Time to first treatment and P53 dysfunction in chronic lymphocytic leukaemia: results of the O‑CLL1 study in early stage patients

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Chronic lymphocytic leukaemia (CLL) is characterised by a heterogeneous clinical course. Such heterogeneity is associated with a number of markers, including TP53 gene inactivation. While TP53 gene alterations determine resistance to chemotherapy, it is not clear whether they can influence early disease progression. To clarify this issue, TP53 mutations and deletions of the corresponding locus [del(17p)] were evaluated in 469 cases from the O‑CLL1 observational study that recruited a cohort of clinically and molecularly characterised Binet stage A patients. Twenty‑four cases harboured somatic TP53 mutations [accompanied by del(17p) in 9 cases], 2 patients had del(17p) only, and 5 patients had TP53 germ‑line variants. While del(17p) with or without TP53 mutations was capable of significantly predicting the time to first treatment, a reliable measure of disease progression, TP53 mutations were not. This was true for cases with high or low variant allele frequency. The lack of predictive ability was independent of the functional features of the mutant P53 protein in terms of transactivation and dominant negative potential. TP53 mutations alone were more frequent in patients with mutated IGHV genes, whereas del(17p) was associated with the presence of adverse prognostic factors, including CD38 positivity, unmutated-IGHV gene status, and NOTCH1 mutations.

Decades of biological and molecular studies have provided extensive information for the understanding of chronic lymphocytic leukaemia (CLL), although many questions remain unanswered and the disease is still considered to be virtually incurable1. Despite the morphological and phenotypic homogeneity of CLL cells, the clinical course of the disease is highly heterogeneous ranging from rapid disease progression, requiring early treatment, to decades of survival with no treatment need2. This clinical course probably reflects the molecular heterogeneity of the disease. Over 80% of CLL cases harbour karyotype aberrations at diagnosis, the most frequent being partial deletions at 13q (~ 55%), 11q (~ 15%), 17p (~ 8%), and gain of chromosome 12 (~ 15%)3. In addition, specific gene mutations (e.g. TP53, SF3B1, BIRC3, NOTCH1, ATM) have been reported4. The incidence of TP53 tumour suppressor gene mutations is low at diagnosis (~ 5 to 7%), but it rises as the disease progresses and reaches approximately 40% in refractory CLL5–9. The European Research Initiative on CLL (ERIC) group recommends TP53 mutational screening for all patients before therapy start to avoid treatment protocols that are ineffective

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in patients with TP53 alterations\(^a\). Furthermore, since mutational status of the immunoglobulin heavy-chain variable region gene (IGHV) and complex karyotype are relevant to improve risk stratification\(^1\), Baliakas et al.\(^1\) have recently incorporated these two markers, and TP53 mutations into a novel prognostic model.

The TP53 tumour suppressor gene encodes a tetrameric transcription factor that controls different pathways, through its ability to transactivate a plethora of downstream effector genes, many of which are relevant for carcinogenesis\(^3\). Disruption of the TP53 protein pathway, which may involve different mechanisms, is highly selected in most tumour types and is frequently due to alterations of both alleles, e.g. deletion of one allele [del(17p)] and mutation of the second allele. Donehower et al.\(^1\), performed a comprehensive assessment of the TP53 pathway involvement in 32 cancers reported by The Cancer Genome Atlas (TCGA) and found that in cancers with TP53 mutations, there was a loss of the second allele due to mutation, chromosomal deletion, or copy-neutral loss of heterozygosity in 91% of the cases\(^14\). The current literature strongly supports the central role of TP53 alterations in CLL\(^35\). Approximately 90% of CLL patients with del(17p) carry a TP53 mutation and ~60% of patients with TP53 mutations also harbour del(17p), as detected by FISH\(^16\). Even in the absence of a del(17p), TP53 mutations appear to have an equally profound impact on outcome and are more frequent in patients with a poor prognosis and a higher genetic complexity\(^4,16\). Moreover, CLL sub-clones carrying TP53 mutations can be positively selected upon treatment, ultimately becoming the prevalent expansion of an initially minor mutant component\(^3,17,21,22\).

The mere presence of TP53 mutations is not synonymous per se of the complete inactivation of the TP53 pathway, since mutant TP53 proteins are functionally and structurally heterogeneous\(^5\) and may impact on important clinical variables, in multiple, often subtle ways, as revealed by cell-based assays and animal models\(^6\). Moreover, while it is widely accepted that alterations of the TP53 gene may influence the clinical course of CLL by causing resistance to chemotherapy, their impact on clonal expansion and disease progression is still not fully defined, especially in the earliest stages of the disease. With the aim of clarifying these points, we studied TP33 alterations in patients enrolled in the O-CLL1 trial (clinicaltrial.gov identifier NCT00917540). In this observational protocol, Binet stage A CLL patients from several Italian Institutions were prospectively enrolled within 12 months of diagnosis (median: 2.3 months) and their evolution was followed. Because the aim of the protocol was to study the disease since the early stages, patients at more advanced stages (Binet stage B and C) were excluded from recruitment. A total of 469 patients from this well characterised cohort were investigated for TP53 mutations using next generation sequencing (NGS) and for del(17p) using FISH. The possible influence of the TP53 alterations on the time to first treatment (TTFT) and on the expansion of individual clones or sub-clones was also investigated.

