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**Genomic Profiling and Directed Ex Vivo Drug Analysis of an Unclassifiable
Myelodysplastic/Myeloproliferative Neoplasm Progressing into Acute Myeloid
Leukemia**

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RUNNING HEAD: Genetic profiling of MDS/MPN-U

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Myelodysplastic/myeloproliferative neoplasms, unclassifiable (MDS/MPN-U) are rare genetically heterogeneous hematologic diseases associated with older age and a poor prognosis. If the disease progresses into acute myeloid leukemia (AML), it is often refractory to treatment. To gain insight into genetic alterations associated with disease progression, we used whole exome sequencing and single nucleotide polymorphism arrays to characterize the bone marrow and blood samples from a 39-year-old woman at MDS/MPN-U diagnosis and at AML progression, in which routine genetic diagnostics had not identified any genetic alterations. Our data revealed the presence of a partial tandem duplication of the *MLL* gene as the only detectable copy number change and 11 non-silent somatic mutations, including *DNMT3A* R882H and *NRAS* G13D. All somatic lesions were present both at initial MDS/MPN-U diagnosis and at AML presentation at similar mutant allele frequencies. The patient has since had two extramedullary relapses and is at high risk of a future bone marrow relapse. A directed ex vivo drug sensitivity analysis showed that the patient's AML cells are sensitive to, e.g., the MEK inhibitor trametinib and the proteasome inhibitor bortezomib, indicating that she may benefit from treatment with these drugs.

INTRODUCTION

Myelodysplastic/myeloproliferative neoplasms, unclassifiable (MDS/MPN-U) are rare heterogeneous diseases associated with older age (≥ 60 years), an inferior overall survival of 12.4-21.8 months, and progression to acute myeloid leukemia (AML) in 23-54% of the cases (Cannella et al., 2008; DiNardo et al., 2014; Wang et al., 2014). MDS/MPN-U belongs to the MDS/MPN World Health Organization category, a group of myeloid neoplasms that have both myelodysplastic and myeloproliferative features at diagnosis. This category also comprises atypical chronic myeloid leukemia, chronic myelomonocytic leukemia, juvenile myelomonocytic leukemia, and refractory anemia with ring sideroblasts and thrombocytosis (Vardiman et al., 2009). Of these, MDS/MPN-U is the least well characterized group and no specific genetic alterations have been found; instead they share many alterations with myeloid disorders, including isolated trisomy 8 and recurrent mutations in, e.g., *DNMT3A*, *JAK2*, *KRAS/NRAS*, *RUNX1*, and *SETBP1* (Ernst et al., 2010; Meggendorfer et al., 2013; DiNardo et al., 2014; Wang et al., 2014). Recently, mutations in *ASXL1*, *TET2*, and *U2AF1* were shown to occur at high frequencies in MDS/MPN-U, with mutations in *U2AF1* being enriched in this subtype (Meggendorfer et al., 2014).

Few studies are available where genetic alterations occurring during the progression of MDS/MPN-U into AML have been studied (Hahm et al., 2015). Herein, we describe a unique case of an MDS/MPN-U, in a 39-year-old woman, that after two months of diagnosis progressed into AML. Using whole exome sequencing and high-resolution single nucleotide polymorphism (SNP) arrays, we identified a partial tandem duplication of the *MLL* gene (also called *KMT2A*) as the only copy number alteration and the presence of 11 validated somatic non-silent mutations, including *DNMT3A* R882H and *NRAS* G13D, all of which were present both at initial MDS/MPN-U diagnosis as well as at AML presentation. Importantly,

we further show that the leukemic cells upon progression into AML were responsive *ex vivo* to, e.g., the MEK inhibitor tramatenib and the proteasome inhibitor bortezomib.

MATERIALS AND METHODS

Patient History

A 39-year-old woman presented with an atypical bone marrow disease with pronounced leukocytosis, neutrophilia, monocytosis, considerable cervical lymphadenopathy, and enlarged submandibular glands. A lymph node biopsy revealed a disrupted architecture with confluent abnormal sheets of highly proliferating left-shifted myeloid cells including dysplastic granulocytes and monocytoid cells (Supplementary Figs. S1A and B and S2) and a low frequency of myeloid blasts, promonocytes, and monocytes (2%, 1%, and 4%, respectively, Supplementary Fig. S3A). The tumor masses were evaluated by a pathologist and determined to be an MDS/MPN-U. Analyses of the bone marrow recapitulated the morphological and immunophenotypic findings of the lymph node, but with a lower frequency of abnormal myeloid blast cells (0.1%; Supplementary Figs. S1C and S3B). Clinical genetic diagnostics revealed a normal female karyotype and lack of *BCR-ABL1*, *FIP1L1-PDGFRA*, and *JAK2* mutations.

The MDS/MPN-U progressed to AML two months after initial diagnosis, with elevated numbers of abnormal promonocytes and monocytes (19% and 23%, respectively; Supplementary Fig. S3C). Except for the loss of CD56 on the promonocyte-like cells in the AML specimen (data not shown), the phenotype of the AML cells was similar to that of the MDS/MPN-U cells (Supplementary Figs. S1D and S3A-C). An allogeneic bone marrow transplantation was performed in first complete remission, but the patient has since had two

extramedullary relapses with infiltrating immature myeloid cells (Supplementary Fig. S4 and Supporting Information for full patient history).

Patient Material

Bone marrow (BM) was collected at the time of presentation, and three months later when the patient had developed AML, a peripheral blood (PB) sample was collected. As constitutional DNA (germline control) for the genomic analyses, a skin biopsy was taken at first complete remission.

DNA Extraction

Mononucleated cells (MNCs) were purified from PB and BM with lymphoprep (AXIS-SHIELD, Oslo, Norway), and DNA and RNA were extracted. DNA from BM, PB, and cultured cells from the skin sample was extracted with the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). Total RNA was extracted from BM and PB with the Trizol Reagent (Life Technologies Corp., Carlsbad, CA). The DNA quantity was assessed with the Qubit Fluorometer (Life Technologies, Paisley, UK). The RNA quantity and quality was assessed by the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA) and the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA) respectively.

Histopathology and Immunohistochemistry

Embedment, sectioning, hematoxylin–eosin staining, and immunohistochemistry of biopsies were performed according to standard clinical protocols. The Ki67 antibody (Dakos, Stockholm, Sweden) was used to estimate proliferation.

