Molecular and functional intra-tumoral heterogeneity in adult Acute Myelogenous Leukemia

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Acute Myeloid Leukemia (AML) is the most common acute leukemia in adults and remains of poor outcome due to high prevalence of disease relapse. Relapse is believed to be associated with a rare, self-renewing phenotypic compartment named Leukemia-Propagating Cells (LPC). Recent studies also reveal that leukemic cells (blasts) actually display different mutation patterns defining molecular subclones in a very AML. Here we give a conceptual review of recent works on these phenotypic, molecular and functional heterogeneities that can be featured by a patient's AML, proposing a novel integrative insight into intra-tumoral disparities.

Introduction

Acute myelogenous leukemia (AML) is the most frequent acute blood cancer in adults. It results from the invasion of bone marrow by undifferentiated proliferating 'blasts' committed in a myelogenous cell lineage. They are able to circulate in the blood and cause a great decrease in the quantity of other lineages (a multi-lineage cytopenia). The resulting immunodeficiency and bleedings quickly threaten the patient's life.

Heterogeneity between different types of AML is already well-known (see French-American-British (FAB) or WHO classifications [1], that are often based on cellular morphologic criteria).

On the other hand, intratumoral heterogeneity has also been studied for almost 50 years [2] and leukaemia is often a focus of major interest, probably due to the tumour accessibility (blood samples, bone marrow aspirations) and its relatively old appreciation. More advanced technologies have allowed researchers to further characterize intraleukemic heterogeneities. Massive parallel sequencing reveal new mutations, even if only 0.1% of a patient's cells are involved. Together with epigenetic marks analysis, they permit further comparisons of genomic and epigenomic landscapes among cells of a tumor, revealing it is composed of clones that evolve in a Darwinian process. Phenotypic single-cell analysis is possible using FACS; this tool also allows definitions of rare cellular compartment with specific phenotypic signature enabling leukemia-initiating cell engraftment into immunodeficient mice, thus defining the leukemic stem cell (as opposed to commonly named "bulk cells", which cannot initiate leukemia).

Despite better insight into molecular and functional heterogeneities in AML, AML's evolution within a patient is still poorly understood. Moreover, few publications consider the association of molecular and functional heterogeneities in AML. Consequently, relationships between them remain poorly understood. The present review offers an insight into recent findings about molecular and functional heterogeneities in adult AML.

Functional Heterogeneity of AML: Different Subsets Defined by Cellular Capacities

To assess what are the relevant functional features that distinguish AML fractions, we first need to explain the clinical impact of AML on a patient. AML is commonly known for inducing a multi-lineage cytopenia, thus implying an anemia, immunodeficiency and coagulation deficiency [3]. Though well-known, these clinical features are actually not fully understood. A frequently raised explanation is that the expanding AML alters normal HSCs (hematopoietic stem cells). Miraki-Moud et al. [4] recently showed that rather than HSC depletion, AML more likely impairs its differentiation, thus decreases progenitor-generation capacities. Such a hypothesis implies it may be relevant to characterize an AML's cells using their functional features: differentiation impairment, frequency of the blasts, self-renewing and survival capacities, and global proliferation rate. More broadly, cytokine dependence, stress and treatment resistance, the need for and the nature of a niche and ultimately the tumor-propagating capability could be added.

