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Possible role of intragenic DNA hypermethylation in gene silencing of the tumor suppressor gene *NR4A3* in acute myeloid leukemia

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Abstract

Expression of the tumor suppressor gene *NR4A3* is silenced in the blasts of acute myeloid leukemia (AML), irrespective of the karyotype. Although the transcriptional reactivation of *NR4A3* is considered to have a broad-spectrum anti-leukemic effect, the therapeutic modalities targeting this gene have been hindered by our minimal understanding of the transcriptional mechanisms regulating its expression, particularly in human AML. Here we show the role of intragenic DNA hypermethylation in reducing the expression of *NR4A3* in AML. Bisulfite sequencing analysis revealed that CpG sites at the intragenic region encompassing exon 3 of *NR4A3*, but not the promoter region, are hypermethylated in AML cell lines and primary AML cells. A DNA methyltransferase inhibitor restored the expression of *NR4A3* following a reduction

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Potential conflict of interest

The authors have no competing financial interests to declare.

Authors contributions

R.S. performed the experiments, analyzed results, created the figures, and wrote the manuscript; T.M. conceived the project, performed the experiments, analyzed the results, secured funding, and wrote the manuscript; A.K. assisted with the experiments; K.C. analyzed TCGA data; M.T. analyzed results and provided critical suggestions to the project; S.K. assisted with the experiments, including gene transduction to leukemia cells; H.N. and I.Y. assisted with the experiments; E.T., C.K., Y.N., S.T., S.S., Y.T., N.M., C.O., E.S. and T.I. collected patient samples; D.S., A.I. and K.Y. provided a critical suggestion to the project; and C.N. directed the project and wrote the manuscript.

in DNA methylation levels at intragenic CpG sites. The *in silico* data revealed an enrichment of H3K4me1 and H2A.Z at exon 3 of *NR4A3* in human non-malignant cells but that was excluded specifically in leukemia cells with CpG hypermethylation. This suggests that exon 3 represents a functional regulatory element involved in the transcriptional regulation of *NR4A3*. Our findings improve the current understanding of the mechanism underlying *NR4A3* silencing and facilitate the development of *NR4A3*-targeted therapy.

Keywords

NR4A3; Tumor suppressor gene; Acute myeloid leukemia; DNA hypermethylation

1. Introduction

Nuclear family 4 subgroup A (NR4A) orphan nuclear receptors have no known natural ligands and consist of three mammalian members: 1) *NR4A1* (Nur77), 2) *NR4A2* (Nurr1), and 3) *NR4A3* (Nor1). All three members share common structural properties in their carboxyl-terminal ligand and central DNA binding domains, while their amino-terminal domains are highly divergent [1–3]. All three receptors are widely expressed in different types of tissues and a variety of physiological signals can induce their expression, leading to the activation of *NR4A* target genes related to the cell cycle, apoptosis, inflammation, atherogenesis, metabolism, or DNA repair in a stimulus- and cell context-dependent manner [1,3,4]. In addition, the NR4A receptors are involved in tumorigenesis [2–5].

NR4A1 and *NR4A3* are reportedly silenced in the blasts of patients with acute myeloid leukemia (AML) irrespective of the karyotype [6]. In line with this finding, *Nr4a1*$^{-/-}$/*Nr4a3*$^{-/-}$ mice rapidly develop AML within one month after birth [6]. These results suggest that *NR4A1* and *NR4A3* function as tumor suppressor genes in myeloid malignancies and that NR4A receptors have a crucial role in the pathogenesis of AML [6]. Thus, unveiling the molecular mechanisms that regulate NR4A expression in AML would facilitate the development of novel therapies, including the transcriptional reactivation of the gene. However, the therapeutic modalities targeting NR4A receptors have been hindered by our minimal understanding of the mechanisms underlying reduced *NR4A1* and *NR4A3* expression, particularly in human AML cells.

Aberrant DNA methylation is a common mechanism in the pathogenesis of several types of cancer [7–13]. It is well-known that the expression of several tumor suppressor genes, such as *p16* and *MLH1*, is repressed due to DNA hypermethylation at their promoter region [9]. Recently, tumorigenesis resulting from DNA hypermethylation at both promoter and transcribed regions has been reported [14]. In addition, several studies have shown both positive and negative correlations between intragenic DNA methylation and gene expression [14–17]. Due to the complex effects of abnormal DNA methylation on gene expression and tumorigenesis, it is essential to investigate the impact of aberrant DNA methylation on gene expression systematically at affected loci by individual tumor suppressors. Given that loss-of-function mutations in *NR4A* have not been reported in AML to date, we hypothesized that abnormal DNA methylation contributes to a reduction in *NR4A* expression in AML. In

this study, we focused on *NR4A3* and analyzed the DNA methylation status of *NR4A3* in human AML cells. DNA hypermethylation at the promoter region of *NR4A3* was not detected, while its intragenic DNA hypermethylation was associated with its reduced expression. Therefore, we propose the potential role of intragenic DNA hypermethylation in the transcriptional repression of *NR4A3* in AML.

