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Asato Kongoji
Toho University Sakura Medical Center
Sanshiro Nakao
sanshiro.nakao@med.toho-u.ac.jp

Toho University Sakura Medical Center
Hidekazu Hasunuma
Toho University Sakura Medical Center
Naomi Shimizu
Toho University Sakura Medical Center

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Research

Changes in oxidative stress markers during azacitidine treatment in patients with myelodysplastic syndromes: a retrospective cohort study

Asato Kongoji[1], Sanshiro Nakao[1,2,3], Hidekazu Hasunuma[3], Naomi Shimizu[1,2,3]

[1] Department of Clinical Laboratory and Experimental-Research Medicine, Toho University Sakura Medical Center, Chiba 285-8741, Japan

[2] Department of Hematology, Toho University Sakura Medical Center, Chiba 285-8741, Japan

[3] Division of Blood Transfusion, Department of Internal Medicine, Toho University Sakura Medical Center, Chiba 285-8741, Japan

Correspondence: Sanshiro Nakao, MD, PhD, Department of Hematology, Toho University Sakura Medical Center, 564-1 Shimoshizu, Sakura, Chiba 285-8741, Japan

Phone: +81-43-462-8811; Fax: +81-43-489-9770; Email: sanshiro.nakao@med.toho-u.ac.jp

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Abstract

Oxidative stress has been implicated in myelodysplastic syndromes (MDS), but its longitudinal behavior during azacitidine (AZA) treatment remains poorly characterized. We retrospectively analyzed 22 patients with MDS who received first-line AZA. Peripheral blood *WT1* mRNA, serum derivatives of reactive oxygen metabolites (d-ROMs), and reactive oxygen species (ROS) in peripheral blood granulocytes were assessed before treatment and at a selected post-treatment assessment. Among 19 patients evaluable by the Revised International Prognostic Scoring System, *WT1* mRNA and d-ROMs levels were higher in the higher-risk group than in the lower-risk group. Log-transformed *WT1* mRNA correlated positively with d-ROMs ($r = 0.4515$, $p = 0.0349$), and d-ROMs correlated positively with granulocyte ROS ($r = 0.4819$, $p = 0.0429$); these nominal associations were exploratory. Overall, d-ROMs and granulocyte ROS decreased during AZA treatment. At the selected post-treatment assessment, both markers decreased within the *WT1*-decrease group. However, at Cycle 3, neither marker changed significantly in the fixed *WT1*-decrease group, whereas RBC transfusion requirements and LDH decreased. Direct between-group comparisons of changes in d-ROMs, granulocyte ROS, RBC transfusion requirements, and LDH were not significant. Because the trajectory groups were defined solely by *WT1* change and did not represent established hematologic response categories, these subgroup findings remain exploratory. Changes in oxidative stress markers were not correlated with changes in RBC transfusion requirements or ferritin. These hypothesis-generating findings indicate that oxidative stress markers decrease during AZA treatment, but whether they track *WT1* trajectory or hematologic response remains uncertain.

Keywords: myelodysplastic syndromes; oxidative stress; reactive oxygen species; azacitidine; *WT1*

Introduction

Oxidative stress–induced DNA damage is an important driver of mutagenesis and carcinogenesis. In a murine model of myelodysplastic syndrome (MDS), increased oxidative stress increased mutation frequency, suggesting that the accumulation of genetic alterations may contribute to disease evolution [1]. MDS comprises a heterogeneous group of clonal hematopoietic stem and progenitor cell neoplasms with an inherent risk of progression to acute myeloid leukemia. Aberrant DNA methylation is an important feature of MDS biology, and oxidative stress may interact with epigenetic dysregulation [2]. Azacitidine (AZA), a DNA hypomethylating agent, improved survival compared with conventional care regimens and is a standard treatment option for patients with higher-risk MDS who are ineligible for allogeneic hematopoietic stem cell transplantation [3].

Patients with MDS often require repeated red blood cell (RBC) transfusions, which can cause secondary iron overload and further increase oxidative stress. Antioxidant approaches and iron chelation have therefore been proposed as rational supportive strategies [4, 5]. Previous studies have reported associations between serum ferritin and circulating reactive oxygen species (ROS) in patients with MDS [4].

Peripheral blood *WT1* mRNA has been investigated as a marker of disease burden and prognosis in MDS [6-9]. Decreases in *WT1* mRNA have also been reported during successful AZA treatment [9]. In addition, case reports have described concurrent increases in *WT1* mRNA and the oxidative stress marker derivatives of reactive oxygen metabolites (d-ROMs) during disease progression [10], and decreased ROS production in peripheral blood leukocyte subsets during a marked response to AZA [11]. However, longitudinal data on oxidative stress markers during AZA treatment remain limited.

We therefore examined relationships between oxidative stress markers and disease-related variables and described changes in d-ROMs and granulocyte ROS during AZA treatment in a retrospective cohort of patients with MDS. We also performed exploratory analyses according to the direction of change in peripheral blood *WT1* mRNA.

Methods

Study design and patients

We retrospectively evaluated patients diagnosed with MDS at Toho University Sakura Medical Center between July 2014 and September 2022 who were ineligible for hematopoietic stem cell transplantation and received at least two cycles of AZA as first-line treatment. Diagnoses were retrospectively classified according to the 2022 World Health Organization classification of myeloid neoplasms [12]. Patients were excluded if pretreatment peripheral blood *WT1* mRNA measurements were unavailable, if they had overt leukemia at the initiation of AZA treatment, defined as ≥20% myeloblasts in either the peripheral blood or bone marrow, or if the C-reactive protein (CRP) concentration at diagnosis was ≥10 mg/dL.

Prognostic risk was assessed using the Revised International Prognostic Scoring System (IPSS-R) [13]. Patients with very low- or low-risk disease were categorized as lower risk, and those with intermediate-, high-, or very high-risk disease were categorized as higher risk. Cytogenetic data required for IPSS-R classification were unavailable for three patients.

