Annals of Oncology 26: 1180–1187, 2015
doi:10.1093/annonc/mdv110
Published online 23 February 2015

A personalised medicine approach for ponatinib-resistant chronic myeloid leukaemia

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Received 9 December 2014; revised 11 February 2015; accepted 17 February 2015

Background: Chronic myeloid leukaemia (CML) is characterised by the presence of a fusion driver oncogene, *BCR-ABL1*, which is a constitutive tyrosine kinase. Tyrosine kinase inhibitors (TKIs) are the central treatment strategy for CML patients and have significantly improved survival rates, but the T315I mutation in the kinase domain of BCR-ABL1 confers resistance to all clinically approved TKIs, except ponatinib. However, compound mutations can mediate resistance even to ponatinib and remain a clinical challenge in CML therapy. Here, we investigated a ponatinib-resistant CML patient through whole-genome sequencing (WGS) to identify the cause of resistance and to find alternative therapeutic targets.

Patients and methods: We carried out WGS on a ponatinib-resistant CML patient and demonstrated an effective combination therapy against the primary CML cells derived from this patient *in vitro*.

Results: Our findings demonstrate the emergence of compound mutations in the BCR-ABL1 kinase domain following ponatinib treatment, and chromosomal structural variation data predicted amplification of *BCL2*. The primary $CD34^+$ CML cells from this patient showed increased sensitivity to the combination of ponatinib and ABT-263, a BCL2 inhibitor with a negligible effect against the normal $CD34^+$ cells.

Conclusion: Our results show the potential of personalised medicine approaches in TKI-resistant CML patients and provide a strategy that could improve clinical outcomes for these patients.

Key words: CML, BCR-ABL1 mutations, whole-genome sequencing, personalised medicine, mechanisms of resistance

introduction

Chronic myeloid leukaemia (CML) is driven by a constitutive tyrosine kinase, *BCR-ABL1*, which results from a reciprocal translocation t(9;22)(q34;q11) in haemopoietic stem cells [1]. Tyrosine kinase inhibitors (TKIs) are the main treatment of CML and have improved disease management and survival [2]. However, resistance is a persistent clinical problem and a significant proportion of patients will progress on first-line therapy due to the emergence of TKI-resistant clones. In some patients, TKI resistance is mediated by BCR-ABL1-independent mechanisms, including increased BCL2 expression and overexpression of LYN kinase, but 20%–30% of patients progress due to acquired point mutations in the *BCR-ABL1* kinase domain that disrupt TKI binding. In particular, BCR-ABL1 p.T315I (the 'gatekeeper' mutation) confers resistance to all approved first- and second-line TKIs, including imatinib, nilotinib, dasatinib, and bosutinib [3–5].

Notably, BCR-ABL1 p.T315I remains sensitive to the TKI ponatinib, a third-line inhibitor [6]. Although an initial response to ponatinib is promising in patients with single mutations in *BCR-ABL1*, response in advanced patients is limited because successive or long-term use of TKIs lead to the evolution of compound *BCR-ABL1* kinase domain mutations that show resistance even to ponatinib [7, 8]. No fourth-line treatments have yet been approved for ponatinib-resistant CML.

We used whole-genome sequencing (WGS) to characterise a ponatinib-resistant CML patient. This revealed a constellation of *BCR-ABL1* mutations that were not detected in diagnostic Sanger sequencing approaches. The WGS approach also revealed amplification of the anti-apoptotic gene *BCL2* and we show that this patient's CML cells were susceptible to a ponatinib/ABT-263 combination, revealing a therapeutic strategy that may have been effective in this patient.

patients and methods

Detailed experimental procedures are in supplementary Methods, available at *Annals of Oncology* online. Briefly, tissues were collected following informed consent and ethical approval from the scientific subcommittee of the Manchester Cancer Research Centre Tissue Biobank and in compliance with the ethical and legal framework of the *Human Tissue Act*, 2004.

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Normal mobilised CD34$^+$ cells surplus to requirements were from patients undergoing chemotherapy and autologous transplantation for myeloma. Their use was authorised by the Salford and Trafford Research Ethics Committee, for samples collected since 2006, and following written informed consent of donors. Isolated CML CD34$^+$ cells were expanded *ex vivo* for genomic DNA extraction. Germline DNA was extracted from the buccal epithelial cells from the patient's fresh saliva. Total RNA from CML CD34$^+$ cells was used for RNA-seq and shot-gun cloning. WGS and RNA-seq were carried out on an Illumina HiSeq2500 in paired-end 100-bp runs, with 65× depth for CML DNA and 35× depth for germline DNA. Raw data are accessible via EGAS00001001150. Detailed computational methods are in supplementary Methods, available at *Annals of Oncology* online. Validation and identification of single and compound mutations in the *BCR-ABL1* kinase domain was by shot-gun cloning and Sanger sequencing [9]. Annexin V apoptosis assays (BD Biosciences) and cell proliferation assays (CellTiter 96 Aqueous One Solution; Promega) were as per manufacturers' instructions. Colony forming cell (CFC) assays were used to examine CML CD34$^+$ cell differentiation following small molecule inhibitor treatments.

