Predictive Biomarkers and Personalized Medicine

Functional Analysis of the ATM-p53-p21 Pathway in the LRF CLL4 Trial: Blockade at the Level of p21 Is Associated with Short Response Duration

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Abstract

Purpose: This study sought to establish whether functional analysis of the ATM-p53-p21 pathway adds to the information provided by currently available prognostic factors in patients with chronic lymphocytic leukemia (CLL) requiring frontline chemotherapy.

Experimental Design: Cryopreserved blood mononuclear cells from 278 patients entering the LRF CLL4 trial comparing chlorambucil, fludarabine, and fludarabine plus cyclophosphamide were analyzed for ATM-p53-p21 pathway defects using an ex vivo functional assay that uses ionizing radiation to activate ATM and flow cytometry to measure upregulation of p53 and p21 proteins. Clinical endpoints were compared between groups of patients defined by their pathway status.

Results: ATM-p53-p21 pathway defects of four different types (A, B, C, and D) were identified in 194 of 278 (70%) samples. The type A defect (high constitutive p53 expression combined with impaired p21 upregulation) and the type C defect (impaired p21 upregulation despite an intact p53 response) were each associated with short progression-free survival. The type A defect was associated with chemoresistance, whereas the type C defect was associated with early relapse. As expected, the type A defect was strongly associated with TP53 deletion/mutation. In contrast, the type C defect was not associated with any of the other prognostic factors examined, including TP53/ATM deletion, TP53 mutation, and IGHV mutational status. Detection of the type C defect added to the prognostic information provided by TP53/ATM deletion, TP53 mutation, and IGHV status.


Introduction

Chronic lymphocytic leukemia (CLL) is a clonal expansion of mature B cells with a distinctive immunophenotype (1). The disease runs a highly variable clinical course, and a number of biologic variables have been identified that correlate with survival (2). Among these, deletion/mutation of TP53 on chromosome 17p is associated with the worst outcome (3).

TP53 encodes p53, a transcription factor that coordinates the cellular response to DNA damage (4). p53 protein is expressed at low levels in resting cells owing to its short half-life. Functional activation of p53 occurs through posttranslational modification that prolongs its half-life and thereby allows its accumulation in the nucleus, where it transactivates genes that regulate apoptosis, cell-cycle arrest, DNA repair, senescence, and angiogenesis (4). p53 also transactivates the E3 ubiquitin ligase MDM2 that targets p53 for proteosomal degradation and thereby limits the duration of the p53 response (5).

TP53 defects are detected in about 10% to 15% of patients with CLL requiring frontline therapy and are strongly linked to chemotherapy resistance, early progression, and short survival (6–16). The majority of TP53 defects consist of a monoallelic TP53 deletion together with a TP53 mutation, although in a minority of cases the defect consists of a TP53 mutation alone (8, 15–21).
Translational Relevance

This study has elucidated the specific clinical problems associated with different types of ATM-p53-p21 pathway defects in a large and uniform cohort of patients with chronic lymphocytic leukemia (CLL) defined by their need for frontline chemotherapy. Four different pathway defects were identified (types A, B, C, and D) based on the expression pattern of p53 and p21 proteins in untreated and irradiated CLL cells. Although 3 of the 4 pathway defects had neutral or redundant prognostic significance, the type C defect (characterized by isolated deregulation of p21) was identified as an independent determinant of early relapse following successful cytoreduction. These findings not only elucidate the clinical relevance of ATM/p53/p21 pathway dysfunction in patients with CLL requiring frontline chemotherapy but also the functional importance of p21 in regulating clonal expansion in CLL with implications for therapeutic intervention.

We previously developed an assay that probes the functional integrity of the p53 pathway by measuring levels of p53 and p21 following exposure to ionizing radiation (IR; ref. 22). The assay exploits the ability of IR to induce double-strand DNA breaks, which activate p53 via ATM-dependent phosphorylation. p21 (CDKN1A) is a consistent transcriptional target of p53 that induces cell-cycle arrest through its function as a cyclin-dependent kinase inhibitor (23). IR-induced upregulation of p21, therefore provides a convenient read-out of p53 transcriptional regulatory activity.

