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The role of molecular biology in the diagnosis of lymphoid neoplasms

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1. ABSTRACT

In recent years, DNA-arrays, gene expression profiling and next-generation sequencing have elucidated the high complexity of genomic alterations occurring in lymphoid malignancies. These studies have also contributed to the identification of new diagnostic and prognostic biomarkers, which may represent possible targets for new therapeutic approaches. Such recent advances have significantly expanded the application of molecular tests to routine diagnostic hematopathology. It is thus conceivable that next-generation assays will soon flank traditional clonality tests and chromosomal translocation assays in the diagnostic work-up of difficult cases. This review is focused on the application of molecular biology techniques in the study of lymphoid tumors. Both conventional and next-generation tests will be addressed, with particular attention to their application to clinical practice.

2. INTRODUCTION

The pathological diagnosis of hematological diseases can be considered as a logical-deductive process, based on the integration of multiple clinical, morphological, immunophenotypical and molecular parameters. The first step is represented by the morphological evaluation of tissue samples, which still represents the crucial starting point for any diagnostic work-up (1). Histological and cytological analyses are however associated with a degree of uncertainty and subjectivity, that can be usually overcome by adequate phenotypical tests (i.e. immunohistochemistry and/or flow cytometry) When even such assays provide inconclusive results, molecular biology investigation can prove crucial to make the correct diagnosis (2).

It is currently estimated that about 10-15% of hematopathological diagnoses relies on clonality tests to

Table 1. Recurrent Chromosomal Translocations in non-Hodgkin lymphomas

Translocation	Fusion Gene	Comment
t(14;18)(q32;q21)	BCL2/IgH@	Found in 85% of FL; also present in some primary DLBCL and in double hit lymphomas.
t(11;14)(q13;q32)	CCND1/IgH@	Characteristic of MCL; also detectable in a subset of MM
t(8;14)(q24;q32)	c-Myc/IgH@	t(8;14) is typical of BL, double hit lymphomas and subsets of DLBCL and PTLD; (2;8) and t(8;22) occur in a significantly lower percentage of cases.
t(2;8)(p12;q24)	c-Myc/IgK@	
t(8;22)(q24;q11)	c-Myc/IgL@	
t(11;18)(q21;q31)	AIP2/MALT1	Characteristic of a subset of extra-nodal MZL;gastric translocated cases do not respond to Helicobacter pylori eradication.
t(11;14)(q32;q21)	MALT1/IgH@	
t(1;14)(q22;q32)	BCL10/IgH@	
t(3;v)(q27;v)	BCL6/v	Observed in some DLBCL, double hit lymphomas, FL and MZL.
t(2;5)(p23;q35)	ALK/NPM	Typical of ALK-positive ALCL; t(2;5) and t(1;2) account for more than 95% of cases; other less frequent translocations involving ALK gene have been reported.

Abbreviations: ALCL= Anaplastic Large Cell Lymphoma; BL= Burkitt Lymphoma; DLBCL= Diffuse Large B-Cell Lymphoma; FL= Follicular Lymphoma; MCL= Mantle Cell Lymphoma; MZL= Marginal Zone Lymphoma; PTLD= post-transplant lymphoproliferative disorder.

ascertain the true nature of a lymphoid infiltrate (3-4). While, in the past, clonality was mainly assessed upon morphological and immunohistochemical grounds, precise and accurate molecular biology techniques have been recently developed to evaluate the presence of chromosomal translocations and antigen receptor gene rearrangements (5).

In recent years, the development of relatively cheap molecular assays, such as polymerase chain reaction (PCR), has represented a turning point in the field of diagnostic hematopathology: many genetic studies, previously performed only in research laboratories, have in fact become available also for routine diagnostic practice, thus improving the reliability of immunohistological diagnoses (6).

The subsequent advent of so called next-generation sequencing assays has deeply contributed to increase our knowledge of hematological malignancies. The better biological characterization of several entities has allowed the identification of new diagnostic and prognostic biomarkers, some of which have been proposed (and are actually used) as targets for new tailored therapies.

The present review is focused on the application of molecular biology techniques in the study of lymphoid tumors. Both conventional and next-generation sequencing tests will be discussed, with particular attention to their clinical applications.

3. MOLECULAR BIOLOGY IN ROUTINE DIAGNOSTIC PRACTICE

Neoplastic diseases can be regarded as uncontrolled proliferations of cells derived from a single common precursor. As such, the neoplastic populations represent clonal processes, characterized by cells sharing identical DNA sequences.

The vast majority of lymphoid malignancies bear clonal rearrangements of either immunoglobulin (*IG*) or T-cell receptor (*TCR*) genes and/or specific chromosomal aberrations, whose detection proves very useful to assess or confirm the neoplastic nature of the disease (Table 1)

In the next paragraphs, the clinical application of such assays will be briefly analyzed. The technical aspects of such techniques and their possible drawbacks will also be addressed.

3.1. Chromosomal aberrations

3.1.1. Lymphomas associated with translocation t(14;18)(q32;q21)

The t(14;18)(q32;q21) is detected in more than 85% of Follicular Lymphomas (FL), in 20% of primary Diffuse Large B-Cell Lymphomas (DLBCL) and in the majority of the so called "double hit" lymphomas (DHL) (7-9). The t(14;18) juxtaposes the *BCL2* gene to the *IgH* locus, leading to the constitutive over-transcription and over-expression of the Bcl2 protein. Although the anti-apoptotic activity of *BCL2* plays an important role in lymphomagenesis, several studies indicate that the t(14;18) is not *per se* sufficient to induce FL. The t(14;18) is thus considered to facilitate rather than directly cause malignant lymphoid transformation (9-11).