2. Materials and methods

2.1. Cells from human subjects and cell lines

We analyzed the bone marrow (BM) from AML patients and control subjects after obtaining written informed consent. Five BM cells from patients diagnosed with lymphoid neoplasia without BM invasion, idiopathic thrombocytopenic purpura, or Kikuchi's disease were used as normal controls. The patient characteristics are shown in Table 1. Procedures were approved by the Human Investigation Review Committee at Chiba University Hospital. Mononuclear cells from BM samples were isolated on Ficoll-Paque PLUS (GE Healthcare, Pittsburgh, PA, USA). Specimens from control patients underwent CD34 positive selection by magnetic antibody-conjugated sorting (Miltenyi Biotech, Bergisch Gladbach, Germany). Primary AML cells were cultured in StemSpan serum-free expansion media (Stemcell Technologies, La Jolla, CA, USA) supplemented with 10 ng/mL recombinant human stem cell factor (SCF), Flt3 ligand (Flt3L), thrombopoietin (TPO), interleukin-3 (IL-3), and IL-6 (PeproTech, Rocky Hill, NJ, USA). All AML cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Thermo Scientific, Waltham, MS, USA) and 1% penicillin–streptomycin at 37 °C in a 5% CO_2 atmosphere.

2.2. Decitabine treatment

Human leukemia cell lines and primary AML cells were treated with different final concentrations of decitabine (DAC; Sigma-Aldrich, St Louis, MO, USA) or the same volume of phosphate buffered saline, replacing the medium and adding DAC or phosphate buffered saline every 24 h until harvested for RNA and DNA extraction.

2.3. Bisulfite sequencing

Genomic DNA was extracted following the procedures outlined in the Wizard Genomic DNA Purification Kit (Promega, San Luis Obispo, CA, USA) for cell lines and AllPrep DNA/RNA Mini Kit (Qiagen, Venlo, Netherlands) for human primary AML cells. The DNA was bisulfite-converted using MethylEasy Xceed Rapid DNA bisulfite modification kit (Human Genetic Signatures, North Ryde, NSW, Australia) according to the manufacture's protocol. The modified DNA was amplified with bisulfite sequencing PCR (BSP). The BSP primers were designed by the online BiSearch software [18]. The sequences of the primers and information regarding the PCR settings are shown in Table 2. The BSP was carried out using EpiTaq HS (Takara Bio, Shiga, Japan). The PCR products were purified using NucleoSpin Gel and PCR clean-up (MACHEREY-NAGEL, Düren, Germany) and then cloned into the T-vector pMD20 (Takara Bio) for sequencing. For each amplicon, 8–12 individual clones were sequenced by Eurofins Genomics (Tokyo, Japan). The final sequence results were processed by online QUMA software [19].

2.4. *In vitro* colony-forming assay

Kasumi-1, THP-1 and MOLM-13 cells were seeded into a methyl-cellulose medium (Methocult M3234; Stemcell Technologies) with 10 ng/mL recombinant human SCF, Flt3L, TPO, IL-3, and IL-6. Colonies propagated in culture were counted on day 10.

2.5. Western blot analysis

Samples were separated by SDS-PAGE, transferred to a PVDF membrane, and detected by Western blotting using a mouse antibody against FLAG M2 (Sigma-Aldrich) and NR4A3 (Perseus Proteomics Inc, Tokyo, Japan).

2.6. Quantitative real-time polymerase chain reaction analysis

The total RNA was isolated using the RNeasy Mini Kit (Qiagen). The reverse transcription step was performed with the Prime-Script RT Reagent Kit (Takara Bio) according to the manufacturer's instructions. The resulting cDNA samples were subjected to quantitative real-time PCR to measure the levels of *NR4A3* mRNA using the TaqMan Assay-on-Demand kit with the StepOne Real-Time PCR System (Applied Biosystems, Norwalk, CT, USA). The Taq-Man probes were purchased from Applied Biosystems (*NR4A3*: Hs00545009 g1; *GAPDH*: Hs99999905 m1).

2.7. Retroviral vectors and virus production

Human *NR4A3* cDNA tagged with a 3 × Flag was subcloned into a site upstream of an *IRES-EGFP* construct in pGCDNsam, a retroviral vector with a long terminal repeat (LTR) derived from a murine stem cell virus [20,21]. A recombinant vesicular stomatitis virus glycoprotein-pseudotyped high-titer retrovirus was generated by a 293GPG packaging cell line [22]. The virus in the supernatants of 293GPG cells was concentrated by centrifugation at 6000 × *g* for 16 h.

2.8. Transduction of AML cells

Kasumi-1, THP-1 and MOLM-13 cells overexpressing *NR4A3* were generated by infection with the supernatants from transfected 293GPG cells in the presence of 5 μg/mL protamine sulfate for 72 h with subsequent sorting of GFP-positive cells using FACS Aria II (BD Biosciences, Bedford, MA, USA).

2.9. DNA methylation profiles and bioinformatics analysis

We used publically available DNA methylation profiles (all based on HumanMethylation450 K BeadChip) of AML patients from The Cancer Genome Atlas (TCGA) [23] and of healthy individuals (GSE40279, GSE35069) [24,25]. Beta values were converted into M-values, on which statistical analysis was performed. For Kaplan-Meier estimation of overall survival and relapse-free survival, we stratified samples by median DNA methylation levels and adjusted the results for multiple testing (log-rank test calculated in R/survival package).

