ORIGINAL ARTICLE

Demethylation and Up-Regulation of an Oncogene after Hypomethylating Therapy

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ABSTRACT

BACKGROUND

Although hypomethylating agents are currently used to treat patients with cancer, whether they can also reactivate and up-regulate oncogenes is not well elucidated.

METHODS

We examined the effect of hypomethylating agents on *SALL4*, a known oncogene that plays an important role in myelodysplastic syndrome and other cancers. Paired bone marrow samples that were obtained from two cohorts of patients with myelodysplastic syndrome before and after treatment with a hypomethylating agent were used to explore the relationships among changes in *SALL4* expression, treatment response, and clinical outcome. Leukemic cell lines with low or undetectable *SALL4* expression were used to study the relationship between *SALL4* methylation and expression. A locus-specific demethylation technology, CRISPR–DNMT1-interacting RNA (CRISPR-DiR), was used to identify the CpG island that is critical for *SALL4* expression.

RESULTS

SALL4 up-regulation after treatment with hypomethylating agents was observed in 10 of 25 patients (40%) in cohort 1 and in 13 of 43 patients (30%) in cohort 2 and was associated with a worse outcome. Using CRISPR-DiR, we discovered that demethylation of a CpG island within the 5′ untranslated region was critical for *SALL4* expression. In cell lines and patients, we confirmed that treatment with a hypomethylating agent led to demethylation of the same CpG region and up-regulation of *SALL4* expression.

CONCLUSIONS

By combining analysis of patient samples with CRISPR-DiR technology, we found that demethylation and up-regulation of an oncogene after treatment with a hypomethylating agent can indeed occur and should be further studied. (Funded by Associazione Italiana per la Ricerca sul Cancro and others.)

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Interest in epigenetic targeting in cancer treatment is increasing. One example is the use of DNA hypomethylating agents. Two hypomethylating agents, 5-aza-2′-deoxycytidine (decitabine) and 5-azacytidine, are being used clinically. There are many mechanisms of action of these drugs, including irreversibly binding to and inhibiting DNA methyltransferases,[1-4] which result in hypomethylation and up-regulation of genes such as tumor suppressors.[5] Other major mechanisms of action include incorporation of the hypomethylating agent into newly synthesized DNA, which triggers a DNA-damage response and leads to cytotoxic effects in cancer cells,[6,7] as well as alteration of immune responses.[8-10]

We hypothesized that the global effect of treatment with hypomethylating agents would not only contribute to the demethylation of tumor-suppressor genes but may also induce demethylation of oncogenes. To test this theory, we focused on candidate genes with the following features: they are aberrantly expressed in human cancers; they are functionally proved to be oncogenes with the use of methods such as murine models; and DNA methylation may be important for the expression of these genes. This led to our current study, in which we examined how hypomethylating agents could activate the known oncofetal protein, spalt-like transcription factor 4 (SALL4), in patients with myelodysplastic syndrome, as a model to examine the effects of hypomethylating agents on up-regulation of oncogenes.

SALL4 plays an essential role in myelodysplastic syndrome and acute myeloid leukemia leukemogenesis[11] and tumorigenesis in several solid tumors, including germ-cell tumors, hepatocellular carcinoma, breast cancer, and lung cancer.[12-14] During development, SALL4 maintains the self-renewal and pluripotency of embryonic stem cells,[15] but it is normally repressed in most adult organs with the exception of germ cells and CD34+ hematopoietic stem cells.[16] *SALL4* is reactivated or aberrantly expressed in a multitude of cancers and has been identified by meta-analysis as a poor prognostic factor. Mechanistically, up-regulation of SALL4 promotes proliferation, metastasis, and drug resistance of cancer cells,[17,18] through a number of mechanisms, such as repression of tumor-suppressor genes[19] and activation of other oncogenes.[20] In hematologic cancers, *SALL4* is aberrantly expressed in high-risk myelodysplastic syndrome,[21] acute myeloid leukemia,[11,20] the blast phase of chronic myeloid leukemia,[22] and precursor B-cell lymphoblastic leukemia or lymphoma.[23] In a murine model with constitutive *SALL4* expression, mice had myelodysplastic syndrome–like features and subsequently leukemic transformation through activation of the Wnt–beta-catenin pathway.[24] Methylation is known to regulate *SALL4* expression and reactivation in solid tumors.[25,26] The expression of *SALL4* in hematologic cancers may also be related to its DNA methylation status. The effect of hypomethylating agents on *SALL4* expression and its clinical implications are unknown.

Hypomethylating agents are now being used to treat blood cancers as well as solid tumors. Because they were first approved by the Food and Drug Administration (FDA) to treat patients with myelodysplastic syndrome more than a decade ago, we therefore retrospectively analyzed *SALL4* expression and clinical survival among 68 patients with myelodysplastic syndrome in two cohorts (25 patients in cohort 1 and 43 patients in cohort 2) with bone marrow samples obtained before and after treatment with a hypomethylating agent. Myelodysplastic syndrome comprises heterogeneous myeloid disorders characterized by cytopenias and dysplasia in peripheral blood and bone marrow, with ineffective hematopoiesis and a variable risk of leukemic transformation.[27,28] To define the mechanisms of altered expression of *SALL4* in response to treatment with a hypomethylating agent, we used a CRISPR-DiR (clustered regularly interspaced short palindromic repeats–DNA methyltransferase 1 [DNMT1]–interacting RNA) approach. First, we identified and demethylated the CpG island that is critical for *SALL4* expression. We then evaluated the effects of treatment with a hypomethylating agent on the methylation status of *SALL4* in samples obtained from patients with myelodysplastic syndrome.

