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REVIEW ARTICLE

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Clonal evolution of acute myeloid leukemia from diagnosis to relapse

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Abstract

Based on the individual genetic profile, acute myeloid leukemia (AML) patients are classified into clinically meaningful molecular subtypes. However, the mutational profile within these groups is highly heterogeneous and multiple AML subclones may exist in a single patient in parallel. Distinct alterations of single cells may be key factors in providing the fitness to survive in this highly competitive environment. Although the majority of AML patients initially respond to induction chemotherapy and achieve a complete remission, most patients will eventually relapse. These points toward an evolutionary process transforming treatment-sensitive cells into treatment-resistant cells. As described by Charles Darwin, evolution by natural selection is the selection of individuals that are optimally adapted to their environment, based on the random acquisition of heritable changes. By changing their mutational profile, AML cell populations are able to adapt to the new environment defined by chemotherapy treatment, ultimately leading to cell survival and regrowth. In this review, we will summarize the current knowledge about clonal evolution in AML, describe different models of clonal evolution, and provide the methodological background that allows the detection of clonal evolution in individual AML patients. During the last years, numerous studies have focused on delineating the molecular patterns that are associated with AML relapse, each focusing on a particular genetic subgroup of AML. Finally, we will review the results of these studies in the light of Darwinian evolution and discuss open questions regarding the molecular background of relapse development.

KEYWORDS

acute myeloid leukemia, clonal evolution, next generation sequencing, relapse, therapy resistance

1 | INTRODUCTION

"According to Darwin's Origin of Species, it is not the most intellectual of the species that survives; it is not the strongest that survives; but the species that survives is the one that is able best to adapt and adjust to the changing environment in which it finds itself." A 100 years after Charles Darwin's *"On the Origin of Species"*, Leon C. Megginson, business professor at Louisiana State University, shared this famous interpretation of Darwin's theory about the evolution by natural selection.[1,2] Today, this theory is not only applicable to the evolution of organisms, but also to the evolution of tumor diseases and the origin of distinct tumor populations in a human individual.

Darwinian evolution by natural selection is described as the result of a two-step process: (a) the acquisition of heritable changes affecting an organism's physical or behavioral traits, that (b) allow the organism to better adapt to its environment, help it to survive and thereby increase the number of offspring. Evolution by natural selection is often referred to as "survival of the fittest," whereas the effect on the fitness by a particular change always depends on the underlying selective pressure, which is given by the surroundings. Thereby, organisms as well as single cells might be perfectly adapted to a particular niche, whereas in a different environment, they would not be able to survive.

In the past, rules of Darwinian evolution have been applied to individual cells and cell populations during their progression to cancer,[3-5] including the malignant transformation of hematopoietic progenitor cells to acute myeloid leukemia (AML).[6-9] The advent of next generation sequencing (NGS) techniques has greatly increased the detection of genetic events that are associated with tumor development and progression. In this review, we will summarize and discuss the results from recent NGS-based studies of AML evolution from diagnosis to relapse and the development of therapy resistance. We will focus on different theoretical models of evolution, the dynamics of evolution, and the methodology to uncover evolutionary events including its strengths, drawbacks, and limitations.

2 | MODELS OF CLONAL EVOLUTION OF AML

AML is the most common acute leukemia in adults and a highly aggressive disease with poor outcome. Although the prognosis of AML patients has improved over the last decades, only one of four patients survives 5 years or longer.[10] In AML, immature blast cells reside in the bone marrow, peripheral blood, and extramedullary tissues, unable to differentiate. These cells are rapidly growing and continuously dividing, lacking maturation and function, thereby suppressing normal hematopoiesis. Even though two-thirds of AML patients respond to induction chemotherapy and achieve complete remission, the majority of these patients will eventually relapse.[11] AML induction chemotherapy has not changed profoundly over the last decades, and the molecular mechanisms mediating the transition of AML cells from therapy sensitive toward resistant are still not fully understood.[12,13] Although several studies have shown relapse-specific gene mutations in individual cohorts of AML patients, no gene mutation was reported to be recurrently gained, while rarely or never lost at relapse across multiple studies. Mutations in *DNMT3A*, *ASXL1*, and *RUNX1* as well as internal tandem duplications in the *FLT3* gene (*FLT3*-ITD) were frequently gained at relapse and associated with poor outcome if present at diagnosis, however, patients with these mutations may still respond to chemotherapy and achieve a complete remission. AML is a genetically heterogeneous disorder, which is characterized by mutations in a variety of genes, encoding for myeloid transcription factors, tumor suppressor genes, epigenetic modifiers, and splicing factors, altering normal hematopoietic function of myeloid progenitor cells. In total, more than 60 genes have been described to be recurrently mutated in AML and we are far from knowing AML genetics in detail as leukemias are highly heterogeneous not just between patients but also within patients.[14-16] Moreover, the molecular profile of AML is changing during the disease, as multiple studies have described genetic and epigenetic evolution of AML from diagnosis to relapse. It is believed that AML originates from a single hematopoietic stem or progenitor cell (HSPC) acquiring somatic mutations over time that lead to a block of differentiation but also provide stem-cell like properties of unrestricted self-renewal, thus enabling mutated HSPCs to grow clonally.[17,18] Prior to leukemia-initiating events, regulators of epigenetic marks (eg, *DNMT3A*, *TET2*, and *ASXL1*) commonly acquire mutations, which may provide a growth advantage, yet they are not sufficient to induce leukemia, and thus commonly described as preleukemic.[19-21] Initiating mutations that provide leukemic potential are often found in the gene nucleophosmin (*NPM1*) and genes associated with signaling activation, for example, *FLT3*.[14,22] Each mutation adds to the genetic complexity and finally, increased clonal heterogeneity is associated with inferior outcome of AML.[23,24]

