

The Prognostic Value of *TP53* Mutations in Chronic Lymphocytic Leukemia Is Independent of Del17p13: Implications for Overall Survival and Chemorefractoriness

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Abstract Purpose: Del17p13 predicts poor outcome and chemorefractoriness in chronic lymphocytic leukemia (CLL). Conversely, it is unknown whether *TP53* mutations carry any prognostic value independent of del17p13. We tested the independent prognostic value of *TP53* mutations in CLL. **Experimental Design:** The study was based on a consecutive series of 308 CLL. DNA sequencing of *TP53* exons 2 to 10 and del17p13 interphase fluorescence *in situ* hybridization were done at CLL diagnosis. Study end points were survival and chemorefractoriness. **Results:** At diagnosis, *TP53* mutations ($n = 32$) occurred in 31 of 308 (10.0%) patients. Of all CLL showing *TP53* disruption by either mutation and/or deletion ($n = 44$), 10 cases (22.7%) showed *TP53* mutations in the absence of del17p13. Multivariate analysis selected *TP53* mutations (hazard ratio, 3.20; $P = 0.002$) as an independent predictor of overall survival after adjustment for del17p13. Also, multivariate analysis selected *TP53* mutations (hazard ratio, 3.97; $P < 0.001$) as an independent predictor of chemorefractoriness after adjustment for del17p13. Compared with cases without *TP53* alterations, CLL harboring any type of *TP53* disruption (mutation only, del17p13 only, or both mutation and del17p13) uniformly displayed a high prevalence of unfavorable prognosticators and poor outcome. Analysis of sequential CLL samples showed the acquisition of new or additional *TP53* alterations at the time of chemorefractoriness. **Conclusions:** These data show that (a) *TP53* mutations are an independent predictor of short survival and chemorefractoriness, and (b) that CLL presenting with *TP53* mutations without del17p13 fare as poorly as CLL carrying del17p13. Because CLL harboring *TP53* mutations without del17p13 are currently not recognized by conventional diagnostic strategies, these results may be relevant for a comprehensive prognostic characterization of CLL.

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Patients with chronic lymphocytic leukemia (CLL) harboring del17p13 display poor survival because of advanced disease at diagnosis, short time to first treatment, and high risk of chemorefractoriness to alkylating agents and purine analogues (1–5). Because of these adverse features, patients harboring del17p13 currently represent a major challenge (6). Given the expanding options available for CLL treatment, early identification of del17p13 is of clinical relevance and has practical implications for therapeutic stratification of patients with CLL (7).

TP53 maps to 17p13.1 and is regarded as the candidate tumor suppressor gene hit by del17p13. Besides deletion, *TP53* may be inactivated through somatic mutations. *TP53* mutations occur in ~10% of CLLs at diagnosis, and have been described within unfavorable CLL subsets such as fludarabine-refractory CLL or CLL with prolymphocytic morphology (8–14). Whereas up to two-thirds of del17p13 CLL also harbor *TP53* mutations, a fraction of CLL carry *TP53* mutations without del17p13 (1, 4, 14). At present, it is unknown whether *TP53* mutations in the absence of del17p13 confer the same biological profile, clinical features, and outcome displayed by

Translational Relevance

Disruption of *TP53* by either mutation, del17p13, or both occurs in a sizeable fraction of chronic lymphocytic leukemias (CLL) at diagnosis. *TP53* disruption by del17p13 predicts poor survival and chemorefractoriness. Conversely, it is unknown whether *TP53* mutations harbor any prognostic relevance that is independent of del17p13. This issue is of potential clinical relevance because patients with *TP53* mutations without del17p13 (a) account for 20% of all patients carrying *TP53* disruption at the time of CLL diagnosis and (b) are not recognized by current diagnostic approaches which recommend screening for del17p13 but not for *TP53* mutations. From a clinical standpoint, assessing the independent prognostic value of *TP53* mutations may clarify whether, analogous to del17p13, *TP53* mutations are an independent predictor of refractoriness to conventional alkylator-based or fludarabine-based regimens. We tested whether *TP53* mutations are an independent predictor of overall survival, treatment-free survival, and chemorefractoriness in a large CLL cohort ($n = 308$). We show that *TP53* mutations are an independent predictor of overall survival, treatment-free survival, and chemorefractoriness. Based on these results, we suggest that *TP53* mutation analysis should be considered for a comprehensive prognostic characterization of patients with CLL who are candidates for treatment.

del17p13 CLL. Also, the independent prognostic relevance of *TP53* mutations in relationship to del17p13 and other biological prognosticators remains undescribed to date.

Current diagnostic guidelines suggest to screen CLL patients for del17p13, but not for *TP53* mutations (7). Therefore, patients with *TP53* disruption due only to mutations are not recognized at diagnosis. We reasoned that detecting all *TP53* genetic abnormalities at the time of diagnosis might be potentially useful for improving CLL clinical management. These considerations prompted us to assess whether *TP53* mutations carry any prognostic value that is independent of del17p13. We show that *TP53* mutations predict poor survival and chemorefractoriness independent of del17p13 in a consecutive series of 308 patients with CLL.

Materials and Methods

Patients. The study was based on a consecutive series of 308 patients with previously untreated CLL who presented for initial evaluation at the Division of Hematology of the Amedeo Avogadro University of Eastern Piedmont, Novara, Italy ($n = 224$) and at the Division of Hematology and Bone Marrow Transplantation of the University of Siena, Siena, Italy ($n = 84$) from January 1996 to June 2006. The database was updated for the analysis in November 2007. Median follow-up of patients that were alive was 54.3 months. No patient was lost at follow-up. All patients provided informed consent in accordance with local institutional review board requirements and Declaration of Helsinki guidelines. Diagnosis of CLL was based on National Cancer Institute Working Group criteria and confirmed by a flow cytometry score of >3 in all cases (15, 16).

The following clinical variables were recorded at presentation: (a) date of diagnosis, age, sex; (b) Binet stage, spleen size (centimeters

below the left costal margin), number of nodal areas involved; (c) lymphocyte count, hemoglobin, platelet count, serum β 2-microglobulin (reference range, 1.8-2.3 mg/dL), lactate dehydrogenase (LDH; reference range, 200-450 units/L).

