GENETIC CHANGES INCLUDING GENE COPY NUMBER ALTERATIONS AND THEIR RELATION TO PROGNOSIS IN CHILDHOOD ACUTE MYELOID LEUKEMIA

Running title: Genetic changes in acute myeloid leukemia

Gemma Armengol[1,2*a], Anna Canellas[1*], Yolanda Álvarez[1], Pilar Bastida[3], José Sánchez de Toledo[3], M. Mar Pérez-Iribarne[4], Mireia Camós[5], Esperanza Tuset[5], Jesús Estella[5], María Dolores Coll[6], María Rosa Caballín[1§], Sakari Knuutila[2§]

[1] Unit of Biological Anthropology, Department of Animal Biology, Plant Biology and Ecology, Faculty of Biosciences, Universitat Autònoma de Barcelona, Barcelona, Spain; [2] Department of Pathology, Haartman Institute and HUSLAB, University of Helsinki and Helsinki University Central Hospital, Helsinki, Finland; [3] Hematology and Oncology Pediatric Department, Hospital Materno-Infantil Vall d'Hebron, Barcelona, Spain; [4] Genetics Department, Hospital Sant Joan de Déu, Barcelona, Spain; [5] Pediatric Hematology Department, Hospital Sant Joan de Déu, Barcelona, Spain; [6] Unit of Cell Biology, Department of Cell Biology, Physiology and Immunology, Faculty of Biosciences, Universitat Autònoma de Barcelona, Barcelona, Spain

* These authors have contributed equally to this work

§ These authors have contributed equally to this work

[a] Corresponding author: Unit of Biological Anthropology, Department of Animal Biology, Plant Biology and Ecology, Faculty of Biosciences, Universitat Autònoma de

Barcelona, 08193-Bellaterra, Barcelona, Spain. Phone number: +34 935812049. Fax number: +34 935811321. e-mail address: gemma.armengol@uab.cat

Keywords: childhood AML, cytogenetics, genetic mutations, genomic imbalances, array CGH, prognosis

ABSTRACT

We studied a series of 68 cases diagnosed with childhood acute myeloid leukemia (AML) using conventional cytogenetics and fluorescence *in situ* hybridization (FISH), polymerase chain reaction (PCR) to analyze mutations in *FLT3* and *NPM1* genes, and/or array comparative genomic hybridization (CGH). Cytogenetic/FISH abnormalities were observed in 71% of cases, *FLT3*-ITD mutations in 15%, and *NPM1* mutations in 13%. Array CGH alterations (average 3.6 per case) were observed in 96% of the tested cases. The most frequent alterations were gains of 8q24.3 and 11p15.5-p15.4 in 16% of the samples. Six genes (*AKT1*, *RUNX1*, *LTB*, *SDC1*, *RUNX1T1* and *JAK2*) from the imbalanced regions have been reported to be involved in AML, whereas other 30 cancer genes, not previously reported in an AML context, were identified as imbalanced. They probably correspond to non passenger alterations that cooperate with the recurrent translocations. Clinical data and genetic changes were tested to find out the possible association with prognosis. Genomic instability (four or more genomic imbalances) was correlated with poor patient outcome (p=0.029).

INTRODUCTION

Childhood acute myeloid leukemia (AML) is a heterogeneous disease that comprises approximately 20% of all pediatric leukemias. Despite improvements in clinical management, the probability of 5-year event free survival (EFS) is about 50% [1, 2]. Relapses and the high frequency of treatment-related deaths remain as the main reasons for treatment failures. Therefore, an appropriate risk-group stratification based upon a better knowledge of pediatric AML biology is needed. The current classification of AML patients at diagnosis mainly depends upon the morphology, immunophenotyping, and especially cytogenetic and molecular abnormalities (French-American-British Cooperative Group (FAB) and World Health Organization (WHO) classifications) [3, 4]. Cytogenetic-molecular findings at diagnosis have been used to classify AML patients into three categories of prognosis: standard or favorable risk group, intermediate risk group and high risk group. The standard risk group (about 30% of patients) is composed of AML cases associated with t(8;21), t(15;17) or inv(16)/t(16;16). The adverse or high risk group can include complex karyotypes, t(6;9), t(9;22), -7, -5, 5q- and/or *FLT3* gene mutation with internal tandem duplications (ITD), depending on the co-operative group. Finally, the intermediate prognosis group (about 40% of the patients) is very heterogeneous and includes all other cases [5]. In addition to *FLT3*-ITD, other molecular abnormalities such as *NPM1* mutations have been recently described as favorable prognostic factors in cytogenetically normal childhood AML [6].

Markedly, there are striking differences between the various AML age-groups. The prognosis varies with age, with the highest survival rate among children (50-70%),

especially those younger than one year of age, followed by young adults (40-50%), and older adults (only 10%). This could be partially explained by the different distribution of cytogenetic subgroups in children and the greater tolerability to chemotherapy, thus allowing the administration of higher doses of treatment [5].

Explicit knowledge regarding childhood AML genetic alterations is required in order to characterize the abnormalities present among different risk-groups and in order to understand the underlying mechanisms of leukemogenesis. For example, some deletions of genes in recurrent chromosomal rearrangements have been observed by fluorescence *in situ* hybridization (FISH) method with variable incidences [7]. To study the genetic features of this disease, genomic technologies, such as microarrays, have proved to allow a high-throughput genome-wide screening. DNA-based microarrays, such as array comparative genomic hybridization (CGH) are useful in the detection of submicroscopic DNA copy number changes and have been applied to study gains and losses of genetic material in many neoplasms. So far, array CGH has been applied for the analysis of adult AML but not for childhood AML [8-11]. A high-resolution array CGH study in a series of adult AML revealed patterns of genomic instability affecting all the cytogenetic risk groups and with an impact on survival [9].

