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“Immunophenotypic and molecular characterization of EBV-driven age-related lymphoproliferative disorders”

Tutor:
Prof. Luigi Ruco

Candidate:
Arianna Di Napoli

Coordinator:
Prof. Angela Santoni

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INDEX

1. INTRODUCTION

1.1 EBV-induced lymphomagenesis	pag. 3
1.2 EBV-associated lymphoproliferative disorders	pag. 7
1.3 Hodgkin Lymphoma and EBV	
1.3.1 Classification and morphology	pag. 15
1.3.2 Epidemiology	pag. 16
1.3.3 Pathogenesis and role of EBV	pag. 17
1.3.4 Genetic alterations and deregulated signalling	pag. 19
1.3.5 Tumor microenviroment	pag. 20

2. PERSONAL CONTRIBUTION

2.1 Aim	pag. 23
2.2 Materials and Methods	pag. 24
2.3 Results	pag. 30
2.4 Discussion	pag. 41

REFERENCES	pag. 48
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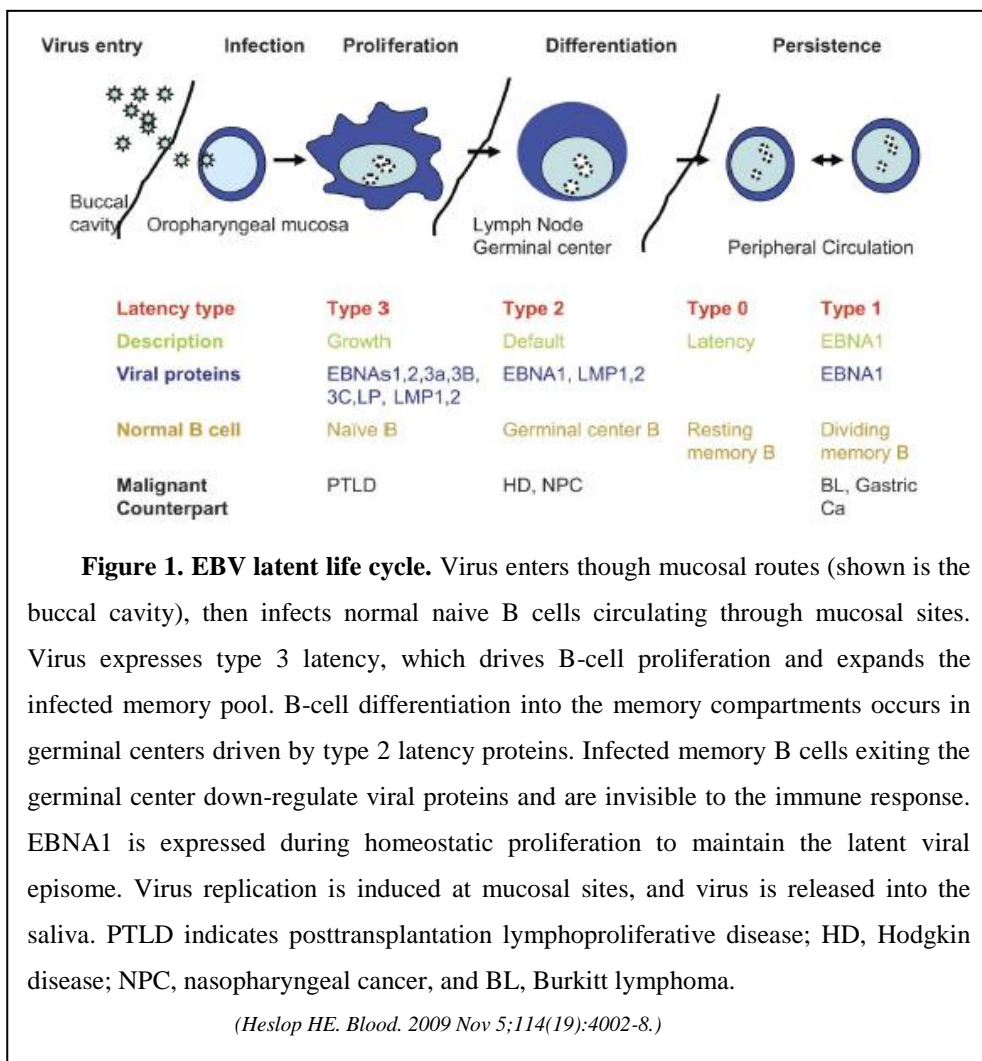
INTRODUCTION

1.1 EPSTEIN BARR VIRUS-INDUCED LYMPHOMAGENESIS

Epstein Barr Virus (EBV) is a member of the herpes virus family that infects more than 90% of the worldwide population (*Rickinson et al, 2006*). In developing countries, primary lytic infection occurs in the oropharynx in early childhood usually asymptotically, while, in developed countries of high socio-economic status it is delayed until adolescence and one third of the cases results in infectious mononucleosis (*Rickinson et al, 2006; Henle et al, 1968*). In the oropharynx, EBV infects via the CD21 (CR2) receptor epithelial cells lining the inner mucosal surfaces of the mouth and nose, and naïve B cells circulating through mucosal site. EBV is highly immunogenic and it induces a vigorous immune response so that, soon after primary infection, EBV persists as an episome in a small proportion of infected B cells, establishing latent infection (*Niedobitek et al, 1997*). There are 4 different types of latency, each characterized by a specific pattern of EBV antigen expression (Figure 1) (*Heslop, 2009*). Most circulating infected memory B cells down-regulate viral antigens (type 0 latency) and are invisible to the immune system. In type 1 latency the nuclear protein EBNA-1 is expressed in proliferating B cells where it induces replication of the viral episome. Type 2 latency is associated with the expression of EBNA-1, and of the membrane proteins LMP-1 and LMP-2 in germinal center B cells. In type 3 latency infected B cells expressed all nuclear proteins (EBNA-1, -2, -3A, 3B, 3C, and LP), LMP-1 and LMP-2, and 2 small RNAs (EBERs). Type 3 latency is the only type able to transform primary B cells *in vitro*; however, it is also the most immunogenic, so that it is rarely detected in healthy persons probably due to a rapid and efficient EBV-specific T-cell response (*Rowe et al, 1992; Ma et al, 2011*).

It has been shown that *in vitro* EBV infects resting B cells and transform them through complex mechanisms into permanently growing lymphoblastoid cell lines. This property makes it a candidate causative for many human cancers including epithelial and haematopoietic tumors (*Rickinson et al, 2006*). Although the true contribution of EBV to the lymphomagenesis remains to be elucidated, it is thought that EBV may enable EBV-infected B cells to exit the cell cycle to become a resting memory B-cell, resulting in their immortalization and continuous proliferation (*Sugden et al, 1989; Saha et al, 2011*) EBNA-1 is a master transcription factor that is required for immortalization; it upregulates p53 expression levels and a variety of other latency genes, as well as host activation proteins (*Yates et al, 1984; Saridakis*

et al, 2005). EBNA-2 has been suggested to be involved in G0 to G1 phase transition however, although it appears essential for initial growth transformation of infected B cells *in vitro*, it seems to have a secondary role in tumor progression (*Johannsen et al*, 1995). EBNA-3A and -3C are also essential for B-cell immortalization acting through different pathways (Table 1) (*Anderton et al*, 2008; *White et al*, 2010). Among EBV-encoded proteins, LMP-1 is the major transforming one; it has been shown to act as an oncogene in rodent fibroblast transformation assay (*Wang et al*, 1985; *Kaye et al*, 1993). There are evidences that LMP1 acts as a constitutively active receptor; in particular, LMP-1 mimics activated CD40, a member of the tumor necrosis factor receptor family, stimulating growth and differentiation responses in B cells (*Uchida et al*, 1999). Moreover, LMP-1 activates the nuclear factor (NF)-kB signalling



cascade (*Zhimin et al*, 2000), and up-regulates the expression of the anti-apoptotic proteins Bcl-2 and A20 stimulating B-cell growth (*Kulwichit et al* 1998; *D'Souza et al*,2004).

An impairment in T-cell response against infected B-cells seems to be crucial for the expansion of transformed cells (*Thorley-Lawson*

et al, 2004; *Hislop et al*, 2007). Immune control of EBV is mediated primarily by T cells; in particular, while CD8⁺ T cells target primarily the EBNA3 and LMP2 proteins, CD4⁺ T cells most frequently react to EBNA1 (*Murray et al* 1992; *Khanna et al*, 1992; *Munz et al*, 2000; *Leen et al*, 2000). Recently, White et al suggested that loss of EBNA3B function in tumor cells

through *EBNA3B* gene mutation cause substantial changes in the character, immune evasion, and aggressiveness of EBV-associated cancer. In this context, escape from HLA-A11–restricted recognition of EBNA3B was previously suggested as the cause of the uncontrolled growth of a post-transplant DLBCL (*White et al, 2012*).

Table 1. Proposed Functions of EBV Latent Antigens and the Associated B-cell Lymphomas

Latent proteins	Functions	Associated lymphomas	Latency type
EBNA-1	Essential for B-cell immortalization, replicates EBV genome, segregates viral episomes at mitosis, blocks interaction between HAUSP and p53 to facilitate p53's degradation	BL Hodgkin's disease AIDS-associated lymphomas Posttransplant lymphoproliferative disorders	I, II, III
EBNA-2	Transcriptional coactivator that upregulates expression of viral (LMP1) and cellular genes (<i>c-myc</i>), essential for B-cell immortalization	AIDS-associated lymphomas Posttransplant lymphoproliferative disorders	III
EBNA-3A	Essential for B-cell immortalization of cell, interacts with RBP-J κ , regulates Notch-signaling pathway	AIDS-associated lymphomas Posttransplant lymphoproliferative disorders	III
EBNA-3B	Not essential for B-cell immortalization, interacts with RBP-J κ	AIDS-associated lymphomas Posttransplant lymphoproliferative disorders	III
EBNA-3C	Essential for B-cell immortalization, overcomes various checkpoints in cell cycle, interacts with RBP-J κ , activates LMP1, blocks p53-dependent apoptosis, enhances kinase activity of both cyclin D1/CDK6 and cyclin A/CDK2 complexes, induces degradation p27 ^{KIP1} and pRb, stabilizes c-Myc, Cyclin D1, and Mdm2, manipulates host chromatin remodeling machinery	AIDS-associated lymphomas Posttransplant lymphoproliferative disorders	III
EBNA-LP	Interacts with EBNA2 to inactivate p53 and pRb, interacts with transcription factors in notch signaling pathway, contributes to B-cell immortalization	AIDS-associated lymphomas Posttransplant lymphoproliferative disorders	III
LMP-1	Mimics CD40 ligand-binding signal, stimulates bcl-2 and $\alpha 20$ expression to block apoptosis, acts as a constitutively active receptor for stimulating many cellular genes, regulates NF- κ B, JAK/STAT, ERK MAPK, IRF, and Wnt signaling pathways, essential for B-cell immortalization	Hodgkin's disease AIDS-associated lymphomas Posttransplant lymphoproliferative disorders	II, III
LMP-2A and -2B	Drives EBV into latency, LMP-2A blocks BCR signaling and -2B assists its function, not essential for B-cell immortalization	Hodgkin's disease AIDS-associated lymphomas Posttransplant lymphoproliferative disorders	II, III
EBERs	Form complexes with La and L22, associate with PKR, induce IFN and IL-10, bind to RIG-1 to activate type I IFNs, not essential for B-cell immortalization	BL Hodgkin's disease AIDS-associated lymphomas Posttransplant lymphoproliferative disorders	I, II, III
BARFs	Protein products may modify Notch signaling, not essential for B-cell immortalization	BL Hodgkin's disease AIDS-associated lymphomas Posttransplant lymphoproliferative disorders	I, II, III

(Saha A. et al. Clin.Cancer Res. 2011 May15;17 (10):3056-3063)

Alternatively, the disruption of the normal balance between latently infected B-cell proliferation and the EBV-cytotoxic T-cell response could be due to an immunodeficient status of the patient or to the administration of immunosuppressive agents (e.g. for organ transplantation). In support of this view, is the frequent evidence of EBV infection in

neoplastic cells of B-cell lymphoproliferative disorders (LPD) occurring in patients with primary immune deficiency, HIV infection, or with iatrogenic immunosuppression (*Swerdlow et al, 2008*).

1.2 EBV-ASSOCIATED LYMPHOPROLIFERATIVE DISORDERS

Since its discovery as the first human tumorigenic virus, EBV has been implicated in the development of a wide range of B-cell (Table 2) and of T/NK-cell lymphoproliferative disorders (Table 3). EBV-associated malignancies arise in both immunosuppressed and immunocompetent individuals, and involve the expression of either some or all of the EBV latent proteins (Figure 1).

1.2.1 EBV-ASSOCIATED B-CELL LYMPHOPROLIFERATIVE DISORDERS

Despite its documented infection in T lymphocytes and epithelial cells, EBV has a major preference for B cells, and under certain circumstances the infected B cells can transform into malignant B-cell lymphomas (*Bajaj et al, 2007*). They include: Hodgkin lymphoma, Burkitt lymphoma, post-transplant lymphoproliferative disorders, lymphomatoid granulomatosis, age-related EBV-associated B-cell lymphoproliferative disorders, diffuse large B cell lymphoma associated with chronic inflammation, and many B-cell lymphomas associated with HIV-infection (*Swerdlow et al, 2008; Carbone et al, 2008*). Different types of EBV-latency are associated with these lymphomas (Figure 1).

Burkitt's Lymphoma

Burkitt's lymphoma (BL) is an aggressive B-cell tumor, often presents in extranodal sites or as an acute leukemia, characterized by the expansion of medium-sized cells with a very high proliferation index (>98%). It includes three variants: endemic (mainly in children from equatorial Africa, Papua, and New Guinea), sporadic (affecting children and young adults throughout the world), and immunodeficiency-related (mostly associated with HIV-infection). EBV has been detected in virtually all cases of the endemic variant, 20%–30% of the cases of the sporadic variant, and 30%–40% of cases of the immunodeficiency-related variant (*Carbone et al, 2008*). In all EBV-positive cases, EBV is found in the majority of the neoplastic cells with a type I latency pattern given by the expression of EBNA-1 and EBERs only (*Weiss et al, 2012*). In endemic Burkitt lymphoma the strong epidemiological link with holoendemic malaria suggests a polymicrobial pathogenesis (*Rochford et al, 2005*). By this model, infection

with Plasmodium Falciparum could reactivate latently infected B cells through toll like receptor 9 (TLR-9) and, at the same time, impact on immunity exhausting EBV-specific T cell response. In all variants, irrespective of EBV status, translocation of the c-myc oncogene into one of the immunoglobulin loci is undoubtedly the key factor in the oncogenesis of Burkitt's lymphoma (*Della Favera et al, 1982; Taub et al, 1982; Hummel et al, 2006*). There is some evidence that EBV-BL arises from a latency pattern-3 progenitor that under a selection pressure down-regulates the c-myc antagonist EBNA-2 (*Weiss et al, 2012*). The detection of somatic hypermutations in the V region of clonally rearranged immunoglobulin genes and the phenotype of the lymphoma cells indicate a germinal center (GC) cell origin of the Burkitt lymphoma (*Chapman et al, 1996*).

