CBFA2T3-GLIS2-positive acute myeloid leukaemia. A peculiar paediatric entity

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Summary

The scenario of paediatric acute myeloid leukaemia (AML), particularly non-Down syndrome acute megakaryoblastic leukaemia (non-DS-AMKL), has been recently revolutionized by the advent of large-scale, genomic sequencing technologies. In this changing landscape, a significantly relevant discovery has been represented by the identification of the CBFA2T3-GLIS2 fusion gene, which is the result of a cryptic inversion of chromosome 16. It is the most frequent chimeric oncogene identified to date in non-DS-AMKL, although it seems not to be exclusively restricted to the French-American-British M7 subgroup. The CBFA2T3-GLIS2 fusion gene characterizes a subtype of leukaemia that is specific to paediatrics, having never been identified in adults. It characterizes an extremely aggressive leukaemia, as the presence of this fusion is associated with a grim outcome in almost all of the case series reported, with overall survival rates ranging between 15% and 30%. Although the molecular basis that underlies this leukaemia subtype is still far from being completely elucidated, unique functional properties induced by CBFA2T3-GLIS2 in the leukaemogenesis driving process have been recently identified. We here review the peculiarities of CBFA2T3-GLIS2-positive AML, describing its intriguing clinical and biological behaviour and providing some challenging targeting opportunities.

Keywords: childhood leukaemia, acute myeloid leukaemia, acute megakaryoblastic leukaemia, leukaemia diagnosis, CBFA2T3-GLIS2.

Childhood acute myeloid leukaemia (AML) is characterized by a relevant cytogenetic and molecular heterogeneity. The past few decades have seen significant improvements in its biological characterization and subsequent risk assessment, with an increasingly molecularly-refined definition of specific subgroups (Pui et al, 2011; Zwaan et al, 2015). The advent of large-scale genomic sequencing technologies has greatly improved the molecular classification of AML and enabled the identification of a subset of paediatric AML associated with a dismal outcome even when high-intensity therapies, including allogeneic haematopoietic stem cell transplantation (allo-HSCT), are employed. This is the case for AML carrying the cryptic inversion of chromosome 16 leading to the CBFA2T3-GLIS2 fusion gene, recently reported in exclusively paediatric settings and unanimously recognized as a type of leukaemia associated with a grim prognosis (Gruber et al, 2012; Thiollier et al, 2012; Masetti et al, 2013a; De Rooij et al, 2017). The identification of this fusion gene is due to the tremendous effort made in the characterization through RNA and exome sequencing of non-Down syndrome acute megakaryoblastic leukaemia (non-DS-AMKL) (Gruber et al, 2012; Thiollier et al, 2012; De Rooij et al, 2013, 2017). Indeed, RNA and exome sequencing allowed the description of recurrent and mutually-exclusive chimeric gene fusions associated with paediatric AMKL that are found in around 60% of cases, including RBM15-MRTFA (MKL1), CBFA2T3-GLIS2, NUP98-KDM5A and KMT2A (MLL) rearrangements (Fig 1) (Gruber & Downing, 2015; De Rooij et al, 2016, 2017). CBFA2T3-GLIS2 is the most frequently identified chimeric oncogene to date in this subset of patients (Gruber & Downing, 2015). Due to the cryptic nature of the CBFA2T3-GLIS2 fusion, this lesion not identifiable by morphology and cytogenetics (Masetti et al, 2013a). The molecular bases for transformation by CBFA2T3-GLIS2 are still unclear; patients carrying this molecular lesion present with a low mutational burden compared with other AML patients (Gruber et al, 2012), thus suggesting that this fusion gene probably represents the founder and sole genomic alteration.

Although CBFA2T3-GLIS2 transcript is present in almost 20% of paediatric non-DS-AMKL, it seems not to be exclusively restricted to the French-American-British (FAB) M7 subgroup (Masetti et al, 2013a). However, the peculiarity of leukaemia carrying this chimeric oncogene goes beyond the considerable incidence in specific subgroups of children with
AML, or the association with a poor prognosis. This lesion seems to distinguish a specific biological and clinical subtype of leukaemia, with intriguing mechanisms driving leukaemogenesis (Thiollier et al, 2012; Thirant et al, 2017a), singular morphological and immunophenotype features, and clinical behaviour.

We here review recent evidence that has emerged over the last few years regarding CBFA2T3-GLIS2-positive AML, providing a comprehensive and detailed clinical and biological picture of this entity.

