haematologica
Journal of The Ferrata Storti Foundation

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Haematologica 2021 [Epub ahead of print]

Citation: Silvia Ramos-Campoy, Anna Puiggros, Sílvia Beà, Sandrine Bougeon, María José Larráyoz, Dolors Costa, Helen Parker, Gian Matteo Rigolin, Margarita Ortega, María Laura Blanco, Rosa Collado, Rocío Salgado, Tycho Baumann, Eva Gimeno, Carolina Moreno, Francesc Bosch, Xavier Calvo, María José Calasanz, Antonio Cuneo, Jonathan C. Strefford, Florence Nguyen-Khac, David Oscier, Claudia Haferlach, Jacqueline Schoumans, and Blanca Espinet. Chromosome banding analysis and genomic microarrays are both useful but not equivalent methods for genomic complexity risk stratification in chronic lymphocytic leukemia patients.
Haematologica. 2021; 106:xxx
doi:10.3324/haematol.2020.274456

Chromosome banding analysis and genomic microarrays are both useful but not equivalent methods for genomic complexity risk stratification in chronic lymphocytic leukemia patients

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Running title: Genome complexity in CLL: karyotype vs microarrays

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Author's contribution

A.P. and B.E. designed the research study. S.R., A.P. and B.E. provided patients data, analyzed the data and wrote the manuscript. S.Bo. and J.S. performed a great proportion of genomic microarrays from cases without this information available. S.Bo., J.S. and S.Be. were involved in analysis, interpretation and critical discussion of the results. S.Be., MJ.L., D.C., H.P., GM.R., M.O., ML.B., R.C., R.S., T.B., E.G., C.M., F.B., X.C., MJ.C., A.C., JC.S., F.NK., D.O. and C.H. provided patients data and samples. All authors read the last version of the manuscript.

Acknowledgements

The authors would like to thank Idoya Ancín, Andrea Campeny, María Dolores García-Malo, Alberto Valiente, Marco Moro, Gonzalo Blanco, Ferran Nadeu and Julio Delgado for their contribution to the study providing patients samples and data and the MARGenomics Platform from Institut Hospital del Mar d'Investigacions Mèdiques (Barcelona) for performing part of the genomic microarrays. This work was partly supported by grants from Generalitat de Catalunya (17SGR437), Gilead Sciences Fellowship (GLD17/00282), Ministerio de Educación, Cultura y Deporte of Spain (FPU17/00361), Fundación Española de Hematología y Hemoterapia (FEHH-Janssen), Instituto de Salud Carlos III/FEDER (PT17/0015/0011) and the "Xarxa de Bancs de tumors" sponsored by Pla Director d'Oncologia de Catalunya (XBTC).

Conflicts of interest

No potential conflicts of interest were disclosed.

Data sharing statement

Detailed chromosome banding analyses and genomic microarrays profiles for selected cases are provided in **Supplementary Tables**. Please contact either bespinet@parcdesalutmar.cat or apuiggros@imim.es for additional data.

Word count abstract: 261

Word count main text: 3997

Table count: 5

Figure count: 3

Abstract

Genome complexity has been associated with poor outcome in patients with chronic lymphocytic leukemia (CLL). Previous cooperative studies established five abnormalities as the cut-off that best predicts an adverse evolution by chromosome banding analysis (CBA) and genomic microarrays (GM). However, data comparing risk stratification by both methods are scarce. Herein, we assessed a cohort of 340 untreated CLL patients highly enriched in cases with complex karyotype (CK, 46.5%) with parallel CBA and GM studies. Abnormalities found by both techniques were compared. Prognostic stratification in three risk groups based on genomic complexity [0-2, 3-4 and ≥5 abnormalities] was also analyzed. No significant differences in the percentage of patients classified into each category were detected, but only a moderate agreement was observed between methods when focusing in individual cases (κ=0.507; p<0.001). Discordant classification was obtained in 100 patients (29.4%), including 3% classified in opposite risk groups. Most discrepancies were technique-dependent and no greater correlation in the number of abnormalities was achieved when different filtering strategies were applied for GM. Nonetheless, both methods showed a similar concordance index for prediction of time to first treatment (TTFT) (CBA: 0.67 vs. GM: 0.65) and overall survival (CBA: 0.55 vs. GM: 0.57). High complexity maintained its significance in the multivariate analysis for TTFT including *TP53* and IGHV status when defined by CBA (HR: 3.23; p<0.001) and GM (HR: 2.74; p<0.001). Our findings suggest that both methods are useful but not equivalent for risk stratification of CLL patients. Validation studies are needed to establish the prognostic value of genome complexity based on GM data in future prospective studies.

Introduction

Deletions of 17p13 region and/or mutations in *TP53* as well as the mutational status of the variable region of the immunoglobulin heavy chain (IGHV) gene constitute the most important prognostic and predictive factors in chronic lymphocytic leukemia (CLL) in the era of chemoimmunotherapy.[1] However, several studies have highlighted the independent clinical significance of genomic complexity, mainly defined by the detection of complex karyotypes (CK) by chromosome banding analysis (CBA), due to its association with an unfavorable clinical outcome. This has been demonstrated in patients treated not only with standard chemoimmunotherapy regimes[2-5] but also in the initial clinical trials with the novel mechanism-based therapeutic agents such as ibrutinib or venetoclax.[6-8]

Early studies in CLL defined CK as the presence of at least three numerical and/or structural chromosomal abnormalities in the same cell clone detected by CBA.[9,10] Of note, the increasing number of chromosomal abnormalities in the karyotype has been correlated with the worsening of clinical evolution of CLL patients.[11,12] In this context, a large retrospective study from the European Research Initiative on CLL (ERIC) has reported that patients with five or more abnormalities (the so-called high-CK) display an adverse outcome independently of other known biomarkers such as *TP53* abnormalities or the IGHV mutational status.[5] On the other hand, it has been demonstrated that CK might have a different clinical impact in CLL patients according not only to the number, but also the type of aberrations detected in the karyotype. In this regard, it has been described that patients with CK carrying +12, +19 display a particularly favorable outcome while the presence of unbalanced rearrangements define a subset with very aggressive disease.[13-15]

Even though CBA has been the gold standard method to identify CK, in the last decade genomic microarrays (GM) have emerged as a valuable tool for genome-wide screening in CLL.[16-20] Indeed, some studies have correlated the genomic complexity detected by GM to progressive disease and poorer response rates to treatment.[21-23] Nonetheless, although some European countries have replaced conventional techniques by GM, standard criteria to analyze and define genomic complexity by GM are still needed. According to the published guidelines for GM analysis in acquired hematologic neoplasms, recurrent aberrations with known clinical relevance in the disease irrespective of their size as well as other copy number abnormalities (CNAs) ≥5Mb should be considered in order to reduce the detection of benign constitutional variants and avoid the reporting of anomalies with uncertain clinical significance.[24]

However, it remains unclear whether this threshold is the optimal to analyze CLL patients or whether potentially relevant chromosomal imbalances are being disregarded by applying this highly conservative size cut-off. Besides, another multicenter study conducted by ERIC suggested that CLL patients could be divided in three distinct prognostic subgroups based on the number of CNAs.[25] According to Leeksma et al, the so-called high genomic complexity (high-GC) subgroup, which is defined by carrying ≥5 CNAs, emerged as prognostically adverse, independently of other biomarkers. Nevertheless, to the best of our knowledge, the comparison of genomic complexity for risk stratification using CBA and GM in parallel has not been performed in a large CLL cohort.

In the present multicenter retrospective study we aimed to compare the usefulness of CBA and GM techniques in a series of 340 CLL patients with and without CK to determine both their concordance and their equivalence in the prognostic stratification of CLL patients with CK. Moreover, we have analyzed the detected aberrations using different counting strategies to ascertain whether other parameters, such as the type of the aberrations or their size, might influence on risk stratification of CLL patients.

Methods

Patient cohort

A total of 340 previously untreated CLL (n=327; 96.2%) and monoclonal B-cell lymphocytosis (n=13; 3.8%) patients from 18 European institutions were included. All had CBA results at diagnosis or before treatment. GM data were already available or obtained from DNA extracted within one year. Analyses were performed on peripheral blood (PB) [n=286] or bone marrow (BM) [n=54]. Due to the purpose of the study, this cohort was enriched in patients with CK (n=158; 46.5%). Demographic, clinical and biological characteristics are summarized in Table 1. The study was carried out in accordance with national and international guidelines (Professional Code of Conduct, Declaration of Helsinki) and approved by Hospital del Mar Ethics Committee (2017/7565/I).

Chromosome banding analyses

CBA was performed on G- or R-banded chromosomes. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature (ISCN 2016).[26] A complex karyotype was defined as the presence of three or more numerical and/or structural chromosomal abnormalities detected in the same cell clone. Patients were stratified in three categories: non-CK (0-2 abn.), low/intermediate-CK (3-4 abn.) and high-CK (≥5 abn.).[5]

Genomic microarray analyses

Microarray platforms used are summarized in Supplementary Table 1. All aberrations found irrespective of size were collected, although non-classical CLL abnormalities (other than gain of chromosome 12 and losses of 11q, 13q and 17p) were filtered in the CNA count for prognostic stratification following the 5Mb cut-off size recommended.[24] Three subgroups were defined according to genomic complexity (GC): low-GC (0-2 CNAs), intermediate-GC (3-4 CNAs) and high-GC (≥5 CNAs).[25] This strategy was compared with other CNA counting methodologies, such as the inclusion of smaller abnormalities (no size filter or 1Mb as cut-off) or counting as a unique CNA small contiguous abnormalities (with a distance ≤1Mb between them) or those included in a chromothripsis event.

Statistical analyses

Descriptive statistics were used to provide frequency distributions of discrete variables while statistical measures were used to provide median values and ranges for

quantitative variables. Groups were compared using Chi-square or Fisher exact tests for discrete variables and Mann-Whitney U test for continuous variables. The Kappa coefficient was used for assessing the agreement in patients categorization among techniques. Survival analysis was restricted to 259 patients. A total of 81 non-CK cases from three institutions were excluded as CBA was performed at recruitment for clinical trials, introducing a bias in the results. Time to first treatment (TTFT), the end point of the study, was calculated from the date of cytogenetic study to the date of first treatment or last follow-up while overall survival (OS) was defined from date of cytogenetic study until last follow-up or death. Kaplan–Meier method was used to estimate the distribution of TTFT and OS. Comparisons among patient subgroups were performed with the Log-rank test. The concordance statistic (C-index) was calculated to assess the accuracy of CBA and GM for predicting TTFT and OS. Multivariate analysis using Cox proportional hazards regression model was used to assess the maintenance of the independent prognostic impact on TTFT and OS. Statistical analyses were performed using SPSS v.23 software (SPSS Inc, Chicago, IL, USA) and R v3.5.2. *P*-values <0.05 were considered statistically significant.

Additional information regarding the methodology of the study is detailed in the Supplementary Data.

Results

Number and type of abnormalities detected by CBA and GM

Regarding CBA, 270/340 (79.4%) patients showed an abnormal karyotype. Overall, 182 were considered non-CK (0-2 abn.) while 158 displayed a CK (≥3 abn.). The vast majority of non-CK aberrant cases carried only one aberration (75/112; 66.9%), while the median number of abnormalities among CK patients was 4 (range: 3-19). Abnormal karyotypes from the non-CK group mainly included known recurrent CLL aberrations, the most frequent being trisomy of chromosome 12 (15.4% patients). In contrast, the CK group showed a wide variety of abnormalities affecting all chromosomes and included unbalanced structural aberrations (552/823; 67.1%), complex abnormalities affecting material of unknown origin (179/823; 21.7%) and monosomies (155/823; 18.8%). In seven of these, a co-existence of +12 and +19, associated with more indolent course, was found (4.4%). Balanced translocations, potentially missed when studied by GM, were present in only 57 patients (11.5% non-CK; 22.8% CK). Even though they were detected in a minority of cases, 13q14 and 14q32 loci were recurrently involved (in 13 and 7 patients, respectively).

GM had the highest detection rate of abnormalities, with 309/340 (90.9%) cases carrying at least one CNA when all the abnormalities, irrespective of their size, were considered. No significant differences were observed among the GM platforms used. Expectedly, the non-CK group showed a significantly lower median number of CNAs compared with CK patients (2 [range: 0-10] vs. 6 [range: 0-51]; $p<0.001$). Nearly half of the patients carried at least one small (<5Mb) non-classical CLL CNA (median size: 5.38Mb [range: 0.019-198Mb]) that would not be taken into consideration following the current microarray recommendations (35.7% non-CK vs. 64.6% CK patients; $p<0.001$). Although they were less frequent, similar results were observed regarding the presence of non-classical CLL CNA below 1Mb (26.9% vs. 44.9%; $p<0.001$). Patterns suggestive of chromothripsis or chromothripsis-like were identified in 30 patients (20 and 10 cases, respectively).

