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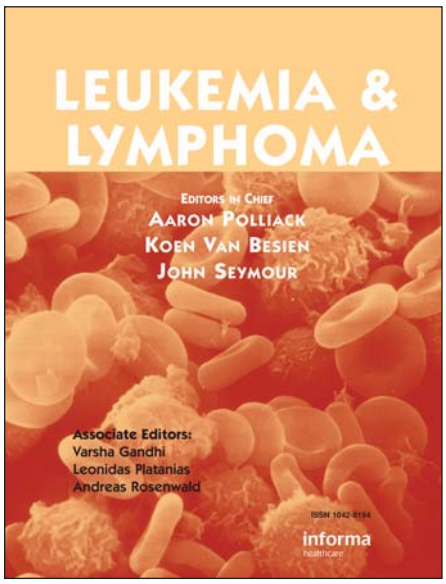

DNMT3A Mutations at the R882 hotspot are only found in the major clones of Acute Myeloid Leukemia

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Letter to the Editor

DNMT3A Mutations at the R882 hotspot are only found in the major clones of Acute Myeloid Leukemia

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Short title: DNMT3A mutations in AML

Acute myeloid leukemia (AML) arises due to the clonal selection and expansion of cells carrying advantageous acquired genetic mutations.[1] Recent whole genome and whole exome sequencing studies of de novo and relapsed AML samples have revealed the clonal architecture of AML.[2,3,4] These studies have demonstrated that the genetic evolution of AML is a dynamic process in which new mutations arise stochastically leading to progressive clonal expansion until the disease becomes clinically manifest.[2] Understanding this paradigm is paramount to improving therapeutic strategies in AML since only treatments targeted at founding clones will lead to eradication of all leukemic cells and potential cure.[1] Hence, one of the goals of current research efforts in AML should be to identify the initiating genetic lesions. Using a highly sensitive method that has potential application as a marker of minimal residual disease (MRD), we demonstrate that, when present, DNMT3A mutations at the R882 hotspot are found at levels representative of the blast count, i.e. the major clone, and more importantly is not detected at low levels, i.e. in sub-clones, in apparently DNMT3A "wild-type" AMLs. This gives weight to the theory that DNMT3A mutations are candidate initiating lesions in AML.

DNMT3A is a de novo DNA methyltransferase that is recurrently mutated in approximately 22% of AML cases and 34% of those with intermediate-risk normal cytogenetic profiles.[5,6] Unlike many other AML gene mutations such as FLT3, DNMT3A mutations do not appear to occur in combination with favorable-risk chromosomal translocations t(8;21), t(15;17) or inv(16) suggesting that DNMT3A mutations represent a distinct alternative route to leukemogenesis.[4,5,7] Importantly, DNMT3A mutations confer an adverse outcome in patients with AML, with median survival of 12.3 versus 41.1 months,[5] and therefore DNMT3A represents an important gene in prognostic profiling in AML.[7-10] Interestingly, patients diagnosed with DNMT3A-mutated AML appear to have improved outcomes with dose-intensified anthracycline induction regimes suggesting that early detection of this mutation has important therapy-related implications as well as impacting on prognosis.[9,11] For this reason we have recently developed a rapid screening test for detection of DNMT3A mutations at the R882 codon mutational hotspot in exon 23, where 60% of the mutations arise.[12] We have since modified this assay to serve as a highly sensitive MRD test for monitoring response to treatment in patients with DNMT3A-mutated AML.

