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Sylvain P. Chantepie[a,b,d,*], Dominique Vaur[a,b], Christoph Grunau[c], Véronique Salaün[e], Mélanie Briand[a], Jean-Jacques Parienti[f], Natacha Heutte[a], Stéphane Cheze[d], Mikel Roussel[g], Pascal Gauduchon[a], Michel Leporrier[d], Sophie Krieger[a,b]

[a] *GRECAN, Centre François Baclesse, Université de Caen Basse-Normandie, Caen Cedex, France*
[b] *Laboratoire de Biologie Clinique et Oncologique, Centre François Baclesse, Caen Cedex, France*
[c] *UMR 5244 CNRS, Perpignan, France*
[d] *Hématologie Clinique, CHU Caen, Caen Cedex, France*
[e] *Laboratoire d'Hématologie, CHU Caen, Caen Cedex, France*
[f] *Service Recherche clinique et biostatistique; CHU Caen, Caen Cedex, France*
[g] *Laboratoire d'Hématologie, CHU Pontchaillou Rennes, France*

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ABSTRACT

ZAP-70 expression is a strong prognostic indicator in chronic lymphocytic leukemia. However, *ZAP-70* quantification by flow cytometry lacks sufficient standardization. Based upon the correlation between ZAP-70 expression and its gene methylation status, we have developed a quantitative pyrosequencing assay for the determination of *ZAP-70* methylation adapted for routine use. Methylation in four CpG pairs (C-223, C-243, C-254, and C-267) in the first intron of *ZAP-70* is associated with repression of *ZAP-70*. Moreover, it correlates with CD38 expression ($n = 111$, $p < .0001$), *IgHv* mutation status ($n = 106$, $p < .0001$), time to treatment ($p < .0001$), and overall survival ($p = .0014$). Pyrosequencing of *ZAP-70* provides a good alternative to flow cytometry.

1. Introduction

B-cell chronic lymphocytic leukemia (B-CLL) is characterized by a monoclonal expansion of mature CD5+ B cells. CLL is a heterogeneous disease with a highly variable clinical course [1]. Three prognosis groups have been defined with clinical classifications [2,3], but these classifications cannot be used to predict the outcome of individual patients at the time of diagnosis, particularly in the early stages of the disease. In recent years, powerful biological prognostic factors have been identified, such as cytogenetic abnormalities [4] and mutations in the immunoglobulin heavy chain variable genes (*IgHv*) [5]. Mutational status in *IgHv* genes, a constitutive feature of each B-CLL clone, is considered to be a major prognostic factor in CLL. DNA micro-array studies have shown that the mutated and unmutated subtypes of CLL can be distinguished by the expression of a small number of genes, in particular *ZAP-70* [6,7], a member of the Syk tyrosine kinase family. *ZAP-70* plays an essential role in signal transduction from normal T-cell receptors (TCR) [8–10]. In healthy individuals, the *ZAP-70* gene is normally expressed in T and natural killer (NK) cells. However, *ZAP-70* is also expressed in pro-/pre-B cells, activated B cells and tumor cells, such as B-cell acute lymphoblastic leukemia (B-ALL) and in CLL cells [6,11–13]. Normal B cells generally lack *ZAP-70* and use Syk for signaling via the BCR. In *ZAP-70+* CLL cells both *ZAP-70* and Syk are phosphorylated after BCR activation. Expression of *ZAP-70* could also explain the more aggressive disease seen in *ZAP-70+* patients. Recent results have shown that *ZAP-70* positive and *ZAP-70* negative CLL cells display a distinct gene expression profile. The implied genes are linked to microenvironment crosstalk [14]. Moreover, CD38 ligation leads to ZAP-70 phosphorylation and increases the ability of CD38+/ZAP-70+ cells to migrate [15]. Current knowledge of the physiopathology of CLL and the major role played by ZAP-70 support the usefulness of analyzing ZAP-70 in CLL. Several studies have demonstrated that there is a close association between *ZAP-70* expression (mRNA and protein) and *IgHv* mutational status, even if this relationship is not absolute, with discordance ranging from

* Corresponding author at: Service d'Hématologie, Université de Caen Basse-Normandie, CHU Caen, Avenue Clémenceau, 14 033 Caen Cedex, France.
Tel.: +33 2 31 27 25 39; fax: +33 2 31 27 25 43.
E-mail address: chantepie-s@chu-caen.fr (S.P. Chantepie).

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8% to 22% of patients [16–20]. In addition, *ZAP-70* expression is recognized as a reliable negative prognostic factor for patients with CLL [16,17,19,21] and a recent study has shown that *ZAP-70* is a stronger risk factor than *IgHv* or CD38 [22].

ZAP-70 expression can be determined by flow cytometry [19], Western-blot [23] and quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR) [21,24,25]. However, the flow cytometry procedure lacks sufficient standardization. RQ-PCR and immunoblot require separation of B-CLL cells from contaminating ZAP-70 positive T and natural killer cells, making these procedures unsuitable for routine laboratory use. In this context, a simplified surrogate method to determine ZAP-70 expression would be beneficial for CLL prognosis assessment.

Epigenetic regulation influences gene expression in normal cells and cancer cells [26]. The latter are highly characterized by hypermethylation of promoters and by methylation of CpG islands, which repress the transcription of genes in cancer and/or hypomethylation of the genome associated with genome instability or up-regulation of gene expression [27,28]. In this setting, methylation of the *ZAP-70* gene appears to be highly predictive of ZAP-70 expression. Corcoran et al. have shown, in 87 CLL cases, that the methylation status of the intron1–exon2 region of *ZAP-70* is associated with protein expression, *IgHv* mutational status, and overall survival [29]. However, methylation was determined by Combined Bisulfite Restriction Analysis (COBRA), which quantifies methylation in only one CpG site. This method is time consuming and not suitable for the medical diagnostic routine. Pyrosequencing is a synthesis method of sequencing that quantifies the incorporation of nucleotides using the enzymatic conversion of released pyrophosphate into a proportional light signal. Pyrosequencing has proven its usefulness in the quantification of DNA methylation.

