## Mutational analysis in serial marrow samples during azacitidine treatment in patients with post-transplant relapse of acute myeloid leukemia or myelodysplastic syndromes

Post-transplant relapse remains a major cause of treatment failure in patients with myeloid malignancies such as myelodysplastic syndromes (MDS) or acute myeloid leukemia (AML). Despite interventions such as withdrawal of immunosuppression, administration of chemotherapy, donor lymphocyte infusion (DLI), and second stem cell transplantation, survival after post-transplant relapse has remained dismal.[1] Several studies, generally retrospective in nature, have reported on the potential benefit of the post-transplant use of hypomethylating agents for the prevention or treatment of relapse.[2-4] We recently presented the results of a prospective phase II trial of azacitidine in patients with MDS or AML with persistent disease or early relapse after hematopoietic cell transplantation (HCT).[5] The trial enrolled 39 patients with MDS or AML, as defined by World Health Organization (WHO) criteria, who had undergone allogeneic HCT from related or unrelated donors following conditioning with various high intensity or reduced intensity regimens. All patients had evidence of persistent or recurrent disease by morphology, cytogenetics, or flow cytometry on marrow samples obtained between day 28 and day 100 post transplant. Treatment consisted of 5-azacitidine, 75 mg/m$^2$/day x 7 days given intravenously every 28 days, starting within two weeks of documentation of disease progression or relapse, until loss of response or documentation of further disease progression. The primary end point was 6-month overall survival (OS). At six months after relapse, 30% of patients had responded to azacitidine, with 3 achieving a complete remission. While these responses were encouraging, the study revealed progressive evolution of cytogenetic abnormalities throughout the course of the disease, from diagnosis to post-transplant relapse.[5] To better define the biology of relapse and the response to azacitidine treatment, we analyzed the mutational profile of paired bone marrow samples obtained at pre-HCT diagnosis, post-HCT relapse, and during subsequent azacitidine treatment. The goal was to identify mutational patterns associated with post-HCT relapse, response to azacitidine and post-relapse survival.

We determined the frequency and chronology of gene mutations using a targeted NGS 54 gene panel on serial bone marrow samples (*Online Supplementary Appendix*). To identify the mutations derived from recipient clones (relapsed disease), we compared the bone marrows at relapse to bone marrows obtained prior to HCT. To approach this analysis conservatively and restrict it to recipient-derived pathogenic mutations, we only considered the mutations that were present in both the bone marrow samples prior to HCT and the bone marrow samples at relapse. We then hypothesized that single nucleotide variants (SNVs) with high frequency of single nucleotide polymorphisms (SNPs) which were specific for the recipient, not for the donor, could determine relative allele frequency in recipient cells at the time of relapse. We first normalized variant allele frequencies (VAFs) by fraction of at least two SNPs consistently restricted to the recipient to compute relative VAFs (Figure 1A).

Samples from 21 patients were available for evaluation of mutation profiles in relation to response to treatment

