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The spectrum of subclonal *TP53* mutations in chronic lymphocytic leukemia: A next generation sequencing retrospective study

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Abstract

Chronic lymphocytic leukemia (CLL) is a hematological disorder with complex clinical and biological behavior. *TP53* mutational status and cytogenetic assessment of the deletion of the corresponding locus (17p13.1) are considered the most relevant biomarkers associated with pharmaco-predictive response, chemo-refractoriness, and worse prognosis in CLL patients. The implementation of Next Generation Sequencing (NGS) methodologies in the clinical laboratory allows for comprehensively analyzing the *TP53* gene and detecting mutations with allele frequencies ≤10%, that is, "subclonal mutations". We retrospectively studied *TP53* gene mutational status by NGS in 220 samples from 171 CLL patients. *TP53* mutations were found in 60/220 (27.3%) samples and 47/171 (27.5%) patients. Interestingly, subclonal mutations could be detected in 31/60 samples (51.7%) corresponding to 25 patients (25/47, 53.2%). We identified 44 distinct subclonal *TP53* mutations

Giovanna Cutrona and Mariella Dono contributed equally to this article.

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clustered in the central DNA-binding domain of p53 protein (exons 5–8, codons 133–286). Missense mutations were predominant (>80%), whereas indels, nonsense, and splice site variants were less represented. All subclonal *TP53* variants but one [p.(Pro191fs)] were already described in NCI and/or Seshat databases as "damaging" and/or "probably damaging" mutations (38/44, 86% and 6/44, 14%, respectively). Longitudinal samples were available for 37 patients. Almost half of them displayed at least one *TP53* mutant subclone, which could be alone (4/16, 25%) or concomitant with other *TP53* mutant clonal ones (12/16, 75%); different patterns of mutational dynamics overtimes were documented. In conclusion, utilization of NGS in our "real-life" cohort of CLL patients demonstrated an elevated frequency of subclonal *TP53* mutations. This finding indicates the need for precisely identifying these mutations during disease since the clones carrying them may become predominant and be responsible for therapy failures.

KEYWORDS

chronic lymphocytic leukemia, heterogeneity, Next Generation Sequencing, subclonal mutations, *TP53*

1 | INTRODUCTION

Chronic lymphocytic leukemia (CLL) is characterized by the monoclonal expansion of mature CD5-, CD23- positive B cells and a highly heterogeneous clinical course reflecting the complexity of genomic alterations.[1–3] The mutational status of the tumor suppressor gene *TP53* is a crucial example of this complexity since it may represent an evolution over time and can be related to selective therapeutic pressure. Notably, *TP53* mutational status is both a prognostic and pharmaco-predictive factor that must be evaluated before the first treatment, and that requires subsequent evaluations to monitor the therapeutic effect and assess the onset of possible resistance mechanisms.[4–8]

Since *TP53* alterations are associated with most chemoimmunotherapy-resistant patients[9] and confer a shorter progression-free survival even in the era of new drugs,[10] accurate analysis of *TP53* mutations and deletion of the corresponding chromosomal locus 17p13.1 (del(17p)) should be incorporated in a diagnostic workup algorithm. There is a generalized consensus, supported by experimental and clinical evidence, that the identification of "clonal" *TP53* mutations (detectable by traditional methods, such as Sanger sequencing) is no longer sufficient since "subclonal" *TP53* mutations, usually considered below Sanger's Limit of Detection (LoD, ≤10% of mutated allele), are very frequently found in CLL patients and could prevail in the course of the disease.[11–17]

Next Generation Sequencing (NGS), with the use of targeted panels, allows deep (LoD ≥1%) or ultra-deep (LoD <1%) sequencing, leading to the detection of mutations far below Sanger's LoD; in addition, NGS methods allow to investigate the exonic regions simultaneously, including the sequencing of all splice sites and part of introns. Thus, NGS approaches could be beneficial for fully genotyping somatic mutations in CLL.[18–26]

This work aimed to retrospectively evaluate the presence of subclonal *TP53* mutations in a real-life cohort of CLL patients by using an NGS targeted panel and following their dynamic molecular evolution throughout the disease utilizing available longitudinal samples.

2 | MATERIALS AND METHODS

2.1 | Specimens

Two hundred twenty samples from 171 patients were investigated. Samples from these patients were sent to the laboratory for routine molecular testing (2003–2020) and were tested for *TP53* mutations by Sanger sequencing (>10% mutated allele cut-off). The overall characteristics of patients to be considered in a "real-life" setting are reported in Table 1.

The genomic DNA was isolated from peripheral blood or bone marrow mononuclear cells, enriched by Ficoll Hypaque separation. At least 50% of CD5-, CD19-positive cells in each sample were assessed by staining the samples with CD19 PE-CY7 (B.D. Biosciences, Inc, San Jose, CA) and CD5 APC (B.D. Biosciences) and by flow cytometry, using a FACS Canto flow-cytometer. Variant allele frequencies (VAFs) from downstream NGS analysis were normalized according to the tumor cell fraction determined in each sample.

The electronic medical records of all patients diagnosed with CLL were reviewed since their use was available in clinical practice.

The *IGHV* mutational status was tested on tumor DNA collected at diagnosis and was assessed according to the European Research Infrastructure Consortium (ERIC) guidelines.[27] Sequences that differed by more than 2% from their corresponding germ-line sequence were mutated.[27–29]

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TABLE 1 Clinical characteristics of chronic lymphocytic leukemia (CLL) patients

Characteristic	Cases (overall population n = 171)
Sex	
Male	107 (62.6%)
Female	64 (37.4%)
Longitudinal patients	37 (21.6%)
Therapy (*n* = 87)[a]	
Yes	*n* = 38 (43.7%) chemo-immunotherapy (bendamustine, rituximab, fludarabine, cyclophosphamide, chlorambucil), BCR signaling inhibitors (ibrutinib/idelalisib), BCL2 signaling inhibitors (venetoclax)
No	49 (56.3%)
IGHV gene status (*n* = 148)[a]	
Mutated	76/148 (51.4%)
Unmutated	72/148 (48.6%)
del(17p) (*n* = 144)[a]	
Present	40/144 (27.8%)
Absent	104/144 (72.2%)

Abbreviations: BCR, B-cell receptor signaling; del(17p), deletion of chromosomal locus 17p13.1; IGHV, immunoglobulin heavy chain.
[a]Number of patients with available data.

