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NUP98-fusion transcripts characterize different biological entities within acute myeloid leukemia: a report from the AIEOP-AML group.

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Running title: NUP98 translocations in pediatric AML

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Letter to the Editor

In the last years, collaborative studies have joined to link the degree of genetic heterogeneity of acute myeloid leukemia (AML) to clinical outcome^{1,2}, allowing risk stratification before therapy and guiding post-induction treatment of children with AML. So far, still half of these patients, whose disease is usually characterized by a grim prognosis, lack a known biomarker offering opportunities of targeted treatment. This relevant limitation prompted to pursue the search of new prognostic biomarkers to address also these forms of still uncharacterized AML. We chose to investigate *NUP98* for its attitude to rearrange with different gene partners and produce oncogenic fusion transcripts often found to be associated with a wide range of hematological disorders, including leukemia³. *NUP98*-rearrangements have been mainly mechanistically associated with oncogenic activation of *HOX-AB* cluster genes, a process largely documented for *MLL*-rearranged AML⁴⁻⁶, in cooperation with other recurrent genetic defects, in particular *FLT3-ITD*⁷. The frequency and prognostic relevance of *NUP98* fusions in a single-center cohort of childhood AML has been recently reported⁸, but neither the frequency nor the role of diverse *NUP98* partners have been investigated. Here, we report an exhaustive molecular screening of twelve *NUP98* rearrangements in the Italian pediatric patients with *de novo* AML enrolled in the AIEOP AML 2002/01 trial⁹. By RT-PCR, we characterized, at diagnosis, 494 patients harboring *CBF* rearrangements (*RUNX1-RUNX1T1* and *CBFB-MYH11*, N=99), *MLL*-translocations (N=86), *FLT3-ITD* (N=54), and rarer mutations/translocations (N=83)^{7,10}, while in 172 patients no mutations were detected (35%). Among these 172 patients without known mutations and 36 patients with isolated *FLT3-ITD*¹¹ mutation we searched for *NUP98* rearrangements with *NSD1*, *HOXC11*, *PHF23*, *HOXA9*, *JARID1A*, *HOXD13*, *LEDGF*, *DDX10*, *HHEX*, *ADD3*, *NSD3* and *LOC348801*, finding 16 (9.3%) with 6 different *NUP98*-fusions (16/172 = 9.3%) and 9 with t(5;11)*NUP98-NSD1*, respectively (9/36=25%, Tab. 1S and 2S). In the 16 patients with *NUP98* fusions, no *CEBPA* mutations were found, while 2 patients had *K-RAS* mutation, one of which concomitant with *NPM1* mutation. Survival analyses revealed that the *NUP98-t* (N=16) had a significant worse event-free survival (EFS, 25%) compared to the rest of AML (49.7%) patients without known mutations (N=156, Neg in Fig. 1A, p<0.01), and significantly higher incidence of relapse (Fig.1A, 66.3% vs 33.6% p-value<0.01, Tab.1S). Reduced EFS and increased CIR were found also in the 9 *NUP98-t* patients with a concomitant *FLT3-ITD* mutation similar to isolated t(5;11) (Fig. 1A). Collectively, in the whole AIEOP-AML trial cohort, we identified 25 *NUP98-t* patients (5%) characterized by a

