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TO THE EDITOR:

Preexisting or therapy-induced mutations in relapsed acute lymphoblastic leukemia?

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We read with interest the paper by Li et al[1] and enthusiastically recommend it to others for its insights into the biology of relapse. Multiagent chemotherapy for acute lymphoblastic leukemia (ALL), developed over decades of clinical trials, is successful for most children and adolescents but fails for others. Despite recent progress,[2,3] the mechanisms of relapse in childhood ALL remain poorly understood.

With whole-genome sequencing at median 30× coverage, Li et al compared diagnostic and relapse ALL sample pairs with times to relapse ranging from 64 to 2410 days and identified several mutations in relapse specimens associated with resistance to specific chemotherapeutic agents. Abnormalities in nucleotide metabolism enzymes NT5C2, PRPS1, and PRPS2, and DNA mismatch repair genes *MSH2*, *MSH6*, and *PMS2*, affect thiopurine response; abnormalities in *FPGS*, a folate metabolism gene linked to methotrexate activity in vivo, may explain escape from conventional maintenance therapy. With these techniques, the mutations identified at relapse were not detected at diagnosis, unlike other cancers. Using published doubling time estimates, Li et al allow 213 days between mutation and clinical relapse with a 5-day doubling time and 374 days with 9-day doubling time. With relapses outside of these limits, the authors conclude that the mutations could not have been present in even a single cell at diagnosis. Li et al suggest that late-appearing mutations may be induced by DNA-damaging therapy.

Mutations may certainly appear after remission but the conclusion that these mutations could not have been present in even a single cell at diagnosis requires further examination. Li et al found that these mutations emerged 213 to 374 days before clinical relapse, during remission, and after induction for all but the earliest relapses. Currently, more than two-thirds of ALL relapses occur after 2 years.[4]

Others have debated whether therapy selects preexisting resistant clones or whether DNA-damaging agents induce such mutations.[5,6] Were mutations therapy induced, reduction or elimination of DNA-damaging agents might enhance cure rates.

Clones that predominate at relapse, or their ancestors, are usually present at diagnosis, though not always detected by "conventional methods." Might these elusive clones already bear the resistance-associated mutations described by Li et al? Late emergence of de novo mutations requires that clones escape primary therapy long enough for later mutations to arise; this phenomenon cannot be explained by resistance mutations only associated with later therapy (eg, postinduction use of thiopurines).

Inability to detect a mutation in the diagnostic specimen does not prove absolute absence; it only proves that the mutation was absent in the particular specimens studied with specific assays. Emerging clones, arising in a single cell, may be anatomically localized and defy limited sampling. Techniques vary in sensitivity.[7] Supplementing polymerase chain reaction with xenograft techniques, Shlush et al identified relapse clones in diagnostic acute myeloid leukemia specimens at a frequency <0.0002% (1 in 500 000).[6] Dobson et al studied 14 paired diagnosis and relapse B-cell ALL specimens with combined genomic and functional analyses.[5] Relapse arose from a minor subclone detectable at diagnosis in 10 of 14 cases and from further evolution of the predominant diagnostic clone in 4 cases. With a limit of detection of a variant allele frequency of 1% with combined sequencing, in 4 of 13 engrafting pairs, "relapse-initiating" clones were identified at diagnosis in xenografts but not by sequencing. Relapse-initiating clones already demonstrated increased tolerance to vincristine, dexamethasone, and L-asparaginase. Dobson et al conclude that their data provide "direct evidence that the Luria-Delbrück principle that resistance in a cell population may be intrinsic occurs in human leukemia."[5(p569)] Choi et al caution that appearance of an apparently de novo clone at relapse may be misinterpreted as a new mutation owing to the limited sensitivity of methods used at diagnosis.[8]

A fragile estimate of doubling times also weakens Li et al's assertion that the mutations must only have emerged at some time after diagnosis. Leukemia is oligoclonal at presentation and relapse.[9] Doubling times are not constant or uniform among the heterogeneous leukemic population. Doubling times do not account for subsets of blasts that have been identified as temporarily quiescent in G0 phase in a hematopoietic niche or elsewhere, neither dividing nor preparing to divide. Quiescence reduces the risk of subsequent mutation and clonal evolution.[10] At some point, quiescent blasts may escape and resume proliferation.

