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Post-azacitidine clone size predicts long-term clinical outcome of patients with myelodysplastic syndromes and related myeloid neoplasms

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1 **ABSTRACT**

2 Azacitidine is a mainstay of therapy for high-risk MDS and other myeloid neoplasms. A significant
3 correlation between azacitidine response and clinical outcome suggests a potential role of mutational
4 profiling based on next-generation sequencing (NGS) before and after therapy in evaluating response
5 and predicting overall survival (OS), which however has not fully elucidated. Here through NGS-based
6 mutational profiling of large cohorts (n=451) of azacitidine-treated patients with high-risk MDS and
7 other myeloid neoplasms, we show significant roles of multi-hit *TP53* and germline *DDX41* mutations
8 in pre-treatment samples and post-treatment clone size in the evaluation/prediction of azacitidine
9 response and OS after azacitidine therapy, which outperformed the prediction based only on clinical
10 response and IPSS-R score. Post-treatment clone size strongly correlated with clinical response with
11 exception of large persistent mutations frequently affecting *TET2* after achieving complete remission
12 and those with *DDX41* mutations, which poorly correlated with clinical response. Our results highlight
13 the importance of evaluating mutations in both pre- and post-treatment samples in the assessment of
14 response and the prediction of OS after azacitidine therapy.

15

INTRODUCTION

Azacitidine, together with another hypomethylating agent (HMA), decitabine, is a standard choice of therapy for patients with higher-risk myelodysplastic syndromes (MDS) and other related myeloid neoplasms, including myelodysplastic/myeloproliferative neoplasms (MDS/MPN), and oligoblastic acute myeloid leukemia (AML). It can induce a clinical remission in 50-60% of treated patients^{1,2} and has been shown to prolong survival compared to standard regimens³ while others respond poorly or not at all and a major clinical question is who best benefits from azacitidine treatment. In fact, many studies have investigated biomarkers that can predict initial response and long-term survival following azacitidine therapy, particularly in terms of gene mutations⁴⁻²⁷. However, despite many efforts, no reliable biomarkers have been identified and results have been inconsistent across studies. Moreover, small numbers of enrolled patients and variable study designs with respect to patient enrollment criteria, definition of outcomes, genotyped genes, and explanatory factors tested do not allow for a direct comparison between or prevented a successful meta-analysis of these results²².

Another problem that complicates the identification of such biomarkers is the lack of reliable measures for the evaluation of the initial response to therapy. To date the IWG criteria have been widely used for the assessment of therapeutic response in MDS and related disorders, in which blast count and hematological improvement are evaluated. Although highly correlated with tumor clearance in AML, blast count may not correctly reflect tumor burden in MDS²⁸, apart from an inter-observer variance in blast cell quantification²⁹. In addition, blood cell counts could be influenced by the use of cytotoxic agents, particularly when they are assessed before BM fully recovers between treatment cycles³⁰. In this regard, next-generation sequencing (NGS) might provide a more robust platform to measure a molecular response or a reduction in tumor burden in terms of a variant allele frequency, or clone size, of somatic mutations detected before and after therapy. In fact, several studies evaluated a molecular response in patients who were treated with hypomethylating agents using NGS^{15,31-35}. However, the size of each study was relatively small to fully evaluate the impact of tumor burden on clinical response and long-term outcomes, although several studies have reported a correlation between a reduced tumor burden and clinical response, particularly in *TP53*-mutated AML and MDS^{14,31,32}.

Here, we investigated the effects of gene mutations on clinical outcomes in the largest cohort of azacitidine-treated patients with MDS, MDS/MPN, and oligoblastic AML (blast percentage $\leq$ 30% at enrollment) ever studied ($n = 341$), where genetic alterations were comprehensively analyzed by targeted-capture sequencing of major drivers for myeloid malignancies. The major results were validated using an independent cohort of azacitidine-treated patients ($n = 110$). In addition, mutational profiles before and after azacitidine treatment were assessed for 290 cases to explore their impact on clinical response and survival. Finally, the role of subsequent transplantation for otherwise intractable *TP53*-mutated cases was investigated in whom a sufficient reduction of *TP53*-mutant clone was obtained with azacitidine treatment.

RESULTS

Study subjects

The discovery cohort consisted of azacitidine-treated patients from the prospective JALSG MDS-212 study³⁶ ($n = 178$) and a retrospective cohort collected at Karolinska Institute and University Hospital ($n = 163$). The validation cohort included 110 azacitidine-treated patients who were retrospectively enrolled from 13 collaborating institutes in Japan (**Supplementary Fig. 1**). Demographic and clinical characteristics, including disease classification, IPSS-R risk, karyotype risk, blood count, marrow blast, of both discovery and validation cohorts are summarized in **Table 1**. The treatment schedule, OS, and other demographic data were largely similar between both cohorts except for an overrepresentation of oligoblastic AML (6.2% versus 13.6%) and underrepresentation of MDS/MPN (7.3% versus 3.6%) in the validation cohort (**Extended Data Fig. 1a, 1b, Table 1**). The response to azacitidine was evaluable in 311 and 85 patients in the discovery and the validation cohorts, respectively (**Table 1**).

Genetic abnormalities in the discovery and the validation cohorts

With a median depth of 565× (range, 81-2,501×) (**Supplementary Fig. 2**), targeted sequencing of the discovery cohort identified 1,069 and 730 single nucleotide variants (SNVs) and short indels in 326 pre- and 209 post-treatment samples, respectively. In sequencing-based copy number analysis, 770 and 352 genomic segments exhibited copy number abnormalities (CNAs) affecting in 207 pre- and 103 post-treatment samples, respectively (**Supplementary Tables 2, 3**). Reflecting an overrepresentation of higher risk patients with indication of azacitidine therapy, -7/del(7q) ($n = 87$), *TP53* mutation ($n = 87$), -5/del(5q) ($n = 80$), *RUNX1* mutation ($n = 73$), loss-of-heterozygosity (LOH) of 17p ($n = 72$), and *TET2* mutation ($n = 71$) were among the most frequent abnormalities (**Supplementary Fig. 3**). Based on our recent report that revealed distinct characterization and outcomes of MDS cases according to allelic state of *TP53* mutation³⁷, we analyzed cases with mono-allelic and multi-hit *TP53* mutation separately. Almost an equivalent coverage (a median depth of 624×; range, 348-1,105×) was obtained in the analysis of the validation cohort, in which we called a total of 290 SNVs or indels and 110 CNAs in 108 pre-treatment samples and 131 SNVs and 98 CNAs in 47 post-treatment samples (**Supplementary Tables 4, 5**). The mutation profile was largely comparable to that of the discovery cohorts, except for a substantial underrepresentation of *SRSF2* and RAS pathway mutations in the validation cohort, likely reflecting a lower representation of MDS/MPN cases in the latter cohort (**Supplementary Fig. 3**). Based on these findings, we first interrogated the best response to azacitidine therapy.

Correlations between genetic abnormalities and treatment response

In univariate analysis, 17p LOH (23/65), multi-hit *TP53* mutation (24/70), higher karyotype (KT) risk (31/112), and -5/del(5q) (21/71) were significantly associated with a higher complete remission (CR) rate, whereas lower hemoglobin levels (<80 g/L) (6/67) and *ASXL1* mutation (4/53) predicted a lower response (**Extended Data Fig. 2a**). These trends were also seen in the validation cohort, although not reaching statistical significance (**Extended Data Fig. 2b**). A lower response in *ASXL1*-mutated cases has also been reported in a previous study of azacitidine-treated patients with chronic myelomonocytic leukemia³⁵. In a multivariate analysis that included significant and sub-significant ($P < 0.10$) factors in the univariate analysis, only multi-hit *TP53* mutation and lower hemoglobin remained significant, although *ASXL1* mutation was still incorporated in the model with a marginal P -value (**Methods, Fig. 1a, Extended Data Fig. 2c**). We observed no significant difference in CR rate itself and other responses between mono-allelic versus multi-hit *TP53* mutation (2/8 versus 24/70 for CR, fisher's exact test, $P = 0.72$) (**Extended Data Fig. 2d**), although this needs further evaluations due to a small number of

patients with mono-allelic mutation ($n = 8$). In contrast to previous studies, none of the genetic abnormalities we evaluated significantly correlated with overall response (as defined by CR, marrow CR (mCR), or partial remission (PR))^{11,18} except for 17p LOH (**Fig. 1b, Extended Data Fig. 3a, 3b**). We also explored the factors predicting progressive disease (PD). Univariate analysis suggested several positive (higher karyotype risk, del(12p), lower absolute nucleated cells (ANC) and hemoglobin, -7/del(7q) and higher age) and negative (*STAG2* mutation) association with PD, of which higher age, higher karyotype risk, and *STAG2* mutation remained significant in multivariate analysis (**Extended Data Fig. 3c, 3d**).

Effects of gene mutations on OS following azacitidine treatment

We next evaluated the effect of genetic alterations on OS. In univariate analysis, multi-hit *TP53* mutation, -5/del(5q), -7/del(7q), 17p LOH, del(18p), 18q LOH, and del(12p) were associated with a shorter OS, together with older age (> 72 years old), higher marrow blast ratio (>10 %), higher KT risk, lower hemoglobin, and lower platelet count ($< 50 \times 10^9/L$). By contrast, mutations in *DDX41* and *SRSF2* predicted a favorable outcome (**Extended Data Fig. 4a**), which was largely recapitulated in the validation cohort (**Extended Data Fig. 4b**). In line with previous reports³⁸, responders showed a significantly longer OS compared with non-responders, with a median OS of 30.5, 22.3, 17.9, and 12.8 months in patients with CR, mCR/PR, SD and PD, respectively (two-sided log-rank test, $P < 0.0001$) (**Extended Data Fig. 4c**). The prolonged OS in responders was seen even among patients with multi-hit *TP53* mutations, which is notoriously associated with very poor outcome, although the prognosis was still dismal with a median survival of 17.0 months for CR cases (**Extended Data Fig. 4d**). Patients with mono-allelic *TP53* mutations had a significantly longer OS that was comparable to that of *TP53*-unmutated patients, compared to those with multi-hit mutations, confirming the effect of allelic state of *TP53* mutations on prognosis also in the context of azacitidine treatment³⁷ (two-sided log-rank test, $P = 0.034$) (**Fig. 1c**). Of note, multi-hit *TP53* mutation further stratified patients in the IPSS-R very high-risk category (two-sided log-rank test, $P = 0.02$) (**Extended Data Fig. 4e**). In accordance with previous reports for azacitidine and decitabine^{9,16,39-41}, the higher CR rate in *TP53*-mutated patients was not necessarily translated into favorable long-term outcomes. This is explained by a shorter progression free survival in *TP53*-mutated patients compared with that in *TP53*-unmutated patients (8.4 versus 36.3 months, two-sided log-rank test, $P < 0.0001$) (**Extended Data Fig. 4f**).

