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CICLO XXVIII

Pediatric AML: from prognostic biomarkers to functional characterization

Direttore della Scuola: Ch.mo Prof. Giuseppe Basso

Coordinatore d'indirizzo: Ch.mo Prof. Giuseppe Basso

Supervisore: Dott.ssa Martina Pigazzi

Dottorando: Valeria Bisio

“Non basta guardare, occorre guardare con gli occhi che vogliono vedere,
che credono in quello che vedono”
Galileo Galilei

A Lucia
che mi ha indicato la strada.

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SUMMARY

Acute myeloid leukemia (AML) is a heterogeneous disease characterized by recurrent genetic aberrations. The prognosis of childhood AML has significantly improved over the last two decades, nevertheless the 30% of cases still relapse[1–3]. Intensive efforts have been devoted to identify new genetic abnormalities and altered signalling pathways to better stratify patients in different risk classes in order to improve children survival treating them with a more specific therapy. However still half of the AML cases do not present a recurrent genetic aberration. Thus, during this PhD I focused on the identification of new molecular markers at diagnosis and the evaluation of known markers during the disease follow up. The prognostic value of these markers has been evaluated to improve patients stratification and whenever possible to suggest novel tailored treatments. The overall goal of this study was also to functionally dissect the leukemogenic mechanism of action of these new molecular markers, in order to find suitable candidate gene/pathway to be targeted in novel personalized therapies.

Initially, a screening of new markers at diagnosis was performed in a large Italian cohort of pediatric AML defining the incidence of genetic abnormalities previously described as single case reports or as novel rearrangements identified by next-generation sequencing. The del(4)(q12)*FIP1L1-PDGFRA*, t(16;21)(p11;q22)*FUS-ERG*, t(8;16)(p11;p13)*MOZ-CB*, t(11;17)(q23;q12-21)*MLL-AF17*, t(4;11)(q35;q23)*MLL-ARGB2*, t(3;5)(q25;q34)*NPM1-MLF1*, *MLLPTD*, and t(11;17)(p15.5;p13)*NUP98-PHF23* were finally classified to be rare events at diagnosis. An exception was the translocation t(5;11)(q35;p15.5)*NUP98-NSD1* which reached an incidence of 4% and was found to occur together with *FLT3-ITD* mutation in more than 50% of cases.

Then, the mutations of the oncogene *c-KIT* were taken into evaluation in a selected subset of *CBF*-rearranged patients since these abnormalities were previously reported to be frequent in adults with CFB-AML at diagnosis[4]. I defined a high frequency of *c-KIT* mutations for *RUNX1-RUNX1T1* (25%) and for *CBFB-MYH11* (18.5%) rearranged pediatric patients. Prognostic value of *c-KIT* mutations was determined only for the *RUNX1-RUNX1T1* rearranged patients, suggesting that they could be further evaluated for a targeted therapy with tyrosine kinase inhibitors.

Then, I take into consideration the evaluation of a molecular marker detected at diagnosis during therapy course, by evaluating the role of monitoring the minimal residual disease (MRD) by Real time RQ-PCR . In pediatric AML, post-treatment MRD monitoring

of biomarkers has been rarely used in the clinical management of patients:. molecular markers suitable for MRD detection still remains debated. I improved knowledge for patients with *AML1-ETO* rearrangement and in *FLT3-ITD* mutation, revealing that MRD levels after induction chemotherapy were prognostically significant in identifying those with higher risk to relapse and die These new group of patients , within the same genetic subgroup, may benefit of novel risk stratification or pre-emptive therapy strategies supporting the t(8;21) and *FLT3-ITD* as reliable molecular markers for disease monitoring also during follow up.

A large part of this PhD program was committed to dissect the biology of some recurrent aberrancies being their functional role investigation mandatory to develop new targeted therapies to improve children cure. I hypothesized that biology might explain the difference in therapy response highlighted in the MRD study performed on *FLT3-ITD*patientswhere half of them was found to reduce MRD levels less than 2 logs from diagnosis with a consequent high risk of relapse and death. By gene expression analyses , I showed that these patients had a different gene expression profile related to epigenetic control, most concerning methylation and acetylation of histones. These findings may suggest that the use of epigenetic drugs, combined with conventional strategies, could be a new therapeutic opportunity for a the *FLT3-ITD* patients showing high MRD levels after the end of first induction course.

A second functional study was carried on the t(6;11)(q27;q23)*MLL-AF6* rearrangement. In the Italian AML cohort, 10% of AML patients are *MLL*-rearranged[5] and among them the t(6;11) cases present the worst prognosis[5,6]. By *in vitro* studies, I found that wild type AF6 protein co-localized with RAS in the bone marrow of healthy donors, while AF6 was sequestered into the nucleus provoking RAS overactivation in t(6;11)(q27;q23) rearranged AML. The role of AF6 in RAS inhibition was confirmed by AF6 silencing or treatment with RAS antagonists, revealing the implication of RAS pathway in the aggressiveness of *MLL-AF6* leukemia. This discovery confirmed the usefulness of Tipifarnib, a drug currently used in RASopathies[7], in this AML subgroup, and opens for new targeted therapies to overcome their poor outcome.

The third functional study was performed on a gene recently found implicated in several translocations being not rare (4.6%) in pediatric AML at diagnosis and with outcome severe prognosis, *NUP98*[8–10]. I deep inside the biology of all the NUP98 detected rearrangements, and identified a specific gene expression pattern as well as a typical outcome. Gene ontology revealed an enrichment in biological processes linked to the

nuclear organization and chromosome instability, confirmed also by in vitro studies on *NUP98-NSD1* rearranged primary cells. Moreover, I reported CREB control in the transcription of *NUP98* and consequently of its chimeras. Altogether, these findings open for further studies into the leukemogenic mechanism of *NUP98*-rearranged AML, and highlight CREB as a possible therapeutic target to decrease the oncogenic properties of NUP98-chimeras.

Finally, during this PhD a variety of molecular lesions were identified permitting a more detailed diagnosis for pediatric AML. The prognostic significance of each marker was evaluated and included in the risk classes stratification of the new AIEOP LAM 2013 protocol, conferring to genetics a strong role in guiding therapeutic strategies. Functional studies were able to characterize new candidate genes that can be specific for a subgroups of AML patients with detrimental prognosis, to be further studied for their therapeutic role and when possible for a even more personalized therapy. All together, this work achieved results that are currently translated into clinical management, and will contribute to the improvement of the outcome of AML children.

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SOMMARIO

La leucemia acuta mieloide (LAM) è una malattia geneticamente eterogenea,caratterizzata da ricorrenti anomalie molecolari. Nonostante la prognosi dei pazienti pediatrici affetti da LAM sia notevolmente migliorata negli ultimi anni, il tasso di ripresa di malattia è di circa il 30% [1–3]. Numerosi studi sono emersi per identificare nuove anomalie genetiche o vie di segnale deregolate nella LAM pediatrica al fine di migliorare la stratificazione dei pazienti nelle diverse classi di rischio, e di conseguenza poter adottare dei percorsi terapeutici specifici e più mirati. Ad oggi tuttavia, per circa il 50% dei casi non si trova alla diagnosi un marcatore molecolare noto in grado di garantire una corretta stratificazione del paziente. Per tale ragione il mio dottorato di ricerca ha avuto come primo scopo la ricerca e l'identificazione di nuovi marcatori molecolari alla diagnosi , e di studiarne il ruolo prognostico affinché si possa garantire una più corretta diagnosi a un più alto numero di pazienti, e si possa valutarne in caso un ruolo anche come marker di monitoraggi durante la terapia del paziente. Infine, allo scopo puramente diagnostico, è stato abbinato uno scopo di ricerca di base, cioè caratterizzare il processo neoplastico mediato da alcuni di questi marcatori molecolari, cercando di identificare dei geni malattia che possano servire essere dei candidati target terapeutici, utili a porre le basi per una gestione sempre più personalizzata e quindi efficace della terapia.

Inizialmente ho effettuato una serie di screening molecolari con l'intento di valutare l'incidenza di alcune anomalie genetiche precedentemente conosciute solo tramite case report o identificate tramite sequenziamento massivo dell'RNA. In particolare, ho definito la del(4)(q12)*FIP1L1-PDGFRA*, la t(16;21)(p11;q22)*FUS-ERG*, la t(8;16)(p11;p13)*MOZ-CBP*, la t(11;17)(q23;q12-21)*MLL-AF17*, t(4;11)(q35;q23)*MLL-ARGB2*, la t(3;5)(q25;q34)*NPM1-MLF1*, il *MLLPTD*, e la t(11;17)(p15.5;p13)*NUP98-PHF23* come eventi mutazionali rari nella coorte pediatrica italiana arruolata nel protocollo LAM 2001-02 (totale pazienti N=482). Al contrario, la t(5;11)(q35;p15.5)*NUP98-NSD1* è stata riscontrata avvenire con una frequenza del 4%, e spesso in concomitanza alla mutazione *FLT3-ITD* (nel 50% dei casi). Tale traslocazione è stata inoltre valutata per il suo peso prognostico, rivelandosi un fattore prognostico negativo perché associato ad un elevato rischio di recidiva e morte.

Poi, un altro screening ha riguardato la valutazione delle presenza di mutazioni a carico del gene *c-KIT*, in un gruppo di pazienti già con riarrangiamento del *CBF*. Le

mutazioni di questo recettore delle tirosin chinasi sono già state ampiamente descritte in numerosi studi soprattutto riguardanti pazienti adulti affetti da LAM[4]. I risultati confermano un'alta frequenza di mutazione di *c-KIT* anche nei pazienti pediatrici con t(8;21)*RUNX1-RUNX1T1* (25%) e con inv(16)*CBFB-MYH11* (18.5%). Il valore prognostico negativo è risultato significativo solo nel gruppo con *RUNX1-RUNX1T1*, per i quali, l'identificazione di queste mutazioni potrebbero supportare l'uso di eventuali terapie con inibitori delle tirosin chinasi per migliorare la loro cura.

Oltre alla diagnosi, il marcatore molecolare può avere un ruolo fondamentale anche durante il corso della malattia. Mi sono occupata di mettere a punto lo studio della malattia residua minima (MRM) mediante PCR quantitativa per tre importanti marker ricorrenti nelle LAM pediatriche. Ad oggi, il monitoraggio della MRM nella LAM pediatrica è scarsamente utilizzato. Qui, si propone il monitoraggio della MRM tramite la RQ-PCR dopo chemioterapia di induzione nei pazienti con t(8;21) e *FLT3-ITD* in grado di individuare i pazienti a più alto rischio di recidivare. Aver identificato la t(8;21) e *FLT3-ITD* come buoni marcatori molecolari per il monitoraggi della MRM, consentirà ai clinici di poter valutare delle strategie alternative in quei pazienti che potrebbero beneficiare di terapie farmacologiche supplementari al fine di evitare la ripresa della malattia.

Infine, molto tempo del mio dottorato è stato impegnato alla caratterizzazione biologica e funzionale di alcuni marcatori molecolari ricorrenti con il fine ultimo di identificare nuovi possibili target terapeutici per migliorare la cure e la sopravvivenza dei pazienti. In primis, ipotizzando che la diversa risposta terapeutica dei casi *FLT3-ITD* abbia origine da una diversità biologia, abbiamo effettuato delle analisi di espressione genica su questo gruppo di pazienti. Questo studio ha permesso di identificare un profilo di espressione genica caratteristico per i pazienti che riducendo meno la malattia dopo l'induzione vanno incontro a un più alto rischio di ricadere. I processi biologici arricchiti in questi pazienti sono risultati riguardare la metilazione e l'acetilazione degli istoni, suggerendo che eventuali agenti deacentilanti o demetilanti, in combinazione con la terapia convenzionale, possano migliorare la sopravvivenza libera da avventi avversi di questi pazienti.

Un altro studio funzionale ha preso in esame la t(6;11)(q27;q23)*MLL-AF6.* Circa il 10% della popolazione pediatrica italiana presenta uno dei riarrangiamenti a carico del gene *MLL* , tra questi la t(6;11) presenta la prognosi peggiore[5,6]. Attraverso studi *in vitro*, ho rivelato che la proteina AF6 endogena si localizza nel citoplasma insieme all'oncogene RAS in cellule di midollo osseo sano. Viceversa, nei pazienti con traslocazione t(6;11),

AF6 è stato riscontrato essere nel nucleo impedendo il fisiologico controllo di RAS nel citoplasma, comportandone un'iper-attivazione della via. Sia il silenziamento di AF6 sia il trattamento con inibitori di RAS hanno confermato il ruolo chiave del pathway di RAS nel sostenere l'aggressività di questa leucemia. Infine, lo studio ha comprovato il Tipifarnib, farmaco già in uso nelle RASopatie[7], come nuovo farmaco utilizzabile nei pazienti pediatrici con t(6;11).

Il terzo studio funzionale ha riguardato un gene molto nuovo nella LAM pediatrica, il gene *NUP98.* Le traslocazioni somatiche associate a questo gene[8–10] si sono riscontrate non rare nella corte pediatrica LAM italiana (4.6%). Lo studio più funzionale ha poi chiarito che ciascuna di queste traslocazioni identificate una diversa biologia così come un diverso ruolo prognostico . Grazie all'analisi di espressione genica ho identificato l'instabilità genetica come il processo biologico maggiormente deregolato in questo gruppo di pazienti con *NUP98*-LAM. Tale processo è stato verificato *in vitro* grazie a colture cellulari primarie di pazienti *NUP98-NSD1* riarrangiati. Inoltre, ho dimostrato che il fattore di trascrizione CREB controlla la trascrizione del gene *NUP98*, cosi come di tutte le oncoproteine che si riscontrano nelle LAM mantenere l'N terminale dello stesso. Questi risultati identificano il ruolo funzionale della chimera NUP98-NSD1, e candidano CREB a possibile bersaglio terapeutico per combattere l'espressione della chimera e quindi la progressione della malattia .

In conclusione, durante i tre anni di dottorato di ricerca ho caratterizzato una serie di marcatori molecolari che hanno permesso una migliore e più dettagliata stratificazione dei pazienti alla diagnosi. Dato il valore prognostico dei vari marcatori, essi sono stati inclusi nel nuovo protocollo terapeutico LAM 2013, che conferisce alla genetica molecolare un ruolo determinante nel guidare la terapia. Infine gli studi funzionali hanno finora portato all'identificazione di nuovi target specifici in vari sottogruppi di LAM a prognosi infausta. Studi futuri sono in corso per valutare questi biomarcatori come target terapeutici da utilizzare per incrementare le possibilità di curare i bambini affetti da LAM .

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CHAPTER 1

Introduction

ACUTE MYELOID LEUKEMIA

Hematopoiesis is a tightly controlled process in which transcription factors and chromatin remodeling genes finally orchestrated the gene expression that defines the phenotype of a blood cell. The hematopoietic hierarchy begins from the pluripotent hematopoietic stem cell (HSC) which, thanks to its hematopoietic potential, gives rise to both the hematopoietic lineages, lymphoid and myeloid. Ineffective hematopoiesis, resulting in homeostatic imbalance in the production of blood cells, led to a series of hematological disorders. Leukemia is the most common hematological malignancy occurring during childhood. Its origin depends on the progenitor cell that is affected for differentiation and proliferation capabilities, that subdivides leukemias in lymphoblastic leukemia , whena lymphoid progenitor cell is mutated, or myeloid leukemia, when the precursor is from the myeloid lineage[1].

Acute Myeloid Leukemia (AML) is relatively rare (15%–20% of overall leukemia in the childhood) characterized by the uncontrolled proliferation, increased survival and impaired differentiation of hematopoiesis as result of distinct but cooperative mutations acquisition. These neoplastic cells, called blast cells, accumulate in the bone marrow and others organs originating the onset of acute leukemia. To be called acute, these abnormal immature leukemic cells, known as blasts, must be present in bone marrow for a percentage greater than >20%[1,2]. The neoplastic myeloblasts can be arrested in a variety of differentiation stages, supporting the loss of the normal hematopoietic function due to alteration of self-renewal, proliferation, and differentiation.

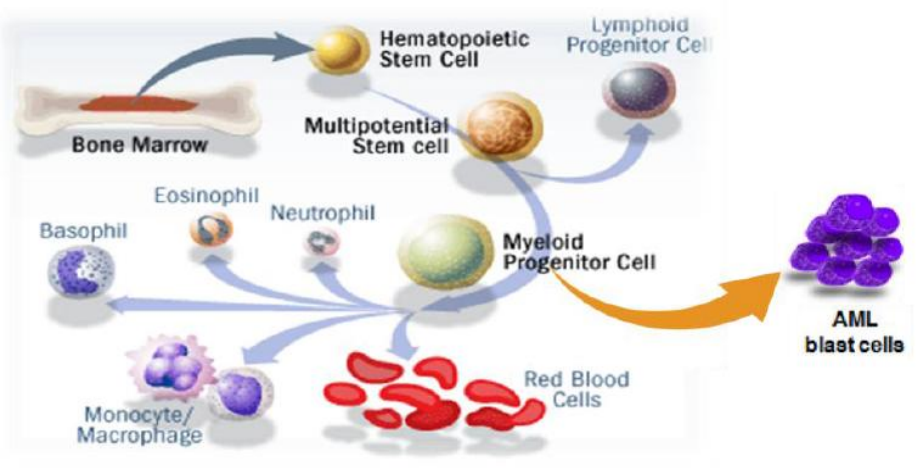

Figure 1. Cellular origin of Acute Myeloid Leukemia shows the differentiation of normal lymphoid and myeloid lineage from hematopoietic stem cells. Yellow arrow points at the abnormal undifferentiated leukemic blast cells (Modified from How stem cells work by Stephanie Watson).

The new era of genomic sequencing and high throughput technology has recently refined the current hypothesis of the AML development. In the first step of the leukemogenesis, a driver mutation occurs within the context of a HSC[3,4]. This alteration confers a proliferative advantage to the cell allowing the clonal expansion, carrying along all the background mutations within its genome (passengers). An additional driver mutation occurs within a committed cell forming the expanding clone which becomes the leukemic "founding" clone detected at the diagnosis. Thus, these cells are supported to contain only a few drivers but many passengers mutations[5–7]. By next generation sequencing studies novel mutations occur at the founding clone that can sustain a relapsing clone able to survive at the chemotherapy[6,8]. Therefore, the AML model is becoming increasingly sophisticated and debated, particularly in pediatric field. Intense efforts have been devoted to identify the genetic mutations require for the malignant transformation. Recent reports highlight that Class I (that confer a proliferative and survival advantage) and Class II (impair differentiation and apoptosis) mutations are only one part of a more complex picture[9]. New mutations have been identify in AML genome that might have a pivotal role in the leukemogenic process and constitute new classes, such as mutations at genes involved in epigenetic modifications (Class III), cell adhesion (Class IV) and DNA repair (Class V)[10–12] (figure 2).

In this new scenario AML constitutes an exceptional biological model of cooperative genetic and epigenetic alterations that initiate the myeloid transformation, a clonal disease and its progression.

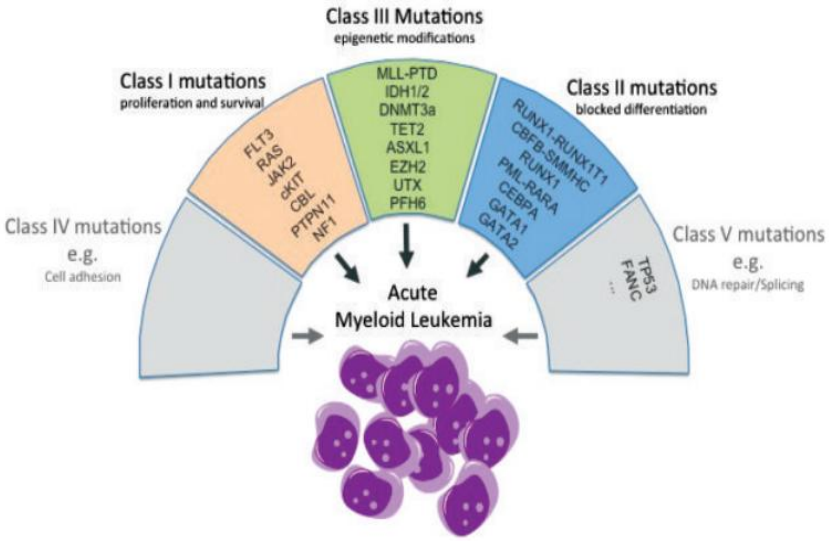

Figure 2. Molecular pathogenesis of AML. Five class of mutations involved in the AML development (from Hematology Education 2012, C.T Hien)

A CLINICAL POINT OF VIEW

AML has an extremely heterogeneous nature recognized as differences in cell morphology, immunophenotype, cytogenetics and molecular genetics. This variability is due to the diversity of myeloid precursor susceptible to malignant transformation, as well as for the multiplicity of the events that orchestrate the genome control. The vast majority of AML cases can be classified according to specific clinical-biological features and genetic abnormalities able to identify distinct subtypes of leukemia[13].

Molecular genetics of AML

Childhood acute leukemia has long been the best characterized malignancies from the genetic point of view. Despite the continuous identification of molecular lesions that guide prognosis and patients clinical management, AML remains highly heterogeneous disease within the 50% of patients that are actually without a known molecular marker. Thus, the identification of novel prognostic factors AML remains one of the main needs for the improvement of AML knowledge and patients survival. Next-generation sequencing of AML has recently shown hundreds of novel genetic lesions within this disease, representing an important advance in order to dissect the leukemogenic process and prognosis and to stimulate the development of targeted therapy. Although, results revealed that the most represented mutations still remain those genomic mutations previously known to occur at *RUNX1, MLL, FLT3, CEBPA, NPM1* and *c-KIT* genes [14,15]. Nevertheless the huge improvements of novel mutations were no recurrent, nor *in vitro* studies are present that can define them as prognostic factor since now. For this, we consider that the 80% of the AML children have disease-associated genomic structural alterations, 65% of those without cytogenetic abnormalities (normal karyotype) have one of known mutations; thus more than 90% of pediatric AML cases are identified to have at least one known genomic alteration (figure 3)[14], and that each individual case of AML harbor a huge number of mutations at specific genes whose role remain elusive[16,17].

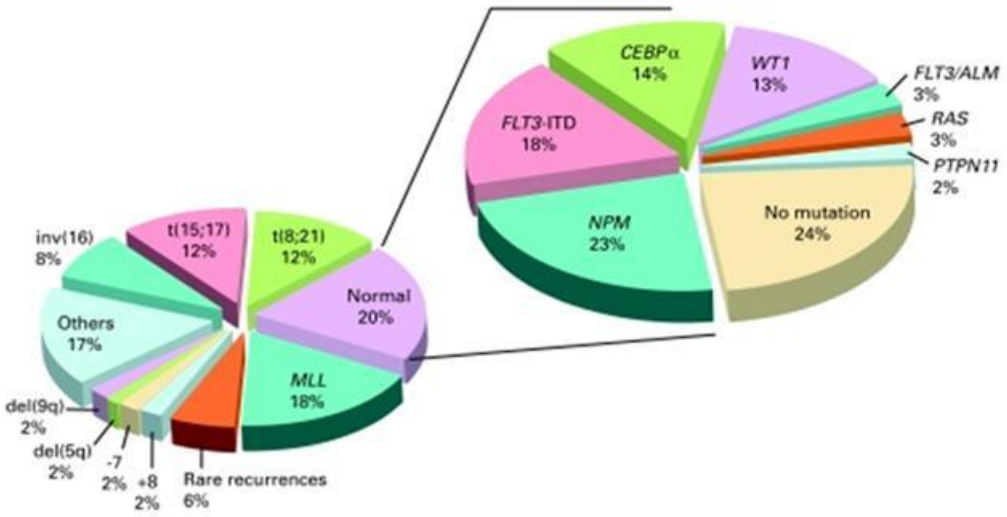

Figure 3. Estimated frequency of specific genotypes in childhood acute myeloid leukemia. Panel to the left demonstrates the most common karyotypic alterations. Eighty percent of all children have disease-associated genomic structural alterations. Mutation profile in those without cytogenetic abnormalities (normal karyotype) is shown in the right panel. Seventy-six percent of those in the normal karyotype population have one of the known mutations; thus, more than 95% of all children with AML have at least one known genomic abnormality[14].

Prognostic Factor and Risk Stratification in Pediatric AML

This genetic characterization is still an open challenge for pediatric AML for both reasons to take new insight into the pathogenesis of AML, but also to improve prognostic risk assessment and subsequently clinical therapeutic strategies. In the past, many clinical biomarkers have been replaced by cytogenetic and molecular features defining a risk-adopted therapy for pediatric AML. The core-binding factor (*CBF*) translocations, such as inv(16)(p13.1;q22)*CBFB-MYH11*, t(16;16)(p13.1;q22)*CBFB-MYH11* and t(8;21)(q22;q22)*RUNX1-RUNX1T1* are classified as standard risk (SR) for their favorable impact by several international groups[15], all of whom reported overall survival (OS) rates of over 85%. Mutations at the *NPM1* and *CEBPA* genes are less common in childhood AML than in adults, they appear to be associated with a similarly favorable outcome[14,15]. Among the molecular markers of high-risk (HR) of relapse there are the *FLT3* mutations, a family that includes internal tandem duplication *(FLT3-ITD)* and point mutations in the kinase domain (*FLT3-KD*). The *FLT3-ITDs* occurs in approximately 10% to 20% of pediatric AML and may be gained or lost at the time of relapse[18,19]. Although these mutations, patients affected with deletion of chromosome 5, 7 (5-,7-), del(5q) share a poor prognosis. One important AML subtype is made up of patients carrying the *MLL*-rearrangements. These AML are the most heterogeneous among all genetic subtype of this disease, and the prognostic impact of *MLL* rearrangement is mostly poor[20]. The *MLL* gene

located at 11q23 is notoriously promiscuous and has more than 120 translocation partners described; AML with t(1;11)(q21;q23) is rare but has a good outcome, whereas disease with t(6;11)(q27;q23), t(10;11)(p12;q23), or t(10;11)(p11.2;q23) had dismal outcome[20,21]. Repetitive rearrangements that involved *NUP98* have been identified in recent works[22–24]. The cryptic translocations *NUP98-NSD*1 has been recently described in AML pediatric patients with a frequency of 4.4% and it occurred frequently with *FLT3-ITD* mutation, mediating a poor outcome[22]. Mutations in Wilms tumor 1(*WT1*) gene have yielded variable outcome reports from different countries[25,26]. Recently novel recurrent gene mutations in adults AML (at *DNMT3a*, *IDH1* and *IDH2*) have been identified with low frequency in pediatric cohorts[27,28]

Treatment and Outcome

Conventional AML therapy is based on intensive use of cytarabine and/or anthracycline, and etoposide that is frequently used in pediatric induction regiments. The clinical outcome of pediatric AML has significantly improved over the past few decades, with current 8 years old EFS and OS of 55% and 68%, respectively, achieved in the multicenter AIEOP AML 2002/01 protocol[29]. The survival rates are similar to those obtained by several cooperative groups (table 1)[15]. This improvement was largely due to a stratification of patients in risk classes with a consequent risk-directed therapy, to the optimization in induction and post remission treatment strategy (high-dose of cytarabine). The SR patients (carrying *CBF* rearrangements) achieved morphological complete remission after the first induction course with idarubicin, cytarabine and etopiside (ICE). Instead for children who require an intensive therapy, the chemotherapeutic regimens consist of 4–5 cycles of intensive chemotherapy, typically including cytarabine combined with an anthracycline. In younger adult patients, results from previous trials suggested that there is a benefit for outcome using high-dose cytarabine in induction, but a similar effect in pediatric AML patients could not be confirmed[29–31]. Beside chemotherapy, the added value of hematopoietic stem cell transplantation (SCT) in newly-diagnosed pediatric AML is becoming stronger. SCT in first CR has been used only for the HR group in the AIEOP AML 2002/01 protocol, and in a more selected subset of high risk cases for others international groups[32]. The Italian studies reveled that the use of auto- or allo-HSCT in HR patients results in lower incidence of leukemia recurrence, with an acceptable treatment-related mortality[29].

Despite intensive treatment, around 30% of the pediatric patients relapse, which confers high morbidity and mortality[14,15]. Notably the outcome in the Italian cohort of SR patients

was found inferior to that reported in other groups. In fact a larger than expected proportion of patients carrying either t(8;21) or inv(16) relapses[33]. To date, there is no explanation for this result. Furthermore, the high frequency of treatment-related deaths (5%–10%), both in treatment protocols for newly-diagnosed, as well as for relapsed disease, the acute toxicity (cardiotoxicity), and the secondary malignancy highlight that an additional intensification of chemotherapy seems no longer feasible[2,34]. Therefore, further knowledge on the molecular and genetic background is urgent in order to detect novel leukemia and patient-specific treatment targets which are less toxic and more effective.

Table 2. Outcomes of Pediatric AML in Recent Collaborative Group Studies

Study Group and Source	Study Acronym and Inclusion Time	No. of Patients	No. (%) of Patients Treated With SCT	Median ± SD EFS (%)	Median ± SD OS (%)	Relapse (%)	Source
AIEOP	AML2002/01 (2002-2011)	482	Allo-SCT: 141 (29) Auto-SCT: 102 (21)	8-year: 55 ± 3	8-year: 68 ± 2	24	Pession et al 2013[11]
BFM-AML SG	AML-BFM 2004 (2004-2010)	521	42 (8)	5-year: 55 ± 2	5-year: 74 ± 2	29	Creutzig et al 2013[6]
COG	AAML03P1 (2003-2005)	340	73 (21)	3-year: 53 ± 6	3-year: 66 ± 5	33 ± 6	Cooper et al 2012[5]
	AAML0531 (2006-2010)	1,022 (ages 0-29 years)	NA	3-year: 53 *v* ≥ 47	3-year: 69 *v* 65	33 *v* 41	Gamis et al 2014[9]
Japan	AML99 (2000-2002)	240	Allo-SCT: 41 (17) Auto-SCT: 5 (2)	5-year: 62 ± 7	5-year: 76 ± 5	32	Tsukimoto et al 2009[15]
JPLSG	AML-05 (2006-2010)	443	54 (12)	3-year: 54 ± 2	3-year: 73 ± 2	30	Tomizawa et al, Leukemia 2013[14] and Int J Hematol 2013[13]
MRC	MRC AML12 (1995-2002)	564	64 (11)	10-year: 54	10-year: 63	35	Gibson et al 2011[10]
EORTC-CLG	EORTC 58,921 (1993-2002)	177	Allo-SCT: 39 (27)	7-year: 49 ± 4	7-year: 62 ± 4		Entz-Werle et al 2005[8]
NOPHO	NOPHO AML 2004 (2004-2009)	151	22 (15)	3-year: 57 ± 5	3-year: 69 ± 5	30	Abrahamsson et al 2011[4], Hasle et al 2012[16]
PPLLSG	PPLLSG AML-98 (1998-2002)	104	Allo-SCT: 14 (13) Auto-SCT: 8 (8)	5-year: 47 ± 5	5-year: 50 ± 5	24	Dluzniewska et al 2005[7]
SJCRH	AML02 (2002-2008)	216	59 (25)	3-year: 63 ± 4	3-year: 71 ± 4	21	Rubnitz et al 2010[12]

Abbreviations: AIEOP, Italian Association for Pediatric Hematology and Oncology; Allo, allogeneic; AML, acute myeloid leukemia; Auto, autologous; BFM SG, Berlin-Frankfurt-Munster Study Group; CLG, Children's Leukemia Group; COG, Children's Oncology Group; EFS, event-free survival; EORTC, European Organisation for Research and Treatment of Cancer; Japan, Japanese Childhood AML cooperative study; JPLSG, The Japanese Pediatric Leukemia/Lymphoma Study Group; MRC, Medical Research Council; NA, not available; NOPHO, Nordic Society for Pediatric Hematology and Oncology; OS, overall survival; PPLLSG, Polish Pediatric Leukemia/Lymphoma Study Group; SD, standard deviation; SCT, stem-cell transplantation; SJCRH, St Jude Children's Research Hospital.

Table 1. Summary of the Major International Cooperative Groups[15]

Minimal Residual Disease

Disease relapse still remains the most important cause of treatment failure in AML. Molecular monitoring of response to treatment by minimal residual disease (MRD) provides important information to tailor treatment in acute lymphoblastic leukemia[35]. On the contrary, on the AML patients MRD has rarely been used in the prospective risk stratification.

The lack of evidence about MRD thresholds, the choice of the most informative MRD time points, and the lack of standardized MRD assays have implied that MRD has never been considered as a prognostic tool directed therapy in the pediatric setting. MRD has variable prognostic power depending on the time of assessment. A rapid evaluation of tumor clearance after induction therapy may be critical, and some investigators have found that post-consolidation MRD levels carry superior prognostic power[36]. Early MRD detection

provided important information not only improving the outcome but also monitoring the excessive therapy toxicities and avoid the patients exposition to unnecessary additional treatment.

The prognostic value of the response measured by flow cytometry after induction and consolidation therapy has been shown to provide independent prognostic information in pediatric AML[37] but few data are available to support the clinical relevance of the molecular MRD in the risk stratification. In the pediatric AML the detection of MRD by a flowcytometric analysis has been associated with adverse prognosis, and MRD evaluation was included in the international clinical trials[36]. Up to now, also the new AIEOP-LAM 2013/01 protocol would consider MRD levels assessed during follow up by flow cytometry technique. The retrospective study performed on 160 patients enrolled at AIEOP AML 2002/01 protocol revealed that MRD at the end of the first induction provides important prognostic information that will be used to improve stratification and to guide the therapy of childhood[38]. Intensive efforts are currently been devoted to the development of molecular methods able to evaluate residual AML burden by fusion transcript detection, that go beyond the sensibility of the flow cytometry (0,01%), and can improve flowcytometric evaluation for the cases where bone marrow regeneration can misinterpret blasts presence.

High throughput approaches to Pediatric AML

In order to provide more insight into the heterogeneity and biology of AML, high throughput technology has been used to allow an unbiased view on small genomic abnormalities, deregulated pathways and drug response. Array-based comparative genomic hybridization (array-CGH) and single-nucleotide polymorphism (SNP) arrays identified several regions with loss of heterozygosity and recurrent copy number variations (CNVs), although with low frequency in AML[39]. Gene expression profiling using microarray-based methodologies has provided new insights into the biology of a variety of hematopoietic malignancies. The gene signatures have proven to be robust discriminators of the specific subtypes of leukemia, showing diagnostic accuracies that, in many cases, exceed those achieved using routine diagnostic approaches[40]. The expression signature for each of the different leukemia subtypes could provide insights into the underlying pathobiology. Furthermore the differentially expressed genes could be evaluated as specific targets to be further investigated[40–42]. Gene expression profiles cannot be considered in cancer biology without the recent predominant discovered role played by the non coding RNAs (ncRNAs). ncRNAs have emerged as crucial regulators of gene expression, epigenetics and cell fate

decisions[43,44]. ncRNAs include highly abundant and functionally important RNAs, such as ribosomal RNAs (rRNAs), transfer (tRNAs), small nuclear RNAs (snRNAs), and small nucleolar RNAs (snoRNAs), and two more important , the microRNAs (miRNAs) and long ncRNAs (lncRNAs), which have been already involved in the regulation of gene expression of cancers. miRNAs are the most studied regulative non-coding RNAs. Differences in miRNAs expression levels have been associated with specific cytogenetic and molecular subsets of AML (miRNAs signature). Changes in the expression of several miRNAs altered in AML have been shown to have functional relevance in leukemogenesis, by acting as oncogenes as well as tumor suppressors[45,46]. If the impact of microRNAs on haematological malignancies has been well described, very little is known about the precise function of the lncRNAs (RNA molecules longer than 200 nucleotides)[47]. The lncRNAs linked to the HOXA cluster called *HOTAIRM1*, *HOTAIR* and *HOTTIP* have been described on leukemia. In particular, they were found to strictly control the expression of different *HOXA* genes, which are important transcriptional regulators in normal and malignant hematopoiesis[48]. Another class of ncRNAs, the snoRNAs, was found misregulated in leukemia but their role nor targets are still unclear, even if they seem to be activators of translation and RNA splicing[44,49,50]. Recently, it has been identified that snoRNAs expression can delineate a specific profile in multiple myeloma and multiple myeloma[51,52]. The ncRNAs aberrant regulation adds a further level of complexity to the heterogeneity of AML, and may be a new biological source to discover new biomarkers and molecular pathways associated to leukemogenesis. Actually, the gene expression[53,54] role in dissecting AML cannot be evaluated without considering the role of epigenetic, including the DNA methylation and histone modifications. Several studies have already disclose the ability of methylation profiles to distinguish cytogenetic subtypes of adult AML and to predict the clinical outcome[9,55]. A central role of epigenetic in AML process has been linked to the presence of mutations and translocations at genes involved in these processes, as *TET2, DNMT3A, IDH1, IDH2*, *EZH2*[56,57]but these aberrancies occur with a very low frequency in AML childhood[27,28]. Further studies are needed to understand epigenetic mechanisms to dissect patients eligible for new treatment opportunities with demethylating agents or histone modification inhibitors currently adopted as important and strategic new drugs in several hematologic diseases[58,59].