The aim of this study was to design a simple, sensitive and quantitative method to determine the methylation status of *ZAP-70* in B-CLL and to evaluate its prognostic value. We therefore developed a straightforward pyrosequencing-based approach to determine the methylation status of *ZAP-70* intron1. The comparison of *ZAP-70* methylation status with ZAP-70 expression by flow cytometry, CD38 expression, the *IgHv* mutation status, and patient outcome shows that quantification of methylation in four CpG sites in *ZAP-70* intron1 is highly predictive for ZAP-70 expression and for disease progression.

2. Materials and methods

2.1. Sample collection

Samples were collected from 123 patients (49 females, 74 males): 117 were diagnosed with B-CLL based on standard NCI criteria [30], 2 with mantle-cell lymphoma (MCL), and 4 with large B-cell lymphoma (DLBCL). Blood samples were obtained from 117 CLL patients. Paraffin-embedded tissue from lymph node biopsies from 8 patients was obtained: 2 patients were diagnosed with CLL, 2 with MCL, and 4 with DLBCL. All patients were untreated at the time of sampling. Binet stages at diagnosis for the 117 CLL were as follows: 114 patients in stage A, 2 patients in stage B, and 1 patient in stage C. The median age and lymphocyte count at diagnosis were 62 years (26–85) and 16×10^9/L, respectively. Median follow-up time was 47 months. Samples were obtained according to French regulations.

Peripheral-blood mononuclear cells were isolated by density-gradient centrifugation using Ficoll-Paque™ Plus (Amersham Biosciences). In normal peripheral blood from healthy controls, fresh B and T lymphocytes were negatively selected using the human B and T cell enrichment kit (Stem Cell technologies, UK). For 17 samples, CD19 positive B-CLL cells were sorted from fresh mononuclear cells using MACS separation columns with CD19 MicroBeads (Miltenyi Biotec, Paris, France). Purity levels of ≥95% were confirmed for all selected samples by flow cytometry with CD5/CD20 positivity for B cells and CD3/CD2 positivity for T cells.

2.2. Determination of ZAP-70 expression

ZAP-70 expression was determined by Western-blot on ten sorted CLL samples (1:1000, 2F3.2, Upstate) or by flow cytometry on thawed cells as previously described [31]. Briefly, 2×10^5 cells obtained from a pool of normal lymphocytes were added to 8×10^5 cells of each B-CLL sample. These cells were then surface stained with FITC-CD3 (UCHT1), PC5-CD19 (J4.119) and PC7-CD5 (BL1a) (Immunotech). After fixation and permeabilization (eBioscience) the cells were incubated with PE-SBZAP (Beckman Coulter) and run on an FC500 flow cytometer (Beckman Coulter). ZAP-70 expression was evaluated as the ratio of mean phycoerythrin fluorescence intensity between CD19+ CD5+ (B-CLL) and CD19+ CD5− (normal control) B cells. A case was considered as ZAP-70 positive for ratios above 1.4, negative for a ratio below 1.3, and intermediate for a ratio between 1.3 and 1.4.

2.3. Determination of CD38 expression

Peripheral-blood mononuclear cells were isolated by density-gradient centrifugation using Ficoll-Paque™ Plus (Amersham Biosciences). The cells were incubated with the following antibody conjugates: anti-CD20 fluoroisothiocyanate (FITC; Becton Dickinson), anti-CD38-phycoerythrin (PE), anti-CD19-PerCPCy5.5, anti-CD5-allophycocyanin (APC). At least 10,000 cells were acquired in the Cellquest program on a FACSCalibur flow cytometer (Becton Dickinson). Samples with >30% of CD19+/CD38+ cells were considered positive for CD38.

2.4. Analysis of IgHv gene mutation status

Determination of the mutational status of *IgHv* was performed and interpreted following the ERIC (European Research Initiative on CLL) recommendations [32].

2.5. Bisulfite sequencing of the ZAP-70 intron1–exon2 region

DNA was isolated using the EZ1™ DNA tissue kit (QIAGEN, Courtaboeuf, France) with the EZ1 BioRobot (QIAGEN), then stored at 4 °C. For paraffin-embedded tissue, a first deparaffination and protein lysis step with G2 solution and proteinase K was carried out (QIAGEN). Five hundred nanograms to 1 μg of DNA was treated with sodium bisulfite using the Epitect™ Bisulfite Kit (QIAGEN) according to the manufacturer's instructions, then stored after washing at −20 °C. The *ZAP-70* intron1–exon2 region was amplified from bisulfite-treated DNA as previously described, using ZAPDN1-F and ZAPDN1-R primers [29] (Fig. 1A). The PCR product was purified with the QIAquick PCR purification kit (QIAGEN, Courtaboeuf, France), ligated into pDrive Cloning Vector. One or 2 μL of ligation product was added to QIAGEN EZ Competent cells and incubated for 5 min on ice. The tubes were heated in a 42 °C water bath. The transformation mixture was directly plated onto LB agar plates containing ampicillin (100 μg/mL). After overnight incubation at 37 C, individual colonies were selected (blue/white screening). The vector DNA was extracted from culture, then insert-sequenced using T7-R primers (T7-R 5′ GTAATACGACTCACTATAG 3′) and ABI PRISM BigDye Terminator Cycle Sequencing Reaction kit v3.1 (Applied Biosystems). The sequencing products were separated on a Hitachi 3130xl Genetic Analyzer (Applied Biosystems) and analyzed with Sequencing Analysis Software v5.2 (Applied Biosystems). The percent methylation was calculated as the average CpG methylation per clone and of all the clones sequenced for each sample (data is available in the DNA methylation database MethDB http://www.methdb.net) [33].