To genetically characterize childhood AML and to ascertain prognostic factors for this disease, we studied a series of consecutive 68 childhood AML cases using conventional cytogenetics, FISH, polymerase chain reaction (PCR), and/or array CGH.

MATERIALS AND METHODS

Patients, Clinical Data and DNA Isolation

Bone marrow (BM) or peripheral blood samples obtained at diagnosis from 68 patients of age less than 17 years with AML were analyzed. Informed consent was obtained from each patient or guardian. Patients were diagnosed and treated at Hospital Vall d'Hebron and Hospital Sant Joan de Déu in Barcelona, Spain, from 1992 to 2002. The study was reviewed and approved by the Research and Ethics Committees of both hospitals. The diagnosis was made according to the FAB and WHO classification. All the patients received similar treatment protocol, except for the cases of acute promyelocytic leukemia (APL), which received all-trans retinoic acid (ATRA)-based therapy. Detailed patients' clinical data and biological characteristics of leukemic cells are shown in Supplementary Table S1.

Genomic DNA was extracted using conventional methods. Reference DNA for CGH hybridizations was extracted from peripheral blood lymphocytes of six healthy male donors and six healthy female donors. In cases where no BM samples were available, cells in fixative were used for PCR analysis as previously described [12].

Cytogenetics

Cultures were harvested after 24 h and processed by standard cytogenetic methods. Karyotype was carried out by G-banding using standard techniques. Whenever possible, 20 metaphases were analyzed. Cytogenetic abnormalities were described according to the International System for Human Cytogenetic Nomenclature [13].

Fluorescence *in Situ* Hybridization Analysis

Interphase FISH analysis was performed in accordance with the manufacturer's guidelines, using the following probes: locus-specific identifier (LSI) probes *AML1/ETO* [t(8;21)] dual color dual fusion translocation probe, *MLL* dual color break apart rearrangement probe, *CBFß* [inv(16)/t(16;16)] dual color break apart rearrangement probe, and *PML/RARα* [t(15;17)] dual color translocation probe; and a probe centromere-specific for chromosome 7 (CEP7). All the probes used were from Vysis, Downers Grove, IL. Two hundred nuclei were analyzed with LSI probes and 300 with the centromeric probe, using an Olympus BX51 fluorescence microscope (Olympus Imaging, Hamburg, Germany). Images were captured using the Cytovision program (Applied Imaging, Newcastle Upon Tyne, UK). The cut-off points for positive values were established as the mean of false positives in 1000 nuclei from BM of five controls with no hematological malignancies plus three standard deviations. These cut-off values were 0% for *AML1/ETO*, 1.5% for *MLL*, 1.1% for *CBFß* rearrangement and 0.9% for *CBFß* trisomy with no rearrangement, 3.2% for *PML/RARα*, and 9.8% for monosomy of chromosome 7.

Detection of *FLT3* and *NPM1* Mutations

Screening for *FLT3*-ITD mutations was performed, as described before, using the previously published primer pairs 11F/12R [14]. Patients were considered positive heterozygous if two bands (a normal-size band of 329 bp and a variable bigger band) were seen in an agarose gel electrophoresis; or positive homozygous if only the bigger band was observed. For the analysis of mutations of exon 12 of *NPM1* gene, 200 ng of genomic DNA or cDNA were amplified using a 6-FAM labeled primer and subsequent analysis of the PCR product in an Automatic sequencer (Abi Prism 310

from Applied Biosystems Inc., Foster City, CA, USA) using the Genescan software as previously described [15]. Only those cases with normal or no results for cytogenetic and FISH analyses were analyzed for *NPM1* mutations.

Array Comparative Genomic Hybridization Analysis

Array CGH was performed for 24 samples on commercial oligonucleotide microarrays (Human Genome CGH 244A microarrays, Agilent Technologies, Palo Alto, CA, USA), which contained probes across 236,000 coding and non-coding human regions (hg18 assembly), providing a representation of the human genome at an average of 8.9 Kb median probe spacing (7.4 Kb in Refseq genes). Labeling of digested and purified sample and reference DNA, hybridization, and washings were done according to the manufacturer's protocols. The images were scanned with Agilent microarray scanner G2565AA (Agilent Technologies) and analyzed with Agilent G2567AA Feature Extraction software (v9.5; Agilent Technologies). DNA copy numbers were detected using the CGH Analytics software v3.5.14 (Agilent Technologies). The DNA analytics settings were as follows: ADM-2, threshold 6.0, and filter 4 probes. All DNA copy number changes observed in the regions known to be polymorphic according to the CGH Analytics software and/or according to the Database of Genomic Variants [16] were excluded from the final results.

Statistical Analysis

Genomic instability, measured as the number of aberrations per tumor detected by array CGH, was tested for association with all other parameters by means of Kruskal Wallis test for nominal variables and Spearman's rho statistics for ordinal variables. Moreover, clinical parameters, as well as the most frequent genetic aberrations

detected by cytogenetics/FISH/PCR and genomic instability were tested for prognostic significance. These potentially prognostic variables were categorized following the limits previously cited [1]. In the case of array CGH data, the median of genomic aberrations per tumor was used (median=3). Therefore, cases with three or less aberrations were grouped apart from cases with four or more. EFS and overall survival (OS) rates (evaluated from the time of diagnosis) were estimated with the Kaplan-Meier method. The survival curves were statistically compared by the log-rank test. Moreover, a multivariate Cox regression was applied. All statistical analyses were performed using SPSS v14.0 (Lead Technologies Inc., Charlotte, NC, USA) and considering a p-value <0.05 as statistically significant. The Bonferroni method was used to adjust for multiple comparisons when needed.

