Received: 25 July 2023 | Accepted: 25 October 2023

DOI: 10.1111/bjh.19191

BJHaem
BRITISH JOURNAL OF HAEMATOLOGY

ORIGINAL PAPER

Haematological Malignancy - Biology

Integrative NGS testing reveals clonal dynamics of adverse genomic defects contributing to a natural progression in treatment-naïve CLL patients

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Funding information
Czech Health Research Council, Grant/Award Number: AZV NU20-08-00314 and NV19-03-00091; European Union—Next Generation EU, Grant/Award Number: Programme EXCELLES, ID Project No. LX22NPO5102; Masaryk University Brno, Czech Republic, Grant/Award Number: MUNI/A/1224/2022; Ministry of Health, Czech Republic, Grant/Award Number: FNBr 65269705

Summary

Large-scale next-generation sequencing (NGS) studies revealed extensive genetic heterogeneity, driving a highly variable clinical course of chronic lymphocytic leukaemia (CLL). The evolution of subclonal populations contributes to diverse therapy responses and disease refractoriness. Besides, the dynamics and impact of subpopulations before therapy initiation are not well understood. We examined changes in genomic defects in serial samples of 100 untreated CLL patients, spanning from indolent to aggressive disease. A comprehensive NGS panel LYNX, which provides targeted mutational analysis and genome-wide chromosomal defect assessment, was employed. We observed dynamic changes in the composition and/or proportion of genomic aberrations in most patients (62%). Clonal evolution of gene variants prevailed over the chromosomal alterations. Unsupervised clustering based on aberration dynamics revealed four groups of patients with different clinical behaviour. An adverse cluster was associated with fast progression and early therapy need, characterized by the expansion of *TP53* defects, *ATM* mutations, and 18p− alongside dynamic *SF3B1* mutations. Our results show that clonal evolution is active even without therapy pressure and that repeated genetic testing can be clinically relevant during long-term patient monitoring. Moreover, integrative NGS testing contributes to the consolidated evaluation of results and accurate assessment of individual patient prognosis.

KEYWORDS
chronic lymphocytic leukaemia, clonal evolution, genomic aberration, integrative NGS testing, prognosis

INTRODUCTION

Cancer evolution is a dynamic process, which includes tumour cell expansion, genetic diversification and progression of aggressive subclones leading to resistance to therapy.[1] Chronic lymphocytic leukaemia (CLL) represents a suitable model to study disease kinetics and its association with genomic features since patients can be easily monitored by sequential blood sampling.[2,3] This B-cell lymphoproliferative disease (LPD) passes through different stages, from premalignant monoclonal B-cell lymphocytosis, through gradual progression to transformation into a more aggressive LPD in some cases.[4,5] The evolution of the disease and vast inter- and intratumour genetic heterogeneity lies behind CLL persistence or refractoriness despite the availability of novel drugs.[6,7]

Over the past decade, genome-wide NGS studies have yielded detailed genetic characterization of CLL.[8–10] The remarkable dynamic of this disease was further ascribed to the

presence of multiple subpopulations with unique molecular aberrations, growth dynamics and response to therapy.[11–14] The clonal expansion of genetic defects and association with resistance to standard and novel therapies was clearly documented.[15–17] However, the clonal dynamics in untreated CLL contributing to natural disease progression and its clinical impact is still less explored.[11,13,18–23] Across the haemato-oncology diagnoses, comprehensive targeted NGS-based panels currently attempt to translate from research to clinical settings.[24–29] In CLL, an appropriate genetic characterization and proper timing of testing can have a clinical impact on prognostication and therapy selection, as the defect composition can change over time.[2,30] Due to the clonal evolution and progression, repeated testing may be warranted beyond the standard initial examination at diagnosis.

Here, we explored changes in the genomic landscape during the pretreatment period of 100 CLL patients using an integrative custom NGS panel LYNX.[24] Our findings provide evidence that dynamic changes (on both gene and chromosomal levels) are frequent and already occur in treatment-naïve CLL. The expansion of adverse alterations may contribute to natural disease progression with early therapy need. Finally, we discuss the timing and benefits of integrative NGS testing for the precise stratification of untreated CLL patients.

MATERIALS AND METHODS

Patients and samples

The retrospective cohort included 100 patients, all providing informed consent with research use (Data S1). Basic characteristics were representative of a standard CLL cohort composition (Table 1; detailed in Table S1). In each patient, serial samples of peripheral blood were collected at two time points before therapy administration, at the diagnosis (TP1) and before the first therapy or at the last available follow-up (TP2) (Data S1). The median interval between TP1 and TP2 was 36 months (range 7–174). About one third of patients (32/100) never received therapy during the follow-up. The timeline of sampling, therapy administration and follow-up are summarized in Figure S1. After B-cell separation, the sample purity was determined by flow cytometry (median 98%, range 95%–99%) and DNA extracted (Data S1).

