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Successful Retreatment With Venetoclax in a Patient With Chronic Lymphocytic Leukemia

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The B-cell lymphoma 2 (BCL2) specific inhibitor, venetoclax, has demonstrated remarkable clinical activity in the treatment of chronic lymphocytic leukemia (CLL), alone or in combination with CD20 antibodies, and is now standard of care for frontline and relapsed/refractory disease.[1,2] Venetoclax promotes rapid CLL cell death in vitro in a tumor protein 53 (*TP53*) independent manner and in vivo with comparable overall response irrespective of *TP53* mutational status.[3,4] However, recent data suggest that loss of *TP53* may impair venetoclax efficacy with suboptimal doses.[5] Nevertheless, some patients relapse and optimal treatment strategies after venetoclax progression remain unclear, particularly if they have also relapsed on tyrosine kinase inhibitors. Acquired resistance mechanisms to venetoclax include mutations to *BCL2*[6] in CLL and the upregulation of alternative anti-apoptotic proteins myeloid leukemia 1 (MCL1) and/or B-cell lymphoma-extra large ($BCLX_L$) in models of lymphoma.[7] Venetoclax retreatment has been successful in CLL where patients have taken "treatment holidays" prior to disease progression.[8] However, the outcome of retreatment following acquired resistance is unknown. Recent data have shown that for some CLL patients, mechanisms of resistance to multiple inhibitors may occur independently in mutually exclusive cells,[9] suggesting retreatment with previously efficacious therapies may be effective in these cases. Here, we report the case of a CLL patient successfully retreated with venetoclax following prior venetoclax relapse and intermediate therapy with a combination of 2 B-cell receptor (BCR) inhibitors (BCRi). To our knowledge, this is the first time such a case has been reported.

This study was approved by local Research Ethics Committee and the University Hospitals of Leicester Nation Health Service Trust (06/Q2501/122). Samples were obtained after written informed consent. Mononuclear cells were separated from whole blood or bone marrow using Ficoll histopaque (10771, Sigma-Aldrich). The proportion of CD19-positive B-cells was confirmed by flow cytometry using anti-human CD19-PE (982402, BioLegend, San Jose, CA). Cells were maintained in RPMI-1640 media (21870076, Gibco; Life Technologies) with 10% fetal calf serum (10270-106, Gibco). Treatment with venetoclax, $BCLX_L$ inhibitor A1331852, or MCL1 inhibitor S63845 (Selleck Chemicals, Houston, TX) was carried out 24 hours before analysis using CellTiter-Glo viability assay.[10] Immunoblot was performed as previously described[11] using antibodies: mouse anti-BCL-2 (M088701-2, Dako Agilent, Hamburg), rabbit anti-BCL-XL (2762S, Cell Signaling, Beverly, MA), rabbit anti-MCL-1 (5453S, Cell Signaling), rabbit anti-Bcl-2-like protein 11 (3183S, Cell Signaling), mouse anti-aTubulin (ab15895, Abcam, Cambridge, United Kingdom). Interphase fluorescence in situ hybridization (FISH) on mononuclear cells was performed as previously described.[12] Details of FISH probes used are given in Suppl. Methods Table S1. DNA was extracted from mononuclear cells and bone marrow using DNeasy tissue kit (Qiagen, Crawley, United Kingdom) according to manufacturer's instructions. Extracted DNA was used to prepare libraries for whole exome sequencing (WES) to assess somatic mutations and copy number variation (CNV) and hybridizing to a CytoScan HD Array (Affymetrix, Santa Clara, CA) for further CNV analysis. Details of sequencing, array and bioinformatics pipelines are provided in SDC Methods. WES data are available in the European Genome-Phenome Archive (EGA; https://ega-archive.org); EGA study accession number: EGAS00001006158.

A 57-year-old lady with refractory CLL to Fludarabine, cyclophosphamide and rituximab, rituximab, cyclophosphamide, hydroxydaunorubicin hydrochloride (doxorubicin hydrochloride), vincristine (Oncovin) and prednisone, and high-dose methylprednisolone was referred to our center unwell with massive lymphadenopathy (Figure 1A-i). FISH analysis with the TP53/myeloperoxidase (MPO) probe showed a heterozygous deletion in 17p13 in 72% of interphase nuclei (Suppl. Table S1) and *TP53* mutant disease [NM_000546.6(TP53):c.329G>T(p.Arg110Leu)] shown by WES. She was entered into a clinical trial assessing single-agent venetoclax in March 2014 (NCT01889186).

