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Targeted therapy and disease monitoring in CNTRL-FGFR1 driven leukaemia

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Abbreviations key:

8p11 MPN	8p11 myeloproliferative syndrome
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
ddPCR	Droplet Digital PCR
FISH	Fluorescence in situ hybridization
RNA-Seq	RNA sequencing
TKIs	Tyrosine kinase inhibitors

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Abstract

We report two patients with leukaemia driven by the rare *CNTRL-FGFR1* fusion oncogene. This fusion arises from a t(8;9)(p12;q33) translocation, and is a rare driver of biphenotypic leukaemia in children. We used RNA sequencing (RNA-seq) to report novel features of expressed *CNTRL-FGFR1*, including *CNTRL-FGFR1* fusion alternative splicing. From this knowledge, we designed and tested a Droplet Digital PCR (ddPCR) assay that detects *CNTRL-FGFR1* expression to approximately one cell in 100,000 using fusion breakpoint specific primers and probes. We also utilised cell-line models to show that effective tyrosine kinase inhibitors (TKIs), which may be included in treatment regimens for this disease, are only those that block FGFR1 phosphorylation.

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Introduction

Oncogenic fusions involving the FGFR1 receptor tyrosine kinase are associated with aggressive haematological malignancies including 8p11 myeloproliferative syndrome (8p11 MPN), acute myeloid leukaemia (AML), acute lymphoblastic leukaemia (ALL), and mixed lineage or biphenotypic leukaemia.[1] FGFR1-driven malignancies have a poor prognosis with current treatment regimens, and more effective therapeutic combinations are needed. Centriolin (*CNTRL*, formerly *CEP110*) is one of 15 genes recognised as an *FGFR1* fusion partner in 8p11 MPN.[2] The *CNTRL-FGFR1* fusion gene was first characterised by Guasch et al[3], arising from a t(8;9)(p12;q33) translocation. Sixteen cases have been reported, all of which describe a common fusion transcript linking exon 40 of *CNTRL* to exon 10 of *FGFR1*.[3-5]

Conventional karyotyping and fluorescence in situ hybridization (FISH) are commonly used to identify *FGFR1* rearrangements.[2] Karyotyping may miss cryptic rearrangements, and FISH analysis depends on prior clinical suspicion. RNA-Seq provides an unbiased method to identify expressed fusion genes.[6] Here we describe two paediatric patients with acute leukaemia harbouring *CNTRL-FGFR1* fusions. RNA-Seq data identified additional structural features of the fusions, including the co-existence of two splice isoforms in a patient, and a transcriptional start site that differs to that previously described.[3,7] We developed a ddPCR assay to detect *CNTRL-FGFR1* fusion transcripts using breakpoint specific probes designed on the basis of RNA-Seq data.

Methods and Results

The clinical details of both patients are outlined in Figure 1 (addition information provided in Supporting Information Table S1). The *FGFR1* aberration in Patient 1 was suspected at diagnosis on the basis of a reported mosaic deletion on chr8p12-p11.23, involving part of the *FGFR1* gene,

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identified on SNP array of the tumour sample. At relapse, the karyotype was 47,XY,+9 and FISH confirmed an *FGFR1* rearrangement (Figs. 1B and 1C). Salvage treatment with vincristine, dexamethasone and imatinib reduced, but did not eradicate, the leukaemic clone. Matched sibling donor allogeneic haematopoietic stem cell transplant was successful in inducing complete remission, which persists five years post-transplant.

The karyotype of the malignant cells from Patient 2 indicated the presence of the reciprocal translocation t(8;9)(p11;q33). The subsequent FISH confirmed an *FGFR1* rearrangement with translocation of 3'FGFR1 to 9q33 (Figs. 1D and 1E). The patient was treated with high dose steroids, hydroxyurea and dasatinib, but an acute respiratory illness led to discontinuation of dasatinib after two weeks. The patient died four months after diagnosis from severe lung disease and generalised myopathy of uncertain aetiology.

