Additional patient information

Cytogenetic analyses of diagnostic samples were performed by Cancer and Leukemia Group B (CALGB)-approved institutional cytogenetic laboratories as part of the cytogenetic companion study 8461 and confirmed by central karyotype review.[1,2] To establish cytogenetically normal acute myeloid leukemia (CN-AML), ≥20 metaphase cells from diagnostic marrow had to be analyzed and the karyotype found to be normal.[2] Patients were treated on one of the following CALGB frontline treatment protocols: 9720,[3] 9420,[4] 8525,[5] 8923[6] and 10201.[7] CALGB 9720 was initiated as a phase III trial in untreated AML patients aged 60 years or older evaluating multidrug resistance (MDR) modulation by valspodar (PSC-833) during induction and consolidation therapy with cytarabine, daunorubicin, and etoposide, and subcutaneous low-dose recombinant interleukin-2 (rIL-2), a regimen previously piloted in older AML patients in CALGB 9420[4] as maintenance therapy. The valspodar (PSC-833)-containing induction and consolidation arm was closed after random assignment of 120 patients because of excessive early deaths.[3] CALGB 9720 continued as a phase III study of rIL-2 maintenance therapy, with all patients receiving cytarabine, daunorubicin, and etoposide chemotherapy, without PSC-833. Notably, patients assigned to the rIL-2 immunotherapy regimen had disease-free survival (DFS) and overall survival (OS) similar to those of patients assigned to no further therapy demonstrating lack of beneficial effects of rIL-2.[8] CALGB 8525[5,9] evaluated three dose schedules of cytarabine (high dose, 3g/m^2; intermediate dose, 400mg/m^2 and low dose, 100mg/m^2) as consolidation treatment for AML patients in first complete remission (CR) and demonstrated a dose-dependent improvement in survival in the core-binding factor and CN-AML cytogenetic subgroups. CALGB 8923 evaluated a regimen of modified high-dose cytarabine plus mitoxantrone compared with a standard lower dose cytarabine scheme; the former failed to show a benefit compared to the latter.[6] CALGB 10201 evaluated the BCL-2 antisense, Oblimersen (Genesense; G3139) administered with induction and consolidation chemotherapy and found no difference in outcome relative to chemotherapy alone.[7] Per protocols, patients enrolled on these studies did not receive allogeneic stem cell transplantation in first CR. Patients enrolled on the treatment protocols were also enrolled on the companion protocols CALGB 9665 (Leukemia Tissue Bank) and CALGB 20202 (molecular studies in AML), and gave informed consent for pretreatment marrow and blood collection and their research use in accordance with the Declaration of Helsinki.

Definition of clinical end points

CR was defined as recovery of an absolute neutrophil count ≥1,500/μL, platelet count ≥100,000/μL, no leukemic blasts in the blood, marrow cellularity >20% with maturation of all cell lines, no Auer rods, <5% marrow blast cells, and no evidence of extramedullary leukemia, all of which had persisted for at least 1 month.[10] Relapse was defined by ≥5% marrow blasts, circulating leukemic blasts, or the development of extramedullary leukemia. OS was measured from the date on study until the date of death, and patients alive at last follow-up were censored. DFS was measured from the date of CR until the date of relapse or death; patients alive and relapse-free at last follow-up were censored. For cumulative incidence of relapse (CIR), time was calculated from date of CR until relapse. Patients alive without relapse were censored. Because in the studied patient group, there were no remission deaths, similar information was attained when DFS or CIR were considered as endpoints (see Figures 1 and S1).

Statistical analyses

Differences for baseline demographic, clinical, and molecular features in patients according to *FLT3*-ITD status were assessed using the Fisher's exact and Wilcoxon rank sum tests for categorical and continuous variables, respectively. CR rates were compared using the Fisher's exact test. Estimated probabilities of OS and DFS were calculated using the Kaplan-Meier method, and the log-rank test evaluated differences between survival distributions. To control for multiple comparisons for the allelic ratio survival analyses, we used the Sidak adjustment.[11] Multivariable proportional hazards models were constructed for OS and DFS to evaluate the impact of *FLT3*-ITD. For the analyses including all 219 patients, factors considered for inclusion in these models were *FLT3*-ITD, *NPM1*, *WT1* and *CEBPA* mutational status, hemoglobin level, platelet count, white blood count (WBC), percentage of blood and bone marrow (BM) blasts, age, race, and sex. Protocol effect (i.e. a categorical variable with five levels, one for each protocol) was evaluated in univariable analyses for outcome, and was not significant in any of the models (CR: P=.85; DFS: P=.76; OS: P=.86). In addition, we also tested whether adding protocol effect to the models evaluating the impact of *FLT3*-ITD on outcome, modified any of the results. Our analyses showed that protocol effect remained insignificant and the effect of *FLT3*-ITD on outcome stayed the same. Thus protocol effect was not included in multivariable modeling. For the subset analyses in the 60-69 year-old patients, factors considered for inclusion in the models were *FLT3*-ITD, *NPM1* and *CEBPA* mutational status, hemoglobin level, platelet count, WBC, percentage of blood and BM blasts, age, race, and sex. Of the above factors, those significant at α=.20 from univariable analyses were used in a limited backwards selection procedure to build multivariable models. Variables remaining in the final models were significant at α=.05. The proportional hazards assumption was checked for each variable individually. If the proportional hazards assumption was not met for a particular variable, then an artificial time dependent covariate was included in all models that contained that variable.[12] For survival end points, hazard ratios with their corresponding 95% confidence intervals were obtained for each significant prognostic factor.