Cytogenetic abnormalities involving deletions at chromosomal loci 11q22.3, 13q14.3, 17p13.1, and trisomy 12 were evaluated by FISH using the protocol provided by the manufacturer of the multi-color probes LSI D13S25/LSI 13q34, LSIp53/CEP17, LSI ATM/CEP11, and CEP12. A total of 200 interphase nuclei were analyzed for each probe set.[30]

2.2 | NGS testing

TP53 mutational status was investigated using the community panel Ion AmpliSeq TP53 panel (ThermoFisher Scientific), a standalone assay covering 100% of exonic positions (2–11 exons) and ±30 bp exon-intron boundaries. This amplicon-based NGS panel comprises 24 primer pairs distributed into two pools already employed in our previous study.[31]

Briefly, eight libraries were automatically prepared on the Ion Chef System (ThermoFisher Scientific) using 10 ng of gDNA and Ion Chef for DL8 kit (ThermoFisher), following the manufacturer's instructions. Up to 32 libraries were diluted to 50 pmol and multiplexed for template preparation and Ion 520 chip loading steps. Sequencing runs were performed on Ion S5 Genestudio (ThermoFisher), and a first analysis locally conducted on Torrent Suite Software (TSS, version 5.14, ThermoFisher) through a predefined bioinformatics pipeline. Coverage and variant calling metrics were determined by running Coverage Analysis and Torrent Variant Caller (TVC) plugins. Variants annotation and filtering were performed by processing Variant Call Format (VCF) files on the Ion Reporter tool (version 5.14, Annotate variants workflow, ThermoFisher). VCF files were reviewed on Integrative Genomic Viewer software (IGV, version 2.7, Broad Institute).

NGS output data files are available at the following link: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA823041.

We established a cut-off of 1% as the minimum VAF to consider a *TP53* mutation. The minimum coverage was 500x amplicon with optimal expected mean depth values of at least 2000x.

To validate subclonal *TP53* mutations, a second independent NGS run was performed starting from library preparation and using the same amount of gDNA as input.

NCI[32] and/or Seshat[33] website tools and relative databases were used to appropriately annotate and characterize the *TP53* mutations. HGVS nomenclature was used to describe the identified mutations.

The synonymous and intronic variants, except those occurring at splice sites, were not considered in our analyses, as recommended by the ERIC consortium.[12]

The possible germline origin of those *TP53* variants with at least 50% VAF was verified through non leukemic cells *TP53* testing (buccal swab and/or CD3+cells) and consequently excluded from the analysis.

3 | RESULTS

3.1 | *TP53* panel performance through coverage analysis

For this study, 220 genomic DNA samples from 171 CLL patients were retrospectively investigated by NGS. We performed optimal sequencing libraries in 100% of cases (*n* = 220); libraries' performance data are reported in Table 2. Uniformity of coverage through the amplicons was >90%, and a high balance of amplification was reached through the two pools of primers.

The median reads number mapped to the full reference hg19 was 211,554 (range 73,551- 359,848) with a median percentage on target (mapped reads target over a target region) of 92.23% (range 68.61%–96.07%) (Table 2).

3.2 | Detection of subclonal TP53 mutations (VAF ≤10%)

In the present study cohort, *TP53* mutations were found in 47/171 (27.5%) patients (Supplementary Table 1). Twenty-two out of 47 (46.8%) patients had only clonal mutations (VAF >10%) (Supplementary Table 2) and 25 (53.2%) reported the following mutational scenario: eight (8/47, 17%) patients displayed only *TP53* subclones (VAF ≤10%) and 17 (17/47, 36.2%) showed both clonal and subclonal

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TABLE 2 Summary of NGS libraries performance

Number of samples = 220 (n° patients = 171)	
Successful sequenced libraries	100%
Uniformity of coverage	>90%
Median mapped reads (range)	211,554 (73,551- 359,848)
Median percentage on target (range)	92.23% (68.61%-96.07%)

TP53 mutations. Table 3 summarizes both clonal and subclonal mutations found in these 25 patients.

Overall, 60/220 (27.3%) samples analyzed were *TP53*-mutated: 29/60 (48.3%) and 14/60 (23.3%) samples harbored only clonal and subclonal mutations, respectively. The remaining 17/60 (28.3%) samples showed both kinds of *TP53* mutations.

Considering all 31 samples with *TP53* subclones (*n* = 25 patients), 16 (51.6%) samples presented a single subclonal variant, whereas the other 15 (48.4%) showed more than one co-occurring *TP53* mutation (range 2–8, median 2). The VAFs of these mutations ranged between 1% and 10%, with a median value of 2%. Of note, the coverage of the *TP53* regions involved in subclonal mutations achieved a mean value of 6713 reads (median 4019), thus indicating that a significantly high level of variant call reliability was reached in terms of "vertical depth".

3.3 | Subclonal TP53 mutations annotation

In total, 25 patients harbored subclonal mutations and 44 subclonal TP53 mutations were counted, mainly missense (*n* = 37; 84.1%), followed by indels (*n* = 4; 9.1%) and nonsense (*n* = 2; 4.6%) types. A single (2.2%) subclonal mutation affecting a splice site was identified (Table 3).

The majority of subclonal *TP53* mutations, as well as the clonal TP53 ones, clustered within exons 5-8 encoding the DNA-binding domain; notably, missense mutations were located within codons 133–286 (Figure 1A). Virtually all substitutions were in the gene's coding region, and either intronic or synonymous mutations were never detected.