Even in those actively proliferating, the published 5- to 9-day doubling times for ALL may be questioned. Supplemental Figure 5C (available on the *Blood* Web site) estimates aggregate doubling times from only 19 B-ALL patients. Only 1 of 19 patients had a late relapse. These aggregate times likely include a far wider range of individual patient values. Supplemental Figure 5D, derived from Tsurusawa et al,[11] shows 9 of 12 samples with doubling times between 5 and 9 days. Two other samples have doubling times of ~10 and 14 days. A doubling time of 14 days would allow for 600 days of geometric proliferation until clinical relapse.

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Furthermore, some subclones may be more or less proliferative than others. Resistant leukemic blasts, like resistant bacteria, may be generally less proliferative than their more sensitive counterparts.[12] Although not universally proven or accepted, this has been shown specifically for NT5C2 mutants.[13] Assignment of an overall doubling time does not account for this proposed "cost of resistance." Partially effective chemotherapy may also slow proliferation (cytostasis) and/or increase cell death short of achieving net cytoreduction in partially resistant subclones, thus increasing effective doubling times.[12] An average doubling time may not fully describe the growth of heterogeneous blast populations in vivo.

Mutations may certainly occur in clinical remission. DNA-damaging chemotherapy may increase the mutation rate. Szikriszt et al[14] surveyed 8 chemotherapeutic agents. Cisplatin had the greatest effect with a 17.3-fold increase over baseline, cyclophosphamide had a 5.4-fold increase, and hydroxyurea, gemcitabine, 5-fluorourucil, etoposide, doxorubicin, and paclitaxel had a 2-fold effect or less. However, McFaul et al[15] studied 29 patients with HIV receiving cytotoxic therapy for lymphoma and found no increased incidence of resistance to HIV therapy.

Assume that blast counts are stable for 2 periods of time, 10^{12} blasts in the 1, 2, or 3 weeks around diagnosis and 10^8 blasts in the 3 years of remission, and the doubling time is 7 days.

If Δt = elapsed time and r = the number of cell divisions per unit time (blast number × divisions/unit time), then $r\,\Delta t$ is the number of cell divisions in time Δt. If p = the risk of mutation per cell division, then $(1 - p)^{r\Delta t}$ is risk of no mutation over $r\,\Delta t$ cell divisions and $1 - (1 - p)^{r\Delta t}$ is the risk of mutation.

The instantaneous rate of mutation is:

$$\lim_{\Delta t \to 0} \frac{1-(1-p)^{r\Delta t}}{\Delta t} = -r \times ln(1-p)$$

For simplicity, call this λ. With a constant number of blasts and constant risk of mutation/unit time over these time intervals, the time to first mutation may be described with an exponential survival function.

As demonstrated by the following equation, with 10^8 blasts over 3 years and $P = 10^{-11}$, the cumulative risk of mutation is 0.14, matching the ~15% relapse rate of childhood ALL, although some relapses are very early and represent persistence of the original blast population:

$$\begin{aligned} Prob(mutation|t &= 52\text{ weeks/year} \times 3\text{ years}) \\ &= 1 - \exp(-\lambda \times 52 \times 3) \\ &= 1 - \exp(r ln(1 - p) \times 52 \times 3) \\ &= 1 - \exp(10^8 \times \ln(1 - 10^{-11}) \times 52 \times 3) \end{aligned}$$

For the 10^{12} blasts at diagnosis, let $P = 10^{-12}$. Allowing for a 10-fold increased mutation rate from cytotoxic chemotherapy, the cumulative risk of mutation is 0.63, 0.86, and >0.95, after 1, 2, or 3 weeks.