The following biological variables were analyzed on fresh or cryopreserved peripheral blood mononuclear cells collected at CLL diagnosis: (a) mutations of *TP53* exons 2 to 10; (b) *IGHV* gene usage and homology to germ line; (c) fluorescence *in situ* hybridization (FISH) karyotype for del13q14, +12, del17p13, and del11q22-q23; (d) CD38 and ZAP70 expression.

During follow-up, the following variables were recorded: (a) date of first-line treatment, (b) type and number of lines of treatment, (c) date of refractoriness to alkylating agents and date of refractoriness to fludarabine-based regimens, and (d) date of death.

Analysis of *TP53* mutations. Mutation analysis of *TP53* exons 2 to 10 was done by DNA direct sequencing on an ABI Prism 3100 automated DNA sequence analyzer (Applied Biosystems; refs. 8, 9). Mutations were confirmed on both strands on independent amplimers and validated by the IARC *TP53* Mutation Database⁴ and the UMD *TP53* Mutation Database⁵ (17, 18). The distribution of mutations on the *TP53* molecule was assessed using the IARC *TP53* Mutation Database (17, 19). The residual transactivation activity of *TP53* alleles carrying missense mutations was estimated *in silico* according to the aforementioned databases (17, 18, 20).

Analysis of *IGHV* rearrangements. *IGHV* rearrangements were amplified from genomic DNA and directly sequenced (21). Sequences were aligned to ImmunoGeneTics directories, and considered mutated if homology to the corresponding germ line gene was $<98\%$.

Flow cytometry. A FACScalibur flow cytometer (Becton Dickinson) was used for flow cytometric analysis. Expression of CD38 and ZAP70 was analyzed as reported (22). Cutoff points of 30% and 20% were used to define positivity for CD38 and ZAP70, respectively.

Interphase FISH. Probes (Vysis) used for FISH analysis were LSI13 and LSID13S319 for detection of del13q14; CEP12 for detection of aneuploidy of chromosome 12; LSIp53 for detection of del17p13; and LSIATM for detection of del11q22-q23. Nuclei were counterstained with 4',6'-diamidino-2-phenylindole and antifade, and signals were visualized using an Olympus BX51 microscope (Olympus Italia). For each probe, at least 500 interphase cells with well-delineated fluorescent spots were examined.

Statistical analysis. Overall survival (OS) was measured from date of CLL diagnosis to date of last follow-up or death. Time to progression (TTP) was measured from date of CLL diagnosis to date of progressive and symptomatic disease according to National Cancer Institute Working Group guidelines, death, or last follow-up (15). Refractory disease was defined as treatment failure (stable disease or progressive disease during treatment) or disease progression within 6 months from antileukemic therapy (7). Time to chemorefractoriness was measured from date of first-line treatment until date of refractoriness to fludarabine-based regimens, date of alkylator refractoriness in patients who had never been exposed to fludarabine, death, or last follow-up. Time to fludarabine refractoriness was measured from date of first exposure to fludarabine-based regimens until date of refractoriness to fludarabine-based regimens, death, or last follow-up.

Categorical variables were compared by χ^2 test or exact tests when appropriate. Continuous variables were compared by Mann-Whitney test. Survival analysis was done by Kaplan-Meier method using log-rank statistics to test for significant associations (23). Multivariate analysis was done by Cox proportional hazard regression (24). A forward stepwise selection algorithm was used. A variable was entered into the model if the probability of its score statistic was ≤ 0.05 . A variable was removed from the model if the probability of its removal statistic was ≥ 0.10 .

⁴ <http://www.p53.iarc.fr/StructureAnalysis.html>

⁵ <http://p53.free.fr/Database/p53recomendations.html>

Table 1. Biological and clinical characteristics of the CLL cohort according to *TP53* mutation status

Characteristics at CLL diagnosis	<i>TP53</i> wild-type	<i>TP53</i> mutated	P
Biological variables at diagnosis			
<i>IGHV</i> homology $\geq 98\%$	99/272 (36.4%)	16/31 (51.6%)	0.098
Normal FISH	77/269 (28.6%)	2/28 (7.1%)	0.014
Number of FISH lesions >1	36/269 (13.4%)	13/28 (46.4%)	<0.001
del13q14	135/269 (50.2%)	16/28 (57.1%)	0.483
+12	56/269 (20.8%)	4/28 (14.3%)	0.413
del11q22-q23	23/269 (8.6%)	1/28 (3.6%)	0.712
del17p13	16/269 (5.9%)	18/28 (64.3%)	<0.001
CD38 $\geq 30\%$	81/272 (29.8%)	11/29 (37.9%)	0.365
ZAP70 $\geq 20\%$	100/241 (41.5%)	13/26 (50.0%)	0.404
Clinical variables at diagnosis			
Age (y)	68 (59-73)	67 (58-77)	0.847
Age >65 y	160/277 (57.8%)	14/31 (54.8%)	0.755
Male	147/277 (53.1%)	17/31 (54.8%)	0.851
Binet B-C	62/277 (22.4%)	15/31 (48.4%)	0.002
Nodal areas ≥ 3	42/276 (15.2%)	9/31 (29.0%)	0.049
Splenomegaly	47/276 (17.0%)	8/31 (25.8%)	0.227
Lymphocytes > $20 \times 10^9/L$	74/277 (26.7%)	8/31 (25.8%)	0.914
Hemoglobin <13 g/dL	79/276 (28.6%)	13/31 (41.9%)	0.125
Platelets < $150 \times 10^9/L$	64/276 (23.2%)	15/31 (48.4%)	0.002
$\beta 2$ -Microglobulin > 2.5 mg/L	108/252 (42.9%)	17/27 (63.0%)	0.046
LDH >1 \times upper limit of normal	50/269 (18.6%)	10/31 (32.3%)	0.072
Treatment			
Lines	2 (1-3)	3 (1-4)	0.205
Lines >2	40/119 (33.6%)	12/22 (54.5%)	0.062
Fludarabine-based	74/119 (62.2%)	14/22 (63.6%)	0.897

NOTE: The 25th to 75th percentiles are reported in parentheses for continuous variables.