RNA-seq of Patient 1 was performed retrospectively on Tissue-banked samples. Patient 2 samples were sequenced during admission. In both instances the precise identity of the *FGFR1* fusion was definitively established using RNA-seq. The full details of the RNA-seq protocol and bioinformatics analyses are provided in Supplemental Methods. We identified fusion transcripts with the JAFFA pipeline[8] in direct mode, and Clinker[9], a fusion visualisation program (Fig. 1F and Supporting Information Figure S1). The malignant cells from Patient 1 expressed two *CNTRL-FGFR1* isoforms, distinguished by alternative splicing of exon 40 of *CNTRL* (Fig. 1F and Supporting Information Figure S2). Only a single expressed isoform was detected in Patient 2, the result of a previously unreported genomic break, which was confirmed by Sanger sequencing (Figs. 1F and 1G).

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The RNA-seq data suggested that the *CNTRL-FGFR1* fusion is transcribed from exon 1 of *CNTRL*, which we confirmed by PCR amplification from Patient 2 cDNA across the breakpoint with reverse PCR primers aligning to exon 10 of *FGFR1* and forward primers targeting exons 1, 4, 8, 10 and 12 of *CNTRL* (Supporting Information Figure S3). The original description of *CNTRL-FGFR1* (or *CEP110-FGFR1*) identified the start site as exon 1 of *CEP110* (GenBank ID = AF083322), which now corresponds to exon 26 of *CNTRL* (Ensembl ID = ENST00000373855.5).[7] We use the nomenclature flCNTRL-FGFR1 (full-length) to distinguish this fusion from the previously described "truncated" version (tCNTRL-FGFR1). We cloned and expressed flCNTRL-FGFR1 in Ba/F3 cells and showed that this form of the fusion is expressed (Fig. 2A) and sufficient to promote IL-3 independent survival (Supporting Information Figure S4).

A ddPCR assay to detect *CNTRL-FGFR1* transcripts was compared to detection of the *FGFR1* kinase domain in Ba/F3 cells infected with retrovirus encoding *CNTRL-FGFR1* serially diluted into wild-type Ba/F3 cells (Fig. 2B). There was a linear relationship between fusion transcript or kinase domain transcript abundance and the proportion of fusion-expressing cells, down to one fusion positive cell in 10^4 wild-type cells (0.01%). Below this dilution, only the fusion-specific probe continued to correlate with the abundance of fusion positive-cells. We also serially diluted cDNA from Patient 1 into cDNA derived from HEK293T cells, and tested fusion isoform specific probes using ddPCR (Fig. 2C). Both isoforms were detectable down to a dilution of 0.01%, with a cDNA input of 100ng.

Previous case reports (including Patient 1 here) described treatment of FGFR1 fusion-driven leukaemias with a range of TKIs, including imatinib.[10-13] We measured the viability of Ba/F3 cells expressing tCNTRL-FGFR1 (Supporting Information Figure S5) or flCNTRL-FGFR1 (Fig. 2D) after TKI treatment, and measured FGFR1 phosphorylation and fusion expression (using a CNTRL

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antibody) by Western blot. Cells were most sensitive to ponatinib, dovitinib and AZD1480. Drugs that induced apoptosis also diminished FGFR1 phosphorylation and over time, decreased FGFR1 fusion expression. Signaling downstream of FGFR1 fusions represses apoptosis, so we sought to understand the association between TKI exposure and abundance of anti-apoptotic proteins known to be critical for oncogenic fusion-immortalised Ba/F3 survival, notably Mcl-1.[14] Whilst TKIs that effectively inhibited tCNTRL-FGFR1 phosphorylation also resulted in diminished Mcl-1 (but not Bcl-2) expression (Supporting Information Figure S5), this association did not hold true in cells expressing flCNTRL-FGFR (Fig. 2E).