Immunophenotypic Analysis by Flow Cytometry

Sample preparation and staining were performed according to standard clinical protocols. Antibodies were purchased from BD (Becton, Dickinson and Company, Franklin Lakes, NJ). Samples were analyzed on a BC Navios (Beckman Coulter, Miami, FL).

High Resolution Genomic Profiling Using SNP Array

The SNP array analysis was performed using the 2.7M. CytoScan HD Array (Affymetrix, Santa Clara, CA) and the CytoScan assay protocol (Affymetrix). Briefly, 250 ng of DNA was fragmented into 150-2000 bp, ligated with adaptors, and PCR-amplified using the Titanium DNA Amplification Kit (Clontech Laboratories Inc., Mountain View, CA), followed by a second fragmentation into 25-125 bp; the DNA was then labeled and hybridized onto the 2.7M. CytoScan HD Array overnight using the Hybridization Oven 645 (Affymetrix). The arrays were next washed and stained in the Fluidics Station 450 (Affymetrix) and scanned in the GeneChip Scanner 3000 7G (Affymetrix). The SNP data were analyzed with the Chromosome Analysis Suite (ChAS; Affymetrix) and annotated based on Hg19. The number of markers required for calling an amplification or deletion was set to 25 and all called variants were then manually inspected.

Exome Capture and Sequencing

Exome capture and sequencing of the MDS/MPN-U, AML, and germline samples were performed by Ambry Genetics (Aliso Viejo, CA). In brief, whole exome target enrichment was performed using the VCRome kit (Roche NimbleGen, Madison, WI). Library preparation was prepared using kits and reagents from Illumina (Illumina, San Diego, CA), and 100 bp paired end sequencing was performed using the Illumina HiSeq platform.

Validation of the *MLL*-PTD

The *MLL*-PTD was validated by RT-PCR using the primer combinations 5.3+3.1c/654c and 5.3+4.1R as described (Caligiuri et al., 1996). Briefly, 2.5 µg RNA was converted into cDNA using standard protocols. The RT-PCR reaction was performed using the Platinum Taq DNA Polymerase kit (Life Technologies) according to the manufacturer's instructions using the following primers: 5.3 (5'-GGAAGTCAAGCAAGCAGGTC-3'), 3.1c/654c (5'-AGGAGAGAGTTTACCTGCTC-3'), 4.1R (5'-GCCTTGTTTCTAGTGACAGG-3') for 35 cycles (95°C, 1 min; 53°C, 1 min; 72°C, 1 min) (Caligiuri et al., 1996). Products were analyzed by agarose gel electrophoresis, purified using the QIAquick Gel Extraction Kit (Qiagen), followed by Sanger sequencing using the BigDye Terminator v1.1 Cycle Sequencing Kit (Life Technologies). Sequencing products were analyzed on the ABI 3130 Genetic Analyzer (Life Technologies; Applied Biosystems). Sequencing primers used: 5.3, 3.1c/654c and 4.1R (see above), 6.1 (5'-GTCCAGAGCAGAGCAAACAG-3'), 3.2c/400c (5'-ACACAGATGGATCTGAGAGG-3'), and 4.2R (5'-GGAGCAAGAGGTTTCAGCATC-3') (Caligiuri et al., 1996).

Validation of Somatic Mutations

For validation, putative non-silent mutations had to fulfill either of the following criteria; the putative mutation was present in both the MDS/MPN-U and the AML sample regardless of mutant allele frequency, or the gene was listed in the Cancer Gene Consensus from COSMIC (https://cancer.sanger.ac.uk/files/cosmic/current_release/cancer_gene_consensus.csv) with a mutant allele frequency of $\geq 5\%$, or the putative mutation had a mutant allele frequency $\geq 20\%$ in either of the samples. This resulted in 58 putative non-silent mutations fulfilling at least one of the above criteria. Validation was performed on the MDS/MPN-U, AML, and germline

samples by PCR amplification of all 58 putative mutations, followed by adaptor ligation and deep sequencing using the Nextera XT DNA Sample Preparation Kit and NextSeq 500 System (Illumina). Primers were designed using Primer 3 (<http://frodo.wi.mit.edu/primer3/>); sequences are available upon request. In brief, 10 ng of DNA was amplified by PCR using the Platinum Taq DNA Polymerase kit (Life Technologies), 10 mM dNTP (GE Healthcare, Pollards Wood, UK), and primers (final conc. of 0.5 μ M) for 30 cycles (94-95°C 0.5-1 min, 57-66°C 0.5-1 min, 72°C 0.5-2 min). Fragment sizes and approximate concentration, as compared with Lambda Hind III (Life Technologies), of PCR products were determined by agarose gel electrophoresis. The PCR products were purified using AMPure XP beads (Beckman Coulter Inc., Brea, CA) and prepared for sequencing using the Nextera XT DNA Sample Preparation Kit and Index Kit (Illumina). Briefly, the input amplicons were fragmented to approximately 300 bp and adapter sequences were added simultaneously using the Nextera XT transposome. Amplicons were then indexed and amplified using PCR and subsequently purified using Ampure XP beads. Sample libraries were pooled and sequencing was prepared according to the MiSeq protocol as described by the manufacturer. In addition, a selected set of 16 putative variants with a mutant allele frequency of $\geq 20\%$ from above was subjected to Sanger sequencing (GATC Biotech AG, Konstanz, Germany).

Sequencing Data Analysis

Paired end reads were aligned to UCSC build Hg19 using the BWA (version 0.6.2) aligner (Li and Durbin, 2009), and Picard (version 1.80) (<http://picard.sourceforge.net>) was used to merge files and deduplicate lanes. GATK (version 2.2.8) was used for quality recalibration and local realignment around insertion deletions (indels) (McKenna et al., 2010; DePristo et al., 2011). Putative sequence variants, single nucleotide variants (SNV), and indels were identified using Strelka (Saunders et al., 2012) and annotated using Annovar

(Wang et al., 2010). Presence of reads in the germline sample that cover the mutant allele is considered a germline variant. The position of a putative mutation is based on Hg19 and the effect at the protein level of a putative mutation was investigated using SIFT or Polyphen (Ng and Henikoff, 2003; Adzhubei et al., 2010).

