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Current Developments in the Management of Leukemia, Lymphoma, and Myeloma

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Molecular Markers in Acute Myeloid Leukemia

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H&O Why is there a need for prognostic molecular markers in acute myeloid leukemia (AML)?

GM Acute myeloid leukemia (AML) is an aggressive cancer in which excessive amounts of myeloblasts are found in the bone marrow and blood. Aberration in the maturation and proliferation of myeloblasts disrupts normal hematopoiesis and eventually leads to bone marrow failure. This results in the high probability for life-threatening infection, bleeding, or potential comorbidities related to anemia. Today, by using a combination of standard-dose chemotherapy and stem cell transplantation, approximately 35–40% of patients younger than 60 years of age are cured. Patients who are 60 years and older have a much smaller chance for being cured, as only approximately 10% or less achieve long-term survival. Therefore, there is clearly a need to improve the current treatment strategies.

In AML, it is now evident that one treatment does not fit all. By classifying patients in outcome-risk groups utilizing cytogenetic and molecular markers that have been shown to be associated with favorable or unfavorable outcomes, we may be able to prospectively differentiate patients who will respond and perhaps be cured with intensified chemotherapy from those who will benefit from allogeneic stem cell transplant and from those who may not benefit from either one and therefore should be candidates for clinical trials with investigational agents. Thus, risk-adapted stratification through a combination

of cytogenetic and molecular markers is giving us a way to predict outcome and allow us to move forth with what we call “personalized treatments”.

H&O What do we know in terms of cytogenetic risk groups?

GM Cytogenetic risk groups have been validated by several different groups around the world. Results have come out consistent, and in general, patients are classified as favorable or unfavorable. The favorable group includes those with acute promyelocytic leukemia (APL), which is defined by the t(15;17) translocation or the molecular equivalent fusion gene *PML/RARA* and those with the core binding factor (CBF) AML, which is defined by t(8;21) and inv(16) or t(16;16) or their molecular equivalent fusion genes *RUNX1/RUNX1T1* and *CBFB/MYH11*, respectively. The unfavorable group includes patients with complex karyotypes, inv(3) or t(3;3), chromosome 5 or 7 abnormalities, or t(6;11), etc. Patients with normal karyotype and those that are not considered in either the favorable or unfavorable groups are included in an intermediate risk group. Although there are minor variations, overall, this classification has been validated across large, multi-institutional studies around the world.

The aforementioned cytogenetic risk groups are mostly applicable to patients younger than 60 years old. There has been an attempt to establish a similar classification for patients older than 60 years, but these patients

do so poorly that even those in the favorable group (ie, CBF AML) have a survival rate of only approximately 30%, and unlike the younger AML patient population, there is overall little survival difference between the intermediate and unfavorable groups.

H&O What molecular markers further refine the cytogenetic risk classification of AML patients?

GM It is now clear that even when we apply cytogenetic risk grouping, we cannot accurately predict the outcome in AML patients. For example, only approximately 50-60% of CBF AML patients who are expected to have a favorable outcome ultimately achieve long-term survival. This means that we fail to accurately predict the outcome in the remaining patients. This realization instigated research in looking for a molecular subset within each cytogenetic group that may allow us to further break down risk classification in patients. Consequently, studies have found that within each cytogenetic group, there is some variation in terms of outcome.

***KIT* Mutations**

In CBF AML, we have found that there is at least one molecular marker that refines the prognostic significance of having t(8;21) or inv(16)/t(16;16): mutations of the *KIT* genes. These mutations occur in approximately 25-30% of CBF AMLs. *KIT* encodes a tyrosine kinase receptor that, when mutated, becomes constitutively activated, thereby leading to abnormal blast proliferation and survival. Patients with *KIT* mutations have a significantly worse outcome than those with wild type *KIT*.

KIT mutations are also a good example of molecular markers that could represent feasible therapeutic targets. As tyrosine kinase inhibitors (TKIs) have been developed, clinical trials that have incorporated these agents in combinations with chemotherapy are being initiated in *KIT* mutated patients. Notably, specific TKIs are selectively active against specific *KIT* mutations. For instance, imatinib (Gleevec, Novartis) is active against various exon 8 mutations and the exon 17 mutation involving codon N822, but not mutations involving codon D816, which can be successfully targeted with dasatinib (Sprycel, Bristol-Myers Squibb) and midostaurin. Therefore, in order to select the appropriate TKI for individual patients, it is necessary to determine the exact type of *KIT* mutation.

***CN-AML* Patients**

Another cytogenetic group that has been well molecularly characterized is the cytogenetically normal AML (CN-AML). The proportion of adults with *de novo* AML and a normal karyotype has varied between 40 and 49% in the largest cytogenetic studies; the outcome of these

patients has varied among studies, with reported 5-year survival rates between 30% and 40%. Recently, it has become evident that CN-AML patients are characterized by molecular heterogeneity that comprises acquired mutations and/or aberrant expression levels of certain genes. These molecular aberrations have been recognized to be associated with clinical outcome, and for some, to also represent therapeutic targets.