Initial application of the assay to a heterogeneous cohort of locally obtained CLL samples revealed 2 abnormal response patterns that we termed A and B (22). Both defects were associated with impaired p21 upregulation. The type A defect, characterized by high baseline p53 expression, was associated with TP53 mutation reflecting the prolonged half-life of most forms of mutant p53 protein. In contrast, the type B defect, characterized by impaired IR-induced p53 accumulation, was associated with ATM mutation reflecting the role of ATM protein in IR-induced p53 activation. Both defects were associated with adverse outcome in this small study.

Application of the assay [adapted for flow cytometry (24)] to a larger cohort of CLL patients showed an imperfect correlation between dysfunction of the ATM-p53-p21 pathway and deletion of TP53 (17p-) and ATM (11q-), indicating that functional and genetic defects only partially overlap (25). We subsequently identified a third ATM-p53-p21 pathway defect (type C) characterized by impaired p21 upregulation despite an intact p53 response. This defect was associated with single-nucleotide polymorphisms in the CDKN1A gene in a proportion of cases (26).

This study was conducted to examine the clinical significance of these various ATM-p53-p21 pathway defects in a large cohort of uniformly treated patients, with CLL defined by the need for frontline therapy.

Patients and Methods

Study population

The LRF CLL4 trial was a multicenter study designed to compare fludarabine or fludarabine plus cyclophosphamide with chlorambucil in patients with CLL requiring frontline therapy (7). A total of 777 patients were recruited between 1999 and 2004. Response was assessed according to National Cancer Institute criteria (27). Samples from 278 patients from the LRF CLL4 trial were included in this study. Cases were selected solely based on the availability of viable mononuclear cells.

Sample collection and storage

Heparinized blood samples were collected at the time of trial entry and sent to the coordinating center. Mononuclear cells were separated by density centrifugation, washed, resuspended in RPMI-1640 medium containing 10% dimethyl sulfoxide and 10% FBS (Sigma), and cryopreserved at 2 x 10⁷ to 3 x 10⁷/mL/vial in liquid nitrogen.

Functional probing of the ATM-p53-p21 pathway

Testing was done as previously described (24) and without knowledge of clinical outcome or any other data. Briefly, thawed mononuclear cells were washed, resuspended in culture medium, and divided into 2 aliquots, one of which was exposed to ionizing radiation (IR, 5 Gy gamma rays). After 15- to 16-hour incubation, the cells were fixed, permeabilized, and incubated with mouse antibodies to p53 or p21 followed by a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody and then PE-conjugated anti-CD19. Analysis was confined to CD19⁺ cells that were alive at fixation according to light scatter criteria, and samples were discarded if the nonirradiated control cells were less than 50% viable. Assays were carried out in batches of 6 to 8 samples, including a control sample with a normal p53/p21 response to ensure consistency between each run. Experiments were rejected if the control sample did not display a normal p53/p21 response.

p53 and p21 levels were expressed as the median fluorescence intensity (MFI). The percent increase in p53 and p21 levels over baseline expression was calculated as [100 x (MFI of irradiated cells – MFI of nonirradiated cells)/MFI of nonirradiated cells]. The upper limit of normal for baseline p53 expression was defined as an MFI value of 15 units. The lower limit of normal for p53 and p21 upregulation was defined as 80% and 25%, respectively. These cut-off values were derived from the separation of data clusters observed in a cohort of locally obtained CLL samples (24). Type A, B, and C defects were defined as follows: (A) high baseline p53 (>15 units), low p21 upregulation (<25%); (B) normal baseline p53 (≤15 units), low p53 upregulation (<80%), low p21 upregulation (<25%); (C) normal baseline p53 (≤15 units), normal p53 upregulation (≥80%), low p21 upregulation (<25%). In addition, we distinguished a further pathway defect (type D) characterized by normal
baseline p53 (≤15 units), low p53 upregulation (<80%) and normal p21 upregulation (≥25%). Representative examples of each type of p53/p21 response patterns are shown in Fig. 1.

During the course of the study, the flow cytometer initially used (Becton Dickinson FACScan) was replaced by a new machine (Becton Dickinson FACSCanto II). To validate the new machine against the old one, 138 samples were tested on both machines. There was almost perfect positive correlation in baseline p53 MFI and percent increase in p53 and p21, with all of the 3 correlation coefficients being greater than 0.90 (Supplementary Fig. S1). The 3 linear regression equations were therefore used to normalize results obtained with the new machine to make them directly comparable with those obtained with the old one (Supplementary Fig. S1).

