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Recurrent *DNMT3A* Mutations in Patients with Myelodysplastic Syndromes

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Abstract

Alterations in DNA methylation have been implicated in the pathogenesis of myelodysplastic syndromes (MDS), although the underlying mechanism remains largely unknown. Methylation of CpG dinucleotides is mediated by DNA methyltransferases, including DNMT1, DNMT3A, and DNMT3B. *DNMT3A* mutations have recently been reported in patients with *de novo* acute myeloid leukemia (AML), providing a rationale for examining the status of *DNMT3A* in MDS samples. Here, we report the frequency of *DNMT3A* mutations in patients with *de novo* MDS, and their association with secondary AML. We sequenced all coding exons of *DNMT3A* using DNA from bone marrow and paired normal cells from 150 patients with MDS and identified 13 heterozygous mutations with predicted translational consequences in 12/150 patients (8.0%). Amino acid R882, located in the methyltransferase domain of *DNMT3A*, was the most common mutation site, accounting for 4/13 mutations. *DNMT3A* mutations were expressed in the majority of cells in all tested mutant samples regardless of blast counts, suggesting that *DNMT3A* mutations occur early in the course of MDS. Patients with *DNMT3A* mutations had worse overall survival compared to patients without *DNMT3A* mutations (p=0.005) and more rapid progression to AML (p=0.007), suggesting that *DNMT3A* mutation status may have prognostic value in *de novo* MDS.

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AUTHOR CONTRIBUTIONS MW, LD, TL, and TG designed the study. DS, JS, MG, MM, RF, HS, JKV, and MO performed research and generated data. MW, LD, DS, JS, JB, PW, JD, EM, RW, TL, and TG analyzed data. MW, LD, DS, and TG wrote the paper.

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Keywords

myelodysplastic syndrome; DNMT3A; mutation

INTRODUCTION

Cancer initiation and progression is caused by genetic and epigenetic alterations in DNA. Cancer genomes are characterized by global DNA hypomethylation with concomitant hypermethylation of gene promoter regions. The skewing of cytosine methylation may contribute to tumor development due to decreased expression of critical tumor suppressor genes that are hypermethylated in cancer cells.[1-3] Currently, the underlying mechanism of altered DNA methylation in cancer genomes and the critical target genes affected by methylation remain largely unknown.[2]

Both acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS) genomes contain different global DNA methylation patterns compared to normal bone marrow cells and compared to each other, suggesting that there may be methylation-specific gene alterations that contribute to these diseases.[4-6] Indirect evidence supporting the hypothesis that DNA methylation changes may be important in AML and MDS genomes comes from the observation that cytosine analog drugs that interfere with methylation (5-azacytidine and decitabine (5-aza-2'-deoxycytidine)) have clinical activity in these diseases.[7-10] However, inhibition of DNA methyltransferases (DNMTs), which are responsible for the covalent linking of methyl groups to the CpG dinucleotide, is only one potential mechanism of action of 5-azacytidine and decitabine.

DNMTs are critical for establishing and maintaining CpG methylation. The major DNMTs with methyltransferase activity in humans are DNMT1, DNMT3A, and DNMT3B. DNMT3A and DNMT3B are the dominant DNA methyltransferases involved in *de novo* DNA methylation and act independent of replication, while DNMT1 acts predominantly during replication to maintain hemimethylated DNA, although their precise roles in cancer cells are less well defined.[11] The link between DNMTs and cancer comes from observations that elevated levels of DNMT proteins and activities occur in many types of cancers, and overexpression of DNMT1 in cells can lead to transformation (reviewed in[3]). In mice, altered DNMTs have variable effects on cancer. The number of colonic polyps that occur in compound *Dnmt1* heterozygous knock-out/Apc^{min} mice is reduced, while T-cell lymphomas are increased in mice carrying a *Dnmt1* hypomorphic allele (~10% normal levels).[12, 13] Inherited mutations in *DNMT3B* are the most common cause of the ICF (immunodeficiency, centrosome instability, and facial anomalies) syndrome in humans.[14] ICF patients have loss of methylated pericentromeric sequences, resulting in chromosomal instability in lymphocytes and a severe immunodeficiency. ICF patients are not prone to cancer, although they tend to die at an early age from infections.[14] Collectively, the data suggest that alterations in DNMTs are associated with cancer, but direct evidence in humans has been lacking.

