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RESEARCH ARTICLE

TP53 mutation analysis in chronic lymphocytic leukemia: comparison of different detection methods

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Abstract *TP53* gene defects represent a strong adverse prognostic factor for patient survival and treatment resistance in chronic lymphocytic leukemia (CLL). Although various methods for *TP53* mutation analysis have been reported, none of them allow the identification of all occurring sequence variants, and the most suitable methodology is still being discussed. The aim of this study was to determine the limitations of commonly used methods for *TP53* mutation examination in CLL and propose an optimal approach for their detection. We examined 182 CLL patients enriched for high-risk cases using denaturing high-performance liquid chromatography (DHPLC), functional analysis of separated alleles in yeast (FASAY), and the AmpliChip p53 Research Test in parallel. The presence of *T53* gene mutations was also evaluated using ultra-deep next generation sequencing (NGS) in 69 patients. In total, 79 *TP53* mutations in 57 (31 %) patients were found; among them, missense substitutions predominated (68 % of detected mutations). Comparing the efficacy of the methods used, DHPLC and FASAY both combined with direct Sanger sequencing achieved the best results, identifying 95 % and 93 % of *TP53*-mutated patients. Nevertheless, we showed that in CLL patients carrying low-proportion *TP53* mutation, the more sensitive approach, e.g., ultra-deep NGS, might be more appropriate. *TP53* gene analysis using DHPLC or FASAY is a suitable approach for mutation detection. Ultra-deep NGS has the potential to overcome shortcomings of methods currently used, allows the detection of minor proportion mutations, and represents thus a promising methodology for near future.

Keywords *TP53* gene · Mutation analysis · Detection methods · Chronic lymphocytic leukemia

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Introduction

Chronic lymphocytic leukemia (CLL), the most frequently diagnosed adult leukemia in Western countries, is characterized by considerable biological and clinical heterogeneity. CLL prognosis is based mainly on clinical staging, detection of recurrent cytogenetic aberrations [del (13) (q14), +12, del (11) (q22), del (17) (p13.1)] and immunoglobulin heavy-chain variable region gene (*IGHV*) mutation status determination [1–4]. Moreover, mutations in the tumor suppressor gene *TP53* have been associated with substantially shortened overall survival, short time to treatment, and resistance to fludarabine-based therapies [5–10], even in cases with low proportion of *TP53*-mutated subclones [11]. In addition, the

cancer cells carrying *TP53* mutations might be selected during CLL course [12, 13]; therefore, a *TP53* mutational status examination is recommended before each therapy [14].

TP53 gene encodes the key transcriptional factor acting in response to genotoxic stress. Defects in p53 pathway impair correct DNA repair and apoptosis and result in increased genomic instability and abnormal cell proliferation. In various cancer types, p53 protein is most often inactivated due to mutation in the *TP53* gene accompanied by deletion of the other allele (locus 17p.13.1). In CLL, *TP53* defects have been observed in ~5–10 % patients at diagnosis, with an increased frequency in progressive and chemo-refractory disease. Moreover, the negative clinical impact of sole *TP53* mutations [in the absence of del (17) (p13.1)] has been shown in CLL [9, 10]; these sole *TP53* mutations cannot be recognized by routinely used cytogenetic examination of 17p locus.

TP53 mutation analysis is becoming routine in research institutes and medical centers examining CLL patients; nevertheless, the methods applied and detection conditions vary considerably, and therefore, somewhat inconsistent results might be achieved [5–8, 14, 15]. To reduce the interlaboratory variability, the European Research Initiative on CLL (ERIC) has recently published criteria for *TP53* analysis together with recommendation of the most suitable methodologies for *TP53* mutation detection. These include (i) direct Sanger sequencing, (ii) denaturing high-performance liquid chromatography (DHPLC) or single-strand conformation analysis, (iii) functional analysis of separated alleles in yeast (FASAY), (iv) chip-based arrays, and (v) next generation sequencing (NGS) [14].

Since each of these methodologies shows some limitations and the optimal approach is still a matter of intensive discussion in CLL community, we present here a follow-up report to the ERIC recommendations [14] comparing the detection efficacy of DHPLC, FASAY, and the AmpliChip p53 Research Test. All three methods were employed in parallel to examine a cohort of 182 CLL patients; additionally, in 69 patients, the results were complemented by ultra-deep NGS.

Materials and methods

Patients' cohort

The cohort examined included 182 CLL patients monitored and treated at the Department of Internal Medicine-Hematology and Oncology, University Hospital Brno in agreement with National Cancer Institute-sponsored Working Group guidelines [16, 17]. In order to collect a sufficient number of clinically relevant mutations, the patients with unfavorable prognosis including advanced disease stage, unmutated *IGHV* gene status, and/or del (17p) were preferentially selected (Table 1). The peripheral blood samples and buccal swabs were obtained between the years 2004–2014 under written informed consent according to the Declaration of Helsinki and University Hospital Brno Ethics Committee regulations.