Results

Incidence and characterization of the TP53 mutational status in O-CLL1 patients. Binet stage A CLL patients from the O-CLL1 trial\(^25\), were characterised for cellular, molecular, and cytogenetic features at diagnosis (Supplementary Table 1A). TP53 mutational status was determined by NGS on highly purified (>95%) CLL B-cells from 475 patients. FISH analysis was used to determine the presence of del(17p) in 469 out of these 475 patients (Supplementary Table 1B). TP53 alterations [del(17p) and/or TP53 mutations] were identified in 31 patients. In five patients a TP53 variant allele frequency (VAF) of approximately 50% was suggestive of a germline mutation. A subsequent analysis of non-tumour samples revealed the presence of the same TP33 mutation. Therefore, these were classified as TP33 germ-line variants (Supplementary Table 1B). The remaining 26 patients were characterised by the presence of: (1) only a TP33 mutation (group Mut/noDel = 15 cases), (2) a TP33 mutation along with the deletion of del(17p) (group Mut/Del = 9 cases), and (3) del(17p) with no TP33 mutation (group noMut/Del = 2 cases) (Table 1). The overall incidence of somatic TP33 alterations in the O-CLL1 cohort [mutations: 9%; 475/475; del(17p): 2.3%; 11/469] and their distribution relative to del(17p) were similar to that of previous reports\(^1,4,8,13,17,21\). The VAF in the Mut/noDel group was below 50% and significantly lower (23.8 ± 18.4 vs 65.7 ± 35.7, \(P = 0.001\)) than that observed in the Mut/Del group. In addition, certain adverse prognostic factors, such as CD38 expression (\(P = 0.0006\)), unmutated-IGHV (UM-IGHV) gene status (\(P = 0.03\)), and NOTCH1 mutations (\(P = 0.0415\)) were significantly less frequent in the cases with TP33 mutation only (Table 1). Conversely, a favourable prognostic factor, such as del(13q), showed a trend toward a higher frequency only in Mut/noDel cases (\(P = 0.06\)) (Table 1).

Influence of the TP53 status on the TTFT in O-CLL1 patients. Next, the influence of the TP53 status on TTFT was evaluated in the O-CLL1 cohort. After a median follow-up of 86 months, TTFT data were available for 462/469 patients, including the 5 cases with a TP53 germline variant. The TTFT of patients with somatic TP33 mutations (Mut = Mut/noDel + Mut/Del: 24 patients) was not statistically different from that of cases with wild-type TP33 (WT/noDel: 431 patients) (HR = 1.5, 95% CI 0.8–2.7, \(P = 0.7\)). In contrast, the TTFT was significantly shorter when patients with del(17p) (Del = Mut/Del + noMut/Del: 11 patients) were compared to those without deletion (noDel: 446 patients, excluding the 5 with TP33 germline variant) (HR = 5.2, 95% CI 2.4–11.2, \(P = 0.0001\)). A multivariate analysis, in which only the presence of del(17p) and of TP53 mutations were introduced, indicated that the presence of the del(17p) at diagnosis constituted a significant risk factor for a shorter TTFT (HR 6.1, 95% CI 2.2–17.0, \(P = 0.001\)), independently of the presence of a TP33 mutation (HR 0.8, 95% CI 0.4–1.8, \(P = 0.6\)). The cohort analysed was also stratified according to the presence/absence of TP33 mutations and/or del(17p) in different combinations (see Fig. 1). The 9 Mut/Del and the 2 WT/Del patients had a significantly shorter TTFT than the 431 WT/noDel cases, whereas a somatic TP33 mutation in the absence of del(17p) (Mut/noDel: 15 patients) was not predictive of a shorter TTFT. A similar result was obtained when the five cases with germ-line allelic variants were compared with the WT TP53 cases. Subsequently we addressed the issue of whether the VAF in the Mut/noDel cases could have an influence on the prediction of the TTFT. To this end Mut/noDel cases were stratified according to high (>10%) and low (<10%) VAF values and the TTFT of the
two groups was analysed. No significant differences were detected in the TTFT in the two groups of patients. The clinical impact of TP53 mutations with different VAF also was analysed in the group of cases with mutated IGHV genes to eliminate a possible confounding factor represented by a strong predictor of prognosis such as IGHV gene mutation status. In this group, there were 9 patients with a V AF > 10% and 4 patients with a V AF < 10%. However, no statistically significant differences in the TTFT were observed (P = 0.5) (Supplementary Fig. 1).

Therefore, the size of the subclone characterised by the presence of a TP53 mutation did not appear to influence the TTFT in Binet stage A CLL. The presence of del(17p), although mostly associated with a TP53 mutation, appears to be more deleterious than the presence of the a TP53 mutation itself in determining a shorter TTFT.

### Functional studies on mutant P53 proteins from O-CLL1 patients.

Next, the functional properties of the P53 proteins encoded by the TP53 mutations detected in our cohort were investigated. A well-established yeast-based functional assay was employed, using four reporter strains. These strains have a different P53 Response Element (RE), which is derived from the promoter of one of four human P53 effector, i.e. P21, BAX, MDM2, and PUMA, respectively. The residual transactivation ability of the mutant P53 proteins compared to that of the WT protein (set as the 100% value) was measured at 30 °C and 37 °C (Table 2). With a threshold of residual WT P53 activity of 20%, all mutant P53 proteins (excluding those encoded by TP53 germline variants) were found inactive at 37 °C, whereas some activity was observed at 30 °C (Table 2; Supplementary Fig. 2A).

