VIRUSES AND LYMPHOMAS

Epstein–Barr virus-associated tumours: an update for the attention of the working pathologist

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Epstein–Barr virus (EBV) is a herpesvirus associated with approximately 1% of tumours worldwide. EBV is the epitome of B lymphotropic viruses, but the spectrum of tumours it is associated with extends to T lymphocyte and NK cell malignancies, various types of carcinomas and smooth muscle tumours. Ubiquitous EBV infection in humans implies that most individuals carry EBV-infected cells. Therefore, mere detection of the virus in individuals with a tumour is not sufficient for establishing a causal relationship between both events, but instead requires unequivocal detection of viral nucleic acids or viral proteins in the tumour cells. Recent controversies about EBV infection in several carcinomas mainly resulted from such technical issues. The gold standard remains in situ EBER detection, but detection of EBNA1 would be an interesting alternative. EBV detection can be helpful for diagnostic, prognostic and therapeutic purposes. The rate of EBV association with entities such as NK/T cell tumours of the nasal type is so high that absence of detection of the virus in such a lesion should cast doubt of the accuracy of the diagnosis. Similarly, diagnosis of EBV-associated follicular pseudo-tumour obviously requires detection of the virus. EBV-positive common gastric adenocarcinomas seem to have a better prognosis than their EBV-negative counterparts and identification of the virus in B cell lymphoproliferations in immunocompromised individuals will guide therapeutic options. In conclusion, EBV-associated tumours are common enough to be relevant for the pathologist in everyday practice, but there is a need to facilitate detection of the virus (eg EBNA1 antibody).

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Epstein–Barr virus (EBV) was discovered in 1964 in African Burkitt lymphoma cell cultures (see Rickinson *et al*[1] for a review). The virus was soon recognised as one of the aetiologies of infectious mononucleosis syndromes and has over the years been found to infect an ever-increasing list of lesions including tumours of the haematopoietic system, carcinomas and even smooth muscle tumours. This led the World Health Organization to classify EBV in 1997 as a tumour virus.[2] EBV infects the large majority of the human population in which the virus establishes a clinically silent lifelong infection.[1] This implies that EBV is a weak transforming agent under physiological circumstances but it would be short-sighted to dismiss it as a mere ''cell passenger''. We know, for example, that less than 1% of women infected with transforming strains of human papillomaviruses will go on to develop cervical cancer, no doubt is cast on their responsibility in the development of this tumour.[3] Defining why certain individuals are susceptible to EBV-mediated transformation remains a central question, even if some conditions such as immunosuppression have long been recognised as a major risk factor.[1] Here we review recent developments in the EBV field. Our objective is to provide an update on the tumours causally linked with the virus and to define the role of EBV detection in the day-to-day practice of working pathologists. Obviously we do not make any pretence of being exhaustive in this short contribution and wish to refer to recent reviews for a more comprehensive treatment of the subject.[1 4]

BACKGROUND

EBV belongs, along with Kaposi-associated virus (HHV8), to the human gamma-herpesvirus subfamily. On infection of its target cells, EBV can undergo lytic replication during which virus progeny are released; or instead it may initiate active latency, a restricted gene expression programme limited to certain members of the Epstein–Barr nuclear antigen (EBNA) and latent membrane protein (LMP) gene families and to the two Epstein–Barr small non-coding RNAs (EBER1 and 2). Three latency types have been described depending on which of these latent genes are expressed.[1] Latency I is limited to EBER and EBNA1 expression; latency II includes LMP1 and 2 in addition; and latency III is defined by expression of EBER, all six EBNA proteins and two LMP proteins. Frontiers between latency types tend to be blurred now that several EBV-positive tumour types have been found to display a viral expression pattern that does not fall into any of these three categories (see below).