Microarray data analyses

Microarray gene and microRNA expression analyses were performed using BRB-ArrayTools version 3.4.0 (R. Simon and A.P. Lam, National Cancer Institute, Bethesda, MD) and using R version 2.3.1 (R Foundation for Statistical Computing, Vienna, Austria).

For gene-expression microarrays, summary measures of gene expression were computed for each probe set using the robust multichip average (RMA) method, which incorporates quantile normalization of arrays.[13] Expression values were logged (base 2) before analysis. A filtering step was performed to remove probe sets that did not display significant variation in expression across arrays. In this procedure, a chi-square test was used to test whether the observed variance in expression of a probe set was significantly larger than the median observed variance in expression for all probe sets using α=.01 as the significance level. A total of 24,437 probe sets passed the filtering criterion. A gene-expression signature was derived by comparing available microarray profiles from *FLT3*-ITD (n=38) versus *FLT3* wild-type (*FLT3*-WT; n=79) patients. Univariable significance levels of α=.001 for gene-expression profiling were used to determine the probe-sets that comprised the signature.

For microRNA microarrays, the signal intensity was calculated for each spot making an adjustment for local background. Intensities were log-transformed and log-intensities from replicate spots were averaged. Quantile normalization was performed on arrays using all human and mouse microRNA probes represented on the array.[14] For each microRNA probe, an adjustment was made for batch effects (ie, differences in expression related to the batch in which arrays were hybridized). Further analysis was limited to the 895 unique human probes represented on the array. A comparison between available patient samples with (n=40) and without (n=80) *FLT3*-ITD was performed, using a univariable significance level of α=.005.

We used GenMAPP version 2.1 and MAPPFinder version 2.0 to assess which biological processes (as designated by the Gene Ontology project at www.geneontology.org) were overrepresented among the genes comprising the *FLT3*-ITD signature. An overrepresented biological process is one with more associated genes (also referred to as members) in the gene-expression signature than expected by chance. In our analysis, we only considered biological processes that were represented by at least five members among the genes that could be analyzed in our microarray-expression database. MAPPFinder uses a permutation procedure to determine overrepresented biological processes. An alpha level of .005 was used for identifying such biological processes. Furthermore, we only report the overrepresented biological processes for which at least half of their members (ie, genes) analyzed in our microarray-expression database were identified as part of the *FLT3*-ITD signature.

To test for an interaction between age group (60-69 vs $\geq$70 years) and *FLT3*-ITD mutation status for each probe-set and microRNA probe studied, an ANOVA model was fitted with expression of the probe-set or microRNA probe as the response variable and age group, *FLT3*-ITD mutation status, and age group by *FLT3*-ITD mutation status interaction as explanatory variables in the model. Global tests were then performed based on a permutation procedure to determine if the number of probe-sets or microRNA probes with a significant interaction effect (*P*-value <.001 for probe-sets, *P*-value <.005 for microRNA probes) was greater than expected by chance.

All statistical analyses were performed by the CALGB Statistical Center.

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Participating institutions

The following Cancer and Leukemia Group B institutions, principal investigators, and cytogeneticists participated in this study:

Wake Forest University School of Medicine, Winston-Salem, NC: David D. Hurd, P. Nagesh Rao, Wendy L. Flejter and Mark J. Pettenati (grant no. CA03927); The Ohio State University Medical Center, Columbus, OH: Clara D. Bloomfield, Karl S. Theil, Diane Minka and Nyla A. Heerema (grant no. CA77658); University of Iowa Hospitals, Iowa City, IA: Daniel A. Vaena and Shivanand R. Patil (grant no. CA47642); North Shore–Long Island Jewish Health System, Manhasset, NY: Daniel R. Budman and Prasad R. K. Koduru (grant no. CA35279); Roswell Park Cancer Institute, Buffalo, NY: Ellis G. Levine and AnneMarie W. Block (grant no. CA02599); Duke University Medical Center, Durham, NC: Jeffrey Crawford, Sandra H. Bigner, Mazin B. Qumsiyeh, John Eyre and Barbara K. Goodman (grant no. CA47577); University of Chicago Medical Center, Chicago, IL: Hedy L. Kindler, Diane Roulston, Yanming Zhang and Michelle M. Le Beau (grant no. CA41287); Ft. Wayne Medical Oncology/Hematology, Ft. Wayne, IN: Sreenivasa Nattam and Patricia I. Bader; Minneapolis VA Medical Center, Minneapolis, MN: Vicki A. Morrison and Sugandhi A. Tharapel (grant no. CA47555); Dana Farber Cancer Institute, Boston, MA: Harold J. Burstein, Ramana Tantravahi, Leonard L. Atkins, Paola Dal Cin and Cynthia C. Morton (grant no. CA32291); University of North Carolina, Chapel Hill, NC: Thomas C. Shea and Kathleen W. Rao (grant no. CA47559); Washington University School of Medicine, St. Louis, MO: Nancy L. Bartlett, Michael S. Watson, Eric C. Crawford, Peining Li, and Jaime Garcia-Heras (grant no. CA77440); Weill Medical College of Cornell University, New York, NY: John Leonard and Ram S. Verma (grant no. CA07968); University of Maryland Cancer Center, Baltimore, MD: Martin J. Edelman, Joseph R. Testa, Maimon M. Cohen, Judith Stamberg, and Yi Ning (grant no. CA31983); Eastern Maine Medical Center, Bangor, ME: Harvey M. Segal and Laurent J. Beauregard (grant no. CA35406); Long Island Jewish Medical Center CCOP, Lake Success, NY: Kanti R. Rai and Prasad R. K. Koduru (grant no. CA11028); Mount Sinai School of Medicine, New York, NY: Lewis R. Silverman and Vesna Najfeld (grant no. CA04457); Rhode Island Hospital, Providence, RI: William Sikov and Teresita Padre-Mendoza, Hon Fong L. Mark and Shelly L. Kerman (grant no. CA08025); SUNY Upstate Medical University, Syracuse, NY: Stephen L. Graziano, Larry Gordon and Constance K. Stein (grant no. CA21060); Dartmouth Medical School, Lebanon, NH: Konstantin Dragnev, Doris H. Wurster-Hill and Thuluvancheri K. Mohandas (grant no. CA04326); Vermont Cancer Center, Burlington, VT: Steven M. Grunberg, Elizabeth F. Allen and Mary Tang (grant no. CA77406); University of Minnesota, Minneapolis, MN: Bruce A. Peterson, Diane C. Arthur and Betsy A. Hirsch (grant no. CA16450); University of Nebraska Medical Center, Omaha, NE: Anne Kessinger and Warren G. Sanger (grant no. CA77298); University of Massachusetts Medical Center, Worcester, MA: William V. Walsh and Vikram Jaswaney (grant no. CA37135); Massachusetts General Hospital, Boston, MA: Jeffrey W. Clark, Leonard L. Atkins, Paola Dal Cin and Cynthia C. Morton (grant no. CA 12449); McGill Department of Oncology, Montreal, Quebec: J. L. Hutchison and Jacqueline Emond (grant no. CA31809); University of Missouri/Ellis Fischel Cancer Center, Columbia, MO: Michael C. Perry and Tim H. Huang (grant no. CA12046); University of Puerto Rico School of Medicine, San Juan, PR: Eileen I. Pacheco, Leonard L. Atkins and Cynthia C. Morton; University of Illinois at Chicago: David J. Peace, Maureen M. McCorquodale and Kathleen E. Richkind (grant no. CA74811); University

of California at San Diego: Barbara A. Parker and Renée Bernstein (grant no. CA11789); Western Pennsylvania Hospital, Pittsburgh, PA: John Lister and Gerard R. Diggans; Walter Reed Army Medical Center, Washington, DC: Brendan M. Weiss and Rawatmal B. Surana (grant no. CA26806); University of Cincinnati Medical Center, Cincinnati, OH: Orlando J. Martelo and Ashok K. Srivastava (grant no. CA47515); Columbia-Presbyterian Medical Center, New York, NY: Rose R. Ellison and Dorothy Warburton (grant no. CA12011); Georgetown University Medical Center, Washington, DC: Minnetta C. Liu and Jeanne M. Meck (grant no. CA77597); SUNY Maimonides Medical Center, Brooklyn, NY: Sameer Rafla and Ram S. Verma (grant no. CA25119); Medical University of South Carolina, Charleston, SC: Mark R. Green and Daynna J. Wolff (grant no. CA03927); Christiana Care Health Services, Inc., Newark, DE: Stephen S. Grubbs and Digamber S. Borgaonkar (grant no. CA45418).