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CHAPTER 2

Aim of the Study

The clinical outcome of pediatric AML has improved significantly over the past few decades but still the 30% of the patients relapse, which confers high morbidity and mortality. Intense efforts have been devoted to molecular classification, however the 50% of children with myeloid leukemia still do not present a known recurrent molecular marker. The aim of this study is to disclose new chromosomal rearrangements, as well as gene mutations and dissect their role of new biomarkers in pediatric AML group, to be used to refine prognostic stratification and suggest differentially tailored treatment, based on integrated genetic profiles. Furthermore, their use as suitable molecular markers for the molecular monitoring of minimal residual disease (MRD) during follow-up would be studied to define treatment response, predict relapse and direct therapy decision. Secondly, these new markers would be biologically and functionally studied to better dissect the pathology of AML. This is translation research, which would provide comprehensive genetic analyses to the clinical setting to enable genotype-specific therapies for a personalized treatment of patients with AML.

CHAPTER 3

Screening of molecular markers in AML

SCREENING OF NOVEL GENETIC ABERRATIONS IN PEDIATRIC ACUTE MYELOID LEUKEMIA: A REPORT FROM THE AIEOP AML-2002 STUDY GROUP.

Martina Pigazzi[1], Elena Manara[1], **Valeria Bisio[1]**, Sanja Aveic[1], Riccardo Masetti[2], Giuseppe Menna[3], Marco Zecca[4], Andrea Pession[2], Franco Locatelli[5], Giuseppe Basso[1]

1) Department of Woman and Child Health, Laboratory of Hematology-Oncology, University of Padova, Padova, Italy

2) Department of Pediatrics, "Lalla Seràgnoli", Hematology-Oncology Unit, University of Bologna, Italy

3) Ospedale Santobono-Pausillipon, Napoli, Italy

4) Oncoematologia Pediatrica, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy

5) Department of Pediatric Hematology-Oncology. IRCCS Ospedale Bambino Gesù, Rome, University of Pavia, Italy

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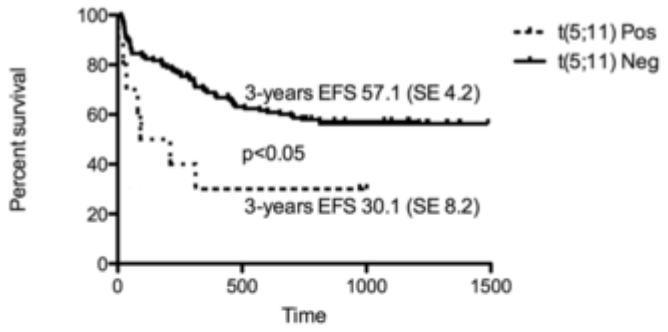
LETTER TO THE EDITOR

Acute myeloid leukemia (AML) is a heterogeneous disease with known specific recurrent genetic aberrations. The continuous and increasing identification of new genetic lesions has permitted to identify new subgroups of patients with different prognosis[1]. In the present work, we evaluated the incidence of rare genetic abnormalities in pediatric AML such as del(4)(q12)FIP1L1-PDGFRA, t(16;21)(p11;q22)FUS/ERG, t(8;16)(p11;p13)MOZ/CBP, t(11;17)(q23;q12-21)MLL/AF17, t(4;11)(q35;q23)MLL/ArgB2, t(5;11)(q35;p15.5)NUP98/NSD1, t(3;5)(q25;q34)NPM1/MLF1, and MLLPTD in 306 children with newly diagnosed *de novo* AML other than acute promyelocytic leukemia enrolled in AIEOP centers from 2000 to 2009,[2] all negative for known recurrent genetic abnormalities involving MLL, CBF-beta and FLT3 genes (77 males and 77 females, median age at diagnosis 7.2 years, range 17 days–17 years). RNA was extracted from fresh bone marrow at diagnosis, and multiplex RT-PCR was employed. Sequencing by Sanger method was applied to all positive cases to characterize fusion breakpoints.

We identified one patient each positive for t(16;21)(p11;q22)*FUS-ERG*, t(11;17)(q23;q12-21)*MLL-AF17*, and t(4;11)(q35;q23)MLL-ArgB2, respectively, this suggesting that these rearrangements are extremely rare in pediatric AML. 2/306 patients had del(4)(q12)*FIP1L1-PDGFRA*, and 4/306 had t(8;16)(p11;p13)*MOZ-CBP*. Interestingly, 6/306 (2%) patients had t(3;5)(q25;q34)NPM1-MLF1, 6/306 (2%) had *MLLPTD*, and 6/306 (2%) were found to carry t(5;11)(q35;p15.5)*NUP98-NSD1*. In our pediatric cohort, the incidence of this last aberration is lower than that previously reported by *Hollink et al*.[3] Subsequently, since a strong association of t(5;11) fusion with *FLT3-ITD* has been described (91%),[3] we extended the screening to 42 children with *de novo* AML harboring the *FLT3-ITD* mutation, enrolled in the AIEOP-LAM 2002 protocol. We found that 6/42 (14%) had the *NUP98-NSD1* fusion. So, six out of 12 NUP98/NSD1-positive patients (50%) were *FLT3-ITD* positive, showing a lower association in our pediatric cohort for these two aberrancies than that reported by Hollink et al.[3] Then, we looked at the event-free survival (EFS) of patients with t(5;11)*NUP98-NDS1* (n=12) and found that it was worse, as compared with patients negative for known molecular lesions and enrolled into the LAM 2002-AIEOP protocol (30.1% vs. 57.1% at 3 years, $p<0.05$).[4] Furthermore, we did not find any difference in either clinical or biological features between patients with isolated t(5;11) and those with t(5;11)+*FLT3-ITD* (Figure 1). The 8-year EFS of *FLT3-ITD*+ children who did or did not carry t(5,11) was 33.3% and 42.7% (p= 0.2),

respectively. This finding suggested that *NUP98-NSD1* fusion protein identifies a previously unrecognized subgroup of *FLT3-ITD* patients with an even worse prognosis.

A



B

NUP98 partner gene	Age,y	Sex	WBC x 10 $^{9/L}$	FAB	Cytogentic aberration(s)	Mutations	CR	Relapse (relapse-free survival in mo.)	Dead (OS in mo.)
NDS1	14	M	404	M2	Normal	FLT-3 ITD	+	-	+ (15.8)
NDS1	15	F	327	M5	Normal	FLT-3 ITD	-		+ (52.4)
NDS1	11	M	78	M2	Normal	FLT-3 ITD	+	-	- (33.3)
NDS1	7	M	202	M5	Normal	FLT-3 ITD	-		+ (14.4)
NDS1	3	F	2.8	M4	Normal	FLT-3 ITD	+	-	- (32.4)
NDS1	3	F	21	M5	Normal	-	+	- (44.0)	- (44.0)
NDS1	9	F	17.9	M2	Normal	-	-		- (10.6)
NDS1	16	M	350	M1	Normal	-	+	+(12.4)	+ (20.2)
NDS1	2	M	4.1	M2	Normal	-	-		+(11.4)
NDS1	2	M	83	M2	Normal	-	-		+(16.3)
NDS1	10	F	218.5	M5	Complex Karyotype	FLT-3 ITD	-		+ (3.3)
NDS1	15	F	62.8	M1	Normal	-	-		+ (17.3)

Figure 1. A) Probability of event-free survival (EFS) in children with *NUP98-NSD1* rearrangement in AML. EFS for patients *NUP98-NSD1*-positive (n = 12, 30.1%) vs. negative patients (n = 142, 57.1%). B) *NUP98-NSD1* rearranged patient's main features.

To test whether *MLLPTD* might also play a role in the occurrence of childhood AML relapse, we analyzed samples from 40 AML at relapse, never finding this abnormality. By contrast, 4 patients harbored at relapse the same *MLLPTD* found at diagnosis, suggesting the stability of this mutation.

In summary, we confirm that t(5,11) is not exceptional in pediatric AML, being frequently associated with *FLT3-ITD,* and identifying patients at high risk of treatment failure. We also suggest a negative role of this translocation in *FLT3-ITD* positive patients to be further considered in the risk stratification of patients. The putative role of the remaining rare abnormalities [5,6] in AML remains to be confirmed in prospective studies with larger cohort of patients.

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IDENTIFICATION OF THE NUP98-PHF23 FUSION GENE IN PEDIATRIC CYTOGENETICALLY NORMAL ACUTE MYELOID LEUKEMIA BY WHOLE-TRANSCRIPTOME SEQUENCING

Marco Togni[1], Riccardo Masetti[1], Martina Pigazzi[2], Annalisa Astolfi[3], Daniele Zama[1], Valentina Indio[3], Salvatore Serravalle[1], Elena Manara[2], **Valeria Bisio[2]**, Carmelo Rizzari[4], Giuseppe Basso[2], Andrea Pession[1] and Franco Locatelli[5]

1) Department of Pediatrics, "Lalla Seràgnoli" Hematology-Oncology Unit, University of Bologna, Bologna, Italy.

2) Department of Paediatric Haematology, University of Padova, Padova, Italy.

3) Giorgio Prodi Cancer Research Centre, University of Bologna, Bologna, Italy.

4) Department of Pediatrics, San Gerardo Hospital, University of Milano-Bicocca, Monza, Italy.

5) Department of Pediatric Hematology-Oncology, IRCCS Ospedale Bambino Gesù, Roma - University of Pavia, Pavia, Italy.

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LETTER TO THE EDITOR

ABSTRACT

The genomic landscape of children with acute myeloid leukemia (AML) who do not carry any cytogenetic abnormality (CN-AML) is particularly heterogeneous and challenging, being characterized by different clinical outcomes. To provide new genetic insights into this AML subset, we analyzed through RNA-seq 13 pediatric CN-AML cases, corroborating our findings in an independent cohort of 168 AML patients enrolled in the AIEOP AML 2002/01 study. We identified a chimeric transcript involving *NUP98* and *PHF23*, resulting from a cryptic t(11;17)(p15;p13) translocation, demonstrating, for the first time, that *NUP98-PHF23* is a novel recurrent (2.6 %) abnormality in pediatric CN-AML.

FINDINGS

Childhood acute myeloid leukemia (AML) is a heterogeneous disease with current survival rates of approximately 60–70 %. Cytogenetics, recurrent molecular abnormalities, and early response to treatment are the main factors influencing outcome[1]. However, around 20 % of pediatric AML do not carry any known cytogenetic abnormality (cytogenetically normal-AML or CN-AML). In order to shed light on this subgroup we performed whole-transcriptome sequencing (WTS) in 13 pediatric CN-AML cases, corroborating relevant findings in an independent cohort of 168 cases. Sequencing was performed on a HiScanSQ sequencer (Illumina), and bioinformatic analysis was performed as previously described[2].

In 2 (CN-AML_54, CN-AML_66) out of 13 cases analyzed, we identified a chimeric transcript involving nucleoporin 98 kDa (*NUP98*) and PHD finger protein 23 (*PHF23*) genes, resulting from a cryptic translocation t(11;17)(p15;p13) (Fig. 1a and Table 1). In both cases, we identified an in-frame fusion between *NUP98* exon 13 and *PHF23* exon 4 (Fig. 1b). To date, the cryptic translocation t(11;17)(p15;p13) has been described only once in an adult AML patient [3], but never in a pediatric AML cohort. Different from what was previously reported by Reader and colleagues [3], in this study the recurrent breakpoint in *PHF23* was always identified at the beginning of exon 4 and not within it (Fig. 1a and b).

Id	Age, years	Gender	WBC, x 10^9/L	FAB	BM blast, % at diagnosis	Extramedullary involvement	HSCT (type)	CR after induction therapy	Relapse (site)	Disease-free duration (months)	Survival duration (months)
CN-AML_54*	2.9	M	187	M1	90	No	Yes (AUTO)	Yes	Yes (BM)	5	† 30
CN-AML_66*	9.0	M	1.2	M0	70	No	Yes (MUD)	Yes	-	65	66
AML_3	9.7	M	6.9	M4	40	No	Yes (MUD)	Yes	-	40	41
AML_4	7.0	M	1.8	M5A	54	No	Yes (AUTO)	Yes	-	103	104

Table 1. Clinical features of pediatric CN-AML patients harboring the *NUP98-PHF23* fusion gene. *, patients identified by RNA-seq; †, dead patient; AUTO, autologous; CR, complete remission; HSCT, hematopoietic stem cell transplantation; MUD, matched unrelated donor; WBC, white blood cells

To assess the incidence of *NUP98-PHF23* fusion in pediatric CN-AML, we examined through RT-PCR analysis and Sanger sequencing a validation cohort of 168 AML children enrolled in the AIEOP AML 2002/01 study[4]; one-hundred thirty-nine patients (76 males and 63 females, median age at diagnosis 7.7 years, range 17 days to 17.9 years) were negative for known recurrent genetic abnormalities involving *MLL*, *CBFB*, and *FLT3*, while the remaining 29 patients (15 males and 14 females, median age at diagnosis 11.8 years, range 3 to 17.4 years) harbored internal tandem duplication of *FLT3* (*FLT3-ITD*), this latter abnormality being chosen because we previously reported a strong association between NUP98-NSD1 rearrangement and *FLT3-ITD*[5]. With the exception of FAB M3 (acute promyelocytic leukemia), all the FAB types were represented in the validation cohort. RNA was extracted from fresh bone marrow at diagnosis, and multiplex RT-PCR was used. Sequencing by Sanger method was applied to all casespositive by PCR to *NUP98-PHF23* fusion gene. Overall, 2 out of 139 CN-AML cases were found to harbor *NUP98-PHF23* (Table 1). *NUP98-PHF23* was not found in any patient harboring *FLT3-ITD*. Fluorescence in-situ hybridization confirmed the cryptic chromosomal translocation t(7;11)(p15;p13) leading to the fusion between *NUP98* and *PHF23* in all cases (Fig. 1c).

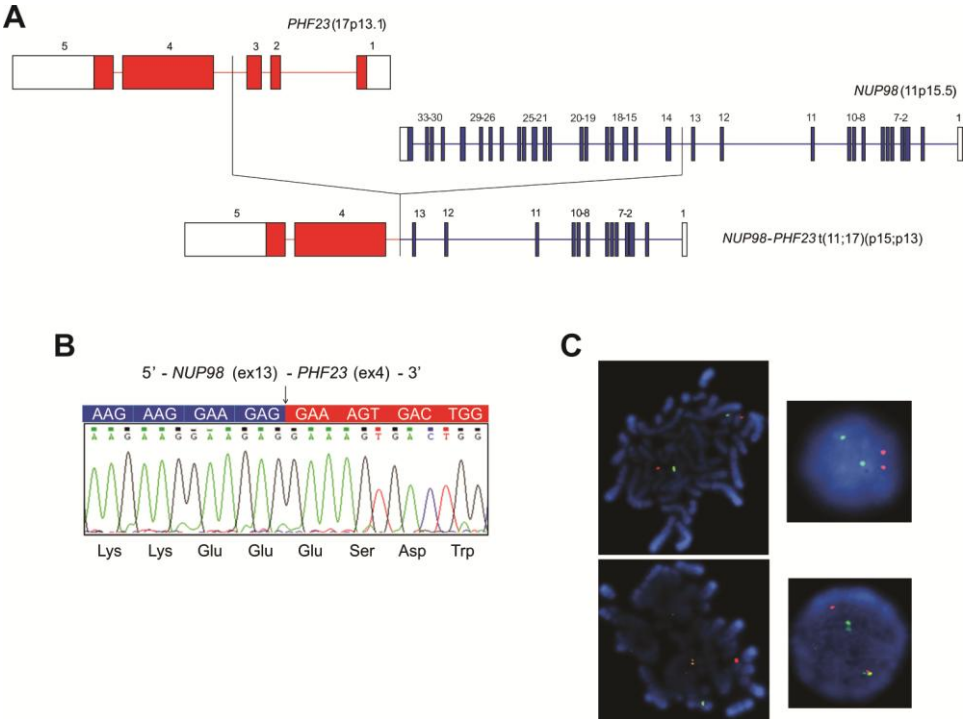

Figure 1. Identification of *NUP98-PHF23* in pediatric CN-AML. A. Schematic representation of *NUP98-PHF23* fusion identified by RNA-seq in pediatric CN-AML. Fusion occurs between exon 13 of *NUP98* and exon 4 of *PHF23*. B. Electropherogram from Sanger sequencing of the region surrounding the breakpoint confirmed the in-frame fusion. A black arrow indicates the fusion breakpoint, predicted sequence of the fusion protein is shown. C. FISH analysis was performed on metaphases and interphase cells using three Blue FISH probes (BlueGnome Ltd., Cambridge), according to the manufacturer's instructions. BAC clones RP11-120E20 and RP11-348A20 (red) were used to probe the *NUP98* gene on chromosome 11, while the BAC clone RP11-542C16 (green) was used to target the *PHF23* gene on chromosome 17. Normal metaphases (upper left) and interphase nuclei (upper right) showed two red signals representing normal copies of *NUP98* and two green signals representing normal copies of *PHF23*. Abnormal metaphases (lower left) and interphase cells (lower right) containing the NUP98-*PHF23* fusion gene showed one red (*NUP98*), one green (*PHF23*) and one yellow fusion signal, which represents the juxtaposition of the translocated portions of the two genes.

So far, many *NUP98*-rearrangements have been found to be associated with both de novo and therapy-related AML but also with T-cell acute lymphoblastic leukemia with over 28 different partner genes [6]. Recently, the fusion *NUP98-JARID1A* has been described to be a recurrent event in pediatric acute megakaryoblastic leukemia (11 %), with a distinct *HOX* gene-expression pattern [7]. Conversely, chromosomal rearrangements and/or mutations of *PHF23* have never been previously described in children with AML. Located on the reverse strand of 17p13.1, *PHF23* encodes for a protein containing a plant homeodomain (PHD) finger [8] involved in chromatin remodeling[3]. Expression of *NUP98-PHF23* has been demonstrated to impair the differentiation of myeloid progenitor cells and promote leukemia development in vitro and in vivo [8–10]. Cells expressing *NUP98-PHF23* are sensitive to disulfiram, an FDA-approved drug, demonstrating the feasibility of

targeting this oncoprotein [9]. In summary, we identified, for the first time in childhood AML, a *NUP98-PHF23* fusion, demonstrating that this genomic aberrancy is not exceptional (tentative frequency of 2.6 %) in pediatric CN-AML. These findings enforce the role of epigenetic regulators in pediatric AML and suggest novel therapeutic targets for this disease.

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CORE BINDING FACTOR ACUTE MYELOID LEUKEMIA IN PEDIATRIC PATIENTS ENROLLED IN THE AIEOP AML 2002/01 TRIAL: SCREENING AND PROGNOSTIC IMPACT OF *C-KIT* MUTATIONS

Elena Manara[1], **Valeria Bisio[1]**, Riccardo Masetti[2], Valzerda Beqiri1, Roberto Rondelli[2], Giuseppe Menna[3], Concetta Micalizzi[4], Nicola Santoro[5], Franco Locatelli[6], Giuseppe Basso[1] , Martina Pigazzi[1]

1) Clinica di Oncoematologia Pediatrica, Università degli Studi di Padova, Padova, Italy;

2) Clinica Pediatrica, Università di Bologna, Ospedale "S. Orsola," Bologna, Italy;

3) Oncoematologia Pediatrica, Ospedale Pausilipon, Napoli, Italy,

4) Oncoematologia Pediatrica, IRCCS Istituto "Giannina Gaslini," Genova, Italy;

5) Clinica Pediatrica, Policlinico di Bari, Bari, Italy;

6) Dipartimento di Oncoematologia Pediatrica, IRCCS Ospedale Pediatrico Bambino Gesù, Roma,Università di Pavia, Italy;

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LETTER TO THE EDITOR

The proto-oncogene *c-KIT*, which encodes a receptor for stem cell factor (SCF), belongs to the type-III receptor of the tyrosine kinase subfamily and is characterized by five extracellular immunoglobulin-like domains, a single transmembrane helix (TM), a cytoplasmic juxtamembrane domain (JMD), and a kinase domain. Abnormal activation of *c-KIT*/SCF growth signal has been frequently documented to occur in cancers, including hematological malignancies, and has been frequently associated with poor prognosis in adults with acute myeloid leukemia (AML) harboring aberrancies at core binding factor genes (*CBF*)[1-3]. *c-KIT* mutations have been reported in pediatric *CBF*-rearranged AML at frequencies ranging from 15 to 54.5%; however, their prognostic significance is still debated [4-7]. Mutations of *c-KIT* occur in the extracellular portion of the receptor implicated in dimerization within exon 8, in the TM-JMD domain within exon 11, and in the activation loop of the tyrosine kinase domain within exon 17, this mediating the constitutive activation of the receptor. The AIEOP AML2002/01 protocol allocated patients with CBF rearrangements in the standard-risk (SR) group, and, although all these patients reached complete remission after the first 2 induction courses, they showed a higher than expected cumulative incidence of relapse (24%)[8]. The identification of new independent prognostic factors and therapeutic targets is desirable to optimize the outcome of this subgroup of childhood AML. In particular, our interest focused on determining whether the presence of *c-KIT* mutation could have a prognostic impact and could allow refining the risk stratification for this subgroup of AML patients.

We retrospectively analyzed the bone marrow at diagnosis of 88 children with CBF-AML enrolled in the SR group of the AIEOP AML2002/01 protocol. Sixty-one patients carried t(8;21)*RUNX1-RUNX1T1,* 26 inv(16)(p13;q22)*CBFB-MYH11* and 1 t(16;16)(p13;q22)*CBFB-MYH11*. Screening for mutations of c-KIT was performed on cDNA by PCR amplification followed by Sanger sequencing of exons 8 and 17, or analysis by the Genescan and Genemapper software (Applied Biosystems Inc) for exon 11. The primers used are listed in Table 1S. Denaturing, annealing and extension step were performed at 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, respectively, for a total of 40 cycles on a thermocycler. PCR products were resolved on a 2% agarose gel. After visual confirmation of amplification, 4 µl of PCR products of exon 8 or 17 were purified with a mixture of 0.5 µl Exonuclease I and 1 µl of FastAP™ Thermosensitive Alkaline Phosphatase (Thermo Scientific) and analyzed by bidirectional sequencing on an ABI310 sequencer, using the BigDye terminator kit v3.1 (Applied Biosystems Inc).

The prognostic impact of *c-KIT* mutation was assessed analyzing overall and event-free survival (OS, EFS) probabilities; the Log-Rank (Mantel-Cox) test was employed to detect differences between subgroups. The screening showed that 5/61 (8%) t(8;21) patients were positive for a point mutation at either codon D816 or N822, affecting the activation loop of the kinase; 1/61 for internal tandem duplication at exon 11, and 9/61 (15%) for small deletions and/or insertions of variable size in the extracellular portion of the receptor (exon 8). Overall, we found that, among the 61 patients harboring the *RUNX1-RUNX1T1* translocation, 15 were mutated for c-KIT (25%). In the cohort of *CBFB-MYH11*-rearranged patients, we documented a lower incidence of *c-KIT* mutations, being found in 5 patients out of the 27 analyzed (18.5%) (Table 1). In inv(16)/t(16;16)-rearranged patients, the mutations were found predominantly in exon 8 (4/5), with just one patient harboring D816V; none had internal tandem duplication.

	Translocation	Age (years)	Sex	WBC x 10 ^{9}/L	RFS (months)	OS (months)	c-KIT mutation
1	inv(16)	15.7	F	48500		Alive	exon 8 - c.1252-1256insAAAA
2	inv(16)	0.8	M	30500	+ (15.3)	Alive	exon 8 - c.1252-1258delTACGACinsTTC
3	inv(16)	11.4	M	15230		Alive	exon 8 - c.1249-1258delACTTACGACinsTTG
4	inv(16)	1.0	F	53080	+ (12)	Dead (16.7)	exon 8 - c.1249-1261delGTCGTAAGTCAGinsGGTTTTCTG
5	inv(16)	11.8	F	68870		Alive	D816V
6	t(8;21)	5.3	M	17590		Alive	exon 8 - c.1255-1261insTTCTTC
7	t(8;21)	7.3	F	4220		Alive	exon 8 - c.1249-1257delGTCGTAAGinsTTCCC
8	t(8;21)	16	M	12050		Alive	exon 8 - c.1249-1255delGTCGTAAGTinsCAAATA
9	t(8;21)	8.1	M	39370		Alive	exon 8 - c.1246-1261delCTGACTTACGACAGGinsCCGAATTTGTGG
10	t(8;21)	11.5	M	10900		Alive	exon 8 - c.1252-1258delTACGACinsTTG
11	t(8;21)	7.4	M	15400		Alive	exon 8 - c.1249-1257delACTTACGACinsATTTCC
12	t(8;21)	5.0	M	105500	+ (6.8)	Dead (13.4)	exon 8 - c.1255-1261delGACAGGinsGCC
13	t(8;21)	9.4	M	30000		Dead (9.3)	exon 8 - c.1249-1258delACTTACGACinsAAGCCTGACTTTG
14	t(8;21)	15.7	F	122400	+ (9)	Dead (12.5)	exon 8 - c.1252-1258delTACGACinsTAG
15	t(8;21)	12.9	F	13700	+ (8.3)	Dead (12.3)	D816Q
16	t(8;21)	16.8	M	72900	+ (5.6)	Dead (11.9)	D816V
17	t(8;21)	2.5	M	1120	+ (6.6)	Dead (13.6)	D816Y
18	t(8;21)	5.30	F	2420		Alive	N822K
19	t(8;21)	7.1	M	19150		Alive	N822K
20	t(8;21)	4.2	M	130800		Alive	ITD - + 97nt

Table 1. Clinical characteristic of *c-KIT* mutated CBF patients. WBC = white blood cell; RFS = relapse free survival; OS = overall survival, ITD = internal tandem duplication, nt = nucleotides

Interestingly, *RUNX1-RUNX1T1*-rearranged patients and *c-KIT*-mutated patients had a significantly worse 4-year OS (51.9%; SE 14.3%) and EFS (51.8%; SE = 14.3%) than patients with isolated t(8;21) (OS = 89.6%, SE = 6.9%, p = 0.0002; Figure 1A; EFS = 78.3%, SE = 6.4%; p = 0.0176; Figure 1B), suggesting that *c-KIT* mutations might contribute to the inferior outcome reported for this subgroup of AML, which has been considered at good prognosis by different collaborative international groups. We highlight that *RUNX1-RUNX1T1*-rearranged patients without *c-KIT* mutations reached the expected survival (90%) for this SR group. The number of *c-KIT*-mutated, *CBFB-MYH11*-rearranged patients was too small for permitting to find, if any, statistical differences in

terms of outcome in comparison to patients who did not carry the *c-KIT* mutation. Noteworthy, there was no statistically significant difference between *c-KIT*-mutated and not mutated *CBF*-rearranged patients in terms of the main clinical features (see also Table 2S).

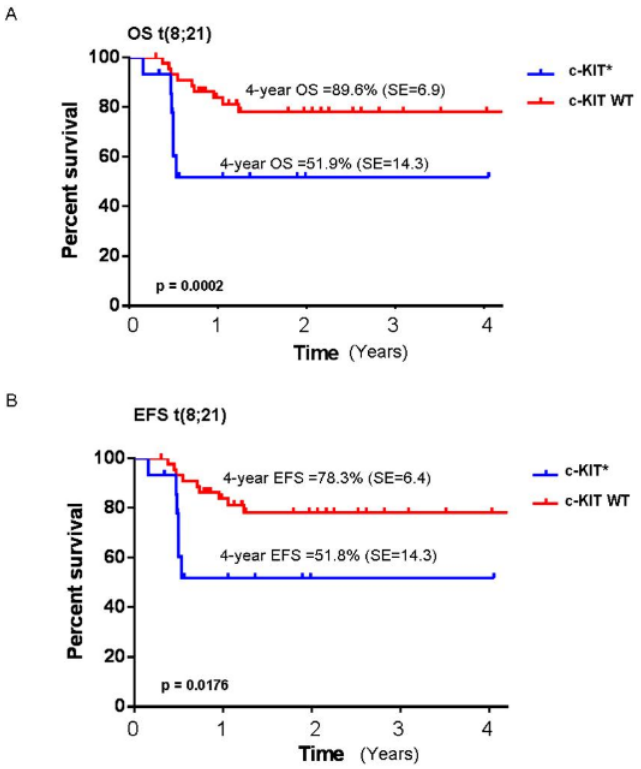

Figure 1. Overall (A) and event free survival (B) probabilities of patients with and without c-KIT mutations harboring RUNX1-RUNTX1 rearrangement.

The prognostic significance of *c-KIT* mutation in other pediatric *CBF*-AML cohorts has been reported to be different in previously published studies. Goemans et al (5) identified *c-KIT* aberrancies in 10/27 children (37%), with a higher incidence of mutation in inv(16) compared to t(8;21) (54.5% vs 31.3%). *Shih et al.*[6] detected abnormalities of c-KIT in 17/41 (41.4%) children with *CBF-AML*: 12/28 (43%) were mutated in *RUNX1-RUNX1T1*-rearranged children, as compared with 5/13 (38.5%) in inv(16). Both studies did not find any statistical influence of *c-KIT* mutation on patient outcome. In addition, Pollard et al (4) analyzed the mutation status of 203 children with *CBF*-AML finding *c-KIT* mutation in 19/94 t(8;21) patients and in 19/71 carrying inv(16). Notwithstanding the large sample size, the results did not reach significance for survival parameters. These findings are in contrast with our data and with the data published by Shimada et colleagues [7] who screened 46 t(8;21) children for *c-KIT* mutations. Significant differences between patients with or without *c-KIT* mutations were observed in the 4-year OS (50.0% versus 97.4%, p =

0.001), disease-free survival (37.5% versus 94.7%, $p < 0.001$) and relapse rate (47.0% versus 2.7%, $p < 0.001$). In view of our data on the incidence and the prognostic impact of *c-KIT* mutations, we believe that t(8;21) and inv(16)/t(16;16) patients should be analyzed separately. In particular, among our c-KIT-mutated patients who experienced relapse we observed that 5 out of 5 t(8;21) patients were dead, whereas 5 out of the 7 children *RUNX1-RUNX1T1*-positive non *c-KIT* mutated who relapsed were rescued by second-line treatment. These findings provide the rationale for considering *c-KIT* mutations as an additional genetic marker to be taken into account in patient stratification. The nature of *c-KIT* mutations offers an attractive target for tyrosine kinase inhibitors. *c-KIT* mutations are associated with a gain of function that induces receptor hyperactivation in response to SCF stimulation, and this leads to a loss of growth factor dependency, increased proliferation and resistance to apoptosis[9]. Notably, different drugs have been developed against tyrosine kinases. Imatinib has been demonstrated to be suitable for mutations at exon 8 and exon 17 involving specifically the codon N822, but not for mutations involving codon D816, which can be successfully targeted with other drugs, such as dasatinib and midostaurin. However, the utility of receptor tyrosine kinase inhibitors for patients with *CBF*-AML and *c-KIT* mutations remains to be further evaluated.

Nowadays, the multistep model of leukemogenesis is widely accepted: class-II mutations affecting genes that impair hematopoietic differentiation, such as *RUNX1-RUNTX1* and *CBFB-MYH11*, cooperate with class-I mutations involving genes, such as *KIT*, *FLT3* and *RAS*, whose mutation leads to increased cell proliferation and survival. Expression of *RUNX1* and *CBF-β* appears to be essential for the development of normal hematopoiesis, since knockout of these genes in animal models leads to lack of definitive hematopoiesis and embryonic animal death (10). Nevertheless, *in vitro* and *in vivo* studies have shown that isolated CBF fusion transcripts were not sufficient to induce leukemogenesis, additional events being necessary for overt leukemia occurrence[11-13]. Recently, 2 different studies reported that patients with t(8;21) treated with a lipid formulation of daunorubicin during induction therapy had a better outcome than those treated with idarubicin[14], and that a second induction course with high-dose cytarabine and mitoxantrone is beneficial for patients with t(8,21)[15]. It remains to be proved whether the advantage of both these therapies mainly concentrates in patients harboring the *c-KIT* mutation.

In summary, we characterized the incidence of *c-KIT* mutations in *CBF* rearranged patients treated with the AIEOP AML2002/01 protocol; it was 25% and 18.5% for children

carrying t(8;21) and inv(16)/ t(16;16), respectively. We document that the detection of a *c-KIT* mutation at diagnosis in t(8;21)-positive patients confers a detrimental prognostic impact. These patients might benefit from a targeted therapy with tyrosine kinase inhibitors.

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SUPPLEMENTARY INFORMATION

c-KIT ex 8 F	GATTAAAAGGCACCGAAGGA
c-KIT ex 8 R	GGTGGCCCAGATGAGTTTAG
c-KIT ex17 F	TTCTTACCAGGTGGCAAAGG
c-KIT ex17 R	AAATGCTTTCAGGTGCCATC
c-KIT ex11 F	6FAM-GTAGCTGGCATGATGTGCATTATT
c-KIT ex11 R	AGGGCTTCCCGTTCTGTCAAA

Table 1S. Primers used for the screening of *c-KIT* mutations.

		t(8;21)			Inv(16) or t(16;16)		
		c-KIT *	c-KIT WT		c-KIT *	c-KIT WT	
#PATIENTS		15	46		5	22	
GENDER	MALE	11 (73%)	25 (55%)	0.2	2 (40%)	14 (64%)	0.25
	FEMALE	4 (27%)	21 (45%)		3 (60%)	8 (36%)	
AGE (years)	<1	0	0	0.15	2 (40%)	1 (5%)	0.08
	1-2	0	0		0	1 (5%)	
	2-10	10 (67%)	21 (45%)		0	8 (36%)	
	>10	5 (33%)	25 (55%)		3 (60%)	12 (54%)	
WBC count (x 10^3/L)	<10	3 (20%)	17 (37%)	0.59	0	5 (22%)	0.3
	10-99	10 (67%)	25 (55%)		5 (100%)	14 (64%)	
	>100	2 (13%)	4 (8%)		0	3 (14%)	

Table 2S. Clinical patient's features.

MINIMAL RESIDUAL DISEASE MONITORED AFTER INDUCTION THERAPY BY RQ-PCR CAN CONTRIBUTE TO TAILOR TREATMENT OF PATIENTS WITH T(8;21)*RUNX1-RUNX1T1* REARRANGEMENT

Martina Pigazzi[1], Elena Manara[1], Barbara Buldini[1], Valzerda Beqiri[1], **Valeria Bisio[1]**, Claudia Tregnago[1], Roberto Rondelli[2], Riccardo Masetti[2], Maria Caterina Putti[1], Franca Fagioli[3], Carmelo Rizzari[4], Andrea Pession[2], Franco Locatelli[5] and Giuseppe Basso[1].