2.6. Pyrosequencing of the ZAP-70 intron1

Bisulfite pyrosequencing of the intron1 region of *ZAP-70* was performed using forward (5′ TTTTATTTATGAGTGAGAAATTTTGG 3′) and reverse (5′ TACAACCCAAACCCCCAACCT 3′ biotinylated) primers, and one sequencing primer (TTTTATTTATGAGTGAGAAA). Bisulfite-treated DNA was added to buffer 10× (1.5 mM $MgCl_2$), dNTP 4 μL, 25 pmol forward primer, 25 pmol of biotinylated reverse primer, Q-solution 5×, $MgCl_2$ 1.5 mM, and 1.25 units of HotStarTaq DNA polymerase (QIAGEN). PCR testing was carried out at 95 °C for 5 min, followed by 45 cycles of 95 °C for 30 s, 55 °C for 2 min, and 72 °C for 90 s, with a final extension of 72 °C for 10 min. Biotinylated single-strand DNA fragments were generated by mixing PCR product with streptavidin-coated paramagnetic beads (GE Healthcare UK) and processing them according to the manufacturer's instructions. A vacuum preparation tool (Biotage) was used to remove salts that inhibit subsequent enzymatic reactions. It made possible the capture and holding of the beads during the different purification steps while allowing the solution to pass easily through the filters. An automated pyrosequencing instrument, PSQ™ 96 MA (Biotage), was used to determine the DNA sequence. Criteria for Pyrogram selection were as follows: sufficient peak heights of >10 units (arbitrary units for light emission, calculated by the software), symmetric peaks without any irregularities or side-peaks, wide reading length with a high reliability throughout the sequence, and absence of significant signals at the positions where a bisulfite treatment control was located or where control nucleotides were dispensed to check for non specific background signals [34].

To examine the linearity and the sensitivity of our new pyrosequencing assay, fully methylated DNA (CpGenome™ Universal Methylated DNA) and fully non-methylated DNA (from selected T lymphocytes) were mixed to obtain different levels of methylation between 0% and 100%. The calibration DNA mixture was bisulfite-treated and PCR-amplified as described above and the methylation of the 4 CpG sites were determined on a PSQ™ 96 MA (Biotage).

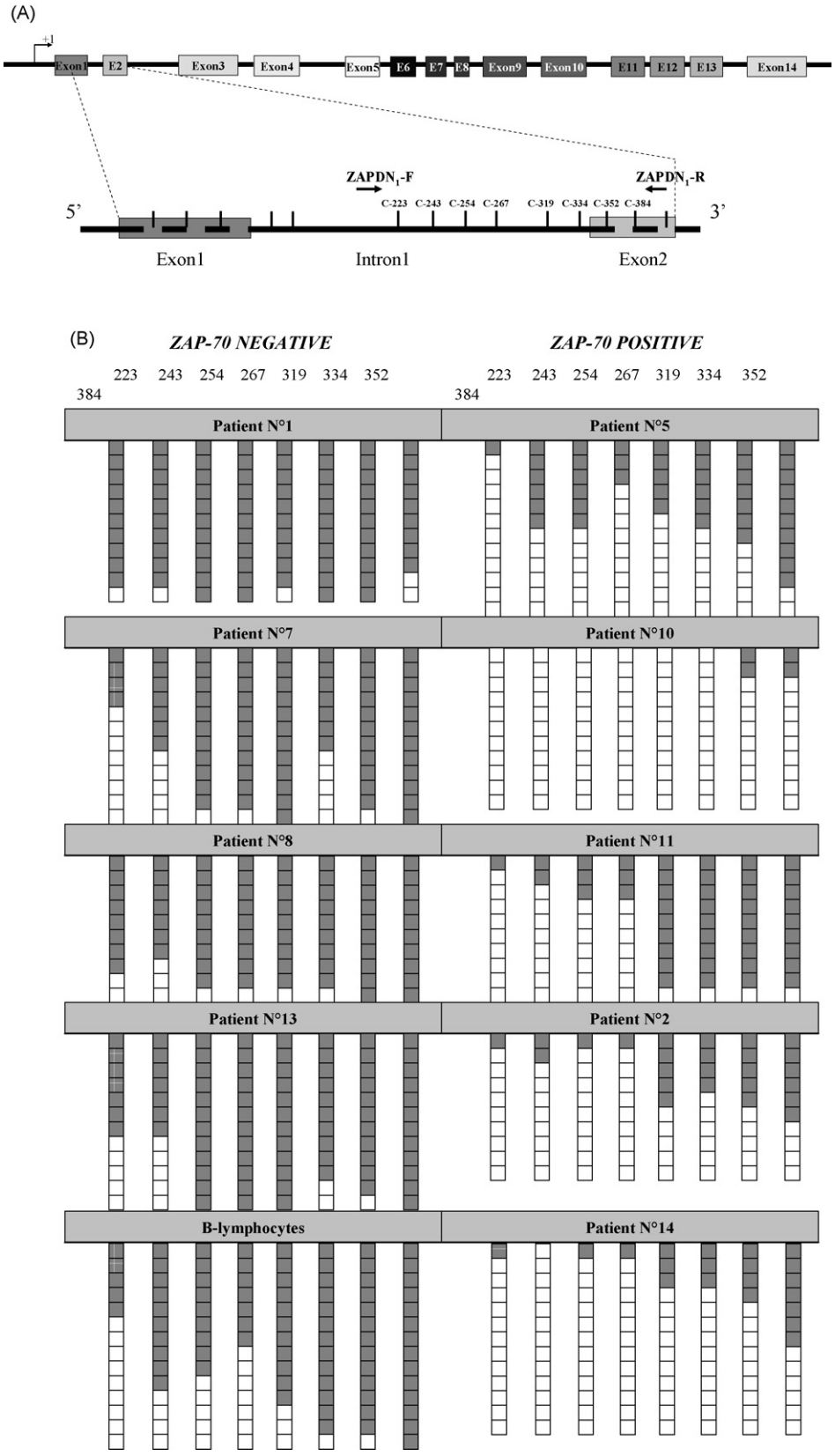

Fig. 1. ZAP-70 intron1–exon2 region methylation is correlated to ZAP-70 expression. (A) Schematic of *ZAP-70* gene: the transcription start site (+1), exons (boxes +/− with dotted lines), introns (horizontal bars), the PCR primers ZAPDN1-F and ZAPDN1-R, CpG dinucleotides (vertical bars) are represented. (B) DNA of ZAP-70 positive or negative CLL patients and B-lymphocytes were bisulfite treated and amplified by ZAPDN1 forward and reverse primers surrounding the CpG: 223-243-254-267-319-334-352-384. PCR products were then cloned and sequenced. Grey boxes represent one methylated CpG and white boxes unmethylated CpG. Each line represents a sequenced clone. The percentage of methylated cytosines loci range from 73% to 94% for the ZAP-70 negative patients and from 0% to 58% for the ZAP-70 positive patients ($p = 0.0043$). The differences of mean of methylation between ZAP-70+ and ZAP-70− patients were statistically significant for the 4 CpG in 5′ of intron1 (C+223 to C+267) ($p = 0.0043$) and for the 4 CpG in the 3′ part of intron1 (C+319 to C+384) ($p = 0.02$).