Cell Culture

Viably frozen PB MNCs collected at AML diagnosis were thawed in IMDM (Sigma-Aldrich, Stockholm, Sweden) containing fetal bovine serum (FBS, Thermo Scientific, South Logan, UT) and DNase I (Sigma-Aldrich) and cells were finally resuspended in AML optimized cell culture medium consisting of IMDM supplemented with 15 % BIT 9500 Serum Substitute (StemCell Technologies, Cambridge, United Kingdom), 100 ng/ml SCF, 50 ng/ml FLT3-L, 20 ng/ml IL3, 20 ng/ml Granulocyte Colony-Stimulating Factor (G-CSF, all cytokines were purchased from Preprotech, Rocky Hill, NJ), 100 μ M β -mercaptoethanol (Sigma-Aldrich), 500 nM StemRegenin (StemCell Technologies), 500 nM UM729 (StemCell Technologies), 100 units/ml Penicillin and 100 g/ml Streptomycin (Thermo Scientific) (Pabst et al., 2014). Primary AML cells were plated in tissue culture-treated 96-well plates at a density of 0.5×10^5 cells / 50 μ l in each well. The cell lines KG-1 and THP-1 (DSMZ, Braunschweig, Germany) were also included and were cultured in RPMI-1640 medium supplemented with 10 % FBS (Thermo Scientific), 100 units/ml Penicillin and 100 g/ml Streptomycin (Thermo Scientific) and were plated in tissue culture-treated 96-well plates at a density of 0.2×10^5 cells / 50 μ l in each well.

Drug Sensitivity Assay

Drugs were dissolved and further diluted in dimethyl sulfoxide (DMSO, Millipore, Darmstadt, Germany) in eight 1:4 dilutions from 10 mM down to 0.6 μ M. Diluted

drugs were further diluted 1:500 in culture medium and 50 μ l were subsequently added to each well containing cells (0.5×10^5 cells / well for the primary AML sample and 0.2×10^5 cells / well for the cell lines KG-1 and THP-1) in 96-well plates, giving a final concentration of 0.1% DMSO and a corresponding drug concentration ranging between 10 μ M and 0.6 nM. Cell viability was determined after 6 days (Lavallée et al., 2015) by the luminescent cell viability assay CellTiter-Glo 2.0 (Promega, Madison, WI), according to the manufacture's protocol. Luminescence was recorded on the Veritas™ Microplate Luminometer (Turner Biosystems, Sunnyvale, CA). Cells treated only with 0.1% DMSO were used as negative control. Each drug concentration was performed in triplicates; negative controls were performed in quadruplicates. The percentage of inhibition was determined by the following formula: $100 - (100 \times (\text{luminescence (drug)} / \text{mean luminescence (negative control)}))$. Half maximal inhibitory concentration (IC₅₀) was calculated using GraphPad Prism 6.0h (La Jolla, CA). The following drugs were used: cytosine β -D-arabinofuranoside (Ara-C, Sigma-Aldrich), daunorubicin hydrochloride (Daunorubicin, Sigma-Aldrich), bortezomib (Santa Cruz Biotechnology, Dallas, TX), SAHA (Vorinostat, Sigma-Aldrich), 5-aza-2'-deoxycytidine (Decitabine, Sigma-Aldrich), and GSK1120212 (Trametinib, Adoq Biosciences, Irwin, CA).

RESULTS

Genomic Analyses at MDS/MPN-U Diagnosis and Progression to AML

To determine the copy number alterations present at AML presentation, high-resolution SNP array analysis was performed, which revealed the presence of an *MLL*-PTD as the only detectable genomic imbalance (Supplementary Fig. S5A). Validation of the rearrangement at the RNA level by PCR followed by Sanger sequencing revealed two variants corresponding to an in-frame duplication of *MLL* exons 2-8 and an out-of-frame

duplication of exons 5-8 and that the *MLL*-PTD was present already at diagnosis of MDS/MPN-U (Supplementary Fig. S5B-D).

The number of coding gene mutations at MDS/MPN-U and at AML presentation was determined by whole exome sequencing (Supplementary Table S1). DNA from cultured cells from a skin biopsy was used as constitutional DNA (referred to as germline sample). Eleven validated somatic non-silent mutations, including *DNMT3A* R882H and *NRAS* G13D (Supplementary Fig. S6A and B), were identified, all of which were present both at initial MDS/MPN-U diagnosis as well as at AML presentation (Table 1). The remaining mutations are not reported in COSMIC, but mutations at other sites in these genes have previously been seen in AML or in other cancer forms (Cancer Genome Atlas Research Network, 2013). The mutant allele frequencies of the identified mutations indicated that they were present in the dominant clone both at MDS/MPN-U diagnosis and at AML presentation (Table 1).

Directed Drug Assay Indicates Potent Response to MEK- and Proteasome Inhibition

We next investigated if we could utilize the genetic information obtained from the genome wide analyses to identify alternative treatment modalities for the patient using US Food and Drug administration (FDA) approved compounds. Six different drugs were chosen based on the literature and previous treatment of the patient; the chemotherapeutic drugs ara-C and daunorubicin, the proteasome inhibitor bortezomib, the histone deacetylase agent vorinostat, the hypomethylating drug decitabine, and the MEK inhibitor trametinib. We also included the AML cell lines THP-1 (expressing *MLL-MLLT3*) and KG-1 (expressing *FGFR1OP2-FGFR1*), both of which harbor mutant *NRAS* (Janssen et al., 1987).

The analysis revealed that the AML cells were highly sensitive towards the MEK inhibitor trametinib (Fig. 2F) and exhibited an IC₅₀ close to that of the *NRAS* mutant

cell line THP-1 (33 nM and 3 nM, respectively). In contrast, despite that KG-1 also has an *NRAS* mutation it was non-responsive towards trametenib. No marked difference in the sensitivity towards ara-C, daunorubicin, bortezomib, and vorinostat was seen among the samples (Fig. 2A-D). Finally, the AML cells were less sensitive towards decitabine as compared with KG-1 (IC₅₀ of 1984 nM and 81 nM, respectively).