severe prognosis (Fig. 1SA-C). We then characterized *NUP98*-rearranged patients (n=19) by using gene expression profiling (GEP) and compared them to 66 cases of AML with various genetic abnormalities (see supplementary methods, GSE75461). Supervised clustering showed that most of the *NUP98*-AML cases grouped independently of the rest of AML cases (Fig. 1B, Fold Change>|1.5|, p-value<0.01, Fig. 2SA, Tab. 3SAB). In particular, the coding transcript clusters revealed 76 differentially expressed mRNAs (p-value<0.05), where most of the upregulated genes were confirmed to belong to the *HOX* family, in particular the *HOX-B* cluster (see non coding clustering analysis at Fig. 2SB), and the most downregulated were all genes involved in mitosis and nuclear division (*CPNE8*, *CPNE3*, *CCNA1*, *FAS*, *DEFA423* being also validated by RQ-PCR Fig. 2SC). By gene ontology, we confirmed their involvement in the regulation of mitosis and chromatin modification (Fig. 2SD, Tab. 4S), this finding supporting the role of *NUP98*-fusions in increasing genome instability¹². We further investigated this finding in *ex vivo* blasts obtained at diagnosis from t(5;11)*NUP98-NSD1*-rearranged patients and implanted in NSG mice (see supplementary methods). We revealed spindle assembly checkpoint (SAC) defects in total cell extracts from *NUP98-t* cells arrested in M-phase after treatment with the microtubule-depolymerizing drug nocodazole (Noco), but no defects were found in BM cells from healthy donors (HBM) used as control. MAD2 and BUB1 protein levels decreased starting from 8 hours post-Noco treatment, while Cyclin B levels increased, indicating that *NUP98-t* cells had an early and uncontrolled entrance in mitosis (Fig. 3SA). Then, we blocked DNA replication using aphidicolin (APH) and found an increased number of mitotic chromosomal breaks 24 hours post treatment in *NUP98-t* cells compared to HBM cells (Fig. 3SB). This latter result was confirmed by protein analysis, which showed PP2A subunit β in the cytoplasm and phosphorylated H2AX (γ H2AX) in the nucleus for the recruitment of enzymes to repair DNA in *NUP98-t* cells. This phenomenon was absent in HBM cells, where γ H2AX was detected in the cytoplasm and PARP was found to be cleaved, suggesting that apoptosis occurred probably due to incapability of healthy cells to repair DNA and survive at the same APH dose (Fig. 1C).

We then moved to *NUP98* partner gene characterization, finding evidence that *NUP98-t* patients significantly differentially expressed 101 transcript clusters independently of what was found in the rest of AML cases (60 coding and 41 non-coding RNAs, Tab. 4S, Kruskal-Wallis test, p-value<0.01) (Fig. 2A, Tab. 5S). We focused our attention on *HOX-AB* cluster genes expression and found that, having *NSD1* as partner gene, conferred the lowest *HOX-AB* expression among the six different *NUP98*-translocations (Fig. 2A, Fig.

4SA, p -value <0.05). Different GEP sustained a different survival depending on partner gene, with *NSD1* mediating the worst prognosis (*NSD1* N=12; *PHF23+JARID1A* N=7, Fig. 4SBC, p <0.05). In order to identify peculiarities among the different *NUP98*-fusions, we performed enrichment analysis for several selected molecular signatures (see supplementary methods). We found that all were enriched in chromosome instability and *HOX/MLL* signatures, with the exception of that involving *PHF23*, which showed correlation with methylation⁶ (Fig. 2B, Tab. 6S), and *NUP98-JARID1A*, confirmed to be typically involved in AML with megakaryoblastic features¹³. This analysis recognized the *NUP98-NSD1* rearranged patients being significantly enriched of the cAMP signaling, the GSK3 inhibitor pathway and CREB targets gene sets, identifying the cAMP response element binding protein (CREB) as a likely key player among these overrepresented signatures (Fig. 2B, Fisher test * p -value <0.05). Gene set enrichment analysis further confirmed that *NUP98-t* transcriptional profiles were enriched in genes dependent on CREB activity, i.e. cAMP and GSK3 signaling (Fig. 5SA). *CREB* is a proto-oncogene in pediatric AML, and its overexpression has been largely demonstrated to induce aberrant cell proliferation and cell-cycle regulation of hematopoietic cells¹⁴. We investigated *in vitro* if CREB could be involved in the transcriptional control of *NUP98* and *NUP98*-fusions strengthened by the presence of CREB consensus regions (the cAMP response elements, CREs) at the site of the *NUP98* promoter (from 1,300 bases upstream of the *NUP98* transcription start site). By chromatin immunoprecipitation, we confirmed that CREB worked as transcription factor at the *NUP98* gene promoter in myeloid leukemia cells (Fig. 5SB). Furthermore, we co-expressed exogenous CREB (pEGFP-flag-CREB) with a reporter gene (LUC) under the control of the wild type-*NUP98* promoter, revealing that CREB mediated LUC over-activation. On the contrary, when we induced mutagenesis at CREs, LUC activity significantly decreased. Moreover, by CREB gene silencing, LUC activity was found to be reduced, as well as *NUP98* protein levels (Fig. 2C, Fig. 5SC), confirming the hypothesis of CREB controlling *NUP98* gene expression. The same experiment of CREB genetic silencing was performed in *ex vivo* cells from three AML patients at diagnosis harboring *t(5;11)NUP98-NSD1*, revealing a reduced *NUP98-NSD1* chimera at both RNA and protein levels (Fig. 6SAB, p -value <0.01). These results for the first time show that inhibiting CREB and its binding to the *NUP98* promoter may constitute a reliable strategy¹⁵ to destabilize *NUP98*-chimera expression.