Covariates at CLL diagnosis that entered multivariate analysis for OS along with *TP53* mutations were *IGHV* gene homology, del17p13, CD38 expression, age, Binet stage, and levels of LDH and $\beta 2$ -microglobulin. Covariates that entered multivariate analysis for time to chemorefractoriness along with *TP53* mutations were *IGHV* gene homology, del17p13, CD38 expression, age, number of lines of treatment, and fludarabine-based treatment. Covariates that entered multivariate analysis for time to fludarabine refractoriness along with *TP53* mutations were *IGHV* gene homology, del17p13, CD38 expression, age, and number of lines of treatment. Stratification of continuous clinical variables was based on the best predictive cutoff value or usual limit of normal. All statistical tests were two-sided. Statistical significance was defined as $P < 0.05$. The analysis was done with the Statistical Package for the Social Sciences software v.15.0.

Results

Characterization of the CLL cohort. The study was based on a consecutive series of 308 (164 males and 144 females) previously untreated CLL. Biological and clinical characteristics at diagnosis are representative of the disease (Table S1). Median age was 67 years (25th-75th percentile, 59-73). According to the Binet staging system, 231 of 308 (75.0%) patients were in stage A, 40 of 308 (13.0%) patients were in stage B, and 37 of 308 (12.0%) patients were in stage C. Median hemoglobin level was 13.6 g/dL (25th-75th percentile, 12.6-14.8), median platelet count was $191 \times 10^9/L$ (25th-75th percentile, 148-236), median serum LDH was 360 units/L (25th-75th percentile, 301-428), and median $\beta 2$ -microglobulin was 2.4 mg/L (25th-75th percentile, 1.8-3.4).

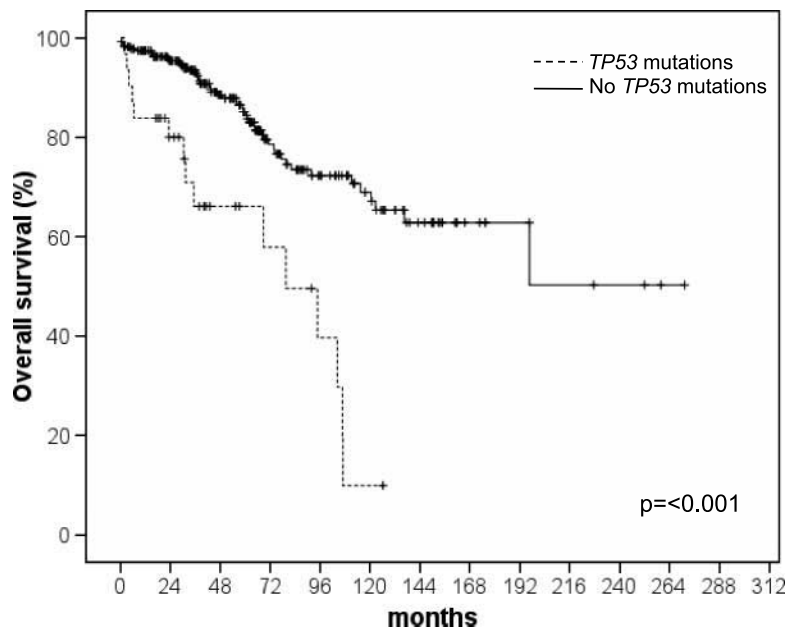
Considering biological prognosticators, *IGHV* homology $\geq 98\%$ occurred in 115 of 303 (38.0%) patients, CD38 $\geq 30\%$

in 92 of 301 (30.6%) patients, and ZAP70 $\geq 20\%$ in 113 of 267 (42.3%) patients. Del13q14 was observed in 151 of 297 (50.8%) cases, normal FISH in 79 of 297 (26.6%) cases, +12 in 60 of 297 (20.2%) cases, del11q22-q23 in 24 of 297 (8.1%) cases, and del17p13 in 34 of 297 (11.4%) cases. CLL with del17p13 displayed a median of 62% (25th-75th percentile, 25-72%) interphase nuclei harboring del17p13.

Molecular and clinical characterization of CLL harboring *TP53* mutations. At CLL diagnosis, *TP53* mutations ($n = 32$) were documented in 31 of 308 (10.0%) patients (Table S2). One patient carried two mutations on independent alleles. All mutations were validated by the IARC *TP53* Mutation Database⁴ and the UMD *TP53* Mutation Database,⁵ and reflected the *TP53* mutation profile reported in CLL (17, 18). All *TP53* mutations predicted functional consequences. Among 26 missense mutations, the median residual transactivation activity compared with germ line *TP53* was 8.9% (25th-75th percentile, 0.6-13.0%), and was $\leq 20\%$ in 22 of 26 (84.6%) mutations (17, 18, 20). All microdeletions and microinsertions (4/32; 12.5%) led to frameshift mutations. One nonsense mutation introduced a stop codon at position 213, and one mutation affected the 3' splicing site of exon 8. *TP53* mutations affected exon 4 in 2 of 32 (6.2%) cases, exon 5 in 6 of 32 (18.7%) cases, exon 6 in 5 of 32 (15.6%) cases, exon 7 in 9 of 32 (28.1%) cases, and exon 8 in 10 of 32 (31.2%) cases. No mutations were found in exons 2, 3, 9, and 10.

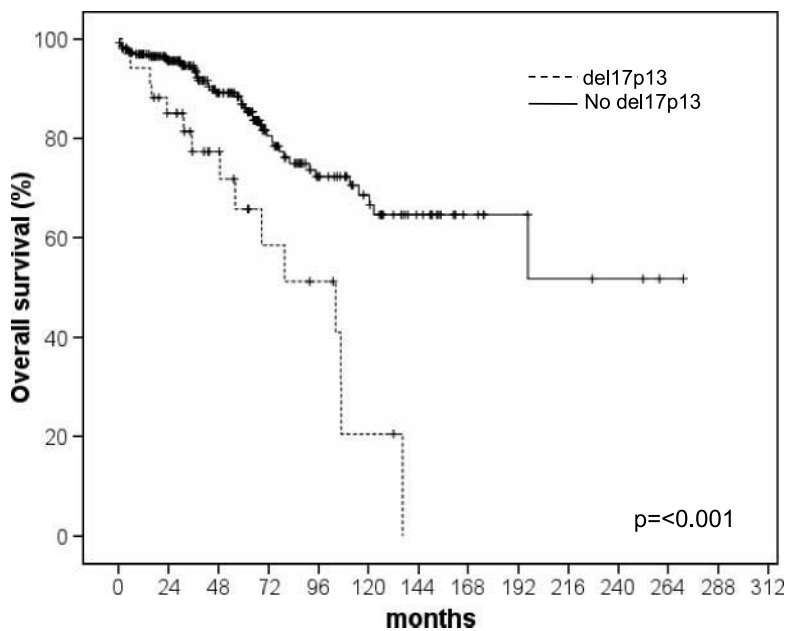
Mutations affecting coding sequences were tested for distribution on *TP53* functional domains (18, 19). Twenty-nine of 31 (93.5%) mutations targeted the *TP53* DNA-binding domain, whereas two frameshift mutations affected the proline-rich motif outside the DNA-binding domain (18, 19). *TP53*

