

ORIGINAL ARTICLE

The detection of *TP53* mutations in chronic lymphocytic leukemia independently predicts rapid disease progression and is highly correlated with a complex aberrant karyotypeF Dicker¹, H Herholz¹, S Schnittger¹, A Nakao², N Patten², L Wu², W Kern¹, T Haferlach¹ and C Haferlach¹¹MLL Munich Leukemia Laboratory, Munich, Germany and ²Roche Molecular Systems Inc., Pleasanton, CA, USA

The poor prognosis of chronic lymphocytic leukemia (CLL) patients with del (17p) is well established. We analyzed whether mutation of *TP53* on the remaining allele adds to the poor prognosis or whether even *TP53* mutation alone may be an adverse prognostic factor. We analyzed *TP53* mutations in 193 CLL patients by denaturing high performance liquid chromatography in combination with direct DNA sequencing and a *TP53* resequencing research microarray. Mutations were correlated to chromosomal aberrations defined by interphase fluorescent *in situ* hybridization and chromosome banding analyses and to the clinical course of patients. *TP53* mutations were detected in 13.5% (26 of 193) of samples, whereas the incidence of del (17p) was 9.3% (18 of 193). *TP53* mutations were significantly associated with del (17p) (concordance 94%, $P < 0.001$) and complex cytogenetic abnormalities (concordance 50%, $P < 0.001$). Among 147 patients whose clinical data were available, patients with *TP53* abnormalities ($n = 20$) had a significantly decreased time to treatment compared to patients without *TP53* aberration ($P < 0.001$). Median time to treatment was short in patients with isolated *TP53* mutation ($n = 6$, 2.0 months) and in those with del (17p) ($n = 14$, 21.3 months) as compared to patients without *TP53* aberration ($n = 127$, 64.9 months, $P < 0.001$). In multivariate Cox regression analysis, *VH* status, *TP53* mutations and also isolated *TP53* mutations independently predicted rapid disease progression.

Leukemia (2009) 23, 117–124; doi:10.1038/leu.2008.274;
published online 9 October 2008

Keywords: *TP53*; CLL; complex karyotype; prognosis; DHPLC

Introduction

The clinical course of chronic lymphocytic leukemia (CLL) patients is highly variable.¹ Several prognostic factors are used, which predict disease progression at diagnosis and which are helpful in guiding treatment decisions.² Screening for cytogenetic aberrations with a selected panel of fluorescent *in situ* hybridization (FISH) probes has identified important prognostic subgroups in CLL.³ Good prognosis CLL patients with deletions of the long arm of chromosome 13 (del (13q)) as the sole aberration are opposed by patients with deletions of the short arm of chromosome 17 (del (17p)), who show poor prognosis. The tumor suppressor gene *TP53* is located at 17p13.⁴ It regulates a network that senses extracellular stress, oncogene activation, as well as DNA damage and enables the cell to react appropriately to such stimuli either by controlling cell cycle arrest or by inducing apoptosis.⁵ Therefore, loss of *TP53*

function is hypothesized to be at least partially responsible for the poor prognosis of del (17p) CLL patients.⁶ The occurrence of del (17p) is associated with short overall survival,³ short treatment-free interval from diagnosis,⁷ short progression-free survival⁸ and resistance to chemotherapy, including chlorambucil and fludarabine.^{7,9}

Studies analyzing *TP53* mutation/overexpression resulted in similar conclusions, even though controversy exists.¹⁰ However, in many of these studies only small case numbers were tested or studies lacked parallel cytogenetic analysis.^{11–13} A very recent report detected a correlation between *TP53* mutations and short survival albeit with a selected study population.¹⁴ In addition, the methods for analyzing the *TP53* status are highly variable ranging from functional analysis of the *TP53* pathway,¹⁵ a functional yeast assay for p53 function (FASAY),¹⁶ gel-based methods,^{12,17} flow cytometry¹⁸ to immunocytochemistry^{12,19} as well as variations in the number of *TP53* exons, which were analyzed.^{9,13} Therefore, several questions still remain to be addressed. These questions pertain to the 'gold standard' for analysis of the *TP53* status for clinical decision making, but also to the interpretation of different patterns of del (17p) and/or *TP53* mutation for the prognosis of individual patients and to coexisting cytogenetic aberrations. To address these questions, we analyzed a large cohort of CLL patients ($n = 193$) for *TP53* mutations by two independent methods, denaturing high performance liquid chromatography (DHPLC) in combination with direct DNA sequencing and a microarray-based resequencing research assay, and correlated the results to cytogenetics and clinical outcome data.

