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A Quantitative Analysis of Subclonal and Clonal Gene Mutations Pre- and Post-therapy in Chronic Lymphocytic Leukemia

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Abstract

Purpose—CLL-associated gene mutations that influence CLL cell fitness and chemotherapy resistance should increase in clonal representation when measured before therapy and at relapse.

Experimental Design—To uncover mutations associated with CLL relapse, we have performed whole exome sequencing (WES) in a discovery cohort of sixty-one relapsed CLL patients identifying eighty-six recurrently mutated genes. The variant allele fractions (VAFs) of nineteen genes with mutations in ≥ 3/61 cases were measured in fifty-three paired pre- and post-treatment CLL samples sorted to purity using panel-based deep re-sequencing or by droplet digital PCR (ddPCR).

Results—We identify mutations in *TP53* as the dominant subclonal gene driver of relapsed CLL often demonstrating substantial increases in VAFs. Subclonal mutations in *SAMHD1* also recurrently demonstrated increased VAFs at relapse. Mutations in *ATP10A, FAT3, FAM50A* and *MGA*, although infrequent, demonstrated enrichment in ≥2 cases each. In contrast, mutations in NOTCH1, SF3B1, POT1, FBXW7, MYD88, NXF1, XPO1, ZMYM3 or CHD2 were predominantly already clonal prior to therapy indicative of a pre-treatment pathogenetic driver role in CLL. Quantitative analyses of clonal dynamics uncovers rising, stable and falling clones and subclones without clear evidence that gene mutations other than in *TP53* and possibly *SAMHD1* are frequently selected for at CLL relapse.

Conclusion—Data in aggregate support a provisional categorization of CLL-associated recurrently mutated genes into three classes i) often subclonal pre-therapy and strongly enriched

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Nisar Amin, Kamlai Saiya-Cork, Brian Parkin and Sami Malek performed the laboratory research.
Kerby Shedden assisted with statistical analysis and software development for data analysis.
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Nisar Amin and Sami Malek wrote the paper.
Sami Malek conceived the study and supervised the work.

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None to declare

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after therapy, or, ii) mostly clonal pre-therapy or without further enrichments at relapse, or, iii) subclonal before and after therapy and enriching only in sporadic cases.

Keywords

relapsed CLL; whole exome sequencing; subclonal gene mutations

INTRODUCTION

Chronic lymphocytic leukemia is a heterogeneous disease caused by marked differences in biology and manifested in varied clinical presentations (1-4). Substantial efforts have been directed at defining the molecular and cellular underpinnings that cause variations in CLL. These efforts have identified molecular traits that associate with initial disease progression, the response durations to up-front therapies and ultimately, differences in overall survival. Most of these efforts have been directed at the characterization of CLL at diagnosis(5-7).

In contrast, comparatively less information is available characterizing CLL that has relapsed from frontline chemo-immunotherapies. What are the determinants or drivers of CLL cell accumulation after therapy? What is the frequency of CLL clonal evolution under therapy and what are the common drivers of therapy resistance? What role do gene mutations as opposed to other CLL traits serve in relapsed CLL? A refined understanding will facilitate development of novel research directions, better diagnostics, risk-adapted counseling and therapy approaches.

With the goal of identifying genomic drivers of CLL progression, investigators have compared the frequencies of selected molecular characteristics in relapsed or refractory CLL patient cohorts with frequencies in unrelated CLL cohorts analyzed at diagnosis. Such efforts, although prone to biases, have implicated *TP53* mutations/del17p in acquired therapy resistance and have generated hypothesis-generating findings about the involvement of specific factors in CLL disease aggressiveness(8-10).

The analysis of paired longitudinal samples procured before and after therapy reduces biases in the discovery of factors driving CLL relapse and clonal evolution. Such paired longitudinal analysis has uncovered the acquisition of selected genomic aberrations as detected through clinical FISH testing in CLL patients over time as well as acquisition of novel acquired copy number aberrations (aCNA) and loss-of-heterozygosity (LOH) in CLL when assayed longitudinally by karyotyping or on high-resolution SNP array platforms(11-18).

With the goals of identifying gene mutations that drive relapsed CLL and to further clarify the biological roles of gene mutations in CLL in general(19-22), we have performed a longitudinal genomic analysis of paired CLL samples procured before and after chemo immunotherapies. Using an experimental approach that combines WES in a discovery cohort of 61 relapsed CLL, followed by deep panel-based re-sequencing in paired pre- and post-treatment samples complemented with droplet digital PCR-measurements of mutated genes, we substantially qualify the role of gene mutations in the pathogenesis of CLL and relapsed CLL.

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Data in aggregate support a provisional categorization of CLL-associated recurrently mutated genes into three classes i) often subclonal pre-therapy and strongly enriched after therapy, or, ii) mostly clonal or major subclonal pre-therapy and without further enrichments at relapse, or, iii) subclonal before and after therapy and enriched only in sporadic cases. Combined, our results suggest mutations in *TP53* and possibly *SAMHD1* as relapse gene drivers in a minority subset (20%) of CLL relapsing after chemo-immunotherapy. The data based on complementary genomic analyses support the refined concept that the majority of CLL undergo clonal evolution after therapy. Importantly, we identify an early pre-treatment pathogenetic role for many known recurrently mutated genes in CLL and provide limits to the hypothesis that sub clonal gene mutations other than in *TP53* frequently drive CLL relapse.

METHODS

Patients

Between January 2005 and June 2011, 300 patients evaluated at the University of Michigan Comprehensive Cancer Center were enrolled onto this study and pre-treatment samples analyzed here were procured at enrollment. As specified in the protocol, patients were resampled, where applicable, at multiple time points following initial enrollment. The trial was approved by the University of Michigan Institutional Review Board (IRBMED #2004-0962) and written informed consent was obtained from all patients prior to enrollment. DNA from 61 relapsed CLL patients that was subjected to WES constituted the discovery cohort. Of these 61 patients, 53 patients had available paired samples procured before therapy and at subsequent relapse from prior chemotherapy (chemo immunotherapy was administered to 93% of these patients) and 8 patients had longitudinal samples analyzed without receiving intercurrent therapy. Of the 53 patients with available paired samples, 41 were untreated at first sampling, while 12 had relapsed at trial enrollment and had undergone additional round(s) of chemotherapy followed by a subsequent relapse (**see Tables 1 and 2; Supplementary Table 1**).

CLL treatment was defined as cytotoxic chemotherapy (usually fludarabine, pentostatin, bendamustine or cyclophosphamide) with or without monoclonal antibody therapy for CLL. Clinical information, including Rai stage and all treatments given, was collected on all patients. Patient samples were characterized for selected CLL-associated chromosomal aberrations on the day of trial enrollment as a routine clinical test at the Mayo Clinic (Rochester, MN) using FISH (CLL-FISH). Measurements of CLL-associated molecular characteristics were as described(6).

Cell Isolation

Flow cytometry sorting of CLL specimens—Cryopreserved PBMCs (frozen after Ficoll-gradient purification) from CLL blood specimens were prepared for FACS sorting into CD19+ and CD3+ cells as previously described(6).

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Preparation of Sample DNA

DNA used for SNP 6.0 profiling was extracted from FACS-sorted CD19+ and CD3+ cells as described(6).

