ORIGINAL ARTICLE

B cell chronic lymphocytic leukaemia/small lymphocytic lymphoma: role of ZAP70 determination on bone marrow biopsy specimens

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Chronic lymphocytic leukaemia/small lymphocytic lymphoma (CLL/SLL) is classified as a mature B cell neoplasm¹; its clinical course is variable,^{2–5} with some patients rapidly progressing despite receiving treatment and others having stopped treatment for a long time. This heterogeneity prompted the search for markers that could predict either course for risk-adapted treatments. Cytogenetic aberrations influence survival,^{6–9} but those with poorer prognosis are often absent at onset. Molecular studies have recently identified two CLL/SLL subgroups, with and without somatic hypermutations in the variable region of immunoglobulin heavy chain genes (IgV_H; respectively, "mutated" and "unmutated"). The latter are generally aggressive diseases with shorter survival, whereas the former are indolent and may never require treatment.^{8 10–14} The gene expression profile of CLL/SLL^{15 16} seems to be distinct from that of other B cell lymphomas or normal B cells, and not significantly different between the two forms, although several genes seem to be differentially regulated; specifically, the gene encoding zeta-associated protein 70 (ZAP70; a cytoplasmic protein present in T/natural killer (NK) cells and activated splenic and tonsillar B lymphocytes)^{17 18} attracted interest because of its significantly higher expression in the unmutated variant.^{15 16 19–22} Several groups applied anti-ZAP70 antibodies to verify whether its detection by western blotting (WB), flow cytometry or, more rarely, immunohistochemistry (IHC) could be used to search for mutations to stratify risk groups.^{19 20 23–30} Notably, in two studies based on a large series of non-Hodgkin's and Hodgkin's lymphomas,^{28 29} ZAP70 was also expressed by some B cell neoplasms, although correlation with the mutational status remained limited to CLL/SLL. Other molecules reported to be

Background: The course of chronic lymphocytic leukaemia/small lymphocytic lymphoma (CLL/SLL) partly depends on the mutational status of the variable region of immunoglobulin heavy chain genes (IgV_H), which defines two subgroups of tumours: mutated and unmutated. The expression of zeta-associated protein 70 (ZAP70) is significantly associated with the more aggressive unmutated forms.

Aims: To assess the feasibility of the ZAP70 immunohistochemical test on bone-marrow biopsy (BMB) specimens and to compare the results with those of western blotting (WB) and IgV_H mutational status assessed on neoplastic cells from peripheral blood.

Methods: 26 patients with CLL/SLL detected on BMB and with known IgV_H mutational status were selected. ZAP70 was determined by immunohistochemistry (IHC) comparing three antibodies from different sources (Upstate, Cell Signaling, Santa Cruz, California, USA) and two different methods (APAAP and EnVision⁺). In 23 cases, ZAP70 WB results were also available.

Results: ZAP70 determination on BMB specimens turned out to be easily feasible with routine procedures with reagents from Upstate and Cell Signaling. The results were concordant with those obtained with WB and mutational status analysis in >80% of the cases with both reagents. Three of four discordant cases were mutated/ZAP70 positive, with two staining weakly for ZAP70 on both WB and IHC.

Conclusions: The study confirms the role of ZAP70 as a possible surrogate of mutational status and emphasises its application in routine diagnostics; it discloses a small subset of discordant cases (mutated/ZAP70 weakly positive) that clinically cluster with the more favourable forms.

indirect indicators of IgV_H mutational status are CD38 and, recently, CD45RA and R0. CD38, although prognostically relevant,^{26 27 31–36} varies with time and is not linearly related to the IgV_H mutations.³⁴ Regarding CD45 subclusters, the expression of CD45RA and CD45R0 have been associated with unmutated and mutated forms, respectively.³⁶

Our aim was to verify how ZAP70 can be routinely determined in bone marrow biopsy (BMB) specimens and whether it represents a surrogate of the mutational status.

MATERIALS AND METHODS

Twenty-six BMB specimens from patients with CLL/SLL were retrieved from the archives of the Haemolymphopathology Service of Bologna University, Bologna, Italy, and selected on the availability of detection of BMB and IgV_H mutational status; data on ZAP70 in peripheral blood obtained by WB were also considered. No marrow clots were available. All patients were followed at the Haematology Service in Reggio, Calabria, Italy.