Primary EBV infection mainly takes place in the oropharyngeal region to which the virus is conveyed by saliva droplets from infected individuals.[1] The nature of the target cells in the oral mucosa is still controversial, but there is agreement that B cells are infected at some stage of the process. If infection is delayed to adolescence or adulthood, it can cause an infectious mononucleosis syndrome (IM), a self-resolving lymphoid disorder that is thought to result from an uncontrolled T cell reaction directed against EBV infected cells.[1] During IM, EBV is exclusively found in B blasts that undergo continuous proliferation under the influence of all latent genes (latency III).[1] EBV induces continuous proliferation of infected B cells in vitro which allows establishment of B cell

lines from a large panel of individuals. Following resolution of primary infection, the virus establishes a lifelong persistence in memory B cells that usually remains clinically silent.[1 5] In this B cell reservoir, viral expression is entirely repressed, a process described as a passive latency or no latency.[1 5]

EBV target cells

The pleiotropic nature of EBV target cells in vivo, including B and T lymphocytes, NK cells, squamous and glandular epithelia, and smooth muscle cells demonstrates that the virus possesses a very large infectious spectrum.[1] However, and with the notable exception of B cells, primary cells and cells lines derived from these cell lineages appear to be paradoxically resistant to virus infection in vitro.[1] If one makes the assumption that the EBV-positive tumours are actually derived from normal cellular counterparts belonging to the cell lineage, this either suggests that in vitro culture conditions are much too crude to allow proper infection, or that the virus accesses its target cells following a more complex path than direct virus infection. Several models have recently been put forward to resolve these issues. Three of these propose that B cells, monocytes or dendritic cells are first infected by the virus, after which these infected cells can establish contact with target epithelial cells.[6–8] A fourth model suggests that the cell lineage of the producer cell line has a significant influence and that viruses produced in the 293 cell line can directly infect primary epithelial cells with high efficiency.[9]

Diagnostic tools

EBV infection can be evidenced by detection of virus-specific nucleic acids. EBERs are expressed at a very high level in infected cells and are therefore easily detected by RNA in situ hybridisation. Viral DNA can also in principle be identified by in situ hybridisation, but this method is much less sensitive than EBER detection because the viral DNA copy number is lower than the EBER copy number by several of orders of magnitude. There are commercially available antibodies against EBNA1, EBNA2 (PE2 clone, Dako) and LMP1 (CS1-4 clone, Dako or S12 clone, BD Pharmingen) that are functional in fixed tissues. The sensitivity of the EBNA1 antibody (1H4 clone) is, however, too limited to be recommended for diagnostic purposes (G Niedobitek, personal communication).

EBV-ASSOCIATED DISEASES

Infectious mononucleosis

EBV is one of a variety of infectious agents that can induce a mononucleosis syndrome. Although it extends beyond the scope of this review, IM remains nevertheless worth mentioning as a source of diagnostic pitfalls, particularly when it arises in older adults, manifesting as an isolated enlarged lymph node.[10] IM may be misdiagnosed as a high-grade large B cell lymphoma, or even Hodgkin lymphoma, particularly in cases containing a large number of B immunoblasts, some of which express CD30, and rare Reed–Sternberg like cells.[10] EBV infected B cells in IM show a typical latency III expression pattern including EBER, EBNA1, EBNA2 and LMP1 which renders virus detection very easy[1] (table 1). The mere presence of EBV does not exclude a large B cell lymphoma, but detection of EBNA2 expression in infected cells strongly favours the diagnosis of IM.[10] Performance of EBV-specific serological studies will, however, establish the proper diagnosis.

EBV-associated tumours in immunosuppressed individuals

The role of the immune system in controlling EBV latent infection is best illustrated by the observation that individuals with a congenital or acquired (eg after HIV infection or organ transplantation) immune deficiency are at increased risk for the development of EBV-associated diseases, the histological type of which varies according to the type of immunodeficiency. Obviously, the clinical context is more suggestive for the diagnosis of this category of tumours than the detection of EBV. However, assessing viral status can have important consequences for treatment as EBV positive-lesions are in principle amenable to EBV-specific T-cell therapy (although this therapy is still limited to a few specialised centres worldwide) or even to anti-viral therapy.[1]