Solution-based exome capture and HiSeq2000-based massively parallel sequencing

Solution-based exome capture and HiSeq2000-based massively parallel sequencing was performed as described(23).

Bioinformatic pipeline analysis of WES data

The exome sequencing data was analyzed by the variant calling pipeline developed by the University of Michigan Bioinformatics Core. For each of the samples, paired-end reads were aligned to the hg19 reference genome using BWA v0.7.8(24), followed by removal of sequence duplicates using PicardTools v1.79 (http://picard.sourceforge.net), local realignment around INDELs and base quality score recalibration using GATK v3.2-2(25). Read coverage on exome capture target regions was calculated using BEDTools v2.20.1(26). Normal-Tumor paired alignment files were submitted to MuTect v1.1.4(27), Strelka v1.0.14(28) and Varscan v2.3.7 (with its false-positive filter)(29) for the detection of somatic and germline SNPs and INDELs.

Candidate variant calls across all samples and patients were merged using Jacquard(30) into a single VCF file that included all variant loci whose filter field passed in MuTect or Strelka or VarScan (VarScan calls were limited to somatic variants confirmed in false-positive filter). Variants were annotated using SnpEff v4.0/hg19(31), dbNSFP v2.4(32), dbSNP v138 (Database of Single Nucleotide Polymorphisms, National Center for Biotechnology Information, National Library of Medicine), and 1000 Genomes v3(33). For variants associated with multiple effects or multiple transcripts, a single “top effect” annotation was nominated based on annotation confidence, predicted impact, gene region, and transcript length. Common variants (at or above 5% overall population allele frequency as reported by 1000 Genomes) were excluded.

Exon resequencing of nominated variants in CLL samples

Primers to amplify and sequence all variants of interest were designed using the primer 3 program (http://primer3.ut.ee/) and sequence information generated using direct sequencing as described(23). Sequence variants were detected using Mutation Surveyor and visible inspection of sequence traces. Mutations were confirmed to be somatically acquired using unamplified CLL CD19+ cell-derived DNA and paired CD3+ cell-derived DNA from sorted cells as templates.

Deep coverage massively parallel re-sequencing of 17 genes and bioinformatics analysis

A customized multiplexed primer panel (Qiagen Gene Read Panel # CNGHS-00586X-849) was used to amplify all coding exons of the genes: TP53, NOTCH1, SF3B1, XPO1, BIRC3, MYD88, NXF1, POT1, CACNA1E, CHD2, EGR2, FAM50A, FAT3, FBXW7, MGA, SAMHD1 and *ZMYM*. PCR products were pooled and sequencing libraries prepared using barcoded adapters. Sequencing was done on a HiSeq2000 sequencer. Bioinformatics

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nomination of sequence variants was performed using Broad GATK HaplotypeCaller v3.3.0 and Varscan. Discordant variant caller results were qualified through ddPCR measurements.

For details of the bioinformatics analyses please see **Supplementary Methods**.

Measurements of VAFs using droplet digital PCR (ddPCR)

Allele-specific fluorescent oligonucleotide probes using minor groove-binding (Life Technologies, Grand Island, NY) or locked nucleic acid (Integrated DNA Technologies [IDT], Coralville, IA) chemistries were developed for each mutation using Primer Express (Life Technologies) and Oligoanalyzer (IDT), respectively. Wildtype and mutant allele-specific probes (5 uM) were combined with allele-independent forward and reverse PCR primers (20 uM) to generate a probe mix. Each probe mix was optimized for allele discrimination using quantitative PCR to derive an optimal annealing temperature (T_A) as follows: 2X Taqman Genotyping Master Mix (Life Technologies) was combined with 40X probe mix and either 30 ng of mutant gDNA derived from a patient sample harboring the mutation of interest or homozygous wildtype genomic DNA (gDNA) derived from the Ly18 cell line and then amplified with a Bio-Rad CFX96 Real-Time System using a T_A gradient for 45 cycles and analyzed with Bio-Rad CFX Manager. The temperature at which both wildtype and mutant probes were amplified in the presence of DNA with a heterozygous mutation but at which the mutant probe was not amplified in the presence of wildtype-only DNA was selected as the optimal T_A to carry forward for ddPCR.

ddPCR was then performed in duplicate for the paired diagnosis and relapsed samples of each case with a mutation of interest as well as a wildtype-only control derived from the Ly18 cell line as follows: a master mix containing 2x Taqman Genotyping Master Mix, 40X probe mix, 25X droplet stabilizer (RainDance Techonologies) and 50X restriction endonuclease (*HaeIII* or *MseI*, New England Biolabs) was added to separate wells containing 50 ng of gDNA for a total reaction volume of 25 uL and then incubated at room temperature for 15 mins. Samples were then pipetted into a RainDrop Source chip (RainDance Technologies) and 3.5-4.5 million droplets were generated with the RainDrop Source machine. The resulting emulsion was amplified for 45 cycles at the previously derived optimal T_A with the following settings: 95^o for 10 min, then 45 cycles of 95° for 15 s and T_A for 60 s, then 98° for 10 min. End-point fluorescence was then measured with a RainDrop Sense machine and analyzed with RainDrop Analyst software. The wildtype and mutant signals of each relapsed sample were used to draw positive gates to then analyze VAF of the diagnosis samples and wildtype-only controls. To call the presence of mutant signal, the mutant gate must have contained at least 5 droplets as well as a ten-fold greater number of mutant droplets than the wildtype control.

RESULTS

Patient Characteristics

To identify gene mutations that may have contributed to CLL that has relapsed from prior chemo immunotherapy and to further qualify the roles served by recurrent gene mutations in CLL pathogenesis and disease evolution, we subjected DNA isolated from FACS-sorted

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CD19+ cells and paired CD3+ cells from 61 relapsed CLL patients (the discovery cohort) to whole exome sequencing (WES). Characteristics of these 61 CLL patients are summarized in **Supplementary Table 1**. Of these 61 patients, 53 patients had available paired samples procured before therapy and at subsequent relapse from prior chemotherapy (chemoimmunotherapy was administered to 93% of these patients; characteristics of these 53 patients and treatment given are summarized in **Tables 1 and 2; Supplementary Table 1**) and 8 patients had longitudinal samples analyzed without receiving intercurrent therapy.

Massively parallel sequencing of the coding genome of relapsed CLL samples

To further our understanding of the genetic basis of relapsed CLL, we used solution exon capture of sheared and processed genomic DNA isolated from highly purified flow-sorted CD19+ B-cells and paired CD3+ T-cells isolated from 61 cases of relapsed CLL followed by paired-end massively parallel sequencing. The very high purity of CLL B-cells and paired CD3+ T-cells afforded by cell sorting improved sensitivity of mutation detection and largely eliminated the distorting effect of cell impurity on VAF estimates. The WES data were characterized by a range of de-duplicated mapped reads per DNA sample of 47,521,667 to 98,812,508 (mean of 71,967,091) and a mean depth of coverage of 72 (range of 52 to 102).