1) Clinica Oncoematologia Pediatrica, Università di Padova, Padova, Italy;
2) Clinica Pediatrica, Università di Bologna, Ospedale "S. Orsola," Bologna, Italy;
3) Oncoematologia Pediatrica, Ospedale Infantile "Regina Margherita", Torino, Italy;
4) Clinica Pediatrica, Università di Milano-Bicocca, Fondazione MBBM, Ospedale S. Gerardo, Monza, Italy;
5) Oncoematologia Pediatrica, IRCCS Ospedale Pediatrico Bambino Gesù, Roma, University of Pavia, Italy;

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LETTER TO THE EDITOR

Disease relapse still remains the most important cause of treatment failure in childhood acute myeloid leukemia (AML). Molecular monitoring of response to treatment by minimal residual disease (MRD) provides remarkable information, widely used to tailor treatment in childhood acute lymphoblastic leukemia[1–3]. On the contrary, prognostic relevance of MRD in pediatric AML has been only recently proposed and needs to be further investigated and confirmed[4–6]. So far, the prognostic impact of the quality of response measured by flow-cytometry after induction and consolidation therapy has been shown to provide independent prognostic information in pediatric AML,[5] able to permit a refinement of risk stratification and to potentially improve AML patient outcome. Intense efforts have been devoted to the development of methods able to measure residual AML burden in support to flow-cytometry, and fusion transcript detection, which is indubitably useful in patient stratification at diagnosis[7], is currently under evaluation as suitable MRD marker to predict relapse in AML[5].

t(8;21)(q22;q22)*RUNX1-RUNX1T1* and inv(16)(p13q22)*CBFB-MYH11* are recurrent somatic lesions detected in about 20% of pediatric AML at diagnosis[8]. In the AIEOP AML 2002/01 protocol, all patients carrying these abnormalities achieved morphological complete remission (CR) after the first induction course with idarubicin, cytarabine and etoposide (ICE) and were grouped as a unique subgroup called standard risk (SR). These children were given, after 2 courses of ICE induction therapy, three post-remissional courses of high-dose cytosine-arabinoside either in combination with etoposide (AVE cycle), or Mitoxantrone (HAM cycle) or administered alone (High dose Ara-c)[7]. A larger than expected proportion of patients carrying either t(8;21) or inv(16) relapsed, this leading to a 8-year probability of event-free survival of 63%. Although many of the relapsing patients were rescued by means of allogeneic hematopoietic stem cell transplantation (HSCT)[8], there is need to identify factors able to predict patients who might not respond to conventional chemotherapy to improve their outcome [9–11]. In this retrospective study, we analyzed the role of MRD monitored by the absolute quantification of *CBF* fusion transcripts early during/after induction treatment, in order to assess its prognostic value in SR childhood AML. We enrolled 76 of the 99 children assigned to the SR group in the AIEOP AML 2002/01 Protocol[8]: 49 carried the t(8;21), 26 inv(16), and one t(16;16) (see Table 1 for details). MRD measurement on bone marrow samples collected at time of diagnosis and after each of the 2 courses of ICE induction chemotherapy was analyzed. It was not possible to study the remaining 23 SR patients due

to insufficient RNA extracted from cells collected after induction courses, but the outcome of patients who were or were not included in the study was comparable (data not shown). We used the Real-time quantitative RQ-PCR (Ipsogene FusionQuant® kit for absolute quantification of fusions on the ABI 7900HD -Applied biosystems) to detect *RUNX1-RUNX1T1* and *CBFB-MYH11* fusions expression following manufacturer's guideline for normalization (with *ABL* gene) and standard curve method for quantification (10^{-5} standard curve sensitivity by using plasmid serial dilutions already included in the kit)[12,13].

	t(8;21)	%	inv(16)	%
Patients	49		27	
Sex				
M	27	55.1	15	55.5
F	22	44.9	12	44.5
Age, years				
< 1	0	0	2	7.4
1-2	0	0	2	7.4
2-10	26	53.1	7	26
> 10	23	46.9	16	59.2
WBC ($x10^9$/L)				
< 20	16	32.6	7	26
20-100	28	57	18	66.6
≥ 100	5	10.4	2	7.4
Outcome				
Relapse	9	18.4	6	22.2
RC post relapse	8	16.3	6	22.2
Death during/post-salvage therapy	4	8.2	0	0
Death after relapse	2	4.1	0	0

Table 1. Patient's clinical characteristics.

For the t(8;21)-rearranged patients, we found a mean number of *RUNX1-RUNX1T1* fusion transcript copies at diagnosis of 643,466 (range from 56,752 to 3,387,522); it decreased to 190,741 mean copies after the I ICE course (range from 11 to 3,062,045), and to 44,671 after the II ICE (range from 0 to 1,671,638). To evaluate the impact of MRD levels measured as copies number during sequential monitoring, the Mantel-Byar test was used to calculate the cumulative incidence of relapse (CIR). We grouped patients in quartiles for copy number measured at diagnosis, after the first and second ICE course. The number of transcript copies at diagnosis, or after induction therapy did not correlate with the probability of both survival and the CIR.

We then considered the logarithmic reduction of MRD after ICE I and II courses calculated with respect to values of copies number of transcript found at diagnosis for each patient (Table 1S). We subdivided patients in three Log-reduction groups: patients who reduced MRD less than 2 Log, between 2 and 3 Log, and those who reduced more than 3

Log in order to investigate whether this distribution into groups for MRD reduction was able to predict a different relapse risk. We interestingly found that 21 (43%) out of the 49 t(8;21) patients enrolled had a slow clearance of blasts after I ICE (< 2 Log with respect to diagnosis), and, at the end of the II ICE course, 10 of them still had a MRD reduction lower than 2 Log. These slow-responding t(8;21) patients at the end of the 2 induction courses had a worse prognosis when compared to patients who reduced MRD more than 3 Log (patients who reduced less than 2 Log showed a 10-year OS of only 58.3% compared to 85.6% of patients who reduced more than 3 Log, p = 0.2, Figure 1).

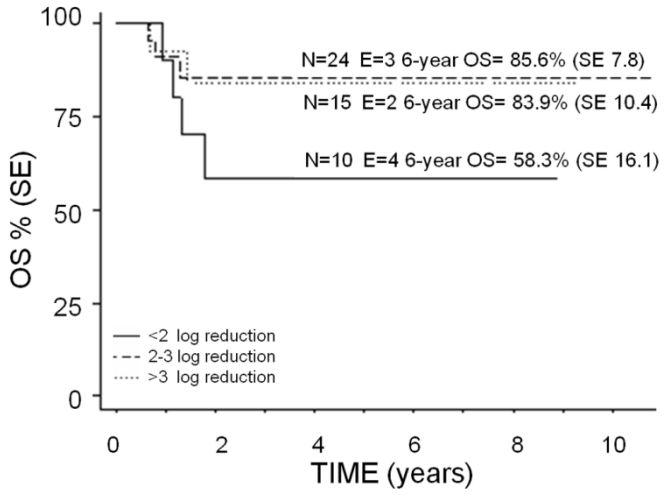

Figure 1. Overall Survival (OS) in *RUNX1-RUNX1T1* patients subdivided into three classes of MRD Log reduction after two induction courses

Next, we investigated if MRD might influence the risk of relapse. Nine out of the 49 patients with t(8;21) relapsed at a median time of 225 days (range 76 to 469) from diagnosis: 7 out of the 9 relapsed t(8;21) patients reduced MRD less than 2 Log after I ICE, and 5 of these 9 relapsed patients still reduced MRD less than 2 Log at the end of the II induction course. The 10-year CIR of these patients after the two induction courses was significantly higher (50%) than that of patients with a MRD reduction greater than 2 Log (17% for 2-3 Log MRD reduction, and 9% for MRD Log reduction >3; Figure 2A I ICE p=0.02; 1B II ICE p=0.004). In univariate analysis both MRD log-reduction after induction therapy and white blood cell (WBC) count at diagnosis >100,000, as recently reported[8], were significant independent factors predicting leukemia relapse; however, they were not confirmed in multivariate analysis, probably due to the limited sample size analyzed. In view of these data, we show that monitoring of molecular MRD levels is instrumental to

predict the risk of relapse for t(8;21)-rearranged patients, thus providing important prognostic information for the therapeutic management of these children.

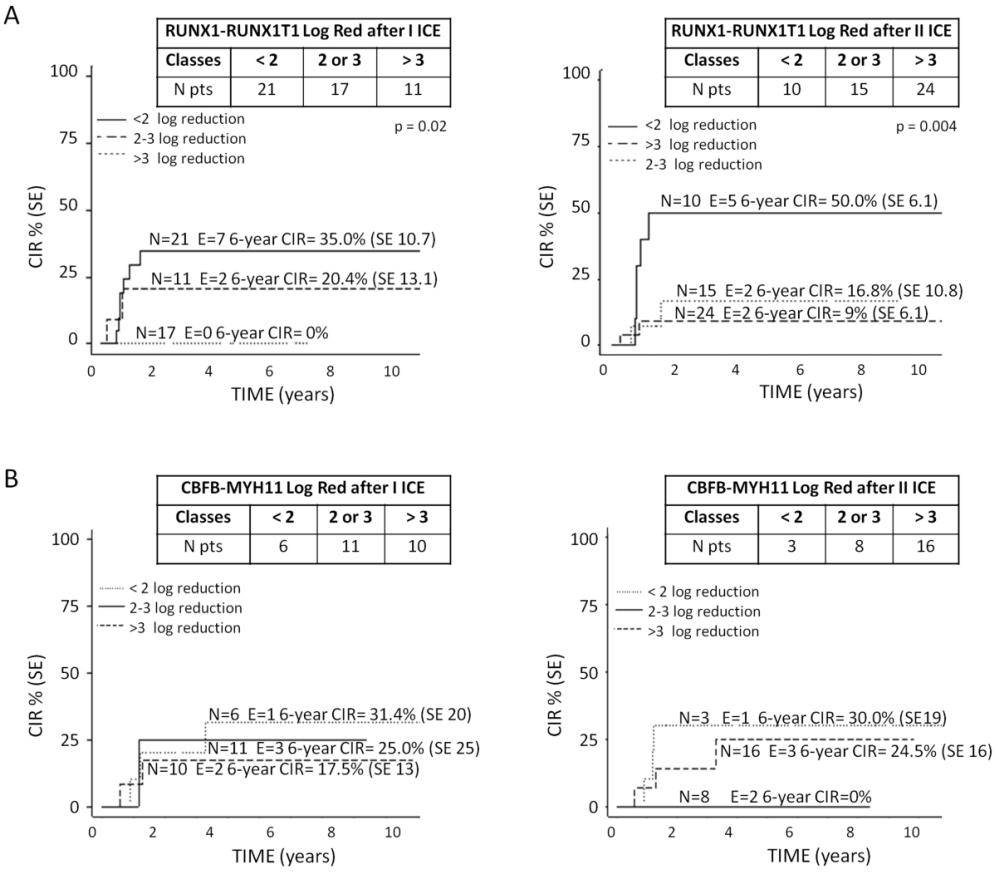

Figure 2. Cumulative Incidence of Relapse (CIR) in patients with *RUNX1-RUNX1T1* (A) and *CBFB-MYH11* (B) subdivided into different subgroups according to MRD Log reduction after I ICE and II ICE.

We then considered inv(16)-rearranged patients and performed similar analyses for the 27 patients enrolled. These patients showed a mean number of transcript copies at diagnosis of 143,015 (range from 102 to 582,426), which decreased to 631 (range from 0 to 3,726) after the I ICE, and to 190 (range from 0 to 686) after the II ICE course, respectively. We evaluated if the copy number of fusion transcripts distributed in quartiles found in BM of patients at diagnosis, and at the end of the two ICE courses may have a prognostic value, but no significant differences were identified neither for CIR nor for OS in inv(16) rearranged patients (Figure 1S). Furthermore, by using the Log MRD reduction distribution, we found that 21/27 patients (78%) after the I ICE, and 24/27 (89%) after the II ICE achieved a MRD reduction greater than 2 Log. At time of last follow-up, 6/27 patients had relapsed; among them, we documented that MRD was reduced more than 2

Log. We conclude that the *CBFB-MYH11* AML showed in most cases a rapid clearance of blasts after induction therapy. Thus, early MRD monitoring does not seem to be useful for predicting relapse occurrence (Figure 2B, not significant at 10 years).

Through this analysis, we document that among pediatric *Core Binding Factor*-rearranged AML two different patterns of molecular response after induction therapy with a different capacity of predicting relapse can be identified. In particular, we found that monitoring of MRD levels after induction provides a reliable prognostic parameter exclusively for the *RUNX1-RUNX1T1*-rearranged patients. These data confirm the clinical usefulness of monitoring MRD levels reported in a recent study on adults with *RUNX1-RUNX1T1* AML[14], although this report differs from our analysis in both timing (after 2 courses of consolidation therapy) and levels of MRD (3 log reduction) cut-off suggested to predict relapse.

In view of these results, we propose a novel MRD-directed risk stratification and treatment of t(8;21) slow-responders to induction therapy. These children can be identified early during treatment and, in light of their high risk of relapse, can be considered candidates to more aggressive therapies, even including allogeneic HSCT. We, thus, propose that RQ-PCR MRD monitoring complemented with the flow-cytometry MRD[14] data might be incorporated directly into clinical practice of the *RUNX1-RUNX1T1* risk class attribution, whereas further studies on extended MRD monitoring for *CBFB-MYH11* rearranged patients are desirable to identify a possible role in predicting the risk of late relapse.

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CHAPTER 4

Translational Research

CHARACTERIZATION OF CHILDREN WITH *FLT3-ITD* ACUTE MYELOID LEUKEMIA. A REPORT FROM THE AIEOP-2002 STUDY GROUP.

Elena Manara[1], Giuseppe Basso[2], Matteo Zampini[1], Barbara Buldini[2], Claudia Tregnago[2], Roberto Rondelli[3],Riccardo Masetti[3], **Valeria Bisio[2]**, Marco Frison[2], Katia Polato[2], Gianni Cazzaniga[4], Giuseppe Menna[5], Franca Fagioli[6], Andrea Biondi[4], Andrea Pession[3], Franco Locatelli[7], Martina Pigazzi[2]

(1) Istituto di Ricerca Pediatrica - Città della Speranza, Padova, Italy.
(2) Dipartimento di Salute della Donna e del Bambino, Clinica di Oncoematologia Pediatrica, Università di Padova, Padova, Italy.
(3) Clinica Pediatrica, Università di Bologna, Ospedale "S. Orsola," Bologna, Italy.
(4) Clinica Pediatrica, Centro Ricerca Tettamanti, Università di Milano-Bicocca, Monza, Italia
(5) Department of Paediatric Haemato-Oncology, Santobono-Pausilipon Hospital, Napoli, Italy.
(6) Paediatric Onco-Haematology, Stem Cell Transplantation and Cellular Therapy Division, Regina Margherita Children's Hospital, Torino, Italy.
(7) IRCCS Bambino Gesù Children's Hospital Rome, Università di Pavia, Rome, Italy.

Submitted

ABSTRACT

Purpose**.** Recurrent molecular markers in AML have been routinely used for risk assessment at diagnosis, whereas their post-treatment monitoring still represents a debated issue in pediatric patients' clinical management.

Experimental design. We evaluated the prognostic value and the biological impact of molecular minimal residual disease (MRD) after induction course I and II and of the Allelic Ratio (AR) at diagnosis of *FLT3*-internal-tandem-duplication (ITD) in childhood AML. We retrospectively screened 494 children with *de novo* AML for *FLT3-ITD* mutation enrolled in the AIEOP-AML 2002/01 protocol. We correlated these parameters with event free survival (EFS), relapse, and gene expression profile (GEP).

Results. Fifty-four patients harboured *FLT3-ITD*. 51% had high-ITD-AR at diagnosis and worse EFS (19.2% versus 63.5% for low-ITD-AR, $p<0.05$). 41% of children with higher levels of MRD after the I induction course had worse EFS (22.2% versus 59.4%, $p<0.05$). GEP showed that patients with high-ITD-AR or persistent MRD had different expression profiles, and that most of deregulated genes were involved in methylation and acetylation. Moreover, ITD-mutation sustained high *CyclinA1* expression, a novel unfavourable prognostic factor (EFS 20.3% versus 51.2% in low *CyclinA1* expression, $p<0.01$), principally by triggering the *B-MYB* oncogenic network.

Conclusions. These findings highlight that ITD-AR levels and MRD after I induction course should be considered in planning clinical post-induction management of *FLT3-ITD* patients to improve their outcome. The evaluation of these parameters suggest different transcriptional activation of epigenetic and oncogenic profiles to be targeted with novel therapeutic approaches in order to overcome variability in outcome among *FLT3-ITD* patients.

INTRODUCTION

Genetic characterization of acute myeloid leukemia (AML) has led to the identification of a great number of molecular markers that are currently used to guide the diagnosis and the clinical management of pediatric patients.[1–4] However, there has been minimal improvement in the outcome of AML, and relapse remains a major cause of treatment failure despite the genetic risk assignment.[5] These molecular markers have further split AML into small patients subgroups for which no studies, providing a more objective assessment of treatment response and targeted therapy, have been reported to date. The monitoring of minimal residual disease (MRD) can be consider one of the most

sensitive approach to detect an early response kinetic to distinguish patients with different risk within the same genetic group, predict relapse and direct pre-emptive MRD-directed therapy decisions.[6]

The Fms-like tyrosine kinase 3 (FLT3) is a class III receptor kinase that is normally expressed by hematopoietic stem cells and progenitor cells and is down-regulated at later differentiation stages, driving early steps of cell proliferation, survival and myeloid lineage differentiation.[7] Abnormalities of the *FLT3* gene occur frequently in AML as a result of internal tandem duplication (ITD) of the juxtamembrane domain-coding sequence in frame with the rest of the gene, or as point mutation within the activation loop domain (*FLT3-ALM*). The mutated *FLT3* gene results in constitutive activation of the kinase domain. In childhood AML, the activating mutations of *FLT3* occur in 10-15% of patients: two-thirds of these harbor ITD while one-third *FLT3-ALM* [8–12].Patients with *FLT3-ALM* have a prognosis comparable to that of patients without *FLT3* mutations;[12] on the contrary, *FLT3-ITD* patients have poor prognosis, mostly for high risk of disease recurrence.[12]

Efforts have been spent in the past decade to identify molecularly adapted treatments, such as those targeting FLT3 activity, and, although some encouraging results have been reported in adults, responses are incomplete and mechanisms of resistance often occur.[13] The degree of reduction of the leukemia-cell burden following the initial courses of induction therapy, as well as the time required to achieve complete remission have been demonstrated in adults to independently impact on prognosis.[14] To date, it remains debated whether the *FLT3-ITD* mutational status affects outcome and whether the specific characteristic of the *FLT3-ITD* mutation, such as the size of the ITD, the number of clones and the allelic ratio (AR), are prognostically relevant factors in children with AML.[15,16]

Furthermore, up to now, the constitutive kinase activity has been commonly targeted with several drugs with a limited clinical success, suggesting that inhibition of other factors contributing to the malignant phenotype is probably required. Here, we report the results of a comprehensive study in a pediatric AML cohort aimed at the characterization of *FLT3-ITD* mutation and its impact on outcome. We investigated the role of the AR at diagnosis. We confirmed the suitability of *FLT3-ITD* aberration as a sensitive molecular marker for MRD monitoring during therapy. Finally, we performed gene expression analysis with the aim of bringing further insight into the biology of the *FLT3-ITD* mutation and its role in explaining the low rate of response to therapy that is observed in a large proportion of *FLT3-ITD* patients.

STUDY DESIGN

Patient samples. Patients with *de novo* AML (N=494) other than acute promyelocytic leukemia, aged 0-18 years, newly diagnosed between 2002 and 2014 in one of the centers associated with AIEOP (Associazione Italiana Ematologia Oncologia Pediatrica) were all treated according to the same protocol AML 2002/01 and all the *FLT3-ITD* mutated patients were considered in this study.[5] In accordance with the Declaration of Helsinki, informed consent, was obtained from patient parents or legal guardians. FAB morphological classification, immunophenotypic analysis and molecular characterization were centrally reviewed at the laboratory of Pediatric Hematology of the University Hospital in Padova, Italy. Follow up samples of BM from *FLT3-ITD*-mutated patients, whenever available after routine analysis, were collected for RNA extraction and MRD analysis at fixed time points: at the end of the first (29 patients) and second (33 patients) course of induction therapy (ICE courses, consisting of a combination of idarubicin, cytarabine and etoposide), and before bone marrow transplantation for patients who underwent to HSCT as defined for the high risk patients by the AML 2002/01 protocol (for 25 patients *FLT3-ITD* who were transplanted in first CR the bone marrow 1 week before transplantation was available and collected for RNA extraction). Details on transplantation procedure and supportive care have been previously described.[17] Bone marrow RNA from 85 patients with *de novo* AML at diagnosis were used for gene expression analysis. This cohort comprised 71 patients harboring recurrent AML abnormalities (*CBF, MLL, NPM1*, *DEK-CAN,* and negative for recurrent molecular aberrancies) and 14 patients with *FLT3-ITD*.

FLT3-ITD **PCR and Allelic Ratio (ITD-AR) determination.** Genomic DNA and total RNA were extracted from BM samples at diagnosis using the Gentra Puregene Cell kit (QIAGEN) and TRIzol (Invitrogen, Carlsbad, CA) as previously described[18]. Reverse transcription was performed using the SuperScript II First Strand Synthesis System (Invitrogen). *FLT3-ITD* screening was performed either on cDNA (N=53, one was not available) or on DNA (N=37) and analyzed with the GeneScan® Analysis Software (see detailed method in supplementary files). We considered the cut off >0.51 as high-ITD-AR for survival analysis [15,19].

Sanger sequencing and Quantitative RQ-PCR. Assessment of *FLT3-ITD* transcript levels was performed with a mutation-patient specific RQ-PCR assay. Briefly, *FLT3-ITD* was sequenced in order to identify and characterize the sequence of the duplication with the 3.1 BigDye Terminator Cycle Sequencing Kit (Applied Biosystems,

Foster City, CA) and analyzed on the 3500DX Genetic Analyzer (Applied Biosystems), according to the manufacturer's instructions. Assessment of *FLT3-ITD* transcript levels was performed on an ABI 7900HD platform (Applied Biosystems) using a Taqman Universal Master Mix (Applied Biosystems). The forward primer and the probe were common while, the reverse primer was designed using Primer Express software (Applied Biosystems) to uniquely select the joining region between the wild-type sequence and the beginning of *FLT3-ITD*, therefore being specific for each patient. All samples were tested in triplicate. Standard curves for *FLT3-ITD* and *GUSB* were established by amplifying a 10-fold serial dilution of target cDNA obtained at diagnosis. MRD levels were normalized to *GUSB* and then measured relative to *FLT3-ITD* transcript levels at diagnosis. RNA in follow-up samples with housekeeping gene *GUSB* Ct >30 were excluded from the study. Expression of *CyclinA1* were measured by RQ-PCR using the Sybr Green PCR master mix (Applied Biosystems) and normalized on *GUSB* using the 2^-DDCt method (see supplementary information for primer sequences).

Gene expression analysis and gene functional enrichment analysis. Bone marrow RNA from 85 patients with *de novo* AML at diagnosis were hybridized on the GeneChip® Human Transcriptome Array 2.0 (Affymetrix), as described in supplementary methods. Microarray expression data are available in the U.S. National Center for Biotechnology Information Gene Expression Omnibus (GEO) database under accession number GSE74183.

Multiparametric flow cytometry (MFC). Immunophenotype was performed at diagnosis and during follow-up on erythrocyte-lysed whole BM by MFC, using a direct immunofluorescence technique with six-color combinations of monoclonal antibodies (MoAbs). Briefly, in each analysis, 0.5 x 10^6 cells were incubated for 10 min with the appropriate combination of MoAbs directly conjugated with FITC, PE, PE-CY5, PE-CY7, APC, APC-CY7. Samples were subsequently lysed using 3 ml of NH_4Cl, then washed in phosphate-buffered saline (PBS), and re-suspended in 0.5 ml of PBS. Intracellular staining was performed by a two-step fixation and permeabilization procedure using a commercial kit (Caltag Laboratories, Fix&PermTM, San Francisco, CA) according to the manufacturer's instructions. Leukemia cells were identified using an immunological gate based on CD45 expression associated with physical parameter.[20] Leukemia associated immunophenotypes (LAIPs), defined by the presence of antigens and flow-cytometry physical abnormalities, absent or very infrequent in normal or regenerating BM, has been created for each patient at diagnosis and used during follow up for MRD monitoring. For

the immunophenotypic characterization of diagnoses 30,000 events for each tube were collected. For MRD detection by MFC, at least 3 x 10^5 ungated events were collected and analyzed. The minimum target sensitivity for quantifying MRD was defined as the ability to detect 50 clustered MRD events in 5 x 10^5 total cellular events (0.01%). Six-color MFC was performed using a BD CANTO II flow cytometer (Becton Dickinson, San Jose, CA).

Statistical methods. Probability of survival was estimated using the Kaplan-Meier method and compared between groups through the log-rank test. Event-Free Survival (EFS) was calculated from date of diagnosis to last follow-up or first event (failure to achieve remission, relapse, death, whichever occurs first). The Mantel-Byar test was used to calculate the cumulative incidence of relapse (CIR). EFS and CIR for patients given HSCT were calculated from the day of transplantation. Significance was tested using Chi-square test and Fischer's exact test. Pearson correlation, Bland-Altman analysis and Spearman's rank correlation tests were also used. Variables having a significant p-value in univariate analysis were included in a multivariate analysis performed using the Cox proportional regression model. A p value ≤ 0.05 was considered to be statistically significant.

RESULTS

Characteristics of the *FLT3-ITD* mutation. *FLT3-ITD* has already been reported to be an adverse prognostic factor in AML.[8,9,12,21,22] Molecular screening for *FLT3-ITD* was performed in 494 patients with *de novo* AML. Fifty-four patients were found to harbor ITD (Table1): these children were older (11.5 versus 7.3 years, $p<0.001$) and had a higher WBC at diagnosis (93 versus 46x10^9 WBC/L, $p<0.01$) than those who did not carry this abnormality.[12] *FLT3-ITD* occurred preferentially in AML without complex karyotype (84.7%),[23,24] and more frequently in patients who did not achieve CR at the end of first induction (59.3%). The ITD length ranged from 18 to 126 base pairs and was not predictive of outcome (data not shown). Forty-five patients were found at diagnosis with a single ITD, while 9 had either 2 or 3 ITDs (Table1S). The clinical significance of the presence of multiple ITD is still debated[9,16], although in our cohort no difference in survival was found (data not shown)[16]. ITD mutation occurred within exon 14 of the *FLT3* gene for all patients analyzed except 6, in whom the duplication interested exon 14 and part of exon 15. 13 had a perfect duplication, while 22 had also an insertion of 3 to 15 random nucleotides maintaining the frame. A common region internal to all the ITDs that covered from aminoacid 591 to 597 is defined (Figure1S)[16,25].

Clinical significance of the *FLT3-ITD* Allelic Ratio. Patient characteristics taken according to ITD-AR measured on cDNA (N=53) revealed that children with high-ITD-AR (>0.51)[15,19] had an increased WBC at diagnosis in comparison to patients with low-ITD-AR (<0.51) (123 versus 77x10^9/L, p<0.05), and carried more frequently a second genetic event, such as a recurrent translocation (55% versus 17%, p<0.01), whereas the percentage of blasts at diagnosis did not significantly influence the AR.[5,10,26] *FLT3-ITD*-AR showed concordance when measured on the 37 patients for whom we had both cDNA and DNA (83%, R=0.68, p<0.001, Table1S). Survival analyses at 3 years on this cohort revealed that high-ITD-AR patients had a worse EFS compared to those with low-ITD-AR when calculated on cDNA (19.2% versus 63.5%, p<0.05, Figure1A), whereas AR performed on DNA was never prognostically significant (Figure1B). This latter observation supports the concept that expression of the mutation is more important than genomic bulk architecture at diagnosis. The incidence of relapse was not influenced by ITD-AR either on cDNA or DNA (CIR not significant, Figure2SAB), mainly because the most frequent event, occurred in patients with a higher-AR, was the failure to achieve CR (No CR = 12/29 (41%) vs 2/24 (8%), p<0.05, Table2).

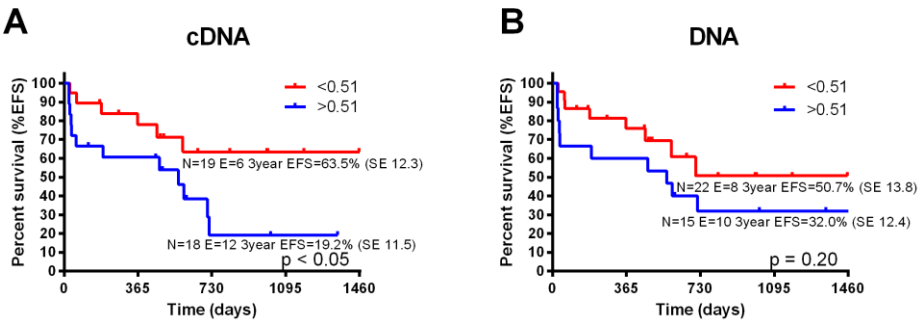

Figure 1. Clinical outcome for FLT3-ITD mutated patients with allelic ratio (ITD/wild type) higher or lower than 0.51 executed for RNA or DNA. A) Event-Free Survival (by AR on cDNA). B) Event-Free Survival (by AR on DNA). Abbreviations: p = p value, N= number of patients, E = number of events, SE = standard error

Impact of MRD after first and second induction courses and before HSCT. It has been reported that *FLT3-ITD* can shift or disappear in the time period elapsing between diagnosis and relapse.[27–29] This phenomenon is due to the outgrowth, owing to treatment, of different clones that evolve in a bulk leukemia.[27–29] In our cohort, 16 out of 19 (84%) paired diagnosis-relapse bone marrow samples maintained the same *ITD* mutation,[27–29] supporting its use as molecular biomarker. To definitely establish the use of *FLT3-ITD* as a *bona fide* biomarker, we confirmed its specificity and sensibility by measuring the MRD

	FLT3-ITD	AML	*p value*
N	**54**	**440**	
Age			*< 0.0001**
<2 yrs	1 (1.8%)	115 (26.1%)	
2-10 yrs	17 (31.5%)	166 (37.7%)	
>10 yrs	36 (66.7%)	159 (36.2%)	
WBC count (x10^9/L)			*< 0.0001**
<10	7 (13.2%)	160 (36.4%)	
10-99	23 (43.4%)	220 (50%)	
>100	23 (43.4%)	60 (13.6%)	
N/A	1	0	
FAB			*0.26*
M0	5 (9.3%)	31 (7%)	
M1	8 (14.8%)	77 (17.5%)	
M2	11 (20.4)	83 (18.9%)	
M4	13 (24%)	73 (16.6%)	
M5	9 (16.7%)	109 (24.8%)	
M6	0	5 (1.1%)	
M7	1 (1.8%)	43 (9.8%)	
N/A	7 (13%)	19 (4.3%)	
Complex Karyotype			*0.01**
YES	7 (15%)	25 (5.7%)	
NO	39 (85%)	414 (94.3%)	
N/A	8	1	
Complete Remission			*< 0.0001**
YES	32 (59.3%)	389 (88.4%)	
Relapse			*0.39*
YES	16 (29.6%)	107 (24.3%)	

Table 1. Characteristics of FLT3-ITD AML patients. Abbreviations: yrs = years; WBC= white blood cell; N/A not available; * $p<0.05$.

by RQ-PCR of the additional fusion gene when present. We selected 8 patients with a concomitant fusion gene, such as the t(5;11)/NUP98-NSD1 (N=4), the t(6;9)/DEK-CAN (N=3) and the t(8;21)/AML1-ETO (N=1), and measured the MRD levels at the same time points (after ICE I and II, and pre HSCT). MRD levels by *FLT3-ITD* or by translocation significantly correlated ($R=0.7$, $p<0.05$) confirming that *FLT3-ITD* can be considered a reliable molecular MRD marker (data not shown).

FLT3-ITD MRD levels reduction compared to diagnosis by RQ-PCR after the first (N=29) and the second (N=33) ICE were analyzed. MRD reduction after the first ICE was

at least 2 logs for 59% of patients (17/29, Table2). Among the 12 patients that presented an MRD reduction less than 2 logs after the first ICE 66.7% relapsed, whereas only 23.5% of those who had > 2logs reduction relapsed ($p<0.05$). Finally, patients who reduced MRD levels less than 2 logs had a significantly worse EFS and higher CIR compared to those who reduced more than 2 logs (EFS: 22.2% versus 59.4 %, $p<0.05$; CIR: 68.7% versus 24.3%, $p<0.05$, Figure2A and Figure3SA). These results indicate that molecular levels of MRD after the first ICE can predict patients outcome anticipating a higher risk of relapse. At the end of the second ICE most of patients had a better disease clearance (26/33 reduced MRD >2logs), however the incidence of relapse did not reach statistical significance (EFS 50.5% versus 14.3%, $p<0.05$; CIR 71.5% versus 43.5%, ns, Figure3SAB). Among the 24 patients eligible to receive HSCT in first CR,[23] the 5 who reduced MRD less than 3 logs showed a trend for a worse outcome when compared to those who reduced more than 3 logs after receiving HSCT (EFS: 40.0% versus 72.9%; CIR: 60.0% versus 27.1%, Figure4SAB).

	High ITD-AR N=29	**Low ITD-AR N=24**	***p value***	**MRD Red < 2logs N=12**	**MRD Red > 2logs N=17**	***p value***
Age mean (years)	11.6	10.2	*0.36*	13.2	10.7	*0.053*
WBC mean (x10^9/L)	123	77	*<0.05**	105	55	*0.16*
No CR	12 (41%)	2 (8%)	*<0.05**	3 (25%)	1 (6%)	*0.27*
Relapse	13 (45%)	9 (37%)	*0.77*	8 (66.7%)	4 (23.5%)	*<0.05**
Associated translocation	16 (55%)	4 (17%)	*<0.05**	5 (42%)	4 (23%)	*0.42*

Table 2. Characteristics of high (>0.51) and low (<0.51) ITD-AR patients on cDNA and of patients with MRD reduction < or > than 2logs. Abbreviations: WBC= white blood cell; CR= complete remission; *p<0.05.

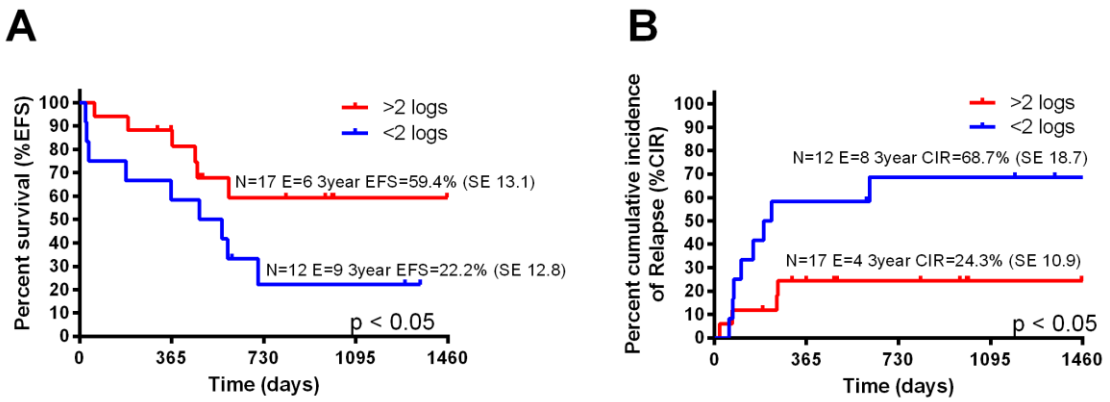

Figure 2. Clinical outcome for patients harboring *FLT3-ITD* mutation that had a minimal residual disease (MRD) reduction greater (>) or lower (<) than 2 logs after the first induction cycle (ICE). The Figure reports data after the first cycle of therapy for A) Event-Free Survival (EFS), B) Cumulative Incidence of relapse. Abbreviation: p = p value, N= number of patients, E = number of events, SE = standard error.