Assay reproducibility was assessed using 22 local CLL samples with normal (n = 4) or type A (n = 3), B (n = 7), C (n = 6), or D (n = 2) responses. Each sample was tested between 3 to 20 times on separate days. Results are summarized in Supplementary Fig. S2 and Supplementary Table S2. The probability of reproducibility was 0.93, 1.00, 0.93, 0.80, and 1.00 for the 5 respective subgroups and 0.92 overall. To confirm that the results obtained using IR were similar to those obtained using etoposide (28, 29), the functional assay was applied to 20 local samples using similar to those obtained using etoposide (28, 29), the overall. To confirm that the results obtained using IR were

**Figure 1.** Representative flow cytometry histograms showing a normal p53/p21 response to IR and examples of each type of functional defect (A, B, C, and D). The histograms show staining for p53 (top) and p21 (bottom) in control (grey line) and irradiated (black line) cells following overnight incubation for 15 to 16 hours.

TP53 mutation analysis

Screening for TP53 mutations was carried out by capillary electrophoresis–single strand conformation analysis (CE-SSCA) as previously reported (16). Any sample producing a mobility shift from the wild-type peaks for any of the exons was considered mutated and subjected to direct sequencing for confirmation (16).

IGHV analysis

The immunoglobulin variable region gene (IGHV) was sequenced from PCR amplified either cDNA or gDNA, as previously described (31). Sequences were aligned to current databases (V-BASE and IMGT). A cut point of 98% homology to the nearest germline sequence was used to define IGHV mutational status (30).

Statistical analysis

Group-wise comparisons of clinical, laboratory, and genetic data were carried out using $\chi^2$ or Fisher exact tests. Overall survival (OS) was calculated from randomization to death from any cause. Progression-free survival (PFS) was timed from randomization to relapse requiring further treatment, progression, or death from any cause. For non-responders and those with progressive disease on first-line treatment, date of progression was when no response or progressive disease was recorded. Survival curves were constructed by the Kaplan–Meier method, and survival distributions compared with the log-rank test. Follow-up was to 31st October 2009, with a median follow up of 6 years 5 months (range: 5 years 1 month–10 years 9 months) for surviving patients. Statistical analyses were conducted with SAS Version 9.1 (SAS Institute Inc.) and in-house programs. All P values given are 2-sided.
Results

Patient characteristics

Data were obtained using 278 pretreatment samples from patients recruited into the LRF CLL4 trial. Of the patients whose samples were used for this study, 77% were male, 29% were age 70 or older, and 32% had Binet stage C disease. Thirty-three percent of patients had adverse chromosomal abnormalities (17p- or 11q-), 64% had unmutated IGHV, 64% were CD38+, and 46% had ZAP-70+. A total of 45%, 26%, and 29% of patients received chlorambucil, fludarabine, or fludarabine plus cyclophosphamide, respectively. The CR/nPR/PR and NR/PD rates in the study cohort were 79% and 21%, respectively. Comparison of the study cohort with that not studied but recruited into the LRF CLL4 trial showed no significant differences in any of these clinical and biologic variables, except that more samples with 11q deletion were included in this study (Supplementary Table S1).

Characterization of ATM-p53-p21 pathway defects

Type A, B, C, and D defects (as defined in the Methods section and illustrated in Fig. 1) were found in 6 (2.2%), 146 (52.5%), 13 (4.7%), and 29 (10.4%) of the 278 samples, respectively. Together, these pathway defects were detected in 194 (70%) of the 278 samples.

The type A defect is associated with short OS

Patients were separated into different groups according to ATM-p53-p21 pathway status and analyzed for OS. No differences were observed when patients were separated according to the presence of absence of pathway dysfunction (Fig. 2A). However, when patients were separated according to the type of dysfunction present, a significant association was observed between the type A defect and shorter OS (Fig. 2B). In contrast, patients with the types B, C, and D defects had an OS similar to that of patients with no pathway dysfunction.

