New flow cytometry in hematologic malignancies

Jan Cools[1,2] and Peter Vandenberghe[2]

[1]Department of Molecular and Developmental Genetics, VIB, Leuven, Belgium; [2]Center for Human Genetics, K.U.Leuven, Leuven, Belgium.

E-mail: jan.cools@cme.vib-kuleuven.be - peter.vandenberghe@med.kuleuven.be. *doi:10.3324/haematol.2009.013482*

Lymphoblastic leukemia/lymphoblastic lymphoma represents a heterogeneous group of neoplasms arising from lymphoblasts committed to either the B- or T-cell lineage. The term acute lymphoblastic leukemia (ALL) is reserved for those cases with extensive involvement of bone marrow or peripheral blood, with 25% bone marrow blasts as a commonly used threshold for defining ALL. The laboratory diagnosis of ALL is complex and is based on cytomorphological, immunological, cytogenetic and molecular analysis. Detailed immunophenotyping of leukemic blasts distinguishes several differentiation stages of B- or T-lymphoblasts: early precursor B-ALL or pro-B-ALL, common B-ALL, and pre-B-ALL for B-lineage lymphoblasts; and pro-T-ALL, pre-T-ALL, cortical T-ALL and medullary T-ALL.[1,2] Over the years, the association between the presence of specific genomic abnormalities with distinct clinical or phenotypic features, or therapeutic responses and survival has led to the recognition of several distinct clinicobiological entities. Among the B-ALL with recurrent genetic abnormalities, the new 2008 WHO classification now recognizes seven genetic entities: B-ALL with t(9;22)(q34;q11); B-ALL with t(v;11q23), B-ALL with t(12;21)(p13;q22), B-ALL with hyperdiploidy, B-ALL with hypodiploidy, B-ALL with t(5;14)(q31;q32) and B-ALL with t(1;19)((q23;p13). In addition to these specific entities, many other recurrent genetic abnormalities have been reported in B-ALL as well as in T-ALL. The prognostic significance of specific genomic lesions has been incorporated into risk stratification and risk-adapted therapy.[3,4] In some cases, they also identify specific subgroups that are candidates for targeted therapies such as imatinib or dasatinib, e.g. B-ALL with t(9;22) or T-ALL with NUP214-ABL1 episomal amplification, or gamma-secretase inhibitors for T-ALL with *NOTCH1* mutations.[5] Finally, genetic analysis at diagnosis may identify specific markers that can be used to follow up the kinetics of the response to treatment and minimal residual disease. Detection of these markers requires higly sensitive technologies.

A standardized protocol for the cytogenetic analysis of B-ALL has been proposed.[6] Standard cytogenetic analysis will readily identify cases with hyperdiploidy (more than 50 but usually less than 66 chromosomes), which have a good prognosis; cases with hypodiploidy (less than 44-46 chromosomes), which have a poor prognosis. Cytogenetic analysis will also easily identify t(9;22), t(1;19) and t(4;11). Additional FISH and PCR analyses are mandatory to identify cytogenetically cryptic aberrations such as t(12;21), or cytogenetically challenging aberrations, especially MLL rearrangements, e.g. t(9;11)(p22;q23) or t(11;19)(q23;p13). These techniques can also be very helpful to resolve the karyotype from poor quality metaphases as often seen in ALL. Thanks to their greater sensitivity, quantitative PCR methods are the method of choice for the follow-up of minimal residual disease.

The complex and heterogeneous biology of B-ALL continues to unfold as more experimental approaches, such as expression profiling, DNA sequencing, and high resolution genomic arrays continue to reveal new recurrent alterations. It is now established that each of the chromosomal rearrangements that occur in ALL are by themselves not sufficient to cause the development of leukemia, and that additional mutations are present in those leukemias. Genome-wide copy number variation screening approaches have identified a number of additional cryptic lesions, such as the deletion and mutation of *IKZF1*, *EBF1*, PAX5 in B-ALL,[7] and the duplication of *MYB* in T-ALL.[8,9] The clinical meaning of these aberrations remains to be fully determined, but it is already clear that IKZF1 lesions have an independent prognostic meaning.[10] In addition, these genome-wide screening approaches have also demonstrated that relapse often arises from pre-leukemic clones, and, hence, that not all mutations present at diagnosis will be found again at the time of relapse.[11,12] The latter observation poses yet another analytical challenge for the diagnostic laboratory in that the use of a single method to follow up the status of a patient during remission may not be sufficient. Rather, a combination of methods is desired so that the development of novel clones at relapse is not missed.

