Clinical Significance of *TP53, BIRC3, ATM* and *MAPK-ERK* genes in Chronic Lymphocytic Leukaemia: Data from the Randomised UK LRF CLL4 Trial

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Abstract

Despite advances in chronic lymphocytic leukaemia (CLL) treatment, globally chemotherapy remains a central treatment modality, with chemotherapy trials representing an invaluable resource to explore disease-related/genetic features contributing to long-term outcomes. In 499 LRF CLL4 cases, a trial with >12 years follow-up, we employed targeted re-sequencing of 22 genes, identifying 623 mutations. After background mutation rate correction, 11/22 genes were recurrently mutated at frequencies between 3.6% (*NFKBIE*) and 24% (*SF3B1*). Mutations beyond Sanger resolution (<12% VAF) were observed in all genes, with *KRAS* mutations principally composed of these low VAF variants**.** Firstly, employing orthogonal approaches to confirm <12% VAF *TP53* mutations*,* we assessed the clinical impact of *TP53* clonal architecture. Whilst ≥12% VAF *TP53*mut cases were associated with reduced PFS and OS, we could not demonstrate a difference between <12% VAF *TP53* mutations and either wild-type or ≥12% VAF *TP53*mut cases. Secondly, we identified biallelic *BIRC3* lesions (mutation and deletion) as an independent marker of inferior PFS and OS. Finally, we observed that mutated *MAPK-ERK* genes were independent markers of poor OS in multivariate survival analysis. In conclusion, our study supports using targeted re-sequencing of expanded gene panels to elucidate the prognostic impact of gene mutations.

Introduction

The application of new technologies continues to reveal the biological basis for the clinical heterogeneity apparent within CLL[1–3]. In particular, next generation sequencing of large patient cohorts has led to the discovery of recurring genomic mutations that cluster into distinct biological signalling pathways. Mutations of specific genes including *TP53*[4–10], *ATM*[9,11–14], *BIRC3*[9,15,16], *SF3B1*[9,17–20], *NOTCH1*[1,9,15,17,20–23] , *RPS15*[2,24], *EGR2*[25,26], and *KRAS*[27,28] are associated with poorer outcome, especially shorter time to first treatment or overall survival (OS). However, numerous factors influence the clinical significance of a driver mutation in an individual patient. These include clinical status, immunogenetic background, clone size, the presence of biallelic abnormalities and co-existing driver mutations or copy number alterations (CNAs). The clinical importance of these potentially confounding factors is most easily established in context of large clinical trials with long follow-up and where data on numerous biomarkers are available. One such study is the phase III UK LRF CLL4 trial (NCT 58585610) that randomly assigned 777 patients to fludarabine (FDR) or fludarabine plus cyclophosphamide (FC) for six courses, or chlorambucil (CHL) for 12 courses, with the primary endpoint of overall survival, and secondary endpoints of response rates, progression-free survival, toxic effects, and quality of life[29]. The trial demonstrated superior response rates and progression-free survival (PFS) for FC-treated patients compared to those patients treated with FDR or CHL. Previous genomic analysis of this trial has shown *TP53*[8], *SF3B1*[17], *NOTCH1* (coding[17] and non-coding[21]), *ATM* plus del(11q)[12], and *EGR2*[26] lesions to have prognostic significance in multivariate analysis (MVA) and of *RPS15*[24] in univariate analysis. The importance of data from CLL4 may be questioned given the studies showing the superior efficacy of FC plus an anti-CD20 antibody (FCR) compared to

chemotherapy alone, with the exception of patients with a *NOTCH1* mutation[20], and emerging data suggesting the superiority of novel agents compared to chemotherapy-based regimens. However, the observation that *TP53*, *SF3B1,* and *RPS15* mutations remain poor risk factors in the German CLL8 trial comparing FCR v FC[20] and the continuing global need for chemotherapy in CLL for the foreseeable future, indicate that genomic data from the UK CLL4 trial will continue to have clinical relevance.

Accordingly, we performed targeted resequencing on all available pre-treatment samples (n=499) from the CLL4 trial to investigate the incidence, clinico-biological associations, and prognostic impact of a panel of 22 genes recurrently mutated in CLL (study overview in **Figure S1**). Important findings include the failure of <12% VAF *TP53* mutations (1.97 – 11.18% Variant Allele Frequency [VAF]) to influence PFS or OS, the importance of 11q deletions on PFS and OS in the context of *ATM* and *BIRC3* mutations, and the reduced OS associated with mutations in the *MAPK-ERK* genes: *BRAF, KRAS,* and *NRAS*.

Methods

Patients and molecular assays

We studied 499 patient samples taken at randomization[29]. Patients were diagnosed using the iwCLL guidelines[30], with informed consent obtained in accordance with the declaration of Helsinki. This study was approved by national/regional research ethics committees. The average lymphocyte percentage of the total white cell count in pre-treatment blood samples was 83.8%. To confirm high tumor load, CD19/CD5 positivity from cases with available flow cytometry data were compared with their matched average lymphocyte percentage (n=233), with an agreement bias of -0.8% (**Figure S2**). Our study cohort did not significantly differ from the entire trial cohort in terms of: treatment allocation, CNAs, age, gender, disease stage, ZAP70/CD38 expression, or IGHV status (**Table S1**). The assessment of established biomarkers was performed as described[31]. All published genetic and biological data on CLL4 patients for genes: *TP53*[8], *ATM*[12,13], *BIRC3*[12], *NOTCH1*[17] (+3'UTR[21]), and *SF3B1*[17], and CNAs: 13q deletion, 17p deletion, 11q deletion, and trisomy 12 (5%, 10%, 5%, and 3% clone size cut-offs, respectively[31]) were integrated into this study, as well as telomere length[32] and levels of prolymphocytes[33].

Targeted re-sequencing, bioinformatics analysis, variant filtering and validation

Mutations in 22 genes were analysed in all 499 patients (TruSeq Custom Amplicon, Illumina, San Diego, CA, USA) (**Table S2**). Libraries were generated from 250 ng or 50 ng (dependent on the amount of available starting material) of DNA according to manufacturer's instructions. The average sequencing yield after Illumina processing (MiSeq, paired-end, 2x150 bp) from 28 runs was 6.9Gbp, with a mean read depth of >1000x (range 502 – 7948)

across all targeted genes, with only 9 amplicons below a mean read depth of 1000 (range 502 – 987) (**Figure S3**).

At this depth subclonal mutations can be detected at the 2% level, assuming a minimum observation of 4 sequencing reads containing the variant base, a Q50 phred like base quality score (p(detected) = 99.999) and a cumulative binomial distribution for n read depth [$\frac{N!}{n!(N-n)!}p^n(1-p)^{N-n}$]. In addition, 6 variants below 2% were included, since the number of sequencing reads in the variant base were more than ten times the assumed minimum observation (range 50 – 126), and the total read depth exceeded 2000 reads in all cases (range 2582 – 6389). Bioinformatic data processing of variants was conducted as previously described[14].

All mutations included in this study are listed in **Table S3**. As the CLL4 cohort lacked germ-line DNA we only considered variants previously observed as somatically acquired in CLL[1,2,14] or annotated in COSMIC (v70)[34], except for specific circumstances regarding *TP53, ATM, BIRC3* and *NOTCH1*. For *TP53*, additional mutations annotated in IARC were re-introduced [35]. Pathogenic *ATM* variants were included if; they were observed in AT families as pathogenic (LOVD [https://databases.lovd.nl/shared/genes/ATM]), they were evolutionary rare missense[36], or were somatically acquired in CLL[13] (**Table S4**). However, this variant strategy does not fully preclude *ATM* variants that exist in germ-line material. For *BIRC3*, only truncating mutations were included[9]. *NOTCH1* PEST domain mutations not predicted to result in protein truncation were removed. All candidate variants were visually inspected in Integrated Genomics Viewer[37]. Genes were defined as recurrent using Tumor Portal (www.tumorportal.org/power), with the background mutation rate for CLL stated on the website, and the number of cases in the study (n = 499) inputted. Mutations were stratified using Sanger sequencing threshold of 12%[5,9].

Thirty-one percent (194/623) of mutations were validated using orthogonal approaches, including Sanger (n=120) and Ion Torrent (19 low-level *TP53* mutations) sequencing, hybridization-based gene enrichment with subsequent sequencing (n=27) and ddPCR (*SF3B1* p.K700E [n=11], *NOTCH1* p.P2415fs [n=19]). 100% of variants were confirmed using this approach. An excellent agreement between TruSeq and orthogonal-derived VAFs was also observed, with an agreement bias of 0.02% (**Figure S4**).

Statistical analysis

Fisher's exact tests were performed for co-occurrence analysis between mutated genes and clinical features. PFS and OS was assessed from randomisation using Kaplan Meier (KM) and Log rank analysis. PFS was defined as time from randomisation to progression (i.e. relapse needing further treatment) or death, or to last follow-up date (Oct 2010; final CLL4 PFS update). OS was defined as time from randomisation to death or to last follow-up date for survivors (August 2016, final CLL4 OS update). Multivariate Cox Proportional Hazard models were generated for OS and PFS using backwards selection (P<0.05), to test the confounding effect of multiple prognostic variables. The Bland-Altman test was used to test agreement between multiple factors, reporting the agreement bias, which is the mean difference between two measurements. All reported *P* values were 2-sided and results were considered significant at the 5% level, using multiple hypothesis testing when appropriate (Benjamini and Hochberg method[38]). Statistical analysis was conducted using R v3.3.0, SPSS v23 (IBM), and Prism v6.0g (GraphPad).