Minimal residual disease evaluation by multiparametric flow cytometry (MFC). Twenty-four follow-up bone marrow samples from *FLT3-ITD* mutated patients were evaluated in parallel with both Multicolour Flow Citometry (MFC) and RQ-PCR analysis at the same time points during treatment. The analysis showed positive concordance between MFC and PCR results (R=0.5, p<0.01, Figure5SAB). To compare the MFC-MRD and PCR-MRD methods more specifically, the Bland-Altman analysis was used.[30] The average of the differences between the two methods was 0.27; thus, the two methods can be considered comparable. Moreover, the difference between measurements using these two methods lies within the limits of agreement more than 95% of the time.

Gene expression profiles of high and low-ITD-AR patients. To gain further insight into the role of *FLT3-ITD* in AML, we analyzed the gene expression profiles of 14 *FLT3-ITD* positive patients. We found 154 transcript clusters (coding genes, lncRNA, microRNA, pseudogenes, rRNA) differentially expressed between *FLT3-ITD* positive and the rest of AML patients (Figure3A and Table2S). GEP by considering coding genes confirmed *ENPP2, CD180, CD86, SORT1*, and *IDH1*, which were up-regulated in *FLT3-ITD* mutated patients, most of them previously described in other reports as confirmed by GSEA (Figure3B)[31,32]. This new platform revealed also a new set of transcripts (N=86) such as lncRNAs and pseudogenes to be further studied within *FLT3-ITD* patients. Expression profiles revealed that *FLT3-ITD* patients clustered according to their ITD-AR status (low versus high, Figure3C). We found 409 transcript clusters with significant different expression between the two sample groups (Table3SA). Among the top-ranking over-expressed genes there was a new methyltransferase *METTL7B* as well as *CyclinA1*, *FOSB* and *JUN*. By GSEA, we found that the main pathways positively enriched in high-

ITD-AR were those connected with H3K27 tri-methylation (Table3SB), which is tightly associated with inactivation of gene promoter. Among the down-regulated genes there was *KMT2A*, a H3K4 methyltransferase, which mediate chromatin modifications associated with epigenetic transcriptional activation. Nonetheless, in high ITD-AR there were down-regulated genes documented to be tumor suppressor (such as *MN1, TP53INP1, MIR181B1*, by TSGene Database).

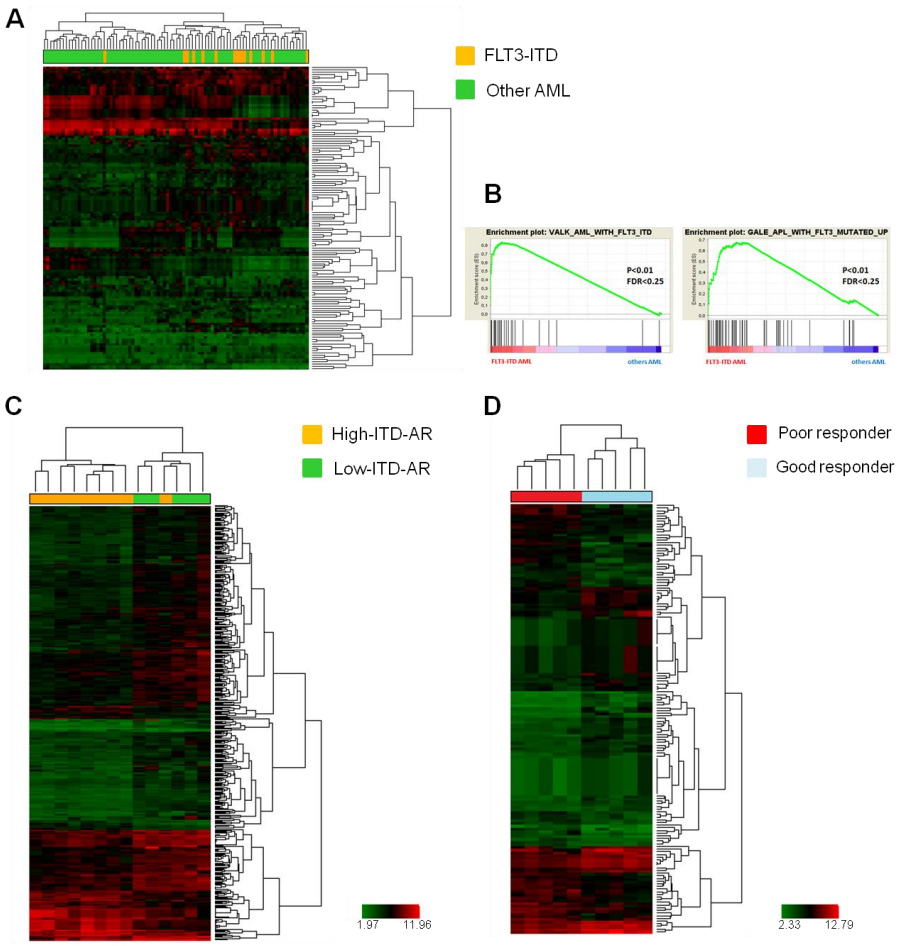

Figure 3. Gene expression profiles of 14 *FLT3-ITD* positive patients. A) Heat map of hierarchical clustering by gene expression signature. In orange are represented mutated patients, while in green other AML including patients with core-binding factor aberrations, *MLL* rearrangements, *NPM1* abnormalities, t(6;9)(p23;q34)*DEK-NUP214* and negative for known recurrent genetic abnormalities previously described. B) Enrichment plots from Gene Set Enrichment Analysis (GSEA). Plots contain profiles of the running enrichment scores (ES) and positions of *FLT3-ITD* related gene set members (indicated by black vertical lines) on the rank ordered list in GSEA. In both plots, we observe a positive enrichment in leukemia samples harboring *FLT3-ITD* mutations gene sets. C) Heat map of hierarchical clustering of patients with high versus low AR ($p<0.05$). In green are represented patients with AR <0.51 (low), while in orange patients with AR >0.51 (high). D) Heat map of hierarchical clustering by gene expression of 5 *FLT3-ITD* positive patients with good or poor minimal molecular disease (MRD) reduction defined as > or < than 2 logs from diagnosis at the end of induction. In red and blue are represented poor and good prognosis patients, respectively.

In addition, Gene Ontology (GO) analysis showed enrichment in biological processes involving nucleosome assembly and DNA organization ($p<0.01$, Table3SC). Interestingly, multivariate analysis showed that high ITD-AR levels significantly contributed to the prognosis of patients with AML ($p<0.05$) more than *CyclinA1* or age, sex and WBC (Table5S).

Gene expression profiles of *FLT3-ITD* patients by MRD levels. In an attempt to explain the different response to induction treatment, we analyzed the *FLT3-ITD*-mutated AML cohort dividing patients according to their MRD clearance (< or > 2logs from diagnosis to completion of ICE, defined as poor or good responders, respectively, Figure3D and Table4SA). Between the two sample groups, 161 transcript clusters with significant differential expression were identified. Among the top ranking over-expressed genes there was *CyclinA1*, this finding supporting the notion that cell cycle is the main actor in drug response,[33,34] and two oncomirs, *MIR21* and *MIR221*, associated with drug resistance.[35,36] These results suggested that reduced response to induction might be sustained by a different transcriptional program already evident at the time of diagnosis. GSEA was then applied revealing a significant positive enrichment in resistance to drugs and, interestingly, in histone acetylation (Table4SB).

Remarkably, *CyclinA1* was significantly overexpressed in *FLT3-ITD*-mutated patients compared to the rest of AML patients (Figure4A, $p<0.05$), in high-ITD-AR compared to low-ITD-AR patients (Figure4B, $p<0.05$) and in poor versus good responders (Figure4C, $p<0.05$). We enlarged the cohort to 48 *FLT3-ITD* patients for *CyclinA1* expression by RQ-PCR (Figure4D). Survival analysis dividing patients according to the mean *CyclinA1* expression value revealed that higher *CyclinA1* expression correlated with poorer EFS (20.3% versus 51.2%, $p<0.05$, Figure4E). Twenty over 24 patients with higher *CyclinA1* expression were also high-ITD-AR, while there were only 8/24 among the low *CyclinA1* expressing group ($p<0.05$). It was previously documented that the CyclinA1/CDK2 complex phosphorylates B-MYB[37] and we found that its signature (deposited data of the B-MYB transcriptional network[38]) when applied to the *FLT3-ITD*-positive patients clustered the high-ITD-AR patients from the low-ITD-AR patients (Figure4F).

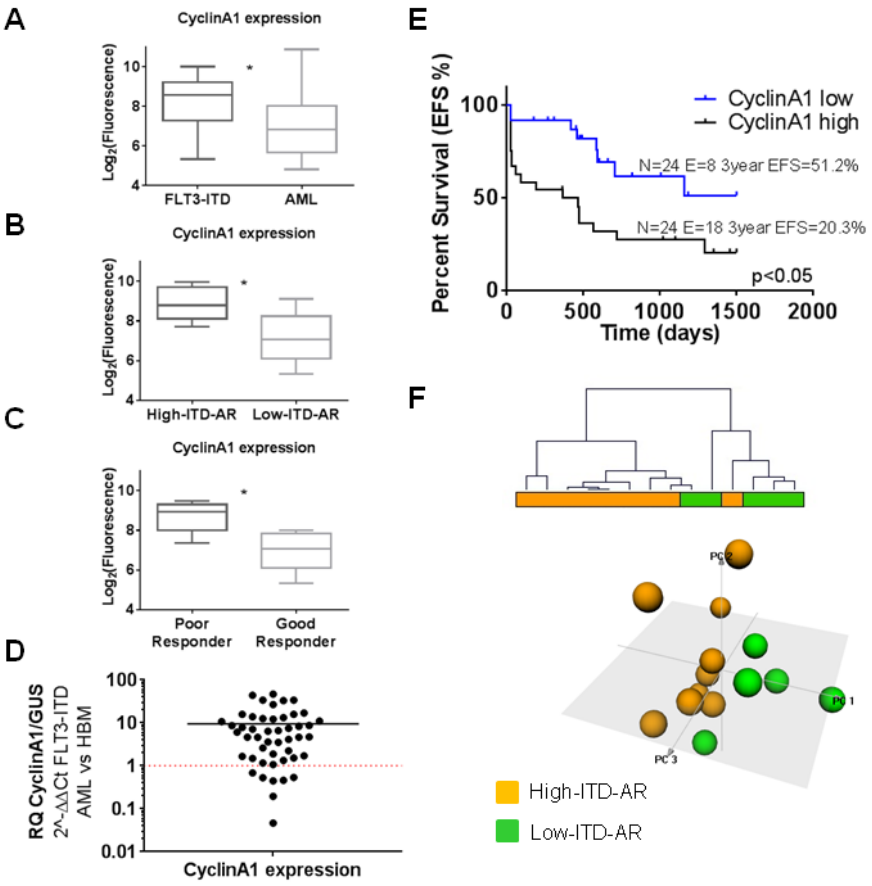

Figure 4. *CyclinA1* in *FLT3-ITD* patients. A) Box Plot of *CyclinA1* (CCNA1) expression in *FLT3-ITD* patients versus patients with other AML variants ($p<0.05$). B) Box Plot of *CyclinA1* expression in High-ITD-AR versus Low-ITD-AR ($p<0.05$) and C) Box Plot of *CyclinA1* expression in Poor versus Good prognosis *FLT3-ITD* patients as assessed by MRD monitoring after induction therapy. D) RQ-PCR expression, calculated with 2^-DDCt of *CyclinA1* in *FLT3-ITD* mutated patients compared to HBM (RQ=1). E) Event-Free Survival for AML patients with high (N = 24) or low (N = 24) level (< or > than median value) of *CyclinA1* expression. F) Heat map of hierarchical clustering (up) and PCA analysis (low) of 14 *FLT3-ITD* positive patients using *B-MYB* signature.([38]) In green are represented patients with AR <0.51 (low), while in orange patients with AR >0.51 (high). Abbreviations: RQ = relative quantity, PCA = Principal component analysis.

DISCUSSION

Recurrent molecular markers have been discovered in AML in the last 3 decades and are now routinely used for risk stratification at diagnosis within clinical protocols. In pediatric AML, post-treatment monitoring of these abnormalities has been rarely used in the clinical management of patients. Up to now, which molecular marker is suitable to be followed during therapy and used to refine remission definition and tailor treatment is still unknown. MRD, largely used in acute lymphoblastic leukemia and acute promyelocytic leukemia[39,40], is gaining popularity also in AML, and its monitoring is mainly based on MFC[41], given the lack of large studies on molecular MRD measurements[42].

FLT3-ITD is one of the most common genetic alterations in childhood AML and is associated with poor prognosis. Even recently, *FLT3-ITD* AML patients showed just a slight improvement in outcome notwithstanding the more intensive chemotherapy adopted in different international protocols[9,10,12]. A large number of studies have been published principally on adult AML[15] with the aim of defining which biological characteristics may be considered in order to improve outcome of *FLT3-ITD* patients, but data are still contradictory.[12,15,16] This study investigated for the first time the prognostic impact of the structural variability of the ITD mutation and of the *FLT3-ITD* AR in a large pediatric cohort on both cDNA and DNA. The ITD-AR measured on cDNA impacts on patient's survival increasing the probability of treatment failure due to a lower rate of CR achievement. The expression of the mutation sustains a different sensitivity of the leukemic clone to therapy and the high-ITD-AR can be considered a reliable independent factor predictive of poor survival since diagnosis. Then, we considered that MRD assessment will be useful to track residual disease at early stage in order to create a molecular profile to personalize treatment and improve survival. The RQ-PCR based method to monitor MRD revealed that *FLT3-ITD*-positive patients since the end of the first induction course present a higher risk of relapse. MRD provide early additional prognostic information within this cohort of patients that can direct a subsequent different therapeutic management of patients. The predictive power of molecular MRD when measured before HSCT was not significant, probably due to the small sample size but suggest that MRD levels identify those patients with a higher risk of post-HSCT relapse. MRD can also at this stage be used for a more informed management of transplantation. Future trials will test whether these patients with positive MRD before HSCT can have a higher chance of success if treated with low intensive GVHD prophylaxis aimed at optimizing the donor graft-versus leukemia effect, or with novel approaches, such as those based on the use of CAR T cells during the conditioning regimen[43].

In an attempt to assess if the different clinical behavior within *FLT3-ITD* patients can have an evident biological basis, we interrogated the expression profile. We found that patients with high-ITD-AR at diagnosis were transcriptionally different from those that had low-ITD-AR, this finding supporting the concept that the expression levels of the mutation orchestrate diverse oncogenic pathways. Differentially expressed genes were found to be significantly connected with tri-methylation of H3K27. Also the expression profile of patients with a worse response to induction therapy was found to be different from that of the good responders, and genes regulated by histone acetylation were found to be

aberrantly expressed. Both analyses converged in sustaining that a high-AR or persistent expression of *FLT3-ITD* mutation exert an aberrant control principally of the epigenetic program that could be responsible of the worse prognosis. Moreover, we found both subunits of the AP-1 complex, JUN and FOSB, were over-expressed in the subgroup of high-ITD-AR patients, this finding sustaining their role in shaping the epigenome of *FLT3-ITD* patients as recently demonstrated.[44] Aberrant histone methylation and acetylation are considered crucial processes during tumorigenesis, and histone deacetylase inhibitors (HDACi) are promising epigenetic cancer drugs.[45,46] Such drugs are currently under evaluation in combination with other chemotherapeutic agents for their antileukemic potential in *FLT3-ITD* adult leukemia showing a synergistic apoptotic induction with FLT3-ITD kinase-inhibitors,[45,46] opening for their further consideration also in pediatric selected subgroups of patients.

In addition, we directed our attention toward *CyclinA1* which was found overexpressed in *FLT3-ITD* patients when compared to the rest of AML. *CyclinA1* has been documented to be leukemogenic *in vitro* and *in vivo,*[35,36,47–49] and to be a biomarker for adults with AML.[44,50] *CyclinA1* has never been explored in pediatric leukemia field. Here, C*yclinA1* presented its highest levels among the patients who either did not respond to induction or had higher ITD-AR, both features of worse prognosis. Then, we found the B-MYB transcription signature differentially expressed among higher ITD-AR patients, supporting that high levels of CyclinA1, in complex with CDK2, overactivates the B-MYB transcription factor and its oncogenic signature in this subgroup of FLT3ITD patients, contributing to their treatment failure.[38]

In conclusion, we report the heterogeneity of *FLT3-ITD* AML patients documenting that evaluation of ITD-AR at diagnosis and molecular monitoring of MRD after the first induction course could be instrumental in stratifying patients in different risk groups, as well as to tailor different treatment within this pediatric AML subtype. We shed light on the role of *FLT3-ITD* mutation, revealing that patients present differences in epigenetic control, mostly in methylation and acetylation pathways, as well as in *CyclinA1* expression, which may explain the variability in outcome among *FLT3-ITD* pediatric patients. These findings suggest the use of epigenetic drugs, combined with conventional strategies, to improve the outcome of pediatric *FLT3-ITD* patients with high ITD-AR and MRD levels.

Finally, this study confirm the role of the molecular MRD approach as a strategy to be further enlarged to all molecular markers for a precise and sensitive track of the leukemia clone in order to adopt a personalized medicine, reduce relapse and increase

children survival. These results wish for future clinical collaborative pediatric trials which will focus on small well-defined and carefully-selected patient cohorts, taking into account the differences in mutation burden and in response to treatment, in order to define standardize protocols and cut-off to be used in routine laboratories.

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SUPPLEMENTARY INFORMATION

Supplementary methods.

***FLT3-ITD* PCR and Allelic Ratio (ITD-AR) determination.** *FLT3-ITD* screening was performed on cDNA of the 54 mutated patients by PCR at exon 14 using the Platinum® PCR SuperMix (Life Technologies) and the following forward (6FAM-GCAATTTAGGTATGAAAGCCAGC) and reverse primers (CACCTGATCCTAGTACCTTCCC). Fragments were run on a 3500DX Genetic Analyzer and analyzed with the GeneScan® Analysis Software; samples showing a mutated profile were then analyzed to determine the ITD allelic ratio (AR). ITD-AR was determined normalizing the peak height of the ITD amplicon to the peak height of the wild-type amplicon. For a large series of patients (37/54), we also measured the ITD-AR on DNA using the same primers. We considered the AR-ITD >0.51 as the cutoff to discriminate patients with high presence of ITD compared to wild type allele as previously defined.[1] The predictive role of the ITD-AR was then calculated on the 37 patients for whom both samples sources, namely cDNA and DNA, were available.

***FLT3-ITD* and *Cyclin A1* RQ-PCR.** *FLT3-ITD* RQ-PCR was performed using a patient specific primer together with a common primer (TCCATAAGCTGTTGCGTTCA) and a common probe (Fam-TTTCCAAAAGCACCTGATCCTAGTACCT-Tamra). As housekeeping gene was used GUSB (F GAAAATATGTGGTTGGAGAGCTCATT, R CCGAGTGAAGATCCCCTTTTTA, probe Fam-CCAGCACTCTCGTCGGTGACTGTTCA-Tamra). *CyclinA1* was amplified with the following primer TCAGTACCTTAGGGAAGCTGAAA and CCAGTCCACCAGAATCGTG and normalized on *GUSB* expression (amplified with the abovementioned primer).

Gene expression analysis. Bone marrow RNA from 85 patients with *de novo* AML at diagnosis were hybridized on the GeneChip® Human Transcriptome Array 2.0 (Affymetrix), following RNA quality evaluation on an Agilent2100 Bioanalyzer (Agilent Technologies). Briefly, 100 ng of total RNA with integrity number (RIN) higher than 8 were labeled and hybridized on the array for 16 h at 45°C using a rotational oven, and washed according to Affymetrix standard protocols using a GC450 Fluidics Station. The Genechips were scanned with an Affymetrix 7G scanner and the CEL files generated were analyzed through Affymetrix Expression Console Software (version 1.3) which normalizes array signals using a robust multiarray averaging (RMA) algorithm. Normalized data were

analyzed using Transcriptome Analysis Console (TAC) 3.0 Software (Affymetrix). An unpaired t-test was applied to identify differentially expressed transcript genes between sample pairs and probes with P-values less than 0.01 and fold-change >2 were declared significant. Whenever possible, the transcript clusters were annotated using the RefSeq database. Microarray expression data are available in the U.S. National Center for Biotechnology Information Gene Expression Omnibus (GEO) database under accession number GSE74183. To visualize similarity of gene expression patterns, we applied hierarchical clustering and principal component analyses methods implemented in *pca3d* package of R 3.1.1 software (http://www.R-project.org).

Gene functional enrichment analysis. We examined whether the differentially expressed genes are biologically meaningful via functional enrichment analysis using the integrative web-based software Enrichr.[2] This tool enables the detection of significant enrichment for a set of genes in different databases: Gene Ontology (GO) Consortium database, Kyoto Encyclopedia of Genes and Genomes (KEGG) database, and Reactome database. We used the whole human genome as a reference set for enrichment analysis and applied a hypergeometric test with a significance level of $p \leq 0.05$. Using Gene Set Enrichment Analysis (GSEA) algorithm with signal-to-noise metric and 1,000 permutations of the genes, we compared the gene expression signatures of patients with *FLT3-ITD* mutation either dividing them in high vs low AR, and then good vs poor responders (where good responders are defined as patients in whom MRD reduction after induction is >2 logs, and poor responders are defined as those in whom MRD reduction after induction is <2 logs). Enrichment of specific genes/pathways were considered statistically significant for p-values <0.05 and FDR <0.25.

Supplementary Figures.

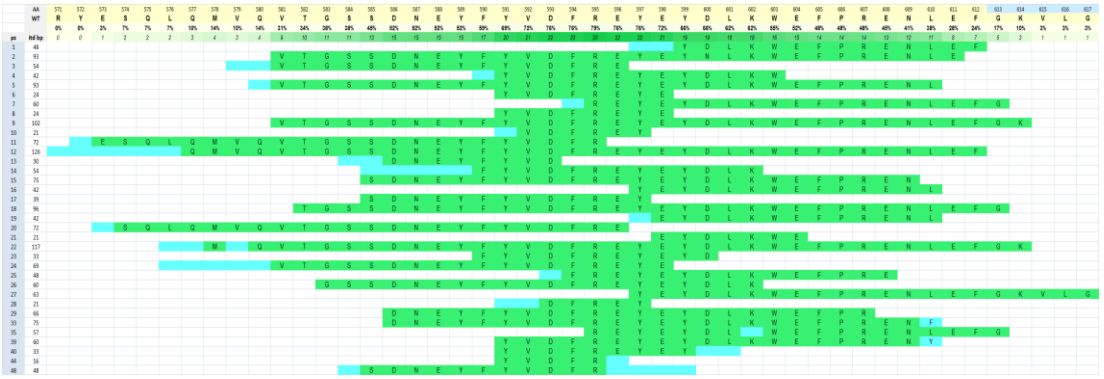

Figure 1S. FLT3 region of internal tandem duplication in the 35 sequenced patients. *FLT3-ITD* mutation, occurred within exon 14 of the *FLT3* gene for all patients analyzed except 6, in whom the duplication interested exon 14 and part of exon 15. 13 had a perfect duplication, while 22 had also an insertion of 3 to 15 random nucleotides maintaining the frame. A common region internal to all the ITDs that covered from aminoacid 591 to 597 is defined. Legend: duplicated aminoacids are in green, inserted mutations are in light blue. Numbers on top represent the wild-type aminoacids from 571 to 617. Exon 14 is represented in yellow, while exon 15 is in bluette

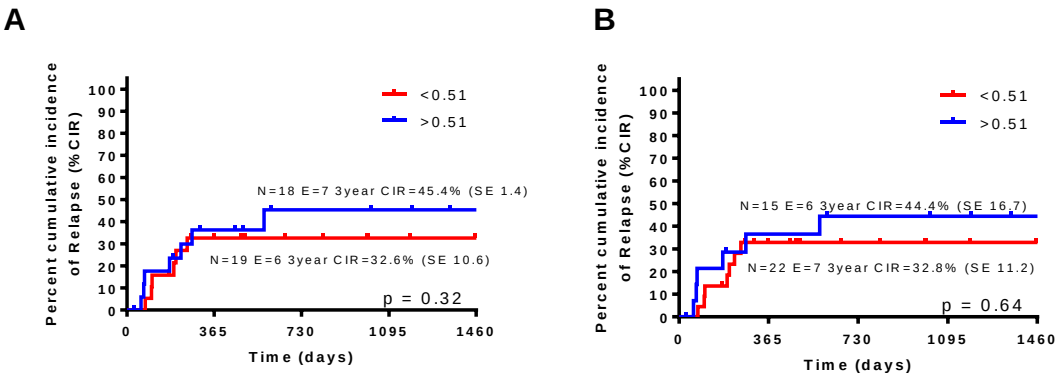

Figure 2S. Clinical outcome for patients harboring *FLT3-ITD* mutation with allelic ratio (ITD/wild type) higher or lower than 0.51 executed for RNA or DNA. A) Cumulative Incidence of relapse (by AR on cDNA). B) Cumulative Incidence of relapse (by AR on DNA). Abbreviations: p = p value, N= number of patients, E = number of events, SE = standard error

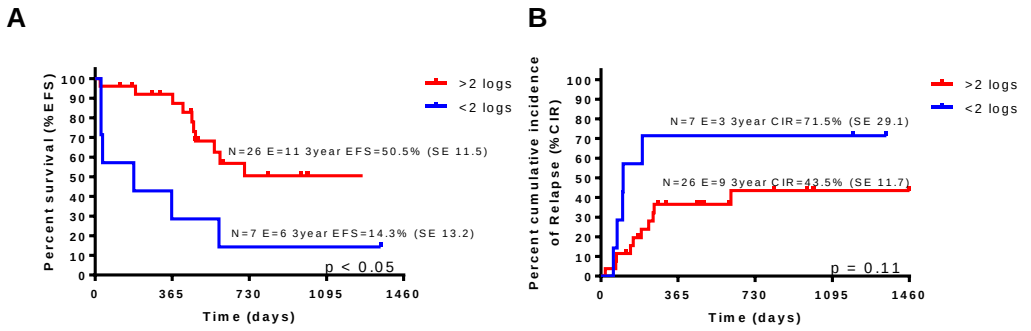

Figure 3S. Clinical outcome for patients harboring *FLT3-ITD* mutation that had a minimal residual disease (MRD) reduction greater (>) or lower (<) than 2 logs after the second induction cycle (ICE). The Figure reports data for A) Event-Free Survival (EFS), B) Cumulative Incidence of relapse. Abbreviation: p = p value, N= number of patients, E = number of events, SE = standard error.

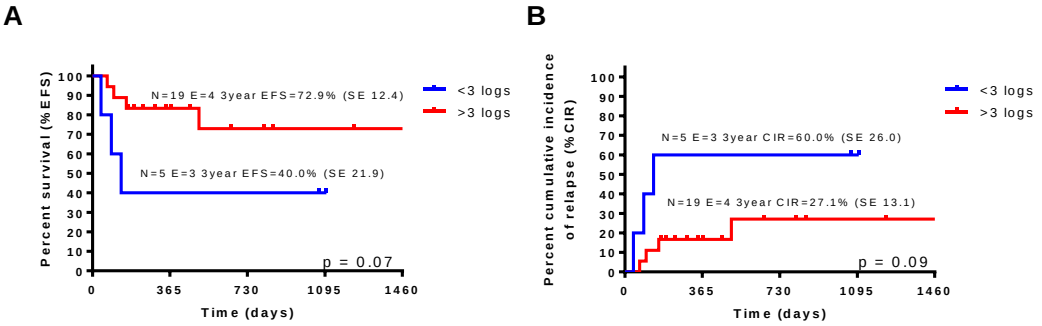

Figure 4S. Clinical outcome for patients harboring *FLT3-ITD* mutation that reduced molecular disease (MRD) more (>) or less (<) than 3 logs before HSCT. In the figure are illustrated data for A) Event Free Survival (p = 0.07) and B) Cumulative incidence of relapse (p = 0.09) . Abbreviation: p = p value, N= number of patients, E = number of events, SE = standard error.

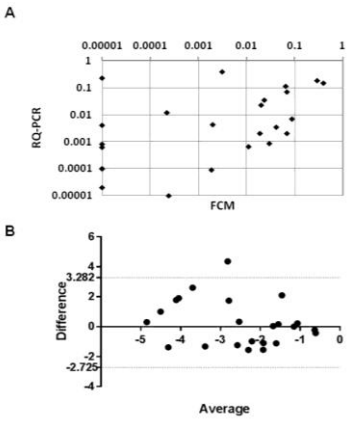

Figure 5S. Quantitative comparison of MRD estimates by MFC and PCR for 24 follow up samples: A) Pearson Correlation and B) Bland-Altman plot of the difference of PCR-MRD and FCM-MRD (Difference) against the mean of the PCR-MRD and FCM-MRD (Mean of differences) after logarithmic transformation. The lines indicate the estimated mean Difference (continuous line) and the upper and lower limits of agreement, mean Difference ± 1.96 SD (dotted lines).

MLL-AF6 FUSION SEQUESTERS AF6 INTO THE NUCLEUS TO TRIGGER RAS ACTIVATION IN MYELOID LEUKEMIA

Manara Elena[1], Baron Emma[1], Tregnago Claudia[1], Aveic Sanja[1], **Bisio Valeria[1]**, Bresolin Silvia[1], Masetti Riccardo[2], Locatelli F[3], Basso Giuseppe[1] , Pigazzi Martina[1]

1) Women and Child Health Department- Hematology-Oncology Laboratory, University of Padova, Padova, Italy
2) Clinica Pediatrica, Università di Bologna, Ospedale "S. Orsola", Bologna, Italy;
3) Oncoematologia Pediatrica, IRCCS Ospedale Pediatrico Bambino Gesù, Roma, University of Pavia, Italy

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ABSTRACT

t(6;11)(q27;q23)*MLL-AF6* is a rare translocation associated with poor outcome in childhood acute myeloid leukemia (AML). The described mechanism by which MLL-AF6, through constitutive self-association and in cooperation with DOT-1L, activates aberrant gene expression does not explain the biological differences existing between t(6;11)-rearranged and other *MLL*-positive patients, nor their different clinical outcome. Here, we show that AF6 is expressed in the cytoplasm of healthy bone marrow cells, and controls RAS-GTP levels. By contrast, in *MLL-AF6*-rearranged cells, AF6 is found localized in the nucleus, leading to aberrant activation of RAS and of its downstream targets. Silencing *MLL-AF6*, we restored AF6 localization in the cytoplasm, this mediating significant reduction of RAS-GTP levels and of cell clonogenic potential. The rescue of RAS-GTP levels after *MLL-AF6* and *AF6* co-silencing, confirmed that MLL-AF6 oncoprotein potentiates the activity of the RAS pathway through retention of AF6 within the nucleus. Exposure of *MLL-AF6*-rearranged AML blasts to Tipifarnib, a RAS inhibitor, leads to cell autophagy and apoptosis, this supporting RAS targeting as a novel potential therapeutic strategy in patients carrying t(6;11). Altogether, these data point to a novel role of the *MLL-AF6* chimera and show that its gene partner, *AF6*, is crucial in AML development.

INTRODUCTION

The mixed lineage leukemia (MLL) protein is a histone H3 lysine 4-specific methyltransferase, commonly associated with transcriptional activation[1]. MLL is essential for both embryonic development and normal hematopoiesis, mainly through transcriptional regulation of the homeobox (*HOX***)** gene[2]. Chromosome translocations involving *MLL* locus are one of the major genetic lesions leading to acute leukemia. *MLL* translocations are detected in up to 80% of infant acute leukemia, and in approximately 10-15% of childhood acute myeloid leukemia (AML)[3,4]. Aberrant proteins resulting from translocations, duplications or amplifications of the *MLL* gene cause alteration of the differentiation program with severe effects on leukemogenesis[5,6]. To date, more than 60 fusion partners of *MLL* have been described, which result in AML, acute lymphoid (ALL), and bi-phenotypic or chemotherapy-related leukemias[7,8]. The underlying mechanisms for *MLL*-mediated leukemogenesis have been extensively studied; however, they still remain elusive for many of the described translocations. *MLL*-rearranged AML is, in fact, a heterogeneous disease, which depends on the *MLL* partner gene for its biological and clinical features, such as gene expression and genomic imbalances[4]. Among diverse fusion

genes, the one that has been consistently associated with the worst outcome both in adult and pediatric AML is *MLL-AF6*[9].

The t(6;11)(q27;q23) translocation is not rare in childhood AML[3,4], and has been demonstrated to impart a worse prognosis with respect to other forms of *MLL*-rearranged AML. AF6 is a cytoplasmic protein with two distinctive features: one single PDZ and two RAS-interacting (RA) domains. The PDZ domain drives AF6 to specialized sites on the membrane, where it can interact with many molecules[10–15]; RA domains are homologous to RA domains of RAS effectors[12,16,17]. Altogether, these characteristics enable AF6, either alone or when fused to MLL, to modulate multiple signal transduction pathways *in vivo*, especially those involving RAS, Notch and Wnt[16]. In the *MLL-AF6* chimera, AF6 protein maintains its functional domains, both PDZ and RA, showing no homology, either for sequence or function, to the product of any other *MLL* partner gene[18,19].

To define the role of *MLL*-fusion genes, several researches were conducted mostly on *MLL-AF9* and *MLL-AF10* chimeras, revealing that a functional hallmark of *MLL* fusion-chimera is a block of hematopoietic differentiation[20–23]. The proposed tumorigenic mechanism of *MLL-AF6* is based on the acquired aberrant transcriptional capability. In fact, the transcriptional activation of MLL is described to be dependent on MLL-AF6 homodimerization mediated by AF6, which acts as a scaffold protein that permits the interaction with target genes[24]. Furthermore, Amstrong's group recently showed that MLL-AF6 requires DOT1L histone-methyltransferase activity to maintain its gene-expression program, which is considered to be its main oncogenic force[25].

In this study, we demonstrate that MLL-AF6 affects AF6 localization, in order to aberrantly activate RAS and its downstream signaling to empower the tumorigenic potential of myeloid cells. In particular, we show that MLL-AF6 sequesters AF6 in the nucleus, leading to increased levels of RAS-GTP in the cytoplasm. Silencing of the chimera re-localized the AF6 protein back into the cytoplasm, this leading to reduction of both RAS levels and activity. These results imply that RAS may play a crucial oncogenic role in AML, prompting us to perform further experiments aimed at disrupting its function. Chemical inhibition of RAS signaling affected the proliferation of t(6;11)-rearranged cells to the same extent as that observed after silencing the chimera. Therefore we explored the effects of a new targeted treatment, namely a farnesyltransferase (FTase) inhibitor, Tipifarnib, and demonstrated its efficacy in primary cultures from patients with t(6;11)-rearranged AML.

MATERIALS AND METHODS

Cell lines, primary cell cultures and patient samples. *MLL-AF6*-rearranged ML2 and SHI-1 cell lines, *MLL-AF9*-rearranged NOMO1 and THP1 cell lines (DSMZ), mononucleated cells obtained from whole bone marrow collected from pediatric healthy donors (HBM), and newly diagnosed t(6;11)*MLL-AF6* and t(9;11)*MLL-AF9*-rearranged AML blasts were cultured in RPMI1640 (Invitrogen-Life Technologies, Monza, Italy) as previously described[26]. Diagnosis of leukemia was established according to standard criteria based on immunohistochemical, immunophenotyping, and cytogenetic studies, as detailed in the AIEOP-2001/02 AML treatment protocol[27]. In compliance with the Helsinki Declaration, informed consent was obtained from patients' parents.