Table 2. EBV-associated lymphoproliferative disorders
EBV-associated B-cell lymphoproliferative disorders
Burkitt's lymphoma
Classic Hodgkin's lymphoma
Post-transplant lymphoproliferative disorders
HIV-associated lymphoproliferative disorders
Primary central nervous system lymphoma
Diffuse large B-cell lymphoma, immunoblastic
HHV-8-positive primary effusion lymphoma and its solid variant
Plasmablastic lymphoma
Other histotypes (rare) ^a
^a Other histotypes include: lymphomatoid granulomatosis, pyothorax-associated lymphoma, senile EBV-associated B-cell lymphoproliferative disorders. Abbreviations: EBV, Epstein-Barr virus; HHV-8, human herpesvirus 8.

(*Carbone A et al. Oncologist. 2008 May;13(5):577-85.*)

EBV-Associated Lymphomas in Immunocompromised Individuals

There exist several distinct EBV-associated lymphoproliferative disorders (LPD) in immunocompromised individuals. Some LPD are associated with primary immune disorders such as X-linked lymphoproliferative syndrome (XLP), severe combined immunodeficiency (SCID), hyper-IgM syndrome, common variable immunodeficiency (CVID), Wiskott-Aldrich syndrome (WAS), while others are associated with immunosuppressive drugs given to transplant recipients (PTLDs) or with HIV-infection. Finally, there are lymphoproliferative

disorders that arise in patients treated with immunosuppressive drugs for autoimmune diseases or for conditions other than in the transplant setting (*Swerdlow et al, 2008*). The most common EBV gene-expression pattern in these disorders is latency III (Figure 1).

Post transplant lymphoproliferative disorders (PTLDs)

PTLDs are lymphoproliferative disorders that develop as a consequence of immunosuppression in transplant recipients of both a solid organ and bone marrow. According to the World Health Organization classification (*Swerdlow et al, 2008*), PTLDs may be classified into: (a) early lesions, generally represented by EBV-driven polyclonal or oligoclonal lymphoproliferations, and (b) true monoclonal diseases, that are not necessarily associated with EBV infection, including polymorphic PTLDs and monomorphic PTLDs. The latter are indistinguishable from those that occur in immunocompetent individuals and, are further distinguished into Burkitt's lymphoma/Burkitt's-like lymphoma, diffuse large B-cell lymphoma (DLBCL), and cHL.

Oncogenic viruses known to be involved in PTLD pathogenesis include EBV and human herpes virus 8 (HHV-8). Several lines of evidence suggest that EBV infection has a major pathogenetic role in PTLDs: (a) EBV infects 70%– 80% PTLD patients, including 100% of early-onset PTLD cases; (b) in many monomorphic PTLD cases, EBV infection is monoclonal, suggesting that the virus might be present in the tumor progenitor cell since the early phases of clonal expansion; (c) an increase in the EBV viral load and a decrease in the number of EBV-specific cytotoxic T lymphocytes (CTLs) have found to be strongly associated with PTLD development; and (d) treatment of PTLDs with autologous EBV-specific CTLs results in viral load control and tumor size reduction (*Davis et al, 2004; Carbone et al, 2008*). Up to 30% of PTLD are EBV-negative, and although some can be driven by HHV-8, others can still be triggered by a no longer detectable EBV or, alternatively, by other unknown viruses (*Swerdlow et al, 2008*).

Other iatrogenic immunodeficiency-associated lymphoproliferative disorders

Other iatrogenic LPD can occur in patients treated with immunosuppressive drugs for autoimmune diseases. It is actually difficult to determine how many LPD are directly related to

the iatrogenic immunosuppression rather than the underlying disorder, however, it is likely that risk and type of LPD depend on the type of immunosuppressive agent (e.g. methotrexate, infliximab, TNF blocks) and on the nature of the underlying disorder (e.g. rheumatoid arthritis, inflammatory bowel disease, dermatomyositis, psoriasis). Most of the cases are diffuse large B cell lymphomas, while others are more polymorphic or resemble Hodgkin lymphoma. In half of the cases the localization is extranodal, and about 50% of the cases are EBV-positive (*Au et al, 2006; Swerdlow et al, 2008*).

HIV-Associated Lymphoproliferative Disorders

HIV-associated lymphoproliferative disorders are a heterogeneous group of diseases that arise in the presence of HIV-associated immunosuppression, a state that permits the unchecked proliferation of EBV-infected lymphocytes. Traditionally, these aggressive disorders include both central nervous system and systemic lymphomas. Pleural effusion lymphomas (PEL) also occurs and often involves EBV in addition to HHV-8 (*Carbone et al, 2008*). The categories of HIV-associated lymphomas included in the latest WHO classification of tumors of haematopoietic and lymphoid tissues are grouped as follows: (a) lymphomas also occurring in immunocompetent patients such as: Burkitt's lymphoma (30% EBV+), systemic DLBCL with centroblastic features (30% EBV+), and DLBCL with immunoblastic features frequently involving the central nervous system (100% EBV+); (b) unusual lymphomas occurring more specifically in HIV-positive patients, including: PEL (80% EBV+ in addition to 100% HHV8+) and plasmablastic lymphoma of the oral cavity (60-70% of the oral cavity); (c) lymphomas also occurring in other immunodeficiency states (*Swerdlow et al, 2008*).

EBV+ diffuse large B cell lymphoma of the elderly

EBV+ diffuse large B cell lymphoma of the elderly is an EBV+ lymphoma occurring in patients over 50 years with no other cause of immunodeficiency. It represents up to 10% of elderly lymphomas in Asian population and about 4% of DLBCL in Western countries (*Gibson et al, 2009; Hoeller et al, 2010*). Its incidence increases with increasing age, and is slightly predominant in male. Most patients (70%) have an extranodal disease, including skin, lung, tonsil, and stomach. The disease is clinically aggressive with a poor prognosis.

Morphologically, it is subclassified in polymorphic and monomorphic. The polymorphic subtype show a proliferation of B cells with a broad range of maturation, whereas in the monomorphic variant neoplastic B cells have either centroblastic or immunoblastic features. In both subtypes are present Hodgkin and Reed Stenberg(H/RS)-like cells. EBER is positive in the neoplastic cells, whereas LMP-1 may be negative. The EBV latency pattern is of type III. It is believed that the development of this lymphoma is related to immunosenescence that is part of the aging process (*Swerdlow et al, 2008*).

Diffuse large B cell lymphoma (DLBCL) associated with chronic inflammation

The disease arise in the context of local long-standing inflammation, usually after decades after the onset of the inflammation. Therefore, patients are usually old with a male predominance. Most cases are reported to be associated with pyothorax occurring many years after artificial pneumothorax for pulmonary tuberculosis. Also, it has been described in bones of patients with chronic osteomyelitis, joints, and soft tissues. It is an aggressive lymphoma with about 25% of 5-year overall survival. Histologically, it resembles EBV+ diffuse large B cell lymphoma of the elderly, and as for it, EBV latency pattern is type III in more than 60% of the cases (*Swerdlow et al, 2008*). It is supposed that local chronic inflammation may favour immune escape of EBV-transformed B cells trough the production of large amount of IL-10, an immunosuppressive cytokine, and that it may also provide autocrine to paracrine tumor growth via the production of IL6 (*Kanno et al, 1996; Kanno et al, 1997*).

Lymphomatoid granulomatosis

Lymphomatoid granulomatosis is a rare angiocentric and angiodestructive lymphoproliferative disease involving extranodal sites (i.e. lung, skin, kidney, brain and, liver). It often develops in patients with an underlying immunodeficiency (e.g. post-organ transplantation therapy, Wiskott-Aldrich syndrome, HIV-infection, and others). Morphologically, It is composed of a small number of large EBV-positive B cells associated with numerous small reactive T cells (*Swerdlow et al, 2008*).

1.2.2 EBV-ASSOCIATED T/NK-CELL LYMPHOPROLIFERATIVE DISORDERS

T-cell lymphoproliferative disorders that have been reported to be EBV associated include a subset of peripheral T-cell lymphomas (PTLC), angioimmunoblastic T-cell lymphoma (AILT), extranodal nasal type NK/T-cell lymphoma, enteropathy-type T-cell lymphoma, hepatosplenic T-cell lymphoma, systemic EBV+ T-cell Lymphoproliferative disease of childhood, Hydroa vacciniforme-like lymphoma, and aggressive NK-cell leukemia/lymphoma. However, many T cell lymphoma may contain scattered EBV+ cells as a reflection of the generalized immunodeficiency that is sometimes part of a peripheral T-cell lymphoma (*Carbone et al, 2008; Swerdlow et al 2008*).

Table 3. EBV-associated lymphoproliferative disorders
EBV-associated T/NK-cell lymphoproliferative disorders
Peripheral T-cell lymphoma, unspecified
Angioimmunoblastic T-cell lymphoma
Extranodal nasal T/NK-cell lymphoma
Other histotypes (rare) ^a
^a Other histotypes include hepatosplenic T-cell lymphoma, nonhepatosplenic $\gamma\delta$ T-cell lymphomas, enteropathy-type T-cell lymphoma. Abbreviations: EBV, Epstein–Barr virus; NK, natural killer.

(*Carbone A et al. Oncologist. 2008 May;13(5):577-85.*)

Angioimmunoblastic T cell lymphoma (AILT)

AILT is one of the most common PTCL subtype. It is characterized by systemic disease, a polymorphous infiltrate primarily involving lymph nodes, and prominent proliferation of high endothelial venules and follicular dendritic cells (*Swerdlow et al 2008*). The clinical behavior is very aggressive with a scarce response to therapy. The molecular pathogenesis of AILT, as in general for all peripheral T-cell neoplasms, is poorly understood. Characteristically, AILT is a lymphoma in which expanding B-cell clones are often present beside the T-cell clones. AILT is also associated with EBV in a high proportion of cases (97%), but in this lymphoma, the EBV is seen mainly in the B lymphocytes and in large atypical H/RS-like B-cells (*Zettl et al,*

2002; Quintanilla-Martinez *et al*, 1999; Smuk *et al*, 2010). The presence of EBV in only a subpopulation of cells suggests that EBV infection is not part of the pathogenesis of the disease, but a manifestation of the diminished local immune surveillance or alternatively, that the viral genome has been lost from the malignant T cells.

Extranodal NK/T-cell lymphoma nasal type

Extranodal NK/T-cell lymphoma nasal type is a predominantly extranodal lymphoma characterized by vascular damage by a population of neoplastic cells characterized by absence of T-cell antigens and by expression of the NK cell marker CD56. Clinically, these tumors occur in the nasal and upper aerodigestive track, and it is more common in Asian and in native Americans of Central and South America. EBV is consistently associated with these lymphomas, regardless of geographical location. Tumor cells are always positive for EBER, and mostly negative for LMPI-1 (Swerdlow *et al* 2008; Weiss *et al* 2012).

Systemic EBV+ T cell lymphoproliferative disease (LPD)

Systemic EBV+ T-cell LPD is a recently recognized clonal proliferation of EBV-infected T cells with an activated cytotoxic phenotype (TIA-1+). It occurs mainly in children and young adults frequently from Asia and Mexico. It can occur shortly after primary acute EBV infection or in the setting of chronic active EBV infection (CAEBV). It is a systemic disease with involvement of liver, spleen, lymph nodes, bone marrow, skin, and lungs. The clinical course is very aggressive with death occurring just after days or weeks. EBER is consistently positive in neoplastic CD8+ T cells. Its association with primary EBV infection strongly suggest the presence of a genetic defect in the host immune response to EBV that may predispose to EBV+ T cell LPD (Swerdlow *et al* 2008; Weiss *et al* 2012).

Hydroa vacciniforme like- lymphoma

It is an EBV+ cutaneous T-cell lymphoma occurring in children and adolescence and associated with hypersensitivity to insect bites and sun. It is seen mainly in Asian and in native

Americans of Central and South America. It involves sun-exposed skin with papulo-vescicular lesions that ulcerates. Clinical course is variable, it may be indolent for several years, or may consists in recurrent skin lesions before progression to systemic involvement. Neoplastic T-cells consistently express EBER, whereas LMP-1 is generally negative(*Swerdlow et al 2008*).

1.3 HODGKIN LYMPHOMA AND EBV

1.3.1 Classification and morphology

Based on differences in histological characteristics, cell of origin, clinical features, and molecular pathogenesis HL is categorized in 2 distinct entities: nodular lymphocyte predominant lymphoma (NLPHL), and classical Hodgkin Lymphoma (cHL). The latter is further subclassified into 4 variants: nodular sclerosis (NS), mixed cellularity (MC), lymphocyte-rich (LR), and lymphocyte-depleted (LD) (*Swerdlow et al 2008*).

A striking feature of both cHL and NLPHL is that in the affected lymph node malignant cells are very rare (0.1-2% of tumor mass) and are scattered in a background rich in small lymphocytes, plasma cells, histiocytes and eosinophils (Figure 2). In cHL the neoplastic cells are referred to as Hodgkin and Reed Sternberg (H/RS) cells. Hodgkin cells are large and mononucleated with prominent eosinophilic nucleoli, whereas Reed Sternberg cells are bi- or multinucleated (Figure 2A). In NLPHL tumor cells are indicated as lymphocyte predominant (LP) cells (formerly called L&H cells) and show a multilobated morphology generally lacking evident nucleoli (“popcorn” cells) (Figure 2B) (*Swerdlow et al 2008*).