DDX41 is one of the most frequent targets of germline mutations responsible for the development of AML and MDS, accounting for 2.4-3.4 % of the cases⁴²⁻⁴⁴. Most *DDX41*-mutated cases have a pathogenic likely-germline variant, of whom 50-79% are accompanied by a somatic mutation, typically the R525H variant⁴²⁻⁴⁴. In total, we found 31 cases with *DDX41* mutations in the discovery cohort, including eight with germline mutation alone, 22 with both germline and somatic mutations, and one with somatic mutations alone (**Supplementary Table 6**). *DDX41*-mutated cases had a significantly longer OS compared with *DDX41*-unmutated cases irrespective of the mutation state (two-sided log-rank test, $P = 0.002$ for unmutated versus germline mutations; $P = 0.001$ for unmutated versus germline plus somatic mutations, **Extended Data Fig. 4g**) or IPSS-R risks (two-sided log-rank test, $P = 0.04$ for IPSS-R very low/low/intermediate, $P = 0.02$ for IPSS-R high, $P = 0.03$ for IPSS-R very high-risk group) (**Extended Data Fig. 4h**). Nevertheless, there was no difference in response profile between *DDX41*-mutated and unmutated cases (**Extended Data Fig. 4i**).

In multivariate analysis, best response (achieved overall response or not), higher age, lower hemoglobin level, lower platelet count, higher blast%, higher KT risk, multi-hit *TP53* mutations, and *DDX41* mutation were independently associated with OS (**Extended Data Fig. 5a, 5b**). Most of the significant variables overlapped with the factors incorporated into the IPSS-R. Indeed IPSS-R score significantly correlated with OS in our cohort (**Extended Data Fig. 5c**), as repeatedly shown in previous reports^{14,45,46}. Thus, we re-constructed a multivariate model by replacing these variables with IPSS-R score and obtained a final model consisting of four independent, significant variables, a multi-hit *TP53* lesion, *DDX41* mutation, best response, as well as IPSS-R (**Fig 1d**). Based on the model, we generated a new scoring system, by which patients were stratified into three risk groups, LOW, INT, and HIGH, with a median survival of 32 (confidential interval (CI): 25-43), 16 (CI: 15-18), and 10 (CI: 8-13) months, respectively (**Fig. 1e**). The model significantly improved compared with an IPSS-R alone model as shown by a higher c-index for the new scoring system (**Extended Data Fig. 5d**). In fact, each IPSS-R group was further stratified into two subgroups with different OS (**Extended Data Fig. 5e**). These results were confirmed in the validation cohort (**Extended Data Fig. 5f**).

Change in clone size during azacitidine therapy

Given that our sequencing panel encompassed most of the driver genes implicated in MDS, we were able to correctly measure the size of both founder clones and subclones before and after azacitidine in most, if not all, cases, which might be used to better evaluated therapeutic response on the basis of the post-treatment reduction of tumor burden. Post-treatment samples were obtained from 290 (64%) cases from the combined discovery and validation cohort after a median of four treatment cycles (**Extended Data Fig. 1c**). In total, we detected 980 driver mutations among samples from 268 cases, of which 851 and 783 were found in 260 pre- and 234 post-azacitidine samples, respectively, with 654 being shared in both samples. Mutations were classified into major clone (MC) mutations when they were contained in the major clone within each of pre- or post-azacitidine samples; otherwise, they were thought to represent non-MC (**Fig. 2a, Methods**). Thus, 448 of the 851 mutations represented MC in pre-treatment samples (MC^{pre}) (**Fig. 2a left panel**). Among these, 387 also represented the largest clone in post-azacitidine samples (MC) (**Fig. 2a right panel, Methods**). MCs were most frequently explained by *TP53* mutations ($n = 68$), followed by those in *TET2* ($n = 39$), *SRSF2* ($n = 36$), *RUNX1* ($n = 25$), and *DDX41* ($n = 19$). By contrast, on the mutated gene basis, *DDX41* (95%), *U2AF1* (82%), *TP53* (81%), and *DNMT3A* (71%) more frequently represented MC mutations, compared to *ASXL1* (31%), *RUNX1* (32%), and *STAG2* (33%) (**Fig. 2b**). Changes in clone size in pre- and post-treatment samples are shown in **Supplementary Fig. 4**.

We next correlated the response to azacitidine with the size of tumor component before and after azacitidine treatment that was estimated by the average size of MC mutations (aveMC^{pre} and aveMC^{post}, respectively) (**Fig. 2a**). We found that aveMC^{post} showed a strong correlation with response (Jonckheere-Terpstra test, $P = 0.0001$) (**Fig. 2c**), while an only modest correlation was noted between aveMC^{pre} and response (Jonckheere-Terpstra test, $P = 0.04$) (**Extended Data Fig. 6a**). Actually, in the 48 cases who achieved CR, the median value of aveMC^{post} was as small as 0.066 including 22 cases that achieved a complete disappearance of the MCs after treatment. Nevertheless, 7 cases showed large clones with >0.40 aveMC^{post} even after achieving CR, of which 4 had subclones that shrank after treatment (**Fig. 2c, cases #1 to #4, Fig. 2d**). MC mutations that persisted in these cases after achieving CR were represented by *TET2* (N=3) and *DNMT3A* (N=1) mutations, both of which are the most

frequently mutated genes in clonal hematopoiesis (CH)⁴⁷⁻⁴⁹. Thus, with a reduced blast count and normalization of blood counts, these cases may represent reversion to CH.

A total of 131 driver mutations were detected in either pre- or post-treatment samples in the 48 CR cases (**Fig. 2e**), in which a distinct pattern of clonal changes was observed according to driver mutations. As expected from a high CR rate of *TP53*-mutated cases, *TP53* was the most frequently mutated gene in CR cases, accounting for 27 mutations in 23 cases. *TP53*-mutated clones were characterized by high percentage to be classified as MCs (24 of 27, 88.9%) and marked reduction in clone size in post-treatment samples (**Fig. 2f**). By contrast, out of 20 *TET*-mutated clones in 11 CR cases, only 8 were classified as MC, where large *TET2*-mutant clones persisted after azacitidine treatment. Of interest, all *RUNX1*-mutated clones (n = 11) detected in 8 pre-treatment samples showed a reduced clone size after treatment (**Supplementary Fig. 5**). However, 8 of these 11 *RUNX1* mutations represented subclones most commonly of *TP53*-mutated MC and the apparent sensitivity of *RUNX1* mutants might be explained by co-occurring *TP53* mutations. Almost always present in a subclone, *NPM1* and *KRAS* mutations in MDS patients have been implicated in progression to secondary AML⁵⁰⁻⁵². In line with this, both mutations in our study disappeared when CR was achieved, which has also been reported in a recent study⁵³. Likewise, a reduce size of *EZH2*-mutated clones also correlated with a favorable clinical response (**Fig. 2f**).

Effect of *DDX41* mutations on azacitidine therapy

In accordance with a strong correlation between response and $\text{aveMC}^{\text{post}}$, patients who failed to achieve response had a large $\text{aveMC}^{\text{post}}$; the median values of $\text{aveMC}^{\text{post}}$ among 158 cases with SD+HI, SD-HI, and PD were 0.65, 0.80, and 0.82, respectively (**Fig. 2c**). Among these, however, 10 showed a small $\text{aveMC}^{\text{post}}$ (<0.10) (**Fig. 2c, cases #5 to #14**). Intriguingly, these cases were highly enriched for germline *DDX41* mutations, which accounted for 8 out of the 10 cases (Fisher's exact test, $P < 0.0001$) (**Fig. 3a**, PD cases; **Fig. 3b**, SD case; **Extended Data Fig. 6b**). Among these 8 cases, only 2 acquired driver mutations in the post-treatment samples, including a novel somatic *DDX41* mutation in both cases (**Extended Data Fig. 6b, cases #6, #9**), while the remaining 6 cases did not acquire novel driver mutations or show an expansion of pre-existing mutant clones. To explore possible mutations in other drivers that were not included in our target panel, we performed whole genome sequencing (WGS) in 4 of these 6 cases. Although a clonal population was suggested from the VAF distribution of somatic mutations, no known driver mutations were identified that should have conferred a survival advantage to these clones. Therefore, the poor response in these cases remains unexplained (**Extended Data Fig. 7, Supplementary Data Table 7**).

In the entire paired cohort, somatic *DDX41* mutations were observed in 22 cases, all of which accompanied a germline *DDX41* mutation. These cases with somatic *DDX41* mutations had distinct features. First, while somatic *DDX41* mutations represented the major clone in most (95%) cases (**Fig. 2b**), their clone size was significantly smaller than expected for a major clone in other cases having MC mutations other than *DDX41* mutations (**Fig. 3c**, *t*-test for clone size of mutations in *DDX41* and the other genes, $P < 0.0001$). This is intriguing, given that *DDX41*-mutated cases tended to have a higher BM blast count than *DDX41*-wild type cases (*t*-test, $P = 0.001$, **Fig. 3d**). In addition, the pre-treatment clone size of somatic *DDX41* mutations was poorly correlated with BM blast count (Pearson correlation, $r = -0.07$, $P = 0.7$, **Fig. 3e**). Two cases with germline *DDX41* mutations showed a rapid increase of blasts

(18% to 37%, 12% to 34%, **Fig. 3e**) before therapy initiation. Somatic *DDX41* mutations showed relatively small changes in clone size during treatment and their post-treatment size poorly correlated with clinical responses (Jonckheere-Terpstra test, $P = 0.2$) or OS (log-rank test, $P = 0.3$). This is in sharp contrast to *TP53* mutations, whose clonal size showed dynamic changes and whose post-treatment clone size significantly correlated with clinical response (Jonckheere-Terpstra test, $P = 2.1 \times 10^{-10}$) and OS (log-rank test, $P = 0.001$) (**Fig. 3f, 3g**).

