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A new methodological approach in the diagnosis of extra-medullary lymphomas: the role of classical and molecular cytogenetics.

Nuovo approccio metodologico nella diagnostica dei linfomi extra-midollari: il ruolo della citogenetica classica e molecolare.

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“γνῶθι σεαυτόν” (*know yourself*)
Apollo Temple, Delphi

To myself

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English summary

Lymphomas are a group of heterogeneous malignant neoplasms involving B, T and Natural Killer (NK) lymphocytes. Their localization is predominantly lymphnode, but frequently they can diffuse to the bone marrow, making the distinction between lymphoma and leukemia more difficult.

The lymphocyte neoplastic transformation is due to genetic alterations that damage its DNA and down regulate cell growth and survival. Cytogenetics played a crucial role in the identification of these chromosomal rearrangements, subsequently allowing the association of these alterations to different tumor subtypes.

Since there is close correlation between chromosomal anomalies and prognostic factors in many haematological tumours, the study of genetic alterations in onco-haematology is now an indispensable tool for the clinician. Cytogenetic analysis is essential for correct diagnosis, prognosis and therapy and it can be used to monitor the patient during the course of therapy.

The incoming of different kinds and more innovative therapies able to eradicate the neoplastic clone, the monitoring of the minimum residual disease (MRD) that is the amount of disease that can only be determined at the molecular level, is increasingly important. As a consequence, the identification of a specific tumour-marker becomes fundamental for the evaluation of remission in the patient.

The cytogenetic investigation, however, is harder when the lymphoma is extra-medullary: in these cases Fluorescence In Situ Hybridization (FISH) analysis is commonly applied, performed on interphase nuclei coming from sections of lymphnode tissue. However, this method has some limitations, because it is based on the use of locus specific probes and it provides targeted information. Furthermore, the outcome of FISH investigations is strongly conditioned by the correct processing of the biopsy sample (time variables and fixation modalities) and by the presence of a sufficient amount of neoplastic cells to be analyzed.

From 2008 at the Division of Pathology and Cytogenetic of the ASST Grande Ospedale Metropolitano Niguarda (Milan) is used a culture method able to further stimulate the proliferation of B lymphocytes.

Cultures of bone marrow (BM) aspirate in patients with suspected extra-nodal lymphoma/lymphoproliferative pathology are added by a specific combination of B cell mitogen named DSP30 (a synthetic oligodeoxynucleotides) associated with Interleukin 2.

The aim of this work is trying to optimize the tissue taken by lymph node biopsy in a patient with suspected extra-medullary lymphoma, producing cell cultures added with the same mitogens for B lymphocytes used in the cultures of BM aspirates.

On this "enriched" and correctly processed sample, we performed a complete analysis of the karyotype that, in some cases, allowed to identify additional chromosomal alterations comparing to those highlighted by FISH analysis and which may be of main importance for the prognostic classification and for monitoring the therapy.

In particular, the aims of the study were the following:

- 1) evaluate the quantity and quality of mitosis in different lymphnode biopsy cultures (fragment vs shaked cultures);
- 2) evaluate if the cytogenetic alterations found are different between the two types of culture;
- 3) evaluate if the alterations found correlate with the immunophenotypical characterization, the morphological analysis and the clinical indication of the sample;
- 4) assess if there is a correlation between cytogenetic and FISH aberrations found;
- 5) evaluate whether our new methodological approach could become relevant or even indispensable in lymphoma diagnosis.

Based on the obtained results, we believe that we have developed an ideal protocol to improve the cytogenetic analysis of lymphomas, including the different types of cell culture, the use of an appropriate mitogen, and the analyses made by conventional and molecular cytogenetics with informative probes. The results are appropriately compared with histological, and immunohistochemical (IHC) data.

Our methodological approach, moreover, allows storing cells suspension, bioptical and histological samples, that can be used also later to perform molecular genetic investigations.

Italian summary

I linfomi sono neoplasie maligne a carico dei linfociti B, T e NK estremamente eterogenee dal punto di vista biologico, clinico e morfologico. La loro localizzazione è prevalentemente linfonodale, ma frequentemente possono diffondersi anche a livello midollare, rendendo ancora più difficile la distinzione tra linfoma e leucemia.

La trasformazione tumorale del linfocita è dovuta ad alterazioni genetiche che danneggiano il suo DNA e interferiscono con i meccanismi che regolano la crescita e la sopravvivenza cellulare. La citogenetica ha avuto un ruolo determinante nell'identificazione di questi riarrangiamenti cromosomici, consentendo successivamente l'associazione di tali alterazioni a sottotipi tumorali diversi.

Poiché è consolidata la stretta correlazione esistente tra alterazioni cromosomiche e fattori prognostici in molti tumori ematologici, lo studio delle alterazioni genetiche in oncoematologia è ormai uno strumento indispensabile per il clinico, in quanto contribuisce all'inquadramento diagnostico, prognostico e terapeutico ed è di ausilio nel monitorare il paziente durante il corso della terapia. Con l'avvento di terapie sempre più innovative capaci di eradicare il clone neoplastico, risulta sempre più importante il monitoraggio della malattia minima residua (MMR) ovvero quella quantità di malattia determinabile solo a livello molecolare. Di conseguenza, l'individuazione di un marcitore genetico che sia tumore-specifico diventa fondamentale per la valutazione della remissione nel paziente.

L'indagine citogenetica risulta però problematica quando il linfoma è extra-midollare: in questi casi si ricorre all'analisi FISH (Fluorescence In Situ Hybridization) eseguita su nuclei in interfase provenienti da sezioni di tessuto linfonodale. Tale metodica però presenta dei limiti, poiché si basa sull'utilizzo di sonde locus specifiche per geni noti e fornisce pertanto informazioni mirate. Inoltre, l'esito delle indagini di FISH è fortemente condizionato sia dal corretto processamento del campione biotecnico (variabili tempo e modalità di fissazione) sia dalla presenza di una quantità sufficiente di cellule neoplastiche da analizzare.

Dal 2008 presso la S.C. Anatomia Istologica Patologica e Citogenetica dell'ASST Grande Ospedale Metropolitano Niguarda viene utilizzato un metodo di coltura in grado di stimolare maggiormente la proliferazione dei linfociti B, avvalendoci dell'aggiunta dell'oligodesossinucleotide DSP30 associato all'Interleuchina 2, in colture di campioni di aspirato midollare in pazienti con sospetto linfoma/patologia linfoproliferativa, quando c'è il sospetto di un'infiltrazione midollare.

Lo scopo di questo lavoro è cercare di ottimizzare il tessuto prelevato tramite biopsia linfonodale in paziente con sospetto di linfoma extra-midollare, attraverso l'utilizzo di

colture cellulari addizionate degli stessi mitogeni per i linfociti B utilizzati nelle colture di aspirato midollare.

Su questo materiale “arricchito” e correttamente processato abbiamo eseguito l’analisi completa del cariotipo che, in alcuni casi, ha permesso di identificare alterazioni cromosomiche aggiuntive rispetto a quelle evidenziate dall’analisi di FISH e che possono essere cruciali per l’inquadramento prognostico e per il monitoraggio della terapia.

In particolare, gli obiettivi dello studio sono stati i seguenti:

- 1) valutare quantità e qualità delle mitosi nelle differenti colture da biopsia linfonodale;
- 2) valutare se le alterazioni citogenetiche riscontrate sono differenti tra le due tipologie di coltura;
- 3) valutare se le alterazioni riscontrate correlano con la caratterizzazione immunofenotipica, l’analisi morfologica e l’indicazione clinica del campione;
- 4) valutare se le alterazioni riscontrate in citogenetica sono le stesse o superiori a quelle riscontrate con l’analisi FISH;
- 5) valutare se questo nuovo approccio metodologico può diventare essenziale e indispensabile nella diagnostica dei linfomi.

Sulla base dei risultati ottenuti, riteniamo di aver sviluppato un protocollo ideale per migliorare l’analisi citogenetica dei linfomi, compresa l’esecuzione di diversi tipi di colture cellulari, l’uso di un mitogeno appropriato e le analisi effettuate mediante citogenetica convenzionale e molecolare con sonde informative. I risultati sono stati opportunamente confrontati con i dati istologici e immunoistochimici.

Il nostro approccio metodologico, inoltre, consente di archiviare campioni sospensivi, biotecnici e istologici, rendendo possibile effettuare indagini genetiche molecolari anche in un secondo momento.

Glossary

BCL2 apoptosis's regulator gene
BCL6 nuclear transcription factor
BCR B-cell receptor
BL Burkitt Lymphoma
BM bone marrow
CD10 cell surface enzyme (also known as CALLA)
CD25 high affinity receptor for interleukin 2
CLL chronic lymphocytic leukemia
CNS central nervous system
CpG DNA regions rich in cytosine and guanine nucleotides
DC Dendritic cells
DLBCL Diffuse Large B-cell Lymphoma
DNA Deoxyribonucleic acid
EBV Epstein-Barr virus
FISH Fluorescence In Situ Hybridization
FFPE formalin-fixed paraffin-embedded
FL Follicular lymphoma
GC germinal center
HL Hodgkin lymphoma
IG immunoglobulin (IgA, IgG, IGH, IGK, IGL, IgM)
IHC Immunohistochemical analysis
MALT mucosa-associated lymphoid tissue
MCL Mantle cell lymphoma
MRD residual disease
NHL non-Hodgkin lymphoma
NK Natural Killer lymphocytes
ODNs Oligodeoxynucleotides
SLL small lymphocytic lymphoma
TLR9 Toll-like receptor 9
WHO World Health Organization

1. Lymphoid neoplasms

Lymphomas are neoplasms of the immune system that originate from lymphocytes at different times of their differentiation. These neoplasms are very heterogeneous regarding biological, clinical and morphological features (Swerdlow et al., 2017).

Clinical presentation mainly includes leukemic forms and tumors affecting predominantly lymphatic organs such as lymph nodes and spleen, but also non-lymphatic organs such as skin and central nervous system (CNS) (Heim & Mitelman, 2015).

Both solid and circulating phases are frequent in lymphoid neoplasms, causing the distinction between lymphoma and leukemia very difficult.

Depending on the phenotype of the cell of origin, lymphomas may be classified as B-cell, T-cell or NK-cell, and they may have an indolent or aggressive clinical course (Robbins & Cotran, 2015).

Historically, there are two main types of mature lymphoid neoplasms: Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL).

About 85–90% of HL and NHL are derived from B cells, whereas remaining lymphomas are derived from T or NK cell lineage.

Epidemiology and etiology of lymphomas

Precursor lymphoid neoplasms, including B lymphoblastic leukemia/lymphoma are primarily childhood diseases, about 75% of cases occur in children aged <6 years. Approximately 85% of cases presenting as lymphoblastic leukemia are of B cell precursor type (Swerdlow et al., 2017).

According to the World Cancer Report 2014 (Stewart & Wild, 2014), there were 566.000 new cases of lymphoma and about 305.000 death due to lymphoma in 2012. Mature B cell neoplasms constitute >90% of lymphoid neoplasms worldwide and account for approximately 4% of all new cancer cases each year (Armitage & Weisenburger, 1998).

The incidence of lymphomas is influenced by geographical, racial and temporal factors and it is higher in industrialized countries, in male and white subjects. The frequency of different B cell neoplasms types is different in various parts of the world. Follicular lymphoma (FL) is more common in USA and western Europe, and is uncommon in South America, eastern Europe, Africa and Asia. Burkitt Lymphoma (BL) is endemic in equatorial Africa, where is the most common childhood malignancy, but it accounts only for 1-2% of lymphomas in the USA and western Europe (Swerdlow et al., 2017).

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Between the 1950s and 1970s in western countries, there was an increase in lymphoma cases, while the incidence of lymphoproliferative diseases has now stabilized. In Italy about 16,000 new cases of lymphoma are diagnosed each year. The median age is 60-70 years and a male predominance has been recorded (52-55% of cases).

The most common lymphoma types are FL and Diffuse Large B cell Lymphoma (DLBCL), which together make up to >60% of all lymphomas other than HL and plasma cell myeloma (International study group classification of non-Hodgkin's lymphoma, 1997) (fig.1).

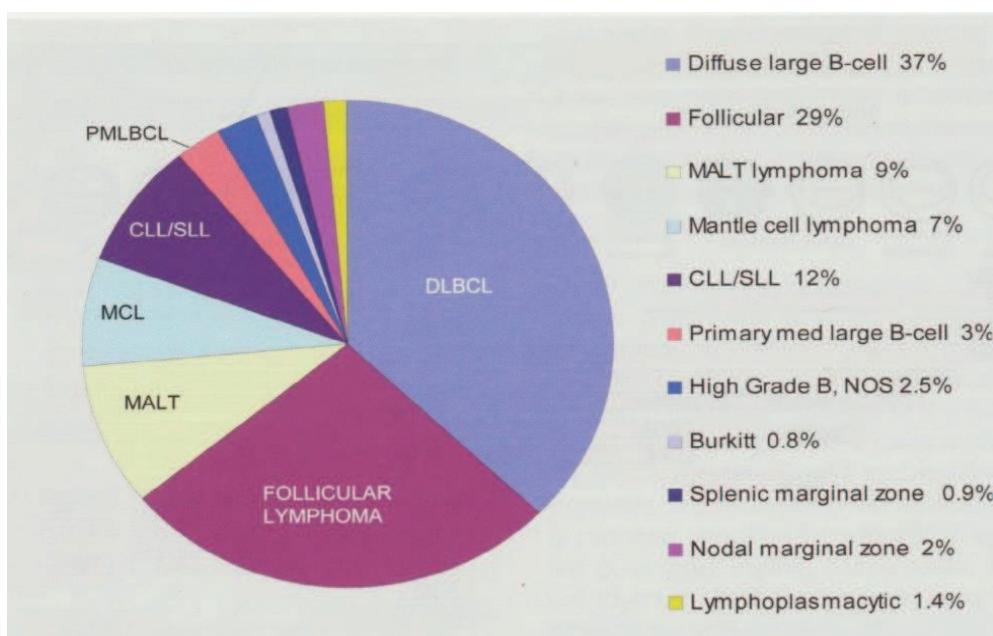


Figure 1: Relative frequencies of B-cell lymphoma subtypes in adults. Note that the incidence of chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) is underestimated, because only patients presenting clinically with lymphoma are included. (data from the Non-Hodgkin's lymphoma classification project; from Swerdlow et al., 2017)

Lymphoma risk factors are not well known. Many cases of NHL arise spontaneously without known causes. However, several factors are related to the development of lymphomas such as alterations in the activity of the immune system, either congenital or acquired immunodeficiency. This group includes patients with Wiskott-Aldrich syndrome (Gore & Trippet, 2010), ataxia-telangiectasia syndrome and HIV-infected individuals. Even patients receiving immunosuppressive agents following organ or BM transplants are at risk of developing post-transplant lymphoproliferative diseases. Patients with autoimmune diseases such as rheumatoid arthritis and Sjogren's syndrome are also at high risk of developing lymphomas.

Introduction

Mutations in genes controlling lymphocyte apoptosis have been linked to increased risk of both autoimmune disease and lymphomas, mainly B cell types. Genome-wide association studies have identified a remarkable number of single nucleotide polymorphisms that are associated with increased risk of lymphoma (Cehran & Slager, 2007; Lan et al., 2007).