Results

Distribution of somatic mutations

We identified 623 mutations (mean = 1.25, min/max = 0/7 per patient) in 335 patients, 398 ≥12% VAF and 225 <12% VAF, with 93% of the entire cohort harbouring ≥1 mutation or CNA (**Figure 1A**). 97 patients without any established CNAs carried 124 mutations (mean = 1.28, min/max = 0/6 per patient), with 22 patients lacking any mutations or CNAs. After background correction (0.5/Mb, ≥3% recurrence, **Table S5**), 11/22 genes were recurrently mutated at frequencies between 3.6% (*NFKBIE*) and 24% (*SF3B1*), (**Figure 1A**, **Table S3, Figure S7**). 121 samples harboured 134 *SF3B1* mutations; 46.3% were the p.K700E variant and 30.6% were other hotspot variants (p.K666X, p.H662X, pG740E, p.G742D). Two or more *SF3B1* mutations were identified in 12 patients (**Figure S5**), with six cases harbouring multiple *SF3B1* mutations present with different VAFs, suggesting the presence of multiple mutated sub-clones. 69 *NOTCH1* mutated patients were identified (13.8%), with 61 mutations in exon 34 (50/61 p.2514fs) and 9 in the 3'UTR. Fifty-five patients carried 59 IARC-annotated *TP53* mutations (exons 4-11, 88% in exons 5-8). Forty pathogenic *ATM* mutations were observed in 37 cases, without evidence of any mutational hotspots. *BIRC3*, *POT, BRAF, XPO1,* and *KRAS* were mutated in 7.2, 6, 6, 5.8, and 5.8% of cases, respectively. Thirty-eight cases harboured a mutation in *BRAF*, with 7 (18.4%) having the p.V600E variant (**Figure S6**).

Clinico-biological features of recurrently mutated genes

Next, we determined statistical associations between these gene mutations, and expansive clinico-biological features, using the Fisher's Exact test (n=1293 tests, **Figure 1B**). 126 associations were observed, including 15 high- (FDR, *Q*>*P* [*P*<0.05]), 35 medium- (*P*<0.01), and 76 low-confidence associations (*P*<0.05). Significant associations between mutations were found in only 10/171 possible associations, such as *NOTCH1*+3'UTR with *BIRC3* (*P*=0.02) and *FBXW7* (*P*=0.01), as well as *BRAF* with *TP53* (*P*=0.03) (**Figure 1B**, **Table S6**).

Distribution of ≥12% VAF and <12% VAF mutations

Next, we classified mutations as Sanger positive (≥12% VAF) or negative (<12% VAF) by accounting for the impact of tumor purity on VAF. Initially, we studied 233 patients with tumor purity derived from CD5/CD19 flow-cytometry. Raw VAFs were compared with purity-adjusted VAFs across all variants (n=288), including <12% VAFs (n=98), and showed an agreement bias of only 5% (**Figure S8A**), which was even lower for <12% VAF mutations (agreement bias <0.82%, **Figure S8B**). Therefore, we analysed all raw VAFs, and observed three variant populations: those found at <12% VAF (1.49-11.56%, n=225), those at larger sub-clonal or clonal levels (12.06-58.15%, n=356), and those concomitant with deletion events (60.19-99.66%, n=42) (**Figure 2A**). *SAMHD1* mutations were exclusively composed of ≥12% VAF (55.3% mean VAF), while *ATM, MYD88, NOTCH1, SF3B1, TP53,* and *XPO1* mutations were found to be contain a significant majority of ≥12% VAF mutations. *KRAS* mutations were more likely to be composed of low VAF variants, with a mean VAF of 10.7% (two-way binomial test*,* False Discovery Rate [FDR], *Q*>*P* [*P*<0.05]) (**Figure 2B**).

Univariate impact of mutated genes on PFS and OS

Clinico-biological features and gene mutations associated with PFS and OS in univariate Cox Proportional Hazards analysis are shown in **Figure 3A**. Gene mutations in *TP53* (with/without del(17p), termed '*TP53*ab') and *EGR2* were associated with reduced PFS (**Figure 3A, Table 1 and Figure S9**). *TP53*ab, and recurrent mutations in *SF3B1, NOTCH1* (+3'UTR)*, EGR2, RPS15, NFKBIE, BRAF, KRAS,* and *NRAS* were associated with reduced OS (**Figure 3A, Table 1 and Figure S10**). As expected, mutations in *MYD88* were confined to IGHV-mutated (IGHV-M) cases, having no significant impact on OS in this subgroup of patients (**Figure S11**). In addition, *TP53* mutations were associated with poor response (**Figure 3B**), *NOTCH1*+3'UTR mutations were associated with death from Richter's syndrome (**Figure 3C**), whilst *TP53, SF3B1, NOTCH1*+3'UTR, *KRAS*, and *EGR2* were significantly associated with <10yr survival (**Figure 3D**). Other significant associations are included in **Figures S12 & S13**.

Clinical relevance of TP53 deletions and mutations

TP53 mutations below the threshold of Sanger sequencing have been associated with inferior survival in retrospective analysis of institutional cohorts[5,9]. We observed 59 *TP53* mutations in 55 patients (**Figure 4A**); all of those tested (n = 51) were confirmed using orthogonal approaches (**Table S3**). These <12% *TP53* mutated were enriched for *BRAF* and *FBXW7* mutations (**Table S8**). *TP53* mutations could be further subdivided into those with <12% VAF (n = 16) or ≥12% VAF (n = 43), with no difference in the site or type of *TP53* mutation between subgroups (**Figure 4A**). After including 17p FISH data, 58 *TP53*ab patients

were identified, divided into cases with sole 17p deletions (n=3), isolated *TP53* mutations (n=27) or both (n=23). Five *TP53* mutated cases lacked FISH data.

Next, we assessed the genomic complexity of *TP53*mut cases. Both <12% VAF and ≥12% VAF *TP53*mut groups had increased mutation/CNA frequency in comparison to *TP53*wt cases (both *P*<0.001) (**Figure 4B**). To further understand the complexity of these two patient subgroups, we inferred the evolutionary history of *TP53*mut cases as previously described in CLL[2]. Both <12% VAF and ≥12% VAF cases exhibited the same heterogeneous pattern of co-existing mutations, where *TP53* mutations were present at higher, or lower VAFS than concomitant driver mutations (**Table S7, Figure 4C, Figure S14**).

Lastly, we assessed the clinical impact of <12% VAF and ≥12% VAF *TP53*mut subgroups in pairwise Kaplan Meier analysis. ≥12% VAF *TP53*mut were associated with reduced PFS and OS compared to cases with wild-type *TP53* (≥12% *TP53*mut = OS: median = 2.18yrs vs. 6.11yrs, *P*<0.001, PFS: median = 0.5yrs vs. 2.17yrs, *P*<0.001). In contrast, we could not demonstrate a significant difference between the <12% VAF *TP53*mut cases and either the wild-type or ≥12% VAF *TP53*mut patients for PFS or OS (<12% *TP53*mut = OS: median = 4.21yrs vs. 6.11yrs, *P* = 0.12, PFS: median = 1.92yrs vs. 2.17yrs, *P* = 0.196) (**Figure 4D & 4E**). These observations held true in 17p deletion stratified analysis (**Figure S15**), confirming the importance of *TP53*mut clone size on survival in this cohort. Stratified <12% VAF vs. ≥12% VAF analysis for other genes with sufficient mutated cases in this cohort can be found in **Figures S16 & S17**.

Biallelic BIRC3 deleted patients infer reduced overall survival in comparison to sole 11q deleted patients

Although neither *ATM* nor *BIRC3* mutations, regardless of their VAF **(Figures S16 & S17),** were associated with reduced PFS or OS in univariate survival analysis (**Figures S12 & S13**), it has previously been demonstrated that the impact of these mutations may be dependent on the presence of a concomitant 11q deletion[12,40]. Therefore, we performed an integrated analysis of the clinical impact of *ATM* and *BIRC3* mutations in the context of 11q-deleted CLL. *ATM* Mutations spanned the entire gene, whilst those targeting *BIRC3* were restricted to the CARD domain, as previously shown[9,11–13,40] (**Figure 5A**, **Figure S7**). Importantly, *ATM* and *BIRC3* mutations were mutually exclusive in our series (**Figure 5B**), suggesting that these mutations may define sub-groups of 11q-deleted CLL. Deletions of 11q were identified using a FISH probe which encompasses the *ATM* but not the *BIRC3* locus. Accordingly, concomitant *BIRC3* loss was defined from previously published SNP6.0 data[12], or where additional DNA was available (n=21), using shallow WGS (positive cases presented in **Figure S18**). Cases (n= 135) were then categorised into five distinct subgroups: sole 11q deletion (n = 71), biallelic *ATM* abnormalities (abs) (n = 12), biallelic *BIRC3* abs (n = 9), sole *ATM* mutations (n = 24) and sole *BIRC3* mutations (n = 19).

After omitting 10 cases with co-existing *TP53*ab[12], we conducted pairwise KM analysis for these five groups compared to cases with no 11q abnormality. (**Figure 5C and 5D; Figure S19**). For both PFS and OS, sole 11q deletion (PFS: median = 1.4yrs vs. 2.5yrs, $P<0.0001$, OS: median = 4.8yrs vs. 6.4yrs, $P=0.002$), as well as biallelic *ATM* (PFS: median = 1yr vs. 2.5yrs, $P=0.001$, OS: median = 4.2yrs vs. 6.4yrs, $P=0.049$) and biallelic *BIRC3* (PFS: median = 1yr vs.