2.10. Statistical analysis

The statistical significance of the data was determined with Student's *t*-test using JMP 11 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Forced expression of NR4A3 by virus-mediated gene delivery suppresses the proliferation activity of human AML cells

We first checked the expression levels of *NR4A3* in 10 AML cell lines and 8 primary AML samples. The expression levels were drastically lower in all cell lines and primary AML samples compared to the controls ($CD34^+$ BM mononuclear cells from control subjects) (Fig. 1A). To evaluate the tumor suppressive function of *NR4A3* in human AML cells, we overexpressed *NR4A3* in Kasumi-1, THP-1 and MOLM-13 cells using a virus-mediated gene delivery system. *NR4A3* was successfully overexpressed in these cells (Fig. 1B), and led to the suppression of their growth as well as a significant reduction in the number of colonies generated in the methylcellulose (Fig. 1C and D). These results indicate that *NR4A3* functions as a tumor suppressor gene in human AML as was reported for murine cells [6,26,27].

3.2. Hypermethylated CpG sites are identified at the intragenic region encompassing exon 3 of NR4A3 in AML cell lines but not at its promoter region

To test our hypothesis that DNA hypermethylation is involved in the mechanism of reduced *NR4A3* expression, we assessed the DNA methylation status of *NR4A3* by bisulfite sequencing analysis. We first investigated the DNA methylation status at the potential promoter sequences—Region 1 (528 bp) and Region 2 (623 bp)—spanning −551 to −24 and −38 to +584 relative to the transcription start site (TSS) of *NR4A3*, respectively (Fig. 2A). Unexpectedly, these regions were not hypermethylated in HL60 and Kasumi-1 cells (Fig. 2A). As intragenic CpG islands can also be preferentially methylated in cancer [28], we next focused on DNA methylation at the transcribed region. Intragenic DNA hyper-methylation affects the gene expression in both a positive and negative manner in cancer cells [14]. Notably, those associated with transcriptional repression have often been reported to be associated with H3K4me1 and H2A.Z enrichment peaks in human mammary epithelial cells (HMECs) [14]. This suggests that some transcribed regions with hypermethylated CpGs may represent functional regulatory elements, such as enhancers or alternative promoters in non-malignant cells [14]. Based on the results of this report, we assessed the distribution of H3K4 me1 and H2A.Z levels using the data from the Encyclopedia of DNA Elements (ENCODE; https://genome.ucsc.edu/ENCODE/, chromatin immunoprecipitation sequencing [ChIP-seq] data) to determine the region of the gene body to be used for DNA methylation analysis. The enrichment peaks of H3K4me1 and/or H2A.Z were observed in HMECs, human monocytes ($CD14^+$ blood cells), and human B cells ($CD20^+$ blood cells) at the region encompassing exon 3 of *NR4A3* (Fig. S1). Thus, we assessed the DNA methylation profile in Region 3 (+6151 to +6535) and Region 4 (+7116 to +7432), including exon 3, which contains 15 and 9 CpG sites, respectively. Bisulfite sequencing analysis revealed that compared to the controls, the CpGs within these intragenic regions were highly methylated in NB4, HL60, Kasumi-1, and THP-1 cells and mildly methylated in MOLM-13 cells (Fig. 2B). Interestingly, H3K4me1 and H2A.Z enrichment peaks were not observed at exon 3 of *NR4A3* in K562 cells from the ENCODE data, suggesting that these histone modifications were replaced by DNA methylation in AML cells (Fig. S1). Taken together, intragenic DNA

hypermethylation of *NR4A3* in AML cells was identified at the region encompassing exon 3, which potentially includes functional elements but not in the promoter region.

3.3. Decitabine restores the expression of NR4A3 and induces DNA demethylation at the region encompassing exon 3

To evaluate the contribution of DNA hypermehylation at the region encompassing exon 3 of *NR4A3* to its transcriptional repression, AML cell lines with heavily hypermethylated CpGs in this region were treated with the DNA methyltransferase inhibitor decitabine (DAC). DAC exposure restored the expression of *NR4A3* in all AML cell lines in a dose- and time-dependent manner (Fig. 3A). Notably, the frequencies of methylated CpG sites in AML cells were reduced after DAC exposure (Fig. 3B). These results suggest that DNA hypermethylation of the region encompassing exon 3 of *NR4A3* is associated with its reduced expression in AML cells.

3.4. DNA hypermethylation at the region encompassing exon 3 of NR4A3 is associated with its reduced expression in human primary AML samples

We next validated the results from AML cell lines in human primary AML samples. Hypermethylated intragenic DNA at the region encompassing exon 3 in AML samples was detected similarly to human AML cell lines (Figs. 4 A and Fig. S2A). Furthermore, DAC exposure restored the expression of *NR4A3* and reduced the methylated levels of the region (Fig. 4B), although the change of its protein level could not be evaluated due to the paucity of our primary samples. Thus, DNA hypermethylation at the region encompassing exon 3 of *NR4A3* is associated with its reduced expression in both AML cell lines and human primary AML samples.

