Acute Myeloid Leukemia • Research Paper

KIT activating mutations: incidence in adult and pediatric acute myeloid leukemia, and identification of an internal tandem duplication

ALESSANDRO BEGHINI
CARLA B. RIPAMONTI
ROBERTO CAIROLI
GIOVANNI CAZZANIGA
PATRIZIA COLAPIETRO
FRANCESCA ELICE
GIANPAOLO NADALI
GIOVANNI GRILLO
OSKAR A. HAAS
ANDREA BIONDI
ENRICA MORRA
LIDIA LARIZZA

From the Department of Biology and Genetics for Medical Sciences, University of Milan, Italy (AB, CBR, PC, LL); Division of Haematology, Niguarda Hospital, Milan, Italy (RC, GG, OAH, EM); Clinica Pediatrica Universita di Milano Bicocca (GG, AB); Dipartimento di Medicina Clinica, Prevenzione e Biotecnologie Sanitarie, Ospedale San Gerardo, Monza, Italy (AB); Division of Haematology, Ospedale S. Bortolo, Vicenza, Italy (FE); Dipartimento di medicina clinica e sperimentale, sezione di ematologia, Policlinico G Rossi, Verona, Italy (GN); CCRI, Children’s Cancer Research Institute, St. Anna Kinderspital, Vienna, Austria (OAH).

Correspondence: Lidia Larizza, Department of Biology and Genetics for Medical Sciences, University of Milan, via Viotti 3/5, 20133 Milan, Italy. E-mail: lidia.larizza@unimi.it

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Background and Objectives. Mutations of KIT receptor tyrosine kinase are involved in the constitutive activation and development of human hematologic malignancies. Gain-of-function mutations in the second intracellular kinase domain (TK2) and in the juxtamembrane domain are described in patients with core binding factor acute myeloid leukemia (CBFL) and are associated with leukocytosis. We evaluated the incidence of KIT mutation in 52 adult patients with de novo CBFL and in 49 FLT3/ITD-negative childhood patients with de novo acute myeloid leukemia (AML), excluding cases of acute promyelocytic leukemia.

Results. Several kinds of gain-of-function KIT mutations were found in 24 of the 52 (46.1%) adult CBFL cases and 6 of the 49 (12.2%) non-APL childhood AML patients. KIT mutations were detected in 4 of the 8 adult patients and one childhood AML case bearing trisomy of chromosome 4 as either the sole cytogenetic aberration or a karyotypic aberration additional to t(8;21). In three of the trisomy 4 cases we demonstrated that trisomy 4 leads to duplication of the KIT mutated allele.

Interpretation and Conclusions. These results underline that the KIT gene is activated in AML characterized by distinct cytogenetic and molecular genetic patterns and represents the most frequently mutated target in adult CBFL.

Key words: KIT mutations, t(8;21), inv(16), trisomy 4, core binding factor leukemia; childhood AML.

Acute myeloid leukemia (AML) is a heterogeneous disease with regard to the morphology, immunophenotype, and genetic rearrangements acquired by leukemic blasts. Multiple recurrent chromosome and gene rearrangements have been identified in AML, and these alterations have been correlated with biological and clinical features of the disease resulting in delineation of prognostically distinct categories of AML. One such category is core-binding factor AML (CBFL) in which core-binding factor (CBF) genes are a frequent target of rearrangements and mutations.

One of the most common targets of translocations that have been implicated in CBFL is the AML1 (RUNX1) gene. AML1 is involved in a number of translocations, including t(8;21) which is found in ~12-15% of AML cases, and occurs in 40% of patients with the M2 subtype of AML. Similarly, a fusion gene CBFβ/MYH11 is produced by juxtaposition of bands 16q22 and 16p13 as a result of inv16 or, less frequently, t(16;16)(p13;q22). Patients with CBF AML constitute approximately 15% to 20% of adults younger than 60 years with de novo AML. It has recently been documented that a single genetic abnormality may not be sufficient to promote leukemogenesis, but this end result may be reached by several different pathways within the same AML clone being simultaneously affected. Abnormalities in genes that are involved in signal transduction pathways are common in AML. Mutations in the KIT and FLT3 receptor tyrosine kinases (RTK) have been described frequently in AML. Intriguingly, KIT mutations have been preferentially associated with AML exhibiting either an inv(16) or a t(8;21) karyotype, i.e. the core binding factor leukemias. Point mutations of KIT have been...
KIT mutation incidence in adult and pediatric AML

