Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Maxson JE, Gotlib J, Pollyea DA, et al. Oncogenic *CSF3R* mutations in chronic neutrophilic leukemia and atypical CML. N Engl J Med 2013;368:1781-90. DOI: 10.1056/NEJMoa1214514

Supplemental Appendix

Supplement to: Maxson J, Gotlib J, et al. Gain-of-function *CSF3R* Mutations in Myeloproliferative Neoplasms

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Full Study Methods

Functional analysis of patient samples

All clinical samples were obtained with informed consent with approval by the Institutional Review Boards of the University of Texas-Southwestern, University of Colorado, Stanford University, Washington University in St. Louis, or Oregon Health & Science University (OHSU). Bone marrow or blood samples from patients with acute leukemia were separated using a Ficoll gradient followed by red blood cell lysis; specimens from patients with CNL or aCML were processed with red blood cell lysis only. Cells were cultured in RPMI-1640 medium (Invitrogen) containing 10% fetal bovine serum (FBS, Atlanta Biologicals), L-glutamine (Invitrogen), fungizone (Invitrogen), penicillin/streptomycin (Invitrogen), and 10^{-4} M 2-mercaptoethanol (Sigma). Screening of primary leukemia cells against panels of siRNA and kinase inhibitors was performed as previously described[1-4].

Genomic analysis of patient samples

Genomic DNA was isolated from cryopreserved patient sample material using Qiagen DNeasy columns. DNA was fragmented by sonication using an S2 Sonicator (Covaris). Fragmented DNA was then processed according to the SeqEZ protocol (Nimblegen/Roche), which is based on the TruSeq protocol (Illumina). Briefly, following fragmentation, DNA was blunt-ended and then 3' tailed with a single "A" nucleotide. Fragments were ligated to adaptors containing indexed barcodes. The library was then size selected using a 2% agarose gel. The recovered library was amplified by limited rounds of polymerase chain reaction (PCR). Solution capture was performed using a custom DNA probe capture library (Supplemental Tables S1 and S2). The library was hybridized to the probe set for 72 hours at 47°C. Captured DNA was then recovered from the probes and amplified by limited rounds of PCR. The amplified library was separated from

unincorporated nucleotides and primers using a QIAquick PCR purification column (Qiagen), followed by verification of the library using the Bioanalyzer (Agilent). Library concentration was determined using real time PCR on a StepOne Real Time System (Life Technologies). The libraries were sequenced on a HiSeq 2000 sequencer (Illumina) followed by FASTQ assembly using the CASAVA pipeline (Illumina). Sequence capture, library preparation, and deep sequencing were performed by the OHSU Massively Parallel Sequencing Shared Resource.

CSF3R mutations were confirmed by PCR amplification of CSF3R exons using M13-tagged primers (exon 14 F GTAAAACGACGGCCAGTCCACGGAGGCAGCTTTAC, exon 14 R CAGGAAACAGCTATGACCAAATCAGCATCCTTTGGGTG, exon 17 F GTAAAACGACGGCCAGTAGTGGCCCAAAGACACAGTC, exon 17 R CAGGAAACAGCTATGACCGGGAGTCCCATAACAGCTCA) followed by purification of PCR products using Amicon Ultra 0.5 mL 30K Centrifugal Filters (Millipore) and Sanger sequencing with M13 forward (GTAAAACGACGGCCAGT) and reverse (CAGGAAACAGCTATGACC) primers. Sanger sequencing was performed by the OHSU DNA Sequence Analysis Shared Resource. Additional sequencing of CSF3R exons 14-17 was performed by Genewiz, Inc (South Plainfield, NJ).

Alignment and analysis of deep sequencing

Reads were aligned using the BWA realignment algorithm[5] and genotyping was performed using the GATK toolkit[6] following the 'Best Protocol' of the 'Best Practice Variant Detection with the GATK v2' (http://www.broadinstitute.org/gatk/). Briefly, genotyping was performed after removing duplicate reads, local realignment around the 1000 Genome's indels and indels inferred from the gapped alignments of all samples (indels found in one sample helped inform the rest). Finally, the quality scores were recalibrated after excluding positions known to vary in dbSNP132.