During the disease, individual AML populations may follow distinct models of clonal evolution and the presence and the abundance of mutations at multiple time points paints a picture of dynamic changes. Linear evolution describes the stepwise acquisition of single mutations, whereas the eradication of the dominant clone, followed by outgrowth of a subclone is termed branching evolution (Figure 1). Without any change in the evolutionary pressure, cell populations are likely to follow a linear evolution, steadily increasing their fitness. Still, branching evolution may follow linear evolution, or vice versa, especially when the evolutionary pressure has changed profoundly (eg, at treatment start or change of therapy). During therapy, the AML cell population may evolve by either acquiring additional mutations mediating therapy resistance (linear evolution, Figure 1A), or by losing mutations that are for example, associated with sensitivity to the treatment (branching evolution, Figure 1B). In summary, AML cell populations at relapse may have evolved from either clonal or subclonal cell populations present at diagnosis, accompanied by potential acquisition of additional mutations. Following either linear or branching evolution, cell populations are steadily undergoing clonal evolution in order to the best adapt to their environment. As the majority of AML patients relapse after initial response to chemotherapy, in most patients a few AML cells find a niche to escape from therapy and eventually grow out again.

In an individual patient, the underlying model of clonal evolution can be assessed by comparing the abundance of each single mutation at multiple time points. In linear evolution, mutations of the major clone present at diagnosis are also present at relapse, accompanied by additional mutations (Figure 2A). Cells of the major clone were sensitive to the treatment, but residual cells have acquired new mutations that provide resistance resulting in therapy escape and development of relapse. New mutations can be both, driver mutations actively providing a growth advantage, or passenger mutations that were already present in a cell prior to the acquisition of a driver mutation. As during each step of linear evolution, a new clone evolves, both the ancestor and the descendent are sharing their particular mutational background

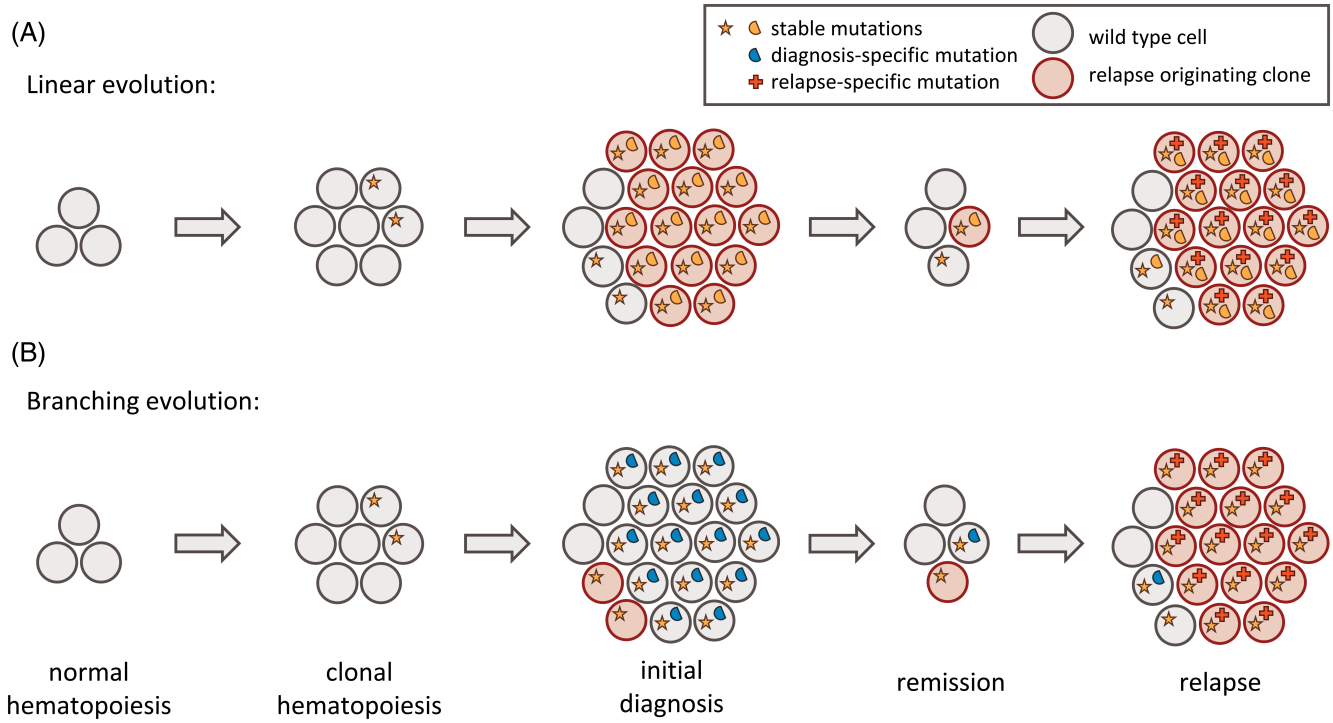

FIGURE 1 Models of clonal evolution of AML over time. Each circle corresponds to an individual cell, multiple identical cells correspond to cell clones, each defined by harboring the identical set of mutations. Cells without symbols refer to wild type cells without somatic mutations. Somatic mutations are represented by different symbols, colored based on their stability: orange (stable mutation), blue (diagnosis-specific mutation), and red (relapse-specific mutation). Cells of the relapse originating clone are highlighted in red. (A) Linear evolution describes the sequential acquisition of mutations. The relapse originating cell is part of the major clone at diagnosis. (B) Branching evolution describes the eradication of the major clone and subsequent outgrowth of a secondary clone. The relapse originating cell is part of a subclone at diagnosis. AML, acute myeloid leukemia

except for the new mutations, including drivers and passengers. During linear evolution, mutations of the major clone are unlikely to get lost, as mutations reverting a mutated allele back to its wild type configuration are very rare events. In contrast, the loss of a mutation is a hallmark of branching evolution. The main clone at diagnosis disappears after treatment and a new clone that is resistant to the therapy is found at relapse. If no additional mutations occur at relapse, this clone likely was present at diagnosis at very low levels (Figure 2B). If additional mutations are detected at relapse, the clone has evolved from a common ancestor that was present at diagnosis (Figure 2C). In neither of the two cases, the relapse has evolved from the dominant clone at diagnosis itself. While initial and relapsed disease may share mutations, branched evolution is characterized by a loss of mutations at relapse.

