Genomic complexity and *IGHV* mutational status are key predictors of outcome of chronic lymphocytic leukemia patients with *TP53* disruption

The clinical course of chronic lymphocytic leukemia (CLL) is extremely heterogeneous and while some patients achieve a normal lifespan, others succumb to the disease shortly after diagnosis. Recurrent chromosomal aberrations as detected by chromosome banding analysis (CBA) or fluorescent *in situ* hybridization (FISH) have a reproducible prognostic power in terms of response to therapy and survival.[1-3] In particular, patients whose tumor cells harbor 17p deletions (17p-) are considered to have a shorter survival and, hence, high-risk CLL. This poor prognosis is, however, not universally true for all patients with 17p- CLL. Indeed, we and others have observed that some clinical-biological features, such as presence of B symptoms, advanced clinical stage, size of the 17p- clone, β2-microglobulin (β2M) concentration and *IGH* mutational status have a significant impact on the outcome of this subgroup of patients.[4,5] Novel molecular studies have helped in the understanding of 17p- CLL. On one hand, *TP53* mutations are present in more than 80% of cases with 17p deletion and in around 5% of patients without 17p deletion.[6,7] On the other hand, next generation sequencing studies have revealed novel genetic aberrations such as *NOTCH1* and *SF3B1* mutations that have a negative impact on survival.[8-10] Finally, genomic complexity, as defined by karyotyping[1] or copy number (CN) arrays, has also been independently associated with disease transformation and poor outcome in patients with CLL.[11,12] The aim of this study was to evaluate the prognostic value of concomitant molecular abnormalities in patients with CLL and *TP53* aberrations as diagnosed by FISH, CBA or DNA sequencing.

From our database, we identified 55 of 763 (7%) CLL patients with *TP53* disruption detected at any time over the course of the disease. Importantly, CBA/FISH and molecular studies were performed on samples drawn on the same date. Other clinical and biological characteristics, such as Binet stage, CD38 and ZAP70 expression or β2M serum concentration, were also analyzed at the time of detection of *TP53* disruption using conventional methods. All patients from this study signed an informed consent and were recruited into the International Cancer Genome Consortium Chronic Lymphocytic Leukemia project, which was reviewed by the Institutional Review Board.

CBA was performed on Giemsa-banded chromosomes obtained after a 72-h culture and stimulation with tetradecanoyl-phorbol-acetate. A complex karyotype was defined as the presence of 3 or more clonal chromosomal aberrations. FISH studies for 11q, 13q and 17p deletions and gains of chromosome 12 were performed using the Vysis CLL probe kit (Abbott, Des Plaines, IL, USA). *IGHV-IGHD-IGHJ*