Patients and methods

Patient cohort

After informed consent, 193 sequential CLL samples that were referred to our institution for diagnostic purpose were included in this study. A diagnosis of CLL was carried out from blood ($n = 150$) or bone marrow ($n = 43$) using the standard criteria.^{20,21} Minimal inclusion criteria were the availability of FISH and *TP53* mutation analyses as described in the Patients and Methods section. Patient characteristics are detailed in Table 1. Chromosome banding analyses as well as analyses of the immunoglobulin heavy-chain variable region (*VH*) gene mutation status were available in 160 and 189 cases, respectively. Clinical outcome data were available in 147 cases with a median follow-up of 58.3 months. The median age at diagnosis was 63.3 years (range: 36.5–84.9) ($n = 180$) with a male to female ratio of 1.88. At diagnosis the distribution of Binet stages A, B and C was 76% ($n = 107$), 18% ($n = 26$) and 6% ($n = 9$), respectively. Approval for this study was obtained from the Bayerische Landesärztekammer (Bavarian Medical Association).

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Received 12 August 2008; revised 2 September 2008; accepted 4 September 2008; published online 9 October 2008

Table 1 Patient characteristics

<i>TP53</i> status	<i>Isolated del(17p)</i>	<i>TP53 mutation with del(17p)</i>	<i>Isolated TP53 mutation</i>	<i>No TP53 aberration</i>
No. of patients (n)	1	17	9	166
<i>Age at diagnosis, years^a</i>				
Median	65	71	63.4	61.9
Range		59.2–81.1	44.9–72.7	36.5–84.9
Male/female	1/0	11/6	7/2	107/59
<i>Binet stage at diagnosis</i>				
A	/	5	3	99
B	1	2	3	20
C	/	3	/	6
Not available	/	7	3	41
<i>Interphase cytogenetics^b</i>				
Del (17p)	1	17	/	0
Del (11q)	/	/	2	22
Del (6q)	/	/	/	13
+12	/	/	/	20
Normal	/	/	3	36
Del (13q) sole	/	/	4	71
IgH	/	/	/	4
<i>Metaphase cytogenetics</i>				
Complex (<i>n</i> = 22)	/	11	/	11
Balanced translocation only (<i>n</i> = 19)	/	1	/	18
Unbalanced translocation only (<i>n</i> = 20)	/	7	1	12
(Balanced + unbalanced) (<i>n</i> = 9)	/	4	/	5
<i>IgVH mutation status</i>				
Unmutated ($\geq 98\%$) (<i>n</i> = 75)	1	13	4	57
Mutated ($< 98\%$) (<i>n</i> = 99)	/	4	4	91
VH3–21 (<i>n</i> = 15)	/	/	1	14
Not available	/	/	/	4
<i>Therapy</i>				
Yes	/	10	5	60
No	1	3	1	68
Not available	/	4	3	39

^aAge at diagnosis was available for 180 patients.^bAccording to the hierarchical classification of Dohner *et al.*³

Analysis of TP53 mutations

Screening for *TP53* mutations was performed from DNA by two independent methods, that is, DHPLC and by a microarray-based resequencing research assay, the AmpliChip p53 Test in development (Roche Molecular Systems Inc., Pleasanton, CA, USA). PCR products for DHPLC analysis of exons 3–9 corresponding to amino acids 26–331 of the 393 amino acids of the human *TP53* gene and the respective exon/intron boundaries were amplified as four separate amplicons with the following oligonucleotides. Exons 3–4 with Ex3-F 5'-aattcatgggactgactttctgtcttctgtc-3' and Ex4-R 5'-gggatacggccaggcattgaa gtctc-3', exons 5–6 with Ex5-F 5'-cttgtgccctgacttcaactctgtctc-3' and Ex6-R 5'-gccactgacaaccaccttaaccctc-3', exon 7 with Ex7-F 5'-gccacaggtctcccaaggc-3' and Ex7-R 5'-tggggcacagcaggcagtg-3' and exons 8–9 with Ex8-F 5'-gtaggacctgattcctta ctgctcttgc-3' and Ex9-R 5'-aactttccactgataagaggccaagac-3'. DHPLC analysis was performed on a WAVE 3500 HT system (Transgenomic Inc., Omaha, NE, USA). Mutations were detected as aberrant elution profile of the PCR product from the cartridge and were verified by direct sequencing using BigDye chemistry (Applied Biosystems, Weiterstadt, Germany). The AmpliChip p53 Test interrogates the coding exons 2–11 of *TP53* and 2 bp of intronic sequence at the exon/intron

boundaries. Exons are amplified in two multiplex PCR reactions from genomic DNA, fragmented, 3' end labeled with a fluorescent dideoxynucleotide and hybridized to the AmpliChip surface. The identity of each nucleotide position of *TP53* is tested by specific oligonucleotides in certain areas (probe cells) of the AmpliChip surface that represent either the wild-type sequence or one of the three possible mismatches or a deletion of the respective nucleotide. The research assay was performed according to the instructions of the manufacturer (Roche Molecular Systems Inc.).

