

Molecular characterization of acute myeloblastic leukemia according to the new WHO classification: a different distribution in Central-West Spain

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Background and Objectives. Molecular analysis has contributed to the identification of several non-random chromosomal translocations, such as t(15;17), t(8;21), inv(16)/t(16;16) and 11q23 abnormalities, typically associated with acute myeloid leukemia (AML). The identification of these chromosomal abnormalities helps not only to define different AML subtypes with distinct prognoses and treatments but also to monitor the disappearance of malignant cells after treatment. Recent reports suggest that the frequency of these alterations may differ according to geographic distribution. However, most of these reports focus on just one or two genetic alterations, which may lead to some selection bias. Appropriate epidemiological studies should be based on unselected consecutive series of patients in which all relevant genes are simultaneously analyzed. The aim of the present study was to explore whether or not the incidence of genetic lesions in Spanish AML patients differs from that reported in other countries.

Design and Methods. In a series of 145 consecutive unselected adult patients with AML we simultaneously analyzed the presence of 4 genetic abnormalities, PML/RAR α for t(15;17), AML1/ETO for t(8;21), CBF β /MYH11 for inv(16)/t(16;16) and rearrangements of the MLL gene for 11q23 abnormalities. AML were classified using the new World Health Organization (WHO) classification for hematologic malignancies. The techniques used were standardized according to the recommendations of the European BIOMED-1 Concerted Action.

Results. The PML/RAR α transcript was present in 34 patients (23.4%) (23 were bcr1, 2 bcr2 and 9 bcr3). The AML1/ETO fusion transcript was detected in only 2 cases (1.4%) both with M2 morphology, but 29 other cases with M2 morphology were negative. CBF β /MYH11 transcript was present in 9 cases (6.2%) eight of them displaying M4Eo morphology. Finally, 5 cases (3.5%) showed rearrangements of the MLL gene. Our results differ from those reported from the United States and North/Central Europe, particularly regarding the incidence of t(15;17) and t(8;21) translocations. In Spain the frequency of t(15;17) is higher while that of t(8;21) is lower.

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Interpretation and Conclusions. These data add epidemiological information about geographic heterogeneity of such chromosome aberrations in AML and would contribute to the design of specific screening strategies adapted to the incidence in each country.

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Key words: acute myeloblastic leukemia, PML/RAR- α , AML1/ETO, CBF- β /MYH11, MLL gene

Several non-random chromosomal translocations are associated with specific morphologic subtypes of leukemia¹ which result in distinctive clinical characteristics, some with important therapeutic implications. This has led the clinical advisory committee (CAC) of the World Health Organization (WHO) to reclassify acute myeloid leukemia (AML) into four different subgroups:² AML with recurrent cytogenetic translocations, AML with multilineage dysplasia, AML and myelodysplastic syndromes (MDS) related to previous therapies, and AML not otherwise characterized. The first group of leukemia is of great importance because it is defined by four different well-characterized types of translocations, which have relevant clinical and therapeutic implications. Thus, AML patients who have the t(15;17)(q22;q11-12) [PML/RAR- α] benefit from the use of all-trans retinoic acid and have a favorable outcome.³⁻⁵ Patients with AML harboring the t(8;21)(q22;q22), [AML1/ETO] or the inv(16)/t(16;16)(p13;q22), [CBF- β /MYH11] have a better prognosis than others lacking these alterations,^{6,7} particularly when treated with high dose ARA-C. Finally, the presence of translocations involving 11q23 (MLL gene rearrangements) confers an intermediate/poor prognosis to the disease.⁶⁻⁸ The new classification of hematologic malignancies recently proposed by the WHO² requires the identification of these translocations as a goal for good clinical practice. In addition, the increasing availability of different techniques to detect such abnormalities [conventional cytogenetics, fluorescence *in situ* hybridization (FISH), and reverse tran-

scriptase-polymerase chain reaction (RT-PCR)] makes this goal possible for all reference centers treating acute leukemias. The European BIOMED-1 group has recently published a standardized RT-PCR protocol to detect several fusion gene transcripts including the majority of those usually present in AML.⁹ This effort will allow many centers to use a well-standardized methodology for diagnosis of AML according to the new WHO classification. The relative frequencies of each AML subtype according to the WHO classification have not been specifically investigated, but they could be extrapolated from data reported in the literature.^{6-8,10,11} However, in order to estimate the incidence of these genetic changes accurately, the studies should have been performed on unselected consecutive series of patients in whom the relevant genes were simultaneously analyzed. Moreover, these frequencies can vary between countries according to their different epidemiological distribution around the world.¹² For instance, acute promyelocytic leukemia (APL) has been shown to be less frequent in USA and central European countries than it is in Latin populations from the USA¹³ and countries such as Brazil, Peru, Mexico and Spain.¹⁴⁻¹⁷ In addition, the t(8;21) has been reported to be more frequent in Japan than in Australia,¹⁸ with an incidence ranging from 18 to 88% within leukemias with FAB-M2 morphology.^{19,20} This supports the view that geographic variations in tumor-associated aberrations in hematologic malignancies exist.^{12,21} Our own group has found an unexpectedly low frequency of t(12;21) in patients with acute lymphoblastic leukemia (ALL) from the western part of Spain.²² In this study, we analyzed a large series of consecutive unselected patients with *de novo* AML from the central-western region of Spain (Castilla y León), in whom we simultaneously investigated the presence of the four types of chromosome aberrations that define the four subgroups of AML with recurrent cytogenetic translocations according to the new WHO classification, using well-standardized molecular methods.

