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ORIGINAL PAPER

Additional lesions identified by genomic microarrays are associated with an inferior outcome in low-risk chronic lymphocytic leukaemia patients

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Summary
We explored the relevance of genomic microarrays (GM) in the refinement of prognosis in newly diagnosed low-risk chronic lymphocytic leukaemia (CLL) patients as defined by isolated del(13q) or no lesions by a standard 4 probe fluorescence in situ hybridization (FISH) analysis. Compared to FISH, additional lesions were detected by GM in 27 of the 119 patients (22.7%). The concordance rate between FISH and GM was 87.4%. Discordant results between cytogenetic banding analysis (CBA) and GM were observed in 45/119 cases (37.8%) and were mainly due to the intrinsic characteristics of each technique. The presence of additional lesions by GM was associated with age > 65 years ($p = 0.047$), advanced Binet stage ($p = 0.001$), CLL-IPI score ($p < 0.001$), a complex karyotype ($p = 0.004$) and a worse time-to-first treatment in multivariate analysis ($p = 0.009$). Additional lesions by GM were also significantly associated with a worse time-to-first treatment in the subset of patients with wild-type *TP53* and mutated IGHV ($p = 0.025$). In CLL patients with low-risk features, the presence of additional lesions identified by GM helps to identify a subset of patients with a worse outcome that could be proposed for a risk-adapted follow-up and for early treatment including targeted agents within clinical trials.

KEYWORDS
arrays, chronic lymphocytic leukaemia, genomic complexity, prognosis

INTRODUCTION

In chronic lymphocytic leukaemia (CLL), fluorescence in situ hybridization (FISH) is the gold standard for the detection of cytogenetic abnormalities including del(13q), del(11q), del(17p) and trisomy 12.[1] However, cytogenetic aberrations occurring in regions uncovered by the standard FISH panel have been shown by conventional cytogenetic banding analysis (CBA) in 20%–30% of patients[2] with 10%–15% of cases presenting a complex karyotype as defined by the presence of three or more abnormalities.[3]

Increasing evidence suggests that genomic complexity (GC) as assessed by CBA might represent in CLL an adverse independent prognostic biomarker.[3–5] For these reasons, CBA was included in the 2018 iwCLL guidelines as a desirable test in the work-up for patients enrolled in clinical trials.[1]

Although CBA is a well-established method that enables a complete overview of the cell genome, it is a rather

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cumbersome procedure requiring fresh dividing cells.[6] Moreover, CBA can only detect gains or losses of chromosome material of at least 10 Mb, being unable to explore small or single-gene rearrangements, which are known to play a role in CLL onset and/or progression.[7] Molecular methods such next generation sequencing (NGS) for gene mutations[8] or GM[9,10] for the detection of DNA gains and losses have, therefore, been used for the study of GC.

At diagnosis, most CLL patients are in an early stage requiring active surveillance and no treatment,[1] as clinical trials enrolling these patients have shown no survival benefit with early intervention[11–13] even with targeted agents.[14] However, the clinical evolution in these patients is quite heterogenous and sometimes unpredictable. In this perspective, a refined genetic work-up at diagnosis could provide relevant prognostic insights to plan risk-adapted follow-up and potential interventions with targeted agents that have shown to be associated with high complete remissions rates with undetectable minimal residual disease.[15] CBA and GM are both valuable tools to assess GC for risk stratification and prognosis, although they are not equivalent towards detecting chromosomal abnormalities, as discordances intrinsic to each technique may be frequently observed.[10] To this end, in the present study we explored the contribution of an extensive genomic assessment including GM in the refinement of prognosis in newly diagnosed CLL patients defined as low risk by the presence of isolated del(13q) or no lesions by standard 4 probe FISH analysis.

MATERIALS AND METHODS

Patients

The study cohort consisted of 119 untreated and consecutive CLL patients followed at the Hematology Unit of Ferrara between 2006 and 2022, with an isolated del(13q) or no lesions by standard 4 probe FISH analysis. Part of these data were previously reported.[16] All patients were diagnosed and treated according to the iwCLL criteria[1] and the CLL-IPI score was calculated as previously reported.[17] The study was approved by the local ethics committee. Fludarabine- or bendamustine-containing regimens, with rituximab, were used as first-line treatment in fit patients; chlorambucil with or without rituximab was used in elderly and/or unfit patients according to the treatment policy adopted at our centre. Since 2015, ibrutinib or idelalisib plus rituximab or venetoclax with or without rituximab were offered to patients according to the approved indications in our country.