Clinical and laboratory variables used for the cross-sectional analyses were assessed at the time of MDS diagnosis, whereas the longitudinal analyses were based on measurements obtained before AZA initiation and at the

selected post-treatment assessment. Therefore, the number of evaluable patients differed among analyses depending on the availability of measurements at each time point.

Azacitidine treatment and supportive care

AZA was administered subcutaneously or intravenously at 75 mg/m²/day for 5 consecutive days every 28 days. Cycles were delayed, or doses were reduced as clinically indicated for severe cytopenia. Treatment was continued until disease progression or unacceptable toxicity.

RBC transfusions were administered when the hemoglobin concentration was <7.0 g/dL or at a higher threshold when clinically indicated because of comorbidities or symptoms. Platelet transfusions were administered when the platelet count was <20 × 10⁹/L or at a higher threshold in patients with bleeding, a high bleeding risk, or concomitant anticoagulant or antiplatelet therapy. Granulocyte colony-stimulating factor was used only for severe, life-threatening infection.

Measurement of peripheral blood *WT1* mRNA and serum d-ROMs

Peripheral blood *WT1* mRNA was measured at LSI Medience Corporation (Tokyo, Japan) using the *WT1* mRNA Assay Kit II (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions, as previously described [14]. The lower limit of measurement was 50 copies/μg RNA. Values below the lower limit of measurement were treated as 50 copies/μg RNA for statistical analyses. *WT1* mRNA values were log10-transformed for analyses requiring logarithmic transformation.

Serum samples collected at diagnosis, before AZA initiation, and during treatment were analyzed in-house using the d-ROMs test kit (Wismerll Co., Ltd., Tokyo, Japan) on a BioMajesty™ JCA-BM6070 automated clinical chemistry analyzer (JEOL Ltd., Tokyo, Japan), according to the manufacturer's instructions [15]. Blood samples were allowed to clot for 15–30 min at room temperature and were then centrifuged at 2,000 ×rpm for ten min at 20 ℃. The separated serum was stored at 4 °C until analysis and was analyzed within 1 week of storage. The assay is based on a colorimetric method and has a measurement range of 40–1,000 U. CARR. Previously reported intra-assay (within-run) and inter-assay (between-run) coefficients of variation for the automated d-ROMs assay were 1.4–4.0% and 2.9–4.7%, respectively [15].

The lowest peripheral blood *WT1* mRNA value measured after AZA initiation was selected as the post-treatment *WT1* mRNA value, and the corresponding assessment time point was used for the post-treatment evaluation of other clinical and oxidative stress variables.

Measurement of ROS production in peripheral blood granulocytes

Reactive oxygen species (ROS) production in peripheral blood granulocytes was assessed by flow cytometry using 2′,7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA; Life Technologies Corporation). A 10 mM $DCFH_2$-DA stock solution was prepared by dissolving 9.74 mg of $DCFH_2$-DA in 2 mL of dimethyl sulfoxide and was stored frozen until use. Immediately before analysis, the stock solution was thawed at room temperature and diluted 1:100 with phosphate-buffered saline (PBS; 5 μL of stock solution plus 495 μL of PBS) to prepare a 100 μM working solution.

Peripheral whole blood (50 μL) from each patient was mixed with 1,000 μL of PBS, then 30 μL of the 100 μM $DCFH_2$-DA working solution was added, yielding a final dye concentration of approximately 2.8 μM. The samples were incubated without agitation for 30 min at 37 °C in a CO_2 incubator and then centrifuged at 1,500 rpm for 5 min. After removal of the supernatant containing excess $DCFH_2$-DA, 2 mL of BD Pharm Lyse™ Lysing Buffer (BD Biosciences), diluted 1:10 with distilled water, was added to each sample. After incubating at room temperature for 7 min, the samples were centrifuged again at 1,500 rpm for 5 min. The supernatant was removed, and the cell pellet was resuspended in 200 μL of PBS.

Fluorescence was measured using a BD FACSVerse™ flow cytometer and analyzed using BD FACSuite™ software (both from BD Biosciences, San Jose, CA, USA). Granulocytes were identified solely as a distinct population based on forward- and side-scatter characteristics; no granulocyte-lineage markers were used. At least 20,000 events were acquired per sample, and the mean fluorescence intensity (MFI) of the gated granulocyte population was calculated. Human IgG was used as an isotype control for each patient sample. ROS production was expressed as the ratio of the MFI obtained from $DCFH_2$-DA-stained granulocytes to that obtained from the corresponding human IgG control.

Assessment of RBC transfusion requirements

RBC transfusion requirements were defined as the total number of RBC units transfused during the 1 month preceding each pretreatment and selected post-treatment *WT1* mRNA measurement.

Exploratory classification by *WT1* trajectory

Because post-treatment bone marrow examinations were not routinely performed, hematologic response could not be systematically assessed according to the International Working Group (IWG) criteria [16]. Therefore, serial

peripheral blood *WT1* mRNA measurements were used to define exploratory molecular subgroups and to examine whether changes in oxidative stress markers accompanied changes in *WT1* during AZA treatment.

Patients whose selected post-treatment *WT1* mRNA value was lower than the pretreatment value were assigned to the *WT1*-decrease group. Patients who showed no decrease in *WT1* mRNA at any assessment after AZA initiation were assigned to the *WT1*-nondecrease group. These categories were defined solely by *WT1* trajectory and were not equivalent to response categories based on established hematologic criteria. Because group assignment was based on the direction of *WT1* change, within-group changes in *WT1* were considered descriptive and were not used to validate the classification.

