Review

Malleable Immunoglobulin Genes and Hematopathology – The Good, the Bad, and the Ugly

A Paper from the 2007 William Beaumont Hospital Symposium on Molecular Pathology

Adam Bagg

*From the Department of Pathology and Laboratory Medicine,
University of Pennsylvania, Philadelphia, Pennsylvania*

Immunoglobulin gene rearrangement analysis is one of the more commonly performed assays available on the hematopathology menu of clinical molecular pathology laboratories. The analysis of these rearrangements provides useful information on a number of different levels in the evaluation of lymphoproliferations. An appreciation of the various mechanisms involved in the numerous physiological pathways affecting the immunoglobulin genes, and hence antibody molecules, is central to an understanding of B-cell development vis-à-vis the generation of immunological diversity. Knowledge about the intricate complexities of these mechanisms is also germane to an evaluation of testing methodologies. With this information, it is easier to develop an understanding of how contemporary molecular testing of immunoglobulin gene rearrangements has evolved, from historically quite heterogeneous, fairly flawed, and rather ugly approaches to current more-standardized protocols. In addition, recognition of how such genetic changes with good intentions can turn bad has fostered increasing insights into the pathogenesis of B-cell lymphomas and leukemias. Despite the significant improvements in the design of immunoglobulin gene rearrangement assays, numerous pitfalls and caveats remain. Accordingly, it is crucial to consider such testing a tool, and although most useful, it is one of many tools that may be required to build cogent diagnoses. (*J Mol Diagn* 2008, 10:396–410; DOI: 10.2353/jmoldx.2008.080061)

The Basics of Diagnostic Molecular Hematopathology

Molecular genetic testing is an integral facet of the evaluation of hematological disorders, in particular in the

work-up of hematological neoplasms.¹ This includes not only the ability to characterize specific leukemias and lymphomas but also to distinguish reactive from neoplastic disorders both lymphoproliferations and, more recently, myeloproliferations.² Allied to its diagnostic utility, molecular analysis is central to contemporary classification and prognostic assignment,^{3,4} and it is particularly well suited to testing for minimal residual disease after therapy.^{5,6}

A variety of molecular phenomena are amenable to routine diagnostic molecular analysis, with an assessment of gene rearrangements currently being the most commonly evaluated phenomenon. Gene rearrangements can be conveniently grouped into two broad categories: physiological and pathological. Physiological gene rearrangements refer to the normal intragenic shuffling of segments of antigen receptor genes, namely immunoglobulin (IG) genes and T-cell receptor (TCR) genes in B and T cells, respectively, that represent a major mechanism in the generation of immunological diversity. By contrast, pathological rearrangements, which are essentially synonymous with chromosomal translocations or inversions, lead to the movement of genes that are physiologically kept separate (ie, this is an intergenic phenomenon). Translocations lead to one of two major consequences that can be considered to have either a qualitative or quantitative effect. Those translocations that cause the disruption of genes, with the subsequent fusion

Supported in part by a Specialized Center of Research grant from the Leukemia and Lymphoma Society of America (to A.B.).

Accepted for publication June 19, 2008.

This article is partly based on material presented by the author at the William Beaumont Hospital 16th Annual Symposium on Molecular Pathology: DNA Technology in the Clinical Laboratory, 2007 September 26–28, Troy, MI.

Address reprint requests to Adam Bagg, MD, Director, Hematology, Department of Pathology and Laboratory Medicine, University of Pennsylvania, 7.103 Founders Pavilion, 3400 Spruce St., Philadelphia, PA 19104-4283. E-mail: adambagg@mail.med.upenn.edu.

Table 1. Possible Indications for Performing Antigen Receptor Gene Rearrangement Analysis

Scenario	Comment
Diagnosis	
Neoplastic versus reactive	Atypical lymphoproliferations in which the lesion is neither definitively neoplastic nor reactive by histomorphology and immunophenotypic analysis
Limited tissue	Small needle biopsies and fine needle aspirations, precluding the ability to evaluate architecture
Evaluating clonal relationships	In different tumors that are separated anatomically and/or chronologically
Equivocal immunophenotype	Numerous caveats to using molecular studies to assign lineage; thus, use this application extremely judiciously
Atypical T-cell lymphoproliferations	Because there is not a similarly useful immunophenotypic marker of clonality as there is for mature B cells ($\kappa:\lambda$ ratio)
Minimal residual disease	
Response to therapy	The rate of clearance of disease may be prognostic
Early recurrence	After attainment of remission
Product contamination	For autologous transplants
Increased precursor B cells	In patients with precursor B-cell neoplasms, to distinguish residual disease from hematogones

of portions of the disrupted genes, resulting in the generation of a novel, pathological chimeric gene and ultimately chimeric oncoprotein, can be considered qualitative. There are numerous well-characterized examples, which include the *BCR-ABL1* fusion generated as a consequence of the t(9;22) in chronic myelogenous leukemia and a subset of (in particular, adult) precursor B-cell acute lymphoblastic leukemia, and the *NPM1-ALK* fusion/t(2;5) found in some cases of anaplastic large-cell lymphoma. By contrast, a translocation resulting in the inappropriate overexpression of an intact gene (which physiologically typically has its expression tightly regulated), often due to the apposition of enhancers or promoters of contextually highly expressed genes, can be considered quantitative. Here, too, there are many well-characterized and recognized examples, such as the overexpression of *BCL2* and *CCND1* as a consequence of being juxtaposed with immunoglobulin heavy chain gene (*IGH@*) in the t(14;18) and t(11;14) associated with follicular and mantle cell lymphoma, respectively. However, this heightened expression is not always a consequence of positive regulation, in that removal of negative regulatory elements may also be operative, as has been demonstrated with translocations involving the *LMO2* gene in T-cell acute lymphoblastic leukemia.⁷

In general terms, the diagnostically relevant end point of analyzing physiological gene rearrangements compared with pathological rearrangements is quite different. For antigen receptor gene rearrangements, the major determination is whether there is homogeneity versus heterogeneity, essentially translating into monoclonality versus polyclonality, which may then be interpreted, with numerous caveats—discussed below, as neoplastic versus reactive. By contrast, the end point of an assessment of diagnostic pathological rearrangements using standard qualitative PCR assays is somewhat different, requiring either a positive (present) or negative (absent) readout. However, such qualitative absolutes, of positive versus negative, although pertinent in the diagnostic setting, are not applicable in the posttherapeutic scenario, where more sensitive and precise quantitative measurements are clearly important.

Clinical Indications for Analyzing Immunoglobulin Gene Rearrangements

There are two broad scenarios in which an analysis of antigen receptor gene rearrangements (ARGRs) in general and *IG* rearrangements in particular can be considered: for initial diagnosis and for subsequent minimal residual disease studies (Table 1). In the diagnostic setting, when evaluating lymphoid tissue microscopically and when the tissue is qualitatively and quantitatively optimal, it is usually quite straightforward to distinguish neoplastic from reactive disorders, in most instances. On such occasions, it is not necessary to perform ARGR analysis for diagnostic purposes. However, in a minor subset of cases, the histomorphological features (and immunophenotypic findings) may not be unequivocal, and it is in these scenarios that ARGR studies may be particularly helpful in providing an assessment of clonality. The frequency with which such cases require clonality testing by ARGR analyses is difficult to define but has been estimated to be required in as many as ~30% of cases in laboratories with limited specialization in hematopathology and ~10% of cases in specialized hematopathology centers.⁸ In addition, with the use of less-invasive diagnostic procedures such as fine needle aspiration and thin needle core biopsies, pathologists are being called on to render diagnoses on lesser amounts of tissue. Although immunophenotypic analysis here is often helpful, the inability to assess architecture, an important facet in the evaluation of lymphoid tissue, may compromise the ability to render a specific or even general (neoplastic versus reactive) diagnosis. An evaluation of ARGR in these situations can provide diagnostically useful information. These studies may also be of value in establishing (or excluding) clonal relationships in two distinct lymphomas that might be separated anatomically and/or chronologically. For example, they may answer such questions as the following: Are the two lymphomas with which the patient presented clonally related? Is this recurrent disease, or a new and different lymphoma? Does this large-cell lymphoma reflect transformation of the small-cell lymphoma?

A fourth context in which ARGR analysis might theoretically be valuable is in the assignment of lineage, when immunophenotypic studies are unhelpful. However, this use of ARGR studies is fraught with caveats and is to be avoided because neoplastic (and sometimes physiological) lymphoid cells can evince cross lineage rearrangements, ie, *IG* gene rearrangements in T cells and *TCR* gene rearrangements in B cells.⁹ Furthermore, with the development of more robust immunophenotypic assays, particularly reagents that are applicable to fixed tissue, situations in which they are unhelpful are less commonly encountered now. Nevertheless, there are scenarios where the judicious use of these studies may be helpful. For example, there is a normal hierarchy of *IG* gene rearrangements, with *IGH@* rearranging before the light chains do in normal B-cell ontogeny. Thus, although *IGH@* gene rearrangements are not uncommon in T-cell malignancies, the finding of a light chain gene rearrangement is less common in these cells, and finding an immunoglobulin light chain gene rearrangement is more likely to reflect the commitment to bona fide B-cell, rather than T-cell, lineage; however, this is certainly not absolute.¹⁰ Finally, although neoplastic B-cell disorders significantly outnumber neoplastic T-cell disorders, particularly in the Western hemisphere (in a ratio of ~6:1), a molecular diagnostic laboratory is perhaps more likely to be called on to perform *TCR* gene rearrangement studies compared with *IG* gene rearrangement analyses. The reason for this is that it is usually possible to assign clonality in (mature) B cells by immunophenotypic, in particular flow cytometric, analysis via an evaluation of κ - and λ -immunoglobulin light chain expression, whereas no similarly useful, simple, and widely available immunophenotypic method of clonality assessment exists for T cells. (There are flow cytometric assays that might facilitate this, by assessing the use of different TCR β chain families, but these approaches are more complex than κ - versus λ -analyses.^{11,12})