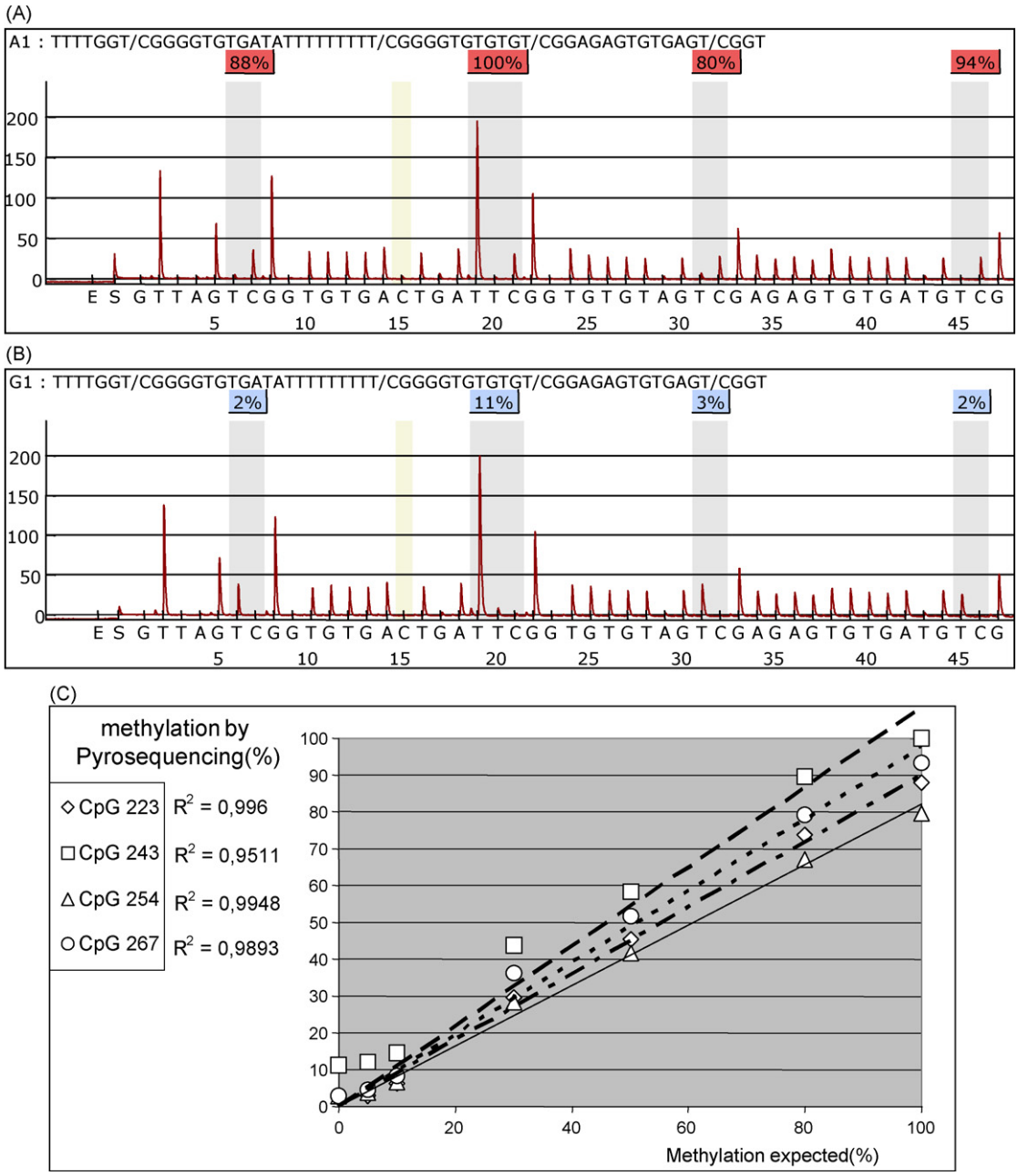

Fig. 2. Pyrosequencing methylation quantification at C-223, C-243, C-254, C-267 of ZAP-70 intron1 in methylated and unmethylated DNA mixture. The sequence in the upper part of each Pyrogram represents the sequence under investigation. The sequence below the Pyrogram indicates the sequentially added nucleotides. The grey regions highlight the analyzed C/T sites, with percentage values for the respective cytosine above them. The degree of methylation is calculated as the ratio of the peak height of the methylated peak to the sum of the peaks corresponding to the methylated and the unmethylated peaks. (A) Pyrogram of fully methylated DNA. *M.Sss*I treated DNA was bisulfite modified, amplified with intron1 forward and reverse biotin primers and then pyrosequenced with sequencing primer. (B) Pyrogram of T-lymphocyte DNA that serves as totally unmethylated control. (C) Standard curves for CpG methylation quantification of known mixture of DNA 100% methylated, 80%, 50%, 30%, 10%, 5% and 0% methylated, obtained by mixing DNA *M.Sss*I treated and DNA from T-lymphocytes known to be unmethylated. Pyrosequencing results of methylation for each CpG (C-223, C-243, C-254, C-267) show good correlation with the expected quantity of methylation ($r^2 \geq 0.95$).

2.7. Results and statistical analysis

The percentage of T + NK cells, constitutively hypomethylated in *ZAP-70*, was assessed by flow cytometry. The percentage of methylation was then calculated using the following formula: methylation of the sample = (100 × %Methylation result)/(100 − %T + NK cells in the sample). Statistical comparison of *ZAP-70* data from Western-blot and pyrosequencing was performed using a two-tailed Student's *t*-test. The χ^2 test was used to test the association between methylation status and other prognostic factors, such as ZAP-70 and *IgHv* mutational status. The percent methylation level cut-off was determined from the specificity [true negative/(true negative + false positive)], sensitivity [true positive/(true positive + false negative)] and accuracy [(true positive + true negative)/total]. Survival time and time to treatment between ZAP-70 groups was compared using Kaplan–Meier plots and the differences were tested using the log-rank test. A p value $\leq .05$ was considered statistically significant. Statistical analysis was performed with SAS (version 9.1) and STATA (version 9.0).