The patient began treatment with 20 mg venetoclax once daily (OD), escalating to 400 mg OD over 4 weeks. Clinical response was observed with a rapid decrease in white cell count (WBC) from 32.5×10^9/L to 2.4×10^9/L in 4 weeks (Figure 1A, Suppl.

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Supplemental digital content is available for this article.

HemaSphere (2022) 6:8(e752).
http://dx.doi.org/10.1097/HS9.0000000000000752.
Received: April 28, 2022 / Accepted: June 8, 2022

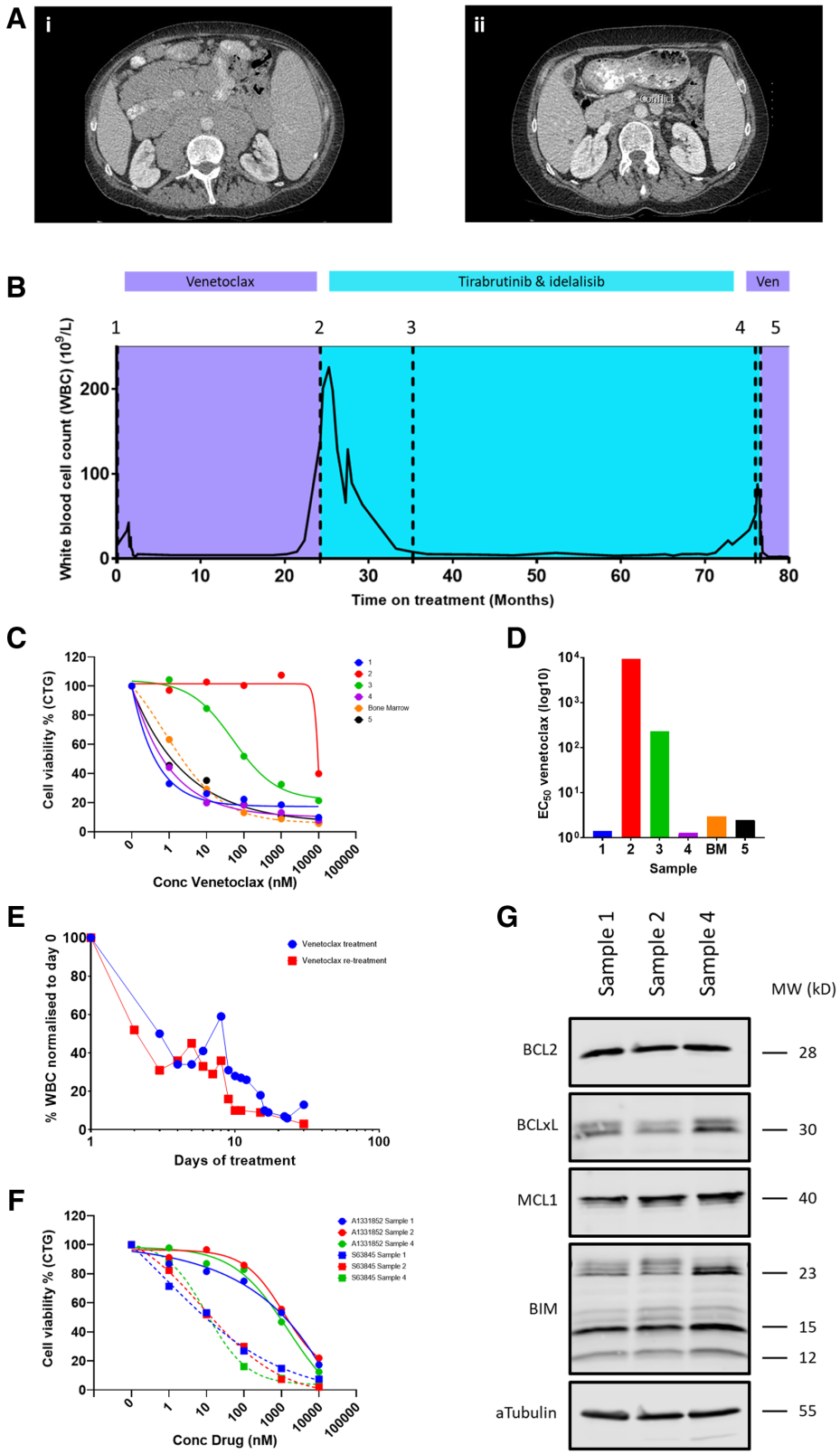

Figure 1. Clinical counts and in vitro analysis. (A), CT images taken before initial venetoclax treatment (i) and 14 wk later (ii) demonstrating clearance of CLL cells observed by reduction in lymphadenopathy. (B), WBC over the course of treatment. Samples used for in vitro studies are indicated with sample numbers (1–5) and times taken are indicated by dashed lines. Venetoclax treatment periods indicated in blue and BCRi indicated in green. (C), CLL cells collected at samples 1–5 (and a bone marrow sample taken at the same time as sample 4) were incubated with increasing concentrations of venetoclax for 24 h before analysis of cell viability by CellTiter-Glo assay (n = 3). (D), EC_{50} values of venetoclax treatment in samples 1–5, calculated using GraphPad Prism. (E), Rate of decrease of WBC over period of venetoclax dose escalation for both initial treatment and retreatment. (F), CLL cells collected at samples 1, 2, and 4 were incubated with increasing concentrations of A1331852 or S63845 for 24 h before analysis of cell viability by CellTiter-Glo assay (n = 2). (G), Immunoblot analysis of lysates prepared from PBMCs of samples 1, 2, and 4. BCL2 = B-cell lymphoma 2; BCLxL = B-cell lymphoma-extra large; BCRi = B-cell receptor inhibitor; BIM = Bcl-2-like 11; BM = bone marrow; CLL = chronic lymphocytic leukemia; CT = computerised tomography; CTG = CellTiter-Glo; EC_{50} = half maximal effective concentration; MCL1 = myeloid leukemia 1; MW = molecular weight; PBMC = peripheral blood mononuclear cells; WBC = white cell count.

