OPEN

Citation: Blood Cancer Journal (2013) **3**, e147; doi:10.1038/bcj.2013.46
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LETTER TO THE EDITOR

Acute promyelocytic leukemias share cooperative mutations with other myeloid-leukemia subgroups

Blood Cancer Journal (2013) **3,** e147; doi:10.1038/bcj.2013.46; published online 13 September 2013

Acute promyelocytic leukemias (APLs) are characterized by the expression of the *PML-RARA* oncogene, which is a product of the 15;17 chromosomal translocation. Two lines of evidence suggest that this genetic alteration is involved in the initiation of promyelocytic leukemogenesis *in vivo*: i) the t(15;17) often represents the only cytogenetic abnormality in an otherwise-normal karyotype; ii) PML-RARA is able to initiate leukemogenesis in mouse models on expression in hematopoietic stem cells (HSCs) or progenitors, giving rise to a disease that recapitulates clinical and morphological features of human APLs.[1] APL onset in mice, however, occurs after a long latency and with a low penetrance, suggesting that, like other cancers, APL is a multistep disease, whereby PML-RARA cooperates with secondary mutations for the full development of the leukemia phenotype. Notably, in the pre-leukemic phase of the disease, PML-RARA expression induces DNA damage in HSCs/progenitors and activates DNA repair, thus imparting a mutator phenotype that might contribute to disease progression.[2,3]

Next-generation sequencing represents a powerful tool for the discovery of genetic alterations at high resolution. To identify gene mutations that might cooperate with PML-RARA in the leukemogenic process, we performed whole-exome sequencing of 5 leukemias that developed in *PML-RARA* transgenic mice (mouse APLs; mAPLs) and 11 patients' leukemias expressing PML-RARA (human APLs; hAPLs) (Supplementary Table 1). For the scope of this investigation, we only considered non-synonymous single-nucleotide variants (SNVs) and small insertions/deletions (indels) occurring in the tumor DNA with an allelic frequency $\geqslant 25\%$ (corresponding to a $> 50\%$ frequency of cells carrying the mutation in the tumor sample, assuming the majority of mutations to be heterozygous). These experimental conditions allow identification of SNVs with a validation rate of 100%, as shown by an independent sequencing approach (Sanger sequencing; unpublished). We identified a total of 18 mutations in the 5 mAPLs (16 SNVs and 2 indels) and 73 mutations in the 11 hAPLs (59 SNVs and 14 indels) (Table 1 and Supplementary Table 2). We validated all the 16 mAPL SNVs by Sanger sequencing (the 2 indels were not amplifiable for technical reasons), 18/18 randomly selected human SNVs and 1/1 indel (Supplementary Table 2).

We next combined the results of our sequencing analyses with those previously published for APLs (1 mAPL and 24 hAPLs),[4–7] obtaining a data set of 41 APL samples (6 mAPLs and 35 hAPLs). Analysis of this data set showed a total of 270 mutations affecting 248 human genes (231 SNVs, 34 indels and 5 splice junction mutations—SJMs), with a low frequency of mutations per case ($\sim$7.1 in hAPLs and $\sim$3.5 in mAPLs). In three cases (two mAPLs and one hAPL), we found no SNVs or indels with a frequency higher than 25%. Surprisingly, two hAPL samples showed several hundreds of low-frequency mutations, whose significance remains unclear (not shown).

Of the 248 mutated genes, 9 were found in $\geqslant 2$ patients (recurrent mutations), and 8 showed a significantly higher mutation rate ($q < 0.005$; *FLT3*; *WT1*; *KRAS*; *CALR*; *CSMD1*; *DDR2*; *REV3L* and *TCERG1L*; Table 2 and Supplementary Table 3). *FLT3* and *KRAS* have been already described as cooperators of PML-RARA in mouse models of APLs,[8] whereas *WT1* is infrequently mutated in hAPLs.[9] Of the remaining five, the *DDR2* (discoidin domain receptor 2) tyrosine kinase is mutated in a small subset of squamous cell lung cancer. Notably, *DDR2* mutations are critical oncogenic events for these tumors and confer high sensitivity to the multi-targeted kinase inhibitor dasatinib.[10] We found a total of 25 mutations affecting these eight genes (APL driver mutations), with *FLT3* and *WT1* being the most frequently involved (Table 2).