found in approximately 5% of AML samples and it has recently been reported that 25% of AML and inv(16) patients have a KIT exon 8 mutation. It may be that the KIT mutation provides the myeloid blasts with the extra hit by conferring proliferation and/or anti-apoptotic activity, as the chimeric transcription factor impairs normal differentiation but has a limited effect on cellular proliferation. Additional chromosomal abnormalities are frequently associated with t(8;21) and trisomy 4 in particular. It has recently been suggested to constitute a distinct subtype of t(8;21) AML. Results of studies investigating the molecular consequences of trisomy 4 focus on the dosage effect resulting from duplication of a mutated KIT allele. We determined the incidence of KIT mutations in adult and childhood AML and analyzed the biological and clinical features of the two groups, defining the molecular and prognostic significance of concomitant trisomy 4.

Our results confirm the previously observed strict correlation between adult CBFL and KIT mutation and document that this mutation also frequently occurs in childhood AML. In both groups trisomy 4 per se or in association with t(8;21) may be a predictor of KIT mutation.

Design and methods

Patients’ selection

We investigated 49 children with newly diagnosed AML. These children did not have acute promyelocytic leukemia, nor did they have FLT3 mutations or internal tandem duplications. We also studied 52 adult patients diagnosed with t(8;21) or inv(16) AML. Three adult t(8;21) patients also carried trisomy 4; one of these patients has been previously described.

The FLT3/ITD-negative children were extracted from a consecutive cohort of cases of pediatric AML. All adult AML patients were recruited consecutively, the only exceptions being 2 cases from St. Anna Kinderspital, Vienna, Austria. Five additional adult AML patients showing only trisomy 4 including two with M1 and one each with M2, M4, and M5 FAB subtypes, were also tested for the c-kit mutation.

Childhood and adult AML were diagnosed according to the French-American-British (FAB) classification. The presence of t(8;2) and inv (16) was assessed by karyotyping, fluorescent in situ hybridization (FISH) and/or reverse transcription polymerase chain reaction (RT-PCR) analysis for the major chimeric transcripts.

Mutational analysis

The exon 17 mutations in the KIT gene were identified by sequencing, as previously described, and by more sensitive assays, such as the Hind assay for Asp816Val as previously described, the Tsp509I assay for Asn822Lys also previously described elsewhere, and ARMS (amplification refractory mutation system) PCR for Asp816Tyr and Asp816His.

Briefly, 178 bp (wild type) and 177 bp (mutated) ARMS PCR products of KIT exon 17 were generated using the following fluorescently labeled primers; the 17A 5'-AGTTTTTACCTTCTTAAG-3' and 17B-Hex-Tyr 5'-TTAGAATCTTGTGATGA-3' for D816Y detection and 17A and 17B-Tet-His 5'-TTAGAATCTTGTGATGTG-3' for D816H detection. ARMS primers for wild type KIT exon 17 were: 17A and fluorescein labeled 17B-Fam-wt 5'-TTAGAATCTTGTGATGC-3'. Denaturing, annealing and extension steps were performed at 94°C for 30 seconds, 48°C for 20 seconds, 72°C for 20 seconds, with a final extension step at 72°C for 4 min. Products were resolved on 1.8% agarose-gel and visualized using a Typhoon 9200 FluorImager system (Amersham Pharmacia). Sequence analysis of exons 2, 8, 10, 11 and 17 of the KIT gene was performed by direct sequencing of PCR products using the following primers: 2A (5'-ATTGTGAGTAGA-CACAAGAC-3') and 2B (5'-TGCAGAAAGCCAAGCATT-3'), 8A and 8B, 10A (5'-GATCCCATCCTGCAAAGGTT-3') and 10B (5'-ATTGTCTCAGTCATTAGAC-3'), 11A (5'-CAG-GTAACATTATTGTATTTG-3') and 11B (5'-TCATCTGTTCAG-GTGGGA-3'), 17A and 17B11 respectively. Direct sequencing of DNA and cDNA products was performed using a Thermo Sequence Dye Terminator sequencing reaction and an ABI Prism 3100 sequencing analyzer (Applied Biosystems).