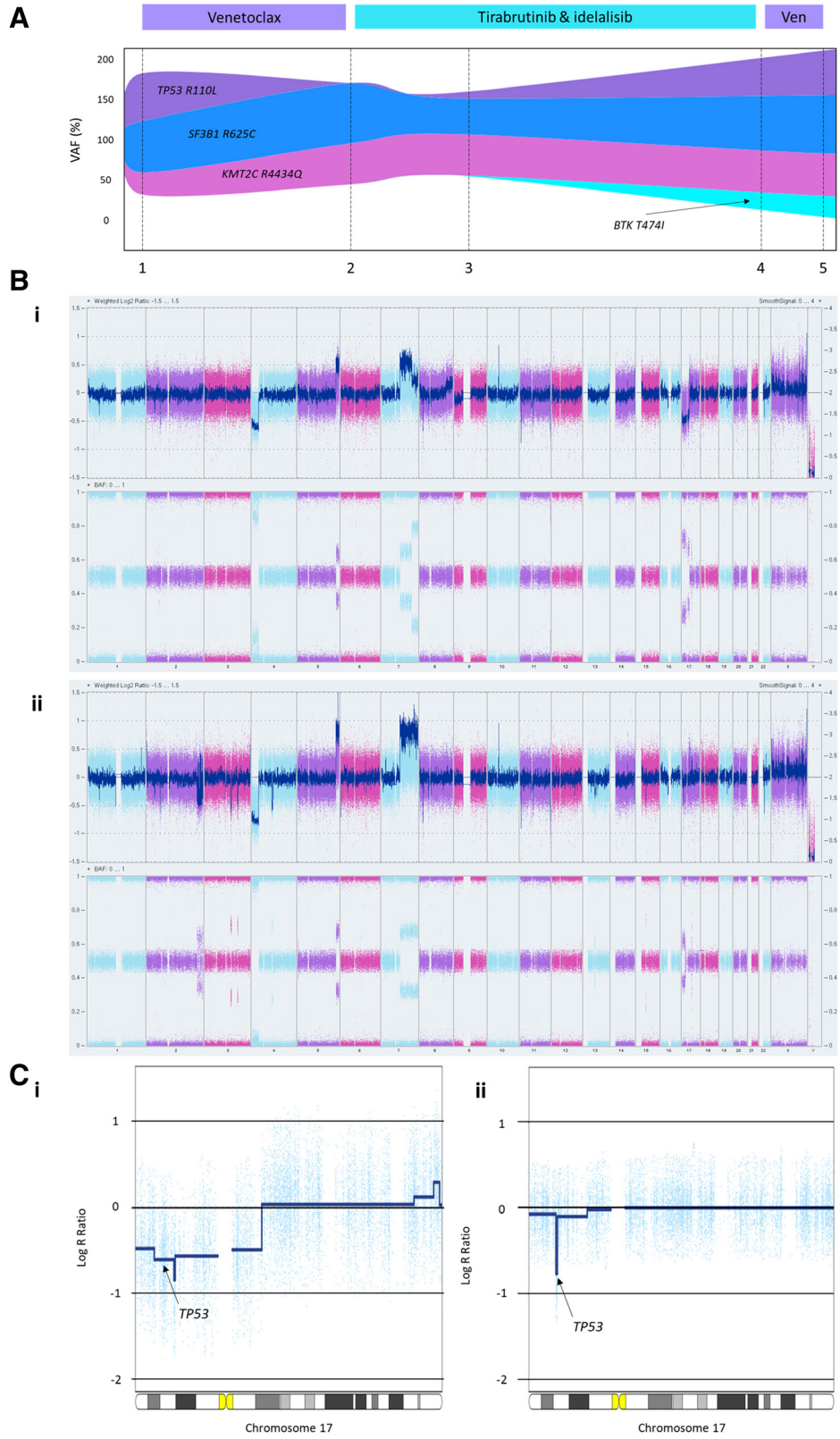

Figure 2. Genomic analysis of PBMC samples. (A), WES was carried out on sequential PBMC samples, with VAFs of driver mutations plotted for each sample. (B), Whole Genome View generated from CytoScan HD Array CNV analysis of sample 1 (i) and sample 2 (ii), with regions of gain indicated by upward deflections and loss by downward deflections in the weighted Log2 ratio. LOH is presented by deviations from the 3 standard lines (0, 0.5, and 1) in the BAF. (C), CNV analysis of chromosome 17 generated from WES data of sample 1 (i) sample 2 (ii) with regions of gain indicated by upward deflections and loss by downward deflections presented as a Log R ratio (a normalized measure of the total signal intensity for 2 alleles). BAF = B-allele frequency; CNV = copy number variation; LOH = loss of heterozygosity; PBMC = peripheral blood mononuclear cells; VAF = variant allele frequency; WES = whole exome sequencing.

Table S2) with rapid resolution of lymphadenopathy (Figure 1A-ii). Venetoclax sensitivity was confirmed in vitro (sample 1, half maximal effective concentration 1.4 nM) (Figure 1C and D).

The patient was treated with venetoclax for 21 months, with low-level (0.01%) disease remaining in the bone marrow, as assessed by multicolor flow cytometry. However, she subsequently progressed very rapidly in August 2015 while still on drug. Disease progression was associated with an increase in lymphocyte count from 17.7×10^9/L to 196.6×10^9/L in just 8 weeks. Clinical resistance was mirrored by an almost 1000-fold increased resistance to venetoclax in vitro (sample 2, EC_{50} 9445 nM).

