Epstein–Barr Virus Distribution in Hodgkin's Disease in an Unselected Swedish Population

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All patients with Hodgkin's disease (HD) (n = 117) identified in the Uppsala/Örebro region of Sweden between 1985 and 1988 were examined for the presence of Epstein–Barr virus (EBV) in the Hodgkin and Reed–Sternberg (HRS) cells. EBV was detected with LMP-1 immunostaining and in situ hybridization for EBERs. Overall, 32 (27%) tumours were EBV-positive but there were significant differences in EBV-positivity between histopathological subgroups (p = 0.03). In MC, 8/21 (38%) were positive, in NS 20/67 (23%), LD 3/3, LP 1/5, and in unclassified 0/1. Patients with EBV-positive tumours were significantly older, mean 52 vs. 42 years (p = 0.02), and were likely to have significantly more B-symptoms or advanced stage disease. Patients with EBV-positive tumours tended to have a poorer survival rate (p = 0.11). The proportion of EBV-positive tumours, and especially the proportion of EBV-positive MC, was lower than previously reported. This could be explained by selection of patients from previous studies, or by differences in EBV-positivity in different geographical or ethnic populations of HD.

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Epstein–Barr virus (EBV) has been implicated in the pathogenesis of Hodgkin's disease (HD) mainly because of seroepidemiological studies (1–6). It has also been possible to demonstrate the presence of EBV DNA in the Hodgkin and Reed Sternberg (HRS) cells, the tumour cells of HD, in 15–80% of the cases (4–10). The EBV in HD tumours has been shown to be monoclonal, but the role of EBV in the pathogenesis of HD is still unclear (9).

The proportion of EBV-positive cases varies between published materials. A possible explanation could be variation in the adopted technique for detection of EBV, but more likely, the selection of the included patients influences the results. The proportion of positive cases has been reported to vary between age groups, geographical regions or ethnic groups, and histopathological subgroups of HD (4, 5, 10). A higher proportion of EBV-positive HD has been found among children and elderly patients, whereas young adults are usually EBV negative (4, 5). In the Western populations, the proportion of EBV-positive HD usually ranges between 30 and 50%, whereas this proportion is higher in Asia and in Latin America (4, 5). A higher association with the mixed cellularity subgroup (MC) has also been reported (4–10). In one study, patients with LMP-1 negative tumours had a poorer survival prognosis (11) but otherwise, no association between EBV-positivity and the clinical course has been described (6, 8).

The proportion of EBV-positive HD in an unselected population has, to our knowledge, not been described. The aim of the present study was to determine the proportion of EBV-positive HD in a defined geographical region and to correlate the findings to clinical parameters.

MATERIAL AND METHODS

Patients

All patients with HD diagnosed between 1985 and 1988 in the Uppsala–Örebro healthcare region in Sweden were traced from the Swedish National Cancer Registry and The National Health Care programme for HD, as previously described (12). After initial re-evaluation of the slides in the late 1980s, 160 patients, including 14 patients diagnosed at autopsy, were accepted as HD cases. In the present study autopsy cases were excluded, since they represent a selection of patients deriving from a relatively low autopsy frequency. All histopathological material was re-examined by two of the authors (GP and CS) using the

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Rye-classification (13) with complementary immunohistochemistry. Discrepancies between the pathologists were resolved by consensus when viewing the slides together. Twenty-two cases were then excluded because of diagnosis of non-Hodgkin's lymphoma (NHL), and 7 cases were also excluded, because of insufficient material, leaving 117 cases to be studied (see Table 1 for a description of the clinical characteristics). The mean age was 45 years (11–87). Stage at diagnosis was defined according to the Ann Arbor classification (14). Complete remission (CR) was defined as absence of all signs of disease on clinical and radiological investigation. The median follow-up for all living patients was 130 months (range 106–153) and none of the patients was lost to follow-up.