The spectrum of missense *TP53* mutations detected in our cohort included those most frequently observed in CLL and involved mainly codons 175, 245, 248, 273, and 282. All but one mutation, p.(Pro191fs), were described already in the official NCI[32] and/or Seshat[33] databases. These databases also predicted that most of the identified mutations (38/44, 86%) were of the "damaging" type. In contrast, the remaining ones (6/44, 14%) could be classified as "probably damaging" (Figure 1B).

The frameshift deletion p.(Pro191fs) (c.570_573delTCCT, exon 6), observed in pt. #2, not present in the current database, has to be considered "likely pathogenic" according to ERIC recommendations[12] and is included in the pie chart of Figure 1B as a "probably damaging" mutation.

3.4 | Longitudinal analysis of TP53 subclones

For 37/171 (21.6%) patients, multiple blood samples obtained at different times were available for longitudinal analysis (range time points 2–4, median 2). Fifteen out of 37 (40.5%) cases were always found wild-type for *TP53* at the different time points (not shown). In 6/37 (16.2%) patients, only clonal *TP53* mutations could be found. Instead, in 16/37 (43.2%) patients, one or more subclonal *TP53* mutations were found either alone (4/16, 25%) or co-occurring with clonal mutations (12/16, 75%), and their longitudinal course is reported in Figure 2.

Figure 3 describes four longitudinal cases (i.e., patients 13, 16, 24, 25) and provides representative results.

Four specimens of patient #13 (Figure 3A), obtained at different time points, were available for NGS testing. Two different subclonal mutations, that is, p.(Asn247Ser) and p.(Ile232Phe), not detected at timepoint 1 (year 2010, patient at diagnosis), became detectable by NGS with a VAF of 1.03% and 1.08%, respectively after a chemotherapy course (year 2016). These mutations changed from subclonal to clonal 3 years later (year 2019) with a VAF of 28% and 29%, respectively. The superimposable behavior suggests that the two mutations were in the *cis* configuration, that is, on the same gene, consistent with a visual inspection of IGV (data not shown). So, both alterations should be reported as p.[(Asn247Ser); (Ile232Phe)].

Patient #16 carried a clonal mutation p.(Arg213Leu) (31.4% VAF) and two subclonal ones, p.(Glu258Lys) and p.(Asp281Gly) (VAF 3.1% and 2.2%, respectively) in the sample obtained in 2008. In the sample obtained in the 2010 sample, an increase of p.(Arg213Leu) (VAF 66.6%) was observed while the two subclonal *TP53* clones did not change their frequencies, 2.5% VAF, and 2.96%, respectively. Between 2010 and 2012, the patient underwent chemotherapy treatment, with a drastic decrease of the dominant clone p.(Arg213Leu) at VAF 4.7%. NGS confirmed this finding. In contrast, the p.(Asp281Gly) mutation was at 10% VAF. Between 2012 and 2016, therapy was continued, and relevant changes in mutational dynamics of *TP53* mutations were observed in the 2016 sample. Specifically, an increase of the p.(Asp281Gly) mutation, which reached 84.1% VAF, an almost complete clearance of the p.(Arg213Leu) (1.13% VAF), and a loss of the p.(Glu258Lys) were detected.

Two samples were available for analysis in patient #24 (Panel 3C). In the year 2011 sample before chemotherapy start, a clonal mutation, p.(Arg273His) (VAF 36.6%), was observed together with the subclonal p.(Tyr236Cys) (VAF 8.5%) mutation. In 2015, after chemotherapy, there was an inversion of frequencies with the p.(Tyr236Cys) mutation that emerged as the dominant clone (90.2% VAF).

Patient #25 (Panel 3D) showed in 2015 only two main subclonal mutations, p.(Val274Asp) and p.(Asp281Asn) (VAF 6.7% and 1.8%, respectively). Instead, in 2016, both mutation frequencies raised to 52% and 29%, respectively. The different VAF levels suggest a trans configuration of mutations, confirmed by IGV visual analysis (data not shown).

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TABLE 3 Characterization of the subclonal *TP53* mutations in 25 patients

pt	Sampling (year)	*TP53* status (mut/wt)	Exon	Nucleotide	Protein change	Variant effect	VAF (%)	Coverage position	Coverage variant
1	2004	wt							
	2016	mut	6	c.626_627delGA	p.(Arg209fs)	Frameshift deletion	5.3	1264	67
2	2016	mut	7	c.751A>C	p.(Ile251Leu)	Missense	2.5	22,025	553
			7	c.701A>G	p.(Tyr234Cys)	Missense	5.95	12,285	731
			6	c.632C>T	p.(Thr211Ile)	Missense	38.8	6402	2483
			6	c.602T>A	p.(Leu201Ter)	Nonsense	40.4	6395	2581
			splicesite_5 (ex.5)	c.376-2A>T		Aberrant splicing	2.43	10,433	254
			6	**c.570_573delTCCT**	**p.(Pro191fs)**	**Frameshift deletion**	6.3	11,723	740
3	2015	mut	7	c.733G>A	p.(Gly245Ser)	Missense	77	17,484	13,458
			6	c.638G>T	p.(Arg213Leu)	Missense	1.65	5260	87
			7	c.742C>T	p.(Arg248Trp)	Missense	1.4	17,286	245
			6	c.590T>A	p.(Val197Glu)	Missense	8	14,971	1199
4	2012	wt							
	2014	mut	5	c.404G>T	p.(Cys135Phe)	Missense	1.26	6207	78
5	2014	wt							
	2016	wt							
	2017	mut	5	c.524G>A	p.(Arg175His)	Missense	1.26	3323	43
			5	c.422G>A	p.(Cys141Tyr)	Missense	1.29	5656	71
6	2008	wt	wt						
	2010	wt	wt						
	2017	mut	8	c.834_851delTGGGAGAGACCGGCGCAC	p.(Gly279_ Thr284del)	In-frame deletion	15.9	11,074	1766
			6	c.641A>G	p.(His214Arg)	Missense	1.66	3379	56
			4	c.224delC	p.(Pro75fs)	Frameshift deletion	51.4	5106	2626
7	2003	wt							
	2016	mut	7	c.713delG	p.(Cys238fs)	Frameshift deletion	3.7	2309	86