Sequencing. Bone marrow samples of t(6;11) pediatric AML were analyzed for mutations affecting p53 (exon 6-exon 8); N- and K-RAS mutation where searched in hotspot region of exon one and two at codon G12, G13 and Q61 by Sanger sequencing.

Immunofluorescence microscopy. Cytospins were incubated overnight at 4°C with 1:500 anti-AF6 (BD Biosciences, Milan, Italy) and anti-RAS (Cell Signaling Technology, Danvers, MA) antibodies. Slides were incubated with secondary antibodies conjugated to Alexa dyes (Invitrogen-Life Technologies). Cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 1:10,000; Sigma-Aldrich, St. Louis, MO) to label nuclei. Staining was visualized by epifluorescence (video-confocal, Vico, Nikon, Tokyo, Japan).

Western blot. Twenty μg of total proteins lysates, isolated as previously described[26], were subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Immunoblots were hybridized with anti-C-terminal portion of AF6 (BD Biosciences); anti-β-actin, anti-c-RAF, anti-MEK1/2 (Sigma-Aldrich); anti-HDAC1 (Santa Cruz Biotechnology, Dallas, TX); anti-RAS, anti-phospho-c-RAF (Ser338), anti-phospho-MEK1/2 (Ser217/221), anti total and phospho-ERK1/2 (Thr202/Tyr204), anti-LC3 (Novus Biologicals, Littelton, CO) and anti-p62 (Abnova, Taipei City, Taiwan). Enhanced chemiluminescence (ECL) western blotting detection reagents and films (GE Healthcare, Cleveland, OH) were used. Densitometric analyses for protein quantification were carried out using the ImageJ 1.38x software (http://rsbweb.nih.gov/ij/index.html). The value of each band was normalized to the value of either β-actin or total-RAS protein.

Reverse phase protein arrays (RPPA). RPPA analysis was performed as described previously.[28,29]

RAS activation assay. A total of $20x10^6$ HBM or transiently silenced ML2 and SHI-1 cells were lysed and 500 μg of protein extract were used for the RAS Activation kit (ENZO Life Sciences, Lausen, Switzerland) according to the manufacturer's instructions. Positive control samples were obtained by treating the lysates with GTPγS at a final concentration of 0.1 mM to activate endogenous RAS.

Immunoprecipitation. ML2, SHI-1 and HBM cells were immunoprecipitated with 8 μg of anti-RAS or anti-AF6 antibody, as previously described[26]. The immunoprecipitates were analyzed by SDS-PAGE.

RNA interference. Two *MLL-AF6*-specific small interfering RNAs (siMAF6; QIAGEN GmbH, Hilden, Germany) were designed on the fusion breakpoint to selectively silence the chimera. To silence *AF6*, siAF6 (FlexiTube siRNA Hs_MLLT4; QIAGEN GmbH) was transfected into HBM cells. Double silencing with both siMLL-AF6 and siAF6 was also performed for rescue experiments. AllStars Neg Control siRNA (scRNA; QIAGEN GmbH) was used as control in each experiment. Cell transfections were performed using the Nucleofector systems (Amaxa Biosystems, Lonza Sales Ltd, Basel, Switzerland) according to the manufacturer's instructions.

RNA isolation and SYBR Green quantitative real-time reverse transcription-PCR assays. Total RNA was extracted with Trizol reagent (Invitrogen-Life Technologies). One μg of RNA was reverse-transcribed using the SuperScript II system (Invitrogen-Life Technologies) and random examers following the manufacturer's instructions. Quantitative RT-PCR (RQ-PCR) reactions were performed in triplicate on an Applied Biosystems 7900 HT (Applied Biosystems-Life Technologies). The comparative Ct method of relative quantification was applied to interpret the results[30].

Luciferase Assay. A mixture of pFOS WT-GL3 (Addgene, #11983[31]) plasmid, Renilla plasmid (REN), siMAF6 or siMAF6 and siAF6 were used to co-transfect cell lines, while a mixture of pFOS WT-GL3, REN, and scRNA was used as control. Real-time quantitative PCR (RQ-PCR) was used to monitor gene silencing. Protein lysates were analyzed for RAS activity by measuring LUC and REN levels using the Dual Luciferase Assay System (Promega Corporation, Madison, WI). LUC activity was normalized to REN activity.

Soft agar colony assay. After MLL-AF6 silencing, a total of $2x10^3$ ML2 and SHI-1 cells were seeded onto a minimum methylcellulose semisolid dish (StemCell Technologies, Vancouver, Canada) and incubated at 37°C. Fourteen days after transduction, colonies were counted by light microscopy after incorporation of MTT.

Apoptosis analysis. Transiently transfected cells were collected, double-stained with Annexin-V/propidium iodide (PI; Immunostep-Valter Occhiena, Turin, Italy) and analyzed using a Cytomics FC500 (Beckman Coulter, Brea, CA). Relative apoptosis was calculated and expressed as the percentage of Annexin-V-positive/PI-positive cells. Cell lines or cultures of primary AML blasts harboring either t(6;11) or t(9;11) were seeded at 10^6 cells per well and treated for 24h with a concentration of Tipifarnib ranging from 0.1 μM to 10 μM.

Microarray analysis. RNA was extracted from bone marrow of 11 *MLL-AF6*-rearranged patients, as well as from a series of 11 HBM. RNA quality was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). The GeneChip Human Genome U133 Plus 2.0 array was used and analyzed as previously described[32]. T-test was used for supervised analysis between t(6;11)-rearranged patients and HBM samples (GSE 19577). To control for false discovery rate (FDR), multiplicity corrections were used; probes with adjusted p-values less than 0.01 were declared significant using Partek Genomic Suite Software. DAVID Functional Annotation Bioinformatics Microarray Analysis was used to identify molecular networks among differentially expressed genes.

Reagents and treatments. ML2 and SHI-1 cell lines were treated with PD98059 (40 μM, Calbiochem, Merck group, Darmstadt, Germany), a MEK inhibitor. ML2, SHI-1, harboring the t(6;11), and NOMO- 1 and THP-1 cell lines harboring t(9;11), as well as primary AML cells from patients with the same two translocations were treated with Tipifarnib (0.1-100 μM, Aurogene srl, Rome, Italy). MTT test was used to assess cell proliferation.

Data analysis. Statistical analysis was performed using Prism 4.02 (Graph Pad Software, San Diego, CA). Experiments were performed in duplicate or triplicate, and results were presented as mean ± standard error of the mean (S.E.M.) of replicate experiments. Statistical significance was evaluated by the unpaired Student's t-test. Differences were considered to be statistically significant at p values <0.05 and were indicated with an asterisk.

RESULTS

MLL-AF6 modifies AF6 localization, maintaining high RAS-GTP levels. There are alternative AF6 isoforms with described specific subcellular localization. In particular, AF6 has been found ubiquitously expressed in different cell types, having a cytoplasmic

localization. A shorter splice variant of AF6 with nuclear localization was reported exclusively in cells of the central nervous system[33]. To define the distribution of AF6 in normal hematopoietic cells, HBM cells were immunostained with an AF6-specific antibody. In these cells, AF6 was expressed within the cytoplasm (Fig.1A). Western blot analysis confirmed its localization; in fact, only the cytoplasmic (Cyt) protein fraction showed AF6 expression. Furthermore, in HBM, AF6 was found to co-localize with RAS; these data were confirmed by co-immunoprecipitation experiments as previously described in other systems (Fig.1B)[14,16,31]. ML2 and SHI-1, two leukemia cell lines carrying the t(6;11)(q27;q23)*MLL-AF6* translocation, were also investigated for AF6 localization. Immunofluorescence showed a nuclear punctate localization for AF6 in both t(6;11)-rearranged cell lines. Nuclear subcellular position of AF6 protein was then confirmed by Western blot. Immunoprecipitation assay demonstrated no interaction between AF6 and RAS in t(6;11)-rearranged cell lines, due to the nuclear localization of AF6 (Fig.1C). We subsequently measured RAS activity in HBM and in leukemia cell lines by GST-pull down of the active form of RAS. ML2 and SHI-1 cells had an elevated amount of RAS in its active GTP-bound status, while the amount of active RAS was low in HBM cells, this supporting the hypothesis that AF6 may act as repressor of RAS activity in normal hematopoietic cells (Fig.1D).

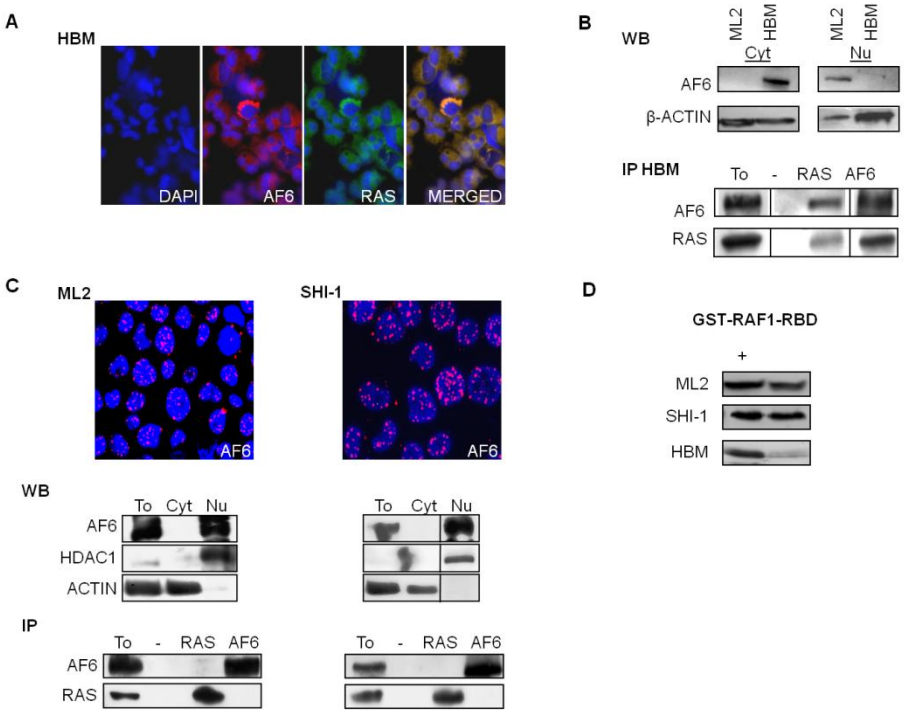

Figure 1. MLL-AF6 modifies AF6 localization from cytosol to nuclear**.** A) AF6 co-localizes with RAS (merged) in cytosol of healthy bone marrow (HBM) cells: (left) immunofluorescence of HBM primary cells

stained with DAPI and respective antibodies against AF6 and RAS (20X zoom). B) (up) Western blot analysis (WB) of AF6 and RAS expression in cytoplasmic (Cyt) and nuclear (Nu) cell extracts. (low) Co-immunoprecipitation (IP) of AF6 and RAS in HBM cells. Total lysates (To) were used as positive controls; negative controls (-). C) (up) Nuclear localization of AF6 in ML2 and SHI-1 cell lines by immunofluorescence (AF6 red, nuclei stained with DAPI in blue, 20X zoom). WB of AF6 and RAS expression in total (To), cytoplasmic (Cyt) and nuclear (Nu) cell extracts; anti-HDAC1 and anti-ACTIN were used as endogenous controls for nuclear and cytoplasmic proteins, respectively. (low) Co-immunoprecipitations (IP) between RAS and AF6 showed no interaction between the two proteins in neither of t(6;11) leukemic cell lines. D) Active RAS-GTP levels in ML2, SHI-1, and HBM cells; positive control +.

Silencing of *MLL-AF6* in t(6;11)-rearranged AML cell lines restores AF6 localization in the cytoplasm and reduces RAS hyperactivation. Silencing of *MLL-AF6* in ML2 and SHI-1 decreased its mRNA expression as compared to cell lines transfected with a non-silencing scramble siRNA (scRNA). In particular, ML2 showed an average reduction of 46% and 38% at 24 and 48 hours, respectively, while SHI-1 showed an average reduction of 53% and 19% at the same time points (by RQ-PCR, data not shown). Chimera protein levels were decreased, as shown by Western blot experiments (Fig.2A). AF6 and RAS localization was investigated after silencing. We demonstrated that AF6 was in the cytoplasm in the *MLL-AF6*-silenced cell lines (Fig.2B) as well as in a primary sample of a t(6;11)-rearranged patient (Fig.2C), whereas it was retained in nuclear foci in scRNA-transfected cells. We also showed a restored co-localization of AF6 and RAS in the cytoplasm (Fig.2B and Fig.2C), as observed in HBM. We measured the active GTP-bound status of RAS and found that it was decreased in cells silenced for *MLL-AF6* compared to scRNA (0.47 vs. 1, respectively; Fig. 2D), suggesting that the chimera maintained AF6 within the nucleus preventing its control over RAS activation.

To confirm our hypothesis, we set up a luciferase assay system in which t(6;11)-rearranged cell lines were co-transfected together with siRNA for *MLL-AF6* and a luciferase reporter of RAS activity. Results showed that luciferase activity was reduced after *MLL-AF6* silencing and AF6 re-localization in the cytoplasm, supporting RAS activity impairment (n=3, p=0.008, Fig. 2E) and the correlation between these events. We then confirmed that co-silencing both *MLL-AF6* and *AF6* increased the luciferase activity, because of RAS rescued expression (n=3, p=0.05, Fig. 2E). This phenomenon was also reinforced by measuring the main RAS target, ERK, which showed a decrease in phosphorylated ERK (p-ERK) form in *MLL-AF6*-silenced ML2, over the total ERK protein which remained at the same levels. By contrast, the ratio p-ERK/ERK increased again after *MLL-AF6* and *AF6* co-silencing (the ratio is represented in the histogram). In line with our hypothesis, the chimera silencing promoted the re-localization of AF6 into the cytoplasm,

thus restoring its control over RAS activity. In the same system, by reducing AF6 levels we rescued RAS activity**.**

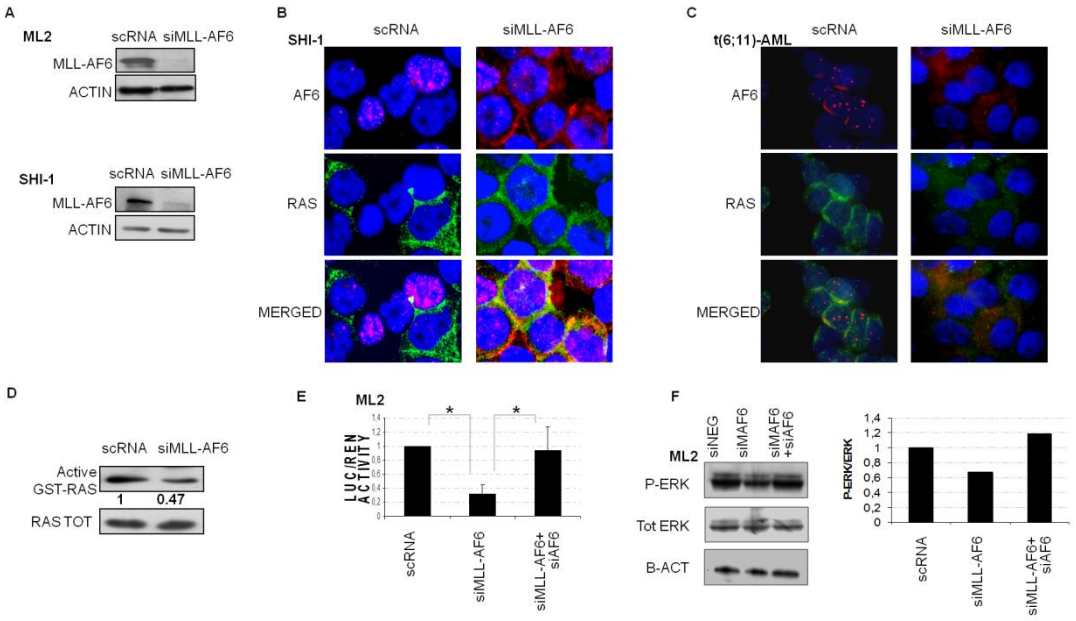

Figure 2. Silencing of MLL-AF6 in t(6;11)(q27;q23) rearranged cells restores AF6 in the cytoplasm. A) Western blot (WB) revealed a decreased levels of MLL-AF6 after silencing (siMLL-AF6) compared to negative controls (scRNA) in both, ML2 and SHI-1. Anti-ACTIN was used as endogenous control. B) siMLL-AF6 cells restored AF6 protein in the cytoplasm. Immunofluorescence shows co-localization of AF6 (red) and RAS (green) in SHI-1 after *MLL-AF6* silencing (merged signals yellow, nuclei blue, 60X zoom). In the negative control (scRNA), the punctuate pattern of AF6 nuclear localization is visible (red AF6, nuclei blue, 60X zoom). C) siMLL-AF6 cells restored AF6 protein in the cytoplasm. Immunofluorescence shows co-localization of AF6 (red) and RAS (green) in primary t(6;11)-AML after *MLL-AF6* silencing (merged signals yellow, nuclei blue, 60X zoom). In the negative control (scRNA), the punctuate pattern of AF6 nuclear localization is visible (red AF6, nuclei blue, 60X zoom). D) Active RAS-GTP levels in ML2 cell line silenced for the chimera showed a decreased activity of RAS (0.47) compared to scRNA..D) Luciferase (LUC) activity of ML2 transfected with a pFOS WT-GL3 plasmid and siMLL-AF6 show a reduction of LUC activity compared to scRNA. Introduction of both siRNA for MLL-AF6 and AF6 show a rescue of LUC activity in ML2. E) WB of P-ERK1/2 and total ERK in ML2 silenced for MLL-AF6 and in double silencing of MLL-AF6 and AF6 compared to scRNA. (right) Histogram represents the ratio between P-ERK and total ERK: a reduction of P-ERK is visble after MLL-AF6 silencing, and a rescue of P-ERK is documented when also AF6 was silenced.

MLL-AF6 **knockdown controls RAS-GTP levels influencing both RAS signaling pathway and cell proliferation.**To demonstrate the relationship between *MLL-AF6* and the RAS pathway, we analyzed the expression of its main downstream targets. Figure 3A shows that, after *MLL-AF6*-silencing, expression of the phosphorylated active form of c-RAF, MEK1/2 and ERK1/2 is decreased in both ML2 and SHI-1. Densitometry was used to assess the extent of protein down-regulation; results are detailed in figure 3A. We confirmed the findings of Western blot analyses by using the sandwich-based enzyme-linked immunosorbent assay (ELISA) technique, since we obtain a 21% reduction of phospho-ERK1/2 levels (p=0.02) in ML2 and SHI-1 cell lines after silencing of the

chimera, whereas other analyzed proteins which are not targets of RAS signaling, such as phospho-p53, phospho-AKT and phospho-JUN, were not affected by silencing (data not shown). Moreover, using an RPPA assay, we demonstrated that silencing of the chimera induces a reduction of p-ERK and p-MEK as well as an increase of the expression levels of proteins involved in apoptosis, such as the cleaved caspase 7 and PARP (Fig. 1S). We confirmed the oncogenic potential of the chimera also by measuring the clonogenic capacity after its silencing. A reduction of about 80% of colony number for ML2 and 55% for SHI-1 was found after silencing (Fig. 3B, n=2; $p<0.05$). Under these conditions, apoptosis was also increased compared with controls for both cell lines (ML2: 37.8% vs. 51.9%, n=3; $p<0.01$, SHI-1: 12.8% vs. 16.0%, n=3; $p<0.05$).

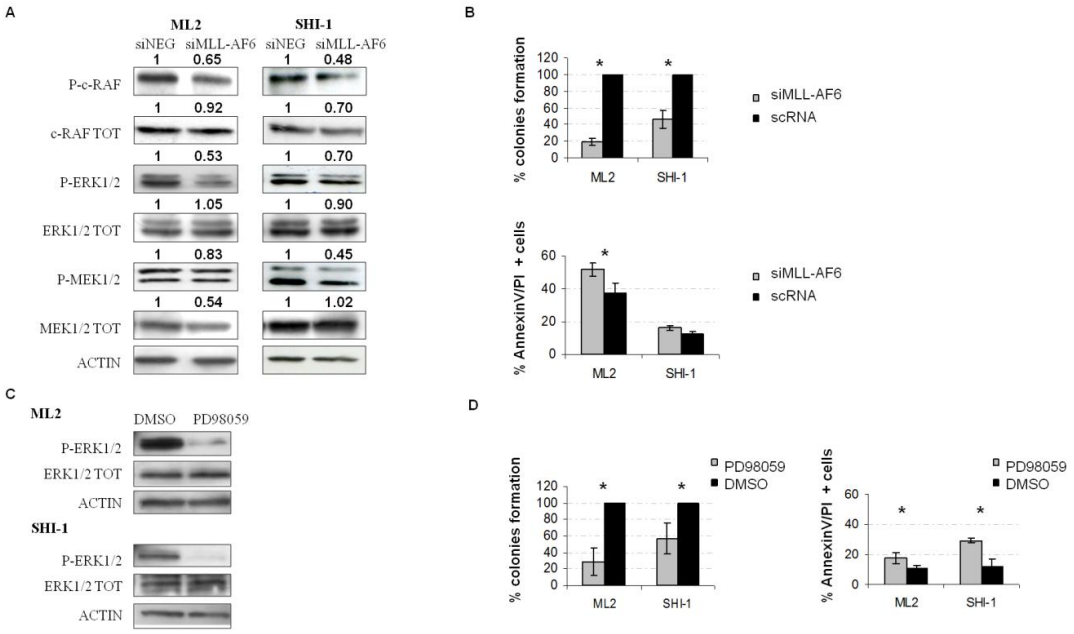

Figure 3. AF6 controls RAS-GTP levels and RAS downstream pathway. A) WB analyses of the RAF/MEK/ERK pathway after silencing of *MLL-AF6* in ML2 and SHI-1 cell lines: a decreased level of phosphorylation for c-RAF, MEK1/2 and ERK1/2 is visible compared to negative controls (scRNA); anti-ACTIN was used as control for total protein amount. B) (up) A decrease in colony formation (% with respect to scRNA) on semisolid medium, and (low) an increase in apoptosis (Annexin and PI positive cells) in ML2 and SHI-1 cell lines after *MLL-AF6* silencing (siMLL-AF6) compared to scRNA are observed ($n = 3$; $p < 0.05$). C) Treatment of ML2 and SHI-1 cells with PD98059, 40 μM. WB analysis shows P-ERK1/2 reduction after treatment. Anti-ACTIN was used as positive control of protein amount. D) (left) An increased apoptosis and (right) a decreased percentage of colony formation is measured after treatment with PD98059 compared to negative controls treated with DMSO (n=2; $p^* < 0.05$).

To ascertain the newly discovered role of RAS pathway activation in childhood AML cells carrying the t(6;11) translocation, we treated ML2 and SHI-1 cells with a chemical inhibitor of RAS signaling, PD980596. In both lines, we confirmed down-regulation of pERK, the main downstream target of RAS (Fig.3C), as well as decreased clonogenicity up to 70% and 30% in ML2 and SHI-1 cell lines, respectively (n=3; $p<0.05$). Furthermore, PD980596 treatment alone increased apoptosis of t(6;11)-rearranged cell lines (ML2: 17.6% vs. 10.9% after DMSO treatment, n=3; $p<0.05$; SHI-1: 29.2% vs. 12.1% of DMSO, n=3; $p<0.05$, Fig.3D). Taken together, these results demonstrate that inhibition of the RAS pathway, either by restoring localization of AF6 in the cytoplasm after chimera silencing or through the use of a chemical compound, concurred to blunt proliferation of *MLL-AF6*-rearranged cells. To further confirm the role of AF6 in controlling RAS activity in hematopoietic cells, we demonstrated increased phosphorylation of RAF/MEK/ERK proteins after AF6 silencing in HBM (30% reduction as determined by RQ-PCR, n=2; $P<0.05$; Fig. 1S), as previously described in other systems[34].

Gene expression profile of t(6;11)-rearranged pediatric patients supports the transcriptional activity of MLL-AF6 on HOXA genes and RAS pathway. Gene expression analysis using Human Genome U133 Plus 2.0 was performed on samples of 11 AML t(6;11)-rearranged patients and of 11 HBM. Supervised analysis between these two groups identified 2,463 differently expressed probe-sets (1,747 genes, FDR <0.01). Among the differentially expressed genes, we found genes typically involved in *MLL*-rearranged leukemia, such as genes belonging to the *MEIS1* and *HOXA* families (Fig.4A). With differentially expressed genes, we performed pathway analysis using the DAVID software, finding that the MAPK pathway and genes related to apoptosis were the most important deregulated pathways in t(6;11)-rearranged cells. Remarkably, in samples with t(6;11) rearrangement, we noted the down-regulation of a gene, *RASA2*, which encodes for a RAS-GAP known to negatively regulate RAS activity, as shown in the box-plot (Fig. 4B, $p<0.001$), and confirmed by RQ-PCR (Fig. 4C, $p<0.01$). These results support the existence of an independent mechanism that enhances RAS activity in this type of leukemia, bringing to light that the RAS pathway sustains the leukemogenic properties of *MLL-AF6*-rearranged leukemia. Further supporting this finding, RASA2 expression is confirmed to be downregulated in t(6;11)-rearranged patients as compared with MLL-other leukemia (Fig. 4D, $p=0.06$ and $p=0.05$).

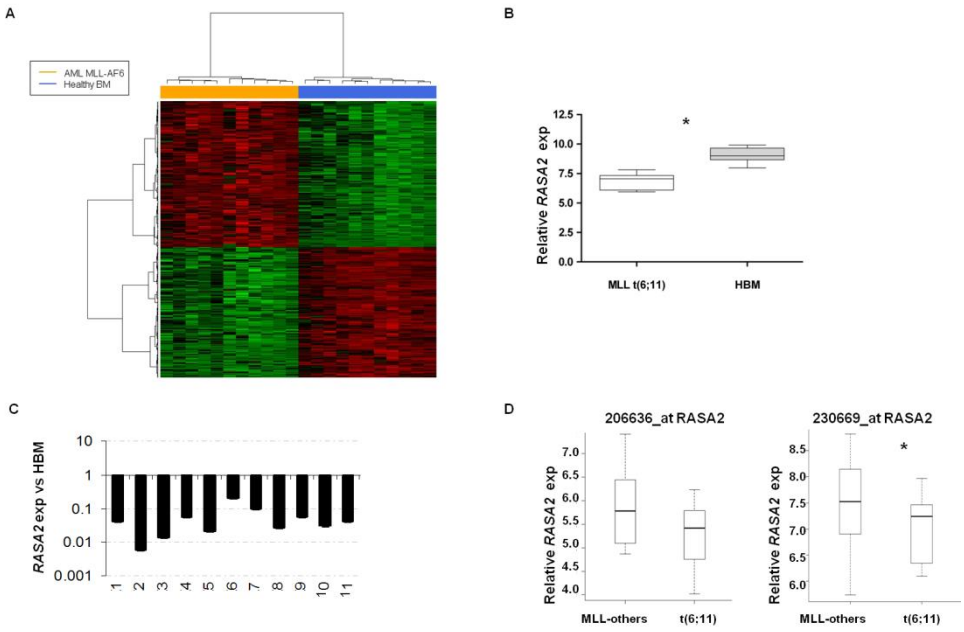

Figure 4. Hoxa genes and RASA2 are differentially expressed between t(6;11) patients and HBM cells**.** A) Hierarchical clustering analysis of 11 patient with t(6;11) (blue) and 11 healthy bone marrow (orange). B) Box-plot of *RASA2* probe sets in t(6;11)-patients (white) vs. HBM (grey) samples generated using Partek Genomic Suite Software. Expression values are indicated in the boxes as the median of each group. Y-axis probe set expression values in log2 scale. C) Histogram confirmed the decreased mRNA levels of *RASA2* by RQPCR (ΔΔCt method) in 11 *MLL-AF6* rearranged patients. Results are calibrated to the 11 HBM (RQ=1). D) Box-plot of *RASA2* probe sets in t(6;11)-patients vs. *MLL*-other leukemic samples generated using Partek Genomic Suite Software. Expression values are indicated in the boxes as the median of each group. Y-axis probe set expression values in log2 scale.

Tipifarnib promotes cell death of t(6;11)-translocated blasts. Since the prognosis of leukemia patients harboring t(6;11) is worse than that of patients with other *MLL* rearrangements[4], we speculated that our findings about the role of the RAS in this subtype of leukemia might lead to novel therapeutic opportunity by using tipifarnib. Tipifarnib is a drug not currently used in treatment of pediatric AML, and currently adopted in clinical trials for different diseases[35–38] for its ability to block the farnesyltransferase enzyme in order to inhibitits targets, among which there is Ras[39,40]. We treated t(6;11)-translocated cell lines, ML2 and SHI-1, as well as cell lines with different rearrangements of *MLL*, i.e. THP1 and NOMO1, both *MLL-AF9* translocated, comparing increasing concentrations of different cytotoxic drugs currently employed for AML treatment, such as doxorubicin (Doxo), cytarabine (Ara-C), etoposide (VP16) with Tipifarnib. We observed that increasing concentrations of Doxo, Ara-C, and VP16 were able to reduce cell proliferation in all cell lines examined, and that ML2 were highly responsive to Tipifarnib, whereas SHI-1 and the two other t(9;11)-rearranged cell lines were not (Fig.5A).

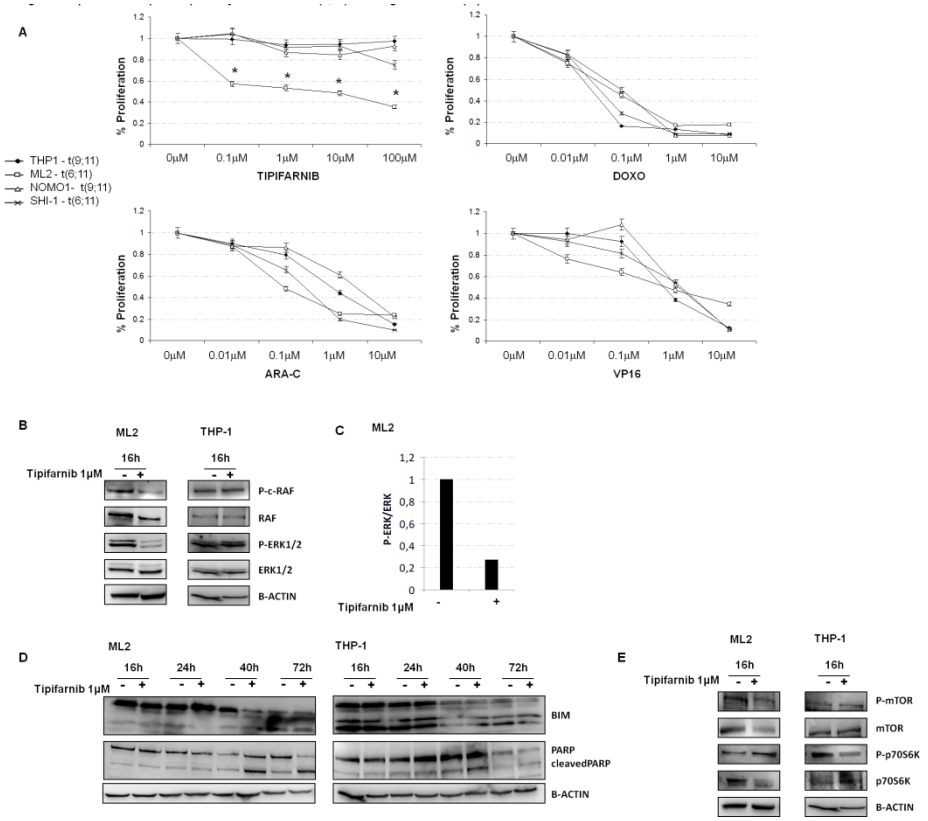

Figure 5. Tipifarnib treatment provoked RAS inhibition and induced apoptosis of t(6;11)-rearranged cell line. A) MLL-rearranged cell lines treated with increasing concentrations of chemotherapics (Doxo, Ara-C or VP16; 0.01 -10 µM) showed a similar reduction in proliferation, while Tipifarnib (0.1 – 100 µM) was specifically reducing ML2 cell proliferation. B) WB analysis showed P-ERK1/2 reduced levels during increasingly Tipifarnib treatment in ML2. (C) Histogram represented the ratio between P-ERK and total ERK in ML2: a reduction of P-ERK was visible after Tipifarnib treatment. (D) WB analysis showed BIM and PARP cleavage increased after Tipifarnib treatment in ML2. (E) WB analysis showed mTOR and p70S6K phosphorylated and total protein after Tipifarnib treatment in ML2 and THP-1.

This different behavior between the two t(6;11)-rearranged cell lines, ML2 and SHI-1, has been already investigated by Chen *et al*., and found to be dependent on the presence of *TP53* mutations, and associated with the documented greater multidrug resistance of this cell line[41]. We performed a western blot to confirm the RAS involvement observing that its pathway, in particular P-ERK1/2, resulted to be decreased at 16h post treatment with 1µM tipifarnib (Fig.5B). We confirmed the downregulation of P-ERK1/2 by observing the upregulation of BIM[39], and the apoptosis induction by PARP cleavage (Figure 5C and figure 5D). To check for off targets effects we analyzed the phosphoryation of mTOR and of the p70S6K, denoting a decrease in the phosphorylated as well as in both total proteins (Fig. 5E).

Consistently with our hypothesis moreover we cannot detect a perturbation of the RAS pathway in Tipifarnib treated THP-1 cells. In view of these findings, we decided to treat with Tipifarnib cultures of primary AML cells obtained from patients with or without

the t(6;11) translocation. Tipifarnib increased cell mortality at 24h of treatment in cultured blasts of *MLL-AF6*-rearranged patients in comparison to cultures from patients with other aberrancies (Fig. 6A, n=2, $p<0.05$). Analyzing Tipifarnib effects, we showed that low concentrations of Tipifarnib (0.1 and 1 μM) increased autophagy, as shown by LC3 and p62 expression; by contrast, this drug at concentrations higher than 10 μM promotes apoptosis, as seen by PARP cleavage (Fig. 6B).

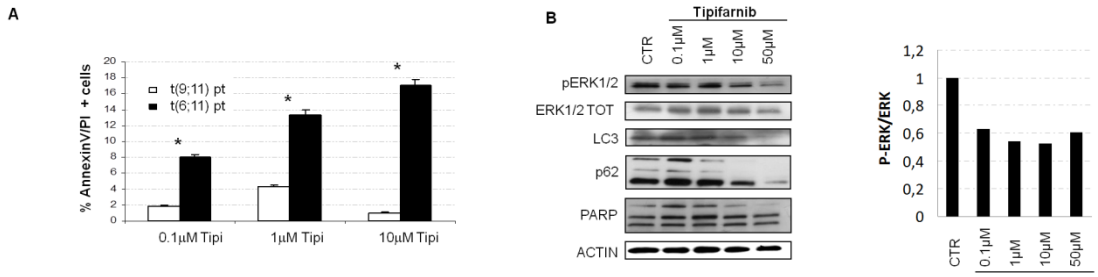

Figure 6. Tipifarnib treatment provoked RAS inhibition and induces apoptosis of t(6;11)-rearranged primary cells. A) t(6;11) primary cell cultures treated with increasing concentration of Tipifarnib showed an accentuated induction of apoptosis (% Annexin V / PI positive cells) especially with increasing drug concentrations with respect to primary AML cultures with different MLL-translocation. B) (left) WB analysis showed P-ERK1/2 reduced levels during increasingly Tipifarnib treatment. LC3 and p62 documented autophagy induction at low Tipifarnib doses, while PARP cleavage confirmed apoptosis when higher doses of Tipifarnib was used. Anti-ACTIN was used as positive control for protein amount. (right) Histogram represented the ratio between P-ERK and total ERK: a reduction of P-ERK was visible after Tipifarnib treatment at any concentration.

These results are consistent with previous reports demonstrating that RAS-induced tumorigenesis both *in vitro* and *in vivo* is always mediated by autophagy[42]. ERK phosphorylation was shown to be inhibited at every concentrations of Tipifarnib (Fig. 6B), confirming the targeting of the RAS pathway.