It is important to distinguish between clonal (present in the majority of cells, ie, the dominant clone) and subclonal mutations (present in a minority of cells). In contrast to the loss of a clonal mutation, the loss of a subclonal mutation does not necessarily imply branching evolution. In this case, a subclone, harboring a particular mutation, is eradicated at a later time point. On the other hand, the major clone is still present and evolves linearly. As linear and branching evolution are defined by the clonal origin of the relapse cell population (clonal or subclonal), it may not be possible to decipher the underlying model of evolution if multiple clones with similar size coexist at diagnosis. The classification of a mutation as clonal or subclonal is derived from the variant allele frequency (VAF), as it provides information about how many cells in a sample carry a particular variant. The VAF is defined as the ratio of sequence reads carrying the mutation to the total number of reads at a given nucleotide position.

As genetic evolution is defined by changes in the mutational profile between diagnosis and relapse, the model of evolution remains unclear in AML patients without detectable differences. If mutations are shared at both time points, relapsed disease is related to the initial disease and has evolved from it (Figure 2D). In contrast, if the mutational profiles at diagnosis and at relapse are completely different, it is unclear if relapse has evolved from the original disease, or if it is an independent, secondary AML (Figure 2E). However, a single shared mutation might not be sufficient to draw any conclusions about genetic evolution, as identical mutations can be found in different, independent malignancies (including solid tumors) such as mutations in *KRAS* (hotspot at residues G12 and G13), *NRAS* (hotspots G12, G13, and Q61), and *BRAF* (hotspot V600). In summary, simple rules can be applied in order to describe clonal evolution from diagnosis to relapse in a particular AML patient (Figure 2F). The presence of common mutations defines the relationship of initial and relapsed AML and the loss of a clonal mutation defines branching evolution, while