In this issue, Raponi and co-workers have studied the efficacy, accuracy and sensitivity of the use of a bead-based flow cytometric immunoassay, yet another method that can be used for the detection of a specific fusion protein in a leukemia sample.[13,14] This method uses a commercially available assay in which the BCR-ABL protein is detected in the lysed blood or bone marrow sample. Here, in contrast to the usual routine hematologic flow cytometry, flow cytometry is used to detect an intracellular protein, not a surface marker. In addition, the assay is performed not on intact cells but on a cell lysate obtained from bone marrow or peripheral blood. BCR-ABL proteins are immunocaptured on beads coupled with an anti-BCR capture antibody, and are subsequently detected using a secondary anti-ABL1 detection antibody. This method is in principle very similar to a classical ELISA test, but uses beads instead of multi-well plates in combination with a readout that is based on flow cytometry. This method was found to be very accurate in samples where the percentage of BCR-ABL positive cells was sufficiently high (over 10%), and highly concordant with molecular assays. Yet, it failed to detect the BCR-ABL protein in 2 steroid treated samples with a very low percentage of BCR-ABL positive cells. In

a diagnostic setting, the rapid turnaround time, ease and specificity of this assay are extremely attractive. It may also provide a *molecular* diagnosis in geographical areas where diagnostic PCR is not available. In principle, the assay could be adapted to detect other types of oncogenic fusion proteins, depending on the availability and/or development of dedicated antibodies. The data reported here by Raponi et al. nicely and independently confirm the data published by the EuroFlow Consortium.[15] As indicated by the two cases with discordant flow cytometric and PCR results, the assay is less sensitive than RT-PCR, which needs to be taken into account when performing these assays, especially during follow-up.

The papers by Raponi[14] and Weerkamp[15] potentially extend the application field of flow cytometry to the detection of the BCR-ABL fusion protein in t(9;22) positive B-ALL and CML, and to the less common cases of t(9;22) positive T-ALL and AML. Flow cytometry has long been an established cornerstone in the diagnosis of hematologic malignancies, mainly to identify the malignant cell type by detection of cell surface proteins that provide information on its differentiation and/or maturation stage.[16] In ALL, detailed antibody panels are used to determine the presence of B- or T-cell markers, and to further delineate the stage of B- or T-cell differentiation. This is important for diagnosis and allows diagnostic molecular and/or FISH algorithms to be rationally triggered to fulfil clinical needs according to modern standards.

The constitutive phosphorylation of the BCR-ABL fusion protein is key to its transforming capacity. In more recent years, powerful assays have been developed to analyze the activation state (phosphorylation state) of signaling proteins within intact cells using fluorescently labeled phospho-specific antibodies. This, so-called, 'phospho-flow' analysis was pioneered by Gary Nolan's group (http://proteomics.stanford.edu/nolan/phosphoflow), and generates novel insights into signaling pathways in growth factor stimulated normal cells and cancer cells.[17] This method takes advantage of the power of flow cytometry to interrogate single individual cells, and thus detects cell populations with different signaling properties within a heterogeneous sample.

In the era of tyrosine kinase inhibitor therapy, rapid analysis of the phosphorylation state of oncogenic proteins such as BCR-ABL, or its downstream effectors such as STAT5 or CRKL, is becoming increasingly important. For example, treatment of a patient with BCR-ABL positive B-ALL with an ABL inhibitor will lead to the dephosphorylation of BCR-ABL and its downstream effectors, except in those cases with BCR-ABL mediated resistance. Thus, the analysis of a blood sample before administration of the first drug dose could provide information on the response to be expected, while subsequent samples under treatment could confirm the response to optimize the drug dose or to suggest switching to another inhibitor if no response can be documented. One example of such a test is the analysis of phospho-CRKL, a surrogate marker for the activation of BCR-ABL. Analysis of the phosphorylation of CRKL can be made by Western blot analysis,[18,19] but could also be performed using 'phospho-flow' analysis.[17] While Western blot analysis is a time consuming method, it is less sensitive to noise, and possible non-specific binding of the antibody used. In contrast, flow cytometric analysis of phospho-CRKL and/or other signaling proteins requires less hands-on time, and can provide results in just one day. Unfortunately, these techniques are more subject to noise and the quality and specificity of the antibodies used are of utmost importance to obtain a sufficient signal to noise ratio. In addition, the assay requires fresh samples with viable cells. At the moment these are still major hurdles for the analysis of phosphoproteins using flow cytometry. Despite these difficulties, a first demonstration of the application of 'phospho-flow' methods for the follow-up of response to ABL inhibitors has been reported,[20,21] and we can expect that further improvements may lead to a more general application of this technology in clinical practice.

