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# Commentary

# Detection of *BCL2* Rearrangements in Follicular Lymphoma

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The identification and characterization of recurrent chromosomal translocations has improved tumor classification and yielded numerous insights into tumor pathogenesis, as such events typically lead to the dysregulation of proteins that control critical cellular processes, such as apoptosis, proliferation, differentiation, and immortalization. A prototypical example of such a chromosomal translocation is the (14;18)(q32;q32), which is highly associated with the B cell neoplasm follicular lymphoma.[1] Molecular analyses of DNA isolated from follicular lymphoma cells in the mid-1980's showed that the t(14;18) creates a derivative chromosome 14 on which the *BCL2* gene is juxtaposed to immunoglobulin heavy chain gene (*IgH*) sequences,[2–5] including enhancer sequences that override normal *BCL2* gene control elements and drive inappropriately high levels of *BCL2* expression in follicle center B cells.[6,7] Subsequently, several groups noted that enforced expression of *BCL2* transgenes in murine hematopoietic cells promoted cell survival,[8,9] the first indication of the anti-apoptotic activity of BCL2. *BCL2* transgenic mice also showed increased susceptibility to autoimmune disease[10] and B cell lymphoma,[11] observations that helped to foster the now generally accepted idea that dysregulation of apoptotic pathways is important in the pathogenesis of many forms of cancer and autoimmune disorders. These effects of BCL2 appear to be mediated through sequestration of "BH3 domain only" pro-apoptotic proteins such as BAD, BIM, and NOXA,[12] which resets the apoptotic "rheostat" toward increased resistance to programmed cell death.

In addition to its impact on our thinking about cancer pathophysiology, the molecular characterization of the t(14;18) and its downstream consequences has influenced our approach to the diagnosis of hematological malignancy in several ways. The t(14;18) was an early example of an acquired genetic lesion that was strongly associated with a particular neoplasm, and thus of utility in tumor classification. Juxtaposed *BCL2* and *IgH* DNA sequences provide a tumor-specific marker that can be exploited in the detection of minimal residual disease.[13,14] Furthermore, because BCL2 is down-regulated in normal germinal center B cells,[15] its expression (or lack thereof) can help to distinguish reactive and neoplastic follicles.[16] Finally, the presence of the t(14;18) has been suggested to be a marker of poor prognosis in diffuse large B cell lymphoma[17,18] (possibly because some such tumors arise through transformation of unrecognized underlying follicular lymphomas), although it must be said that more recent studies have not detected such a correlation.[19–21]

These relationships have prompted the development of multiple methods that aim to detect the t(14;18) in tissue specimens. In this issue of *The American Journal of Pathology*, Albinger-Hegyi et al[22] report a new set of polymerase chain reaction (PCR)-based tests for detection of the *BCL2/IgH* fusion genes that offer some significant advantages in sensitivity over other more commonly used PCR-based methods. This commentary will attempt to place this contribution in context by discussing the advantages and limitations of various tests that aim to detect the t(14;18) or its downstream consequences.

## *What Is the True Incidence of the t(14;18) in Follicular Lymphoma?*

Determination of the sensitivity of any test obviously requires one to be able to identify true positives, but the reported incidence of the t(14;18) in follicular lymphoma varies widely. The original gold standard for detection of the t(14;18) was karyotypic analysis. In early karyotypic studies from Yunis' group,[1] >80% of follicular lymphomas were associated with the t(14;18). However, few centers send lymphoma specimens for routine karyotyping, at least in part because of issues related to reimbursement and the labor-intensive nature of metaphase

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chromosome preparation and analysis. Karytoypic analysis may also be prone to false negative results when applied to tumors, such as follicular lymphoma, with low growth fractions.

