EBV-Associated Malignancies


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Abstract: Epstein-Barr virus (EBV) infection has been implicated in the aetiopathogenic mechanisms of several neoplastic and non-neoplastic disorders. Although the precise mechanisms of the tumourigenic actions of EBV have not yet been fully elucidated, this virus has been strongly linked to subtypes of Hodgkin’s and non-Hodgkin’s lymphomas (especially Burkitt’s lymphoma), HIV/AIDS lymphomas, nasopharyngeal carcinoma and gastric carcinoma, among others. The fact that persistent infections occur in greater than 95% of adults with an overall relatively low incidence of EBV related tumours compared with the prevalence of infection shows that there are definitely many other factors (genetic and environmental) that contribute to tumour development in EBV positive individuals. In this article, we review some of the currently available knowledge about these relationships in the commonly encountered EBV-associated malignancies. It is hoped that with continued research in the pathogenic mechanisms of EBV, specific roles will be identified that will facilitate the development of specific targeted therapy.

Keywords: Epstein-Barr virus, association, malignancies, targeted therapy.

INTRODUCTION

Tumours arise as a result of genetic and epigenetic alterations which transform the normal cell into an immortalized proliferating cell, the mass of the tumour being a product of clonal expansion of this transformed cell. Many factors are responsible for this transformation and some of the most important ones are viruses [1]. The discovery of the role of viruses in the causation of certain animal cancers with the discovery of viral oncogenes laid an important foundation for the understanding of the mechanism of tumours generally. Oncogenes were first discovered as part of the viral genome of oncogenic animal viruses [1, 2]. As a consequence of the understanding gained from the actions of viral oncogenes, cellular oncogenes were later discovered.

Viruses act in inducing cancers through different methods. Transforming retroviruses, for instance, carry cellular genes which are transcribed by the virion-associated reverse transcriptase, followed by integration of the double stranded DNA copy into the chromosomal DNA of the cell [1, 2]. Several different mechanisms are encountered before cancers are eventually formed.

Epstein Barr Virus (EBV or Human herpesvirus 4) belongs to the genus Lymphocryptoviridae, the gamma 1 subtype of the subfamily Gammaherpesvirinae and is one of the most common viruses in humans. It is present in all populations, infecting more than 95% of all individuals within the first four decades of life. In developing countries, infections occur very early in life with no specific characteristics other than the general symptoms of acute viraemia. In developed countries however, the infection is usually delayed until adolescence or early adulthood years where it causes infectious mononucleosis, a benign self-limiting lymphoproliferative disorder [3]. Though the infection with EBV is benign in the acute stages and latent in the chronic phase in the vast majority of people, the virus has been demonstrated to be involved in the development of many malignancies with the list of such malignancies progressively increasing. The first association was with the endemic Burkitt’s lymphoma. Subsequently, other lymphomas (subtypes of both Hodgkin’s and non-Hodgkin’s lymphomas) are also now known to be associated with EBV infection. Epithelial malignancies such as lymphoepitheliomas of nasopharynx and stomach are currently included in the list of EBV associated tumours. This review discusses the role of EBV in the causation of various malignancies and the modern approaches to specific diagnosis and targeted treatments. A list of some EBV-associated neoplasms and their peculiar characteristics is shown in Table 1.

EBV AND CARCINOGENESIS

Establishment of Persistent Infection

The mechanism of tumour causation by EBV varies, depending on tumour type. However, certain basic general characteristics of EBV biology are found in the various EBV-associated cancers. The first stage in the mechanisms of EBV tumourigenesis is the establishment of a persistent infection [4].
Persistent infections occur in greater than 95% of adults. The relatively low incidence of EBV related tumours compared with this prevalence of infection shows that there are definitely many other factors that contribute to tumour development. The primary infection with EBV is believed to start within the oropharyngeal epithelial cells with viruses subsequently passing to subepithelial B cells through direct contact. The invasion of the immune system by EBV infection of B cells stimulates a vigorous CD8+ T cell response. The development of a virus-specific adaptive immune response is largely responsible for the elimination, to a large extent, of EBV infection by reducing the number of EBV infected B cells. This elimination is however incomplete. Persistent EBV infection remains as a latent infection in peripheral blood lymphocytes and as a lytic infection in the oral cavity, which results in the shedding of infectious viruses via oral secretions [4].

EBV infects resting B cells and turns them into continuously proliferating lymphoblastoid cell lines that express nine latency-associated viral proteins, including six nuclear antigens (Epstein-Barr nuclear antigen (EBNA)-1, -2, -3A, -3B, -3C and -LP) and three membrane proteins (latent membrane protein (LMP)-1, -2A and -2B) [5]. However, Only some of these proteins are expressed in EBV-carrying malignancies. Genomic instability is a hallmark of malignant transformation and is frequently associated with chromosomal aberrations, including reciprocal translocations, deletions, inversions and duplications, that deregulate the expression of oncogenes or tumour suppressor genes [6]. These aberrations are clonally transmitted and constitute useful cytogenetic markers of malignancy. In addition, nonclonal chromosome aberrations, such as dicentric chromosomes, chromosome fragments, gaps, double minutes and rings, are generated by inappropriate repair of DNA breaks. These aberrations are usually lethal to dividing cells and are therefore generated de novo during each cell cycle. Several studies have reported the presence of clonal chromosome translocations in EBV associated malignancies [7, 8]. In their very recent publication, Gruhne et al. showed the specific roles of three EBV latency proteins [7]. They demonstrated that the EBV nuclear antigens, EBNA-1 and EBNA-3C, and the latent membrane protein 1, LMP-1, independently promote genomic instability, as detected by nonclonal chromosomal aberrations, DNA breaks and phosphorylation of histone H2AX. EBNA-1 promotes the generation of DNA damage by inducing reactive oxygen species (ROS), whereas DNA repair is inhibited in LMP-1-expressing cells through downregulation of the DNA damage-sensing kinase, ataxia telangietasia mutated (ATM), reduction of phosphorylation of its downstream targets Chk2 and inactivation of the G2 checkpoint. EBNA-3C enhances the propagation of damaged DNA through inactivation of the mitotic spindle checkpoint and transcriptional down regulation of BubR1. Thus, multiple cellular functions involved in the maintenance of genome integrity seem to be independently targeted by EBV, pointing to the induction of genomic instability as a critical event in viral oncogenesis. Additionally, in several cancer cells types, the EBV genome is heavily methylated and few viral genes are expressed. Methylation of DNA is usually associated with inhibition of gene expression; partly mediated by the association of specific methyl-CpG-binding proteins with methylated DNA, leading to transcriptional silencing and chromatin remodelling; and also by the inhibition of DNA binding of some transcription factors through DNA methylation [9].