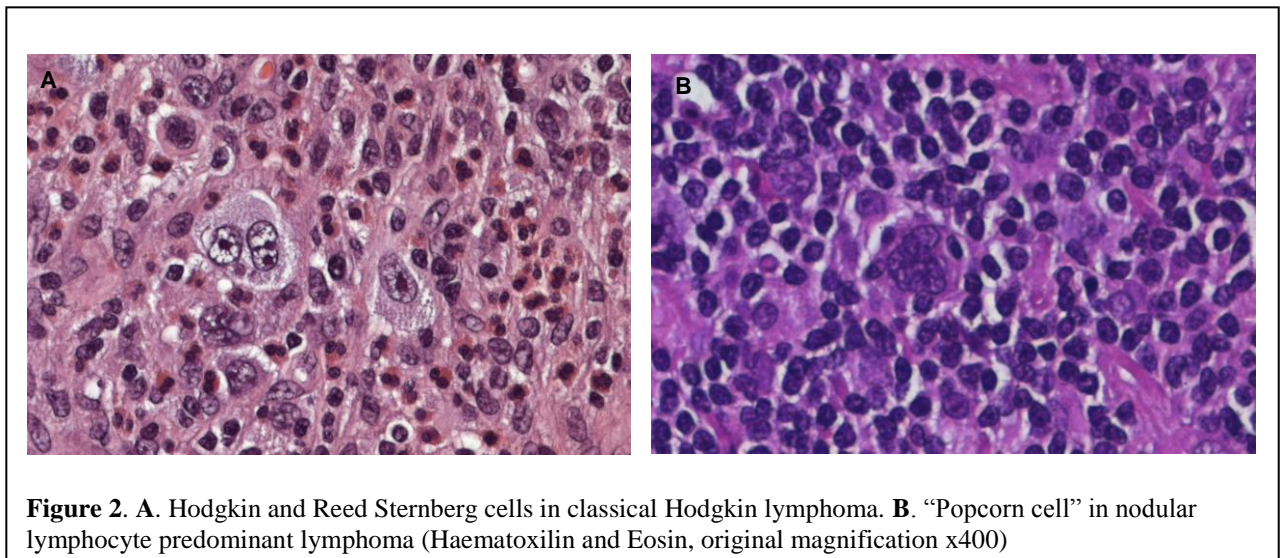


Figure 2. A. Hodgkin and Reed Sternberg cells in classical Hodgkin lymphoma. B. “Popcorn cell” in nodular lymphocyte predominant lymphoma (Haematoxylin and Eosin, original magnification x400)

The cellular origin of the tumor cells in HL remained elusive for many years, since

The cell of origin of HL tumor cells was elusive for many years until analysis of immunoglobulin gene rearrangement in microdissected tumor cells revealed that these cells are

frequently clonal B cells with somatically mutated immunoglobulin V genes (*Kuppers et al, 1994; Kanzler et al, 1996; Kuppers et al, 1994*). However, except of LP cells, that consistently express the B-cell markers CD20, CD79a, CD19, and the transcription factors PAX5, OCT2 and BOB1, the phenotype of the H/RS does not reveal its B cell origin. Indeed, apart from low expression of PAX5 and a focal staining for CD20 in about 20% of the cases, all the other markers of B cell lineage are down regulated in cHL (*Tzankov et al, 2003; Saez et al, 2002; Kuppers 2009 Hematol; Browne et al, 2006*). Characteristically, H/RS cells and not LP cells express CD30 a member of the tumor necrosis factor receptor (TNFR) family. Also, in about 85% of the cases there is coexpression of CD15, a myeloid-associated marker. Aberrant expression of T-cell (*Dallenbach et al, 1989; al Saati et al, 1997; Tzankov et al, 2005*) or dendritic cell-associated markers (*Pinkus et al, 1997; Sorg et al, 1997; Peh et al, 2001*) has also been reported in a minority of cHL. Although most of the cases expressing a T-cell phenotype are also found to be of B-cell origin based on molecular analysis, a T-cell origin has also been suggested in few reported cases based on the presence of T cell receptor gene rearrangements in tumor cells (*Muchen et al 2000; Seitz et al, 2000*).

1.3.2 Epidemiology

Hodgkin lymphoma (HL) is one of the most common lymphomas in the Western World with an incidence of 3 cases per 100 000 persons-year (*Farrell et al, 2011*). Classical Hodgkin lymphoma accounts for more than 95% of all HL and is associated with EBV in about 30-40% of the cases (*Swerdlow et al, 2008*). The highest incidence of cHL is in Caucasians, followed by African Americans and Hispanics, with the lowest observed in Orientals. However, an increasing

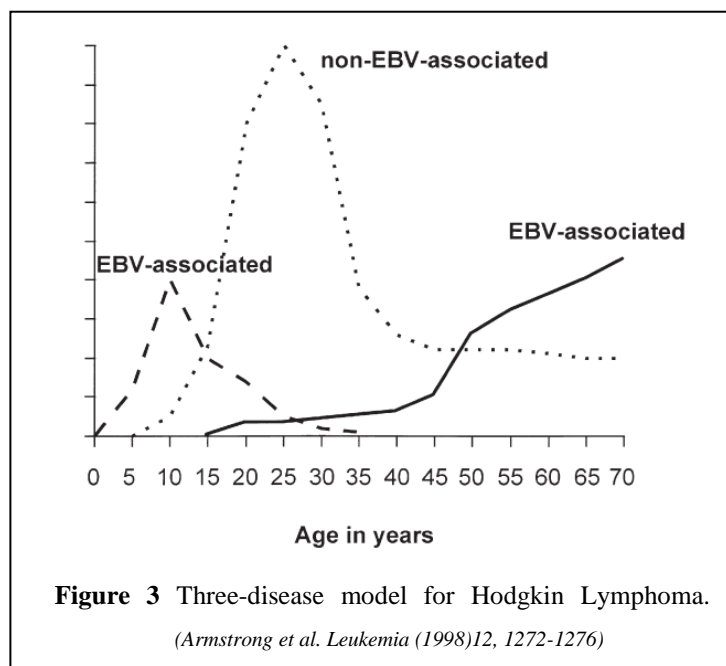


Figure 3 Three-disease model for Hodgkin Lymphoma. (*Armstrong et al. Leukemia (1998)12, 1272-1276*)

incidence of cHL was reported among Chinese immigrants in Western Countries (*Huang et al,*

2011). Epidemiologic and molecular findings suggest that cHL is not a single disease but consists of more than one entity. Indeed, cHL may occur in different geographic and socioeconomic settings with distinct pathological and clinical characteristics (Carbone *et al*, 2011). In 1998 Armstrong *et al*. proposed a “three-disease model” based on age presentation, histological type and EBV association (Figure 3) (Armstrong *et al*, 1998). The first entity is largely an EBV-associated disease of childhood, with higher incidence in developing countries and of usually MC type. The second entity predominantly affects older adults, and is also usually of the MC subtype and EBV associated. The third one occurs in young adults, is more prevalent in developed countries, is not associated with EBV, and is usually of the NS subtype. Harris *et al*. suggested three epidemiological patterns of cHL based on socioeconomic level. The first pattern is seen in poorly developed countries, occur in early childhood, and is mainly of the MC subtype; the second pattern is observed in developing and transitional economies, affects both children and adolescence, and shows equal frequency of MC and NS subtypes; the third one occurs in developed countries and display a third decade peak and a predominance of NS subtype (Harris *et al*, 1998). Another HL classification is based on patients’s immunological status, by which cHL may occur: a) in the general population, or b) in immunosuppressed hosts (associated with HIV-infection or with iatrogenic immunosuppression) (Carbone *et al*, 2011). However, despite all these categorizations, there are several lines of evidence that suggest that these epidemiological patterns are not tight and that transition may exists among different forms of the disease.

1.3.3 Pathogenesis and role of EBV

EBV is found in up to 40% of cHL, whereas is rarely found in NLPHL (Kuppers *et al*, Nature 2009). The association varying with age (more frequent in children and older adults), gender (more frequent in males), geography and socioeconomic status (higher in Asia and in Central and South America), histology (more likely in MC and LD subtypes), and host conditions (nearly all cases of HL occurring in patients with AIDS are EBV+) (Kuppers *et al*, Nature 2009; De Re *et al*, 1993). In EBV+ tumors EBV is detected in the majority of H/RS cells and it is found to be clonal, suggesting that infection occurred prior to transformation of B cells (Weiss *et al*, 1987; Weiss *et al*, 1989; Gulley *et al*, 1994).

EBV+ H/RS cells exhibit a type II latency phenotype, with the expression of a limited number of latency genes, including EBNA1, LMP1, LMP2 and EBERs. EBNA 1 is essential

for maintaining viral genome as an episome. Moreover, it may support tumor development upregulating CCL20, an attracting chemokine for Tregs, and downregulating the protein-tyrosine phosphatase-k, which is a putative tumor suppressor gene (*Baumforth et al, 2008; Flavell et al, 2008*). LMP-1 is an oncogene that mimicking an active CD40 receptor constitutively activates NF- κ B in tumor cells (*Kilger et al, 1998*). LMP-2 can replace the function of a BCR, and *in vitro* is essential for the rescue and transformation by EBV of germinal center (GC) B cells lacking functional BCRs (*Bechter et al, 2005*). Thus, it appears that through the BCR and the CD40 signaling EBV may play a critical role in the initial events of HL pathogenesis, rescuing from apoptosis EBV-infected GC cells carrying BCR destructive mutations (*Mancao et al, 2005; Chaganti et al, 2005*). In addition, LMP-1 and LMP-2 contribute to the downregulation of the B-cell phenotype in EBV+ H/RS cells through activation of the Notch1 pathway (*Portis et al, 2003; Vockerodt et al, 2008*).

The demonstration that a virus strain carrying a 30bp deletion in the LMP1 gene was more tumorigenic than the prototype B95.8 LMP1 led to numerous studies of the prevalence of this virus strain within EBV-associated cancers, including HL. In general, virus strains carrying this 30bp deletion occur with a similar frequency in virus-positive tumour patients and in healthy donors from the same geographical region. The exception to this is HL, where some studies have shown an increased incidence of this deletion variant in HIV-positive HL compared to HIV-negative HL, and in pediatric HL compared to normal controls (*Hu et al, 1993; Santon et al, 1998*).

Nevertheless, most adults that carry EBV never develop cHL, and in up to 60% of cHL there is no evidence of EBV in malignant cells. Although the morphology, phenotype and gene expression profile of EBV-associated and EBV-negative cases of cHL appear similar, there is increasing evidence that the molecular pathogenesis of these two conditions may be distinct (*Farrell et al 2011*). Based on epidemiological observations, in 1996 Mac Mahon has suggested that Hodgkin's disease may not be a single entity but a syndrome comprising at least two, and possibly three, entities with different etiologies (*MacMahon, 1996*). This hypothesis was further supported by *Armstrong et al* with the "three-disease model" with HL occurring in childhood (EBV+, MC type), HL of young adults (EBV-, NS type) and HL of older adults (EBV+, MC type) (*Armstrong et al, 1998*). In 2002 based on studies assessing that infectious mononucleosis is associated with higher risk of developing HL, *Jarrett et al.* has extended the "three-disease model" to include a fourth entity represented by EBV-associated cases occurring in young adults with delayed exposure to EBV (*Jarrett et al, 2003; Jarrett et al, Leuk Lymphoma 2003*). However, the infrequent association of EBV with HL in young adulthood,

suggested that EBV is mainly not pathogenetic in young patients and that another currently unknown virus might be involved in the development of the disease (Kapatai *et al* 2007, Jarrett *et al*, *Leuk Lymphoma* 2003). Conversely, the “hit and run hypothesis” (Ambinder, 2000; Jox *et al*, 1997; Trivedi *et al*, 1995) suggested that EBV may contribute early on the pathogenesis of EBV-negative HL. Based on this hypothesis after EBV has infected and transformed B cells it is lost because of the presence of a defective integrated and rearranged viral DNA. The partial elimination of defective EBV episomes from infected cells has been previously detected in some cases of EBV-negative sporadic Burkitt lymphoma (Trivedi *et al*, 1995); this prompted to the search for EBV DNA in H/RS cells. Although Gan *et al*. found defective EBV genome in 2 cases of EBV-negative HL, others did not find any trace of EBV in H/RS to support a “hit-and-run” mechanism. Moreover, because some young patients with HL have no evidence of previous EBV-infection, it seems unlikely that EBV can be responsible for EBV-negative HL. In this view, EBV-positive and EBV-negative HL may simply represent two distinct diseases (Gan *et al*, 2002; Gallagher *et al*, 2003). Whether or not EBV-positivity in HL has prognostic significance remains controversial, with data on both sides of the question (Table 4) (Jarrett *et al*, 2005); nevertheless, the expression of viral antigens on tumor cells may still be crucial posing theoretical targets for anti-cancer therapies, including vaccination.

Reference	Population-based	No. of Patients	Age range, y	Median follow-up, mo	NLPHL included	% EBV-associated	Prognostic significance of EBV-association on OS
Armstrong <i>et al</i> ⁵	No	59	Not stated	Not stated	Yes	36	None
Axdorph <i>et al</i> ⁶	No	95	14–77	145	No	33	None
Enblad <i>et al</i> ¹¹	Yes	117	11–87	130	Yes	27	None
Flavell <i>et al</i> ⁸	No	273	≥15	60	Yes	29	None
Murray <i>et al</i> ⁷	No	190	22–49	86	Yes	27	None
Vestlev <i>et al</i> ⁴	No	66	9–78	22	Yes	41	None
Krugmann <i>et al</i> ⁹	No	119	14–83	122	No	26	None
Herling <i>et al</i> ¹⁰	No	303	Adults	65	No	21	None
Vassalo <i>et al</i> ¹⁵	No	78	15–75	Not stated	No	64	Favorable (assessed by LMP1 expression)
Glavina-Durdov <i>et al</i> ¹⁶	No	100	13–84	60	Yes	26	None. Positive impact on DFS in those ≤30 y
Engel <i>et al</i> ¹³	No	47	≤14	Not stated	No	68	Favorable
Morente <i>et al</i> ¹²	No	140	5–83	>24	Yes	51	Favorable
Naresh <i>et al</i> ¹⁴	No	110	4–61	57	No	78	Favorable
Stark <i>et al</i> ¹⁸	Yes	102	≥60	62	Yes	34	Unfavorable
Clarke <i>et al</i> ¹⁷	Yes	311	19–79	73	Yes	17	Unfavorable in those ≥45 y

NLPHL indicates nodular lymphocyte predominant Hodgkin lymphoma; EBV, Epstein Barr virus; OS, overall survival; LMP1, Epstein-Barr virus latent membrane protein 1; and DFS, disease-free survival.

Table 4. Summary of previous studies that have examined the effect of EBV status on clinical outcome in Hodgkin lymphoma. (Jarrett RF *et al*. *Blood* 2005 106: 2444-2451)

1.3.4 Genetic alterations and deregulated signalling

H/RS cells carry rearranged and somatically mutated Ig V genes in nearly all cases; however in 25% of the cHL clearly destructive somatic mutations were found that rendered originally functional IgV region non functional (*Kuppers et al, 1994; Kanzler et al, 1996*). Such mutations happen in germinal centre (GC) B cells, that normally rapidly undergo apoptosis. Thus, in HL pathogenesis critical steps most likely occur in GC to enable H/RS precursors with defective BCR to escape apoptosis. Nevertheless, it can not be rule out that additional transforming events could affect H/RS cells before they enter the GC and even after they have left it. Supporting this multistep process is the evidence that HL tumor cells usually show multiple chromosomal abnormalities and subclonal aberrations, indicating chromosomal instability (*Steidl et al, 2010; Weber-Matthiesen et al, 1995*).

Chromosomal translocations involving the Ig loci were detected in about 20% of cHL. Some of them involve the oncogenes BCL2, BCL6 and MYC, but for most cases partner gene is unknown (*Martin-Subero et al, 2006*). Considering the general silencing of the Ig loci in H/RS cells, it might be possible that these translocations may be relevant just in the early stages of the disease, when H/RS cells still have a B-cell phenotype. Conversely, translocations involving BCL6 are frequently found in LP cells (*Wlodarska et al, 2003*).

A rescue of H/RS from apoptosis is probably a key event in HL pathogenesis hence, activators or inhibitors of apoptosis were extensively studied for genetic aberrations in H/RS cells. The genetic lesions most frequently found in cHL involve members of two signalling pathways: JAK-STAT and NF- κ B. In about 20% of HL cases there are genomic gains of JAK2 in H/RS cells, and in 40% of the cases inactivating mutations of SOCS1, a negative regulator of JAK-STAT signalling, can be found (*Joos et al, 2000; Weniger et al, 2006*). Multiple genetic lesions in the NF- κ B pathway seems to contribute to its deregulation in H/RS cells. Interesting, among these an inhibitor of NF- κ B activity A20, is inactivated in 40% of mainly EBV-negative HL (*Schmitz et al, 2009*). This suggest that A20 inactivation and EBV infection are mutually exclusive transforming events in cHL. Other cell signalling pathways known to be aberrantly activated in HL include the PI3K/AKT pathway and the MAPK/ERK pathway. Both may be critical for H/RS cell survival and proliferation (*Dutton et al, 2005; Zheng et al, 2003*).

