ZAP-70 methylation status is associated with *ZAP-70* expression status in chronic lymphocytic leukemia

Martin Corcoran
Anton Parker
Jenny Orchard
Zadie Davis
Michaela Wirtz
Oliver J. Schmitz
David Oscier

Background and Objectives. ZAP-70 expression is a recognized prognostic marker in chronic lymphocytic leukemia (CLL). The aim of this study was to analyze whether the methylation status of the *ZAP-70* gene is associated with expression of the ZAP-70 protein.

Design and Methods. Patients with CLL (n=87), acute lymphoblastic leukemia (n=13), mantle cell leukemia (n=13) and splenic marginal zone lymphoma (n=14) of known immunoglobulin gene mutation (*IgVH*) status were studied. The methylation status of the 5' region of *ZAP-70* was analyzed by combined bisulphite restriction analysis (COBRA), southern blotting and bisulphite sequencing in 10 CLL patients and in normal T/NK and B cells. Further COBRA of a single CpG site located 334bp downstream of the *ZAP-70* transcription start site (C-334) was then performed on all patients.

Results. ZAP-70 expression status in CLL and normal peripheral blood lymphocytes is associated with the methylation status of the intron1-exon2 boundary region of *ZAP-70* and methylation status of C-334 determined by COBRA is representative of methylation in this region. Of 87 CLL patients, 51/53 ZAP-70 negative patients had methylation at C-334 and 30/32 ZAP-70 positive patients did not have methylation (p<0.0001); a similar association was seen in all other diseases. Median survivals of methylated and unmethylated CLL patients were 211 and 85 months, respectively (p<0.0001).

Interpretation and Conclusions. Measuring *ZAP-70* methylation status at C-334 is a simple and reproducible method for predicting prognosis in CLL, which is closely associated with *ZAP-70* expression and *IgVH* gene mutational status. Methylation of a highly conserved intronic region of *ZAP-70* may be responsible for regulation of expression in normal and neoplastic cells.

Key words: CLL, *ZAP-70*, methylation.

Haematologica 2005; 90:1078-1088

From the Departments of Molecular Biology and Hematology, Royal Bournemouth Hospital, Bournemouth, UK (MC, AP, JO, ZD, MW, DO); Department of Oncology and Pathology, Cancercentrum, Karolinska Hospital, Stockholm, Sweden (MC); Department of Analytical Chemistry, University of Wuppertal, Wuppertal, Germany (MW, OJS).

Correspondence:
David Oscier, Department of Haematology, Royal Bournemouth Hospital, Bournemouth, Dorset, BH7 7DW, UK.
E-mail: david.oscier@rbch.nhs.uk

Heterogeneity in the clinical course of chronic lymphocytic leukemia (CLL) is a consequence of differences in cell biology between cases and the arbitrary time of diagnosis in a condition that is usually discovered incidentally from a routine blood count. In multi-variate analyses which include clinical stage, expression of CD38, cytogenetic abnormalities and immunoglobulin heavy chain gene (*IgVH*) mutation status, the last is the best predictor of overall survival.[1,2] Although other prognostic factors such as deletion of 17p or 11q and usage of the V(H)3-21 gene segment, have been associated with short survival times independently of the *IgVH* mutational status,[2,3] patients whose CLL cells have 98% or greater homology to the germ line *IgVH* gene sequence have a significantly worse prognosis than those whose *IgVH* genes have undergone a somatic hypermutation process.[4,5] However this assay is both expensive and labor intensive, limiting its routine use.

Gene expression profiling has shown that the genetic signature of the two mutational subsets of CLL is similar. However approximately 175 genes are differentially expressed with a statistical significance of p<0.001 using the lymphochip array system. Mean expression of the gene encoding ZAP-70, a zeta-chain associated protein tyrosine kinase, is 4.3 fold higher in unmutated than mutated cases and discriminates between the two mutational subsets of CLL.[6] We and others have shown that both ZAP-70 mRNA and protein expression, measured by flow cytometry, predicts time to treatment, progression-free survival and overall survival in both Binet stage A and all stages of CLL, with equivalent prognostic significance to *IgVH* gene mutational status.[7-11] ZAP-70 is essential for signaling through the T-cell receptor as well as other receptors expressed on T cells, NK cells, eosinophils and mast cells.[12-14] Mutation of the *ZAP-70* gene in humans results in severe combined immune deficiency with

absence of CD8 cells and a signal transduction defect in CD4 cells.[15] Experimentally, a spontaneous mutation within the *ZAP-70* gene causes chronic autoimmune arthritis in mice, resembling human rheumatoid arthritis.[16] ZAP-70 is not expressed in circulating normal human B cells, and signaling through the B cell receptor utilizes a related and more potent protein tyrosine kinase, Syk.

There is increasing evidence that regulation of gene expression during normal lymphocyte development is mediated through changes in chromatin structure, differential compartmentalization of active and silent genes within the nucleus and possibly through CpG methylation.[17] It is also well established that abnormal methylation is frequent in malignancy. Hypermethylation of CpG islands within gene promoters results in gene silencing, while hypomethylation may cause genomic instability or the up-regulation of gene expression.[18] CpG island hypermethylation and global hypomethylation may occur independently within the same tumor.

We hypothesized that the differential expression of ZAP-70 in CLL may be related to the methylation status of the *ZAP-70* gene. The data presented here confirm a close association between ZAP-70 expression, *ZAP-70* methylation status and *IgVH* gene status in CLL. In order to further elucidate the significance of *ZAP-70* methylation in CLL we also studied ZAP-70 expression and methylation in normal circulating B cells and T cells, B-cell acute lymphoblastic leukemia and the B-cell tumors, mantle cell lymphoma and splenic marginal zone lymphoma, in which *IgVH* genes may be either mutated or unmutated.

Design and Methods

Samples from patients

Peripheral blood samples were collected from 87 patients with a diagnosis of CLL based on standard NCI criteria. The median age of these patients was 77 years (range 48-95), and the series included 53 males. The mean follow-up time from diagnosis was 106 months (range 27-323 months). Binet stage at diagnosis was as follows: 78 patients were in stage A, 5 patients in stage B, 3 patients in stage C and in 1 case the stage was unknown. Thirty-six patients had received treatment but samples were all taken at least 3 months after any treatment had been completed. Genomic aberrations were as follows: trisomy 12 in 15 patients; del 11q in 11 patients; abnormalities of p53 in 9 patients.

Blood samples were also obtained from 3 healthy controls; 8 patients with common or pre B cell acute lymphoblastic leukemia (B-ALL) and 5 patients with T-cell acute lymphoblastic leukemia (T-ALL), all diagnosed by immunophenotyping; 13 patients with a t(11;14) translocation with morphology, immunophenotype and histology (5 cases) typical of mantle cell lymphoma (MCL); and 14 patients with peripheral blood morphology, immunophenotype and splenic histology (4 cases) consistent with splenic marginal zone lymphoma (SMZL). Written consent to participation in the study was obtained from all patients and controls. ZAP-70 protein expression had previously been determined on all patients and *IgVH* gene mutational status was previously established in all 87 CLL, 11 MCL and 13 SMZL patients.

Mononuclear cells from peripheral blood samples were isolated on a Histopaque gradient (Sigma, Dorset, UK) and stored at –20°C for up to a year prior to DNA extraction. Normal CD2 positive T/NK-cells and CD19 positive B-cells were sorted from fresh mononuclear cells pooled from the three healthy controls using MACS Separation Columns with CD2 and CD19 MicroBeads (Miltenyi Biotec GmBH, Bergisch Gladbach, Germany). Post-sort purity levels of 95% were confirmed for both cell subsets by flow cytometry. Mixing of these pure samples and further flow cytometric analysis were used to produce a range of CD19 positive B-cell samples with known CD2 positive T/NK cell contamination.