Using deep sequencing approaches to define mutational clonal evolution in AML, Ding et al. found DNMT3A mutations in 2 out of 8 samples.[2] In both cases, the variant allele was present at high frequency in the sample suggesting the DNMT3A mutation was present in all leukemic cells and therefore likely to have arisen in the founding clone. Using mutational clustering for one of the DNMT3A-mutated samples, the authors were able to dissect out four distinct clonal populations according to each clone's repertoire of mutations and thereby estimate their relative sizes. They calculated the major clone size was 93.72% of total cells and contained three minor and genetically distinct sub-clones of 53.12%, 29.04% and 5.10% respectively with the smallest clone giving rise to the major clone at time of relapse. Assuming this particular AML sample reflects

the typical clonal heterogeneity of the disease at time of diagnosis, and moreover that gene mutations are acquired at random, we reasoned that DNMT3A mutations should have an equal likelihood of arising at each step of clonal evolution. If this is so, then we would expect to find minor DNMT3A-mutated clones arising more frequently than major clones at time of diagnosis and also newly arising DNMT3A mutations at time of relapse.

We sought to test the hypothesis that mutations in DNMT3A are initiating events in AML by showing that low level mutated sub-clones are never found in apparently DNMT3A wild-type AMLs. Hitherto, most studies have looked at DNMT3A-mutated AMLs and attempted to show that the mutation is present in all leukemic cells.[5,13] Other studies have demonstrated that DNMT3A-mutated AMLs consistently retain the mutation at relapse unlike notable other gene mutations such as FLT3.[14,15] However, we elected instead to focus on AML samples that were wild-type for DNMT3A at codon R882 by standard Sanger sequencing of PCR-amplified DNA and to screen these for low level sub-clones of R882-mutated cells. In order to achieve this goal, we used our MRD assay which reliably detects DNMT3A-R882 mutations down to less than 1% of total cells, and therefore able to detect any mutations in all of the sub-clone populations demonstrated by Ding et al. Our method detects all mutations affecting the R882 codon by taking advantage of the fact that they all perturb the CCGC restriction site for AciI at this locus (Figure 1a). Mutations at codon R882 occur in approximately 60% of all DNMT3A-mutated AMLs and typically arise due to a C-to-T transition at the CpG dinucleotide on either the coding or non-coding strands leading to R882H and R882C mutations respectively.[5] Briefly, we extracted genomic DNA from our AML samples and generated a PCR amplicon of 444 base pairs that encompassed the R882 codon of the DNMT3A gene. The amplicon was then digested using AciI which is unable to cut the DNA molecule if an R882 codon mutation is present. Three internal non-overlapping amplicons were then generated in independent quantitative PCR reactions using a 1:1000 dilution of the AciI-digested DNA. The three primer pairs were specifically designed to allow the R882 mutational frequency to be calculated according to the relative ratios of the middle amplicon to an upstream “loading” control and a downstream “digestion” control (Figure 1b). We used the OCI-AML3 cell line, which we have previously shown to contain a heterozygous DNMT3A-R882C mutation, as a positive control.[12] PCR-amplified DNA from the OCI-AML3 cell line yields an upstream amplicon that contains no AciI sites, a middle amplicon that contains 50% AciI sites, and a downstream amplicon that contains 100% AciI sites. A standard curve was generated using serial dilutions of OCI-AML3 genomic DNA mixed with DNA from KG-1 cells, which are wild-type for the DNMT3A-R882 codon (Figure 1c). The mutational frequency at the DNMT3A-R882 codon of the patient samples were then calculated according to the relative Ct values of the three primer pairs and using the standard curve (Figure 1d).

We analyzed bone marrow samples from a total of 19 AML patients following informed consent and under institutional ethical approval. 11 of the patients had intermediate risk disease (mostly normal cytogenetics), 3 had unfavorable risk disease, 1 had favorable risk disease (t(8;21) translocation),

and 4 had indeterminate risk disease due to absent cytogenetic data. 3 of the patients with normal cytogenetic AML also had samples re-analyzed at time of relapse yielding a total of 22 samples. Using our assay, we identified DNMT3A-R882 mutations in 2 of our AML samples that were both confirmed as R882H mutations by Sanger sequencing. The clone sizes in these two cases were estimated at 97.6% and 71.1% of total cells (or 48.8% and 35.55% variant allele frequency respectively in view of presumed mutational heterozygosity) which was commensurate with the leukemic blast percentage in each sample (estimated at 94% and 67% myeloid respectively) and thus representative of the major leukemic clone. More importantly, our assay excluded the presence of DNMT3A-R882 mutations in all the other 20 AML samples, including the 3 relapse samples, down to a sensitivity of <1% of total cells. The clinical details of all the samples including age, sex, type, cytogenetic, molecular and prognostic data are contained in Table I along with the DNMT3A-R882 mutational frequency for each sample.