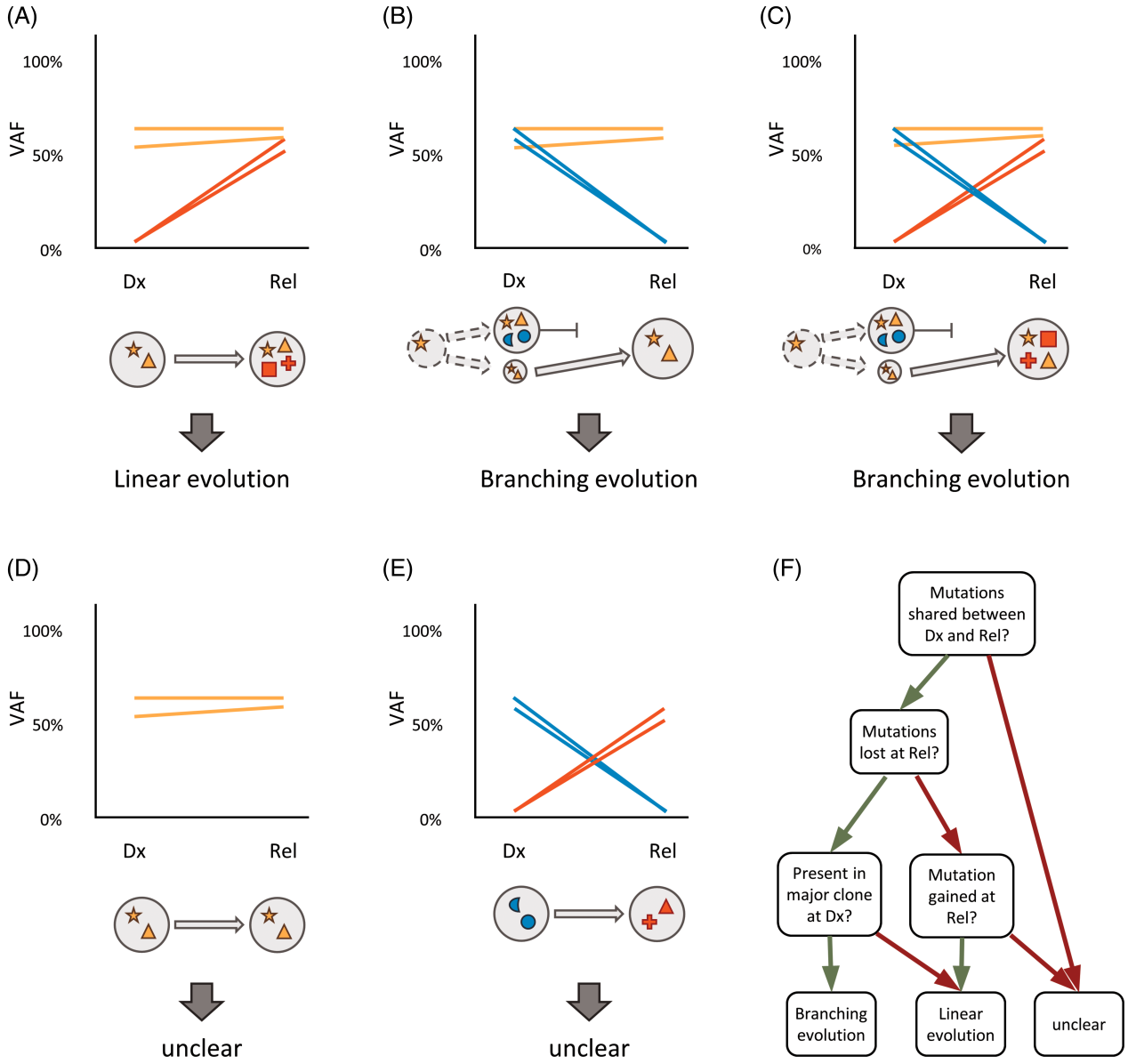

FIGURE 2 Examples of clonal evolution between diagnosis and relapse in a single patient. Each line corresponds to an individual mutation and illustrates the presence of the mutation at both time points, colored by its stability. Each circle corresponds to an individual cell clone, defined by harboring the identical set of mutations. Somatic mutations are represented by different symbols, colored based on their stability. Cells without symbol refer to wild type cells without somatic mutations. VAF, variant allele frequency; Dx, diagnosis; Rel, relapse; orange, stable at Dx and Rel; blue, lost at Rel; red, gained at Rel. (A) Genetic evolution with stable mutations and gained mutations. (B) Genetic evolution with stable mutations and lost mutations. (C) Genetic evolution with stable mutations, gained mutations, and lost mutations. (D) Genetic evolution with stable mutations only. (E) Genetic evolution with gained mutations and lost mutations only. (F) Decision tree to define the underlying model of genetic evolution

mutation gains are possible in both, linear and branching evolution. As the loss of a clonal mutation is the hallmark of branching evolution, it is necessary to include as many genomic target regions as possible in order to describe the evolutionary model appropriately.

Finally, clonal evolution occurs at multiple levels. Relapsed AML must not necessarily differ genetically from initial AML. Clonal evolution may also take place on the epigenetic level, when different epigenetic profiles confer therapy resistance. Changes in epigenetic profiles may be detected between diagnosis and relapse using appropriate methods. A recent study suggests that both genetic and epigenetic evolution may complement each other, and thus a lack of genetic evolution can be compensated by increased epigenetic evolution.[25]

3 | AML RELAPSE AFTER CHEMOTHERAPY

Since the first AML genomes were sequenced about 10 years ago,[26,27] numerous researchers have made efforts to catalogue the genetic

lesions in AML introducing a huge number of genetic subclasses of AML with distinct clinical characteristics such as response to therapy and risk of relapse.[15,16,22] By comparing diagnostic and relapse samples, differences in the mutational landscape can be detected and recurrent changes are very likely to contribute to the development of resistance. In about 30%-40% of AML cases, cytogenetic alterations at relapse not present at initial diagnosis were described as potential mechanism of AML relapse.[28-31] In the majority of these cases, a more complex karyotype was detected at relapse, which is generally associated with inferior survival. However, in <10% of AML cases, the karyotype found at relapse was described to be less complex. Trisomies 8 and 21, as well as deletions affecting the long arm of chromosome 9 are recurrently gained at relapse, still their association with therapy resistance remains unclear, as these alterations are not described as prognostically relevant if present at diagnosis.[32-34] In contrast, deletions of the long arm of chromosomes 5 or 7 are both reported to be recurrently gained at relapse and associated with poor outcome at diagnosis.[16,35] Moreover, NGS enabled the investigation of AML relapse at single nucleotide resolution. Based on whole genome sequencing (WGS) of paired diagnosis/relapse samples, AML relapse has been described to evolve either from the initial founding clone or from a subclone present at diagnosis.[36] Over the past years, numerous studies have delineated the molecular patterns associated with AML relapse (Table 1).

These studies have focused mostly on well-defined and clinically meaningful molecular subclasses of AML. The number of genes analyzed ranged from as little as five by targeted gene panel sequencing (GPS) up to >20 000 by whole exome sequencing (WES). The number of paired patient samples collected at time points of initial diagnosis and relapse after chemotherapy ranged from 11 to 129 (GPS) and 5 to 50 (WES), respectively (Table 1). WES is commonly applied to a limited number of matched diagnosis/relapse patient samples, while targeted GPS is usually performed in larger cohorts in order to estimate the frequency of gene mutations. To detect the frequency of individual mutations at diagnosis and/or relapse, it might be sufficient to collect any diagnosis and relapse samples, even if they do not stem from the same individuals. However, to draw meaningful conclusions about the clonal evolution from diagnosis to relapse, it is imperative to sequence longitudinal samples from the same patients. So far, most studies focused on the detection of mutation frequencies rather than the longitudinal analysis of individual mutations. Moreover, as GPS studies are focusing on