METHODS

PATIENTS AND SAMPLE COLLECTION

Bone marrow samples in cohort 1 were obtained from 37 patients with newly diagnosed myelodysplastic syndrome who were enrolled in the BMT-AZA trial.[29,30] The current study was over-

seen and approved by the University of Rome Tor Vergata. Written informed consent was obtained from the patients before inclusion in the study. CD34– bone marrow mononuclear cells obtained from 10 healthy donors and CD34+ cells obtained from 5 healthy donors were used as control cohorts. Of the 37 patients with myelodysplastic syndrome, 25 had paired bone marrow samples collected before and after four cycles of azacytidine. In cohort 2, a total of 43 bone marrow samples were obtained from the Institute of Hematology and Blood Diseases Hospital in Tianjin, China, before or after three to five cycles of treatment with a hypomethylating agent. Patient samples were obtained with written informed consent in accordance with the Declaration of Helsinki and approval of the human research ethics committee at the Institute of Hematology and Blood Disease Hospital, Chinese Academy of Medical Sciences. Bone marrow mononuclear cells were isolated by Ficoll gradient centrifugation with the use of Lympholyte-H (Cedarlane), in accordance with the instructions of the manufacturer. Myelodysplastic syndrome was diagnosed in accordance with the 2016 World Health Organization (WHO) classification.[31] Other clinical characteristics, including age at diagnosis, sex, Revised International Prognostic Scoring System risk status, WHO Classification-based Prognostic Scoring System risk status, response after treatment with a hypomethylating agent, peripheral-blood counts, bone marrow blast counts, and cytogenetic features, were also reviewed. The definition of first response follows the International Working Group criteria.[32] Patients who had a response included those having complete remission, partial remission, or hematologic improvement, whereas patients who did not have a response included those with stable disease or progressive disease.

STATISTICAL ANALYSIS

Clinical characteristics and mutational profiling are presented as the number and percentage of patients. Data are presented as medians and interquartile ranges for skewed data. Categorical variables were compared with the use of Fisher's exact test or the chi-square test, as appropriate, and continuous variables were compared with the use of the Wilcoxon rank-sum test. We also used the Wilcoxon rank-sum test for paired observations and factor change in *SALL4* messenger RNA (mRNA) levels in patients who had a response as compared with those who did not have a response.

Progression-free survival was defined as the time from treatment with a hypomethylating agent to disease progression or death from the treatment, and overall survival was defined as time from treatment with a hypomethylating agent to death. Overall and progression-free survival were analyzed with the use of the Kaplan–Meier product-limit method with censoring for patients who did not have disease progression or who did not die during the treatment period. A log-rank test was used to compare survival curves for statistical significance. Hazard ratios and 95% confidence intervals were calculated with the use of a Cox proportional-hazards model. All prognostic factors with a P value of less than 0.1 in the univariate model were further entered into the multivariate analysis.

All statistical testing was performed with the use of two-tailed tests; a P value of less than 0.05 was considered to indicate statistical significance. All analyses were performed with the use of SPSS statistical software, version 22 (SPSS). A droplet digital polymerase-chain-reaction (ddPCR) experiment was repeated three times for each group of experiments, and Student's t-test was used. Additional materials are described in the Supplementary Appendix, available with the full text of this article at NEJM.org.

RESULTS

HYPOMETHYLATING AGENTS AND UP-REGULATION OF *SALL4*

To evaluate the effect of hypomethylating agents on *SALL4* expression after treatment, we first measured the baseline *SALL4* expression at diagnosis in bone marrow mononuclear cells obtained from patients with myelodysplastic syndrome before azacytidine treatment in cohort 1. Levels of *SALL4* mRNA were significantly higher in 37 patients with myelodysplastic syndrome than in healthy donors (P=0.002) (Fig. S1A in the Supplementary Appendix), a finding similar to what has been reported previously.[21] In cohort 1, a total of 25 patients had available paired bone marrow samples at diagnosis and after four cycles of azacytidine. Of these 25 patients, 12 (48%) were classified as having had a response: 9 (36%) had a complete response, 2 (8%)

had a partial response, and 1 (4%) had hematologic improvement. The remaining 13 patients (52%) were classified as not having had a response: 10 (40%) had stable disease, and 3 (12%) had progressive disease. Baseline *SALL4* expression at diagnosis did not differ substantially among these groups. In cohort 2, of the 43 patients, 20 (47%) were classified as having had response: 15 (35%) had a complete response, and 5 (12%) had hematologic improvement. The remaining 23 patients (53%) were classified as not having had a response: 18 (42%) had stable disease, and 5 (12%) had progressive disease.

