Clinical impact of small TP53 mutated subclones in chronic lymphocytic leukemia

Davide Rossi, Hossein Khliabani, Valeria Spina, Carmela Ciardullo, Alessio Bruscaggin, Rosella Famà, Silvia Rasi, Sara Monti, Clara Deambrogi, Lorenzo De Paoli, Jiguang Wang, Valter Gattei, Anna Guarini, Robin Foà, Raul Rabadan, and Gianluca Gaidano

Division of Hematology, Department of Translational Medicine, Amedeo Avogadro University of Eastern Piedmont, Novara, Italy; Department of Systems Biology and Department of Biomedical Informatics, Center for Computational Biology and Bioinformatics, Institute for Cancer Genetics, Columbia University, New York, NY; Clinical and Experimental Onc-Hematology, Centro di Riferimento Oncologico, Aviano, Italy; and Division of Hematology, Department of Cellular Biotechnologies and Hematology, Sapienza University, Rome, Italy

TP53 mutations are strong predictors of poor survival and refractoriness in chronic lymphocytic leukemia (CLL) and have direct implications for disease management. Clinical information on TP53 mutations is limited to lesions represented in >20% leukemic cells. Here, we tested the clinical impact and prediction of chemorefractoriness of very small TP53 mutated subclones. The TP53 gene underwent ultra-deep-next generation sequencing (NGS) in 309 newly diagnosed CLL. A robust bioinformatic algorithm was established for the highly sensitive detection of few TP53 mutated cells (down to 3 out of ~1000 wild-type cells). Minor subclones were validated by independent approaches. Ultra-deep-NGS identified small TP53 mutated subclones in 28/309 (9%) untreated CLL that, due to their very low abundance (median allele frequency: 2.1%), were missed by Sanger sequencing. Patients harboring small TP53 mutated subclones showed the same clinical phenotype and poor survival (hazard ratio = 2.01; P = .0250) as those of patients carrying clonal TP53 lesions. By longitudinal analysis, small TP53 mutated subclones identified before treatment became the predominant population at the time of CLL relapse and anticipated the development of chemorefractoriness. This study provides a proof-of-principle that very minor leukemia subclones detected at diagnosis are an important driver of the subsequent disease course. (Blood. 2014;123(14):2139-2147)

Introduction

TP53 mutations represent strong predictors of poor survival and refractoriness in chronic lymphocytic leukemia (CLL) and, for these reasons, they have a well-established clinical relevance and direct implications for the management of this leukemia. To date, information on the clinical relevance of TP53 mutations in CLL is limited to lesions that are clonally represented in the leukemic population, as revealed by Sanger sequencing that is the most widely adopted method to assess TP53 mutation status in this leukemia. Next generation sequencing (NGS) technologies provide a novel opportunity to examine in depth the clonal heterogeneity of the CLL genome, with the potential for sensitive detection of mutations restricted to a small fraction of the total tumor cell population. Exploiting these approaches, recent genomic studies have disclosed the complexity of CLL clonal architecture and provided the proof-of-principle that genetically diverse subclones may be admixed with a dominant leukemic clone. Although genomic studies have depicted the landscape of the clonal complexity of CLL, little is known about the clinical implications and dynamics of very small subclones that may be present, but are commonly undetected, in the leukemic cell population.

Understanding the significance of small CLL subclones might be particularly important if they are driven by genetic lesions associated with treatment resistance, such as TP53 mutations. In this respect, analysis of the subclonal architecture of TP53 mutations in the early disease phases may help anticipate the genetic composition of later phases of the disease, including chemorefractoriness and relapse, and may also predict the disease ultimate clinical course. In this study, by using a highly sensitive ultra-deep-NGS approach capable of detecting few mutated cells, we tested the clinical impact of small TP53 mutated subclones on CLL outcome.

Patients and methods

Patients

The study population was a consecutive series of 309 newly diagnosed CLL patients (Table 1) who were prospectively registered in the Amedeo

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Table 1. Characteristics of the whole CLL series of patients harboring solely subclonal TP53 mutations, and of patients harboring clonal TP53 mutations

<table>
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<th>Characteristics*</th>
<th>All (n = 309)</th>
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<th>Clonal TP53 mutations (n = 28)</th>
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*IGHV, immunoglobulin heavy variable gene; CDR3, complementarity determining region 3.
†IGHV mutation status was assessable in 304 patients; 5 patients lacked productive IGHV-IGHD-IGHJ rearrangements.
‡P value for the comparison between cases harboring solely subclonal TP53 mutations vs clonal TP53 mutations.

