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The *MLL* partial tandem duplication in adults aged 60 years and older with *de novo* cytogenetically normal acute myeloid leukemia

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Cytogenetically normal acute myeloid leukemia (CN-AML) comprises nearly half of AML diagnoses annually, and historically, patients in this cytogenetic subgroup have been considered as being at intermediate risk for clinical outcomes. However, the outcome of these patients varies considerably based on the presence or absence of non-random genetic aberrations that can be used as risk stratification factors.¹

The partial tandem duplication of the *MLL* gene (*MLL*-PTD) was the first somatic mutation associated with a trisomy aberration in AML¹ and the first molecular prognostic marker identified in CN-AML² where it was associated with shorter disease-free survival.^{2–4} We recently reported that the adverse prognostic impact of *MLL*-PTD in younger (<60 years) adults with *de novo* CN-AML may be abrogated when more intensive consolidation regimens are implemented.⁵ However, the patients with *MLL*-PTD who relapsed generally had a second adverse molecular marker, such as *FLT3*-ITD.⁵ In contrast, the clinical and biological impact of *MLL*-PTD in older patients with CN-AML is not yet established. Thus,

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CONFLICT OF INTEREST

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we evaluated blood or bone marrow samples from 226 consented newly diagnosed *de novo* CN-AML patients aged ≥ 60 years and treated on Cancer and Leukemia Group B (CALGB) protocols for the presence of an in-frame *MLL*-PTD transcript using PCR/sequencing and confirmed using real-time PCR.^{2,5} Assays to detect other molecular aberrations with prognostic significance in CN-AML, and genome-wide gene and microRNA-expression profiling, were carried out centrally (Supplementary Information).

Baseline characteristics were compared between *MLL*-PTD and *MLL* wild-type (WT) patients using Fisher's exact test for categorical and the Wilcoxon rank-sum test for continuous variables. Clinical endpoints were defined according to published recommendations (Supplementary Information). Achievement of complete remission was compared between *MLL*-PTD and *MLL*-WT patients using the Fisher's exact test. For time-to-event analyses (that is, disease-free, overall and event-free survival), survival estimates were calculated using the Kaplan–Meier method. Survival data for *MLL*-PTD and *MLL*-WT patients were compared using the log-rank test. Statistical analyses were carried out by the Alliance for Clinical Trials in Oncology Statistics and Data Center.

MLL-PTD was present in 13 of the 226 (6%) patients analyzed. The frequency of *MLL*-PTD in the current study is slightly lower than that in our previous report of younger adults with CN-AML (10%)⁵ but is in line with reports analyzing exclusively older patients (4%)⁶ or both younger and older CN-AML patients as a single cohort (7.5%).⁷ Compared with *MLL*-WT patients, *MLL*-PTD patients had lower hemoglobin levels ($P = 0.001$; Table 1), lacked *CEBPA* and *IDH2*R172 mutations, and 11 of the 13 *MLL*-PTD patients lacked *NPM1* mutations (*NPM1*-WT) ($P = 0.002$; Table 1 and Supplementary Figure S1). Approximately one-third of *MLL*-PTD patients also harbored a *FLT3*-ITD, which is similar to our previous findings in younger CN-AML adults.⁵ However, contrary to another study reporting frequent co-existence of mutated *RUNX1* and *MLL*-PTD in AML,⁸ we found these two molecular aberrations concurrently present only in three older patients with primary CN-AML. Aside from one patient, mutations in the genes encoding chromatin or epigenetic modifiers (*DNMT3A*, *TET2* and *ASXL1*) appeared to be exclusive of each other in the *MLL*-PTD patients (Supplementary Figure S1). However, the majority of the older patients with *MLL*-PTD had at least one additional mutation in an epigenetics and/or chromatin remodeling-associated gene. This further supports the view that altered epigenetics and/or chromatin remodeling may constitute a crucial mechanism in AML leukemogenesis and is consistent with our previous report of an association between *MLL*-PTD and increased global DNA methylation.⁹ Importantly, therapeutic targeting of the involved epigenetic and chromatin remodeling factors and/or pathways can potentially prolong survival in these older adults who generally cannot tolerate current chemotherapies and/or transplantation. Other clinical and molecular features studied did not differ significantly between the *MLL*-PTD and *MLL*-WT patients (Table 1).

Complete remission rates were similar (69 and 67%) between patients with and without *MLL*-PTD, respectively ($P = 1.00$; Table 1). Median disease-free survival was 0.7 and 0.8 years for *MLL*-PTD patients as compared with the *MLL*-WT patients ($P = 0.45$) and the percentages of patients disease-free at 3 years were 22% and 18%, respectively. With a median follow-up for those alive at 5.5 years (range: 2.3–11.6 years) ($n = 19$), overall survival was also not significantly different between *MLL*-PTD and *MLL*-WT groups (median OS: 1.1 and 1.1 years, respectively; $P = 0.38$). By 3 years, the percent of *MLL*-PTD patients alive was 15%, whereas that of *MLL*-WT patients was 19% (Table 1).