Post-transplant lymphoproliferative disorders

Haemopoietic lesions that arise in transplant recipients are grouped under the generic name of post-transplant lymphoproliferative disorders (PTLD). Obviously, transplant recipients can develop lymphomas unrelated to their condition but it is still unclear which percentage of EBV-negative lymphomas arising in transplant recipients are PTLD. However, the observation that some of the tumours regress on withdrawal of immunosuppressive regimens certainly supports the idea that EBV-negative PTLD exist. Analysis of EBV-positive PTLD has recently shown that many PTLD derive from ''forbidden'' B cells that do not carry surface immunoglobulins as a result of crippling mutations in the immunoglobulin receptor.[11 12] This establishes a parallel with Hodgkin disease and angioimmunoblastic T cell lymphoma (AITL), in which similar observations were made, and underlines the high sensitivity of these abnormal B cells to transformation on EBV infection.[1] PTLD are classically of B cell origin, but recent work has established that 7–15% of them belong to the T cell and NK lineages.[13] The rate of EBV association is much higher in B cell PTLD (80% in late onset lesions, 100% in early onset lesions) than in non B-cell tumours (37%) (reviewed in Swerdlow[13]). Although classical early onset EBV-positive B cell PTLD shows a latency III expression pattern, more restricted types of latencies have also been described.[14]

HIV-associated lymphomas

HIV infection predisposes to a large number of haemopoietic malignancies that only partially overlap with PTLDs. In addition to diffuse large B cell lymphomas, HIV-infected patients frequently develop Burkitt lymphomas that are associated with EBV in 40% of cases. Primary effusion lymphomas, Hodgkin lymphomas, and anaplastic large cell lymphomas have also been described. Plasmablastic lymphoma (PBL), a more recently individualised entity, was initially described in the oral cavity[15] as a tumour that mainly arose in HIV-infected individuals. Since its initial description, more than 100 cases of PBL have been published, two-thirds of which involved the oral mucosa.[16–22] PBL are therefore not restricted to the oral cavity, but can instead be observed nearly everywhere with a possible predilection for skin, bone marrow and lower gastrointestinal tract. Subsequent reports confirmed tight association of PBL with immunodeficiencies and in particular with HIV infection. Nearly three-quarters of PBL are EBER positive, but only a minority of those that carry the virus express LMP1.[15]

Hairy leukoplakia

This benign lesion of the tongue is typically encountered in HIV-infected individuals and is clinically and histologically characterised by hyperplasia and hyperkeratosis of the tongue squamous epithelium. EBV infection is located in the upper layers of the affected epithelium and leads to an active viral lytic replication. These characteristics, viral tropism for differentiated epithelial squamous cells and spontaneous EBV replication in infected cells, reflect intrinsic properties of virus–cell interactions as recently described in an in vitro model using primary epithelial cultures.[9]

Table 1 Entities for which Epstein–Barr virus (EBV) detection is of diagnostic value

	EBER1* (%)	LMP1† (%)	EBNA2† (%)
Infectious mononucleosis	100	100	100
Smooth muscle tumour	100	0	100
Gastric lymphoepithelial carcinoma	100	weak and inconstant	0
Gastric adenocarcinoma	5–15	0	0
Hodgkin lymphoma	40–60	100	0
Lymphomatoid granulomatosis (B type)	90	100	66
Angioimmunoblastic T cell lymphoma	81–95	60	0
Richter syndrome	15–20	90	NA
NK leukaemia	100	NA	NA
Nasal type T/NK	100	100 (weak and focal)	0
Liver follicular dendritic cells	100	100 (weak and focal)	0

The results given here are only indicative as they are sometimes based on the analysis of a small number of cases. NA, data not available.
*The percentage of tumours belonging to a given entity that express EBV EBER is indicated.
†The percentage of EBV-positive tumours that express LMP1 or EBNA2 is indicated.