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TABLE 3 (Continued)

pt	Sampling (year)	*TP53* status (mut/wt)	Exon	Nucleotide	Protein change	Variant effect	VAF (%)	Coverage position	Coverage variant
			8	c.817C>T	p.(Arg273Cys)	Missense	5.2	2711	140
			splicesite_5 (ex.8)	c.783-1G>T		Aberrant splicing	23	2708	623
			7	c.710T>A	p.(Met237Lys)	Missense	1.53	2360	36
			6	c.670G>T	p.(Glu224Ter)	Nonsense	5.41	1459	79
			6	c.626_627delGA	p.(Arg209fs)	Frameshift deletion	4.51	1440	65
			5	c.469G>T	p.(Val157Phe)	Missense	2.07	3535	73
			5	c.495G>T	p.(Gln165His)	Missense	1.52	2035	31
			7	c.734_735delGCinsTT	p.(Gly245Val)	Missense	6.6	4483	297
8	2003	wt							
	2010	mut	8	c.844C>T	p.(Arg282Trp)	Missense	1.08	3698	40
9	2019	mut	7	c.751A>T	p.(Ile251Phe)	Missense	6.85	39,329	2704
10	2018	mut	7	c.751A>C	p.(Ile251Leu)	Missense	3.57	9921	354
11	2008	wt							
	2014	mut	8	c.818G>A	p.(Arg273His)	Missense	28.3	15,992	4523
	2016	mut	8	c.818G>A	p.(Arg273His)	Missense	4.1	14,140	575
12	2010	wt							
	2016	mut	7	c.725G>T	p.(Cys242Phe)	Missense	26.4	23,414	6185
			5	c.398T>A	p.(Met133Lys)	Missense	3.1	6104	191
13	2010	wt							
	2016	mut	7	c.740A>G	p.(Asn247Ser)	Missense	1.03	28,779	297
			7	c.694A>T	p.(Ile232Phe)	Missense	1.08	16,932	183
	2018	mut	7	c.740A>G	p.(Asn247Ser)	Missense	7.79	7755	604
			7	c.694A>T	p.(Ile232Phe)	Missense	8.35	7531	629
	2019	mut	7	c.740A>G	p.(Asn247Ser)	Missense	28	14,867	531
			7	c.694A>T	p.(Ile232Phe)	Missense	29.1	14,904	513

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TABLE 3 (Continued)

pt	Sampling (year)	*TP53* status (mut/wt)	Exon	Nucleotide	Protein change	Variant effect	VAF (%)	Coverage position	Coverage variant
14	2015	mut	6	c.578A>T	p.(His193Leu)	Missense	21.3	4824	1028
	2017	mut	6	c.578A>T	p.(His193Leu)	Missense	78.5	5564	4368
			6	c.560G>T	p.(Gly187Val)	Missense	3.7	5654	210
	2019	mut	6	c.578A>T	p.(His193Leu)	Missense	84.98	1857	1578
15	2016	mut	7	c.751A>C	p.(Ile251Leu)	Missense	3.77	2018	76
			8	c.826G>A	p.(Ala276Thr)	Missense	47.4	4247	2013
16	2008	mut	7	c.772G>A	p.(Glu258Lys)	Missense	3.1	3976	122
			8	c.842 A>G	p.(Asp281Gly)	Missense	2.2	8235	177
			6	c.638G>T	p.(Arg213Leu)	Missense	31.4	1241	389
	2010	mut	6	c.638G>T	p.(Arg213Leu)	Missense	66.6	850	566
			7	c.716A>C	p.(Asn239Thr)	Missense	1.56	1343	21
			8	c.842A>G	p.(Asp281Gly)	Missense	2.96	6244	185
			7	c.772G>A	p.(Glu258Lys)	Missense	2.5	1808	45
	2012	mut	8	c.842A>G	p.(Asp281Gly)	Missense	10	6846	682
			6	c.638G>T	p.(Arg213Leu)	Missense	4.7	1572	74
	2016	mut	8	c.842A>G	p.(Asp281Gly)	Missense	84.1	7100	5971
			6	c.638G>T	p.(Arg213Leu)	Missense	1.13	1499	17
17	2016	mut	6	c.535C>T	p.(His179Tyr)	Missense	1.68	1550	26
			6	c.584T>A	p.(Ile195Asn)	Missense	86	2644	2275
18	2017	mut	8	c.857A>G	p.(Glu286Gly)	Missense	12.5	8198	164
			6	c.580C>T	p.(Leu194Phe)	Missense	17.5	7250	204
	2019	mut	8	c.857A>G	p.(Glu286Gly)	Missense	2.8	4771	45
			6	c.580C>T	p.(Leu194Phe)	Missense	5.7	2475	43
19	2017	mut	4	c.266_267delCC	p.(Pro89fs)	Frameshift deletion	58.84	3071	1807
			6	c.643A>G	p.(Ser215Gly)	Missense	5.59	2130	119
			6	c.632C>T	p.(Thr211Ile)	Missense	3.14	2135	67
			5	c.490A>G	p.(Lys164Glu)	Missense	4.32	1761	76

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TABLE 3 (Continued)