Recently the molecular heterogeneity with respect to *NPM1* and *CEBPA* mutations and *FLT3*-ITD has also been used by the European LeukemiaNet (ELN) to classify CN-AML patients into two distinct Genetic Groups for reporting and comparing AML studies.¹⁰ When

we evaluated the prognostic significance of *MLL*-PTD in the ELN Genetic Groups, the *MLL*-PTD patients were more frequently classified in the ELN Intermediate-I Genetic Group, which is defined by the absence of *CEBPA* mutations and co-presence of mutated *NPM1* and *FLT3*-ITD or presence of *NPM1*-WT with or without *FLT3*-ITD ($P = 0.007$). However, as in the overall analysis, the presence of *MLL*-PTD did not impact on outcome endpoints in this Genetic Group (Table 1). Although not directly comparable to ours, in a study that included both CN-AML patients and those with abnormal cytogenetics, Schlenk *et al.*⁶ found no prognostic impact of *MLL*-PTD in patients older than 60 years receiving all-*trans* retinoic acid in addition to intensive chemotherapy. Likewise, Steudel *et al.*⁷ reported that *MLL*-PTD did not impact on outcomes of a cytogenetically heterogeneous group of adults with AML. The lack of prognostic impact by the *MLL*-PTD in older *de novo* CN-AML patients may be related to the overall poor prognosis of this age group of patients regardless of the presence or absence of the mutation.

To gain insights into *MLL*-PTD-associated biology in older CN-AML patients, we performed microarray gene- and microRNA-expression profiling analyses. No gene expression signature was associated with *MLL*-PTD, despite the established transcriptional and epigenetics roles of normal and abnormal MLL protein in hematopoiesis and leukemia, respectively. However, the current finding is consistent with a study that included adult AML patients with normal and abnormal cytogenetics, in which no gene expression clusters or signatures associated with *MLL*-PTD were identified.¹¹ Although the reasons of the failure to derive a *MLL*-PTD-associated gene expression signature are unknown, they might be related to the presence of other molecular markers with stronger biological impact on gene expression.

However, when we examined the expression of the eight *MLL* probe-sets on the microarray, three (212078_s_at, 212079_s_at and 1565436_s_at) were homologous to the regions encompassing the commonly duplicated exons in *MLL*-PTD patients (GenBank accession no. NM_005933). These three probe-sets showed evidence of upregulation in *MLL*-PTD patients relative to the *MLL*-WT patients (PTD:WT fold-changes of 1.41, 1.74 and 1.61, respectively; $P < 0.005$, each; Supplementary Table S1). In contrast, none of the remaining five probe-sets were differentially expressed between *MLL*-PTD and *MLL*-WT patients (Supplementary Table S1). These results correlate well with PCR/sequencing of the same regions of the *MLL* transcript, validating our *MLL*-PTD assay.

Contrary to the global gene-expression analysis, a microRNA signature was obtained when comparing *MLL*-PTD and *MLL*-WT patients' microRNA profiles (Table 2; global test of differential microRNA expression, $P = 0.01$). The microRNA-expression signature comprised 23 probes, representing 21 microRNAs, many of which have reported roles in hematopoiesis and/or leukemia. The minor transcript *miR-196b**, underexpressed in *MLL*-PTD patients (Table 2), is derived from the antisense strand of the *miR-196* gene located within the *HOXA* gene cluster. Although a strong correlation between *HOXA9* expression and expression of the major transcript, *miR-196b*, and an association of upregulation of *miR-196b* with reduced overall survival in AML have been reported,¹² the role of the related *miR-196b** is unknown. The most underexpressed microRNA was *miR-197* and *in silico* analyses predict *ASXL1*, a recently identified adverse prognostic marker in CN-AML (Supplementary ref. no. 8), to be targeted by *miR-197*.