Treatment

All patients were treated in accordance with a National Health Care programme for HD (15). Briefly, the treatment was as follows: Adult patients (16–60 years) in clinical (CS) and pathological (PS) stages IA and PS IIA were treated with radiotherapy only (mantle field or inverted Y-field, 40 Gy). Patients with CS IIA, and in more advanced stages of disease were treated with 6–8 courses of chemotherapy, mainly MOPP/ABVD followed by involved field radiotherapy to bulky sites. Patients with bulky disease in stages CS and PS IA and PS IIA and some patients in PS IIB received two courses of chemotherapy before radiotherapy (mantle field or inverted Y-field, 40 Gy). Patients < 16 years were mainly treated with chemotherapy regardless of stage. For patients > 60 years of age, the treatment recommendations were basically the same as those for the younger adults, but it was seldom possible to give the treatment to a sufficient level, mainly because of toxicity (16).

Detection of EBV in tumour specimens

Immunostaining for latent membrane protein-1 (LMP-1) was performed in all cases. In cases with negative LMP staining, in situ hybridization (ISH) for EBV early RNAs (EBERs) was performed. In three cases where the initial LMP-1 staining was negative and EBER ISH was positive, the LMP-1 staining was carried out again, with a positive result.

LMP-1 staining. The paraffin-embedded tissue was sectioned (3 μm) and deparaffinized. A cocktail of commercially available (Dakopatts, Copenhagen, Denmark) monoclonal antibodies (clones CS. 1–4) reactive with the EBV encoded LMP-1 was used. After microwave pretreatment, the sections were incubated with the antibodies and stained using the immunoperoxidase technique (17).

EBER-1 in situ hybridization. EBER-1 was detected with RNA/RNA ISH using the single-stranded digoxigenin-labelled riboprobes complementary (anti-sense probe) to EBER-1 RNA transcripts. Probes were generated by *in vitro* transcription from a transcription vector containing an EBER-1 specific insert probe preparation. The ISH procedure has been described elsewhere (18). Paraffin-embedded pellets of EBV-infected lymphoid cell lines (Raji), EBV-positive AIDS-related NHL or Danish HD cases served as positive controls for EBER ISH.

Statistical methods

In order to compare differences in proportions, χ^2-square analyses were carried out and an unpaired two-tailed Student's t-test was undertaken to calculate differences in mean ages. Survival analyses with the log-rank significance test and uni- and multivariate analyses with the Cox proportional hazard model were performed. Disease-free survival (DFS), HD-specific and overall survival were analysed. DFS was defined as freedom from any recurrence, where patients who never reached a CR had a DFS of zero months. HD-specific survival was defined as patients dead from or with HD, whereas patients who died from other causes without any evidence of HD were censored after their deaths.

RESULTS

EBV in relation to clinical characteristics

Overall, 32 (27%) of the 117 patients had EBV-positive tumours. The results of the EBV-detection in relation to clinical characteristics are presented in Table 1. EBV-positivity was significantly more common in patients with B-symptoms and advanced-stage disease.

EBV in relation to different age groups

The mean age of the patients with EBV-positive tumours was 52 years as compared with 42 years for the EBV-

Table 1

Clinical characteristics in relation to EBV status of the tumours

Clinical characteristic	Total number	EBV+(%)	p-value
Male	66	21 (32)	0.2
Female	51	11 (22)	
Stage I	23	4 (17)	
Stage II	39	10 (25)	0.5
Stage III	31	10 (32)	
Stage IV	23	8 (33)	
unknown	1	0	
Stage IA+IIA	46	7 (15)	
Stage IIB–IV	70	25 (36)	<0.01
Unknown	1	0	
A-symptoms	66	12 (18)	<0.01
B-symptoms	50	20 (40)	
Unknown	1	0	
Bulky disease	29	6 (21)	0.3
Non-bulky disease	87	26 (30)	
Unknown	1	0	
Total	117	32 (27)	