Analysis of cytogenetic aberrations and VH status

Fluorescent *in situ* hybridization, chromosome banding analyses and *VH*-gene sequencing were performed as previously described.^{22,23} The FISH panel included probes for the detection of trisomy 12, *IGH* rearrangements and deletions of 6q21, 11q22.3 (*ATM*), 13q14 (*D13S25* and *D13S319*) and 17p13 (*TP53*) (Abbott, Wiesbaden, Germany). Chromosomes were classified according to the International System for Human Cytogenetic Nomenclature (ISCN).²⁴ A sequence identity cutoff of 98% was used to define an unmutated ($\geq 98\%$) and a mutated ($< 98\%$) *VH*-mutation status.^{25,26}

Statistical analysis

The correlation between *TP53* mutation status and cytogenetic analysis was assessed with the Fisher's exact test. All tests were two-sided and an effect was considered significant at $P < 0.05$. The primary clinical end point was time from diagnosis to treatment (TTT). The differences in TTT were calculated by log-rank statistics and curves were plotted using the Kaplan–Meier estimates. Cox models (dependent variable: TTT) were used for multivariate analyses. The covariates included in these models were those out of the following parameters which significantly correlated with TTT in univariate analysis with FISH normal, FISH del (11q), FISH del (17p), *VH*-mutation status, *TP53* mutations but also isolated *TP53* mutations as dichotomous variables. For statistical analysis SPSS (version 14.0) software (SPSS, Chicago, IL, USA) was used.

Results

TP53 mutation screening and comparison of two methods

In 193 CLL samples *TP53* mutations were screened by DHPLC and the AmpliChip p53 Test in parallel. DHPLC analysis detected 24 mutations in 20 different patients (10.4%), which could be confirmed by DNA sequencing (Table 2). We simultaneously detected two different mutations in 4 of these 20 patients (Table 2, patients 4, 6, 17, 25). In parallel, the same samples were screened with the AmpliChip p53 Test, which analyzes the entire coding region of *TP53* plus two intronic nucleotides at the exon/intron boundaries. The AmpliChip identified a total of 30 mutations in 25 different patients (13%) (Table 2). In three patients, we simultaneously detected two different mutations and three mutations in one patient (Table 2). However, the AmpliChip p53 Test did not detect three mutations that were detected by DHPLC/sequencing (Table 2, patients 1, 4, 7). These were composed of two small deletions (1 bp and 4 bp) and a 1 bp insertion. On the other hand, the AmpliChip p53 Test detected one additional mutation in exon 10 (Table 2, patients 26), an exon that was not analyzed by DHPLC. Furthermore, it detected eight additional mutations not called by direct DNA sequencing (Table 2, patients 7, 11, 12, 13, 16, 17, 18, 21). This discrepancy is most likely due to the lower sensitivity of DNA sequencing (10%) compared to the resequencing array, as the results of the AmpliChip p53 Test matched DHPLC analysis (Table 2).

Taking together the results of both methods, *TP53* mutations were detected in 26 out of the 193 CLL patients (13.5%). The distribution of the mutations within single exons and the affected amino-acid codons are shown in Figure 1.

*Correlation of cytogenetic aberrations and *VH* status to *TP53* mutations*

Del (17p) was significantly associated with *TP53* mutation on the second allele ($P < 0.001$) in 17 of 18 cases with del (17p) (94%) (Figure 2a). Other categories as defined by FISH that carried *TP53* mutations had either no aberrations ('normal karyotype') ($n = 3$, 7.7%), del (13q) sole ($n = 4$, 5.3%) or del (11q) ($n = 2$, 8.3%) (Figure 2a). Notably, two patients carried two *TP53* mutations without concomitant del (17p), suggesting that both alleles might be affected by mutations (patients 4, 6), making a total of 19 patients that were affected by aberrations on both alleles. In another patient (no. 21), del (17p) was detected in only 4 out of 100 interphases, however, the deletion was confirmed on metaphase spreads. A small aberrant peak

was detected in DHPLC analysis in the same patient, indicating that loss of *TP53* has occurred only in a very small subclone. Overall, 9 of the 193 (4.7%) CLL samples carried *TP53* mutations without additional del (17p) as investigated by FISH (clinical and laboratory characteristics are detailed in the Supplementary Table).

Additional poor prognostic risk groups that have been identified recently by chromosome banding analyses are patients with translocations or with complex karyotype, defined by ≥ 3 aberrations.²⁷ A total of 160 CLL samples were analyzed by chromosome banding (Table 1). A complex aberrant karyotype was detected in 22 patients (13.8%) and translocations in 48 patients (30%). These cytogenetic subgroups were in part overlapping. Patients showing translocations were further subdivided into samples with unbalanced ($n = 20$) or balanced translocations ($n = 19$) or samples carrying both ($n = 9$) (Figure 2b). Each cytogenetic subgroup was compared to the remaining patient population. A complex aberrant karyotype and unbalanced translocations were significantly associated with *TP53* mutations (50%, $n = 11$; $P < 0.001$ and 40%, $n = 8$; $P = 0.001$, respectively; Figure 2b, Table 1). Samples with balanced translocations had a low incidence of *TP53* mutations ($n = 1$; 5.3%; $P = 0.472$), whereas samples with unbalanced in combination with balanced translocations had a similar frequency of *TP53* mutations ($n = 4$; 36.4%; $P = 0.015$) compared to unbalanced translocations (Figure 2b). Interestingly, samples with complex aberrant karyotype, which were associated with *TP53* aberration, always had a *TP53* mutation/deletion genotype ($n = 11$). Vice versa, samples with this genotype did not necessarily have a complex aberrant karyotype ($n = 6$ out of 17). However, this finding has to be confirmed in a larger series of clinical samples.

