RESEARCH BRIEF

Acquisition of the Recurrent Gly101Val Mutation in BCL2 Confers Resistance to Venetoclax in Patients with Progressive Chronic Lymphocytic Leukemia

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ABSTRACT The BCL2 inhibitor venetoclax induces high rates of durable remission in patients with previously treated chronic lymphocytic leukemia (CLL). However, despite continuous daily treatment, leukemia recurs in most patients. To investigate the mechanisms of secondary resistance, we analyzed paired pre-venetoclax and progression samples from 15 patients with CLL progression enrolled on venetoclax clinical trials. The novel Gly101Val mutation in BCL2 was identified at progression in 7 patients, but not at study entry. It was first detectable after 19 to 42 months of therapy, and its emergence anticipated clinical disease progression by many months. Gly101Val reduces the affinity of BCL2 for venetoclax by ~180-fold in surface plasmon resonance assays, thereby preventing the drug from displacing proapoptotic mediators from BCL2 in cells and conferring acquired resistance in cell lines and primary patient cells. This mutation provides new insights into the pathobiology of venetoclax resistance and provides a potential biomarker of impending clinical relapse.

SIGNIFICANCE: Why CLL recurs in patients who achieve remission with the BCL2 inhibitor venetoclax has been unknown. We provide the first description of an acquired point mutation in BCL2 arising recurrently and exclusively in venetoclax-treated patients. The mutation reduces venetoclax binding and is sufficient to confer resistance.

See related commentary by Thangavadivel and Byrd, p. 320.

INTRODUCTION

Venetoclax is a potent and highly selective BCL2 inhibitor (1) approved for the treatment of patients with previously treated chronic lymphocytic leukemia (CLL), as monotherapy (2–5) or in combination with rituximab (6, 7). Venetoclax is taken continuously once daily until disease progression. Clinical responses are rapid, with ~80% of patients achieving substantial cytoreduction (2, 3). Complete remissions are seen in ~20% of patients with monotherapy and 20% to 50%

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when combined with rituximab (2, 3, 6–8). Further, a minority achieve remissions that are measurable residual disease (MRD)–negative by highly sensitive multicolor flow cytometry (MFC; refs. 7, 9). However, disease relapse ultimately occurs in the majority of patients who were heavily pretreated prior to commencing venetoclax. The median response duration in patients with deletion 17p CLL receiving venetoclax monotherapy is 33.2 months, highlighting the significant challenge secondary resistance poses (9).

Disease progression on venetoclax may manifest as transformation to diffuse large B-cell lymphoma [Richter transformation (RT)] in a small proportion of patients, but usually presents as a recurrence of CLL (CLL-type progression) without morphologic or immunophenotypic change (10, 11). The underlying biological mechanisms leading to CLL progression in venetoclax-treated patients remain largely unknown. Acquired abnormalities in *BTG1, TP53, CDKN2A/B, SF3B1,* and *BRAF* have been reported in a small cohort of 8 patients with deletion 17p CLL progressing on venetoclax, 4 with RT (12). However, a causal relationship has yet to be established, particularly for lesions that are common in CLL (e.g., *TP53* and *SF3B1*), and the mechanisms that result in CLL-type progressions on venetoclax therapy may well be distinct from those emerging as RT.

Venetoclax inhibits BCL2's function by binding its critical hydrophobic groove, the same site that sequesters its physiologic ligands, BH3 domain–containing proapoptotic proteins (13). If unconstrained by BCL2, these BH3-domain proteins (e.g., BIM, BAX, and BAK) are free to drive apoptosis. Acquired mutations in the BH3-binding groove of BCL2 have been reported in a murine cell line where venetoclax refractoriness was induced through continuous drug exposure (BCL2 Phe101Cys and Phe101Leu, analogous to human BCL2 Phe104Cys and Phe104Leu; ref. 14). However, *BCL2* mutations have yet to be described in patients treated with venetoclax.

Using a cohort of 67 patients (10) with relapsed CLL treated with venetoclax on 3 early-phase clinical trials, we have performed a focused genomic evaluation in those with CLL-type progressions, specifically excluding those with RT (where genomic heterogeneity is well recognized). Among the CLL progressions on venetoclax, we have discovered and functionally characterized a recurrent novel *BCL2* mutation [NM_000633.2:c.302G>T, p.(Gly101Val)] present at disease progression but not at treatment initiation. Gly101Val markedly reduces the affinity of venetoclax for BCL2 and confers acquired resistance *in vitro* and *in vivo* in patients with CLL.

RESULTS

Twenty-one patients treated with venetoclax in the study cohort (10) have experienced CLL-type progression after achieving an initial response, whereas 18 developed RT. CLL-type progression manifested clinically as a gradual increase in morphologic CLL burden over months to years, typically after a period when subclinical disease was initially detected in the bone marrow (and subsequently blood) by MFC. The median time on venetoclax to CLL-type clinical progression in this cohort was 36 (range, 6.5–73) months.

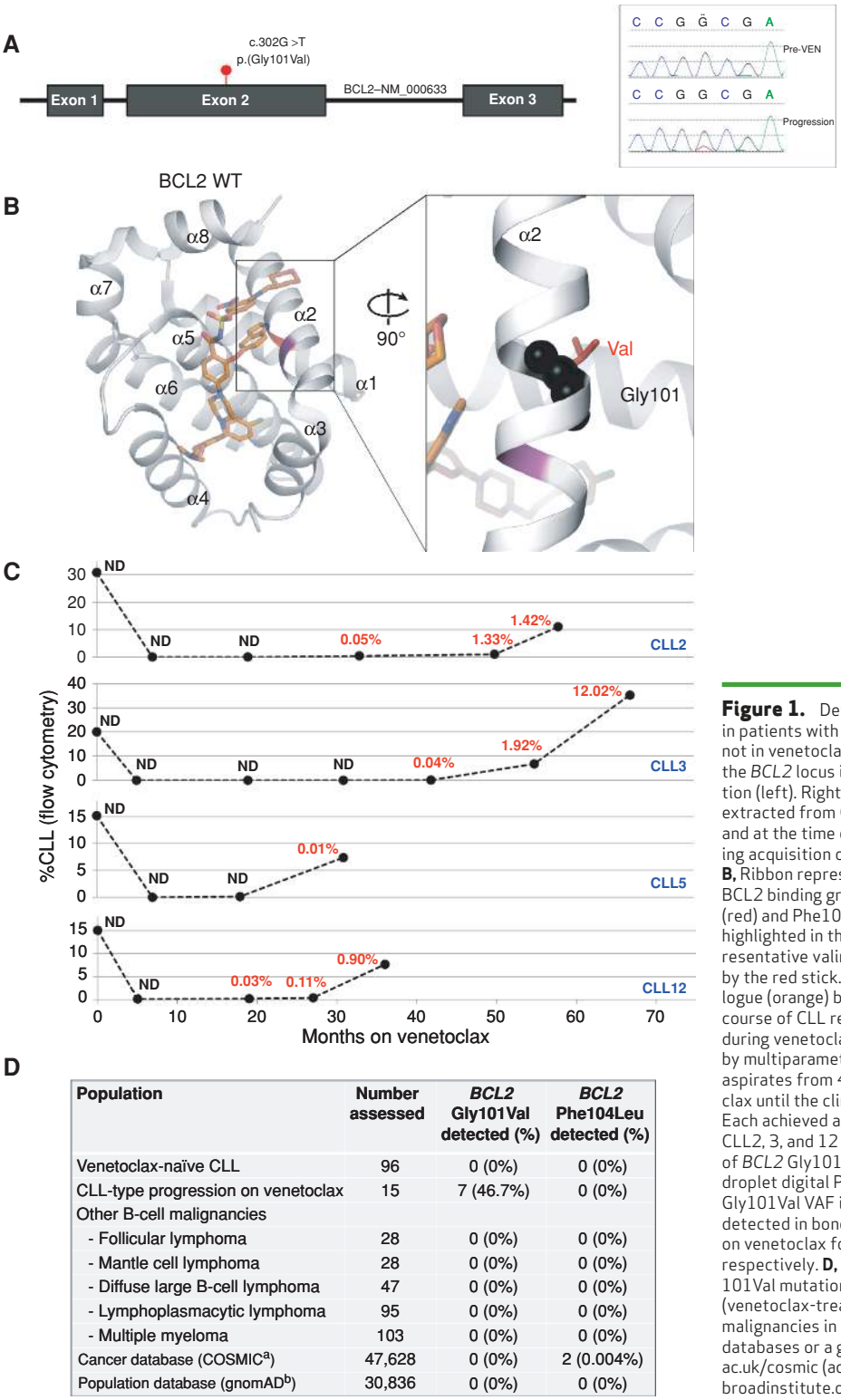
Genomic Assessment of Patient Samples Pre– and Post–Venetoclax Treatment