High frequency of *PPM1D*-mutations in newly emerging clones

Out of the 783 driver mutations detected in post-treatment samples, 129 from 85 cases were not detected in pre-treatment samples and thus were considered newly emerging clones. They were most commonly explained by *RUNX1* mutations ($n = 11$), followed by *TET2* ($n = 9$), *CBL*, *DDX41*, *PPM1D*, *TP53* ($n = 8$), and other genes. On gene basis, mutations in *PPM1D* (47%), *CBL* (38%), and *DDX41* (29%) were most frequently detected as newly emerging clones (**Fig. 4a**). Although as a whole, newly emerging clones did not necessarily predicted PD (**Fig. 4b**), they still correlated with treatment response depending on the type of mutated gene and their clone sizes. For example, newly emerging clones with a large (>0.30) clone size predicted poor response (5 PD and 3 SD-HI) (**Fig. 4c**), while those with *PPM1D*, *TET2*, and *DDX41* mutations tended to have a favorable response with 88% (7/8), 75% (6/8), and 50% (3/6) of patients achieving CR/mCR/PR, respectively (**Fig. 4d**). *PPM1D*, a *TP53*-induced serine/threonine phosphatase, negatively regulates *TP53* pathway, whose mutations are preferentially detected in therapy-related myeloid neoplasms (t-MN)⁵⁴ as well as in clonal hematopoiesis in healthy individuals or those who are treated by chemotherapy. In the current cohort, *PPM1D* mutations were found in 9 cases, including 3 cases with t-MN, in newly emerging ($n = 8$) or expanding ($n = 1$) clones during azacitidine therapy (**Fig. 4e**). Of particular interest is a finding that *PPM1D*-mutated clones co-existed with *TP53*-mutated clones in 6 out of the 9 cases, all of which showed a favorable molecular response to azacitidine. Single cell-sequencing analysis confirmed that *TP53* and *PPM1D* mutations occur in distinct cells (**Fig. 4f**). In addition, many of these *PPM1D* mutations ($n = 7$) were confirmed in pre-treatment samples mostly in a small cell fraction (**Extended Data Table 1**). Thus, it might be speculated that in many of these patients, there was some common selective pressure that favored selection of both *TP53*- and *PPM1D*-mutated clones in a manner that initially led to the predominance of *TP53*-mutants and that an azacitidine-induced effacement of *TP53*-clones thereafter allowed for clonal expansion of pre-existing *PPM1D*-mutants.

OS model after azacitidine therapy

We next evaluated whether or not the multivariate model of OS constructed in Fig. 1 could be improved by including post-treatment tumor burden as expressed by the largest clone size in post-treatment sample ($\text{maxVAF}^{\text{post}}$). In multivariate analysis, $\text{maxVAF}^{\text{post}}$ was shown to be independent significant predictor of OS, together with the presence of multi-hit *TP53* alteration and *DDX41* mutation before therapy, best response (overall response or not), and IPSS-R score (**Fig. 5a**). Based on the multivariate model, we revised the scoring system by including $\text{maxVAF}^{\text{post}}$ to define three risk groups (**Fig. 5b**). The improvement of the model was illustrated by showing a significant increment of model fitness as each of gene mutations (multi-hit *TP53* alteration and *DDX41* mutations)(G), clinical response (R), and $\text{maxVAF}^{\text{post}}$ (P) were included in the IPSS-R alone model (I) in this order (**Fig. 5c**). We further tested the improvement of the mode by cross-validation, in which we split the entire cohort into 75% training and 25% validation subsets and for each split, c-index was calculated for the

validation set based on the model constructed for the training set (**Fig. 5d**). The mean c-index in 10,000 cross validations increased 0.025 by adding response data to IPSS-R score (I versus IR in **Fig. 5e**). Addition of gene mutation and maxVAF^{post} data further improved prediction of the model as seen by an increased c-index by 0.034 (IR versus IGR) and 0.010 (IGR versus IGRP), respectively.

Outcome of allogeneic stem cell transplantation (allo-SCT)

Among the paired cohort, 73 patients received allo-SCT a median of 5.2 (range, 1.7–22) months after the initiation of azacitidine therapy. The median post-transplant OS was 82.6 (range, 30–58.1) months (**Extended Data Fig. 8a**) after a median follow-up period of 49 months. Post-treatment clone size was evaluable for 53 cases and the optimal threshold of maxVAF^{post} to stratify post-transplant OS was 0.13, which significantly stratified OS after allo-SCT (log-rank test, $P = 0.046$) (**Extended Data Fig. 8b**). Next, we focused on *TP53*-mutated cases who are expected to have a dismal prognosis even with allo-SCT⁵⁵⁻⁵⁷. Among 13 cases with multi-hit *TP53* mutations who were transplanted, a median OS after the initiation of azacitidine therapy and allo-SCT were 24.3 and 19.2 months, respectively (**Extended Data Fig. 8c, 8d**). Six cases (**Fig. 5e**, cases 1-5, 13) were progression-free after a median follow-up of 23.7 (range, 7.3–25.0) months. Case 1 died 7 months after allo-SCT with no evidence of relapse. Notably, all 4 cases in which the mutant *TP53* clone size decreased to <0.10 maintained CR/mCR with a median follow-up of 23.7 (range, 7.3–24.9) months (**Fig. 5f**). This suggests that a significant reduction of *TP53*-mutant clone size with azacitidine therapy could improve clinical outcomes of cases with multi-hit *TP53* mutation when consolidated with allo-SCT.

DISCUSSION

NSG-based mutational profiling has successfully been applied to longitudinal disease monitoring to depict clonal dynamics in representative patients with myeloid neoplasms who were treated with a variety of drugs, including azacitidine^{32,34,37,58,59}. However, the role of NGS-based profiling in the evaluation of therapeutic response has not fully been investigated in a large cohort of patients uniformly evaluated with standardized response criteria. In the current study, enrolling a large number of patients who were treated with azacitidine and uniformly assessed using the IWG criteria, we have delineated the impact of NGS-based mutation profiling of both pre- and post-treatment samples on short-term response and long-term survival after azacitidine therapy.

Compared with the conventional assessment based only on blast count and hematological recovery using the IWG criteria, NGS-based mutation profiling before and after azacitidine therapy enables a more detailed assessment of molecular response and a better prediction of OS, where the mutation status of *TP53* and *DDX41* and post-treatment clone size play significant roles. The mutation status of *TP53* and *DDX41* in pre-treatment samples improves the prediction of OS compared to using IPSS-R alone. The mutation status of both genes also improves the post-treatment OS model based only on IPSS-R and clinical response and the prediction is further improved by including post-treatment clone size (maxVAF^{post}), even though the latter itself strongly correlates with clinical response, suggesting that a reduced tumor burden contributes to the efficacy of azacitidine. Of particular interest in this regard, a subset of patients who achieved CR showed a complete or almost complete clearance (<10%) of tumor cells, or ‘molecular CR’, which was also seen in patients with mCR, even though hematological recovery was incomplete. Molecular CR was previously reported in decitabine-treated

AML/MDS patients with *TP53* mutations³¹. In our study using azacitidine, it was most frequently, though much less so than in the decitabine study, seen in patients with multi-hit *TP53* mutations associated with CR/mCR responses (18/46, 39%), but was also seen in many *TP53*-unmutated patients carrying *RUNX1* (n = 7), *SRSF2* (n = 7), *TET2* (n = 5), and *DDX41* (n = 5) mutations in MC. Taken together, these results suggest an importance of the NGS-based assessment of post-treatment samples for evaluation of azacitidine therapy.

Despite an excellent correlation between post-treatment clone size and clinical response overall, discrepancy was seen between both in several cases. CR in most cases accompanied a substantial reduction of MCs. However, it can be achieved even with persistent large MCs having multiple mutations, typically including those affecting *TET2*, in a minority of cases. This confirmed a previous observation that multiple residual and/or newly emerging mutations are compatible with completely normal hematopoiesis, as reported in CR in AML⁶⁰ or clonal hematopoiesis in healthy individuals^{47,49}. By contrast, other cases showed PD even though a complete clearance of major clones was obtained. This was typically seen in cases with somatic *DDX41* mutations. The clearance (<10% clone size) of somatic *DDX41* mutations were found in 14 cases, of which seven achieved CR/mCR, whereas five remained SD-HI or even showed PD. However, in line with a favorable prognostic impact of *DDX41* mutations in our OS models, even these cases had a relatively longer OS. Finally, while representing the major clone in most cases and associated with elevated blast counts, somatic *DDX41*-mutants had a significantly smaller clone sizes than expected for a major clone having other driver mutations, which together with their unique response to azacitidine, is among unique features of *DDX41*-mutated myeloid neoplasms. In contrast to *DDX41* mutations, multi-hit *TP53* lesions predict a dismal prognosis even with allo-SCT. As previously demonstrated, the excellent initial response to HMA in many cases with multi-hit *TP53* lesions is not translated into an improvement of long-term outcomes, although those who achieved CR still had a significantly better OS than those who did not. Nevertheless, the excellent initial response, once obtained, might be exploited to improve long-term OS in combination with allo-SCT if performed in a timely manner. In fact, our preliminary result suggests a possibility that when performed for patients in whom the size of *TP53*-mutated clone was reduced to <0.10 before transplantation, allo-SCT could result in a long-term outcome, which needs a further evaluation.

