

**Review Article** 

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# Molecular Biomarkers in Cytogenetically Normal – Acute Myeloid Leukemia: harnessing the targets

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# Abstract

Cytogenetics based risk adapted therapy has set the stage for personalized medicine for Acute Myeloid Leukemia but 50% of Acute Myeloid Leukemia cases exhibit normal cytogenetics. With the molecular techniques mutations like NPM1, CEBPA and FLT3-ITD have been discovered and are recommended by WHO and ELN. These are molecular prognostic biomarkers which have refined the risk stratification resulting in better but still very variable outcome and there is always a risk of over treating some cases using the conventional 3+7 regimen. Thus there is a need to identify disease specific predictive biomarkers and translate them to targeted therapy. The implementation of high throughput molecular diagnostics has provided various biomarkers in the form of gene mutation and over expression, mi RNA and epigenetics which can be used for diagnosis, screening, monitoring, surveillance, or for providing predictive or prognostic information. Molecular biomarkers also play a role in development of targeted therapies. Some markers like FLT3-ITD have been identified against which specific inhibitors have been developed but many of these molecular lesions represent cooperating rather than initiating genetic events which cannot be targeted.

Eventually the cost and time to completion of whole-genome sequencing will decrease and it will be more convenient to sequence the entire genome from an individual patient rather than to screen for various mutations. Hopefully, in the future a ubiquitous target will be discovered that would be used across all the subgroups of Acute Myeloid Leukemia.

**Keywords:** CN-AML; Biomarkers; Prognostic markers; Predictive markers; Targeted therapy; Personalized medicine

AML: Abbrevations: Acute Myeloid Leukemia; CN-AML: Cytogenetic Ally Normal Acute Myeloid Leukemia; IR-AML: Intermediate Risk - Acute Myeloid Leukemia; NPM1: Nucleophosmin-1; CEBPA: CCAAT/Enhancer Binding Protein Alpha; FLT3-ITD: Fms-Like Tyrosine Kinase 3-Internal Tandem Duplication; WHO: World Health Organization; ELN: European Leukemia Net; CGH: Comparative Genome Hybridization; SNP: Single Nucleotide Polymorphism; mi RNA: Micro RNA; FAB: French American British; M3, M3v, and M4Eo: FAB categories; RT-PCR, qRT-PCR: Reverse Transcriptase Polymerase Chain Reaction, Quantitative RT-PCR; FISH: Florescence in situ Hybridization; MRD: Minimal Residual Disease; IDH1/2: Isocitrate Dehydrogenases1/2; DNMT3A: DNA methyltransferase 3A; TET2: tet oncogene family member 2, or Ten-Eleven Translocation; EZH2-: Enzymatic Component Of The Polycomb Repressive Complex; BCOR: BCL6 co repressor; allo HSCT, SCT: Allogenic Hematopoietic Stem Cell Transplant; MLL-PTD: Mixed Lineage Leukemia Partial Tandem Duplication; ASXL1: Additional Sex Combs Like 1; HDACi: Histone Deacetylase Inhibitors; ECOG E1900 trial: Eastern Cooperative Oncology Group; RAS, N RAS, K RAS: Rat Sarcoma/ Kirsten Ras Oncogene/ Neuroblastoma RAS Viral (v-ras) Oncogene Homolog; KIT: v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog; WT1 mutation: Wilm's Tumor 1; RUNX1 mutations: Runt-Related Transcription Factor 1; MEK: Mitogen-Activated Protein Kinase Kinase-1; TP53 gene/P 53: Tumor Protein P53 Gene/ Protein 53; BAALC gene: Brain And Acute Leukemia, Cytoplasmic; MN1 gene: Meningioma 1; ERG- v: Ets Erythroblastosis Virus E26 Oncogene Homolog, Avian; EVI1: Ecotropic viral integration site 1; PI3/AKT/mTOR Pathways: Phosphatidylinositol-3-Kinase/ Akt, Also Known As Protein Kinase B/ Mammalian Target Of Rapamycin/ Phosphatase And Tensin Homologue Deleted On Chromosome Ten; NF-kB: Nuclear Factor Kappa From B Cells; Wnt/beta-catenin pathways: Wingless-Related Integration Site; MDR1: Multi Drug Resistance Gene; P-gp: P Glycoprotein; LRP: Lung Resistance Protein; MRP1: MDR Associated Protein 1; Bcl-2: B-Cell Lymphoma 2; LSC: Leukemia Stem Cell; VEGF: Vascular Endothelial Growth Factor; CR: Complete Remission; EFS: Event Free Survival; RFS: Relapse Free Survival; OS: Overall Survival; DFS: Disease Free Survival; Ara-C: Cytosine Arabinoside