Smooth muscle tumours

EBV-positive smooth muscle tumour (SMT) is a rare lesion and was initially described in an organ transplant recipient.[23] SMT have so far been exclusively reported in patients with congenital or acquired immune deficiencies. Consistent EBV infection in SMT developed by organ transplant recipients and HIV-infected children was demonstrated two decades later.[24 25] SMT are unusual in that they show an isolated expression of EBNA2[25] (table 1). SMT are well-differentiated smooth cell tumours that can be observed in a large number of anatomical sites, but lung, liver, soft tissue and gastrointestinal tract seem to be sites of predilection.[26] Some SMT show prominent intra-tumoural T lymphocytes which might be directed against EBV proteins. Although SMT frequently present as multiple lesions, their clinical prognosis is much more favourable than for classical leiomyosarcomas.[26] Assessment of EBV status allows easy distinction between both entities.

EBV and carcinomas

Nasopharyngeal carcinoma (NPC), a tumour occurring at high incidence in South-East Asia, at intermediate incidence in Northern Africa and at a low frequency in populations of Caucasian origin, is consistently associated with EBV, at least in its undifferentiated form.[1] The majority of tumour cells express EBER, EBNA1, and LMP2.[1] LMP1 is inconsistently and generally weakly expressed in these tumours, and even then found only in a subset of tumour cells.[1] NPCs showing various degrees of keratinisation have also been described, but only those occurring in high-incidence areas carry the virus.[27] Outside these regions, only a third of NPCs with features of keratinisation are EBV positive.[27] Whether EBV-positive and EBV-negative keratinising NPCs share common pathogenetic features or represent fully distinct entities is unclear. Lymphoepithelial carcinomas that exhibit histological features similar to those of NPC have been described in various anatomical locations, but only those arising in the stomach show a high rate of EBV association.[27] EBV detection in lymphoepithelial carcinoma does not seem to be relevant for diagnostic purposes, except possibly in cases of metastatic lymphoepithelial carcinomas for which the primary tumour site is unknown. An association with the virus would then first orientate clinical investigations towards the oronasopharyngeal and upper gastrointestinal regions.[27]

It has recently become clear that not only lymphoepithelial, but also some 5–10% of well-differentiated gastric adenocarcinomas carry the viral genome.[28–31] Infected cells express the EBERs, EBNA1, BARF1 and LMP2-specific transcripts[32] (table 1). Interestingly, no evidence for LMP1 transcripts or proteins could be found in these tumours. EBV-positive classical adenocarcinoma have been found to have a better prognosis than their EBV-negative counterparts. This is related to a lower propensity of EBV-associated tumours to invade adjacent lymph nodes, perhaps as a result of an immune response directed against the EBV proteins expressed by tumour cells.[33] The observation that involved intra-tumoural T lymphocytes show an activated phenotype is in favour of this hypothesis.[31] Figure 1 illustrates these histological features.

EBV and haematological diseases

The viral genome has been detected in a large number of haemopoietic disorders that we briefly review.

B cell lymphomas

Systematic testing of a large panel of B cell lymphomas has shown that the EBV genome is found mainly in high grade lymphomas, though rare cases of partial EBV infection of low grade B cell lymphomas have been observed.[34] Among EBV-positive high grade B cell lymphomas, *Burkitt lymphoma* (BL) occupies a particular position as being the tumour type in which the virus was discovered. Only a minority of BL encountered outside Africa are EBV-positive. Detection of EBV in BL has not been found to have any relevance for pathologists so far.

The virus has also been detected in several *large B cell lymphoma* types, including large diffuse B cell lymphomas and their anaplastic variants.[34] Approximately 15% of this group of lymphomas contain the EBV genome, although they only inconsistently produce LMP1 and never produce EBNA2.[34] Large B cell lymphomas that develop in the settings of chronic inflammation, including pyothorax-associated lymphomas,[35 36] have been well characterised. More than two thirds of these lymphomas carry EBV-specific DNA sequences, the large majority of which express EBERs and LMP1. EBNA2 expression is more inconsistent, but EBNA2 transcript-positive tumours appear to be associated with a better clinical prognosis.[37] More recently, the so-called ''senile large cell lymphomas'', which arise in patients older than 70 and show histological features of anaplastic large B cell lymphomas, have been individualised. These tumours stained positive for LMP1 in all tested cases and for EBNA2 in one third of the cases.[38 39]