Association with microbiological agents has been described. Helicobacter Pylori may be associated with gastric NHL. Epstein-Barr virus (EBV) infection is a predisposing factor for the development of the endemic form of BL in Africa (detected in nearly 100% of cases). EBV is also involved in the pathogenesis of many B-cell lymphomas arising in immunosuppressed or elderly patients. Hepatitis C virus has been supposed to be the infectious agent in some cases of indolent lymphomas in patients suffering from hepatitis or healthy carriers. The molecular mechanism following the viral infection involves integration of the viral genome into nuclear DNA, favouring the establishment of the neoplastic transformation process.

Moreover, some chemical and physical agents such as pesticides, benzene, nitrites, ionizing radiation and wrong lifestyle, like cigarette smoking, have been correlated to the onset of lymphomas (Colt et al., 2006; Hartge et al., 2005).

Clinical presentation

Lymphomas typically cause enlargement of lymph node, but higher digestive tract, intestine, bone marrow (BM), CNS and skin can be affected in one third of the cases. The enlargement of lymph nodes is frequently painless. Lymphoma can broaden from the site of origin to other lymph nodes and finally to the spleen, liver and bone marrow. The dissemination of neoplastic cells can also occur in the blood and mimic leukemia. While the HL is spread by contiguity, NHL can occur in remote lymph node stations.

Patients may report systemic symptoms that are referred to as “B symptoms”: such as fever higher than 38°C, nocturnal profuse sweats, weight loss above 10%. Another frequent systemic symptom is itching. Other signs of NHL may include bone pain, chest pain, abdominal pain, rash, enlarged spleen, tiredness.

However, it is very difficult to identify a specific symptomatology for lymphoma and this makes diagnosis rather difficult.

Diagnosis

The confirmation of the diagnosis of lymphoma is performed usually by biopsy of an involved lymph node/organ. Cytological examination on needle biopsy is not sufficient for

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the diagnosis of lymphoma, since histological examination of the biopsy preparation is needed to define the subtype of lymphoma. This typing is important especially for a detailed characterization of the disease leading to a suitable and optimal therapeutic choice. After the diagnosis, it is necessary to establish the staging of the disease and identify all the involved sites. Therefore, the following exams are mandatory:

- computed tomography of the thorax abdomen, pelvis and neck to evaluate all the lymph node involved;
- BM biopsy, to evaluate if the BM is involved;
- BM aspirate to identify genetic alterations important for prognosis and therapy;
- spinal tap for chemical-physical and cytological examination of the liquor, when the involvement of CNS is suspected, or in case of BM disease;
- total body positron emission tomography.

All these investigations make possible to establish the stage of the disease.

The staging method commonly used for NHL and HL is *Ann-Arbor method*, based on the number of tumour localization sites (lymph node and extra lymph node) and on the presence or absence of systemic symptoms (Corradini & Foà, 2015).

2. Classification of B cell neoplasms

B-cell neoplasms usually mimic various stages of normal B-cell differentiation and this is an important criterion for their classification and nomenclature (Swerdlow et al., 2017).

The classification of lymphoid neoplasms is based on all available information to define disease entities. Thus, sufficient available tissue is critical for this multiparameter approach. There is no specific antigenic marker for any neoplasms, hence a combination of morphological features and antigenic markers panel are required for a correct diagnosis. Genetic features are playing an increasingly important role in the classification of lymphoid malignancies. However, the molecular pathogenesis of many types of lymphoma are yet unknown yet. Genetic studies, in particular molecular analysis of immunoglobulin (IG) genes and fluorescence in situ hybridization (FISH) are effective diagnostic tools for the clonality determination in B cell proliferation and for identification of cytogenetic aberrations associated with some disease subtypes (Swerdlow et al., 2017).

The World Health Organization (WHO) classification of Tumours of Haematopoietic and Lymphoid tissues emphasizes the importance of a global knowledge, to achieve either an accurate diagnosis or the characterization of some diseases. The 2017 revision is based on the 2008 WHO classification and incorporates information from clinical findings,

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morphology, immunophenotyping and genetics, to refine previous entities that were considered to represent heterogeneous conditions, to describe new provisional entities based on knowledge accrued during the past years, and to use new findings from next-generation sequencing studies that have provided substantial insight into disease biology.

B-cell neoplasms are divided into the following groups:

Mature B-cell neoplasms	
Chronic lymphocytic leukemia/small lymphocytic lymphoma	
Monoclonal B-cell lymphocytosis*	
B-cell polylymphocytic leukemia	
Splenic marginal zone lymphoma	
Hairy cell leukemia	
<i>Splenic B-cell lymphoma/leukemia, unclassifiable</i>	
<i>Splenic diffuse red pulp small B-cell lymphoma</i>	
<i>Hairy cell leukemia-variant</i>	
Lymphoplasmacytic lymphoma	
Waldenström macroglobulinemia	
Monoclonal gammopathy of undetermined significance (MGUS), IgM*	
μ heavy-chain disease	
γ heavy-chain disease	
α heavy-chain disease	
Monoclonal gammopathy of undetermined significance (MGUS), IgG/A*	
Plasma cell myeloma	
Solitary plasmacytoma of bone	T-cell/histiocyte-rich large B-cell lymphoma
Extraosseous plasmacytoma	Primary DLBCL of the central nervous system (CNS)
Monoclonal immunoglobulin deposition diseases*	Primary cutaneous DLBCL, leg type
Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma)	<i>EBV⁺ DLBCL, NOS*</i>
Nodal marginal zone lymphoma	<i>EBV⁺ mucocutaneous ulcer*</i>
<i>Pediatric nodal marginal zone lymphoma</i>	DLBCL associated with chronic inflammation
Follicular lymphoma	Lymphomatoid granulomatosis
<i>In situ</i> follicular neoplasia*	Primary mediastinal (thymic) large B-cell lymphoma
Duodenal-type follicular lymphoma*	Intravascular large B-cell lymphoma
Pediatric-type follicular lymphoma*	ALK ⁺ large B-cell lymphoma
Large B-cell lymphoma with <i>IRF4</i> rearrangement*	Plasmablastic lymphoma
Primary cutaneous follicle center lymphoma	Primary effusion lymphoma
Mantle cell lymphoma	<i>HHV8⁺ DLBCL, NOS*</i>
<i>In situ</i> mantle cell neoplasia*	Burkitt lymphoma
Diffuse large B-cell lymphoma (DLBCL), NOS	<i>Burkitt-like lymphoma with 11q aberration*</i>
Germinal center B-cell type*	High-grade B-cell lymphoma, with <i>MYC</i> and <i>BCL2</i> and/or <i>BCL6</i> rearrangements*
Activated B-cell type*	High-grade B-cell lymphoma, NOS*
	B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and classical Hodgkin lymphoma

Figure 2: 2017 WHO classification of mature B cell neoplasms. Provisional entities are listed in italics.

*Changes from 2008 classification (from Swerdlow et al., 2017).

3. B cell lymphomas and lymphocyte differentiation

The lymphatic system is composed by a network of lymphatic collectors, along which lymph nodes are located. Each lymph node is surrounded by a fibrous capsule, which extends inside the lymph node to form trabeculae. The substance of the lymph node is divided into the outer *cortex* and the inner *medulla*. The cortex is continuous around the medulla except where the medulla comes into direct contact with the hilum. Thin reticular

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fibers of connective tissue and elastin form a supporting meshwork inside the node. B cells are mainly found in the superficial cortex where they are clustered together in *lymphoid follicles* while T cells are mainly in the *paracortex* (fig. 3) (Abbas et al., 2002).

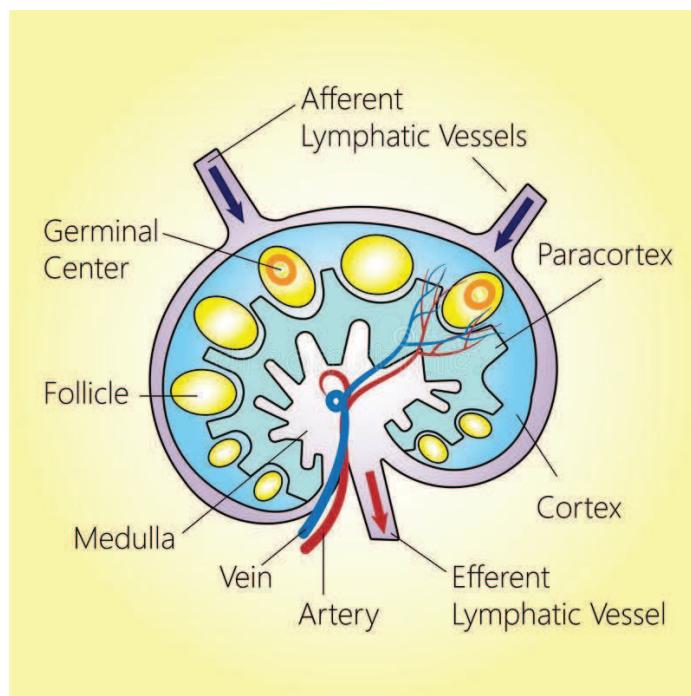


Figure 3: Schematic representation of a lymph node section. Afferent and efferent lymphatic vessels, veins and arteries are indicated. Each lobule is composed by a cortical region, a paracortical region and a basal region in the medulla. B cells are clustered into follicles, while T cell are located mainly into the paracortex (from www.istockphoto.com)

Different lymphocyte populations are located in different areas of the lymph node. Follicles represent B areas. Some follicles contain a central area called *germinal center* (GC). Follicles number and composition can change especially either when stimulated by an antigen, or when they develop a GC. Follicles without a GC are called primary follicles, while the others are secondary follicles. Primary follicles are predominantly populated by mature naive B lymphocytes. GC develops in response to antigenic stimulation. Inside them are found many different cellular tasks typical of the lymph node: a notable B proliferation, the selection of B lymphocytes that produce high-affinity antibodies and the generation of memory B cells. GC predominantly consists of B lymphocytes and a smaller number of macrophages, dendritic cells and T lymphocytes. It is divided into two areas (fig.4): a *dark area* located in the basal zone of GC, occupied by proliferating centroblasts, whom shift in the light area turning into centrocytes; a *light area* located in the inner part of the follicle and occupied by centrocytes which yield memory B lymphocytes or plasma cells. Only 10% of them will remain in the lymph node to produce antibodies, the

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remaining 90% will move into the BM (Abbas et al., 2002). The GC is a unique physiological structure, that supports rapid B-cell proliferation and two physiological genetic processes: somatic hypermutation in the IGV genes and class-switch recombination from IgM production to IgG or IgA production, both requiring double-stranded DNA breaks (Armitage et al., 2017). Through these mechanisms, the GC gives rise to the higher-affinity IgG or IgA antibodies of the immune response (MacLennan et al., 1990). The ability of GC to support these processes is potentially lethal because although these processes are required for the generation of antibody diversity, they are also error-prone and probably underlie lymphomagenesis.

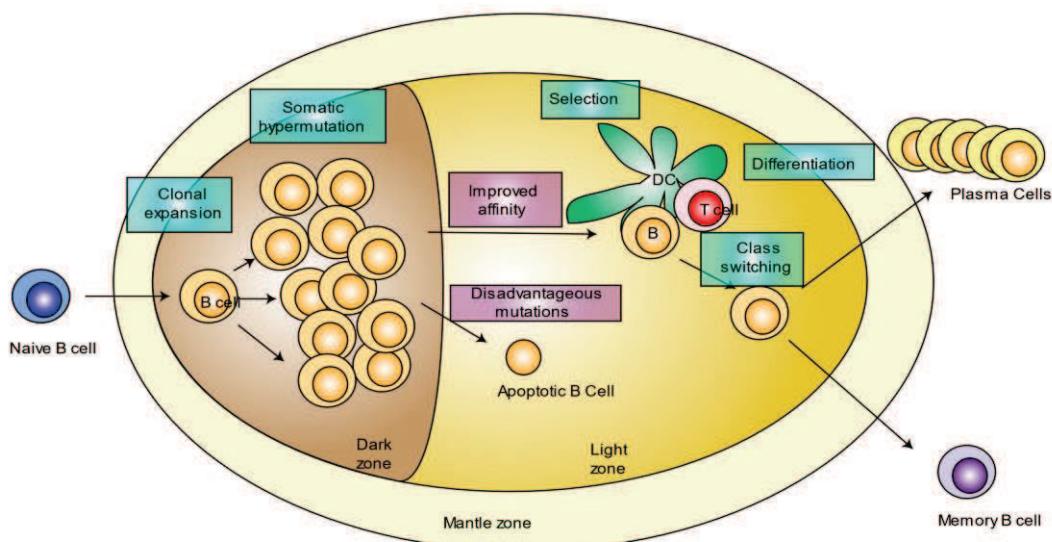


Figure 4: Schematic representation of a germinal centre (GC). The dark area is occupied by centroblasts and a light area occupied by centrocytes. Dendritic cells (DC) and T lymphocytes as supporting structure network (from Commons Wikimedia.org).

As part of the reticular network there are follicular dendritic cells and fibroblastic reticular cells. The reticular network not only provides the structural support, but also the surface for adhesion of dendritic cells, macrophages and lymphocytes. It allows material's exchanging with blood through the high endothelial venules and provides the growth and regulatory factors necessary for activation and maturation of immune cells.

The development and maturation of B lymphocytes are regulated process that take place in the BM, peripheral blood and lymphoid organs. Every maturation stage is characterized by physiological genetic rearrangements (Heim & Mitelman, 2015).

The capacity of the immune system cells to recognize multiple antigens through some different antigen-specific receptors is due to intrachromosomal rearrangements of the

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genes encoding Ig in B lymphocytes. Ig are glycoproteins composed of 4 chains, 2 called “heavy chain” (H) and 2 called “light chain” kappa (κ) or lambda (λ). All these proteins are encoded by the Ig genes, IGH at chromosome 14q32, IGK at 2p12 and IGL at 22q11 (Heim & Mitelman, 2015). Every locus Ig contains gene segments for variability (V), diversity (D), joining (J) and constant (C) sequences. All Ig loci are subjected to multiple physiological breaks as part of recombination events during B cell development. Rearrangements of the VDJ genes are connected to the great variability and specificity of the immune system (fig.5) (Abbas et al., 2002).

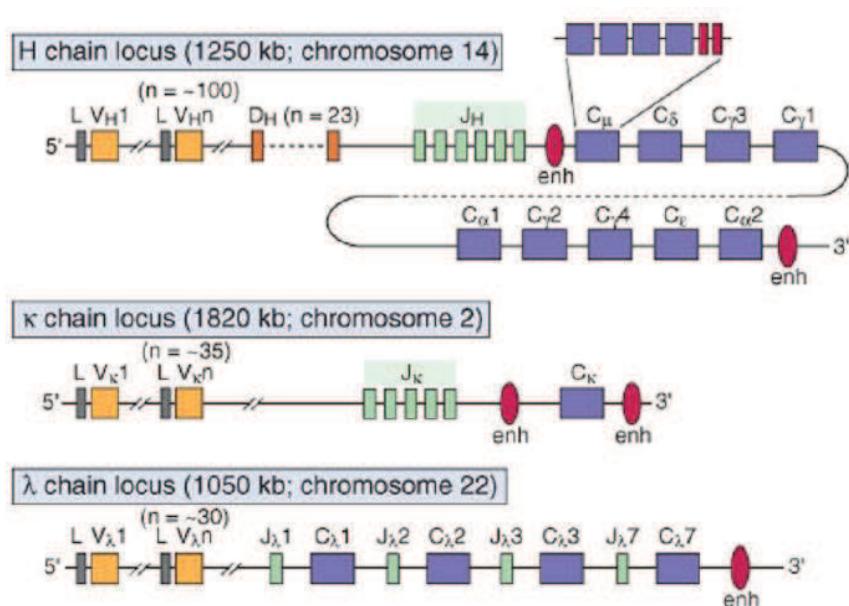


Figure 5: Ig loci organization. There are 3 different gene loci on different chromosomes that code for the three Ig chains: IGH at 14q32, IGK at 2p12, IGL at 22q11. Each locus contains a constant region (C) that encodes part C of the chains and a variable region (V) made of multiple copies of V and J segments. The IGH locus also contains D segments (Abbas et al., 2002).