An unmutated *VH*-mutation status was significantly associated with del (17p) ($P = 0.002$) as previously reported.²⁸ Similarly, *TP53* mutation also showed a significant association with an unmutated *VH* status in our cohort with 18 of the 26 *TP53* mutated samples being *VH* unmutated ($P = 0.003$) and to Binet stages B/C with 8 of the 32 Binet stage B/C patients carrying a *TP53* mutation (25%, $P = 0.025$).

*Prognostic impact of *TP53* aberrations*

The time from diagnosis to initial treatment (TTT) was used as the primary study end point for evaluation of the clinical significance of *TP53* aberrations, that is, mutations and/or deletions. In a first step, to validate our cohort with respect to known prognostic markers, we analyzed TTT in relation to the *VH* status. Patients with an unmutated *VH* ($n = 60$) and patients using the *VH3–21* gene ($n = 15$) had a significantly reduced TTT (median of 23.0 and 22.3 months, respectively) compared to a median TTT of 91.8 months in patients with a mutated status ($n = 69$) ($P < 0.001$) (Figure 3a).

The total of patients with *TP53* aberrations, that is, *TP53* mutation and/or del (17p) ($n = 20$), also had a significantly reduced TTT compared to those without these aberrations ($n = 127$) (median of 13.2 months vs a median of 64.9 months, $P < 0.001$) (Figure 3b). The entire patient population ($n = 147$) was further subdivided into patients with del (17p) ($n = 14$) as detected by FISH, which is presently the standard in routine diagnostics, and patients with isolated *TP53* mutations without concomitant del (17p) ($n = 6$) as detected by DHPLC or array. These were compared to patients without such aberrations ($n = 127$) (Figure 3c). The clinical courses of patients with del (17p) and patients with isolated *TP53* mutations were similarly unfavorable with a median TTT of 21.3 and 2 months,

Table 2 Characterization of the TP53-mutation status and of cytogenetic aberration

Patient	Mutation ^a		Exon	Del (17p) interphases	FISH	Karyotyping	Mutation screening			IARC-DB
	Protein	DNA					(DHPLC	+ Seq) ^b	p53 array	
1	D61KfsX60	12105_12108del	4	67/100	Del (17p)	Complex	Positive	Positive	Negative	/
2	P75LfsX47	12148delC	4	54/100	Del (17p)	ND	Positive	Positive	Positive	/
3	S96LfsX26	12211delC	4	92/100	Del (17p)	ND	Positive	Positive	Positive	/
4	P58QfsX64 Q165LfsX20	12097delC 13172_13173insT	4 5	0/100	Normal	ND	Positive Positive	Positive Positive	Positive Negative	/
5	P151S	13130C>T	5	70/100	Del(17p)	Complex	Positive	Positive	Positive	Missense
6	S183X R209KfsX5	13227C>G 13385_13386delAG	5 7	0/100	Normal	Other	Positive Positive	Positive Positive	Positive Positive	/ /
7	E204SfsX42 Y234C	13370delG 14028A>G	5 7	66/100	Del (17p)	ND	Positive Positive	Positive Negative	Negative Positive	/ Missense
8	R213X	13397C>T	6	0/100	Del (11q)	ND	Positive	Positive	Positive	/
9	Y220C	13419A>G	6	74/100	Del (17p)	Complex	Positive	Positive	Positive	Missense
10	Y220C	13419A>G	6	50/100	Del (17p)	Complex	Positive	Positive	Positive	Missense
11	Y236C	14034A>G	7	0/100	Normal	Other	Positive	Negative	Positive	Missense
12	Y234C	14028A>G	7	0/100	Del (13q)	Other	Positive	Negative	Positive	Missense
13	N239I	14043A>T	7	0/100	Del (13q)	Other	Positive	Negative	Positive	Missense
14	R248Q	14070G>A	7	0/100	Del (13q)	Other	Positive	Positive	Positive	Missense
15	Y236C	14034A>G	7	95/100	Del (17p)	Complex	Positive	Positive	Positive	Missense
16	R248Q R273H	14070G>A 14487G>A	7 8	30/100	Del (17p)	ND	Positive Positive	Negative Positive	Positive Positive	Missense Missense
17	R248Q C275F Y327X	14070G>A 14493G>T 14742T>G	7 8 9	30/100	Del (17p)	Other	Positive Positive n.a.	Positive Positive Negative	Positive Positive Positive	Missense Missense /
18	E286K	14525G>A	8	0/100	Del (11q)	Other	Positive	Negative	Positive	Missense
19	R290C	14537C>T	8	0/100	Del (13q)	Other	Positive	Positive	Positive	Missense
20	C275Y	14493G>A	8	89/100	Del (17p)	Complex	Positive	Positive	Positive	Missense
21	G262V	14454G>T	8	4/100 ^c	Del (17p)	Complex	Positive	Negative	Positive	Missense
22	R273C	14486C>T	8	18/100	Del (17p)	Complex	Positive	Positive	Positive	Missense
23	R280K	14508G>A	8	45/100	Del (17p)	Other	Positive	Positive	Positive	Missense
24	R273L	14487G>T	8	83/100	Del (17p)	Complex	Positive	Positive	Positive	Missense
25	A276P R282P	14495G>C 14514G>C	8 8	78/100	Del (17p)	Complex	Positive Positive	Positive Positive	Positive Positive	Missense Missense
26	R337C	17587C>T	10	94/100	Del (17p)	Complex	ND	ND	Positive	Missense