Statistical analysis

Continuous variables are reported as medians and interquartile ranges (IQRs). Independent groups were compared using the Wilcoxon rank-sum test, whereas paired pretreatment and post-treatment values were compared using the exact two-sided Wilcoxon signed-rank test. For paired analyses, only patients with both measurements for the relevant parameter were included, and missing data were excluded on a pairwise basis. Correlations among baseline continuous variables were assessed using Pearson correlation coefficients. Because peripheral blood *WT1* mRNA values were highly skewed, they were log10-transformed before correlation analyses. Exploratory correlations between changes in clinical variables and changes in oxidative stress markers were assessed using Spearman rank correlation coefficients.

Changes were calculated as pretreatment minus post-treatment values; therefore, positive values indicated decreases during AZA treatment. Changes in d-ROMs levels, granulocyte ROS production, RBC transfusion requirements, and LDH levels were compared between the *WT1*-decrease and *WT1*-nondecrease groups using the exact Wilcoxon rank-sum test. Between-group differences were reported as Hodges–Lehmann estimates of location shift (*WT1*-decrease minus *WT1*-nondecrease), with 95% confidence intervals.

To evaluate changes at a uniform early treatment time point, an additional paired analysis was performed using Cycle 3 measurements. The *WT1* trajectory group assignments used in the primary subgroup analysis (Table 5) were retained, and patients were not reclassified according to their Cycle 3 *WT1* mRNA values. The paired-analysis procedure described above was applied within each fixed group.

All tests were two-sided, and $p < 0.05$ was considered nominally significant. No adjustment was made for multiple testing; accordingly, all inferential results were considered exploratory. A significant result within one subgroup and a nonsignificant result within another were not interpreted as evidence of a difference between the subgroups. Analyses were performed using JMP software, version 14 (SAS Institute Inc., Cary, NC, USA).

Results

Patient characteristics

Patient characteristics were summarized using descriptive statistics; continuous variables were reported as medians and interquartile ranges, and categorical variables as numbers and percentages. The study included 22 patients (Table 1). The median age was 77 years (IQR, 70–81 years), and 20 patients were male. According to the 2022 WHO classification, 10 patients (45%) had MDS with low blasts, 7 (32%) had MDS with increased blasts-1, and 5 (23%) had MDS with increased blasts-2. Cytogenetic evaluation was unavailable for three patients. Among the 19 patients evaluable by IPSS-R, 1 (5%) had very low-risk disease, 7 (37%) had low-risk disease, 2 (10%) had intermediate-risk disease, 3 (16%) had high-risk disease, and 6 (32%) had very high-risk disease.

At the time of MDS diagnosis, the median peripheral blood *WT1* mRNA level was 1,600 copies/μg RNA (IQR, 390–6,450), the median d-ROMs level was 422 U. CARR (IQR, 331–512), and the median granulocyte ROS ratio was 1.87 (IQR, 1.30–2.95; $n = 18$).

Comparison by IPSS-R risk category

Comparisons between the lower- and higher-risk groups were performed using the Wilcoxon rank-sum test. Among the 19 patients evaluable by IPSS-R, 8 were categorized as lower risk and 11 as higher risk (Table 2). Median peripheral blood *WT1* mRNA was higher in the higher-risk group than in the lower-risk group (4,000 vs. 295 copies/μg RNA, $p = 0.0286$). Median d-ROMs was also higher in the higher-risk group (429 vs. 312 U. CARR, $p = 0.0431$). Granulocyte ROS and the other evaluated clinical variables did not differ nominally between risk groups.

Baseline associations among *WT1* mRNA, oxidative stress markers, and clinical variables

Associations among baseline continuous variables were assessed using Pearson correlation coefficients. As shown in Table 3, log-transformed peripheral blood *WT1* mRNA correlated positively with d-ROMs ($r = 0.4515$, $p = 0.0349$)

and CRP ($r = 0.4502$, $p = 0.0355$). It was not significantly correlated with granulocyte ROS ($r = 0.3873$, $p = 0.1123$) or ferritin ($r = 0.0385$, $p = 0.8756$).

d-ROMs correlated positively with granulocyte ROS ($r = 0.4819$, $p = 0.0429$) and CRP ($r = 0.4872$, $p = 0.0215$), but not with ferritin ($r = -0.0713$, $p = 0.7719$). Because these correlations were not adjusted for multiple testing, nominally significant results should be interpreted as hypothesis-generating.

Changes during AZA treatment in the overall cohort

The selected post-treatment assessment was performed after a median of 3 AZA cycles (range, 2–16 cycles). Paired changes from pretreatment to the selected post-treatment assessment were evaluated using the Wilcoxon signed-rank test and are summarized in Table 4. Median peripheral blood *WT1* mRNA decreased from 1,600 copies/μg RNA (IQR, 250–15,500) before treatment to 510 copies/μg RNA (IQR, 170–3,025) after treatment ($p = 0.0223$). Median d-ROMs decreased from 421 U. CARR (IQR, 331–483) to 326 U. CARR (IQR, 257–365; $p = 0.0024$). Among 17 patients with paired ROS measurements, the median granulocyte ROS ratio decreased from 1.31 (IQR, 1.13–2.43) to 1.19 (IQR, 1.02–1.58; $p = 0.0267$). RBC transfusion requirements decreased from 6 units/month (IQR, 2–8) to 1 unit/month (IQR, 0–4; $p = 0.0053$), and lactate dehydrogenase decreased from 261 U/L (IQR, 198–316) to 208 U/L (IQR, 177–225; $p < 0.0001$). CRP and ferritin did not change significantly.

Correlations between changes from pretreatment to post-treatment were assessed using Spearman rank correlation coefficients. In exploratory analyses, changes in RBC transfusion requirements were not significantly correlated with changes in d-ROMs (Spearman $\rho = -0.2963$, $p = 0.1806$) or granulocyte ROS ($\rho = -0.1650$, $p = 0.5129$). Changes in ferritin were not significantly correlated with changes in d-ROMs ($\rho = -0.2722$, $p = 0.2744$) or granulocyte ROS ($\rho = 0.0738$, $p = 0.7858$).

