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BCL-2 dependence is a favorable predictive marker of response to therapy for chronic lymphocytic leukemia

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Abstract

Background Established genetic biomarkers in chronic lymphocytic leukemia (CLL) have been useful in predicting response to chemoimmunotherapy but are less predictive of response to targeted therapies. With several such targeted therapies now approved for CLL, identifying novel, non-genetic predictive biomarkers of response may help to select the optimal therapy for individual patients.

Methods We coupled data from a functional precision medicine technique called BH3-profiling, which assesses cellular cytochrome *c* loss levels as indicators for survival dependence on anti-apoptotic proteins, with multi-omics data consisting of targeted and whole-exome sequencing, genome-wide DNA methylation profiles, RNA-sequencing, protein and functional analyses, to identify biomarkers for treatment response in CLL patients.

Results We initially studied 73 CLL patients from a discovery cohort. We found that greater dependence on the anti-apoptotic BCL-2 protein was associated with prognostically favorable genetic biomarkers. Furthermore, BCL-2 dependence was strongly associated with gene expression patterns and signaling pathways that suggest a more targeted drug-sensitive milieu and was predictive of drug responses. We subsequently demonstrated that these associations were causal in cell lines and additional CLL patient samples. To validate the findings from our discovery cohort and in vitro studies, we utilized primary CLL cells from 54 additional patients treated on a prospective, phase-2 clinical trial of the BTK inhibitor ibrutinib given in combination with chemoimmunotherapy (fludarabine, cyclophosphamide, rituximab) and confirmed in this independent dataset that higher BCL-2 dependence predicted favorable clinical response, independent of the genetic background of the CLL cells.

Conclusion We comprehensively defined BCL-2 dependence as a potential functional and predictive biomarker of treatment response in CLL, underscoring the importance of characterizing apoptotic signaling in CLL to stratify patients beyond genetic markers and identifying novel combinations to exploit BCL-2 dependence therapeutically. Our approach has the potential to help optimize targeted therapy combinations for CLL patients.

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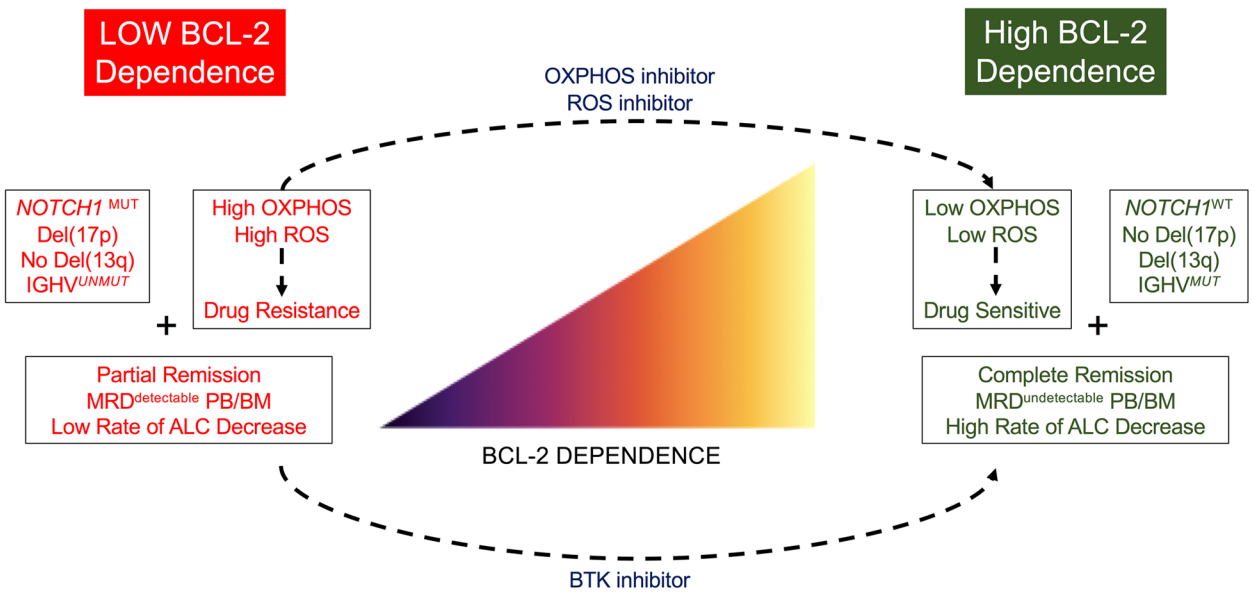
Graphical Abstract



Introduction

Over the last decade, targeted agents have transformed the therapeutic landscape in chronic lymphocytic leukemia (CLL). Although predictive of response to chemoimmunotherapy, established genetic biomarkers such as deletion-17p (del(17p)), mutation of tumor protein-53 (*TP53*), and the mutational status of immunoglobulin heavy chain variable gene (IGHV) are less useful in predicting response to targeted agents [1]. For example, patients with CLL who are treated with continuous Bruton Tyrosine Kinase inhibitors (BTKi) have similar median progression-free survival (PFS) irrespective of IGHV mutational status [1–3]. Hence, additional biomarkers that predict response to targeted therapy would be useful in CLL to rationally guide therapy selection and optimize outcomes for individual patients.

Several important studies have dissected the diverse array of genomic aberrations observed in CLL [4, 5]. These rich datasets help us understand CLL biology; however, they do not fully explain the heterogeneity in response observed with targeted agents. Functional precision medicine may provide an additional layer of information to complement genomics, but until recently has not been widely studied in CLL. Initial work in this area utilized ex vivo sensitivity testing alongside genome, transcriptome, and DNA methylome analyses as a drug-perturbation strategy to classify CLL in phenotypic subgroups with characteristic dependences on the B-cell receptor (BCR) pathway, mammalian target of Rapamycin (mTOR), and mitogen-activated protein kinase kinase (MEK) [6]. Subsequent efforts using ex vivo profiling of response to individual drugs or combinations revealed synergistic drug effects and genetic dependences in CLL [7] as well as guided treatment with improved clinical benefits in certain patients with aggressive hematologic malignancies [8]. Despite the helpful insights gained from these studies, they did not specifically provide functional characterization of the intrinsic pathway of mitochondrial apoptosis, known to be a fundamental aspect of CLL pathophysiology [9–11].

The mitochondrial apoptotic pathway is regulated by the complex interactions between pro- and anti-apoptotic proteins of the B-cell leukemia/lymphoma-2 (BCL-2) family. The interactions of these proteins determine cell fate by governing the threshold of mitochondrial outer-membrane permeabilization and cytochrome *c* (CytC) release, followed by caspase-dependent apoptosis. BH3-profiling is a functional assay developed to interrogate the interactions of BCL-2 family members and thereby measure the proximity of a cell to the apoptotic threshold (known as "priming"), as well as to identify specific anti-apoptotic proteins a cell depends on for survival [12, 13].

To more completely characterize functional BCL-2 family dependence in CLL and begin to unravel the relationship of apoptotic priming to common genomic

aberrations and treatment response in this disease, we studied primary samples from 127 patients with CLL using BH3-profiling. This included an initial discovery cohort of 73 patients with available genomic data including targeted-sequencing (targeted-Seq), whole-exome sequencing (WES), genome-wide DNA methylation profiles and RNA-sequencing (RNA-Seq). Our findings suggest that dependence on the anti-apoptotic protein BCL-2 for survival is a functional predictive biomarker for treatment response, which we further validated experimentally in cell lines and ex vivo CLL patient samples. Using a validation cohort of 54 additional primary samples from CLL patients treated uniformly in a phase-2 clinical trial of the Bruton Tyrosine Kinase (BTK) inhibitor ibrutinib with the chemotherapy regimen fludarabine, cyclophosphamide, and rituximab (FCR) [14], we confirmed our findings in vivo in patients to demonstrate the potential clinical applicability of this approach.

Methods

Patient samples isolation and usage

Peripheral blood mononuclear cells (PBMCs) were collected and analyzed from 73 patients of our discovery cohort, and 54 samples of our validation cohort, with all patients fulfilling diagnostic and immunophenotypic criteria for CLL. Cells were isolated by density gradient centrifugation by Ficoll-Paque (GE Healthcare), viably frozen in FBS (Gibco) supplemented with 10% DMSO. Samples with > 60% viability and > 85% $CD19^+$ $CD5^+$ cells (PBMCs) were used for analysis.

Baseline characteristics of the discovery cohort are in supplementary material 2 and cited here [6]. For validation cohort, samples were collected from previously untreated patients in an investigator-initiated trial of CLL for frontline therapy with ibrutinib + FCR (iFCR) at pretreatment and after one-week of ibrutinib monotherapy (just prior to the first cycle of FCR). Baseline patient characteristics, study design, and full clinical results were previously reported [14]. Samples from both discovery and validation cohorts were BH3-profiled. Detailed BH3-profiling technique is in the supplemental information.

Multi-omics profiling

Multi-omics profiling, including WES, targeted-Seq, DNA methylation profiling and RNA-Seq, were previously performed on the same set of patient samples from the discovery cohort [6]. Clinical outcomes of these samples were recorded. Data are available in the R data package BloodCancerMultiOmics2017 from the Bioconductor project (http://bioconductor.org).

Integrative data analysis

Analyses were performed using R3.4, which include univariate association tests, multivariate regression with and without lasso penalization, Cox regression, generalized linear models, principal component analysis, and gene set enrichment analysis. For association tests between bioenergetic features and genetic variants (i.e. copy number variants and gene mutations), only those with five or more variant cases were included. All *P*-values from association tests were adjusted for multiple testing by applying the Benjamini–Hochberg procedure to control false discovery rate (FDR). The full analysis walkthrough and scripts as Rmarkdown files are available at (GitHub:https://github.com/Lu-Group-UKHD/CLL_BH3_Profiling).

Statistical analysis

Dotted graph bars depict mean ± SD. Significance tests were displayed in figure legends together with the number of replicates for each experiment. Significant *P*-value was set at < 0.05. Asterisks on graph bars indicate $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, or otherwise specified in the figures.

A more comprehensive method section could be found in the supplemental information file.