She next received a combination of tirabrutinib (20 mg twice daily [BD]) and idelalisib (50 mg BD) (NCT02968563). She remained well without significant toxicity for 47 months, attaining stable partial remission. However, she developed pancytopenia due to progressive bone marrow infiltration in November 2019. At relapse to BCRi, there was no recurrence of the bulky lymphadenopathy observed prior to initial venetoclax therapy.

In vitro analysis on sample 3 taken 11 months after starting BCRi treatment, while the patient was still responding, showed a partial regain of sensitivity to venetoclax (EC_{50} 227.9 nM). Furthermore, blood and bone marrow samples taken at BCRi progression showed comparable venetoclax sensitivity as sample 1 (sample 4, EC_{50} 1.3 nM) (Figure 1C and D).

Based on these results, she was retreated with venetoclax in December 2019. Within 4 weeks, her WBC recovered with rapid clearance of CLL cells at a comparable rate to that observed initially (Figure 1E). There was a recovery of normal hematological indices and resolution of splenomegaly. Unfortunately, she died in April 2020 due to coronavirus disease 2019-associated pneumonitis. A timeline of WBC count with duration of treatments is presented (Figure 1B).

To understand the causes of variable venetoclax sensitivities, WES was performed on the same CLL samples as in vitro venetoclax sensitivity analyses.

A productive unmutated *IGHV* rearrangement (1–69), possible driver mutations in *SF3B1*, *KMT2C*, *FANCA*, and *SPEN*, and copy number alterations to chromosomes 4p16.3-p15.1, 5q34-q35.3, 7q21.11-q32.3, and Xq28-q28 were detected at comparable frequencies across all samples (Figure 2A and B, Suppl. Figures S1 and S2, Suppl. Tables S3 and S4).