The landscape of recurrently mutated genes in relapsed CLL

We identified and subsequently confirmed through Sanger sequencing in paired CD19+ and CD3+ cell-derived DNA 86 genes that were recurrently mutated in the discovery cohort of 61 relapsed CLL. The confirmed mutations were also analyzed through Sanger sequencing in paired pre-treatment DNA. Details of these results are summarized in **Supplementary Table 2**. Of the 86 genes, 19 were mutated in ≥ 3/61 (~≥5%) of cases in rCLL. In descending order of frequency of CLL cases involved these are: *NOTCH1* (13/61), *xTP53* (8/61), *SF3B1* (8/61), *POT1* (8/61), *XPO1* (7/61), *SAMHD1* (6/61), *CHD2* (6/61), *FAM50A* (4/61), *MYD88* (4/61), *NXF1* (4/61), *ZMYM3* (4/61), *APT10A* (3/61), *ATRX* (3/61), *CACNA1E* (3/61), *EGR2* (3/61), *FAT3* (3/61), *IRF4* (3/61), *MGA* (3/61) and *FBXW7* (3/61). Some of these genes have not previously been recognized as recurrently mutated in CLL.

To complement the WES data, we re-sequenced *RPS15* and *NFKBIE* exon 1 spanning the previously identified c.759_762delTTAC in 294 consecutively enrolled, unselected CLL patients: We detected 7/294 (2.4%) *RPS15* mutations and 3/294 (1%) *NFKBIE* mutations(34-36). We had previously reported on sporadic (<2%) *BRAF* mutations in CLL(37).

In the 61 relapsed CLL subjected to WES we detected 2/61 (3%) *RPS15* mutations, both of which were present before and after therapy. We detected 1/61 *BRAF* mutation (we sequenced the hotspot exons 11 - 14), which was acquired after therapy. We detected no (0/61) *NFKBIE* mutations. Finally, review of *ATM* mutations as detected by WES and confirmed by Sanger sequencing uncovered 2/61 mutations, one acquired at relapse and one present before and after therapy.

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Measurement of variant allele fractions in paired pre- and post-treatment CLL samples of 19 recurrently mutated genes

Aided by cell sorting to achieve maximal CLL cell and DNA purity, we proceeded with custom primer panel-based deep re-sequencing of all coding exons of NOTCH1, TP53, SF3B1, POT1, XPO1, SAMHD1, CHD2, FAM50A, MYD88, NXF1, ZMYM3, BIRC3, CACNA1E, EGR2, FAT3, MGA and *FBXW7* in 53 paired DNA samples isolated from CLL samples procured before and at relapse from chemo immunotherapy. For two genes (*APT10A* and *ATRX*), we resorted to ddPCR-based VAF estimations. For the analysis of deep panel re-sequencing data and VAF estimations we employed two separate bioinformatics approaches in parallel that overall demonstrated excellent concordance. For selected gene mutations and for instances of discordant bioinformatics results, these estimates were further qualified using ddPCR. Finally, a few selected mutations were manually identified using review of BAM files and the fraction of mutated reads was used as a VAF estimate. A summary of VAFs in paired pre- and post-treatment samples for the gene mutations analyzed is outlined in **Supplementary Table 3**.

We proceeded with SNP 6.0 array profiling of the CLL cases that were subjected to WES analysis, extending previously published measurements(38). We find that almost all gene mutations in CLL (other than in *TP53* or *ATM*) are not associated with either copy loss or gain of the wild type alleles, providing additional evidence that these lesion types independently influence CLL pathogenesis. VAF estimates for gene mutations as presented here were therefore not in need of correction for aCNAs and can be used to directly estimate clone sizes.

The recurrent emergence of clonal *TP53* and *SAMHD1* mutations at relapse due to enrichment of pre-existing subclones

A comparison of VAFs pre- and post-therapy and the identification of VAF increases post-therapy can identify candidate genes involved in chemotherapy resistance resulting in preferential clonal outgrowth at relapse. In this large longitudinal CLL genomics study, we identified only two genes that frequently enriched at relapse: *TP53* and *SAMHD1* **(Figure 1)** (39, 40). Overall, 13% (7/53) of rCLL demonstrated enrichment of *TP53* mutations from preexisting subclones at relapse and no case demonstrated a decline (please note that 2 CLL cases carried more than one *TP53* mutation: CLL90 acquired a del17p at relapse and carried two *TP53* mutations in two distinct clones, while CLL117 acquired a del17p at relapse and carried three *TP53* mutations in 2 or possibly 3 distinct clones; **see Supplementary Table 3**). Mutations in *TP53* detected at relapse were all present at diagnosis allowing for the novel conclusion that CLL therapy did not induce such mutations directly. Most such pre-treatment *TP53* mutations existed as subclones at times characterized by very minor clonal representation (VAF range 0.0002 – 0.1448) as measured through ddPCR. In two CLL cases, *TP53* mutations that were already clonal did enrich further at relapse, indicating conversion to full homozygosity. One CLL case that was assayed without receiving intercurrent therapy maintained a subclonal *TP53* mutation status and showed no enrichment **(Supplementary Figure 1)**.

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The second mutated gene that frequently enriched after therapy was *SAMHD1*(40). Four CLL cases demonstrated substantial enrichment from pre-existing subclones of *SAMHD1* mutations at relapse. Two additional cases that were analyzed without having received intercurrent therapy but were already in relapse from chemo immunotherapy at first sampling were already clonal and did not enrich further **(Supplementary Figure 1)**. Finally, one case (CLL17) that contained 4 distinct *SAMHD1* mutations demonstrated enrichment for 2 such mutations and depletion of two other ones, indicative of two co-existing subclones carrying such mutations.

To provide additional context to these findings and to identify potential interactions with other genomic events, we reviewed the six CLL cases with *SAMHD1* mutations in greater detail: CLL17 carried 4 *SAMHD1* mutations likely in two subclones and harbored no aCNAs in either disease phase. The rising subclone was also identified by mutations in *ATP10A* and *CACNA1E* (see below); CLL 77 (no intercurrent therapy) carried 1 *SAMHD1* mutation, displaying a VAF of ~ 0.5 in both phases and harbored no aCNA in either phase; CLL 188 (no intercurrent therapy) carried 1 *SAMHD1* mutation, displaying a VAF of ~0.4 in both phases and harbored no aCNA in the diagnosis sample; CLL 205 had 1 *SAMHD1* mutation at increasing VAFs of 0.02 and 0.14 and carried a short del13q-I. Interestingly, this case also demonstrated enrichment in an *ATP10A* mutation. CLL 218 carried 2 *SAMHD1* mutations each displaying strong VAF gains (both ~0.05 to 0.5) indicative of one clone and lacked aCNAs in either phase. CLL218 at relapse also lost a clone containing a *SF3B1* mutation. Finally, CLL 296 had 1 *SAMHD1* mutation with increasing VAFs (0.02 – 0.22) and carried a del11q pre-treatment but no post treatment SNP array data were available. CLL296 also carried a separate rising dominant clone marked by a *NOTCH1* mutation.

These data in aggregate identify *SAMHD1* mutations as a candidate gene conferring some degree of in vivo resistance to standard CLL therapies.

Of additional interest is the fact that both *TP53* and *SAMHD1* mutations were still often present in subclones (VAFs <0.5) post therapy, suggesting that a true bottleneck does not exists in the evolution of CLL but instead multiple CLL clones survived and emerged in parallel post-therapy together constituting the clinically apparent relapsed disease.