The breakpoints in the *BCL2* gene are mainly grouped within three regions: (i) the *major breakpoint region* (MBR), (ii) the *minor cluster region* (MCR) and (iii) the *intermediate cluster region* (ICR) though other possible *variants* are recognized. The MBR is involved in the majority of cases, whereas the MCR and ICR account for most of the remaining ones (12-15). The most effective methods for the detection of t(14;18) at diagnosis are represented by Southern blot and fluorescence *in situ* hybridization (FISH). These tests manage to cover very large gene portions, being thus capable to detect most *BCL2* rearrangements. PCR has a relatively low sensibility in this field, due to the intrinsic characteristics of the available primers, which are not designed to recognize all the possible *BCL2* breakpoint regions (7, 16).

The detection of t(14;18) may significantly contribute to the diagnosis of FL, as it allows the distinction from cases of atypical florid follicular hyperplasia with follicle lysis, which feature a misleading Bcl2 staining, but are consistently negative for the translocation. The molecular assessment of t(14;18) is also helpful in determining post-therapy minimal residual disease (MRD): the high sensitivity of molecular assays may in fact allow to detect very small amounts of

translocated cells, even in the complete absence of clinically evident residual disease. Of note, since translocation-positive B lymphocytes can occasionally be found in otherwise healthy patients (11), the mere demonstration of t(14;18)-positive B-cells in the peripheral blood or in bone marrow aspirates cannot be reliably assumed as indicative of disease persistence. In such cases, a clonal relationship between the original neoplastic population and the post-therapy translocation-positive B cells should be demonstrated by sequencing the translocated genes.

3.1.2. Lymphomas associated with translocation t(11;14)(q13;q32)

Despite the t(11;14)(q13;q32) is considered the genetic hallmark of mantle cell lymphoma (MCL), this translocation has been reported in a substantial fraction of plasma cell myelomas (PCM) (17-19).

In plasma cell neoplasms, the presence of chromosome 11 aberrations has been originally associated with a better prognosis (20). Subsequent studies have failed to confirm such association, indicating that t(11;14)-bearing PCMs probably represent a subset of plasma cell neoplasms with distinct immunological and immunophenotypic features, but with an outcome comparable to that of the non-translocated cases (21).

The t(11;14)(q13;q32) juxtaposes the *CCND1* gene (on chromosome 11q13) to the *IgH* locus (on chromosome 14q32), leading to the up-regulation of cyclin D1, a cell-cycle regulator. The latter, not expressed in normal B-lymphocytes, has been shown to confer a proliferative advantage at least in MCL cells (18).

Since *CCND1* breakpoints are variable and scattered over a very long stretch of DNA (*major translocation cluster)*, both Southern blot probes and PCR primers are largely ineffective in detecting *CCND1* rearrangements. Conversely, FISH analysis can document t(11;14) in almost 100% of MCLs (22-23). Nonetheless, the current availability of an anti-cyclin D1 monoclonal antibody for immunohistochemical use limits the application of molecular testing to few selected cases (24).

3.1.3. Lymphomas associated with BCL6 translocations

BCL6 is a zinc finger transcription factor that controls the expression of several genes involved in lymphocyte maturation and differentiation. It also exerts a fundamental role in the development of germinal centers by interacting with genes like *BLIMP1*, *IRF4*, *p21*, *p27* and *TP53* (25-26). In animal models, the deregulation of *BCL6* leads to the development of DLBCL-like lymphoproliferative disorders (27); in humans, *BCL6* translocations have been reported in 30-40% of DLBCL and in a subset of FL and marginal zone lymphomas (MZL) (28-29).

The *BCL6* locus is located on the short arm of chromosome 3 (3p27) and can be involved in several translocations with both immunoglobulin and non-immunoglobulin partners. The breaks on *BCL6* gene usually occur within a *major breakpoint region* (MBR) located in the 5'-portion of the gene (28). *BCL6* translocations can be detected by Southern blot (using probes that hybridize to the MBR) or by FISH analysis (30). In the latter instance, *BCL6* break-apart probes are strongly preferable over translocation-specific probes, given the high number of possible translocation partners.

Of note, *BCL6* deregulation is not limited to chromosomal translocations: aside from major genetic rearrangements, *BCL6* can indeed harbor several somatic mutations, that have been reported both in germinal center-derived lymphomas and in primary mediastinal DLBCL (31-32).

3.1.4. Lymphomas associated with MYC translocations

Translocations of *MYC* gene characterize a subset of high grade B-cell lymphomas with a particularly dismal prognosis. First described in Burkitt lymphoma (BL), *MYC* translocations have been later documented in DHLs, in 10-15% DLBCLs and in a subset of post-transplant lymphoproliferative disorders (9, 33-34).

The *MYC* gene is located on chromosome 8q24 and encodes a transcription factor involved in cell-cycle regulation and cell survival (35). *MYC* translocation partners are represented by the *IGH* locus on chromosome 14q32 or more rarely by the *IGK* or *IGL* loci on chromosomes 2q12 and 22q11, respectively. The translocations can be detected by Southern blot or FISH analysis. The latter provides the best results by using break-apart probes, that flank *MYC* breakpoints (36). The presence of any of such translocations leads to c-Myc protein over-expression, which, in turn, provides a proliferative advantage to neoplastic cells (extremely high mitotic activity and nuclear Ki67 proliferation index).

