Cytogenetic and molecular testing in lymphoma patients

Part 1: (Cyto)genetic methods, B- and T-cell clonalities and aberrations in mature B-cell lymphomas

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On behalf of the BHS Lymphoproliferative Disease Committee 2017:

SUMMARY
It is now well demonstrated that cytogenetic and molecular testing are valuable tools for the diagnostic, prognostication, and decision of treatment strategy in lymphoproliferative disorders. Here, we will give an overview of the genetic tests that represent current and future clinical assessment tools in the context of lymphoid malignancies. This review has been divided into two distinct but complementary parts. Part I will address the genetic aspects of low grade B-cell lymphomas and will very briefly describe the different technical methods that can be used in routine practice for the clinical management of lymphoid malignancies. Part II will cover aggressive B- and T/NK-cell lymphomas as well as Hodgkin lymphoma and will be published subsequently. (BELG J HEMATOL 2018;9(6):225-36)

INTRODUCTION
Cytogenetic and molecular testing plays a major role for the diagnostic and prognostic assessment in lymphomas as well as for selection of therapeutic strategies. According to the 2008 and the more recent 2017 revision of the World Health Organization (WHO) classification of lymphoid neoplasms, genetic markers are as important as clinical, morphologic and immunophenotypic features to define some unique entities.\textsuperscript{1} First, we will briefly describe the main characteristics of the different genetic tests (conventional and molecular karyotype, fluorescence in situ hybridisation (FISH), polymerase chain reaction (PCR), massive parallel sequencing (MPS), gene expression profile (GEP), circulating tumour DNA, B- and T-cell clonality tests). Afterwards, we will review the most common lymphoma subtypes and the indications for the different genetic tests that are currently used or could be potentially relevant in clinical practice in the near future, knowing that the field and the techniques are rapidly evolving.

In the different tables proposed throughout this review, we have classified the use of genetic markers (cytogenetic and molecular) in three categories according to evidence based medicine criteria:

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Keywords: Cytogenetics, mature B-cell lymphoma, molecular testing.
- Mandatory = the test has demonstrated its clinical utility and is needed for the clinical management of the patient (diagnosis, prognosis, predictive value and/or theranostics).
- Optional or recommended = although the test has demonstrated its clinical significance, it is not mandatory for diagnosis or choosing frontline treatment nowadays.
- Under development = the test need further clinical assessment to demonstrate its clinical significance and utility.

WHAT METHOD SHOULD BE USED IN ROUTINE PRACTICE?

All the mentioned methodologies are complementary and the role of each of them in the work up (diagnosis, prognosis, predictive value, theranostics) will depend on the genetic landscape that shapes each lymphomatous entity. However, some of these techniques are unequivocally part of the current technical panel used whereas the usefulness of other methods needs further evaluation and are not currently exploited despite the very promising results they can bring.

KARYOTYPE

Although conventional karyotype or metaphase conventional cytogenetics is sometimes considered as an “old fashion” technology, it still remains a very powerful tool because it offers a broad view of the tumoural genomic landscape, including balanced (translocations or inversions) with breakpoints involving specific loci and unbalanced (trisomies, monosomies, duplications, and deletions) chromosomal changes. This analysis allows the assessment of the degree of complexity of the genomic aberrations: in lymphoproliferative disorders, the karyotype is considered as ‘simple’ if there are less than three chromosomal aberrations or ‘complex’ if there are three or more chromosomal aberrations (each abnormality being counted as one alteration). Conventional karyotype is also useful to decipher genomic aberrations observed by FISH when this last method is used as first genetic test. The main limitations of karyotyping are i) the need for actively dividing cells (adequate fresh tissue and successful cell culture are essential prerequisites), ii) the limited resolution (of approximately 5-10 megabases (Mb)), and iii) the inability of identifying the exact nature of complex rearrangements including marker chromosomes.

FLUORESCENT IN SITU HYBRIDISATION (FISH)

FISH testing bridges the area between conventional cytogenetic analysis and molecular diagnostics by providing higher sensitivity and resolution (up to 50 kilobase (Kb) resolution). FISH can bypass the need of cell culture and is a very robust method for detecting well documented specific chromosomal abnormalities on non-dividing cells (so-called ‘interphase cells’) provided by smears, cytopsins, or paraffin-embedded tissues. Commercial FISH probes are usually several hundred Kb in length and will thus cover all possible scattered breakpoints located within and in the vicinity of a gene involved in a chromosomal translocation. This means that the rearrangement of a specific gene will be identified in a single FISH experiment whereas several independent PCR tests will be needed to cover all existing breakpoints. This explains why FISH will be more sensitive than PCR in detecting BCL1, BCL2 or BCL6 gene rearrangements, as an example. Even more, and unlike reverse transcriptase (RT)-PCR, FISH offers the advantage of detecting in a single experiment all recurrent rearrangements of a gene involved in translocations with multiple different gene partners (‘break-apart strategy’). It is thus the method of choice to detect rearrangement of genes such as BCL6 or ALK, for example, without the need to know the gene partner. Beside chromosomal translocations, FISH can also detect specific chromosomal aberrations such as del(7q) or trisomies 3 and 18 in marginal zone lymphoma (MZL).

Karyotype and FISH remain currently the basic complementary technologies to identify lymphoma-associated chromosomal aberrations.