A



	Events/N	Median OS	SE±	5-year OS	SE±
TP53 mutations	15/31	79.6 months	17.6	66.2%	0.095
No TP53 mutations	50/277	Not reached	-	85.2%	0.026

B



	Events/N	Median OS	SE±	5-year OS	SE±
del17p13	15/34	104.2	16.7	65.8	0.100
No del17p13	44/263	Not reached	na	86.9	0.025

Fig. 1. Kaplan-Meier curves of OS according to TP53 mutations and del17p13. Univariate analysis identified TP53 mutations (A) and del17p13 (B) as risk factors of short OS.

Table 2. Multivariate analysis for OS, chemorefractoriness, and fludarabine refractoriness in CLL

	HR (95% CI)	P
OS*		
Age >65 y	4.98 (2.45-10.10)	<0.001
Binet B-C	3.01 (1.67-5.42)	<0.001
TP53 mutations	3.20 (1.52-6.70)	0.002
Time to chemorefractoriness [†]		
TP53 mutations	3.97 (2.09-7.51)	<0.001
Lines of treatment >2	2.55 (1.39-4.66)	0.002
Age >65 y	2.27 (1.28-3.85)	0.004
IGHV homology ≥98%	1.88 (1.02-3.44)	0.041
Time to fludarabine refractoriness [‡]		
TP53 mutations	6.72 (2.61-17.21)	<0.001

Abbreviations: 95% CI, 95% confidence interval; P, P value calculated by Cox analysis.

*Covariates at CLL diagnosis that entered the analysis were TP53 mutations, IGHV gene homology, del17p13, CD38 expression, age, Binet stage, and levels of LDH and β2-microglobulin.

[†]Covariates that entered the analysis were TP53 mutations, IGHV gene homology, del17p13, CD38 expression, age, number of lines of treatment, and fludarabine-based treatment.

[‡]Covariates that entered the analysis were TP53 mutations, IGHV gene homology, del17p13, CD38 expression, age, and number of lines of treatment.

codons directly involved in DNA binding or zinc atom ligation were targeted by mutations in 7 of 31 (22.5%) cases (19).

The biological and clinical features of CLL harboring TP53 mutations are summarized in Table 1. At diagnosis, TP53 mutations associated with advanced Binet stage ($P = 0.002$), thrombocytopenia ($P = 0.002$), elevated β2-microglobulin ($P = 0.046$), higher number of FISH lesions ($P < 0.001$), and occurrence of del17p13 ($P < 0.001$). Despite the statistical association between TP53 mutations and del17p13 in the whole cohort, the overlap between these two genetic lesions was restricted to a fraction of patients. By combining the results of FISH and TP53 mutation analyses in cases for which both data were available ($n = 297$), 44 of 297 (14.8%) CLL carried TP53 inactivation through mutation and/or deletion. Of these, 18 of 44 (40.9%) cases showed TP53 mutation paired to del17p13, 10 of 44 (22.7%) cases showed TP53 mutations in the absence of del17p13, and 16 of 44 (36.3%) cases showed del17p13 in the absence of TP53 mutations.

TP53 mutations predict OS independent of del17p13. After a median follow-up of 54.3 months from diagnosis, 65 of 308 patients had died. Median OS of the whole series was 196.6 (SE ± 47.3) months. Univariate log-rank analysis identified both TP53 mutations and del17p13 as risk factors for short OS. Median OS for patients with TP53 mutations was 79.6 months (5-year OS, 66.2%), whereas median OS for patients without TP53 mutations was not reached and the 5-year OS was 85.2% ($P < 0.001$; Fig. 1A). Median OS for patients with del17p13 was 104.2 months (5-year OS, 65.8%) versus not reached (5-year OS, 86.9%) for patients devoid of del17p13 ($P < 0.001$; Fig. 1B). Other variables associated with short OS in univariate log-rank analysis are listed in Table S3.

Multivariate analysis selected TP53 mutations [hazard ratio (HR), 3.20; $P = 0.002$], along with age >65 years (HR, 4.98; $P < 0.001$), and advanced Binet stage (HR, 3.01; $P < 0.001$) as

independent predictors of OS (Table 2). Other covariates included in the multivariate analysis and not selected as predictor of OS were IGHV gene homology ≥98% ($P = 0.116$), del17p13 ($P = 0.246$), CD38 ≥30% ($P = 0.229$), LDH >1× upper limit of normal ($P = 0.510$), and β2-microglobulin >2.5 mg/L ($P = 0.694$).

TP53 mutations predict chemorefractoriness independent of the concomitant occurrence of del17p13. At the time of last follow-up, 141 patients had been treated (Table S1). Lines of treatment were 1 in 52 of 141 (36.8%) patients, 2 in 37 of 141 (26.2%) patients, 3 in 23 of 141 (16.3%) patients, and >3 in 29 of 141 (20.5%) patients. Fludarabine-based regimens were used in 88 of 141 (62.4%) patients. No patient received anti-CD20 or anti-CD52 antibodies before the definition of chemorefractoriness.

After a median follow-up of 35.0 months from the date of first-line treatment, chemorefractoriness was documented in 64 of 141 (45.4%) patients. Median time to chemorefractoriness was 51.7 (SE ± 14.0) months in the whole CLL series. Univariate log-rank analysis identified both TP53 mutations and del17p13 as risk factors of short time to chemorefractoriness. Median time to chemorefractoriness for patients with TP53 mutations was 6.3 versus 72.7 months for patients without TP53 mutations ($P < 0.001$; Fig. 2A). Median time to chemorefractoriness for patients with del17p13 was 21.4 versus 66.3 months for patients without del17p13 ($P = 0.002$; Fig. 2B). Other predictors of short time to chemorefractoriness identified by univariate log-rank analysis are listed in Table S4.

