Value of Multifaced Approach Diagnosis and Classification of Acute Leukemias

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The aims of this report are to asses the present status of conventional (morphological and cytochemical) methods in diagnosing acute leukemias (AL), to mention additional characteristic profiles utilizing composite, cytogenetic, immunophenotypic and molecular data, in the attempt to reaffirm concordance difficulties between the acute leukemia French-American-British (FAB) (1), World Health Organisation (WHO) classifications (2) and EGIL (European Group for the Immunological Characterisation of Leukemias) (3).

The FAB classification system for ALs was based on morphological and cytochemical criteria. The classification of AL proposed by EGIL was based on immunophenotyping, targeting a more precise delineation of the hematological lineage and differentiation stage of specific types of leukemia. Immunophenotyping has become fundamental for the classification and essential for the recognition of the various subtypes of AL. The WHO classification of AL (2008-2009) has incorporated cytomorphology, immunophenotyping, cytogenetic and molecular changes.

This report deals, from the laboratory point of view, with issues and controversies related to AL classification and how immunophenotyping and cytogenetic/molecular information is continuously changing this classification.

Diagnosis of Acute Leukemia

The diagnosis of AL undergoes a stepwise approach. First is the distinction of an AL from other hematological neoplastic diseases and reactive disorders. Second is differentiating acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL). The third step is the classification of AML and ALL into categories that define treatment and prognostic groups.

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Morphology and cytochemistry in the diagnosis of acute leukemia

In most cases, the first two facets of the diagnosis of AL can be achieved by careful morphological assessment of blood and bone marrow smears (4).

In AL, the morphologic identification of cells is sometimes difficult, due to a marked similarity between earlier precursors of different cells series. In cases of poorly differentiated AL, the morphologic features may be equivocal, requiring additional studies. In these cases the cytochemical stains are of great help in recognizing the type of precursor cells, especially when there is asynchronism between nuclear and cytoplasmic maturation. The presence of such cytochemically stainable components, in early precursors, indicates specific cellular differentiation, which the precursors are undergoing, thus making the identification easier.

The myeloperoxidase (MPO), Sudan Black B, Periodic acid Schiff (PAS) and non specific esterase (alpha naphtyl-acetate esterase -ANAE) are the most commonly used and the most valuable cytochemical stainings in distinguishing AML from ALL. Distinct cytochemical patterns were observed in different types of leukemias (4). MPO positive blasts are quite specific for AML. PAS block positivity is seen in ALL, which has significance in absence of MPO positivity. Diffuse or granular PAS positivity has no significance. ANAE positivity permits a distinction between monocyte lineage and neutrophil lineage (4).

With the addition of cytochemistry to the morphologic assessment, most cases of acute leukemia can be appropriately designated as AML or ALL. However, there remains a minority of cases that cannot be definitively diagnosed by these methods.

In these cases the blastic cells are completely undifferentiated and cytochemistry cannot aid in the diagnosis since they have not yet developed their normal complement of enzymes and metabolic products.

Immunophenotyping in the diagnosis of acute leukemia

The lineage of most cases of morphologically and cytochemically poorly differentiated AL can be accurately characterized by immunophenotyping. Additionally, immunophenotypic subsets of AML and ALL can be determined (3) (5). Multiparametric flow cytometry is the preferred method for immunophenotyping AL.

The lineage of hematopoietic cells is defined both by antigens expressed and the absence of expression of antigens associated with a different lineage. Leukemia cells, however, may aberrantly express some antigens of another lineage or lack expression of an expected antigen (6). It is important, therefore, to use panels that include sufficient numbers of antibodies to assess a spectrum of both myeloid and lymphoid antigens. The imunophenotying analyses must undergo some steps: lineage assignment, maturational analysis, complete characterization of blast cells and normal cells.

For ALLs, the immunophenotypic categories are particularly important because they identify distinctive treatment and prognostic groups (6).

In AMLs, immunophenotyping is most important in distinguishing poorly differentiated cases from ALL, in characterizing a few AML subsets and biphenotypic acute leukemia (BAL). Often confused with acute bilineal leukemia (BLL) that is composed of a mixed population of leukemia cells of two different lineages BAL refers to acute leukemia with a single population of blasts coexpressing markers of two different lineages. To define BAL, a scoring system was proposed by the EGIL in 1995. This scoring system assigned different scores to several immunological markers based on their lineage specificity.The 2008 WHO Classification acknowledged the limitations of the EGIL and proposed to define BAL as "a single population of blasts that would meet criteria for B-ALL or T-ALL but that also express myeloperoxidase (MPO)" or have "unequivocal evidence of monoblastic differentiation" based on specific requirements (7). However, the new WHO Classification did not address the clinical significance of the proposed new definition of BAL, nor the implications for treatment.