Genomic analyses

FISH, CBA and immunoglobulin heavy chain variable region gene (IGHV) sequencing analyses were performed on peripheral blood (PB) samples as described.[8,16] Gene mutations were analysed by NGS using the Ion Torrent PGM platform (Life Technologies), as described.[8]

Microarrays

DNA from PB samples was extracted and processed as previously described[8] and analysed using the Cytoscan HD Array (Affymetrix, Thermo Fisher Scientific). The array contains more than 2.6×10^6 copy number markers, including 750 000 single nucleotide polymorphism probes. Intensity data files (CEL) were analysed using the Chromosome Analysis Suite (ChAS) v 4.0 software (Affymetrix, Thermo Fisher Scientific), applying the Single Sample Analysis Workflow. All gains and losses identified by ChAS were annotated using GRCh37/hg19. Data were visually inspected and manually revised to check breakpoints and identify low-level mosaic aberrations. For classical CLL-associated genomic rearrangements (deletion in chromosomes 11q22.3, 13q14 and 17p13), all copy number variants (CNVs) were considered, despite their length and overlapping with polymorphic structural variants annotated in the Database of Genomic Variant (DGV).[18] In the rest of the genome, rearrangements overlapping at least for 70% to DGV records were filtered out, as well as rearrangements smaller than 100 kb in length and/or characterized by less than 50 markers. For risk stratification and in order to evaluate concordance with CBA results in non-classical CLL-associated regions, we considered only gains and losses ≥5 Mb in length.[10] To identify recurrent rearrangements, we considered at least partially overlapping CNVs present in >1 patient, without applying a minimal reciprocal overlap. The minimal deleted and the minimal amplified region were calculated. Recurrences involving potential germline variants (47,XXX) or frequently observed somatic changes (45X,-Y) without a clear link with cancer occurrence were not included in the analysis.

Statistical analysis

Fisher's exact test was applied for categorical variables. Time-to-first treatment (TTFT) was calculated as the interval between diagnosis and the start of first-line treatment. Overall survival (OS) was calculated from the date of diagnosis until death due to any cause or until the last patient follow-up. Survival curves were compared by the log-rank test. Proportional hazards regression analysis was used to identify the significant independent prognostic variables on TTFT and OS. Concerning TTFT, formal tests for interactions were performed for subgroups. For TTFT prediction, the Harrell index (*c*-index) and the Akaike information criterium were used to compare the prognostic models. Statistical analysis was performed using Stata: release 17.0 (Stata Corp.). A $p > 0.05$ was considered as not significant.

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RESULTS

Patients

The main clinical and biological characteristics of the CLL patients are reported in Table 1. The median age of patients was 67 years (range 34–89 years) with 52.1% of patients older than 65 years. An isolated 13q deletion by FISH was present in 70 patients (58.8%). Seventy-four patients (67.9%) had a mutated IGHV configuration. *TP53* mutations were observed in 8 cases (6.7%) while mutations of *SF3B1* or *NOTCH1* genes were observed in 10 (8.4%) and 9 (7.6%) cases, respectively. Overall, by NGS (cut-off 5%) gene mutations were observed in 45 out of 119 patients (37.8%; Table S1). Patients' distribution according to CLL-IPI was as follows: 55 low (51.4%), 25 intermediate (23.4%) and 27 high/very high risk (25.2%). By CBA, a complex karyotype as defined by the presence of three or more chromosomal abnormalities in the same clone was observed in nine cases (7.6%).

Genomic microarrays

Results of GM and CBA are shown in Table S2. Compared to FISH results, additional lesions were detected by GM in 27 patients (22.7%). In seven out of 119 cases (5.9%) GM revealed high-risk lesions involving regions undetected by standard FISH (3 del17p13, 2 del11q22, 1 del11q22 and del17p13, 1 trisomy 12). Of note, three of the four cases with del17p13 undetected by FISH had a *TP53* mutation by NGS. In six cases GM revealed 13q14 deletions undetected by FISH while in four cases the del13q14 detected by FISH was not detected by GM. Overall, the concordance rate between FISH and GM was 87.4% (104 out of 119 cases).