The type A and type C defects are associated with short PFS

Patient outcome was also examined using PFS as an endpoint. In keeping with the OS analysis, there was no difference in the PFS of patients with or without ATM-p53-p21 pathway dysfunction (Fig. 2C). In contrast, when patients were separated according to the type of dysfunction present, both type A and type C defects were associated with a short PFS, whereas the PFS of patients with types B and D

Figure 2. Effects of ATM-p53-p21 pathway defects on OS (A and B) and PFS (C and D). Cases are separated according to the presence or absence of functional defects (A and C) or according to the type of defect (B and D).
defects was similar to that of patients with no pathway dysfunction (Fig. 2D).

**The type A defect is associated with chemotherapy resistance, whereas the type C defect is associated with short response duration**

Because short PFS can reflect failure of cytoreduction or rapid disease progression following successful cytoreduction, the different patient groups were compared for therapeutic response and response duration. No difference in response was observed when patients were separated according to the presence or absence of ATM-p53-p21 pathway dysfunction (Table 1). This observation held true for the entire cohort and also within each treatment arm. However, separating patients according to the type of dysfunction present showed a significant association between the type A defect and failure to achieve a CR, nPR, or PR with any treatment (Table 2). These findings implicated primary chemoresistance as an explanation for the short PFS of patients with type A dysfunction.

With regard to response duration, no difference was seen when patients were separated according to the presence or absence of ATM-p53-p21 pathway dysfunction (Fig. 3A). However, when patients were separated according to the type of dysfunction present, a significant association was observed between the type C defect and short response duration (Fig. 3B). These findings suggested that the short PFS of patients with the type C defect reflects early relapse following successful cytoreduction.

**The type A defect is associated with TP53 mutation/deletion, whereas the type C defect is not associated with other prognostic factors**

We next addressed the possibility that the adverse outcome associated with the type A and type C defects might reflect an association with other adverse features. The relationship between ATM-p53-p21 pathway status and other currently used prognostic factors is shown in Table 3. As expected, there was a strong association between the type A defect and TP53 deletion/mutation (Table 3). Specifically, among the 6 patients with the type A defect, 5 (83%) had a TP53 deletion and 6 (100%) a TP53 mutation. The respective figures in the rest of the cohort were 13 of 238 (5.5%) and 15 of 235 (6.4%). Therefore, the adverse outcome associated with the type A defect can be readily explained by its association with TP53 mutation/deletion. In contrast, there was no association between the type C defect and TP53 mutation, TP53 deletion, other chromosomal abnormalities, age, gender, stage, IGHV status, or treatment allocation. Together, these findings indicated that the adverse outcome associated with the type C defect cannot be explained by an association with other adverse features.

### Table 1. Effect of ATM-p53-p21 pathway dysfunction on response to chemotherapy

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Response</th>
<th>Total n</th>
<th>Normal n (%)</th>
<th>Dysfunction n (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorambucil</td>
<td>CR/nPR/PR</td>
<td>88</td>
<td>31 (74)</td>
<td>57 (70)</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>NR/PD</td>
<td>35</td>
<td>11 (26)</td>
<td>24 (30)</td>
<td></td>
</tr>
<tr>
<td>Fludarabine</td>
<td>CR/nPR/PR</td>
<td>52</td>
<td>16 (76)</td>
<td>36 (82)</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>NR/PD</td>
<td>13</td>
<td>5 (24)</td>
<td>8 (18)</td>
<td></td>
</tr>
<tr>
<td>Fludarabine + cyclophosphamide</td>
<td>CR/nPR/PR</td>
<td>69</td>
<td>20 (100)</td>
<td>49 (89)</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>NR/PD</td>
<td>6</td>
<td>0 (0)</td>
<td>6 (11)</td>
<td></td>
</tr>
<tr>
<td>Any treatment</td>
<td>CR/nPR/PR</td>
<td>209</td>
<td>67 (81)</td>
<td>142 (79)</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>NR/PD</td>
<td>54</td>
<td>16 (19)</td>
<td>38 (21)</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. Effect of specific ATM-p53-p21 pathway defects on response to chemotherapy

