ZAP-70 expression and prognosis in chronic lymphocytic leukaemia

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Summary

Background Chronic lymphocytic leukaemia (CLL) is a heterogeneous disease; many patients never need treatment, whereas some have poor outcomes. New treatments, which can induce complete remissions, allow patients with poor outlook to be treated while they are still asymptomatic. Whether or not the IgVH gene is mutated is the best predictor of clinical outcome, but this assay is unsuited to the routine laboratory. The gene coding for ZAP-70, a tyrosine kinase protein normally expressed in T and NK cells, has been shown by gene-expression profiling to be differentially expressed between patients with mutated and unmutated IgVH genes. We assessed whether ZAP-70 could be used as a prognostic marker in CLL.

Methods We developed a flow cytometry assay for ZAP-70 protein expression and investigated its concordance with ZAP-70 mRNA expression, IgVH gene mutational status, and clinical outcome in 167 patients with CLL.

Findings We showed high concordance between ZAP-70 protein expression and IgVH gene mutations. 108 patients (65%) had mutated IgVH genes and were ZAP-70 negative; 46 (28%) had unmutated IgVH genes and were ZAP-70 positive. Findings were discordant in 13 patients: six (4%) had mutated IgVH genes but were ZAP-70 positive, and seven (4%) had unmutated IgVH genes and were ZAP-70 negative. Expression of mRNA showed 97% concordance with ZAP-70 protein. Median survival was 24·4 years (95% CI 15·1–33·8) in ZAP-70 negative patients and 9·3 years (7·0–11·5) in those who were ZAP-70 positive (hazard ratio 5·5, 2·8–10·8).

Interpretation ZAP-70 protein, which can be measured by flow cytometry in the general laboratory, is a reliable prognostic marker in CLL, equivalent to that of IgVH gene mutational status.

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Introduction

The clinical course of chronic lymphocytic leukaemia (CLL) is heterogeneous: many patients have indolent disease that never needs treatment, but others have an aggressive clinical outcome.1 Asymptomatic patients with low tumour burden (Binet stage A) derive no benefit from treatment with chlorambucil.2,3 However, newer treatments, such as fludarabine and alemtuzumab, with the potential to induce complete remission, could allow early treatment for asymptomatic patients whose disease is likely to progress.2 Accurate identification of these patients is therefore increasingly important.

Markers of poor prognosis in both stage A and advanced CLL include an absence of mutations in the variable region of the immunoglobulin heavy chain gene (IgVH gene),13,14 deletion of chromosome 11q23, loss or mutation of the p53 gene, CD38 positivity, and raised concentrations of serum markers such as thymidine kinase.15 In multivariate analyses, IgVH gene mutational status is the best predictor of clinical outcome: patients whose CLL cells have unmutated IgVH genes have a significantly worse prognosis than those whose IgVH genes have undergone somatic mutation (median survivals of 6–9–9·9 years vs up to 24 years, respectively).17 However, the assay for the mutation is both expensive and labour intensive, precluding its routine use.

Gene expression profiling with cDNA microarrays has been used in CLL to compare patients with mutated and unmutated IgVH genes. In two initial studies11,12 the genetic signature of the two CLL groups was shown to be similar. However, about 30 genes did show strong differential expression in the Lymphochip array system.19 Expression of the gene coding for ZAP-70, a tyrosine kinase protein essential for T cell signalling but not found in normal B cells, was fivefold higher in patients with unmutated IgVH genes than in those with mutated genes, and was the best discriminator between the two groups.

We aimed to develop a flow cytometry assay for ZAP-70 protein expression and to assess whether differential gene expression for ZAP-70 accords with differences in protein expression, whether ZAP-70 protein expression has prognostic importance in CLL, and to what extent the protein can be used as a surrogate for IgVH gene mutation status.