In addition to the above diagnostic scenarios, an assessment of ARGR can be particularly useful in the assessment of minimal residual disease (MRD), when extremely sensitive PCR-based approaches can detect neoplasia well below the level that can be appreciated morphologically (and sometimes immunophenotypically). Thus, for patients in whom therapy is given with curative intent and/or in whom the measurement of MRD is clinically relevant (ie, predictive of outcome, with the potential for meaningful therapeutic intervention), it is useful to evaluate a diagnostic specimen for its specific ARGR(s). This is performed not necessarily for primary diagnostic purposes but rather to provide a molecular fingerprint that can be used for subsequent tracking of MRD. There are a number of somewhat distinct settings in which MRD assessment can be performed and may be clinically useful. This includes the initial phases of chemotherapy for acute lymphoblastic leukemia, in which the ability to reduce the level of disease below a certain threshold (typically of the order of a 4-log reduction from diagnosis) is considered an extremely favorable prognostic variable. By contrast, cases in which there is a less than 2 log reduction, reflective of possible chemotherapy resis-

tance, fare much more poorly and may be considered for alternative therapeutic modalities.¹³ A second context in which MRD testing may be of value is in monitoring patients after remission, including molecular remission, has been attained to assess for early relapse.¹⁴ Therapeutic intervention here, when the tumor burden is relatively low, may be more effective than when attempting to treat at the time of florid hematological or clinical relapse, as has been demonstrated in some myeloid leukemias.^{15,16} Third, MRD testing can be applied to stem cell products that are to be used for autologous transplantation, to ensure that the reinfused material is free of contaminating tumor.¹⁷ Finally, in addition to the above three contexts in which MRD testing exploits the sensitivity of testing, the specificity of ARGR can also be a valuable phenomenon. This refers to patients with precursor B-cell lymphoblastic leukemias, in which in posttherapy bone marrow specimens, it may be challenging to distinguish physiological, regenerating precursor B cells (hematogones) from neoplastic precursor B cells (residual disease). Although flow cytometric analysis ought to be able to make this distinction, a simple PCR for ARGR may be just as informative and potentially more specific.¹⁸

Importantly, however, the family-specific primers used for qualitative diagnostic testing (see Evolution of Testing Methodologies to Assess Immunoglobulin Gene Rearrangements) do not have sufficient sensitivity to allow for meaningful MRD testing, in that they are able to detect only ~5 to 10% clonal cells. By contrast, for MRD measurements to be clinically relevant, sensitivities need to be achieved on the order of 0.01%, levels that cannot be attained with the primers used in diagnostic testing. Accordingly, analysis must be performed by quantitative (typically real-time) PCR, using reagents (primers and/or probes) that are specific for the *IGH@* gene rearrangement present in the B-cell neoplasm that is being monitored. The production of such reagents is laborious, requiring the cloning and sequencing of DNA sequences in each individual case, in and around the region of the *IGH@* gene that is essentially specific for each B cell and hence each B-cell neoplasm.

Physiology of Immunoglobulin Genes and the Generation of Immunological Diversity

Any understanding of the utility (and pitfalls) of clinical diagnostic *IG* analysis is predicated on an understanding of the physiological mechanisms involved in the generation of diverse *IG* genes and ultimately Ig molecules (antibodies). Similar mechanisms underlie the generation of the diversity of T-cell repertoire, via the generation of numerous T-cell receptors, the analysis of which is equally invaluable in the determination of clonality of T cells; however, the focus of this discussion is B cells and their *IG* genes.

It is perhaps not too oversimplified to consider that the *raison d'être* of a B cell is to generate a functional and specific antibody molecule. Collectively, B cells are called on to generate a plethora of antibodies to account for all possible foreign antigens to which the human or-

Figure 1. Major stages of development of the *IG* repertoire. Three major phases during which *IG* gene diversity and effector function is generated are noted at the **top**: VDJ recombination, SHM (somatic hypermutation), and CSR (class switch recombination), and the enzymes mediating these events (RAG1/2, recombinase-activating genes 1 and 2; AID, activation-induced cytidine deaminase). The generation of combinatorial diversity is not noted (see text for details). The two major sites at which these events occur are in the **middle**: bone marrow and germinal center of lymph nodes. The nongerminal center (T-cell independent) pathway for acquisition of memory (ie, a B cell can acquire memory and become a marginal zone cell, independent of the germinal center) is not shown. The respective stages of B-cell maturation are noted at the **bottom**.

ganism might be exposed. However, there are insufficient genes in the whole human genome (never mind within the *IG* genes themselves) to account for the vast number of antibodies required. Thus, there are a number of different mechanisms that allow for the creation of a multitude of different *IG* genes and ultimately antibody molecules (Figure 1). These mechanisms include combinatorial diversity, junctional diversity, somatic hypermutation, and class switch recombination. They are mediated by a variety of key enzymes, including recombinase-activating genes (RAG) 1 and 2, terminal deoxynucleotidyl transferase, and activation-induced cytosine deaminase.

The first step in the generation of diversity occurs very early in B-cell differentiation, while it is still developing in the bone marrow, at the pro B-cell stage of maturation.¹⁹ Importantly, this apparently stochastic rearrangement of disparate segments within the *IG* genes is antigen independent, occurring long before exposure to antigen; the B cell at this stage, lacking the ability to synthesize and express Ig molecules, is not yet equipped to even recognize antigens. In the germline of such B cells, the *IGH@* gene contains numerous disparate V (variable), D (diversity), and J (joining) gene segments. All other cells, of course, contain these gene segments in the germline configuration, too; however, their subsequent rearrangement is essentially exclusive to B cells. There are ~38 to 46 functional V segments (there are many more pseudo and rearrangeable, but nonfunctional V segments), ~23 D segments, and 6 J segments [Immunogenetics Repertoire (IG and TR); http://imgt.cines.fr/textes/IMGTrepertoire/LocusGenes/locusdesc/human/IGH/Hu_IGHdesc.html, last accessed April 28, 2008] with one segment from each of these three different regions randomly recombining to generate a specific VDJ gene rearrangement. Flanking the V, D, and J segments are recombination signal sequences, consisting of conserved heptamers and nonamers, separated by nonconserved spacers, typically of 12 or 23 nucleotides. These recombination signal sequences are important in ensuring legitimate recombination, and they provide binding sites for the primary enzyme complex (recombinase activating genes RAG1/

RAG2) that mediates the initial DNA cleavage and synapsis required for this event. A variety of other DNA repair factors are involved in this reaction, including DNA-PKc, Ku70, Ku80, Artemis, DNA ligase IV, XRCC4, and Cernunnos. There is a hierarchy both in the order of *IG* gene rearrangements at the different loci (*IGH@* on 14q32, then *IGK@* on 2p11, and then *IGL@* on 22q11) and within the *IGH@* locus. Thus, with regard to the *IGH@* locus, DJ rearrangement occurs first, and only once this is completed is V to DJ rearrangement able to occur.²⁰

In addition to this important apparently random shuffling mechanism for the generation of immunological diversity (so called combinatorial diversity), nucleotides are also randomly both deleted and added at the sites of V to D and D to J fusion. This process is primarily mediated by the enzyme terminal deoxynucleotidyl transferase (TdT) and is referred to as generating junctional diversity, with the added nucleotides creating N regions between V and D and between D and J. Additional mechanisms contribute to junctional diversity, such as the inclusion of P (for palindromic) nucleotides by RAG1/2 and the exonuclease activity of the DNA repair machinery. Based on the number of functional *IGH@* V, D, and J segments, there are on the order of 10^4 possible recombination events; together with VJ rearrangements at each of the *IGK@* and *IGL@* loci (both of which lack D segments), a total of ~ 10^6 different antibody molecules can be yielded via combinatorial mechanisms.²¹ Junctional diversity (through the actions of both TdT and the other noted mechanisms) is responsible for at least 6 additional orders of magnitude of diversity, thus allowing for the potential of $>10^{12}$ different antibody molecules.²² Thus, two major and temporally related diversification mechanisms, combinatorial and junctional, mediated by RAG1/RAG2 and TdT, respectively, occur in the early bone marrow phase of B-cell development, before antigen exposure.

The fully rearranged *IGH@* gene (VNDNJ) contains a number of complementarity determining regions (CDRs) and framework regions (FRs) (Figure 2). As the name suggests, the CDRs encode for those components of the antibody molecule that are most intimately involved with

Figure 2. *IGH@* VDJ recombination and detection of PCR products. **A:** D-J rearrangement precedes full V-DJ rearrangement (see text for details). The intervening DNA sequences are deleted. In between the rearranged V and D, and D and J, are N (nucleotide) sequences, inserted by TdT (terminal deoxynucleotidyl transferase). Only a rearranged VDJ gene can be PCR amplified with V and J primers, since in the germline (non-rearranged) configuration the V and J gene segments are too far apart to be successfully amplified. The primers are directed at the FRs (framework regions), with most of the length and composition heterogeneity generated by CDR3 (complementarity determining region 3). **B:** PCR products analyzed by gel electrophoresis. **Lane 1**, size markers; **lane 2**, polyclonal smear; **lane 3**, two monoclonal bands, consistent with bi-allelic rearrangement; **lane 4**, specimen from same patient as analyzed in **lane 3**, now 2 years later, with the apparently identical rearrangements most suggestive of relapse rather than a second primary; **lane 5**, monoclonal; **lane 6**, polyclonal smear; **lane 7**, neither discrete band nor smear, suggestive of either an absence of B cells or failure of these primers (seen as band at the bottom) to amplify a monoclonal rearrangement. **C:** PCR products analyzed by capillary electrophoresis. A Gaussian distribution is seen with polyclonal, reactive B cells, using FR1, FR2, and FR3 primers. The x axis reflects the lengths of the products in base pairs, whereas the y axis reflects the height of the peaks in arbitrary fluorescent units. Original images kindly provided by Jason Merker, MD, Stanford University. **D:** PCR products analyzed by capillary electrophoresis. A discrete peak in the absence of a Gaussian distribution is seen with monoclonal B cells, using FR1, FR2, and FR3 primers. The axes are as noted above in **C**. Original images kindly provided by Jason Merker, MD, Stanford University.

antigen recognition and are the most diverse between anti-*hy*bodies and *IGH@* genes. By contrast, the FRs are typically quite similar between different antibodies and *IGH@* genes. An appreciation of these differences (CDRs versus FRs) is central to understanding the PCR-based detection of *IGH@* gene rearrangements, as discussed under Evolution of Testing Methodologies to Assess Immunoglobulin Gene Rearrangements. There are three CDRs in the *IGH@* gene, with the most distal (3') CDR3 being the most heterogeneous, because it is affected by both recombinatorial and junctional diversification mechanisms described above. By contrast, the more proximal (5') CDR1 and CDR2 are encoded for in the germline (as are all of the FRs) and are not affected by either recombination or the action of TdT. The CDRs are flanked by the total of four FR regions, with FR1, FR2, and FR3 being encoded by the V segment and FR4 residing in the J segment. The whole D segment, flanked on both sides by the extremely heterogeneous N region, fully resides in CDR3.