Table 1 Clinical and biological characterization of 182 CLL patients

Characteristics	Number of patients
Median age at diagnosis (n=180)	61 (33–78)
Sex (n=182)	
Male	115 (63 %)
Female	67 (37 %)
Stage at the time of *TP53* analysis (n=178)	
Rai 0	26 (15 %)
Rai I/II	55 (31 %)
Rai III/IV	97 (54 %)
IGHV gene mutation status (n=182; 98 % germline sequence identity cut-off)	
Mutated	33 (18 %)
Unmutated	140 (77 %)
IGHV3-21 (irrespective of the germline identity)	8 (5 %)
Hierarchical cytogenetics at the time of *TP53* analysis (I-FISH; n=179)[a]	
del (13) (q14) sole	47 (26 %)
+12	22 (12 %)
del (11) (q22.3)	63 (35 %)
del (17) (p13.1)	32 (18 %)
Normal	32 (18 %)
Therapy before *TP53* analysis (n=180); chemotherapy and/or immunotherapy	
No	87 (48 %)
Yes	93 (52 %)

I-FISH interphase fluorescence in situ hybridization, *IGHV* immunoglobulin heavy-chain variable region

[a] More than one cytogenetic aberration present in 37 % (66/179) of patients

TP53 mutation analysis using a combination of detection methods

Mutations in the *TP53* gene were examined using FASAY, DHPLC, and the AmpliChip p53 Research Test in 182 CLL patients in parallel. Since both FASAY and DHPLC represent highly sensitive but only prescreening detection methods [10, 14, 18], mutations in the samples manifesting *TP53* variations in the analyses were confirmed by conventional Sanger sequencing. Moreover, in 69 patients examined (32 *TP53*-wild-type and 37 *TP53*-mutated), the results were also verified using ultra-deep NGS. The nucleic acid samples were isolated from peripheral blood mononuclear cells (Histopaque®-1077; Sigma Aldrich) and/or separated CD19+ cells of the patients analyzed (Ficoll-Paque PLUS; GE Healthcare, complemented with RosetteSep™ kits; Stemcell™ Technologies). In the two cases harboring *TP53* mutations without functional impact, as

assessed by FASAY and the International Agency for Research on Cancer (IARC) *TP53* database [19], the corresponding buccal swabs were sequenced to verify the somatic origin of the mutations.

Yeast functional analysis

The experimental setting of FASAY with appropriate modifications for CLL patients including details about the optimization process was previously described [10, 18]. Briefly, the cDNA amplified using proof-reading Pfu DNA polymerase (exons 4–10; Agilent Technologies, Inc.) was transformed into ADE2^{-} LEU2^{-} modified yeast strain together with an open reading frame expression vector containing *ADE2* gene under the control of p53-responsive promoter and selectable LEU marker. In a medium deficient for adenine, transcripts coding for transcriptionally inactive p53 give rise to easily distinguishable growth-restricted red colonies in contrast to white ones harboring wild-type p53. The background of FASAY was determined at 10 % of red colonies covering the alterations caused by sample processing, low input RNA quality, or PCR amplification errors [18]. The presence of *TP53* mutations was confirmed using direct Sanger sequencing of corresponding DNA isolated from the red colonies (Big Dye chemistry; Applied Biosystems).

DHPLC and DNA sequencing

Mutational screening using DHPLC (Varian Inc.) encompassed exons 4–9 and bordering intron *TP53* gene sequences. DNA samples were amplified according to the IARC recommendations [19] using proof-reading Optimase Polymerase (Transgenomic) to minimize artificial mismatches. Each sample was prepared in duplicate; one aliquot was mixed with 25 % of *TP53*-wild-type DNA to efficiently recognize fully selected mutations. After renaturation, the samples harboring sequence variations were distinguished from wild-type PCR products based on the different column-retention time upon partially denaturing conditions (available upon request).

Among 728 PCR products tested using DHPLC, 154 *TP53* wild-type amplicons and all 172 *TP53*-aberrant amplicons were sequenced on an ABI PRISM® 3700 Genetic Analyzer (Applied Biosystems) and compared to the reference sequences [GenBank: NG_017013.2; NC_000017: c7531642-7512445]. The functional effect of the *TP53* variants was verified using the IARC *TP53* database [19] to distinguish polymorphisms and functional variants from deleterious mutations. The estimated threshold for direct Sanger sequencing was ~10 % of mutated DNA (Mutation Surveyor DNA Variant Analysis Software; Softgenetics®).

