The Journal of Molecular Diagnostics, Vol. 17, No. 6, November 2015

ELSEVIER

the Journal of Molecular Diagnostics

jmd.amjpathol.org

Detection of Dual *IDH1* and *IDH2* Mutations by Targeted Next-Generation Sequencing in Acute Myeloid Leukemia and Myelodysplastic Syndromes

Mia Y. Platt,[*†] Amir T. Fathi,[†‡] Darrell R. Borger,[*†] Andrew M. Brunner,[†‡] Robert P. Hasserjian,[*†] Leonora Balaj,[†§] Amy Lum,[¶] Stephen Yip,[¶] Dora Dias-Santagata,[*†] Zongli Zheng,[*†] Long P. Le,[*†] Timothy A. Graubert,[†‡] A. John Iafrate,[*†] and Valentina Nardi[*†]

From the Departments of Pathology, Medicine,[‡] and Neurology,[§] Massachusetts General Hospital, Boston, Massachusetts; the Harvard Medical School,[†] Boston, Massachusetts; and the Department of Pathology and Laboratory Medicine,[¶] University of British Columbia, Vancouver, British Columbia, Canada*

Accepted for publication June 17, 2015.

Address correspondence to Valentina Nardi, M.D., Department of Pathology, Massachusetts General Hospital, 55 Fruit St., GRJ 245, Boston, MA 02114. E-mail: vnardi@partners.org.

Studies in myeloid neoplasms have described recurrent *IDH1* and *IDH2* mutations as primarily mutually exclusive. Over a 6-month period of clinical testing with a targeted next-generation sequencing assay, we evaluated 92 patients with acute myeloid leukemia, myelodysplastic syndrome, and chronic myelomonocytic leukemia and identified a subset of 21 patients (23%) who harbored mutations in either *IDH1* or *IDH2*. Of the 21 patients with IDH mutations, 4 (19%) were found to have single nucleotide variants in both *IDH1* and *IDH2*. An additional patient included in the study was found to have two different *IDH2* mutations. The mutations were typically present at different variant allelic frequencies, with one predominating over the other, consistent with the presence of multiple subclones in a single patient. In one case, the variant allelic frequencies in both *IDH1* and *IDH2* were equally low in the setting of a high percentage of blasts, suggesting that the *IDH* mutations were unlikely to be present in the founding clone. Given these data, we conclude that dual *IDH1/2* mutations likely were previously underestimated, a finding that may carry important treatment implications. *(J Mol Diagn 2015, 17: 661—668; http://dx.doi.org/10.1016/j.jmoldx.2015.06.004)*

In recent years, whole-genome sequencing of acute myeloid leukemias (AMLs) led to the identification of frequent heterozygous mutations in isocitrate dehydrogenase 1 gene (*IDH1*).[1,2] Subsequent exome sequencing and targeted resequencing studies in AML also identified frequent *IDH2* mutations.[3,4] Overall, approximately 15% to 30% of AML have mutations in *IDH1* or *IDH2*, almost exclusively at codon Arg132 for *IDH1* and at codons Arg140 and Arg172 for *IDH2*.[1,3–6] Patients with cytogenetically normal AML are enriched for these mutations. The mutations confer neomorphic enzyme activity through the NADPH-dependent reduction of the normal end-product α-ketoglutarate to the putative oncometabolite 2-hydroxyglutarate. The accumulation of high levels of 2-hydroxyglutarate in the *IDH1/2*-mutant tumor provides an important mechanism of cellular transformation through the targeting of epigenetic regulators.[7]

IDH mutations were also identified in preleukemic clonal malignancies, including myelodysplastic syndromes (MDSs) and myeloproliferative neoplasms (approximately 5% of MDS/myeloproliferative neoplasm and approximately 20% of AML arising from MDS or myeloproliferative neoplasm).[1,4,8] They occur early in disease pathogenesis in the founding clone[9] and act as major initiating mutations.[10] Mutations in the two *IDH* genes are likely mutually exclusive,[11] with only rare reports of concurrent *IDH1* and *IDH2* mutations[12] at unknown allelic frequency (AFs).

Testing for these mutations is becoming increasingly important to patient care, given that first-generation IDH inhibitors

Supported by T32 training/NIH grant T32 CA 71345-18 (A.M.B.) and Vancouver Coastal Health Research Institute Mentored Clinician Scientist Award, BrainCare BC (S.Y.).

Disclosures: A.T.F. has participated in advisory boards for Agios Pharmaceuticals; Z.Z., L.P.L., and A.J.I. hold patents for the Anchored Multiplex PCR technology, licensed to ArcherDx; Z.Z., L.P.L., and A.J.I. hold stocks in ArcherDx; and L.P.L. and A.J.I. were consultants for Enzymatics, which had acquired ArcherDx.

http://dx.doi.org/10.1016/j.jmoldx.2015.06.004

were shown to suppress the growth of 2-hydroxyglutarate-producing IDH-mutant tumor cells both *in vitro* and *in vivo*.[13–15] Clinical trials with IDH1 and IDH2 inhibitors for patients with *IDH1/2* mutations are ongoing.[16–20]

Because our laboratory launched a next-generation sequencing (NGS)-based tumor genotyping assay in early 2014, we found that *IDH1* and *IDH2* mutations co-occur in the same tumors more frequently than was reported. This study is a detailed description of five cases of dual mutations we have thus far identified and of the potential implications of these results.

Materials and Methods

Identification of Cases

The Partners HealthCare Institutional Review Board granted approval for this study before its initiation. The electronic files of the Massachusetts General Hospital Pathology Department were queried for all cases run with the use of the NGS assay (SNAPSHOT NGS) since its launch in April 2014 until October 3, 2014, with a primary diagnosis of AML, MDS, or chronic myelomonocytic leukemia. A subsequent query was run to identify the subset of cases with either *IDH1* or *IDH2* single nucleotide variants (SNVs). Unique patients were enumerated such that multiple specimens sent on a single patient were not individually counted.