In the BM, B-cell development is initiated by rearrangements of IGH and IGK/IGL loci in B-cell progenitors. Those cells that produce non-functional rearrangements die by apoptosis. These naive B cells exit the BM to seed secondary lymphoid organs such as lymph nodes, spleen and mucosa-associated lymphoid tissue (MALT). Here, they interact with antigen and form primary and subsequently secondary lymphoid follicles forming a GC. Healthy GC represent sites of affinity maturation, a process that results in the selection of B cells that secrete high-affinity antibodies (Abbas et al., 2002).

B cells present several antigenic surface markers depending on the stage of development to which they are. For example, GC centroblasts switch off expression of BCL2 (apoptosis's regulator gene), therefore they and their progeny are susceptible to apoptosis.

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Conversely, GC centroblasts express the two important markers CD10 (a cell surface enzyme also known as CALLA) and BCL6 (a nuclear transcription factor) (Saito et al., 2007; Pittaluga et al., 1996) (fig.6).

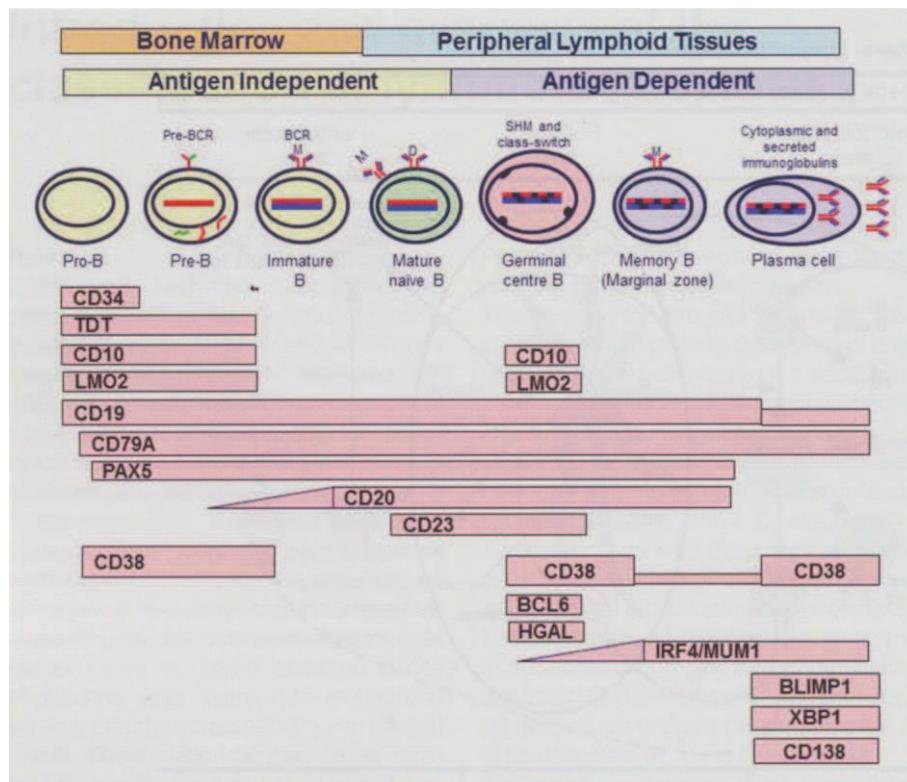


Figure 6: B cell differentiation. B cells show different stages of differentiation from pro-B cells to plasma cells. B cells encounter antigen in the germinal centre and then they start the differentiation. Red bar in nucleus indicates heavy chain gene rearrangements; blue bar indicates light chain rearrangements; in black the somatic hypermutation. BCR, B cell receptor; D, surface IgD; M, surface IgM; SHM, somatic hypermutation (Swerdlow et al., 2017).

Most DLBCL are composed of cells that in part resemble centroblasts and have mutated IGV genes, consistent with a derivation from cells that have been exposed to the GC. BL cells are BCL6 positive and have mutated IGH genes and therefore they are thought to correspond to a GC centroblast. Both BL and DLBCL correspond to proliferating cells and are clinically aggressive tumours. FL are tumours of GC B cells in which the GC cells fail to undergo apoptosis, in most cases due to a chromosomal rearrangement t(14;18) (q32;q21), that prevents the normal switching off of BCL2 expression. Centrocytes usually predominate over centroblasts and these cases neoplasms tend to be indolent (Swerdlow et al., 2017). Post germinal centre B cells retain the ability to home to tissue in which they have undergone antigen stimulation, so that B cells that arise in MALT tend to return there, whereas B cells that arise in the lymph nodes home to nodal sites and BM (Butcher, 1990). Marginal zone lymphomas of the MALT type, splenic type and nodal type come

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from post-GC memory B cells of marginal zone type that derive from and proliferate specifically in extranodal, splenic and nodal tissue. Plasma cell myeloma corresponds to a bone marrow-homing plasma cells (Swerdlow et al., 2017) (fig. 7).

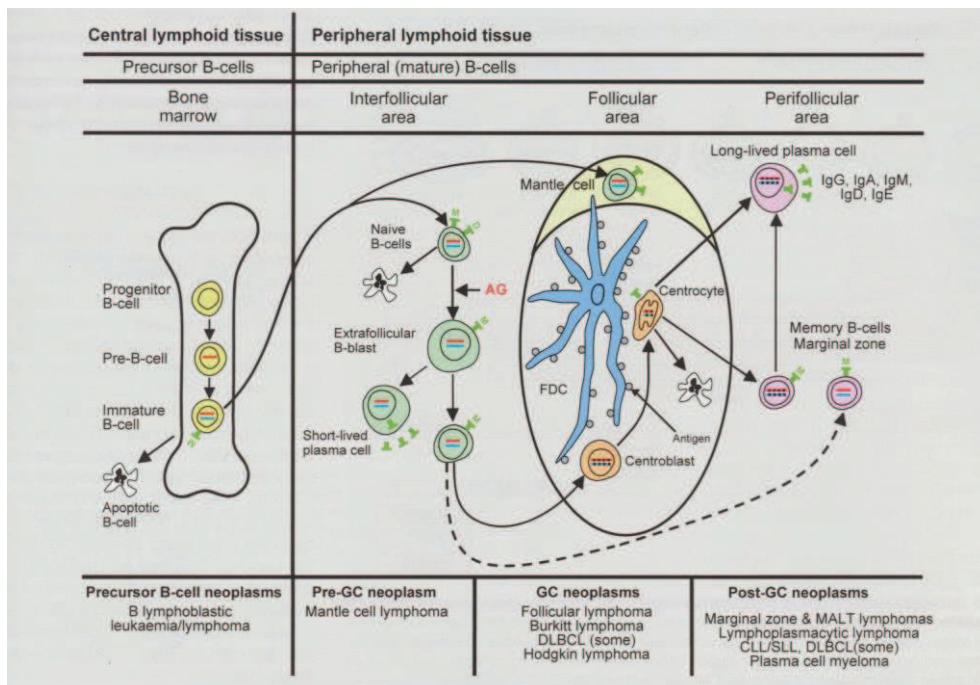


Figure 7: B cell normal development and its relationship to major B cell neoplasms. B cell neoplasms correspond to various stages of normal B cell maturation. Precursor B cells, which mature in bone marrow, may undergo apoptosis or develop into mature naïve B cell which after exposure to antigen (AG) may develop into short-lived plasma cells or enter the germinal centre (GC). In the GC, the centroblasts either undergo apoptosis or develop into centrocytes. Post GC cells include long-lived plasma cells and memory B cells. Red bars indicate IGH gene rearrangement and blue bars IG light rearrangement; somatic hypermutation is indicated in black (modified from Swerdlow et al., 2017).

4. IG loci translocations and cytogenetic evolution in B cell neoplasms

In normal immune responses, polyclonal populations of lymphocytes are present and express many different antigen receptors, through physiological rearrangements of the VDJ genes. Conversely in most lymphoid neoplasms are found pathological rearrangements of the antigen receptor genes, commonly preceding the neoplastic transformation. Hence, all cells derived from the malignant progenitor have the same configuration and sequence of the antigen receptor and synthesize receptor proteins for the identical antigen (clonal growth). Therefore, the analysis of the antigen receptor genes or their products is used to distinguish reactive (polyclonal) lymphoid proliferations from neoplastic ones (monoclonal). Furthermore since several mature B-cell neoplasms have

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characteristic genetic abnormalities that are important in determining their biological features, the genetic characterization is useful in differential diagnosis.

A great number of chromosomal aberrations have been described in mature lymphoid neoplasms and many of them are non-random (Mitelman Database of chromosome aberrations in cancer). Cytogenetics has played crucial role in providing substantial insight into the genetic mechanisms of lymphomagenesis. Novel chromosomal rearrangements have been continuously identified using different cytogenetic approaches such as FISH and array comparative genomic hybridization. The identification of chromosomal alterations in lymphomas, has greatly impacted on the classification, especially in NHL, and has concurred to establish a distinct subtype (Bhavana et al., 2011).

The IGH, IGK and IGL loci are frequently involved in many recurrent chromosomal translocations in B-cell malignancies (Heim & Mitelman, 2015) (fig. 8). The frequency of IG translocations in different B cell malignancies subtypes is variable: the presence of IG translocations can be pathognomonic for the disease and detectable in more than 95% of cases, as reported in BL with the t(8;14)(q24;q32) and Mantle cell lymphoma (MCL) with the t(11;14)(q13;q32) (Fu et al., 2005; Hummel et al., 2006). Moreover, IG translocations in B cells malignancies may be multiple and may involve both IGH and IGL/IGK loci. Sometimes, different partners genes are involved in the same translocation, like BCL2 and MALT1, which rearrange with IGH in t(14;18)(q32;q21) in FL and MALT lymphoma respectively (Tsujimoto et al., 1985; Sanchez-Izquierdo et al., 2003; Streubel et al., 2004).

translocations	Lymphoma/leukemia subtype	Translocations partner
t(1;14)(p22;q32)	MALT lymphoma	BCL10
t(1;14)(q21;q32)	DLBCL	MUC1
t(1;22)(q21;q11)	FL	FCGR2B
t(2;14)(p13;q32)	CLL	BCL11A
t(3;14)(q27;q32)	DLBCL	BCL6
t(6;14)(p21;q32)	DLBCL and other B-cell lymphoma	CCND3
t(7;14)(q21;q32)	Splenic lymphoma	CDK6
t(8;14)(q24;q32)	BL, DLBCL, B-cell prolymphocytic leukemia, MM	MYC
t(9;14)(p13;q32)	Lymphoplasmacytoid immunocytoma, other B NHL	PAX5
t(10;14)(q24;q32)	DLBCL	NFKB2
t(11;14)(q13;q32)	MCL, CLL, B-cell prolymphocytic leukemia	CCND1
t(11;14)(q23;q32)	DLBCL	RCK
t(12;14)(p13;q32)	MCL	CCND2
t(12;14)(q24;q32)	BL, MM	BCL7A

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del(14)(q24q32)	CLL	ZEP36L1
t(14;15)(q32;q11-q13)	DLBCL	BCL8
t(14;18)(q32;q21)	FL	BCL2
t(14;18)(q32;q21)	MALT	MALT1
t(14;19)(q32;q12)	DLBCL	CCNE1
t(14;19)(q32;q13)	DLBCL	SPIB
t(14;19)(q32;q13)	CLL, other B-NHL	BCL3
t(14;22)(q32;q11)	B-NHL low grade	IGL?

Figure 8: Chromosomal translocations and other structural anomalies affecting the IGH locus (14q32), IGK locus (2p12) and IGL locus (22q11) in B cell mature neoplasms other than multiple myeloma (from Heim & Mitelman, 2015).

It is supposed that pathological chromosomal translocations are derived from mistakes in physiological recombination process. Depending on whether mistakes occur in the VDJ joining process, the chromosomal breakpoints are found in different regions of the IG loci. It is also supposed that the location of IG breakpoint reflects the developmental stage at which the translocation occurs in B cell (Heim & Mitelman, 2015). The consequence of IG translocations is the activation of intact oncogenes through dysregulation mediated by enhancer segments of the IG loci. Moreover, the breakpoint on the derivative partner chromosome can be located at 3' or 5' end of the target oncogene where the IG enhancer is aberrantly juxtaposed, causing a dysregulated oncogene expression, leading to the transforming mechanism (Heim & Mitelman, 2015).

It is well known that a primary genetic alteration of a cell initiates lymphomagenesis (Heim & Mitelman, 2015). Frequently, chromosomal translocations involving IG loci are primary genetic alterations and can be used as diagnostic markers. The primary genetic alteration is often not sufficient to drive lymphomagenesis (Willis & Dyer, 2000). Moreover, several primary genetic alterations like t(14;18)(q32;q21), t(11;14)(q13;q32) and t(2;5)(q23;q35) are found in healthy individuals by sensitive molecular techniques, (Maes et al., 2001; Basake et al., 2002; Biagi & Seymour, 2002). Then, additional genetic changes are required for the lymphoid malignancy development: these anomalies are called secondary genetic changes. Consequently, most lymphomas with a defined primary change like t(14;18)(q32;q21) in FL or t(11;14)(q13;q32) in MCL, carry also secondary alterations. These alterations predominantly lead to gain or loss of genetic material and are associated with the transformation of an indolent malignancy to a more aggressive forms. Moreover, the number of secondary genetic aberrations increases during disease progression and they can be different also in tumors of the same type (Heim & Mitelman, 2015). Nevertheless, the pattern of secondary changes in mature lymphoid neoplasms is not random, but rather seems to depend on the nature of the primary event (Ott et al., 1997)

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(fig. 9). However, some chromosomal alterations are common in different types of lymphoid neoplasms. These might constitute primary aberrations in some of them but are more likely secondary or even tertiary aberrations in the majority. The best example of such low-specific change is deletion of the long arm of chromosome 6. This anomaly is frequent in all types of mature lymphoid neoplasms including HL, with an incidence between 5% in chronic lymphocytic leukemia (CLL) and 30% in DLBCL. Numerous studies suggested that there are three minimally common deleted regions not always related to the type of neoplasia: a distal one encompassing 6q25-27, a second one involving the more proximal band 6q21 and a third involving band 6q23 (Heim & Mitelman, 2015).

