anemia, decreased platelet counts, and liver enzymes, mice with double and triple mutations generally displayed intermediate values, relative to *Prf1*-deficient animals that all died. In immunological terms, double and triple mutations amounted to increased serum levels of interferon-γ, interleukin-6, and tumor necrosis factor, representing cytokines that mediate pathology in HLH. Notably, cytotoxic granule trafficking and exocytosis was reduced in mice carrying heterozygous mutations in both *Stx11*, and *Rab27a*, but only to 2/3 of wild-type mouse levels. In summary, Sepulveda and colleagues thus found that an accumulation of heterozygous mutations in HLH-associated genes in mice correlated with transient HLH manifestations following viral infection and a fatal outcome in a small proportion of animals. The number and severity of disease features thus correlated with gene dosage.

The findings by de Saint Basile and coworkers[3] have several implications for human disease. Their study supports the concept of a polygenic inheritance of HLH, where accumulation of partial genetic defects in cytotoxic granule-dependent pathway may increase the likelihood of developing disease (see figure). Of note, in a cohort of 2701 patients with a clinically suspected diagnosis of HLH, Zhang and colleagues found that only 1% of patients carried heterozygous mutations in 2 or more different HLH-associated genes.[6] Because noncoding aberrations represent a significant cause of HLH that were not assessed in the study by Zhang et al, this figure likely represents an underestimate.[6,7] In individual cases, calculating the risk of HLH based on genetic information is complicated. First, the effect of a specific mutation is not easily quantified. Distinct HLH-associated genes are associated with different severities. Some mutations may even display dominant negative effects, which are determined through advanced cellular assays.[8] Second, with less severe impairments in lymphocyte cytotoxicity, environmental factors play an increasing role in determining HLH susceptibility.[3] Nonetheless, the concept of gene dosages in determining the efficacy of target cell killing may have wider implications for the risk of developing a spectrum of diseases associated with impaired lymphocyte cytotoxicity. As the authors point out,[1] lymphocyte cytotoxicity gene dosages may combine with mutations in genes regulating inflammation, thereby contributing to the risk of developing inflammatory syndromes such as macrophage activation syndrome. Moreover, biallelic mutations in HLH-associated genes have been linked to cancer susceptibility.[4] Relatives of primary HLH patients, likely carriers of heterozygous mutations, display a 3-fold increased risk of cancer, specifically in female carriers.[9] It is therefore conceivable that an accumulation of genetic lesions in the molecular pathway required for lymphocyte cytotoxicity may substantially increase the risk of developing cancer (see figure). Large-scale genome projects have revealed that 16% of the human population carries at least 1 predicted damaging mutation in a gene required for lymphocyte cytotoxicity.[10] Accumulation of mutations in such genes may therefore signify an important mechanism for HLH as well as the spectrum of related diseases in a relatively large number of individuals.