Both single nucleotide variants (SNVs) and small indels were detected. The effect of the variants was initially assessed relative to Ensembl build 56 gene models[7].

Vectors and cloning

CSF3R transcript variant 1 (NM_000760.2) pDONR vector was purchased from GeneCopoeia. CSF3R mutations were introduced using the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies). CSF3R was cloned into the MSCV-IRES-green fluorescent protein (GFP) plasmid using the Gateway Cloning System (Invitrogen).

Cell culture and retrovirus generation

293T17 cells were obtained from American Type Culture Collection (ATCC) and grown in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) containing 10% FBS, L-glutamine, fungizone and penicillin-streptomycin. 293T17 cells were transfected with FuGENE 6 (Promega). To generate murine retrovirus, 293T17 cells were transfected with MSCV-IRES-GFP constructs and EcoPac helper plasmid (a gift from Dr. Rick Van Etten, Tufts University, Boston, MA). Ba/F3 cells were obtained from ATCC and grown in RPMI 1640 medium with 10% FBS, L-glutamine, fungizone, penicillin-streptomycin, and 15% WEHI-conditioned medium (a source of IL3). Stable Ba/F3 cell lines were generated by infection of 3 x 10^6 cells with 1 mL of murine retrovirus expressing WT CSF3R, CSF3R mutants or an empty control vector, followed by sorting GFP positive cells by FACSaria (BD Biosciences).

Ba/F3 transformation assays

Parental Ba/F3 cells or those stably expressing WT CSF3R or CSF3R mutants were washed three times and re-suspended in RPMI 1640 with 10% FBS, L-glutamine, fungizone and

penicillin/streptomycin at a density of 5 x 10^5 cells per mL. Viable cell counts were obtained using propidium iodide exclusion on a Guava Personal Cell Analysis System (Millipore).

Immunoblotting

Ba/F3 cells stably expressing CSF3R constructs were lysed in Cell Lysis Buffer (Cell Signaling) containing Complete Mini Protease Inhibitor Cocktail Tablets (Roche), Phosphatase Inhibitor Cocktail 2 (Sigma) and Phenylmethanesulfonyl fluoride solution (Sigma) and then quantitated using the Pierce BCA Protein Assay Kit (Thermo Scientific). Lysates were diluted in sample buffer (75 mM Tris pH 6.8, 3% SCS, 15% glycerol, 8% B-mercaptoethanol, 0.1% bromophenol blue). Lysates were then separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore) and subjected to immunoblot analysis using the following antibodies: anti-GCSF Receptor (CSF3R) antibody (abcam, ab126167), anti-ACK1 (TNK2) antibody (abcam, ab74091), Phospho-STAT3 (Tyr705) (Cell Signaling Technologies (CST), 9131), Stat3 Antibody (CST, 9132), Phospho-Jak2 (Tyr1007/1008) Antibody (CST, 3771), JAK2 (D2E12) XP Rabbit mAb (CST, 3230), Phospho-Src Family (Tyr416) Antibody (CST, 2101), Src Antibody (CST, 2108), Anti-Actin (Ab-1) Mouse mAb (JLA20) (Calbiochem, CP01). All primary antibodies were used at a 1:1000 dilution, except for Actin, which was used at a 1:5000 dilution. Anti-mouse or anti-rabbit IgG HRP conjugate secondary antibodies (Promega) were used at a 1:5000 dilution and immunoblots were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) on a Lummi Imager (Boehringer Mannheim).

Murine hematopoietic colony formation assays

All mouse work was performed according to a protocol approved by the OHSU Institutional Animal Care and Use Committee. Bone marrow was isolated from 6-10 week old BALB/c mice