Table S1. Multivariable Cox regression models for outcome in all patients

End point/variables in final models	HR	95% CI	*P*
Disease-free survival			
FLT3-ITD*, positive vs negative	2.10	1.36-2.23	<.001
NPM1, mutated vs wild-type	0.59	0.41-0.85	.005
WBC*, continuous, 50-unit increase	1.44	1.07-1.95	.028
Hemoglobin*, continuous	1.27	1.01-1.60	.045
Overall survival			
FLT3-ITD, positive vs negative	1.97	1.45-2.68	<.001
NPM1, mutated vs wild-type	0.54	0.40-0.73	<.001

HR indicates hazard ratio; CI, confidence interval; DFS, disease-free survival; OS, overall survival; WBC, white blood count; *FLT3*-ITD, internal tandem duplication of the *FLT3* gene; and *NPM1*, nucleophosmin 1.

A hazard ratio greater than 1 (less than 1) corresponds to a higher (lower) risk for higher values of continuous variables and the first category listed of a dichotomous variable. Variables considered in the multivariable models were those significant at α=.20 from the univariable models. Variables considered in the models were as follows: for DFS, *FLT3*-ITD (positive vs negative), *NPM1* (mutated vs wild-type), *WT1* (mutated vs wild-type), hemoglobin, WBC and race (white vs nonwhite); for OS, *FLT3*-ITD (positive vs negative), *NPM1* (mutated vs wild-type), *WT1* (mutated vs wild-type), platelets, WBC, race (white vs nonwhite) and performance status (Grade 2, 3 or 4 vs Grade 0 or 1).

* *FLT3*-ITD, WBC and hemoglobin did not meet the proportional hazards assumption for DFS. Initially, *FLT3*-ITD significantly increases the risk for relapse or death, but the strength of its impact seems to lessen after about 9 months; the HR for *FLT3*-ITD is provided at 6 months. Higher WBC is associated with worse DFS until about 5 months; the HR for WBC is provided at 3 months. Higher hemoglobin is associated with worse DFS until about 3 months; the HR for hemoglobin is provided at 2 months.

Table S2. Comparisons of DFS and OS of older primary CN-AML patients according to *FLT3*-ITD allelic ratio

End point	High* (n=36)	Low* (n=36)	Wild-type* (n=147)	*P*† High vs low	*P*† High vs wild-type	*P*† Low vs wild-type
Disease-free survival				.95	.01	.09
Median, y	0.4	0.5	1.0			
Disease-free at 3 y, % (95% CI)	10 (2-26)	11 (3-26)	18 (12-26)			
Overall survival				.38	<.001	.04
Median, y	0.5	0.8	1.4			
Alive at 3 y, % (95% CI)	14 (5-27)	14 (5-27)	23 (16-30)			

* High and low *FLT3*-ITD allelic ratios are based on the median (0.615) *FLT3*-ITD/WT allelic ratio; wild-type is *FLT3*-ITD/WT allelic ratio equal to zero.

† *P*-values for DFS and OS variables were from the log-rank test. These *P*-values were adjusted for multiple comparisons using the Sidak procedure (Westfall PH, Tobias RD, Rom D, Wolfinger RD, Hochberg Y. Multiple Comparisons and Multiple Tests Using the SAS System. Cary, NC: SAS Institute Inc; 1999).

Median follow-up was 3.8 years (range, 2.3-11.6) for patients alive.

Table S3. Multivariable Cox regression models for outcome in patients age 60–69 years

End point/variables in final models	HR	95% CI	*P*
Disease-free survival			
FLT3-ITD*, positive vs negative	2.94	1.67-5.18	<.001
Hemoglobin*, continuous	1.50	1.05-2.15	.018
Overall survival			
FLT3-ITD, positive vs negative	2.79	1.85-4.20	<.001
NPM1, mutated vs wild-type	0.62	0.41-0.93	.021

HR indicates hazard ratio; CI, confidence interval; DFS, disease-free survival; OS, overall survival; and *FLT3*-ITD, internal tandem duplication of the *FLT3* gene.

A hazard ratio greater than 1 (less than 1) corresponds to a higher (lower) risk for higher values of continuous variables and the first category listed of a dichotomous variable. Variables considered in the multivariable models were those significant at α=.20 from the univariable models. Variables considered in the models were as follows: for DFS, *FLT3*-ITD (positive vs negative), hemoglobin, and WBC; for OS, *FLT3*-ITD (positive vs negative) and *NPM1* (mutated vs wild-type).

* *FLT3*-ITD and hemoglobin did not meet the proportional hazards assumption for DFS. Initially, *FLT3*-ITD significantly increases the risk for relapse or death, but the strength of its impact seems to lessen after about 9 months; the HR for *FLT3*-ITD is provided at 6 months. Higher hemoglobin is associated with worse DFS until about 3 months; the HR for hemoglobin is provided at 2 months.

