Infant acute leukemia with lineage switch at relapse expressing a novel t(4;11)(q21;q23) MLL-AF4 fusion transcript

Detection of a transcript MLL-AF4 in an infant with acute leukemia at diagnosis and relapse

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Abstract

Background. A high occurrence of translocation t(4;11)(q21;q23) was reported in infant acute lymphoblastic leukemia (ALL) leading to the fusion of the mixed lineage leukemia (MLL) gene on chromosome 11 and the AF4 gene on chromosome 4. More than 50 distinct MLL-AF4 types of fusion have been previously identified, none of those reported matching the peculiarities found in an infant ALL case to be reported below. Materials and methods. Molecular tests were performed for the detection of TEL-AML1, BCR-ABL(p190), E2A-PBX1, and MLL-AF4 in the peripheral blood sample of a 21 days new-born boy suspected of ALL. An unexpected MLL-AF4 fragment was identified, further purified, and later analyzed by sequencing. Flow cytometry analyses were carried out at diagnosis and relapse on a FACSCanto-II cytometer (Becton-Dickinson). Results. The patient was found to be positive for the MLL-AF4 transcript, with an unusually long-sized product and a previously undescribed sequence (in-frame fusion between exon 12 of MLL and exon 4 of the AF4 gene). The immunophenotypic analyses also showed a particular development: while at diagnosis a dominant malignant clone displaying a B lymphoid precursor phenotype was described, at relapse a malignant monocytoid population predominantly expanded. The presence of MLL-AF4 e12-e4 transcript was still manifest at relapse, without other transcript characteristic for myeloid lineage. Conclusions. To our knowledge, this is the first report of a MLL-AF4 rearrangement revealing this complex transcript with new breakpoints in MLL. Its early detection may predict an immunophenotypic switch and may assist the clinicians in designing optimized therapies.

Keywords: acute lymphoid leukemia, fusion proteins, immunophenotypic switch

Rezumat

Introductie. Pacienții cu leucemie acută limfoblastică (LAL) neonatală prezintă în mod frecvent, o translocație particulară, t(4;11)(q21;q23), care ducă la fuziunea genei MLL (mixed lineage leukemia) de pe cromozomul 11 cu gena AF4 de pe cromozomul 4. Deși în literatura de specialitate au fost descrise anterior cazuri de

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Introduction

Translocations employing mixed lineage leukemia (MLL) gene on 11q23 occur in different categories of leukemia with a high frequency (1, 4). One specific type of translocation, t(4;11) (q21;q23), involving the AF4 gene partner on chromosome 4, is reported to occur in 50–70% cases of infant acute lymphoblastic leukemia (ALL), while in pediatric and adult ALL cases its frequency is only 5% (2, 5-8). The breakpoint region of the MLL gene with the highest occurrence has been described as being located on a fragment of 8.3 kb, between exons 8 and 12. While breakage occurs most frequently in introns 9 and 10 in pediatric and adult ALL, in infant ALL the intron 11 is most commonly involved (9). The breakpoint cluster region of the AF4 gene is also described, and found to be larger and located within a region of 40 kb. The most frequent fusion point in MLL-AF4 products is exon 4; rarely, exons 5, 6 and 7 are also involved (2, 9).

Reports of dissimilar MLL-AF4 transcripts, with diverse length and sequence due to different inclusion of exons within the breakpoint regions, have been already published (1, 2, 10). About 55% of infant MLL-AF4 transcripts contain exon 11 of the MLL gene and exon 4 of the AF4 gene (8). Another common phenomenon reported occurs as a consequence of differential splicing, leading to more than one transcript in the same leukemia patient (11, 12).

From an immunophenotypic perspective, the translocation t(4;11)(q21;q23) has been associated with a pro-B-ALL maturation stage (CyCD79a+, CD19+, CD34+, CD10−, CD24−) (7, 13-15), co-expression of myeloid antigens (CD15 and CD65) (14, 16-18), as well as NG2 co-expression (19). According to the most recent edition of the WHO Classification of Hematopoietic and Lymphoid Tissues (20, 21), MLL-AF4 positive ALL cases belong to a subset of mixed phenotype acute leukemias (MPAL), formerly referred to as either ALL with aberrant expression of myeloid markers or biphenotypic leukemias (22, 23).

