin, and Bcl-2.¹⁶

The role that HHV-8 plays in the pathogenesis of Kaposi's sarcoma is unclear, but it is likely that cytokines¹⁷⁻¹⁹ and cell cycle regulatory genes play a central role, as do anti-apoptosis proteins, which might be encoded by the viral genome. However, recent work on the role of Bcl-2 in Kaposi's sarcoma has produced conflicting results.²⁰⁻²² Therefore, we wished to explore Bcl-2 expression in Kaposi's sarcoma, and to determine whether there was a correlation with HHV-8 status, using a unique set of HHV-8 positive and negative cases. In addition, we hoped to elucidate whether there was a relation between these two variables and p53 expression, as assessed by immunohistochemistry.

Materials and methods

HHV-8 AMPLIFICATION

Representative formalin fixed, paraffin wax embedded tissue (table 1) was cut into sterile Eppendorf tubes, dewaxed, and suspended in 200 µl proteinase K (Boehringer Mannheim, Mannheim, Germany) digestion buffer (100 mM NaCl, 10 mM Tris, 25 mM EDTA, 0.5% sodium dodecyl sulphate (SDS), pH 8.4), with a final proteinase K

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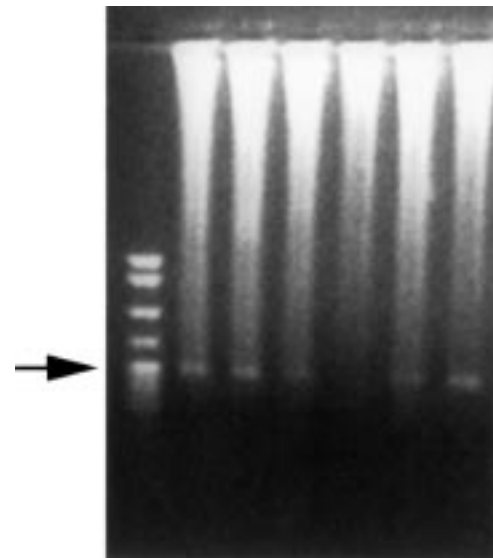


Figure 1 Two per cent agarose gel demonstrating characteristic 233 bp product (ORF26).

Table 1 Clinical details (including HHV-8 status) and analysis of Bcl-2 and p53 protein products

Case no.	HHV-8	Sex	HIV status	Site	p53*	Bcl-2*
1	+ve	Female	+ve	Skin patch	-ve sp -ve endo	+ sp -ve endo
	+ve			Nodular	++ sp + endo	+ sp + endo
2	+ve	Male	+ve	Skin patch	-ve sp -ve endo	+ sp -ve endo
3	+ve	Male	+ve	Skin nodular	+++ sp +++ endo (patch component only)	+ sp -ve endo
4	+ve	?	?	Skin patch	+ sp	ND
5	+ve	?	?	Skin patch	-ve endo -ve sp	+ sp -ve endo
6	-ve	Male	-ve	Lymph node	++ sp ++ endo	+ sp -ve endo
7	+ve	Male	-ve	Skin patch	-ve sp -ve endo	-ve sp -ve endo
8	+ve	Male	-ve	Skin patch	+ sp + endo	+ sp -ve endo
9	+ve	Male	+ve	Skin patch	+ sp + endo	++ sp -ve endo
10	+ve	Male	+ve	Skin patch	++ sp + endo	+ sp -ve endo
11	-ve	Male	-ve	Skin nodular	+++ sp ++ endo (focal)	++ sp ++ endo (focal)
12	+ve	Male	+ve	Lymph node	++ sp ++ endo (focal)	+ sp -ve endo
13	+ve	Male	+ve	Lung	++ sp ++ endo (focal)	++++ sp ++++ endo
14	ND	Male	+ve	Mouth	++ sp ++ endo (focal)	++ sp + endo
15	+ve	Male	+ve	Stomach	+ sp -ve endo	-ve sp -ve endo
16	+ve	?	?	?	+++ sp + endo (focal)	ND
17	-ve	Female	+ve	Skin nodular	+++ sp +++ endo	+ sp -ve endo

*Positive staining for p53 and Bcl-2 on a scale of + (weak) to ++++ (very strong). +ve, positive; -ve, negative; end, endothelium; ND, not determined; sp, spindle cell.

concentration of 0.1 mg/ml. After a three day incubation, the sample DNA was purified and precipitated using a phenol/chloroform extraction method followed by ethanol/3 M sodium acetate. Amplification of β globin was performed to confirm the amplifiability of the extracted DNA.