ARRAY-BASED KARYOTYPING (OR “MOLECULAR KARYOTYPING”)

In this method, tumour DNA is hybridised to high-density arrays of probes distributed over the entire genome. The probes type used in high density arrays are bacterial artificial chromosome (BAC) clones or oligonucleotides. The fluorescent signal can be interpreted in comparison to a control DNA co-hybridised with the tumour DNA [so called ‘Comparative Genomic Hybridisation (CGH) arrays’]. Oligonucleotides including single nucleotide polymorphisms (SNP) do not need a control DNA [so called ‘SNP arrays’]. Array-based karyotyping has the great advantage of circumventing the need for tumour cell metaphases and to detect DNA copy number changes - amplified (or duplicated) and deleted genomic regions - in a much higher resolution than conventional karyotype or even FISH. It can be used to assess the degree of genomic complexity in aggressive B-cell lymphomas and it is the best technique to detect the interstitial 11q aberration in Burkitt-like lymphoma with 11q aberration (BLL 11q), as an example. Array-based karyotyping remains nevertheless an expensive technology and should be used cautiously. Moreover, its application is limited by the inability to detect balanced chromosomal translocations and the failure to reveal DNA copy number variations when the sample analysed contains less than 20-30% of tumoral cells.
PCR is the method of choice to detect a single specific gene mutation in lymphomas. The typical example is the detection of the MYD88 gene mutation in lymphoplasmocytic lymphoma (LPL) or a search for B/T-cell clonality. PCR should not be the upfront method for identification of specific chromosomal translocations because of the significant incidence of false negative results (see chapter IV). If possible, a chromosomal translocation should first be identified by FISH and, in case of a positive result, be picked up by PCR in order to get a specific biomarker for the molecular follow up (minimal residual disease (MRD)). However, the timing, the indication and the PCR methods (Long-distance (LD)PCR, allele-specific oligonucleotide (ASO) PCR, etc.) to be used for MRD assessment are still under evaluation in the frame of international clinical trials.2

MASSIVE PARALLEL SEQUENCING (MPS)
(ALSO NAMED NEXT GENERATION SEQUENCING (NGS))

Various examples are given in literature demonstrating that it is a set of genomic alterations (‘mutational landscape’) rather than one single abnormality which will allow making an accurate diagnosis or determining the outcome and/or the therapeutic sensitivity of the tumour. MPS is the current methodology that allows performing a comprehensive study of a tumour genome in one single test and this method is now replacing the Sanger sequencing for the identification of gene mutations. Although the data given by MPS are very promising in terms of diagnosis, prognosis and theranostics, they nevertheless require further validation before being used in daily clinical practice in the context of lymphoma.

GENE EXPRESSION PROFILE (GEP)
(MICROARRAYS)

GEP studies have undoubtedly contributed to the establishment of a better diagnostic classification, not only for diffuse large B-cell lymphoma (DLBCL) but also for T-cell lymphoma (TCL). In TCL, GEP has also identified molecular subgroups with oncogenic pathways that are specific to different subgroups and could serve as diagnostic tool and/or be considered as potential targets to novel and more efficient therapies. These methods are nevertheless not suitable for routine use and have been replaced by other techniques easier to apply in daily routine such as immunohistochemistry (IHC).

CIRCULATING TUMOUR DNA (LIQUID BIOPSY)

Monitoring of circulating tumour DNA by MPS or PCR for absolute quantification such as digital droplet PCR (ddPCR) seems to hold tremendous promise as tools for response-adapted therapy and for MRD studies. Nevertheless, it needs further evaluation in clinical trials before introducing the liquid biopsy in clinical practice.

B- AND T-CELL CLONALITY TEST

In daily practice, B- and T-cell clonality tests (Immunoglobulin (Ig) and/or T-cell receptor (TCR) gene rearrangements) are not required for the diagnostic work up of every lymphoma case. The use of these clonality tests should be limited to samples with a diagnostic difficulty:
- suspicion of lymphoma clinically despite reactive morphological features.
- and/or inconclusive diagnosis despite of extensive morphological and immunophenotypic analysis.
- B-cell versus T-cell lineage orientation in case of a morphologically mature lymphoid proliferation with uninterpretable lymphoid markers by immunophenotypic analysis.
- Doubt on the clonal origin of a lymphoid disorder during follow up (relapse of the initial lymphoma versus an unrelated lymphoma).

Also, the clonality tests could be repeated at a distant relapse because of the risk of loss of the primary monoclonal profile due to a possible sub-clonal evolution. Of course, it should be performed only if it will change further decisions.

Ig/TCR gene rearrangements occur in the earliest stages of lymphoid differentiation and thus are present in almost all immature and mature lymphoid cells. They nevertheless occur sequentially in a hierarchical order with IG heavy chains (H) genes being first rearranged followed by rearrangement of IG kappa (K) and IG lambda (λ) genes during B-cell development. IGH gene rearrangements thus are most widely used in order to cover the broadest spectrum of B-cell proliferative disease, followed by IγK and IGL.3,4 During T-cell ontogenesis, TCR delta (δ) gene rearrangement occurs first in early thymocytes, followed by TCR gamma (γ) gene rearrangement and then, in most thymocytes, followed by TCR beta (β) and subsequent TCR alpha (α) rearrangements. However, the TCRα rearrangement leads to the deletion of the TCRδ gene because this locus is located within the TCRα gene. This explains why virtually all the αβ TCR and γδ TCR expressing T-cell lymphomas will have at least a rearranged TCRγ gene. Therefore, TCRγ gene is most widely used, followed by TCRβ and then TCRα gene for T-cell clonality.4,5 In practice, most molecular laboratories use commercial assays allowing to recognize as many different rearrangements as possible among the broad spectrum of B- and T-cell lymphoproliferative disorders. The EuroClonality (BIOMED-2) consortium has proposed an assay which is now commercially available as IdeiClone clonality assay (Invivoscribe technologies Inc.,
Clinicians must be aware of several limitations and pitfalls of molecular IGH and TCR analysis in order to adequately interpret the results provided by the molecular laboratory (the reliability of the results depending also on the quality of the sample received):

