UNIVERSITA' DEGLI STUDI DI VERONA

DIPARTIMENTO DI
PATOLOGIA E DIAGNOSTICA

SCUOLA DI DOTTORATO DI
SCIENZE BIOMEDICHE TRASLAZIONALI

DOTTORATO DI RICERCA IN
PATOLOGIA ONCOLOGICA E FISIOPATOLOGIA RIGENERATIVA TISSUTALE UMANA

CICLO /ANNO XXIV/ 2009

TITOLO DELLA TESI DI DOTTORATO

"*IN VITRO* AND *IN VIVO* MODEL OF EBV-POSITIVE ACTIVATED DIFFUSE LARGE B CELL LYMPHOMA WITH PLASMACYTIC DIFFERENTIATION"

S.S.D. MED/08

Coordinatore: Prof. ALDO SCARPA

Tutor: Dott. MAURO KRAMPERA

PhD Student: Dott.ssa ILARIA NICHELE

TABLE OF CONTENTS

RIASSUNTO

Le malattie linfoproliferative croniche possono evolvere istologicamente in linfomi ad alto grado di malignità. E' noto come il virus di Epstein-Barr giochi un ruolo importante nella patogenesi di alcuni linfomi aggressivi e come sia associato alla trasformazione istologica di forme indolenti. E' nota inoltre la sua capacità trasformante di cellule in vitro. Le linee cellulari rappresentano un importante strumento di ricerca poiché facilitano lo studio della biologia di molte malattie e l'applicazione di nuove terapie mirate. VR09 é una nuova linea cellulare di linfoma B diffuso a grandi cellule (DLBCL) con differenziazione plasmacitica realizzata nel nostro laboratorio e derivante da un caso di malattia linfoproliferativa B. Abbiamo dettagliatamente studiato la linea cellulare attraverso la caratterizzazione immunofenotipica, immunoistochimica, molecolare, citogenetica e mediante FISH. Abbiamo inoltre testato il potenziale tumorigenico di VR09 in vivo. Le cellule in sospensione hanno mostrato capacità proliferativa in vitro dopo alcuni mesi e capacità di sviluppare una massa sottocutanea quando inoculate sottocute in topi immunodeficienti Rag2-/- γ-chain-/- . Sia le cellule in vitro che quelle derivate dalla massa sottocutanea hanno mostrato un profilo immunoistochimico corrispondente ad uno stadio "attivato" della maturazione B con aspetti di differenziazione plasmacitica (CD19+, CD20+, CD79a+, CD79b+/-, CD138+/-, cyclina D1-, Ki67 80%, IgM+, IgD+, MUM1+, MDNA+, CD10-, CD22+, CD23+, CD43+, K+, λ-, Bcl2+, Bcl6-) e la presenza di EBV in forma episomica. E' stata inoltre identificata la trisomia del cromosoma 12, la presenza di ipermutazioni somatiche nella regione VH, la mutazione dei geni Card11 e CD79B, e

la presenza di p53 in forma wild-type. Questa nuova linea cellulare potrebbe essere utile per caratterizzare in modo più approfondito le forme di DLBCL con differenziazione plasmacitica e testare in esse nuove terapie mirate.

ABSTRACT

Background: B-cell lymphoproliferative diseases can show plasmacytic differentiation and may potentially progress to diffuse large B cell lymphoma (DLBCL). Epstein-Barr virus infection may cause the transformation of malignant cells *in vitro*.

Design and Method: we established VR09 cell line, a DLBCL cell line with plasmacytic differentiation, obtained from a case of atypical B-cell chronic lymphoproliferative disease with plasmacytic features. We used flow cytometry, immunohistochemistry, polymerase chain reaction, cytogenetic analysis and florescence *in situ* hybridization to characterize this cell line. We also assessed whether VR09 has tumorigenic potential *in vivo*.

Results: cells in suspension revealed plasmacytic features and grew as spherical tumors when inoculated subcutaneously into immunodeficient $Rag2^{-/-}$ γ-$chain^{-/-}$ mice. VR09 cell line and tumors displayed the phenotype of activated stage of B cell maturation, with secretory differentiation (CD19+ CD20+ CD79a+ CD79b+/- CD138+/- cycline D1- Ki67 80% IgM+ IgD+ MUM1+ MDNA+ CD10- CD22+ CD23+ CD43+ K+, λ- Bcl2+ Bcl6-); in addition they displayed episomal EBV genome, chromosome 12 trisomy, absence of c-MYC rearrangement, presence of somatic hypermutation in the VH region, mutations of Card 11 and CD79B genes, and wild-type p53.

Conclusion: This new EBV-positive cell line may be useful to further characterize activated DLBCL with plasmacytic features.

1 INTRODUCTION

1.1 Primitive diffuse large B cell lymphoma: general aspects

Diffuse large B cell lymphoma (DLBCL) is the most common form of non-Hodgkin Lymphoma in the western countries with age-related increasing incidence, from 2 cases per 100,000 at 20-24 years of age to 112 cases per 100,000 by 80-84 years (Yancik, 2004); however, DLBCL incidence is 1.5-fold higher in males and there are significant racial differences, with increase rate in Caucasians (Fisher RI, 2004). It is characterized by aggressive evolution, with a median survival of less than one year in untreated patients.

On the whole, the incidence of non-Hodgkin lymphomas increased dramatically in the last three decades, thus making these diseases the seventh most common cancer type. Several factors have contributed to this increase, including more sensitive methods to make proper diagnosis, improvements in reporting of haematological malignancies, changes in the classification systems used for lymphoid malignancies and human immunodeficiency virus (HIV) infection that is associated with the increase in HIV-associated lymphomas (Friedberg, 2008).

Although the etiology of DLBCL still remains unknown for the majority of patients, increased risk of lymphoma, including DLBCL, has been observed in association with viral infections, chemotherapy and conditions affecting immune system, such as autoimmune diseases, organ transplantation and primary or acquired immunodeficiencies. In particular, several infectious agents have been linked to the risk of lymphoma, such as Epstein-Barr virus (EBV), human herpes virus 8 (HHV 8), Helicobacter pylori, Chlamydia psittaci and hepatitis C virus (HCV) (Fisher SG, 2004).

Clinical, morphological and molecular heterogeneity of DLBCL has been known for a long time. The attempts to define DLBCL subgroups simply on the basis of morphology have largely failed due to the wide diagnostic discrepancies among histologists. Thus, the classification of human lymphomas has evolved constantly during decades. Recently, DLBCL nomenclature in 2008 World Health Organization (WHO) classification of the lymphoid tissue tumors have been modified to include some newly defined entities on the basis on distinctive clinical, pathological or biological features (**Table 1**).

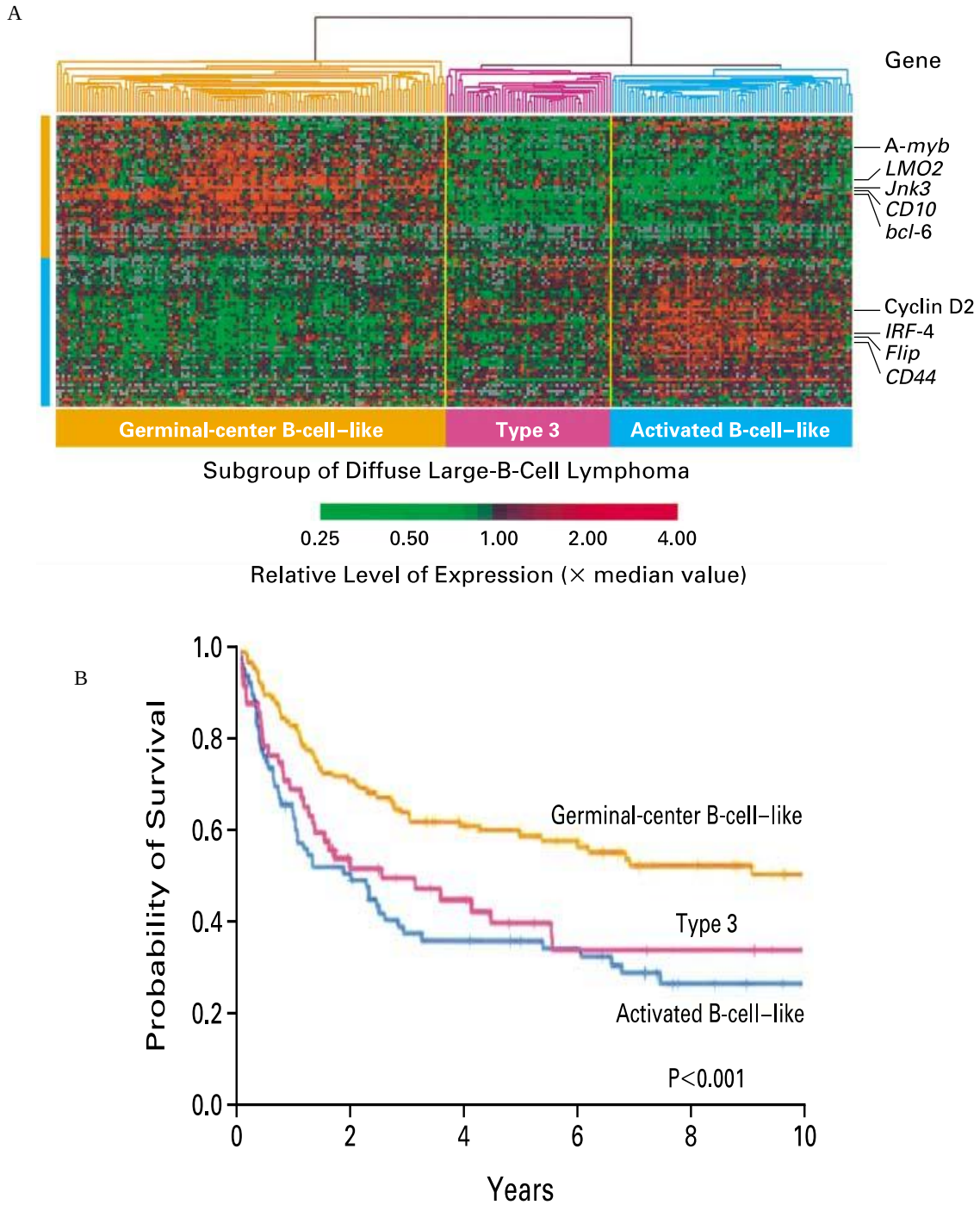
CLASSIFICATION
Diffuse Large B cell Lymphoma (DLBCL), not otherwise specified
Diffuse Large B cell Lymphoma subtypes
T-cell/histiocyte rich large B cell lymphoma
Primary DLBCL of the CNS
Primary cutaneous DLBCL, leg type
EBV positive DLBCL of the elderly
Other Lymphomas of large B cells
Primary mediastinal large B cell lymphoma
Intravascular large B cell lymphoma
DLBCL associated with chronic inflammation
Lymphomatoid granulomatosis
ALK positive large B cell lymphoma
Plasmablastic lymphoma
Large B-cell lymphoma arising in HHV8-associated multicentric Castleman disease
Primary effusion lymphoma
Borderline cases
B cell lymphoma, unclassifiable, with features intermediate between DLBCL and Burkitt lymphoma
B cell lymphoma, unclassifiable, with features intermediate between DLBCL and classical Hodgkin's lymphoma

Table 1. World Health Organization (WHO) Classification of DLBCL

However, most DLBCL do not possess specific clinical or pathological features and, therefore, are included in the group of DLBCL not otherwise specified (NOS) (Campo, 2011).

By the gene expression profiling technology (GEP) developed in the

last decades, DLBCL have been further classified into distinct molecular subgroups. The first subgroup have the gene expression profile clustering with that of normal germinal center B (GCB) cells and has been named ‘GCB variant’. The second group has a set of signature genes similar to that of mitogenically activated blood B cells (ABC) and has been termed ‘ABC variant’. These subsets are associated with specific genetic alterations, different molecular signalling pathways and different clinical outcome (Rosenwald, 2002) (**Figure 1**).



N Engl J Med, Vol 346, N 25, June 2002

Figure 1. Subgroups of DLBCL according to gene expression profiling.
A. Hierarchical clustering of DLBCL according to the level of expression of 100 genes. Red areas indicate increased expression, and green areas decreased expression. Each column represents a single DLBCL type and each row represents a single gene. **B.** Overall survival after chemotherapy among untreated patients according to gene expression subgroup.

