NF1 Inactivation in Adult Acute Myelogenous Leukemia

Brian Parkin¹, Peter Ouillette¹, Yin Wang⁴, Yan Liu⁴, Whitney Wright¹, Diane Roulston⁵, Anjali Purkayastha³, Amanda Dressel⁵, Judith Karp⁵, Paula Bockenstedt¹, Ammar Al-Zoubi¹, Moshe Talpaz¹, Lisa Kujawski¹, Yang Liu⁴, Kerby Shedden², Sajid Shakhan¹, Cheng Li⁶, Harry Erba¹, and Sami N. Malek¹,³

Abstract

Purpose: This study was conducted to identify novel genes with importance to the biology of adult acute myelogenous leukemia (AML).

Experimental Design: We analyzed DNA from highly purified AML blasts and paired buccal cells from 95 patients for recurrent genomic microdeletions using ultra-high density Affymetrix single nucleotide polymorphism 6.0 array–based genomic profiling.

Results: Through fine mapping of microdeletions on 17q, we derived a minimal deleted region of ~0.9-Mb length that harbors 11 known genes; this region includes Neurofibromin 1 (NF1). Sequence analysis of all NF1 coding exons in the 11 AML cases with NF1 copy number changes identified acquired truncating frameshift mutations in two patients. These NF1 mutations were already present in the hematopoietic stem cell compartment. Subsequent expression analysis of NF1 mRNA in the entire AML cohort using fluorescence-activated cell sorting sorted blasts as a source of RNA identified six patients (one with a NF1 mutation) with absent NF1 expression. The NF1 null states were associated with increased Ras-bound GTP, and short hairpin RNA–mediated NF1 suppression in primary AML blasts with wild-type NF1 facilitated colony formation in methylcellulose. Primary AML blasts without functional NF1, unlike blasts with functional NF1, displayed sensitivity to rapamycin-induced apoptosis, thus identifying a dependence on mammalian target of rapamycin (mTOR) signaling for survival. Finally, colony formation in methylcellulose ex vivo of NF1 null CD34+/CD38− cells sorted from AML bone marrow samples was inhibited by low-dose rapamycin.

Conclusions: NF1 null states are present in 7 of 95 (7%) of adult AML and delineate a disease subset that could be preferentially targeted by Ras or mammalian target of rapamycin–directed therapeutics. Clin Cancer Res; 16(16); 4135–47. ©2010 AACR.

Cytogenetics and mutations in selected genes are of dominant importance to the biology and clinical outcome of patients with acute myelogenous leukemia (AML; refs. 1–7). Further, genomic changes based on karyotyping of blood or marrow specimens from patients with AML directly affect AML treatment decisions. Although the anatomy of recurrent chromosomal translocations and the functional consequences of translocation-associated fusion proteins are well studied, relatively less is known about the genes that are mutated or deregulated as a consequence of subchromosomal copy number changes. Efforts at mapping subchromosomal genomic copy number changes using array-based comparative genomic hybridization (CGH) or single nucleotide polymorphism (SNP) arrays in AML have identified genomic losses and gains, and candidate genes have been proposed (8–11).

Authors’ Affiliations: ¹Department of Internal Medicine, Division of Hematology and Oncology, Departments of ²Statistics, ³Pathology, and ⁴Surgery, Division of Immunotherapy, University of Michigan, Ann Arbor, Michigan; ⁵Department of Oncology, Johns Hopkins Cancer Center, Baltimore, Maryland; and ⁶Departments of Biostatistics and Biostatistics and Computational Biology, Harvard School of Public Health and the Dana-Farber Cancer Institute, Boston, Massachusetts

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

B. Parkin and P. Ouillette contributed equally to this work.

Peter Ouillette, Yin Wang, Yan Liu, Yang Liu, Whitney Wright, and Sami Malek performed the laboratory research.

Kerby Shedden, Cheng Li, and Sajid Shakhan assisted with statistical methods.

Brian Parkin, Amanda Dressel, Lisa Kujawski, Paula Bockenstedt, Ammar Al-Zoubi, Moshe Talpaz, Harry Erba, and Sami Malek enrolled patients and contributed and analyzed the clinical data.

Diane Roulston and Anjali Purkayastha assisted with cytogenetics/FISH data interpretation.

Judith Karp assisted with questions related to AML biology and classification.

Sami Malek conceived the study.

Peter Ouillette, Brian Parkin, and Sami Malek wrote the paper.

Corresponding Author: Sami N. Malek, Department of Internal Medicine, Division of Hematology and Oncology, University of Michigan, 1500 East Medical Center Drive, Ann Arbor, MI 48109-0936. Phone: 734-763-2194; Fax: 734-647-9654; E-mail: smalek@med.umich.edu.

