Acute Undifferentiated Leukemia or Minimally Differentiated Acute Myeloid Leukemia: Further Emphasis on Molecular Analysis in Leukemia Diagnosis

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Acute undifferentiated leukemia (AUL) is a rare subset of acute leukemias, being included in the 2008 edition of the World Health Organization Classification of Tumors of Hematopoietic and Lymphoid Tissues as a special category called ‘acute leukemias of ambiguous lineage’, in which the leukemic cells express no more than one membrane marker of any given lineage. We report a rare case of therapy-related acute myeloid leukemia (AML), in which a strict myeloid marker myeloperoxidase (MPO) expression was confirmed by real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis but not immunophenotyping. Although the diagnosis of AUL was initially considered, the lineage of leukemic cells was demonstrated by qRT-PCR analysis. Our observations suggest that molecular analysis of lineage markers may help to establish the diagnosis of acute leukemias.

Key words: WHO classification, acute undifferentiated leukemia, acute myeloid leukemia, myeloperoxidase gene, molecular analysis

Introduction

The diagnosis and classification of acute leukemias rely on morphology, immunophenotyping with the expression of surface or cytoplasmic (cy) antigens, chromosomal abnormalities, and specific molecular genetic analyses. The classification of acute leukemias established by the French-American-British (FAB) Cooperative Study Group has long been used, being based on traditional morphologic and cytochemical criteria1). The FAB classification also includes minimally differentiated acute myeloid leukemia (AML-M0), where the criteria consist of the presence of less than 3% myeloperoxidase (MPO) – and/or sudan black-B-positive blasts in the bone marrow by light microscopy, at least one myeloid-associated antigen positivity (CD13 and/or CD33), and the absence of lymphoid antigens, with the exception of TdT and CD72). In addition, an immunocytochemistry and/or electron microscopy analysis with MPO expression is optional2), but the latter is by no means practical. There are some cases where the lineage origin of leukemic blasts cannot be established by the associations of morphology, cytochemistry, and immunophenotyping with an extensive panel of monoclonal antibodies, leading to the tentative category of acute undifferentiated leukemia (AUL), which is unclassifiable by the FAB classification3)-5). The European Group for the Immunological Characterization of Leukemias (EGIL) also recognized a rare subtype of acute leukemias as ‘undifferentiated acute leukemias’, where the leukemic cells do not express lineage-specific markers6).

The 2008 edition of the World Health Organization (WHO) Classification of Tumors of Hematopoietic and Lymphoid Tissues recognizes a special category called ‘acute leukemias of ambiguous lineage’, which encompasses those leukemias showing no clear evidence of differentiation along a single
lineage and includes AUL\textsuperscript{7,8}. By the definition, AUL expresses no markers considered specific for either lymphoid or myeloid lineage, being necessary to exclude leukemias of unusual lineages, such as those derived from myeloid or plasmacytoid dendritic cell precursors, NK-cell precursors, basophils, or even non-hematopoietic tumors. Thus, the diagnosis of AUL may be complex and required for analyses other than conventional immunophenotyping.

In this paper, we report a rare case of therapy-related AML, in which MPO expression in the patient’s leukemic cells was confirmed only by real-time quantitative reverse transcription–polymerase chain reaction (qRT–PCR) analysis and not immunophenotyping. Our observations suggest the need for further emphasis on molecular analysis of lineage markers in leukemia diagnosis.

