Clinical impact of the subclonal architecture
and mutational complexity in chronic lymphocytic leukemia

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Next generation sequencing approach

Deep-targeted next-generation sequencing (NGS) of POT1 (exons 5-19), NFKBIE (exons 1-2), ZNF292 (exons 6, 8), XPO1 (exon 15), EGR2 (exons 1-2), FBXW7 (exons 2-12), MGA (exons 2-24), KLHL6 (exon 1), RPS15 (exons 3-4), MYD88 (exons 2-5), DDX3X (exons 1-17), BRAF (exons 12, 15), NFX1 (exons 2-21), DTX1 (exon 1), BCor (exons 4, 8), CCND2 (exon 5), KRAS (exons 2-3), IRF4 (exon 3), MED12 (exon 2), ZMYM3 (exons 15, 21, 23), NRAS (exons 2-3), TRAF3 (exon 11), and PIM1 (exons 2-4) was performed (Supplementary Table S2). NGS libraries were performed using the Access Array system (Fluidigm, South San Francisco, CA, USA) and/or the Nextera XT DNA library preparation kit (Illumina, San Diego, CA, USA). Specific primers for the target regions amplified in the Access Array libraries were designed with the D3 Assay Design tool (https://www.fluidigm.com/assays) (Supplementary Table S3). Once the libraries were generated using the Access Array system and pooled, they were sequenced with a paired-end run of 210 bp in a MiSeq equipment (Illumina). On the other hand, the Primer3^1,2 program was used to design the specific primers for the amplification of the target regions included in the Nextera XT libraries (Supplementary Table S4). Long polymerase chain reaction (PCR) amplifications were performed using the KAPA HiFi DNA Polymerase HotStart ReadyMix (Kapa Biosystems, Wilmington, MA, USA) and normalized with the SeqalPrep Normalization Plate kit (Invitrogen, Waltham, MA, USA).^3 Libraries were afterwards generated with the Nextera XT DNA Library Preparation kit and sequenced in a MiSeq (run of 2x150 bp).

Bioinformatic analysis

The complete bioinformatic analysis was performed as previously described.4 Briefly, quality control and trimming of the raw data was done using the FastQC (version 0.11.2), Flexbar (version 2.5),5 and Trimmomatic (version 0.32).6 Sequencing reads were subsequently aligned to the human reference genome (GRCh37/hg19) using the Burrows-Wheeler Aligner–MEM algorithm (version 0.7.10).7 The indel realignment and base quality score recalibration steps were applied as described in the Genome Analysis Toolkit (GATK) Best Practice recommendations using the RealignerTargetCreator, IndelRealigner, BaseRecalibrator, PrintReads, and AnalyzeCovariates from the GATK (version 3.3-0).8–10 Variant calling was performed independently using three different algorithms: VarScan2 (version 2.3.7),11 UnifiedGenotyper (GATK version 3.3-0), and HaplotypeCaller (GATK version 3.3-0). The post processing filter variant-filter (fpffilter.pl) was used to filter the point mutations detected by
VarScan2 while the VariantFiltration tool from GATK was used to hard-filter the variants identified by both UnifiedGenotyper and HaplotypeCaller algorithms. Additionally, the entire pipeline established on the MiSeq Reporter Software (MSR, version 2.4.60) was run independently. In this case, upon the supplied aligned files, the Somatic Variant Caller was used for variant detection. All variants detected for any of the variant callers applied were combined and annotated using ANNOVAR (version 2016Feb01)\textsuperscript{12} as well as custom scripts before filtering. All programs were executed following the authors’ recommendations.

**Confirmation of the somatic origin of the mutations**

Synonymous variants and polymorphisms described either in the Single Nucleotide Polymorphism Database (dbSNP142) with a European population frequency higher than 1% (1000 Genomes Project database) or in our CLL-genome project database\textsuperscript{13} were automatically removed by the bioinformatic pipeline. All variants that passed the previous filter were considered somatic if they were truncating, affected splicing sites, or were identified as previous somatic mutations in COSMIC (http://cancer.sanger.ac.uk/cosmic, v72) or in our own CLL database.\textsuperscript{13} Next, 219 variants not fulfilling the previous criteria were investigated in the germ line DNA of the patients by NGS, Sanger sequencing (Supplementary Table S5) or allele-specific (AS)-PCR (Supplementary Table S6). Among them, 145 variants were not detected in the normal DNA and therefore considered somatic. Among the 74 variants classified as germ line, and therefore not considered in this study, 27 were analyzed by NGS, 33 by Sanger sequencing, and 14 by AS-PCR. All germ line variants identified by NGS where present at >35% of VAF in the normal DNA, indicating that they were *bona fide* individual polymorphisms. Overall, only somatic/truncating mutations were finally considered in the analyses.

**Variant allele frequency variability due to the NGS approach**

The estimated variant allele frequency (VAF) from a NGS study may have some intrinsic variability due to the library preparation and sequencing. Analyzing the VAF detected for the same mutation in two independent NGS rounds we could estimate the expected variance on VAF due to the NGS approach. In this line, 643 variants (179 somatic mutations and 464 variants annotated as polymorphisms) detected in two independent runs of NGS were used to estimate this variability (Supplementary Figure S3). Due to the fact that there was heteroscedasticity related to the VAF value (lower variance in extreme values of VAF), we used the msir R package (version 1.3.1) to fit a Loess smooth curve and estimate the deviation of the VAF of a second
round of NGS in respect to the first one, represented by the 90% predictive interval (Supplementary Figure S3). The obtained predictive intervals at each specific VAF were converted to a cancer cell fraction (CCF) variability range using the formula used to estimate the CCF of the mutations. In this regard, two mutations where considered to have similar CCFs when their intervals overlapped.

**Verification of low variant allele frequency mutations**

Due to the existent baseline noise when performing a next-generation sequencing experiment, all mutations identified at low VAF (VAF <12%) were verified by a second independent NGS experiment and/or confirmed by AS-PCR (Supplementary Table S6), as described. In this line, 135 mutations were verified by AS-PCR, 94 by NGS, and 22 by both approaches.

**Sanger sequencing**

Amplification of the fragment of interest by PCR was performed using 1.25U AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA, Estados Unidos), PCR buffers (Applied Biosystems), 200µM dNTP mix (Invitrogen), 100nM forward and reverse primers (Supplementary Table S5), 1.5mM MgCl2, and 50ng of DNA in a final reaction volume of 50µl. PCR products were cleaned using ExoSAP-IT (USB) and sequenced using ABI Prism BigDye terminator (version 3.1) (Applied Biosystems) with 5pmol of each primer. Sequencing reactions were run on an ABI-3730 automated sequencer (Applied Biosystems). All sequences were visually examined with the Mutation Surveyor® software (v3.24, SoftGenetics, State College, PA, USA).

**Sequence analysis of IGHV-IGHD-IGHJ rearrangements**

The sequence analysis of IGHV-IGHD-IGHJ rearrangements were performed on either genomic DNA or complementary DNA using leader or consensus primers for the IGHV FR1 along with appropriate consensus constant primers, as previously reported. Only productive rearrangements were evaluated. Sequences with ≥98% identity to the germline were considered unmutated.
DNA copy number analysis

Copy number alterations (CNA) were investigated by high density SNP arrays (Genome-wide Human SNP Array 6.0, Affymetrix) and evaluated using Nexus Copy Number Discovery edition software (version 7.5, Biodiscovery, Hawthorne, CA, USA) in 376 cases, as described elsewhere. In order to quantify the CCF carrying each CNA, a dilutional experiment mixing DNA from MEC-1 CLL cell line (Supplementary Figure S1A) with normal DNA (Human Genome DNA: Male. Reference: G147A. Promega, Madison, WI, USA) was performed analyzing samples containing 100%, 90%, 80%, 60%, 40%, and 20% of MEC-1 DNA (Supplementary Figure S1B). Twenty copy number (CN) losses and 12 CN gains clonally represented in MEC-1 cell line were used to correlate the probe median score of each CNA to its known tumor cell content (or CCF) (Supplementary Figure S1C-D). After modeling a Loess fitted curve for the losses and a second model for the gains we were able to predict the CCF of the CNA (losses and gains) identified in our tumor samples. A 90% predictive interval was calculated in order to obtain a variability range of CCF due to the SNP array technology (Supplementary Figure S1C-D). The predicted CCFs were finally corrected by the tumor purity of the respective samples.

The CCF of the CNA were also used to calculate the CCF carrying each somatic mutation in order to correct for the ploidy of the locus when a given somatic mutation and a CNA affecting the same region co-occur in the same patient. Mutations and CNA were then classified as clonal or subclonal if their CCF was ≥85% or <85%, respectively. Subclonal mutations were further subclassified in subclonal with high CCF (subclonal-high) or subclonal with low CCF (subclonal-low), being the cut-off ≥25% of CCF. The quantification of the CCF carrying each specific mutation allowed us to quantitatively analyze the effect of the mutations on clinical outcome being not limited to study only the effect of their presence or absence (see next section for further details).

Algorithm for the identification of specific gene CCF patterns with impact on clinical outcome

To determine the clinical impact of the specific gene CCF we selected genes mutated in at least 10 cases and designed an algorithm based on the CCF of each mutated gene that combined maximally selected rank statistics (maxstat R package), Fine-Gray/Cox regression, and Gray’s/log-rank test (Supplementary Figure S11). We first used maxstat to determine the CCF cut-off that offered the best prediction of outcome for each gene. If the cut-off obtained was <15% of CCF, we performed a univariate analysis with the mutated cases considering the CCF of the mutations as a continuous variable using Fine-Gray/Cox regression. If the P-value of this
continuous analysis was < 0.1, we considered that the effect of the mutation was continuous, i.e. the more cells carrying the alteration, the worse the prognosis of the patient. These genes were considered to have a prognostic impact with a CCF-gradual pattern when the Gray’s/log-rank test between mutated and unmutated subgroups was significant ($P < 0.05$). Conversely, if $P > 0.1$ (i.e. not a continuous effect) in the univariate continuous analysis, we also performed a univariate categorical analysis (all mutated cases vs unmutated) to check if gene mutations at any CCF impacted the outcome of the patients. If in this analysis the mutated gene had a prognostic impact (Gray’s/log-rank test $P < 0.05$), the gene was classified as having a CCF-independent pattern as all mutations, even the ones at very low CCF, had similar prognostic value. If the mutated gene did not have prognostic impact ($P > 0.05$), the gene was considered as not having a prognostic impact at any CCF.