(The Jackson Laboratory), red blood cells were lysed using ammonium chloride solution (Stem Cell Technologies), cells were then washed and incubated overnight in pre-stimulation medium, DMEM, 10% FBS, L-glutamine, penicillin/streptomycin, 15% WEHI conditioned medium, 7 ng/mL recombinant murine IL-3 (Peprotech), 12 ng/mL recombinant murine IL-6 (Peprotech) and 56 ng/mL recombinant murine SCF (Peprotech). Cells were infected on two successive days with murine retrovirus expressing WT CSF3R, CSF3R S783fs, CSF3R T618I or an empty vector control. Four hours after the second infection, cells were washed three times and 2.5 x 10^4 cells were plated in 1 mL of MethoCult® M3234 Methylcellulose Medium for Mouse Cells without cytokines (Stemcell Technologies) in triplicate. Recombinant human GCSF (Peprotech), dasatinib (LC laboratories) and ruxolitinib (Selleck Chemicals) were added to the medium prior to plating as described in the figures. Colony formation was scored manually by light microscopy one week after plating. Differences in response to dasatinib or ruxolitinib of CSF3R membrane proximal or truncation mutant colonies were evaluated for significance using a paired t-test with p-values as reported in the legend for Figure 2.

J.E.M., J.G., B.J.D., and J.W.T. designed the study, all authors gathered, analyzed, and vouch for the data and the analysis, J.E.M., J.G., D.A.P., C.A.E., C.E.T., B.J.D., and J.W.T. wrote the paper, and all authors reviewed and decided to publish the paper. J.E.M. and J.W.T. wrote the first draft of the paper. There were no agreements concerning confidentiality of the data between the sponsor and the authors or the institutions named in the credit lines.

Supplemental Figures

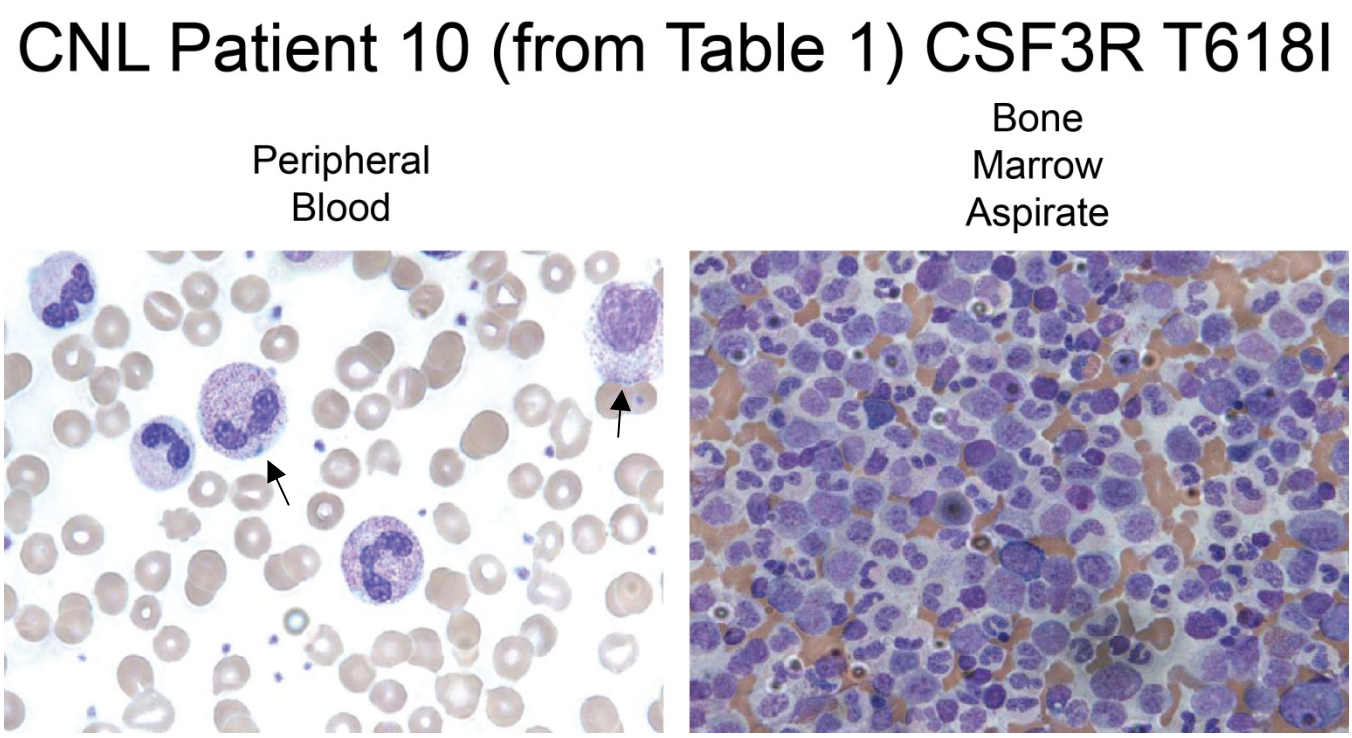

Figure S1. Histopathology of peripheral blood and bone marrow aspirate from a patient with CNL