Transformation of Cells In Vitro

When peripheral blood lymphocytes from chronic virus carriers are placed in culture, the few EBV-infected B cells that are present regularly give rise to spontaneous outgrowth of EBV-transformed cell lines, known as lymphoblastoid cell lines (LCLs), provided that immune T cells are either removed or inhibited. These cells all carry multiple copies of the viral episome and demonstrate the latency III program (described earlier). EBNA-2 and LMP-1 have been demonstrated to be absolute requirements for this in vitro transformation while EBNA leader protein, EBNA-3A, EBNA-3C, and LMP 2A play important roles in the process [10].

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Tumour Formation in Immune Suppressed Individuals

As noted earlier, EBV infected B cells grow into LCLs in vitro when cultured in the absence of T cells. This implies that the prevention of spontaneous generation of B cell tumours from infected B cells in vivo is related to the intact immune system. It follows therefore that B cell tumours will develop in conditions of severe immune suppression. This has been demonstrated experimentally in animal models in cotton top tamarins and in severe combined immunodeficient (SCID) mice [11]. In these cases the proliferation can be oligoclonal or monoclonal and they all resemble LCLs latency III pattern of EBV gene expression and in cell surface phenotype.

The obvious inference from the remarkable efficiency of tumour development in these experimental models is that the development of immunoblastic lymphomas in severely immunosuppressed humans only requires EBV induced B cell transformation, with no necessity for secondary genetic or epigenetic changes. It is also notable that tumour regression usually follows reduction in the level of immunosuppression if the cancer is therapy induced. These particular lesions are therefore quite different in pathogenesis from other EBV positive malignancies, where viral infection is but one event in a complex multistep process of cancer development.

Three main types of lymphomas are seen in different categories of immunocompromised persons: (1) Lymphomas in individuals with inherited immunodeficiency states referred to as X-linked lymphoproliferative disorders. (2) Lymphomas associated with iatrogenic immunosuppressant therapy given to transplant recipients, and (3) AIDS-associated lymphoproliferative disorders.

Post-Transplant Lymphoproliferative Disorders (PTLDs)

It is now well established that there is a high incidence of lymphoproliferative disorders following solid organ and haematopietic stem cell transplantation (HSCT). PTLDs are made up of a variety of almost exclusively B cell proliferations ranging from spontaneously regressing, polytypic lymphoplasmacytoid B cell expansions which
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Pathogenesis of PTLD

Oncogenic viruses known to be involved in the pathogenesis of PTLD include EBV and HHV-8 [10]. These viruses act through direct mechanisms, directly infecting the tumour clone and exerting a transforming effect upon B cells. Using different strategies, they ensure persistent infection. These strategies include prevention of death of infected cells, enhancement of their proliferation to maintain the infected reservoir, and evasion of the immune system [15-17]. Several lines of evidence suggest that EBV infection has a major pathogenic role in PTLDs: (a) EBV infects ~60%-80% of PTLD patients, including 100% of early-onset PTLD patients [18]; (b) in many cases of monomorphic PTLD, EBV infection is monoclonal, consistent with the hypothesis that the virus has been present in the tumour progenitor cell since the early phases of clonal expansion; (c) a decrease in EBV-specific cytotoxic T lymphocytes (CTLs) and an increase in the EBV viral load are strongly associated with PTLD development [19]; and (d) treatment of PTLDs with autologous EBV-specific CTLs results in viral load control and tumour size reduction.

Normally, a balance exists between viral load and immune mechanisms which maintains persistent infection at a subclinical level in immunocompetent EBV-positive individuals. However, immunosuppressive therapy used to prevent graft rejection usually leads to loss of cytotoxic T lymphocytes which allows infected cells to persist. As a result, EBV infected cells accumulate in the body with enhanced virus replication. The amount of EBV shed in saliva following transplantation as well as the amount of EBV infected B cells in blood and level of EBV DNA in peripheral blood are increased following transplantation [20-23]. This combination of findings is referred to as a “reactivated” EBV infection. In most cases, there is no associated disease, however, in a minority of patients, uncontrolled EBV driven B cell proliferation occurs, leading to clinical PTLD [17]. In a donor-recipient pair with an EBV positive donor and an EBV negative patient, the donor organ can act as the source of infection [24, 25]. On the other hand, most cases of PTLD occurring in previously EBV seropositive individuals do not develop for over a year, and may present many years post-transplant [17, 26]. Following solid organ transplantation, PTLD can arise early or late in the post-transplant period, unlike in haemopoietic stem cell transplantation where PTLD generally arises early and is rapidly fatal [27]. In most cases of PTLD, tumour cells express the latency III pattern of EBV gene expression, with some cells undergoing lytic replication [28]. The expression of the latent antigens (particularly the viral oncoproteins LMP-1 and EBNA-2) has been found to have profound effects on the infected cell, with loss of growth control and induction of proliferation. This strongly suggests that EBV is directly responsible for polyclonal B cell hyperplasia and PTLD. However, within a particular tumour, the pattern of viral gene expression may vary between individual cells [17] and occasionally latency type I and II patterns have also been detected in PTLD biopsies [29]. In these cases additional genetic or epigenetic changes are probably required for tumour outgrowth.