Successful *IGH@* VDJ recombination and *IG* light chain gene rearrangement are required for B cells to express intact and functional Ig molecules on their surface mem-

branes, at which stage they may exit the bone marrow, having now developed their primary or central repertoire. Although these circulating B cells are now "mature," as evidenced by their expression of Ig molecules on surface membranes, they are still considered naïve in terms of mostly not yet having been exposed to antigen. These naïve B cells, which account for ~60 to 70% of circulating B cells, must now migrate to lymph nodes, the spleen, and mucosa-associated lymphoid tissue to generate their secondary or peripheral repertoire. Specifically, they must relocate to specialized regions of these secondary lymphoid organs, the primary follicles. Here, via intricate and complex cellular and humoral interactions, they undergo the germinal center reaction, generating secondary follicles, from which memory B cells and plasma cells will ultimately emerge. The cellular partners in these germinal center events include T cells and antigen-presenting follicular dendritic cells. However, transiting the germinal center is not an absolute requirement for the attainment of memory, in that some (marginal zone) B cells can acquire memory in a T-cell-independent manner.²³

The Ig molecules generated by VDJ recombination in the bone marrow are of low affinity, because they are

created in an apparently random fashion. For these to neutralize and clear pathogenic antigens in an efficient manner, they must acquire higher affinity for these antigens and be able to perform different effector functions. To this end, and although rather oversimplified, there are two regions within the germinal center, in which two somewhat distinct DNA breakage events occur, which are central to this fine-tuning of the humoral immune response (Figure 1).²⁴ The dark zone is characterized by proliferating larger centroblasts, and it is predominantly here that somatic hypermutation (SHM) happens; whereas it is in the light zones, dominated by smaller centrocytes, that class switch recombination (CSR) typically occurs. Both of these phenomena are mediated by the enzyme activation-induced cytosine deaminase (AID).²³ AID was only identified less than a decade ago, and its expression appears to be restricted to germinal center (GC) B cells; however, there is evidence that it can also be expressed in interfollicular large B cells, outside the GC and in the medulla of the thymus.²⁵ Curiously, although AID functions in the nucleus, it is predominantly located in the cytoplasm, and it is unclear how nuclear access is mediated. However, its localization is more likely to be nuclear in centrocytes.²⁶ Its expression in GC B cells is transiently induced by a number of factors, including CD40, interleukin-4, B-cell activation factor belonging to the tumor necrosis factor family, a proliferation-inducing ligand, transforming growth factor- β , and lipopolysaccharide, whereas its expression is negatively regulated by B lymphocyte induced maturation protein 1, inhibitor of DNA binding/differentiation, and CD45. As its name indicates, AID deaminates cytidine, resulting in uracil:guanine mismatches; these are then repaired by either mutation (leading to SHM) or nonhomologous end joining (leading to CSR). Importantly, both of these events appear to specifically target single-stranded DNA and hence are transcription dependent.

The primary function of SHM is to convert low-affinity into high-affinity Igs. Although B cells are exposed to antigens within the GC, the mutations induced by SHM are random (ie, they are not directly induced by specific antigens). However, mutations in *Ig* genes occur approximately 6 orders of magnitude more often than spontaneous mutations at other loci.²⁷ These mutations are predominantly point mutations, although insertions and deletions may also occur. Transition mutations (pyrimidine to another pyrimidine, or purine to purine) are approximately twice as common as transversions (pyrimidine to purine or vice versa). Although targeted i) toward certain hotspot motifs such as DGYW [D = adenosine (A), guanosine (G), or thymidine (T); Y = cytidine (C) or T; W = A or T], indicating that it is somewhat influenced by the primary sequence of the DNA, and ii) to a ~1- to 2-kb region downstream of the transcription start site (after which SHM exponentially decreases), affecting certain CDRs more than others, SHM is essentially a random phenomenon. This is evidenced by the fact that most of the mutations do not increase affinity in the antigen binding sites. Most actually decrease affinity or even abrogate expression of Ig molecules on surface membranes by generating stop codons or frame shifts. Such GC B cells

are unable to optimally bind antigen, so that they do not receive an appropriate survival signal, and consequently more than 90% of these cells die. This is facilitated by the physiological down-regulation of antiapoptotic mediators (such as BCL2) in GC B cells and accounts for the characteristic histological prominence of tingible-body macrophages in reactive GCs. Although SHM is traditionally tied to the GC reaction, it is also possible for this to occur outside of the GC.²⁸

CSR, also mediated by AID, does not actually add to immunological diversity, in that it does not expand the range of foreign antigens that can be detected by different Igs. Rather, it results in the important altered effector function of the Ig molecule without any alteration in antigenic specificity. This region-specific recombination event replaces the μ - and δ -constant region segments (encoding IgM and IgD) with a downstream constant region, resulting in the ultimate synthesis of IgG, IgA, or IgE, each with specialized functional properties. This phenomenon occurs mostly in the centrocytes in the light zone of the GC. SHM and CSR are not temporally linked, and there are distinct domains of AID required for these two different processes.²⁹ Like SHM, CSR can occur both within the GC, in a T-cell-dependent manner, and outside the GC, in a T-cell-independent manner³⁰; the latter, however, occurs quite extensively. CSR requires the presence of specific repetitive regions known as switch regions; these regions of several hundred base pairs precede each of the *Ig* constant regions. Like VDJ recombination in the marrow—but unlike SHM—CSR results in the deletion of quite large intervening regions of DNA, and which appears to be repaired using similar pathways.

Gene conversion is a third process mediated by AID and is involved in Ig diversification in chicken and sheep; however, it appears not to occur in humans and will not be discussed further here.³¹ Receptor editing, mediated by RAG1/RAG2, appears to be important in humans with regard to developing central tolerance. This process occurs primarily in the bone marrow, and it offers B cells with self-reacting antibodies a second shot at life. These enzymes, the expression of which was once thought to be restricted to the bone marrow in the generation of initial recombinatorial diversity, as discussed above, can apparently be reactivated much later in B-cell ontogeny, specifically in the GC.³² However, this putative extramedullary RAG1/RAG2 reactivation is controversial.³³

Evolution of Testing Methodologies to Assess Immunoglobulin Gene Rearrangements

Of the numerous physiological mechanisms involved in the generation of a diverse immune response detailed above, it is the phenomenon of V(D)J recombination, together with the junctional diversity generated by N nucleotide insertion and excision, that is the basis of clinical analysis of *Ig* gene rearrangements. The latter, TdT-mediated mechanism is particularly pertinent to PCR testing. Historically (in the 1980s, after the cloning of the antigen receptor genes), testing was performed by

Southern blot analysis, which is largely based on the creation of different restriction fragment lengths generated as a consequence of the rearrangement process.^{34,35} Although still considered the “gold standard” for such analyses (with this designation, perhaps as much affectionate as it is currently clinically relevant), this approach has a number of drawbacks. These include i) the need for high-molecular weight DNA, which is not routinely obtainable from a frequent source of pathological material, namely formalin-fixed paraffin-embedded tissue; ii) the need for relatively large amounts (~10 µg per restriction enzyme digest) of this good-quality DNA further limiting its use to sizable amounts of not-always-available fresh or frozen tissue; iii) the common use of radioactive materials, with its attendant risks, but noting that chemiluminescent hybridization is an alternative to radioactivity; iv) its fairly laborious and technically demanding nature, affecting the cost of the assay; v) the slow turnaround time, often on the order of 2 to 3 weeks, precluding the ability to apply the results to clinical decision-making in a useful period of time; and vi) its relative insensitivity, requiring the presence of at least ~5 to 10% clonal cells for their detection.

Thus, there was great anticipation and excitement when the first PCR-based assays were described in the early 1990s.^{36–38} PCR certainly has numerous advantages over Southern blot analysis and essentially overcomes all of the drawbacks alluded to above. In contrast to Southern blot analysis, which is largely based on the loss of restriction enzyme sites as a consequence of DNA loss during the course of VDJ recombination, the primary attribute that PCR analysis of the *IGH@* gene exploits is the extreme heterogeneity in size (and, to some degree, nucleotide composition, depending on the mode of readout) of different CDR3 regions between different *IGH@* genes. In addition to using different V, D, and J segments, polyclonal B cells will, even more importantly for the purposes of understanding the basis of this assay, have differently sized N regions due to the random actions of TdT. Thus, PCR analysis of CDR3 performed on such a population of B cells, using a 5' FR3 V primer (or primers) and a 3' J primer, would generate a heterogeneous population of different sized products (Figure 2). When run on a gel (typically then either an agarose or acrylamide gel), this would yield a smear, reflecting the products of various length. By contrast, a monoclonal population of B cells, derived from a single neoplastically transformed daughter cell, would all harbor the identical CDR3 region, and hence the analysis of the PCR products would typically yield a single sharp band, reflecting the identically sized PCR products. Not uncommonly, two bands can be seen in a monoclonal process, not because of biallelicity, but, rather, reflective of biallelic rearrangements. Such a phenomenon is not that uncommon, because the process, as alluded to above and in particular due to the actions of TdT, is error prone and has a better than 1 in 2 chance of generating a nonfunctional rearrangement at first attempt. This would then prompt the need for the other allele to attempt a successful rearrangement; however, both would be retained and be PCR-able in a neoplasm derived from that cell.

However, the initial excitement of moving into PCR bases diagnostics of *IG* analysis was tempered by the rather alarming inconsistency in testing strategies and hence results, with a number of studies highlighting the need for standardization.^{39–41} The problems with the first-generation PCR assays were numerous, with many suffering from issues that resulted in both false negativity and false positivity. False negativity was often due to inadequate primer annealing, typically related to the use of limited consensus primers or the effects of SHM, whereas false positivity typically emanated from suboptimal readout of the amplified PCR products. Because the majority of B-cell malignancies arise from GC or post-GC B cells, many of their *IGH@* genes will have undergone SHM, with this being a major explanation for false negatives. By contrast, there is a much lower frequency of this phenomenon in pre-GC malignancies, such as lymphoblastic leukemia/lymphomas and mantle cell lymphomas. This accounts for the observation that false negative *IGH@* PCR assays are much more infrequent in these lymphomas. Many of these shortcomings have been addressed at a number of levels by numerous investigators over the past decade and overcome through rigorous optimization and standardization of such testing, spearheaded by a highly collaborative European consortium (BIOMED-2 Concerted Action BHM4-CT98-3936), which was initiated in the late 1990s and culminated in a number of key publications in 2003¹⁰ and 2007.^{8,42}