DISCUSSION

MPD/MDS-U is a rare group of hematopoietic diseases with a poor prognosis that progress to AML in 23-54% (Cannella et al., 2008; DiNardo et al., 2014; Wang et al., 2014). To gain insight into the genetic changes associated with evolution to AML, we determined the coding genetic lesions of hematologic samples from a single patient at MPD/MDS-U diagnosis and at AML presentation. Our analyses identified an *MLL*-PTD as well as 11 additional non-silent mutations, including mutations in *NRAS* and *DNMT3A*. An *MLL*-PTD is present in approximately 8% of AML cases with a normal karyotype and is typically considered an adverse prognostic factor (Caligiuri et al., 1998; Döhner et al., 2002; Schlenk et al., 2008). However, younger patients with an *MLL*-PTD treated with an intensified consolidation therapy including autologous peripheral stem cell transplantation had similar outcomes to that of AML patients with normal karyotype lacking *MLL*-PTD, showing that intensified treatment may modulate the historically poor outcome of this leukemia subtype (Whitman et al., 2007).

Mutations in *DNMT3A* were first identified as part of a whole genome sequencing effort of an AML patient with a normal karyotype; mutations in this gene were highly recurrent in an extended cohort, in particular among patients with a cytogenetic profile associated with an intermediate risk (34%), including patients with a normal cytogenetics (37%) (Ley et al., 2010). Although mutations are found across the entire *DNMT3A* gene, the

R882 codon, identified also in our patient, is a known hot spot, with R822H being the most common substitution (Supplementary Fig. S6A). Several studies have shown that mutations in *DNMT3A* correlate with an inferior outcome (Ley et al., 2010; Thol et al., 2011; Yan et al., 2011); however, the largest study to date could not verify this; only among cytogenetically normal patients in the unfavorable subgroup, as defined by the European LeukemiaNet, had the presence of a R882 mutation a moderate negative prognostic effect (Gaidzik et al., 2013). *DNMT3A* mutations in secondary AML have been described to be present in the corresponding antecedent MDS or MPN, in a series of ten patients, suggestive of an early event (Fried et al., 2012). In addition to the *DNMT3A* mutation, our patient had an *NRAS* G13D; *NRAS* mutations occur in around 10-13% of patients with AML but do not confer any significant prognostic impact (Bacher et al., 2006; Schlenk et al., 2008).

All mutations identified at initial MDS/MPN-U diagnosis were present at AML presentation and no additional mutations were identified in the AML specimen. Furthermore, at the level of detection of our analysis, the mutant allele frequencies indicated that all mutations were present in the dominant leukemia clone both at initial MDS/MPN-U diagnosis as well as at AML presentation. Thus, it appears as if all of the mutations needed for AML were there, already two months before the AML diagnosis.

Despite a good initial response to chemotherapy, the patient experienced two extramedullary relapses with infiltrating immature myeloid cells (Supporting Information). To identify alternative treatment modalities that could be used at a future potential bone marrow relapse, we investigated the response of her AML cells ex vivo to a selected set of drugs. Because it is difficult to culture primary AML cells ex vivo without spontaneous differentiation (Mayani et al., 2009), we implemented the recently optimized culture conditions proposed to maintain AML self-renewal, mainly by antagonizing the aryl hydrocarbon receptor pathway (Pabst et al., 2014).

We found that that the patient's AML cells were sensitive towards inhibition of MEK ex vivo, suggesting a potential clinical benefit of this treatment. The AML cells had an IC50 for trametinib similar to that of *RAS* mutant primary *MLL*-rearranged AML samples (Lavallée et al., 2015). Likewise, the *MLL* positive *NRAS*-dependent cell line THP-1 (Burgess et al., 2014) proved highly sensitive, whereas KG-1 was non-responsive despite that fact that it harbors mutant *NRAS* (Long et al., 2014). Trametinib is a potent MEK inhibitor approved for *BRAF* mutant unresectable or metastatic melanoma, and has been proposed to have an increased efficacy in *NRAS* mutant AML (Burgess et al., 2014; Lavallée et al., 2015) and is in clinical trial for AML with mutant *NRAS* (clinicaltrials.gov identifier NCT01907815). Our data further demonstrate that the AML cells, as well as THP-1 and KG-1, were sensitive towards bortezomib (Horton et al., 2006; Oerlemans et al., 2008). Bortezomib in combination with vorinostat have shown high efficacy in primary infant *MLL*-rearranged acute lymphoblastic leukemia drug assays (Koss et al., 2014) and are in clinical trial as a combinatorial therapy for younger patients with leukemia (clinicaltrials.gov identifier NCT02419755). Similar to the ara-C resistant cell line THP-1 (Ge et al., 2004), the AML cells had relatively high IC50 for this drug. Finally, decitabine is approved for treatment of high-risk MDS (Im et al., 2014) and may improve the complete remission rates in AML (Metzeler et al., 2012) and MDS patients (Traina et al., 2014) with *DNMT3A* mutations. Notably, in our analyses, the AML sample was the least sensitive sample towards decitabine despite the fact that it harbors a *DNMT3A* mutation; the *DNMT3A* wild-type cell line KG-1 was more sensitive (Tiacchi et al., 2012).

Taken together, genomic characterization using high resolution SNP array profiling and whole exome sequencing of the leukemic specimens from a woman presenting with MDS/MPN-U revealed the presence of twelve genetic lesions including a partial tandem duplication of *MLL* and missense mutations in *DNMT3A* and *NRAS*. The results of the

extended genetic analysis performed as part of this study can be used to confirm and monitor a possible bone marrow relapse, which would be helpful to make a decision on the need of systemic therapy. Her disease course together with her high-risk genetic lesions suggests a poor prognosis and that she may benefit from alternative treatment modalities, potentially by targeting the RAS signaling pathway using, e.g., the MEK inhibitor trametinib or by proteasome inhibition through bortezomib.

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FIGURE LEGEND

Figure 1. Ex vivo drug sensitivity assay of the AML sample. Dose-response curves and half maximal inhibitory concentration (IC₅₀) for the primary AML sample (AML-D) and the AML cell lines THP-1 and KG-1 after 6 days of drug treatment revealed similar responses for all samples to (A) ara-C, (B) daunorubicin, (C) bortezomib, and (D) vorinostat. (E) Decitabine showed increased efficacy in KG-1 and (F) trametinib in THP-1 and the AML sample. Data presented as mean $\pm$ s.e.m.

TABLE 1. Validated Somatic Mutations in the Diagnostic MDS/MPD-U Specimen and in the AML Sample.