Incidence

The CBFA2T3-GLIS2 fusion gene was initially reported to occur only in the paediatric non-DS-AMKL subgroup (Gruber et al, 2012; Thiollier et al, 2012). As already mentioned, this is the most frequent chimeric oncogene identified in this subset of patients. In a cohort including 22 non-DS paediatric AMKL, 9 DS AMKL and 8 adult AMKL cases, Thiollier et al (2012) first identified the presence of the fusion gene in 31% (N = 7) of non-DS paediatric AMKL patients, a fusion never detected in DS-AMKL and adult AMKL patients. In an almost concomitant report, Gruber et al (2012) performed transcriptome sequencing on leukaemia cells collected at diagnosis from a discovery cohort of 14 paediatric non-DS-AMKL cases and a recurrence/validation cohort of 34 paediatric and 28 adult non-DS-AMKL cases. CBFA2T3-GLIS2 fusion was found in 13 of 48 paediatric samples (27%), confirming that this lesion is restricted to the paediatric population. Our group identified the CBFA2T3-GLIS2 fusion gene in 20 of 237 patients with cytogenetically normal (CN) AML (8.4%). Among the positive cases, only a half belonged to FAB M7 subgroup, the remaining cases were associated with other morphological FAB subgroups, such as M0, M1, M2, M4 and M5 AML (Masetti et al, 2013a). In the two largest cohorts of paediatric AMKL screened by deep-sequencing approaches and collected thanks to international collaborative efforts, the presence of the CBFA2T3-GLIS2 fusion gene was detected in 16% and 18.6% of cases (De Rooij et al, 2016, 2017). Recently, a higher percentage (27%) of transcript-positive patients was reported in paediatric non-DS-AMKL enrolled in two Japanese clinical trials, whilst it was not identified in any of the other paediatric AML cases studied (Hara et al, 2017).

Molecular structure of CBFA2T3-GLIS2 fusion gene

The chimeric transcript involving CBFA2T3 and GLIS2 results 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. The most common chimeric CBFA2T3-GLIS2 transcript lies between exon 11 of CBFA2T3 and exon 3 of GLIS2 (Gruber et al, 2012; Thiollier et al, 2012; Masetti et al, 2013a) (Fig 2). Other rare chimeric transcripts have been reported: CBFA2T3-ex10/GLIS2-ex3 (Gruber et al, 2012), CBFA2T3-ex12/GLIS2-ex1 (Gruber et al, 2012) and CBFA2T3-ex10/GLIS2-ex2 (Masetti et al, 2013a).

CBFA2T3 is a member of the RUNX1T1 (previously termed ETO/CBFA2T1/MTG8) complex and was initially
identified as a fusion partner of RUNX1 in rare cases of therapy-related AML harbouring t(16;21)(q24;q22) (Gamou et al., 1998). Later, it was implicated in the maintenance of haematopoietic stem cell quiescence (Chyla et al., 2008). In particular, CBFA2T3 participates in high-molecular-weight complexes and its expression is essential for haematopoietic stem cell self-renewal and differentiation. Furthermore, it plays a critical role in the development of megakaryocyte-erythrocyte progenitors in-vivo (Fischer et al., 2012; Leung et al., 2013). The genomic structure and amino acid sequence of CBFA2T3 are highly similar to that of the other two CBFA2T-family members (RUNX1T1, previously termed CBFA2T1, and CBFA2T2). In particular, CBFA2T2 is known to bind to the RUNX1/RUNX1T1 complex and its relevance in leukaemogenesis is reported in both paediatric and adult AML (Richkind et al., 2000; Guastadisegni et al., 2010; Bolouri et al., 2018).

GLIS2 (GLI-similar 2) is a member of the Krüppel-like zinc finger transcription factor group, which is closely related to the GLI family of proteins mediating the transcriptional response to the Hedgehog pathway activation (Lamar et al., 2001; Kim et al., 2007). GLIS2 is highly expressed in the adult kidney, where it suppresses the GLI1-activated transcriptome and maintains homeostasis. GLIS2 inactivating mutations have been found in nephronophthisis, an autosomal recessive renal disease (Attanasio et al., 2007). Before 2012, GLIS2 had never been previously implicated in leukaemogenesis. Its pivotal role for haematopoietic stem cell repopulation in mice has been recently suggested (Holmfeldt et al., 2016). However, it is not expressed in differentiating haematopoietic cells, suggesting that its fusion with CBFA2T3 leads to ectopic GLIS2 activity (Thirant et al., 2017b).

Because of the fusion, CBFA2T3 loses the MYND (myeloid, nervy and DEAF-1 domain) class of zinc finger domain reported to interact with the nuclear receptor co-repressor (NCoR) repressor complex. In contrast, the GLIS2 gene maintains the zinc finger domain and, consequently, the ability to interact with DNA (Thiollier et al., 2012) (Fig 2).

**Gene expression profile**

Patients with CBFA2T3-GLIS2 positive AML present a clearly peculiar expression signature clustering them apart from other non-DS-AMKL, including up-regulation of genes involved in the Hedgehog, JAK-STAT and wingless/integrated (WNT)/ß-catenin pathways. In particular, analysis of the gene expression signatures of CBFA2T3-GLIS2 expressing AMKLs revealed altered expression of a number of genes in the sonic hedgehog (SHH) and WNT pathways, such as GATA3, IGFBP7, CCND2, PTCH1 and HHIP, as well the bone morphogenic protein (BMP) pathway BMP2 and BMP4, which is directly influenced by SHH signalling (Gruber et al., 2012). In an elegant series of experiments, Thirant et al (2017a) recently elucidated the transcriptional programme and unique functional properties induced by CBFA2T3-GLIS2 in the leukaemogenesis-driving process. To define the effect of CBFA2T3-GLIS2 and the contribution of CBFA2T3 and GLIS2 moieties to haematopoietic differentiation and self-renewal, CBFA2T3-GLIS2+, CBFA2T3- or GLIS2-encoding retroviruses have been transduced into murine bone marrow progenitors and in-vitro cultures were performed. The expression of GLIS2 or CBFA2T3-GLIS2 induced megakaryocytic differentiation in primary haematopoietic progenitor cells, but only CBFA2T3-GLIS2 resulted in increased self-renewal capacity. Furthermore, the ectopic expression of CBFA2T3, GLIS2 or CBFA2T3-GLIS2 in the HEL cell line identified 3,798 differentially expressed genes significantly correlated with a published CBFA2T3-GLIS2