Our group has recently found that *DNMT3A* mutations are common (22% frequency) in *de novo* AML and are associated with poor survival,[15] providing a rationale for screening

patients with *de novo* MDS and secondary AML for mutations in *DNMT3A*. In this study, we performed total exonic resequencing of *DNMT3A* using DNA from 150 *de novo* MDS patients, of which 46 developed secondary AML, and identified 13 somatic mutations in 12 patients (8.0% of cases).

MATERIALS AND METHODS

Patients

Adults diagnosed with myelodysplastic syndrome and no prior history of exposure to chemotherapy and/or radiotherapy were included in the study. A punch biopsy of normal skin and a bone marrow aspirate were obtained with informed consent in accordance with a tissue acquisition protocol approved by the Human Research Protection Office at the Washington University School of Medicine.

DNA Samples

Genomic DNA samples were extracted from the skin and unfractionated bone marrow specimens and then subjected to whole genome amplification, as previously described.[16]

Sequencing

Primers were designed to amplify the coding sequences and splice sites of *DNMT3A* (provided in[15]). Amplicons were sequenced using BigDye chemistry and analyzed on an ABI 3730 sequencer. Sequence variants, including single nucleotide variants (SNVs) and insertion/deletion events (indels) were called by The Genome Center's mutational profiling pipeline and manually reviewed. Potential somatic mutations (present in tumor, but not in the matched normal sample) were confirmed by independent PCR and sequencing.

Expression profiling and cDNA analysis

$CD34^+$ cell purification, RNA preparation, hybridization to the Affymetrix U133plus2 arrays, and analysis of data were performed as previously described for these samples.[16] cDNA was prepared according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA). Primers for cDNA amplification and sequencing crossed exon boundaries in all cases.

Statistical Analysis

Differences in clinical characteristics of patients with or without *DNMT3A* mutations were assessed using the Fisher's exact or Mann-Whitney tests. Survival data are current to 01/06/2011. Survival curves were generated using the Kaplan-Meier method and differences were assessed by Log-Rank analysis. The analyses were generated using SAS/STAT software (Version 9.2 of SAS System for Windows, SAS Institute Inc., Cary, NC). Association testing of germline polymorphisms was performed using the Fisher's exact or Chi-square tests. The predicted severity of *DNMT3A* mutations with translational consequences was evaluated using the SIFT and Polyphen2 algorithms.[17, 18]

RESULTS

Patient Characteristics

We identified a total of 150 patients with de novo MDS who had paired bone marrow (tumor) and skin (normal) DNA samples available. Cases were classified according to the French-American-British (FAB) system upon banking of their bone marrow specimens. The diagnoses included refractory anemia (RA; n=67), RA with ringed sideroblasts (RARS; n=5), RA with excess blasts (RAEB; n=72), and RA with excess blasts in transformation (RAEB-T; n=6) (Table 1). The median International Prognostic Scoring System (IPSS) score was 1 (range 0-3), and the median myeloblast count was 5 (range 0-28%).

DNMT3A mutations in MDS

We designed and validated 28 primer pairs covering the coding sequences and splice sites of all 23 exons for *DNMT3A*. To ensure comprehensive coverage of *DNMT3A* we performed bidirectional sequencing of all amplicons in the 150 paired DNA samples, producing 16,890 reads. At least one sequencing read (forward or reverse) was obtained for 83% of the 460.1 kbp of target sequence. Only two amplicons failed to produce adequate sequence coverage (amplicon 0001788_083, covering 5' UTR in exon 1;amplicon 0001788_055, covering exon 14). The Genome Center's analysis pipeline[16] was used to identify sequence variants and we restricted our analysis to changes with predicted translational consequences (i.e., nonsynonymous substitutions, insertion/deletions, and splice site nucleotide changes) and identified 13 mutations in 12 bone marrow samples from 150 MDS samples (8.0% of cases) (Table 2). All 13 mutations were confirmed as somatic by repeat sequencing of the paired tumor/normal DNA samples (Supplemental Figure 1). All 13 mutations were heterozygous and 7 mutations have not been previously described.[15, 19] One patient (UPN 379929) had two mutations in different exons. Four of the 13 mutations occurred at amino acid R882, which was also the most common mutation site in AML.[15, 19] Another mutation (at amino acid P904) is also recurrent in MDS and was detected in one *de novo* AML patient.[15] To identify tumor samples that might contain a somatic deletion or copy number neutral loss of heterozygosity (LOH) involving the *DNMT3A* gene, we used the Affymetrix SNP array 6.0 to analyze three samples containing two or more consecutive SNPs that appeared to be heterozygous in the germline (skin) and homozygous in the paired tumor sample. No deletions or LOH events were detected in these three samples (suggesting that the apparent LOH at these isolated SNPs were sequencing artifacts).