results

We report on a patient in their 70s who presented with chronic phase CML. The patient was initially treated with imatinib (400 mg/day), which resulted in a complete haematological response (CHR), but failed to achieve a complete cytogenetic response (CCR) at 6 months (Figure 1 A). Dose escalation of imatinib (600 mg/day), followed by the second-generation TKI dasatinib (100 mg/day), each for 6 months also failed to produce a CCR (Figure 1A) and due to nilotinib and interferon intolerance, the patient was again placed on standard-dose imatinib. *BCR-ABL1* mutation analysis at this time demonstrated a C>T, p.T315I mutation, but the patient was not considered suitable for allogeneic bone marrow transplantation due to co-morbidities. In the absence of haematological response, imatinib was discontinued and the patient was treated with hydroxycarbamide. Three years following diagnosis, the patient was treated with ponatinib (45 mg/day) and despite achieving a temporary CHR, a CCR was never

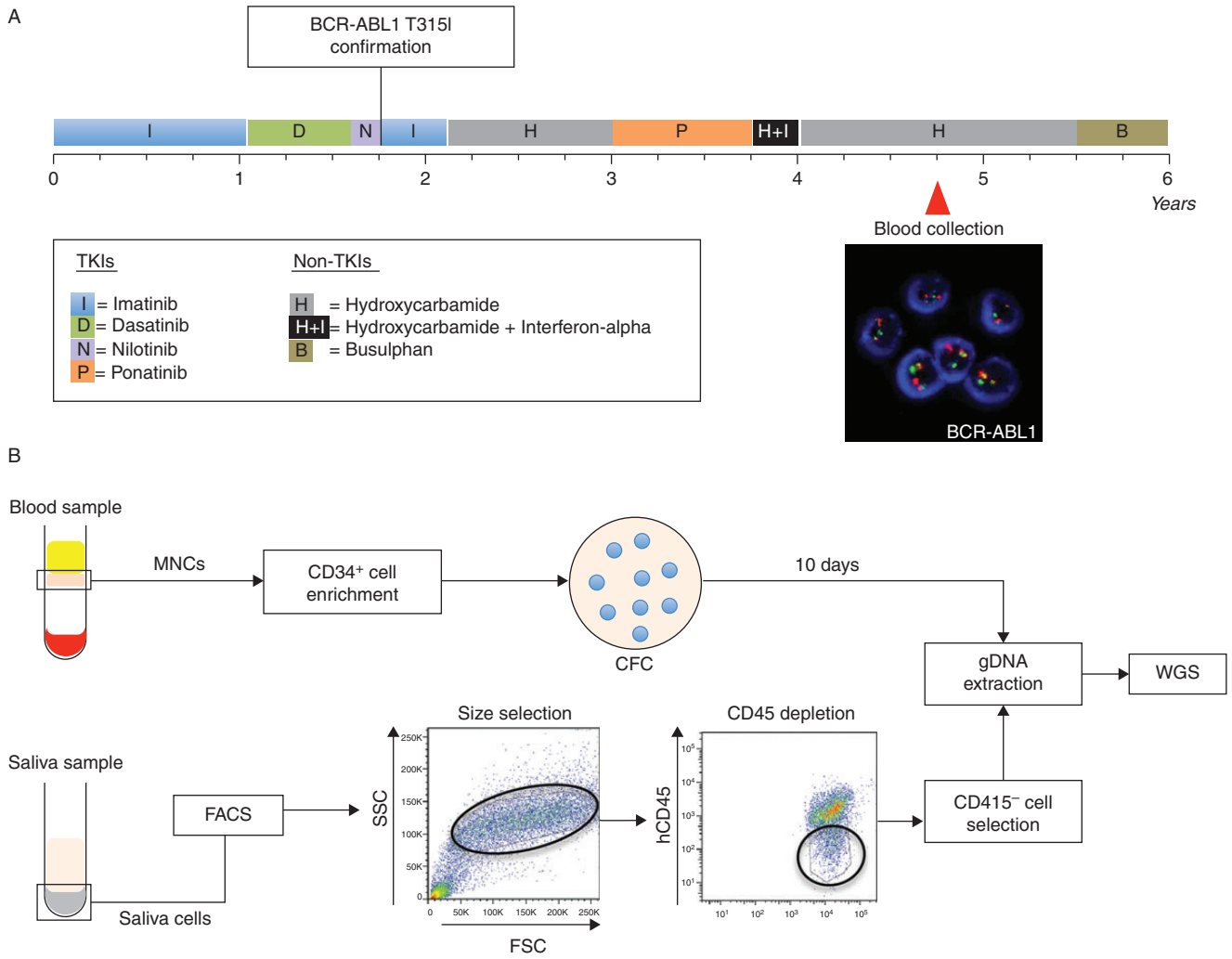

Figure 1. Timeline of CML patient's treatments and the study design. (A) TKI and non-TKI treatments shown as coloured boxes over the horizontal timeline axis. The time point of blood sample collection for the WGS study is indicated (red triangle). Inset shows the *BCR-ABL1* FISH results for this blood sample. (B) Schematic diagram showing the extraction of tumour genomic DNA (gDNA) from CD34$^+$-enriched peripheral blood mononucleated cells (MNCs) that are expanded in a colony forming cell (CFC) assay, and the extraction of germline gDNA from buccal epithelial cells using FACS, for WGS. CML, chronic myeloid leukaemia; TKIs, tyrosine kinase inhibitors.

achieved, and within 9 months the CHR was lost, as revealed by a rising white blood cell count. The patient was retreated with hydroxycarbamide, but developed leg ulceration necessitating discontinuation (Figure 1A). The patient remains in chronic phase with disease control being achieved with intermittent etoposide (Bristol-Myers Squibb Pharmaceuticals Limited, Uxbridge).