As an alternative, molecular diagnostic laboratories began to offer a variety of tests for the t(14;18), which take advantage of the fact that in the vast majority of t(14;18)s, the chromosomal breakpoints on chromosome 18 fall within non-coding sequences lying 3′ of the *BCL2* locus. Prior work revealed two breakpoint clusters; a major breakpoint region (MBR) located within exon 3 of *BCL2*, and a minor cluster region (mcr) located ~25 kb 3′ of exon 3.[23] The breakpoints on chromosome 14 occur most commonly just 5′ of *IgH* $J_H$ segments within sequences that typically show evidence of exonucleolytic "nibbling," N-bp additions, and (in a subset of cases) D segment addition,[13,24] events that occur normally during attempted V(D)J recombination. Less commonly, *IgH* breakpoints may occur at sites 3′ of the $J_H$ segments, or rarely even in Ig switch regions. *BCL2* mRNA expression is up-regulated in the translocated allele through the action of *IgH* E[$\mu$] enhancer sequences, which are highly active in germinal center B cells. In the cases of rearrangements falling in the MBR, a *BCL2/IgH fusion* mRNA transcript is produced [6,7] whereas rearrangements in the mcr lead to increased levels of a normal BCL2 mRNA. In contrast to the t(14;18) allele, the untranslocated *BCL2* allele is transcriptionally silent.[6,7]

Initial molecular tests relying on Southern blot hybridization with probes homologous to MBR and mcr sequences confirmed the strong association of *BCL2* rearrangements and follicular lymphoma. For example, Cleary's group[6] detected *BCL2* rearrangements in 64 of 72 (89%) of grade I to III follicular lymphomas, as well as ~30% of diffuse large B cell lymphomas. As Albinger-Heygi et al[22] point out, the detection rates were significantly lower in Europe (41 to 61%) and in the Far East (32 to 39%) using both cytogenetic and molecular techniques. These discrepancies led to the suggestion that follicular lymphoma is associated more commonly with the t(14;18) in the United States than Europe or elsewhere, and implied that differences in the background incidence of the t(14;18) might explain the remarkable geographic variation in follicular lymphoma incidence.

More recent analyses, however, suggest that the variation in the incidence of the t(14;18) in follicular lymphoma across studies is technical rather than real, at least when speaking of differences between U.S. and European studies. These new data were obtained using methods that eliminate most false negative results by detecting rearrangements involving *BCL2* sequences outside of the MBR and mcr. Albinger-Heygi et al (based in Switzerland)[22] identified the t(14;18) in 42 of 59 cases (71%) of follicular lymphoma using a long-range PCR method. Of note, all of these rearrangements would have also been detected by Southern blot hybridization as described by Cleary et al[23] if the restriction enzyme *Bam*HI was used to digest genomic DNA. In contrast, only 50% of these rearrangements were detected using standard PCR conditions and MBR/mcr-specific primer pairs, indicating that a high fraction of rearrangements occur outside of the MBR and mcr in the population under study. A similar recent analysis by a Japanese group using long-range PCR concluded that ~one-third of t(14;18) breakpoints fall outside of the MBR and mcr,[25] indicating that alternative breakpoints occur commonly in diverse populations.

The occurrence of the t(14;18) at high frequency in follicular lymphoma in Europe has been independently supported in another recent study. Vaandrager et al (based in the Netherlands)[26] used an elegant dual segregation fluorescence *in situ* hybridization (FISH) approach that permits the identification of *BCL2/IgH* fusion genes with high sensitivity and specificity in interphase nuclei. This method detected t(14;18)s in 35 of 40 (88%) of follicular lymphomas, an incidence nearly identical to that observed in California 15 years ago. Further work is necessary to clearly delineate the strength of association of the t(14;18) with follicular lymphoma in other populations, such as Asia, where follicular lymphoma is relatively rare. However, in the West, it appears that the t(14;18) is present in 70 to 90% of follicular lymphomas.

## *Which Test Is Best?*

Although the majority of cases of follicular lymphoma can be diagnosed by conventional morphology, the detection of t(14;18) is diagnostically useful in some cases where atypical morphological patterns yield a differential diagnosis of reactive hyperplasia or alternatively another non-Hodgkin's lymphoma with a nodular architecture. Detection of t(14;18) is also important in the detection of minimal residual disease post-therapy. In the diagnostic setting, Albinger-Hegyi et al[22] demonstrate that long-range PCR methods can be as sensitive as Southern blotting according to Weiss et al.[23] Both methods are limited in that they require high molecular weight DNA, which can only be prepared from fresh or fresh-frozen tissue. Long-range PCR offers substantial advantages over Southern blotting in principle, as the analysis can be completed in several days (versus 1 to 2 weeks for Southern blotting) and does not require the use of radioisotopes. A relative disadvantage is that PCR methods (even conventional methods) do not always "travel well" from laboratory to laboratory and PCR machine to PCR machine, and the general applicability of long-range PCR tests in molecular diagnostic laboratories remains to be determined.

