Leukemia Research 35 (2011) 1432–1435

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

Invited review

Searching for surrogates for *IGHV* mutations in chronic lymphocytic leukemia

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ARTICLE INFO

Article history:
Received 5 July 2011
Received in revised form 11 July 2011
Accepted 16 July 2011
Available online 12 August 2011

Keywords:
CLL
IGHV mutations
Prognosis
CD38
ZAP-70
Immature laminin receptor
Stereotypy

ABSTRACT

Despite a startling separation of chronic lymphocytic leukemia (CLL) into two clinically different diseases with average survivals of 8 years and 25 years, the mutational status of immunoglobulin variable region (*IGHV*) genes has not entered routine clinical practice to assess prognosis, although its assessment is regarded as an essential for clinical trials. Instead, surrogates that may be measured by flow cytometry have been sought. Measurements of the expression of CD38 and ZAP-70 have been the most popular assays for prognosis although both are in their own ways unsatisfactory. Many other candidates have emerged, but none has been universally endorsed.

As the assay for *IGHV* mutations has been standardized the level of difficulty has diminished and as greater numbers of cases have been assessed it has become clear that there is even more information to be gathered from the study of the sequence of *IGHV* genes. It has been recognized that stereotypy within CLL is associated with more specific clinical features than mere longevity and an even greater heterogeneity has been revealed. It seems clear that the search for surrogacy is futile and that *IGHV* mutational status should become a routine investigation in CLL.

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1. Introduction

More than a decade ago the simultaneous publication of two papers seemed to explain the marked heterogeneity that clinicians had observed in the natural history of chronic lymphocytic leukemia (CLL) [1,2]. The rearrangement of immunoglobulin (Ig) genes is an essential process in the maturation of normal B cells, whereby each lymphocyte acquires a unique B cell receptor (BCR) formed by the selection and recombination of individual gene segments from a large repertoire.

For the heavy chain of Ig, selection and recombination takes place from one each from 51 V_H genes, 27 *D* genes and 6 J_H genes [3]. The junctions of V_H to *D* and *D* to J_H are imprecise, with the deletion by exonucleases of templated nucleotides or the insertion by terminal deoxytransferase (TdT) of non-templated nucleotides in a random manner [4]. This introduces a further huge diversity into the shape of the Ig molecule, especially as the *D* segment can be read in any of the three frames [5]. The consequence is that the third complementarity-determining region (CDR3) of any given lymphocyte is virtually unique, and provides a clonal signature for any tumor deriving from it.

Rearrangement of the light chain variable region genes occurs in a similar manner, involving single-step recombinations of *V*/*J* gene segments but with no *D* segments.

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doi:10.1016/j.leukres.2011.07.020

On completion of this maturation the B-cell leaves the bone marrow for the periphery where it may encounter antigen. It then undergoes affinity maturation, usually in the germinal centers of the peripheral lymphoid organs. Here, somatic mutation is induced under the influence of CD40+ve T cells, cytokines and antigen-bearing follicular dendritic cells [6]. The rate of introduction of base pair changes is of the order of 10^{-4}–10^{-3} per generation. The mutations tend to cluster in the CDRs, possibly for structural reasons and possibly because of antigenic selection.

Interest in immunoglobulin genes in CLL originally stemmed from attempts to recognize the normal cell counterpart to the CLL cell. Because CLL cells express CD5 many authorities had accepted that CLL cells derived from the minor population of CD5+ve naïve B cells. Early sequences of the *IGHV* genes of tumor cells from patients with CLL found them to be in germline configuration [7–9] tending to confirm their origin from a naïve B cell. However, reports began to appear in the literature detailing cases with evidence of somatic mutation culminating in 1994 with a review of the literature by Schroeder and Dighiero [10] which found that 36/75 reported cases had *IGHV* genes with less than 98% sequence homology to the appropriate germline gene. The figure of 98% was chosen because polymorphisms, which are quite common in *IGHV* genes, can account for that degree of disparity [11]. Subsequently, a multicenter study of 64 patients with undoubted and classical CLL also found two groups of roughly equal numbers with respectively mutated and unmutated *IGHV* genes [12].

2. *IGHV* genes as prognostic factors

The first suggestion that *IGHV* mutations might have prognostic significance came from Oscier et al. [13] who examined 22 patients with classical CLL segregated according to karyotype. Tumors with trisomy 12 had unmutated *IGHV* genes but those with 13q14 abnormalities detected by conventional cytogenetics had evidence of somatic mutations. By 1998 this series had been extended and presented at several meetings, demonstrating a more aggressive disease and a shorter survival for patients with unmutated *IGVH* genes [14–16] before the definitive publication of the two papers that documented and mutually corroborated the finding that *IGVH* mutational status identifies two subsets of CLL, one with a median survival of 8 years and one with a median survival of approximately 25 years [1,2].