1.3.5 Tumor microenviroment

The microenviroment in HL is unique among lymphomas both for the complexity of cell types involved, and for the fact that non-tumor cells represent the majority of the cells,

accounting for 99% of all the cells in the tumor. H/RS cells grow in a typical microenvironment that is composed of several types of cells, including B cells, T cells, plasma cells, eosinophils, mast cells, and fibroblasts (Swedlow *et al*, 2008). This microenvironment is a critical determinant for the initiation and progression of HL. It is likely essential for H/RS cell survival, as indicated from the difficulty to grow H/RS cells in culture or in immunodeficient mice or to find H/RS cells in the peripheral blood (Kapp *et al*, 1993; Kapp *et al*, Blood 1993). H/RS cells appear to regulate this microenvironment by secretion of cytokines and chemokines (Figure 4) (Steidl *et al* 2011). For example, H/RS cells attract eosinophils by granulocyte-macrophage colony-stimulating factor, IL5, IL9, CCL5, and CCL28, and they attract TH2-type T helper cells and Treg cells by secretion of CCL5, CCL17, and CCL22 (Skinnider *et al*, 2002; Aldinucci *et al*, 2008; Fisher *et al*, 2003).

CD4+ T cells usually represent the largest population of cells in the lymphoma tissue. A fraction of these cells are CD4+ T helper cells. These cells are in close contact with H/RS cells and may play a pathogenetic role by stimulating the survival and growth of tumor cells. Indeed, although H/RS cells do not express most B cell-associated genes, they retained expression of MHC class II, CD40, CD80 and CD86, key molecules for an interaction of B cells with T helper cells. The interaction between H/RS cells and T cells is histologically typical; in HL biopsies H/RS cells are surrounded by CD40 ligand-expressing CD4+ T cells forming with them “rosettes” structures. The CD40 stimulation mediated by T cells leads to the activation of NF-kB, a survival signal for H/RS cells (Carbone *et al*, 1995; Nozawa *et al*, 1998).

H/RS cells can also orchestrate the cellular infiltrates to evade an attack by cytotoxic T cells (CTL) or NK cells. In this regard, it has recently become clear that many of the CD4+ T cells in classical HL are not helper but regulatory T cells. H/RS cells not only can attract these cells but also can induce the differentiation of naïve CD4+

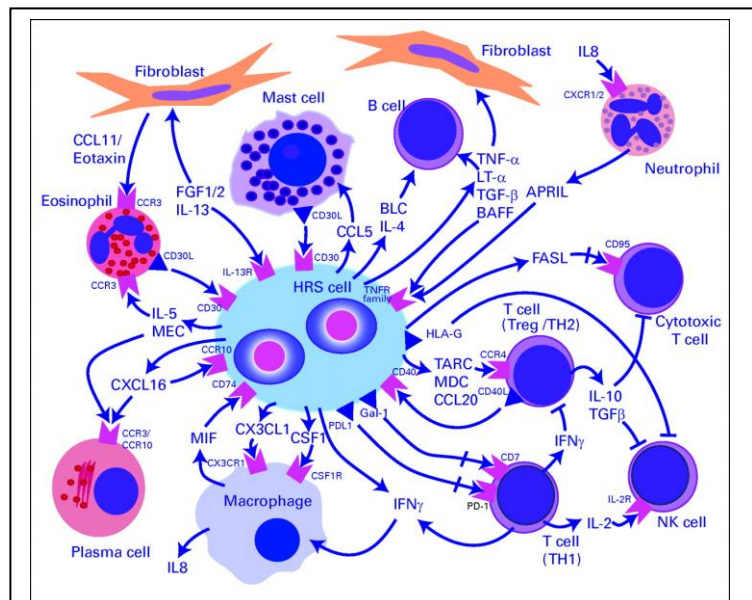


Figure 4. Schematic of the crosstalk between malignant Hodgkin Reed-Sternberg (H/RS) cells and the tumor microenvironment in classical Hodgkin's lymphoma. (Steidl C *et al*. JCO 2011;29:1812-1826)

T cells into Treg cells (Tanijiri *et al*, 2007). Tregs may have a pathogenetic role, as there is

indication that they have immunosuppressive activity on HL-infiltrating CTL cells (Marshall *et al*, 2004). However, high number of Treg cells in HL microenvironment has been reported to be associated with a better prognosis, indicating that an excess of these cells may have suppressive effects also on H/RS survival (Alvaro *et al*, 2005; Kelley *et al*, 2007). Furthermore, H/RS cells might inhibit cytotoxic cell functions through the expression of PD1 and CD95, and the secretion of the immunosuppressive cytokines IL-10, TGF β , galectine-1 and prostaglandin E2 (Chemintz *et al*, 2007; Gandhi *et al*, 2007; Aldinucci *et al* 2010; Kuppers *et al* 2012).

EBV infection might also affect the microenvironment composition by increasing the production of molecules involved in immune escape and T cell recruitment. For example, IL10 was found to be expressed in 66% of EBV+ cHL cases but in only 16% of EBV-negative cases (Skinnider *et al* 2002). *In vitro* HL cells can process and present epitopes from LMP1 and LMP2A in the context of multiple class I alleles (MHC) and are sensitive to lysis by EBV-specific CTLs. However, EBV-infected H/RS cells survive *in vivo*, probably because H/RS cells are able to counteract EBV-specific CTL responses. Surprisingly, EBV+ HL have been shown to contain more activated CTLs and express higher levels of MHC class I than EBV-negative cases (Kapatai *et al*, 2007). Moreover, Chetaille *et al*. described a molecular signature of EBV+ cHL, characterized by genes associated with Th1 and antiviral responses (Chetaille *et al*, 2009). However, CTL cells might not be effective in the immune response against EBV in H/RS cells. Recent data have shown a strong association between EBV+ HL and human leukocyte antigen (HLA) class I genotype. Increased risk was associated with HLA-A*01 and decreased risk with HLA-A*02. Whereas CTL responses to many HLA-A*02 restricted EBV epitopes have been described, there are no confirmed HLA-A*01-restricted responses to epitopes derived from either lytic or latent viral proteins. This raises the suspicion that the increased risk of EBV-associated cHL is related to a weak EBV-specific CTL response (Hjalgrim *et al*, 2010; Straathof *et al*, 2005).

Several studies point to an adverse prognosis for EBV-association in adult cHL; however, physiological and age-related changes of the immune system may also play an important role in modulating the tumour microenvironment in HL. Supporting this, a favourable outcome was described for EBV+ cHL occurring in young patients (Barros *et al*, 2012). Also, another study has demonstrated that there is an age-dependent relationship between tumor EBV status and clinical outcome in cHL. In particular, it was found that in patients aged 16 to 49 years EBV status has not significant impact on prognosis, whereas in patients over 50 years EBV status was significantly associated with poorer outcome, suggesting that an age-related decrease in

immunity may contribute to the age effect on prognosis in patients with EBV+ cHL (*Jarrett et al, 2005*).

2. PERSONAL CONTRIBUTION

2.1 AIM

Based on epidemiological studies, EBV-positive lymphoproliferative disorders (LPD) are associated with patient's age, immune system status of the host, geography, and socioeconomic conditions. In particular, in immunocompetent patients EBV+ cHL occur more frequently in children from poorly developed countries and in older adults from developed countries, whereas EBV-negative cases are more frequent among young adults of developed countries (*Armstrong et al 1998, Jarrett et al 2003*). These differences have questioned the effective role of EBV in the pathogenesis of the disease, and have raised the possibility that EBV-positive and EBV-negative cHL may represent two distinct diseases (*Harris et al 1998*).

It has been suggested that different factors may contribute to the development of EBV+ cHL in children and older patients. In the former, early age of EBV infection has been reported to greatly affect the association of EBV with cHL (*Glaser et al 1997*) while, in the latter immunosenescence related to patient's age has been proposed as a key factor for the development of EBV+ cHL (*Jarrett et al 2005, Dojcinov et al 2011*). In both cases, it has been suggested that an impaired immune status of the host may contribute to the development of EBV+ cHL. However, whether or not EBV+ cHL occurring in children and in old patients represent the same disease remains to be clarified.

To address this issue, we characterized and compared the immunophenotypic and molecular features of 57 cases of HL occurring in pediatric patients from Baghdad with those of 30 cases of HL diagnosed in old Italian patients.

2.2 MATERIALS AND METHODS

Patients

Paraffin blocks of 57 cases of pediatric HL first diagnosed at the Children Welfare Teaching Hospital of Baghdad in the period 2008-2012 were sent to the Pathology Unit of Sant'Andrea Hospital of Rome for a second opinion. Clinico-pathological features of the patients are summarized in Table 5. Histologically, 51 cases were classified as cHL (89%), of which 35 as MC (69%), and 16 as NS (31%). Six cases were classified as NLP-HL. Moreover, 30 cases of Hodgkin Lymphoma diagnosed in caucasian Italian patients aged 50 years or older in the period 2003-2012 at Sant'Andrea Hospital of Rome were enrolled in the study (Table 7). A control group of 10 cases of EBV-negative cHL from caucasian Italian adult young patients (age range 17-32 year old) was included in the study.

Immunohistochemistry

Phenotypic characterization of tumor cells was performed by immunohistochemistry on formalin-fixed paraffin-embedded (FFPE) serial sections of the involved lymph node using the following antibody: CD3, CD4, CD8, CD20, (Novocastra, UK), CD30, CD79a, CD15, (Dako, Denmark), PAX5 (Thermo Scientific, USA), OCT2 and BOB1 (Santa Cruz Biotechnology Inc., USA). The number of stained cells with H/RS morphology was determined at 400X. Cases were classified as negative; or as having <50% H/RS cells stained; or as having >50% H/RS cells stained.

T-cell components present in the tumor microenvironment of cHL was investigated by immunohistochemistry for CD3, CD4, CD8, CD56, and Granzyme B (Dako, Denmark). Data were obtained by counting 5 separate 1000× high-power fields (HPFs) and calculating the mean number of positively stained cells per HPF.

FFPE tissue sections were immunostained on an automated immunostainer (Dako Corp., Carpinteria, CA) using a dextran polymer-peroxidase-DAB detection kit (Dako EnVision™ FLEX+ kit) according to the manufacturer's instructions. Briefly, using a 3-in-1 procedure, deparaffinization, rehydration and heat-induced epitope retrieval was performed incubating 4 μm thick paraffin sections with the Envision pre-heated target retrieval solution for 20 minutes at 97°C. Sections were then cooled at room temperature, immersed in a washing

buffer for 5 min, and then incubated with an optimal dilution of the primary antibody for 20 min. After a washing bath, sections were incubated for 20 min with a FLEX HRP-conjugated (horse radish peroxidase) linker composed by secondary antibodies with anti-mouse and anti-rabbit Ig specificity. Immunohistochemical reactions were subsequently developed with diaminobenzidine as chromogenic peroxidase substrate, and slides were counterstained with haematoxylin.

Tests for the detection of EBV in tumor cells

To investigate the presence of EBV infection in H/RS cells immunohistochemistry for LMP-1 protein (Dako, Denmark) was performed in all the pediatric and adult cHL. In addition, all the cases were also tested by in situ hybridization (ISH) for EBV-encoded RNAs (EBERs). EBER-ISH was performed on paraffin sections using a cocktail of EBER1 and EBER2 fluorochrome-conjugated riboprobes (EBER PNA Probe/Fluorescein, Dako, Denmark), and a polyclonal rabbit anti-FITC/HRP antibody (Dako, Denmark). Briefly, deparaffinized 2 µm thick sections were pre-treated with a pepsin solution (proteinase K) for 6 min at room temperature in a humid chamber. Proteolytic process was stopped with 95% ethanol for 10 secs. Sections were washed in distilled water, and air-dried. Sections were then incubated with the EBER PNA probe fluorescein-conjugated for 2 h at 37°C in a hybridization oven and covered with a coverslip. Slides were washed for 25 min with a stringent washing solution, air-dried, and then incubated in a humidity chamber with an anti-FITC/HRP rabbit polyclonal antibody for 30 min. After a washing step with PBS buffer, a 5 min incubation with the HRP-substrate (diaminobenzidina) was performed. Slides were counterstained with haematoxylin.

Genomic DNA extraction

Genomic DNA was purified from formalin-fixed paraffin embedded tissues using the QIAamp DNA mini kit (Qiagen). The QIAamp DNA purification procedure was carried out using QIAamp Mini spin columns. For each tumor a paraffin block was cut at 10 µm and collected in an autoclaved plastic microtube (1.5 ml). For each microtube, 2 sections of total 20 µm thickness were carefully collected. Deparaffinization was carried out by adding 1 ml xylene to each microtube for 30 min for two changes, followed by 100% and 75% ethanol for 30 min

with two changes. After a washing step with PBS for 15 min in two changes, 200 µl of lysis buffer containing proteinase K was added and incubated at 56 °C overnight using a heat block. When all tissue fragments were dissolved completely, the lysate was loaded onto the QIAamp Mini spin column. A brief centrifugation was carried on to allow DNA to be adsorbed onto the QIAamp silica membrane. DNA bound to the QIAamp membrane was washed in 2 centrifugations using 2 different washing buffers. Purified DNA was eluted in AE from the QIAamp Mini spin column in a concentrated form. Using a spectrophotometer (Nanodrop, ThermoScientific), the amount of DNA yield was measured according to the standard protocol recommended by the manufacturer.

Polymerase Chain Reaction (PCR) analysis of the LMP-1 gene polymorphism

To investigate LMP-1 gene polymorphism in HL biopsies of Iraqi children, the DNA was extracted from 25 EBV+ cHL (21 MC and 4 NS) as previously described. The DNA from B95.8 and Raji cell lines was used as LMP1 wild-type control. The DNA from AG876 and Rael cell lines was used as LMP1 deleted variant control. A DLBCL cell line, U2932 was used as EBV negative control of the PCR reaction. For the PCR reaction, following primers were designed: forward primer: 5' GTG GGG GTC GTC ATC ATC TC 3' (B95.8 coordinates 168190-168209); reverse primer: 5'CGG AAG AGG TTG AAA ACA AA 3' (B95.8 coordinates 168331-168350). The DNA was amplified using the following PCR conditions: initial denaturation step at 94°C for 5 minutes, 40 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 1 minute, extension at 72°C for 1 minute, final extension step at 72°C for 10 minutes. PCR products were visualised on a 2% agarose gel. The product length for the wild type LMP1 amplicon was 161 bp, and for the deleted LMP1 variant amplicon 131 bp.