Leukemia-propagating cells have been identified when successfully engrafted in an immunodeficient mice 20 years ago, thus identifying the first functional subset in AML [5]**. In that case again, the field was led by hematological cancer and then supported by discoveries of many cancer propagating cell among different tissues such as colon [6], breast [7], or brain [8]. The leukemic propagating cell theory states that from a long-term self-renewing cell, an entire hierarchically organized leukemia can be maintained.
and propagated [9]. This very cell is then the origin of the tumor, as a normal stem cell initiates novel tissue through progenitors. Its characteristics generally encompass low frequency in samples (0.01 to 1% for AML [10]) and a poor proliferation rate [11]. Specifically in AML, the LPC pool showed evidence of treatment resistance. For example, chemotherapies that are currently used (cytarabine, anthracyclines [3]) target dividing cells. Therefore, it is likely that these drugs have little impact on slow-dividing cells such as LPC. Because of its very nature -stem cell-like- the LPC is also believed to have an intrinsic resistance through expression of drug-efflux proteins (members of ABC, ATP-Binding Cassette transporters family for example) [12]. Moreover, LPC probably depends on a niche since it only localizes in bone marrow [13]. The very LPC pool in AML also appears heterogenous, both phenotypically [10] (see 'Phenotypical Heterogeneities') and functionally, as many studies exhibited differences of tumor-initiation [14]. This last point specifically needs comparisons between the different immuno-deficient mouse models (NSG, NOD-SCID, etc.) investigating the differential engraftment they enable.

Considering this evidence, we cannot but imagine a more advanced functional AML structure that would include not only LPC, but also the bulk (currently defined by default of initiating capacity). If we consider an AML as a tissue that follows a hierarchical organization, it is very likely to comprise more functional subpopulations than the classical LPC-bulk segregation. Interestingly, Patel et al. [15] recently showed that some of relapse-AML patients displayed changes in their compositions regardless of stem-cell activities; they differed in chemotherapy-resistance gene expression and were consequently functionally heterogenous.

Phenotypical Heterogeneities

Defining a functional structure of leukemia relies on our ability to sort and collect subtypes of its living cells in a flow cytometry platform, using fluorescence-labelled antibodies to target cell-surface markers (mainly CD, or cluster of differentiation). Hence, patterns of phenotypes among blasts is of importance since it may help further diagnosis and primary tumour prognosis guidelines (including clonal specific treatment), as envisaged by the EuroFlow program [16].

If CD45 and SSC (sideward light scatter) are two criteria commonly used for isolation of blasts in any type of AML [17], many papers suggest that a more diversified immunostaining allows MFC (multi-parameter flow cytometry) to sort AML blasts in clusters based upon surface markers [18]. Indeed, numerous CD show differences in expression among a patient's cell population. CD34 and CD38 are heterogeneously expressed within an AML [5] and so are CD11b and CD45RA [10]. Hoffmann et al. [18] further underline five CD featuring major disparities: CD11b, CD14, CD58, CD90 and CD117. Based on these markers, these studies highlight the phenotypic heterogeneity that a patient's AML can display. It also provides a tool that questions the current cytogenetic classification, since the intermediate cytogenetic risk patients (Table 1) presenting heterogeneity in the five markers mentioned in Hoffmann's study actually showed poorer prognosis than adverse cytogenetic risk patients.

These markers are originally inspired from normal hematopoietic hierarchy in human bone marrow. CD34 is a classical immature HSC or progenitor marker and has long been known for being recurrently positive in cells first called SL-IC (SCID mice leukemia-initiating cells) then LSC (leukemic stem cells). The latter were first isolated for their low expression of CD38 [5] but when engrafted in more immunodeficient mice, CD34-CD38- cellular fraction may also be able to initiate AML. In that case for example, the anti-CD38 antibodies used could have misled researchers in properly identifying this fraction, as shown by Taussig et al. [19]. Then, it seems sensible that a CD34- LPC not only can derive from a HSC but also from a GMP (granulocyte-macrophage progenitor)-like or LMP (lymphoid-primed multipotent progenitor)-like progenitor, as demonstrated by Goardon et al. [10]. AML appears here as a progenitor disease, where LMP- like can give rise to GMP but not the opposite. Finally, Taussig et al. [20] even showed LPC can reside in a CD34- compartment when isolated from CD34 low-expressing AML.