EBV positive cells are detected in 15–20% of cases of *Richter syndrome*, ie high-grade transformation of B chronic lymphocytic lymphoma into a large cell lymphoma. Detection of EBV EBERs or less frequently of LMP1 in these blasts might be indicative of poorer prognosis.[40]

Hodgkin lymphoma carries the virus in 40% of sclero-nodular forms and 80% of mixed cellularity forms. Infected tumours show a latency II viral expression pattern with strong LMP1

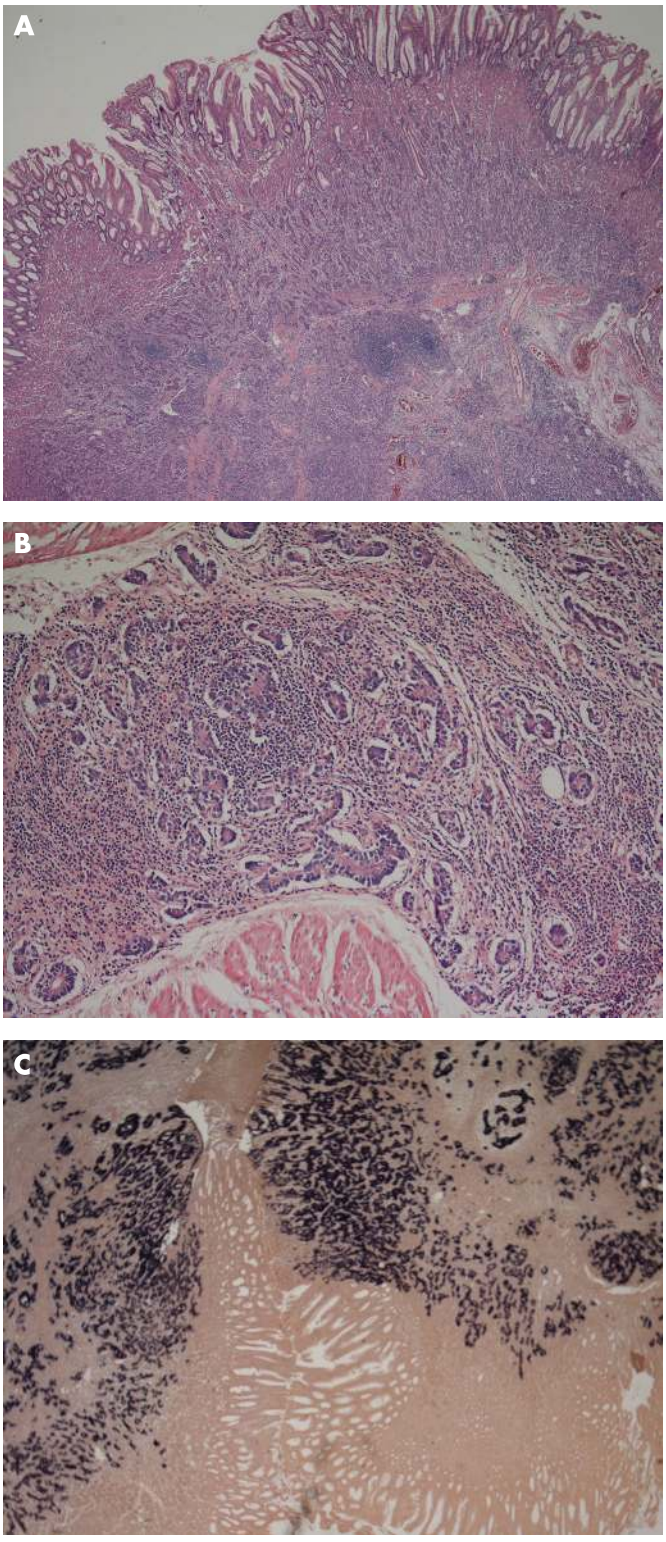

Figure 1 Gastric adenocarcinoma associated with Epstein–Barr virus. (A) and (B) H&E staining showing moderately to poorly differentiated gastric adenocarcinoma, intestinal-type with marked lymphocytic infiltrate (×20 and ×200 respectively). (C) In situ hybridisation with EBER probes revealing extensive positivity in the tumour cells (×20).

