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Letter
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The Landscape of *KMT2A*-PTD AML: Concurrent Mutations, Gene Expression Signatures, and Clinical Outcome

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Acute myeloid leukemia (AML) patients with partial tandem duplications (PTDs) in the *Mixed Lineage Leukemia* (*MLL*) officially known as the Lysine (K)-specific Methyltransferase 2A (KMT2A) gene, generally have adverse outcomes. Previous mouse studies have shown that Kmt2a-ptd is insufficient to cause AML, indicating additional mutations are required for leukemogenesis. Herein, we evaluated the mutational landscape, gene expression signatures and prognosis of *KMT2A*-PTD adult AML in comparison to a well-characterized adult AML cohort without *KMT2A*-PTD. Our study demonstrates that *KMT2A*-PTD AML has a distinct gene expression signature and that concomitant *DNMT3A* and *NRAS* mutations were associated with adverse clinical outcome in this subset of AML.

AML patients with *KMT2A*-PTD is characterized by an internal duplication spanning exon 3 to 9, exon 3 to 10, or exon 3 to 11 (Fig. S1A, Supplemental Digital Content 1, http://links.lww.com/HS/A25).[1] *KMT2A*-PTDs occur in 3.2 to 11% of adult *de novo* AML and are more frequently present in AML with normal cytogenetics and AML with trisomy of chromosome 11 as a sole cytogenetic aberration.[2] The presence of a *KMT2A*-PTD has been shown to associate with adverse outcome in AML.[2,3]

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The partial-tandem duplications within *KMT2A* result in in-frame additions of extra N-terminal amino acids and maintain functional proteins, which contribute to leukemogenesis.[4] Under normal physiological conditions, the *KMT2A* gene encodes a SET domain-containing protein, which mediates methylation of histone 3 lysine 4 (H3K4).[5] The effect of the PTD on normal KMT2A function and its role in leukemogenesis are currently unknown. 11q23-rearrangements involving *KMT2A* gene (3–4% of adult AML) result in abrogation of KMT2A transactivation and histone methyltransferase function.[6] *Kmt2a*-PTD alone appeared insufficient to cause AML.[7] These findings support the notion that additional genetic hits are required for the development of *KMT2A*-PTD leukemia.[7,8]

We aimed to evaluate the mutational landscape, gene expression signatures and prognosis of *KMT2A*-PTD adult AML in comparison to a well-characterized adult AML cohort without *KMT2A*-PTD (hereafter referred as reference cohort) treated according to the international multicenter HOVON-SAKK AML clinical trials (www.hovon.nl).

cDNA from 1998 AML patients was screened for *KMT2A*-PTD mutations using RT-PCR and confirmed by Sanger sequencing (Fig. S1B and Supplementary method, Supplemental Digital Content 1 and 2, http://links.lww.com/HS/A25, http://links.lww.com/HS/A26). *KMT2A*-PTDs were present in 5.5% (109 out of 1998) of all AML cases. The *KMT2A*-PTD was examined in the context of a number of clinical parameters (summarized in Table S1, Supplemental Digital Content 3, http://links.lww.com/HS/A27, and detailed in Table S4, Supplemental Digital Content 4, http://links.lww.com/HS/A28). The median age of AML patients with or without a *KMT2A*-PTD was 56 and 51 years, respectively ($p = 0.0016$). The majority of *KMT2A*-PTD AML patients had a normal karyotype (57.3%). However, this did not significantly differ from AML patients without a *KMT2A*-PTD (48.8%) ($p = 0.158$). In line with previous studies, the presence of *KMT2A*-PTDs was significantly associated with a concurrent trisomy 11 as compared to the *KMT2A* wild-type AML reference cohort (7.3% vs 1.1%; respectively, $p = 0.002$).