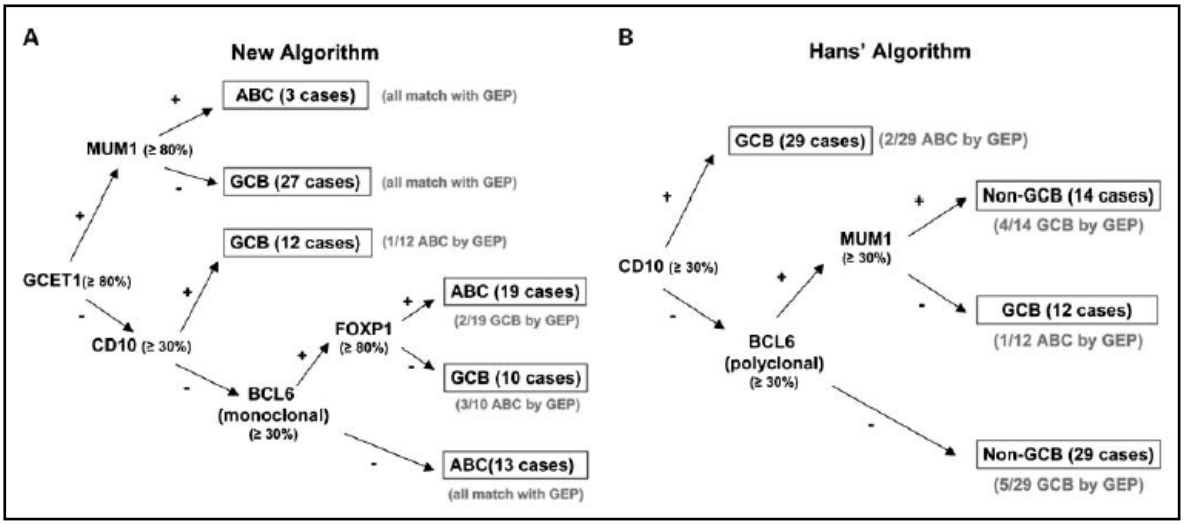
Clinical presentation of DLBCL is usually characterized by rapidly enlarging lymph nodes. However, the main localization may be extranodal in up to 40% of patients, commonly involving skin, gastrointestinal tract, central nervous system, lungs, genitourinary tract or bones. Approximately 15% of patients may have bone marrow involvement, about one-third B symptoms (fever, night sweats and weight loss), nearly 50% Ann Arbor system stage III/IV disease and more than 50% elevated serum lactate dehydrogenase (LDH) levels (Flowers, 2010). However, DLBCL patients normally have highly variable clinical course and treatment responses.

Although DLBCL is normally characterized by aggressive development, it is commonly curable with conventional anthracycline-based chemotherapy. Advances in the management of DLBCL during the last decade, including the advent of monoclonal antibodies, have led to excellent outcome for many patients. It is now clear that rituximab-containing regimens improve response rate and survival of DLBCL patients regardless of age. However, relapse remains a consistent clinical problem that needs a better understanding of disease biology to improve current strategies for salvage therapy based on targeted therapy approaches (Flowers, 2010).

The efforts to predict DLBCL outcome led to propose in 1993 the International Prognostic Index (IPI), which remains nowadays the primary clinical tool used to predict outcome. IPI score is based on the consideration of 5 risk parameters, with one point given for each factor, i.e. stage III/IV disease, elevated LDH, age >60 years, Eastern Cooperative Oncology Group (ECOG) performance status and involvement of > 1 extranodal site. This way, IPI scoring system stratify patients into 4 risk groups with a 5-year overall survival (OS) ranging from 26% to 73% (Shipp, 1993).

However, outcome prediction has been recently improved by the addition of biological prognostic factors. As previous mentioned, DLBCL have been divided into two subgroups, GCB and ABC types, according to different gene expression profiles. GCB and ABC subtypes have different pathogenetic mechanisms that have impact on the development of target therapies. When treated with CHOP (Cyclophosphamide, Doxorubicin, Vincristine and Prednisone) or CHOP-like chemoterapeutic regimens, patients with GCB DLBCL have a better survival independently from IPI score (Rosenwald, 2002). The prognostic value of GEP classification remains significant also for DLBCL patients treated with Rituximab plus CHOP or CHOP-like therapy (Lenz, 2003).

As gene expression profiling technology has not easily tranferred into common practice, an immunohistochemical algorithm have been proposed and validated for classification of DLBCL into GCB and ABC subtypes. The initial immunohistochemical algorithm proposed by Hans et al. (Hans, Blood 2004) was improved by a consortium of hematopathologists, leading to a new algorithm with 93% concordance with gene expression profiling (**Figure 2**). Independently of IPI score, GCB and ABC subtypes, as defined by IHC, showed significant differences in event-free survival and overall survival among patients treated with and without Rituximab-based regimens. (Choi, 2009).



Choi, Clin Cancer Res 2009

Figure 2. The new algorithm and the Hans' algorithm.

1.2 Biology and pathogenetic pathways in diffuse large B cell lymphoma

DLBCL grows diffusely, completely subverting the normal lymph node architecture. It expresses different pan-B markers, such as CD19, CD20, CD22 and CD79a, but may lack one or more of them. Surface and/or cytoplasmic immunoglobulins can be demonstrated in 50-70% of cases. Some cases express CD5 (10%) or CD10 (25-50%). CD5+ DLBCL do not express cyclin D1, thus differing from blastoid variants of mantle cell lymphoma. BCL2 is expressed in approximately 30-50% of cases and nuclear BCL6 expression is observed in a very high proportion of cases. Expression of plasma cell-associated markers, such as CD138, is rare. The proliferative fraction as detected by Ki-67 staining is usually high and may be greater than 90% in some cases (Gatter, 2001).

Peripheral B cells of either germinal or post-germinal centre are supposed to be the cells of origin of DLBCL, thus suggesting that this

tumor can derive from two different maturation stages of B cell development. As previously mentioned, this distinction reflects the two major molecular categories that have been recognized over the past decades according to gene expression profiling studies, i.e. GCB and ABC subtypes (Alizadeth, 2000).

Germinal Center (GC)

In the physiologic development of B cells, GC is the hallmark of T cell-dependent immune responses. Once naïve B cells encounter their antigen, a rapid and intense cell proliferation occurs, leading to the formation of histologically well-defined structures (MacLennan, 1994). Within the GC, the immunoglobulin genes of the B cells are modified through the process of somatic hypermutation and class switch recombination, two events aimed at favouring the emergence of cells producing antibodies with increasing antigen affinity and capable of distinct effector functions. GC can be divided into two anatomically distinct areas: the dark zone, populated by rapidly dividing centroblasts, and the light zone, which is composed of smaller non-dividing centrocytes in close contact with follicular dendritic cells (Klein, 2003). Centroblasts are characterized by elevated expression of BCL6, a powerful transcriptional repressor that modulates the expression of a set of genes, including those involved in B cell receptor (BCR) and CD40 signalling, T cell-mediated B cell activation, induction of apoptosis, response to DNA damage, negative regulation of cell cycle progression, several cytokines pathways and plasma cell differentiation. This complex transcriptional program driven by BCL-6 plays a critical role in the GC and in DLBCL is frequently targeted by genetic lesions (Pasqualucci, 2011). When centroblasts stop proliferating, they differentiate into centrocytes that are rechallenged in light zone by the

antigen. Centrocytes expressing BCR either with reduced affinity for the antigen or self-reactive undergo apoptosis, while a few cells with high affinity antigen receptors are selected to differentiate into plasma cells or memory B cells (Klein, 2008).

A key concept for the understanding of DLBC pathogenesis is the relationship between these tumors and the events that take place in normal B cell counterpart. GCB-DLBCLs appear to derive from proliferating CB, as their expression profile includes genes that are specific for GC B cells, such as BCL-6 (Alizadeth, 2000). Genetic lesions that are specific for GCB-DLBCL include: 1. t(14,18) translocation, which deregulates the expression of the anti-apoptotic BCL2 protein in 35-45% of cases, thus becoming insensitive to BCL-6-mediated transcriptional repression (Ci, 2009); 2. MYC oncogene involvement altering cell proliferation and growth; 3. somatic heterozygous mutations in histone methyltransferases; 4. alterations inactivating acetyltransferase genes that impair the ability of this enzyme to acetylate known substrates, such as BCL-6 and p53, leading to constitutive activation of the oncoprotein and decreased p53 tumor suppressor function; 5. somatic mutations affecting BCL6 self control circuits, thereby causing its deregulated expression. Other lesions consist in mutations and deletions of the p53 tumor suppressor gene, often detected in cases deriving from the transformation of low grade lymphoma and associated with chromosomal traslocation of BCL2 and deletions of the tumor suppressor PTEN (Pasqualucci, 2011).

Activated B Cell (ABC)

Plasma cells represent the final step of B cell differentiation as cell mediators of humoral immunity. After antigen exposure, naïve B cells can differentiate rapidly into short-living plasma cells secreting

unmutated immunoglobulin within the medullary zone of lymph nodes and splenic red pulp. In case of antigen persistence, B cells expressing high-affinity Ig are selected within GC and give rise to early plasma cells. Some plasma cell precursors can migrate to the bone marrow or to the lamina propria of mucosae, where they differentiate into long-living plasma cells. Whereas terminally differentiated plasma cells do not divide, maturing plasma cell precursors, i.e. plasmablasts, undergo cell division (Tarte, 2003). In humans, three subsets of normal plasma cells have been isolated, representing a gradient in plasma cells maturation: early plasma cells in tonsils, transitional plasma cells in peripheral blood and mature plasma cells in bone marrow (Medina, 2002). In particular, peripheral blood B cells differentiating into polyclonal plasmablastic cells are highly proliferative, produce large amount of Ig, have the morphologic and phenotypic features of plasma cells and express high levels of plasma cells specific transcription factors, such as XPP-1, PRDI-BF1 and IRF-4 (Tarte, 2003).

The ABC-DLBCL subgroup displays a transcriptional signature related to either plasmablastic B cells presumably blocked during plasma cell differentiation or mitogenically activated peripheral blood B cells. Their gene expression profile suggests that the putative cell of origin has received signals determining the downregulation of the GC specific program and the upregulation of genes required for terminal B cell differentiation, such as IRF 4 or XBP1. Thus, ABC DLBCL arises from post-germinal center B cells that stopped their plasmacytic differentiation (Alizadeh, 2000).

Several genetic abnormalities are observed almost exclusively in ABC-DLBCL, such as: 1. amplification of the BCL2 locus with the consequent inhibition of apoptosis; 2. mutations within NF-kB, BCR and JAK-STAT signalling pathways; 3. inactivating mutations and

deletions of PRDM1 tumor suppressor gene; 4. deletion or lack of expression of the CDkN2A tumor suppressor gene (Pasqualucci, 2011). In particular, a characteristic feature of ABC DLBCL is the constitutive activation of the NF-kB pathway, which plays a pivotal role in proliferation, differentiation and survival of normal lymphoid cells (Davis, 2001). This abnormality has been linked to malfunctioning of a variety of upstream proteins including CARD11. In normal B cells, CARD11 is engaged during antigenic stimulation of BCR signalling and acts as signalling scaffold protein to coordinate the activation of IkB kinase beta, a positive regulator of the NF-kB pathway. About 10% of ABC DLBCLs have mutant CARD11 isoforms that activate NF-kB. Missense mutations have been detected, all whitin exons encoding the coiled-coil domain (Lenz, 2008). For the majority of ABC DLBCL cases, NF-kB activation can be observed in absence of CARD11 mutations. In these cases, NF-kB activation may be linked to persistently active BCR signalling. Somatic mutation affecting the immunoreceptor tyrosine-based activation motif (ITAM) of CD79B and CD79A was detected frequently in ABC DLBCLs, but rarely in other lymphomas. These mutations increase surface BCR expression and modulate Lyn kinase, a feedback inhibitor of BCR signalling (Davis, 2010).

1.3 Diffuse large B cell lymphoma derived from lymphoproliferative diseases

Chronic B cell lymphoproliferative disorders are a biologically heterogeneous group of malignant diseases characterized by accumulation of mature B cells in bone marrow, peripheral blood and

lymphoid tissues (Pangalis, 1999). They are often diagnosed by flow cytometric immunophenotyping that identifies a clonal light chain-restricted population expressing B cell markers in the blood or bone marrow (Dronca R, 2010) (**Table 2**).

CONDITION	SmIg	CD5	CD43	CD22	CD23	CD25	FMC7	CD103	CD11C	CD10	CYTOGENETIC ABNORMALITIES AND MOLECULAR CHARACTERISTICS
CLL	-/+	+	+	-/+	+	+/-	-/+	-	-/+	-	+12, de13q14, 6q-, 11q+, 14q+
Lymphoplasmacytoid lymphoma or immunocytoma	++	-/+	+/-	+	-	-/+	+	-	-/+	-	(CIg+, 100%), +12, 13q-, 14q+,11q+
Prolymphocytic leukemia	++	-/+	+	+	-/+	-	+	-	-	-/+	t(11;14) (q13;q32), *bcl-1*
Hairy-cell leukemia	++	-	+	+	-	+	+	+	+	-	HC2+,+5
Splenic lymphoma with villous lymphocytes	++	-/+	+	+	+/-	-/+	+	-/+	+/-	-/+	t(11;14) (q13;q32), del/t 7q22-35, 2p11
Marginal-zone B-cell lymphoma	++	-	-/+	+/-	+/-	-	+	-	+/-	-	(CIg+, 40%), +3, t(11;18)
Mantle-cell lymphoma	++	+	+	+/-	-	-	+/-	-	-	-/+	t(11;14) (q13;q32), *bcl-1*
Follicular lymphoma	++	-/+	-	+/-	-/+	-	+	-	-	+/-	t(14;18) (q32;q21), *bcl-2*

Rozman, N Engl J Med 1995

Table 2. Immunophenotypic, cytogenetic and molecular features of malignant conditions affecting mature B cells.