Bonardi et al. [21] further analyzed transcriptome-proteome correlations that revealed CD34- LPC-enriched fractions displayed high level of CD135, CD47, and many more. Interestingly, CD135 (or FLT3, fms-like tyrosine kinase 3) was the most discriminant marker between CD34+ leukemic and CD34- normal cells. Commonly known for undergoing fusion or over-expression, both leading to over-activation, FLT3 anomalies like FLT3-ITD (internal tandem duplication) are associated with an unfavorable prognosis (especially reduced OS, overall survival [22,23]). This study also reveals CD47 expression (associated with decreased OS too [24]) is heterogenous even among CD34- cells. Together, these two markers give us an insight into immunophenotypic heterogeneity both within an AML and a so-called compartment (LPC fraction).

Such links between phenotype and functional heterogeneities find further applications on treatment assays, as shown by Jin et al. [25] that targeted CD123 (ILR3a, interleukin 3 receptor γ chain) or Majeti et al. [24] with CD47. Indeed, such surface markers are targets for monoclonal antibodies, that impair LPC homing (CD123) or help for their clearance (CD123 and 47, see 'Functional Implications').

On the other hand, as stressed by Majeti & Weissman [26] in response to Goardon's study, the immunophenotype is currently not sufficient to perfectly characterize a patient's AML since we hardly grasp all the fractions of which it is composed and the markers they express. Moreover, AML blasts are cancerous cells and thus may display aberrant phenotype markers.
because of dysregulated expression and variations through time and treatment. Zeijlmaker et al. [27] specifically reviewed loss and gain of immuno-phenotypic markers in cases of relapse AML compared to the primary tumor. In that case, surface markers would not be sufficient to isolate its compartments; this perspective requires further investigations.

Cytogenetic Heterogeneities

Well-known and used in both diagnosis and prognosis (Table 1), the cytogenetic characteristics of leukemic and more specifically AML cells may constitute the first step among molecular heterogeneities. Indeed, Bochtler et al. [28]*• showed very recently in prospective randomized multi-center trials involving more than 2,600 non-M3 AML (FAB classification) patients that up to 16% of the population (and 32% of aberrant cytogenetic cases) displayed different karyotypes in their AML. Then, it implies that heterogeneities are quite frequent at a cytogenetic level. Noteworthy, it also constitutes an additional adverse prognosis marker since it reduces overall survival. The different possible profiles are either composite karyotypes when clones are too different to be clonally related, or subclones obviously derived from the same founder in a mother-daughter process (when two clones) or branched evolution (when ≥ 3). Strikingly, very few other papers [29–31] have shown similar results in adult AML since 1979 [32], and none in equivalent population size.

Though Paulsson refutes cytogenetic heterogeneities in AML [33], she underlines 17.6% of all AML patients referenced with chromosome abnormalities in the Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer [34] display two or more cytogenetically-characterized subclones. Besides, cytogenetics is still largely used in diagnosis and prognosis, especially in 2008 WHO classification. And more advanced cytogenetic technologies such as FISH could help completing karyotype-based studies like Bochtler’s one in heterogeneity characterization. Nevertheless, these studies are often limited to proliferating cells (mostly composing the bulk, see above ‘Functional Heterogeneity in AML’).

Table 1. Cytogenetic-based risk classification of AML cases.

<table>
<thead>
<tr>
<th>Risk Category</th>
<th>5-year survival</th>
<th>5-year relapse rate</th>
</tr>
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<tbody>
<tr>
<td>Good</td>
<td>65%</td>
<td>35%</td>
</tr>
<tr>
<td>Intermediate</td>
<td>41%</td>
<td>51%</td>
</tr>
<tr>
<td>Adverse</td>
<td>14%</td>
<td>76%</td>
</tr>
</tbody>
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Based on Grimwade et al. [54], these statistics are still current. Categories are based upon correlations between cytogenetic anomalies and 5-years survival and relapse rates.