3. Searching for surrogates

From the beginning it was recognized that sequencing of *IGVH* genes would not be available to most laboratories, especially if the technique involved a post-doctoral student, a sheet of X-ray film, a ruler and a lot of patience. One of the original papers proposed the percentage of B-cells expressing CD38 measured by flow cytometry as a surrogate measurement [1]. Despite its initial attraction CD38 expression later proved to be discordant with *IGHV* mutational status in 30% of cases and vary during the course of the disease in up to 25% of cases [17]. In fact, CD38 expression is an independent prognostic variable that can be combined with *IGHV* mutational status to enhance prognostic predictability [17].

Once *IGHV* mutational status had so comprehensively separated CLL into two clinical types, the question was asked as to whether it was one or two diseases [18]. Gene expression profiling demonstrated a distinct pattern for CLL distinguishing it from other lymphoid tumors and any particular B-cell population, although the closest normal cell profile was that of a memory B-cell [19,20]. However, the expression of a small number of genes in the mutated and unmutated subtypes was different, with *ZAP-70* standing out as the gene that most stringently separated the subsets.

Flow cytometric assays for ZAP-70 expression have proved difficult, especially as it is an intracellular antigen so that the cells require permeabilization, but at least three different methods have been reported [21–23]. Seen as surrogate assays for *IGHV* gene mutations, the first two assays performed similarly with around 94% concordance, but the third, which used a different and directly conjugated antibody, had only a 77% concordance with *IGHV* gene mutations. On the other hand, in this study ZAP-70 expression performed better than *IGHV* mutations in predicting treatment-free survival. Patients who were ZAP-70 positive; *IGHV* mutated had a worse survival than those who were ZAP-70 negative; *IGHV* unmutated.

Nevertheless, establishing a standardized assay for ZAP-70 expression has proved difficult [24] and many of the most experienced laboratories have dropped it from their repertoire as being unreliable. The need for a dependable surrogate for *IGHV* mutational status remains. Although no other prognostic factor has been widely adopted, there has been no shortage of candidates. A recent review described nineteen new prognostic markers: *TCL1* gene expression, CLLU1 expression, miRNA signature, mRNA signature, and expression of Lipoprotein lipase A, *ADAM29*, *HEM1*, *Septin 10*, *DMD*, and *PEG 10*, levels of VEGF and thrombopoietin; telomere length and activity; surface expression of CD49d, CD69 and FCRL, expression of anti-apoptotic genes such as *MCL-1* and the *Bcl-2/Bax* ratio, *MDR1/MDR-3* genes, and *AID* mRNA [25]. Even an observation of something as simple as the percentage of 'smudge' cells on the blood film has proved to be an independent prognostic factor, though not alas a substitute for *IGHV* gene mutational status [26].

Combinations of prognostic factors might be more useful than individual factors. CD38 and *IGHV* mutations [17] or CD38 and ZAP70 [27,28] both perform better than any one factor. A scoring system based on six surface molecules (CD62L, CD54, CD49c, CD49d, CD38, and CD79b) detectable by flow cytometry has been proposed [29].

4. Immature laminin receptor

In a recent issue of *Leukemia Research*, Friedrichs et al. [30] proposed that as high expression of the immature laminin receptor (iLR) protein predicted a favorable prognosis for CLL correlating with mutated *IGHV* genes and being detectable by flow cytometry, it might be used as a surrogate. ILR is an oncofetal antigen overexpressed in several tumor tissues but absent in the normal differentiation process. It is the precursor molecule of the mature 67 kDa protein that plays a role in the cell adhesion-associated processes initiated by laminin binding.

Friedrichs et al. studied 134 patients with CLL. The flow cytometry assay used direct staining with a FITC-conjugated mouse anti-ILR monoclonal antibody and cells were double stained with an anti-CD19-PE antibody or an isotope control in the conventional way. ILR scores over CD38 and ZAP-70 in not being present on T cells nor on normal B cells. A cut-off 30% cells staining was determined best to distinguish between positive and negative. 41% of CLLs in their series were judged to be positive. There was a correlation between the expression of iLR and low ZAP-70 expression, mutated *IGHV* status and low CD38 expression. There was no change in iLR expression over time as there sometimes is with CD38 and ZAP-70 expression [17,31]. However, the correlation of high expression of iLR with mutated *IGHV* genes was no better than for ZAP-70 or CD38. Only 78.9% of patients with mutated IGHV genes were iLR positive and 81.7% of unmutated cases were iLR negative. ILR negativity therefore behaves with a similar degree of concordance to *IGHV* mutations as ZAP-70 or CD38 expression. In a multivariate analysis iLR expression retained some independent predictive value for prognosis.