Flow cytometric analysis of ZAP-70

ZAP-70 protein was measured as previously described.[10] Briefly, separated lymphoid cells were placed in 0.5% paraformaldehyde for 30 minutes, washed with phosphate-buffered saline containing 0.05% Tween and 2% human serum albumin (PBSAT) and stored in 80% ethanol at -20°C for periods between 24 hours and 4 weeks. After storage the cells were washed in PBSAT and stained with 1.5 μL of the primary antibody, clone 2F3.2 (Upstate Biotechnology, Charlottesville, USA); all samples were run with an isotype control, mouse IgG2a (DakoCytomation Ltd., Cambridgeshire, UK). The secondary antibody was fluoroscein isothiocyanate (FITC)-conjugated (SAM, Novocastra Laboratories Ltd, Newcastle Upon Tyne, UK) and surface staining with CD2-phycoerythin (DakoCytomation Ltd.) was used to delineate T and NK cells. A minimum of 5000 cells was acquired in the Cell Quest program on a Becton Dickinson FacsCalibur flow cytometer (BD Biosciences, Palo Alto, CA, USA). As previously established, the cut-off for ZAP-70 positivity was 10%.

IgVH gene analysis

Immunoglobulin variable region genes were sequenced as previously described.[4] Briefly, the variable region was amplified by polymerase chain reaction (PCR) using a mixture of oligonucleotide 5'

primers specific for each leader sequence of the VH families 1-7, together with a 3' constant region primer or a 5' framework 1 (FW1) consensus primer and a 3' consensus JH primer. PCR products were purified (Qiagen, West Sussex, UK) and sequenced directly using an ABI 377/310 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). Nucleotide sequences were aligned to EMBL/GenBank and V-BASE(16), using MacVector 4.0 (International Biotechnologies, New Haven, CT, USA), or Lasergene (DNASTAR, Madison, WI, USA) sequencing analysis software. As previously determined, a cut-off of ≥98% germ-line homology was taken to define the unmutated sub-set.

Combined bisulphite restriction analysis of the 5' region of the ZAP-70 gene

The *ZAP-70* gene was annotated and entered into the NCBI third party annotated database as sequence BK005537. Initial screening was performed using the combined bisulphite restriction analysis (COBRA) technique.[19] Genomic DNA was obtained from mononuclear cell samples by phenol-chloroform extraction and approximately 1μg was treated with bisulphite as described previously, cleaned up using wizard clean up kit (Promega, Madison, WI, USA) and stored at –20°C for up to 2 months.[20] COBRA was performed on seven CpG loci spanning a 2.5 kb region from upstream of the predicted promoter region to intron 1 of the *ZAP-70* gene. The positions of the seven CpG loci relative to the *ZAP-70* TSS, the primer pairs used to amplify the sites, and the methylation sensitive restriction enzyme used (HpyCH4IV or Taqα1 (New England Biolabs, Beverly, MA, USA) are shown in Table 1. All primers were specific for the bisulphite modified sequence, contained no CpG dinucleotides and were designed using the program Methprimer.[21] Amplification was performed using Titanium Taq DNA polymerase (BD Biosciences) under standard PCR conditions with an annealing temperature of 54°C. Agarose gel electrophoresis, ethidium bromide staining and densitometry using AlphaEaseFC software (AlphaInnotec, San Leandro, CA, USA) was used for restriction fragment analysis of each PCR product.

Southern analysis of the intron1-exon2 boundary region of the ZAP-70 gene

Five micrograms of untreated genomic DNA were digested overnight with the restriction enzyme EcoR1 (New England Biolabs). Samples were then digested overnight with the methylation sensitive restriction enzyme Sma1 (New England Biolabs) that only cuts at restriction sites containing unmethylated cytosine. The resulting digests were run on a 1% agarose gel, transferred to Hybond-N nylon membrane (Amersham Phamacia Biotech UK Ltd., Buckinghamshire, UK) and hybridized with the PCR derived probe pZAP70. Primers for pZAP70 are as follows, pZAP70F CTCAGCAGACACCAGGCCTTC and pZAP70R GCTTACAAAGCCGGTTTGGATTTCC. Probe pZAP70 recognizes a 295 bp segment of the 5' region of ZAP-70 encompassing the 3' part of exon 1 and the 5' part of intron 1. The probe spans a CpG containing Sma1 site 243bp downstream of the TSS (C-243) (Figure 1).

Table 1. PCR based-methylation sensitive restriction analysis targets CpG loci within the 5' region of the *ZAP-70* gene.

Position of CpG	*Restriction Enzyme Name*	*Primer*	*Sequence*	*Amplicon Size (bp)*
+2122	HpyCH4IV	ZAPUP1-F	GAGGTATTTTGTTTTATAGTTGGTTTAG	138
		ZAPUP1-R	ATTACAACCCAAACAACCTAACC	
+1610	Taq1	ZAPUP2- F	GGTAGGGTGTGAGGGGATTTTTAGG	205
		ZAPUP2-R	CCCACCTATCCTTTCTAAACC	
+871	Taq1	ZAPUP3- F	GTGTAGGGGTTTAAAATTTATTATAAGG	540
		ZAPUP3-R	CTCTCAAAAAAAAACCTACAAAACCAC	
+631	HpyCH4IV	ZAPUP4- F	GAGAGAGTGTATGAGGTGTG	347
		ZAPUP4-R	ACCCTTACTCTCCAATAACTC	
+336	HpyCH4IV	ZAPUP5- F	GATGTTTGGTATAGAAGGGGT	301
		ZAPUP5-R	AACAAAAACAAAATTTCAAAAAAA	
+88	HpyCH4IV	ZAPUP6- F	TAGTTTTATTTTGTAGTTGGG	322
		ZAPUP6-R	AACCCCCTAAAATAACCCTAATAA	
-334	Taq1	ZAPDN1-F	TTTTATTTATGAGTGAGAAATTTTGG	213
		ZAPDN1-R	TATCCAAACAAACAAACCACTTAC	

The table shows the position of CpG loci in relation to the ZAP-70 transcription start site (TSS), restriction enzymes with a recognition site spanning CpG loci, the primers used for amplification of loci and PCR amplicon sizes.

Bisulphite sequencing of the ZAP-70 intron1-exon2 boundary region

The 213bp PCR product amplified from bisulphite-treated DNA using primers ZAPDN1-F and ZAPDN1-R, which span the *ZAP-70* intron1-exon2 boundary (Table 1, position –334) was cloned using pCR®4-TOPO® Vector and One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen, Paisley, UK). After overnight incubation at 37°C on LB agar plates containing 100 μg/mL ampicillin, individual discrete colonies were picked and cultured overnight in LB broth containing 100 μg/mL ampicillin, at 37°C. The vector DNA was then extracted from the cultures using a standard rapid alkaline lysis method and Wizard® DNA clean up columns (Promega) and the insert sequenced using a standard M13-R primer (M13-R, 5'CAGGAAACAGCTATGAC) and ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit v1.1 (Applied Biosystems). The sequencing prod-