Sequencing approach and variant categorization

Samples were analysed using our custom capture-based NGS panel LYNX[24] aimed at the simultaneous detection of variants in selected genes (SNVs and indels), genome-wide chromosomal defects (copy-number alterations, CNAs; copy-neutral loss of heterozygosity, CN-LOH), IG/TR rearrangements and common translocations; described briefly in Data S1.

TABLE 1 Patients' baseline characteristics.

Parameter	Category	CLL patients ($n = 100$)
Gender	Male/female	61/39
Age (years)	Median (range)	63 (30–83)
Rai stage	0	63
	I–II	34
	III–IV	3
IGHV status	Mut/unmut	46/54
Treatment during follow-up	Yes/no	68/32
TTFT (months)	Median (range)	38 (11–141)
Follow-up, overall (months)	Median (range)	86 (21–271)
Follow-up, untreated (months)	Median (range)	80 (21–268)

Abbreviations: IGHV, immunoglobulin heavy-chain variable-region genes; TTFT, time to first treatment.

The LYNX panel design enables the assessment of the status of both alleles simultaneously. Each mutation's cancer cell fraction (CCF) was calculated as described previously.[13] An aberration (CNA or gene variant) was considered clonal if CCF was >80%. Aberrations at a subclonal level were classified as high CCF or low CCF if the value was >30% or <30% respectively.

The comparison of consecutive samples enabled the individual aberration categorization into three groups: (i) genesis/growth, (ii) loss/drop and (iii) stable when there was no significant change in the proportion. In the case of mutations, the criterion for a dynamic change (growth and loss) was a significant difference in the number of reference and variant reads (Fisher test, $p < 0.05$) and at least a 20% change of VAF in TP2 compared to TP1 to comprehend biological importance. A difference of 20% in CCF was evaluated as a change for CNAs.

Statistical methods and clustering

All data analyses were performed in R software.[31] The Fisher test was used to assess the association between categorical data. Mann-Whitney test and Kruskal-Wallis ANOVA followed by adjusted post-hoc Dunn test were applied to determine relationships between continuous and categorical variables. The log-rank test and Kaplan–Meier curves served to compare and visualize survival data. p-Values <0.05 were considered statistically significant for all statistical tests.

A weighted unsupervised clustering with an agglomerative approach and complete linkage was employed to stratify the patients according to the presence and dynamics of genomic defects (Data S1). The associations of individual clusters with clinical and genetic data were confirmed by the Fisher exact test for categorical variables and the Kruskal–Wallis ANOVA with the post hoc Dunn test for continuous variables. Clinical parameters evaluated within the clusters are described in Data S1.

RESULTS

Common co-occurrence of clonal and subclonal genomic alterations underline extensive heterogeneity at the diagnosis of CLL

At the baseline, our comprehensive LYNX panel detected 182 variants in 51 of 70 tested genes (Table S2) and 134 chromosomal aberrations (Table S3). The spectrum of genomic defects and their abundance (Figure 1) is typical for diagnostic CLL samples and corresponds with previous reports.[8,10,12]

Briefly, gene variants above the detection limit of 5% were found in 83/100 patients with the most frequently affected *NOTCH1* (including 3′UTR variants), *SF3B1* and *ATM* genes. The *NOTCH1, KMT2D, MYD88* and *XPO1* genes manifested predominantly clonal or high-CCF variants (30%–80%), *BIRC3* subclonal variants (<30%), and the rest showed diversity in CCF at the diagnosis (Table S2; Figure S2). Chromosomal defect/s were detected in 85/100 patients. As expected, 13q−, 11q− and trisomy 12 were the most frequent. Regarding known CNA CLL drivers,[8,9,32] we also identified single cases of 2p+, 8q+, 8p− and +18. CN-LOHs were distributed on various chromosomes, with those recurring on chr13 and chr9. At the diagnosis, 70% (94/134) of CNAs/CN-LOHs were clonal, dominantly represented by trisomy 12 (87%; 13/15), aberrations of chr17 (75%; 3/4) and chr13 (67%; 43/64) (Table S3; Figure S3).