- **False-negative results** are mainly caused by two factors.
  - First, the primers used in PCR-based clonality test do not cover all IGH and TCR rearrangements generated in lymphoproliferative disorders.
  - Second, physiologic phenomenon arising in mature B-cells (somatic hypermutation and isotype class switching in rearranged IG gene) can hamper the proper annealing of PCR primers. Therefore, false-negative results are more likely to be encountered in mature B-cell lymphomas of germinal centre or post-germinal centre origin such as follicular lymphomas (FL), MZL and DLBCL. In TCL, monoclonality will be detected in nearly 100% of cases (regardless of whether they express T-cell antigens) when using the combined TCRγ and TCRβ targets, except for anaplastic large cell lymphoma (ALCL) where no TCRγ and TCRβ rearrangements will be observed in 20-25% of cases and in some cases of natural killer (NK) cell lymphomas.

- **False-positive results** can be produced especially with TCRγ as PCR target and even if technologies of good resolution such as gene scanning are used. Also, samples containing few lymphocytes (as observed in case of slight inflammation) may generate a B-or T-cell pseudo-monoclonal pattern. It is nevertheless most likely that the use of MPS for clonality tests will, in most cases, overcome these issues. Lastly, one must be aware that expanded T-cell clones are frequently detected in the blood of normal elderly subjects. Therefore, a clonal TCR rearrangement profile observed in the peripheral blood sample of a patient with suspected lymphoma should be interpreted with caution (and, if possible, compared with the B- or T-cell monoclonal profile detected in a morphologically infiltrated sample before claiming blood involvement).

- **Conventional PCR-based clonality tests cannot afford an accurate and sensitive detection** as the primers used are not specific to the malignant clone related -IGH or TCR rearrangement. These tests have a detection limit of 1%-10%, depending on the background of non-neoplastic B- and T-cells present in the sample analysed. They are thus certainly not adequate for a sensitive follow up of the minimal residual disease which should be assessed using molecular tools such as IG/TCR ASO-PCR. Although relevant and sensitive for the MRD, IG/TCR ASO-PCR is a laborious and time-consuming methodology that needs a specific development for each patient and is therefore not suitable in routine molecular laboratories. It may be that new technologies such as high-throughput sequencing, offering high sensitivity and of easier applicability than IG/TCR ASO-PCR, will be used in the future for the detection of small B/T-cell clones.

- **Clonality is not equivalent to malignancy.** Some lymphoproliferative disease (monoclonal gammopathy of undetermined significance (MGUS), Monoclonal B-cell lymphocytosis (MBL), etc.) and non-neoplastic lymphoproliferations (viral or bacterial infections, autoimmune diseases and immunodeficiencies) may display monoclonality because they can harbour several antigen-specific sub clones or reduced diversity of B- or T-cell repertoire.

- **IG and TCR gene rearrangements are not markers for lineage.** Cross lineage IG/TCR gene rearrangements are not uncommon in immature T-or B-cell malignancies (i.e. acute lymphoblastic lymphoma (ALL)). TCR gene rearrangements occur in 10-20% of B-cell malignancies and IG gene rearrangements occur in 5-10% of T-cell malignancies. Although this cross lineage phenomenon is much less observed in mature B- and T-cell lymphomas, individual IG and TCR gene rearrangements cannot be formally used as markers for B/T-lineage assignment, but the complete IG/TCR-gene rearrangement pattern of a lymphoid malignancy might support lineage assignment.

In conclusion, these Ig/TCR clonality tests should be limited to the samples with a diagnostic difficulty, and the results should always be interpreted in the context of morphological, immunophenotypical, and clinical features of a patient.

**CYTOGENETIC AND MOLECULAR MARKERS IN LOW GRADE LYMPHOMA**

**FOLLICULAR LYMPHOMA (FL)**

The translocation t(14;18)[IGH/BCL2] and the rare variant translocations t(2;18)[IGK/BCL2] and t(18;22)[IGL/BCL2] are encountered in up to 90% of FL. This so-called ‘BCL2 rearrangement’ (leading to a BCL2 overexpression) is essentially observed in histological grades 1, 2, and 3A FL. FL negative for BCL2 rearrangement more frequently belong to grade3B category and express late germinal centre markers. However, most of them (70-85%) express the BCL2 protein. The absence of BCL2 rearrangement does not seem to have an impact on prognosis.
Karyotype analysis is very powerful in detecting the t(14;18) and variant translocations. However, these chromosomal abnormalities will be much easily detected by FISH with the use of a BCL2 break-apart or fusion probe. The FISH methodology is therefore the golden standard tool to identify all 18q21/BCL2 rearrangements (whatever the partner gene) and to distinguish IGH-BCL2 from IGH-MALT1 gene rearrangements (both leading to a t(14;18)(q24;q21) translocation); the genomic PCR is less sensitive as it will be positive in only 70% of FISH positive cases. If FISH analysis gives a positive result, genomic PCR can then be tested on the tumour DNA sample and, when positive, be used as a marker for MRD in the future.

Several variants of FL such as paediatric FL, primary intestinal FL and extra nodal FL (usually of the skin) typically lack the t(14;18) translocation and are usually BCL2 negative on IHC. The paediatric-type FL has not to be confused with the new provisional entity in the 2017 WHO classification, namely the IRF4-positive large B-cell lymphoma. An IRF4 gene rearrangement must be suspected in rare cases of large B-cell lymphoma (diffuse, follicular or follicular and diffuse growth pattern) observed in children and young adults and with predominantly involvement of Waldeyer ring and head and neck nodes. This gene aberration defines a disease with favourable prognosis.