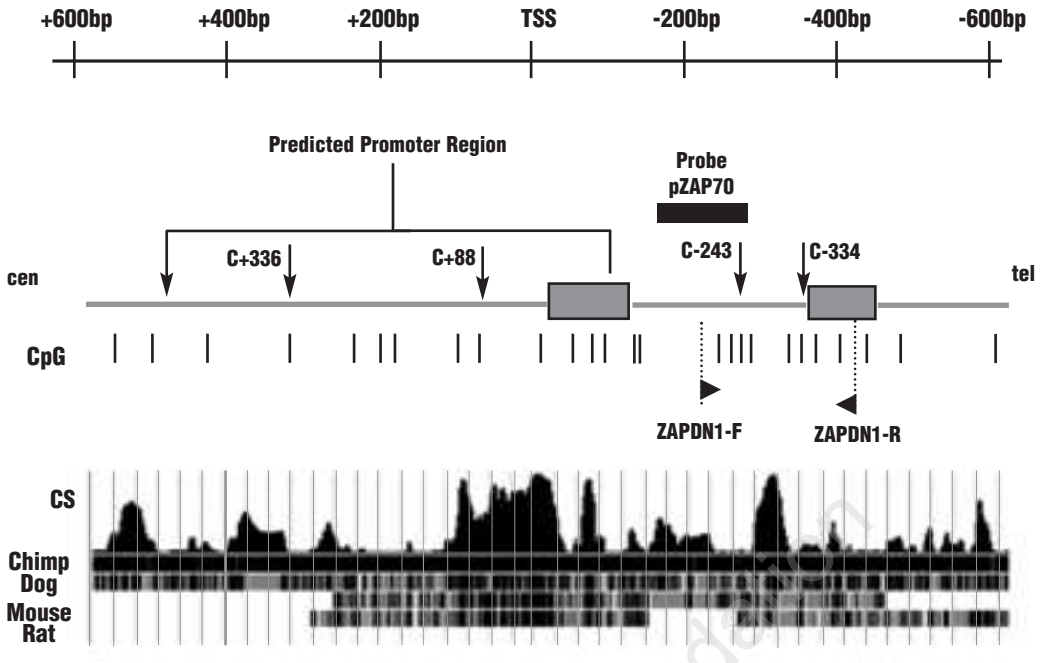


Figure 1. Schematic of the *ZAP-70* transcription start site (TSS). The position of exon 1 and exon 2 of the *ZAP-70* gene (gray boxes), the PCR primers ZAPDN1-F and ZAPDN1-R, CpG dinucleotides (vertical bars), CpG loci within restriction digest sites (C+336, C+88, C-334), the Sma1 restriction site (C-243) and the promoter region predicted using the ElDorado extended genome annotation facility (*http://www.genomatix.de/cgi-bin/eldorado/main.pl*) are shown. The BLAT analysis conservation score (CS) is displayed as a histogram indicating conservation of the human *ZAP-70* 5' region across all chimp, mouse, rat, dog, chicken, fugu, and zebrafish genomes. Conserved regions between the human *ZAP-70* 5' region and that of chimpanzee, dog, mouse and rat are also shown (*http://genome.ucsc.edu/cgi-bin/hgBlat?command=start&db=mm4*).

ucts were run on an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems) and analyzed using SeqManII Sequence Analysis Software (DNASTAR Inc).

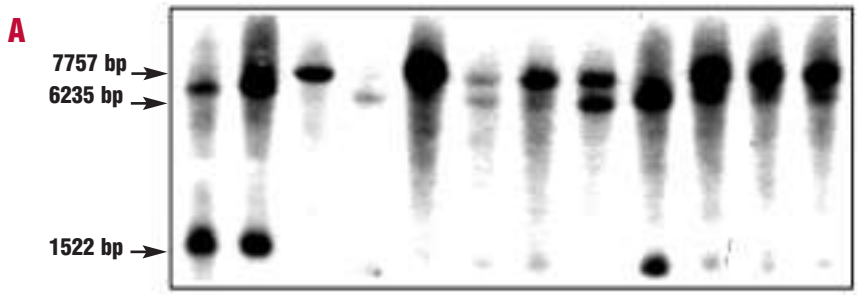
Capillary electrophoretic analysis of genomic DNA methylation levels

Determination of the genomic DNA methylation level was carried out by the previously published capillary electrophoresis and laser-induced fluorescence (CE-LIF) detection method, which is based on DNA hydrolysis, DNA derivatization, capillary electrophoretic analysis and the determination of the derivatization factors.[22] The CE instrument used for the analysis was a PACE™ MDQ system with a laser-induced-fluorescence detector (Beckman Coulter, Munich, Germany). The separation was carried out at 25°C on an untreated fused-silica capillary (CS-Chromatography-Service, Langerwehe, Germany); the field strength was 400 V/cm. The samples were injected hydrodynamically and the capillary outlet was the cathode in all runs. While we used 1 or 10 µg of genomic DNA for the analyses described here more recent experiments have shown that reproducible results can also be obtained with only 100 ng DNA.[23]

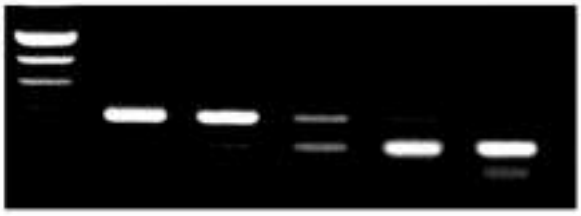
Results

Methylation status of CpG loci within the 5' region of the ZAP-70 gene in CLL

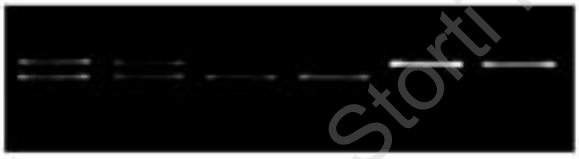
COBRA of seven CpG loci across the 5' region of *ZAP-70* was performed on five ZAP-70 positive patients (cases 2, 4, 18, 69 and 70) and five ZAP-70 negative CLL patients (cases 17, 32, 37, 43 and 79). Only PCR product amplified from genomic DNA with 5-methyl cytosine at a CpG locus would retain the restriction site after bisulphite treatment and be cut. No restriction, and therefore no methylation, was observed in any of the patients' samples at the CpG sites +2122bp, +1610bp, +871bp, +336bp or +88bp to the TSS. Some restriction and therefore methylation was observed in four cases at the CpG site +631bp to the TSS, but no association was seen between the methylation status of this site and ZAP-70 expression status. At the CpG site located in intron 1, -334bp to the TSS (C-334), the restricted fraction (RF) ranged from 73-96% (mean, 83%±9%) for the five ZAP-70 negative patients and from 4-21% (mean, 13%±6%) for the five ZAP-70 positive cases. Using an unpaired two-tailed Student t-test, the difference between the restricted fractions seen for the two groups of patients



	Patient Number											
	2	4	17	18	C	32	43	37	69	79	81	87
ZAP-70	Pos	Pos	Neg	Pos	N/A	Neg	Neg	Neg	Pos	Neg	Neg	Neg
Interpretation	R	R	U	R	U	I	U	I	R	U	U	U



	Size	Sample Number				
	Marker	1	2	3	4	5
B-cells (%)	–	5	26	51	75	>95
RF (%)	–	8	30	59	77	94



	Patient Number					
	3	12	24	28	58	80
ZAP-70	Neg	Neg	Pos	Pos	Neg	Neg
RF (%)	59	64	94	97	0	25

Figure 2. Southern based hybridization analysis and combined bisulphite restriction analysis (COBRA) of the intron 1/exon 2 boundary region of the *ZAP-70* gene. **Panel A.** The results of analysis at the CpG site 243bp downstream of the ZAP-70 transcription start site (C-243) in 11 CLL patients with known ZAP-70 protein expression. The hybridization results indicate complete restriction (R) in patients 2, 4, 18 and 69 at the methylation sensitive SmaI site spanning C-243, while patients 17, 43, 79, 81 and 87 are completely unrestricted (U). Patients 32 and 37 gave an intermediate result (I) with approximately 50% restriction at this site. Normal B-lymphocyte control DNA (sample C) was digested with the restriction enzyme EcoR1 only. **Panel B.** Results of COBRA of the CpG site 334bp downstream of the *ZAP-70* transcription start site (C-334) for pure T/NK cells, pure B cells and mixed B/T cell samples, showing percentage B cells and the fraction of the 213bp ZAPDN1-F - ZAPDN1-R PCR product restricted by TaqαI as calculated by densitometric analysis (RF). **Panel C.** COBRA of C-334 for six CLL patients. The results of ZAP-70 protein expression by flow cytometry and the fraction of the 213bp ZAPDN1-F - ZAPDN1-R PCR product restricted by TaqαI, as calculated by densitometric analysis (RF), are shown.