Exploratory analyses by *WT1* trajectory

Within each trajectory-defined group, paired pretreatment and post-treatment values were compared using the Wilcoxon signed-rank test. Sixteen patients were classified in the *WT1*-decrease group (Table 5). By definition, median *WT1* mRNA decreased from 4,150 to 510 copies/μg RNA. Median d-ROMs decreased from 421 to 332 U. CARR ($p = 0.0182$), and among 12 patients with paired measurements, the median granulocyte ROS ratio decreased from 1.87 to 1.29 ($p = 0.0342$). RBC transfusion requirements decreased from 7 to 0 units/month ($p = 0.0073$), and lactate dehydrogenase decreased from 270 to 199 U/L ($p = 0.0003$). Individual changes in *WT1* mRNA, d-ROMs, and granulocyte ROS are shown in Fig. 1.

Six patients were classified in the *WT1*-nondecrease group (Table 5). By definition, median *WT1* mRNA increased from 160 to 840 copies/μg RNA. Median d-ROMs changed from 406 to 304 U. CARR ($p = 0.0938$), and among five patients with paired measurements, the median granulocyte ROS ratio changed from 1.19 to 0.87 ($p = 0.625$). RBC transfusion requirements changed from 1 to 2 units/month ($p = 0.6875$). Individual changes are shown in Fig. 2.

Changes from pretreatment to Cycle 3 according to the fixed *WT1* trajectory groups

The Cycle 3 analysis was conducted using the fixed *WT1* trajectory group assignments defined in Table 5. The fixed groups comprised 16 patients in the *WT1*-decrease group and six patients in the *WT1*-nondecrease group. For each parameter, only patients with paired pretreatment and Cycle 3 measurements were included.

Within the *WT1*-decrease group, median *WT1* mRNA decreased from 4,150 to 540 copies/μg RNA ($p = 0.0004$). RBC transfusion requirements decreased from 7 to 2 units/month ($p = 0.0067$), and LDH levels decreased from 270 to 182 U/L ($p = 0.0010$). Although median d-ROMs levels decreased numerically from 421 to 361 U.CARR, the change was not statistically significant ($p = 0.0833$). Among the 12 patients with paired granulocyte ROS measurements, the median ROS ratio changed from 1.87 to 1.63 without statistical significance ($p = 0.4238$). No significant changes were observed in CRP or ferritin levels.

Within the *WT1*-nondecrease group, median *WT1* mRNA increased numerically from 160 to 690 copies/μg RNA, but the change did not reach statistical significance ($p = 0.0625$). No significant within-group changes were observed in d-ROMs levels, granulocyte ROS production, RBC transfusion requirements, CRP levels, ferritin levels, or LDH levels (all $p > 0.05$) (Table 6).

Comparison of changes between the *WT1*-decrease and *WT1*-nondecrease groups

Direct between-group comparisons showed no statistically significant differences in changes in d-ROMs, granulocyte ROS, RBC transfusion requirements, or LDH between the *WT1*-decrease and *WT1*-nondecrease groups (Table 7). All 95% confidence intervals for the Hodges–Lehmann estimates included zero and were wide, indicating substantial uncertainty in both the magnitude and direction of the estimated effects. Therefore, these findings do not

provide sufficient evidence that changes in oxidative stress markers differ according to *WT1* trajectory, although clinically meaningful differences cannot be excluded because of the limited sample size.

Discussion

In this retrospective cohort, d-ROMs and peripheral blood *WT1* mRNA were higher in patients with higher-risk MDS than in those with lower-risk disease, and d-ROMs correlated with both *WT1* mRNA and granulocyte ROS. d-ROMs and granulocyte ROS also decreased during AZA treatment in paired analyses. These observations suggest a possible association between oxidative stress and MDS-related disease activity, although further studies are needed to determine whether oxidative stress markers may reflect hematologic response.

Oxidative stress has been implicated in genomic instability and disease evolution in MDS [1, 17]. The higher d-ROMs values in the higher-risk group and their positive correlation with *WT1* mRNA are consistent with this biological framework. However, both d-ROMs and *WT1* mRNA also correlated with CRP, and d-ROMs are not disease-specific. Residual inflammation, infection, comorbidities, or other systemic factors may therefore have contributed to the observed cross-sectional associations despite exclusion of patients with CRP ≥10 mg/dL.

This longitudinal pattern is consistent with a previous case report describing reduced ROS production in peripheral blood leukocyte subsets during a marked response to AZA [11]. The present cohort provides additional longitudinal observations, but the mechanism cannot be determined from these data. Oxidative stress has been associated with methylation of the *p15* and *p16* promoters in MDS [2], and AZA could influence oxidative stress indirectly through changes in the abnormal clone, marrow function, inflammation, or transfusion requirements. Because methylation and clonal burden were not measured, a direct effect of AZA-mediated hypomethylation on oxidative stress cannot be inferred.

Transfusion-related iron overload is another potential contributor to oxidative stress in MDS [4, 5]. RBC transfusion requirements decreased during AZA treatment, whereas ferritin levels did not change significantly. Furthermore, neither changes in transfusion requirements nor changes in ferritin were correlated with changes in d-ROMs or granulocyte ROS. These exploratory results suggest that transfusion burden and ferritin alone may not fully explain the observed changes. However, the small sample size, limitations of ferritin as a marker of iron overload, and absence of serial measurements of non-transferrin-bound iron (NTBI), particularly its redox-active component, labile plasma iron (LPI), preclude firm conclusions. Although speculative, these findings leave open the possibility that

MDS-related pathobiological processes, beyond transfusion-related iron overload, may contribute to changes in oxidative stress during AZA treatment.