We next investigated the APL specificity of the identified mutations, as compared with other subgroups of acute myeloid leukemias (AMLs). First, we generated a data set of mutations in all the available AML samples ($n = 206$; 196 previously published samples[7,11–14] and 10 new samples from this study (Supplementary Tables 1 and 2)) and divided the AML samples in different genetic/cytogenetic subgroups: i) samples with mutations of nucleophosmin (NPM1^{+}: 58 cases); ii) normal karyotype without NPM1 mutations (NK-NPM1^{-}: 58 cases); iii) complex karyotype ($n = 22$); iv) translocations or inversions affecting CBFB/MYH11 ($n = 11$); v) t(8;21) RUNX1/RUNX1T1 ($n = 7$); vi) trisomy 8 ($n = 8$); vii) MLL-X translocations ($n = 10$). Twenty-seven cases did not fall into any of these categories ('Other'). As for PML-RARA, indirect evidence from mouse models suggests that mutated *NPM1*[15] and fusion proteins of *CBFB*, *RUNX1* and *MLL* are initiating mutations for AMLs (reviewed in McCormack *et al.*[8]).

AML mutations were separately analyzed in each of the AML subgroups. We found a total of 1360 mutated genes ($\sim$9 mutations per case), of which 153 were recurrently mutated ($\geqslant 2$ patients in the same subgroup) and 40 showed a significantly higher mutation rate ($q < 0.005$; Table 2 and Supplementary Table 3). Notably, included in the 40 genes were 21 of the 22 significantly mutated genes identified in a recent analysis of 200 AMLs (also part of our data set[7]), and additional mutated genes critical for AMLs (*BCOR*, *ASXL1*, *GATA2*, *SUZ12* and *DDX41*) or for selected epithelial cancers (*CTCF*, *PLCE1* and *CHD4*). The most frequently mutated genes were also significantly associated with specific AML subgroups: *FLT3*, *IDH1*, *DNMT3A* and *PTPN11* with NPM1^{+} AMLs; *RUNX1*, *CEBPA* and *GATA2* with NK-NPM1^{-} AMLs; *TP53* with AMLs with complex karyotypes; *KIT* with CBFB/MYH11 AMLs; *IDH1* with AMLs with trisomy 8 (Table 2). Moreover, mutations in *SF3B1*, *PTPN11*, *DNAH9*, are present in both human and mouse leukemias.

We then analyzed the distribution of the significantly mutated APL and AML genes ($n = 44$) across all samples ($n = 239$). Twenty-eight genes ($\sim$64%) were mutated in more than one cytogenetic subgroup, covering 383 of the 416 mutations identified in all samples ($\sim$92%). In the remaining 16 genes ($\sim$36%), mutations were instead associated with a specific subgroup, corresponding to just 33 of the identified mutations ($\sim$8%) and suggesting that subgroup specificity might be due to their low frequency. Indeed, we found a significant correlation between frequency of mutations per gene and numbers of subgroups where it is mutated (Spearman's coefficient value of 0.93). As regards APLs, 5 of the 8