# Introduction

Pretreatment cytogenetics is the backbone of current risk stratified but non specific treatment of Acute Myeloid Leukemia (AML). Nearly 50% of AML cases have no abnormality on karyotyping, even with comparative genomic hybridization (CGH) or SNP arrays [1-3]. Cytogenetically normal (CN-AML) patients, who comprise the largest subgroup and have been assigned an 'intermediate' prognosis, with five-year survival rates varying between 24% and 42% [4]. This clinical heterogeneity is related to the genomic heterogeneity of this cytogenetic subgroup in which several molecular aberrations have been identified.

Molecular characterization of CN-AML has been a major advancement in the management of AML patients. These mutations are the prognostic biomarkers and they influence treatment decisions

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#### in terms of better prognostic stratification but lack predictability. Thus even when multitude of mutations have been discovered they have not been translated into effective targeted therapy and non specific 3+7 regimen is still being used to treat this very heterogeneous category. There is always a risk of over treating many patients because of the lack of more specific drugs which require predictive biomarkers for their development.

The use of specific nontoxic drug All Trans Retinoic Acid (ATRA) in Acute Promyelocytic leukemia only proves that 'one drug fits all' practice should now give way to customized targeted therapy or personalized medicine. Personalized medicine has its underpinnings in the clinical molecular testing of diagnostic, prognostic, and predictive biomarkers.

Remarkable advancement in molecular diagnostics and highthroughput DNA sequencing has provided many biomarkers in the form of gene mutations and expressions, epigenetics and miRNA. There is a need to identify predictive biomarkers amongst them which can accurately predict the outcome in AML patients and can be matched with customized therapy based on individual prognostic risk and therapeutic response.

This review is an overview of the various genetic and epigenetic variations which have impact on the course of the disease along with the various available techniques and also some deterrents which have to be overcome for the genetic biomarkers to become standard of care.

# It is Important to Ensure that the Patient's Karyotype is Truly Normal

Before proceeding for the molecular studies it is imperative to ensure that the karyotype of the CN-AML patient is truly normal. This is because rarely a CN AML case may harbor fusion genes due to cryptic recurrent translocations. To reliably pick up such a case it is recommended that analysis of at least 20 metaphases from a marrow sample cultured in vitro for 24 to 48 hours is performed [5-7].

Such an investigation is warranted especially in CN-AML patients with FAB categories M3, M3v, and M4Eo morphology but is otherwise not routinely recommended outside of a clinical trial [8]. The RT-PCR and FISH can detect the cryptic recurrent translocations while spectral karyotyping [9], FISH with a comprehensive set of genomic DNA probes [10], and comparative genomic hybridization [11] can confirm the absence of any other unrecognized chromosome aberrations.

# Various Categories of Mutations with its Respective Targeted Therapy

# Mutations with widely recognized clinical impact

Molecular markers that have been incorporated in WHO and ELN classification systems are [12] are NPM1, FLT3-ITD and CEBPA. They are recommended to be used in routine in order to identify patients who would benefit from allo SCT. The FLT3-ITD is associated with aggressive disease and poor outcome while biallelic CEBPA mutation and NPM1 without FLT3-ITD have favorable outcome. The FLT3-ITD and NPM1 mutations frequently coexist and their interaction influences the prognosis hence both should be tested upfront in all patients with CN-AML and when both NPM1 and FLT3-ITD mutations are negative, CEBPA mutations should be tested for further risk stratification.

