Background and Objectives. p53 status and CD38 antigen are biological factors influencing response to therapy and clinical course in B–cell chronic lymphocytic leukemia (B–CLL). This study tests the hypothesis that soluble p53 alone and in association with CD38 can enucleate B–CLL subsets at worse prognosis.

Design and Methods. Wild and mutant forms of p53 protein were evaluated in 197 B–CLL patients at diagnosis or before progression by an immunoenzymatic method in plasma using an anti–p53 monoclonal antibody. CD38 expression was analyzed by a multicolor flow cytometric assay.

Results. Higher levels of both soluble p53 (sp53) and CD38 were significantly correlated with intermediate and high Rai stages, with higher β2–microglobulin and soluble CD23 values, determined at diagnosis. Shorter overall survival (OS) and progression-free survival (PFS) were both observed in sp53+ and CD38+ patients (p<0.0001). Simultaneous positivity or negativity for sp53 and CD38 identified two subsets of patients, the former with a worse prognosis and the latter with a better prognosis with regard to PFS (p<0.0001) and OS (p<0.0001). The predictive value of sp53 and CD38 was retained among the patients within the intermediate Rai risk group.

Interpretation and Conclusions. sp53 and CD38 together with ZAP–70 were confirmed to be independent prognostic factors in multivariate analysis. With regard to PFS, ZAP–70, sp53 and CD38 were confirmed to be independent prognostic factors. Concerning OS, ZAP–70, CD38 and age (< or > 60 years) were independent prognostic factors whereas sp53 showed only a tendency towards statistical significance.

Key words: soluble p53, CD38, prognosis, B–CLL.
tochemistry, was found to be correlated with p53 gene mutations, progressive disease and short survival.\textsuperscript{18} The analysis of p53 status by ELISA demonstrated that 60% of progressive cases of B-CLL were positive, whereas none of the non–progressive ones was positive.\textsuperscript{19} The aim of the present study was to analyze the clinical predictive value of soluble p53 (sp53) plasma levels, alone and in combination with other parameters, such as CD38 antigen, ZAP-70 and cytogenetics, in B-CLL.

Design and Methods

Patients

Approval for this study was obtained from the institutional review board. Informed consent was provided according to the Declaration of Helsinki. One hundred and ninety-seven consecutive B-CLL patients were admitted to this study. There were 104 men and 93 women with a median age of 65 years (range, 37 to 84). Clinical follow–up for all patients ranged from 13 to 157 months, with a median follow-up of 55 months. Patients were considered to have progressive disease if they had at least one of the following parameters: a lymphocyte doubling time of less than 1 year, progression to a more advanced Rai modified stage, development of systemic symptoms or Richter’s syndrome. Fresh or cryopreserved B-CLL cells were obtained for CD38 marker analysis in 197 patients. Plasma samples were available for sp53 analysis in 197 patients. All these samples were collected on a single day for each patient and were evaluated at initial diagnosis in 102 cases and during an indolent follow–up before progression in 95 patients. All these determinations were performed in triplicate and at least three times along the course of the disease at diagnosis or before disease progression. No particular variation was seen during the course of the follow–up and therefore the reported results derived from the value at diagnosis or during indolent follow–up. We tried empirically to set various cut–off points for each biological variable (sp53, CD38) and the selected thresholds were sufficient to identify accurately patients at poor prognosis based on differences in progression and survival. Moreover, we applied a discriminant function analysis based on the squared Mahalanobis distances of each case from its group centroids (sp53, CD38) to verify the selected cut points. The percentage correct (observed classification versus predicted classification) was 84% for sp53 and 99% for CD38.

Fifty–three patients had a low modified Rai stage, 138 had an intermediate stage and 6 had a high stage. The stage of the disease was defined at diagnosis. One hundred and ten of 197 patients underwent chemotherapy for their disease. Of the 110 treated patients, 50 were intermittently treated with chlorambucil at convention-al doses often combined with prednisone. The other 60 patients received and completed 6 courses of fludarabine monophosphate (Fludara, Schering AG, Berlin, Germany) at 25 mg/m$^2$/day for 5 days.