The subgroup analyses provided a more nuanced view of the changes in oxidative stress markers during AZA treatment. At the selected post-treatment assessment, significant reductions in d-ROMs levels and granulocyte ROS production were observed within the *WT1*-decrease group (Table 5). However, when the same *WT1* trajectory group assignments were retained and the analysis was restricted to the uniform Cycle 3 time point, neither d-ROMs levels nor granulocyte ROS production changed significantly, whereas reductions in RBC transfusion requirements and LDH levels remained significant (Table 6). These findings indicate that changes in oxidative stress markers were not consistently detectable at this early treatment time point. The discrepancy between the two analyses may reflect variability in the timing of oxidative stress responses, the heterogeneous timing of the selected post-treatment assessments, missing measurements, or limited statistical power. It should not be interpreted as evidence that oxidative stress markers change only after prolonged treatment. Furthermore, a significant within-group change in the *WT1*-decrease group and a nonsignificant change in the *WT1*-nondecrease group do not establish a difference between the groups. Consistent with this consideration, direct comparisons of the magnitudes of change did not demonstrate significant between-group differences (Table 7). Thus, the subgroup findings remain exploratory and do not establish that changes in d-ROMs levels or granulocyte ROS production track the *WT1*-defined treatment trajectory.

This study has several additional limitations. It was a small, single-center retrospective study without an untreated or comparator group. Patients were required to receive at least two AZA cycles, which may have introduced survivor and selection biases. The timing of post-treatment sampling was not standardized, ROS data were missing for some patients, and residual day-to-day assay variability may not have been fully eliminated. Furthermore, selecting the post-treatment assessment time point based on the lowest observed *WT1* mRNA value may have introduced regression-to-the-mean bias and outcome-dependent time-point selection. The limited sample size may have reduced the statistical power to detect between-group differences, and these subgroup findings should be interpreted as exploratory. Multiple comparisons were conducted without multiplicity adjustment, and Pearson correlations in small samples may be sensitive to outliers. Finally, established hematologic response criteria were not used, and inflammation and other confounding factors could not be adequately controlled.

In conclusion, oxidative stress markers decreased during AZA treatment and were cross-sectionally associated with MDS-related variables. Whether changes in d-ROMs or granulocyte ROS differ according to *WT1* trajectory or track established hematologic response remains undetermined.

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Author contributions: Asato Kongoji and Hidekazu Hasunuma performed the experiments and collected the clinical data. Sanshiro Nakao supervised the preparation of the manuscript. Naomi Shimizu critically revised the manuscript and performed the final review. All authors read and approved the final manuscript.

Ethics approval: This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Toho University Sakura Medical Center (approval no. S24067_S22005(S17087)_S16015 and 2014-069).

Consent to participate: Written informed consent to participate in the study was obtained from all participants.

Consent for publication: Not applicable.

Data availability: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figure legends

Fig. 1. Individual paired changes from pretreatment to the selected post-treatment assessment in (a) peripheral blood *WT1* mRNA, (b) serum derivatives of reactive oxygen metabolites (d-ROMs), and (c) granulocyte reactive oxygen species (ROS) production in the *WT1*-decrease group. The selected post-treatment assessment corresponded to the time point at which the lowest post-AZA *WT1* mRNA value was recorded. Changes in *WT1* mRNA are shown descriptively because group assignment was defined by the direction of *WT1* change. d-ROMs levels ($p = 0.0182$) and granulocyte ROS production ($p = 0.0342$) decreased in paired analyses using the exact two-sided Wilcoxon signed-rank test. An asterisk indicates nominal statistical significance ($p < 0.05$).

Fig. 2. Individual paired changes from pretreatment to the selected post-treatment assessment in (a) peripheral blood *WT1* mRNA, (b) serum derivatives of reactive oxygen metabolites (d-ROMs), and (c) granulocyte reactive oxygen species (ROS) production in the *WT1*-nondecrease group. The selected post-treatment assessment corresponded to the time point at which the lowest post-AZA *WT1* mRNA value was recorded. Changes in *WT1* mRNA are shown descriptively because patients in this group showed no decrease in *WT1* mRNA at any post-treatment assessment. Within-group changes in d-ROMs levels ($p = 0.0938$) and granulocyte ROS production ($p = 0.625$) did not reach nominal statistical significance in paired analyses using the exact two-sided Wilcoxon signed-rank test. An asterisk indicates nominal statistical significance ($p < 0.05$).

Fig. 1 Individual changes in WT1 mRNA and oxidative stress markers during azacitidine treatment in the *WT-1*-decrease group