Gene drivers of early CLL pathogenesis identified through high VAFs pre-therapy

One of the important findings resulting from our VAF measurements pre- and post-therapy in a large CLL cohort relates to the common pre-therapy clonal representation of the following gene mutations: NOTCH1, SF3B1, XPO1, NXF1, MYD88, ZMYM3, CHD2, POT1 and *FBXW7* **(Figure 2)**. The quantitatively most common genotype was a clonal (as opposed to subclonal) mutation status pre-therapy, providing support to a revised model in which most of these gene mutations serve an early therapy-independent role in CLL pathogenesis. Although occasional CLL cases for some genes showed enrichment at relapse other cases revealed depletion of pre-exiting mutations thus demonstrating no consistent trends (for instance see data for *NOTCH1* or *SF3B1*).

To provide additional context to these later findings we reviewed the five CLL cases that demonstrated substantial changes in *NOTCH1* mutation VAFs before and after therapy.

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CLL117 lost the *NOTCH1* mutant clone and acquired a dominant *TP53* mutant clone; CLL270 demonstrated a drop in *NOTCH1* VAFs from 0.48 to 0.32 and expanded a minor clone identified through a mutation in *MGA* that must have been *NOTCH1* wt; CLL185 expanded a *NOTCH1* mutant clone (VAFs: 0.048-0.255) while acquiring a large del13q-II lesion, a lesion type previously implicated in CLL relapse(38). CLL195 demonstrated a substantial expansion of the *NOTCH1* mutated clone (VAFs: 0.095-0.389) while also carrying a del11q at both disease phases. By FISH del11q was 43% pre-therapy indicating it was acquired before the *NOTCH1* mutation. Finally, CLL296 demonstrated a very strong *NOTCH1* mutant clone expansion (VAFs: 0.07-0.83).

For the six CLL cases that demonstrated substantial changes in *SF3B1* mutation VAFs we find the following genomic context: CLL11 (declining mutant *SF3B1* VAFs: 0.296-0.147) maintained a clonal *POT1* mutation in both phase and gained a separate clone identified through a mutation in the gene *TENM2* (0.02-0.238); CLL36 (declining mutant *SF3B1* VAFs: 0.396-0.143) had no additional markers for the rising *SF3B1* wt clone identified; CLL209 (declining mutant *SF3B1* VAFs: 0.476-0.329) maintained clonal *NOTCH1* and *XPO1* mutations and gained an 11q deletion; CLL218 largely lost the *SF3B1* mutant clone (declining mutant *SF3B1* VAFs: 0.387-0.002) and acquired a clone marked by mutations in *SAMHD1, MGA* and *FAT3*. Finally, two CLL cases (CLL72 and CLL78) demonstrated minor enrichments in *SF3B1* mutant VAFs post therapy without attaining clonal representation (**see Supplementary Table 3)**.

Further, we find that the VAFs for occasional CLL cases demonstrated subclonal status pre-therapy (for instance, see data for *CHD2* or *POT1*) but very little or no enrichment was seen for most such cases post-therapy. Finally CLL #219 demonstrated a strong *CHD2* mutation enrichment that occurred together with a *TP53* mutation enrichment.

Identification of candidate subclonal gene drivers of relapsed CLL

Within the group of infrequently mutated genes for which we determined VAFs at diagnosis and relapse we identified a subset that demonstrated enrichment at relapse in ≥2 cases for each gene. These are: *ATP10A, ATRX, CACNA1E, FAT3, FAM50A* and *MGA* **(Figure 3**) and together they constitute candidate genes that may contain true, albeit infrequent drivers of CLL relapse. Once we excluded gene mutations demonstrating VAF increases in CLL cases that also demonstrated the simultaneous emergence of a *TP53* mutation, only the genes *ATP10A, ATRX, FAM50A, FAT3* and *MGA* remained, and as outlined above, *ATP10A* mutations occurred twice in the setting of acquired *SAMHD1* mutations. Notwithstanding these caveats, some of these candidate genes are deserving of further study in much larger relapsed CLL cohorts.

Analyses of clonal dynamics uncovers rising, stable and falling clones without clear evidence that recurrent subclonal gene mutations other than in *TP53* frequently drive CLL relapse

The ultra-high purity of our isolated CLL cell DNA paired with the fact that the commonly mutated genes other than *TP53* were almost never affected by genomic gains or losses as measured through SNP 6.0 profiling **(Supplementary Table 3)** allowed us to track clonal

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dynamics directly through the VAF measurements. For heterozygous gene mutations, which constitute the vast majority of genes in this study, the cell fraction carrying the mutations is twice the VAF measurement (e.g. VAF = 0.5; cell fraction with mutations 1; the VAF for X-linked genes in males were corrected by a factor of 0.5). Representative results for CLL cases are displayed in Figure 4 (A: rising subclone scenario; B: stable clone scenario and C: complex and falling clones scenario). From this data and the summary data displayed in **Figure 2** it is clear that genes commonly mutated in CLL, including NOTCH1, SF3B1, XPO1, NXF1, MYD88, ZMYM3, CHD2, POT1 and *FBXW7* serve early pre-treatment roles in CLL pathogenesis and often are already present in almost all tumor cells pre-therapy.

Review of the various clone tracking scenarios displayed in **Figure 4A-D** also clearly demonstrates limits to the hypothesis that recurrent subclonal gene mutations frequently are responsible for CLL relapse because i) rising, stable and falling clones were identified in multiple cases and ii) subclonal CLL relapse drivers other than *TP53* and possibly *SAMHD1* were not commonly detected, and, iii) rising CLL subclones were marked by a variety of uncommon or non-recurrent gene mutations likely indicative of passenger status(22, 41).

The majority of relapsed CLL demonstrates clonal evolution from pre-treatment states

We proceeded with SNP 6.0 array profiling of the CLL cases that were subjected to sequence analysis, extending previously published measurements(38). Subsequently, we combined the CLL sequencing data with the SNP array profiling data and categorized the emergence or loss of any genomic lesion at relapse as a clonal evolution event. Of the 53 CLL patients subjected to longitudinal analysis that received intercurrent therapy, 62% (33/53) developed genomic changes (almost always in the form of acquisitions) in aCNAs or copy neutral LOHs or gene mutations, while 38% (20/53) did not demonstrate such changes **(Table 2)**. Therefore, CLL relapse in the majority of CLL cases is due to the emergence of novel clones that differ from the quantitatively dominant pre-treatment clones usually through acquisition of additional genomic changes. In a minority of cases the CLL relapse is apparently due to proportional regrowth of the pre-existing dominant clone or to changes not captured through WES and SNP array profiling.

DISCUSSION

In this study, we have performed WES followed by Sanger sequence validation to interrogate the exome of 61 relapsed CLL patients for somatically acquired gene mutations. Next, we employed custom panel-based deep re-sequencing of all exons of the genes most frequently mutated in this discovery cohort on paired CLL samples procured before therapy and at relapse to measure variant allele fractions (VAFs). For selected genes we validated and/or resorted to droplet digital PCR for VAF estimations allowing for confirmation of rare subclones at a depth not previously reported. Quantitative measurements further allowed for analyses of clone and subclone dynamics in 30 informative cases.