Avogadro University CLL-database from December 1996 through October 2011. CLL diagnosis was according to International Workshop on CLL-National Cancer Institute (IWCLL-NCI) criteria.5 Fifty-three cases presented with symptomatic disease according to guidelines2 and were therefore treated at diagnosis. The study population was provided with sequential tumor samples and clinical information prospectively collected at clinically relevant time points. The database was updated in May 2013. The median follow-up of living patients was 8.1 years. No patient was lost to follow-up.

The study was designed to assess differences in overall survival (OS) between cases harboring a wild-type TP53 gene and cases harboring small TP53 mutated subclones. The exact prevalence of small TP53 mutated subclones in CLL is currently unknown. Assuming that small TP53 mutated subclones occur in at least 10% of the population, we estimated that 309 patients harboring small TP53 mutations (power = 81%; α = 0.01).

The REMARK criteria were followed throughout this study (supplemental Table 1, available on the Blood Web site).18 Patients provided informed consent in accordance with local institutional review board requirements and the Declaration of Helsinki. The study was approved by the Ethical Committee of the Ospedale Maggiore della Carità di Novara associated with the Amedeo Avogadro University of Eastern Piedmont (protocol code 59/CE; study number CE 8/11).

Specimen characteristics

TP53 mutation screening was performed on peripheral blood (PB) mononuclear cell samples collected at CLL diagnosis. Clonal evolution analysis was performed on PB mononuclear cell samples collected at progression requiring treatment, relapse, and last follow-up. In all cases, the fraction of tumor cells corresponded to 70% to 98% as assessed by flow cytometry. To account for tumor representation, the frequency of the mutant TP53 alleles provided by ultra-deep-NGS was corrected for the proportion of CD19+/CD5+ cells in each sample.

TP53 sequencing

Ultra-deep-NGS of the TP53 mutation hotspots (exons 4-8, including splicing sites) was performed using the 454 chemistry and was based on amplicon libraries. The TP53 region of interest was covered by 6 sequence-specific primer pairs, each flanked by tagged sequences to barcode the samples (supplemental Table 2). In each experiment, 60 amplicons, corresponding to the TP53 region of interest of 10 distinct patients, were amplified from genomic DNA by using a high-fidelity Taq polymerase (FastStart High Fidelity PCR System; Roche Diagnostics) and subjected to ultra-deep-NGS on the Genome Sequencer Junior (454 Life Sciences) to obtain a ~2000-fold coverage per amplicon. Through this approach, the average sequencing coverage across TP53 target regions was 2660× and >87% of the sequenced amplicons had sequence coverage of >1000×. TP53 mutation analysis was also performed in parallel by Sanger sequencing as previously reported.4 Further details are available in the supplemental Appendix.

Establishment of a bioinformatic approach to call subclonal TP53 mutations out of the background error noise

To establish a robust bioinformatic approach to call subclonal TP53 mutations of low abundance out of the background error noise of deep-NGS, a pivotal dilution experiment was performed. This experiment allowed us to: 1) calibrate ultra-deep-NGS for systematic biases that lead to sequencing errors; 2) derive the distribution of sequencing errors; 3) determine the sequencing depth required for a highly sensitive detection of small (<0.5% allele frequency) mutations out of the background error noise; and 4) statistically test the confidence in discovering subclonal events (supplemental Figures 1-4). The dilution experiment established the negative binomial distribution as the best fit for ultra-deep-NGS error distribution (supplemental Methods; supplemental Figure 2) that was used to estimate statistical frequency thresholds above which true subclonal mutations are distinguished from the background error noise (supplemental Figures 3 and 4).19,24 Based on these observations, a robust bioinformatic workflow was established to call subclonal TP53 variants from ultra-deep-NGS experiments in patient samples. By this approach, we were able to detect subclonal TP53 mutations represented in at least 0.3% of the alleles (3 mutated alleles out of 1000 alleles) (supplemental Figures 2 and 3). Further details of the bioinformatic algorithm are available in the supplemental Appendix.