Targeted DNA-Seq Using Anchored Multiplex PCR

The SNAPSHOT NGS assay uses a multiplex PCR technology called Anchored Multiplex PCR for SNVs and insertion/deletion detection in genomic DNA with the use of NGS.[21] Briefly, genomic DNA was isolated from blood or bone marrow aspirates (QIAcube; Qiagen, Valencia, CA). The genomic DNA was sheared with the Covaris (Woburn, MA) M220 instrument followed by end-repair, adenylation, and ligation with an adapter. A sequencing library that targeted hotspots and exons in 39 genes (Supplemental Table S1) was generated with two hemi-nested PCR reactions with the use of one primer specific to a sequence in the gene of interest and one specific to a universal sequence in the adapter, for each PCR reaction.[21] Illumina (San Diego, CA) MiSeq 2 × 151 bp paired-end sequencing results were aligned to the hg19 human genome reference with the use of BWA-MEM.[22] PCR/optical duplicates were removed on the basis of unique start sites of sequenced molecules. MuTect[23] was used for SNV detection and Oncotator was used for mutation annotation (*http://www.broadinstitute.org/oncotator*, last accessed May 6, 2015). A minimum threshold of five unique reads that supported the canonical mutation and visualization in JBrowse[24] were required to make a variant call.

Focused Ultra-Deep Amplicon Sequencing Using the Ion Torrent PGM Platform

Barcoded and tagged unidirectional PCR primers targeting codons 132 of *IDH1* and 140 of *IDH2* were designed to generate amplicons approximately 100 bp in length (Table 1). Genomic DNA samples were quantitated with the Qubit high-sensitivity DNA assay kit (Thermo Fisher Scientific, Waltham, MA), individually amplified with Platinum Taq HiFi, purified with Ampure XL beads (Agencourt, Brea, CA), and quantitated with High-Sensitivity DNA chip on the Agilent Technologies BioAnalyzer (Santa Clara, CA). The amplicons were normalized and pooled to generate emulsion PCR libraries on the Ion Torrent OneTouch2 platform with the use of the Ion PGM Template OT2 400 kit (Thermo Fisher Scientific). The libraries were enriched with the Ion Torrent ES platform, and all sequencing was performed with the Ion PGM Hi-Q Sequencing kit on the Ion Torrent PGM platform, analyzed, and visualized with the IGV version 2.3. The average base pair coverage of the amplicon was approximately 100,000 times. Relative frequencies of the mutant alleles were derived from dividing the number of mutant calls by total calls of the relevant position (Supplemental Table S2).

Droplet Digital PCR for *IDH1* c.395G>A, p.Arg132His

Genomic DNA was used as input for digital PCR at 10 ng per reaction. Primers spanned an intron/exon junction and were as follows: *IDH1* forward primer, 5′-CTGCAAAAATATC-CCCCGGC-3′; *IDH1* reverse primer, 5′-CAAGTTGGAAA-TTTCTGGGCCAT-3′; mutant probe, 5′-ATCATAGGTCG-TCATGCTTAT-3′; and wild-type probe, 5′-ATCATAGGT-CATCATGCTTAT-3′. Reactions were performed in a 20-μL reaction with the use of the droplet digital PCR Supermix for Probes (no dUTP; Bio-Rad, Hercules, CA). Final concentration of primers and probes were 900 nmol/L and 250 nmol/L, respectively. The relation between the presence or absence of mutant or wild-type molecules in a droplet is defined by the Poisson distribution and allows robust, digital quantification of the two molecules relative to one another (Supplemental Table S3). Droplet generation was performed according to the manufacturer's instructions. Cycling conditions were 95°C for 10 minutes, followed by 40 cycles of 94°C for 30 seconds and 64°C for 2 minutes, and then a final 10-minute incubation at 98°C. The temperature ramp rate was 2.5°C/second. Droplet reading was performed on a QX200 droplet digital PCR droplet reader (Bio-Rad), and the analysis was performed with manual mode on a QuantaSoft Analysis software version 1.4 (Bio-Rad) by adjusting the gates to the no template control samples.

FLT3, *NPM1*, and *CEBPA* Mutation Analysis

Specimens were sent to LabCorp (Research Triangle Park, NC) for *FLT3*, *NPM1*, and *CEBPA* mutation analysis. Briefly, PCR amplification for the detection of *FLT3* internal tandem duplication mutations (*FLT3*-ITDs) was performed, and the products were run on the ABI 3500xl genetic analyzer (Thermo Fisher Scientific) for size determination. The *ITD* wild-type produces a fragment that is approximately 327 bp, whereas the presence of an insertion produces a fragment that is ≥330 bp. PCR and size determination was also performed for *NPM1* analysis to detect a

Table 1 PCR Primers for Focused Ultra-Deep Amplicon Sequencing Using the Ion Torrent PGM Platform