3. Results

3.1. Methylation status of the CpG locus within the intron1–exon2 region of the ZAP-70 gene in CLL

Bisulfite sequencing of at least 8 clones for each of 4 ZAP-70 negative, 6 ZAP-70 positive CLL patients, and B lymphocytes

from a healthy donor are shown in Fig. 1. Complete bisulfite conversion was confirmed by the absence of cytosine residues at non-CpG loci. Considering all CpG in all clones from each patient, the percentage of methylated cytosine loci ranged from 73% to 94% (mean; 84.6%) for the ZAP-70 negative patients whereas it ranged from 0% to 58% (mean; 4.6%) for the ZAP-70 positive patients. The difference in methylation between the 2 ZAP-70 positive and negative groups was statistically significant ($p = .0043$). When we considered the percentage of clones methylated at each locus, the difference between the ZAP-70 positive and the ZAP-70 negative groups was statistically significant for the 8 CpG loci (C+223, C+243, C+254, C+267): for each CpG; $p = .0043$, C+319; $p = .009$, C+334; $p = .02$, C+354; $p = .03$, C+384; $p = .05$). The mean methylation of the first 4 CpG (223–267) clearly distinguished between ZAP-70 positive and negative patients ($p = .0043$). However, the differences in mean methylation in the 4 last CpG (319 to 384) were found to be less distinct, but still significantly different between positive and negative ZAP-70 patients ($p = .02$). *ZAP-70* positive patients 11 and 2 had an unexpectedly high degree of methylation in sites C+319 to C+384. Consequently, the first four CpG sites (C+223, C+243, C+254, C+267) were selected for further analysis.

3.2. Pyrosequencing of intron1 of ZAP-70: assay development and validation

Based on the intron1 bisulfite sequencing results (Fig. 1B) and the size limitation of pyrosequencing, a fragment spanning the first 4 CpG dinucleotides (C-223 to C-267) was studied using a pyrosequencing assay. Using the PSQ assay design software™ (Biotage), primers were designed to amplify this intron1 region of *ZAP-70*. One sequencing primer with high-quality Pyrograms™ was selected (Fig. 2A and B). To validate these quantitative pyrosequencing assays, calibration mixtures of DNA with known %methylation values were analyzed using pyrosequencing. As expected, results showed that T-lymphocyte DNA was hypomethylated with a mean of 4% (range; 2–12%, mean (SD) 0.41%) whereas M.SssI treated DNA was nearly fully methylated in the 4 CpG sites with a mean of 89% (range; 73–100%, mean (SD); 1.54%, data not shown). Standard curves generated from analysis of the DNA mixtures were linear ($r^2 > .95$, Fig. 2C). Sensitivity of pyrosequencing was 5% (data not shown). An excellent correlation was observed between methylation quantification results with bisulfite sequencing and pyrosequencing for the 4 CpG sites ($r^2 > .95$). Pyrosequencing assay on 8 DNA samples extracted from paraffin-embedded tissue (2 CLL, 2 MCL, 4 DLBCL) resulted in pyrograms of the same quality. Examples of the high reproducibility of the pyrosequencing assay are shown in Table 1. The intra-series reproducibility standard deviation was between 0.44% and 3.41% and the inter-series reproducibility standard deviation was between 0.1% and 7.1% for the 4 CpG sites.

3.3. Comparison between intron1-methylation status and ZAP-70 protein expression

Determination of ZAP-70 expression by Western-blot was performed in 24 cases (data not shown). Among the ZAP-70 positive cells ($n = 13$), the methylation average of the four CpG sites was 21% (range; 5–47%). For the ZAP-70 negative samples ($n = 11$) average methylation was 71.9% (range; 58–81%). The difference in the average methylation between the two groups was statistically significant ($p < .0001$, Fig. 3A). The difference was also significant for each CpG (data not shown). Maximal prediction accuracy, specificity and sensitivity of the methylation assay were observed using a mean methylation threshold of 50–55% mean methylation (Fig. 3B). Samples with more than 50% intron1-methylation of *ZAP-70* were considered as ZAP-70 negative, and samples with less than 50% methylation as ZAP-70 positive (sensitivity = 100%, specificity = 100%, accuracy = 100%). Further statistical analyses were based on the 50% cut-off.

Table 1
Repeatability (A) and reproducibility (B) of pyrosequencing assay.

No. of patients	Mean methylation at the 4 CpG sites: % methylation (SD) $n = 4$			
	CpG+223	CpG+243	CpG+254	CpG+267
A				
43	72.45 (0.96)	91.94 (3.41)	66.51 (2.17)	80.53 (1.3)
60	8.46 (1.72)	27.57 (2.63)	10.92 (1.28)	14.54 (1.56)
75	76.2 (0.44)	67.93 (2.13)	58.14 (1.85)	84 (0.93)
B				
13	62.6 (2.7)	74.45 (7.1)	64.12 (3.7)	83.11 (2.6)
14	8.13 (4.7)	15.1 (4)	6.86 (2.9)	7.98 (4.4)
43	72.8 (1.2)	92.3 (3)	67 (2.1)	82.1 (3.2)
48	48.6 (1.2)	63.2 (1.7)	45.1 (1.3)	55.9 (2.1)
58	44 (1.9)	82.8 (4.1)	60.5 (0.5)	76.4 (0.7)
60	9.2 (1.8)	27.5 (2.3)	11 (1.1)	14.8 (1.4)
61	5 (1)	22.9 (2)	9.2 (0.8)	7.8 (1.5)
68	4 (0.1)	11 (1)	4.6 (0.7)	5.7 (1.3)
70	44 (1.9)	82.3 (4.1)	60.5 (0.5)	76.4 (0.7)
81	71.4 (2.2)	88.6 (5.1)	52.9 (0.4)	77.1 (1.4)

Determination of ZAP-70 expression by flow cytometry was performed on 43 CLL patient cells independently of the 24 Western-blot patient cells (data not shown). Eleven were ZAP-70 positive and 32 negative. The mean methylation of *ZAP-70* intron1 was 60.9% for the ZAP-70 negative group and 31.6% for the ZAP-70 positive group. The difference between the two groups was statistically significant at the $p = .002$ level. For 10 cases (23%), ZAP-70 determination by flow cytometry was discordant with methylation status. Eight out of the ten cases had low methylation levels (<50%), but ZAP-70 expression could not be detected by flow cytometry.