Cytogenetics in the diagnosis of acute leukemia

Clone cytogenesis abnormalities are identified in 60-80% of cases of AML and approximately 80% of case of ALL (8). Both numerical and structural abnormalities are common. Recurring balanced translocations, particularly t(8; 21), t(15;17), inv(16)/t(16;16), and 11q23 translocations, represent a substantial percentage of cytogenetic abnormalities in AML. Additional abnormalities, including trisomies, deletions,

and complex karyotypes, contribute to another large percentage of adult AMLs and show overlap with cases of myelodysplasia (8).

Bone marrow cytogenetic findings are a major independent indicator of prognosis for both AML and ALL and define treatment groups (9, 10). They are essential in the assessment of patients with AL and should be performed in every case. Hyperdiploidy with >50 chromosomes, often an extra copy of chromosome(s) 4 and/or 10, has a particularly favorable prognosis. Structural abnormalities in childhood B-cell precursor ALL are more often associated with an intermediate or poor prognosis with one exception [t(12;21)(p12;q22)], which has a high rate of complete remission and presumably a high incidence of long-term disease-free survival. Patients with B-cell precursor ALL with a 9;22 translocation [t(9/22)(q24;q11)] or abnormalities involving chromosome 11q23, most often a t(4;11)(q21;q23), have an unfavorable prognosis.

Molecular analysis of acute leukemia

In the diagnosis of AL, molecular analysis may be used to establish clonality or to identify molecular translocations producing fusion gene products (11,12). Molecular studies are also powerful tools for the identification of minimal residual disease and early relapse (13). Techniques for molecular analysis of leukemia include Southern blot, PCR, and FISH. Gene rearrangements may serve as a fingerprint for molecular changes and identification of minimal residual leukemia, when there are too few leukemic cells present to be recognized by morphologic examination or immunophenotyping. In some cases, molecular translocations are present when karyotypic changes are not evident. An example is the TEL-AML1 fusion gene resulting from the t(12;21)(p12;q22) translocation. This chromosomal translocation generally is cryptic and can only be identified by molecular analysis (PCR or FISH) (14). This is occasionally the case with well-established translocations, in which the involved chromosome segments are too small for detection by karyotyping or because the translocation is complex and involves several chromosomes. It is important, therefore, to perform molecular analysis when the presence of a fusion gene that would impact treatment decisions is suspected. One unresolved issue is the nature of the underlying genetic alterations in approximately 40% of AML case with normal karyotype (15).

Novel approaches in genomics, such as surveying the expression levels of thousands of genes in parallel using DNA microarray technology, open possibilities to further refine the studies on AL. Today, gene expression profiling in AL is becoming well established and has already been proven to be valuable in diagnosing different cytogenetic subtypes, discovering novel subclasses and predicting clinical outcome (16).

Classification of Acute Leukemia

Human ALs are broadly classified as myeloid or lymphoid according to the expression of surface and cytoplasmic antigens. Uncommonly, the lineage of origin is not clear; either two separate blast populations are encountered, one myeloid and the other lymphoid, or, a single blast population demonstrating evidence of both myeloid and lymphoid differentiation concurrently.

FAB classification of acute leukemia

The FAB classification of AML is a lineage-based morphologic classification that categorizes cases according to the degree of maturation of the leukemic cells and their lineage differentiation and the bone marrow blast cell percentage must be at least 30%.

The FAB classification of ALL (Table 2) is simpler than for AML (Table 1), but the criteria that distinguish the categories are less precise.

WHO classification of acute leukemia

In the mid 1990s, the Society for Hematopathology in the United States and the European Association for Hematopathology were enlisted to update the WHO classification of hematopoietic neoplasms. This classification eliminates the problems of an exclusively lineage-based or an exclusively cytogenetic/molecular classification by combining the best features of both. The WHO classification of AMLs includes traditional FAB-type categories of disease, as well as additional disease types that correlate with specific cytogenetic findings and AML associated with myelodysplasia. The result is a classification that enhances clinical and prognostic utility and retains usability. In the WHO classification of AML there are four major categories: AML with recurrent cytogenetic translocations, AML with multilineage dyspla-