The presence of MLL-AF4 has been identified as an unfavorable prognostic factor in infant leukemia (5, 6, 24-26), while in pediatric cases, different age groups have different prognosis (5, 6). Similarly, all MPAL subsets have been reported to have a dismal prognosis (21, 26, 27), therefore, an intensive multi-agent chemotherapy, age-related dose adjustment or treatment intensification may be essential when such a subtype is identified.
Material and methods

Patient clinical status and initial laboratory findings

A 21 days new-born boy, was transferred from the Intensive Care to the Hemato-Oncology Unit of “St. Mary” Clinical Emergency Hospital for Children, Iasi, Romania, with poor general status, pallor, micro-poly-adenopathies, hepatosplenomegaly, and moderate dehydration due to refusal of breastfeeding. An abrupt increase of the White Blood Cell Count (WBC) (from 20000/mm c to 40000/mm c in only 24 hours) was associated with severe thrombocytopenia (19000/mm c) and a substantial presence of blasts with lymphoid morphology both in the bone marrow (92%) and peripheral blood (PB) (52%).

Immunophenotyping by flow cytometry

At diagnosis, the immunophenotypic expression of the following markers was investigated by flow cytometry in a PB sample (40999 cells/mm c), at the cell surface (s): CD45, CD14, CD71, CD5, CD10, CD19, CD33, CD13, HLA-DR, CD34, CD117, CD4, CD8, CD3, CD16+56, CD20, CD22, IgM CD38, and intracellularly (ic): CD20, IgM, TdT, CD79a, myeloperoxidase – MPO. When the patient relapsed, the expression of CD15, CD36, CD11b, CD64, CD16 and all of the markers stated above was evaluated by flow cytometry in a PB sample (101850 cells/mm c). Up to six colour panels and a FACSCantoII Cytometer equipped with FACSDiva 6.1.2. Software (Becton Dickinson) were used.

RNA extraction

Molecular tests were performed at diagnosis for the detection of 4 fusion genes: TEL-AML1, BCR-ABL(p190), E2A-PBX1 and MLL-AF4 (routinely evaluated when a lymphoid lineage is involved). At relapse, the presence of an additional set of transcripts (routinely evaluated when a myeloid lineage is involved) was investigated: PML-RARA, AML-ETO, and CBFB-MYH11. PB lymphocytes isolated by red blood lysis (Promega Inc, Madison, WI, USA), were washed with phosphate buffer saline solution (PBS), resuspended at a concentration of 3x10⁷ cells per 1 mL of Guanidin Thiocianate reagent (EZ-RNA Total RNA Isolation Kit - Biological Industries), and stored at −70°C until use.

Reverse transcription and polymerase chain reaction (PCR) analysis

cDNA was synthesized from 4 µg of total RNA (500ng/µl) using the following mix: 4 µl Improm™ 5 X Reaction Buffer (Promega), 0.5 mM of each dNTP, 20 U Recombinant RNasin® Ribonuclease Inhibitor (Promega) and 1 µl Improm II™ Reverse Transcriptase (Promega). Fifteen µl of the reverse transcription reaction mix described above were added onto RNA, only after the template and primers (0.5 µg random hexamers-Promega) were heated at 70°C/5 min and then chilled on ice for 5 min. The annealing, extension, and enzyme inactivation parameters were 25°C/5 min, 42°C/one hour, and 70°C/15 min, respectively. Then the cDNA was diluted to a final volume of 100 µl.

Five µL of cDNA (equivalent of 100ng of RNA) was PCR amplified in a 25 µL reaction volume either to check the integrity of cDNA (with the ABL reference gene) (28) or for the detection of fusion gene transcripts, using, 5 X Green GoTaq® Flexi Buffer (Promega), 200 µM dNTPs, 10 pmol of forward and reverse primers (Table 1) and 1U GoTaq® Hot Start Polymerase (Promega). All amplification steps were carried out conform to the standardized BIOMED-1 RT-PCR protocols (8).

cDNAs from patients previously diagnosed in our center as positive for each of the fusion proteins assessed were used as positive controls.