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**Fig 2.** Representation of CBFA2T3-GLIS2 fusion protein. Top panels: Common breakpoints in CBFA2T3 and GLIS2 genes at exons 11 and 3, respectively. Bottom panel: CBFA2T3-Ex11/GLIS2-Ex3 fusion protein with the retained domains. MYND, myeloid, nervy, and DEAF-1; NHR, nervy homology regions; TAD, topologically associating domain; TRD, trans-Repression Domain; ZF, zinc finger.
patient signature and contained most of the genes regulated by CBFA2T3 \((n = 1125)\) or GLIS2 \((n = 1173)\), while 2405 genes were specifically deregulated by the CBFA2T3-GLIS2 fusion. Interestingly, several transcription factors known to interact with CBFA2T3 were down-regulated by the fusion transcript, including GATA1 \((-9\text{-fold})\), while the megakaryocytic oncogene ERG was up-regulated upon CBFA2T3-GLIS2 and GLIS2 expression \((+9.44\text{- and }+2.88\text{-fold, respectively})\).

Chromatin immunoprecipitation (ChIP) sequencing demonstrated that the fusion transcript binds CBFA2T3 known sites. Moreover, ChIP experiments confirmed that the fusion transcript interacts with motifs of factors known to complex with CBFA2T3, including those for RUNX1, ERG and GATA1. Interestingly, de novo binding sites tended to be located in super-enhancer regions and to be co-occupied by ERG and these sites are associated with strong up-regulation. As a result, the CBFA2T3-GLIS2 gene blocks in a unique hit the differentiation of megakaryocytic cells, inhibiting the expression of GATA1, and increases self-renewal capacity by inducing the expression of ERG (Thirant et al, 2017a) (Fig 3). Based on this evidence, targeting integrity of the CBFA2T3-GLIS2 complex may represent an appealing opportunity for developing targeted therapeutic strategies (Thirant et al, 2017b).

**Mutation pattern in CBFA2T3-GLIS2 positive leukaemia**

Despite the poor prognosis and low response to conventional chemotherapy, transplantation of fusion gene-modified bone marrow cells into animal syngeneic recipients failed to induce overt leukaemia transformation, consistent with a requirement for cooperative mutations (Gruber et al, 2012; Dang et al, 2017).

However, the total burden of somatic mutations associated with CBFA2T3-GLIS2 is significantly lower than that found in other subgroups of AMKL \((7.17 \pm 3.60 \text{ vs. } 16.60 \pm 5.13, P = 0.009)\) (Gruber et al, 2012; De Rooij et al, 2017). Indeed, genetic mutations involving GATA1, KIT and FLT3 genes are less frequent in CBFA2T3-GLIS2-positive than in negative patients, being detected only in few cases. In the study reported by Hara et al (2017), genetic mutations were found in 14% of AMKL CBFA2T3-GLIS2-positive patients as compared to 35% of negative patients. The genes commonly involved are FLT3, GATA1 and KIT (Hara et al, 2017) as well as RAS mutations and mutations in the JAK/STAT pathway (Table I) (Gruber et al, 2012; De Rooij et al, 2017). So far, an impact of the mutation burden on the outcome of CBFA2T3-GLIS2 positive AML has not been demonstrated.

**Cytogenetic pattern of abnormalities in CBFA2T3-GLIS2 positive leukaemia**

Concomitant cytogenetic abnormalities are rare in patients expressing CBFA2T3-GLIS2. In the Japanese cohort, 33% of fusion-positive patients had a normal karyotype (i.e. CN-AML) as compared to 9% in fusion-negative patients \((P = 0.075)\) (Hara et al, 2017). When not associated with normal karyotype, CBFA2T3-GLIS2 was associated with trisomy 21 (Gruber et al, 2012; De Rooij et al, 2017; Hara et al, 2017).
Recent studies compared the clinical features of patients with CBFA2T3-GLIS2 fusion gene, involving Desert Hedgehog (DHH), a member of Hedgehog family, and Ras Homologue Enrich in Brain Like 1 (RHEBL1), a gene coding for a small GTPase of the Ras family (Masetti et al, 2013b). We detected DHH-RHEBL1 fusion in 8 out of 20 (40%) CBFA2T3-GLIS2-rearranged patients. Gene expression analysis performed on RNA-seq data revealed that DHH-RHEBL1-positive patients exhibited a specific signature (Masetti et al, 2013b).