Results

Overall apoptotic priming and functional dependence correlate with distinct molecular features

As the heterogeneity of ex vivo drug responses in CLL has been found to be partly explained by the genomic background of patient samples [7], we first evaluated whether differences in mitochondrial priming and functional dependence on BCL-2 family anti-apoptotic proteins could complement genomic data to better understand this variation. We performed BH3-profiling on primary CLL cells derived from the peripheral blood of 73 patients in our discovery cohort, using 7 different BH3 peptides and mimetic drug in multiple concentrations each to capture the full range of different anti-apoptotic protein dependences (Fig. 1A, S1). These 7 BH3 peptides and mimetic drug have previously been characterized and tested to ensure their specificity to binding and inhibiting their respective anti-apoptotic proteins to elucidate survival dependences [15, 16]. Several concentrations of peptides and mimetic were included to ensure sufficient coverage of their effects and reliability in inducing CytC loss across a dynamic range. Cells were first incubated with individual BH3 peptides and BH3 mimetic drug that recognize specific anti-apoptotic proteins to induce CytC release. Greater CytC release

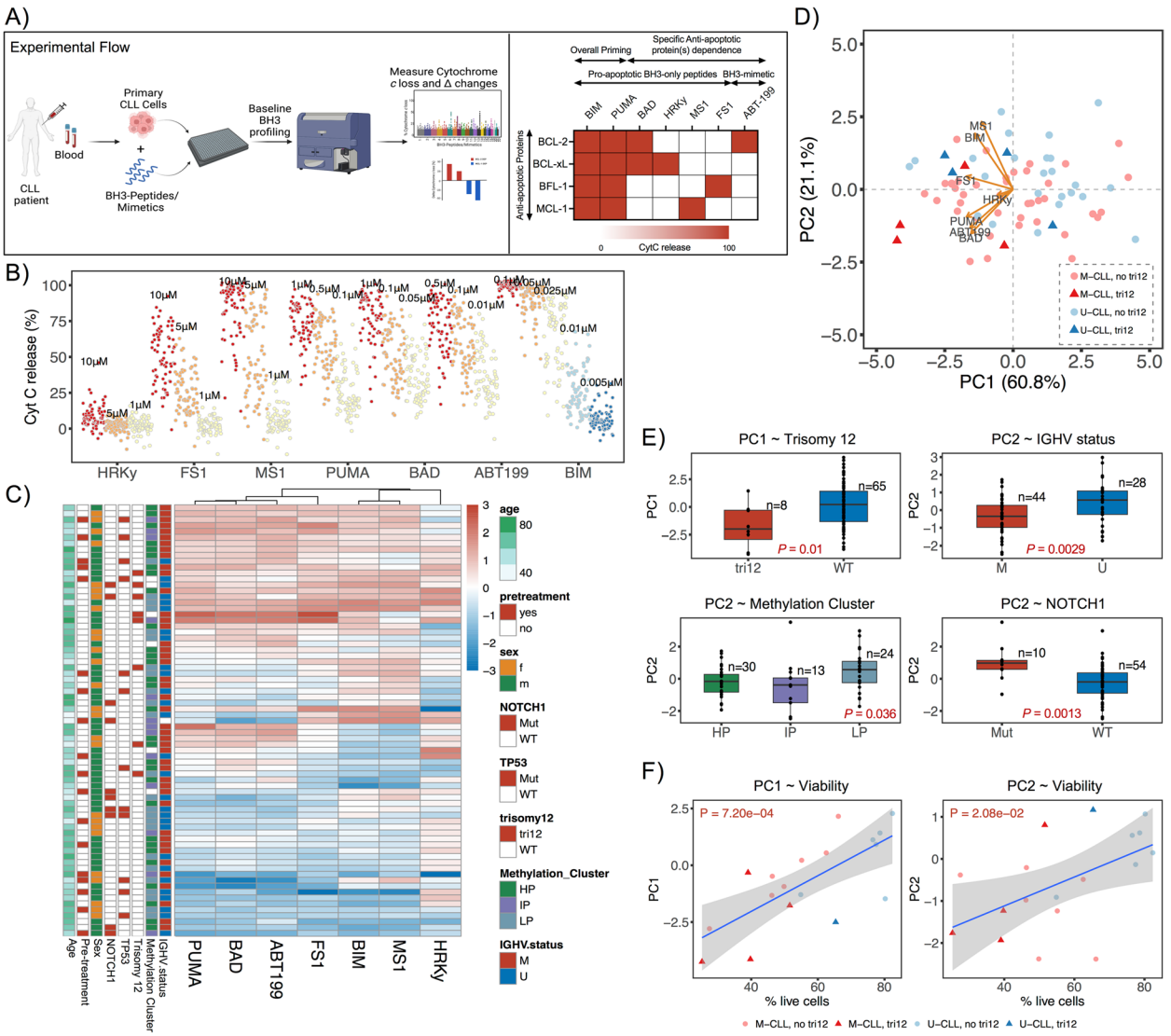


Fig. 1 BH3-profiling overview and its correlations with clinical and genetic backgrounds in primary CLL cells. **A** BH3-profiling is a technique used to measure how close a cell is to the threshold of apoptosis, and to identify how dependent a cell is on certain BCL-2 family anti-apoptotic protein(s) for survival. Measurements by flow cytometry are determined based on the level of cytochrome *c* (CytC) release, induced by specific BH3 peptides or mimetic drug, whereby the higher the CytC is released or loss (%), the higher the dependence(s) is towards its respective anti-apoptotic protein(s). The heatmap shows peptides or mimetic drug that measures the overall apoptotic priming of cells and the specific anti-apoptotic dependences of cells. Diagrams were created either in BioRender. Chamberlain, S. (2025) https://BioRender.com/y06s314 (left panel) or Powerpoint software (heatmap). **B** Measurement of CytC release (%) of 73 CLL patient samples from the discovery cohort, induced by the BH3 peptides or mimetic. **C** Heatmap showing BH3-profiling pattern with heterogenic clinical and genetic background. The CytC values (area under curve) were centered by mean and scaled by standard deviation column-wise in order to reveal the pattern among patient samples with heterogenic clinical and genetic background. **D** The PCA biplot shows CLL samples (points) and BH3 peptides or mimetics (arrows) on the first two principal components (PC1 and PC2). Points represent individual CLL samples, with distances indicating similarities, while arrows show variable (BH3 peptides or mimetic) contributions, with direction and length indicating correlations and influence (strength of correlations) on the components. Closer points suggest similar samples and angles between arrows indicate variable correlations. **E** Boxplots showing the significant associations (nominal *P* value < 0.05, Student's t-test) between principal components and patient genetic background. Tri12 – Trisomy 12, WT – wild type, M – mutated, U – unmutated, HP – highly programmed, IP – intermediately programmed or LP – lowly programmed CLL based on methylation clusters, Mut – mutated. **F** Significant correlations (nominal *P* value < 0.05, Student's t-test) between principal components and the % baseline viabilities of the cells from CLL patient samples (% live cells) after 48-h culture in media

indicates higher dependence(s) on specific anti-apoptotic protein(s), depicted by the heatmap (Fig. 1A, right panel). Significant heterogeneity was observed, with CLL cells displaying a wide range of baseline priming and marked variation in dependence on different anti-apoptotic BCL-2 family members (Fig. 1B, S1). Nonetheless, CLL cells have a predominant pattern of high BCL-2 dependence as shown by the significant CytC release even at low concentrations of ABT199 (VEN, BCL-2 specific inhibitor) or BAD (BCL-2 and BCL-xL-specific peptide), as compared to HRKy (BCL-xL-specific peptide), MS1 (MCL-1-specific peptide) or FS1 (BFL-1-specific peptide). The results from BAD peptide here specifically reflect BCL-2 dependence due to the low HRKy-dependent CytC release and lack of correlation to HRKy (BCL-xL dependence) (Fig. S2-S3).

To assess the molecular determinants of anti-apoptotic protein dependence and mitochondrial priming, we integrated our BH3-profiling data with targeted-Seq, WES, DNA methylation profiles and RNA-Seq data that were previously reported for this same patient cohort [6]. To characterize the overall landscape in an unbiased fashion, we first performed unsupervised hierarchical clustering of all pro-apoptotic peptides and CLL samples based on the BH3-profiling data (Fig. 1C). This clustering did not reveal distinct groups significantly related to genomic or clinical features but showed a continuous gradient that reflects a response pattern of CLL samples to pro-apoptotic peptides. This common trend was also observed in the principal component analysis (PCA), where the first principal component (PC1), which explains 60.8% of the variance in the BH3 profile, was negatively associated with the priming of all BH3 peptides and thus indicated a more resistant phenotype (Fig. 1D). By correlating PC1 with individual genomic backgrounds, we observed that CLL cells with trisomy 12 show significantly lower PC1 values, indicating that these samples are more responsive to BH3 peptide-induced CytC release and potentially to drug-induced mitochondrial apoptosis (Fig. 1D, E). This is in line with our previous observation that samples with trisomy 12 show higher ex vivo drug sensitivity to multiple kinase inhibitors [6].

In contrast, PC2, which explains 21.1% of the variance (Fig. 1D), distinguished the populations of CLL samples that fall under ABT199, BAD, and PUMA peptides, which indicate higher CytC release and apoptotic priming (Fig. 1B), or HRKy, FS1, MS1, which indicate relatively lower CytC release and hence more apoptotic-resistant (Fig. 1B). PC2 shows significant correlations with IGHV mutational status, and with the epigenetic sub-types of CLL (methylation clusters) (Fig. 1E). Mutated IGHV status correlating with negative PC2 values suggest that these cells are more responsive to ABT199, BAD or PUMA (Fig. 1E), corresponding to stronger CytC release with these BH3 mimetic/peptides (Fig. 1B). This is consistent with the more indolent nature of the disease in CLL patients with mutated IGHV [17]. In contrast, lowly programmed (LP) CLL or hypomethylation cluster correlated with higher PC2 values, suggesting that this cluster is less primed for apoptosis, which corresponds to our recent publication that CLL cells with high proliferative drive possess hypomethylation profile and worst outcome [18, 19]. PC2 also showed significant correlation with *NOTCH1* mutational status, whereby wild-type *NOTCH1* correlated with negative PC2 values (Fig. 1E). This is in line with the typically less aggressive behavior of CLL with wild-type *NOTCH1* [20, 21].

We also found that the viability of these primary CLL cells was positively correlated with PC1 and PC2, both of which have decreased apoptotic priming at their positive values, following 48 h of culturing in media (Fig. 1F). This validates our BH3-profiling data as a robust measurement of the propensity of CLL cells to undergo apoptosis, and also suggests that samples that are sensitive to BH3 peptide-induced CytC release (negative PC1 or 2 values) are likely to undergo cell death, as they are more readily primed for apoptosis and potentially more susceptible to therapeutic intervention.