production (table 1). Detection of the virus is a strong argument in favour of a diagnosis of Hodgkin lymphoma, in particular in cases where differential diagnosis with a Hodgkin-like anaplastic large cell lymphoma is difficult (see also section on controversies).

Lymphomatoid granulomatosis (LG) is a rare systemic lymphoid tumour that mainly affects skin, brain, lungs and kidneys.[41] Histological examination reveals features of a frequently necrotic angiocentric and angiodestructive lymphoid infiltrate admixed with variable proportions of T cells, plasma cells, histiocytes and eosinophils.[42] EBV EBER has been detected in nearly all pulmonary LG cases which contain large B cells and a florid T cell reaction, but is absent from T cell or indeterminate lymphomas presenting as a LG.[43] Similarly, only cutaneous LG with B cell monoclonal populations carry the virus.[41] This diagnosis is rarely established clinically and lesions can show extensive necrosis that render diagnosis difficult.[44] There is limited information about EBV protein expression pattern in LG, but in one study all three EBV-positive LG cases studied expressed LMP1, two of which were also EBNA2 positive[45] (table 1). EBV detection in these lesions can be very helpful for diagnostic purposes as shown in fig 2.

Angioimmunoblastic T cell lymphoma (AITL) is characterised by a polymorphic cellular infiltrate including T and B cells, hyperplastic small vessels and a variable degree of follicular disorganisation. A majority of AITL cases (81–95%) contain a variable number of EBV EBER-positive large B blasts but also some EBV-positive T cells.[46–48] Roughly 60% of EBV-positive AITL produce LMP1[47 48] (table 1). In a further study, three of the four tested AITL cases were LMP1-positive, two were LMP2-positive and none produced EBNA2.[49] Interestingly, EBV infection associated with B cell expansion of infected B cells was a feature of AITL, but not of unspecified peripheral T-cell lymphomas, which were all EBV-negative.[50] The detection of the virus can therefore contribute to the diagnostic process.

Tumours derived from T cells and NK cells

This category of tumours is certainly the one for which EBV detection can contribute most to the diagnosis. The rate of association of the virus with these tumours is so high that the absence of the virus virtually excludes diagnosis. All described entities, mostly encountered in Japan and South-East Asia, are closely related and can arise in a single patient.

Peripheral T cell lymphoma, NK tumours and EBV-associated haematophagocytic syndrome (HS): HS is frequently observed in infants with immune deficiencies and primary or chronic EBV infection is one of its well recognised aetiologies.[1] In this case, T lymphocytosis and EBV-positive T cells are frequently observed in the blood and tissues.[1] Affected individuals are at high risk of subsequently or concurrently developing EBV-positive T-cell lymphomas and NK cell tumours.[1] Detection of EBV in either normal T lymphocytes or tumour cells establishes the diagnosis. Tumour cells express EBER and produce LMP1.[1]

NK leukaemias are mainly observed in the Far East in adolescents that present with hepatosplenomegaly and multiple cutaneous lesions.[10] Histological examination identifies large granular lymphocytes expressing NK markers.[10] The rate of EBV association with these lesions approaches 100%[10] (table 1).

