MYELOID NEOPLASIA

CBFA2T3-GLIS2 fusion transcript is a novel common feature in pediatric, cytogenetically normal AML, not restricted to FAB M7 subtype

Riccardo Masetti,[1] Martina Pigazzi,[2] Marco Togni,[1] Annalisa Astolfi,[3] Valentina Indio,[3] Elena Manara,[2] Rita Casadio,[4] Andrea Pession,[1] Giuseppe Basso,[2] and Franco Locatelli[5]

[1]Department of Pediatrics, Lalla Seràgnoli, Hematology-Oncology Unit, University of Bologna, Italy; [2]Department of Woman and Child Health, Laboratory of Hematology-Oncology, University of Padova, Padova, Italy; [3]Giorgio Prodi Cancer Research Center, University of Bologna, [4]Biocomputing Group, Department of Biological, Geological and Environmental Sciences, University of Bologna, Bologna, Italy; and [5]Department of Pediatric Hematology-Oncology, Istituto di Ricovero e Cura a Carattere Scientifico Ospedale Bambino Gesù, Rome, University of Pavia, Italy

Key Points

- The *CBFA2T3-GLIS2* fusion transcript is common in pediatric cytogenetically normal AML and not restricted to FAB M7 subtype.
- The *CBFA2T3-GLIS2* fusion transcript is associated with poor prognosis in pediatric patients with AML.

Pediatric cytogenetically normal acute myeloid leukemia (CN-AML) is a heterogeneous subgroup of myeloid clonal disorders that do not harbor known mutations. To investigate the mutation spectrum of pediatric CN-AML, we performed whole-transcriptome massively parallel sequencing on blasts from 7 CN-AML pediatric patients. In 3 patients we identified a recurrent cryptic inversion of chromosome 16, encoding a *CBFA2T3-GLIS2* fusion transcript. In a validation cohort of 230 pediatric CN-AML samples we identified 17 new cases. Among a total of 20 patients with *CBFA2T3-GLIS2* fusion transcript out of 237 investigated (8.4%), 10 patients (50%) did not belong to the French–American–British (FAB) M7 subgroup. The 5-year event-free survival for these 20 children was worse than that for the other CN-AML patients (27.4% vs 59.6%; $P = .01$). These data suggest that the presence of *CBFA2T3-GLIS2* fusion transcript is a novel common feature of pediatric CN-AML, not restricted to the FAB M7 subtype, predicting poorer outcome. (*Blood*. 2013;121(17):3469-3472)

Introduction

Pediatric acute myeloid leukemia (AML) is a molecularly heterogeneous disease that arises from genetic alterations of pathways that regulate self-renewal and myeloid differentiation. While the majority of patients carry recurrent chromosomal translocations, almost 20% of childhood AMLs do not show any recognizable cytogenetic alteration and are defined as cytogenetically normal AML (CN-AML).[1] Many genetic abnormalities have been identified in AML with normal karyotype, with the most frequent affecting genes such as *NPM1*, *FLT3*, *CEBPA*, and *WT1*.[1-5]

Genome-wide analyses have been used with the aim of determining the full array of genetic lesions of CN-AML. Recent studies have provided new insight into the molecular genetics and biology of AML, confirming both the complexity and the heterogeneity of this disease.[6] Novel lesions such as mutations in *IDH1* and *DNMT3A* have been identified.[7,8] However, these alterations are rare in pediatric AML, with *IDH1*/*IDH2* accounting for 2% to 4% of cases[9,10] and *DNMT3A* not even being found mutated in childhood AML.[11] Recently, 2 studies identified a novel recurrent translocation involving *CBFA2T3* and *GLIS2* in about 30% of children with non-Down syndrome acute megakaryoblastic leukemia (non-DS AMKL, AML French–American–British [FAB] M7).[12,13] Nevertheless, there are many children with CN-AML in whom no genetic abnormality has been detected. The identification of the different genetic profiles characterizing this subgroup is a primary objective to be pursued. To this end, we performed whole-transcriptome massively parallel sequencing of 7 cases of pediatric CN-AML with the aim of identifying recurrent somatic mutations or genomic rearrangements. Subsequently, we validated our findings in a larger cohort of 230 pediatric CN-AML patients.