There was no association between mutation detection and the myeloblast count of the banked bone marrow specimen (median blast count was 5% in patients without *DNMT3A* mutations, vs. 7% in patients with *DNMT3A* mutations, p=0.31), suggesting that mutations were not missed due to the cellular heterogeneity in the samples. In fact, the mutant allele trace peak heights were consistently equal to the normal allele trace peak heights for each sample and independent of the myeloblast count (p=0.40) (Supplemental Figure 1). This supports our previous observation that the myeloblast count underestimates the size of the mutant clone in MDS[16] and further suggests that hematopoiesis is clonal in early stage MDS and that the *DNMT3A* mutations are acquired early in MDS pathogenesis.

Consequences of DNMT3A mutations

Mutation of the R882 position in *DNMT3A* has previously been shown to reduce the methyltransferase activity of the protein and reduces its ability to bind DNA.[19, 20] The functional consequences of the remaining mutations has not been studied, therefore we applied a computational algorithm to assess the consequences of the remaining missense mutations. The frameshift and nonsense mutations were not testable using this approach. Of the remaining 6 mutations, SIFT uniformly predicted the mutations to be damaging (Table 2), whereas PolyPhen2 predicted all but one, S714C, to be damaging.[17, 18] In addition to the R882 position, only the P904 position has been previously reported to be mutated.[15] The locations of the remaining missense mutations fall within the methyltransferase domain (Figure 1). *DNMT3A* mRNA expression measured by microarray was similar in normal CD34+ bone marrow cells and in CD34-selected cells from MDS samples with (n=3) or without *DNMT3A* mutations (n=20) (Supplemental Figure 2). In 8 samples from MDS patients with *DNMT3A* mutations, cDNA sequencing detected both the mutant and wildtype alleles, suggesting that both were expressed (Table 2, Supplemental Figure 3).

Clinical significance of DNMT3A mutations in MDS

The clinical characteristics of the 12 patients with *DNMT3A* mutations were similar to that of the 138 patients without mutations (Table 1). *DNMT3A* mutations occurred in all FAB subtypes tested and were not associated with a specific karyotype. Six patients with mutant *DNMT3A* had low or INT-1 IPSS scores, and 6 patients had INT-2 or high scores, indicating that mutations occurred in patients that were low and high risk for AML progression based on their IPSS. We compared the overall survival (OS) of 12 patients with *DNMT3A* mutations vs. 138 patients without a mutation and observed significantly worse OS in patients with mutations (log-rank p=0.005), although our sample size is small and transplantation status was not considered (Figure 2A). There was also worse event-free survival (EFS) for patients with mutations (log-rank p=0.009) (Figure 2B). 7/12 (58%) of patients with a *DNMT3A* mutation progressed to AML, compared to 39/138 (28%) of patients without a mutation (Figure 2C, log-rank p=0.007). The median survival of patients with a mutation was 433 days compared to 965 days for patients without a mutation (Table 1, log-rank p=0.005). A multivariate analysis for outcomes could not be performed due to the small sample size of patients with mutations, indicating that a larger cohort from a clinical trial will be needed to definitively address the effect of *DNMT3A* mutations on outcomes. 8 of 12 MDS patients with *DNMT3A* mutations received some treatment with the DNMT inhibitors, azacitidine or decitabine (Supplemental Table 1). The small sample size and non-uniform treatment precludes an assessment of the impact of *DNMT3A* mutations on response to DNMT inhibitors in this cohort.

No novel coding SNPs were detected in *DNMT3A*. We examined the allele and genotype frequencies of two known synonymous SNPs in our cohort (rs2276598, rs41284843) and found no significant differences, comparing either MDS patients to race-matched control subjects (Supplemental Table 2) or comparing MDS patients with *DNMT3A* mutations to MDS patients without *DNMT3A* mutations (Supplemental Table 3).

DISCUSSION

In this study, we identified 13 somatic mutations in the DNA methyltransferase gene *DNMT3A* in 12/150 (8.0%) *de novo* MDS patients. The mutations were heterozygous and were computationally predicted to alter the protein function. A mutation hot spot at amino acid R882 occurred in 4 MDS samples and is located in the methyltransferase domain of the protein. The mutations occurred in all MDS FAB subtypes (excluding CMML, which was not examined) as well as IPSS scores ranging from 0-3. MDS patients harboring a mutation in *DNMT3A* appear to have worse overall survival and more rapid progression to AML, suggesting that this mutation may have prognostic value in MDS.