BCL-2 dependence is positively correlated with known prognostic markers in CLL

Given that the PCA demonstrated correlations with mutational status of CLL, we next sought to evaluate the correlation between dependence on specific anti-apoptotic proteins and established genomic markers (Fig. 2A, S4). The most significant association was observed between the prognostically favorable mutated IGHV [17] and higher BCL-2 dependence, followed by trisomy 12 and higher BCL-2 dependence (Fig. 2A, B, S4). Lower BCL-2 dependence also correlated with CLL cells harboring the poor prognostic marker *NOTCH1* mutation (Fig. 2B) [20–22]. Notably, pre-treatment status was not a confounding factor when included as a co-variate in our linear models for association, as the association tests results were largely similar to when only previously untreated patients were included (Fig. S5). We next performed multivariate regression with LASSO penalty to select genomic and demographic features that can explain the heterogeneity of BH3 profiles. Among the selected features, poor prognostic factors such as del(17p) and *NOTCH1* mutation were correlated with lower BCL-2 dependence, while more favorable prognostic factors, such as del(13q) and trisomy 12 [23, 24], were correlated with higher BCL-2 dependence (Fig. 2C). Conversely,

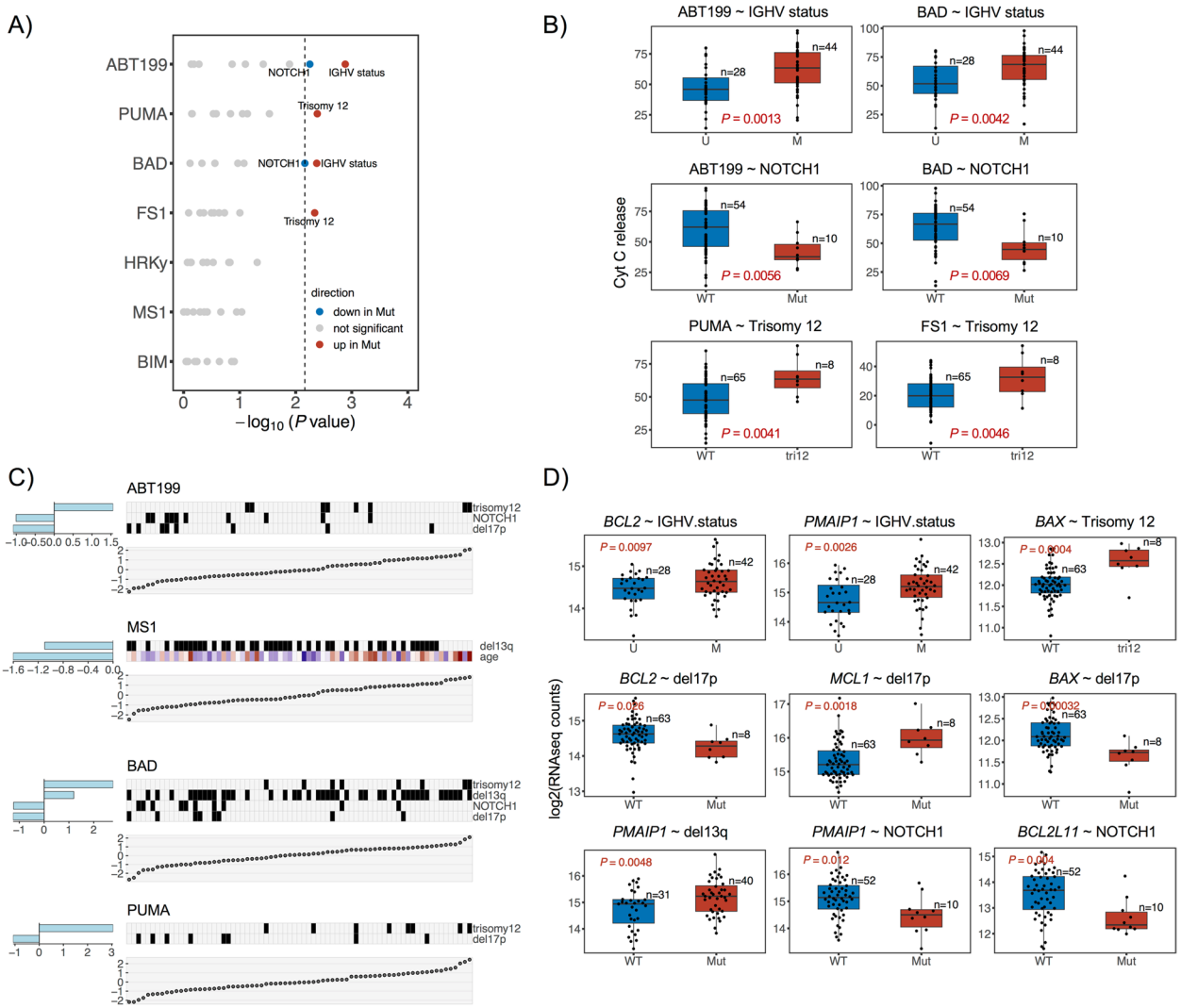


Fig. 2 Specific genetic determinants through BH3-profiling of CLL cells. **A** Summary of significant associations between genetic variations and BH3 profiles (Student's t-test). Dashed line is 10% FDR. **B** Boxplot of significant associations between BH3 profile and genetic variations. Only associations passed 10% FDR control are shown. (BAD, ABT199, PUMA = BCL-2 dependence, FS1 = BFL-1 dependence). **C** Genetic and demographic features selected by regularized multi-variate linear regression (LASSO) for explaining BH3 profile pattern. Horizontal bars on the left indicate the average feature coefficients in the regression models and scatter plot at the bottom indicates z-score of CytC release following peptide treatment. **D** Boxplot of significant associations between *BCL2* family gene expressions and genetic variations. Only associations above the 10% FDR control are shown. (*BCL2* – BCL-2; *MCL1* – MCL-1; *BAX* – BAX; *BCL2L11* – BIM; *PMAIP* – NOXA)

only MCL-1, with lower dependence, was correlated with del(13q) (Fig. 2C). These findings suggest that anti-apoptotic dependence, particularly BCL-2 dependence, could be favorable predictive biomarkers, based on correlation with various prognostic genetic biomarkers. Interestingly, the predictive potential of BCL-2 dependence was further supported by the correlations between relevant apoptotic gene expressions and established genomic markers (Fig. 2D). For example, *BCL2* expression was higher in mutated IGHV or non-del(17p) CLL samples. Relevantly, these samples also express higher pro-apoptotic genes such as *PMAIP* (NOXA), *BAX* or *BCL2L11* (BIM), indicating that these BCL-2 dependent CLL cells are again more readily primed for apoptosis due to the higher expression of pro-apoptotic genes. *PMAIP* expression was also higher in del(13q) cells, which aligns with our earlier observation of lower MCL-1 dependence in del(13q) cells, as NOXA is known to degrade MCL-1 [25, 26] (Fig. 2D). This serves as a control to indicate that not

all dependence, but BCL-2 dependence specifically, is associated with favorable genomic markers.

To further evaluate the predictive potential of BCL-2 dependence, we analyzed the correlation between BH3 profiles and prognostic outcomes. We found that only ABT199 and BAD (corresponding to BCL-2 dependence) were significantly associated with time to treatment (TTT) in our univariate analysis (P value = 0.012 for BAD and 0.030 for ABT199) (Fig. S6). Overall survival (OS) data were not mature enough to be evaluated, as only 8 out of 73 patients were deceased in the cohort.

High BCL-2 dependence is associated with gene expression patterns and functional pathways that reflect favorable treatment response

Apart from our robust correlations between BCL-2 dependence and favorable prognostic markers, our PCA previously suggested that cells that are sensitive to BH3 peptide-induced CytC release were more readily primed for cell death, hence suggesting more responsiveness to treatment intervention (Fig. 1F). To further investigate this finding, we used available clinical data from 6 patients with CLL previously treated with a purine-analog based chemoimmunotherapy combination of pentostatin, cyclophosphamide, rituximab (PCR) to examine whether priming or specific anti-apoptotic protein dependence could predict for response to treatment. As one measure of clinical activity, we studied the rate of change of absolute lymphocyte counts (ALC) during the course of this therapy. We observed that BCL-2 dependence ($R = 0.89$, $P = 0.0161$), and to a lesser extent BFL-1 dependence ($R = 0.86$, $P = 0.0274$), were positively correlated with the lymphocyte count drop rate after treatment (Fig. 3A). This finding provided preliminary support that anti-apoptotic protein dependence, particularly on BCL-2, could predict for a favorable treatment response.

To further understand the functional aspects of anti-apoptotic protein dependence as a predictive biomarker for treatment response, we evaluated the association between gene expression levels and dependence on various anti-apoptotic BCL-2 family members. We found that only BCL-2 dependence (ABT199 or BAD) was associated with differential RNA expression (Fig. S7-S9). Importantly, we observed positive correlations between BCL-2 dependence and expression of *BCL2L11* (BIM) and *PMAIP1* (NOXA)(Fig. 3B, S8-S9), which were among the strongest correlations in our analyses. Notably, BIM and NOXA proteins are a pro-apoptotic sensitizer and activator, respectively, that promote apoptotic-cell death. Other pro-cell death genes were also positively correlated with BCL-2 dependence, such as the inter-strand crosslink-repair protein, *FANCI* and androgen transmembrane protein, *PMEPA1* [27–29]. In contrast, BCL-2 dependence correlated inversely with expression of *COX5A*, *LAG3*, *MYLK* (Fig. 3B, S8-S9), genes that are related to CLL and cancer cell survival or progression [30–32]. These observations suggest that CLL cells with high BCL-2 dependence undergo apoptosis more readily and therefore may be more susceptible and responsive to drug treatment.

Pathway enrichment analysis further suggest that BCL-2 dependence is positively correlated with TNFα/NFκB-signaling, while inversely correlated with oxidative phosphorylation (OXPHOS) (Fig. 3C, D). The positive correlation between BCL-2 dependence and the TNFα/NF κ B-regulated mitochondrial anti-oxidant superoxide dismutase-2 (SOD2), indicates that with higher SOD2 expression, more intracellular superoxide ($O_2^{\cdot -}$) is removed to induce a lower redox milieu in BCL-2-dependent cells (Fig. 3D). This supports a more drug-sensitive phenotype, as increased reactive oxygen species (ROS), $O_2^{\cdot -}$ or redox milieu have been implicated in drug resistance, improved cancer cell survival and proliferation [31, 33–36]. Other notable gene expression under TNFα/NF κ B-signaling is ATPase plasma membrane Ca^{2+} transporter-1, *ATP2B1* (Fig. 3D), previously reported to promote apoptosis [37, 38].

The inverse correlation between BCL-2 dependence and OXPHOS from our pathway enrichment (Fig. 3C) is also consistent with the inverse correlation between BCL-2 dependence and the mitochondrial complex-IV subunit gene, *COX5A* (Fig. 3B), which positively affects mitochondrial respiration (OXPHOS) and redox metabolism [31, 33]. Given that high OXPHOS and mitochondrial $O_2^{\cdot -}$ are implicated in drug resistance, for example in CLL cells resistant to ABT199 (VEN) [36], our finding of high BCL-2 dependence correlating with low *COX5A*, OXPHOS and high SOD2 provides some initial mechanistic insight into the reason why BCL-2 dependence is a predictive biomarker of drug sensitivity. Thus, these data suggest that high BCL-2 dependence could be a functional biomarker that predicts for a favorable treatment response, due to its correlation with drug-sensitive phenotypes.