After an initial denaturation (95°C/2 min), 35 cycles of denaturation (94°C/30 sec), annealing (55°C, for ABL and 65°C, for fusion genes/60 sec), and extension (72°C/1 min), followed by a final extension (72°C/10 min), were performed (PalmCyclerTM, Corbett, LifeSciences/Qiagen, Germantown, MD, USA).

The PCR products were size-fractionated by electrophoresis on a 2% agarose gel and stained with ethidium bromide in Sub-Cellp
System for Submerged Horizontal Electrophoresis (Bio-Rad Laboratories Inc, Hercules, CA, USA) at 5 V/cm. Following electrophoresis, gels were visualized under UV in a G:BOX Chemi TM Gel Documentation System (Syngene, Cambridge, UK) and interpreted with GeneSnapTM and GeneTools TM softwares.

To confirm the presence of the uncommon MLL-AF4 fragment identified, additional primer pairs and standards were used (MLL-AF4 e11-e5, e10-e4, e9-e5 from IPSOGEN, Luminy Biotech Enterprises, Marseille, France) (Table 2).

The primer sequences were previously described (29). The amplification parameters were: initial denaturation (95°C/ 2 min), followed by 35 cycles of denaturation (94°C/ 30 sec), annealing (60°C/ 60 sec), and extension (72°C/ 1 min), with a final extension (72°C/ 10 min), using PalmCyclerTM (Corbett, LifeSciences/ Qiagen, Germantown, MD, USA).

**Sequence analysis**

The MLL-AF4 fragment was purified from gel, with Wizard® SV Gel and PCR Clean-up System Promega Inc, Madison, WI, USA (according to the manufacturer’s instructions) and then analyzed by Sanger sequencing.

The product was sequenced in forward and reverse reactions, using a Beckman Coulter kit (Dye Terminator Cycle Sequencing - DTCS, Quick Start Kit), a Beckman Coulter analysis software (CEQ8000 Investigator), primers previously described (29), and the following se-
sequencing parameters: 30 cycles of 96°C/20 sec, 50°C/20 sec, 60°C/4 min.

When the molecular response to treatment was investigated, RealTime PCR was performed using the same primers, a TaqMan sonde (6-Fam/Tamra labeled) with the following sequence: 5’-CATGCGCGCTCCTTTGAAGC-3’ (29) and the same standards described above as positive controls (Ipsogen, Luminy Biotech Enterprises, Marseille, France) (Table 2). In parallel it was used a reference ABL kit from IPSOGEN (Luminy Biotech Enterprises, Marseille, France). All protocols used have been approved by the Ethics Committee of the institution within which the tests were undertaken.

Results

Patient management and clinical evolution

Based on the cyto-morphologic, molecular, and immunophenotypic assays performed with the occasion of patient’s first hospital admission, the initial diagnosis was ALL with B-cell precursors and aberrant expression of the myeloid marker CD33. The treatment was established according to the INTERFANT 99 protocol. During the cortico-sensitivity test, the number of leukocytes increased to 135000/mm c, therefore the patient was assigned to the high-risk group, being considered a “poor responder” to prednisone. The patient attained morphological and molecular remission subsequent the induction chemotherapy.

One year since diagnosis the patient presented in the Hemato-Oncology Unit with bad clinical status and fever. When re-evaluated, the patient presented hyperleucocytosis (101850/mm c), a high number of blasts in the PB (~80%), and a morphologic and immunophenotypic blast description attributed to both, the monocytoid lineage (predominant) and the lymphoid lineage (subdominant). At this point the diagnosis was Acute leukemias of ambiguous lineage, MPAL subtype, with rearranged MLL and early medullary relapse. As a consequence, the patient received allopurinol, hyperhydration, supportive therapy and corticotherapy. No remission was obtained and the patient died two months after relapse.

Detection of the MLL-AF4 transcript with an unusual in-frame fusion pattern

Molecular tests performed at first admission for the detection of TEL-AML1, BCR-ABL(p190), E2A-PBX1, and MLL-AF4, revealed the occurrence of a MLL-AF4 fusion transcript in a PB sample from the infant investigated. None of the other transcripts evaluated was found to be positive. The MLL-AF4 amplified product was around 800 bp in size, which was nearly 370 bp larger than the e9e4 amplifier used as standard in the assay (Figure 1), and about 120 bp larger than the biggest expected amplicon, e11e4 (Table 1).

