DNA Damage Response (DDR) Is Associated With Treatment-free Remission in Chronic Myeloid Leukemia Patients

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Tyrosine kinase inhibitors (TKI) drastically improved outcome in chronic myeloid leukemia (CML): selected patients with optimal response may suspend the therapy indefinitely, after a prolonged remission.[1] However, about 50% patients relapse rapidly and must resume treatment. Relapses are likely due to residual TKI-insensitive quiescent leukemic stem cells (LSCs) that persist under TKI.[2] These cells aberrantly express CD26 (dipeptidyl-peptidase IV), a cell surface marker that can be used to distinguish them from normal hematopoietic stem cells (HSCs).[3] DNA damage response (DDR) is constitutively triggered in cancer cells in response to oncogenic stress[4] and the accumulation of DNA damage can generate genetic abnormalities favoring disease recurrence and transformation to advanced phase.[5] On the other hand, extensive DNA damage may cause stem cell exhaustion, leading to inadequate clonogenic potential even after the release of drug pressure.[6] We investigated whether DDR in CML LSCs at diagnosis can be associated to treatment-free remission (TFR).

In a cohort of 72 Ph+ CML patients treated with frontline TKIs (Suppl. Figure S1 and Tables S1–S2), 39 have been under therapy for over 5 years (median, 7 years) with a deep molecular remission (DMR, defined as MR4 or better) lasting >18 months, and were eligible for discontinuation. Of these, 34 patients agreed to discontinue and 13 of 34 (38%) relapsed (recurrence was defined as a confirmed loss of MR4); median time to failure was 4.5 months (interquartile range [IR], 4.0–7.2) and in 11 of 13 cases (85%), the relapse occurred within

8 months from discontinuation, in line with previous observations. Cumulative incidence of molecular relapse was 34% at 24 months and 49% at 100 months (Suppl. Figure S2A). All relapsed patients resumed therapy and achieved a second molecular remission; no clinical progressions were observed. At the time of reporting, 21 patients were in TFR with a median follow up of 37 months (IR, 19–56). We obtained 49 bone marrow (BM) samples at onset (10 from patients that later relapsed after discontinuation, 14 from nonrelapsed patients, 5 from eligible patients who decided not to stop treatment, 16 from patients who did not meet the criteria for discontinuation ["continued"], and 4 currently under TKI since <5 years; see Suppl. Figure S1) and 20 samples collected during TKI treatment (range, 3 months–6 years). We sorted CD45+CD34+CD38-CD26+ LCSs and CD45+CD34+CD38-CD26- HSCs from these BM samples (Figure 1A). Fluorescent in-situ hybridization for breakpoint cluster region::Abelson proto-oncogene 1 (BCR::ABL1) fusion performed on 10 representative samples confirmed that all cells in the CD26+ fraction carried the t(9;22) translocation, indicating that CD26 expression identifies CML LSCs within the CD34+CD38- stem cell population.