Diagnostics

Enzyme-linked immunosorbent assays

Wild and mutant forms of human p53 were detected and quantified by ELISA carried out according to the manufacturer’s instructions (Bender MedSystems, Vienna, Austria). An anti-p53 monoclonal coating antibody (clone B-F 34) was absorbed onto microwells and p53 present in the plasma of patients bound to antibodies adsorbed to the microwells; a biotin–conjugated monoclonal anti-p53 antibody (clone BP-3) was added and bound to the p53 captured by the first antibody. Streptavidin–horseradish–peroxidase (HRP) was added and bound to the biotin–conjugated anti-p53. Following incubation unbound streptavidin–HRP was removed by washing, and substrate solution (tetramethyl–benzidine plus 0.02% buffered hydrogen peroxide) reactive with HRP was added to the wells. A colored product was formed in proportion to the amount of p53 present in the sample. The reaction was terminated by addition of phosphoric acid and absorbance was measured at 450 nm. For reference, the mean of 65 normal plasma samples was 1.8 U/mL (SD=0.7), the range being between 0.02 and 3.0 U/mL. The threshold of positivity was set at the sp53 value of more than 3.5 U/mL.

The immunoenzymometric assay for soluble CD23 was performed as described elsewhere.\textsuperscript{2} The threshold of positivity was set at the sCD23 value of more than 60 U/mL and the reported results derived from the value at diagnosis.

Cellular immunophenotypic analysis

The following antibody conjugates were used: anti-CD23-PE, anti-CD5-FITC, anti-CD38-PE, anti-CD19-APC, anti-CD45-FITC, anti-CD14-PE, anti-CD95-PE, and anti-CD10-FITC (Becton Dickinson Immunocytometry Systems, San José, CA, USA). Peripheral blood mononuclear cells were analyzed for surface expression of CD19/CD5/CD38 and CD19/CD5/CD23 by triple color immunofluorescence. ZAP-70 protein determination was also performed by flow cytometry, as described elsewhere.\textsuperscript{16} Flow cytometric analyses were performed on a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry Systems) and CellQuest software was used to acquire and analyze data.

Interphase FISH

Separate hybridizations were carried out for loci on chromosomes 11, 12, 13 and 17. For chromosomes 11 (q23), 13 and 17 commercial probes (ATM-2, Rb-1 and p53, respectively) were used (Vysis, Inc, London, UK). An
satellite DNA probe CEP12, directly labeled with SpectrumGreen, was used to detect aneuploidy of chromosome 12. LSIp53, labeled with SpectrumOrange (Vysis), was evaluated to detect chromosome deletion at 17p13.1. We used peripheral blood lymphocytes, which were separated by density gradient centrifugation, treated with hypotonic solution (KCI) and fixed with methanol-acetic acid. The slides were then aged for 20 min at 80°C on a hot plate and dehydrated for 2 min in 70%, 80% and 100% ethanol and air dried. Gene frames were applied to dried slides in order to mark and separate the hybridization areas of single probes. Slides were placed on a hot plate at 37°C and 5 µL of each probe buffer solution were applied inside the area of the slides delineated by the frame. The slides were then sealed with a 22x22 mm gene-frame plastic coverslip and placed in the Vysis Hybrite™ machine. Co-denaturation was carried out at 68°C for 5 min and hybridization at 37°C over-night. The slides were washed in 0.4xSSC/0.3% NP-40 for 2 min at 71°C, followed by 1 min washing in 2xSSC at room temperature. Finally, the nuclei were counterstained with 4’, 6-diamidino-2-phenylindole (DAPI) and signals were visualized using an Olympus BX51 microscope (Olympus Italia, Milan, Italy). Two hundred interphase cells with well-delineated fluorescent spots were examined.