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Translational Relevance

Acute myelogenous leukemia (AML) has an incidence of ~12,000 new cases in the United States per year with ~9,000 untimely deaths. Cytogenetics and mutations in selected genes are of importance to the biology, therapy selection, and clinical outcome of patients with AML. Ras pathway activation due to either Ras mutations or upstream gene mutations is common in AML, but knowledge about the role of the tumor suppressor NF1, a negative Ras regulator, in AML is still evolving. In this study, we provide a detailed analysis of NF1 aberrations in a large cohort of AML and have identified NF1 null AML blasts and NF1 null CD34+/CD38− cells to be sensitive to mammalian target of rapamycin inhibition, thus providing a refined molecular rationale for clinical applications of mammalian target of rapamycin inhibitors in AML.

Ultimately, however, the combination of high-resolution DNA copy number and loss of heterozygosity analysis, combined with DNA sequence and gene expression analysis, and followed by functional analysis is needed to identify novel genes with pathologic significance in AML.

Recent developments in genome-wide high-resolution copy number analysis using SNP arrays have aided better definitions of the pathologic anatomy of cancer genomes, and application of SNP array technology to hematologic cancers has refined knowledge of the anatomy of clinically important chromosomal lesions (12–19). For this study, we have used Affymetrix SNP 6.0 arrays to interrogate the genomes of a large panel of adult AML cases for recurrent microdeletions and have identified a minimal deleted region of 0.9 Mb on 17q that spans NF1. Through combined sequence and expression analysis, we identified NF1 null states in ~7% of adult AML and have traced NF1 mutations to the hematopoietic stem cell compartment. Importantly, in addition to Ras pathway activation, NF1 null blasts showed a sensitivity to low-dose rapamycin-induced apoptosis, thus identifying a dependence on mammalian target of rapamycin (mTOR) signaling for survival. Furthermore, CD34+/CD38− cells isolated from NF1 null AML bone marrow were inhibited by low-dose rapamycin in methylcellulose colony assays. Combined, these data provide the most complete description yet of the incidence and consequences of NF1 functional loss in AML and suggest that a subset of AML patients are good candidates for clinical applications of mTOR inhibitors.

Materials and Methods

Patients

Between March 2005 and August 2008, 95 patients with AML that were evaluated at the University of Michigan Comprehensive Cancer Center were enrolled into this study. The study was approved by the University of Michigan Institutional Review Board (IRBMED #2004-1022), and written informed consent was obtained from all patients before enrollment. Of these 95 patient samples, 92 resulted in array-based data for paired samples (blasts and buccal DNA) and 3 resulted in tumor data only.

Cell isolation

Ficoll gradient separation and cryopreservation. Peripheral blood or bone marrow mononuclear cells from AML patients were isolated by Ficoll-paque gradient centrifugation (GE Healthcare), aliquoted into FCS with 10% DMOSO, and cryopreserved in the liquid phase of a liquid nitrogen tank.

Microbead-based negative selection and subsequent flow cytometry sorting of leukemia specimens. Liquid nitrogen-cryopreserved (liquid phase) peripheral blood or bone marrow mononuclear cells derived from AML patients were washed and recovered by centrifugation and then treated with anti-human CD3, anti-human CD14 microbeads (if blasts were negative for CD14 expression), anti-human CD19 (if blasts were negative for CD19 expression), and anti-human CD235a per manufacturer’s recommendations (all microbead products: Miltenyi Biotec). Cell suspensions were run through Miltenyi MACS LS separation columns to negatively enrich for AML blasts. All blast preps were analyzed by cytopsins for purity. This schema always resulted in >90% blast purity.

AML blast DNA used for SNP 6.0 profiling and RNA used for expression analysis were extracted from samples that were further purified as follows: post-Miltenyi column samples were washed and stained with FITC-conjugated anti-CD33, phycoerythrin-conjugated anti-CD13, and allophycocyanin (APC)-conjugated anti-CD45 (all antibodies: eBioscience). After final washing, propidium iodide (PI) was added to a concentration of 1 μg/mL to discriminate dead cells. Sorting of cells was done on a FACS Aria high-speed flow cytometer (Becton Dickinson). Live cells (PI negative) were gated for blasts by identifying those cells with intermediate intensity staining for CD45 and low to moderate intensity side scatter (20). CD33 and CD13 were then used to further discriminate blasts versus erythroid lineage and mature myeloid lineage cells.

Preparation of sample DNA

Highly pure blast cells or buccal swabs were digested overnight in 100 mmol/L Tris (pH 8.0), 50 mmol/L EDTA, 50 mmol/L NaCl, 0.5% SDS, and 100 μg/mL of Proteinase K (Invitrogen) at 56°C. DNA was extracted using phenolchloroform and precipitated using ammonium acetate, ethanol, and glycogen.