Finally, it would be worth mentioning the emergence of newly evolving clones carrying *PPM1D* mutations. Although rarely found in pre-treatment samples in conventional NGS platforms (< 4%), they are frequently detected in post-treatment samples as multiple parallel mutations suggestive of strong positive selection. Of interest, *PPM1D* mutations, escaping mutation calls, were already present in a small fraction before therapy, indicating that such selection, if ever, would not be caused by azacitidine, but likely to be intrinsic to pre-treatment BM microenvironment. Of note in this regard, the majority (6/9) of newly emerging *PPM1D* mutations were found in patients with multi-hit *TP53* mutations with a substantially reduced clone size in response to azacitidine, in which *TP53* and *PPM1D* mutations are likely to be clonally independent. Because mapped on the same functional pathway, both genes are recurrently mutated in clonal hematopoiesis in association with a previous exposure to cytotoxic stress, we speculate that the similar mechanism operated in the early selection of both mutants, of *PPM1D*-mutants compatible with normal hematopoiesis replaced the *TP53*-mutant that are sensitive to azacitidine, through a bottle-neck effect.

AUTHOR CONTRIBUTIONS

Y.N., M.Tobiasson, Y.M., E.H.-L., and S.Ogawa. conceived and designed the study. M.Tobiasson, S.S., B.E., S.Ohtake, J.T., M.C., L.Z., M.K., Y.Shibata., N.N., Mizuki W., H.Hiramoto, K.U., Mitsumasa W., K.I., H.Handa, M.Taguchi, T.K., K.O., T.I., A.K.T., H.Tsurumi, S.K., S.C., T.N., S.M., P.E., Y.M. provided patient samples and clinical data. Y.N., M.Tobiasson, J.T., Y.Shiozawa, Y.Shiraishi, H.Tanaka, K.Y., H.M., M.N., S.M., E.P., Y.M., E.H.-L., and S.Ogawa. performed bioinformatics analysis and interpreted data. Y.N., M.Tobiasson, M.C., E.H.-L., and S.Ogawa. prepared the manuscript. All authors participated in discussions and interpretation of the data and results.

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COMPETING INTEREST DECLARATION

The authors declare the following competing interests. Y.N. has received consultancy fee from Otsuka Pharmaceutical. K.U. received research funding from Otsuka, Nippon-Shinyaku, and has served on speaker bureaus for Otsuka, Nippon-Shinyaku. K.I. received honoraria from Nippon Shinyaku. T.K. received grants and lecture fees from Nippon Shinyaku. K.O received honorarium from Nippon Shinyaku. Y.M. received honorarium from Nippon Shinyaku.

METHODS

Study subjects

The discovery cohort included patients enrolled from 1) Japan Adult Leukemia Study Group MDS 212 (JALSG-MDS-212) study³⁶ and 2) retrospectively collected consecutive patients treated with azacitidine at the Karolinska University Hospital. The validation cohort included retrospectively collected patients treated at Japanese institutes. The details of these cohorts are depicted in **Supplementary Data**. The discovery cohort and the validation cohort were analyzed separately for determining the impact of pre-treatment factors on response and survival, while a combined cohort of discovery and validation cohorts was used to explore the effect of clonal change post-treatment factors and transplantation. For details, see **Supplementary Fig. 1**. Patients in the discovery cohort were treated with 1 - 47 cycles of 75 mg/m² azacitidine for 5 to 7 consecutive days with a median of 6 in those who have completed the treatment protocol. At the time of the last follow-up, 19 and 17 patients were still on treatment in the discovery and the validation cohorts, respectively (**Extended Data Fig. 1a**).

Clinical data

Data collection

For JALSG-MDS212 cohort, clinical data were collected using an electric data capture system specifically designed for this study. For Karolinska cohort, we extracted clinical data from the institutional electric health record system at Karolinska Institute. For the validation cohort, we used a questionnaire to collect clinical data from collaborative institutions.

Baseline parameter

Baseline parameters included patient age at azacitidine administration, sex, history of chemotherapy or radiotherapy for other malignancies, disease subtype according to WHO 2016 classification⁶¹, risk category by IPSS-R (not applied to chronic myelomonocytic leukemia (CMML) cases with WBC 12.0×10^9 /L), karyotype (KT), absolute neutrophil counts (ANC), hemoglobin, platelet and bone marrow (BM) blast ratio. KT risk was evaluated according to IPSS-R-based KT risk classification⁶² and classified into higher (poor and very poor) and lower (the others) groups. Continuous variables from laboratory data were divided into lower and higher groups by clinically significant thresholds in reference to the criteria applied in IPSS-R⁶². Specifically, ANC $< 8.0 \times 10^9$ /L, hemoglobin level < 80 g/L, platelet counts $< 50 \times 10^9$ /L, were assumed lower and BM blast ratio $> 10\%$ as higher. Age was classified into higher and lower groups by the median value of 72-year-old in the entire cohort. We did not include marrow cellularity because bone marrow biopsy was not conducted in many Japanese samples. We also excluded transfusion dependencies from the analyses because transfusion policy is different between Japan and Sweden. IPSS-R was calculated using the laboratory data before azacitidine administration.

Response assessment.

Treatment response was assessed according to the International Working Group (IWG) response criteria 2006⁶³. The best response that was achieved from azacitidine treatment and the response at the time of post-treatment sampling were recorded. The former was assessed except for the analysis that explored the correlation between post-treatment clone size and clinical response. Patients without response information were not included in the section that explore the factors predicting

clinical response. Hematological improvement (HI) included achieving improvement in either of erythroid (HI-E), platelet (HI-P) or neutrophil (HI-N) lineage.

Targeted sequencing

Sampling of tumor cells

For JALSG-MDS212 cohort, bone marrow was collected just before start of azacitidine treatment and after 4 courses according to the study protocol unless patients were dropped out before course 4. Bone marrow samples were collected at LSI Medience Corporation, where DNA was extracted and stored. For Karolinska cohort and the validation cohort, bone marrow samples collected and analyzed before course 1 and around course 4 (**Extended Data Fig. 1c**). In this paper, we refer to the samples collected before azacitidine administration and around course 4 as 'pre-treatment' and 'post-treatment' samples, respectively.

Panel information for targeted-capture sequencing

We applied targeted-capture sequencing using either of 5 panels designed for genetic study of myeloid malignancies at Kyoto University ($n = 4$) or Memorial Sloan Kettering Cancer Center (MSKCC) ($n = 1$). 66 genes were commonly included in all the panels and were investigated in this study (**Supplementary Table 1**). All the panels also included 1,158 to 1,428 SNPs probes so that we can detect genome-wide copy number changes and allelic imbalances.

Sequencing method

97 of 163 pre-treatment samples from Karolinska Institute cohort were sequenced at MSKCC lab. For library construction, 11 to 800 ng of genomic DNA was used using the KAPA Hyper Prep Kit (Kapa Biosystems KK8504) with 7 to 12 cycles of PCR. After sample barcoding, 10 to 1,610 ng of each library were pooled and captured by hybridization. Captured pools were sequenced with paired-end Illumina HiSeq at 100 bp or 125 bp read length. A set of normal control was built by sequencing 48 blood samples from cases who did not have hematological disease. Fastq files were shared with Kyoto University Lab. All the other samples were sequenced at Kyoto University. 50 ng or 200 ng of genomic DNA were enriched for target regions by liquid-phase hybridization using the SureSelect custom kit (Agilent Technologies®), according to the manufacturer's protocol optimized for automated sample processing, as previously described⁶⁴. Purified library was subjected to high-throughput sequencing analysis with Illumina HiSeq 2500 or NovaSeq 6000 using 125 bp or 150 bp pair-end mode. We also sequenced 106 blood samples from the subjects who do not have hematological diseases and used as normal controls for exclusion of sequencing errors.

Mutation call

Sequencing reads were aligned to the human genome reference (hg19) using Burrows-Wheeler Aligner, version 0.7.8 with default parameter settings. All the bam files generated at MSKCC and Kyoto University were collected and analyzed together at Kyoto University. Quality of the sequencing data are summarized in **Supplementary Fig. 2**. Mutation calling was performed through our established pipeline (genomon pipeline 2.6.0, <https://github.com/Genomon-Project>), as previously reported⁶⁴⁻⁶⁷ using the following parameters.

Adopt variants fulfilling the following criteria:

- 459 (i) Mapping Quality score ≥ 20
- 460 (ii) Base Quality score ≥ 15
- 461 (iii) Number of total reads ≥ 100
- 462 (iv) Number of variant reads ≥ 4
- 463 (v) Variant allele frequency ≥ 0.02
- 464 (vi) Fisher's p-value for specific presentation compared with normal controls < 0.02

465

466 Following candidates were excluded:

- 467 (i) Synonymous and ambiguous (unknown) variants
- 468 (ii) Variants which were read only from one direction
- 469 (iii) Single nucleotide substitutions in which other mutations were called at the same position
- 470 and their variant allele frequency was ≥ 0.1 .

471 Mapping errors were removed by visual inspection on the Integrative Genomics Viewer (IGV) browser
 472 (<http://software.broadinstitute.org/software/igv/>). Structural variants were also called using
 473 genomon pipeline 2.6.0.

474 **Curation of oncogenic variants**

475 The significant variants that fulfill the quality filter noted above were further assessed for oncogenicity
 476 based on in-house curation program. The curation policy was determined individually for each gene
 477 based on previous reports and databases after exclusion of variants registered in public SNPs
 478 databases (the 1000 genomes project as of 2014 Aug, ESP6500, Human Genome Variation Database)
 479 and call errors using EB call⁶⁸ and in-house blacklist of error calls. All the pathogenic calls are listed in
 480 **Supplementary Table 2 and 3.**

481 **Copy number and allelic imbalance**

482 We included 1,158 to 1,428 SNPs probes to allow for detection of copy number changes and allelic
 483 imbalances. This technique, called CNACS is implemented in the program available at
 484 https://github.com/papaemmelab/toil_cnacs. Manual inspection of the result was conducted to
 485 discriminate call errors.