Fifteen of 21 patients with CLL-type progression had suitable pre-venetoclax and progression specimens available for analysis. Targeted amplicon next-generation sequencing (NGS) was performed on paired samples assessing the entire coding region of *BCL2.* In 4 patients (CLL2, CLL3, CLL5, and CLL12), a single-nucleotide variant was detected in *BCL2* [NM_000633.2:c.302G>T, p.(Gly101Val)] in samples at, or following, CLL-type progression (Fig. 1A and B). This variant was undetectable in paired pre-venetoclax samples. Digital droplet PCR (ddPCR) was used to characterize the emergence of the mutation in serial archived samples from these patients (Fig. 1C) alongside bone marrow disease burden measured by MFC. Gly101Val was first detected at low variant allele frequency (VAF) after 19 to 42 months on venetoclax, up to 25 months earlier than when standard disease progression criteria were met.

$CD19^+$-selected tumor cells of 92.8% purity collected at CLL progression from CLL3 had a Gly101Val VAF of 32.49% by ddPCR. No copy-number loss or acquired uniparental disomy at the *BCL2* locus was observed (Supplementary Fig. S1A). This is consistent with Gly101Val being present in the heterozygous state in the majority (~70%) of CLL cells in this patient at the time of progression. Expression of the Gly101Val mutation was confirmed in this patient by RNA sequencing (Supplementary Fig. S1B). Using sample CLL burden (determined by MFC) and ddPCR VAF, we estimated the proportion of CLL cells carrying Gly101Val in patients CLL2, CLL5, and CLL12 (assuming heterozygosity) to be approximately 26%, 46%, and 60%, respectively (Supplementary Table S1).

The observation of subclonality and detection of Gly101Val in preprogression samples at frequencies lower than detectable by targeted amplicon NGS led us to test whether it could be detected in other CLL-type progressors using ddPCR. Gly101Val was detected in 3 other patients (CLL6, CLL8, and CLL14) at low VAF (0.01%–0.4%) in post-venetoclax samples (Supplementary Table S1). The CLL disease burden assessed by MFC in these post-venetoclax samples ranged from 3.3% to 57.5% of leukocytes. Therefore, the estimated proportion of CLL cells carrying the Gly101Val mutation in these patients at progression was 1.4% to 4.3%. Importantly, Gly101Val was not detected in paired pre-venetoclax samples from any of the 7 Gly101Val-mutated patients using ddPCR (limit of detection 0.01%). After cessation of venetoclax, a stable proportion of CLL cells bearing Gly101Val persisted for at least 6 months in 5 patients who received BTK inhibitor therapy (Supplementary Fig. S2).

Targeted amplicon NGS of 32 other genes commonly mutated in CLL and lymphoid malignancy was also performed on pre- and postprogression samples (Supplementary Table S1). In 8 of 15 patients with CLL-type progressions, different sequence variants were detected (not including *BCL2* Gly101Val) at progression compared with baseline. Five had *TP53* variants at baseline that were not detected at CLL-type progression, 3 had new *TP53* variants at progression; variants in *NOTCH1, KRAS,* and *BIRC3* were lost in single patients at progression, and there were acquisitions of *SF3B1* or *KRAS* variants in single patients.



Population	Number assessed	*BCL2* Gly101Val detected (%)	*BCL2* Phe104Leu detected (%)
Venetoclax-naïve CLL	96	0 (0%)	0 (0%)
CLL-type progression on venetoclax	15	7 (46.7%)	0 (0%)
Other B-cell malignancies			
- Follicular lymphoma	28	0 (0%)	0 (0%)
- Mantle cell lymphoma	28	0 (0%)	0 (0%)
- Diffuse large B-cell lymphoma	47	0 (0%)	0 (0%)
- Lymphoplasmacytic lymphoma	95	0 (0%)	0 (0%)
- Multiple myeloma	103	0 (0%)	0 (0%)
Cancer database (COSMIC[a])	47,628	0 (0%)	2 (0.004%)
Population database (gnomAD[b])	30,836	0 (0%)	0 (0%)

Figure 1. Detection of the BCL2 Gly101Val mutation in patients with progressive CLL on venetoclax (VEN), but not in venetoclax-naïve patients. **A,** Genomic structure of the *BCL2* locus indicating the position of the point mutation (left). Right, Sanger sequencing traces from DNA extracted from CLL3 before venetoclax treatment (top) and at the time of CLL-type progression (bottom) showing acquisition of NM_000633.2; c.302G>T, p.(Gly101Val). **B,** Ribbon representation of α-helices 1–8 that form the BCL2 binding groove, indicating the location of Gly101 (red) and Phe104 (purple) on the α2 helix. Gly101 is highlighted in the black sphere representation, and a representative valine side chain at this position is indicated by the red stick. The structure is that of a venetoclax analogue (orange) bound to BCL2 (PDB: 4MAN) (1). **C,** Time course of CLL response and subsequent reemergence during venetoclax therapy. The CLL burden was measured by multiparameter flow cytometry in serial bone marrow aspirates from 4 patients from the initiation of venetoclax until the clinical diagnosis of progressive disease. Each achieved a clinical response: complete remission for CLL2, 3, and 12 and partial remission for CLL5. The VAF of *BCL2* Gly101Val in bone marrow samples measured by droplet digital PCR is overlaid. ND, not detected. *BCL2* Gly101Val VAF is indicated in red. The mutation was first detected in bone marrow after 33, 42, 31, and 19 months on venetoclax for patients CLL2, CLL3, CLL5, and CLL12, respectively. **D,** Summary of the incidence of *BCL2* Gly101Val mutation and *BCL2* Phe104Leu mutation in CLL (venetoclax-treated, venetoclax-naïve) and other B-cell malignancies in this study; and in publicly available cancer databases or a general population. [a]https://cancer.sanger.ac.uk/cosmic (accessed August 25, 2018). [b]http://gnomad.broadinstitute.org/ (accessed August 25, 2018).

BCL2 Gly101Val was not found in searches of either general population or cancer patient public databases (Fig. 1D). Importantly, review of *BCL2* sequencing data from 96 patients with venetoclax-naïve CLL (63 untreated, 33 with relapsed/refractory disease; 23 with previous fludarabine-based treatment) did not reveal any cases bearing the Gly101Val mutation (Fig. 1D), and it was not observed in the limited number of biopsies analyzed from patients who progressed with RT ($n = 5$). Further, Gly101Val was not detected in targeted amplicon sequencing data from 301 patients with other B-cell

malignancies. Nor did we detect any cases with the *BCL2* Phe104Leu mutation analogous to that reported to confer venetoclax resistance in murine cell lines (14). Given the observation of acquisition of Gly101Val only in the setting of CLL-type progression on venetoclax treatment, we hypothesized that this variant contributes to venetoclax resistance.