The three areas of improvement formalized by BIOMED-2, as they pertain to the *IG* genes, included the i) design of complete sets of primers, in particular numerous V-family specific primers, rather than consensus primers, to cover all possible *IGH@* V-segments; ii) inclusion of an assessment of incomplete DJ *IGH@* rearrangements; and iii) addition of multiple *IG* targets, now including *IGK@* and *IGL@*. With regard to the readout of the amplified product, the value of both heteroduplex analysis and capillary electrophoresis was emphasized. (Much of the same pertains to TCR analysis, which is not discussed further in this review.) Although the various facets of the BIOMED-2 approach have been described and implemented by others, a major attribute is the effort involved in rigorously designing and testing a highly complex assay. Analysis of the *IG* genes is possible through a total of eight separate multiplexed assays; however, it is not always necessary for all eight assays to be performed ab initio, as detailed below. There are five *IGH@* tubes, two *IGK@* tubes, and one *IGL@* tube. Three of the *IGH@* analyses evaluate complete V(D)J rearrangements, whereas the other two evaluate incomplete DJ rearrangements. Using the complete set of primers, clonality can be documented in 96 to 99% of all B-cell lymphomas in both frozen and paraffin-embedded tissues.^{42–44}

The upstream primers of the different three VJ tubes target the three FRs in the V segment (six for FR1 and seven each for FR2 and FR3, to cover all rearrangeable V families); a single downstream J consensus primer suffices for all six J segments. Although each of the sets of FR1, FR2, and FR3 primers essentially ultimately amplify the same CDR3 region (because there is no significant additional length diversity upstream of CDR3), they are

often complementary, rather than being redundant. This is because although SHM targets most of the rearranged VDJ, its activity appears to be greatest in and around CDR3; accordingly, the flanking FRs, in particular FR3, are most vulnerable to being caught in the mutational cross-fire.⁴⁵ Such mutations in the typically bland and homogeneous FRs may then compromise primer annealing, yielding a false-negative PCR outcome. By contrast, FR1 and FR2 are subjected to less SHM, and thus a clonal rearrangement missed with FR3 primers may well be detected with FR2 or FR1 primers. Conversely, however, one potential limitation is that with the progression from using FR3 to FR2 to FR1 primers, there is the need to amplify increasingly greater sized products, with the approximate ranges of the amplifiable products being ~100 to 170, ~250 to 295, and ~310 to 360 bp, respectively. Although this is unlikely to be an issue when using fresh or frozen tissue, it may become challenging (but certainly not impossible) to amplify to the size range required using FR1 primers, when the source is formalin-fixed paraffin-embedded tissue. The BIOMED-2 protocol includes a useful multiplexed control gene PCR generating a 100-bp ladder of PCR products to enable an assessment of the integrity of the extracted DNA.

The added value of the inclusion of the two DJ tubes (one containing six D primers, the other a single D primer, both with a single J primer as above) is twofold. First, these primers will detect very early pro-B acute lymphoblastic leukemias that have been arrested at such an early stage of B-cell development that they have not yet undergone full V to DJ rearrangement, so that standard VJ primers would fail to detect such incomplete rearrangements.⁴⁶ Second, and more pertinent, is the fact that ~30% of B-cell neoplasms overall harbor incomplete *IGH@* DJ rearrangements. This phenomenon is particularly common in plasma cell myeloma, where it occurs in up to 60% of cases,⁴⁷ and chronic lymphocytic leukemia, where it is seen in more than 40% of cases.⁴² This is observed less frequently in follicular and mantle cell lymphomas,⁴² perhaps because one *IGH@* allele is likely to be involved in a disease-associated translocation that characterizes these two lymphomas, whereas the other needs to be completely and functionally rearranged to allow for expression of Ig molecules on surface membranes. Nonetheless, targeting incomplete *IGH@* DJ rearrangements for PCR is particularly appealing, because given their nonfunctional nature, they are not transcribed, and the absence of single stranded DNA prevents AID from inducing SHM, ultimately allowing for optimal primer annealing.

The added value of incorporating *IG* light chain analysis has been borne out in a number of studies.^{42,43,48–50} In particular, *IGK@* PCR has been particularly gratifying in the evaluation of the clonality of follicular lymphoma, that lymphoma with the historically highest false-negativity rate when analyzed with *IGH@* primers, due not only to SHM per se, but the added nemesis of ongoing SHM, in this lymphoma that quite faithfully recapitulates the physiology of normal GC cells. Adding *IGK@* PCR to *IGH@* PCR results in a dramatic improvement in the detection of clonality in follicular lymphomas, increasing the yield by

up to 60%.^{42,43,48} A number of possible explanations have been proposed for this phenomenon,⁴⁸ including the following: i) *IGK@* PCR includes the use of a Kde primer, to amplify a unique deletion found at this locus, and which is not subjected to SHM¹⁰; ii) even a functional *IGK@* VJ rearrangement may be subjected to less SHM than an *IGH@* rearrangement⁵¹; iii) the product size of the most frequently amplified *IGK@* V-segment is relatively small (~150 bp), allowing for enhanced amplification compared with the larger FR1 and FR2 *IGH@* products; and iv) one of the *IGH@* genes will likely have been “consumed” in the prototypic t(14;18) of follicular lymphoma, rendering only one *IGH@* locus (compared with potentially two *IGK@* loci) amenable to analysis.

Despite the advantages of *IGK@* PCR, especially in the analysis of lymphomas arising from GC or post GC cells, it is still essential to retain *IGH@* PCR, given the complementary nature of these approaches. Thus, when all major mature B-cell lymphoma types were evaluated in two large series,^{42,43} *IGH@* PCR detected clonality in 86 and 91% of lymphomas, and *IGK@* PCR detected clonality in 72 and 88% of lymphomas, but the two combined increased detection rates to 96 and 98%, respectively. In addition, in precursor B-cell leukemias/lymphomas, maturation arrest may occur before *IGK@* gene rearrangement, further emphasizing the need to retain *IGH@* PCR analysis in diagnostic testing.

The studies noted above^{42,43} also highlight the limitations of *IGL@* testing, with this analysis detecting clonality in only 28 to 29% of lymphomas. Whereas essentially all mature B-cell lymphomas will have rearranged their *IGK@* gene, far fewer will have rearranged their *IGL@* gene, based on the hierarchical order of *IG* gene rearrangements. Thus, clonality of mature B-cell lymphomas can be detected in 96 to 98% of cases without the inclusion of *IGL@* PCR. Accordingly, an evaluation of *IGL@* gene rearrangements appears not to provide sufficient additional information to warrant inclusion in most diagnostic testing algorithms.

With regard to PCR product readout when using capillary electrophoresis, it is usually quite straightforward to distinguish polyclonal from monoclonal rearrangements, with the former yielding a Gaussian distribution of fragments, whereas monoclonality is evident when there is one (or two) discrete peak (Figure 2). This is quite analogous to the interpretation of serum protein electrophoresis. A major attribute of capillary electrophoresis is its ability to accurately size, in base pairs, a monoclonal rearrangement, which then affords the neoplasm with an objective fingerprint of value both for MRD testing and for comparison of anatomically or chronologically disparate neoplasms, to determine whether they are clonally related. Additionally, it is sometimes possible to evaluate semiquantitatively the size (not in base pairs on the horizontal axis, but in fluorescent units on the vertical axis) of a peak, which might be of value in assessing its “monoclonality” when it exists in a polyclonal background; however, despite numerous attempts to apply this clinically, it remains a somewhat controversial application.⁸ Because heteroduplex analysis involves denaturation and rapid annealing steps, it has the added value of assessing the

“quality” (ie, nucleotide composition) of CDR3, more so than its quantity (ie, nucleotide length). Accordingly, this modality of readout might be of value when capillary electrophoresis is equivocal. This seems to be an issue with *IGK@* analysis, in which the restricted CDR3 repertoire can confound clonality assessment when evaluated by fragment length determination.^{10,42}

Although it might be considered ideal, when assessing *IG* gene clonality, to perform all eight multiplex reactions described by BIOMED-2, such an approach may be neither practical nor necessary. Accordingly, attempts have been made at developing clinically meaningful algorithms, which might be more applicable to routine hematopathological practice, by determining the highest diagnostic yield from a minimal number of reactions. Such an approach would also have potential economic benefits. Thus, the BIOMED-2 group themselves have suggested the following algorithm for assessing clonality in suspected B-cell neoplasms.⁴² First, run the three *IGH@* VJ tubes, preferably with the two *IGK@* tubes. If no clonality is discerned, the second step is to run one *IGH@* DJ tube (that which contains the D1–D6 primers), preferably with the 1 *IGL@* tube. With this approach, monoclonality was identified with the first five tubes in 98% of lymphomas, with the additional two tubes raising the yield to 99%. By contrast, another study⁴³ proposed a first step using three tubes (one *IGH@* VJ tube—FR2 only—plus two *IGK@* tubes), with the second step using an additional three tubes (the other two *IGH@* VJ tubes—FR1 and FR2—plus the one *IGH@* DJ tube noted above). With this approach, monoclonality was documented in 91 and 96% of cases respectively. There are, however, some key differences between the two studies. Most importantly, the former was performed on frozen tissue, whereas the latter was done on formalin-fixed, paraffin-embedded tissue. However, in the latter study, cases that lack the ability to amplify a 300-bp control gene were excluded from analysis (21% of the cases were excluded for this reason). Additionally, the different mixture of cases—in particular those of GC or post-GC origin—between the two studies might also account for the somewhat (but certainly not dramatically) different outcomes. Nevertheless, these studies highlight the added value of *IGK@* and *IGH@* DJ (D1 to D6) PCR, and suggest that two reactions (the *IGH@* DJ tube with the single D7 primer and the *IGL@* tube) provide limited added value. Even with this somewhat restricted approach, it is possible to determine clonality in more than one reaction in around 80% of cases. This apparent but useful redundancy and the ability to detect clonality in two or more independent PCR assays are particularly reassuring in the documentation of bona fide neoplasia and perhaps carry more diagnostic weight than finding clonality in only one reaction, which might provide less compelling evidence of overt neoplasia.

Although the widespread availability of a standardized assay, such as that developed by the BIOMED-2 consortium is most welcome, somewhat more contentious is the application of a patent to this assay. Thus, the patents for *IG* PCR were exclusively licensed to a company, InVivoScribe (San Diego, CA) in 2002, with these patents cur-

rently being enforced in the United States of America, Japan, and Australia. Clinical laboratories, including academic medical centers, are required to obtain sublicenses to perform *IG* PCR analyses, entailing the payment of royalties. Alternatively, laboratories may pay lower royalties if they use InVivoScribe kits.

A number of alternative approaches for the evaluation of *IG* gene rearrangements have been described, including the use of the ligase chain reaction,⁵² assessing the peak area on a melting curve analysis,⁵³ and using oligonucleotide microchips for *TCR* gene rearrangements.⁵⁴ However, it remains to be determined if these strategies will have more to offer than, and as great applicability as, the BIOMED-2 approach.