BCL6/3q26 rearrangement is found in 5-15% of FL and in 25% of FL negative for BCL2 rearrangement especially in testicular FL, seen mainly in young boys. A 1p36 deletion (encompassing the TNFRSF14 gene) is observed in rare but distinct cases of diffuse-appearing FL without BCL2 rearrangement and presenting as a large but localized inguinal mass. IRF4, BCL6 and 1p36 aberrations can be detected by FISH. Aside from the identification of a t(14;18)/BCL2+ translocation, conventional karyotyping or array-based CGH might be useful in detecting additional chromosomal abnormalities which are found in 90% of FL [such as 1p36, 9p21 or 17p13(TP53) deletions]. The number of additional aberrations increases with histological grade and transformation. The mutational landscape in FL is starting to be better understood, but the clinical impact of this molecular profile remains to be determined. Mutations affecting genes involved in epigenetic regulation are extremely common in FL (including KMT2D/MLL2, CREBBP, EZH2, ARID1A, MEF2B and EP300) and could represent key therapeutic targets in the future. Deregulations of genes involved in cell cycle regulation and DNA damage responses (e.g. CDKN2A/p16, TP53) or in the NF-κB pathway (CARD11, TNFAIP3/A20, MYD88) are linked to the FL transformation. A new prognostic model called ‘m7-FLIPI’ integrates mutation status of seven of the above-mentioned genes (EZH2, ARID1A, MEF2B, EP300, FOXO1, CREBBP, and CARD11) with clinical risk parameters (namely, the “follicular lymphoma international prognostic index” (FLIPI)). This prognostic index would be able to identify patients at high risk for early failure of first-line chemo-immunotherapy (CIT) and death. Nevertheless, this model requires further validation before being used in daily routine.

A diagnosis of “in situ” follicular neoplasm is given when atypical lymphoid B-cells display a BCL2 bright overexpression due to a t(14;18) translocation in some germinal centres of lymphoid follicles. Those cells harbour few or no additional alterations in contrast to overt FL and the progression of these “in situ” lesions to frank FL is very low. However, the coexistence of a simultaneous disseminated FL (~ 20% of cases) and/or the association with another subtype of low grade B-cell lymphoma justify further clinical monitoring. IGH/BCL2-positive circulating blood cells can be detected in otherwise healthy individuals and the frequency of these so-called “FL-like cells” increases with age and exposure to pesticides. The malignant potential of those cells is very low. Their identification is nevertheless a potential disruptive factor for MRD monitoring in FL patients.

MANTLE CELL LYMPHOMA (MCL)
The t(11;14)[IGH/CCND1] translocation is observed in more than 95% of cases of MCL. It is considered as the primary genetic event and is very specific for MCL. This CyclinD1 gene rearrangement can be easily identified by FISH which is more sensitive than PCR since the last methodology will detect the rearrangement in only +/- 60% of FISH positive cases. Diagnosis of MCL is made mostly by CCND1 protein overexpression on IHC. A CCND2 gene rearrangement will be detected in about half of the cyclin D1 negative MCL and a CCND3 gene rearrangement has been described in a single cyclin D1 negative MCL case.

Nuclear overexpression of the neural transcription factor SOX11 is observed in >90% of MCL cases and, in particular, in all MCL cyclin D1 negative cases. Although SOX11 overexpression can be observed in some cases of other mature B-cell neoplasms such as splenic marginal zone B-cell lymphoma (SMZL), SOX11 remains a very good diagnostic marker for MCL. A reliable diagnosis of MCL can thus be done in almost all MCL cases by IHC (CCND1 and/or SOX11 overexpression). FISH will be used if immuno-morphological profiles are doubtful. FISH analysis can also be performed in order to get a marker for follow up. If a CCND1 gene rearrangement is detected, genomic PCR can therefore be tested on the tumour DNA sample and, when positive, be used as a marker for MRD.

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The nodal forms of MCL carry mostly unmutated IGVH genes whereas a small subset of MCL carrying mutated IGVH genes are known as leukemic non-nodal MCL with an indolent course.22,23 Although the lack of SOX11 expression has been associated with the indolent form of MCL, the prognostic value of this biomarker is still a matter of debate. Therefore, at this time, SOX11 expression is not considered as a prognostic biomarker in MCL.24

Aside from the identification of a t(11;14)/IGH-CCND1+ translocation, cytogenetic profile may reflect the outcome of the disease as a complex karyotype will most likely be in keeping with a poor outcome whereas a karyotype with a single t(11;14) is usually associated with the indolent form of MCL.25 Among additional chromosome abnormalities, losses of 17p13 and 9p21 chromosomal regions (TP53 deletion/mutation and P16 deletion, respectively) are closely related to the more aggressive form of MCL (blastoid MCL). Cytogenetic tools (such as karyotype, FISH or, copy number-arrays) thus might be useful in identifying MCL with a more aggressive course.