WHO	FAB	
AML with recurrent genetic abnormalities		
AML with t(8;21)(q22;q22); RUNX1-RUNX1T1		
AML with inv(16)(p13.1q22) or t(16;16) (p13.1;q22) CBFB-MYH11		
Acute promyelocytic leukemia with t(15;17) (q22;q12) PML-RARA	Acute promyelocytic leukemia -microgranular variant	M3
AML with t(9 ;11)(p22;q23); MLLT3-MLL		
AML with t(6;9)(p23;q34); DEK-NUP214		
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2) RPN1-EVI1		
AML (megakarioblastic) with t(1;22)(p13;q13) RBMI5-MKLI	Megakaryoblastic leukemia	M7
AML with mutated NPM1		
AML with mutated CEBPA		
AML with myelodysplasia-related changes		
Therapy-related myeloid neoplasms		
AML not otherwise specified		
AML wiyh minimal differentiation	Myeloblastic leukemia minimally differentiated	M0
AML without maturation	Myeloblastic leukemia without maturatio	M1
AML with maturation	Myeloblastic leukemia with maturation	M2
Acute myelomonocytic leukemia	Myelomonocytic leukemia	M4
	-with bone marrow eosinophilia	M4Eo
Acute monoblastic/monocytic leukemia	Monocytic leukemia	M5
	Poorly differentiated	M5a
	Differentiated	M5b
Acute erytthroid leukemia	Erytroleukemia	M6
Pure erythroid leukemia		
Erithroleukemia, erythroid/myeloid		
Acute megakarioblastic leukemia	Megakaryoblastic leukemia	M7
Acute basophilic leukemia		
Acute panmyelosis with myelofibrosis		
Myeloid sarcoma		
Myeloid proliferations related to Down syndrome		
Transient abnormal myelopoiesis		
Myeloid leukemia associated with Down syndrome		
Blastic plasmacytoid denderitic cell neoplasm		

TABLE 1. Comparative WHO and the FAB classifications of acute myeloid leukemia.

sia, therapy-related (secondary) AML, AML not otherwise categorized (includes the former FAB categories). Table 1 represents an attempt to compare classifications of AML using WHO and FAB criteria.

The FAB (Table 2) and WHO (Table 3) classifications of ALL cannot be compared; the first one is mainly morphological, while the second one is more complex.

The major categories of ALL in the revised WHO classification (Table 3), defined by im-

munophenotype are three: B-cell precursor, T-cell precursor and mature B-cell ALL (Burkitt lymphoma leukemia). Within the B-cell precursor category there are several subtypes identified by cytogenetic/molecular abnormalities. The WHO classification has changed the grouping of ALL to reflect increased understanding of the biology and molecular pathogenesis of the diseases. The major treatment and prognostic groups in childhood ALL are identified in this classification.

European Group for the Immunological classification of Leukemia (EGIL)

EGIL has proposed that AL should be classified on the basis of immunophenotype alone. This classification has the strength that suggests standardized criteria and guidelines for defining a leukemia as myeloid, T lineage, B lineage, or biphenotypic, based on marker expression. It also suggests criteria for distinguishing BAL from AML with aberrant expression of lymphoid antigens, and from ALL with aberrant expression of myeloid antigens.

Within the AMLs, only three subtypes as defined by the FAB classification: M0-AML, M6-AML and M7-AML, can be unequivocally defined by immunological markers; prospective studies are undertaken to see whether characteristic immunological profiles are associated with particular AML subtypes defined by specific cytogenetic abnormalities. Lymphoid antigen expression is relatively common in AML. Some lymphoid antigens, such as CD19 and CD2, are expressed commonly in specific subtypes of AML in this system, and such antigen expression should not be regarded as evidence of BAL. Criteria for the definition of (BAL) are devised (7) and a scoring system is outlined aimed to distinguish BAL from those AL with expression of a marker from another lineage. As seen above, this scoring was improved by WHO system in 2008.

In addition, an uncommon subset of AL with no evidence of lymphoid or myeloid differentiation is recognized and the useful panel of markers to investigate and establish the cell nature of the AL is outlined.

European Leukemia Network (ELN) is an EU-funded organization which integrates European expertise, explores and incorporates translational research to applications in public health. The most important results of the ELN are the guidelines and management recommendations for virtually every leukemia and interdisciplinary specialty which proposed the groundwork for uniform definitions and standards required for diagnoses, classifications, therapy or common clinical trials and projects (17,18). ❑

DISCUSSIONS

The FAB classification of ALL and AML is based on morphology and cytochemical staining of blasts and provides a common language for comparing and treating AL. The major advantage of the FAB lineage-based classification system is its ease of use. The cytologic criteria are well defined; they do not require high technology and can be applied in most laboratories throughout the world. Application of WHO classification requires more complex investigations, so there is necessarily some delay in making a definitive diagnosis. A preliminary morphological diagnosis based on the FAB classification therefore remains appropriate. The FAB classification is also applicable to the majority of cases of AL, and they partially define prognostic groups.

A major difficulty of the FAB classification is encountered in cases with negative cytochemical staining and in distinguishing M1 from L2 or M1 from M2 or M2 from M4. Another disadvantage of FAB classification is the modest clinical relevance by not adequately defining biologic and treatment groups.