Study design

Patient samples

Patients analyzed either in the parallel sequencing screening or in the validation cohort are children with newly diagnosed de novo AML other than promyelocytic leukemia. The patients are enrolled in the Associazione Italiana Ematologia Oncologia Pediatrica 2002/01 Protocol, which was approved by the institutional review

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R.M. and M.P. contributed equally to this study. G.B. and F.L. are co-senior authors.

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Table 1. Clinical features of the CN-AML patients harboring the *CBFA2T3-GLIS2* fusion gene

ID	Age, years	Gender	WBC, × 10^9/L	FAB	BM blast, percentage at diagnosis	Extramedullary involvement	HSCT (type)	CR after induction therapy	Relapse (site)	Disease-free duration (months)	Survival duration (months)
1*	0.8	M	13.63	M2	52	No	Yes (MFD)	Yes		46.7	+48.0
2*	0.3	F	7.52	M5A	88	No	Yes (AUTO)	Yes		90.6	+91.4
3	1.4	F	65.08	M0	98	No	Yes (AUTO)	Yes	Yes (BM)	9.3	13.0
4	12.7	F	22.8	M1	61	No	Yes (AUTO)	Yes		70.1	+74.5
5	17.0	F	0.91	M1	98	No	Yes (AUTO)	Yes	Yes (BM + skin)	38.7	50.4
6	13.3	M	7.52	M5	90	No	No	No			2.6
7	0.9	M	7.53	M5	60	No	Yes (MUD)	Yes	Yes (BM)	15.1	19.3
8	12.1	M	20.82	M0	70	No	Yes (AUTO)	Yes	Yes (BM)	22.7	24
9	17.2	M	35.7	M0	85	No	No	No			3.2
10	16.4	F	26.4	M4	70	No	Yes (AUTO)	Yes	Yes (BM)	8.2	12.1
11	3.2	F	24.2	M7	70	No	Yes (MUD)	Yes	Yes (BM)	12.2	24.7
12	4.0	M	12.79	M7	74	No	Yes (MUD)	Yes	Yes (BM)	8.6	10.7
13	0.7	M	13.6	M7	95	Yes (CNS)	Yes (MUD)	No			9.4
14	0.7	F	9.6	M7	90	No	Yes (MUD)	No	Yes (BM)		21.1
15	2.0	F	7.4	M7	95	No	Yes (MFD)	Yes		109.2	+111.8
16	1.4	F	128.6	M7	50	No	No	No			2.1
17*	0.5	F	13.25	M7	90	No	No	Yes	Yes (BM + CNS)	3.2	5.7
18	3.0	F	13.63	M7	40	No	Yes (MUD)	Yes		37.2	+45.2
19	1.6	F	115	M7	90	No	Yes (MFD)	Yes	Yes (BM)	17.7	24.7
20	1.9	F	56.5	M7	30	No	Yes (MFD)	Yes		13.5	+15.6

+, patients alive and in CR; AUTO, autologous; CNS, central nervous system; CR, complete remission; HSCT, hematopoietic stem cell transplantation; MFD, matched family donor; MUD, matched unrelated donor; WBC, white blood cells.

*Patients identified in the RNA-seq screening.

board of the Sant' Orsola-Malpighi Hospital, Bologna, Italy.[14] Patients gave informed consent in accordance with the Declaration of Helsinki. FAB morphological classification and immunophenotypic analysis were centrally established at the laboratory of Pediatric Hematology of the University Hospital in Padova, Italy. Chromosome analysis was performed on bone marrow (BM) aspirates using standard laboratory procedures. Karyotypes were reported according to the International System for Human Cytogenetic Nomenclature.[15] For fluorescence in situ hybridization, an *MLL* locus-specific dual-color probe for 11q23 (Abbott-Vysis, Downers Grove, IL) was used. All CN patients were also negative for known recurrent genetic abnormalities involving *MLL*, *CBFB*, *NPM1*, and *FLT3* genes.