2.5yrs, *P*<0.0001, OS: median = 3.3yrs vs. 6.4yrs, *P*=0.001), were associated with a significantly reduced survival.

The outcome of cases with biallelic abs was then compared to those with del(11q) only. There were no significant differences in PFS (biallelic *ATM* vs. 11q = 1yr vs. 1.4yrs, *P* = 0.336; biallelic *BIRC3*: 1yr vs. 1.4yrs, *P* = 0.178); however cases with biallelic *BIRC3* abs had a significantly reduced OS, whilst cases with biallelic *ATM* abs did not significantly differ in median survival times compared to sole 11q deleted cases (biallelic *ATM* vs. 11q = 4.2yrs vs. 4.8yrs, *P* = 0.493; biallelic *BIRC3*: 3.3yrs vs. 4.8yrs , *P* = 0.03). This suggests that biallelic loss of *BIRC3* represents the subgroup of 11q deleted CLL with the worst outcome following initial treatment with chemotherapy.

MAPK-ERK pathway members: BRAF, KRAS, and NRAS, all infer poor overall survival in CLL4

Mutations in *MAPK-ERK* genes, *BRAF* (38 mutations/30 cases), *KRAS* (34/29) and *NRAS* (11/10), were principally composed of specific hotspot variants (*BRAF*: p.G469A/E, *KRAS*: p.G13D, *NRAS*: p.Q61K/R) (**Figure S7**), and the majority of *MAPK-ERK* mutated cases (87%) only harboured a mutation in one of these genes (**Figure 6A**). Interestingly, *MAPK-ERK* mutated patients displayed an increased frequency of mutated genes and CNVs per case versus *MAPK-ERK* wild-type patients (**Figure S20**). In univariate analysis, each mutation was associated with a shorter median OS than wildtype: *BRAF* (OS median: 3.92yrs vs. 6yrs, *P* = 0.009), *KRAS* (OS median: 3.83yrs vs. 5.89yrs, *P*<0.001), and *NRAS* (OS median: 4.24yrs vs. 5.88yrs, *P* = 0.01) (**Figure 6B-D**). Stratified <12% VAF vs. ≥12% VAF analysis indicated that the outcome of *KRAS* mutated cases was independent of VAF while shorter OS in *BRAF* mutated cases was associated with <12% VAF **(Figure S16; Table S9)**. Taken together,

MAPK-ERK mutations exhibited inferior OS compared to wildtype cases (OS median: 3.83yrs vs. 6.10yrs, *P*<0.001), and were negatively associated with long-term survival (Odds Ratio = 0.19, *P* = 0.0003) (**Figure 6E**), with only 4/60 mutated cases defined as long-term survivors. Furthermore, *MAPK-ERK* mutated patients were more likely to carry IGHV-U genes (IGHV-U Odds Ratio = 4.29, *P* <0.0001; IGHV homology >99% Odds Ratio = 3.51, *P* = 0.0002), and significantly less likely to harbour del(13q) as a sole aberration (Odds Ratio = 0.23, *P*<0.0001, **Table S10**).

Multivariate modelling identifies TP53ab, biallelic BIRC3, SF3B1, EGR2, and MAPK-ERK gene mutations as independent markers of inferior OS.

Finally, we constructed comprehensive multivariate Cox Proportional Hazards models for PFS and OS (**Table 2**) which included those clinical and genetic variables significant in univariate analysis, as well as biallelic *ATM* and *BIRC3* they emerged from our stratified 11q deletion analysis, and short telomeres based on our previous paper on the topic[32]. A backwards selection approach was applied, until all variables within the model had a *P* value <0.05. For PFS, the final model was constructed from 225 patients and 210 events (274 were excluded due to missing data) and showed that *TP53*ab (HR = 4.98, *P*<0.001), biallelic *BIRC3* (HR = 3.83, *P* = 0.004), short telomeres (HR = 1.96, *P*<0.001), sole 11q deletion (HR = 1.82, *P* = 0.003), and increased prolymphocytes (HR = 1.51, *P* = 0.033) were independent markers of PFS. For OS, the final model was constructed from 391 patients and 323 events (108 observations were excluded due to missing data). *TP53*ab (HR = 4.25, *P*<0.001), biallelic *BIRC3* (HR = 2.76, *P* = 0.004), mutations in *EGR2* (HR = 2.19, *P* = 0.015), *MAPK-ERK* genes (HR = 1.68, *P* = 0.002), *SF3B1* (HR = 1.54, *P* = 0.001), as well as IGHV-U genes (HR = 1.83, *P*<0.001) and Binet stage B&C (HR = 1.45, *P* = 0.008), were all observed as independent

markers of OS. This data confirms our univariate survival analysis, showing that cases with biallelic *BIRC3* deletions exhibit reduced PFS and OS, and that mutations in the *MAPK-ERK* pathway lead to reduced OS.

Discussion

We report targeted re-sequencing analysis of 22 genes known to be recurrently mutated in CLL in the UK CLL4 clinical trial. CLL4 represents an ideal candidate for such an analysis, with expansive clinical and biological description[8,12,33, 13,17,21,24,26,29,31,32,41,42] and protracted clinical follow-up. Our study confirms previous studies incorporating samples from this patient cohort showing the impact of *TP53*ab on PFS and OS in MVA, *SF3B1, EGR2*[25,26], *RPS15*[1,24] and *NFKBIE*[25,28,43] mutations on OS in univariate analysis, with *SF3B1* and *EGR2* mutations retained as independent markers of OS in multivariate analysis.

The literature suggests that patients with *MAPK-ERK* mutations represent a biologically distinct subgroup, where *MAPK-ERK* mutations are frequently mutually exclusive, are enriched for trisomy 12, unmutated IGHV genes and other adverse biological markers (e.g. CD38, ZAP-70, CD49d), and are linked to inferior time to first treatment in retrospective cohorts [42,44–46]. We now show the *MAPK-ERK* genes, *BRAF, KRAS,* and *NRAS* (collectively representing 12.2% of patients) are also independently associated with short OS in a cohort of patients requiring treatment. Vendramini *et al.* showed a similar frequency of mutations in these genes (14%)[45], while Giménez and co-workers found that 5.5% of CLL cases harbours functionally deleterious mutations in 11 genes involved in the *MAPK-ERK* pathway[46], the latter likely reflects the early-stage composition of the cohort. In support of the biological impact of these mutated genes in CLL, 1) Analysis of mutated patients exhibit an enrichment of gene sets associated with transcriptional activation of the MAPK-ERK

pathway [45], 2) preliminary *in vitro* analysis suggests cells from these patients are prone to killing with ERK inhibitors[46], 3) *BRAF* mutations accelerated disease progression in Eμ-TCL1 mice[47], 4) mutant *BRAF* has been implicated in venetoclax resistance [48], and 5) *KRAS* mutated cases associated with poor response to chemoimmunotherapy[27] and lenalidomide[49].

Screening for *TP53*ab using FISH and Sanger sequencing has known prognostic value[6,8,20,31], and predicts for resistance to chemo-immunotherapy[50]. *TP53* mutations that present at low VAFs, below the detection limit of conventional Sanger sequencing may also be positively selected by chemotherapy, and also predict inferior survival, at least in retrospective, institutional cohorts[3,5,9]. The *TP53* Network of ERIC provide expansive guidelines on the most suitable approach for *TP53* mutational analysis, but also conclude that the clinical importance of low-level *TP53* clones remains an unresolved issue, requiring validation in clinical trials[50]. We demonstrated inferior PFS and OS only for those patients with ≥12% VAF *TP53* mutations, but we could not demonstrate inferior survival associated with cases harboring <12% VAF *TP53* mutations, the inference perhaps is that these cases represent an intermediate-risk group. Given the unexpected nature of this finding, we also conducted stratified 17p deleted survival analysis, identifying the same result for <12% VAF *TP53* mutations without 17p deletion. Furthermore, we proceeded to show that our observation was not associated with any differences in the type of *TP53* mutation, their co-existence with other more clonal prognostically-important gene mutations or biological features, nor the enrichment of any specific treatment. As a consequence, we feel that our observation is technically sound, and warrants confirmation in further studies.

There remains disagreement regarding the relative clinical significance of deletion and mutation of the *BIRC3* and *ATM* genes, both mapping to the long arm of chromosome 11.

The *ATM* gene is mutated in 30-40% of 11q deleted patients[11,13], where it results in biallelic inactivation of *ATM*, driving an impaired DNA damage response[51]. The prognostic impact of *ATM* mutations is controversial in unselected cohorts[9], with the strongest impact when the wild-type allele is lost. In our study, whilst we triaged *ATM* mutations based on their putative pathogenicity, several are reported in both somatic (i.e. COSMIC) and germline (i.e. dbSNP, EXAC, ClinVar) databases, lending uncertainty to their prognostic impact. The sequencing of matched germ-line material would provide additional clarity, but was not possible due to the historical nature of CLL4. Preliminary studies support a pathogenetic role of *BIRC3*[16,40], more recent studies provide less certainty. For example, in the RESONATE clinical trial[52] and the large retrospective study coordinated by ERIC[53], *BIRC3* mutations were not linked to inferior PFS or TTFT, respectively. Another comparator would be the RESONATE2 trial, which compared first line treatment with Ibrutinib v chlorambucil[54]. The 24 month PFS for 11q deleted patients in the Ibrutinib arm was 97%. Further studies are required to determine if the long-term outcome of biallelic *BIRC3* cases is equally good under modern small molecule inhibition. In our previous CLL4 analysis, we demonstrated that *BIRC3* dysfunction (defined as deletion AND/OR mutations of *BIRC3*) did not impact survival in 11q deleted CLL, while biallelic *ATM* lesions remained informative[12]. However, this analysis utilized Sanger sequencing, and hence only identified a small number of *BIRC3* mutations. Our current study, therefore aimed to expand the analysis with a larger patient cohort with significantly improved technology. This approach permitted the identification of a meaningful number of cases with loss and mutation of *BIRC3*. As neither *ATM* nor *BIRC3* mutations were linked to survival in univariate analysis, we performed a stratified analysis in 11q-deleted cases. In so doing, we show that biallelic *BIRC3* cases have a further reduction in survival in comparison to sole 11q deleted cases and were found to be independent

prognostic markers for PFS and OS in MVA. Finally, *ATM* and *BIRC3* mutated cases without 11q deletion have a similar survival to wildtype cases.