RESULTS

A total of 68 patients with AML were analyzed, from which five corresponded to secondary AML and the rest to *de novo* AML. Three patients presented Down syndrome and one of them died shortly after diagnosis; therefore, a transient leukemia could not be discarded. The age range was from 1 day to 17 years old and the male/female proportion was 34/34. The number of patients for each FAB subtype was 6 M1 (9%), 17 M2 (25%), 8 M3 (12%), 1 M3v (2%), 9 M4 (13%), 2 M4eo (3%), 18 M5 (26%), 4 M6 (6%), and 3 M7 (4%). The secondary leukemias were M5 (four cases) and M6 (one case). Table I shows the results from cytogenetics, FISH, PCR and array CGH of the 24 cases with array CGH data; all other results are shown in Supplementary Table S2.

Detection of Cytogenetic/FISH Abnormalities

Cytogenetic results were obtained from 59 patients, 43 (73%) of them exhibited chromosomic abnormalities and 16 (27%) showed a normal karyotype (Supplementary Table S2). FISH analysis for the main AML cytogenetic alterations confirmed the cytogenetic results for 24 cases; sixteen cases had other cytogenetic abnormalities differing from those analyzed by FISH, and FISH experiments did not give additional information, with the exception of one case (*MLL* rearrangement); and three samples could not be analyzed by FISH because of insufficient sample material. In the 16 cytogenetically normal cases, no genetic abnormalities were detected by FISH. In those nine cases with no cell growth, FISH analysis was useful for detecting rearrangements in five cases, whereas no rearrangement was noticed in the other four cases. Overall, seven patients showed the t(8;21) or *AML1*/*ETO* rearrangement, 13 had *MLL* rearrangements, four had inv(16)/t(16;16) or *CBFB* rearrangements, seven had t(15;17) or *PML/RARα* rearrangement, two had a loss of chromosome 7, five presented complex karyotype (three or more numerical/structural cytogenetic changes), and 26 had other alterations (Supplementary Table S2).

Detection of *FLT3* and *NPM1* Mutations by PCR

A total of 54 patients (79%) were analyzed for *FLT3*-ITD mutations; forty six of these patients (85%) had the wild type of this gene whereas rest of the eight subjects (15%) presented the mutation (Supplementary Table S2). Four of these cases with mutations had shown a normal karyotype in the cytogenetics and FISH analysis. Moreover, two out of eight cases with *FLT3*-ITD were diagnosed for acute promyelocytic leukemias. In addition, 15 cases (cytogenetically normal or with no

cytogenetic/FISH results) were analyzed for *NPM1* mutations and two of them exhibited the mutated alleles (cases 4 and 27).

Detection of Genomic Imbalances by Array CGH

A molecular genome-wide approach, such as array CGH, was performed to identify copy number changes in 24 samples. All cases except one (case 42) showed one or several regions with gains and/or losses of genetic material (Table I) with an average of 3.6 altered regions (range 0-11). Overall, 87 genomic imbalances were detected; of which 59 were gains (68%) and 28 were losses (32%). All genomic imbalances detected by cytogenetics were recognized by array CGH, whereas most of the altered regions revealed by array CGH were undetectable by conventional karyotyping. Most of the observed alterations were not recurrent. The only recurrent changes were present in few cases; gains of 8q24.3 or 11p15.5-p15.4 in four cases (16%) and gains of 2p21 or 2q37.1 or losses of 4p16.3, 7q31.1 or chromosome Y in 2 cases (8%). The genes involved in each of the imbalanced regions are reported in Table I (except for regions with more than 15 genes), with special marks for those known to be related to AML or cancer. Interestingly, the deletion of *RUNX1T1* (*ETO*) gene in case 45 was confirmed by FISH analysis with the AML1/ETO Dual Color, Dual fusion Translocation Probe. Nearly half of the cells displayed only one fusion signal, two green (*AML1*) signals and one orange (*ETO*) signal, representing a translocation plus deletion of the 5' *ETO* region (Supplementary Figure S1). Moreover, genomic instability, measured as the number of array CGH alterations, did not show any significant association with all other patient and clinical characteristics (Supplementary Table S3).

Correlation of Clinico-Biological Parameters and Genetic Changes for Survival

The potential prognostic variables, such as clinical parameters, genetic aberrations detected by cytogenetics, FISH, or PCR and the number of array CGH aberrations per tumor, were correlated to OS and EFS. Table II shows the data of 5-year OS and EFS for each factor. Clinical characteristics, such as gender, age at diagnosis, white blood cell (WBC) count, hemoglobin, and platelet count did not show a significant association with OS nor EFS. The same happened with FAB type; however, in this case it was possible to clearly observe three subgroups with different survival trends (Supplementary Figure S2). Later, cases were grouped according to these arbitrary FAB type groups even though they are genetically and biologically distinct: M3/M4, M5/M6, and M1/M2/M7, and their survival curves showed to be significantly different (Figure 1) (p=0.01 for OS; p=0.005 for EFS). After pairwise comparisons at the adjusted 0.017 level of significance, M3/M4 appeared to have statistically significant better prognosis than M1/M2/M7 subgroup (p=0.003 for OS; p=0.001 for EFS). Four out of five patients diagnosed with secondary leukemias died of disease (the fifth case was censored at month 52) (p=0.028 for OS). Apart from clinical features, genetic alterations detected by cytogenetics and/or FISH analysis did not show any significant correlation with survival (Supplementary Figure S3). However, the OS curves showed a trend as follows (from highest survival to lowest survival): inv(16)/t(16;16), t(15;17), *MLL* rearrangements, monosomy 7, other karyotypes, t(8;21), and complex karyotypes. The EFS curves showed this trend: inv(16)/t(16;16), t(15;17), *MLL* rearrangements, other karyotypes, t(8;21), complex karyotypes, and monosomy 7. The single case with del(5q) was censored at month 49. Later, patients were grouped according to these trends: favorable [inv(16)]; adverse (t(8;21) or complex karyotype); and intermediate (all other groups), and borderline correlations