Peripheral blood (patient #10, Table 1) showing segmented and band neutrophils with toxic granulation and Döhle bodies (arrow), common morphologic features among CNL and aCML patients carrying *CSF3R* mutations (Wright Giemsa stain, 1000x). The bone marrow aspirate from patient 10 reveals a marked myeloid hyperplasia with full spectrum maturation (Wright Giemsa stain, 400x). Courtesy Dr. Tracy George, University of New Mexico.

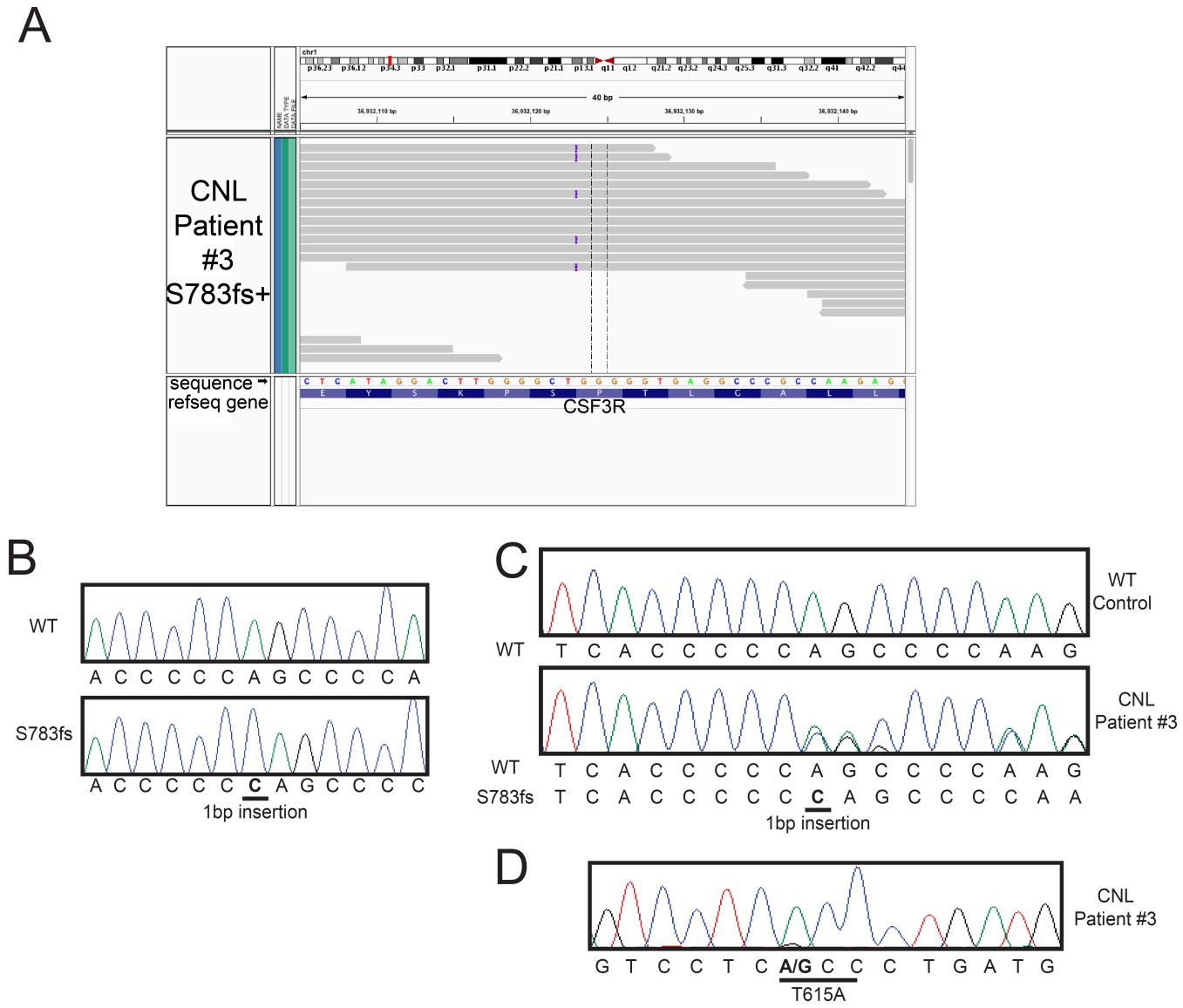

Figure S2. *CSF3R* deep sequencing and Sanger validation for Patient #3

A. The reads for *CSF3R* for the sample from Patient #3 (Table S3; CSF3R S783fs) at the point of insertion are shown. The T-->TG insertion is shown by the purple lines in some of the reads (the genomic sequence is annotated in the opposite orientation on the genome browser as the transcript, in the transcript the mutation corresponds to A --> CA. Note that the dotted lines are simply a reference point. **B.