#### Targeted therapy

CN-AML patients harboring CEBPA and particularly NPM1

mutations without FLT3-ITD mutations are sensitive to the induction therapy [13] and standard induction chemotherapy followed by three to four cycles of high-dose cytarabine is recommended in younger adults (<60 years old) with CN-AML. No targeted therapy so far exists for the two favorable mutations but Blasts with NPM1 mutations exhibit high density of CD33 and addition of anti-CD33 immunoconjugate to chemotherapy has been tried [14]. Also ATRA with induction therapy has been tried in elderly CN-AML expressing NPM1 mutations [15]. Small molecules which can interfere with the oligomerization properties of NPM1 have shown anti-leukemic activity in vitro and may enter in clinical trial [16]. Thus CEBPA and particularly NPM1 mutations are only prognostic biomarkers the only predictive marker in use currently is the FLT3-ITD mutation against which multi-targeted TKI are in early phases of clinical development viz sorafenib, lestaurtinib, midostaurin, quizartinib and tandutinib. All exert some anti-leukemic activity, but when used as single agents have been disappointing hence FLT3 inhibitors have been tried combined with chemotherapy, in Phase III trials [17,18]. FLT3 mutation because of its mutability is not good target for MRD [19] but it is expressed on the LSC and FLT3 inhibitors are being tried [20].

# **Epigenetic modifier genes**

A significant class of the new mutations is epigenetic modifier genes which include the IDH1, IDH2, DNMT3A and TET2 genes. With the exception of TET2 mutations, all other mutations have been identified by massively parallel sequencing.

# IDH1 and IDH2 mutations

Mutations in IDH1 and IDH2 occur in 10-12% CN-AML patients [21]. Mutant IDH enzymatic activity in AML converts alpha ketoglutarate to 2 hydroxyglutarate (2-HG) which is the oncometabolite associated with leukemogenesis. Raised 2-HG can be detected in the serum and BM of AML patients with IDH1/2 mutations and serves as a biomarker for this group of patients and also as a measure of minimal residual disease [22,23]. There is a mutual exclusivity between IDH1/2 and TET2 mutations in AML and significant co occurrence with NPM1 mutations [24]. Mutations in the IDH1 occur at R132 while at IDH2 at R172. Where mutations at IDHR172 do not correlate with outcome or response to therapy, IDHR132 mutation had worse outcome in patients with the FLT3 wild type genotype, in contrast IDH R140Q mutation conferred improved OS [25].

# **DNMT3A mutations**

DNMT3A mutations have been identified in 20% acute myeloid leukemia (AML). It is the second most common somatic mutation [21] after FLT3 ITD mutations, within CN-AML.The DNMT3A R882H mutations is associated with NPM1, FLT3-ITD and IDH1 mutations and is also with BCOR mutations.

DNMT3A mutations can identify new subgroups of CN-AML patients with shorter overall and disease free survival. In fact, DNMT3A mutations appear to exert their negative impact mostly in the high-risk category of CN-AML (wild-type NPM1 and wild-type FLT3), for which more intensive treatments, including allo HSCT, are already recommended [26-28].

# **TET2 mutations**

The Tet oncogene family member 2 (TET2) encodes for proteins that are involved in epigenetic regulation. TET2 mutations have been detected in 7.6% of AML and also in association with CN-AML. The

enzymatic activity of TET2 is impaired by 2-HG produced by mutant IDH1/2 hence mutations of IDH1/2 are mutually exclusive with mutations of TET2 [18,29]. The prognostic impact of TET2 mutations in AML is unclear [18].

#### **MLL mutations**

Partial Tandem Duplication (PTD) of MLL has been detected in 5-11% of CN-AML [4,24]. It is proposed to contribute to AML development through DNA hypermethylation and epigenetic silencing of tumor suppressor genes [30]. MLL-PTD may be associated with FLT3-ITD and FLT3 point mutations and have shortened remission duration and shorter Disease-Free Survival (DFS) [31].

#### **ASXL1 mutations**

ASXL1 mutations are much more common in elderly than younger AML patients (16% vs. 3-5%) [18]. Although rare in AML ASXL1 mutation is associated with adverse overall survival [21].

#### Targeted therapy

Unlike chromosomal deletions where there is an irreversible loss of function, epigenetic mutations can be reversed using HDACi and hypomethylating agents like 5-aza-2'-deoxycytidine (decitabine) and 5-azacytidine [32].

No specific targeted therapy apart from the hypomethylation therapy is available for this subset of AML. A small molecule inhibitor has been developed which would block the synthesis of 2-HG and potentially be the specific drug for IDH1/2 mutations [33,34]. For MLL mutations a specific therapy has been developed to target DOT1L, which is a histone methyltransferase utilized by the rearranged/ mutated MLL. Recently small molecule inhibitor of DOT1L namely EPZ-5676 has entered human clinical trial [35].

