Venetoclax resistance and acquired *BCL2* mutations in chronic lymphocytic leukemia

Venetoclax is a first in class, highly specific antagonist of BCL2 and supersedes current standard treatments in haematologic malignancies as recently demonstrated in the MURANO trial.[1] Remarkable efficacy has been shown particularly in chronic lymphocytic leukemia (CLL) with a significant clearance of minimal residual disease (MRD). In contrast to combination therapy, continuous treatment with venetoclax as a single agent results in up to 50% of patients becoming refractory after 2-3 years as reported in trials with heavily pretreated CLL patients.[2–4] Very recently a potential resistance mechanism to venetoclax has been identified as an acquired mutation in *BCL2*.[5] Here we independently demonstrate the appearance of the *BCL2* mutation G101V in three out of four patients that became refractory to venetoclax. Moreover, we have also identified a second unreported *BCL2* mutation D103Y in one of these patients with venetoclax resistance.

From 10/2013 to 04/2015 15 heavily pretreated, high-risk CLL patients with a deletion of chromosome 17p were enrolled in our center in the pivotal phase 2 trial M13-982[4] with continuous venetoclax treatment. Over time most patients stopped treatment due to various reasons, however four out of six patients treated with venetoclax for a duration longer than 3 years developed progressive CLL.

One of these patients was a 78 year-old woman diagnosed with CLL in 2004. She presented with a second relapse in February 2013 after two different chemo (immuno) therapy regimens (Figure 1A). Genetic analyses showed unmutated IGHV (V2-05) and a deletion of chromosome 17p. A partial response was achieved on a single agent venetoclax treatment with shrinking mediastinal lymph nodes and a normalization of blood counts. However, a routine Computed Tomography (CT) scan in March 2016 (week 120 on treatment) showed increasing lymphadenopathy with histologic confirmation of CLL in lymph node and bone marrow biopsies. As progression was asymptomatic, the patient opted to remain on venetoclax under close monitoring according to the protocol. In February 2017, night sweats and fatigue developed, accompanied by neutropenia and thrombocytopenia. Positron Emission Tomography (PET) -CT scan confirmed splenomegaly and generalized lymphadenopathy with moderate fludeoxyglucose uptake. Cutting needle biopsy of a spleen and bone marrow biopsy confirmed infiltration by CLL and no evidence of Richter transformation. Flow cytometry, fluorescence *in situ* hybridization and *IGHV* sequencing from peripheral blood confirmed CLL with baseline features ($CD19^+CD5^+CD23^+$, deletion 17p), although the lymphocyte count remained low (1.2 g/L). The patient was switched to ibrutinib treatment, but died only a few days later of pneumonia during neutropenia.

In order to identify genomic variants underlying the development of venetoclax resistance, we performed