486 **CNAs and definition of arm level CNAs**

487 Total copy number (TCN) of 2.22 or larger was assumed CN-gains, and TCN < 1.88 was assumed CN-loss.
 488 Copy number neutral LOH was called when B-allele frequency was < 0.90 with TCN between 1.88 and
 489 2.22. Arm level CNA were called when the total length of affected region within the arm is > 1 M bp
 490 for 17p and > 3 M bp for the other arms. For gains in chromosome 8, CNA events in long and short arms
 491 are counted together. Partial tandem duplication in *KMT2A* (*KMT2A*-PTD) was detected using CNA data.
 492 Specifically, *KMT2A*-PTD was called when copy number gain (TCN > 2.5) was detected within the region
 493 between intron 1 and intron 19 of *KMT2A* (NM_005933) gene. All the CNAs are listed in **Supplementary**
 494 **Tables 4 and 5.**

495 **Calculation of clone size for each mutation**

496 The clone size of the relevant mutation was calculated from VAF values accounting for chromosomal
 497 ploidy considering sex, copy number changes, and allelic imbalances that span the mutated region.
 498 The details of adjustment calculation are described before⁵⁵.

Germline versus somatic origins of *DDX41* mutations

DDX41 represents one of the largest inheritable risk for myeloid malignancies and about 50 to 79 % of the MDS or AML patients with *DDX41* germline variants acquire somatic mutations in *DDX41* gene^{42,43}. The *DDX41* variants were assumed somatic when the variant allele frequency (VAF) value was < 0.34 for indels and < 0.39 for single nucleotide variants (SNVs). For the cases with CNAs in the *DDX41* region, CNA-adjusted VAF was calculated. All the others were judged likely germline. In the analyses of evaluating clone size changes after azacitidine administration, only somatic mutations were considered. The *DDX41* variants were listed with VAF values in **Supplementary Table 6**.

Curation of mutations in *TP53*

We consulted IARC *TP53* database (<http://p53.iarc.fr/>) for pathogenicity⁶⁹. Specifically, we checked the transcriptional activity (TA) for each SNVs in the 'Gene variation' database, which summarizes the change of TA on 8 different promoters by introducing artificially constructed vectors harboring all possible amino acid substitutions ($n = 2,314$) into yeast⁷⁰. Interestingly, we found that none of the non-damaging mutations in this database were associated with either loss of heterozygosity (LOH) in 17p or complex karyotype unless they possessed other pathogenic *TP53* mutations. Finally, we judged the alterations is the following list as non-pathogenic: E11Q, V31I, A76G, A129P, A189V, N235S, N310K, G360V. We assumed all the mutations resulting in gene truncation and those in the splicing regions as pathogenic. In addition, we discriminated multi-hit cases from mono-allelic case based on the presumed number of alleles affected. Briefly, multi-hit mutation is judged when a patient has either of 1) two of more *TP53* mutations, 2) one *TP53* mutation accompanied by either of copy number neutral LOH or deletion of 17p. The significance of discriminating these two allelic statuses of *TP53* mutation is described in our recent paper³⁷.

Definition of major clones (MC)

In the analysis of the clonal changes, *FLT3*-ITD and *KMT2A*-PTD were removed from the mutation list because the mutated clone size cannot be adequately assessed with targeted-sequencing data for these alterations. The germline *DDX41* mutations were also removed. All the mutations were classified into MC or non-MC. First, mutated clone size was assessed for all the mutations detected in pre-treatment samples, and the largest clone group in pre-treatment samples (MC^{pre}) was defined to include the largest and near largest clones (with the difference in clone size from the largest one being < 0.10. This threshold corresponds to difference of 0.05 in VAF sizes for autosomes without CNAs). Next, mutated clone size in post-treatment samples was assessed for all MC^{pre} and the subset of MC^{pre} showing the largest and near largest size after treatment were defined as MC. Again, the difference in clone size of near largest clones from the largest one was set < 0.10. Specifically, we assigned 448 MC^{pre} and 387 MC among 851 mutated clones detected in pre-treatment samples. A set of MCs in a case was defined as a 'MC group' and its size was defined to be the average value of the clones included in the MC group (**Fig. 2a**). Note that post-treatment size of MC group can be 0 when all MC^{pre} in a case disappeared after treatment, and actually this was achieved in 44 cases.

Single-cell sequencing library preparation and genotyping

Patient samples were thawed and washed with PBS supplemented with EDTA and 5% FCS (FACS buffer). Cells were stained with APC-Cy7-conjugated CD45 for 15 min at 4 °C. Cells were then washed and resuspended in FACS buffer with DAPI and sorted to isolate viable blood cells (DAPI-, CD45+) using

FACSAria III Cell Sorter (Beckton & Dickinson). Single cells were encapsulated using a Tapestry microfluidics cartridge, lysed, and barcoded. Barcoded samples were then subjected to targeted PCR amplification of a custom 312 amplicons covering 45 genes implicated in myeloid malignancies included in Tapestry® Single-Cell DNA Myeloid Panel. PCR products were merged, purified with Ampure XP beads (Beckman Coulter), and used as a template for PCR to incorporate Illumina i5/i7 indices. PCR products were quantified using TapeStation 4400 (Agilent Technology) and subjected to sequencing on an Illumina NovaSeq 6000 (Illumina). FASTQ files were analyzed through the Tapestry pipeline to generate a loom file. Filtering low-quality genotypes or cells and defining clones were performed with Tapestry Insights following manufacturer's instruction.

Whole-genome sequencing

50 ng DNA were subjected to library preparation using KAPA Hyper Prep Kit followed by sequencing using DNBSEQ-G400 (MGI Tech) with target depth of 100× in 150-bp paired-end mode. The target depth was 100×, and the actual depth was 133× (60–293×). The mean depth in germline controls from 122 individuals was 140× (55–204×). Mutation calling was performed using the Genomon2 pipeline (v.2.6), as previously described⁷¹. Somatic mutations were detected by eliminating polymorphisms and sequencing errors. To achieve this, we first applied default Genomon 2.6.0 settings and further removed low-quality, unreliable reads and variants according to the following criteria: (i) a sufficient number of reads (total reads > 30 and variant reads > 3), (ii) variant alleles are observed at a significantly higher frequency than expected by error (number of supporting reads in controls < 5, Fisher *P*-value <10⁻¹), (iii) significant variants on the basis of an empirical distribution of VAFs as determined using WGS data of non-paired peripheral blood samples (EBCall algorithm, *n* = 16)⁶⁸, (iv) not included in common SNPs (frequency in 1000 genome project 2014 Oct all database < 0.001, <https://www.internationalgenome.org/>). Only single nucleotide variants are assumed.

Statistical analysis

All statistical calculations were performed using R version 4.0.3. Differences in distribution of categorical and continuous variables were assessed with Fisher's exact test and Student's t-test, respectively. All the explanatory variables were dichotomized if not specified using the thresholds presented above. Time was assessed from the start of azacitidine treatment to the events of interest unless otherwise specified. Time-to-event data were analyzed using the Kaplan–Meier method and assessed for difference among groups by log-rank test using survival_3.2-7 package. For the analyses to explore the factors associated with clinical response, Fisher's exact test and logistic regression model was applied for univariate and multivariate analysis, respectively. For the analyses to explore the factors associated with survival, Cox proportional hazard model was applied for both univariate and multivariate model. Significant and sub-significant (*P* < 0.10) factors in univariate analyses with incidence of > 10% in the target cohort were subjected to a multivariate analysis followed by parameter selection with Akaike's information criteria (AIC) using step function in R base functions. Multiple collinearity among explicative variables was assessed before parameter selection by calculating generalized variance-inflation factor (gVIF) using vif function of car_3.0-10 package for the multivariate model. If the largest gVIF is > 3, the explicative variable showing the second largest gVIF value was removed and reconstructed the multivariate model with the residual variables. Stratified OS model was constructed using the covariates that were significant in multivariate analysis. The weight of each covariate was determined based on the coefficients of Cox proportional hazard model

(log(hazard ratio)) with a scaling factor and large rounding. The cut off values for risk stratification were determined on the basis of grid search for the two thresholds that most significantly stratified the discovery cohort into three groups in terms of OS. Differences in likelihood between two regression models were compared by anova. Concordance index (c-index) of survival data was calculated using estC function of compareC_1.3.1 package. The optimal cut point of $\max(VAF^{post})$ was determined by surv_cutpoint function of survminer_0.4.8 package. In addition, R packages ggplot2_3.3.2, forestmodel_0.6.2, ggpubr_0.4.0, ggsci_2.9, patchwork_1.0.1, RColorBrewer_1.1-2 packages were used for graphical presentation.

Ethical issue

This study was approved by the institutional ethical committee at Kyoto University (G0608), Karolinska Institute (Dnr 2017/1090—31/4) and all the participating institutes and hospitals. All patients provided fully informed consent.

Data accessibility

Tables containing clinical data, results of univariate analysis for response or OS, clone size in pre-and post-treatment samples are available on Github at <https://github.com/ynanya/MDS-postAZA-clone>.

Code reproducibility

All computational code is available at <https://github.com/ynanya/MDS-postAZA-clone>.

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Table 1. Characteristics of the study cohort