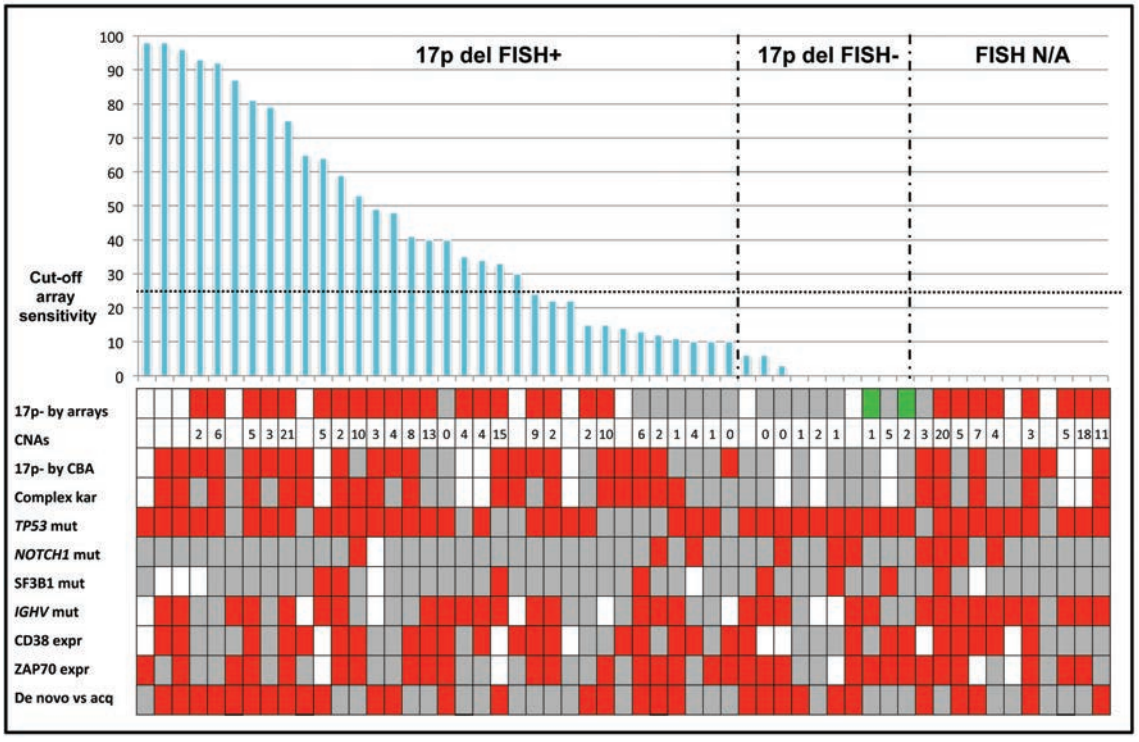

Figure 1. Graphical representation of the whole cohort of patients according to *TP53* alterations. The percentage of cells with 17p deletion by FISH is illustrated in the vertical bar plots. The squares below indicate the presence of 17p losses by copy number arrays (red: loss; green: copy number neutral loss of heterozygosity; gray: wild type; white: not available); copy number alterations; 17p deletions by chromosome banding analysis (red: presence; gray: absence; white: not available); complex karyotype by chromosome banding analysis (red: presence; gray: absence; white: not available); *TP53* mutations by sequencing (red: presence; gray: absence; white: not available); *NOTCH1* mutations by sequencing (red: presence; gray: absence; white: not available); *SF3B1* mutations by sequencing (red: presence; gray: absence; white: not available); IGHV mutational status by sequencing (red: unmutated; gray: mutated; white: not available); CD38 expression (red: high; gray: low; white: not available); ZAP70 expression (red: high; gray: low; white: not available); type of *TP53* disruption (red: *de novo*; gray: acquired).

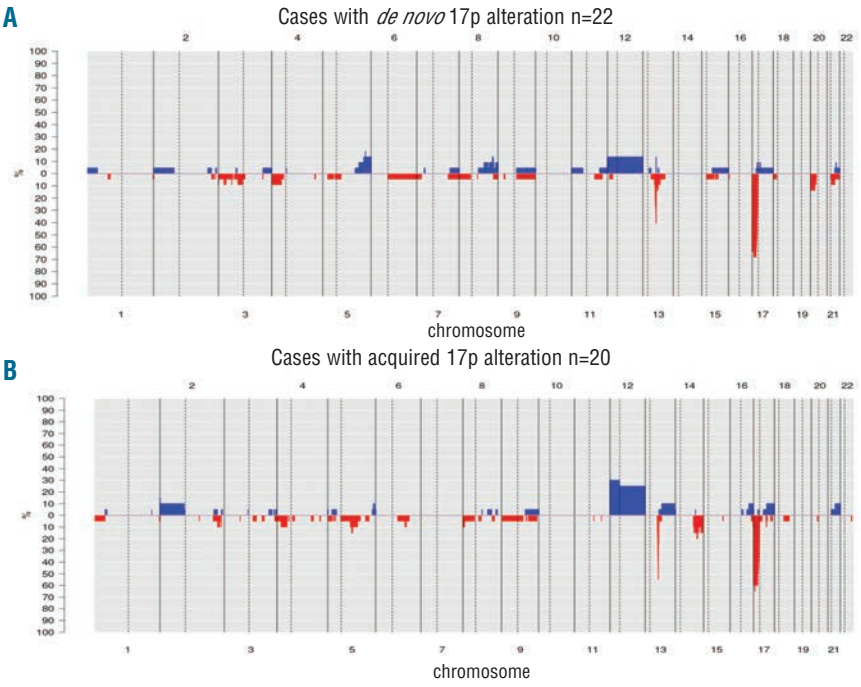

Figure 2. Copy number profiles of cases with *de novo* (A) and acquired (B) *TP53* disruption. In the x-axis the chromosomes are represented horizontally from 1 to 22, in the y-axis the percentage of cases showing the copy number alterations. Gains are represented in the positive y-axis and colored in blue, whereas losses are represented in the negative y-axis in red.