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Response</th>
<th>Normal n (%)</th>
<th>Type A n (%)</th>
<th>Type B n (%)</th>
<th>Type C n (%)</th>
<th>Type D n (%)</th>
<th>P (versus normal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorambucil</td>
<td>CR/nPR/PR</td>
<td>31 (74)</td>
<td>0 (0)</td>
<td>41 (66)</td>
<td>6 (86)</td>
<td>10 (100)</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>NR/PD</td>
<td>11 (26)</td>
<td>2 (100)</td>
<td>21 (34)</td>
<td>1 (14)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Fludarabine</td>
<td>CR/nPR/PR</td>
<td>16 (76)</td>
<td>0 (0)</td>
<td>27 (79)</td>
<td>2 (100)</td>
<td>7 (100)</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>NR/PD</td>
<td>5 (24)</td>
<td>1 (100)</td>
<td>7 (21)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Fludarabine + cyclophosphamide</td>
<td>CR/nPR/PR</td>
<td>20 (100)</td>
<td>1 (100)</td>
<td>38 (80)</td>
<td>2 (67)</td>
<td>8 (89)</td>
<td></td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>NR/PD</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>4 (10)</td>
<td>1 (33)</td>
<td>1 (11)</td>
<td></td>
</tr>
<tr>
<td>Any treatment</td>
<td>CR/nPR/PR</td>
<td>67 (81)</td>
<td>1 (25)</td>
<td>106 (77)</td>
<td>10 (83)</td>
<td>25 (96)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NR/PD</td>
<td>16 (19)</td>
<td>3 (75)</td>
<td>32 (23)</td>
<td>2 (17)</td>
<td>1 (4)</td>
<td></td>
</tr>
<tr>
<td><strong>P (versus normal)</strong></td>
<td></td>
<td></td>
<td>0.03</td>
<td>0.50</td>
<td>0.99</td>
<td>0.07</td>
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The type C defect adds to the prognostic information provided by TP53/ATM deletion, TP53 mutation, and IGHV status

We next sought to establish whether the type C defect adds to the prognostic information provided by currently available genetic markers. When the prognostic effect of the type C defect was examined in the subset of patients lacking deletion of TP53 and ATM, the type C defect was still associated with a significantly shorter PFS and response duration (Fig. 3C and D). Furthermore, differences in PFS and response duration between patients with the type C defect and the remainder were still significant after adjusting for TP53 mutation (P = 0.004 and P < 0.00001, respectively) or for both TP53 mutation and IGHV mutational status (P < 0.0001 for both endpoints). These observations suggested that detection of the type C ATM-p53-p21 pathway defect does indeed add to the prognostic information provided by TP53/ATM deletion, TP53 mutation, and IGHV mutational status.

Discussion

Deletion/mutation of TP53 is a well-established adverse prognostic factor in CLL. However, previous studies from this and other groups have shown that p53 pathway defects can be caused by other mechanisms (25, 26, 32). This study therefore sought to establish whether functional analysis of the ATM-p53-p21 pathway has prognostic value in CLL and, if so, whether it adds to the information provided by currently available prognostic factors. The question was addressed using stored samples from a representative subset of patients recruited into the LRF CLL4 trial of frontline chemotherapy. Among the 4 types of dysfunction identified, only the type A and type C defects were associated with adverse outcome, both being associated with short PFS. The type A defect was associated with chemoresistance, whereas the type C defect was associated with early relapse. The type A defect was strongly associated with TP53 deletion/mutation, but the type C defect was not associated with any of the other prognostic factors examined. Furthermore, the short response duration associated with the type C defect was observed in patients without TP53/ATM deletion and was independent of TP53 or IGHV mutational status.

It may seem surprising that only 6 of the 21 cases with TP53 mutation were classified as type A in the functional assay, the rest being classified as type B (n = 10), normal (n = 3), or type C (n = 2). However, there are at least...
3 possible explanations for this observation. First, different TP53 mutations result in varying degrees of baseline p53 protein overexpression (32, 33). In keeping with this idea, all 3 samples in this study with frameshift or truncation mutations were classified as type B in the functional assay, likely reflecting the lack of expression of full-length p53 protein from the mutated allele in these cases (Supplementary Table S3). By the same token, missense TP53 mutations could potentially produce a type C defect if the full-length mutant p53 protein retained a relatively normal pattern of expression. Second, it is well established that mutant p53 proteins derived from missense mutations retain a varying amount wild-type p53 activity (The TP53 Web Site. http://p53.free.fr/index.html). In this study, the predicted transcriptional activity of the mutant p53 protein was lost (<20% of wild-type p53 activity) in 15 of 16 informative samples. However, the one case with a p53 mutant protein that was predicted to retain 73% of wild-type p53 activity was classified as normal in the functional assay. Furthermore, the only case in which the abnormal SSCA screening results could not be confirmed by sequencing was also classified as functionally normal. Third, it should be noted that TP53 defects usually affect only a proportion of cells in the malignant clone. This raises the possibility that the TP53-defective subclone might evade detection if it is below a certain size. In keeping with this idea, the mean proportion of cells with a TP53 deletion among TP53-mutant samples classified as type A, B, C, and functionally normal was 67.7%, 54.5%, 43.5%, and 12.4%, respectively. In summary, TP53-mutant CLL samples can appear as normal or type A, B, or C in the functional assay depending on the type of TP53 mutation present, the amount of residual wild-type p53 function retained by the mutant p53 protein, the size of the TP53-defective clone, and the coexistence of other defects in the ATM-p53-p21 pathway.

