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.

Haematologica 2006; 91:1283-1284
(*http://www.haematologica.org/journal/2006/09/1283.html*)

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.[1] Translocation (8;21)(q22;q22) and inv(16)(p13q22) are found respectively in approximately 7% and 8% of adults with *de novo* AML.[2] 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.[3] 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.[4] 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.[5-12] Although several mutations in the extracellular and transmembrane domain of the *c-KIT* gene have been described,[7,8] the most common activating alleles in the context of *AML1-ETO* leukemias are D816V, D816Y, D816H and N822K. These alelles are located in the second tyrosine kinase (TK2) domain of the *c-KIT* gene[5] which is a hot spot for mutation searching. Beghini *et al.*[9] detected Asp816 mutations in 32.6% of their CBF-positive AML patients, and Wang *et al.*[10] in 31%. Nanri *et al.* concluded that 27% of their series had a mutation in the TK2 domain of the *c-KIT* gene.[11] Recently, Cairoli *et al.*[12] 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 –20°C or –80°C, respectively. The *AML1-ETO* rearrangement was investigated and quantified according to the BIOMED protocol.[13] 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*[14] 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.[10] 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

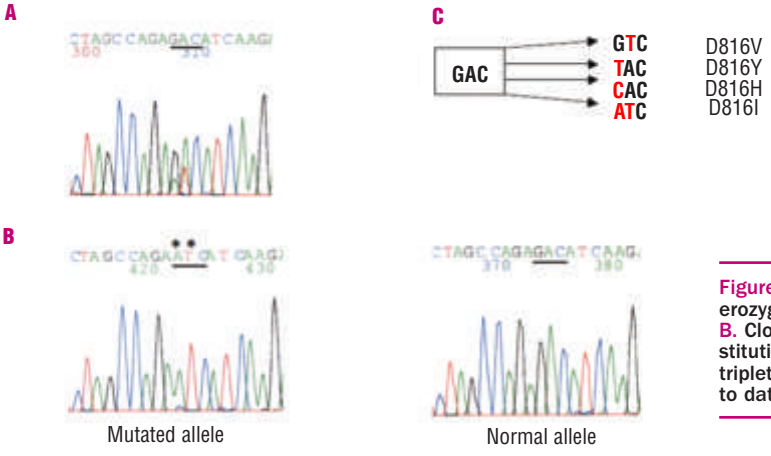

Figure 1. A. Sequence analysis showed two heterozygous changes in amino acid 816 (underlined). **B.** Cloning of the PCR products revealed both substitutions in the same allele. **C.** The normal 816 triplet and the four missense mutations described to date.

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 pCR®4-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 HinfI 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,[6-8] 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*.[15] DNA was amplified using primers covering exon 17 and intron boundaries, and two hybrydization 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 derivate 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 50ºC. The final melting curve analysis was performed from 40ºC to 85ºC with a slope of 0.5ºC/sec. In this mutational study, we identified melting temperatures that indicated various D816 mutations.[15] Twenty-eight samples had a common peak at 62ºC (representing the normal allele), one sample had an extra peak at 56ºC and corresponded to the patient with the D816I mutation in a heterozygous state, and another presented a peak at 58ºC. No sample from this last case was available for sequencing, but in accordance with the results of Schnittger *et al*.,[15] we could expect that this profile corresponded to a D816H mutation.

The patient with the D816I *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*.[12] It would be interesting to study whether other *AML1* 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. Minimal residual disease studies at the end of chemotherapy help to identify subgroups of patients with an increased risk of relapse.[17] 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 RNA 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 Spanish *AML1-ETO* leukemias (6.6%), but that it was able to identify patients with a poor outcome. Schnittger *et al*.[15] suggested that exon 8 mutations are frequent in *CBFB-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.

Adriana Lasa, Maria T. Carricondo, Maria J. Carnicer, Granada Perea, Anna Aventín, Josep F. Nomdedeu

Laboratori d'Hematologia, Hospital de la Santa Creu i Sant Pau, Avda Sant Antoni M. Claret 167, 08025, Barcelona, Spain

Funding: supported by FIS grant PI031396.

Acknowledgments: we wish to thank all the CETLAM centers for their participation.

Key words: D816, c-KIT, AML1-ETO leukemia, refractory disease.