DNMT3A, in conjunction with DNMT3B, is critical for proper *de novo* DNA methylation and development of mammals. Mice that are homozygous null for *Dnmt3a*$^{-/-}$ are born runted, die of aganglionic megacolon, and males lack germ cells.[21] Conditional knockout of *Dnmt3a* in the germ cells showed lack of methylation of normally imprinted genes *H19* and *Dlk1-Gtl2*.[22] *Dnmt3b*$^{-/-}$ mice die at 9.5 days post coitus (dpc) and display demethylation of minor satellite repeats, and the double mutant *Dnmt3a*$^{-/-}$/*Dnmt3b*$^{-/-}$ mice are embryonic lethal at day 8.5 dpc with global demethylation.[21] Conditional deletion of *Dnmt3a*$^{-/-}$ or *Dnmt3b*$^{-/-}$ individually in murine Kit^{+}/Lineage^{-}/Sca^{+} (KLS) bone marrow cells did not affect myeloid colony formation, multilineage differentiation, or self-renewal, but did alter methylation patterns in differentiating hematopoietic cells.[23] In contrast, *Dnmt3a*$^{-/-}$/*Dnmt3b*$^{-/-}$ KLS cells were unable to contribute to long-term hematopoiesis in a murine transplantation model, suggesting that either *Dnmt3a*$^{-/-}$ or *Dnmt3b*$^{-/-}$ is necessary to maintain hematopoietic stem cell self-renewal.[23] It remains unclear how acquired mutations in *DNMT3A* may alter hematopoiesis, but murine knockout models suggest that simple loss of function may not be the predominant mechanism. In fact, overexpression of a *Dnmt3a* isoform (*Dnmt3a1*) was recently shown to increase penetrance and reduce latency of *PML/RARA*-initiated myeloid leukemia in a murine transplantation model.[24]

All the *DNMT3A* mutations in MDS are heterozygous and it is not known whether these nucleotide mutations result in a loss of function, gain of function, or have a dominant negative property. The missense mutations involve highly conserved residues within the methyltransferase domain of *DNMT3A*, suggesting that they may not be simple loss of function mutations but may confer a novel protein function. In contrast, the frameshift and nonsense mutations occur upstream of the methyltransferase domain and are likely to be loss of function mutations. Although previous reports suggest that the R882 mutation has loss of function properties,[19, 20] the R882 mutant was not tested in the presence of the wild-type allele, which may alter the mutant phenotype. The DNA and cDNA sequencing data revealed that the mutant *DNMT3A* allele was present and expressed in nearly all cells in the MDS samples, even though the myeloblast count was far less than <30% for most samples. This suggests that *DNMT3A* mutations are very early genetic events in MDS, and may confer a clonal advantage to cells bearing a mutation in this gene. Ultimately, modeling these mutations in an organism will be required to understand their transforming potential.

This report and the recent identification of mutations and deletions in histone modifying genes (*UTX, ASXL1, TET2, EZH2*) in MDS patients provides compelling evidence that

epigenetic alterations contribute to MDS pathogenesis.[25-32] *UTX* is a histone demethylase, *EZH2* is a histone methyltransferase, *ASXL1* helps recruit polycomb and trithorax complexes to chromatin, and *TET2* converts methylcytosine to hydroxymethylcytosine.[26, 33-37] Many, but not all, of the somatic variants in *TET2*, *ASXL1*, and *EZH2* result in frameshifts and nonsense mutations, suggesting that they result in loss of function. In addition, *ATRX* is a member of the SWI/SNF group of proteins that associate with chromatin and *ATRX* is mutated in a rare form of MDS that is associated with alpha-thalassemia (ATMDS).[38] Given that these genes affect epigenetic pathways, it will be important to know whether mutations in these genes are mutually exclusive, or whether the genes could have nonoverlapping functional consequences. Likewise, it is unknown whether mutations in different genes will impact the expression of a common set of genes, or whether each mutation results in a unique gene expression signature. These questions will need to be addressed using larger cohorts of patients that are comprehensively genotyped for mutations in all the genes.