was significant ($p<0.001$). Southern analysis was performed to confirm the differential methylation seen at the C-334 location. The CpG methylation sensitive SmaI restriction site, spanning the CpG locus –243bp relative to the TSS (C-243), was chosen for its close proximity to C-334. Analysis was performed on nine of the ten CLL patients previously studied (cases 2, 4, 17, 18, 32, 37, 43, 69 and 79), together with two additional ZAP-70 negative patients (cases 81 and 87). The C-243 locus was undigested and therefore methylated in five of seven ZAP-70 negative patients and showed partial methylation in the other two cases. In contrast, all four ZAP-70 positive cases demonstrated complete digestion, indicating unmethylated cytosine at C-243 (Figure 2A). As further confirmation of the variable methylation results seen at C-243 and C-334 and to assess the methylation status of the six other CpG sites in the intron 1-exon 2 boundary region of *ZAP-70*, bisulphite sequencing was performed on eight clones from each of the five ZAP-70 positive and five ZAP-70 negative CLL patients previously analyzed by methylation sensitive restriction (Table 2). Sequence analysis showed no cytosine residues at non-CpG loci, indicating complete bisulphite conversion. Analysis of all CpG loci (deleted CpG sites were excluded from analysis) in all clones from each patient, showed that the percentage of cytosine residues methylated ranged from 52 - 97% (mean, 74±19%) for each of the ZAP-70 negative patients and from 3-13% (mean, 7±4%) for each of the ZAP-70 positive patients. Using an unpaired two-tailed Student t-test, the difference between the two groups of patients was statistically significant ($p<0.001$). Looking in turn at the percentage of clones methylated for each patient at each CpG locus, there was a significant difference between the ZAP-70 positive and ZAP-70 negative patients at C-319, C-334, C-352 and C-384 ($p<0.001$).

Table 2. Bisulphite sequencing of the intron 1/exon 2 boundary region.

	ZAP-70 NEGATIVE *Cytosine loci (bp downstream of TSS)*									***ZAP-70 POSITIVE*** *Cytosine loci (bp downstream of TSS)*							
	223	*243*	*254*	*267*	*319*	*334*	*352*	*384*		*223*	*243*	*254*	*267*	*319*	*334*	*352*	*384*
	Patient 17 Clone number									**Patient 2 Clone number**							
1	U	**M**	U	U	U	U	U	U	1	U	U	U	U	U	U	U	U
2	**M**	**M**	**M**	**M**	**M**	**M**	**M**	**M**	2	U	U	U	U	U	U	U	U
3	**M**	**M**	**M**	**M**	**M**	**M**	**M**	**M**	3	U	U	U	U	U	U	U	U
4	U	**M**	U	**M**	**M**	**M**	**M**	**M**	4	U	U	U	U	U	U	U	U
5	U	U	U	U	U	U	U	**M**	5	U	U	U	U	U	U	U	U
6	**M**	**M**	U	U	**M**	**M**	**M**	**M**	6	U	U	U	U	U	U	U	U
7	**M**	**M**	**M**	**M**	**M**	**M**	**M**	**M**	7	U	U	U	**M**	U	U	U	U
8	U	**M**	**M**	U	U	**M**	**M**	U	8	U	U	U	U	U	U	U	**M**
	Patient 32 Clone number									**Patient 4 Clone number**							
1	**M**	**M**	U	U	U	U	**M**	**M**	1	U	U	U	U	U	U	U	U
2	U	U	U	U	U	U	M	**M**	2	U	U	U	U	U	U	**M**	U
3	**M**	**M**	del	U	**M**	**M**	U	**M**	3	U	U	U	U	U	U	U	**M**
4	**M**	U	del	U	**M**	**M**	**M**	**M**	4	U	U	U	U	U	U	U	**M**
5	**M**	**M**	del	U	**M**	**M**	**M**	**M**	5	U	U	U	U	U	U	U	U
6	**M**	**M**	del	M	**M**	**M**	**M**	**M**	6	U	U	U	U	U	U	U	U
7	**M**	U	U	U	U	**M**	**M**	**M**	7	U	U	U	U	U	U	U	U
8	**M**	**M**	del	U	**M**	**M**	**M**	**M**	8	U	U	U	U	U	U	U	U
	Patient 37 Clone number									**Patient 18 Clone number**							
1	U	U	U	U	U	U	U	**M**	1	U	U	U	U	U	U	**M**	**M**
2	U	U	U	U	M	**M**	**M**	**M**	2	U	U	U	U	U	U	U	U
3	U	U	**M**	U	M	**M**	**M**	**M**	3	U	U	U	U	U	U	U	U
4	U	**M**	U	**M**	M	**M**	**M**	**M**	4	U	U	U	U	U	U	U	U
5	U	**M**	**M**	**M**	M	**M**	**M**	**M**	5	U	U	U	U	U	U	U	U
6	U	U	U	U	M	**M**	**M**	**M**	6	U	U	U	U	U	U	U	U
7	U	U	U	U	U	U	U	**M**	7	U	U	U	U	U	U	U	U
8	U	**M**	U	U	M	**M**	**M**	**M**	8	**M**	**M**	U	**M**	**M**	**M**	**M**	U
	Patient 43 Clone number									**Patient 69 Clone number**							
1	**M**	**M**	**M**	**M**	**M**	**M**	**M**	**M**	1	U	U	U	U	U	U	U	U
2	**M**	U	**M**	**M**	**M**	**M**	**M**	**M**	2	U	U	U	U	U	U	U	U
3	**M**	**M**	**M**	**M**	**M**	**M**	**M**	**M**	3	U	U	U	U	U	U	U	**M**
4	**M**	**M**	**M**	**M**	**M**	**M**	**M**	**M**	4	U	U	U	U	U	U	U	U
5	**M**	**M**	**M**	**M**	**M**	**M**	**M**	**M**	5	U	U	U	U	U	U	U	**M**
6	**M**	**M**	**M**	**M**	**M**	**M**	**M**	**M**	6	U	U	U	U	U	U	U	U
7	**M**	**M**	**M**	**M**	**M**	**M**	**M**	**M**	7	U	U	U	U	U	U	U	U
8	U	**M**	**M**	**M**	**M**	**M**	**M**	**M**	8	U	U	U	U	U	U	U	U
	Patient 79 Clone number									**Patient 70 Clone number**							
1	U	U	U	U	U	U	U	U	1	U	U	U	U	U	U	**M**	**M**
2	**M**	**M**	**M**	**M**	**M**	**M**	**M**	**M**	2	U	U	U	U	U	U	U	**M**
3	**M**	**M**	**M**	**M**	**M**	**M**	**M**	**M**	3	U	U	U	U	U	U	**M**	U
4	**M**	**M**	**M**	**M**	**M**	**M**	**M**	**M**	4	U	U	U	U	U	U	U	**M**
5	**M**	**M**	**M**	**M**	**M**	**M**	**M**	**M**	5	U	U	U	U	U	U	U	U
6	**M**	**M**	**M**	**M**	**M**	**M**	**M**	**M**	6	U	U	U	U	U	U	U	**M**
7	**M**	**M**	**M**	**M**	**M**	**M**	**M**	**M**	7	U	U	U	U	U	U	U	U
8	**M**	**M**	**M**	**M**	**M**	**M**	**M**	**M**	8	U	U	U	U	U	U	U	U

Sequencing results for eight ZAPDN1-F/ZAPDN1-R PCR products, cloned from each of five ZAP-70 negative and five ZAP-70 positive CLL patients. The position of the CpG loci relative to the ZAP-70 transcription start site (TSS) and the methylation status of the CpG loci is shown (M: methylated; U: unmethylated).

