

## Impact of cytogenetic and molecular prognostic markers on the clinical management of chronic lymphocytic leukemia

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The prognosis of patients with B-cell chronic lymphocytic leukemia (B-CLL) has long been determined by the clinical staging systems of Binet and Rai.<sup>1-4</sup> Unfortunately, these systems fail to identify patients in early stages whose disease will rapidly progress. Neither can this approach clearly predict the response of individual patients to specific therapies. In recent years, numerous genetic approaches have provided new markers for prognosis and response prediction.<sup>5</sup> The predictive potential of genetic factors associated with CLL outcome is determined by (i) its accessibility for routine use; (ii) its stability throughout the course of disease; and (iii) its sensitivity and specificity. Prognostic factors are largely dependent on the conventional therapeutic regimens used (i.e. chemotherapy) as well as on the availability of specific targeting drugs. There is increasing evidence that the behavior of the leukemia is not only dependent on genetic factors within the CLL clone, but also on the genetic background of the host in general (*susceptibility*) and in particular of the microenvironment.

We will discuss advances in the genetics of CLL with a special focus on molecular markers. We will first focus on well-established prognostic markers and then review the current status of molecular predictive markers and their potential influence on treatment decisions.

### Prognostic markers

#### Conventional cytogenetics

Karyotypes from CLL cells collected from peripheral blood or bone marrow are difficult to assess. However, stimulation by CD40L or CpG oligonucleotides has, in most cases, yielded positive results.<sup>6</sup> One of the most important findings was that the number of chromosomal translocations has largely been underestimated. Patients with translocations have a poor prognosis with short treatment free and overall survival times.<sup>6</sup> Recent advances were also made in identifying chromosomal regions predisposing for familial CLL. Regions on chromosomes 13q as well as 1, 3, 6, 12, and 17 have been linked to pathogenesis of inherited disease.<sup>7-9</sup> However, while relatives of CLL patients have increased numbers of circulating CD19<sup>+</sup>/CD5<sup>+</sup> B-cells (a phenomenon called *B-cell monoclonal lymphocytosis*), the risk factors leading to the final development of overt CLL are still unclear.<sup>10</sup> Furthermore, familial CLL does not seem to be associated with a shorter survival in affected individuals.<sup>11</sup> Micro RNAs (MiRs) are also connected to familial disease<sup>12</sup> although their impact is not yet clear.

#### Fluorescence in situ hybridization

The prognostic value of aberrations has long been established following the hierarchical model described by Döhner *et al.*<sup>13,14</sup> Half of the B-CLL cases have a del 13q, indicating good prognosis. By contrast, 11q deletions are associated with bulky disease, and are associated with short survival times. This is even more pronounced for 17p deletions which predict for very poor outcome.<sup>15,16</sup> Interphase cytogenetics have been extended to a molecular level by microarray analysis in several studies.<sup>17-19</sup> Therefore, a number of genes over- or under-expressed in association with chromosomal aberrations have been identified. Some of these are directly related to the location on deleted or duplicated chromosomes (*gene dosage effect*). However, a number of genes from unaffected chromosomes change their expression, suggesting trans-acting effects. These genes have only partially been evaluated.<sup>20</sup>

#### Molecular prognostic markers

Molecular markers can be classified according to their presence on the DNA level (mutations, polymorphisms) or on the RNA level (gene expression) (Table 1). Most markers are predictive at diagnosis, but dynamic markers predicting response to therapy, such as minimal residual disease, are also available.