Altogether, 97 patients carried at least one alteration with frequent co-occurrence of defects (Table S1). A variety of affected genes and chromosomes with diverse clonality reflects and confirms the extensive molecular heterogeneity of CLL and the existence of subclonal events. Clonal genomic alterations prevailed at the diagnosis of CLL, as they were

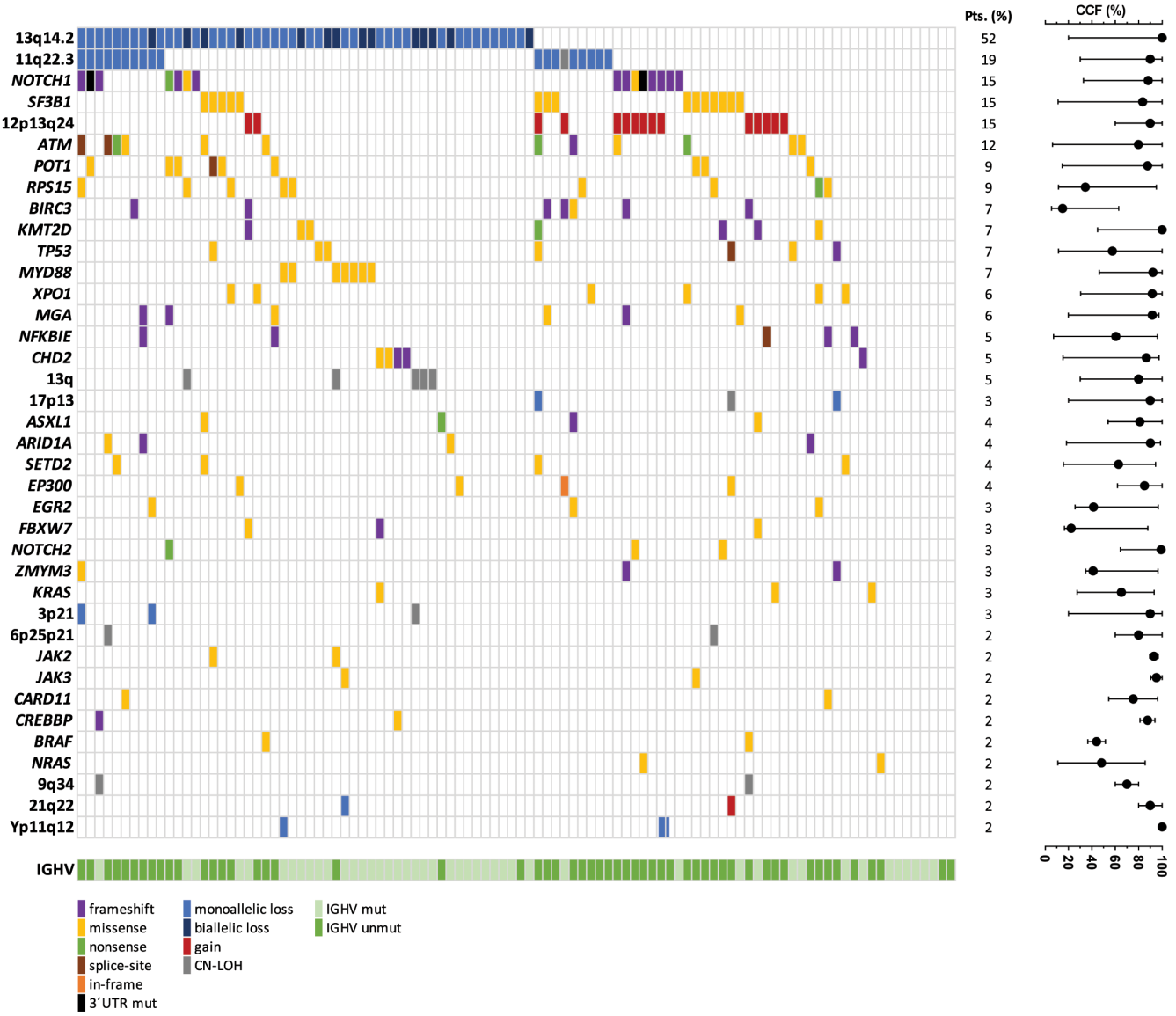

FIGURE 1 The distribution of genomic defects detected at the diagnosis in 100 untreated CLL patients. Only aberrations with the occurrence in >1% of patients are included. The bottom part shows the IGHV status.

observed in more than half of the patients. Predominant clonal occurrence of *NOTCH1* and *MYD88* mutations, +12, and 13q− suggests early involvement in CLL development.

The dynamic changes of genomic alteration before the first therapy are frequent, with the superiority of gene variants over chromosomal defects

The analysis of consecutive samples collected at TP2 identified 206 gene variants (Table S2) and 162 chromosomal aberrations (Table S3), revealing a gain of new alterations (Figure S4). Dynamic changes in mutations and CNAs/CN-LOHs are graphically summarized in the integrative oncoplot (Figure 2).

The analysis of gene variants dynamics disclosed 60% (124/206) stable mutations, 25% (52/206) variants with increasing VAF, 7% (12/206) showing a drop in VAF and 8% (18/206) variants newly emerged above the panel sensitivity in TP2 (Table S2). Three mutations in the genes *SF3B1* (VAF 27%), *KMT2D* (VAF 15%) and *SETD2* (VAF 16%) were not detected in TP2. Among 70 expanding or newly emerged variants, the most abundant were *BIRC3*, *NOTCH1*, *ATM*, *SF3B1* and *TP53* mutations; the majority of them (51/70; 73%) remained or occurred in subclonal condition. Variants evolving into clonal during the natural progression of the disease were present in a variety of genes, but the *TP53* gene most often (three variants).