with OS (p=0.067) and with EFS (p=0.079) were observed. In relation to *FLT3*, cases with ITD and those without ITD did not show significant differences in the survival among this series, even after removing APL cases. Three out of the four cases with normal karyotype and *FLT3*-ITD died of disease. One of these patients with poor prognosis (case 49) showed both alleles with the length mutation. The *NPM1* mutation was observed in two out of 15 patients and one of them died of disease. This patient also showed *FLT3*-ITD mutation. The other patient with the *NPM1* mutation could not be analyzed for *FLT3*-ITD mutations because of insufficient sample.

Genomic instability was observed to be a statistically significant prognostic factor. Cases with four or more genomic aberrations had worse prognosis than those with less than four (p=0.029 for OS, and p=0.041 for EFS) (Figure 2). The multivariate analysis was unable to contribute new information, other than the bivariate analysis with the Kaplan-Meier curves, due to the relatively small number of patients with childhood AML on which array CGH was performed.

DISCUSSION

Nowadays, childhood AML is managed through the use of risk-adapted therapy, requiring explicit knowledge of clinical and biological data that may help in stratifying patients by risk groups. Therefore, studies are required to describe clinical and genetic characteristics of childhood AML patients and to correlate these factors with the outcomes.

The correlation of the overall outcome of patients with their clinical characteristics was not significant, except for FAB type and secondary leukemias. The age has been identified as a prognostic factor in childhood AML, but we detected its association with the survival at only 10% of significance level. In several reports published since 2000, the age effect is not observed to be large, even though the older age tends to be indicative of adverse evolution [2, 17-20]. In relation to FAB type, associations between FAB subtype and prognosis have been variable. APL (AML M3) is usually associated with good outcome and M7 with poor survival. Our series confirms these correlations.

In the present study cytogenetic and molecular characteristics showed survival trends that correlated with what has been previously published, except for t(8;21) [1]. However, the differences were not significant, most probably due to the availability of fewer samples belonging to each category. In majority of the studies, though t(8;21) and inv(16) are grouped together as core-binding factor AML with relatively favorable prognosis, patients with t(8;21) have shown a shorter survival rate as compared to those with inv(16) [20-22]. In our series, five out of seven patients with t(8;21) died of disease, four of which expired due to transplant complications (cases 30, 34, 44, and 45). These patients underwent transplant following the protocol established in the hospital until 2002, when a stratification protocol was implemented. Therefore, post BM transplant complications may be responsible for the poor prognosis observed in these patients, rather than the cytogenetic translocation. Moreover, the poor outcome of patients with t(8;21), may be related to the absence of high-dose cytarabine in the

treatment regimens, as suggested in other childhood AML series [21]. Finally, mutations in the *KIT* gene (not analyzed in the present study) have been associated with inferior OS and relapse in pediatric patients with t(8;21) [23]. However, comprehensive studies are required to confirm the prognostic significance of these mutations.

In addition, *FLT3* mutations were not correlated with poor survival rate, contrary to some of the previous observations [24, 25]. The small number of cases assessed in this research work may be responsible for these differences. Moreover, some recent studies have reported that poor prognosis is only conferred by high *FLT3* mutant to *FLT3* wild-type allelic ratio [26]. Unfortunately, we could not calculate the allelic ratio but the only case with one *FLT3*-ITD allele and loss of the wild-type allele died of disease. As regards to *NPM1* mutation, it was observed in only two patients among whom one died of disease. This case also had *FLT3*-ITD mutation. It has been suggested that patients with *NPM1* gene alterations in the absence of *FLT3*-ITD mutations have better long-term survival chances as compared to others [27].

To our knowledge, this is the first study in which array CGH analysis has been carried out in childhood AML patients, providing a comprehensive high resolution description of gains and losses of genetic material in leukemic cells. Genomic imbalances were detected in 23 out of 24 childhood AML cases representing all AML subtypes, and most of them were not recurrent, what agrees with previous studies carried out on adult patients [9, 11]. The most frequent alterations were gains of 8q24.3 and of 11p15.5-p15.4 each in four cases out of 24. In both regions, there

were many genes involved, making it difficult to distinguish the gene/s that may promote or contribute to AML development.

Overall, few genes from the imbalanced regions have been reported in the literature to be related to AML: *AKT1* (gained in case 31), *RUNX1* (lost in case 33, an M2 AML), *LTB* (lost in case 37), *SDC1* and *RUNX1T1* (gained and lost in case 45, an M2 AML, respectively), and *JAK2* (gained in case 48). Interestingly, *RUNX1* and *RUNX1T1* are the key factors in M2 AML, characterized by the t(8;21)(q22;q22), which gives rise to the transcription factor fusion protein, RUNX1-RUNX1T1 (also known as AML1-ETO). It has been observed that RUNX1 protects hematopoietic stem cells from oncogenic insult [28] and its haploinsufficiency is associated with AML development [29]. *RUNX1* deletion has been observed in about 4% of AML cases with t(8;21) [7, 30]. Moreover, *RUNX1T1* deletion has been observed in 9% of patients with t(8;21) [31]. So far, no prognostic information has been obtained from the reported cases with *RUNX1* or *RUNX1T1* deletions, but in our series both patients died of disease. Regarding *AKT* gene, the PI3-kinase/Akt pathway is constitutively active in primary AML cells [32]. It is known that constitutive activation of the PI3K-Akt pathway occurs due to the amplification of the *PIK3* or the *AKT*, or as a result of mutations in the components of the pathway [33]. Lymphotoxin β (LTB) expression is minimized in malignant myeloid cells [34], which can be perfectly correlated with the loss of this gene observed in one case. Expression of syndecan-1 (SDC1) has been observed to be stronger in AML cells than in normal myeloid cells [35], that can be correlated with the gain of this gene in case 45. To our knowledge, no reports about *LTB* loss or *AKT1* or *SDC1* gains in AML cases have been reported up to now. Finally, a low incidence level of single mutations of *JAK2* gene with