AmpliChip p53 Research Test

TP53 gene analysis using the AmpliChip p53 Research Test, a microarray-based assay, was performed according to the manufacturer s instructions in cooperation with Roche Molecular Systems Inc. Pleasanton, CA. Briefly, exons 2–11 of the *TP53* gene (including splicing sites) were amplified in two master mix reactions. The generated PCR products were fragmented with DNase I, end-labeled using TdT enzyme, and then loaded onto the Amplichip p53 microarray containing oligonucleotide probes corresponding to the wild-type and mutant *TP53* sequences. Hybridization, staining, and washing procedures were performed using a GeneChip® Fluidics Station 450 (Affymetrix); the microarrays were scanned on an Affymetrix GeneChip® Scanner 3000 7G. Data were analyzed, and results were evaluated by Roche Molecular Systems Inc. Pleasanton, CA.

The AmpliChip p53 Research Test was designed to detect single base pair substitutions and single nucleotide deletions in the whole coding region and splice-sites of the *TP53* gene [8, 20]. The declared threshold for the *TP53*-specific resequencing microarray was ~25 % of mutated DNA.

Ultra-deep NGS

Ultra-deep next generation sequencing of *TP53* exons 4–10 and corresponding splicing sites was performed on a MiSeq platform (Illumina). The experimental design and reaction conditions followed the manufacturer recommendations. Briefly, DNA samples were amplified using proof-reading Q5® High-Fidelity DNA Polymerase (New England Biolabs; primers' sequences in Online Resource, Table 1S). Each PCR product was purified separately with Agencourt® AMPure® XP (Beckman Coulter) and quantified using a Qubit® dsDNA HS Assay Kit (Life Technologies). The purified amplicons were mixed at equimolar ratios according to the number of molecules and diluted to a final amount of 1 ng. The indexed paired-end library was prepared with a Nextera XT DNA Sample Preparation Kit (Illumina) and sequenced using a MiSeq Reagent Kit v2 (300 cycles; Illumina). The achieved median per base coverage was 27,538 reads (range 2096–88, 976).

To call the sequence variants, an in-house bioinformatics pipeline was established [21]. Sequencing reads were preprocessed and aligned to the reference sequence [GenBank: GRCh37.p9] using CLC Genomic Workbench version 6.0.4 (CLC Bio). Variant calling was performed using the deepSNV R-package [21] with a statistical approach applying the shearwater algorithm to compute Bayes classifiers based on a betabinomial model [22, 23]. From the reproducibility test, we disclosed that we were able to reliably distinguish point mismatches and ≥2 nucleotide insertions/deletions (indels) at the level of 0.2 % of variant reads, and 1-nucleotide

deletions at the level of 1 % of variant reads as these may be artificially introduced during the sequencing and alignment process [21].

Cytogenetic analysis using I-FISH and SNP-based arrays

The del (17) (p13.1) was examined together with other common cytogenetic aberrations [i.e., del (13) (q14), +12 and del (11) (q22.3)] in 179/182 patients investigated using interphase fluorescence in situ hybridization (I-FISH) with locus-specific probes (Abbott Molecular Inc.) [10]. In 15 of the *TP53*-mutated patients, *TP53* gene abnormalities or copy-neutral loss of heterozygozity (cn-LOH) were analyzed using Cytogenetics Whole-Genome 2.7 M Array (n=9; Affymetrix) and CytoScan® High Density Array (n=6; Affymetrix). The cytogenetic array analyses were performed according to the manufacturer s instructions.

Results

Frequency and localization of *TP53* mutations

The presence of *TP53* mutations was examined in all patients (n=182) using three methods in parallel: FASAY and DHPLC both complemented with direct Sanger sequencing and the AmpliChip p53 Research Test. Moreover, ultra-deep NGS was performed in 32 *TP53* wild-type and 37 *TP53*-mutated patients. Only *TP53* mutations identified using at least two different methodologies were considered as true variants.

In total, 79 *TP53* mutations in 31 % (57/182) of patients were detected; multiple *TP53* mutations were found in 17 of them (2–3 mutations per patient; Online Resource, Table 2S). The silent mutations and intron variants (with the exception of splice-site mutations) were not further considered since their prognostic impact is mostly unknown and it is generally supposed to be minimal. The observed high *TP53* mutation frequency attributes to the unfavorable profile of analyzed cohort (Table 1).

Among all *TP53* mutations detected, missense substitutions predominated (n=54; 68 %), followed by frameshift mutations (n=12; 15 %), splice-site mutations (n=6; 8 %), in-frame deletions (n=4; 5 %), and nonsense mutations (n=3; 4 %). The occurrence of the mutations identified was limited to exons 4–10 (between amino acids 109–346; Fig. 1). In exons 2, 3, and 11, which were analyzed using the AmpliChip p53 Research Test only, no *TP53* mutation was detected confirming that they are rare in these loci [8, 24].