Array data analysis

The DNA was prepared for hybridization to SNP 6.0 arrays according to the manufacturers’ recommendations. Affymetrix CEL files for each blast and buccal sample were analyzed using the Genotyping Console software for initial quality control, followed by use of the Affymetrix “Birdseed” algorithm to generate tab-delimited SNP call
files in text format. Call rates for the entire group of samples included in this report were between 93.57% and 99.45% with mean and median call rates of 98.35% and 98.58%, respectively; none of the DNA samples gave out-of-bounds results. Sample copy number heatmap displays were obtained from CEL files through use of the freely available software dChip adapted to operate in a 64-bit environment (21). To generate functional and practical displays of LOH, a two-step, internally developed, Java-based software analysis system was used. The Pre-LOH Unification Tool served to align all individual patient SNP calls to their respective dbSNP rs ID numbers and genomic physical positions before incorporation into the LOH tool version 2, an updated version of the LOH tool able to accommodate Affy SNP 6.0 array data (22). SNP 6.0 data files from all samples described in this study have been deposited with Geo accession number GSE21107.

**Cytogenetic analysis and fluorescence in situ hybridization**

Cytogenetic analysis was done on direct and 24-hour cultures of leukemic cells without mitogens from bone marrow and peripheral blood samples that were prepared and fixed using standard techniques. Cryopreserved purified blast cells used for fluorescence in situ hybridization (FISH) analysis were thawed and cultured at 1 × 10⁶ cells/mL for 24 hours at 37°C. Following incubation, the cells were prepared as for cytogenetics samples using standard techniques. Interphase FISH analysis for the **NF1** locus was done using BAC probe RP11-241P14 labeled with the fluorophore Green (BlueGnome) and a SpectrumOrange-labeled control probe that hybridizes to the subtelomeric region at 17q25 (TelVysion17q, Abbott Molecular). The BAC probe RP11-241P14 hybridizes to chromosome band 17q11.2 (from nucleotide 27,081,373-27,261,539); it is 180 kb in length. FISH was done following the procedures recommended by the manufacturers. A 4′-6-diamidino-2-phenylindole counter-stain was used to visualize interphase nuclei. The expected normal signal pattern is two orange and two green signals. Slides were examined using a Leica DMRA fluorescence microscope equipped with a filter set including TexasRed/FTIC dual-band pass and 4′-6-diamidino-2-phenylindole filters. A total of 200 interphase cells were analyzed per patient. Negative controls were prepared from cryopreserved purified AML blast cells without evidence of deletion of **NF1** by SNP 6.0 array profiling, and were run in parallel with each hybridization.

**Quantitative real-time PCR analysis of genomic copy number changes at 17q**

Primers and Taqman-based probes for quantitative polymerase chain reaction (Q-PCR) applications were purchased from Applied Biosystems. Primers/probe mixtures included Hs00169714_m1 and Hs00175238_m1. PCRs were done using DNA extracted from sorted blasts as template. Standardization of relative copy number estimates for genomic regions of interest was done using the ΔΔCt method with the Ct values for the **RAG2** locus as reference.

**Exon resequencing of **NF1**, **NPM1**, **Flt3**, **p53**, **N-Ras**, and **K-ras****

Primers to amplify and sequence exons 1 to 58 of human **NF1** (labeled 1-57 plus 31a), exon 12 of human **NPM1**, exons 13 to 15 and 20 of human **Flt3**, exons 2 and 3 of **N-ras** and **K-ras**, and exons 5 to 9 of human **p53** and adjacent intronic sequences were designed using the primer 3 program (http://frodo.wi.mit.edu/primer3/). The sequences of the **NF1** primers used are tabulated in Supplementary Table S1. High quality sequence tracings were obtained for 63 of (11 × 58 = 638) 99% of exons covered. PCR products were generated using Repli-g (Qiagen)-amplified DNA from highly pure blast cells as templates. Amplifications were done using Taq polymerase. PCR amplicons were prepared for direct sequencing with internal nested sequencing primers using the exonuclease/shrimp alkaline phosphatase method (USB). Mutation Surveyor (SoftGenetics LLC) software was used to compare experimental **NF1** sequences against Refseq Genbank sequences as well as by visual inspection of sequence tracings.

**Measurements of normalized **NF1** or **IRF8** expression using Q-PCR**

RNA was prepared from approximately 2 × 10⁵ to 2 × 10⁶ ultrapure blasts, purified as outlined above, using the Trizol reagent, and resuspended in 50 µL DEPC-treated water. Complementary DNA was made from ~20 ng of RNA using the SuperScript III first strand synthesis kit (Invitrogen) and oligo-RT priming. Primers and Taqman-based probes were purchased from Applied Bio-systems (Primers-on-demand). Primer/probe mixtures included **NF1** (Hs00169714_m1), **IRF8** (Hs00175238_m1), and **PGK1** (Hu PGK1). Duplicate amplification reactions included primers/probes, Taqman 2× Universal PCR Master Mix, No AmpErase UNG, and 1 µL of cDNA in a 20-µL reaction volume. Reactions were done on an ABI 7900HT machine. Normalization of relative copy number estimates for **NF1** or **IRF8** mRNA was done with the Ct values for **PGK1** as reference (Ct mean gene of interest – Ct mean **PGK1**).