Fluorescence *in situ* hybridization (FISH), the gold standard method for the detection of the four genetic abnormalities included in Döhner et al prognostic hierarchical model[27], confirmed the higher incidence of high-risk aberrations in the CK group. Specifically, del(11q) was found in 12.4% (22/177) of non-CK patients and 32.2% (49/152) of CK patients ($p<0.001$) while del(17p) was present in 4.5% (8/177) and 40.1% (61/152), respectively ($p<0.001$). Detection of del(13q), del(11q) and del(17p) was lower by CBA compared to FISH although these loci were involved in different type of abnormalities

(Supplementary Table 2). GM showed a high concordance with FISH results (Supplementary Table 3).

With regard to commonly detected non-classical CLL abnormalities, similar results were observed by both CBA and GM among non-CK and CK patients. The only recurrent aberrations detected by CBA within the non-CK group were deletions in the long arm of chromosome 14 (6.6%) and unbalanced translocations affecting 2p arm (5.5%), which were detected as losses at 14q and gains of 2p region by GM, respectively. Likewise, despite being distributed along the genome, gains of chromosome arms 2p, 3q, 8q, 15q, 17q and 19q and losses at 3p, 4p, 6q, 8p, 14q, 15q and 18p, usually involved in unbalanced translocations or simple deletions in the karyotype, were the most recurrent CNAs detected in CK patients (Supplementary Figure 1). Detailed information regarding recurrent CNAs found by GM is shown in Table 2.

Risk stratification of the genomic complexity observed by CBA and GM

In order to compare the concordance among risk stratification based on CBA and GM data, patients were classified into those categories suggested by previous large-scale studies.[5,25] Notably, both techniques did not significantly differ in the percentage of patients classified into intermediate-risk categories (3-4 abnormalities; 23.8% by CBA vs. 24.4% by GM; p=0.923) or those showing the highest risk (≥5 abnormalities; 22.6% and 19.1%, respectively; p=0.299). However, when focusing in individual cases, only a moderate agreement was observed between methods (κ=0.507; p<0.001). Discordant classification was obtained in 100 patients (29.4%), including eight cases with ≥5 abnormalities in the karyotype which would not be considered complex by GM and two patients with high-GC who did not have CK (2.9%) (Table 3).

Next, we evaluated if the CNA filtering strategy used for GM results could underlie the differences observed in the assessment of the complexity. Nevertheless, the proportion of patients with CNAs <5Mb was similarly represented among those patients with increased complexity scored by CBA (n=58) or by GM (n=42) (55.2% vs. 64.3%, respectively; p=0.413). When less strict filtering strategies were applied for GM, no greater correlation in the number of abnormalities counted by CBA and GM was achieved. Similar results were observed when including all the abnormalities irrespective of their size, using 1Mb as cut-off for non-classical CLL CNAs, or if CNAs separated by <1Mb or chromothripsis patterns were counted as one event to evaluate the effect of joining consecutive aberrations (Figure 1).

Parallel analyses of the abnormalities detected by CBA and GM were also performed to identify other potential causes of discrepancy. Among those abnormalities recorded only by CBA, differences were mainly due to the presence of balanced translocations (n=28 patients) or abnormalities represented in a minor proportion of tumor cells probably expanded during the cytogenetics culture which were missed by GM (n=40 patients). Moreover, FISH with chromosome painting probes performed in two high-CK cases by CBA who showed low-GC revealed that some apparently unbalanced abnormalities were complex balanced rearrangements that ultimately did not lead to loss of material (Supplementary Figure 2). On the other hand, when assessed by GM, most of the more complex cases showed aberrations <10Mb, which is the resolution threshold of CBA, or multiple CNA that corresponded to complex rearrangements recorded as single abnormalities in the karyotype (73 and 19 cases, respectively). No division of the tumor clone during the cytogenetics culture is the most feasible explanation for 50 patients who carried CNAs ≥10Mb that should have been identified by CBA, of which 17 presented a normal karyotype. Detailed comparison for the ten patients who only displayed high complexity by one method is shown in Supplementary Table 4.

The genetic analysis using both methods allowed the correction of the karyotype in six patients after GM interpretation (Supplementary Table 5). Although it resulted in a change of the number of abnormalities recorded by CBA for three of them, initial counts were considered for the present analysis.

Prognostic impact of CK stratification by CBA and GM

As previously stated in the ERIC studies, significant differences in terms of TTFT were observed within the three risk groups according to the number of aberrations found by CBA and GM.[5,25] Whereas the highest risk group defined by both techniques displayed a similar short median TTFT (5 and 3 months by CBA and GM, respectively), TTFT was shorter for the intermediate risk group when defined by CBA (18 vs. 35 months) (Figure 2A). Indeed, both methods showed a similar accuracy for predicting TTFT (C-index: 0.67 by CBA vs. 0.65 by GM). With regard to OS, only the highest risk groups defined by each technique displayed a poorer outcome (68 months in both cases) (Figure 2B) although differences were only statistically significant in GM defined groups. Equivalent C-indexes were obtained for OS (0.55 by CBA vs. 0.57 by GM).

In order to compare the discriminatory power for outcome prediction of both techniques, patients were first classified according to CBA to assess TTFT of GM defined groups within each category. Of note, those non-CK and low/intermediate-CK patients by CBA who carried ≥5 CNAs (high-GC) showed a poor outcome equivalent to that observed in the high-CK by CBA (median TTFT: 2 and 1 months, respectively). However, within the high-CK group, low-GC patients did not show a better evolution (TTFT: 2 months) while cases with intermediate-GC displayed an unexpected median TTFT of 22 months (Figure 3A). When these analyses were performed in the reverse order, CBA reclassification within the low-GC patients allowed the distinction of three risk categories showing similar outcomes to those observed when applied to the global cohort (p<0.001). No significant differences were observed when the intermediate-GC and high-GC categories were reclassified (Figure 3B). It is noteworthy that the ten cases categorized in opposite risk groups displayed the poor prognosis predicted by the technique that classified them in the higher risk category.

Expectedly, the frequency of *TP53* abnormalities (deletions and/or mutations) increased together with the complexity by both methods. In contrast, intermediate and high risk categories showed a similar increased proportion of unmutated IGHV (U-IGHV) and del(11q) compared with non-CK/low-GC patients (Supplementary Table 6). Despite being highly associated with these known prognostic factors, three groups with significant differences on TTFT could be established by CBA and GM when patients were categorized depending on their *TP53* status (Supplementary Figure 3). Regarding IGHV, similar results were obtained within the mutated IGHV (M-IGHV) group while no clear discrimination was observed in the U-IGHV subset (Supplementary Figure 4). No prognostic impact was observed for del(11q) in the entire cohort (Table 4). High complexity defined by both CBA and GM maintained its significance when a multivariate analysis for TTFT including *TP53* and IGHV status was performed. Conversely, genomic complexity by GM lost its significance in the multivariate analysis for OS (Table 5).

Finally, the impact of other genetic findings was also analyzed. In this regard, the presence of unbalanced rearrangements was associated with shorter TTFT in the entire cohort (11 months vs. NR; p<0.001) and within the non-CK subgroup (10 months vs. NR; p=0.001) (Supplementary Figure 5). A negative impact was also observed for chromothripsis (2 months vs. 37 months; p<0.001), which was mainly found among CK patients (29/30). Indeed, tendency to this worse evolution was maintained within this subset (5 months vs. 15 months; p=0.062) (Supplementary Figure 6). As expected, these cases showed a high frequency of abnormalities in *TP53* (22/30; 73.3%) and U-

IGHV (21/29; 72.4%). In the multivariate analysis including these features and genomic complexity, only the latter defined by both CBA and GM retained its negative impact (Supplementary Table 7).

Discussion

In recent years, there has been a rising interest in identifying CLL patients with CK since they may pursue a more aggressive clinical course and respond less well to treatment.[2,4,28] Large cooperative studies within the ERIC have demonstrated that five abnormalities is the optimal cut-off which better predicts an impaired outcome by both CBA and GM.[5,25] However, data comparing the risk stratification based on genomic complexity by both methods in the same patients are scarce. Indeed, a small cohort of 122 patients from Leeksma et al study was analyzed by GM and CBA, but the proportion of CK cases was very low, as expected in an unselected CLL population. To the best of our knowledge, the present study is the largest report conducted to date in which a cohort of CLL patients enriched in CK cases has been simultaneously analyzed by CBA and GM, comparing the usefulness of both methods in their prognostic stratification.

By clustering patients according to ERIC previously defined criteria, we confirmed that both techniques did not differ in the proportion of patients classified into each risk category. Notwithstanding, it should be pointed out that only moderate agreement was observed between them. Discordances in the risk assigned to nearly one third of patients were found, including around 3% of patients classified in either high or low risk groups depending on the methodology employed for their study. We have demonstrated that most of these discordances are consequence of known limitations intrinsic to each technique. In this regard, the clonal architecture in the sample could mask some alterations by GM, if present in low percentages below their limited sensitivity (~20%), while the CBA result would rely on the *in vitro* proliferative capacity of the altered clones.[29,30] In addition, balanced abnormalities are only detectable by CBA and, contrarily to the expected, our FISH painting studies confirmed that not all the complex rearrangements described in the karyotype ultimately imply gain or loss of genomic material. On the contrary, some abnormalities recorded as a single monosomy or unbalanced translocation in the karyotype turned out to be multiple CNA or even chromothripsis events when assessed by GM. Thus, our results suggested that discrepancies were not predictable by the type of abnormalities detected by any of the methods. Conversely, we discarded a global underestimation of the genomic complexity associated with the application of the recommended filtering criteria for non-classical CLL CNAs by GM.[24] Small abnormalities (<5Mb) were equally found by GM among concordant and discordant patients, and greater agreement in the number of abnormalities could not be achieved when smaller CNAs were also considered. Thus, we have confirmed that the present recommendations for GM analysis are robust for

complexity assessment.[24] The observed differences did not represent a poorer performance for CBA or GM in patients risk stratification. In both cases, the established risk groups showed significant differences in terms of TTFT, which were independent of *TP53* and IGHV mutational status. Concerning OS, only high complex groups displayed a dismal evolution. In addition, the heterogeneity regarding the GM platforms employed could be a limitation of this study. However, all GM results were reviewed and uniformly interpreted using the same criteria to filter CNAs and similar findings were obtained among different GM companies.

Regarding CBA data, previous publications have investigated whether specific cytogenetic patterns not identifiable by GM (presence of balanced or unbalanced rearrangements) may correlate with dismal outcome. Initial studies suggested that carrying chromosomal translocations was associated with poorer clinical course.[31] More recently, this negative impact has been attributed to the presence of unbalanced rearrangements and its association with CK.[2,32] Indeed, Rigolin et al proved that CK carrying unbalanced rearrangements constituted a very poor risk subset with particular features such as an increased proportion of *TP53* aberrations and a lower frequency of 11q deletions. The presence of these aberrations has also been associated with a deregulated expression of genes involved in cell cycle control and DNA damage response.[14] Visentin et al showed that the combination of the presence of CK and/or unbalanced rearrangements by CBA and IGHV mutational status improved their risk stratification.[15] In our cohort, we observed a shorter TTFT for those patients with unbalanced rearrangements but the poor outcome was not confirmed within CK group. Unexpectedly, GM were not able to detect CNAs related to all the apparently unbalanced rearrangements. Indeed, the eight patients with high-CK classified as low-GC by GM carried this type of abnormalities and showed a dismal evolution. On the other hand, our GM analyses identified patients with patterns of chromothripsis who showed a short TTFT. As previously reported, there was a high association between chromothripsis and CK or *TP53* aberrations.[19,33,34] Our study is based in a retrospective cohort highly enriched in patients carrying CK, which was necessary to extensively compare both genetic methodologies in the detection of these prognostically relevant highly complex cases. Therefore, as it is not representative of a real-life CLL cohort, it hinders the development of more accurate genetic prognostic scores. Additionally, potential confounding effects of different therapeutic agents could be attributed to the retrospective and multicenter nature of the cohort enriched in treated patients. These could underlie the lack of statistical significance of genome complexity in the analyses for OS.

To date, most of the survival analyses of genomic complexity included in clinical trials have been reported using CBA data. Even though the prognostic/predictive value of CK for TTFT and progression-free survival in patients treated with chemoimmunotherapy has been extensively demonstrated[2-5,10], its clinical relevance in patients receiving the new treatment modalities has not been fully established. Initial data from clinical trials with novel agents, mainly developed in older relapsed/refractory and/or in high risk patients (*TP53* del/mut, U-IGHV) suggested an adverse significance of CK.[6-8,35] In contrast, a number of recent studies including extended follow-ups of older trials, pooled analyses or new drug combinations have not confirmed its adverse significance.[36-43] However, most of these studies have analyzed CK impact taking into account patients with ≥3 aberrations but not those with high complexity (≥5 aberrations), compared a low number of patients and showed relatively short follow-ups.[44] Thus, additional analyses are needed to clarify the prognostic/predictive impact of genomic complexity.