Primers	Sequence
IDH1 primers	
IDH1_R132_1F	5′-**CCATCTCATCCCTGCGTGTCTCCGACTCAGGCATG**TCACATTATTGCCAACATGACT-3′
IDH1_R132_2F	5′-**CCATCTCATCCCTGCGTGTCTCCGACTCAGCGATC**TCACATTATTGCCAACATGACT-3′
IDH1_R132_3F	5′-**CCATCTCATCCCTGCGTGTCTCCGACTCAGAGCTA**TCACATTATTGCCAACATGACT-3′
IDH1_R132_4F	5′-**CCATCTCATCCCTGCGTGTCTCCGACTCAGACACA**TCACATTATTGCCAACATGACT-3′
IDH1_R132_5F	5′-**CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTCT**TCACATTATTGCCAACATGACT-3′
IDH1_R132_6F	5′-**CCATCTCATCCCTGCGTGTCTCCGACTCAGTCAGT**TCACATTATTGCCAACATGACT-3′
IDH1_R132_7F	5′-**CCATCTCATCCCTGCGTGTCTCCGACTCAGTATAT**TCACATTATTGCCAACATGACT-3′
IDH1_R132_8F	5′-**CCATCTCATCCCTGCGTGTCTCCGACTCAGAGTCA**TCACATTATTGCCAACATGACT-3′
IDH1_R132_9F	5′-**CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGCT**TCACATTATTGCCAACATGACT-3′
IDH1_R132_10F	5′-**CCATCTCATCCCTGCGTGTCTCCGACTCAGTGTGT**TCACATTATTGCCAACATGACT-3′
IDH1_R132_R	5′-**CCTCTCTATGGGCAGTCGGTGATGCATG**CGGTCTTCAGAGAAGCCATT-3′
IDH2 primers	
IDH2_R140_1F	5′-**CCATCTCATCCCTGCGTGTCTCCGACTCAGGCATG**CTAGGCGTGGGATGTTTTTG-3′
IDH2_R140_2F	5′-**CCATCTCATCCCTGCGTGTCTCCGACTCAGCGATC**CTAGGCGTGGGATGTTTTTG-3′
IDH2_R140_3F	5′-**CCATCTCATCCCTGCGTGTCTCCGACTCAGAGCTA**CTAGGCGTGGGATGTTTTTG-3′
IDH2_R140_4F	5′-**CCATCTCATCCCTGCGTGTCTCCGACTCAGACACA**CTAGGCGTGGGATGTTTTTG-3′
IDH2_R140_5F	5′-**CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTCT**CTAGGCGTGGGATGTTTTTG-3′
IDH2_R140_6F	5′-**CCATCTCATCCCTGCGTGTCTCCGACTCAGTCAGT**CTAGGCGTGGGATGTTTTTG-3′
IDH2_R140_7F	5′-**CCATCTCATCCCTGCGTGTCTCCGACTCAGTATAT**CTAGGCGTGGGATGTTTTTG-3′
IDH2_R140_8F	5′-**CCATCTCATCCCTGCGTGTCTCCGACTCAGAGTCA**CTAGGCGTGGGATGTTTTTG-3′
IDH2_R140_9F	5′-**CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGCT**CTAGGCGTGGGATGTTTTTG-3′
IDH2_R140_10F	5′-**CCATCTCATCCCTGCGTGTCTCCGACTCAGTGTGT**CTAGGCGTGGGATGTTTTTG-3′
IDH2_R140_R	5′-**CCTCTCTATGGGCAGTCGGTGATGCATG**TCTGTCCTCACAGAGTTCAAGC-3′

Bold text indicates sequence of the adapter and barcode.

4-bp insertion at nucleotide position 959 (exon 12). For *CEBPA* analysis, the *CEBPA* coding region was PCR-amplified and was Sanger sequenced to identify sequence variations.

Immunohistochemistry

Two micron-thick formalin-fixed, paraffin-embedded bone marrow (BM) core biopsy sections were stained with hematoxylin and eosin. Immunohistochemistry for IDH1 was performed on 5-μm–thick formalin-fixed, paraffin-embedded BM biopsy sections with an antibody specific for the mutant IDH1 p.Arg132His protein (dilution 1:150; H09; Dianova, Hamburg, Germany). A labeled streptavidin biotin kit was used as a detection system (Leica Biosystem, Nussloch, Germany). Combined cytoplasmic and nuclear staining was interpreted as immunopositive. Currently, antibodies are only available against IDH1 p.Arg132His mutant protein; no antibodies specific for any of the IDH2 mutated proteins are available for use in immunohistochemistry.

Results

Clinical and Cytogenetic Characteristics

Clinical, cytogenetic, and molecular characteristics of the patients with dual mutations in *IDH1* and *IDH2* are listed in Table 2. Three of the five patients, all men, exhibited diagnoses of AML. Two additional patients (one female and one male) were diagnosed with MDS (both refractory anemia with excess blasts-2; RAEB-2). The age at diagnosis ranged from 61 to 73 years. The AML patients had BM aspirate or (when not available) peripheral blood blast counts that ranged from 22% to 33% at diagnosis. Blast counts from the RAEB-2 patients were not available for the sequenced specimens. All five patients had normal karyotypes.

Of note, patient 2 had a history of bladder cancer, treated with surgery and Bacille Calmette-Guérin. None of the patients had any reported environmental exposures or a family history of blood disorders or hematologic neoplasms.

Frequency of *IDH1/2* Mutations

This study included all AML ($n = 53$), MDS ($n = 34$), and chronic myelomonocytic leukemia ($n = 5$) patients with specimens submitted for the SNAPSHOT NGS assay during the 6-month period from the assay's launch in April 2014 through October 2014 (total $n = 92$). Of these 92 patients, 21 (23%) harbored *IDH* mutations (14 AML, 6 MDS, 1 chronic myelomonocytic leukemia). Of these 21 patients, 4 (19%) had coexisting *IDH1* and *IDH2* mutations. A fifth patient (with MDS) who was included in our study was previously identified as harboring two different *IDH2* mutations on a previously used hotspot profiling platform, which were confirmed by the current SNAPSHOT NGS assay. The dual *IDH1* and *IDH2*