subsequent activation of tyrosine kinase has been reported in AML [36]. Interestingly, numerical gains of *JAK2* gene, ranging from 3 to 25 copies, have been observed in myeloproliferative disorders [37]. This gene has been suggested as a candidate amplified gene in AML cases with complex karyotypes after microarray-based molecular characterization [8]. Apart from this, there are other 30 genes gained or lost in our series (marked in Table I) related to tumorigenesis, according to the literature (cancer genes). These cancer genes are involved in some of the acquired functional characteristics of the tumoral process, such as cell growth, evasion of apoptosis, limitless replicative potential and metastasis [38]. Half of the 52 imbalanced regions (apart from those with more than 15 genes and those without known genes) harbored at least one of these cancer genes. Moreover, 15 out of 24 cases exhibited at least one cancer related gene gained or lost and this number increases to 20 out of 24 (83%) if regions with more than 15 known genes are included. Notably, the cancer gene imbalances identified in this study have not been described so far in AML children or adult patients.

We suggest that the genomic instability shown by leukemic cells in all cases except one, involves gain or loss of one or more than one cancer genes, which would represent cooperative events to the known recurrent translocations or to other genetic alterations, according to the multi-step model of cancer. The cancer gene imbalances identified in 83% of cases could correspond to driver alterations, while the other alterations could be passengers, with no contribution to cancer development [39].

An interesting finding in the present study was the correlation of genomic instability with poor survival rate in patients with childhood AML. The presence of four or more genomic imbalances was one of the most important prognostic factors, and it was independent of other clinical and genetic features. This corroborates another study that demonstrated the prognostic importance of genomic imbalances detected by array CGH in adult patients with AML [9]. Moreover, association of genomic instability with poor survival has been observed in other tumors, such as Ewing's sarcoma [40, 41], bladder cancer [42] and breast cancer [43].

In conclusion, we were able to genetically characterize a series of childhood AML by cytogenetics, FISH, PCR, and/or array CGH. Genomic imbalances detected by array CGH were present in all cases except one. Many of the altered regions harbored cancer genes, not previously reported in an AML context and probably corresponding to non passenger alterations that cooperate with the recurrent translocations. Moreover, the genomic instability appeared as a very important prognostic factor for this disease. However, prospective and confirmatory studies are needed.

Declaration of interest: *The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.*

ACKNOWLEDGEMENTS

The authors wish to thank Jessica Martínez, Susana Gaitán, Ángeles Pérez, Laia Puig and Lorena Ripollés for their help in the experiments. The research group has been recognized as a high quality research group by *Generalitat de Catalunya*

(2005SGR 00164). This work was partially financed by the Spanish National Cancer Network (RD060020/1020) from *Ministerio de Sanidad y Consumo*.

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Table I. Results of cytogenetics, FISH analysis, PCR for detection of *FLT3*-ITD, and array CGH.

Case	Karyotype	*AML1/ ETO*	*MLL*	*CBFβ*	*PML/ RARα*	CEP 7	*FLT3*-ITD	array CGH (genes involved)[a]
14	47,XY,inv(16)(p12q22),+22[21] /46,XY[1]			+ (69,5%)			wt	2q35+ (*STK36*, *TTLL4*), 12q13.2+ (*TIMELESS*), +22 (many)
15	47,XX,+8,der(11)t(11;?)(q23;?) [16]/46,XX[3]		+ (50%)				wt	+8 (many)
20	46,XY,t(8;21)(q22;q22)[3]/47, XY,+4,t(8,21)(q22;q22)[15]/46, XY[1]	+ (93,5%)						2q35+ (unknown), +4 (many), 15q21.3+ (*CGNL1*)
22					+[b]			7q22.3- (*COG5*), 13q21.1-31.3+ (many), 14q32.31+ (unknown), 17q24.1+ (*POLG2*, *DDX5*, *CCDC45*), 20q11.22+ (*RBM39*)
23	46,XX,inv(16)(p13q22)[13]/46, XX[13]			+ (78%)			wt	1p22.1- (*FNBP1L*, *BCAR3*, *DNTTIP2*, *GCLM*), 2q33.3- (*MDH1B*, *FASTKD2*, *CPO*, *KLF7*, *CREB1*, *FAM119A*, *CCNYL1*, *FZD5*)
24			+ (53%)				wt	7p22.2- (unknown)
25[c]	47,XX,+21c[28]		–					5q35.3+ (*BTNL3*), 18q21.32+ (*RPS3A*), +21, 22q13.1+ (*MAP3K7IP1*, *MGAT3*)
26	46,XY,t(8;21)(q22;q22)[6]/45, XY,t(8;21)(q22;q22)[3]/46,XY [14]	+ (99,5%)					wt	8q24.3+ (many), 9p21.2-p21.1+ (*LINGO2*), 12q13.3+ (*LRP1*, *NXPH4*, *SHMT2*, *NDUFA4L2*, *STAC3*), -Y (many)
28	47,XX,+mar1,+mar2[5]/46,XX [35]	–	–	–			wt	7q31.1- (unknown)
29		–	+ (65%)				wt	18q12.2- (unknown), 19q13.41- (unknown)
30	45,XY,t(8;21)(q22;q22)[19]/46, XY[6]	+ (99%)					wt	8q22.2- (*STK3*), Xq21.31-q21.32- (*PABPC5*, *PCDH11X*), -Y (many)
31	46,XY,t(11;22)(q23;q23)[20]		+ (68%)				wt	1p31.1- (*SLC44A5*), 14q32.33+ (*ADSSL*,