However, in a minority of patients, no specific pathological diagnosis can be done. Indeed, some entities may have overlapping features, frequently presenting diagnostic problems and leading to the simple definition of atypical lymphoproliferative disease (Rozman, 1995). Moreover, several types of small B cell neoplasms can display plasmacytic differentiation without specific markers of lymphoplasmacytic lymphoma. Thus, sometimes only a partial diagnosis of small B cell lymphoma with plasmacytic differentiation

can be done (Campo, Blood 2011).

Histologic transformation from indolent to more aggressive lymphoma is a common feature of virtually all histologic subtypes of chronic B cell lymphoproliferative disorders. Sometimes, the morphologic characterization of DLBCL subtypes can be complicated by the presence at diagnosis of small B cell components. In most cases, this presentation occurs in patients with history of indolent lymphoma, thus permitting the diagnosis of histologic transformation. However, a proportion of DLBCL are unknown indolent lymphomas diagnosed only at the time of their transformation (Ghesquieres, 2006).

Histologic transformation of follicular cell lymphoma is the most frequently described and studied entity (Yuen, 1995). However, histologic transformation can occur in other low grade B cell lymphoproliferative diseases, such as marginal zone lymphoma (Dungarwalla, 2008), MALT lymphoma (Liu, 2010) lymphoplasmacytic lymphoma / Waldenstrom macroglobulinemia (Shiseki, 2011) and small lymphocytic lymphoma/B cell chronic lymphocytic leukemia (Rossi, 2009).

The accumulation of additional genetic abnormalities are responsible for histologic transformation of indolent lymphomas to diffuse aggressive histotypes, mainly in clonally-related histologic transformation that happens in most cases of DLBCL evolving from follicular lymphoma, marginal zone lymphoma and lymphoplasmacytic lymphoma (Freedman, 2005) and in about 80% of cases of DLBCL evolving from chronic lymphocytic leukemia (Richter syndrome) (Rossi, 2009). The specific genetic lesions identified include alterations in proliferation-regulating genes (C-MYC), cell cycle regulators (CDKN2a, CDKNb), and programmed cell death inducers (TP53, C-MYC, BCL2). Moreover, acquisition of 17p13 deletion is a

frequent molecular event in Richter syndrome and in other types of transformation from indolent to aggressive B cell malignancies (Rossi, 2008). However, chromosomal aberrations usually found in the related indolent lymphomas have been detected also in DLBCL, such as t(14;18) in cases originated from follicular lymphoma, aberration of chromosomes 3, 18 and 7 in cases derived from marginal zone lymphoma, and deletions of 11q, 13q, 6q or trisomy 12 in cases derived from chronic lymphocytic leukemia (Ghesquieres, 2006).

The pathogenetic mechanisms of not clonally-related histologic transformation cannot be directly ascribed to the accumulation of genetic lesions in the low-grade clone, but rather to alterations of the host genetic background and immunologic functions, or microenvironment dysfunctions enhancing the probability of DLBCL to develop (Rossi, 2009).

DLBCL developing in a patient with previous indolent lymphoma is usually resistant to chemotherapy and the prognosis is poorer than in *de novo* DLBCL. Median overall survival ranges from 2.5 to 22 months, although is generally shorter than one year (Bastion, 1997).

1.4 EBV infection and malignancies

Epstein-Barr virus is a gamma-herpesvirus with 172-kb DNA genome, which infects more than 90% of world population. It has a tropism for epithelial cells (e.g oral and nasopharyngeal mucosa), lymphocytes (B and T cells) and myocytes, but it predominantly infects, replicates and persists in B cells (Thorley-Lawson, 2001). Primary infection of EBV may cause short-term proliferation of B cells in human hosts. The infection is usually self-limited and controlled by the strongly elevated

T cell immune response (Chen, 2011). Then, EBV persists latently in the host inside long-life memory B cells. These cells can undergo limited expansion or extrafollicular proliferation and persist in the host for a long time. EBV can reactivate periodically and infect new hosts through saliva. Thus, like other herpesvirus, it has both a latent phase of infection and a lytic (productive) phase that produces new infectious virions (Michelow, 2012). If the infection occurs in adolescence or adulthood, it may cause the clinical symptoms of infectious mononucleosis, with up to 50% of T cells in the host specific to the virus; however, most EBV infections are initially asyntomatic (Chen, 2011). *In vivo* and *in vitro* EBV latent state is characterized by the absence of viremia and maintenance of the viral genome into the cell nucleus, where persists as extrachromosomal circular molecule named episome (Tsimberidou, 2005).

During latency, a limited number of EBV-encoded proteins and several non-coding RNAs are expressed. These include two EBV encoded small RNAs (EBER1 and EBER2), nuclear antigens and membrane proteins (Chen, 2011). In the latent form, EBV-encoded genes ensure the survival of the viral genome, allowing it to circumvent host's immune surveillance by limited expression of viral proteins.

Genetic and biochemical experiments demonstrated that several viral proteins directly contribute not only to latency of infected cells *in vitro* and *in vivo*, but also to growth transformation (Michelow, 2012).

In vitro, EBV infects resting human B lymphocytes and transforms them into clonal lymphoblastoid cells, a hallmark process termed growth transformation. After infection, B cells become activated as a result of the expression of EBV latent antigens and may proliferate indefinitely (Kelly, 2006). Nuclear antigens, such as EBNA 1, -2, -3A, 3B, 3C, -LP, and membrane proteins including LMP1, PMP2A and -B

are required for this transformation process and the coding genes are consistently expressed in lymphoblastoid cells. Typically, EBV latent genes mimic cellular functions; for instance, LMP1 indirectly inhibits apoptosis by upregulating several cellular antiapoptotic genes presumably through the induction of the NF-kB pahway, thus promoting cell proliferation and targeting physiological signalling pathways (Altmann, 2005).

Transforming capacity of EBV suggests its oncogenic role. Indeed, EBV is highly associated with several neoplastic diseases, such as endemic Burkitt Lymphoma, T cell lymphoma, nasopharyngeal carcinoma, Hodgkin Lymphoma and immunoblastic lymphoma in immunocompromised patients (Young, 2004).

EBV has been also detected in low-grade lymphoproliferative diseases, such as B-cell chronic lymphocytic leukemia (CLL), where it has been shown to correlate in some cases with disease transformation into Richter syndrome (Jeffrey 2010, Rossi 2009, Ansell 1999). The importance of EBV in the pathogenesis of lymphoma emerges also in the last update of WHO classification of lymphoid neoplasms, which recognizes several DLBCL entities characterized by EBV infection of tumor cells, including EBV positive-DLBCL of the elderly that are still considered a provisional entity requiring a more precise definition (Campo, 2011).

The mechanisms by which EBV infection causes lymphomagenesis are complex. In many malignancies EBV presence in episomal form within tumor cells indicates that the virus entered these cells prior to their clonal expansion. In the immunocompetent host, latent EBV infection is under control of the immune system and the vast majority of the individuals never develops EBV-associated tumors. Neverthelss, viral genome, once integrated inside B cell DNA without killing the cells,

may both affect the control pathways of cell growth, thus favouring clonal B cell expansion, and prevent them from being destroyed by the immune system (Thompson, 2004). A percentage of EBV-infected patients with lymphoma express LMP and EBNA2 viral antigens, which are well known transforming factors for B cells. However, patients with Burkitt lymphoma fail to express these proteins, thus suggesting distinct roles of viral genes in transformation process (Hamilton-Dutoit, 1992). Additional factors may play a role in the development of EBV-associated neoplasms, including genetic susceptibility, environmental factors such as parasitic infections, nutritional issues and pathological conditions affecting host immune status (Michelow, 2012).

EBV identification in neoplastic cells can be very helpful for diagnosis and can be obtained through different methods, including commercial antibodies against EBNAs (detected by immunofluorescence) and LMP-1 (detected by membrane and cytoplasmic immunohistochemical staining), RNA in situ hybrization to detect EBERs and/or polymerase chain reaction (PCR) (Gulley, 2002).

In situ hybridization for EBER is perhaps the best test to detect and localize latent EBV in tissue and cell samples (Cohen, 2000).

1.5 Human cell lines and animal models

Human lymphoma cell lines and animal models have contributed significantly to better understand the physiopathology of hematopoietic tumors. However, the number of established cell lines from *ex vivo* tumor cells is quite low due to the difficulty in reproducing *in vitro* the

microenviromental conditions supporting *in vivo* cell growth (Drexler, 2000).

EBV-associated lymphoblastoid cell lines have been the first to be achieved; their phenotype corresponds to activated B cells with non-malignant properties, as they are polyclonal, diploid and do not form tumors after subcutaneous inoculation in nude mice (Nilsson, 1992). In addition, several hematopoietic cell lines of malignant origin, including lymphoma, myeloma and leukemia cell lines, have been established and studied; some of them are EBV-positive (Nilsson, 1975). Malignant cell lines are generally characterized by monoclonal origin, aneuploidy, genetic alterations, differentiation arrest, sustained proliferation *in vitro* and capability of maintaining most cellular features and specific genetic alterations of the primitive tumor; nevertheless, primitive malignant cells may often change some morphological and phenotypic features once cultured *in vitro*, especially if EBV-positive. Leukemia-lymphoma cell lines are genetically unstable at both cytogenetical and molecular levels; they may acquire additional numerical and structural chromosome alterations and various types of point mutations along *in vitro* expansion, thus becoming more and more different from primary *ex vivo* tumor cells (Drexler, Leuk Res 2000).

The first malignant hematopoietic cell line was obtained from a Burkitt's lymphoma patient in 1963. Since then a large number of cell lines has been obtained, although not all of them have been thoroughly characterized. Cell lines have been recently described for multiple myeloma, Waldenstrom's macroglobulinemia (Hodge, 2011), mantle cell lymphoma (Zamò, 2006), chronic myeloid leukemia (Park, 2010) and acute lymphoblastic leukemia (Okabe, 2010); by contrast, cell lines from lymphoproliferative diseases were obtained only if infected by

EBV or derived from CLL in prolymphocytoid transformation (Stacchini, 1999).

Cell lines have been used also *in vivo* as xenografts into immunodeficient animals to study the different phases of tumor development and the role of host immune system. Severe combined immune deficient (SCID) mice, lacking mature T and B cells (but still showing NK cell activity), have been employed as *in vivo* model for multiple myeloma (Ashmann, 1995) or CLL (Mohammad, 1996).

Recently, genetically-modified immunodeficient mice have been transplanted with human-derived hematopoietic stem cells *('humanized mice')*. This model is becoming a powerful tool to investigate human diseases, as it recapitules entirely human tumor development, without any interference by host immune system. Several immunodeficient mice have been developed: NOD/Scid mice exhibit deficiencies in macrophage, dendritic cell, T, B and NK cell and complement functions. Rag2-/- γ-chain-/- mice are also commonly used to establish humanized mice, as their genetic background is associated to high efficiency of human cell engraftment (Ono, 2011).

CHAPTER 2

2 AIMS

When considering the treatment of malignant diseases, both in clinical and research setting, it is crucial to understand their pathobiological features. The availability of stable human leukemia-lymphoma cell lines and animal models have contributed significantly to this aim, thus representing an important tool for therapeutic advances.

Biological features of DLBCL have been recently included in the last WHO classification and they are used to differentiate the treatment. However, some DLBCL subtypes have not been completely studied yet and sometimes their diagnosis is still problematic because of the lack of extensive analysis, i.e. DLBCLs displaying phenotype that resembles late stage of B cell differentiation.

We have established a new cell line, called VR09, which is an EBV-positive DLBCL with activated phenotype and plasmacytic differentiation, derived from a patient with low-grade lymphoproliferative disease. Aims of this study were:

1. to characterize VR09 cell line by means of different approaches, such as immunophenotyping, immunohistochemistry, PCR, cytogenetic analysis and fluorescence in situ hybridation (FISH);
2. to test VR09 tumorigenic potential *in vivo* into Rag2-/- γ-chain-/- mice through primary and secondary transplants;
3. to provide a new model of DLBCL with plasmacytic differentiation for further studies *in vitro* and *in vivo*.