#### Gene mutations

Immunoglobulin VH-mutational status is now well established as a strong predictor of outcome.<sup>21-28</sup> A 98% sequence homology to germline has been defined as a useful clinical cut-off between good (mutated) or poor (unmutated) prognosis. More detailed analysis has revealed that the 98% threshold is indeed relevant, but that some variation exists.<sup>29</sup> Further analysis has revealed the prognostic relevance of specific VH-families: VH 1-69 cases are always unmutated CLL and mutated cases with a VH 3-21 have a poor outcome.<sup>30,31</sup> In addition, immunoglobulin receptors coding for particular antigen-binding sites can be critical in determining clinical features and outcome for at least some CLL patients.<sup>32</sup>

A number of non-immunoglobulin genes have been searched for mutations in CLL. Germ-line or somatic mutations were found in 5 out of 42 sequenced microRNAs (miR) in 11 out of 75 patients with CLL.<sup>12</sup>

#### Gene expression

Perhaps the most important value of IgVH mutational status was its ability to discriminate between patient

subgroups for further analysis in microarray studies. Gene expression profiling revealed the association of a number of genes previously thought to be unrelated to CLL. Depending on the use of CD19-selected/T-cell depleted or unpurified B-CLL samples, a variety of markers have been found since the initial experiments of Klein and Rosenwald in 2001.<sup>19,52-54</sup> A comprehensive analysis of more than 500 patients suggests a complex expression pattern with many CLL subgroups and overlaps.<sup>55</sup> Furthermore, Fernández *et al.* have shown considerable intra-individual modulation of gene expression patterns during the course of disease.<sup>56</sup>

The first established marker emerging from microarray analysis was the receptor kinase ZAP-70. The prognostic value of this genetic marker has now been well established by FACS-analysis or PCR.<sup>33,34,57-62</sup> ZAP-70 protein expression predicts for treatment-free survival (TFS) and overall survival independently of mutation status. While convincing results were obtained by several groups using different protein detection methods, efforts by the European Initiative in CLL Research (ERIC) to standardize their study approach have proved problematic.<sup>63</sup> Furthermore, investigations into the association with IgVH mutational status have yielded differing results, particularly 17p- samples cluster in the ZAP-70 negative group.<sup>64</sup> Assessment of ZAP-70 mRNA expression by real time PCR requires positive (CD19) or negative selection of B-cells.<sup>33,34</sup> Despite these drawbacks, ZAP-70 is a useful clinical marker and may also serve as a future target for specific signal transduction inhibitors.

Among the markers with an even stronger correlation to IgVH mutational status,<sup>52,53</sup> lipoprotein lipase has been extensively studied.<sup>35-40</sup> Its association with other markers (cytogenetic risk groups, molecular markers) as well as patient outcome (time to treatment, overall survival) is also strong. Lipoprotein lipase is a stable marker which has been studied by real-time PCR in several large series using purified or unpurified CLL cells or even whole blood. No difference between purified and unpurified samples was observed in several studies, indicating its potential for easy and general use. Its specificity regarding IgVH mutational status is 89%, with a sensitivity of 68%, a positive predictive value of 83% and a negative predictive value of 78%.<sup>39</sup> Discordant results are also observed.<sup>36</sup> Lipoprotein lipase can be combined with a downregulated marker (ADAM29) to increase specificity.<sup>35</sup> While lipoprotein lipase protein can also be detected on normal B-cells, its cytoplasmatic expression correlates well with RNA levels.<sup>36</sup> We have used the level of lipoprotein lipase-expression as a discriminator for microarray analysis.<sup>19</sup> Several markers emerging from this experiment have been validated by real time PCR. Among those, septin 10 and dystrophin (DMD) were strongly associated with time to treatment. The prognostic significance of some of these factors have also been confirmed by other groups (septin 10, DMD, AKAP12/gravin)<sup>35-39,52-54</sup> (Table 1). In addition, lipoprotein

**Table 1. Cytogenetic and molecular markers in CLL with prognostic significance.**

Cytogenetic markers		Ref.
Conventional cytogenetics	Translocations	6
FISH	17p-, 11q-, 13q-, +12	13-19
Molecular markers		
Mutations	IgVH-status	21-32
	Micro RNAs	12
mRNA expression	ZAP-70	33,34
	LPL	35-40
	PEG 10	41
	Sarcoglycan $\epsilon$	41
	Septin 10	19
	Dystrophin	19
	Activation-induced cytidine deaminase	42-45
	Telomerase	46
	L-selectin	20
Integrin- $\beta$ 2	20	
	<i>CLLU1</i>	47
Minimal residual disease	ASO-PCR	48-51

lipase expression correlated with several functional modules including the MTA3 and fatty acid degradation pathways.<sup>19</sup> Using special statistical methods exploiting the lipoprotein lipase-associated gene expression signature we have shown that lipoprotein lipase-positive CLL cells show similarities to other tissues like fat, muscle and dendritic cells. This body of information suggests that lipoprotein lipase mRNA and protein expression may be of functional importance.