Genomic and Epigenomic Heterogeneities

As primarily an adult cancerous disease, AML is classically considered to be caused by somatic mutations. The genomic and epigenomic studies are undoubtedly the most recent and flourishing ones in terms of heterogeneities. These studies generally analyze Copy Number Variations (CNV), Single Nucleotide Variations (SNV), insertions and deletions (indels) and gene fusions. Interestingly, it seems that de novo AML displays fewer gene mutations (13 per AML on average) than other adult cancers [35]*•. Still, the authors develop interesting concepts and converge on many lines about molecular heterogeneities.

First, they all conclude to a clonal structure of AML, whichever type it is; clusters of mutations from genomics reveal that different subclones (up to 5) co-exist in the same AML sample and derive (directly or not) from a common ancestor (a founder clone, thus confirming clonal theory) when there are ≥ 2 clones (Figure 1).

Second, this structure results from a branching, Darwinian-type evolution (Figure 1) as demonstrated by ‘primary tumor vs relapse’ comparisons [36]**. Indeed, a treatment constitutes a selective pressure on a population of subclones developed from a founder through genetic events, thus differing in fitness in such an environment. Noteworthy, the authors discovered many more mutations in relapse than in primary tumor, partly attributed to toxicity of the treatment on DNA.

Figure 1. Clonal evolution in Acute Myeloid Leukemia. AML imitates normal tissue born from a common normal ancestor (HS(PC), dark grey cell), through Darwinian evolution. Schematically, the ancestor (that has already undergone several mutations) produces a genetic favorable event and transforms into a leukemia initiating cell (red fraction) giving birth to different subclones that have different destinies. As they undergo a selective pressure in the organism (e.g. a treatment), they can either survive without change in fitness (LiC); survive with a higher fitness (orange subclone cells that proliferate), or lower one and not survive (purple subclone, disappearing). In the end, an AML sample only provides evidence of subclones currently existing at a given time (blue frame), and needs further explorations to retrace the whole evolution. HS(PC)=Hematopoietic Stem (Progenitor) Cell.
Similarly, Walter et al. [37] focused on the transition from MDS (myelodysplastic syndrome, clonal hematological disorder without blockade of differentiation) to its frequent evolution in secondary AML. In that case, the sampled cellular population in secondary AML had at least one more clone than MDS, as well as more mutations (SNV).

Interestingly, Jan et al. [38] showed that residual HSCs in FLT3-ITD (see Phenotypic Heterogeneities) AML constitute a reservoir of preleukemic HSCs that harbor founder mutations but still lack in abnormalities required to generate AML. The authors thus consider the clonal development of AML even before the effective transformation. This suggests that there may be more diverse founding clones than currently showed, and that normal cellular state is to be refined.

Which genes were mutated? The genes referenced by the 2008 WHO classification are mostly signaling genes and/or oncogenes such as NPM1 (coding Nucleophosmin 1) and FLT3, less commonly CEBPA (coding CCAAT enhancer-binding protein alpha, a transcription factor), KIT (codes for stem cell growth factor receptor) and CD117), MLL (mixed-lineage leukemia), WT1 (codes Wilms tumor protein 1), NRAS and KRAS (neuroblastoma and Kirsten rat sarcoma). In addition to these, more recent papers show many more genes mutated. Some of them (about 5 per patient) are also recurrently mutated. Interestingly, intermediate cytogenetic risk samples did not display less recurrent mutations than adverse ones. Then, AML might be characterized by mutations clusters exhibited by a patient, beyond the WHO classification. To reach such a goal, the Cancer Genome Atlas Research Network (CGARN) sorted mutations discovered in their study in protein classes. They encompass spliceosome proteins, cohesin, chromatin modifiers, myeloid transcription factors, activated signaling pathways, DNA methylation-linked proteins, tumor suppressors, NPM1 mutations and transcription factor-fusions, signaling mutations being the most represented. We notice here that SNV are not the only type of mutation included, what is a big step toward integrative analysis of molecular heterogeneities of an AML.