Collectively, the growing list of somatic mutations in MDS that affect histone modifying proteins, and now the DNA methyltransferase gene *DNMT3A*, may provide a potential mechanism for the clinical activity observed in some MDS patients treated with histone deacetylase inhibitors and DNA methyltransferase inhibitors. The response to hypomethylating agents will be an important association to explore in large retrospective studies, and prospectively in future clinical trials. The potential utility of *DNMT3A* mutation status for determining prognosis in *de novo* MDS is compelling, but will need to be validated in larger clinical studies. If reproduced, *DNMT3A* mutation status could help risk stratify *de novo* MDS patients for more aggressive treatment early in their disease course, such as allogeneic transplant for eligible candidates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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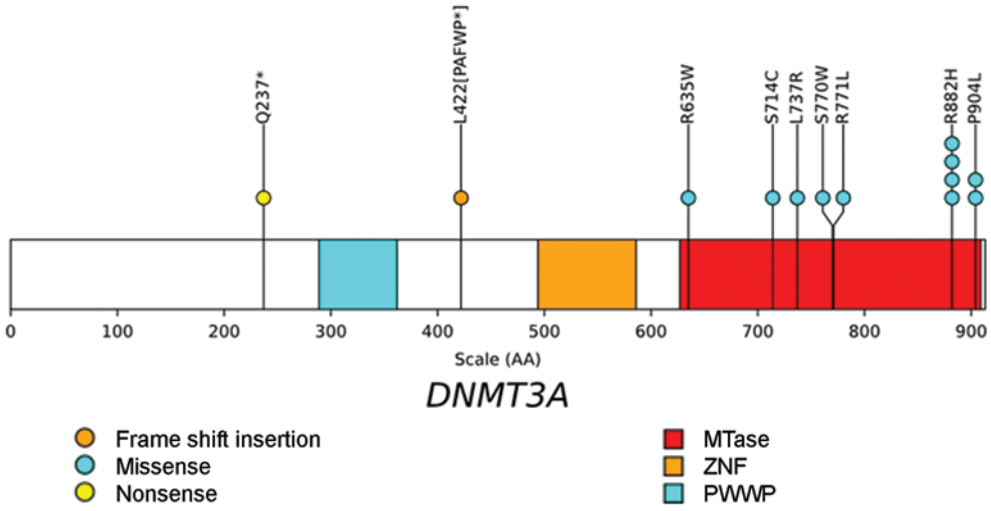

Figure 1. Location of mutations in the *DNMT3A* gene

The positions and predicted translational consequences of *DNMT3A* mutations detected in 150 MDS samples are shown. Each circle is a mutation found in one patient. One patient has two mutations in *DNMT3A*. The conserved proline-tryptophan-tryptophan-proline (PWWP), zinc finger (ZNF), and methyltransferase (MTase) domains are shown.

*, stop codon.