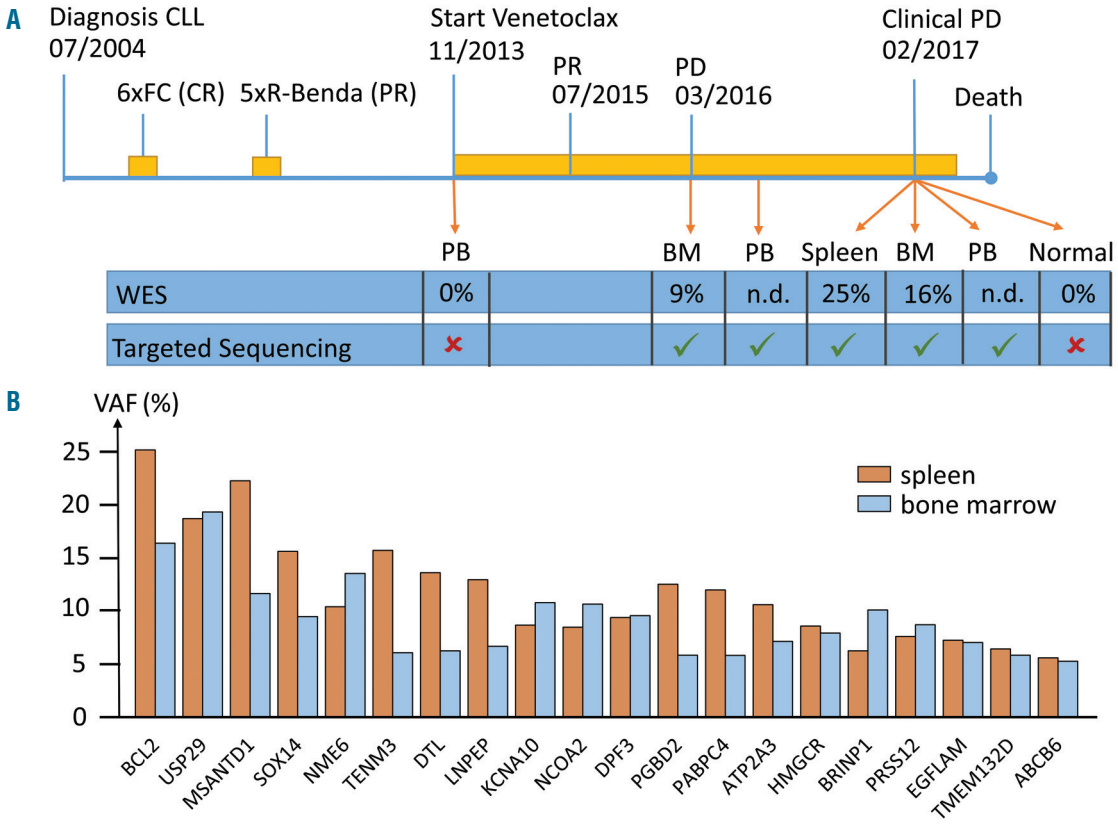

Figure 1. History of an exemplary patient that acquired a *BCL2* G101V mutation during treatment with venetoclax. A. Timeline of patient treatment (yellow bars) and sample collection time points (orange arrows). Variant allelic fraction (VAF) of G101V *BCL2* in different samples measured via WES. Targeted NGS was used to confirm the presence (✓) or absence (✗) of the variant. B. Prevalence of all mutations found in the spleen (red) and bone marrow (blue) at relapse, but not at baseline. Mutations are sorted based on the mean VAF of the spleen and bone marrow samples.

whole exome sequencing of non-malignant and tumor samples from different time points.

Tumor samples were collected and CD19$^+$ cells enriched to enhance tumor purity at baseline (11/2013), first progression (03/2016) and final staging (02/2017) (Figure 1A). Sequence analysis revealed a missense mutation in *BCL2* in spleen and bone marrow samples from the time point of refractory CLL, but neither in baseline CLL samples nor in non-malignant cells obtained from negative CD19 selection from peripheral blood. The acquired variant in codon 302 was predicted to replace the amino acid glycine at position 101 with valine (G101V). Targeted amplicon sequencing confirmed the presence of the G101V *BCL2* variant in all tissues of refractory CLL including the bone marrow sample from 2016 and the peripheral blood sample from 2016 and 2017, but neither at baseline (0/568 reads) nor in non-malignant cells (0/403 reads). The variant allele fraction (VAF) of G101V increased in the bone marrow from 9% in 2016 to 16% in 2017 and was highest in the spleen with 25%. The *BCL2* mutation showed the highest increase of all novel or accumulating variants present in both the spleen and bone marrow samples at the time of the refractory disease (Figure 1B).

To demonstrate that the acquisition of *BCL2* G101V is associated with resistance in our unbiased whole exome sequencing (WES) approach, we wanted to evaluate the role of acquired *BCL2* mutations via targeted next generation sequencing (NGS) of the 3 additional patients with acquired resistance to venetoclax. In two cases we confirmed the presence of *BCL2* G101V at refractory CLL disease stage but not before treatment initiation.