On the other hand, when the maxstat analysis indicated a CCF cut-off higher than 15% and the univariate categorical analysis based on this cut-off (cases with mutations at CCF ≥ cut-off vs unmutated/mutated at CCF < cut-off) had a significant impact on outcome (Gray’s/log-rank test $P < 0.05$) the gene was classified as having a CCF-dominant pattern, i.e. only cases carrying the mutation at a higher frequency than the cut-off had a poor outcome. Finally, the resulting classification was represented and visually inspected by survival curves (Figure 4, Supplementary Figure S12). A summary of the output of the algorithm for each gene is shown in Supplementary Table S16.
### SUPPLEMENTARY TABLES

**Table S1.** Molecular and clinical characteristics of the 48 CLL cases included in the longitudinal study.

*Supplementary file: Supplementary Table S1. Molecular and clinical characteristics of the 48 CLL cases included in the longitudinal study (XLSX, 19kB)*

**Table S2.** Target regions, transcripts used, library strategy, and coverage report.

| Target gene | RefSeq transcript | Target regiona | Chr | Start position (hg19) | End position (hg19) | Library strategy | Mean coverage | % samples covered >100x in >75% region | % samples covered >1000x in covered |
|-------------|------------------|----------------|-----|----------------------|---------------------|------------------|--------------|----------------------------------------|-------------------------------------|
| **BCOR**    | NM_001123385     | Exon 8         | chrX| 39922858             | 39923106            | Access Array     | 1128         | 73.9                                   | 46.1                                |
| **BCOR**    | NM_001123385     | Exon 4         | chrX| 39931599             | 39934436            | Nextera XT/ Access Array | 1904/1313   | 83/41.6                                | 61.6/3.4                            |
| **BRAF**    | NM_004333        | Exon 15        | chr7| 140453072            | 140453196           | Access Array     | 2897         | 98.3                                   | 97.5                                |
| **BRAF**    | NM_004333        | Exon 12        | chr7| 140477788            | 140477878           | Access Array     | 3851         | 98                                     | 95.6                                |
| **CCND2**   | NM_001759        | Exon 5         | chr12| 4409023              | 4409178             | Access Array     | 4092         | 97.3                                   | 95.6                                |
| **DDX3X**   | NM_001193416     | Exon 1         | chrX| 41193503             | 41193553            | Access Array     | 2121         | 58.1                                   | 53.9                                |
| **DDX3X**   | NM_001193416     | Exon 2         | chrX| 41196658             | 41196721            | Access Array     | 3002         | 96.6                                   | 95.3                                |
| **DDX3X**   | NM_001193416     | Exon 3         | chrX| 41198286             | 41198339            | Access Array     | 156          | 46.6                                   | 0                                   |
| **DDX3X**   | NM_001193416     | Exon 4         | chrX| 41200734             | 41200872            | Access Array     | 3093         | 97.3                                   | 94.3                                |
| **DDX3X**   | NM_001193416     | Exon 5         | chrX| 41201745             | 41201909            | Access Array     | 5567         | 97.5                                   | 96.1                                |
| **DDX3X**   | NM_001193416     | Exon 6         | chrX| 41201987             | 41202092            | Access Array     | 3199         | 98.3                                   | 95.6                                |
| **DDX3X**   | NM_001193416     | Exon 7         | chrX| 41202466             | 41202607            | Access Array     | 3245         | 96.6                                   | 94.8                                |
| **DDX3X**   | NM_001193416     | Exon 8         | chrX| 41202987             | 41203078            | Access Array     | 4899         | 98.5                                   | 98.3                                |
| **DDX3X**   | NM_001193416     | Exon 9         | chrX| 41203280             | 41203384            | Access Array     | 2779         | 98.3                                   | 97.3                                |
| **DDX3X**   | NM_001193416     | Exon 10        | chrX| 41203489             | 41203655            | Access Array     | 3307         | 98                                     | 92.9                                |
| **DDX3X**   | NM_001193416     | Exon 11        | chrX| 41204430             | 41204580            | Access Array     | 3746         | 98.3                                   | 95.1                                |
| **DDX3X**   | NM_001193416     | Exon 12        | chrX| 41204654             | 41204804            | Access Array     | 3054         | 96.3                                   | 85.9                                |
| **DDX3X**   | NM_001193416     | Exon 13        | chrX| 41205479             | 41205666            | Access Array     | 4470         | 98.5                                   | 92.1                                |
| **DDX3X**   | NM_001193416     | Exon 14        | chrX| 41205755             | 41205878            | Access Array     | 5522         | 98.5                                   | 98.3                                |
| **DDX3X**   | NM_001193416     | Exon 15        | chrX| 41206109             | 41206268            | Access Array     | 4596         | 98                                     | 98                                  |
| **DDX3X**   | NM_001193416     | Exon 16        | chrX| 41206562             | 41206707            | Access Array     | 3120         | 59.9                                   | 51.7                                |
| **DDX3X**   | NM_001193416     | Exon 17        | chrX| 41206890             | 41206975            | Access Array     | 4517         | 98.5                                   | 98.3                                |
| **DTX1**    | NM_0004416       | Exon 1         | chr12| 11349599             | 11349625            | Nextera XT/ Access Array | 1862/516    | 95.3/17.7                               | 55.4/9.4                             |
| **EGR2**    | NM_000399        | Exon 2         | chr10| 64572964             | 64574231            | Nextera XT/ Access Array | 3056/2083   | 94.6/56.9                               | 88.4/48.3                            |
| **EGR2**    | NM_000399        | Exon 1         | chr10| 64575618             | 64575792            | Nextera XT/ Access Array | 2324/2372   | 94.6/88.3                               | 82.3/78.3                            |
| **FBXW7**   | NM_033632        | Exon 12        | chr4| 153244030            | 153244304           | Access Array     | 2479         | 94.3                                   | 88.4                                |
| **FBXW7**   | NM_033632        | Exon 11        | chr4| 153245333            | 153245549           | Access Array     | 3998         | 97.3                                   | 95.6                                |
| **FBXW7**   | NM_033632        | Exon 10        | chr4| 153247155            | 153247386           | Access Array     | 1969         | 85.5                                   | 56.7                                |
| **FBXW7**   | NM_033632        | Exon 9         | chr4| 153249357            | 153249544           | Access Array     | 2527         | 95.3                                   | 70                                   |
| **FBXW7**   | NM_033632        | Exon 8         | chr4| 153250821            | 153250940           | Access Array     | 2101         | 87.7                                   | 67                                   |
A page from a document with various gene names and exon numbers listed, along with genomic coordinates and array access information.
| Gene | Access Array | Exon | Chr | Start | End | Access Array | Mean Coverage | 69% Coverage |
|------|--------------|------|-----|-------|------|---------------|---------------|--------------|
| NFX1 | NM_006362    | Exon 15 | chr11 | 62563756 | 62563820 | Access Array | 1565 | 70.7 | 65.5 |
| NFX1 | NM_006362    | Exon 14 | chr11 | 62563929 | 62564042 | Access Array | 1998 | 96.3 | 91.9 |
| NFX1 | NM_006362    | Exon 13 | chr11 | 62564652 | 62564713 | Access Array | 1849 | 93.1 | 85.2 |
| NFX1 | NM_006362    | Exon 12 | chr11 | 62564787 | 62564861 | Access Array | 1515 | 89.4 | 68  |
| NFX1 | NM_006362    | Exon 11 | chr11 | 62566008 | 62566050 | Access Array | 1969 | 93.6 | 88.4 |
| NFX1 | NM_006362    | Exon 9  | chr11 | 62568563 | 62568676 | Access Array | 2868 | 97.3 | 95.1 |
| NFX1 | NM_006362    | Exon 8  | chr11 | 62568798 | 62568892 | Access Array | 4312 | 97.8 | 95.8 |
| NFX1 | NM_006362    | Exon 7  | chr11 | 62569031 | 62569106 | Access Array | 5241 | 96.8 | 95.8 |
| NFX1 | NM_006362    | Exon 6  | chr11 | 62569208 | 62569294 | Access Array | 707  | 96.1 | 17.5 |
| NFX1 | NM_006362    | Exon 5  | chr11 | 62569411 | 62569521 | Access Array | 3119 | 97.8 | 95.3 |
| NFX1 | NM_006362    | Exon 4  | chr11 | 62569646 | 62569735 | Access Array | 2118 | 96.3 | 86.7 |
| NFX1 | NM_006362    | Exon 3  | chr11 | 62570888 | 62571047 | Access Array | 4617 | 98.5 | 98.3 |
| NFX1 | NM_006362    | Exon 2  | chr11 | 62571261 | 62571453 | Access Array | 2089 | 96.1 | 59.1 |
| PIM1 | NM_001243186 | Exon 2  | chr6  | 37138546 | 37138658 | Nextera XT/Access Array | 403/6 | 83.3/0.2 | 6.4/0.2 |
| PIM1 | NM_001243186 | Exon 3  | chr6  | 37138754 | 37138810 | Nextera XT/Access Array | 590/803 | 89.2/36.7 | 14.5/33 |
| PIM1 | NM_001243186 | Exon 4  | chr6  | 37138898 | 37139270 | Nextera XT/Access Array | 1377/2202 | 94.3/56.4 | 39.4/53.4 |
| POT1 | NM_015450    | Exon 19 | chr7  | 124464013 | 124464131 | Access Array | 4022 | 98.3 | 95.1 |
| POT1 | NM_015450    | Exon 18 | chr7  | 124465303 | 124465414 | Access Array | 2942 | 97.8 | 94.6 |
| POT1 | NM_015450    | Exon 17 | chr7  | 124467265 | 124467362 | Access Array | 4006 | 98.3 | 97.8 |
| POT1 | NM_015450    | Exon 16 | chr7  | 124469305 | 124469399 | Access Array | 3102 | 97.8 | 93.3 |
| POT1 | NM_015450    | Exon 15 | chr7  | 124475330 | 124475471 | Access Array | 4184 | 97.8 | 93.8 |
| POT1 | NM_015450    | Exon 14 | chr7  | 124481024 | 124481235 | Access Array | 4555 | 98.5 | 97.8 |
| POT1 | NM_015450    | Exon 13 | chr7  | 124482858 | 124483020 | Access Array | 4511 | 98.5 | 98.3 |
| POT1 | NM_015450    | Exon 12 | chr7  | 124486993 | 124487055 | Access Array | 2534 | 98.5 | 93.8 |
| POT1 | NM_015450    | Exon 11 | chr7  | 124491923 | 124492008 | Access Array | 2432 | 96.3 | 93.3 |
| POT1 | NM_015450    | Exon 10 | chr7  | 124493023 | 124493195 | Access Array | 3942 | 98.3 | 97.3 |
| POT1 | NM_015450    | Exon 9  | chr7  | 124499008 | 124499169 | Access Array | 3531 | 98 | 95.1 |
| POT1 | NM_015450    | Exon 8  | chr7  | 124503401 | 124503697 | Access Array | 5562 | 96.8 | 95.1 |
| POT1 | NM_015450    | Exon 7  | chr7  | 124510962 | 124511098 | Access Array | 2804 | 96.1 | 82  |
| POT1 | NM_015450    | Exon 6  | chr7  | 124532317 | 124532437 | Access Array | 1532 | 96.1 | 78.3 |
| POT1 | NM_015450    | Exon 5  | chr7  | 124537216 | 124537230 | Access Array | 2792 | 98.5 | 97.3 |
| RS15 | NM_001018    | Exon 3  | chr19 | 1440015  | 1440255  | Access Array | 2247 | 70  | 57.1 |
| RS15 | NM_001018    | Exon 4  | chr19 | 1440345  | 1440464  | Access Array | 1864 | 76.4 | 71.7 |
| TRAF3| NM_003300    | Exon 11 | chr14 | 103371547 | 103372124 | Access Array | 2717 | 85.7 | 70.4 |
| XPO1 | NM_003400    | Exon 15 | chr2  | 61719457 | 61719619 | Access Array | 5546 | 98.5 | 98.3 |
| ZMYM3| NM_005096    | Exon 23 | chrX  | 70462017 | 70462277 | Access Array | 1797 | 27.1 | 1.5  |
| ZMYM3| NM_005096    | Exon 21 | chrX  | 70463676 | 70463833 | Access Array | 5311 | 97.8 | 95.8 |
| ZMYM3| NM_005096    | Exon 15 | chrX  | 70466200 | 70466365 | Access Array | 1545 | 0  | 0  |
| ZNF292| NM_015021   | Exon 6  | chr6  | 87953190 | 87953332 | Access Array | 2825 | 96.8 | 94.6 |
| ZNF292| NM_015021   | Exon 8  | chr6  | 87964365 | 87971522 | Access Array | 3149 | 95.1 | 82.3 |