High BCL-2 dependence is an underlying mechanism of increased drug sensitivity

To better understand the mechanistic underpinnings of our hypothesis that BCL-2 dependence predicts favorable treatment response, we devised an experimental system utilizing two diffuse large B-cell lymphoma (DLBCL) cell lines – one with high dependence on BCL-2 (OCI-Ly1) and another with lower dependence on BCL-2 (OCI-Ly3), demonstrated by BAD- and ABT199-induced CytC release and its delta CytC release (values of CytC release

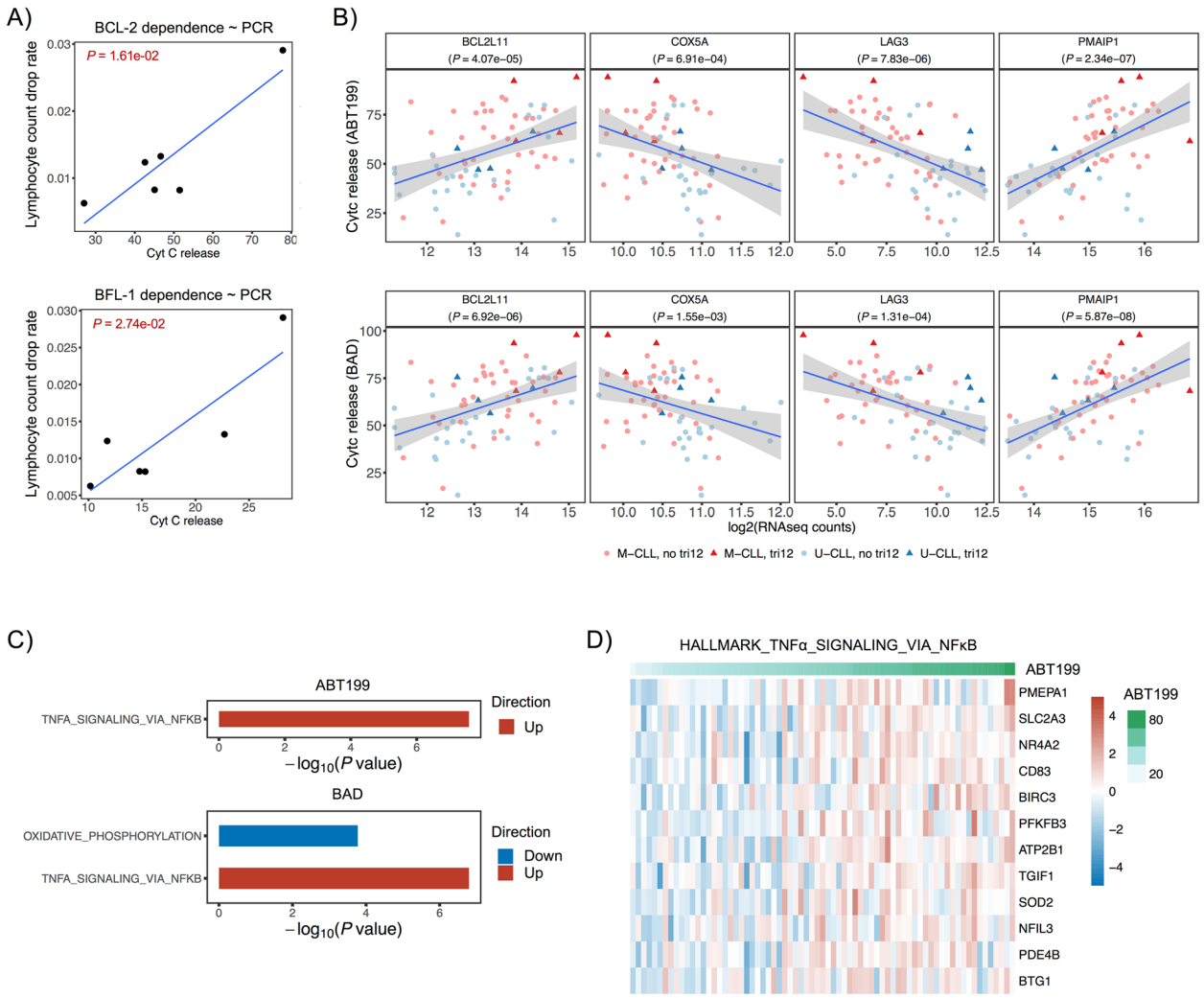


Fig. 3 Specific gene expression, signaling-pathway patterns and in vivo drug response are correlated with BCL-2 dependence. **A** Associations between BH3 profile and in vivo drug response estimated from retrospective clinical data (lymphocyte drop rate) (Pearson's correlation test). The lymphocyte drop rate was calculated by dividing the change in lymphocyte count (in millions) between the start and end of therapy by the duration of the therapy (in days) for each patient. **B** Example of significant correlations between BCL-2 dependence (reflected by ABT199, BAD or PUMA-induced CytC release) and mRNA expressions of *PMAIP1* (NOXA), *BCL2L11* (BIM), *COX5A*, *LAG3*, measured by RNA-seq. **C** Cancer hallmark pathways enrichment analysis for mRNAs correlated with BCL-2 dependence. Enrichment results passed 10% FDR control are shown. **D** A heatmap showing the mRNA expression of the genes (Blue to red bar indicates expression levels), both associated with BCL-2 dependence (Green bar indicates increasing BCL-2 dependence) and belong to the TNFα/NFκB-signaling pathway

derived from CytC release values of OCI-Ly3 minus that of OCI-Ly1 cells) (Fig. 4A, B). Of note, cell lines more typically utilized in other CLL studies such as OSU-CLL, HG3, and MEC-1, were not used here, as these cell lines generally have markedly low dependence on BCL-2 as compared to primary CLL cells, and are therefore relatively resistant to BCL-2 inhibition as compared to primary CLL cells (Fig. S10) [39, 40]. Hence, the two DLBCL lines with drastically different BCL-2 dependences were selected as experimental tools to delineate the different responses toward drug treatment(s), since one line is highly BCL-2 dependent (like primary CLL cells) and the other is distinctly not BCL-2 dependent, but is otherwise similar to its BCL-2 dependent cell line counterpart.

We found that OCI-Ly1 (higher BCL-2 dependence) cells, relative to OCI-Ly3 (lower BCL-2 dependence) cells, have higher BIM protein expression and TNFα/NF κ B-signaling by Western blot analysis (Fig. 4C), recapitulating our transcriptomic analyses of primary CLL cells (Fig. 3B-D). Consistent with our transcriptomic data showing lower *COX5A* and higher *SOD2* expression in highly BCL-2-dependent primary CLL cells

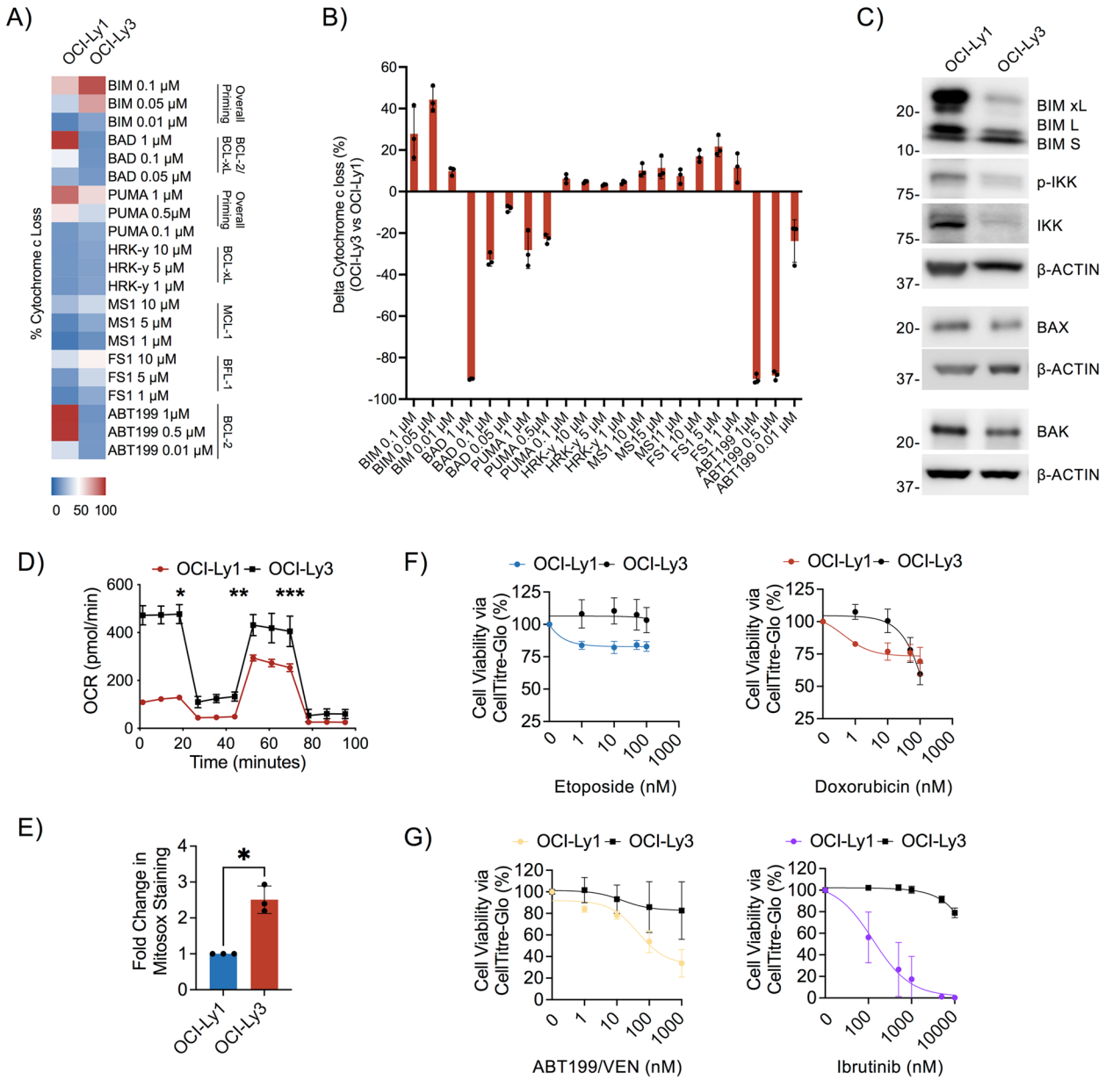


Fig. 4 Correlations between BCL-2 dependence and multi-omics analyses were experimentally re-capitulated in cell lines. **A** Baseline BH3-profiling was performed for two DLBCL cell lines OCI-Ly1 and OCI-Ly3. High CytC release by BH3 peptides or mimetic drug indicates inclined dependence towards its respective anti-apoptotic protein(s) for survival ($n=3$). Specific dependences as reflected on the right. **B** Delta CytC release between OCI-Ly3 and OCI-Ly1 (CytC release % value of OCI-Ly3 minus CytC release % value of OCI-Ly1) was calculated to reflect absolute changes in dependence to specific anti-apoptotic protein(s) for survival. Net + % indicates relative increased or better dependence and net—% suggests relative decreased dependence ($n=3$). **C** Western blot analysis showing protein expression levels of BIM-xL, L, S, phosphor-IKK, total IKK, BAX, BAK and respective β-Actin(s) of OCI-Ly1 and OCI-Ly3. **D** Mitochondrial respiration or OXPHOS measurement between OCI-Ly1 and OCI-Ly3. Asterisks indicate addition of oligomycin (*), FCCP (**), antimycin-A/rotenone (***) to indicate basal and maximal respirations. Representative graph is shown here ($n=3$). **E** Mitosox staining was measured and quantified between OCI-Ly1 and OCI-Ly3 ($n=3$). Unpaired t-test was used. **F**, **G** Cell viability by CellTiter-Glo® was measured between OCI-Ly1 and OCI-Ly3 following 48-h treatment with increasing doses of ABT199 (VEN) ($n=4$), ibrutinib ($n=3$), etoposide ($n=4$) or doxorubicin ($n=4$)

(Fig. 3B, D), we observed lower mitochondrial respiration/OXPHOS and mitochondrial $O_2^{\cdot-}$ levels in OCI-Ly1 compared to OCI-Ly3 (Fig. 4D, E). Importantly, OCI-Ly1 cells possessed higher pro-apoptotic BAX and BAK protein expression (Fig. 4C) and were more sensitive to various targeted and chemotherapeutic drug treatments,