To characterise further the patient's disease, we carried out WGS on $CD34^+$ cells isolated from blood 57 months after diagnosis (Figure 1A and B). The $CD34^+$ cells were 99.5% *BCR-ABL1*$^+$ by FISH, and germline DNA was isolated from buccal epithelial cells (Figure 1A and B). We observed 12 466 predicted somatic single-nucleotide variants (SNVs) in the patient's CML cells, resulting in 23 predicted non-synonymous mutations (Table 1), a modest mutational burden that is similar to that seen in acute myeloid leukaemia (AML), but considerably lower than that in cutaneous melanoma, colorectal adenocarcinoma, and lung cancers, which are associated with known carcinogens (Figure 2A). Approximately 40% of the somatic mutations were C>T (G>A) transitions and approximately 20% T>C (A>G) transitions (Figure 2B), also a common mutation signature in AML [10]. Trinucleotide analysis of the mutant base and its flanking nucleotides (Figure 2C) revealed a match to the recently described mutational signature 1B, which is common in several cancers including AML, acute lymphoblastic leukaemia (ALL), chronic lymphocytic leukaemia (CLL), and B-cell lymphoma [11].

Among the 23 predicted somatic coding region SNVs, 10 occur at a frequency of <0.05% in the dbSNP database (Table 1 and Figure 3A). Notably, RNA-seq analysis revealed that only 12 of the 23 predicted SNVs were expressed and of the 4 previously reported in cancers (COSMIC, http://cancer.sanger.ac.uk/cosmic), only *ABL1* (C>T, p.T315I) and *ABL1* (T>G, p.F359V) were expressed (Table 1). The *ABL1* mutations are also the only missense SNVs that have been reported in CML (COSMIC) and have a damaging effect on protein function as predicted by SIFT and PolyPhen scores (Table 1). We used a 10% threshold to call the SNVs (supplementary Methods, available at *Annals of Oncology* online), so to detect lower-frequency SNVs in the *ABL1* kinase domain we set the detection limit to 0.1%, but did not detect any additional mutations. However, when we manually scanned this region in the Integrative Genomic Viewer (IGV v2.3.3.38), we observed an additional 53 low-frequency SNVs, but only 2 of these were validated by Sanger sequencing (supplementary Table S1, available at *Annals of Oncology* online). To investigate this region further, we carried out PCR-mediated shot-gun cloning and Sanger sequencing of the *BCR-ABL1* kinase domain [12], comparing a pre-ponatinib sample from 21 months to the post-ponatinib sample obtained at 57 months. The p.T315I mutation was present in 100% of the clones from the pre-ponatinib sample and ~21% of these carried additional point mutations (Figure 3B), including p.E225G/T315I and p.T315I/F359C compound mutations (supplementary Table S2, available at *Annals of Oncology* online), which have been shown to mediate ponatinib resistance [8]. Intriguingly, following ponatinib treatment, we observed a reduction of p.T315I to ~17% of the clones, with emergence of a new dominant clone (~53%) carrying a p.F359V mutation (Figure 3B). Moreover, following ponatinib, the number of compound mutations increased to ~45% and included p.T315I/M351V which also confers ponatinib resistance [8] (supplementary Table S2, available at *Annals of Oncology* online).

We predicted 58 loss of heterozygosity (LOH) SNVs in the coding regions, 20 of which have been reported (COSMIC; Figure 3A and supplementary Table S3, available at *Annals of Oncology* online). We predicted 183 somatic small insertions or deletions (indels), but none in the coding regions. We also predicted 12 chromosomal translocations with the potential to generate fusion genes, 2 of which were the balanced t(9;22)(q34;q11) reciprocal translocations that create *BCR-ABL1* (Figure 3A and supplementary Table S4, available at *Annals of Oncology* online). However, our RNA-seq revealed that only *BCR-ABL1* and *ABL1-BCR* were expressed, strongly suggesting that *BCR-ABL1* was the driver oncogene (supplementary Table S4, available at *Annals of Oncology* online) and that the other potential fusions were not pathogenic.