Further analyses also comprise mutual exclusivity and co-occurrence of mutations and reveal for example that FLT3, NPM1 and DNMT3 are often mutated together and that this trio appears in intermediate cytogenetic-risk. Thus, it may define a new genuine type of AML that needs to be more functionally and clinically studied, so as to be systematically investigated at diagnosis.

Many recent studies reveal inter-individual epigenetic heterogeneities in AML, notably DNA methylation profiles (for review, see Schoofs et al. [39]) and miRNA and mRNA expression. The CGARN study thus found interesting correlations between clusters of samples (defined on CpG-sparse DNA regions), mutations and miRNA profiles and mRNA profiles, especially in samples displaying well-known mutations including IDH1/2, PML-RARA (promyelocytic leukemia; retinoic acid receptor alpha) fusion, and co-occurrence of FLT3, NPM1 and DNMT3 discussed earlier. However, little is shown there about intra-tumoral epigenetic heterogeneities.

Functional Implications of Molecular Heterogeneities

When we consider the range of mutations related to AML (Figure 2), we understand that better analyses of

![Figure 2. Overview of mutations' targets and their functional role in AML blasts.](https://example.com/aml-functional-implications)

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gene functions and dysregulations need to follow mutations discoveries. A given mutation is not a proof of an impact on cellular functions before further investigations (is the mutation exonic? If yes, does it really impair the protein's function or localization? If it is not, does it have a quantitative and/or significant impact on gene expression regulation?). Furthermore, most mutations found in AML blasts seem to be events that occur even before the transformation of the HS(P)C (hematopoietic stem (progenitor) cell) in leukemia-initiating cell [40] (Figure 1). How can we determine their real impact? Studying their recurrence over blasts of an AML and comparing them with HS(P)C's is a solution. The CGARN study thus concludes that most of their cases include an average of 5 mutations, but it does not point out which ones. Nevertheless, PML-RARA, IDH1, TET2 (Seven translocation), DNMT3A and NPM1 are extensively mutated in the population studied (up to 27% of cases), and in other studies [36][40]. Together with comparisons with HS(P)C, these mutations are thought to be initiating events.

Mutations in genes coding for chromatin modifiers such as H3 methyl-transferases EZH2 (enhancer of zest homolog 2) or MLL (Mixed Lineage Leukemia) are proved to impact on leukemogenesis. MLL translocations responsible for loss of the methyl-transferase activity are well-known since most of them are sufficient to transform hematopoietic cells into LPCs [40]. Moreover, patients with MLL fusions-AML may significantly display high mutation rate of genes involved in RAS signaling pathway [41]. Heterogeneity in AML epigenetic landscapes is then likely to define heterogeneity in functional subsets of AML cells.

Similarly, mutations in genes coding DNA methyl-transferases such as DNMT3 and methylcytosine dioxygenase TET were proven to significantly impact on both epigenetic profile and prognosis [39]. Besides, mutations of IDH1 are already known to disrupt normal methylation pathways through metabolic availability of methyl donors [42]. Since these mutations are diverse, transformed cells are very likely to display heterogeneities in their epigenetic landscapes. Moreover, comparisons between gene mutations in epigenetic pathways and actual epigenetic modifications start being explored. For example, methylation of DNMT3A intern promotor biologically mimics mutation of the gene itself [43]. As DNMT3A mutations could be included in AML classifications, epigenetic studies are proved to be of relevant interest. Though demonstrated at the inter-individual scale by Akalin et al. [44], epigenetic heterogeneities may need better intra-tumoral characterization as support.

Another well-known translocation linked with pathogenesis is PML-RARA; this fusion leads to HDAC (histone deacetylase) recruitment [46] and defines a subtype of AML -acute promyelocytic leukemia.

Mutations in nucleophosmin 1 (NPM1) are also very common and proved to be associated with better prognosis in non-FLT3-ITD AML patients, possibly because of increased T-cell-mediated immuno-susceptibility [47] (Figure 3).