Design and Methods

Patients and samples

From August 1994 to April 1999, samples from 233 newly diagnosed adult patients with AML were received at the laboratory of the University Hospital of Salamanca for immunophenotypic, cytogenetic and molecular studies. This is the reference laboratory for a region of 2.2 million inhabitants in the central-western part of Spain (Castilla y León). From them, 77 patients were excluded from the analysis because they were referred in a non-consecutive way from other regions of Spain. Therefore, 156 cases were included in the study with the guarantee that their diagnostic samples were consecutive and unselected. Patients with secondary AML after chemotherapy, myelodysplasia or myeloproliferative disorders were also excluded.

Diagnostic criteria

Patients were initially diagnosed using the standard morphologic criteria used by the FAB group.²³ Once the status of the different translocations was known, all

cases were reviewed in order to assign the AML subtype definitively according to the WHO classification.²

Immunophenotypic studies

Immunophenotypic studies were performed in erythrocyte lysed samples using a large panel of monoclonal antibodies (MoAbs) in triple-staining combinations analyzed by flow cytometry as previously described.^{24,25}

RT-PCR assay

Total RNA was extracted from bone marrow (BM) and/or peripheral blood (PB) samples by the guanidinium thiocyanate/phenol chloroform method.²⁶ The mRNA quality was always assessed in all cases by RT-PCR amplification of the ABL transcript, and was acceptable in 145 out of the 156 cases (93%). All these samples were analyzed for presence of the PML/RAR- α , AML1/ETO and CBF- β /MYH11 fusion transcripts according to the primers, protocols and criteria of the *European BIOMED 1 Concerted Action for standardization of MRD studies in acute leukaemia*.⁹ All assays were carried out at least twice, using appropriate positive (cDNA from the NB4, KASUMI1 & ME-1 cell lines) and negative controls (cDNA from a healthy donor and H2O). PCR products were electrophoresed on 2% NuSieve agarose gels and visualized by ethidium bromide staining under UV light.

Southern blot assay (MLL rearrangements)

High molecular weight DNA was isolated by standard proteinase K digestion, phenol-chloroform extraction and ethanol precipitation according to standard procedures.²⁷ For Southern blot analysis, 10 μ g of DNA were digested to completion with the BamHI restriction enzyme, size fractionated in 0.8% agarose gel, denatured, neutralized, and transferred to nylon membranes (Hybond-N+, Amersham, Little Chalfont, UK). The blots were hybridized with the appropriate ³²P-labeled probe, washed twice in 0.1X SSC, 1% sodium dodecyl sulfate (SDS) for 30 minutes at 65°C, and autoradiographed between 48 and 96 hours at -80°C, according to previously published standard methods.²⁷ The probe used in this study was a 0.74 kb BamHI cDNA fragment which spans the 8.3 kb breakpoint cluster region within the MLL gene.²⁸

Results

The distribution of the 145 cases that were finally included in the study, according to the FAB classification was as follows: M0 in eight cases, M1 in 20, M2 in 32, M3 in 37 (32 hypergranular and 5 microgranular), M4 in 24 (including 8 M4Eo), M5 in 19, M6 in four and M7 in only one case. There were 87 males and 58 females, with a median age of 63 years (range 6-90). Four patients were aged <14 years.

The relative frequencies of each group according to the WHO classification within the 145 consecutive valid cases are shown in Table 1. Thirty-four patients (23.5%) were found to have the PML/RAR- α fusion transcript (23 with type bcr1, two bcr2 and nine bcr3) that identifies the presence of t(15;17). Of these, 33 (97%) corresponded to cases identified as having a M3 morphology

Table 1. Relative frequencies of the different AML subtypes both in consecutive patients and in the whole series of patients.