Our data show a consistent relationship between the capacity of each TKI to specifically target FGFR1 and their effectiveness in killing CNTRL-FGFR1 expressing Ba/F3 cells. However, this is at odds with reports of apparent clinical responses to combination treatment regimens that included imatinib or dasatinib with vincristine, including in Patient 1.[10] Ba/F3 cells expressing flCNTRL-FGFR1 were treated with ponatinib or imatinib, together with vincristine and dexamethasone, and imatinib induced no additional decrease in cell viability (Supporting Information Figure S6). Ponatinib effectively killed fusion-expressing cells, alone or in combination with vincristine and dexamethasone.

Discussion

In this study, we present two new cases of the *CNTRL-FGFR1* fusion. Using RNA-seq, we have identified a novel *CNTRL-FGFR1* fusion transcript, linking exon 39 of *CNTRL* to exon 10 of *FGFR1*, and have shown that this transcript arises through either a different genomic breakpoint or alternative splicing. We show that the *CNTRL-FGFR1* fusion is transcribed from exon 1 of *CNTRL*, and differs

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from that previously described[7]. Despite these structural differences, flCNTRL-FGFR1 has the same functional activity as the tCNTRL-FGFR1, and is sufficient to induce cytokine independent survival.

It is unlikely that the alternate splicing of *CNTRL-FGFR1* has any significant functional impact, as there are no alterations to functional domains, and both isoforms drive elevated expression of the FGFR1 kinase domain. However, alternate splicing and the novel genomic breakpoints we have identified, will influence the design of molecular assays for fusion detection, such as ddPCR, critical for minimal residual disease monitoring.

Despite reports of clinical responses to treatment regimens that included imatinib or dasatinib, our data suggests that these responses were most likely the result of the inclusion of drugs such as vincristine. The use of a TKI that effectively targets FGFR1 (such as ponatinib) may be a more rational therapeutic choice for treating CNTRL-FGFR1 driven leukaemia.

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Authorship Contributions

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Contribution: L.M.B. and R.C.B. performed experiments; N.D. and B.S. assisted in bioinformatics analysis; I.B., J.C. and V.P. performed karyotype and FISH analysis; J.C. and V.P. performed SNP array analysis; D.A.K.Q., S.L.K. and F.M. provided patient clinical information and insight, and helped draft the manuscript; I.J.M. and A.O. provided intellectual advice and assisted with bioinformatics interpretation; L.M.B. and P.G.E. conceived the study, prepared figures and wrote the manuscript.

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Figure 1. Patient clinical characteristics and RNA sequencing analysis