Table 1. Mutations identified by next-generation sequencing in APLs

Cases	*Mutations*				*Study*
	nsSNVs	*Indels*	*SJMs*	*Total*	
Mouse APLs					
mAPL#Mi1	0	0	0	0	Present
mAPL#Mi2	1	0	0	1	Present
mAPL#Mi3	9	1	0	10	Present
mAPL#Mi4	6	1	0	7	Present
mAPL#Mi5	0	0	0	0	Present
mAPL	3	0	0	3	Wartman *et al.*[4]
Total mutations (per Pt.)	19 (3.16)	2 (0.33)	0	21 (3.50)	6 cases total
Human APLs					
hAPL#Mi1	5	0	0	5	Present
hAPL#Mi2	13	3	0	16	Present
hAPL#Mi3	2	1	0	3	Present
hAPL#Mi4	3	3	0	6	Present
hAPL#Mi5	0	0	0	0	Present
hAPL#Mi6	5	1	0	6	Present
hAPL#Mi7	12	2	0	14	Present
hAPL#Mi8	1	1	0	2	Present
hAPL#Mi9	4	1	0	5	Present
hAPL#Mi10	7	1	0	8	Present
hAPL#Mi11	7	1	0	8	Present
hAPL#1	5	2	0	7	Greif *et al.*[5]
hAPL#2	3	0	0	3	Greif *et al.*[5]
hAPL#3	4	0	0	4	Greif *et al.*[5]
hAPL	12	0	0	12	Welch *et al.*[6]
TCGA-AB-2803	12	1	0	13	TCGA[7]
TCGA-AB-2804	7	1	0	8	TCGA[7]
TCGA-AB-2823	0	1	0	1	TCGA[7]
TCGA-AB-2840	0	1	0	1	TCGA[7]
TCGA-AB-2841	4	0	0	4	TCGA[7]
TCGA-AB-2862	7	0	0	7	TCGA[7]
TCGA-AB-2872	9	1	0	10	TCGA[7]
TCGA-AB-2897	5	0	1	6	TCGA[7]
TCGA-AB-2905	15	2	0	17	TCGA[7]
TCGA-AB-2906	9	1	0	10	TCGA[7]
TCGA-AB-2980	3	1	0	4	TCGA[7]
TCGA-AB-2982	1	1	0	2	TCGA[7]
TCGA-AB-2991	10	0	0	10	TCGA[7]
TCGA-AB-2994	6	1	0	7	TCGA[7]
TCGA-AB-2997	9	1	0	10	TCGA[7]
TCGA-AB-2998	4	1	0	5	TCGA[7]
TCGA-AB-2999	9	0	0	9	TCGA[7]
TCGA-AB-3001	8	0	2	10	TCGA[7]
TCGA-AB-3007	5	1	1	7	TCGA[7]
TCGA-AB-3012	6	2	1	9	TCGA[7]
Total in hAPLs (per Pt.)	212 (6.06)	32 (0.91)	5 (0.14)	249 (7.11)	35 cases
Total h + mAPLs (per Pt.)	231 (5.63)	34 (0.83)	5 (0.12)	270 (6.59)	41 cases

Abbreviations: APL, acute promyelocytic leukemia; h, human; indels, small insertion/deletions; m, mouse; nsSNV, non-synonymous single nucleotide variant; per Pt., per patient; SJM, splice junction mutation; TCGA, The Cancer Genome Atlas.

significantly mutated genes were also found in other AML cytogenetic subgroups (*FLT3*; *WT1*; *KRAS*; *CSMD1* and *DDR2*), covering a total of 100 mutations, while 3 (*CALR*; *REV3L* and *TCERG1L*) were only found in APLs, covering 6 mutations. Three genes that were found significantly mutated in other AML subgroups were also mutated in APLs (*PHF6*, *FAM5C* and *PTPN11*).

Together, these results imply that different myeloid leukemias, including APLs, share the same subset of cooperating mutations, and are consistent with a scenario whereby specific initiating mutations interact with a common pool of highly heterogeneous, yet phenotypically equivalent, cooperating mutations. Indirect evidence, however, suggests that the pool of cooperating mutations in AMLs is not yet entirely defined, and that sequencing of additional AMLs is needed. In fact, due to the limited size of some samples (for example, in selected AML subgroups such as those with rearrangements of *CBFB*, *RUNX1* or *MLL*), it is likely that our statistical analyses do not allow the identification of all the driver mutations in AML. Among all the mutated genes ($n = 1559$), $\sim 3\%$ ($n = 45$) were mutated at statistically significant frequency (driver mutations). Among the others (passenger mutations), however, we identified mutations that have been causally implicated in the pathogenesis of AMLs (for example, *ETV6*, *JAK2*, *NOTCH1*, *NUMA1*, *PRDM16*, *CBL*, *CBFB*, *CHIC2*, *ELF4*, *NSD1* and *PDGFRB*) or other cancers (for example, *PTEN*, *MYC*, *ARID1A*, *SF3B1*, *EGFR*, *NF1*, *THRAP3*, *MED12*, *KDR*, *IKZF1*, *DAXX* and *SETD2*).