In fact, five mutants encoded P53 proteins with residual activity (p.Ala161Thr, p.Pro191del, p.Arg213Leu, and p.Arg282Trp within the Mut/noDel group and p.Val272Leu within the Mut/Del group). Of note, the P53 protein encoded by all TP53 germline variants (p.Asn235Ser, p.Arg283Cys, and p.Gly360Val) behaved similarly to the WT P53 and were active at both 30 °C and 37 °C (Supplementary Table 3).

### Table 1. Biologic and molecular features of the 26 CLL patients harbouring somatic TP53 alterations.

| ID* | cDNA variant* | Protein variant* | VAF%a | del(17p) | del(13q) | +12 | del(11q) | CD38 | ZAP-70 | IGHVb | NOTCH1 | SF3BI |
|-----|---------------|------------------|-------|---------|---------|-----|---------|------|--------|-------|--------|-------|
| 15 patients with Mut/noDel | | | | | | | | | | | | |
| MS0273 | c.524G>A | p.Arg175Hs | 11.7 | − | + | − | − | − | − | M | WT | WT |
| CA0082 | c.638G>T | p.Arg213Leu | 7.7 | − | + | − | − | − | − | M | WT | WT |
| CP0003 | c.833C>G | p.Pro278Arg | 4.1 | − | + | − | − | − | − | M | WT | WT |
| GM0252 | c.584T>C | p.Ile195Thr | 23.9 | − | + | − | − | − | + | M | WT | WT |
| MG0248 | c.844C>T | p.Arg282Trp | 44.6 | − | + | − | − | − | + | M | WT | WT |
| DD0478 | c.568_570delCCT | p.Pro191del | 18.3 | − | + | − | − | − | + | M | WT | WT |
| GC0448 | c.742C>T | p.Arg248Trp | 2.8 | − | + | − | − | − | − | M | WT | WT |
| PA0254 | c.481G>A | p.Ala161Thr | 38.0 | − | + | − | − | + | M | WT | WT |
| AR0222 | c.578A>G | p.His193Arg | 41.5 | − | + | − | − | − | − | M | WT | WT |
| CG0620 | c.626_627delIGA | p.Arg209fs | 48.7 | − | + | − | − | − | − | M | WT | WT |
| AA0396 | c.479T>A | p.Val157Asp | 4.4 | − | + | − | − | − | + | UM | WT | WT |
| CR0115 | c.338T>C | p.Phe113Ser | 2.0 | − | − | − | − | − | − | M | WT | WT |
| AG2064 | c.517G>A | p.Val173Met | 48.5 | − | + | − | − | − | − | M | WT | WT |
| SG0168 | c.260_261dupC | p.Ala88fs | 34.8 | − | + | − | − | − | − | M | WT | WT |
| DA0455 | c.818G>A | p.Arg273Hs | 18.0 | − | − | − | − | − | + | M | WT | WT |
| 9 patients with Mut/Del | | | | | | | | | | | | |
| CR0203 | c.772G>T | p.Glu258Ter | 97.7 | + | − | − | + | + | + | UM | Mutb WT |
| IF0044 | c.842A>G | p.Asp281Gly | 95.1 | + | − | − | − | − | + | UM | WT | WT |
| DA0094 | c.833C>G | p.Pro278Arg | 98.3 | + | − | − | − | − | − | + | UM | WT | WT |
| CS0290 | c.814G>T | p.Val272Leu | 36.6 | + | − | − | − | − | − | M | WT | WT |
| DG0193 | c.584T>C | p.Ile195Thr | 77.3 | + | − | − | − | − | + | UM | Mutb WT |
| FD0404 | c.824G>A | p.Cys275Tyr | 63.6 | + | − | − | − | − | + | UM | Mutb WT |
| PG0208 | c.524G>A | p.Arg175Hs | 16.4 | + | − | − | − | − | + | UM | WT | WT |
| CG0622 | c.497C>A | p.Val272Leu | 36.6 | + | − | − | − | − | − | M | WT | WT |
| DS0264 | c.622_623insr | p.Asp208Glufs | 95.1 | + | − | − | − | − | − | UM | WT | WT |
| 2 patients with noMut/Del | | | | | | | | | | | | |
| CP0036 | WT | WT | n.a | + | − | − | − | − | + | − | M | WT | WT |
| NT0628 | WT | WT | n.a | + | − | − | − | − | + | − | UM | WT | WT |

*Patient identification; *Based on HGVS (Human Genome Variation Society) nomenclature; VAF, Variant allele frequency; IGHV-UM, unmutated; IGHV-M, mutated; insertion of AATTTGGATG; Mut, NOTCH1 coding mutation c.7541_7542delCT, p.P2515fs*4; + 12, trisomy 12; WT, wild-type; n.a., not applicable.