#### Expression of other markers was also linked to prognosis

The gene responsible for somatic mutations in immunoglobulins (activation-induced cytidine deaminase, *AICDA*) is overexpressed in high-risk (unmutated) CLL cases.<sup>42-45</sup> We have investigated the prognostic significance of *PEG10*. This maternally-expressed gene is overexpressed in high-risk CLL in parallel with sarcoglycan  $\epsilon$ , which resides within the same locus on chromosome 7q21. Knock-down of *PEG10* expression in primary CLL patient cells results in increased apoptotic cell death.<sup>41</sup>

Another important functional target is the telomerase gene which is overexpressed in CLL cells of patients with poor prognosis.<sup>46</sup> Additional prognostic factors include L-selectin and integrin- $\beta$ 2.<sup>20</sup> A very CLL-specific gene is *CLLU1* whose mRNA expression level can predict time to initiation of treatment and survival in CLL patients.<sup>47</sup>

One of the most significant findings in the field of CLL research was the detection of certain microRNA genes which are over- or underexpressed in conjunction with certain chromosomal aberrations.<sup>12,18</sup> Specific miR expression signatures are correlated with IgVH mutational status, ZAP-70 expression and treatment-free survival indicating prognostic importance.

**Minimal residual disease**

Molecular monitoring of residual CLL cells during therapy has been used as a dynamic marker during therapy. Eradication of MRD below detection levels of tailored PCR (ASO-PCR) or multicolour FACS is a predictor of favourable outcome.<sup>48,49</sup> This has been shown for induction therapy with the antibody alemtuzumab as well as for autologous or allogeneic stem cell transplantation.<sup>50,51</sup>

**Prediction of response and influence on treatment decisions**

**Cytogenetics/FISH**

Chromosome 17p deletions or p53 mutations result in poor response to fludarabine and rituximab which can be overcome by therapy with alemtuzumab<sup>65,66</sup> (Table 2). Alemtuzumab is particularly effective when combined with high-dose steroids.<sup>67</sup> A novel apoptogenic mechanism, cytosolic Histone H1.2 release, was also shown to be p53 dependent.<sup>68</sup> The predictive value of trisomy 12 is abrogated when fludarabine-containing regimens are used (*S. Stilgenbauer, personal communication*). Patients with deletions in 17p or 11q have been shown to respond to flavopiridol.<sup>69</sup> While combination chemoimmunotherapy with rituximab, pentostatin and cyclophosphamide was not effective in 17p- patients, it was very effective in patients with 11q deletions.<sup>70</sup> These novel data will obviously have consequences for treatment selection and expected response and will pave the way to tailored therapy.

**Minimal residual disease**

Minimal residual disease detection may not only be used as a measure of outcome, but could potentially serve to guide therapy in individual patients. In particular, response assessment after induction therapy could be used to tailor maintenance treatment with alemtuzumab or rituximab.<sup>51,78</sup> Since the clinical outcome of patients who become minimal residual disease-negative is substantially improved these molecular data may also impact on transplant decisions. Thus, minimal residual disease data will contribute to challenge the paradigm that CLL should not be treated aggressively.<sup>49,78-81</sup>

**In vitro evidence for response to specific targeting drugs**

Molecular markers have led to the detection of potentially active novel agents or off-label use of known drugs against CLL (Table 2). While these data are still based on *in vitro* or *ex vivo* observations, some of the agents used are close to clinical testing. Inhibitors of activated heat shock protein 90 (HSP90) have been shown to influence survival of B-cells expressing high levels of ZAP-70.<sup>72</sup> Specific ZAP-70 inhibitors are currently being developed. Since there is strong evidence for the functional importance of lipoprotein lipase in high-risk CLL, it is noteworthy that lipoprotein lipase inhibitors are already