These 68 patients were then stratified according to the factor change in *SALL4* mRNA expression before and after treatment with a hypomethylating agent. A waterfall plot depicting the factor changes in *SALL4* mRNA levels showed that patients can be separated into two groups, one with *SALL4* up-regulation and the other with *SALL4* down-regulation. In cohort 1, a total of 10 of the 25 patients (40%) had an increase in *SALL4* expression and 15 patients (60%) had a decrease in *SALL4* expression after four cycles of azacytidine treatment (Fig. 1A). The median $\log_2$ factor change was 2.78 (interquartile range, 2.15 to 5.65) in patients with *SALL4* up-regulation and –2.25 (interquartile range, –1.26 to –4.45) in those with *SALL4* down-regulation. In cohort 2, a total of 13 patients (30%) had *SALL4* up-regulation and 30 patients (70%) had *SALL4* down-regulation after three to five cycles of treatment with a hypomethylating agent (Fig. 1B). The median $\log_2$ factor change was 1.99 (interquartile range, 0.70 to 3.05) in patients with *SALL4* up-regulation and –1.99 (interquartile range, –0.84 to –2.71) in those with *SALL4* down-regulation. No significant difference in the factor change in *SALL4* mRNA was noted between patients who had a response and those who did not have a response in cohort 1 (Fig. S1B) and cohort 2 (Fig. S1C).

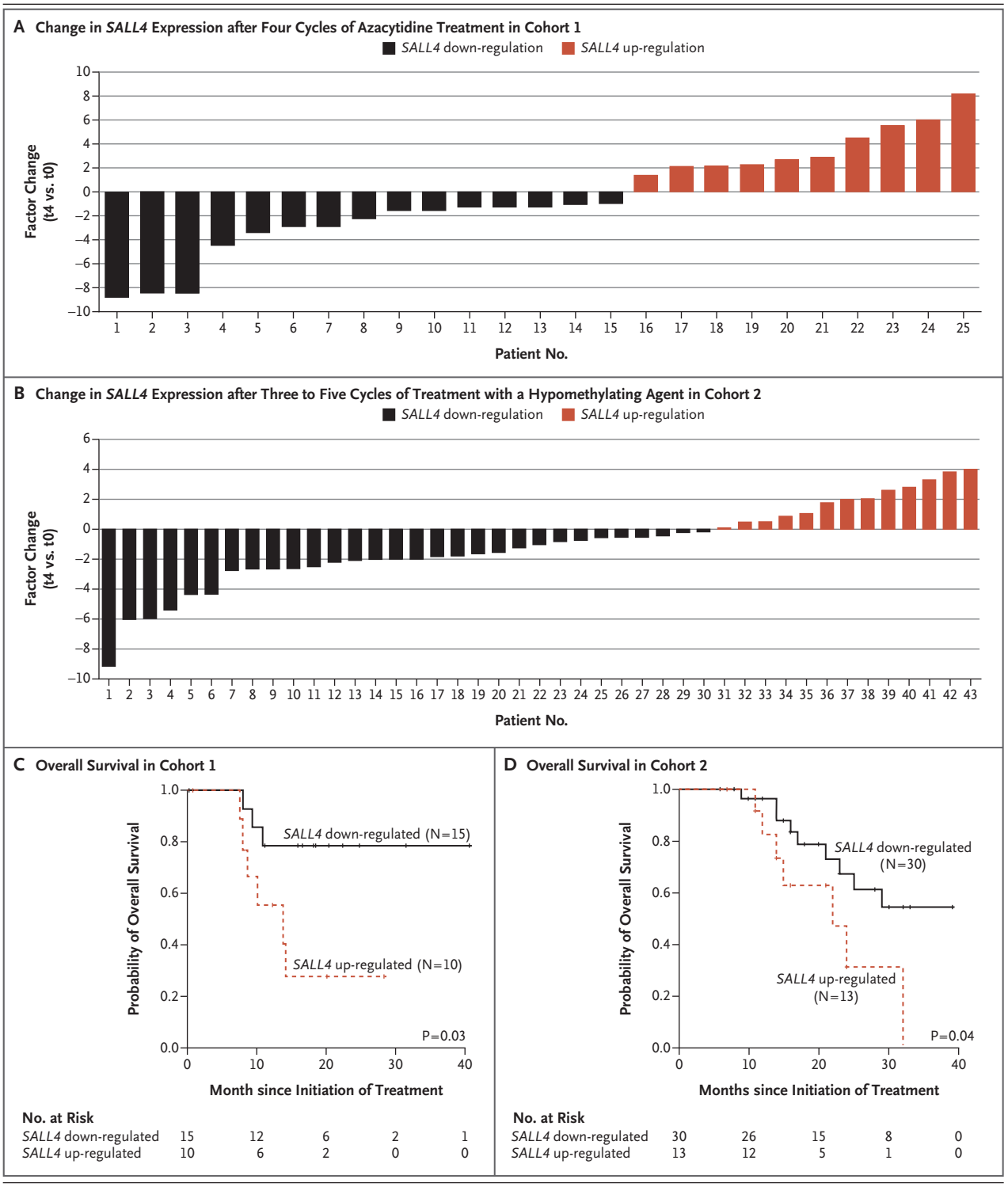
When overall survival was compared between the patients with *SALL4* up-regulation and those with *SALL4* down-regulation in both cohort 1 and cohort 2, those with *SALL4* up-regulation had poorer overall survival than those with *SALL4* down-regulation ($P=0.03$ in cohort 1 and $P=0.04$ in cohort 2) (Fig. 1C and 1D). These findings indicate that *SALL4* up-regulation may be associated with worse survival.