With regard to recurrent molecular abnormalities of non-DS-AMKL, the CBFA2T3-GLIS2 fusion gene is mutually exclusive with other cryptic chimeric fusion genes recurrently detected in this AML FAB variant, namely RBM15-MRTFA and NUP98-KDMSA (De Rooij et al, 2017).

**Clinical characteristics of patients carrying the CBFA2T3-GLIS2 fusion gene**

Recent studies compared the clinical features of patients with and without CBFA2T3-GLIS2, either belonging or not to the AMKL subgroup, to establish if the fusion gene confers a distinct clinical profile to these patients (Masetti et al, 2013a; De Rooij et al, 2016; Hara et al, 2017). No significant differences in gender were found between the two groups (De Rooij et al, 2016). Regarding age, as already mentioned, the CBFA2T3-GLIS2 fusion has never been found in adult AML patients (Gruber et al, 2012; Thiollier et al, 2012; De Rooij et al, 2017) and, in childhood, fusion-positive patients have been found to be significantly younger than fusion-negative patients (Masetti et al, 2013a; De Rooij et al, 2016; Hara et al, 2017). In particular, most patients with CBFA2T3-GLIS2 were younger than 5 years of age (Gruber et al, 2012; Masetti et al, 2014).

In the AMKL study cohort reported by Hara et al (2017), patients were divided into early-onset (N = 41, 0–4 years) and late-onset (N = 3, 12–13 years), and fusion genes, namely CBFA2T3-GLIS2, NUP98-KDMSA, RBM15-MRTFA, KMT2A-MLLT3 and KMT2A-MLLT10, were only present in early-onset patients. Comparing CBFA2T3-GLIS2-positive (N = 12) and -negative (N = 32) patients, all positive patients were early-onset, i.e. aged 0–4 years, whereas negative patients reflected the bimodal distribution that characterizes the age of AMKL patients. Additionally, 7 out of 12 early-onset CBFA2T3-GLIS2 fusion-positive patients were infants (<1 year) (58%), confirming data previously reported by our group (Masetti et al, 2013a, 2014).

In the study cohort reported by De Rooij et al (2016), median age at diagnosis was 1.5 years, similar to the other paediatric AMKL cases, but all of the CBFA2T3-GLIS2-positive patients were aged <4 years, whereas other recurrent mutations were associated with a wider age-range at diagnosis. CBFA2T3-GLIS2 is more common in younger patients not only in non-DS-AMKL, but also in paediatric de novo non-AMKL CN-AML (Masetti et al, 2013a).

No significant differences were found between fusion-positive and fusion-negative AMKL paediatric patients in terms of leucocyte count at diagnosis, but, in AMKL, CBFA2T3-GLIS2-positive patients tended to have a higher percentage of bone marrow blasts at diagnosis compared to fusion-negative patients (De Rooij et al, 2016; Hara et al, 2017).

Extramacular involvement is more frequent in patients expressing the CBFA2T3-GLIS2 chimeric gene (25%) compared to the frequency reported for paediatric AML in general (Masetti et al, 2013a; Pession et al, 2013). In some of these cases, extramedullary involvement can be initially confused with a non-haematopoietic tumour, especially in the presence of cranial bone, ribs and lumbosacral column involvement. To better characterize the molecular and clinical features of AMKL patients, Thiollier et al (2012) developed a xenotransplantation model in which human AMKL cells were injected into immunodeficient mice. Mice injected with CBFA2T3-GLIS2-positive cells (AMKL7) presented spleen nodular infiltration, a finding not observed in animals transplanted with AMKL blasts not carrying this fusion gene. Recipients of CBFA2T3-GLIS2-positive cells also more frequently showed macroscopic focal spinal cord infiltration of megakaryoblastic cells and cerebral leptomeningeal infiltrates. In one case, primary, secondary and tertiary recipients of

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**Table I. Frequency of cooperating mutations in CBFA2T3-GLIS2 positive leukaemia in comparison with other subgroups of paediatric AML.**

| Aberration | CBFA2T3-GLIS2 positive AML | CBFA2T3-GLIS2 negative AML | References |
|------------|---------------------------|---------------------------|-----------|
| FLT3      | 17%                       | 7–10%                     | Hara et al (2017); Meshinchi et al (2006); Bolouri et al (2018); Manara et al (2017) |
| JAK/STAT  | 13–23%                    | 15%*                      | Gruber et al (2012); De Rooij et al (2017) |
| RAS       | 6%                        | 17–19%                    | De Rooij et al (2017); Goemans et al (2005); Bolouri et al (2018) |
| GATA1     | 17%                       | 7–9%*                     | Hara et al (2017); Gruber et al (2015); De Rooij et al (2017) |
| KIT       | 8%                        | 44%‡ of CBF-AML           | Hara et al (2017); Manara et al (2014) |

AML, acute myeloid leukaemia; CBF, core-binding factor; non-DS-AMKL, non-Down syndrome acute megakaryoblastic leukaemia.

*The percent is restricted to non-DS-AMKL.