When touch preparations are available or can be made from fresh-frozen tissue, new dual segregation interphase FISH tests (which can be conducted using commercially available probes from Vysis, Downer's Grove, IL) have very high sensitivity and specificity. FISH has a 2-day turn-around, assuming that molecular cytogenetics expertise is available locally. The test is best performed on air-dried touch preparations, which can be stored indefinitely at room temperature in a dessicator. Analysis of interphase nuclei for specific chromosomal aberrations by FISH is less laborious than the preparation and analysis of metaphase chromosomes, but still requires substantial time at the fluorescent microscope analyzing results. This limitation may be obviated by robotic FISH

workstations that are now under development, which promise to provide higher throughput at lower cost in the future.

Because many hematolymphoid malignancies are associated with specific chromosomal aberrations that are amenable to detection by FISH (eg, t(8;14) in Burkitt lymphoma, t(11;14) in mantle cell lymphoma, etc), we currently prepare touch preparations from all fresh hematopathology specimens in the eventuality that FISH analysis proves to be warranted. While it is also feasible to perform FISH for the t(14;18), as well as other cytogenetic aberrations, on nuclei isolated from paraffin-embedded tissues, this type of analysis is much more labor intensive and susceptible to interpretive difficulties than FISH performed on touch preparations.

The identification by Albinger-Heygi et al[22] of an additional cluster of breakpoints within the region lying between the MBR and mcr is of value when considering the analysis of those cases in which DNA can only be isolated from paraffin-embedded tissue. Using primers specific for this intermediate breakpoint cluster (icr), 6 of 21 rearrangements missed with MBR and mcr primers were identified. A total of only two rearrangements were detected with mcr primers, suggesting that breaks occur in the icr more commonly than the mcr. Breaks in the icr have also been detected in other parts of the world, such as Japan.[25] Practically, in diagnostically challenging cases, the molecular diagnostic laboratory will often have only the paraffin block available for testing. The addition of icr primer sets to t(14;18) PCR assays should significantly increase detection rates in the routine setting. This is of added importance since standard PCR analyses using consensus primers to detect clonal *IgH* rearrangements have limited sensitivity in follicular lymphoma, in part because somatic hypermutation of *IgH* sequences may prevent primer annealing. As a result, the diagnostic lab is often reliant on t(14;18) detection to confirm the diagnosis of lymphoma.

Sensitive detection of the t(14;18) for purposes of measurement of minimal residual disease continues to fall within the realm of the PCR. To date, there are no studies using long-range PCR in minimal residual disease studies, and it remains to be seen if long-range PCR methods can be as sensitive or specific as conventional PCR methods. Both methods are theoretically prone to false negative results stemming from the surprisingly frequent occurrence of nontransformed B cells bearing the t(14;18), which is often detectable within the reactive lymph nodes[27] or peripheral blood lymphocytes of normal individuals,[28,29] even in populations in which follicular lymphoma is relatively rare.[30] Such false-positive results can be minimized if the *BCL/IgH* fusion sequences found in tissues tested for minimal disease are confirmed to be present in diagnostic lymph node specimens from the same patients.

## *Can BCL2 Immunohistochemistry Be Used as a Surrogate Test for the Presence of the t(14;18)?*

Because *BCL2* expression is down-regulated in normal germinal centers, the presence of BCL2 protein can help to distinguish follicular lymphomas from reactive follicular hyperplasias.[16] In other lymphomas, such as anaplastic large cell lymphoma associated with *ALK* rearrangements and mantle cell lymphoma associated with BCL1 rearrangements, immunohistochemical detection of the *ALK* and *CyclinD1* gene products is tightly correlated with the presence of rearrangements involving these genes. Does a similar relationship exist between BCL2 protein and *BCL2* gene rearrangement in follicular lymphoma?