Based on the expected ratio of minor to major clones of 3:1 from the published example by Ding et al.,[2] and calculating the frequency of mutations involving the R882 codon at 13.2% in all unselected AML cases (60% of all DNMT3A mutations, which occur at 22%),[5] we used the Fisher Exact Test to compare our measured mutation rates to those predicted. Our findings were not significantly different from that predicted in the major clones (2 measured vs 3 predicted of 19 samples, p=1.00). However, in the minor clones we found the difference to be highly statistically significant (0 measured vs 8 of 57 expected, p=0.006).

We conclude that R882 mutations in DNMT3A are not found in secondary sub-clones, but rather only in the dominant leukaemic clone. We therefore propose that DNMT3A mutations, at least at the R882 hotspot, are candidate initiating lesions in AML. We see no reason for the other mutations reported in DNMT3A to behave in a different fashion and we believe that deep sequencing approaches designed to map out the mutational architecture of AML will vindicate our data confirming DNMT3A as a key therapeutic target in this disease. Finally, we recognize that our assay is a highly sensitive method for rapid detection of DNMT3A-R882 mutations in newly diagnosed AML patients with important implications for prognosis, treatment stratification and disease monitoring.

Author contributions

KEB performed the research and analysed the data; JNB and AM contributed to data analysis; GAH, JNB and TR contributed to the research; HJS, BHR and TJC designed the study and procured samples; TJC obtained ethical approval and funding for the study; all authors contributed to writing and review of the manuscript. There are no conflicts of interests to declare.

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Figure Legends

Figure 1A

DNA sequencing traces for wild-type and mutated forms of DNMT3A at codon 882 showing loss of the AciI restriction site for both R882H and R882C.

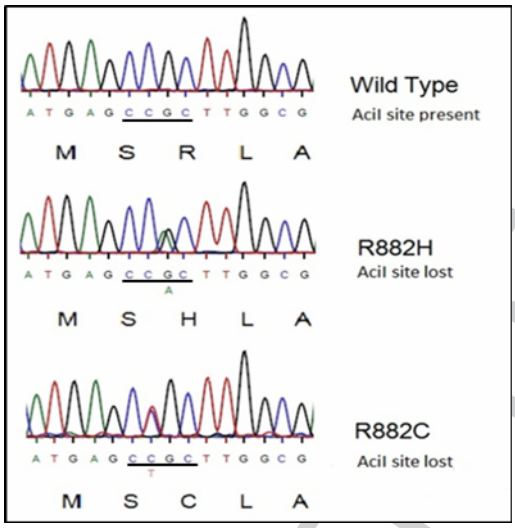

Figure 1B

Illustration showing the relative positions of the qPCR primer pairs and Aci restriction sites within the DNMT3A gene for the OCI-AML3 cell line which carries a heterozygous R882C mutation. Forward and reverse primer sequences for each amplicon are shown below.

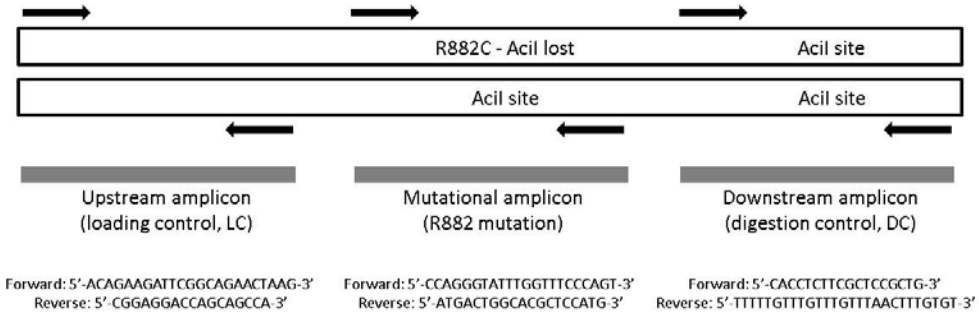

Figure 1C

Standard curve showing linear correlation between serial dilutions of mutated OCI-AML3 DNA and level of DNMT3A-R882C mutation detected by the assay.