3 METHODS

3.1 Cell collection and culture

A 75-year old Caucasian man was admitted to hospital in September 2008 for fever, neutropenia and lymphocytosis (WBC $20.1x10^9$/L, with neutrophils $0.8x10^9$/L and lymphocytes $18.2x10^9$/L), and moderate anemia and trombocytopenia (Hb 9 g/dl, PLTS $93x10^9$/L). No significant superficial lymphoadenopathy or splenomegaly were found. A bone marrow sample was sent to our laboratory for first level-immunophenotyping by flow cytometry (FACSCanto, Becton Dickinson Biosciences, CA, USA). Bone marrow smear appeared infiltrated (60%) by a cell population of lymphoplasmocytoid elements of small-medium size. First level-immunophenotyping showed the presence of a cell population expressing CD19, CD20, CD22, CD138, surface immunoglobulins (sIg) at high level, and negative for CD5, CD10, ZAP-70, thus suggesting the diagnosis of atypical B-cell chronic lymphoproliferative disease with plasmacytic features. No other exams could be performed, as the patient died of sepsis the following day.

Mononuclear cells were purified from bone marrow sample by Ficoll-Hypaque centrifugation (Lymphoprep, Fresenius Kabi Norge AS for Axis-Shield Poc AS, Oslo, Norway), washed in phosphate-buffered saline solution (PBS) and resuspended at $1x10^6$/mL concentration in RPMI 1640 + GlutaMAX 1X containing 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin (all from GIBCO, Invitrogen). Cells were cultured in 75 cm^2 flask and incubated in humidified 5% CO_2 atmosphere at 37°C. Half of culture medium was

replaced every 3-4 days maintaining the same cell density of $1x10^6$ cells/mL.

To determine growth kinetics, cells were seeded at lower density (350,000/mL) and counted at 0, 24, 48 and 72 hours by flow-cytometry (FACSCanto, Becton Dickinson, Italy). No mitogens or growth factors were added during culture. Cells were maintained in culture up to one year. Aliquots of cells were periodically frozen in culture medium supplemented with 10% dimethyl-sulfoxide (DMSO) and stored in liquid nitrogen.

Cell morphology was evaluated on cytospins stained with May-Grunwald Giemsa dye.

3.2 Immunophenotypic analysis

Cell vitality was assessed by acridine-orange/ethidium bromide staining and epifluorescent microscopy. Aliquots of $3x10^5$ cells were incubated for 15 minutes at room temperature with three-color combinations of appropriate monoclonal antibodies anti-human CD3, CD19, CD20, CD22, CD23, CD25, CD38, CD43, CD45, FMC-7, CD79b 7AAD (Becton Dickinson, Italy), CD5, CD10, K, λ, IgG, IgM, IgD (Dako, Italy), CD103 (Beckman Coulter, Italy), CD138 (Cytognos, Italy), and isotype controls (Becton Dickinson, Italy). Samples were analyzed by FacsCANTO flow cytometer with BD FACSDiva software. Data analysis was carried out using the laboratory's template for CLL; lymphocytes were identified by a gate made on their CD45 expression and morphologic parameters (side and forward scatters, SSC and FSC). The expression of antigens examined was assessed relatively to their respective isotypes controls.

3.3 Determination of DNA content

DNA staining solution including 5 ml of hypotonic solution, 50 µg of propidium iodide (Bender MedSystems) and 20 µg of RNAse was prepared. An aliquot of the cells suspension was centrifuged and resuspended in PBS 100 µl; then, 1.5 x 10^5 cells were incubated with 1 ml of staining solution for two hours at 4°C. Samples were analyzed by FacsCANTO flow cytometer and analyzed by FacsCANTO flow cytometer with BD FACSDiva software.

3.4 DNA and RNA extraction, cDNA synthesis

DNA and RNA were obtained from 10^7 cells by AllPrep DNA/RNA/Protein Mini Kit (Quiagen, Hilden, Germany). DNA quality was verified by spectrophotometry and RNA quality by the Agilent Bionanalyzer 2100; 1 µg of total RNA was reverse-transcribed by using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, California) and cDNA was used as a template for RT-PCR amplification using listed in **Table 3**.

Primers	Sequence	Ta°	Amplicon lenght
P53-1-F	AAGTCTAGAGCCACCGTCCA	55°C	771 bp
P53-1-R	AAGTGTTTCTGTCATCCAAATACTC		
P53-2-F	AGCCAAGTCTGTGACTTGCA	55°C	851 bp
P53-2-R	GGGGAACAAGAAGTGGAGAA		
Card11-1-F	AGATGCAACGGGAGCCTGGC	55°C	631 pb
Card11-1-R	AGGTTAGCAGCTCCACGCGC		
Card11-2-F	GGCCAAGGACCTGCAACGCT	55°C	703 pb
Card11-2-R	CCGCTCCACCTCCTCCAGCT		
Card11-3-F	GAGGCCCTGGAGGACAGGCA	55°C	690 pb
Card11-3-R	TCCGCAGGAGCTAGGGCTGG		
Card11-4-F	TCCTGCCCTACCATCCGCCC	55°C	671 pb
Card11-4-R	CAGCAGCTGGTGGCCCTCAC		
Card11-5-F	TCCCAGCTCACCCTGCTGGG	55°C	739 pb
Card11-5-R	CCGAGATGATGCGGACCCGC		
Card11-6-F	CCCGTCTCTCGCGAGCAAGC	55°C	928 pb
Card11-6-R	CGTCTGCTGGGGCAGCTCTG		
CD79B-1-F	GCCTCGGACGTTGTCACGGG	55°C	858 pb *
CD79B-1-R	TGGGCCAGCTTCAGAGGCCA		

* Isoform 1: 858 pb; Isoform 2: 546 pb; Isoform 3: 861 pb

Table 3. Primers used to amplify and sequence P53, Card 11 and CD79B genes

3.5 Analysis of VH rearrangement, p53, CD79B and Card 11 mutation

Rearranged *VH* genes were analyzed as previously described (Zamò, 2006), while *TP53, CD79B* and *CARD11* genes were amplified and sequenced by using the primers reported in **Table 3**. VH and CD79B PCR bands were excised from agarose gels and purified by using spin columns (PureLink Quick Gel Extraction and PCR Purification Combo Kit, Invitrogen). PCR products were sequenced by dye terminator reaction (Big Dye Terminator Cycle Sequencing Kit v.3.1, Applied Biosystems, Warrington, UK) on AB3130XL automated sequencer (Applied Biosystems). P53 and CARD11 PCR products were directly purified with magnetic beads (Agencourt AMPure XP, Beckman

Coulter Genomics, Beverly, Massachussets, USA) and sequenced as reported above and by outsourcing to Base Clear BV (Leiden, The Netherlands).

VH genes sequences were compared to published germline sequences using IgBLAST (*http://www.ncbi.nlm.nih.gov/igblast/*). Sequences with 2% or less deviation from any germline IgVH sequence were considered unmutated. *TP53*, *CD79B* and *CARD11* amplified sequences were compared to reported sequences by using Geneious software (Biomatters Ltd., Auckland, New Zeland).

3.6 Preparation of formalin-fixed paraffin-embedded (FFPE) cell block and immunohistochmemical staining

Briefly, $12x10^6$ cells were centrifuged, washed once in PBS and then incubated for 15 minutes in 4% buffered formalin. Cells were centrifuged again and resuspended in 1 ml of 1% low melting point agarose at 37°C; then, they were poured into disposable plastic moulds and cooled for ten minutes at +4° C. Solid agarose blocks were put inside a histological cassette, with 4% buffered formalin at room temperature, and then processed routinely as tissue block; 4 □m-thick sections were cut from the block and stained with ematoxilin-eosin to verify the quality of the cell inclusion. Many sections from the block were immunolabeled with the broad panel of antibodies described in **Table 4**. All samples were processed by using a sensitive 'Bond polymer Refine' detection system in automated Bond immunostainer (BondMax, Vision-Biosystem, Menarini, Florence, Italy).

Antibody	Species	Clone	Source	Diluition	VR09 cell line	Tumor masses
Annexin a1	mouse	B01P	Abnova	100	neg	neg
Bcl-2	mouse	124	Dako	40	pos	pos
Bcl-6	mouse	LN22	Novocastra	20	neg	neg
Cyclin D1	rabbit	SP4	Neomarkers	10	neg	neg
CD03	rabbit	SP7	Labvision	150	neg	neg
CD05	mouse	4C7	Novocastra	200	neg	neg
CD10	mouse	56C6	Novocastra	50	neg	neg
CD19	mouse	LE-CD19	Serotec	200	pos	pos
CD20	mouse	L26	Novocastra	100	pos	pos
CD22	mouse	FPC1	Novocastra	20	pos	pos
CD23	mouse	1B12	Novocastra	100	pos	pos
CD25 IL2R	mouse	4C9	Novocastra	100	pos/neg	pos/neg
CD38	mouse	38C03	Neomarkers	50	neg	pos
CD43 (T cell)	mouse	MT1	Novocastra	30	pos	pos
CD79alfa	mouse	JCB117	Dako	100	pos	pos
CD79beta	mouse	JS01	Novocastra	50	pos/neg	pos/neg
CD123	mouse	7G3	Bd	100	neg	pos/neg
CD138 (syndecan-1)	mouse	BB4	Serotec	50	pos/neg	pos/neg
DBA44 hairy cell	mouse	DBA.44	Dako	20	neg	neg
FOXP1	mouse	JC12	Abcam	500	neg	pos/neg
EBV (EBER)		mRNA probe *	Vision Byosistems		pos	pos
GCET1	mouse	RAM341	Abcam	500	neg	neg
HHV-8	mouse	13B10	Novocastra	50	neg	neg
IgD	rabbit	polyclonal	Dako	20	pos	pos/neg
IgG	rabbit	polyclonal	Dako	10000	neg	neg
IgM	rabbit	polyclonal	Dako	5000	pos/neg	pos/neg
Ki-67	mouse	MM1	Novocastra	20000 (15' incubation)	pos (40%)	pos (80%)
ZAP-70	mouse	2F3.2	Upstate	200	neg	neg
κ chains	rabbit	polyclonal	Dako	50	pos	pos
λ chains	rabbit		Dako	30000	neg	neg
MNDA	mouse	235	provided by CNIO **	2	pos	pos
MUM1 Protein	mouse	MUM1p	Dako	50	pos	pos
PAX 5	mouse	1EW	Novocastra	50	pos/neg	pos/neg
P53 Protein	mouse	DO-7	Novocastra	20	pos/neg	pos/neg
Sox11	rabbit	polyclonal	Atlas Sigma	50	pos/neg	pos/neg
TCL1A	mouse	1-21	Santa Cruz	100	pos/neg	pos/neg
TdT	rabbit	polyclonal	Dako	30	neg	neg
TRAcP	mouse	26E5	Neomarkers	30	neg	neg

* see Materials and Methods ** Reference 20

Table 4. Marker expression by VR09 cell line and tumor masses, as assessed by immunohistochemistry performed on FFPE cell block.

3.7 Establishment of mouse in vivo model

Three month-old immunodeficient $Rag2^{-/-}$ γ-$chain^{-/-}$ mice (Taconic animal models, New York's River Valley, NY USA) were injected either subcutaneously (s.c.) (n=6) with $5x10^6$ VR09 cells, resuspended in 0.2 ml PBS, or intravenously (i.v) (n=6) with 0.5 x 10^6 VR09 cells resuspended in 0.1 ml PBS. Animals were checked three times a week for the development of tumors. When s.c. tumors developed up to approximately 2 cm of diameter, corresponding to a spheric volume of approximately 4.187 cm^3, mice were sacrified. Half of the tumor mass was frozen and half included in paraffin according to standard procedures. Sections of 5-8 μm from each tumoral mass were prepared and analyzed for morphology, immunohistochemistry and FISH. Paraffin sections for immunohistochemistry were stained with a panel of antibodies, as described above (**Table 4**). Moreover, a little portion of the mass from two of six s.c injected mice, was dissected and mechanically dissociated into single-cell suspension; $1.5x10^6$ cells from each tumor were cultured in RPMI + 10% FBS and incubated as described above. Immunophenotipic analysis was performed on secondary cell culture after two months of culture, as previously described.

Three of six mice injected i.v. were euthanized at +30 days, while the others were sacrificed after four months from the treatment. Spleen, liver, femurs, lymph nodes, lungs and bowel of the i.v. injected animals were collected and included in paraffine. Tissue sections were immunostained with antibodies against CD20 and CD138 to verify the presence on tumor infiltration. In addition, immunophenotyping with anti-human CD19 and CD45 monoclonal antibodies and isotype

controls was performed by standard procedures on peripheral blood mononuclear cells of all mice injected both s.c. and i.v.

The *in vivo* studies were approved by Verona University Ethical Committee for experimentation on animals (Prot. n°51 del 16/06/11, D.lgs 116/92).