The most frequently mutated regions included the well-known codons 234 (n=3), 248 (n=3), 249 (n=3), 273 (n=4), 277 (n=4) [19, 24], and unexpectedly also the splice site c.673-2 nt (n=4) leading to the aberrant splicing of exon 7. Similar to the *TP53* mutation pattern in other cancers [19], 95 % of mutations (n=75) occurred within the p53 DNA-binding domain (codons 101–300 including splice-sites). The remaining 4 mutations were found outside this region, namely in exons 9 (n=3) and 10 (n=1) (Figs. 1 and 2). Interestingly, we observed a high proportion of non-missense *TP53* mutations in exon 6 (6/12 mutations detected in this region); in contrast, in exon 7, only missense substitutions were found (n=15) (Fig. 2).

Association between *TP53* mutations and del (17) (p13.1)

Using I-FISH, del (17) (p13.1) was observed in 18 % (32/179) of patients examined; in one patient (no. 287), the del (17) (p13.1) was selected later during the disease course in addition to the already present *TP53* mutation. The del (17) (p13.1) significantly correlated with the presence of *TP53* mutations (P<0.0001); 81 % (26/32) of patients with del (17) (p13.1) harbored *TP53* mutations, and reciprocally, in 46 % (26/56) of patients with *TP53* mutations, the del (17) (p13.1) was found. Overall, the concurrent presence of *TP53* mutations and del (17) (p13.1) was observed in 42 % (26/62) of patients with *TP53* defects (Fig. 3).

Within the group harboring *TP53* mutation (s) but not del (17) (p13.1) (n=30), two or more different *TP53* mutations were found in 33 % (10/30) of patients. Using FASAY, which is based on subcloning, we were able to confirm that in all cases with two *TP53* mutations, they were present on separate alleles. However, unless single-cell analysis is used, it is not possible to decide whether the mutations are present in different subclones or if both *TP53* alleles in one cell are affected. In the remaining 20 patients without del (17) (p13.1), a single *TP53* mutation was observed (Fig. 3).

To investigate the occurrence of cn-LOH or additional defects in the *TP53* gene, the SNP-based arrays were performed in available samples from 15/30 patients with sole *TP53* mutation (s). Using this approach, the deletion of exon 11 in the *TP53* gene was detected in one patient (patient no. 373). The cn-LOH was observed in 6/30 *TP53*-mutated patients without del (17) (p13.1) and corresponded to the DNA sequencing results (mutation proportion >50 % of DNA determined using Mutation Surveyor DNA Variant Analysis Software; Online Resource, Table 2S).

Screening of *TP53* mutations using FASAY

Mutation analysis using FASAY detected 78 % (62/79) of *TP53* mutations in 93 % (53/57) of *TP53*-mutated patients. The causal *TP53* mutations leading to transcriptional p53-inactivation were identified by DNA sequencing from FASAY-generated red colonies. We particularly noticed a decreased ability of FASAY to detect truncating *TP53* mutations presumably causing nonsense-mediated mRNA decay

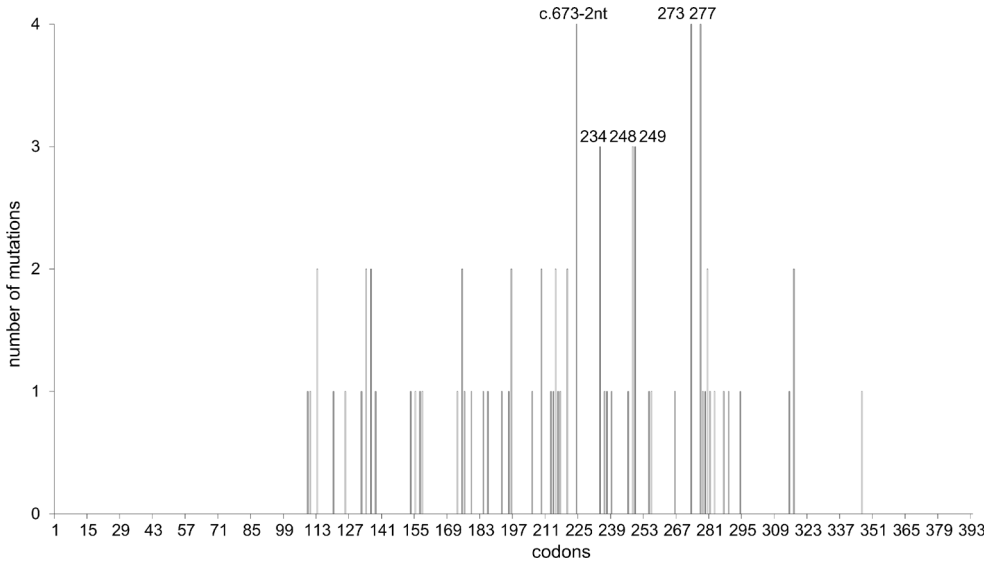

Fig. 1 Localization and frequency of 79 identified *TP53* mutations according to the codon distribution including splice-sites. In case of deletions and insertions, only the first affected codon was considered. The region examined using all three detection methods spanned codons 35–331. The most frequently mutated loci are shown