We predicted 53 large chromosomal deletions and 7 large chromosomal amplifications (Figure 3A). Two of the deletions and three of the amplifications affected coding regions (supplementary Table S5, available at *Annals of Oncology* online). A large deletion of 5q23 is similar to that previously described in a blast crisis patient (supplementary Table S5, available at *Annals of Oncology* online), but we noted an ~8 Mb amplification of chromosome 18q that harbours the anti-apoptotic gene *BCL2*. *BCL2* upregulation is associated with sensitivity to BCL2 inhibitors, so we modelled this using two *BCR-ABL1*$^+$ cell lines, BV173R and K562 cells. BV173R cells expressed approximately seven-fold more *BCL2* than K562 cells (Figure 4A) and were significantly more sensitive to a BH3-mimetic BCL2 inhibitor, ABT-263 [13] (Figure 4B). We confirmed that *BCL2* expression was 14-fold higher in the patient's $CD34^+$ CML cells than normal mobilised $CD34^+$ cells (Figure 4A), and that the patient's $CD34^+$ cells were more sensitive to ABT-263 (Figure 4B). Moreover, ponatinib and ABT-263 cooperated to increase apoptosis in the patient's $CD34^+$ cells (Figure 4C). These results were reflected in viability assays. Ponatinib and ABT-263 did not significantly suppress the viability of normal $CD34^+$ cells (Figure 4D), but both inhibited growth of the patient's $CD34^+$ cells and they cooperated to suppress the growth of these cells (Figure 4E). We also examined ABT-199, a second BCL2 inhibitor [14]. ABT-199 was less potent than ABT-263 at inducing apoptosis in the patient's $CD34^+$ cells (supplementary Figure S1A, available at *Annals of Oncology* online), but it nevertheless cooperated with ponatinib to enhance apoptosis (supplementary Figure S1B, available at *Annals of Oncology* online). Next, we examined the differentiation capacity of the patient's $CD34^+$ cells that survived the drug treatments. In CFC assays, only ~5% of the control colonies were erythroid and ~95% were granulocytic, but the granulocytic fraction decreased to ~85% in the presence of ponatinib, to ~60% in the presence of ABT-263, and to only 24% in the presence of both drugs, a trend that was also observed with ABT-199 (Figure 4F and G; supplementary Figures S1C and S2, available at *Annals of Oncology* online). Thus, in addition to inducing apoptosis and reducing viability, the ponatinib/ABT-263 combination selectively reduced production of granulocytic lineage cells, either by inducing lineage-specific apoptosis or differentiation of surviving cells down the erythroid lineage.

discussion

We report WGS analysis of a CML patient and observe a mutation signature predominated by C>T transitions and a

Table 1. List of the genes with missense somatic SNVs in CML cells.

Gene	Genomic variant[a]	Protein variant	SIFT	PolyPhen	COSMIC	dbSNP (%MAF)	CML DNA (%MAF)	Normal DNA	CML RNA #1 (%MAF)	CML RNA #2 (%MAF)
ABL1	chr9:133748283C > T	T315I	0	1	COSM12560, COSM12603	rs121913459 (N/A)	11/65 (16.92%)	0/37	11/145 (7.59%)	21/142 (14.79%)
ABL1	chr9:133748414T > G	F359V	0	0.999	COSM12605, COSM133668	rs121913452 (N/A)	21/70 (30%)	1/28	29/150 (19.33%)	29/173 (16.67%)
C1orf168	chr1:57233559A > G	Y336H	0.02	0.931			24/63 (38.09%)	2/41	0	0
CANT1	chr17:76993184T > C	N174S	0.08	0.326			40/71 (40.84%)	0/36	91/184 (49.46%)	86/190 (45.26%)
CNTNAP3	chr9:39132947G > A	R688W	0	0.934			29/54 (53.7%)	0/23	0	0
DPY19L2	chr12:63964599T > C	I647V	1	0.006	COSM1128502		7/53 (13.2%)	1/33	0	0
GTSE1	chr22:46724662C > T	P601L	0	0.139			45/80 (56.25%)	0/35	131/248 (52.82%)	117/226 (51.77%)
IGFN1	chr1:201178965A > G	I1648M	0.05	0.007		rs12758143 (N/A)	9/47 (19.14%)	0/25	0	0
ITIH1	chr3:52821993T > C	L5P	0.11	0		rs71299618 (N/A)	6/34 (17.64%)	0/15	0	0
KIAA1522	chr1:33207790G > A	G79S	0.07	0			36/72 (50%)	0/14	0	0
LARS	chr5:145499963C > T	R1072H	0.04	0.978		rs145644461 (0.05%)	15/79 (18.98%)	0/44	77/551 (13.97%)	70/614 (11.38%)
MARCH9	chr12:58152559C > T	T307M	0	0.026		rs370203524 (0.01%)	45/85 (52.94%)	0/32	19/47 (40.43%)	17/28 (60.71%)
MT-ND1	chrM:3448A > C	Q47H	0	0.991			1326/9888 (13.41%)	747/5180	0	0
NBPF1	chr1:16891365G > A	T1038M	0.1	0.01		rs201912185 (N/A)	73/607 (12.02%)	17/280	6/43 (13.64%)	8/31 (25.81%)
NBPF10	chr1:145323656A > T	I1165F	0.19	0.493	COSM895149	rs75252120 (N/A)	58/420 (13.8%)	21/207	0	0
NCKAP5	chr2:133542514C > T	G624R	0.43	0.066			34/65 (52.3%)	1/29	0	0
SCG2	chr2:224462853C > T	R383Q	0.9	0.001		rs139176325 (0.04%)	12/65 (18.46%)	2/41	0	0
SMPD2	chr6:109763241A > G	I97V	0.18	0			48/75 (50.66%)	0/30	42/75 (56%)	47/88 (53.41%)
SNRPA1	chr15:101825161C > T	M135I	0.22	0			31/80 (38.75%)	0/31	0	4/13 (30.77%)
UACA	chr15:70980121C > A	D142Y	0	1			37/87 (42.52%)	0/36	4/12 (33.33%)	4/11 (36.36%)
XIRP1	chr3:39230480C > T	V153I	0	0.996		rs201031340 (0.05%)	28/64 (43.75%)	0/29	0	0
ZBTB46	chr20:62407126C > T	S376N	0.41	0.597			49/90 (54.44%)	0/19	5/12 (41.67%)	0
ZZEF1	chr17:3999258T > C	I594V	0.17	0.995			32/64 (50%)	1/36	38/97 (39.18%)	41/88 (46.07%)