Discordant results between CBA and GM for non-classical CLL rearrangements were observed in 45 out of 119 cases (37.8%). Reasons for discordances (sometimes referred to more than one rearrangement in the same patient) were abnormalities detected by CBA in a minor proportion of tumour cells (i.e. <25% cells with abnormal karyotype, or likely to have expanded in the CBA cell culture, $n=24$) and missed by GM, by apparently unbalanced rearrangements that ultimately might not have led to loss of material and not detectable by GM ($n=11$), apparently balanced translocations in CBA and missed by GM ($n=21$ patients), gains or losses sized below CBA resolution (<10 Mb, $n=8$), no division of the tumour clone in CBA cell culture ($n=16$), low-level mosaicism (~10%–15%, $n=6$), multiple CNAs interpreted by karyotype as one rearrangement ($n=2$). Reasons for discrepancies between GM, FISH or CBA in cases with further rearrangements than del13q14 are reported in Table S3. Recurrent abnormalities detected by GM in non-classical CLL-associated regions were detected in 22 patients (18.5%) and are reported in Table S4. Non-recurrent non-classical CLL rearrangements identified by GM, are reported in Table S5.

TABLE 1 Demographics and comparison between patients with and without additional lesions by GM.

Variable	Total, $n=119$ (%)	GM; no lesions, $n=92$ (77.3%)	GM: ≥1 lesion, $n=27$ (22.7%)	p
Age >65/≥65 years	57 (47.9)/62 (52.1)	49 (53.3)/43 (46.7)	8 (29.6)/19 (70.4)	**0.047**
Sex M/F	72 (60.5)/47 (39.5)	55 (59.8)/37 (40.2)	17 (63.0)/10 (37.0)	0.826
Stage Binet A/B-C	92 (77.3)/27 (22.7)	79 (85.9)/13 (14.1)	13 (48.2)/14 (51.8)	**<0.001**
b2m <3.5 yes/no	97 (82.2)/21 (17.8)	75 (86.2)/12 (13.8)	22 (71.0)/9 (29.0)	0.098
FISH del13q/normal	70 (58.8)/49 (41.2)	58 (63.0)/34 (37.0)	12 (44.4)/15 (55.6)	0.119
IGHV mut/unmut	74 (67.9)/35 (32.1)	61 (72.6)/23 (27.4)	13 (52.0)/12 (48.0)	0.086
TP53 mut/WT	8 (0.6.7)/111 (93.3)	4 (4.4)/88 (95.6)	4 (14.8)/23 (85.2)	0.077
SF3B1 mut/WT	10 (8.4)/109 (91.6)	9 (9.8)/83 (90.2)	1 (3.7)/26 (96.3)	0.452
NOTCH1 mut/WT	9 (7.6)/110 (92.4)	6 (6.5)/86 (93.5)	3 (11.1)/24 (88.9)	0.422
ATM mut/WT	5 (4.2)/114 (95.8)	3 (3.3)/89 (96.7)	2 (7.4)/23 (92.6)	0.318
MYD88 mut/WT	5 (4.2)/114 (95.8)	2 (2.2)/90 (97.8)	3 (11.1)/24 (88.9)	0.076
POT1 mut/WT	4 (3.4)/115 (96.6)	4 (4.4)/88 (95.6)	0 (0.0)/27 (100.0)	0.573
BIRC3 mut/WT	2 (1.7)/117 (98.3)	2 (2.2)/90 (97.8)	0 (0.0)/27 (100.0)	1.000
Gene muts yes/no	45 (37.8)/74 (62.2)	33 (35.9)/59 (64.1)	12 (44.4)/15 (55.6)	0.500
CLL-IPI low/int/≥high	55 (51.4)/25 (23.4)/27 (25.2)	50 (60.2)/19 (22.9)/14 (16.9)	5 (20.8)/6 (25.0)/13 (54.2)	**<0.001**
CK no/yes	110 (92.4)/9 (7.6)	89 (96.7)/3 (3.3)	21 (77.8)/6 (22.2)	**0.004**
GM lesions 0/1/2/≥3	92 (77.4)/14 (11.8)/7 (5.9)/7 (5.9)	—	—	—
Treatment yes/no	61 (51.3)/58 (48.7)	40 (43.5)/52 (56.5)	21 (77.8)/6 (22.2)	**0.002**
Alive/dead	72 (60.5)/47 (39.5)	63 (68.5)/29 (31.5)	9 (33.3)/18 (66.7)	**0.002**

Abbreviations: CK, complex karyotype; GM, genomic microarrays; WT, wild type.