Table 2 Patient Characteristics and *IDH1* and *IDH2* Single Nucleotide Variants

Case No.	Dx	Age, years/ sex	Specimen type	Blast, %	*IDH1* mutation (allelic frequency, %)	No. of Reads (ALT/REF)	*IDH2* mutation (allelic frequency, %)	No. of Reads (ALT/REF)	Other (allelic frequency, %)	Karyotype	*FLT3*-ITD, *NPM1*, *CEBPA* status
1	AML	63/M	BM	22	p.Arg132His (c.395G>A) (12)	588/4228	p.Arg140Gln (c.419G>A) (2)	232/12310	*SMAD4* p.Ala458Val (c.1373C>T) (45), *CDH1* p.Ala592Thr (c.1774G>A) (46)	46,XY	*FLT3* wild-type *NPM1* wild-type *CEBPA* wild-type
2	AML	73/M	BM	30* (PB)	p.Arg132His (c.395G>A) (44)	191/256	p.Arg140Trp (c.418C>T) (5)	50/1044		46,XY	*FLT3* wild-type *NPM1* mutated (4 bp insertion at c.959) *CEBPA* wild-type
3	AML	61/M	BM	33	p.Arg132Cys (c.394C>T) (3)	7/237	p.Arg140Gln (c.419G>A) (3)	19/589	*NRAS* p.Gly12Asp (c.35G>A) (34), *NRAS* p.Gly12Ser (c.34G>A) (2)	46,XY	*FLT3*-ITD mutated [PCR product of 429 bp (wild-type ~327 bp)] *NPM1* wild-type *CEBPA* double mutant [p.Asn74X (c.219insT), p.Ala295Thr (c.883G>A)]
4	RAEB-2	68/F	PB	13†	p.Arg132His (c.395G>A) (4)	8/199	p.Arg140Gln (c.419G>A) (12)	60/482		46,XX	NA
5	RAEB-2	64/M	BM	13†			p.Arg140Gln (c.419G>A) (27); p.Arg140Trp (c.418C>T) (13)	337/943, 169/1138		46,XY	*FLT3* wild-type *NPM1* wild-type *CEBPA* wild-type

*BM was unavailable for use to determine blast percentage by structural characteristics because of extensive necrosis. Instead, the blast percentage from PB is shown.

†The blast count was unavailable for the specimen submitted for sequencing. The blast percentage shown is from a different specimen on a different day (from PB 16 days before the sequencing sample for patient 4, and from BM 13 days before the sequencing sample for patient 5.).

F, female; M, male; ALT, alternative allele; AML, acute myeloid leukemia; BM, bone marrow; Dx, diagnosis; ITD, internal duplication mutation; NA, not applicable; PB, peripheral blood; RAEB-2, refractory anemia with excess blasts-2; REF, reference allele.

variants identified were missense mutations at the canonically mutated codons, Arg132 and Arg140, respectively (Table 2).

Although several *IDH* mutations were observed at low AF, our confidence in these calls was based on a high depth of coverage (average target region coverage for each of the five samples, 3453; range, 690 to 8909), with a high number of unique molecules supporting the calls (well above background), the mutations' location at canonical sites, and the identification of the mutations on multiple samples from the same patient on repeat testing in some instances (data not shown). In addition, the mutations were verified by an orthogonal sequencing method (see *Focused Ultra-Deep Amplicon Sequencing Using the Ion Torrent PGM Platform*) (Supplemental Table S2). The

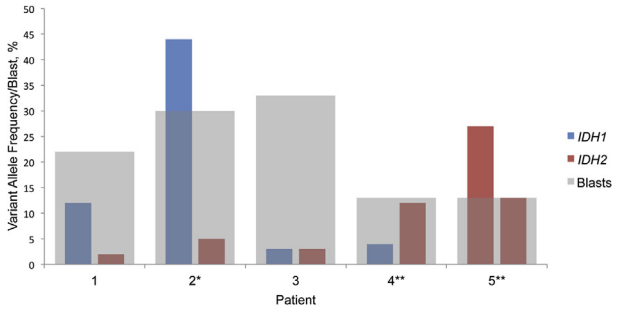


Figure 1 Relation between variant allelic frequency and blast percentage. The histogram displays the variant allelic frequency in the context of the blast count for each of the five patients. The **asterisk** affixed to Patient 2 indicates blast count was obtained from PB. The **double asterisk** affixed to Patients 4 and 5 indicate blast count was obtained from a different specimen or date (PB 16 days and BM 13 days before the sequencing sample, respectively). BM, bone marrow; IDH, isocitrate dehydrogenase; PB, peripheral blood.

IDH1/2 mutational burden for each case (as represented by the variant AF) is described in the sections below along with non-*IDH* mutations.

Patient 1

Patient 1 had AML with normal karyotype and without *FLT3*, *NPM1*, or *CEBPA* mutations and showed an *IDH1* p.Arg132His (c.395G>A) mutation at 12% AF and an *IDH2* p.Arg140Gln (c.419G>A) mutation at 2%, in the setting of a marrow blast percentage of 22% (Table 2 and Figure 1). Although the blast count does not necessarily reflect the size of the clonal population, if we assumed a heterozygous *IDH1* mutation were present in every cell of the blast population, the marrow involvement by 22% blasts would predict a variant AF of approximately 11%. The calculated AF of 12% by SNAPSHOT NGS, combined with the observation of mutant IDH protein by immunohistochemistry in nearly all of the blasts, is consistent with this assumption (Supplemental Figure S1). SNVs in SMAD family member 4 and *CDH1* genes were also detected by the SNAPSHOT NGS assay. The variant AFs of the SNVs in SMAD family member 4 and cadherin 1, type 1 genes, however, were close to 50% (45% and 46%, respectively), suggesting these were not somatic tumor mutations.

Patient 2

Patient 2 had AML with normal karyotype and mutated *NPM1*, but wild-type *FLT3* and *CEBPA*, and showed a predominant *IDH1* p.Arg132His (c.395G>A) mutation on the BM aspirate with a 44% AF, whereas an *IDH2* p.Arg140Trp (c.418C>T) mutation was present at 5% AF. The marrow blast percentage could not be determined because of extensive necrosis, but a concurrent peripheral blood sample showed a blast percentage of 30% (Table 2 and Figure 1). No other SNVs were detected by SNAPSHOT NGS.