Acute myeloid leukemias	Consecutive cases n = 145 %	Whole series n = 222 %
AML with recurrent cytogenetic translocations	50 34.5%	98 43.8%
AML with t(15;176) PML/RAR- α	34 23.5%	78 35.1%
AML with t(8;21) AML1/ETO	2 1.4%	3 1.4%
AML with inv(16) or t(16;16) CBF- β /MYH11	9 6.2%	11 5.0%
AML with 11q23 abnormalities (MLL)	5 3.4%	6 2.7%
AML not otherwise characterized	95 65.5%	124 56.2%
AML minimally differentiated	7 4.8%	8 3.6%
AML without maturation	21 14.5%	29 13.1%
AML with maturation	29 20.0%	42 18.9%
Acute myelomonocytic leukemia	15 10.5%	19 8.5%
Acute monocytic leukemia	18 12.4%	20 9.0%
Acute erythroid leukemia	4 2.8%	5 2.3%
Acute megakaryocytic leukemia	1 0.7%	1 0.5%
Acute basophilic leukemia	0 0.0%	0 0.0%
Acute pan-myelosis with myelofibrosis	0 0.0%	0 0.0%

Table 2. Distribution of chromosomal translocations in AML according to patients' age.

Acute myeloid leukemias	6-35 years	36-60 years	>60 years	Total
AML with t(15;176) PML/RAR- α	14 (42%)	10 (29%)	10 (29%)	34 (23.5%)
AML with t(8;21) AML1/ETO	0 (0%)	1 (50%)	1 (50%)	2 (1.4%)
AML with inv(16) or t(16;16) CBF- β /MYH11	4 (44%)	2 (23%)	3 (33%)	9 (6.2%)
AML with 11q23 abnormalities (MLL)	1 (20%)	1 (20%)	3 (60%)	5 (3.4%)
AML not otherwise characterized	8 (8%)	21 (22%)	66 (70%)	95 (65.5%)
Total	27 (18%)	35 (24%)	83 (56%)	145

(30 hypergranular, 3 microgranular), while there was one case initially diagnosed as having an M0 morphology. In this specific patient, the positive PCR result was confirmed by using different sets of primers and cDNAs, as well as a FISH technique.²⁵ The presence of the AML1/ETO fusion transcript which results from the t(8;21) was detected in only 2 cases (1.4%). Both cases displayed a M2 morphology, while the remaining 29 cases with such morphology were negative. The presence of the CBF- β /MYH11 fusion transcript was detected in 9 cases (6.2%). Eight of them corresponded to the 8 AML cases with FAB-M4Eo morphology. The other remaining CBF- β /MYH11 positive case was identified as a FAB-M4, but without a sufficient number of eosinophils to be classified as M4Eo. The type of fusion transcript was A in seven cases, C in one and D in the other.

The MLL gene was rearranged by Southern blot analy-

sis in 5 cases (3.5%). These included one M1, two M2, one M4 and one M5.

As far as the age distribution was concerned, the study sample was representative of the population of our region, characterized by advanced age (mean 63 years; range, 14-90). Upon dividing the series into three age groups (14-35, 36-60 and >60 years), we observed that the overall frequency of leukemia displaying one of the four recurrent cytogenetic translocations was much higher in the youngest age group. By contrast, the frequency of AML not otherwise characterized increased with age (8%, 22% and 70%) ($p < 0.05$, Table 2). It should be noted that this difference was mainly due to the higher incidence of PML/RAR α positive cases among young AML patients, while 11q23 abnormalities were more frequent in the advanced age group.

In addition, considering the whole series (consecutive unselected patients plus referred patients), 222 cases presented valid data. In this series, as might be expected, the incidence of PML/RAR- α positive cases was higher than in the unselected series, but the incidence of the other genetic abnormalities did not differ (Table 1).

Discussion

In the present study we prospectively analyzed, in a series of AML patients representative of the population from the central-western region of Spain, the incidence of the most frequent chromosome abnormalities described in AML. Two important pre requisites of this study were: i) the series of patients was based on consecutive unselected AML cases and ii) in all cases the four genetic abnormalities were simultaneously explored, using well-standardized molecular techniques according to BIOMED-1 protocols.⁹ Our data show that in Spain the t(15;17) is more frequent and the t(8;21) is rarer than in other countries (USA and North-Central Europe). Although many cases were also analyzed by cytogenetics, there were a substantial number of cases lacking these data due to inappropriate storage or transportation, or due to the lack of metaphases or clonal cytogenetic abnormalities after the analysis. So, no epidemiological data can be added based on conventional cytogenetics, although we can say that no differences were observed between the two techniques when cytogenetics was available.