The mutational profile observed in MCL is made of various mostly non specific somatic mutations affecting many different genes involved in cell cycle (TP53, ATM, CCND1, being the most frequent), anti-apoptotic genes (such as BIRC3, TLR2), chromatin modifiers (such as WHSC1, MLL2, MEF2B) and the NOTCH pathway (NOTCH1, NOTCH2).26 Mutations in both NOTCH genes are associated with a dismal outcome and offer potential therapeutic targets.26 Those NGS data still need further validation before being used outside a research context although the specific search for TP53 disruption starts to be a current clinical indication in (blastoid) MCL.15,27

MARGINAL ZONE B-CELL LYMPHOMA (MZL)

Nodal marginal Zone B-cell Lymphoma (NMZL) and Splenic Marginal MZL (SMZL) exhibit different cytogenetic profiles compared to extra nodal MZL of Mucosa-associated Lymphoid Tissue (MALT). NMZL and SMZL are associated with numerical and structural abnormalities whereas MALT will rather exhibit chromosomal translocations. All these aberrations are detectable by karyotype and/or FISH. NMZL displays most often numerical abnormalities (trisomy 3/3q, 6p, 7q, 12q and 18/18q) and del(6)(q23-24) but no specific cytogenetic aberrations have been really identified so far.30 Nevertheless, the co-occurrence of trisomy 3 and 18 (observed in out of 20% of cases) is rather specific to a MZL and allow distinguishing this entity from other mature B-cells lymphomas. None of these chromosomal abnormalities seems to have an adverse prognostic impact, and the genes targeted have not been yet identified. Lastly, most of the cases show a mutated IGHV status.

MALT lymphomas may exhibit structural or numerical chromosomal abnormalities (6q23 deletion or trisomy 3 and 18 being observed in 20-30 % of the cases) but they are essentially characterised by four main recurrent chromosomal translocations that demonstrate a site-specificity in terms of their incidence: t(11;18)(q21;q21), t(14;18)(q32;q21), t(1;14) (p22;q32) and t(3;14)(p14.1;q32) (Table 1).29 The t(11;18)/

### TABLE 1. Four main recurrent chromosomal translocations observed in MALT lymphomas.

<table>
<thead>
<tr>
<th>Translocation</th>
<th>Product</th>
<th>% of cases</th>
<th>Main localizations</th>
</tr>
</thead>
<tbody>
<tr>
<td>t(11;18)(q21;q21)</td>
<td>BIRC3-MALT1 fusion protein</td>
<td>15-40%</td>
<td>Stomach, lung, intestine</td>
</tr>
<tr>
<td>t(14;18)(q32;q21)</td>
<td>Overexpression of MALT1</td>
<td>~20%</td>
<td>Ocular adnexa, salivary glands, skin, lung, liver</td>
</tr>
<tr>
<td>t(1;14)(p22;q32)</td>
<td>Overexpression of BCL10</td>
<td>~5%</td>
<td>Intestine, lung</td>
</tr>
<tr>
<td>t(3;14)(p14.1;q32)</td>
<td>Overexpression of FOXP1</td>
<td>&lt; 5%</td>
<td>Thyroid, ocular adnexa, skin</td>
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</tbody>
</table>

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BIRC3-MALT1 translocation is more often observed in *H pylori* negative forms of gastric MALT lymphoma (60 vs 15%). When present in *H pylori* positive cases, it is associated with a lack of response to anti-*H pylori* therapy in nearly 100% of cases.24 The t(3;14) translocation in MALT lymphoma has been associated with a risk of transformation to high-grade tumors.29

SMZL shows a characteristic 7q32 chromosomal deletion, observed in approximately 30-40% of cases.30 Although several genes such as the POT1, SHH or IRF5 have been proposed as putative candidate genes, no molecular target subjacent to the 7q deletion has been currently identified with certainty.31 A small number of SMZL carry a recurrent t(2;7)(p12;q21) which activates the CDK6 gene through juxtaposition with the IGK locus.

NGS studies have demonstrated that constitutive activation of the nuclear factor-κB (NF-κB) signalling pathway (through activating of inactivating mutations of MALT1, BCL10, IKKKB, TNFAIP3, MAP3K14, BIRC3 or CARD11 genes) appears to be a major driver for the development of MALT lymphoma, NMZL and SMZL and could therefore represent a key therapeutic target in the future.30,32-34 NOTCH2 mutations are relatively specific for both SMZL and NMZL (~20-40%) among mature B-cell tumors.30 They thus represent a good biomarker for non-MALT MZL. Mutations of the PTPRD gene seem to be observed most exclusively in NMZL among other mature B-cells neoplasms (including other MZL).30 They may represent a genetic biomarker with positive predictive value for NMZL. NOTCH2 and KLF2 mutations (10-40% of cases) are associated with poor survival and transformation to aggressive lymphoma.30 They represent promising prognostic biomarkers. Lastly, a MYD88 mutation can be observed in rare cases of MZL with some plasmacytic differentiation but those lymphomas remain histologically and phenotypically distinguishable from classical LPL.

HAIRY CELL LEUKAEMIA (HCL)

Hairy cell leukaemia can be, although rarely, confused with ‘HCL-like diseases’ such as SMZL, HCL variant (HCL-v) and splenic diffuse red pulp small B-cell lymphoma (SDRPL). The distinction is clinically relevant because the treatment for HCL will be different from HCL-like disorders. The BRAFV600E mutation is observed in >97% of HCL cases and is very specific for this entity among B-cell chronic lymphoproliferative disorders.35 Also, the identification of this mutation opens new therapeutic opportunities for targeted treatment using BRAF inhibitors. It can be detected in bone marrow or peripheral blood samples but sensitive PCR-based techniques such allele-specific oligonucleotide (ASO)-PCR are usually required because the HCL cells clone can be minimal.35

HCL-v does not exhibit a BRAFV600E mutation and can display a MAP2K2 gene mutation in 50% of cases.36 In contrast with other types of small B-cell lymphomas, SDRPL shows a CCND3 expression in the majority of cases (sometimes associated with a missense mutation in this CCND3 gene).37

CHRONIC LYMPHOCYTIC LEUKAEMIA (CLL) AND MONOCLONAL B-CELL LYMPHOCYTOSIS (MBL)