Genomic DNA (gDNA) was available for 85 out of the 109 *KMT2A*-PTD AML cases. We performed next-generation sequencing (NGS) on the 85 *KMT2A*-PTD AML cases to determine the concurrent driver mutations, with as reference the *KMT2A* wild-type AML cohort ($n = 561$). All AML cases were sequenced using the Illumina TruSight Myeloid Sequencing Panel

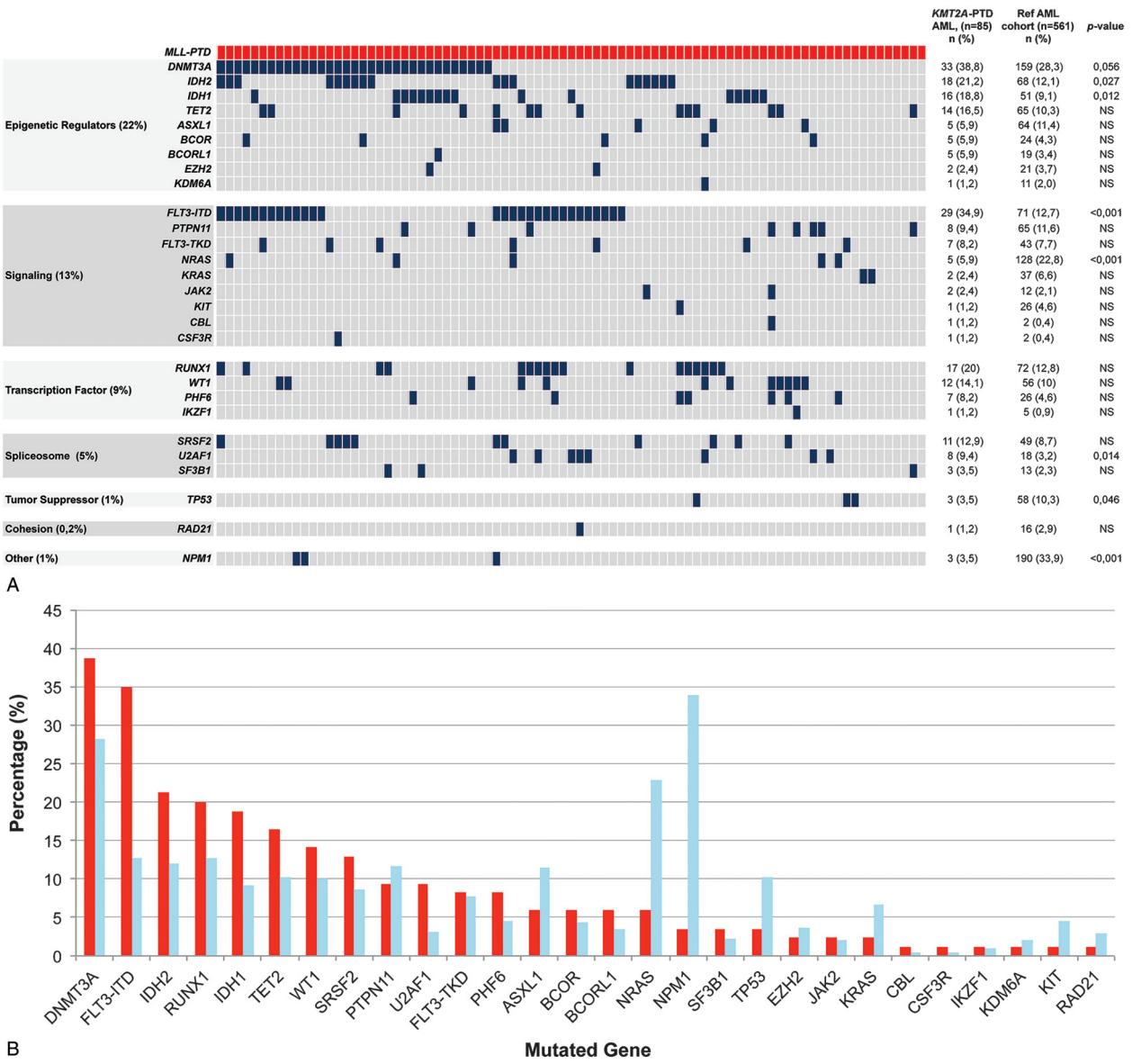

Figure 1. Mutational landscape and gene expression changes of *KMT2A*-PTD AML. A) *KMT2A*-PTD mutation landscape. Each column represents an individual AML patient. The gene mutations are categorized according to gene function and family. The number and the percentage of the gene mutations in *KMT2A*-PTD AML and the AML reference cohort and p-value are indicated. B) The frequency of concurrent mutations of *KMT2A*-PTD AML (red) and the reference cohort (blue).