**Cell fractionation of AML cases with **NF1** mutations for **NF1** sequence analysis**

AML cells were stained with anti-CD34 and CD38, and sorted into CD34+CD38+, CD34–CD38–, and CD34–CD38+ subsets using FACSAria (Becton Dickinson).

**Ras activity measurements in AML blasts**

Analysis of Ras activation was done using a Ras Activation Assay kit from Millipore according to the manufacturer’s instructions. Briefly, two million leukemia blast cells from selected patients (see Supplementary Table S2) isolated using bead-based depletion of nonblasts, as
above, were lysed in 500 µL of lysis buffer that was included in the Ras activity assay kit. The lysates were cleared through centrifugation and subsequently incubated at 4°C overnight with glutathione agarose beads bound to a glutathione S-transferase fusion protein of the human Ras binding domain of Raf-1. Following washings with lysis buffer, the bound GTP-Ras was liberated through boiling in SDS-PAGE loading buffer and then resolved by SDS-PAGE. A pan-anti-Ras antibody able to recognize total H-, K-, and N-Ras was used to measure Ras protein amounts. A GTPγS-loaded cell supernatant was used as a positive assay control. The same amount of lysate for each sample before the bead pull-down was analyzed in parallel by immunoblotting for total Ras. Blots were subjected to densitometric quantitation using an Alpha Imager transilluminator (Cell Biosciences) and the SpotDenso feature of this company's AlphaEase FC software. An image of each blot film was captured using identical camera exposure settings for all films. For each blot image, identically sized density measurement rectangles were placed around each protein band of interest, and local background was subtracted for each individual rectangle. Resulting band density values were imported into Microsoft Excel. For each sample, the protein band density values for GTP-Ras and total Ras were divided by the respective values for GTP-Ras and total Ras in one selected sample (indexed as 100%) as indicated in Fig. 3A and B.

**NF1 short hairpin RNA–mediated interference and blast colony formation assays**

The lentiviral vector plent6/V5-TOPO, obtained from Invitrogen, was modified to express either two scrambled short hairpin RNAs (shRNA; Sr1: ggcgttggctcgtttagta, and Sr2: aaataatagcctggaaaagg [ref. 24]), according to our published procedure (25).

Ficoll-prepared cells from bone marrow or peripheral blood from five AML patients (blast percentage range, 40-90%; see Supplementary Table S2) that were all wild-type for NF1 were spin inoculated with recombinant lentiviruses and green fluorescent protein–positive cells purified through FACS ~36 hours postinfection. RNA was prepared, and NF1 and PGK1 mRNA were quantified using Q-PCR as above.

**Rapamycin treatment and AML blast apoptosis assays**

Bead-purified blasts (2.5 × 10⁶) were incubated in 100 µL RPMI with 10% FCS and analyzed by Annexin V– and PI–based flow cytometry after 24 hours. Cells were incubated in DMSO only or with 20 nmol/L rapamycin (LC Laboratories). The fractions of cells still viable after 24 hours (Annexin V negative, PI negative) were normalized to the nontreated percent viable cell fraction for each patient, and results were plotted.

**Ex vivo colony growth assays of purified CD34+/CD38− bone marrow cells in the presence of rapamycin**

Ficoll gradient–prepared, cryopreserved AML marrows (see Supplementary Table S3) were thawed, washed, blocked, and stained with anti–CD34-phycoerythrin and anti–CD38-APC (both from eBioscience). The CD34+/CD38− fraction of each marrow was sorted on a FACSARia flow sorter (Becton Dickinson). Purified cells were introduced, in duplicate, into semisolid methylcellulose medium supplemented with multiple cytokines (Methocult H4535, StemCell Technologies) at concentrations of 1,000 and 5,000 cells/mL and using rapamycin (LC Laboratories) concentrations from 0 to 100 nmol/L. Compact colonies were counted in duplicate at 7 days.

**Ex vivo colony growth assays of AML marrow or blood cells**

Ficoll gradient–prepared, cryopreserved AML marrows with >50% blasts (see Supplementary Table S4) were thawed, washed, and introduced directly, in duplicate, into methylcellulose medium (Methocult H4230, StemCell Technologies) containing either a range of GM-CSF from 0 to 1 ng/mL only, or containing both GM-CSF and a constant 50 ng/mL of SCF (#215-GM and 255-SC, respectively, both from R&D Systems). Final concentration of cells in methylcellulose was 20,000/mL. Colonies were counted at 7 days. Marrows were prescreened whenever possible to exclude cases with prevalent FLT3 ITD mutations or lack of IRF-8 expression to minimize confounding effects of these genes on the assay (26).