The demographic and clinical characteristics of the patients with *SALL4* up-regulation and those with *SALL4* down-regulation are shown in Tables 1 and 2. The median follow-up after the initiation of therapy was 14.1 months (interquartile range, 9.0 to 20.4) in cohort 1 and 17.0 months (interquartile range, 12.0 to 28.0) in cohort 2. We further conducted a Cox proportional-hazards model analysis of prognostic factors for overall survival on the basis of patient characteristics at diagnosis, mutational profile,[30] and *SALL4* expression changes. In a multivariate analysis, *SALL4* up-regulation was a common independent negative predictor of overall survival (cohort 1: hazard ratio for death, 6.48; 95% confidence interval [CI], 1.06 to 39.67; cohort 2: hazard ratio, 2.74; 95% CI, 0.93 to 8.03) (Tables S1 and S2). In addition, the presence of a *RUNX1* mutation in cohort 1 and the lack of a treatment response in cohort 2 were also negative predictors of overall survival in a multivariate analysis.

Because somatic mutations, including epigenetic factors such as *DNMT3A*, *IDH1*, *IDH2*, and *TET2*, are common in patients with myelodysplastic syndrome,[33] we evaluated whether *SALL4* up-regulation or down-regulation in patients after treatment was related to their preexisting mutation status. Common somatic mutations in 30 genes, known to be frequently mutated in patients with myelodysplastic syndrome, are reported in Figure S3. At diagnosis, we identified at least one mutation in 22 of 25 patients (88%) in cohort 1 and in 32 of 41 patients (78%) in cohort 2. A total of 19 of 22 patients (86%) in cohort 1 and 16 of 32 patients (50%) in cohort 2 had more than one gene mutation. From these observations, it appears that *SALL4* up-regulation or down-regulation in patients after treatment with a hypomethylating agent was not related to their preexisting mutation status.

DEMETHYLATION OF A CRITICAL CPG REGION AND UP-REGULATION OF *SALL4*

We next investigated the mechanisms of *SALL4* up-regulation after treatment with a hypomethylating agent. One possibility is that such treatment can lead to demethylation of a CpG island that is critical for *SALL4* expression. The differential methylation of the *SALL4* locus has been observed in K562-induced pluripotency reprogrammed cells.[34] The major CpG island of *SALL4* is located across the 2000-bp locus, including 5′ untranslated region (5′UTR)–Exon 1–Intron 1.



Within this CpG island, methylation of 5′UTR–Exon 1–Intron 1, a specific 500-bp DNA segment, was further observed to be negatively correlated with *SALL4* expression in patients with hepatocellular carcinoma and in hepatocellular-carcinoma cell lines[25] (Fig. 2A). We hypothesized that demethylation of this region could lead to up-regulation of *SALL4*.

Figure 1 (facing page). Increased *SALL4* Expression in Patients with Myelodysplastic Syndrome after Treatment with a Hypomethylating Agent.

Panel A shows a waterfall plot of the $\log_2$ factor change in *SALL4* expression in 25 patients after four cycles of azacytidine treatment in cohort 1. Panel B shows a waterfall plot of the $\log_2$ factor change in *SALL4* expression in 43 patients after three to five cycles of treatment with a hypomethylating agent in cohort 2. The term t0 denotes before any cycles of treatment, and t4 after four cycles of treatment. Panel C shows overall survival among patients with *SALL4* up-regulation and those with *SALL4* down-regulation in cohort 1, and Panel D shows the corresponding data in cohort 2. In Panels C and D, tick marks indicate censored data. A log-rank test was used to compare survival curves for statistical significance in Panels C and D.

To localize the critical CpG region responsible for regulation of *SALL4* expression and to test the causal relationship between DNA methylation of *SALL4* and RNA expression, we applied the CRISPR-DiR technique.[35] This approach uses induction of locus-specific demethylation by blocking DNMT1 activity[36] in cell lines with undetectable or low *SALL4* expression.

In the CRISPR-DiR method, two loops of the guide RNA that are not required for guide function have been replaced with sequences that specifically interact with and inhibit DNMT1.[35,36] This modified guide RNA can achieve site-specific demethylation.[35] We tested several site-specific guide RNAs around the major CpG island of *SALL4*, and only sgSALL4_1 (named as DiR_SALL4 here), targeting around the 5′UTR CpG 11 region, showed effective demethylation ability.[25] We further monitored the methylation of the 5′UTR CpG region in HL-60 cells (with no or low *SALL4* expression) after treatment with CRISPR-DiR (Fig. 2A). On transduction of HL-60 cells with DiR_SALL4, substantial demethylation changes were observed after 8 days (Fig. 2B) as well as increased SALL4 transcript levels (Fig. 2C), as compared with a control scrambled guide RNA (DiR-NT). Similar demethylation results by DiR_SALL4 were observed in other SNU387 cells expressing low or no *SALL4*, whereas CRISPR-DiRs targeting neighboring regions could not demethylate.[25] These findings show that demethylation of this region by *SALL4* locus-specific CRISPR-DiR can lead to up-regulation of *SALL4*.

HYPOMETHYLATING AGENTS AND DEMETHYLATION OF *SALL4*

We then tested whether *SALL4* could be up-regulated by hypomethylating agents. Using HL-60 cells, we first evaluated the dynamics of *SALL4* mRNA levels through a cycle of decitabine, using a dosage range of 100 to 500 nmol per liter. The number of mRNA copies per cell (assessed by means of ddPCR assay) and protein level (assessed by means of Western blot analysis) for SALL4 was measured at day 5. We noticed a dose-dependent up-regulation of *SALL4* mRNA expression at day 5 (Fig. 3A and B).