One particular finding of this study is that, even though overall concordance between FISH and GM is high (90%), GM do not detect around 20% of cases with *TP53* deletion due to their low sensitivity.[23,29] The presence of 17p13 deletions and/or mutations in *TP53* predicts the poorest outcome and its assessment is currently mandatory in CLL study. Our results confirm that FISH should be maintained for the study of CLL patients complemented with one genome-wide technique to assess genome complexity for risk stratification. The choice between CBA and GM will depend on each laboratory, which should take into account the methods and equipment availabilities, personnel expertise and the economic costs, among others.

In conclusion, we have confirmed that both CBA and GM are valuable tools to assess the prognosis of CLL patients based on genomic complexity. Nevertheless, a considerable proportion of cases are discordantly classified depending on the technique employed. Consequently, previous findings generated from CBA, currently the gold standard for cytogenetic assessment, are not directly applicable to GM or other promising cytogenomic methodologies such as optical genome mapping. Additional validation studies are needed to establish the prognostic value of genomic complexity by GM in future prospective studies and clinical trials.

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TABLES

Table 1. Baseline characteristics of patients at diagnosis and last follow-up.

	NON-CK GROUP n = 182; n (%)	**CK GROUP** n = 158; n (%)	***p*-value**
Gender			
Men	118 (64.8%)	113 (71.5%)	0.115
Median age at diagnosis	66 years [29-89]	68 years [33-96]	0.056
Stage at diagnosis			
MBL	11 (6.0%)	2 (1.3%)	0.024
CLL	171 (94.0%)	156 (98.7%)	
Binet A	117/159 (73.6%)	80/136 (58.8%)	0.009
Binet B/C	42/159 (26.4%)	56/136 (41.2%)	
Common CLL genomic aberrations*			
del(13)(q14)	103/182 (56.6%)	96/158 (60.8%)	0.437
isolated del(13)(q14)	70/103 (67.9%)	25/96 (26.0%)	<0.001
Trisomy 12	29/182 (15.9%)	27/158 (17.1%)	0.775
del(11)(q22q23)	25/182 (13.7%)	49/158 (31.0%)	<0.001
Aberrations in *TP53***	21/164 (12.8%)	70/156 (44.9%)	<0.001
del(17)(p13)	8/182 (4.4%)	65/158 (41.1%)	<0.001
TP53 mutation	15/161 (9.3%)	45/147 (30.6%)	<0.001
Unmutated IGHV	80/169 (47.3%)	92/138 (66.7%)	<0.001
Median follow-up [range]	68 months [0-261]	29 months [0-160]	<0.001
Time from diagnosis to cytogenetic study	3.5 months [0-242]	0 months [0-298]	<0.001
Treatment			
Treated patients	32/101 (31.7%)	103/151 (68.2%)	<0.001
Median time to first treatment [95% CI]	NR	13 months [8-18]	<0.001
Survival			
Median overall survival [95% CI]	102 months [82-121]	81 months [58-103]	0.367

*Deletions and trisomy detected by FISH and/or genomic microarrays.
**Cases in which *TP53* mutation screening was not performed and FISH and/or genomic microarrays were negative for deletion were not considered.
Abbreviations: MBL = monoclonal B-cell lymphocytosis, CI = confidence interval, NR: not reached.

Table 2. Recurrent copy number abnormalities found by genomic microarrays within the non-CK and CK subgroups and minimal common altered regions.

CNA	Non-CK		
	n (%)	Cytogenetic bands	Minimal deleted/amplified region (GRCh37/hg19)
Gain 2p	16 (8.8)	p25.3-p21	chr2: 29,477 - 45,859,076
Loss 14q	13 (7.1)	q24.1-q32.11	chr14: 69,272,718 - 91,882,259

CNA	CK		
	n (%)	Cytogenetic bands	Minimal deleted/amplified region (GRCh37/hg19)
Gain 2p	39 (24.7)	p24.3-p23.1	chr2: 15,664,402 - 30,125,169
Gain 2p		p22.3-p15	chr2: 32,877,675 - 62,206,329
Loss 3p	13 (8.2)	p21.31-p21.31	chr3: 47,084,224 - 48,321,854
Gain 3q	11 (6.9)	q26.1-q29	chr3: 165,375,394 - 196,284,424
Loss 4p	15 (9.5)	p16.2-p15.2	chr4: 5,481,786 - 25,640,042
Loss 6q	15 (9.5)	q16.3-q21	chr6: 103,468,966 - 112,256,460
Loss 8p	16 (10.1)	p23.1-p22	chr8: 12,617,155 - 15,933,687
Gain 8q	17 (10.8)	q24.21-q24-21	chr8: 128,286,744 - 130,380,043
Loss 14q	13 (8.2)	q24.2-q24.3	chr14: 70,711,555 - 77,202,084
Loss 15q	16 (10.1)	q15.1-q15.1	chr15: 41,755,587 - 42,090,500
Gain 15q	11 (6.9)	q22.31-q26.3	chr15: 66,265,674 - 99,711,975
Gain 17q	12 (7.6)	q22-q25.1	chr17: 56,560,919 - 71,135,799
Loss 18p	24 (15.2)	p11.31-p11.23	chr18: 4,853,926 - 7,717,988
Gain 19q	12 (7.6)	q13.41-q13.42	chr19: 51,943,080 - 54,499,334

In non-CK group, aberrations were considered recurrent if present in at least 10 patients while in CK group, recurrence was set at 10 and 15 patients for gains and losses, respectively.
Abbreviations: CK = complex karyotype, CNA = copy number abnormality

Table 3. Classification of patients in the previously suggested risk categories according to the number of aberrations detected by chromosome banding analysis and genomic microarrays.

		CHROMOSOME BANDING ANALYSIS			
		Non-CK (0-2 abn.)	**Low / Intermediate-CK** (3-4 abn.)	**High-CK** (≥5 abn.)	**Total**
GENOMIC MICROARRAYS	**Low-GC** (0-2 CNA)	**157**	**27**	**8**	192 (56.5%)
	Intermediate-GC (3-4 CNA)	**23**	**37**	**23**	83 (24.4%)
	High-GC (≥5 CNA)	**2**	**17**	**46**	65 (19.1%)
	Total	182 (53.5%)	81 (23.8%)	77 (22.6%)	340

Abbreviations: CK = complex karyotype, GC = genomic complexity; CNA = copy number abnormality. A moderate agreement was observed between methods (κ=0.507; p<0.001).

Table 4. Univariate and multivariate analysis for time to first treatment (TTFT)

Variable	Univariate analysis		Multivariate analysis for CBA		Multivariate analysis for GM	
	Median TTFT in months (95% CI)	***p*-value**	**Hazard ratio (95% CI)**	***p*-value**	**Hazard ratio (95% CI)**	***p*-value**
CBA						
low/intermediate-CK vs. non-CK	18 (11-25) vs. NR	<0.001	2.54 (1.47-4.41)	<0.001	-	-
high-CK vs. non-CK	5 (1-9) vs. NR	<0.001	3.23 (1.81-5.76)	<0.001	-	-
GM						
intermediate-GC vs. low-GC	35 (0-74) vs. NR	0.022	-	-	1.24 (0.76-2.04)	0.395
high-GC vs. low-GC	3 (0-6) vs. NR	<0.001	-	-	2.74 (1.61-4.67)	<0.001
Del/mut *TP53*	4 (0-9)	<0.001	1.72 (1.14-2.60)	0.010	1.44 (0.92-2.26)	0.109
U-IGHV	12 (4-20)	<0.001	1.71 (1.12-2.61)	0.012	2.12 (1.39-3.22)	<0.001
del(11)(q22q23)	17 (9-25)	0.111	NA	NA	NA	NA

Abbreviations: CBA = chromosome banding analysis, CK = complex karyotype, non-CK = 0-2 abnormalities detected by CBA, low/intermediate-CK = 3-4 abnormalities, high-CK = ≥5 abnormalities, GM = genomic microarrays, GC = genomic complexity, low-GC = 0-2 copy number abnormalities (CNA) detected by genomic microarrays, intermediate-GC = 3-4 CNA, high-GC = ≥5 CNA, del/mut *TP53* = abnormalities in *TP53* include deletion in 17p13 and/or mutation in *TP53* gene, U-IGHV = CLL with unmutated IGHV, NR = not reached, NA = not assessed.

Table 5. Univariate and multivariate analysis for overall survival (OS)

Variable	Univariate analysis		Multivariate analysis for GM*	
	Median OS in months (95% CI)	*p*-value	Hazard ratio (95% CI)	*p*-value
CBA				
low/intermediate-CK vs. non-CK	114 (65-163) vs. 102 (83-121)	0.729	-	-
high-CK vs. non-CK	68 (25-111) vs. 102 (83-121)	0.133	-	-
GM				
intermediate-GC vs. low-GC	114 (64-164) vs. 103 (55-151)	0.741	0.69 (0.36-1.34)	0.275
high-GC vs. low-GC	68 (32-104) vs. 103 (55-151)	0.003	1.51 (0.76-3.01)	0.244
Del/mut *TP53*	50 (29-71)	<0.001	1.89 (1.05-3.42)	0.034
U-IGHV	79 (58-100)	0.008	1.97 (1.15-3.36)	0.013
del(11)(q22q23)	79 (53-105)	0.255	NA	NA

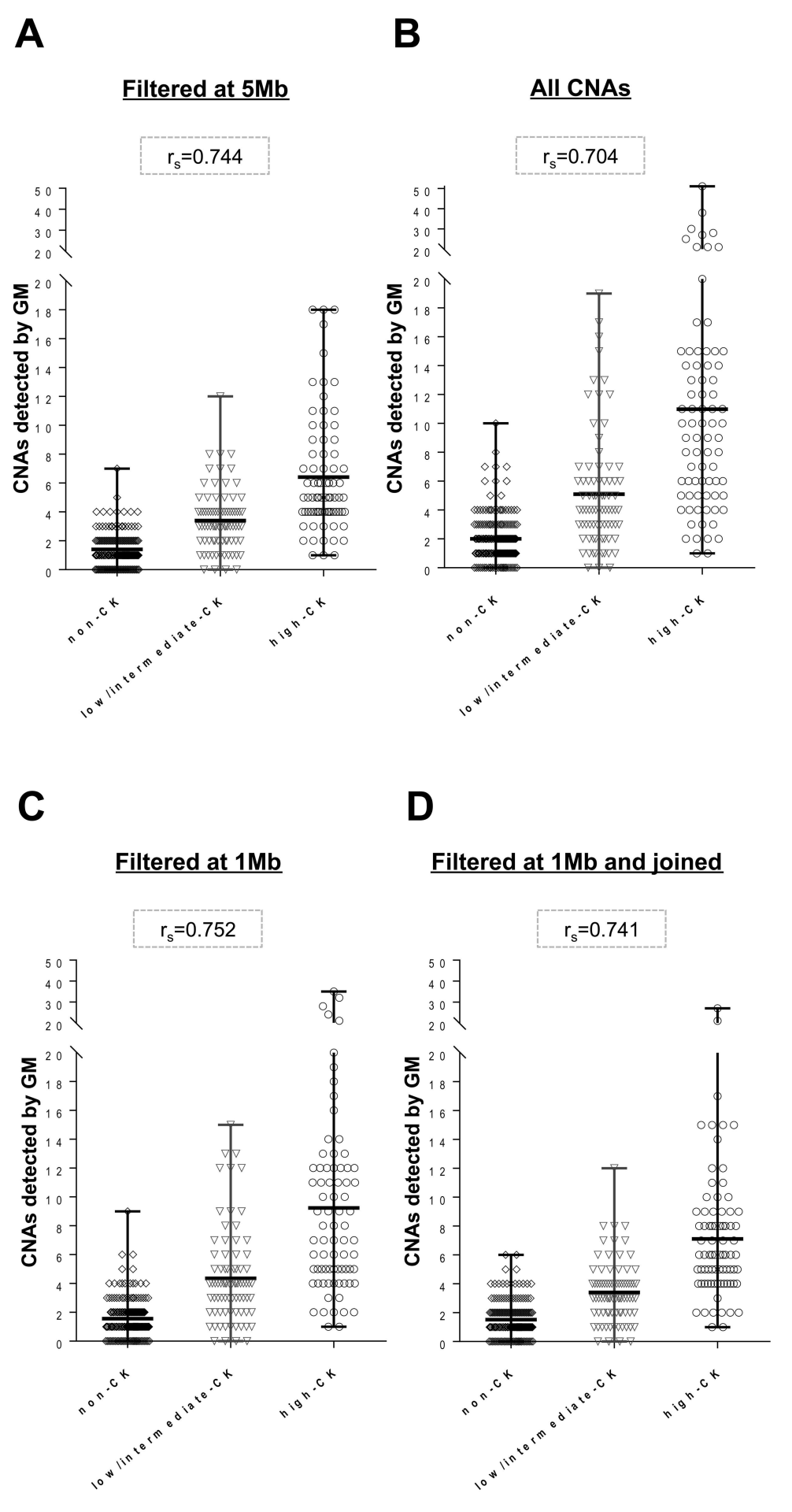
*Multivariate analysis for CBA-defined risk categories was not performed due to the lack of statistical significance in the univariate analysis.
Abbreviations: CBA = chromosome banding analysis, CK = complex karyotype, non-CK = 0-2 abnormalities detected by CBA, low/intermediate-CK = 3-4 abnormalities, high-CK = ≥5 abnormalities, GM = genomic microarrays, GC = genomic complexity, low-GC = 0-2 copy number abnormalities (CNA) detected by genomic microarrays, intermediate-GC = 3-4 CNA, high-GC = ≥5 CNA, del/mut *TP53* = abnormalities in *TP53* include deletion in 17p13 and/or mutation in *TP53* gene, U-IGHV = CLL with unmutated IGHV, NR = not reached, NA = not assessed.