Colour coding: red cells, missense mutations with damaging effect on protein function; green cells, tolerated missense variants.

[a] hg19 genomic coordinates.

% MAF, percentage of mutant allele frequency.

doi:10.1093/annonc/mdv110 | 1183

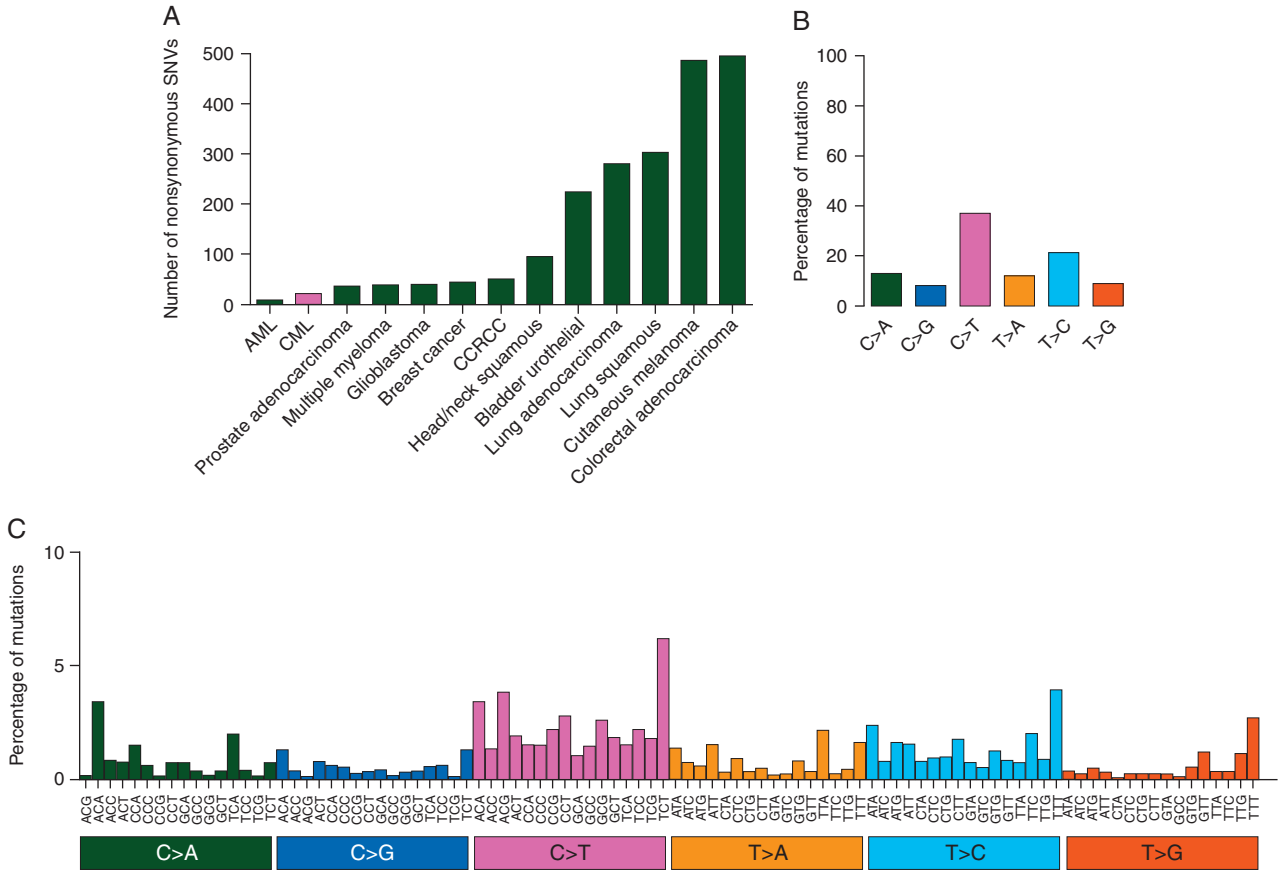

Figure 2. Mutational signature and mutational burden of CML. (A) Average number of non-synonymous SNVs found in different cancers (The Cancer Genome Atlas, www.cbioportal.org) compared with the CML patient. CCRCC, clear cell renal cell carcinoma; SNVs, single-nucleotide variants. (B) Mutational signature of the predicted 12 466 SNVs in both strands. (C) Trinucleotide mutational signature based on the 96 substitution classification defined by the type of substitution and the immediate 3′ and 5′ bases adjacent to the mutated base. Vertical axes in (B) and (C) show the percentage of each mutation class.

trinucleotide signature 1B. These mutations are thought to result from spontaneous deamination of 5-methyl-cytosine in germline and normal somatic cells [11]. Although the aetiology of these cells is unclear, this signature is age-related and particularly common in haematological malignancies including ALL, AML, CLL, and B-cell lymphoma [11], suggesting a common somatic mutation mechanism in these diseases.