**Case report**

The patient was an 88–year–old male who presented with anemia in October 2009. Two years before admission to the hospital, he had been treated with 6 courses of the R–CHOP chemotherapy regimen for nasopharyngeal non–Hodgkin’s lymphoma (NHL) and achieved complete remission (CR) in another hospital. Hematologic findings upon admission were: hemoglobin (Hb), 5.9 g/dL; platelets, \( 7.4 \times 10^9/L \); and white blood cells (WBC), \( 28.1 \times 10^9/L \) with 1% band forms, 46% segmented neutrophils, 1% eosinophils, 3% monocytes, and 49% lymphocytes. A bone marrow aspirate showed hypocellularity with 31.0% blasts, 1.8% promyelocytes, 1.8% myelocytes, 1.6% metamyelocytes, 10.0% neutrophils, 0.6% eosinophils, 26.8% lymphocytes, 4% monocytes, 1.8% plasma cells, and 18.6% erythroblasts. As shown in Figure-1A, blasts were characterized by a large size, modest amount of cytoplasm without granules, and prominent nucleolus. No dysplastic change was observed in the erythroid, megakaryocytic, or granulocytic/monocytic lineages. The cytochemistry of blasts showed negativity for MPO and esterase stain.

Immunophenotyping of bone marrow cells at diagnosis was performed using two-color flow cytometry and a panel of monoclonal antibodies. Labeled cells were analyzed after the blast cell population was gated in a dot plot displaying the linear forward (FSC) and side scatter (SSC) properties of the cells. We used a CD45 intensity expression with right–angle light scatter to detect leukemic blasts and separate them from the normal bone marrow populations\textsuperscript{9} (Figure 1–B). Antigen expression was considered positive if 20% or more of blast cells reacted with a particular antibody\textsuperscript{10}. As shown in Figure–1B, leukemic cells of the patient were positive for CD34 (75%), CD117 (93%), and HLA–DR (88%) antigens (none are lineage–specific), but negative for CD13 (15%) and CD33 (3%) antigens (both are myeloid markers). The percentage of cyMPO–positive cells was 4.6% (being negative) with gating set for whole mononuclear cells (Figure–1C) and 19.2% with gating set for larger blast cell populations (Figure–1D). The fluorescence intensity of cyMPO expression was very weak, displaying a position near the negative control (Figure–1E). Immunophenotyping by flow cytometry may be operator–dependent, especially in the case of cytoplasmic antigen expression. In this case, whereas the cyMPO expression provides evidence of myeloid lineage commitment\textsuperscript{11}, the faint cyMPO expression in the patient’s leukemic blasts was inconclusive for establishing a myeloid origin.

Cytogenetic analysis of bone marrow cells by conventional G–banding showed 47, XY, +8, inv (9) (p12q13) in 3 out of 20 metaphase cells analyzed (Figure–2A), and the remaining 17 cells were 46, XY, inv (9) (p12q13), where the latter was considered to be a normal variation. To confirm the presence of trisomy 8, we performed fluorescence in situ hybridization (FISH) analysis using the CEP 8 SpectrumOrange Direct Labeled Fluorescent DNA Probe Kit (Abbott Laboratories, Abbott Park, IL, USA), as described previously\textsuperscript{12}. As shown in Figure–2B, a three–orange signal pattern was detected in 35% of the interphase nuclei analyzed, indicating the presence of trisomy 8.

Taking account of the inconclusive cyMPO expression, we performed qRT–PCR analysis. Total RNA was extracted from bone marrow cells using the RNeasy Mini Kit (Qiagen, Maryland, CA, USA). Complementary DNA was synthesized from cellular RNA using the 5X PrimeScript\textsuperscript{TM} RT Master Mix, and real–time PCR was performed using the Thermal Cycler Dice\textsuperscript{TM} Real Time System II and SYBR\textregistered{} Premix Ex Taq\textsuperscript{TM} II (all from Takara Bio, Otsu, Japan). Primer sequences were as follows: MPO–1 forward: 5’–CTG CAT CAT CGG TAC CCA GTT C–3’, reverse: 5’–GAT GCC TGT GTT GTT
**Figure 1**  A. Three large leukemic blasts with a modest amount of cytoplasm, no granules, and a prominent nucleolus in the bone marrow at diagnosis (original magnification × 1,000). B. Immunophenotyping of bone marrow cells at diagnosis using two-color flow cytometry and a panel of monoclonal antibodies. For cyMPO expression, whole mononuclear cells (C) or a larger blast cell population (D) were gated in a dot plot with displaying linear forward scatter (FSC) and side scatter (SSC) properties of the cells. E. The fluorescence intensity of cyMPO expression showed a position near the negative control.