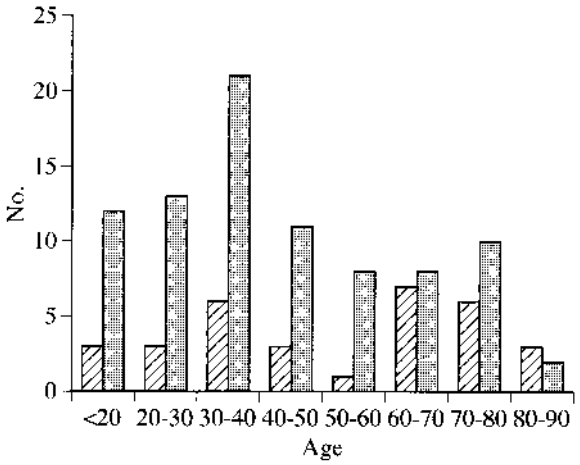

Fig. 1. Age distribution of EBV-positive (▨) and EBV-negative cases (□).

negative patients ($p = 0.02$). The numbers of EBV-positive patients in different age intervals are presented in Fig. 1. Both EBV-positive and EBV-negative patients appeared to show a bimodal age distribution. The numbers of EBV-positive tumours in the MC and NS subgroups in different age groups are presented in Fig. 2a and b.

EBV in relation to histopathological subgroup

There was a statistically significant difference in EBV positivity between histological subgroups. In MC 8/23 (38%) were positive, in NS 20/87 (23%), in LD 3/3 and in LP 1/5 ($p = 0.03$). When the NS and MC groups were analysed separately, it appeared that the differences in clinical characteristics between EBV-positive and EBV-negative cases were only present in NS HD (Table 2). The number of patients with MC was, however, low. For example, EBV positivity in the NS group was significantly more common in advanced stages, in patients with B-symptoms and in elderly patients (Table 2).None of the patients with NS histology in stages IA or IIA below the age of 60 years was EBV-positive.

Survival analyses

Patients with EBV-positive tumours had a poorer survival, but the difference was not statistically significant (DFS: $p = 0.17$, HD-specific survival: $p = 0.10$, overall survival: $p = 0.11$) (Fig. 3a). When the survival was analysed separately for patients above and below the age of 60 years, it seemed that the survival difference was only present in the elderly age group, although no statistically significant difference was seen even in that group (Fig. 3b and c). A multivariate analysis of HD-specific survival for all patients was performed for the following factors: EBV status, stage (IA-IIA vs. IIB–IV), histology (NS vs. MC), age, gender and bulky disease. Age was then the only significant factor ($p = 0.01$).

DISCUSSION

Hodgkin's disease is characterized by a bimodal age distribution and three distinctive epidemiological patterns of HD have been proposed (5, 19). One pattern with a high incidence of HD, mainly MC, in children in developing countries, one with a high incidence of NS in young adults in countries with high economic development and a third, again with mainly MC with increasing incidence in higher age groups (4, 5). These epidemiological patterns correspond to different pattern of EBV-positivity of the tumours in these groups. A high proportion of EBV-positive HD has been described in children, especially in Latin America but also in Western populations (4, 5). HD in children is often of MC histology, but in children also NS is often EBV-positive (4, 5). In the