Abbreviations: DHPLC, denaturing high performance liquid chromatography; NA, not applicable; ND, not determined; Seq, DNA sequencing. Bold and italic entries are mutations that were detected by DHPLC or chip.

^aTP53 sequence according to X54156 at GenBank1114.

^bDHPLC-positive samples were analyzed by DNA sequencing. Due to the lower sensitivity of DNA sequencing compared to DHPLC analysis, DNA sequencing could not confirm all DHPLC-positive results.

^cDel (17p) was confirmed on metaphases (patient 21).

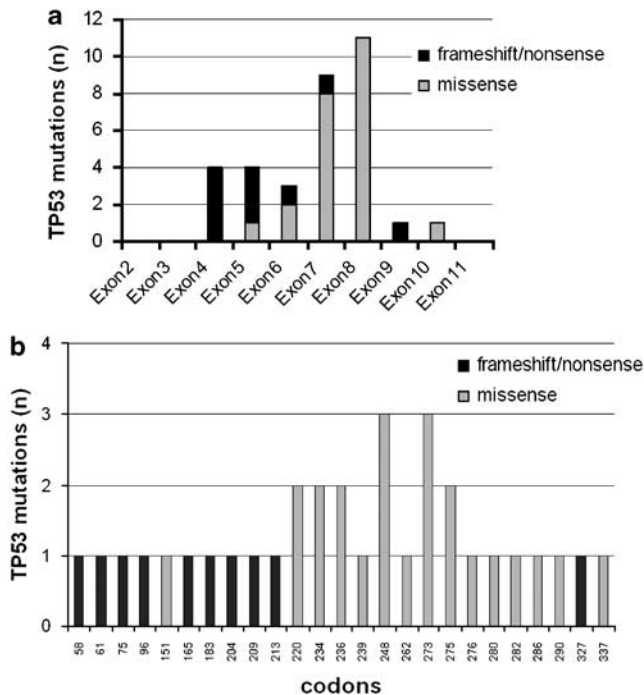


Figure 1 Incidence of *TP53* mutations in the different, coding *TP53* exons. (a) The exon or (b) the codon number is indicated at the x axes of the bar graph, whereas the number of *TP53* mutations (*n*) is indicated at the y axes. Missense mutations are indicated in gray, frameshift or nonsense mutations are indicated in black. Exon boundaries are bordered by the following amino acids (aa): exon 4 (aa33–126), exon 5 (aa126–187), exon 6 (aa187–224), exon 7 (aa225–261), exon 8 (aa261–307), exon 9 (aa307–331), exon 10 (aa332–367). The DNA-binding domain and the tetramerization domain are located between aa102–292 and 325–356, respectively.

respectively, compared to 64.9 months in patients without aberrations ($P < 0.001$). As it is still a matter of debate, if *TP53* aberrations are induced²⁹ or selected for by chemotherapy, we excluded patients with earlier therapy before the time of study inclusion resulting in a patient population of 125 cases. With this selected patient population, results were very similar compared to the unselected population (data not shown).

Different classes of FISH aberrations (normal, del (13q), trisomy 12, del (11q) and del (17p)), *VH*-mutation status and isolated *TP53* mutations were included in univariate Cox regression analyses (Table 3). The study population ($n = 143$) included six samples with isolated *TP53* mutations, where clinical data were available (see Supplementary Table). All prognostic parameters that were identified in univariate analyses to carry significant prognostic impact (isolated *TP53* mutations, *VH* status, FISH normal, FISH del (11q) and FISH del (17p)) were further evaluated in multivariate analyses (Table 4). As already reported by others,^{26,30} the *VH*-mutation status carried independent prognostic impact with regard to TTT ($P < 0.001$) (Table 4). Importantly, isolated *TP53* mutations also proved to be of independent prognostic value ($P < 0.001$), whereas del (17p) in this analysis of our study population lost its independent prognostic value ($P = 0.153$) (Table 4). In a similar analysis with the entire group of *TP53* mutations ($n = 19$) instead of only isolated mutations, again *VH* status as well as *TP53* mutations had independent prognostic value ($P < 0.001$ and < 0.001 , respectively). When Binet stage (stage A vs B/C) was included into the multivariate analyses, the study population was reduced to only 113 patients including 5 patients with an isolated *TP53*