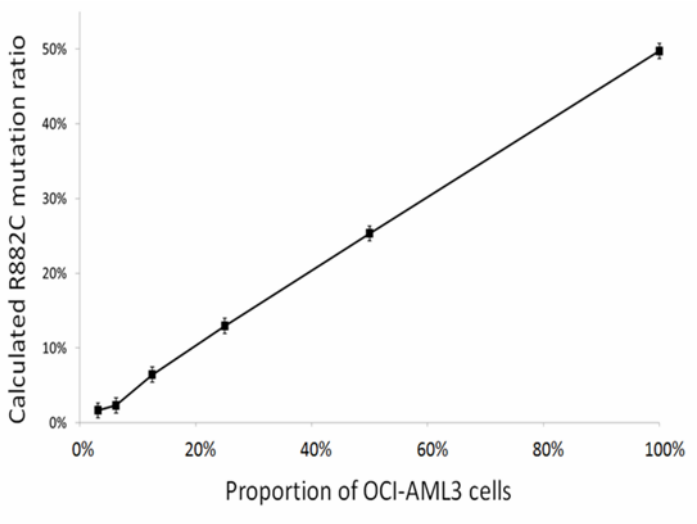

Figure 1D

Graphs showing qPCR curves for the 3 primer pairs for the mutated OCI-AML3 and control KG1 cell lines (top) and for R882H-mutated and wild-type AML patient samples (below), with calculated relative variant allele frequency (LC = loading control; DC = digestion control).