**Results**

**Patient characteristics**

Included in this analysis are data from 95 patients, of which 76 patients were previously nontreated and 19 patients had relapsed AML. Sixty nine percent, 13%, and 18% of cases were primary AML, secondary AML, or treatment-related AML, respectively. Data are summarized in Table 1.

**SNP 6.0 array–based data analysis**

In support of the analyses outlined below, we developed and refined previously validated software tools that
can be used for display of data resulting from SNP 6.0 array profiling. These are the pre-LOH Unification Tool, the LOHtool version 2, and an updated version of dChipSNP (18, 21, 27). Lesion calling relied on visual inspection of copy number and LOH displays for paired tumor and buccal DNA samples within dChipSNP, a conservative and very specific method of lesion detection (see Materials and Methods). To obtain additional experimental verification that this approach would reliably detect single copy number changes, we initially measured genomic copy number estimates in 10 cases with heterozygous NF1 deletions (see below) and 10 NF1 undeleted cases using a Taqman-based Q-PCR probe for NF1 (located in intron 1 at physical position 26,447,140, based on the National Center for Biotechnology Information build 36) compared with a reference gene (RAG2). Data are summarized in Supplementary Table S5, showing excellent agreement (~1.8-fold mean copy number differences between deleted and undeleted samples) with SNP array–based genomic copy number estimates (case #76 displayed a 17q deletion that may not have included the NF1-cn probe location, and case #147 displayed a heterozygous deletion of RAG2 located on chromosome 11 at approximately 36.57-36.576 Mb).

We also used FISH with probes located within NF1 and a reference probe (see methods) on seven cases with available material for study [cases # 7, 45, 87, 140, 147, 157, and 132 (NF1 gain)] and, for each case, confirmed the SNP 6.0 array findings (Supplementary Table S6A and B). Finally, the complete list of acquired chromosomal copy number changes and acquired UPD for the 92 samples subjected to paired analysis can be found in Supplementary Table S7.

**Detection of microdeletions spanning the NF1 tumor suppressor gene on 17q in AML**

Copy number analysis for chromosome 17p and 17q detected large deletions and microdeletions in genomic regions where deletions of various lengths have previously been described (8, 9, 11) and two minimal regions of deletion (MDR) could be deduced (Supplementary Table S8; Fig. 1A and B). These are 17q-MDR1 (spanning ~0.3 Mb), which contained five genes as tabulated in Fig. 1D, and 17q-MDR2 (spanning ~0.9 Mb), which contained 11 genes as tabulated in Fig. 1D. 17q-MDR2 was present in 10 of 95 (11%) cases and invariably included the NF1 gene. An additional case (#132) had a gain of NF1 as part of a larger gain on 17p (see Fig. 1A-B). All cases of LOH at 17q-MDR2 (Fig. 1C) were associated with copy loss; thus, copy neutral LOH (acquired UPD) at the NF1 locus did not exist in this AML cohort. Review of cytogenetics data for the AML cases with microdeletions at 17q (#7, 45, 87, 140, and 157) showed that only one case was detected using routine karyotyping.

Given that NF1 is a known tumor suppressor in humans and a negative regulator of Ras signaling, and given that mutations in NF1 have been described in sporadic cases of pediatric leukemias [T-cell acute lymphoblastic leukemia (T-ALL) and one confirmed case of AML] and frequently in cases of juvenile myelomonocytic leukemia, we proceeded to sequence all 58 coding exons of NF1 in all 11 affected AML cases using direct exon resequencing (28–31). These efforts uncovered a 7-bp insertion in NF1 in exon 41 in case #7 (heterozygous NF1 deletion) resulting in a truncated NF1 protein (NF1 δ amino acid 2,058-2,818) and a heterozygous frame-shift mutation in NF1 in exon 42 in case #132 (a case with three NF1

| Table 1. Baseline characteristics of patients analyzed in this study |
|-----------------------|-----------------|-----------------|
| **Characteristic**    | Treatment naïve at enrollment, no. (%) | Previously treated and relapsed at enrollment, no. (%) |
| Sample size (95 total) | 76 (79) | 19 (21) |
| Age, y                | 64 | 65 |
| Range                 | 20-85 | 21-79 |
| Sex                   | Male | 45 (59) | 11 (58) |
|                       | Female | 31 (41) | 8 (42) |
| FAB classification*   | M0 | 10 (16) | 0 (0) |
|                       | M1 | 13 (20) | 3 (20) |
|                       | M2 | 12 (19) | 3 (20) |
|                       | M4 | 24 (37) | 6 (40) |
|                       | M5 | 5 (8) | 3 (20) |
|                       | M6 | 0 (0) | 0 (0) |
|                       | M7 | 0 (0) | 0 (0) |
| Immunophenotype       | CD13 positive | 67 (88) | 16 (84) |
|                       | CD33 positive | 66 (87) | 19 (100) |
|                       | CD34 positive | 60 (79) | 10 (53) |
|                       | CD117 positive | 70 (92) | 17 (89) |
| Cytogenetic class†    | Favorable | 6 (8) | 0 (0) |
|                       | Intermediate | 38 (51) | 16 (84) |
|                       | Unfavorable | 31 (41) | 3 (16) |
| No. of karyotypic abnormalities | Three or more | 16 (20) | 1 (5) |
|                       | Less than three | 59 (80) | 18 (95) |
| Pathogenesis          | De novo | 51 (67) | 15 (78) |
|                       | Prior | 10 (13) | 2 (11) |
|                      | myelodysplasia | 15 (20) | 2 (11) |


*The FAB subclassification was unspecified in 16 samples. The M3 subtype was not included in this study.