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GM and outcome

For further analyses, patients were stratified into two subgroups based on the presence of additional non-classical rearrangements by GM. The presence of at least one additional lesion was significantly associated with age >65 years ($p = 0.047$), advanced Binet stage ($p < 0.001$), CLL-IPI score ($p < 0.001$), the presence of a complex karyotype ($p = 0.004$) and need of treatment ($p = 0.002$). No association was found between the presence of a gene mutation by NGS and additional lesions by GM (Table 1). After a median follow-up of 102 months (range: 1–204 months), 61 patients (51.2%) received a first-line treatment and 47 (39.5%) died. Median time-to-first treatment was 103 months (95% CI 75–166 months) while median OS for the all population was 175.6 months (95% CI 133.2-NR). In univariate analysis, a significant shorter TTFT (Table 2) was associated with the presence of additional lesions by GM ($p < 0.001$, Figure 1A) and with CLL-IPI ($p = 0.001$ and $p < 0.001$ for intermediate and high/very high-risk groups in comparison to low-risk cases, Figure S1), with mutations of SF3B1 ($p = 0.008$), NOTCH1 ($p = 0.007$) and ATM ($p = 0.012$) and with the presence of at least one mutation of the 20 gene NGS panel ($p < 0.001$).

Multivariate analysis (Table 2) confirmed the independent prognostic significance on TTFT of both additional lesions by GM ($p = 0.009$), CLL-IPI ($p = 0.002$ and $p < 0.001$ for intermediate and high/very high-risk groups in comparison to low-risk cases) and SF3B1 mutations ($p = 0.010$).

A subanalysis was conducted in 69 patients with isolated del(13q) or no lesions by standard 4 probe FISH analysis, WT *TP53* and mutated IGHV. Patients' characteristics are reported in Table S6. When considering TTFT, univariate and multivariate confirmed the independent prognostic value of additional lesions by GM ($p = 0.025$, Table 3 and Figure 1B).

Concerning TTFT, no interactions were observed for subgroups of significant variables (Table S7). For the risk of TTFT, the *c*-indexes and the Akaike information criteria indicated that our models had a higher prediction accuracy and performance than the models without GM (Table S8).

TABLE 2 Univariate and multivariate analysis for TTFT.

Variable	Univariate, HR (95% CI)	*p*	Multivariate, HR (95% CI)	*p*
Sex M/F	0.76 (0.45–1.30)	0.316	—	—
FISH normal/13q	0.85 (0.51–1.45)	0.559	—	—
SF3B1 mut/WT	2.64 (1.29–5.39)	0.008	3.38 (1.34–8.50)	0.010
NOTCH1 mut/WT	2.99 (1.34–6.66)	0.007	3.04 (0.92–10.01)	0.068
ATM mut/WT	3.32 (1.30–8.48)	0.012	0.73 (0.21–2.54)	0.624
MYD88 mut/WT	0.99 (0.22–3.79)	0.912	—	—
Gene mutations yes/no	2.59 (1.55–4.35)	<0.001	1.07 (0.54–2.12)	0.837
GM 0/≥1 lesion	3.66 (2.10–6.40)	<0.001	2.45 (1.26–4.79)	0.009
CLL-IPI Int versus low	3.48 (1.69–7.16)	0.001	3.34 (1.54–7.25)	0.002
CLL-IPI ≥ high versus low	7.22 (3.71–14.03)	<0.001	5.92 (2.76–12.68)	<0.001
CK no/yes	1.99 (0.79–5.02)	0.145	—	—