Multivariate analysis selected TP53 mutations (HR, 3.97; $P < 0.001$), lines of treatment >2 (HR, 2.55; $P = 0.002$), age >65 years (HR, 2.27; $P = 0.002$), and IGHV homology ≥98% (HR, 1.88; $P = 0.041$) as independent predictors of short time to chemorefractoriness (Table 2). Other covariates included in the multivariate analysis and not selected as predictors of short time to chemorefractoriness were del17p13 ($P = 0.210$), CD38 ≥30% ($P = 0.645$), and fludarabine-based treatment ($P = 0.867$).

The value of TP53 mutations in predicting chemorefractoriness was also assessed within the group of patients who had been exposed to fludarabine ($n = 88$). In this patient subgroup, multivariate analysis selected TP53 mutations as the sole independent predictor of short time to fludarabine refractoriness (HR, 6.72; $P < 0.001$; Table 2). Other covariates included in the multivariate analysis and not selected as predictors of short time to fludarabine refractoriness were IGHV homology ≥98% ($P = 0.231$), del17p13 ($P = 0.117$), CD38 ≥30% ($P = 0.629$), age >65 years ($P = 0.343$), and lines of treatment >2 ($P = 0.765$).

CLL harboring any type of TP53 disruption share unfavorable biological and clinical features at diagnosis and display similar outcome. Biological and clinical features at diagnosis as well as outcome were compared in CLL groups characterized by (a) TP53 mutations without del17p13 ($n = 10$), (b) del17p13 without TP53 mutations ($n = 16$), (c) concomitance of TP53 mutations and del17p13 ($n = 18$), and (d) absence of TP53 disruption, i.e., absence of both TP53 mutations and del17p13 ($n = 253$). CLL harboring any type of TP53 disruption were biologically and clinically similar. First, compared with CLL without TP53 disruption, cases harboring TP53 mutations only, del17p13 only, or both TP53 mutations and del17p13, uniformly displayed a higher prevalence of unfavorable

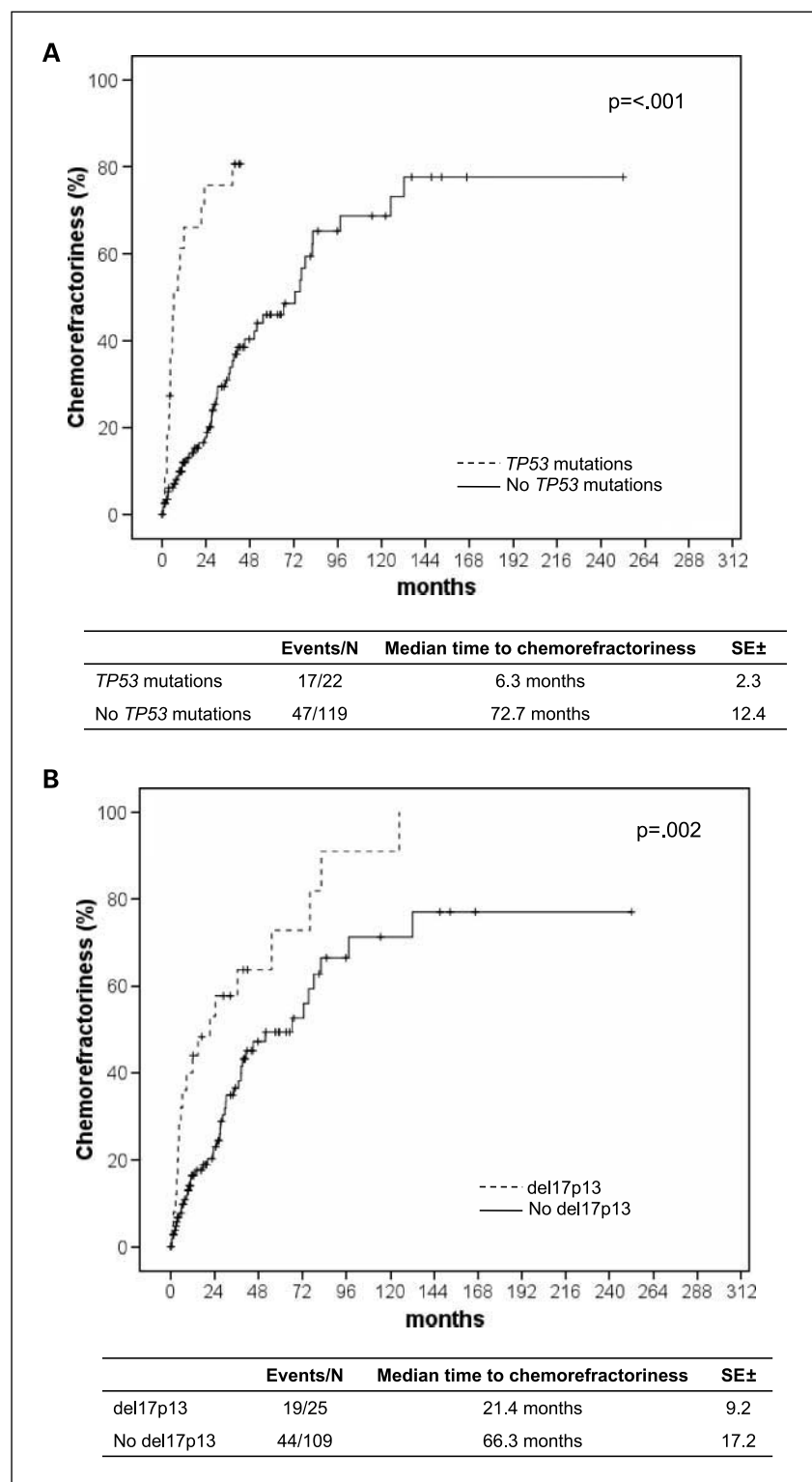


Fig. 2. Kaplan-Meier curves of time to chemorefractoriness according to TP53 mutations and del17p13. Univariate analysis identified TP53 mutations (A) and del17p13 (B) as risk factors of short time to chemorefractoriness.