								SIVA, ***AKT1***, *PLD4*, *CDCA4*, *GPR132*, *JAG2*, *NUDT14*, *TBTD6*, *BRF1*, *PACS2*, *MTA1*, *CRIP1*, *CRIP2*), Xp22.31+ (*HDHD1A*, *PNPLA4*)
33[d]	45,XY,add(3)(q29),-7[7]					+ (88%)		1q44+ (*SH3BP5L*, *ZNF672*, *ZNF692*), 3q24-q29+ (many), -7 (many), 21q22.12- (***RUNX1***)
35	46,XX,t(15;17)(q22;q21)[20]				+ (54%)		FLT3-ITD	4p16.3- (unknown), 15q15.1-q15.2- (*CAPN3*, *ZFP106*, *SNAP23*, *LRRC57*, *CEP27*, *CDAN1*, *TTBK2*), 17q12- (*NLE1*, *UNC45B*, *AMAC1*, *SLFN5*, *SLFN11*, *SLFN12*, *SLFN13*, *PEX12*, *AP2B1*, *RASL10B*, *GAS2L2*, *MMP28*, *TAF15*, *CCL5*, *RDM1*)
36	46,XY[20]		–				wt	3p21.31+ (many), 4p16.3- (unknown), 5q35.3+ (*PRR7*, *DBN1*, *DDX41*), 6p22.1+ (*HIST1H* cluster), 9q33.3-q34.11+ (many), 16p13.3+ (many), 17q21.32+ (*KPNB1*, *OSBPL7*, *MRPL10*, *SCRN2*, *CDK5RAP3*, *PNPO*, *ATAD4*, *NFE2L1*, *CBX1*), 19q13.2+ (*POU2F2*, *DEDD2*, *ZNF526*, *GSK3A*, *CIC*, *PAFAH1B3*, *ERF*), 21q22.11+ (*GART*, *SON*), Xp11.23+ (many)
37			+ (69%)				FLT3-ITD	2q31.1+ (unknown), 3p21.31+ (*COL7A1*, *UQCRC1*, *SLC26A6*, *CELSR3*), 6p21.33- (*TNF*, ***LTB***), 7q31.1- (unknown)
40	48,XX,+9,+22[20]		–					2p21+ (*HAAO*), 8q24.3+ (many), +9 (many), 11p15.5-p15.4+ (many), 11q12.3- (*SLC22A10*, *SLC22A9*), 13q34+ (*FAM706*), 16p11.2- (*ITGAM*), +22 (many), Yp11.2+ (unknown)

41	46,XY[20]	–	–	–			wt	1p36.13+ (*CLCNKB*, *FAM131C*), 1q22- (*GBA*), 3q23+ (*TFDP2*, *GK5*, *XRN1*)
42	46,XY,t(11;17)(q23;q25)[20]		+ (95%)				wt	NONE
43			+ (26,5%)					9q32+ (*KIF12*, *COL27A1*), 10p13- (*CDC123*), 11p15.5-p15.4 + (many)
44	46,XX,t(8;21)(q22;q22)[20]	+ (99%)					wt	2q37.1+ (*ALPPL2*, *ALPI*, *ECEL1*, *CHRND*, *CHRNG*, *TIGD1*, *EIF4E2*), 8q24.3+ (many), 11p15.5-p15.4+ (many), 17q25.3+ (many), Xq26.2-q26.3- (*PHF6*, *PLAC1*, *HPRT1*, *MOSPD1*)
45	46,XY,del(7)(q33),t(8;21)(q22; q22)[20]	+[e] (81,5%)					wt	1q42.13+ (*GUK1*, *GJA12*), 2p24.1+ (***SDC1***), 2p21+ (*OXER1*, *HAAO*), 2q37.1+ (*DIS3L2*, *ALPP*, *ECEL1*, *ALPPL2*), 3q21.3+ (*H1FOO*, *PLXND1*), 7q31.32-q36.3- (many), 8q21.3-q22.1- (***RUNX1T1***), 8q24.3+ (many), 10q24.32+ (*FGF8*, *NPM3*), 11p15.5-p15.4+ (many), 11p11.2+ (*ZNF289*, *PACSIN3*)
47	46,XX[30]	–	–	–	–		FLT3-ITD	13q14.2-q14.3- (*CAB39L*, *SETDB2*, *PHF11*, *RCBTB1*), 13q14.3- (*UTP14C*, *NEK5*)
48	50,XY,+5,del(6)(q15q24),+10, der(12)t(1;12)(q25;q24.3),+13, +18[6]/50,XY,+5,del(6)(q15q24),i(7)(q10q10),+10,der(12)t(1; 12)(q25;q24.3),+13,+18[7]/46, XY[7]		–				wt	9p24.1+ (***JAK2***, *INSL6*)

The empty cells are the experiments not done because of lack of cell growth (for cytogenetics), not performed (for FISH), or lack of material (for FISH or PCR).
wt: wild type; many: more than 15 genes involved

[a] genes involved in each region are in parenthesis, genes known to be involved in AML are marked in bold, and those involved in cancer are underlined; [b] performed by RT-PCR; [c] patient with Down syndrome; [d] patient with Fanconi anemia; [e] 17% of cells with translocation (der(8) and der(21)), 47% of cells with translocation plus deletion at chromosome 8 (der(8) but no der(21))

Table II. Comparative 5-year overall survival (OS) and event free survival (EFS) by clinical and molecular characteristics of AML patients.