(nonsense, frameshift, and splice-site mutations); only 9/21 of these mutations were found (Table 2). However, 10/12 undetected truncating mutations accompanied other *TP53* mutation (s) identified by FASAY; therefore, the patients carrying these mutations were recognized as *TP53*-mutated (Online Resource, Table 2S). Moreover, owing to the high detection efficacy of FASAY followed by cloned DNA sequencing (background ~10 % of red colonies), all 4 in-frame deletions and 49/54 missense substitutions were detected (Table 2). Among five unidentified missense substitutions, two of them (p.Q317K, p.R283C) were *TP53* variants with preserved transcriptional activity (patient no. 194, 868; Online Resource, Table 2S). In line with its functional read-out, these mutations were present in white FASAY colonies and were not therefore recognized in the original analysis. In these cases, the paired nontumor DNA was analyzed using Sanger sequencing, and the germinal origin of the mutations was proven. The other three undetected missense substitutions were present in a small proportion of cancer cells (<10 % of DNA) and accompanied dominant *TP53* mutation (s) recognized by FASAY (patient no. 480, 653, 414; Online Resource, Table 2S).

Analysis of *TP53* mutations using DHPLC and DNA sequencing

Mutational screening based on DHPLC combined with direct Sanger sequencing detected 87 % (69/79) of *TP53* mutations in 95 % (54/57) of *TP53*-mutated patients; in 95 DHPLC-

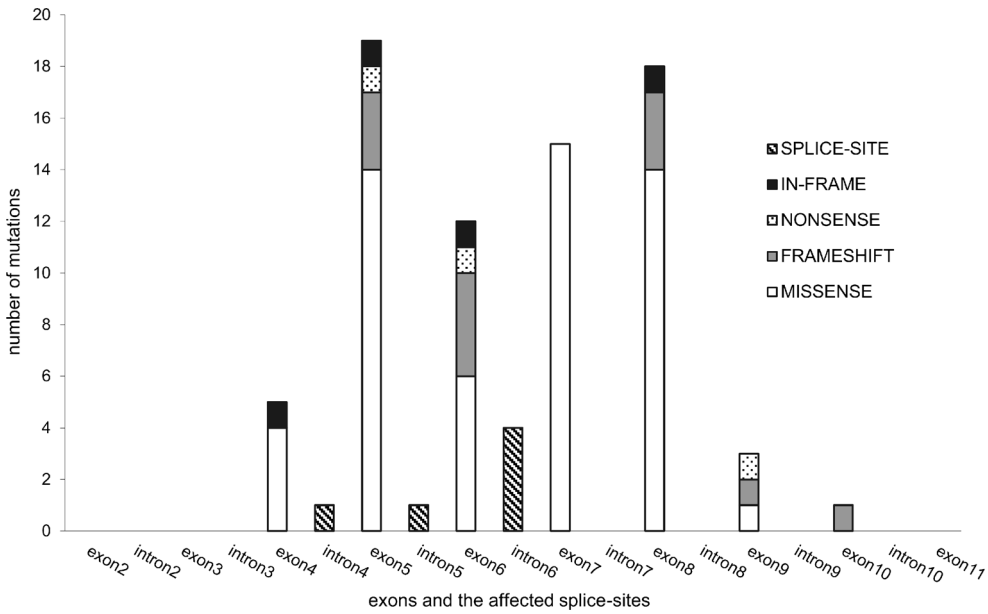

Fig. 2 Localization and frequency of 79 detected *TP53* mutations according to the exon-intron distribution. In case of introns, only splice-site mutations were considered. The region examined using all detection methods spanned exons 4–9

