Papers

Deletions in the Epstein–Barr virus latent membrane protein-1 oncogene in Hodgkin's disease

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Abstract

Aims—To analyse the latent membrane protein-1 (LMP-1) gene in a series of patients with Epstein–Barr virus (EBV) positive LMP expressing ordinary and HIV associated Hodgkin's disease to detect possible genetic alterations and particularly the existence of deletions near the 3′ end of the gene.

Methods—Expression of the EBV LMP-1 was assessed using immunohistochemistry in 186 cases of Hodgkin's disease and 31 cases of HIV associated Hodgkin's disease. Genomic DNA was extracted from frozen lymph node biopsy specimens from 25 cases of Hodgkin's disease and 11 of HIV associated Hodgkin's disease, all of whom expressed the LMP-1 protein within diagnostic Hodgkin and Reed-Sternberg (HRS) cells, and amplified by polymerase chain reaction (PCR) using primers specific for the different LMP-1 regions.

Results—LMP-1 expression was observed in 106 of 186 Hodgkin's disease cases and in all 31 HIV associated Hodgkin's disease cases. Molecular analysis of the LMP-1 gene showed a high degree of genetic heterogeneity in the carboxy-terminal domain compared with the prototype B95-8 EBV strain, specially in the patients with HIV associated Hodgkin's disease. Variation in the size of the repeated region was found in 17 of 25 Hodgkin's disease and nine of 11 HIV associated Hodgkin's disease cases. Deletions of 30 base pairs near the 3′ end of the gene were detected in all cases of HIV associated Hodgkin's disease and in six Hodgkin's disease. In one case of Hodgkin's disease a larger deletion was observed. In all patients with LMP-1 deletion mutants, 50–90% of the diagnostic HRS cells expressed the LMP-1 protein.

Conclusions—The presence of the 30 base pair deletion in all cases of HIV associated Hodgkin's disease supports previous studies that reported aggressive histological and clinical behaviour in tumours harbouring this deletion. This deletion may prolong the half-life of the protein which would explain the high levels of LMP-1 expressing HRS cells in those cases carrying LMP-1 deletions. That the 30 base

pair deletion was present in all of the HIV associated Hodgkin's disease specimens suggests that impairment of immune function is a stringent requirement for the expansion of malignant cells infected by EBV strains containing the deleted LMP-1 gene.

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Epstein–Barr virus (EBV) is an ubiquitous human herpesvirus associated with several malignancies including Burkitt's lymphoma, particularly in the high incidence areas of tropical Africa, nasopharyngeal carcinoma, lymphoproliferative disorders in immunocompromised patients, and Hodgkin's disease.[1]

EBV has been detected in up to 93% of Hodgkin's disease cases using the highly sensitive Polymerase Chain Reaction (PCR).[2] Patients with EBV positive Hodgkin's disease often express latent membrane protein-1 (LMP-1), this expression being restricted to Hodgkin and Reed-Sternberg (HRS) cells.[3 4] LMP-1 can transform rodent fibroblasts and render them tumorigenic in nude mice.[5 6] In B cells, LMP-1 induces expression of different activations markers, adhesion molecules, NF-κB, and the *bcl*-2 oncogene.[7–9] Genetic analyses with EBV recombinants indicate that LMP-1 is essential for primary B lymphocyte transformation.[10]

Structurally, LMP-1 can be divided into three domains: a short intracytoplasmic N terminal domain, six membrane spanning segments and a C terminal region 200 amino acids in length located in the cytoplasm.[11] LMP-1 is present in patches in the membrane and is associated with the cytoskeleton.[12 13] LMP-1 is characterised biochemically by serine/threonine phosphorylation and rapid turnover because of specific cleavage of the protein at Leucine 242. Cleavage results in release of phosphorylated fragment (p25) into the cytoplasm.[14–17] EBV recombinants with deletions in any part of the LMP-1 N terminal domain have substantial growth transforming activity for primary B lymphocytes;[18] however, those lacking the first 44 amino acids of the LMP-1 C terminal domain are incapable of transforming

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primary B lymphocytes.[19] It has been shown recently that these 44 amino acids interact with a protein related to the tumour necrosis factor receptor associated factors. This interaction links LMP-1 transforming activity to the signaling pathways of the tumour necrosis factor receptors.[20]

The presence of 30 base pair (bp) deletions in the C terminal end of LMP-1 (amino acids 343 to 352 of the B95-8 strain) in Hodgkin's disease seems to be associated with aggressive disease.[21 22] Therefore, we have analysed the LMP-1 gene in a series of patients with EBV positive ordinary and HIV associated Hodgkin's disease expressing LMP-1 in order to detect possible genetic alterations and particularly the 30 bp deletion.