We next examined the methylation status of the *SALL4* 5′UTR CpG island region after decitabine treatment at a dose of 250 nmol per liter and found that this region was demethylated in HL-60 cells, in accordance with our CRISPR-DiR result (Fig. 3C). Similar results were also observed in another *SALL4*-low leukemic cell line, K562 (Fig. S4A, S4B, and S4C).

We next examined the methylation changes in bone marrow samples from patients with myelodysplastic syndrome before and after treatment with a hypomethylating agent. In six patients with *SALL4* up-regulation, we noticed decreased methylation at the critical 5′UTR CpG region (Fig. 3D and 3E). Conversely, we did not observe changes in the methylation at the same CpG region in nine patients with *SALL4* down-regulation after treatment with a hypomethylating agent (Fig. 3D and 3E).

DISCUSSION

Since its first approval by the FDA to treat myelodysplastic syndrome in 2004, hypomethylating-agent therapy has been used in patients with hematologic cancers and solid tumors. In this study, we were interested in investigating whether hypomethylating agents could activate oncogenes as an unintended consequence. We used myelodysplastic syndrome as the disease model to test our hypothesis because of the long-standing use of hypomethylating-agent therapy in this patient population and because *SALL4* has been shown to act as an oncogene in experimental animal models of myelodysplastic syndrome and acute myeloid leukemia. Although continuous hypomethylating-agent therapy in patients with myelodysplastic syndrome who had a response could improve their clinical characteristics, over-

Table 1. Baseline Characteristics of 25 Patients Treated with a Hypomethylating Agent in Cohort 1.*

Characteristic	*SALL4* Up-Regulation (N=10)	*SALL4* Down-Regulation (N=15)
Median age (IQR) — yr	59.6 (46.2–61)	59.5 (45–61.5)
Sex — no. (%)		
Male	9 (90)	8 (53)
Female	1 (10)	7 (47)
IPSS risk status — no. (%)		
Low or intermediate-1	1 (10)	1 (7)
Intermediate-2 or high	9 (90)	13 (87)
WPSS risk status — no. (%)		
Very low or low	1 (10)	0
Intermediate	1 (10)	2 (13)
High or very high	8 (80)	10 (67)
IPSS-R risk status — no./total no. (%)		
Very low	0/9	1/13 (8)
Low	0/9	1/13 (8)
Intermediate	2/9 (22)	2/13 (15)
High	3/9 (33)	2/13 (15)
Very high	4/9 (44)	7/13 (54)
Treatment response — no. (%)		
Yes	5 (50)	7 (47)
No	5 (50)	8 (53)
Median white-cell count (IQR) — per mm^3	2450 (1900–4015)	3320 (2280–10,410)
Median absolute neutrophil count (IQR) — per mm^3	400 (215–1490)	700 (400–2000)
Median hemoglobin level (IQR) — g/dl	9.9 (8.6–10.6)	9.4 (8.7–11.5)
Median platelet count (IQR) — per mm^3	49,500 (26,750–164,250)	77,000 (49,000–129,000)
Median percentage of blasts in bone marrow aspirate (IQR)	13 (11–15)	12 (3.8–17.8)
Cytogenetic features of MDS — no. (%)†		
Good karyotype	4 (40)	5 (33)
Intermediate karyotype	2 (20)	2 (13)
Poor karyotype	3 (30)	6 (40)
Mutational profiling — no. (%)‡		
TET2 mutation	1 (10)	6 (40)
ASXL1 mutation	3 (30)	6 (40)
RUNX1 mutation	2 (20)	4 (27)
SETBP1 mutation	2 (20)	4 (27)
TP53 mutation	3 (30)	2 (13)
ZRSF2 mutation	4 (40)	1 (7)
DNMT3A mutation	1 (10)	3 (20)
SRSF2 mutation	1 (10)	3 (20)
SF3B1 mutation	1 (10)	2 (13)
U2AF1 mutation	3 (30)	0
CEBPA mutation	0	3 (20)

Table 1. (Continued.)

Characteristic	*SALL4* Up-Regulation (N=10)	*SALL4* Down-Regulation (N=15)
CBL mutation	1 (10)	1 (7)
ETV6 mutation	0	2 (13)
IDH2 mutation	1 (10)	1 (7)
NRAS mutation	0	2 (13)
CSF3R mutation	1 (10)	0
JAK2 mutation	1 (10)	0

* IPSS denotes International Prognostic Scoring System, IPSS-R Revised IPSS, IQR interquartile range, MDS myelodysplastic syndrome, and WPSS World Health Organization Classification-based Prognostic Scoring System.
† Three patients (one with *SALL4* up-regulation and two with *SALL4* down-regulation) had no metaphase of cytogenetic analysis.
‡ No mutations in *ABL1*, *BRAF*, *CALR*, *EZH2*, *FLT3*, *HRAS*, *IDH1*, *KIT*, *KRAS*, *MPL*, *NPM1*, *PTPN11*, and *WT1* were observed in either group.

all survival after such therapy in a "real-world" evaluation of patients with high-risk myelodysplastic syndrome or low-blast-count acute myeloid leukemia was lower than that expected in clinical trials,[37] and the average prognosis after failure of a hypomethylating agent was less than 6 months.[38] The mechanisms for primary and secondary drug resistance to hypomethylating agents and associated prognostic factors are still under active investigation.