Strikingly, we identified an additional acquired variant in *BCL2* of patient 3. This second variant, *BCL2* D103Y, was confirmed at two independent time points with two independent NGS assays each time and presented after 39 months of venetoclax treatment in peripheral blood with a VAF of 7% which increased to 18 % at month 44 (Figure 2). Patient cells from a later timepoint displayed the *BCL2* G101V mutation in addition to D103Y. Of note the *BCL2* G101V variant was not detectable at 39 months and only present at the latest time point with a VAF of 14%. Importantly, both *BCL2* variants are on different reads, suggesting that they occur within two distinct subclones at different time points of onset and with different growth rates, possibly pointing to different degrees of clonal fitness.

Sequencing of 546 venetoclax-naïve CLL patients with the same targeted next generation sequencing (tNGS) assay did not identify the *BCL2* variants G101V nor D103Y in any of the cases. We also sequenced four patients that relapsed after time-limited venetoclax therapy without the identification of any acquired *BCL2* variant.

In contrast to the G101 mutation, the D103 mutation is part of the BH3 binding pocket of BCL2 (Figure 3A, B) and is one of the few amino acids within the BH3 binding domain that differs between BCL2 and BCL-XL.[6] The aspartate in position 103 is an important amino acid for the binding of venetoclax to BCL2 and the affinity of that

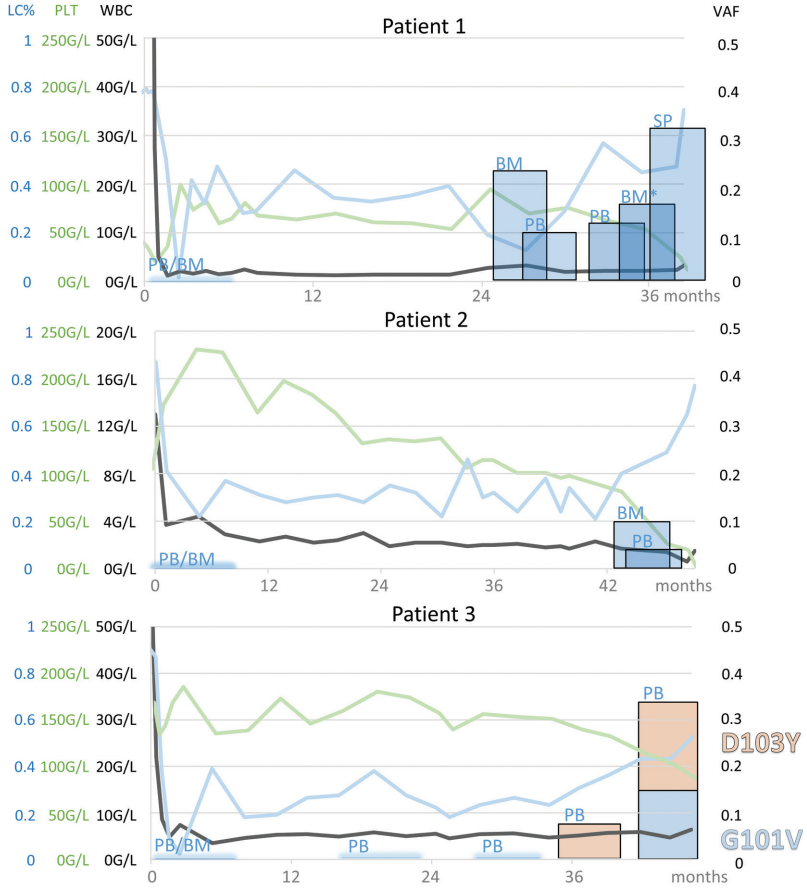

Figure 2. Treatment course and appearance of *BCL2* mutations in three CLL patients. Relative lymphocyte count (LC%, blue), white blood count (WBC, black) and platelets (PLT, green) are shown for 3 different CLL patients from the initiation of the treatment with venetoclax to progression. Variants in *BCL2* are shown for different time points and in different tissues are shown (PB=peripheral blood, BM=bone marrow, SP=spleen). G101V is depicted in blue, D103Y in red. The VAF is estimated from targeted sequencing (except for bone marrow sample 2 of patient 1 [BM*], in which it is estimated from WES).