Chronic Lymphocytic Leukaemia (CLL)/ Small lymphocytic lymphoma (SLL)

CLL/SLL are both characterised by recurrent genomic imbalances observed in approximately 80% of cases and detectable by conventional karyotype, FISH, copy-number arrays (SNP-CGH arrays) or Multiplex Ligation-dependent Probe Amplification (MLPA) analysis. None are specific to CLL/SLL. Five prognostic categories have been identified already in 2000 by simply using FISH. The best outcome was seen for patients with a sole 13q14 deletion, followed by those without any FISH aberration or a trisomy 12, and a bad outcome for patients with a 11q or 17p deletion.38

A deletion of the long arm of chromosome 13, involving band 13q14, represents the single most common alteration (~55%), usually heterozygous but sometimes homozygous. The minimum common deleted region (type 1) included two miRNA, miR-16-1 and miR15a which favour apoptosis by negatively regulating the BCL2 gene expression, and the DLEU2 (‘deleted in lymphocytic leukaemia’) gene whose function is not well known although it is thought that it could inhibit the cell cycle progression.39 Although the sole 13q deletion was historically associated with a favourable course, CLL with a 13q deletion in a high proportion of cells and/or with a large sized 13q deletion (namely, the type II deletion that includes also the retinoblastoma (RBI) gene) seems not to retain this good prognosis.39 Trisomy 12/12q13 (11-25%) is frequently associated with atypical morphological and/or immunophenotypic features. The prognostic relevance of +12 remains a matter of debate.40 It is still considered as intermediate. The genes involved in the pathogenesis of CLL with trisomy 12 have not been identified yet.

A deletion of the long arm of chromosome 11 (6-20%) frequently encompasses bands 11q22-23. It typically confers bulky lymphadenopathies, rapid progression and reduced overall survival, although the prognosis associated with del(11q) has improved in recent years with the use of chemoimmunotherapy (CIT) (combined rituximab-fludarabine-endoxan regimens).41 The deletion usually involves the ATM (ataxia-telangiectasia-mutated) tumour suppressor gene which
TABLE 2. Relevant genetic biomarkers for the clinical management of mature B-cell lymphomas.

<table>
<thead>
<tr>
<th>Lymphoma entities</th>
<th>Mandatory</th>
<th>Optional or recommended</th>
<th>Under development</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL</td>
<td>If suspicion of paediatric FL: BCL2/18q21 R (1,2)</td>
<td>BCL2/18q21 R (1,2)</td>
<td>Mutational landscape (6)</td>
</tr>
<tr>
<td></td>
<td>If BCL2 not rearranged: → BCL6/3q27 R (1,2)</td>
<td></td>
<td>“m7-FLIPI” (EZH2, ARID1A, MEF2B, EP300, FOXO1, CREBBP, and CARD11) + BCL2, KMT2D/MLL2, CREBBP, MEF2B, EZH2, EP300, TNFRS14, MAP2K1, EPH17, BCL6, TNAIP3, CDKN2A, TP53, etc.</td>
</tr>
<tr>
<td></td>
<td>Degree of complexity of the genomic aberrations (1,3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Transformed FL, lymphoblastic features: → MYC/8q24 R (1,2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>If case of diffuse-appearing FL without BCL2 R: → del(1p36) (1,2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>If suspicion of IRF4 rearranged Large B-cell lymphoma: → IRF4/6p25.2 R (1,2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Degree of complexity of the genomic aberrations (1,3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCL</td>
<td>CCND1/11q13 R (1,2)</td>
<td>CCND1/11q13 R (1,2)</td>
<td>Mutational landscape (6)</td>
</tr>
<tr>
<td></td>
<td>Degree of complexity of the genomic aberrations (1,3)</td>
<td></td>
<td>(NOTCH1/2, MLL2, TP53, BIRC3, etc.)</td>
</tr>
<tr>
<td></td>
<td>If CCND1 not rearranged: → CCND2/2p13 R (1,2,3,4)</td>
<td></td>
<td>IGVH mutational status (2,9)</td>
</tr>
<tr>
<td></td>
<td>If blastoid MCL: → MYC/8q24 R (1,2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>→ TP53 disruption (2,3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MZL</td>
<td>If nodal MZL: → Trisomy 3/3q, 18/18q (1,2)</td>
<td>Trisomy 3/3q, 18/18q or t(2;7)/CDK6 R (1,2)</td>
<td>Mutational landscape (6)</td>
</tr>
<tr>
<td></td>
<td>If splenic MZL: → del(7q32), Trisomy 3/3q, 18/18q or t(2;7)/CDK6 R (1,2)</td>
<td>Trisomy 3/3q, 18/18q or t(2;7)/CDK6 R (1,2)</td>
<td>(NOTCH1/2, MLL2, TP53, BIRC3, etc.)</td>
</tr>
<tr>
<td></td>
<td>If MALT lymphoma: → MALT1/18q21 R (1,2,4)</td>
<td>MALT1/18q21 R (1,2,4)</td>
<td>IGVH mutational status (2,9)</td>
</tr>
<tr>
<td></td>
<td>→ BCL10/1p22 (1,2)</td>
<td>BCL10/1p22 (1,2)</td>
<td></td>
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<tr>
<td></td>
<td>→ FOXP1/3p14.1 (1,2)</td>
<td>FOXP1/3p14.1 (1,2)</td>
<td></td>
</tr>
<tr>
<td>LPL/WM</td>
<td>MYD88 mutation (1)</td>
<td>MYD88/CXCR4 co-mutation status (6)</td>
<td>Mutational landscape (6)</td>
</tr>
<tr>
<td></td>
<td>del(1q) (1,2,3)</td>
<td>del(1q) (1,2,3)</td>
<td>(CXCR4, ARID1A, BTK, CARD11, TP53, CD79A/B, PLCγ2, etc.)</td>
</tr>
<tr>
<td></td>
<td>del(17p13) / TP53 mutation (1,2,3)</td>
<td>del(17p13) / TP53 mutation (1,2,3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trisomy 4</td>
<td>Trisomy 4</td>
<td></td>
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<tr>
<td>HCL</td>
<td>BRAF V600E (4)</td>
<td>→ MAP2K1 mutation (6)</td>
<td>Mutational landscape (6)</td>
</tr>
<tr>
<td></td>
<td>If suspicion of HCL:</td>
<td></td>
<td>(ATM, NOTCH1, SF3B1, BIRC3, POT1, MYD88)</td>
</tr>
<tr>
<td>CLL</td>
<td>del(17p13) (1,2,3,4)</td>
<td>ATM/11q22-23, +12, del(13)(q14), del(6)(q21) (1,2,3,4)</td>
<td>Mutational landscape (6)</td>
</tr>
<tr>
<td></td>
<td>IGHV mutational status (2,9)</td>
<td>BCL3/19q13 (1,2,4)</td>
<td>(ATM, NOTCH1, SF3B1, BIRC3, POT1, MYD88)</td>
</tr>
<tr>
<td></td>
<td>If no 17p13 deletion, TP53 mutation (1,2,3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBL</td>
<td>If “atypical CLL or non CLL type phenotype”: → CCND1/11q13 R (1,2)</td>
<td>→ del(7q), t(12;7) (1,2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>If suspicion of HCL:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-/T-cell clonality</td>
<td>in case of diagnostic difficulties</td>
<td></td>
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</tbody>
</table>