*Target regions also include the 2-bp splice sites at 5' and 3' of each exon.

aNFKBIE hotspot mutation (chr6:44232739_44232742delGTAA, p.Y254fs*13) was covered with a mean coverage of 295x, with 69% of the samples having this region covered at >100x by Nextera XT. Using the Access Array system (Fluidigm), this region was covered at a mean coverage of 1846x, with 69% of the samples having this region covered at >1000x. Combining
both techniques, but considering only the technique that better covers each sample, the mean coverage was 1923x and 91% and 70% of the samples had a coverage >100 and 1000x, respectively. Region considered for these calculations: chr6:44232734-44232743.

56% of the NRAS exon 3 region (chr1:115256418-115256520) was covered with a mean coverage of 415x, and 90% of the samples had this region covered at >100x.

58% of the ZMYM3 exon 23 region (chrX:70462126-70462277) was covered with a mean coverage of 3009x, and 98% of the samples had this region covered at >1000x.

61% of the ZMYM3 exon 15 region (chrX:70466200-70466300) was covered with a mean coverage of 2419x, and 89% of the samples had this region covered at >100x.

Table S3. Access Array (Fluidigm) primers for deep-targeted NGS.

Supplementary file: Supplementary Table S3. Access Array (Fluidigm) primers for deep-targeted NGS (XLSX, 40kB)

Table S4. Specific primers for Nextera XT library preparation for deep-targeted NGS.

| Target gene | Target region | Chr | Start position (hg19) | End position (hg19) | Forward primer (5' → 3') | Reverse primer (5' → 3') | Amplicon length (bp) |
|-------------|---------------|-----|-----------------------|---------------------|--------------------------|--------------------------|--------------------|
| BCOR        | Exon 4        | chrX | 39931193              | 39934693            | CATGAATCCCCAGACATGC      | TATGGGCTCAAGTCCAAAG     | 3501               |
| DTX1        | Exon 1        | chr12 | 113495836             | 113496416           | TAACGGAGCTCTAGGCTC       | GGACAAGGTTAGACTG6CCT    | 575                |
| EGR2        | Exon 2-3      | chr10 | 64572337              | 64576487            | TCTCCCCATCACATTGTCA      | GAAAGTCCCCAGAGAAGGGA   | 4151               |
| NFKBIE      | Exon 1        | chr6  | 44232605              | 44234489            | AGACCGAATGGGGACCTGAGA    | TAGTGCCAGGAAATGAGGGA   | 1885               |
| PIM1        | Exon 2-4      | chr6  | 37138343              | 37139441            | GAGGTGGGGATGCTTTGTCC     | AAATCCCCGGCTTTACTACG   | 1099               |
Table S5. Primers used for Sanger sequencing.

| Target gene | Target region | Chr  | Start position (hg19) | End position (hg19) | Forward primer (5' → 3') | Reverse primer (5' → 3') | Amplicon length (bp) |
|-------------|---------------|------|-----------------------|---------------------|--------------------------|--------------------------|----------------------|
| BCOR        | Exon 4        | chrX | 39932996              | 39933364            | CCCAGTCCAATGCCTTGGTTT    | AGACAGCGGTCCAAGACAGA    | 369                  |
| BCOR        | Exon 4        | chrX | 39933787              | 39934176            | GAAGGTTCGCCAAGACAGA      | CCCAATGCTCAGATGCTTCT    | 390                  |
| BCOR        | Exon 8        | chrX | 39922658              | 39923441            | TGGAGGCTCAGAAGGTAATTC    | ACTCCTCCATCCACCACTC     | 784                  |
| BRAF        | Exon 12       | chr7 | 140477816             | 140478120           | AGGCCTTGAATGCTGAGG       | GGAACAAAGAGGTGGCTT      | 305                  |
| DDX3X       | Exons 9-10    | chrX | 41200631              | 41201147            | CCCGGATCTTGGGCTTGGG      | GGAACAAAGAGGAGGAGG      | 720                  |
| DTX1        | Exon 1        | chr12| 113495836             | 113496410           | TAACGGAGGTCTCTAGGGAGG    | GGACAAGGTTTAGACTG       | 575                  |
| EGR2        | Exon 1        | chr12| 46575472              | 46576021            | CTGGGGCTCGCTCGGAGG       | GAAAACATGAGGAGGAGG      | 550                  |
| EGR2        | Exon 2        | chr12| 46573406              | 46573920            | GCCTTCCCCCTCCTC          | CTGGGGCTCGCTCGGAGG      | 517                  |
| KLHL6       | Exon 1        | chr12| 183273077             | 183273531           | TGGGGCTCGCTCGGAGG       | GAAAACATGAGGAGGAGG      | 713                  |
| MGA         | Exon 8        | chr12| 41203116              | 41203835            | GCCTTCCCCCTCCTC          | CTGGGGCTCGCTCGGAGG      | 474                  |
| MGA         | Exon 17       | chr12| 42042271              | 42042681            | GCCTTCCCCCTCCTC          | CTGGGGCTCGCTCGGAGG      | 411                  |
| MYD88       | Exon 2        | chr12| 38181251              | 38181784            | GCCTTCCCCCTCCTC          | CTGGGGCTCGCTCGGAGG      | 534                  |
| NXF1        | Exon 19       | chr12| 62561652              | 62562126            | GCCTTCCCCCTCCTC          | CTGGGGCTCGCTCGGAGG      | 475                  |
| NXF1        | Exon 2        | chr12| 62571036              | 62571452            | GCCTTCCCCCTCCTC          | CTGGGGCTCGCTCGGAGG      | 475                  |
| POT1        | Exon 11       | chr12| 62568893              | 62569414            | GCCTTCCCCCTCCTC          | CTGGGGCTCGCTCGGAGG      | 522                  |
| POT1        | Exon 11       | chr12| 124491714             | 124492168           | ACTCCTCCCAATGAGGAGG      | GCCTTCCCCCTCCTC          | 455                  |
| POT1        | Exon 6        | chr12| 124532243             | 124532585           | ACTCCTCCCAATGAGGAGG      | GCCTTCCCCCTCCTC          | 455                  |
| POT1        | Exon 7        | chr12| 124510843             | 124511258           | ACTCCTCCCAATGAGGAGG      | GCCTTCCCCCTCCTC          | 455                  |
| POT1        | Exon 8        | chr12| 124503233             | 124503763           | ACTCCTCCCAATGAGGAGG      | GCCTTCCCCCTCCTC          | 531                  |
| POT1        | Exon 9        | chr12| 124498661             | 124499154           | ACTCCTCCCAATGAGGAGG      | GCCTTCCCCCTCCTC          | 494                  |
| TRAF3       | Exon 11       | chr12| 103371498             | 103372126           | ACTCCTCCCAATGAGGAGG      | GCCTTCCCCCTCCTC          | 434                  |
| ZMYM3       | Exon 23       | chrX | 70461863              | 70462201            | GCCTTCCCCCTCCTC          | CTGGGGCTCGCTCGGAGG      | 416                  |
| ZNF292      | Exon 2        | chr7 | 87965855              | 87966308            | GCCTTCCCCCTCCTC          | CTGGGGCTCGCTCGGAGG      | 454                  |
| ZNF292      | Exon 8        | chr7 | 87967480              | 87968198            | GCCTTCCCCCTCCTC          | CTGGGGCTCGCTCGGAGG      | 719                  |
| ZNF292      | Exon 8        | chr7 | 87968378              | 87968922            | GCCTTCCCCCTCCTC          | CTGGGGCTCGCTCGGAGG      | 545                  |
| ZNF292      | Exon 8        | chr7 | 87970836              | 87971163            | GCCTTCCCCCTCCTC          | CTGGGGCTCGCTCGGAGG      | 328                  |
Table S6. AS-PCR primers used to verify the mutations called by the bioinformatic pipeline.