The most overexpressed was *miR-150* (2.1-fold); this microRNA targets *c-MYB* translation associated with altered erythroid–megakaryocyte progenitor commitment and B-cell maturation.¹³ WT MLL and c-MYB proteins functionally interact to regulate *HOXA9* gene expression,¹⁴ a known transcriptional target of *MLL*-PTD. Another overexpressed

microRNA was *miR-142*, previously reported to be a transcriptional target of the *MLL* protein.¹⁵

We conclude that *MLL*-PTD does not have prognostic impact in older CN-AML patients treated with cytarabine/anthracycline-based chemotherapy. This may be related to the overall poor prognosis in this age group and/or simultaneous presence of other genetic aberrations that have stronger clinical impact that masks the influence *MLL*-PTD may have on outcome. Our work contributes to the understanding of the possible role of microRNAs in older AML by reporting the first microRNA-expression signature associated with *MLL*-PTD. The hope is that these data will aid in the development of novel approaches that improve the otherwise poor outcome of these older patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Comparisons of clinical and molecular features and the outcomes in overall patient cohort and within the ELN Intermediate I Genetic Group at presentation by *MLL* status

<i>Characteristic</i>	<i>MLL-PTD (n = 13)</i>	<i>MLL-WT (n = 213)</i>	<i>P-value^a</i>
<i>Age, y</i>			0.25
Median	65	68	
Range	60–77	60–83	
<i>Sex, no. (%)</i>			0.08
Male	10 (77)	106 (50)	
Female	3 (23)	107 (50)	
<i>Race, no. (%)</i>			0.61
White	13 (100)	192 (91)	
Non-white	0 (0)	19 (9)	
<i>Hemoglobin, g/dl</i>			0.01
Median	8.5	9.5	
Range	6.0–11.7	5.4–15.0	
<i>Platelet count, × 10⁹/l</i>			0.41
Median	53	70	
Range	20–246	11–850	
<i>WBC count, × 10⁹/l</i>			0.16
Median	9.1	28.4	
Range	1.3–434.1	0.8–450.0	
<i>Blood blasts, %</i>			0.44
Median	32	52	
Range	0–96	0–99	
<i>Bone marrow blasts, %</i>			0.93
Median	47	68	
Range	17–97	4–97	
<i>FAB, no. (%)^b</i>			0.68
M0	0 (0)	3 (2)	
M1	3 (33)	32 (23)	
M2	1 (11)	44 (31)	
M4	3 (33)	31 (22)	
M5	2 (22)	27 (19)	
M6	0 (0)	3 (2)	
<i>Extramedullary involvement, no. (%)</i>	2 (15)	51 (25)	0.74
<i>ELN Genetic Group, no. (%)^c</i>			0.007
Favorable	1 (8)	97 (47)	
Intermediate-I	12 (92)	109 (53)	
<i>FLT3-ITD, no. (%)</i>			1.00
Present	4 (31)	70 (34)	
Absent	9 (69)	137 (66)	

<i>Characteristic</i>	<i>MLL-PTD (n = 13)</i>	<i>MLL-WT (n = 213)</i>	<i>P-value^a</i>
<i>NPM1, no. (%)</i>			0.002
Mutated	2 (15)	126 (61)	
Wild type	11 (85)	80 (39)	
<i>CEBPA, no. (%)</i>			0.37
Mutated	0 (0)	26 (13)	
Single mutated	0	16	
Double mutated	0	10	
Wild type	13 (100)	180 (87)	
<i>RUNX1, no. (%)</i>			0.43
Mutated	3 (25)	31 (16)	
Wild type	9 (75)	159 (84)	
<i>WT1, no. (%)</i>			0.18
Mutated	2 (15)	11 (5)	
Wild type	11 (85)	195 (95)	
<i>FLT3-TKD, no. (%)</i>			1.00
Present	1 (8)	22 (11)	
Absent	12 (92)	184 (89)	
<i>IDH1, no. (%)</i>			1.00
Mutated	1 (8)	25 (12)	
Wild type	12 (92)	179 (88)	
<i>IDH2, no. (%)</i>			0.51
IDH2	4 (31)	47 (23)	
R140	4	38	
R172	0	9	
Wild type	9 (69)	157 (77)	
<i>TET2, no. (%)</i>			1.00
Mutated	3 (23)	58 (29)	
Wild type	10 (77)	144 (71)	
<i>ASXL1, no. (%)</i>			0.23
Mutated	4 (31)	31 (15)	
Wild type	9 (69)	171 (85)	
<i>DNMT3A</i>			1.00
Mutated	4 (33)	65 (33)	(mut vs wt)
R882	3	38	
Non-R882	1	27	
Wild type	8 (67)	135 (67)	
<i>ERG expression, no. (%)^d</i>			0.47
High	3 (38)	75 (54)	
Low	5 (62)	64 (46)	
<i>BAALC expression, no. (%)^d</i>			0.28
High	6 (75)	69 (51)	
Low	2 (25)	66 (49)	