Patient 3

Patient 3 had AML with normal karyotype, a *FLT3* ITD mutation, two *CEBPA* mutations, and no *NPM1* mutation. Both an *IDH1* p.Arg132Cys (c.394C>T) and an *IDH2* p.Arg140Gln (c.419G>A) mutation were present, each at an equally low AF of 3%, in the setting of 33% blasts in the marrow (Table 2 and Figure 1). SNAPSHOT NGS also detected two neuroblastoma RAS viral (v-ras) oncogene homolog (*NRAS*) gene mutations that were present in *trans*, both at the hotspot codon 12 [p.Gly12Asp (c.35G>A) and p.Gly12Ser (c.34G>A)] at AFs of 34% and 2%, respectively (Supplemental Figure S2).

Patient 4

Patient 4, diagnosed with RAEB-2 with normal karyotype, harbored a predominant *IDH2* p.Arg140Gln (c.419G>A) mutation at 12% AF and an *IDH1* p.Arg132His (c.395G>A) mutation at 4% AF (Table 2 and Figure 1). The peripheral blood for this patient had 13% blasts 16 days before the NGS testing. No other SNVs were detected by SNAPSHOT NGS. Mutational analyses for *FLT3*, *NPM1*, and *CEBPA* mutations were not performed.

Patient 5

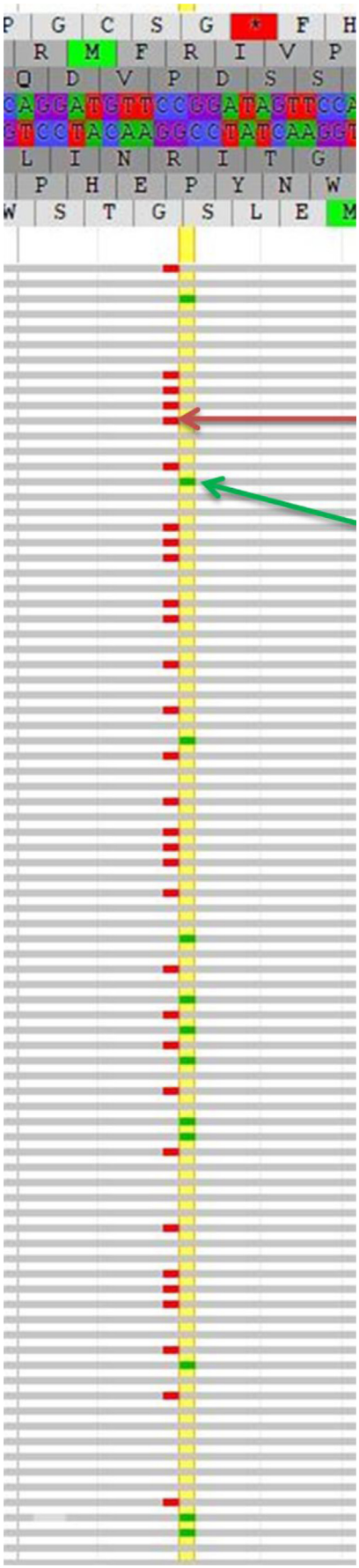
Patient 5, diagnosed with RAEB-2 with normal karyotype, had two separate *IDH2* mutations with AFs of 27% and 13%. The BM aspirate for this patient had 13% blasts 13 days before the NGS testing. The two mutations represented different SNVs that affected the same hot spot codon 140 [p.Arg140Gln (c.419G>A) and p.Arg140Trp (c.418C>T), respectively] and were shown to be in *trans*, confirmed by visualization on independent sequencing reads (Figure 2). No other SNVs were detected by SNAPSHOT NGS. Mutational analyses by PCR for *FLT3*, *NPM1*, and *CEBPA* did not identify mutations in these genes.

Validation of Low Allelic Frequency Mutations

The four patient samples harboring mutations at ≤5% AF were resequenced with a second NGS assay that uses a different platform, the Ion Torrent PGM platform. Ultra-deep sequencing (average base coverage, approximately 100,000 times) verified the presence of the mutations at similar AF to the original method (Supplemental Table S2). For patients with the *IDH1* Arg132His mutation (patients 1, 2, and 4), droplet digital PCR, an additional orthogonal method, was used to confirm the mutations, one of which was ≤5% AF (Supplemental Table S3).

Discussion

Recurrent *IDH* mutations were described in a variety of malignancies, including gliomas,[25,26] chondrosarcomas,[27–29]



cholangiocarcinomas,[30] and breast carcinomas,[31] and also in a number of myeloid neoplasms such as AML, MDS, and myeloproliferative neoplasm.[1,32] Both *IDH1* and *IDH2* encode IDH enzymes that normally function to convert isocitrate to α-ketoglutarate. Mutant enzymes, however, reduce α-ketoglutarate to the oncometabolite 2-hydroxyglutarate, which in turn goes on to inhibit α-ketoglutarate−dependent enzymes, such as *TET2*, resulting in aberrant epigenetic modifications.[11] Mutant IDH1 and IDH2 enzymes thus serve as promising therapeutic targets, and specific inhibitors are currently in various stages of development.[13−20,33,34]

With the use of a sensitive NGS mutation panel, we identified dual mutations in *IDH1* and *IDH2* that occur at greater frequency than previously described. Among the *IDH*-mutated myeloid neoplasms ($n = 21$), nearly one-quarter were found to have dual *IDH* mutations (5 of 21 patients, including one patient who harbored two *IDH2* mutations.) The previous under-recognition of dual mutations is likely because of the lack of highly sensitive sequencing assays with the ability to routinely detect variants that are only present at an AF of 1% to 5% of reads. Although in some cases the detected *IDH1/2* mutations were present at an AF as high as 44%, we were also able to identify low-level mutations at 2% to 3% AF that would have otherwise been missed by less-sensitive assays. Importantly, we verified the mutations with ultra-deep sequencing with the use of an alternative NGS platform, an orthogonal method, to help rule out the possibility that the low-level mutations were a result of sequencing error.[35] Furthermore, dual *IDH* mutations were not detected in 1390 other tumor samples that concurrently underwent SNAPSHOT NGS sequencing between April and October 2014 (data not shown).