In conclusion, our study makes three main contributions to the field. We show an expansive analysis of the impact of clinico-biological disease features on the clinical importance of important gene mutations, including *SF3B1, EGR2,* and the *MAPK-ERK* genes. Our analysis suggests that <12% VAF *TP53* mutations are an intermediate survival group. Finally, we show that biallelic *BIRC3* aberrations identify a novel patient subgroup with poor survival, inferior to those with 11q-deletions alone. Taken together, we demonstrate that a more expansive genomic screening approach provides additional clinical information, thereby helping to establish the precise importance of genetic alterations in the context of other established and emerging biomarkers. Furthermore, our work will facilitate the development of international standards for the detection and interpretation of somatic mutations in CLL.

Declaration of interests

The authors declare no conflict of interest.

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Author contributions

SJB, MJJR-Z, RC, AS, and JCS designed the research. SJB, MJJR-Z & JCS analysed the data. SJB, MJJR-Z, DGO & JCS and wrote the paper. SJB, RC, HP, PA, ES-D. ML, ZD, LK PR, DV, JF, AB, RM, DC, ME, DB, HMC, DGO, RJW, AJS, MSC, MJJRZ, and AS performed the research and/or contributed patient samples and associated data. All authors read and agreed to the final version of the manuscript.

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Figure Legends

Figure 1. Mutation landscape and co-occurrence associations of the CLL4 cohort.

A Mutational Landscape of CLL4. In the Waterfall plot, known recurrently mutated genes and copy number alterations are shown, hierarchically clustered by mutation frequency (vertical bar chart, right). The mutation burden captured by the study is shown in the bar chart above the heat map. Mutation types are depicted in the above key. The inset vertical bar chart represents the distribution of the number of mutated genes/CNAs per case. **B** Co-occurrence of all available clinico-biological features from the CLL4 clinical trial. The co-occurrence (red) or mutual exclusivity (green) is plotted per interaction in the graph based on the level of significance (from light to dark: $P<0.05$, $P<0.01$, $Q>P$ [$P<0.05$], $Q>P$ [$P<0.01$]).

Figure 2. CLL4 mutation architecture.

A Distribution of mutation variant allele frequency. Scatter plot of all variants by read depth and VAF (red dots = <12% VAF [left of dotted line], blue dots = >12% VAF). **B** Distribution of ≥12% and <12% variants. Top: Proportion of ≥12% and <12% variants ranked by highest proportion of ≥12% VAF variants. Two-way binomial distribution used to test whether genes contained significantly more ≥12% VAF or <12% VAF mutations, with asterisks representing genes which retained significance after multiple hypothesis testing ($Q>P$ [$P<0.05$]). Bottom: VAF distribution of variants per gene. Variants with loss of the other allele (identified by FISH), shown in red for biallelic *TP53,* turquoise for biallelic *ATM* and pink for biallelic *BIRC3.*

Figure 3. Clinical outcome of mutated genes, CNAs, and clinical features in CLL4.

A Forest plot showing the hazard ratios of 26 significant variables for either overall survival (left; black) or progression free survival (right; red) in univariate survival analysis. Variables sorted by the hazard ratio values for overall survival. **B** Bar chart showing the mutation frequency difference between *TP53*mut cases who achieved CR/NodPR or NR/PD. **C** Bar chart showing the *NOTCH1*+3'UTR mutation frequency in relation to Death from Richter's syndrome. **D** Bar chart showing the mutation frequency in relation to patients termed 'long-term survivors' for *TP53, SF3B1, NOTCH*+3'UTR, *KRAS,* and *EGR2.*

Figure 4. Clinical relevance of <12% VAF *TP53* mutations in CLL4.

A Mutation Lolliplot displaying the *TP53* mutations observed in CLL4, stratified by Sanger sequencing threshold. **B** Mutated genes/CNVs per *TP53*mut subgroup. One-way ANOVA conducted vs. *TP53*wt cases. **C** Examples of In-going and out-going edges drawn from each *TP53*mut subgroup, with patient ID number and IGHV status defined above each graph. **D** OS pairwise KM plot comparing ≥12% VAF *TP53*mut cases (red), <12% VAF *TP53*mut cases(green), and *TP53*wt cases (black). **E** PFS pairwise KM plot comparing ≥12% VAF *TP53*mut cases (red), <12% VAF *TP53*mut cases(green), and *TP53*wt cases (black). Inset table in D&E displays pairwise log rank *P* values between each variable vs. wild type.

Figure 5. Importance of 11q deletion in the context of *ATM* and *BIRC3* mutations in CLL4.

A Mutation Lolliplot of *ATM* (upper) and *BIRC3* (lower) mutations observed in CLL4. **B** Heat map of *ATM* and *BIRC3* mutated cases stratified by 11q deletion status. **C** OS pairwise KM plot comparing mutated *ATM* (left) and *BIRC3* (right) in the context of 11q deletion. **D** PFS pairwise KM plot comparing mutated *ATM* (left) and *BIRC3* (right) in the context of 11q deletion. Inset table in C&D displays pairwise log rank *P* values between each variable vs.

591 wild type for combined pairwise KM analysis of *ATM* and *BIRC3* in the context of 11q
592 deletion.

593

594 **Figure 6. *MAPK-ERK* genes predict poor OS in CLL4.**

595 **A** Heat map of *BRAF* (blue)*, KRAS* (green)*, NRAS* (red), and co-mutated genes of *MAPK-ERK*
596 mutated cases (black). Cases wildtype for each gene represented by grey bars. **B-E** Overall
597 survival univariate KM plots for *BRAF* (**B**), *KRAS* (**C**), *NRAS* (**D**), and a combined variable of
598 *APK-ERK* (**E**). Coloured line represents mutated cases, black line represents wild type cases.

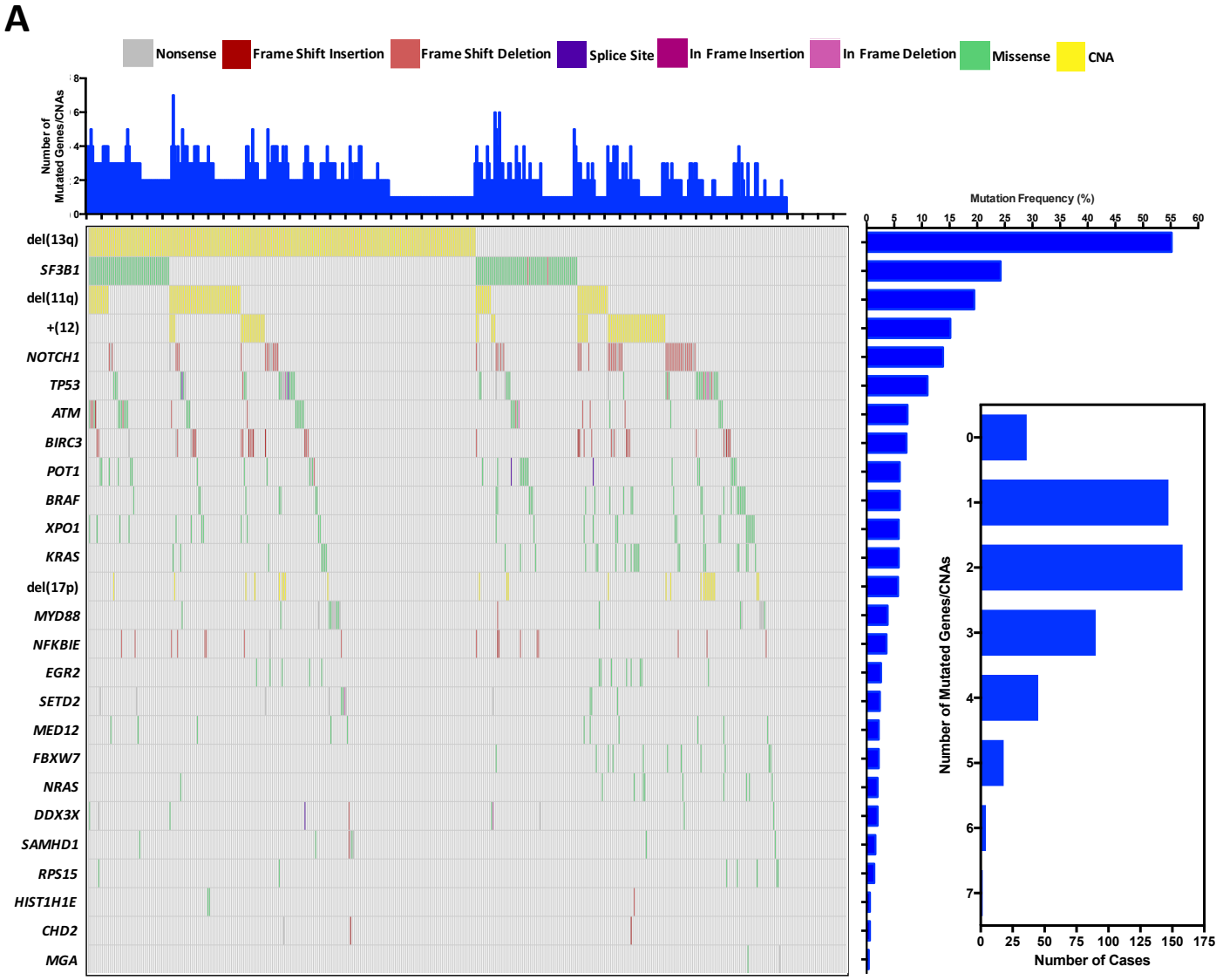
Table 1. Univariate survival association analysis for overall survival and progression free survival in CLL4.