HIV/AIDS-Associated Lymphomas

HIV/AIDS-associated lymphomas constitute a heterogeneous group of lymphoproliferative disorders arising in the setting of profound immunosuppression in the terminal phases of HIV infection. This severe immunosuppression allows uncontrolled proliferation of EBV infected lymphocytes. This group of lymphomas include primary central nervous system (CNS) lymphomas, diffuse large B cell lymphomas (DLBCL), Hodgkin’s lymphoma (HL), Burkitt’s lymphoma (BL) or BL-like lymphomas, and primary effusion lymphomas. Variations exist in the frequency of EBV infection in AIDS-related lymphomas depending on the subtype of tumour involved. CNS lymphomas in AIDS patients have been mostly affected with EBV infection. One potential explanation for this finding is that HIV-induced immunodeficiency may increase the trafficking of EBV-infected B cells into the brain, or the combined immunodeficiency and immunoprivileged nature of the CNS favours EBV-driven B lymphoproliferations [4]. Most cases of DLBCL with immunoblastic morphology and HL in AIDS patients are EBV related. These cases appear in the final stage of AIDS when the patient is severely immunocompromised. In contrast, only 30%-50% of cases of Burkitt or Burkitt-like lymphomas in AIDS patients are associated with EBV infection [30-32]. In these cases, EBV infection and immunosuppression may increase the pool of B cells at risk for a c-MYC translocation and may not be the primary mechanism for driving malignant proliferation. EBV infection can be detected in some primary effusion
lymphomas, but these B cell lymphoproliferations are linked consistently to KSHV infection, which suggests that the gamma-2 herpesvirus is more important for this malignancy than EBV infection [4].

**EBV-ASSOCIATED SMOOTH MUSCLE TUMOURS IN IMMUNOCOMPROMISED INDIVIDUALS**

Immunocompromised patients have in rare cases developed smooth muscle neoplasms that harbour clonal EBV genomes. These tumours occur typically in children who are on immunosuppressive therapy or with severe immunodeficiency from HIV infection. They are more commonly located within the gastrointestinal tract or pulmonary tree [33, 34].

**EBV-ASSOCIATED MALIGNANCIES IN THE IMMUNOCOMPETENT HOST**

The three most important malignancies in the immunocompetent host are Burkitt’s lymphoma, Hodgkin’s lymphoma and nasopharyngeal carcinoma. It would however appear that EBV, as a causative agent, is just one of the factors involved in the complex multifactorial mechanisms involved in the multistep pathogenesis of these neoplasms. Typically, these malignancies occur many years after primary EBV infection [4].

**Burkitt’s Lymphoma**

Burkitt’s lymphoma (BL) is an aggressive B cell tumour. The different types include the endemic type, the sporadic type and the immunodeficiency associated type. Whatever the type, the fundamental transforming event in BL is the translocation of the MYC gene. This translocation is to one of 3 chromosomes from the normal position on chromosome 8. The 3 destination chromosomes are 14 (to the region of the Ig heavy chain), 2 (to the region of the Ig kappa genes) and 22 (to the region of the Ig lambda genes). In 80% of cases the t(8;14)(q24;q32) translocation occurs with the IGK gene. The remaining 20% of cases are split between the translocations with the IGK and IGL (corresponding to t(2;8) and t(8;22) respectively) [35].

The effect of the reciprocal translocation is to create a constitutively activated MYC gene. The MYC proto-oncogene plays a critical role in regulating cell proliferation, differentiation and apoptosis depending on the type of cell or other situations [36]. In BL, the outcome of the MYC mutation can be summarized as cell growth, uncontrolled proliferation, and increased number of subsequent mutations occurring in the genome. Not all BL are EBV-associated but the chromosomal translocations remain fairly constant, differing only in the breakage points on the Ig gene. It is clear that EBV does not act in BL as a direct transforming virus but through an indirect manner. Its role appears to be in stimulating B cell proliferations thereby increasing the risk of spontaneous translocations in the B cell line. It has also been shown to increase the rate of somatic hypermutations due to virus induced extended expression of the enzyme Activation Induced Deaminase (AID). EBV also acts by obstructing the apoptotic pathways that are normally activated when there is excessive MYC activity. In endemic BL, it acts in concert with other factors like malaria which seem to act by reducing T cell response to EBV antigens through a non EBV-specific activity. The events that bring about this translocation and those that allow cells to survive with the constitutive expression of MYC have been the subject of intense investigations. EBV infection, malaria, immunodeficiency and spontaneous somatic mutations all contribute to the origin and maintenance of this cancer [32]. It is also important to note that the inactivation of two common tumour suppressor genes (TSGs), the cyclin dependent kinase inhibitors (p16/CDK4A and p15/CDK4B), through promoter hypermethylation, and the inactivation of the p53 homologue p73 are frequently observed in BL. Further, the epigenetic inactivation of members of the BCL2-family or other pro-apoptotic gene families is a common event in BL. A couple of broad-spectrum TSGs and genes involved in cell cycle control, apoptosis, intracellular signaling, proliferation, and surface adhesion are also frequently methylated in BLs [37]. BL, first described by Dr Dennis Burkitt, in 1958, was initially thought to be a sarcoma of the jaws [38]. Subsequently however, further studies revealed that this tumour was a unique type of non-Hodgkin’s lymphoma with a peculiar epidemiology and a very strong association with EBV [39, 40]. The disease originally described is the Endemic BL which is largely found in Africa. It typically affects the facial skeleton, particularly the jaw bones, in children between 2 and 9 years. Endemic BL occurs frequently (in 50-100 cases per 1,000,000 individuals) in the equatorial regions of Africa and Papua, New Guinea. Most cases of endemic BL are associated with the presence of EBV in the tumour cells [4]. The earliest descriptions of the geographical distribution of BL coincided with the so-called lymphoma belt of Africa which was associated with malaria hyper- and holo-endemicity [41]. This led to the hypothesis that malaria or some other infectious agent carried by mosquitoes was responsible for the tumour. The roles of malaria in BL causation are not fully understood. The most common hypothesis in the relationship is the profound immunosuppression caused by malaria. Malaria suppresses T-cell responses including those directed against EBV [42]. Sporadic Burkitt’s lymphoma was described outside the African region, but it is morphologically similar to endemic BL. It predominantly affects the organs of the abdominal cavity. Sporadic BL can be found in any age group and has not been associated with any specific co-factor. The incidence of sporadic BL is much lower than the endemic disease, with 2-3 cases per 1,000,000 individuals in the equatorial regions of Africa and Papua, New Guinea. In the United States and Europe the association with EBV is low (15-30% of cases) [40, 43, 44]. HIV-associated BL is the third variant that was subsequently described in the era of HIV infection. Although it has been commonly reported in the developed world and associated with HIV in some adults in Africa, the childhood variety of the disease among HIV infected children has not been well characterized. However, it can be seen in any geographical location and in all age groups [35, 45, 46].