IgV<sub>H</sub> mutation analysis

Total RNA was extracted and reverse-transcribed as previously reported. The resulting cDNA, checked for first strand synthesis, was amplified using a mixture of sense primers annealing either to the V<sub>1</sub> through V<sub>6</sub> leader sequences or to the 5’ end of V<sub>1</sub>-V<sub>6</sub> FR1, as reported. These primers were used in conjunction with a mixture of antisense primers complementary to the germ line JH regions. The purified amplified products, inserted into the PCR2.1-TOPO vector (Invitrogen, Milan, Italy) were expanded in TOP10 One Shot™ competent cells (Invitrogen) and cloned. Plasmid DNA was isolated from overnight cultures of randomly selected colonies and sequenced using an automatic DNA sequencer (Beckman CEQ2000XL, Beckman Coulter, Hialeah, FL, USA). Comparisons between the obtained sequences and those of the various germ line IgV<sub>H</sub> genes were performed with the IgBLAST directory (http://www.ncbi.nlm.nih.gov/igblast) using the Mac Vector 7.1 sequence analysis software (Accelrys, Syrnantec Co., San Diego, CA, USA). Only when the same V-D-J rearrangement was identified in at least 5–10 clones, was a given IgV<sub>H</sub> sequence further analyzed. Alignment of the IgV<sub>H</sub> sequences available for each patient often revealed, along with mutations shared by all the transcripts analysed, a number of unique or partially shared mutations. For this reason, all mutational analyses were carried out in each IgV<sub>H</sub> transcript separately, and the percent mutation assigned to a given B-CLL was the mean value of the percent mutations found in each transcript. V<sub>H</sub> gene sequences deviating more than 2% from the corresponding germline gene were defined as mutated.

p53 expression by flow cytometry

Mononuclear cells, prefixed with a 0.5% paraformaldehyde, were incubated with a mouse anti-human p53 IgG2b monoclonal antibody (clone DO-7, Dako, Glostrup, Denmark) recognizing both mutated and wild-type forms, diluted in a 0.1% saponin solution and then incubated with 100 µL of RPE-conjugated rabbit anti-mouse F(ab’): fragment antibody (Dako). Finally, the cells were analyzed on an Epics XL flow cytometer (Beckman Coulter). An isotype-matched IgG2b monoclonal antibody was used as the negative control. The cut-off value based on ten patients who had no mutations on sequencing (data not shown) was 20% and values higher than 20% were considered to be positive (Figure 1).

Statistical analysis

Data were analyzed using Statistica® for Windows Version 6.0. All statistical evaluations were performed at the end of data collection. Associations between the sp53 plasma levels or CD38 and clinical variables, such as modified Rai stages and response to fludarabine, were assessed by the Mann-Whitney test. To quantify the degree of association between sp53 values and other biological variables, such as β2-microglobulin and CD38 percentages, Spearman’s coefficient was calculated. Correlations between lymphadenopathy/splenomegaly and the sp53 values, CD38 or ZAP-70 expression, were based on the two-tailed Fisher’s exact test. Response was assessed based on the National Cancer Institute Working Group criteria. Progression-free survival and overall survival, measured from diagnosis, were estimated by the Kaplan-Meier method and compared by the log-rank test. Cox proportional hazards regression model was used to assess the independent effect of covariables, treated as dichotomous, both on progression-free survival and on overall survival.

Results

Profiles of soluble p53 protein, CD38, ZAP-70, IgV<sub>H</sub> mutation and FISH

The proportion of B cells expressing CD38 varied from 0 to 87% and the threshold was set at 30%. The association between CD38 percentages and the modified Rai stages was statistically significant (Figure 2A, p=0.0002). Fifty-six patients (28.4%) were found to have sp53 plasma levels higher than 3.5 U/mL (range 0-23.7). When the levels of sp53 and the modified Rai stages were repre-
sented as a biparametric dot-plot, two sets could be separated, one with more than 3.5 U/mL sp53 (sp53+) and the other with less than 3.5 U/mL sp53 (sp53−). The comparisons between low, intermediate, high modified Rai stage and sp53 were very significant (Figure 2B, \(p<0.0001\)). The threshold of positivity for ZAP-70 was set at 20% of CD19−CD5− B cells and a significant correlation was found between ZAP-70 expression and the modified Rai stages (\(p=0.00001\)).