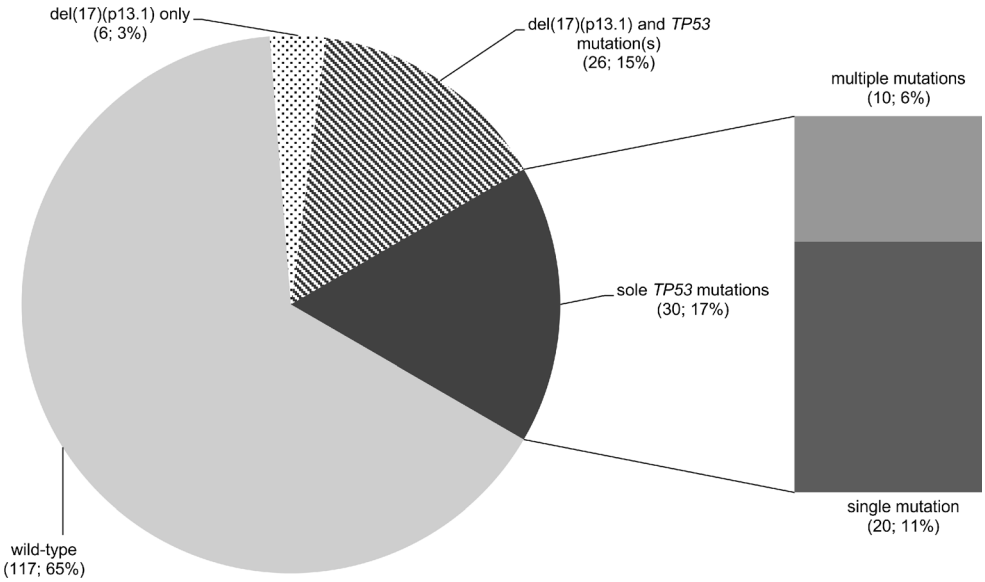

Fig. 3 Characterization of CLL patients according to the presence and type of *TP53* defects. Concurrent data on the 17p13.1 locus deletion (examined by I-FISH) and *TP53* mutational status (investigated using FASAY, DHPLC, and the AmpliChip p53 Research Test) were available in 179 of 182 analyzed patients. The presence of cn-LOH assessed by SNP-based arrays and direct sequencing and the occurrence of additional low-level *TP53* mutations detected using ultra-deep NGS only are not shown

abnormal PCR products, annotated *TP53* polymorphisms were found (p.P36P; p.R72P; p.R213R). DHPLC supplemented with Sanger sequencing identified all frameshift mutations, in-frame deletions, and nonsense mutations (n=19; Table 2). On the other hand, this approach not detected 9/54 missense substitutions and 1/6 splice-site mutations most likely due to the lower detection efficacy of conventional Sanger sequencing utilized as a confirmatory method (~10 % of mutated DNA; Online Resource, Table 2S). None of the 154 amplicons determined as *TP53* wild-type in DHPLC analysis carried *TP53* variations according to Sanger sequencing.

Detection of *TP53* mutations using the AmpliChip p53 Research Test

The AmpliChip p53 Research Test was developed as a specific array for *TP53* mutation detection and was performed in collaboration with Roche Molecular Systems Inc. In total, 71 % (56/79) of *TP53* mutations in 81 % (46/57) of *TP53*-mutated patients were detected using this array (Table 2). Considering these results in the context of the detection limits declared (threshold 25 % of mutated DNA, recognition of single base pair substitutions and deletions), the AmpliChip p53 Research Test correctly identified 91 % (31/34) of the mutations in 91 % (29/32) of *TP53*-mutated patients. The *TP53* mutations undetected included single nucleotide deletions (n=2; patient no. 91, 399) and one missense substitution (n=1; patient no. 6007). Interestingly, among the remaining 45 *TP53* mutations that were present under the declared limit of the AmpliChip p53 Research Test detection, this approach recognized 25 of them (19 missense substitutions, 3 splice-site mutations, 2 multiple nucleotide deletions, and 1 duplication; Online Resource, Table 2S). The spectrum and frequency of *TP53* polymorphisms identified using this method were exactly the same as in the DHPLC analysis combined with DNA sequencing (see above).

Confirming the *TP53* mutations' presence using ultra-deep NGS

To verify the results obtained using FASAY, DHPLC, and/or the AmpliChip p53 Research Test, ultra-deep NGS on the Illumina MiSeq platform was performed in 69 patients. In

Table 2 Efficiency of *TP53* mutation identification (n=79) using different detection methods

Mutation type	Total number	FASAY	DHPLC+sequencing	AmpliChip p53 Test
Missense mutations	54	49 (91 %)	45 (83 %)	46 (85 %)
Non-missense mutations	25	13 (52 %)	24 (96 %)	10 (40 %)
- frameshift mutations	12	8	12	3
- splice-site mutations	6	1	5	4
- in-frame deletions	4	4	4	0
- nonsense mutations	3	0	3	3
Total number of mutations	79	62 (78 %)	69 (87 %)	56 (71 %)
Total number of mutated patients	57	53 (93 %)	54 (95 %)	46 (81 %)

DHPLC denaturing high-performance liquid chromatography, *FASAY* functional analysis of separated alleles in yeast

total, 58 *TP53* mutations found by the three tested methods in 37 patients were examined, and the presence of all these mutations was confirmed using ultra-deep NGS. Moreover, in 24 of these patients, additional low-level *TP53* mutations occurring below 10 % of DNA were detected (Online Resource, Table 2S) [21].

In the 32 *TP53* wild-type patients analyzed, the ultra-deep NGS revealed very low level *TP53* mutations occurring under the detection limit of all tested methods in 8 of them (mutation proportion 0.2–3.8 %). In 5 of these patients, an expansion of the particular *TP53* mutations was noticed using FASAY in the available follow-up sample (data not shown) [21].