| Target gene | Chr  | Genomic position (hg19) | Ref | Alt  | Amino acid | Forward primer WT (5’ → 3’) | Forward primer Mut (5’ → 3’) | Reverse primer WT (5’ → 3’) | Reverse primer Mut (5’ → 3’) |
|-------------|------|------------------------|-----|------|------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| BCor        | chrX | 39932628               | G   | T    | p.Y657*   | TCAGGGGCTGGGTAAGGAGGAGG     | TCAGGGGCTGGGTAAGGAGGAGG     | CAGCAACCCAAGACAGGAGTG      | -                           |
| BCor        | chrX | 39932621               | AAGGGAGGT A | p.Y657fs*5 | GGCTGCTTAAGGAGGAGGTA   | GGCTGCTTAAGGAGGAGGTA        | GCAACCCAAGACAGGAGTCT       | -                           |
| BCor        | chrX | 39932231               | T    | P.N790fs*27 | TCCCTTGATCCAGTGGGGG    | TCCCTTGATCCAGTGGGGG         | ATGAGAGAGCGGCTAGGAG        | -                           |
| CDN2        | chr12| 4409027                | A    | C    | p.D241A   | GTCTGGACCACCTGCTAGG         | GTCTGGACCACCTGCTAGG         | ATATCCCGACACTGCCAG         | -                           |
| CDN2        | chr12| 44090097               | C    | T    | p.Q265*   | CAGCACTACCCGCTAGGACC       | CAGCACTACCCGCTAGGACC       | AGGTGGCTTCCATCCCCAAA       | -                           |
| CDN2        | chr12| 4409107                | G    | C    | p.G268R   | TGTGGGAGTGGCTGAAGTGAGG     | TGTGGGAGTGGCTGAAGTGAGG     | GGACAAGGACGCAAGCAC       | -                           |
| DDX3X       | chrX | 41205649               | T    | G    | p.L495V   | GCTCAGGAAAAAGGAGAATT       | GCTCAGGAAAAAGGAGAATT       | TTTCCTGACACACATCCAGC      | -                           |
| DDX3X       | chrX | 41205532               | T    | C    | p.S456fs  | GACCAAAAAGGAGTAGATT       | GACCAAAAAGGAGTAGATT       | TTTCCTGACACACATCCAGC      | -                           |
| DDX3X       | chrX | 41203031               | C    | T    | p.N26fs   | CTTCTGGCCCATCTTGAAGT       | CTTCTGGCCCATCTTGAAGT       | TTTCCTGACACACATCCAGC      | -                           |
| DDX3X       | chrX | 41196690               | T    | A    | p.T280N   | ACTGGCAACAGCCACGAC         | ACTGGCAACAGCCACGAC         | AGGTGGCTTCCATCCCCAAA       | -                           |
| DDX3X       | chrX | 41203018               | C    | T    | p.Q241    | TCAGGGGCTGGGTAAGGAGGAGG    | TCAGGGGCTGGGTAAGGAGGAGG    | CAGCAACCCAAGACAGGAGTG      | -                           |
| DDX3X       | chrX | 41196690               | T    | C    | p.N26fs   | CTTCTGGCCCATCTTGAAGT       | CTTCTGGCCCATCTTGAAGT       | TTTCCTGACACACATCCAGC      | -                           |
| DDX3X       | chrX | 41205652               | G    | A    | p.V496M   | GCTCAGGAAAAAGGAGAATT       | GCTCAGGAAAAAGGAGAATT       | TTTCCTGACACACATCCAGC      | -                           |
| DTX1        | chr12| 113496096              | G    | C    | p.E33D    | TTGGAGAGTGGCTGAAGTGAGG     | TTGGAGAGTGGCTGAAGTGAGG     | GGACAAGGACGCAAGCCCT       | -                           |
| EGR2        | chr10| 64573167               | T    | C    | p.D411H   | GGTTGGCCCTCCTCTCATC        | GGTTGGCCCTCCTCTCATC        | AAGTTGACCCACAGCCCT        | -                           |
| EGR2        | chr10| 64573332               | C    | T    | p.E356K   | GGATGTGCCGTGCTAGG          | GGATGTGCCGTGCTAGG          | ACCAGGCACTCCTCCTCCATG      | -                           |
| EGR2        | chr10| 64574168               | CTGGGATA | p.Y75fs | GGAGCAAAGTGCTGGGAGA    | GGAGCAAAGTGCTGGGAGA    | AAGGCGGAGAAGGATGGGA       | -                           |
| EGR2        | chr10| 64573060               | GC   | C    | p.E33D    | TTGGAGAGTGGCTGAAGTGAGG     | TTGGAGAGTGGCTGAAGTGAGG     | GGACAAGGACGCAAGCCCT       | -                           |
| EGR2        | chr10| 64573248               | T    | T    | p.H384N   | GGATATGGTGCTGCTAGG         | GGATATGGTGCTGCTAGG         | GGATATGGTGCTGCTAGG         | -                           |
| EGR2        | chr10| 64573326               | C    | T    | p.T358A   | GGATGGCCCTCCTCTCCATC       | GGATGGCCCTCCTCTCCATC       | ACCAGGCACTCCTCCTCCATG      | -                           |
| EGR2        | chr10| 64573679               | C    | T    | p.G240D   | GGCTGGGAGGAGGAGGAGGAGG    | GGCTGGGAGGAGGAGGAGGAGG    | ACCAGGCACTCCTCCTCCATG      | -                           |
| FBXW7       | chr4  | 153253760              | ATT  | A    | p.S114R   | AGGAACCTGGTGGAGGGAGGAGG    | AGGAACCTGGTGGAGGGAGGAGG    | TGACCCACATAACTCAGC        | -                           |
| IRF4        | chr6  | 394946                 | C    | A    | p.S114R   | AGGAACCTGGTGGAGGGAGGAGG    | AGGAACCTGGTGGAGGGAGGAGG    | TGACCCACATAACTCAGC        | -                           |
| Locus | Chromosome | Position | Genotype | Protein Change | Transcript 1 | Transcript 2 |
|-------|------------|----------|----------|---------------|-------------|-------------|
| IRF4  | chr6       | 394951   | T        | p.L116R       | CTCTCATTCTTTCCCACCGG | -           | TGTACGGGTTCTGAGATGTCACCA |
| IRF4  | chr6       | 394993   | AG       | p.G131fs*18   | TTGGGTTGAGAATGTTGCGC | -           | CCCCTACCTTTTTGGCTCTCTTT |
| KHL6  | chr3       | 183273248| A        | p.L65P        | TCAGAGGGTTTCTCATTCGGA | TCAGACGTTTCTCATTCGCG | GTTGATGCGAGGACAAAGGG |
| KHL6  | chr3       | 183273269| G        | p.L58R        | GGTGTTGCCGAGCATTGGAAGA | GGTGTTGCCGAGCATTGGAAGA | AGCTGTTAGAATGTTGAGG |
| KHL6  | chr3       | 183273255| C        | p.E63*        | GTTTCCTCCAGCAGGTTTGA | GTTTCCTCCAGCAGGTTTGA | GTTGATGCGAGGACAAAGGG |
| KRAS  | chr12      | 25398282 | A        | p.G131fs*18   | TTGGGTTGAGAATGTTGCGC | -           | CCCCTACCTTTTTGGCTCTCTTT |
| KLHL6 | chr3       | 25398284 | T        | p.G12D        | CACTCTTGGCTAGGAATGGGTTC | CACTCTTGGCTAGGAATGGGTTC | AAGGCACGATGAGGAGGAGT |
| KLHL6 | chr3       | 25398284 | C        | p.P2156fs*23  | GCAGCAATCTAATCTACAGCA | GCAGCAATCTAATCTACAGCA | TAACTGATGCTACTAGCAATAG |
| MGAT  | chr15      | 42005588 | A        | p.V1109fs*34  | GCACCTTGTTTCTCTAGG   | GCACCTTGTTTCTCTAGG   | ACATGCGGAACTCAGAAA |
| MGA   | chr15      | 42042270 | G        | p.P2156fs*23  | GCAGCAATCTAATCTACAGCA | GCAGCAATCTAATCTACAGCA | TAACTGATGCTACTAGCAATAG |
| MYD88 | chr3       | 38182641 | T        | p.L273P       | GTGCCCATCAGAAGCGACT  | GTGCCCATCAGAAGCGACT  | AGATACACGACACACACAGG |
| MYD88 | chr3       | 38182032 | C        | p.S219C       | ATCATCTTGGGAAGGGTGCA | -           | CTACAGATGAGCCCTCCCAG |
| NFKBIE| chr6       | 44232738 | T        | p.Y254fs*13   | GGCACTGGTTTCTCTAGG   | GGCACTGGTTTCTCTAGG   | AAGCGTGATGAGGAGGAGT |
| NXF1  | chr11      | 62569722 | C        | p.G127V       | ATGCCTTGTCATACTTTCTGC | ATGCCTTGTCATACTTTCTGC | AGATACACGACACACACAGG |
| NXF1  | chr11      | 62568570 | T        | p.N301S       | TCCGAGCTATCAATTCTCAGC | TCCGAGCTATCAATTCTCAGC | AGATACACGACACACACAGG |
| POT1  | chr7       | 124493170| T        | p.Y242C       | GATTGAAGTTTGGTATGAAGGCTAT | GATTGAAGTTTGGTATGAAGGCTAT | AGATACACGACACACACAGG |
| POT1  | chr7       | 124503553| C        | p.Y43C        | TCCACAATAGTTACAACTGAGCAAT | TCCACAATAGTTACAACTGAGCAAT | AGATACACGACACACACAGG |
| POT1  | chr7       | 124503666| C        | p.R80H        | ATACCTTGGCTAGTGAGG    | ATACCTTGGCTAGTGAGG    | AGATACACGACACACACAGG |
| POT1  | chr7       | 124510981| C        | p.N54fs*2     | TGGGTTAAGAAGGAGAGG    | TGGGTTAAGAAGGAGAGG    | AGATACACGACACACACAGG |
| POT1  | chr7       | 124511058| AT       | p.Y493P       | GGGGGAAGGGGAGAGG    | GGGGGAAGGGGAGAGG    | AGATACACGACACACACAGG |
| POT1  | chr7       | 124511092| T        | p.Q223P       | CTGAGGCTGCAAGGAGG    | CTGAGGCTGCAAGGAGG    | AGATACACGACACACACAGG |
| POT1  | chr7       | 124532325| T        | p.G145*       | CACAGTTTTTAAACTACAGGCTTC | CACAGTTTTTAAACTACAGGCTTC | TGCCTTTATGACTAGTGTG |
| POT1  | chr7       | 124532362| C        | p.V281        | GGGGCTTAAAAGAAGTCTACACAC | GGGGCTTAAAAGAAGTCTACACAC | TGCCTTTATGACTAGTGTG |
| POT1  | chr7       | 124532666| C        | p.G331D       | CAAAGGCAGAGGCTGTTGATA | CAAAGGCAGAGGCTGTTGATA | AGGAGACTACTACACTAGC |
| POT1  | chr7       | 124532347| G        | p.K33Q        | TTTGCTTGATAGTTGGGCTT | TTTGCTTGATAGTTGGGCTT | TGCCTTTATGACTAGTGTG |
| POT1  | chr7       | 124510985| C        | p.Y43C        | TCCACAATAGTTACAACTGAGCAAT | TCCACAATAGTTACAACTGAGCAAT | TGCCTTTATGACTAGTGTG |
| POT1  | chr7       | 124475360| A        | p.L493P       | GGGGAAAGGAGAGGAGGAGG | GGGGAAAGGAGAGGAGGAGG | TGCCTTTATGACTAGTGTG |
| RPS15 | chr19      | 1440456  | A        | p.K145*       | CCCGCTTCTACCTCTCCCTCA | CCCGCTTCTACCTCTCCCTCA | AGACTGCTAAACAGGCTTGG |
| XPO   | chr2       | 61719472 | C        | p.E571K       | TGGCATACCTCTCAAACCT | TGGCATACCTCTCAAACCT | AGACTGCTAAACAGGCTTGG |