There was a very significant correlation between sp53 values and p53 percentages (66 patients), calculated by flow cytometry (\(r=0.68, p<0.0001\)). Spearman’s correlation between the plasma levels of sp53 and the percentages of CD38+ cells was \(r=0.27 (p=0.0001)\), indicating a moderate direct relationship. The level of ZAP-70 protein, determined in 188 patients, was significantly correlated with sp53 values: 100/141 patients with lower sp53 levels had ZAP-70 values <30% showed low- sp53 levels and the serum levels of β2-microglobulin, calculated at diagnosis, \((r=0.33, p<0.0001)\) or soluble CD23 (sCD23) levels \((r=0.37, p<0.0001)\). Moreover, in 79 patients analyzed both for \(IgVH\) mutations and sp53 levels, 48/61 patients with lower sp53 levels had \(IgVH\) mutations >2% \((p=0.05)\). Finally, the close relationship between CD38 and \(IgVH\) mutational status was confirmed (54/65 patients with CD38 <30% showed \(IgVH\) mutations >2%, \(p=0.003\)). Important correlations were found between sp53 levels and the serum levels of β2–microglobulin, calculated at diagnosis, \((r=0.33, p<0.0001)\) or soluble CD23 (sCD23) levels \((r=0.37, p<0.0001)\). Moreover, in 79 patients analyzed both for \(IgVH\) mutations and sp53 levels, 48/61 patients with lower sp53 levels had \(IgVH\) mutations >2% \((p=0.05)\). Finally, the close relationship between CD38 and \(IgVH\) mutational status was confirmed (54/65 patients with CD38 <30% showed \(IgVH\) mutations >2%, \(p=0.003\)). One hundred and thirty-eight patients were analyzed by interphase FISH in order to evaluate deletions in chromosome bands 17p13, 11q23, 13q14 and trisomy of band 12q13. With regard to cytogenetic groups, 73 patients (52.9%) had a normal karyotype and 35 (25.4%) had 13q-. Thirty (21.7%) patients had trisomy 12 (n=14), 11q− (n=9) and 17p− (n=4) or other rare abnormalities (\(light\) gene rearrangement, \(n=2\) and 6p− deletion, \(n=1\)). There was only a trend towards correlation between poor risk cytogenetic groups, such as trisomy 12, 11q− and 17p− and higher p53 values \((12/37, p=0.16)\).

**Clinical course and outcome**

With regard to the clinical course, the presence of three or more important intrathoracic/abdominal lymphadenopathies (>3 cm in diameter) and/or splenomegaly was found in 60.7% of patients with sp53+ B-CLL (\(p=0.00001\)). Likewise, higher CD38 percentages and higher ZAP-70 expression were significantly more frequent among patients with a large tumor burden (data not shown).

We also investigated sp53 as a predictor of clinical outcome. Sixty patients underwent 6 monthly courses of fludarabine monophosphate at 25 mg/m² for 5 days as their first-line chemotherapy; the global complete remission (CR) rate was 40% (24 of 60 patients). A higher CR rate was found both in sp53+ patients (59% vs 35%; \(p=0.005\)) and in CD38− patients (78% vs 22%; \(p=0.004\)).