The chromosomal defects (Table S3) were stable in most cases (67%; 108/162), followed by newly gained alterations in 19% (31/162) of cases that were primarily subclonal (26/31; 84%). Alterations on chr13, chr17 and chr9 were the most frequent among expanding or emerging defects (16/44, 6/44 and 5/44 respectively). Aberrations with growing CCF occurred in TP1 in a subclonal state (11/13) and 7 (64%) expanded into clonal. Three alterations were not found in TP2 (11q−, 13q−, +12; each in an individual patient).

The number of genomic alterations increased during the pretreatment period, with a significant rise in the number of defects per patient (Figure S4). Stable composition of genomic defects without dynamic changes in their proportion was observed only in 38% (38/100) of patients (Table S1), including three patients that did not harbour any genomic variants. Thus, the majority of untreated patients showed clonal evolution of various extents, from dynamics in gene variants (34%) or chromosomal defects (10%) only to dynamics in both (18%). Importantly, subclones with gene mutations were significantly more susceptible to evolution than those with disrupted chromosomes (66/93 and 20/40, respectively; $p = 0.029$).

A long pretreatment period provides a space for the clonal evolution of genomic defects (IG rearrangements undergo clonal shifts in a minority of patients)

Due to the observed frequent clonal shifts, we explored the association with the length of the tracking period before therapy initiation. We compared the dynamic composition of genomic defects with static composition, defined as stable defects with none or one dynamic defect (i.e. genesis/growth or loss/drop). Patients with dynamic composition showed significantly extended monitoring periods than patients with static defects (median 45 and 29 months, respectively; $p < 0.05$) (Figure S5A). Detailed analysis of the time needed for the evolution of individual aberration did not reveal significant results except for the *MYD88* mutations and chr13 defects. Again, a trend favouring longer time required for dynamic changes was observed compared to static variants and/or the absence of any variant (Figure S5B).

The LYNX panel versatility allowed us the evaluation of IG rearrangements to better understand the clonal composition in individual cases. In TP1, we identified 498 IG heavy and light chain gene rearrangements (i.e. clonotypes) characterizing patients' CLL clones (range 2–15 clonotypes per case, median 4). Typically, single productive IGH and one or two productive IGK/IGL clonotypes were detected per case (90/100), denoting the monoclonal disease. In the minority of cases, various clonotypes were identified, suggesting the presence of multiple independent clones (6/100) or IG intraclonal diversification (IG ICD; 4/100) (Table S4). Following the clonal evolution in TP2, we observed clonal shifts in 9/10 multiclonal patients, and an additional case with IG ICD emerged in TP2.

Unsupervised clustering distinguished a group of patients with an adverse clinical course of the disease

Genomic aberrations and their dynamics assigning patients to individual clusters are schematically depicted in Figure 2. Five aberration profiles were included (i.e. stable clonal, stable subclonal, genesis/growth, loss/drop and no defect; Data S1), and four distinct clusters were recognized (Figure S6).

The main clinical and genetic characteristics of clusters are summarized in Figure 3. Four defined clusters significantly differed in the presence and evolution of *TP53* defects (mutations and 17p−/CN-LOH), *ATM* defects (mutations and 11q−/CN-LOH), +12 and *BIRC3* mutations ($p < 0.001$), followed by 13q−/CN-LOH, 8q+, 18p−, *NOTCH1* mutations ($p < 0.01$) (Table S5). Cluster 1 was characterized by the predominance of stable *NOTCH1* mutations plus 12+ and expanding *BIRC3* mutations. In cluster 2, the most abundant were stable 11q−/CN-LOH and expanding *EGR2* mutations, whereas, in cluster 3, stable defects of chr13 dominated. Cluster 4 harboured adverse abnormalities like *TP53* defects (stable and expanding), expanding *ATM* mutations and 18p−, and dynamic *SF3B1, ZMYM3, ASXL1* mutations and 8q+ (Table S5). Separate clustering in TP1 and TP2 based only on genomic defects presence (Figure S7A,B respectively) with the subsequent analysis of their difference among clusters showed significant clonal shifts from diagnoses to the