Validation of small TP53 mutated subclones

Small TP53 mutated subclones called by the bioinformatic algorithm were validated by a double-step experimental approach. In the first step,25 subclonal
variants were subjected to independent polymerase chain reaction (PCR) amplification and ultra-deep-NGS sequencing experiments using the same experimental conditions and coverage described above. In the second step, subclonal TP53 variants were further validated by allele specific PCR (AS-PCR). Further details are available in the supplemental Appendix.

Databases

TP53 mutations were annotated using the IARC TP53 database. For each TP53 missense mutation, the CDKN1A promoter-specific transcriptional activity measured in yeast functional assays was extracted and expressed as percent of wild-type activity. The molecular and functional profiles of clonal TP53 mutations in CLL were derived from public databases.

Statistical analysis

OS from diagnosis was the primary end point and was measured from date of initial presentation to date of death from any cause (event) or last follow-up (censoring). Analysis of OS from first treatment was a secondary and exploratory end point, included 53 newly diagnosed patients who presented with symptomatic disease requiring treatment, and was measured from date of first treatment to date of death from any cause (event) or last follow-up (censoring). Molecular studies were blinded to the study end points. Survival analysis was performed by the Kaplan-Meier method. To test the independence of the prognostic value of small TP53 mutated subclones on OS, we performed a comprehensive multivariate Cox analysis with backward-stepwise elimination of nonsignificant covariates. None of the covariates violated the proportional hazard regression assumptions. Bias corrected prediction accuracy of the Cox model to protect against overfitting. The maximally selected rank statistics was used to identify a cutoff in the size of the 50% CI range: 0.9% to 20.5%) compared with wild-type TP53 (Figure 1C). Overall, the molecular and functional profiles of subclonal TP53 mutations did not significantly differ from those of TP53 variants that gained clonal representation in CLL (Figure 1). These data indicate that ultra-deep-NGS significantly adds to the analysis of TP53 genetic defects in CLL by allowing the identification of small TP53 mutated subclones among patients that would be otherwise considered wild type for the TP53 gene according to Sanger sequencing.

Subclonal mutations have the same detrimental impact on TP53 function as clonal defects

The molecular profile and functional impact of subclonal TP53 mutations was dissected to discriminate whether they act as pathogenic drivers or as irrelevant passengers in CLL. Subclonal TP53 mutations were mainly missense substitutions (78%, 39/50) mapping in the DNA-binding domain of the TP53 protein and recurrently affecting hotspot codons (ie, 179 and 248) that are known to be required for DNA binding (Figure 1C-E). These features predicted impairment of the transcriptional activation of TP53 response genes. Consistently, the median residual transactivationial activity of subclonal TP53 mutations toward the CDKN1A (p21) promoter was only 14.5% (interquartile range: 9.9% to 20.5%) compared with wild-type TP53 (Figure 1F). The remaining subclonal TP53 mutations were splice site (10%, 5/50), nonsense (8%, 4/50), and indel (4%, 2/50) variants that were selected to truncate or entirely remove the DNA binding domain of TP53 (Figure 1C). All subclonal variants were further validated by allele specific PCR (AS-PCR). These data indicate that subclonal TP53 mutations do not represent random passenger events but instead negatively impact on TP53 function as clonal TP53 variants.

Small TP53 mutated subclones have the same unfavorable prognostic impact as clonal defects

Cases harboring solely subclonal TP53 mutations and cases harboring clonal TP53 variants shared the same clinical and immunogenetic picture at presentation (Table 1; supplemental Figure 6) and showed a similarly poor clinical course. By univariate analysis, the OS of cases harboring solely subclonal TP53 mutations was significantly shorter (5-year OS: 46.3%; P = .0042) than that of cases with an unmutated TP53 gene (5-year OS: 75.1%) and was similar to that of cases harboring clonal TP53 mutations (5-year OS: 34.6%; P = .6926) (Table 2; Figure 3A).

To assess the impact of the TP53 mutation load on CLL survival, we divided patients into subgroups according to their TP53 mutation abundance (0.3% to 1%, 1.1% to 10%, >10% of the variant allele frequency). By this analysis, patients harboring TP53 mutations, independent of the size of the clone, were characterized by a homogeneously poor outcome and showed an OS shorter than that of patients with a wild-type TP53 gene (supplemental Figure 7). Consistently, the maximally selected rank statistics failed to identify a cutoff in the size of the TP53 mutated clone capable of best predicting CLL OS (supplemental Figure 8). This analysis is indicative of a “yes/no effect” of TP53 mutations on CLL outcome irrespective of the abundance of the mutated clone.