A shorter PFS was observed both in sp53+ patients (5% vs 67% at 9 years; \(p<0.0001\), Figure 3A) and in CD38+ patients (13% vs 51% at 9 years; \(p<0.0001\), Figure 3B). Likewise, a shorter OS was found both in sp53− patients (15% vs 97% at 11 years; \(<0.0001\), Figure 4A) and in CD38− patients (28% vs 81% at 11 years; \(<0.00001\), Figure 4B). Interestingly, the simultaneous positivity or negativity for sp53 and CD38 identified two subsets of patients, the former with a worse prognosis and the latter with a better prognosis with regard to both PFS (5% vs 84% at 4 years, \(p<0.0001\), Figure 5A) and OS (0% vs 98% at 10 years, \(<0.0001\), Figure 5B). The subgroup with mixed positivity and negativity [CD38+sp53−]/ [CD38−sp53+] showed an intermediate outcome (PFS=60% at 4 years, Figure 5A; OS=55% at 10 years, Figure 5B). ZAP-70 positivity also allowed us to identify a subset of patients with a poor prognosis with regard to
to PFS (36% vs 78% at 5 years, \( p < 0.0001 \)) and OS (58% vs 92% at 10 years, \( p = 0.00002 \)). Furthermore, within the low Rai stage, sp53+ patients showed a better outcome with regard to PFS (84% vs 33% at 5 years, \( p = 0.0004 \)) and OS (97% vs 50% at 8 years, \( p = 0.0001 \)).

The predictive value of sp53 levels and CD38 with regard to both PFS and OS was maintained among the patients within the intermediate Rai risk group (Figures 6A-D). We performed multivariate Cox regression analysis of PFS and OS including, as co-variates, age, modified Rai stage, lymphocyte cell counts, \( \beta_2 \)-microglobulin, sCD23, ZAP-70, sp53 and CD38. With regard to PFS, ZAP-70, sp53 and CD38 were confirmed to be independent prognostic factors (Table 1). Concerning OS, ZAP-70, CD38 and age (< or > 60 years) were independent prognostic factors, while sp53 showed only a trend towards statistical significance (Table 2).

**Discussion**

Two aims of the present study were to validate an immunoenzymatic test for sp53 and to explore how sp53 plasma levels and CD38 expression affect prognosis. A particular subset of B-CLL with higher sp53 values was identified and was characterized by advanced Rai stage, higher \( \beta_2 \)-microglobulin and higher sCD23 concentrations.

p53 overexpression may be more reliable and sensitive than a mutational study for predicting the risk of progression or survival in B-CLL.\(^{35,36} \) We noted that no particular variation in the level of sp53 occurred during the course of follow-up, before any sign of progression, indicating the stability of this marker. Among the various possible mechanisms by which p53 overexpression could have occurred in our patients, we should consider the higher frequency of patients with poor risk cytogenetic abnormalities, such as trisomy 12, 11q- and 17p-, within the sp53+ subgroup, and inactivation by regulator genes, some of which reside on chromosome 11q.\(^{37} \) As a matter of fact, a simple screening test that detects p53 dysfunction due to mutation of the genes encoding either p53 or ATM, a kinase that regulates p53, has been developed.\(^{38} \) This dysfunction was characterized by increased baseline levels of p53, determined by Western blot, and there was a close relationship between p53 dysfunction and \( IgVH \) mutation. Similarly, in our study, many patients with lower sp53 levels presented \( IgVH \) mutations >2% (\( p = 0.05 \)).

Moreover, sp53 levels showed significant correlations with both CD38 expression (\( p = 0.0001 \)) and ZAP-70 (\( p = 0.00005 \)). All these strong relationships confirm the role of sp53 expression as an important factor for a novel risk stratification also including CD38, ZAP-70/\( IgVH \) mutational status and FISH data.

sp53 levels and CD38 expression were used to predict the chemosensitivity of B-CLL patients: infact, complete response to front-line fludarabine therapy was significantly correlated with lower sp53 (\( p = 0.005 \)) and CD38 negativity (\( p = 0.004 \)). That CLL patients with high levels of sp53 have a poor response to fludarabine is in keeping with the established importance of the p53 pathway in maintaining genomic integrity and mediating the action of certain cytotoxic agents, including purine