PCR detection of Epstein Barr virus (EBV)-encoded RNA 1 (EBER1)

In order to further confirm the presence of EBV-infection in HL biopsies of two elderly patients with methachronous B-cell lymphoma a PCR for the detection of EBER1 gene was performed on total DNA extracted from the whole paraffin tissue sections. For the PCR reaction, the following primers were designed: forward primer 5'AGGACCTACGTGCCCTAGA3', reverse primer 5'AAAACATGCGGACCACCAGC3'.

The DNA from U2932 EBVGFP c11 (DLBCL, EBV+) and RAJI (BL, EBV+) cell lines was used as EBER1-positive control. U2932 (DLBCL, EBV-) and BC3 (PEL, EBV-) cell lines were used as EBV-negative controls of the PCR reaction. PCR products were visualised on a 2% agarose gel. The product length for EBER1 amplicon was 167 bp.

T-cell receptor-gamma (TCR- γ) gene rearrangements analysis

In order to investigate the status of the TCR γ gene in both the pediatric and the elderly cases of HL, a TCR γ gene clonality assay was carried out using the “TCR γ rearrangements molecular analysis kit” (Master Diagnostica, Spain) in 38 of 51 Iraqi cHL and in 21 of 30 cases of adult cHL. This test utilizes the BIOMED-2 multiplex PCR master mixes targeting the variable (V) and joining (J) regions that flank the unique hypervariable antigen-binding region 3 (CDR3) of the TCR γ gene locus, which is rearranged early during T-cell development (*van Dongen et al 2003*). In particular, genomic DNA extracted from paraffin tissue sections was used in two independent PCR amplification reactions. One reaction amplified DNA sequences between the V gamma segments 1-8 and 10, and all J gamma segments, while the other amplified DNA sequences between the V gamma segments 9 and 11, and all J gamma segments. One monoclonal and one polyclonal control samples were included in each reaction. Following PCR amplification, the fluorochrome-labeled single-strand (denatured) PCR products were separated in a capillary sequencing polymer as function of size and detected automatically with a laser scanning on the ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, California). This analysis is based on the principle that primers conjugated with different fluorescent dyes produce different emission spectra upon excitation by a laser in a capillary electrophoresis instrument (GENESCAN). In this manner, different fluorophors can correspond to different targeted regions. GeneMapper software (ABI) was then used to visualize and analyze PCR products, allowing detection of clonal cells. In case of polyclonal lymphoproliferation many different PCR products of different sizes resulted in a gaussian distribution of homogeneous peaks. One type of PCR product due to a fully monoclonal lymphoid population gave a single prominent peak. When two unequivocal peaks were observed the case was considered as bi-allelic monoclonal. Samples in which more than 2 discrete peaks (2.5-fold higher than the adjacent peaks that represent the polyclonal background) were observed were scored as oligoclonal (Figure 6). The limit of detection of this assay has been determined by the manufacturer to be approximately 1 clonal cell in 100

hundred normal cells (1%), and inter-assay and intra-assay reproducibility in size determination using capillary electrophoresis has been considered to be approximately 1-2 basepairs.

Heteroduplex analysis of TCR γ gene rearrangements

PCR amplifications products of TCR γ gene were also evaluated by heteroduplex analysis on a non-denaturing polyacrylamide gel according to BIOMED-2 report (*van Dongen et al 2003*). Twenty μ l of PCR product was denatured at 94°C for 5 minutes and re-annealed at 4°C for 60 minutes. Five μ l of ice-cold non-denaturing bromophenol blue loading buffer was added to samples. Then 20 μ l of mixture was loaded into the wells of 6% non-denaturing polyacrylamide TBE gel (Invitrogen) with a 0.5X TBE running buffer (Invitrogen) and runned at 110V for 3 hours. Gels were stained in 0.5 μ g/ml EtBr in water for 5-10 minutes, and washed twice in water for 5-10 minutes. UV illumination was used for visualization. Gel was photographed and data were interpreted. In heteroduplex analysis, PCR products are heat denatured and subsequently rapidly cooled to induce duplex (homo or heteroduplex) formation. In samples that contain polyclonal lymphoid cells PCR fragments of rearranged TCR γ genes form heteroduplexes, which result in a background smear of slow migrating fragments. PCR products from monoclonal or oligoclonal lymphoid cell populations give rise to homoduplexes bands (Figure 6).

Immunoglobulin (IGH) gene rearrangements analysis

We performed molecular evaluation of IGH gene rearrangements in 38 of 51 Iraqi cHL and in 21 of 30 cases of adult cHL using the “Identyclone IGH gene clonality assay” (Invivoscribe, California). This test, based on BIOMED 2 protocol (Ref 15), amplify the DNA between primers that target the three conserved framework regions (FR1, FR2, and FR3) of the variable (V) segments and the conserved joining (J) regions of the IGH gene locus. These regions rearrange during B-cell differentiation generating VDJ products of unique length and sequence. Genomic DNA extracted from FFPE tissues was amplified using three master mixes that combine 3 different sets of VH primers with 1 JH consensus primer. PCR amplifications products were then analyzed on the ABI 3130 Genetic Analyzer (Applied Biosystems, Foster

City, California) using GeneMapper program (as previously described for the TCR γ gene clonality assay). One monoclonal and one polyclonal control samples were included in each reaction. Clonal rearrangements were identified as prominent, single-sized peaks in the expected range, whereas samples were scored as polyclonal when a gaussian distribution of homogeneous peaks was observed.

Laser Capture Microdissection and TCR γ gene rearrangement analysis

Laser capture microdissection was performed using the microdissection laser system SL CUT (NIKON Instruments, Italy). Large atypical cells with Hodgkin and Reed-Stenberg-like morphology were isolated on Haematoxylin and Eosin stained sections collecting approximately 200 cells per sample. Moreover, about 300 morphologically typical lymphocytes were picked from the same tissue section. DNA extraction was performed using the Pico pure isolation Kit (ARCTURUS, Bioscience Inc., Mountain View, CA, USA). TCR γ clonality assay was performed on microdissected samples as previously described for the whole tissue sections.

Statistical analysis

The association between clinical, pathological, and molecular variables (tumor histology, EBV-infection, TCR γ and IGH clonality) was assessed using Fisher's exact test. Student's t-test was used to test the correlation between patient's age and tumor histology. Associations were considered to be statistically significant with p values < 0.05 .

2.3 RESULTS

Clinico-pathological features of HL occurring in Iraqi children

In a collaborative study between the Children Welfare Teaching Hospital of the University of Baghdad and the Sapienza University of Rome, 57 cases of Hodgkin lymphoma

Table 5. Age-related histology and EBV infection of 51 cases of pediatric cHL.

Groups	M:F Ratio	n. of cases	MC-HL	NS-HL	EBV+
3-5y	5:1	18	16 (89%)	2 (11%)	18 (100%)
6-10y	3:1	24	14 (58%)	10 (42%)	20 (83%)
11-13y	2:1	9	5 (56%)	4 (44%)	6 (67%)
Total	3.25:1	51	35 (69%)	16 (31%)	44 (86%)

(HL) affecting Iraqi children under 14 years of age were reviewed at the Sant'Andrea Hospital of Rome (Table 5).

Histologically, 51 cases were classified as cHL (MC = 69%; NS = 31%), and 6 cases as Nodular Lymphocyte Predominance HL. The children with MC were predominantly male (ratio M:F MC=6:1 versus NS=1.3:1; $p=0.033$), and were younger than those with NS (mean age = 6.9 year old versus 8.75 year old; $p=0.016$).

To detect EBV infection of H/RS cells EBER hybridization and LMP-1 immunostaining was performed in all the cases (Fig. 5a). EBV infection of H/RS cells was demonstrated in 44 of 51 cases of cHL (86%), and was more common in MC than in NS (97% versus 63%; $p=0.0025$); the 6 cases of NLP-HL were all EBV-negative. In all EBV+ cases, LMP-1/EBER reactivity was detected in CD30-positive cells with typical H/RS morphology (Figure 5); EBV+ H/RS cells were 60% of CD30+ cells in MC and 53% in NS. When children were stratified according to age it was found that all cases of cHL in the 3-5 years age range were EBV+. Moreover, with the increase of the age, there was a gradual decrease of EBV+ cases (from 100% to 67%), and an increase in NS cases (from 11% to 44%). These data indicate a progressive age-related switch from an EBV+ MC type of cHL to an EBV-negative NS type.

Immunohistochemistry for CD20, CD79a, PAX-5, OCT-2, and BOB-1 was used to assess the immunophenotypic profile of H/RS cells. Expression of B cell markers by H/RS cells was similar in MC and NS, and was not influenced by EBV infection. In fact, H/RS cells were

PAX-5 positive in the majority of cHL cases (MC 94% versus NS 87%), and expressed at a lesser degree other B-cell markers including CD20 (MC 26% versus NS 25%), CD79a (MC 37% versus NS 25%), OCT-2 (MC 31% versus NS 12%), and BOB-1 (MC 11% versus NS 12%) (Table 6). The six cases classified as NLP-HL were all EBV-negative, and contained LP cells intensely positive for all the B–cell markers and negative for CD30 and CD15 (Table 6).

Table 6. Expression of B cell markers by H/RS cells in 57 cases of HL from Iraqi children.

Histology	n. of cases	PAX5			CD20			CD79a			OCT-2			BOB-1		
		Neg	<50%	>50%	Neg	<50%	>50%	Neg	<50%	>50%	Neg	<50%	>50%	Neg	<50%	>50%
cHL-MC	35	2	10	23	26	9	0	22	13	0	24	8	3	31	4	0
		6%	28%	66%	74%	26%	-	63%	37%	-	69%	23%	8%	89%	11%	-
cHL-NS	16	2	3	11	12	4	0	12	3	1	14	1	1	14	2	0
		13%	19%	68%	75%	25%	-	75%	19%	6%	88%	6%	6%	88%	12%	-
cHL-EBV-pos	44	4	11	29	33	11	0	27	16	1	32	8	4	39	5	0
		9%	25%	66%	75%	25%	-	61%	36%	2%	73%	18%	9%	89%	11%	-
cHL-EBV-neg	7	0	2	5	5	2	0	7	0	0	6	1	0	6	1	0
		-	29%	71%	71%	29%	-	100%	-	-	86%	14%	0%	86%	14%	-
NLP-HL	6	0	1	5	0	0	6	0	0	6	0	0	6	0	0	6
		-	17%	83%	-	-	100%	-	-	100%	-	-	100%	-	-	100%

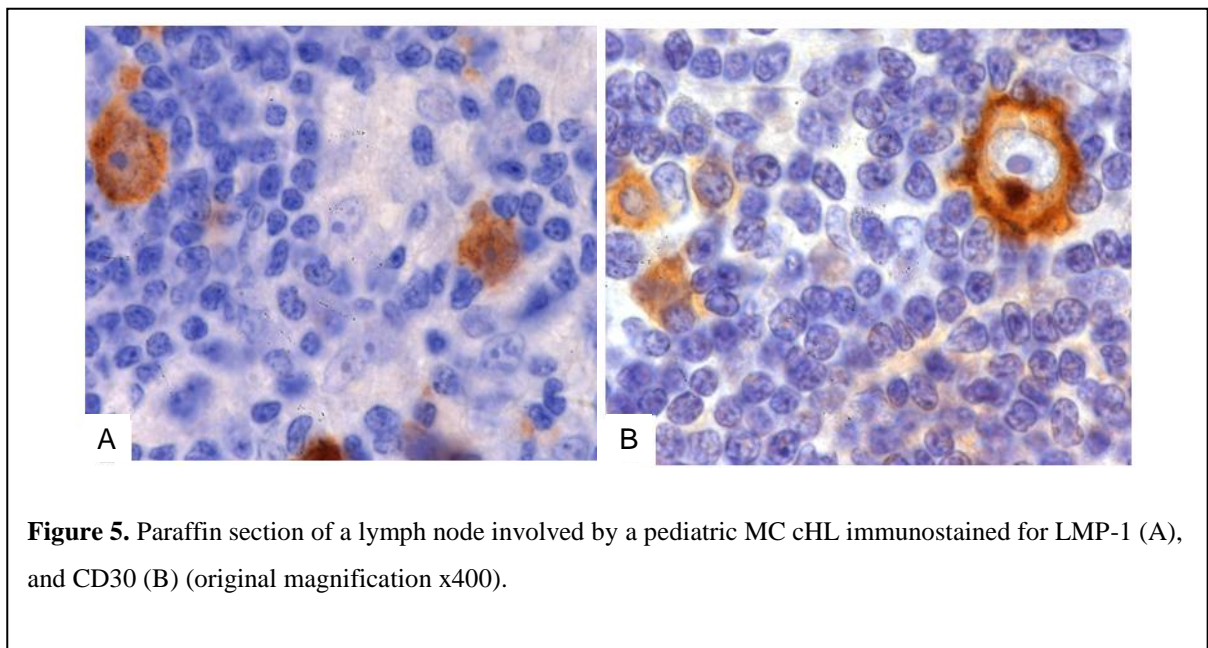


Figure 5. Paraffin section of a lymph node involved by a pediatric MC cHL immunostained for LMP-1 (A), and CD30 (B) (original magnification x400).

Table 7. Histology and EBV infection in 30 Italian cHL stratified according to age

Groups	M:F ratio	n° of cases	MC-HL	NS-HL	LR-HL	Uncl-HL	EBV+
50-59y	1:1	8	2 (25%)	3 (38%)	2 (25%)	1 (13%)	3 (38%)
60-69y	1:1	10	2(20%)	6 (60%)	2 (20%)	0	4 (40%)
70-79y	7:1	8	4 (50.0%)	2 (25.0%)	1 (12.5%)	1 (12.5%)	5 (62.5%)
≥80y	0:4	4	3 (75%)	1 (25%)	0	0	4 (100%)
Total	-	30	11 (37%)	12 (40%)	5 (17%)	2 (7%)	16 (53%)

Clinico-pathological features of HL occurring in Italian old adults

Thirty cases of classical Hodgkin lymphoma diagnosed in patients over 50 years of age at Sant'Andrea Hospital of Rome were further characterized. Histologically, they were subclassified as MC in 11 cases (37%), as NS in 12 cases (40%), and as LR in 5 cases (17%). In 2 cases biopsy samples were too small to assess HL variant and were identified as Unclassified-HL. Compared to NS, MC cases were slightly more common in older patients (mean age MC = 71.6 year old versus NS = 64.3 year old; $p=0.057$), and were not significantly associated with patients gender (ratio M:F MC=1.3:1 versus NS=1:1; $p=1$).

EBV infection of H/RS cells was found in 16 of 30 cHL (53%), and was more common in MC than in NS (82% versus 33%; $p=0.036$). Among the EBV+ cases there were also the 2 Unclassified HL (100%) and 1 case of LR (20%). LMP-1/EBER reactivity was detected in cells with typical H/RS morphology in all the 16 EBV+ cases. EBV+ H/RS cells were 100% of the CD30+ cells in MC cases and 75% in NS cases. When patients were stratified according to age (Table 7), it was found that with increasing age there was a progressive transition from EBV-negative to EBV-positive cases (from 38% to 100%), and an increase in MC cases (from 25% to 75%). This result was exactly the opposite of what we observed in Iraqi children, where it was found an inverse age-related transition from EBV+ MC cases to EBV-negative NS cases.