**IRF4**, **KHL6**, **KLHL6**, **KRAS**, **MGA**, **MYD88**, **NXF1**, **POT1**, **XPO** are genes listed in the table. The table lists the chromosomes, positions, genotypes, protein changes, and transcripts for each gene.
| Gene   | Chromosome | Position | Mutation  | Mutant Allele 1 | Mutant Allele 2  | Normal Allele 1 | Normal Allele 2 |
|--------|------------|----------|-----------|----------------|-----------------|----------------|----------------|
| XPO1   | chr2       | 61719471 | T>C       | p.E571G       | TGGCATCACCTCTCAAACCT | ACTGTAGTAACAGCTGTCGA | ACTGTAGTAACAGCTGTCGG |
| XPO1   | chr2       | 61719471 | T>A       | p.E571V       | TGGCATCACCTCTCAAACCT | ACTGTAGTAACAGCTGTCGA | ACTGTAGTAACAGCTGTCGT |
| ZMYM3  | chrX       | 70466233 | C>T       | p.V848M       | CTTCCTTTGGACCTCTCTCAC  | CTTCCTTTGGACCTCTCCAT  | AAAACCCAGACACCCCTCC  |
| ZNF292 | chr6       | 87969505 | C>G       | p.I1376M      | CTCTTCCCTCACCAGCAGA | GCCCTGAACACCGCTCACAG | GCCCTGAACACCGCTCACAC |
| ZNF292 | chr6       | 87967475 | C>G       | p.I1376M      | CTCTTCCCTCACCAGCAGA | GCCCTGAACACCGCTCACAG | GCCCTGAACACCGCTCACAC |
**Table S7.** Mutations identified by deep-targeted NGS in 406 untreated CLL patients.

*Supplementary file: Supplementary Table S7. Mutations identified by deep-targeted NGS in 406 untreated CLL patients (XLSX, 65kB)*

**Table S8.** Comparison of clinical and biological characteristics between mutated cases with or without convergent mutational evolution (CME).

| Parameter         | Category              | Mutated cases without CME (n=192) | Cases with CME (n=66) | P-value |
|-------------------|-----------------------|-----------------------------------|-----------------------|---------|
| Binet stage       | A (%)                 | 72.9                              | 65.2                  | 0.271   |
| Age (years)       | Median (range)        | 67 (25-91)                        | 66 (45-94)            | 0.806   |
| IGHV mutational status | Mutated (%)    | 41.7                              | 39.7                  | 0.661   |

**Table S9.** Mutations identified in the sequential samples analyzed in the longitudinal study.

*Supplementary file: Supplementary Table S9. Mutations identified in the sequential samples analyzed in the longitudinal study (XLSX, 23kB)*

**Table S10.** CNA identified in 376 untreated CLL patients by SNP arrays.

*Supplementary file: Supplementary Table S10. CNA identified in 376 untreated CLL patients by SNP arrays (XLSX, 44kB)*
## Table S11. Temporal classification of CLL driver alterations.

| Alteration                  | Out-going edges | In-going edges | P-value | Q value | Classification |
|-----------------------------|-----------------|----------------|---------|---------|----------------|
| del(13q14)                  | 111             | 18             | < 0.001 | < 0.001 | Early          |
| tri(12)                     | 55              | 2              | < 0.001 | < 0.001 | Early          |
| del(11q)                    | 46              | 11             | < 0.001 | < 0.001 | Early          |
| del(17p)                    | 24              | 5              | < 0.001 | 0.002   | Early          |
| amp(8q23-q24)               | 30              | 5              | < 0.001 | < 0.001 | Early          |
| del(10q24.32-NFKB2)         | 19              | 3              | < 0.001 | 0.003   | Early          |
| del(14q24.1)                | 14              | 0              | < 0.001 | < 0.001 | Early          |
| NOTCH1                      | 44              | 80             | 0.002   | 0.006   | Late           |
| TP53                        | 12              | 55             | < 0.001 | < 0.001 | Late           |
| NFKBIE                      | 5               | 52             | < 0.001 | < 0.001 | Late           |
| ZNF292                      | 8               | 31             | < 0.001 | 0.002   | Late           |
| BIRC3                       | 3               | 33             | < 0.001 | < 0.001 | Late           |
| amp(2p16)                   | 17              | 23             | 0.430   | 0.696   | Int./not powered |
| del(18p)                    | 10              | 6              | 0.454   | 0.696   | Int./not powered |
| del(8p)                     | 7               | 12             | 0.359   | 0.696   | Int./not powered |
| del(20p)                    | 7               | 11             | 0.481   | 0.696   | Int./not powered |
| del(6q15-ZNF292)            | 8               | 8              | 1       | 1       | Int./not powered |
| del(3p21.31-SETD2)          | 4               | 7              | 0.549   | 0.737   | Int./not powered |
| del(15q15.1-MGA)            | 12              | 8              | 0.503   | 0.696   | Int./not powered |
| del(2q37.1-SP100)           | 9               | 3              | 0.146   | 0.312   | Int./not powered |
| amp(3q26.1)                 | 8               | 2              | 0.109   | 0.271   | Int./not powered |
| amp(5q34)                   | 6               | 1              | 0.125   | 0.280   | Int./not powered |
| del(6p21.1-NFKBIE)          | 1               | 3              | 0.625   | 0.773   | Int./not powered |
| tri(18)                     | 1               | 0              | 1       | 1       | Int./not powered |
| SF3B1                       | 29              | 37             | 0.389   | 0.653   | Int./not powered |
| ATM                         | 31              | 48             | 0.071   | 0.221   | Int./not powered |
| POT1                        | 16              | 23             | 0.337   | 0.633   | Int./not powered |
| XPO1                        | 7               | 17             | 0.064   | 0.215   | Int./not powered |
| EGR2                        | 9               | 16             | 0.229   | 0.490   | Int./not powered |
| FBXW7                       | 13              | 7              | 0.263   | 0.538   | Int./not powered |
| MGA                         | 8               | 18             | 0.075   | 0.222   | Int./not powered |
| KLIHL6                      | 4               | 5              | 1       | 1       | Int./not powered |
| RPS15                       | 12              | 8              | 0.503   | 0.696   | Int./not powered |
| MYD88                       | 3               | 10             | 0.092   | 0.241   | Int./not powered |
| DDX3X                       | 10              | 16             | 0.327   | 0.633   | Int./not powered |
| BRAF                        | 3               | 10             | 0.092   | 0.241   | Int./not powered |
| NFX1                        | 4               | 3              | 1       | 1       | Int./not powered |
| DTX1                        | 3               | 1              | 0.625   | 0.773   | Int./not powered |
| BCOR                        | 2               | 4              | 0.687   | 0.829   | Int./not powered |
| CCND2                       | 4               | 4              | 1       | 1       | Int./not powered |
| KRAS                        | 1               | 4              | 0.375   | 0.653   | Int./not powered |
| IRF4                        | 0               | 7              | 0.016   | 0.056   | Int./not powered |
| MED12                       | 2               | 3              | 1       | 1       | Int./not powered |
| ZMYM3                       | 1               | 3              | 0.625   | 0.773   | Int./not powered |
| NRAS                        | 0               | 1              | 1       | 1       | Int./not powered |
| TRAF3                       | 0               | 1              | 1       | 1       | Int./not powered |
| PIM1                        | 2               | 0              | 0.500   | 0.696   | Int./not powered |

