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Short Report

TP53 mutations are early events in chronic lymphocytic leukemia disease progression and precede evolution to complex karyotypes

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TP53 **abnormalities lead to resistance to purine analogues and are found in over 40% of patients with refractory chronic lymphocytic leukemia (CLL). At diagnosis, no more than 5% of patients carry the 17p deletion, most cases harbour mutations within the other *TP53* allele. The incidence of a *TP53* mutation as the only alteration is approximately 5%, but this depends on the sensitivity of the technique. Recently, having a complex karyotype has been considered a strong adverse prognostic factor. However, there are no longitudinal studies simultaneously examining the presence of the 17p deletion, *TP53* mutations and karyotype abnormalities. We conducted a retrospective longitudinal study of 31 relapsed/refractory CLL patients. Two to six blood samples per patient were analyzed, with a median follow-up of 8 years. In this report, we assessed the sequence of events of *TP53* clonal evolution and correlated the presence of *TP53* abnormalities to genetic instability during progression and treatment. Next-generation sequencing allowed the early detection of *TP53* mutated clones and was able to be performed on a routine basis, demonstrating an excellent correlation between the Illumina and Ion Torrent technologies. We concluded that *TP53* mutations are early events and precede clonal evolution to complex karyotypes. We strongly recommend the early and iterated detection of *TP53* mutations in progressive cases.**

TP53 mutations and 17p deletion are associated with a poor prognosis in CLL.[1–4] *TP53* alterations are observed in approximately 10% of treatment-naïve CLL patients, but in up to 40–50% of fludarabine-refractory CLL patients.[5] *TP53* mutations are detected in over 80% of cases with 17p deletions,[1,4] but *TP53* mutations are also observed in a significant proportion of CLL with no 17p deletion with similar adverse outcomes.[1] Small subclones with *TP53* mutations, detected only by sensitive techniques, are associated with the same adverse prognosis as large clones.[6] As new targeted therapies are changing the treatment landscape,[7] it becomes increasingly important to detect *TP53* abnormalities in a sensitive manner. However, use of next-generation sequencing (NGS) in clinical practice is not yet established.[8]

Moreover, complex karyotypes (≥3 abnormalities) have recently been noted as having a major prognostic impact,[1,9] but the sequential occurrence of *TP53* abnormalities and complex karyotypes has not been addressed in a longitudinal study so far.

In this report, we assessed the sequence of events of clonal evolution through a longitudinal study and correlated the presence of *TP53* abnormalities to genetic instability during progression and treatment.

Key words: CLL, TP53, clonal evolution
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What's new?

For patients with chronic lymphocytic leukemia, possessing multiple chromosomal abnormalities means bad news. Could there be a way to predict genomic instability before it happens? These authors investigated whether mutations in *TP53* might presage the onset of chromosomal abnormalities. They conducted a longitudinal study, in which they followed 31 CLL patients over a period of several years. It turned out that mutations in *TP53* occur early and precede the genomic instability that generates chromosomal abnormalities and hinders recovery. Thus, searching for *TP53* mutations could identify those patients likely to develop additional chromosomal defects and poor outcomes.

Material and Methods

We conducted a longitudinal retrospective study in a high-risk cohort of 31 relapsed/refractory patients (Supporting Information Table 1). Two to six blood samples per patient (Median 3) were analyzed, with a median follow-up of 8 years (2.3–22.5 years).

For conventional cytogenetic analysis and fluorescence *in situ* hybridization, blood samples were incubated in RPMI medium supplemented with 10% fetal calf serum at 37°C for 48 to 96 h and stimulated with 12-O-tetradecanoylphorbol-13-acetate (TPA) or CpG-oligonucleotides and interleukin 2. Cells were prepared using routine cytogenetic techniques. Chromosomes were identified by R-banding. Cytogenetic abnormalities were described according to the International System for Human Cytogenetic Nomenclature.

FISH was performed using *TP53* probe associated with alpha-satellite from chromosome 17 as control (Cytocell, Cambridge). Metaphases and at least 200 interphase nuclei were analyzed. The cutoff level for the diagnosis of del(17p) was 10.2%.[10,11] Cell images were acquired with microscope Axioplan 2 Imaging (Carl Zeiss, GmbH, Jena) and analyzed by CytoVision image analysis system ISIS (Metasystems). An abnormality was considered clonal when at least two metaphases had the same aberration in case of a structural abnormality or an extra chromosome. For classification as a monosomy, the monosomy had to be present in at least three metaphases.