Concordantly with the literature (Swerdlow *et al* 2008; Mani *et al* 2009) expression of B-cell markers was found to be downregulated in H/RS cells. In fact, tumor cells were

consistently CD30 and CD15 positive (100% of the cases), expressed frequently PAX-5 (MC 100% versus NS 92%), and at a lesser degree other B-cell markers including CD20 (MC 36% versus NS 8%), CD79a (MC 27% versus NS 25%), OCT-2 (MC 73% versus NS 50%), and BOB-1 (MC 54% versus NS 16) (Table 8). As for pediatric cHL, it was found that the immunophenotypic profile of H/RS cells was similar in MC and NS, and was not influenced by EBV infection. When results were compared to that of Iraqi children, a significantly higher expression of OCT-2 and BOB-1 was found in MC cHL occurring in elderly Italian patients than that found in MC pediatric cases (Table 9).

Table 8. Expression of B cell markers in 30 cHL of Italian old adult patients

Histology	n. of cases	PAX5			CD20			CD79a			OCT-2			BOB-1		
		Neg	<50%	>50%	Neg	<50%	>50%	Neg	<50%	>50%	Neg	<50%	>50%	Neg	<50%	>50%
cHL-MC	11	0	1	10	7	3	1	8	1	2	3	3	5	5	4	2
		-	9%	91%	64%	27%	9%	73%	9%	18%	27%	27%	45%	45%	36%	18%
cHL-NS	12	1	3	8	11	0	1	9	3	0	6	3	3	10	1	1
		8%	25%	67%	92%	-	8%	75%	25%	-	50%	25%	25%	84	8%	8%
cHL-LR	5	1	1	3	2	2	1	2	2	1	2	2	1	4	1	0
		20%	20%	60%	40%	40%	20%	40%	40%	20%	40%	40%	20%	80%	20%	-
cHL-Unclass	2	0	1	1	0	1	1	1	0	1	1	0	1	1	0	1
		-	50%	50%	-	50%	50%	50%	-	50%	50%	-	50%	50%	-	50%
cHL-EBV+	16	0	3	13	8	4	4	9	3	4	4	4	8	10	4	2
		-	19%	81%	50%	25%	25%	56%	19%	25%	25%	50%	63%	25%	13%	
cHL-EBV-neg	14	2	3	9	12	2	0	11	3	0	8	4	2	10	2	2
		14%	21%	64%	86%	14%	-	79%	21%	-	57%	29%	14%	71%	14%	14%

Tumor microenvironment composition in pediatric and elderly cHL

Recently Barros *et al* have shown that in EBV+ pediatric cHL the tumor microenvironment is characterized by a cytotoxic/T-helper cell 1 (Th1) profile (Barros *et al*, *IJC* 2011). The different T cell components present in the tumor microenvironment of our series of cHL were investigated by immunohistochemistry for CD4, CD8, CD56, and Granzyme B to identify activated cytotoxic T/NK lymphocytes (CTLs).

In the pediatric cases increased GrB+ CTLs were significantly associated with patient's age of 3–5 years as compared to over 6 years (mean value 129/HPF vs 27/HPF, $p=0.002$), with the histological subtype MC as compared to NS (mean value 104/HPF vs 26/HPF, $p=0.02$) and EBV+ versus EBV-negative status (mean value 95/HPF vs 21/HPF, $p=0.04$); no statistically

significant correlation was found between the number of intratumoral GrB+ CTLs and TCR γ clonality. Consistently with data obtained in the pediatric cases, in cHL of the elderly we found a significant increase of GrB+ CTLs in the MC as compared to the NS subtype (mean value 125/HPF vs. 23/HPF, $p=0.005$). Conversely, in cHL of the elderly the number of GrB+ CTLs was not associated with EBV-status, age of the patient, and TCR γ gene clonality. No statistically significant differences were found between cHL of Iraqi children and that of old Italian patients (mean value 73/HPF vs 66/HPF; $p=0.425$).

The number of CD4+, CD8+ and of CD56+ cells was comparable between pediatric and elderly cHL. Furthermore, for both pediatric and old patients no association was found between the number of the CD4+, CD8+, and CD56+ cells and histological variant, EBV status and TCR γ gene rearrangements.

Table 9. Expression of B cell markers in MC and NS cHL occurring in Iraqi children and in Italian old patients

Histology	n. of cases	PAX5			CD20			CD79a			OCT-2			BOB-1		
		Neg	<50%	>50%	Neg	<50%	>50%	Neg	<50%	>50%	Neg	<50%	>50%	Neg	<50%	>50%
cHL-MC old	11	0	1	10	7	3	1	8	1	2	3	3	5	5	4	2
		-	9%	91%	64%	27%	9%	73%	9%	18%	27%	27%	45%	45%	36%	18%
cHL-MC children	35	2	10	23	26	9	0	22	13	0	24	8	3	31	4	0
		6%	29%	66%	74%	26%	-	63%	37%	-	69%	23%	9%	89%	11%	-
		$p=0.343$			$p=0.338$			$p=0.024$			$p=0.009$			$p=0.003$		
cHL-NS old	12	1	3	8	11	0	1	9	3	0	6	3	3	10	1	1
		8%	25%	67%	92%	-	8%	75%	25%	-	50%	25%	25%	84	8%	8%
cHL-NS children	16	2	3	11	12	4	0	12	3	1	14	1	1	14	2	0
		13%	19%	69%	75%	25%	-	75%	19%	6%	88%	6%	6%	88%	13%	-
		$p=0.999$			$p=0.06$			$p=0.999$			$p=0.072$			$p=0.560$		

LMP-1 gene polymorphism in EBV+ cHL of Iraqi children

Sequence analyses have found several nucleotide variations in the EBV LMP1 gene which has been reported to have greater tumorigenic potential (*Santon et al 1998*). We have investigated the EBV-LMP-1 gene polymorphism in 25 cases of cHL of Iraqi children to demonstrate the characteristic 30 basepair deletion in the 3' end of EBV LMP-1 gene. Wild type LMP-1 gene was found in 17/25 cases (68%), whereas the LMP-1 deleted variant of EBV was detected in 8/25 cases (32%); no significant association was found between the presence of LMP-1 deletion and any of the clinico-pathological features of the disease. Furthermore, the

observed incidence of EBV LMP-1 gene deletion (32%) is similar to that previously reported for USA and Brazil (33%) (Hayashi *et al* 1997).

Table 10. IGH and TCR γ gene rearrangements in cHL of Iraqi childhood

Groups	n. of cases	IGH		TCR γ			IGH + TCR γ
		Monoclonal	Polyclonal	Monoclonal	Oligoclonal	Polyclonal	Monoclonal
cHL all cases	38	14 37%	24 63%	10 26%	18 47%	10 26%	4 10%
cHL-MC	26	10 38%	16 62%	4 15%	14 54%	8 31%	2 8%
cHL-NS	12	4 33%	8 67%	6 50%	4 33%	2 17%	2 20%
cHL-EBV+	33	13 40%	20 60%	6 18%	17 52%	10 30%	3 9%
cHL-EBV-	5	1 20%	4 80%	4 80%	1 20%	0 -	1 20%

IGH and TCR γ gene rearrangements in elderly and pediatric cHL

IGH and TCR γ gene rearrangements were investigated in 38 of 51 cases of pediatric cHL (Table 10) and in 21 of 30 cases of adult cHL (Table 11) using a multiplex PCR on DNA extracted from whole paraffin sections. In the remaining cases (13 pediatric cHL and 9 cHL of the elderly) the analysis was inconclusive, probably because of poor quality DNA. PCR amplifications products of TCR γ gene were also evaluated by heteroduplex analysis on a polyacrilamide gel to confirm the existence of oligoclonal and monoclonal gene rearrangements (Figure 6).

Among the 38 cHL of childhood clonal IGH rearrangements were detected in 14 cases (37%), and oligoclonal/monoclonal TCR γ rearrangements in 28 cases (74%), including 5 of 5 EBV-negative cases. Dual IGH and TCR γ clonal rearrangements were detected in 4 of 38 cases (10%).

In the Italian elderly cases IGH monoclonal rearrangements were found in 10 of 21 cHL (48%). Mono/oligoclonal TCR γ rearrangements were detected in 7 of 21 cases (33%); this pattern was significantly different from what observed in cHL of Iraqi children ($p=0.005$), where a restricted TCR γ was found in the majority of the cases (74%). In 3 elderly cHLs (14%) a concomitant IGH and TCR γ monoclonal rearrangement was detected.

In both, cHL of childhood and cHL of the elderly, no association was found between IGH and/or TCR γ clonality and histological subtype, EBV status, or patient's age (Table 12). Nevertheless, it is interesting to note that the pattern of TCR γ clonality identified in our series of pediatric and elderly cHL was profoundly different from what we observed in 10 cases of EBV-negative cHL-NS occurred in Italian young adult patients, which exhibited polyclonal TCR γ rearrangements in all the cases (Table 12).

Table 11. IGH and TCR γ gene rearrangements in elderly Italian cHL

Groups	n. of cases	IGH		TCR γ			IGH + TCR γ
		Monoclonal	Polyclonal	Monoclonal	Oligoclonal	Polyclonal	Monoclonal
cHL all cases	21	10 48%	11 52%	5 24%	2 9%	14 67%	3 14%
cHL-MC	8	3 38%	5 62%	2 25%	1 13%	5 62%	1 13%
cHL-NS	8	4 50%	4 50%	2 50%	1 33%	5 17%	1 13%
cHL-LR	3	1 33%	2 67%	0 -	0 -	100%	0 -
cHL-UNCL	2	2 100%	0 -	1 50%	0 -	1 50%	1 50%
cHL-EBV+	10	5 50%	5 50%	3 30%	1 10%	6 60%	2 20%
cHL-EBV-	11	5 45%	6 55%	2 18%	1 9%	8 73%	1 9%

Immunophenotypic characterization and single-cell PCR analysis of cHL with TCR γ monoclonality

TCR γ gene rearrangements are likely to be related to the non-neoplastic T-cell population that characterized Hodgkin lymphomas however, rarely, T-cell markers-positive H/RS cells showed rearranged TCR genes, suggesting a T-cell derivation in a minority of the cases (*Seitz et al 2000*). To clarify the phenotype of H/RS cells in our series of pediatric and elderly HL with evidence of TCR γ gene monoclonality we investigated the immunohistochemical expression of several B-cell (CD20, CD79a, OCT2, BOB1) and T-cell markers (CD3, CD4, CD4) on H/RS cells. In 2 out of 10 pediatric cases (20%) and in 5 of 5

elderly patients (100%) H/RS showed expression of at least one of the B-cell marker tested; in contrast, none of the cases showed expression of T-cell associated markers on H/RS cells.

To investigate the possibility that TCR γ monoclonality may also be a specific characteristic of H/RS cells, a single-cell PCR analysis of TCR γ gene clonality was performed in 4 EBV+ MC-HL of Iraqi children and in 3 elderly EBV+ MC-HL with evidence of restricted TCR γ gene rearrangements and no expression of T-cell markers on tumor cells. H/RS cells were easily recognizable by morphology, and could be singly collected with laser capture microdissection. DNA was obtained from about 200 microdissected H/RS cells and from 300 microdissected surrounding small lymphocytes per sample. H/RS-like cells and neoplastic T-lymphocytes microdissected from an angioimmunoblastic T-cell lymphoma were used as controls. Interpretable results were yielded only in one case of Iraqi children because of poor quality of the DNA extracted. Both genescan and heteroduplex analyses revealed the presence of a clonal TCR γ rearrangement in the microdissected H/RS cells undistinguishable from that observed in the whole tissue section (Figure 7A). This result suggests that H/RS cells might be able to rearrange both IGH and TCR γ genes. This observation is in keeping with what we have found in a previous study of a case of EBV+ mucocutaneous ulcer (*Di Napoli et al, 2011*). The mucocutaneous ulcer is a newly described EBV-related lymphoma (*Dojcnov et al, 2010*) that affects immunosuppressed or elderly people. It consists of an isolated ulcer, involving the skin or the mucosa of the gastrointestinal tract, composed by a polymorphous inflammatory-like infiltrate, in which are admixed atypical large EBV+ B cells with H/RS-like morphology. IGH and TCR γ genes clonality has been reported in 39% and 38% of the case respectively. We provided evidence of monoclonal TCR γ rearrangement in the microdissected neoplastic cells with H/RS-like morphology and not in the surrounding lymphocytes (Figure 7B).

Methachronous EBV-positive HL and EBV-negative non-Hodgkin lymphomas

In our series of cHL occurred in elderly patients we found that two patients had a prior history of lymphoma diagnosed by our Institution. The first patient had an extranodal diffuse large B cell lymphoma (DLBCL) of the lung treated with R-CHOP 5 years earlier; the other patient had a small B-cell lymphoma of the MALT type of the left kidney treated with surgery alone 2 years earlier. Both patients had a nodal EBV+ cHL; however, when we look for EBV-infection in the prior extranodal non-Hodgkin B-cell lymphomas we did not find any EBER and/or LMP1 positive cell. To confirm the data we performed PCR analysis for the detection of

EBER-1 gene on the DNA extracted from the tissue biopsies of the methachronous lymphomas. In both patients molecular analysis confirmed the absence of EBER-1 in the previous non-Hodgkin B-cell lymphomas (Figure 8).

IGH gene clonality assay showed monoclonal rearrangements in both patients with EBV+ cHL. In order to establish a possible clonal relationship with previous non-Hodgkin B cell lymphomas we compared rearrangements patterns between the methachronous tumors. In the MALT B cell lymphoma pherograms showed an identical pattern of IGH clonality between the methachronous tumors (Figure 9 case B); whereas, in the large B cell lymphoma the position of the IGH clonal peak differ from the one detected in the subsequent HL for 3 base pairs (Figure 9 case A).