Significant P-values regarding the comparison of Mut/noDel vs Mut/Del patients for the presence of del(13q), CD38, UM-IGHV and NOTCH1 mutation are the following: P = 0.06, P = 0.0006, P = 0.003 and P = 0.0415, respectively.
Mutant P53 proteins were also transiently expressed in HCT116 TP53−/− cells and were tested for their ability to transactivate the luciferase reporter gene carrying a fragment of the P21 promoter (Supplementary Fig. 2B). The results obtained were consistent with those obtained in yeast at 30 °C, confirming that some mutants (e.g. p.AlaA161Thr, p.Pro191del and p.Arg282Trp) had a partial activity. Finally, the TP53 germ-line allelic variants identified in our cohort were found to encode proteins with a high functional efficiency of transactivation (p.Asn235Ser, p.Arg283Cys, and p.Gly360Val) in both mammalian cell- and yeast-based assays (Supplementary Fig. 2B and Supplementary Table 3).

It is known that certain mutant P53 proteins have the capacity of inhibiting the activity of the WT protein when heterozygous at TP53 locus [i.e. they are Dominant Negative (DN)]26. Therefore, the DN potential of each mutant P53 protein of the Mut/noDel group was determined using one of the reporter strains (i.e. yLFM-P21-5’) (Table 3). None of the mutant P53 proteins with partial residual function (i.e. p.Ala161Thr, p.Pro191del, p.Arg213Leu, p.Val272Leu and p.Arg282Trp) exhibited DN potential (Table 3). Of note, all of the TP53 germine variants identified in patients without del(17p) encoded a recessive mutant P53 (Supplementary Table 4).

Influence of the residual transactivation ability and DN potential of mutant P53 proteins on TTFT in O-CLL1 patients.

Given the functional heterogeneity of P53 proteins in our cohort, we asked whether such heterogeneity could influence the TTFT. This analysis was carried out on the Mut/noDel patients’ group (15 cases). Neither the transactivation ability (Fig. 2A) nor the DN potential (Fig. 2B) appeared to affect the Kaplan–Meier curves of these patients’ groups, which were similar to those of the WT/noDel patients (431 patients). Although potentially interesting, these tests could not be carried out in the Mut/Del patients’ group since the residual transactivation activity was < 20% in 8/9 available cases and ≥ 20% in a single case. Thus, this issue warrants re-evaluation in a larger study sample.

Multivariate analysis.

CD38 and ZAP-70 expression, β2-microglobulin (β2M) serum levels, Rai stage, IGHV mutational status, del(11q), NOTCH1 and SF3B1 mutations, MBL classification were confirmed to have a significant prognostic power on univariate analysis27 (Supplementary Table 5). A Cox multivariate analysis was then performed by introducing into the model markers indicative of TP53 alterations together with the variables with a significant prognostic power for TTFT determined in the univariate analysis. Both del(17p) together with TP53 mutations, and del(17p) alone, retained an independent prognostic power, whereas TP53 mutation alone did not. Other markers with an independent prognostic power were IGHV mutational status, del(11q), NOTCH1 mutation, β2M levels, and Rai stage (Fig. 3).

Changes in the TP53 alterations pattern with time.

TP53 mutational status was re-assessed in 171 patients after 36 months from diagnosis or at the time of therapy need. Of these, 140 re-tested at 36 months after diagnosis, did not present any new TP53 alteration. Another 23 cases, without TP53 alterations at diagnosis, were retested at the time of therapy need; again, no new TP53 alterations were recorded. Eight cases with a TP53 mutation at diagnosis were re-tested at 36 months (6 cases) or at the time of therapy need (2 cases). All of the 8 cases with a somatic TP53 mutation continued to express the same mutation seen at diagnosis, although with some changes in their VAF (Fig. 4). The 163 patients, negative for somatic TP53 mutations at diagnosis, still presented WT TP53 gene (or the same germline TP53 allelic variant) after 36 months. None of these 161 patients...
harboured del(17p) at diagnosis or at re-testing. The two cases re-tested at the time of therapy need presented p.Arg175His or p.Val157Asp, respectively. Of these, one [with p.Arg175His, and a del(17p) both at diagnosis and at re-testing] exhibited an over fourfold VAF increase, while the other [with p.Val157Asp mutation and no del(17p) also at re-testing] did not manifest any change in VAF.

Discussion
The present study investigated the influence on disease progression of TP53 alterations in Binet A CLL patients, enrolled in the multicentre prospective observational O-CLL1 trial with a long follow-up (median ~7 years). This influence was measured by determining the TTFT, which represents a rather accurate approach to assess disease progression. TP53 mutations and del(17p) were determined in 98.7% of patients (469/475). There are differences in the thresholds distinguishing positive from negative cases for the FISH and NGS analysis (i.e. measurement of del(17p) and TP53 mutations, respectively) due to the different sensitivity of each methodology. Since the sensitivity is somewhat imbalanced in favour of NGS, it cannot be excluded that certain patients harboured a del(17p), in addition to the TP53 mutation, but that the former lesion goes undetected by FISH being present at sub-threshold level in a small sub-clonal component. Therefore, all the categories of TP53 alterations dealt within the present paper refer to those detected within these technical limits. This admittedly may represent a limitation, especially in cases with VAF < 10%.

