Diagnostic Algorithm in Acute Leukemia

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Acute leukemia (AL) is a disease characterized by abnormal proliferation of primitive hemopoietic cells outcrowding normal marrow elements. When first described it was inevitably fatal within a very short period. The marked progress in the field of chemotherapy and other therapeutic modalities has changed the outcome of acute leukemia, at least in some subtypes, from a fatal to a potentially curable disease. This was associated with trials to classify acute leukemia into different subtypes looking for proper diagnosis which would determine the line of therapy. Expressions like species specific and personalized therapy have been introduced. Species specific therapy involves broad classification into subtypes while personalized therapy requires in depth characterization of the malignant cells at the individual case level. Such an approach requires initially an accurate diagnosis and requires, as well, defining disease marker(s) that would predict response to therapy upfront or during the course of treatment.

Diagnostic and prognostic workup

After clinical examination, the first task is to confirm the diagnosis and make a proper classification of the type of leukemia. The second task is to look for prognostic markers that would help determining the proper therapeutic regimen. The third task is to follow up the patient for response and probably change the treatment plan.

A diagnostic workup starts basically with a full blood count, a bone marrow (BM) aspirate and cytochemistry. Samples are taken, as well, for immunophenotyping, cytogenetics and molecular genetics.

Morphology and Cytochemistry

Examination of peripheral blood (PB) and BM smears gives the first clue to diagnosis. BM is essential to fulfill the criterion of ≥ 20% blast to establish the diagnosis of acute leukemia (AL) (1, 2). The morphology of the blast cells, though not sufficient in most cases, is still a corner stone in diagnosis. The presence of Auer rods would exclude acute lymphoblastic leukemia (ALL). Certain types of acute nonlymphoid leukemia (ANLL) have characteristic morphology namely monocytic leukemia (M4 and M5), the classical hypergranular form of acute promyelocytic leukemia (M3), and most cases of erythroid leukemia (M6). Acute Megakaryoblastic leukemia (M7) cannot be diagnosed but may be suspected from morphology. The next step in the diagnostic workup is cytochemistry which is the main tool to classify AL into its two main categories, ALL and ANLL. A positive staining of the blast cells for myeloperoxidase (MPO), chloracetate esterase and/or Sudan Black would confirm a diagnosis of ANLL. A negative staining however would not exclude such a diagnosis. It is worth mentioning that a common mistake is done at this stage by reporting the case as ALL on account of negative cytochemistry. The case can still be an M0 which is by definition lacking Auer rods and negative for all myeloid specific cytochemical stains (1, 2). This subtype can only be diagnosed by immunophenotyping (IPT) as will be mentioned later. Cytochemistry can further establish the FAB subtype. M3 is associated with very strong MPO which will also stain the Fagots. It is especially important in the diagnosis of the hypogranular variant which could be morphologically misinterpreted as M5. Cytochemistry is the main diagnostic tool in monocytic leukemia where M5 shows strong staining for nonspecific esterase that is completely inhibited by fluoride while the myeloid elements in M4 show weak staining that is not inhibited by fluoride. Dual esterase for monocytic and myeloid elements on the same smear is preferred in cases of M4 for accurate determination of the relative percentage of each component. Periodic acid Schiff (PAS) staining is the diagnostic tool in M6 with its characteristic block positivity. Acid phosphatase (AP) is of limited value except in case of TALL where it shows its characteristic paranuclear dot like positivity in the place of Golgi apparatus. Though not diagnostic, AP has a characteristic pattern in M7 with strong dot like positivity distributed all over the cell. With a negative cytochemistry ALL may be highly suspected but not definitely diagnosed. With the exception of AP+ TALL, ALL is an exclusion diagnosis that has to be confirmed by immunophenotyping. After morphology and cytochemistry, most ANLL cases (except M0 and M7) would have been properly categorized, or at least suspected, according to FAB classification. The other cases are most probably ALL.