BMB specimens were fixed in B5 solution for 2 h, soaked in 70% alcohol for at least 30 min, and then decalcified in an EDTA-based solution for 2.5 h.³⁷ Sections of 3 µm thickness were cut for histological examination (H&E, Giemsa, Gomori silver impregnation) and IHC.

Abbreviations: APAAP, alkaline phosphatase anti-alkaline phosphatase; BMB, bone marrow biopsy; CLL/SLL, chronic lymphocytic leukaemia/small lymphocytic lymphoma; IHC, immunohistochemistry; IgV_H, immunoglobulin heavy chain genes; NK, natural killer; WB, western blotting; ZAP70, zeta-associated protein 70

Table 1 Antibodies and antigen retrieval strategies

Antigen	Source/clone	Dilution	Type of antigen unmasking
CD3	Neomarkers/SP7	1:300	A
CD20	Dako/L26	1:500	A
CD79a	Dako/JCB117	1:10	A
CD5	Dako/54F6	1:5	B
CD45RA	Dako/4KB5	1:20	A
CD45RO	Dako/UCHL1	1:120	A
CD23	Novocastra/1B12	1:30	B
CD38	Novocastra/SPC32	1:10	A
Cyclin D1	Neo Markers/cyclin D1 SP4	1:10	B
ZAP70	Santa Cruz Polyclonal	1:100	A, B, C, D
		1:200	
		1:400	
		1:800	
ZAP70	Upstate/2F3.2 Monoclonal	1:20	A
ZAP70	Cell Signaling/99F2 Polyclonal	1:40	B

Three cycles of 5 min each of microwaving at 750 (A) and 900 (B) W power with sections soaked in 1 μ M EDTA solution (pH 8.0); three cycles of 5 min each of microwaving at 750 W power with sections soaked in citrate buffer (C); proteolytic treatment at 37°C for 4 min with protease XIV (D).

The latter was performed by using the alkaline phosphatase anti-alkaline phosphatase complexes (APAAP) technique,³⁸ and table 1 lists the antibodies. Regarding ZAP70, the EnVision⁺ method³⁹ was also used and three different antibodies were compared (table 1): all of them were previously tested at different dilutions on formalin- and B5-fixed BMB specimens with reactive T cells and hyperplastic lymph nodes.

IHC results were scored as follows: +, >75% positive cells; +/-, 50–75% positive cells; -/+, 25–50% positive cells; rare, 10–25% positive cells; -, <10% positive cells. The staining intensity was recorded as moderate or weak (W) by comparison with the

strong positivity of T lymphocytes (endogenous positive control).

Determination of ZAP70 was carried out by WB as described previously.⁴⁰ Constant amounts (10 μ g/10 μ l) of protein from cell lysates were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis in 10% acrylamide, transferred to polyvinylidene difluoride Hybond-P membranes (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK), and exposed to primary mouse monoclonal antibody against ZAP70 (clone 2F3.2; Upstate, Lake Placid, New York, USA) or actin (Sigma, St Louis, Missouri, USA) and then to horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, California, USA). Peroxidase activity was revealed by chemiluminescence and the membrane was exposed to the film for 5 min. If the B-CLL cells in the suspensions were <90%, T cells, NK cells and monocytes were removed by CD3, CD56, CD16 and CD14 monoclonal antibody treatment (Becton Dickinson, San Diego, California, USA), followed by magnetic beads (Goat Anti-Mouse IgG Dynabeads; Dynal Biotech ASA, Oslo, Norway). H9 T cells expressing ZAP70 were used as positive controls of the antibody reactivity. The ZAP70 band intensity of CLL/SLL samples was compared with that of suspensions of purified T cells admixed with different proportions of mononuclear cells depleted of T and NK cells. These suspensions were obtained from the peripheral blood of the same healthy donor as described previously.⁴⁰ Accordingly, CLL/SLL samples were divided into three groups based on the levels of ZAP70 expression in the control suspensions that contained different percentages of ZAP70-positive cells: strong (corresponding to 40–100% positive cells), weak (corresponding to 20–40% positive cells) and negative (corresponding to <20% positive cells; fig 1). The CLL/SLL IgV_H rearrangement and mutations were determined using cDNA as described previously.⁴¹ The cut-off value used to assign IgV_H mutational status was 2%.