ECOG E1900 trial was conducted using several of the above markers and survival was seen to be improved with escalated dose of daunorubicin among patients with DNMT3A or NPM1 mutations or MLL translocations [36]. Using gene mutations to segregate patients who may benefit from higher doses of chemotherapy was the step in the direction of personalized therapy for AML.

The mutations cooperate in the leukemogenesis and understanding of which mutations occur together can provide important prognostic information. More extensive mutational analysis differentiates otherwise 'unremarkable on cytogenetics' AML category into clinically relevant risk groups [37]. FLT3-ITD has been shown to be an independent prognostic factor for poor outcome in AML in many studies hence using it as the starting point the following risk stratification has been proposed for CN-AML [21].

# FLT3-ITD-negative

- 1. NPM1/IDH mutant patients represent a favorable-risk AML.
- 2. NPM1 mutant patients without concurrent IDH mutations have less favorable outcome.
- 3. Presence of TET2, ASXL1,and/or MLL-PTD is associated with very adverse overall survival.

#### **FLT3-ITD mutant**

1. Concurrent mutations in TET2, DNMT3A, MLL-PTD, forms a very-high-risk subset which may impact the treatment decisions. Large number (approximately 47%) of the total intermediate-risk cases fall into this category which harbor any one of the above four mutations.

2. In the absence of the above 4 genetic abnormalities outcome is same as that of FLT3-ITD/CEBPa double-mutant patients.

This mutationally defined risk stratification would be clinically significant to identify patients with favorable risk who would have better outcome than even core binding factor-positive AML cases simply with standard induction and consolidation. Patients with adverse-risk AML fare badly with standard therapy and require SCT or investigational therapy for cure [21].

In a large study [38] using multiplexed mass spectrometry, 344 mutations across 31 genes were screened which are associated with leukemia. It was observed that between the normal and abnormal cytogenetic risk groups, the mutation frequency and distribution is very different, suggesting different biology. Not only was the mutation frequency in CN AML at 78%, twice that seen in abnormal cytogenetics. The mutation distribution was also distinct which included more frequent IDH1, NPM1, and NPM1-FLT3 overlapping mutations in the normal cytogenetic group and increased RAS, KIT, and isolated FLT3-ITD mutations in the abnormal cytogenetic group. Concurrent mutations in 2 or more genes were seen almost exclusively in patients with normal cytogenetics In the CN-AML cases, FLT3, NPM1, IDH1, and CEBPA mutations were seen more commonly as multiple mutations than in isolation [38]. Such mutational profiling not only helps to understand the biology and prognosticate the patients it may also help selecting patients for clinical trials with novel inhibitors.

# **Other Mutations**

Apart from the above mutations there are some newer less common ones which also influence the prognosis.

# WT1 gene

These mutations are detectable in 10-13% of CN-AML [39-43] and in multivariable analysis, WT1mutation was an independent adverse prognostic factor and the WT1mut/FLT3-ITD<sup>pos</sup> genotype appeared to be associated with worse clinical course. Gaidzik et al. suggested that the negative impact of WT1 mutations as reported by others may be overcome by the use of repetitive cycles of high-dose cytarabine [41]. SNP rs16754 located in the mutational hot spot of WT1 in exon 7 has been shown to be associated with favorable outcome in patients with CN-AML and increased chemo sensitivity [44].

#### RUNX1 gene

Mutations usually cluster in the Runt domain of the gene. Frequencies of RUNX1 mutations within CN-AML are variable, ranging from 6.3% [45] to 26.3% [46]. The Mutations are associated with resistance to chemotherapy and inferior EFS, RFS, and OS [45-47]; however an improved RFS is seen with allo HSCT.

#### BCOR (BCL6 co repressor) gene

Mutations of the BCOR gene were discovered by whole exome sequencing of a single CN-AML patient [48]. Since then has been found to occur in about 4% of all CN-AML and occur with DNMT3A mutations. They predict a poorer prognosis [48] and act by interfering with epigenetic mechanisms [49].

# **RAS** gene

NRAS mutation in CN-AML has been found in 9% adult and 14% younger 60 years, patients [50]. Although NRAS mutations have been

known to have no prognostic impact they provide a target for molecular therapy [33]. More recently, Neubauer et al. showed a predictive impact of RAS mutations in that patients receiving high-dose cytarabine in consolidation therapy had a significantly lower probability of relapse as compared with patients receiving standard dose cytarabine [51]. RAS mutations also render the cells sensitive to MEK inhibitors and currently clinical trials investigating MEK inhibitors in myeloid malignancies with NRAS and/or KRAS mutations are underway [52].