**EBV and Burkitt’s Lymphoma**

The stories of EBV and Burkitt’s lymphoma (BL) are interrelated as the discovery of EBV was a direct outcome of continuous research and clinical descriptions of BL. The initial restricted African geographical distribution of BL was intriguing prompting studies searching for the possible viral
cause, eventually leading to the discovery of the novel human herpesvirus. EBV is a lymphotropic gamma human Herpesvirus, the first virus to be associated with a human tumour [47]. In a recent review, Thorley-Lawson et al. [43] described the curious relationship between EBV infection and Burkitt’s lymphoma. The most compelling argument for a direct role for EBV in BL pathogenesis was the initial discovery that almost 100% of BL in equatorial Africa was associated with EBV infection. It was also shown that children who were infected early in life and who produced the highest antibody titres to the virus were at highest risk for developing the tumour. The tumour cells were found to express EBNA (EBV nuclear antigen), a serologically defined, putative tumour antigen, in their nuclei, although, this antigen was later shown to be composed of six components, of which only one, EBNA-1, was expressed in EBV-positive BL [43, 48]. Subsequently, EBV was firmly linked to other proliferative diseases, including acute infectious mononucleosis, nasopharyngeal carcinoma, Hodgkin’s disease, immunoblastic lymphoma in individuals who are immunosuppressed, a subset of gastric carcinomas, rare T- and NK-cell lymphomas and leiomyosarcoma [43, 49]. The surprising result that Epstein-Barr virus (EBV) was not restricted to BL patients as initially believed, but was not only associated with various other cancers but also found to be widespread with prevalence in about 95% of the adult population made it an even more exciting subject of research. One observation that favoured the carcinogenic role of EBV in BL was the finding that EBV was a potent transforming virus in culture for the same cell type that develops into BL, the B lymphocyte [43]. EBV appears to be an extremely efficient transforming virus in culture, being able to convert >50% of all target cells (the resting B cell) into continuously proliferating, latently infected lymphoblastoid cell lines (LCLs) within a few days [43]. In spite of the very close association between EBV and BL, the precise roles of EBV in BL tumourigenesis remained difficult to understand for a very long time. This was because, although EBV has been detected in virtually all cases of endemic BL, most cases of sporadic and HIV related BL were not EBV associated. Further studies therefore led to the understanding that EBV is a cofactor in the pathogenesis of BL.

The Interrelationships Between c-myc/Ig Translocations and EBV Infection in the Pathogenesis of BL

The current knowledge is that c-myc/Ig translocations are the hallmark of BL and EBV is a cofactor in the pathogenesis of endemic BL [50]. Irrespective of its geographical location and its relationship with EBV infection, BL is invariably characterized by recurrent specific reciprocal chromosomal translocations involving the long arm of chromosome 8 carrying the c-myc gene and one of the immunoglobulin (Ig) heavy or light chain loci on chromosome 14 (heavy chain), 2 or 22 (j or k light chain) [46, 51, 52]. Although not sufficient on its own, c-myc activation by chromosomal translocation appears to be a requirement for BL carcinogenesis [46] This is supported by the notion that cmyc activation is a hallmark of retrovirus or pristane-induced B cell malignancies in chicken, mice and rats [46, 47], and that transgenic mice expressing c-myc under the control of the Ig heavy or light chain enhancers develop malignant B cell lymphomas with high incidence and short latency [46, 53], some of which are histologically indistinguishable from human BL. A difference however exists between human BL and the BL in mouse. Human BL arises from germinal center cells that have encountered antigen and undergone somatic hypermutation (SHM), whereas the tumours arising in transgenic mice originate from naive B or even pre-B cells [54]. Thorley-Lawson et al. have proposed a mechanism for the pathogenesis of EBV positive BL [43]. According to their proposal: A naive B cell that is newly infected with Epstein-Barr virus (EBV) in the tonsils expresses the growth transcription programme that is driven by EBNA-2 (EBV nuclear antigen 2) to become a B blast (latency 3). Proliferation of the infected B blasts is driven in part by EBNA-2 activation of MYC [43]. EBNA-2 activates the transcription of EBNA-3A and EBNA-3C, which in turn induce the epigenetic repression of BCL2L11 (Bcl2-like protein 11; also known as BIM), thereby allowing the cell to tolerate activated MYC [43]. In parallel, EBNA-3A and EBNA-3C autoregulate cell growth through epigenetic repression of the growth-programme, allowing the cell to differentiate into a germinal-centre (GC) cell with a repressed BIM gene. The MYC translocation occurs at the GC stage, which leads to uncontrolled proliferation. Normally this would induce apoptosis through the activation of BIM. However, the prior repression of BIM allows the cell to survive and continue proliferating [43]. The infected GC cell then follows its normal behaviour and leaves to become a memory cell. During this transition the virus also follows its normal behaviour for EBNA1, which is required to replicate the viral genome when the cell proliferates. MYC-driven proliferation prevents the latently infected cell from becoming a resting memory cell, allowing it to grow as a Burkitt’s lymphoma tumour that expresses the viral EBNA1-only programme [43]. It is clear that the interrelationship between EBV and tumourigenesis in BL will continue to be a focus of research.

Hodgkin’s Lymphoma

Hodgkin’s lymphoma (HL) has been referred to as an unusual neoplasm due to the fact that the malignant cells constitute only a minority of cells in the tumour mass. Classical HL (cHL) is characterized by the presence of clonal, malignant multinucleated Reed Sternberg giant cells in a background of reactive inflammatory cells that includes lymphocytes, plasma cells, granulocytes, and histiocytes. Most of the proliferations within these tissues result from the reactive inflammatory cells that accompany the Reed Sternberg cells. The Reed Sternberg cells typically constitute less than 1% of the cellularity. The presence of the Reed Sternberg cells is pathognomonic for this tumour. HL exists in 5 forms: mixed-cellularity, nodular-sclerosis, lymphocyte-rich, lymphocyte-depleted and lymphocyte-predominant subtypes. All of these subtypes apart from the lymphocyte-predominant HL (LPHL) constitute what is now known as the classical HL (cHL). LPHL is a unique clinicopathological entity not associated with EBV.