In addition to identifying low-level mutations, we also found that multiple *IDH1/2* mutations may be present at substantially different AFs. In fact, in four of the five patients, one *IDH1/2* mutation predominated compared with the other, with a twofold to ninefold difference between the mutations' AFs (patient 1, *IDH1* 12% versus *IDH2* 2%; patient 2, 44% versus 5%; patient 4, 4% versus 12%; patient 5, *IDH2* p.Arg140Gln 27% versus *IDH2* p.Arg140Trp 13%) (Table 2). Recognition of the high frequency of concomitant mutations in *IDH1* and *IDH2* underscores the utility of panel testing and of sufficient read coverage for accurate and

Figure 2 Screenshot of the dual mutations visualized in JBrowse. In the upper portion of the figure is the hg19 reference genome sequence (and corresponding amino acid translation) for codons 137 to 143. The horizontal **gray rows** indicate individual reads (ie, individual molecules) sequenced in the forward direction. The small **red bars** indicate a nucleotide change (SNV) from the reference nucleotide G to A (c.419G>A) [the **red arrow** points to example IDH2 p.Arg140Trp (c.418C>T)]; the small **green bars** indicate a nucleotide change (SNV) from the reference nucleotide C to T (c.418C>T) [the **green arrow** points to example *IDH2* p.Arg140Gln (c.419G>A)]. The **yellow vertical highlight line** marks the nucleotides at position 419 in the coding sequence where an SNV was detected. IDH, isocitrate dehydrogenase; SNV, single nucleotide variant.

comprehensive molecular diagnosis. It may also affect decisions about conventional or targeted therapy.

The identification of mutations at low AFs, often in the setting of relatively extensive myeloblast marrow infiltration, may suggest that *IDH1/2* mutations do not always occur in the founding neoplastic clone. This may have important implications about the role of mutant *IDH1* and *IDH2* in the clonal evolution of myeloid neoplasms and their targeted inhibition. Of note, in our study, two of the five patients had co-occurring non-*IDH* mutations. Patient 2 harbored an *NPM1* mutation (AF unknown), and patient 3 had two canonical mutations in neuroblastoma RAS viral (v-ras) oncogene homolog gene (p.Gly12Asp, p.Gly12Ser), of which one was at much higher AF (34%) than the concurrent *IDH* mutations (3% each), as well as *FLT3*-ITD and dual *CEBPA* mutations (AF unknown) (Table 2). Although the remaining three patients did not harbor other somatic mutations among the 39 genes tested in our NGS panel, the panel did not assess for a number of genes relevant to myeloid neoplasms such as *DNMT3A*, *TET2,* or *ASXL1* (Supplemental Table S1). Overall, our findings suggest that mutations in *IDH1/2* may not always be present in the founding clone and rather may represent simultaneous or sequential events throughout clonal evolution.

In summary, we report that concomitant mutations in *IDH1* and *IDH2* are not infrequent events in *IDH*-mutated myeloid neoplasms. The use of panel testing that has sufficient depth of coverage, along with the reporting of allele frequencies of identified mutations, may have considerable impact on both the initial diagnostics and subsequent clinical decision making about therapy. Finally, although it remains to be definitively demonstrated whether *IDH1/2* mutations co-occur in the same tumor cells or in discrete tumor cell subsets, our data support the co-existence of multiple tumor subclones within individual patients. This finding may have practical consequences; although the initial clinical data with the use of IDH-specific inhibitors may be encouraging, other strategies such as simultaneous inhibition of both IDH1 and IDH2 activity, in addition to other antileukemic therapies, may be necessary for patients with both *IDH1*- and *IDH2*-mutation−positive subclones.

Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.jmoldx.2015.06.004*.