Whole-transcriptome sequencing and bioinformatics analyses

Total RNA was extracted from BM leukemia cells of CN-AML patients by TRIzol, following the manufacturer's protocol (Invitrogen, Karlsruhe, Germany); 250 to 1000 ng of total RNA was used for the synthesis of cDNA libraries with TruSeq RNA Sample Prep Kit v2 (Illumina, San Diego, CA) according to the manufacturer's recommendations. Sequencing by synthesis was performed on HiScanSQ sequencer (Illumina) at 75bp in paired-end mode. Reads were aligned with TopHat2/BowTie2[16] to the reference human genome hg19/GRCh37. deFuse[17] and ChimeraScan[18] packages were used to detect chimeric transcripts from RNA-seq data.

Screening of *CBFA2T3-GLIS2* fusion transcript in the validation patient cohort

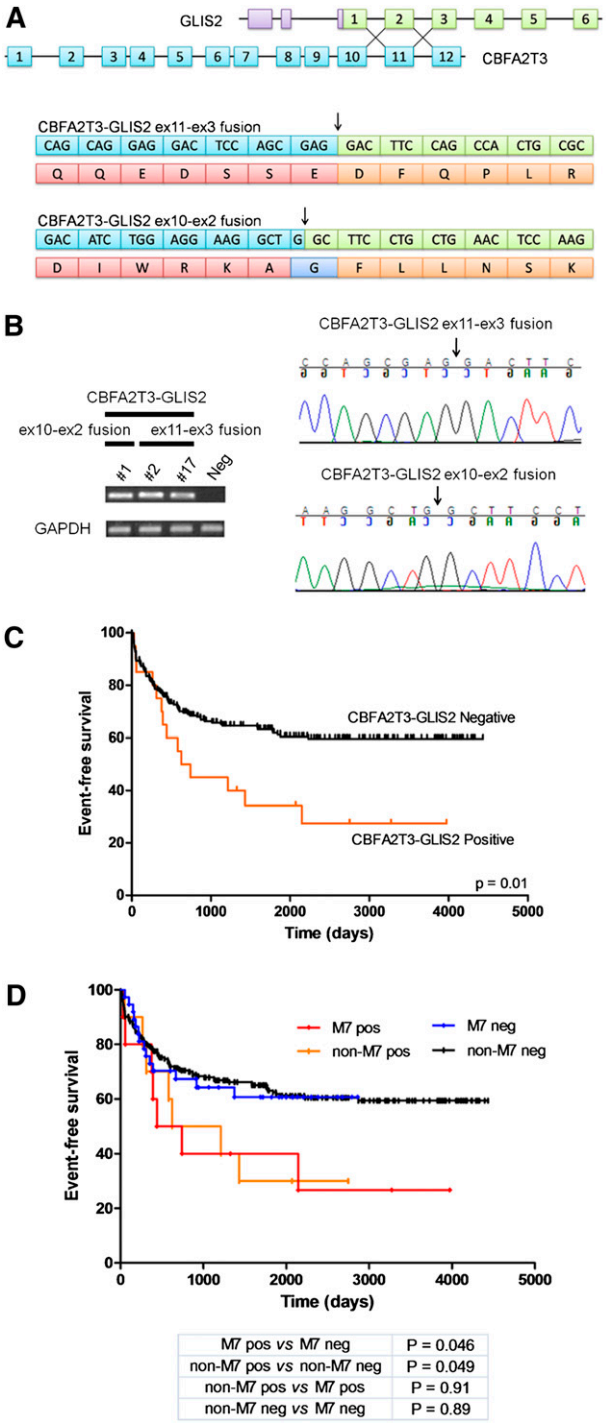
Total RNA was extracted from BM leukemia cells of all samples using TRIzol. *CBFA2T3-GLIS2* fusion transcript was detected with reverse-transcription polymerase chain reaction (RT-PCR) and sequenced with the BigDye terminator v3.1 Cycle Sequencing kit (PE Applied Biosystems, Foster City, CA) on an Applied Biosystems 310 analyzer. The following primers were used: forward 5′-CGAAGGGCCTCAGC TAGACGT-3′, reverse 5′-AGCCACTGCGCTATTTGGAT-3′.