Methods

Patients and samples

We investigated 167 patients with CLL from a total of 608 who were followed up in Bournemouth between October, 1983, and July, 2002, the only criterion for selection being the availability of fresh or dimethylsulfoxide-frozen cells. All patients gave written consent to participation in the study, which was approved by the local ethics committee. Diagnosis of CLL was based on standard morphological and immuno-
phenotypical criteria. Disease stability and disease-related deaths were assessed as previously described. Briefly, progressive disease was defined as an increase in stage, a lymphocyte doubling time of shorter than 12 months, or systemic B symptoms necessitating treatment. Some patients were treated after a long wait-and-watch period, usually with white-cell counts that had risen slowly to greater than $100\times10^9/L$, without fulfilling criteria for progressive disease. These patients were designated slowly progressive. Among the 123 survivors, the median follow-up time from diagnosis was 7.3 years (lower quartile 3.8 years, upper quartile 11.5 years). Samples from treated patients were all taken at least 3 months after the last treatment.

We measured ZAP-70 in both fresh and frozen cells in 25 patients, and results were concordant. In 20 patients, we measured ZAP-70 in sequential samples. At least $10^8$ fresh separated CLL cells were needed for cDNA microarray analysis; this amount was available for 64 otherwise unselected patients, and hence we were able to obtain data on ZAP-70 mRNA expression and contemporaneous ZAP-70 protein expression for this group. B lymphocytes from five healthy adults were also separated and assayed for ZAP-70 protein expression.

Figure 1: Dot plots from flow cytometry showing ZAP-70 protein expression in typical patients

Patient 1: ZAP-70 negative, mutated IgVH gene. (A) Side scatter (SSC-H) vs isotype control (IGG2a FITC). (B) Side scatter vs ZAP-70 FITC. (C) CD3-PE vs ZAP-70 FITC. Patient 2: ZAP-70 positive, unmutated IgVH gene. (A) Side scatter vs isotype control. (B) Side scatter vs ZAP-70 FITC. (C) CD2-PE against ZAP-70 FITC confirms the population in R2 to be T and NK cells.
previously established criteria.15

30% or greater as a CD38-positive result, on the basis of isotype control was run in all cases. We denoted a value of FITC+(CD19 PE–Cy5)+lymphocytes. An appropriate gating on forward-side scatter and subsequently on (CD5

of cells positive for CD38 (HB-7 PE, Becton Dickinson, purified (Qiagen, Crawley, UK) and sequenced directly

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mixture of oligonucleotide 5

leader sequence of the V H families 1-7, together with a 3’

constant region primer or a 5’ framework 1 consensus primer and a 3’ consensus J H primer. PCR products were purified (Qiagen, Crawley, UK) and sequenced directly with an automated DNA sequencer (ABI 377/310, Applied Biosystems, Foster City, USA). Nucleotide sequences were aligned to EMBL/GenBank and V-BASE16 by use of MacVector 4.0 (International Biotechnologies, New Haven, USA), or Lasergene (DNASTAR, Madison, USA) sequencing analysis software. As previously decided, a cutoff of 98% or greater germline homology defined the group with unmutated IgVH. This limit was set to allow for the possibility of a 2% deviation from the germline sequence due to polymorphisms rather than to somatic mutation.

The cDNA microarray methods have been described in detail elsewhere.17,18 Briefly, CLL cells were purified by magnetic selection for CD19+ cells (Miltenyi Biotech, Bergish Gladbach, Germany) at 4°C and frozen within 6 h of venesection, before extraction of mRNA (Fast Track, Invitrogen, Paisley, UK) and microarray analysis. CD19 selection typically resulted in greater than 98% purity as measured by flow cytometry. Fluorescently labelled cDNA probes were generated from mRNA; Cy-5 dye was used to label cDNA from the CLL samples, and Cy-3 dye to label cDNA from a reference pool of mRNA prepared from nine lymphoma cell lines. Lymphochip cDNA microarrays containing 13 868 human cDNAs were prepared and used as previously described.12,18 We based the initial selection of microarray data on fluorescence signal intensity, with the requirement of 50 relative fluorescent units above background in both the Cy-3 and Cy-5 channels, or 500 relative fluorescent units above background in either channel alone. The correlation between expression of ZAP-70 mRNA in this microarray and real-time quantitative PCR has been shown to be good (Pearson coefficient r=0.901).15