TP53 mutations were screened on DNA using Sanger (Exons 4–9) (3500 Dx Series Genetic Analyzer ThermoFisher, Saint Aubin, France)[12] and NGS of the entire coding region of *TP53 (*Ion AmpliSeq^{TM} TP53 Panel ThermoFisher). The results were validated on two different platforms: Ion PGM system (ThermoFisher) and Miseq (Illumina GmbH Munich, Germany). The results were validated when >90% amplicons showed at least 100 reads, 100% target base coverage were over 20x and a minimum of 11 reads supported the variant. *TP53* variants were analyzed using the IARC (www-p53.iarc.fr) and the UMD *TP53* mutation databases (p53.free.fr). Each *TP53* variant was validated by TP53_MUT_ASSESSOR, a multicriteria algorithm specifically developed for the analysis of TP53 mutation and based on the 2015 issue of the UMD TP53 database containing 60,000 TP53 mutations.[13]

Results and Discussion

Among the relapsed/refractory cohort, *TP53* was found to be mutated in 16/31 patients. A total of 29 mutations were detected in Exons 4 to 9; they were not clustered in any known hotspot (Supporting Information Table 2). These mutations were validated as being pathogenic using the TP53_MUT_Assessor algorithm; 27 had been previously described in other tumors and were known to correspond to impaired transcriptional activity. The two novel variants (deletions of 8 and 22 nucleotides) were likely pathogenic, as they impaired P53 protein expression.

NGS technology detected small *TP53* mutated clones that were not evidenced by Sanger sequencing (Supporting Information Table 3). All mutations identified using Sanger methodology, were confirmed using NGS Ion Torrent and analyzed according to the European Research Initiative on CLL (ERIC) recommendations.[8] Illumina and Ion PGM NGS sequencing generated highly similar results with notably an identical percentage of variant allele frequency (VAF). Only one deletion, of 24 bp covering a splice site, was missed by PGM due to the design of the primers which overlapped the deleted bases both in forward and reverse strands. Moreover, a subclonal deletion of 5 bp, only detected using Ion Torrent, and a 8 bp subclonal deletion, only detected using Sanger technology, were discarded by PGM and Illumina variant calling algorithms because of the low quality score of the alignment between two homopolymers.

At the time of diagnosis, *TP53* mutations were detected in 10/31 (32%) patients. Three of these mutations were detected by NGS technology only as allele frequency ranged from 3.5 to 20%.

Deletion of 17p was systematically investigated by FISH and was present in 7/31 (23%) patients. All the patients with 17p deletions carried a mutation on the other allele. This is in concordance with the publication by Landau *et al.*[14] suggesting that *TP53* is an exception among tumor suppressor genes which are essentially haplo-insufficient to begin with. As described by Landau *et al.*[14] we have no evidence that del(17p) alone could be detrimental as we did not detect any in our series. Three additional cases harbored only mutations; two of which had more than one mutation. Eight patients harbored a complex karyotype at diagnosis, for five patients it was associated with *TP53* abnormalities.

During progression, a clonal evolution with either *TP53* alterations or the development of a complex karyotype was observed in 14/31 (45%) patients. Among patients who had *TP53* abnormalities at diagnosis, the acquisition of further *TP53* subclonal alterations was very frequent, that is, 5/10 (3 novel *TP53* mutations, one

