Identification of a germline F692L drug resistance variant *in cis* with *Flt3*-internal tandem duplication in knock-in mice

Internal tandem duplication (ITD) mutations in the juxtamembrane domain of the fms-like tyrosine kinase 3 (*FLT3*) gene occur in approximately one quarter of cases of acute myeloid leukemia (AML),[1] are associated with constitutive activation of the kinase[2] and confer a poor prognosis.[3] Our understanding of the molecular consequences of these mutations has benefited from studies of bespoke mouse models.[4-6] Herein we report the identification of the germline variant F692L *in cis* with the *Flt3*-ITD allele of the widely studied *Flt3*^{tm1Dgg} mouse.[4] As this variant is analogous to the human FLT3 F691L "gatekeeper" mutation[3] we investigated this finding further. We found that primary AML cells from *Npm1cA*; *Flt3*^{tm1Dgg} double-mutant mice are resistant to sorafenib and quizartinib (AC220), but sensitive to ponatinib. The same pattern of sensitivity was observed when we tested these tyrosine kinase inhibitors against isogenic Ba/F3 cells transfected with the murine *Flt3*-ITD F692L or the reverted *Flt3*-ITD wild-type cDNA, confirming that *Flt3*-ITD F692L is responsible for recapitulating the resistance pattern of the human F691L mutation. The presence of this *Flt3* tyrosine kinase inhibitor-resistant variant needs to be considered when interpreting data using this model, but also makes the model an extremely useful tool for studying tyrosine kinase inhibitor resistance.

The molecular consequences of *FLT3*-ITD are pleiotropic and include activation of STAT5 *via* the SRC kinase, phosphorylation of the transcription factor FOXO3A with associated activation of Akt and induction of reactive oxygen species, among others (reviewed by Yeung *et al.*[3]). Knock-in mouse models have been instrumental in deciphering the hematopoietic and leukemogenic effects of *FLT3*-ITD alone[4-6] and in combination with mutations co-occurring with *FLT3*-ITD in human AML[7-12]; as well as in studying the effectiveness of different therapeutic approaches in *FLT3*-ITD-positive leukemias.[11,13] As part of these efforts, we studied the powerful collaboration between *FLT3*-ITD and mutant *NPM1*, by crossing our conditional *Npm1*$^{flox\text{-}cA}$ knock-in[14] with the *Flt3*-ITD knock-in mice (*Flt3*^{tm1Dgg}) generated by Lee *et al.*[4] Triple transgenic *Npm1*$^{flox\text{-}cA/+}$; *Flt3*$^{ITD/+}$; *Mx1-Cre*$^{+}$ animals rapidly succumbed to AML with a median latency of 49 days, exhibiting full recombination of the conditional *Npm1*$^{flox\text{-}cA}$ allele without the need to induce *Mx1-Cre* expression with polyinosinic-polycytidylic acid as previously published.[8] To identify or exclude additional