We observed a modest mutational burden, characteristic of cancers that are not associated with known carcinogens such as ultraviolet light or cigarette smoke. Moreover, the WGS revealed an ABL1 p.F359V mutation that was not detected by diagnostic Sanger sequencing. We confirmed the presence of this mutation by shot-gun cloning and Sanger sequencing, and it is unclear why the diagnostic Sanger sequencing did not reveal this mutation, but one possibility is that the wild-type codon is favoured for base incorporation and thereby masks the mutant allele. The shot-gun sequencing also revealed several compound mutations that were not identified by WGS, because read-length limitations only reveals these when they are within ~100 bases of each other. Finally, the shot-gun sequencing identified many low-frequency *BCR-ABL1* kinase domain mutations that were not revealed by WGS, possibly because of low read-depth in our WGS or because they are PCR-generated artefacts of the shot-gun cloning. These observations highlight why a combination of sequencing approaches is desirable when performing these studies, but these considerations aside, our data suggest that there was dramatic treatment-driven clonal evolution. This presumably accounts for the failure to achieve a CCR despite the initial haematological response and is in agreement with recent data, showing that p.T315I TKI-resistant CML patients fail to respond to ponatinib due to emergence of compound *BCR-ABL1* kinase domain mutations [8]. It has also been reported that sequential TKI treatments in relapsed CML patients can drive clonal selection of *BCR-ABL1* mutants that were present at a low frequency before treatment commenced [12, 15].

The reciprocal t(9;22)(q34;q11) translocation is the hallmark of CML [1]. It was predicted by our WGS and RNA-seq results, and validated by our shot-gun cloning approach. The BCR-ABL1 fusion gene in this patient is the e14a2 isoform that occurs between exons 14 and 15 of *BCR*, generating the BCR-ABL1 p210 fusion protein. The importance of e13a2 and e14a2 isoforms to risk stratification at the time of diagnosis is debated, but it has been suggested that the e14a2-type CML cells can activate HLA-mediated T-cell response that can be exploited in IFN-α therapy [16].

It is unclear what contribution the large chromosomal structural variations (SVs) made towards the disease. A putative amplification of ~42 Mb on chromosome 6 harbours 370 genes,