Figure 1 Demonstration of zeta-associated protein 70 (ZAP70) by western blotting (WB). (A) Ten different chronic lymphocytic leukaemia (CLL) cases are shown classified as strongly positive (S), weakly positive (W) and negative (N). In lane 7, there are H9 control T cells, and in lane 11 the T and NK cells are separated from the peripheral blood cells of a CLL case, the purified B cells of which are shown in lane 10. All lanes are loaded with 10 μ g/10 μ l sample total protein lysates. The membrane was exposed to chemiluminescence. The second panel shows the same membrane subsequently incubated with an anti-actin monoclonal antibody and exposed to chemiluminescence. (B) Graded dilutions of peripheral blood T cells and ZAP70-negative cells to determine the intensity of the WB bands corresponding to the indicated percentages of ZAP70-positive cells.

RESULTS

Immunohistochemistry

All biopsy specimens were infiltrated by a lymphoid infiltrate histologically consistent with CLL/SLL,¹ which always showed positivity for CD79a and CD20; CD20 stained at lower intensity than CD79a, and stained mainly pro-lymphocytes and para-immunoblasts, as reported previously.^{42–43} CD3 and CD45R0 were negative in neoplastic cells, being expressed by T lymphocytes. CD5 was evaluable in 25 cases (10 cases +, 8 +/-, 5 -/+, 2 rare), its intensity ranging from weak to moderate. CD23 was evaluable in 23 cases (22 cases +, 1 -). CD38 staining was always weaker than in control plasma cells and scored 20 cases -, 3 rare, 1 +/-, 2 +/-; CD45RA was evaluable in 19/26 cases and scored + to +/-, with a staining pattern similar to that of CD20. Cyclin D1 was always negative.

ZAP70 results (table 2) varied depending on the antibodies and detection method applied. The antibody from Santa Cruz failed to stain both T lymphocytes and lymphomatous cells, despite all attempts. The remaining two antibodies worked on all tissues tested, producing the correct cytoplasmic staining, stronger in intensity on T lymphocytes and weaker on neoplastic cells. On reactive tissues, no substantial differences were recorded between the antibodies and the detection methods.

The evaluable cases with Upstate ZAP70 were 25/26 with APAAP (10 scored +, 15 -) and 22/26 with EnVision⁺ (8 scored +, 1 +/-, 13 -; fig 2A–D); those with Cell Signaling ZAP70 were 20/24 with APAAP (6 scored +, 2 +/-, 12 -; 2 cases were exhausted) and 19/24 with Envision⁺ (6 scored +, 1 +/-, 12 -; 2 cases were exhausted; fig 2E,F). In the 20 comparable cases, the results with the two antibodies overlapped substantially (table 2). Somewhat surprisingly, given our previous experience³⁹ and that observed on reactive tissues, EnVision⁺ gave no advantage over APAAP, often providing either diffuse background staining or weak reactivity on control T cells.

Considering the results reported above and the higher number of evaluable cases with Upstate ZAP70 in the APAAP technique, the latter will be used as term of comparison for IHC in the following.

Peripheral blood: ZAP70 WB and mutational status

WB data were available in 23 cases, of which 13 were positive (9 strong, 4 weak) and 10 negative (table 2). On PCR, all cases were clonally rearranged for IgV_H: of these, 17 turned out to be mutated and 9 unmutated. In the former group, the percentage deviation of mutations ranged from 2.6 to 11.6, with an average value of 6.335 (table 2).

Comparison among the techniques used

ZAP70 IHC and WB were concordant in 19/23 cases (82.6%), although performed on different sample types (bone marrow and blood, respectively). Interestingly, 3/4 discordant cases were mutated and this result was consistent with IHC as opposed to WB.

When ZAP70 IHC was evaluated versus mutational status, concordance was found in 21/25 cases (84.9%; 87.5% for unmutated/ZAP70 positive and 82.3% for mutated/ZAP70 negative).

Regarding the four discordant cases, one was unmutated/ZAP70 negative and three mutated/ZAP70 positive, two of which were scored weak on IHC; interestingly, one of them was weakly positive with Upstate and negative with Cell Signaling antibodies (see the Discussion section). WB and mutational status were concordant in 16/23 cases (69.5%): 6/7 discordant cases were mutated/ZAP70 positive (85.7%) and 4 of them were scored weak.