Procedures
To assess ZAP-70 by flow cytometry, lymphoid cells from peripheral blood (163 patients), bone marrow (one patient), or disaggregated lymphoid tissue (three patients) were separated on lymphocyte separation medium and either used fresh, or frozen in dimethylsulphoxide and subsequently thawed. We placed 5×10⁶ cells in 0·5% paraformaldehyde for 30 min, washed them with phosphate buffered saline containing 0·05% Tween and 2% human serum albumin, and stored them in 80% ethanol at –20ºC for between 24 h and 4 weeks. Cells were washed with the same buffer before incubation with primary antibody (ZAP-70, clone 2F3-2, Upstate Biotechnology, Milton Keynes, UK) or isotype control (Mouse IgG2a, Dako, Ely, UK) for 20 min followed by secondary antibody (Sheep-anti-mouse fluorescein-isothiocyanate [FITC]-conjugate, Novocastra, Newcastle Upon Tyne, UK) for 15 min. At least 5000 cells were acquired in the Cell Quest program on a Becton Dickinson FacsCalibur flow cytometer.

CLL cells that are positive for ZAP-70 stain homogeneously and consistently more weakly than T and NK cells, which can be readily identified as a separate, intensely staining population and which, in ZAP-70 negative patients, provide a useful positive control for antibody activity. The T and NK cells were gated and this value was subtracted from the total ZAP-70 positive population to give a value for CLL cells. We used this one-colour assay for most samples, but validated the gating procedure by addition of a second colour surface antibody (CD3-PE or CD2-PE, Dako, 2% deviation from the germline sequence due to polymorphisms rather than to somatic mutation.

We measured CD38 by a three-colour technique as previously described.15 Briefly, we measured the proportion of cells positive for CD38 (HB-7 PE, Becton Dickinson, Oxford, UK) in the tumour cell population, defined by gating on forward-side scatter and subsequently on (CD5

FITC)+/(CD19 PE–Cy5)+lymphocytes. An appropriate isotype control was run in all cases. We denoted a value of 30% or greater as a CD38-positive result, on the basis of previously established criteria.15

We sequenced IgVH genes as previously described. Briefly, the variable region was amplified by PCR with a mixture of oligonucleotide 3’ primers specific for each leader sequence of the V H families 1-7, together with a 3’ constant region primer or a 5’ framework 1 consensus primer and a 3’ consensus J H primer. PCR products were purified (Qiagen, Crawley, UK) and sequenced directly

Figure 2: Expression of ZAP-70 protein and mRNA in patients with mutated and unmutated IgVH genes
ZAP-70 mRNA reported as log2 relative mRNA expression.

Figure 3: ZAP-70 protein expression versus IgVH gene mutation status
Homology with germline IgVH (%)
Statistical analysis
We analysed data with SPSS (version 10) with a 5% significance level. Data for time to death from diagnosis were analysed with Kaplan-Meier survival curves. Comparison of survival curves between two or more groups of patients were made with log-rank tests. We used Youden’s index\(^1\) to assess the optimum cutoff point for ZAP-70 positivity by flow cytometry. Since many of the predictors of survival are inter-related, Cox proportional hazard regression was used to calculate adjusted and unadjusted hazard ratios for each factor, together with 95% CIs. First order interactions between factors were also tested and none were significant. We assessed sensitivity, specificity, positive predictive values, and negative predictive values to summarise how closely the results of the ZAP-70 test agree with the gold standard IgVH gene test.

Role of the funding source
The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, writing of the report, or in the decision to submit the manuscript for publication.

Results
102 patients (61%) were male; median age at diagnosis was 67 years (lower quartile 53, upper quartile 76). Binet stage at presentation was A in 143 patients (86%), B in 12 (7%), C in seven (4%), and unknown in five (3%). The most common cytogenetic abnormalities were: evidence of p53 mutation or loss in six patients (4%), deletion of chromosome 11q in 14 patients (8%), trisomy 12 in 49 patients (29%), and 13q deletion in 71 patients (43%); 13q deletion was the sole abnormality in 50 (30%).

IgVH genes were mutated in 114 patients (68%) and unmutated in 53 (32%); of the latter, 42 (25%) showed 100% homology to the germline sequence. CD38 data were available in 162 patients; 59 (36%) were positive and 103 (64%) were negative. Disease stability was known in 164 patients; it was stable in 87 patients (53%), progressive in 42 (26%), and slowly progressive in 35 (21%). 92 patients (55%) were never treated, and 75 (45%) had disease progression that needed treatment at some stage during follow-up. There were 42 deaths (25%), of which 22 were related to CLL.