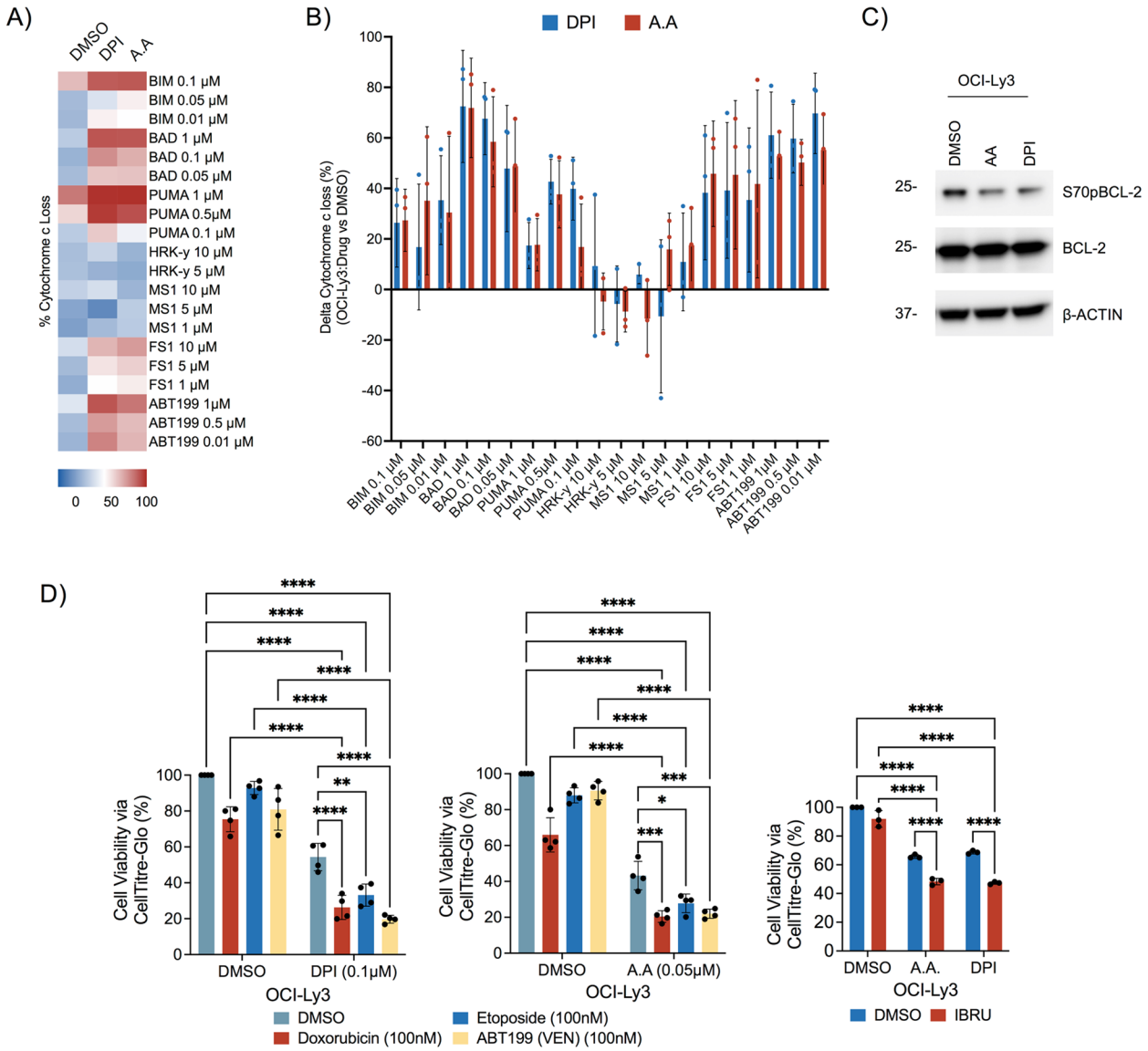


Fig. 5 BCL-2 dependence is a causal function of drug sensitivity in cancer cells in vitro. **A** BH3-profiling performed following treatment with DPI (0.1 μM) or AA (0.05 μM) for 15 h for OCI-Ly3 cells ($n=3$). **B** Delta CytC release between DPI/AA- and DMSO-treated OCI-Ly3 (CytC release % value of DPI/AA minus CytC release % value of DMSO) was calculated to reflect absolute changes in dependence to specific anti-apoptotic protein(s) for survival ($n=3$). **C** Western blot analysis showing S70pBCL-2, BCL-2 and β-Actin levels of OCI-Ly3. **D** Cell viability by CellTiter-Glo® was measured for OCI-Ly3 following 2-h pre-treatment with DPI (0.1 μM) or AA (0.05 μM) and subsequent 48-h co-treatment with either doxorubicin (0.1 μM, $n=4$), etoposide (0.1 μM, $n=4$), ABT199 (VEN) (0.1 μM, $n=4$) or ibrutinib (1 μM, $n=3$). Tukey's multiple comparisons test was used

including ibrutinib, ABT199 (VEN), doxorubicin and etoposide relative to OCI-Ly3 cells (Fig. 4F, G). These findings in DLBCL cell lines reflect the phenotypes and potential treatment response of primary CLL cells with different degrees of BCL-2 dependence.

To further examine if these associations with BCL-2 dependence were causal, we proceeded to investigate the therapeutically and immediately actionable targets or functions from transcriptomic and pathway enrichment analyses, namely OXPHOS and mitochondrial $O_2^{\cdot-}$ production, which are associated with BCL-2 dependence. We treated the relatively drug-resistant and lower BCL-2-dependent OCI-Ly3 cells with an inhibitor of NAPDH oxidase-dependent $O_2^{\cdot-}$ production, diphenyleneiodonium (DPI), or a mitochondrial complex-III/OXPHOS inhibitor, antimycin-A (AA), and observed that this led to an increase in BCL-2 dependence (Fig. 5A), and a clear switch of net negative to positive BCL-2 dependence

when comparing DPI or AA with DMSO control (Fig. 5B, delta values derived from drug-treated minus control groups). We recently reported that BCL-2 phosphorylation negatively regulates the dependence of BCL-2 in DLBCL and CLL cells [13]. Our data further confirm that AA and DPI could reduce BCL-2 phosphorylation in OCI-Ly3 cells (Fig. 5C), thus corroborating the increased dependence on BCL-2 for survival. DPI is also used here as a positive control for the reduction in BCL-2 phosphorylation [41]. The decrease in BCL-2 phosphorylation by AA/DPI alone corresponds to a slight drop in cell survival (Fig. 5D). Importantly, increase in BCL-2 dependence was further accompanied by a significant increase in sensitization to chemo- and targeted drugs doxorubicin, etoposide, ABT199 (VEN) and ibrutinib (Fig. 5D). These findings thus validate the associations between BCL-2 dependence and multi-omics analyses in primary CLL cells and confirm that high BCL-2 dependence is one mechanism underlying improved treatment response.

To corroborate these observations from cell lines, we profiled 8 additional primary CLL samples and clustered them based on their degrees of BCL-2 dependence (patients 1–8). We observed that patients 5–8 had relatively higher BCL-2 dependence as compared to patients 1–4 (Fig. 6A). We further observed that patients 5–8 also had higher pro-apoptotic BIM and BAX protein expressions (Fig. 6B). Notably, strong and significant correlations between BCL-2 and BIM ($r=0.9011$, $P=0.0022$) or BCL-2 and BAX ($r=0.8166$, $P=0.0134$), but not MCL-1 and BIM ($r=0.3919$, $P=0.3369$) or MCL-1 and BAX ($r=0.1708$, $P=0.686$), were observed (Fig. 6C, D). Importantly, BCL-2 dependence, but not MCL-1 dependence (Fig. S11), was highly associated with BCL-2 ($r=0.8166$, $P=0.0138$), BAX ($r=0.8138$, $P=0.014$) and BIM ($r=0.882$, $P=0.0038$) protein expressions (Fig. 6E). These data again corroborate our correlative data on BCL-2 dependence and RNA-Seq (Fig. 3B), as well as our recent study on defining anti-apoptotic protein dependences by specific exogenous BCL-2 family protein expression [13], and support the hypothesis that BCL-2-dependent cells are more primed for apoptosis due to the higher levels of pro-apoptotic proteins.

To further investigate the correlation between BCL-2 dependence and treatment response, we analyzed patients 1 and 7, who both had samples with sufficient cell numbers but also had opposite anti-apoptotic dependences (Fig. 6F) We treated these cells with a chemotherapeutic agent, fludarabine, or targeted agent, ibrutinib ex vivo and measured cell viability (Fig. 6G). We observed again that primary CLL cells from patient 7 with higher BCL-2 dependence were more susceptible to apoptotic cell death when treated with fludarabine or ibrutinib, as compared to those of patient 1 with lower BCL-2 dependence (Fig. 6F, G). In contrast, the higher MCL-1 dependence in patient 1's cells did not respond as well, hence indicating that drug sensitivity is not arbitrarily governed by any high anti-apoptotic protein dependence (Fig. 6F, G).

Finally, we treated cells from 8 additional patients with CLL of varying BCL-2 dependences with AA or DPI ex vivo, and observed that these drugs could similarly increase BCL-2 dependence (Fig. 6H, S12A), which further translated to enhanced apoptotic cell death when combined with ibrutinib or fludarabine (Fig. 6I, S12B). We demonstrated that BCL-2 dependence and cell death were again enhanced by the decrease in BCL-2 phosphorylation in primary CLL cells (Fig. 6J, S12C). To ensure reproducibility and clinical relevance, we studied an OXPHOS inhibitor, IACS-010759, that has undergone clinical trials [42]. Indeed, IACS-010759 was capable of increasing BCL-2 dependence in CLL patient samples as well as sensitivity to both targeted agents, ABT199 (VEN) and ibrutinib (Fig. 6K, L, S12D). Collectively, our in vitro and ex vivo data demonstrate consistently that higher BCL-2 dependence underlies better response to various treatment modalities.

Functional BCL-2 dependence predicts clinical response of CLL patients to a targeted chemoimmunotherapy-based regimen

Having mapped the landscape of functional BCL-2 family dependence across a range of CLL genetic backgrounds with transcriptomic and proteomic analyses, and having performed experimental validation to unravel the causal relationship between BCL-2 dependence, pro-cell death gene expressions, drug sensitivity and response, we next applied this technique to a validation cohort of CLL samples from 54 patients treated uniformly on a prospective, phase-2 trial of first-line ibrutinib plus FCR (iFCR) [14]. Our aim was to validate that with higher BCL-2 dependence, patients will be more responsive to treatment.