It may also seem surprising that cases with the type B defect comprised more than half of the entire cohort. It was beyond the scope of this study to screen for ATM mutations, but their frequency has been reported as 17.1% among patients with 11q deletions and 4.5% in patients without 11q deletions (34). Because only 30% of the type B cases had an 11q deletion (Table 3), ATM inactivation due to deletion and/or mutation is unlikely

### Table 3. Relationship between ATM-p53-p21 pathway defects and other biologic variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>Total n</th>
<th>Normal n (%)</th>
<th>Type A n (%)</th>
<th>Type B n (%)</th>
<th>Type C n (%)</th>
<th>Type D n (%)</th>
<th>P&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>Gender</td>
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<td>Female</td>
<td>64</td>
<td>16 (19)</td>
<td>0 (0)</td>
<td>33 (23)</td>
<td>5 (38)</td>
<td>10 (34)</td>
<td>0.18</td>
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<tr>
<td>Male</td>
<td>214</td>
<td>66 (81)</td>
<td>6 (100)</td>
<td>113 (77)</td>
<td>8 (62)</td>
<td>19 (66)</td>
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<tr>
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</tr>
<tr>
<td>&lt;60</td>
<td>84</td>
<td>24 (29)</td>
<td>1 (17)</td>
<td>43 (29)</td>
<td>3 (23)</td>
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<td>60–</td>
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<td>38 (45)</td>
<td>3 (50)</td>
<td>56 (38)</td>
<td>7 (54)</td>
<td>9 (31)</td>
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<tr>
<td>70+</td>
<td>81</td>
<td>22 (26)</td>
<td>2 (33)</td>
<td>47 (32)</td>
<td>3 (23)</td>
<td>7 (24)</td>
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<td>Binet stage</td>
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<tr>
<td>B</td>
<td>116</td>
<td>35 (42)</td>
<td>1 (17)</td>
<td>64 (44)</td>
<td>5 (38)</td>
<td>11 (38)</td>
<td>0.33</td>
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<tr>
<td>C</td>
<td>90</td>
<td>22 (26)</td>
<td>1 (17)</td>
<td>52 (36)</td>
<td>3 (23)</td>
<td>9 (31)</td>
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<td>TP53 gene</td>
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<tr>
<td>Wild type</td>
<td>235</td>
<td>76 (96)</td>
<td>0 (0)</td>
<td>122 (92)</td>
<td>10 (83)</td>
<td>27 (100)</td>
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<tr>
<td>Mutated</td>
<td>21</td>
<td>3 (4)</td>
<td>6 (100)</td>
<td>10 (8)</td>
<td>2 (17)</td>
<td>0 (0)</td>
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<td>IGHV mutation</td>
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<tr>
<td>&lt;2%</td>
<td>156</td>
<td>43 (60)</td>
<td>5 (83)</td>
<td>87 (66)</td>
<td>6 (55)</td>
<td>15 (60)</td>
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<td>≥2%</td>
<td>89</td>
<td>29 (40)</td>
<td>1 (17)</td>
<td>44 (34)</td>
<td>5 (45)</td>
<td>10 (40)</td>
<td></td>
</tr>
<tr>
<td>17p (TP53) deletion&lt;sup&gt;b&lt;/sup&gt;</td>
<td>≤10%</td>
<td>238</td>
<td>73 (97)</td>
<td>1 (17)</td>
<td>126 (93)</td>
<td>12 (92)</td>
<td>26 (96)</td>
</tr>
<tr>
<td>&gt;10%</td>
<td>18</td>
<td>2 (3)</td>
<td>5 (83)</td>