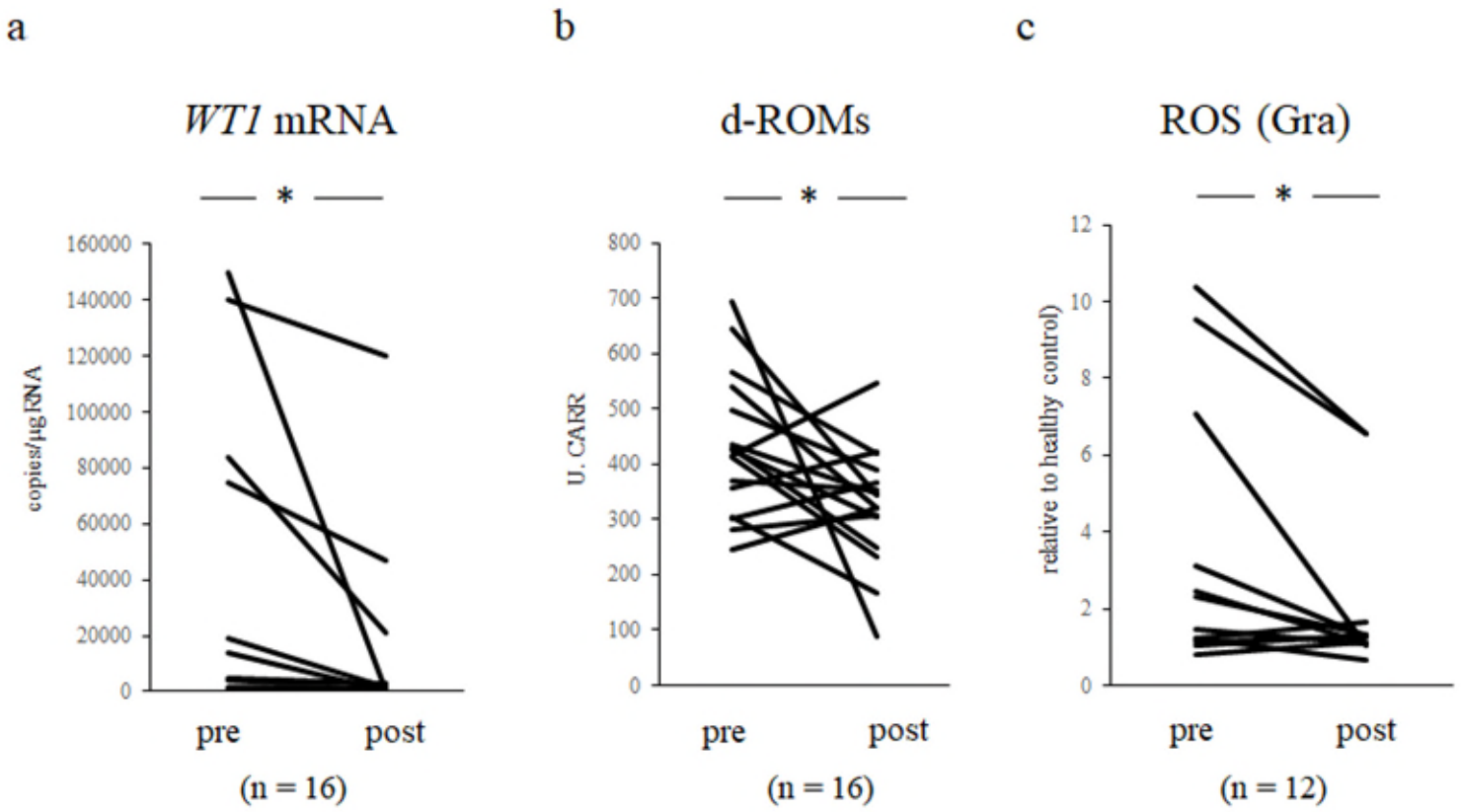


Fig. 2 Individual changes in WT1 mRNA and oxidative stress markers during azacitidine treatment in the *WT-1*-nondecrease group