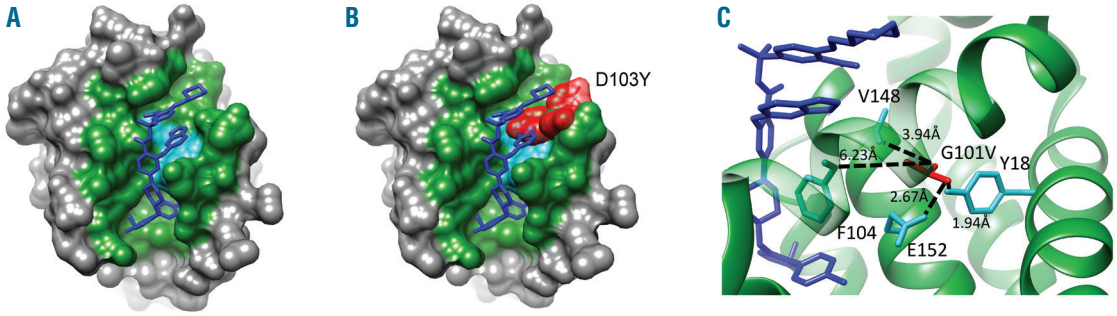

Figure 3. A. Surface model of BCL2 (grey) with areas in close contact to venetoclax in green and residues V148 and F104 highlighted in cyan. B. Surface model of A) with superimposed D103Y point mutation (red) showing three different conformational states. C. BCL2 ribbon structure (green), superimposed G101V point mutation (red) and close neighboring amino acids (cyan) with distances indicated. The crystal structure was obtained and modified from PDB:4MAN.

binding is based on a hydrogen bond to the indole ring of venetoclax. The D103Y replacement of aspartate by tyrosine results in the extension of the bulkier amino acid into the binding pocket as shown by the three different conformational states of the tyrosine sidechain exemplifying the reach of this aberrant tyrosine and its potential to inhibit venetoclax binding (Figure 3B).

Conversely, the amino acid at position 101 is located on the counterside of a critical alpha helix that consists of a significant proportion of the BH3 binding pocket. The mutational change from glycine to valine results in the presence of a bulkier sidechain within the interior of the globular BCL2 protein. Dynamic modeling of G101V leads to a conformational shift due to the overcrowding of neighboring residues E152, V148, F104 and Y18 that are only between 1.94 and 6.23 Å away and consist in part of the BH3 binding pocket (Figure 3C). Residue F104 is a key structural player within the modeled venetoclax docking site and in close proximity to the G101V mutation. These structural observations provide an explanation of how the G101V mutation impairs the binding affinity of venetoclax recently shown *in vitro*.[5]

In our case series these resistance variants were detected in three out of four venetoclax-refractory CLL patients and in accordance to the recent publication of Blombery *et al.*,[5] after more than 2.5 years of successful therapy. For at least two of these patients we could show, that the mutations were detectable up to 1 year before subsequent treatment was required. Interestingly, lymphocytosis was not the major feature of disease progression but rather thrombocytopenia and neutropenia suggesting an affinity of the resistant cells for bone marrow infiltration. Indeed, in patient 1 the allele fractions of the bone marrow and spleen was higher than in the peripheral blood at any time point, while no biopsy was available for the other two patients. In further concordance with Blombery *et al.*,[5] the subclonal character of the *BCL2* mutation variants was comparable and below 50%, which implies the presence of further resistant mechanisms deriving from a diminished clonal fitness due to a shift of the competitive conditions in the BH3 binding pocket. This could be caused by retaining the binding of anti-apoptotic molecules to G101V BCL2 while D103Y might impede the binding of anti-apoptotic proteins to BCL2 leading to a reduced fitness. This theory is supported by recently published functional analyses from primary cells and cell lines with the G101V variant showing lower binding affinity to venetoclax and navitoclax but only marginally compromised BIM and BAX binding.[5,7,8] *In silico* modeling supports these results and illustrates the likely underlying cause: an application of directional forces caused by the bulkier valine residue leads to a predicted minor shift of the α helices of BCL2 with minor but effective conformational changes in several critical amino acids that are predicted to reduce the binding of venetoclax. The specific substitution in all reported resistant CLL cases of glycine 101 for valine and not for other residues that could be caused by a missense variant in the same codon and *e.g.* result in the substitution of G101 to serine, alanine, aspartic acid, cysteine or asparagine is also suggestive of the highly specific molecular mechanism of reduced venetoclax binding. Valine is the only amino acid with protruding bulky methyl groups in close proximity to the BCL2 helix backbone that provides a stable extension of the helical structure and allows helical shifts. Such shifts between BCL2 helices are possibly more tolerated than missense mutation within the BH3 pocket, because the latter likely impede the binding of other BH3 ligands. However, with D103Y we identified a variant which may mainly affect the binding to venetoclax based on the interaction of its indole ring at the p4 spot, which will need to be investigated in more detail.