<td>9 (7)</td>
<td>1 (8)</td>
<td>1 (4)</td>
<td></td>
</tr>
<tr>
<td>11q (ATM) deletion&lt;sup&gt;b&lt;/sup&gt;</td>
<td>≤5%</td>
<td>189</td>
<td>59 (79)</td>
<td>6 (100)</td>
<td>95 (70)</td>
<td>10 (77)</td>
<td>19 (70)</td>
</tr>
<tr>
<td>&gt;5%</td>
<td>67</td>
<td>16 (21)</td>
<td>0 (0)</td>
<td>40 (30)</td>
<td>3 (23)</td>
<td>8 (30)</td>
<td></td>
</tr>
<tr>
<td>6q deletion&lt;sup&gt;b&lt;/sup&gt;</td>
<td>≤5%</td>
<td>184</td>
<td>63 (95)</td>
<td>5 (100)</td>
<td>85 (97)</td>
<td>10 (100)</td>
<td>21 (100)</td>
</tr>
<tr>
<td>&gt;5%</td>
<td>5</td>
<td>6 (3)</td>
<td>0 (0)</td>
<td>3 (3)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Trisomy 12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>≤3%</td>
<td>230</td>
<td>64 (85)</td>
<td>6 (100)</td>
<td>125 (93)</td>
<td>11 (85)</td>
<td>24 (89)</td>
</tr>
<tr>
<td>&gt;3%</td>
<td>26</td>
<td>11 (15)</td>
<td>0 (0)</td>
<td>10 (7)</td>
<td>2 (15)</td>
<td>3 (11)</td>
<td></td>
</tr>
<tr>
<td>13q deletion&lt;sup&gt;b&lt;/sup&gt;</td>
<td>≤5%</td>
<td>159</td>
<td>42 (56)</td>
<td>6 (100)</td>
<td>86 (64)</td>
<td>9 (69)</td>
<td>16 (59)</td>
</tr>
<tr>
<td>&gt;5%</td>
<td>97</td>
<td>33 (44)</td>
<td>0 (0)</td>
<td>49 (36)</td>
<td>4 (31)</td>
<td>11 (41)</td>
<td></td>
</tr>
<tr>
<td>Fluda + Cyclo</td>
<td>82</td>
<td>21 (25)</td>
<td>2 (33)</td>
<td>44 (30)</td>
<td>4 (31)</td>
<td>11 (38)</td>
<td></td>
</tr>
<tr>
<td>Treatment allocation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlor</td>
<td>125</td>
<td>42 (50)</td>
<td>2 (33)</td>
<td>63 (43)</td>
<td>7 (54)</td>
<td>11 (38)</td>
<td>0.90</td>
</tr>
<tr>
<td>Fluda</td>
<td>71</td>
<td>21 (25)</td>
<td>2 (33)</td>
<td>39 (27)</td>
<td>2 (15)</td>
<td>7 (24)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Stage A progressive disease.<br>
<sup>b</sup>FISH data were sorted according to Dohner’s hierarchical model (30, 45).<br>
<sup>c</sup>P values were calculated using the Fisher–Freeman–Halton exact test.
to account for the type B defect in the majority of affected cases. However, the ATM-p53 signaling pathway is highly complex, with much scope for repression through mechanisms other than ATM mutation/deletion. For example, our group has previously shown that IR-induced p53 activation in CLL cells can be suppressed by bFGF (35). We have also shown that CLL cells express constitutively activated Akt that represses p53 activation via phosphorylation of its inhibitory partner, MDM2 (36). Others have reported that the activity of MDM2 in repressing p53 is influenced by a polymorphism in MDM2 (37), whereas hypermethylation of the TP53 promoter has been implicated as a cause of p53 dysfunction in another study (38). In summary, there are a number of potential mechanisms that could produce the type B defect and thereby explain its high frequency in this study.

The type D functional response was unexpected and at first sight seems difficult to explain, given that the transcriptional upregulation of p21 by IR is p53 dependent. One possible explanation for this defect is that it reflects unusually rapid reversal of IR-induced accumulation of p53 protein following transactivation of its target genes.