3.5. DNA methylation status at the region encompassing exon 3 of NR4A3 shows a trend for survival association

To assess the association between DNA methylation status at the region of exon 3 of *NR4A3*, and the prognosis and genetic background, we analyzed DNA methylation profile of 194 AML patients from The Cancer Genome Atlas (TCGA) [23]. In line with the findings in the current study, the region encompassing exon 3 of *NR4A3* in AML patients was significantly hypermethylated compared with normal controls (Fig. 5A). Although the methylation status was not associated with relapse-free survival, higher methylation at the region encompassing exon 3 of *NR4A3* was associated with a trend for poor overall survival (Fig. 5B). Furthermore, increased methylation at the region was significantly associated with mutations in *TP53* and *RUNX1*, and inversely correlated with mutations in *NPM1* and *DNMT3A*, and *PML-RARA* rearrangements (Fig. 5C).

4. Discussion

AML is a heterogeneous disease with a multitude of chromosomal abnormalities and gene mutations [23,29]. This heterogeneity is a formidable obstacle in the development of therapies applicable for a wide range of AML patients. From this standpoint, transcriptional reactivation of the tumor suppressor *NR4A3* is advantageous because its expression has been reported to be commonly silenced in the blasts of patients with AML, irrespective of

karyotype [6]. However, the underlying mechanism of the reduced expression of *NR4A3* in AML is poorly understood. To our knowledge, this is the first study demonstrating the relationship between *NR4A3* silencing and DNA methylation in human AML cells.

In this study, we first confirmed the tumor suppressive function of *NR4A3* in human AML cells. To overcome the limitations of standard plasmid transfection approaches for NR4A3, Boudreaux et al. established a tumor suppressive function of NR4A3 in human AML cell lines using *in vitro* transcribed RNA electroporation system [26]. However, gene expression by electroporation is transient and therefore not suitable for long-term expression in human AML cells. In addition, there are concerns regarding the possibility that electroporation damages the cells and consequently affects the gene expression profile of these cells. In this study, we used a retroviral gene transfer approach for stable and robust expression of NR4A3, and unequivocally demonstrated a tumor suppressive function of NR4A3 in human AML cells.

DNA methylation analysis by bisulfite sequencing in the current study identified hypermethylated CpG sites at an intragenic region that included exon 3 of *NR4A3*, although not at its promoter region, in both human AML cell lines and primary human AML samples. Notably, the exposure of DAC restored the expression of *NR4A3* and was accompanied by DNA demethylation in the region encompassing exon 3. These findings suggest a role of intragenic DNA hypermethylation in the transcriptional repression of *NR4A3* in AML. The role of DNA methylation in cancer, particularly in the gene body, is complex. Recently, Yang et al. reported that DAC-induced demethylation of the gene bodies in tumor cells led to the downregulation of a cohort of genes, many of which are involved in metabolic processes regulated by MYC [14]. They also identified a group of genes showing a negative correlation between gene-body methylation and gene expression [14]. These findings suggest that gene-body methylation plays an important role not only in the upregulation of genes related to oncogenesis but also in the transcriptional repression of tumor suppressor genes.

The ChIP-seq data from the ENCODE database showed H3K4me1 and H2A.Z enrichment peaks at the region including exon 3 of *NR4A3* in HMECs, human monocytes, and human B cells but not in K562 cells (Fig. S1). These data indicate that histone modifications in the region are replaced by DNA methylation in AML cells. H2A.Z is enriched at active promoter and enhancer regions and is mutually exclusive with DNA methylation [30]. In addition, H2A.Z mediates nucleosome depletion and contributes to an open and accessible chromatin structure in embryonic stem cells, while DNA methylation often occurs at nucleosome-occupied regions [30–36]. Thus, these effects on nucleosome structure by different epigenetic modifications may account for the different expression levels of *NR4A3* between normal hematopoietic cells and AML cells. Alternatively, given that H3K4me1 marks the enhancer region, this area may function as an enhancer [28,37]. It is also possible that exon 3 functions as an alternative promoter [15,28]. These aspects should be investigated in future studies of AML.

Notably, *NR4A3* is also silenced and functions as a tumor suppressor gene in aggressive B-cell lymphomas. Intriguingly, Deutsch et al. performed DNA methylation analyses of the

coding sequences and the regulatory region of *NR4A3* in lymphoma cells from patients and did not find any significant alterations [38]. These results may suggest that different mechanisms operate to reduce the expression of *NR4A3* in AML and B-cell lymphoma even though *NR4A3* functions as a tumor suppressor gene in both diseases.

Despite our findings in the current study, gene-body methylation may not be the only mechanism responsible for silencing of *NR4A3* in AML. For instance, *NR4A3* has been reported to be a direct, positively regulated transcriptional target of RUNX1 in hematopoietic progenitor cells [39]. Entinostat, a histone deacetylase inhibitor, has also been reported to restore the expression of *NR4A3* in AML [40]. Several upregulated microRNAs which target *NR4A3* are also reported in primary myelofibrosis [41]. These reports imply that a single therapy with a DNA-demethylating agent is insufficient to restore *NR4A3* expression completely in AML cells. In addition, the DNA methylation status at exon 3 of *NR4A3* in AML patients is not limited to a single genetic event (Fig. 5C), suggesting that the mechanism of *NR4A3* transcriptional repression is likely complex.

In summary, this is the first report showing an inverse relationship between the expression of *NR4A3* and intragenic DNA methylation. Our findings suggest the role of intragenic DNA hyper-methylation as a mechanism underlying the reduced expression of *NR4A3* in AML. However, the regulatory mechanisms of *NR4A3* expression are assumed to be complex and variable depending on the type of AML. As the reactivation of *NR4A3* in AML cells is a promising therapeutic strategy, further study is required to unveil the mechanisms that silence *NR4A3* in AML.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2016.09.018.