Patient CLL Cells with Gly101Val at Progression and Gly101Val-Expressing Cell Lines Are Intrinsically Less Sensitive to Venetoclax

Freshly collected CLL cells from progression on venetoclax for the 4 patients with significant proportions of leukemic cells harboring Gly101Val were much less sensitive to venetoclax killing *in vitro* than their CLL cells collected at study entry (Fig. 2A). A similar acquisition of reduced sensitivity was observed when the cells were exposed to ABT-737, a dual BCL2/BCLxL inhibitor with a similar chemical scaffold (ref. 15; Supplementary Fig. S3).

To determine whether the Gly101Val mutation alone is sufficient to confer resistance to venetoclax, the mutant was overexpressed in two human B-lineage cell lines. RS4;11 Gly101Val cells were approximately 30-fold less sensitive to venetoclax than RS4;11 wild-type (WT) BCL2 cells (Fig. 2B and D), and similar results were observed using KMS-12-PE (Fig. 2C and D). The Phe104Leu mutation also induced similar resistance in these cell lines (Fig. 2B and C).

BCL2 Prosurvival Function Is Maintained Despite the Gly101Val Mutation

We next tested whether the prosurvival function of BCL2 was affected more generally by the Gly101Val mutation. High levels of BCL2 are associated with resistance to DNA-damaging cytotoxics and other anticancer drugs, as demonstrated when WT BCL2 was overexpressed in RS4;11 cells (Fig. 2E). Gly101Val similarly reduced *in vitro* sensitivity to each of etoposide, cytarabine, fludarabine, and dexamethasone, as did Phe104Leu (Fig. 2E). Following the same pattern, Gly101Val overexpression was as effective as WT BCL2 overexpression in KMS-12-PE cells at protecting against killing by bortezomib (Supplementary Fig. S3B). Thus, the normal function of BCL2 to block apoptosis is preserved despite the Gly101Val mutation.

Venetoclax Binding to BCL2 Is Markedly Reduced by the Gly101Val Mutation

Our functional studies in cell lines and the detection of the Gly101Val mutation exclusively in venetoclax-treated patients developing progressive CLL imply that the effect of this mutation is likely to be highly specific for the inhibition of BCL2 by venetoclax. To test this, we examined how the Gly101Val mutation affects the ability of BCL2 to bind either venetoclax or its physiologic ligands, the key proapoptotic proteins such as BIM, BAX, and BAK.

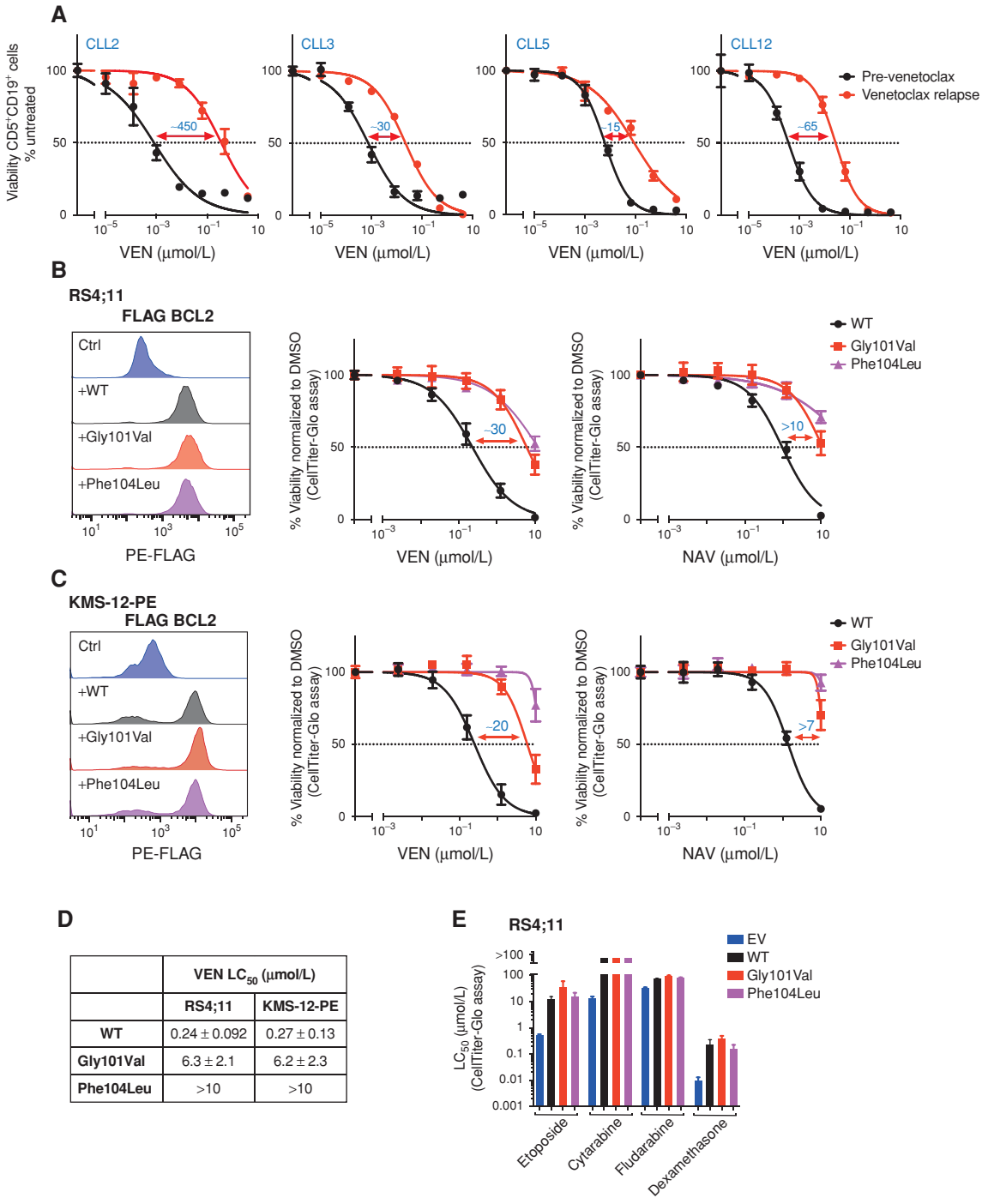
The Gly101Val mutation did not significantly affect the ability of BCL2 to bind a BIMBH3 peptide (Fig. 3A and B), consistent with our findings that the mutant broadly retains WT prosurvival activity (Fig. 2E). However, we saw a striking difference when we assessed the ability of venetoclax to compete with BIM for BCL2 binding (Fig. 3D and E). Competition surface plasmon resonance (SPR) experiments showed venetoclax binds avidly to WT BCL2, preventing BIM binding. This is markedly reduced (~180-fold) with the Gly101Val mutant, as indicated by the flattening of the sigmoidal curves toward the hyperbola in the case of the mutation compared with WT protein (Fig. 3E). A modest reduction in BAXBH3 binding was also observed (Fig. 3A and C). Although the magnitudes varied, similar trends were observed with the Phe104Leu mutant. Given that the principal impact of these mutations is reduction in venetoclax binding to BCL2, we predicted that venetoclax would be less able to displace proapoptotic molecules from BCL2 Gly101Val in cells.