Given the cooccurrence of TP53 mutations with 17p13 deletion (Table 1), this lesion was incorporated in survival analysis to assess...
the impact of subclonal mutations as the sole TP53 defect. Also by this approach, patients harboring solely subclonal TP53 mutations showed a significantly shorter OS than cases devoid of TP53 abnormalities. Conversely, the OS of patients carrying solely subclonal TP53 mutations was similar to that of cases with clonal TP53 genetic defects (Figure 3B).

The impact of small TP53 mutated subclones on CLL survival was independent of the potential confounding effects of other variables that are clinically relevant in this leukemia. By multivariate analysis for OS, CLL patients harboring small TP53 mutated subclones had a 2.0-fold increased risk of death (hazard ratio = 2.01; 95% CI, 1.24-4.38; \( P = .0250 \)) after adjusting for age, gender, disease stage, co-occurrence of clonal TP53 lesions, and other biological prognostic factors (ie, IGHV mutation status, 11q22-23 deletion, mutations of NOTCH1 and SF3B1, and BIRC3 disruption) (Table 2). Notably, the adjusted hazard of death marked by small TP53 mutated subclones was similar in magnitude to that of clonally represented TP53 lesions (Table 2).

Overall, these data document that CLL patients harboring small TP53 mutated subclones have the same clinico-biological phenotype and poor outcome as patients in whom TP53 lesions are clonally represented in the tumor.

Small TP53 mutated subclones detected at diagnosis subsequently expand under the selective pressure of treatment

The dynamics of small TP53 mutated subclones was assessed by longitudinal ultra-deep-NGS analysis of sequential PB samples collected from patients immediately before first treatment and then at disease relapse (n = 13 patients; 61% treated with immunochemotherapy regimens). Among these cases, the small TP53 mutated subclones identified before treatment became the predominant tumor cell population at the time of CLL relapse (Figure 4). Selection of the small TP53 mutated subclones occurred independent of the type of treatment (ie, FCR, fludarabine-based combinations or alkylating agents). This may have resulted from the removal of the dominant
TP53 wild-type clones by cytotoxic treatment, allowing the expansion of TP53 mutated subclones whose selection, because of their chemoresistance, was favored by ineffective therapies. In these patients, the expansion of small TP53 mutated subclones invariably paralleled the development of a chemorefractory phenotype.

We also examined sequential samples from 2 patients (interval between sampling 48 and 38 months, respectively) who harbored solely small TP53 mutated subclones at diagnosis and who did not require treatment during the clinical follow-up. In these 2 patients managed only by a watch-and-wait policy, the small TP53 mutated subclones did not increase in their size during the course of the disease (supplemental Figure 9).

Overall, these data indicate that chemotherapy is the major selective pressure favoring the expansion of TP53 mutated clones in CLL.

Small TP53 mutated subclones detected before treatment anticipate the development of a chemorefractory phenotype

Among CLL investigated at the time of treatment requirement (n = 53; 36% treated with FCR; supplemental Table 4), patients harboring small TP53 mutated subclones failed treatment and died of chemoresistance in a proportion similar to that of cases with clonal TP53 variants (Figure 3C). In fact, based on an exploratory analysis, the OS of cases harboring solely subclonal TP53 mutations was significantly shorter (5-year OS: 0%; P = .0171) than that of cases with an unmutated TP53 gene (5-year OS: 54.3%), and was similar to that of cases harboring clonal TP53 mutations (5-year OS: 12.1%; P = .4170) (Figure 3C).

These data indicate that, among patients requiring treatment, the detection of small TP53 mutated subclones in the early disease phases invariably anticipates the genetic composition of the disease at relapse and the development of a chemorefractory phenotype.

Discussion

This study shows that small TP53 mutated subclones detected by highly sensitive ultra-deep-NGS occur in a significant fraction of...
Table 2. Univariate and multivariate analysis of OS

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<th>UCI</th>
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Total number of patients included in the multivariate analysis: 304; Events: 104; 5 patients lacked productive IGHV-IGHD-IGHJ rearrangements. Shrinkage coefficient of the final model: 0.92.