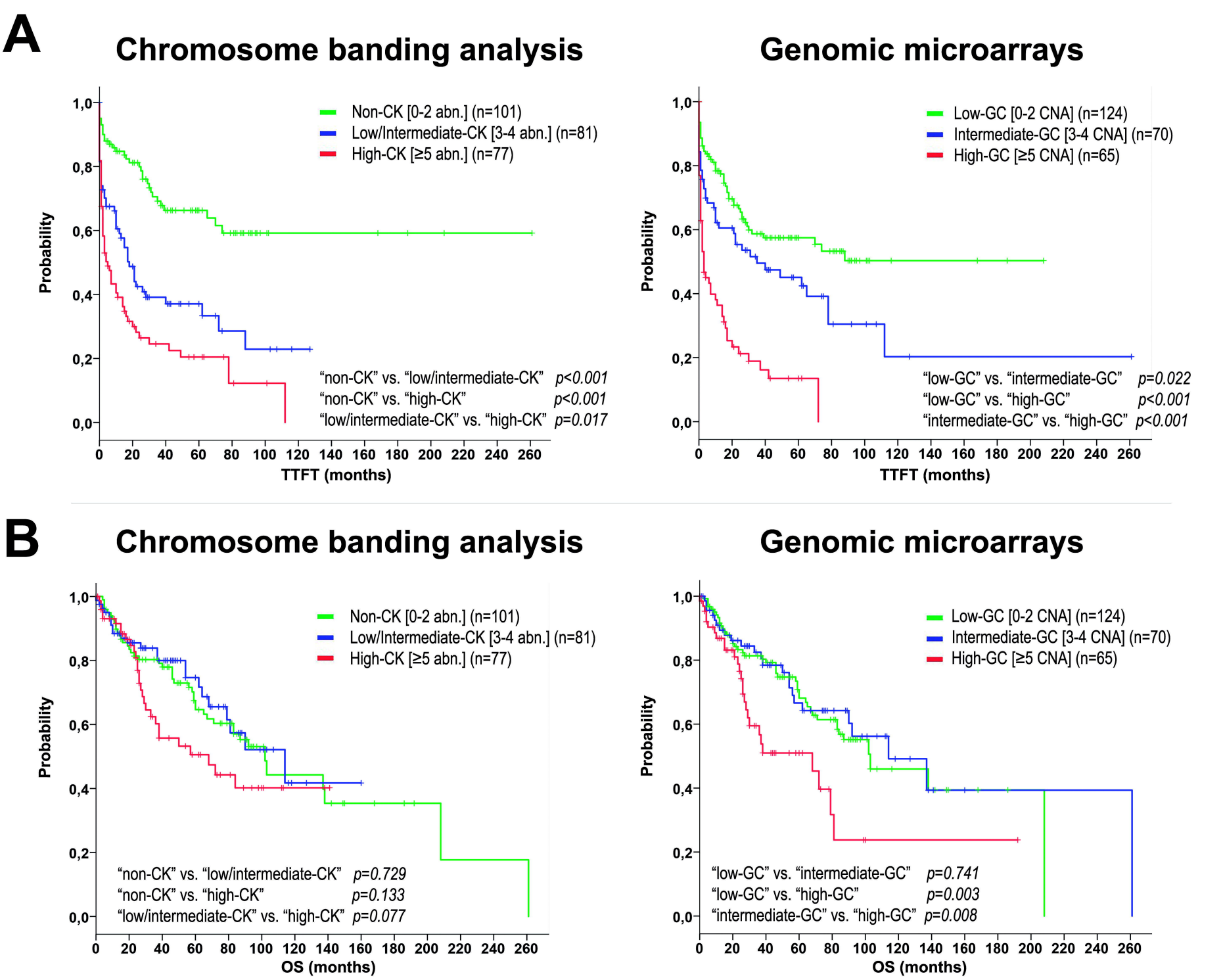
Figure legends

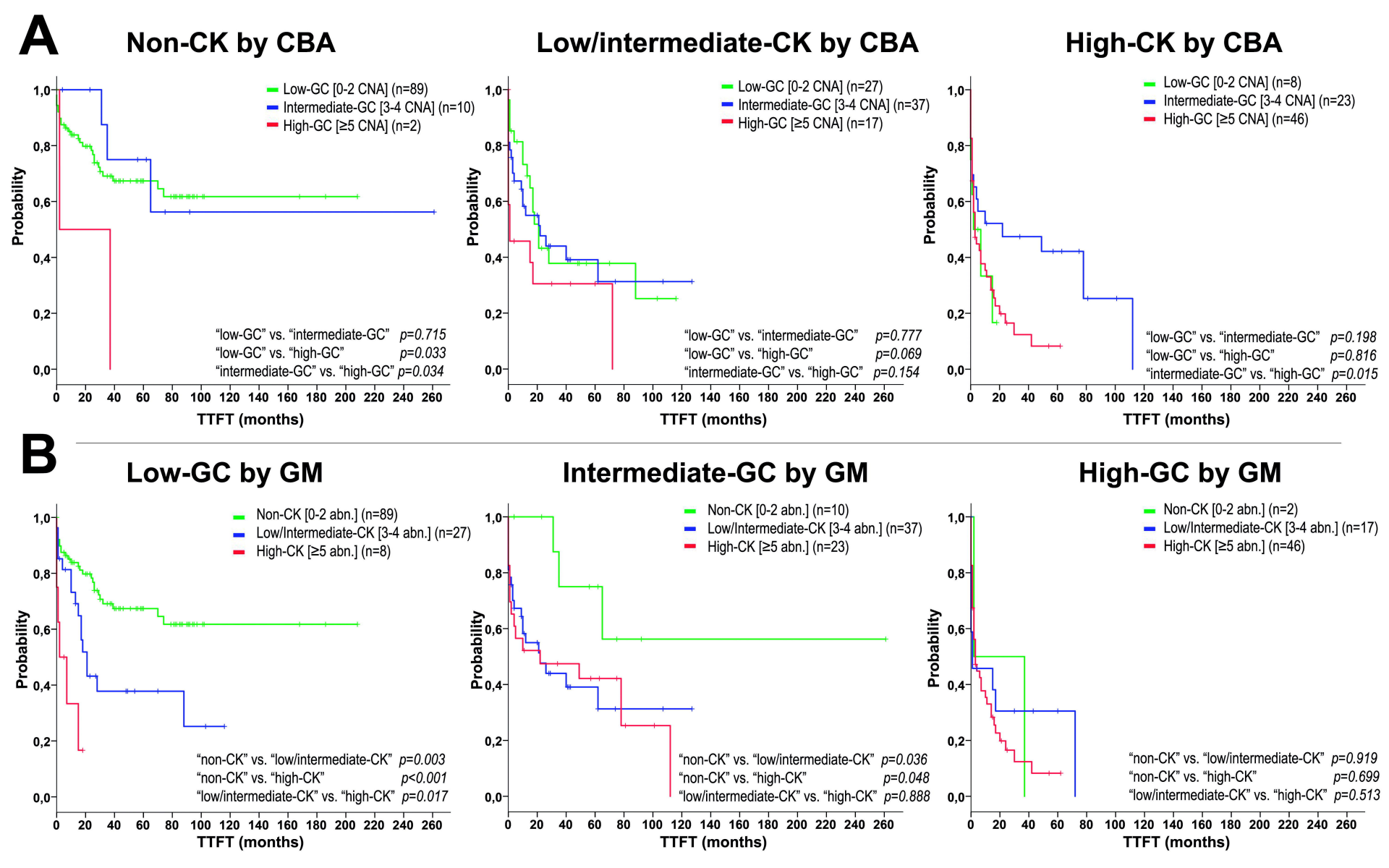
Figure 1. Distribution of the number of CNAs detected by genomic microarrays among the groups identified by chromosome banding analysis. Patients were divided according to the risk groups defined by CBA in non-CK (0-2 abn.), low/intermediate-CK (3-4 abn.) or high-CK (≥5 abn.). Each plot represents CNA counts found when non-classical CLL abnormalities were filtered by different strategies: (A) Current recommended criteria for GM analysis (cut-off size: ≥5Mb); (B) Considering all the CNAs irrespectively of their size; (C) Considering only those CNAs ≥1Mb; (D) Filtering by 1Mb cut-off and grouping small contiguous abnormalities or considering those CNAs included in a chromothripsis event as a unique CNA. Spearman correlation coefficient between CBA and GM counts is shown for each GM analysis.

Figure 2. Kaplan-Meier plots for time to first treatment (TTFT) and overall survival (OS) based on genomic complexity stratification assessed by chromosome banding analysis and genomic microarrays. Kaplan-Meier estimation for TTFT (A) and OS (B) in patients classified in each category based on total number of aberrations found by CBA [non-CK (0-2 abn.), low/intermediate-CK (3-4 abn.) or high-CK (≥5 abn.)] (plots on the left) and based on total number of copy number aberrations (CNA) detected by GM [low-GC (0-2 CNA), intermediate-GC (3-4 CNA) or high-GC (≥5 CNA)] (plots on the right).

Figure 3. Kaplan-Meier plots for time to first treatment (TTFT) of the genomic risk stratification within each category defined by the alternative technique. (A) Patients classified in each category based on total number of aberrations found by CBA [non-CK (0-2 abn.), low/intermediate-CK (3-4 abn.) or high-CK (≥5 abn.)] are represented in different plots. TTFT of GM defined groups was assessed. Within non-CK and low/intermediate-CK, cases classified as high-GC (≥5 CNA by GM) showed a poor outcome. In the high-CK group, those low-GC patients did not display a better evolution while intermediate-GC cases showed an unexpected median TTFT of 22 months. (B) Each plot represents patients classified in each category based on total number of copy number aberrations (CNA) detected by GM [low-GC (0-2 CNA), intermediate-GC (3-4 CNA) or high-GC (≥5 CNA)]. Within each subgroup, TTFT of CBA defined groups was assessed. Low-GC patients could be stratified in three risk categories when reclassified by CBA, while no significant differences were observed when intermediate-GC and high-GC subsets were reclassified.