The overall incidence of somatic TP53 mutations [5% (24/469 patients)] and del(17p) [2.3% (11/469 patients)] in the O-CLL1 cohort and their relative distribution are similar to that of previous reports.4,16,17,21 The majority of TP53 mutations were point mutations, causing a single amino-acid substitution in the P53 protein DNA binding domain. This was true for both the Mut/noDel and the Mut/Del patient groups. Although over 2,000 amino acid substitutions caused by a point mutation in the TP53 gene have been identified, 3 amino acid substitutions (p.Arg175His, p.Pro278Arg, and p.Ile195Thr) were shared by the Mut/noDel and Mut/Del patients’ groups.

| ID     | Protein variant | Mutant P53 residual activity (% of WT P53) |
|--------|-----------------|--------------------------------------------|
|        |                 | 30°C P21 PUMA MDM2 BAX 37°C P21 PUMA MDM2 BAX |
| 15 patients with Mut/noDel |                 |                                           |
| MS0273 | p.Arg175His     | 0 1 1 1 0 1 1 1 1 1 1 1 |
| CA0082 | p.Arg213Leu     | 54 8 21 7 2 2 3 3 3 |
| CF9003 | p.Pro278Arg     | 0 1 1 1 1 3 3 3 3 3 |
| GM0252 | p.Ile195Thr     | 6 2 2 2 1 3 3 4 4 |
| MG0248 | p.Arg282Trp     | 70 67 50 52 1 2 3 3 3 3 |
| DD0478 | p.Pro191del     | 67 38 55 42 2 3 4 5 5 5 |
| GC0448 | p.Arg248Trp     | 0 1 1 1 1 2 3 3 3 3 |
| PA0254 | p.Ala161Thr     | 96 62 77 74 2 2 4 4 4 4 |
| AR0222 | p.His193Arg     | 0 1 1 1 1 1 3 3 |
| CG0620 | p.Arg209fs      | 0 1 1 1 1 1 2 3 3 3 |
| AA0396 | p.Val157Asp     | 0 1 1 1 1 2 3 3 3 |
| CR0115 | p.Phe113Ser     | 3 1 1 1 1 3 3 3 3 3 |
| AG0464 | p.Val173Met     | 0 1 1 1 1 3 3 3 3 3 |
| SG0168 | p.Ala88fs       | 1 1 1 1 2 4 5 7 7 7 |
| DA0455 | p.Arg273His     | 0 1 1 1 1 1 3 3 |

9 patients with Mut/Del

| ID     | Protein variant | Mutant P53 residual activity (% of WT P53) |
|--------|-----------------|--------------------------------------------|
| CR0203 | p.Glu258Ter     | 0 1 1 1 0 1 1 1 3 |
| IF0044 | p.Asp281Gly     | 0 1 1 1 1 3 3 3 |
| DA0094 | p.Pro278Arg     | 0 1 1 1 1 3 3 3 3 |
| CS0290 | p.Val272Leu     | 87 64 77 74 4 3 3 3 |
| DG0193 | p.Ile195Thr     | 6 2 2 2 1 3 3 4 4 |
| FD0404 | p.Cys275Tyr     | 0 1 1 1 1 3 3 3 3 |
| PG0028 | p.Arg175His     | 0 1 1 1 0 1 1 1 1 1 |
| CG0622 | p.Ser166Ter     | 1 1 1 1 1 1 1 3 3 3 |
| DS0264 | p.Asp208Glufs   | 0 1 1 1 1 3 3 |

Table 2. Evaluation of the transactivation ability of somatic TP53 mutations using a yeast-based assay. Results are shown as residual activity of the mutant P53 protein with respect to wild-type (WT) P53 protein set as 100%. Each single mutant P53 was expressed in four different reporter yeast strains identified by the P53 Response Element (RE) from the promoter of the P21, PUMA, MDM2 or BAX effector genes. Transactivation ability was determined by growing yeast at 30°C and 37°C.
mutations (P = 0.0415), were less frequently observed in the Mut/noDel patients' group. We also observed that the TTFT of patients with mutated or WT TP53 gene was similar (P = 0.7), a finding which is in line with that of Brieghel et al.28, who demonstrated that TP53 mutations, determined by NGS, did not influence the clinical course of patients, in the absence of del(17p); this was true for cases with high (V AF ≥ 10%) and low (V AF < 10%) TP53 mutation burden28. We also did not find differences in the TTFT of Mut/noDel patients when they were stratified into two groups based upon high or low V AF, and this was also true for the patients with TP53 mutations from the mutated IGHV group stratified according to V AF values. Therefore, the size of the cell sub-clone bearing the TP53 mutations does not appear to influence disease progression for Binet stage A CLL patients. In this study, the TTFT of the patients with del(17p) was significantly shorter than that of patients without deletion (P<0.0001), while del(17p) concomitant with a TP53 mutation represented, an additional and independent prognostic factor associated with shorter TTFT in multivariate analyses. However, in interpreting these data, it should be noted that most patients with del(17p) also had a TP53 mutation, and that only two cases had del(17p).

### Table 3. Evaluation of the dominant-negative potential of somatic TP53 mutations using a yeast-based assay. Results are shown as percentage of the activity of the co-expression of wild-type (WT) and mutant P53 proteins with respect to the expression of the single WT P53 set as 100%. P53 proteins (WT and mutant) were co-expressed in yLFM-P21-5' reporter strain and grown at 30 °C. Mutant P53s are classified as dominant (D) or recessive (r), when the net activity is below or above 100%, respectively.

