Acute Myeloid Leukemia

A new D816 c-KIT gene mutation in refractory AML1-ETO leukemia

One of the most common genetic events in acute myeloid leukemia (AML) is the t(8;21) (q22;q22) translocation, which contributes to leukemic transformation. However, different lines of evidence suggest that the AML1-ETO rearrangement is not sufficient to cause the full leukemic phenotype. Secondary genetic alterations such as mutations in receptor tyrosine kinases are thus required to induce overt AML. The incidence of c-KIT mutations in exon 17 was evaluated in 37 Spanish patients with AML1-ETO+ leukemias. c-KIT mutations were present in only two cases (6.6%) and were shown to be associated with an adverse outcome. The frequency of c-KIT mutations described here is much lower than in other reports.

Acute myeloid leukemia (AML) is a heterogeneous disease with regard to the morphology, immunophenotype, and genetic aberrations acquired by the leukemic blasts. Mutations or rearrangements of genes encoding transcription factors and tyrosine kinases represent two classes of the most frequent genetic aberrations found in leukemia. Translocation (8;21)(q22;q22) and inv(16)(p13q22) are found respectively in approximately 7% and 8% of adults with de novo AML. At the molecular level, t(8;21) and inv(16) result in the creation of the fusion genes AML1-ETO and CBFB/MYH11 which respectively disrupt the α and β subunits of the core binding factor (CBF), a heterodimeric transcription factor involved in the regulation of hematopoiesis. Mutations in the c-KIT and FLT3 receptor tyrosine kinases (RTK) have also been described frequently in AML, and c-KIT mutations have been preferentially associated with AML harboring t(8;21) or inv(16). Clinically, patients with these two forms of AML show a high rate of complete remission and their prognosis is considered better than that of patients with a normal karyotype or other chromosomal aberrations. It has been postulated that one of each class of these mutations (transcription factors and tyrosine kinases) is insufficient to cause overt leukemia but that they cooperate in a two-hit model to initiate a leukemic phenotype. It has previously been documented that the incidence of point mutations in the tyrosine kinase gene c-KIT ranges from 11% to 40% in newly diagnosed AML with t(8;21) translocations. Although several mutations in the extracellular and transmembrane domain of the c-KIT gene have been described, the most common activating alleles in the context of AML1-ETO leukemias are D816V, D816Y, D816H and N822K. These alleles are located in the second tyrosine kinase (TK2) domain of the c-KIT gene which is a hot spot for mutation searching. Beghini et al. detected Asp816 mutations in 32.6% of their CBF-positive AML patients, and Wang et al. in 31%. Nanri et al. concluded that 27% of their series had a mutation in the TK2 domain of the c-KIT gene. Recently, Caironi et al. also evaluated the prognostic significance of 816 c-KIT mutations CBF in leukemia. They showed a negative impact on the outcome of t(8;21) AML, although the prognostic implications of c-KIT mutations remained unclear. The aim of the present study was to examine the TK2 domain of the c-KIT gene for mutations in a consecutive series of Spanish AML patients harboring the AML1-ETO rearrangement. A total of 37 patients with t(8;21)(q22;q22) but without FLT3 mutations (internal tandem duplication and D835V) were selected from the group of AML patients at Hospital Santa Creu i Sant Pau and examined for the presence of mutations in the TK2 domain (exon 17) of the c-KIT gene. Paired DNA/RNA samples were available from 23 of the 37 patients, DNA samples from seven cases and RNA samples from the remaining seven patients. DNA and RNA were extracted following standard protocols and stored at –20ºC or –80ºC, respectively. The AML1-ETO rearrangement was investigated and quantified according to the BIOMED protocol. All samples showed c-KIT over-expression assessed by CD117 antigen reactivity on leukemic cells. The human Kasumi1 cell line with the N822K mutation in c-KIT was used as a positive control.