*P-trend.
†Clonal TP53 mutations and/or 17p13 deletion.
‡BIRC3 mutations and/or BIRC3 deletion.

HR, hazard ratio; IGHV, immunoglobulin heavy variable gene; LCI, 95% lower CI; UCI, 95% upper CI.
newly diagnosed CLL, have the same unfavorable prognostic impact as clonal TP53 defects, and anticipate the development of a chemorefractory phenotype among CLL patients requiring treatment.

TP53 mutation analysis is currently advised for proper management of CLL patients,\textsuperscript{8-11} and Sanger sequencing is the approach currently recommended by guidelines.\textsuperscript{11} Due to its limited sensitivity,\textsuperscript{11} conventional Sanger sequencing misclassifies as wild type; 5\% of newly diagnosed CLL otherwise harboring TP53 mutations.\textsuperscript{8} Thanks to its high sensitivity (down to 1\% to 0.1\%),\textsuperscript{38,39} ultra-deep-NGS is capable of detecting such minor, but clinically relevant, TP53 mutated subclones. Therefore, ultra-deep-NGS should be considered as a useful tool for a comprehensive assessment of TP53 disruption in CLL. Though the retrospective design represents a limitation of this analysis, a strong biological rationale supports the clinical relevance of subclonal TP53 mutations in CLL and their more general application as a biomarker in this disease. Subclonal TP53 variants show molecular and functional clues that are highly consistent with those of TP53 mutations with known pathogenicity,\textsuperscript{28} thus indicating that they have been selected to damage the TP53 protein.\textsuperscript{28} The pathogenic effect of subclonal TP53 mutations is confirmed by the observation that, in patients, small TP53 mutated subclones are resistant to chemo+/- immunotherapy and are positively selected by treatments to progressively become the dominant leukemic population at the time of CLL relapse. Ultra-deep-NGS may capture newly born and highly fit TP53 mutations at the initial phases of their clonal selection. In this scenario, beside the intrinsic fitness imposed by the TP53 variant, the small TP53 mutant subclone needs further environmental pressures/constraint (ie, microenvironmental interactions, chemotherapy) to overcome and substitute the TP53 wild-type cell population.\textsuperscript{14-17}

![Figure 3](http://www.bloodjournal.org/content/123/14/2145/F3)

**Figure 3.** Kaplan-Meier estimates of OS of patients harboring small TP53 mutated subclones. (A) Comparison of OS from CLL diagnosis between patients harboring solely subclonal TP53 mutations, cases harboring clonal TP53 mutations, and cases harboring an unmutated TP53 gene. (B) Comparison of OS from CLL diagnosis between patients harboring solely subclonal TP53 mutations, cases harboring solely clonal TP53 lesions (ie, mutations or deletions), cases harboring clonal TP53 lesions coexisting with subclonal TP53 mutations, and cases harboring a wild-type TP53 gene. (C) Comparison of OS from first treatment between patients harboring solely subclonal TP53 mutations, cases harboring clonal TP53 mutations, and cases harboring an unmutated TP53 gene. **p**, P values by log-rank test.
therefore represent the best candidates for new treatment strategies or stem cell transplant. Because patients harboring small TP53 mutated subclones have the same risk of failing and dying as patients harboring clonal TP53 defects, both at diagnosis and at treatment requirement, their identification is advisable in order to manage them as high-risk CLL. Treatment approaches should be selected to target both the major TP53 wild-type clone as well as small TP53 mutated subclones to avoid their subsequent selection and outgrowth that is otherwise destined to occur in all cases exposed to ineffective treatments, as documented by the current study. Given their promising activity against CLL cells with TP53 defects, new targeted drugs (eg, ABT-199 and ibrutinib) may represent a rational treatment to suppress or even eradicate small subclones harboring TP53 lesions.

**Acknowledgments**

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Clinical impact of small TP53 mutated subclones in chronic lymphocytic leukemia

Davide Rossi, Hossein Khiabanian, Valeria Spina, Carmela Ciardullo, Alessio Bruscaggin, Rosella Famà, Silvia Rasi, Sara Monti, Clara Deambrogi, Lorenzo De Paoli, Jiguang Wang, Valter Gattei, Anna Guarini, Robin Foà, Raul Rabadán and Gianluca Gaidano