Characteristic	MLL-PTD (n = 13)	MLL-WT (n = 213)	P-value ^a
<i>MNI expression group, no. (%)^d</i>			1.00
High	6 (55)	69 (51)	
Low	5 (45)	67 (49)	
<i>Complete remission rate, no. (%)</i>	9 (69)	142 (67)	1.00
<i>Disease-free survival^e</i>			0.45
Median, y	0.7	0.8	
Disease-free at 3 y, % (95% CI)	22 (3– 51)	18 (12– 24)	
<i>Overall survival^f</i>			0.38
Median, y	1.1	1.1	
Alive at 3 y, % (95% CI)	15 (2– 39)	19 (14– 24)	
<i>Event-free survival</i>			0.60
Median, y	0.6	0.6	
Event-free at 3 y, % (95% CI)	15 (2– 39)	12 (8– 16)	
<i>ELN Intermediate-I Genetic Group</i>			
No. of patients	12	109	
<i>Complete remission rate, no. (%)</i>	8 (67)	62 (57)	0.56
<i>Disease-free survival^g</i>			0.88
Median, y	0.6	0.6	
Disease-free at 3 y, % (95% CI)	13 (1– 42)	10 (4– 19)	
<i>Overall survival^h</i>			0.92
Median, y	1.0	0.8	
Alive at 3 y, % (95% CI)	8 (1– 31)	10 (5– 17)	
<i>Event-free survival</i>			0.88
Median, y	0.6	0.3	
Event-free at 3 y, % (95% CI)	8 (1– 31)	6 (2– 11)	

Abbreviations: CI, confidence interval; ELN, European LeukemiaNet; FAB, French-American-British classification; *FLT3*-ITD, internal tandem duplication of the *FLT3* gene; *FLT3*-TKD, tyrosine kinase domain mutation in the *FLT3* gene; WBC, white blood cell.

^a *P*-values for categorical variables are from Fisher's exact test, *P*-values for continuous variables are from Wilcoxon rank sum test and *P*-values for time to event variables are from the log-rank test.

^b FAB are centrally reviewed.

^c The ELN Favorable Genetic Group is defined as patients with mutated *CEBPA* or mutated *NPM1* without *FLT3*-ITD; Intermediate-I Genetic Group is defined as patients that are not classified in the Favorable Genetic Group, that is, those with wild-type *CEBPA* who are either *FLT3*-ITD-positive with or without an *NPM1* mutation or *FLT3*-ITD-negative with wild-type *NPM1*.

^d The median expression value was used as a cut point.

^e The median follow-up for those who have not had an event is 5.5 years, range: 4.6 – 11.6 years (*n* = 15).

^f The median follow-up for those alive is 5.5 years, range: 2.3 – 11.6 years (*n* = 19).

^g The median follow-up for those who have not had an event is 7.3 years, range: 5.5 – 7.4 years (*n* = 3).

^hThe median follow-up for those alive is 7.3 years, range: 5.5 – 7.4 years ($n = 3$).

Table 2Differentially expressed microRNAs associated with *MLL*-PTD in older, *de novo* CN-AML patients^a

<i>Target microRNA</i>	<i>Fold-change: MLL-PTD/MLL-WT</i>	<i>P-value</i>
<i>MicroRNAs downregulated in MLL-PTD patients</i>		
hsa-miR-96	0.59	0.0013752
hsa-miR-130b*, ^b	0.76	0.0037523
hsa-miR-185*, ^b	0.69	0.0009761
hsa-miR-196b*, ^b	0.70	0.0034725
hsa-miR-197	0.65	2.45E-05
hsa-miR-205	0.70	0.0045768
hsa-mir-320a (prec)	0.67	0.0008229
hsa-mir-320a (prec)	0.67	0.0009745
hsa-miR-326	0.57	0.0015483
hsa-miR-328	0.64	0.0003135
hsa-mir-329-1 (prec)	0.61	0.0004629
hsa-mir-331 (prec)	0.55	0.0011029
hsa-mir-422a (prec)	0.68	0.002089
hsa-miR-497	0.62	0.0012652
hsa-miR-596	0.67	0.0020937
<i>MicroRNAs upregulated in MLL-PTD patients</i>		
hsa-miR-26a	1.65	0.0024263
hsa-miR-26a	1.75	0.0019854
hsa-miR-122	1.32	0.0049319
hsa-miR-142-5p	1.56	0.0027454
hsa-miR-150	2.14	0.000397
hsa-miR-185	1.52	0.0010639
hsa-miR-202	1.54	0.0017005
hsa-mir-640 (prec)	1.67	0.0006557

Abbreviation: prec, the precursor microRNA sequence is detected by the probe.

^aA total of 23 of 460 microRNA probes tested were significant ($P < 0.005$; global test; P -value = 0.011). Probes are grouped by direction of fold-change and ordered by target microRNA.

^bAn asterisk behind the microRNA's symbol indicates that this microRNA is the minor sequence generated from the antisense strand; the asterisk is part of the standard nomenclature for naming microRNAs.