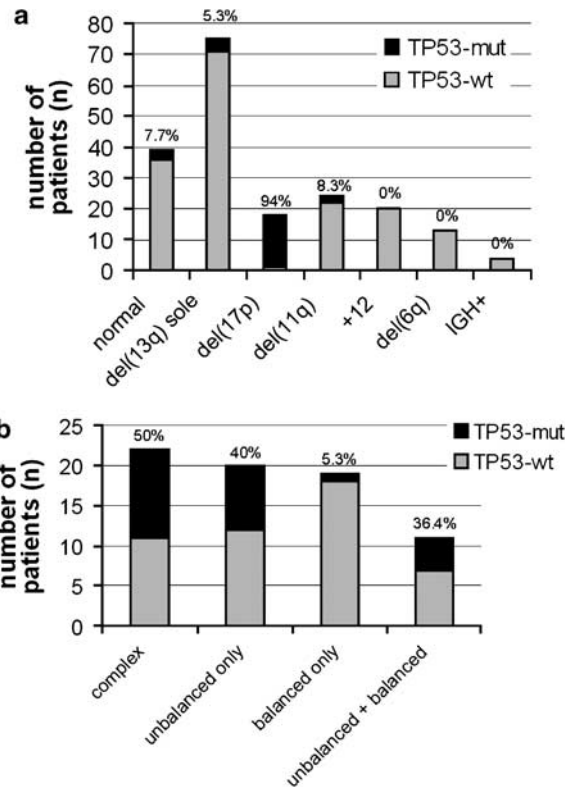


Figure 2 Correlation of cytogenetic aberration with the number of patients affected by *TP53* mutations. The different fluorescent *in situ* hybridization (a) or cytogenetic categories (b) are indicated at the bottom of the bar graphs. The number of patients in each category is given by the columns. *TP53*-mutated patients are indicated in black. Percent *TP53*-mutated patients per category are indicated.

mutation. However, the parameters *VH*-mutation status as well as isolated *TP53* mutations retained their independent, significantly shorter TTT ($P = 0.04$ and 0.027 , respectively) (not shown).

Discussion

In this study, we analyzed the incidence of *TP53* mutations in CLL in the context of cytogenetic aberrations, defined by FISH and also by chromosome banding analysis, and further correlated these data to *VH*-mutational status and time from diagnosis to first treatment.

Therefore, we performed the *TP53* mutation screening on two independent techniques, that is, by DHPLC/sequencing or with the AmpliChip p53 Test. Even if monitoring of p53 protein expression by flow¹⁸ or immunocytochemistry¹⁹ seems easier to perform and may also identify patients with poor prognosis,¹⁹ these methods almost certainly underestimate the true incidence of *TP53* aberrations.¹² This was clearly proven also by our study, as splice site, frameshift or nonsense mutations, which occurred with an incidence of 30% (10/33) in our cohort (Table 2), are very likely to remain undetected by methods relying on p53 overexpression only. Furthermore, DHPLC compared to gel-based screening^{12,17} is a fast method, which offers the possibility of standardization and automatization, and is at least as or even more sensitive in mutation detection.^{31,32} In our study, the sensitivity of detecting single-nucleotide substitutions was similar between DHPLC screening and the AmpliChip p53 Test