‡The percent is restricted to paediatric CBF-AML (inv(16), t(16;16), t(8;21)).
AMKL7 cells showed hind leg paralysis and abnormal magnetic resonance imaging signals suggestive of leukaemia spinal cord infiltration (Thiollier et al., 2012). In our case series of fusion-positive patients, we also found central nervous system involvement more likely than in fusion-negative patients, but this does not seem to affect the prognosis (Masetti et al., 2013a; Creutzig et al., 2017).

**Morphological characteristics of patients with the CBFA2T3-GLIS2 fusion gene**

Bone marrow smears of CBFA2T3-GLIS2-positive patients show morphological features of the different FAB subtypes. No specific morphological aspects associated with the fusion gene have been documented. In AMKL CBFA2T3-GLIS2-positive patients, megakaryoblasts are the predominant component of the blast population in bone marrow and/or peripheral blood. These are markedly pleomorphic, ranging from small, round cells with scanty cytoplasm and inconspicuous nucleoli, resembling haematogones, to large cells with abundant cytoplasm and prominent nucleoli. They often display cytoplasmic blebs or pseudopods and may appear in clusters mimicking metastatic tumours.

In an Italian paediatric cohort, 50% (N = 10) of the fusion-positive patients had a non-FAB M7 subtype, distributed as follows: M5 15%, M0 15%, M1 10%, M2 5%, M4 5% (Masetti et al., 2013a). No morphological differences were found compared to the AML CBFA2T3-GLIS2-negative FAB counterpart (Masetti et al., 2013a).

**Immunophenotypic characteristics of patients with the CBFA2T3-GLIS2 fusion gene**

The CBFA2T3-GLIS2 fusion oncogene is associated with a distinct immunophenotype characterized by overexpression of CD56 (NCAM1) and under-expression of both HLA-DR and CD38, similar to the one described as RAM phenotype (Eidenschink Brodersen et al., 2016). It has been demonstrated, through immunophenotypic analysis combined with morphological, genetic and clinical features, that the RAM phenotype (bright CD56 expression at minimum 2 log10 units greater than normal myeloid progenitors, dim-to-negative expression of CD45 and CD38, and lack of HLA-DR) is a unique entity, distinct from CD56+ non-RAM and CD56-negative leukaemias. This phenotype has been associated with younger age, high rate of induction failure and poor outcome (Eidenschink Brodersen et al., 2016). Interestingly, the RAM phenotype is more prevalent in FAB M7 patients (Eidenschink Brodersen et al., 2016).

These results suggested that, although CBFA2T3-GLIS2-positive patients are not identifiable by morphology or cytogenetics, a large portion of them can be detected by flow-cytometry thanks to the combination of CD56/HLA-DR/CD38, CD56/HLA-DR and CD56/CD38 (Eidenschink Brodersen et al., 2016).

Analysing the distinct gene expression profile of CBFA2T3-GLIS2 fusion-positive samples, Thiollier et al. (2012) observed that the surface marker CD56 presented a mean differential expression of 35-fold by microarray and >200-fold by RNA-seq. Flow-cytometry analysis revealed that CBFA2T3-GLIS2-positive AMKL blasts were CD41+CD56+ and that CD56 was significantly more expressed than on NUP98-KDM5A-expressing AMKL leukaemia cells (Thiollier et al., 2012).

Using ChIP, our group showed a significant enrichment of CBFA2T3-GLIS2 fusion protein on the NCAM1 (CD56) promoter, confirming that CBFA2T3-GLIS2 directly regulates the expression of CD56 (Masetti et al., 2017).

Future prospective studies are warranted to definitively validate the role of flow-cytometry for identifying children with CBFA2T3-GLIS2–positive AML; in particular, these investigations should have the goal to assess whether the combination of bright expression of CD56 with low to negative expression of HLA-DR and/or CD38 enables a faster and reliable identification of CBFA2T3-GLIS2-positive patients.

**Clinical outcome of patients with the CBFA2T3-GLIS2 fusion gene**

In almost all of the case series reported so far, the presence of the CBFA2T3-GLIS2 fusion transcript is associated with a worse outcome as compared to fusion-negative AML paediatric patients (Gruber et al., 2012; Masetti et al., 2013a; De Rooij et al., 2017; Hara et al., 2017).

Historically, non-DS-AMKL has a poorer outcome when compared to other AML subtypes, with survival rates ranging between 15% and 50% (Creutzig et al., 2005; Inaba et al., 2015; Schweitzer et al., 2015), but the prognostic impact of specific cytogenetically (Inaba et al., 2015) and molecularly defined subgroups in AMKL has been better clarified only in the last few years (De Rooij et al., 2017). Recent studies reporting the clinical outcome of non-DS-AMKL children according to the different specific recurrent genetic abnormalities showed that CBFA2T3-GLIS2 has the strongest negative impact on patient outcome (De Rooij et al., 2016, 2017), with the 5-year probability of overall survival (OS) ranging between 15 and 30% (De Rooij et al., 2016, 2017). This is mainly due to the high frequency of non-response to induction therapy (primary induction failure, PIF) and cumulative incidence of relapse (CIR) (De Rooij et al., 2016). The presence of CBFA2T3-GLIS2 was shown to confer a cumulative incidence of either relapse or non-response of 86% and was the highest among non-DS-AMKL when compared with other genetic subgroups (De Rooij et al., 2017). In our cohort of Italian children, the evaluation of response to initial therapy by multidimensional flow-cytometry showed that majority of fusion-positive patients continued to have detectable disease (i.e. minimal residual disease) at the end of induction treatment (R. Masetti, unpublished data), which resulted in a CIR of around 50% (Masetti et al., 2013a). In the same cohort of children, the time elapsing between diagnosis and
This research paper analysed only normal karyotype patients.