Furthermore, we ruled out that RAS hyperactivation is dependent on gene mutations, since none of the 11 t(6;11)-rearranged patients had known mutations in hotspot region of *N-RAS,K-RAS* nor *TP53*, as expected in pediatric AML[43,44].

DISCUSSION

The *MLL-AF6* fusion transcript has been found in a significant proportion of children with AML, and is associated with the worst prognosis among all variants of *MLL*-positive leukemia[2,4,6]. *MLL* partner genes are broadly classified into two distinct groups based on their structural characteristics and cellular localization: gene codifying for the nuclear partner proteins with features of putative transcriptional regulators, and genes codifying for cytoplasmic partners, associated with intracellular signaling[24]. AF6 protein is

the most frequent cytoplasmic partner in AML and has been previously found to ultimately orchestrate the aberrant transcription of *MLL* target genes as described by Cleary's and Amstrong's groups[24,25].

In this study, we identified a novel role for MLL-AF6 fusion protein in pediatric AML. We showed that AF6 and RAS co-localize and interact in the cytoplasm of healthy hematopoietic cells, while in t(6;11)-rearranged leukemia cells the two proteins have different localization, and hence the inhibitory effect of AF6 over RAS activation is lost. Liedtke *et al*. showed that AF6 exerts its function primarily as a scaffold protein for dimerization and activation of the transcriptional activity of *MLL-AF6*[24]. These authors demonstrated the ability of the chimera to homodimerize and activate its oncogenic potential trough the $AF6^{RA1}$ domain which is capable of mediating self-association *in vitro* and is responsible of the self-association in the context of the MLL-AF6 fusion protein. Being documented the ability of RA1 to self-associate, we infer that AF6 can also heterodimerize with MLL-AF6, leading to a previously unrecognized and parallel function of this chimera, able to delocalize the wild-type AF6 to the nucleus, this enhancing the activation of RAS and its downstream pathway[24]. Here, we showed that AF6 shuttling from cytoplasm to nucleus determines the level of the RAS-GTP active form, contributing to the tumorigenic effect exerted by *MLL-AF6* as a transcription factor.

Gene expression analysis, in fact, revealed that primary blasts of *MLL-AF6*-rearranged patients showed a gene expression profile typical of patients with other *MLL*-associated rearrangements, in which *HOXA* cluster is mainly overexpressed[24]. However, we revealed that the MAPK pathway, acting downstream of RAS activation, is altered and that, among differentially expressed genes, *RASA2*, a known RAS inhibitor, is strongly down-regulated in t(6;11)-rearranged cells, supporting the aberrant activation of RAS signaling. The hypothesis that RAS levels depend mainly on AF6 expression in hematopoietic cells has been here for the first time addressed and confirmed by rescue experiments. In t(6;11)-positive AML, AF6 is found within the nucleus, and after silencing of both *MLL-AF6* and AF6, an evident increase of RAS activity was documented, with the concomitant rescue also of phospho–ERK levels when compared to silencing of *MLL-AF6* alone.

Several studies previously demonstrated that uncontrolled RAS activation is one of the most common genetic alterations associated with development of several human cancers, including adult *MLL*-rearranged leukemias[45–48]. This uncontrolled RAS activation

is mainly due to known somatic activating mutations[49,50], or to the constitutive activation of several receptor tyrosine kinases, such as CSF-1 and FLT3, or derives from the loss of function of tumor suppressor genes, such as *NF1* and *PTPN11*[51]. Even though the RAS pathway is over-activated in *MLL-AF6*-positive pediatric patients, in our cohort we never found mutations in the *RAS* gene, confirming our hypothesis of an active role of the chimera *MLL-AF6* as the driving force of the observed aberrant RAS pathway activation.

Taken together, these results should encourage further studies aimed at evaluating novel treatment modalities in the t(6;11)(q27;q23) subgroup of childhood AML, considering that this subgroup still carries a dismal prognosis[4]. To this purpose, we investigated the effect of the FTase inhibitor Tipifarnib[52], which hampers the attachment of the farnesyl moiety to the RAS protein, thereby repressing its activation. This drug is currently under evaluation in hematological diseases with high rates of RAS mutation and activation[35–38,53,54]. A phase-1 study showed that pediatric patients with resistant or refractory AML well tolerated Tipifarnib, although a poor clinical response was observed, partially due to the fact that RAS hyperactivation is not demonstrable in all AML variants. Support to the rationale of using Tipifarnib is provided by the observation that apoptosis after drug exposure was enhanced in primary blasts carrying t(6;11), while blasts carrying other *MLL* rearrangement of childhood AML never showed relevant sensitivity to this drug. Notably, leukemia cells were sensitive to high concentrations of the drug, whereas low concentrations failed to induce apoptosis caused by a parallel activation of the autophagy process. This finding has been discussed in several works showing that many cancer cells with aberrant RAS activation have a high basal autophagy, and some depend on autophagy for normal growth[55]. Autophagy is a dynamic process that prolongs survival for a short time under stress conditions[56]; therefore, blocking autophagy with conventional inhibitors[37], together with the use of Tipifarnib, could be considered for t(6;11)-rearranged patients.

Since most patients with t(6;11) succumb within 1 year from diagnosis due to resistance to conventional cytotoxic therapy, RAS targeting promises to be a new valuable option for this subset of childhood AML.

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SUPPLEMENTARY INFORMATION

Supplementary figures

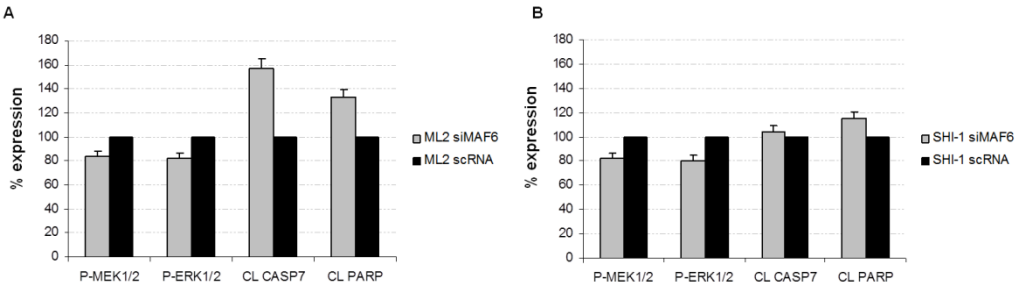


Figure 1S. Elisa assay in ML2 and SHI-1 after *MLL-AF6* silencing denoted decreased expression of proteins involved in RAS signaling pathway and increased levels of cleaved caspase 7 and PARP in ML2 and SHI-1 after *MLL-AF6* silencing.

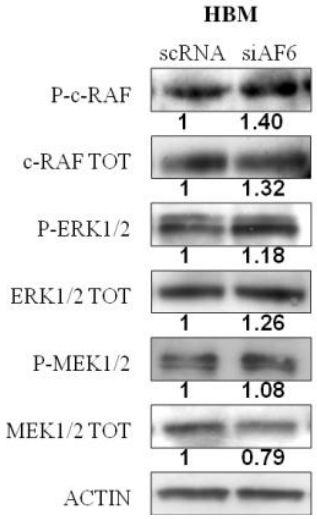


Figure 2S. AF6 silencing in primary culture of healthy bone marrow enhanced RAS pathway activation. Western blot analyses on HBM silenced for AF6 (siAF6) showed increasing levels of P-c-RAF, P-MEK1/2 and P-ERK1/2 proteins with respect to negative controls (scRNA); anti-ACTIN was used as positive control of protein amount. Values depicted in the figure represent densitometry of the bands obtained with ImageJ software.

NUP98 FUSION PROTEINS ARE RECURRENT ABERRANCIES IN CHILDHOOD ACUTE MYELOID LEUKEMIA: A REPORT FROM THE AML 2002/01 STUDY GROUP

Bisio Valeria[1], Zampini Matteo[2], Manara Elena[2],Tregnago Claudia[1], Lezsl Anna[1], Di Meglio Anna Maria[1], Masetti Riccardo[3], Togni Marco[3], Salsi Valentina[4], Zappavigna Vincenzo[4], Locatelli Franco[5], Basso Giuseppe[1] and Pigazzi Martina[1].

1) Department of Woman and Child Health, Haemato-Oncology Division, University of Padova, Azienda Ospedale Padova, Padova, Italy.

2) Istituto di Ricerca Pediatrica - Città della Speranza, Padova, Italy.

3) Paediatric Oncology and Haematology Unit "Lalla Seragnoli", Department of Paediatrics, University of Bologna Sant'Orsola-Malpighi Hospital, Bologna, Italy.

4) Department of Pediatric Hematology-Oncology, IRCCS, Bambino Gesù Children's Hospital , Rome, Italy

5) Department of Pediatric Hematology-Oncology, IRCCS Ospedale Bambino Gesù, Roma University of Pavia, Pavia, Italy.

Submitted

ABSTRACT

Oncogenic fusion proteins are often key players in childhood acute myeloid leukemia (AML), and *Nucleoporin 98* (*NUP98*) is described to translocate at least with 29 different partner genes. However, no comprehensive studies have investigated the incidence or the prognostic relevance of most of the *NUP98*-fusions, nor analogy and dissimilarity in their clinic and leukemogenic role. Here, we screened the pediatric Italian AML cohort (N=482) for 12 *NUP98* partners finding 22 positive patients (4.6%) harboring *NSD1*, *PHF23*, *JARID1A*, *DDX10*, *HOXD13*, *LEDGF* characterized by a dismal 8 years event free survival (27% versus 56.2% of other AML). Gene expression profiling (GEP) highlighted that this newly identified AML entity present a significant different coding as well as a non coding signature compared to others genetic subtypes of AML (*CBF*, *MLL*, *FLT3-ITD*). Each partner gene was revealed unique for GEP and outcome. *NUP98-NSD1* was the most represented fusion, with the worst outcome (16.6%) and the lowest *HOXA* and *B* cluster genes expression among all *NUP98*-AML, supporting alternative co-operating processes to cause this aggressive type of leukemia. We underpinned in *NSD1-NUP98* rearranged primary cells the involvement of both, the cAMP/CREB signaling and chromosome instability (CIN), being the main networks enriched by gene ontology. An enhanced CIN and a pivotal role of the transcription factor CREB in controlling *NUP98*-chimeras expression were demonstrated. These findings declare *NUP98* rearrangements a not rare high risk AML category defined by diverse leukemogenic processes occurring for each oncogenic protein. We support CREB inhibitors clinical investigation for a more targeted *NUP98*-AML treatment.

INTRODUCTION

Chromosomal rearrangements involving the human *NUP98* (Nucleoporin 98kDa) gene have been recently found associated with a wide range of hematological disorders, including leukemia[1–3]. *NUP98*, like *MLL*, is a promiscuous gene, reported to mate with more than 29 distinct partners, forming fusion oncoproteins that concur to acute myeloid leukemia (AML) development[1], thus *NUP98* rises as a key player in this cancer. Among the different *NUP98* rearrangements, *NUP98-NSD1* and *JARID1A* were the only fusions described in childhood AML presenting an aggressive leukemia and disappointing treatment outcome[2,3].

Of note, all fusions have the same structure: the amino terminal portion of NUP98, containing the Gly-Leu-Phe-Gly (GLFG) residues, interacts with histone-modifying

enzymes such as CBP-p300, and joins in frame the C-terminal of different proteins. Most of the NUP98 known partner genes harbor the homeodomain (HD), several others without HD can directly or indirectly activate gene transcription[1]. Indeed, *NUP98*-translocations have been associated with the deregulation of *HOXA* and *HOXB* cluster genes, both important for normal hematopoiesis.[4] Moreover, the abnormal expression of the *HOXA* genes has been demonstrated to impair normal cell differentiations and enhances self renewal of hematopoietic stem cells, as reported for the leukemic transformation induced by *MLL* rearrangements[4–6]. This differentiation defects confer to the *NUP98*-translocations characteristics of type II aberrations and leads in mice, after a long latency, to an impaired myelopoyesis with the development of a hematopoietic diseases[7–9]. Cooperating type I molecular aberrations, such as *FLT3-ITD* or *RAS* mutations, concur to disease progression from MDS to leukemia[7,10]. In pediatric AML, indeed, it has been reported a frequent association between *FLT3-ITD* and *NUP98-NSD1*[2,11].

Despite recent reports, no comprehensive studies have been undertaken to investigate the incidence or the prognostic relevance of *NUP98*-fusions in childhood AML or to uncover similarities and differences in their neoplastic mechanism. In addition, it is not yet clear whether *NUP98*-partner gene reflects diversity in initial fusion events, cell type or mechanism of action.

Here, to increase the clinical and biological understanding of *NUP98*-fusions, we report the results of an exhaustive study in a pediatric AML cohort (N=482) enrolled in a single clinical protocol (AIEOP 2002/01)[12]. Moreover, we took advantage of a genome-wide technology (HTA 2.0, Affymetrix) in order to bring further insight into the biology of this *NUP98*-translocated leukemia, focusing on its characterization either by a RNA coding and non coding expression profile. Results firstly elucidated the main altered oncogenic transcriptional programs induced by the presence of each *NUP98*-fusion going beyond the already reported *HOX* overexpression, and shedding light on a possible route to treatment of these patients.

METHODS

Patients. Bone marrow (BM) sample from patients with *de novo* AML at diagnosis (N= 482), aged from 0 to 18 years, enrolled in the AIEOP LAM 2001-2002 protocol were included in this study[12]. Patients with granulocytic sarcoma, secondary AML, secondary myelodysplastic syndrome (MDS), or Down syndrome or patients with acute promyelocytic leukemia were excluded from the present analysis. In agreement with the Declaration of Helsinki, written informed consent was obtained from their parents. The initial diagnosis of AML according to the FAB classification, immunophenotype and molecular characterization was centrally reviewed in the Laboratory of Pediatric Hematology at the University Hospital in Padova. 172 of the 482 patients were found negative for the recurrent genetic abnormalities involving *MLL, CBF, ,FLT3-ITD and some other rare aberrancies* included in the protocol screening panel for risk stratification[11–13]. The molecularly negative and the 42 *FLT3-ITD* rearranged were screened for 12 NUP98 fusions.

RNA isolation, polymerase chain reaction (PCR) analysis and Real Time-PCR. Total RNA was isolated using Trizol (Invitrogen-Life Technologies, Monza, Italy). One microgram of RNA was reverse-transcribed into cDNA using SuperScript II (Invitrogen) according to the manufacturer's instructions. For molecular screening multiplex RT-PCR were performed using Platinum® PCR SuperMix (Invitrogen-Life Technologies, Monza, Italy). Sanger sequencing was executed to all positive cases to characterize fusion breakpoints and to asses genes mutations (c-KIT, IHD1, NPM1, N and K RAS). Briefly, 3.1 BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) was used and reaction was analyzed on the 3500DX Genetic Analyzer (Applied Biosystems). Expression of mRNA were measured by Real Time PCR (RQ-PCR) on an ABI 7900HD platform (Applied Biosystems) using the Syber Green PCR master mix (Applied Biosystems) and normalized on GUSB using the 2^-DDCt method[14]. See Supplementary Methods for primer sequences.

Gene expression analysis. RNA from bone marrow of patients with de novo AML at diagnosis (N=85) were hybridized on the GeneChip® Human Transcriptome Array 2.0 (Affymetrix, Santa Clara, CA)) containing >6.0 million probes covering coding and non coding transcripts. The analysis was performed for 19 patients harboring *NUP98*-translocations (*NUP98*-t), 20 patients with core-binding factor (*CBF*) aberrations (*RUNX1-RUNX1T1* and *CBFB-MHY11*), 7 with *MLL*-rearrangements, 7 with t(6;9)(p23;q34)*DEK-NUP214*, 8 carrying *FLT3-ITD* mutation and 24 patients negative for known recurrent

genetic abnormalities screened. RNA quality was assessed on an Agilent2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Details are described in Supplementary Methods. Microarray expression data are available in the U.S. National Center for Biotechnology Information Gene Expression Omnibus (GEO) database under accession number GSEXXXX.

Plasmid construction. EGFP-N1-ΔGFP-CREB plasmid was obtained as previously described[14]. Reporter construct for the wild type human NUP98 promoter containing the CREB binding sites (CRE) was PCR amplified from genomic DNA and cloned into the pXP2 promoter-less vector (pXP2-NUP98prom)[15].

Cell culture, transfection and treatment. Leukemia cell lines (HL60, U937; DSMZ) were cultured in RPMI1640 (Invitrogen-Life Technologies, Monza, Italy) supplemented with 10% fetal bovine serum (FBS; Invitrogen-Life Technologies, Monza, Italy) while human embryonic kidney cells (HEK293T) were cultured in DMEM (Invitrogen-Life Technologies, Monza, Italy) with 10% FBS as previously described[16,17]. Newly diagnosed t(5;11)*NUP98-NSD1* rearranged AML blasts and *ex vivo* expanded *NUP98-NSD1* cells were cultured in RPMI1640 supplemented with 10% FBS and cytokines rhIL-3 (20ng/mL), rhIL-6 (20ng/mL), rhSCF (50ng/mL), rhTPO (50ng/mL) and rhFLT-3 Ligand (50ng/mL). HEK293T transfections were carried out by $CaPO_4$ precipitation either using 1.0–4.0μg of expression construct pEGFP-N1-ΔGFP-CREB or negative control (pEGFPΔ-N1) per 6cm dish or to selectively silence CREB, a 400pmol mix of two small interfering RNAs (QIAGEN GmbH, Hilden, Germany) previously used by *Pigazzi et al*[14] and a all Stars Neg Control siRNA (QIAGEN GmbH, Hilden, Germany) used as control. Primary AML cells from patients with NUP98-t and healthy bone marrow (HBM) were transfected using the Nucleofector system (Amaxa Biosystems, Lonza Sales Ltd., Basel, Switzerland) according to the manufacturer's instructions.) and CIN was evaluated by nocodazole (300nM) and aphidicolin (0.5μM) (Sigma-Aldrich, St. Louis, MO) treatment for 24 hours.

Cytogenetic and molecular analysis. Chromosomes analysis was performed was performed on metaphases on bone marrow cell (BM), using standard laboratory procedures. The karyotypes were reported according to the International System for Human Cytogenetic Nomenclature (ISCN 2013)[18]. The probe used for Fluorescence in situ hybridization (FISH) was a NUP98 Break Apart (BA) Probe Set dual color probe locus specific for 11p15 (Empire Genomics LLC).

Luciferase assay. HEK293T were transfected with a Renilla plasmid (REN), a Luciferase reporter containing either the wild type *NUP98* promoter sequence (pXP2-NUP98prom), or a mutated at CRE sequences of *NUP98*-promoter (pXP2-NUP98prom CREB CRE mut) or with the empty vector as internal control. Luciferase activity was determined by Dual-Luciferase® Reporter (DLR™) Assay System following Promega manufacturer's instructions.

Chromatin immunoprecipitation. HL60 and U937 cell lines were processed for chromatin immunoprecipitation assay (Millipore, Darmstadt, Germany) as previously described[19]. Briefly, the immunoprecipitation was performed overnight at 4°C with rotation using two independent CREB antibodies (Sigma-Aldrich, St. Louis, MO /Millipore, Darmstadt, Germany) and Immunoglobulin (Ig), as negative controls. Input DNA of HL60 cell line was used as positive control. DNA was recovered and amplified by PCR with selected primers (see tables of primer for sequences).

Immunoblotting analysis. Total proteins lysates (20μg) were isolated as previously described[16] and were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blot were hybridized with anti-β-ACTIN, anti-HDAC, anti-PARP, anti-NUP98 L205, anti-ϒH2AX (Ser139), anti-H2AX, anti-PP2A B55 (Cell Signaling Technology, Danvers, MA), anti-CREB (Sigma-Aldrich, St. Louis, MO /Millipore, Darmstadt, Germany), anti-BUB1 (Abnova, Taipei City, Taiwan), anti-MAD2L1 (Genetex, Irvine, CA), anti-CYCLIN B (BD Transduction Laboratories); the horseradish peroxidase–conjugated secondary antibody was either anti-goat, rabbit or mouse (Millipore, Darmstadt, Germany).

Data evaluation and statistical analyses. Probability of survival was established using Kaplan-Meier method and compared between groups through the log-rank test. EFS was calculated from date of diagnosis to last follow-up or first event (failure to achieve CR, relapse, second malignancy, or death due to any cause, whichever occurred first). Statistical comparisons between the study groups were also made. Pearson chi-square statistic was used to test for differences in the distribution of categorical variables. Statistical analyses for microRNA level were performed using the Mann-Whitney unpaired two-tailed t test. A p-value of $p\text{-value}<0.05$ was considered statistically significant.

RESULTS

Identification of Nup98 fusion genes. In order to evaluate the incidence of 12 different *NUP98*-fusions, previously described implicated in AML or in other myeloid

disorders[1], a large cohort of pediatric *de novo* AML enrolled in the AIEOP AML-2002/01 protocol[12] was screened by RT-PCR for *NUP98* rearranged with *NSD1, HOXC11, PHF23, HOXA9, JARID1A, HOXD13, LEDGF, DDX10, HHEX, ADD3, NSD3 and LOC348801*. Altogether *NUP98*-fusion transcripts were detected in 22 cases with a frequency of 4.6% in the total AIEOP cohort (Fig.1A, Tab.1A). t(5;11)(q35;p15.5)*NUP98-NSD1* was the translocation majorly detected (12 patients), followed by t(11;17)(p15.5;p13)*NUP98-PHF23* in 4 diagnosis, and t(11;21)(p12;p13)*NUP98-JARID1A* in 3 children; while t(2;11)(q31;p15)*NUP98-HOXD13*, t(9;11)(p22;p15.5)*NUP98-LEDGF* and inv(11)(p15q22)*NUP98-DDX10* were found in just one case each. Specifically, 16 out of 22 positive cases were identified among the 172 AML patients (9.3%) previously found negative for known recurrent genetic abnormalities involving *CBF, MLL* and *FLT3* genes (Tab.1B). These *NUP98*-rearranged patients were found similar at diagnosis for sex distribution, median age and white blood cell count, and FAB to the rest of molecularly negative patients (Tab.1B), however all *NUP98-JARID1A* patients presented exclusively a M7 morphology confirming previously reported data[3]. Interestingly, 56% of *NUP98*-rearranged patients presented a complex karyotype, defined with 3 or more structural or numerical chromosome aberrations, compared to 11% of the negative cohort (p-value<0.0001, Tab.1B).

MARKER	AGE, Y	SEX	WBC count (x10^6/L)	FAB	Karyotype	Mutations
NUP98-LEDGF	5	F	200000	M2	normal	none
NUP98-NSD1	13	M	404000	M2	normal	FLT3/ITD
NUP98-NSD1	15	F	327000	M5	normal	FLT3/ITD
NUP98-NSD1	2	F	21440	M5	complex	none
NUP98-NSD1	10	M	78000	M2	complex	FLT3/ITD
NUP98-NSD1	8	F	17900	M2	normal	none
NUP98-NSD1	6	M	202000	M5	normal	FLT3/ITD
NUP98-NSD1	15	M	350000	M1	complex	none
NUP98-NSD1	2	M	4100	M2	normal	NPM1
NUP98-NSD1	2	F	286	M4	normal	FLT3/ITD
NUP98-NSD1	2	M	83070	M2	normal	none
NUP98-NSD1	9	F	218580	M5	complex	FLT3/ITD
NUP98-NSD1	15	F	62800	M1	complex	none
NUP98-HOXD13	1	M	24530	M1	complex	none
NUP98-JARID1A	2	F	5830	M7	complex	none
NUP98-JARID1A	2	F	28400	M7	complex	none
NUP98-JARID1A	0	M	188000	M7	normal	none
NUP98-PHF23	9	M	6950	M4	normal	none
NUP98-PHF23	6	M	1850	M5	normal	none
NUP98-PHF23	2	M	187900	M1	normal	none
NUP98-PHF23	9	M	1220	M0	normal	none
NUP98-DDX10	11	M	29700	M5	complex	none

Table 1. Clinical and molecular characteristics of *NUP98*-traslocated patients. Individual characteristics of *NUP98*-translocated AML patients (N=22). Abbreviations: Y, years at diagnosis; M, male; F, female; WBC, white blood cell count; FAB= French–American–British classification.

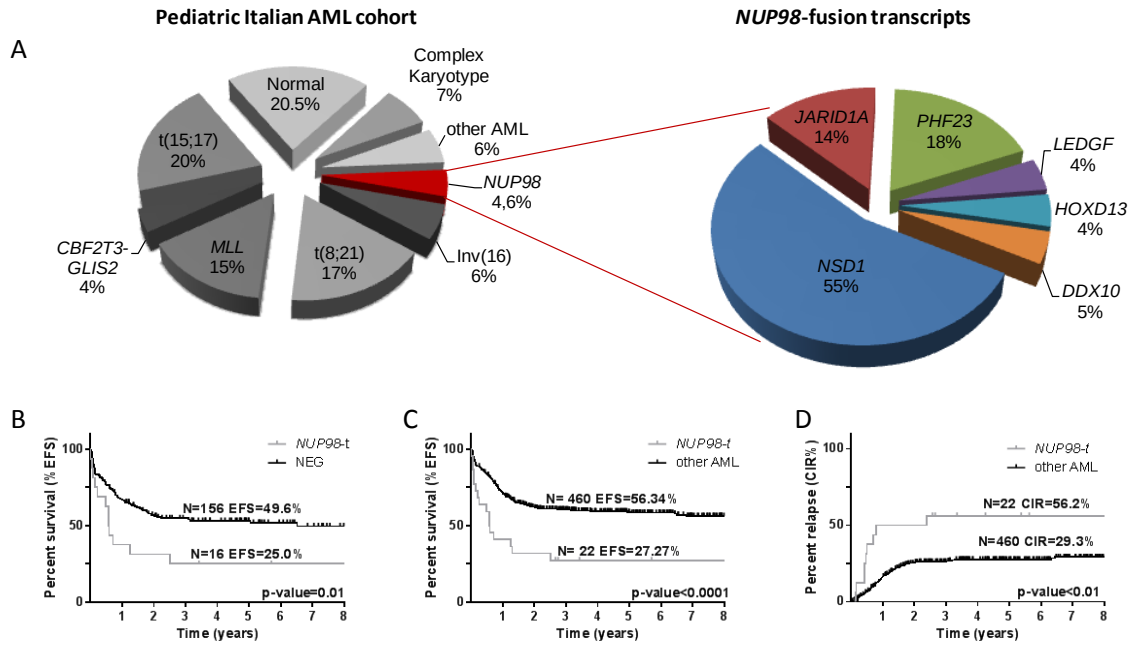


Figure 1. *NUP98*-translocations are frequent events in pediatric AML. A) Pie charts showing the percentage of different type-II aberrations and of different *NUP98*-fusions in the pediatric Italian AML cohort (N=482, AML2002/01). B) Probability of event-free survival (EFS) in children with *NUP98*-translocations versus AML patients negative for recurrent molecular aberrations (NEG in the graph); C) or versus the entire Italian AML cohort enrolled; D) Cumulative incidence of relapse (CIR) in children harboring *NUP98*-rearrangements and in the rest of the Italian AML cohort. Abbreviations: N, number of cases.

Considering the clinical impact, we revealed that the *NUP98* positive patients had a significantly dismal event free survival (N=16, EFS at 8 years=25%) compared to the rest of molecularly negative patients (N=156, EFS=49.6%; p-value<0.05, Fig.1B). Indeed, despite the high complete remission rate of *NUP98*-translocated (*NUP98*-t) patients (75%) similar to the negative cases (83.9%), 9 out of 16 *NUP98* cases (56.0%) incurred in a relapse versus just the 25% in the juxtaposed group (data not shown, p<0.01). Six *NUP98*-t cases were identified among the 42 *FLT3-ITD* patients (14.2%), and they carried exclusively the t(5;11)*NUP98-NSD1*. No differences were found either in clinical features or survival between patients with isolated t(5;11) and those carrying the *FLT3-ITD* and *NUP98-NSD1*[11]. Finally, we confirmed the worse outcome and the higher cumulative incidence of relapse (CIR) of the *NUP98*-AML subgroup (N=22, EFS at 8 years=27.27%, CIR at 8 years=56.2%) compared to the entire Italian cohort of pediatric AML patients enrolled in the AIEOP AML-2002/01 protocol (N=460, EFS 56.3% Fig.1C, CIR=29.3% p-value<0.01, Fig.1D).

	NUP98-t, (%)		AML molecular negative, (%)		p-value
TOTAL	16	(9.3%)	156	(90.7%)	
AGE, Y					0.33
<2	8	(50.0%)	54	(34.6%)	
2-10	5	(31.2%)	46	(29.5%)	
>10	3	(18.8%)	56	(35.9%)	
SEX					0.57
Female	10	(62.5%)	86	(55.1%)	
Male	6	(37.5%)	70	(44.9%)	
WBC					0.28
<10,000	5	(31.2%)	62	(39.8%)	
10,000-99,000	7	(43.8%)	74	(47.4%)	
>100,000	4	(25.0%)	20	(12.8%)	
FAB					0.70
M0	1	(6.2%)	19	(12.2%)	
M1	4	(25.0%)	38	(24.4%)	
M2	4	(25.0%)	18	(11.5%)	
M4	1	(6.2%)	22	(14.1%)	
M5	3	(18.8%)	29	(18.6%)	
M6	0	(0.0%)	3	(1.9%)	
M7	3	(18.8%)	18	(11.5%)	
NA	0	(0%)	9	(5.8%)	
Karyotype					**< 0.00001**
Complex	9	(56.2%)	18	(11.5%)	
Normal	7	(43.8%)	138	(88.5%)	
CR					0.36
	12	(75.0%)	131	(83.9%)	
RELAPSE					**<0.01**
	9	(56.2%)	40	(25.6%)	

Table 2. Clinical and molecular characteristics of selected AML cohort (N=172 molecular negative) for *NUP98*-t screening.Characteristics of *NUP98*-traslocated positive versus negative cases in the pediatric AML enrolled at the AIEOP AML-2002/01 protocol. Abbreviations: Y, years at diagnosis; WBC, white blood cell count; FAB, French–American–British classification; NA, not available; CR, complete remission.

***NUP98* rearranged patients expression profile**. In order to characterize the new group of *NUP98*-t patients, we performed gene expression profiling using the new GeneChip® Human Transcriptome Array 2.0 (Affymetrix) for 19 *NUP98* and compared them to 66 cases of *de novo* pediatric AML selected to provide a representation of the known morphologic, genetic, and prognostic subtypes. The unsupervised cluster analysis showed heterogeneity within AML either performed on the totality of probes (Fig.1SA), only on the coding transcript clusters (Fig.1SB), or the non coding portion (Fig.1SC) at exception of the *AML1-ETO* and *MLL* rearrangements, which clustered as previously reported[20,21]. Additionally, we identified the *NUP98*-t patients who gathered almost together revealing a new AML entity for gene expression (Tab.1SA-B, p-value<0.01). We then used a supervised analysis, and by analyzing the coding transcript cluster we identified 76 differentially expressed mRNAs specific for the *NUP98*-t patients (fold change>|1.5|, p-value<0.01, Fig.2A, Tab.1SA). The most discriminative unregulated genes were implicated in protein trafficking (*CPNE8, CPNE3*), in cell cycle progression (*CCNA1*), in regulation of cell death (FAS) and, in agreement with other reports, most of

them belonged to the *HOX* family (*HOXB5, HOXB4, HOXB6, MEIS1, NKX2-3*[2,3,21,22]) or were genes such defensins (*DEFA4*)[23].

Gene Ontology (GO) analysis in the *NUP98*-t showed enrichment in comparison with others AML in biological processes linked to the nuclear organization and chromosome assembling (regulation of mitotic metaphase/anaphase transition, of regulation of nuclear division, mitosis chromatin modification; Hypergeometric Test, p-value<0.05, Fig.2B, Tab.2S). To confirm the fidelity of the microarray data, we validated the differential expression of several genes related to regulation of mitotic transition (such as *CUL7, CUL9, HERC2*), or to chromatin organization (KDM4B, CENPV and BCORL1) by RQ-PCR. All the analyzed genes were downregulated in *NUP98*-t patients consistently with the results obtained from microarray analysis (Fig.3S).

We focused on genes of the *HOXA* and *HOXB* clusters, already known for their role in AML[24,25], and we performed an unsupervised cluster analysis based on their expression. We revealed that their expression can split AML in three main branches: all the different *NUP98*-partners had a specific *HOX* signature, not only *NUP98-NSD1* or *JARID1A* as previously reported[2,3], showed an overexpression of *HOXA* and *HOXB* genes, clustering together with *DEK-NUP214* rearranged patients and some molecularly negative cases. *CBF* rearrangements and *FLT3-ITD* mutation had a low or absent expression of both *HOXA* and *HOXB* genes, while *MLL*-rearranged cases were characterized solely by the presence of high *HOXA* genes (Fig.2C)[5,26,27]. Interestingly, concurrently to *HOX* cluster overexpression, we noted an upregulation of antisense transcripts genes belonging to *HOXA* (*HOXA-AS4,-AS2,-AS3*) and *HOXB* (*HOXB-AS1,-AS2,-AS5*) clusters within the *NUP98*-t patients. On the contrary, the remaining two groups lacked the expression of the *HOXB* antisense probes, while *HOXA-AS* overexpression was restricted to the *MLL* subgroup.