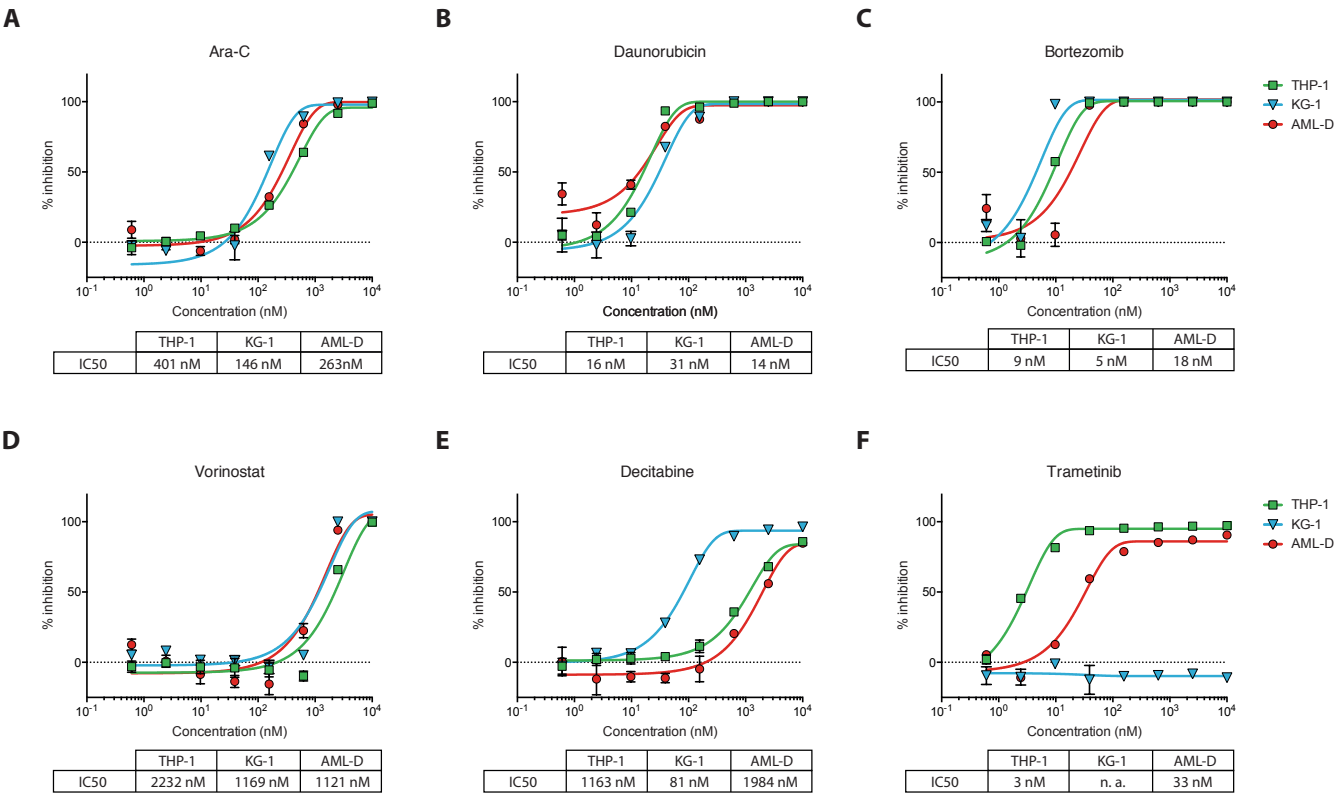
Gene symbol	RefSeq	Amino acid change	Chromosomal position ^a	Mutation type	Mutation present in Cosmic ^b	Mutant allele frequency MDS/MPN-U	Mutant allele frequency AML
<i>TTC39A</i>	NM_001080494	Q524fs	Chr1: 51754552	Framshift deletion	No	43%	37%
<i>NRAS</i>	NM_002524	G13D	Chr1: 115258744	Missense	Yes	43%	38%
<i>DNMT3A</i>	NM_022552	R882H	Chr2: 25457242	Missense	Yes	52%	37%
<i>POTEE</i>	NM_001083538	R795H	Chr2: 132021412	Missense	No	56%	48%
<i>RNF123</i>	NM_022064	S432F	Chr3: 49738941	Missense	No	47%	52%
<i>CNTN3</i>	NM_020872	R206X	Chr3: 74420389	Nonsense	No	37%	35%
<i>COL19A1</i>	NM_001858	F1015S	Chr6: 70900035	Missense	No	49%	37%
<i>POLR3A</i>	NM_007055	P607S	Chr10: 79769385	Missense	No	42%	ND ^c
<i>BUD13</i>	NM_001159736	R132C	Chr11: 116633911	Missense	No	37%	33%
<i>C19orf80</i>	NM_018687	R85W	Chr19: 11350566	Missense	No	42%	36%
<i>SRPX2</i>	NM_014467	R110H	ChrX: 99917338	Missense	No	53%	44%

^a The chromosomal position is given based on Hg19.

^b <http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/>.

^c ND, not detected in the whole exome sequencing data for this sample; however, this mutation was validated both with Sanger and deep sequencing and was thus present also in the AML sample.