The presence of the uncommonly larger MLL-AF4 fragment was confirmed with a second amplification, based on additional primer pairs MLL-1/AF4 and MLL-2/AF4, and with
three different MLL-AF4 standards used for comparison (e11-e5, e10-e4, e9-e5) (Figure 2).

As all amplified fragments were clearly size-fractionated by electrophoresis on the agarose gel, the MLL-AF4 fragments of interest were easily cut and purified, in order to allow for the analysis of their particular sequence. Sequence analysis revealed a MLL-AF4 product resulting from in-frame fusion between exons 12 and 4 of the MLL and AF4 genes, respectively (Figure 3).

This sequence was deposited in the international genetic sequence database GeneBank and received the accession number JN169752.1.

Molecular tests performed one year later, at relapse, aimed at the detection of the same TEL-AML1, BCR-ABL(p190), E2A-PBX1, and MLL-AF4 fusion transcripts. Due to the lineage switch noted at relapse, the presence of an additional set of transcripts (routinely evaluated when a myeloid lineage is involved), was investigated: PML-RARa, AML-ETO, and CBFB-MYH11. The presence of the same MLL-AF4(e12e4) transcript was identified at relapse and none of the other transcripts evaluated (either with a lymphoid, or with a myeloid lineage association) was found to be positive.
The lineage switch at relapse

Both immunophenotypic (Figure 4) and cyto-morphologic assays performed at diagnosis revealed the presence of approximately 50% of B-lymphoid precursors in a PB sample with 40000 WBC/ mmc. The immunophenotype of these cells, as assessed by flow cytometry, was suggestive for a pro-B blockage: CD45+ CD19+ HLA/DR+ CD10- CD34+ CD22+- CD20- IgM+ CD79a+ TdT+ CD38+. Among the three myeloid antigens investigated at diagnosis (MPO, CD13, and CD33), only the CD33 antigen was partially positive (on more than 60% of the blast population), indicating either an illegitimate/
aberrant expression of myeloid markers, or a mixed phenotype. We mention that no CD15 or CD64 immuno-staining was performed at diagnosis. Subdominant adult (8%) and less-differentiated (9%) monocytoid cell populations were also noticeable in the PB sample evaluated by flow cytometry at diagnosis (Figure 4).

Immunophenotypic (Figure 5) examination carried out one year later, at relapse, revealed the presence of mixed malignant lineages (lymphoid and monocytoid), in a PB sample, with the predominance of the later: 62% monocytoid cells (promonocytes and monocytes: CD45+ HLA/DR+ CD33+ CD13+ CD117-CD34- CD14+/ CD64+high CD36+ CD13+ CD11b+/ CD2- CD15+ CD16- MPO-); 17% B lymphoid precursor cells (CD45+low CD34+ HLA/DR+ CD19+ CD20s- CD22s- MPO- TdT+low with mixed lineage phenotype: CD64+low CD15+low CD33+/-); 11% granulocytes; 7% lymphocytes.

Discussions

The importance of genetic events in the classification, therapy, and prognosis of ALL has gained recently growing credit. The most up to date WHO Classification of Hematopoietic and Lymphoid Tissues delineates several new-defined ALL entities, such as neoplasias having a strong association with specific recurrent genetic abnormalities or of ambiguous lineage, each having several related subtypes, depending on the presence of distinctive molecular events: t(9;22)(q34;q11.2); BCR-ABL1/ t(v;11q23); MLL rearranged/ t(12;21)(p13;q22); TEL-AML1 (ETV6-RUNX1)/ hyperdiploidy/ hypodiploidy/ t(5;14)(q31;q32); IL3-IGH/ t(1;19) (q23;p13.3); E2A-PBX1 (TCF3-PBX1) (20). Most of these clinical entities associate with particular biological or phenotypic properties and have significant prognostic connotations (30).

Similar to other reports, our case had an aggressive clinical evolution (32-35). However, whether there is any correlation between prognosis and different types of MLL-AF4 fusion genes (based on their breakpoint regions) remains to be established.