Summarizing the major findings from this study, we i) identify recurrent strong enrichment of *TP53* mutations from pre-existing rare subclones in CLL at relapse(42, 43); suggesting *TP53* mutations were the dominant gene driver of rCLL; ii) recurrent enrichment of subclonal *SAMDH1* mutations at relapse nominating this gene as an in vivo therapy

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resistance factor in CLL; iii) identify a few candidate CLL relapse-associated mutated genes that enriched at relapse albeit present individually in only a few cases each. Some of these genes are deserving of expansion studies once much larger cohorts of rCLL become available; iv) importantly, we identify many of the known recurrently mutated genes in CLL as often already clonal prior to therapy (NOTCH1, SF3B1, POT1, FBXW7, MYD88, NXF1, XPO1, ZMYM3 or CHD2), or without consistent enrichment/depletion trends at relapse providing clear evidence that these genes serve an early therapy-independent role in CLL pathogenesis(35), v) through analyses of clone and subclone dynamics we identify patterns of clonal enrichment, or stability or complex patterns including clonal decline that together do not support recurrent subclonal gene drivers (other than in *TP53*) as frequent causes of CLL relapse, and, vi) confirm through a more complete genomic analysis the frequent occurrence of genomically altered CLL clones that dominate the disease at relapse.

This study is characterized by various methodological strengths that support the overall conclusions drawn, including: i) the use of high-purity, flow-sorted CD19+ and CD3+ cells as a source of DNA therefore effectively eliminating confounding effects of impure CLL tumor cell populations on estimations of VAFs and clone sizes; the very high tumor cell purity is evidenced by VAFs clustering around 0.5 for many of the studied genes (equal to 100% of cells carrying heterozygous mutations given the lack of genomic alterations affecting mutated gene loci), ii) a large patient cohort that was relatively uniformly treated and sampled longitudinally before and after therapy reducing the effect of biases; iii) genomic aCNA/LOH and gene resequencing analyses that were based on paired DNA samples (tumor and paired normal) for all CLL samples; and, iv); use of various complementary highly sensitive techniques to quantify VAFs in paired CLL diagnosis and relapse samples.

The understanding of the role of gene mutations in the pathogenesis of CLL is undergoing steady refinements. Here we demonstrate that many previously identified recurrently mutated genes are already clonal pre- therapy in CLL, which implies a role in disease initiation possibly from pre-leukemic cells or outgrowth of dominant CLL clones from MBL(35, 44, 45). Findings may partly inform conflicting reports of prognostic implications of gene mutations given that prognosis is influenced by factors governing cell accumulation and therapy efficaciousness and less likely so by early biological events in CLL(46-50). The overarching finding that only a minority of relapsed CLL acquired recurrent clonal gene mutations after potent chemotherapy supports the conclusion that gene mutations as a group, with the exceptions of *TP53* and possibly *SAMHD1*, do not markedly affect chemotherapy efficaciousness in patients and that molecular contributors to relapse reside within acquired copy number aberrations and hitherto unidentified molecular changes worthy of additional studies.

It is however worth mentioning that future studies could aim at unbiased exome-wide ultra-deep analysis of genomic lesions and subclones in CLL at diagnosis and again at relapse. While such studies are highly likely to identify additional complexity, the critical question would be how much of that complexity translates into disease progression and subclonal outgrowth or, alternatively, just represents subclonal diversity present in all cancers.

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In summary, based on the largest reported longitudinal multi-dimensional genomic analysis in relapsed CLL to date, we demonstrate that the emergence of novel dominant clones is frequent in relapsed CLL. The origin of most genomic changes, including mutations in genes like *TP53* or *SAMHD1*, is likely therapy-independent and due to random mutagenesis. Our data support a revised appreciation of an early pathogenetic role for many of the more commonly mutated genes in the pathogenesis of CLL and qualify the roles of subclones as marked by gene mutations in CLL pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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TRANSLATIONAL RELEVANCE

The molecular determinants that influence outgrowth of CLL from monoclonal B cell lymphocytosis (MBL) pre-therapy and the determinants of relapse following therapy are under active investigations. In particular, the discovery of multiple recurrently mutated genes in CLL motivates research into their contribution to CLL disease biology and progression after therapy. In this study, we have used complementary experimental approaches to study recurrent gene mutations in paired CLL samples procured before and after therapy. Using DNA isolated from flow-sorted CLL cells for all analyses, we find that many gene mutations in CLL are already clonal prior to therapy or show no consistent enrichment post-therapy indicative of an early pathogenetic and therapy-unrelated role in CLL. In contrast, through use of highly sensitive droplet digital PCR able to detect mutations at 0.01% allele frequency, we find that clones carrying *TP53* or *SAMHD1* mutation enrich post-therapy often from deep subclones and are not therapy-induced.

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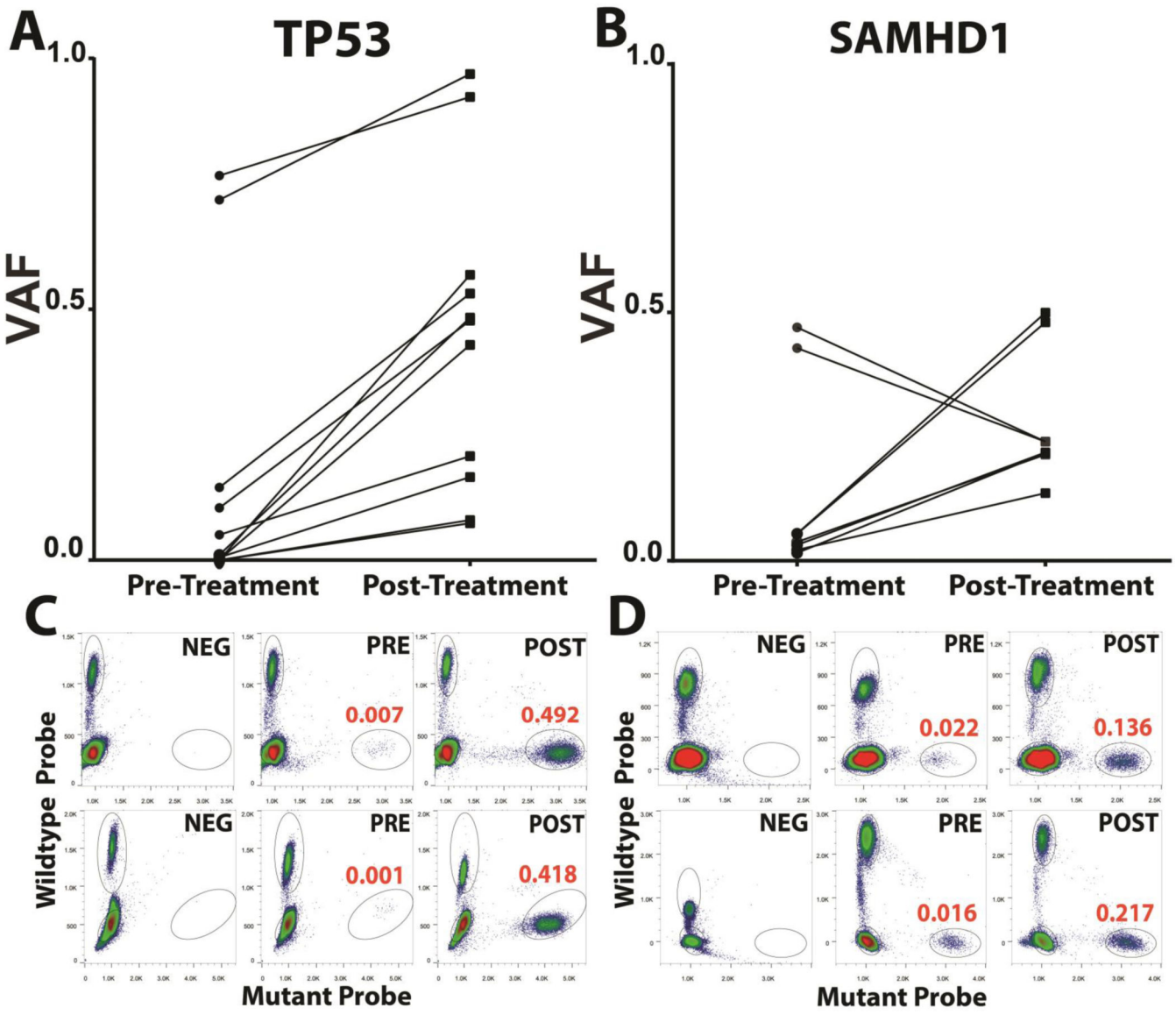