3.4. Correlation of ZAP-70 methylation status with peripheral-blood lymphocyte count

After having validated the quality of our bisulfite-pyrosequencing procedure and correlation with ZAP-70 expression, *ZAP-70* methylation levels were determined in CLL cells from 117 patients (55 patients with methylation < 50%; mean 20.6%, and 62 patients with methylation ≥50%; mean = 68.9%). Methylation levels were corrected for T- and NK-cell contamination (47 patients with methylation < 50%, mean = 19.7%, 70 patients with methylation ≥50%, mean = 78.3%). However, for only 8 patients T/NK cell counts were high enough to change them from "hypomethylated" to "methylated" status. Six out of the 8 patients had lymphocyte counts of under 10×10^9/L and the other 2 patients had lymphocyte counts of 12×10^9/L, and 29×10^9/L, respectively. Mean methylation levels were between 45% and 50% before T/NK correction (see Supplementary SI Data).

3.5. Comparison of intron1-methylation status and IgHv mutation

The percentage of methylation determined by pyrosequencing was compared with *IgHv* status available for 106 out of 117 CLL samples. Comparison with *IgHv* mutational status shows that the percentage of methylation for the mutated CLL and unmutated CLL samples was 20% and 73%, respectively ($p < .0001$). Sixty-two out of 70 (88%) patients with mutated *IgHv* genes had methylation at intron1 of *ZAP-70* and 35 of 36 (97%) patients with unmutated *IgHv* genes had a hypomethylated intron1 ($p < .0001$, Fig. 4). Discordance between *IgHv* and *ZAP-70* methylation status determined by pyrosequencing of intron1 was 8.5% (9/106): 8 cases were mutated

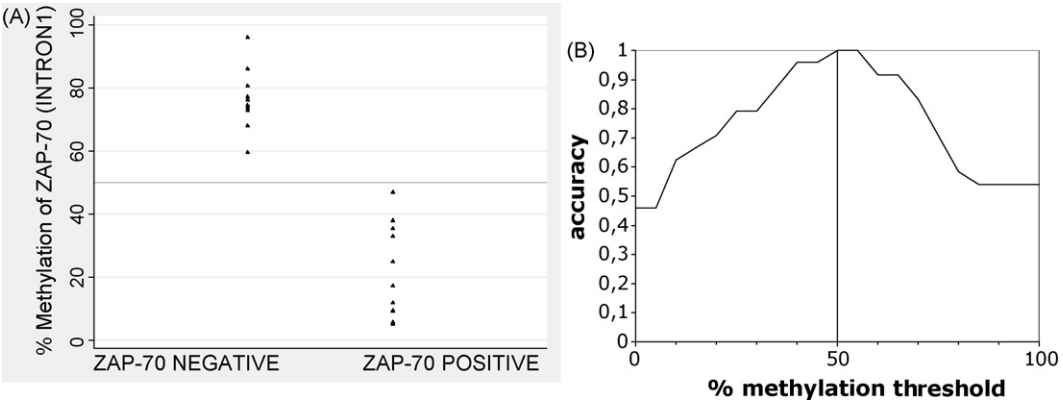

Fig. 3. The relationship between intron1-methylation status and ZAP-70 expression. Percentage of methylation determined by pyrosequencing was corrected with T/NK-cell contamination. (A) Results of the average methylation of the 4 CpG loci 223, 243, 254 and 267 bases downstream of the ZAP-70 transcription start site for ZAP-70 negative and positive patients ($n = 24$). (B) Accuracy of diagnostic value of DNA methylation for ZAP-70 expression as determined by Western-blot obtained by varying the decision threshold of mean %methylation of the four CpG 223, 243, 254 and 267.

and hypomethylated (ZAP-70 positive) and one case was unmutated and methylated for intron1 of *ZAP-70*.

3.6. Comparison of intron1-methylation status and CD38 expression

Comparison with CD38 expression ($n = 111$) showed that means of intron1-methylation of *ZAP-70* for CD38 positive and negative groups were 27% and 68%, respectively ($p < .0001$). Using the cut-off of 50% to distinguish between methylated and hypomethylated patients for *ZAP-70* intron1, 59 out of 76 CD38 negative patients (78%) were found to be methylated and 29 out of 35 CD38 positive patients (83%) were hypomethylated ($p < .0001$).

3.7. Intron1-methylation status of ZAP-70 and survival in CLL

Patients with intron1-methylation had significantly longer treatment-free survival (TFS) and overall survival (OS) than patients with intron1 hypomethylation ($p < .0001$ and $p = .0014$, respectively, Fig. 5A and B). The median survival of patients with methylated intron1 of *ZAP-70* is beyond the time-scale of this study, whereas it was only 92 months for patients who were hypomethylated in intron1 of *ZAP-70*.

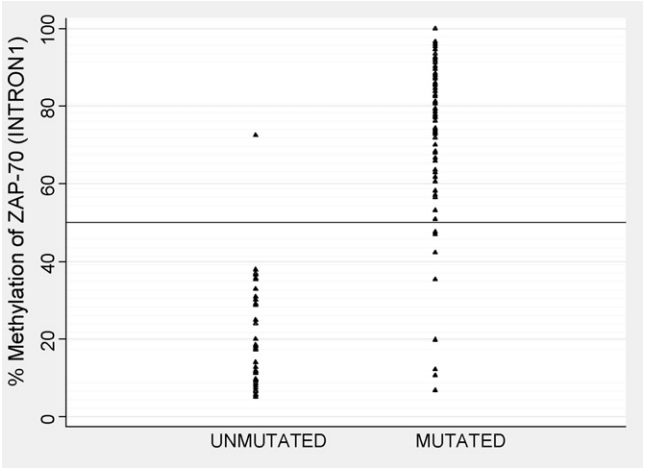

Fig. 4. The relationship between intron1-methylation status, *IgHv* mutation status. The percentage of methylation of *ZAP-70* intron1 in patients with mutated and unmutated *IgHv* genes ($n = 106$). *ZAP-70* intron1-methylation is associated with *IgHv* mutation status in 106 CLL ($p < 0.0001$ χ^2 test) with 8.5% of discordance.