#### TP53 gene

P53 gene mutations are found in less than 10% of de novo AML and are associated with older age, resistance to chemotherapy and very short survival [53].

# **Over Expression of Genes**

#### The BAALC (brain and acute leukemia, cytoplasmic) gene

High BAALC expression levels predict lower CR rates and also low disease-free and overall survival (OS) [42,54].

#### MN1 (meningioma 1) gene

MN1 over expression is associated with poor response to induction chemotherapy higher relapse rate and worse OS. While low MN1 expression has been correlated with response to ATRA in elderly non APML patients, thereby suggesting that MN1 expression is not only a prognostic but also a predictive marker for response to treatment. In younger than 60 years CN-AML cases, higher MN1 expression is associated with NPM1 wild-type status and increased BAALC expression [42,55].

# ERG (v-ets erythroblastosis virus E26 oncogene homolog, avian)

The adverse prognostic significance of over expression of ERG has been established in CN-AML [56]. High ERG expression levels influences outcome of low molecular risk CN-AML (mutated NPM1 without FLT3-ITD). In a GEP study ERG expression was the strongest negative prognostic factor and provided prognostic information in addition to established parameters (eg, FLT3-ITD) [42].

#### EVI1 (ecotropic viral integration site 1)

High EVI1 expression predicts poor outcome in the cytogenetic intermediate-risk group. Patients with high EVI1 expression had significantly better 5-year relapse-free survival and OS who received allo HSCT in the first CR [55, 57].

#### **EZH2** mutations

Recently found in a male with CN-AML [58]. Over expression of EZH2 has been reported in both solid tumors and blood cancers [59,60].

# Targeted therapy

Recently an S-adenosyl homocysteine hydrolase inhibitor named 3-Deazaneplanocin A (DZNep) has been shown to induce selective apoptosis in cancer cells sparing the normal cells and removed EZH2 [61-64]. This compound has not reached yet the clinical trial setting. Apart from this the above markers are investigational and so far have had no bearing on treatment decisions but are important prognostic markers. For many of them, there are still not enough studies evaluating their prognostic and predictive impact.

# **Other Molecular Biomarkers**

## Micro RNA (miR)

Recent studies have also shown that changes in microRNA expression can affect clinical outcome in AML. Garzon et al. [65] reported that, across all cytogenetic subgroups, over expressed miR-20a, miR-25, miR-191, miR-199a, and miR-199b adversely affected OS. Using 12 micro RNA probes an expression signature which associated with event-free survival has been defined in patients with CN-AML belonging to the molecular high-risk group (FLT3-ITD<sup>pos</sup> + NPM1 <sup>wild-type</sup>). Five probes in the signature represented miR-181a and miR-181b; down regulation of the members of miR-181 family attributed to the aggressive leukemia phenotype and high levels of miR-181 expression is associated with less aggressive disease and predicts favorable outcome in CN-AML [64].

#### Therapeutic implications

Synthetic oligonucleotides targeting specific miRNAs have been proposed as a therapy with potential anti-leukemic activity.

# PI3K/AKT/mTOR pathways

Most AML cases show activated PI3K/AKT/mTOR pathways [65-67]. PI3 kinase inhibitors such as LY294002 and PI-103, have shown to induce apoptosis in AML cells ex vivo [68,69]. Inhibition of Akt with perfosine [70] when combined with MEK inhibitors in preclinical studies has shown kill AML cells ex vivo [71]. Although rapamycin, an mTORC1 inhibitor, failed to show responses in clinical trial as a single agent [72] it may have potentially have some role as a chemo sensitizing agent [73]. PI3K and mTORC1/2 inhibitors like temsirolimus when combined with chemotherapy like clofarabine in elderly high risk AML has been observed to achieve much better clinical responses than either agent alone [74-76]. mTOR activation which is the downstream of PI3K and AKT results in the phosphorylation ribosomal S6 protein which is a robust marker and can be noted by flow cytometry and can be used as a molecular marker to develop drugs that would target this pathway [77].

# **Response to Therapy**

#### Minimal residual disease (MRD)

The MRD is an in vivo measure of drug sensitivity and provides a patient-specific parameter predictive of risk of relapse. The newly discovered molecular prognostic biomarkers are being tested for their viability to be used for MRD measurement. For this purpose, it is important to choose a marker that is measureable and stable throughout the disease course. An NPM1 mutation has been proved to be a suitable biomarker since it remains stable throughout the course of the disease including relapse [78].