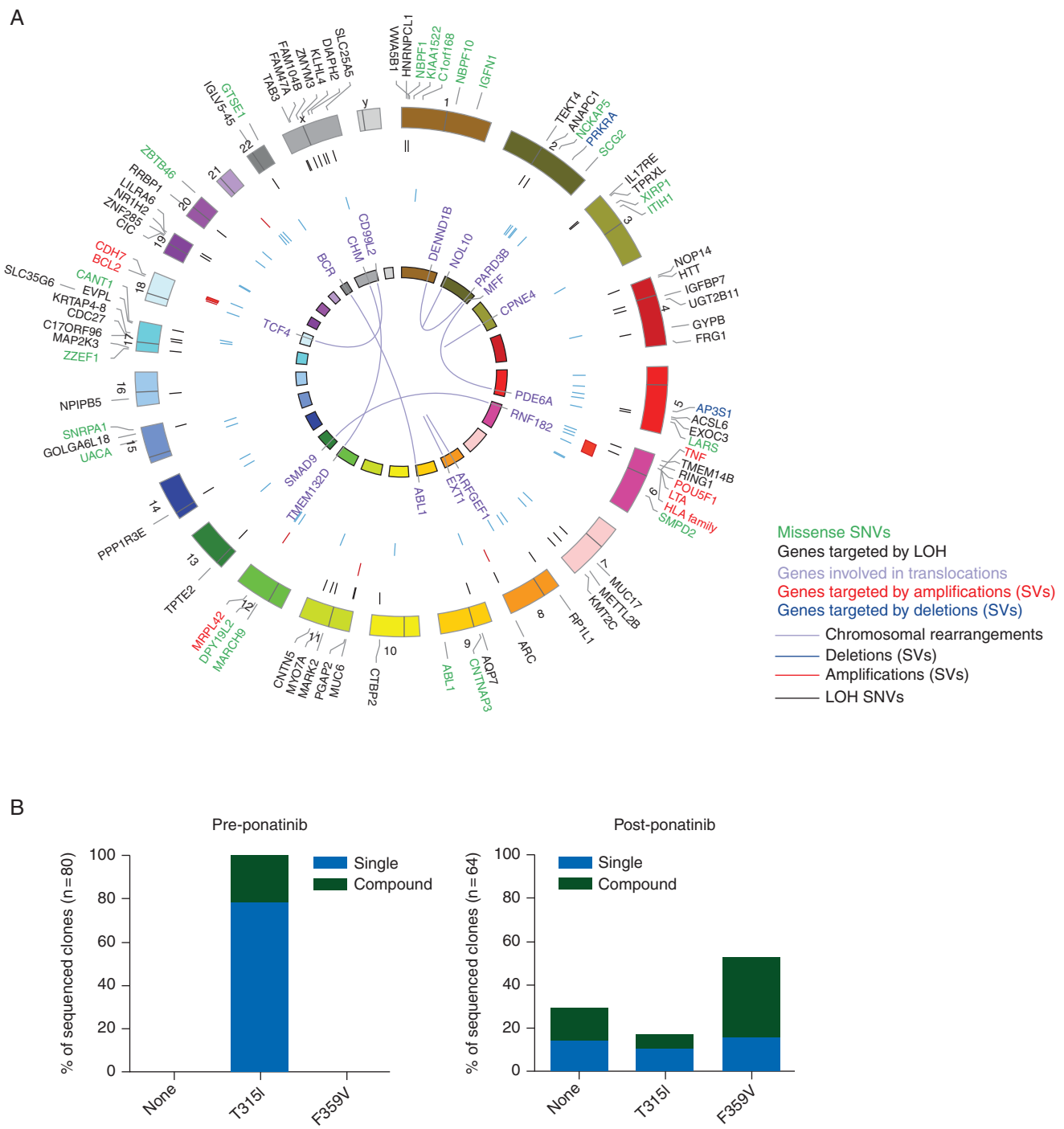

Figure 3. Genomic characterisation of patient's CML. (A) Circos plot illustrates the predicted somatic variants including gene-specific SNVs (green gene names), genes affected by LOH (black gene names), genes affected by translocations (purple gene names), and the key genes targeted by SV amplifications (red gene names) and SV deletions (blue gene names) predicted in CML cells compared with germline. Inter- and intrachromosomal rearrangements are shown by purple lines. The genomic locations of LOH SNVs (black lines), amplification (red line), and deletion (blue line) SVs are indicated. Grey lines mark the position of centromeres in each chromosome. (B) Shot-gun cloning and sequencing of the *BCR-ABL1* kinase domain in the samples obtained before and after ponatinib treatment. Each bar represents the percentage of single or compound mutations of the total number of sequenced clones. SNVs, single-nucleotide variants; LOH, loss of heterozygosity; SV, structural variation; CML, Chronic myeloid leukaemia.

including 16 HLA family genes that modulate T-cell response towards leukaemic cells [16]. This region also includes *TNF*, which modulates CML CD34$^+$ cell survival, and whose co-inhibition with BCR-ABL1 induces CML cell death [17]. Importantly, we observed a putative ~8 Mb amplification on chromosome 18 that harbours *BCL2*, a pro-survival driver oncogene in B-cell lymphomas [18]. *BCL2* is important in CML pathogenesis, progression to blast crisis [19, 20], and its