Extranodal NK/T cell lymphoma, nasal type consists of an angioinvasive and angiodestructive lymphoid infiltrate with necrosis that affects and can cause destruction of the nasal cavity and of several anatomical structures of the mid-face (reviewed in Al-Hakeem *et al*[51]). These tumours arise most commonly in South-East Asia but also in Central and South America. EBV is present in virtually all cases (EBER positive, EBV proteins rarely expressed, but in this case a more or less complete latency II pattern is observed).[1] Patients with this tumour can develop EBV-associated HS.[51]

Inflammatory pseudotumour-like follicular dendritic cell tumour (IPLFD) or follicular dendritic cell proliferation associated with EBV preferentially develops in liver and spleen, or more rarely in lymph nodes.[10] This exceptional tumour is characterised in

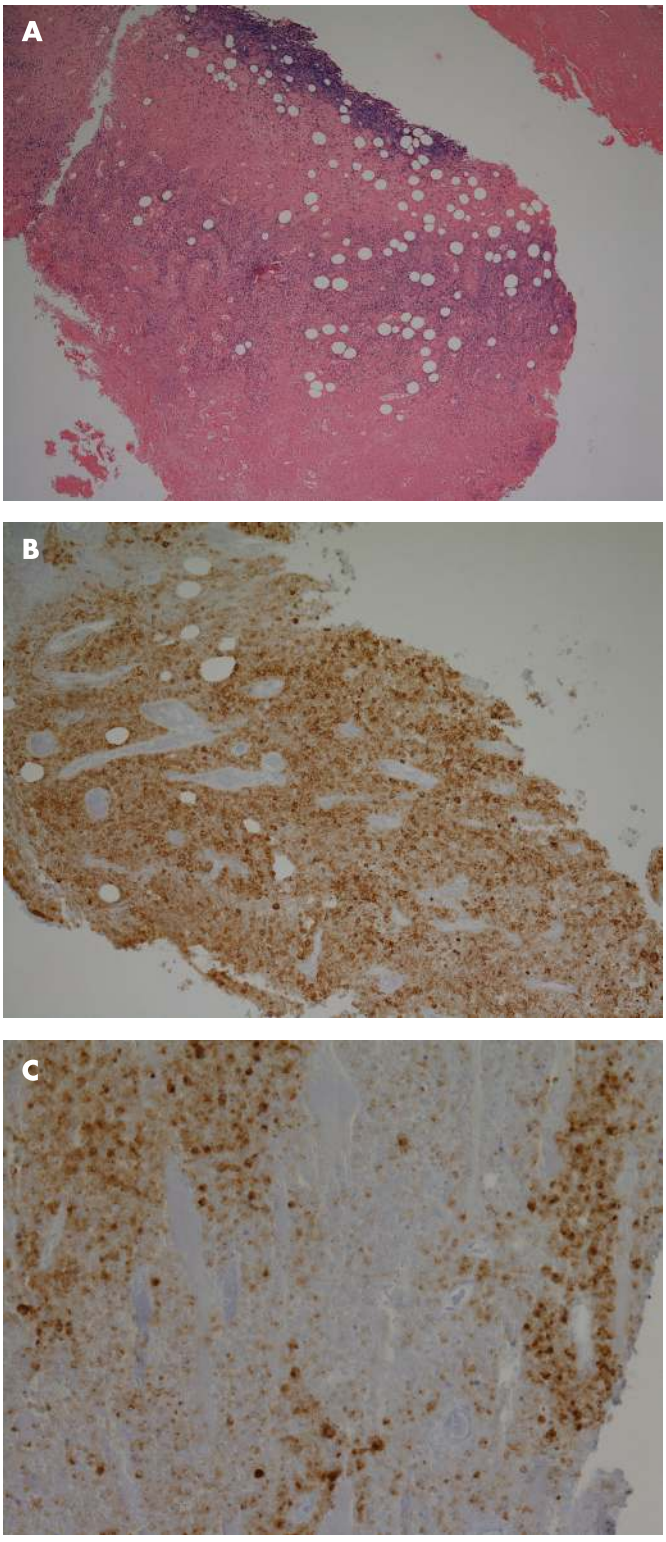

Figure 2 Lymphomatoid granulomatosis. (A) Biopsy of a subcutaneous soft tissue mass. H&E stain shows extensive tumour necrosis (×20). (B) CD20 immunostaining reveals abundant population of altered B-cells in necrotic material (×100). (C) LMP immunostaining shows diffuse positivity in the necrotic material (×200).