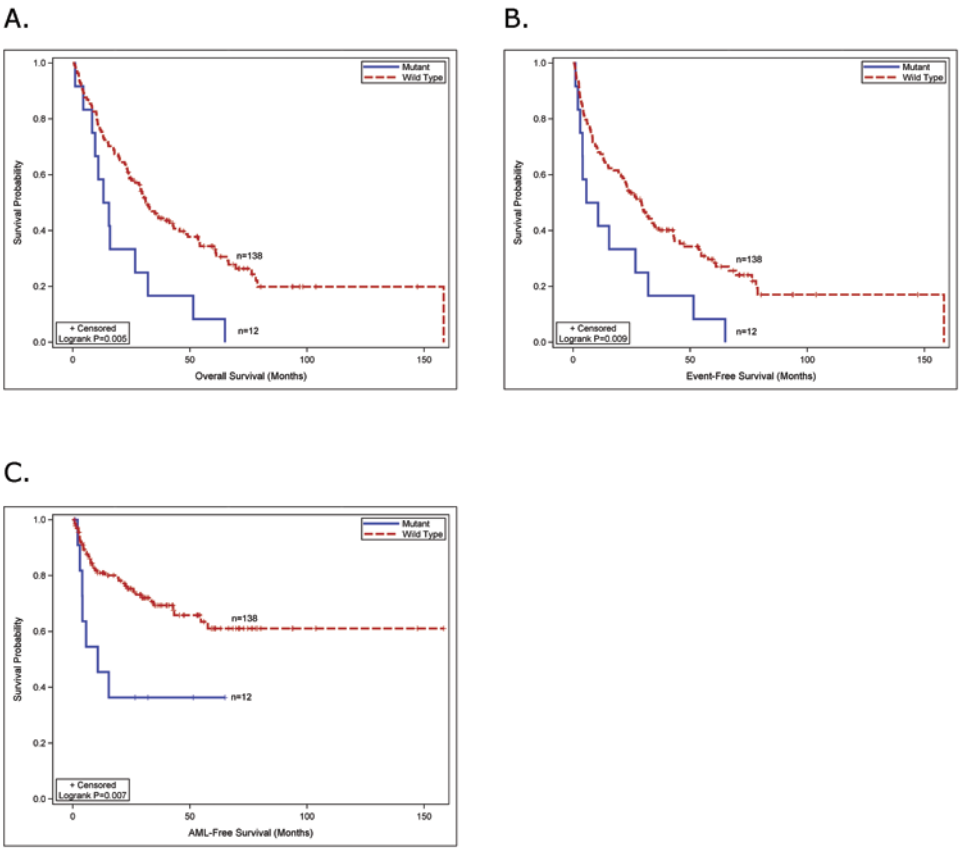

Figure 2. Survival analysis of MDS patients with *DNMT3A* mutations

(A) the overall survival of MDS patients with *DNMT3A* mutations compared to MDS patients without *DNMT3A* mutations. (B) the event-free survival of MDS patients with or without *DNMT3A* mutations. (C) progression to AML in MDS patients with or without *DNMT3A* mutations.

Table 1

Patient Characteristics

	DNMT3A wildtype no. (%)	*DNMT3A* mutant no. (%)	P-value
Total (n=150)	138 (92.0)	12 (8.0)	
Age at diagnosis	60	69	0.03†
range (median)	20-87 (62)	39-86 (71)	
Median Survival	965 days	433 days	0.02*
			0.005**
Gender			
Male	83 (60)	9 (75)	0.31
Female	55 (40)	3 (25)	
FAB subtype			
RA	63 (45)	4 (33)	0.30
RARS	4 (3)	1 (8)	
RAEB	66 (48)	6 (50)	
RAEB-T	5 (4)	1 (8)	
CMML	0 (0)	0 (0)	
Blood count mean			
Hb (g/dL)	10.1	9.7	0.50†
ANC (K/mcL)	3.0	2.2	0.90†
Plt (K/mcL)	97.0	68.3	0.85†
Cytogenetics			
normal	62	7	0.39
-Y only	2	0	1.00
-5, del(5q)	28	2	1.00
-7, del(7q)	16	1	1.00
-17, del(17q)	4	1	0.34
-20, del(20q)	11	1	1.00
+8	17	1	1.00
complex (≥ 3)	34	2	0.74
other	17	1	1.00
not available	4	0	1.00
IPSS			
low	22 (17)	1 (8)	0.82
INT-1	55 (41)	5 (42)	
INT-2	36 (27)	3 (25)	
high	20 (15)	3 (25)	
not available	5	0	

all comparisons performed using two-sided Fisher's Exact Test, except:

† two-sided Mann-Whitney *U* test;

*Wilcoxon-Gehan;

**log-rank.

Hb, hemoglobin; ANC, absolute neutrophil count; Plt, platelet count

Table 2

DNMT3A Mutations in MDS Patients.

UPN	Genome coordinate	Nucleotide change	Mutation in cDNA?	Zygosity	Exon	Protein change	SIFT
176267	2:25,310,746	G>A	Yes	Het	23	R882H	Damaging
317598	2:25,310,746	G>A	Yes	Het	23	R882H	Damaging
457721	2:25,310,746	G>A	NE	Het	23	R882H	Damaging
958595	2:25,310,746	G>A	NE	Het	23	R882H	Damaging
319179	2:25,310,680	C>T	Yes	Het	23	P904L	Damaging
379929	2:25,310,680	C>T	NE	Het	23	P904L	Damaging
379929	2:25,316,787	T>G	NE	Het	19	L737R	Damaging
988428	2:25,316,685	G>T	Yes	Het	19	R771L	Damaging
989739	2:25,316,688	C>G	Yes	Het	19	S770W	Damaging
207282	2:25,317,045	C>G	Yes	Het	18	S714C	Damaging
917011	2:25,320,304	C>T	Yes	Het	16	R635W	Damaging
690100	2:25,323,007	INS [GGCCCTTAGGGCCAGAAGGCTG]	NE	Het	10	L422 [PAFWP*]	NE
975079	2:25,324,556	C>T	Yes	Het	7	Q237*	NE

UPN, unique patient number; Het, heterozygous; INS, insertion; SIFT, sorts intolerant from tolerant substitutions; NE, not evaluable.

Note: *DNMT3A* is transcribed from the “minus” strand.