HHV-8 PCR

The presence of HHV-8 was detected using nested primers to the open reading frame (ORF) 26 of the minor capsid protein (VP23) using the following reagents: 0.5 μ M each primer, 200 μ M dNTPs, 1.5 mM MgCl₂, 1 \times PCR buffer II (Perkin Elmer, New Jersey, USA) and 2.5 U Amplitaq DNA polymerase (Perkin Elmer). Primers comprised the outer primer pair, KS4: 5'-AGCACTCGCAGG GCAGT ACG-3', KS5: 5'-GACTCTTCG CTGATGAACTGG-3'; and the inner primer pair, KS1: 5'-AGCCGAAAGGATTCCAC CAT-3', KS2: 5'-TCCGTGTTGTCTACGT CCAG-3'.

All reactions were performed in a 480 DNA thermal cycler (Perkin Elmer) with cycling parameters as follows: 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. A 94°C 30 second "hot start" was used during both steps. Reaction products were separated on a 2% ethidium bromide stained agarose gel (Sigma, Poole, Dorset, UK) (fig 1).

TaqMan PCR

Cases were reamplified using the newly described technique of TaqMan PCR. This has been described elsewhere,²³ and refers to the addition of a target specific oligonucleotide probe to the PCR reaction, which also contains a conventional primer pair. The intact probe has a fluorescent reporter molecule at its 5' end, which is normally "quenched" by a quencher sequence situated at its 3' end. When amplification occurs, the probe is cleaved as a result of the 5' exonuclease activity of the AmpliTaq DNA polymerase. This results in the release of reporter fluorescence that can be estimated using a luminescence spectrometer. An increase in sample fluorescence occurs only when there is specific amplification, thus obviating the need to perform Southern blot analysis.

For the detection of HHV-8, reaction conditions were as described previously,²³ using 300 nM of both primers (KS1: 5'-AGCCG AAAGGATTCCACCAT-3', KS2: 5'-TCCGT GTTGTCTACGTCCAG-3') and 200 nM of the TaqMan probe (5' F-CGCTATTCTG CAGCAGCTGTTGGTGTACCA-T 3', where F = 6-carboxy-fluorescein (FAM) and T = 6-carboxy-tetramethyl-rhodamine (TAMRA) (Perkin Elmer).

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Representative sections from 16 cases (15 patients) were cut on to slides, dewaxed, and hydrated (table 1). Sections were immersed in 0.01 M citrate buffer (pH 6.0) and the slides