pt	Sampling (year)	*TP53* status (mut/wt)	Exon	Nucleotide	Protein change	Variant effect	VAF (%)	Coverage position	Coverage variant
20	2008	mut	10	c.1021T>A	p.(Phe341Ile)	Missense	50.1	8229	4122
			7	c.722C>T	p.(Ser241Phe)	Missense	36.6	5341	1953
			6	c.659A>G	p.(Tyr220Cys)	Missense	3.5	4171	146
	2016	mut	10	c.1021T>A	p.(Phe341Ile)	Missense	51	3810	1942
			7	c.722C>T	p.(Ser241Phe)	Missense	35.2	3464	1219
			6	c.659A>G	p.(Tyr220Cys)	Missense	12.6	2197	276
21	2013	wt							
	2020	mut	splicesite_3 (ex.5)	c.376-1_377delGTA		Aberrant splicing	72.44	3948	2860
			7	c.736T>C	p.(Met246Val)	Missense	1.6	9997	159
			7	c.742C>T	p.(Arg248Trp)	Missense	7.12	3876	276
			6	c.626_627delGA	p.(Arg209Lysfs)	Frameshift deletion	6.36	3960	252
			8	c.838A>T	p.(Arg280Ter)	Nonsense	4.88	3958	193
22	2016	mut	6	c.568_570delCCT	p.(Pro190del)	In-frame deletion	1.52	2828	43
			5	c.527G>A	p.(Cys176Tyr)	Missense	1.71	2867	49
			6	c.659A>G	p.(Tyr220Cys)	Missense	19.4	2168	420
23	2017	mut	5	c.472C>A	p.(Arg158Ser)	Missense	1.56	5973	93
24	2011	mut	7	c.707A>G	p.(Tyr236Cys)	Missense	8.5	2364	200
			8	c.818G>A	p.(Arg273His)	Missense	36.6	2617	959
	2015	mut	7	c.707A>G	p.(Tyr236Cys)	Missense	90.2	2780	2507
			8	c.818G>A	p.(Arg273His)	Missense	2	3097	61
25	2015	mut	8	c.821T>A	p.(Val274Asp)	Missense	6.7	4062	272
			8	c.841G>A	p.(Asp281Asn)	Missense	1.77	8235	146
	2016	mut	8	c.821T>A	p.(Val274Asp)	Missense	52	2556	1329
			8	c.841G>A	p.(Asp281Asn)	Missense	29	6292	1825

Note: In bold, the only *TP53* mutation not yet described in NCI/Seshat databases.
Abbreviations: mut, mutated; VAF, variant allele frequency; wt, wild type.

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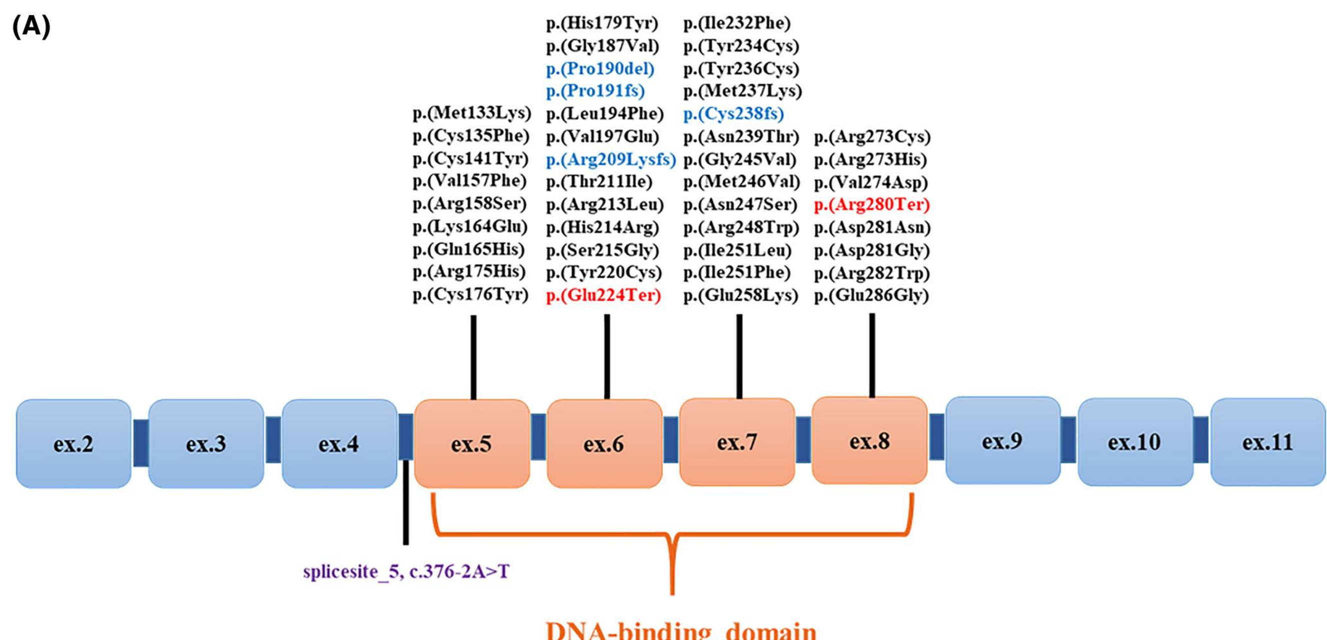