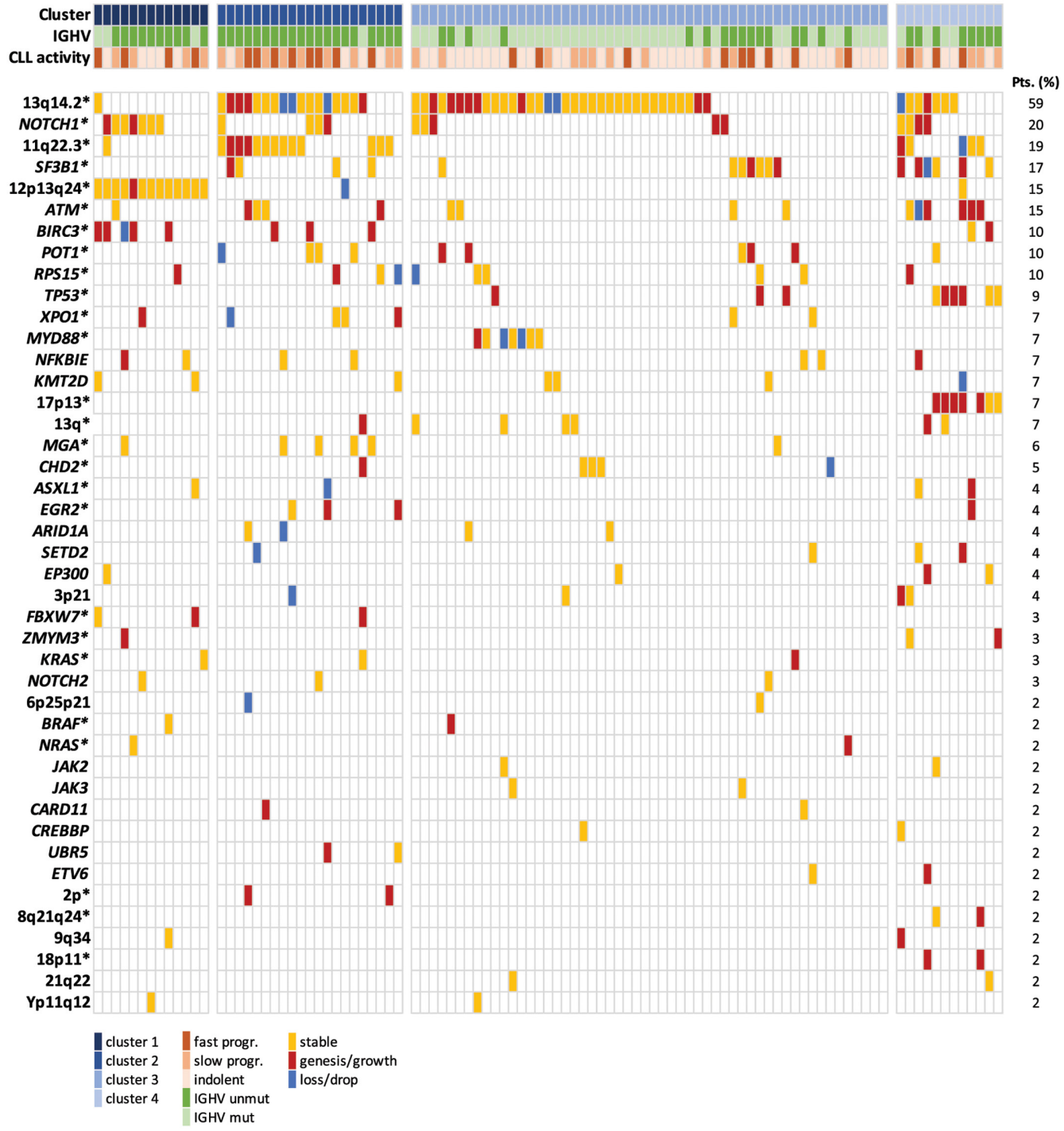

FIGURE 2 Integrative oncoplot representing the dynamics of genomic defects and patient assignment to clusters. The upper part shows defined clusters, IGHV status and CLL activity according to the therapy need (<2 years for fast progression, 2–5 years for slow progression, and >5 years or never for indolent disease). Only aberrations with the occurrence in >1% of patients are included. If ≥2 concurrent variants in one gene occur, the dynamic change is preferentially depicted than the stability. CLL driver aberrations are labelled with an asterisk.

first therapy for *ATM* mutation, 13q−/CN-LOH, 17p−/CN-LOH and 18p− (Table S6).