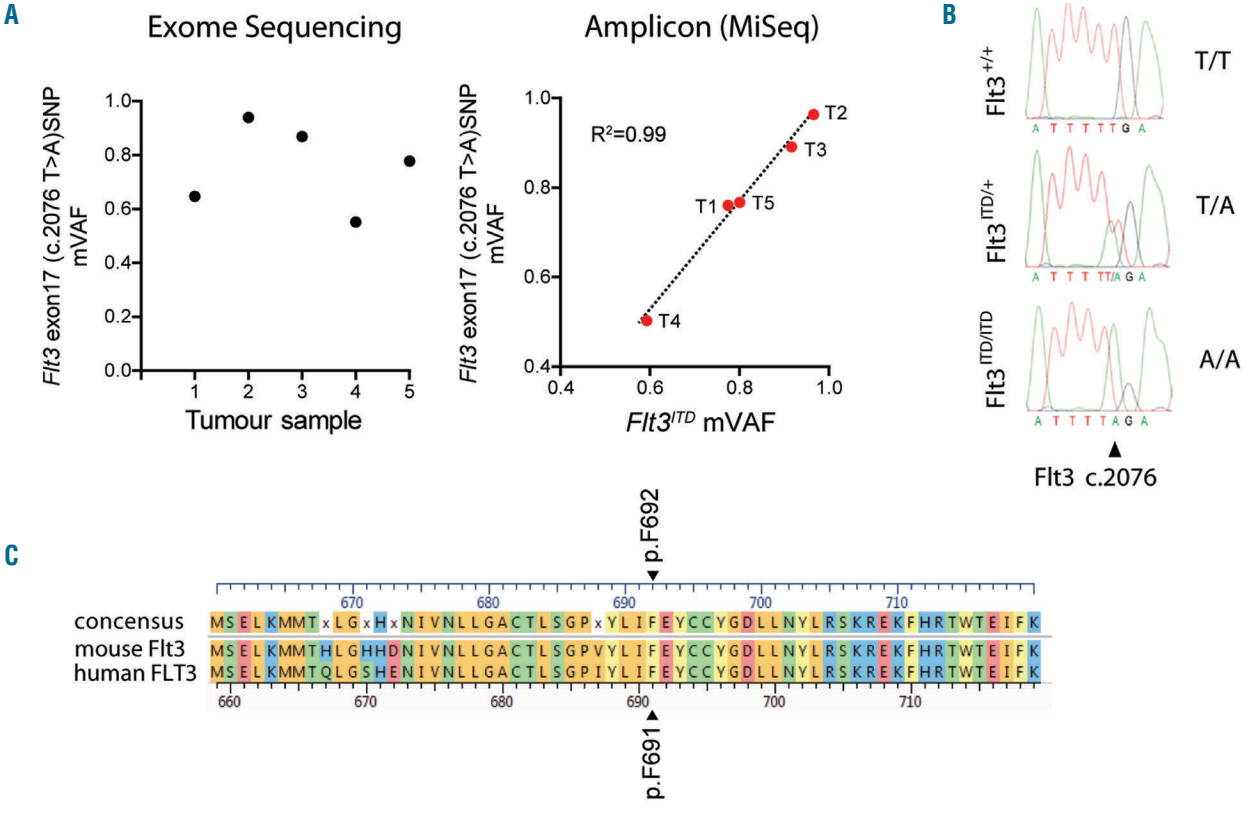

Figure 1. Identification of the *Flt3* c.2076 T>A (F692L) variant *in cis* with *Flt3*-ITD in knock-in mice. (A) The *Flt3* c.2076 T>A exon 17 single nucleotide variant was identified in five of five exomes of *Npm1*$^{cA/+}$; *Flt3*$^{ITD/+}$; *Mx1-Cre*$^{+}$ acute myeloid leukemia (AML) samples at varying variant allele frequencies (VAF) (left panel). These results were confirmed by polymerase chain reaction-amplification followed by MiSeq sequencing of AML DNA samples (T1-T5) and this also revealed a close correlation between the single nucleotide variant with the ITD VAF (right panel). (B) Sequencing of tail DNA from *Flt3*$^{+/+}$, *Flt3*$^{ITD/+}$ and *Flt3*$^{ITD/ITD}$ mice confirmed that the single nucleotide variant is present *in cis* with the *Flt3*^{tm1Dgg} allele and was not particular to the *Npm1*^{cA}; *Flt3*^{tm1Dgg} double mutant cohort. (C) Protein sequence alignment of mouse and human FLT3 protein demonstrates that the mouse p.F692 corresponds to human p.F691, target of the F691L gatekeeper tyrosine kinase inhibitor resistance mutations.