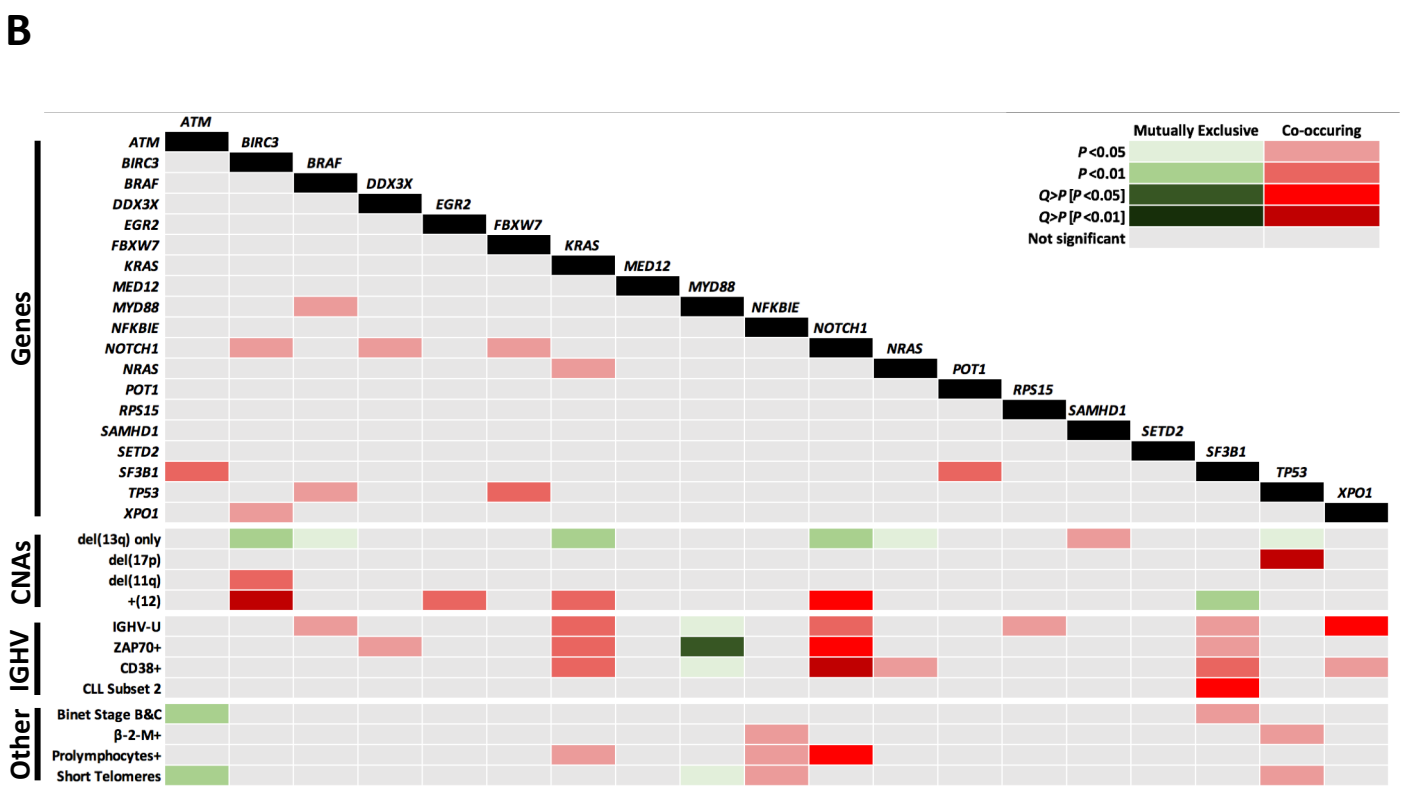
		Overall Survival									Progression-Free Survival								
Variable	**Status**	**Total**	**Events**	**Median (years)**	**LCI**	**UCI**	**HR**	**LCI**	**UCI**	***P* Value**	**Total**	**Events**	**Median (years)**	**LCI**	**UCI**	**HR**	**LCI**	**UCI**	***P* Value**
BRAF	Unmutated	469	383	6	5.39	6.55	-	-	-	-	469	429	2	1.83	2.33	-	-	-	-
	Mutated	30	28	2.87	6.25	7.5	1.66	1.13	2.44	0.009	30	30	2.12	1.42	3.33	1.11	0.77	1.61	0.586
EGR2	Unmutated	486	398	5.95	5.08	6.39	-	-	-	-	486	446	2.08	22	28	-	-	-	-
	Mutated	13	13	3.45	3.85	-	2.91	1.67	5.1	<0.0001	13	13	0.415	10	NA	2	1.15	3.49	0.012
KRAS	Unmutated	470	383	5.89	5.32	6.44	-	-	-	-	470	430	2.04	22	28	-	-	-	-
	Mutated	29	28	3.83	2.79	6.86	1.96	1.33	2.89	<0.001	29	29	1.92	18	35	1.29	0.89	1.89	0.18
MYD88	Unmutated	480	401	5.67	4.96	6.25	-	-	-	-	480	442	1.96	21	27	-	-	-	-
	Mutated	19	10	10.3	7.15	-	0.42	0.22	0.79	0.005	19	17	2.67	27	68	0.76	0.47	1.23	0.262
NFKBIE	Unmutated	481	393	5.9	5.32	6.47	-	-	-	-	481	441	2	22	28	-	-	-	-
	Mutated	18	18	3.61	2.77	6.98	2.01	1.25	3.23	0.003	18	18	1.83	16	35	1.59	0.99	2.55	0.054
***NOTCH1*+3'UTR**	Unmutated	375	306	6.22	5.56	6.73	-	-	-	-	430	391	2.17	22	28	-	-	-	-
	Mutated	69	62	4.28	3.62	6.03	1.47	1.12	1.94	0.005	69	68	1.92	17	30	1.24	0.96	1.61	0.099
NRAS	Unmutated	489	401	5.88	5.23	6.44	-	-	-	-	489	450	2	22	28	-	-	-	-
	Mutated	10	10	4.24	2.05	-	2.21	1.18	4.16	0.011	10	9	2.54	19	NA	0.9	0.47	1.75	0.758
RPS15	Unmutated	492	404	5.86	5.3	6.42	-	-	-	-	492	452	2.08	22	28	-	-	-	-
	Mutated	7	7	2.89	2.18	-	2.37	1.12	5.03	0.02	7	7	1.75	3	NA	1.99	0.94	4.21	0.067
SF3B1	Unmutated	378	301	6.33	5.64	6.99	-	-	-	-	378	344	2.17	22	29	-	-	-	-
	Mutated	121	110	4.49	3.92	5.65	1.48	1.19	1.85	<0.001	121	115	1.92	17	28	1.19	0.96	1.47	0.112
TP53	Unmutated	444	360	6.15	5.64	6.7	-	-	-	-	451	413	2.17	23	29	-	-	-	-
	Mutated	55	51	2.65	1.47	3.87	2	1.49	2.69	<0.001	48	46	0.5	5	15	1.95	1.27	2.34	<0.0001

Table 2. Multivariate Cox model for overall survival and progression free survival in CLL4.

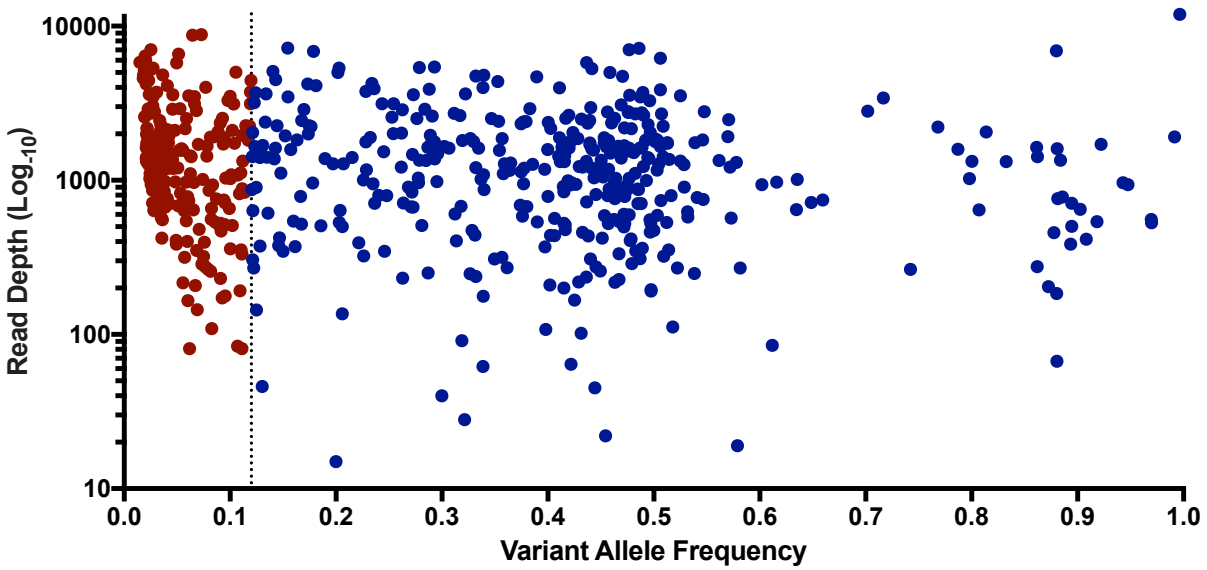
Survival	Variable	HR	LCI	UCI	*P*
Overall	***TP53*ab**	4.247	2.932	6.151	<0.0001
	Biallelic *BIRC3*	2.756	1.397	5.438	0.003
	***EGR2* mutated**	2.188	1.167	4.099	0.015
	IGHV-U	1.831	1.417	2.364	<0.0001
	***MAPK-ERK* mutated**	1.683	1.202	2.356	0.002
	***SF3B1* mutated**	1.544	1.191	2.002	0.001
	Binet Stage B & C	1.454	1.102	1.918	0.008
	11q deletion	1.431	1.081	1.895	0.012
Progression-Free	***TP53*ab**	4.975	3.049	8.118	<0.001
	Short Telomeres	1.964	1.466	2.629	<0.001
	11q deletion	1.816	1.226	2.688	0.003
	Biallelic *BIRC3*	3.833	1.537	9.557	0.004
	Prolymphocytes	1.508	1.034	2.198	0.033