Of note, *MYC* translocations do not represent the only source of c-Myc protein over-expression and recent studies conducted on large series of DLBCLs clearly demonstrate that Myc-positive cases (irrespective of the presence of translocations) experience a shorter overall and progression-free survival, especially when Bcl2 protein is also over-expressed (33).

3.1.5. MALT lymphoma-associated translocations

Three main translocations have been reported in mucosa-associated lymphoid tissue (MALT) lymphomas. The t(11;18)(q21;q31) is by far the most frequent, being documented in 48% of gastric MALT lymphomas. Such translocation juxtaposes *API2* gene on chromosome 11q31 to *MALT1* gene on chromosome 18q21 (37). Despite *API2* promotes cell survival by inhibiting apoptosis, the major pathogenetic role seems to be played by *MALT1*, that activates the *NF-kB* pathway (38).

The NF-kB transcription factor is physiologically activated by several proinflammatory cytokines (IL-1, TNF-a) or pathogen-associated molecular patterns, via the recruitment of the IKK complex that induce the NF-kB cytoplasmic factor to translocate into the nucleus. Here it activates target genes involved in the development,

proliferation, and survival of both T- and B-cells. Its constitutive activation by *MALT1* can thus promote the proliferation of specific lymphoid clones, eventually leading to their neoplastic transformation (39).

The documentation of t(11;18)(q21;q31) has important clinical implications because translocation-positive MALT lymphomas do not indeed respond to *Helicobacter pylori* eradication (40). Despite this, their evolution rate to secondary large B cell lymphomas is significantly lower than that reported for un-translocated cases (40).

In addition to t(11;18)(q21;q31), *MALT1* gene can be involved in translocations encompassing the *IGH* locus as a partner: the resulting t(14;18)(q32;q21) has been documented in ocular and skin, but not gastric MALT lymphomas (41). Of note, t(11;18)(q31;q21) and t(14;18)(q32;q21) seem to be mutually exclusive and have never been documented in the same neoplasm.

The third most common translocation in MALT lymphomas is the t(1;14)(q22;q32), which juxtaposes the *BCL10* gene to the *IGH* locus. *BCL10* contributes to the activation of the NF-kB pathway by binding to MALT1 and inducing the activation of the I?B kinase complex (42).

MALT lymphoma-associated translocations can be assessed by both real-time PCR (RT-PCR) and FISH analysis, but their use in routine diagnostic hematopathology is still limited to very selected cases. In fact, despite MALT translocations should be theoretically assessed at diagnosis in each and every case, PCR and/or FISH analyses are usually performed only at re-evaluation, if the lymphoid infiltrate persists even after *Helicobacter pylori* eradication.

3.1.6. Translocations associated to ALK-positive Anaplastic Large Cell Lymphoma

Anaplastic large cell lymphoma (ALCL) is an aggressive CD30-positive T-cell lymphoma, further sub-classified in two distinct clinical and biological entities, upon ALK protein over-expression (43-44).

ALK-positive ALCL bears chromosomal translocations involving the *ALK* gene on chromosome 2p23: the most common translocation partner is the *nucleophosmin* (*NPM*) gene on chromosome 5q35 (more than 80% cases), but other partner genes have been reported on chromosomes 1, 2, 3, 17, 19, 22 and X (43, 45). The *ALK* gene product is a receptor tyrosine kinase that controls cell proliferation and survival by directly activating the *STAT3*, *AKT*, *MAPK* and sonic hedgehog pathways. Translocations lead to *ALK* constitutive activation, that is in turn responsible for the proliferation and survival of the neoplastic cells (43).

The detection of *ALK* translocations is of paramount importance to differentiate ALK-positive from ALK-negative ALCL ,since the latter features a significantly worse prognosis than ALCL-positive cases (46). Although molecular techniques can promptly identify these translocations, their use has been limited by the availability of polyclonal and monoclonal antibodies against ALK protein. The pattern and sub-cellular distribution of ALK immuno-staining, which normally stains only the cell nucleus, apparently correlates with the underlying chromosomal translocations (43). In particular, the classic t(2;5)(p23;q35) is associated with both nuclear and cytoplasmic staining, while most variant translocations display a diffuse cytoplasmic or membranous pattern. A unique granular cytoplasmic expression has been linked to the t(2;17)(p23;q11), which juxtaposes *ALK* to *clathrin heavy polypeptide* (*CLTC)*, a structural component of intra-cytoplasmic coated vesicles (47).

3.2. Antigen receptor genes

Compared to either chromosomal aberrations, fusion transcripts or breakpoint fusion regions, the determination of antigen receptor gene rearrangements represents a cheap and easily performable test to assess the clonal nature of a lymphoid proliferation.

Ig and TCR receptors are heterodimeric proteins with common structural and functional properties: surface Igs are heterodimers of heavy (*IGH*) and light (*IGK* or *IGL*) chains, whereas TCRs are composed of either a/ß or ?/d dimers. These components are transcribed by genes consisting of different segments (the variable (V), joining (J), constant (C) and diversity (D) regions), that are sequentially and randomly rearranged during lymphopoiesis. The resulting gene sequence will be then translated into a surface receptor on B and T lymphocytes (48). The random recombination of gene sequences, the junctional deletion/insertion of nucleotides and the imprecise DNA alignment allow to develop a virtually unlimited repertoire of unique receptors, capable to recognize foreign antigens (49). As a result, the chance for two different lymphocytes to bear the same antigen receptor is virtually negligible and the documentation of identical *Ig* or *TCR* rearrangements is indicative of a clonal lymphoid population.