3.8 EBV status evaluation

The presence of EBV was assessed on both cells cultured *in vitro* and cells obtained from the tumoral mass developing after *in vivo* injection of VR09 cell line. Epstein-Barr virus-encoded RNA (EBER) hybridization was performed on cytoinclusion by means of specific fluorescein-conjugated EBER probes (Bond ISH EBER Probe, Vision-Biosystem, Menarini, Florence, Italy). EBER sequence was detected by anti-fluorescin antibody associated with a sensitive 'Bond polymer Refine' chromogenic detection system in an automated immunostainer (Bondmax, Vision-Biosystem, Menarini, Florence, Italy).

3.9 Karyotyping

Cytogenetic analysis was performed with standard methods on both cells cultured *in vitro* and cell suspensions from tumors developing after *in vivo* injection of VR09 cell line: 10- $20x10^6$ cells were centrifuged, resuspended in RPMI 1640 medium containing 20% fetal bovine serum (FBS, Lonza, Verviers, Belgium) and cultured for 24 hours. Before harvest, 0.15 µg/ml colchicin (Eur-Clone, Italy) was added. Chromosome preparation and staining using QFQ technique was performed according to standard protocols. Karyotypes were scored

according to the International System for Human Cytogenetic Nomenclature (ISCN) (Brothman, 2009). Images were captured with a ZEISS Axioplan microscope (ZEISS, Jena, Germany) and evaluated by Cytovision applied imaging system (Molecular Devices, New Milton).

3.10 Fluorescence in situ hybridization (FISH)

Interphase cytogenetic FISH was performed on nuclei from VR09 cell suspension and formalin-fixed and paraffin-embedded tissue sections from *in vivo* tumor masses developing after *in vivo* injection of VR09 cell line. The following kits were used: LSI BCL-2 dual color probes for 14q32;18q21 (Abbott-Vysis, Olympus), LSI CCND1 (11q13) break-apart probes (Abbott-Vysis, Olympus), LSI IGH/CCND1 probe for 11q13-14q32.3 (Abbott-Vysis, Olympus), LSI MYC dual color break-apart probe for 8q24 (Abbott-Vysis, Olympus), LSI C-MYC dual color probes for 8q24-14q32 (Abbott-Vysis, Olympus) and CEP (centromeric) mapping 12p11.1-q11.1 (Abbott-Vysis, Olympus). Chromosome 9 probes from human painting box kit (Spectral Imaging) were also used to identify whole chromosome 9 on metaphases of cell derived from *in vivo* tumor masses. The procedure was performed according to the methods described elsewhere (Brunelli, 2008).

FISH slides were examined by using either Axioplan (Zeiss, Germany) or Olympus BX61 (Olympus, Hamburg, Germany) epifluorescent automated microscopes. The signals were recorded using a CCD camera (Axiocam HRm, Zeiss and Digital Camera Olympus), and score was assigned according to manufacturer's instructions, available in each commercial FISH kit.

CHAPTER 4

4 RESULTS

4.1 Establishment and characterization of VR09 cell line

Primary cells proliferated very slowly during the first six weeks of culture, with a consequent decline in cell number. Remaining cells formed a few clusters of proliferation and started growing in suspension forming small round clumps (**Figure 3A**). At ten weeks of culture a stable proliferating cell line (designed VR09) was established, with a doubling time of approximately 84 hours. May-Grünwald-Giemsa staining showed a wide cellular size spectrum, from medium to large cells, with predominantly plasmacytoid features and occasional bizarre shapes: cells had a round-ovoid, often eccentric or moderately irregular nucleus, with compact chromatin and abundant basophilic cytoplasm. (**Figure 3B**).

At present, VR09 cells grow in suspension in RPMI 1640 medium supplemented with 10% heat-inactivated FBS without requiring any other supplements, and can be vitally stored in a medium consisting of 60% RPMI 1640 medium, 30% heat-inactivated FBS and 10% DMSO. Once thawed and put in culture, VR09 cell line grows rapidly and maintains the same doubling time of the early culture. Cells are optimally maintained at 500,000 and 1,000,000/mL cell density and may be split 1:5 every 72 hours.

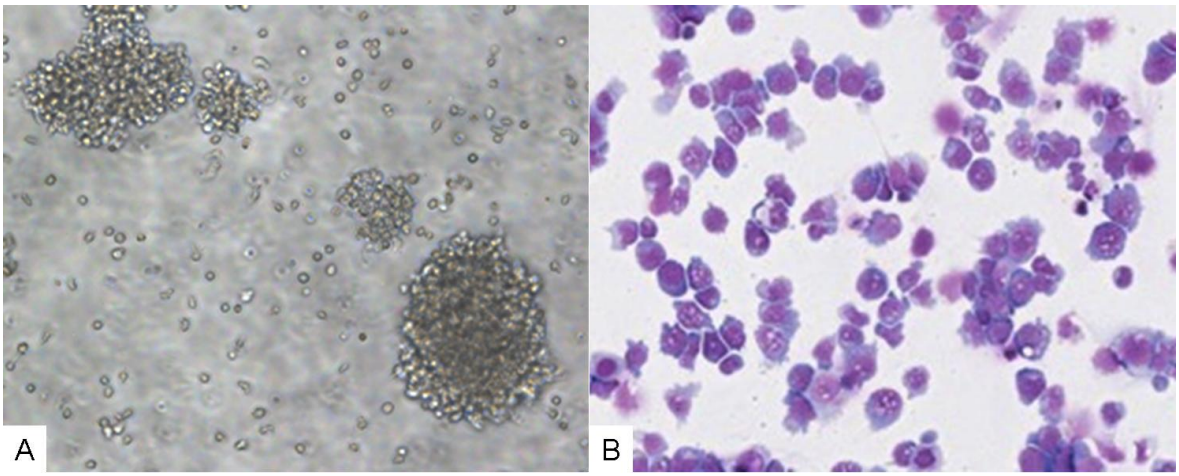


Figure 3. VR09 cell growth in suspension and morphological features, as assessed by May-Grünwald-Giemsa staining. **A**. Small and round clumps in suspension. **B**. Plasmacytoid appearance, with irregular nucleus, compact chromatin and abundant basophilic cytoplasm.

4.2 Immunophenotyping and immunohistochemistry

Immunophenotyping by flow cytometry was performed on VR09 cell line at 10 weeks and one year after continuous culture, resembling the phenotype of atypical B-cell chronic lymphoproliferative disease with plasmacytic features found in the patient. In particular, cells were $CD45^{+}$, $CD19^{+}$, $CD20^{+}$, $CD22^{+}$, $CD23^{+}$, $CD43^{+}$, $CD38^{+}$, $CD138^{+/-}$, IgD^{+}, IgM^{+}, IgG^{+} and kappa $chains^{+}$, and negative for CD3, CD5, CD10, CD25, CD79b, CD103, FMC7 and lambda chains (**Figure 4**); Zap-70, as expected, was not expressed.

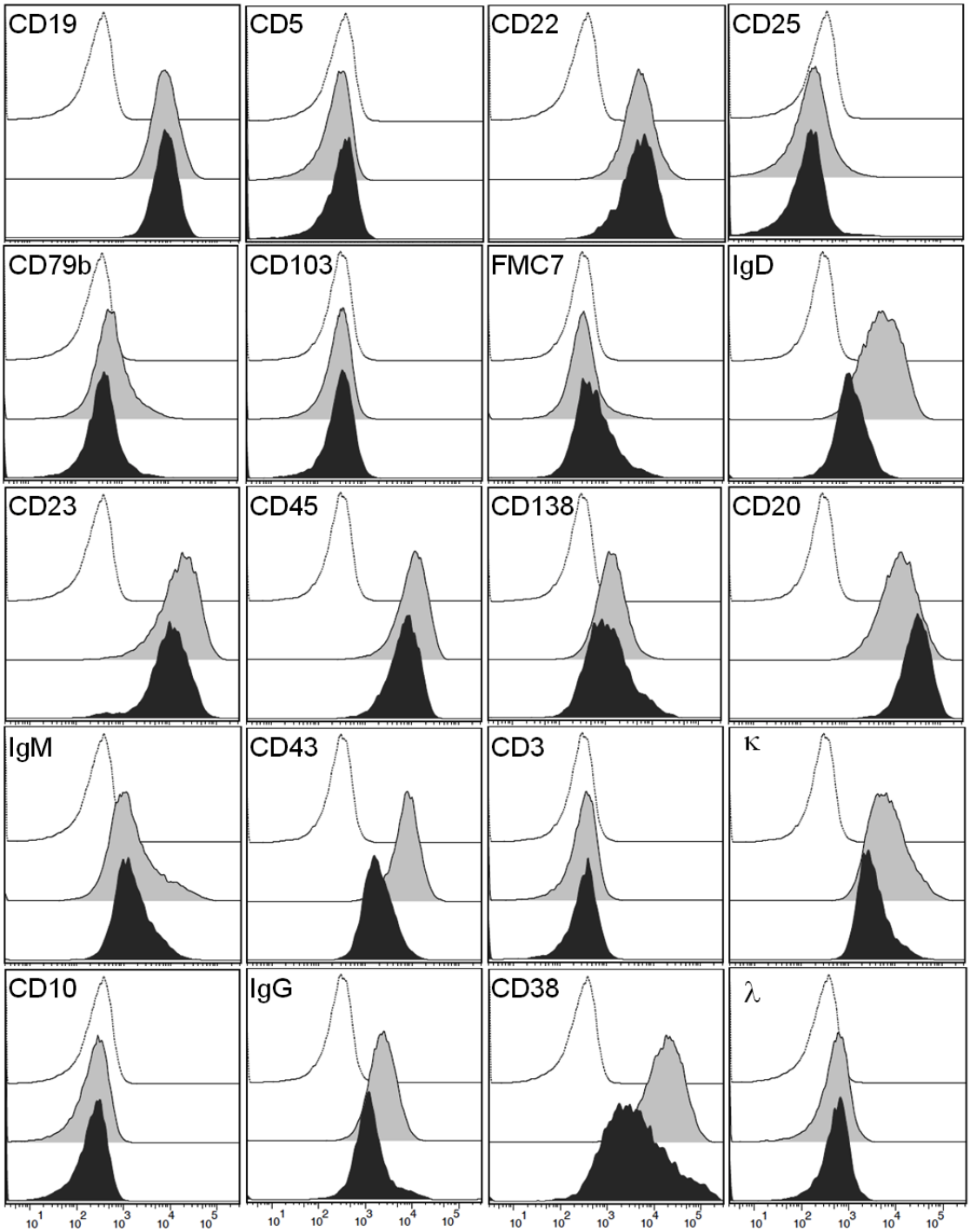


Figure 4. Immunophenotyping of VR09 cell line (grey and filled curves) and cell suspensions from *in vivo* tumor mass (black and filled curves), as compared with isotype control (white curves).

VR09 surface markers remained unchanged over time, with the exception of CD20, whose expression had a 1 log increase as compared to starting culture.

Results of immunohistochemistry performed on FFPE cell block are summarized in **Table 4**: all the markers shown by flow cytometry was confirmed, with the exception of IgG and CD38 expression that resulted negative by immunohistochemistry; proliferation index (Ki-67+ cells) was about 40%. In addition, cells were positive for Bcl-2, MNDA (Kanellis, 2009) and MUM1, and negative for Bcl-6, Cyclin D1, Annexin A1, DBA44, FOXP1, GCET1, ZAP-70, TdT and TRAP; other markers, such as PAX-5, Sox11 and TCL1, were weakly and variably expressed. In summary, neoplastic cells displayed a late B-cell phenotype (MNDA+, FOXP1+, IRF4/MUM1+, cyK+, CD138+/-), while germinal center markers (Bcl-6, CD10, TCL1, GCET1) and immature B-cell markers (TdT) were negative. Cyclin D1 was included to exclude the remote possibility of a blastoid variant of mantle cell lymphoma, and also because plasma cell myeloma can be cyclin D1-positive. Hairy cell leukemia markers (Annexin A1, DBA44, TRAP) were investigated to exclude the very remote possibility of evolution from hairy cell leukemia (in consideration of the clinical history).

4.3 Cell cycle

DNA content of VR09 cell line was analyzed at six months and one year of culture. High S-phase rate was observed (diploid S 22.66 %), in absence of tetraploid or aneuploid peak, as compared to the control (diploid S: 1.64%).

4.4 IGVH, TP53, CARD11 and CD79B mutation analysis

VR09 cell line showed VH3-7/D4-23/JH4 gene rearrangement. The comparison of the sequence with published germline sequences showed 95% identity, with evidence of somatic hypermutation (SHM) in the VH region, defined as more than 2% mutations as compared to germline sequences. The analysis of the sequences obtained from both strands of cDNA and compared with the published *TP53* sequence (U94788.1) showed that this sequence in VR09 cell line was wild-type. *CARD11* sequence was compared to the published one (NM_032415): VR09 cell line showed a silent heterozygous mutation leading to D533D variation in the protein linker region. Finally, VR09 cell line expressed all the three isoforms of the CD79B gene (NM_000626.2; NM_021602.2; NM_001039933.1): cDNA sequence analysis showed that variants 1 and 3 had an homozygous silent mutation in exon 3 leading to C122C variation in the Ig-like V-type protein domain, involving the disulfide bond; moreover, variant 2 had an Alanine insertion in the exon 2b corresponding to the protein signal peptide region.