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**Figure 2. CD38 expression and soluble p53 (sp53) levels in B-CLL patients among the modified Rai stages.** There was a significant association between CD38 percentages and the modified Rai stages (A). Although there was not a clear cut between the cases with sp53 > 3.5 U/mL and those expressing lower than 3.5 U/mL within each modified Rai stage, these comparisons were statistically very significant (B).
Soluble p53 in B-CLL

analogs.39,40 sp53+ patients progressed more rapidly to advanced Rai stages (Figure 3A) and had a significantly shorter survival (Figure 4A), confirming the possibility that sp53 could predict the clinical outcome already at diagnosis. Moreover, CD38 expression and sp53 lev-

Table 1. Cox regression analysis of progression-free survival.

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Table 2. Cox regression analysis of overall survival.

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Figure 3. Progression-free survival curves based on sp53 and CD38 expression. Kaplan-Meier plots comparing progression-free survival in patients with sp53 greater than 3.5 U/mL or less than 3.5 U/mL (A), or more than 30% or less than 30% CD38+ B-CLL cells (B). As shown, progression-free survival was significantly shorter in patients with sp53 >3.5 U/mL and in those with >30% CD38 cells.

Figure 4. Overall survival curves based on sp53 and CD38 expression. Kaplan-Meier plots comparing overall survival in patients with sp53 greater or less than 3.5 U/mL (A), or more than 30% or less than 30% CD38+ B-CLL cells (B). As shown, overall survival was significantly shorter in patients with sp53 >3.5 U/mL and in those with >30% CD38 cells.

Figure 5. Progression-free survival and overall survival curves based on combined sp53 and CD38 expression. By combining sp53 and CD38 expression, we were able to identify three classes of patients with significantly different outcomes: 1) [sp53 (<3.5 U/mL)/CD38 (<30%)] showing the best outcome; 2) [sp53 (>3.5 U/mL)/CD38 (>30%)] showing the worst outcome and 3) [sp53/CD38+] or [sp53+/CD38+] showing an intermediate outcome, with regard to (A) progression-free survival and (B) overall survival.
els provided additive prognostic information, since B-CLL patients with CD38 >30% and sp53 >3.5 U/mL progressed very rapidly (Figure 5A) and had a very short OS (Figure 5B). The patients who were sp53 CD38− or sp53− CD38+ had an intermediate outcome. Furthermore, the prognostic relevance of sp53 and CD38 values with regard to progression and survival was the same within the intermediate Rai stage, confirming their independence from clinical stage (Figure 6). This observation is important for clinicians who have to choose which patients in this group need treatment, because they can show either an indolent course or aggressive disease. The prognostic relevance of sp53 and CD38 together with ZAP-70 was also confirmed in multivariate analysis (Tables 1 and 2).

In conclusion, we suggest sp53 determination in B-CLL as a novel prognostic indicator, which can be obtained easily with a standard immunoenzymatic procedure. This test should be integrated with other important determinations, such as CD38 and ZAP-70/IgVH, in order both to delineate a precise biological profile of B-CLL patients, and to stratify them better in different risk classes. Improved knowledge of B-CLL subgroups with a predictable, unfavorable outcome could allow us to include these patients in clinical trials and to use earlier experimental targeted approaches.

GDP, MIDP: conception, design, analysis and interpretation of data (in particular statistical analysis of clinical data); AV, LM, FB: revising the article for important intellectual content (biological and clinical data); RM, GS, PP, MCC, AB: analysis and interpretation of data (in particular flow cytometry, immunoenzymatic procedures and FISH analysis); VG, MD: analysis and interpretation of data (in particular IgVH mutational studies); SA: final approval of the version to be published. The authors reported no potential conflicts of interest. Supported in part by MURST, Programmi di ricerca di interesse nazionale, 2003. Presented in part at the 44th annual meeting of the American Society of Hematology, Philadelphia, PN, USA December 7-11, 2002. Manuscript received July 29, 2004. Accepted September 15, 2004.
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