3.2.1. PCR clonality tests

In the last decades, PCR has gradually replaced more expensive and time-consuming techniques for the assessment of lymphoid clonality in the routine diagnostic practice. The high sensibility and consistency of PCR tests has also extended the application of clonality tests to formalin-fixed paraffin-embedded tissue samples (generally characterized by little and/or poorly preserved DNA).

PCR clonality tests are based on the amplification of antigen receptor genes, whose subsequent migration on gel-electrophoresis provides useful information on the presence and nature of *IG* or *TCR* rearrangements.

A reactive lymphoid infiltrate is composed of hundreds of lymphocytes expressing different antigen receptors. The associated PCR pattern is characterized by multiple bands or by several peaks graphically defining a Gaussian curve at gene scanning (Figure 1A). This pattern

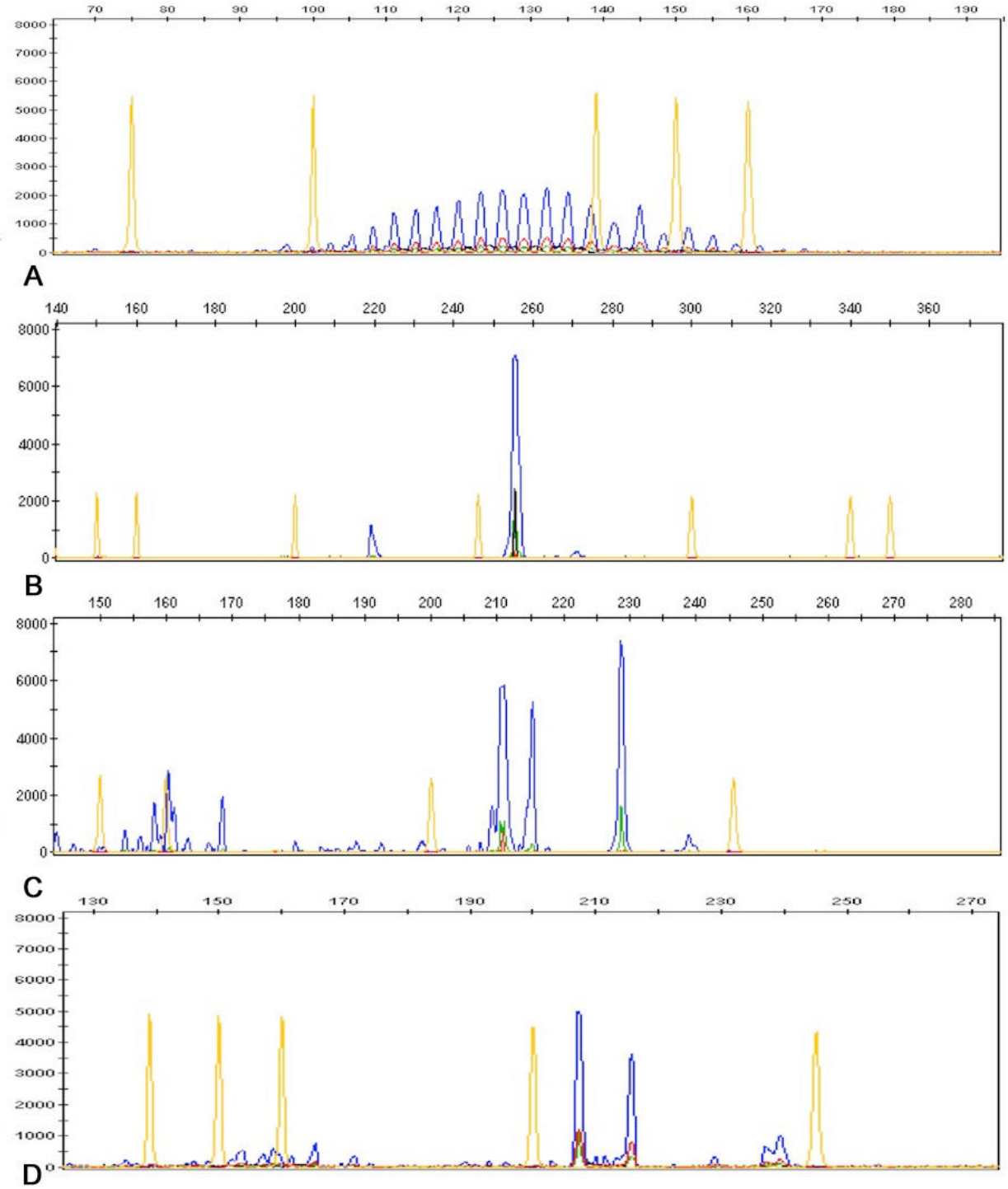

Figure 1. Patterns of PCR clonality tests for B- and T-cell neoplasms. A. The presence of multiple peaks graphically defining a Gaussian curve is considered the molecular hallmark of any reactive lymphoid infiltrate. This policlonal pattern reflects the amplification of several gene products characterized by variable molecular weights. B. The presence of only one sharp peak (monoclonal pattern) is highly suggestive of a lymphoid neoplasm. This pattern results from the amplification of an antigen receptor derived from genetically identical neoplastic cells. C-D. On occasion, intermediate patterns can be found: oligoclonal patterns (C) are defined as the presence of three to five peaks over a policlonal background, while biclonal/biallelic patterns (D) are characterized by the presence of just two sharply defined peaks. (Gel-electrophoresis and gene scan).