The correlation with other biological and clinical patients' characteristics showed significant differences among the clusters in the IGHV status ($p < 0.001$), CLL activity ($p = 0.02$), absolute lymphocyte count (ALC) in TP2 ($p < 0.001$, whereas in TP1 $p = 0.18$), rate of ALC increase per month ($p < 0.01$) and time to first treatment (TTFT; $p < 0.001$) (Figure 4A–E). Rai stage and lymphocyte doubling time (LDT) were not significantly different among clusters (Table 2). The adverse outcome was observed for clusters 4 and 2 with a predominance of unmutated IGHV, early

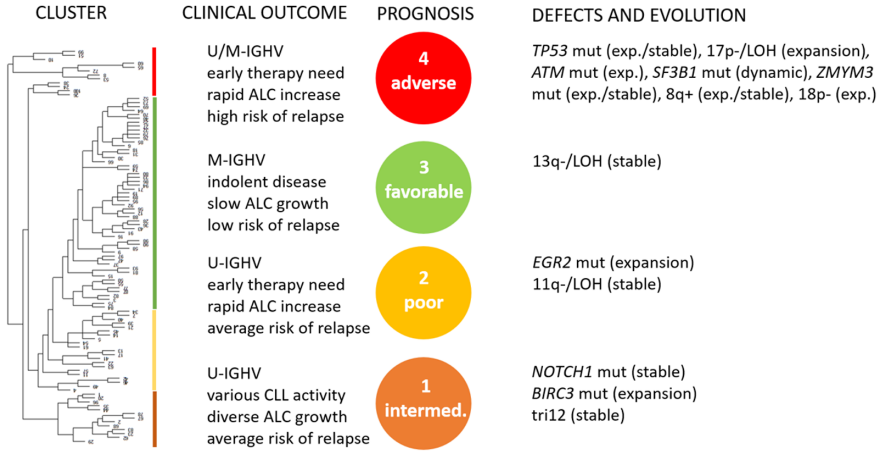

FIGURE 3 The clinical and genetic characteristics of four defined clusters among untreated CLL patients. Group comparisons were performed (Table 2; Table S5) and only significant differences and the most abundant defects are depicted.

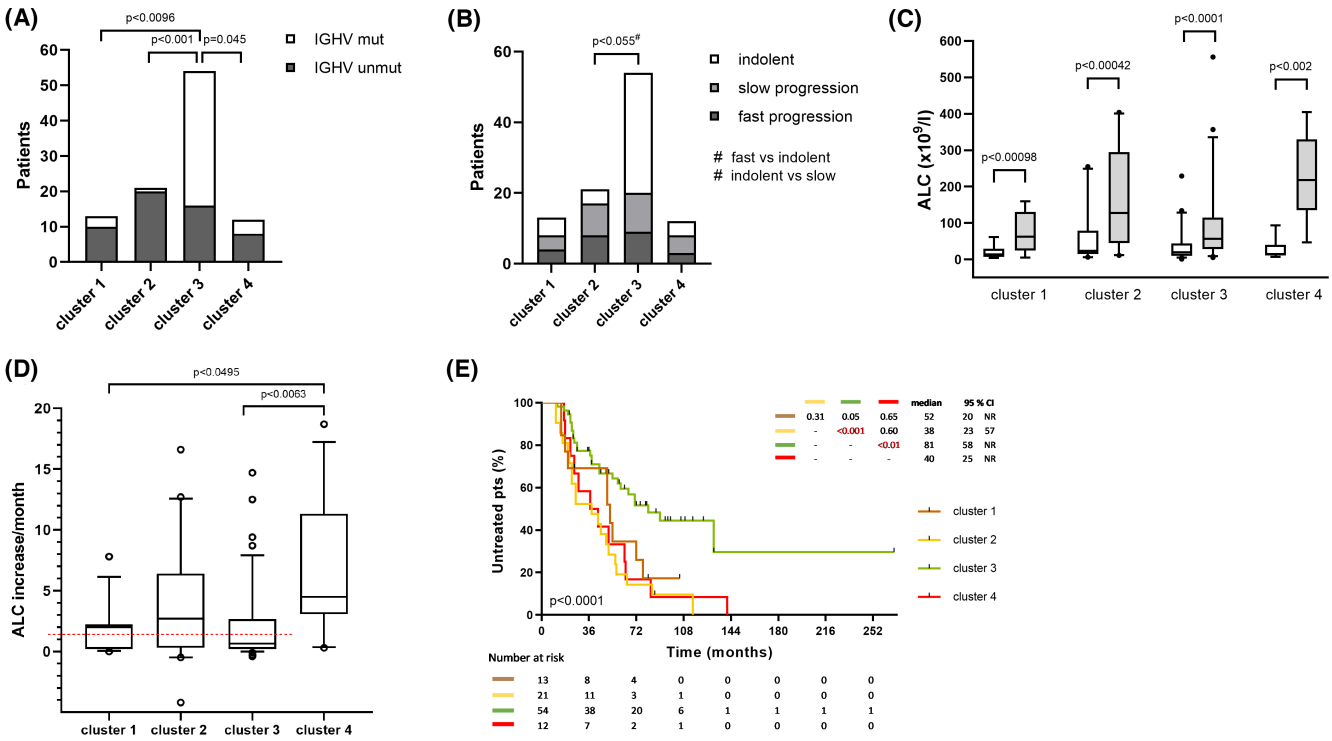

FIGURE 4 Clinical differences among defined clusters. (A) IGHV status ($p < 0.001$), (B) CLL activity ($p = 0.01$), (C) absolute lymphocyte count (ALC) in TP1 and TP2 (white and grey box, respectively; $p = 0.18$ in TP1 and $p < 0.01$ in TP2 among all clusters), (D) rate of ALC increase per month (red line represents the median of the whole cohort, and also the cut-off for slow and rapid ALC increase/month; $p < 0.01$), and (E) time to first treatment (TTFT) in defined clusters (NR: not reached).