4.5 Mouse Model

All six mice injected s.c. with VR09 cell line developed spherical tumor mass around the site of injection by 34-74 days after treatment and were sacrificed 10 days later. Subcutaneous mass and tumor incidence *in vivo* are shown in **Figure 5**.

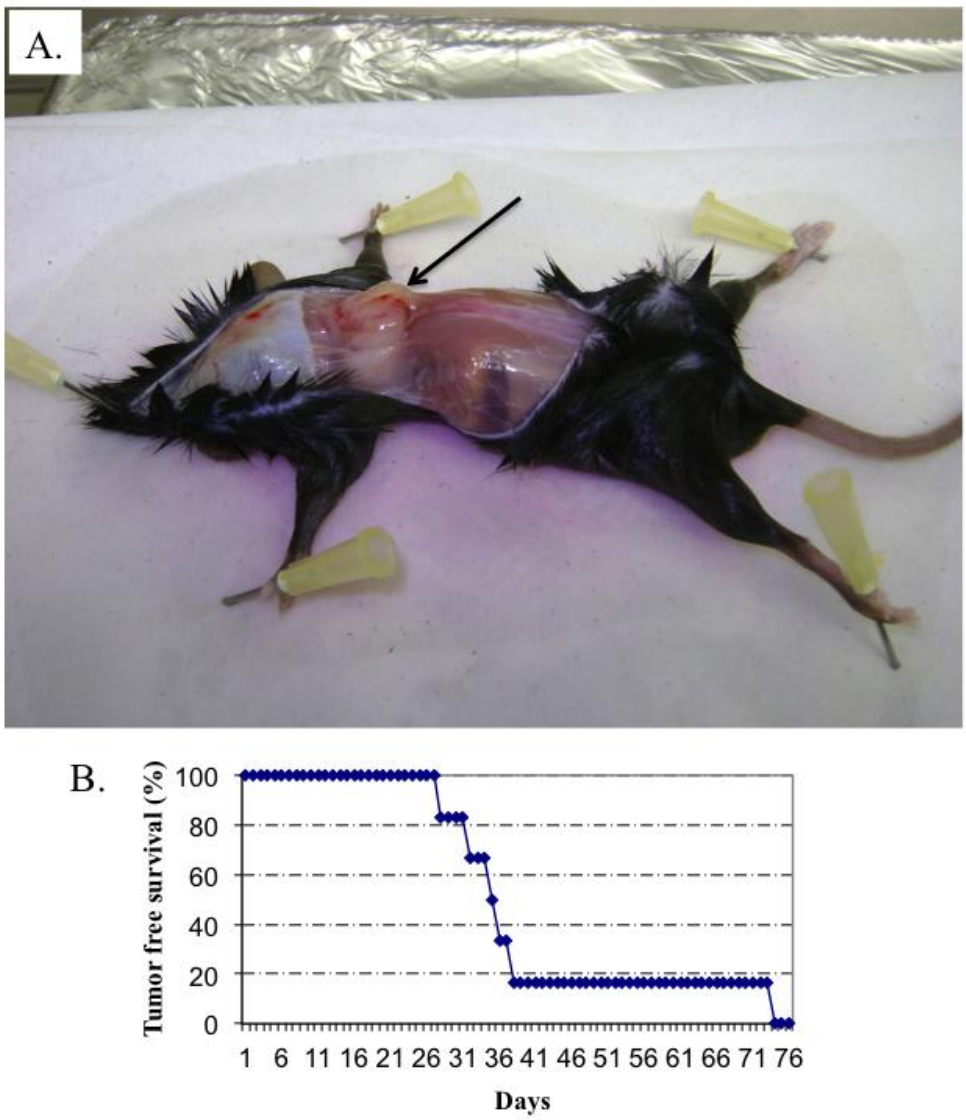


Figure 5. A. Spherical subcutaneous mass (arrow) in Rag-$2^{-/-}$ γ-$chain^{-/-}$ mice 36 days after VR09 cell line injection (representative case). B. Timing of tumor development *in vivo* (6 mice s.c. injected with $5x10^6$ cells/mouse).

Cells from subcutaneous masses were evaluated by H&E and Giemsa staining: cells ranged from medium to large size, with irregular nuclei, condensed chromatin without nucleoli and basophilic cytoplasm. Immunohistochemistry showed no significant differences in tumor cell immunophenotype as compared to VR09 cell line in culture (**Table 4** and **Figure 6**).

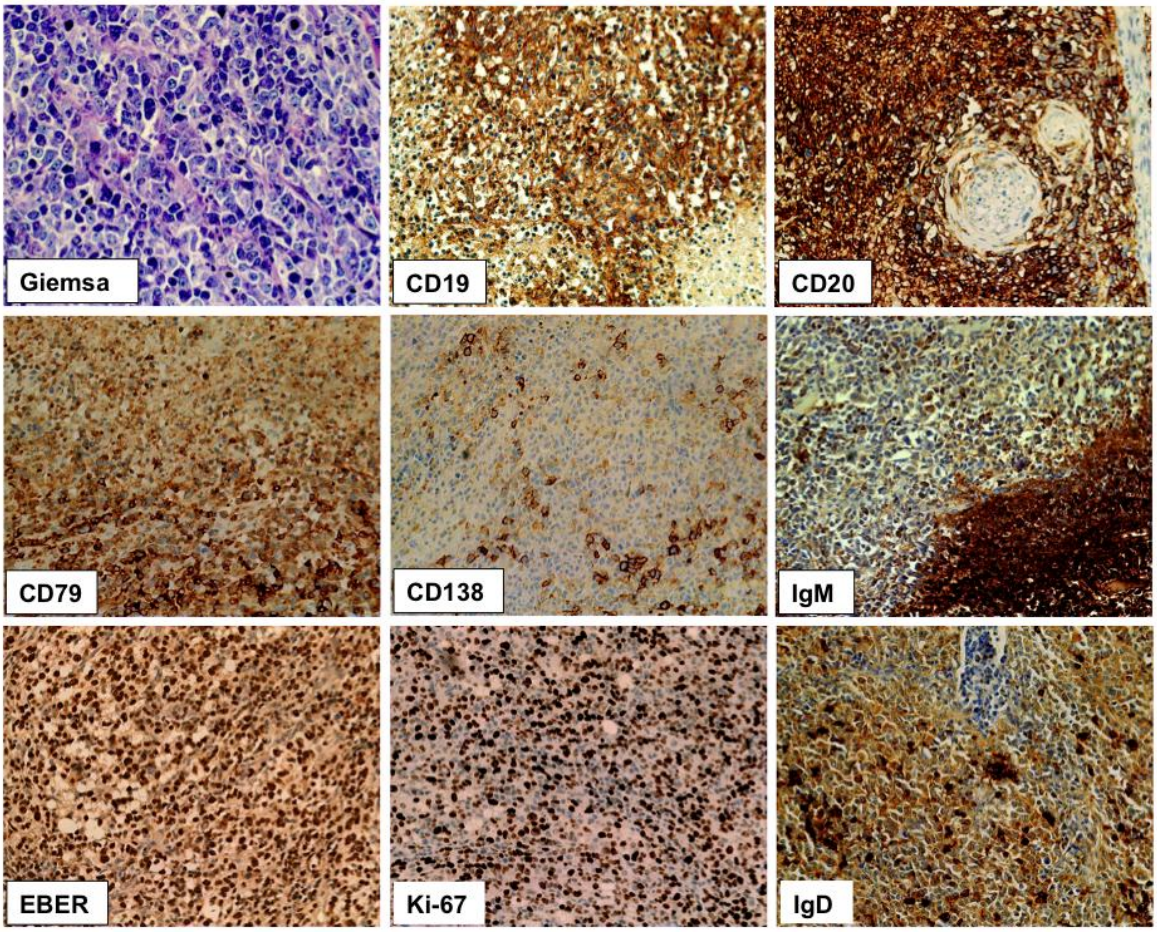


Figure 6. High magnification (20X) of histological sections of tumors developing after *in vivo* injection of VR09 cell line. Lymphoid infiltrates display large size, plasmablastic-plasmacytic features and high Ki-67 index. Cells express CD19, CD20, CD138, CD79a, IgM, IgG and EBV protein (EBER).

Cells suspensions obtained by disaggregation of *in vivo* tumor masses showed proliferative capacity when cultured with the same procedure used for primary VR09 cell line. Again, cells appeared as small and round clumps with plasmacytoid features and bizarre shape: immunophenotyping on these cells after two months of continuous secondary culture did not show any significant change of cell surface marker expression as compared to primary VR09 cell line (**Figure 4**).

Of the six i.v injected mice, three were euthanized after 30 days from injection and the other 3 after four months: histopathologic examination of spleen, liver, femurs, lymph nodes and lungs did not show any evidence of tumor infiltration. In addition, no circulating human

CD45/CD19-positive cells were detected in peripheral blood of both s.c. and i.v. injected mice.

4.6 EBV status

EBV RNA evaluation was performed on FFPE cell blocks and tumoral masses by using a fluorescein-conjugated probe coupled to chromogenic detection (Bond ISH EBER Probe, Vision-Biosystem, Menarini, Florence, Italy). In situ hybridization for EBV-encoded RNA (EBER) showed a clear nuclear signal in both VR09 cell line and tumors (**Table 4** and **Figure 6**).

4.7 Karyotyping

Chromosome analysis performed at 10 weeks and one year of continuous culture showed a male karyotype with 47 chromosomes. All metaphases exhibited trisomy of chromosome 12. Karyotype was repeated on secondary cell cultures obtained by disaggregation of *in vivo* tumor masses, thus revealing the presence of additional structural chromosome aberration involving chromosomes 7 and 9, i.e. 47, XY, der(7)(9qter->9p23::7p13->7qter), +der(7)(9qter->9p23::7p13->7qter), -9, +12 (**Figure 7A**).

4.8 Fluorescence in situ hybridization (FISH)

Adjacent or fused fluorescent signals (LSI BCL-2) were found in 5% of nuclei on tissue sections; this percentage is below the established 10%

signal threshold for considering a case positive (adjacent signals are generated by random ovelapping of genomic regions). No break-apart fluorescent signals were found for LSI CCND1 (11q13) and LSI MYC (8q) probes on tissue sections. No adjacent or fused fluorescent signals were found for LSI IGH/CCND and LSI C-MYC dual color probes (4% of nuclei, below threshold). Trisomy of chromosome 12 was found in 45% of nuclei. The rearrangement involving chromosomes 9 and 7 detected by karyotyping on cell suspension from *in vivo* tumor mass was confirmed by fluorescent signals derived from hybridization of chromosome 9 DNA probes to target chromosome 9 (chromosome 9 paint) (**Figure 7B**).