References

1. Mardis ER, Ding L, Dooling DJ, Larson DE, McLellan MD, Chen K, et al: Recurring mutations found by sequencing an acute myeloid leukemia genome. N Engl J Med 2009, 361:1058−1066
2. Cancer Genome Atlas Research Network: Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. N Engl J Med 2013, 368:2059−2074
3. Ward PS, Patel J, Wise DR, Abdel-Wahab O, Bennett BD, Coller HA, Cross JR, Fantin VR, Hedvat CV, Perl AE, Rabinowitz JD, Carroll M, Su SM, Sharp KA, Levine RL, Thompson CB: The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting alpha-ketoglutarate to 2-hydroxyglutarate. Cancer Cell 2010, 17:225−234
4. Marcucci G, Maharry K, Wu YZ, Radmacher MD, Mrozek K, Margeson D, Holland KB, Whitman SP, Becker H, Schwind S, Metzeler KH, Powell BL, Carter TH, Kolitz JE, Wetzler M, Carroll AJ, Baer MR, Caligiuri MA, Larson RA, Bloomfield CD: IDH1 and IDH2 gene mutations identify novel molecular subsets within de novo cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study. J Clin Oncol 2010, 28:2348−2355
5. Green CL, Evans CM, Zhao L, Hills RK, Burnett AK, Linch DC, Gale RE: The prognostic significance of IDH2 mutations in AML depends on the location of the mutation. Blood 2011, 118:409−412
6. Patel JP, Gonen M, Figueroa ME, Fernandez H, Sun Z, Racevskis J, Van Vlierberghe P, Dolgalev I, Thomas S, Aminova O, Huberman K, Cheng J, Viale A, Socci ND, Heguy A, Cherry A, Vance G, Higgins RR, Ketterling RP, Gallagher RE, Litzow M, van den Brink MR, Lazarus HM, Rowe JM, Luger S, Ferrando A, Paietta E, Tallman MS, Melnick A, Abdel-Wahab O, Levine RL: Prognostic relevance of integrated genetic profiling in acute myeloid leukemia. N Engl J Med 2012, 366:1079−1089
7. Losman JA, Looper RE, Koivunen P, Lee S, Schneider RK, McMahon C, Cowley GS, Root DE, Ebert BL, Kaelin WG Jr: (R)-2-hydroxyglutarate is sufficient to promote leukemogenesis and its effects are reversible. Science 2013, 339:1621−1625
8. Chotirat S, Thongnoppakhun W, Wanachiwanawin W, Auewarakul CU: Acquired somatic mutations of isocitrate dehydrogenases 1 and 2 (IDH1 and IDH2) in preleukemic disorders. Blood Cells Mol Dis 2015, 54:286−291
9. Lin TL, Nagata Y, Kao HW, Sanada M, Okuno Y, Huang CF, Liang DC, Kuo MC, Lai CL, Lee EH, Shih YS, Tanaka H, Shiraishi Y, Chiba K, Lin TH, Wu JH, Miyano S, Ogawa S, Shih LY: Clonal leukemic evolution in myelodysplastic syndromes with TET2 and IDH1/2 mutations. Haematologica 2014, 99:28−36
10. Welch JS, Ley TJ, Link DC, Miller CA, Larson DE, Koboldt DC, et al: The origin and evolution of mutations in acute myeloid leukemia. Cell 2012, 150:264−278
11. Figueroa ME, Abdel-Wahab O, Lu C, Ward PS, Patel J, Shih A, Li Y, Bhagwat N, Vasanthakumar A, Fernandez HF, Tallman MS, Sun Z, Wolniak K, Peeters JK, Liu W, Choe SE, Fantin VR, Paietta E, Lowenberg B, Licht JD, Godley LA, Delwel R, Valk PJ, Thompson CB, Levine RL, Melnick A: Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. Cancer Cell 2010, 18:553−567
12. Paschka P, Schlenk RF, Gaidzik VI, Habdank M, Kronke J, Bullinger L, Spath D, Kayser S, Zucknick M, Gotze K, Horst HA, Germing U, Dohner H, Dohner K: IDH1 and IDH2 mutations are frequent genetic alterations in acute myeloid leukemia and confer adverse prognosis in cytogenetically normal acute myeloid leukemia with NPM1 mutation without FLT3 internal tandem duplication. J Clin Oncol 2010, 28:3636−3643
13. Popovici-Muller J, Saunders JO, Salituro FG, Travins JM, Yan S, Zhao F, Gross S, Dang L, Yen KE, Yang H, Straley KS, Jin S, Kunii K, Fantin VR, Zhang S, Pan Q, Shi D, Biller SA, Su SM: Discovery of the first potent inhibitors of mutant IDH1 that lower tumor 2-HG in vivo. ACS Med Chem Lett 2012, 3:850−855
14. Rohle D, Popovici-Muller J, Palaskas N, Turcan S, Grommes C, Campos C, Tsoi J, Clark O, Oldrini B, Komisopoulou E, Kunii K, Pedraza A, Schalm S, Silverman L, Miller A, Wang F, Yang H, Chen Y, Kernytsky A, Rosenblum MK, Liu W, Biller SA, Su SM, Brennan CW, Chan TA, Graeber TG, Yen KE, Mellinghoff IK: An inhibitor of mutant IDH1 delays growth and promotes differentiation of glioma cells. Science 2013, 340:626−630