In our two cohorts of patients with myelodysplastic syndrome from distinct ethnic backgrounds, we observed that after treatment with a hypomethylating agent, 23 of 68 patients (34%) had *SALL4* up-regulation and 45 (66%) had *SALL4* down-regulation. We observed that a poor long-term outcome correlates with *SALL4* up-regulation. The important role of SALL4 in myelodysplastic syndrome and acute myeloid leukemia has been shown in previous studies.[11] In SALL4 transgenic (Tg) mice, ineffective hematopoiesis and myelodysplastic syndrome–like features were observed: increased apoptosis with decreased complete blood-cell counts and abnormal morphologic features of blood cells. Impairment of DNA-damage repair was also noted in these Tg mice. Therefore, we hypothesize that up-regulation of SALL4 can lead to defective hematopoiesis, accumulation of mutations, and progression to acute myeloid leukemia, which may in part explain the poorer prognosis in patients with myelodysplastic syndrome and *SALL4* up-regulation. Identifying other oncogenes that are similarly activated by hypomethylating-agent therapy and evaluation of these and *SALL4* expression in larger prospective trial samples in the future will be necessary to validate this notion.

Another unique aspect of *SALL4* is that its expression is associated with DNA methylation status. Previously, a major challenge in the field of DNA methylation was to show a causal relationship between the DNA methylation and gene expression at a locus-specific and cellular level. Recent advances in functional genomic approaches such as the use of the CRISPR–Cas9 nuclease system have provided us with additional tools to address this question. Using the locus-specific CRISPR-DiR approach, we have identified a critical CpG island responsible for *SALL4* expression and, more importantly, shown that treatment with a hypomethylating agent leads to demethylation of this region and up-regulation of *SALL4*. Demethylation of this same region was observed in patients with myelodysplastic syndrome having increased *SALL4* expression after hypomethylating-agent therapy. These observations support our hypothesis that treatment with a hypomethylating agent can up-regulate oncogenes such as *SALL4*.

It is unclear why only a subgroup of patients had up-regulation of *SALL4* after treatment with a hypomethylating agent, and future studies will be necessary to fully understand the mechanism or mechanisms. It is possible that DNA demethylation is the first step for gene activation, and additional chromatin remodeling and interaction between enhancer and promoter are needed to maintain gene expression. For example, in a re-

Table 2. Baseline Characteristics of 43 Patients Treated with a Hypomethylating Agent in Cohort 2.

Characteristics	*SALL4* Up-Regulation (N=13)	*SALL4* Down-Regulation (N=30)
Median age (IQR) — yr	65 (60–69)	62.5 (59.8–67.5)
Sex — no. (%)		
Male	11 (85)	17 (57)
Female	2 (15)	13 (43)
IPSS risk status — no./total no. (%)		
Low or intermediate-1	8/11 (73)	17/26 (65)
Intermediate-2 or high	3/11 (27)	9/26 (35)
WPSS risk status — no./total no. (%)		
Very low or low	1/11 (9)	2/26 (8)
Intermediate	2/11 (18)	9/26 (35)
High or very high	8/11 (73)	15/26 (58)
IPSS-R risk status — no./total no. (%)		
Very low	0/13	1/26 (4)
Low	1/13 (8)	1/26 (4)
Intermediate	4/13 (31)	7/26 (27)
High	6/13 (46)	13/26 (50)
Very high	2/13 (15)	4/26 (15)
Treatment response — no. (%)		
Yes	5 (38)	15 (50)
No	8 (62)	15 (50)
Median white-cell count (IQR) — per mm^3	2220 (1310–4310)	2180 (1830–2750)
Median absolute neutrophil count (IQR) — per mm^3	780 (330–2240)	900 (630–1370)
Median hemoglobin level (IQR) — g/dl	7.0 (6.2–10.2)	8.1 (7.0–9.6)
Median platelet count (IQR) — per mm^3	47,000 (31,000–120,000)	70,000 (38,000–107,000)
Median percentage of blasts in bone marrow aspirate (IQR)	6 (5.5–10.8)	7.3 (5.4–11.1)
Cytogenetic features of MDS — no./total no. (%)*		
Good karyotype	10/11 (91)	22/26 (85)
Intermediate karyotype	1/11 (9)	0/26
Poor karyotype	0/11	4/26 (15)
Mutational profiling — no./total no. (%)†		
TET2 mutation	1/13 (8)	2/28 (7)
ASXL1 mutation	4/13 (31)	5/28 (18)
RUNX1 mutation	3/13 (23)	3/28 (11)
SETBP1 mutation	0/13	2/28 (7)
TP53 mutation	1/13 (8)	4/28 (14)
DNMT3A mutation	0/13	1/28 (4)
SRSF2 mutation	0/13	1/28 (4)
SF3B1 mutation	3/13 (23)	2/28 (7)
U2AF1 mutation	3/13 (23)	4/28 (14)
CEBPA mutation	0/13	1/28 (4)
IDH2 mutation	1/13 (8)	0/28

Table 2. (Continued.)

Characteristics	*SALL4* Up-Regulation (N=13)	*SALL4* Down-Regulation (N=30)
NRAS mutation	0/13	3/28 (11)
JAK2 mutation	1/13 (8)	1/28 (4)
EZH2 mutation	1/13 (8)	1/28 (4)
FLT3 mutation	1/13 (8)	0/28
IDH1 mutation	1/13 (8)	2/28 (7)
KRAS mutation	1/13 (8)	0/28
NPM1 mutation	0/13	2/28 (7)

* Two patients with *SALL4* down-regulation had no metaphase of cytogenetic analysis. Inadequate Giemsa banding in cytogenetic analysis was seen for one patient with *SALL4* up-regulation and two patients with *SALL4* down-regulation.
† No mutations in *ABL1*, *BRAF*, *CALR*, *CBL*, *CSF3R*, *ETV6*, *KIT*, *MPL*, *PTPN11*, *WT1*, and *ZRSF2* were observed in either group. Two patients with *SALL4* down-regulation did not undergo next-generation sequencing.