We generated two datasets by BH3-profiling these samples: (i) baseline CytC release data of pretreatment patient samples, and (ii) delta values from CytC release data of paired patient samples after 1 week of ibrutinib monotherapy minus CytC release data of pretreatment patient samples (Fig. 7A). The latter dataset was assessed to inform how ibrutinib alters BH3-profiling results. To characterize the anti-apoptotic dependence patterns of these two datasets, we initially performed an unsupervised hierarchical clustering analysis (Fig. S13A). Similar to our initial discovery cohort, we observed that CLL patients whose CLL cells had high BCL-2 dependence at baseline as well as CLL patients with a further net

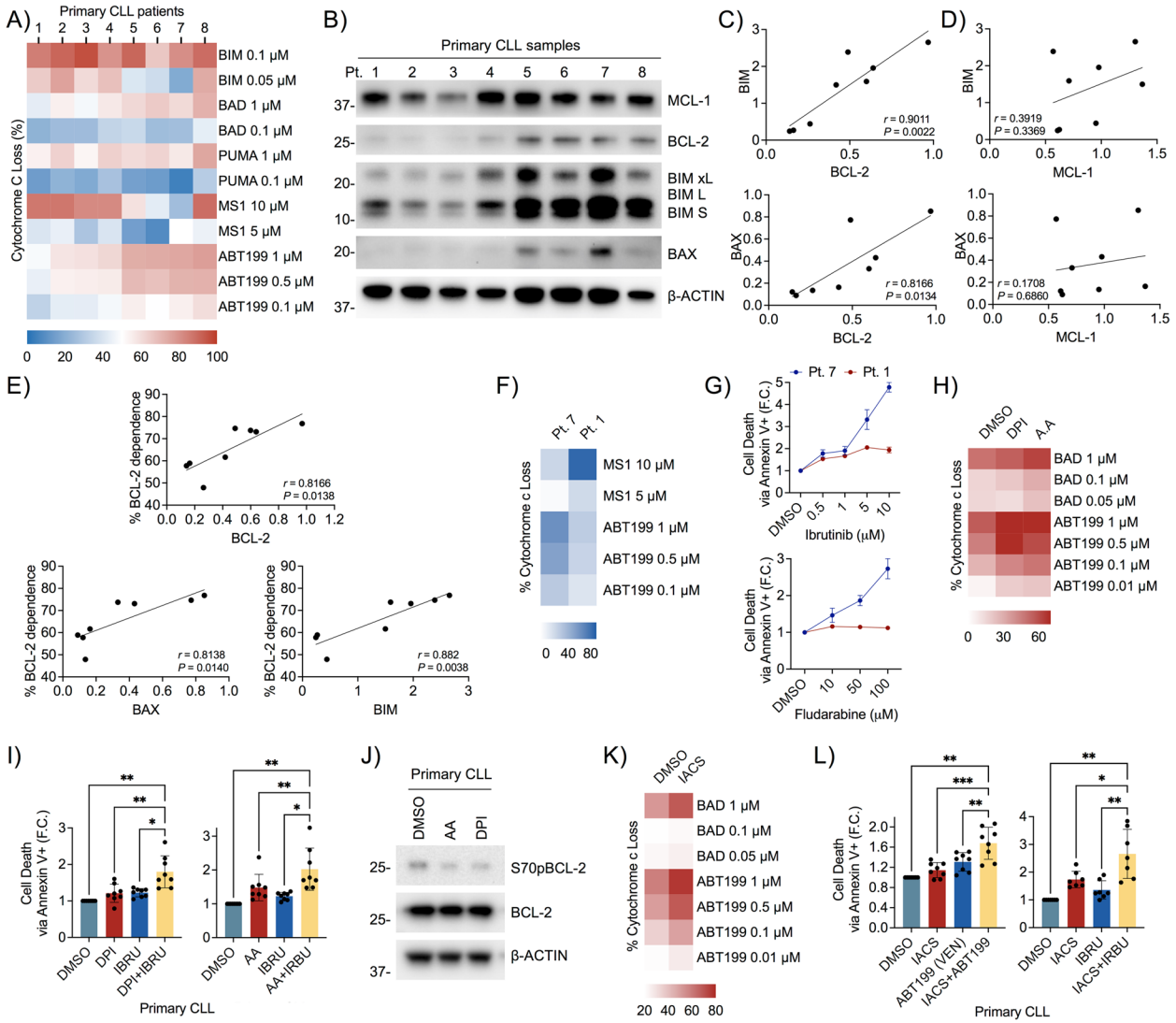


Fig. 6 High BCL-2 dependence predicts favorable drug response in primary CLL cells ex vivo. **A** Baseline BH3-profiling was performed on primary CLL cells. Cells were incubated in specific BH3 peptides or mimetic for 1 h prior to determining CytC loss. Patients arbitrarily labeled as patient 1–8. **B** Western blot analysis showing MCL-1, BCL-2, BIM xL, L, S, BAX and β-Actin levels of the same 8 CLL patient samples in Fig. 6A. **C**, **D** Pearson correlation coefficient analyses between BCL-2 family protein expressions, normalized to β-Actin levels from Fig. 6B. **E** Pearson correlation coefficient analyses of BCL-2 dependence and normalized BCL-2, BAX or BIM protein expression levels. **F** Replot of baseline BH3-profiling of primary CLL cells from patient 1 and 7 with different degrees of MCL-1 and BCL-2 dependences. **G** Cell death levels by fold change (F.C.) of annexin V staining of the same patient 1 and 7 following 24-h ex vivo fludarabine or ibrutinib treatment (with stroma NKtert co-culture) (Viability was measured in duplicates of the same patient, $n = 2$). **H** BH3-profiling performed following ex vivo treatment with DPI or AA for 6 h in 8 CLL patient primary cells to evaluate BCL-2 dependence level. **I** Cell death levels by fold change of annexin V staining of CLL patient primary cells following 2-h pre-treatment with AA or DPI followed by 24-h ibrutinib ($n = 8$) (with stroma NKtert co-culture). Sidak's multiple comparisons test was used. **J** Western blot analysis showing S70pBCL-2, BCL-2 and β-Actin levels of 8 CLL patient primary cells following ex vivo treatment with DPI or AA for 6 h. A representative patient shown. Densitometry analysis is in supplemental figure S12C. **K** BH3-profiling performed following ex vivo treatment of 0.1 μM IACS-010759 for 6 h in 8 CLL patient primary cells to evaluate BCL-2 dependence level. **L** Cell death levels by fold change of annexin V staining of CLL patient primary cells following 2-h pre-treatment with 0.1 μM IACS-010759 followed by 24-h 2.5 nM ABT199 (VEN) ($n = 8$) or 1 μM ibrutinib ($n = 7$) (with stroma NKtert co-culture). Sidak's multiple comparisons test was used

increase in BCL-2 dependence following ibrutinib treatment (positive delta CytC values) were both associated with the more favorable mutated IGHV status (Fig. 7B). Importantly, results from the 1 week of ibrutinib dataset confirmed that as long as BCL-2 dependence is high, CLL cells will still be associated with favorable genetic markers such as mutated IGHV.

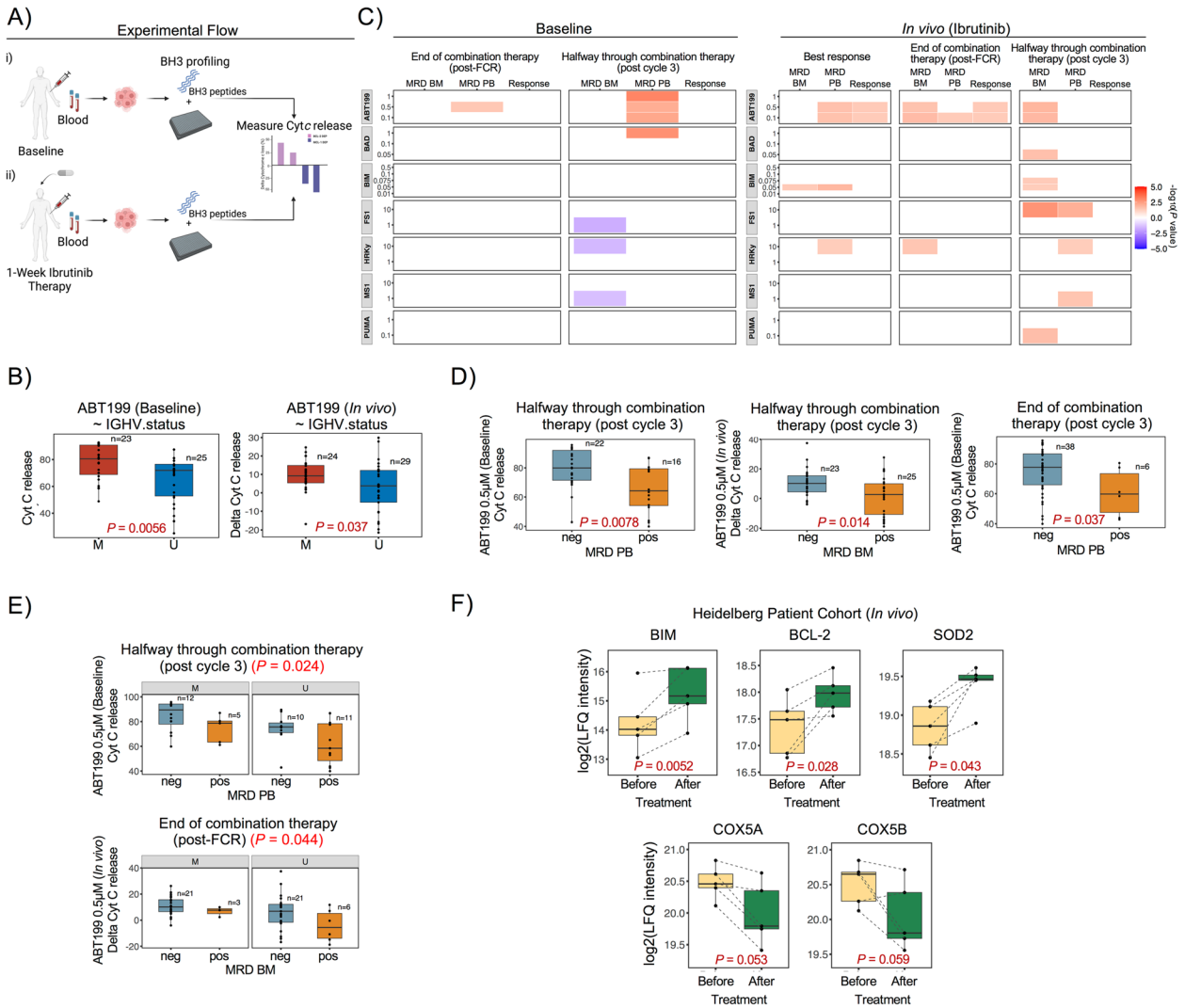


Fig. 7 BCL-2 dependence predicts for complete remission and undetectable minimal residue disease in validation cohort. **A** Paired blood samples from patients with CLL were drawn pre-treatment and after 1 week of ibrutinib therapy in a frontline iFCR phase-2 clinical trial. PBMCs from these patients were isolated and used directly for in vivo BH3-profiling. Diagram was created in BioRender. Chamberlain, S. (2025) https://BioRender.com/h74g455. **B** Associations between the levels of CytC release by ABT199 (BCL-2 dependence) and IGHV mutational status in baseline patients as well as association between the delta levels of CytC release by ABT199 (BCL-2 dependence) and IGHV mutational status in in vivo ibrutinib-treated patients. Positive delta values from in vivo ibrutinib group indicates further net increase in BCL-2 dependence following ibrutinib treatment in comparison to baseline screening and vice versa for negative delta values. **C** *P* value heatmap plot summarizing the *P* values of association between BH3 profile and various clinical outcomes. Positive *P* values (red) indicating higher CytC release in "neg" group for MRD or "PR" group for response. MRD - minimal residue disease, BM - bone marrow, PB - Peripheral Blood. **D** Boxplots showing the examples of associations between baseline or delta BCL-2 dependence (represented by ABT199) and clinical outcomes. The *P* values were calculated by Student's t-test. **E** Boxplots showing the examples of IGHV-independent associations between baseline or delta BCL-2 dependence and clinical outcomes. The *P* values were calculated by two-way ANOVA tests including IGHV mutational status as an additional covariate. **F** Boxplot of significant associations between protein expressions of paired CLL patient samples before and after in vivo ibrutinib therapy additional cohort (n = 5). Only associations above the 10% FDR control are shown