Alterations with a Q value < .05 were classified as early or late events, regarding their out/in-going edges.
Table S12. Temporal classification of CLL driver alterations considering only the mutations present at a CCF ≥25%.

| Alteration                | Out-going edges | In-going edges | P-value   | Q value | Classification            |
|---------------------------|-----------------|----------------|-----------|---------|---------------------------|
| del(13q14)                | 49              | 18             | < 0.001   | 0.004   | Early                     |
| tri(12)                   | 21              | 2              | < 0.001   | 0.004   | Early                     |
| del(14q24.1)              | 10              | 0              | 0.002     | 0.020   | Early                     |
| amp(8q23-q24)             | 18              | 5              | 0.011     | 0.072   | Early (non-significant trend) |
| del(17p)                  | 16              | 5              | 0.027     | 0.109   | Early (non-significant trend) |
| TP53                      | 5               | 25             | < 0.001   | 0.004   | Late                      |
| MGA                       | 0               | 8              | 0.008     | 0.064   | Late (non-significant trend) |
| BIRC3                     | 0               | 7              | 0.016     | 0.091   | Late (non-significant trend) |
| ATM                       | 11              | 26             | 0.020     | 0.102   | Late (non-significant trend) |
| del(20p)                  | 2               | 11             | 0.022     | 0.102   | Late (non-significant trend) |
| amp(2p16)                 | 10              | 23             | 0.035     | 0.131   | Late (non-significant trend) |
| del(11q)                  | 23              | 11             | 0.058     | 0.197   | Int./not powered           |
| del(8p)                   | 4               | 12             | 0.077     | 0.225   | Int./not powered           |
| amp(3q26.1)               | 4               | 0              | 0.125     | 0.320   | Int./not powered           |
| del(3p21.31-SETD2)        | 2               | 7              | 0.180     | 0.409   | Int./not powered           |
| del(10q24.32-NFKB2)       | 8               | 3              | 0.227     | 0.489   | Int./not powered           |
| amp(5q34)                 | 3               | 1              | 0.625     | 0.915   | Int./not powered           |
| del(6p21.1-NFKBIE)        | 1               | 3              | 0.625     | 0.915   | Int./not powered           |
| del(18p)                  | 6               | 6              | 1         | 1       | Int./not powered           |
| del(6q15-ZNF292)          | 7               | 8              | 1         | 1       | Int./not powered           |
| del(15q15.1-MGA)          | 6               | 8              | 0.790     | 1       | Int./not powered           |
| del(2q37.1-SP100)         | 4               | 3              | 1         | 1       | Int./not powered           |
| tri(18)                   | 1               | 0              | 1         | 1       | Int./not powered           |
| NFKBIE                    | 0               | 5              | 0.062     | 0.197   | Int./not powered           |
| DDX3X                     | 2               | 8              | 0.109     | 0.299   | Int./not powered           |
| SF3B1                     | 15              | 7              | 0.134     | 0.323   | Int./not powered           |
| ZMYM3                     | 0               | 3              | 0.250     | 0.513   | Int./not powered           |
| FBXW7                     | 4               | 1              | 0.375     | 0.699   | Int./not powered           |
| KLRH6                     | 1               | 4              | 0.375     | 0.699   | Int./not powered           |
| BCO1                      | 0               | 2              | 0.500     | 0.854   | Int./not powered           |
| IRF4                      | 0               | 2              | 0.500     | 0.854   | Int./not powered           |
| BRAF                      | 1               | 3              | 0.625     | 0.915   | Int./not powered           |
| XPO1                      | 1               | 3              | 0.625     | 0.915   | Int./not powered           |
| NOTCH1                    | 17              | 18             | 1         | 1       | Int./not powered           |
| POT1                      | 4               | 6              | 0.754     | 1       | Int./not powered           |
| ZNF292                    | 3               | 5              | 0.727     | 1       | Int./not powered           |
| EGR2                      | 4               | 3              | 1         | 1       | Int./not powered           |
| RPS15                     | 2               | 3              | 1         | 1       | Int./not powered           |
| DTX1                      | 1               | 0              | 1         | 1       | Int./not powered           |
| CCND2                     | 2               | 2              | 1         | 1       | Int./not powered           |
| TRAF3                     | 0               | 1              | 1         | 1       | Int./not powered           |
| MYD88                     | 0               | 0              | -         | -       | Int./not powered           |
| NXF1                      | 0               | 0              | -         | -       | Int./not powered           |
| KRAS                      | 0               | 0              | -         | -       | Int./not powered           |
Alterations with a Q value < .05 were classified as early or late events, regarding their number of out/in-going edges. Alterations with a Q value < .15 were considered as having a non-significant trend.

Table S13. Temporal relationship between specific pairs of driver alterations.

| Alteration A | Alteration B | Alteration A → Alteration B (CCF A > CCF B) | Alteration B → Alteration A (CCF A < CCF B) | P-value | Q value |
|--------------|--------------|--------------------------------------------|--------------------------------------------|---------|---------|
| del(13q14)   | NOTCH1       | 15                                         | 1                                         | < .001  | 0.013   |
| del(13q14)   | SF3B1        | 9                                          | 1                                         | 0.022   | 0.062   |
| del(13q14)   | TP53         | 10                                         | 2                                         | 0.039   | 0.084   |
| del(13q14)   | ATM          | 7                                          | 0                                         | 0.016   | 0.051   |
| del(13q14)   | POT1         | 6                                          | 0                                         | 0.031   | 0.074   |
| del(13q14)   | NFKBIE       | 7                                          | 0                                         | 0.016   | 0.051   |
| del(13q14)   | ZNF292       | 7                                          | 0                                         | 0.016   | 0.051   |
| del(13q14)   | BIRC3        | 7                                          | 0                                         | 0.016   | 0.051   |
| tri(12)      | NOTCH1       | 9                                          | 0                                         | 0.004   | 0.051   |
| tri(12)      | TP53         | 5                                          | 0                                         | 0.062   | 0.108   |
| tri(12)      | BIRC3        | 6                                          | 0                                         | 0.031   | 0.074   |
| del(11q)     | amp(2p16)    | 5                                          | 0                                         | 0.062   | 0.108   |
| del(11q)     | ATM          | 11                                         | 1                                         | 0.006   | 0.051   |
| del(17p)     | TP53         | 8                                          | 0                                         | 0.009   | 0.051   |
| NFKBIE       | RPS15        | 0                                          | 5                                         | 0.062   | 0.108   |
| del(13q14)   | amp(2p16)    | 4                                          | 1                                         | 0.375   | 0.464   |
| del(11q)     | SF3B1        | 4                                          | 1                                         | 0.375   | 0.464   |
| amp(2p16)    | ATM          | 5                                          | 2                                         | 0.453   | 0.512   |
| del(10q24.32-NFKB2) | NOTCH1 | 5                                          | 1                                         | 0.219   | 0.335   |
| NOTCH1       | SF3B1        | 2                                          | 5                                         | 0.453   | 0.512   |
| NOTCH1       | TP53         | 6                                          | 2                                         | 0.289   | 0.418   |
| NOTCH1       | ATM          | 3                                          | 3                                         | 1       | 1       |
| NOTCH1       | POT1         | 1                                          | 5                                         | 0.219   | 0.335   |
| NOTCH1       | NFKBIE       | 4                                          | 1                                         | 0.375   | 0.464   |
| NOTCH1       | MGA          | 4                                          | 2                                         | 0.687   | 0.715   |
| NOTCH1       | DDX3X        | 4                                          | 2                                         | 0.687   | 0.715   |

Pairs of alterations with at least 5 connections with different CCFs were analyzed. Relationships with Q values < .15 were considered.
### Table S14. Temporal acquisition of gene mutations in specific biological pathways.

| Pathway                        | Out-going edges | In-going edges | P-value | Q-value | Classification               |
|--------------------------------|-----------------|----------------|---------|---------|-----------------------------|
| NF-κB signaling                | 9               | 26             | 0.006   | 0.042   | Late                        |
| B-cell signaling               | 7               | 3              | 0.343   | 0.873   | Int./not powered            |
| RNA metabolism                 | 34              | 27             | 0.443   | 0.883   | Int./not powered            |
| Notch signaling                | 31              | 25             | 0.504   | 0.883   | Int./not powered            |
| Cell cycle                     | 6               | 8              | 0.790   | 1       | Int./not powered            |
| DNA damage response            | 31              | 29             | 0.897   | 1       | Int./not powered            |
| Chromatin structure            | 1               | 1              | 1       | 1       | Int./not powered            |

NF-κB signaling: *BIRC3, TRAF3, EGR2, NFKBIE*; B-cell signaling: *BCOR, IRF4, KLHL6, MYD88*; RNA metabolism: *SF3B1, XPO1, RPS15, DDX3X, ZNF292, MED12, NXF1, MGA*; Notch signaling: *NOTCH1, FBXW7, DTX1*; Cell cycle: *KRAS, NRAS, BRAF, CCND2*; DNA damage response: *TP53, ATM, POT1*; Chromatin structure: *ZMYM3*.