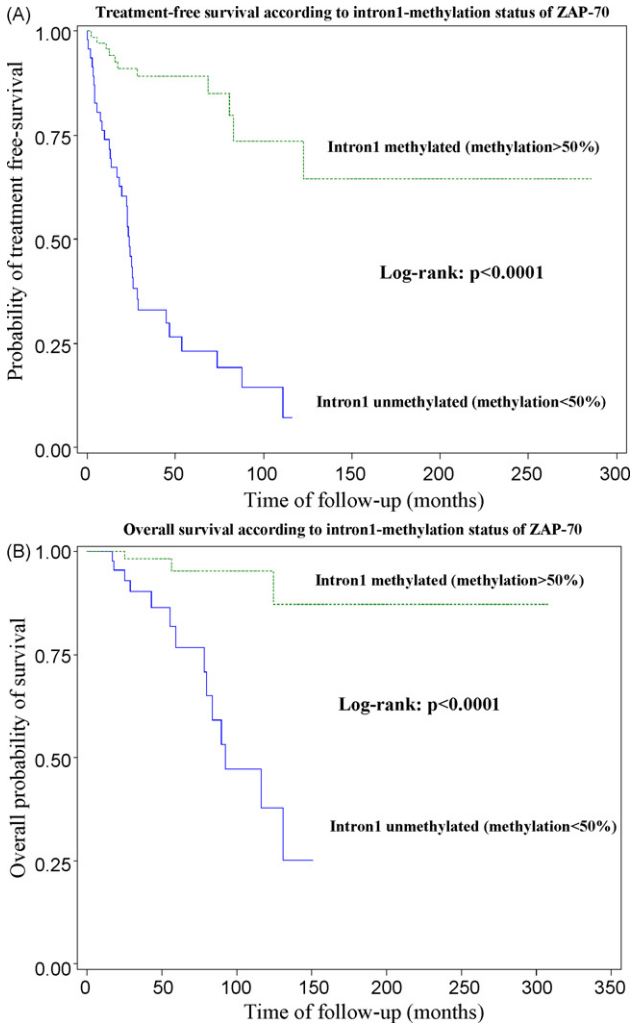

Fig. 5. Survival curves for 117 patients with B-CLL according to ZAP-70 intron1-methylation. Kaplan–Meier plot comparing treatment-free survival based on the presence of intron1-methylation of *ZAP-70* greater than 50% or lower than 50%. Patients with methylated DNA (>50%) in CLL cells experienced a longer TFS ($p < 0.0001$). (B) Kaplan–Meier plot comparing overall survival of CLL patients with methylated (>50%) and unmethylated (<50%) *ZAP-70* intron1 in CLL cells. Methylation level of *ZAP-70* intron1 greater than 50% characterized patients with a longer OS ($p = 0.0014$).

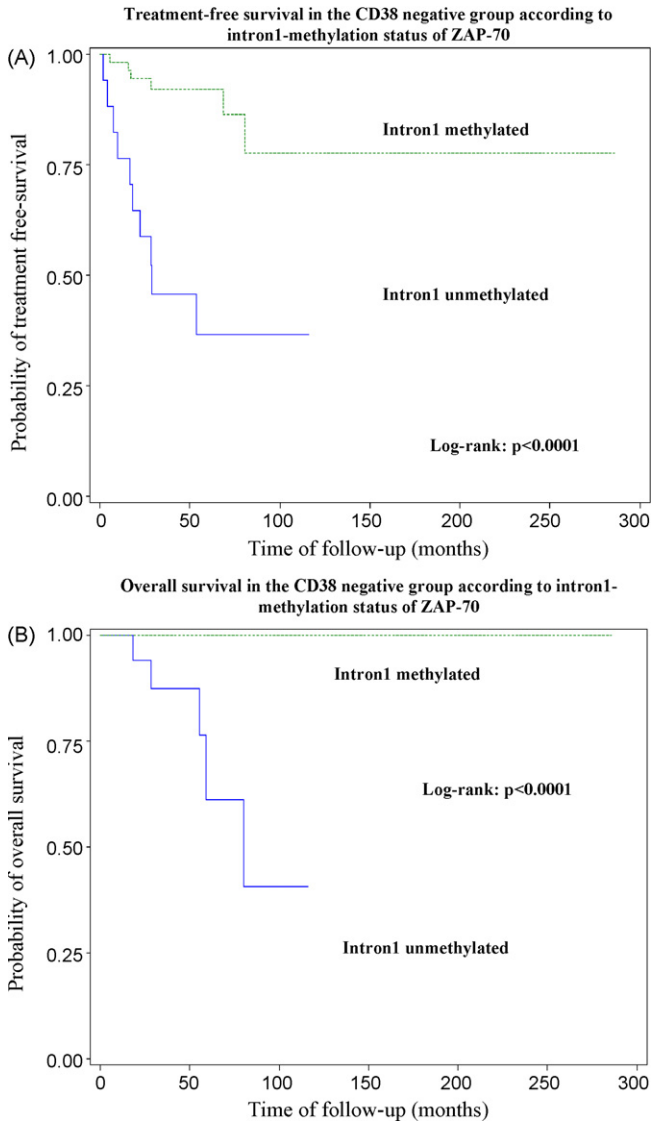

Fig. 6. Treatment-free and overall survival for the CD38 negative group according to ZAP-70 intron1-methylation. (A) Kaplan–Meier plot comparing treatment-free survival according to the methylation status of ZAP-70 intron1. CD38 negative and intron1 ZAP-70 methylated CLL patients have a significantly higher TFS than CD38 negative and intron1 ZAP-70 unmethylated patients ($p < 0.0001$). (B) Kaplan–Meier plot comparing overall survival of CD38 negative CLL patients with methylated (>50%) and unmethylated (<50%) *ZAP-70* intron1 in CLL cells. Methylation level of *ZAP-70* intron1 greater than 50% characterized patients with a longer OS ($p < 0.0001$).