Provided cells or lysates can be stored under conditions that preserve the phosphorylation status, the flow cytometric assay of Raponi and co-workers could potentially be adapted to measure phosphorylated BCR-ABL instead of or in addition to the presence of BCR-ABL. Although at the expense of single cell analysis, a bead assay for BCR-ABL phosphorylaton could obviate the cumbersome need for viable cells in 'phospho-flow' analysis, and might provide a more convenient approach to functional assessment of cellular responses to tyrosine kinase inhibitors, that might be applicable on a wider scale.

Dr. Cools is Professor at the Center for Human Genetics of the University of Leuven (K.U.Leuven), and group leader at the Department of Molecular and Developmental Genetics of the VIB, Leuven, Belgium. Dr. Vandenberghe is Professor at the Center for Human Genetics of the University of Leuven (K.U.Leuven), and Head of the Laboratory for Cytogenetic and Molecular Diagnosis of Malignant Disorders of the University Hospital Leuven (UZ Leuven), Leuven, Belgium.

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The significance of monoclonal gammopathy of undetermined significance

Robert A. Kyle and Shaji Kumar

Department of Medicine, Division of Hematology, Mayo Clinic, Rochester, MN, USA

E-mail: kyle.robert@mayo.edu. *doi:10.3324/haematol.2009.013961*

Monoclonal gammopathy of undetermined significance (MGUS) is characterized by a serum M protein concentration of less than 30 g/L, fewer than 10% clonal plasma cells in the bone marrow, and the absence of end-organ damage that can be attributed to the plasma cell proliferative disorder. End-organ damage is defined by hypercalcemia, renal insufficiency, anemia, or bone lesions (CRAB) related to the plasma cell proliferative disease.[1]

The prevalence of MGUS was 3.2% in 21,463 predominantly white residents of Olmsted County, Minnesota, who were 50 years of age or older.[2] The prevalence was 4.0% in men and 2.7% in women, 5.3% in persons 70 years of age or older, and almost 9% in men older than 85 years of age. Despite the common occurrence of MGUS, it is markedly underdiagnosed in the general population because this condition is asymptomatic and does not produce the signs or symptoms of multiple myeloma or related disorders. We found that the prevalence of MGUS in Olmsted County was 3.8% in persons 70 years of age, but that the prevalence of clinically detected cases at this age was only 0.8%. Thus, only 21% of patients with MGUS at the age of 70 were detected by clinical practice in Olmsted County.[3] In contrast, at the age of 80, 33% of patients with MGUS were detected by routine clinical practice, while the clinical detection rate was only 8% in those 50 years old. Overall, only 22% of patients with a known MGUS were recognized by routine clinical practice in Olmsted County, Minnesota.

The prevalence of MGUS in African Americans[4,5] and Africans[6] is approximately double that in whites. The prevalence in Japan is lower than in whites.[7]

The cause of MGUS is not known. In a report of atomic bomb survivors, those exposed to high levels of radiation at a young age had an increased risk of MGUS. Pesticides have also been implicated. In a study of pesticide applicators living in Iowa or North Carolina, the age-adjusted prevalence of MGUS was 1.9-fold higher than in men from Minnesota.[8] A 3-fold or greater risk was found in users of dieldrin, a chlorinated insecticide and the carbon-tetrachloride-carbon disulfide fumigant mixture. There was also an increased risk of MGUS in those exposed to the fungicide chlorthalonil. There is also a genetic element. A report on 247 first-degree relatives of 97 MGUS patients showed an approximate 2-fold higher risk of MGUS in first-degree relatives.[9]

What is the importance of MGUS? Is it simply an interesting laboratory finding or is it of importance to the patient? Prior to 1978, the presence of an asymptomatic M protein was often referred to as *benign monoclonal gammopathy*. In that year, we published the findings of a study of 241 patients with a monoclonal gammopathy but no evidence of multiple myeloma, Waldenström's