Regarding signal transduction, the most striking example may be FLT3 (or CD135, tyrosine-kinase membrane receptor activating MAPK and Ras pathways), whose gene mutations are associated with reduced OS as mentioned above.

Together, these data provide another insight of how functional heterogeneities can be displayed by an AML case. Again, if a sample shows heterogeneity in its genomic landscapes, i.e. in its mutation patterns, it is likely to exhibit variations in its mRNA splicing activity, its chromatin state at specific loci (then its chance of mutations), its immuno-susceptibility, or its signaling activation.

![Heterogeneities in AML, K. Sermet](https://example.com)

**Discussion**

We showed that increasing progress is being made in phenotypic, molecular and functional dissection of AML. Massive parallel sequencing and cytogenetics revealed molecular heterogeneities that constitute the basis for clonal model of tumor (Figure 1) [28,35]. This model was first envisaged by Nowell in 1976 [48][49] through the case of hematological malignancies. AML is defined as a set of subclones deriving from a founder by a branching clonal evolution. The relapse is due to the expansion of a treatment-resistant clone (founder or not) that gains mutations and is responsible for poor outcome [36][40]; Understanding the sequence of molecular events will help to identify and target the founder anomaly.

Through improvements of xenograft techniques and functional assays, a more accurate perception of the LPC roles in the development of AML has permitted a better understanding of this disease kinetics. LPCs display self-renewal and treatment-resistance and thus are good candidates in causing relapse.

Moreover, the identification of the functional properties of subclones depends on our ability to sort and isolate them. Then, finding new surface markers and regrouping them into proper patterns is of outstanding interest; it can also provide physicians with new therapeutic targets. New mass cytometry tools such as viSNE (visualizing stochastic neighborhood embedding) could then use these markers to help practitioners picture phenotypic distances between subclones in a two-dimension scatter plot at diagnosis or relapse [49].

Subclones displaying low frequency in a sample such as LPCs need a single-cell analysis to be identified and truly characterized. Flow cytometry solves the problem at the phenotypic scale but may be not sufficient. Integrated microfluidic circuit and library preparation, (RT)PCR and sequencer at single-cell level will permit co-analysis of genomics, epigenomics, and soon proteomics. Guo et al. [50][51] thus demonstrate the clonal development at this high resolution that informs directly on processes of normal and diseased hematopoiesis. This possibility to study at once clonal development state and molecular and functional data in each cell of a tumor brings new perspectives. It will help us tackle the issues of intra-tumoral heterogeneity,
better detect subclone(s) responsible for relapse, and ultimately offer new treatments.

Major efforts are being made to develop targeted therapies, not only considering inter-individual heterogeneities but also intra-individual ones (see Guzman et al. for review [51]). Therapeutic targets such as CD123 and DNMT3 are currently in clinical trials, which would not exist without the recent studies on LPC. We can additionally suspect other types of heterogeneity, for example spatial and temporal. Even if AML is not a solid tumor, this does not obviate the risk of misinterpretation on bone marrow samples if they appear to be spatially heterogeneous. Moreover, as shown in Merlo et al. [52], a sample only reflects the state of an AML at a given time and the disease may evolve spontaneously, even without considering treatments.

This review gives an insight in the current understanding of AML heterogeneities (Figure 3). It also underscores the potential that needs to be developed by studying correlations between molecular and functional heterogeneities. Beyond hematological malignancies, further investigations in other cancers -including solid ones- can also be expected.

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References and recommended reading
Papers of particular interest have been highlighted as:
● of special interest
●● of outstanding interest


This paper describes an excellent example of therapeutic
targeting in LPC.


Heterogeneities in AML, K. Sermet

   - This report is now considered as a founder of clonal evolution theory in cancer.


   - By applying single-cell genomics to an AML mouse model, the authors mapped the HSC differentiation and the development of a hierarchy with two different self-renewing lineages in a hematological malignancy.