Correspondence: Josep F. Nomdedeu, Laboratori d'Hematologia, Hospital de la Santa Creu i Sant Pau, Avda Sant Antoni M. Claret 167, 08025, Barcelona, Spain. E-mail: jnomdedeu@hsp.santpau.es

References

1. Yuan Y, Zhou L, Miyamoto T, Iwasaki H, Harakawa N, Hetherington CJ, et al. AML1-ETO expression is directly involved in the development of acute myeloid leukemia in the presence of additional mutations. Proc Natl Acad Sci USA 2001;98:10398-403.
2. Byrd JC, Mrçozek K, Dodge RK, Carroll AJ, Edwards CG, Arthur DC, et al. Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with the novo acute myeloid leukemia: Results from Cancer and Leukemia Group B (CALGB 8461). Blood 2002;100:4325-36.
3. Speck NA. Core binding factor and its role in normal hematopoietic development. Curr Opin Hematol 2001;8:192-6.
4. Kelly LM, Gilliland DG. Genetics of myeloid leukemias. Annu Rev Genomics Hum Genet 2002;3:179-98.
5. Beghini A, Peterlongo P, Ripamonti C, Larizza L, Cairoli R, Morra E, et al. C-KIT mutations in core binding factor leukemias. Blood 2000;95:726-7.
6. Gari M, Goodeve A, Wilson G, Winship P, Langabeer S, Linch D, et al. c-KIT proto-oncogen exon 8 in-frame deletion plus insertion mutations in acute myeloid leukaemia. Br J Haematol 1999;105:894-900.
7. Care RS, Valk PJ, Goodeve AC, Abu-Duhier FM, Geertsma-Kleinekoort WM, Wilson GA, et al. Incidence and prognosis of c-KIT and FLT3 mutations in core binding factor (CBF) acute myeloid leukaemias. Br J Haematol 2003;121:775-7.
8. Kohl TM, Schnittger S, Ellwart A, Hiddemann W, Spiekermann K. KIT exon 8 mutations associated with core-binding (CBF)-acute myeloid leukemia (AML) cause hyperactivation of the receptor in response to stem cell factor. Blood 2005;105:3319-21.
9. Beghini A, Ripamonti CB, Cairoli R, Cazzaniga G, Colapietro P, Elice F, et al. KIT activating mutations: incidence in adult and pediatric acute myeloid leukemia, and identification of an internal tandem duplication. Haematologica 2004;89:920-5.
10. Wang YY, Zhou GB, Yin T, Chen B, Shi JY, Liang WX, et al. AML1-ETO and C-KIT mutation/overexpression in t(8;21) leukemia: Implication in stepwise leukemogenesis and response to Gleevec. Proc Natl Acad Sci USA 2005;102:1104-9.
11. Nanri T, Matsuno N, Kawakita T, Suzushima H, Kawano F, Mitsuya H, et al. Mutations in the receptor tyrosine kinase pathway are associated with clinical outcome in patients with acute myeloblastic leukemia harboring t(8;21)(q22;q22). Leukemia 2005;19:1361-6.
12. Cairoli R, Beghini A, Grillo G, Nadali G, Elice F, Ripamonti CB, et al. Prognostic impact of c-KIT mutations in core binding factor leukemias. An Italian retrospective study. Blood 2006;107:3463-8.
13. Gabert J, Beillard E, van der Velden VH, Bi W, Grimwade D, Pallisgaard N, et al. Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia – a Europe Against Cancer program. Leukemia 2003;17:2318-57.
14. Larizza L, Magnani I, Beghini A. The Kasumi-1 cell line: a t(8;21)-kit mutant model for acute myeloid leukemia. Leuk Lymphoma 2005;46: 247-55.
15. Schnittger S, Kohl TM, Haferlach T, Kern W, Hiddemann W, Spiekermann K, et al. KIT-D816 mutations in AML1-ETO positive AML are associated with impaired event-free and overall survival. Blood 2006; 107:1791-9.
16. Brunet S, Esteve J, Berlanga J, Ribera JM, Bueno J, Marti JM, et al. Treatment of primary acute myeloid leukemia: results of a prospective multicenter trial including high-dose cytarabine or stem cell transplantation as post-remission strategy. Grupo Cooperativo de Estudio y Tratamiento de las Leucemias Agudas y Mielodisplasias. Haematologica 2004;89:940-9.
17. Perea G, Lasa A, Aventin A, Domingo A, Villamor N, Queipo de llano MP, et al. Prognostic value of minimal residual disease (MRD) in acute myeloid leukemia (AML) with favorable cytogenetics [t(8;21) and inv(16)]. Leukemia 2006;20:87-94.