FIGURE 1



Supporting Information for

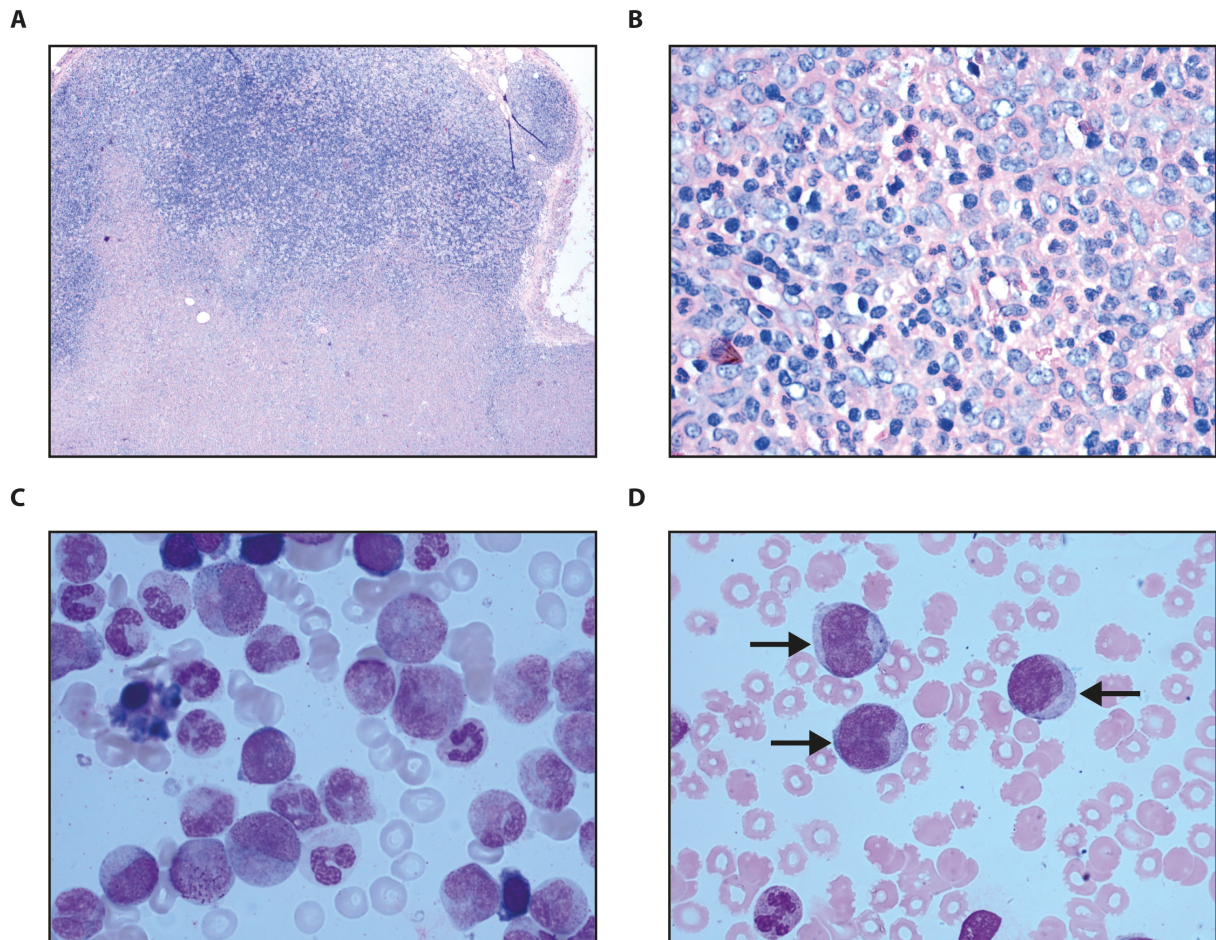
Genomic Profiling and Directed Ex Vivo Drug Analysis of an Unclassifiable

Myelodysplastic/Myeloproliferative Neoplasm Progressing into Acute Myeloid

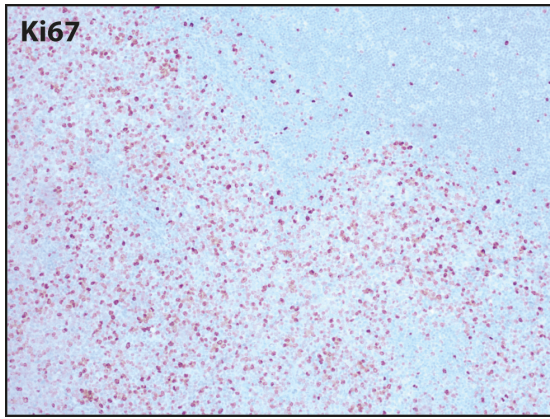
Leukemia

Supplementary Table S1. Whole Exome Sequencing Coverage Data

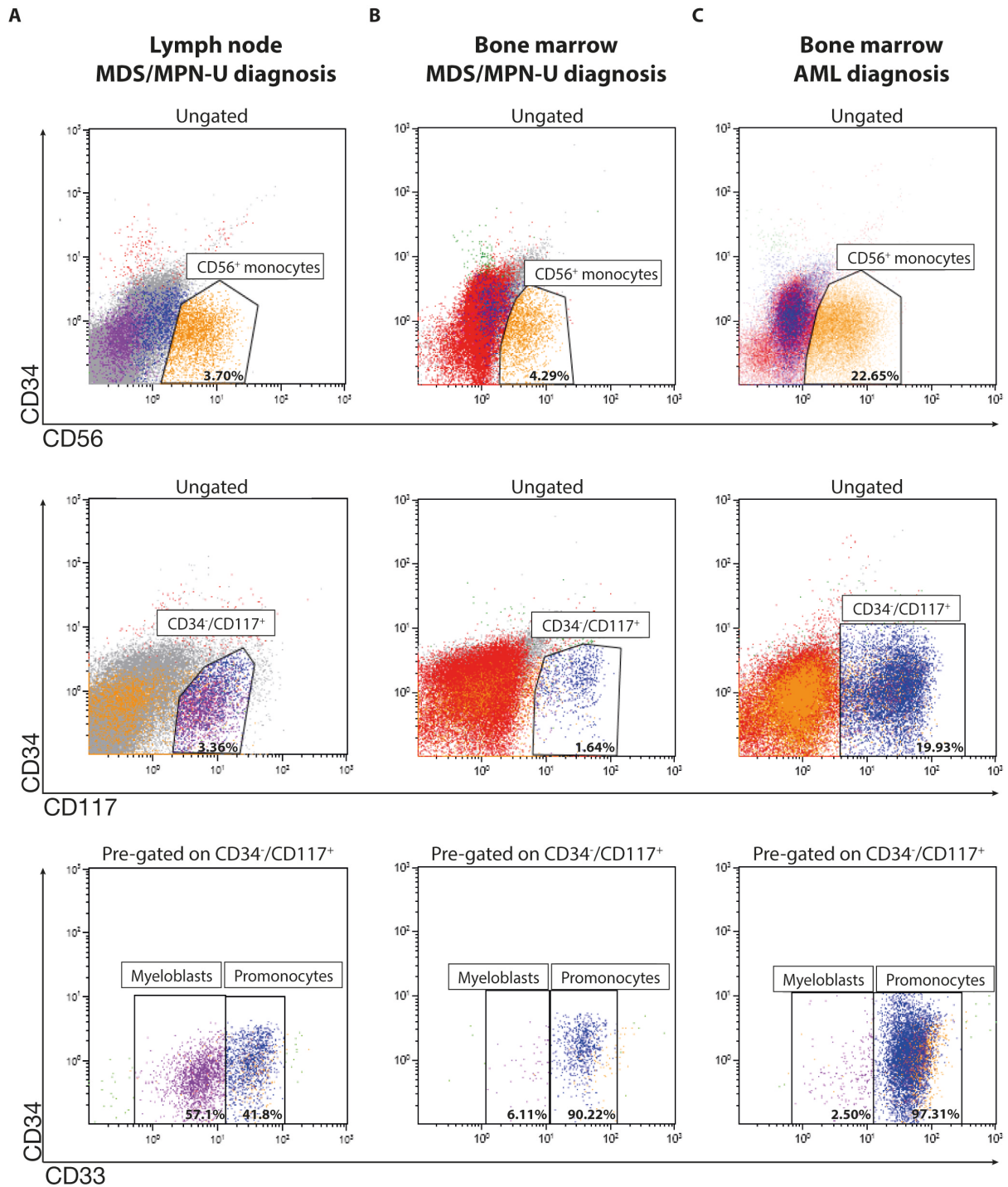
Patient	Type	Nucleotides sequenced	% of coding exons with coverage				
			>5x	>10x	>20x	>30x	>50x
AML01D	MDS/MPD –U diagnosis	2 771 631 010	98.8	96.9	88.6	76.0	50.7
AML01A	AML diagnosis	2 724 132 239	98.6	96.4	86.9	73.7	48.9
AML01G	Germline	2 702 500 189	99.1	97.6	90.9	79.1	52.6