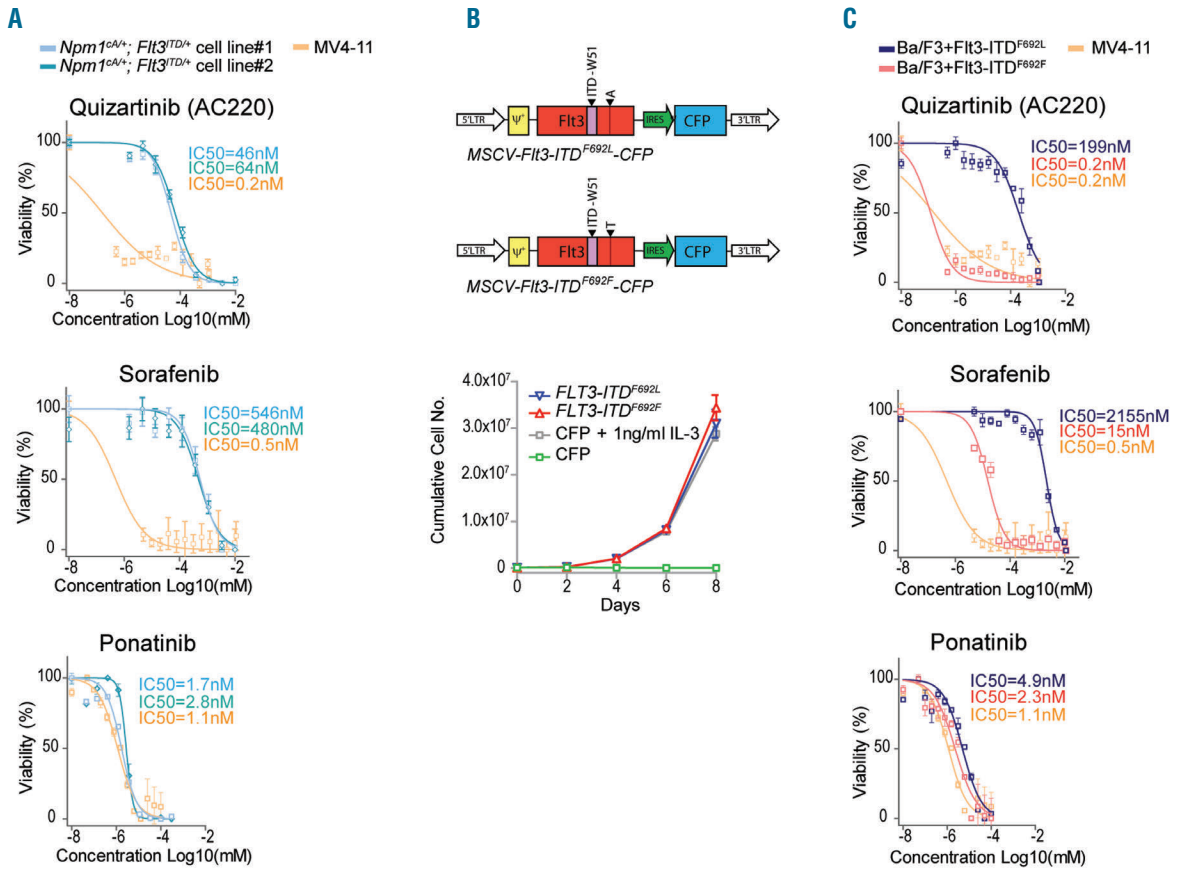

Figure 2. The *Flt3* c.2076 T>A (F692L) variant confers resistance to quizartinib and sorafenib. (A) Two independent leukemic cell lines, derived from *Npm1*$^{cA/+}$; *Flt3*$^{ITD/+}$ mice, were cultured in X-VIVO-20 medium (Lonza) (supplemented with 5% fetal calf serum, 10 ng/mL murine interleukin-6 and 50 ng/mL murine stem cell factor) and incubated with Flt3 tyrosine kinase inhibitors over a range of concentrations. IC_{50} values for each murine leukemia and for the control cell line MV4;11, in response to each treatment are displayed. (B) *MSCV-Flt3*-ITD-*CFP* retroviral constructs used to stably transduce CellSensor® irf-bla Ba/F3 cells. Both the *Flt3*-ITD^{F692L} and the reverted *Flt3*-ITD^{F692F} cDNAs transformed the Ba/F3 cells to interleukin-3 (IL-3) independent growth with growth kinetics similar to those of IL-3-stimulated cells transduced with an MSCV-CFP (empty) vector (see also *Online Supplementary Figure S2*). (C) Stably transduced mutant Ba/F3 cell lines and the *FLT3*-ITD-positive AML cell line MV4;11 were incubated with Flt3 inhibitors at a range of concentrations. The MSCV-*Flt3*-ITD^{F692L}-CFP construct conferred Ba/F3 resistance to both quizartinib and sorafenib, but not to ponatinib; cells transduced with the MSCV-*Flt3*-ITD^{F692F}-CFP construct remained sensitive to all three inhibitors. IC_{50} values for *Flt3*ITD$^{p.F692L}$ (blue) or the reverted *Flt3*-ITD^{F692L} cDNA (red) transduced cells are displayed inset for each drug. All assays were performed in triplicate.