When Good Immunoglobulin Genes Go Bad

Given the degree to which *IG* genes are physiologically assaulted, being subjected to DNA damage at specific stages in the bone marrow and germinal center during the lifespan of a B cell, it should come as no surprise that this intended benevolent process, for the generation of immunological diversity, might occasionally have malevolent consequences, such as oncogene activation, leading to neoplasia. There are three specific phases when the *IG* genes are particularly vulnerable and when physiological changes with good intentions can go awry, leading to neoplastic transformation. These three stages are V(D)J recombination (primarily if not exclusively in the bone marrow), SHM, and CSR (the latter two primarily if not exclusively in the germinal centers). Of note, although there are approximately equal numbers of B cells and T cells in the human body and although both are subjected to similar, potentially error-prone V(D)J recombination within the marrow and thymus, respectively, B-cell neoplasms outnumber T-cell neoplasms ~6:1. This is almost certainly due to the unique exposure of B cells to the particular treacherous germinal center environment, from which, not surprisingly, many B-cell lymphomas emanate.

Nevertheless, there are at least two quite common B-cell lymphomas in which the disease-defining (if not absolutely disease-specific) translocation appears to arise at the time of VDJ recombination in the bone marrow. These are follicular lymphoma and mantle cell lymphoma and their hallmark t(14;18)/*BCL2-IGH@* and t(11;14)/*CCND1-IGH@* translocations, resulting in the dysregulated expression of the *BCL2* and *CCND1* genes, respectively. This is due to the juxtaposition of these normally strictly regulated genes with *IGH@* enhancer elements, with this *IGH@* transcriptional machinery being quite, and appropriately, active in B cells. The unscheduled overexpression of *BCL2* leads to protection from apoptosis, whereas that of *CCND1* leads to increased cell cycling, both key steps in (but not, in isolation, sufficient for) neoplastic transformation. The evidence that these translocations arise from errors in RAG1/2-mediated VDJ recombination has been documented by sequencing the breakpoints, demonstrating the presence of *IGH@* J segments juxtaposed with the *BCL2* gene, noncoded (presumably TdT-mediated) nucleo-

tides, and structures in the *BCL2* breakpoint that are cut by RAG1/2.^{55–59}

Although the dogma has generally been that such RAG1/2-induced translocations lead to oncogene activation via juxtaposition with enhancers or other regulatory elements of actively transcribing antigen receptor genes, they may also, on occasion, lead to oncogene activation via the removal of negative regulatory elements.⁷ In addition to these two different mechanisms leading to the broad quantitative/gene dysregulation translocation consequences alluded to in the introduction, there are other ways in which RAG1/2 may be involved in neoplastic transformation. Thus, it has been proposed that this enzyme complex is also involved in the alternative group of qualitative translocations, leading to the formation of chimeric oncogenes (*E2A-PBX1*), and the inactivation of tumor suppressor (*CDKN2A*) and micro-RNA loci.⁵⁹ Additionally, events leading to oncogene activation (quantitative translocation) need not involve an antigen receptor gene, as has been demonstrated with *TAL1* activation.⁶⁰ More recently, it has been shown that the deletion of *IKZF1*, a frequent phenomenon in *BCR-ABL1*+ acute lymphoblastic leukemia is likely to be mediated by RAG1/RAG2.⁶¹

Once in the GC, when the B cell is subjected to two distinct phases of DNA breakage, namely SHM and CSR—both mediated by AID, it is once again possible that these events with good intentions can go bad. Both of these phenomena can result in chromosomal translocations, with each associated with distinct translocations in distinctive B-cell and plasma cell neoplasms.⁶² Thus, the t(8;14) of endemic Burkitt lymphoma and t(3;14) associated with a subset of diffuse large B-cell lymphoma (DLBCL), leading to the unscheduled overexpression of *MYC* and *BCL6*, via mechanisms similar to those alluded to above, are both initiated by SHM. Interestingly, the cytogenetically identical t(8;14) associated with sporadic Burkitt lymphoma (which, in its classical form, differs from endemic Burkitt lymphoma in terms of geography, clinical presentation and Epstein Barr Virus association), while also juxtaposing *MYC* and *IGH@*, does so at the time of CSR rather than being initiated by SHM as in the endemic form (the *MYC* breakpoints are also typically different). The activation of *BCL6* expression in the t(3;14) translocation can also occur via this additional mechanism at the time of CSR. Other examples of translocations associated with CSR are those seen in plasma cell myeloma, such as the t(4;14) and t(11;14), which may result in dysregulated expression of *FGFR3* and *CCND1*, respectively.⁶³

Intriguingly, SHM is not restricted to the *IG* genes, and a number of other genes can be physiologically mutated in normal B cells.²³ These include *BCL6*, *CD10*, *CD79A*, *CD79B*, and *CD95*. It seems as if these genes are targeted for SHM because i) they are actively being transcribed in the GC, and ii) they may contain some of the same mutational hotspots, as seen in *IG* genes, that partially direct the actions of AID. However, the rate of mutation in these genes is approximately 2 orders of magnitude less than what occurs in the *IG* genes but is still 4 orders of magnitude greater than background. This is not a rare event because about one-third of normal GC

and memory B cells harbor *BCL6* mutations. The physiological benefit of these mutations is unclear but may reflect the collateral damage of heightened AID activity at a time when they are vulnerable, due to their active transcription.

In addition to this physiological mutation of non-*IG* genes, these genes may also be mutated pathologically. Here, and in contrast to the apparent lack of functional consequences with physiological mutations, mutations are now associated with a phenotype. These include the disruption of normal transcriptional regulation by mutations in their 5' nontranscribed regions or amino acid changes that affect the function of the gene product.²² Thus, *BCL6* can, in addition to being subjected to physiological mutations, undergo pathological mutations that allow for its increased expression. One of the physiological functions of the BCL6 protein is to suppress cell cycle and apoptotic responses to the normal physiological DNA damage (SHM and CSR) that is central to the biology of the GC. It does this by inhibiting the transcription of key factors involved in the response to genotoxic stress, including P53⁶⁴ and ATR,⁶⁵ thus facilitating physiological genomic remodeling. However, exposure of the B cell to this excessive protection from DNA damage is presumably permissive for the development of translocations and other dangerous DNA damage. In addition to *BCL6*, other genes that undergo pathological mutations include *MYC*, *PIM1*, *PAX5*, and *Rho/TTP*, each of which is mutated in a large number of lymphomas, in particular DLBCLs.⁶⁶

DLBCLs can be divided, based on their expression profiles, into a number of subtypes, the most frequent of which are those of GC B-cell and activated B-cell histogenic origin, with the latter believed to be derived from cells at a somewhat later, post-GC stage of B-cell development.⁶⁷ Accordingly, one might anticipate that that GC B-cell cases are more likely than activated B-cell cases to evince the features described above. However, the opposite has been observed, with aberrant CSR leading to translocations more commonly in activated B-cell compared with GC B-cell DLBCLs.⁶⁸ This is thought to be due to the paradoxically increased expression of AID in activated B-cell DLBCLs, as a consequence of elevated IRF4 expression in such cases, with this being related to the constitutively activated nuclear factor- κ B pathway that characterizes this subset of DLBCL.^{67,69} Another apparent AID paradox is evident in chronic lymphocytic leukemia. Chronic lymphocytic leukemia has been shown to encompass two forms, based on whether the *IGH@* genes do or do not display physiological SHM. A reasonable expectation might be that those with SHM express AID, whereas those that do not would not. In fact, the opposite has been observed.⁷⁰ Putative explanations include the suggestion that the SHM occurred at an earlier stage of B-cell development in those that are AID negative, whereas an impaired AID machinery or alternative splice variants might account for the lack of SHM in those cases that are AID positive.

Whatever the explanation for the activity and function (or lack thereof) of AID, accumulating data underscore the central role that this enzyme has in the genesis of a

large proportion of B-cell lymphomas and perhaps many other neoplasms as well.⁷¹ When ectopically expressed in mice, lung tumors and T-cell lymphomas (but curiously not B-cell lymphomas) develop. In humans, AID can be expressed by testicular germ cell tumors. Its expression in follicular dendritic cells may be associated with the development of MALT (mucosa-associated lymphoid tissue) lymphomas.⁷² AID expression can also be induced by a number of viruses, including EBV, HIV, and HCV, each of which have been implicated in the pathogenesis of B-cell lymphomas. In general, those B-cell lymphomas that are AID positive tend to be associated with an adverse prognosis.⁷¹ Furthermore, chronic inflammation can induce AID expression. This is especially intriguing because there is an extensive body of literature proposing the role of chronic inflammation in the development of cancer, with the proposal that >15% of human tumors can be attributed to chronic inflammation.⁷³ Although it is not apparently the target of translocations or mutations itself, it might not be too speculative to consider AID as an oncogene, given its central role in tumorigenesis, by virtue of its ability to cause both translocations and mutations of bona fide oncogenes.^{74,75} Allied to this, AID might well be a promising therapeutic target, which might lead to the suppression of oncogenic DNA damage. Of note, inhibition of this key enzyme might not lead to any anticipated serious immunological toxicities, because patients with congenital AID deficiency causing the autosomal recessive form of hyper-IgM syndrome actually show minimal lethal manifestations.⁷⁶

Pitfalls and Caveats of Immunoglobulin Gene Clonality Testing

As alluded to in the introduction, there are well-defined diagnostic applications of *IG* clonality assessment in the clinical laboratory. With the numerous refinements of testing methodologies, the added value of such analyses has become increasingly robust. Nevertheless, there are a variety of circumstances in which such assays, interpreted in molecular isolation, can be misleading, and thus it is important to be aware of the occurrence of both false-positive and false-negative results.