Increased level of MRD is an indication for either allogeneic transplantation or other investigational therapies. But MRD to be clinically relevant must be used along with pretreatment variables to designate patients into specific risk categories [19].

#### Multidrug resistance

It is the levels of multidrug resistance gene 1 (MDR1) encoding P-glycoprotein (Pgp) as well as the MDR associated protein 1 (MRP1) and lung resistance protein (LRP) which mediate drug resistance and correlate with response to chemotherapy and clinical outcome.

Various studies have evaluated the prognostic significance of

expression of MDR genes [42,79-81]. Early studies demonstrated significant association between expression of MDR genes and clinical outcome [82-84], while the later studies [85] demonstrated that though MDR genes may not be an independent prognostic factor, but it can be a potential therapeutic target. Several agents have been developed to impair the function of proteins encoded by MDR genes, [86-89] and in combination with conventional chemotherapy, these agents sensitize the cells to the chemotherapy and thus improve clinical outcome [89].

Evasion of apoptosis is also connected to drug resistance. Bcl-2 is an apoptosis inhibitor protein when over expressed renders tumor cells resistant to induction of apoptosis. As high levels of bcl-2, in AML has been pursued as a potential drug target using agents such as antisense oligonucleotides and small molecular inhibitors [90-92]. Another strategy to induce apoptosis is by the novel compound APR-246, which restores the function of p53 which has been tested in a clinical trial in patients with hematological malignancies [93].

#### The Leukemic Stem Cells (LSCs)

LSCs represent a reservoir of leukemia and current treatment is cytotoxic to the tumor bulk, but not to the leukemic stem cells that culminate into relapse and resistance to chemotherapy [94,95]. The signaling pathways like NF-kB, PI3K/Akt/mTOR, and Wnt/betacatenin pathways are important for the survival of LSCs and have obvious therapeutic implications [96]. Parthenolide has been found to induce apoptosis in AML; it is a potent inhibitor of NF-kB, and compared to cytarabine much more selectively eliminates LSCs thus sparing normal hematopoietic cells [97]. The PI3/AKT/mTOR pathway, constitutively activated in most AMLs, is important for the LSCs and is being targeted by mTOR inhibitors, three of which rapamycin, temsirolimus, and everolimus, are undergoing clinical trials [72,73]. Pharmacologic inhibition of activated phosphatidylinositide-3 kinase by LY294002 is also being studied which would survival of LSCs [98]. Relationship of LSC with its microenvironment is the new target for drug development. Antiangiogenic drugs [99,100] are the main drug of interest. Trials of angiogenesis inhibitors (angiostatin, endostatin, and platelet factor-4), antibodies to VEGF or fibroblast growth factor, or inhibitors of endothelial cell proliferation, such as SU5416, are underway [101]. Agents such as granulocyte colony stimulating factor cause LSCs to cycle, disrupting them from their vascular niche and increased their sensitivity to cytarabine [102]. Rakicidin A is another compound which may target a hypoxic microenvironment [103]. Several FLT3 inhibitors, including CEP701and PKC412, are also being tried in phase II clinical trials in AML targeting the markers on the LSC [104].

Cyclosporine A which is a P-glycoprotein inhibitor when used along with standard cytotoxic chemotherapy was found to improve clinical outcomes in poor risk patients with AML [105]. However, studies with another P-glycoprotein inhibitor valspodar (PSC-833) failed to replicate the results [106]. Currently P-glycoprotein inhibitor zosuquidar is undergoing clinical trial in combination with standard cytotoxic agents. Breast cancer resistance protein inhibitors are much more fastidious and effective, but are still in nascent stage of clinical development [107,108]. The differential expression of miRNAs on the LSCs is being targeted for the eradication of LSCs [109,110].

#### Pharmacogenomics

It is the study of how genetic variants influence drug efficacy and toxicity. By sequencing the germline and leukemia genomes in patients with AML, it would be possible to identify genetic variants known to affect the efficacy and toxicity of chemotherapeutic agents which subsequently influence the choice and dose of drugs used [111]. The pharmacogenetics testing can be implemented even before the treatment begins. Pharmacogenomics thus makes it possible to optimize the dosage of existing therapeutics as has been studied with Ara-C [112]. Detection of various polymorphisms in genes involved in metabolism using the newer high-throughput genomics will increase in the utility of pharmacogenomics.