Table S5. Overrepresented biological processes in the *FLT3*-ITD-associated gene-expression signature

GO ID	GO Name	Percentage of members studied that appear in the *FLT3*-ITD signature	*P*
48291	isotype switching to IgG isotypes	100.00	0.003
48302	regulation of isotype switching to IgG isotypes	100.00	0.003
18196	peptidyl-asparagine modification	90.00	<0.001
18279	protein amino acid N-linked glycosylation via asparagine	90.00	<0.001
9081	branched chain family amino acid metabolic process	81.82	<0.001
9083	branched chain family amino acid catabolic process	77.78	0.002
6778	porphyrin metabolic process	76.47	<0.001
33013	tetrapyrrole metabolic process	76.47	<0.001
42168	heme metabolic process	73.33	<0.001
6779	porphyrin biosynthetic process	71.43	<0.001
33014	tetrapyrrole biosynthetic process	71.43	<0.001
51187	cofactor catabolic process	71.43	<0.001
9451	RNA modification	69.23	0.001
6783	heme biosynthetic process	66.67	0.003
6099	tricarboxylic acid cycle	66.67	<0.001
9109	coenzyme catabolic process	66.67	<0.001
46356	acetyl-CoA catabolic process	66.67	<0.001
6084	acetyl-CoA metabolic process	62.50	<0.001
42440	pigment metabolic process	61.54	<0.001
9063	amino acid catabolic process	59.52	<0.001
46483	heterocycle metabolic process	58.82	<0.001
6487	protein amino acid N-linked glycosylation	58.62	0.002
9310	amine catabolic process	58.33	<0.001
19748	secondary metabolic process	57.58	<0.001
46148	pigment biosynthetic process	56.52	0.003
44270	nitrogen compound catabolic process	56.00	<0.001
6096	glycolysis	55.56	<0.001
6007	glucose catabolic process	52.17	<0.001
6418	tRNA aminoacylation for protein translation	51.16	<0.001
43038	amino acid activation	51.16	<0.001
43039	tRNA aminoacylation	51.16	<0.001
46164	alcohol catabolic process	50.00	<0.001

Gray-shaded rows indicate GO terms involved in cellular metabolic processes.

Table S6. *FLT3*-ITD-associated microRNA expression-signature derived from a comparison of *FLT3*-ITD *versus* *FLT3*-WT patients in older primary CN-AML, sorted by decreasing fold-change

Target microRNA[†]	Fold-change: *FLT3*-ITD/*FLT3*-WT	*P*
Overexpressed in *FLT3*-ITD patients		
miR-155	2.81	< 1e-07
miR-125b-2*	1.96	< 1e-07
miR-146b-5p	1.60	4.67E-05
miR-378	1.52	0.00023
miR-136*	1.47	0.00260
miR-196a	1.46	0.00082
miR-135a	1.36	0.00098
miR-20a	1.35	1.00E-07
miR-196a*	1.34	0.00389
miR-16-2*	1.33	3.01E-05
miR-20b	1.30	7.80E-06
miR-106a[‡]	1.28	0.00019
miR-92a	1.25	0.00149
miR-17	1.23	0.00338
miR-106a[‡]	1.22	0.00036
miR-106b	1.21	0.00055
miR-19a	1.19	0.00384
Underexpressed in *FLT3*-ITD patients		
miR-107[‡]	0.79	0.00080
miR-107[‡]	0.79	0.00028
miR-103-as	0.78	0.00014
miR-185	0.77	0.00154
mir-640 (precursor)	0.74	0.00257
mir-514 (precursor)	0.70	0.00175
miR-342-3p	0.69	0.00012
mir-451 (precursor)	0.68	0.00154
miR-512-5p	0.65	0.00485
miR-150	0.60	6.79E-05
miR-144	0.53	0.00198
miR-451	0.40	9.00E-07
miR-144*	0.36	1.00E-06
miR-488	0.36	6.00E-06
miR-486-5p	0.33	1.40E-06

[†]Target microRNAs are annotated using standard nomenclature (Griffiths-Jones S. miRBase: microRNA sequences and annotation. *Curr Protoc Bioinformatics.* 2010, Chapter 12:Unit 12.9.1-10).
[‡] Target microRNA represented by two probes in the signature.