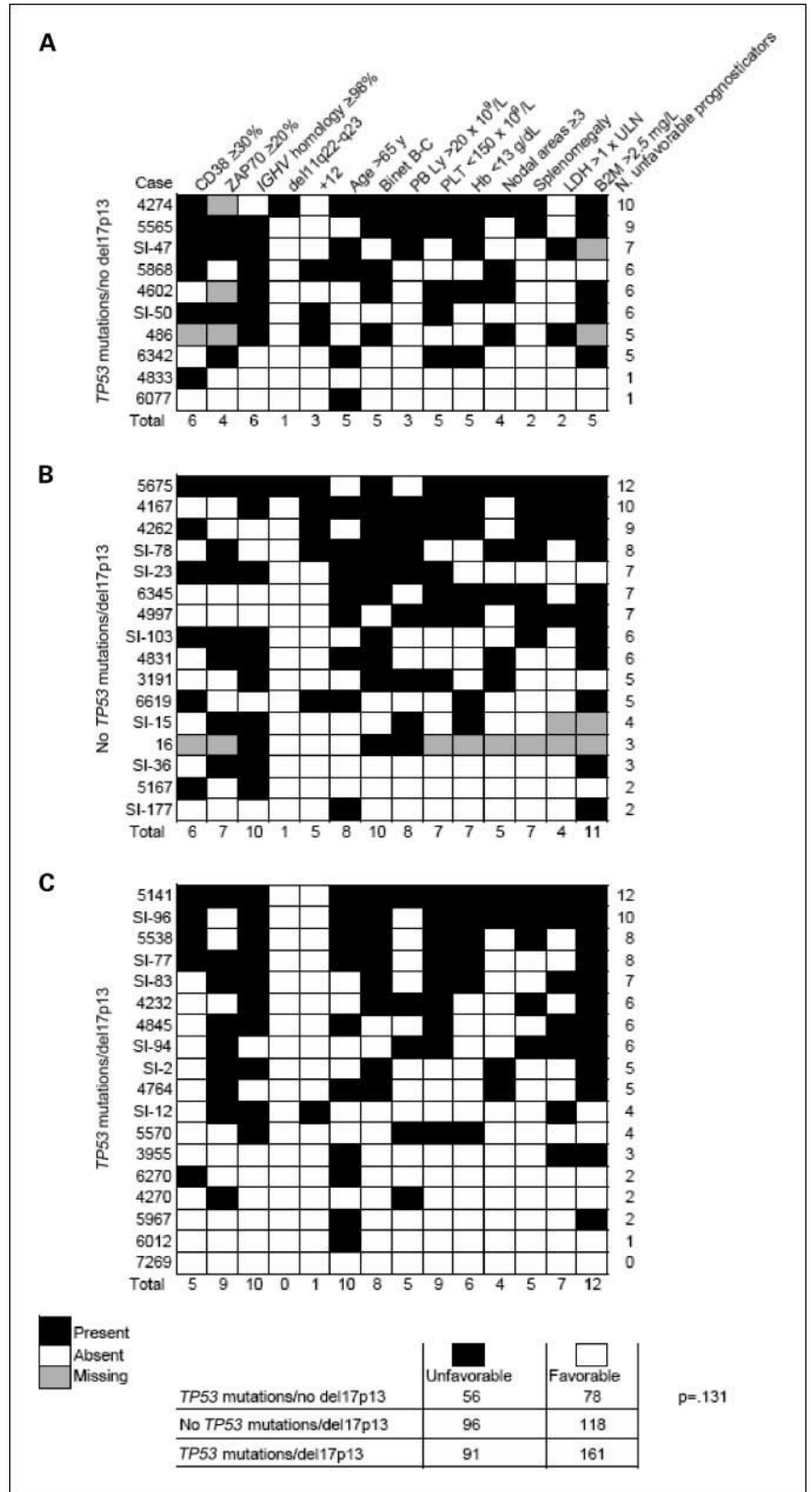
prognosticators, including *IGHV* homology of $\geq 98\%$ (86/248, 34.7% versus 6/10, 60.0% versus 10/16, 62.5% versus 10/18, 55.6%, respectively; $P = 0.021$), advanced Binet stage (49/253, 19.4% versus 5/10, 50.0% versus 10/16, 62.5% versus 8/18, 44.4%, respectively; $P < 0.001$), and elevated $\beta 2$ -microglobulin (93/232, 40.1% versus 5/8, 62.5% versus 10/13, 76.9% versus 10/16, 62.5%, respectively; $P = 0.013$; Fig. 3). The overall

distribution of unfavorable prognosticators did not differ among CLL harboring TP53 mutations only, del17p13 only, or both lesions ($P = 0.131$; Fig. 3). Second, compared with CLL without TP53 disruption, cases harboring TP53 mutations only, del17p13 only, or both lesions uniformly showed an increased risk of progression to symptomatic disease requiring treatment according to National Cancer Institute Working

group criteria ($P < 0.05$ for pairwise comparisons; Fig. 4A; ref. 15). Third, compared with CLL without TP53 disruption, OS was uniformly poor in all CLL subgroups harboring any type of TP53 disruption ($P < 0.05$ for pairwise comparisons; Fig. 4B).

With respect to chemorefractoriness, patients harboring TP53 mutations only and patients harboring both TP53 mutation and del17p13 were characterized by short time to chemorefractoriness compared with patients without any TP53

Fig. 3. Overall distribution of unfavorable prognosticators among CLL harboring TP53 mutations only, del17p13 only, or both lesions. Grids detail the distribution of 14 prognostic markers, i.e., CD38 expression $\geq 30\%$, ZAP70 expression $\geq 20\%$, IGHV homology $\geq 98\%$, presence of del11q22-q23, presence of +12, age >65 years, Binet stage B-C, peripheral blood lymphocytes (PB Ly) $>20 \times 10^9/L$, platelets (PLT) $<150 \times 10^9/L$, hemoglobin (Hb) <13 g/dL, involvement of more than three nodal areas, presence of splenomegaly, LDH $>1 \times$ upper limit of normal (ULN), $\beta 2$ -microglobulin (B2M) >2.5 mg/L, in CLL harboring TP53 mutations only ($n = 10$; A), del17p13 only ($n = 16$; B), or both TP53 mutations and del17p13 ($n = 18$; C). Black boxes, presence of the unfavorable prognostic marker; open boxes, absence of the unfavorable prognostic marker; gray boxes, missing data. χ^2 test compares the distribution of unfavorable prognostic markers among CLL harboring TP53 mutations only, del17p13 only, or both lesions.



alteration or to patients harboring del17p13 only ($P < 0.05$ for pairwise comparisons; Fig. 4C). CLL that at diagnosis harbored del17p13 without *TP53* mutations showed a significantly longer time to chemorefractoriness compared with CLL who at diagnosis carried both del17p13 and *TP53* mutations (median, 55.2 versus 5.5 months, respectively; $P = 0.003$; Fig. 4C). Notably, at diagnosis, the percentage of deleted nuclei was significantly lower in CLL harboring del17p13 only (median, 45.0%; 25th-75th percentiles, 7.3-67.4%) compared with CLL with both del17p13 and *TP53* mutations (median, 66.5%; 25th-75th percentiles, 52.1-77.9%, respectively; $P = 0.020$).