(1) classical karyotype, (2) fluorescent in situ hybridization (FISH), (3) molecular karyotyping (based on the CGH-array or the SNP-array approach), (4) conventional PCR, (5) MPS, (6) MLPA. * these aberrations are rare to very rare, and their search can be replaced by a SOX11 immunohistochemistry staining. ** a search for a t(11;18) should be performed in gastric MALT lymphoma when there is a lack of response to anti-H pylori therapy. *** to perform before initiating a therapy and at relapse.
encodes a protein involved in DNA damage repair and acting upstream to the TP53 gene pathway. Mutation of the remaining ATM allele (“biallelic inactivation”) is detected in about 30% of del(11q) cases and appears to be a major determinant of chemo-refractoriness in CLL.\(^{41}\) Lastly, the del(11q) can also involve the BIRC3 gene located close to the ATM locus which negatively regulates the non-canonical NF-κB pathway. Aside from deletion, inactivating mutations of BIRC3 are observed in about 5% of CLL cases. They seem to be associated with increased resistance to chemotherapy and are mutually exclusive to TP53 inactivation.\(^{42}\) The 17p deletion accounts for ~7% of CLL patients and leads to the loss of the TP53 tumour suppressor gene. Most of the cases (~90%) show inactivation of both TP53 copies through deletion of one allele and inactivating mutation of the second one or, more rarely, inactivating mutations of both alleles (+/- 5%).\(^{40}\) The TP53 inactivation is usually associated with higher genetic complexity (complex karyotype) and displays a dismal prognosis with a rapid progression and short survival. It is also predictive of refractoriness to standard chemotherapy or CIT and justify the use of the novel oral therapies, such as irutinib, idelalisib or venetoclax.\(^{45}\) The TP53 mutation rate increases with time, with an incidence being +/- 4% at diagnosis, 10-12% at disease progression (first-line treatment) and up to 40% in case of refractory CLL.\(^{43}\) Due to these observations, the European Research Initiative on CLL (ERIC) recommends TP53 mutational screening (exon 4-9) for all patients before the start of any therapy, in order to select TP53-independent therapy, where appropriate.\(^{43}\) The screening has to be performed with the use of deep sequencing methodologies such as MPS and not by classic Sanger sequencing (limit of detection, 15-20% allele frequency) since patients harbouring small TP53 mutated sub clones (less than 1% allele frequency) will show the same unfavourable outcome and poor response to treatment as those of patients carrying a TP53 aberration present in a major clone.\(^{44}\) In other words, the clinical impact of TP53 alterations in CLL will be the same irrespective of the size of the mutated cell populations. This could be explained by the fact that an even minor TP53 mutated sub clone will become the predominant population at time of relapse due to chemo refractoriness. However, in a small proportion of cases, a TP53 mutated sub clone can remain indolent during follow-up. Therefore, it is recommended a closer monitoring of asymptomatic patients with a TP53 mutation but without treatment indication (i.e. every three months). Several studies have shown that a complex karyotype (≥ 3 chromosomal aberrations) as observed in ~20% of CLL patients, is associated with an adverse outcome, even in the absence of TP53/ATM deletions and is a strong predictor of refractoriness to conventional CIT.\(^{46}\) These observations imply that a genetic work up of CLL should not be limited to FISH analysis only and that conventional chromosome banding analysis (karyotype) is still useful. Reciprocal chromosomal translocations are detected in ~20% of cases.\(^{48}\) Few of them are recurrent and involve the IG loci in CLL (4-9%). The prognosis varies according to the gene partner. The t(14;19)(q32;q13)/IGH-BCL3 and the translocations involving the MYC locus (8q24) are associated with an adverse prognosis while the t(14;18)(q32;q21)/IGH-BCL2 does not seem to alter the disease evolution. Akin to TP53, the IGHV (immunoglobulin heavy chain variable genes) mutational status is one of the major prognostic biomarkers in CLL, allowing to divide the disease into two main clinical subsets with significantly different behaviors.\(^{47}\) CLL cases with unmutated IGHV (defined as ≥ 98% germ-line sequence homology) are characterised by enhanced B-cell activation (BCR signalling) and a poor prognosis while mutated IGHV CLL cases (<98% germ-line sequence homology) are less likely to proliferate in response to BCR signalling (anergy) and are associated with a favourable outcome. The survival of patients with unmutated IGHV gene is significantly shorter irrespective of the disease stage although those patients tend to present initially with a more advanced disease stage. It is noteworthy that ‘apparently mutated’ CLL cases showing a IGHV3-21 usage (more precisely CLL with immunoglobulin heavy chains encoded by mutated IGHV3-21 and immunoglobulin light chains encoded by unmutated IGLV3-21) will have an outcome similar to unmutated IGHV CLL (3% of all CLL cases).\(^{48}\) Like cytogenetic abnormalities that show evolution during the course of the disease, the IGVH status also can change over time. IGVH mutational status represents a reliable predictive biomarker for identifying patients that may benefit the most from CIT with FCR.\(^{47}\) Lastly, the recent characterisation of the genomic landscape of CLL has revealed recurrent mutated genes; the most common being SF3B1 (21%), ATM (15%), NOTCH1 (6%), BIRC3 (4%), POT1 and MYD88 (3-5%), in addition to TP53.\(^{49,40}\) The mutated profile varies during the evolution of the disease with the TP53, BIRC3, NOTCH1 and SF3B1 mutation rate being higher at relapse. Although most of these mutated genes are associated with adverse outcome (apart for MYD88), detection of mutations other than TP53 is not currently included in routine clinical practice as it is not known that specific therapeutic interventions can change the dismal prognosis of these aberrations. Monoclonal B-cell lymphocytosis (MBL) In roughly 75% of cases, MBL will share the immunopheno-
type of CLL (‘CLL-type MB’). In other cases, MBL will carry an immunophenotypic profile different from CLL and will be categorised as ‘atypical CLL MBL’ (CD5+, CD20 bright and/or lacking CD23 expression) and ‘CD5 negative (non-CLL) MBL’.15 In atypical CLL MBL, a FISH for t(11;14) must be performed to rule out MCL. The same recommendation must be applied to CD5 negative MBL knowing that the leukemic, small variant, MCL with indolent behaviour can be sometimes CD5 negative.