Figure 1. Frequent enrichment of mutations in *TP53* and *SAMHD1* after therapy in CLL

Displayed are variant allele fractions (VAFs) for A: *TP53* (N=7 CLL carrying a combined total of 11 mutations, CLL90 carried 2 mutations and CLL117 carried 3 mutations) and, B: *SAMHD1* (N=4 CLL carrying a combined total of 8 mutations; CLL 17 carried 4 mutations and CLL 218 carried 2 mutations; the two declining SAMHD1 VAFs are both from CLL 17) in paired CLL samples procured before and after therapy. VAFs for *TP53* were not corrected for presence of del17p. C: Representative droplet digital PCR results for NEG: unmutated control DNA, PRE: CLL DNA procured pre-therapy and POST: CLL DNA procured post-therapy. Red numbers indicate the mean VAFs based on duplicate measurements. Results for two representative CLL cases are shown each for *TP53* and *SAMHD1*.

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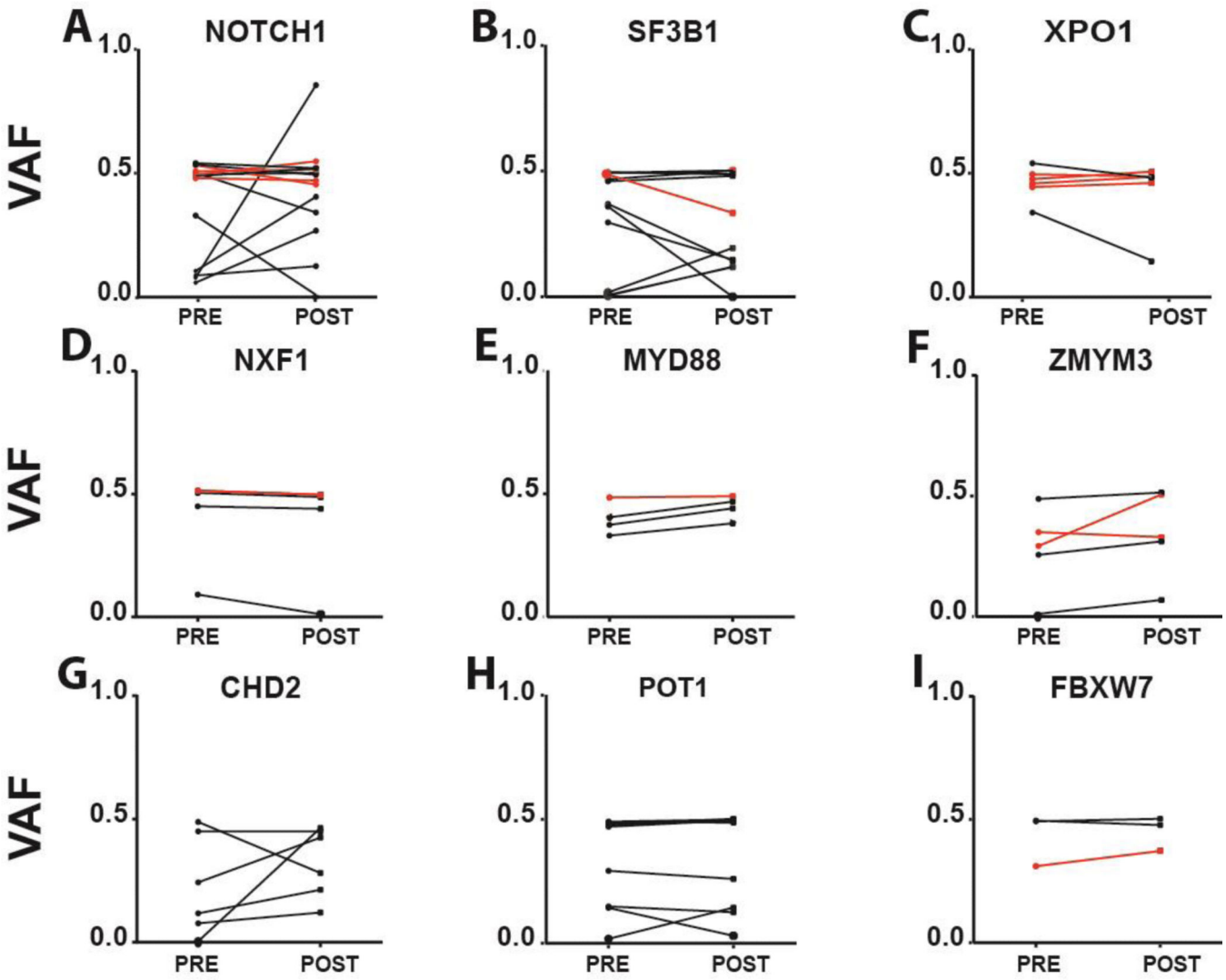

Figure 2. Evidence for an early pathogenetic role of various recurrent gene mutations in CLL

Displayed are variant allele fractions (VAFs) for A: *NOTCH1*; B: *SF3B1*; C: *XPO1*; D: *NXF1*; E: *MYD88*; F: *ZMYM3*; G: *CHD2*; H: *POT1* and, I: *FBXW7* in paired samples procured before and after therapy. Black: CLL samples untreated at first measurements; Red: CLL samples that had relapsed at first measurement and that received additional therapy followed by relapse.