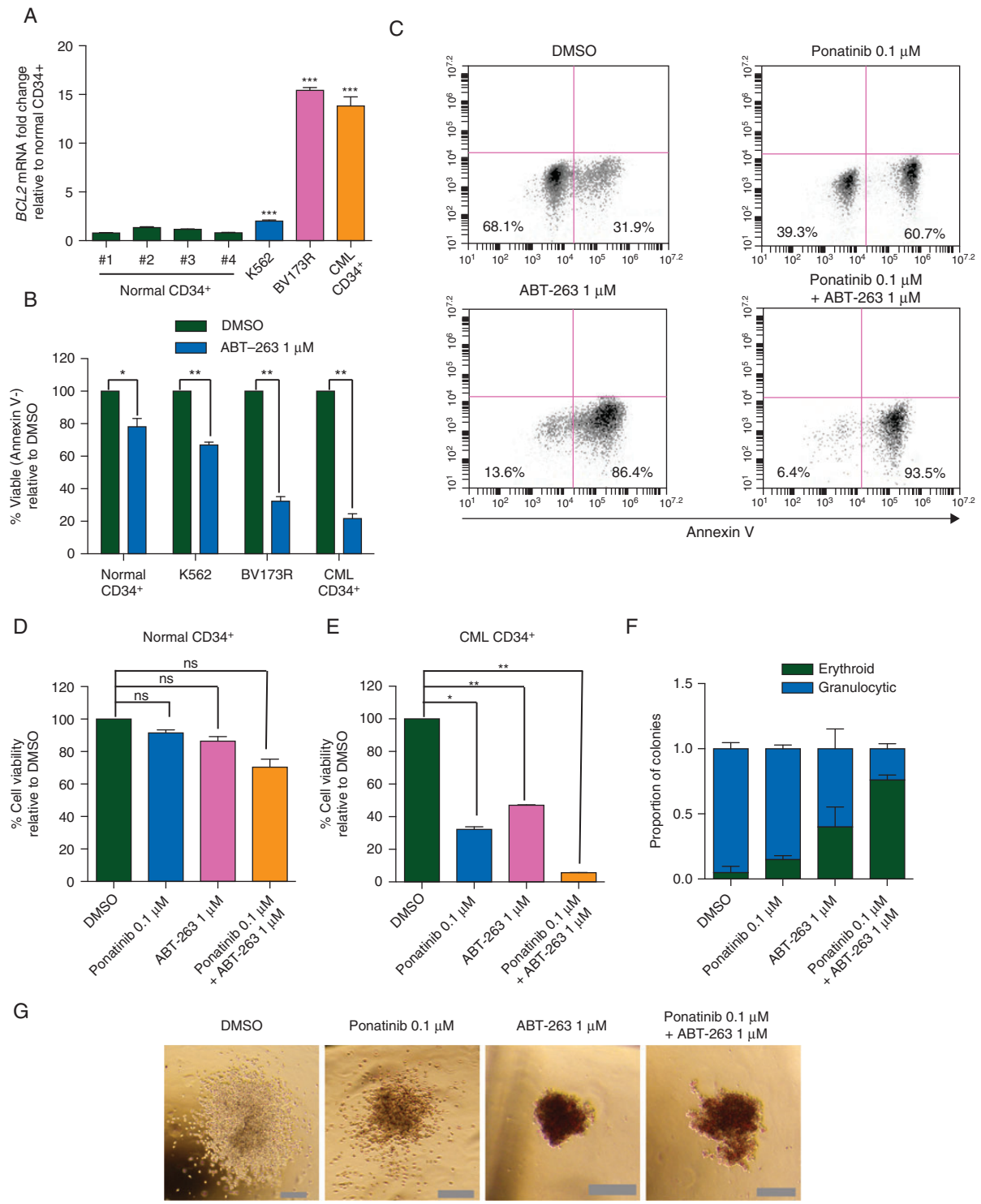

Figure 4. Ponatinib and ABT-263 co-operate to induce death or differentiation of CD34$^+$ chronic myeloid leukaemia (CML) cells. (A) *BCL2* mRNA levels in the patient's CD34$^+$ CML cells, K562 and BV173R cells, and normal donor CD34$^+$ cells. *P*-values were calculated relative to the average normal CD34$^+$ *BCL2* mRNA levels. ***$P < 0.0001$. (B) Normalised percentage of viable cells (Annexin V$^-$) following 72 h treatment with DMSO (control) or ABT-263. *$P < 0.05$; **$P < 0.01$. (C) FACS dot plots showing changes in the percentage of apoptotic cells (Annexin V$^+$; horizontal axes) in CD34$^+$ CML cells treated with DMSO, ponatinib, ABT-263, and the combination for 72 h. (D) Cell proliferation assays for normal CD34$^+$ cells, and (E) CML patient's CD34$^+$ cells treated with DMSO, ponatinib, ABT-263, or the combination for 72 h. *$P < 0.05$; **$P < 0.01$; ns, not significant. (F) Proportion of granulocytic and erythroid colonies following a 14-day colony forming cell (CFC) assay using CD34$^+$ CML cells that survived 72 h of treatment with DMSO, ponatinib, ABT-263, or the combination. (G) Colonies representative of each of treatment arm in (F). Scale bars: 200 μm. Error bars indicate standard error of mean (SEM) obtained from triplicates.

overexpression is associated with TKI resistance [3]. BCL2 inhibitors such as ABT-263 and ABT-199 are in phase 2 clinical trials [14, 21]. We find that the chromosomal SV that leads to *BCL2* amplification in this patient was associated with elevated BCL2 expression and sensitivity to ABT-263 as has been reported for B-cell lymphomas and ALL cells harbouring an *IGH-BCL2* fusion or trisomy 18 [13]. However, in other reports, only a modest induction in apoptosis was seen with ABT-263 in *BCR-ABL1*$^+$ CML cells without any effects on stem cell survival or self-renewal capacity [13, 22]. Using cell lines, we show that the response to ABT-263 is correlated to high levels of *BCL2* and independent of *BCR-ABL1* expression, supporting the conclusion that the response to ABT-263 we observed in the CML patient's CD34$^+$ cells is due to the *BCL2* amplification. This observation suggests BCR-ABL-dependent and BCR-ABL-independent mechanisms of resistance in this patient and notably, the patient's CML cells were highly sensitive to combined inhibition of both resistance pathways. Moreover, the cells that survived the combination treatment favoured erythroid over granulocytic lineage differentiation.

Thus, in summary, we describe the first WGS of CML. The patient developed ponatinib-resistant disease and our data suggest that the underlying mechanism was driven by compound *BCR-ABL1* kinase domain mutations and *BCL2* overexpression. Our study describes an approach to personalised medicine for CML and we provide a proof-of-principle that the integration of WGS and functional studies can identify new combination treatments for patients who have failed even third-generation BCR-ABL1 inhibitors. Unfortunately, in this case, we were unable to translate our findings into patient's benefit because ABT-263 is not currently licensed.

acknowledgements

We thank Jane Rogan and Deepti Wilks from the Tissue Biobank for collection of blood and saliva samples, Jeff Barry and Michael Hughes for flow cytometry, Yvonne Hey for WGS and RNA-seq, Tim Somerville for normal CD34$^+$ cDNA, Nick Telford and Mike Green (Department of Oncology and Cytogenetics, Christie NHS Foundation Trust) for FISH, and Nicholas Donato (University of Michigan Ann Arbor, MI, USA) for BV173R cells.

funding

This work was supported by the Kay Kendall Leukaemia Fund (KKL631) and Cancer Research UK (C15759/A12328).

disclosure

The authors have declared no conflicts of interest.

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