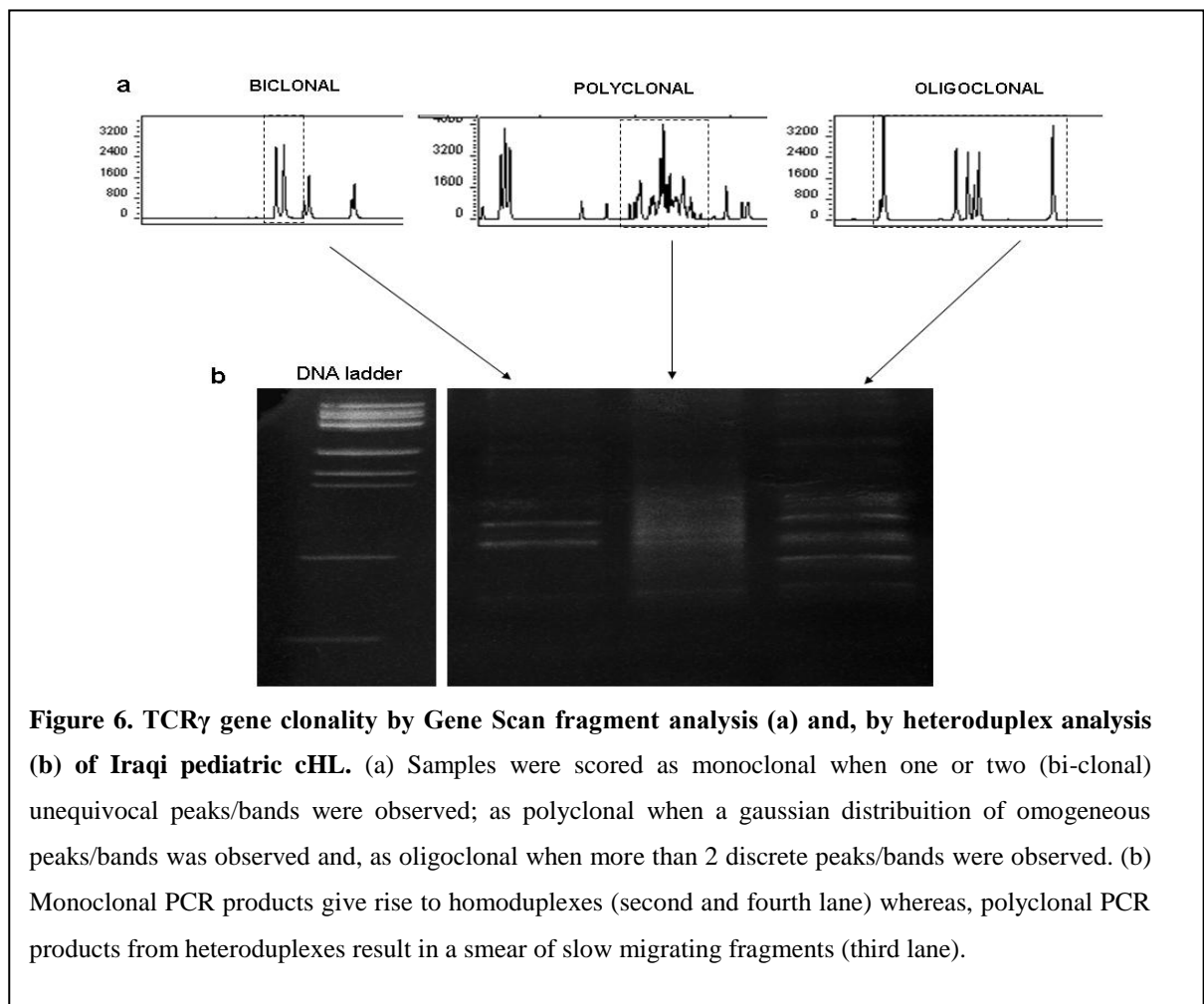


Table 12. TCR γ gene rearrangements in cHL grouped by patients age

Groups	n. of cases	TCR γ		
		Monoclonal	Oligoclonal	Polyclonal
cHL-old 50-69y	13	2 15%	2 15%	9 70%
cHL-old ≥ 70 y	8	3 37%	0 -	5 63%
cHL-child 3-5y	13	2 15%	6 46%	5 39%
cHL-child 6-13y	25	8 32%	12 48%	5 20%
cHL-young 17-32y	10	0 -	0 -	10 100%

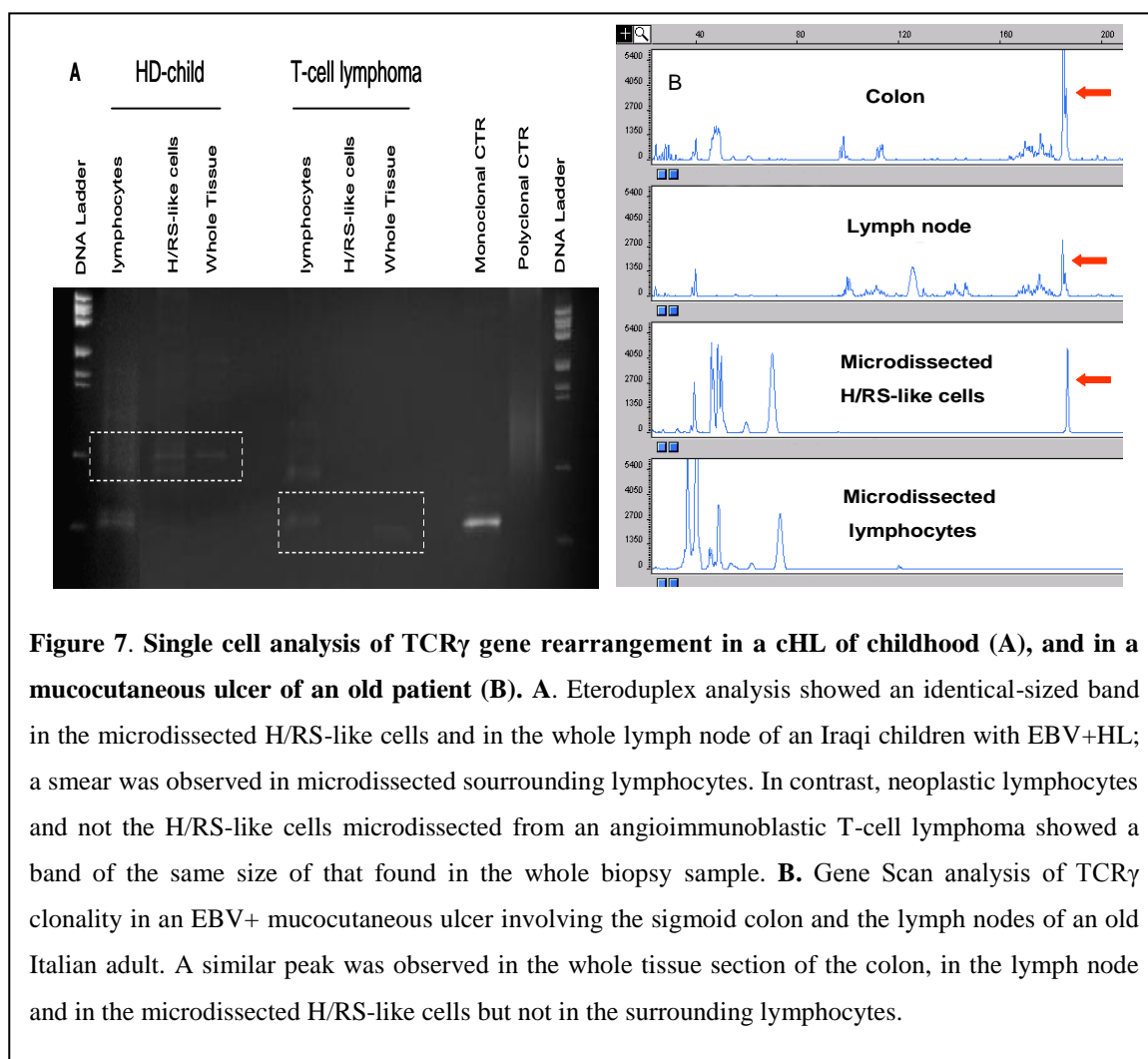
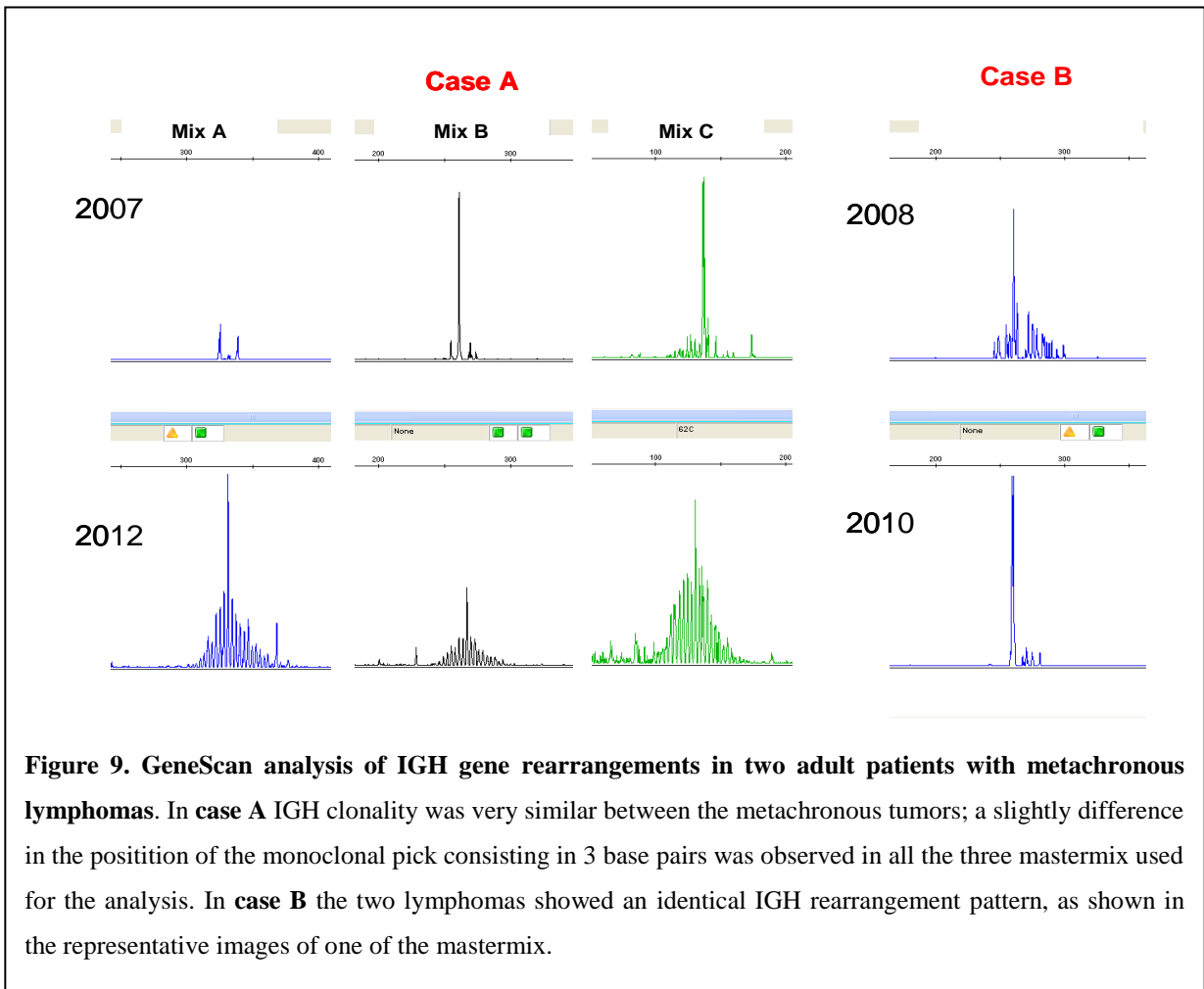
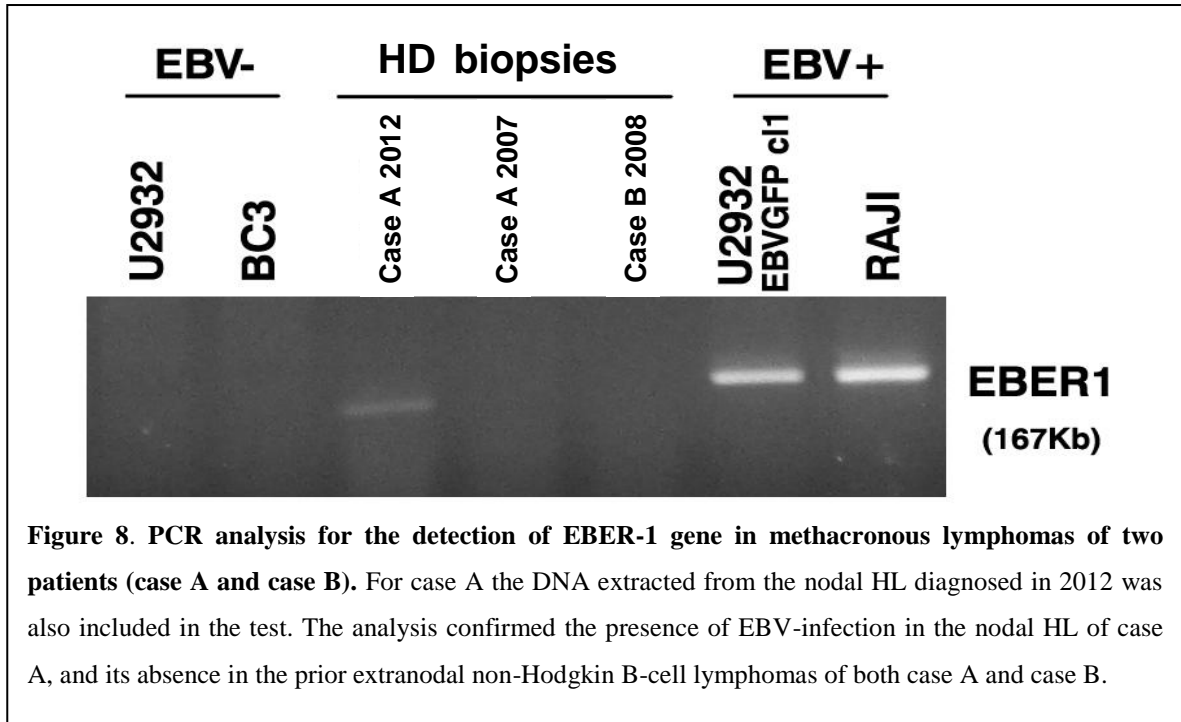


Figure 7. Single cell analysis of TCR γ gene rearrangement in a cHL of childhood (A), and in a mucocutaneous ulcer of an old patient (B). **A.** Eteroduplex analysis showed an identical-sized band in the microdissected H/RS-like cells and in the whole lymph node of an Iraqi children with EBV+HL; a smear was observed in microdissected surrounding lymphocytes. In contrast, neoplastic lymphocytes and not the H/RS-like cells microdissected from an angioimmunoblastic T-cell lymphoma showed a band of the same size of that found in the whole biopsy sample. **B.** Gene Scan analysis of TCR γ clonality in an EBV+ mucocutaneous ulcer involving the sigmoid colon and the lymph nodes of an old Italian adult. A similar peak was observed in the whole tissue section of the colon, in the lymph node and in the microdissected H/RS-like cells but not in the surrounding lymphocytes.



DISCUSSION

cHL in Iraqi children under 6 years of age is strictly associated with EBV-infection, MC-subtype and a cytotoxic background

In 1997 Glaser et al (*Glaser et al 1997*) published a meta-analysis of the epidemiologic characteristics of EBV-associated HL based on international data concerning 1546 HL patients of any age. They reported the existence of a higher risk of EBV+ cHL in children for both NS and MC subtypes; it was suggested that timing of infection greatly affects the association of EBV with cHL in children, with early age at infection strongly predicting EBV+ disease for both these histological subtypes. Our findings are consistent with this interpretation. In fact, we have observed that EBV infection of H/RS cells is present in 97% MC-HL and in 63% NS-HL of Iraqi children, whereas EBV+ NS in western countries accounts for approximately 10-25% of cases.