To investigate this, we assessed the ability of venetoclax to free proapoptotic BIM, BAX, or BAK already bound to BCL2 in cells. BIM binding to BCL2 (both WT and mutant) was very tight and was largely unaffected by the addition of venetoclax (Fig. 4A; Supplementary Fig. S4). In contrast, venetoclax could free BAX and BAK from WT BCL2, but was ineffective when these proapoptotic molecules were bound to the Gly101Val or the Phe104Leu mutants. Thus, in the biochemical and cellular assays, we found venetoclax to be less effective against the Gly101Val mutant, and this probably accounts for the reduced sensitivity of the cells to venetoclax. Interestingly, we also observed increased binding of BAK to the Gly101Val mutant, and reduced BIM and BAX binding in cells. Potentially, the impact of the mutation on the propensity of BCL2 to bind the various BH3 domain–containing proapoptotic proteins might also contribute to the resistance observed.

The Gly101Val Mutation Confers a Selective Advantage during Continuous Exposure to Sublethal Concentrations of Venetoclax

As our biochemical and short-term cellular studies highlight how the Gly101Val mutation impairs the sensitivity of BCL2 to venetoclax, we next explored the consequences of continuous exposure to venetoclax (as used clinically) in long-term cultures. In the absence of venetoclax, the WT and Gly101Val-expressing cells grew at similar rates. However, in the presence of a sublethal dose of venetoclax, the latter outcompeted WT cells. This occurred rapidly in KMS-12-PE cells and more slowly in RS4;11 (Fig. 4B), reflecting the 25% shorter doubling time and greater sensitivity to venetoclax of parental KMS-12-PE cells compared with RS4;11.

We next sought to understand how the presence of the Gly101Val mutation might interact with exogenous factors that also protect CLL cells from killing by venetoclax *in vivo*. Using an established model of the CLL microenvironment (16), we cocultured CLL cells from progression on CD40 ligand–expressing stromal cells. For comparison, we cocultured CLL cells with exclusively WT BCL2 from 3 venetoclax-naïve patients. As shown in Fig. 4C, freshly collected CLL3 cells were substantially less sensitive than venetoclax-naïve CLL cells prior to coculture. After 1 week on stroma, venetoclax-naïve CLL cells were less sensitive to venetoclax, displaying similar sensitivity to fresh CLL3 cells prior to coculture. With 1 week of coculture, cells from CLL3, CLL2, and CLL5 (Supplementary Fig. S5A) became markedly resistant to venetoclax, even at concentrations between 1 and 10 μmol/L, the latter higher than achievable clinically (2).



	VEN LC_{50} (μmol/L)	
	RS4;11	KMS-12-PE
WT	0.24 ± 0.092	0.27 ± 0.13
Gly101Val	6.3 ± 2.1	6.2 ± 2.3
Phe104Leu	>10	>10

Figure 2. Cells bearing BCL2 Gly101Val have attenuated sensitivity to venetoclax. **A,** Leukemic cells from patients CLL2, CLL3, CLL5, and CLL12 are markedly less sensitive to venetoclax at progression. Mononuclear cells prepared prior to the patients commencing venetoclax (VEN; black) or at CLL progression (red) were incubated for 24 hours with venetoclax *in vitro* (0–4 μmol/L). Data represent means ± 1 SD of triplicate measurements in single experiments for viable (PI^-) CLL cells ($CD5^+$ $CD19^+$); fold changes in LC_{50} values are indicated. **B,** Expression of BCL2 Gly101Val or BCL2 Phe104Leu in the RS4;11 cell line reduces sensitivity to venetoclax. Each of these mutants or WT BCL2 were equally expressed (see FACS profiles on left), and the *in vitro* sensitivities to venetoclax (0–10 μmol/L; middle) or to navitoclax (NAV; 0–10 μmol/L; right) measured 24 hours later. The fold reductions in LC_{50} values with the Gly101Val mutation are indicated. **C,** Similar to **B,** but in another B-lineage cell line, KMS-12-PE. **D,** Summary of the LC_{50}s (**B**, **C**) for WT BCL2 and each of the mutants. **E,** Like WT BCL2, the BCL2 mutants afforded protection against killing by other anticancer drugs. Control RS4;11 cells, or ones expressing WT or mutant BCL2 were incubated with etoposide (0–100 μmol/L, 24 hours), cytarabine (0–100 μmol/L, 24 hours), fludarabine (0–100 μmol/L, 24 hours), or dexamethasone (0–10 μmol/L, 48 hours), and LC_{50} values were calculated. The expression of WT BCL2 significantly inhibited killing by these drugs; the mutants (Gly101Val or Phe104Leu) were as potent as the WT protein. Data in **B**–**E** represent means ± 1 SD of three independent experiments.

A

BCL2 protein	BIMBH3 K_D (nmol/L ± SD, n = 3)	BAXBH3 K_D (nmol/L ± SD, n = 3)
WT	**0.29** ± 0.17	**1.4** ± 0.2
Gly101Val	**0.84** ± 0.04	**11.7** ± 1.6
Phe104Leu	**0.039** ± 0.001	**6.1** ± 1.0

D

BCL2 protein	VEN K_I (nmol/L ± SD, n = 3)
WT	**0.018** ± 0.014
Gly101Val	**3.2** ± 1.1
Phe104Leu	**0.46** ± 0.12