(i.e. polyclonal pattern) results form the amplification of several gene products with different molecular weights and constitutes the molecular evidence of the reactive nature of a lymphoid infiltrate (50).

Conversely, a neoplastic lymphoproliferative disorder is composed of cells with identical antigen receptors, that lead to one or very few sharply defined bands or peaks on gel electrophoresis (i.e. monoclonal pattern) (Figure 1B). Intermediate patterns are referred to as oligoclonal rearrangements (Figure 1C).

While evaluating monoclonal gene rearrangements, it is of pivotal importance to bear in mind that the presence of monoclonality does not directly indicate the presence of a neoplastic disease. In fact, several non-neoplastic lymphoid disorders (i.e. infectious mononucleosis, monoclonal lymphoid disease of uncertain significance and monoclonal gammopathy of uncertain significance) have been associated to monoclonal IgH and/or TCR rearrangements (5-6).

One of the most critical issue related to these tests is the choice of the adequate primers, capable to recognize and properly anneal to target DNA sequences. Any inconsistency in such fundamental step can in fact lead to false negative (or false positive) results. To overcome these potential pitfalls, international protocols have been recently proposed for PCR-based clonality tests (50).

For the assessment of B-cell clonality, the most commonly employed primers are targeted against the J and V regions of *IGH* gene (JH and VH). The shortness of the obtained amplicon (less than 150 base pairs) makes the test applicable also to archival paraffin-embedded tissues, which are often characterized by poorly preserved DNA and short DNA extracted fragments. The main limitation of this approach is its relatively low sensibility: this especially occurs in tumors with a high burden of somatic mutations which can affect the target sequences, thus impeding appropriate annealing of the primers (51-52). This drawback can be overcome by using additional primers for different *IGH* gene regions (D_H and J_H) and, preferably, for *IGK* and *IGL* genes (5, 53-55).

The assessment of T-cell clonality is based on similar assumptions. Since rearrangements between Va and Ja regions cover very large DNA segments, *TCRa* genes are not suitable for PCR testing. The same holds true for *TCRd* genes, which are located within the *TCRa* genes and are frequently deleted during their rearrangement (56-57). The structural simplicity of *TCR?* (absence of D regions) makes it more amenable for PCR testing than the *TCRß* genes. However, since the amplification of *TCRß* is slightly more informative than that of *TCR?*, these genomic sequences should be jointly investigated (53, 58).

As previously partially outlined, clonality tests may provide false positive or false negative results due to technical or biological pitfalls. In some instances, false negative results can be the consequence of poor DNA preservation: a DNA quality check (with control multiplex PCR) is thus always strongly advisable.

While interpreting PCR clonality assays, it has also to be bore in mind that lymphocytes undergo rearrangements on one or both alleles of the variable *IG* and *TCR* loci: the latter event may give rise to two (or more) clonal peaks, that represent biallellic rearrangements and not different clones (Figure 1D) (59-60).

In addition, the availability of small or inadequate biopsy samples may lead to the selective amplification of a limited number of lymphocytes, which results in a pseudo-clonal pattern easily mis-interpretable as a truly monoclonal proliferation. Likewise, oligo/mono-clonal peaks may be obtained from florid inflammatory lymphoid infiltrates as a result of the wider expansion of some sub-clones over others (61). In these cases a careful revaluation of the original immunohistology is recommended as well as the acquisition of complete clinical information. Since pseudo-clonal bands are usually not reproducible and tend to disappear (or to have different sizes) in the repeated molecular assays , it is also strongly advisable to perform duplicate PCR analyses (28).

In conclusion, PCR clonality tests are a powerful tool in the diagnostic work-up of lymphoid malignancies. Proper knowledge of their biological bases and of their technical limits may help to improve the reliability of such technique, although routine duplicate PCR analysis is recommended.

4. NOVEL MOLECULAR APPROACHES IN THE STUDY AND DIAGNOSIS OF LYMPHOID NEOPLASM

In the very last years, few fields of anatomic pathology have experienced so many and great progresses as hematopathology. New entities have been characterized and many old ones have been re-interpreted according to new more defined pathobiologic data. These advances have been sustained by an impressive development of molecular biology techniques, which have allowed to highlight the genetic mechanisms of lymphocyte physiology and pathophysiology. In particular, high throughput technologies have led to design microarray platforms for global DNA analysis and gene expression profiling (GEP) (62). Moreover, the development of next-generation sequencing has made it possible to extensively study the whole spectrum of genomic derangements occurring during lymphomagenesis (63). Though translation into clinical practice is not a straightforward process, several of such findings have led to important diagnostic, prognostic and therapeutic implications. The next paragraphs will address the major contributions of the three most updated molecular technologies (DNA microarrays, gene expression profiling and next-Generation sequencing) in the study of lymphoid neoplasms.

4.1. DNA Microarrays in the study of lymphoid neoplasms

The recent development of comparative genomic hybridization (CGH) and single-nucleotide polymorphism (SNP) arrays have provided important insights into the molecular biology of lymphoid neoplasms.

4.1.1. The role of CGH-arrays in the study of lymphoid neoplasms

The first DNA-arrays were based on the technology used for CGH studies. CGH allows the detection of DNA copy number variations in test probes (usually neoplastic tissue) by comparison with a reference DNA sample. In brief, the test and reference probes are labelled with different fluorophores, denaturated and hybridized to normal metaphase chromosomes or cloned DNA fragments (61). Depending on the DNA copy number present in the test and reference probe, different fluorescent signals are obtained for each chromosome segment: gain of genetic material will correspond to a stronger signal in the test sample compared to the reference probe, while loss of genetic sequences will be pinpointed by a weaker fluorescence of the test sample. If the DNA content of the test and reference probe is equal, a neutral signal will be observed (64).