Neoplasm	Cytogenetic aberration	% Frequency
B cell chronic lymphocytic leukemia	del(13q) +12 del(11q) del(6q) del(17p) t/der(14q32)	~50 ~15 15-20 ~5 4-20
Mantle cell lymphoma	t(11;14)(q13;q32) del(11q) del(13q) der(3q) +12 del(1p) del(6q) del(9p) del(17p)	>95 10-50 5-15
Extranodal marginal zone B cell lymphoma of MALT type	t(11;18)(q21;q21) t(14;18)(q32)(q21) involving MALT t(3;14)(p14;q32) t(1;14)(p22;q32) +3/3q +18/18q	15 11 <10 <2 30
Follicular lymphoma	t(14;18)(q21;q32) and variants t(3q27) +X +7 +12/12q +18/18q +der(18)t(14;18) del(6q) del(10q) del(17p) dup(1q) der(1p)	80-90 <10 (FL 1/2) 55 (FL3B) >10
Diffuse large B cell lymphoma	t(3q27) t(14;18)(q21;q32) t(8;14)(q24;q32) t(8q24) +3/3q +18/18q +19q del(6q) del(9p) +1q +2p13-16 +7 +11q +12/12q +9/9p23-24 +2p13-16	20-40 20-30 5-10 10-40 10-40 50-90
Burkitt lymphoma	t(8;14)(q24;q32) and variants dup(1q) +7 +12	100 ~30-50 ~10-30
Plasma cell myeloma/plasmacytoma	t(4;14)(p16;q32) t(11;14)(q13;q32) t(14;16)(q32;q23) t(6;14)(p25;q32) t(8;14)(q24;q32) and other t(14q32) -13/del(13q) dup(1q) del(6q) del(11q)	~20 5 15-50 5-20

Figure 9: Frequent and diagnostically relevant chromosomal anomalies in the most common mature B cell neoplasms (modified from Mitelman, 2015).

5. The DSP30 mitogen

The DeoxyPhosphorothioate 30 (DSP30) is a synthetic Oligodeoxynucleotides (ODNs), containing non-methylated CpG islands, having immunostimulatory properties on B lymphocytes, monocytes and dendritic cells. It is able to stimulate cell proliferation through a molecular mechanism that mimics the action of a bacterial infection (Krieg et al., 1995).

Numerous studies have shown that:

- bacterial DNA causes the proliferation of the immune system cells that become functionally active (Krieg et al., 1995);
- the immune system stimulation is mediated by non-methylated CpG dinucleotides that are present at high frequency in the bacterial DNA but not in mammalian DNA. Hence, human immune system cells can distinguish between self DNA and non-self DNA (Krieg et al., 1995);
- many ODNs, different in length, sequence and structure, had been tested in vitro, but the highest stimulation index was obtained with a DNA composed by many CpG islands having two purine at 5' and two pyrimidine at 3' (Krieg et al., 1995; Decker et al., 2000).

The immunostimulatory effect by DSP30 is sequence-specific: stimulation does not occur if the critical "motif CpG" is destroyed by inversion or by nucleotide methylation (Takeshita et al., 2004). While in the mouse the DNA containing CpG stimulates all the B cells, in humans the stimulation occurs preferentially in the activated B cells and/or in the memory cells.

The DSP30 consists of 27 bp whose sequence is:

5' TCGTCGCTGTCTCCGCTTCTTGCC 3'

The ODN structure has been modified: in every phosphodiesteric bond, the free oxygen is replaced with a sulphur atom and this modification makes the ODN resistant to the action of the deoxyribonuclease present in cell cultures (fig. 10).

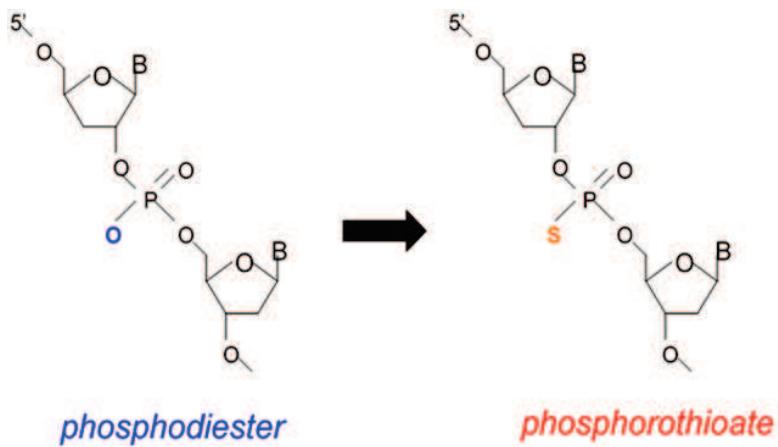


Figure 10: Scheme of the DSP30 structural change.

The DSP30 immunostimulatory effect is mediated by its link with the Toll-like receptor 9 (TLR9), a protein belonging to a class of single-segment transmembrane receptors (TLRs) that play a key role in immunity. They are located either on the cell surface or on the surface of cellular compartments (Takeshita et al., 2004). Its name derives from the structural analogy with the gene *Toll* identified in Drosophila. At first *Toll* was identified for its role in development, later its role in innate immunity was also discovered. TLRs protect the animal from pathogenic agents' infection by activating the synthesis of antimicrobial proteins. The TLRs are present in vertebrates and invertebrates and are one of the oldest and most preserved immune system elements. It has been estimated that most of the mammals have between 10 and 15 TLRs. To date, 10 have been identified in humans (fig.11).

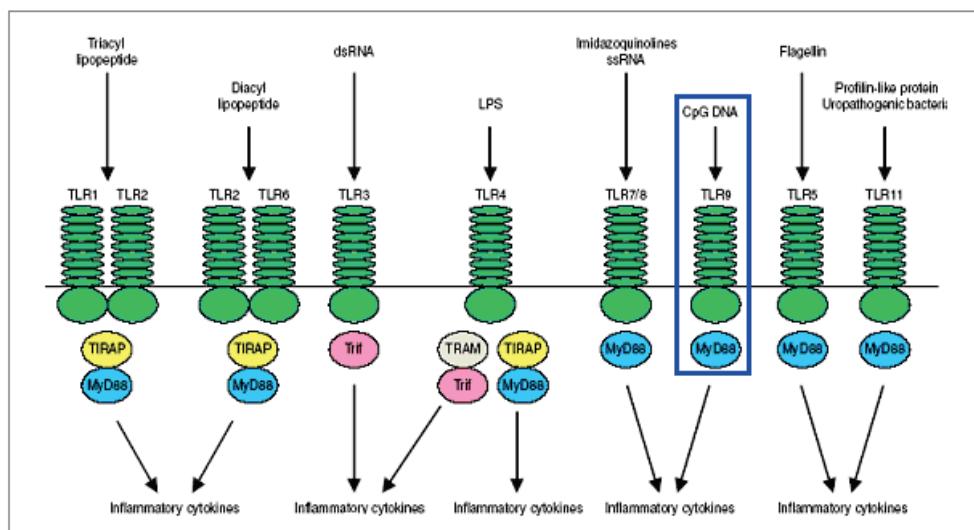


Figure 11: Tool Like Receptors and ligands (from Peng SL, 2005).

TLRs structure includes three protein domains: an extracellular domain (ECD), a transmembrane domain (TMD) and an intracellular domain (ICD) containing a region called TIR (Toll-IL 1 Receptor). In the carboxy-terminal part (-COOH), there are 32 amino acids that form 1/5 of the α -helix structure which is critical for the activation of the immunostimulatory signal (Takeshita et al., 2004) (fig.12).

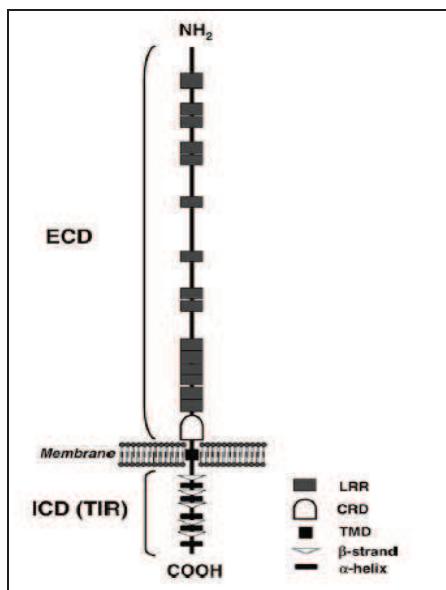


Figure 12: TLRs structure. ECD extracellular domain, ICD intracellular domain, TMD transmembrane domain (Takeshita et al., 2004).

TLRs recognize common molecules expressed in high quantities during infections with pathogenic microorganisms. Genetic studies have shown that there is a different and specific ligand for each TLR (Takeda & Akira, 2005). Both in humans and mouse the recognition of non-methylated CpG dinucleotides is mediated by TLR 9.

The model proposed to explain the activation mechanism in B lymphocytes is as follows: TLR9 molecules are synthesized in the endoplasmic reticulum and then they are located inside endocellular compartments that are immediately under the membrane (endosome). The DNA containing CpG is incorporated by endocytosis in the cell after binding to the B cell receptor (BCR), and the whole complex is transported within the endosome. After the interaction between CpG-DNA and TLR 9, the endosome is relocated in regions more inside the cell and MyD88, an "adapter" protein, is recruited. A cascade of signals is initiated via the IRAK-TRAF6-TAK1 mediated pathway, which ends in the activation of NF-

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kB and AP1, which migrate into the nucleus and regulate the gene expression of some cytokines / chemokines. (Takeshita et al., 2004; Peng, 2005).

Therefore B lymphocytes:

- proliferate, differentiate and secrete Ig (Takeshita et al., 2004; Peng, 2005);
- express CD25 on the surface, a high affinity receptor for interleukin 2 (IL-2). This production is greater in the cells of patients with LLC-B than in B cells of normal controls (Decker et al., 2000). For this reason, the IL-2 addition in vitro cell cultures cause an increase in cell proliferation. It is well described that a stimulation with both DSP30 and IL-2 allows to obtain a greater number of cells in mitosis and does not induce the appearance of chromosomal aberrations (Decker et al., 2006).

Aim of work

The WHO classification of tumours of Haematopoietic and Lymphoid tissues, 3rd edition, published in 2001 reflected a paradigm shift in the approach to classification of neoplasms. A multiparameter approach to define diseases has been adopted that uses all available information, that is clinical features, morphology, immunophenotype, and genetic data. For the first time genetic information was incorporated into diagnostic algorithms provided for the various entities. As underlined by the Editors of the fourth edition, “classification is the language of medicine: diseases must be described, defined and named before they can be diagnosed, treated and studied. A consensus on definitions and terminology is essential for both clinical practice and investigations.”

The importance of combining clinical, pathological and genetic data is highlighted in the last two editions of WHO classification of tumours, 4th and 5th edition, published in 2008 and 2017 respectively.

We remind that about 85–90% of HL and NHL are derived from B cells, whereas the remaining lymphomas are derived from T or NK-cell lineage. Their localization is mainly in the lymph node. Therefore, the genetic characterization is often performed by FISH on formalin-fixed paraffin-embedded (FFPE) lymph node tissue sections, using a locus specific probe. This method is very useful to investigate gene rearrangements directly on tissues processed for histopathological diagnostics, but the analysis is thus performed only on interphase nuclei and does not allow a “global view” as instead cytogenetics does.

Conventional chromosome analysis on BM aspirate is useless in extra-medullary lymphoma diagnosis. Nevertheless, standard cytogenetic analysis should be carried out because it can disclose the presence of additional chromosome anomalies, not identified by FISH performed with locus specific probes, which could involve important genes and consequently change the response to therapy.

The aim of this work is to provide a new method that allows the integration of cytogenetic analysis in the genetic characterization of the extra-medullary lymphomas. The increasingly remarkable genotype/phenotype relationship is generating new diagnostic approaches, improved prognostic/predictive models, and hopefully innovative therapeutic approaches according to the principles of precision medicine.

The goals we intend to reach in this study are the following:

- 1) evaluate quantity and quality of nuclei and mitoses in two different type of lymph node biopsy cultures added with the mitogen DSP30 + IL-2 (“shaken” versus “fragment” culture, as it will be explained in Methods);

Aim of work

- 2) evaluate whether the cytogenetic alterations found are different between the two types of culture;
- 3) evaluate whether the abnormalities found correlate with the morphological analysis, immunophenotypic characterization of the sample (including the one performed on cell block after cultured lymph node) and clinical features;
- 4) assess if there is a correlation between cytogenetic and FISH aberrations found;
- 5) evaluate whether our new methodological approach could become relevant or even indispensable in lymphoma diagnostics.

Moreover, this method allow the storage of biological sample, in addition to that processed for histological analysis, to carry out further cytogenetic/molecular investigations, in a future time.

Method and material

1. Case selection

This study includes cases with cytogenetic studies from 42 lymph node biopsy specimens (out of a total of 82 in 2016-2018 period) received for a complete hematopathologic evaluation at the S.C. Anatomia Istologia Patologica e Citogenetica of ASST Grande Ospedale Metropolitano Niguarda. As long as there was sufficient material, we set up cytogenetic cultures and performed karyotypes. We ruled out some cases in which the patient's anamnestic history reported a pathology not B cells-related.

We selected 42 cases classified by histological analysis as follows:

- 2 metastases of other neoplasms
- 1 case without disease localization
- 4 reactive lymphadenitis
- 7 Hodgkin's lymphomas
- 17 mature B-cell lymphomas, follicular type (FL)
- 1 Burkitt lymphoma (BL)
- 4 chronic lymphocytic leukemia (CLL)
- 4 diffuse large B cell lymphoma (DLBCL)
- 1 plasmacytoid lymphoma
- 1 plasmablastic lymphoma

Cytogenetic and molecular cytogenetic protocols were applied to all these samples, even when the histopathological diagnosis was yet in progress.

2. Lymph node biopsy cell cultures

Lymph node samples is collected by Pathologist in a sterile manner and send as soon as possible to the Cytogenetic laboratory (the tissue sample must be fresh). The sample selected should be "pure" tumour, without necrosis.

Disaggregate with a scalpel the lymphoid tissue sample and proceed to the preparation of two different types of cultures:

Shaked culture technique

1. put a portion of the fragments into a tube containing Hank's and shake vigorously for at least 2 minutes (if the sample is adequate it should numb quickly)
2. centrifuge for 5' to 1200 rpm

Method and material

3. eliminate the Supernatant
4. resuspend the cells suspension and put it in a flask with 5 mL of CHANG BMC medium (Irvine Scientific) + DSP30 (2 μ M) (TIBMolBiol, Genova, Italy) and IL2 [5ng/mL] (Gibco by Life Technologies, Italy)

Fragment culture technique

Take the remaining part of lymph node fragments and put in a flask with 5 mL of Chang BMC medium + DSP30 + IL2 (same concentrations as explained above).

3. Bone marrow aspirate cell cultures

BM aspirate cultures are prepared putting 0.5 mL of BM aspirate in a flask with 5 mL of medium Chang BMC + DSP30 + IL2 (same concentrations as explained above).

All the lymph node cells and BM aspirate cultures are incubated in humid 5% CO₂ incubator at 37°C for 72-120 hours and 24h-48 hours respectively.