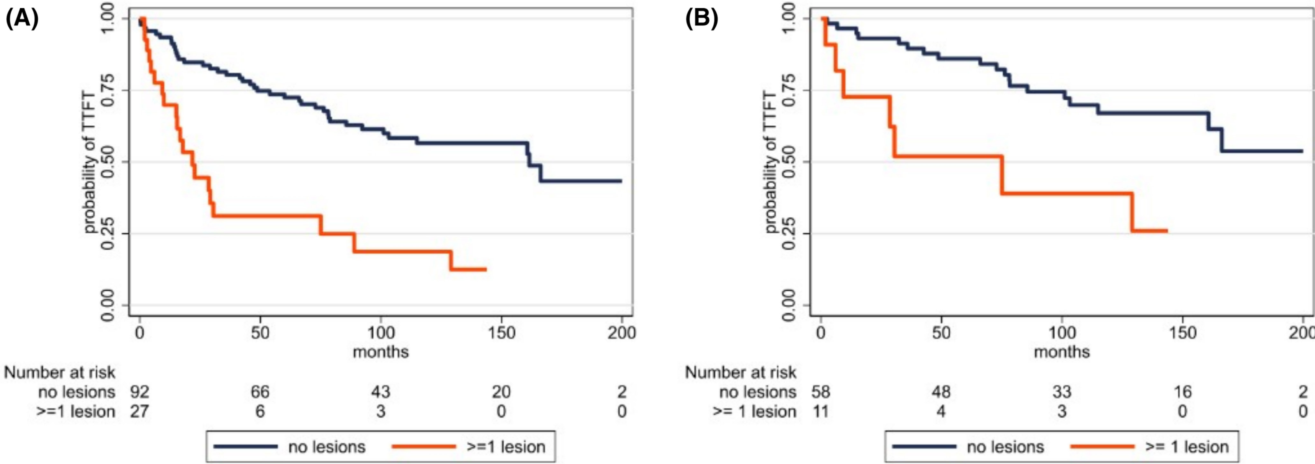

FIGURE 1 TTFT in low-risk patients by fluorescence in situ hybridization (FISH) (A) and in patients with low-risk FISH, WT *TP53* and mutated IGHV (B), respectively. TTFT, time-to-first treatment. [Colour figure can be viewed at wileyonlinelibrary.com]

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TABLE 3 Univariate and multivariate analysis for TTFT in patients with low-risk FISH and WT *TP53* and mutated IGHV.

Variable	Univariate, HR (95% CI)	p	Multivariate, HR (95% CI)	p
Sex M/F	0.79 (0.36–1.74)	0.553	—	—
FISH normal/13q	0.57 (0.25–1.31)	0.184	—	—
***SF3B1* mut/WT**	3.40 (1.16–9.93)	0.025	3.61 (0.81–16.14)	0.092
NOTCH1 mut/WT	2.06 (0.48–8.83)	0.330	—	—
MYD88 mut/WT	1.74 (0.41–7.44)	0.453	—	—
Gene mutations yes/no	2.57 (1.16–5.69)	0.020	1.35 (0.49–3.73)	0.563
GM 0/≥1 lesion	3.69 (1.52–8.96)	0.004	3.18 (1.16–8.76)	0.025
CLL-IPI Int versus low	3.244 (1.07–9.86)	0.038	2.39 (0.67–8.48)	0.177
CLL-IPI ≥ high versus low	9.46 (2.97–30.06)	<0.001	7.95 (2.13–29.76)	0.002
CK no/yes	1.02 (0.14–7.57)	0.984	—	—

When considering OS (Table S9), the presence of additional lesions by GM was significantly associated with a worse OS in univariate analysis ($p = 0.001$, Figure S2), while in multivariate analysis only CLL-IPI (Figure S3) independently conferred a worse outcome.

DISCUSSION

In recent years, several reports have outlined, in CLL, the prognostic and predictive relevance of GC as assessed by different methods including CBA, NGS and GM.[3,5,8–10,19,20] At present, guidelines advise the study of GC by CBA within clinical trials while other genetic biomarkers including FISH analysis, the IGHV and *TP53* status are recommended at the time of disease progression for cases requiring treatment. However, treatment is not necessary at diagnosis in the majority of CLL cases and the clinical evolution in some patients is quite heterogenous. At diagnosis, a refined genetic assessment could, therefore, represent a useful tool to drive a risk-adapted follow-up and, possibly, early intervention with targeted agents within clinical trials in specific subsets of patients at risk of a rapid progression. Several prognostic indexes, that include both clinical and biologic biomarkers, have been developed to allow a rationale management of patients with CLL in the clinical practice and in clinical trials.[17,21,22] However, few studies have addressed the prognostic relevance of GC in relation to prognostic indexes,[4,22,23] particularly in the setting of low-risk CLL patients.

In this study, we have compared FISH and GM results to detect GC in relation to CLL-IPI in the assessment of prognosis in newly diagnosed CLL patients with low-risk cytogenetic findings as defined by isolated del(13q) or no lesions by the standard 4 probe FISH approach. For this analysis, GM study was conducted by using a recommended approach[10,24] that considers only CNAs ≥5 Mb to reduce the reporting of small anomalies with uncertain clinical significance. Moreover, it has been reported that lowering the CNA size cut-off to 1 MB does not significantly improve risk stratification.[9] We confirmed the overall good concordance (87.4%) between GM and FISH results. This figure is in line with a previous report showing a concordance between the two techniques ranging from 82.6% to 98.2% according to the different probes.[10] At variance with previous studies,[9,10] we found GM abnormalities in a smaller proportion of patients (22.7%) reflecting the low-risk profile of our CLL patients characterized by no lesions or isolated del(13q) by FISH. This is in line with a previous report from our group, in which CBA identified 1–2 lesions (other than del13q) in 26% and a complex karyotype in 5% of 120 low-risk FISH patients, with prognostic relevance in terms of TTFT and OS.[25]