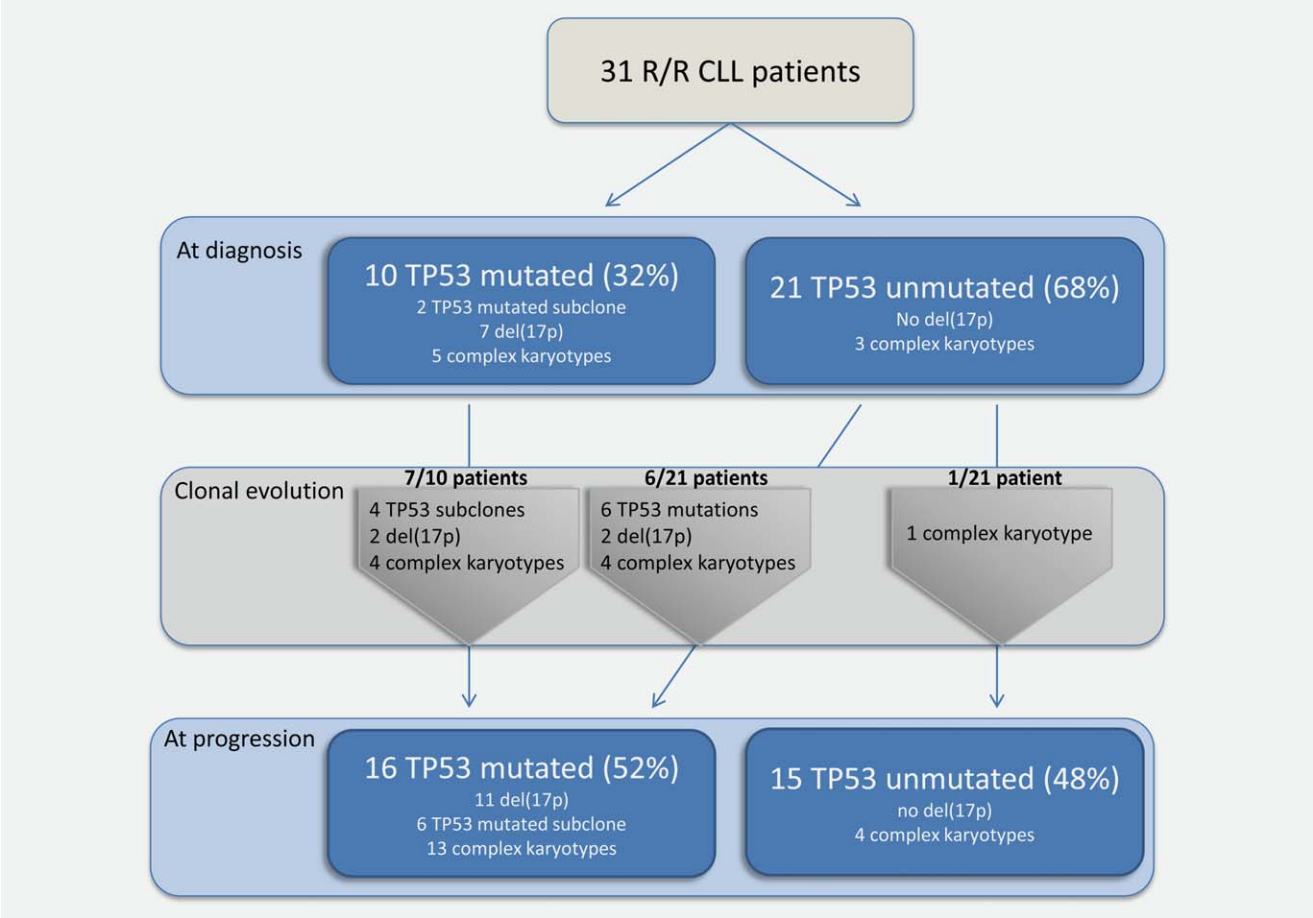

Figure 1. *TP53* abnormalities and karyotype evolution of the relapse/refractory (R/R) CLL patients. This chart shows the evolution of *TP53* abnormalities (mutation and 17p deletion) and complex karyotypes (≥3 abnormalities) from diagnosis to progression in our relapse/refractory cohort. Clonal evolutions include either additional *TP53* sub-clonal mutations or 17p deletion and the evolution of the karyotype to a complex karyotype. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

17p deletion, and one with both a *TP53* mutated subclone and 17p deletion), in agreement with what previously described.[15] A complex karyotype emerged in four cases. In 6/21 (28%) patients without *TP53* alterations at diagnosis, a *TP53* alteration was found during progression; two of these patients also acquired a 17p deletion, and four acquired a complex karyotype. Of note, only one patient with no *TP53* mutation acquired a complex karyotype (Fig. 1).