** Genomic DNA flanking the 1 bp insertion in *CSF3R* from patient #3 was amplified and cloned into plasmids such that single clones could be sequenced to confirm the presence of a 1 bp insertion. Representative electropherograms of clones exhibiting the WT and S783fs alleles are shown. **C.** Direct Sanger sequencing of amplified genomic DNA from Patient #3 shows that the S783fs mutation is present at nearly 50% frequency, while the T615A mutation (**D**) is a very low frequency mutation. This is further supported by cloning and enumeration of sequenced PCR products from this same specimen as described in Table S4. In all electropherograms, A is denoted as a green line, C as a blue line, G as a black line and T as a red line.

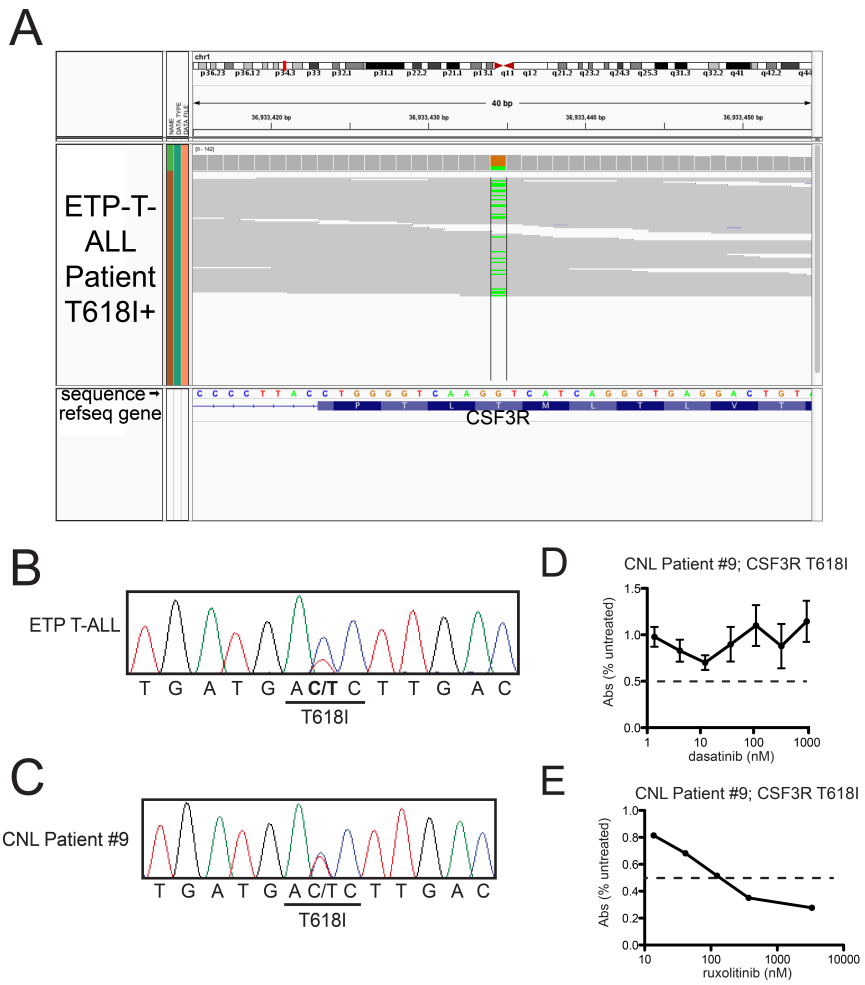

Figure S3. *CSF3R* deep sequencing and Sanger validation for T618I-positive ETP-T-ALL and CNL Patient #9