Louis Hospital.

applicable; non-DS-AMKL; non-Down syndrome acute megakaryoblastic leukaemia; SJRH, St. Jude Children’s Research Hospital; SLH, Saint

AIEOP, Italian Association of Paediatric Haematology and Oncology (Associazione Italiana Ematologia Oncologia Pediatrica); COG, Children’s Oncology Group; DCOG, Dutch Childhood Oncology Group; I-BFM, International Berlin-Frankfurt-Munster Study Group, Dutch Childhood Oncology Group, Saint Louis Hospital) and the Children’s Oncology Group in the US (De Rooij et al, 2016) evaluated the outcome of non-DS-AMKL associated with different recurrent genetic lesions; patients expressing $CBFA2T3-GLIS2$ showed a 4-year probability of OS and event-free survival (EFS) of 38 ± 10% and 33 ± 10%, respectively, with a CIR of 42 ± 10%. The small difference between OS and EFS probability of children with $CBFA2T3-GLIS2$ fusion transcript indicates that, once relapsed, these patients have a dismal chance of rescue. This outcome was not significantly different from that of NUP98-KDM5A-positive patients (36 ± 13%, 36 ± 13% and 36 ± 14% respectively), and from that of patients harbouring $KMT2A$-rearrangements (33 ± 13%, 34 ± 13% and 51 ± 15% respectively). All of these three subgroups showed a significantly poorer outcome than that of patients expressing RBM15-MRTFA and of patients without translocations (their 4-year probability of OS, EFS and CIR being 70 ± 5% $P = 0.0013$, 62 ± 5% $P \leq 0.0001$ and 19 ± 4% $P = 0.003$, respectively) (Table II).

In a previously published study evaluating 40 non-DS-AMKL children treated at multiple centres with different treatment approaches, Gruber et al (2012) reported a significantly worse 5-year OS of $CBFA2T3-GLIS2$-positive patients as compared to patients with AMKL lacking this chimeric transcript (28.1% vs. 41.9%; $P = 0.05$). Similar results were obtained when the analysis was restricted to 19 non-DS-AMKL patients from St. Jude Children’s Research Hospital, with a 5-year OS of 34.3% and 88.9% for $CBFA2T3-GLIS2$-positive and -negative patients, respectively ($P = 0.03$) (Gruber et al, 2012).

The most recent Japanese AML99 and AML-05 experience showed a significantly lower 4-year EFS and a worse CIR for fusion-positive than fusion-negative patients (EFS: 16% vs. 35% and CIR: 7% vs. 41%; respectively) (Hara et al., 2017). Interestingly, six (86%) out of seven $CBFA2T3-GLIS2$-positive infants relapsed and all the $CBFA2T3-GLIS2$-positive patients with PIF were infants, suggesting that fusion-positive infants may have an even worse prognosis than older fusion-positive paediatric patients (Hara et al., 2017). The molecularly defined subgroup of patients carrying the $DHH-RHEBL1$ fusion transcript among the $CBFA2T3-GLIS2$-positive patients (Masetti et al, 2013b) seems to be associated with a particularly dismal outcome, although this finding has to be confirmed in a larger cohort of patients. The published case series of $CBFA2T3-GLIS2$-positive patients are not sufficiently large enough to clarify if

### Table II. Prognostic and patients feature of non-DS-AMKL with $CBFA2T3-GLIS2$, NUP98-KDM5A, and $KMT2A$ rearrangements.