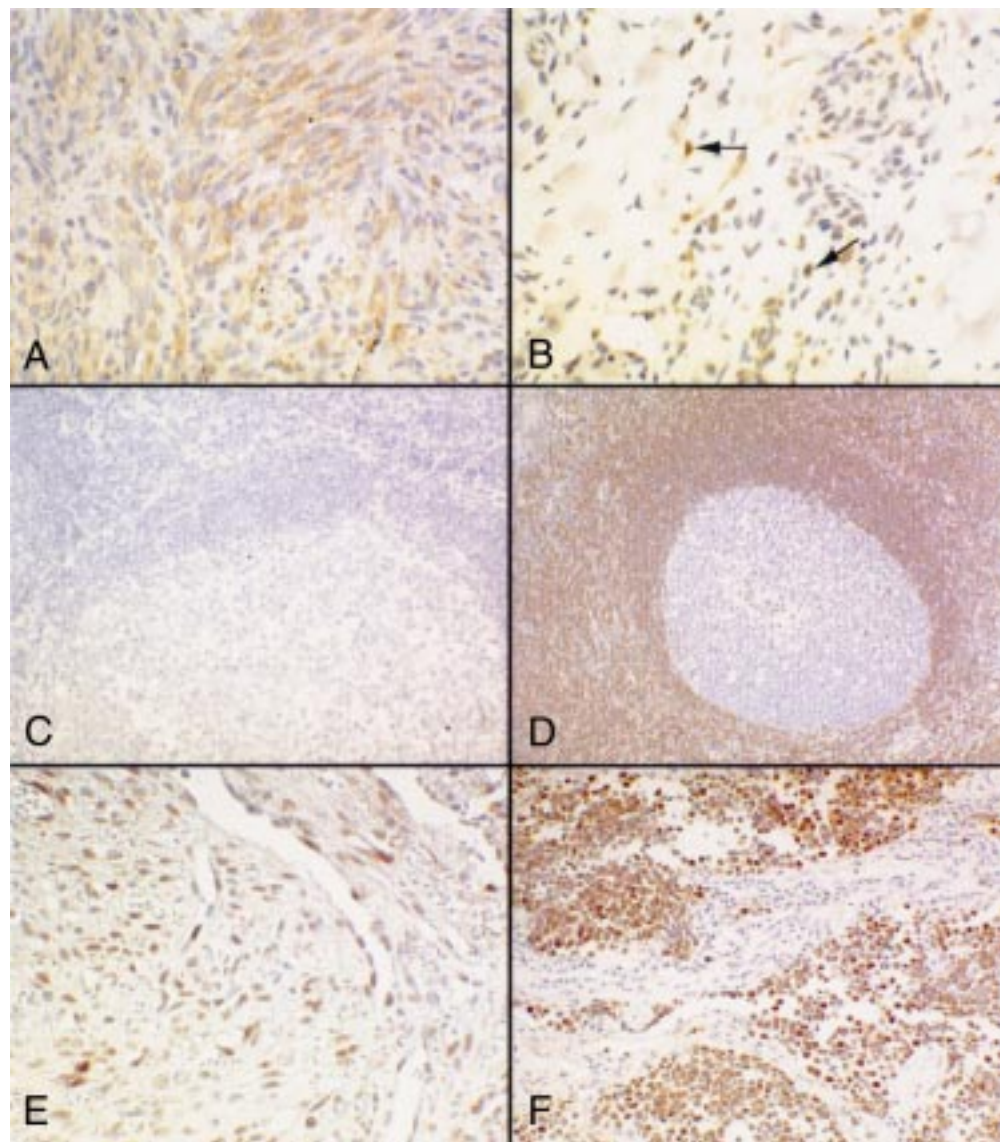


Figure 2 (A) Immunohistochemical detection of Bcl-2 in Kaposi's sarcoma spindle cells (nodular Kaposi's sarcoma) with (B) positive control lymphocytes (arrows); (C) and (D) negative and positive Bcl-2 controls, respectively; (E) p53 nuclear staining (endothelial and spindle cells) with (F) p53 positive control.

were microwaved for 15 minutes. Endogenous peroxidase activity was blocked with 0.6% hydrogen peroxide in absolute methanol for 15 minutes. The sections were then washed in phosphate buffered saline (PBS), followed by a 10 minute block in normal horse serum (Vector Quick Kit PK8800; Vector, Peterborough, UK), after which the anti-Bcl-2 antibody (M887; Dako, Cambridge, UK) was applied (1/50 dilution in PBS). After 30 minutes incubation at room temperature, the slides were washed and incubated in universal secondary antibody (Vector Quick Kit PK8800; Vector). This was followed by a further incubation step in streptavidin-peroxidase (Vector Quick Kit PK8800; Vector) and the reaction was visualised in DAB (SK4100; Vector). Tonsil tissue was used as the positive control.

A similar protocol was used for p53 immunohistochemistry but heat mediated antigen retrieval was used and the primary antibody was DO7 (Dako; 1/50 dilution). A case of adenocarcinoma was used as the positive

control tissue. Eighteen representative sections from 17 patients were selected.

Results

Immunohistochemical estimation of the p53 and Bcl-2 protein products were assessed in up to 18 specimens from 17 patients (table 1). Fourteen specimens contained amplifiable HHV-8 DNA sequences and three patients were negative for HHV-8 DNA. Staining was assessed semiquantitatively on a scale from + (weak) to ++++ (strong). Both spindle cells and endothelial cells were assessed independently. One of 16 cases stained strongly for Bcl-2, with the remaining cases being either negative or weakly stained. Internal control lymphocytes were positive (fig 2). There was no association with HHV-8 status and/or with stage. Four cases stained moderately strongly for p53 (+++) (fig 2). Interestingly, although there was no obvious relation to HHV-8 status, all HHV-8 negative cases showed moderate p53 expression (++) . In addition, although

there was a tendency for moderately strong p53 staining in nodular samples, protein synthesis was confined to the patch component only in one case (case 3). Finally, there was no apparent relation between Bcl-2 and p53 expression.