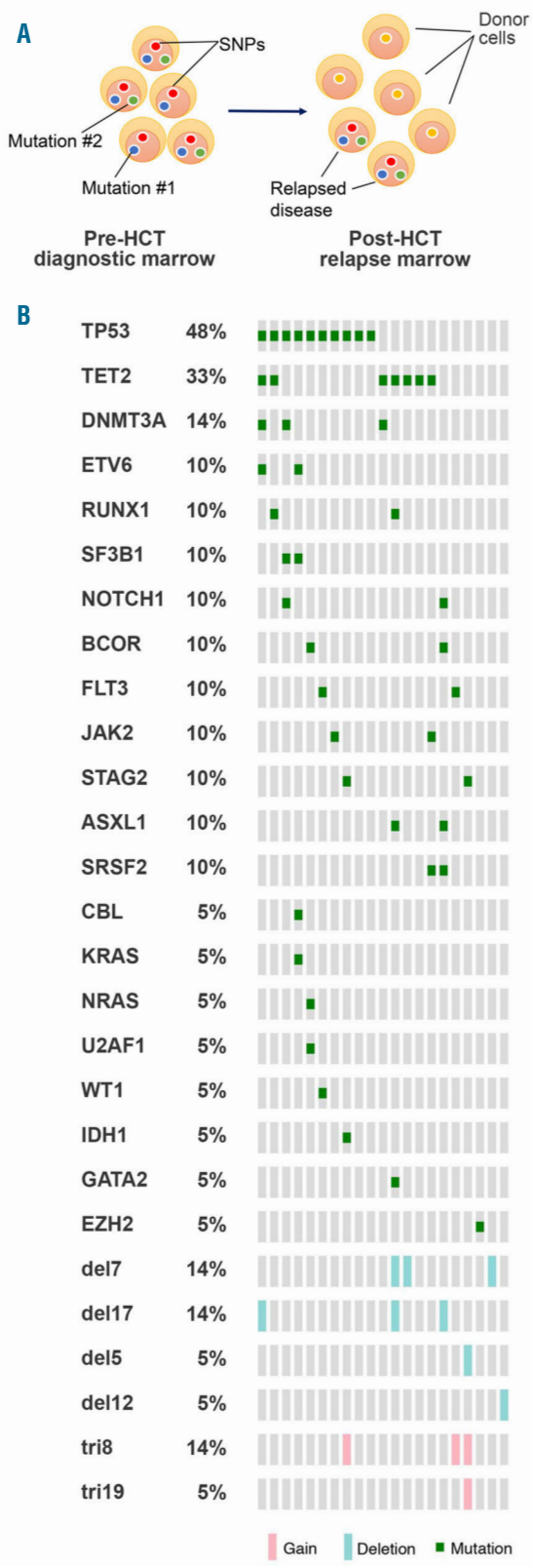


**Figure 1. Spectrum of mutations and common cytogenetic abnormalities in 21 patients with post-hematopoietic cell transplantation (post-HCT) relapse.** Only mutations that were present pre-HCT and persisted in post-HCT relapse were selected. (A) Schematic illustration of determining mutations in post-HCT relapse. (B) Mutation profile in 21 patients. Each column represents an individual patient sample, and each colored cell represents mutation of the gene listed to the left of that row.