In this high-risk cohort, unfavorable prognostic factors such as unmutated IGHV, mutations of *SF3B1* and *NOTCH1*, or the presence of an 11q deletion (Supporting Information Table 1) had a similar incidence whether *TP53* abnormalities were present or not. Conversely, the incidence of complex karyotypes was strikingly different in both groups. During progression, eight patients carrying *TP53* mutations acquired a complex karyotype, as compared to only one in the group without *TP53* alterations ($p = 0.011$). Interestingly, in 5/8 cases, the longitudinal analysis indicated that the acquisition of *TP53* mutations clearly preceded karyotype evolution (Fig. 2). Altogether, a complex karyotype was observed in 13/16 patients with *TP53* mutations, whereas it was present in 4/15 patients without *TP53* mutations ($p = 0.0038$). This observation highlights the genetic instability related to the presence of a *TP53* mutation, and its likely role in the development of a complex karyotype.[16]

It is widely accepted that chemotherapy plays a key role in driving the selection of clones carrying *TP53* mutations.[17] In our cohort, after treatment, 6/21 patients with no *TP53* alteration at diagnosis acquired one and 7/10 patients with *TP53* mutations from diagnosis acquired at least one additional subclone. This significant association between *TP53* abnormalities and clonal progression after chemotherapy ($p = 0.045$) is on line with the impact of purine analogues. However, three patients acquired a *TP53* mutation before any treatment (UPN 4, 6 and 11, Fig. 2). This suggests that *TP53* mutations detected after therapy are selected rather than directly induced by therapeutic agents, which is in agreement with the scenario proposed by Malcikova *et al.*[17] Moreover, UPN 4 carrying both a del(17p) and *TP53* mutation at diagnosis remained stable and untreated over a period of 13 years. It is noteworthy that this patient had mutated IGHV genes. This is in agreement with Delgado *et al.*[18] showing