In our assessment of ZAP-70 protein expression, two different approaches were used to determine the best cutoff point for ZAP-70 positivity as 10%. Firstly, Youden’s index\(^1\) was calculated in all patients to optimise sensitivity and specificity of ZAP-70 in distinguishing between those with mutated and unmutated IgVH genes. This method determined the cutoff value as between 10% and 15%. Secondly, there were 47 patients who were judged by cDNA microarray to have negative IgVH gene ZAP-70 Stage CD38 Karyotype Disease homology protein at (%) (%)

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<tr>
<th>Patient</th>
<th>IgVH homology (%)</th>
<th>ZAP-70 Stage</th>
<th>CD38 (%)</th>
<th>Karyotype</th>
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<tr>
<td>1</td>
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<td>12</td>
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<td>100</td>
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<td>5</td>
<td>+12</td>
<td>S (7-3)</td>
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P=progressive disease. SP=slowly progressive disease. S=stable disease; in stable patients, length of follow-up in years shown in brackets.

Table 1: Details of patients with discordant findings

Figure 4: Effects of ZAP-70, IgVH gene mutation, and CD38 on survival
expression, reported as log2 relative mRNA expression, in samples were available, similar amounts of ZAP-70 were (ZAP-70 negative) in whom both blood and lymph node 10% boundary. In nine patients (five ZAP-70 positive, four over a 24-month follow-up period from less than 10% to 29 months (range 12–48). In one patient the value changed measurements of ZAP-70 were made in 20 patients (14 and IgVH gene status on survival Table 2: expression. In two patients ZAP-70 mRNA was between ZAP-70 mRNA expression and ZAP-70 protein figure 2. In 62 patients (97%), there was concordance 107 patients with CLL and shown high concordance We have profiled gene expression in purified lymphocytes from healthy individuals all showed ZAP-70 values of less than 10%, in accord with previous findings that normal B cells do not express ZAP-70. Serial measurements of ZAP-70 were made in 20 patients (14 ZAP-70 positive, six ZAP-70 negative) over a mean time of 29 months (range 12–48). In one patient the value changed over a 24-month follow-up period from less than 10% to 15%; in the other 19 patients the values did not cross the 10% boundary. In nine patients (five ZAP-70 positive, four ZAP-70 negative) in whom both blood and lymph node samples were available, similar amounts of ZAP-70 were obtained in both tissues. We obtained measurements of ZAP-70 mRNA expression, reported as log, relative mRNA expression, in 64 (patients. In 17 patients (27%) mRNA was overexpressed and in 47 (73%) mRNA was low or absent. Comparison of ZAP-70 mRNA expression and ZAP-70 protein expression by flow cytometry in this group of 64 patients is shown in figure 2. In 62 patients (97%), there was concordance between ZAP-70 mRNA expression and ZAP-70 protein expression. In two patients ZAP-70 mRNA was overexpressed but protein expression was negative; one of these patients had mutated IgVH genes and one did not. The relation between ZAP-70 protein expression and IgVH gene mutational status is shown in figure 3. 108 patients (65%) had mutated IgVH genes and were ZAP-70 negative. 46 patients (28%) had unmutated IgVH genes and were ZAP-70 positive, giving a concordance of 92% (95% CI 87–95). Six (4%) patients had mutated IgVH genes but were ZAP-70 positive, and seven (4%) had unmutated IgVH genes and were ZAP-70 negative. Details of these 13 discordant instances are shown in table 1. Patients 1–6 all had borderline mutational status, with five showing 97% germline homology and the sixth, with 96% homology, showing the VH 3–21 gene segment, which is associated with poor prognosis. Five of the six had progressive or slowly progressive disease. Patients 7–13 had unmutated IgVH genes but were ZAP-70 negative (confirmed by mRNA expression data in three patients). Five patients had progressive or slowly progressive disease and two were stable. For all 143 patients who presented with stage A disease, we compared the prognostic value of ZAP-70 positivity with that of unmutated IgVH genes. 43 patients who developed progressive or slowly progressive disease could be correctly identified by presence of unmutated IgVH genes, and four patients who had stable disease after more than 5 years of follow-up were misclassified by this marker. The corresponding numbers for ZAP-70 positivity were 44 and four patients, respectively. Concordance was shown between CD38 and IgVH gene mutational status in 122 of 162 patients (75%; 95% CI 68–81) and ZAP-70 protein expression in 121 of 162 patients (75%; 68–81), respectively. Median survival was 9·3 years (95% CI 7·0–11·5) in ZAP-70 positive patients and 24·4 years (15·1–33·8) in the ZAP-70 negative group (p<0·0001; figure 4A and B). When only disease-related deaths were included, the corresponding values were 10·7 years (6·8–14·5) and 25·8 years (95% CI could not be calculated because there were no subsequent deaths) respectively (p<0·0001). Median survival was 24·4 years (95% CI 15·1–33·7) in patients with mutated IgVH genes and 9·8 years in those without the mutation (p<0·0001, figure 4C). Mean survival was 24·0 years (95% CI 17·2–30·8) in CD38-negative patients and 13·6 years (9·4–17·7) in those who were CD38-positive (p=0·008; figure 4D).

