Papers

Cyclin D1 expression and HHV8 in Kaposi sarcoma

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Abstract

Background—Human herpesvirus 8 (HHV8) appears to be the agent responsible for Kaposi sarcoma. The mechanism remains undetermined but may involve cell cycle regulating genes including D type cyclins which are pivotal in cell cycle progression. Recent HHV8 genetic analysis has revealed the presence of a v-cyclin which is homologous to D type cyclins.

Aims—First, to assess whether there is an independent relation between endogenous cyclin D1 expression in Kaposi sarcoma and HHV8 status; second to determine whether v-cyclin mRNA expression varies with Kaposi sarcoma stage.

Methods—Cyclin D1 immunohistochemistry was performed on 17 paraffin embedded Kaposi sarcoma samples from 16 patients. HHV8 status was assessed in 15 of these using nested polymerase chain reaction (PCR) to ORF 26 and the newly described technique of TaqMan® PCR. An additional 10 fresh Kaposi sarcoma samples (early and nodular) were examined for HHV8 v-cyclin RNA.

Results—One case, which did not contain amplifiable HHV8, showed strong cyclin D1 staining. The remaining cases were negative or weakly staining; v-cyclin transcript load was higher in early Kaposi sarcoma.

Conclusions—While endogenous cyclin D1 expression is independent of HHV8 status, v-cyclin transcription is higher in early lesions, supporting the "viral hit" hypothesis.

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Keywords: Kaposi sarcoma; cyclin D1; HHV8 v-cyclin

Kaposi sarcoma, a tumour of uncertain histogenesis, has long been considered to be caused by a transmissible infectious agent.¹ Human herpesvirus 8 (HHV8)/Kaposi sarcoma associated herpesvirus (KSHV)² is generally accepted to be the most likely candidate agent, and this is supported by its strong association with all subtypes of Kaposi sarcoma.³⁻⁶ It has also been documented in

multicentric Castleman disease (MCD)⁷ and primary effusion lymphoma (PEL)(8). However, the mode by which HHV8 is involved in the pathogenesis of Kaposi sarcoma remains unresolved and it is known that many factors are implicated including cytokines,^{9,10} anti-apoptosis genes,¹¹ and cell surface receptors, for example CD40.¹²

HHV8 genome analysis has shown that many homologues of cellular genes are present including genes which encode Bcl-2,¹³ cytokines,¹⁴ G protein coupled receptors,¹⁵ and a v-cyclin.¹⁵⁻¹⁸ The latter is known to be homologous to D2 cyclins and has been shown to induce hyperphosphorylation of retinoblastoma protein in transfected cell lines,¹⁶ which suggests that HHV8 might play a direct role in cell cycle deregulation. The D type cyclins are pivotal molecules in cell proliferation governing cell cycle progression in the G1 phase. Cyclins form complexes with cyclin dependent kinases and mediate their proliferative effect by hyperphosphorylation of checkpoint molecules, for example retinoblastoma protein, thereby inhibiting the function of the latter.^{19,20} As cyclins are key molecules in cell cycle progression, we decided to investigate the expression of cyclin D1 in specimens of Kaposi sarcoma from different anatomical sites to establish whether there was a relation with the presence or absence of HHV8 within these lesions. In addition, we hoped to determine whether v-cyclin RNA expression varied with tumour stage.

Methods

CYCLIN D1 IMMUNOHISTOCHEMISTRY

Immunohistochemical estimation of the cyclin D1 protein product was assessed in 17 specimens from 16 patients (table 1). Eleven patients were HIV positive and the three known HIV negative cases were of African endemic origin. Representative sections were mounted on glass slides coated with Vectabond (Vector Laboratories), dewaxed, and rehydrated. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide for 20 minutes.

The sections were placed in a domestic pressure cooker in 0.01 M sodium citrate (pH 6) buffer and boiled at full pressure for 90

Table 1 Clinical details (including HHV8 status) and analysis of cyclin D1 protein product