Methylation status of CpG loci within the intron 1-exon 2 boundary region of the ZAP-70 gene in normal T/NK and B cells

Having shown differential methylation between ZAP-70 positive and ZAP-70 negative CLL cases, we investigated the methylation status of the eight CpG intron1-exon 2 boundary sites in normal B cells that do not express ZAP-70 and normal T/NK cells that are ZAP-70 positive. Sixteen clones were sequenced from purified normal $CD19^+$ B cells and a further sixteen from $CD2^+$ T/NK cells. Again, complete bisulphite conversion was confirmed by the absence of cytosine

residues at non-CpG loci. Considering the percentage of CpG loci methylated in each clone, there was interclonal variation among the B-cell clones (range 0-100%, mean 68%, SD 30%) and T/NK-cell clones (range 0-100%, mean 18%, SD 28%) and a significant difference between the two populations, using an unpaired two-tailed Student's t-test ($p<0.001$). The sequencing results showed variable methylation of the C-334 locus between the normal B and T/NK cells, allowing the use of mixed lymphocyte samples to assess the sensitivity of the C-334 restriction analysis assay. COBRA of >95% pure normal T/NK-cells showed an RF of 8% and for >95% pure normal B cells the RF was 94%; mixed samples containing 26%, 51% and 75% B cells showed RF values of 30%, 59% and 77% respectively, as demonstrated in Figure 2B. Comparing the RF values to the percentage B cells gave a correlation coefficient greater than 0.99. These findings suggest that in patient samples undergoing COBRA of C-334 the percentage of normal cell contamination would be directly reflected in the RF value.

COBRA of C-334 methylation status in CLL

Cut-offs for the COBRA technique were set inline with the RF values seen at C-334 for the ten patients whose methylation status in the intron 1-exon 2 region had been confirmed by bisulphite sequencing. Patients with RF ≥ 70% were classed as methylated, those with RF ≤ 30% were classed as unmethylated and values from 30 to 70% were classed as equivocal.

In all 87 CLL samples tested by COBRA, 53 were methylated at C-334 with RF ranging from 73 to 99% (mean, 89±10%) and 32 were unmethylated at C-334 with RF ranging from 2 to 23% (mean, 11±7%). In two patients, cases 3 and 12, the restriction analysis was equivocal with RF values of 59% and 64%, respectively (see Figure 2C). The two patients with equivocal values were not included in the CLL patient cohort for statistical analysis but were investigated further by bisulphite sequencing, yielding results similar to the previous sequencing results from the five ZAP-70 negative patients (*data not shown*). Looking specifically at the C-334 locus in the sequences, the frequency of methylation was 63% for patient 3 and 50% for patient 12, these values were again similar to the RF values obtained for both patients (64% and 59%, respectively).

C-334 methylation status is associated with ZAP-70 protein expression status and with IgVH gene mutational status in CLL

The relationships between methylation status at C-334, ZAP-70 protein expression, and IgVH gene mutational status, are shown in Figure 3A. Using a 10% cut-off for ZAP-70 protein expression, 51 of 53 (96%) ZAP-70 negative patients had methylation at C-334 and 30 of 32 (94%) ZAP-70 positive patients did not have methylation at C-334 (Fisher's exact test, $p<0.0001$). All four patients with discordance between ZAP-70 expression and C-334 methylation had unmutated *IgVH* genes; two were ZAP-70 negative and unmethylated at C-334 and two were ZAP-70 positive and methylated at C-334. Bisulphite sequencing of these four patients again showed that the RF values obtained by COBRA of C-334 were representative of the methylation status of the intron

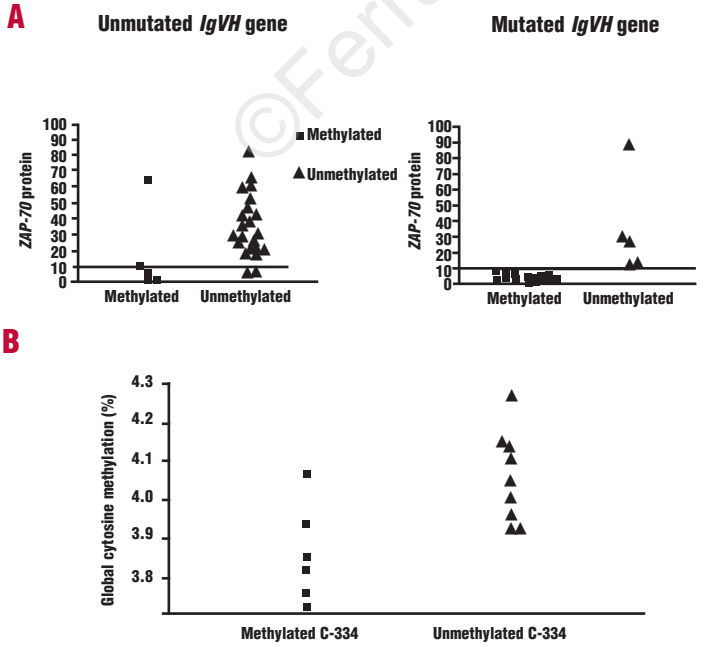


Figure 3. The relationships between methylation status at C-334, ZAP-70 protein expression, *IgVH* gene mutational status and global cytosine methylation levels in CLL patients. Panel A. Methylation status at the CpG loci 334 bases downstream of the *ZAP-70* transcription start site (C-334) against ZAP-70 protein expression, in two groups of CLL patients with unmutated or mutated *IgVH* genes. Panel B. The percentage of cytosine residues methylated in Ficoll-separated lymphocyte DNA samples from six CLL patients with methylated cytosine at the C-334 and nine CLL patients with unmethylated cytosine at C-334.

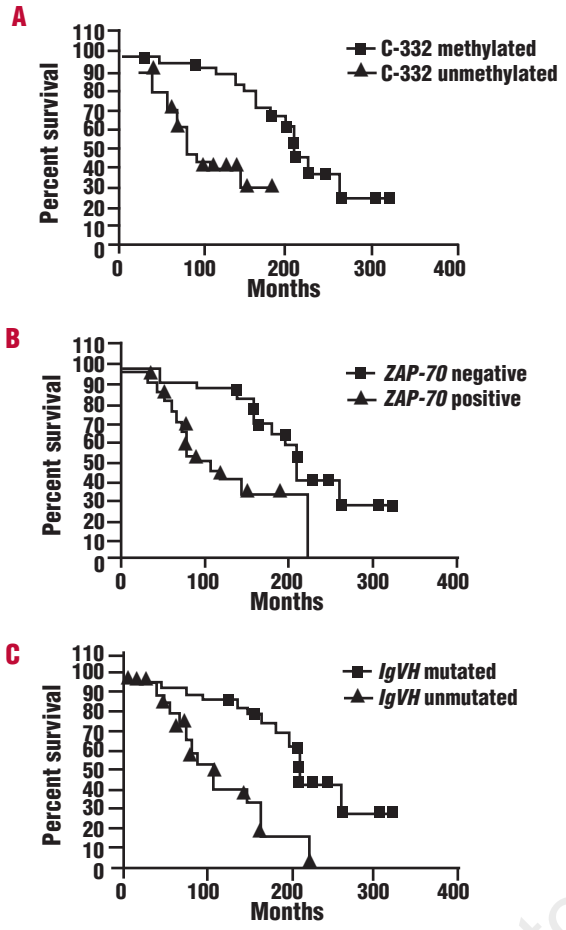


Figure 4. Kaplan-Meier survival curves for 85 CLL patients. Panel A. Patients with methylated cytosine at the CpG loci 334 bases downstream of the ZAP-70 transcription start site (C-334) and C-334 unmethylated CLL patients had median survivals of 211 months and 85 months, respectively (95% CI). Panel B. ZAP-70 negative and ZAP-70 positive CLL patients had median survivals of 211 months and 110 months, respectively. Panel C. *IgVH* gene mutated CLL patients and *IgVH* gene unmutated CLL patients had median survivals of 211 and 110 months, respectively.