4. Lymph node biopsy and bone marrow aspirate cultures processing for cytogenetic analysis

The method is as follows:

1. add Colcemid [10ug/ML] (Roche) to each culture and incubate for 5 hours;
2. transfer the cell suspension into a 15 mL Falcon tube;
3. centrifuge 5' at 2000 rpm and discard the supernatant;
4. add 5 mL of 0.56% KCl solution and incubate 10';
5. gently shake by inversion for few seconds;
6. centrifuge 5' at 2000 rpm and discard the supernatant;
7. add 5 mL of 5% Acetic Acid water solution;
8. gently shake by inversion for few seconds;
9. centrifuge 5' at 2000 rpm and discard the supernatant;
10. add 5 mL of Fixative Solution composed of Acetic acid and Methanol (1:3)
11. gently shake by inversion for few seconds;
12. centrifuge 5' at 2000 rpm;
13. discard the supernatant and add some drops of Fixative Solution to reach the right concentration of cells suspension for a suitable spreading;
14. spread the cells suspension on slides and let air dry;
15. stain the cells plunging the slide in Quinacrin mustard [0.5%] (ICN biomedical Inc);

Method and material

16. remove the excess of Quinacrin mustard rinsing the slide in McIlvaine buffer solution (200 mM Disodium Hydrogen phosphate and 100 mM Citric Acid).

Cytogenetic analysis was performed by fluorescence microscope Axio Imager Z1 (Carl Zeiss MicroImaging GmbH, Gottingen, Germany), equipped with a UV 100-W lamp (Osram, Augsburg, Germany), ProgRes MF CCD camera (Jenoptik AG, Jena, Germany), IKAROS System Software (MetaSystems Hard & Software, Altlussheim, Germany). Karyotypes results were described according to International System for Human Cytogenetic Nomenclature (ISCN 2016).

5. Preparation of Bio-Agar cell block from lymph node biopsy cell culture

Additional shaked and fragment culture were set up on 10 samples of lymph node biopsies to proceed to cell block preparation.

The Bio-Agar (Bio-Optica Milano s.p.a.) is an aggregating medium that is solid at room temperature and became fluid after heating at 60°C. Once dissolved, the agar remains fluid for about 15 minutes.

Sample preparation

1. transfer the fresh cell suspension from flask to a 50 mL Falcon tube, centrifuge 5' at 2000 rpm and discard the supernatant;
2. add 5 mL of 10% buffered formalin and fix from 45' to 3 hours;
3. centrifuge 10' at 2500 rpm;
4. discard the supernatant;
5. add 3-5 drops of liquefied Bio-Agar;
6. mix with vortex for few seconds;
7. put the sample at 4°C for 5' to solidify;
8. remove the solidified sample from the bottom of the tube, place it in a histological cassette and incubate in 10% neutrally buffered formalin for at least 1 hour;
9. proceed to standard histological processing, paraffin inclusion and section cutting.

6. Fluorescence in situ hybridization (FISH) on cytogenetic samples and formalin-fixed paraffin-embedded (FFPE) sections

We performed FISH analysis both on BM cultures, lymph node cytogenetic cultures and FFPE lymph node sections.

Method and material

We used only commercial FISH probes (Tab 1).

Table 1: Probes used to perform fluorescence in situ hybridization analysis.

Probe	Manufacturer	Type of probe
MYC (8q24)	Vysis/Abbott, Illinois, USA	break apart probe
BCL2 (18q21.33)	Vysis/Abbott, Illinois, USA	break apart probe
BCL6 (3q27)	MetaSystems, Altlussheim, Germany	break apart probe
MYC (8q24)	MetaSystems, Altlussheim, Germany	break apart probe triple-color
t(8;14)(q24;q32) IGH/MYC	Cytocell Ltd., Cambridge, UK	dual color dual fusion probe
t(14;18)(q32;q21) IGH/BCL2	Cytocell Ltd., Cambridge, UK	dual color dual fusion probe
t(11;14)(q13;q32) IGH/CCND1	Vysis/Abbott, Illinois, USA	dual color dual fusion probe
TP53 (17p13)/D17Z1 (17p11-q11)	MetaSystems, Altlussheim, Germany	deletion probe
ATM (11q22.3)/D11Z1(11p11-q11)	MetaSystems, Altlussheim, Germany	deletion probe
D13S319 (13q14)/LAMP1 (13qter)	Vysis/Abbott, Illinois, USA	deletion probe
D12Z1 (12p11-q11)	Vysis/Abbott, Illinois, USA	enumeration probe

FISH on cytogenetic preparation of bone marrow and lymph node biopsy cultures

Pre-treatment and hybridization (day 1)

Spread the cells suspension on slides

1. dry the sample in an incubator 37°C over night;
2. immerse the slide in a 2X SSC buffer (NaCl 3M, sodium citrate 0.3M) for 2' at room temperature;
3. dispense the probe on the sample and put a coverslip.

Sample and probe were co-denatured and hybridized on StatSpin ThermoBrite System (Iris Sample Processing Inc., Massachusetts, U.S.A.) following the manufacturer's instructions.

Post-hybridization washing (day 2)

4. Gently remove the coverslip and wash the slide in a 0.4X SSC solution for 2' at 73°C;
5. wash the slide in a 2X SSC 0.05% Tween 20 solution for 1' at room temperature;
6. wash quickly in distilled water and dehydrate the slide in 70%, 85% and 100% ethanol solutions;
7. let the sample air dry in the darkness;

Method and material

8. dispense DAPI II counterstain (4,6-diamidino-2-phenyindole) [125ng/ml] (Abbott Molecular Inc) for nuclear counterstaining and apply a coverslip.

FISH on formalin-fixed paraffin-embedded (FFPE) samples

Selection and preparation of the sample

- fixation time of histological samples must not exceed 24-48 hours
- histological sections must be 3 µm in thickness and placed on a silanized or positively charged slide
- histological sections must be air-dried over night or in a oven at 60°C for 15'.

Pre-Treatment and hybridization (day 1)

1. put the sample in Xylene for 10' at room temperature. Repeat the step n. 1 for 3 times
2. put the slide in Ethanol 100% for 5' at room temperature for 2 times;
3. put the slide in Ethanol 96% for 5' at room temperature for 2 times and air-dry;
4. put the slide in the pre-treatment solution (Histology FISH Accessory kit, Dako Denmark A/S, Denmark) for 15' at 96°C;
5. wash the slide in distilled water for 2' at room temperature;
6. dispense the digestion solution (Histology FISH Accessory kit, Dako Denmark A/S, Denmark), incubate for at least 3-5' and control at the microscope the right digestion of cytoplasm;
7. wash the slide in distilled water;
8. dehydrate the slide in 70%, 85% and 100% ethanol solutions;
9. dispense the probe and put a coverslip.

Sample and probe were co-denatured and hybridized on StatSpin ThermoBrite System (Iris Sample Processing Inc., Massachusetts, U.S.A.) following the manufacturer's instructions.

Post-hybridization washing (day 2)

10. wash slide in a 0.4X SSC solution for 2' at 73°C;
11. wash slide in a 2X SSC 0.05% Tween 20 solution for 1' at room temperature;
12. wash quickly in distilled water and dehydrate the slide in 70%, 85% and 100% ethanol solutions;
13. let the sample air dry in the darkness;
14. dispense DAPI II counterstain (4,6-diamidino-2-phenyindole) [125ng/ml] (Abbott Molecular Inc) and apply a coverslip.

Method and material

FISH analysis was performed by fluorescence microscope Axio Imager Z1 (Carl Zeiss MicroImaging GmbH, Gottingen, Germany), equipped with a UV 100-W lamp (Osram, Augsburg, Germany), ProgRes MF CCD camera (Jenoptik AG, Jena, Germany), ISIS System Software (MetaSystems Hard & Software, Altlussheim, Germany).

7. Immunohistochemical analysis on cell block sections

Immunohistochemical (IHC) analysis is a technique in the pathological anatomy routine that allows identifying intra and extra cellular location of a specific protein. This technique is based on the antigen-antibody link that is referred to be as one of the most specific reaction in cellular biology.

IHC investigations are mainly performed on FFPE sections. The quality of the results depends on the amount of the original material, on the preservation of the tissue antigenicity and on the signal amplification power by the systems used to highlight the antigen-antibody reaction.

Details of the method are not reported because IHC analysis are performed by another laboratory of the department using the DakoAutostainer automatic processor (Dako Denmark A/S, Denmark).

For each cell block we prepared histological sections of 3 µm thickness placed on appropriate polarized slides. Subsequently, histological sections are dried at 60°C for 20 minutes. The antibodies used in this study are indicated in the following table.

Table 2: Antibodies used to perform IHC analysis on cell block sections.

Antibody	Localization	Dilution	Manufacturer
Bcl6	nuclear	ready to use	Dako Denmark A/S, Denmark
Bcl2	membrane	ready to use	Dako Denmark A/S, Denmark
Cd30	membrane	ready to use	Dako Denmark A/S, Denmark
fascin	cytoplasmatic	1:100	Dako Denmark A/S, Denmark
CD10	membrane	ready to use	Dako Denmark A/S, Denmark

Results

At least one slide for cytogenetic analysis was set up for each lymph node biopsy culture, “shaked” and “fragment” techniques. Cellularity and metaphase quantity and quality of each culture type (“shaked” versus “fragment”) are shown in Table 3.

The number of metaphases was between 5 and 10 in the “shaked” cultures in 14 samples out of 42 showed and in the “fragment” cultures the same amount of metaphases was found in 15 samples out of 42. A good quantity of nuclei and metaphases (more than 10) was found in 8 samples out of 42 in the “shaked” cultures and in 7 samples out of 42 in the “fragment” cultures. Slight variations may be related to a different amount of lymph node material at the time of culturing. Only two cases, n. 35 (reactive lymphadenitis) and n. 37 (HL) present a noticeable difference between the two different cultures. So, no significant differences in quantity and quality of nuclei and metaphases were observed, as well as any difference in term of chromosome anomalies found.

All the cytogenetic results obtained are shown in Table 4. Cytogenetic analysis of lymph node biopsy cultures failed in 7 cases (16%), 4 of which were metastases of other neoplasms or reactive lymphadenitis. Lymph node karyotype showed chromosomal anomalies in 16 cases out of 35 (46%) (samples n. 3, 8, 13, 20, 23, 24, 25-27, 31-33, 37-38, 40-41): 9 were mature B-cell lymphoma FL type, with grade between 1-2 and 3B, 4 were CLL, 1 was plasmacytoid lymphoma, 1 was HL, 1 was DLBCL (then revised as double hit lymphoma, DH) (see Table 4). In 10 out of 16 samples (62%) (samples n. 3, 8, 13, 20, 23, 24-25, 31, 38, 41) chromosomal anomalies found were closely related to the definite histological diagnosis (e.g. t(14;18)(q32;q21) in FL cases, +12 in CLL cases). In the last 6 samples (cases n. 26, 27, 32, 33, 37, 40) karyotype showed different kind of chromosomal anomalies (e.g. deletion of short arm of chromosome 17, trisomy 12 and undefined chromosome markers) that are not related to a specific type of neoplasm (Table 4). In 18 cases the analysis was also performed on BM aspirate concurrently taken with the lymph node biopsy (cases n. 2, 6, 8, 10, 16-20, 22-26, 31, 39-40).

FISH analysis on FFPE lymph node sections were performed for samples n. 2, 8, 13, 27, and 38 (see Table 4). Whenever performed, FISH analysis on lymph node and/or BM cultures and/or FFPE sections confirmed abnormal lymph node karyotype (cases n. 8, 13, 20, 23-25, 31, 38).

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Table 3: comparison between the two types of lymph node cultures, shaked versus fragment: – sign indicates rare nuclei and rare/absent metaphases; + sign indicates sufficient nuclei and sufficient metaphases (from 5 to 10 metaphases); ++ sign indicates good quantity of nuclei and metaphases.

sample	shaked culture		fragment culture	
	nuclei quantity	metaphases quantity and quality	nuclei quantity	metaphases quantity and quality
1	+	+	+	+
2	+	-	+	-
3	+	+	+	+
4	-	-	-	-
5	-	-	-	-
6	+	+	+	+
7	+	+	+	+
8	+	+	+	+
9	++	++	++	++
10	-	-	-	-
11	-	-	-	-
12	-	-	-	-
13	++	++	++	++
14	+	-	+	-
15	-	-	-	-
16	+	+	+	+
17	+	+	+	+
18	+	+	+	+
19	+	-	+	-
20	+	+	+	+
21	-	-	-	-
22	-	-	-	-
23	+	-	+	-
24	++	-	++	-
25	++	++	+	+
26	+	+	+	+
27	+	-	+	-
28	++	-	++	-
29	+	+	+	+
30	-	-	-	-
31	++	++	++	++
32	++	+	++	++
33	++	++	++	++
34	++	++	++	++
35	-	-	+	+
36	-	-	-	-
37	++	++	-	-
38	++	++	++	++
39	+	-	+	-
40	++	+	++	+
41	+	-	+	-
42	+	+	+	+

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*Table 4: Cytogenetic results of lymph node (*from shaked and fragment cultures) and bone marrow (BM) cultures. N.d. not done analysis. DLBCL, diffuse large B-cell lymphoma. HL, Hodgkin lymphoma. CLL, chronic lymphocytic leukemia. DH, double hit lymphoma.*

sample	lymph node karyotype*	BM aspirate karyotype	FISH on lymph node culture	FISH on BM culture	FISH on FFPE lymph node section	histological diagnosis
1	46,XY[8]	n.d.	n.d.	n.d.	n.d.	DLBCL
2	absent metaphases	46,XY[20]	nuc ish (MYCx2)(5' MYCsep3' MYCx1)[50/100]; (IGH,MYC)x3(IGHconMYCx2)[47/100]	nuc ish(IGH,MYC)x2[100]	nuc ish (MYCx2)(5' MYCsep3' MYCx 1)[49/100]; (IGH,MYC)x3(IGHconMYCx 2)[51/100]; (BCL2x2)[100] (BCL6x2)[100]	BL
3	48,XY,+12,+mar[4]	n.d.	n.d.	n.d.	n.d.	FL grade 1-2
4	46,XY[7]	n.d.	n.d.	n.d.	n.d.	HL
5	absent metaphases	n.d.	n.d.	n.d.	n.d.	metastasis
6	46,XY[8]	46,XY[20]	n.d.	n.d.	n.d.	HL
7	46,XY[12]	n.d.	n.d.	n.d.	n.d.	HL
8	47,XY,+12[10]	47,XY,+12[3]/47,XY,add(4) (q3?5),+12[4]/46,XY[2]	n.d.	nuc ish (D12Z1x3)[80/100]	nuc ish (TP53,D17Z1)x2[100]	CLL
9	46,XY[20]	46,XY[20]	n.d.	nuc ish (IGH,BCL2)x2[100]	n.d.	FL grade 1-2
10	absent metaphases	46,XY[20]	n.d.	n.d.	n.d.	no disease recurrence
11	absent metaphases	n.d.	n.d.	n.d.	n.d.	metastasis
12	46,XY[10]	n.d.	n.d.	n.d.	n.d.	reactive lymphadenitis