Supplementary Figure S1. Pathological evaluation by immunohistochemistry and flow cytometry. (A) Hematoxylin-eosin stain of the lymph node biopsy at MDS/MPN-U diagnosis revealed a disrupted cellular architecture as indicated by the presence of the lighter stained area in the lower half of the image (10x). (B) High power (60x) of the hematoxylin-eosin stain of the lymph node revealed a diffuse infiltrate of highly proliferating myeloid cells that stained positive for Ki67 (see Supplementary Fig. S2), including blast-like immature cells, monocytoid cells, and dysplastic granulocytes. (C) Bone marrow smear at MDS/MPN-U diagnosis shows dysplastic granulocytes including many eosinophils. (D) The bone marrow smear at AML diagnosis revealed promonocytes (left center, two cells) and monoblasts (right center, one cell) at a high frequency.

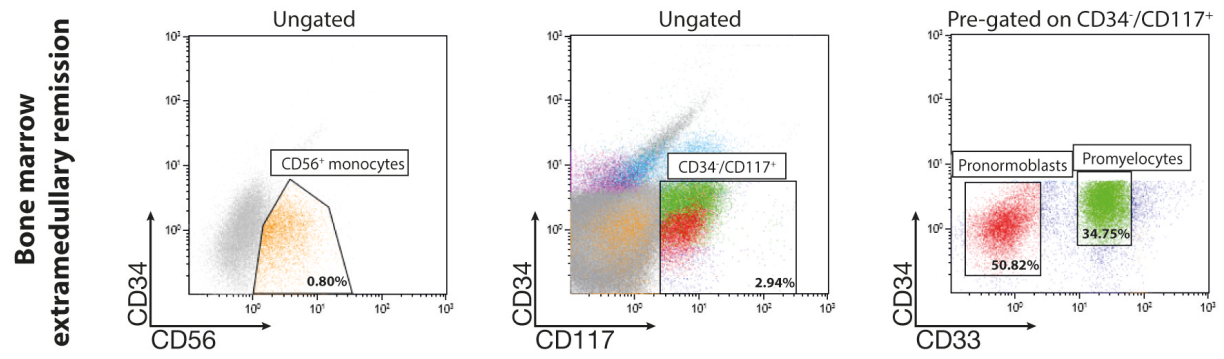


Supplementary Figure S2. The lymph node at MDS/MPN-U diagnosis contained a high number of proliferating cells, as determined by Ki67 staining.

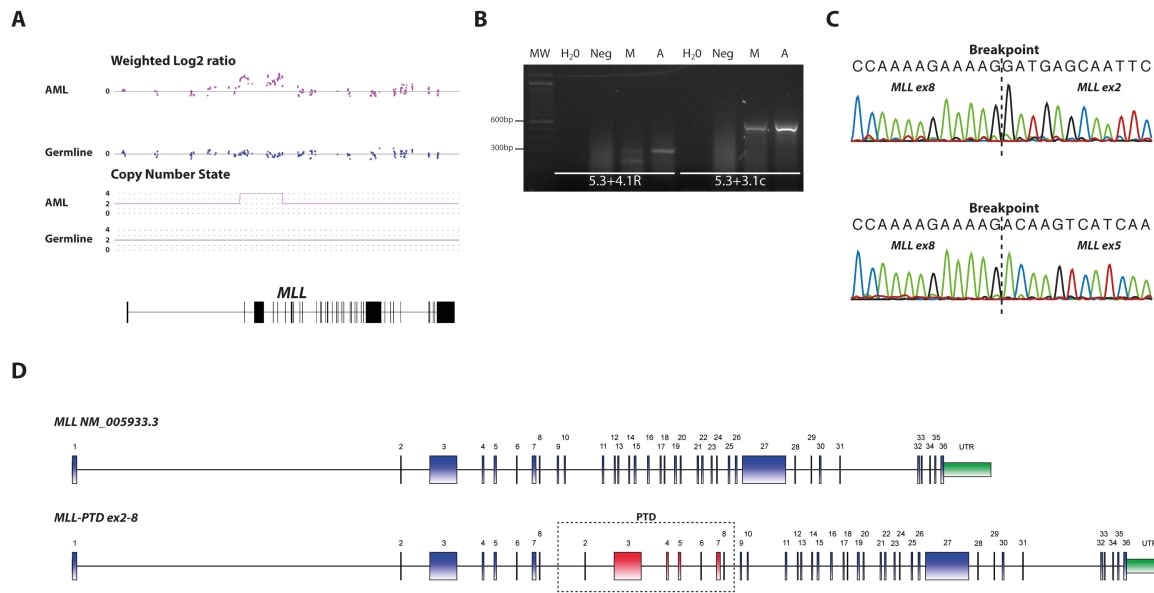


Supplementary Figure S3. (A) At MDS/MPN-U diagnosis, flow cytometric analysis of the lymph node revealed an abnormal amount of monocytes (3.7%), promonocytes (41.8%, i.e. 1.4% of the total population) and myeloblasts (57.1%, i.e. 1.9% of the total population). (B) Flow cytometric analysis of the bone marrow at MDS/MPN-U diagnosis closely resembled that of the lymph node given abnormal amount of monocytes (4.3%) and promonocytes (90.2%, i.e. 1.5% of the total population) but with a much lower frequency of myeloblasts