In the case reported here, the immunophenotypic analyses also revealed an infrequent development, showing a lineage switch at relapse. At diagnosis, a dominant malignant clone displaying a B precursor lymphoid phenotype was described in the patient’s PB sample evaluated and, although initially not taken into account, subdominant monocytoid cell populations were also noticeable. The bilineage nature of the case suggests that the t(4;11) transforms an uncommitted, multipotential progenitor. Nevertheless, at relapse, a malignant monocytoid population predominantly expanded, while

Most intriguingly, the genetic anomaly found in our case (an unexpectedly large-sized MLL-AF4 transcript), initially thought to result either due to different breakpoints, or to the insertion of an intronic fragment, was finally found to involve novel breakpoints within exon 12 of MLL and exon 4 of AF4. This particular translocation is reported here in premiere, since the most frequent breakage in infant ALL were found to occur between intron 11 in MLL and exon 4 in AF4 (2, 9).

The active involvement of MLL gene in early hematopoiesis has been suggested by studies carried out on MLL-gene counterparts in various animal experimental models (Drosophila or mice) (31). Although not mandatory for terminal myeloid differentiation, MLL function was found to influence the survival and expansion of multipotent progenitors. Therefore, since MLL is essential for normal, early, hematopoiesis and since all of the nearly 30 partner genes, reported to participate with MLL in reciprocal chromosomal translocations, encode for either signaling molecules or nuclear factors (31), such genetic anomalies are certainly linked to leukemogenesis.
de lymphoid progenitor cells (with aberrant myeloid marker expression) became less frequent. According to some authors, the retention of the same genetic anomaly at relapse may be an indication that the switch involved the original leukemic clone and did not reflect the selection of a co-existing, sub-dominant subclone (32).

However, as acute leukaemias of ambiguous lineage are rare subtypes, the pathogenic mechanisms triggering their development have remained obscure. It is still unclear whether the presence of two distinctive malignant

Figure 5. The immunophenotypic profile of cell populations detected by flow cytometry in a peripheral blood sample from an infant acute leukemia case (relapse). Monocytoid cells, monocytes (blue) and promonocytes (violet), were found to be the dominant (36% and 26%, respectively) cell populations at relapse. A subdominant (17%) population of B cell precursors (orange) was also identified as having an aberrant phenotype (co-expression of myeloid markers: CD15, CD64, CD33). Other cell populations may also be distinguished with the marker combinations used: lymphocytes (dark green) and granulocytes (light green). Data were acquired with a FACSCanto II cytometer (BD Biosciences) and analysed with FACS Diva software, version 6.1.2.
clones represents connected disease entities or the consequences of different degrees of maturation from a common precursor.

Cases of infant ALL that demonstrated a switch to a monocytoid lineage have been previously reported, harboring distinct genetic lesions, such as the MLL gene translocation with the CREP-binding protein gene (32), or MLLT10 gene (35), or other types of MLL rearrangements (31, 33, 4), with some of these authors implying that the MLL gene rearrangement occurred in precursor cells having a double differentiation potential (towards either B lymphocytes or monocytes) (35).

Sensitive detection of MLL rearrangements, accurate lineage assignment, and early lineage switch prediction may have a crucial clinical impact, supporting the clinician in treatment making decisions and increasing the precision of minimal residual disease detection techniques.

Conclusions

We have described here a novel MLL-AF4 fusion transcript, undoubtedly difficult to detect with conventional diagnostic methods. Sequence analysis of such atypical products should be customarily performed for various reasons: the early prediction of an immunophenotypic switch, a more accurate prognostic assessment, input to the understanding of the underlying molecular mechanism and assistance in designing optimized therapies.

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Conflicts of interest

The authors declare that there are no conflicts of interest concerning this paper.

Abbreviations

ALL - acute lymphoblastic leukemia
bp - base pairs
HR - patient code
ic - intracellular
MLL - mixed lineage leukemia
MPAL - mixed phenotype acute leukemias
NTC - no template control
PB - peripheral blood
PBS - phosphate buffer saline solution
PCR - polymerase-chain reaction
s - surface
STD - standard
WBC - White Blood Cell Count

References

33. Jiang JG, Roman E, Nandula SV, Murty VV, Bhagat G, Alobeid B. Congenital MLL-positive B-cell acute lymphoblastic leukemia (B-ALL) switched lineage at relapse to acute myelocytic leukemia (AML) with persistent t(4;11) and t(1;6) translocations and JH gene rearrangement. Leuk Lymphoma 2005; 46: 1223-7