rearrangements and mutational status and TP53 mutation analysis were analyzed following ERIC recommendations.[13,14] *NOTCH1* and *SF3B1* mutations were evaluated as previously described.[8,9] CN analysis was performed using two different platforms: a custom Agilent 8x60K oligonucleotide array (I Salaverria *et al.*, manuscript in preparation) and the Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA, USA). Nexus 6.0 Discovery Edition (Biodiscovery, El Segundo, CA, USA) was used for global analysis and visualization. All data have been up-loaded to the GEO database (GSE56277).

Comparison between groups was performed using Fisher's exact and Mann-Whitney tests. Overall survival (OS) and time to first treatment (TTFT) were calculated from the date of sampling to the date of death or front-line treatment, respectively, or last follow up. Appropriate cut-off points for copy number alterations (CNAs) were calculated using maximally selected rank statistics. Cox regression multivariate models were fitted in order to assess the independent prognostic value of those covariates that were significant by univariate analysis.

Data were collected from 55 patients with *TP53* disruption (Figure 1). Thirty-four patients had 17p- diagnosed by interphase FISH and 6 by CBA before the availability of FISH. The remaining 15 patients were identified to have *TP53* mutations by Sanger sequencing but had no identifiable 17p deletion by CBA and/or FISH. Thirty (55%) patients had *de novo* aberrations (i.e. detected within 6 months of CLL diagnosis) and 25 (45%) acquired them at a median of 58 months from diagnosis (range 8-194 months), 23 of these patients (92%) after CLL-specific therapy. Median age of the entire population was 67 years (range 30-98 years) when the *TP53* disruption was detected. Patients with acquired aberrations had a higher incidence of Binet stage B-C disease and elevated β2M concentration at the time of detection of *TP53* disruption, consistent with a more advanced disease (*Online Supplementary Table S1*). CBA was performed in 48 patients and yielded adequate metaphases in 45 of them (Figure 1). Chromosome abnormalities involving 17p were observed in 27 of 45 (60%)

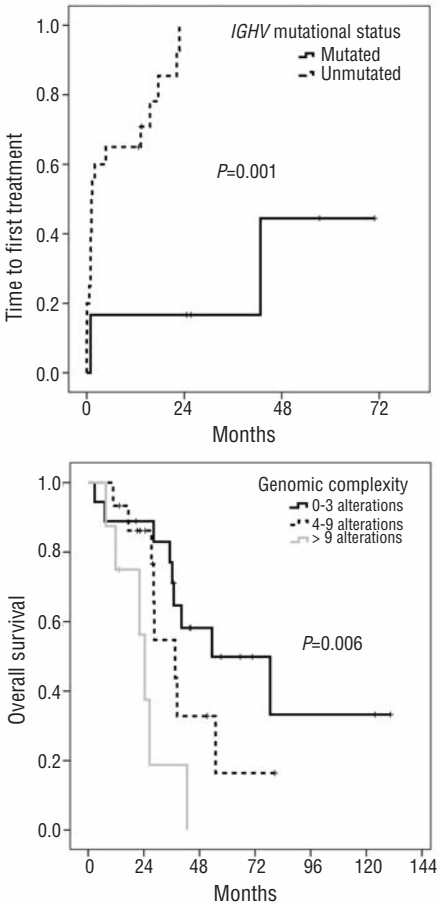

Figure 3. (A) Time to first treatment in patients with *de novo TP53* disruption according to *IGHV* mutational status [(mutated (n = 6) *vs.* unmutated (n = 20)]; (B) overall survival in patients with *TP53* disruption according to the number of copy number alterations [0-3 (n=18) *vs.*4-9 (n=15) *vs.* more than 9 (n=8)].

patients (*Online Supplementary Table S1*), and 23 of 45 (52%) patients had a complex karyotype.