A
Filtered at 5Mb
r_s=0.744
CNAs detected by GM
non-CK
low/intermediate-CK
high-CK
B
All CNAs
r_s=0.704
CNAs detected by GM
non-CK
low/intermediate-CK
high-CK
C
Filtered at 1Mb
r_s=0.752
CNAs detected by GM
non-CK
low/intermediate-CK
high-CK
D
Filtered at 1Mb and joined
r_s=0.741
CNAs detected by GM
non-CK
low/intermediate-CK
high-CK


A
Chromosome banding analysis
Non-CK [0-2 abn.] (n=101)
Low/Intermediate-CK [3-4 abn.] (n=81)
High-CK [≥5 abn.] (n=77)
"non-CK" vs. "low/intermediate-CK" p<0.001
"non-CK" vs. "high-CK" p<0.001
"low/intermediate-CK" vs. "high-CK" p=0.017
Probability
TTFT (months)
Genomic microarrays
Low-GC [0-2 CNA] (n=124)
Intermediate-GC [3-4 CNA] (n=70)
High-GC [≥5 CNA] (n=65)
"low-GC" vs. "intermediate-GC" p=0.022
"low-GC" vs. "high-GC" p<0.001
"intermediate-GC" vs. "high-GC" p<0.001
Probability
TTFT (months)
B
Chromosome banding analysis
Non-CK [0-2 abn.] (n=101)
Low/Intermediate-CK [3-4 abn.] (n=81)
High-CK [≥5 abn.] (n=77)
"non-CK" vs. "low/intermediate-CK" p=0.729
"non-CK" vs. "high-CK" p=0.133
"low/intermediate-CK" vs. "high-CK" p=0.077
Probability
OS (months)
Genomic microarrays
Low-GC [0-2 CNA] (n=124)
Intermediate-GC [3-4 CNA] (n=70)
High-GC [≥5 CNA] (n=65)
"low-GC" vs. "intermediate-GC" p=0.741
"low-GC" vs. "high-GC" p=0.003
"intermediate-GC" vs. "high-GC" p=0.008
Probability
OS (months)


A
Non-CK by CBA
Low-GC [0-2 CNA] (n=89)
Intermediate-GC [3-4 CNA] (n=10)
High-GC [≥5 CNA] (n=2)
"low-GC" vs. "intermediate-GC" p=0.715
"low-GC" vs. "high-GC" p=0.033
"intermediate-GC" vs. "high-GC" p=0.034
Low/intermediate-CK by CBA
Low-GC [0-2 CNA] (n=27)
Intermediate-GC [3-4 CNA] (n=37)
High-GC [≥5 CNA] (n=17)
"low-GC" vs. "intermediate-GC" p=0.777
"low-GC" vs. "high-GC" p=0.069
"intermediate-GC" vs. "high-GC" p=0.154
High-CK by CBA
Low-GC [0-2 CNA] (n=8)
Intermediate-GC [3-4 CNA] (n=23)
High-GC [≥5 CNA] (n=46)
"low-GC" vs. "intermediate-GC" p=0.198
"low-GC" vs. "high-GC" p=0.816
"intermediate-GC" vs. "high-GC" p=0.015
Probability
TTFT (months)
B
Low-GC by GM
Non-CK [0-2 abn.] (n=89)
Low/Intermediate-CK [3-4 abn.] (n=27)
High-CK [≥5 abn.] (n=8)
"non-CK" vs. "low/intermediate-CK" p=0.003
"non-CK" vs. "high-CK" p<0.001
"low/intermediate-CK" vs. "high-CK" p=0.017
Intermediate-GC by GM
Non-CK [0-2 abn.] (n=10)
Low/Intermediate-CK [3-4 abn.] (n=37)
High-CK [≥5 abn.] (n=23)
"non-CK" vs. "low/intermediate-CK" p=0.036
"non-CK" vs. "high-CK" p=0.048
"low/intermediate-CK" vs. "high-CK" p=0.888
High-GC by GM
Non-CK [0-2 abn.] (n=2)
Low/Intermediate-CK [3-4 abn.] (n=17)
High-CK [≥5 abn.] (n=46)
"non-CK" vs. "low/intermediate-CK" p=0.919
"non-CK" vs. "high-CK" p=0.699
"low/intermediate-CK" vs. "high-CK" p=0.513
Probability
TTFT (months)

Chromosome banding analysis and genomic microarrays are both useful but not equivalent methods for genomic complexity risk stratification in chronic lymphocytic leukemia patients

Ramos-Campoy et al.

SUPPLEMENTARY DATA

Supplementary Methods

Patient cohort

Patients were diagnosed between 1983 and 2018 according to current guidelines.[1-4] Clinical information collected at diagnosis included demographics (age and gender), Binet stage, genetic and molecular data. Regarding information on evolution, dates of treatment administration and last follow-up were collected. Of note, data from CBA or GM of some patients have been included in previous publications although they were not used with the same purpose as the present study.[5-13]

Cytogenetic analyses

Peripheral blood (PB) or bone marrow (BM) samples cultures using either phorbol-12-myristate-13-acetate (TPA) (n=228; 67%), immunostimulatory cytosine guanine dinucleotide (CpG)-oligonucleotide DSP30 plus interleukin 2 (IL-2) (n=19; 5.6%) or both (n=93; 27.4%) as mitogens were established following standard procedures.[14] At least 20 metaphases were analyzed in cases with normal karyotype while for abnormal karyotypes, the minimum were 10. Number and type of abnormalities were recorded. Balanced rearrangements included translocations and inversions, while chromosome additions, duplications, insertions, isochromosomes, as well as derivative, dicentric, ring and marker chromosomes were considered unbalanced rearrangements and were counted as one aberration.

Interphase fluorescence *in situ* hybridization (FISH) results were available in 320/340 (94.1%) cases using probes for the chromosomal regions 13q14, 11q22 (*ATM*) and 17p13 (*TP53*) and the centromere of chromosome 12 (CEP 12). In five cases, whole chromosome painting was performed in order to study the discrepancies observed between CBA and GM.

Genomic microarray analyses

Genomic microarrays data were already available or obtained from DNA extracted in a period of time less than one year from the date of CBA in order to avoid the emergence of additional abnormalities (median time from CBA to GM=0 months; range: 0-12). GM

were assessed on DNA from whole PB (n=113; 33%), PB mononuclear cells (n=63; 19%), PB CD19+ purified cells (n=110; 32%) or from BM samples (n=54; 16%). Only DNA that fulfilled quality controls required was amplified, labelled and hybridized to different genomic microarray platforms according to the manufacturer's protocols. Obtained data were visually revised and copy number variants found as benign polymorphisms in the Database of Genomic Variants (http://dgv.tcag.ca/dgv/app/home) were excluded. For defining genome coordinates, annotations of genome version GRCh37/hg19 were used. Chromothripsis-like and chromothripsis patterns were defined by the presence of ≥7 and ≥10 oscillating switches, respectively, between two or three copy number states on an individual chromosome.[7,8,15]

Although the objectives of the study did not consider the analysis of copy-number neutral loss of heterozygosity (CN-LOH), in those cases in which the microarray platform included single nucleotide polymorphisms (SNP) probes, a global screening for CN-LOH was performed. CN-LOH were recorded when detected in a region larger than 10Mb and extending to chromosome telomeres. They were not included in the counting of CNAs.

TP53 mutation analysis

A total of 308 (90.6%) cases were screened for *TP53* mutations. For the assessment of *TP53* mutations exons 4-8 were sequenced (exons 9-10 were also included in some centers) following ERIC recommendations.[16] Sixty (19.5%) cases were screened by Sanger sequencing whereas the remaining (n=248; 80.5%) were analyzed by next-generation sequencing. Only mutations with a variant allele frequency >10% were considered.

IGHV mutational analysis

IGHV mutational status was analyzed in 307 (90.3%) patients following established international guidelines.[17] Sequences were examined and interpreted using the IMGT database and the IMGT/V-QUEST tool. Clonotypic IGHV gene sequences with <98% germline identity were defined as mutated (M-IGHV) whereas those with ≥98% identity were classified as unmutated (U-IGHV).

Statistical analyses

As different European centers were involved in the present study, before performing the survival analyses we evaluated the homogeneity of the results in terms of time to first treatment (TTFT). We found out that in three institutions, TTFT in the non-CK group was notably shorter than previously reported in other studies[11] because CBA in

these centers were mainly performed at recruitment for clinical trials. Therefore, in order to avoid biases in the results reported herein, 81 cases were not included in the survival analyses. As for the CK group, no differences were observed between the collaborating centers. Consequently, survival analyses were performed in 259 patients.

Supplementary Results

Risk stratification of the genomic complexity observed by CBA and GM

Regarding CBA, when results obtained with each mitogen were considered separately, those cases stimulated with IL-2+DSP30 exhibited a higher proportion of complex cases. Significant differences were observed in the percentage of patients classified into intermediate-risk categories (3-4 abnormalities; 20.6% with TPA vs. 32.1% with IL-2+DSP30) or those showing the highest risk (≥5 abnormalities; 14.9% and 27.7%, respectively) (p<0.001). However, when comparing with GM classification, both methods presented a similar moderate agreement (TPA: κ=0.464; IL-2+DSP30: κ=0.530).

Number and type of abnormalities detected by CBA and GM

Regions with CN-LOH were detected in 23 (7.5%) patients as the microarray platform used in 306 cases also contained SNP probes. Median size of CN-LOH was 50.1Mb (range: 11.9-159Mb) and they were found in several chromosomes. Notably, two of the three cases with CN-LOH affecting 17p arm and the only case with CN-LOH involving *ATM* gene had *TP53* and *ATM* genes mutated, respectively. Nevertheless, CN-LOH data were not included in the analyses.

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Supplementary Tables

Table S1. Genomic microarray platforms used in this study.

Genomic microarray platform	n (%)
Whole-Genome 2.7M (ThermoFisher)	62 (18.2)
CytoScan HD array (ThermoFisher)	87 (25.6)
Affymetrix SNP6.0 (ThermoFisher)	82 (24.1)
SurePrint G3 Human CGH 8x60K (Agilent)*	11 (3.2)
SurePrint G3 ISCA CGH+SNP Bundle, 4x180K (Agilent)	75 (22.1)
Illumina Human Omni1-Quad array (Illumina)	12 (3.5)
Illumina Human Omni2.5-Quad array (Illumina)	11 (3.2)

*Custom design described in Salaverria I, Martín-Garcia D, López C, et al. Detection of chromothripsis-like patterns with a custom array platform for chronic lymphocytic leukemia. Genes Chromosomes Cancer. 2015;54(11):668-80.

Table S2. Detection of the four classical CLL abnormalities by chromosome banding analysis in those patients with FISH results available.

	FISH		CHROMOSOME BANDING ANALYSIS				
Locus affected	**Patients tested**	**Altered cases** *n* (%)	**Abnormalities in CLL *loci*** *n* (%)	**Detail of the abnormalities**	n (%)	**Confirmed by FISH**	**Overall concordance**
13q14	327	188 (57.5)	97 (29.7)	Monosomy 13	13 (13.4)	12/13 (92.3%)	50.0%
				Deletion 13q14	66 (68.0)	64/66 (97.0%)	
				Balanced translocation involving 13q14	9 (9.3)	9/9 (100%)	
				Unbalanced translocation in 13q14	9 (9.3)	9/9 (100%)	
Chromosome 12	327	56 (17.1)	54 (16.5)	Trisomy 12	54 (16.5)	54/54 (100%)	96.4%
11q22q23 (*ATM*)	329	71 (21.6)	67 (20.4)	Monosomy 11	1 (1.5)	1/1 (100%)	87.3%
				Deletion 11q22q23	58 (86.6)	55/58 (94.8%)	
				Additional material in 11q22q23	1 (1.5)	1/1 (100%)	
				Balanced translocation involving 11q22q23	2 (2.9)	1/2 (50.0%)	
				Unbalanced translocation in 11q22q23	5 (7.5)	4/5 (80.0%)	
17p13 (*TP53*)	329	69 (21.0)	67 (20.4)	Monosomy 17	14 (20.9)	13/14 (92.9%)	91.3%
				Deletion 17p13	10 (14.9)	10/10 (100%)	
				Additional material in 17p	16 (23.9)	14/16 (87.5%)	
				Isochromosome (17)(q10)	5 (7.5)	5/5 (100%)	
				Dicentric chromosomes affecting 17p	4 (5.9)	4/4 (100%)	
				Unbalanced translocation in 17p	18 (26.9)	17/18 (94.4%)	

Table S3. Detection of the four classical CLL abnormalities by genomic microarrays in those patients with FISH results available.

	FISH		GENOMIC MICROARRAYS				
Locus affected	**Patients tested**	**Altered cases** n (%)	**Altered cases** n (%)	**Median size** Mb (range)	**Minimal abnormal region** Cytobands (coordinates: GRCh37/hg19)	**Overall concordance**	**Detail of discordant cases**
13q14	327	188 (57.5)	171 (52.3)	1.94 (0.035-95.7)	q14.2-q14.2 (50,632,951 - 50,659,544)	88.8% (167/188)	- 21 only positive by FISH (20/21 <30% nuclei) - 4 only positive by GM
Chromosome 12	327	56 (17.1)	55 (16.8)	133.60 (132.3-133.8)	p13.33-q24.33 (192,539 - 132,349,534)	98.2% (55/56)	- 1 only positive by FISH (7% nuclei)
11q22q23 (*ATM*)	329	71 (21.6)	68 (20.7)	25.81 (0.151-54.5)	q22.3-q22.3 (108,125,328-108,276,581)	91.5% (65/71)	- 6 only positive by FISH (5/6 <30% nuclei) - 3 only positive by GM
17p13 (*TP53*)	329	69 (21.0)	58 (17.6)	21.47 (0.470-25.9)	p13.1-p13.1 (7,481,305-7,678,604)	82.6% (57/69)	- 12 only positive by FISH (11/12 <20% nuclei) - 1 only positive by GM

Table S4. Abnormalities detected by chromosome banding analysis and genomic microarrays in the ten patients classified in opposite risk categories depending on the technique employed for their study.