Discussion

Kaposi's sarcoma is a tumour of uncertain origin and complex pathogenesis. Multiple factors are implicated, including cytokines¹⁷⁻¹⁹ and anti-apoptosis genes,²⁰⁻²² which might interact with an infectious agent. HHV-8² is thought to be the most likely infectious cause of Kaposi's sarcoma. This idea is supported by its strong association with all Kaposi's sarcoma subtypes.²⁻⁶ The way in which it causes Kaposi's sarcoma remains undetermined, but it is probable that HHV-8 interferes with the cell cycle by promoting cell survival and thus the accumulation of tumorigenic genetic events. This is supported by recent viral genome analysis, which has revealed the presence of a Bcl-2 homologue that can inhibit Bax mediated/Sindbis virus induced apoptosis.²⁴⁻²⁵

Cell survival is governed by an increasingly recognised family of apoptosis regulating genes,²⁶⁻²⁷ of which the prototype is Bcl-2, an anti-apoptosis gene, first described in relation to t(14;18), which characterises follicular lymphomas.²⁸ It is structurally homologous to other, related proteins including Bcl-X_L, Bcl-X_S, Bax, and Bad. The Bcl-2 family are characterised by their ability to form hetero/homodimers with each other, thus promoting either cell survival and/or apoptosis. Other classes of proteins are also implicated in cell survival. These include c-myc²⁹ and wild-type p53, which promotes cell cycle arrest and apoptosis on exposure to injurious agents such as ultraviolet radiation,³⁰ an effect that might be mediated by Bax.³¹

During viral infection, many viruses use specific anti-apoptosis strategies to facilitate survival and viral replication.³² This might be directed at p53 induced apoptosis as a result of p53 binding by viral products (such as human papilloma virus (HPV) E6 and EBV EBNA-5 and BZLF1). Alternatively, virally encoded proteins, such as the adenovirus E1B 19 kDa protein, might inhibit apoptosis in a manner analogous to Bcl-2.³³ Similarly, the EBV BHRF1 protein is functionally homologous to Bcl-2, promoting cell survival³⁴ and oncogenesis, as does the EBV encoded latent membrane protein 1 (LMP-1).³⁵ In accord with this, recent HHV-8 viral genome analysis has indicated the presence of a v-Bcl-2, which has been mapped to ORF16. The encoded protein is homologous to human Bcl-2, Bcl-X_L, and Bax, as well as to other Bcl-2-type proteins encoded by related viruses, such as HVS ORF16 and EBV BHRF1.²⁴ It is functionally homologous to human Bcl-2 because it can inhibit Bax mediated apoptosis and forms heterodimers with human Bcl-2. Cell localisation studies indicate that it is an intracellular protein, suggesting that it may be localised to organelle membranes.²⁴ However, v-Bcl-2 does not heterodimerise with Bax or Bak.²⁵

Our results suggest that Bcl-2 expression is weak in most of our Kaposi's sarcoma samples, being found in both HHV-8 positive and negative cases. This is in accord with the previous observations by Foreman and colleagues,²¹ who found that Bcl-X_L was more likely to be expressed than Bcl-2, both in tissue sections and in in vitro studies. This may relate to the fact that v-Bcl-2 appears to be expressed primarily during the lytic phase,²⁴⁻²⁵ which is supported further by the demonstration that low concentrations of Bcl-2 mRNA are found in AIDS Kaposi's sarcoma cell lines.³⁶ In addition, RNA analysis indicates that most Kaposi's sarcoma cells are latently infected, with lytic viral replication being confined to a minority of cells.³⁷ Finally, we did not demonstrate an association between Bcl-2 and p53 expression, which often shows an inverse relation.³⁸

Abrogation of p53 function is the most common abnormality in human tumours,³⁰ and recent evidence indicates that p53 mutations might underlie a subgroup of AIDS associated Kaposi's sarcoma,³⁹ which is clinically more aggressive than other forms of Kaposi's sarcoma. Our findings support those of Bergman and colleagues⁴⁰ and also of Dada *et al*,²² who found that most Kaposi's sarcoma lesions exhibited weak p53 staining. In addition, strong p53 expression was seen in two cases that were clinically aggressive.²² Similarly, three of our nodular Kaposi's sarcoma samples showed moderately strong expression, even though staining was confined to the patch component only in one case (case 3). We also failed to demonstrate an association with HHV-8/or human immunodeficiency virus (HIV) status. However, it is well recognised that p53 immunohistochemical staining interpretation is fraught with difficulty, being governed by both protein stabilisation and/or genetic derangements.⁴¹

It is probable that HHV-8, like other herpes viruses, promotes viral replication and infectivity by inhibiting host induced apoptosis following viral infection, which might be mediated by v-Bcl-2. However, other mechanisms are likely to operate, and recent work indicates that an IL-6 viral homologue has similar activity to human IL-6 in preventing apoptosis in a mouse plasmacytoma cell line.⁴² The mode by which HHV-8 causes Kaposi's sarcoma is yet to be elucidated, but it is likely to involve anti-apoptotic mechanisms and abrogation of p53 function and cell cycle deregulation,⁴³ thereby enhancing cell survival and viral replication in tandem with effective evasion from host defence mechanisms.

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