Feature	Number (%)	% OS ± SE	*P* value	% EFS ± SE	*P* value
Gender					
Male	34 (50)	47.1 ± 8.6	0.188	41.2 ± 8.4	0.145
Female	34 (50)	64.4 ± 8.3		58.2 ± 8.6	
Age					
≤ 2 years	17 (25)	76.5 ± 10.3	0.087	64.7 ± 11.6	0.217
> 2 years	51 (75)	48.6 ± 7.1		44.1 ± 7.1	
WBC					
≤ 50 x 10^9 /L	46 (70)	54.1 ± 7.4	0.504	47.2 ± 7.5	0.405
> 50 x 10^9 /L	20 (30)	65.0 ± 10.7		60.0 ± 11.0	
Hemoglobin					
< 8 g/dL	20 (32)	65.0 ± 10.7	0.697	48.1 ± 11.7	0.844
8-10 g/dL	27 (44)	51.6 ± 9.7		47.9 ± 9.7	
>10 g/dL	15 (24)	60.0 ± 12.6		60.0 ± 12.6	
Platelet					
≤ 50 x 10^9 /L	30 (48)	56.0 ± 9.2	0.772	48.6 ± 9.4	0.721
> 50 x 10^9 /L	33 (52)	60.6 ± 8.5		54.5 ± 8.7	
FAB type					
M1	6 (9)	33.3 ± 19.2	0.203	16.7 ± 15.2	0.065
M2	16 (24)	36.5 ± 12.3		30.0 ± 11.8	
M3	9 (13)	77.8 ± 13.9		77.8 ± 13.9	
M4	11 (16)	72.7 ± 13.4		72.7 ± 13.4	
M5	18 (26)	61.1 ± 11.5		50.0 ± 11.8	
M6	5 (7)	60.0 ± 21.9		60.0 ± 21.9	
M7	3 (4)	33.3 ± 27.2		33.3 ± 27.2	
FAB type group					
M3/M4	21 (31)	76.2 ± 9.3	0.010*	70.3 ± 10.3	0.005*
M5/M6	23 (34)	60.9 ± 10.2		52.2 ± 10.4	
M1/M2/M7	24 (35)	32.8 ± 9.7		28.6 ± 9.4	
Secondary leukemias					
secondary	5 (7)	20 ± 17.9 (last case alive censored at 52 months)	0.028*	20 ± 17.9 (last case alive censored at 52 months)	0.108
non-secondary	63 (93)	58.5 ± 6.2		51.9 ± 6.4	
Cytogenetics/FISH					
inv(16)/t(16;16)	4 (6)	100.0 ± 0.0	0.324	100.0 ± 0.0	0.110
t(8;21)	7 (11)	28.6 ± 17.1		28.6 ± 17.1	
MLL rearrangement	13 (20)	61.5 ± 13.5		61.5 ± 13.5	
t(15;17)	7 (11)	71.4 ± 17.1		71.4 ± 17.1	
monosomy 7	2 (3)	50.0 ± 35.4		0.0 ± 0.0	
del(5q)	1 (2)	censored at 49 months		censored at 49 months	
complex karyotype	5 (8)	20.0 ± 17.9		20.0 ± 17.9	
other alterations	25 (39)	48.0 ± 10.0		36.0 ± 9.6	
Genetic groups[a]					
favorable	11 (17)	100.0 ± 0.0	0.067	100.0 ± 0.0	0.079
intermediate	45 (70)	55.3 ± 7.3		46.5 ± 7.3	
adverse	8 (13)	25.0 ± 12.5		25.0 ± 12.5	
FLT3					
wt	46 (85)	56.1 ± 7.4	0.672	49.2 ± 7.5	0.400

ITD	8 (15)	50.0 ± 17.7		37.5 ± 17.1	
Array CGH					
<4 aberrations	15 (63)	66.7 ± 12.2	0.029*	66.7 ± 12.2	0.041*
>=4 aberrations	9 (38)	33.3 ± 15.7		33.3 ± 15.7	

[a] Favorable: inv(16)/t(16;16); intermediate: *MLL* rearrangement, t(15;17), monosomy 7, or other karyotypes; adverse: t(8;21), or complex karyotype. *: statistical significance ($p<0.05$).