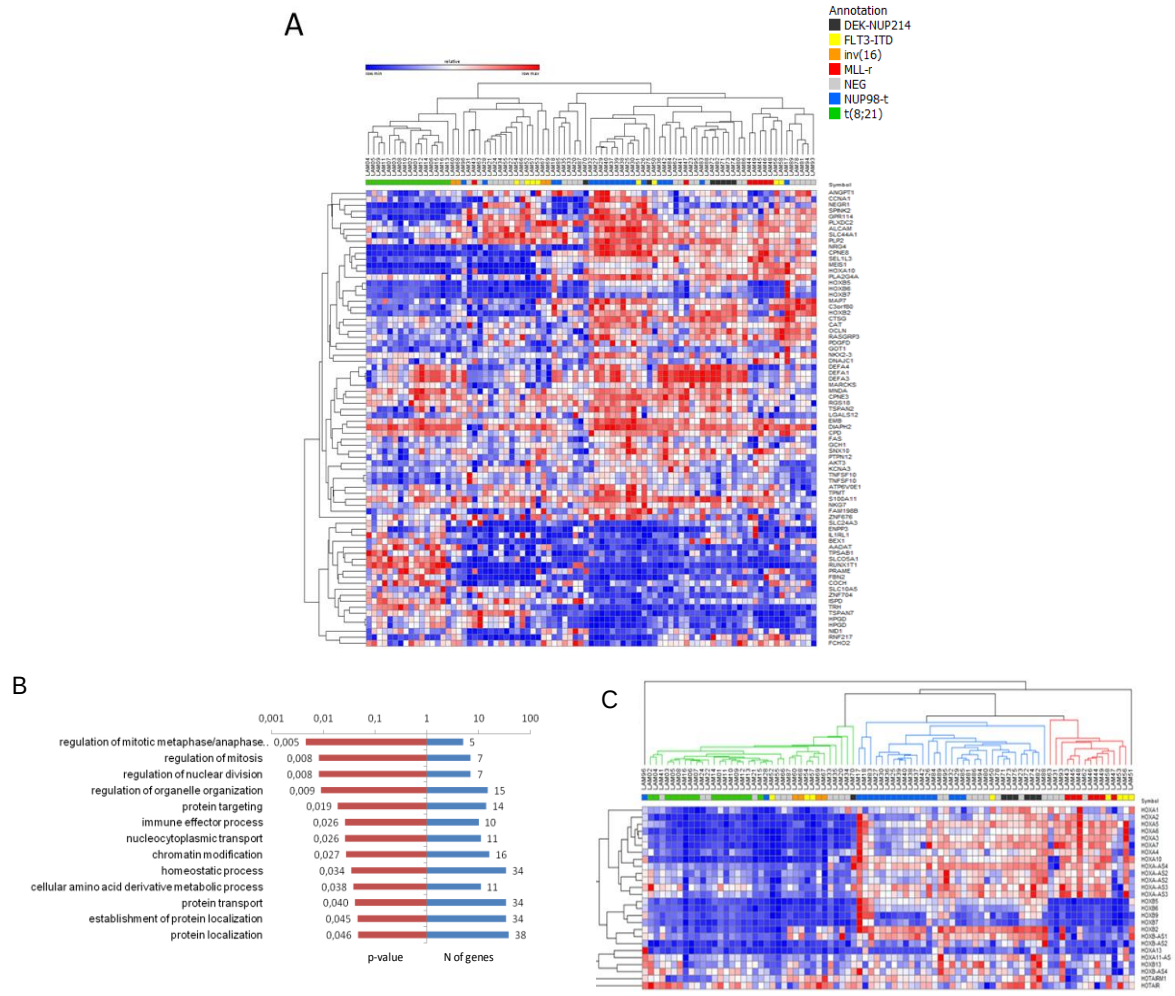
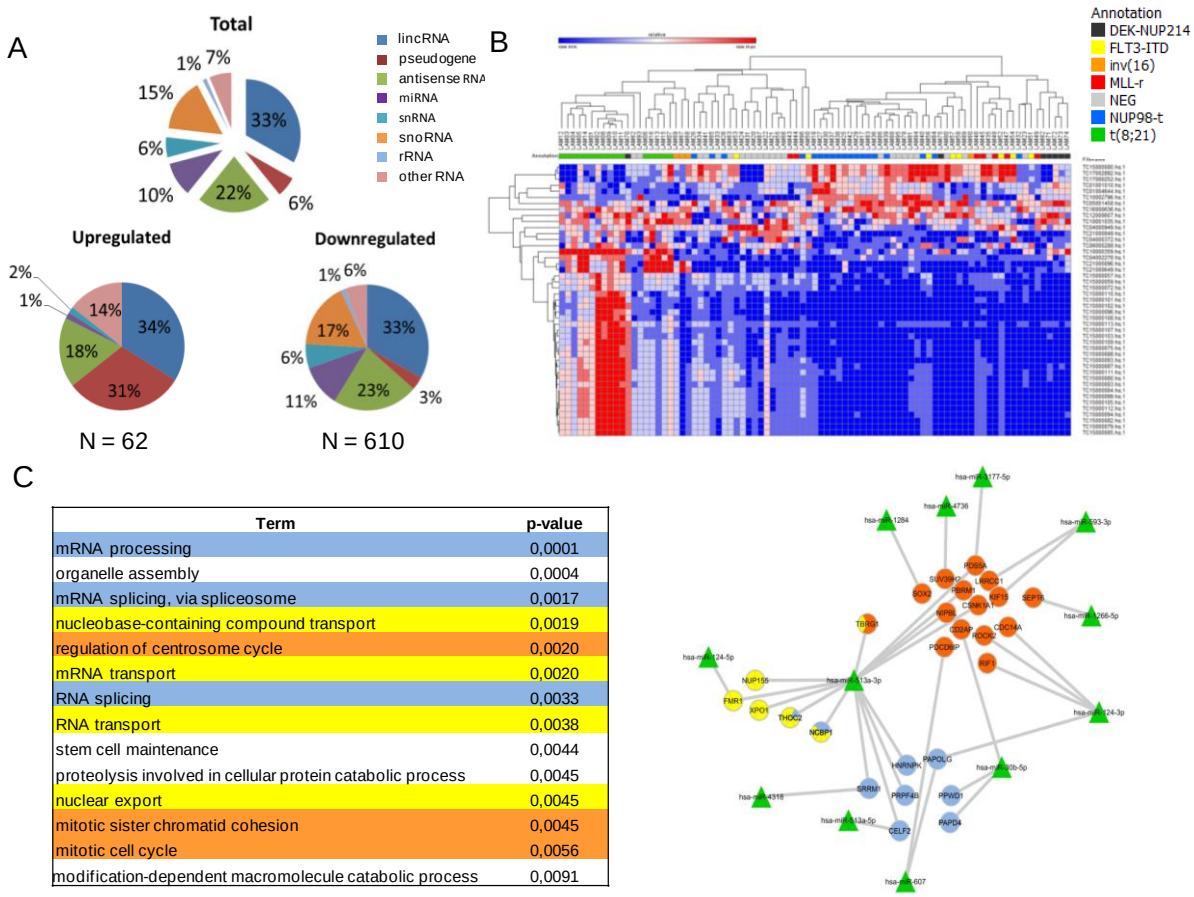


Figure 2. Gene expression analysis of coding transcript clusters in pediatric AML. A) Supervised hierarchical clustering analysis using the 76 differentially expressed coding RNAs identified among 19 *NUP98*-t and 66 *de novo* AML patients (t-test, p-value<0.01). Genetic AML subtypes are indicated in the legend with color codes. B) Gene Ontology annotation of biological process enrichment of *NUP98*-patients performed on differentially expressed coding genes (p-value<0.05). Values on red column represent p-values while on blue column the number of genes, data are computed using Enrichr with the default association rules. C) Unsupervised cluster analysis of pediatric AML based on all *HOXA* and *HOXB* annotating probe sets present on the HTA 2.0 microarray. Genetic AML subtypes are indicated with color codes (as in panel A). Three cluster were observed as indicated by the different colors of the dendrogram (green=*CBF* rearrangements and *FLT3-ITD* mutation; blue= *NUP98*-t and *DEK-NUP214*, red= *MLL*-rearranged cases).

Non coding RNAs in *NUP98*-t patients. Taking advantages of this new HTA platform, we could analyze the contribution of the aberrant expression of the non coding RNAs (ncRNAs), including small nucleolar RNAs (snoRNAs), microRNAs (miRNAs) and long ncRNAs (lncRNAs): 672 differentially expressed ncRNAs between *NUP98*-t and the AML cohort were revealed (p-value<0.01, Tab.1S). The ncRNAs most represented were lncRNAs (33%), followed by antisense RNA (22%), snoRNAs (15%), miRNAs (10%), snRNA (6%), rRNA (1%) and others unknown ncRNAs (7%) (Fig.3A). Most of the probe sets (610 over 672, 90.7%) were downregulated while just 62 ncRNAs were upregulated in

NUP98-t. Interestingly, miRNAs (11%) and snoRNAs (17%) were highly depicted in the downregulated RNAs and they were absent in the upregulated list. On the contrary, pseudogenes were mainly upregulated in the *NUP98*-t patients (31%) compared to the rest of AML (3%). Therefore, patients with *NUP98*-t had a strong and distinctive ncRNAs signature, highlighting the important function that those control deputy RNA might play in the *NUP98*-leukemia (Fig.3B).



Term	p-value
mRNA processing	0,0001
organelle assembly	0,0004
mRNA splicing, via spliceosome	0,0017
nucleobase-containing compound transport	0,0019
regulation of centrosome cycle	0,0020
mRNA transport	0,0020
RNA splicing	0,0033
RNA transport	0,0038
stem cell maintenance	0,0044
proteolysis involved in cellular protein catabolic process	0,0045
nuclear export	0,0045
mitotic sister chromatid cohesion	0,0045
mitotic cell cycle	0,0056
modification-dependent macromolecule catabolic process	0,0091

Figure 3. Gene expression analysis of non coding transcript clusters (ncRNAs) in pediatric AML. A) Pie chart show the 672 differently expressed ncRNAs identified by supervised hierarchical clustering analysis (t-test, p-value<0.01) among the 19 *NUP98*-t and the 66 *de novo* AML patients. B) Heat map of hierarchical clustering analysis using the 44 most differentially expressed ncRNAs identified (t-test, p-value<0.01, FC>|1.5|). Genetic AML subtypes are indicated with color codes reported in the legend. C) (left) Gene Ontology annotation enrichment analysis for biological processes of coding genes negatively correlated with differentially expressed miRNAs (11%) in patients harboring *NUP98*-fusions (Pearson r<-0.4, p-value<0.01) and identified as targets in DIANA-microT web server computed using Enrichr with the default association rules. (right) miRNA-target interaction network shows interaction between the most deregulated miRNAs and their targets involved in the main biological processes such as mitotic cell cycle (in orange), mRNA processing (in blue), and nuclear transport (in yellow)

The 44 most expressed transcript clusters identified between the two sample groups (p-value<0.01; foldchange>|1.5|) were able to cluster *NUP98*-t patients and the other recurrent aberrations with the same strength of coding genes (Fig.3B; Tab.1SB). Among the top-ranking overexpressed ncRNAs, we identified some *HOX* antisense transcripts (*HOXB-AS1, HOXA-AS4*), and the pseudogene *CCDC144B* which is a well known stem cell marker[28]. In addition, we highlighted a strong downregulation of several members of the SNORD family belonging to a cluster located at 15q11 such as *SNORD115, SNORD116*, known to be implicated in hematological disorder[29,30] (Tab.1SB).

MicroRNA showed a general downregulation and, in order to investigate their involvement in *NUP98*-leukemogenesis, we correlated their expression levels with the upregulated genes of the *NUP98*-t being also predicted as microRNA targets by DIANA microT-CDS software[31]. We found significant negative correlations between 26 miRNAs and 167 predicted mRNA targets (Pearson correlation<-0.4; p-value<0.01, Tab.3SA). Gene Ontology (GO) analysis performed on these genes showed a significant enrichment in biological processes linked to nuclear organization and chromosome assembling in agreement with previous GO analysis from GEP (mitotic cell cycle, nuclear transport, and mRNA processing) (Hypergeometric Test, p-value<0.01). Cytoscape software was used to construct the miRNA-target regulation network involved in *NUP98*-t leukemogenesis (Fig.3C). Interestingly, in the differentially expressed miRNAs list, miR-513a was found as a key gene putatively involved in the control of several genes involved in all the main biological processes of the *NUP98*-t. In addition, we highlighted that miR-124, a miRNA already found involved in AML[32], was inversely correlated to *SNAI2, CDK13, ROCK2, FOXQ1, genes* previously identified having a role in different cancers[33–36].

Partner genes characterization. Then, we investigated within the NUP98--t patients with particular attention to each fusion partner gene. We found 101 significantly differentially expressed transcript clusters (60 coding and 41 non coding RNAs, Tab.4SAB, Kurskal-Wallis test, p-value<0.01) among the *NUP98* partners, revealing that each chimera could drive a different gene expression profile (Fig.4A). The same grouping was obtained either with the coding or the non coding genes (Fig.4SAB) supporting the importance of both transcript cluster classes in the *NUP98*-t AML. Interestingly, we found a distinctive expression of both the *HOXA* and *HOXB* cluster genes among the *NUP98*-t. The heat map depicted in Fig.4B showed that *NUP98-NSD1* rearranged patients presented a specific downregulation of both the *HOXA* and *HOXB* genes compared to the rest of the *NUP98*-partners (Fig.5S).

To emphasized other partner driven differences, we compared, through Nearest Template Prediction algorithm (NTP), the significant enrichment of single patients with a list of molecular signatures related to *NUP98*-AML and cancer biological processes (Tab.5S) such as chromosomal instability, methylation, cell signaling and differentiation[3,10,17,22,37]. All partners, with the exception of *PHF23,* resulted to be more enriched in processes related to chromosome instability, while the former showed correlation to methylation[22]. Moreover, only *JARID1A* rearranged patients were enriched in megakaryocytic lineage as previously reported[3]. Focusing on the most represented partner in our cohort, the *NSD1,* we highlighted that it was mainly built up of signature involved in the translation initiation, indeed, among the top ranking overexpressed genes there was the eukaryotic initiation factors 4 (*EIF4*) as well as the Poly(A) Binding Protein, Cytoplasmic 1 (*PABPC1*); furthermore, the signatures involved in activation of CREB signaling[38,39] (cAMP and GS3K) as well as signature of dismal prognosis (Fig.4C) were found statistically significant when compared to the rest of interrogated signaling (Fisher test p-value<0.05).

The identification of specific biological features associated with different *NUP98*-translocation suggested that each partner drives a specific oncogenic signaling that may contribute to different prognostic implications. Indeed, the EFS of *NUP98-NSD1, NUP98-JARID1A* and *NUP98-PHF23* patients were 16.6%, 33.3% and 75% respectively (Fig. 6S, p-value<0.05) showing, among all fusions, the worst clinical outcome for *NUP98-NSD1* chimera and the best survival for *NUP98-PHF23* patients. Even if survival analysis need to be validated in enlarged cohorts, our data cannot be ignored becausea follow up of 8 years contribute to consider reliable the prognostic value of at least the main representative three *NUP98* partners. A more robust analysis confirmed that *NUP98-NSD1* presented the worst EFS compared to the remaining groups, that may be differently considered for a less severe clinical management (Fig.4D, p-value<0.05).

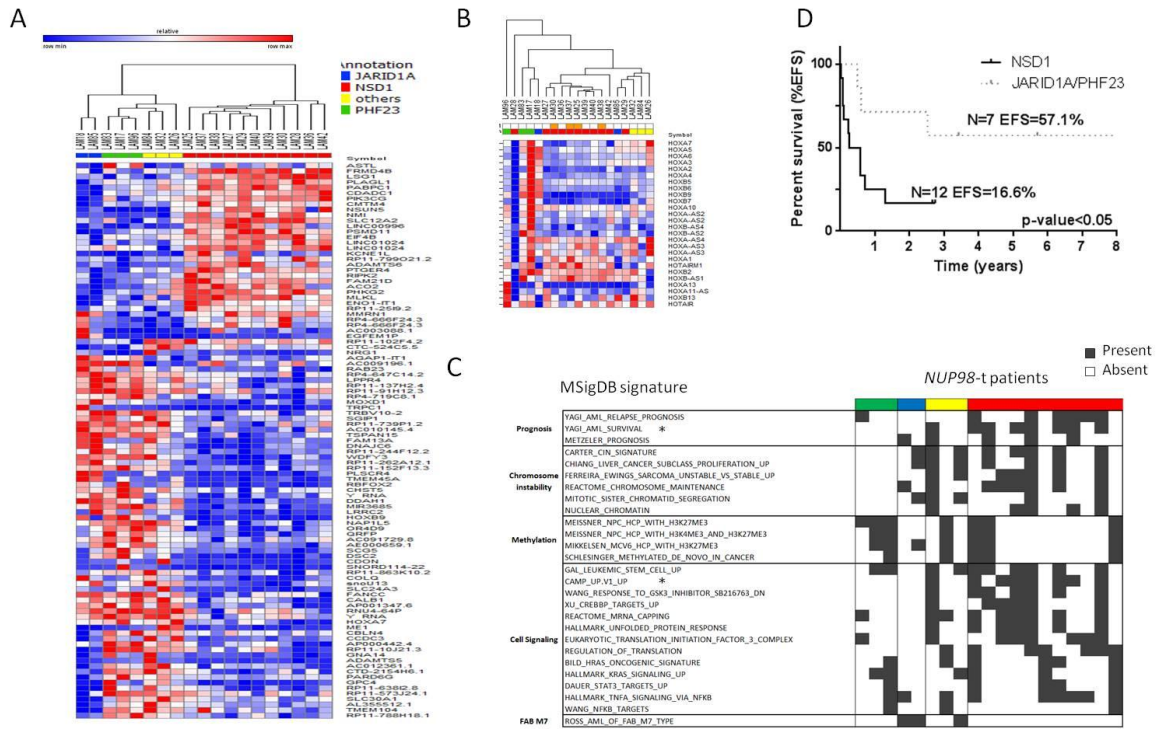


Figure 4. *NUP98* partner genes clustering analysis. A) Supervised hierarchical clustering analysis using the 101 probe sets identified by Kruskal-Wallis test among 19 *NUP98*-t patients (p-value<0.01) that comprehend 2 t(11;21)(p12;p13)*NUP98-JARID1A*, 11 t(5;11)(q35;p15.5)*NUP98-NSD1*, 3 t(11;17)(p15.5;p13)*NUP98-PHF23*, and 3 others fusion (1 t(2;11)(q31;p15)*NUP98-HOXD13*, 1 t(9;11)(p22;p15.5)*NUP98-LEDGF* and *1* inv(11)(p15q22)*NUP98-DDX10*). Different *NUP98* partner genes are indicated below the dendrogram with different colors. B) Unsupervised cluster analysis of pediatric *NUP98*-t patients based on all *HOXA* and *HOXB* clusters annotating probe sets present on the HTA 2.0 microarray. (C) The presence of significant Molecular Signature DataBase (MSigDB p-value<0.05) gene patterns identified by NearestTemplatePrediction (NTP) algorithm are indicated by black boxes (*p-value<0.05 by Fisher test for *NUP98-NSD1* versus others). D) Event-Free Survival (EFS) calculated for patients which harbor *NSD1* as partner gene (N=12) or *JARID1A* and *PHF23* as partner genes (N=7). The difference between Kaplan-Meier curves results statistically significant (Log-rank test, p-value<0.05).

CREB directly targets NUP98 chimeras. The most important signaling enriched in *NUP98-NSD1* patients was the cAMP/CREB[38,39], an important pathway already described contributing to leukemia[40–42]. CREB is a transcription factor with several binding sites in *NUP98* promoter (CRE region around 1300 bases before NUP98 transcription start site; Match ver 1.0 tool analysis, TRANSFAC MATRIX TABLE, Release 7.0, Fig.5A) and we confirmed its transactivational role by chromatin immunoprecipitation in AML cell lines (HL60 and U937)(Fig.5B). Furthermore, a luciferase reporter gene (LUC) being under the control of *NUP98* promoter, confirmed CREB dependent modulation of the reporter gene expression (Fig. 5C). Results showed that reporter activity (pXP2-NUPPROM) significantly decreased in combination with CREB silencing compared to controls (either the vector alone ,pXP2; the vector with CRE mutated sequence,pXP2-NUPPROM CREB BS MUT: and siRNA CTR). In particular, after 24h of CREB siRNA

transfection, LUC activity was decreased. On the other hand, an increase in LUC activity was seen following CREB overexpression (pEGFP-flagCREB) in comparison with controls (pXP2, pXP2-NUPPROM CREB BS MUT)(Fig.5C), all results were double-checked by western blot analysis (Fig.5D).

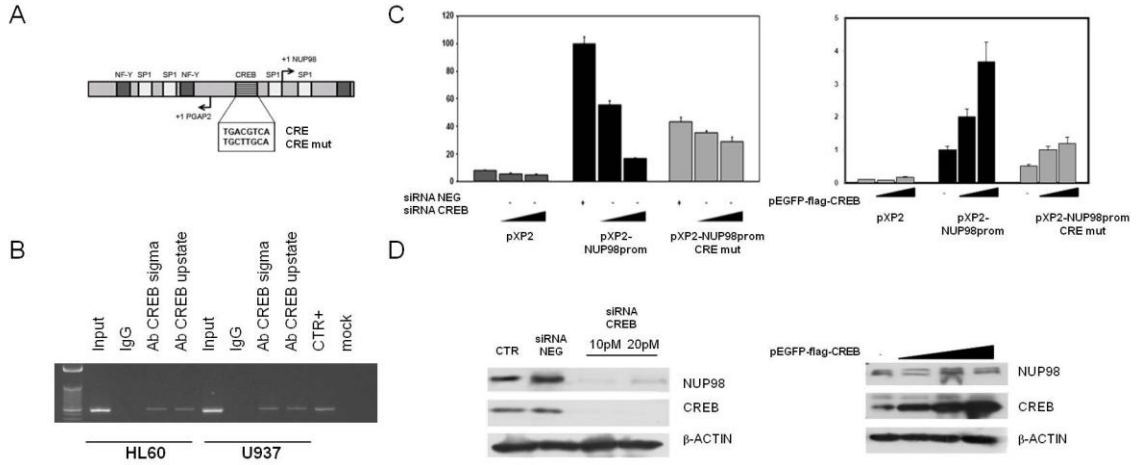


Figure 5. CREB regulates *NUP98* endogenous expression. A) A schematic structure of *NUP98*-promoter 1300 bases before transcription start site identified by Match ver 1.0 tool analysis, TRANSFAC MATRIX TABLE, Release 7.0. The wild type (top) and the mutated (bottom) sequence of the cAMP response element (CRE) recognized by CREB, is highlighted in the box. Mutations in the sequence were inserted between the nucleotides 3-6. B) PCR of the 200-bp *NUP98* promoter fragment amplicon after chromatin immunoprecipitation (ChIP) for CREB in AML cell lines, HL60 and U937 (input=cell lysates before immunoprecipitation; CTR+=cell line DNA; IgG is the negative control of ChIP, mock= PCR mix without DNA). C) The portion of the wild type CRE promoter depicted in the cartoon was cloned in the LUC vector pXP2-NUP98prom, while the mutated one in pXP2-NUP98prom CRE mut. Luciferase (LUC) activity was measured in HEK293T transient cotransfection of pXP2-NUP98prom with siRNA CREB/siRNA NEG or with pEGFP-flag-CREB plasmid to silence or overexpress CREB respectively. The vector alone (pXP2) or the mutated CRE vector were used as controls. Results show a significant decrease and increase in LUC activity after CREB levels modulation (N= 3, normalized with Renilla (REN) activity). D) Representative Western blot analysis of NUP98 protein level after CREB silencing (siRNA CREB) or overexpression (pEGFP-flag-CREB) in HEK293T cell line. NUP98, CREB and β-ACTIN proteins were revealed.

Given the fact that NUP98 maintains its N-terminus during somatic translocation, its promoter guide fusions expression[1], and CREB can be considered the candidate in maintaining chimera expression along AML course. To substantiate this hypothesis we engrafted NSG mice with patients harboring t(5;11)*NUP98-NSD1* translocation and, after confirming the same leukemia phenotype of diagnosis, we silenced CREB gene. Twenty-four hours and 48h post CREB silencing (RQ=0.5, Fig.6A), we attested a strong reduction of *NUP98-NSD1* expression (RQ =0.03) and of some of its target genes. NUP98-NSD1 protein showed the same reduction seen for mRNA since 24h (Fig.6B). Cells were

confirmed to increase apoptosis and reduce clonogenicity after CREB and chimera reduction levels, as expected (data not shown)[14,19,41,43].

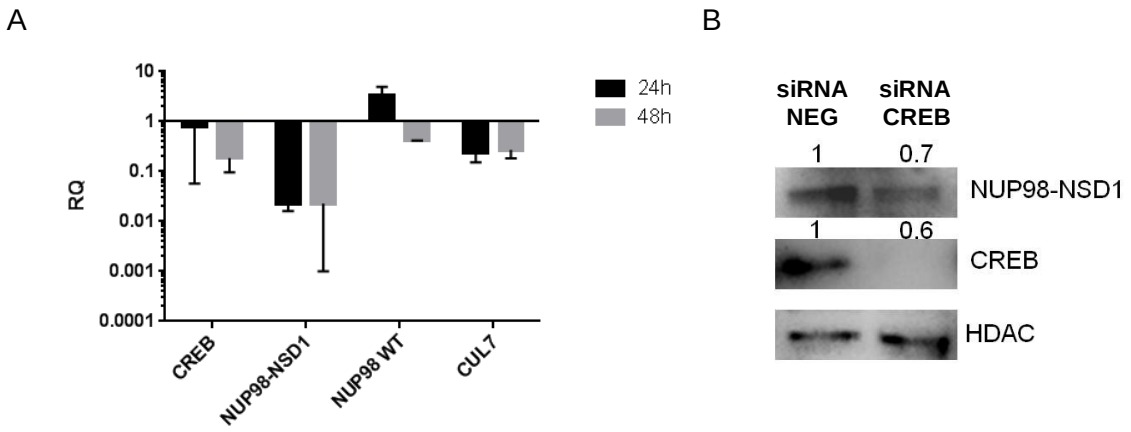


Figure 6. CREB silencing in *NUP98*-AML cultures. A) Decrease of *NUP98-NSD1* mRNA levels 24 and 48 hours post CREB silencing was detected by RQ-PCR (RQ=1 for siRNA NEG, N=3, p-value<0.05). B) Western blot analysis confirmed the reduction of NUP98-NSD1 chimeric protein levels in primary cell lines after CREB silencing, number in the figure represent the densitometric quantification mean of Western blot band normalized to siRNA NEG (N=3, p-value<0.05)

NUP98-NSD1 enhance genome instability. To gain into the leukemogenic role mediated by these *NUP98*-chimeras we used results obtained by GEP and GO, where chromosome instability was revealed as one main feature. The previous observation that *NUP98-HOXD13* overexpression in fibroblasts caused substantial perturbation of chromosome segregation[17] together with the fact that almost the 60% of *NUP98*-translocated patients presented a complex karyotype at diagnosis, forced our attention on this mechanism. In *vitro* studies on *NUP98-NSD1* primary cells and healthy bone marrow (HBM) were conducted. Cells were arrested in M phases by treatment with the microtubule-depolymerizing drug nocodazole and harvested at different time point (0-24h) to analyze spindle assembly checkpoint (SAC) defects in total cell extracts. Western blot analysis showed since 8h of treatment a decrease in MAD2 and BUB1 protein levels compared to healthy bone marrow cells, confirming the lack of control on sister chromatids separation in *NUP98*-translocated cells. Indeed, an increase in the expression of Cyclin B is revealed at the same time points, indicating an early and uncontrolled entrance in mitosis for leukemic cells (Fig.7A).

To address the possibility that *NUP98*-t chimera are more prone to chromosomal double strand break, we blocked DNA replication by exposing cells to aphidicolin (APH) treatment and measured the number of mitotic chromosomal breaks 24h post treatment. *NUP98*-translocated cells exhibited more visible chromosomal breaks (Fig.7B). This latter result was confirmed by western blot analysis, after 24h of APH treatment, PP2A β subunit

was found in the cytoplasm, with a consequent increase in H2AX phosphorylation (γH2AX) more evident in the *NUP98*-t cells. In response to genotoxic stress, in fact, PP2A translocate into the cytoplasm, allowing the activation of ATM which in turn phosphorylate H2AX, the docking site for DNA repair enzymes recruitment. Interestingly, only in HBM cells the γH2AX was detected in the cytoplasmic fraction to sustain the incapability of healthy cells to survive after a strong not repaired DNA damage, activating the apoptotic pathway (Fig.7C)[44].

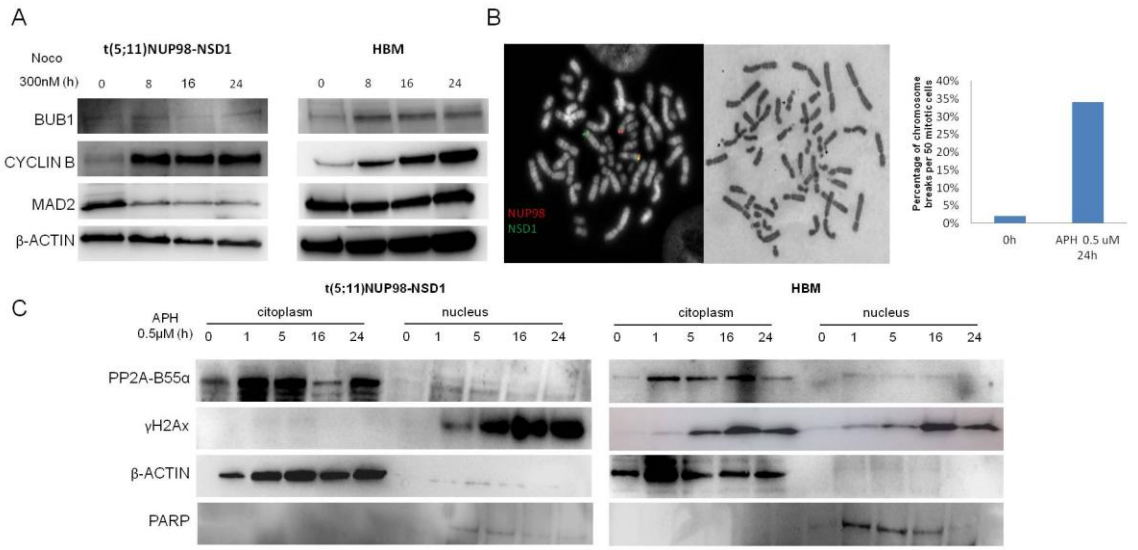


Figure 7. NUP98 fusion oncoproteins cause genomic instability. A) Western Blot analysis showing a decrease of mitotic proteins levels in nocodazole treated NUP98-NSD1 rearranged primary cells and HBM. Cyclin B1 (CCNB1), mitotic arrest deficient 2 (MAD2), and BUB1 were detected impaired in t(5;11). B) Dual color FISH analysis using a red probe for *NUP98* and green probe for *NSD1* in ex vivo *NUP98-NSD1* cells. To evaluate the Frequency of chromosomal aberrations (CAs) 50 mitosis for each cases were counted. G-banded karyotype showing the chromosome aberrations (arrows) in *NUP98-NSD1* primary cells and HBM before and after treatment with aphidicolin (APH) for 24h at 0.05μM are shown. C) After APH treatment the expression of PP2A-B55α in the cytoplasm and γH2Ax in the nucleus of *NUP98-NSD1* primary cells measured by western blotting revealed chromosome instability; whereas cytoplasmic γH2Ax in the HBM confirmed activation of apoptosis

DISCUSSION

In the era of personalized therapy, where specific molecule can be targeted, it is becoming increasingly important to identify genetic abnormalities that may dictate the disease and treatment course[45]. However, despite recent progresses in the identification of molecular lesions, still more than the 50% of patients with AML lack of a known genetic biomarker at diagnosis, remaining a heterogeneous disease[46,47]. The outcome of children with AML has significantly improved over the past two decades, however a remarkable contribution to the cure has been given only by the wide use of hematopoietic stem cell transplantation, since chemotherapy consisting in repeated courses of intensive consolidation has not been changed. Thus, the identification of novel prognostic and also therapeutic markers constitute an urgent need to overcome AML obsolete cure and improve children outcome.

In this study, we pursued the discovery of new biomarkers for pediatric AML at diagnosis focusing on *NUP98* gene, and then we tried to dissect the mechanism of action to indicate novel targeted strategies. Results revealed that *NUP98* rearrangements were not rare events (4.6%) among Italian children with leukemia, and identified, both clinically and biologically, a new relevant class of pediatric AML with severe prognosis and specific leukemogenic process. *NUP98*-rearrangements incidence is comparable with those identified by the Japanese (4.8%) and the BFM cohort (5.7%) that took in consideration only the t(5;11)(q35;p15.5)*NUP98-NSD1*, with the addition of t(11;21)(p12;p13)*NUP98-JARID1A* just for the BFM[2,3,21]. Indeed, *NUP98-NSD1* is the majorly found rearrangement also in our cohort, that is also characterized by other five partners gene. *NUP98*-rearrangements have been considered type II abnormalities[4] to impair the differentiation process , and according to Kelly and Gillard hypothesis[48], an additive type I abnormality, such as *FLT3-ITD* or *RAS* mutations[2,4,10,49] may occur to develop full-blown leukemia. Interestingly, in our cohort we found *FLT3-ITD* exclusively within the *NUP98-NSD1* rearranged patients, as *Hollink et al.*[2] and in contrast with *Taketani et al.*[10] that found *FLT3-ITD* mutation in other *NUP98*-translocations, probably due to the uniqueness of the Japanese leukemia[10]. However, in our study, others collaborative type I events, such as *N RAS, K RAS, IDH1* and *c-KIT* mutations were never found in the whole cohort of *NUP98*-rearranged patients, and new mutational events that could contribute to leukemia with *NUP98* translocations remains to be elucidated. Altogether, *NUP98*-patients were characterized by a detrimental clinical outcome, with a higher than expected frequency of relapse (60%), also if compared with high risk AML[12], suggesting that current therapy has

a very low efficiency in blasts clearance, contributing to disease recurrence. Moreover, we did not find any difference in either clinical or biologic features between patients with isolated t(5;11) and those with t(5;11) and *FLT3-ITD*, sustaining that the chimera superimposes its role in determining the leukemia gene expression profile and aggressiveness. Nevertheless, the type of partner gene confers specificity in defining patients outcome[2,3,11], indeed, those harboring t(5;11) present the worst survival, while those characterized by *NUP98-PHF23* display a better prognosis. Prospective and cooperative studies are desirable to confirm these results and improve risk stratification within *NUP98*-rearranged patients.

Here, we attempted to comprehend if differences in outcome found within the *NUP98*-subgroups could be explained by a different tumor biology. We interrogated gene expression profiling trying to find candidate genes involved in *NUP98* translocations. In the last decades, there has been an effort to integrate gene expression globally in AML prognosis; the identification of distinct gene expression signatures has already showed its impact on patients' clinical management, including determining sensitivity to specific therapies[13,50,51]. Here, *NUP98*-rearranged patients were revealed to have a specific coding and non coding gene expression profile, confirming the homogeneity of this newly identified AML entity with respect to the rest of AML, such as *CBF-MLL-NUP214*-rearranged as well as *FLT3-ITD* and the rest of molecularly negative AML. This is the first time where a specific non coding signature is given for a comprehensive expressional study of all the non coding RNAs (ncRNAs) among pediatric AML. We revealed that ncRNAs signature clustered *NUP98*-t patients and others known cytogenetic AML subgroups at the same strength of coding genes, although sustaining the role of ncRNAs as important mediators of the AML phenotype[52–54]. This analysis revealed that microRNAs, the most characterized ncRNAs class[14,30,55–57], were found significantly downregulated in *NUP98*-t patients with respect to the rest of AML. Therefore, we hypothesized that their downregulation may explain the uncontrolled expression of putative oncogenes in *NUP98* leukemias. By correlating microRNAs and gene expression we generated a list of new candidate couples of tumor suppressors and oncogenes that may be involved in *NUP98* mediated leukemogenesis. Among them, we consider miR-513a the most intriguing microRNA for its role in different biological process involved in *NUP98*-t patients, as well as for its previous role found in the block of cellular differentiation in other cancers[58,59]. Functional studies should be conducted to address if miR-513a could be a good therapeutic

candidate in this AML context[60], and which is or which are its putative targets among the several overexpressed genes found in *NUP98* leukemia.

Looking for biological processes enriched in the *NUP98*-t patients, the gene expression signatures of both coding or non coding probes converged in the identification of the regulation of mitosis and chromosome segregation[17,61–63]. Thus, moving on *NUP98*-t primary cells, we observed uncontrolled mitosis in the presence of an unsatisfied spindle assembly complex, and we detected a higher number of chromosomal breaks and an increased protein levels involved in DNA damage in response to genotoxic stress. On the contrary, same treatments performed in healthy hematopoietic cells correlated with an accumulation of a pro-apoptotic signal due to the translocation of γH2AX in the cytoplasm, and lead to cell death[44]. All these findings converged to sustain that DNA repair, chromosome segregation and cell cycle control, already known for their role in genomic instability and in tumors cell biology[64,65], are processes straightly involved in *NUP98* leukemogenesis. In proof of these deregulated processes, we highlighted that *NUP98*-translocated patients present in the 56.2% of cases a complex karyotype compared to the 11.5% of the rest of AML. It is thus tempting to speculate that the progressive genomic instability observed in these *NUP98* model, due to failure of DNA repair and to spindle assembly defects, may be the pivotal mechanism leading to the acquisition of cooperating molecular aberrations that may lead to disease progression from MDS to leukemia[7,66,67]. However, the exact underlying mechanism that takes place in *NUP98* leukemia will require further investigations, but these novel findings sustain the chimeras role mediating different leukemogenic pathways as previously proposed[68].