†One sample had no cytogenetic data available.
Fig. 1. A and B, fine mapping of minimal deleted regions on chromosome 17q in AML uncovers frequent NF1 deletions (heat map display). A, copy number heat map display for a region on chromosome 17p and 17q for DNA from AML-derived blasts and paired buccal DNA based on SNP 6.0 array profiling. The approximate location of p53 and NF1 is indicated. B, copy number display for a region on chromosome 17q for DNA from AML-derived blasts and paired buccal DNA from AML patients. Each column represents one patient. Minimal deleted regions and boundaries are marked. C, LOH display for AML blasts versus buccal DNA for the genomic region corresponding to B. D, genes located within 17q-MDR 1 and 2.
copies), resulting in a truncated NF1 molecule (NF1 δ amino acid 2,137-2,818, based on NM_000267.2) encoded by the mutated alleles (Fig. 2B and C). Confirmatory sequencing of paired buccal DNA confirmed the somatic nature of these mutations (Fig. 2B and C).

Given the absence of mutations in NF1 in the majority of AML cases with one retained NF1 copy, we proceeded with measurements of NF1 expression in all AML blast specimens for which sufficient intact RNA was available (Fig. 2A). Interestingly, this analysis disclosed three AML cases with heterozygous NF1 deletions (including case # 7) and three AML cases without NF1 deletions with absent NF1 expression and thus complete loss of NF1 activity (in addition to cases with low relative NF1 expression) and, further, reduced mean NF1 expression in the remaining AML cases with heterozygous NF1 deletion (n = 7) compared with all AML cases without NF1 deletion (n = 77; δ CT mean NF1-PGK1 of 6.3 versus 5.0 in heterozygously deleted NF1 versus undelthed NF1 blasts; P < 0.001). A summary of clinical and genomic characteristics of the AML cases with NF1 null states and the cases with heterozygous NF1 deletion but preserved NF1 expression from the retained allele can be found in Table 2.

To address the question of the presence of NF1 mutations in leukemia progenitor cells, we repeated sorts for AML-derived bone marrow cells from the two cases with NF1 mutations using CD34 and CD38 as markers and analyzed the resulting CD34+/CD38− cell populations for NF1 mutations (Fig. 2B and C). As can be seen in Fig. 2B and C, NF1 mutations were found in the CD34+/CD38− cell population known to harbor the leukemia initiating cells.

**NF1 regulates the activation state of Ras in primary AML blasts and NF1 downregulation increases blast colony formation in methylcellulose**

Given that NF1 regulates the activation states of Ras, we measured GTP-bound Ras by immunoblotting in
bead-purified AML blasts with various NF1 and Ras states and wild-type Flt3 status (Fig. 3A), and normalized data based on the signals detected in Ras mutant blasts (#57). As can be seen in Fig. 3A, GTP-Ras was substantially increased in AML cases with absent NF1 expression and in cases with activating N-Ras or K-Ras mutations, respectively, if compared with blasts that were wild-type for NF1 and Ras (all cases were wild-type for Flt3). Thus, NF1 deficiency results in an increase in Ras activity in affected AML cases. Next, we proceeded to estimate the effect of heterozygous NF1 deletions but preserved NF1 mRNA expression from the retained NF1 allele on GTP-bound Ras. As can be seen in Fig. 3B, whereas NF1 null blasts again showed an approximately fivefold increase in GTP-Ras compared with blasts that were wild-type for NF1 and Ras (based on newly bead-purified AML blasts from cases #9 and 110), the effect of heterozygous NF1 deletions on the amount of GTP-Ras was not substantially different from NF1 nonaberrant blasts in three of the four cases tested, with only one case (#45) showing a mild increase. Thus, within the limits of these measurements (all done in bead-purified primary human leukemia blasts), one can conclude that complete NF1 loss is required to result in substantial Ras activation and that heterozygous NF1 states that preserve some NF1 expression are not sufficient for robust Ras activation.

Next, we asked whether NF1 deficiency had an effect on blast colony formation as a readout for pro-proliferative activities of lower NF1 levels. Aliquots of blasts from five AML cases with wild-type Ras and wild-type Flt3 were infected with lentiviruses carrying transcriptional units for validated NF1suppressing shRNA or scrambled shRNAs.