Because these are PCR-based assays, with the ability to amplify single templates many orders of magnitude, false-positive monoclonality may occur through contamination. However, this ought not to be an issue in contemporary practice, with the concurrent evaluation of numerous negative controls, including i) polyclonal genomic DNA controls, ii) controls without any template, and iii) genomic DNA controls that do not harbor rearrangements, polyclonal or otherwise. Pseudoclonality can be confounding in scenarios in which there is a paucity of lymphocytes; this ought to be recognized both by integration with the tandem histology and by a failure to demonstrate reproducibly the same clonal rearrangement on repeat analysis.⁷⁷ There are a variety of reactive inflammatory and infectious scenarios in which dominant clones can emerge. These include *H. pylori*-induced gastritis, numerous viral infections, and autoimmune diseases such as Sjögren syndrome and

rheumatoid arthritis.^{78–82} Many of these reactive conditions, however, are associated with the subsequent development of bona fide lymphomas, underscoring the need for especially careful histological correlation in such instances. However, with improved clonality testing and more rigorous adherence to histological criteria, it is possible to identify more robust correlations between clonality detection and overt, or emerging, lymphoma.⁸³ Other conditions that might be associated with clonality in the absence of neoplasia include immune reconstitution after stem cell transplant and immune responses to tumors, although these are typically associated with the emergence of *TCR*-detectable, rather than *IG*-detectable, clones.^{84,85} False-positivity may also occur as a consequence of contamination by chemicals used in the routine processing of the tissue, including eosin⁸⁶ and zinc formalin.⁸⁷ It is also not altogether surprising that the complexity of the BIOMED-2 reactions might also yield false-positive products, and thus it is crucial to be aware of such recurrent spurious results.¹⁰

The basis for false-negative results might be best considered in three broad categories: preanalytic, technical, and biological. Preanalytic issues that may compromise the ability to detect a clonal rearrangement (or any molecular genetic phenomenon) include template degradation, the effects of fixation, and lack of representative sampling. Although DNA is relatively robust compared with RNA, suboptimal fixation may compromise the ability to extract sufficiently intact DNA; as a corollary, when PCR outcomes are compared on tandem fixed and frozen specimens, it is clear that fixation in general compromised the yield of DNA.⁸⁸ It is also well recognized that certain fixatives, in particular the preferred fixative of hematopathologists, B5, compromise the ability to perform PCR. Similarly, the modality of decalcification of bone marrows can impact the ability to obtain intact DNA for PCR, with neutral pH EDTA far preferable to HCl with its attendant depurination activity. Without assessing appropriate histology, it is not always clear that appropriate lesional tissue has been submitted for molecular analysis, and it has been recommended by some that H&E-stained slides be prepared on sections obtained immediately prior and subsequent to those used for DNA extraction.⁸⁹ The use of consensus, rather than family-specific *IGH@ V* primers, and targeting of CDR3 only had been a major cause of false-negative results; however, this ought to be less of an issue with the use of the improved and standardized BIOMED-2 primers.

Numerous biological factors may compromise *IG* PCR analysis. For example, partial DJ rearrangements, which may be seen in pro-B-cell acute lymphoblastic leukemias and in variable degrees in mature B-cell lymphomas, would not be detected with assays that only use V primers, to detect V(D)J rearrangements.^{46,47} Theoretically, excess TdT-mediated endonuclease activity might result in the removal of part of the FR3 or FR4 (J) region, thus compromising primer annealing; however, primers have been designed to render this possibility rather remote. Oligoclonality, especially in precursor B-cell acute lymphoblastic leukemias can result in the failure to detect a clone that becomes dominant at relapse.⁹⁰ Ongoing rearrangements in a precursor B-cell acute lymphoblastic

leukemia can similarly compromise detection at relapse.⁹¹ Although the establishment of reference ranges for the expected sizes of rearranged bands or peaks is central to the interpretation of *IG* PCR studies, it is worth recalling that such reference ranges only encompass the 5th to 95th percentiles. Accordingly, it is not always necessarily appropriate to dismiss dominant bands or peaks that reside just beyond these boundaries.¹⁰ Furthermore, even products considerably smaller or larger than expected might have a biological basis and should not necessarily be dismissed as being spurious and inappropriately scored as negative. Thus, undersized products might be the result of deletional events,⁹² whereas oversized products may occur due to primers annealing to a J segment downstream of the one used in the VDJ rearrangement.⁹³ Perhaps the most problematic cause of false negatives is due to SHM, which, as alluded to under Evolution of Testing Methodologies to Assess Immunoglobulin Gene Rearrangements, is largely (but not exclusively) a limitation in the evaluation of follicular lymphoma, with the annealing of primers, especially FR3 primers, being severely compromised. However, and as noted, this is mostly circumventable by an inclusion of both *IGK@* and, to some degree, *IGH@* DJ analysis. Deletion of large portions of the *IGH@* gene that is often allied to SHM and that may be seen in up to 10% of lymphomas can also result in false-negative results.⁹⁴ Losses of DNA that accompany physiological VDJ rearrangements may even compromise FISH analyses, creating a false impression of pathological 14q32 deletions.⁹⁵

The multitude of scenarios in which false-positive and false-negative results may arise underscores the need to always interpret molecular data in context. Interpretation in isolation might well lead to misdiagnoses. Accordingly, it is essential that *IG* PCR data always be integrated with clinical, pathological, and immunophenotypic data to best harness the value of such analyses. Depending on the specific scenario, however, it is incumbent on a clinical molecular diagnostics laboratory to minimize false calls. Thus, a simple strategy of performing all analyses in duplicate might avoid misconstruing pseudoclonality. Undersized and oversized products, which might well represent bona fide clonal products, should perhaps be confirmed by sequencing, depending on the clinicopathological context. It might also be prudent, with the ability of current PCR protocols to now target a multitude of loci, to consider the value of finding evidence of clonality at more than one locus to bolster a call of bona fide clonality.

In summary, an appreciation of the mechanisms involved in the numerous physiological pathways affecting the shape of the immunoglobulin genes (and hence antibody molecules) is central to an understanding of both B-cell development, with regard to the generation of immunological diversity, and how these pathways can go awry, as a step down the road of neoplastic transformation. With this background, an evaluation of the evolution of immunoglobulin clonality testing can be placed in perspective. Furthermore, recognition of how these genetic changes with good intentions can turn bad is paramount to contemporary views of B-cell lymphomagenesis. Al-

though there have been significant improvements in assay design and the advantages afforded by clonality assessment of the immunoglobulin genes, it is important to remain cognizant of the numerous associated diagnostic pitfalls.

Acknowledgments

I thank Eline Nina Luning Prak, MD, PhD, for her critical reading of this manuscript and her numerous extremely helpful comments and suggestions.

References

1. Paessler ME, Bagg A: Use of molecular techniques in the analysis of hematologic diseases. *Hematology: Basic Principles and Practice*, ed 4. Edited by R Hoffman, SJ Shattil, B Furie, HJ Cohen, LE Silberstein, P McGlave, M Strauss, EJ Benz. Elsevier, Philadelphia 2005, pp 2713–2726
2. Tefferi A, Vardiman JW: Classification and diagnosis of myeloproliferative neoplasms: the 2008 World Health Organization criteria and point-of-care diagnostic algorithms. *Leukemia* 2008, 22:14–22
3. Leich E, Hartmann EM, Burek C, Ott G, Rosenwald A: Diagnostic and prognostic significance of gene expression profiling in lymphomas. *APMIS* 2007, 115:1135–1146
4. Bagg A: Role of molecular studies in the classification of lymphoma. *Expert Rev Mol Diagn* 2004, 4:83–97
5. Szczepański T: Why and how to quantify minimal residual disease in acute lymphoblastic leukemia? *Leukemia* 2007, 21:622–626
6. Ou J, Vergilio JA, Bagg A: Molecular diagnosis and monitoring in the clinical management of patients with chronic myelogenous leukemia treated with tyrosine kinase inhibitors. *Am J Hematol* 2008, 83:296–302
7. Dik WA, Nadel B, Przybylski GK, Asnafi V, Grabarczyk P, Navarro JM, Verhaaf B, Schmidt CA, Macintyre EA, van Dongen JJ, Langerak AW: Different chromosomal breakpoints impact the level of LMO2 expression in T-ALL. *Blood* 2007, 110:388–392
8. van Krieken JH, Langerak AW, Macintyre EA, Kneba M, Hodges E, Sanz RG, Morgan GJ, Parreira A, Molina TJ, Cabeçadas J, Gaulard P, Jasani B, Garcia JF, Ott M, Hannsmann ML, Berger F, Hummel M, Davi F, Brüggemann M, Lavender FL, Schuurin E, Evans PA, White H, Salles G, Groenen PJ, Gameiro P, Pott Ch, Dongen JJ: Improved reliability of lymphoma diagnostics via PCR-based clonality testing: report of the BIOMED-2 Concerted Action BHM4-CT98-3936. *Leukemia* 2007, 21:201–206
9. Bagg A: Immunoglobulin and T-cell receptor gene rearrangements: minding your B's and T's in assessing lineage and clonality in neoplastic lymphoproliferative disorders. *J Mol Diagn* 2006, 8:426–429
10. van Dongen JJ, Langerak AW, Brüggemann M, Evans PA, Hummel M, Lavender FL, Delabesse E, Davi F, Schuurin E, García-Sanz R, van Krieken JH, Droese J, González D, Bastard C, White HE, Spaargaren M, González M, Parreira A, Smith JL, Morgan GJ, Kneba M, Macintyre EA: Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia* 2003, 17:2257–2317
11. Morice WG, Kimlinger T, Katzmann JA, Lust JA, Heimgartner PJ, Halling KC, Hanson CA: Flow cytometric assessment of TCR-Vbeta expression in the evaluation of peripheral blood involvement by T-cell lymphoproliferative disorders: a comparison with conventional T-cell immunophenotyping and molecular genetic techniques. *Am J Clin Pathol* 2004, 121:373–383
12. Beck RC, Stahl S, O'Keefe CL, Maciejewski JP, Theil KS, Hsi ED: Detection of mature T-cell leukemias by flow cytometry using anti-T-cell receptor V beta antibodies. *Am J Clin Pathol* 2003, 120:785–794
13. van der Velden VH, Panzer-Grümayer ER, Cazzaniga G, Flohr T, Sutton R, Schrauder A, Basso G, Schrappe M, Wijkhuijs JM, Konrad M, Bartram CR, Masera G, Biondi A, van Dongen JJ: Optimization of PCR-based minimal residual disease diagnostics for childhood acute lymphoblastic leukemia in a multi-center setting. *Leukemia* 2007, 21:706–713