Next, we correlated anti-apoptotic dependence data generated through BH3-profiling with the clinical responses (complete response, CR vs. partial response, PR) and minimal residual disease (MRD) status halfway through combination therapy (post-cycle 3), at the end of combination therapy (post-cycle 6), and at time of best response on trial (including patients who went on to receive ibrutinib monotherapy maintenance). We hypothesized that BCL-2 dependence would predict depth of clinical response in the iFCR clinical trial cohort

Table 1 AUROC of baseline BCL-2 dependence for the prediction of treatment response and outcome

FEATURE	AUROC (VALUE)	AUROC (SD)	TREATMENT TIMEPOINT	RESPONSE (AREA)
ABT199 (1 μM)	0.802	0.082	Post Cycle 3	MRD PB
ABT199 (1 μM)	0.783	0.117	Post FCR	MRD PB
ABT199 (0.1 μM)	0.773	0.118	Post FCR	MRD PB
BAD (0.1 μM)	0.760	0.112	Post FCR	MRD PB
ABT199 (0.5 μM)	0.752	0.133	Post FCR	MRD PB

Post Cycle 3: Halfway through combination therapy

Post FCR: End of combination therapy

MRD PB: Minimal Residue Disease Peripheral Blood

[14]. We observed that there were significant correlations between the different peptides/BH3 mimetic at various endpoints. Specifically, higher BCL-2 dependence in CLL cells from patients at baseline was indeed associated with increased likelihood of achieving undetectable MRD (uMRD) and CR at the halfway mark on treatment, at the end of combination therapy and at the time of best response (Fig. 7C, D). Additionally, CLL cells with further net increase in BCL-2 dependence (positive delta CytC values) after 1 week of ibrutinib treatment were similarly associated with increased likelihood of achieving uMRD and CR at these endpoints (Fig. 7C, D, S13B). These findings were consistent with our initial observations of the 6 PCR-treated patients (Fig. 3A) as well as our in vitro and ex vivo studies (Figs. 4– 6). Interestingly, both BFL-1 and BCL-xL dependence were also associated with response and uMRD status in the in vivo ibrutinib-treated group, though these associations were only apparent at earlier timepoints (Fig. 7C). In our uni- and multivariate analyses, the association between BCL-2 dependence and uMRD was not confounded by IGHV mutational status (Fig. 7E), and IGHV mutational status alone did not predict uMRD at any specified timepoint (Fig. S14). This suggests that BCL-2 dependence itself is a primary factor associated with likelihood of achieving uMRD, supporting our overall hypothesis that BCL-2 dependence is a potential independent biomarker of favorable response to various therapies in CLL. In addition, proteomics data from an additional, independent in vivo ibrutinib-treated patient cohort demonstrated that ibrutinib could increase BCL-2, BIM, SOD2, while decrease COX5A/B (Fig. 7F), proteins corresponding to our earlier observations that support BCL-2 dependence in association with better apoptotic priming and treatment response (Fig. 3B, 4C, 6B). This further supports our finding that ibrutinib increases BCL-2 dependence in our ibrutinib-treated cohort (Fig. 7C), and corroborates our previous study demonstrating that ibrutinib increases BCL-2 dependence [12]. Finally, to investigate the reliability of the BH3-profiling test in predicting treatment response and thus the validity of BCL-2 dependence in predicting favorable treatment response, we calculated area under the receiver operating characteristic (AUROC) curves for each of the BH3 features when predicting each clinical outcome. We displayed the top 5 features from baseline or in vivo ibrutinib treatment (Tables 1 and 2, complete list in supplementary material 1). We observed that these top features from both baseline and in vivo ibrutinib sets with good to excellent prediction powers were all features of

Table 2 AUROC of BCL-2 dependence following ibrutinib treatment for the prediction of treatment response and outcome

IN VIVO IBRUTINIB				
FEATURE	**AUROC (VALUE)**	**AUROC (SD)**	**TREATMENT TIMEPOINT**	**RESPONSE (AREA)**
PUMA (1 μM)	0.827	0.127	Post FCR	MRD PB
ABT199 (0.1 μM)	0.779	0.171	Post FCR	MRD PB
BAD (1 μM)	0.778	0.125	Best Response	MRD PB
ABT199 (0.1 μM)	0.774	0.130	Best Response	MRD BM
ABT199 (1 μM)	0.758	0.149	Post FCR	MRD PB

Post FCR: End of combination therapy

MRD PB: Minimal Residue Disease Peripheral Blood

MRD BM: Minimal Residue Disease Bone Marrow

BCL-2 dependence; for example, baseline ABT199 at 1 μM (AUROC = 0.802 ± 0.082) and in vivo PUMA peptide at 1 μM (AUROC = 0.827 ± 0.127). In contrast, features of BCL-xL or BFL-1 dependence were displayed at lower rankings (Supplemental Table 1). The AUROC analysis therefore provides validation of BH3-profiling and confirms our observations that BCL-2 dependence is a potential predictive biomarker of favorable response for different treatments in CLL.

Discussion

We used BH3-profiling to unlock the key molecular features defining anti-apoptotic protein dependence and response to treatment in CLL. In our discovery cohort of 73 CLL patients, we found that higher BCL-2 dependence was associated with favorable genetic prognostic factors and lesser reliance on OXPHOS and mitochondrial $O_2^{\cdot-}$. In contrast, lower BCL-2 dependence was associated with poor prognostic factors, high OXPHOS and mitochondrial $O_2^{\cdot-}$ reliance. BCL-2 dependence was correlated with transcriptomic signatures that indicate a more apoptotic-ready and drug-sensitive milieu. We further confirmed that these correlations were underlying regulatory factors for BCL-2 dependence and subsequent drug response in cell lines and ex vivo patient samples. In our uniformly-treated iFCR clinical trial, we ascertained that primary CLL cells from patients at baseline or after 1 week of ibrutinib treatment with higher BCL-2 dependence in vivo independently predicted depth of clinical response, while IGHV mutational status alone was unable to do so. The latter analysis is consistent with our recent publication that clinical response in patients with CLL treated with iFCR was not affected by IGHV mutational status [43]. Overall, our study is the first to comprehensively define BCL-2 dependence as a potential functional and predictive treatment response biomarker in CLL, integrating multi-omics data and BH3-profiling to validate its clinical utility.

Our analysis not only demonstrated that BCL-2 dependence and associated expression of apoptotic genes correlated positively with favorable prognostic biomarkers, but also identified associations of BCL-2 dependence with drug response and a drug-sensitive transcriptome, which collectively provided us an angle to investigate the possibility of BCL-2 dependence in predicting treatment response in CLL patients. For example, higher BCL-2 dependence was associated with increased expression of the pro-apoptotic BH3-only protein NOXA. NOXA is known to prevent the anti-apoptotic MCL-1 from binding to the pro-apoptotic activator BIM and effector BAK [25, 44–46]. It has also been suggested that NOXA may increase proteasomal degradation of MCL-1 [25, 26], which leads to increased sensitivity to BCL-2 inhibition [13, 36]. Hence, increased NOXA expression may reduce MCL-1 protein, increasing CLL cell dependence on BCL-2 for survival. Indeed, NOXA genetic amplification has recently been suggested to increase sensitivity to BCL-2 inhibitors in DLBCL [47]. Importantly, we found that BCL-2 dependence, together with BCL-2 protein expression, were positively associated with BAX/BAK and BIM expression. Higher BAX, BAK and BIM expression suggest that the impact of the canonical role of BCL-2 in sequestering and inhibiting BIM/BAX/BAK-induced apoptosis may increase, thus translating to an enhanced dependence on BCL-2 for survival. When these pro-apoptotic protein levels are high, in association with high BCL-2 and its dependence for survival, this indicates that BCL-2 is loaded with pro-apoptotic proteins, and when triggered by drugs, may release a floodgate of active pro-apoptotic effectors to induce apoptotic cell death. This provides a mechanistic rationale for our finding that BCL-2 dependence, in positive association with BAX/BAK and BIM expression, predicts better response to treatment. Our findings thus collectively begin to elucidate the mechanisms underlying high BCL-2 dependence in CLL, and how BCL-2 dependence may predict for treatment response.

BCL-2 dependence also positively correlated with TNFα/NFκB-signaling. While TNFα/NFκB-signaling is frequently associated with cell survival, evolving evidence has implicated its involvement in cell death regulation [37, 38, 48–51]. For example, TNFα-induced apoptosis may first be inhibited by the activation of Rel/NFκB, but over time increases spontaneous apoptosis in cancer cells. This was shown to be due to the upregulation of SOD2, which serves as an anti-oxidant protein to convert $O_2^{\cdot-}$ to hydrogen peroxide (H_2O_2) [49]. Our findings demonstrating higher BCL-2 dependence in associated with higher SOD2 expression and lower mitochondrial $O_2^{\cdot-}$ levels in vitro support this prior observation. We hypothesize that tumor cells may survive due to SOD2 upregulation, which rapidly detoxifies the initial apoptogenic burst of drug/TNFα-induced $O_2^{\cdot-}$ to H_2O_2, but they eventually succumb to the progressive build-up of H_2O_2 that leads to apoptosis [49]. Interestingly, TNFα and NFκB have both been shown to positively regulate *BCL-2* transcription, and we therefore anticipate that an increase in BCL-2 dependence is due in part to an increase in *BCL-2* transcription by TNFα/NFκB signaling [52, 53].