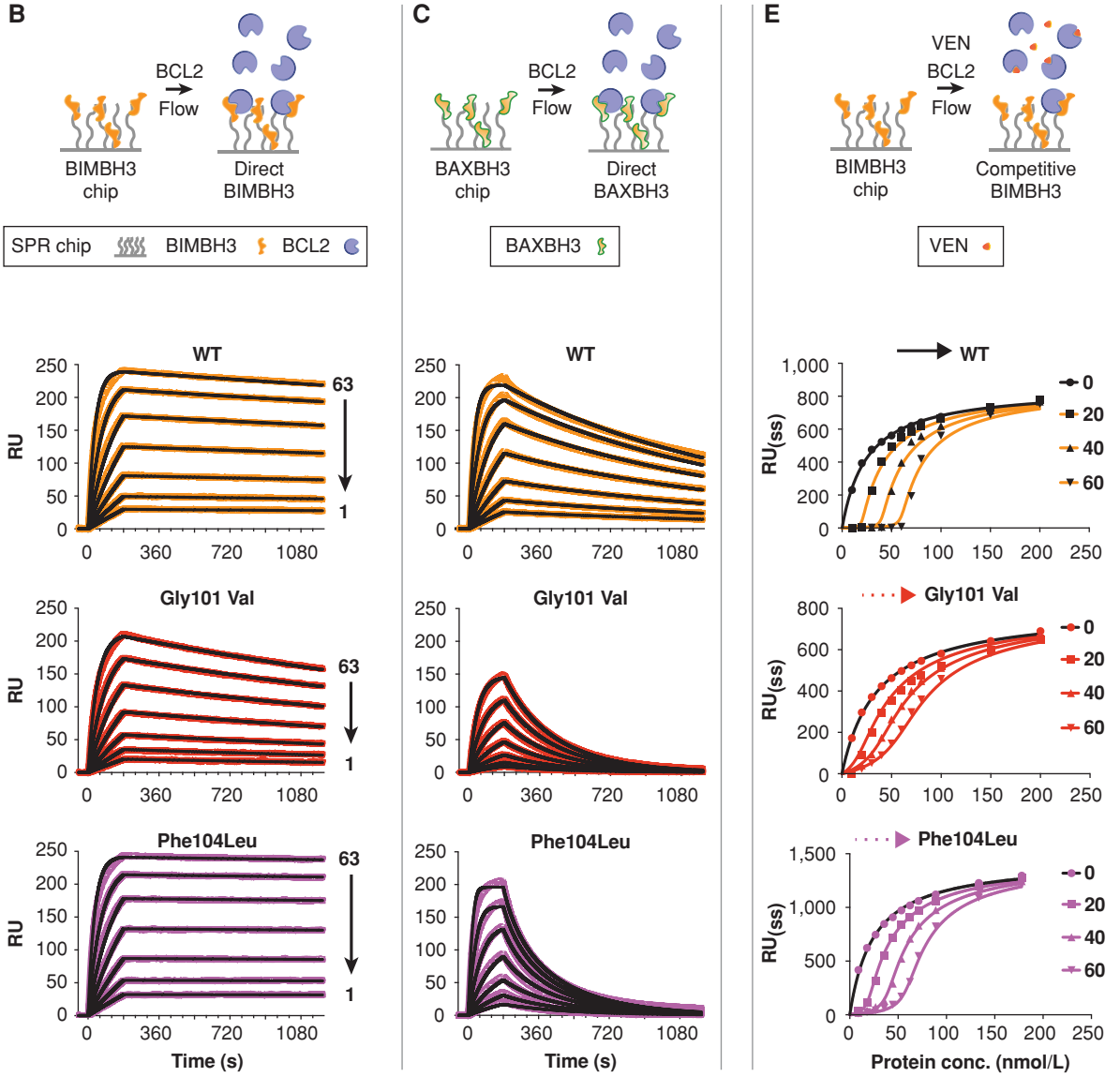


Figure 3. Impact of the Gly101Val mutation on the ability of BCL2 to bind BH3 ligands. **A,** Table summarizing the binding affinities of BH3 peptides derived from BIM or BAX for WT or mutant BCL2 as determined by direct binding assays shown in **B** and **C**. Data represent means ± SD of three independent experiments. **B,** BIMBH3 binding. 0–62.5 nmol/L WT or mutant BCL2 were used as analytes against the BIMBH3 peptide immobilized on an SPR; BIAcore) sensor chip, the top panel illustrating the experimental conditions used. The raw response (RU) curves (colored curves) from a representative experiment were fitted to a one site-specific kinetic model (black curves) to derive on and off rates, and hence to calculate K_D values for interactions with WT BCL2 (second panel), Gly101Val (third panel), and Phe104Leu (fourth panel). **C,** Similar to **B,** with the same BCL2 concentrations indicated in **B,** but using a BAXBH3 immobilized sensor chip to determine BAXBH3 binding affinities. **D,** Reduced affinity of venetoclax for the Gly101Val mutant. Table summarizing the steady-state binding affinities of venetoclax determined in competition assays (**E**). Protocols to immobilize venetoclax, unlike the BIMBH3 peptide, to sensor chips have not been optimized, hence precluding affinity determination by direct binding. Data represent means ± SD of three independent experiments. **E,** Solution competition assays to determine venetoclax binding affinity. WT or mutant BCL2 (0–250 nmol/L) was preincubated with 0, 20, 40, or 60 nmol/L of venetoclax, before flowing the analyte over a BIMBH3 sensor chip. The data were fitted to a steady-state competition equation to derive K_I for venetoclax (summarized in **D**) and the fitted curves for a representative experiment shown below. As venetoclax bound avidly to WT BCL2, the reduced amount of free BCL2 available to bind BIMBH3 on the sensor chip was clearly evident by a drop in the steady-state response ($RU_{(ss)}$) at low BCL2 concentrations proportional to venetoclax concentration (black arrow), whereas this was reduced with the Gly101Val mutant (red arrow) reflecting the weak affinity of venetoclax for the mutant.