In lymphoid malignancies, CGH arrays have reported a much higher burden of chromosomal alterations than the one previously observed by conventional cytogenetics. Nonetheless, the overall genomic profile of each lymphoma entity appears to be rather unique, so that minor (almost undetectable) differences may be responsible of a great biological and clinical variability (65-67). Among B-cell lymphomas, such features are well exemplified by MZL and DLBCL of the activated B-cell type (ABC DLBCL): despite both entities display gains in chromosome 3 and 18q, molecular events such as deletions of 6q, 9p and 19q are more specific of ABC-DLBCLs and may account for their aggressive clinical course (68). Similar considerations can be made for T-cell lymphomas: although several common genetic derangements have been described in ALK-positive and ALK-negative ALCL, gains of 17p and losses of 4q or 11q14 are more frequent in ALK-positive cases. On the other hand, gains of 1q and 6p21 have only been found in ALK-negative ALCL and may correlate with their poorer outcome (69).

The DNA-array technology has also identified previously unknown genes, which may represent new diagnostic and therapeutic targets for the treatment of specific entities. One of the major acquisitions in this field is the discovery of *MIR17HG* as the only codified gene within the 13q31 minimal amplified region of MCL (70-71). *MIR17HG* encodes a polycistronic miRNAs oncogene (miR-17-92), that has been recently related to the molecular pathogenesis of various high-grade lymphomas (72).

CGH-arrays can finally highlight chromosomal derangements which correlate with prognosis and outcome. Some of these alterations affect genes whose prognostic value is largely ascertained (e.g. inactivation of *TP53* on 17p), while others require further characterization (e.g. gains of chromosome 3 in aggressive DLBCL and MCL) (68,73).

4.1.2. The role of SNP-arrays in the study of lymphoid neoplasms

The molecular features of lymphoid neoplasms can be investigated also by means of single-nucleotide polymorphisms (SNP). The latter are defined as variants of single nucleotides within a specific DNA sequence. SNPs are located in both coding and non-coding regions and may variably affect cell biology and metabolism (74).

Despite most SNPs are biologically and clinically silent, polymorphisms occurring in coding genome regions may lead to missense or non-sense mutations, which eventually result in structural and functional changes of the coded proteins. Similarly, SNPs located in non-coding regions may impair gene splicing or alter the translation of messenger RNAs (74).

SNP-array studies are based on the hybridization of test DNA to oligonucleotide probes, corresponding to allelic variants of selected SNPs. The presence of hybridization signals from both allelic probes indicates genetic heterozygosity, while selective hybridization to only one SNP variant is consistent with homo- or hemi-zygosity (75). In recent years, variable SNP profiles have been associated with specific lymphoma entities.

The SNPs most commonly associated with non-Hodgkin lymphomas are located within genes that regulate cell-cycle and cell survival (e.g. FL and *rs7567444/rs3789068* SNP in *BCL2L11*; DLBCL and *rs1880030* SNP in *BCL7A*) (76). Other commonly reported SNPs affect genes that belong to the *TNF/NF-kB* pathways (e.g. *rs2844484* SNP in *LTA* gene; *rs4934436* SNP in *FAS* gene; *rs12211228* SNP in *IRF4* gene) (77). More recently, SNP-arrays have also disclosed a relationship between non-Hodgkin lymphomas and genes related to the innate immune response: variants of *MBP* (*rs8094402*) and *MASP2* (*rs12711521*) have been associated with a reduced risk of developing DLBCL, while specific SNPs of *DEFB126* (*rs6054706*) seem to directly correlate with the risk of developing FL (78).

4.2. Gene expression profiling (GEP) in the study of lymphoid neoplasms

The development of microarray technology has made it possible to study the RNA expression profiles of tumors. Gene expression profiling (GEP) technology is based on the hybridization of labeled RNAs to DNA probes, which have been previously immobilized on a solid surface. The signal obtained by fluorochrome-labeled RNAs is proportional to the quantity of the corresponding transcripts and provides an overall measure of gene expression within the tested tissues (79).

The major challenge of GEP technology resides in the huge amount of data which have to be analyzed by bioinformatic tools and subsequently validated by PCR or immunohistochemistry. The interpretation of GEP is mainly performed according to unsupervised or supervised analyses. While unsupervised analysis looks for the natural grouping of gene expression profiles on the base of molecular similarities/dissimilarities, supervised analysis is aimed to identify differences in the molecular profile of already known disease entities (80). In the study of lymphoid tumors, GEP analysis has greatly contributed to better characterize the pathobiology of several entities and to find out new diagnostic and therapeutic biomarkers.

4.2.1. GEP in the study of non-Hodgkin B-cell lymphomas

The role of GEP studies in refining the characterization of known lymphoid malignancies is well exemplified by DLBCL, not otherwise specified (DLBCL, NOS). In such tumor, GEP analysis has highlighted two different RNA expression signatures, corresponding to biologically and prognostically different subgroups: (i) germinal B cell-like DLBCL (GBC DLBCL), that expresses genes related to germinal center B-cells; and (ii) activated B cell-like DLBCL (ABC DLBCL), whose expression profile is similar to that of post-germinal center activated B-cells (81-82). ABC DLBCL are characterized by a more aggressive clinical course, that could be partially related to the constitutive expression of genes belonging to the *NF-kB* pathway (83).