Differences observed between samples included the variant allele frequency (VAF) of the loss-of-function *TP53 R110L* mutation[13] and the size of the chromosome 17p deletion. Prior to initial venetoclax treatment (sample 1), *TP53 R110L* had a VAF of 42.7%. CNV analysis demonstrated a heterozygous chromosome 17p13 deletion extending across the centromere (Figure 2C-i). At venetoclax relapse, *TP53 R110L* was not detected by WES (sample 2). CNV analysis at this time showed a *TP53* focal copy number loss, and not the previously observed arm loss, extending from base pairs (bp) 7,168,109-7,593,927 (425,818bp, hg19), which was detected in all subsequent samples (Figure 2C-ii). This likely represents the emergence of a new subclone selected for during venetoclax exposure. This was not detected by FISH as the commercial probes used (TP53/MPO and locus specific identifier TP53) extended beyond the focal loss. Venetoclax resistance was not associated with detectable mutations in *BCL2* or genes of *BCL2* interacting proteins. Similar findings have been observed in the MURANO (venetoclax and rituxumab in relapsed/refractory CLL, NCT02005471) trial, where they observed a lack of acquired *BCL2* mutations.[14] In addition, there were no changes in protein expression of BCL2 or other anti-apoptotic proteins (Figure 1G), nor altered sensitivity to $BCLX_L$ or MCL1 specific inhibitors (Figure 1F).

After 11 months of BCRi therapy during clinical response (sample 3), *TP53 R110L* became detectable again at low level (VAF of 4.2%). This coincided with the increased in vitro venetoclax sensitivity observed. These changes in VAF occurred despite observing no altered VAFs in other driver mutations, allowing a distinction between the subclonal *TP53 R110L* mutation and other likely clonal driver mutations (Figure 2A, Suppl. Table S3). Unexpectedly the reemergence of *TP53 R110L* was not accompanied by the reemergence of the 17p13 deletion. Instead, the *TP53* focal copy number loss remained, suggesting greater subclonal complexity than just 2 competing subclonal populations.

At this point, no BTK inhibitor (BTKi)-associated *BTK* or *PLCG2* mutations were detected. After another 36 months of therapy at BCRi disease progression (sample 4), *BTK T474I* (associated with BTKi resistance[15]) was detectable at a VAF of 16.9%. The VAF of *TP53 R110L* increased to 35.0% and remained stable until time of death.

In summary, we have demonstrated that clonal evolution and genetic complexity in a patient with CLL allowed for sensitivity to venetoclax to be regained after initial venetoclax progression and subsequent prolonged BCRi therapy. The venetoclax-sensitive subclone could be characterized by WES, and its reemergence tracked throughout BCRi treatment. Retreatment with venetoclax was informed by in vitro drug testing,[16] which may therefore have clinical utility to monitor and predict in vivo responses. We note that these observations were made during intermediate combination therapy of 2 BCRi, and it is unclear if similar clonal selection would occur with single-agent BCRi.

This study is limited to observations in just 1 case of venetoclax progression. Retrospective in vitro analysis of CLL cases treated with sequential targeted therapies may help to provide further evidence of treatment resensitization in a larger patient cohort. In addition, there was only a short follow-up of 4 months after commencing venetoclax retreatment, so it remains unclear how durable and comparable to the initial venetoclax treatment the response would have been.

These data suggest that consecutive exposure to different classes of targeted agents may allow the reemergence of drug-sensitive subclones, providing rationale for retreatment with previously efficacious therapies, or the use of combination therapies early to target multiple subclones with distinct sensitivities.

ACKNOWLEDGMENTS

We would like to thank the research nurses at the Hope Clinical Trial Unit, Leicester Royal Infirmary, as well as the staff of the tumor genetics laboratories of the Universities Kiel and Ulm. This research used the ALICE High Performance Computing Facility at the University of Leicester.

AUTHOR CONTRIBUTIONS

RAJ and VMS performed research. All authors involved in data analysis and interpretation. RAJ and VMS drafted the article that was revised and approved by all authors (article writing). MJSD and HSW designed and supervised the study.

DISCLOSURES

MJSD has received research funding from Gilead Sciences, and honorarium and travel grants from AbbVie. HSW has received research funding from Gilead Sciences, and honorarium and travel grants from AbbVie. All the other authors have no conflicts of interest to disclose.

SOURCES OF FUNDING

This work was supported by funds from the Scott Waudby Trust, the Hope Against Cancer charity, the University of Leicester, the Kay Kendall Leukemia Fund, Gilead Sciences, Inc, and the Deutsche Forschungsgemeinschaft through SFB1074 (project B10).

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