Case	CHROMOSOME BANDING ANALYSIS		GENOMIC MICROARRAYS					
	Karyotype	Number of aberrations	Type	Chr.	Start-end	Size (Mb)	Number of CNA	Number of CNA ≥5Mb
#37	47,XY,add(8)(p23),-9,add(12)(q24),-13,-18,+4mar[41]/46,XY[8]	9	GAIN	12	p13.33-q24.33 (173786-133777902)	133,604	2	1
			GAIN	18	p11.31-p11.23 (6929190-8087455)	1,158		
#38	47,XY,-4,del(7)(p?),+12,add(15)(q26),+mar[20]/47,XY,+12[20]/ 46,XY[10]	5	GAIN	12	p13.33-q24.33 (173786-133777902)	133,604	1	1
#100	46,XY,add(19)(q13.4)[25]/45,XY,der(2)t(2;6)(p23;p12),del(6)(q?), -20,del(21)(q22)[5]/46,XY[21]	5	GAIN	2	p25.3-p13.3 (0-70151030)	70,151	1	1
#119	46,XX,i(17)(q10)[7]/45,XX,-13,add(14)(q11),i(17)(q10)[4]/ 44,XX,del(6)(q14q24),add(7)(q36),-12,-15,i(17)(q10)[1]/ 43,XX,-5,del(6)(q14q24),add(7)(q36),add(10)(q22),-12, -15,i(17)(q10)[7]/46,XX[4]	9	GAIN	17	q11.1-q25.3 (25270397-81041938)	55,772	2	2
			LOSS	17	p13.3-p11.1 (525-22261792)	22,261		
#121	47,XY,+12[9]/47,XY,del(X)(q25),add(5)(q31),add(8)(q24),+12, del(14)(q22q32)[4]/46,XY[7]	5	GAIN	12	p13.33-q24.33 (173786-133777902)	133,604	2	2
			LOSS	14	q23.2-q32.12 (63953105-93505497)	29,552		
#152	46,XX,del(14)(q24q32)[1]/44,XX,der(4;21)(q10;q10),del(14)(q24q32), der(15;22)(q10;q10)[5]/43,XX,der(1)t(1;17)(p11;q11), der(4;21)(q10;q10),der(11)t(1;11)(p11;q25),del(14)(q24q32), der(15;22)(q10;q10),-17[5]/ XY 46,XX[6]	6	LOSS	4	p16.3-p14 (0-39309957)	39,310	4	2
			LOSS	4	p12-p11 (45427534-49174296)	3,747		
			LOSS	4	q11-q12 (52697788-55087565)	2,390		
			LOSS	14	q24.1-q32.33 (69262059-106067093)	36,805		
#180	46,Y,der(X)t(X;2)(q26;p15)[5]/46,XY,der(2)t(2;2)(p24;p15)[5]/ 46,XY,der(5)t(2;5)(p15;q35),r(8)(p11q24),der(11)t(5;11)(?;q24)t(2;5) (p15;?)[4]/46,XY,der(6)t(2;6)(p15;q27)[2]/46,XY[14]	6	GAIN	2	p25.3-p15 (0-62206329)	62,206	2	2
			LOSS	13	q14.2-q14.3 (49874813-51747327)	1,873		
#228	45,X,-Y,del(11)(q?14),del(13)(q14q22)[7]/ 44,idem,der(12)t(12;13)(p?13;q?12)[5]/45,idem,t(1;11)(p?36;q13)[3]/ 46,XY[2]	5	LOSS	11	q14.1-q23.3 (79261152-116172518)	36,911	3	2
			LOSS	13	q14.11-q21.33 (43292880-72523559)	29,231		
			LOSS	X	q27.3-q27.3 (145094655-145177733)	0,083		

#c362	44,XY,der(5;17)t(5;17)ins(5;17)(q1?3;p11q25)[2]/46,XY[12]	1	LOSS	5	q12.3-q13.1 (64558088-66809076)	2,251	10	7
			LOSS	5	q13.2-q23.1 (70908308-119198005)	48,290		
			LOSS	5	q31.3-q32 (144329951-149326255)	4,996		
			LOSS	5	q33.1-q33.2 (149834719-155509902)	5,675		
			LOSS	11	q22.3-q23.3 (105315158-115742438)	10,427		
			LOSS	13	q14.2-q14.3 (48675135-51631607)	2,956		
			LOSS	17	p13.3-p11.2 (1-19149275)	19,149		
			LOSS	17	q11.1-q11.2 (25270425-26179601)	0,909		
			LOSS	18	p11.32-p11.21 (454728-14318059)	13,863		
			LOSS	20	p13-p11.1 (60001-26107860)	26,048		
#c377	46,XY[29]	0	LOSS	1	q42.12-q42.12 (225692953-225845385)	0,152	7	5
			LOSS	1	q42.12-q44 (225961441-249224401)	23,263		
			LOSS	4	p16.3-p15.2 (10001-27127332)	27,117		
			LOSS	4	p14-q21.21 (39184089-81142337)	41,958		
			LOSS	4	q31.3-q31.3 (153332112-154672325)	1,340		
			LOSS	13	q14.13-q14.3 (46725019-52636989)	5,912		
			LOSS	13	q14.2-q14.3 (50339821-51605362)	1,266		

Those CNA highlighted in grey were non-classical CLL abnormalities smaller than 5Mb.

Table S5. Initial and rewritten karyotypes from six patients in which the formula was modified after genomic microarrays analysis.

Case	Initial karyotype	N abn.	Abnormalities by genomic microarrays				Rewritten karyotype	N abn.
			Type	Chr.	Start-end	Size (Mb)		
#16	45,XY,-13,add(15)(p11),der(17)t(13;17)(q11;p11)[10]/ 46,XY[10]	3	GAIN	3	q11.1-q29 (93626178-197851986)	104,226	45,XY,-13,der(15)t(3;15)(q11;p11), der(17)t(13;17)(q11;p11)[10]/46,XY[10]	3
			LOSS	3	p26.3-p26.1 (311066-6061949)	5,751		
			LOSS	13	q14.2-q14.3 (50595391-51485770)	0,890		
			GAIN	15	q22.2-q26.3 (60417426-102345371)	41,928		
			LOSS	17	p13.3-p11.1 (9474-22227062)	22,218		
#43	47,XX,del(11)(q23),-14,+2mar[10]/46,XX[20]	4	GAIN	2	p25.3-p11.2 (12770-89129064)	89,116	47,XX,+i(2)(p10),del(11)(q23),del(13)(q14q22)[10]/ 46,XX[20]	3
			LOSS	11	q14.1-q23.3 (77108160-117201998)	40,094		
			LOSS	13	q14.2-q22.3 (49894796-77764277)	27,869		
			LOSS	14	q23.2-q24.1 (64199833-69665479)	5,466		
#48	47,XY,der(12)(q?),+der(12)(q?),del(13)(q?)[30]	3	GAIN	12	p13.33-q23.2 (173786-102013163)	101,839	47,XY,der(12)(q?),+der(12)t(12;13)(q23;q21), del(13)(q14q21)[30]	3
			GAIN	13	q21.32-q34 (67265752-115107733)	47,842		
			LOSS	13	q14.13-q21.31 (46950690-63774667)	16,824		
			LOSS	17	p12-p12 (14000097-14622477)	0,622		
#58	46,XY,-5,-6,-14,+3mar[9]/46,XY[11]	6	LOSS	5	q14.2-q23.1 (82410385-115495863)	33,085	46,XY,del(5)(q14q23),der(6)inv(6)(?),del(13)(q12q14)[9]/ 46,XY[11]	3
			LOSS	13	q14.2-q14.3 (50140480-51426156)	1,286		
			LOSS	13	q12.3-q14.3 (32145265-52311881)	20,167		
#61	43-45,X,-X,del(2)(p15),+4,-7,add(11)(q21),-12,-13, add(14)(q32),add(17)(p11)[cp6]/46,XX[9]	9	GAIN	2	p25.3-p13.1 (12770-73803026)	73,790	45,X,-X,del(2)(p15),+4,der(7)t(2;7)(p13;q36), del(11)(q21q23),-12,del(13)(q14),der(14)t(2;14)(p13;q32), dic(17;18)(p10;p10),+3mar[6]/46,XX[9]	12
			LOSS	11	q21-q23.2 (95086750-112810693)	17,724		
			LOSS	13	q14.2-q14.3 (50691182-51659251)	0,968		
			LOSS	17	p13.3-p11.2 (525-21565553)	21,565		
			LOSS	18	p11.32-p11.21 (136226-13719291)	13,583		
#62	46,XX,t(2;5)(p16;p15),del(4)(q31),add(15)(q26)[6]/ 46,XX[18]	3	No aberrations				46,XX,t(2;15)(p16;q26),t(4;5)(q31;p15)[3]/46,XX[18]	2

Abbreviations: Abn.= Abnormalities, Chr.= Chromosome

Table S6. Frequency of different genetic features in the three subgroups defined by chromosome banding analysis and genomic microarrays (n=259)

	CHROMOSOME BANDING ANALYSIS					GENOMIC MICROARRAYS				
	Non-CK	**Low/ intermediate-CK**	**High-CK**	***p*-value**	***p*-value** low/int vs high-CK	**Low-GC**	**Intermediate-GC**	**High-GC**	***p*-value**	***p*-value** intermediate vs high-GC
Known CLL genetic prognostic factors										
Del(13)(q14)	63 (62.4%)	47 (58.0%)	49 (63.6%)	0.744	0.470	68 (54.8%)	50 (71.4%)	41 (63.1%)	0.071	0.301
Trisomy 12	18 (17.8%)	19 (23.5%)	8 (10.4%)	0.095	0.029	28 (22.6%)	15 (21.4%)	2 (3.1%)	0.002	0.001
Del(11)(q22)	12 (11.9%)	26 (32.1%)	23 (29.9%)	0.002	0.762	13 (10.5%)	28 (40.0%)	20 (30.8%)	<0.001	0.263
Del(17)(p13)/mutation *TP53* (n=239)	7 (8.4%)	23 (28.7%)	47 (61.8%)	<0.001	<0.001	11 (10.3%)	21 (30.9%)	45 (70.3%)	<0.001	<0.001
U-IGHV (n=227)	25 (28.1%)	42 (61.8%)	50 (71.4%)	<0.001	0.229	40 (36.7%)	35 (60.3%)	42 (70.0%)	<0.001	0.271
Type of abnormality by CBA										
Unbalanced rearrangements	3 (3.0%)	58 (71.6%)	76 (98.7%)	<0.001	<0.001	26 (21.0%)	49 (70.0%)	62 (95.4%)	<0.001	<0.001
Presence of material from unknown origin	1 (1.0%)	32 (39.5%)	45 (58.4%)	<0.001	0.017	15 (12.1%)	26 (37.1%)	37 (56.9%)	<0.001	0.021
Clonal evolution	1 (1.0%)	40 (49.4%)	41 (53.2%)	<0.001	0.627	20 (16.1%)	29 (41.4%)	33 (50.8%)	<0.001	0.277
Type of abnormality by GM										
Common CNA										
Gain 2p	2 (2.0%)	12 (14.8%)	27 (35.1%)	<0.001	0.003	3 (2.4%)	19 (27.1%)	19 (29.2%)	<0.001	0.788
Loss 3p	1 (1.0%)	5 (6.2%)	8 (10.4%)	0.021	0.335	3 (2.4%)	3 (4.3%)	8 (12.3%)	0.015	0.089
Gain 3q	0	6 (7.4%)	5 (6.5%)	0.024	0.822	0	4 (5.7%)	7 (10.8%)	0.002	0.283
Loss 4p	1 (1.0%)	7 (8.6%)	8 (10.4%)	0.019	0.708	1 (0.8%)	2 (2.9%)	13 (20.0%)	<0.001	0.002
Loss 6q	1 (1.0%)	5 (6.2%)	10 (13.0%)	0.004	0.144	1 (0.8%)	5 (7.1%)	10 (15.4%)	<0.001	0.128
Loss 8p	0	6 (7.4%)	10 (13.0%)	0.001	0.245	0	5 (7.1%)	11 (16.9%)	<0.001	0.079
Gain 8q	1 (1.0%)	8 (9.9%)	9 (11.7%)	0.010	0.713	0	4 (5.7%)	14 (21.5%)	<0.001	0.007
Loss 14q	2 (2.0%)	4 (4.9%)	9 (11.7%)	0.021	0.123	5 (4.0%)	4 (5.7%)	6 (9.2%)	0.348	0.521
Loss 15q	0	5 (6.2%)	11 (14.3%)	<0.001	0.091	1 (0.8%)	3 (4.3%)	12 (18.5%)	<0.001	0.009
Gain 17q	0	3 (3.7%)	9 (11.7%)	0.001	0.058	1 (0.8%)	3 (4.3%)	8 (12.3%)	0.002	0.089
Loss 18p	3 (3.0%)	6 (7.4%)	18 (23.4%)	<0.001	0.005	2 (1.6%)	8 (11.4%)	17 (26.2%)	<0.001	0.028
Gain 19q	1 (1.0%)	6 (7.4%)	6 (7.8%)	0.059	0.927	1 (0.8%)	8 (11.4%)	4 (6.2%)	0.004	0.282
Chromothripsis	1 (1.0%)	7 (8.6%)	22 (28.6%)	<0.001	0.001	1 (0.8%)	6 (8.6%)	23 (35.4%)	<0.001	<0.001