TABLE 1

Publication	AML subtype	N total patients	N matched Dx/Rel	Sequencing strategy	Models of evolution	Dx associated mutations	Rel associated mutations
Garg et al.,[37] Blood 2015	AML with FLT3-ITD	80	WES: 13 (+CR), GPS: 37 (+CR)	Initial WES, extended GPS	Linear (n = 2), branching (n = 5)	***NPM1***, *CEBPA*, *GATA2*, *SRCAP*	*SETD1A*, ***ASXL1***
Madan et al.,[38] Leukemia 2016	APL	212	WES: 8 (+CR), GPS:22 (-CR)	Initial WES, extended GPS	Linear (n = 5), branching (n = 1)	***FLT3*-SNV**, ***NRAS***, *KRAS*	*PML*, *RARA*, ***RUNX1***, *ARID1B*
Sood et al.,[39] Leukemia 2016	AML with inv(16) or t (8;21)	13	10 (+CR), 3 (-CR)	WES	Linear (n = 7), branching (n = 4)	***NRAS***	*GATA2* deletions-
Sun et al.,[40] Leukemia 2017	AML with MLL-PTD	85	WES: 5 (+CR), GPS: 8 (-CR)	Initial WES, extended GPS	NA	*STAG2*	*FAT1*
Greif et al.,[41] Clin Can Res 2018	CN-AML	50	50 (+CR)	WES, validated by GPS	Linear (n= 33), branching (n = 11)	***NPM1***, ***FLT3*-SNV**, ***PTPN11***, ***NRAS***	***FLT3*-ITD**, *IDH1*, *WT1*, *KDM6A*
Höllein et al.,[43] Blood Adv 2018	AML with NPM1 mut	104	11 (-CR)	GPS	NA	***NPM1***, ***PTPN11***	***RUNX1***, *TP53*
Christen et al.,[44] Blood 2019	AML with t(8;21)	331	19 (+CR)	GPS	NA	***ASXL2***	*G2E3*
Höllein et al.,[45] Hemasphere 2019	AML with RUNX1-RUNX1T1	94	17 (-CR)	GPS	NA	***ASXL1***, ***ASXL2***, ***NRAS***	*KIT*
Cocciardi et al.,[42] Nat Commun 2019	AML with NPM1 mut	129	WES: 20 (+CR), GPS: 109 (-CR)	Initial GPS, selected WES	Linear (n = 5), branching (n = 15)	***NPM1***, ***NRAS***, ***FLT3*-SNV**	***FLT3*-ITD**, *MLL*-PTD, ***RUNX1***

Note: Gene symbols in bold represent common events reported at least in two independent cohorts.
Abbreviations: Dx, diagnosis; Rel, relapse; WES, whole exome sequencing; GPS, gene panel sequencing; CR, complete remission.

mutations that are well-known to be acquired somatically in AML, germline control samples are usually not included. In WES studies, the larger number of detected mutations, including passenger mutations, helps to assess clonal evolution more precisely. Thus, in this review, the estimation of linear vs branching evolution in individual cohorts was limited to WES cases only. However, to assign the somatic status of mutations detected by WES, it is necessary to include a germline control sample, for example, a sample at complete remission. Due to limited sample availability and increased sequencing costs, WES studies commonly include a limited number of patients only.

Several studies have applied WES in order to genetically characterize AML at diagnosis and relapse (Table 1). In 2015, the genetic profiles of 13 matched diagnosis/remission/relapse triplets of AML patients carrying an *FLT3*-ITD were described based on WES analysis. Further, an additional 67 patients including 37 diagnosis/relapse pairs were analyzed using GPS, covering a total of 50 paired diagnosis/relapse sample pairs.[37] In another study from the same group, 12 relapsed acute promyelocytic leukemia (APL) patients were analyzed using WES (including eight diagnosis/remission/relapse triplets), followed by the comparison of 153 relapsed APL cases with 69 non-relapsed APL cases using GPS (including 30 diagnosis/relapse pairs).[38] Sood and colleagues described the mutational landscape of relapsed core binding factor (CBF) leukemia, defined by either inv(16) or t(8;21) and a total of 13 patients was characterized by WES, including 10 diagnosis/remission/relapse triplets and three diagnosis/relapse pairs.[39] AML relapse with partial tandem duplication of the *MLL* gene (*MLL*-PTD) was evaluated by sequencing samples from 85 patients. Five diagnosis/remission/relapse triplets were analyzed by WES and eight diagnosis/relapse pairs were analyzed by GPS.[40] Relapse of the largest cytogenetic subgroup of AML, patients with normal karyotype, was characterized by 50 matched diagnosis/remission/relapse triplets using WES, followed by GPS for variant validation.[41] Very recently, Cocciardi et al. described the mutational spectrum at diagnosis and relapse of *NPM1* mutated AML in 129 cases using GPS of five commonly mutated genes (*DNMT3A*, *FLT3*, *NRAS*, *IDH1*, and *IDH2*). In addition, they performed WES of 10 cases positive for *NPM1* mutation at diagnosis with loss of the *NPM1* mutation at relapse (here referred to as *NPM1*$^+$/*NPM1*$^-$ AML) and 10 cases with stable *NPM1* mutation.[42]

In general, larger cohorts of relapsed AML patients have been characterized using GPS only. Another cohort of 104 *NPM1* mutated AML patients at diagnosis was described, however, at relapse only 11 matched samples were included, all with loss of *NPM1* mutation (*NPM1*$^+$/*NPM1*$^-$ AML).[43] A study on CBF leukemia, focusing on t(8;21) positive AML only, covered a total of 331 patients, including 19 diagnosis/remission/relapse triplets.[44] CBF leukemia defined by *RUNX1-RUNX1T1* fusion was characterized in 94 patients, including 17 diagnosis/relapse pairs.[45]

In summary, the major limitation of most studies describing the genetic evolution from AML diagnosis to relapse is either the focus on a small set of genes only, or the limited number of paired samples. Only few studies included a reasonable number of patients and comprehensive genetic profiling by WES.