### Table S15. CNA identified in the sequential samples analyzed in the longitudinal study.

| Case | Chr  | Start position (hg19) | End position (hg19) | Event   | CCF |
|------|------|-----------------------|---------------------|---------|-----|
| 24-2 | chr12| 145739                | 133779461           | CN Gain | 100 |
| 24-2 | chr17| 52844401              | 56619391            | CN Loss | 100 |
| 27-2 | chr13| 48496748              | 51570061            | CN Loss | 100 |
| 27-3 | chr9 | 70907292              | 141153431           | CN Loss | 79.24 |
| 27-3 | chr13| 48474078              | 51571454            | CN Loss | 100 |
| 27-3 | chr17| 0                     | 19751800            | CN Loss | 89.17 |
| 29-2 | chr1 | 28003685              | 39385712            | CN Loss | 52.18 |
| 29-2 | chr11| 81997865              | 114232574           | CN Loss | 38.92 |
| 29-2 | chr13| 43373412              | 52583981            | CN Loss | 47.26 |
| 47-2 | chr5 | 90159528              | 112894641           | CN Loss | 99.1 |
| 47-2 | chr12| 145739                | 133779461           | CN Gain | 100 |
| 47-2 | chr13| 48856202              | 52981500            | CN Loss | 95.46 |
| 47-2 | chr17| 0                     | 26415213            | CN Loss | 94.07 |
| 47-2 | chr17| 29541908              | 32028411            | CN Loss | 82.16 |
| 47-2 | chr17| 35216940              | 35459748            | CN Loss | 92.44 |
| 47-2 | chr17| 49727681              | 54754350            | CN Loss | 76.85 |
| 47-2 | chr17| 67175999              | 72209853            | CN Loss | 69.08 |
| 48-2 | chr2 | 232952935             | 243102476           | CN Loss | 79.67 |
| 48-2 | chr3 | 75467674              | 75931704            | CN Gain | 99.02 |
| 48-2 | chr3 | 118956344             | 197962430           | CN Gain | 87.49 |
| 48-2 | chr7 | 16913652              | 17416937            | CN Gain | 100 |
| 48-2 | chr7 | 143218633             | 143532988           | CN Gain | 100 |
| 73-2 | chr3 | 6647527               | 52513610            | CN Loss | 27.5 |
| 73-2 | chr13| 44652243              | 74733583            | CN Loss | 72.67 |
| 73-2 | chr17| 1                     | 22263006            | CN Loss | 38.93 |
| chr  | CN   | CN   | Homozygous Copy Loss | Homozygous Copy Loss |
|------|------|------|----------------------|----------------------|
| chr9 | 6977976 | 77079354 | 97.85 | CN Loss |
| chr13 | 47930454 | 65230248 | 100 | CN Loss |
| chr13 | 47435512 | 50532216 | 24.36 | CN Loss |
| chr13 | 50532217 | 51495664 | na | CN Loss |
| chr16 | 47415392 | 47669196 | 100 | CN Loss |
| chr15 | 93148606 | 102420705 | 100 | CN Loss |
| chr8  | 67438475 | 146259574 | 100 | CN Gain |
| chr13 | 50522257 | 51529916 | 100 | CN Loss |
| chr13 | 41973384 | 51846014 | 100 | CN Loss |
| chr8  | 48292859 | 48675393 | 100 | CN Loss |
| chr12 | 145739  | 133779461 | 100 | CN Gain |
| chr13 | 47531603 | 51471176 | 71.34 | CN Loss |
| chr12 | 131732300 | 131834302 | 100 | CN Loss |
| chr13 | 50546382 | 51480442 | na | CN Loss |
| chr13 | 47624417 | 51636090 | 100 | CN Loss |
| chr10 | 102843647 | 135449667 | 100 | CN Loss |
| chr12 | 46377041 | 133779461 | 100 | CN Gain |
| chr14 | 62327857 | 78130190 | 100 | CN Loss |
| chr13 | 50591783 | 51347782 | na | CN Loss |
| chr13 | 50590765 | 51347782 | na | CN Loss |
| chr2  | 10000   | 92285930 | 66.96 | CN Gain |
| chr13 | 41514889 | 42764265 | 67.37 | CN Loss |
| chr13 | 48415495 | 50553238 | 85.22 | CN Loss |
| chr13 | 50553239 | 51775052 | na | CN Loss |
| chr13 | 51775053 | 77166122 | 74.85 | CN Loss |
| chr18 | 130072  | 13715376 | 60.68 | CN Loss |
| chr2  | 231042372 | 231602800 | 100 | CN Loss |
| chr3  | 26037098 | 26168215 | 100 | CN Loss |
| chr6  | 162775040 | 163136852 | 100 | CN Gain |
| chr13 | 50496333 | 51438125 | 100 | CN Loss |
| chr13 | 48700382 | 52903825 | 100 | CN Loss |
| chr12 | 145739  | 133779461 | 100 | CN Gain |
| chr13 | 50245847 | 51570061 | 84.4 | CN Loss |
| chr13 | 50575189 | 51477334 | 100 | CN Loss |
| chr7  | 143914613 | 144117765 | 100 | CN Gain |
| chr13 | 47936555 | 56914417 | 100 | CN Loss |
| chr13 | 50560863 | 51461403 | 100 | CN Loss |
| chr4  | 160762679 | 160973516 | 100 | CN Loss |
| chr6  | 168335948 | 168583553 | 100 | CN Gain |
| chr9  | 20356788 | 30356439 | 100 | CN Loss |
| chr9  | 70986320 | 141074404 | 100 | CN Loss |
| chr11 | 2       | 61601850 | 100 | CN Loss |
| chr12 | 145739  | 133779461 | 100 | CN Gain |
| chr13 | 42763930 | 50107904 | 100 | CN Loss |
| chr14 | 22474859 | 23018097 | 100 | CN Loss |
| chr15 | 24349824 | 24726268 | 100 | CN Gain |
| chr9  | 20412119 | 22449551 | 100 | CN Loss |
| Position | Chromosome | Start | End | Type       |
|----------|------------|-------|-----|------------|
| 456-2    | chr13      | 50546382 | 51182569 | CN Loss | 69.99 |
| 456-2    | chr19      | 20581725 | 20721342 | CN Loss | 100  |
| 456-2    | chr19      | 43292575 | 43612172 | CN Loss | 96.85|
| 589-2    | chr2       | 10000   | 92326171 | CN Gain | 100  |
| 589-2    | chr10      | 102689838 | 10455106 | CN Loss | 100  |
| 589-2    | chr20      | 60000   | 26319569 | CN Loss | 100  |
| 618-2    | chr8       | 10001   | 43838887 | CN Loss | 99.68|
| 618-2    | chr17      | 0       | 22152702 | CN Loss | 99.56|
| 701-2    | chr2       | 10000   | 92326171 | CN Gain | 100  |
| 701-2    | chr9       | 20501591 | 20625551 | CN Loss | 100  |
| 745-2    | chr13      | 44617371 | 50538562 | CN Loss | 100  |
| 745-2    | chr13      | 50538562 | 51420125 | Homozygous Copy Loss | na |
| 759-2    | chr12      | 145739  | 133779461 | CN Gain | 99.29|
| 787-2    | chr1       | 156287292 | 161110488 | CN Loss | 100  |
| 787-2    | chr1       | 223797846 | 227689207 | CN Loss | 100  |
| 1076-2   | chr11      | 98133733 | 121464062 | CN Loss | 98.57|
| 1076-2   | chr12      | 204302  | 9354560  | CN Gain | 48.81|
| 1076-2   | chr12      | 11964269 | 18749512 | CN Loss | 40.76|
| 1076-2   | chr13      | 19815048 | 24959490 | CN Loss | 56.38|
| 1076-2   | chr9       | 79126120 | 124400641 | CN Gain | 26.13|
| 1076-2   | chr14      | 99754406 | 107289540 | CN Loss | 99.34|
| 1076-2   | chr16      | 78197385 | 78352939 | CN Gain | 100  |
| 1076-2   | chr16      | 79495915 | 79688205 | CN Gain | 73.94|
| 1076-2   | chr3       | 154659178 | 154932978 | CN Loss | 100  |
| 1078-2   | chr13      | 32737317 | 33359001 | CN Loss | 67.6 |
| 1078-2   | chr13      | 50475234 | 51534610 | CN Loss | 87.06|
| 1189-2   | chr13      | 50538562 | 51526306 | CN Loss | 100  |
| 1313-2   | chr13      | 43033142 | 53610920 | CN Loss | 100  |

CCF, cancer cell fraction; CN, copy number; na, not available.
Table S16. Summary of the results of the algorithm applied to determine the pattern of CCF of each gene mutation.