The histogenetic sub-classification of DLBCL has gained an even greater prognostic importance after the advent of immunochemotherapy, since GCB DLBCLs feature a significantly better response to rituximab-based treatments compared to non-GCB DLBCLs (84).

Among small B-cell neoplasms, GEP analysis of chronic lymphocytic leukemia (B-CLL) has pointed out significant differences between mutated and un-mutated B-CLL subtypes, that nonetheless display a common B-cell origin from lymphocytes genetically close to post-germinal centre memory cells (85). RNA expression profiles have also indicated *ZAP70* as a relatively specific marker of un-mutated B-CLL, whose immunohistochemical expression is currently assessed in routine diagnostic practice as a valid surrogate of more expansive molecular analyses (86).

Regarding MCL, some cases lack cyclin D1 expression, as also confirmed by GEP studies (87); the latter have also reported *SOX11* up-regulation in most MCLs, irrespective of cyclin D1 status. As such, *SOX11* represents a sensible and specific biomarker of MCL, whose immunohistochemical assessment has become part of the diagnostic work-up in cyclin D1-negative challenging cases. In addition, *SOX11* down-regulation in MCL has been associated with a more favorable outcome (88).

In recent years, GEP analysis has also contributed to highlight the molecular biology of BL. Endemic, sporadic and immunodeficiency-associated cases are indeed characterized by striking similar RNA expression profiles, which significantly differ from those of other B-cell malignancies. These findings corroborate the hypothesis that the different subtypes of BL actually belong to a unique biological entity, characterized by the over-expression of *MYC*-related genes (89).

Finally, GEP studies have identified a subset of aggressive B-cell lymphomas whose molecular signature is intermediate between DLBCL and BL (90). In the current WHO Classification, such neoplasms are classified as a provisional entity and their true biological nature is still under debate. In fact, several data indicate that aggressive B-cell lymphomas with features intermediate between DLBCL and BL probably represent a heterogeneous group of aggressive lymphomas and not a single biological entity (90).

4.2.2. GEP in the study of non-Hodgkin T-cell lymphomas

In the setting of T-cell lymphomas, GEP analysis has recognized specific expression patterns for peripheral T-cell lymphomas, not otherwise specified (PTCL, NOS), angioimmunoblastic T-cell lymphoma (AITL) and ALCL (91-93). These studies have also demonstrated that PTCLs, NOS with a follicular T-helper phenotype are biologically and clinically distinct from classical AITLs, despite their common follicular helper T-cell derivation. The recent development of an accurate GEP-based molecular classifier may represent an additional tool in the diagnostic workup of nodal peripheral T-cell lymphomas (94).

RNA expression profiles have also demonstrated clear-cut differences among ALK-negative ALCL, ALK-positive ALCL and CD30-positive PTCL, NOS. Such a biological diversity bears significant clinical implications, since CD30-positive PTCLs, NOS are associated with a much worse prognosis than both ALK-positive and ALK-negative ALCLs (94).

Finally, the application of GEP technology to the study of T-cell lymphomas has recently disclosed several previously unknown molecular pathways (e.g. aberrant tyrosine kinase signaling or altered histone deacetylase activity) which may represent new molecular targets for next-generation tailored therapies (95).

4.3. Next-generation sequencing in the study of lymphoid neoplasms

The recent introduction of next-generation sequencing (NGS) has further contributed to highlight the biological features of lymphoid tumors. A comprehensive characterization of specific genome sequences (e.g. antigen receptor genes) may indeed provide a high-resolution picture of lymphoid cells upon physiological and pathological conditions.

In recent years, the sequencing and comparison of tumor and constitutional DNA has allowed to detect different types of genetic derangements (point mutations, insertions and/or deletions, amplifications, translocations or other large structural alterations), that specifically affect neoplastic cells. Such molecular features represent the genetic basis of tumors and may help to define new diagnostic and therapeutic approaches for their treatment (63). NGS can be applied to either the whole genome or to selected DNA regions, such as the coding exons (exome sequencing) or the transcribed RNAs (transcriptome sequencing). While whole genome sequencing provides the most comprehensive information on tumor-associated mutations, exome and transcriptome only analyze regions known to play a key role in tumor development and maintenance.

NGS studies are based on the fragmentation of target DNA probes, with subsequent amplification and

sequencing of the obtained DNA fragments. The massive production of parallel sequences generates several reads for each genome position: the number of reads per stretch of DNA is referred to as "coverage". A high coverage improves the detection of point mutations and small insertions/deletions by filtering out the noise due to possible DNA contaminants. A number of reads above or below the mean coverage respectively indicates gains or losses of DNA material, whereas the mapping of variable reads in two distant regions stands for the presence of chromosomal translocations (63).

The study of somatic mutations in lymphoid neoplasms has disclosed a complex panorama, with different entities being characterized by remarkably variable mutagenic potential (low mutagenic potential: B-CLL; high mutagenic potential: PCM). The majority of mutations occurs at low frequencies, with only few of them being reported in a large number of cases. Important exceptions to this rule are represented by $BRAF^{V600E}$ and $MYD88^{L256P}$ mutations, that have been reported in virtually all cases of HCL and Waldenstrom's macroglobulinemia, respectively (96-97).