Methods
One hundred and eighty six cases of ordinary Hodgkin's disease and 31 cases of Hodgkin's disease associated with HIV infection were selected from several hospitals in Spain. Diagnosis of Hodgkin's disease was performed routinely on 5 μm lymph node sections stained with haematoxilin and eosin. The Hodgkin's disease specimens were subclassified according to the Rye modification of the Lukes and Butler classification.[23] The clinical stage at presentation was established according to the Ann Arbor System.[24]

Fresh frozen material for DNA extraction was available in 25 ordinary Hodgkin's disease cases (two lymphocyte predominant, 14 nodular sclerosis, nine mixed cellularity), and in 11 patients with HIV associated Hodgkin's disease patients (two nodular sclerosis, six mixed cellularity, two lymphocyte depletion, and one not classifiable). All 11 patients with HIV associated Hodgkin's disease were male and intravenous drug misusers. The degree of infiltration by HRS cells in the group of patients for which DNA extraction was possible was as follows: eight of the ordinary Hodgkin's disease cases contained numerous HRS cells (5·25%) and the other 17 showed a weak infiltration (<5%); in the HIV associated Hodgkin's disease cases, three samples were heavily infiltrated (>25%), seven contained numerous HRS cells and one showed a weak infiltration.

IMMUNOHISTOCHEMISTRY
Immunohistochemical analysis was performed on routinely processed paraffin wax sections with the CS1-4 monoclonal antibody (Dako, Glostrup, Denmark) and standard alkaline phosphatase–antialkaline phosphatase methods. Paraffin wax sections were pretreated in a microwave oven to enhance antigen retrieval. LMP-1 expression was restricted to HRS cells.

DNA ISOLATION AND PCR AMPLIFICATION
Total genomic DNA was extracted from frozen lymph node biopsy specimens following as described previously.[25]

EBV typing was performed by amplifying DNA from the EBV 3C nuclear antigen

(EBNA-3C). Amplification of type A EBV DNA gave rise to a 153 bp fragment and type B EBV DNAs to a 246 bp product. Primers and PCR conditions are described elsewhere.[26]

The LMP-1 gene was amplified using different primer pairs specific for the N terminal, transmembrane and C terminal domains (for detailed information about PCR conditions and primer sequences see [27]). Each PCR product (5 μl) was electrophoresed on a 6% polyacrylamide gel for one hour at 150 volts at room temperature to check the amount, specificity, and size of the PCR product. DNA extracted from the B95-8 and AG876 cell lines was used to control for types A and B, respectively, EBV. The AG876 LMP-1 gene contains the same 30 bp deletion (amino acids 343 to 352 of the B95-8 sequence) reported in some EBV positive Hodgkin's tumours.[21 22 28 29]

Results
IMMUNOHISTOCHEMISTRY
The LMP-1 protein was detected within HRS cells in 106 (57%) of 186 cases of ordinary Hodgkin's disease. In all 31 cases of HIV associated Hodgkin's disease HRS cells exhibited a strong, well defined signal for LMP-1: 50–90% of HRS cells were positive in each case. This extensive expression of LMP-1 in tumour cells in patients with HIV associated Hodgkin's disease has been reported previously.[30]

ANALYSIS OF LMP-1 GENE
Amplification of the DNA region coding for the N terminal and the three transmembrane domains was unsuccessful in six of 25 patients with ordinary Hodgkin's disease and in one of 11 cases of HIV associated Hodgkin's disease. Amplification products of the expected size were detected in the remaining cases. Lack of successful amplification was probably because of polymorphisms or deletions at primer region 1 (N terminal region of LMP-1).