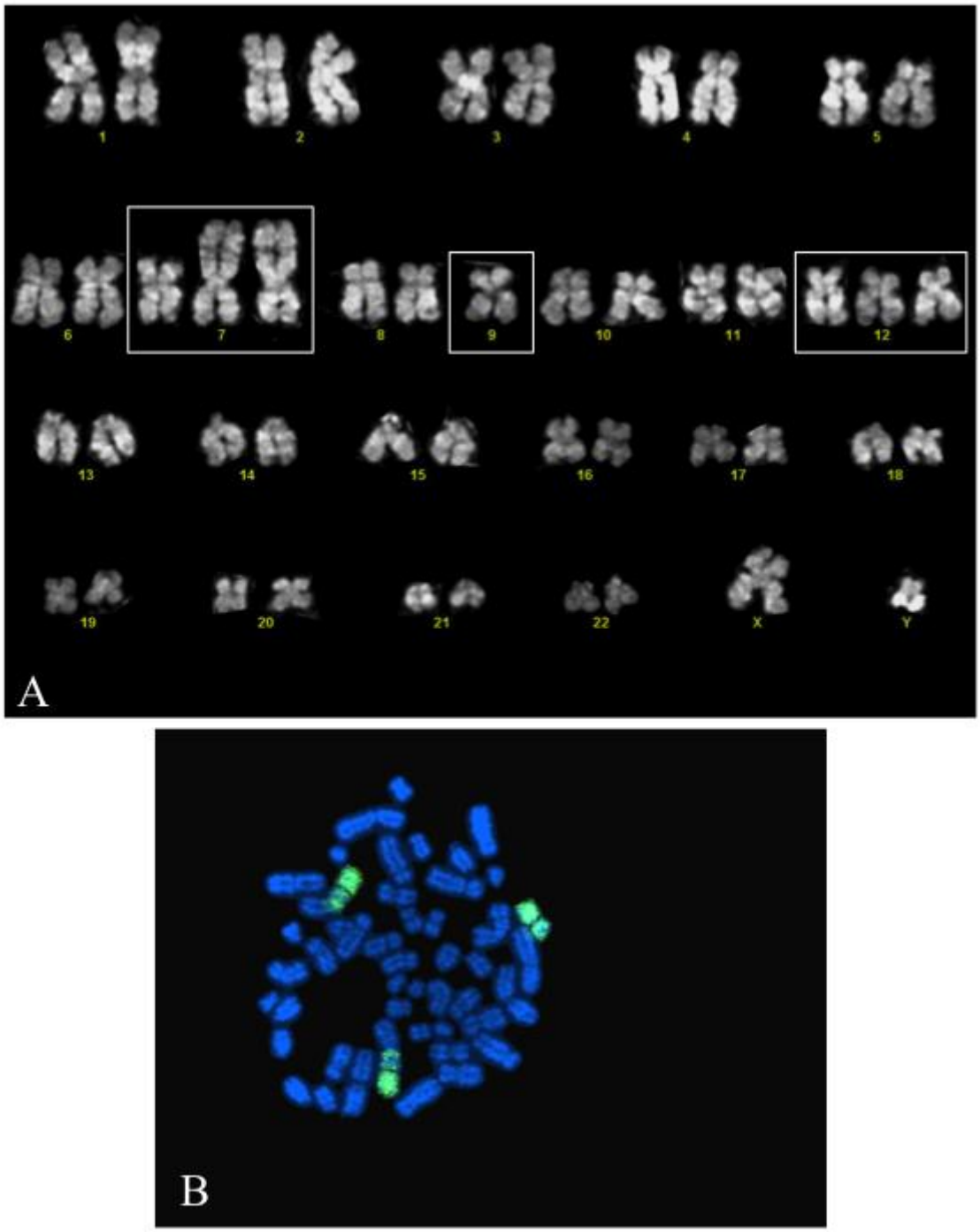


Figure 7. Karyotyping (A) and chromosome 9 painting by FISH (B).

5 DISCUSSION

Several lymphoma cell lines have been obtained so far, but cell lines of DLBCL with plasmacytic differentiation are still lacking. We have established and characterized of novel human EBV-positive DLBCL cell line with plasmacytic differentiation (VR09) obtained from a patient with atypical B-cell chronic lymphoproliferative disease with plasmacytic features.

VR09 cell line consists of cells with medium-large size and plasmacytic (rarely plasmablastic) features. Such a morphologic pattern may wrongly induce to consider VR09 as a variant of plasmablastic lymphoma that is, indeed, characterized by plasmablastic cells mixed with a variable number of mature plasma cells. However, VR09 phenotype does not correspond to plasmablastic lymphoma, due to strong expression of B cell markers CD20 and CD79 that are usually absent in the latter (Taddesse-Heath, 2010). Besides expressing other pan-B markers, such as CD19 and CD22, VR09 displays high degree of Ki-67 proliferation marker and the immunohistochemical pattern of activated DLBCL, i.e. the expression of MUM1 and the negativity for GCET1, CD10, BCL-6 markers (Choi, 2009). Moreover, the positivity of MUM1, the expression of immunoglobulins, and the partial expression of CD38 and CD138 suggest the status of terminal B cell differentiation. Consequently, VR09 may be considered a DLBCL variant corresponding to the intermediate step between activated B cells and plasmablasts, i.e. a late activated stage of B cell maturation, not entirely completed (Jourdan, 2009).

Some evidence suggests that DLBCL with plasmablastic and plasmacytic differentiation represents a clinically and pathologically

heterogeneous spectrum of entities, whose diagnosis may often represents a particular challenge (Teruya-Feldstein, 2005). Only one study reported cases of DLBCL showing a phenotype similar to VR09 cell line, i.e. CD20+, MUM-1+, CD138+/-, BCL-6-, IgM+. The Authors explained this entity as a result of transformation of transient B cell populations characterized by plasmablastic morphology and increasing plasmacytic features, and associated to the loss of GC and acquisition of post-GC immunophenotypic features (Simonitsch-Klupp, 2004). VR09 cell line may be close to this entity, although the VR09 morphology resembles activated B cells with plasmacytic features rather than plasmablastic cells. Thus, VR09 would represent an earlier stage in the continuum spectrum of neoplastic differentiation that ranges from conventional activated DLBCL to plasma cell disorders (Montes-Moreno, 2010).

All the markers shown by flow cytometry was confirmed by immunohistochemistry, with the exception of CD38 that resulted negative by immunohistochemistry. However, the latter is less sensitive than immunophenotyping. Furthermore, tissue sections of tumor masses obtained from VR09 resulted positive for CD38 also by immunohistochemistry.

When compared to the cell population collected from the patient, VR09 cells showed larger size, but also plasmacytoid features and all the markers of the peripheral blood B cells, i.e CD19, CD20, CD22, CD138, surface immunoglobulins at high level, and clonal restriction for k surface light chain, while confirming its negativity for CD5 and CD10 markers. Morphology and immunophenotype of patient's peripheral blood cells were in agreement with B-cell chronic lymphoproliferative disease with plasmacytic features, without peculiar patterns that suggested alternative diagnosis. Low-grade B-cell

lymphoproliferative disorders, such as CLL/small lymphocytic lymphoma and marginal zone B-cell lymphoma, sometimes may have overlapping features, thus hampering differential diagnosis (Rozman, 1995; Pangalis, 1999). Furthermore, many types of small B cell lymphoid neoplasms can display plasmacytic differentiation and phenotype resembling lymphoplasmacytic lymphoma (Campo, 2011; Hjalmar 1998). Lymphoplasmacytic lymphoma consists of small lymphocytes, plasmacytoid lymphocytes and plasma cells, and express IgM and pan-B-cell antigens, such as CD19, CD20 and CD22. Moreover, it is usually negative for CD5, CD10 and variably to CD23 (Lin P, 2003). Although this phenotype is similar to the cells from which VR09 derived, we concluded for atypical B-chronic lymphoproliferative disease with plasmacytic differentiation rather than plasmacytic lymphoma, due to the lack of IgM paraprotein.

Other chronic lymphoproliferative diseases may undergo transformation into DLBCL, although it occurs more frequently in CLL (Rossi, 2009). Thus, we think that VR09 cell line is the result of *in vitro* transformation of the original atypical chronic lymphoproliferative disease due to the presence of latent EBV infection in the primary cells (Nilsson, 1992). The association between EBV infection and indolent B-cell lymphoma has been reported (Jeffrey, 2010; Varghese, 2008); some cases of EBV-positive DLBCL developed from chronic lymphoproliferative diseases, and it is well known that EBV may have a potential role in the progression of the indolent disease (Tsimberidou 2005; Lin 2003). Thus, the positivity of EBV in VR09 cell line is not surprising.

We found the presence of chromosome 12 trisomy in VR09. This aberration is often detected in CLL (Stilgenbauer, 1998) and in other chronic B cell malignancies, including sometimes lymphoplasmacytic

lymphoma (Hjalmar, 1998). Moreover, it has been reported that chromosomal aberrations usually found in indolent lymphoma are detectable also in related DLBCL (Ghesquires, 2006). These features confirm that VR09 cell line originated from a low grade lymphoproliferative disease. The additional chromosomal abnormalities found in VR09 cell line after secondary culture is not surprising, as cell lines are usually genetically unstable and may acquire numerical and structural chromosomal alterations (Drexler, 2002).

Interestingly, VR09 cell line grows subcutaneously without either dissemination in peripheral blood or engraftment when administrated i.v. This strange peculiarity could be simply due to the *in vivo* model used. $Rag2^{-/-}$ γ-$chain^{-/-}$ is a strain of immunodeficient mice with C57B16/10 background devoid of functional B and T cells because of the complete lack of function of the V(D)J recombinase enzyme system, and displaying deficient innate immunity due to the absence of γ-chain of interleukin-2-receptor (Shinkai, 1992). This model has the advantage to show a stable phenotype without developing spontaneous tumors, as compared to SCID mice (Van Rijn, 2007). Indeed, the injection of VR09 cell line into mice led to 100% incidence of developing tumors *in vivo*, always maintaining the same features.

Another important finding is the detection in VR09 cells of a synonymous CD79B variant involving the insertion of Alanine in the exon 2b of variant 2. This variant may be physiological, as it has been detected in other isoforms; however, it has never been described before in variant 2. A recent study suggested that also synonymous variants may be involved in the pathogenesis of some diseases (Brest, 2011). Somatic mutations affecting the immunoreceptor tyrosine-based activation motif (ITAM) of CD79B signalling sequence have been detected in ABC DLBCLs, but rarely in other lymphomas; this

evidence further supports the activate pattern of VR09 (Davis 2010). As the constitutive activation of the NF-kB pathway, due to abnormalities in a variety of upstream proteins including Card-11, is a characteristic feature of ABC DLBCL (Lenz, 2008), we studied this protein. VR09 cell line did not display the common missense mutations of Card11; however, the detection of synonymous mutations of the protein confirms the accuracy of sequencing method.

In conclusion, VR09 is a new cell line of activated DLBCL with plasmacytic differentiation that grows as solitary tumor once injected s.c. in immunodeficient mice. This model could be useful for further studies about the development of DLBCL in patients with low-grade B-cell lymphoproliferative disorders with plasmacytic differentiation, which represents a rare but possible event in clinical practice. Furthermore, it may represent a model for the development of target therapies in ABC lymphomas.

REFERENCES

Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature. 2000; 403: 503-11.

Altmann M, Hammerschmidt W. Epstein-Barr virus provides a new paradigm: a requirement for the immediate inhibition of apoptosis. PloS Biol. 2005 Dec; 3(12):e404. Epub 2005 Nov 15.

Ansell SM, Li CY, Lloyd RV, Phyliky RL. Epstein-Barr virus infection in Richter's transformation. Am J Hematol. 1999; 60:99-104.

Ashmann Ej, Van Tol Mj, Oudeman-Gruber J et al. The SCID mouse as a model for ultiple myeloma. Br J Haematol. 1995; 89:319-27.

Bastion Y, Sebban C, Berger F, Felman P, Salles G, Dumontet C et al. Incidence, predictive factors and outcome of lymphoma transformation in follicular lymphoma patients. J Clin Oncol. 1997; 15:1587-1594.

Brest P, Lapaquette P, Souidi M, Lebrigand K, Cesaro A, Vouret-Craviari V *et al*. A synonymous variant in IRGM alters a binding site for miR-196 and causes deregulation of IRGM-dependent xenophagy in Crohn's disease. Nat Genet 2011; 43:242-5.

Brothman AR, Persons DL, Shaffer LG. Nomenclature evolution: Changes in the ISCN from the 2005 to the 2009 edition. Cytogenet Genome Res 2009; 127:1-4.

Brunelli M, Eccher A, Gobbo S, Ficarra V, Novara G, Cossu-Rocca P *et al*. Loss of chromosome 9p is an independent prognostic factor in patients with clear cell renal cell carcinoma. Mod Pathol. 2008; 21:1-6.

Campo E, Swerdlow SH, Harris NL, Pileri S, Stein H, Jaffe ES. The 2008 WHO classification of lymphoid neoplasms and beyond: evolving concepts and practical applications. Blood 2011; 117:5019-32.

Chen MR. Epstein-Barr virus, the immune system and associated diseases. Front Microbiol. 2011; 2:5.

Choi WW, Weisenburger DD, Greiner TC, Piris MA, Banham AH, Delabie J et al. A new immunostain algorithm classifies diffuse large B-cell lymphoma into molecular subtypes with high accuracy. Clin Cancer Res 2009; 15:5494-502.

Ci W, Polo JM, Cerchietti L, Shaknovich R, Wang L, Yang SN et al. The BCL6 transcriptional program features repression of multiple oncogenes in primary B cells and is deregulated in DLBCL. Blood. 2009; 113: 5536-48.

Cohen JI. Epstein-Barr virus infection. N Engl J Med. 2000; 343:481–492.

Davis R, Ngo V, Lenz G, Tolar P, Young R, Romesser P et al. Chronic active B-cell-receptor signalling in diffuse large B-cell lymphoma. Nature. 2010; 463: 88-92.

Davis RE, Brown KD, Siebenlist U, Staudt LM. Constitutive nuclear factor kappaB activity is required for survival of activated B cell-like diffuse large B cell lymphoma cells. J Exp Med. 2001; 1861-74.

Drexler HG, Matsuo AY, MacLeod RA. Continuous hematopoietic cell lines as model systems for leukemia-lymphoma research. Leuk Res. 2000; 24:881-911.