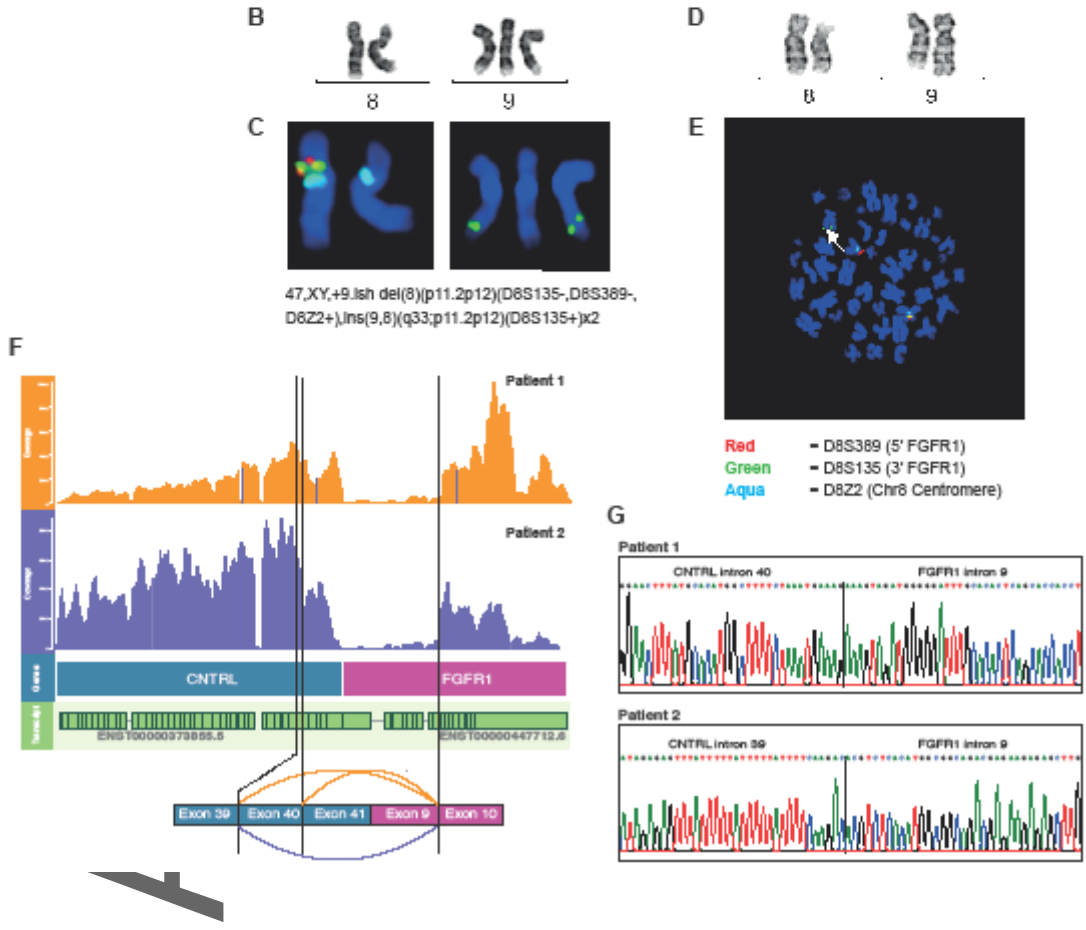
(A) Shows table describing clinical characteristics of Patient 1 and Patient 2. The karyotype of Patient 1 was 47,XY,+9 at relapse (B) and FISH analysis confirmed a cryptic *FGFR1* rearrangement (C). For Patient 2, a reciprocal translocation t(8;9)(p11;q33) was reported on the karyotype (D), and the subsequent FISH confirmed an *FGFR1* rearrangement with translocation of 3'*FGFR1* to 9q33 (indicated by the white arrow) (E). FISH probes are shown in the key below figure E. (F) Schematic of the *CNTRL-FGFR1* fusions identified in two patients by RNA sequencing. The Clinker gene fusion visualizer (https://github.com/Oshlack/Clinker) was used to illustrate the coverage of RNA sequencing reads across the *CNTRL* and *FGFR1* genes from patients 1 and 2. The Gene track shows the 2 genes involved in the fusion with each exon of the gene shown in the exon (green) track. The reference transcripts shown are *CNTRL* Ensembl transcript ID = ENST00000373855.5 and *FGFR1* Ensembl transcript ID = ENST00000447712.6. The vertical black lines show the breakpoints of the fusion transcripts. These are connected to the exon schematic at the bottom of this panel which shows that two distinct breaks occur in *CNTRL* that lead to the formation of alternate transcripts, resulting in the fusion of either exon 39 or 40 to exon 10 of *FGFR1*. (G) Chromatogram of Sanger sequencing from patient 1 and 2 genomic DNA identified different breakpoints in *CNTRL* and *FGFR1* intronic regions.