TKI therapy drastically reduced the number of CD26+ cells in the CD34+CD38- stem cell fraction, indicating that they are selectively targeted by TKIs, in contrast to normal CD26- HSCs; we could appreciate this result by comparing samples from untreated and treated groups (median %CD26+ cells, 27% versus 3%; $P < 0.0001$; Figure 1B) as well as by longitudinal analysis of BM samples collected from the same patient before and after treatment (Figure 1C). The intra-patient trend of CD26+ cells number correlated very well with molecular response evaluated by quantitative polymerase chain reaction (qPCR) (Figure 1C). Similar results were obtained when the frequency of CD26+ cells was measured as a fraction of the whole CD45+ hematopoietic compartment (Suppl. Figure S2B). These data suggest that TKI treatment shrinks the CD26+ LSC population and that these cells represent a reliable hallmark of CML.[7] We then compared BM samples collected at diagnosis from 29 patients who later achieved the criteria for TKI stop ("eligible" patients) versus 16 patients that did not ("continued" patients): the latter showed a higher percentage of CD26+ LCSs at onset (Figure 1D and Suppl. Figure S2C). Of note, the 2 groups spent a comparable number of years on TKI treatment (continued versus eligible, mean ± SD: 8.5±2.9 versus 7.4±2.2, respectively;
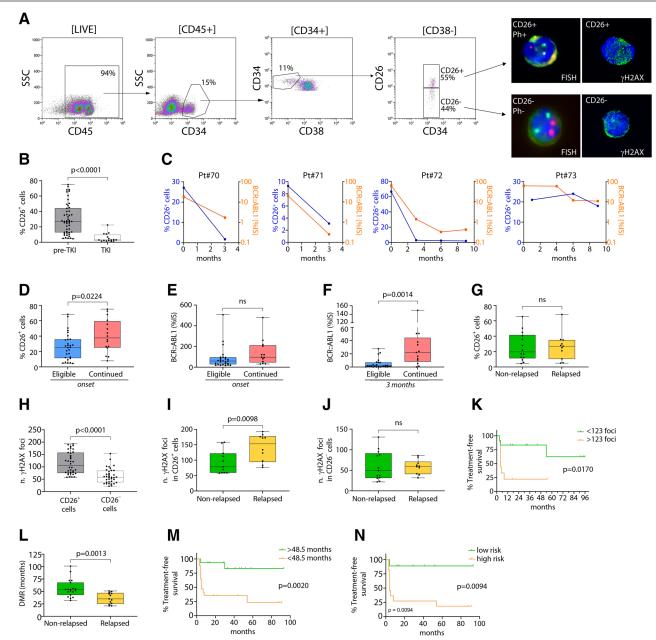
Figure 1. (A) FACS sorting protocol employed to isolate CD45+CD34+CD38−CD26+ and CD45+CD34+CD38−CD26− cells from BM CML samples. One representative onset BM sample is shown. The intensity of CD26 staining displayed a continuous pattern, rather than a clear partition into CD26+ and CD26− populations; indeed, in some patients, a minor fraction of CD26lo/Ph+ cells was noted, representing on average 6% of the sorted CD26− population. Representative FISH and γH2AX staining images are shown on the right, in sorted CD26+ and CD26− cells. Nuclei from CD26− cells display 2 green signals where the probe has hybridized to the BCR region; and 2 orange signals, where the probe has hybridized to the ABL1 region. Nuclei from CD26+ cells show green-orange fusion signal (yellow). (B) Frequency of CD26+ LSCs within the CD45+CD34+CD38− cell fraction, in untreated (pre-TKI) and treated (TKI) patients (box and whiskers plot, min to max). (C) Percentage of CD26+ LSCs (blue line, left y-axis) and BCR::ABL1 mRNA levels (orange, right y-axis) in 3 responding and 1 nonresponding CML patients at diagnosis and after starting TKI. (D) Percentage of CD26+ LSCs at onset, in patients who achieved eligibility criteria for treatment discontinuation ("Eligible," blue box; n = 29) vs patients that did not obtained sustained MR4 ("Continued" patients, red box; n = 16). (E−F) IS-adjusted quantitative PCR analysis of BCR::ABL1 transcript levels at onset and after 3 mo on therapy, in patients who could not stop and in patients who achieved the minimal criteria to discontinue. (G) Number of CD26+ cells in relapsed vs nonrelapsed discontinued patients. (H) γH2AX foci number is higher in CD26+ than in CD26− cells (mean ± SD: 115±44 vs 63±30; P < 0.0001). (I) CD26+ LSCs from patients relapsing after discontinuation (n = 10) show increased γH2AX compared with LSCs from nonrelapsed patients (n = 12), at diagnosis. (J) γH2AX foci in CD26− normal HSCs cells from relapsed and nonrelapsed patients (mean ± SD: 60±35 vs 58±17; P = 0.886). (K) Treatment-free survival in discontinued patients with high (>123 foci, green line) or low γH2AX foci counts (<123 foci, orange line). The cutoff was estimated by ROC analysis. (L) Duration of DMR before discontinuation in relapsed (n = 13) vs nonrelapsed (n = 18) patients. (M) Treatment-free survival in patients who stopped after more (green line) or less (orange line) than 48.5 mo in DMR. The probability of TFR at 24 mo is 93% vs 38% in the group discontinuing after >4 vs <4 y, respectively. (N) Treatment-free survival of high- and low-risk groups, clustered by Cox regression hazard considering γH2AX foci number and DMR. Survival curves were compared by log-rank test. Group comparisons were made by Mann-Whitney test (P values shown above graphs); ns indicates nonsignificant comparisons (P > 0.05). BM = bone marrow; BCR = breakpoint cluster region; CML = chronic myeloid leukemia; DMR = deep molecular remission; FACS=fluorescence-activated cell sorting; FISH = fluorescent in-situ hybridization; HSC = hematopoietic stem cell; LSC = leukemic stem cell; PCR = polymerase chain reaction; ROC = receiver operating characteristic; TFR = treatment-free remission; TKI = tyrosine kinase inhibitors.