Semi quantitative PCR amplification and evaluation of allelic imbalance for D816Y and D816V mutations

ARMS D816Y/WT PCR were performed using primers 17A and both 17B-Hex-Tyr and 17B-Fam-wt primers for 25 cycles under the above described conditions. Quantitative measurements were obtained after capillary electrophoresis of PCR products using the ABI 3100 genetic analyzer and Gene Scan software (Applied Biosystems) for each sample. Semi quantitative analysis of D816V allelic imbalance was obtained after capillary electrophoresis of Hind1-digested PCR products performed using the 17A and 17B-Fam-wt primers and the above described conditions.

All PCR reactions were performed in triplicate and were repeated twice.

Results

Incidence of KIT mutant receptor in CBFL

One of the aims of our study was to examine the incidence of KIT mutations in adult CBFL. To this purpose we considered 37 new, consecutively recruited patients in addition to 15 previously reported CBFL patients and
correlated the presence/absence of mutation with the clinical data, FAB classification and cytogenetic findings. The overall series could be divided into two groups: group I (32 patients) carrying t(8;21) with or without other karyotypic abnormalities and group II (20 patients) carrying inv(16). Among these 52 CBFL patients we found 17 patients with D816 missense mutations (10 with D816V, 5 with D816Y, and 2 with D816H) accounting for 32.6% of the total, 5 patients (#9, 13, 32, 38 and 45 in Figure 1A) carrying a new type exon 8 in-frame deletion plus insertion mutations (9.6%), one patient with a previously described (V530I)12 transmembrane mutation (V530I),12 and in one patient we observed for the first time an ITD mutation within exon 11 of the KIT gene (Figure 1B).

The KIT mutated t(8;21) patients were mainly characterized by having a white cell count > 40×10⁹/mL. Extramedullary disease was observed only in patients with KIT mutation. It is of note that KIT activating mutations were found in all three patients with trisomy 4 associated with t(8;21), two of whom had extramedullary disease. Therefore KIT mutations were detected in 46.1% of the CBFL adult patients studied.

**Table 1. Clinical and genetic features of the 52 AML study patients recruited according to CBF rearrangements.**

<table>
<thead>
<tr>
<th>t(8;21)</th>
<th>inv(16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>Positive</td>
<td>15</td>
</tr>
<tr>
<td>Negative</td>
<td>17</td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
</tr>
<tr>
<td>Positive</td>
<td>6</td>
</tr>
<tr>
<td>Negative</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
</tr>
</tbody>
</table>

**Age (years)***

| ≤ 60 | 14 | 51.8 |
| > 60 | 4 | 80.0 |

**Sex**

| Male | 11 | 47.8 |
| Female | 3 | 33.3 |

**WBC***

| ≤ 40×10⁹/L | 9 | 36 |
| > 40×10⁹/L | 4 | 0 |

**FAB subtype***

| M1 | 1 | 0 |
| M2 | 14 | 17 |
| M4Eo | 9 | 47.3 |

**Trisomy of chromosome 4***

| 3 | 3 |

**Extrad.**

| 3 | 0 |

**Incidence of KIT mutant receptor in FLT3/ITD-negative childhood AML***

A further aim of our study was to verify the incidence of KIT mutations in childhood AML. We did this by analyzing a cohort of children previously screened for FLT3 mutations.23 Results from 49 childhood leukemias (excluding acute promyelocytic leukemia) are given in Table 2. Six KIT mutations were identified, thus giving a mutation incidence of 12.2%. Mutations were equally distributed among M1 and M2 FAB subtypes. No correlation between KIT mutation and an elevated white blood cell

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**Figure 1. Novel KIT mutations in adults (#a) and children (#c) with AML. (A) Exon 8 in-frame deletions plus insertions affecting amino acids from 416 to 419. The nucleotide changes in each patient are indicated in parentheses underneath the resulting new sequence. The inserted nucleotides are indicated by bold underlined characters. (B) PCR amplification of exon 11 DNA showing a longer product, in addition to the 325 bp wild-type KIT (case #a34). Lanes C1 and C2, control DNA. The amino acid sequence of the juxtamembrane portion duplicated in KIT/ITD is shown. The duplicated sequence, in single letter code, is shown in bold letters and underlining indicates the inserted extra amino acid.
count was observed. We found four patients with KIT missense mutations of D816 (1 with D816V, 2 with D816Y and 1 with D816H), and two with exon 8 in-frame deletion and insertion (#c35 and c49 in Figure 1A). The KIT N822K mutation, which was reported in childhood AML,14 was searched for but not detected in our cohort. Two KIT mutated cases had t(8;21) and a third patient carried a trisomy 4 karyotype.