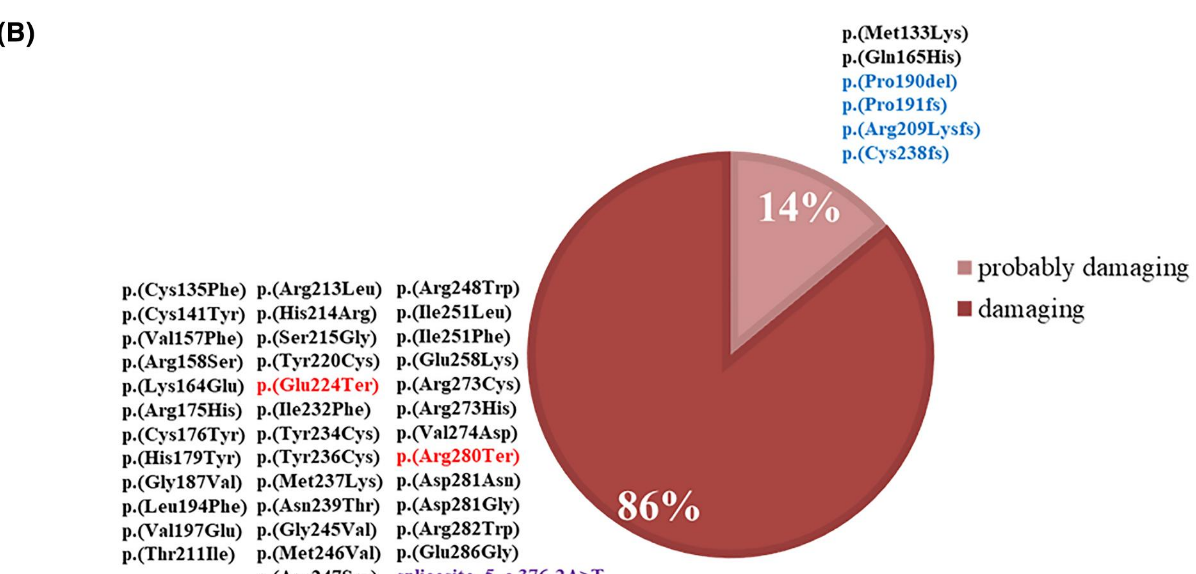

FIGURE 1 Subclonal *TP53* mutations characterization. (A) describes the localization of each specific *TP53* mutation within the *TP53* gene and the corresponding exons. For each mutation, P53 protein prediction is detailed in (B), classified as "damaging" and "probably damaging" according to the NCI[31] and/or Seshat[32] databases. Black, light blue, red, and purple colors refer to missense, indels, nonsense, and splice-site mutations, respectively

3.5 | Correlation between TP53 mutations occurrence, del(17p), and treatment

We tested del(17p) structural aberration by FISH in 144/171 (76.6%) patients and 170 samples. We detected del(17p) in 40/144 (27.8%) patients and 42/170 (24.7%) samples. In 22 patients, we documented both *TP53* mutations and del(17p), whereas, in the remaining 18 patients, only the del(17p) was found, and data were reported in Supplementary Table 3.

Additional information about other cytogenetic abnormalities, that is, deletions at chromosomal loci 11q22.3, 13q14.3, and trisomy 12, were separately reported (Supplementary Table 4).

Considering longitudinal cases, we have available FISH data for 36/37 of them. Within these 36 patients, del(17p) was present in 17 (47.2%); furthermore, analyzing also the *TP53* status, it turned out that 21/36 (58.3%) patients were *TP53* mutated, while the remaining 15/36 (41.7%) cases harbored wild-type *TP53* (Table 4).

The analysis of the 21 *TP53* mutated patients revealed that 12/21 (57.1%) carried a del(17p), whereas 9/21 (42.9%) were del(17p)-negative (Table 5). Among the 12 double TP53/del(17p)- positive cases, 8/12 showed both clonal and subclonal mutations, 3/12 harbored only clonal TP53 mutations and one displayed only subclonal TP53 mutation (Table 5). Furthermore, interestingly, 8/12 *TP53* mutated/del(17p) positive patients acquired both alterations

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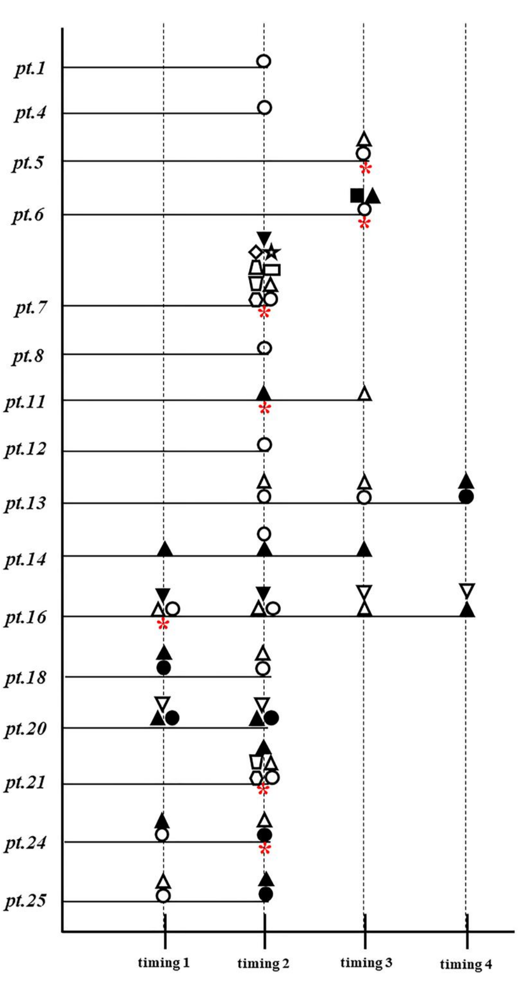

FIGURE 2 Simplified timeline of subclonal *TP53* mutations appearance in the 16 longitudinal patients in whom these subclonal mutations could be observed. Each lane represents one patient. Empty and full shape symbols represent subclonal and clonal *TP53* mutations, respectively. Different symbols are used to indicate other *TP53* mutations; the same symbol is used to indicate the mutational evolution longitudinally. Changing a given symbol from empty to full or vice-versa denotes a difference in a given patient of the same mutation from subclonal to clonal or vice-versa. A red asterisk indicates the timing when the del(17p) was revealed for the first time (when data was available)

during the same timepoint, with six patients also developing concomitant clonal and subclonal *TP53* mutations (not shown).

Then, we attempted to associate the clinical follow-up history with *TP53* mutational and del(17p) status in 31/36 patients from whom clinical data were available. Among these 31 patients, 24/31 (77.4%) underwent first-line chemo-immunotherapy regimens. Eighteen out of 24 patients (75%) were treated before *TP53* mutation detection. For most of them (12/18), at least a pre-treatment sample was available and found wildtype for *TP53* by NGS testing, possibly suggesting a therapeutic pressure to develop *TP53* mutations. Of the remaining six treated cases, no *TP53* mutations were detectable through the different samplings regardless of the specific treatment regimen.

Fifteen longitudinal patients with available treatment data were also del(17p)-positive, and 10/15 were also TP53-mutated. A cumulative analysis of *TP53* mutations, del(17p), and treatment data are reported in Figure 4.

It is also relevant to highlight that among treated patients showing a mutated *TP53* profile, subclonal mutations were appreciable in 14 cases, and their presence was exclusive in three of them (patients #1, #5, and #8).