cooperating somatic mutations involved in leukemic progression, we performed whole exome sequencing of five cases of *Npm1*$^{flox\text{-}cA/+}$; *Flt3*$^{ITD/+}$; *Mx1-Cre*$^{+}$ AML. Comparison with constitutional (tail) exome sequence from C57BL/6N mice identified a common variant located at Chr5:147349699 (GRCm38), within exon 17 of the *Flt3* gene. The variant, *Flt3* c.2076 T>A (p.F692L), was present at allele frequencies of 0.55-0.94. Using polymerase chain reaction amplification followed by MiSeq next-generation sequencing we found that these variant allele frequencies correlated strongly with those of the *Flt3*-ITD allele itself (Figure 1A). As the *Flt3*-ITD mutation is located at exon 14 and frequently exhibits loss of heterozygosity in our model[8] this indicated that the F692L variant is present *in cis* with the knock-in ITD. We went on to confirm this by genotyping germline DNA (tail) from *Flt3*$^{ITD/+}$ and *Flt3*$^{ITD/ITD}$ single transgenic mice from our cohort (Figure 1B). Furthermore, we identified the same variant in germline DNA from the cohorts of two separate research groups who obtained the *Flt3*-ITD mouse model independently (*Online Supplementary Figure S1*).

As the murine F692L variant is equivalent to the human F691L gatekeeper mutation, which confers AML resistance to multiple FLT3 tyrosine kinase inhibitors,[3,15] we proceeded to test whether this was also true for the murine variant. To do this we cultured AML cells from two independent *Npm1*$^{cA/+}$; *Flt3*$^{ITD/+}$; *Mx1-Cre*$^{+}$ mice[8] in X-VIVO medium (Lonza, Switzerland) supplemented with murine inteleukin-3, interleukin-6 and stem cell factor (Miltenyi, Germany). After 72 h in culture, we removed the interleukin-3 and exposed the cells to varying concentrations of quizartinib (AC220), sorafenib and ponatinib for 3 days, using the human FLT3-ITD-positive AML cell line MV4-11 as a positive control. After 3 days of treatment, cell viability was assessed using the CellTiter 96® AQueous assay (Promega, USA). We found that, compared to MV4-11, *Npm1*$^{cA/+}$; *Flt3*$^{ITD/+}$; *Mx1-Cre*$^{+}$ AML cells exhibited resistance to quizartinib and sorafenib, but not to ponatinib which is known to retain activity against the human FLT3-ITD F691L mutant isoform[15,16] (Figure 2A).

To exclude the possibility that our murine leukemias

and MV4-11 displayed different resistance patterns for reasons other than the F692L mutation, we amplified the full open-reading frame of the *Flt3* cDNA from bone marrow mRNA of homozygous *Flt3*$^{ITD/ITD}$ mice and cloned this into an MSCV-CFP retroviral backbone (kind gift from Dr. Brian Huntly, University of Cambridge, UK) (Figure 2B). We then also generated a reverted c.2076A>T (*Flt3*-ITD^{F692F}) construct in the same MSCV-CFP retroviral backbone using Gibson assembly (see *Online Supplementary Methods*). The two retroviruses were each used to transduce interleukin-3-dependent CellSensor® irf-bla Ba/F3 cells (Invitrogen, Carlsbad, USA), which were subsequently sorted to >95% purity (*Online Supplementary Methods and Online Supplementary Figure S2A*). Both the *Flt3*-ITD^{F692L} and *Flt3*-ITD^{F692F} cDNA-transfected cells displayed comparable interleukin-3-independent growth patterns (Figure 2B) and Forster resonance energy transfer assays showed concomitant activation of the JAK/STAT pathway, recapitulating the effects of human *FLT3*-ITD mutants (*Online Supplementary Figure S2B*). Next, we treated the transformed Ba/F3 cell lines as described above for the primary AML cells. This demonstrated that cells transformed with *Flt3*-ITD^{F692L} were resistant to quizartinib and sorafenib, but not ponatinib, unlike wild-type *Flt3*$^{ITDp.F692F}$ cells that were sensitive to all three compounds (Figure 2C). These data, taken together with our results with the primary AML cells, confirm that the murine F692L mutation mimics the properties of the human F691L mutant *FLT3*-ITD and confers a similar profile of resistance to FLT3 inhibitors.[16]