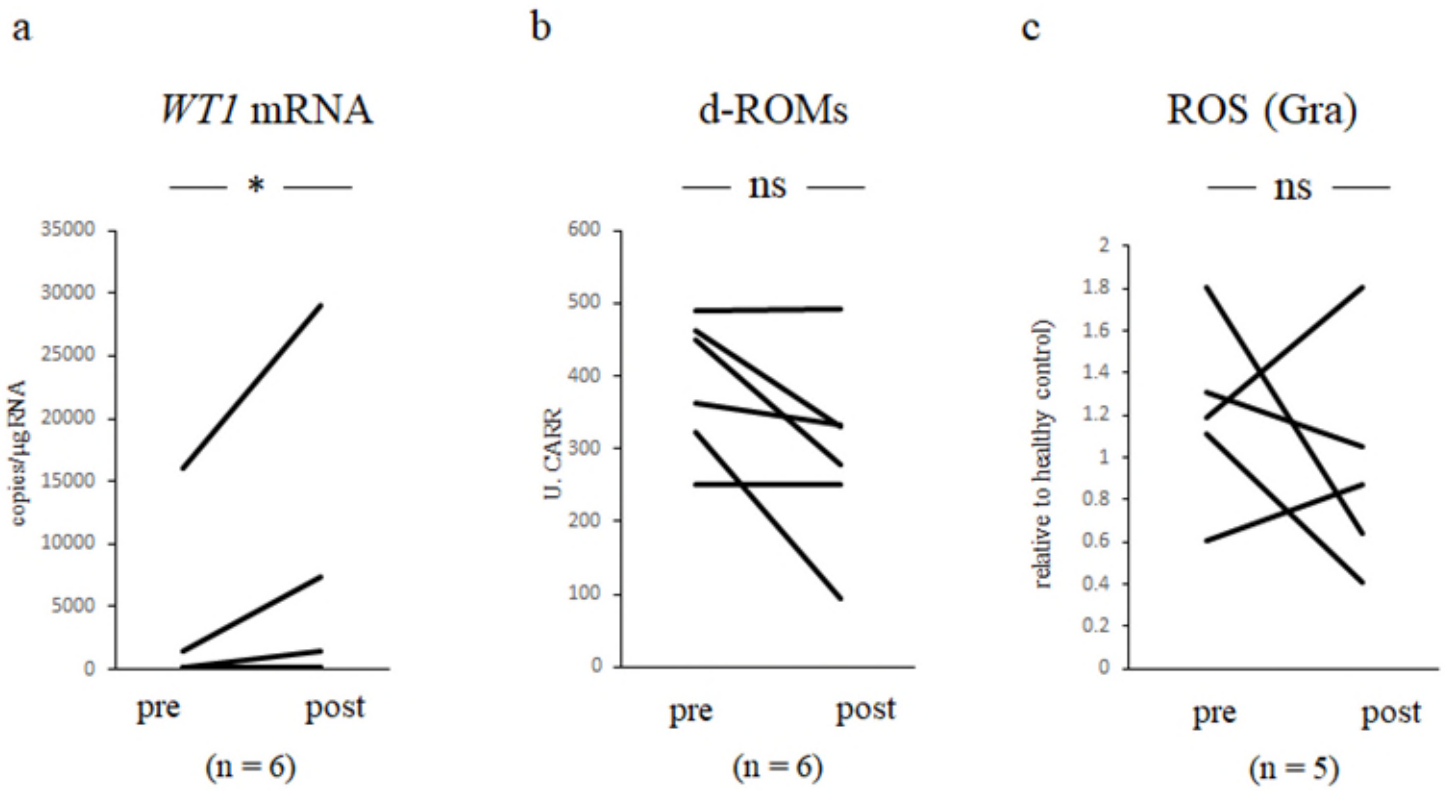


Table 1. Patient characteristics and clinical parameters at diagnosis

Clinical condition	n	Ratio (%)	Variable	Unit	Median (IQR)
Total	22	100	Age	years	77 (70–81)
MDS-with low blasts	10	45	Gender	m/f	20/2
MDS-with increased blasts-1	7	32	WBC (n = 22)	10^3/μL	2.27 (1.62–4.43)
MDS-with increased blasts-2	5	23	RBC (n = 22)	10^4/μL	250 (219–290)
			Hemoglobin (n = 22)	g/dL	7.8 (6.9–9.2)
Revised International Prognostic Scoring System	19		Platelets (n = 22)	10^4/μL	9.4 (6.4–14.5)
Very Low	1	5	LDH (n = 22)	U/L	249 (183–295)
Low	7	37	UA (n = 22)	mg/dL	6.0 (5.0–6.6)
Intermediate	2	10	Ferritin (n = 19)	ng/mL	695.5 (364.4–1,237.2)
High	3	16	CRP (n = 22)	mg/dL	0.37 (0.13–1.13)
Very high	6	32	*WT1* mRNA (n = 22)	copies/μg RNA	1,600 (390–6,450)
			d-ROMs (n = 22)	U. CARR	422 (331–512)
			ROS (Gra) (n = 18)	relative to control	1.87 (1.30–2.95)

MDS, myelodysplastic syndrome; IPSS-R, Revised International Prognostic Scoring System; IQR, interquartile range; WBC, white blood cells; RBC, red blood cells; LDH, lactate dehydrogenase; UA, uric acid; CRP, C-reactive protein; PB-*WT1*, peripheral blood-*Wilms tumor 1*; d-ROMs, derivative of reactive oxygen metabolites; Gra, granulocyte.

Table 2. Comparison of clinical parameters and oxidative stress markers between IPSS-R lower-risk and higher-risk groups

Clinical parameter	Unit	n	Low risk Median (IQR)	n	High risk Median (IQR)	*p* value
Age	years	8	77 (70–79)	11	75 (70–82)	1.0000
WBC	$10^3/\mu L$	8	2.13 (1.55–5.22)	11	3.09 (1.60–4.33)	0.7726
Hemoglobin	g/dL	8	7.6 (6.8–8.5)	11	7.9 (7.3–9.2)	0.8042
Platelets	$10^4/\mu L$	8	10.5 (9.1–14.4)	11	7.2 (5.8–14.8)	0.3421
LDH	U/L	8	183 (178–211)	11	255 (214–315)	0.0899
UA	mg/dL	8	6.0 (4.9–6.8)	11	6.4 (5.3–6.8)	0.6494
Ferritin	ng/mL	7	530.59 (364.40–1,005.51)	11	754.9 (358.79–1,068.60)	0.7859
CRP	mg/dL	8	0.27 (0.09–0.30)	11	0.81 (0.16–1.90)	0.1074
PB-*WT1*	copy/μg RNA	8	295 (99–1,800)	11	4,000 (810–16,500)	0.0286
d-ROMs	U.CARR	8	312 (292–394)	11	429 (392–525)	0.0431
ROS (Gra)	expression/control	6	1.77 (1.59–2.89)	10	1.83 (1.21–2.37)	1.0000

Abbreviations are the same as those in Table 1.

Table 3. Association of *WT1* mRNA with oxidative stress markers and other clinical parameters

	log *WT1* mRNA	d-ROMs	ROS	Ferritin	CRP
d-ROMs	$r = 0.4515$ $p = 0.0349$* (n = 22)				
ROS	$r = 0.3873$ $p = 0.1123$ (n = 18)	$r = 0.4819$ $p = 0.0429$* (n = 18)			
Ferritin	$r = 0.0385$ $p = 0.8756$ (n = 19)	$r = -0.0713$ $p = 0.7719$ (n = 19)	$r = -0.4940$ $p = 0.0613$ (n = 15)		
CRP	$r = 0.4502$ $p = 0.0355$* (n = 22)	$r = 0.4872$ $p = 0.0215$* (n = 22)	$r = 0.0850$ $p = 0.7374$ (n = 18)	$r = 0.3446$ $p = 0.486$ (n = 19)	

Abbreviations are the same as those in Table 1.

*An asterisk indicates statistical significance ($p < 0.05$).

Table 4. Changes in each clinical test value for the entire treatment group before and after administration of azacitidine

Clinical parameter	Unit	n	Time point	Median (IQR)	*p* value
WT1 mRNA	copies/μg RNA	22	Pre	1,600 (250–15,500)	0.0223
			Post	510 (170–3,025)	
d-ROMs	U. CARR	22	Pre	421 (331–483)	0.0024
			Post	326 (257–365)	
ROS (Gra)	relative to control	17	Pre	1.31 (1.13–2.43)	0.0267
			Post	1.19 (1.02–1.58)	
Dose of RBC transfusion requirements	units	22	Pre	6 (2–8)	0.0053
			Post	1 (0–4)	
CRP	mg/dL	22	Pre	0.37 (0.13–1.13)	0.195
			Post	0.13 (0.02–0.47)	
Ferritin	ng/mL	18	Pre	695.46 (364.40–1,237.24)	0.3247
			Post	647.40 (287.01–1,144.30)	
LDH	U/L	22	Pre	261 (198–316)	<0.0001
			Post	208 (177–225)	

Abbreviations are the same as those in Table 1.