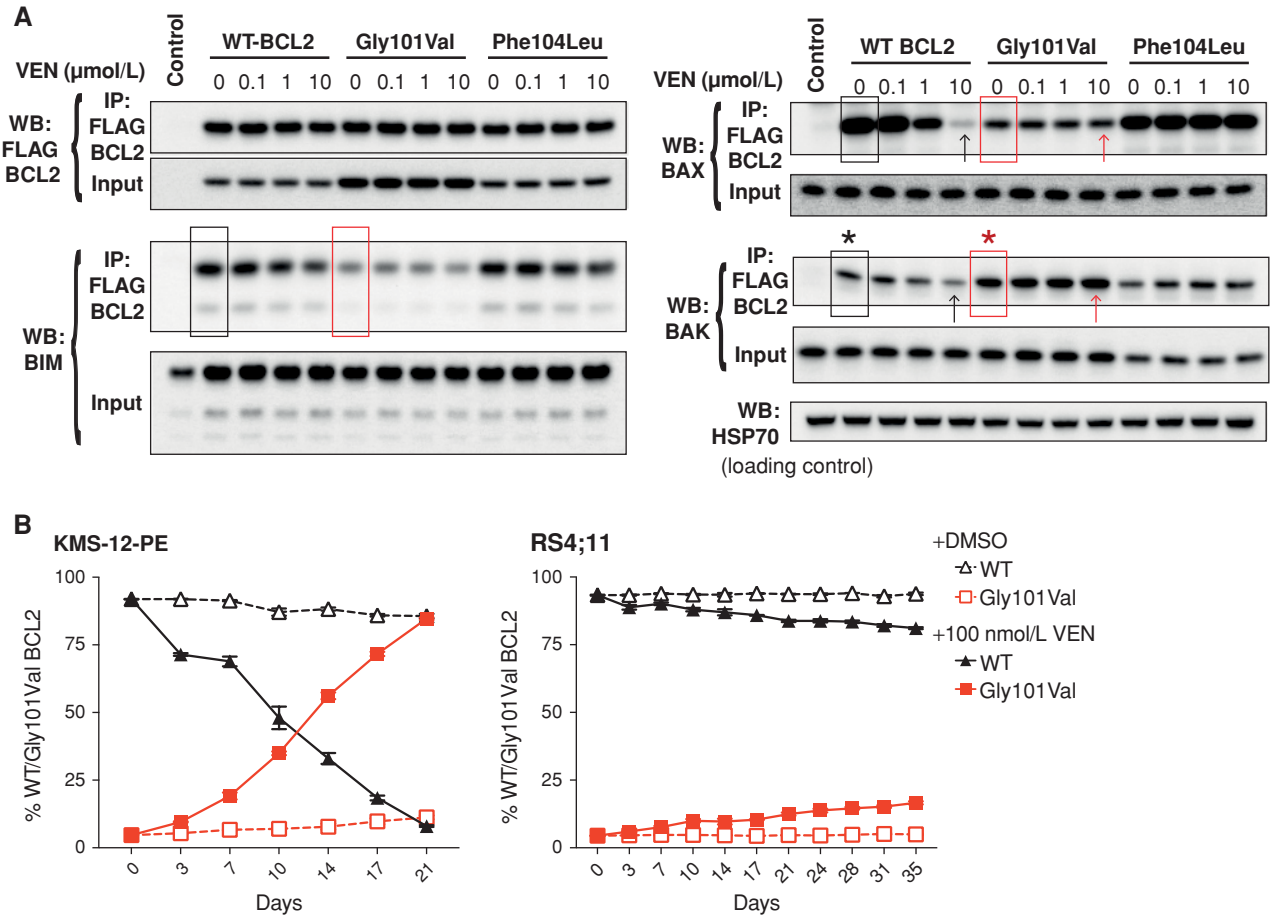


Figure 4. Consequences of the BCL2 Gly101Val mutation. **A,** Venetoclax is less able to compete endogenous BH3 ligands off mutant BCL2 in cells. Equivalent lysates prepared from KMS-12-PE cells ectopically expressing FLAG-tagged WT or mutant BCL2 (Fig. 2C) were immunoprecipitated with the FLAG antibody. The amount of FLAG-tagged BCL2 proteins was comparable, and expression of the binding partners BIM, BAX, or BAK was unaffected (corresponding input lanes). Association of BIM and BAX with the Gly101Val mutant was reduced (compare red with black boxes), probably because of increased BAK binding to the mutant (compare red with black asterisks). In the WT BCL2 cells, BIM binding was very tight and was largely unchanged by venetoclax treatment, but bound BAX and BAK was reduced by venetoclax treatment (see black arrows). However, BAX or BAK remained tightly bound to the Gly101Val or Phe104Leu mutants. Data shown are from a representative of three experiments; control: FLAG immunoprecipitation from parental KMS-12-PE cells. **B,** To varying degrees, cells expressing the Gly101Val mutant outgrow those expressing WT BCL2 when exposed to sublethal concentrations of venetoclax. KMS-12-PE cells (left) or RS4;11 cells (right) expressing WT (GFP labeled) or mutant (BFP labeled) BCL2 were mixed in a 95:5 ratio and grown for the indicated periods in 100 nmol/L venetoclax (filled symbols) or under control conditions (DMSO; open symbols). The relative percentage of the GFP^+ (WT BCL2) or BFP^+ (Gly101Val) cells were monitored by flow cytometry every 3 to 4 days. Data represent means ± 1 SD of three independent experiments. (*continued on following page*)

Additional Disease Resistance Mechanisms Can Coexist with the Gly101Val Mutation

Unlike cell line cultures, the patient samples showed significant subclonality for the Gly101Val mutation (e.g., 70% of malignant cells in CLL3 and 25% in CLL2). Cytotoxicity experiments in mixed populations of BCL2 WT and BCL2-mutant cell lines confirmed that the presence of subclones can shift the observed LC_{50} of the whole population (Supplementary Fig. S5B) by similar extent to that observed between study entry and progression for 3 patients (CLL3, CLL5, and CLL12; Fig. 2A). However, for CLL2, where the Gly101Val mutation–containing subclone comprised only 25% of the resistant leukemic population, the LC_{50} shift was ~450-fold, markedly higher than observed in either the cell lines or the other 3 patients. We investigated prosurvival BCL2 family expression in CLL-progression cells from this patient by mass cytometry and observed bimodal expression of another prosurvival protein, BCLxL, not observed in the other patient samples (Fig. 4D; Supplementary Fig. S6). Cells with high BCLxL expression had much lower BCL2 expression than in either the BCLxL-low subpopulation or other CLL samples. When cells from CLL2 were flow-sorted based on BCLxL expression, the Gly101Val mutation was almost exclusively confined to the population with low BCLxL expression, indicating that distinct mechanisms of resistance were operating in these two populations (Fig. 4E). Notably, CLL2 was observed to be more sensitive to a BCLxL-specific inhibitor (A-1331852; ref. 17) than other CLL samples. Modeling of the data was consistent with the presence of two subpopulations,