Patient No	HHV8	HIV status	Sex	Site	Cyclin D1
1	Pos	Pos	F	Skin, patch	Neg sp Neg endo Neg sp Neg endo
2	Pos	Pos	M	Skin, nodular Skin, patch	++ sp + endo + sp Neg endo
3	Pos	Pos	M	Skin, nodular	Neg sp Neg endo +++ sp +++ endo (focal)
4	Pos	?	?	Skin, patch	+ sp Neg endo Neg sp Neg endo
5	Neg	Neg	M	Lymph node	Neg sp Neg endo Neg sp Neg endo
6	Pos	Neg	M	Skin, patch	Neg sp Neg endo Neg sp Neg endo
7	Pos	Pos	M	Skin, patch	Neg sp Neg endo Neg sp Neg endo
8	Pos	Pos	M	Skin, patch	Neg sp Neg endo Neg sp Neg endo
9	Neg	Neg	M	Skin, nodular	Neg sp Neg endo Neg sp Neg endo
10	Pos	Pos	M	Lymph node	Neg sp Neg endo Neg sp Neg endo
11	Pos	Pos	M	Lung	Neg sp Neg endo Neg sp Neg endo
12	N/D	Pos	M	Stomach	Neg sp Neg endo Neg sp Neg endo
13	N/D	Pos	M	Mouth	+/- sp +/- endo Neg sp Neg endo
14	Pos	Pos	M	Stomach	Neg sp Neg endo Neg sp Neg endo
15	Pos	?	?		Neg sp Neg endo Neg sp Neg endo
16	Neg	Pos	F	Skin, nodular	+ sp Neg endo (focal)

endo, endothelial cell; F, female; HHV8, human herpesvirus 8; M, male; N/D, not done; Neg, negative; Pos, positive; sp, spindle cell; +/- to +++, degree of positivity.

seconds.²¹ On removal from the pressure cooker, slides were immediately immersed in distilled water and transferred to phosphate buffered saline (PBS).

The sections were subsequently incubated at room temperature for one hour in NCL-cyclin-D1-GM monoclonal antibody (clone P2D11F11; 1:25) (Novocastra Laboratories). The slides were then washed and incubated in biotinylated goat antimouse antibody (Dako code No E0433; 1:400) for 30 minutes, followed by a further incubation in streptavidin-HRP (Dako code No P0397) employing diaminobenzidine (DAB) as the chromagen.

Cells were regarded as cyclin D1 positive when there was nuclear staining. Staining was assessed semiquantitatively on a scale from +

(weak) to ++++ (strong). Both spindle cells and endothelial cells were assessed independently. A case of mantle cell lymphoma was used as positive control material. The primary antibody was omitted in the negative controls.

HHV8 PCR AMPLIFICATION

Representative formalin fixed, paraffin embedded tissue from 15 specimens was dewaxed and placed for three to five days at 37°C in proteinase K (0.1 mg/ml) digestion buffer (100 mM NaCl, 10 mM Tris, 25 mM ethylene diamine tetra-acetic acid (EDTA), and 0.5% sodium dodecyl sulphate (SDS), pH 8.4). The DNA was purified by phenol/chloroform extraction followed by overnight precipitation in 3 M sodium acetate/ethanol. Samples were then centrifuged and resuspended in high performance liquid chromatography (HPLC) water. The quality of DNA was assessed by β globin amplification (yielding a 268 base pair fragment).

Nested polymerase chain reaction (PCR) amplification of the open reading frame (ORF) 26 of HHV8 was conducted in a 480 DNA thermal cycler (Perkin Elmer) using the following primer pairs:

Outer set:

KS4 5'-AGCACTCGCAGGGCA GTACG-3'

KS5 5'-GACTCTTCGCTGATGAACTGG-3'

Inner set:

KS1 5'-AGCCGAAAGGATTCC ACCAT-3'

KS2 5'-TCCGTGTTGTCTACGTCCAG-3'

The cycling protocol was as follows:

Outer set (94°C × 45 s; 60°C × 30 s; 72°C × 45 s) for 25 cycles;

Inner set (94°C × 45 s; 55°C × 30 s; 72°C × 45 s) for 35 cycles, with a soak file of 4°C.

Reaction products (233 base pair fragment) were run on a 2% agarose gel (Sigma) (fig 1).

TaqMAN® PCR

Negative cases were subsequently reamplified using TaqMan PCR which we have described in detail elsewhere.²² This technique employs conventional primer pairs in the amplification reaction as well as a fluorescent labelled target specific oligonucleotide probe. During amplification the fluorescent reporter molecule is released by exploiting the 5' nuclease activity of *AmpliTaq* DNA polymerase. The increase in sample fluorescence correlates with the amount of specific product generated and may be detected by a luminescence spectrometer.