The inverse correlation between BCL-2 dependence and OXPHOS from our enrichment pathway analysis is also supported by our in vitro data. As high OXPHOS and the subsequent increase in mitochondrial $O_2^{\cdot-}$ production have been implicated in resistance to various therapeutic drugs [31, 33, 34, 36, 41, 54], the lower

OXPHOS and mitochondrial $O_2^{\cdot-}$ levels, and consequentially higher BCL-2 dependence, suggest a more drug-sensitive milieu and favorable treatment response, as observed in our experimental data in cell line and primary CLL samples. We previously demonstrated that COX5A/$O_2^{\cdot-}$ could activate the anti-apoptotic activity of BCL-2 via its phosphorylation [31, 35] and that BCL-2 phosphorylation could suppress BCL-2 dependence [13]. Here, we confirmed that inhibiting OXPHOS or preventing $O_2^{\cdot-}$ production could reduce BCL-2 phosphorylation and reciprocally increase BCL-2 dependence and drug sensitivity. Interestingly, TNFα/NFκB is known to positively regulate both *SOD2* and *BCL2* transcriptions, and previous work demonstrated that SOD inhibition increases $O_2^{\cdot-}$ levels, thereby increasing BCL-2 phosphorylation and reducing drug sensitivity [41]. These previous findings corroborate our work here, and provide a potential link between TNFα/NFκB signaling and BCL-2 phosphorylation via SOD2. We further hypothesize that the increase in TNFα/NFκB signaling would increase expression of both SOD2 and BCL-2, potentially allowing more SOD2 to reduce $O_2^{\cdot-}$ levels and BCL-2 phosphorylation, ultimately making cells more dependent on BCL-2 and sensitive to drug treatments. Collectively, our findings support the observation that a favorable treatment response to therapy is more likely when CLL cells have higher BCL-2 dependence, and our data provide initial insights into the mechanisms underlying BCL-2 dependence.

Our study further suggests that BCL-2 dependence should be explored as a predictive biomarker to identify CLL patients who are more likely to respond favorably not only to conventional chemotherapy but also to targeted therapy. We observed that CLL cells with higher BCL-2 dependence, treated with targeted agents such as ABT199 (VEN), ibrutinib or conventional chemotherapy such as fludarabine, doxorubicin, etoposide, had higher cell death, and that CLL patients treated with iFCR were likely to have improved clinical response. Our observations here support exploring alternative therapeutic approaches for CLL patients with lower levels of BCL-2 dependence. Such patients may benefit from a combination treatment approach incorporating drugs targeting one or more of the therapeutically actionable nodes, which may increase BCL-2 dependence. One such example is the use of protein phosphatase 2A activator drugs (PADs) [13, 55], that we recently demonstrated increase BCL-2 dependence through the activation of protein phosphatase 2A and reduction of BCL-2 phosphorylation [13]. Given that our transcriptomics analysis also provided multiple pharmacologically-actionable targets that are linked to lower BCL-2 dependence (Fig. 3B, C, S8-S9), this suggests several new potential combination partners that could increase BCL-2 dependence and thereby sensitize CLL cells to various targeted and/or chemotherapy regimens. For example, targeted treatment combinations with low doses of OXPHOS inhibitors (including several currently under clinical evaluation) [42]) or ROS scavengers (*i.e.* SOD2 mimetic, GC4419) [56] with ibrutinib and/or ABT199 (VEN) could be explored. Thus, in addition to BCL-2 dependence serving as a potential predictive biomarker of treatment response, understanding the mechanisms underlying its modulation may help to potentially identify new therapeutic combinations.

It is worth noting the possibility that some CLL cells may depend more on MCL-1 than on BCL-2. We observed in a previous study that CLL and DLBCL cells with lower BCL-2 dependence, display elevated MCL-1 dependence, which translates to higher sensitivity to MCL-1 inhibitor, S63845 [13]. Nonetheless, as toxicity issue has to date impeded the clinical development of MCL-1 inhibitors, our data here provide a potential alternative to increase BCL-2 dependence in CLL patients with initially lower BCL-2 dependence. Another important factor worth mentioning is the ability of ibrutinib to induce tumor cell release from lymph nodes [57], raising the question of whether the in vivo ibrutinib treatment in our iFCR cohort would influence the BH3-profiling results. Similar to our baseline profiling showing the association between BCL-2 dependence and better treatment response, the 7-day treatment with ibrutinib showed very similar results, in which our profiling at this timepoint showed that higher BCL-2 dependence is associated with better treatment response. As such, the ability of ibrutinib to release tumor cells from the lymph nodes does not seem to affect our BH3-profiling results, including BCL-2 dependence predicting treatment response. In addition, as our previous work showed that ibrutinib could increase BCL-2 dependence, we further hypothesize that CLL cells that are released into circulation would still be exposed to the effects of ibrutinib to increase BCL-2 dependence. Taken together, the effects of ibrutinib treatment before administering FCR would still allow us to properly predict treatment responses via BH3-profiling. A final factor worth mentioning is the translational implications of BH3-profiling as a tool for clinical practice. Efforts are currently well underway to further standardize the technique. Automated techniques have been employed pertaining to preparation, handling and measurement, to minimize potential for human error and facilitate possible future regulatory approval. We note that BH3-profiling is particularly well-suited for circulating diseases such as CLL due to easy sample availability and abundance from just a routine blood draw. The very rapid turnaround time of just a few hours and relatively low cost of the assay may facilitate uptake of the

technique in practice as additional validation studies continue to read out.

Though our findings are robust, our study does have limitations to highlight. For example, our validation cohort from the iFCR trial is a population treated with a targeted therapy plus chemoimmunotherapy combination. Future studies will be needed to validate whether our results also apply in cohorts of patients treated with targeted therapies alone. Additionally, the clinical data from both our discovery and validation cohorts were not mature enough to analyze time-to-event endpoints such as PFS and OS, which will be important to evaluate in our future studies. Secondly, although our discovery cohort of 73 patients encompasses various diverse patients characteristics (*i.e.* genetic anomalies, prior treatment status, demographic diversity) we acknowledge that there may still be potential selection biases in populations of patients followed at tertiary academic hospitals compared to patients followed in community practice. Finally, while a group of untreated patients only was included in our iFCR cohort to control for potential confounding effects from a mixed group of previously treated or untreated patients, it would also be informative to include patients that were previously treated in our future studies to evaluate the potential confounding effects of prior treatments.

Conclusion

Unlike in many cancers, the novel agents approved in CLL do not target recurrent mutations; rather, these new drugs targets pathways such as BCR signaling and the BCL-2 family that are functionally important for the survival of CLL cells. Thus, although genomic biomarkers will continue to be an important way to understand the biology of CLL, functional precision medicine strategies will also be crucial to help optimize the efficacy of targeted agents in this disease. By directing these agents preferentially to the patients most likely to benefit, multidimensional functional and omics mapping will bring us closer to the goal of personalizing therapy for patients with CLL. We identified and defined BCL-2 dependence as a potential functional predictive biomarker of conventional and targeted treatment response in CLL. This underscores the importance of characterizing functional apoptotic signaling in CLL to stratify patients beyond established genetic biomarkers, which currently is less useful to guide treatment with targeted agents, as well as to identify novel combinations to exploit BCL-2 dependence therapeutically.

Abbreviations

AA	Antimycin-A
ABT199	Venetoclax or VEN
ALC	Absolute lymphocyte counts
AUROC	Area under the receiver operating characteristic
BCR	B-cell receptor
BH3	BCL-2 homology domain 3
BTKi	Bruton Tyrosine Kinase inhibitor
CLL	Chronic lymphocytic leukemia
CR	Complete remission
CytC	Cytochrome *c*
Del(13q)	Deletion-13q
Del(17p)	Deletion-17p
Delta CytC release	Values derived from % values of CytC release of cell A minus that of cell B.
DLBCL	Diffuse large B-cell lymphoma
DPI	Diphenyleneiodonium
iFCR	Ibrutinib, fludarabine, cyclophosphamide, rituximab
IGHV	Immunoglobulin heavy chain variable gene
MEK	Mitogen-activated protein kinase kinase
MRD	Minimal residual disease
mTOR	Mammalian target of rapamycin
$O_2^{\cdot -}$	Superoxide
OS	Overall survival
PBMC	Peripheral blood mononuclear cells
PC1	First principal component
PC2	Second principal component
PCA	Principal component analysis
PCR	Pentostatin, cyclophosphamide, rituximab
PFS	Progression-free survival
PR	Partial remission
RNA-Seq	RNA sequencing
ROS	Reactive oxygen species
SOD2	Superoxide dismutase-2
Targeted-Seq	Targeted sequencing
TP53	Tumor protein-53
uMRD	Undetectable MRD
WES	Whole-exome sequencing

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12943-025-02260-7.

Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

Supplementary Material 4.

Supplementary Material 5.

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Authors' contributions

SJFC, JL, RV, TZ, MSD conceptualized and designed the study. SJFC, JL, RV, TZL, WH, TZ, MSD developed the methodology. SJFC, RV, TZL, JQE, FZ, JW, JZ acquired the data. SJFC, JL, RV, TZL, JQE, FZ, JW, JZ, CH, LRK, MSD analyzed and interpreted the data. All authors wrote, reviewed and/or revised the manuscript. SF, JRB, BCG, CUN, MSD provided the administrative, technical and/or material support. SCJF, WH, TZ, MSD supervised the study.

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Data availability

Source data for in vitro and ex vivo analyses are provided separately as supplemental supporting data excel file. Figure 7F dataset is accessible through the RPIDE database: https://www.ebi.ac.uk/pride/archive/projects/PXD022198. All other multi-omics profiling data are available in the R data package BloodCancerMultiOmics2017 from the Bioconductor project (http://bioconductor.org). Materials are available from authors upon reasonable request.

Declarations

Ethics approval and consent to participate

Informed consent has been obtained from patients and approval have been granted by the Ethics Committee Heidelberg (University of Heidelberg, Germany; S-206/2011;S-356/2013) and Dana-Farber/Harvard Cancer Center Institutional Review Board; #99-224).

Competing interests

RV is a current employee of Genmab Biotechnology Company, Copenhagen, Denmark. TZL is a current employee at Casma Therapeutics, Inc, Cambridge, MA, USA. CH has received honoraria from AbbVie, Janssen, Roche, Gilead, Takeda, Daiichi and personal fees from AbbVie, Janssen, Roche, Gilead, Takeda. BCG consults and serves on the Advisory board for MSD, Merck KGaA, Novartis, Adagene Inc., Bayer Healthcare and receives research funding from Taiho pharmaceuticals, Otsuka pharmaceuticals, Adagene Inc., Bayer Healthcare, Alx Oncology, Sanofi and Amgen. JRB has served as a consultant for Abbvie, Acerta/Astra-Zeneca, BeiGene, Genentech/Roche, Grifols Worldwide Operations, Hutchmed, iOnctura, Janssen, Kite, Loxo/Lilly, MEI Pharma, Numab Therapeutics, Pfizer, Pharmacyclics and received research funding from BeiGene, Gilead, iOnctura, Loxo/Lilly, MEI Pharma, TG Therapeutics. CUN has received consultancy fees and/or travel grants from Janssen, Abbvie, Novartis, Roche, Sunesis, Gilead, AstraZeneca, and CSL Behring, research support from Abbvie, AstraZeneca, Janssen and Novo Nordisk Foundation (NNF16OC0019302). TZ has received honoraria from Roche, Abbvie, Gilead, Janssen, AstraZeneca, Eli Lilly and Janpix. MSD has received institutional research funding from Ascentage Pharma, MEI Pharma, Novartis, Surface Oncology, TG Therapeutics and personal consulting income from AbbVie, Adaptive Biosciences, Ascentage Pharma, AstraZeneca, BeiGene, BMS, Eli Lilly, Genentech, Genmab, Janssen, Merck, Mingsight Pharmaceuticals, Secura Bio, TG Therapeutics, and Takeda. Other authors have no potential conflicts of interest or relevant disclosures.

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