FLT3-ITD* and *FLT3-TKD

Among the most common mutations found in CN-AML are those of FMS-like tyrosine kinase 3 (*FLT3*) genes—namely *FLT3*-internal tandem duplication (ITD) and *FLT3* tyrosine kinase domain mutations (TKD). Both of these mutations induce change in *FLT3*, which is a gene that encodes for a tyrosine kinase receptor. When it is mutated, the mechanism is similar to the mutated *KIT*: it activates the abnormal mechanism of myeloblast proliferation and survival. *FLT3*-ITD is probably one of the most important molecular prognostic markers in AML, particularly in CN-AML, where it has an incidence rate of approximately 20-30% and invariably predicts poorer outcome.

The incidence of *FLT3*-TKD is much less: approximately 5-6% in CN-AML. The jury is still out on the prognostic significance of *FLT3*-TKD. A study conducted by the Medical Research Council suggested that the presence of *FLT3*-TKD was an indicator of better prognosis among AML patients,¹ whereas the Cancer and Leukemia Group B (CALGB) has reported it to be an unfavorable marker.² We are currently trying to reconcile the differences between these 2 studies.

***NPM1* Mutations**

Another important prognostic marker worth mentioning are the mutations of the nucleophosmin gene (*NPM1*). These mutations were initially reported by Falini and colleagues who discovered that more than 50% of CN-AML cases may harbor these mutations.³ *NPM1* mutations cause alterations in the encoded protein that lead to its aberrant cytoplasmic localization, which in nonmutated cells, is instead mostly located in the nucleolus. Altered distribution of this protein in cell compartments most likely interferes with the normal functions of nucleophosmin, which include preventing nucleolar protein aggregation, regulation of ribosomal protein assembly and their nucleocytoplasmic transport, as well as the regulation of the ARF and p53 tumor-suppressor pathways.

There has been some controversy in recent years on what prognostic significance *NPM1* mutations have, but the fact is that the true prognostic meaning for *NPM1* mutation is evident when *NPM1* mutations are considered along with *FLT3*-ITD. Based on the presence or

absence of these mutations, patients with CN-AML can be divided into 2 subsets: one is the molecular low risk group (ie, CN-AML patients with *NPM1* mutations and without *FLT3-ITD*) who has a better outcome, and the other is the molecular high risk group (ie, patients with *FLT3-ITD* or those without *FLT3-ITD* and with wild-type *NPM1*).⁴ However, even when we consider the dual mutation status, we are not completely accurate in our outcome prediction, as approximately 40% of the molecular low risk patients relapse, and approximately 30% of the molecular high risk patients remain event-free.

CEBPA Mutations

We are currently trying to refine the *FLT3/NPM1*-based classification by taking other molecular markers into account. One such molecular marker is the CCAAT/enhancer binding protein alpha (*CEBPA*) mutations, which has been mostly associated with CN-AML. The incidence of *CEBPA* mutations is approximately 15%, and they have invariably been reported to be predictive of favorable outcomes. In the last year, CALGB showed that *CEBPA* mutations are mostly restricted to CN-AML molecular high risk patients (ie, patients with *FLT3-ITD* or those without *FLT3-ITD* and with wild-type *NPM1*). It has been reported that 92% of patients with *CEBPA* mutations belong to the molecular high risk group.

Therefore, it is possible to further divide the molecular high risk patients into 2 subsets: one with *CEBPA* mutations with outcomes similar to the molecular low risk group, and the other with *CEBPA* wild type with a very unfavorable outcome. Importantly, *NPM1* and *CEBPA* mutations along with *FLT3-ITD* have recently been recognized as molecular aberrations characterizing specific AML biologic entities by the 2008 World Health Organization classification for myeloid neoplasms.

ERG Mutations

For CN-AML patients with molecular low risk (ie, CN-AML patients with *NPM1* mutations and without *FLT3-ITD*), CALGB investigators have found that there is another gene that further refines risk classification. If patients who harbor *NPM1* mutations and lack *FLT3-ITD* also have a high expression of a gene called *ERG*, they have a negative clinical outcome that is similar to that of molecular high risk patients. In contrast, patients who have *NPM1* mutations and lack *FLT3-ITD* would have a very favorable outcome if they express low levels of *ERG*.

Evidently in CN-AML, we started with 1 molecular marker—*FLT3-ITD*—and further refined risk classification with the addition of considering *NPM1* mutations; currently we are trying to further refine this by taking *CEBPA* mutations and *ERG* expressions into account.

However, the situation is even more complicated because there are additional markers to consider: *WT1* mutations or overexpressions of *BAALC* or *MNI* have been reported to predict an unfavorable outcome.