### Table 2. Clinical and genomic characteristics of AML patients with NF1 null states due to mutations or absent expression and of AML patients with heterozygous NF1 deletions and retained NF1 expression

<table>
<thead>
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<th>AML case no.</th>
<th>NF1 status (mutations/ expression)</th>
<th>NF1 status</th>
<th>Age</th>
<th>Disease status</th>
<th>AML type</th>
<th>FAB type</th>
<th>Cytogenetics</th>
<th>p53 status</th>
<th>Flt3 status</th>
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Abbeviation: Wt, wild-type.
Compact colony numbers were scored after 7 days in culture. As can be seen in Fig. 3C, four of five AML cases showed a substantial and significant increase in AML blast colony formation in methylcellulose when NF1 was downregulated, whereas the remaining AML case showed already relatively elevated colony formation propensities at baseline (and was characterized by the lowest NF1 mRNA expression at baseline compared with the other studied cases). Thus, NF1 directly influences AML blast proliferation/growth (see also Fig. 4B). Finally, given that baseline colony numbers were not influenced by the cell source (bone marrow versus peripheral blood), that the colonies did not have the appearance of blast-forming unit (erythroid), and given that colony-forming unit numbers in nonmalignant blood are substantially lower than the colony numbers observed here, we conclude that the majority of colonies are AML blast derived (32).

**Colony formation of AML blasts in methylcellulose supplemented with escalating doses of GM-CSF with or without SCF**

Hypersensitivity of myeloid cells derived from patients with juvenile myelomonocytic leukemia has been described, and murine cells with engineered complete NF1 inactivation are hypersensitive to GM-CSF (30, 33, 34). Therefore, we measured colony formation of
AML blasts from NF1 null cases \( (n = 4) \), heterozygous (1N) NF1 cases with preserved NF1 expression from the retained allele \( (n = 3) \), and NF1 nonaberrant cases \( (n = 8) \) in methylcellulose only supplemented with either escalating doses of GM-CSF, or escalating doses of GM-CSF and a fixed dose of SCF. As can be seen in Supplementary Figs. S1 and S2, most of the cases with the ability to form colonies under these conditions displayed some sensitivity to GM-CSF, although one tested NF1 null case and one 1N NF1 case with preserved NF1 mRNA expression from the retained allele appeared particularly sensitive.

**NF1 null AML-derived blasts are sensitive to rapamycin-induced apoptosis**

An additional important function of NF1 as a negative regulator of mTOR signaling has been described (24). Based on this observation, we reasoned that AML blasts that may have arisen in the setting of NF1 deficiency would be more dependent on mTOR signaling for survival and growth than NF1 wild-type blasts. We therefore treated purified primary AML blasts with rapamycin and measured apoptosis induction after overnight cultures. Data are summarized in Fig. 4A. Importantly, AML blasts without functional NF1 \( (n = 5) \) were substantially and...
significantly ($P < 0.001$) more sensitive to mTOR inhibition than NF1 wild-type blasts ($n = 12$) or blasts with one preserved NF1 copy ($n = 6$) and retained NF1 expression.

**Ex vivo colony formation of NF1 null AML-derived CD34+/CD38− cells is inhibited by rapamycin**

Next, we asked whether the cellular compartment that harbors the leukemia-initiating cells in AML (CD34+/CD38− cells) would be sensitive to rapamycin treatment. CD34+/CD38− cells from AML bone marrow samples that were either NF1 null or had lost one NF1 gene copy but displayed NF1 expression from the retained allele, or were 2N at the NF1 locus and expressed NF1 (nonaberrant cases) were sorted using FACS, and cells subsequently plated in supplemented methylcellulose medium in the presence of varying concentrations of rapamycin (0-100 nmol/L). Colony formation was assessed after 7 days in culture. As can be seen in Fig. 4B, colony formation in the absence of rapamycin treatment (nontreated samples) was robust in three of four cases each that were either NF1 null (red lines) or 1N NF1 with retained NF1 expression (blue lines), whereas only two of seven NF1 nonaberrant cases displayed appreciable colony formation, suggesting that lower or absent NF1 expression facilitates ex vivo growth of affected cells (and complementing the experimental observations displayed in Fig. 3C). Furthermore, cells from all informative NF1 null cases or 1N NF1 cases with retained NF1 expression and, to a lesser degree, cells from the two informative NF1 nonaberrant cases displayed sensitivity to low-dose rapamycin ex vivo. Thus, in summary, a fraction of blast cells and leukemia-initiating cells from AML patients with NF1 null states are growth inhibited or killed by low-dose rapamycin.