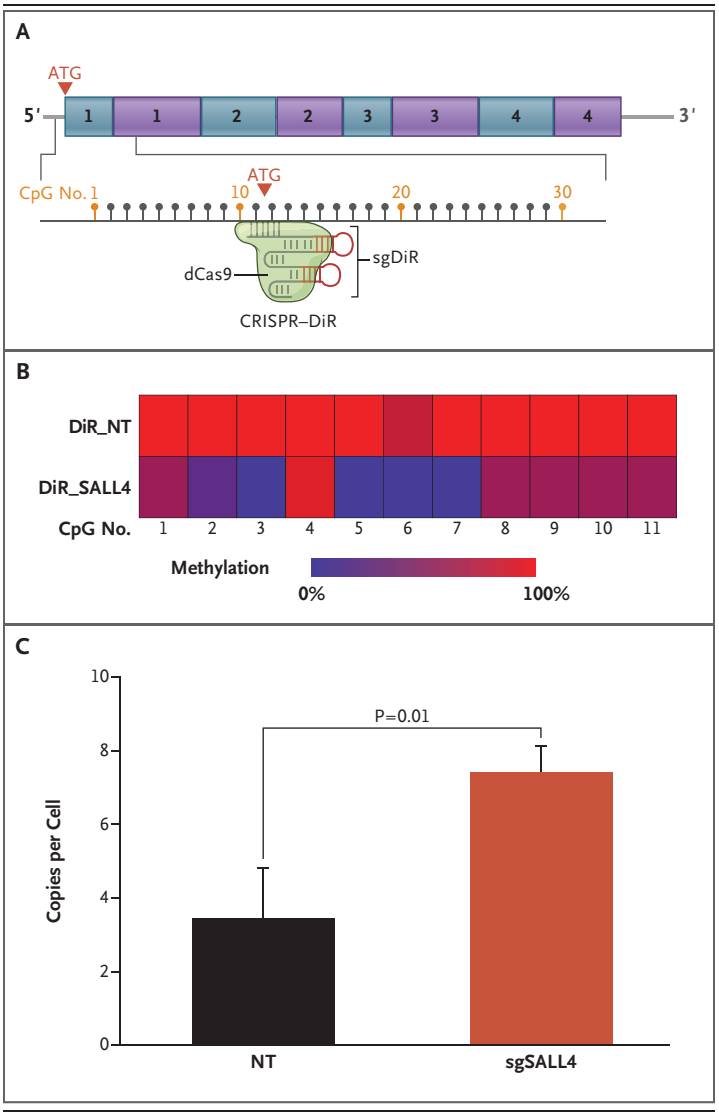


Figure 2. Demethylation of a Critical CpG Island and *SALL4* Expression in Leukemic Cells Treated with CRISPR-DiR.

Panel A shows the CpG region within *SALL4* 5′ untranslated region (5′UTR)–Exon 1–Intron 1. Depicted at the top of the diagram are the 30 CpG residues; depicted below are the four *SALL4* exons (in blue) and introns (in purple), with the location of the 30 CpG residues in the Exon 1–Intron 1 region. The location of the target of the guide RNA for CRISPR-DiR (clustered regularly interspaced short palindromic repeats–DNA methyltransferase 1 [DNMT1]–interacting RNA) is shown below, targeting CpG number 11. The term dCas9 denotes nuclease-dead Cas9, and sgDiR single-guide DNMT1-interacting RNA. Panel B shows bisulfite sequencing after CRISPR-DiR of HL-60 cells transduced with either a nontargeting negative control guide RNA (DiR_NT) or a targeting guide RNA (DiR_SALL4), leading to demethylation, with higher methylation indicated by redder shading. Panel C shows up-regulation of *SALL4* transcript (copies per cell assessed by droplet digital polymerase-chain-reaction [ddPCR] assay) in HL-60 cells treated with CRISPR-DiR 8 days after transduction of the CRISPR-DiR. A Wilcoxon rank-sum test was used to evaluate the statistical difference in copies per cell between NT (in three patients) and sgSALL4 (in three patients). Data are shown as means, and error bars indicate the standard deviation.