4 | DISCUSSION

Currently, *TP53* alterations in CLL represent the rationale for using novel therapeutic options, such as ibrutinib/idelalisib/acalabrutinib and venetoclax, targeting the B-cell receptor signaling and the BCL-2, respectively.[10,34–37]

It is accepted that the positive clonal expansion of subclonal *TP53* mutations, that is, those with ≤10% VAF, represents one of the main drivers of chemoimmunotherapy failure.[13,16,17] Their relevance in CLL consists of the likelihood of allowing the outgrowth of resistant cellular clones, for example, due to chemotherapy pressure or other changes in the environment[4,15,17] Therefore, the detection of subclonal *TP53* mutations may change the treatment paradigm, also in light of some recent evidence indicating that ibrutinib does not significantly contribute to the positive selection of pre-existing *TP53* subclones in CLL patients but rather lead to a decrease in terms of the number of mutations and related allele frequency.[38–41]

Implementing sensitive approaches, such as NGS, in clinical laboratories dealing with *TP53* mutational assessment has improved the detection of low-burden *TP53* mutations. Although current guidelines for clinical reports are based on *TP53* mutations detectable by Sanger sequencing (>10% VAF), recent ERIC recommendations strongly suggest that mutations detected at a VAF range of 5%–10% could be mentioned in the final report.[12] However, the clinical relevance is still doubtful due to a lack of clinical evidence.[11,13,17,39,42] Our results reported at least one subclonal *TP53* mutation in half of the overall *TP53* mutated samples tested (31/60), supporting the evidence that the presence of subclones is not a rare event.[17,43]

Of note, mutated *TP53* subclones found in our study seem to follow the same mutational pattern of clonal variants already documented in CLL patients (i.e., codons 175, 245, 248, 273, and 282). Although we are aware of the lack of correlation of our *TP53* mutational data with clinical information, this finding corroborates the hypothesis reported in other studies that subclonal mutations may significantly impact CLL management and disease course.[13,15,17]

By prediction, it was impressive that most subclonal *TP53* mutations could be classified as damaging (38/44; 86%) and that no subclonal intronic or synonymous mutations were found in our samples, further suggesting that the occurrence of low abundance mutations affecting the coding sequence of the *TP53* gene is an event that can contribute to worse the prognosis.

NGS procedure was beneficial for the longitudinal follow-up of the 16/25 patients where subclones occurred since different changes

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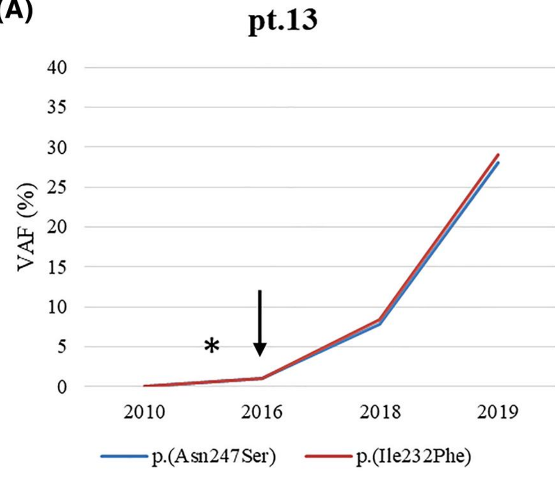

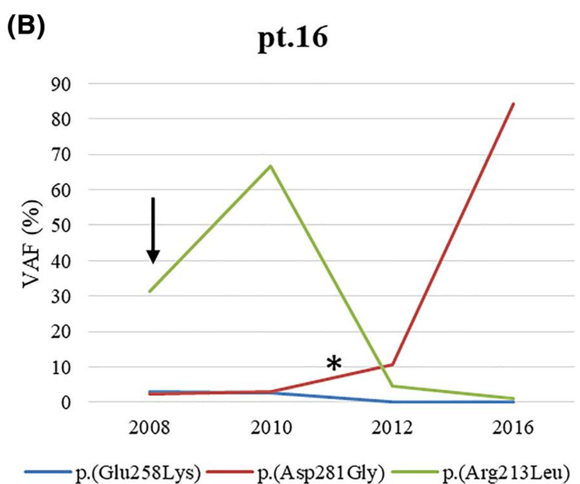

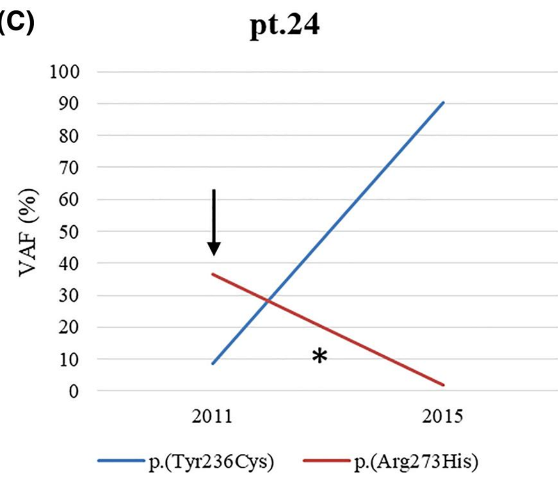

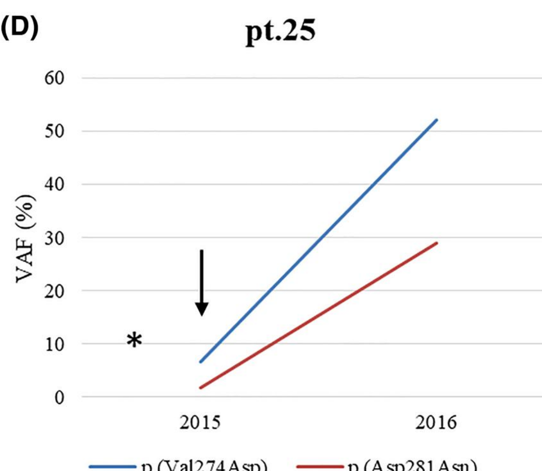

FIGURE 3 Longitudinal pattern of subclonal *TP53* mutations studied in four selected patients. The evolution of variant allele frequencies of the *TP53* subclones was plotted at the four different time points analyzed for patients 13 and 16 (Panel A and B, respectively). Only two timings were available for patients 24 and 25 (Panel C and D, respectively). Each colored line represents a specific *TP53* mutation. The black arrow indicates the first time at least one subclonal *TP53* mutation was detected by NGS testing. The asterisk indicates the moment of treatment respect to time of *TP53* analysis. VAF, variant allele frequency; pt, patient

TABLE 4 Analysis of *TP53* and del(17p) status in 36 longitudinal cases

	TP53		
del(17p)	**pos**	**neg**	**Total**
pos	12	5	17
neg	9	10	19
Total	21	15	36

Abbreviations: del(17p), deletion of chromosomal locus 17p13.1; neg, negative; pos, positive.