	<i>Discovery cohort</i>		<i>Validaiton cohort</i>		<i>P-values</i>
No. of patients	341		110		
Age at enrollement (median [range]) – yrs	72	(19–91)	69	(16–84)	0.0011 *
Gender – no. (%)					0.10 †
Male	224	(65.7)	82	(74.5)	
Female	117	(34.3)	28	(25.5)	
WHO–classification at enrollment –no. (%)					0.024 †‡
MDS	295	(86.5)	91	(82.7)	
isolated del(5q)	3	(0.9)	2	(1.8)	
MDS-SLD	1	(0.3)	1	(0.9)	
MDS-MLD	19	(5.6)	18	(16.4)	
MDS-RS	1	(0.3)	3	(2.7)	
MDS-U	4	(1.2)	0	(0.0)	
MDS-EB1	123	(36.1)	26	(23.6)	
MDS-EB2	144	(42.2)	41	(37.3)	
AML	21	(6.2)	15	(13.6)	
MDS/MPN	25	(7.3)	4	(3.6)	
Atypical CML, <i>BCR-ABL1</i> negative	0	(0.0)	1	(0.9)	
CMML	21	(6.2)	2	(1.8)	
MDS/MPN-U	4	(1.2)	1	(0.9)	
IPSS-R - no. (%)§					0.14 †
Very Low	1	(0.3)	3	(2.8)	
Low	11	(3.4)	6	(5.7)	
Intermediate	73	(22.8)	19	(17.9)	
High	96	(30.0)	30	(28.3)	
Very High	139	(43.4)	48	(45.3)	
Karyotype risks (IPSS-R–based) – no. (%)					0.021 †
Very Good	3	(0.9)	5	(4.4)	
Good	148	(43.5)	36	(33.6)	
Intermediate	63	(18.5)	16	(15.0)	
Poor	38	(11.2)	16	(14.2)	
Very Poor	88	(25.9)	37	(32.7)	
Peripheral Blood					
WBC (median (range)) x10 ⁹ /L	2.8	(0.3–107.9)	2.4	(0.4–57.8)	0.044 *
ANC (median (range)) x10 ⁹ /L	1.0	(0–38.2)	1.0	(0.05–31.8)	0.39 *
HB (median (range)) g/dL	9.2	(4.1–15)	8.2	(4.2–14.9)	0.0014 *
PLT (median (range)) x10 ⁹ /L	69.0	(5–1237)	73.5	(4–709)	0.93 *
Bone Marrow					
Blast (median [range]) - %	10	(0–37.0)	9.0	(0–30.0)	0.33 *
Post aza samples - available no. (%)	237	(69.5)	53	(48.18)	0.00017 †
RBC transfusion dependent – no. (%)	166	(48.7)	47	(44.3)	0.44 †
PLT transfusion dependent – no. (%)	29	(8.5)	19	(17.6)	0.012 †
Response (best response)					0.0005 †
CR (%)	59	(19.0)	13	(14.9)	
mCR (%)	69	(22.2)	6	(6.9)	
PR (%)	2	(0.6)	19	(21.8)	
SD (%)	110	(35.4)	33	(37.9)	
PD (%)	71	(22.8)	16	(18.4)	

* Student's t-test was applied to compare continuous values. †Fisher's exact test was applied to compare categorical variables. ‡

Distribution among major categories are assessed. § IPSS-R was not calculated for CMML cases with WBC $\geq 12 \times 10^9/L$. Abbreviations: MDS-SLD, MDS with single lineage dysplasia; MDS-MLD, MDS with multilineage dysplasia; MDS-RS, refractory anemia with ring sideroblasts; MDS-U, MDS unclassifiable; MDS-EB1/2, MDS with excess blasts 1/2; MDS, myelodysplastic syndromes; AML, acute myeloid leukemia; MDS/MPN, Myelodysplastic/myeloproliferative neoplasms; CML, chronic myeloid leukemia; CMML, chronic myelomonocytic leukemia; MDS/MPN-U, Myelodysplastic/myeloproliferative neoplasms unclassifiable; IPSS-R, International Prognostic Scoring System Revised; WBC, white blood cell; ANC, absolute neutrophil count; HB, hemoglobine; PLT, platelet; CR, complete remission; mCR, marrow CR; PR, partial remission; SD, stable disease; PD, progressive disease.

Figure 1

Figure 1 | Genetic and clinical factors associated with response and overall survival.

a, A forest plot showing the result of a multivariate logistic regression analysis for the factors associated with achieving CR performed on the discovery cohort with complete data for response analysis ($n = 311$). Explanatory variables are hemoglobin level, mutations in *TP53* and *ASXL1*. Hemoglobin level was dichotomized with a threshold of 80 g/L; the x-axis is log10 scaled.

b, A bar plot showing the number of cases with the response indicated by color that have the genetic alterations indicated on the x-axis.

c, Kaplan-Meier estimates of overall survival per *TP53* allelic state. The number of the cases at risk at each time is indicated in the tables below. P values are derived from two-sided log-rank tests.

d, A forest plot showing the result of Cox proportional hazards regression for overall survival (OS) on the discovery cohort with complete data for OS analysis ($n = 341$). Explanatory variables are IPSS-R raw score, multi-hit *TP53* mutation, *DDX41* mutation, and clinical response (achievement of overall response (OR) or not). The x-axis is log10 scaled. Weight indicates coefficient to construct a novel risk-stratification model based on this multivariate model. Specifically, total score of the novel scoring model is calculated as $1 \times (\text{IPSS-R raw score}) + 2 \times (\text{Multi-hit } TP53 \text{ mutation}) - 4 \times (\text{DDX41 mutation}) + 3 \times (\text{no achievement of OR})$.

e, All the cases in the discovery cohort were stratified into three risk classes (LOW: total score ~7/INT: total score 7.5~11.5/HIGH: total score 12~) based on the total score calculated in **Fig. 1d**. The number of the cases at risk at each time is indicated in the tables below. P values are derived from two-sided log-rank tests.

CR, complete remission; mCR, marrow CR; PR, partial remission; SD, stable disease; HI, hematological improvement; PD, progressive disease; HB, hemoglobin; PLT, platelet; Del, deletion.

Figure 2

Figure 2 | Clone changes during azacitidine treatment and genetic mechanisms of CR.

a, A schematic explanation of major clones (MC) determined accounting for the clone size in pre- (x-axis) and post-treatment (y-axis) samples from a patient. A set of mutations showing the largest and near largest size (difference in size is < 0.10 compared with the largest mutation) in a pre-treatment sample were defined as MC^{pre} (left panel). Among the MC^{pre} , a set of mutations showing the largest and near largest size in a post-treatment sample were assumed MC (right panel). The detailed description of definition appears in **Methods**. **b**, Bar plots showing the numbers of major clones (MC) (left axis) represented by the genes indicated on x-axis. Filled circles indicate the proportion of the variants (right axis) classified as MC out of all the mutations in the paired cohort. **c**, Box plots showing the MC group sizes of post-treatment samples in the paired cohort having the response indicated on x-axis. $P = 0.0001$, Jonckheere-Terpstra tests. **d**, Fish plots showing the estimated transition of the clone structure during pre- and post-treatment periods for the 4 CR cases that simultaneously had large MC group sizes (> 0.40) and shrinking subclones. Sample IDs correspond to those indicated in **Fig. 2c**. **e**, A bar plot showing the number of clones represented by the genes indicated on x-axis. Only clones seen in CR cases are shown. Colors indicate whether each clone was classified as MC or not. **f**, Paired box plots showing the changes in clone size between pre- and post-treatment samples with mutations in indicated genes. The same mutations are connected by lines. Colors of lines represent whether they are MC or not.

Figure 3

Figure 3 | Somatic DDX41-mutated clone size does not correlate with blast ratio or response to treatment.

a, Percentage of the cases that have *DDX41* germline variants is shown among the cases with stable diseases (SD) or progressive disease (PD) having major clone (MC) group sizes < 0.10 ($n = 10$) and ≥ 0.10 ($n = 148$). $P = 1.22 \times 10^{-7}$, Fisher's exact test. **b**, Fish plots showing estimated transition of the clone structure throughout the pre- and post-treatment periods for the 4 PD cases that had post-treatment MC group sizes < 0.10 . Sample IDs corresponds to those indicated in **Fig. 2c**. **c**, Box plots showing the size of major clone (MC) groups in pre-treatment samples that have mutations in the genes indicated on x-axis. A box plot representing *DDX41*-mutated MC groups is highlighted with red color. P value is derived from a two-sided t-test for comparison of pre-treatment MC sizes represented by *DDX41* and the other genes. **d**, Box plots showing marrow blast percentage for the cases with or without *DDX41* germline mutations. P value is derived from a two-sided t-test. **e**, A scatter plot showing marrow blast percentage (x-axis) and size of clones represented by somatic *DDX41* mutations (y-axis). P value is derived from a Pearson's correlation test and r value indicates Pearson's product moment correlation coefficient. **f**, Paired box plots showing the changes in clone size between pre- and post-treatment samples with mutations in *DDX41* (upper panel) and *TP53* (lower panel). The same mutations are connected by lines. Colors of lines represent whether they are MC or not. P values are derived from Jonckheere-Terpstra tests under the hypothesis that there is no correlation between clinical response and post-treatment clone sizes. **g**, Kaplan-Meier estimates of overall survival (OS) per clone sizes in post-treatment samples. Cases with somatic *DDX41* mutations (left, $n = 28$) and *TP53* mutations (right, $n = 68$) are shown. The clone sizes were divided by the median value for each gene. The number of the cases at risk at each time is indicated in the table below. P values are derived from two-sided log-rank tests.

Figure 4

Figure 4 | Property and role of newly emerging clones (NECs) after azacitidine treatment.

a, Bar plots showing the numbers of newly emerging clones (NEC) (left axis) represented by the genes indicated on x-axis. Filled circles indicate the proportion of the mutations (right axis) classified as NEC out of all the mutations in the paired cohort. **b**, Bar graphs showing the proportion of the cases with or without NEC showing responses indicated by color. P value is derived from two-sided Fisher's exact test to estimate the frequency of the cases having progressive disease (PD). **c**, Jitter plots showing the post-treatment clone size of NECs represented by the genes indicated on x-axis. Colors indicate clinical response. **d**, Bar graphs showing the proportion of the cases with NEC represented by the genes indicated on x-axis having the response indicated by colors. **e**, Clone size transition in pre- and post-treatment samples of *TP53* (black) and *PPM1D* (red) in the 9 cases having mutations in *PPM1D* classified as DEC. Bold lines indicate variant classified as DEC. **f**, Single cell analysis of the case #PPM1D-1 showing variant allele frequency of mutations in *TP53* and *PPM1D* in 5,080 cells. Shaded colors indicate the variant allele frequencies of the variants.