Classical HL (cHL) is a complex and multifactorial disease. EBV infection is associated with approximately 40% of cHL cases most frequently with the mixed-cellularity subtype. This subtype is more common in males and demonstrates a bimodal age distribution (<10 and >50 years
of age). EBV-negative cHL is most common in adolescents and young adults, does not show a gender predisposition, and is associated with a high standard of living in childhood [4]. It was in 1966 that MacMahon first proposed that an infectious agent might be a possible agent in the pathogenesis of HL [55]. This was supported by subsequent findings in which raised antibody titres to EBV antigens were found more in HL compared with other lymphoma patients [56]. The fact that the detection of raised levels of EBV preceded the development of HL by several years also supported the growing suspicion that EBV contributed to the carcinogenesis in HL [57]. Another significant contribution was the study by Gutensohn N et al. in which it was reported that, following infectious mononucleosis, the relative risk of developing HL compared to individuals without prior history ranged between 2.0 and 5.0 [58]. The first report of the successful demonstration of EBV DNA in HL tissue specimens was by Weiss et al. in 1991 using the cloned BamHI W fragment of EBV, as an in situ hybridization probe [59].

When present, EBV is clonal, which indicates clonal expansion of single EBV-infected cells. In this cells EBV infection exhibits a type II form of latency, EBV gene expression being limited to the EBERs, Epstein-Barr nuclear antigen 1 (EBNA1), latent membrane protein 1 (LMP-1) in high levels, LMP2, and the Bam HIA transcripts [60].

In situ hybridization probes were developed to target highly abundant EBERs which provided a reliable and simple method for the detection of EBV in archival HL specimens [61]. Initial studies showed that EBV DNA was detected in 20-25% of HL tumour specimens [61]. The detection of EBV DNA by in situ hybridization provided the first demonstration of its existence in the Reed Stenberg giant cells [53, 62]. Subsequently, the demonstration of the abundant EBV early RNA (EBER-1 and EBER-2) sequences in Reed Stenberg cells provided a sensitive method for detecting latent infection in situ. This technique is generally accepted as the "gold standard" for the detection of latent EBV infection in clinical samples [53].

The mechanism of transformation in HL appears also to be indirect. The malignant cells of HL are believed to derive from the germinal centre B cell [63]. These cells are those that have acquired serious Ig mutations resulting in non-functional Igs. These types of damaged cells would under normal circumstances have undergone apoptotic elimination. This apoptosis is overcome if there is a high level of activation of NFkB. The EBV LMP-1 is a potent activator of NFkB, and the activation of NFkB is an important step for EBV-induced B cell immortalization. In addition, the EBV LMP-2A can mimic signalling effectively through the Ig receptor [64].

Reed Stenberg cells exhibit a type II form of latency, EBV gene expression being limited to the EBERs, EBNA-1 [10], LMP-1 " LMP-2 [52], and the Bam HIA transcripts [52].

It has been variously reported that several epidemiological factors such as sex, age, ethnicity, country of residence and histological subtype have roles to play in the association between EBV and HL. In particular, in developed populations, the association between EBV and HL is less, with percentages of between 20% and 50% for North American and European cases [52, 65, 66], 57% for China but much higher rates in underdeveloped countries such as Peru [67] and Kenya [68-70]. It has been postulated that the increased incidence of EBV positive HL in underdeveloped countries is a consequence of the existence of an underlying immunosuppression similar to that seen in African BL in a malaria infected population [61]. In spite of the increased knowledge about the contributory roles of the various carcinogenic mechanisms in HL, the precise contribution of EBV remains yet to be fully understood. In particular, it is very important to identify the roles of latent virus products, particularly LMP-1 and LMP-2. A number of TSGs involved in cell cycle control, apoptosis, and surface adhesion was frequently silenced by methylation in HL tumours: p16 and p15 were methylated at higher frequency in relapsed tumours than in primary tumours. Studies have shown that the pro-apoptotic CHK2 kinase was sporadically silenced in various carcinomas and lymphomas, but was completely silenced in HD cell lines. Contrary to BL, the “death associated protein kinase” (DAPK) was methylated at a low frequency, and mainly at advanced tumour stages. Like in BL cells, PCDH10 was highly methylated in HD cell lines. Impaired Ig production in RS cells despite their mostly functional gene rearrangements, has also been attributed to probable epigenetic closedown of the IgH promoter [37].

**T/NK NASAL TYPE LYMPHOMA**

Apart from B cells which are known to be primarily infected by EBV, other non-B-cell NHLs associated with EBV infection include T-cell lymphoproliferative disorders which include a subset of peripheral T-cell lymphomas such as extranodal nasal type NK/T-cell lymphoma. Several unique genotypic and phenotypic features have been identified in this subset of EBV associated lymphomas, some of which are absence of T-cell antigens, the expression of the NK cell marker CD56, and the absence of T-cell receptor gene rearrangement. These tumours typically occur in the nasal and upper aerodigestive area. EBV infection has been found to be consistently associated with these lymphomas, irrespective of geographical location [10, 71].

**EPITHELIAL MALIGNANCIES**

EBV has been demonstrated in many epithelial malignancies, the most consistent one and arguably the most important in this regard is the undifferentiated (non-keratinizing) carcinoma of the nasopharynx, which is also called lymphoepithelioma because of the diffuse lymphocytic infiltration that accompany the tumour cells. Here the association is such that regardless of geography nearly 100% of the tumours and all the tumour cells have been demonstrated to be monoclonally EBV-positive. Lymphoepithelioma-type tumours occurs in many organs, and EBV has been shown to be positively associated with a large number of these tumours, including those in the salivary gland, stomach, middle ear, lungs, and thymus [72, 73]. Some other non-lymphoepithelioma-type epithelial malignancies have also been shown to be EBV-positive though the role of the virus in carcinogenesis remains controversial in many of these tumours.
**Nasopharyngeal Carcinoma (NPC)**

Nasopharyngeal carcinoma has been reported in almost all parts of the world, however, most cases are found in South East Asia, Southern China (including Hong Kong), North Africa and in the Eskimo population of Alaska, USA [74-77]. The incidence reaches a peak of around 20-30 cases per 100,000 with rates being highest in individuals of Chinese descent irrespective of where they live, and particularly in Cantonese males. It has been found out that c-kit is highly expressed in the juvenile form of North African nasopharyngeal carcinomas with a significant association between LMP-1 and c-kit expression [78].

There is no doubt that NPC has a definite association with the EBV [79]. The persisting controversy, however, is the specific pathogenic mechanism by which EBV causes NPC. This is in spite of the fact that virtually all cases of non keratinizing NPC are EBV positive in all geographical locations. Specifically, the direct role of EBV in the carcinogenesis of NPC is strongly disputed.