In the 34 patients with a positive FISH test, the median percentage of 17p- cells was 40% (range 10-98%). Forty-two cases were studied by CN-arrays: 27 using SNP arrays 6.0 and 15 using a customized 8x60K array (Figure 2 and *Online Supplementary Table S2*). CN-arrays revealed 17p losses in 27 of 42 (64%) patients. Furthermore, 17p losses were more frequent in patients with 25% or more cells with deletion by FISH compared to patients with a lower allelic burden (93% *vs.* 24%; *P*<0.001). Indeed, CN-arrays only failed to detect a 17p loss in one out of 15 patients with 25% or more 17p- cells by FISH, which is considered the limit of detection of CN-arrays.[11] In addition, CN-arrays were able to detect a copy number neutral loss of heterozygosity (CNN-LOH) of the 17p region in 2 of 14 (14%) patients who had *TP53* mutations without 17p deletion by FISH (*Online Supplementary Figure S1*). The presence of other genomic aberrations that have been reported to confer prognostic value independently of *TP53* disruption, such as gains at 2p and 8q or losses at 8p,[15] was too low in our series (6%, 9% and 6%, respectively) to have any prognostic impact.

The number of CNAs was equally distributed among patients with *de novo* and acquired *TP53* disruption (*Online Supplementary Table S1*). The maxstat analysis revealed two possible cut offs for CNAs with a high prognostic power: three and nine CNAs per case (*Online Supplementary Figure S2*). For the validation of the cut offs, prediction error curves for their different values were estimated using a .632+ bootstrap strategy. The integrated Brier score between time 0 and time 54 of each estimated curve was used as a performance measure of the corresponding cut offs (*Online Supplementary Figure S3*).

There was a significant correlation between the presence or absence of a complex karyotype by CBA and the number of CNAs by CN-array (P=0.011, Mann-Whitney test). *TP53* mutations were detected in 43 of 55 (78%) patients. *NOTCH1* and *SF3B1* mutations were identified in 10 of 54 (19%) and 8 of 49 (16%) patients, respectively, but no clear association was evident between the presence of these mutations and any other genomic aberration and/or prognostic marker. The great majority (86%) of patients with 17p disruption by CN-arrays (either 17p loss or CNN-LOH) also had concurrent *TP53* mutations.

Among patients with *de novo TP53* disruption, 21 of 30 (70%) required CLL-specific therapy (*Online Supplementary Table S4*). Median TTFT was nine months, and covariates predictive of a shorter TTFT were unmutated *IGHV* genes (*P*=0.011) and high ZAP70 expression (*P*=0.011). Multivariate analysis revealed that *IGHV* mutational status was the only variable with independent prognostic value in terms of TTFT (hazard ratio [HR] 13.8, 95% confidence interval [CI] 1.7-112.9; *P*=0.014) (Figure 3A). Median overall survival for the entire cohort was 37 (95%CI: 34-41) months from the time of sampling. We tested both possible cut offs for CNAs (three and nine alterations) and found that both were equally significant (*P*=0.024). By multivariate analysis, the only factor with independent prognostic value was the number of CNAs (0-3 *vs.* 4-9 *vs.* >9; P=0.024) (Figure 3B). Hazard ratios for CNAs were 7.63 (95%CI: 1.6-37.0; *P*=0.011) for the 0-3 *versus* 4-9 comparison and 7.35 (95%CI: 1.62-33.3; *P*=0.010) for the 4-9 *versus* >9 comparison (*Online Supplementary Table S5*).

In conclusion, the prognosis of CLL patients with a *TP53* disruption is modulated by their genomic complexity as assessed by CN-arrays but also by additional molecular features such as *IGHV* mutations. Genomic complexity as determined by CN-arrays was predictive of OS and *IGHV* mutational status was predictive of TTFT, which is in keeping with previous results.[11] Finally, SNP-arrays were very helpful in the detection of 17p CNN-LOH. These results require validation but provide further evidence of the expanding role of CN-arrays and molecular testing in the prognostic workup of patients with CLL.

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