RNA obtained from 30 AML1-ETO patients at diagnosis and from the Kasumi cell line was retrotranscribed and amplified using primers covering amino acids 653 to 857 of the c-KIT gene. Polymerase chain reaction (PCR) products were purified and directly sequenced with 3.3 pmol of each forward and reverse primer using the Rhodamine Terminator Cycle Sequencing Ready Reaction kit. Sequence analysis was performed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Our analysis of 30 samples of RNA from AML patients detected only one case with a mutation (Figure 1). Sequence analysis of the mutated sample revealed two heterozygous

![Sequence analysis](image)

**Figure 1.** A. Sequence analysis showed two heterozygous changes in amino acid 816 (underlined). B. Cloning of the PCR products revealed both substitutions in the same allele. C. The normal 816 triplet and the four missense mutations described to date.
changes (G→A and A→T) at amino acid D816. To determine whether or not these substitutions were in the same allele, the PCR product was cloned in a pCR4-TOPO® plasmid (Invitrogen, Netherlands). The sequence of eight different clones located the mutation in the same allele and was consistent with a substitution of two nucleotides, GA→AT. Interestingly, this new mutation was located in the most frequently mutated amino acid, identifying a new missense mutation at the same position: D816I. This new mutation would have been detected by another more sensitive technique such as the Hinf I restriction assay, but without sequencing, it would have been wrongly classified as D816V. A feature of the Kasumi N822K mutation, not observed in our patient with the D816I mutation, is the enhanced dosage of the mutated c-KIT allele resulting from the concomitant trisomy of chromosome 4, described by Larizza et al.14

In the light of the low frequency of mutations observed in this series (3.3%), which is in sharp contrast with findings from other groups,6,11 we decided to use the Light Cycler employing fluorogenic probes, a more sensitive approach. D816 c-KIT mutations were screened in the 30 available DNA samples using a melting curve based assay with the primers and hybridization probes described by Schnittger et al.11 DNA was amplified using primers covering exon 17 and intron boundaries, and two hybridization probes were used for the post-PCR melting analysis. Data were analyzed using the Lightcycler 4.0 software (Roche Diagnostics, Mannheim, Germany) and the second derivative maximum method. DNA was amplified in a 20 µL reaction volume with 0.5 µM of each forward and reverse primer, 0.25 µM Hyb-Probes, 4 µL LightCycler® FastStart DNA Master Plus Hybridization Probes (Roche Diagnostics, Mannheim, Germany) and 200-500 ng of DNA. Amplification was performed with 36 cycles using an annealing temperature of 50°C. The final melting curve analysis was performed from 40°C to 85°C with a slope of 0.5°C/sec. In this mutational study, we identified melting temperatures that indicated various D816 mutations.25 Twenty-eight samples had a common peak at 62°C (representing the normal allele), one sample had an extra peak at 56°C and corresponded to the normal peak at 62°C (representing the normal allele), one sample from this last case was available for sequencing, but in accordance with the results of Schnittger et al.,11 we could expect that this profile corresponded to a D816H mutation.

The patient with the D816 c-KIT mutation had refractory disease and died 1 year later, whereas the patient with the other mutation achieved a complete remission but relapsed 25 months later. These results are in accordance with those published by Cairoli et al.11 It would be interesting to study whether other AML cases without the AML1-ETO rearrangement and refractory to treatment harbor a c-KIT mutation. Clinical studies have often grouped patients with CBF AML into the favorable-risk prognostic category. Using a novel disease stage study at the end of chemotherapy help to identify subgroups of patients with an increased risk of relapse.12 We screened 37 patients for KIT mutations in exon 17 of the tyrosine kinase domain, using DNA and/or RNA (depending on the availability) as the starting material. The first mutation screening was performed on DNA from 30 patients. Using this technique we detected only one mutation, representing an incidence of 3.3%, in AML1-ETO patients. When a more sensitive technique was employed, no additional mutation was detected in 23 of these 30 patients, but the technique did identify one more mutation in seven other AML1-ETO cases. Overall, the mutation frequency in exon 17 of c-KIT was 6.6%. Our data show that the c-KIT mutation in the TK2 domain was not common in this consecutive series of AML1-ETO leukemias (6.6%), but that it was able to identify patients with a poor outcome. Schnittger et al.12 suggested that exon 8 mutations are frequent in CBF-MYH11 positive AML, whereas c-KIT D816 mutations represent a frequent molecular event in AML1-ETO leukemias (10.5%).

In line with the results observed in the present series, techniques for identifying and quantifying c-KIT mutations should be compared in order to obtain clinically relevant information concerning AML1-ETO leukemias.

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