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13	86,XXY,+del(1)(q?)x2,+2x2 ,+3x2,+4x2,+5x2,+7,+8, +9x2,+10x2,+11x2,+12x3, +13x3,t(14;18)(q32;q21), +16x2,+17,+18x2,+19x2, +20x2,+21x2,+22,+3 mar[12]/46,XY[3]	n.d.	n.d.	n.d.	nuc ish (BCL2x2)(5'BCL2sep3'BCL2 x1)[80/100]	FL grade 3a
14	46,XY[10]	n.d.	n.d.	n.d.	n.d.	HL
15	46,XX[5]	n.d.	n.d.	n.d.	n.d.	HL
16	46,XY[12]	46,XY[11]	n.d.	n.d.	n.d.	FL grade 1
17	46,XX[10]	46,XX[24]	n.d.	nuc ish (IGH,BCL2)x2[100]	n.d.	FL grade 1-2
18	46,XX[14]	46,XX[22]	n.d.	nuc ish (IGH,BCL2)x2[100]	n.d.	FL grade 1-2
19	46,XY[3]	45,XY,del(13)(q13), der(17)t(17;22)(p11;q11), -22[16]/46,XY[5]	n.d.	nuc ish (D13S319x1,LAMP1x 2)[75/100]; (TP53x1,D17Z1x2)[76 /100]	n.d.	DLBCL evolved from Richter syndrome
20	46,XX,t(14;18) (q32;q21)[8]/46,XX[9]	46,XX,t(14;18)(q32;q21)[1]/ 46,XX[22]	nuc ish (IGH,BCL2)x3(IGHconBCL2x2 [87/100])	nuc ish (IGH,BCL2)x3(IGHco nBCL2x2)[90/100]	n.d.	plasmacytoid lymphoma
21	46,XY[1]	n.d.	n.d.	n.d.	n.d.	DLBCL
22	46,XY[3]	47,XY,del(2)(q2?3),add(8) (q2?4),add(9)(p2?1),add(18) (q2?3),+mar[3]/46,XY[20]	n.d.	nuc ish (MYCx2)[100]; (IGH,BCL2)x2[100]	n.d.	plasmablastic lymphoma
23	47,XY,+12[2]/46,XY[2]	47,XY,+12[5]/46,XY[15]	n.d.	nuc ish (D12Z1x3)[58/100]	n.d.	CLL
24	50,XX,+3,t(14;18)(q32;q21) ,+17,+18,+21 inc[1]/46,XX[2]	51,XX,+3,t(14;18)(q32;q21), +17,+18,+21,+mar[6]/ 46,XX[22]	n.d.	nuc ish (IGH,BCL2)x3(IGHco nBCL2x2)[75/100]; (TP53,D17Z1)x3[74/1 00]	n.d.	FL grade 2

Results

25	46,XY,del(11)(q22)[9]/46,X, idem,add(Y)(q12)[1]/46, idem,i(2)(q10)[3]/45,idem, t(12;15)(p13;q14), t(14;21)(q21;q22) [12]	46,XY,del(11)(q22)[5]/46,X, idem,add(Y)(q12)[2]/46,XY,i dem,t(12;15)(p13;q14), t(14;21)(q21;q22) [12]	n.d.	nuc ish (ATMx1,D11Z1x2)[60 /100]; (D13S319x1,LAMP1x 2)[70/100]	n.d.	CLL
26	46,XY,add(X)(p?22)[2]/46,X Y,del(2)(q31),i(17)(q10)[3], 46,XY,del(5)(q14q33),i(17)(q10)[cp18]/ 46,XY[1]	46,XY,i(17)(q10)[10]/46,idem ,del(5)(q14q33)[2]/46,XY,t(1 1;14)(q14;q32),add(15)(q26)[10]/46,XY[2]	nuc ish (TP53x1,D17Z1x2)[85/100]; (IGH,CCND1)x3 (IGHconCCND1x2)[7 5/100]	nuc ish (IGH,CCND1)x3 (IGHconCCND1x2)[7 5/100]	n.d.	CLL
27	46,XY,del(17)(p1?)2/ 46,XY[2]	n.d.	nuc ish (IGH,BCL2)x3(IGHconBCL2x2) [70/100]	n.d.	nuc ish (BCL2x2)(5'BCL2sep3'BCL2 x1)[82/100]	FL grade 3
28	absent metaphases	n.d.	n.d.	n.d.	n.d.	reactive lymphadenitis
29	46,XY[16]	n.d.	n.d.	n.d.	n.d.	reactive lymphadenitis
30	absent metaphases	n.d.	n.d.	n.d.	n.d.	FL grade 3A
31	49~50,X,- Y,der(1)t(1;?)(p;?),del(2)p? ,+13x2,+14x2,t(14;18) (q32;q21),+15x2,+2~3mar [cp14]/46,XY[2]	46,XY[20]	n.d.	nuc ish (IGH,BCL2)x3(IGHco nBCL2x2)[92/100]	n.d.	FL grade 2
32	42~43,XY,-10,-11,-17, -18[cp7]/46,XY[6]	n.d.	n.d.	n.d.	n.d.	FL grade 3B
33	47,XX,9qh+,+12[6]/ 46,XX,9qh+[14]	n.d.	n.d.	n.d.	n.d.	FL grade 1-2
34	46,XY[18]	n.d.	n.d.	n.d.	n.d.	HL
35	46,XY[10]	n.d.	n.d.	n.d.	n.d.	reactive lymphadenitis

Results

36	absent metaphases	n.d.	n.d.	n.d.	n.d.	FL grade 2 with areas 3A
37	47,XX,+mar[2]/46,XX[16]	n.d.	n.d.	n.d.	n.d.	HL
38	45,X,-Y,-6,add(8)(q2?4), t(14;18)(q32;q21),add(18) (q2?1)-19,-22,+3~4mar[16]	n.d.	nuc ish (POU5F1Bx3,MYCx2,MYCdim x1,D8S1207x2)(POU5F1Bcon MYCdimx1,POU5F1BconMYC conD8S1207x2)[98/100]; (BCL2x2)(5'BCL2sep3'BCL2x1) [60/100]/(5'BCL2x1,3'BCL2x2) (5'BCL2con3'BCL2x0)[75/100]	n.d.	nuc ish (5'MYCx3,3'MYCx2)(5'MYC con3'MYCx2)[73/100]; (POU5F1Bx3,MYCx2,MYC dimx1,D8S1207x2)(POU5F 1BconMYCdimx1,POU5F1 BconMYCconD8S1207x2) [74/100]; (BCL2x2)(5'BCL2sep3'BCL2 x1)[60/100]/(5'BCL2x1,3'BC L2x2)(5'BCL2con3'BCL2x0) [25/100]; (BCL6x2)[100]	DLBCL/DH
39	46,XX[3]	46,XX[23]	n.d.	n.d.	n.d.	FL grade 2
40	45,XX,-1,+mar[2]/46,XX[16]	46,XX,-1,+mar[1]/46,XX[19]	n.d.	nuc ish (IGH,BCL2)x3(IGHco nBCL2x2)[70/100]	n.d.	FL grade 2, with focal areas 3A
41	46,XY,-12,t(14;18)(q32;q21), +mar[2]/ 46,XY[5]	n.d.	n.d.	n.d.	n.d.	FL grade 2
42	46,XY[15]	n.d.	n.d.	n.d.	n.d.	FL grade 1-2

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10 out of 18 cases didn't show any differences between lymph node karyotype and BM aspirate karyotype (samples n. 6, 9, 16, 17, 18, 20, 23, 24, 39, 40). Analysing the BM aspirate and lymph node cultures, differences in either karyotype or FISH analysis were detected in 7 patients (samples n. 2, 8, 19, 22, 25, 26, 31).

For example, sample n. 2 was FISH-positive for MYC gene rearrangement and translocation $t(8;14)(q24;q32)$ both on FFPE section and lymph node culture, but not in BM culture (fig.13).

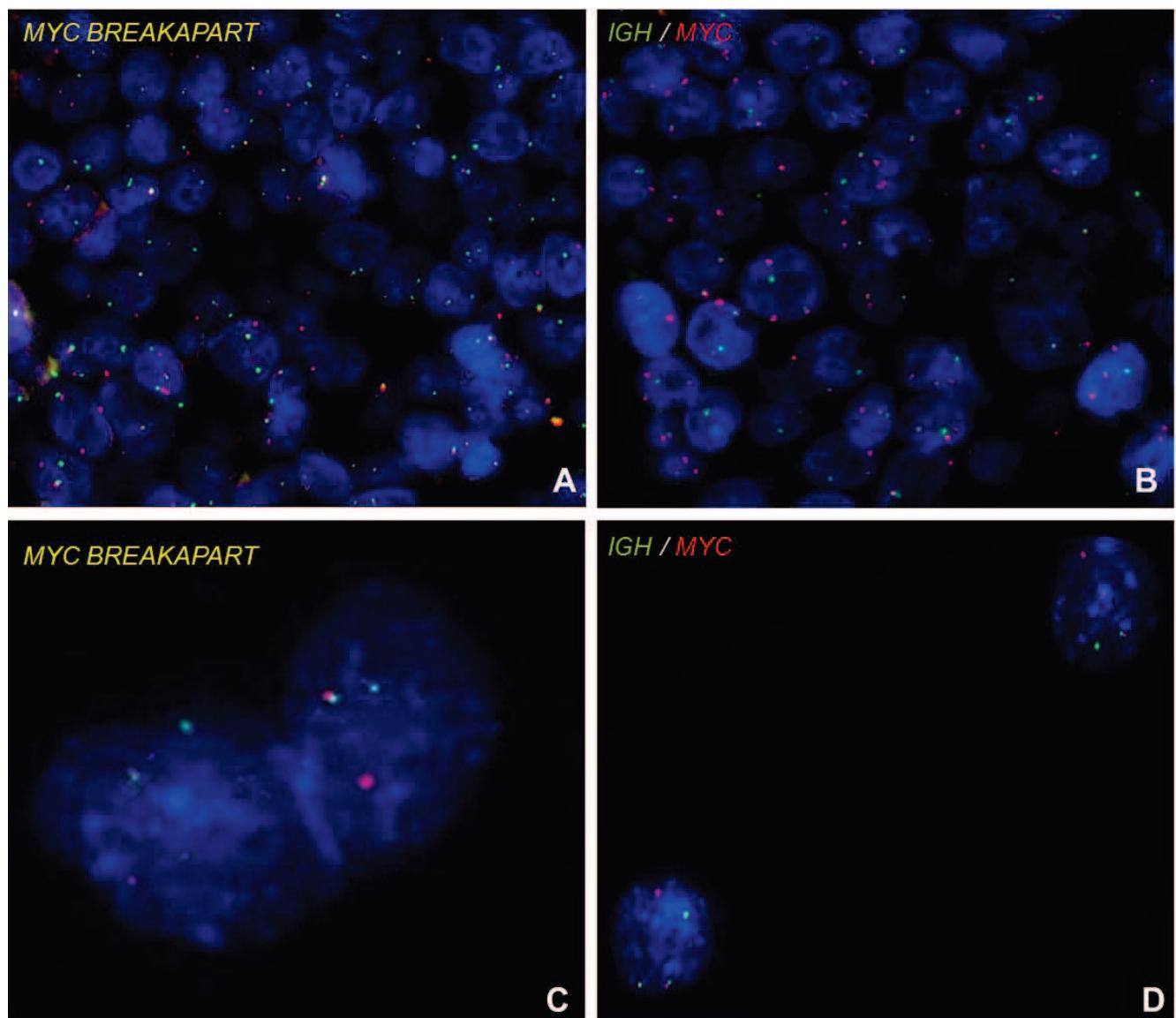
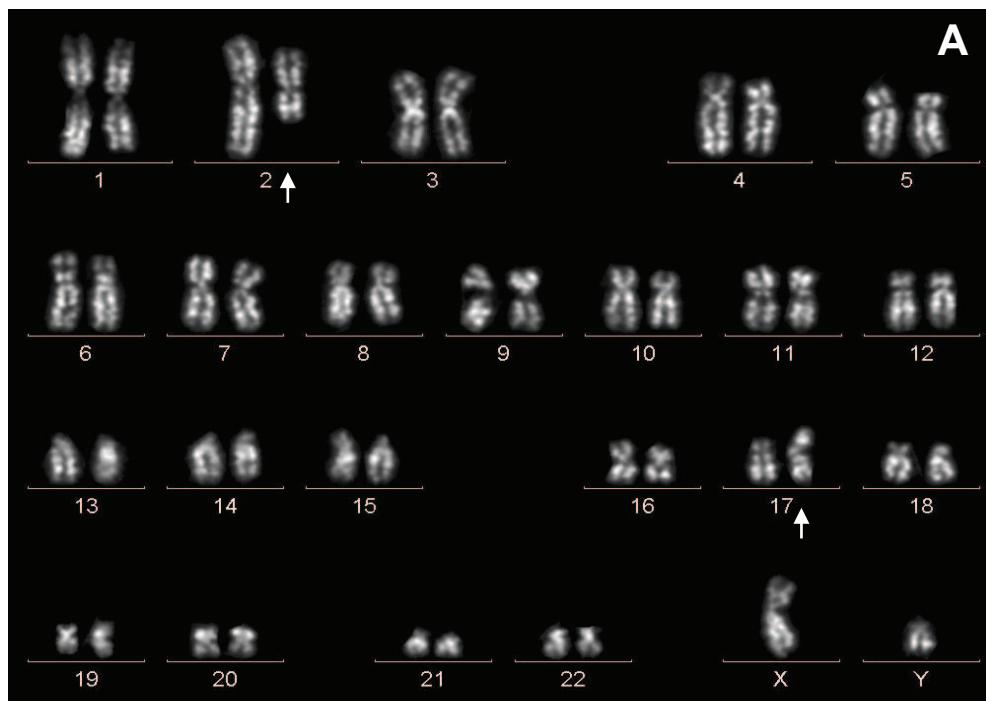


Figure 13: FISH performed on sample n. 2: (A) MYC break apart and (B) $t(8;14)$ /IGH/MYC on FFPE lymph node sections; (C) MYC break apart and (D) $t(8;14)$ /IGH/MYC on lymph node culture.

Furthermore, lymph node and BM karyotype may also share only some abnormalities and differ for others (samples n. 8, 25, 26) (Table 4). Sample n. 26, e. g., showed a complex karyotype both on lymph node and BM cultures: besides some other structural anomalies,

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the two type of cultures shared the i(17)(q10), but the translocation t(11;14)(q14;q32) was present only in BM culture (data confirmed also by FISH) (fig. 14).