Using cytogenetic and morphologic data it was reported that the incidence of M3/t(15;17) positive AML was between 5%^{23,29-31} and 11%³²⁻³⁴ in United States and North-Central Europe. More recent data confirm that the frequency is around 10%^{6,8} as it is in Australia and Japan.¹⁹ In a British series including 1,612 AML patients and based on cytogenetic data, the incidence of t(15;17) is 13%.⁷ However, there are some data suggesting that the frequency of the t(15;17) can be higher in some countries and/or specific populations. Thus, this frequency increases to 17% in Italian children³⁵ and seems to be over 20% in countries such as Brazil, Mexico, Peru and Spain.¹⁴⁻¹⁷ Even more significantly, this frequency has been shown to be as high as 37.5% in

patients of Latin origin from Los Angeles.¹³ However, as mentioned above, these findings are mainly based on morphologic and cytogenetic data, so some variations could be expected upon using molecular analysis. Our study confirms that AML with t(15;17) represents one quarter of all *de novo* AML, which actually means that it is the most frequent type of acute leukemia in our population. Moreover, the incidence is greater in younger patients, which makes this epidemiological data even more valuable, since such patients can take advantage of curative treatments⁵ and quick screening tests for the disease.²⁵ The second observation of note in the present study is the extremely low frequency of t(8;21) AML1/ETO found: only 1.4%, even when we included specially referred patients (whole series of 222 patients). So far, we have only seen 5 cases in more than 400 AMLs already screened in our laboratory for this genetic abnormality. The initial reports showed a global frequency of t(8;21) of around 15%, increasing within those cases with FAB-M2 morphology.³⁶⁻³⁸ However, marked geographic variations have been shown. Thus, the reported incidence of t(8;21) within the FAB M2 subtype ranges from 58-88% in Asian patients, 19-54% in other European countries and 12-27% in USA.^{12,18,39} In addition, the most recent data from European countries based on cytogenetic or molecular studies have shown that the frequency of this aberration is around 8% of all AMLs.^{7,8} These data indicate that, according to the frequency detected in the present study (1.4% of all AMLs; 6% of all FAB M2 morphologies), this genetic abnormality is more unusual in Spain than in other countries, although apparently the incidence of t(8;21) is higher in young AML patients,⁴⁰ and our series could be biased by the advanced age of our population. Nevertheless, this factor is not strong enough to justify the great variation that has been obtained compared to previous reports and moreover, our analysis according to age subgroups did not show any difference.

Finally, the frequencies of the CBF- β /MYH11 fusion gene and MLL rearrangements (6% and 4%, respectively) coincide with data reported in the literature.⁶⁻⁸

In summary, the results presented here, especially those referring to the t(15;17) and t(8;21) translocations, support the existence of epidemiological/geographic variations in the presentation of AML. Awareness of these variations should contribute to the design of cost-effective screening strategies, adapted by individual National Health systems according to the incidence of locally detected genetic aberrations.

Contributions and Acknowledgments

MCC, RGS, MG, AB and JFSM belong to the University Hospital of Salamanca and to the Centro de Investigación del Cáncer de Salamanca, contributing with the inclusion of 57 patients and performance of all molecular studies. MCC carried out all the sample manipulations, including sample reception and labeling, RNA extraction and RT-PCR development, data interpretation and emission of information. She also received and formatted the clinical data, and contributed to the literature reviewing. AB helped her

in all the technical aspects and carried out the analyses in case of substitution requirements. RGS and MG were the laboratory leaders and should be considered as the main investigators. They designed the study, performed the literature review and wrote the paper. They were also responsible for the final data interpretation, direct supervision and day-to-day contact with the participants. AO belongs to the Unidad de Citometría de flujo of the Salamanca University, where immunophenotypic studies of all patients were carried out. FR is the clinician of the Hospital Virgen Blanca de León, which contributed with the inclusion of 22 patients. JFC is the clinician of the Hospital Universitario de Valladolid, which contributed with the inclusion of 22 patients. MJR is the clinician of the Hospital Nuestra Señora de Sonsoles de Ávila, which contributed with the inclusion of 15 patients. AS is the clinician of the Hospital General Yagüe de Burgos, which contributed with the inclusion of 14 patients. AC is the clinician responsible for the Hospital Nuestra Señora de la Concha de Zamora, which contributed with the inclusion of 9 patients. MJC is the clinician of the Hospital General de Segovia, which contributed with the inclusion of 7 patients. In addition, the Hospitals Camino de Santiago de Ponferrada, Nuestra Señora del Puerto de Plasencia and Río Carrion de Palencia, whose clinicians were J. Galende, GA Martín Núñez and F. Ortega, contributed with five, three and two patients, respectively. JFSM was responsible for the whole group, contributed to the writing of the paper and gave final approval of the version to be submitted. In addition, the authors would like to thank Mark Anderson and F. García for their technical assistance.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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Potential implications for clinical practice

The findings presented here constitute an epidemiological tool for the diagnosis of AML in Spain and may be very useful in the design of diagnostic strategies in our country. In addition, this information must be taken into account during the development of therapeutic protocols, since the data reflect the distribution of some leukemias, such as promyelocytic leukemias, with specific therapeutic strategies.⁴¹

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