**Discussion**

In this study, we have used ultra-high-density SNP arrays to interrogate the genomes of highly purified leukemic blasts and paired buccal DNA from 95 AML patients for chromosomal copy number changes. Through comparison of blast-derived and paired buccal DNA in this analysis, we were able to unequivocally identify small somatically acquired losses at unprecedented resolution. Focusing on small deletions (submegabase to a few megabases) that occurred recurrently or that occurred at positions that are spanned by recurrent larger deletions, we have identified a minimal deleted region of ~0.9 Mb on chromosome 17q that spans NF1, a disease-causing gene in neurofibromatosis. NF1 copy number alterations were found in 11 of 95 (12%) patients in this cohort (10 cases with heterozygous NF1 deletions and 1 case with a large chromosomal gain that harbored mutated NF1 alleles) and in 6 of 86 patients recently described by Walter et al. (29). Sequence-based analysis identified two AML cases with mutated acquired NF1 alleles that predicted for truncated NF1 proteins. As these NF1 mutations were also identified in CD34+/CD38− cell populations, we can presume that NF1 mutations contribute to the biological phenotype of leukemia-initiating cells, possibly through control of proliferation. In this context, it seems important that AML blasts with shRNA-mediated NF1 knockdown showed improved growth and colony formation ex vivo and that most NF1 null AML-derived CD34+/CD38− cells grew robustly in cytokine-supplemented methylcellulose.

Although sequencing of all coding exons of NF1 in nine additional AML cases with heterozygous NF1 deletions did not reveal inactivating gene mutations, expression analysis of NF1 in a large collection of AML cases showed lower expression in cases with heterozygous NF1 deletions as opposed to wild-type NF1 cases. The accuracy of this expression analysis is further strengthened by the fact that we used FACS-sorted AML blasts as a source of RNA, effectively eliminating contributions of nonblast cells to the NF1 mRNA quantitations. Lower NF1 mRNA expression may contribute to AML pathogenesis, given prior evidence for NF1 haploinsufficiency in some biological systems (35–37). Our study of AML cases with heterozygous NF1 deletions and preserved NF1 expression from the retained allele uncovered some evidence for haploinsufficiency only in the setting of ex vivo blast colony formation. The activity of Ras was not substantially influenced in blasts with heterozygous NF1 deletions that preserved NF1 expression from the retained allele. Importantly, six AML cases (three cases with heterozygous NF1 deletions and three cases without NF1 deletions) did not express NF1 mRNA, thus identifying a subset of adult AML with NF1 null states. Where measured, NF1 null states were associated with substantially (~5-fold) increased Ras activity. Identification of AML cases with Ras activation due to complete NF1 inactivation may be of clinical importance, given (a) findings of increased sensitivity of AML blasts from such patients to Cytarabine (1-β-D-arabinofuranosylcytosine; refs. 38, 39) and (b) the potential for identifying additional subsets of AML for Ras-directed therapeutics.

In this study, we have identified for the first time a sensitivity of NF1 null AML blasts to rapamycin-induced apoptosis. Blasts with preserved NF1 expression independent of NF1 copy number status did not undergo rapamycin-induced apoptosis, in agreement with prior observations (40). Subsequent analysis of the ex vivo colony formation potential of CD34+/CD38− cells isolated from AML cases with various NF1 states uncovered an inhibitory effect of low-dose rapamycin on NF1 null blasts and on blasts with heterozygous NF1 deletion, and preserved expression from the retained allele, although some of the informative NF1 nonaberrant cases were also inhibited (41). Together, these findings of sensitivity of NF1 null AML blasts to rapamycin extends observations of regulation of mTOR by NF1 to leukemia and opens possibilities for patient selection for clinical mTOR inhibitor applications based on NF1 status (42–44).

NF1 mutations have long been implicated in the pathogenesis of juvenile myelomonocytic leukemia, but evidence for a role of NF1 in adult AML has been sporadic. Using a very large collection of >600 cases of hematologic malignancies and focusing on a subset of 34 cases with...
myelodysplastic syndrome (MDS)/AML and AML/RUNX1 mutations. Niimi et al. (45) identified one case of high-grade MDS and one case of secondary AML following MDS with NF1 sequence changes predicting for truncated NF1 alleles, although evidence that these changes were acquired in tumor cells was not provided. Recently, Balgobind et al. (28) examined 103 pediatric T-ALL cases and 71 pediatric AML cases with mixed lineage leukemia (MLL) rearrangements and identified two AML cases with 17q microdeletions and NF1 mutations, of which one was confirmed to be acquired in tumor cells. An expression analysis of NF1 in this AML cohort was not reported. In contrast with these two reports, the seven AML cases in this cohort that were NF1 null did not have MLL rearrangements, and all were primary AML.

In summary, this study advances our knowledge of the incidence of NF1 aberrations and NF1 null states in AML, and provides experimental evidence that NF1 null states are functionally relevant in AML. Finally, we show that AML cases with absent NF1 function are sensitive to mTOR inhibition, thus providing a rationale for the study of mTOR inhibitors in NF1 null AML subsets.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Brian Parkin, Peter Ouillette, Yin Wang, et al.

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