In summary, we screened twelve *NUP98* rearrangements in a large pediatric cohort of Italian children with AML and found that six of them identified a new subgroup of recurrent

somatic translocations with a total frequency of 5%. *NUP98*-rearranged patients had an incidence of relapse higher than that of the general high-risk AML, resulting into poorer EFS. We found that *NUP98*-leukemia arises as a sequence of biologically distinct phenomena where each partner gene plays a unique role and that *NUP98*-fusions identify a novel AML subgroup functionally independent from the rest of AML. To the best of our knowledge, this is the first study documenting in primary *NUP98-NSD1*-rearranged leukemia cells an altered spindle assembly checkpoint and an aberrant response to DNA damage. These evidences confirmed that *NUP98* cells suffer from enhanced genomic instability, which may contribute to a more pronounced transcriptional variability and to the acquisition of cooperating mutations, which can concur to the severe prognosis. Collaborative studies in larger cohorts are warranted to yield additional insights into the clinical and biological role played by *NUP98* fusion transcripts. Notably, we documented that CREB is the transcriptional factor controlling *NUP98* expression; thus, its targeting could be further investigated as a novel potential strategy for the treatment of this subtype of AML.

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Authorship Contributions: VB, EM, CT, VS, DDG, MT, performed *in vitro* experiments; MZ performed GEP analysis; AL, ADM, CM, VZ performed and interpreted cytogenetic analysis; SM performed xenotransplantation in NSG mice; RR, EM, RM performed the statistical analysis; FL, GB, MP designed the research, analyzed and interpreted data, wrote the manuscript.

Conflict of Interest Disclosure:

The authors declare no competing financial interests

Supplementary information is available at Leukemia’s website.

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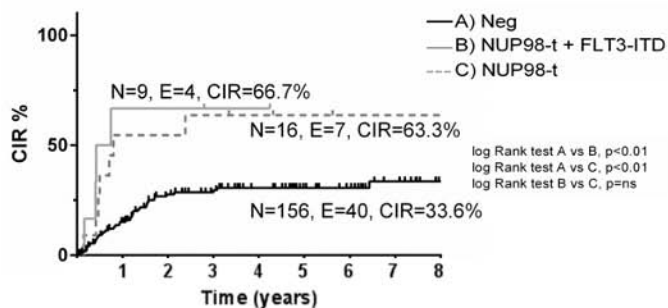
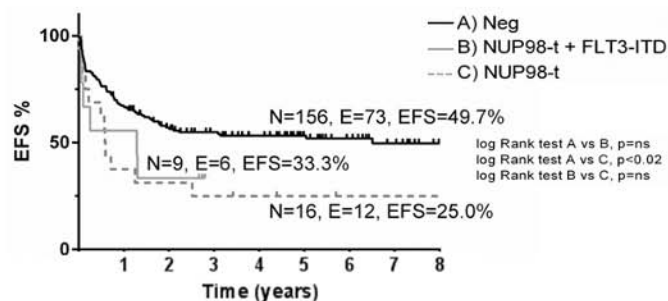
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Figure legends

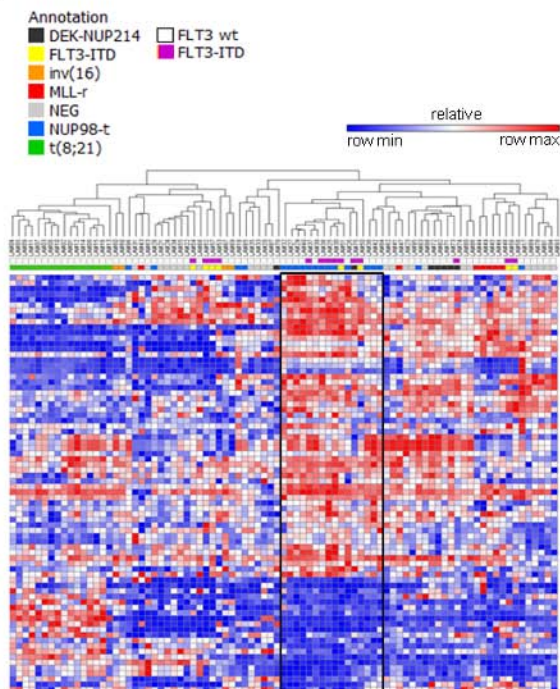
Figure 1. Characterization of *NUP98-t* pediatric AML. A) EFS probability and CIR in 9 and 16 children with *NUP98-t* with and without *FLT3-ITD*, respectively, as compared to the molecularly negative patients enrolled in the Italian AML cohort (p-value < 0.05). Abbreviations: N, number of cases; EFS, event free survival; CIR, cumulative incidence of relapse. B) Supervised hierarchical clustering analysis (using 76 differentially expressed coding RNAs (p-value < 0.01). C) Western blot of lysates after APH treatment. The expression of PP2A-B55 α in the cytoplasm and γ H2Ax in the nucleus of *NUP98-NSD1* primary cells revealed cell integrity and chromosome instability, whereas cytoplasmic γ H2Ax and the increased PARP cleaved (cl) in the HBM revealed that apoptosis occurred. β -ACTIN and PARP1 total (total) represent the loading controls of cytoplasmic and nuclear extracts, respectively.