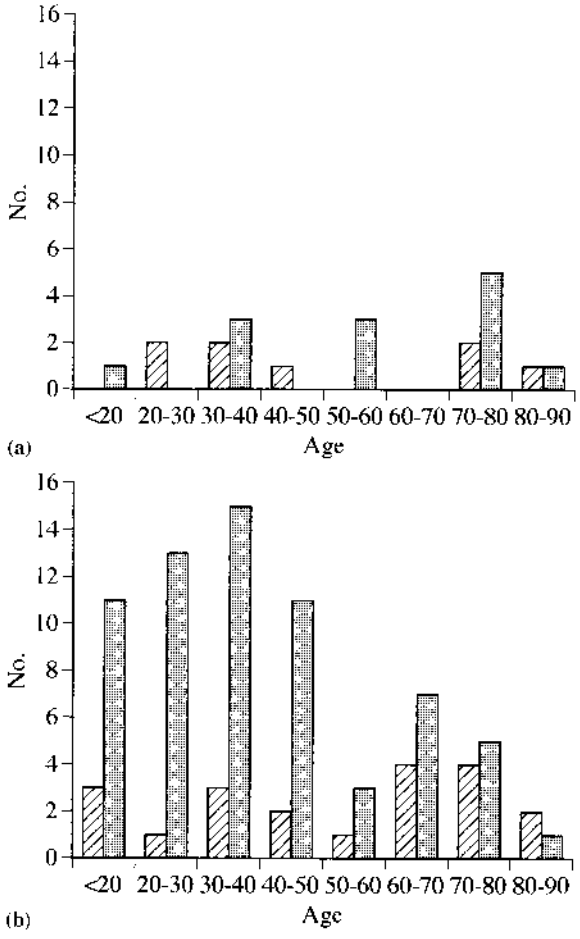

Fig. 2. a: Age distribution of EBV-positive and EBV-negative MC cases. ▨ MC EBV-positive. MC EBV-negative. b: Age distribution of EBV-positive and EBV-negative NS cases. ▨ NS EBV-positive. NS EBV-negative.

Table 2

The distribution of EBV-positive cases in the NS and MC subgroups

	NS			MC		
	Total No.	EBV+(%)	p-value	Total No.	EBV+(%)	p-value
St IA-IIA	30	2 (7)		10	3 (30)	
St IIB-IV	56	18 (32)	<0.01	11	5 (45)	0.46
A-symptoms	66	26 (39)		15	5 (33)	
B-symptoms	20	15 (75)	<0.01	6	3 (50)	0.48
< 60 years	64	10 (16)		12	5 (42)	
≥ 60 years	23	10 (43)	<0.01	9	3 (33)	0.69
Men	46	13 (28)		15	7 (47)	
Women	41	7 (17)	0.20	6	1 (17)	0.20
St IA–IIA, <60 years	23	0		5	2	
St IIB–IV, <60 years	41	10 (24)	<0.01	7	3	0.90
Men<60 years	26	8 (24)		10	5 (50)	
Women<60 years	30	2 (7)	0.06	2	0	0.19

Western adult population, the second proposed epidemiological HD entity dominated by NS histology has a low proportion of EBV-positive tumours, but the proportion of EBV-positive MC is also lower (4, 5). A higher proportion of EBV-positivity is then again seen in the older adults.

In our population-based study the proportion of elderly HD patients is greater than that in any other previously published studies (4, 5). The mean age was 45 years compared with below 40 years and often below 35 years in most other studies. Patients of over 60 years constitute 31% of the present material. The high mean age is of course also influenced by the fact that HD is extremely rare in children in Sweden. The youngest patient in this material was 11 years old. In the light of previous studies (4, 5) one would thus expect a high proportion of EBV-positivity if the epidemiological pattern of EBV in HD in the Western population was applicable to the Swedish cases. In contrast, the proportion of EBV-positive HD and especially MC was lower than in most other studies. It is unlikely that this is due to technical reasons or differences in classification, since one of the authors (GP) has studied EBV in many different HD materials using the same technique and strict application of the classification. An alternative, and very likely explanation, is that there are geographical differences in the number of EBV-positive HD also within the Western population. The geographical distribution of EBV-positive HD should be further investigated in unselected materials in order to clarify this issue.

There were significant differences in EBV-positivity in different clinical presentations of HD. This has, to our knowledge, not previously been described and raises questions about the role of EBV in the pathogenesis of HD. For example, a comparably large group of patients could be identified where no case was EBV-positive: the patients below 60 years of age in low stages with a NS histology. If this is a true phenomenon, it contradicts the hypothesis that EBV is involved in the aetiology of NS in the younger patients, at least in low-stage disease. The difference in EBV-positivity between patients in low and advanced stages and with or without B-symptoms was more pronounced in the NS group, but the number of MC cases was low in our study. The differences in these respects between NS and MC patients must therefore be interpreted with caution. A possible explanation for a higher EBV-positivity in advanced-stage disease could be that the previously described immune deficiency in HD (20) is more pronounced in patients in the advanced stages. A pre-existing immune deficiency might favour EBV-induced HD, which is also apparent in HIV-infected patients who frequently develop EBV-positive HD, often MC or LD (21). Whether this is a relevant parallel remains to be investigated. This hypothesis is contradicted by the finding that even though EBV-positive NS patients generally had a more advanced disease, they did not have a poorer survival. The influence of EBV on the survival of HD in this material seems complex. On the one hand, EBV is related to older age and presumably by the age relation to a poorer prognosis. On the other hand, younger HD patients with EBV-positive HD have a more advanced disease but not a poorer prognosis. This material is too small to solve the issue and larger materials need to be studied. A relationship between EBV-positivity and a better survival has previously been shown in one study (11) in HD and in one study for LMP-1 expression in nasopharyngeal carcinoma (22).

