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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
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 leukemia\(^3\). NUP98-rearrangements have been mainly mechanistically associated with oncogenic activation of HOX-AB cluster genes, a process largely documented for MLL-rearranged AML\(^4-6\), 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 reported\(^8\), 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 trial\(^9\). 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
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 instability. 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 (γH2AX) in the nucleus for the recruitment of enzymes to repair DNA in NUP98-t cells. This phenomenon was absent in HBM cells, where γH2AX 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.
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 methylation\(^6\) (Fig. 2B, Tab. 6S), and NUP98-JARID1A, confirmed to be typically involved in AML with megakaryoblastic features\(^{13}\). 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 cells\(^{14}\). 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 strategy\(^{15}\) 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.
References


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-B55α in the cytoplasm and γH2Ax in the nucleus of NUP98-NSD1 primary cells revealed cell integrity and chromosome instability, whereas cytoplasmic γH2Ax 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-NUP98prom and pXP2-NUP98prom CRE mut, respectively. Luciferase (LUC) activity was measured in HEK293T transient co-transfection of pXP2-NUP98prom 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).
A) Neg
B) NUP98-t + FLT3-ITD
C) NUP98-t

N=156, E=73, EFS=49.7%
N=9, E=6, EFS=33.3%
N=16, E=12, EFS=25.0%

log Rank test A vs B, p=ns
log Rank test A vs C, p<0.02
log Rank test B vs C, p=ns

A) Neg
B) NUP98-t + FLT3-ITD
C) NUP98-t

N=9, E=4, CIR=66.7%
N=16, E=7, CIR=63.3%
N=156, E=40, CIR=33.6%

log Rank test A vs B, p<0.01
log Rank test A vs C, p<0.01
log Rank test B vs C, p=ns

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