14. Raff T, Gökbüget N, Lüschen S, Reutzel R, Ritgen M, Irmer S, Böttcher S, Horst HA, Kneba M, Hoelzer D, Brüggemann M, GMALL Study Group: Molecular relapse in adult standard-risk ALL patients detected by prospective MRD monitoring during and after maintenance treatment: data from the GMALL 06/99 and 07/03 trials. *Blood* 2007, 109:910–915
15. Cervetti G, Galimberti S, Andreazzoli F, Fazzi R, Cecconi N, Caracciolo F, Petrin M: Rituximab as treatment for minimal residual disease in hairy cell leukaemia. *Eur J Haematol* 2004, 73:412–417
16. Kim YJ, Kim DW, Lee S, Chung NG, Hwang JY, Kim YL, Min CK, Kim CC: Preemptive treatment of minimal residual disease post transplant in CML using real-time quantitative RT-PCR: a prospective, randomized trial. *Bone Marrow Transplant* 2004, 33:535–542
17. Corradini P, Carrabba MG, Farina L: Molecular methods used for the detection of autologous graft contamination in lymphoid disorders. *Methods Mol Med* 2007, 134:179–196
18. Kallakury BV, Hartmann DP, Cossman J, Gootenberg JE, Bagg A: Post-therapy surveillance of B-cell precursor acute lymphoblastic leukemia: the value of polymerase chain reaction and limitations of flow cytometry. *Am J Clin Pathol* 1999, 111:759–766
19. van Zelm MC, van der Burg M, de Ridder D, Barendregt BH, de Haas EF, Reinders MJ, Lankester AC, Révész T, Staal FJ, van Dongen JJ: Ig gene rearrangement steps are initiated in early human precursor B cell subsets and correlate with specific transcription factor expression. *J Immunol* 2005, 175:5912–5922
20. Kipps TJ: Human B cell biology. *Int Rev Immunol* 1997, 15:243–264
21. Janeway C: Immunobiology. The Immune System in Health and Disease, ed 6. New York, Garland Science, 2005
22. Sagaert X, Sprangers B, De Wolf-Peeters C: The dynamics of the B follicle: understanding the normal counterpart of B-cell-derived malignancies. *Leukemia* 2007, 21:1378–1386
23. Klein U, Dalla-Favera R: Germinal centres: role in B-cell physiology and malignancy. *Nat Rev Immunol* 2008, 8:22–33
24. Ramiro A, San-Martin BR, McBride K, Jankovic M, Barreto V, Nussenzweig A, Nussenzweig MC: The role of activation-induced deaminase in antibody diversification and chromosome translocations. *Adv Immunol* 2007, 94:75–107
25. Moldenhauer G, Popov SW, Wotschke B, Brüderlein S, Riedl P, Fissolo N, Schirmbeck R, Ritz O, Möller P, Leithäuser F: AID expression identifies interfollicular large B cells as putative precursors of mature B-cell malignancies. *Blood* 2006, 107:2470–2473
26. Cattoretti G, Büttner M, Shaknovich R, Kremmer E, Alobeid B, Niedobitek G: Nuclear and cytoplasmic AID in extrafollicular and germinal center B cells. *Blood* 2006, 107:3967
27. Odegard VH, Schatz DG: Targeting of somatic hypermutation. *Nat Rev Immunol* 2006, 6:573–583
28. William J, Euler C, Christensen S, Shlomchik MJ: Evolution of auto-antibody responses via somatic hypermutation outside of germinal centers. *Science* 2002, 297:2066–2070
29. Barreto V, Reina-San-Martin B, Ramiro AR, McBride KM, Nussenzweig MC: C-terminal deletion of AID uncouples class switch recombination from somatic hypermutation and gene conversion. *Mol Cell* 2003, 12:501–508
30. Toellner KM, Gulbranson-Judge A, Taylor DR, Sze DM, MacLennan IC: Immunoglobulin switch transcript production in vivo related to the site and time of antigen-specific B cell activation. *J Exp Med* 1996, 183:2303–2312
31. Tang ES, Martin A: Immunoglobulin gene conversion: synthesizing antibody diversification and DNA repair. *DNA Repair* 2007, 6:1557–1571
32. Han S, Zheng B, Schatz DG, Spanopoulou E, Kelsoe G: Neoteny in lymphocytes: rag1 and Rag2 expression in germinal center B cells. *Science* 1996, 274:2094–2097
33. Gärtner F, Alt FW, Monroe RJ, Seidl KJ: Antigen-independent appearance of recombination activating gene (RAG)-positive bone marrow B cells in the spleens of immunized mice. *J Exp Med* 2000, 192:1745–1754
34. van Dongen JJ, Wolvers-Tettero IL: Analysis of immunoglobulin and T cell receptor genes. Part II: possibilities and limitations in the diagnosis and management of lymphoproliferative diseases and related disorders. *Clin Chim Acta* 1991, 198:93–174
35. Papadopoulos KP, Bagg A, Bezwoda WR, Mendelow BV: The routine diagnostic utility of immunoglobulin and T-cell receptor gene rearrangements in lymphoproliferative disorders. *Am J Clin Pathol* 1989, 91:633–638
36. McCarthy KP, Sloane JP, Wiedemann LM: Rapid method for distinguishing clonal from polyclonal B cell populations in surgical biopsy specimens. *J Clin Pathol* 1990, 43:429–432
37. Sioutos N, Bagg A, Michaud GY, Irving SG, Hartmann DP, Siragy H, Oliveri DR, Locker J, Cossman J: Polymerase chain reaction versus Southern blot hybridization: detection of immunoglobulin heavy-chain gene rearrangements. *Diagn Mol Pathol* 1995, 4:8–13
38. Trainor KJ, Brisco MJ, Story CJ, Morley AA: Monoclonality in B-lymphoproliferative disorders detected at the DNA level. *Blood* 1990, 75:2220–2222
39. González M, González D, López-Pérez R, García-Sanz R, Chillón MC, Balanzategui A, Mateos MV, Alaejos I, Langerak AW, Orfão A, Van Dongen JJ, San Miguel JF: Heteroduplex analysis of VDJ amplified segments from rearranged IgH genes for clonality assessments in B-cell non-Hodgkin's lymphoma: a comparison between different strategies. *Haematologica* 1999, 84:779–784
40. Derksen PJ, Langerak AW, Kerkhof E, Wolvers-Tettero IL, Boor PP, Mulder AH, Vrints LW, Coebergh JW, van Krieken JH, Schuurin E, Kluin PM, van Dongen JJ: Comparison of different polymerase chain reaction-based approaches for clonality assessment of immunoglobulin heavy-chain gene rearrangements in B-cell neoplasia. *Mod Pathol* 1999, 12:794–805
41. Bagg A, Brazier RM, Arber DA, Bijwaard KE, Chu AY: Immunoglobulin heavy chain gene analysis in lymphomas: a multi-center study demonstrating the heterogeneity of performance of polymerase chain reaction assays. *J Mol Diagn* 2002, 4:81–89
42. Evans PA, Pott CH, Groenen PJ, Salles G, Davi F, Berger F, Garcia JF, van Krieken JH, Pals S, Kluin P, Schuurin E, Spaargaren M, Boone E, González D, Martinez B, Villuendas R, Gameiro P, Diss TC, Mills K, Morgan GJ, Carter GI, Milner BJ, Pearson D, Hummel M, Jung W, Ott M, Canioni D, Beldjord K, Bastard C, Delfau-Larue MH, van Dongen JJ, Molina TJ, Cabecadas J: Significantly improved PCR-based clonality testing in B-cell malignancies by use of multiple immunoglobulin gene targets: Report of the BIOMED-2 Concerted Action BHM4-CT98–3936. *Leukemia* 2007, 21:207–214
43. Liu H, Bench AJ, Bacon CM, Payne K, Huang Y, Scott MA, Erber WN, Grant JW, Du MQ: A practical strategy for the routine use of BIOMED-2 PCR assays for detection of B- and T-cell clonality in diagnostic haematopathology. *Br J Haematol* 2007, 138:31–43
44. McClure RF, Kaur P, Pagel E, Ouillette PD, Holtegaard CE, Treptow CL, Kurtin PJ: Validation of immunoglobulin gene rearrangement detection by PCR using commercially available BIOMED-2 primers. *Leukemia* 2006, 20:176–179
45. Jolly CJ, Wagner SD, Rada C, Klix N, Milstein C, Neuberg MS: The targeting of somatic hypermutation. *Semin Immunol* 1996, 8:159–168
46. Szczepański T, Willemse MJ, van Wering ER, van Weerden JF, Kamps WA, van Dongen JJ: Precursor-B-ALL with D(H)-J(H) gene rearrangements have an immature immunogenotype with a high frequency of oligoclonality and hyperdiploidy of chromosome 14. *Leukemia* 2001, 15:1415–1423
47. González D, Balanzategui A, García-Sanz R, Gutiérrez N, Seabra C, van Dongen JJ, González M, San Miguel JF: Incomplete DJH rearrangements of the IgH gene are frequent in multiple myeloma patients: immunobiological characteristics and clinical implications. *Leukemia* 2003, 17:1398–1403
48. Halldórsdóttir AM, Zehnauer BA, Burack WR: Application of BIOMED-2 clonality assays to formalin-fixed paraffin embedded follicular lymphoma specimens: superior performance of the IGK assays compared to IGH for suboptimal specimens. *Leuk Lymphoma* 2007, 48:1338–1343
49. Gong JZ, Zheng S, Chiarle R, De Wolf-Peeters C, Palestro G, Frizzera G, Inghirami G: Detection of immunoglobulin kappa light chain rearrangements by polymerase chain reaction: an improved method for detecting clonal B-cell lymphoproliferative disorders. *Am J Pathol* 1999, 155:355–363
50. Catherwood MA, Gonzalez D, Patton C, Dobbin E, Venkatraman L, Alexander HD: Improved clonality assessment in germinal centre/post-germinal centre non-Hodgkin's lymphomas with high rates of somatic hypermutation. *J Clin Pathol* 2007, 60:524–528
51. Kosmas C, Stamatopoulos K, Papadaki T, Belessi C, Yataganas X, Anagnostou D, Loukopoulou D: Somatic hypermutation of immunoglobulin variable region genes: focus on follicular lymphoma and multiple myeloma. *Immunol Rev* 1998, 162:281–292
52. Jilani I, Keating M, Day A, William W, Kantarjian H, O'Brien S, Giles FJ,