Figure 5

Figure 5 | The role of post-treatment clone size on improvement of OS model and predicting post-transplant outcomes.

a, A forest plot showing the result of Cox proportional hazards regression for overall survival (OS) performed on the paired cohort with complete data for OS analysis ($n = 267$). N indicates the number of cases having the variables. Explicative variables are International Prognostic Scoring System Revised (IPSS-R) raw score, pre-treatment mutations (multi-hit *TP53* mutations, *DDX41* mutations), the largest variant allele frequency (VAF) values adjusted for copy number alterations in post-treatment samples ($\text{MaxVAF}^{\text{post}}$), and overall response according to the International Working Group (IWG) 2006 criteria. Overall response includes complete remission (CR), marrow CR (mCR) and partial remission (PR); the x-axis is log10 scaled. Weight indicates coefficient to construct a novel risk-stratification model based on this multivariate model. Specifically, total score of the novel model is calculated as $1 \times (\text{IPSS-R raw score}) + 2 \times (\text{Multi-hit } TP53 \text{ mutation}) - 3 \times (\text{DDX41 mutation}) + 3 \times (\text{MaxVAF}^{\text{post}} \text{ value}) + 1 \times (\text{no achievement of OR})$. **b**, All the cases were stratified into three risk classes (LOW: total score ~ 7 /INT: total score $7 \sim 11$ /HIGH: total score $11 \sim$) based on the total score calculated in **Fig. 5a**. Kaplan-Meier estimates of overall survival per risk classes of the proposed model is presented. The number of the cases at risk at each time is indicated in the tables below. *P* values are derived from two-sided log-rank tests. **c**, Bar graphs showing log-converted likelihood of Cox proportional hazards regression for overall survival (OS) models including the explicative variables symbolized by the four capital letters. Each of the symbols represents I, IPSS-R raw score; G, pre-treatment gene mutations in which multi-hit *TP53* and *DDX41* mutations are included; R, overall response; P, the largest variant allele frequency (VAF) values adjusted for copy number alterations in post-treatment samples. **d**, A schematic presentation of the analysis that examines the improvement of predictability. The paired cohort was randomly split into 75% training and 25% validation subsets 10,000 times and constructed a model with each of the training set and fit the model and calculated the concordance index (c-index) for the corresponding validation set. **e**, Box plots indicating the distribution of c-index in the validation cohorts of 10,000 simulations. The symbols that represent models are the same as in **Fig. 5c**. **f**, A swimmer's plot showing the clinical course for the 16 transplanted cases with *TP53* mutations. Red and black asterisks indicate *TP53* allelic state. Colors of horizontal bars indicate the period having the clinical state. Vertical bars show the clone size of *TP53* variants in pre- and post-treatment samples. CR, complete remission; mCR, marrow CR; PR, partial remission; SD, stable disease; HI, hematological improvement; PD, progressive disease.

Extended Data Figure 1

Extended Data Figure 1 | Azacitidine treatment profile, overall survival and time to response.

a, Histograms showing the number of all azacitidine treatment courses for the discovery (upper panel, $n = 336$) and the validation (lower panel, $n = 101$) cohorts with complete data for analysis. Colors indicate whether the cases are still on azacitidine treatment or not. **b**, Kaplan-Meier estimates of overall survival (OS) on 341 cases in the discovery cohort and 110 cases in the validation cohort. The numbers of the cases at risk at each time are indicated in the tables below. P value is derived from a two-sided log-rank test. **c**, Histograms showing the number of azacitidine treatment courses when post-treatment samples were collected for the discovery (upper panel, $n = 265$) and the validation (lower panel, $n = 68$) cohorts. The sampling time was fixed as after course 4 for JALSG-MDS-212 cohorts, while it was not fixed in the other cohorts.

Extended Data Figure 2

Extended Data Figure 2 | Factors associated with achieving complete remission (CR).

a, A volcano plot showing the result of univariate analyses for the factors associated with achieving CR. X- and y-axis show odds ratio and *P*-values derived from two-sided Fisher's exact tests. The x- and y-axis are log2 and log10 scaled, respectively. Area of circles indicates the number of samples having the features indicated by the covariates and color of circles indicates the attribute of the covariates. Dotted lines indicate significant ($P = 0.05$) and sub-significant ($P = 0.10$) levels. Hemoglobin level (HB) was dichotomized with a threshold of 80 g/L and karyotype (KT) risks were classified into higher (poor and very poor, according to IPSS-R-based karyotype risk classification) and lower (intermediate, good and very good) groups. **b**, A forest plot showing the result of univariate analyses for the factors associated with achieving CR for the validation cohort with complete data for response analysis ($n = 90$). Explanatory variables are limited to those showing significant ($P < 0.05$) or sub-significant ($P < 0.10$) association with achieving CR in univariate analyses for the discovery cohort (**Extended Data Fig. 2a**); x-axis is log10 scaled. **c**, Bar plots showing generalized variance inflation factor (gVIF) of the covariates indicated on x-axis in the multivariate logistic regression model for achieving CR. Left and right panels show gVIF profiles before and after correction for multiple collinearity, respectively (**Methods**). The variables after the correction (right panel) were subjected to multivariate analysis followed by AIC-based covariate selection. **d**, Bar plots showing the proportion of cases having the response indicated by color with the *TP53* allelic status indicated on the x-axis.

Extended Data Figure 3

a

b

c

d

Variable	Odds ratio for PD (95% CI)	P-value
Higher age	1.73 (1.00, 3.02)	0.05
Lower ANC	1.54 (0.88, 2.67)	0.13
Higher KT risk	1.80 (1.02, 3.16)	0.04
STAG2	0.28 (0.07, 0.85)	0.05

Extended Data Figure 3 | Factors associated with achieving overall response (OR) and having progressive disease (PD).

a, Bar plots showing the proportion of cases having the response indicated by color with the genetic alterations indicated on the x-axis. **b,c**, A volcano plot showing the result of univariate analyses for the factors associated with achieving OR (**b**) and having PD (**c**). X- and y-axis show odds ratio and *P* values derived from two-sided Fisher's exact tests. The x- and y-axis are log2 and log10 scaled. Area of circles indicates the number of samples having the features indicated by the covariates and color of circles indicates the attributes of the covariates. Dotted lines indicate significant ($P = 0.05$) and sub-significant ($P = 0.10$) levels. Hemoglobin (HB) level and absolute neutrophil counts (ANC) were dichotomized with a threshold of 80 g/L and $8.0 \times 10^9/L$, respectively. Karyotype (KT) risks were classified into higher (poor and very poor, according to IPSS-R-based karyotype risk classification) and lower (intermediate, good and very good) groups. **d**, A forest plot showing the result of a multivariate logistic regression analysis for the factors associated with having PD performed on the discovery cohort with complete data for response analysis ($n = 311$).

Extended Data Figure 4

Extended Data Figure 4 | Univariate analysis for overall survival (OS).

a, A volcano plot showing the result of univariate analyses for the factors associated with OS. X- and y-axis show hazard ratio and P-values derived from Cox proportional hazards model. The x- and y-axis are log2 and log10 scaled. Area of circles indicates the number of samples having the features indicated by the covariates and color of circles indicates the attributes of the covariates. Dotted lines indicate significant ($P = 0.05$) and sub-significant ($P = 0.10$) levels. Age, hemoglobin level (HB), platelet level (PLT), marrow blast ratio (blast%) were dichotomized with a threshold of 72-year-old, 80 g/L, $50 \times 10^9/L$, 10%, respectively. Karyotype (KT) risks were classified into higher (poor and very poor, according to IPSS-R-based karyotype risk classification) and lower (intermediate, good and very good) groups. b, A forest plot showing the result of univariate analysis for the factors associated with OS performed on the validation cohort with complete data for survival analysis ($n = 110$). Explicative variables are limited to those showing significance ($P < 0.05$) in univariate analysis in the discovery cohort (Extended Data Fig. 4a); x-axis is log10 scaled. c, d, Kaplan-Meier estimates of OS per clinical responses on the 311 cases in the entire discovery cohort (c) and on the 70 cases in the discovery cohort having multi-hit TP53 mutations (d) with complete data for the analyses. The numbers of the cases at risk at each time are indicated in the tables below. P-value is derived from a two-sided log-rank test. e, Kaplan-Meier estimates of OS per TP53 allelic state on the 139 cases in the discovery cohort with IPSS-R very high risk. The numbers of the cases at risk at each time are indicated in the table below. P-values are derived from two-sided log-rank tests. f, Kaplan-Meier estimates of progression free survival per TP53 mutation status on the 59 cases who achieved CR. The numbers of the cases at risk at each time are indicated in the table below. P value is derived from a two-sided log-rank test. g, Kaplan-Meier estimates of OS per DDX41 mutational state on the 341 cases in the discovery cohort. The numbers of the cases at risk at each time are indicated in the table below. P values are derived from two-sided log-rank tests. h, Kaplan-Meier estimates of overall survival per DDX41 mutation status for IPSS-R very low/low/intermediate (left), high (middle) and very high (right) risk cases. The number of the cases at risk at each time is indicated in the tables below. P values are derived from two-sided log-rank tests. i, Bar plots showing the proportion of the cases having responses indicated by color in DDX41-mutated and unmutated cases. P value is derived from a Fisher's exact test.

Extended Data Figure 5

Extended Data Figure 5 | Factors associated with overall survival (OS).

a, Bar plots showing the generalized variance inflation factor (gVIF) of the covariates indicated on x-axis that are included in the multivariate Cox proportional hazards model for OS. Left and right panels show gVIF values before and after selection of parameter sets to circumvent multiple collinearity (**Methods**). **b**, A forest plot showing the result of Cox proportional hazards regression for overall survival (OS) on the discovery cohort with complete data for OS analysis ($n = 341$). Explicative variables are age, hemoglobin level, platelet level, marrow blast ratio (blast%), karyotype (KT) risks, multi-hit *TP53* mutation, *DDX41* mutation, del(18p), and -7/del(7q). Age, hemoglobin, platelet count, blast% were dichotomized with thresholds of 72-year-old, 80 g/L, $50 \times 10^9/L$, 10%, respectively. KT risks were classified into higher (poor and very poor) and lower (intermediate, good and very good) groups; the x-axis is log10 scaled. **c**, Kaplan-Meier estimates of OS per IPSS-R risks on the 320 cases in the discovery cohort with complete data for the analysis. The numbers of the cases at risk at each time are indicated in the tables below. *P* value is derived from a two-sided log-rank test. **d**, Concordance index (C-index) of IPSS-R alone model and the novel scoring system for the discovery (left) and the validation (right) cohorts, respectively. **e**, Kaplan-Meier estimates of OS per risk groups of the novel scoring model for IPSS-R very low/low/intermediate (left panel), high (middle panel), and very high (right panel) risk groups, respectively. The numbers of the cases at risk at each time are indicated in the tables below. *P* value is derived from a two-sided log-rank test. **f**, Kaplan-Meier estimates of overall survival per risk classes of the proposed model for the validation cohort. Risk classes are determined according to the formula presented in legends for **Fig. 1d** and **Fig. 1e**. The number of the cases at risk at each time is indicated in the tables below. *P* values are derived from two-sided log-rank tests.

t-MN, treatment related myeloid neoplasm; AZA, azacitidine, CR, complete remission; mCR, marrow CR; PR, partial remission; SD, stable disease; HI, hematological improvement; PD, progressive disease; WT, wild type.