Results and discussion

Identification of *CBFA2T3-GLIS2* fusion transcript in children with CN-AML by whole-transcriptome sequencing

Whole-transcriptome massively parallel sequencing in the 7 children with CN-AML yielded an average of 88.3 million mapped reads per patient, thus reaching an average coverage of 36×. Single nucleotide variant calling confirmed the absence of mutations in genes such as *NPM1*, *CEBPA*, *FLT3*, *MLL*, *IDH1*, *IDH2*, and *DNMT3A*.

Two algorithms, ChimeraScan[17] and deFuse,[18] were used to identify new putative fusion transcripts. In 3 of 7 patients (1, 2, 17; Table 1) we identified a chimeric transcript involving *CBFA2T3* and *GLIS2*, resulting from a cryptic inversion of the telomeric region of chromosome 16 that fuses the 5′ portion of *CBFA2T3* in frame with the 3′ region of *GLIS2*. *CBFA2T3*, also known as *MTG16* or *ETO2*, is a member of the myeloid translocation gene family that is fused to *AML1* in the *t*(16;21) (q24;q22) translocation that is identified in therapy-related AML.[19,20] *GLIS2* is a transcription factor of the GLI-similar Krüppel-like zinc finger protein family that acts as an inhibitor of the hedgehog signaling pathway.[21] This fusion was the only recurrent one, and its identification was supported by an average of 42 span and 153 split reads. Two patients showed the fusion between exon 11 of *CBFA2T3* and exon 3 of *GLIS2*, while the third showed *CBFA2T3* exon 10 fused to exon 2 of *GLIS2* (Figure 1A). These data were also confirmed by RT-PCR analysis and Sanger sequencing (Figure 1B). Interestingly, we identified 1 novel breakpoint for the *CBFA2T3-GLIS2* fusion transcript (*CBFA2T3*-ex10/ex2-*GLIS2*), which is different from breakpoints already described in the 2 recent



M7 pos *vs* M7 neg	P = 0.046
non-M7 pos *vs* non-M7 neg	P = 0.049
non-M7 pos *vs* M7 pos	P = 0.91
non-M7 neg *vs* M7 neg	P = 0.89

Figure 1. *CBFA2T3-GLIS2* fusion transcript is a novel common feature of pediatric CN-AML, predicting poorer outcome. (A) Schematic representation of the fusion between *CBFA2T3* and *GLIS2* and predicted sequence of the fusion proteins found in CN-AML. The exon-intron gene structures are indicated. The purple blocks represent untranslated exons. Black arrows indicate the fusion breakpoint. (B) RT-PCR analysis and Sanger sequencing performed in order to validate the detection of the *CBFA2T3-GLIS2* fusion. Detection of the GAPDH transcript was used as an RNA quality control. A library negative for the *CBFA2T3-GLIS2* fusion transcript was used as negative control (Neg). Black arrows indicate the fusion breakpoint. (C) Probability of 5-year EFS in children with *CBFA2T3-GLIS2* fusion transcript in CN-AML. EFS of *CBFA2T3-GLIS2*–positive patients (27.4%, SE 10.5) vs *CBFA2T3-GLIS2*–negative patients (59.6%, SE 3.6; $P = .01$). (D) Probability of 5-year EFS in pediatric CN-AML with or without *CBFA2T3-GLIS2* fusion transcript stratified according to FAB subgroups (M7 vs non-M7): EFS of non-M7 CN-AML without *CBFA2T3-GLIS2* = 59.4%, SE 3.5 vs EFS of non-M7 CN-AML with *CBFA2T3-GLIS2* = 30.0%, SE 14.4 ($P = .04$). EFS of FAB-M7 CN-AML without *CBFA2T3-GLIS2* = 60.7%, SE 8.3 vs EFS of FAB-M7 CN-AML with *CBFA2T3-GLIS2* = 26.6%, SE 15.0 ($P = .04$).

reports on non-DS AMKL.[12,13] Taken together, these data suggest that the cryptic inv(16) generates a *CBFA2T3-GLIS2* fusion gene that can be truncated at different positions.