Clinical data and ZAP70 IHC

The median age at onset was 65 years and the male:female ratio was 15:11 (table 2). The clinical outcome was available in 21 cases, with a 47-month median follow-up (range 1–225 months); in 10/21 cases first-line chemotherapy was required. In cases that were ZAP70 negative (n = 13) or weakly positive (n = 1) on BMB, there was a significantly longer time to the first treatment than in the positive cases (123 vs 21 months, p < 0.001).

DISCUSSION

The aims of this study on 26 CLL/SLL cases were to assess IHC determination of ZAP70 on BMB specimens and to compare IHC with WB data and IgV_H mutational status assessed on neoplastic cells from peripheral blood.

We first showed that IHC is easily applicable to routine BMB specimens and powerful in discriminating mutated from unmutated cases. In fact, ZAP70 (Upstate) results on BMB specimens were concordant with mutational status in 84.9% of the cases, with a higher rate than that obtained when WB data were compared with mutational status (69.5%). IHC also showed a high concordance with WB (82.6%) and was particularly reliable in the four discordant cases, where it was consistent with the mutational results, as opposed to WB.

It would certainly have been ideal to have WB and mutational status data from bone marrow aspirates also: however, in the daily management of patients with CLL/SLL, such analyses are routinely performed on peripheral blood. Our case series did not differ in this regard. In addition, as far as we know, there is no evidence from the literature that ZAP70 WB and mutational status analyses do provide different results in CLL/SLL elements when assessed in different sample types.

As far as we know, only five reports have been published on the comparison between IHC, WB and/or fluorescence-activated cell sorting analysis and mutational status.^{19–23, 28–30} In the papers from Crespo *et al*²³ and Carreras *et al*,²⁹ although the concordance between ZAP70 (in peripheral blood and tissue biopsy specimens) and mutations was high and similar to ours, data on BMBs were either lacking²³ or failed²⁹: specifically, Carreras *et al*²⁹ did not obtain evaluable results in 30 Bouin-fixed BMB specimens, despite the strong reactivities in non-decalcified samples with the Upstate monoclonal antibody.

Wiestner *et al*¹⁹ studied formalin-fixed peripheral blood mononuclear cell clots and B5-fixed BMB specimens with the Upstate antibody and an avidin–biotin–peroxidase method, obtaining 86% concordance between IHC and mutational status. However, the exact number of tested BMB specimens is unspecified and there is a major issue with clots. Admirand *et al*²⁸ found 85% concordance between IHC (performed with the Upstate monoclonal and a peroxidase-based labelled streptavidin–biotin method) and mutations in 22 CLL/SLL samples, 14 of which were formalin-fixed BMB specimens and clots, stating that interpretation of the latter results was easier. The latter statement is worthy of attention, as clots are not decalcified and are thus devoid of the related tissue artefacts that can impair the quality of IHC. O'Connor *et al*³⁰ found no correlation between ZAP70 IHC on BMB specimens and mutational status, but their use of methylmethacrylate resin for BMB processing could explain the discrepancy.

Our study compares the results for ZAP70 in CLL/SLL cases using antibodies from Upstate (monoclonal) and Cell Signaling (polyclonal), confirming their reliability in terms of sensitivity and specificity, and their optimal management on both B5- and formalin-fixed tissues, either decalcified or not.

In addition, the study supports the use of the APAAP technique rather than the peroxidase-based Envision⁺ method

Figure 2 (A) Bone-marrow infiltrate with overt positivity for zeta-associated protein 70 (ZAP70; Upstate monoclonal antibody; alkaline phosphatase anti-alkaline phosphatase [APAAP] technique; Gill's haematoxylin nuclear counterstaining; $\times 200$); inset: bone-marrow infiltrate with ZAP70 negativity: note the staining of reactive T-lymphocytes that represent the internal control (Upstate monoclonal antibody; APAAP technique; Gill's haematoxylin nuclear counterstaining; $\times 200$). (B) Bone-marrow infiltrate with weak positivity for ZAP70 (Upstate monoclonal antibody; APAAP technique; Gill's haematoxylin nuclear counterstaining; $\times 200$). (C) Bone-marrow infiltrate with overt positivity for ZAP70 (Upstate monoclonal antibody; EnVision⁺ technique; Gill's haematoxylin nuclear counterstaining; $\times 200$). (D) Bone-marrow infiltrate with weak positivity for ZAP70; note the moderate background staining (Upstate monoclonal antibody; EnVision⁺ technique; Gill's haematoxylin nuclear counterstaining; $\times 200$). (E) Bone-marrow infiltrate with overt positivity for ZAP70 (Cell Signaling polyclonal antibody; APAAP technique; Gill's haematoxylin nuclear counterstaining; $\times 200$). (F) Bone-marrow infiltrate with moderate positivity for ZAP70 (Cell Signaling polyclonal antibody; EnVision⁺ technique; Gill's haematoxylin nuclear counterstaining; $\times 200$).