Table 5. Changes in clinical parameters and oxidative stress markers during azacitidine treatment by *WT1* trajectory group

Clinical parameter	*WT1* trajectory group	n	Pretreatment Median (IQR)	Post-treatment Median (IQR)	*p* value
WT1 mRNA (copies/μg RNA)	*WT1*-decrease	16	4,150 (695–33,000)	510 (113–2,875)	<0.0001
	WT1-nondecrease	6	160 (110–1,103)	840 (173–5,850)	0.0313
d-ROMs (U. CARR)	*WT1*-decrease	16	421 (343–509)	332 (290–373)	0.0182
	WT1-nondecrease	6	406 (333–460)	304 (257–333)	0.0938
ROS (Gra) (relative to control)	*WT1*-decrease	12	1.87 (1.19–4.11)	1.29 (1.10–1.66)	0.0342
	WT1-nondecrease	5	1.19 (1.11–1.31)	0.87 (0.64–1.05)	0.625
RBC transfusion requirements (units/month)	*WT1*-decrease	16	7 (5–9)	0 (0–5)	0.0073
	WT1-nondecrease	6	1 (0–5)	2 (1–4)	0.6875
CRP (mg/dL)	*WT1*-decrease	16	0.45 (0.25–1.23)	0.10 (0.03–0.58)	0.2293
	WT1-nondecrease	6	0.20 (0.09–0.49)	0.25 (0.10–0.44)	0.875
Ferritin (ng/mL)	*WT1*-decrease	12	725.18 (391.17–1,228.62)	872.14 (624.74–1,550.75)	0.6772
	WT1-nondecrease	6	360.40 (287.01–498.62)	415.40 (315.74–1,566.98)	0.4375
LDH (U/L)	*WT1*-decrease	16	270 (202–312)	199 (178–223)	0.0003
	WT1-nondecrease	6	220 (198–300)	223 (186–258)	0.4375

Data are presented as median (interquartile range). *P* values are for within-group comparisons between pretreatment and post-treatment values using the Wilcoxon signed-rank test. Abbreviations are the same as those in Table 1.

Table 6. Changes in clinical parameters and oxidative stress markers from pretreatment to Cycle 3 of azacitidine treatment by *WT1* trajectory group

Clinical parameter	*WT1* trajectory group	n	Pretreatment Median (IQR)	Cycle 3 Median (IQR)	*p* value
WT1 mRNA (copies/μg RNA)	*WT1*-decrease	16	4,150 (695–33,000)	540 (124–2,900)	0.0004
	WT1-nondecrease	6	160 (110–1,103)	690 (263–12,218)	0.0625
d-ROMs (U. CARR)	*WT1*-decrease	16	421 (343–509)	361 (311–396)	0.0833
	WT1-nondecrease	6	406 (333–460)	387 (302–416)	0.4375
ROS (Gra) (relative to control)	*WT1*-decrease	12	1.87 (1.19–4.11)	1.63 (1.34–2.76)	0.4238
	WT1-nondecrease	5	1.19 (1.11–1.31)	1.62 (1.03–2.84)	0.4375
RBC transfusion requirements (units/month)	*WT1*-decrease	16	7 (5–9)	2 (0–5)	0.0067
	WT1-nondecrease	6	1 (0–5)	1 (0–4)	0.6875
CRP (mg/dL)	*WT1*-decrease	16	0.45 (0.25–1.23)	0.24 (0.03–1.12)	0.2769
	WT1-nondecrease	6	0.20 (0.09–0.49)	0.36 (0.16–0.54)	0.1875
Ferritin (ng/mL)	*WT1*-decrease	10	720.70 (286.32–1,316.09)	964.87 (546.18–1,337.23)	0.8457
	WT1-nondecrease	5	402.69 (326.10–530.59)	405.94 (343.24–476.97)	0.8125
LDH (U/L)	*WT1*-decrease	16	270 (202–312)	182 (173–224)	0.0010
	WT1-nondecrease	6	220 (198–300)	193 (179–206)	0.4375

Data are presented as median (interquartile range). Only paired pretreatment and Cycle 3 observations were included for each parameter. *P* values are for within-group comparisons using exact two-sided Wilcoxon signed-rank tests. *WT1* trajectory groups were defined using the selected post-treatment *WT1* mRNA assessment, as in Table 5, and the same group assignments were retained for the Cycle 3 analysis. Abbreviations are the same as those in Table 1.

Table 7. Comparison of changes between the *WT1*-decrease and *WT1*-nondecrease groups

Variable	*WT1*-decrease group		*WT1*-nondecrease group		Between-group difference (95% CI)	Exact *p* value
	n	Median (IQR)	n	Median (IQR)		
Change in d-ROMs, U.CARR	16	116 (−37.5 to 180)	6	80 (8.5 to 162)	−11 (−120 to 140)	0.9144
Change in granulocyte ROS ratio	12	0.769 (−0.190 to 2.141)	5	0.255 (−0.263 to 0.698)	0.623 (−0.602 to 2.716)	0.3284
Change in RBC transfusion requirements units/month	16	3 (1.5 to 8)	6	1 (−1.5 to 3.5)	3 (−2 to 8)	0.2114
Change in LDH, U/L	16	65.5 (28.5 to 104.8)	6	21 (1.5 to 21)	44.5 (−9 to 105)	0.0828

Data are presented as median (interquartile range).

Change was calculated as pretreatment minus post-treatment; positive values indicate decreases during AZA treatment.

Between-group differences are Hodges–Lehmann estimates of the location shift (*WT1*-decrease minus *WT1*-nondecrease) with 95% confidence intervals.

P values were calculated using exact two-sided Wilcoxon rank-sum tests. No adjustment for multiple testing was performed; all analyses were exploratory. Abbreviations are the same as those in Table 1.