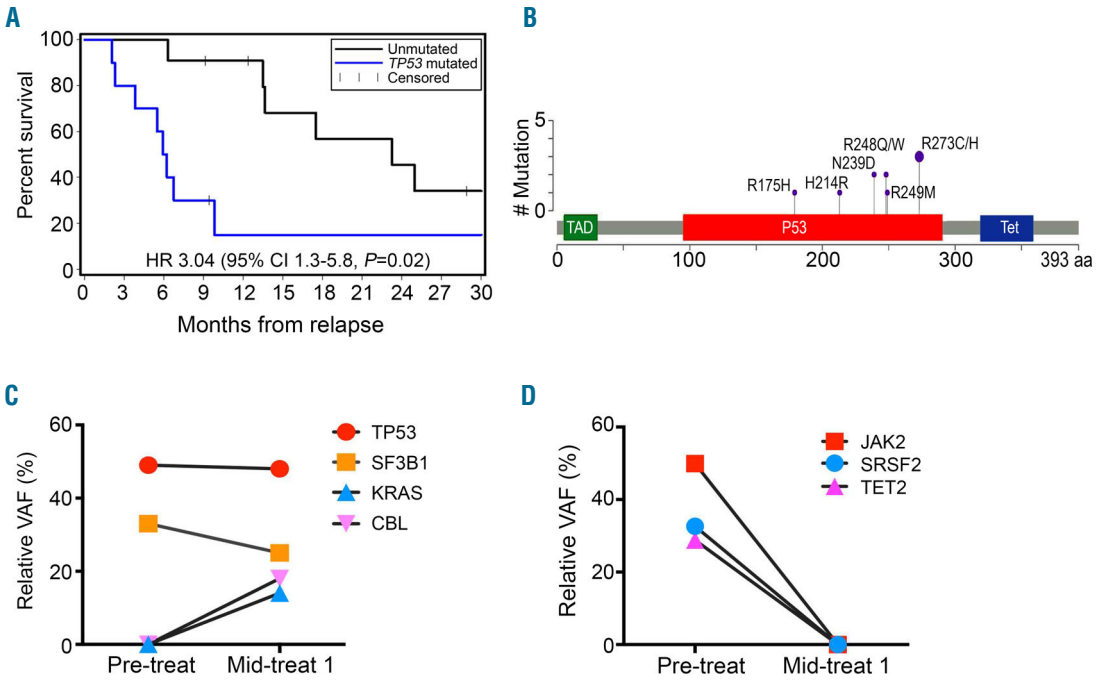


**Figure 2. Persistent *TP53* mutation in post-hematopoietic cell transplantation (post-HCT) relapse and unfavorable survival.** (A) Kaplan-Meier estimates of OS for patients with and without the *TP53* mutation, and multivariate analysis (hazard ratio, HR). Patients with persistent *TP53* mutation had unfavorable survival despite azacitidine treatment. (B) Mutations and frequency of each individual mutation in the *TP53* gene on a linear protein with its domains and frequency of the mutations on the y axis. Mutations were present in pre-HCT diagnostic marrow and persisted in post-HCT relapse. TAD: p53 transactivation motif; P53: p53 DNA-binding domain; Tet: p53 tetramerization motif. (C and D) Progressive changes in mutational burden during treatment with hypomethylating agents. Serial bone-marrow samples were analyzed while patients received azacitidine. Relative variant allele frequency was calculated within recipient cells by comparing known recipient specific single nucleotide variants present in pre-transplant samples and post-HCT relapse samples. Representative mutation profiles in individual patients during treatment. (C) Mutations in TP53 persisted and mutations in the diagnostic samples re-emerged later in non-responders. (D) Clonal mutations disappeared in responders.

and survival (Figure 1B). At post-HCT relapse, but prior to initiation of treatment with azacitidine, the average number of mutations in 21 patients was 2.2 (range 0-5). The number of mutations among responders (complete and partial responses) was slightly lower than the number of mutations among non-responders, but the difference was not statistically significant (1.8 *vs.* 2.9; P=0.09). The most commonly mutated genes at the time of relapse included *TP53* (48%), *TET2* (33%) and *DNMT3A* (14%). These mutations persisted through the course of HCT from pre-HCT to post-HCT relapse. Mutational profiles in relapsed disease after HCT corresponded to those found to be associated with unfavorable prognosis in previous studies, measuring mutations in pre-HCT samples.[6,7] These findings support the concept that clones containing certain mutations, e.g. in *TP53*, survive conditioning therapy and the allogeneic effect mediated by donor cells, and prevail through clonal selection and expansion during disease progression and relapse.

We next asked which mutation might predict response to azacitidine and affect survival (Table 1). Among the genes under consideration, mutations in *TP53* were significantly associated with poor responsiveness to azacitidine [Odds Ratio (OR) 3.08, 95%CI: 1.1-9.0; *P*=0.04] and inferior survival [Hazard Ratio (HR) 3.04, 95%CI: 1.3-5.8; *P*=0.02] (Figure 2A). Most mutations in *TP53* occurred in the DNA binding domain (Figure 2B). Conversely, mutations in *TET2* were associated with a trend toward favorable response to azacitidine (OR 0.27, 95%CI: 0.1-1.0; *P*=0.06) and superior survival (HR 0.2, 95%CI: 0-1.6; *P*=0.12) (*Online Supplementary Figure S1*). Mutations in *TET2* have also been associated with superior response to hypomethylating agents in the non-transplant setting,[8] while they predicted unfavorable outcomes in patients who underwent HCT.[9] Our data may suggest that azacitidine in the post-HCT relapse setting can greatly improve outcomes in patients with *TET2* mutated disease. However, the power of a *post hoc* subgroup analysis in the small cohort was limited.

To characterize clonal changes in individual patients during azacitidine therapy, we examined paired marrow samples from 7 patients who failed to respond to treatment and from 4 patients who responded (Figure 2C and D and *Online Supplementary Figures S2* and *S3*). We asked whether the burden of each mutated gene changed during the course of treatment, and if such changes correlated with clinical responses. Relative VAFs were determined by normalization with the fraction of recipient specific SNVs. Recipient-specific mutations were also present in the pre-HCT bone marrows, and VAFs of the mutations in relapse were equal or smaller than the frequency of recipient specific SNVs. Relative VAFs of the mutations in *TP53*, with one exception, remained unchanged over the course of treatment, suggesting that clones with *TP53* mutations were refractory to azacitidine, consistent with the unfavorable prognosis of patients with *TP53* mutations.[9,10] This mutation profile also implies molecular evolution at the clonal level that may account for the significantly inferior outcomes in patients with *TP53* mutation in relapse (Figure 2A). In patients who did not respond to azacitidine, most mutations persisted, and on occasions mutations that were not

detectable in relapse, but present in pre-HCT, reappeared later during treatment, mirroring clinical outcomes. Simultaneous abnormal cytogenetics, for example, del 7q, were present and could certainly contribute to the inferior response (Figure 2C and *Online Supplementary Figure S2*). Such a pattern would imply an underlying genetic mechanism of resistance to azacitidine *via* persistence of pre-existing resistant clones (such as clones with mutation in *TP53*). In contrast, bone marrow from patients whose disease responded to azacitidine exhibited complete loss of clones as identified by disappearance of mutations (Figure 2D and *Online Supplementary Figure S3*). One patient among responders did not have detectable mutations, but a cytogenetic abnormality, del (11q), disappeared, and this was consistent with the findings in mutation profiles. These data support the concept that mutational profiles mirror clinical responses to azacitidine, and that mutations in *TP53* are significantly associated with unfavorable outcomes, regardless of treatment.