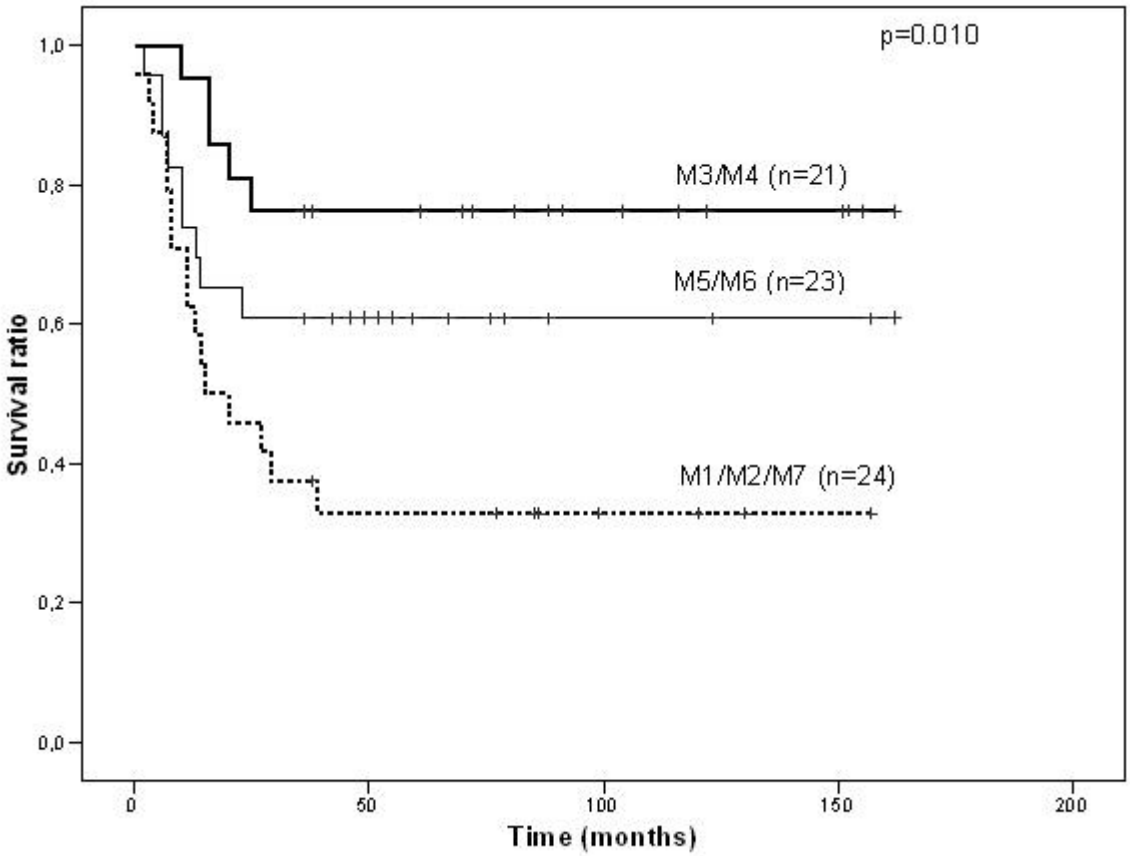

Figure 1. Overall survival Kaplan–Meyer plot of childhood AML according to FAB type groups.

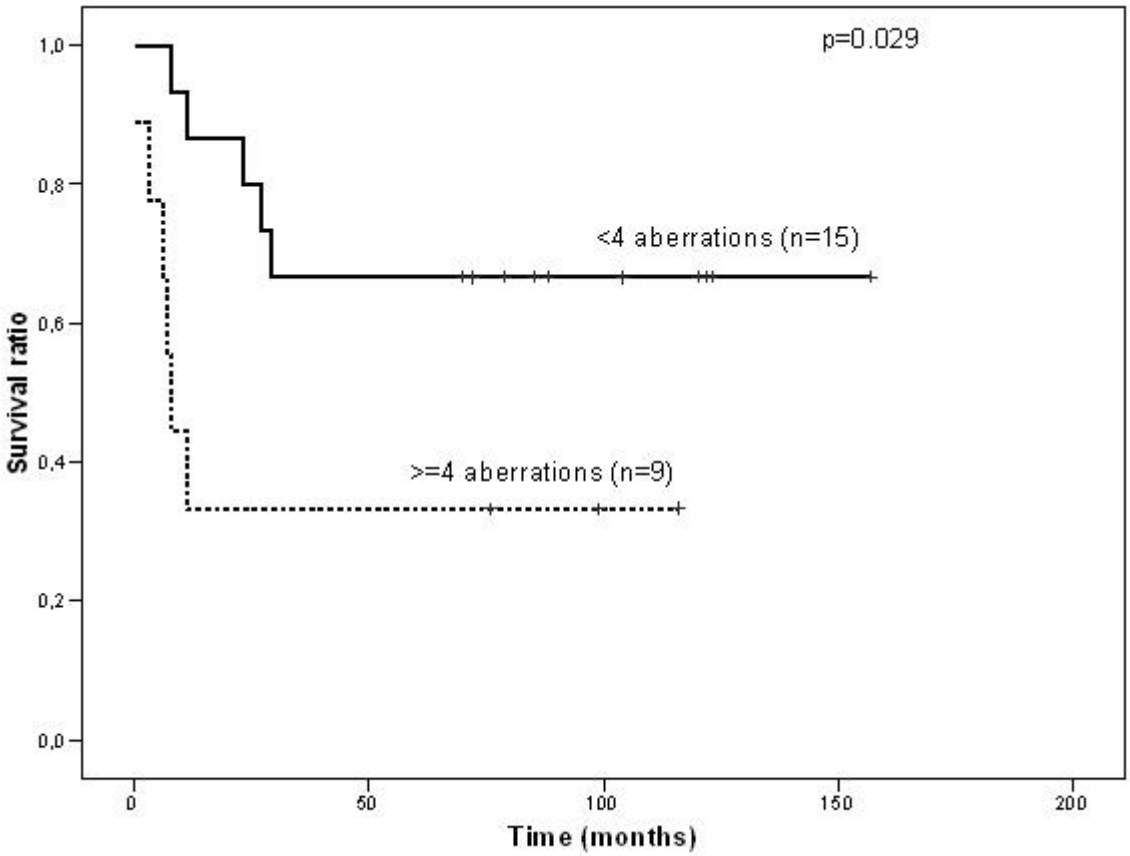

Figure 2. Overall survival Kaplan–Meyer plot of childhood AML according to array CGH results (four or more genomic imbalances vs. less than four).