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	Patient 1	Patient 2
Age at Diagnosis	10-year-old male	2 month-old male
Diagnosis (WHO 2008)	Acute leukaemic presentation of myeloid and lymphoid neoplasm with *FGFR1* abnormalities	Myeloid and lymphoid neoplasms with *FGFR1* abnormalities
Initial Presentation	FBC: Hb 138 g/L, plt 210x10^9/L, WBC 32.9x10^9/L, Neut 9.54x10^9/L, Blast 3.62x10^9/L. Bone marrow was hypercellular with eosinophilia and 40% blasts.	FBC: Hb 83 g/L, plt 11x10^9/L, WBC 51.1x10^9/L, Neut 9.20x10^9/L. Normocellular bone marrow aspirate with trilineage haematopoiesis.
Immunophenotype	Expression of CD45, HLA-DR, CD34, CD4, CD13, CD36, CD71, CD123 with some expression of CD7, intra CD79a, TDT and borderline expression of CD15, CD33, CD2, CD64 and intra CD22	Small population of T cells present (1.2%) with weak CD3, CD5, CD8 and CD7 expression, and gain of CD56. Small (0.69%) population of T-cells with a lymphoblastic phenotype also present.
Initial Treatment	Following initial poor response to corticosteroids, the patient was treated on an AML protocol (COG AAML0531).	Hydroxyurea and high dose steroids. Dasatinib (8.5mg/kg) once daily for two weeks was added after diagnosis was known.
End of cycle 1 MRD/BMA	0.25% of lymphoblasts by flow cytometry	Persistance of t(8;9)(p11;q33) without blastic transformation
End of cycle 2 MRD	0.08% of lymphoblasts by flow cytometry	*NA*
Relapse	Yes (87 days-post initial diagnosis)	No
Immunophenotype	Identical immunopheonotype to diagnosis	*NA*
MRD	3.379% by flow cytometry. 60% blasts by morphological analysis.	*NA*
Treatment	Vincristine (1.5mg/m2 once weekly), dexamethasone (23 mg/m2 once daily), Imatinib (460 mg/m2 per day twice daily) for two months. MRD = 0.5% by flow cytometry at completion.	*NA*
Outcome	Matched sibling donor allogeneic transplant (HSCT). In remission 5 years post-transplant.	Died of severe lung disease 4 months post-diagnosis



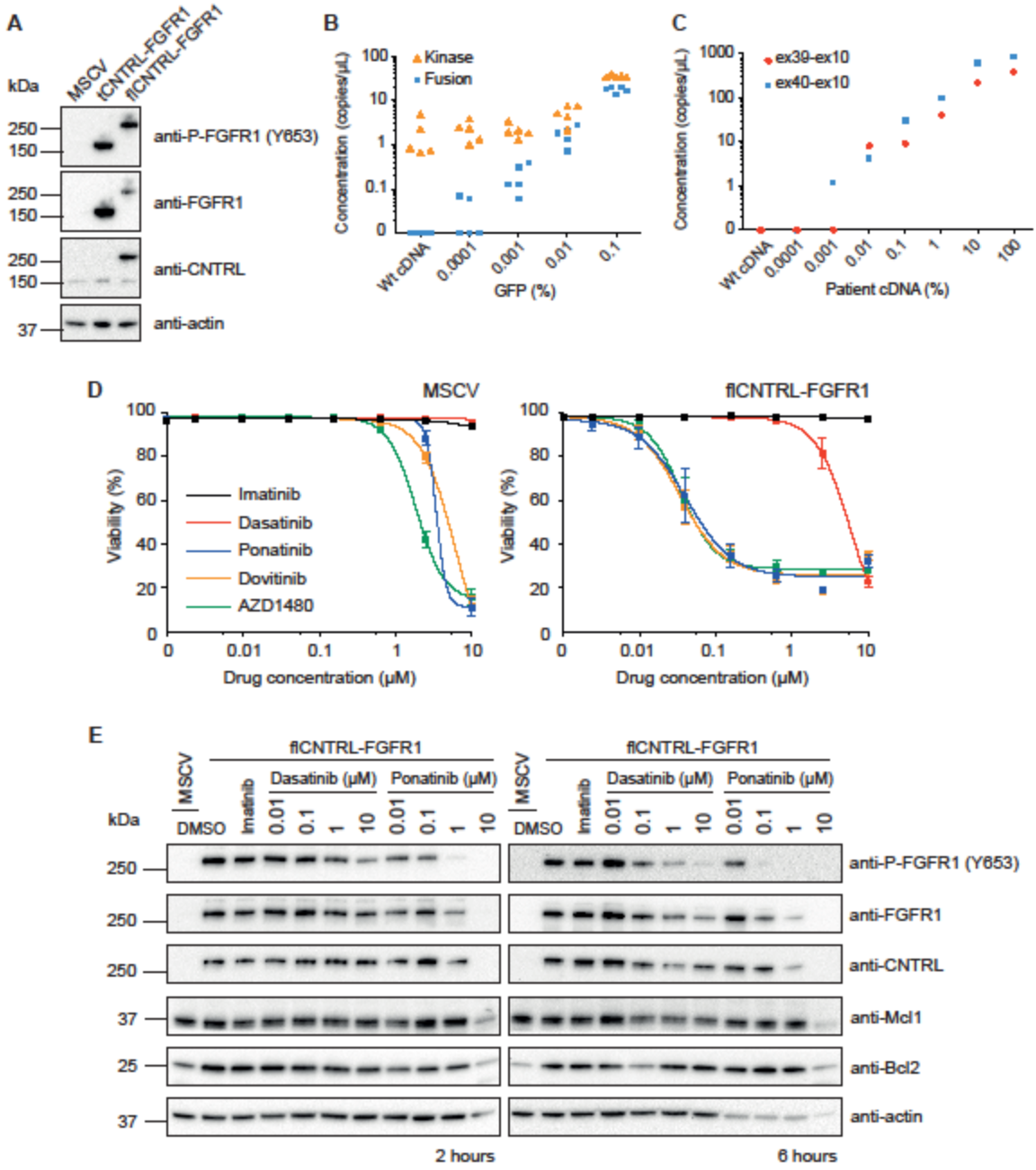
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Figure 2. Minimal residual disease monitoring by ddPCR, and therapeutic targeting of CNTRL-FGFR1 in Ba/F3 cells