One of the problems confronting the explanation of the EBV in the pathogenesis of the tumour is the fact that mature nasopharyngeal cells are not usually infected with EBV in vivo and in vitro. Yet the tumours have been shown to be infected with EBV before transformation. It has been shown that the immature epithelial cells carry CD21 and can be infected by the virus. It is therefore postulated that EBV infects nasopharyngeal cells that have been stimulated by other environmental factors. The area of emphasis appears to be the importance of dietary carcinogens, such as salted fish products [68, 70]. Salted fish and preserved food top the list of dietary agents implicated in NPC [80, 81]. Areas that have been found to be mostly affected are southern China, Tunisia and Greenland, where the diet contain large amounts of salted fish and preserved food rich in N-nitrosodimethylamine (NDMA), N-nitrosopyrrolidene (NPyR) and N-nitrosopiperidine (NPIP) [68, 82]. Exposure to smoke or chemical pollutants, including trace elements (e.g. nickel) are some of the environmental factors which have also been reported to be associated with the development of NPC [68, 83].

It appears that latency gene expression in NPC is intermediate between that seen in BL (latency I program) and HL (latency II program). The expression of latent viral EBNA-1 and the EBER genes have been confirmed in all EBV-positive NPC cases. It also appears that LMP-2A may have growth-promoting effects in epithelial cells, and LMP-2A and LMP-2B transcripts are amplifiable in most tumours [4, 84]. However, reports show that LMP-2A protein can be detected in only about 50% of NPC [85]. LMP-1 mRNA is more difficult to detect, and protein is identified readily in only 35% of cases. LMP-1 has been identified in all pre-invasive lesions, suggesting that its expression is necessary in early lesions but may not be as essential in established carcinomas [86]. EBV-induced proliferation of epithelial cells may increase the risk of other genetic and epigenetic events that may contribute to tumourigenesis [4]. Elevated titers of IgA antibody to EBV viral capsid antigen (VCA) are usually found in patients with NPC, therefore this method of measuring patients’ EBV-specific IgA antibodies is useful in screening for early detection of NPC [70]. EBNA-1 and EBERs are expressed in all EBV positive cases of NPC [87, 88]. EBV associated NPC has been proposed as a promising target for virus specific immunotherapy, but this can only be successful after the various controversies regarding the roles of EBV and NPC carcinogenesis is resolved.

Though several epigenetic alterations have been observed in NPC, however LMP-1 and E-cadherin (CDH1) appear to be central to the pathology. LMP-1 transfection into carcinoma cells suppressed CDH1 expression, thereby facilitating a more invasive growth. The CDH1 promoter methylation was found in about 50% of primary tumours, increasing with advanced and invasive tumour stages; and correlating strongly with EBV-infection. Thus, EBV appears to contribute to the rapid metastasis, especially when the tumour expresses LMP-1. However, some NPCs have been known to have low-level LMP-1 expression [37].

**Gastric Carcinoma (GC)**

Adenocarcinomas make up about 95% of gastric carcinomas. There are generally 2 distinct types of GCs: intestinal and diffuse. EBV-positive GC make up a little less than 10% of all GCs [89] and they are found in both types. When detected early in any of these types, they have a characteristic diffuse ‘lace pattern’ [90].

The lymphoepithelioma-like morphology with the typical histology of poorly differentiated carcinoma with dense infiltration of lymphocytes is seen in only about 20% of all EBV-positive tumours [91].

EBV positive gastric carcinoma is a non-endemic disease distributed throughout the world with only variations in the proportion of GC that it constitutes [92].

The exact role of EBV in GC is still not fully understood especially when other factors which are known to be important in the development of the tumours are considered. These factors include heredity, H. pylori infection, ingestion of certain food types and occupational exposures to such things as wood dust [93].

Analysis of EBV in GC biopsies indicates that carcinoma is formed by the proliferation of a single EBV-infected cell which indicates that the infection is not subsequent upon tumour development but a crucial part of the carcinogenetic process of EBV positive GCs. The EBV genes expressed are EBNA-1, EBER-1 and EBER-2, and the transcripts from the TGFβ and NF-κB signalling. The LMP-2A-induced apoptosis, and caused anchorage-independent growth. In gastric carcinogenesis, an accidental EBV infection of gastric epithelial cells may occur, through a direct contact with EBV-infected activated lymphocytes. When epithelial latent infection is established after all, the host cell may start methylation of the viral genome, but also its own cellular TSGs. The epigenetically disrupted cell may,
supported by the EBV latency functions, suppress apoptosis, and stimulate proliferative factors and cytokines which attract infiltrating lymphocytes and cause the expansive growth typical for GC [90].

**DIAGNOSIS OF EBV-ASSOCIATED MALIGNANCIES**

The diagnosis of EBV latency or disease from clinical samples has long been a subject of controversy, largely because EBV contributes to morbidity and mortality in healthy subjects and particularly in the immunocompromised hosts. The bone marrow and solid organ transplant recipients represent a special class of immunocompromised patients; the former being without immunocompetent specific anti-EBV lymphocytes for a variable period, while the latter has severely incapacitated lymphocytes, as a direct effect of iatrogenic immunosuppression.

**In Situ Hybridization**

The detection of EBER transcripts by *in situ hybridization* (ISH) is widely regarded as the gold standard and is rightly, the most common method for the molecular diagnosis of EBV-associated malignancies [94]. The EBV DNA genome can also be detected using probes that recognize its BamHI W internal repeat sequences [95], but this is less sensitive. ISH further serves to localize EBV activity to specific cell types within the lesion.

**Immunohistochemistry and Immunocytochemistry**

Latent membrane protein-1 (LMP-1) immunocytochemistry has been shown to be just as effective as EBER-ISH in detecting EBV in PTLD, Hodgkin’s lymphoma, and infectious mononucleosis [96]. However, it is less useful in non-Hodgkin’s lymphoma (NHL) or carcinomas. Other immunostain targets include EBNA-1, EBNA-2, LMP-1, and LMP-2A [87]. Of these factors, only BZLF1 is detectable in lytic infections as in oral hairy leukoplakia.