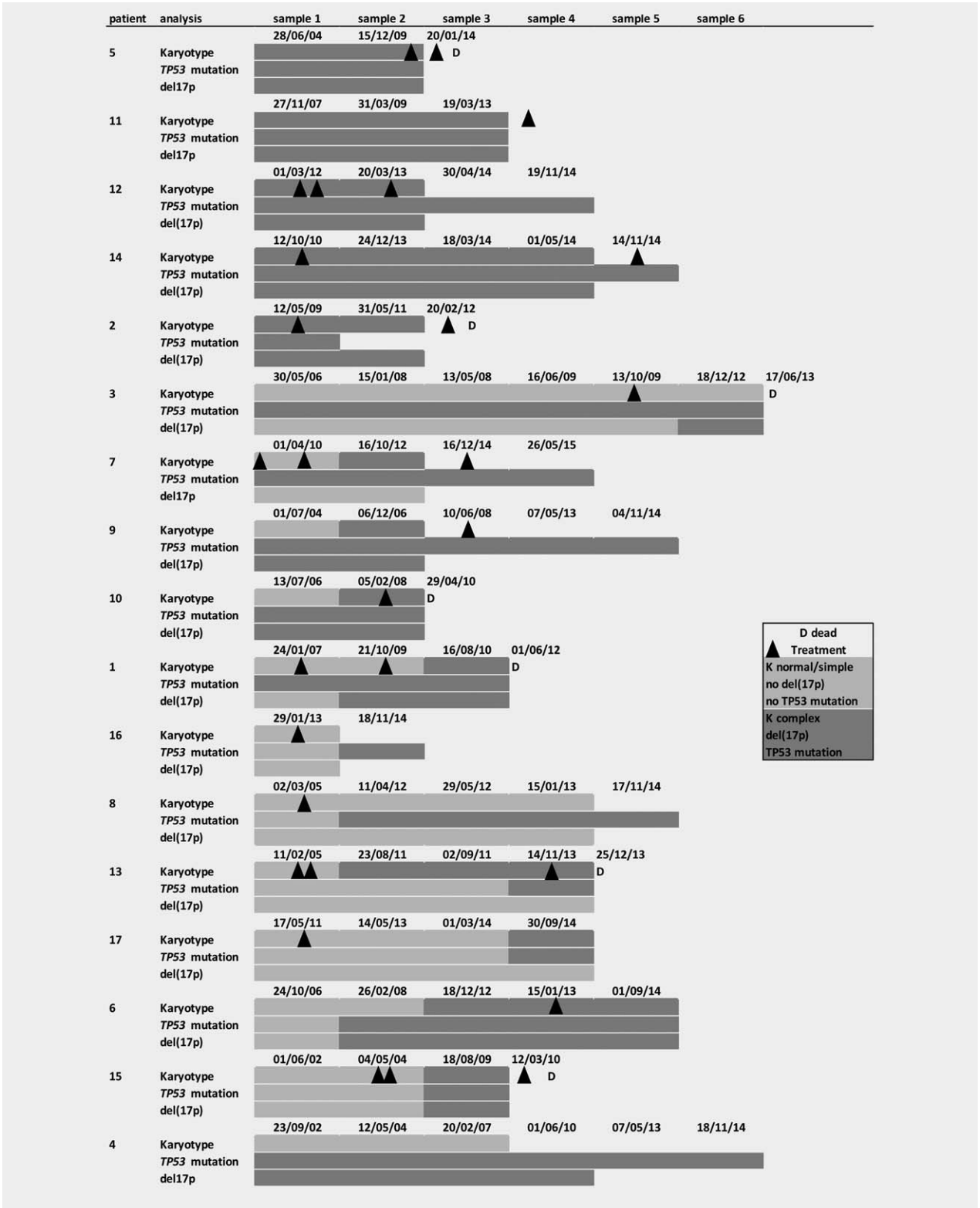

Figure 2. Longitudinal analysis of *TP53* mutations, 17p deletions and karyotypes. Follow-up of the 16 patients with *TP53* mutations was conducted at any time in their disease. Two to six blood samples per patient (Median 3) were analyzed, with a median follow-up of 8 years (2.3–22). Dark grey bars indicate the presence of *TP53* mutations, 17p deletion, or a complex karyotype. Light grey bars represent the absence of *TP53* mutations, the absence of 17p deletion, or a normal or simple karyotype. Triangles represent lines of treatment and D represents the date of death. Patient 4, who remains untreated and is not included in this R/R cohort, was added to the chart as an example of the acquisition of subclonal mutations independent of any treatment.

that absence of IGHV mutations is a key predictor of poor outcome of CLL patients with TP53 disruption.

Each patient received 1 to 3 treatment lines. Both, time to first treatment (TFT) and overall survival were identical for the group with or without *TP53* abnormalities (mean TFT 4.4 and 3.9 years, respectively). Sixteen patients received fludarabine as a first-line treatment. In three cases with isolated *TP53* mutations at diagnosis, the *TP53* mutated clone expanded at relapse (Patients 1, 3, 7). In four other patients, *TP53* alterations were only detectable after fludarabine treatment (Patient 8, 13, 15, 17). Eight patients known to have mutated *TP53* before treatment received Campath-Dexamethasone; in five of them tested at relapse, the *TP53* mutation clone had expanded. Four patients with *TP53* mutations received ibrutinib as a second or third treatment (Patients 7, 12, 14, 16). After one year on ibrutinib, the percentage of VAF in the residual lymphocytosis was stable. These patients are now in clinical remission reflecting the efficacy of ibrutinib on the mutated clones. However, the persistence of the mutated clones shows the failure of eradicating the disease.

In conclusion, the occurrence of complex karyotypes is significantly associated with *TP53* abnormalities. *TP53* mutations are related to genetic instability, clearly preceding the onset of complex karyotypes. Chemotherapy favors the selection and expansion of mutated clones. Thus, we recommend early and iterative screening for *TP53* abnormalities during follow-up and before each new line of treatment. NGS dedicated sequencing assays are doable on a routine basis, and could represent the technique of choice in clinical practice.

Authorship Contributions

G.L. collected, analyzed, and interpreted the data and wrote the paper. E.T. analyzed and interpreted the data. V.E. analyzed and interpreted the data. A.S., V.B., V.L. and J.F.C. performed the sequencing. R.L. interpreted the data. L.G. co-wrote the manuscript. N.V.B. reviewed the study. T.S. analyzed the data. S.S. designed and interpreted the data. F.C. designed the research; collected, analyzed and interpreted the data; and wrote the paper. F.B.M. designed the research; collected, analyzed and interpreted the data; and wrote the paper.

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