Extended Data Figure 6

Extended Data Figure 6 | Major clone size in pre-treatment samples and clonal structure in stable disease (SD) cases with small post-treatment major clone (MC) size.

a, Box plots showing the MC group sizes of pre-treatment samples in the paired cohort having the response indicated on x-axis. $P = 0.04$, Jonckheere-Terpstra trend tests. **b**, Fish plots showing estimated transition of the clone structure during azacitidine for the six cases with SD that had post-treatment MC group sizes < 0.10 . Sample IDs correspond to those indicated in **Fig. 2c**.

CR, complete remission; mCR, marrow CR; PR, partial remission; HI, hematological improvement; PD, progressive disease.

Extended Data Figure 7

Extended Data Figure 7 | Whole genome sequencing to search for potential drivers.

We conducted whole genome sequencing for the 4 cases that had no overall response after azacitidine treatment although MC group sizes were < 0.20. All these cases had germline *DDX41* mutations. **a**, Filtering condition of somatic mutations of whole genome sequencing to curate somatic mutations. **b**, Variant allele frequency (VAF) histogram of somatic mutations of the 4 cases. Only single nucleotide variants were included from the somatic mutation list because indels show deviated VAF values from true ratio of altered alleles. **c**, A table showing the clinical and post-treatment clonal composition of the 4 cases. All these cases show small major clone (MC) group sizes in post-treatment samples. 1000g2014oct_all, 1000 Genomes Project (2014 Oct) annotations; EBCall, an empirical Bayesian framework for somatic mutation call. See Nucleic Acids Res 41:e89, 2013; SD, stable disease; HI, hematological improvement; PD, progressive disease.

Extended Data Figure 8

Extended Data Figure 8 | Outcomes of transplanted cases.

a, Kaplan-Meier estimates of OS on the 73 cases who received allogeneic stem cell transplantation (allo-SCT). **b**, Kaplan-Meier estimates of OS per maxVAF_{post} values (> 0.13 or ≤0.13) on the 53 cases who received allogeneic stem cell transplantation (allo-SCT) after azacitidine treatment. The numbers of the cases at risk at each time are indicated in the table below. *P* value is derived from a two-sided log-rank test. **c**, **d**, Kaplan-Meier estimates of OS on the 13 cases with *TP53* mutations who received allo-SCT. Time is calculated after azacitidine administration (**c**) or allo-SCT (**d**). The numbers of the cases at risk at each time are indicated in the table below.

Extended Data Table 1

Sample	TP53	PPM1D Variant	Pre-treatment			
			Detected by pipeline	IGV		
				VAF	Number of supporting reads	Number of total reads
#PPM1D-1	Multihit	p.S432fs	Yes	0.046	25	548
#PPM1D-2	Multihit	p.I436fs	No	0.005	7	1367
#PPM1D-3	Multihit	p.P471fs	No	0.001	2	1429
#PPM1D-4	Multihit	p.N477fs	No	0.011	10	938
#PPM1D-5	Wt	p.C478X	No	0.000	0	883
#PPM1D-6	1-hit	p.D488fs	No	0.000	0	2138
#PPM1D-7	Wt	p.M508fs	No	0.005	10	2107
#PPM1D-8	Wt	p.R552X	No	0.003	2	589
#PPM1D-9	Multihit	p.R572X	No	0.001	1	924

Extended Data Table 1 | Strenuous search for PPM1D-mutated clones by integrative genomics viewer (IGV) in pre-treatment samples that expanded their sizes in post-treatment samples.

A table showing the result of strenuous search for the supporting reads in pre-treatment samples by IGV. Case IDs correspond to those presented in **Fig. 4e**.

VAF, variant allele frequency; WT, wild type.

Supplementary Appendix

Supplementary Data

Supplementary Figures

Supplementary Figure 1 | Summary of the study cohorts subjected to each analysis.

Supplementary Figure 2 | Quality control of targeted sequencing.

Supplementary Figure 3 | Mutation Profile of discovery and validation cohorts.

Supplementary Figure 4 | Paired box plots showing clone sizes in pre- and post-treatment samples.

Supplementary Figure 5 | Fish plots of CR cases harboring mutations in *RUNX1*.

Supplementary Tables

Supplementary Table 1 | Panel of genes applied in this study.

Supplementary Table 2 | List of pathogenic variants in the pre-treatment samples.

Supplementary Table 3 | List of pathogenic variants in the post-treatment samples.

Supplementary Table 4 | List of CNVs in the pre-treatment samples.

Supplementary Table 5 | List of CNVs in the post-treatment samples.

Supplementary Table 6 | List of *DDX41* mutations with VAF values and expected genetic mode.

Supplementary Table 7 | List of somatic mutations in 4 WGS for germline *DDX41*-mutated cases with poor response.

Supplementary data

Details of study cohort

JALSG-MDS-201

JALSG-MDS-212 study is a multi-center prospective, randomized phase 3 trial conducted by Japan Adult Leukemia Study Group (JALSG). The major inclusion criteria is myelodysplastic syndromes (MDS) refractory anemia with excess of blasts (RAEB) or RAEB in transformation (RAEB-T) in FAB classification². The patients were excluded who were assumed to be candidates for allogeneic stem cell transplantation (allo-SCT), who is younger than 16 years old, who has dysfunction in major organs (liver, kidney, heart, and lung), and who had received any of the following therapies: azacitidine, anti-cancer drugs, allo-SCT, lenalidomide. The registered subjects were randomly assigned to either of 5-day and 7-day schedule of azacitidine at 75 mg/m² daily dose. The primary endpoint is to examine the non-inferiority of 5-day cohort to 7-day cohort in two-year overall survival (OS). This study is registered at UMIN-CTR (UMIN000009633). We excluded the subjects from this study when the patient dropped out before start of azacitidine, both response and survival data are missing, genetic data were not available for pre-treatment samples. Although marrow blast ratio increased >30% (34% and 37%) in 2 subjects between enrollment to JALSG-MDS212 study and start of azacitidine, they were included in this study following the intention-to-treat policy of JALSG-MDS212 study. Both of the 2 cases had germline *DDX41* mutations.

Karolinska University retrospective cohort.

Retrospectively collected consecutive patients with MDS, MDS/MPN, and oligoblastic secondary AML (marrow blast < 30% and transformed from MDS or MDS/MPN) who had received at least 1 cycle of azacitidine were included. Patients were excluded from this study who received azacitidine therapy as a consolidation therapy following intensive chemotherapies or as a palliative treatment. The patients were treated uniformly according to the European label.

The validation cohort.

For the validation cohort, we enrolled subjects from the hematological malignancy biobank at Kyoto University, collected from collaborative institutes in Japan. We enrolled the cases meeting the following criteria: 1) MDS, MDS/MPN, or AML-MRC < 30% blast that have progressed from MDS or MDS/MPN. 2) received at least one

course of azacitidine. 3) azacitidine was not administered as a consolidation therapy following intensive chemotherapies or as a palliative treatment.

Supplementary Fig. 1

Supplementary Fig. 1 | Summary of the study cohorts subjected to each analysis.

a, The discovery cohort was enrolled from JALSG-MDS-212 prospective study (n=178) and from consecutively collected retrospectives cohort from Karolinska Institute (n=163). As a validation cohort, additional 110 cases were retrospectively collected biobank of hematological malignancies at Kyoto University. Pre-treatment samples were collected and subjected for sequencing analysis. Among them, post-treatment samples were also subjected for sequencing analysis for 237 and 53 cases in the discovery and the validation cohort, respectively. **b**, Illustrative explanation showing the cohorts that were subjected to each analysis represented by the indicated data.

Supplementary Fig. 2

Supplementary Fig. 2 | Quality of sequencing.

a, Depth and %duplicated ratio of the targeted sequencing. Samples are arranged in the ascending order of average depth and color-coded by the cohort. **b**, Ratio of the targeted genomic region covered by the depths indicated in each color, where samples are arranged in the same order in the panel **a**.

Supplementary Fig. 3

a Pre-treatment samples

b Post-treatment samples

Supplementary Fig. 3 | Distribution of frequently observed genetic alterations.

The distribution of genetic alterations detected in discovery cohort (upper panel) and validation cohort (lower panel) in pre-treatment samples (a), and post-treatment samples (b). Y-axes of the bar plots indicate the number of samples harbouring the corresponding variants. CNA, copy number alteration; LOH, loss of heterozygosity.

Supplementary Fig. 4

Supplementary Fig. 4 | Paired box plots showing clone sizes in pre- and post-treatment samples.

Paired box plots showing the change in clone sizes represented by indicated genes. The data are shown for each response and colors of line indicate whether the clone is classified as a major clone (MC, black lines) or not (red lines).

Supplementary Fig. 5

Supplementary Fig. 5 | Fish plots of CR cases harboring mutations in RUNX1.

Fishplots showing changes in clonal structures assumed from variant allele frequency (VAF) values including mutations in *RUNX1* gene in pre-treatment samples that achieved complete remission (CR). Clones marked with underline represent major clones (MC). All the 11 clones represented by *RUNX1* gene mutations reduced their sizes. Three of these clones were MC (#R1:*RUNX1*, p.Q335X, #R5:*RUNX1*, p.R201X, #R6:*RUNX1*, p.P103S) and the other 8 clones were non-MC and were assumed to be subclones. Six out of these non-MC (#R2:*RUNX1*, p.S322X, #R4:*RUNX1*, p.R204Q, #R5:*RUNX1*, p.D198N, *RUNX1*, p.S141fs, #R6:*RUNX1*, p.Y189X, #R7:*RUNX1*, p.R166X) were included by parent clones that also reduced their sizes.

Supplementary Files

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- [SupplementaryTables.xlsx](#)
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