(Table S6, Supplemental Digital Content 4, http://links.lww.com/HS/A28) on the HiSeq 2500 in Rapid mode following the manufacturer's recommendations (Illumina, San Diego, CA). *FLT3* internal tandem duplications (ITD) were determined with fragment length analyses as previously described.[9] The number of mutations detected in the *KMT2A*-PTD AML cohort ranged from 0 to 6 mutations, with an average of 2.7 mutations per *KMT2A*-PTD AML patient, whereas, the AML reference cohort carried 0 to 9 mutations with an average of 3.0 mutations per AML patient. The average number of mutations was not significantly different between both cohorts (p=0.1).

The most frequently mutated genes in *KMT2A*-PTD AML were *DNMT3A* (38.8%), *FLT3*-ITD (34.9%), *IDH2* (21.2%), *RUNX1* (20%), *IDH1* (18.8%), *TET2* (16.5%), *WT1* (14.1%), and *SRSF2* (12.9%) (Fig. 1). In contrast to what has been reported, we found three cases with a concurrent mutation in the *NPM1* gene (Fig. 1).[10,11]

We next examined whether the concurrently mutated genes were significantly associated with *KMT2A*-PTD AML compared to the *KMT2A* wild-type AML reference cohort. *KMT2A*-PTD concurrent mutations were present in proteins involved in epigenetic regulation, signaling, transcription, splicing, chromosome segregation, and tumor suppression (Fig. 1A). None of these mutational categories was significantly associated with *KMT2A*-PTD AML. However, a number of mutated genes were significantly more frequent in *KMT2A*-PTD AML compared to other types of AML, i.e., *FLT3*-ITD (34.9% vs. 23%; p=0.028) as well as mutations in *IDH1* (18.8% vs 9.1%; p=0.012),