Minor proportion *TP53* mutations

Among 79 *TP53* mutations detected, 16 low-level mutations present in <10 % of DNA were observed in 26 % (15/57) of *TP53*-mutated patients using a combination of FASAY, DHPLC, and the AmpliChip p53 Research Test. As assessed by ultra-deep NGS and FISH analyses, 2 of these patients carried the low-level *TP53* mutation as a single abnormality; in 13 patients, the minor proportion *TP53* mutations were accompanied by other *TP53* defects [*TP53* mutation (s) and/or del (17p)]. The low-level *TP53* mutations identified included 12 missense substitutions, 1 frameshift mutation, 2 splice-site mutations, and 1 in-frame deletion. The presence of all these mutations was independently confirmed by ultra-deep NGS (Online Resource, Table 2S).

Comparing the efficiency of the methods applied with respect to the minor proportion mutations' detection, FASAY achieved the best results, identifying 63 % (10/16) of the low-level mutations, and importantly, all 15 of the patients examined were recognized as *TP53*-mutated (Table 3). DHPLC combined with Sanger sequencing showed a similar detection efficacy to the AmpliChip p53 Research Test in these cases (recognition of 50 and 50 % minor proportion *TP53* mutations and identification 12 and 10 patients as *TP53*-mutated, respectively; Table 3).

Discussion

The independent poor prognostic impact of *TP53* defects (gene deletion and/or mutations) on disease course and patient prognosis has been repeatedly proven in CLL [5–8, 25, 26]. The proper assessment of *TP53* status is especially crucial in CLL therapy management as patients with *TP53* defects should be considered for allogeneic stem cell transplantation or enrollment to clinical trials testing new perspective drugs [2–4]. Regarding this, the detection limit of a particular methodology is important as the clinical impact of small *TP53*-mutated subclones has very recently been proven in CLL [11, 21]. Besides standardized investigation of *TP53* allele deletion at 17p locus using interphase FISH, various approaches with different detection efficacy have been applied to examine *TP53* mutations [5–8, 14, 15]. Since an optimal methodology has been intensively discussed in the CLL community during the last few years because of *TP53* defects' heterogeneity [14], we report here a single-center study reflecting on the limitations of the major methods recently used in *TP53* mutation analysis.

To explore the disparity in *TP53* mutation detection, we examined a cohort of 182 CLL patients enriched for unfavorable cases using FASAY, DHPLC, and the AmpliChip p53 Research Test in parallel. In total, 79 *TP53* mutations were identified and verified in 57 (31 %) patients. The localization and spectrum of the detected mutations were comparable to other CLL cohorts, including, e.g., 2 nt deletion in codon 209 [19, 24]. Of note, we observed quite a high frequency of *TP53* mutations in the splice-site c.673-2 nt (n=4). Mutations in this position lead to the aberrant splicing of *TP53* exon 7 and have been sporadically observed in solid tumors, e.g., in lung or urinary tract cancers [19, 27].

TP53 gene analysis generally focuses on exons 4–10 and the corresponding splice-sites, where up to 95 % of mutations is supposed to be detected [19, 24]. In CLL, the presence of *TP53* mutations in exons 2, 3, and 11 has been shown to be extremely rare, and their examination is not recommended in

Table 3 Efficiency of minor proportion *TP53* mutations (n= 16) identification using different detection methods

Mutation type	Total number	FASAY	DHPLC + sequencing	AmpliChip p53 Test
Missense mutations	12	9 (75 %)	5 (42 %)	7 (58 %)
Non-missense mutations	4	1 (25 %)	3 (75 %)	1 (25 %)
- frameshift mutations	1	0	1	0
- splice-site mutations	2	0	1	1
- in-frame deletions	1	1	1	0
Total number of mutations	16	10 (63 %)	8 (50 %)	8 (50 %)
Total number of mutated patients	15	15 (100 %)	12 (80 %)	10 (67 %)

routine practice [8, 14]. No mutation in this region was also detected in our patients using the AmpliChip p53 Research Test.

In accordance with the reported results [7, 9, 10, 24], the presence of *TP53* mutations was significantly associated with del (17) (p13.1) in our cohort ($P<0.0001$). However, in 30 *TP53*-defected patients, the sole *TP53* mutation (s) occurred without del(17) (p13.1). In addition to the dominant *TP53* mutations detected, minor proportion *TP53* mutations (present in <10 % of DNA) were observed in 16 *TP53*-mutated patients using FASAY, DHPLC, and the AmpliChip p53 Research Test. Nevertheless, due to the low mutation load, the detection of minor proportion *TP53* mutations might be problematic using conventional methods, and a more sensitive approach, e.g., next generation sequencing should be applied in these cases [11, 21].