Figure S1. Cumulative incidence of relapse (CIR) for patients with or without *FLT3*-ITD

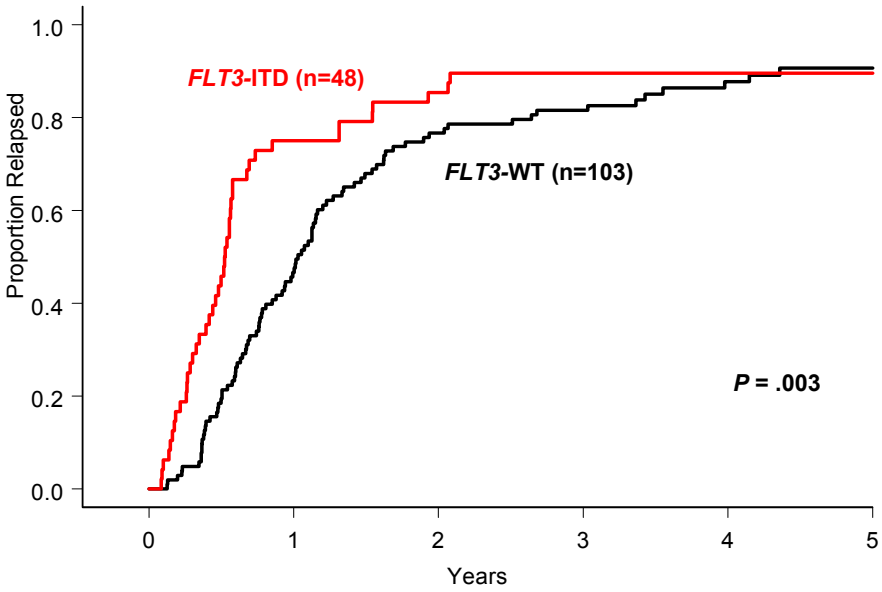


Figure S2. Kaplan-Meier survival plots of older (≥60 years) patients with cytogenetically normal, primary acute myeloid leukemia according to *FLT3*-ITD: wild-type (WT) allelic ratio

(A) Disease-free and (B) overall survival.

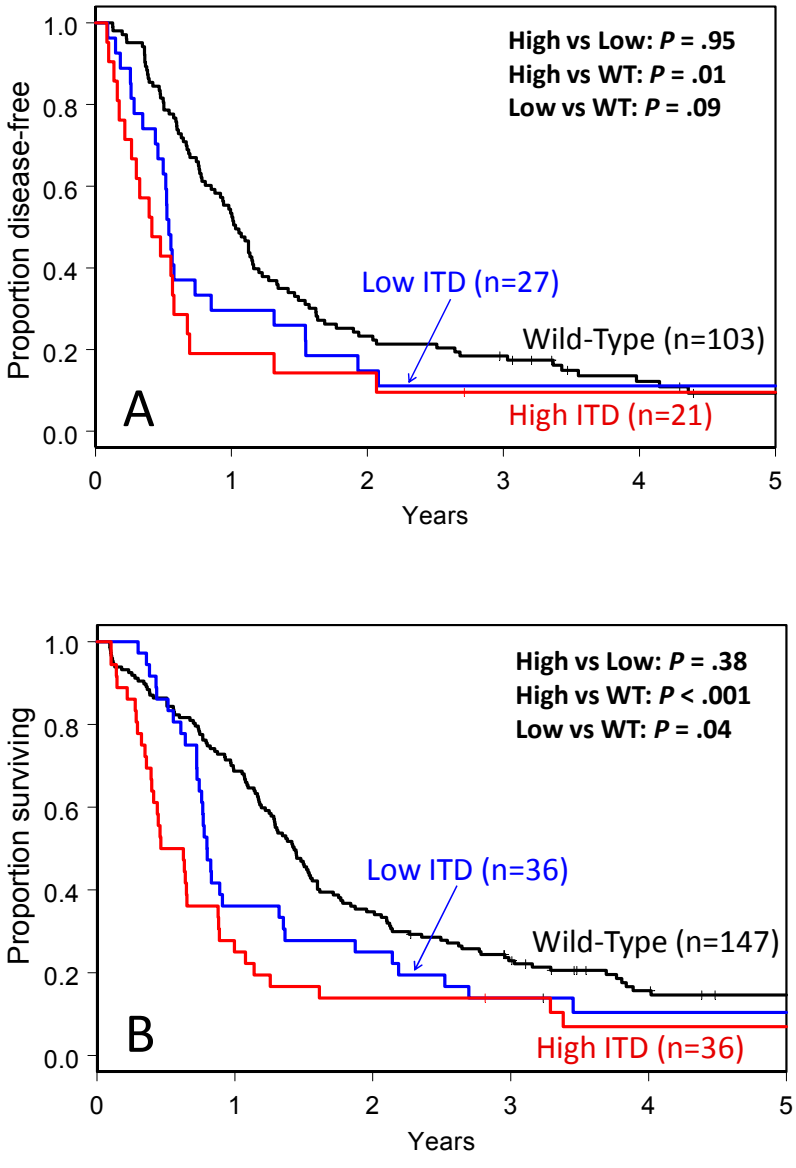


Figure S3. Heat maps of the gene- and microRNA-expression signatures derived from a comparison of *FLT3*-ITD and *FLT3*-WT primary CN-AML patients ≥60 years of age

Rows represent A) probe-sets or B) microRNA probes and columns represent patients. Patients are grouped by *FLT3*-ITD status and, within the *FLT3*-ITD group, ordered from left to right by increasing ITD/WT allelic ratio indicated by the triangle. Genes and microRNAs are ordered by hierarchical cluster analysis. The complete list of genes corresponding to each row is given in Table S4, Excel worksheet 2. Expression values of the probe-sets and microRNA probes are represented by color, with blue indicating expression less than and red indicating expression greater than the median value for the given probe-set or microRNA probe. Arrows indicate genes or microRNAs that are discussed in the text. Four microRNAs are listed in duplicate because they are represented by two distinct probes in the signature.