The OS model was built using the following starting variables: *MAPK-ERK*mut, *TP53*ab (after removal of <12% *TP53* mutations), *EGR2*mut, *RPS15*mut, *NFKBIE*mut, *MYD88*mut, *SF3B1*mut, *NOTCH1*+3'UTRmut, Binet Stage B&C, 11q deletion, biallelic *ATM*, biallelic *BIRC3*, sole 13q deletion, trisomy 12, IGHV-U. The final model for OS consisted of 391 patients and 323 events. The PFS model was built using the following starting variables: *TP53*ab, *EGR2*mut, biallelic *ATM*, biallelic *BIRC3*, 11q deletion without *ATM* or *BIRC3* mutations, sole 13q deletion, Short Telomeres, Prolymphocytes+, and IGHV-U. The final model for PFS consisted of 225 patients and 210 events. Variables for both OS and PFS MVA models were removed using the backwards selection method. HR = Hazard Ratio, LCI = Lower Confidence Interval, UCI = Upper Confidence Interval, *P* = Multivariate Log Rank *P* value.

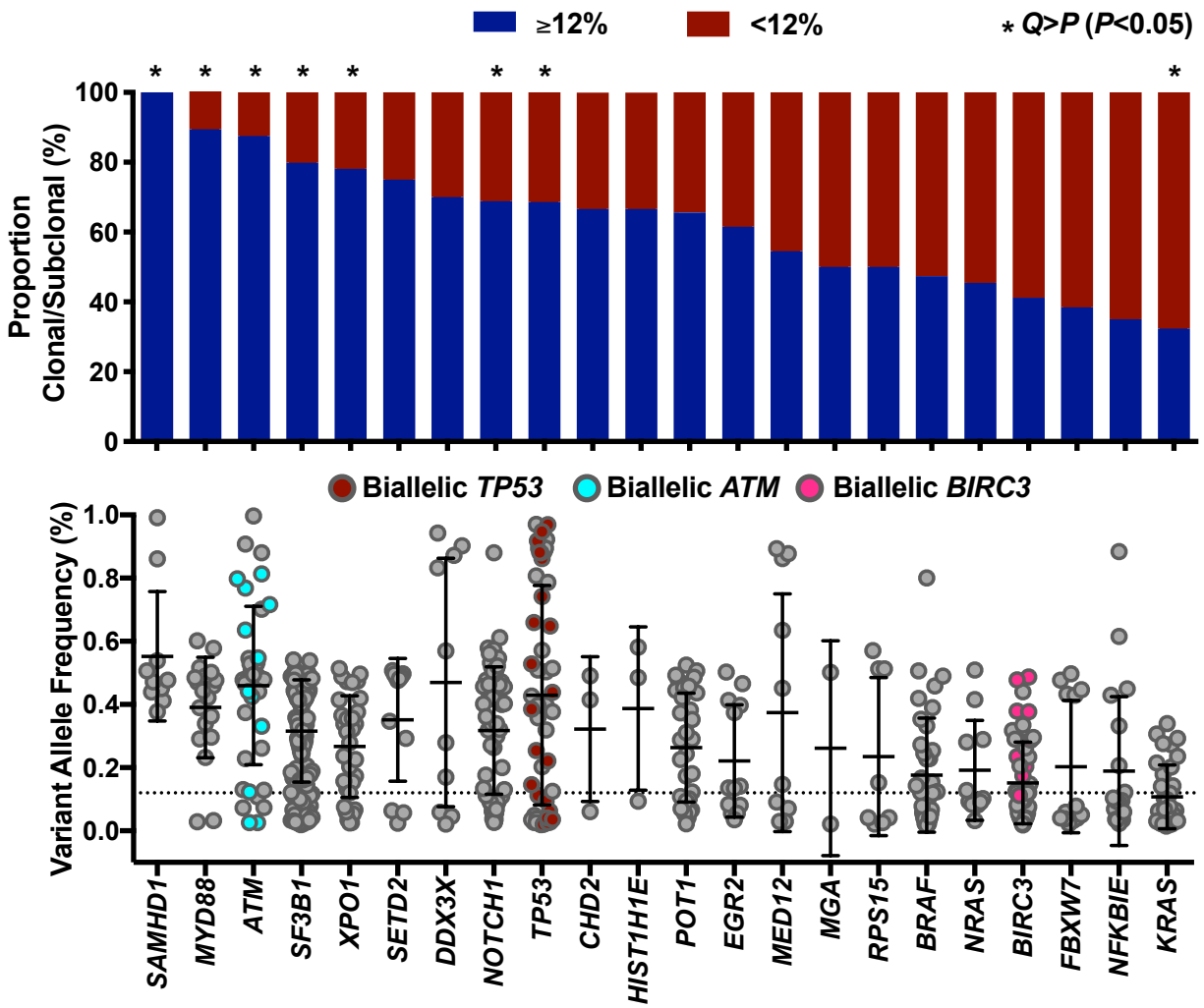

A
Nonsense
Frame Shift Insertion
Frame Shift Deletion
Splice Site
In Frame Insertion
In Frame Deletion
Missense
CNA
Number of Mutated Genes/CNAs
Mutation Frequency (%)
del(13q)
SF3B1
del(11q)
+(12)
NOTCH1
TP53
ATM
BIRC3
POT1
BRAF
XPO1
KRAS
del(17p)
MYD88
NFKBIE
EGR2
SETD2
MED12
FBXW7
NRAS
DDX3X
SAMHD1
RPS15
HIST1H1E
CHD2
MGA
Number of Mutated Genes/CNAs
Number of Cases

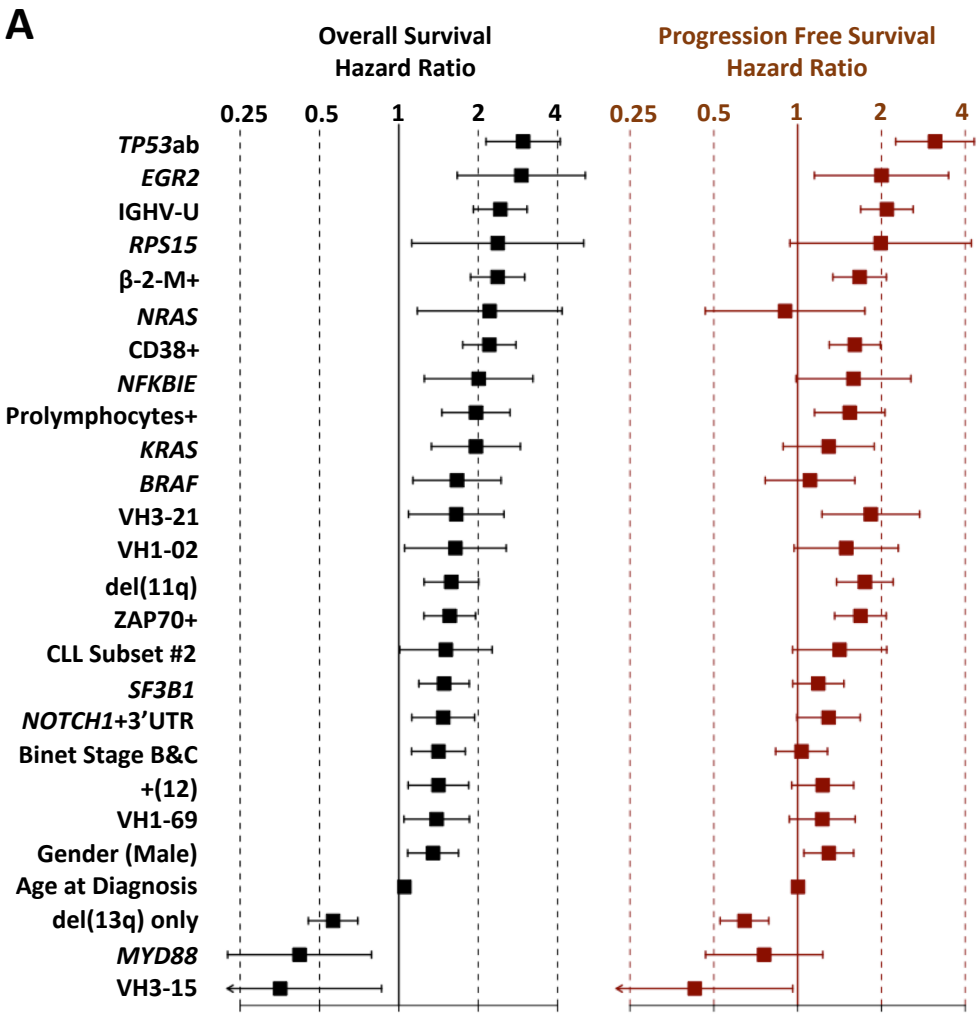

B
Genes
CNAs
IGHV
Other
Mutually Exclusive
Co-occuring
P<0.05
P<0.01
Q>P [P<0.05]
Q>P [P<0.01]
Not significant
del(13q) only
del(17p)
del(11q)
+(12)
IGHV-U
ZAP70+
CD38+
CLL Subset 2
Binet Stage B&C
β-2-M+
Prolymphocytes+
Short Telomeres