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● ● ● LYMPHOID NEOPLASIA

Comment on Nadeu et al, page 2122

Not all subclones matter in CLL

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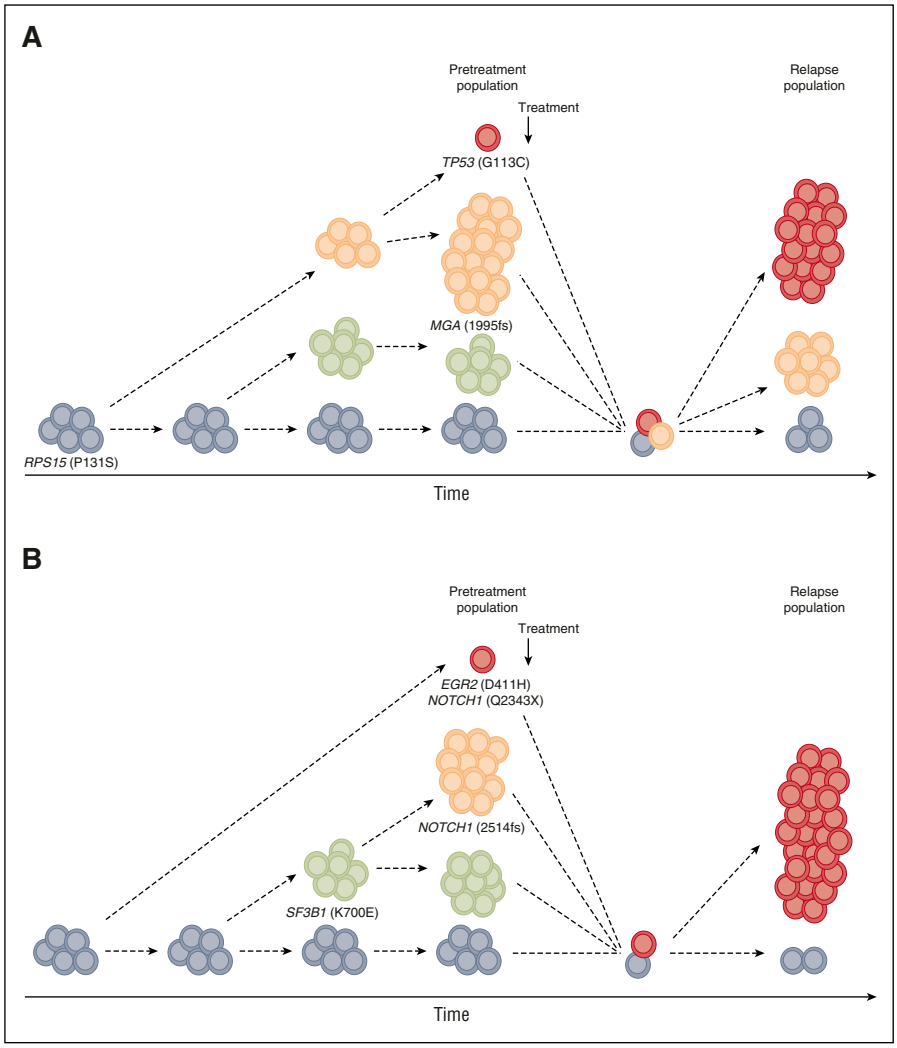
In this issue of *Blood*, Nadeu et al delve deep into the genomes of patients diagnosed with chronic lymphocytic leukemia (CLL) and report on the clinical implications of tumor heterogeneity in the era of next-generation sequencing (NGS).[1] While clonal evolution is common in hematologic tumors, it remains challenging to detect; however, this difficulty can be circumvented by performing ultradeep sequencing, which substantially improves the discovery of variants across a range of variant allelic frequencies (VAFs). This strategy was adopted by the Spanish group, which enabled them to not only detect subclonal mutations but also demonstrate that the presence of subclonal mutations within certain genes (ie, *TP53* and *NOTCH1*) is clinically relevant.[1]

Enabled by technological advances, the full compendium of common, recurrent somatic mutations in the coding genome of CLL is nearing completion and has revealed that this malignancy is not characterized by a single unifying pathological mutation. Instead, a relatively restricted number of genes are mutated at a high frequency in CLL (*TP53*,



Clonal evolution and intraclonal dynamics in 2 CLL patients relapsing after fludarabine, cyclophosphamide, and rituximab therapy. Examples based on whole-exome sequencing data from our recent study (Ljungström et al).[5] (A) In addition to the founding clone that harbors a *RPS15* mutation, 2 subclones were observed before treatment initiation, one of which carried a frameshift deletion within *MGA*. At relapse, the *MGA*-deleted subclone disappeared, while a new subclone containing a *TP53* mutation emerged. (B) A marked shift in subclonal populations was observed; while 2 subclones harboring the classical 2-bp *NOTCH1* deletion and the recurrent p.K700E mutation in *SF3B1* were eradicated after treatment, a new subclone emerged harboring a stop-gain *NOTCH1* mutation and an *EGR2* mutation.

ATM, *NOTCH1*, and *SF3B1*), with most recurrently affected genes located in the so-called long tail of the mutation distribution, occurring at a low frequency (<5%).[2,3] We have also recently begun to understand the clinical impact of these mutations, with a number of genes harboring alterations linked to poor clinical outcome and enriched in patients with a clinically aggressive or chemorefractory disease (eg, *NOTCH1*, *ATM*, *SF3B1*, *NFKBIE*, *BIRC3*, and *RPS15*).[4,5] In a recent multicenter study conducted by the European Research Initiative on CLL, the impact of *TP53*, *NOTCH1*, *SF3B1*, *BIRC3*, and *MYD88* mutations were investigated in 3490 patients, and the study revealed that *TP53* and *SF3B1* mutations, but not *NOTCH1* mutations, remained as independent markers of short time to first treatment (TTFT) in multivariate analysis.[6] Studies have also suggested a predictive role for additional genes harboring mutations, but these have to be studied further before firm conclusions can be reached.