Table 2 Summary of the analysed cases and main results of immunohistochemistry, western blotting and mutational studies

N° BX	Sex	Age (years)	ZAP70 ENV Upst	ZAP70 APAAP Upst	ZAP70 ENV Cell Sig	ZAP70 APAAP Cell Sig	ZAP70 WB	MS (% deviation)	IHC/MS*	CD45RA	CD45RO
1	F	74	—	—	—	—	NA	Mu (7.3%)	Concordant	NE	—
2	M	81	—	—	NE	NE	—	Mu (11.6%)	Concordant	—/+W	—
3	M	68	—	—	—	—	+S	UM	Discordant	NE	—
4	M	72	—	—	—	—	—	Mu (4.4%)	Concordant	NE	—
5	F	68	—/+	—	—	—	+W	Mu (5.6%)	Concordant	NE	—
6	M	62	—	—	EXH	EXH	—	Mu (5.5%)	Concordant	NE	—
7	F	74	—	—	—	—	—	Mu (7%)	Concordant	NE	—
8	F	67	NE	—	NE	NE	—	Mu (7.4%)	Concordant	+	—
9	M	33	—	—	—	—	—	Mu (7.1%)	Concordant	—/+	—
10	F	72	—	—	NE	NE	NA	Mu (3.8%)	Concordant	+	—
11	F	59	NE	+W	—	—	+W	Mu (3%)	Discordant	+	—
12	F	63	—	—	—	—	—	Mu (7.5%)	Concordant	—/+	—
13	M	68	—	—	—	—	+W	Mu (7.8%)	Concordant	+/-W	—
14	M	72	—	—	—	—	—	Mu (6%)	Concordant	NE	—
15	F	68	NE	+	+	+W	+S	UM	Concordant	+	—
16	M	81	+	+	NE	NE	+S	Mu (2.6%)	Discordant	—	NE
17	M	55	NE	—	—	—	+S	Mu (6.1%)	Concordant	+/-W	NE
18	F	73	—	—	—	—	—	Mu (5%)	Concordant	+	—
19	F	49	+	+	+	+W	+S	UM	Concordant	—/+ W	—
20	M	75	—	NE	EXH	EXH	—	UM	Concordant	+	—
21	M	47	+	+	+	+/-	+S	UM	Concordant	—/+	—
22	M	66	+	+	NE	+	+S	UM	Concordant	+	—
23	M	80	+	+	+	+	+S	UM	Concordant	+/-	—
24	M	65	+	+	+	+	+S	UM	Concordant	+	—
25	M	67	+	+W	+/-W	+/-W	+W	Mu (10.3%)	Discordant	+	—
26	F	56	+	+	+	+	NA	UM	Concordant	+/- W	—

APAAP, alkaline phosphatase anti-alkaline phosphatase; BX, biopsy; Cell Sig, Cell Signaling; EXH, material exhausted; ENV, envision; F, female; IHC, immunohistochemistry; M, male; MS, mutation status; NE, not evaluable; NA, not available; Mu, mutated; UM, unmutated; S, strong intensity; Upst, Upstate; W, weak intensity; WB western blotting; ZAP70, zeta-associated protein 70; scores: see text

*Concordance is defined between IHC (Upstate ZAP70 in APAAP) and MS.

and this may confirm and explain the difficulties in the IHC interpretation reported by others.^{19–28}