For the detection of HHV8, reaction conditions were as previously described²² with 300 nM of each primer (KS1 5'-AGCCGA AAGGATTCCACCAT-3'; KS2 5'-TCCGTG TTGTCTACGTCCAG-3') and 200 nM of TaqMan probe (5' F-CGCTATTCTGCA GCAGCTGTTGGTGTACCA-T 3', where F=FAM (6-carboxy-fluorescein) and T=TAMRA (6-carboxy-tetramethyl-rhodamine)). No template controls were performed in triplicate.

V-CYCLIN RNA AMPLIFICATION

Ten fresh Kaposi sarcoma skin cases (five early/five nodular) were selected (table 2). RNA was extracted using the RNAzol B

Figure 1 2% agarose gel electrophoresis (HHV8 KS 330 Bam).

Table 2 Clinical details and HHV8 v-cyclin RNA analysis according to Kaposi sarcoma stage

Case No	Stage	Sex	HIV status	v-Cyclin transcripts /100 ng tRNA
1	Early	M	Pos	600
2	Early	M	Pos	850
3	Early	M	Pos	450
4	Early	M	Pos	1050
5	Early	M	Pos	1167
6	Nodular	M	Pos	210
7	Nodular	F	Pos	50
8	Nodular	M	Pos	230
9	Nodular	M	Pos	258
10	Nodular	M	Pos	140

F, female; M, male; Pos, positive.

method (Molecular Research Centre Inc). All gene quantitations were performed using the 7700 DNA sequence detector (Perkin Elmer) using 100 ng of extracted total RNA. The following protocol was employed: primers 200 nM, deoxynucleotide triphosphates 300 µM, TaqMan probe 100 nM, rTth polymerase 0.1 U/µl, uracil N-glycosylase 0.01 U/µl, 1 × TaqMan EZ buffer, manganese acetate 3 mM (Perkin Elmer), with DNase and RNase free water to 50 µl.

HHV8 v-cyclin gene

Forward primer: 5' ACC AGT TCA CTT TGC TAT GCC 3'

Reverse primer: 5' GCT TTT GTA ATC AGG GTG TTG AC 3'

TaqMan probe: 5' FAM-CAG ACT CCT TTT CCC GCC AAG AAC TTA TAG-TAMRA 3'

Reference gene for total RNA

GAPDH was used as the reference gene for tRNA:

Forward primer: 5' GAA GGT GAA GGT CGG AGT 3'

Reverse primer: 5' GAA GAT GGT GAT GGG ATT TC 3'

TaqMan probe: 5' JOE-CCG ACT CTT GCC CTT CGA AC-TAMRA 3'

Thermocycling conditions

The following thermocycling conditions were applied:

50°C × 2 min; 60°C × 30 min; 95°C × 5 min; [94°C × 20 s; 60°C × 1 min (v-cyclin HHV8)/ 62°C × 1 min (GAPDH)] × 40 cycles.

v-Cyclin transcript number was calculated using TaqMan PCR employing cloned v-cyclin gene in PCR 2.1 as standards. This assay was repeated in triplicate. GAPDH copy number was then calculated for each 100 ng of RNA used. Sample variability was assessed by reference to the GAPDH result. As the copy number of GAPDH is 2×10^7 per 200 ng of tRNA, the v-cyclin value can then be expressed as the number of transcripts/100 ng total RNA.

STATISTICAL ANALYSIS

The association between viral v-cyclin expression and stage of disease was analysed using the standard *t* test.

Results

Of the 17 specimens examined by cyclin-D1 immunohistochemistry, one case showed moderately strong staining (fig 2). This staining was focal and is it noteworthy that the patient was negative for HHV8, which was detected in 12 of 15 cases examined. Five specimens showed weak focal positivity (table 1). All other cases were negative. There was minimal or negative staining in adjacent uninvolved tissue. There was no relation to the disease stage or anatomical site. v-Cyclin RNA transcript load was higher in early Kaposi sarcoma lesions (table 2). A significant difference was found between HHV 8 v-cyclin gene expression in early and late Kaposi sarcoma biopsy samples ($0.01 < p < 0.001$).