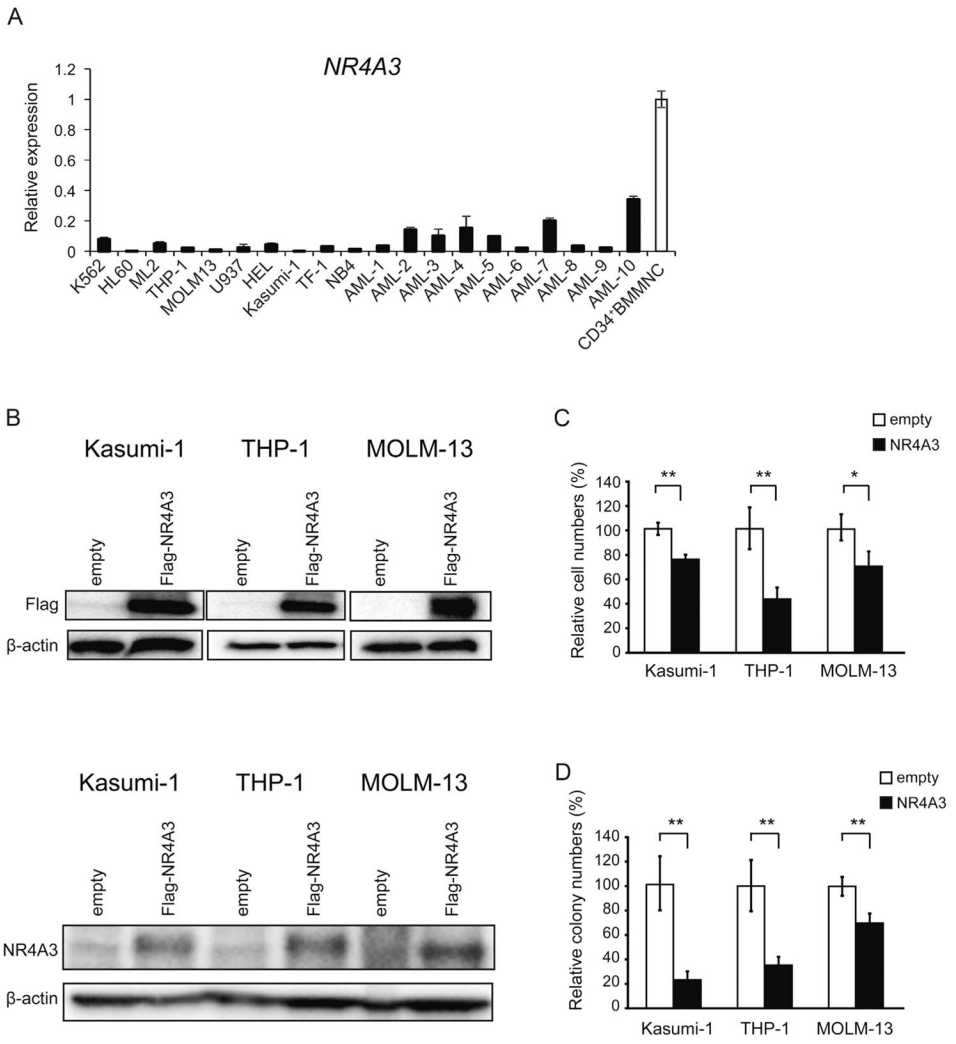

Fig. 1.

NR4A3 functions as a tumor suppressor gene in human acute myeloid leukemia (AML) cells. (A) Quantitative real-time PCR analysis of *NR4A3* expression in AML cell lines, human primary AML samples and $CD34^+$ bone marrow mononuclear cells (BMMNC) from control subjects (Control-3, Control-4 and Control-5). mRNA levels were normalized to *GAPDH* expression. The expression level in $CD34^+$BMMNC is shown as the mean ± S.D. (n = 3). The expression level in AML cell lines and human primary AML samples relative to the mean level in $CD34^+$BMMNC is represented as the mean ± S.D. for triplicate analyses. (B-D) Kasumi-1, THP-1 and MOLM-13 cells were transduced with either the control or *NR4A3* retrovirus and were purified for the experiments by cell sorting using green fluorescent protein (GFP) as a marker 72 h after infection. (B) NR4A3 expressions 72 h after GFP sorting were detected by Western blotting using the anti-FLAG antibody (upper panel) and the anti-NR4A3 antibody (lower panel). (C) The growth of the AML cells transduced with *NR4A3*. The data are shown as the means ± S.D. for triplicate analyses. (D) Colony formations in methylcellulose were determined for AML cells transduced with *NR4A3*.