In all of these studies, the vast majority of patients share at least one mutation between diagnosis and relapse. This suggests that, in general, initial and relapsed AML have a common origin, defined by these founding mutations, and relapse has not developed as an independent leukemia. Both evolutionary models, linear and branching evolution, were found across all cytogenetic subgroups (Table 1). On average, the number of mutations was similar at diagnosis and relapse in all cohorts, although in *FLT3*-ITD positive AML, a lower mutational load at relapse was reported,[37] while in the CN-AML cohort, the mutation load increased.[41] The frequency of transversions (C > A and C > G changes) was commonly higher among relapse-specific mutations,[37,41,42,44] which is in line with previous reports and may be due to the mutagenic effect of cytarabine treatment.[36,46] Of note, this was not observed in APL, however, APL treatment does not include cytarabine.[38] The most frequently mutated genes, *NPM1*, *FLT3*, and *DNMT3A*, show variable evolutionary patterns in different AML cohorts. Mutations in *NPM1* are rather stable in all cohorts, lost only in 2/36 CN-AML patients[41] and 4/21 *FLT3*-ITD positive patients,[37] which is in line with the reported frequency of *NPM1* mutation loss at relapse in general.[42,43,47] Similarly, *FLT3*-ITDs have a rather stable mutation pattern in the CN-AML cohort, while they are gained at relapse in 6/25 patients,[41] which is in line with the ELN classification that associates *FLT3*-ITD with poor prognosis.[12] Interestingly in *NPM1*$^+$/*NPM1*$^-$ AML patients, *FLT3*-ITDs were not detected at relapse although present in 18/61 *NPM1*$^+$ diagnostic samples, pointing towards the development of an independent AML at relapse.[42,43] *FLT3* point mutations are often lost in the CN-AML cohort[41] and *NPM1*$^+$ patients,[42,43] while gained in patients of the *FLT3*-ITD AML cohort.[37] *DNMT3A* is stably mutated in most of the patients without any loss in the CN-AML cohort,[41] still recurrently lost in 2/18 *FLT3*-ITD patients[37] and 6/91 *NPM1*$^+$ patients.[42,43] Additional examples for stable mutations detected in multiple cohorts include *IDH1* and *IDH2*, although these mutations are also gained in several patients.[37,40,41,43] Genes specifically mutated at diagnosis include *NRAS*, *KIT*, and *PTPN11*.[38,39,41,43] In contrast, *FLT3*-ITDs and mutations in *WT1*, *KDM6A*, and *RUNX1* are often gained at relapse.[38,41-43] Interestingly, mutations in *ASXL1* were found to be relapse-specific in *FLT3*-ITD AML,[37] but stable in CN-AML[41] and diagnosis-specific in *NPM1*$^+$/*NPM1*$^-$ AML.[43] Similarly, *TET2* mutations were stable in the CN-AML cohort[41] but frequently gained and lost in APL.[38]

In summary, mutations in *NPM1* and in signaling genes (eg, *FLT3*, *NRAS*, *KIT*, and *PTPN11*) are often found in AML at diagnosis but are frequently lost at relapse. This is true for CBF leukemia as well as for CN-AML, although these two subtypes of AML generally have distinct mutational profiles. Interestingly, *FLT3* point mutations are recurrently lost at relapse, while *FLT3*-ITDs are recurrently gained. In contrast, epigenetic regulators show a variety of evolutionary patterns. While regulators of DNA methylation (eg, *DNMT3A*, *TET1/2*, and *IDH1/2*) are rather stable but also recurrently gained at relapse, regulators of chromatin remodeling and histone modifiers (eg, *ASXL1/2*, *ARID1A/B*, *KDM6A*, and *MLL2/3*) may be recurrently gained but also lost at relapse. Taken together, while deregulation of signaling pathways is critical for AML initiation, epigenetic regulation might play an essential role in the evolution towards relapse. In particular, reprogramming the DNA