Discussion

The D cyclins (D1–3) are key cell cycle regulatory molecules governing cell cycle progression in G1 phase. This is mediated by hyperphosphorylation of the retinoblastoma protein, thereby removing the inhibitory function of the latter.^{19–20} Cyclin D1, also known as PRAD1, has been localised to chromosome 11q13²³ and is overexpressed in parathyroid tumours as a result of chromosomal rearrangement. D class cyclins form complexes with cyclin dependent kinases (CDK) which may be inhibited by CDK inhibitors (for example, WAF1-p21) either directly or by a cyclin–CDK complex.²⁰ Deregulation of cell cycle control has been implicated in oncogenesis, and abnormal cyclin D1 expression has been documented in diverse tumours including mantle cell lymphoma^{24–26} and carcinomas of the ovary,²⁷ endometrium,²⁸ and breast.^{29–30} Immunohistochemical analysis shows that cyclin D1 is expressed at low levels in normal tissue, being mainly restricted to proliferative zones, for example in stratified squamous epithelia. In addition, there appears to be minimal expression within lymphoid tissue including tonsil, spleen, and lymph node.³⁰

Figure 2 Cyclin D1 immunohistochemistry: nuclear positivity in Kaposi sarcoma nodule (patient No 5).

It is known that following viral infection, host cell defences are activated. These include cell cycle shutdown, apoptosis, and induction of cell mediated immunity.³¹ It is probable that v-cyclins act by inhibiting cell cycle arrest by binding to the retinoblastoma protein.

Recent sequence analysis of the HHV8 genome indicates that ORF 72 encodes a D type cyclin homologue.¹⁵⁻¹⁸ This ORF shows homology to the ORF 72 of *Herpesvirus saimiri* which similarly encodes a cyclin D homologue.³² In addition, transfected osteosarcoma cell lines have shown that HHV8 v-cyclin is functional and induces hyperphosphorylation of wild type retinoblastoma protein consistent with the inactive form of the protein.¹⁶ v-Cyclin transcripts have been demonstrated both in Kaposi sarcoma biopsies and effusion lymphomas.^{15 16 33 34} This argues strongly in favour of a direct role for HHV8 in cell cycle deregulation and tumour development.

As cyclin D1 is implicated in the pathogenesis of diverse tumour types,²³⁻³⁰ the aim of our study was to assess endogenous cyclin D1 expression in Kaposi sarcoma and to determine whether there was a relation with HHV8 status independent of v-cyclin. While the number of examined cases is small, we have been unable to show a correlation between cyclin D1 expression (as measured by immunohistochemistry) and the presence of HHV8. On the contrary, the single strongly positive specimen was a lymph node which was HHV8 negative on PCR analysis using two different techniques, including TaqMan PCR which we estimate to have a detection sensitivity of one copy in 10⁶ contaminating DNA sequences (submitted for publication). The remaining cases were either negative or showed weak focal staining only. In addition, cyclin D1 expression was independent of tumour stage. In contrast, Horenstein and colleagues found that endogenous cyclin D1, which they showed to be antigenically distinct from HHV8 v-cyclin, was more likely to be expressed in late Kaposi sarcoma.³⁵

The nature of Kaposi sarcoma is enigmatic and whether it represents a neoplastic or an atypical reactive process is unresolved.³⁶ The role of HHV8 is unclear but it is likely that the virus infects target endothelial cells, promoting an inflammatory cascade mediated by cytokines which in some cases allows the emergence of a malignant clone.³⁷ HHV8 v-cyclin is functional, most homologous to cyclin D2, and known to complex with CDK 6.^{38 39} In contrast to cellular cyclins, v-cyclin/CDK complexes are more resistant to inhibition by CDK inhibitor proteins.³⁹ We have shown that HHV8 v-cyclin transcripts are higher in early Kaposi sarcoma lesions, which would tend to support the hypothesis that the virus operates a "hit and run" mechanism. At variance with our findings, two separate groups have shown by in situ studies that HHV8 v-cyclin expression is greater in advanced Kaposi sarcoma.^{33 34} By contrast, this would support a direct role for HHV8 in Kaposi sarcoma pathogenesis. Additionally, Davis *et al* conclude that increased

HHV8 v-cyclin expression in late Kaposi sarcoma is likely to promote cell cycle progression, which is in keeping with the higher S-phase fractions documented in nodular lesions.³³

In summary, we have been unable to correlate the expression of cyclin-D1 with the presence of HHV8 or with tumour stage. In addition, we have shown that v-cyclin expression is greater in early lesions. It is likely that the pathogenesis of Kaposi sarcoma is a complex process involving cell cycle deregulation, cytokines, and apoptosis, functions that may be mediated by viral genes.

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