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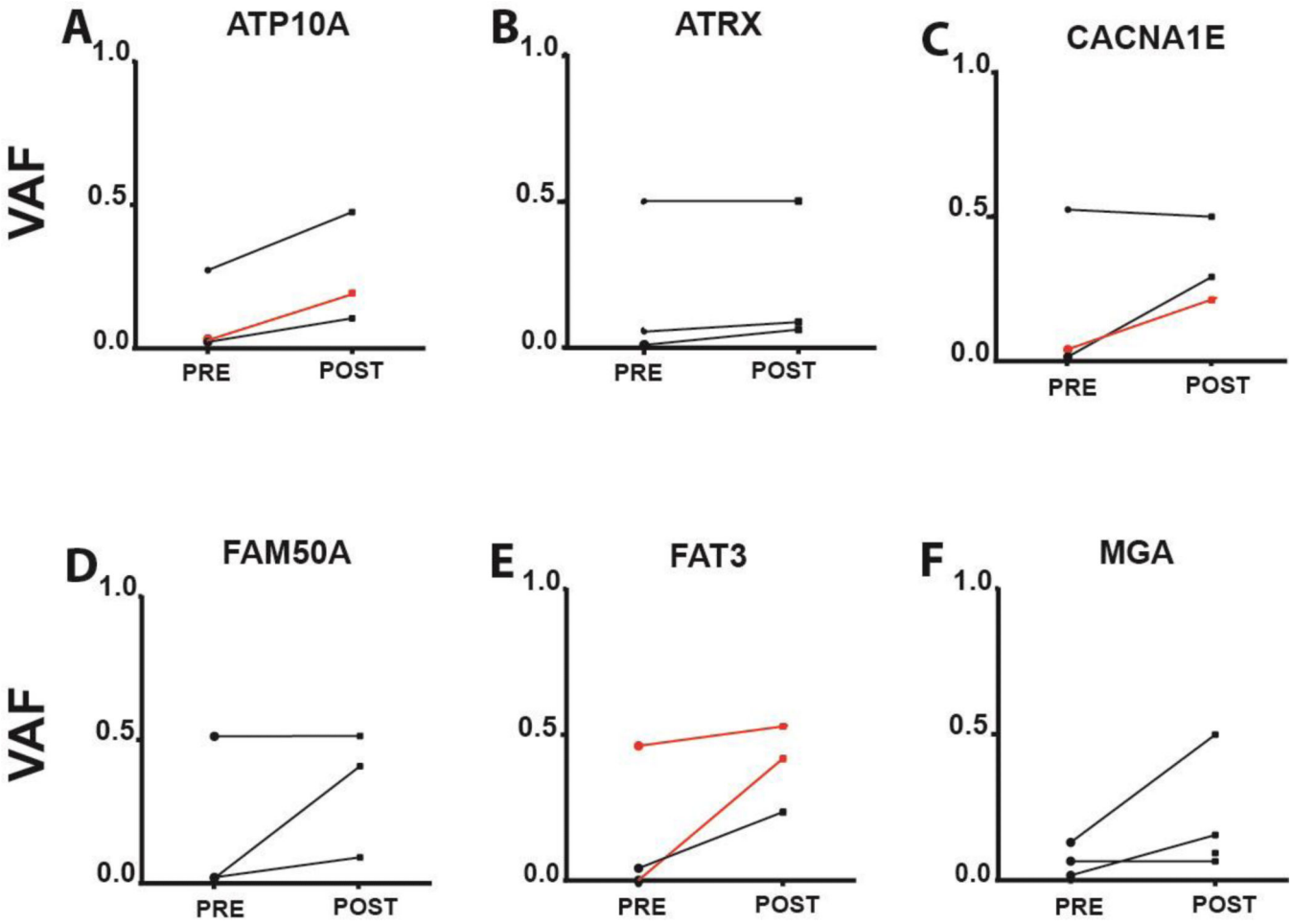

Figure 3. Candidate gene drivers of CLL relapse

Displayed are variant allele fractions (VAFs) for A: *ATP10A*; B: *ATRX*; C: *CACNA1E*; D: *FAM50A*; E: *FAT3* and, F: *MGA* in paired samples procured before and after therapy. Black: CLL samples untreated at first measurements; Red: CLL samples that had relapsed at first measurement and that received additional therapy followed by relapse.

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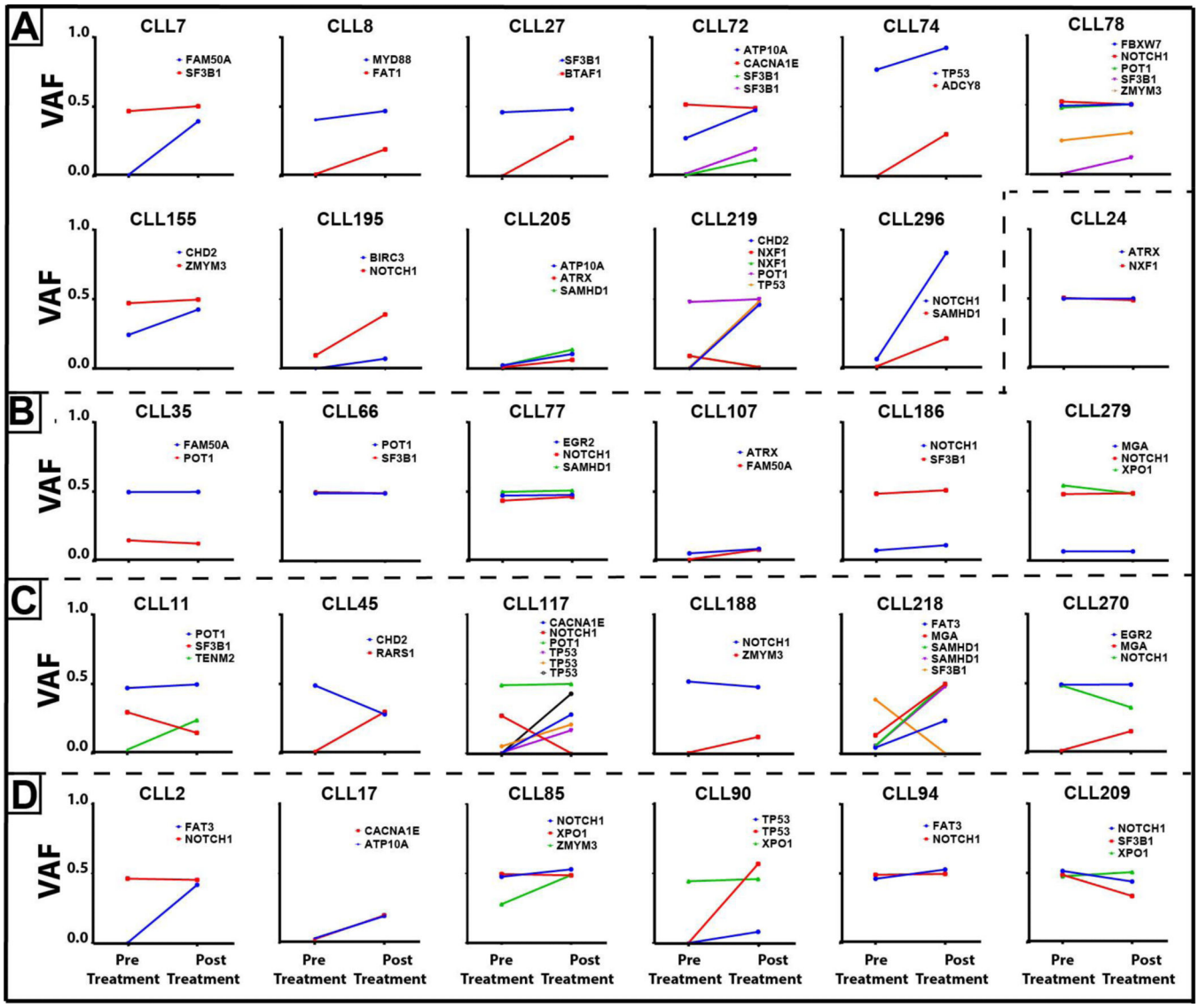

Figure 4. CLL clone dynamics in individual patients identifies rising, stable and falling clones and subclones post therapy

A-C: All CLL cases were untreated at first measurements. A: CLL cases with rising subclones; B: CLL cases with stable clones; C: CLL cases with complex clone dynamics including rising and falling clones. D: CLL samples that had relapsed at first measurement and that received additional therapy.