In the CD38 negative subgroup, median TFS and OS outcomes were not attained in *ZAP-70* methylated patients due to the time-scale of the study. Hypomethylated patients had median TFS and OS of 29 and 80 months, respectively ($p < .0001$, Fig. 6).

For the CD38 positive group, *ZAP-70* methylated patients had higher TFS than hypomethylated patients (not attained versus 23 months ($p = .0091$)).

4. Discussion

This study confirms that the intron1–exon2 region of *ZAP*-70 is hypomethylated in T lymphocytes (expressing ZAP-70) and methylated in non-expressing B lymphocytes from healthy donors. These results show that the difference in methylation between ZAP-70 positive and negative CLL was significant for the 8 CpG sites (C+223 to C+384 to the TSS). Differences between the average methylation in ZAP-70 positive and negative patients are very close to results published by Corcoran et al. [29]. In their study, methylation of the ZAP-70 positive and negative samples showed significant correlation between ZAP-70 expression and hypomethylation for all CpG sites, with results showing better resolution with CpG+319 to CpG+384. Taken together, the study of Corcoran et al. and this study demonstrate that there is a close association between ZAP-70 expression and the methylation status of the intron1–exon2 of *ZAP-70*. Considering the results of bisulfite sequencing for patients 2 and 11 (Fig. 1) and the lower *p*-value, the average methylation of the first 4 CpGs was chosen rather than methylation of a single CpG. Indeed, aberrant methylation more often concerns, not a particular CpG site, but rather groups of several CpGs located in the 5′-untranslated region [26,27]. Bisulfite sequencing is a common method for the quantification of DNA methylation in specific genomic regions [35]. It relies on the chemical modification of hypomethylated cytosines and subsequent PCR amplification of the region of interest. In PCR products, methylated cytosines are displayed as cytosines and non-methylated cytosines such as thymines. Sequencing of subcloned PCR products delivers high-resolution methylation maps, but the method is too laborious for routine clinical use. In order to analyze the methylation of 4 CpG pairs in a 54 pb region (which is predictive of *ZAP-70* status) without cloning, we chose a method that delivers sequence reads and methylation quantification directly from PCR products. Pyrosequencing is a sequencing-by-synthesis method that relies on the release of pyrophosphate (Pp_i) during incorporation of nucleotides. Released Pp_i are quantitatively converted into a light flash resulting from an enzymatic cascade. These light signals are measured by a charge-coupled device camera and are displayed as peaks in a pyrogram. Pyrosequencing combines the qualities of direct quantitative sequencing, reproducibility, speed, and ease-of-use. It has many advantages compared to other techniques for methylation studies, e.g., bisulfite sequencing, COBRA, methyl-specific PCR (MSP), and quantitative-MSP (Q-MSP). COBRA is a quantitative method, but requires time-consuming enzymatic digestion and reduces the choice of analyzable CpG sites to those recognized by restriction enzymes. MSP uses several primers and probes to study methylation of all CpG dinucleotide combinations. The pyrosequencing assay, described here, only requires small quantities of DNA. It is a straightforward method that is fast, highly reproducible, inexpensive and can be used with paraffin-embedded tissue or frozen cells, making it the method of choice in the molecular laboratory routine.

DNA methylation analysis of CLL samples by pyrosequencing can be done without purification of B cells. Immunophenotyping of the sample determines the relative amount of T cells, NK cells and B cells in blood samples. It can be used to determine the number of contaminating (hypomethylated) T and NK cells and to correct the methylation results. To use DNA methylation of the *ZAP-70* gene as a predictive marker of ZAP-70 expression, we define a threshold of 50% as the value below which CLL cells are considered ZAP-70 positive. This threshold is in agreement with the results of Parker et al. It is possible that this empirically determined threshold value of 50% methylation corresponds to monoallelic methylation [36].

IgHv mutated and unmutated groups have similar median survival when compared with methylated and hypomethylated groups (outcome not attained versus 92 months). Because of this close relationship between *IgHv* mutation status and methylation status of *ZAP-70* intron1, multivariate analysis was not performed. Discordances between *ZAP-70* methylation status and *IgHv* mutational status were observed in 8.5% of the cases. This result is very close to that published by Corcoran et al. and is consistent with the 8–22% discordant cases between ZAP-70 expression and *IgHv* mutational status found in the literature. Additionally, the methylation status retains its prognostic value on TFS regardless of CD38 expression.

A lack of agreement between methylation and ZAP-70 expression determined by flow cytometry was observed in 23% of the patients. Eight cases (80% of the discordant cases) were non-methylated and ZAP-70 negative. Similar discordance was also observed by Corcoran et al. In a recent study, ZAP-70 mRNA expression predicted time to progression in CLL, even though a difference of 17.7% was observed between ZAP-70 mRNA and ZAP-70 protein expression [37]. This difference may be due to post-transcriptional regulation. Despite these discordant results, *ZAP-70* methylation status is associated with survival in CLL as shown by this study and by that of Corcoran et al.

In conclusion, the methylation status of *ZAP-70* intron1, analyzed by pyrosequencing, predicts ZAP-70 expression and overall survival in CLL. Pyrosequencing is a relatively inexpensive, easy to use, sensitive and reproducible method that can be performed in the molecular laboratory. Consequently, this new assay provides an alternative to currently used methods for ZAP-70 based prognosis.

Conflict of interest

The authors declare that there are no competing financial interests.

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Contribution: S.P.C., D.V., P.G., M.L., S.K. designed the project and directly participated in the drafting/writing/editing of the manuscript; S.C. and S.K. performed bisulfite sequencing, *ZAP-70* mRNA quantification, ZAP-70 immunoblot, pyrosequencing and analyzed data; D.V. was responsible for *IgHv* analysis and interpretation; C.G. contribute to the design of methylation analysis assay; V.S. and M.R. directed flow cytometry of samples and analysis; M.B. performed ZAP-70 immunoblot; Statistical analysis was carried out by N.H. and J.J.P.; S.C. contributed to immunoblot.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.leukres.2009.10.018.

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