Even though there are many open questions with regard to the molecular mechanism of the resistance to venetoclax, we see in our small case series that mutations in *BCL2* are frequent in CLL patients becoming refractory to continuous venetoclax treatment. It is likely that time-restricted venetoclax treatment in combination with a second drug (*i.e.* antibody) will lower the risk to generate such resistance variants. Therefore the design of the MURANO trial could be a model to effectively eliminate CLL and prevent resistance to venetoclax caused by G101V and D103Y.[1,3]

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Funding: this work was supported by the Else Kröner-Fresenius-Stiftung (2010_Kolleg24), EC (01KT1601, FIRE CLL), BMBF (031L0076C PRECISe), and Deutsche Forschungsgemeinschaft (SFB 1074 projects B1, B2.

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doi:10.3324/haematol.2019.222588

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Kater AP, Seymour JF, Hillmen P, et al. Fixed Duration of Venetoclax-Rituximab in Relapsed/Refractory Chronic Lymphocytic Leukemia Eradicates Minimal Residual Disease and Prolongs Survival: Post-Treatment Follow-Up of the MURANO Phase III Study. J Clin Oncol. 2019;37(4):4:269-277.
2. Roberts AW, Davids MS, Pagel JM, et al. Targeting BCL2 with Venetoclax in Relapsed Chronic Lymphocytic Leukemia. N Engl J Med. 2016;374(4):311-322.
3. Seymour JF, Kipps TJ, Eichhorst B, et al. Venetoclax–Rituximab in Relapsed or Refractory Chronic Lymphocytic Leukemia. N Engl J Med. 2018;378(12):1107-1120.
4. Stilgenbauer S, Eichhorst B, Schetelig J, et al. Venetoclax for Patients With Chronic Lymphocytic Leukemia With 17p Deletion: Results From the Full Population of a Phase II Pivotal Trial. J Clin Oncol. 2018;36(19):1973-1980.
5. Blombery P, Anderson MA, Gong J, et al. Acquisition of the recurrent Gly101Val mutation in BCL2 confers resistance to venetoclax in patients with progressive chronic lymphocytic leukemia. Cancer Discov. 2018;9(3):342-353.
6. Souers AJ, Leverson JD, Boghaert ER, et al. ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. Nat Med. 2013;19(2):202–208.
7. Fresquet V, Rieger M, Carolis C, García-Barchino MJ, Martinez-Climent JA. Acquired mutations in BCL2 family proteins conferring resistance to the BH3 mimetic ABT-199 in lymphoma. Blood. 2014;123(26):4111-4119.
8. Tahir SK, Smith ML, Hessler P, et al. Potential mechanisms of resistance to venetoclax and strategies to circumvent it. BMC Cancer. 2017;17(1):399.
9. Pettersen EF, Goddard TD, Huang CC, et al. UCSF Chimera: a visualization system for exploratory research and analysis. J Comput Chem. 2004;25(13):1605-1612.
10. Schrödinger Release 2018-4: Maestro, Schrödinger, LLC, New York, NY, 2018.