Here, we show that the *Flt3*^{tm1Dgg} knock-in mice carry the *Flt3* F692L resistance variant in cis with the human W51 *Flt3*-ITD mutation. The variant corresponds to the human F691L and shares at least some of its drug sensitivity/resistance profile. The origin of the c.2076A>T (p.F692L) variant within the *Flt3*^{tm1Dgg} allele is not clear. Examination of the strategy and design of the original gene targeting construct revealed that this did not extend to exon 17, which contains codon F692. It does, therefore, appear unlikely that the c.2076T>A single nucleotide variant was introduced into the mouse *Flt3* locus during gene targeting.[4] Furthermore, the presence of the variant in three out of three independent *Flt3*^{tm1Dgg} mouse colonies tested rules out the possibility that the variant was acquired uniquely in our cohort. The single nucleotide variant was, therefore, either present in the original embryonic stem cells used to generate the knock-in mice or was acquired in the germline during the expansion of the original colony.

Notably this single nucleotide variant is not present in any of the 48 sequenced mouse strains according to the mouse genome informatics (MGI) database (*http://www.informatics.jax.org/javawi2/servlet/WIFetch?page=snpQF*). Nevertheless, regardless of its origin, the presence of this variant needs to be taken into account when interpreting the findings of the many studies using Flt3^{tm1Dgg} mice[7-13] particularly those investigating therapeutic approaches.[11,13] Additionally, our findings make the *Flt3*^{tm1Dgg} model particularly useful for studying novel tyrosine kinase inhibitors such as ponatinib and PLX3397, which retain activity against the human F691L gatekeeper mutation.[15] Moreover, our findings emphasize the importance of careful characterization of genetically modified mice before drawing conclusions about the pathologies they model, particularly when targeted therapeutic interventions are being investigated. Although the genetic variant influencing drug response was not present in wild-type mice in our study, in other cases it may represent a consistent human - mouse difference, as was recently described for lenalidomide and a mouse-specific variant in Cereblon (*Crbn*) that makes murine cells insusceptible to the drug.[17] Given the availability and relative affordability of next-generation sequencing, we propose that genomic characterization, such as exome sequencing, of frequently-used or novel mouse models should be applied to identify coding variants in order to avoid misinterpretation of findings from these otherwise highly valuable biological reagents.

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doi:10.3324/haematol.2016.146159

Acknowledgments: We thank Professor Gary Gilliland for his careful review of the manuscript and helpful discussions. We thank Drs Adam Mead and Wallace Langdon for providing us with tail DNA from their Flt3-ITD (Flt3^{tm1Dgg}*) homozygous mice, and Dr Brian Huntly for providing us with the MSCV-CFP retroviral backbone.*

Funding: BC is funded by the "China Scholarship Council" for his visiting studies in UK. AM is funded by the Kay Kendall Leukaemia Fund project grant. CG was funded by a Bloodwise Clinical Research Training Fellowship. IV is funded by Spanish Ministerio de Economía y Competitividad subprograma Ramón y Cajal. We thank Servicio Santander Supercomputación for their support. OMD, JLC and GSV are funded by a Wellcome Trust Senior Fellowship in Clinical Science (WT095663MA) and this work was also funded by the Wellcome Trust Sanger Institute.

Key words: acute myeloid leukemia, molecular pharmacology, tyrosine kinase inhibitor resistance.

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.

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