**Table 2. Impact of selected genetic markers on treatment decisions.**

	Prognostic	Influence of treatment decision, response to specific drugs	Reference
<b>Cytogenetics/FISH</b>			
17p-	yes	effective: alemtuzumab, flavopiridol, high-dose steroids; ineffective: fludarabine, rituximab	65-68
11p-	yes	effective: flavopiridol, pentostatin (PCR) not very effective: alemtuzumab	69, 70
+12	(yes)	prognostic power lost after treatment with FC	
<b>Molecular markers</b>			
<i>Mutation</i>			
IgVH	yes	unclear	
MiR genes	yes	unclear	
FCγReceptor IIIA	no	effect of rituximab dependent on genotype	71
<i>Expression</i>			
ZAP70	yes	HSP90 inhibitors effective in vitro, direct inhibitors in development	72
LPL	yes	LPL inhibitor orlistat effective in vitro	73
PI3-Kinase	no	Antiapoptotic effect of Wortmannin, LY294002 in vitro	74
Fibromodulin	no	Elicits autologous CD8 <sup>+</sup> T-cell response in vitro	75
MDR1	yes	Multidrug resistance, target for specific agents	76
<b>Dynamic markers</b>			
microarray p53 signature	no	fludarabine response influenced	77
Minimal residual disease	yes	maintenance treatment with alemtuzumab	50,78

on the market for metabolic disorders. There is preliminary evidence that one of these agents (orlistat) leads to apoptosis in B-CLL cells.<sup>73</sup> Since orlistat has low toxicity, its off-label use in B-CLL seems warranted. We have shown that PI3-kinase/AKT inhibitors (Wortmannin, LY294002) also effectively induce apoptosis of primary B-CLL cells.<sup>74</sup> A number of genes important for CLL survival have been knocked down by siRNA technology. Thus, even miR genes or others may eventually serve as targets for therapeutic interventions.<sup>12,18</sup>

Molecules detected by molecular methods may also serve as targets for T-cell immunotherapy. One such example is the CLL specific antigen fibromodulin which allows expansion of specific CD8<sup>+</sup> autologous T lymphocytes *in vitro*.<sup>75</sup>

**Pharmacogenomics**

In addition to general static and dynamic prognostic markers related to the disease, several genetic markers associated with response to specific therapies have been investigated (Table 2). Polymorphisms of the FC recep-

tor  $\gamma$ IIIa (FCGR3A) generally predict response to rituximab.<sup>71</sup> Rosenwald *et al.* have studied the gene expression pattern of CLL cells after therapy with fludarabine disease *in vivo* and *in vitro* and have shown that fludarabine induced changes result in a p53-related expression signature.<sup>77</sup> The results predict that fludarabine treatment will lead to selection of p53-mutated CLL clones. The phenomenon of multi-drug resistance has recently been investigated.<sup>76</sup>

### Conclusions

Genetic markers have already contributed many important insights into the biology of B-CLL. Some genetic markers have been established as routinely used prognostic factors in addition to the traditional staging systems.<sup>82</sup> This is particularly true for cytogenetic aberrations detected by FISH; other markers like ZAP-70 have shown their prognostic power in large patient series, but

need to be more thoroughly harmonized for standard use.<sup>83</sup> Markers like IgVH mutational status and minimal residual disease detection by PCR are harmonized, but cannot easily be used in daily routine.<sup>48,83</sup> The least developed group, including lipoprotein lipase, activation-induced cytidine deaminase, or micro RNAs, have not reached either one of these stages, but are potentially interesting prognostic markers.

Currently, the use of genetic and molecular markers could be restricted to patients who will receive more than palliative treatment. There is strong evidence that several of the predictive markers will influence treatment decisions for tailored therapy.<sup>84,85</sup> While generalized routine use of these data cannot be recommended to date, we are soon expecting results from randomized trials powered to answer questions related to the rational use of specific therapies subsequent to the identification of molecular genetic markers.

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