With this model, any mutation that might appear over 3 years after remission was more likely to have appeared earlier around diagnosis. Should cytotoxic chemotherapy increase the mutation rate <10-fold, the relative likelihood of early appearance is yet greater.

Mutations may emerge despite remission. A mutation and its specific clone may be eradicated only to reemerge later in therapy from some elusive precursor population. The risk of breakthrough in chronic myelogenous leukemia is related to disease burden. In chronic-phase chronic myeloid leukemia, maintaining disease burden below 0.1% assures less chance of clonal evolution and clinical progression.[16] Mutations may enhance sensitivity to chemotherapy as well as resistance. Gaynon and Sun have proposed that the predominant clones at relapse may differ from the occult clones that escape primary therapy.[17] Regimens with substantial response rates after relapse in ALL, acute myeloid leukemia, anaplastic large cell lymphoma, Hodgkin disease, and rhabdomyosarcoma have all failed to prevent relapse when included in earlier therapy.

Cytotoxic chemotherapy exploits a presumed proliferative difference between highly proliferative cancer cells and less highly proliferative host cells.[18] However, not all leukemic clones are highly proliferative and some may escape cytotoxic chemotherapy, only to reemerge with a subsequent mutation or epigenetic change increasing proliferation and restoring some degree of chemosensitivity.

Most likely, resistant clones are already present at diagnosis, though elusive, and selected by therapy.[8,19,20] We should be very slow to exclude the presence of relapse mutations at presentation, recognizing the oligoclonality of leukemia and the limitations of our methods. The article by Li et al nonetheless provides an important insight into the complex biology of relapse.

Authorship

Contribution: P.S.G., E.O., and L.J. conceptualized ideas, wrote the paper, and approved the final draft in detail; and L.J. formulated the statistical assertions.

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Footnotes

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The online version of this article contains a data supplement.

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RESPONSE

Therapy-induced mutagenesis in relapsed ALL is supported by mutational signature analysis

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We appreciate the interest of Gaynon et al[1] in our study on acute lymphoblastic leukemia (ALL) relapse.[2] They argued against our finding that therapy-induced drug resistance mutations occur in ALL and stated that these mutations were potentially preexisting at diagnosis but undetectable. They focused primarily on our mathematical modeling of ALL doubling times, while ignoring other important biological evidence from our study.

First, our strongest evidence of therapy-induced drug resistance mutations came from mutational signature analysis, not the modeling of doubling times. We showed that >25% of relapsed ALLs bore 1 or 2 treatment-induced mutational signatures, referred as novel signatures A and B. We showed experimentally that novel signature B was induced by thiopurine treatment, and the therapy-induced mutations were clonal in most relapses (supplemental Figure 10); hence therapy does not simply induce minor subclonal variants.

Second, drug resistance mutations in relapsed ALL preferentially occurred at trinucleotide contexts mutated by therapy. For example, relapse-specific *NT5C2* R367Q mutations were C>T mutations occurring at the center of TCG trinucleotides. We showed experimentally that thiopurines cause this type of mutation, and *NT5C2* R367Q mutations were significantly enriched in relapses that bore the thiopurine signature (supplemental Figure 16). We further analyzed the probability that each drug resistance mutation was caused by individual signatures present in each leukemia sample, based on each signature's preference to mutate at specific trinucleotides, using a published method.[3] This indicated that *NT5C2*, *TP53*, *NR3C1*, and *PRPS1* drug resistance mutations were likely induced by treatment in a subset of patients. A separate manuscript in preparation strongly reinforces this paradigm.

Gaynon et al also misinterpreted our conclusions; we did not dismiss the contribution of preexisting clones to relapse. They cited the study of Dobson et al,[4] in which we participated as collaborators, including part of the genomic analysis, as evidence that a minor subclone is usually present at diagnosis, which leads to the eventual relapse. Similarly, we also showed in supplemental Figure 17 that in 80% of patients the relapse-fated clone was detectable at diagnosis. Indeed, our Visual Abstract

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