(A) Western blot analysis of Ba/F3 cells retrovirally infected with MSCV (empty vector control), tCNTRL-FGFR1 MSCV and flCNTRL-FGFR1 MSCV constructs. Western Blot probed with anti-phospho-FGFR1 (P-FGFR1) antibody detecting phosphorylation at Y653 (upper panel), anti-FGFR1 (second panel), and anti-CNTRL antibody that binds to epitope in exon 4 of CNTRL (third panel). Anti-ß-actin antibody was used as a loading control. (B) The number of positive droplets detected using the *FGFR1* kinase probe (orange) and the *CNTRL-FGFR1* breakpoint specific ex40-ex10 probe (blue), using ddPCR assay. The concentration of *FGFR1* kinase or *CNTRL-FGFR1* in the unit of copies/µL is shown on the y-axis, and the percentage of GFP positive cells in the sample is on the x-axis (n=5). (C) Shows detection of *CNTRL-FGFR1* with ex39-ex10 (red) and ex40-ex10 (blue) probes. Concentration of each transcript is shown on the y-axis, and the percentage of patient cDNA in each sample is labelled on the x-axis. (D) Ba/F3 cells transformed with fl*CNTRL-FGFR1* (flCNTRL), as well as MSCV empty vector control cells, were treated with: imatinib, dasatinib, ponatinib, dovitinib and AZD1480. Graph shows viability (%) on the y-axis, determined by flow cytometry and PI exclusion, and the x-axis depicts increasing drug concentration in µM. Data presented as mean ± SEM (n=3). (E) Western blot analysis of fl*CNTRL-FGFR1* expressing Ba/F3 cells treated with four doses of dasatinib or ponatinib for two (left panel) and six (right panel) hours. Vehicle treated empty vector control (MSCV) and flCNTRL-FGFR1 Ba/F3 cells are shown in the left two lanes (DMSO), as well as flCNTRL-FGFR1 cells treated with 10µM imatinib. Increasing doses are labelled in µM concentration. Western blot probed with anti-phospho-FGFR1 (P-FGFR1, Y653), anti-FGFR1, anti-CNTRL, anti-Mcl1, and anti-Bcl2, labelled on the right side of each panel. Anti-ß-actin antibody was used as a loading control.

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