Dronca RS, Jevremovic D, Hanson CA, Rabe KG, Shanafelt TD, Morice WG. CD5-positive chronic B-cell lymphoproliferative disorders: diagnosis and prognosis of a heterogeneous disease entity. Cytometry. 2010; 78:35-41.

Dungarwalla M, Appiah-Cubi S, Kulkarni S, Saso R, Wotherspoon A, Osuji N et al. High grade transformation in splenic marginal zone lymphoma with circulating villous lymphocytes: the site of transformation influences response to therapy and prognosis. Br J Haematol. 2008; 143:71-4.

Fisher RI, Miller TP, O Çonnor OA. Diffuse aggressive lymphoma. Hematology Am Soc Hematol Educ Program. 2004:221-36

Fisher SG, Fisher RI. The epidemiology of non-Hodgkin lymphoma. Oncogene. 2004; 23-6524-34.

Flowers CR, Sinha R, Vose JM. Improving Outcome for patients with diffuse large B-cell lymohoma. CA Cancer J Clin. 2010; 60:393-408.

Freedman AS. Biology and management of histology transformation of indolent lymphoma. Hematology Am Soc Hematol Educ Program. 2005; 314-20.

Friedberg JW, Fisher RI. Diffuse large B-cell lymphoma. Hematol Oncol Clin North Am. 2008. 22; 941-52, ix.

Gatter KC, Warnke RA. Diffuse Large B-cell lymphoma. In: Jaffe ES, Harris NL, Stein H, Vardiman JW editors. Pathology and genetics of tumours of haematopoietic and lymphoid tissues. Lyon: IARCPress; 2001. p. 171-74.

Ghesquieres H, Berger F, Felman P, Callet-Bauchu E, Bryon P, Traverse-Glehen A et al. Clinicopathologic characteristics and outcome of diffuse large B cell lymphomas presenting with an associated low-grade component at diagnosis. J Clin Oncol. 2006; 24:5234-41.

Gulley ML, Glaser SL, Craig FE, Borowitz M, Mann RB, Shema SJ,

Ambinder RF. Guidelines for interpreting EBER in situ hybridization and LMP1 immunohistochemical tests for detecting Epstein-Barr virus in Hodgkin lymphoma. Am J Clin Pathol. 2002; 117:259-67.

Hamilton-Dutoit SJ, Pallesen G. A survey of Epstein-Barr virus gene expression in sporadic non Hodgkin's lymphomas. Detection of Epstein-Barr virus in a subset of peripheral T-cell lymphomas. Am J Pathol. 1992; 140:1315-1325.

Hans CP, Weisenburger DD, Greiner TC, Gascoyne RD, Delabie J, Ott G et al. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood. 2004; 103:275-82.

Hjalmar V, Kimby E, Matutes E, Sundstrom C, Jacobsson B, Arvidsson I, Hast R. Trisomy 12 and lymphoplasmacytoid lymphocytes in chronic leukemic B-cell disorders. Haematologica. 1998; 83: 602-9.

Hodge LS, Novak AJ, Grote DM, Braggio E, Ketterling RP, Manske MK. Establishment and characterization of a novel Waldenstrom's macroglobulinemia cell line, MWCL-1. Blood. 2011; 117:e190-7.

Jeffrey J, Tarrand JJ, Keating MJ, Tsimberidou AM, O'Brien S, LaSala RP et al. Epstein-Barr virus latent membrane protein 1 mRNA is expressed in a significant proportion of patients with chronic lymphocytic leukemia. Cancer. 2010; 116:880-7.

Jourdan M, Caraux A, De Vos J, Fiol G, Larroque M, Cognot C et al. An in vitro model of differentiation of memory B cells into plasmablasts and plasma cells including detailed phenotypic and molecular characterization. Blood. 2009; 114: 5173-81.

Kanellis G, Roncador G, Arribas A, Mollejo M, Montes-Moreno S, Maestre L, et al. Identification of MNDA as a new marker for Nodal Marginal Zone Lymphoma. Leukemia. 2009; 23:1847-57

Kelly GL, Milner AE, Baldwin GS, Bell Al, Rickinson AB. Three restricted forms of Epstein-Barr virus latency counteracting apoptosis in c-myc-expressing Burkitt lymphoma cells. Proc Natl Acad Sci. 2006; 103:14395-40.

Klein U, Dalla-Favera R. Germinal centres: role in B-cell physiology and malignancy. Nat Rev Immunol. 2008; 8: 22-33.

Klein U, Tu Y, Stolovitzky GA, Keller JL, Haddad J Jr, Milikovic V et al. Transcriptional analysis of the B cell germinal center reaction. Proc Natl Acad Sci USA. 2003; 100:2639-44.

Lenz G, Davis R, Ngo V, Lam L, George T, Wright G et al. Oncogenic CARD11 mutations in human diffuse large B cell lymphoma. Science. 2008; 319:1676-1679.

Lenz G, Davis RE, Ngo VN, Lam L, George TC, Wright GW *et al*. Oncogenic CARD11 mutations in human diffuse large B cell lymphoma. Science 2008; 319(5870):1676-9.

Lenz G, Wright G, Dave SS, Xiao W, Powell J, Zhao H et al. Stromal gene signatures in large-B-cell lymphomas. N Engl J Med 2008; 359:2313-23.

Lin P, Mansoor A, Bueso-Ramos C, Hao S, Lai R, Medeiros LJ. Diffuse large B-cell lymphoma occurring in patients with lymphoplasmacytic lymphoma/Waldenström macroglobulinemia. Clinicopathologic features of 12 cases. Am J Clin Pathol. 2003; 120:246-53.

Liu TY, Dei PH, Kuo SH, Lin CW. Early low-grade gastric MALToma

rarely transforms into diffuse large cell lymphoma or progresses beyond the stomach and regional lymph nodes. J Formos Med Assoc. 2010; 109:463-71.

MacLennan IC. Germinal centers. Annu Rev Immunol. 1994; 12: 117- 39.

Medina F, Segundo C, Campos-Caro A, Gonzalez-Garcia I, Brieva JA. The heterogeneity shown by human plasma cells from tonsil, blood, and bone marrow reveals graded stages of increasing maturity, but local profiles of adhesion molecule expression. Blood. 2002; 99:2154-61.

Michelow P, Wright C, Pantanowitz L. A review of the cytomorphology of Epstein-Barr Virus-associated malignancies. Acta Cytol. 2012; 56:1-14.

Mohammad Rm, Mohamed AN, Hamdan MY et al. Establishment of a human CLL xenograft model: utility as a preclinical therapeutic model. Leukemia. 1996; 10: 130-7.

Montes-Moreno S, Gonzalez-Medina AR, Rodriguez-Pinilla SM, Maestre L, Sanchez-Verde L, Roncador G. Aggressive large B-cell lymphoma with plasma cell differentiation: immunohistochemical characterization of plasmablastic lymphoma and diffuse large B-cell lymphoma with partial plasmablastic phenotype. Haematologica. 2010; 95:1342-9.

Nilsson K, Ponten J. Classification and biological nature of established human hematopoietic cell lines. Int J Cancer. 1975; 15:321-41.

Nilsson K. Human B-lymphoid cell lines. Hum Cell. 1992; 5:25-41.

Okabe S, Tauchi T, Ohyashiki K. Establishment of a new Philadelphia chromosome-positive acute lymphoblastic leukemia cell line (SK-9) with T315I mutation. Exp Hematol. 2010; 38:765-72.

Ono A, Hattoi S, Kariya R, Iwanaga S, Taura M, Harada H et al.

Comparative study oh human hematopoietic cell engraftment into Balb/c and C57BL/6 strain of Rag-2/Jak3 double-deficient mice. J Biomed Biotechnol. 2011; 2011:539748

Pangalis GA, Angelopoulou MK, Vassilakopoulos TP, Siakantaris MP, Kittas C. B-chronic lymphocytic leukemia, small lymphocytic lymphoma and lymphoplasmacytic lymphoma, including Waldenstrom's macroglobulinemia: a clinical, morphologic, and biologic spectrum of similar disorders. Semin Hematol. 1999; 36:104-14.

Park J, Kim KI, Koh Y, Won NH, Oh JM, Lee DS et al. Establishment of a new Glivec-resistant chronic myeloid leukemia cell line, SNUCML-02, using an in vivo model. Exp Hematol. 2010; 38:773-81.

Pasqualucci L. Relevant pathogenetic pathways in diffuse large B cell lymphoma. Hematology Education. 2011; 5:191-198.

Rosenwald A, Wright G, Chan WC, Connors JM, Campo E, Fisher RI et al. The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. N Engl J Med. 2002; 346:1937-1947.

Rossi D, Cerri M, Capello D, Deambrogi C, Rossi FM, Zucchetto A et al. Biological and clinical risk factors of chronic lymphocytic leukemia transformation to Richter syndrome. Br J Haematol 2008; 142:202-215.

Rossi D, Gaidano G. Richter syndrome: molecular insight and clinical perspectives. Hematol Oncol. 2009; 27:1-10.

Rozman C, Montserrat E. Chronic lymphocytic leukemia. N Engl J Med. 1995; 33:1052-7.

Shinkai Y, Rathbun G, Lam KP, Oltz EM, Stewart V, Mendelsohn M, Charron J, Datta M, Young F, Stall AM, Alt FW. RAG-2-deficient mice

lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. Cell 1992; 68:855-67.

Shipp et al. The international Non-Hodgkin's Lymphoma prognostic factors project. A predictive model for aggressive non-hodgkin's lymphoma. N Engl J Med. 1993; 329:987-94.

Shiseki M, Masuda A, Watanabe N, Fujii M, Kimura T, Yoshinaga K. Development of diffuse large B-cell lymphoma in a patient with Waldenstrom's macroglobulinemia/lymphoplasmacytic lymphoma: clonal identity between two B-cell neoplasms. Hematol Rep. 2011. 31; 3(2):e10.

Simonitsch-Klupp I, Hauser I, Ott, G, Drach J, Ackermann J, Kaufmann J et al. Diffuse large B-cell lymphomas with plasmablastic/plasmacytoid features are associated with TP53 deletions and poor clinical outcome. Leukemia. 2004; 18:146-55.

Stacchini A, Aragno M, Vallario A, Alfarano A, Circosta P, Gottardi P et al. MEC1 and MEC2: two new cell lines derived from B-chronic lymphocytic leukaemia in prolymphocytoid transformation. Leuk Res. 1999; 23:127-36.

Stilgenbauer S, Döhner K, Bentz M, Lichter P, Döhner H. Molecular cytogenetic analysis of B-cell chronic lymphocytic leukemia. Ann Hematol 1998; 76:101-10.

Taddesse-Heath L, Meloni-Ehrig A, Scheerle J, Kelly JC, Jaffe ES. Plasmablastic lymphoma with MYC translocation: evidence for a common pathway in the generation of plasmablastic features. Mod Pathol. 2010; 23(7):991-9.

Tarte K, Zhan F, De Vos J, Klein B, Shaughnessy J Jr. Gene expression profiling of plasma cells and plasmablasts: toward a better understanding of the late stage of B-cell differentiation. Blood. 2003; 102:592-600.

Teruya-Feldstein J. Diffuse large B cell lymphomas with plasmablastic differentiation. Curr Oncol Rep. 2005; 5:357-63.

Thompson MP, Kurzrock R. Epstein-Barr virus and cancer. Clin Cancer Res. 2004; 10:803-821.

Thorley-Lawson D.A. Epstein-Barr virus: exploiting the immune system. Nat. Rev. 2001; 1:75-82.

Tsimberidou AM, Keating MJ. Richter syndrome: biology, incidence and therapeutic strategies. Cancer. 2005; 103:216-28.

Van Rijn RS, Simonetti ER, Hagenbeek A, Bonyhadi M, Storm G, Martens AC *et al.* Quantitative assessment of human T lymphocytes in RAG2(-/-) gammac(-/-) mice: the impact of ex vivo manipulation on *in vivo* functionality. Exp Hematol 2007; 35:117-27.

Varghese AM, Sayala H, Evans PA, O'Connor SJ, Patmore R, Hillmen P. Development of EBV-associated diffuse large B-cell lymphoma in Waldenstrom macroglobulinemia and mantle cell lymphoma. Leuk Lymphoma. 2008; 49:1618-9.

Yancik R, Ries L.A. Cancer in Older persons: an international issue in an aging world. Semin Oncol. 2004; 31:128-36.

Young LS, Rickinson AB. Epstein–Barr virus: 40 years on. Nat. Rev. Cancer. 2004; 4: 757–768.

Yuen AR, Kamel OW, Halpern J, Horning SJ. Long-term survival after histologic transformation of low-grade follicular lymphoma. J Clin Oncol.

1995; 13:1726-33.

Zamo' A, Ott G, Katzenberger T, Adam P, Parolini C, Scarpa A et al. Establishment of the MAVER-1 cell line, a model for leukemic and aggressive mantle cell lymphoma. Haematologica 2006; 91:40-7.