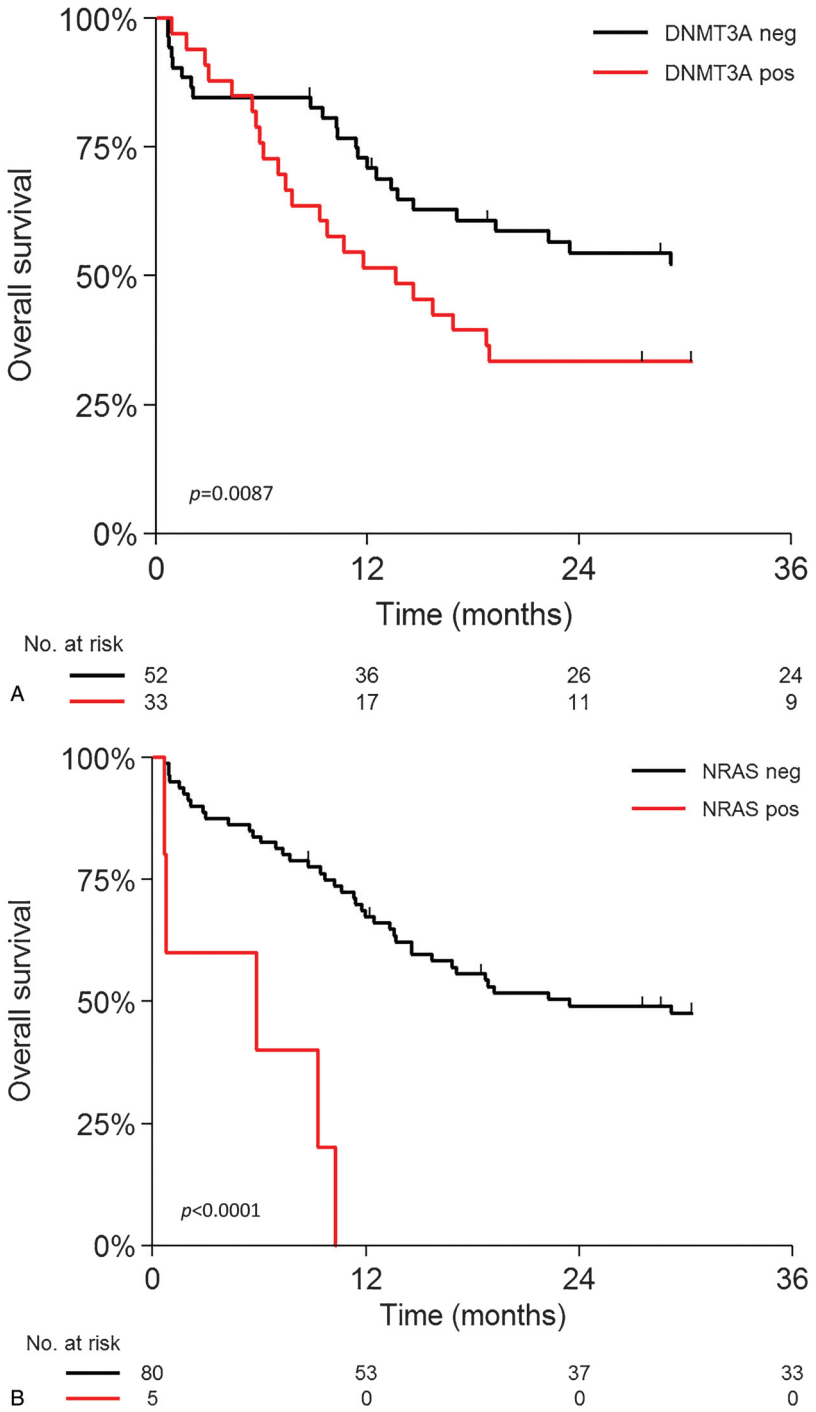

Figure 2. Overall survival analysis *KMT2A*-PTD AML and concurrent mutations. A) *KMT2A*-PTD with and without *DNMT3A* mutations ($P = 0.0087$). B) *KMT2A*-PTD with and without *NRAS* mutations ($P < 0.0001$).

U2AF1 (9.4% vs 3.2%; p = 0.014) and *IDH2* (21.2% vs 12.1%; p = 0.027) [Fig. 1B and Fig. S2, Supplemental Digital Content 1, http://links.lww.com/HS/A25]. Similar associations with *IDH2* and *U2AF1* mutations were demonstrated by Papaemmanuil et al[12] however, *DNMT3A, RUNX1,* and *STAG2* mutations were not significantly associated with *KMT2A*-PTD AML in our cohort. Sun *et al.* demonstrated that *KMT2A*-PTD AML carried more frequently *FLT3, DNMT3A, RUNX1, IDH1* and *IDH2* mutations,[10] whereas Kao et al showed this correlation for *FLT3, U2AF1, RUNX1, STAG2, PTPN11, WT1* and *EZH2* mutations.[11] However, in the latter 2 studies results were not compared to an internal *KMT2A* wild-type AML cohort, which could potentially result in misinterpretation as a result of selection biases regarding these positive associations. In our AML cohort, we did see trends for associations, for example, mutations in *DNMT3A*, *RUNX1, PTPN11,* and *WT1*, but these did not

reach statistical significance. In contrast, mutations in *NPM1* (3.5% vs 33.9%; $p < 0.001$), *TP53* (3.5% vs 10.3%; $p = 0.046$) and *NRAS* (5.9% vs 22.8%; $p < 0.001$) were significantly less frequent in *KMT2A*-PTD AML in our cohort (Fig. 1 and Fig. S2, Supplemental Digital Content 1, http://links.lww.com/HS/A25). Mutual exclusivity between *NPM1* mutations and *KMT2A*-PTDs have been shown before in AML.[10–12]

We next investigated which genes were differentially expressed between *KMT2A*-PTD AML and all other AMLs, in particular, AML with t(11q23), using our previously published gene expression profile (GEP) dataset (n = 513 AML).[13] Interestingly, multiple homeobox-related gene family members were consistently overexpressed in *KMT2A*-PTD AML. The top-35 differentially expressed genes included HOX- and TALE-related genes, such as *HOXB5*, *HOXB6*, *HOXB7*, *HOXB8*, *HOXB9,* and *NKX2.3*, whereas *KMT2A* itself appeared to be the most consistently overexpressed gene (Table S2, Supplemental Digital Content 3, http://links.lww.com/HS/A27). Since mutations in *NPM1* are also associated with dysregulation of *HOX* gene expression, we next used an association model to see if those differentially expressed *HOX* genes were also dysregulated in *NPM1* mutant AML.[14,15] In this association model, which takes a number of relevant clinically and genetically defined subsets of AML into account, *HOX*-related genes, such as *HOXA7*, *HOXA9,* and *HOXA5*, seemed to be more significantly differentially expressed in mutant *NPM1* AML (Table S2, Supplemental Digital Content 3, http://links.lww.com/HS/A27). In contrast, these specific gene expression changes were absent when a similar analysis was performed in AML with t(11q23) involving *KMT2A* (Table S2, Supplemental Digital Content 3, http://links.lww.com/HS/A27). Thus, *KMT2A*-PTD may induce overexpression of *HOX*-related genes in different ways than t(11q23)-related fusion proteins, suggesting that the *KMT2A*-PTD induces leukemogenesis by mechanisms distinct from t(11q23) abnormalities involving *KMT2A*.