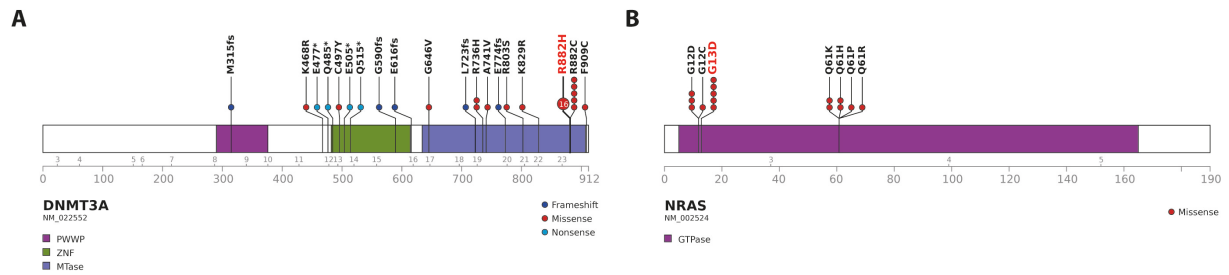
(6.1%, i.e. 0.1% of the total population). (C) The same populations were found at high frequencies in the bone marrow at AML diagnosis; monocytes (22.7%), promonocytes (97.3%, i.e. 19.4% of the total population), and myeloblasts (2.5%, i.e. 0.5% of the total population). However, there was a loss of CD56 on the promonocyte-like cells (data not shown).



Supplementary Figure S4. Three months post radiation therapy of the first extramedullary relapse, no leukemic cells could be detected in the bone marrow.



Supplementary Figure S5. (A) High resolution SNP array analysis revealed a partial-tandem duplication of *KMT2A/MLL* (*MLL*-PTD) as the only detectable copy number alteration. (B) The *MLL*-PTD was confirmed by RT-PCR using two different primer pairs (5.3 + 4.1R and 5.3 + 3.1c) on the MDS/MPN-U (M) and the AML sample (A), respectively, followed by Sanger sequencing. (C) Sequencing revealed the presence of two different variants, one fusing *MLL* exon 8 to exon 2 (in-frame, primer pair 5.3 + 3.1c) and the other *MLL* exon 8 to exon 5 (out-of-frame, primer pair 5.3 + 4.1R) and that the *MLL*-PTD was present also at initial MDS/MPN-U diagnosis. (D) Schematic DNA structure of the *MLL*-PTD, wild-type exons are shown in blue and duplicated exons in red.



Supplementary Figure S6. (A) Mutations in *DNMT3A* among AML cases with a normal karyotype as reported by the Cancer Genome Atlas network (Cancer Genome Atlas Research Network, 2013), with the variant detected in our patient (R882H) marked in red, showing that this is the most frequent *DNMT3A* mutation. (B) The same data shown for *NRAS*, but here, mutations occurring in the whole AML cohort are depicted, again with the specific mutation found in our patient highlighted in red.

Patient history

A 39-year-old woman presented with an atypical bone marrow disease in March 2012. One year before initial presentation, she experienced joint aches and borderline anemia (Hb of 116 g/l); six months before initial diagnosis, she became very tired and two months before she quickly lost 13 kg of weight. At diagnosis, she had pronounced leukocytosis, with increased neutrophils, monocytes, and appearance of immature blood cells including blasts. Her peripheral blood cell counts at diagnosis were: white blood cells $45 \times 10^9/l$ (72% neutrophils, 7% monocytes, 1.8% promyelocytes, 1.8% metamyelocytes, 0.4% myelocytes, and 0.9% blasts), platelets $61 \times 10^9/l$, and Hb 68 g/l. She presented with cervical lymphadenopathy and a soft tissue mass in the submandibular gland. The tumor masses were evaluated by a pathologist and determined to be a myelodysplastic/myeloproliferative neoplasm, unclassifiable (MDS/MPN-U). A bone marrow aspirate showed 1.5% promonocytes (CD117⁺/CD33⁺/CD34⁻/HLA-DR⁺/CD135⁺/CD133⁺) and 4.3% abnormal monocytes (CD56⁺/CD123⁺/CD135⁺).

Clinical genetic diagnostics revealed a normal female karyotype and lack of the t(9;22)(q34;q11)/*BCR-ABL1* fusion gene, del(4)(q12)/*FIP1L1-PDGFR*A rearrangement and *JAK2* mutations (codon V617 and exon 12 investigated). Treatment was started with hydroxyurea and she received multiple blood transfusions. Two months after initial presentation (in May) she developed a further increase in leukocytes ($90.7 \times 10^9/l$), monocytes ($16.1 \times 10^9/l$), and blast cells ($4.8 \times 10^9/l$), fever, and gingivitis. A bone marrow aspirate showed high cellularity, 24% of myeloid blast cells, and a high frequency of atypical monocytes and eosinophils. The immunophenotype was similar to the initial bone marrow examination with 19.4% promonocytelike cells CD117⁺/CD33⁺/CD34⁻/HLA-DR⁺/CD135⁺/CD133⁺ and 22.7% abnormal monocytes (CD56⁺/CD123⁺/CD135⁺); AML was diagnosed. Induction and consolidation therapy was given with daunorubicin and cytarabine.

She subsequently underwent allogeneic bone marrow transplantation in first complete remission in September 2012 and was allografted with cells from an unrelated donor after a myeloablative conditioning with total body irradiation and high dose cyclophosphamide. The patient developed a skin graft-versus-host-disease that was treated with steroids.

Twenty-one months after initial MDS/MPN-U diagnosis, in December 2013, the patient relapsed with a local tumor mass in her right breast and underwent local radiation therapy. Pathological evaluation revealed infiltration of immature myeloid cells (MPO⁺, CD117⁺, CD68⁺). At that time, no leukemic cells could be detected in the bone marrow. Twenty-one months after her first relapse, she developed a second extramedullary relapse with a parasternal soft tissue tumor mass, and was treated with local radiation therapy.