Immunophenotyping

The next step is to perform immunophenotyping. It is performed using monoclonal antibodies (Mo Abs) and multiple color (3-4 colors) flow cytometry. IPT is a must in ALL; cases have to be classified as TALL, precursor B-ALL or mature B- ALL. Each of these three categories has a different treatment protocol and they vary in prognosis (3, 4). IPT value in ANLL used to be confined to the diagnosis of M0 and M7 by detecting myeloid antigen expression on the cytochemically negative blast

cells in case of M0 and the megakaryocytic-specific markers (CD41, CD42 and/or CD61) in case of M7. However IPT is currently an integral part of the diagnostic workup of both ALL and ANLL to be used as a marker for minimal residual disease (MRD) detection. At least two 4-color panels expressed on ≥ 50% of the blast cells at diagnosis have to be specified for each case; this is achievable in > 94% of cases (5).The use of two panels will overcome the possible Immunophenotypic shift that might occur in some cases (6).

The panel of Mo Abs used for ALL should include

- T Lineage: cytoplasmic CD3 + CD5, CD2 or CD7. CD4 and CD8 are essential to exclude residual normal T cells; they also indicate the stage of T cell differentiation.

For determination of the stage of T cell differentiation, CD1 is added to the panel.

- B Lineage: cytoplasmic CD22 + CD19 (pan B), CD10 for CALL, cytoplasmic μ for pre-B as well as κ and λ for mature-B.
- Myeloid Markers: CD13, CD33, MPO to exclude M0 and diagnose biphenotypic leukemia, CD14 for M4 and M5 and CD41/CD42/CD61 for M7
- Others: CD45, CD34 and anti class II MHC.
- Markers for MRD: TDT, CD66c, NG-2, CD21, CD38 and CD58 (7)

According to marker expression, cases are classified into: (1) precursor-B including Pro B: CD19+, Cyt CD22+, cyt CD79α+ and CD10 -, CALL: CD10+, immunoglobulin (Ig)- and Pre B: cyt μ+ and sIg-. (2) Mature-B: CD19+, CD22+, cyt CD79α+ and CD10+, sIg κ or λ +. (3) TALL: CD3 + CD5, CD2 and/or CD7.

Flow cytometry is also useful to measure the DNA index. An index of ≥ 1.16 <1.6 is a good prognostic parameter; the good prognosis has been attributed to double trisomy of chromosome 4 and 10 which has to be looked for by FISH technique in any case with DNA index > 1 (8)

Cytogenetics

Conventional Karyotyping is essential for all cases. The tremendous progress in molecular genetics did not undermine its role in the workup of acute leukemia. It gives an overview of the findings including multiple and complex translocations. It is also the first step in the discovery of any possible new genes. Marked progress in the techniques used from simple G-banding to FISH, to whole chromosomal staining, spectral karyotyping (Sky) technique and comparative genomic hybridization has added more to its value. Karyotyping can detect both numerical and structural abnormalities. However there is a number of translocations that are cryptic and cannot be detected by conventional karyotyping e.g. t(12;21). Others may be missed in certain cases by Karyotyping and detected only by molecular techniques e.g. t(9;22). In other situations, still, the translocation has to be confirmed at the molecular level for targeted therapy e.g. t(15;17) (PML, RARA) for all-trans-Retinoic Acid (ATRA) therapy.

Molecular Genetics of ALL

Molecular genetics of Precursor-B ALL

About 30-35% of ALL cases show one or the other of four common translocations. The t(12;21)(p13;q22) ETV6/RUNX1a "cryptic" chromosome rearrangement is associated with favorable prognosis using treatment protocols for low risks ALL (9). The t(1;19)(q23;p13) TCF3/PBX1 fusion gene is usually associated with cyt μ positive precursor B ALL (pre-B ALL). This translocation has been associated with a poor response to antimetabolites therapy and an unfavorable outcome in the past. Recent treatment protocols for higher risk ALL have significantly improved the long term disease free survival rate (10). The t(9;22)(q32;q11) BCR/ABL, or Philadelphia (Ph) chromosome is usually detected at the cytogenetic level. However molecular analysis to detect BCR/ABL1 fusion mRNA product identifies additional cases that are missed by conventional karyotyping and either p210 or p190 might be detected. The prognosis is unfavorable in both children and adults (11). The last group of chromosomal translocations with a known prognostic significance is the rearrangement of chromosome 11q23; t(4;11)(q21;q23) MLL/AF4 is the most common among this subgroup accounting for 60-70% of these cases. This translocation is associated with poor prognosis (12).