| Reference | Group/treatment protocol | Variable | Frequency (%) | Age at diagnosis, median (range) | OS | EFS |
|-----------|--------------------------|----------|---------------|-------------------------------|-----|-----|
| Gruber et al (2012) | SJRH | $CBFA2T3-GLIS2$ | 13/48 (27%) | 1-5 (0-6-4-7) | 5 years 28% | N/A |
|  |  | NUP98-KDM5A | 4/48 (8.3%) | N/A | N/A | N/A |
|  |  | $KMT2A$ | N/A | N/A | N/A | N/A |
| De Rooij et al (2013) | DCOG | $CBFA2T3-GLIS2$ | 13/105 (12-3) | 1-4 (0-6-3-4) | 5 years 19% | 5 years 35% |
|  |  | NUP98-KDM5A | 11/105 (10-5%) | 1-8 (0-9-4-8) | 5 years 22% | 5 years 22% |
|  |  | $KMT2A$ | 13/96 (13-5%) | 1-8 (0-7-12) | 5 years 27% | 5 years 28% |
| Masetti et al (2013a)* | AIEOP 2002/01 AML trial | $CBFA2T3-GLIS2$ | 20/237 (8-4%) | 1-9 (0-5-4) | N/A | N/A |
|  |  | NUP98-KDM5A | N/A | N/A | N/A | N/A |
|  |  | $KMT2A$ | N/A | N/A | N/A | N/A |
| De Rooij et al (2016) | AIEOP | $CBFA2T3-GLIS2$ | 24/153 (16%) | 1-5 (0-5-4-0) | 4 years 38-6% | 4 years 33% |
|  |  | NUP98-KDM5A | 14/193 (9%) | 1-9 (0-8-8-5) | 4 years 36% | 4 years 36% |
|  |  | $KMT2A$ | 14/193 (9%) | 1-9 (0-7-12-0) | 4 years 33% | 4 years 34% |
| Hara et al (2017) | JCACS AML99 trial | $CBFA2T3-GLIS2$ | 12/44 (27%) | 0 (0-2) | 4 years 41-7% | 4 years 16-7% |
|  |  | JPLSG AML-05 trials | NUP98-KDM5A | 4/44 (9%) | N/A | 4 years 50% |
|  |  | $KMT2A$ | 3/44 (7%) | N/A | N/A | N/A |
| De Rooij et al (2017) | Multiple Institutions | $CBFA2T3-GLIS2$ | 16/87 (18%) | 1-3 (0-5-2-8) | 5 years 14% | 5 years 8% |
|  |  | NUP98-KDM5A | 10/87 (11-3%) | 2-6 (1-1-8-5) | 5 years 35% | 5 years 27% |
|  |  | $KMT2A$ | 15/87 (17-2%) | 2-5 (0-7-7-4) | 5 years 27% | 5 years 25% |

AIEOP, Italian Association of Paediatric Haematology and Oncology (Associazione Italiana Ematologia Oncologia Pediatrica); COG, Children’s Oncology Group; DCOG, Dutch Childhood Oncology Group; I-BFM, International Berlin-Frankfurt-Munster Study Group, Dutch Childhood Oncology Group, Saint Louis Hospital) and the Children’s Oncology Group in the US (De Rooij et al, 2016) evaluated the outcome of non-DS-AMKL associated with different recurrent genetic lesions; patients expressing $CBFA2T3-GLIS2$ showed a 4-year probability of OS and event-free survival (EFS) of 38 ± 10% and 33 ± 10%, respectively, with a CIR of 42 ± 10%. The small difference between OS and EFS probability of children with $CBFA2T3-GLIS2$ fusion transcript indicates that, once relapsed, these patients have a dismal chance of rescue. This outcome was not significantly different from that of NUP98-KDM5A-positive patients (36 ± 13%, 36 ± 13% and 36 ± 14% respectively), and from that of patients harbouring $KMT2A$-rearrangements (33 ± 13%, 34 ± 13% and 51 ± 15% respectively). All of these three subgroups showed a significantly poorer outcome than that of patients expressing RBM15-MRTFA and of patients without translocations (their 4-year probability of OS, EFS and CIR being 70 ± 5% $P = 0.0013$, 62 ± 5% $P \leq 0.0001$ and 19 ± 4% $P = 0.003$, respectively) (Table II).
there are any additional clinical or molecular risk factors predicting outcome.

The poor outcome was confirmed also in CBFA2T3-GLIS2 patients with AML (Masetti et al, 2013a). In the Italian cohort (Masetti et al, 2013a), the probability of EFS was lower for fusion-positive non-M7 patients as compared to fusion-negative non-M7 patients (30.0% vs. 59.4%, \( P = 0.04 \)), this suggesting that the grim prognosis conferred by the presence of the fusion transcript is not influenced by the FAB group (Masetti et al, 2013a).

In light of all these findings, CBFA2T3-GLIS2-positive patients are currently allocated to the high-risk group of many paediatric treatment protocols and are considered candidates to receive allog-HSCT- in first complete remission (CR1). Although there is clear evidence that allo-HSCT has a greater anti-leukaemic potential than chemotherapy as post-remission treatment, its role for children with AML in CR1 is still debated (Hasle, 2014; Zwaan et al, 2015). Despite the lack of robust data showing an undisputable advantage for CBFA2T3-GLIS2-positive patients given an allograft in CR1, it is reasonable to speculate that, after achieving remission, a consolidation approach including allo-HSCT could be the best strategy to avoid recurrence (De Rooij et al, 2017; Hara et al, 2017).

**Targeting opportunities for patients with the CBFA2T3-GLIS2 fusion gene**

Given the grim prognosis of CBFA2T3-GLIS2-positive patients, it is clear how much this leukaemia subgroup needs new, targeted therapeutic approaches. Nowadays, a deeper knowledge of the expression pathways induced by the fusion gene opens new potential therapeutic strategies (Fig 4).