1-exon 2 boundary region (*data not shown*). Using the established 98% homology to the germline sequence cut-off for *IgVH* gene mutational status, 49 of 54 (91%) patients with mutated *IgVH* genes had methylation at C-334 and 27 of 31 (87%) patients with unmutated *IgVH* genes had an unmethylated C-334 locus (Fisher's exact test, $p<0.0001$). Of the nine patients showing discordance between ZAP-70 expression and *IgVH* gene mutational status, there was concordance between ZAP-70 expression and methylation status in seven (five unmutated and two mutated *IgVH* genes). Patients 3 and 12, who displayed equivocal C-334 methylation results by restriction analysis but methylation of the 5' region by bisulphite sequencing, were ZAP-70 negative and had mutated *IgVH* genes.

C-334 methylation status is associated with survival in CLL

The median survival of patients with methylation at C-334 was 211 months whereas it was 85 months for patients unmethylated at the same locus ($p<0.0001$) (Figure 4A). Patients negative for ZAP-70 expression also had a median survival of 211 months, compared to 110 months for those expressed ZAP-70 ($p<0.0001$) (Figure 4B), while patients with either mutated or unmutated *IgVH* genes had identical median survivals of 211 and 110 months, respectively ($p<0.0002$) (Figure 4C).

C-334 methylation and global genomic DNA methylation in CLL

Global cytosine methylation was analyzed in 15 of 87 CLL patients, six were ZAP-70 negative, with mutated *IgVH* genes and methylated C-334, and nine were ZAP-70 positive, with unmutated *IgVH* genes and unmethylated C-334. The global cytosine methylation level for each patient ranged from 3.7 to 4.1% (mean, 3.9±0.15%) in the C-334 methylated patients and from 3.9 – 4.3% (mean, 4.1±0.12%) in the C-334 unmethylated patients, the difference between the two patient groups being significant ($p\leq0.001$) (Figure 3B).

ZAP-70 methylation, expression, and IgVH gene mutational status in non-CLL cases

COBRA of C-334 was performed on all 40 non-CLL patients and the results are shown in Table 3. Of eight B-ALL patients, five ZAP-70 positive cases were unmethylated at C-334 and of three ZAP-70 negative cases two were methylated and one was unmethylated. All five cases of T-ALL were ZAP-70 positive and of these four were unmethylated and one was methylated at C-334. In the 13 cases of MCL, all 12 ZAP-70 negative cases were methylated, while the one remaining ZAP-70 positive case was unmethylated. Of the SMZL cases, all 13 ZAP-70 negative cases were methylated but the 1 ZAP-70 positive case was also methylated. Considering the MCL and SMZL patients, all of the seven patients with unmutated *IgVH* genes and 16 of 17 patients with mutated *IgVH* genes had methylation at C-334.

Discussion

IgVH gene mutational status is well established as a powerful independent prognostic marker in CLL, although this does not appear to be the case in other lymphoproliferative disorders such as MCL or SLVL in which *IgVH* genes may also be mutated or unmutated. In CLL ZAP-70 expression at both mRNA and protein

Table 3. *ZAP-70* methylation, expression, and *IgVH* gene mutational status in non-CLL cases.

Disease	*Total Cases*	*ZAP-70 +VE Status*	*Methylation (ZAP-70 +VE)*	*ZAP-70 -VE Status*	*Methylation (ZAP-70 -VE)*
B-ALL	8	5	5 U	3	1U,2M
T-ALL	5	5	4U, 1M	0	–
MCL	13	1	1U	12	12M
SMZL	14	1	1M	13	13M

ZAP-70 protein expression as determined by flow cytometry and corresponding methylation status of the C-334 loci in 13 cases of common or pre-B-cell acute lymphoblastic leukemia (B-ALL), 5 cases of T cell acute lymphoblastic leukemia (T-ALL), 13 patients with mantle cell lymphoma (MCL) and 14 cases with splenic marginal zone lymphoma (SMZL). M: methylated at C-334, unmethylated at C-334.

levels has shown a close association with *IgVH* gene mutational status, although the percentage of discordant cases varies between series depending on the patient population and the *ZAP 70* assay employed. We now show that *ZAP 70* is differentially methylated in CLL and that a close association exists between *ZAP 70* methylation and expression, and with *IgVH* gene mutation status. Specifically, the majority of cases expressing ZAP-70 protein show lack of methylation in the intron 1-exon 2 boundary region of the *ZAP-70* gene and have unmutated *IgVH* genes. We also show that this region of *ZAP-70* is unmethylated in circulating T cells expressing ZAP-70, but methylated in ZAP-70 negative circulating B cells derived from healthy controls.

The assay for *IgVH* gene mutational status remains technically difficult and expensive and many assays for ZAP-70 expression are problematic because of the much greater intensity of expression in contaminating T/NK cells than in tumor cells (e.g. quantitative reverse transcription-PCR, Western blotting). Measuring ZAP-70 expression by flow cytometry avoids this problem, but since the protein is a weakly expressed intracellular antigen in tumor cells, this assay has yet to be standardized. The methylation assay we now describe appears to be tolerant of levels of T/NK cell contamination routinely seen in CLL and so may be valuable as a prognostic marker and surrogate for analysis of *IgVH* gene mutational status in cases for which only small quantities of DNA, such as from archival samples, are available for analysis. The difference in median survival between unmethylated and methylated cases (85 versus 210 months) was very similar to that found for ZAP-70 expression or *IgVH* gene mutational status. There are a number of reasons why the *ZAP-70* gene may be differentially methylated in CLL. One possibility is that this reflects the normal pattern of *ZAP-70* methylation expression during B-cell maturation. Support for a possible role of *ZAP-70* in B-cell development comes from murine data.[23,24] In the absence of Syk, murine B-cell development is partially blocked at the pro-B to pre-B-cell transition. However, mice lacking both Syk and *ZAP-70* have a complete block in B-cell maturation at the pro-B-cell stage and a failure of heavy chain allelic exclusion. There is preliminary data both from our own laboratory (*data not shown*) and from analysis of two normal marrow samples by Crespo *et al.*, that ZAP-70 is expressed in immature B ($CD19^+$, $CD10^+$) cells.[25] We also found *ZAP-70* to be unmethylated and expressed not only in cases of T-ALL but also in B-ALL. ZAP-70 expression in cases of CLL with unmutated *IgVH* genes may, therefore, reflect the origin of this sub-type of CLL from an immature B cell normally expressing ZAP-70. However, evidence against this hypothesis comes from the observation of biased *IgVH* gene usage in unmutated CLL that strongly suggests that this subset of CLL arises from an antigen experienced B cell.[26] Another possibility is that ZAP-70 positive CLL arises from a rare subset of mature ZAP-70 positive B cells. Although we have found no evidence for a circulating ZAP-70 positive B cell, Nolz *et al*. have recently shown that ZAP-70 is expressed in a subpopulation of normal tonsillar and splenic B-lymphocytes that express an activated phenotype.[27] In contrast, cases of ZAP-70 negative CLL could arise from a mature B cell which has usually undergone somatic hypermutation of the *IgVH* genes and methylation of the *ZAP-70* gene.