Total colonies were scored on day 10. The percentage of colony numbers relative to the controls is shown as the means ± S.D. for triplicate analyses. *, $P < 0.05$; **, $P < 0.01$

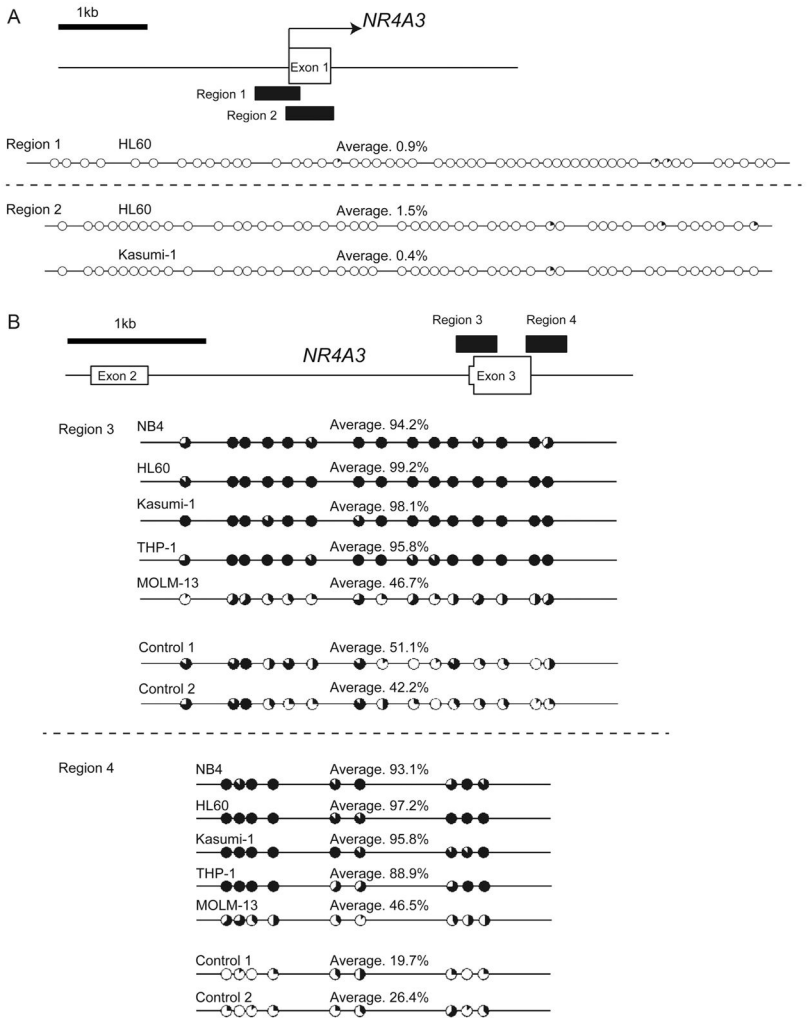

Fig. 2.

The hypermethylated region was identified at the region encompassing exon 3 of *NR4A3* but not its promoter region.

(A–B) Targeted regions of bisulfite sequencing PCR are shown as black boxes. The methylation status of CpG sites at the potential promoter region (A) and at the region encompassing exon 3 (B) of *NR4A3* was analyzed in AML cell lines and control samples (n = 2) using bisulfite sequencing. The percentages of methylation at each CpG site are indicated by black coloring within a pie chart. The percentages of methylation of the total CpG sites in the region of PCR products are also indicated as an average.

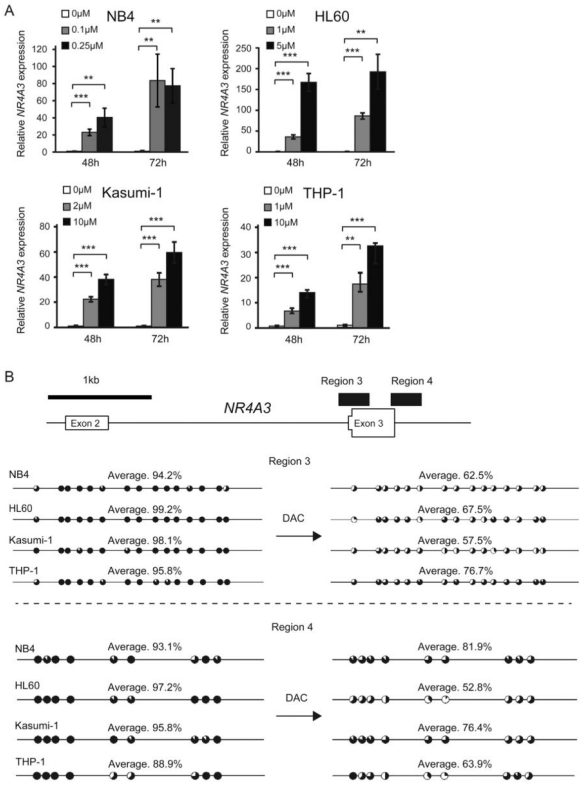

Fig. 3.