- Albitar M: Simplified sensitive method for the detection of B-cell clonality in lymphoid malignancies. *Clin Lab Haematol* 2006, 28:325–331
53. Retamales E, Rodriguez L, Guzman L, Aguayo F, Palma M, Backhouse C, Argandona J, Riquelme E, Corvalan A: Analytical detection of immunoglobulin heavy chain gene rearrangements in gastric lymphoid infiltrates by peak area analysis of the melting curve in the LightCycler System. *J Mol Diagn* 2007, 9:351–357
54. Gra OA, Sidorova JV, Nikitin EA, Turygin AY, Surzhikov SA, Melikyan AL, Sudarikov AB, Zasedatelev AS, Nasedkina TV: Analysis of T-cell receptor-gamma gene rearrangements using oligonucleotide microchip: a novel approach for the determination of T-cell clonality. *J Mol Diagn* 2007, 9:249–257
55. Welzel N, Le T, Marculescu R, Mitterbauer G, Chott A, Pott C, Kneba M, Du MQ, Kusec R, Drach J, Raderer M, Mannhalter C, Lechner K, Nadel B, Jaeger U: Templated nucleotide addition and immunoglobulin JH-gene utilization in t(11;14) junctions: implications for the mechanism of translocation and the origin of mantle cell lymphoma. *Cancer Res* 2001, 61:1629–1636
56. Tsujimoto Y, Gorham J, Cossman J, Jaffe E, Croce CM: The t(14;18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining. *Science* 1985, 229:1390–1393
57. Tsujimoto Y, Louie E, Bashir MM, Croce CM: The reciprocal partners of both the t(14;18) and the t(11;14) translocations involved in B-cell neoplasms are rearranged by the same mechanism. *Oncogene* 1988, 2:347–351
58. Raghavan SC, Swanson PC, Wu X, Hsieh CL, Lieber MR: A non-B-DNA structure at the Bcl-2 major breakpoint region is cleaved by the RAG complex. *Nature* 2004, 428:88–93
59. Haines BB, Ryu CJ, Chen J: Recombination activating genes (RAG) in lymphoma development. *Cell Cycle* 2006, 5:913–916
60. Aplan PD, Lombardi DP, Ginsberg AM, Cossman J, Bertness VL, Kirsch IR: Disruption of the human SCL locus by "illegitimate" V-(D)-J recombinase activity. *Science* 1990, 250:1426–1429
61. Mullighan CG, Miller CB, Radtke I, Phillips LA, Dalton J, Ma J, White D, Hughes TP, Le Beau MM, Pui CH, Relling MV, Shurtleff SA, Downing JR: BCR-ABL1 lymphoblastic leukaemia is characterized by the deletion of Ikaros. *Nature* 2008, 453:110–114
62. Küppers R: Mechanisms of B-cell lymphoma pathogenesis. *Nat Rev Cancer* 2005, 5:251–262
63. Chng WJ, Glebov O, Bergsagel PL, Kuehl WM: Genetic events in the pathogenesis of multiple myeloma. *Best Pract Res Clin Haematol* 2007, 20:571–596
64. Phan RT, Dalla-Favera R: The BCL6 proto-oncogene suppresses p53 expression in germinal-centre B cells. *Nature* 2004, 432:635–639
65. Ranuncolo SM, Polo JM, Dierov J, Singer M, Kuo T, Grealley J, Green R, Carroll M, Melnick A: Bcl-6 mediates the germinal center B cell phenotype and lymphomagenesis through transcriptional repression of the DNA-damage sensor ATR. *Nat Immunol* 2007, 8:705–714
66. Pasqualucci L, Neumeister P, Goossens T, Nanjangud G, Chaganti RS, Küppers R, Dalla-Favera R: Hypermutation of multiple proto-oncogenes in B-cell diffuse large-cell lymphomas. *Nature* 2001, 412:341–346
67. Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, Boldrick JC, Sabet H, Tran T, Yu X, Powell JL, Yang L, Marti GE, Moore T, Hudson J Jr, Lu L, Lewis DB, Tibshirani R, Sherlock G, Chan WC, Greiner TC, Weisenburger DD, Armitage JO, Warnke R, Levy R, Wilson W, Grever MR, Byrd JC, Botstein D, Brown PO, Staudt LM: Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 2000, 403:503–511
68. Lenz G, Nagel I, Siebert R, Roschke AV, Sanger W, Wright GW, Dave SS, Tan B, Zhao H, Rosenwald A, Muller-Hermelink HK, Gascoyne RD, Campo E, Jaffe ES, Smeland EB, Fisher RI, Kuehl WM, Chan WC, Staudt LM: Aberrant immunoglobulin class switch recombination and switch translocations in activated B cell-like diffuse large B cell lymphoma. *J Exp Med* 2007, 204:633–643
69. Wright G, Tan B, Rosenwald A, Hurt EH, Wiestner A, Staudt LM: A gene expression-based method to diagnose clinically distinct subgroups of diffuse large B cell lymphoma. *Proc Natl Acad Sci USA* 2003, 100:9991–9996
70. McCarthy H, Wierda WG, Barron LL, Cromwell CC, Wang J, Coombes KR, Rangel R, Elenitoba-Johnson KS, Keating MJ, Abruzzo LV: High expression of activation-induced cytidine deaminase (AID) and splice variants is a distinctive feature of poor-prognosis chronic lymphocytic leukemia. *Blood* 2003, 101:4903–4908
71. Okazaki IM, Kotani A, Honjo T: Role of AID in tumorigenesis. *Adv Immunol* 2007, 94:245–273
72. Bombardieri M, Barone F, Humby F, Kelly S, McGurk M, Morgan P, Challacombe S, De Vita S, Valesini G, Spencer J, Pitzalis C: Activation-induced cytidine deaminase expression in follicular dendritic cell networks and interfollicular large B cells supports functionality of ectopic lymphoid neogenesis in autoimmune sialoadenitis and MALT lymphoma in Sjögren's syndrome. *J Immunol* 2007, 179:4929–4938
73. Lu H, Ouyang W, Huang C: Inflammation, a key event in cancer development. *Mol Cancer Res* 2006, 4:221–233
74. Pasqualucci L, Bhagat G, Jankovic M, Compagno M, Smith P, Muramatsu M, Honjo T, Morse HC III, Nussenzweig MC, Dalla-Favera R: AID is required for germinal center-derived lymphomagenesis. *Nat Genet* 2008, 40:108–112
75. Chesi M, Robbiani DF, Sebag M, Chng WJ, Affer M, Tiedemann R, Valdez R, Palmer SE, Haas SS, Stewart AK, Fonseca R, Kremer R, Cattoretti G, Bergsagel PL: AID-dependent activation of a MYC transgene induces multiple myeloma in a conditional mouse model of post-germinal center malignancies. *Cancer Cell* 2008, 13:167–180
76. Revy P, Muto T, Levy Y, Geissmann F, Plebani A, Sanal O, Catalan N, Forveille M, Dufourcq-Labeau R, Gennery A, Tezcan I, Ersoy F, Kayserili H, Ugazio AG, Brousse N, Muramatsu M, Notarangelo LD, Kinoshita K, Honjo T, Fischer A, Durandy A: Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). *Cell* 2000, 102:565–575
77. Elenitoba-Johnson KS, Bohling SD, Mitchell RS, Brown MS, Roboto-rye RS: PCR analysis of the immunoglobulin heavy chain gene in polyclonal processes can yield pseudoclonal bands as an artifact of low B cell number. *J Mol Diagn* 2000, 2:92–96
78. Landau DA, Saadoun D, Calabrese LH, Cacoub P: The pathophysiology of HCV induced B-cell clonal disorders. *Autoimmun Rev* 2007, 6:581–587
79. Dong L, Masaki Y, Takegami T, Jin ZX, Huang CR, Fukushima T, Sawaki T, Kawanami T, Saeki T, Kitagawa K, Sugai S, Okazaki T, Hirose Y, Umehara H: Clonality analysis of lymphoproliferative disorders in patients with Sjögren's syndrome. *Clin Exp Immunol* 2007, 150:279–284
80. Moshynska OV, Saxena A: Clonal relationship between Hashimoto thyroiditis and thyroid lymphoma. *J Clin Pathol* 2008, 61:438–444
81. Engels K, Oeschger S, Hansmann ML, Hillebrand M, Kriener S: Bone marrow trephines containing lymphoid aggregates from patients with rheumatoid and other autoimmune disorders frequently show clonal B-cell infiltrates. *Hum Pathol* 2007, 38:1402–1411
82. Nam-Cha SH, San-Millán B, Mollejo M, García-Cosío M, Garijo G, Gomez M, Warnke RA, Jaffe ES, Piris MA: Light-chain-restricted germinal centres in reactive lymphadenitis: report of eight cases. *Histopathology* 2008, 52:436–444
83. Hummel M, Oeschger S, Barth TF, Loddenkemper C, Cogliatti SB, Marx A, Wacker HH, Feller AC, Bernd HW, Hansmann ML, Stein H, Möller P: Wotherspoon criteria combined with B cell clonality analysis by advanced polymerase chain reaction technology discriminates covert gastric marginal zone lymphoma from chronic gastritis. *Gut* 2006, 55:782–787
84. Gorochov G, Debré P, Leblond V, Sadat-Sowti B, Sigaux F, Autran B: Oligoclonal expansion of CD8+ CD57+ T cells with restricted T-cell receptor beta chain variability after bone marrow transplantation. *Blood* 1994, 83:587–595
85. Lim SH, Badros A, Lue C, Barlogie B: Distinct T-cell clonal expansion in the vicinity of tumor cells in plasmacytoma. *Cancer* 2001, 91:900–908
86. Murphy KM, Braylan R, Almasri N, Foss R, Rimsa L: IgH PCR of zinc formalin-fixed, paraffin-embedded non-lymphomatous gastric samples produces artifactual "clonal" bands not observed in paired tissues unexposed to zinc formalin. *J Mol Diagn* 2002, 4:159–163
87. Arber DA, Brazier RM, Bagg A, Bijwaard KE: Evaluation of T cell receptor testing in lymphoid neoplasms: results of a multicenter study of 29 extracted DNA and paraffin-embedded samples. *J Mol Diagn* 2001, 3:133–140

89. Langerak AW, Groenen PJTA, van Krieken JHJM, van Dongen JJM: Immunoglobulin/T-cell receptor diagnostics. *Expert Opin Med Diagn* 2007, 1:451–461
90. Choi S, Henderson MJ, Kwan E, Beesley AH, Sutton R, Bahar AY, Giles J, Venn NC, Pozza LD, Baker DL, Marshall GM, Kees UR, Haber M, Norris MD: Relapse in children with acute lymphoblastic leukemia involving selection of a preexisting drug-resistant subclone. *Blood* 2007, 110:632–639
91. Panzer-Grümayer ER, Cazzaniga G, van der Velden VH, del Giudice L, Peham M, Mann G, Eckert C, Schrauder A, Germano G, Harbott J, Basso G, Biondi A, van Dongen JJ, Gadner H, Haas OA: Immunogenotype changes prevail in relapses of young children with TEL-AML1-positive acute lymphoblastic leukemia and derive mainly from clonal selection. *Clin Cancer Res* 2005, 11:7720–7727
92. Vargas RL, Felgar RE, Rothberg PG: Detection of clonality in lymphoproliferations using PCR of the antigen receptor genes: does size matter? *Leuk Res* 2008, 32:335–338
93. Langerak AW: Undersized, oversized? It is not one-size-fits-all in lymphoid clonality detection. *Leuk Res* 2008, 32:203–204
94. Goossens T, Klein U, Küppers R: Frequent occurrence of deletions and duplications during somatic hypermutation: implications for oncogene translocations and heavy chain disease. *Proc Natl Acad Sci USA* 1998, 95:2463–2468
95. Wlodarska I, Matthews C, Veyt E, Pospisilova H, Catherwood MA, Poulsen TS, Vanhentenrijk V, Ibbotson R, Vandenberghe P, Morris TC, Alexander HD: Telomeric IGH losses detectable by fluorescence in situ hybridization in chronic lymphocytic leukemia reflect somatic VH recombination events. *J Mol Diagn* 2007, 9:47–54