We conclude that the number of patients with EBV-positive HD in this population-based study was lower than in most studies, especially for MC. There were also patterns of EBV-association correlated to different clinical presentations, i.e. EBV-positive patients were older, were usually in the advanced stages of disease, and frequently had B-symptoms.

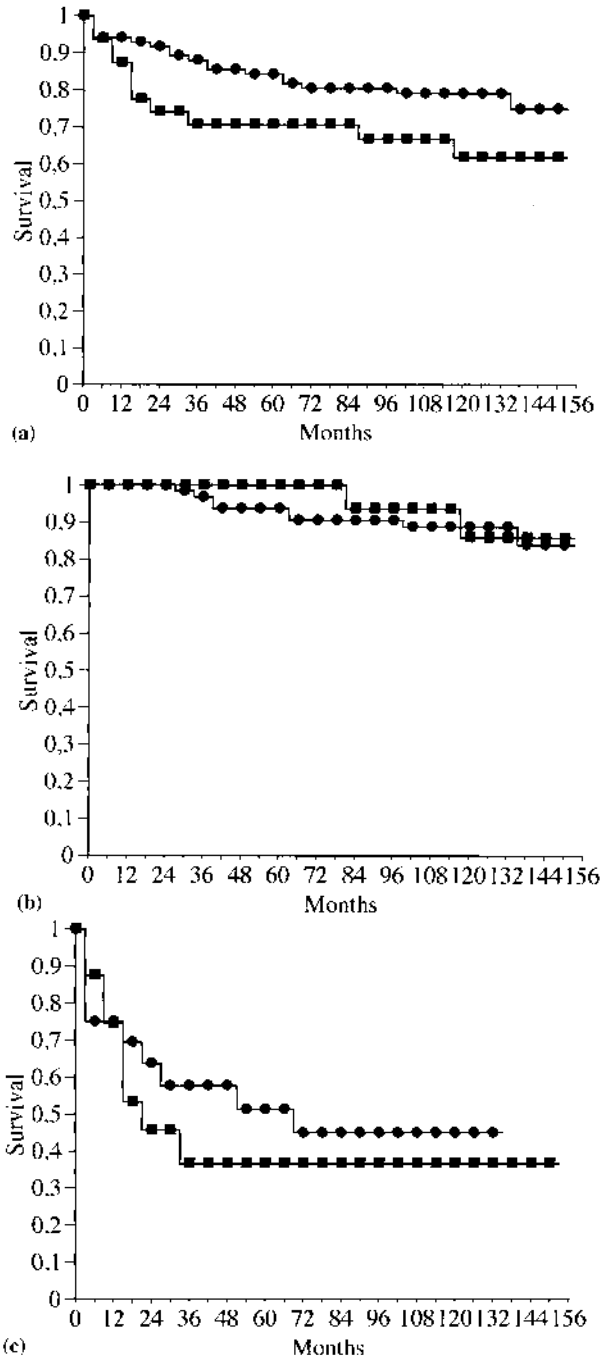

Fig. 3. a: HD-specific survival of EBV-positive (■) and EBV-negative patients (●); b: HD-specific survival of EBV-positive (■) and EBV-negative (●) patients below the age of 60 years; c: HD-specific survival of EBV-positive (■) and EBV-negative (●) patients above the age of 60 years.

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