15. Wang F, Travins J, DeLaBarre B, Penard-Lacronique V, Schalm S, Hansen E, Straley K, Kernytsky A, Liu W, Gliser C, Yang H, Gross S, Artin E, Saada V, Mylonas E, Quivoron C, Popovici-Muller J, Saunders JO, Salituro FG, Yan S, Murray S, Wei W, Gao Y, Dang L, Dorsch M, Agresta S, Schenkein DP, Biller SA, Su SM, de Botton S, Yen KE: Targeted inhibition of mutant IDH2 in leukemia cells induces cellular differentiation. Science 2013, 340:622−626
16. Hansen E, Quivoron C, Straley K, Lemieux RM, Popovici-Muller J, Sadrzadeh H, Fathi AT, Gliser C, David M, Saada V, Micol J-B, Bernard O, Dorsch M, Yang H, Su M, Agresta S, de Botton S, Penard-Lacronique V, Yen K: AG-120, an oral, selective, first-in-class, potent inhibitor of mutant IDH1, reduces intracellular 2HG and induces cellular differentiation in TF-1 R132H cells and primary human IDH1 mutant AML patient samples treated ex vivo. Blood 2014, 124:3734
17. Shih AH, Shank KR, Meydan C, Intlekofer AM, Ward P, Thompson CB, Melnick AM, Travins J, Straley K, Gliser C, Yen K, Levine RL: AG-221, a small molecule mutant IDH2 inhibitor, remodels the epigenetic state of IDH2-mutant cells and induces alterations in self-renewal/differentiation in IDH2-mutant AML model in vivo. Blood 2014, 124:437
18. Quivoron C, David M, Straley K, Travins J, Kim H, Chen Y, Zhu D, Saada V, Bawa O, Opolon P, Polrot M, Micol J-B, Willekens C, Bernard O, Yang H, Agresta S, de Botton S, Yen K, Penard-Lacronique V: AG-221, an oral, selective, first-in-class, potent IDH2-R140Q mutant inhibitor, induces differentiation in a xenotransplant model. Blood 2014, 124:3735
19. Stein EM, Altman JK, Collins R, DeAngelo DJ, Fathi AT, Flinn I, Frankel A, Levine RL, Medeiros BC, Patel M, Pollyea DA, Roboz GJ, Stone RM, Swords RT, Tallman MS, Agresta S, Fan B, Yang H, Yen K, de Botton S: AG-221, an oral, selective, first-in-class, potent inhibitor of the IDH2 mutant metabolic enzyme, induces durable remissions in a phase I study in patients with IDH2 mutation positive advanced hematologic malignancies. Blood 2014, 124:115
20. Fan B, Chen Y, Wang F, Yen K, Utley L, Almon C, Biller S, Agresta S, Yang H: Evaluation of pharmacokinetic-pharmacodynamic (PKPD) relationship of an oral, selective, first-in-class, potent IDH2 inhibitor, AG-221, from a phase 1 trial in patients with advanced IDH2 mutant positive hematologic malignancies. Blood 2014, 124:3737
21. Zheng Z, Liebers M, Zhelyazkova B, Cao Y, Panditi D, Lynch KD, Chen J, Robinson HE, Shim HS, Chmielecki J, Pao W, Engelman JA, Iafrate AJ, Le LP: Anchored multiplex PCR for targeted next-generation sequencing. Nat Med 2014, 20:1479−1484
22. Li H, Durbin R: Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 2009, 25:1754−1760
23. Cibulskis K, Lawrence MS, Carter SL, Sivachenko A, Jaffe D, Sougnez C, Gabriel S, Meyerson M, Lander ES, Getz G: Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. Nat Biotechnol 2013, 31:213−219
24. Westesson O, Skinner M, Holmes I: Visualizing next-generation sequencing data with JBrowse. Brief Bioinform 2013, 14:172−177
25. Yan H, Parsons DW, Jin G, McLendon R, Rasheed BA, Yuan W, Kos I, Batinic-Haberle I, Jones S, Riggins GJ, Friedman H, Friedman A, Reardon D, Herndon J, Kinzler KW, Velculescu VE, Vogelstein B, Bigner DD: IDH1 and IDH2 mutations in gliomas. N Engl J Med 2009, 360:765−773
26. Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Siu IM, Gallia GL, Olivi A, McLendon R, Rasheed BA, Keir S, Nikolskaya T, Nikolsky Y, Busam DA, Tekleab H, Diaz LA Jr, Hartigan J, Smith DR, Strausberg RL, Marie SK, Shinjo SM, Yan H, Riggins GJ, Bigner DD, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu VE, Kinzler KW: An integrated genomic analysis of human glioblastoma multiforme. Science 2008, 321:1807−1812
27. Kerr DA, Lopez HU, Deshpande V, Hornicek FJ, Duan Z, Zhang Y, Rosenberg AE, Borger DR, Nielsen GP: Molecular distinction of chondrosarcoma from chondroblastic osteosarcoma through IDH1/2 mutations. Am J Surg Pathol 2013, 37:787−795
28. Arai M, Nobusawa S, Ikota H, Takemura S, Nakazato Y: Frequent IDH1/2 mutations in intracranial chondrosarcoma: a possible diagnostic clue for its differentiation from chordoma. Brain Tumor Pathol 2012, 29:201−206
29. Amary MF, Bacsi K, Maggiani F, Damato S, Halai D, Berisha F, Pollock R, O'Donnell P, Grigoriadis A, Diss T, Eskandarpour M, Presneau N, Hogendoorn PC, Futreal A, Tirabosco R, Flanagan AM: IDH1 and IDH2 mutations are frequent events in central chondrosarcoma and central and periosteal chondromas but not in other mesenchymal tumours. J Pathol 2011, 224:334−343
30. Borger DR, Tanabe KK, Fan KC, Lopez HU, Fantin VR, Straley KS, Schenkein DP, Hezel AF, Ancukiewicz M, Liebman HM, Kwak EL, Clark JW, Ryan DP, Deshpande V, Dias-Santagata D, Ellisen LW, Zhu AX, Iafrate AJ: Frequent mutation of isocitrate dehydrogenase (IDH)1 and IDH2 in cholangiocarcinoma identified through broad-based tumor genotyping. Oncologist 2012, 17:72−79
31. Fathi AT, Sadrzadeh H, Comander AH, Higgins MJ, Bardia A, Perry A, Burke M, Silver R, Matulis CR, Straley KS, Yen KE, Agresta S, Kim H, Schenkein DP, Borger DR: Isocitrate dehydrogenase 1 (IDH1) mutation in breast adenocarcinoma is associated with elevated levels of serum and urine 2-hydroxyglutarate. Oncologist 2014, 19:602−607
32. Kosmider O, Gelsi-Boyer V, Slama L, Dreyfus F, Beyne-Rauzy O, Quesnel B, Hunault-Berger M, Slama B, Vey N, Lacombe C, Solary E, Birnbaum D, Bernard OA, Fontenay M: Mutations of IDH1 and IDH2 genes in early and accelerated phases of myelodysplastic syndromes and MDS/myeloproliferative neoplasms. Leukemia 2010, 24:1094−1096
33. Chaturvedi A, Araujo Cruz MM, Jyotsana N, Sharma A, Yun H, Gorlich K, Wichmann M, Schwarzer A, Preller M, Thol F, Meyer J, Haemmerle R, Struys EA, Jansen EE, Modlich U, Li Z, Sly LM, Geffers R, Lindner R, Manstein DJ, Lehmann U, Krauter J, Ganser A, Heuser M: Mutant IDH1 promotes leukemogenesis in vivo and can be specifically targeted in human AML. Blood 2013, 122:2877−2887
34. Pirozzi CJ, Reitman ZJ, Yan H: Releasing the block: setting differentiation free with mutant IDH inhibitors. Cancer Cell 2013, 23:570−572
35. Quail MA, Smith M, Coupland P, Otto TD, Harris SR, Connor TR, Bertoni A, Swerdlow HP, Gu Y: A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. BMC Genomics 2012, 13:341