Abbreviations: CK = complex karyotype, non-CK = 0-2 abnormalities detected by chromosome banding analysis, low/intermediate-CK = 3-4 abnormalities, high-CK = ≥5 abnormalities, GC = genomic complexity, low-GC = 0-2 copy number abnormalities (CNA) detected by genomic microarrays, intermediate-GC = 3-4 CNA, high-GC = ≥5 CNA, U-IGHV = CLL with unmutated IGHV

Table S7. Univariate and multivariate analysis for time to first treatment (TTFT)

Variable	Univariate analysis		Multivariate analysis for CBA		Multivariate analysis for GM	
	Median TTFT in months (95% CI)	***p*-value**	**Hazard ratio (95% CI)**	***p*-value**	**Hazard ratio (95% CI)**	***p*-value**
CBA						
low/intermediate-CK vs. non-CK	18 (11-25) vs. NR	<0.001	2.85 (1.53-5.31)	<0.001	-	-
high-CK vs. non-CK	5 (1-9) vs. NR	<0.001	4.54 (2.18-9.44)	<0.001	-	-
GM						
intermediate-GC vs. low-GC	35 (0-74) vs. NR	0.022	-	-	1.60 (1.05-2.43)	0.029
high-GC vs. low-GC	3 (0-6) vs. NR	<0.001	-	-	3.52 (2.27-5.46)	<0.001
Unbalanced rearrangements	11 (5-17)	<0.001	0.97 (0.53-1.77)	0.916	-	-
Chromothripsis	2 (0-6)	<0.001	-	-	1.35 (0.83-2.20)	0.228

Abbreviations: CBA = chromosome banding analysis, CK = complex karyotype, non-CK = 0-2 abnormalities detected by CBA, low/intermediate-CK = 3-4 abnormalities, high-CK = ≥5 abnormalities, GM = genomic microarrays, GC = genomic complexity, low-GC = 0-2 copy number abnormalities (CNA) detected by genomic microarrays, intermediate-GC = 3-4 CNA, high-GC = ≥5 CNA, CI = confidence interval, NR = not reached.

Supplementary Figures

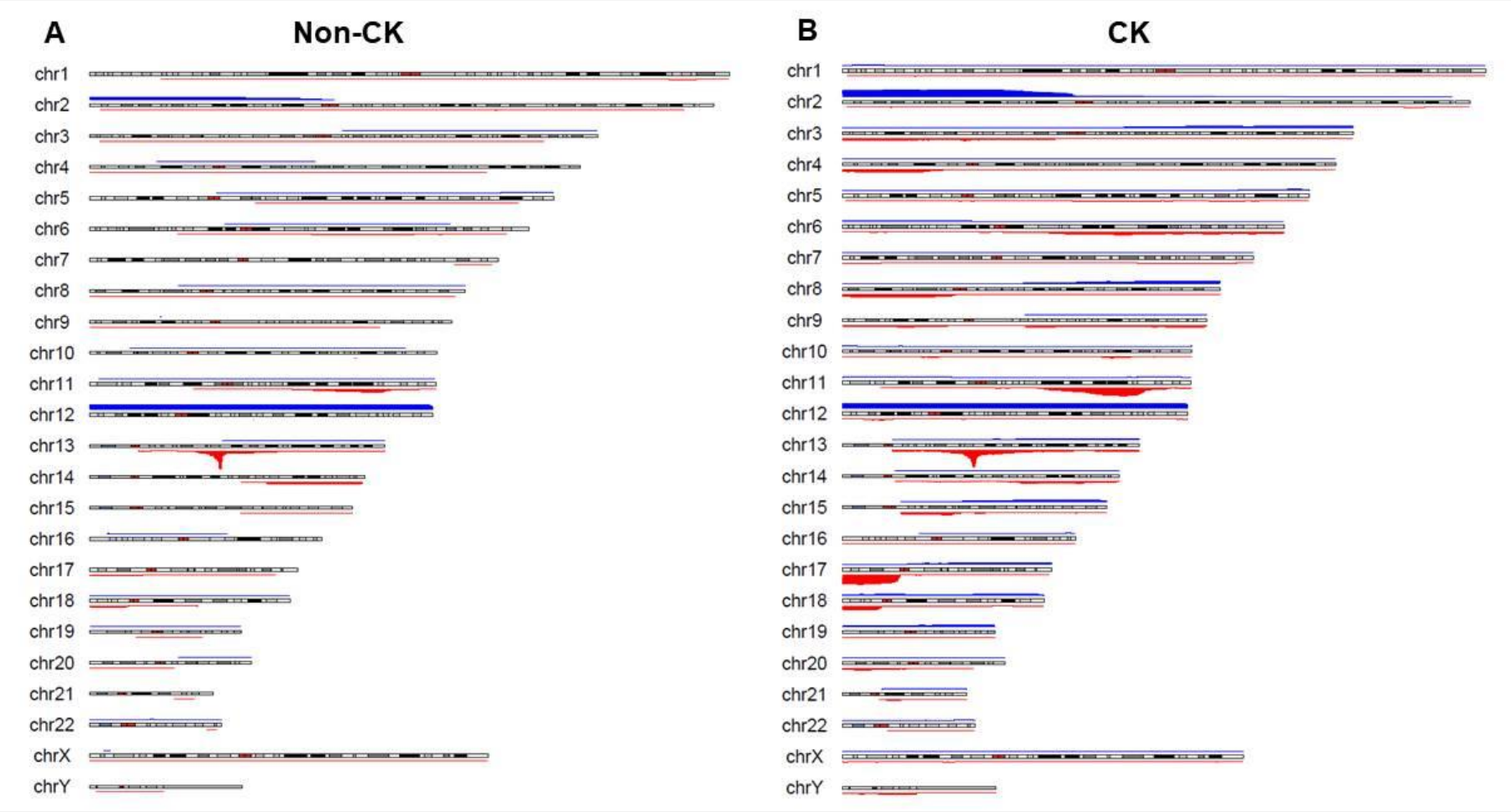

Figure S1. **Distribution of the copy number aberrations detected by genomic microarrays in non-CK and CK groups.** (A) Non-CK subgroup (0-2 abnormalities), (B) CK subgroup (≥3 abnormalities). Gains are represented in blue above and losses in red below the affected chromosomal regions. The thickness of the bars represents the number of cases showing the respective gain or loss. Figures were created by using KaryoploteR package of R.

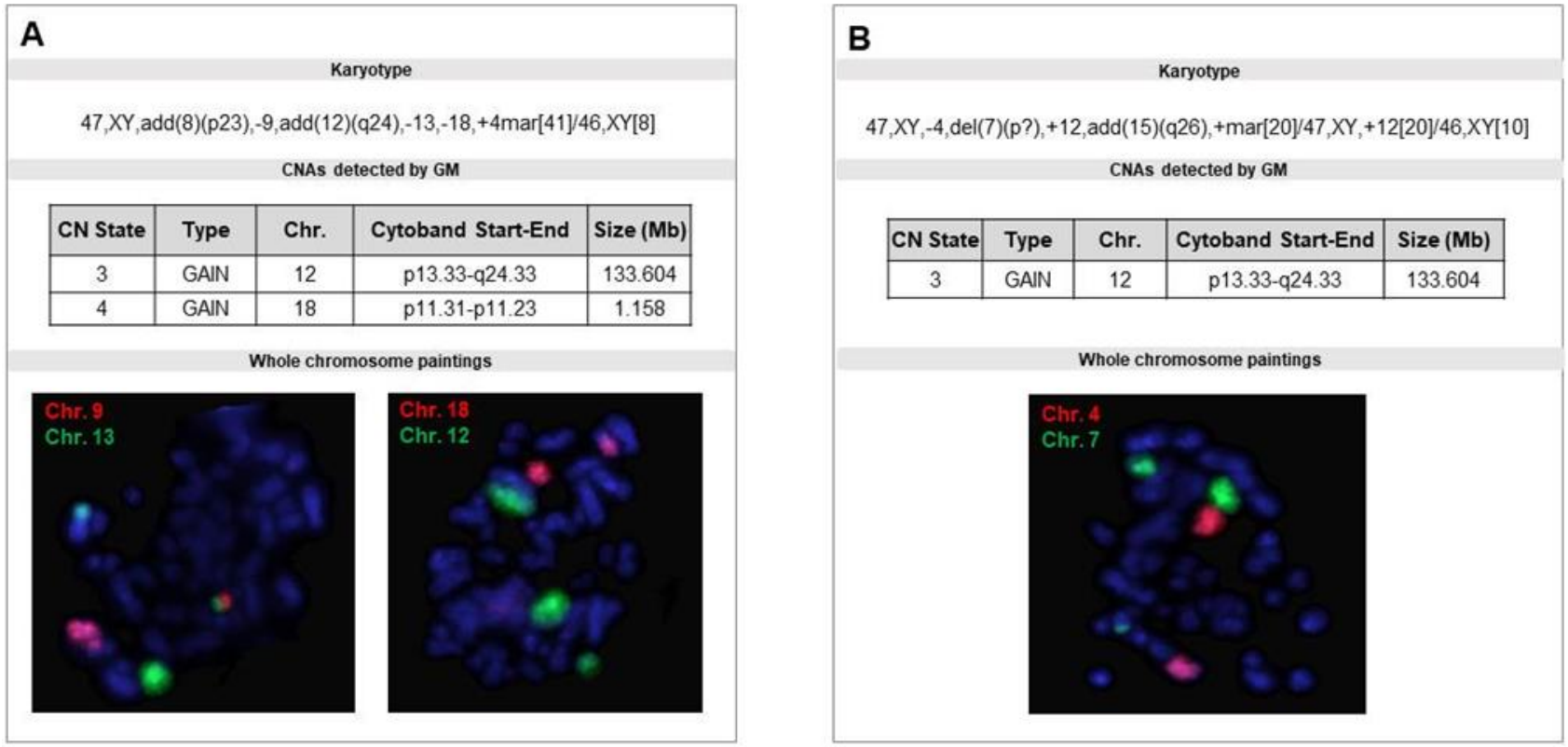

Figure S2. Whole chromosome painting FISH images of two high-CK cases classified as low-GC by genomic microarrays. (A) Nine aberrations were detected by CBA while only two were observed by GM. FISH was performed using chromosome painting probes for chromosomes 9 (red) and 13 (green), on the left image, and for chromosomes 12 (green) and 18 (red), on the right image. FISH revealed that chromosomes apparently lost in the karyotype appeared to be fragmented, either constituting the additional material of other chromosomes or being part of marker chromosomes. (B) Five aberrations were detected by CBA while only gain of chromosome 12 was detected by GM. FISH was performed using chromosome painting probes for chromosomes 4 (red) and 7 (green). According to FISH images, both chromosomes were present in the analyzed metaphases but were fragmented (chr.7) or considered as marker chromosomes (chr.4). Chromosomes were stained with DAPI.