The strong association observed between the type A defect and primary chemoresistance likely reflects the fact that all type A cases harbored a TP53 mutation and is in keeping with the established importance of wild-type p53 in mediating the cytotoxicity of DNA-damaging chemotherapy (39–41). However, in the light of this consideration, the lack of chemoresistance in patients with the type B ATM-p53-p21 defect, which is also associated with failure of p53 activation, was unexpected. The likely explanation for this discrepancy is that the upstream mechanisms responsible for p53 activation by IR (or etoposide) are not the same as those responsible for p53 activation in response to alkylating agents and purine analogs, and that the latter drugs are capable of inducing p53 activation and apoptosis in CLL cells that do not accumulate p53 in response to IR (or etoposide). It is also possible that the relationship between response and specific ATM-p53-p21 pathway defects might differ between the 3 treatment arms. However, the study was not powered to address this question.

The association between the type A defect and short OS is in keeping with the importance of wild-type p53 in maintaining genomic stability through the coordination of DNA repair (42). Thus, the genomic instability associated with loss of wild-type p53 is likely to increase the probability of the CLL clone acquiring new mutations that confer a survival/growth advantage. This in turn would be expected to result in the emergence of increasingly aggressive and therapy-resistant subclones.

The most novel and interesting finding in this study was the association between the type C defect and short response duration—a reflection of rapid clonal expansion following successful cyto reduction. This could not be explained by an association with other risk factors but is in keeping with the pivotal role of p21 in limiting cell-cycle progression through its function as an inhibitor of cyclin-dependent kinases (CDK) 2, 3, 4, and 6 (43, 44). However, this idea needs to be reconciled with the neutral effect on response duration of the type B defect, which is also associated with impaired p21 upregulation. The likely explanation for this apparent contradiction is that failure of p21 upregulation in the type B defect reflects upstream blockade of the ATM/p53 pathway, whereas the type C defect, at least in some patients, seems to reflect a defect of p21 itself with broader implications for cell-cycle regulation. Thus, we have previously reported an association between the type C defect and 2 functionally interacting polymorphisms in the CDKN1A gene that affect p21 mRNA levels (26).

Although functional analysis of the ATM-p53-p21 pathway adds to the prognostic information provided by TP53 mutation and deletion of 17p and 11q, it should be noted that these genetic defects have a dominant effect on prognosis. Thus, among the 21 patients with TP53 mutation/deletion, no association was observed between p53 functional status and outcome. In contrast, among patients with the type B functional response, those with TP53 mutation/deletion had a significantly worse response, PFS, and OS (P values 0.015, 0.031, and 0.048, respectively). 11q deletion was also associated with a shorter OS among patients with the type B defect (P = 0.029).

With regard to how functional analysis of the ATM-p53-p21 pathway might be used to guide patient management, the assay is actually very straightforward to carry out and should consequently be well within the capabilities of most large centers. However, it should be noted that, with the possible exception of TP53 deletion/mutation, testing for prognostic biomarkers of any kind has not yet been shown to improve outcome in CLL. This reflects the fact that the specific clinical problems associated with particular biomarkers, or therapeutic strategies that address these problems, have not yet been properly evaluated. It is therefore premature to recommend the application of ATM-p53-p21 functional analysis in routine clinical practice. However, given the ability of the test to identify patients at high risk of early relapse following successful induction therapy, its most logical use at present would be as an entry criterion for clinical trials of postinduction maintenance therapy.

To conclude, this study has elucidated the specific clinical problems associated with different types of ATM-p53-p21 pathway defects in a large and uniform cohort of patients with CLL defined by their need for frontline chemotherapy. Although 3 of the 4 pathway defects had neutral or redundant prognostic significance, the type C defect was identified as an independent determinant of early relapse following successful cyto reduction. From a biologic perspective, our study sheds light on the functional importance of p21 in regulating clonal expansion in CLL and in doing so implicates cell-cycle control as a potential target for therapeutic intervention.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Lin, J. Adamson, A. Carter, R. Wade, D. Gonzalez, A.R. Pettitt

Writing, review, and/or revision of the manuscript: K. Lin, J. Adamson, A. Carter, R. Wade, S. Richards, D. Gonzalez, C. Dearden, D.G. Oscier, D. Cavoysky, A.R. Pettitt

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Adamson, M. Oates, S. Richards

Study supervision: A.R. Pettitt

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