In addition to somatic mutations leading to p53 dysfunction, two germinal missense *TP53* substitutions with a preserved p53 transcriptional-activation function [19] were identified in two patients using DHPLC and the AmpliChip p53 Research Test. Q317K, the first of the *TP53* mutations identified, has been sporadically observed in some solid tumors [19]. The second, R283C, has already been detected in a CLL patient, who had acquired the mutation after allogeneic stem cell transplantation from the sibling and the mutation was proven to be present in the donor DNA without any effect on donor health [28]. The impact of these mutations and other functional *TP53* variants including germinal mutations on CLL prognosis is highly improbable, and their reporting without the appropriate description might be misleading.

TP53 mutational analysis using FASAY, DHPLC, and the AmpliChip p53 Research Test revealed 93 %, 95 %, and 81 % of *TP53*-mutated patients, respectively. Despite the high detection efficacy, some mutations may escape identification using any of these methods [14] due to the *TP53* mutations' heterogeneity. Direct Sanger sequencing is considered to be the gold standard for *TP53* mutation analysis [14]. Nevertheless, the low sensitivity of this approach, generally reaching ~20 % of mutated DNA and a relatively high direct cost per sample, has resulted in a combination of Sanger sequencing with prescreening methods. Among them, DHPLC and FASAY are frequently used for *TP53* testing in CLL as they are able to detect even subclonal *TP53* mutations [8, 10, 14].

Considering the limits of the methods examined, FASAY detected fewer truncating *TP53* mutations leading to nonsense-mediated mRNA decay than DHPLC and the AmpliChip p53 Research Test. On the other hand, only FASAY identified all 15 patients carrying minor proportion *TP53* mutations. FASAY can also recognize patients with multiple low-level *TP53* mutations in the absence of any dominant *TP53* mutation as the overall percentage of red colonies equals the sum of all present mutations [29]. In addition, only deleterious *TP53* mutations leading to the transcriptional p53-inactivation and therefore a presumed negative impact on disease prognosis are identified using FASAY [10, 18].

DHPLC complemented with DNA sequencing showed the best efficacy from the three methods tested for non-missense *TP53* mutations detection. However, utilizing direct Sanger sequencing as a confirmatory method in DHPLC analysis somewhat reduces the sensitivity of this approach [8]. Of note, using prescreening methods such as DHPLC and FASAY, the detection limit reached strongly depends on the input sample quality and the precise experimental setting optimization [8, 10, 18], which may be relatively laborious. Since false positive or false negative results caused by inadequate sample preparation and processing might be produced by these methods, it is recommended to perform external data validation in cooperation with centers experienced in *TP53* mutational analysis [14].

In contrast, it is not necessary to optimize any ready-to-use method for *TP53* analysis such as the AmpliChip p53 Research Test, which has recently been tested especially in leukemia and breast cancer patients [8, 15, 20]. However, this approach fails to recognize insertions and multiple nucleotide deletions as the array is not designed for their detection [15].

In view of the reported limitations in each method tested [14], utilizing NGS seems to be a promising approach for *TP53* mutation detection. In our study, the ultra-deep NGS confirmed the presence of all *TP53* mutations assessed; of note, this methodology enabled the detection of additional low-level *TP53* mutations in CLL cells, which might be the subject of further clonal selection [11–13, 21, 30, 31]. However, using NGS, determining the noise level is important as errors might be induced during PCR amplification of the samples [11, 21]. Despite the obvious benefits such as sensitivity and time efficiency, the expensive laboratory equipment and high direct costs together with the necessity of a background in bioinformatics still represent the major limitations of wider NGS utilization in *TP53* mutation analysis [14].

Since *TP53* gene analysis itself cannot resolve all CLL treatment-refractory cases [32], a lot of functional tests have been developed to study ATM-p53 DNA-damage-response pathway impairment [33]. Among them, the most clinically applicable assays include the following: (i) p53-target genes' expression analysis (e.g., *CDKN1A*, *MIR34A*) using real-time polymerase chain reaction [32, 34]; (ii) p53-regulated apoptotic gene expression examination (e.g., BAX, PUMA, CD95) using reverse transcription-multiplex ligation-dependent probe amplification [35]; (iii) p53-p21 protein level detection using Western blot or fluorescence activated cell sorting [34–36]. However, in the case of low sample purity or when a small proportion of *TP53*-mutated clones or *TP53* truncating mutations are present, the sensitivity of the functional tests have been reported to be considerably reduced [35, 36];

therefore, their routine utilization is rather questionable [14, 35].

We conclude that DHPLC or FASAY followed by mutation presence confirmation and identification using direct Sanger sequencing represent suitable methods for *TP53* mutation analysis, since both approaches detected *TP53* mutations in more than 90 % of *TP53*-mutated patients. Owing to the adverse prognostic impact of *TP53* mutations on CLL prognosis, it is strongly recommended to check the functionality of mutations and the frequency of their occurrence using the IARC *TP53* database [19] or The *TP53* mutant Web site [27]. In the near future, next generation sequencing including commercially available assays is likely to become a standard approach for *TP53* mutation analysis as its costs are supposed to decrease and appropriate statistical tools are being developed and widely tested.

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