A. Reads for *CSF3R* in the region of the T618I mutation are shown collapsed due to the large number of reads. The green bars indicate the reads with the G-->A mutation corresponding to T618I (Note: since CSF3R is oriented in the reverse direction in the genome alignment, on the mRNA transcript the corresponding change is A**C**C (T) to A**T**C (I)). Coverage is shown above the reads and the rectangles are colored in the proportion to the reads bases. The colors are shown in the Sequence track. **B**. Sequencing of the ETP-T-ALL patient from (A) revealed a C to T mutation resulting in a T618I mutation in *CSF3R*. In the electropherogram, A is denoted as a green line, C as a blue line, G as a black line and T as a red line. **C.** Sequencing of the CNL patient #9 specimen from (D, E) revealed a T618I point mutation in *CSF3R*. The region surrounding the *CSF3R* T618I mutation was PCR amplified from genomic DNA and subjected to Sanger sequencing. A single base pair mutation (C to T) was found at approximately 50% frequency and the electropherograms for the region surrounding the mutation are shown. **D.** Cells from CNL patient #9 (Table S3) were tested for sensitivity to dasatinib. The dasatinib curves were run in triplicate and did not reach an IC50, even at the highest concentration tested (1000 nM). Values represent mean percent cell viability ± s.e.m. **E.** The same CNL patient sample (patient #9) was tested for sensitivity to ruxolitinib. The patient specimen was sensitive to ruxolitinib with an IC50 of 127 nM. Values represent mean percent cell viability.

Supplemental Tables

Table S1. Gene List for Exon Sequence Capture. (see separate file)

Table S2. Genomic Coordinates for Exon Sequence Capture. (see separate file)

Patient #	Diagnosis	CSF3R Mutation	Gender/Age	(% Segmented + Band Neutrophils) / % Precursors	Highest recorded WBC (/μL)	WBC (/μL)	ANC (/μL)	Hb (g/dL) / Hct	Platelets (/μL)	MCV (fL)
1	aCML favored over CNL[1]	D771fs	M/67	82 (82 + 0) / <5	20,000	15,900	13,038	9.5/28.2	210,000	100.9
2	aCML	G683R[2]	F/50	70 (58 + 12) / 10	42,000	13,200	7,700	14.1/42.6	54,000	89.5
3	CNL	S783fs/T615A	M/73	82 (82 + 0) / <5	64,000	8,100	6,642	9.2/26.4	415,000	97.7
4	aCML	T615A/Y752X	M/70	42 (20 + 22) / 48	270,000	37,100	15,582	9.0/27.2	126,000	95.9
5	CNL	T618I/D771fs	F/76	77 (47 + 30) / 19	94,500	94,500	72,750	12.3/35.0	288,000	91.3
6	CNL	T618I/E808K[3]	M/73	94 (94 + 0) / 0	271,000	8,300	7,802	10.3/30	30,000	100.9
7	aCML favored over CNL[1]	T618I/W791X	M/71	94 (89 + 5) / 0	83,800	56,700	53,298	10.4/32.3	215,000	90.5
8	aCML	T618I	M/88	81 (65 + 16) / 7	175,900	38,100	24,800	8.8/24.5	98,000	92.3
9	CNL	T618I	M/53	86 (81 + 5) / 9	108,100	96,500	87,800	10.8/33.6	65,000	94.9
10	CNL	T618I	F/73	96 (96 + 0) / 0	178,600	178,600	171,456	7.5/22.2	83,000	111.3
11	aCML favored over CNL[1]	T618I	M/77	95 (75 + 20) / <5	39,100	39,100	24,992	8.4/26.0	69,000	108.7
12	CNL	T618I	M/69	87 (80 + 7) / 7	250,000	115,500	100,485	8.3/ NA	129,000	N/A[4]
13	aCML	T618I	F/73	81 (58 + 23) / 6	211,000	98,700	79,947	11.2/34.6	271,000	93.2
14	CNL	T618I	M/57	93 (93 + 0)/ 0	63,800	48,500	45,100	11.0/32.6	108,000	92.2
15	CNL	T618I	M/67	88 (83 + 5)/ 3	272,400	163,000	154,900	8.8/25.8	22,000	90.5
16	aCML	T618I	F/74	72 (62 + 10)/ 24	177,000	105,000	75,600	9.9/29.6	88,000	78.2
17	aCML	None	F/48	55 (40 + 15) / 7	96,000	80,100	51,264	10.2/32.5	204,000	96.3
18	aCML	None	F/73	60 (53 + 7) / 26	252,600	133,200	79,920	8.3/27.8	117,000	85.1
19	aCML	None	F/62	64 (64 + 0) / 0	42,900	12,900	8,256	12.1/36.3	378,000	98.7
20	aCML	None	M/87	50 (36 + 14) / 43	47,000	38,900	19,450	9.3/27.2	50,000	91.7
21	CNL	None	M/86	92 (92 + 0) / 0	130,000	73,200	67,344	11.2/36.7	190,000	81.4
22	aCML	None	M/86	48 (42 + 6) / 49	114,000	114,000	54,720	7.7/23.7	8,000	90.8
23	aCML	None	M/80	58 (30 + 28) / 18	64,100	64,100	37,178	13.1/38.6	180,000	86.3
24	aCML	None	M/74	89 (89 + 0) / 6-20	41,300	41,300	36,757	12.1/38.2	38,000	104.0
25	aCML	None	F/49	48 (32 + 16) / 36	69,100	69,100	33,168	9.8/28.2	223,000	99.4
26	aCML	None	M/71	61 (35 + 26) / 20	102,000	102,000	52,020	9.1/10.3	272,000	101
27	aCML	None	F/76	69 (63 + 6) / 9	65,000	18,000	12,420	9.2/26.1	295,000	84.3