Figure 2. *NUP98* partner genes confer different expression profiles and CREB drives their expression. A) Left panel shows supervised hierarchical clustering analysis using the 101 probe sets identified by Kruskal-Wallis test among 19 *NUP98-t* patients (p-value < 0.01). Right panel shows *HOXA-B* cluster analysis of pediatric *NUP98-t* patients. B) Molecular signature enrichment analysis. A significant molecular signature is indicated by a black box (*p-value < 0.05 for *NUP98-NSD1* cases versus all the others). C) *NUP98*-wild type and mutated promoter sequence was cloned in the LUC-vector-pXP2-*NUP98*prom and pXP2-*NUP98*prom CRE mut, respectively. Luciferase (LUC) activity was measured in HEK293T transient co-transfection of pXP2-*NUP98*prom with pEGFP-flag-CREB plasmid or siR-NEG and SiR-CREB. The vector alone (pXP2) has been used as control. (N=3, normalized with Renilla (REN) activity).

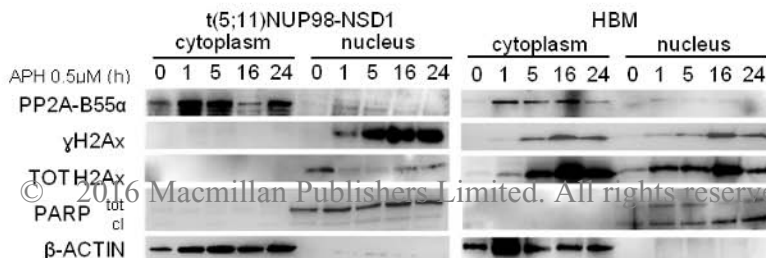
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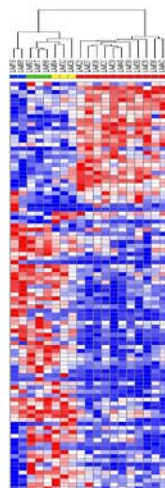
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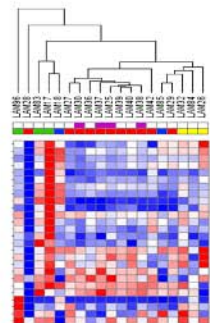
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Annotation

- JARID1A
- NSD1
- others
- PHF23

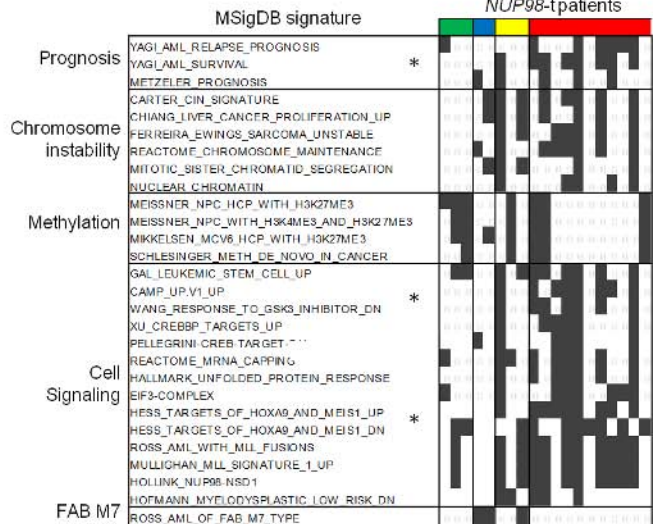
- FLT3 wt
- FLT3-ITD



Symbol

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