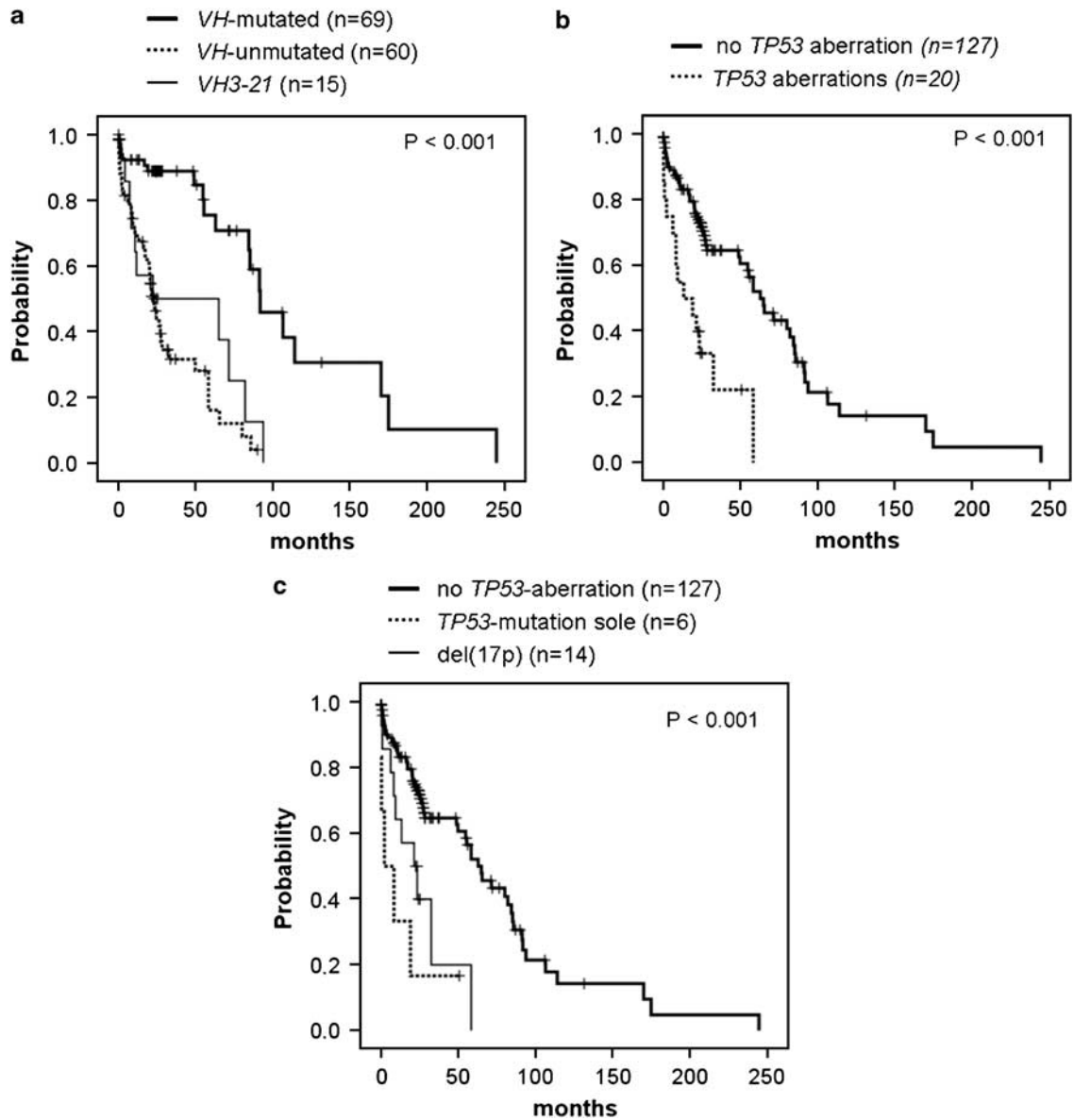


Figure 3 Kaplan-Meier curves of time from diagnosis to treatment (TTT) according to the VH status (a), TP53 aberrations (TP53 mutation and/or del (17p)) (b) or isolated TP53 mutation or del (17p) (c).

Table 3 Univariate analyses according to time from diagnosis to treatment (n = 143)

Parameter	Hazard ratio	95% CI	P-value
Isolated TP53 mutation	4.437	1.756–11.210	0.002
VH unmutated	3.933	2.354–6.572	<0.001
FISH normal	0.448	0.239–0.840	0.012
FISH del (13q)	0.925	0.570–1.502	0.753
FISH +12	0.779	0.283–2.146	0.629
FISH del (11q)	2.477	1.403–4.374	0.002
FISH del (17p)	2.486	1.249–4.946	0.009

Abbreviations: CI, confidence interval; FISH, fluorescent *in situ* hybridization.

Table 4 Multivariate analyses according to time from diagnosis to treatment (n = 143)

Parameter	Hazard ratio	95% CI	P-value
Isolated TP53 mutation	6461	2.409–17.330	<0.001
VH unmutated	3444	1.918–6.182	<0.001
FISH normal	0.589	0.297–1.136	0.112
FISH del (11q)	1128	0.574–2.216	0.726
FISH del (17p)	1733	0.816–3.682	0.153

Abbreviations: CI, confidence interval; FISH, fluorescent *in situ* hybridization.

as both methods showed overlapping results. The AmpliChip p53 Test, however, is not designed to detect deletions larger than one nucleotide or insertions and consequently did not

detect a 4 bp deletion and a 1 bp insertion, but also a 1 bp deletion (Table 2). On the other hand, the chip is more sensitive in calling mutations in small clones, it detected eight single base changes which were missed by direct sequencing (Table 2).