P = 0.124; Suppl. Figure S2D). This result points to a possible correlation between the number of CD26+ LSCs in patients' BM at diagnosis and the probability to obtain a deep remission. Patients who met criteria for discontinuation had lower BCR::ABL1 transcript levels by qPCR after 3 months on TKI treatment, but not at the time of diagnosis (Figure 1E, F), suggesting that measuring LSCs may be more sensitive than qPCR as a biomarker of response at diagnosis.

Next, BMs from 10 patients who relapsed after discontinuation were compared with samples from 14 nonrelapsed patients. The number of LSCs at onset did not differ between the 2 groups (mean ± SD: 26 ± 18 versus 28 ± 17; P = 0.803; Figure 1G). We hypothesized that their DDR status, rather than their number, may correlate with relapse post-discontinuation. The number of γH2AX foci was determined in stem cells by immunofluorescence, as a surrogate marker of DNA damage. Overall, CD34+CD38−CD26+ cells had approximately 2-fold higher γH2AX foci compared with CD34+CD38−CD26− cells, not only on average, but in every Ph+ CML patient analyzed, regardless of clinical response or disease status (Figure 1H). The γH2AX staining pattern was a uniform punctate distribution of foci (Figure 1A), consistent with oncogene-induced replication and oxidative DNA damage (double strand breaks);[5,8] we never observed a diffused pan-nuclear nor a nuclear periphery staining, suggesting that H2AX phosphorylation in these cells may not be associated with extreme replicative stress or cell death, at least in the majority of cells.[9,10] Further investigation, using additional markers, will be needed to analyze these processes more in depth. Interestingly, the number of foci was significantly higher in CD26+ LSCs isolated from patients who later failed discontinuation in comparison with LSCs from patients that achieved durable TFR (median 152.9 versus 78.5; Mann-Whitney U test P < 0.01; Figure 1I), while no differences were observed in normal (CD26−) HSCs between the 2 groups (Figure 1J). Receiver operating characteristic curve analysis and Youden's index estimation indicated that patients with a high number of γH2AX foci (more than 123 foci) were more likely to lose molecular response, with 22% versus 83% patients in TFR at 24 months (Figure 1K). By contrast, BCR::ABL1 qPCR at onset was not different between relapsed and nonrelapsed patients (Suppl. Figure S2E) indicating that γH2AX foci number in CD26+ cells is not a mere representation of disease burden. The number of γH2AX foci was similar in LSCs of patients that did not achieve criteria for TKI interruption compared with those who could discontinue (Suppl. Figure S2F). Finally, by univariate analysis, we found that TFR patients had been in DMR for longer time, compared with relapsed ones, before stop (median DMR, 54 versus 35 months, P = 0.001): a 4 years' cutoff discriminated nonrelapsing from relapsing patients (Figure 1L, M). Furthermore, a higher proportion of nonrelapsed patients compared with relapsed ones had sustained MR4.5 at discontinuation (100% versus 50%, respectively; median DMR4.5, 49 versus 18 months, P < 0.001).

To confirm our observations, we assessed the association of 8 meaningful clinical and biological variables (age at discontinuation, Sokal score, duration of TKI, duration of DMR, duration of DMR4.5, qPCR response, γH2AX foci, %CD26+ cells) with disease relapse, by a combined regularized Cox regression and Kaplan-Meier survival analysis, in 24 patients for whom all these data were available. This multivariate analysis selected 2 variables: γH2AX foci as negatively associated and DMR as positively associated with treatment-free survival (Suppl. Table S3). Using the regularized Cox regression proportional hazard, estimated with the 2 selected variables, we could stratify patients into 2 risk groups (high versus low risk) with significantly different survival (P = 0.009; Figure 1N).

In conclusion, in CML patients at diagnosis, we identified links between: (a) the number of CD26+ LSCs at onset and the probability of prolonged MR4; (b) the level of DDR in LSCs and the risk of relapse after TKI discontinuation. These results identify γH2AX as a possible marker of durable TFR. Genomic instability is an early event in CML, promoting BCR::ABL1 translocation; it is then sustained by BCR::ABL1 kinase activity, causing progression to advanced phase.[11] Higher DDR has been documented in CML versus healthy donor stem cells and in blast versus chronic phase CML.[8] Inhibition of BCR::ABL1 kinase has a modest effect on reactive oxygen species production and DNA damage in TKI-refractory quiescent LSCs: hence, primitive LSCs have been postulated as the source of genomic instability that lead to recurrent disease.[5] The possibility to sort LSCs from HSCs within the same sample by CD26 expression allowed us to establish that Ph+ LSCs display higher DDR than Ph− HSCs at single patient level. We demonstrated that higher γH2AX in LSCs is associated with recurrence of leukemia on TKI discontinuation. Therefore, we propose that γH2AX foci number may represent a prognostic molecular marker for treatment discontinuation outcome. Confirmation studies will be necessary to validate such an indication.