In non CLL-type MBL, the presence of a CD5-/CD10+ profile should prompt the investigators also to search for cytogenetic aberrations associated with non-MALT MZL, in particular, a 7q deletion and t(2;7) translocation.15 An isochromosome 17q and a translocation involving the 14q32 chromosomal region (IGH locus) are also related to CD5 negative MBL and non-MALT MZL cases.

If the cytogenetic investigations are positive, a more intensive diagnostic work-up including bone marrow biopsy and CT scans could be performed. If no MCL or MZL related cytogenetic aberrations have been detected, the probability of progressing to an overt lymphoma is low and the monoclonal B-cell expansion could be transient and self-limiting.15

LYMPHOPLASMOCYTIC LYMPHOMA (LPL) AND WALDENSTRÖM MACROGLOBULINEMIA (WM)

LPL with peripheral IgM paraprotein or WM display recurring activating somatic mutations including MYD88 (95% to 97%), CXCR4 (30% to 40%), ARID1A (17%), and CD79A/CD79B (8% to 15%).19 CXCR4 mutation is observed almost
The MYD88 and CXCR4 mutation status is an important determinant of clinical presentation and impact overall survival in WM/LPL, with the MYD88(265P>CXCR4)WHRMEN2 mutational pattern associated with the most aggressive form of the disease. 52

The MYD88 and CXCR4 mutation status may also alter the treatment outcome. 52,53 Patients with a mutated MYD88 without a CXCR4 mutation exhibit the best responses to ibrutinib. Lower and delayed response rates to ibrutinib are associated with mutated CXCR4 WM. The CXCR4 mutation seems also to reduce the efficacy to everolimus or treatment with inazoxan, dexamethasone and rituximab. 49 Resistance to ibrutinib therapy seems also to be linked to the emergence of a BTK gene mutation, most often in the setting of a CXCR4 mutated status. 54

Nowadays, mutational analysis in WM/LPL are limited to the MYD88 gene status (L265P hotspot mutation) but it may be that, in the near future, broader mutational analysis including CXCR4, BTK (and other genes such as CARD11 or PLCγ2, etc.) will be required for personalised treatment approaches.

Among cytogenetic aberrations, trisomy 4 and deletion 6q are recurrent in WM/LPL. 11,55 The detection of their association may help in diagnosis of WM/LPL. Chromosomal profile may also help with prognostication. 35 Deletions of 6q and 11q as well as trisomy 4 are associated with poor outcome but this prognostic impact must be confirmed in patients treated with new therapeutic approaches. Lastly, patients with del(17p)/TP53 deletion will have short progression-free and disease-free survivals. 55

CONCLUSION

The importance of genetic markers in the diagnosis of low grade B-cell lymphomas is now recognised as a component of the current sub classifications in the recent 2016 revision of the World Health Organization (WHO) classification of lymphoid neoplasms. Those markers are complementary to the morphological work up and represent valuable tools for the diagnostic and/or prognostic assessment in lymphomas as well as for therapeutic strategies.

Several methodologies such as karyotype, FISH, array-based karyotyping, massive parallel sequencing or circulating tumour DNA can now be used in routine practice for the clinical management of B-cell lymphoma patients. These techniques have their respective benefits and limitations that we need to know in order to perform an appropriate genetic work up.

REFERENCES