Adjusted and unadjusted hazard ratios for ZAP-70, CD38, and IgVH gene mutational status are shown in table 2. In univariate analyses, ZAP-70 and IgVH gene status were shown to be strong prognostic factors for survival, and CD38 was also significant. Adjustment of ZAP-70 for CD38 resulted in very little change in the hazard ratio for ZAP-70, but CD38 was no longer significant; similar results applied when adjusting IgVH gene mutational status against CD38. Proportional hazards models including both ZAP-70 and IgVH gene status are not presented because the high concordance between the two factors means that each became not significant when adjusted for the other.

Discussion

We have profiled gene expression in purified lymphocytes from 107 patients with CLL and shown high concordance between ZAP-70 mRNA expression and IgVH gene mutational status; the markers were similar in their ability to identify a subgroup of patients with short treatment-free survival. We developed a flow cytometric assay in which contaminating T and NK cells can readily be identified by the high intensity of ZAP-70 in these cells compared with CLL cells. Our findings show a close correlation between expression of ZAP-70 mRNA and protein, and between ZAP-70 protein expression and IgVH gene mutational status. Furthermore, both ZAP-70 protein expression and IgVH gene mutational status were strong predictors for overall survival. CD38, which we also measured by flow cytometry, had weak prognostic value in univariate analysis but did not improve the predictive power of either ZAP-70 or IgVH gene mutational status in multivariate analysis. The case-mix in the present study is that of a district general hospital rather than a tertiary referral centre, and the preponderance of patients with stage A indolent disease contributed to the long median survival (24 years) in the ZAP-70 negative group. Additionally, the concordance between ZAP-70 protein expression and IgVH gene mutational status might be reduced in a series with fewer indolent cases, since concordance was especially good in patients with more mutations. However, this flow cytometry assay is well suited to use in district general hospitals, which might be expected to have referral patterns similar to our own.

A similar study of ZAP-70 protein expression measured by flow cytometry has been reported by Crespo and colleagues who used the same antibody as that used in the present study and investigated 56 patients with CLL, of whom more than 50% had unmutated IgVH genes. They also showed a strong correlation between ZAP-70 protein expression and IgVH gene mutational status, although the effect of ZAP-70 positivity on survival only reached

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<th>Table 2: Hazard ratios for the effects of ZAP-70 protein, CD38, and IgVH gene status on survival</th>
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<td>Unadjusted hazard ratio (95% CI)</td>
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<tr>
<td>ZAP-70 and CD38</td>
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<td>IgVH gene status and CD38</td>
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significance in the subgroup of patients who had stage A disease at diagnosis.

Of particular interest in both studies are the individuals in whom \textit{IgVH} gene mutational status and ZAP-70 expression are discordant. In our study, five of the six patients who expressed ZAP-70 and had mutated genes had 97% homology to the germline sequence, and one (using the poor prognosis V3-21 gene) had 96% homology. These patients would be judged to have a poor outlook in studies in which either 95% or 97% homology is used to define prognostic subgroups. All patients in both studies with less than 96% homology failed to express ZAP-70, emphasising the correlation between ZAP-70 expression and low mutational load rather than an absence of mutations. Of greater biological interest are our seven patients with 98% or greater homology to the germline sequence who did not express ZAP-70. Crespo and colleagues\textsuperscript{23} also identified three patients in their unmuted group who were ZAP-70 negative. This finding suggests that these patients represent a real biological occurrence rather than a technical difficulty in ZAP-70 measurement. Longer follow-up of many more discordant cases will be needed to ascertain whether ZAP-70 expression or \textit{IgVH} gene mutational status is the better predictor of their clinical course.