It is important to note that the samples used in this study were taken at diagnosis, while the best time to analyze CML stem cells and their functional status would ideally be at the time of discontinuation, which is however more challenging due to the extremely low number of residual CD26+ cells. We hypothesize that the amount of γH2AX foci in LSCs at diagnosis represents the individual basal level of DNA damage and captures a critical intrinsic phenotype of leukemia-promoting cells of each patient. Thus, we suggest that DDR in CML LSCs at diagnosis could represent a marker of genetic instability, particularly in quiescent LSCs that survive TKI treatment[2] and keep accumulating DNA damage, until they start proliferating again and cause the relapse: therefore, the higher the damage at onset, the more genetic aberrations could be present at discontinuation and the more likely LSCs could gain the ability to regenerate a proliferating leukemic progenitor pool after treatment discontinuation. It will be important to isolate the pure TKI-insensitive quiescent stem cell fraction from the whole CD34+CD38−CD26+ population that includes also TKI-sensitive cycling leukemic progenitors,[12] to increase resolution of the analysis. It would also be interesting to integrate these data with other chromatin marks, as epigenetic modifiers have recently been associated with TFR.[13]

Interestingly, γH2AX foci levels observed before TKI treatment did not impact on treatment efficacy, they rather seem to be strictly connected to TFR. Therefore, this feature may uniquely affect the ability of persistent CML stem cells to reinitiate leukemic hematopoiesis on drug withdrawal, rather than providing a drug resistant phenotype, as also suggested by the fact that patients responded very nicely to reintroduction of TKIs.

REFERENCES

1. Inzoli E, Aroldi A, Piazza R, et al. Tyrosine kinase inhibitor discontinuation in chronic myeloid leukemia: eligibility criteria and predictors of success. *Am J Hematol.* 2022;97:1075–1085.

2. Corbin AS, Agarwal A, Loriaux M, et al. Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. *J Clin Invest*. 2011;121:396–409.

3. Herrmann H, Sadovnik I, Cerny-Reiterer S, et al. Dipeptidylpeptidase IV (CD26) defines leukemic stem cells (LSC) in chronic myeloid leukemia. *Blood*. 2014;123:3951–3962.

4. Bartkova J, Horejsí Z, Koed K, et al. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature*. 2005;434:864–870.

5. Bolton-Gillespie E, Schemionek M, Klein HU, et al. Genomic instability may originate from imatinib-refractory chronic myeloid leukemia stem cells. *Blood*. 2013;121:4175–4183.

6. Caocci G, Greco M, Delogu G, et al. Telomere length shortening is associated with treatment-free remission in chronic myeloid leukemia patients. *J Hematol Oncol*. 2016;9:63.

7. Bocchia M, Sicuranza A, Abruzzese E, et al. Residual peripheral blood CD26(+) leukemic stem cells in chronic myeloid leukemia patients during TKI therapy and during treatment-free remission. *Front Oncol*. 2018;8:194.

8. Popp HD, Kohl V, Naumann N, et al. DNA damage and DNA damage response in chronic myeloid leukemia. *Int J Mol Sci*. 2020;21:1177.

9. Moeglin E, Desplancq D, Conic S, et al. Uniform widespread nuclear phosphorylation of histone H2AX is an indicator of lethal DNA replication stress. *Cancers (Basel)*. 2019;11:355.

10. Solier S, Pommier Y. The apoptotic ring: a novel entity with phosphorylated histones H2AX and H2B and activated DNA damage response kinases. *Cell Cycle*. 2009;8:1853–1859.

11. Stetka J, Gursky J, Liñan Velasquez J, et al. Role of DNA damage response in suppressing malignant progression of chronic myeloid leukemia and polycythemia vera: impact of different oncogenes. *Cancers (Basel)*. 2020;12:903.

12. Warfvinge R, Geironson L, Sommarin MNE, et al. Single-cell molecular analysis defines therapy response and immunophenotype of stem cell subpopulations in CML. *Blood*. 2017;129:2384–2394.

13. Adnan Awad S, Brück O, Shanmuganathan N, et al. Epigenetic modifier gene mutations in chronic myeloid leukemia (CML) at diagnosis are associated with risk of relapse upon treatment discontinuation. *Blood Cancer J*. 2022;12:69.