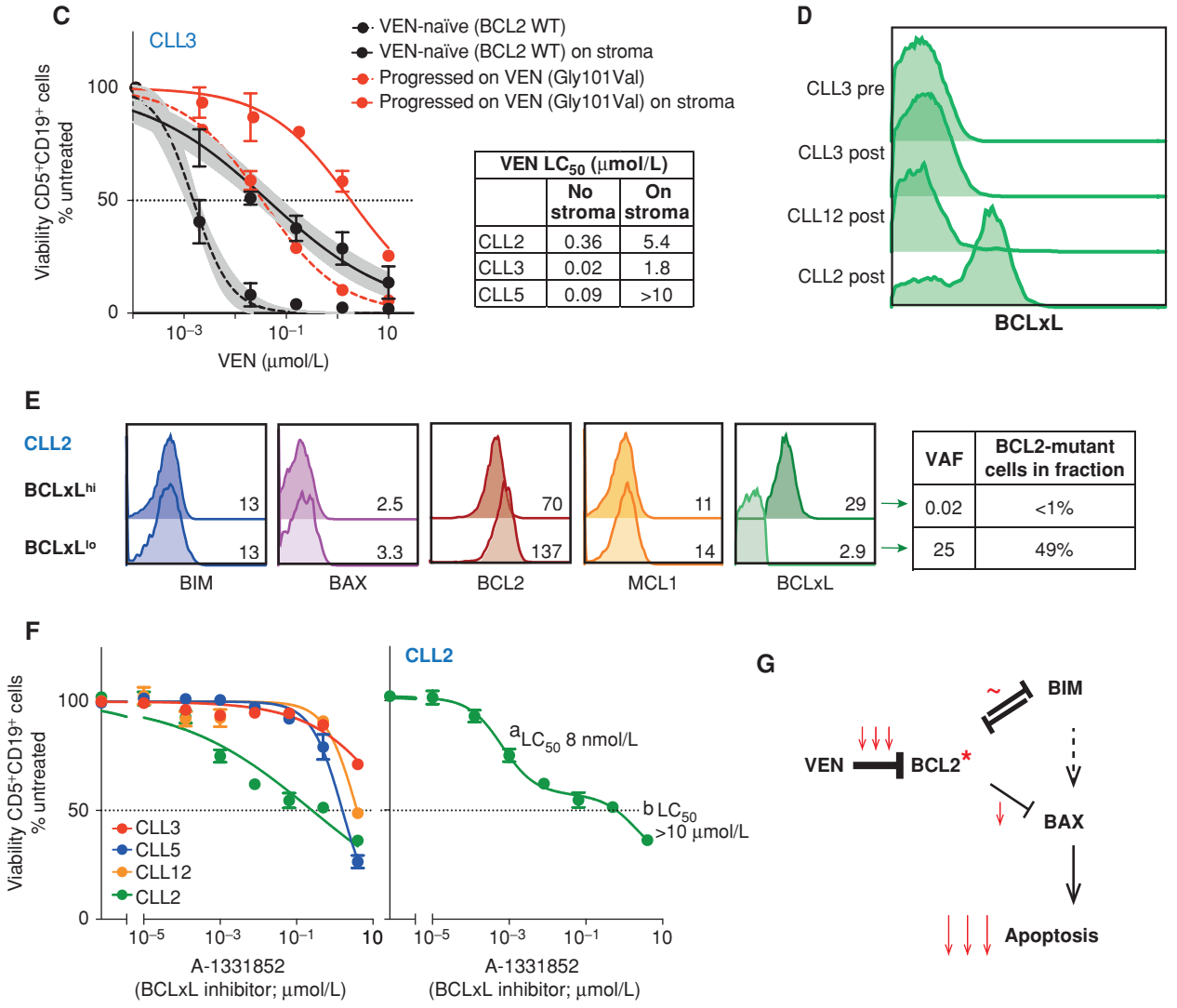


Figure 4. ***(Continued)*** **C,** CLL cells from patients who progressed on venetoclax became highly resistant to venetoclax when cultured under conditions mimicking the stroma. Samples from 3 venetoclax-naïve patients (black lines) became less sensitive when grown for 1 week on human CD40 ligand-expressing feeder cells supplemented with recombinant human IL21 and anti-IgM: compare continuous black line with dashed black line. Starting from a higher baseline, CLL3 patient sample (red lines) also showed a marked attenuation in venetoclax sensitivity when cultured under these conditions, such that it became almost completely resistant. This was not due to selection for Gly101Val-expressing cells because the VAF was unchanged. Data represent means ± 1 SD of triplicate measurements in single experiments for viable (PI^-) CLL cells ($CD5^+$ $CD19^+$) in each sample. The table summarizes the impact of culturing samples from 3 patients who had progressive CLL while on venetoclax. **D,** Histograms of mass cytometric analysis of BCLxL expression in viable (cisplatin^{lo}) $CD5^+CD19^+$ peripheral blood mononuclear cells (PBMC) from 3 patients at study entry (CLL3) and upon relapse (CLL2, 3, and 12). See also Supplementary Fig. S6. **E,** The high BCLxL-expressing subclone in CLL2 does not bear the BCL2 Gly101Val mutation, whereas the BCL2-mutant clone expresses low levels of BCLxL. Thawed postprogression PBMCs from CLL2 were permeabilized to enable flow-cytometric intracellular measurement of BCLxL, then sorted for $CD19^+$ cells with either high or low BCLxL expression (45% and 38% of cells, respectively). The mean intensity value (arbitrary units) is reported for each histogram. The VAF for Gly101Val mutation was measured for each fraction by ddPCR, and is tabulated to the right. The VAF of the input $CD19^+$ cells was 13.78%, indicating that approximately 27.5% of input CLL cells carried the mutation. **F,** *In vitro* sensitivity of progressive CLL to the BCLxL inhibitor A-1331852. Cell samples from patients after progression on venetoclax were incubated with varying concentrations of A-1331852 for 24 hours. Left, cells from patients CLL2, 3, 5, and 12. Only CLL2 showed substantial sensitivity to this drug. However, visual inspection of the shape of the CLL2 curve suggested the presence of more than one population of cells. Right, improved curve fit when CLL2 data were reanalyzed in a two-population model (Prism). The data are consistent with the presence of two populations, one sensitive (a) and the other insensitive (b) to A-1331852. The calculated LC_{50} for each is noted on the graph. Data represent means ± 1 SD of triplicate measurements in single experiments for each patient sample. **G,** Model of how the BCL2 Gly101Val mutation (indicated by red asterisk) affected the induction of apoptosis by venetoclax. The predominant effect is likely to be the marked reduction in the ability of venetoclax to bind to mutant BCL2.

one sensitive to BCLxL inhibition and the other insensitive (Fig. 4F). Mass cytometry did not identify significant alterations in MCL1 or BCLxL expression in CLL cells in the other patient samples tested (CLL3, CLL5, and CLL12).

DISCUSSION

We have identified and functionally characterized a novel recurrent *BCL2* mutation (Gly101Val) emerging in a cohort of patients with CLL-type progressions treated with venetoclax. Our data demonstrate that the Gly101Val mutation impairs binding of venetoclax to BCL2, confers resistance to venetoclax in both patient leukemia cells and engineered cell lines, and provides a selective growth advantage over WT cells when maintained in the presence of the drug *in vitro*.

CLL cells are universally addicted to the high levels of BCL2, which keep proapoptotic relatives such as BIM, BAX, and BAK in check by direct sequestration through its BH3-binding groove, thereby permitting their inappropriate survival (13, 18). By binding BCL2 tightly in the same groove, venetoclax effectively displaces these proapoptotic proteins, freeing them to trigger apoptosis of the CLL cells *in vitro* and *in vivo* (1, 19). Through competitive binding experiments, we showed that venetoclax has markedly decreased binding (~180-fold) to Gly101Val, thereby compromising its ability to displace proapoptotic proteins such as BAX (Fig. 4A) and hence the induction of apoptosis (Figs. 2A–C and 4G). Because BCL2 binding to proapoptotic BIM and BAX was only marginally compromised, the ability of the Gly101Val mutant to function normally to protect cells from apoptosis in the absence of venetoclax was unaffected (Fig. 2E). Further investigation is required to formally establish whether Gly101Val has any modest gain or loss of function when assessed under different physiologic or pathologic circumstances.

Thus, the Gly101Val mutation specifically affects the action of venetoclax (Fig. 4E) and compounds with a similar chemical scaffold, such as ABT-737 and navitoclax (ref. 13; Fig. 2B and C; Supplementary Fig. S3A). Surprisingly, venetoclax does not directly contact Gly101, at least in the available structure of a venetoclax analogue bound to BCL2 (ref. 1; Fig. 1B). This residue is highly conserved among BCL2 prosurvival family members, and the binding groove is malleable such that it can accommodate multiple ligands, including a variety of BH3 domains and BH3-mimetic drugs (15, 20). We speculate that the mutation must indirectly affect the binding groove to selectively impair venetoclax binding. Gly101Val represents an early example of a mutation that induces resistance to a targeted therapy by modifying a protein–protein interaction site, rather than an enzyme active site (e.g., mutations in BCR–ABL1, ref. 21; and BTK, ref. 22).

Although our experimental data indicate that the Gly101Val mutation is sufficient to confer resistance to venetoclax and outgrowth of mutation-bearing clones in the presence of continuous venetoclax exposure *in vitro* and in patients, our data also indicate that other mechanisms for development of resistance are clinically important. The wide range of subclonality of Gly101Val in our cohort (1.4%–70%) suggests that other acquired changes must confer resistance in non–BCL2 mutation–bearing subclones. Further research is required to identify these alternative mechanisms. However, in 1 patient with cryopreserved cells available for analysis, we were able to demonstrate increased BCLxL expression in a major subclone without the Gly101Val mutation. Increased BCLxL expression has been described recently as a mechanism of resistance to venetoclax in mantle cell lymphoma (23, 24) and appears to be a major contributor to resistance in patient CLL2. Whether changes in expression of BCLxL or other BCL2 family proteins are common in CLL progression on venetoclax remains to be established. Of broad relevance, we also demonstrated that exogenous microenvironmental support amplified the intrinsic resistance of Gly101Val mutation–bearing CLL cells in all samples available for testing. This combined effect probably explains the scenario observed in our patients where BCL2-mutant clones with LC_{50} values of 100 nmol/L to 1 μmol/L accumulated despite ongoing exposure to the typical low micromolar (approximately 1–3 μmol/L) steady-state concentrations of venetoclax observed *in vivo* with continuous therapy (2).

Like others (12), we observed genomic changes between treatment commencement and progression, particularly acquisition and loss of *TP53* variants, but no compelling candidates that could directly account for resistance to venetoclax. We have previously shown that venetoclax-mediated CLL cell death *in vitro* is independent of *TP53* mutation and function (19). Most likely, the association of *TP53* aberrations with venetoclax resistance is functionally indirect, reflecting the underlying genomic instability in CLL cells from this heavily pretreated, relapsed/refractory cohort of patients (10). Transcriptomic or epigenomic profiling of suitable clinical specimens when they become available may assist in the identification of additional causes of venetoclax resistance, as may broader genomic profiling with whole-exome and whole-genome sequencing. Although further research is required into the development of RT as an avenue to clinical resistance to venetoclax, we have not observed *BCL2* mutations to be contributory. Reassuringly, recent clinical data suggest that RT is infrequently observed on venetoclax in less heavily pretreated patients (7).

Our data support the concept that venetoclax resistance in CLL-type progression is a heterogeneous phenomenon, both between and within individual patients. As we have observed disease progression in every case where Gly101Val was found and that this mutation could be detected before overt clinical relapse in patients through sensitive allele-specific ddPCR, this discovery may have clinical application. Detection of the Gly101Val could potentially serve as an early biomarker of impending disease progression, allowing early therapeutic intervention such as the addition of another drug with a different mechanism of action.

METHODS

See also Supplementary Materials for additional details.

Clinical Cohort

The patient cohort of the first 67 consecutive patients with relapsed CLL/small lymphocytic lymphoma treated with venetoclax on clinical trials at our two institutions has been previously described (10). All provided written informed consent, and the studies were conducted in accordance with the Declaration of Helsinki and after Human Research

Ethics Committee approval. Patients were enrolled from June 2011 to March 2015 and were reviewed at a minimum every 3 months until progression, death, or discontinuation from trial. Outcome data were updated to June 1, 2018.