The prognostic impact of *KMT2A*-PTD on overall survival (OS) and event-free survival (EFS) of this AML cohort appeared to be not significantly different to wild-type *KMT2A* AML ($p = 0.44$) (Figure S3, Supplemental Digital Content 1, http://links.lww.com/HS/A25). This is in contrast to what has been shown before but in line with more recent publications.[2,3] In normal karyotype AML, this association was also absent ($p = 0.7$, data not shown). We next addressed the question whether the concurrent mutations might carry prognostic value within *KMT2A*-PTD AML. Interestingly, in *KMT2A*-PTD AML, coexisting *DNMT3A* mutations were significantly associated with inferior overall survival (HR: 2.06; 95%CI: 1.19–3.58; $p = 0.010$) (Fig. 2A), as was suggested before.[11] Moreover, *KMT2A*-PTD AML patients that harbor *NRAS* mutations also have an inferior outcome (HR: 6.54; 95%CI: 2.45–17.49; $p < 0.001$) (Fig. 2B). RAS-related mutations such as FLT3 TKD mutations recently were shown to confer an inferior outcome to patients with *KMT2A*-PTD AML. Survival analysis of *DNMT3A* and *NRAS* mutations in *KMT2A* wild-type AML patients revealed that mutations in *DNMT3A* were not associated with treatment outcome ($p = 0.99$) (Fig. S4, Supplemental Digital Content 1, http://links.lww.com/HS/A25), whereas mutations in *NRAS* only showed a borderline association with positive outcome ($p = 0.044$) (Fig. S5, Supplemental Digital Content 1, http://links.lww.com/HS/A25). In multivariable analysis, including white blood cell count (WBC) and cytogenetics, *DNMT3A* and *NRAS* mutations remained significantly associated with adverse outcome (Table S3, Supplemental Digital Content 3, http://links.lww.com/HS/A27). Thus, although *KMT2A*-PTD did not associate with outcome in AML in general, specific mutational subtypes within *KMT2A*-PTD AML appear to carry poor prognostic value.

To validate our findings in *KMT2A*-PTD AML, we investigated an independent *KMT2A*-PTD AML cohort of patients included in the treatment protocols of the CETLAM cooperative group. The validation cohort contained 27 *KMT2A*-PTD AML patients with a median age of 56 years (Table S5, Supplemental Digital Content 4, http://links.lww.com/HS/A28), who were sequenced using Qiagen Human Myeloid Neoplasms GeneRead DNAseq Targeted Panel V2 (Table S6, Supplemental Digital Content 4, http://links.lww.com/HS/A28) (Qiagen, Hilden, Germany) on the MiSeq (Illumina, San Diego, CA) as per manufacturer instructions. The most frequently mutated genes in *KMT2A*-PTD AML validation cohort were similar to our 85 AML patients cohort (*DNMT3A* (48%), *FLT3*-ITD (37%), *IDH2* (22.2%), *WT1* (14.8%), *IDH1* (14.8%) and *RUNX1* (11.1%)) (Fig. S2, Supplemental Digital Content 1, http://links.lww.com/HS/A25). In the validation cohort, we confirmed that *KMT2A*-PTD AML patients with concurrent mutant *DNMT3A* have inferior outcome (13 out of 27; $p = 0.0017$; Fig. S6, Supplemental Digital Content 1, http://links.lww.com/HS/A25). Unfortunately, the low numbers in the validation cohort precluded survival analyses of coexisting *NRAS* mutations, which should be confirmed in a larger *KMT2A*-PTD AML cohort.

In summary, we revealed within the molecular landscape of *KMT2A*-PTD AML, which carry specific *HOX* gene expression signatures, that concurrent *DNMT3A* mutations and *NRAS* mutations are associated with an adverse outcome.

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