CBFA2T3-GLIS2 fusion transcript is recurrent in pediatric CN-AML

To assess the prevalence of *CBFA2T3-GLIS2* fusion, we then examined a validation cohort of 230 children with newly diagnosed de novo CN-AML, also negative for known recurrent genetic abnormalities involving *MLL*, *CBFB*, *NPM1*, and *FLT3* genes. Globally, the *CBFA2T3-GLIS2* rearrangement was detected in 20 of 237 cases (8.4%) with CN-AML. RT-PCR analysis and Sanger sequencing confirmed that all positive cases in the validation cohort carried the *CBFA2T3* exon 11-*GLIS2* exon 3 fusion. Fifty percent (N = 10) of the positive patients belonged to the M7 FAB subgroup, while the remaining patients (N = 10) were distributed among the other FAB classes (see Table 1). These results indicate that the *CBFA2T3-GLIS2* fusion transcript, recently described as a distinctive feature of pediatric non-DS AMKL,[12,13] should be more broadly considered as a genetic abnormality that is shared with other FAB subgroups of pediatric CN-AML.

CBFA2T3-GLIS2 fusion transcript identifies a subset of childhood CN-AML with poor outcome

We evaluated whether the presence of *CBFA2T3-GLIS2* fusion product influences patients outcome. The 5-year event-free survival (EFS) of the 20 patients with the *CBFA2T3-GLIS2* fusion gene was significantly worse than that of the 217 pediatric CN-AML patients not harboring the translocation (27.4%, standard error [SE] 10.5 vs 59.6%, SE 3.6; $P = .01$; Figure 1C). We also stratified the patients with respect to FAB subgroups (M7 vs non-M7; Figure 1D). The 5-year EFS of FAB M7 patients with or without the *CBFA2T3-GLIS2* fusion gene was significantly different: 26.6% (SE 15.0) and 60.7% (SE 8.3; $P = .04$), respectively. Similar results were obtained in non-M7 patients; the 5-year EFS of patients assigned to other FAB categories with or without the *CBFA2T3-GLIS2* fusion transcript was 30.0% (SE 14.4) and 59.4% (SE 3.5; $P = .04$), respectively. No statistically significant difference in EFS of non-M7 and M7 patients harboring the *CBFA2T3-GLIS2* fusion transcript (30.0%, SE 14.4 vs 26.6%, SE 15.0; $P = .91$) was found, suggesting that FAB classification does not interact with the*CBFA2T3-GLIS2* fusion product in influencing outcome. Taken together, these data indicate that the *CBFA2T3-GLIS2* fusion transcript is a novel common feature in pediatric CN-AML that is not restricted to the FAB M7 subtype, predicting poor outcome.

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Authorship

Contribution: R.M. and M.P. equally coordinated the work, analyzed data, performed statistical analyses, and wrote the paper; M.T. and A.A. performed the whole-transcriptome massively

parallel sequencing; V.I. and R.C. performed bioinformatics analyses; E.M. performed mutation analyses on the validation cohort; and A.P., G.B., and F.L. designed and supervised the research. A.P. and F.L. equally contributed to the critical revision and writing of the manuscript. All authors read and approved the final version of the manuscript.

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Correspondence: Andrea Pession, Paediatric Oncology and Haematology Unit, Lalla Seràgnoli, Sant'Orsola-Malpighi Hospital, Via Massarenti 11, 40137 Bologna, Italy; e-mail: andrea.pession@unibo.it.

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