Of the 6 children with KIT mutations, 5 are alive (four of them after bone marrow transplantation), and one died after 7 months from disease progression during chemotherapy, never having achieved a clinical remission. Of the 5 survivors with KIT mutations, one relapsed after 3 years, received an autologous BMT and is currently alive 8 years after this transplant. Two were positive for t(8;21): one received an allogeneic BMT in first remission and is now alive 5 years after BMT; the other was treated with chemotherapy and is also alive 5 years after diagnosis. The other two patients received autologous BMT in first remission: one relapsed and then underwent allogeneic BMT and is currently alive 2 months after this allogeneic graft. Of the 43 children with wild type KIT, 13 relapsed; 22 underwent either autologous or allogeneic BMT; overall, 14/43 died of disease progression and 2 of transplant-related causes.

**Trisomy 4 in KIT mutated patients and duplication of the mutated allele**

The finding of the peculiar association of trisomy 4 and t(8;21) in KIT mutated CBFL raised the question of whether trisomy 4 might be a predictive marker of KIT mutation even if it were separated from t(8;21). We thus screened 5 adult AML patients whose only karyotypic abnormality was trisomy 4 and detected one D816V mutated case of M2 subtype (data not shown) besides the child listed in Table 2.

### Table 2. Clinical and genetic features of the 49 children with AML selected for the absence of FLT3/ITD mutations.

<table>
<thead>
<tr>
<th>KIT mutation positive</th>
<th>KIT mutation negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td>12.2</td>
</tr>
</tbody>
</table>

**Age (years)**

| ≤ 10 | 5 | 11.9 | 37 | 88 | 42 |
| > 10 | 1 | 14.2 | 6  | 85.7 | 7  |

**Sex**

<table>
<thead>
<tr>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>17.6</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
</tr>
</tbody>
</table>

**WBC**

| ≤ 40×10^9/L | 3 | 10.3 | 26 | 89.6 | 29 |
| > 40×10^9/L | 2 | 11.7 | 15 | 88.2 | 17 |

**FAB subtype**

| M1 | 3 | 25 | 9 | 75 | 12 |
| M2 | 3 | 21.4 | 10 | 71.4 | 14 |
| M4 | 8 | 8 |
| M4Eo | 1 | 1 |
| M5 | 11 | 11 |
| M6 | 1 | 1 |
| M7 | 2 | 2 |

| t(8;21)§ | 2 | 25 | 6 | 75 | 8 |
| inv(16)# | 0 | 1 | 1 |

| Trisomy of chromosome 4 | 1 | 0 | 1 |

*Data not available for three patients; §presence of t(8;21) as an additional chromosome aberration; #presence of inv(16) as an additional chromosome aberration; *presence of trisomy 4 as an additional chromosome aberration.
We previously described a CBFL patient (here included among the 3 adult patients reported) in whom trisomy 4 led to duplication of the KIT mutated allele. In order to generalize this finding we quantified the dosage of the D816Y and D816V alleles carried by the two new adult CBFL patients (Table 1) and the child with AML and trisomy 4 (Table 2).

As can be seen in Figures 2A and 2B the ratio of the mutated D816Y or D816V allele to the wild-type KIT allele turned out to be 2:1 in all three samples investigated. Direct sequencing of cDNA from two of the above patients showed that the D816Y and D816V alleles were expressed (data not shown).

**Discussion**

The association between KIT mutation and CBFL, exhibiting either an inv(16) or t(8;21), was first documented as a non-random event favored by the differentiation block of KIT expressing myelomonoblasts. The association was subsequently discussed in the context of accumulating evidence showing that the expression of transcription factor fusion proteins may contribute to expansion of the stem cell pool, but is not sufficient to induce a leukemia phenotype, as one or more additional mutations are required for leukemogenesis.