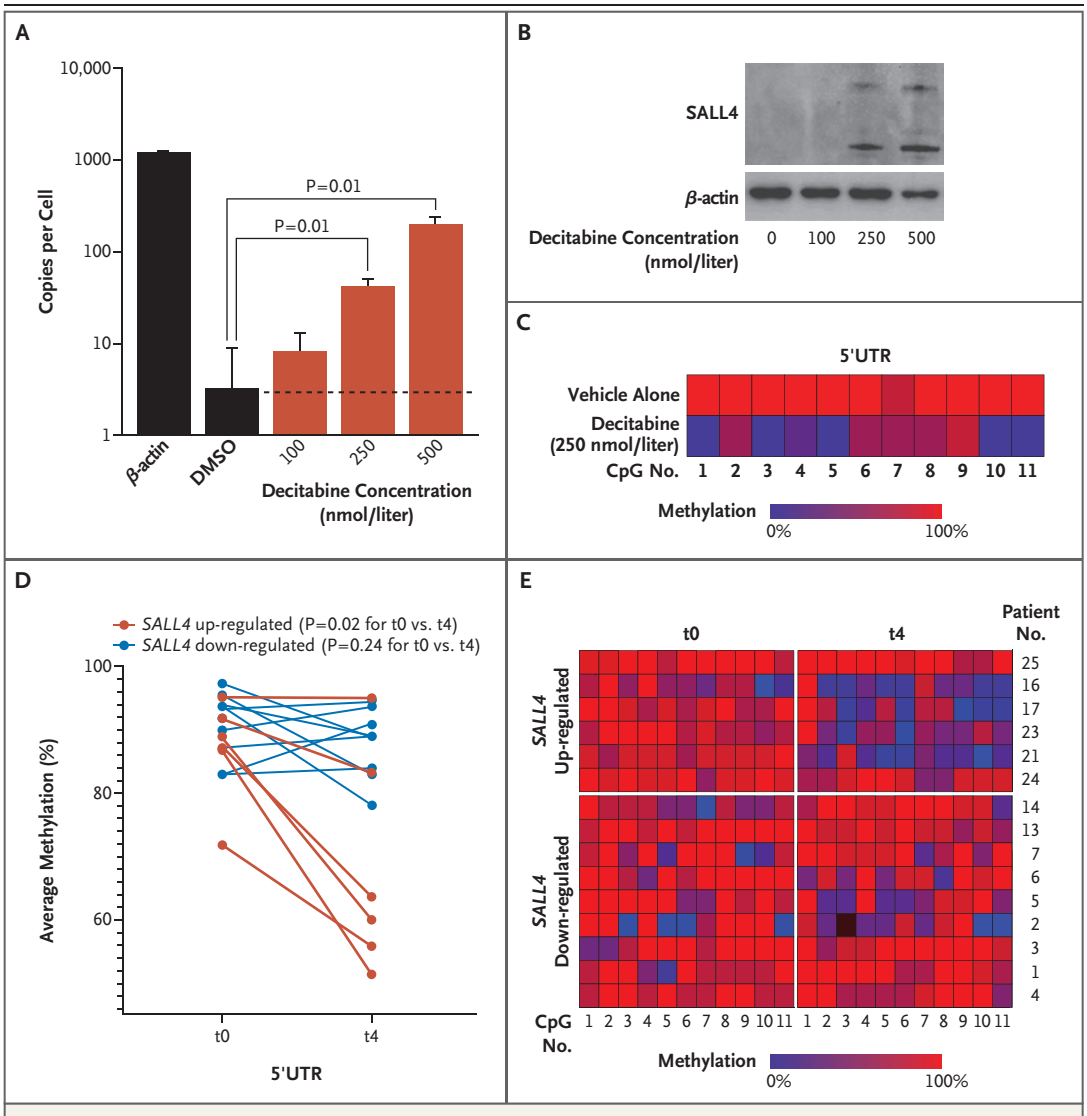


Figure 3. Demethylation of a Critical CpG Island and *SALL4* Expression in Leukemic Cells and Patients with Myelodysplastic Syndrome Treated with a Hypomethylating Agent.

Panel A shows SALL4 copies per cell assessed by ddPCR assay in HL-60 cells treated with decitabine. A Wilcoxon rank-sum test was used to evaluate the statistical difference in copies per cell between dimethylsulfoxide (DMSO) and decitabine treatments. Data are shown as means, and error bars indicate standard deviations of three biologic replicates. Panel B shows Western blot analysis of HL-60 cells treated with decitabine. Panel C shows methylation profiling in HL-60 cells treated with vehicle alone as compared with those treated with decitabine at a dose of 250 nmol per liter. Treatment with a hypomethylating agent leads to demethylation of *SALL4* in patients with myelodysplastic syndrome. Panel D shows average methylation across *SALL4* 5′UTR CpG loci (1 through 11) in patients with myelodysplastic syndrome before any cycles of azacytidine treatment (t0) and after four cycles of such treatment (t4). Red dots indicate patients with up-regulation of *SALL4* expression after azacytidine treatments, and blue dots indicate patients with down-regulation of *SALL4* after azacytidine treatments. Methylation values at t0 were compared with methylation values at t4 in each up- and down-regulated group separately. The average difference in methylation between the two time points was calculated with the use of a Wilcoxon matched-pairs signed-rank test (one-tailed) for each up- and down-regulated group separately. Panel E shows methylation changes in six patients with *SALL4* up-regulation and nine patients with *SALL4* down-regulation at t0 and t4.

cent study, we found that demethylation and subsequent binding of methylation-sensitive regulators could lead to long-range interactions between gene locus and distal regulatory elements, resulting in sustained up-regulation of gene expression.[35]

Using patient samples and a targeted demethylation assay, we found that monotherapy with a hypomethylating agent can activate or up-regulate oncogenes such as *SALL4*. Up-regulation of *SALL4* probably influences the clinical progression of the disease; similar biologic effects may accompany treatment with a hypomethylating agent in patients with cancers other than myelodysplastic syndrome. Although the up-regulation of *SALL4* may be associated with a worse prognosis, it may also provide an additional treatment option on the basis of *SALL4*-mediated cancer vulnerability. We are currently exploring the concept that if *SALL4* expression is up-regulated, a concomitant targeted therapy that directly or indirectly mitigates *SALL4* expression, function, or both could be added to the treatment plan.[39]

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APPENDIX

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