Figure S3A

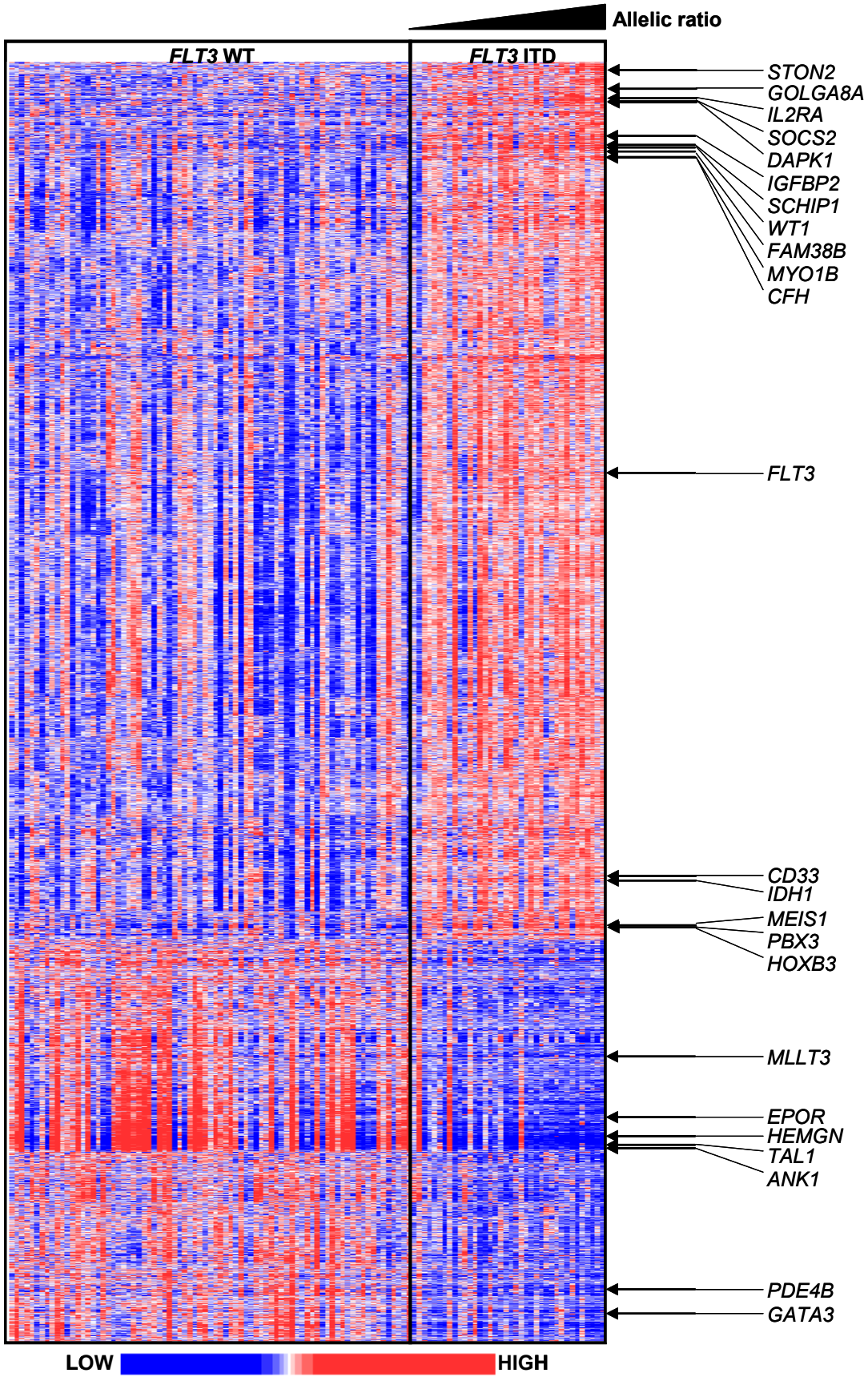
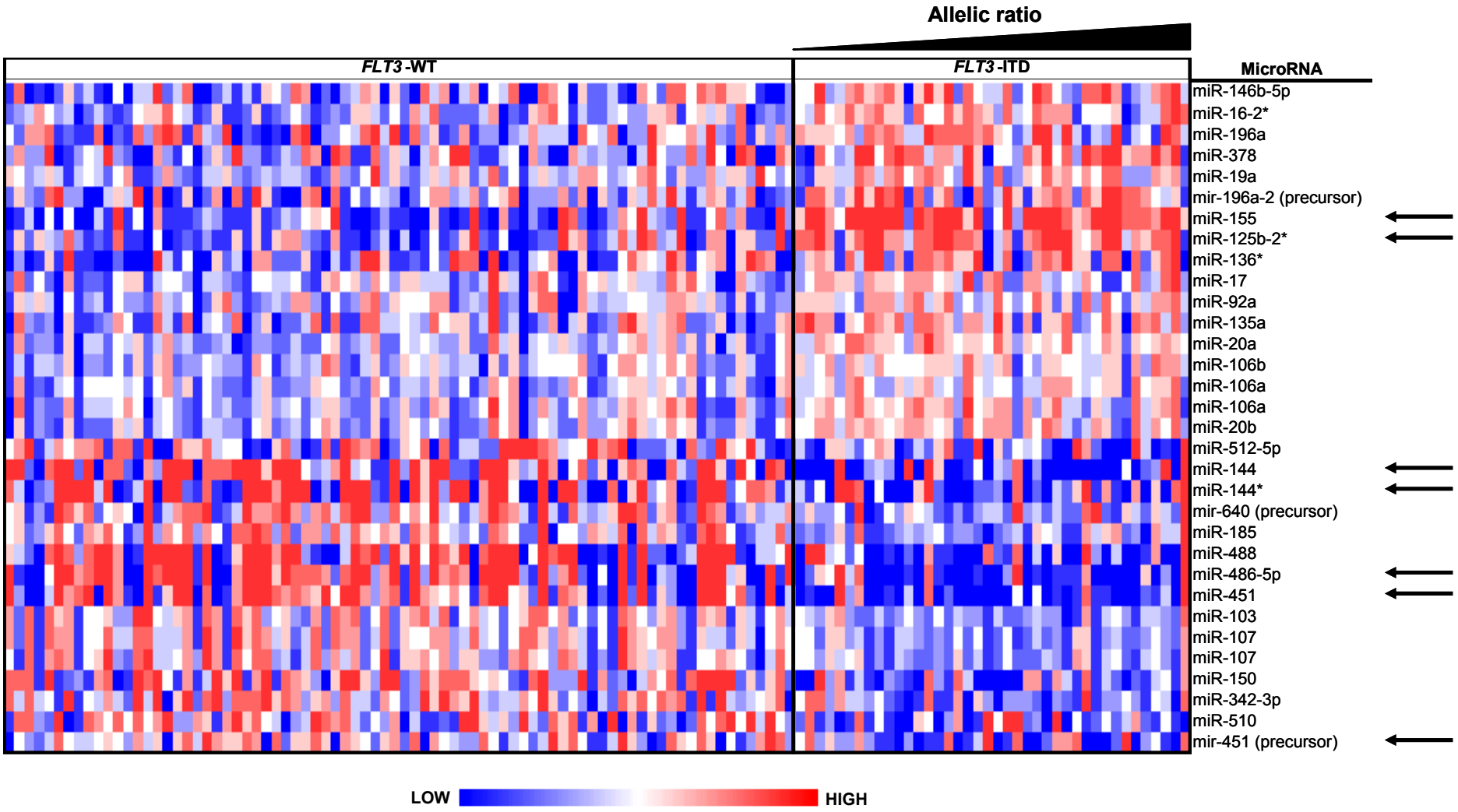

Allelic ratio
FLT3 WT
FLT3 ITD
STON2
GOLGA8A
IL2RA
SOCS2
DAPK1
IGFBP2
SCHIP1
WT1
FAM38B
MYO1B
CFH
FLT3
CD33
IDH1
MEIS1
PBX3
HOXB3
MLLT3
EPOR
HEMGN
TAL1
ANK1
PDE4B
GATA3
LOW
HIGH

Figure S3B


Allelic ratio
FLT3-WT
FLT3-ITD
MicroRNA
miR-146b-5p
miR-16-2*
miR-196a
miR-378
miR-19a
mir-196a-2 (precursor)
miR-155
miR-125b-2*
miR-136*
miR-17
miR-92a
miR-135a
miR-20a
miR-106b
miR-106a
miR-106a
miR-20b
miR-512-5p
miR-144
miR-144*
mir-640 (precursor)
miR-185
miR-488
miR-486-5p
miR-451
miR-103
miR-107
miR-107
miR-150
miR-342-3p
miR-510
mir-451 (precursor)
LOW
HIGH