TABLE 5 Correlation of clonal and subclonal *TP53* mutations with del(17p) status in longitudinal cases

	del(17p)	
***TP53* type**	**pos**	**neg**
Clonal	3	3
Subclonal	1	3
Clonal AND subclonal	8	3
Total	12	9

Abbreviations: del(17p), deletion of chromosomal locus 17p13.1; neg, negative; pos, positive.

in the *TP53* mutational scenario over time could be observed. Four different mutational patterns were identified, and of note, NGS analysis demonstrated that early identified subclonal *TP53* mutations become predominant in subsequent time points and, vice versa, that clonal mutations turned out to be subclonal ones. This finding may represent a critical point in the early detection of de novo mutations and managing minimal residual disease, as NGS can reveal low-burden *TP53* mutations that may affect treatment effectiveness and anticipate the disease progression.[44–48]

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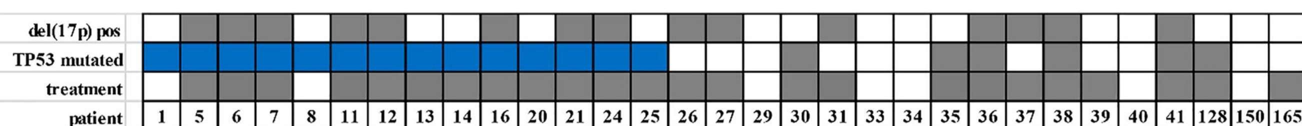

FIGURE 4 Heatmap representation of treatment, *TP53* mutational, and del(17p) status in 31 longitudinal patients. Blue boxes indicate the presence of at least a subclonal *TP53* mutation

The analysis of longitudinal patients with available clinical data made it possible to correlate the mutational status of *TP53* and del (17p), also merging treatment data. Interestingly, it was found that 18/24 of patients treated with chemoimmunotherapy and followed over time developed at least one *TP53* mutation and 15/18 a del (17p). Ten of 24 treated patients shared TP53 mutation/s as well as del(17p). Both events were detected at the same time for all but one patient (case #36, del(17p) not determined at 2016 timing), thus revealing a possible role in therapeutic pressure in establishing these alterations in the disease course, as already documented.[16,49–51]

Furthermore, following dynamics over time, the appearance of subclonal *TP53* mutations was observed in 14 patients along with other *TP53* clonal mutations in all cases but three (patients #1, #5, and #8). This evidence suggests that the subclonal *TP53* mutations usually develop later with respect to clonal mutations and del(17p).

Although *TP53* mutations were already present before treatment in three patients, #16, #20, and #24, in two (#16 and #20), the therapy may have led to a dramatic evolution of aggressive subclones (see Table 3 and Figure 3). The main evolution pattern seems to be the replacement of a predominant mutated *TP53* clone with a new subclone that grows over time, becoming the prevalent one.

Overall, our study performed in a real-life clinical setting from a single institution further supported the importance of detecting subclonal *TP53* mutants in CLL patients. Although our study is based on a small cohort of CLL patients and the correlation with clinical and FISH data was not available for all patients, we believe that it contributes to a complete view of the *TP53* mutational profile and its clinical role. Indeed, although clonal *TP53* mutations are still considered the only biomarker for treatment with targeted agents, there is the need to reevaluate the clinical relevance of subclonal mutations in order to address more patients to this type of treatment.

5 | CONCLUSIONS

The clinical significance of the subclonal *TP53* mutations needs further efforts to be accepted and implemented as routine testing; our study represents a valuable contribution even if correlation of molecular findings with clinical data is lacking. Our study demonstrated that NGS is suitable for detecting subclonal *TP53* mutations and following the mutational dynamics in CLL patients. It likely represents the most sustainable methodology for *TP53* testing before any line of treatment, as well as for real-time therapy monitoring and early detection of disease progression and relapse.

AUTHOR CONTRIBUTIONS

Giuseppa De Luca, Mariella Dono, Giannamaria Cerruti, and Giovanna Cutrona had full access to all the data in the study and took responsibility for the integrity and the accuracy of the data analysis. Study concept and design: Giuseppa De Luca and Giovanna Cutrona. Acquisition of clinical data: Adalberto Ibatici, Nikki Di Felice, Fortunato Morabito, and Antonino Neri. Acquisition, analysis of molecular data: Sonia Fabris, Serena Matis, Monica Colombo and Giuseppa De Luca. Interpretation of data: Giuseppa De Luca, Mariella Dono, Alessia Ciarrocchi, Giovanna Cutrona and Gilberto Fronza. Drafting of the article: Mariella Dono, Giuseppa De Luca, Gilberto Fronza, Paola Monti, Paola Menichini, and Giovanna Cutrona Critical revision of the article for important intellectual content: Paolo Nozza, Antonino Neri, Manlio Ferrarini, Fortunato Morabito and Franco Fais. Administrative, technical, or material support: Romana Conte and Sonia Lastraioli. Study supervision: Giovanna Cutrona and Mariella Dono.

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CONFLICT OF INTEREST

Nothing to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in TP53 CLL sequencing at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA823041, reference number PRJNA823041.

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Additional supporting information can be found online in the Supporting Information section at the end of this article.

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