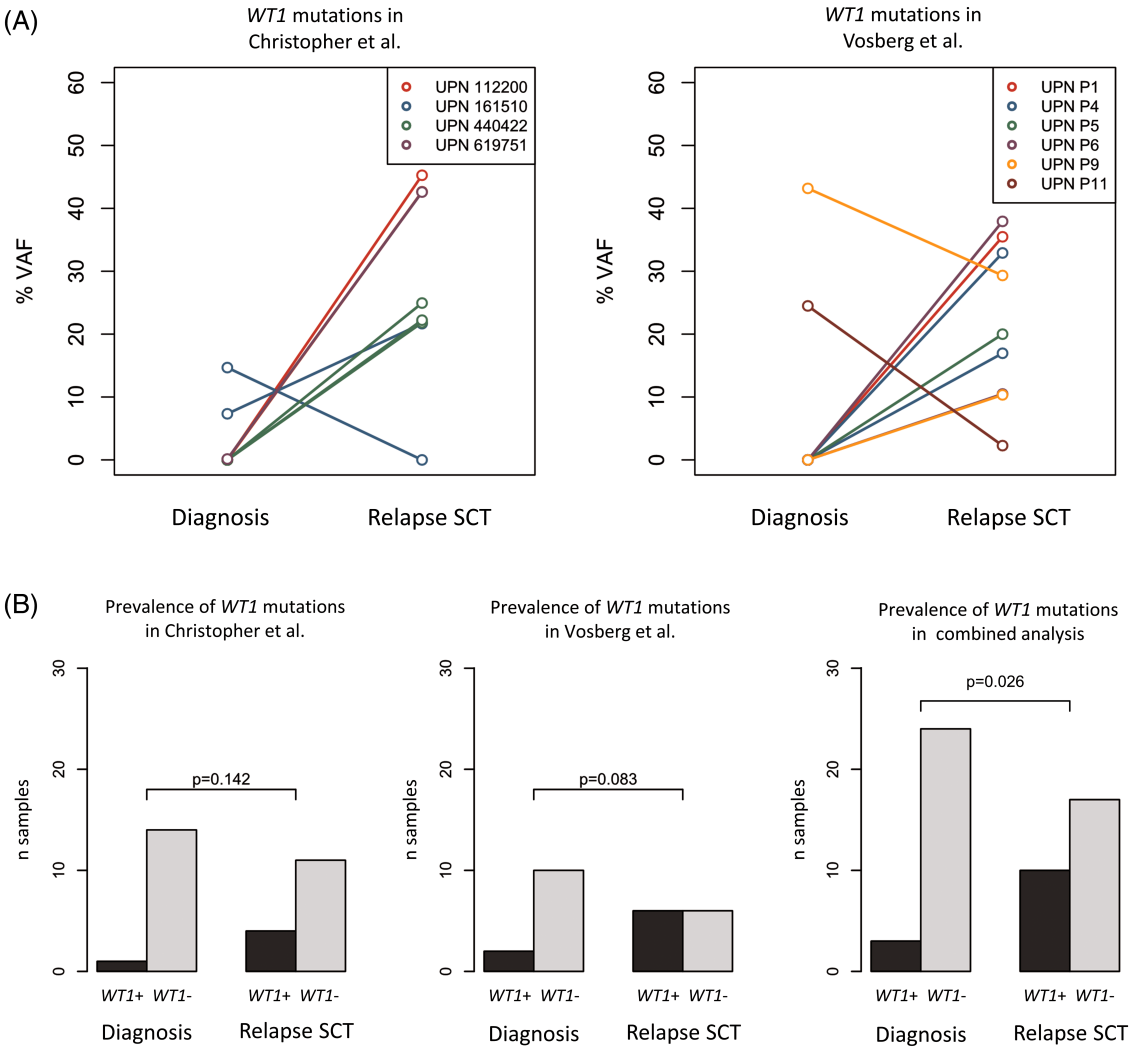

FIGURE 3 The frequency of *WT1* mutations increase upon relapse after allogeneic stem cell transplantation (relapse SCT) in studies of Christopher et al. and Vosberg et al. (A) Variant allele frequencies (VAF) of *WT1* mutations before and after relapse SCT in single data sets. (B) Prevalence of *WT1* mutations in the single data sets and in the combined analysis

methylation landscape during chemotherapy treatment might be the key for AML cells to find their niche, develop resistance, and escape therapy.

4 | MOLECULAR MECHANISMS OF CHEMOTHERAPY RESISTANCE

Several mechanisms of resistance development have been proposed, based on the deregulation of individual genes in relapsed AML, including *SAMHD1*, *EZH2*, and *KDM6A*. *SAMHD1* encodes for a deoxynucleoside triphosphate triphosphohydrolase and is commonly mutated in T cell lymphomas, chronic lymphocytic leukemia, and colon cancer.[48-50] Although *SAMHD1* has been shown to sensitize cancer cells towards chemotherapy,[51] it was reported that in AML, expression of *SAMHD1* is inversely correlated to cytarabine response in vitro and in vivo.[52] *EZH2* encodes a histone methyltransferase and is commonly mutated at AML diagnosis and at relapse. Inactivation of EZH2 has been associated with poor prognosis in AML as well as in other hematologic malignancies.[53-56] Loss of EZH2 is commonly found in AML relapse and induces resistance towards multiple drugs, including tyrosine kinase inhibitors. Different from its role in AML, *EZH2* mutations are associated with better outcome of patients with follicular lymphomas.[57] The histone H3 lysine 27 demethylase *KDM6A* is commonly inactivated in several types of cancer, including leukemia.[58-60] In AML, mutations in *KDM6A* are recurrently gained at relapse. In vitro knock out of KDM6A results in increased resistance to cytarabine and daunorubicin, while re-expression of KDM6A sensitizes cells to treatment.[61]

5 | AML RELAPSE AFTER ALLOGENEIC STEM CELL TRANSPLANTATION

Recently, it was reported that in AML cells after allogeneic stem cell transplantation (SCT), no relapse-specific mutational patterns were

observed.[62] In contrast, other studies found an enrichment of *WT1* mutations in AML relapse after chemotherapy as well as after SCT.[41,63] Although the gain of *WT1* mutations were not significant in any of the studies, combined analysis of two independent cohorts showed a significant association of *WT1* mutations with relapse after SCT suggesting a common evolutionary pattern (Figure 3). While *WT1* mutations are not relapse-specific, they are recurrently gained at relapse pointing towards a role in disease progression. WT1 constitutes an important epitope for immune response to leukemia, mediating the graft-vs-leukemia effect and providing the rationale for vaccination strategies.[64,65] However, somatic mutations may disrupt the immunogenic potential of WT1, thereby contributing to immune escape after allogeneic transplantation.

This exemplary meta-analysis demonstrates the potential to obtain more conclusive results upon integrative analysis of independent studies. So far, individual studies of AML evolution have not been very conclusive, especially if they are limited to few patients only.

6 | SUMMARY, DISCUSSION, OUTLOOK

Over the last years, several studies have focused on deciphering the genetic differences of AML at diagnosis and at relapse. By comparing these studies, several common features can be described. In the vast majority of cases, AML relapse evolved from the initially diagnosed disease, as the paired samples share common founding mutations. This process can follow either linear or branching evolution. Mutations in genes associated with signaling activation are commonly found at diagnosis but frequently lost at relapse, suggesting a major role in the development of leukemia but a minor role in escape from chemotherapy. Mutations in epigenetic regulators, especially regulators of DNA methylation, are generally stable and commonly gained at relapse, which points towards an important role of epigenetic reprogramming in therapy resistance. Nevertheless, the overall number of matched samples at both time points was limited in most studies, and thus the detection of true relapse drivers for all patients may not be possible. As AML samples are genetically highly heterogeneous, common features are rare and the picture of how AML clones evolve over time under the pressure of chemotherapy is still incomplete. Additional studies with increasing patient numbers will be necessary in order to bridge this gap.