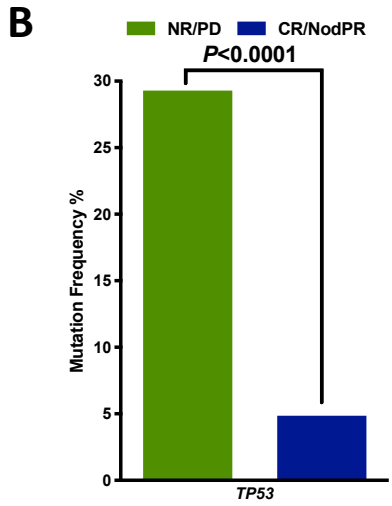
A

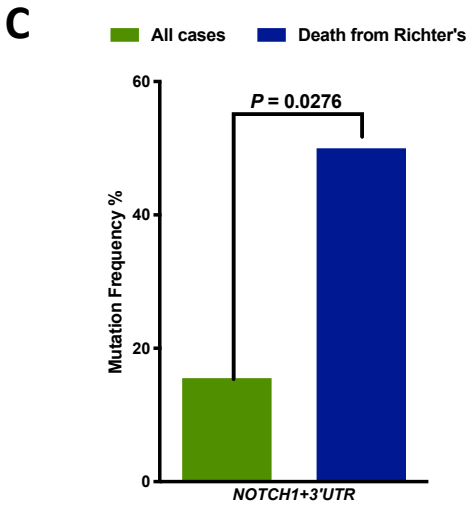

Read Depth (Log10)
10000
1000
100
10
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Variant Allele Frequency

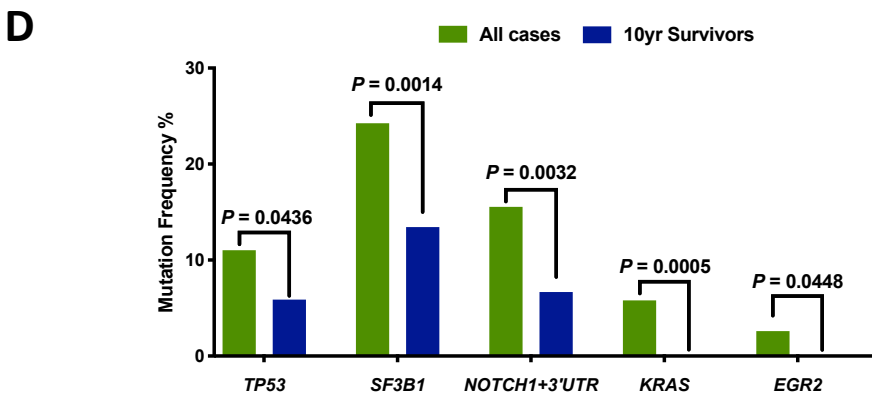
B

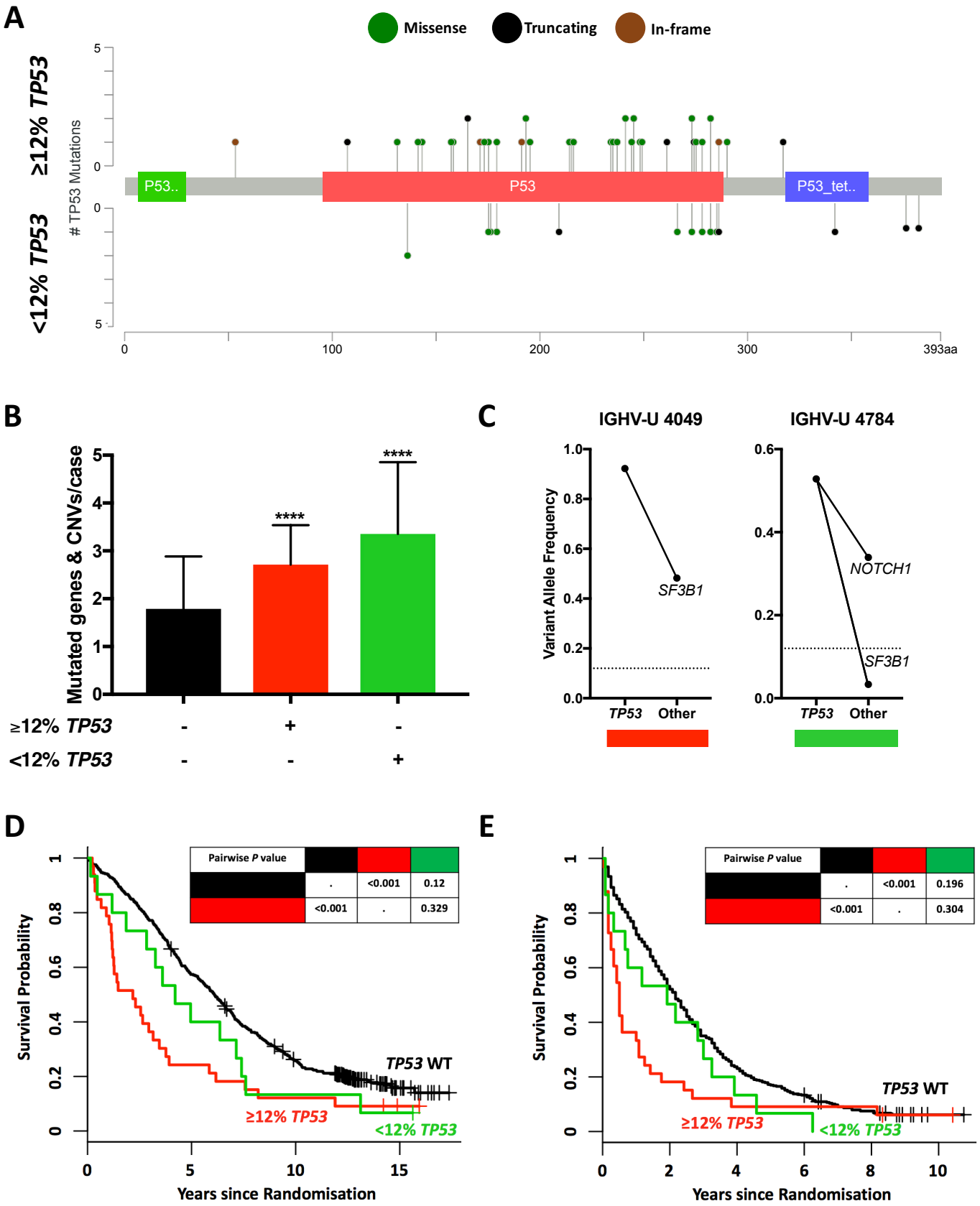

≥12%
<12%
* Q>P (P<0.05)
Proportion Clonal/Subclonal (%)
100
80
60
40
20
0
Biallelic TP53
Biallelic ATM
Biallelic BIRC3
Variant Allele Frequency (%)
1.0
0.8
0.6
0.4
0.2
0.0
SAMHD1
MYD88
ATM
SF3B1
XPO1
SETD2
DDX3X
NOTCH1
TP53
CHD2
HIST1H1E
POT1
EGR2
MED12
MGA
RPS15
BRAF
NRAS
BIRC3
FBXW7
NFKBIE
KRAS