**Viral Nucleic Acid Amplification**

The detection of EBV nucleic acids can be done using PCR and nucleic acid sequence-based amplification (NASBA); both of which cannot sufficiently differentiate between latency and disease. However, they have found some use in the differential diagnosis of metastatic undifferentiated carcinomas of unknown primary [97] and CNS lymphomas [98]. On the other hand, quantitative PCR has been used quite efficiently for EBV viral load assays; especially in the diagnosis and monitoring of PTLD and nasopharyngeal carcinoma [99, 100]. The test is sufficiently rapid, sensitive and specific.

**Serology**

In the immunocompetent host, EBV-specific ELISA or immuno-fluorescent essays can reliably distinguish acute from previous EBV infection [101]. EBV-associated

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**Table 1. List of Some EBV-Associated Neoplasms and their Peculiar Characteristics**

<table>
<thead>
<tr>
<th>EBV-Associated Neoplasms</th>
<th>Prevalence of EBV</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burkitt’s lymphoma</td>
<td>EBV has been detected in virtually all cases of the endemic variant, 15%-20% of cases of the sporadic variant, and 30%-40% of cases of the immunodeficiency-related variant</td>
<td>only EBNA-1 is detected together with two untranslated RNAs (EBERs) and several micro-RNAs. EBNA-1 enhances tumor progression by causing DNA damage and genomic instability through induction of reactive oxygen species (ROS)</td>
</tr>
<tr>
<td>Nasopharyngeal carcinoma (NPC)</td>
<td>nearly 100% of nonkeratinising NPC tumors carry clonal EBV genomes and express EBV proteins</td>
<td>LMP-1 is expressed in a sub-set of NPC</td>
</tr>
<tr>
<td>Postransplant lymphoproliferative disorders (PTLD)</td>
<td>EBV infects ~60%- 80% PTLD patients, including 100% of early-onset PTLD patients</td>
<td>Most PTLD tumour cells express the latency III pattern of EBV gene expression, with a small proportion of cells undergoing lytic replication. Occasionally latency type I and II patterns have also been detected in PTLD biopsies. In these cases additional genetic or epigenetic changes are probably required for tumour outgrowth</td>
</tr>
<tr>
<td>Classical Hodgkin Lymphoma</td>
<td>EBV infection is associated with approximately 40% of cHL cases, most frequently with the mixed-cellularity subtype</td>
<td>Type II latent infection gene products are typically expressed, i.e., EBERs, EBNA-1, LMP1, and LMP-2A</td>
</tr>
<tr>
<td>Gastric Carcinoma</td>
<td>10% of all cases of gastric carcinoma</td>
<td>all cases of EBV associated gastric carcinoma express latent membrane protein 2A (LMP-2A) which up-regulates the cellular <em>survivin</em> gene through the NFkB pathway, conferring resistance to apoptotic stimuli on the neoplastic cells. EBERs are also expressed in almost every EBV gastric carcinoma cell, suggesting its importance for the development and maintenance of this carcinoma</td>
</tr>
<tr>
<td>T-cell lymphoma</td>
<td>10%</td>
<td>EBNA-1, LMP-1, LMP-2A, LMP-2B</td>
</tr>
<tr>
<td>T/NK nasal type lymphoma</td>
<td>~100%</td>
<td>Absence of T-cell antigens, the expression of the NK cell marker CD56, and the absence of T-cell receptor gene rearrangement.</td>
</tr>
<tr>
<td>B lymphoproliferative diseases</td>
<td>90%</td>
<td>EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, LMP-1, LMP-2A, LMP-2B</td>
</tr>
</tbody>
</table>

EBV = Epstein Barr Virus, EBNA = EBV nuclear antigen, LMP = latent membrane protein, RNA = ribonucleic acid, EBER = Epstein Barr Virus RNA.
malignancies are often characterized by markedly high titres against early antigen and IgG viral capsid antigen. EBNA titres are however reduced, making it nonspecific for malignancy, and thus inadequate for diagnosis [87]. On the contrary, nasopharyngeal carcinoma is usually associated with elevated titres against multiple viral antigens, especially IgA antibodies against lytic antigens, reflecting the tumour’s mucosal origin [102].

Other Methods

Several other techniques have been used to study the EBV-associated malignancies including Southern blot analysis of EBV DNA, culture of EBV or EBV-infected lymphocytes and electron microscopy to examine the detailed morphologic changes associated with EBV infection. Some of these techniques remain relevant even today, for understanding EBV biology and pathology; but are seldom required in the clinical setting. Newer technologies such as gene expression profiling and array are also being refined to further subclassify and prognosticate these diseases [87].

TREATMENT STRATEGIES FOR EBV POSITIVE TUMOURS

Several treatment strategies have been tried in the management of EBV positive tumours. Some of these treatment modalities include.

Combination Cytotoxic Therapies

Combination cytotoxic chemotherapy is still highly favoured in the treatment modalities for PTLDs. The overall survival and response rates have varied in different study groups. There has also been no consensus as to the best chemotherapy regimens and periods of chemotherapy administration. Knowing that standard chemotherapy protocols are associated with high rates of morbidity and mortality, research scientists have tended to favour the use of low dose regimens. The drawback of this is increased relapse rates associated with low dose protocols. In high grade PTLDs therefore, standard regimens such as CHOP are therefore preferred [101-104]. In various other EBV associated malignancies, chemotherapy is also playing significant roles in the treatment protocols. In a recent publication, Kawada et al. [105] showed that tubacin, a small molecule inhibitor of histone deacetylase 6 that also blocks aggresome activity killed Epstein-Barr virus (EBV)-positive Burkitt lymphoma (BL) cells better than EBV-transformed lymphoblastoid cells (LCLs) or EBV-negative BL cells. They showed that tubacin killed EBV-positive BL cells in a caspase-3-independent pathway that involved reactive oxygen species and was blocked by butylated hydroxyanisole. Their study suggested that the combination of a proteasome inhibitor and an HLA-C6 inhibitor may represent a useful strategy for the treatment of certain EBV-associated B cell lymphomas. Further studies using this strategy will need to be carried out.