NGS

Sequences of targeted regions within 33 genes (listed in Supplementary Methods) were analyzed. Indexed, amplicon-based libraries were prepared using Access Array methodology (Fluidigm) and sequenced to a depth of ~1,000× on a MiSeq instrument using v2 chemistry (Illumina). Alignment, variant calling, and annotation were performed using an in-house pipeline. Variants were evaluated using multiple functional and quality filters to identify likely pathogenic variants. Sanger sequencing was used to confirm selected variants. Sample CLL3 was sequenced with a hybridization-based (Agilent SureSelect) NGS panel targeting 363 genes with mean target read depth of ~600×, as described previously (25). Whole-transcriptome sequencing was performed using TruSeq RNA Library Prep Kit v2 on patient CLL3 using RNA extracted from $CD19^+$ selected tumor cells.

Digital Droplet PCR

A ddPCR assay to detect the *BCL2* NM_000633.2:c.302G>T, p.(Gly101Val) variant using forward (5′-CTGGACATCTCGGCGAAG-3′) and reverse (5′-HEX-CC+G+G+CGAC+GA-IABkFQ-3′) oligonucleotide primers with locked nucleic acid probes against WT and mutant sequence was designed. PCR reactions were carried out with 1× ddPCR Supermix for Probes (no dUTP), 2 μL of 360 GC enhancer (Applied Biosystems), primers (900 nmol/L), probes (250 nmol/L), and 50 ng of DNA template to a final volume of 25 μL. PCR cycling conditions were activation (95°C, 10 minutes), 40 cycles of denaturation (94°C, 30 seconds), annealing/extension (60°C, 1 minute), and enzyme deactivation (98°C, 10 minutes). All steps had a ramp rate of 2°C/second. Droplets were generated using the Automatic Droplet generator QX200 AutoDG (Bio-Rad) and read on the QX200TM Droplet Reader with data acquired and analyzed by QuantaSoft software (Bio-Rad).

Cell Biology

Cell Lines. KMS-12-PE (sourced from DSMZ in 2013; cat. #ACC606) and RS4;11 (sourced from ATCC in 2014; cat. #CRL-1873) were cultured with HTRPMI/10% fetal bovine serum. Early passages (P5–P7) after purchase were cryopreserved and thawed for the experiments. Cells beyond passage 15 were not used. Monthly tests for *Mycoplasma* were consistently negative (MycoAlert mycoplasma detection kit; Lonza). *In vitro* killing assays (19) and coculture (16) were performed as previously described, with minor adjustments (see Supplementary Materials).

Plasmids, Retrovirus Production, and Infection. WT *BCL2* construct was reported previously (26); the mutants were introduced by PCR using primers with the desired mutation. cDNAs encoding either WT or mutant FLAG-tagged *BCL2* were inserted into the MSCV-IRES-hygromycin retroviral construct; retroviral production and generation of cell lines was performed as previously described (27).

Cell Viability Assays. Sensitivity of the engineered cell lines to cytotoxics was determined in cell viability assays (CellTiter-Glo assay; Promega, cat. #G9241) as previously described (27). For the competition experiments, WT (GFP^+) or Gly101Val BCL2 (BFP^+) cells were mixed in a 95:5 ratio, and their growth in 100 nmol/L venetoclax or DMSO was monitored by flow cytometry.

SPR Binding Experiments

Experiments were performed using a BIAcore 4000 using a SA sensor chip (GE Healthcare) immobilized with biotinylated BIMBH3 or BAXBH3 peptides, with BIMBH3-4A peptide as a nonbinding reference. Peptide affinities were determined by direct binding with BCL2 (0–63 nmol/L) as the analyte. Venetoclax affinity was determined by competition against immobilized BIMBH3 peptide, using BCL2 (0–250 nmol/L) premixed with venetoclax (0, 20, 40, 60 nmol/L) as the analyte. Direct binding experiments were fitted to a 1:1 binding site model and competition to a steady-state competition model; see also Supplementary Methods.

Coimmunoprecipitation and Mass Cytometry

FLAG-tagged WT or mutant BCL2 was immunoprecipitated from equivalent lysates prepared from venetoclax-treated or control cells with the rat anti-FLAG antibody (clone 9H1, WEHI). The coprecipitated proteins were detected with antibodies to BIM (clone 3C5, WEHI), BAX (clone 21C10, WEHI), or BAK (clone 7D10, WEHI).

For mass cytometry, fresh or thawed cryopreserved patient cells were incubated with cisplatin to allow detection of nonviable cells, then fixed with paraformaldehyde (PFA; Electron Microscopy Sciences), pelleted, washed, and stored at −80°C.

Cell samples for batch analysis were barcoded using the 20-plex palladium barcoding kit according to the manufacturer's instructions (Fluidigm). Following barcoding, cells were pelleted, washed, and then permeabilized at 4°C with methanol for 10 minutes. After washing, cells were incubated with specific antibody–metal conjugates (see Supplementary Methods for specific listings), then washed and stained with 125 nmol/L $^{191}Ir/^{193}Ir$ DNA intercalator (Fluidigm) overnight. After washing, cells were resuspended with EQ normalization beads immediately before analysis using a Helios mass cytometer (Fluidigm). Throughout the analysis, cells were maintained at 4°C and introduced at a constant rate of ~300 cells/second. Data concatenation, normalization, and debarcoding were done with the Helios software, version 6.7.1014 (Fluidigm). Single cells were gated using the FlowJo (version 10.5) and Cytobank software (http://www.cytobank.org) based on event length and $^{191}Ir/^{193}Ir$ DNA content to avoid debris and doublets. Following single-cell gating, $CD5^+CD19^+$ live nonapoptotic $cisplatin^{lo}$ cells were analyzed for levels of the various metal conjugates.

Disclosure of Potential Conflicts of Interest

The Walter and Eliza Hall Institute receives milestone and royalty payments related to venetoclax, and employees may be eligible for benefits related to these payments. M.A. Anderson is a hematologist at the Department of Haematology, Royal Melbourne Hospital and Peter McCallum Cancer Centre; is an employee of the Walter and Eliza Hall Institute which receives milestone and royalty payments related to venetoclax; and has received honoraria from the speakers bureau of AbbVie. J.-n. Gong, R. Thijssen, R.W. Birkinshaw, C.E. Teh, Z. Xu, C. Flensburg, and I.J. Majewski have received remuneration from the Walter and Eliza Hall Institute. T.E. Lew is an employee of the Walter and Eliza Hall Institute of Medical Research and receives milestone and royalty payments related to venetoclax. D.H.D. Gray reports receiving commercial research support from Servier Pharmaceuticals and has received other remuneration from the Walter and Eliza Hall Institute. C.S. Tam has received honoraria from the speakers bureau of AbbVie. J.F. Seymour reports receiving commercial research support from AbbVie, Celgene, Janssen, and Roche; has received honoraria from the speakers bureaus of AbbVie and Roche; and is a consultant/advisory board member for AbbVie, Acerta, Celgene, Janssen, Morphosys, Roche, Sunesis, and Takeda. P.E. Czabotar reports receiving a commercial research grant from Anaxis, is a consultant/advisory board member for Anaxis, and has received other remuneration from the Walter and Eliza Hall Institute. D.C.S. Huang reports receiving a commercial research grant from Servier, has received honoraria from the speakers bureau of AbbVie, and has received other remuneration from the Walter and Eliza Hall Institute of Medical Research. A.W. Roberts receives milestones and royalties related to venetoclax from the Walter and Eliza Hall Institute of

Medical Research, reports receiving commercial research grants from AbbVie and Janssen, and has received other remuneration from the Walter and Eliza Hall Institute of Medical Research. No potential conflicts of interest were disclosed by the other authors.

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