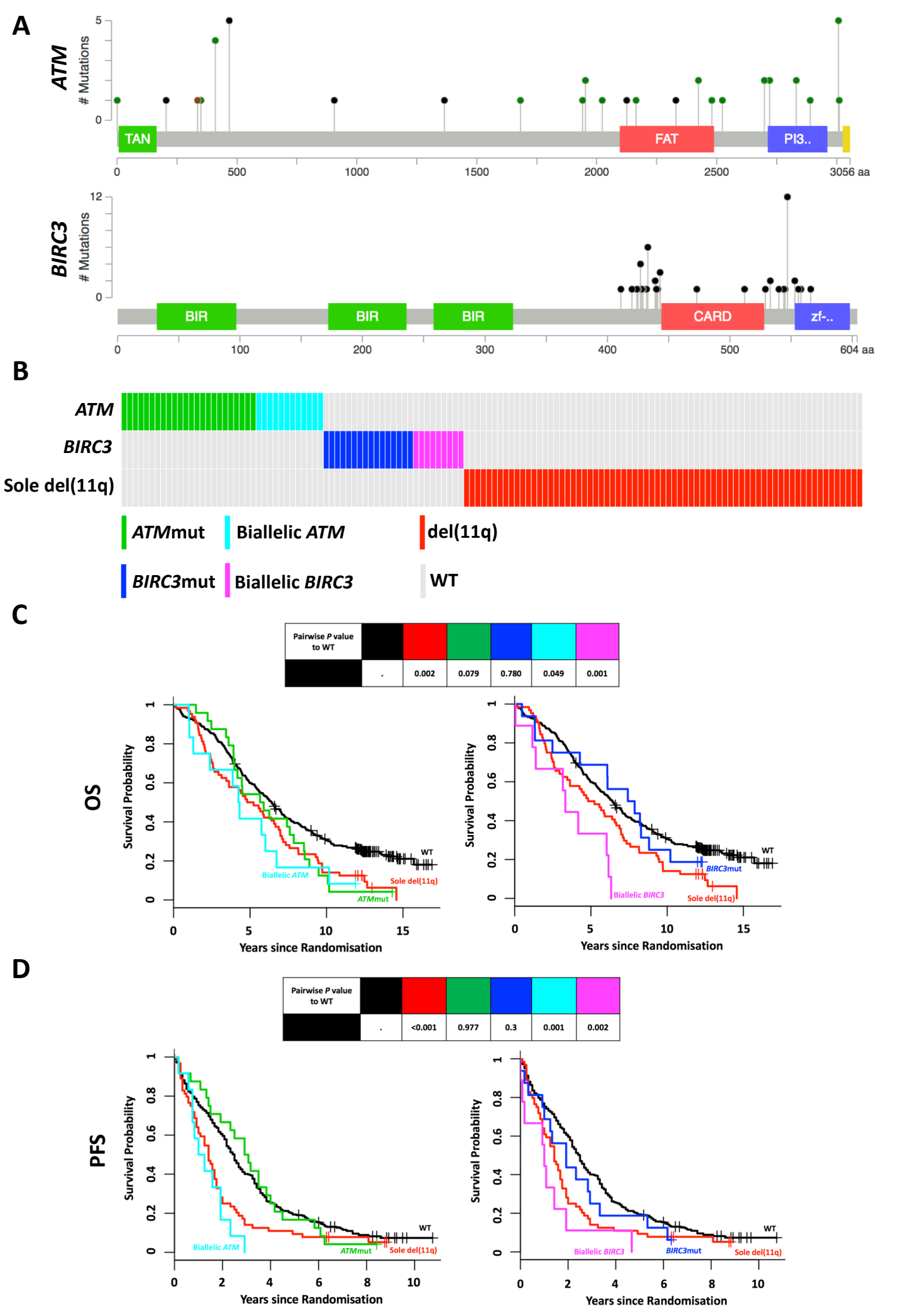

A
Overall Survival
Hazard Ratio
Progression Free Survival
Hazard Ratio
0.25
0.5
1
2
4
TP53ab
EGR2
IGHV-U
RPS15
β-2-M+
NRAS
CD38+
NFKBIE
Prolymphocytes+
KRAS
BRAF
VH3-21
VH1-02
del(11q)
ZAP70+
CLL Subset #2
SF3B1
NOTCH1+3'UTR
Binet Stage B&C
+(12)
VH1-69
Gender (Male)
Age at Diagnosis
del(13q) only
MYD88
VH3-15

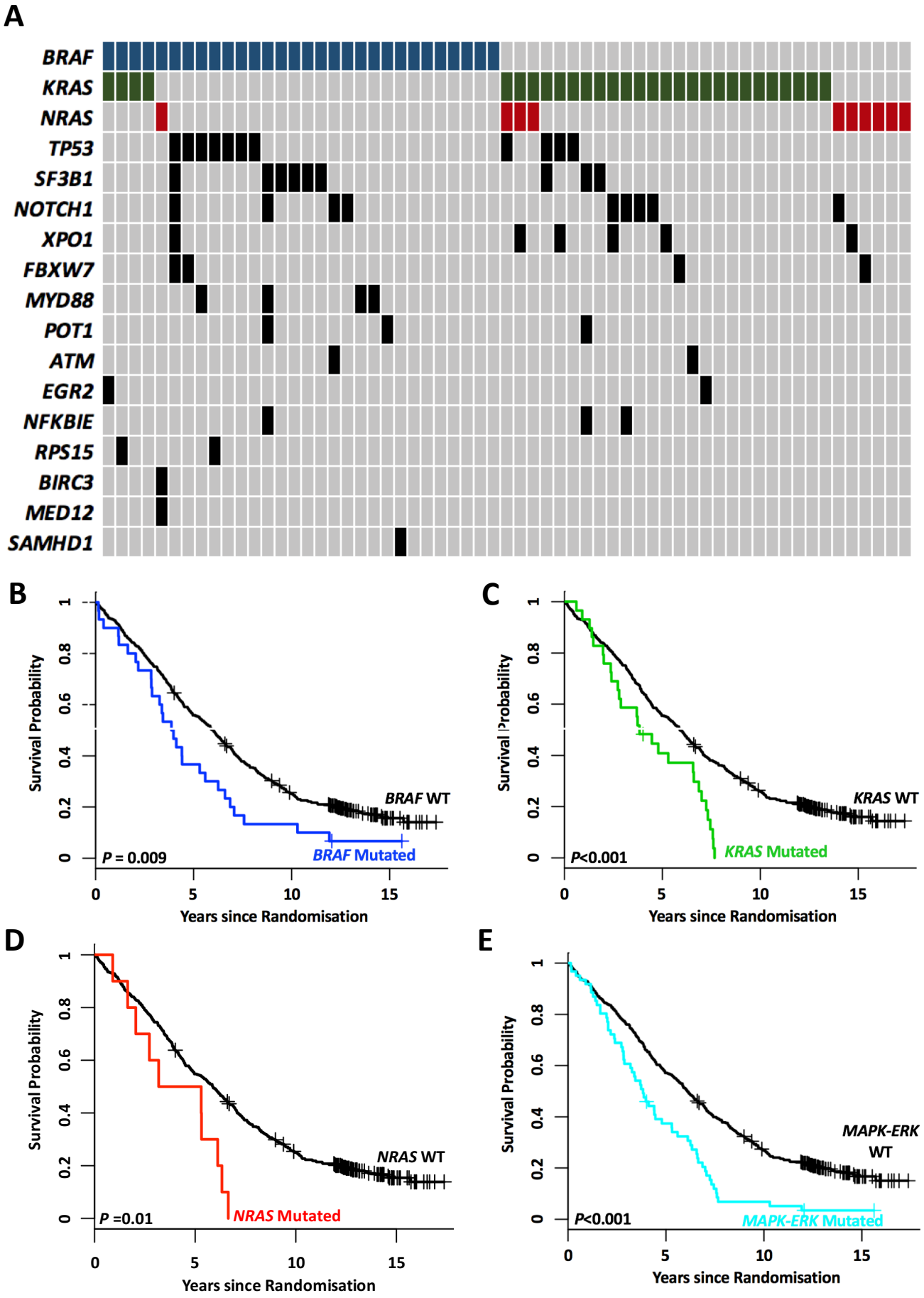

B
NR/PD
CR/NodPR
P<0.0001
Mutation Frequency %
30
25
20
15
10
5
0
TP53


C
All cases
Death from Richter's
P = 0.0276
Mutation Frequency %
60
40
20
0
NOTCH1+3'UTR


D
All cases
10yr Survivors
Mutation Frequency %
30
20
10
0
P = 0.0436
P = 0.0014
P = 0.0032
P = 0.0005
P = 0.0448
TP53
SF3B1
NOTCH1+3'UTR
KRAS
EGR2


A
Missense
Truncating
In-frame
≥12% TP53
<12% TP53
TP53 Mutations
P53..
P53
P53_tet..
393aa
B
Mutated genes & CNVs/case
****
****
≥12% TP53
<12% TP53
C
IGHV-U 4049
IGHV-U 4784
Variant Allele Frequency
SF3B1
NOTCH1
TP53
Other
D
Pairwise P value
<0.001
0.12
0.329
Survival Probability
TP53 WT
≥12% TP53
<12% TP53
Years since Randomisation
E
Pairwise P value
<0.001
0.196
0.304
Survival Probability
TP53 WT
≥12% TP53
<12% TP53
Years since Randomisation


A
ATM
Mutations
5
0
TAN
FAT
PI3..
0
500
1000
1500
2000
2500
3056 aa
BIRC3
Mutations
12
0
BIR
BIR
BIR
CARD
zf-..
0
100
200
300
400
500
604 aa
B
ATM
BIRC3
Sole del(11q)
ATMmut
Biallelic ATM
del(11q)
BIRC3mut
Biallelic BIRC3
WT
C
Pairwise P value to WT
.
0.002
0.079
0.780
0.049
0.001
OS
Survival Probability
Years since Randomisation
WT
Biallelic ATM
Sole del(11q)
ATMmut
Biallelic BIRC3
BIRC3mut
D
Pairwise P value to WT
.
<0.001
0.977
0.3
0.001
0.002
PFS
Survival Probability
Years since Randomisation
WT
Biallelic ATM
ATMmut
Sole del(11q)
Biallelic BIRC3
BIRC3mut


A
BRAF
KRAS
NRAS
TP53
SF3B1
NOTCH1
XPO1
FBXW7
MYD88
POT1
ATM
EGR2
NFKBIE
RPS15
BIRC3
MED12
SAMHD1
B
Survival Probability
BRAF WT
BRAF Mutated
P = 0.009
Years since Randomisation
C
Survival Probability
KRAS WT
KRAS Mutated
P<0.001
Years since Randomisation
D
Survival Probability
NRAS WT
NRAS Mutated
P =0.01
Years since Randomisation
E
Survival Probability
MAPK-ERK WT
MAPK-ERK Mutated
P<0.001
Years since Randomisation