With the possible exception of rare cases in which intraclonal diversification might be sufficient to change a patient from the unmuted to the mutated subgroup,\textsuperscript{24} \textit{IgVH} gene mutational status is fixed and therefore useful as a prognostic marker in early disease, in contrast to chromosome abnormalities or CD38 expression which can change with time. In the present study we did not measure concentrations of ZAP-70 protein at presentation. Nevertheless, in the 20 patients in whom serial measurements were possible, all but one (with a maximum of only 15% ZAP-70) showed no change over 2–3 years of follow-up. Larger studies with longer follow-up are needed to confirm the stability of ZAP-70 expression, both with time and in relation to treatment. If either ZAP-70 expression or \textit{IgVH} gene mutation had been used as the sole prognostic marker in stage A patients as the entry criterion for a study to assess intensive therapy in newly-presenting asymptomatic individuals with poor outlook, then four patients who had stable disease after more than 5 years of follow-up would have received inappropriate treatment. If both markers had been used, three patients would have been misclassified.

ZAP-70 expression is an unexpected finding in a B-cell tumour, since the protein has not been reported in normal circulating B cells. B cells use a related protein tyrosine kinase, Syk, which is activated through the B-cell antigen receptor (BCR) complex. There are no data to indicate whether ZAP-70 expression in a subset of CLL reflects ZAP-70 expression during human B-cell ontogeny or is the consequence of aberrant malignancy-related overexpression, although ZAP-70 is needed for pre-B cell development in Syk-deficient mice.\textsuperscript{25} In T cells, the functional importance of ZAP-70 is shown by the finding of immunodeficiency in rare individuals with ZAP-70 mutations.\textsuperscript{26,27} There is evidence that Syk and ZAP-70 are functionally homologous\textsuperscript{28} and that expression of ZAP-70 can reconstitute BCR function in Syk-negative B cells.\textsuperscript{29} Syk has been shown to be important in TCR signalling in ZAP-70-deficient individuals.\textsuperscript{22} Some studies of signalling through BCR in CLL patients have shown that unmuted B-CLL cells expressing ZAP-70 induced greater phosphorylation of cytosolic proteins on BCR stimulation than did mutated, ZAP-70 negative cells.

Moreover, in the ZAP-70 positive cells, BCR ligation resulted in rapid phosphorylation of ZAP-70 and its association with surface immunoglobulin and CD79b, suggesting a functional role of ZAP-70 in BCR signalling. If there are important differences in BCR signalling between the two mutational subsets in CLL, investigation of patients who are ZAP-70 negative, with normal \textit{IgVH} gene mutations but progressive disease, might elicit different abnormalities in these signalling pathways.

Many questions remain. Why is ZAP-70 expressed in a subset of CLL but not in normal B cells, nor in other common B-cell cancers such as follicular lymphoma or diffuse large cell lymphoma? Why is ZAP-70 expression so closely associated with \textit{IgVH} gene mutational status? Is ZAP-70 protein functional in CLL and does its overexpression contribute to poor prognosis? Clearly much more work on ZAP-70 expression in CLL is needed, but our study represents the successful translation of cDNA microarray findings to a flow cytometry assay that can be widely used and is of reliable prognostic value.

Contributors

D G Oscier and L Staudt provided the original concept for the study. J A Orchard and R E Ibbs obtained ZAP-70 flow cytometry data. A Wiestner, A Rosenwald, and L Staudt did ZAP-70 microarray analyses. Z Davis and R E Ibbs investigated \textit{IgVH} gene status. P W Thomas did statistical analyses. J A Orchard and D G Oscier drafted the manuscript. D G Oscier, T J Hamblin, J A Orchard provided clinical care and recorded clinical data.

Conflict of interest statement

None declared.

Acknowledgments

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