| ID       | Protein variant | % Net activity (P21, 30 °C) | Classification |
|----------|-----------------|-----------------------------|----------------|
| MS0273   | p.Arg175His     | 61                          | D              |
| CA0082   | p.Arg213Leu     | > 100                       | r              |
| CF0003   | p.Pro278Arg     | 73                          | D              |
| GM0252   | p.Ile195Thr     | > 100                       | r              |
| MG0248   | p.Arg282Trp     | > 100                       | r              |
| DD0478   | p.Pro191del     | > 100                       | r              |
| GC0448   | p.Arg248Trp     | 39                          | D              |
| PA0254   | p.Ala161Thr     | > 100                       | r              |
| AR0222   | p.His193Arg     | 59                          | D              |
| CG0620   | p.Arg209fs      | > 100                       | r              |
| AA0396   | p.Val157Asp     | 61                          | D              |
| CR0115   | p.Phe113Ser     | > 100                       | r              |
| AG0464   | p.Val173Met     | 40                          | D              |
| SG0168   | p.Ala88fs       | > 100                       | r              |
| DA0455   | p.Arg273His     | 32                          | D              |

Figure 2. Influence of residual functionality of mutated P53 proteins on TTFT in O-CLL1 patients. Kaplan–Meier curves of cases belonging to the MUT/noDel group, clustered on the basis of (A) residual transactivation ability of the mutated P53 protein (≥ 20% versus < 20% with respect to WT P53) as determined in the yeast reporter strain yLFM-P21-5'; (B) dominant-negative (DN) or recessive (rec) classification of the mutated P53 protein, as determined in the yeast reporter strain yLFM-P21-5'.
without a TP53 mutation. Therefore, it is difficult to precisely evaluate the contribution of del (17p) alone to disease progression with this low number of cases. However, it is of note that Yu et al.\textsuperscript{29} reported that Mut/Del\textsubscript{CLL} patients had a TTFT shorter than that of the Mut/noDel patients. Moreover, Hoechstetter et al.\textsuperscript{30} showed that del(17p) is the highest weighted factor of the six considered in a multivariate analysis to predict TTFT and OS in Binet stage A CLL, although it requires the cooperation of additional factors to determine progression.

Unlike that reported in this and in the other studies quoted above, Dicker et al.\textsuperscript{18} and Rossi et al.\textsuperscript{5} reported that TP53 mutations alone were capable of predicting a shorter TTFT. These discrepancies are difficult to explain.

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**Figure 3.** Analysis of the prognostic impact of TP53 alterations in O-CLL1 cohort study. Cox multivariate analysis of biological and clinical variables found to be significant on univariate analysis. Presence of a TP53 mutation associated with del(17p) was forced in the model as measure of P53 dysfunction.

**Figure 4.** Determination of variant allele frequency (VAF) at 36 months after diagnosis or at progression in O-CLL1 cohort study. TP53 mutational status was re-assessed in 171 patients by NGS after 36 months from the initial test or at disease progression. Presented are the 8 cases with a TP53 mutation at diagnosis, which continued to express the same mutation, albeit with some degree of clonal expansion. FISH analysis for del(17p) was repeated in all cases after 36 months (36 m, red bars) or at the time of therapy need (prog, red bars): none of the cases negative for del(17p) (Mut/noDel) became del(17p) positive. Percentage increase in the VAF of the samples analysed at 36 m/prog, compared to the samples at T0 (grey bars), are indicated in the figure only for cases in which it clearly occurred (CA0082, DD0478, GM0252, PA0254, PG0028). The increment was calculated according to the formula: (VAF 36 m/prog–VAF T0)/VAF 36 m/prog × 100. Of the two cases that experienced progression, PG0028 presented an increase of the percentage of del(17p) positive cells and of VAF (78%). The other case (AA0396) showed no increase in VAF.
P53 proteins can acquire new functions favouring tumour cell expansion. These properties, indicated collectively cases and further analyses in larger cohorts seem to be needed. Furthermore, it is worth recalling that mutant However, the interpretation of these data requires caution because of the relatively low number of Mut/noDel cases and further analyses in larger cohorts seem to be needed. Furthermore, it is worth recalling that mutant P53 proteins can acquire new functions favouring tumour cell expansion. These properties, indicated collectively as gain of function, may in principle be present in CLL patients and perhaps have a higher incidence in more advanced cases. These too deserve analyses in large cohorts of patients.

IGHV mutational status represents a valuable prognostic marker for risk progression and outcome. In the O-CLL1 cohort, the majority of cases with del(17p) had unmutated IGHV genes, whereas the majority of Mut/noDel cases had mutated IGHV genes. This finding may concur to explain why Mut/noDel cases progressed more slowly to the more advanced stages requiring therapy (Table 1). Furthermore, Mut/noDel cases were mostly negative for other unfavourable prognostic markers, whereas this was not the case for the group of patients with del(17p) (Mut/Del and WT/Del) (Table 1). These considerations may also help explain the discrepancies in the prognostic role of TP53 mutations between this study and those including Binet B and C patients. Since unmutated-IGHV cases are likely to progress more rapidly towards advanced stages, it is possible that cases with mutated IGHV genes and TP53 mutations, that may not progress, are less frequently found in cohorts comprising numerous advanced cases.

Re-evaluation of 171 patients from this study following a 36 months interval or at the time of therapy need did not reveal any changes in TP53 alterations status. Specifically, there was neither an acquisition/loss of a TP53 mutation nor of del(17p). Only in one patient an expansion of a sub-clone carrying a non-functional TP53 mutation was observed at progression.

In conclusion, the present study based on a clinically and molecularly well-characterised Binet stage A CLL cohort demonstrates that the occurrence of del(17p) significantly predicted TTFT, while that of a TP53 mutation alone, was unable of such prediction.