# **Molecular Biomarker Detection Methodologies**

Although karyotyping is the basis for classification in 2008 World Health Organization classification scheme and it is possible to categorize over two-thirds of AML compared to only one-third in the 2001 World Health Organization scheme. But it was the molecular technologies which has catapulted the categorization of the otherwise cytogenetically unremarkable intermediate risk CNAML cases into risk adapted categories thus allowing not only for better understanding of leukemogenesis but also biomarker discovery and potential for targeted therapy.

The implementation of DNA and protein microarrays has accelerated the biomarker discovery like never before. The ultimate goal is to identify sets of disease specific biomarkers and then to combine them with a robust screening technology. Though this technique is not yet applicable for MRD detection for which PCR is preferred. It can also be an alternative to microarray- especially when genes in very low copy number have to be confirmed. Quantitative RT-PCR (qRT-PCR), where signals are simultaneously generated in an exponential way is the most sensitive technique for mRNA detection and quantitation [113]. Hundreds of SNPs across the whole genome can be analyzed with the help of SNPs arrays. DNA copy number, loss of heterozygosity and chromosomal modifications can also be picked up using array technology. Gene expression profiling based on array technology not only identifies potential disease biomarkers but also improves the understanding of the molecular biology of the disease.

Although automated sequencing instruments (sequencers) allow high-throughput nucleotide sequencing full-genome sequencing and exonic sequencing is now feasible with rapidly decreasing turnover time and may be in near future available for clinical use to identify druggable targets and markers for monitoring tumor burden. Unique subtypes of AML have been identified on the basis of DNA methylation signatures, which have been found to be predictive of survival but not yet of response to hypomethylating agents [114].

Newer high throughput technologies have made it possible to categorize 80% of AML cases based on molecular biomarkers to predict outcome and select appropriate treatment but more than 25% of AML patients still remain uncategorized which exhibit no mutations in the known leukemia-associated genes [115]. More sophisticated technology needs to be developed for these patients.

#### Deterrents in the Development of Targeted Therapy

Biomarker-based drug development is a proven strategy in the development of new drugs. The implementation of DNA and protein microarrays has accelerated the biomarker discovery. The ultimate goal is to identify sets of disease specific biomarkers which would allow stratification based on clinical molecular testing thus evading the toxic effects and morbidity by patient selection.

Despite the rapid developments of molecular technologies and

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newer biomarkers being discovered it has not translated into the targeted drug development. This has been marred by many problems.

- 1. Due to their relative infrequency of the mutations conclusions about their significance cannot be made with great confidence.
- Genetic complexity of AML is characterized by several coexisting mutations hence interactions with other molecular markers would be important but were not taken into account.
- 3. Their mere discovery offers potential targets for novel therapies, but to identify the truly 'druggable' ones would require delineating the driver mutations where most of the mutations which have been identified are passenger mutations.
- 4. The prognostic impact of the markers has been mostly evaluated in retrospective studies, involving low patient numbers.
- 5. Turnaround time for their results is still not appropriate to plan the induction regimen based on the genetic profile of patients.
- 6. The cancer biomarkers in clinical use are not suitable for population screening or for early diagnosis.
- 7. Prohibitive cost of genotyping or sequencing deters the progress of biomarker discovery and utilization.
- 8. Targeting essential cell functions creates drugs with narrow therapeutic indices. These new agents are unlikely to be curative when administered as monotherapy. Thus, new compounds with an antileukemic activity need to be tested in combination with other new agents or with already approved conventional drugs.
- Drugs targeting the regulators of cellular proliferation, survival, angiogenicity or immunogenicity of leukemic cells may produce collateral damage in normal cells which share these properties.

Large prospective multicentric studies perhaps on international scale are clearly warranted to clarify these caveats.

#### Conclusion

In conclusion, the most immediate application of molecular prognostic markers is in the decision whether or not to proceed to SCT in first CR based on a risk-adapted approach. The molecular biomarkers also have immense role in the design and implementation of targeted therapies which is making its way from disease-based therapeutic regimens to molecular target-based protocols. Hopefully a more ubiquitous 'fit for purpose' biomarker will be discovered that can be used across the AML subgroups and there will not be a requirement for a unique therapy matched for each subgroup.

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