MLL-PTD Patients

The partial tandem duplication (PTD) involving the mixed lineage leukemia gene (*MLL*) was previously determined to be an adverse prognostic marker in CN-AML. However, we have recently found that if *MLL*-PTD patients are treated intensively with at least an autologous stem cell transplant, they may have a very similar outcome to CN-AML patients without this mutation; the strategy does not necessarily lead to a better outcome, but the marker no longer predicts worse outcome. *MLL*-PTD may now be considered as a type of neutral marker whose previous unfavorable significance has been modified by changes in therapeutic approaches.

This emphasizes that all these molecular markers are predictive of outcomes based on the treatment that are administered to the patients. Hopefully in the future, when our treatment is refined and prognostic markers are also used as therapeutic targets, some of the unfavorable predictors may become neutral or even favorable. For example, if *FLT3-ITD* patients will be found to benefit from TKI therapy, *FLT3-ITD* may no longer be considered an unfavorable marker but rather a favorable predictor for a specific treatment outcome. The ongoing CALGB 10603 trial is stratifying patients with *FLT3-ITD* to receive either a TKI plus chemotherapy treatment or chemotherapy plus placebo treatment; it would be interesting to see whether there is a substantial increase in survival after TKI treatment.

Needless to say, the study of molecular markers in AML is a complicated research area, as new markers being discovered lead to new ways to refine our classifications. Many markers have been explored, and new treatments are upcoming. However, I think that these markers have substantially improved our approach with AML for 2 reasons: 1) we understand the biology of AML and their clinical implication much better, and 2) we start to understand how we can target, from a treatment standpoint, some of these biologic aberrations that nonrandomly occur in AML blasts.

It is important to underscore that in order to fully implement all these markers for risk stratification and treatment guidance outside of clinical trials still requires further investigation. Although gene mutational analyses can be performed without major difficulties by specialized molecular diagnostic laboratories, the analyses of genes that predict clinical outcome based on expression levels require standardization studies yet to be conducted.

H&O Is there a prognostic significance in the allelic ratio of these mutations?

GM This concept is important and may be applicable to several mutations, but currently *FLT3*-ITD is the only example. The question is whether expression levels of a mutated allele with respect to the wild type allele has prognostic significance and therefore can further refine the use of a specific marker as an outcome predictor. It has been found in retrospective studies that patients with *FLT3*-ITD who have a low allelic ratio (the ratio between the mutated allele *FLT3*-ITD vs the *FLT3* wild type) have a better outcome than patients with a high allelic ratio.⁵ Then the question becomes where to prospectively draw the line to select a specific cut-off that defines high versus low risk for patients enrolled in ongoing clinical trials. Further research is needed to determine the absolute allelic ratio cut off that can be widely utilized to guide patient treatment upfront; some validation on this topic is going on in the CALGB 10603 trial.

H&O What do we know of microRNAs as molecular markers for AML?

GM MicroRNAs are short, noncoding RNAs that hybridize to complementary messenger RNA (mRNA) and therefore induce the degradation or translation inhibition of their targets and the downregulation of corresponding encoded proteins. Thus, if microRNAs that target oncogenes are depleted in leukemia cells, the targeted oncogene expression will be upregulated. On the other hand, if microRNAs that target tumor suppressor genes are upregulated, the expression of tumor suppressor genes becomes downregulated. Both these mechanisms have been shown to contribute to leukemogenesis.

In 2008, we reported the first validated prognostic signature of microRNAs in CN-AML.⁶ By using microRNA expression profiling on CN-AML patients younger than 60 years who were similarly treated on CALGB protocols, we derived a microRNA signature that was associated with outcome in CN-AML molecular high risk patients. The prognostic significance of this signature was initially demonstrated in a training set of patients and

subsequently validated in an independent set of patients. We found that this microRNA expression signature was associated with outcome independent of other molecular and clinical prognosticators.

Interestingly, by combining the results of the microRNA expression profiling with those of gene expression profiling in the same patient group, we also showed that the expression levels of microRNAs constituting the prognostic signature negatively correlated with that of predicted target genes, which encoded proteins that were involved in certain innate immunity pathways such as TLR4 and IL1 β . The lower the expression of the targeting microRNA, the higher the expression of the target genes and the chance for unfavorable outcome. Indeed, high expression of proteins that regulate inflammatory responses in a normal host have been shown to promote uncontrolled AML blast survival and proliferation. These studies are good examples of how it is possible to identify, through studies that integrate cytogenetic and molecular prognostic information with biologic findings, potentially novel subsets of AML and relevant therapeutic targets. Indeed, I believe that similar strategies supported by rapidly developing technologies will allow us to search the whole genome of leukemia cells for gene mutations and aberrant expression levels and guide the future treatment for AML patients.

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