Materials and methods.

All methods were carried out in accordance with relevant guidelines and regulations.

Patients and CLL cells preparation. Only previously untreated Binet stage A CLL patients not requiring therapy according to NCI guidelines were prospectively enrolled within 12 months of diagnosis (O-CLL1 protocol, clinicaltrial.gov identifier NCT00917540). This protocol was presented by the Gruppo Italiano Studio dei Linfomi (GISL) on behalf of several Italian participating institutions and approved by the Ethics Review Committee (Comitato Etico Provinciale, Modena, Italy). Written informed consent was obtained from all patients in accordance with the declaration of Helsinki. The ethics committees from each participating centre (listed in the acknowledgements) approved this study. The median time between diagnosis and patient enrolment in the study was 2.3 months. A total of 420 (89.6%) and 386 (82.3%) patients had follow-up data at 1- and 2-year, respectively. Recruitment began in January 2007 and the criteria for CLL diagnosis employed followed the 1996 NCI/IWCLL classification. Treatment was decided uniformly for all participating centres based on documented progressive and symptomatic disease according to National Cancer Institute-sponsored working guidelines. CLL cell phenotypes, CD38, and ZAP-70 expression, and IGHV mutational status assessment was centralised in the laboratory in Genoa, while all FISH assays were performed in Milan.

The median age of the entire cohort was 61.2 years, 214 cases (41%) were female. Finally, at the time of the present analysis, 179 cases (35.2%) progressed and were treated.

Ethical parameters. Ethical parameters, included in the synopsis of O-CLL1 protocol, clinicaltrial.gov identifier NCT00917540 were the following: It is responsibility of the investigator(s) to submit a copy of the protocol and detailed patient information sheet-consent form to an Independent Ethics Committee or Institutional Review Board in order to obtain independent approval to conduct the study. It is responsibility of the investigator(s) to ensure that the study is conducted in full conformance with the principles of the current version of the Declaration of Helsinki and to ensure that the study is performed in accordance with the international Good Clinical Practice (GCP) standards and according to all local laws and regulations concerning clinical studies.

CLL cells preparations. Peripheral blood mononuclear cells (PBMCs) from patients with CLL were isolated by Ficoll-Hypaque (Seromed, Biochrom) density gradient centrifugation. CD19-positive CLL cells were enriched by negative selection with the EasySep-Human B-cell Enrichment Kit without CD43 depletion (STEM-CELL Technologies, Voden Medical Instruments S.p.A.), using the fully automated protocol of immunomagnetic cell separation with RoboSep (Stem Cell Technologies). The percentage of purified B Cells (CD19+) exceeded 95%, as detected by flow cytometry.
Amplification Refractory Mutation System (ARMS)-PCR as previously described. c.7541_7542delCT mutation was tested by NGS using Roche 454 technology and subsequently validated by the Ion Torrent platforms (Thermo Fisher Scientific Carlsbad, CA) in 241 cases (see https://tools.thermofish er.com/content/sfs/manuals/MAN0013432_ION_AmpliSeq_Library_Prepare_on_Ion_Chef_UG.pdf for further details). Sixteen cases were tested using both platforms with 100% concordance. Briefly, TP53 libraries were prepared using genomic DNA extracted from CD19+ purified B cells (QIAamp DNA Blood Mini Kit, Qiagen Hilden, Germany) according to the respective protocols (see above) and then sequenced with both NGS platforms taking into account the achievement of a threshold of 500 reads for each TP53 amplicon. Cases with VAF ≥ 2% were considered positive for TP53 mutation; in addition, samples with TP53 variant <10% VAF were re-evaluated by a second NGS run. The panel utilized for the Ion torrent NGS study was the Ion AmpliSeq™ TP53 Panel comprising 24 primer pairs across 2 pools that provides 100% of coverage of exons 2–11 and exon–intron boundaries (± 30 bp padding). The TP53 primer panel applied to Roche NGS technology spanned exons 4 to 9. Of the 171 cases sequenced (131 with Roche J, 51 with Ion Torrent, 11 were subsequently analysed using both sequencing approaches with concordant results), TP53 status was determined not only at the time of diagnosis, but also after 36 months or at the time of therapy need. Any TP53 variant identified [exonic, intronic and frequent SNPs (e.g. Pro72Arg)] identified by NGS, was recorded for each sample. For all samples in which the variant was verified, and the VAF was approximately 50%, suggesting a germline origin, the patient-matched normal tissue DNA was subsequently analysed by NGS. For patients CG0015 and RA0023, DNA was obtained from buccal swabs (3 separate swabs per patient). For GS0473, RO0479, and NF0056 non-tumour DNA was obtained from CD3+ cells obtained by triple staining with CD19, CD5, and CD3 mAbs (Becton Dickinson) followed by cell sorting (FACS ARIA II, Becton Dickinson) of CD3+CD5+ cells to avoid any contamination of CD19+CD3+CD5+ neoplastic cells. No mutations in exons 2, 3, or 11 were identified by Ion Torrent NGS among the 241 CLL cases analysed using this platform, while in only one case (1/241, 0.4%) a germline variant, was discovered in exon 10. This suggests that there was a number of mutated samples, possibly not being detected by sequencing of exons 4–9 with the Roche platform, that was negligible or non-existing, and that the results obtained with the two methods were comparable. Overall, sequencing of the entire coding portion of the gene using the Ion AmpliSeq™ TP53 Panel indicated that exons 4–8 were the most recurrently mutated. The functional analysis, performed using a yeast-based assay, was applied, to those TP53 variants causing either amino-acid substitutions or coding for a truncated P53 protein.