an inflammatory background including numerous lymphocytes and plasma cells, among which neoplastic follicular dendritic cells (FDC) can be identified.[10] Tumour cells have been described as spindle cells resembling fibroblasts that express FDC markers.[52 53] EBV infection of these lesions was first evidenced in a study of inflammatory pseudo-tumours, a large and heterogeneous group that includes IPLFD.[54] Viral infection is readily established with an EBER assay showing intense signals in all tumour cells; LMP1 staining, although consistently positive, appears to be weak and focal[52 53 55] (table 1). Due to the consistent association with EBV, diagnosis of this entity requires detection of the virus.[10]

CONTROVERSIES

Whereas EBV infection in certain tumour types has been firmly established by several independent research groups, its association with other tumour entities is still controversial.[27 56]

Two reports in the early 1990s assessing EBV infection in enteropathy-associated T cell lymphomas reported discrepant results. One study found EBER-positive cells and EBV DNA by Southern blot hybridisation analysis in one third of cases, but these findings could not be confirmed by the other research group.[57 58]

Another controversial issue concerns the associations between EBV infection and anaplastic large cell lymphomas (ALCL). Earlier reports drew discrepant conclusions in this regard (reviewed in Herling *et al*[59]), but more recent contributions have concluded that both systemic and primary cutaneous types of ALCL arising in Western patients are not associated with the virus.[59 60] A certain degree of geographical heterogeneity might, however, exist as rare EBV-positive cases of cutaneous ALCL in Korean patients were recently reported.[61] Importantly, a series of 10 ALK-positive ALCL showing histological features reminiscent of nodular sclerosis Hodgkin lymphomas were found to be EBV-negative, confirming the reliability of EBV detection as a potentially helpful diagnostic tool to distinguish between ALCL and Hodgkin disease.[62]

Studies assessing EBV association with breast adenocarcinomas have reported contradictory results.[56] This is probably at least partly related to the antibody directed against EBNA1 (clone 2B4). Indeed, EBNA1 protein shares a peptide sequence with the MAGE-4 cellular protein, and the 2B4 EBNA1 antibody recognises this peptide.[63] Cross-reaction between anti EBNA1 antibodies and MAGE-4 can therefore lead to false positive results. Some studies have nevertheless identified a minority of breast cancer cases containing rare EBER-positive cells.[64–67] The current body of evidence therefore favours the view of EBV infection being a rare event. It remains possible, however, that some breast cancer cases could show partial EBV infection whose significance for tumour pathogenesis remains to be determined.

Another controversial issue has resulted from claims of EBV infection in hepatocarcinomas that occurred in Japanese patients.[68] Interestingly, these tumours were found to be EBER negative. This would mean that yet another type of latency exists in which EBERs are not expressed. However, analysis of a panel of hepatocarcinomas that occurred in European and Northern American patients could not find any evidence for EBV infection in these lesions.[69] The possibility of geographic heterogeneity remains; DNA in situ hybridisation should settle the issue.

Several studies on the links between EBV infection and squamous cell carcinomas of the oral cavity have been published, with highly variable results. Not taking into account studies performed with PCR analysis alone, one report found a high rate of association (16/24 EBER positive cases) with EBV positive cases producing EBNA2 and LMP1.[70] Another report found a low rate of association (20%), the majority of which were also LMP1-positive cases.[71] In contrast, Iamaroon *et al* could not find any evidence for EBV infection in 19 tested cases.[72] More work appears to be required to clarify these issues.

Take-home messages

- Epstein–Barr virus (EBV) is a herpesvirus associated with a variety of tumours including B and T lymphomas, NK cell malignancies, carcinomas and smooth muscle tumours.
- In situ hybridisation of viral EBERs remains the gold standard for virus detection as LMP1 is only inconsistently detected in EBV-positive tumours.
- EBV detection can be helpful for diagnostic, prognostic and therapeutic purposes.

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