Analysis of the last intracytoplasmic region of LMP-1 was of particular interest. Amplification of the region containing the 33 bp repeat element displayed considerable variation in the size of the PCR product among different samples: 15 of 25 cases of ordinary Hodgkin's disease and six of 11 cases of HIV associated Hodgkin's disease had longer amplification products than expected, whereas shorter products were obtained in two ordinary and three HIV associated Hodgkin's disease cases. The remaining samples gave rise to specific PCR products (fig 1). In each patient a single band was present indicating the presence of clonal EBV DNA.[28]

When the last intracytoplasmic portion was amplified no product was found in four of 25 cases of ordinary Hodgkin's disease. In the remaining 21 cases a product of 286 bp resulting from the presence of the 30 bp deletion was obtained in six (28·6%) cases. In patient HD66 a smaller PCR product was obtained, indicating a longer delection (fig 2). Interestingly, in the seven patients with ordinary Hodgkin's disease harbouring the LMP-1 de-

Table 1 *Clinical findings in patients with ordinary Hodgkin's disease carrying deletions in the C terminal region of the LMP-1 gene*

Case No.	Sex/age (years)	Stage	Type	% HRS cells	EBV type	Current status
14	M/5	I·A	NS-I	<5	B	Alive
54	M/50	III·B	MC	5–25	A	Relapse with LCAL
66	F/56	III·B	MC	5–25	A	Died
133	M/43	II·B	MC	<5	A	Died
178	M/22	III·B	MC	5–25	A	Alive
179	M/71	III·B	NS	<5	A	Died
182	M/30	II·A	NS-I	<5	B	Alive

MC = mixed cellularity; NS = nodular sclerosis; LCAL = large cell anaplastic lymphoma. Case 66 had a deletion more than 30 bp long.

Table 2 *Clinical findings in patients with HIV associated Hodgkin's disease*

Case No.	Origin	Stage	Type	% HRS cells	EBV type	Current status
1	Madrid	IV·B	MC	<5	A	Lost to follow up
2	Madrid	III·B	NS-II	5–25	B	Died
3	Madrid	III·B	NS-II	5–25	A	Died
4	Madrid	IV·B	MC	<5	A	Alive
5	Madrid	IV·B	LD	>25	B	Died
6	Barcelona	III·B	MC	5–25	B	Died
7	Barcelona	IV·B	LD	>25	A	Alive
8	Barcelona	III·B	MC	5–25	A	Lost to follow up
9	Barcelona	IV·B	MC	5–25	B	Lost to follow up
10	Barcelona	III·B	nc	>25	B	Lost to follow up

All the patients were male and intravenous drug misusers. The 30 bp deletion was present in all patients. MC = mixed cellularity; NS = nodular sclerosis; LD = lymphocyte depletion; nc = not classifiable.

Figure 1 Variation in the size of the LMP-1 region containing the repeat element. PCR was used to amplify genomic DNA extracted from fresh, frozen tissue samples. PCR products were separated by electrophoresis on 6% polyacrylamide, ethidium bromide stained gels. Lanes 1 to 4, cases 1, 2, 3, and 4 (HIV associated Hodgkin's disease); lane 5, B95-8; lane 6, case 5 (HIV associated Hodgkin's disease); lane 7, case 54 (Hodgkin's disease); lane 8, case 66 (Hodgkin's disease); lane 9, PCR reaction without DNA.

letion mutants immunohistochemical analysis revealed strong labelling, with 50–90% of HRS cells exhibiting a positive reaction. In the HIV associated Hodgkin's disease group, amplification was unsuccessful in one case and the remaining 10 cases carried the 30 bp deletion (fig 2). Clinical findings in patients carrying LMP-1 deletions are presented in tables 1 and 2.

EBNA-3C TYPING

In the group of patients carrying LMP-1 deletions, five of seven cases of ordinary Hodgkin's disease and five of 10 cases of HIV associated Hodgkin's disease gave rise to a 153 bp product from the type A EBNA-3C gene; a 246 bp fragment from type B EBNA-3C was amplified in the remaining two cases of ordinary Hodgkin's disease and five of HIV associated Hodgkin's disease (tables 1 and 2).

Discussion

Analysis of the LMP-1 gene in EBV positive Hodgkin's tumours has revealed existence of deletions near the C terminal end of the protein, which seem to be associated with aggressive disease.[21 22] These deletions, corresponding to amino acids 343 to 352 of the prototype B95-8 EBV strain, were also detected in the AG876 Burkitt's lymphoma cell line[28 29] and in the tumorigenic nasopharyngeal carcinoma cell line CAO.[31] The frequency of the 30 bp deletion in the Spanish cases of ordinary Hodgkin's disease (28·6%) is similar to that reported by Sandvej et al[22] in Danish patients with Hodgkin's disease. It is of interest that in this study this deletion was found in all cases of HIV associated Hodgkin's disease. Moreover, we found a statistically significant difference (p<0·001) in the percentage of cases of HIV associated Hodgkin's disease with deletions in the EBV LMP-1 oncogene compared with HD occurring in immunocompetent subjects.