Because most follicular lymphoma series have relied on short-range PCR methods to identify tumors with the t(14;18), relatively few studies have addressed this question in a rigorous way. One of the best from Europe is the study of Vaandrager et al,[26] who correlated immunohistochemical staining for BCL2 with FISH analyses looking for the t(14;18). They found that 32 of 35 follicular lymphomas with the t(14;18) were positive for BCL2 protein expression, indicating a strong (but not absolute) correlation between immunohistochemistry and *BCL2* gene rearrangement. Of interest, all five lymphomas that were negative for the t(14;18) were also negative by immunohistochemistry for BCL2 protein expression. In another recent study from Vancouver, Skinnider et al found that 89% of follicular lymphomas associated with the t(14;18) stained for BCL2 protein, whereas 25% of follicular lymphomas lacking the t(14;18) showed BCL2 immunoreactivity.[31] The basis for the lack of BCL2 protein staining in some t(14;18)-positive tumors is unclear, but it again supports the conclusion that immunohistochemistry is imperfectly correlated with the presence of the t(14;18). The existence of BCL2-positive, t(14;18)-negative tumors is not surprising, as it has long been recognized that BCL2 expression is up-regulated independently of *BCL2* gene rearrangement in many other forms of lymphoma.

A practical take-home message from these studies is that the failure to detect BCL2 protein in a follicular proliferation cannot be taken as an assurance of benignity (or even the absence of the t(14;18)). The distinction between follicular lymphoma and reactive hyperplasia should be based on morphological features (which are sufficient to diagnose follicular lymphoma in $>$80% of cases), BCL2 protein staining, and tests that determine clonality. These studies also provide further evidence for the existence of a small subset (10 to 30%) of follicular lymphomas that lack the t(14;18) and frequently fail to express BCL2 protein. Whether the natural histories of t(14;18)-positive and -negative follicular lymphomas vary is a matter of current investigation.

## *What Do the New Breakpoints Tell Us about the Mechanism of BCL2 Rearrangement in Follicular Lymphoma?*

Since the acquisition of the t(14;18) is a common step in follicular lymphomagenesis, it is of interest to consider whether the responsible chromosomal breakpoints occur randomly, or through single or multiple directed mechanisms. The vast majority of breaks in the IgH locus show the molecular fingerprints of V(D)J recombinase activity, which led to the early hypothesis that ectopic V(D)J re-

combinase activity causes the breaks in *BCL2.*[4]. As noted, mapping of BCL2 breakpoints have detected three distinct breakpoint clusters, indicating that breakage is non-random. As Albinger-Heygi et al[22] point out, however, while breaks in *BCL2* do occur adjacent to sequences closely resembling recombinase recognition sequences, the breakpoints are infrequent. Breaks occur most commonly within 3 tight "microclusters" in a 150-bp region of the MBR which bears no significant homology to recombinase recognition sequences.[30] The MBR is flanked by an AT-rich sequence resembling a matrix attachment site, and associates with several polypeptides, including SATB1[32] and Ku (which is required for V(D)J recombination).[33] It is hypothesized that the MBR AT-rich sequence has a high propensity to unwind and may be susceptible to torsion-related breakage. The mcr and icr do not show homology to the MBR; breaks in this region presumably occur through other mechanisms. Hence, current data suggest that most breaks in *BCL2* are recombinase-independent and diverse in mechanism.

## *Final Perspectives*

We appear to be entering an era where therapies will be increasingly directed at the molecular lesions that cause cancer. As a result, diagnosticians will be pressed to provide information rapidly to clinicians about specific therapeutic targets (such as BCL2 protein in follicular lymphoma) and their molecular correlates (such as *BCL2* rearrangements) that may be predictive of therapeutic response. The work of Albinger-Hegyi et al[22] highlights the limitations of standard PCR-based methods for robust detection of *BCL2* rearrangements in follicular lymphomas, but it seems unlikely that long-range PCR will have much of an impact on molecular diagnostics generally. FISH analysis of interphase nuclei (or metaphase chromosomes) can provide rapid information about genomic rearrangements, amplifications or deletions, assuming that one knows what region of the genome to interrogate. However, it may prove more important to examine the pattern of gene expression (the transcriptosome) or protein expression and activation (the proteome) in tumor cells when considering which drugs are appropriate for which tumors. The challenge for the future will be to find cost-effective, flexible, reliable diagnostic approaches that provide information relevant to targeted therapeutic approaches. It is too early at present to predict where new therapies will take diagnostics, or how fast the changes will come; the only thing that is certain is that molecular tests will eventually, inevitably, become much more important in the day-to-day practice of diagnostic pathology.

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