In conclusion, these data from a prospective phase II trial show continuous clonal evolution of post-HCT relapsed MDS and AML during treatment with azacitidine. *TP53*, *TET2* and *DNMT3A* mutations present before transplantation, persisted after relapse. In particular, mutations in *TP53* were associated with inferior prognosis. In general, progressive changes in mutational burdens during treatment mirrored the pattern of clinical response. Clones with *TP53* mutations mostly remained unchanged throughout treatment. These findings provide a molecular basis for the clinical observations with azacitidine therapy in previous trials and in the present study, i.e. a lesser likelihood of eradication of the underlying malignancy containing those mutations and progressive clonal evolution during treatment. Further investigations of the clonal architecture in the context of therapeutic interventions in larger cohorts of patients are warranted to define functional mechanisms of individual mutations such as those in *TET2* and *DNMT3A*. These studies should lead to a greater understanding of disease biology and the mechanism of treatment responses, and should lead to novel treatment modalities.

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**Table 1.** Multivariable analysis of mutations and clinical outcomes: azacitidine response (Odds Ratio; OR) and survival (Hazard Rate).

| Mutation and azacitidine response (present *vs.* absent) | OR | 95% CI | *P* |
|---|---|---|---|
| TP53 | 3.08 | 1.1-9.0 | 0.04 |
| TET2 | 0.27 | 0.1-1.0 | 0.06 |
| Mutation and survival (present *vs.* absent) | HR | 95% CI | *P* |
| TP53 | 3.04 | 1.3-5.8 | 0.02 |
| TET2 | 0.20 | 0.0-1.6 | 0.12 |

HR: Hazard Ratio for mortality; OR: Odds Ratio for non-response.

## References

1. Mielcarek M, Storer BE, Flowers MED, Storb R, Sandmaier BM, Martin PJ. Outcomes among patients with recurrent high-risk hematologic malignancies after allogeneic hematopoietic cell transplantation. Biol Blood Marrow Transplant. 2007;13:1160-1168.
2. Craddock C, Labopin M, Robin M, et al. Clinical activity of azacitidine in patients who relapse after allogeneic stem cell transplantation for acute myeloid leukemia. Haematologica. 2016;101(7):879-883.
3. de Lima M, Giralt S, Thall PF, et al. Maintenance therapy with low-dose azacitidine after allogeneic hematopoietic stem cell transplantation for recurrent acute myelogenous leukemia or myelodysplastic syndrome: a dose and schedule finding study. Cancer. 2010; 116(23):5420-5431.
4. Schroeder T, Rautenberg C, Krüger WH, et al. Decitabine as salvage therapy for relapse of AML and MDS after allogeneic stem cell transplantation - a retrospective multicenter analysis on behalf of the German Cooperative Transplant Study Group (Abstract #3446). Blood. 2016;128(22):3446.
5. Woo J, Deeg HJ, Storer B, Yeung C, et al. Factors determining responses to azacitidine in patients with MDS and AML with early post-transplant relapse: a prospective trial. Biol Blood Marrow Transplant. 2017;23(1):176-179.
6. Cancer Genome Atlas Research N. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. New Engl J Med. 2013;368(22):2059-2074.
7. Papaemmanuil E, Gerstung M, Malcovati L, et al. Clinical and biological implications of driver mutations in myelodysplastic syndromes. Blood. 2013;122(22):3616-3627.
8. Bejar R, Lord A, Stevenson K, et al. TET2 mutations predict response to hypomethylating agents in myelodysplastic syndrome patients. Blood. 2014;124(17):2705-2712.
9. Bejar R, Stevenson KE, Caughey B, et al. Somatic mutations predict poor outcome in patients with myelodysplastic syndrome after hematopoietic stem-cell transplantation. J Clin Oncol. 2014; 32(25):2691-2698.
10. Wong TN, Ramsingh G, Young AL, et al. Role of TP53 mutations in the origin and evolution of therapy-related acute myeloid leukaemia. Nature. 2015;518(7540):552-555.