An alternative explanation for ZAP-70 expression in a subset of CLL is a malignancy-related abnormality of *ZAP-70* gene methylation. A recent study, utilizing restriction landmark genomic scanning as a global marker for hypermethylation, showed 4.8% of CpG islands were aberrantly and non-randomly methylated in CLL cells from 10 cases compared to CD19 positive B cells from normal controls and that treating a cell line, possessing an immunophenotype and genetic abnormalities typical of CLL, with a demethylating agent resulted in up-regulated expression of several genes, which were hypermethylated in clinical samples.[28] Previous studies had noted hypermethylation of the E cadherin and p57 KIP2 genes in some cases of CLL, but gene expression in these patients was not studied.[29]

Global hypomethylation studied with a variety of techniques has been well documented in CLL.[22,30-32] Using capillary electrophoretic analysis, Stach *et al*. found high variability of methylation levels among 81 patients with CLL but a general trend towards hypomethylation compared to levels in normal CD19 positive B cells.[30] Preliminary data from our patients using the same method show an association between global cytosine hypomethylation and *ZAP-70* methylation and lack of expression. This implies that if *ZAP-70* methylation is malignancy-related, then hypomethylation of *ZAP-70* in a subset of CLL arises as a consequence of gene-specific rather than global hypomethylation. Gene-specific hypomethylation has previously been documented for a number of genes in CLL including ornithine decarboxlyase, Erb A1, BCL2, hTERT and TCL1, with evidence of an association

between hypomethylation and gene expression in the last two genes.[28,29,33-38]

Our initial studies showed no variation in methylation between ZAP-70 positive and negative CLL patients at sites located within the predicted promoter region of ZAP-70. An association between gene expression and methylation changes within the first intron and second exon of a gene is unusual. However, a recent study has shown that expression of the methylation controlled DNAJ (*MCJ*) gene depends on the methylation status of a CpG island within exon 1 and is independent of methylation in the promoter region.[39] *MCJ* is methylated and not expressed in both normal epithelial cells and ovarian cancer cell lines but is unmethylated and expressed in normal lymphocytes and fibroblasts. In an ovarian cancer cell line, in which *MCJ* was methylated and unexpressed, reduced histone acetylation was found not only within the CpG island in exon 1 but also within the gene promoter suggesting a link between changes in chromatin configuration within 5 prime exons and the promoter region. It is also interesting to note that the 3 prime end of intron 1 of *ZAP-70*, which we have shown to be differentially methylated, shows a similar level of cross species conservation to parts of the predicted promoter and is more highly conserved than any of the 5 prime non-coding exons of the *ZAP-70* gene (Figure 1). Transcription factor binding site analysis predicts several Sp1 binding sites within this highly conserved region (*data not shown*) suggesting a possible role as part of the true minimal promoter or as a separate enhancer region for the expression of ZAP-70. Sp1 has been shown to have both a marked enhancer effect on the transcription of genes and sensitivity to methylation of its binding sites.[40-42]

Data on ZAP-70 expression in B-cell tumors other than CLL are limited.[43,44] In contrast to CLL and B-ALL, we found ZAP-70 to be unexpressed and methylated in the majority of cases of mantle cell lymphoma and splenic marginal zone lymphoma. The association between ZAP-70 expression and *IgVH* gene mutational status in CLL, and the observation in transgenic mice that somatic hypermutation is linked to methylation, raised the possibility that other mature B-cell tumors with unmutated *IgVH* genes might express ZAP-70. However none of our cases of MCL or SMZL with unmutated *IgVH* genes had unmethylated *ZAP-70* genes, nor expressed ZAP-70 protein.

Further work is clearly required to elucidate the mechanisms controlling ZAP-70 expression in normal lymphoid cells and in CLL. The absence, in a minority of cases, of an association between ZAP-70 expression and methylation status at C-334 clearly indicates that other factors affect ZAP-70 expression. This may also have practical significance since ZAP-70 expression may have a functional role in CLL and contribute to the poorer prognosis of cases expressing ZAP-70.[45,46] Histone deacetylase (HDAC) inhibitors induce histone H3 and H4 acetylation in CLL cells *in vitro*, and result in caspase-8-dependent apoptosis utilizing a tumor necrosis factor receptor pathway. HDAC inhibitors are entering clinical practice for the management of CLL and it will be important to ensure that these agents, used singly or in combination with demethylating agents, do not result in ZAP-70 expression in the benign subset of CLL with mutated *IgVH* genes and methylated *ZAP-70* genes.[47,48]

MC, AP: contributed equally to this study and should be credited jointly with first authorship. MC was responsible for the design of the methylation analysis assays used. He carried out a large part of the COBRA and all Southern analyses, as well as contributing heavily to the critical revision and final approval of the manuscript; AP carried out a substantial amount of the COBRA and all of the bisulphite sequencing. He was also responsible for the interpretation of all of the collated data and the overall writing of the manuscript and the generation of the figures and tables. He oversaw all revisions and gave final approval of the manuscript; all ZAP-70 flow cytometry analyses and data interpretation and the related written methods were contributed by JO, who also made a significant contribution to the writing of the results section and the critical revision and final approval of the manuscript; all Vh-gene analysis and data interpretation and the related written methods where contributed by ZD, who also gave final approval of the manuscript. MW, OS: performed all global cytosine methylation analyses and data interpretation and the related written methods; both authors were also involved in the critical revision and final approval of the manuscript. DO: responsible for the original conception of the study. As well as drafting large sections of the 'background and objectives' and 'interpretation and conclusion' sections, he contributed heavily to the critical review of the manuscript and gave final approval for its submission.

We are indebted to Professor Peter Thomas for statistical advice and to Mary Tiller for her technical assistance.

Manuscript received April 20, 2005. Accepted June 21, 2005.

References

1. Oscier DG, Gardiner AC, Mould SJ, Glide S, Davis ZA, Ibbotson RE, 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.
2. Krober A, Seiler T, Benner A, Bullinger L, Bruckle E, Lichter P, et al. V (H) mutation status, CD38 expression level, genomic aberrations and survival in chronic lymphocytic leukemia. Blood 2002;100:1410-6.
3. Tobin G, Soderberg O, Thunberg U, Rosenquist R. V(H)3-21 gene usage in chronic lymphocytic leukemia-characterization of a new subgroup with distinct molecular features and poor survival. Leuk Lymphoma 2004;45:221-8.
4. Hamblin T, Davis Z, Gardiner A, Oscier D, Stevenson F. Unmutated IgVH genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood 1999;94:1848-54.
5. Damle RN, Wasil T, Fais F, Ghiotto F, Valetto A, Allen SL, et al. IgV gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. Blood 1999; 94:1840-7.
6. Rosenwald A, Alizadeh AA, Widhopf G, Simon R, Davis RE, Yu X, et al. Relation of gene expression phenotype to immunoglobulin mutation genotype in B cell chronic lymphocytic leukemia. J Exp Med 2001;194:1639-47.
7. Wiestner A, Rosenwald A, Barry TS, Wright G, Davis RE, Henrickson SE, et al. ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglublin genes, inferior clinical outcome and distinct