The 4 discordant cases deserve attention: 3 of 4 showed a mutated/ZAP70-positive pattern, and only in 2 of 4 (3 and 16) could discordance be reliably assessed. In fact, case 25 was scored weak with both antibodies, whereas case 11 only stained positive with the Upstate monoclonal antibody, being negative with the one from Cell Signaling. Considering the large overlap obtained with the two antibodies and the clear-cut positivity in discordant case 16, we think that the results in cases 25 and 11 are probably related to either the reagent or the tissue, and we speculate that weak ZAP70 staining should not be regarded as a reliable surrogate for mutations. Although clinicopathological correlations are beyond the scope of this study, this suggestion finds support in the “time to treatment period” known for one of our patients, which parallels that of the mutated/ZAP70-negative ones (see the Results section). In addition, this case was included in a series of 125 patients published by Morabito *et al.*³¹ where the discordant mutated/ZAP70 weakly positive cases (on WB) clustered with the concordant mutated/ZAP70-negative cases in terms of “actuarial risk of treatment requirement” and “3-year therapy-free survival”. Of course, this finding needs confirmation, as Wiestner *et al.*¹⁹ reported progressive disease in two of four similar patients: notably, both cases showed a mutated VH3-21 gene, absent in the remaining two “favourable” ones.

Vilpo *et al.*³⁶ analysed the phenotype of 32 CLL/SLL cases by flow cytometry and found that the only antigens differentially expressed in the two molecular forms were CD45R0 (evaluated as number of positive cells and associated with mutated cases) and CD45RA (evaluated as geometric mean fluorescence and associated with unmutated variants). We could not reproduce these data in our series, as our results do not discriminate mutated from unmutated forms; however, our evaluation of CD45RA was not successful in the older cases (before 1996) and our approach was different from that of Vilpo *et al.*³⁶

Finally, as stated in the introduction, ZAP70 positivity is not exclusive to CLL/SLL, but can also occur in other B cell lymphomas.^{28–29} The latter finding, which has no prognostic relevance, can, however, be diagnostically misleading. Thus, it is advisable to search for ZAP70 when the diagnosis of CLL/SLL is confirmed and other B cell lymphomas excluded (ie, mantle cell lymphoma by negativity for cyclin D1, follicular lymphomas

by positivity for bcl-6 and/or CD10, marginal zone lymphoma and lymphoplasmacytic lymphomas mainly by negativity for CD5).

In conclusion, we show that ZAP70 can be optimally detected on B5-fixed, decalcified and paraffin-wax-embedded BMB specimens using antibodies from Upstate or Cell Signaling, and the APAAP technique: this can be performed as reliably as WB and fluorescence-activated cell sorting analysis and in concordance with the mutational status. The recognition of weakly positive cases is of interest, as they more often correspond to mutated forms with a “time to treatment” closer to that of ZAP70-negative cases²⁷: in such instances, additional molecular studies should be advised.¹⁹

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REFERENCES

- Müller-Hermelink HK, Monserrat E, Catovsky D, *et al.* Chronic lymphocytic leukaemia/small lymphocytic lymphoma. In: Jaffe ES, Harris NL, Stein H, Vardiman JW, eds. *Pathology and genetics of tumours of haematopoietic and lymphoid tissues*. Lyon: IARC Press, 2001:127–30.
- Rozman C, Monteserrat E. Chronic lymphocytic leukemia. *N Engl J Med* 1995;**333**:1052–7.
- Kipps TJ. Chronic lymphocytic leukemia. *Curr Opin Hematol* 2000;**7**:223–34.
- Hamblin T. Chronic lymphocytic leukaemia: one disease or two? *Ann Hematol* 2002;**81**:299–303.
- Rai KR, Wasil T, Iqbal U, *et al.* Clinical staging and prognostic markers in chronic lymphocytic leukemia. *Hematol Oncol Clin North Am* 2004;**18**:795–807.
- Döhner H, Stilgenbauer S, Benner A, *et al.* Genomic aberrations and survival in chronic lymphocytic leukemia. *N Engl J Med* 2000;**343**:1910–16.
- Chevallier P, Penhler D, Avet-Loiseau H, *et al.* CD38 expression and secondary 17p deletion are important prognostic factors in chronic lymphocytic leukaemia. *Br J Haematol* 2002;**116**:142–50.
- Krober A, Seiler T, Benner A, *et al.* V(H) mutation status, CD38 expression level, genomic aberrations and survival in chronic lymphocytic leukemia. *Blood* 2002;**100**:1410–16.
- Stilgenbauer S, Döhner H. Molecular genetics and its clinical relevance. *Hematol Oncol Clin North Am* 2004;**18**:827–48.
- Damle RM, Wasil T, Fais F, *et al.* Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. *Blood* 1999;**94**:1840–7.
- Hamblin TJ, Davis Z, Gardiner A, *et al.* Unmutated IgV(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood* 1999;**94**:1848–54.
- Tobin G, Thunberg U, Johnson A, *et al.* Somatic mutated Ig V(H) 3–21 genes characterize a new subset of chronic lymphocytic leukemia. *Blood* 2002;**99**:2262–4.
- Keating MJ, Chiorazzi N, Messmer B, *et al.* Biology and treatment of chronic lymphocytic leukemia. *Hematology (Am Soc Hematol Educ Program)* 2003;**1**:153–75.
- Oscier DG, Gardiner AC, Mould SJ, *et al.* Multivariate analysis of prognostic factors in CLL: clinical stage, IGVH gene mutational status, and loss or mutation of the p53 gene are independent prognostic factors. *Blood* 2002;**100**:1177–84.
- Klein U, Tu Y, Stolovitzky GA, *et al.* Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneous phenotype related to memory B cells. *J Exp Med* 2001;**194**:1625–38.
- Rosenwald A, Alizadeh AA, Windhopf G, *et al.* Relation of gene expression phenotype to immunoglobulin mutation genotype in B cell chronic lymphocytic leukemia. *J Exp Med* 2001;**194**:1639–47.