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Table 2. Genes with a significantly higher mutation rate in APLs and AMLs

Mutated Gene	PML-RARA	NPM1$^+$	NK-NPM1$^-$	Complex karyotype	CBFB/ MYH11	MLL-X	Trisomy 8	RUNX1/ RUNX1T1	Other	Total[a]	TCGA[b]
FLT3	9	32	9	1	3	2	1	1	5	63	x
CSMD1	3	1	0	1	0	0	0	0	1	6	
WT1	3	5	5	0	1	1	0	0	2	17	x
DDR2	2	0	1	0	0	0	0	0	0	3	
KRAS	2	3	2	2	0	0	1	0	1	11	x
CALR	2	0	0	0	0	0	0	0	0	2	
REV3L	2	0	0	0	0	0	0	0	0	2	
TCERG1L	2	0	0	0	0	0	0	0	0	2	
FAM5C	1	2	0	1	0	0	0	0	1	5	x
PHF6	1	1	2	1	0	0	1	0	1	7	x
PTPN11	1	7	0	2	1	0	0	0	0	11	x
ASXL1	0	0	3	0	0	0	2	0	1	6	
CEBPA	0	2	12	1	0	0	1	0	3	19	x
DNMT3A	0	29	15	3	0	0	5	0	4	56	x
EZH2	0	0	1	0	0	0	0	0	3	4	x
GATA2	0	1	6	0	0	0	0	0	0	7	
GRIK2	0	2	0	0	0	1	0	0	0	3	
IDH1	0	12	2	1	0	0	5	0	3	23	x
IDH2	0	5	8	1	0	0	3	0	4	21	x
KIT	0	1	0	1	3	0	0	2	1	8	x
NRAS	0	5	4	2	2	1	0	0	2	16	x
PHACTR1	0	0	0	1	0	0	0	0	2	0	
PLCE1	0	0	1	0	0	0	0	1	2	4	
RAD21	0	3	1	0	0	0	0	1	0	5	x
RUNX1	0	0	12	1	0	0	2	0	7	22	x
SMC1A	0	4	0	0	0	1	1	1	0	7	x
SMC3	0	3	2	1	0	0	1	1	0	8	x
STAG2	0	3	3	0	0	0	0	0	1	7	x
SUZ12	0	0	1	2	0	0	0	0	0	3	
TET2	0	2	9	1	0	0	1	1	4	18	x
TP53	0	0	1	12	0	0	0	0	2	15	x
U2AF1	0	1	2	0	0	0	2	0	3	8	x
BCOR	0	0	3	0	0	0	0	0	0	3	
CHD4	0	0	0	2	0	0	0	0	0	2	
CTCF	0	0	2	0	0	0	0	0	0	2	
DDX41	0	0	2	0	0	0	0	0	0	2	
FAM57B	0	2	0	0	0	0	0	0	0	2	
GIGYF2	0	0	0	0	0	0	0	2	0	2	
GJB3	0	0	0	2	0	0	0	0	0	2	
KDM3B	0	0	0	2	0	0	0	0	0	2	
KRT13	0	2	0	0	0	0	0	0	0	2	
PCDHA13	0	0	2	0	0	0	0	0	0	2	
SCN1A	0	0	0	2	0	0	0	0	0	2	
TBX15	0	2	0	0	0	0	0	0	0	2	
TCEB3B	0	0	2	0	0	0	0	0	0	2	

🟩 found in ≥2 AML subgroups
🟧 found in only one AML subgroup
🟦 associated to a specific AML subgroup with q≥0.01
⬜ associated to a specific AML subgroup with q≥0.05

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[a] Total number of mutations identified for each mutated gene. [b] Significantly mutated genes identified by The Cancer Genome Atlas Research network (TCGA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

LR was supported by a Reintegration AIRC/Marie Curie International Fellowship in Cancer Research. This work was supported by grants from Fondazione Cariplo to LR; Associazione Italiana Ricerca sul Cancro (AIRC) and Italian Ministry of Health to PGP; European Commission (FP7) to GM. We thank Paola Dalton for critical review of the manuscript.

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