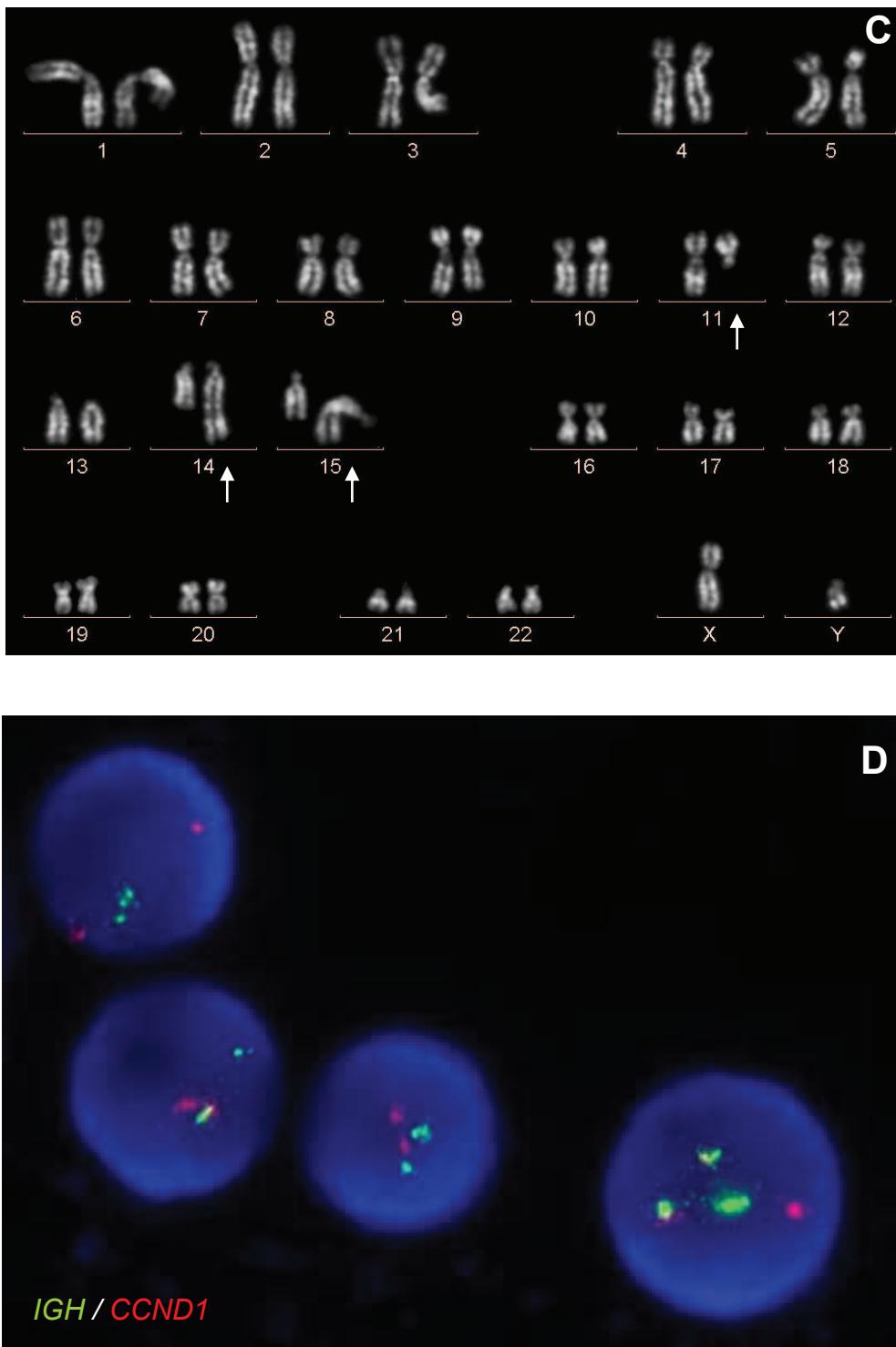


Figure 14: Sample n. 26. (A) Karyotype of lymph node culture showing cell clone with del(2)(q31) and i(17)(q10). (B) Karyotype of BM showing cell clone with del(5)(q14q33) and i(17)(q10) and (C) cell clone with t(11)(q14;q32) and add(15)(q26), not present in lymph node cultures. Chromosome anomalies are indicated by arrows. (D) FISH on BM interphase nuclei showing the presence of t(11;14)(q14;q32): IGH probe labelled in green and CCND1 probe labelled in red. The t(11;14)-positive cell showed a signal hybridization pattern with 2 fusion signals yellow (Y), 1 green (G) and 1 red (R).

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Complex karyotypes (more than 3 chromosome anomalies) were observed in 8 lymph node samples (n. 13, 24, 25, 26, 31, 32, 38, 41): 4 of them were diagnosed as FL with grade ranging from 2 to 3B.

In t(14;18)(q32;q21)-positive cases n. 13, 24, 31, 38, 41, in addition to FL more common characteristic translocation, other additional anomalies were observed. One of them, sample n. 38, showed a cell clone with karyotype:

45,X,-Y,-6,add(8)(q2?4),t(14;18)(q32;q21),-19,-22,+3~4mar (fig.15).

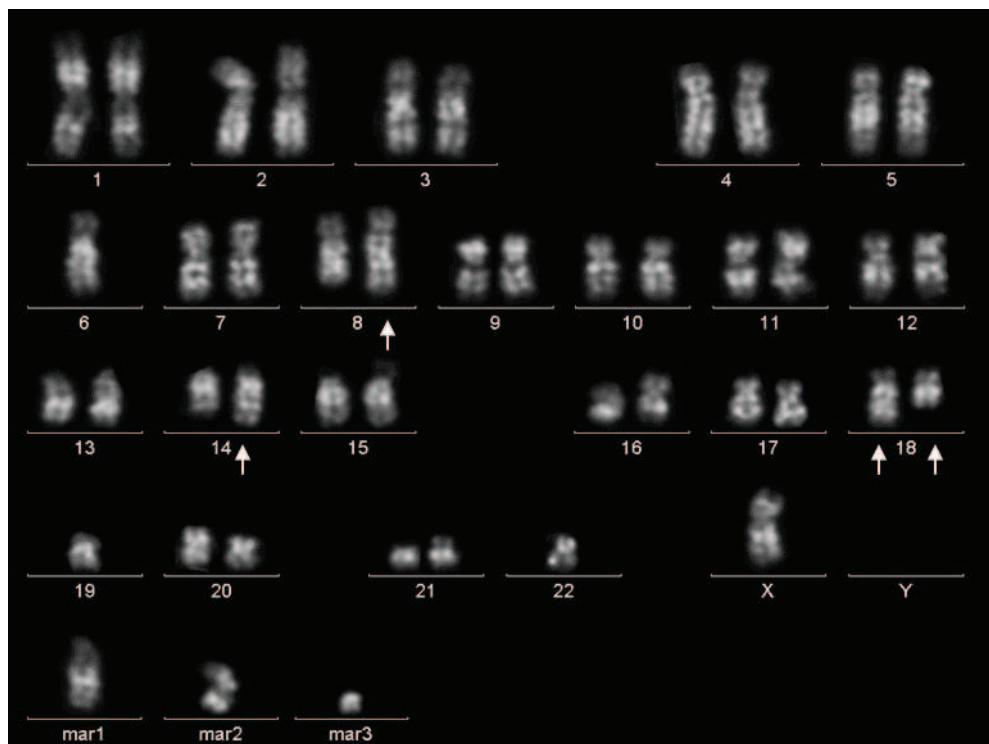


Figure 15: Lymph node karyotype of sample n. 38. 45,X,-Y,-6,add(8)(q2?4),t(14;18)(q32;q21),add(18)(q2?1),-19,-22,+3~4mar. Chromosome anomalies are indicated by arrows.

On this sample we performed FISH on FFPE to investigate the status of MYC and BCL2 genes. MYC signals hybridization pattern displayed the presence of an atypical pattern with an additional 5'end signal very close to an intact MYC signal (fig. 16A). BCL2 probe showed two different hybridization patterns (fig. 16B), both positive for gene rearrangement.

Results

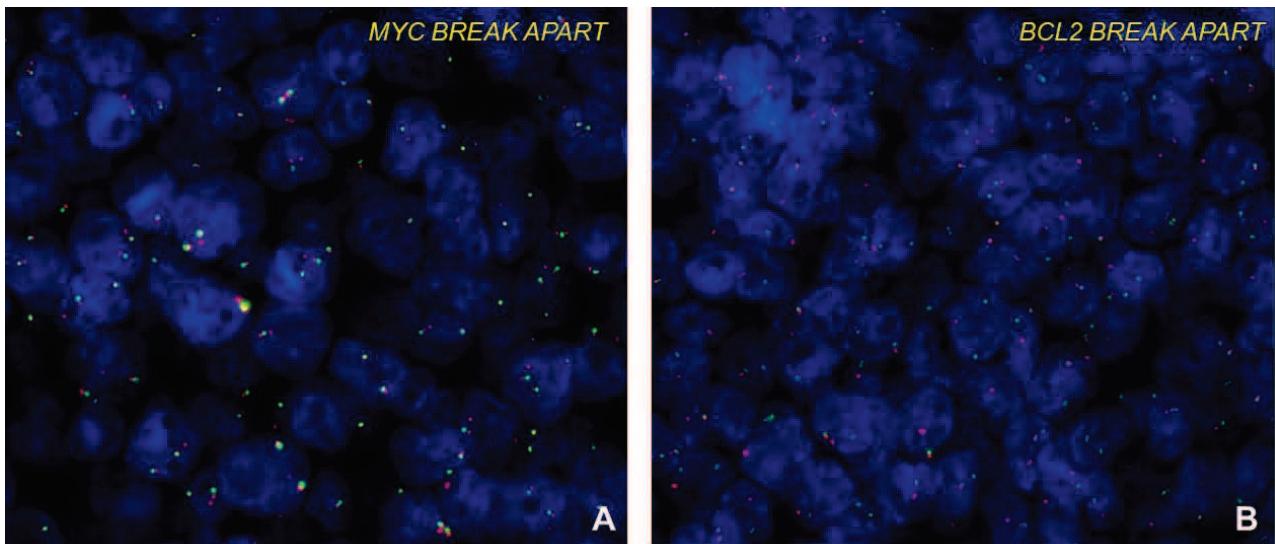
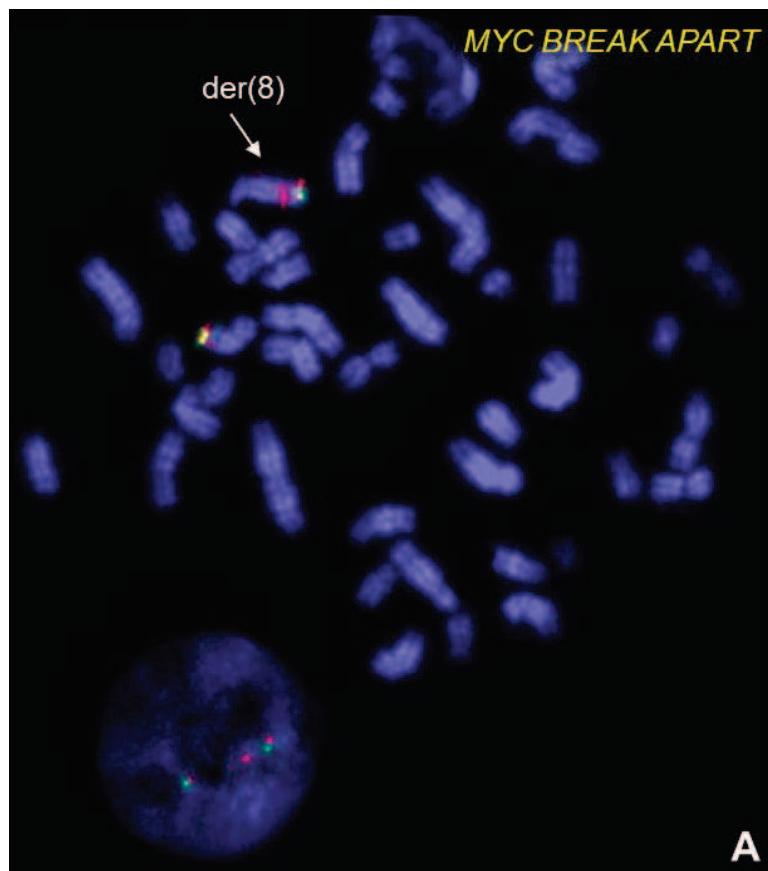


Figure 16: Sample n. 38. FISH performed on FFPE lymph node sections. (A) MYC break apart probe displayed the presence of an additional 5'end (R) signal of the gene. (B) BCL2 break apart probe showed the presence of two different hybridization patterns (1Y 1G 1R signals; 2G 1R and no fusion signals) which correspond to the presence of two different cell clones.

Thus, MYC and BCL2 status were tested also on chromosomes obtained from lymph node culture (fig. 17).



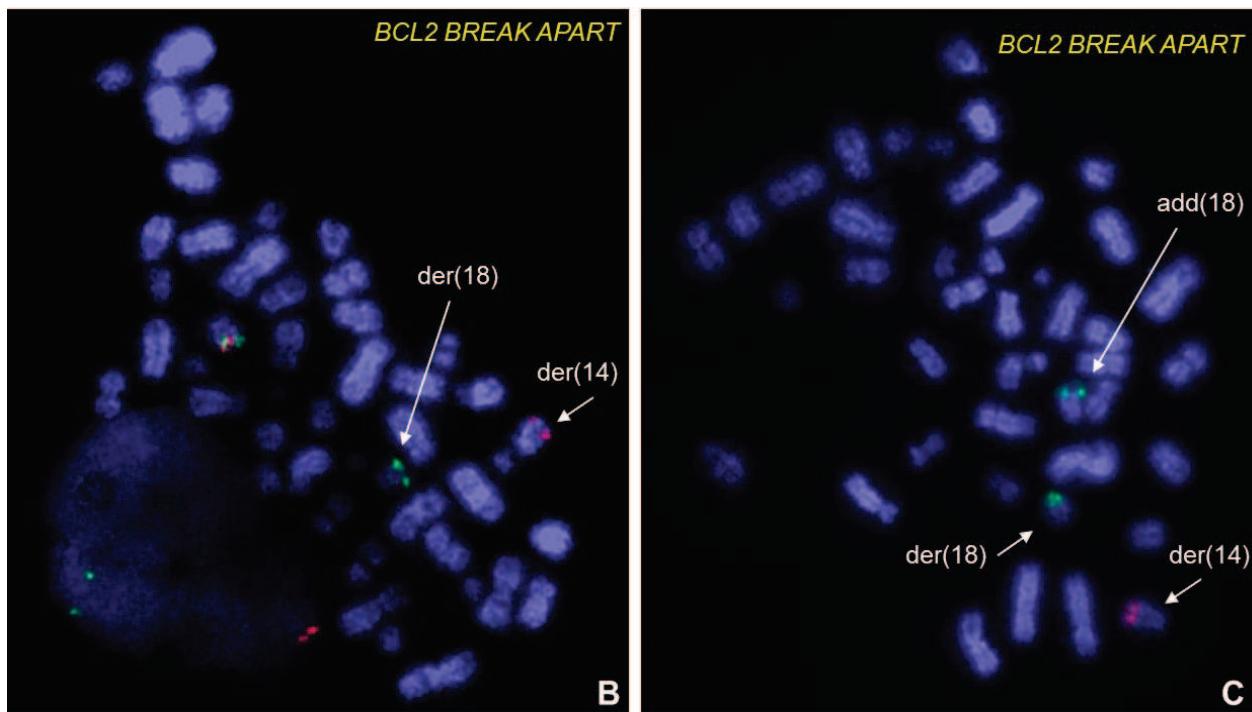


Figure 17: Sample n. 38. FISH on lymph node cultures. (A) MYC break apart probe showed a partial duplication of 5'end of gene on der(8)(q2?4). BCL2 break apart probe showed two abnormal patterns: (B) pattern 1Y1G1R corresponding to the t(14;18)(q32;q21); (C) pattern 2G1R without any fusion signal corresponding to the presence of translocation associated with an additional chromosome anomaly on homologous chromosome 18, defined as add(18)(q2?4).