No association has been found between the presence of these deletions and the EBV subtype. In the present study deletions were detected more frequently in type A isolates because most of the cases of ordinary Hodgkin's disease belonged to this EBV subtype. However, type B EBV is found more frequently in HIV associated Hodgkin's disease (50% of cases). The association between type B disease and compromised immunity has been described elsewhere.[32]

The data presented here confirm the previously reported relation between partial deletions at the C terminal region of the LMP-1 gene and aggressive behaviour in Hodgkin's disease: most of our patients carrying LMP-1 deletions presented with advanced clinical disease at diagnosis, belonged to unfavorable histological subtypes, and contained numerous (5–25%) HRS cells or were heavily infiltrated (>25% in three cases of HIV associated Hodgkin's disease cases). This features of aggressiveness are greater in the cases of HIV associated Hodgkin's disease (table 2). It is well known that Hodgkin's disease is more aggressive, with a poor response to therapy and
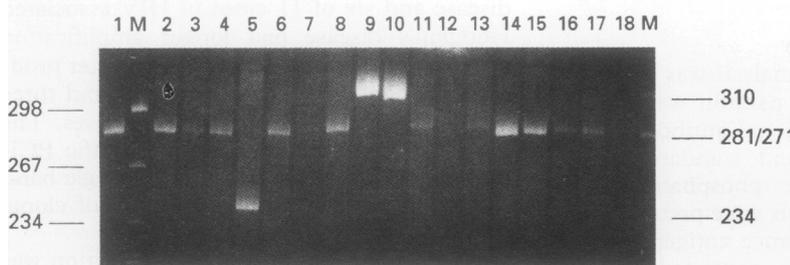
Figure 2 Detection of the 30 bp deletion at the C terminal region of the LMP-1 gene. In isolates containing a deletion between amino acids 343 and 352 a 286 base pair band was detected. In isolates containing these amino acids a 316 bp fragment was detected. The AG876 cell line was used to control for the presence of the 30 bp deletion. Lane 1, AG876; lanes 2 to 4, cases 1, 2, and 3 (HIV associated Hodgkin's disease); lanes 5 to 7, cases 66, 133 and 178 (Hodgkin's disease); lane 8, case 4 (HIV associated Hodgkin's disease); lane 9, B95-8; lane 10, case 150 (Hodgkin's disease); lanes 11 to 17, cases 5, 11, 6, 7, 8, 9, and 10 (HIV associated Hodgkin's disease); lane 18, PCR reaction without DNA; M, molecular weight marker. The prototype B95-8 (lane 9) and case 150 (lane 10) gave a 316 bp fragment. Case 66 (lane 5) carried a deletion longer than 30 bp and case 11 (lane 12) did not amplify.

bad prognosis in patients with HIV infection compared with that in HIV negative patients.[33 34]

Transformation assays with deletion mutants have demonstrated that the sequence between amino acid 334 and 364 is required for rapid protein turnover.[35] Deletions located in this region may affect the turnover of the LMP-1 protein prolonging its half-life and, therefore, promoting a higher level of cellular expression. This hypothesis may explain the strong, well defined signal obtained on immunohistochemistry in patients carrying the deletion mutants.

Although some authors suggest that forms of LMP-1 containing these deletions lack the immunogenic epitopes present in wild-type LMP-1 and therefore escape HLA restricted cytotoxic T lymphocytes;[36] however, we think that these highly expressed forms of LMP-1 conserve their immunogenic properties and that unrestricted growth of malignant cells infected by EBV LMP-1 deletion mutants occurs mainly in immunodeficient patients. This may explain the presence of deletions in all cases of HIV associated Hodgkin's disease together with the high proportion of HRS cells (50–90%) expressing the protein in patients with LMP-1 deletions. The need for impaired immune function does not preclude the existence of deletions in cases of ordinary Hodgkin's disease. The immunological derangement exhibited by untreated patients with Hodgkin's disease is widely documented.[37]

In conclusion, EBV strains containing partial deletions in the C terminal region of the LMP-1 gene may play an important role in the pathogenesis of HIV associated Hodgkin's disease. Furthermore, these deletions could be related to the more aggressive course of Hodgkin's disease in immunodificient patients. Future efforts should be directed towards elucidating whether EBV strains carrying deletions in the LMP-1 gene are important aetiological agents in the development of lymphoproliferative disorders in immunocompromised patients.

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