**Figure 2**  A. A representative karyotype of bone marrow metaphase cells at diagnosis by G-banding analysis. The arrow represents trisomy 8. B. FISH analysis using a probe specific for chromosome 8, showing a three-orange signal pattern in 35% of the interphase nuclei analyzed.
GTC GCA GA-3'; MPO-2 forward: 5'-TAC CAG
GAA GCC CGG AAG AT-3', reverse: 5'-TGA GTC
ATT GTA GGA ACG GTACGT G-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
forward: 5'-GCA CCG TCA AGG CTG AGA AC-3',
reverse: 5'-TGG TGA AGA CGC CAG TGG A-3'.

qRT-PCR analysis showed MPO gene expression in
the bone marrow cells of the patient, compared with
the positive control of HL60 and buffy coat cells and
the negative control of K562 cells (Figure-3A). The
relative expression of the MPO gene was near the
same level as in HL-60 cells when both MPO-1 and
MPO-2 primers were used (Figure-3B). These find-
ings suggest that the leukemic blasts of the patient
might be an earlier myeloid differentiation stage
before the full expression of myeloid markers.

The patient was treated with AML-directed
combination chemotherapy and achieved CR. In
September 2010, the patient was readmitted to the
hospital because of recurrence of the disease. He
received salvage chemotherapy, but became refrac-
tory to it, and died of disease progression and sepsis
in January 2011.

Discussion

Among the various types of AML, AML-M0 is
regarded as a relatively rare disorder with an
incidence of 2–4%. AML-M0 blasts exhibit large
and agranular features, and frequently lack those
of a specific cell lineage, precluding diagnosis on
morphological grounds alone. The criteria include
the expression of at least one myeloid-associated
antigen (CD13 and/or CD33). As for MPO expres-
sion, cyMPO antigens may be positive by immuno-
phenotyping with anti-MPO monoclonal antibodies.
In the present case, leukemic blasts were positive
for CD34, CD117, and HLA-DR antigens, but nega-
tive for CD13 and CD33 antigens, and showed faint
cyMPO expression with weak fluorescence inten-
sity. The latter might be inconclusive as evidence of
myeloid differentiation. The immature nature of leu-
kemic blasts has been associated with the expres-
sion of markers for hematopoietic progenitor cells,
such as CD34, CD117, and HLA-DR. Although the
diagnosis of the present case was initially consid-
ered as AUL, qRT-PCR analysis revealed MPO
gene expression, being of myeloid origin and
presumably categorized as AML-M0. These findings suggest that immunophenotyping alone might not be sufficient for establishing a diagnosis of acute leukemias with immature phenotypes.

The diagnosis and classification of acute leukemias affect patient management options. The prognosis of AUL patients is generally considered as poor, although the information is too scanty to make any definitive statements. AML-M0 has also been reported to be associated with a poor prognosis compared with non-M0 counterparts. Factors that may be responsible for the poorer prognosis include the high frequency of unfavorable cytogenetic changes, the high expression of immature markers, and the frequent expression of multidrug resistance protein. Furthermore, treatment outcomes in AML patients continuously decline with aging. In the present case, our patient was an 88-year-old male with prior chemotherapy for NHL and his leukemic blasts exhibited an immature phenotype. The diagnosis of AML was made by qRT-PCR analysis and he achieved CR with AML-directed intensive chemotherapy.

In conclusion, qRT-PCR analysis successfully detected MPO gene expression in the patient’s leukemic cells. Our observations suggest the usefulness of qRT-PCR analysis of lineage markers in leukemia diagnosis.

Conflict of interests

The authors declare that there are no conflicts of interest regarding the publication of this paper.

References