therapy need, and rapid increase in ALC/month. An intermediate cluster 1 was associated with unmutated IGHV, diverse ALC increase per month and CLL activity. Finally, cluster 3 was favourable, having mutated IGHV, slow ALC increase and predominantly indolent disease course with the prolonged TTFT. Considering the CLL course after the first therapy, the analysis of time to second-line treatment (TTST) during the follow-up period further confirmed the inferior prognosis of cluster 4. Cluster 3 showed the lowest rate of relapses (Figure S8).

DISCUSSION

Numerous studies have attempted to describe molecular features of CLL and link their dynamics and evolution with clinical behaviour in individual patients.[10,11,13,19] The selection of minor preexisting subclones with resistance-conferring defects was described in patients treated with chemoimmunotherapy[14] and novel targeted therapies such as ibrutinib[33] and venetoclax.[34] On the contrary, it was assumed that stable genetic alterations predominate in the

TABLE 2 Clinical differences among groups of patients after unsupervised clustering.

Parameter	Category	Pts. in cluster 1 ($n=13$)	Pts. in cluster 2 ($n=21$)	Pts. in cluster 3 ($n=54$)	Pts. in cluster 4 ($n=12$)	*p* value
Rai stage	0	8	14	36	5	0.51
	1	5	6	15	5	
	2	0	0	2	1	
	3	0	1	0	0	
	4	0	0	1	1	
IGHV status	Mutated	3	1	38	4	<0.001
	Unmutated	10	20	16	8	
CLL activity	Fast progression	4	8	9	3	0.02
	Slow progression	4	9	11	5	
	Indolent	5	4	34	4	
LDT	<12 months	2	3	13	5	0.63
	>12 months	11	18	41	7	
ALC increase/month	Rapid	7	14	19	10	0.0052
	Slow	6	7	35	2	
ALC in TP1	Median ALC	14	23	19	15	0.18
ALC in TP2	Median ALC	62	127	56	218	0.0013
TTFT	Median (months)	52	38	81	40	<0.0001

Abbreviations: ALC, absolute lymphocyte count; IGHV, immunoglobulin heavy-chain variable-region genes; LDT, lymphocyte doubling time; TTFT, time to first treatment.

pretreatment period of CLL.[10,13,19] Nevertheless, recent studies showed some extent of clonal evolution in the absence of selective therapy pressure.[11,18,20]

We performed a targeted-sequencing study on 200 consecutive samples from 100 untreated CLL patients to explore the extent of natural clonal evolution. Similarly to published data,[13,22,23] we observed extensive heterogeneity in the presence, proportion and dynamics of genomic defects. Our results showed that gene and chromosomal alterations are dynamic and frequently evolve in more than half of treatment-naïve CLL patients. In a minority of patients, the dynamics in genomic abnormalities could be ascribed to the clonal drifts among multiple clonotypes or the intraclonal diversification in line with our and other previous reports.[35–38] Our findings are consistent with the model of gradual cancer progression[1] and studies showing natural CLL progression.[11,18,20] Small cohort studies by Ramassone et al.[20] ($n=28$) and Hernandez et al.[18] ($n=35$) observed continuous evolution irrespective of clinical outcome suggesting that the acquisition of aberrations occurs in the stable phase. In contrast, Smith et al.[19] showed rare pretreatment clonal evolution but significant DNA methylation changes involved in the disease course of 27 patients.

CNAs are generally considered early clonal events followed by the acquisition of somatic mutations. However, the hierarchical pattern determining the order of genomic changes has not been described yet, as the results of the studies are inconsistent.[9,13] We and Landau et al.[9] showed the early occurrence of *NOTCH1* mutations alongside the +12, while Nadeu et al.[13] described them as late events. In general, we have observed various scenarios of aberration dynamics, which supports individual evolution rather than the uniform hierarchical model. Conclusively, the assessment of natural CLL progression should include both chromosomal and gene defects and also IG clonality since they evolve and contribute together to the disease development.