The location of *TP53* mutations ranged from exons 4 to 10 (Figure 1). In our cohort, no mutations were found in exons 2, 3 and 11, which was also observed by others³³ and which is in agreement with recommendations for mutation screening in the IARC database.³⁴ Most *TP53* mutations (82%, $n=27$) in our study were located inside the DNA-binding domain, which is defined between amino acids 102–292,³⁵ whereas four frameshift mutations leading to premature stop codons were located N-terminal to the DNA-binding domain and two mutations (one missense, one nonsense) in the C-terminal tetramerization domain.³⁶ Most mutations inside the DNA-binding domain were missense (81%, $n=22$), which included mutations in the hot spot positions R248 ($n=3$) and R273 ($n=3$).³⁴ In addition, many of the codons of missense mutations described in this study (P151, Y220, Y234, Y236, N239, G262, R273, A276, E286) have been shown in a yeast reporter system to be transcriptionally defective in activating different p53 response elements.³⁷ The difference between patients being affected by missense or frameshift/nonsense mutations of *TP53* is presently not clear. Early studies have shown gain of function of certain missense mutations resulting in an enhanced tumorigenic potential compared to the cells lacking p53.³⁸ Thus, the clinical impact of any specific mutation has to be clarified in larger series and was not in the focus of our approach.

Del (17p) as detected by FISH may serve not only as a predictor of poor prognosis, but also as a predictor of poor response to conventional purine-analog based regimen⁷ and might influence treatment decisions to consider alternative treatments such as alemtuzumab and bone marrow transplantation.^{39,40} Considerable interest exists to confirm *TP53* as the gene on the short arm of chromosome 17, which is responsible for poor prognosis. This assumption is challenged by the fact that chromosome banding analysis of del (17p) mostly resulted in the detection of loss of the entire short arm by translocations or isochromosome formation,^{22,41} indicating that additional genes might be involved. Few CLL studies have carried out parallel assessment of 17p deletion and *TP53* mutation.^{9,11,14,42} One of these studies detected 8 cases with mutations among the 11 cases with parallel del (17p) (73%).⁴² Similarly, the other studies also showed a trend toward a significant correlation between *TP53* mutation and deletion, even though, case numbers were small or cytogenetic analyses was available on only part of the study population.^{9,11} Our study is in line with these previous observations with a concordance rate of 94% between del (17p) and *TP53* mutation, underscoring a strong selective pressure for loss of *TP53* function on both alleles and confirming the role of *TP53* aberration for the poor prognosis of CLL patients.

Another consequence of loss of *TP53* function is genetic instability,⁴³ and consistently, karyotype analysis in our study indicated a significant correlation to a complex aberrant karyotype (≥ 3 chromosomal aberrations) (Figure 2b). Mutations in genes in addition to *TP53* might be the driving forces for such chromosomal defects; however, a defective p53-mediated cell cycle checkpoint might allow such cells to continue proliferation.⁴³ The significant correlation of *TP53* mutations with unbalanced translocations is likely to be associated with defective DNA-damage checkpoints (Figure 2b). Unrepaired DNA double-strand breaks during mitosis are possibly the reason for recombination events leading to chromosomal rearrangements.⁴⁴ Other mechanisms for genetic instability might be active in samples with complex aberrant karyotype lacking *TP53* aberrations ($n=11$). At least three of these samples carried an *ATM* deletion, a gene that senses DNA double-strand breaks.⁴⁵ Patients with complex aberrant karyotype or translo-

cations have been associated with poor prognosis previously.²⁷ Therefore, *TP53* mutations might be one of the factors that confer poor prognosis in these patients.

In our study, a significant number of samples ($n=9$; 4.7%) carried a *TP53* mutation without del (17p), and the clinical consequences for these patients have not been evaluated separately in previous studies.^{7,9,13,19} A recent report correlated genetic and molecular marker, including *TP53* mutations, with response to fludarabine or fludarabine/cyclophosphamide treatment and progression-free survival.¹⁰ *TP53* mutations in contrast to del (17p) in the latter study did not attain significant prognostic value, however, apparently patients with non-silent but also with silent *TP53* mutations were considered for inclusion into the *TP53* mutated group.¹⁰ In this study, time from diagnosis to initial treatment (TTT) was selected as the primary clinical end point. In our study population, *TP53* aberrations ($n=20$) (*TP53* mutation and/or del (17p)) predicted earlier TTT compared to patients without *TP53* mutations ($n=127$) (Figure 3b). We can show that in the absence of del (17p), CLL patients with *TP53* mutation alone require early treatment, not significantly different from patients with del (17p) (Figure 3c). This important finding was not different in a selected patient population that had not received therapy before analysis of *TP53* mutation status (data not shown). The relevance of screening *TP53* mutations was further underscored by its independent prognostic impact in Cox regression analysis (Table 4). Here, patients with isolated *TP53* mutations appeared to be clinically equally poor compared to patients with *TP53* mutation plus del (17p), which is in line with a very recent publication.¹⁴ The prognostic impact of isolated del (17p) could not be evaluated separately due to the small case number ($n=1$). Therefore, screening for *TP53* mutations adds prognostic information for individual CLL patients. The prognostic impact of *TP53* mutations on clinical parameters in addition to TTT has to be clarified in larger prospective studies. Also in the era of compounds, which target the p53 pathway in tumor cells and which require an intact p53,⁴⁶ screening for *TP53* mutations might help to identify patient not eligible for therapy.

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