| Gene   | n  | CCF pattern | Cut-off (% CCF) | P-value continuous analysis | P-value mutated vs unmutated | P-value mutated CCF ≥ cut-off vs unmutated + (mut CCF < cut-off) | CCF pattern | Cut-off (% CCF) | P-value continuous analysis | P-value mutated vs unmutated | P-value mutated CCF ≥ cut-off vs unmutated + (mut CCF < cut-off) |
|--------|----|-------------|-----------------|-----------------------------|-----------------------------|-----------------------------------------------------------------|-------------|-----------------|-----------------------------|-----------------------------|------------------------------------------------------------------|
| NOTCH1 | 86 | CCF-gradual | 6.69            | 0.085                      | < 0.001                     | -                                                               | CCF-dominant | 63.27 | -              | -                          | < 0.001                                                             |
| SF3B1  | 51 | CCF-gradual | 7.39            | 0.006                      | < 0.001                     | -                                                               | CCF-independent | 2.71 | 0.86          | 0.045                      | -                          |
| ATM    | 36 | CCF-gradual | 5.86            | 0.024                      | < 0.001                     | -                                                               | No impact    | 47.16 | -              | -                          | 0.80                                                                  |
| POT1   | 27 | CCF-independent | 0             | 0.19                       | < 0.001                     | -                                                               | No impact    | 13.85 | 0.47          | 0.57                      | -                          |
| NFKBIE | 27 | CCF-independent | 3.13            | 0.18                       | 0.004                       | -                                                               | No impact    | 2.70  | 0.30          | 0.79                      | -                          |
| ZNF292 | 27 | No impact   | 0               | 0.62                       | 0.25                        | -                                                               | No impact    | 1.25  | 0.54          | 0.32                      | -                          |
| XPO1   | 22 | CCF-independent | 0             | 0.26                       | < 0.001                     | -                                                               | No impact    | 4.63  | 0.67          | 0.77                      | -                          |
| EGR2   | 20 | No impact   | 2.17            | 0.20                       | 0.08                        | -                                                               | No impact    | 20.27 | -              | -                          | 0.33                                                                  |
| FBXW7  | 19 | No impact   | 50.63           | -                          | -                           | 0.58                                                            | CCF-dominant | 52.22 | -              | -                          | < 0.001                                                             |
| MGA    | 18 | CCF-independent | 0             | 0.29                       | < 0.001                     | -                                                               | No impact    | 74.87 | -              | -                          | 0.68                                                                  |
| BIRC3  | 17 | No impact   | 22.17           | -                          | -                           | 0.63                                                            | No impact    | 9.10  | 0.61          | 0.19                      | -                          |
| KLHL6  | 17 | No impact   | 46.59           | -                          | -                           | 0.57                                                            | No impact    | 94.31 | -              | -                          | 0.82                                                                  |
| RPS15  | 15 | CCF-gradual | 1.41            | 0.053                      | < 0.001                     | -                                                               | No impact    | 46.52 | -              | -                          | 0.28                                                                  |
| MYD88  | 15 | No impact   | 81.31           | -                          | -                           | 0.26                                                            | No impact    | 6.90  | 0.52          | 0.21                      | -                          |
| DDX3X  | 14 | CCF-independent | 0             | 0.38                       | 0.001                       | -                                                               | No impact    | 5.32  | 0.42          | 0.42                      | -                          |
| BRAF   | 11 | CCF-independent | 6.27            | 0.69                       | 0.004                       | -                                                               | Not analyzed | 52.59 | -              | -                          | 0.34                                                                  |
| NXF1   | 7  | Not analyzed | -               | -                          | -                           | -                                                               | Not analyzed | -    | -              | -                          | -                          |
| DTX1   | 6  | Not analyzed | -               | -                          | -                           | -                                                               | Not analyzed | -    | -              | -                          | -                          |
| BCOR   | 6  | Not analyzed | -               | -                          | -                           | -                                                               | Not analyzed | -    | -              | -                          | -                          |
| CCND2  | 6  | Not analyzed | -               | -                          | -                           | -                                                               | Not analyzed | -    | -              | -                          | -                          |
| KRAS   | 5  | Not analyzed | -               | -                          | -                           | -                                                               | Not analyzed | -    | -              | -                          | -                          |
| Gene | CCF | Analyzed | Univariate analysis |
|------|-----|----------|---------------------|
| IRF4 | 5   | Not analyzed | -                   |
| MED12| 3   | Not analyzed | -                   |
| ZMYM3| 3   | Not analyzed | -                   |
| NRAS | 2   | Not analyzed | -                   |
| TRAF3| 2   | Not analyzed | -                   |
| PIM1 | 2   | Not analyzed | -                   |

a No impact, no impact of the gene mutations at any CCF for the outcome analyzed (TTFT or OS).

b Univariate continuous analysis considering the mutations as a continuous numeric variable based on their CCF. Only mutated cases were included in this analysis to better capture the possible gradual effect of the mutations.

c Univariate categorical analysis comparing unmutated cases vs mutated cases independently of the CCF of the mutations.

d Univariate categorical analysis in which only those patients carrying the mutation at a CCF ≥ cut-off were considered mutated.
Table S17. No independent prognostic value of the subclonal diversity for OS.

| Variable                        | HR (95% CI)     | P-value |
|---------------------------------|-----------------|---------|
| IGHV (unmut vs. mut)            | 2.72 (1.73-4.27)| < 0.001 |
| Age at sampling (>65 vs. ≤65 years) | 2.69 (1.66-4.33) | < 0.001 |
| TP53 (mut/deletion vs. wt)      | 2.86 (1.69-4.86) | < 0.001 |
| SF3B1 (mut vs. unmut)           | 1.73 (0.99-3.01) | 0.051   |

N=307, events=79. Starting model: IGHV, Binet stage, age at sampling, gender, TP53 aberration (mutation/deletion), ATM aberration (mutation/deletion), SF3B1 mutation, number of drivers in subclonal tumors, and number of drivers in clonal tumors. The number of driver alterations did not include TP53, ATM, and SF3B1 mutations, neither del(17p) nor del(11q).
Figure S1. Dilutional experiment of MEC-1 cell line DNA for CNA quantification. (A) Copy number (CN) profile of MEC-1. Chromosomes are aligned from 1 to 22, X, and Y, from p to q. (B) Graphical representation of the CN losses (depicted in red) and gains (in blue) identified in MEC-1 CLL cell line (100% MEC-1). The intensity of the color represents the clonality (or abundance) of the alteration in the tumor population, being the color darker when it is more clonally represented. The diluted samples mixing MEC-1 DNA with normal DNA are also plotted showing a gradual decrease of the intensity in which the alteration is detected when increasing the...
amount of normal mixed DNA. (C-D) Graphical representation of the probe median obtained using Nexus Copy Number Discovery (x-axis), and the known tumor abundance (cancer cell fraction, CCF, y-axis) for 20 CN deletions (C) and 12 CN gains (D) clonally represented in MEC-1. Each dot corresponds to one alteration. Black lines show the fitted Loess curves (obtained using the msir R package) for each scenario. Dashed lines represent the 90% predictive intervals. The cut-off point of 85% of CCF, which was used to classify alterations as clonal or subclonal, is represented in green.

Figure S2. Summary of the variant calling. (A) Venn diagram showing the degree of overlap on the identification of the 609 somatic/truncating mutations considered between the different variant callers used. SVC, Somatic Variant Caller (MiSeq, Illumina); UG, UnifiedGenotyper (GATK); HC, HaplotypeCaller (GATK); VS2, VarScan2. (B) Bar plot representing the total number of mutations identified by each variant caller. (C) Histograms showing the minimum variant allele frequency (VAF) of a mutation identified for each gene. Blue bars correspond to genes in which the minimum VAF was ≥12% whereas orange bars to genes with a VAF <12%. This cut-off value represents the detection threshold of mutations by Sanger sequencing. Number of mutations identified in each gene is shown next to its name on the x-axis.
Figure S3. Variant allele frequency variability due to the sequencing methodology. Graphical representation of the variant allele frequency (VAF) obtained for 643 variants (represented as gray dots) when sequenced in two independent NGS rounds (run 1, x-axis; run 2, y-axis). Perfect correlation is shown in green (VAF run 1 = VAF run 2) while the fitted Loess model is represented in black. The 90% predictive interval at each VAF is shown using dashed black lines.
Figure S4. No influence of the time of sampling on clinical outcome. Comparison of TTFT (left) and OS (right) among patients the sample of which was obtained within the first year of diagnosis (gray line) vs. after the first year (brown). The global $P$-values by Gray’s test (TTFT) and log-rank test (OS) are shown on the top-right corner of each curve. The number of patients at risk at each time point is shown at the bottom.
Figure S5. Gene maps of the 28 CLL driver genes analyzed. Coding exons are represented by gray boxes and the main protein domains are colored. Mutations types are represented in different shapes and the color code represents their cancer cell fraction (CCF). The y-axis on the left of each gene map reflects the maximum number of mutations affecting a single amino acid position. The number on the right represents the length of the resulting protein in amino acids.
Figure S6. Comparison of clinico-biological features between cases carrying subclonal-low and subclonal-high/clonal mutations. Comparison of the age at sampling (A), percentage of cases with Binet A (B), and average number of driver alterations (mutations and CNA) (C) between cases carrying subclonal-low and subclonal-high/clonal mutations in each of the genes mutated in more than 10 cases. No significant differences were observed after correcting for multiple comparisons.

Figure S7. Clinical implications of convergent mutational evolution (CME). (A) Comparison of the number of genes mutated and driver CNA between cases with (orange) or without (yellow) CME. (B) Comparison of TTFT (left) and OS (right) among unmutated patients (gray line), mutated patients without CME (yellow), and mutated patients with CME (orange). The global P-values by Gray’s test (TTFT) and log-rank test (OS) are shown on the top-right corner of each curve. P-values of each pairwise comparisons are also shown inside the plotting areas. The number of patients at risk at each time point is shown at the bottom.
Figure S8. Incidence and CCF of the CLL driver CNA. (A) Pie chart representing the distribution of patients according to the clonality of their CNA (top-right corner). Percentage of cases carrying each driver CNA colored based on its clonality (subclonal, light red; clonal, red; or homozygous deletion, dark red). (B) Distribution of the CCFs of each driver CNA where each dot corresponds to the CCF of the alteration in one patient.
Figure S9. CCF distribution of gene mutations and CNA, and proposed evolution route. (A) Density plot of the CCFs of the CNA and gene mutations. (B) Cartoon illustrating the proposed evolution route of formation of CLL tumors carrying CNA and gene mutations.
Figure S10. CCF of TP53 aberrations in CLL. (A) Distribution of the CCF of del(17p) and TP53 mutations for each patient carrying a TP53 aberration. Cases are grouped according to the presence of del(17p) and/or TP53 mutations. (B) Clonal evolution observed for three cases with concomitant evolution of del(17p) and TP53 mutation. Two cases received treatment between samples (CLL27 and CLL73).
Figure S11. Schematic representation of the algorithm used for the identification of specific gene CCF patterns with impact on clinical outcome. Schematic diagram of the algorithm applied to quantitatively analyze the clinical implications of each mutated gene based on the CCF of the mutations (see Supplementary Methods for a detailed explanation).
Figure S12. Survival curves of the patterns of CCF with impact on clinical outcome. Time to first treatment (TTFT) and overall survival (OS) curves to graphically represent the CCF patterns with impact on clinical outcome identified for each gene: CCF-independent pattern (A) or CCF-gradual pattern (B). P-values for all pairwise comparisons are shown inside the plot area. P, P-values by Gray’s test (TTFT) or log-rank test (OS).
Figure S13. Clinical impact of the presence of driver subclones and mutational complexity. (A) Impact on TTFT (left) and OS (right) of the presence of at least one subclonal driver alteration. (B) Increasing impact of the accumulation of driver alterations (mutational complexity) on TTFT (left) and OS (right). *P*, *P*-values by Gray’s test (TTFT) or log-rank test (OS).
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