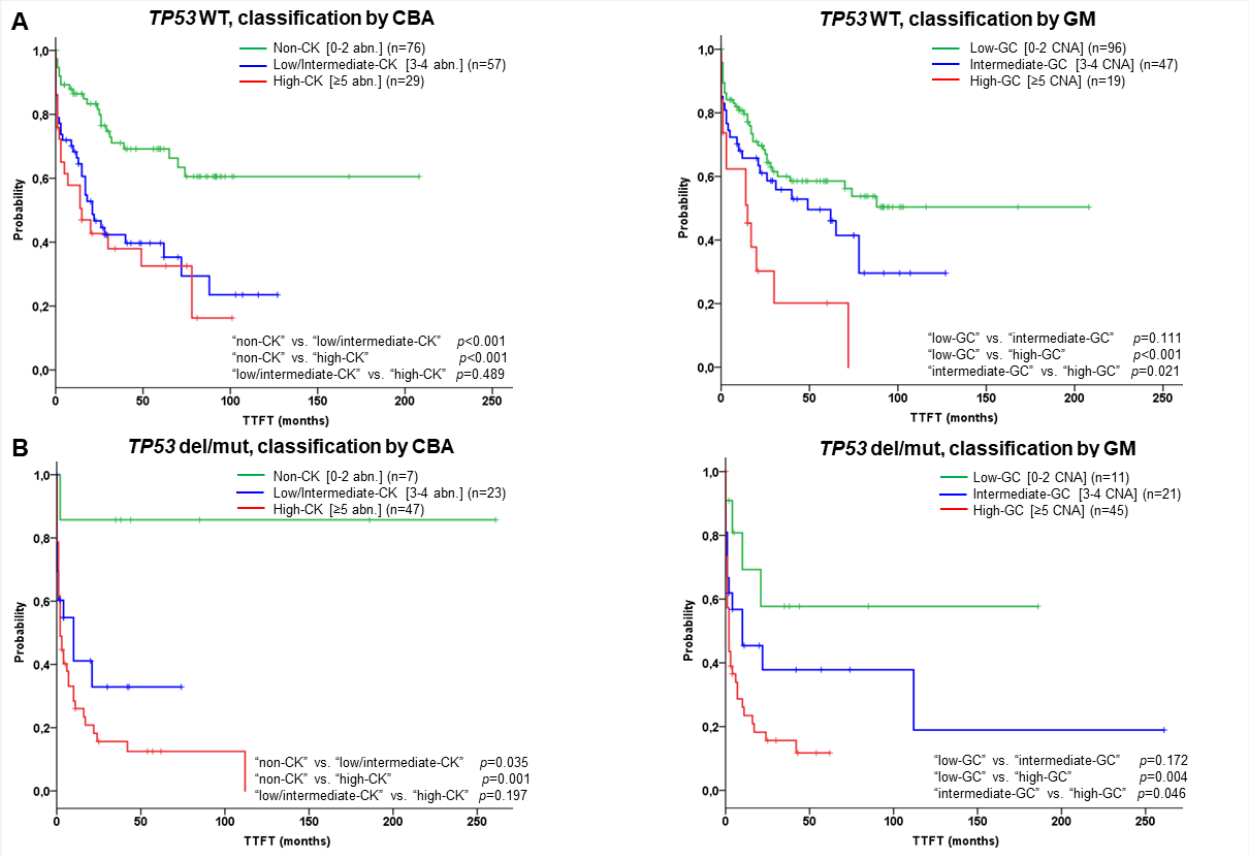

Figure S3. Effect on TTFT of risk categories defined by chromosome banding analysis and genomic microarrays in patients with abnormal *TP53* (deleted and/or mutated). Kaplan-Meier estimation for TTFT in risk categories defined by CBA (plots on the left) or GM (plots on the right) in patients with normal *TP53* (A) and in patients with deleted and/or mutated *TP53* (B).

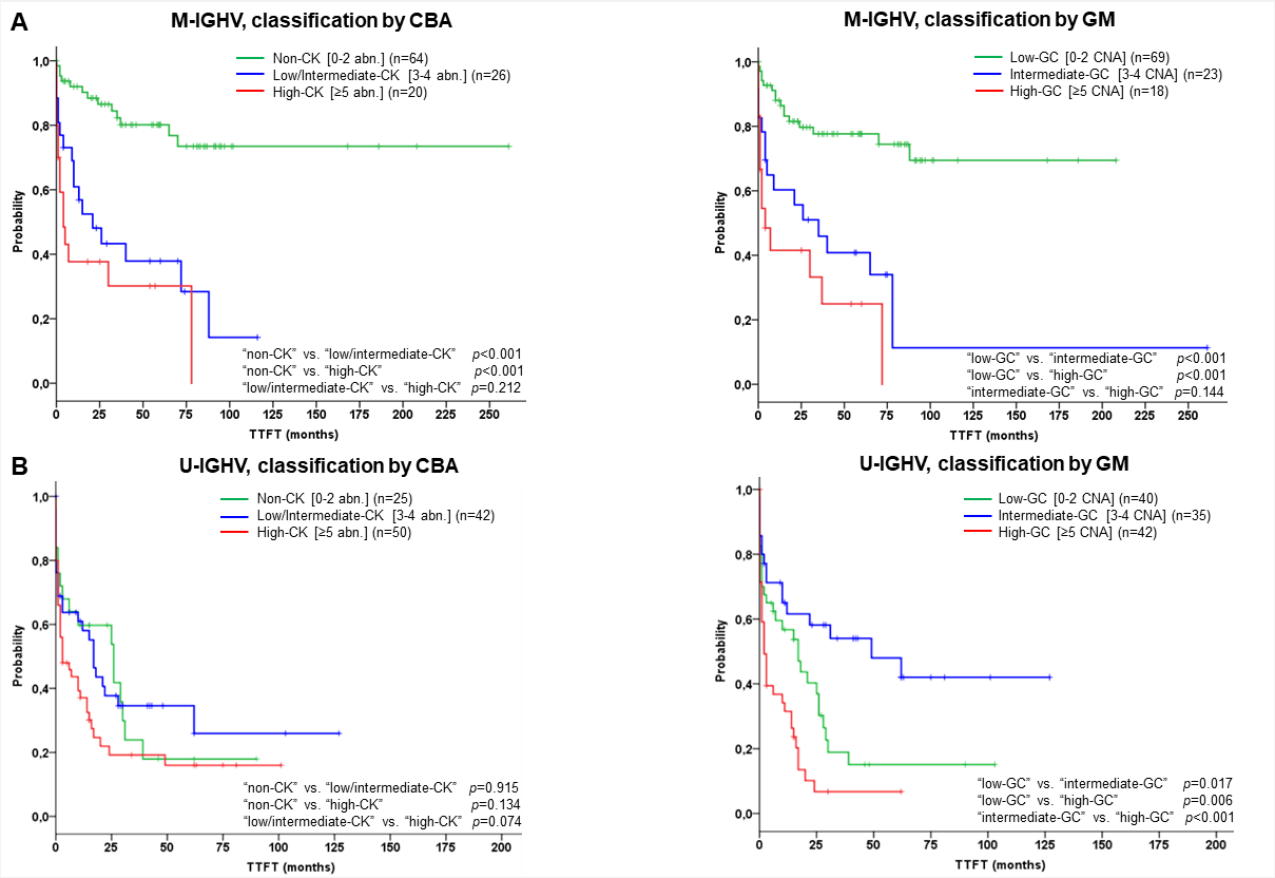

Figure S4. Effect on TTFT of risk categories defined by chromosome banding analysis and genomic microarrays in patients with M-IGHV or U-IGHV. Kaplan-Meier estimation for TTFT in risk categories defined by CBA (plots on the left) or GM (plots on the right) in patients with M-IGHV (A) and in patients with U-IGHV (B).

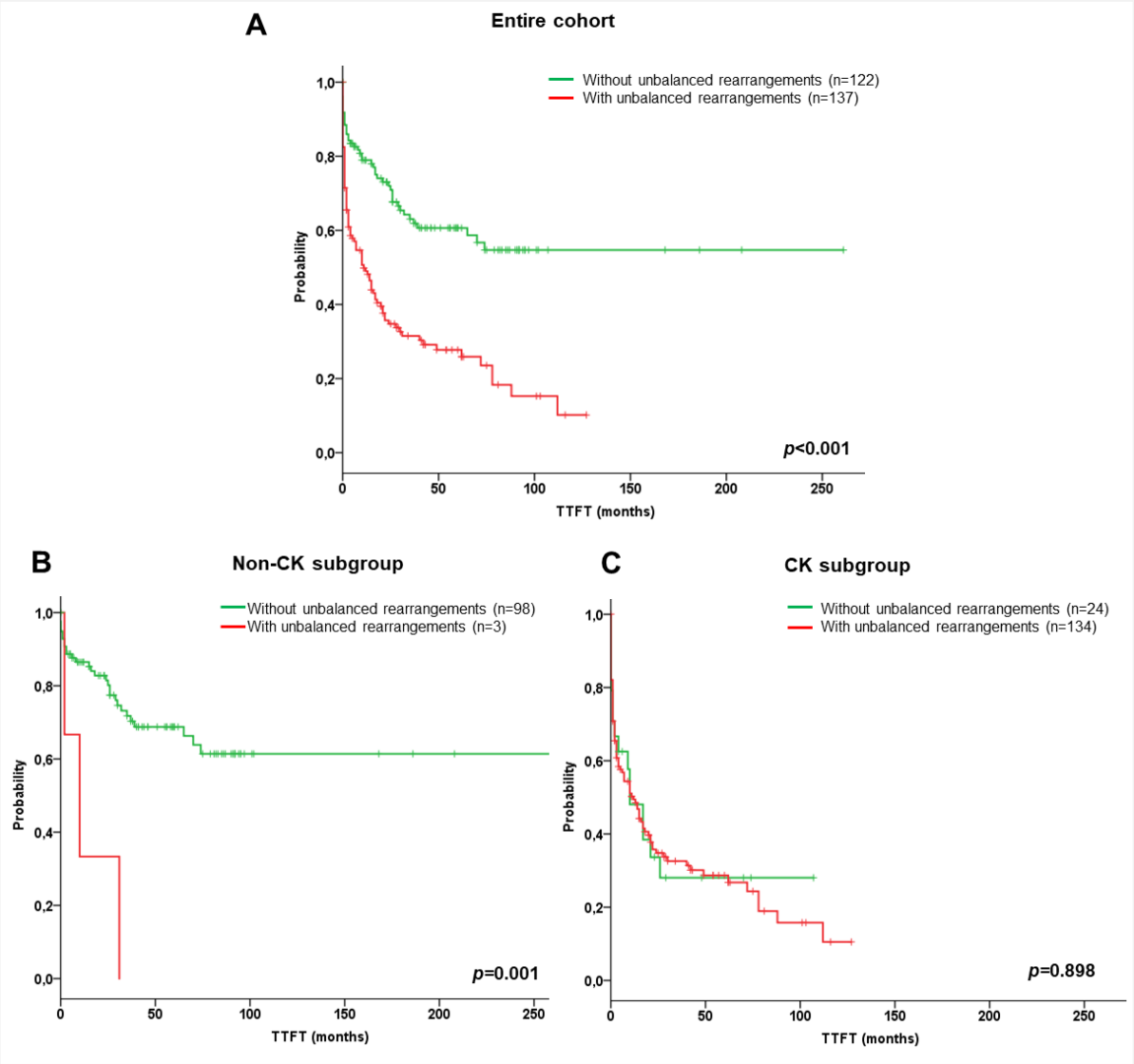

Figure S5. Effect on TTFT of unbalanced rearrangements detected by chromosome banding analysis in the entire cohort and within the non-CK and CK subgroups. Kaplan-Meier estimation for TTFT in patients with and without unbalanced rearrangements in the entire cohort (A) and in non-CK (B) and CK subgroups (C).

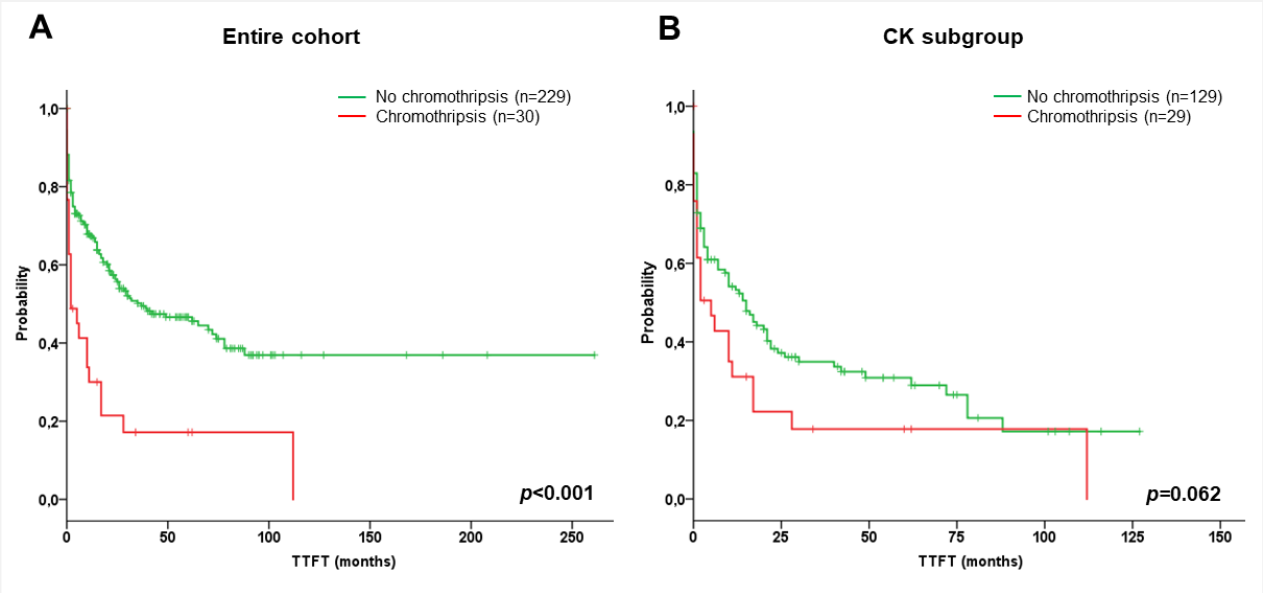

Figure S6. Effect on TTFT of chromothripsis in the entire cohort and within the CK subgroup. Kaplan-Meier estimation for TTFT in patients with and without chromothripsis in the entire cohort (A) and in CK subgroup (B). Survival plot for non-CK subgroup is not shown as only one case displayed chromothripsis.