An important insight into the nature of the secondary mutation that co-operates with AML1-ETO arose from the observation that leukemia cells derived from an induced transgenic mouse model expressing the fusion protein were growth factor-independent in contrast to AML1-ETO-expressing non-tumorigenic cells.

Moreover chimeric mice harboring the CBFβ-MYH11 knock-in gene provided evidence that the fusion protein blocks differentiation of the myeloid cells at the level of KIT progenitors. In order to assess the contribution of the KIT mutation to CBFL we performed an extensive mutation analysis on a group of 52 adult CBFL patients and 49 FLT3/ITD-negative, non-APL pediatric AML patients. We found that 46.1% of adult CBFL patients and 12.2% of non-APL pediatric AML patients carry a KIT activating mutation. Preliminary findings on KIT mutations in childhood leukemia showed a quite similar mutation incidence (11.3%) with this mutation apparently as all cases of adult t(8;21), but only one pediatric case were available for the molecular test of chimeric transcripts that can only occasionally be detected when conventional cytogenetic analysis fails to show the t(8;21). While t(8;21) is found in M2 and M1 subtypes of childhood leukemia it predominantly occurs in the M2 subtype in adults. Considering both M1 and M2 subtypes of childhood leukemia, the percentage of KIT mutation appears similar to that found in adult patients.

All four t(8;21) adult patients with a high white blood cell count > 40×10^9/L were KIT mutated, which is consistent with previously reported observations. This correlation was not evidenced in the KIT mutated pediatric patients, at difference to what has been reported in the FLT3/ITD subgroup.

The Asp816 residue in the second TK domain was confirmed to be a mutation hot spot, being the prevalent KIT mutation in our sample (32.6%), followed by in frame deletion/insertion affecting exon 8 (9.6%), which has recently been estimated to occur in 25% of inv(16) patients.

The observed differences between our study and the cited one in the overall KIT mutation incidence and the prevalence of mutations in the CBFL subtypes may be explained by our mutation screening approach which targeted exons 2, 10 and 11 in addition to exons 8 and 17 and applied highly sensitive techniques such as ARMS to detect D816Y/H mutations and the HinfI assay for D816V. Geographical mutation gradients and different sample sizes may also play some role in the observed differences. We detected a KIT/ITD mutation with three extranucleotides inserted between a 45bp-duplication. This affected KIT region is also involved in the pathogenesis of gastrointestinal stromal tumors.

Most importantly, the same highly conserved residues from 599 to 607 involved in the FLT3 ITD are included in the corresponding (from 578 to 586) KIT ITD. This observation links KIT and FLT3 genes, showing that the same critical residues are involved in the same pathogenetic mechanism.

Our study also shows that t(8;21) AML and trisomy 4, which confers a relatively poor prognosis compared to that of t(8;21) AML patients, represents an association predicting a KIT activating mutation. So far this particular association has been demonstrated in three of our patients (two adult and one child) and in one of the trisomy 4 patients reported by Nishii available for KIT mutation screening. These observations, although limited to a small number of patients, suggest a strict association which might foretell a poor prognosis. An adverse effect of this additional aberration is predicted by the frequently observed duplication of the mutated KIT allele and enhanced mutation effects as foreseeable by cDNA sequencing, confirming previous findings in a CBFL patient.

The same intriguing association was also recently...
described in the CBFL cell line Kasumi-1 and may constitute a positively-selected mechanism playing an important role in the poor prognosis of patients with t(8;21) and trisomy 4 possibly due to drug resistance.

The overall results demonstrate that the KIT gene is the target most frequently mutated to become constitutively active in CBFL, and that KIT mutations appear to be associated with distinct genetic patterns in AML characterized by the presence of AML1 or CBFB fusion proteins, and possible concomitant trisomy 4.

The molecular subtyping of different KIT mutations and their possible combination with other genetic features, such as trisomy 4, might have clinical implications, not only related to prognosis but also to patients’ management. CBF leukemias with KIT exon 8 activating or ITD mutations might be treated effectively with specific TK inhibitors which are not beneficial for cases with Asp816 mutations or any type of Asp816 change. Follow-up studies are needed on AML adult and childhood patients with and without KIT mutations to assess the clinical significance of the different types of mutation and design the most well-targeted treatment for the different molecular subtypes.

References