Table S3. Mutations in CSF3R are enriched in patients with neutrophilic leukemia.

Leukemia patient samples were sequenced for CSF3R exons 14-17 encompassing the membrane proximal extracellular domain, transmembrane and cytoplasmic domains. CSF3R membrane proximal mutations are blue and truncation mutations are red. Compound mutations listed in predicted temporal order of acquisition by leukemic cells.

[1]aCML favored over CNL because of the presence of granulocytic dysplasia and/or prior WBC differentials showing > 10% immature precursors

[2]G683R not evaluated in Ba/F3 cells; this mutation is annotated in CSF3R transcript variant 3, because it is not present in variant 1; all other variants are annotated in transcript 1

[3]E808K Reported in[8]

[4]Unable to report due to high WBC indices

CNL – chronic neutrophilic leukemia; aCML – atypical chronic myeloid leukemia; WBC – White Blood Cell Count; ANC – Absolute Neutrophil Count; Hb/Hct – Hemoglobin and Hematocrit; MCV – Mean Corpuscular Volume

Patient Number	CSF3R Mutations	Frequency
3	None	3/9
3	T615A only	0/9
3	S783fs only	4/9
3	S783fs/T615A	2/9
5	None	2/11
5	T618I only	3/11
5	D771fs only	0/11
5	T618I/D771fs	6/11

Table S4. *CSF3R* Compound mutations are on the same allele

RNA was isolated from cryopreserved patient samples. cDNA was then synthesized and used to amplify a region of CSF3R encompassing both mutations. The PCR products were TOPO TA cloned (Invitrogen), transformed into *E. coli*, and individual clones were isolated from bacterial colonies and sequenced. The frequency of the mutations is listed as a fraction of the total colonies sequenced. Both patients had clones with both the membrane proximal point mutations and the truncation mutations, indicating that they were on the same allele. Patient #3 had some clones with only the S783fs mutation but not the T615A mutation, indicating that the S783fs mutation most likely arose first. This is consistent with the sequencing results from genomic DNA indicating that the T615A mutation was present at very low frequency (Figure S2). Patient #5 had clones with T618I only but not D771fs only, indicating that the T618I mutation likely preceded the D771fs mutation.

Supplemental References

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