Decitabine restored the silenced expression of *NR4A3* accompanied by demethylation at the region encompassing exon 3 of *NR4A3.*

(A–B) AML cell lines, in which the region encompassing exon 3 is heavily methylated, were treated with different doses of decitabine (DAC) for 48 and 72 h. (A) mRNA levels of *NR4A3* in the cells were evaluated by quantitative real-time PCR and normalized to *GAPDH* expression. Data are shown as the mean ± S.D. for triplicate analyses. (B) The methylation status of CpG sites at the region encompassing exon 3 of *NR4A3* in AML cell lines was analyzed before and after DAC exposure using bisulfite sequencing. The percentages of methylation at each CpG site are indicated by black coloring in pie charts. The percentages of methylation of the total CpG sites in the region of PCR products are also indicated with an average. **, $P < 0.01$; ***, $P < 0.001$

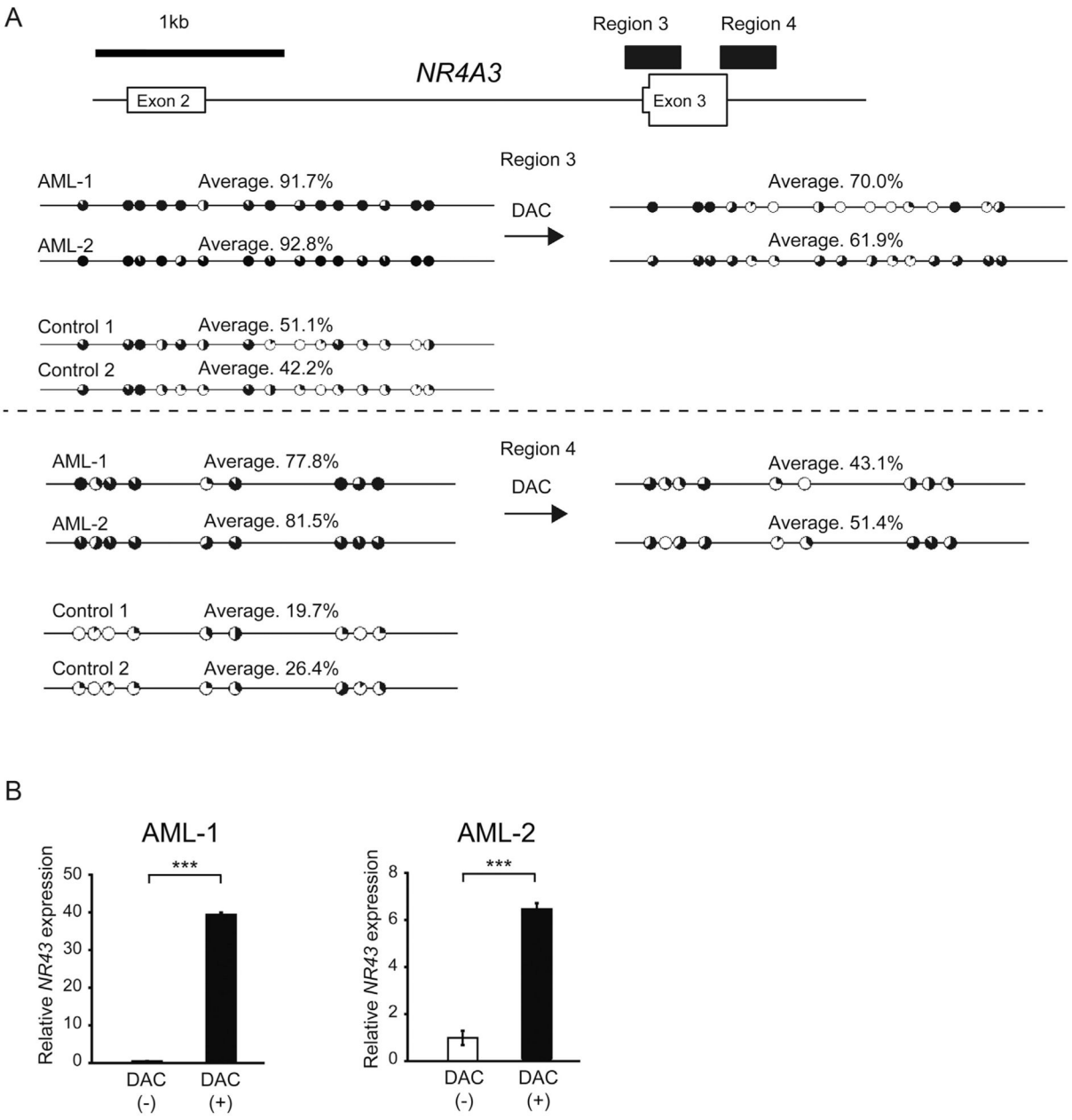

Fig. 4.

DNA hypermethylation at the region encompassing exon 3 of *NR4A3* is associated with its reduced expression in human primary AML samples.

(A) Two human primary AML cell samples were treated with 0.33 μM (AML-1) or 1 μM (AML-2) of DAC for 72 h. The methylation status of CpG sites at the region encompassing exon 3 of *NR4A3* in human primary AML samples and control samples (n = 2) was analyzed before and after DAC exposure using bisulfite sequencing. The percentages of methylation at each CpG site are indicated by the black coloring on the pie charts. The percentages of methylation of the total CpG sites in the region of PCR products are also indicated with an average. (B) Two human primary AML cell samples were treated with

0.33 μM (AML-1) or 5 μM (AML-2) of DAC for 72 h. mRNA levels of *NR4A3* in primary human cells were evaluated by quantitative real-time PCR and normalized to *GAPDH* expression. Data are shown as the mean ± S.D. for triplicate analyses. **, $P < 0.01$, ***, $P < 0.001$.