Acquisition of *TP53* mutations is a mechanism of chemorefractoriness in CLL. Clonal evolution leading to acquisition

of *TP53* mutations or deletion may be a mechanism of chemorefractoriness in CLL otherwise devoid of *TP53* abnormalities at diagnosis (6, 25, 26). Sequential samples were available for 14 CLL who had no *TP53* abnormalities at diagnosis and who later developed chemorefractoriness. In these patients, samples obtained at the time of chemorefractoriness showed acquisition of *TP53* abnormalities in 5 of 14 (35.7%) cases. Two patients acquired *TP53* mutations (Arg273His and del13069-13071), two patients acquired del17p13, and one patient acquired both *TP53* mutations (Arg213stop) and del17p13.

The acquisition of additional *TP53* abnormalities at the time of chemorefractoriness was also documented in patients with

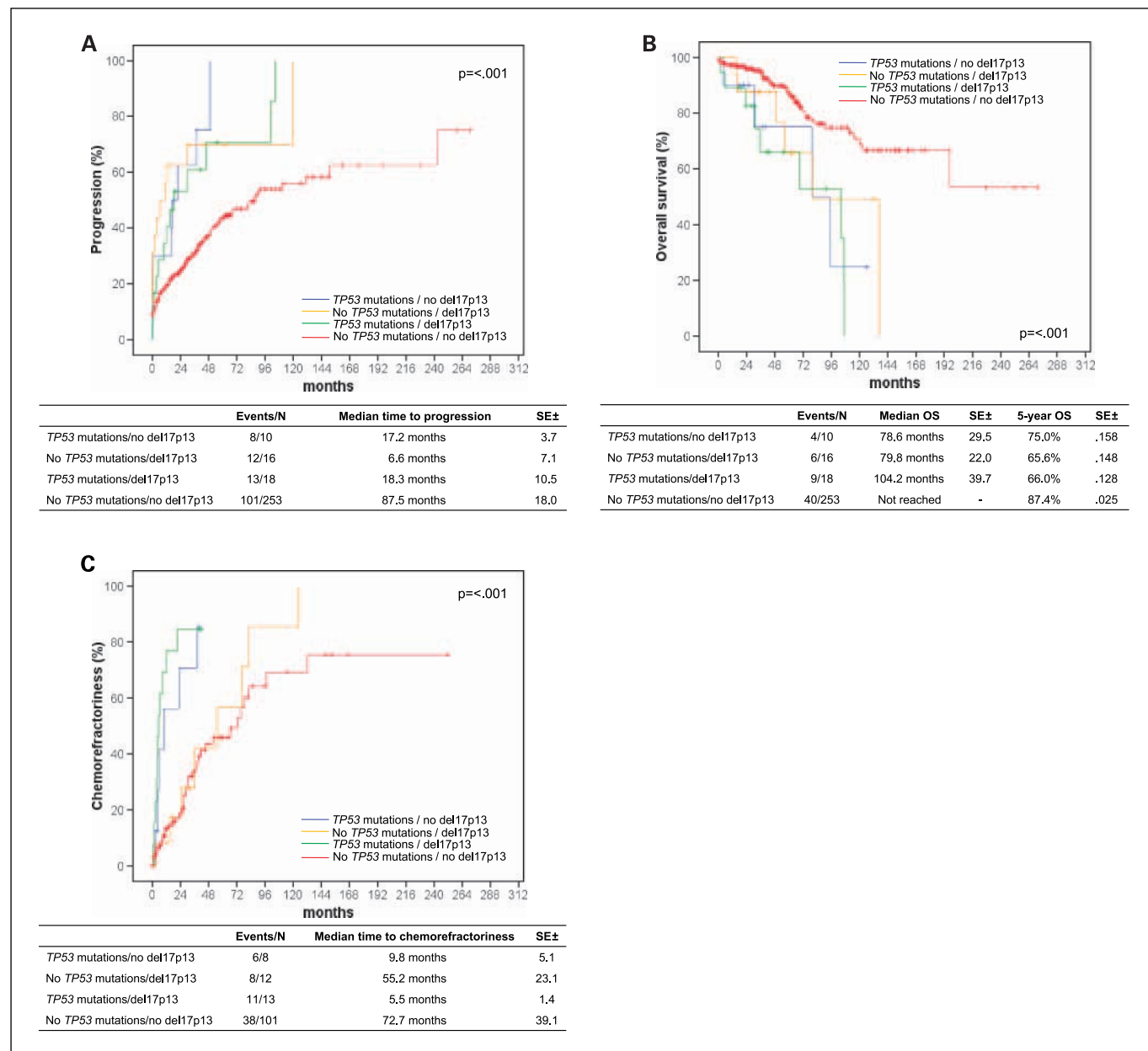


Fig. 4. Kaplan-Meier curves of time to progression, OS, and time to chemorefractoriness in CLL stratified according to *TP53* mutations and del17p13. Compared with CLL without *TP53* disruption, cases harboring *TP53* mutations only, del17p13 only, or both lesions showed short time to progression (A) and short OS (B; $P < 0.05$ for pairwise comparisons). Patients harboring *TP53* mutations only and patients harboring both *TP53* mutation and del17p13 showed short time to chemorefractoriness compared with patients without any *TP53* alteration or with patients harboring del17p13 only (C; $P < 0.05$ for pairwise comparisons).

CLL who at diagnosis harbored a single alteration affecting the *TP53* locus. In fact, three of three patients with CLL who at diagnosis harbored del17p13 but no *TP53* mutations acquired *TP53* mutations (Pro177Ser, Met237Ile, His179Leu) at the time of chemorefractoriness.