gene expression profile. Blood 2003; 101:4944-51.
8. Durig J, Nuckel H, Cremer M, Fuhrer A, Halfmeyer K, Fandrey J, et al. ZAP-70 expression is a prognostic factor in chronic lymphocytic leukemia. Leukemia 2003;17:2426-34.
9. Crespo M, Bosch F, Villamor N, Bellosillo B, Colomer D, Rozman M, et al. ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. N Engl J Med 2003;18:1764-75.
10. Orchard JA, Ibbotson RE, Davis Z, Wiestner A, Rosenwald A, Thomas PW, et al. ZAP-70 expression and prognosis in chronic lymphocytic leukaemia. Lancet 2004;363:105-11.
11. Rassenti LZ, Huynh L, Toy TL, Chen L, Keating MJ, Gribben JG, et al. ZAP-70 compared with immunoglobulin heavy-chain mutation status as predictors of disease progression in chronic lymphocytic leukemia. N Engl J Med 2004; 351:893-901.
12. Chan AC, Irving BA, Fraser JD, Weiss A. The z chain is associated with a tyrosine kinase and upon T-cell antigen receptor stimulation associates with ZAP-70, a 70-kDa tyrosine phosphoprotein. Proc Natl Acad Sci USA 1991; 88:9166-70.
13. Vivier E, da Silva AJ, Ackerly M, Levine H, Rudd CE, Anderson P. Association of 70-kDa tyrosine phosphoprotein with the CD16: zg complex expressed in human natural killer cells. Eur J Immunol 1993;23:1872-6.
14. Ticchioni M, Charvet C, Noraz N, Lamy L, Steinberg M, Bernard A, et al. ZAP-70 is required for CXCL12 mediated T-cell transendothelial migration. Blood 2002;99:3111-8.
15. Elder ME, Hope TJ, Parslow TG, Umetsu DT, Wara DW, Cowan MJ. Severe combined immunodeficiency with absence of peripheral blood CD8+ T cells due to ZAP-70 deficiency. Cell Immunol 1995;165:110-7.
16. Sakaguchi N, Takahashi T, Hata H, Nomura T, Tagami T, Yamazaki S, et al. Altered thymic T-cell selection due to a mutation of the ZAP-70 gene causes autoimmune arthritis in mice. Nature 2003;426:454-60.
17. Mostoslavsky R, Alt FW, Bassing CH. Chromatin dynamics and locus accessibility in the immune system. Nature Immunology 2003;4:603-6.
18. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 2003; 349:2042-54.
19. Xiong Z, Laird PW. COBRA: a sensitive and quantitative DNA methylation assay. Nucleic Acids Res 1997;25: 25324.
20. Clark S, Harrison J, Paul C, Frommer M. High sensitivity mapping of methylated cytosines. Nucleic Acids Res 2004; 22:2990-7.
21. Li LC, Dahiya R. MethPrimer: designing primers for methylation PCRs. Bioinformatics 2002;11:1427-31.
22. Wirtz M, Stach D, Kliem HC, Wiessler M, Schmitz OJ. Determination of the DNA methylation level in tumor cells by capillary electrophoresis and laser-induced fluorescence detection. Electrophoresis 2004;25:839-45.
23. Schweighoffer E, Vanes L, Mathiot A, Nakamura T, Tybulewicz VLJ. Unexpected requirement for ZAP-70 in pre-B cell development and allelic exclusion. Immunity 2003;18:523-33.
24. Meade J, Tybulewicz VLJ, Tuner M. The tyrosine kinase Syk is required for light chain isotype exclusion but dispensable for the negative selection of B cells. Eur J Immunol 2004;34:1102-10.
25. Crespo M, Villamor N, Bosch F, Giné E, Campo E, Montserrat E. ZAP-70 is expressed in normal B cells of immature phenotype and in some B acute lymphoblastic leukemias. Hematol J 2004;Suppl2:S19.
26. Chiorazzi N, Ferrarini M. B cell chronic lymphocytic leukemia: lessons learned from studies of the B cell antigen receptor. Annu Rev Immunol 2003;21:841-94.
27. Nolz JC, Tschumper RC, Pittner BT, Darce JR, Kay NE, Jelinek DF. ZAP-70 is expressed by a subset of normal human B-lymphocytes displaying an activated phenotype. Leukemia 2005; 31:[Epub ahead of print]
28. Rush LJ, Raval A, Funchain P, Johnson AJ, Smith L, Lucas DM, et al. Epigenetic profiling in chronic lymphocytic leukemia reveals novel methylation targets. Cancer Res 2004;64:2424-33.
29. Li Y, Nagai H, Ohno T, Yuge M, Hatano S, Ito E, et al. Aberrant DNA methylation of p57KIP2 gene in the promoter region in lymphoid malignancies of B-cell phenotype. Blood 2002;100:2572-7.
30. Stach D, Schmitz OJ, Stilgenbauer S, Benner A, Dohner H, Wiessler M, et al. Capillary electrophoretic analysis of genomic DNA methylation levels. Nucleic Acids Res 2003;31:E2.
31. de Capoa A, Grappelli C, Febbo FR, Spano A, Niveleau A, Cafolla A, et al. Methylation levels of normal and chronic lymphocytic leukemia B lymphocytes: computer-assisted quantitative analysis of anti-5 methylcytosine antibody binding to individual nuclei. Cytometry 1999;36:157-9.
32. Wahlfors J, Hiltunen H, Heinonen K, Hamalainen E, Alhonen L, Janne J. Genomic hypomethylation in human chronic lymphocytic leukemia. Blood 1992;80:2074-80.
33. Melki JR, Vincent PC, Brown RD, Clark SJ. Hypermethylation of E-cadherin in leukemia. Blood 2000;95: 3208-13.
34. Crossen PE, Morrison MJ. Hypermethylation of the M27, (DXS255) locus in chronic B-cell leukemia. Br J Haematol 1998;100:191-3.
35. Hanada M, Delia D, Aiello A, Stadtmauer E, Reed J C. Bcl-2 gene hypomethylation and high level expression in B-cell chronic lymphocytic leukaemia. Blood 1993;82:1820-8.
36. Lipsanen V, Leinonen P, Alhonen L, Janne J. Hypomethylation of ornithine decarboxylase gene and erb-Al oncogene in human chronic lymphatic leukemia. Blood 1988;72:2042-4.
37. Bechter OE, Eisterer W, Dlaska M, Kuhr T, Thaler J. CpG island methylation of the hTERT promoter is associated with lower telomerase activity in B-cell lymphocytic leukemia, Exp Hematol 2002; 30:26-33.
38. Yuille MR, Condie A, Stone EM, Wilsher J, Bradshaw PS, Brooks L, et al. TCL1 is activated by chromosomal rearrangement or by hypomethylation. Genes Chromosomes Cancer 2001; 30:336-41.
39. Strathdee G, Davies BR, Vass JK, Siddiqui N, Brown R. Cell type-specific methylation of an intronic CpG island controls expression of the MCJ gene. Carcinogenesis 2004;25:693-701.
40. Pang RT, Lee LT, Ng SS, Yung WH, Chow BK. CpG methylation and transcription factors Sp1 and Sp3 regulate the expression of the human secretin receptor gene. Mol Endocrinol 2004; 18:471-83.
41. Georgopoulos S, Kan HY, Reardon-Alulis C, Zannis V. The SP1 sites of the human apoCIII enhancer are essential for the expression of the apoCIII gene and contribute to the hepatic and intestinal expression of the apoA-I gene in transgenic mice. Nucleic Acids Res 2000;28:4919-29.
42. Clark SJ, Harrison J, Molloy PL. Sp1 binding is inhibited by (m)Cp(m)CpG methylation. Gene. 1997;195:67-71.
43. Sup SJ, Domiati-Saad R, Kelley TW, Steinle R, Zhao X, Hsi ED. ZAP-70 expression in B-cell hematologic malignancy is not limited to CLL/SLL. Am J Clin Pathol 2004;122:582-7.
44. Carreras J, Villamor N, Colomo L, Moreno C, Ramon y Cajal S, Crespo M, et al. Immunohistochemical analysis of ZAP-70 expression in B-cell lymphoid neoplasms. J Pathol 2005;205: 507-13.
45. Chen L, Widhopf G, Huynh L, Rassenti L, Rai KR, Weiss A, et al. Expression of ZAP-70 is associated with increased B-cell receptor signaling in chronic lymphocytic leukemia. Blood 2002;100:4609-14.
46. Chen L, Apgar J, Huynh L, Dicker F, Giago-McGahan T, Rassenti L, et al. ZAP-70 directly enhances IgM signaling in chronic lymphocytic leukemia Blood 2005;105:2036-41.
47. Byrd JC, Shinn C, Ravi R, Willis CR, Waselenko JK, Flinn IW, et al. Depsipeptide (FR901228): a novel therapeutic agent with selective, in vitro activity against human B-cell chronic lymphocytic leukemia cells. Blood 1999; 94:1401-8.
48. Aron JL, Parthun MR, Marcucci G, Kitada S, Mone AP, Davis ME, et al. Depsipeptide (FR901228) induces histone acetylatiuon and inhibition of histone deacetylase in chronic lymphocytic leukemia cells concurrent with activation of caspase 8 – mediated apoptosis and down-regulation of c-FLIP protein. Blood 2003;102:652-8.