The development of NGS has changed the comprehensiveness of human genetic analysis and provided us with insights into subclonal complexity and the dynamic changes that occur over time and in relation to therapy (see figure).[7] Recent studies have revealed that minor *TP53* subclones (ie, subclones with a VAF not detected by Sanger sequencing) are as equally important to detect as clonal *TP53* mutations (ie, detected by Sanger sequencing), because CLL patients harboring minor subclones at diagnosis exhibited a poor outcome similar to patients carrying clonal *TP53* mutations.[8,9] Nevertheless, the impact of recurrent mutations in other genes deemed to be potential "drivers" has not yet been investigated.

To clarify this matter, Nadeu et al performed targeted ultradeep NGS in a large, well-characterized cohort of 406 untreated CLL patients and investigated *TP53*, *ATM*, *BIRC3*, *SF3B1*, and *NOTCH1*.[1] The authors initially made a series of important validation experiments to ensure high sequence depth/coverage and reproducibility and to determine the minimal VAF cutoff at which they could detect a mutation with their NGS strategy (range, 0.3% to 2%, depending on the specific gene analyzed). Using this approach, they identified a substantial proportion of subclonal mutations within *TP53* (4.2%), *SF3B1* (5.2%), and *NOTCH1* (7.6%), while fewer subclonal *ATM* (1.3%) and *BIRC3* (2.2%) mutations were observed. Confirming the known clinical impact of clonal mutations within *TP53*, *ATM*, *NOTCH1*, *SF3B1*, and *BIRC3*, they proceeded to demonstrate that CLL patients possessing subclonal *NOTCH1* mutations had a significantly shorter TTFT, while subclonal *TP53* mutations were associated with a shorter overall survival (OS). However, subclonal *SF3B1* mutations did not appear to influence disease outcome (too few subclonal *ATM* and *BIRC3* mutations were detected to allow for a meaningful analysis). Genomic analyses also permits inference about the temporality of mutational events, and by monitoring a subset of patients longitudinally, Nadeu et al were able to gain insight into the complex clonal evolution of CLL. They observed a temporal acquisition of mutations and a gradual genomic degeneration with subclones expanding, disappearing, or remaining stable. The presence of clonal evolution was also associated with a shorter OS, which is in line with recent studies.

However, as is often the case, the road from genetic discovery to understanding the biological context, and ultimately to patient therapy, is not always clear-cut and is often plagued by hurdles along the way. In the setting of subclonal mutations in CLL, it is relevant to note that Rasi et al very recently reported no impact of subclonal *SF3B1*,

NOTCH1, and *BIRC3* mutations on OS, suggesting that subclonal *NOTCH1*, *SF3B1*, and *BIRC3* mutations may not provide the same "fitness advantage" as subclonal *TP53* mutations.[10]

Henceforth, given the relative rarity of cases harboring subclonal mutations and the differing clinical impact observed between the 2 aforementioned studies, it is evident that large-scale ultradeep NGS studies must be performed in order to analyze subclonal mutations in CLL and definitively draw conclusions regarding the relative impact of each type of mutation in conjunction with the presence of other genetic events. In other words, we can no longer simply think of mutations as being present or absent, it will be vital to know what proportion of cells harbor a mutation not only to predict outcome but also to optimize therapy. Along these lines, it will be important to address the issue of subclonal mutations in homogeneously treated patients particularly in the era of targeted therapy using B-cell receptor inhibitors (ibrutinib and idelalisib).

As a final remark, while the study by Nadeu et al elegantly demonstrates that as the scope of possibilities increases through technological developments, we are able to ask increasingly sophisticated questions, raising questions regarding (1) how to establish a cutoff value for VAFs, (2) whether this value has to be gene specific, and (3) what the best methodologies are for detecting subclonal mutations. Hence, there is an evident, unmet need to harmonize the methodologies used to detect minor subclones, and rigorous testing is required to establish the technology and minimal requirements for the standardized assessment of such subclones, which will be particularly relevant in the multicenter setting and to ensure clinical benefit before widespread introduction.

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