The WHO system has incorporated cytomorphology, immunophenotyping, cytogenetic and molecular changes. The result is a classification that enhances the clinical and prognostic relevance. Still, some disease types (e.g. myeloid sarcoma) cannot be diagnosed without detailed clinical or histopathological information.

Detection of various cytogenetic abnormalities in AML is known to have prognostic significance, but some of them do not correlate well with FAB classification disease groups and also

Small blasts, scant cytoplasm, inconspicuous cytoplasm	L1
Large, often heterogeneous blasts, moderately abundant cytoplasm	L2
Variable sized blasts, dark blue cytoplasm cytoplasmic vacuoles	L3

TABLE 2. FAB classification of acute lymphoblastic leukemia.

Precursor lymphoid neoplasms
B-lymphoblastic leukaemia/lymphoma not otherwise specified
B-lymphoblastic leukaemia/lymphoma with recurrent genetic abnormalities:
t(9;22) BCR/ABL: 25% of adult B-ALL, <5% childhood B-ALL
t(v;11q23) 11q23 MLL rearrangement: especially infants <1 year,
rare in children; increased frequency in adults
t(12;21) ETV6/RUNX1: 25% of childhood B-ALL
hyperdiploidy (>50 chromosomes): 25% of childhood B-ALL
hypodiploidy (<45 chromosomes)
t(5;14) IL3/IGH
t(1;19) E2A/PBX1.
T-lymphoblastic leukaemia/lymphoma
Mature B-cell leukemia/lymphoma Burkitt

TABLE 3. WHO classification of acute lymphoblastic leukemia.

a substantial subset of cases shows no karyotypic abnormalities. Cryptic translocations are present in some of these cases, and other molecular genetic abnormalities presumably are present in the leukemic cells of these patients.

Another issue requires the additional evaluation of the leukemic blasts by molecular analysis and flow cytometry. Though in WHO system AML is defined by the presence of ≥20% blasts in blood or bone marrow, clonal, recurring cytogenetic abnormalities should be considered AML regardless of blast percentage.

The most significant differences between the FAB and the WHO classifications are:

- A lower blast threshold for the diagnosis of AML: The WHO defines AML when the blast percentage reaches 20% in the bone marrow, while FAB specifies a 30% limit.
- Patients with recurring clone cytogenetic abnormalities like t(8;21)(q22;q22), t(16;16)(p13;q22), inv(16)(p13;q22), or t(15;17) (q22;q12) should be considered to have AML regardless of the blast percentage.
- While immunologic classification of ALL has proven to be a reproducible and valuable clinical management tool, immunologic marker expression is far more heterogeneous in AML, which makes it difficult to incorporate this information into a classification system that is reproducible and clinically relevant. Thus, the major clinical role for immunophenotyping in AML is still to differentiate it from ALL and, to define BAL(7) based on the degree of lineage specificity of particular antigens.
- Although substantially improved in relation to the EGIL, the WHO classification is still not optimal for guiding the clinical management of patients with BAL.

However, a purely immunological classification has the disadvantage that discrete entities may be included into wrong categories; for example some cases of AML of FAB M2 subtype associated with t(8;21)(q22;q22) would be classified as "AML of myelomonocytic lineage", while others would be classified as "AML with lymphoid antigen expression," depending on whether or not a case showed aberrant expression of CD19. In addition, rare cases of AL have been described as clearly myeloid when assessed by cytology and cytochemistry, but which did not express any of the commonly investigated myeloid antigens.

The poor correlation between the FAB myeloid subtypes and antigenic phenotype in AML is a disadvantage, since the vast majority of hematologists still use the FAB classification for a preliminary, quick diagnosis.

Several clinically important categories of AL have been defined by cytogenetic/molecular studies during the past two decades. As a result, there are proponents for abandoning the lineage-based classifications and developing an exclusively cytogenetic/molecular analysis-based classification of AL. Clearly, cytogenetic/molecular analysis-based groupings better define biologic and prognostic groups, but requirement for technology that is not always available at present is a negative aspect of an exclusively cytogenetic/molecular classification. In addition, currently the majority of cases of AML do not express recurrent cytogenetic changes. ❑

CONCLUSIONS

Diagnosing AL is a multistep process, a team work, in which clinical, morphological, cytochemical, immunophenotypical, cytogenetic and molecular investigation, bring together valuable information for a precise diagnostic conclusion. These methods are complementary rather than competitive and offer a flexible approach to diagnosis.

An ideal classification is one which recognizes real entities with fundamental biological differences, prognostic implications and therapeutic relevance, but as seen above, none of the classification is perfect.

Pathologic classifications must change continuously to reflect advances in our understanding of disease. In addition, evaluation of the reproducibility of these systems among pathologists would be needed and clinical studies are warranted to validate any proposed classification. ❑

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