Take-home messages

- Immunohistochemical determination of the zeta-associated protein 70 (ZAP70) protein is easily and reliably applicable to decalcified, B5- or formalin-fixed, and paraffin-wax-embedded bone marrow biopsy specimens.
- Of the three antibodies tested (polyclonal/Santa Cruz, monoclonal/Upstate, rabbit monoclonal/Cell Signaling), those of choice are the monoclonal ones.
- The concordance between the determination of ZAP70 by immunohistochemistry and the mutational status analysis is almost 85%, thus consistent with that reported in the literature for other means of investigation such as western blotting and flow cytometry.
- The exact meaning of weak ZAP70-positive staining, more often found in discordant cases (mainly with a ZAP70-positive/mutated pattern), is still debatable and should be definitively assessed by expanding the number of observations.

- 17 **Chan AC**, Iwashima M, Turck CW, *et al*. ZAP-70: a 70-kd protein-tyrosine kinase that associates with TCR zeta chain. *Cell* 1992;**71**:649–62.
- 18 **Nolz JC**, Tschumper RC, Pittner BT, *et al*. ZAP-70 is expressed by a subset of normal human B-lymphocytes displaying an activated phenotype. *Leukemia* 2005;**19**:1018–24.
- 19 **Wiestner A**, Rosenwald A, Barry TS, *et al*. ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. *Blood* 2003;**101**:4944–51.
- 20 **Orchard JA**, Ibbotson RE, Davis Z, *et al*. ZAP-70 expression and prognosis in chronic lymphocytic leukemia. *Lancet* 2004;**363**:105–11.
- 21 **Calin GA**, Liu CG, Sevignani C, *et al*. MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias. *Proc Natl Acad Sci USA* 2004;**101**:11755–60.
- 22 **Ferrer A**, Ollila J, Tobin G, *et al*. Different gene expression in immunoglobulin-mutated and immunoglobulin-unmutated forms of chronic lymphocytic leukemia. *Cancer Genet Cytogenet* 2004;**153**:69–72.
- 23 **Crespo M**, Bosch F, Villamor N, *et al*. ZAP-70 expression as a surrogate for immunoglobulin variable region mutations in chronic lymphocytic leukemia. *N Engl J Med* 2003;**348**:1764–75.
- 24 **Rassenti L**, Huynh L, Toy TL, *et al*. ZAP-70 compared with immunoglobulin heavy chain gene mutation status as a predictor of disease progression in chronic lymphocytic leukemia. *N Engl J Med* 2004;**351**:893–901.
- 25 **Kim SZ**, Chow KU, Kukoc-Zivojnov N, *et al*. Expression of ZAP-70 protein correlates with disease stage in chronic lymphocytic leukemia and is associated with, but not generally restricted to, non-mutated Ig VH status. *Leuk Lymphoma* 2004;**45**:2037–45.
- 26 **Schroers R**, Griesinger F, Trumper L, *et al*. Combined analysis of ZAP-70 and CD38 expression as a predictor of disease progression in B-cell chronic lymphocytic leukemia. *Leukemia* 2005;**19**:750–8.
- 27 **Morabito F**, Cutrona G, Matis S, *et al*. Relationship between ZAP-70 and CD38 expression, and mutational status of immunoglobulin heavy-chain variable region in predicting treatment free survival in chronic lymphocytic leukemia [abstract]. *Hematologica* 2005;**27**.