In order to improve clonality analysis in AML patients, it is necessary to maximize the sequencing coverage, the number of target genes, and the number of longitudinal samples from a single patient. Although GPS commonly offers higher sensitivity to detect subclonal mutations as compared to WES, in most cases we are still not able to distinguish between the outgrowth of a very small subclone present at diagnosis and the de-novo acquisition of a mutation in a single cell that survived chemotherapy using either of these techniques. Thus, additional and more detailed studies with increased sequencing coverage are necessary in the future. Current NGS techniques using unique molecular identifiers offer the possibility to precisely detect a single mutation in more than 10 000 cells. However, they are still limited to very few target regions only.[66,67] Ultimately, single cell studies may provide the sensitivity required to precisely track individual mutations.[68] Regarding the genes of interest, most studies focus on a limited target region, either all genes by WES or a subset of previously described genes using GPS, as high coverage WGS is still expensive. Of note, in any kind of targeted sequencing, the selection of the enrichment method is critical for estimating the underlying clonality. As during each step of polymerase chain reaction (PCR), which is necessary for target enrichment, sequencing artifacts may be introduced and subsequently amplified. As a consequence, these PCR artifacts bias the computation of the VAF, resulting in incorrect estimation of the clonality. Using hybridization capture-based enrichment methods only few PCR steps are involved and moreover, artifacts introduced by PCR can be detected more readily allowing for their removal from further analysis. In contrast, correcting for PCR artifacts is not possible when using amplicon-based enrichment strategies as here the sequence of interest is intended to be enriched by PCR amplification. Thus, during subsequent data analysis it is not possible to distinguish desired PCR amplification from PCR artifacts. Finally, in order to correctly estimate clonality, it is critical to set the VAFs of individual mutations in relation to each other. Even in high-depth sequencing data, the VAF may not perfectly represent the number of cells carrying a particular mutation or the VAFs of multiple mutations may not be distinguishable from each other in order to assign the mutations to separate clones and multiple constellations of clonality are possible. Tracking these mutations over time in as many longitudinal samples as possible increases the power to correctly assign common mutations shared by the same individual clone. In each sample, the VAFs may be corrected for the individual tumor burden. However, the number of tumor cells may be unknown and may also not be deduced from mutation analysis because preleukemic variants are also present in non-leukemic blast cells to an unknown extent and the founding mutation, present in all leukemic cells, might be unknown.

The model of evolution is playing a major role not just biologically but also clinically. The evolution from treatment-sensitive AML toward treatment-resistant AML is based on losing a particular feature that provides sensitivity and/or gaining a feature that provides resistance. AML cells that followed linear evolution have been in the need of acquiring a mutation that provides resistance, which was not yet present in the main clone at diagnosis. This process, involving erroneous DNA replication during multiple cell divisions, may need some time, while the cells are under the pressure of chemotherapy. If residual leukemia cells are not able to gain the mutation quickly enough, they may be eradicated by the therapy. Thus, mutation clearance is directly correlated to the risk of AML relapse and its dynamics.[69-72] On the other hand, as the subclonal diversity of AML at diagnosis is commonly high, individual cells capable of giving rise to relapse may already exist at diagnosis.[73,74] If a particular mutation that provides resistance is already present in a single cell, this cell has the strongest fitness of all cells under the new selective pressure of chemotherapy. Within this niche, the adapted cell survives and may grow out, while all its competitors are eradicated by the therapy, thereby resulting in

early relapse. In contrast, if AML cells at diagnosis harbor a feature that provides sensitivity, these cells may either lose it by back-mutation, or an ancestral clone which is not carrying this mutation may grow out under therapy. Whether a resistance-mediating mutation is needed, or a sensitivity-mediating mutation must get lost in order to find a niche which allows for leukemia cell survival, the presence of a corresponding subclone at diagnosis leads to rapid development of relapse. Generally, the model of selective outgrowth in branching evolution may follow faster dynamics compared to the acquisition of de-novo mutations in linear evolution, as this usually takes time and the number of tumor cells that may possibly acquire such mutation has been reduced to a minimum by the therapy. Indeed, when correlating clinical outcome with evolutionary models, early relapsing AML is often associated with a loss of mutations, the major hallmark of branching evolution.[41] Although linear evolution can also be detected in AML patients with early relapse, branching evolution is a very rare event in patients with late relapse.

Within the model of branching evolution, it is important to differ between the outgrowth of a common ancestor of initial and relapsed AML, and the evolution of an independent leukemia due to pre-existent and probably preleukemic clonal hematopoiesis. In clonal hematopoiesis, very early mutations, commonly in epigenetic regulators, are present before the onset of the disease, sometimes decades before the diagnosis of leukemia. Although clonal hematopoiesis is associated with an increased lifetime risk of developing hematologic malignancies, these mutations are not sufficient to induce leukemogenesis. Thus, relapsed AML sharing only preleukemic mutations with diagnostic AML should not be viewed as an example of branching evolution but rather as independent AML. Further, to clearly describe different mechanisms of AML evolution, it is also important to distinguish between preleukemic mutations, founding mutations, driver mutations and passenger mutations when looking at stable mutations between diagnosis and relapse.

Although previous studies have shown that a transformation from therapy-sensitive towards therapy-resistant AML may be mediated by a change in the mutational profile, still in about 25% of AML patients no mutational gain at relapse was detected. Moreover, these patients relapsed significantly earlier, indicating an even more aggressive disease. Thus, it is important to search for drivers of AML relapse not only at the level of genetics, but also at additional biological layers like epigenetics, especially as mutations in epigenetic regulators are relatively stable over time and moreover frequently gained at relapse.

Although every study cohort of relapsed AML helps to identify common features and mechanisms, they also add to the complexity of AML genetics. When looking deep enough, each AML (epi) genome is as individual as the patient itself and moreover, each evolutionary process from diagnosis to relapse is defined by multiple patient- and treatment-specific features. Meta-analyses of multiple studies will help to detect common mechanisms of therapy resistance. Finally, we are facing a paradigm shift from classifying AML into common genetic subgroups towards treating AML as a genetically individual disease that evolves over time.

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