In a recent study comparing the results of CBA and GM in the detection of GC,[10] only a moderate agreement was observed between the two methods, although both techniques did not differ in risk assessment categorization. Discordances were observed in nearly one-third of cases and they were mainly a consequence of known characteristics of these techniques. In agreement with this report,[10] we found that in nearly one-third of patients GM and CBA gave discordant results mainly due to technical reasons.[10,26–28] When considering the number of lesions revealed by GM results, it must be pointed out that the optimal threshold for prognostic correlations is still a matter of debate. In previous studies including unselected CLL patients,[9,10] low-risk CLL were defined by the presence of 0–2 lesions by GM, including 13q deletion. In our low-risk patients, we used for clinical correlations a stratification based on the presence of a single non-classical additional lesion by GM. This approach is justified because it may increase the specificity of the prognostic correlations by excluding the presence of the 13q14 deletion, a well-known favourable prognostic parameter.

Interestingly, we observed a significant correlation between the presence of additional lesions by GM and adverse prognostic features including advanced stage, a high-risk CLL-IPI score and the presence of a complex karyotype, while no association was found with the presence of gene mutations by NGS using a panel of 20 genes frequently involved in CLL.

Moreover, we also found that in low-risk CLL by FISH, the presence of additional lesions by GM is an independent biomarker predicting a shorter TTFT along with CLL-IPI

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and *SF3B1* mutations. Interestingly, *SF3B1* mutations were also confirmed as an independent adverse prognostic factor on TTFT in a recent large multicentre study conducted by ERIC in Harmony.[29] The presence of GM lesions was also associated with a worse OS at univariate analysis although we have to consider that many patients were treated before the introduction of new target agents and that, in some cases, these patients would today be ineligible to chemoimmunotherapy, due to their genetic profile.

Of interest is also the observation that GM appears to overcome, in multivariate analysis, the prognostic relevance of the complex karyotype suggesting that, at least in this subset of patients, GM might represent a surrogate biomarker for GC instead of CBA. Moreover, the prognostic significance of additional GM lesions was also confirmed in the subset of patients with very low-risk genetic features including isolated 13q or no lesions by FISH, WT *TP53* and mutated IGHV. Overall, these findings could be of relevance from a practical point of view particularly for CBA, a demanding technique requiring fresh dividing cells that are not necessary when applying the GM.[6] Noteworthy, GM analysis can be performed on the same sample used for molecular characterization including IGHV and *TP53* status making this assessment more feasible than CBA not only in clinical trials but also in clinical practice. Moreover, recent data suggest that GC, as assessed by arrays, could also be used as a predictive biomarker for measurable residual disease conversion and disease progression within clinical trial.[20]

The strength of this paper relies not only on the extensive genomic analysis including FISH, CBA, GM and NGS in a homogenous subset of low-risk CLL patients as defined by FISH but also on the correlation of GM results with clinical findings and with a validated CLL prognostic index, the CLL-IPI. Larger numbers of patients are, however, needed to confirm these data, particularly concerning OS in homogenously treated patients.

Overall, our analysis suggests that, among low-risk CLL, a refined genomic assessment by GM might identify a subset of patients with peculiar biological features that may predict a worse outcome independently of other biomarkers of GC. These patients may benefit of a risk-adapted follow-up and may represent a patient population candidate to early treatment including targeted agents within prospective clinical trials.[11–14]

AUTHOR CONTRIBUTIONS

Gian Matteo Rigolin, Viviana Caputo, Ilaria Del Giudice, Robin Foà and Antonio Cuneo were responsible for designing the study, analysing data, interpreting results and writing the manuscript. Alice Traversa, Viviana Caputo, Agnese Giovannetti and Antonio Pizzuti performed GM analysis. Antonella Bardi performed CBA analyses. Sara Raponi, Caterina Ilari, Luciana Cafforio, Agnese Giovannetti and Elena Saccenti performed molecular analyses. Gian Matteo Rigolin performed statistical analysis. All authors provided feedback on the report, reviewed the manuscript for important intellectual content and approved the final version.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest for this paper.

DATA AVAILABILITY STATEMENT

Data are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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