The unsupervised clustering revealed four groups that significantly differed in biological and clinical features. Our adverse cluster resembles the group with exponential leucocyte growth determined by Gruber et al.[11] as it comprised patients with a fast-growing progressive disease with early therapy need and a high risk of relapse. This cluster was enriched with the expansion of *TP53* defects, *ATM* and *SF3B1* mutation, and 18p−, similar to the above-mentioned group except for +12. In our study, stable +12 and *NOTCH1* mutations were associated with the intermediate cluster representing a mixture of clinical manifestations. The favourable cluster corresponds with the logistic growth group described by Gruber and colleagues.[11] Although subclones with well-established CLL drivers show a growth advantage and their accumulation influence rapid therapy need,[11,13] their kinetics does not always match the growth characteristic of the overall tumour.[11,18] We also observed patients with slow lymphocyte count increase in clusters with inferior prognoses (clusters 2 and 4) and vice versa. Therefore, anticipating disease progression only from WBC/ALC is insufficient, and monitoring genetic alteration evolution would be beneficial.

The impact of emerging clonal evolution on prognosis and therapy intervention in early-stage CLL patients is a clinically important question. The prognostic scoring systems integrating genomic defects[39–43] could be distorted due to vast intraclonal heterogeneity and evolving subpopulations. Indeed, our results showed that the diagnostic genomic composition does not have to correspond to the pretreatment state, and

driver defects can emerge in long-standing disease despite clinically indolent course. This creates an opportunity for a personalized consideration of repeated examination. In addition, the integration of sequencing data with tumour burden measurement (i.e. WBC count) may be of clinical relevance in improving prognostication and therapeutic strategy.[11] The recent CLL12 trial[44] assessing the use of ibrutinib in early-stage patients with increased risk of progression has not proved better overall survival. However, identifying high-risk patients, especially with emerging clonal evolution, in early intervention clinical trials is still in need.[45,46]

Our data accentuate the benefit of comprehensive NGS testing over employing separate methods for evaluating genomic complexity. Although FISH analysis is routinely used,[21] it alone does not provide an accurate insight since a poor prognosis for patients with a complex karyotype was recently described.[40] Indeed, we detected 46 additional chromosomal defects beyond the standard FISH panel. Moreover, it is desirable to examine both alleles of the *TP53* and *ATM* genes, representing adverse prognostic factors.[30,47–49] Integrative targeted NGS testing in CLL can overcome the drawbacks of parallel genetic tests by simultaneous analysis of multiple genetic markers, resulting in faster and consolidated interpretation of results.[24,25]

In summary, our data extend the previous reports on CLL evolution and dynamics of genomic defects during the pretreatment period of the disease. We emphasize the relevance of reassessing genetic status by integrative NGS testing when an updated prognosis is required, as some of the aberrations can develop over time, especially in patients with a long 'watch and wait' phase. It also advocates expanding the testing scope of adverse defects, at least to mutations in *ATM* and *SF3B1* genes, and 18p−. Our findings contribute to ongoing efforts of precision medicine, where an individual and comprehensive assessment of genomic defects may predict the disease course and therapeutic strategy.

AUTHOR CONTRIBUTIONS

Veronika Navrkalova and Jana Kotaskova were responsible for the study design, experiments, data analysis and wrote the manuscript; Karla Plevova performed the data analysis and wrote the manuscript; Lenka Radova executed the statistical analyses; Jakub Porc and Karol Pal were responsible for the bioinformatic analyses; Michael Doubek and Anna Panovska provided the patient samples and clinical data; Jitka Malcikova, Sarka Pavlova and Sarka Pospisilova edited the manuscript.

ACKNOWLEDGEMENTS

We acknowledge the CF Genomics CEITEC MU supported by the NCMG research infrastructure (LM2018132 funded by MEYS CR) for their support in obtaining scientific data presented in this paper. Core Facility Bioinformatics of CEITEC Masaryk University is gratefully acknowledged for the obtaining of the scientific data presented in this paper. Part of the work was carried out with the support of research infrastructure EATRIS-CZ, ID number LM2018133, funded by MEYS CR.

FUNDING INFORMATION

This work was supported by the projects AZV NV19-03-00091, AZV NU20-08-00314 and Conceptual development of research organization (FNBr 65269705) provided by the Ministry of Health of the Czech Republic, by the project MUNI/A/1224/2022 and National Institute for Cancer Research (Programme EXCELLES, ID Project No. LX22NPO5102) funded by the European Union—Next Generation EU.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

ETHICS STATEMENT

The Ethics Committees of the University Hospital Brno and Masaryk University approved the study.

PATIENT CONSENT STATEMENT

Informed consent for specimen storage and research use was obtained in accordance with the Declaration of Helsinki from all 100 CLL patients analysed in our study.

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SUPPORTING INFORMATION

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

How to cite this article: Navrkalova V, Plevova K, Radova L, Porc J, Pal K, Malcikova J, et al. Integrative NGS testing reveals clonal dynamics of adverse genomic defects contributing to a natural progression in treatment-naïve CLL patients. Br J Haematol. 2024;204(1):240–249. https://doi.org/10.1111/bjh.19191