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Table 1

Characteristics of fifty-three relapsed CLL patients analyzed by WES, deep custom panel-based resequencing and ddPCR.

Patient Characteristics	Intercurrent therapy no. (%)
Sample size N= 53 patients	53 (100%)
Age at enrollment, years	
Median	64
Range	39 - 84
Gender	
Female	18 (34%)
Male	35 (66%)
Rai stage at enrollment	
Low, 0	12 (23%)
Intermediate, I-II	36 (67%)
High, III-IV	5 (10%)
NOTCH1 exon 34 mutations at enrollment (Sanger)	
Wild-type	43 (81%)
Mutated	10 (19%)
SF3B1 exons 13-17 mutations at enrollment (Sanger)	
Wild-type	45 (85%)
Mutated	8 (15%)
P53 exons 2-10 mutations at enrollment (Sanger)	
Wild-type	51 (96%)
Mutated	2 (4%)
Prioritized interphase FISH-25 *	
17p deletion	2 (4%)
11q deletion	6 (11%)
Trisomy 12	11 (21%)
Normal FISH	13 (25%)
13q deletion (sole abnormality)	21 (40%)
IgV_H mutational status	
Unmutated (≥ 98% homology to germline)	31 (58%)
Mutated (< 98% homology to germline)	18 (34%)
Not evaluable	4 (8%)
ZAP-70 expression	
Positive (> 20%)	33 (62%)
Negative (≤ 20%)	16 (30%)

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Patient Characteristics	Intercurrent therapy no. (%)
Not available	4 (8%)
Treatment status at enrollment	
Not treated	41 (77%)
Treated	12 (23%)
Number of prior therapies	
0	41 (77%)
1	7 (13%)
2	3 (6%)
>2	2 (4%)

* FISH findings in ≥25% of nuclei. Order of prioritization: 17p > 11q > trisomy 12 > 13q > Ig translocations > normal FISH. Please see Supplementary Table 1 for details.

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Table 2

Listing of therapies received by CLL patients between sample procurements, response types and clonal evolution events.

CLL ID	Treatment status at trial enrollment	Intercurrent therapy	Response type to intercurrent therapy	Clonal evolution (CE) or stability?
CLL002	T	R → FCR	PR	CE (+aCNA; +M)
CLL004	T	FR	PR	stable
CLL007	UT	FR	CR	CE (+aCNA, −aCNA; +M)
CLL008	UT	FR	CR	CE (+aCNA; +M)
CLL010	UT	FR	PR	CE (+aCNA; +M)
CLL011	UT	FR	CR	CE (+aCNA; +M)
CLL013	T	R-CVP	PR	stable
CLL017	T	FR → FCR	SD/PR	CE (+M)
CLL021	UT	R-CVP → BEXXAR	CR/CR	CE (+M)
CLL024	UT	FR	CR	CE (+aCNA)
CLL027	UT	FR	PR	CE (+M)
CLL029	UT	FR	CR	CE (+M)
CLL035	UT	FR	PR	stable
CLL036	UT	FR	PR	CE (+cnLOH)
CLL058	UT	FR	CR	CE (+M)
CLL060	T	PCR	PR	stable
CLL064	T	PCR	PR	stable
CLL066	UT	R → PCR	PR	stable
CLL072	UT	FR	PD	CE (+M)
CLL074	UT	FR	CR	CE (+aCNA; +M)
CLL078	UT	FR	CR	stable
CLL085	T	FCR	CR	CE (+M)
CLL090	T	BR	CR	CE (+aCNA; +M)
CLL094	T	A	PR	stable
CLL104	UT	F → A	PR/CR	CE (+aCNA, −aCNA; +M)
CLL107	UT	PCR	CR	CE (+M)
CLL109	UT	FR	PR	stable
CLL112	UT	FR	CR	stable
CLL116	UT	FR	CR	CE (+cnLOH; +M)
CLL117	UT	FCR → BR	CR/CR	CE (+aCNA, +M, +cnLOH, −M)
CLL120	UT	FR	PR	stable
CLL121	UT	FCR	PR	CE (+aCNA; +M)
CLL125	UT	F → B	SD/SD	CE (+M)
CLL135	UT	FR	PR	stable
CLL155	UT	FCR	CR	stable

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CLL ID	Treatment status at trial enrollment	Intercurrent therapy	Response type to intercurrent therapy	Clonal evolution (CE) or stability?
CLL170	T	PCR → flavopiridol → RICE	PD/PD/PD	stable
CLL176	UT	FCR	CR	stable
CLL185	UT	FR	CR	CE (+aCNA, −aCNA; +M)
CLL186	UT	FR, BR	PR,PR	CE (+M)
CLL189	UT	FR	PR	CE (+M)
CLL195	UT	FCR	PR	CE (+M)
CLL205	UT	FCR	PR	CE (+M)
CLL209	T	PCR	PR	CE (+aCNA; +M)
CLL211	UT	FCR	CR	CE (−M)
CLL212	T	BR	PR	stable
CLL218	UT	FR	PR	CE (+M)
CLL219	UT	R-CVP → B	CR/CR	CE (+M)
CLL230	UT	BR → BR	PR/PR	stable
CLL242	UT	F → FC → BR	PD/PR	stable
CLL270	UT	R-CHOP	PD	CE (+M)
CLL271	UT	B	PR	stable
CLL279	UT	FR	CR	stable
CLL296	UT	BR	CR	CE (+M)
CLL018	T	NONE		stable
CLL045	T	NONE		CE (+M)
CLL077	T	NONE		stable
CLL123	T	NONE		stable
CLL164	T	NONE		stable
CLL165	T	NONE		stable
CLL188	T	NONE		stable
CLL220	T	NONE		stable

Please see Supplementary Table 1 for details.

F: fludarabine; C: cyclophosphamide; R: rituximab; A: alemtuzumab; O: ofatumumab; V: vincristine; P: prednisone; P: pentostatin; M: methylprednisolone; I: ifosphamide; E: etoposide. CR: complete remission; PR: partial remission; SD: stable disease; PD: progressive disease. CE: clonal evolution; aCNA: acquired genomic copy number aberration; (+M): acquisition of a gene mutations; cnLOH: copy-neutral LOH. UT: untreated at sample procurement; T: previously treated prior to sample procurement.