Molecular genetics of TALL

These may be classified into three groups (13).

I- Fusion transcript:

- CALM-AF10: It has an incidence of 10% in both children and adults
- SIL-TAL1: It has an incidence of 5-10% in children and 25% in adults

The clinical relevance of both is still controversial with claimed good prognosis in children

II- Translocations:

- TLX1 (HOX11): It has an incidence of 5-10% in children and 15-20% in adults. : It is claimed to be associated with poor prognosis though it is still controversial.
- TLX3 (HOX11L2): It has an incidence of 25% in children and 5-10% in adults. It is associated with good prognosis both in children and adults
- MYB: It has an incidence of 5-10% in both children and adults.
- LMO1 and LMO2: Its incidence is about 45% (including deletions)

III- Point mutations/deletions:

- NOTCH1: It has an incidence of 50% in both children and adults. Its prognostic impact is controversial
- FBW7: It has an incidence of 25-30% in both children and adults.
- CDKN2A deletions: Rare

Molecular genetics of ANLL

At the cytogenetic level, ANLL is extremely heterogeneous with > 200 reported structural and numerical aberrations (14). Cytogenetics at diagnosis is amongst the strongest independent prognostic factors (15). However, 40-45% of ANLL have no cytogenetic abnormalities, what is known as cytogenetically normal (CN) ANLL.

Translocations t(8; 21) (AML1/ETO) Runx1- RUNX1T1, Inv (16) (CBFB/MYH11) and t(15;17) (PML/RARA) are associated with good prognosis. Conversely, translocations involving band 3q26 (with overexpression of EVI gene or those with complex karyotype are generally associated with inferior outcome (14, 15). The CN-AML group is associated with intermediate prognosis. Recently, however, a prognostic role of molecular genetics within cytogenetically defined groups of ANLL patients is more and more appreciated (16).

Genetic stratification of AML with core-binding factor: t(8;21) & Inv(16)

This group of patients has a favorable prognosis; Still 50% are not cured with contemporary chemotherapy. It was found that the presence of KIT mutations would affect the prognosis in these cases. KIT mutations occur mainly in exon 8 and 17. It has an incidence of 20-45% and claimed to have variable geographical distribution. It is associated with adverse prognosis in the t(8;21) group and a higher incidence of relapse in Inv (16)(17, 18). The other aspect of the coin, however, is that it constitutes a potential therapeutic target for tyrosine kinase inhibitors (TKIs). In a recent study the presence of either KIT, FLT3 internal tandem duplication (ITD) or JAK2 V617F mutations were shown to be associated with bad prognosis in this group of patients (19).

Molecular Markers in Cytogenetically Normal ANNL (CN-ANLL)

About 40% of patients with CN AML leukemia can be cured but until recently this subgroup could not be recognized. A number of prognostic markers can now be used to subcategorize this group of patients. These include gene mutations and overexpression of single genes.

Mutations of the FLT3 gene

Internal tandem duplications (ITDs) of the FLT3 gene occur within the juxtamembrane domain (exon 14 and 15). The duplication can vary in length from 3 to more than 400 nucleotides. FLT3-ITDs are detected in 28-33% of CN ANLL patients (20, 21). Further 5-14% of CN-ANLL patients carry missense mutations in exon 20 of FLT3 i.e. within the activation loop of the tyrosine kinase domain (FLT3-TKD). FLT3-ITD has been found to be an independent prognostic factor associated with shorter complete remission duration (CRD), disease free survival (DFS) and overall survival (OS). There is evidence that the outcome may be related to the level of the mutant allele than just to its mere presence; only patients with loss of wild type FLT3 have dismal outcome and not those with heterozygous mutations (20). The prognostic significance of isolated FLT3-TKD remains controversial.