For each disease entity, mutations tend to cluster within genes linked to specific molecular pathways. Examples of such convergence can be found in PCM (mutations of genes involved in protein translation and chromatin remodeling) (98), in B-CLL (mutations of *NOTCH1* signaling, RNA splicing, inflammatory response and cell-cycle control) (99-100), and in splenic MZL (mutations in marginal zone-specific genes and in *NOTCH2* and *NF-kB* pathway) (101).

The convergence of different gene mutations into common molecular pathways is further emphasized by recent studies conducted on DLBCLs, which have reported frequent deregulations in chromatin remodeling, immune recognition and post-germinal center differentiation genes (102-103). In particular, recurrent mutations of *S1P1*, *GNA13* and *GNAI2* have been shown to converge on the regulation of B-cell homing and germinal center physiology (104).

With respect to T-cell lymphomas, NGS has greatly contributed to highlight the pathobiology of ALK-negative ALCL, by indicating the presence of recurrent t(6;7) chromosomal translocations and the frequent loss of *TP53* and *PRDM1* (105-106).

In the setting of AITL and PTCL, NOS with a follicular T-helper phenotype, NGS have reported recurrent *TET2* mutations, which have been shown to correlate with an adverse clinical outcome and a poor prognosis (107). Similar studies have also documented recurrent *DNMT3A*, *IDH2* and *RHOA* mutations in AITL and, less frequently, in PTCL, NOS (108-110**).** Of note, the vast majority of such mutations are associated with *TET2* derangements, which indicates a possible pathogenetic link for all these genetic events. Finally, among T/NK-cell tumors, NGS studies have recently expanded our understanding of large granular lymphocytic leukemia, by indicating the presence of recurrent *STAT3* mutations in about 40% of cases (111).

From a clinical perspective, NGS may further improve the sensitivity and efficacy of diagnostic clonality tests. In particular, specific NGS techniques have been recently developed, which allow direct sequencing of rearranged *IGH* and *TCR* genes by using primers targeted against each pair of V and J segment. Such techniques, aimed to directly amplify and sequence all possible gene rearrangements, are suitable to be further optimized by ensuring better coverage of all possible gene rearrangements (i.e. partial *IGHD-IGHJ* rearrangements or *IGK* locus rearrangements involving the kappa-deleting element) (112).

Although translation of NGS studies into clinical practice still represents a challenge, recent evidence indicates that wide genome sequencing may provide useful information also regarding the prognosis and therapy of lymphoid tumors. As example, *NOTCH1* mutations have been associated to a worse outcome in a subset of B-CLL and MCL (113-114). In such cases, the administration of *NOTCH1*-inhibitors could represent a valid therapeutic approach, but further studies are needed to test this hypothesis. The same holds true for the presence of activating *BRAF* mutations in 100% of HCL, which provides an additional diagnostic marker and a potential target for tailored therapies (96, 115).

Finally the discovery that DLBCLs harbor mutations in genes not previously considered as participating to their pathogenesis (e.g. histone/chromatin modification genes; chronic active B-cell-receptor and altered *MEF2B* signaling) has disclosed new target therapies for their treatment (103, 116-117).

5. CONCLUSIONS

In the last decades, the development of molecular biology techniques has greatly contributed to improve the diagnosis of lymphoid neoplasms and our understanding of lymphopoiesis and lymphomagenesis. Clonality tests are integrated into the routine diagnostic practice and molecular data are one of the crucial parameters for the diagnosis of particularly difficult cases.

On the other hand, the development of DNA microarrays, GEP and NGS technology have greatly contributed to better characterize several entities, also identifying new diagnostic and prognostic biomarkers. Different molecular techniques can be even matched together in order to provide the best prognostic information and to identify possible targets for future therapeutic approaches (118).

This impressive progress is attributable to several independent factors: (i) the existence of adequate *in vivo* and *in vitro* models; (ii) the peculiar biology of lymphoid tumors; (iii) the relatively user-friendly application of phenotypical and molecular tests to hematological diseases. A major contribution to the development of our knowledge has been also provided by the close collaboration between traditional methods and new techniques. In fact, the histological and immunohistochemical evaluation of lymphoid tumors still represents the cornerstone for the proper interpretation of both DNA-arrays, GEP and NGS

results (119). This integrative approach is the paradigm for any further research activity and constitutes the basis for any progress in our understanding of lymphoid tumors.

6. ACKNOWLEDGEMENT

The first two authors have equally contributed to this manuscript.

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Abbreviations: ABC: activated B cell-like; AITL: angioimmunoblastic T-cell lymphoma; ALCL: anaplastic large cell lymphoma; GBC DLBCL: germinal B cell-like DLBCL; B-CLL: B-cell chronic lymphocytic leukemia; BL: Burkitt lymphoma; CGH: comparative genome hybridization; DHL: double hit lymphoma; DLBCL: diffuse large B-cell lymphoma; FISH: fluorescence *in situ* hybridization; FL: follicular lymphoma; GEP: gene expression profiling; HCL: hairy cell leukemia; MALT: mucosa-associated lymphoid tissue; MCL: mantle cell lymphoma; MZL: marginal zone lymphoma; NGS: next-generation sequencing; PCM: plasma cell myeloma;PTCL,

NOS: peripheral T-cell lymphoma, not otherwise specified; PTLD: post-transplant lymphoproliferative disorder; SNP: single nucleotide polymorphism; TCR: T-cell receptor

Key Words: Non-Hodgkin Lymphoma, Molecular Biology, Clonality Test, DNA-arrays, Genome Expression Profiling, Next-Generation Sequencing, Review

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