Evaluation of P53 function in a yeast-reporter assay. Yeast strains and media. The yLFM-P21-5', yLFM-PUMA, yLFM-MDM2P2C, and yLFM-BAX A+B yeast strains were used to assess the functionality of TP53 variants: all strains were isogenic except for the different P53 response element (RE) located upstream of the luciferase reporter gene (LUC1). Cells were grown inYPD medium (1% yeast extract, 2% peptone, 2% dextrose, 200 mg/L adenine) or in selective medium (with or without 2% agar) containing dextrose or raffinose as a carbon source plus adenine (200 mg/L), but in the absence of tryptophan and/or leucine (Sigma-Aldrich, Saint Louis, Missouri, USA; Biokar Diagnostics, Allonne, France). Galactose (Sigma-Aldrich, Saint Louis, Missouri, USA) was added to the medium to modulate P53 expression under the inducible GAL1,10 promoter. \n
Yeast vectors. For the transactivation assay, human WT and mutant P53 proteins were expressed using a pTSG-based vector (TRP1). The P53 mutants were constructed in the pTSG-based vector (through SgrAl/Stul digestion and subsequent ligation) (New England Biolabs) from available pLS-based vector. When cloned mutants were not available, P53 mutants were firstly constructed in a pLS- or pTSG-based vector expressing in vivo yeast
homologous recombination, as previously described\(^{42}\) and then cloned (through SgrAI/StuI or XhoI/NotI digestion and subsequent ligation) (New England Biolabs) in the pTSG-based vector.

For the dominance assay, P53 mutant protein was expressed using a pLS- or pTS-based vector; the pLS89 (TRP1) or pLS89 (LEU2) expressing WT P53 protein under the inducible GAL1,10 promoter was co-transformed in yeast, based on the previously used selection marker. Plasmid pRS314 (TRP1) and pRS315 (LEU2) were used as empty vectors. The list of primers used to construct pLS- or pTS-based P53 mutant vectors is available upon request.

**Yeast functional assay.** Quantitative functional assays (evaluation of transactivation ability and DN potential) were performed according to the miniaturized protocol we developed\(^{43}\). The transactivation activity of a mutant P53 was measured by calculating the percentage with respect to WT P53 (set as 100%); the DN potential was calculated by comparing the net activity of WT and mutant P53 co-expression with respect to the expression of WT P53 alone (set as 100%). A mutant P53 was defined as recessive or dominant when the net activity was above or below 100%, respectively.

**Evaluation of P53 function in a mammalian reporter-assay.** Cell line and media. HCT116 TP53\(^{−/−}\) cells (human colon carcinoma) were obtained from Dr. B. Vogelstein (The Johns Hopkins Kimmel Cancer Center, Baltimore, MD). Cells were grown in RPMI containing 10% foetal bovine serum, L-glutamine, and a penicillin–streptomycin antibiotic mixture (Euroclone, Milano, Italy), and maintained at 37 °C in 5% CO2 at 100% humidity.

*Mammalian expression and reporter vectors.* A pCMvneo-based (Promega) vector was used to express WT and mutant P53 in mammalian cells. Plasmids expressing mutant P53 were constructed as previously described\(^{42}\). The pGL3-1138 and the pRL-SV40 plasmids were used as reporter (P21 promoter) and normalization vectors, respectively\(^{49}\).

*Mammalian functional assay.* HCT116 TP53\(^{−/−}\) cells transfected with pCI-neo-based P53 expression vectors, the reporter, and normalization plasmids, were collected and washed with cold PBS. Lysis was performed in 1X PLB buffer (Passive Lysis Buffer, Promega). Luciferase assays were conducted as previously described\(^{49}\).

**Statistical analysis.** Statistical analyses were performed as previously described\(^{25,27}\). Briefly, SPSS for Windows, v13.0, 2004 software (SPSS, UK) was used for all the analyses. We performed, statistical comparisons, for categorical variables, using two-way tables for the Fisher's exact test, while multiway tables were used for the Pearson's Chi-square test. TTFT analyses were performed using the Kaplan–Meier method in patients with a minimum follow-up. Using the log-rank test, we calculated the statistical significance of associations between individual variables and survival. We investigated the prognostic impact for the outcome variable by univariate and multiple Cox regression analysis. Data were expressed as hazard ratio (HR) and 95% confidence interval (CI). A value of \(P<0.05\) was considered statistically significant.

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Author contributions

M.L., G.DeL., S.M.M.C.S.F., M.B., S. Z., A.G.R., and M.D. performed biological characterization (including NGS sequencing and cytogenetic analysis) of CLL samples. PMo PMe, and AS were involved in the functional characterisation of mutant P53; M.G., A.I., A.N. M.F., and F.M. were involved at the clinical level; M.F. e F.M. performed NGS sequencing and cytogenetic analysis) of CLL samples. PMo PMe, and AS were involved in the functional characterisation of mutant P53; M.G., A.I., A.N. M.F., and F.M. were involved at the clinical level; M.F. e F.M. conceived

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Competing interests
The authors declare no competing interests.

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