Discussion

In this study, we document that (a) the association of *TP53* mutations with short survival and chemorefractoriness is independent of del17p13 and other biological prognosticators; (b) CLL presenting with *TP53* mutations in the absence of del17p13 fare as poorly as CLL carrying del17p13.

Del17p13 is a widely accepted marker of poor CLL outcome and chemorefractoriness (1–6). In contrast, the prognostic relevance of *TP53* mutations in CLL is poorly characterized, and it is currently unknown whether *TP53* mutations carry prognostic value that is independent of del17p13 (10, 11). Here, we extensively screened a large CLL cohort for both *TP53* mutations and del17p13. The approach chosen for mutational analysis encompassed the whole *TP53* mutational hotspot (exons 5-8) and extended to flanking both exon 5' (exons 2-4) and exon 3' (exons 9 and 10). Our results show that *TP53* mutations at CLL diagnosis predict short survival and high risk of chemorefractoriness in a manner that is independent of del17p13 and of other potential confounding covariates, both biological and clinical.

At diagnosis, the distribution of *TP53* mutations and del17p13 in individual patients overlaps only partially. Accordingly, a fraction of CLL with *TP53* disruption present only with *TP53* mutation in the absence of del17p13. These patients are not recognized by routine diagnostic approaches to CLL, which includes screening of del17p13 but not of *TP53* mutations. At diagnosis, patients with *TP53* mutations but no del17p13 represent 3.4% of all CLL. This frequency should not be disregarded because it accounts for 20% of CLL carrying *TP53* disruption at diagnosis, and is superimposable to the frequency of CLL carrying del17p13 in the absence of *TP53* mutations.

CLL harboring *TP53* mutations without del17p13 share similar clinico-biological features and outcome with the more frequent subset of del17p13 CLL. First, CLL harboring *TP53* mutations without del17p13 display a profile of unfavorable prognosticators similar to that of del17p13 CLL, and including *IGHV* homology of $\geq 98\%$, frequent presentation in advanced stage, and association with proliferation markers. Second, CLL harboring *TP53* mutations without del17p13 as well as del17p13 CLL are characterized by a similar risk of progression to symptomatic disease requiring treatment. Third, both CLL harboring *TP53* mutations without del17p13 and del17p13 CLL are characterized by similarly short OS.

The only other study that explored the prognostic relevance of *TP53* mutations in relation to del17p13 failed to identify the negative effect on outcome of *TP53* mutations in the absence of del17p13 (27). Grever et al. (27) identified 28 *TP53* mutations among 228 symptomatic and previously untreated CLL entering the Intergroup Trial E2997. Of the 22 *TP53* mutated patients who were also studied by FISH, 7 cases carried both *TP53* mutations and del17p13, whereas the remaining 15 patients harbored *TP53* mutations but not del17p13 (27). Several key features might explain the discrepancy between our

study and the study by Grever et al. (27). In our cohort, all mutations (a) affected *TP53* coding exons or splicing sites, (b) were confirmed by DNA direct sequencing, and (c) were validated by the IARC *TP53* Mutation Database for CLL⁴ and the UMD *TP53* Mutation Database⁵ (17, 18). In contrast, of the 28 *TP53* sequence variations reported by Grever et al. (27), only 11 were *TP53* mutations confirmed by DNA sequencing, whereas 11 variations were known polymorphisms, 2 were intronic changes, and 4 were detected by denaturing gradient gel electrophoresis but not confirmed by DNA sequencing. Both in CLL and in other types of cancers, the prognostic effect of *TP53* sequence variations other than true mutations is doubtful (28–30). Differences in the choice of clinical end points may further explain the discrepancy between the two studies. In fact, our primary end point was OS, whereas the end point chosen by Grever et al. (27) was progression-free survival after first-line treatment with fludarabine or fludarabine plus cyclophosphamide.

In addition to survival, *TP53* mutations seem to be a powerful predictor of chemorefractoriness. This association is not only documented by actuarial analysis of cases harboring *TP53* mutations at CLL diagnosis, but is also reinforced by the finding that patients acquire *TP53* alterations at the time of chemorefractoriness. *In vitro* models are in support of our clinical observation because *TP53* mutant CLL are refractory *in vitro* to purine analogues and alkylators, and display a defect in activating proapoptotic responses after DNA damage (10, 31–33).

CLL that at diagnosis harbored del17p13 without *TP53* mutations displayed a significantly longer time to chemorefractoriness than CLL with *TP53* mutations already at diagnosis. Notably, CLL with del17p13 only showed a significantly lower percentage of deleted nuclei compared with CLL with both del17p13 and *TP53* mutations. In addition, CLL with del17p13 only acquired *TP53* mutations at chemorefractoriness. Based on these data, clonal evolution with the acquisition of a second *TP53* event may be required for the development of a refractory phenotype.

Our results indicate that an *IGHV* homology of $\geq 98\%$ is also an independent predictor of chemorefractoriness along with *TP53* mutations. An *IGHV* homology of $\geq 98\%$ identifies a subgroup of CLL at higher risk of clonal evolution, and therefore, is more prone to acquire genetic lesions that in turn may favor chemorefractoriness (26, 34). Indeed, in our study all eight cases that acquired *TP53* mutations or deletion at the time of chemorefractoriness displayed *IGHV* homology of $\geq 98\%$.

Besides *TP53* mutations and deletion, other mechanisms of *TP53* dysfunction may be operative in CLL (28, 35–38). These mechanisms may involve the *ATM* and *MDM2* genes that regulate *TP53* function at the protein level. Notably, *ATM* mutations and *MDM2* polymorphisms causing aberrant *MDM2* expression have been shown to harbor prognostic relevance in CLL (35, 37, 38). Because *TP53* mutations and deletion did not recapitulate all cases of chemorefractoriness in our study, an integrated evaluation of all mechanisms of *TP53* dysfunction should be done in future studies.

These results may be relevant for the clinical management of CLL. Because current diagnostic practices screen only for *TP53* genetic abnormalities that are detectable by FISH (7), patients presenting with *TP53* mutations in the absence of del17p13 at

diagnosis are not recognized. More than 95% of *TP53* mutations detected in this study and of those registered in public CLL databases affect exons 5 to 8, whereas mutations at other sites are exceptional (17, 18). On these grounds, mutation analysis of exons 5 to 8 may represent a practical strategy for detecting *TP53* mutations, and should be consid-

ered for a comprehensive prognostic characterization of patients with CLL who are candidates for treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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