On 10 samples of lymph node biopsies, additional “shaked” and “fragment” culture were set up to proceed to cell block preparation. The first and the last sections were coloured with ematoxilin-eosin (EE) (fig.18) to evaluate cells quality and quantity together with the pathologist. Based on IHC profile at the histological diagnosis, we performed IHC analysis also on cell block lymph node sections. All results are shown in Table 5, the antibodies used for the IHC profile are also listed. Referring to cell quality, the cell block preparations made up from the two kinds of culture are perfectly overlapping. IHC analysis from both types of culture are quite the same, and similar to those carried out on histological sections (fig.19) even if IHC on “shaked” culture showed a higher percentage of positive nuclei in 3 samples (cases n. 30-31, 40) and a lower percentage of positive nuclei in 1 sample (case n. 33). Samples n. 35 and 36 were very poor: the first one was a reactive lymphadenitis and in the second one the initial material was really scarce. IHC was not performed on both these cases.

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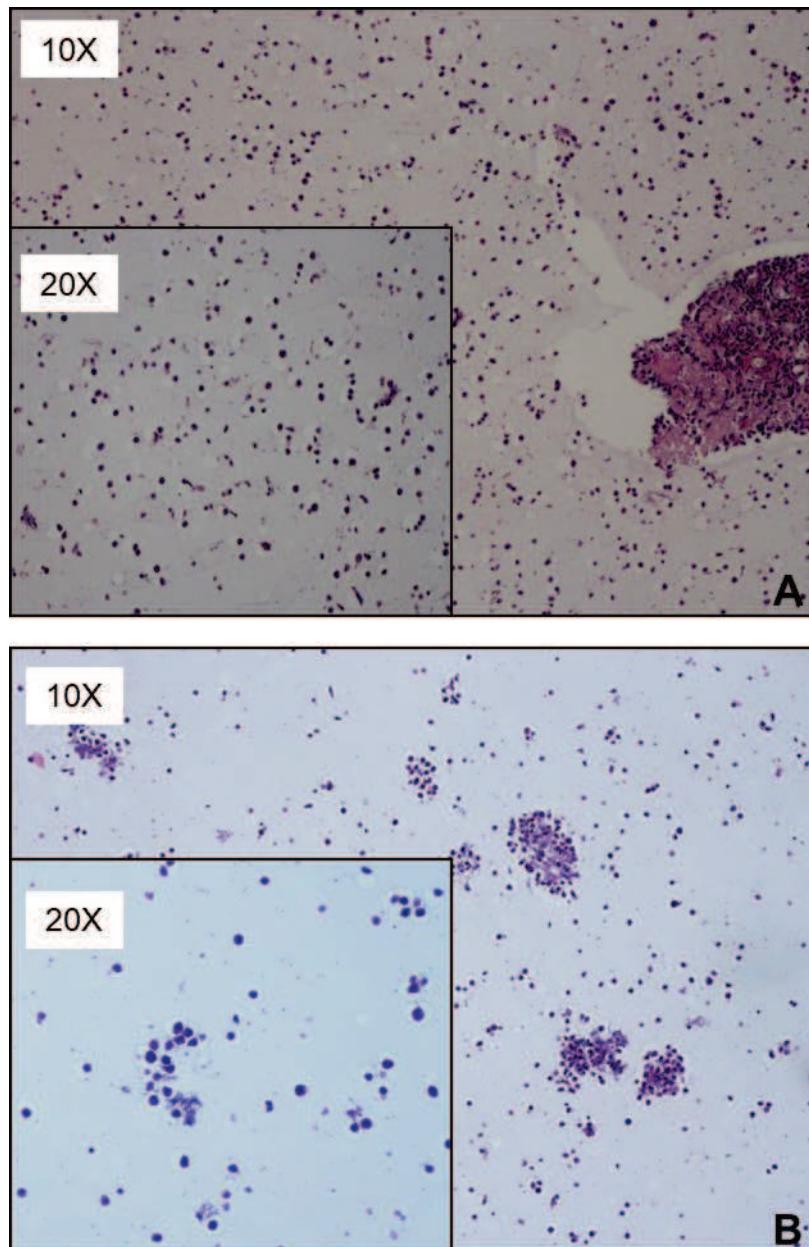


Figure 18: Ematossilin-eosin (EE) of cell-blocks of (A) fragment and (B) shaked lymph node cultures of sample n.38 (10X and 20X magnification).

Results

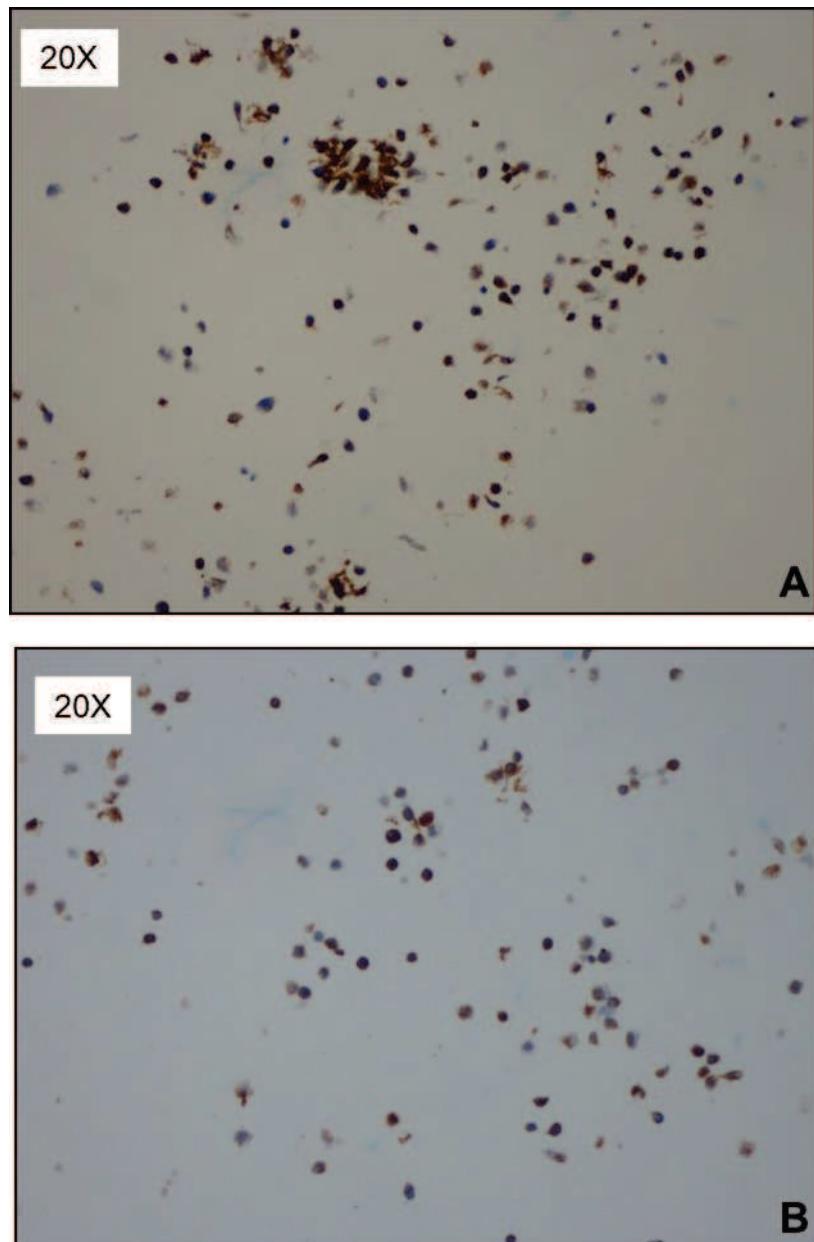


Figure 19: *Bcl2 IHC analysis performed on cell-blocks of (A) fragment and (B) shaked lymph node cultures of sample n.38 (20X magnification).*

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*Table 5: Comparison between cell block obtained from shaked and fragment cultures. IHC, immunohistochemical analysis. N.d. not done analysis. EBV, Epstein Barr virus; *due to poor quality of IHC analysis, the 41 sample evaluation was predominantly morphological and highlighted 70% of neoplastic cells.*

sample	cell block fragment culture quality	cell block shaked culture quality	IHC on fragment culture	IHC on shaked culture	IHC on histological section	histological diagnosis
30	good	good	bcl6+ 30%	bcl6+ 50%	CD20+, bcl2-, bcl6+, CD10+, CD5-, Myc 5-10%, CD30+/-, Ki67 80%	FL grade 3A
31	good	good	bcl6+ 30%	bcl6+ 35-40%	CD20+, Bcl2+, Bcl6+, CD10+, CD23+, CD5-, cyclin D1-, Ki67: 45-50%.	FL grade 2
33	good	good	bcl6+ 40-50%	bcl6+ 40%	CD20+, bcl2+, bcl6+, CD10+, CD5-, Ki67 20-35%	FL grade 1-2
34	poor	poor	cd30+ 30% fascina+	cd30+30% fascina+	CD30+, fascin+, PAX5+/-, CD20+/-, CD79a-, CD15-, CD45-, EBV-.	HL
35	very poor	very poor	n.d.	n.d.	n.d.	reactive lymphadenitis
36	very poor	very poor	n.d.	n.d.	CD20+, CD10+, bcl6+, bcl2+ (negative in 3A areas), CD5-, Ki67 from 15 to 50-60%	FL grade 2 with areas 3A
37	poor	poor	cd30+ rare cells	cd30+ rare cells	PAX5+, CD30+, CD15+, CD20-, EBV/EBER-.	HL
38	good	good	cd10+ and bcl2+90%	cd10+ and bcl2+90%	CD20+, CD79a+, bcl2+, bcl6+, CD10+, MUM1-, CD30- (<1%), CD5-, Cyclin D1-, c-Myc 35%, Ki67 40-70%	DLBCL/DH
40	good	good	cd10+ and bcl2+ 50% bcl6+/-	cd10+ and bcl2+ 65-70% bcl6+/-	CD20+, CD10+, bcl6+/-, bcl2+, CD23, Cyclin D1-, CD5-, Ki67 15-40%	FL grade 2, with focal areas 3A
41*	scarce	scarce	bcl2+ and cd10+	bcl2+ and cd10+	CD20+, bcl2+, CD10+, bcl6+ (feeble), CD23-, CD5-, Myc- (10%), Ki67 30%	FL grade 2

Discussion and conclusion

The 2017 update of WHO classification of tumours of Haematopoietic and Lymphoid tissues highlights the clinical relevance of the characterization of molecular features of these disorders. Among these, especially for lymphomas a precise genetic characterization is required for a correct histopathological definition and a more appropriate therapeutic treatment.

Cytogenetic analysis of lymphomas has given many relevant results and over time has allowed the identification of specific chromosome alterations according to different types of tumours (Mitelman Database of chromosome aberrations in cancer). In particular, NHL show a large number of recurrent chromosome rearrangements, especially translocations involving IGH gene like t(11;14)(q13;q32) in MCL, t(14;18)(q32;q21) in FL, t(8;14)(q24;q32) in BL, as discussed above. However, in the case of a suspected lymphoma, cytogenetic analysis performed on lymph node biopsy specimens is not widely used as diagnostic test. In literature few data are reported regarding the protocols used and when it is useful to perform cytogenetic analysis for diagnostic and prognostic purposes (Cook, 2004; Wang, 2017; Nardi, 2015). To date, no data are available about the use of mitogen DSP30 in lymph node cultures in order to optimize lymph node metaphases quality and allow the detection of all possible significant chromosomal anomalies.

In this thesis we suggest the developing of an algorithm specific for B-cell lymphomas diagnosis, which are the majority: this will allow to improve and make more specific and sensitive the detection of cytogenetic abnormalities. For this purpose, in relation to the aims listed above, we have used:

- conventional cytogenetic studies, that have been widely proposed to be discarded in favour of molecular analysis techniques. To reach better results for cytogenetic investigations, we used two different lymph node culture techniques “shaked” and “fragment” in order to verify which is more suitable. In the meantime, we tested the addition of mitogen DSP30 with IL2, already used for CLL samples (Decker et al., 2006). As summarized in table 3, we did not find any evident difference between “shaked” and “fragment” cultures, both in term of quality and quantity of metaphases/nuclei and in term of chromosome anomalies found (Table 4), although the “shaked” culture preparation is more user-friendly. The results also confirm that only B-cells were in mitosis (under the effect of DSP30 with IL2) and could be analysed even in the fragment culture.

Discussion and conclusion

According to the obtained cytogenetic results (Table 4), we can assess that the use of lymph node cultures added with DSP30 + IL-2, has proved useful to a better analysis also for B-cell lymphomas, besides CLL. In particular, among FL cases, a complete cytogenetic analysis was more informative than FISH performed on interphase nuclei (both on lymph node cytogenetic preparation and FFPE sections), especially in the more aggressive forms with grade ranging from 2 to 3.

In some cases, we have obtained karyotypes from lymph nodes with a more precise definition of whole genomic alterations. For example, in the case n. 38 (Table 4), FISH performed on FFPE lymph node sections showed an unusual FISH pattern for MYC and BCL2 genes rearrangement. In the same analysis performed on lymph node, conventional and molecular cytogenetic techniques highlighted that the atypical hybridization pattern of MYC corresponded to a partial duplication involving 5'-end MYC. In addition, BCL2 hybridization pattern clarified the presence of two cell clones detected on FFPE sections: both had the t(14;18)(q32;q21) but one of them had also an alteration of the homologous chromosome 18, defined as add(18)(q2?1). The presence of both MYC and BCL2 rearrangements modified the histological diagnosis from DLBCL to Double Hit Lymphoma (DHL).

- molecular cytogenetic studies by FISH with specific probes for the most common B-cell lymphoma anomalies involving well identified genes (e. g. MYC and/or BCL2) and with probes for anomalies not common, but that may be related to the type of lymphoma under evaluation (e. g. TP53 gene). As described in Table 4, only few cases showed a great difference between lymph node karyotype and FISH results (samples 19, 26, 27).

All these results highlighted the importance of a complete cytogenetic analysis (karyotype and FISH) to better define the neoplastic clones.

In addition, IHC analysis performed on selected samples of lymph node cultures have shown that the addition of DSP30+IL2 to the two types of culture does not modify the immunophenotype of the tumour, as evidenced at the definitive histological analysis (Table 5). The correlation between the immunophenotype and type of lymphoma was so confirmed.

In conclusion, we believe that the results here reported demonstrated that our protocol is perfectly suitable to improve the cytogenetic analysis of lymphomas, including the

Discussion and conclusion

performing of different types of cell culture, the use of an appropriate mitogen, and the analysis made by conventional and molecular cytogenetics. It is worthy of note that the results are appropriately compared with histological, and IHC data, giving relevant informations. Our methodological approach, moreover, allows to store cells suspension, bioptical and histological samples, making possible to perform molecular genetic investigations even at a later time.

In synthesis, the take home messages are:

- do not forget conventional cytogenetics,
- "shaked" culture has to be preferred when the lymph node sample is scarce,
- add the mitogen DSP30 + IL2 to the lymph node cultures when there is a suspicion of a B-cell lymphoma.

All the genetic characterization data of the lymphomas are a precious tool for a better diagnosis, prognosis, therapy, and they also impact on the patients' outcome.

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