- 28 **Admirand JH**, Rassidakis GZ, Abruzzo LV, *et al*. Immunohistochemical detection of ZAP-70 in 341 cases of non-Hodgkin and Hodgkin lymphoma. *Mod Pathol* 2004;**17**:954–61.
- 29 **Carreiras J**, Villamor N, Colomo L, *et al*. Immunohistochemical analysis of ZAP-70 expression in B-cell lymphoid neoplasms. *J Pathol* 2005;**205**:507–13.
- 30 **O'Connor SJM**, Starczynski J, Teasdale J, *et al*. Can ZAP-70 protein expression in B-CLL as detected by immunohistochemistry predict VH mutation status? [Abstract]. *Blood* 2003;**102**:34.
- 31 **Morabito F**, Mangiola M, Oliva B, *et al*. Peripheral blood CD38 expression predicts survival in B-cell chronic lymphocytic leukemia. *Leuk Res* 2001;**25**:927–32.
- 32 **Del Poeta G**, Maurillo L, Venditti A, *et al*. Clinical significance of CD38 expression in chronic lymphocytic leukemia. *Blood* 2001;**98**:2633–9.
- 33 **Ibrahim S**, Keating M, Do KA, *et al*. CD38 expression as important prognostic factor in B-cell chronic lymphocytic leukemia. *Blood* 2001;**98**:181–6.
- 34 **Hamblin TJ**, Orchard JA, Ibbotson RE, *et al*. CD38 expression and immunoglobulin variable region mutations are independent prognostic variables in chronic lymphocytic leukemia but CD38 expression may vary during the course of disease. *Blood* 2002;**99**:1023–9.
- 35 **Mainou-Fowler T**, Dignum HM, Proctor SJ, *et al*. The prognostic value of CD38 expression and its quantification in B cell chronic lymphocytic leukemia (B-CLL). *Leuk Lymphoma* 2004;**45**:455–62.
- 36 **Vilpo J**, Tobin G, Hulkkonen J, *et al*. Surface antigen, expression and correlation with variable heavy-chain gene mutation status in chronic lymphocytic leukemia. *Eur J Haematol* 2003;**70**:53–9.
- 37 **Pileri SA**, Ascani S, Milani M, *et al*. Acute leukaemia immunophenotyping in bone marrow routine sections. *Br J Haematol* 1999;**105**:394–401.
- 38 **Cordell JL**, Falini B, Erber WN, *et al*. Immunoenzymatic labelling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). *J Histochem Cytochem* 1984;**32**:219–29.
- 39 **Sabattini E**, Bisgaard K, Ascani S, *et al*. The EnVisionTM system: a new immunohistochemical method for diagnostics and research. Critical comparison with the APAAP, ChemMateTM, CSA, LABC and SABC techniques. *J Clin Pathol* 1998;**51**:506–11.
- 40 **Messmer BT**, Messmer D, Allen SL, *et al*. In vivo measurements document the dynamic cellular kinetics of chronic lymphocytic leukemia B cells. *J Clin Invest* 2005;**115**:755–64.
- 41 **Fais F**, Ghiotto F, Hashimoto S, *et al*. Chronic lymphocytic leukemia B cells express restricted sets of mutated and unmutated antigen receptors. *J Clin Invest* 1998;**102**:1515–25.
- 42 **Tefferi A**, Bartholomai BJ, Witzig TE, *et al*. Heterogeneity and clinical relevance of the intensity of CD20 and immunoglobulin light-chain expression in B-cell chronic lymphocytic leukemia. *Am J Clin Pathol* 1996;**106**:457–61.
- 43 **Pileri SA**, Sabattini E, Agostinelli C, *et al*. Histopathology of B-cell chronic lymphocytic leukemia. *Hematol Oncol Clin North Am* 2004;**18**:807–26.

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