Table 13. EBV+ classic Hodgkin lymphoma in 300 Asian children in relation to age

Age range	Iraq		China ^a		India ^b		Total cases	EBV+ cases
	n. of cases	EBV+ cases	n. of cases	EBV+ cases	n. of cases	EBV+ cases		
3-5 y	18	18	33	32	17	17	68	67
	35% ^c	100% ^d	32%	97%	12%	100%	23%	99%
6-10 y	24	20	58	54	93	89	175	163
	47%	83%	56%	93%	64%	96%	58%	93%
11-14 y	9	6	13	7	35	28	57	41
	18%	67%	12%	54%	24%	80%	19%	72%
Total cases	51	44	104	93	145	134	300	271
		86%		89%		92%		90%

^aZhou XG et al. *Cancer* 2001; 92:1621-1631

^bDinand V et al. *Eur. J. Cancer* 2007 ; 43 :161-168

^cPercent of the total number of cHL cases

^dPercent of EBV+ cases/total number of classic HL in the age-range

Moreover, we have noted that there is a progressive age-related decrease in the percentage of EBV+ cases (from 100% to 67%), and a switch in the histological types from MC to NS. The epidemiology of cHL in Iraq is similar to that reported for China (*Zhou et al 2001*) and India (*Dinand et al 2007*). When the data of the three Asian countries are pooled together for a total of 300 children under 14 years of age (Table 13), it becomes clear that virtually all cases (99%)

of cHL under 6 years of age were EBV+, and that the percentage of EBV+ cases significantly decreased to 72% in the 11-14 years age-range. At present there is no definitive explanation for these findings. EBV-infection is strictly associated with MC histology, and it was speculated that a defective immune response, due to immaturity of the immune system might represent one of the triggering events (*Harris et al, 1998; Barros et al, 2012; Barros et al, 2011*). This interpretation is supported by the observation that the inflammatory background of MC-HL in children under 10 years is profoundly different from that of older children with NS subtype. In particular, *Barros et al.* found that the inflammatory background of EBV+ MC-HL-cases in children under 10 years was characterised by the presence of an intense T cell infiltrate exhibiting a cytotoxic/Th1 profile, whereas in the NS subtype of older children and in EBV-negative cases there is a higher number of CD4+ T cells with a more regulatory/Th2 profile (*Barros et al, IJC 2011*). In keeping with *Barros et al*, we found that in our series of pediatric cHL the number of GrB+ CTL cells was significantly higher in MC subtypes, in EBV-positive cases, and in patients of 3-5 years old.

LMP-1 gene polymorphism is not a frequent feature of EBV+ cHL of Iraqi children

EBV LMP-1 polymorphisms have been reported to have a greater tumorigenic potential than wild type LMP-1 gene. Among these a characteristic 30 basepair deletion in the 3' end of EBV LMP-1 gene has been found to be essential for the transformation activity of LMP-1 oncogene in transfection studies. The 30-bp deletion has been demonstrated in 4/12 (33%) EBV+ cHL cases from USA and in 12/26 (46%) cases from Brazil (*Hayashi et al 1997*). An higher incidence of EBV LMP-1 deletion has been found in HL occurring in HIV patients (89%) (*Docetti et al 1997; Bellas et al 1996*) and in Spanish children (79%) (*Santon et al 1998*). In our series of EBV+ HL of Iraqi children, we have found the presence of the 30-bp LMP-1 gene deletion in 32% of the cases. Our data are similar to what previously reported in USA and Brazil but dramatically different to that observed among Spanish children. It may be possible that geographical and ethnical factors may influence the incidence of EBV infection with EBV strains carrying distinct LMP-1 genes.

Aging is associated to the development of an EBV+ MC-HL subtype

Epidemiological studies reported that EBV+ HL of the mixed cellularity type shows a peak in older adults (≥ 55 years) in developed countries (*Armstrong et al, 1998, Jarrett et al,*

2003; Jarrett *et al*, 2005). Consistently with these reports, we detected EBV infection in 16 of 30 cHL (53%) occurred in elderly Italian patients, with a significant predilection for MC than NS histology (82% vs 33%; $p=0.036$). Moreover, the percentage of EBV-associated cases, and of MC HL subtype gradually increased with advancing of patients age, supporting the hypothesis that EBV infection, histology and patients age are closely related. Jarrett *et al*. indicated that the decline in EBV-specific cellular immunity that occurs with age may be responsible of EBV reactivation in infected B-cells, thus increasing the risk of their malignant transformation (Jarrett *et al*, 2005). Ageing is known to be accompanied by thymic involution and also by a chronic low-grade inflammation state; these determine a progressive exhaustion of the CD8+ T-cell population and predispose elderly people to tumor development (Vasto *et al*, 2007; Candore *et al*, 2010). A major force able to drive a chronic pro-inflammatory state during aging may be represented by persistent infections by EBV and Cytomegalovirus virus (CMV). In support of this view in our series of cHL occurring in Italian old patients we observed a significant higher number of GrB+ CTL cells in the MC cases, which were found to be more commonly associated with EBV-infection.

cHL of elderly Italians retains higher expression of B-cell markers than cHL of Iraqi children

It is widely accepted that H/RS derive from mature B cells, although they show global loss of the B-cell phenotype. Mechanisms causing this extensive reprogramming of the tumor cells are mostly unknown. One of the contributing factor identified is given by the downregulation of the expression of several transcription factors in H/RS cells, including OCT2 and BOB1, that regulates the expression of many B-cell specific genes. An exception of this rule is represented by PAX5, the main B-cell lineage commitment factor. In fact, although PAX5 is found to be expressed in H/RS, many of its target genes are found to be down regulated, suggesting that its activity might be somehow impaired (Saez *et al*, 2002; Kuppers, *Hematol* 2009; Browne *et al*, 2006). Our data are consistent with what previously reported in the literature; in the series of Hodgkin lymphomas we investigated H/RS cells were usually positive for PAX5, and exhibited downregulation of the other B-cell markers CD20, CD79a, OCT2 and BOB1. However, when stratified for histology and patients age, our data showed that MC-HL of old Italian adults retain an higher expression of OCT-2 (73%) and BOB-1 (55%) compared to MC-HL of Iraqi children (OCT2 31%; BOB1 11%). The expression of OCT2 and BOB1 in elderly MC-HL was also greater than that reported in the literature (OCT-

2 12-63%; BOB-1 25-37%) (Cosio *et al*, 2004; Browne *et al*, 2006). This difference may be due to the fact that in previous studies HL of different subtypes or from patients with different age were pooled together, whereas in our study we sub-group HL cases by histology and patient age. Moreover, we considered both strong and weak, but clearly positive, staining patterns, whereas others may have considered as positive only strong intensity staining. Alternatively, our findings may raise the possibility that some of the elderly MC-HL are instead EBV+ DLBCL of the elderly. However, not all the HL of old adults that express OCT-2 and/or BOB-1 were EBV+. Furthermore, the consistent expression of CD15, together with the infrequent positivity for CD20 or CD79a, on morphologically typical H/RS cells argues against this hypothesis. Finally, Adams *et al.*, have found that BOB-1, OCT-2, CD79a and CD20 were more commonly expressed in post-transplant Hodgkin lymphoma (ptHL) versus cHL, suggesting that ptHL and cHL are closely related but not identical neoplasm, differing in the strict association with EBV infection, persistent phenotypic B-cell signature, and high expression of PI3K as well as the slightly CD4-depleted but TIA-1/Granzyme B-enriched cellular background composition in ptHL (Adams *et al*, 2009).

Restricted TCR γ gene rearrangements are a distinctive feature of cHL of Iraqi children

We have found a monoclonal IGH gene rearrangement in 37% of pediatric HL and in 48% of elderly cases using the BIOMED2 system applied to total DNA extracted from whole paraffin sections. This percentage is similar to that reported by Chute *et al.* (Chute *et al*, 2008) who could demonstrate IGH monoclonality in 24% of cHL cases, and borderline clonality in 17%.

An interesting finding of our study is the demonstration of monoclonal/oligoclonal TCR γ , suggestive of restricted T cell responses, in a large proportion of pediatric cHL (74%). This percentage is considerably higher than that reported for France, where only 13 of 85 (15%) cHL cases exhibited clonal TCR γ gene rearrangements (Al Saati *et al*, 1997). However, the age of the French patients was not reported in the paper, so that it can not be excluded the existence of a possible association between patient's age and TCR γ gene status.

The biological significance of restricted T cell responses in HL involved tissues may be only matter of speculation. Restricted T cell responses against EBV antigens may be probably indicative of a poorly efficient immune response. In fact, during primary EBV infection, recovery from the symptoms is coincident with broad T cell reactivity to multiple epitopes whereas, in patients with a delayed healing a narrowly focused response has been observed

(*Bharadwaj et al, 2001*). More recently, it has been shown that a distinctive serologic response to EBV latent antigens, indicative of immune dysfunction, is associated with an increased risk to develop EBV+ cHL (*Levin et al, 2012*). Moreover, T cell restricted responses were previously observed in about 30% of EBV-driven lymphoproliferative disorders secondary to iatrogenic immunosuppression (*Au et al, 2006; Dojcinov et al, 2010*) and to immunosenescence owing to ageing (*Dojcinov et al, 2011*). In this latter condition T cell responses to viruses are profoundly affected due to accumulation of mature CD8+ T cells with diminished functionality. A similar condition was described in early infancy, where cytotoxic T lymphocyte responses to viral infections remain low as compared with adults (*Kovarik et al, 1998*). Furthermore, we detected a consistent polyclonal TCR γ rearrangements in 10 cases of EBV-negative NS-HL of Italian young adults. This supports the hypothesis that high incidence of T cell restricted responses in cHL of childhood might be the expression of a defective local immune response, which might be particularly pronounced in children under 14 years of age.

In contrast to Iraqi children, our series of cHL occurring in old Italian adults showed monoclonal/oligoclonal TCR γ rearrangements only in 33% of the cases. This difference might be related to a different tumor microenvironment. It is possible that children and older adults differ in their response to the tumor not only with respect to the recruited cell types but also to the functional status of these cells. *Barros et al.* have recently found that tumor microenvironment composition in pediatric cHL is distinct from adults, with a higher number of CD14+ cells and of CD3+, CD8+, TIA-1+, and TBET+ lymphocytes in children under 10 years (*Barros et al, IJC 2011; Barros et al, 2012*). In our series of pediatric and elderly cHL we did not find any significant difference in the number of CD3+, CD4+, CD8+, CD56+, GrB+ cells, with both pediatric and elderly cHL, having a more cytotoxic background in MC as compared to NS subtype. Nevertheless, we have no data on the functionality of these cells to exclude that different TCR γ clonality pattern may reflect diverse states of immune responsiveness.

Concomitant IGH and TCR γ rearrangements occurrence in H/RS cells

A small proportion (10-14%) of HL cases from both pediatric and elderly patients showed a concomitant IGH and TCR γ clonality. Dual IGH and TCR γ clonality has been previously reported by *Saati et al.* in 6% of a series of 85 HL. Reports on single cell analysis of IGH and TCR γ rearrangements in HL showed that clonality of IGH and TCR γ genes in H/RS cells are mutually exclusive, with evidence of TCR genes clonality in H/RS cells expressing T-

cell markers (Seitz *et al*, 2000; Müschen *et al*, 2000). Thanks to laser capture micro-dissection we provide evidence that TCR γ clonal rearrangement may occur in neoplastic B cells with H/RS-like morphology lacking expression of T-cell associated markers. Thus, our findings raise the possibility that in H/RS cells a still unrecognized transforming event might cause deregulation/impairment of the RAG (Recombination Activating Gene) enzyme system inducing TCR gene rearrangements in B cells which have already undergone IGH rearrangements. A similar pathogenetic mechanism has already been proposed to explain the presence of concomitant IGH and TCR rearrangements in a consistent number of cases of precursor-B-ALL (Szczepański *et al*, 1999).

Composite EBV+ HL and EBV-negative non-Hodgkin B cell-lymphoma

It is interesting to note that in our series of elderly EBV+ HL two patients had a prior history of an EBV-negative extranodal non-Hodgkin B-cell lymphoma. GeneScan analysis demonstrated an identical monoclonal IgH rearrangement in the methachronous lymphomas of one of the two patients, suggesting derivation from the same neoplastic clone. In the other patient, a very similar IgH clonality pattern was observed between the two lymphomas. Although IGH somatic mutation seems not to be a characteristic feature of cHL (Kuppers *et al*, 1994), in this patient it can be possible that additional mutations in the rearranged IGH gene may have occurred in the subsequent tumor. Alternatively, it might be possible that the two tumors represent unrelated neoplasms. To address this question, we are presently undergoing IGH mutational analysis of the methachronous lymphomas. Recently, a single cell analysis have been conduct to investigate the clonal relationship of a series of cHL and its recurrences (Oberman *et al*, 2011). The authors found both related and unrelated relapses, and observed an EBV-association switch in a proportion of the clonally unrelated HL. Similarly, Tinguely *et al*. described a composite lymphoma consisting of a mantle cell lymphoma (MCL) and an EBV+ HL, in which single cell analysis of rearranged V genes revealed a clonal relationship between the two lymphomas. Moreover, the authors detected somatic mutations in H/RS but not in MCL cells, and noticed that only a subclone of the H/RS population, with a particular mutation pattern in the V genes, was EBV-infected. This finding represented for Tinguely *et al* a strong indication that EBV infection of the H/RS cell precursor occurred in the germinal center (GC) (Tinguely *et al*, 2003). The identification of composite synchronous and methachronous tumors suggests the possibility of the existence of a common tumor stem or precursor cell that already carried some transforming events. Nevertheless, it might be possible that unknown genetic or

environmental factors (i.e. chemotherapies, aging) predispose an individual to the development of clonally unrelated lymphomas.

The presence of EBV exclusively in the relapsed HL in our cases and in the ones described in the literature (*Oberman et al, 2011*) raises concern about the role of EBV in the pathogenesis of the disease. It can be speculated that a common precursor might be subsequently infected by EBV during the remission period between the two methachronous lymphomas, probably through virions released by non-neoplastic memory B cells. Alternatively, based on the “hit and run” theory, we could assume that EBV infection occurred early during tumorigenesis, and that only tumor cells with a defective EBV genome that loss the virus might have had a selective advantage in the prior lymphoma (*Jox et al, 1997; Trivedi et al, 1995*). However, this theory is difficult to prove because we and others have failed to find any trace of previous EBV infection in H/RS cells of EBV-negative lymphomas (*Staratschek-Jox et al, 2000*). In contrast, arguments in favour of the theory are the observation that EBV+ HL cases may relapse as EBV-negative cases (*Delecluse et al, 1997; Nerurkar et al, 2000*), the notion that infectious mononucleosis represents a risk factor also for the development of EBV-negative HL (*Jarrett et al, 2002; Sleckman et al, 1998*), and the demonstration of defective forms of EBV which progressively lose the ability to infect host cells (*Gan et al, 2002*).

In conclusion, our data suggest that cHL occurring in Iraqi children and in Italian older adults are very similar with an age-depend variation in histology and EBV-infection, suggesting that in both class of patients an impaired immune system may be critical for the development of the disease. However, significant differences in B-cell phenotype of H/RS cells and in pattern of TCR γ gene rearrangements suggest that cHL of pediatric and elderly patients are closely related but not identical neoplasm, probably representing a continuum spectrum.

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