In fact, *NUP98*, like *MLL*, has many partner genes characterized by different domains that define the leukemogenic potential of the fusion[68,69]. In our cohort we identified 6 partner genes: 3 of them, the most frequent, NSD1,PHF23 and JARID1A, present a chromatin recognition domain (PHD) designated to interpret H3K4me3 marks[22,70] while the other partners are constituted by DNA binding domain as a homeodomain (HOXD13)[9,68] or coiled-coil domain (LEDGF and ADD3)[1]. Of note, we evidenced the overexpression of *HOX* genes in *NUP98* rearranged patients compared to the rest of AML cases as already reported in other cohorts [2,3,22]. Since now, the deregulation of the *HOX* genes has been straightly correlated with leukemogenesis of NUP98-chimeras[4]. Nevertheless, by studying *HOX A* and *B* genes expression among different *NUP98*-chimeras, we revealed that in *NUP98-NSD1* cases their expression was extremely lower suggesting that probably others co-operating oncogenic processes are occurring to cause

this aggressive type of leukemia. In particular, *NUP98-NSD1* patients were found particularly enriched for CREB signaling[38,39]. This important network has been validated in patient's primary cells: CREB was revealed to directly control NUP98 promoter; its silencing severely decreased *NUP98-NSD1* chimera and its target genes expression, finally proving that CREB controls the transcriptional regulation of *NUP98-NSD1*. The finding points to possible CREB-NUP98 axis as pivotal leukemogenic mechanism in this N*UP98*-AML subgroup, that together with chromosome instability, define a novel mechanisms in the pathogenesis of this disease. In conclusion, the identification of *NUP98* rearrangements as not rare biological and clinical entity in childhood AML raises important improvements in the genetic landscape and management of pediatric AML. The specific gene expression profiling such as a different clinical outcome for each partner gene reveal a distinct nature of each subtype of *NUP98*-rearranged AML. CREB involvement and the enhanced genomic instability among these patients opens for further evaluation on the leukemogenic role of *NUP98-NSD1* oncoprotein, that can be determinant for the aggressive nature of this leukemia. The identification of this mechanism unclose new therapeutic strategies, such as CREB inhibitors, combined with conventional therapy to improve the outcome of these subsets of childhood AML.

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SUPPLEMENTARY INFORMATION

Supplementary methods.

Gene expression analysis. Briefly, 100 ng of total RNA were labeled and hybridized to Affymetrix GeneChip® Human Transcriptome Array 2.0 (HTA 2.0) for 16 h at 45°C using a rotational oven and washed according to Affymetrix standard protocols using a GC450 Fluidics Station. The Genechips were scanned with an Affymetrix 7G scanner and the CEL files generated were analyzed through Affymetrix Expression Console Software (version 1.3) which normalizes array signals using a robust multiarray averaging (RMA) algorithm. Probes were re-annotated using GENECODE v.19 gene annotation database (www.genecodegenes.org)[1] in order to identify both coding and non-coding RNAs covered by probes. Transcripts were included if at least the 95% of nucleotides overlapped with probes. In miRNA analysis, for every probe which recognizes pre-miRNA, we used both associated miRNA mature, where available. Normalized data were analyzed using GENE-E analysis platform (Broad Institute of Harvard and MIT, Boston, MA). Cluster analysis was performed using distances based on Pearson Correlation or Spearman Rank coefficient. An unpaired t-test was applied to identify differentially expressed genes between sample pairs and probes with P-values less than 0.01 were declared significant.

Gene functional enrichment analysis. We examined whether the differentially expressed genes are biologically meaningful via functional enrichment analysis using the integrative web-based software Enrichr[2]. This tool enables the detection of significant enrichment for a set of genes in Gene Ontology (GO) Consortium database. We used the whole human genome as a reference set for enrichment analysis and applied a hypergeometric test with a significance level of $p \leq 0.05$. To understand which biological processes were regulated by differentially expressed miRNAs, we identified the predicted miRNA–target interactions through DIANA microT-CDS software[3] with a prediction threshold of 0.8. We examined the Pearson's correlation (r) of miRNA-target pairs with *micrographite* R package[4]. Only pairs with $r < -0.4$ and p-value < 0.01 were considered negatively correlated. Gene identified were undergo to functional enrichment analysis as previously described. miRNA-target interactions network was designed through Cytoscape v3.3.0 software[5].

Gene Set Enrichment Analysis (GSEA) software version 4.0[6] was used to identify gene sets in the public domain that share the expression pattern found in the NUP98 translocated patients respect to an eterogeneous cohort of pediatric AML patients. For each

group of gene sets, GSEA calculates and evaluates the statistical significance of an enrichment score (ES). The ES reflects the degree to which a gene set is over represented. We compared the gene expression signatures collapsing the probe sets to gene vectors and using the signal-to-noise metric, the gene-set permutation type and 1,000 permutations. As recommended by GSEA guidelines, only gene sets with a P-value < 0.05 and a false discovery rate (FDR) *q*-value < 0.25 were considered.

For help with interpreting the gene set enrichment analysis go to http://www.broadinstitute.org/gsea/doc/GSEAUserGuideFrame.html%20Interpreting_GSEA_Results. The NearestTemplatePrediction algorithm (NTP)[7] implemented as module of the Gene Pattern software (Broad Institute of Harvard and MIT, Boston, MA) was used to predict the proximity of the expression pattern of molecular signatures deposited in the Molecular Signature Database (www.broadinstitute.org/gsea/msigdb) to each single patient's gene-expression data using cosine distance. Only prediction with statistical significance (p-value < 0.05) were used for the heatmap generation.

Primers sequences

NUP98F	GCTGGACAGGCATCTTTGTT
NUP98 988F	ACCAGCCTCTTCAGCAAACCATTTG
NSD1 R1	TGCACCTGCTCCTGTACCTTC
NSD1 R2	TCCAAAAGCCACTTGCTTGGC
NSD3 R1	AATGCTTTCTCTGCATGGGC
NSD3 R2	CACAGATCTTGGTCGTCGGG
JARID1A R1	TAGCTTCCGTTTCCGTTTCT
JARID1A R2	TCAGCTCCTTTGATTTGTCT
PHF23 R1	GGCCCCAGTAGCTTTGACAGAT
PHF23 R2	GCAGACGAGAGAAAGTGGACCT
HOXA9R1	CCTGCGGTCCCTGGTGAGGT
HOXA9R2	GCACCGCTTTTTCCGAGT
HOXD13 R1	CAGGAGACAGTATCTTTGAGCTTGG
HOXD13 R2	AAGCTGTCTGTGGCCAACC
HOXC11 R1	TGCAGCCGCTTCTCTTTGTT
HOXC11 R2	TTACAGCAGAGGATTTCCCG
ADD3 R1	ACCTACTCACTCGCTTAGCA
ADD3 R2	CTTCTTCGATTTTCTCTGGAGACTT
HHEX R	ATTTAGCGCGTCGATTCTGA
HHEX R1	TCTCGGGCGGAGAGAGATATT
DDX10 F	AAATATACTCGTGTGCACACCAGG
DDX10 1078 R	TGCTCTTCTTCTTCAGATGGCTTC
LEDGF R	AACAGATGCTGTTGCTGTTGTCAC
NSD3 R	AATGCTTTCTCTGCATGGGC
LOC348801 R	TCTCTCCTTTCTGCTTTCAGGT
LOC348801 R2	AAGAGGCAGAACGCTTGGTT
NUP98 CHIP F	AGTTACCATGCCATTCTGGGG
NUP98 CHIP R	CATTGGGCAACGCGTCTTTT
CREB F	CGGTGCCAACTCCAATTTAC
CREB R	ATTGCTCCTCCCTGGGTAAT
NUP98 F RQ-PCR	CCATCTATGGATGACCTTGCTAAA
NUP98 R RQ-PCR	TCCGACCAATAGTGAAATCAGAGA
CUL7 F RQ-PCR	CCTACCTGAGGGGCACTTTG
CUL7R RQ-PCR	CGCCTTCAGGTCGTTGAGAT
NUP98-NSD1 F RQ-PCR	CTTGTACAGGAGCCTTTGG
NUP98-NSD1 R RQ-PCR	GGCTTCCTAAGGCGTTTCTT

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Suplementary figures

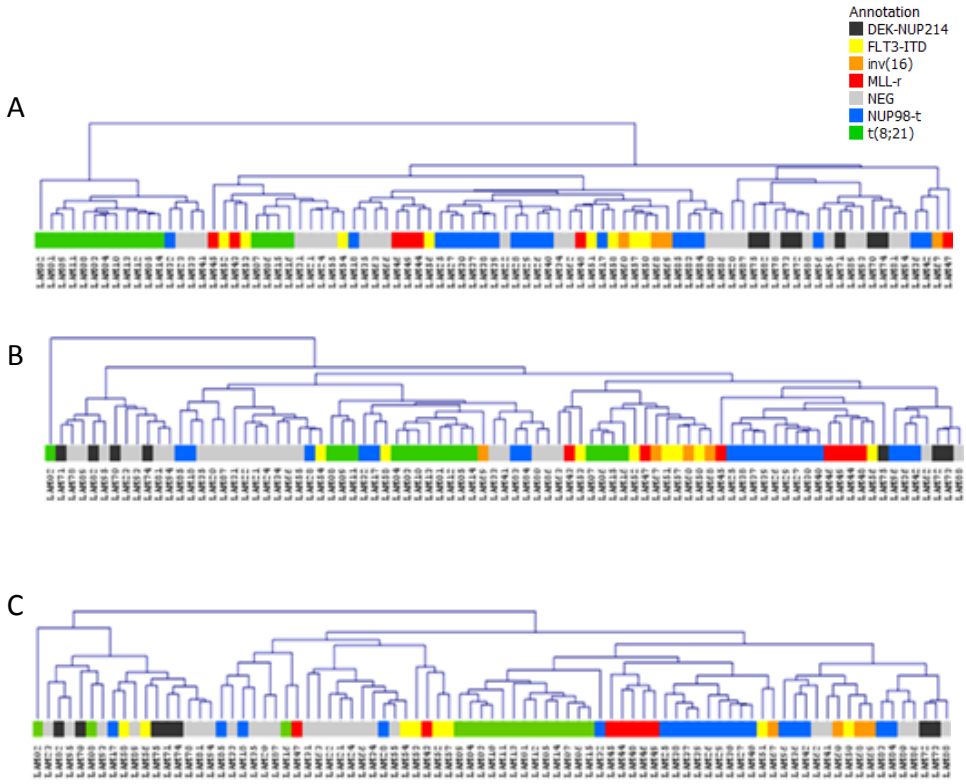

Figure 1S. Unsupervised clustering of pediatric *de novo* AML. Cluster analysis using Spearman correlation distance among the gene expression profiles of 85 *de novo* pediatric AML samples (p-value<0.01). Samples are color-coded according to their cytogenetic subtype. The heatmaps identify 3 probe matrix : A) Total RNA, B) coding RNA C) non-coding RNA.

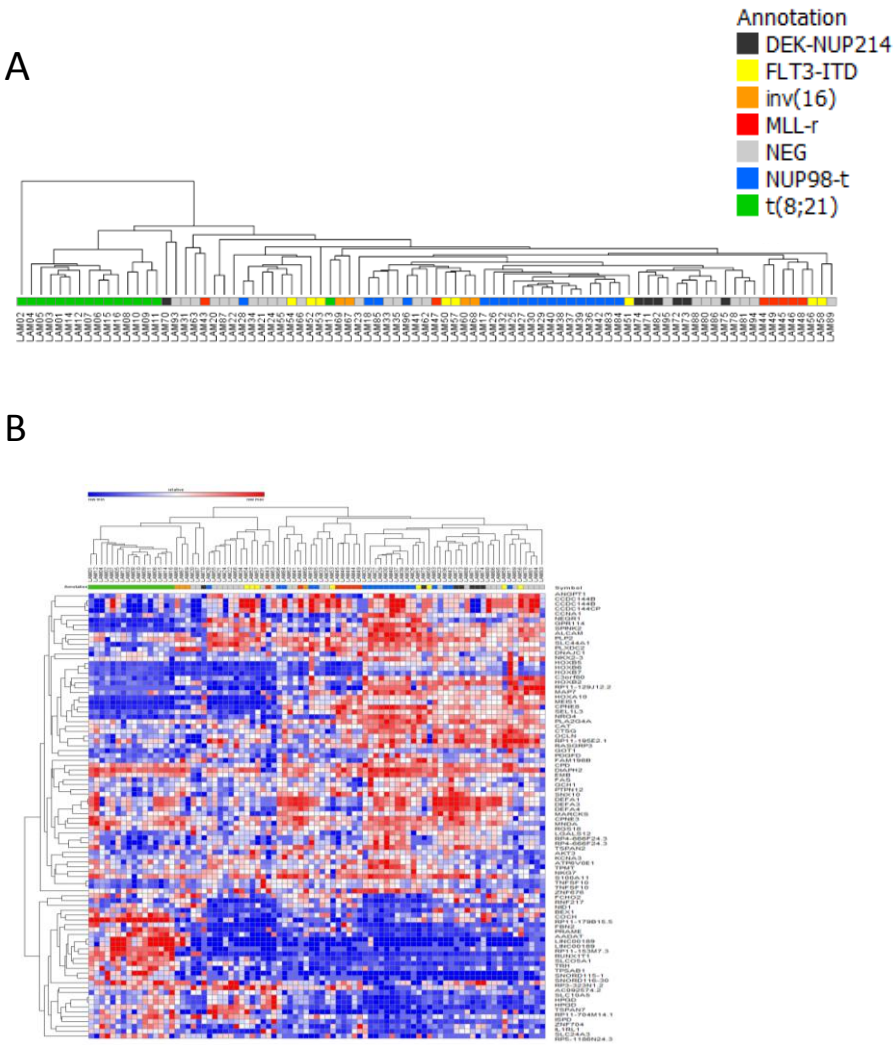

Figure 2S. Gene expression profile of 19 *NUP98*-traslocated patients. A) Supervised hierarchical clustering analysis using the 1291 probe sets identified among 19 *NUP98*-t and 66 *de novo* AML patients. B) Heat map of the 120 probe sets most differentially expressed (FC>1.5) between *NUP98-t* and all AML rearrangements studied. The genetic subtypes were indicated with color codes. The differently expressed genes between the two groups were identified by gene selection based on t-test (*p*-value<0.01).

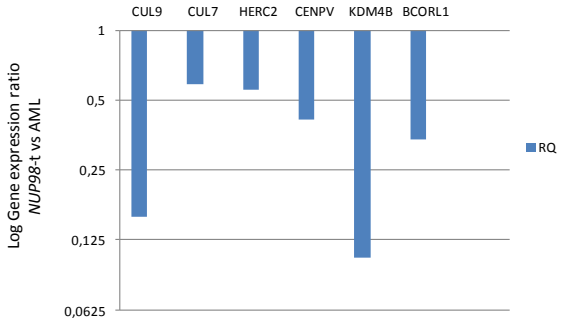

Figure 3S. Real time quantitative-PCR (RQ-PCR) validation of microarray data. Six genes were selected to validate microarray data by real time-PCR. *CUL7, CUL9, HERC2* are related to regulation of mitotic transition while KDM4B, CENPV and BCORL1 are involved in chromatin organization. cDNAs from bone marrow of 19 NUP98-t patients and other 22 *de novo* AML (including patients with core-binding factor aberrations, MLL rearrangements, t(6;9)(p23;q34)*DEK-NUP214* and negative for known recurrent genetic abnormalities previously described) were used for the RQ-PCR reaction. RQ expression, calculated with 2^-DDCt of genes in 19 *NUP98*-t patients compared to other AML (RQ=1) is presented

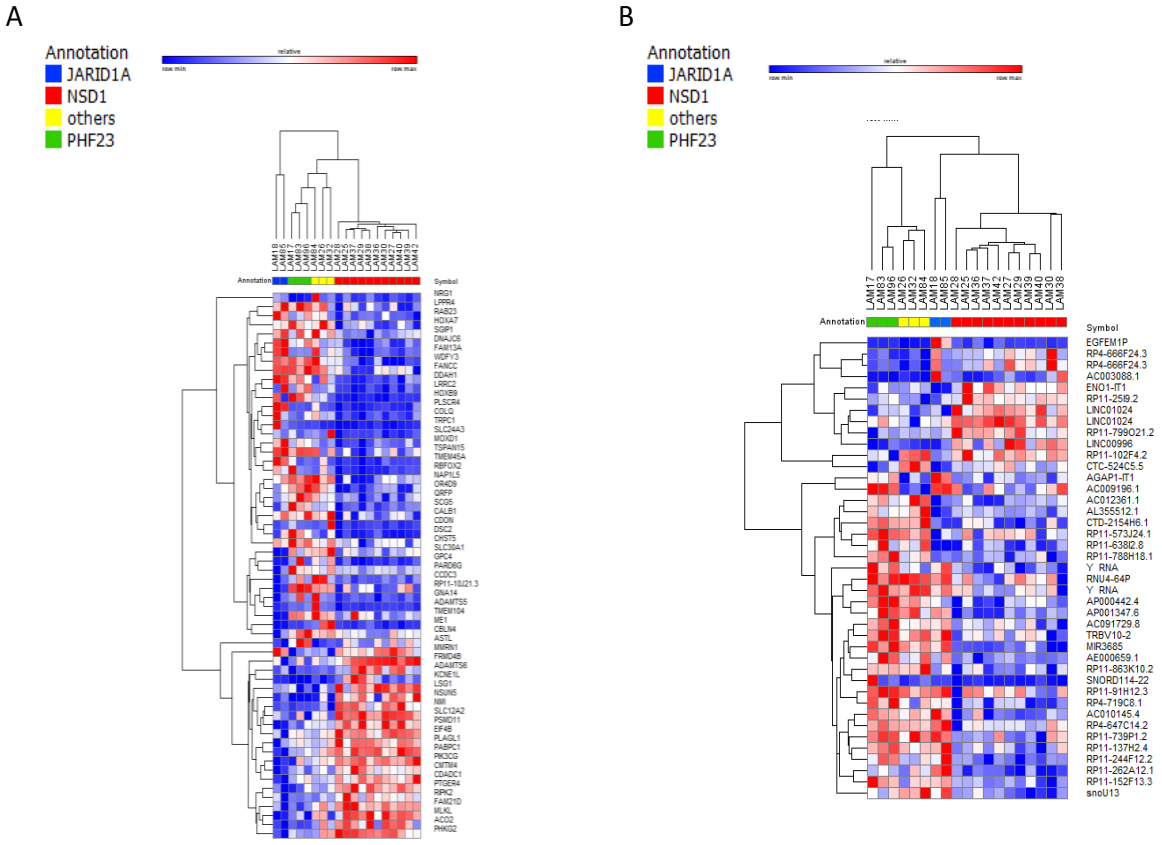

Figure 4S. Coding and non-coding RNA cluster analysis of *NUP98*-AML patients. Supervised hierarchical clustering analysis using A) the 60 differentially expressed coding RNAs and B) the 41 non-coding RNAs identified among the 19 *NUP98*-t patients (Kruskal Wallis, p-value<0.01). Different *NUP98* partner genes were indicated below the dendrogram with different colors: blue for JARID1A, red for NSD1, green for PHF23 and yellow for other partner genes.

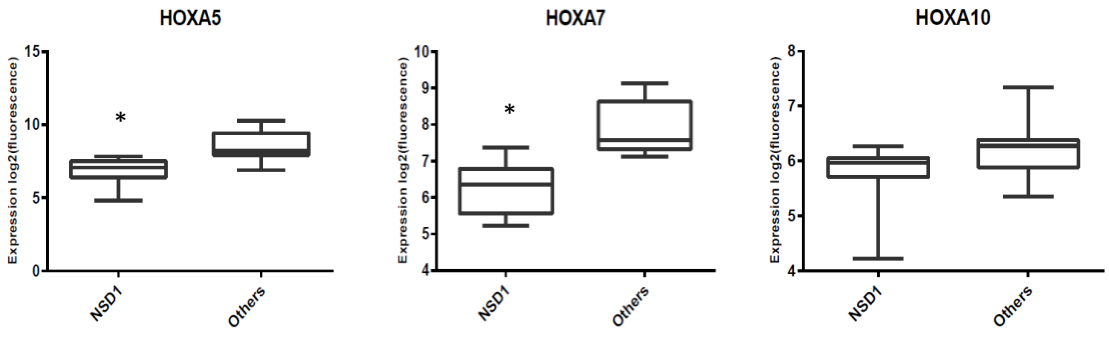

Figure 5S. HOXA genes expression in *NUP98*-t patients. Box Plot of *HOXA* genes expression in patients harboring *NSD1* (N=11) as partner gene versus others *NUP98*-t positive patients (N=8, p-value<0.05).

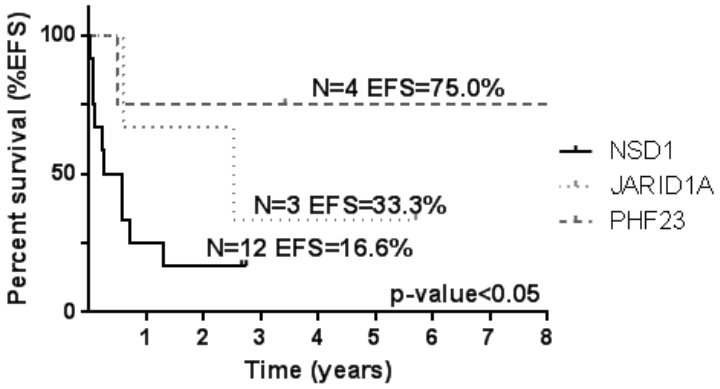

Figure 6S . Probability of event-free survival (EFS) in *NUP98*-AML characterized by different partner genes. EFS was calculated for patients with *NUP98* and *NDS1*, *JARID1A* and *PHF23* (p-value<0.05) at 8 years of follow up.

CHAPTER 5

Conclusions

Acute leukemia is the most common form of pediatric malignancy and numerous events are currently known to occur and finally contribute to promote the onset of this disease. Despite leukemias have been well characterized from the genetic view point, acute myeloid leukemia (AML) remains the most heterogeneous disease with just the 50% of patients presenting an available biomarker that can direct therapy and guide prognosis[1,2]. During this PhD my main interest was to increase the number of molecular markers available for pediatric AML patients, considering a large cohort of AML enrolled in AIEOP-LAM 2001-02 protocol from 2002 to 2012[3].

At first, I evaluated the incidence of rare genetic abnormalities in pediatric AML such as del(4)(q12)*FIP1L1-PDGFRA*, t(16;21)(p11;q22)*FUS-ERG*, t(8;16)(p11;p13)*MOZ-CBP*, t(11;17)(q23;q12-21)*MLL-AF17*, t(4;11)(q35;q23)*MLL-ARGB2*, *MLLPTD* t(5;11)(q35;p15.5)*NUP98-NSD1* and t(3;5)(q25;q34)*NPM1-MLF1*. These rearrangements were described mainly as case reports in literature but their incidence in a pediatric cohort was never considered. Thanks to the large retrospective screening I established that the 2% of cases presented the t(3;5)(q25;q34)*NPM1-MLF1*, the 1.3% of patients harbored the t(8;16)(p11;p13)*MOZ-CREBBP*, while 2% carried *MLLPTD*. These results reveal that such rearrangements remain rare in the pediatric field, however further prospective studies will permit to enlarge these small cohorts of new AML subgroups, allowing in the near future their characterization also for a prognostic value. The best result was obtained for the translocation t(5;11)*NUP98-NSD1*, which reached an incidence of 4% in AML patients, and moreover was found associated to the *FLT3-ITD* mutation. I established that also in the Italian cohort this marker is associated to a very low survival, as previously reported for another European cohort[4].

Then I also evaluated the incidence of *c-KIT* mutations, already known to occur in different cancers as well as in adults AML[5,6]. I considered a selected group of AML, the *CBF*-rearranged, because *c-KIT* mutations were previously found frequent within patients harboring these molecular lesions[7]. I confirmed a strong incidence of *c-KIT* mutations in pediatric patients with the t(8;21) *RUNX1-RUNX1T1* (25%), and with the inv(16)/t(16;16) *CBFB-MYH11* (18.5%). The predictive value of a worst outcome of *c-KIT* mutations was confirmed only for the t(8;21) patients. This result permits the identification of a group of patients, within the same genetic background, with a higher risk of relapse and that may be considered for alternative therapeutic strategies.

Finally, these molecular screenings performed at diagnosis of AML confirmed that the identification of a marker is mandatory for a more precise patient stratification in

appropriate risk class with the final aim to better manage their cure and improve their survival.

Then I evaluated if the crucial role of a molecular marker at diagnosis could persist also during therapy course. At first, I focused on the Italian standard-risk (SR) group of patients, as defined by the protocol AIEOP 2002/01 harboring the isolated *CBF*-rearrangements, in which the outcome was found less than the expected[3,8]. I performed the molecular monitoring of minimal residual disease (MRD) levels after induction chemotherapy for both, the *RUNX1-RUNX1T1* and *CBFB-MYH11* fusion transcripts. Results revealed that MRD levels provided reliable prognostic parameters to manage therapy decisions also in childhood AML, in particular for the *RUNX1-RUNX1T1* rearranged patients. I reproduced the same results when molecular MRD levels after induction were measured on *FLT3-ITD* marker.

These new findings on the role of molecular MRD, contribute to overcome the lack of studies on post-treatment monitoring of genetic abnormalities and confer, also in pediatric AML, a new role to MRD molecular monitoring.

I dedicated most of my PhD to perform clinical translational research: this branch of medical science aims to expedite the translation of scientific discovery into new or improved standards of care. This definition fit well with my personal feeling that drove my PhD experience towards a research that has to be instrumental to improve children survival and fight cancer. When a molecular marker is defined for its diagnostic role, it is important to underpin the leukemogenic and biological mechanism involved with it. With this aim, I performed several investigations to better comprehend *FLT3-ITD* mutation, *MLL-AF6* fusion, and the *NUP98*-rearrangements.

The first functional study was a comprehensive study on *FLT3-ITD* mutation in a large pediatric AML cohort (482 patients) enrolled in a single clinical protocol[3]. We tried to discuss the significance of the allelic burden of the mutation, as well as the minimal residual disease after induction treatment, and defined both features as strategic tools to refine risk stratification and direct pre-emptive therapy. We used this two parameters to divide patients in two groups and investigated the disease biology by gene expression profile. Results revealed that patients with high allelic ratio of ITD mutation as well as persistent residual disease activated different signaling pathways concerning methylation, acetylation, and the *CyclinA1/cMYB* oncogenic pathway. These findings open for further experimentation of novel epigenetic drugs, combined with conventional strategies, that can

be adopted to improve the survival of a subgroup of *FLT3-ITD* patients who shares a high risk of relapse and die[9,10].

The investigation on *MLL-AF6*-rearranged patients was suggested by the very low percentage of survival that this marker conferred to this subgroup of AML (22% at 8 years)[11,12]. The functional study was addressed to comprehend the role of the chimera in order to find new targeted therapies. Results demonstrated that the cytoplasmatic interaction of AF6 and RAS was responsible to maintain low levels of RAS-GTP in hematopoietic cells, while the occurrence of MLL-AF6 resulted in an de-localization of AF6 into the nucleus with the consequent RAS activation, which contributed to the aggressiveness of this leukemia subtype. These findings were confirmed *in vitro,* where we used specific RAS-inhibitors to increase AML blasts susceptibility to death. A new era of new therapeutic opportunities against RAS signaling can be supported for this subgroup of leukemia with a very detrimental destiny.

The last functional study sought to refine the role of various *NUP98*-rearrangements found in pediatric AML[4,13]. The fact that *NUP98* can arrange with several genes is extremely interesting: above all because it reflects the properties of *MLL,* one of the key-gene in pediatric leukemia. To discover if *NUP98* plays a leading role in AML, I looked at several fusions involving its N-terminus with the C-terminus of *HOXC11, HOXA9, HOXD13, HHEX* (sharing a HD domain), *LEDGF*, *DDX10, ADD3, LOC348801* (sharing a CC domain), and NSD3, *JARID1, PHF23* (sharing a PHD domain)[14,15]. All together, these aberrancies allowed to define a novel Italian *NUP98*-AML subgroup (4.6%) with dismal outcome. Then, biological implications were interrogated in order to dissect the mechanism of action of *NUP98* translocations. I found a unique expression pattern with both coding and non coding transcripts with respect to other AML selected to provide a good representation of the known morphologic, genetic, and prognostic subtypes of *de novo* pediatric AML. Genes enrichment analysis showed that *NUP98*-AML were particularly characterized by processes correlated to nuclear organization and chromosomes assembly. By gene expression analysis I also established that the *NUP98* partner gene drove a specific gene signature as well as a different outcome. *In vitro* analysis chromosome instability being involved in this type of leukemia was confirmed, and also I revealed CREB as the transcription factor responsible for the chimera expression. This study provides important clinical and biological implications: such as the identification of new markers at diagnosis that can assign patients to specific risk-classes to

overcome their dismal prognosis, and the definition of the leukemic mechanism behind NUP98-oncoprotein that could be targeted to improve patients cure.

In conclusion of this PhD, I can sustain that pediatric AML is still a very heterogeneous disease. The discovery that within the same genetic group, that is expected to present a concordant outcome, it is possible to identify a wide spectrum of additive markers, at diagnosis and during follow up, that concur in defining new and distinct prognosis, reveals a even more complex picture of pediatric AML. The fact that most differences in clinical outcome can derive from differences in response to therapy, and that it is often guided by a specific patient-biology support the further evaluation of the crucial role of defining a even more personalized therapy to improve children survival. The fact that some of my results have been included in the AIEOP LAM 2013 protocol and consist in the characterization of new markers that will improve the risk stratification at diagnosis for a subset of Italian patients (e.g. *FUS-ERG, NPM1-MLF1, MLL-ARGB2, NUP98-NSD1, c-KIT*) and refine post-remission course thanks to disease monitoring during follow-up (e.g. *RUNX1-RUNX1T1* and *FLT3-ITD*) (figure 1) confers to this PhD great satisfactions.

Notwithstanding the effort spent in identifying new markers, still a large part of patients remains not characterized for the genetic aberrations that induced their leukemia. Therefore, the application of high throughput technology will likely accelerate our insight in the genetic landscape of AML allowing the identification of new aberrations. Deciphering the functional consequences of the abovementioned aberrations still remains a challenge, that has to be explored in order to develop new therapies to improve the outcome of pediatric AML as I found for *MLL-AF6* and *NUP98*-rearrangements. Indeed, the preliminary results that suggest a more personalized therapy, such as RAS inhibitors or CREB inhibitors, may be deeply investigated to change the fate of these subsets of childhood AML.

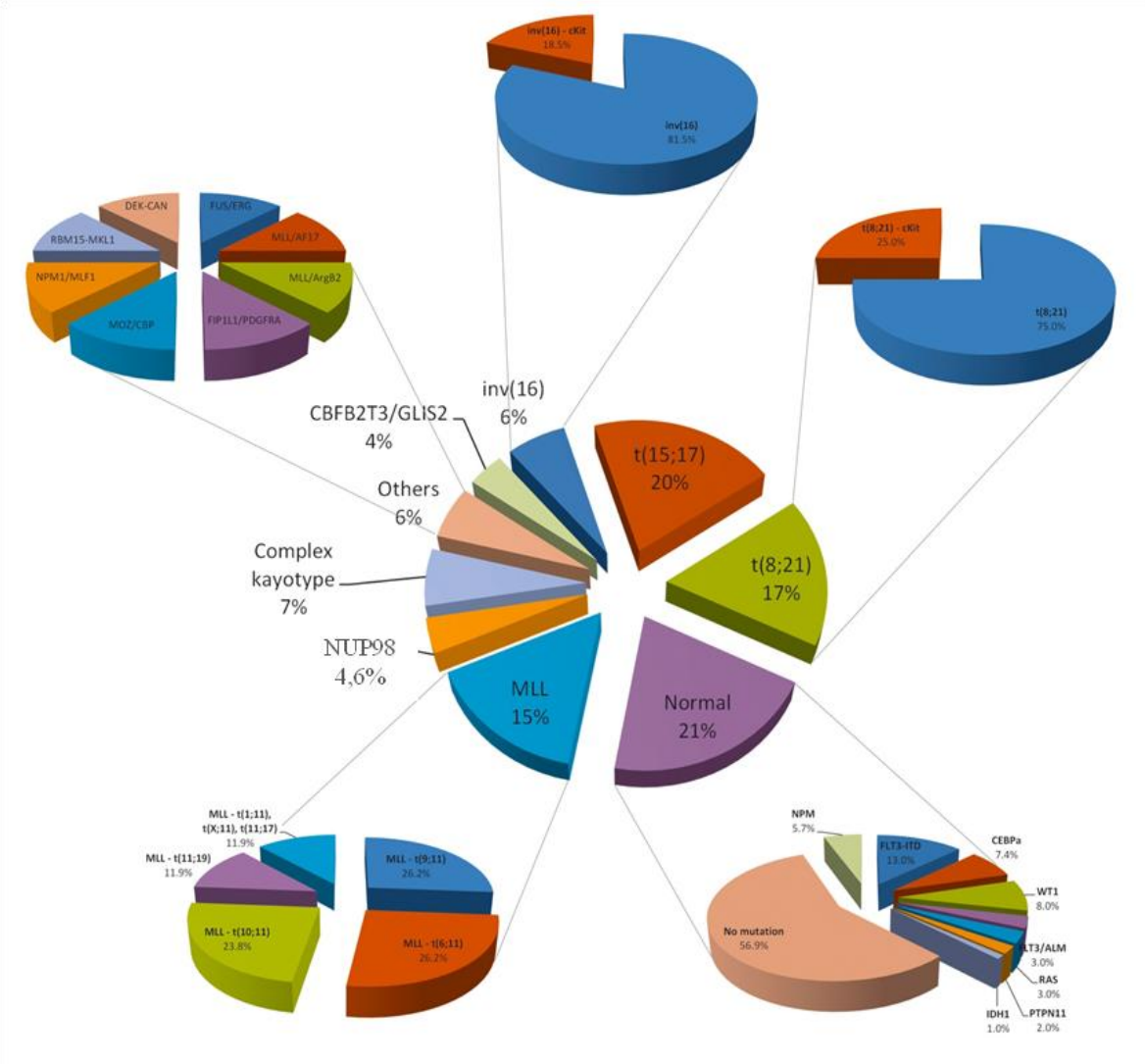

Figure 1. Estimated frequency of genetic abnormalities in childhood AML in the Italian population. The most common karyotypic alterations are shown in the center, the others are made up of recently identified rare abnormalities. (Right) Mutational profile of patients without cytogenetic abnormalities (normal) and the incidence of *cKIT* mutations in t*(8;21)* rearranged patients; 43.1% of those in the normal population have one of the known mutations. The most frequent MLL partner genes found in AML Italian cohort are also shown. Reported frequencies derived from the Italian AIEOP AML 2002/01 protocol that enrolled 482 patients from 2002 to 2012[3]. Modified from G. Basso Hematology Education 2014.

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ABOUT THE AUTHOR

She achieved her Master Degree in Biology at the University of Padova in 2011. During her thesis she trained in the laboratory of Dr M. Carneiro at CNC (Center for Neuroscience and Cell Biology) Coimbra, Portugal. After graduation, she continued her research in the same lab obtaining a fellowship from University of Coimbra. In 2013, she decided to implement her scientific education starting a PhD program in the Onco-hematology laboratory of Professor Giuseppe Basso. She mainly focused her attention on the identification of new genetic abnormalities and altered signaling pathways in pediatric acute myeloid leukemia (AML) to better classify and stratify patients in different risk classes. She strongly believes in the importance of what she is doing. Her research was published in 5 international papers and was presented to several poster sessions in national and international congresses as EHA (European Hematology Association) or ASH (American Society of Hematology) where she received an ASH Abstract Achieved Award in 2014.

Publications

Togni M, Masetti R, Pigazzi M, Astolfi A, Zama D, Indio V, Serravalle S, Manara E, **Bisio V**, Rizzari C, Basso G, Pession A, Locatelli F. Identification of the NUP98-PHF23 fusion gene in pediatric cytogenetically normal acute myeloid leukemia by whole-transcriptome sequencing. J Hematol Oncol. 2015 Jun 12;8:69.

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