As early as 2012, the efficacy of Dimethylfasudil (DiMF) was demonstrated in the treatment of AMKL, particularly AMKL with CBFA2T3-GLIS2 (Thiollier et al, 2012). In particular, DiMF, an inhibitor of Aurora kinase A (AURKA), was shown to efficiently induce differentiation and polyplidization of leukaemia blasts and drastically inhibited proliferation in-vitro (Wen et al, 2012). In addition, in-vivo treatment with AURKA inhibitor of mice xenografted with human AMKL cells bearing the CBFA2T3-GLIS2 fusion significantly reduced the disease burden and prolonged survival of the mice (Thiollier et al, 2012) (Fig 4).

Another attractive target is the protein complex recruited by the fusion described by Thirant et al (2017a). In particular, it is known that ETO proteins depend on the NHR2 domain for their ability to oligomerize and recruit co-factors and can be inhibited by the expression of a peptide called NC128, which contains the NHR2 domain (Wichmann et al, 2007). Indeed, NC128 expression in cell lines and CBFA2T3-GLIS2 blasts derived from AMKL patients decreased proliferation, reduced cell-cycle progression and increased cell death, and was associated with significant down-regulation of ERG and up-regulation of GATA1 expression in-vitro and in-vivo (Thirant et al, 2017a) (Fig 4). These data support the concept that the NHR2 domain is essential for the establishment of CBFA2T3-GLIS2 transcriptional alterations and for the in-vivo maintenance of human CBFA2T3-GLIS2-expressing AMKL cells. In recent years, our group has been working to specifically counteract the leukaemia-promoting effect of the fusion gene by inhibiting its binding to DNA through targeting of the GLIS2 zinc finger domain (Masetti et al, 2017). Indeed, the GLIS2 protein shares a highly homologous zinc finger domain with members of the GLI proteins (Vasanth et al, 2011).
GLI family proteins are the final effectors of the classic Hedgehog pathway, and some preclinical studies provided evidence of the inhibition of their activity by some molecules (Pan et al., 2012; Agyeman et al., 2014; Wellbrock et al., 2015).

The GLI inhibitor GANT61 is a small molecule that inhibits DNA binding of GLI family proteins, and it is mainly used in preclinical studies (Pan et al., 2012; Wellbrock et al., 2015). Considering the high homology of the DNA-binding domain between GLIS2 and GLI family proteins, we hypothesized that GANT61 might be used to specifically target the CBFA2T3-GLIS2 fusion in childhood AML (Masetti et al., 2017). Experimental results showed that, after exposure to GANT61, both cell lines and primary AML cells carrying CBFA2T3-GLIS2 showed a higher sensitivity to undergo apoptosis and to display G1 cell-cycle arrest than AML cells without the GLIS2 fusion (Masetti et al., 2017) (Fig 4). Moreover, GANT61 treatment induced down-regulation of some genes directly regulated by the fusion gene, including ERG, GATA3, DNMT1 and DNMT3B, suggesting the specificity of GANT61 treatment to block the DNA binding (Masetti et al., 2017). Although further studies are required to confirm these results, it is reasonable to hypothesize that inhibition of GLIS2 transcription activity could represent a valid therapeutic approach for targeting CBFA2T3-GLIS2 positive leukaemia and for improving the dismal outcome of young children carrying this peculiar molecular lesion (Fig 4).

Conclusion

CBFA2T3-GLIS2 represents one of the most important, recently identified, recurrent molecular lesions in the evolving molecular landscape of paediatric AML. Its discovery significantly contributed to re-categorize the heterogeneous scenario of paediatric non-DS AMKL. Moreover, as this fusion transcript has been found in non-M7 patients, we suggest extending the screening of this fusion to all newly diagnosed children below the age of five and not showing recurrent molecular lesions typical of paediatric AML. Given that conventional karyotyping will not reveal cryptic translocations like CBFA2T3-GLIS2, this should be included in the panel of routinely screened aberrations through a reverse transcription polymerase chain reaction approach using specific primers (Masetti et al., 2013a).

The relevance of CBFA2T3-GLIS2 for paediatric haematologists is not only related to its frequency or to the fact that this characterizes a specific leukaemia entity of childhood. In fact, given that CBFA2T3-GLIS2 positive leukaemia is an extremely aggressive disease, the detection of this lesion is of paramount importance for the proper risk-based allocation of patients in future clinical trials. Most of the patients harbouring CBFA2T3-GLIS2 present with a resistant disease and have a high propensity to relapse; thus, allocation of patients to high-risk treatment and use of HSCT in consolidation therapy are strongly recommended. The contribution to leukaemogenesis of the cryptic fusion transcript has been gradually elucidated over recent years (Thiollier et al., 2012; De Rooij et al., 2017; Thirant et al., 2017a) and it has been speculated that this aberration could represent an attractive option for targeted, more effective therapies. This approach is more than desirable, in a context such as paediatric AML, in which, despite the use of intensive chemotherapy and allogeneic HSCT, the survival rate so far is still unsatisfactory.

Author Contribution

MR performed the literature review and wrote the paper. SNB contributed to write the paper. AP and FL conceived the idea and edited the manuscript. All authors approve the submitted version of the manuscript.

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