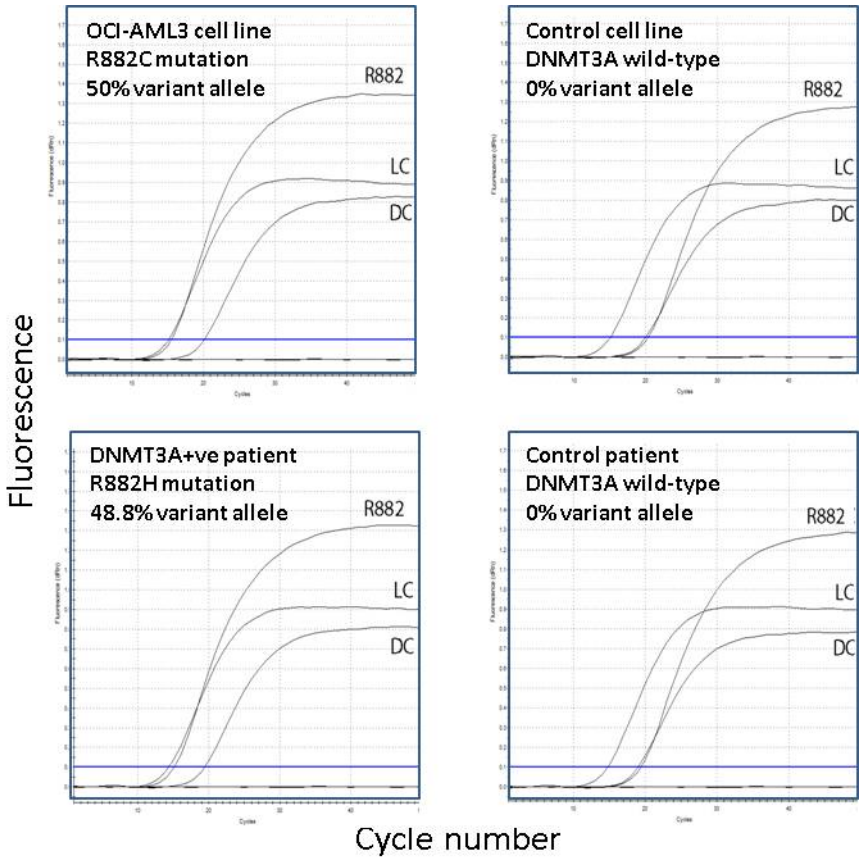

Table I

Table showing clinical details of all the AML samples analysed including cytogenetic, molecular and prognostic data alongside the variant DNMT3A-R882 allele frequency. Two samples revealed major R882H-mutated clones (71.1% and 97.6%). No mutations were detected in the other 20 samples including 3 relapse samples indicating no minor clones detected with a sensitivity threshold of 1% of total cells (ND = not detected).

Patient ID	Age	Sex	Type of AML	Cytogenetics	Molecular marker detected	Risk status	DNMT3A R882 mutation present	Mutation frequency
BSMS 32	76	F	Secondary AML (MDS)	45,XX,inv(3),del(5)	Not available	Unfavourable	No	ND
BSMS 33	76	M	De novo AML	46,XY,del(5)	FLT3 ITD +ve, NPM1 -ve	Intermediate	Yes – R882H	71.1%
BSMS 71	70	M	Secondary AML (MDS)	Normal cytogenetics	FLT3 ITD -ve, NPM1 -ve	Intermediate	No	ND
BSMS 73	77	F	De novo AML	46,XX,t(10;12)	FLT3 ITD -ve, NPM1 -ve	Intermediate	No	ND
BSMS 74	76	F	De novo AML	Normal cytogenetics	FLT3 ITD +ve, NPM1 +ve	Intermediate	No	ND
BSMS 82	62	M	Secondary AML (MPN)	Normal cytogenetics	FLT3 ITD -ve, NPM1 -ve, JAK2 +ve	Intermediate	No	ND
BSMS 85	80	M	Secondary AML (MDS)	Normal cytogenetics	FLT3 ITD -ve, NPM1 -ve	Intermediate	Yes – R882H	97.6%
BSMS 87	31	M	De novo AML	Not available	Not available	Indeterminate	No	ND
BSMS 89	23	M	De novo AML	Complex	MLL gene rearrangement	Unfavourable	No	ND
BSMS 95	78	M	De novo AML	Normal cytogenetics	Not available	Intermediate	No	ND
BSMS 95 (relapse)	78	M	Relapsed AML	Normal cytogenetics	Not available	Intermediate	No	ND
BSMS 103	74	M	De novo AML	Normal	Not available	Intermediate	No	ND

				cytogenetics				
BSMS 115	63	F	De novo AML	Not available	Not available	Indeterminate	No	ND
BSMS 116	55	M	De novo AML	Normal cytogenetics	Not available	Intermediate	No	ND
BSMS 116 (relapse)	55	M	Relapsed AML	Normal cytogenetics	Not available	Intermediate	No	ND
BSMS 122	74	M	Secondary AML (MDS)	Complex	Not available	Unfavourable	No	ND
BSMS 127	62	F	De novo AML	45,X,-X,t(8;21)	Not available	Favourable	No	ND
BSMS 129	78	M	De novo AML	Normal cytogenetics	Not available	Intermediate	No	ND
BSMS 131	71	F	Secondary AML (MDS)	Normal cytogenetics	FLT3 ITD -ve, NPM1 -ve	Intermediate	No	ND
BSMS 131 (relapse)	71	F	Relapsed AML	Normal cytogenetics	FLT3 ITD -ve, NPM1 -ve	Intermediate	No	ND
BSMS 136	64	F	De novo AML	Not available	Not available	Indeterminate	No	ND
BSMS 180	40	M	De novo AML	Not available	Not available	Indeterminate	No	ND