Induction of Lytic EBV Infection

A very useful strategy currently being tried is the development of EBV-based therapy that involves the purposeful induction of lytic EBV infection in tumours. It has been shown that induction of lytic EBV infection in cancers activates expression of EBV-encoded kinases that convert the prodrug, ganciclovir, to its active cytotropic form. In mouse models for EBV-positive tumours, combining a lytic-inducing chemotherapy with ganciclovir has been found to be much more effective than either agent alone for treating tumours [106]. CD70 cellular protein has been found to be another potential EBV-based target. EBV-positive tumours commonly express CD70, while CD70 expression in normal cells is restricted to a few highly activated B cells and T cells. Anti-CD70 monoclonal antibody inhibits the growth of CD70-positive (but not CD70-negative) Burkitt’s lymphomas in SCID mice. Finally, while completely lytic EBV infection is clearly incompatible with tumour cell growth, Kenney et al. have reported that small numbers of lytically-infected cells actually promote the growth of EBV-immortalized lymphocytes in SCID mice, through the release of paracrine growth factors as well as angiogenic factors. Thus, agents that prevent the earliest stage of lytic EBV infection (such as fatty acid synthase inhibitors), rather than the later stage of viral replication, might also be useful in the treatment of early-stage EBV positive tumours [96].

Kenney et al. have done extensive studies on the use of EBV-targeted therapies in the treatment of EBV-positive tumours using various therapeutic lytic induction strategies. They have described three strategies including gene delivery techniques to induce lytic infection in EBV-tumours and cell lysis, induction of lytic viral gene expression by chemotherapy and radiation therapy, and use of ganciclovir (GCV) to enhance the cytotoxic effect of chemotherapy and radiation in an EBV-dependent manner [96]. It has been observed that lytic induction strategies are most effective for inhibiting tumour growth when combined with the antiviral drug, ganciclovir [107]. Current exciting area of research is the development of therapeutic monoclonal antibodies directed against proteins preferentially expressed in EBV-positive cancers. In this regard, CD20 monoclonal antibody (rituximab), directed against the B-cell specific protein, is now widely used to treat posttransplant lymphoproliferative disorders. In the pharmacologic approaches to the treatment of EBV associated Hodgkin’s lymphoma, butyrate is being combined with ganciclovir as a treatment strategy. The viral thymidine kinase phosphorylates ganciclovir. Ganciclovir, when phosphorylated, inhibits the cellular DNA polymerase, leading to apoptosis in cycling cells. It is hoped that in the combination with butyrate and ganciclovir, butyrate would upregulate these viral enzymes and that ganciclovir would kill EBV-associated tumours [108, 109]. Early results appear to be encouraging, but further studies are required.

Immunotherapy Using EBV-Specific Cytotoxic T Cells

Adoptive immunotherapy with cytotoxic T cells specifically targeting EBV antigens is currently being tried. Several of these strategies have produced impressive results and are being tried not only to treat but also to prevent EBV related lymphoproliferative diseases [110]. In the recent landmark report by Heslop et al. [109], they studied 114 patients who had received infusions of EBV-specific cytotoxic T lymphocytes (CTLs) at three different centers to prevent or treat EBV-positive lymphoproliferative disease arising after hematopoietic stem cell transplantation. Toxicity was minimal, consisting mainly of localized swelling at sites of responsive disease. None of the 101

peptide
patients who received CTL prophylaxis developed EBV-positive LPD while 11 of the 13 patients treated with CTLs for boipsy-proven or probable LPD achieved sustained complete remissions. In 2002, Comoli et al. [111], also reported impressive reports. Their study was to assessed whether transfer of EBV-specific cytotoxic T lymphocytes (CTLs) generated in vitro from the peripheral blood of allograft recipients receiving immunosuppression could increase EBV-specific killing in vivo without augmenting the probability of graft rejection. Autologous EBV-specific CTLs were generated for 23 patients who were identified as being at risk of developing PTLD through the finding of elevated EBV DNA load. Of the 23 patients, 7 received 1 to 5 infusions of EBV-specific CTLs. CTL transfer was well tolerated, and none of the patients showed any evidence of rejection. An increase of the EBV-specific cytotoxicity was observed after infusion, notwithstanding continuation of immunosuppressive therapy. EBV DNA levels had a 1.5- to 3-log decrease in 5 patients, whereas in the other 2 graft recipients CTL transfer had no apparent stable effect on EBV load. They concluded by suggesting that the infusion of autologous EBV specific CTLs obtained from peripheral blood mononuclear cells recovered at the time of viral reactivation is able to augment virus-specific immune response and to reduce viral load in organ transplant recipients. Also, as far back as 1998, Rooney et al. [112] had reported excellent results when they studied the safety and efficacy of adoptively transferred gene-marked virus-specific cytotoxic T lymphocytes (CTLs) as prophylaxis and treatment of EBV associated lymphoproliferative disorders in recipients of T-cell-depleted allogeneic bone marrow. In 42 patients treated prophylactically, no toxicity was experienced. None of these patients developed EBV associated lymphoproliferative disorders, in contrast with eight of 53 (15%) patients who did not receive prophylactic CTL. Three patients who had not received CTL developed aggressive disease and received CTL as treatment. Gene-marked CTL homed to tumour sites and selective accumulation of marker gene was detected in tumour tissues. Tumours regressed completely in two patients, but the third died of respiratory failure. Infused CTLs persisted for up to 3 years in vivo, they rapidly reconstituted EBV-specific immune responses to levels seen in normal individuals, and they reduced high viral titers by two to three logs. Since then, their team has continued using autologous EBV-specific CTL to treat patients with relapsed EBV-positive Hodgkin’s disease. However Rooney et al. [111] cautioned that the prophylactic use of EBV-specific cytotoxic T cells is safe and effective, but that its infusion as a therapeutic strategy in rapidly progressive and invasive disease can cause damage and may be ineffective in advanced cases. All these various reports confirm the promising role of immunotherapy in the treatment of EBV positive tumours. However, further studies are required to optimize this treatment strategy especially in advanced aggressive diseases.

CONCLUSION

Epstein-Barr virus infection has been implicated in the pathogenesis of several cancers. Recent data indicates the role of EBV in the epigenetic alterations in several types of cancers including gastric carcinoma, nasopharyngeal carcinoma, and lymphomas. Several trials in the treatment of EBV associated cancers include the use of combination cytotoxic chemotherapy, purposeful induction of lytic EBV infection in tumours and adoptive immunotherapy using EBV-specific cytotoxic T cells. Further trials should be aimed at optimising these various treatment protocols.

REFERENCES

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