Mutations of the MLL gene

Partial Tandem Duplication spanning exons 5 through 11 or less frequently 12 occurs in about 8% of adult de novo ANLL. It is associated with shorter remission duration but not OS. Improved outcome has been recently reported in young adults treated with autologous SCT in first remission (22)

Mutations of the CEBPA gene

The CEBPA gene encodes C/EBP α protein. It occurs at a frequency of 15-19% and is associated with longer CRD and OS (23)

Mutations of NPM1 gene

NPM1 encodes nucleophosmin; it occurs at a frequency of 45-64% of CN-AML and is associated with better CR rate, EFS, RFS, and OS. Forty per cent of patients with NPM1 mutations have FLT3-ITD. Its good prognostic impact is effective only in absence of FLT3-ITD; it has no effect in presence of FLT3-ITD (18). NPM1 mutations are associated with a beneficial effect of ATRA given after intensive conventional chemotherapy in elderly patients with non-APL AML. The effect is also confined to the FLT3-ITD negative patients (24, 25, 26).

Mutations of the WT1 gene

It has been suggested that WTI protein could promote stem cell proliferation and induce a block in differentiation. It occurs at a frequency of 10% in CN-ANLL. Its association with FLT3-ITD is especially detrimental with failure to achieve CR (27)

Mutations in AML1

Other than the t(8;21) (AML1/ETO) Runx1- RUNX1T, mutations of the AML1 have been shown to be involved in other AML subsets. They are found in nearly all cases with trisomy 13, in a large number of cases with trisomy 21, and also in 10% of CN-AML (28). No data is, currently, available on its clinical relevance.

Mutations in NRAS gene

NRAS mutations have not been shown to be of prognostic significance, yet they may provide a target for molecular therapy (29).

Overexpression of BAALC gene

BAALC expression is mostly detected in hematopoietic precursors and neuroectoderm-derived tissues. High expression is encountered in ALL, AML, ABC but not CML. High expression in CN-ANLL is associated with primary resistance, shorter DFS, OS and higher cumulative incidence of relapse (CIR). It is especially predictive in absence of FLT3-ITD & CEBPA mutations (30). It has been suggested that patients with high BAALC expression might benefit from allogeneic SCT (31)

Overexpression of the ERG gene

ERG is one of > 30 members of ETS gene family. In CN-ANLL high ERG expression is associated with adverse impact on CIR and EFS. Its adverse impact on OS is observed only, in patients with low BAALC (32).

Overexpression of MN1 gene

Recently, overexpression of MN1 was found to be associated with shorter OS and RFS in CN-ANLL (33). These results need further confirmation (29).

To evaluate the impact of each individual marker in the concert of all other markers it will be necessary to analyze large cohorts of homogenously treated patients for the various genetic changes (29). In a metaanalysis of 872 younger adults with CN-AML (34), mutations in NPM1, FLT3, CEBPA, MLL and NRAS were analyzed. Two genotypes consistently appeared in multivariate analysis as markers of favorable prognosis associated with good response to therapy, favorable relapse-free survival and overall survival. These are CEBPAmutant associated with 62% 4 year survival and NPM1mutant/ FLT3-ITDnegative associated with 60% 4 year survival. The latter subgroup does not benefit from allogeneic SCT in first line therapy (2 9).

An algorithm for laboratory diagnostic and prognostic workup of Acute Leukemia

Basic workup

Complete blood picture, bone marrow aspirate (Biopsy may be done in selected cases especially M7), cytochemistry as indicated, immunophenotyping and conventional karyotyping. Cases will be categorized into ALL and AML with proper FAB classification for AML and immunophenotype for ALL. Further molecular testing depends on the subtype.

Precursor B ALL

- RT-PCR for t(12;21); t(1;19); t(9;22) (both p190 and p210)
- FISH for MLL gene rearrangement.

TALL

- HOX11, HOX11L2 and SIL-TALL expression.

AML

All cases should be tested for t(8;21), Inv (16) and t(15;17).

- **AML with core-binding factor [t(8;21) and Inv (16)]:**
  - FLT3-ITD.
  - KIT mutations
  - JAK2-V617F mutation
- **Cytogenetically normal AML (CN-AML):**
  - Test for FLT3-ITD, CEBPA, MLL and WT1 mutations in all cases.
  - Test for NPM1 mutations and BAALC expression in FLT3-ITD negative patients.
  - Test for ERG expression in BAALC low patients.

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