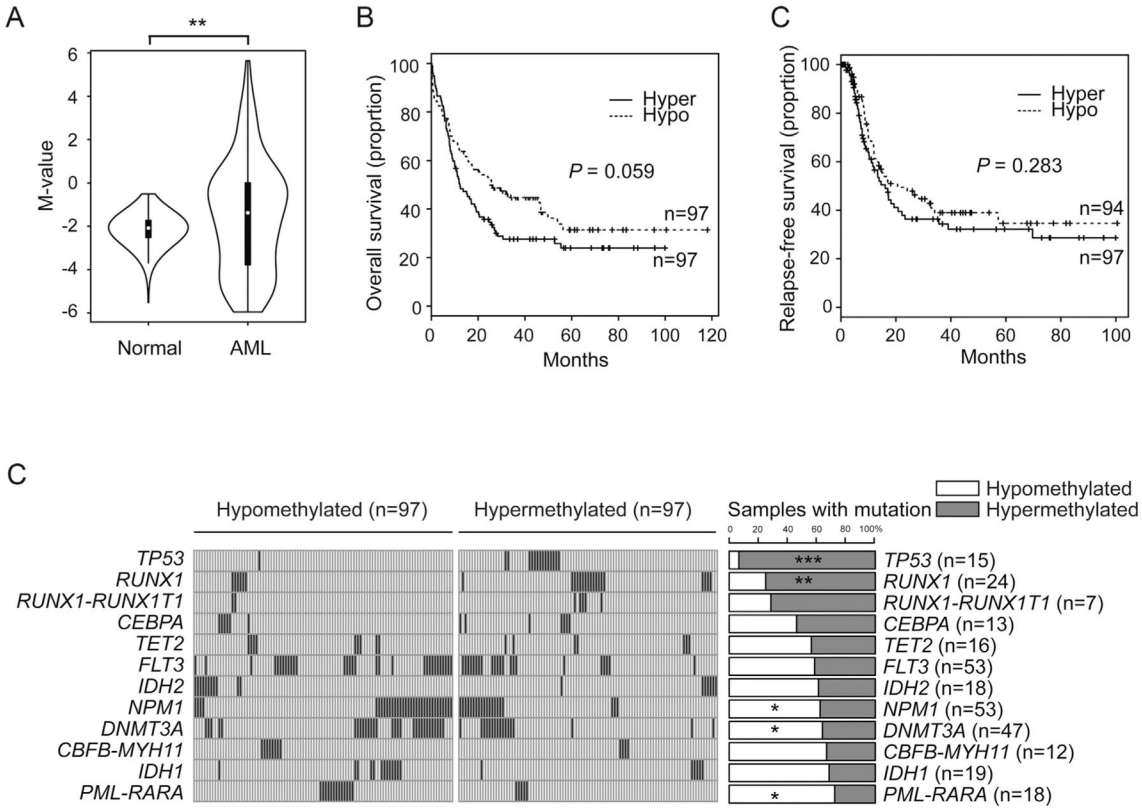

Fig. 5.

DNA methylation level at exon 3 of *NR4A3* influences the prognosis of AML patients, and is associated with genetic abnormalities.

(A) Violin plots showing the M-value distribution of DNA methylation in exon 3 of *NR4A3* of 656 healthy controls (GSE40279, GSE35069) [24,25] and of 194 AML patients from The Cancer Genome Atlas (TCGA) database [23]. (B) Overall survival curve (left panel) and relapse-free survival curve (right panel) of TCGA samples classified by median DNA methylation level at exon 3 of *NR4A3*. (C) Enrichment of specific mutations according to DNA methylation level at exon 3 of *NR4A3*. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Table 1

Patient Characteristics.

FAB, French–American–British classification; WBC, white blood cell; AML, acute myeloid leukemia; BM, bone marrow; F, female; M, male; DLBCL, diffuse large B-cell lymphoma; AITL, angioimmunoblastic T-cell lymphoma; ITP, idiopathic thrombocytopenic purpura.

ID	Age	Sex	Disease	FAB	WBC (/μl)	Cytogenetics	Blasts in BM (%)
AML-1	59	M	AML	M2	22000	Normal	88.0
AML-2	39	M	AML	M3	5300	t(15;17) (q22;q12)	77.0
AML-3	64	F	AML	M4	144100	Normal	82.2
AML-4	58	F	AML	M5a	78500	Normal	80.8
AML-5	72	M	AML	M0	17500	Complex karyotype	91.6
AML-6	39	F	AML	M4	77800	Complex karyotype	83.8
AML-7	50	F	AML	M1	30700	Normal	77.2
AML-8	41	M	AML	M5a	129700	Normal	88.2
AML-9	69	M	AML	M5a	19000	46,XY,t(9;11)(p22;q23)	92.2
AML-10	58	F	AML	M2	8100	45,X,-X,t(8;21)(q22;q22), del(9)(q)	74.2
Control-1	69	M	DLBCL	–	–	–	–
Control-2	16	F	AITL	–	–	–	–
Control-3	54	F	ITP	–	–	–	–
Control-4	51	M	DLBCL	–	–	–	–
Control-5	27	M	Kikuchi’s disease	–	–	–	–

Table 2

The sequences of primers and conditions for bisulfite-sequencing PCR.

Name	Analyzed regions relative to transcriptional start site of *NR4A3*	No. of analyzed CpGs	Forward (5′ → 3′)	Reverse (5′ → 3′)	Annealing temperature (°C)	Cycle	Length(bp)
NR4A3-Region 1	−551 to −24	51	TTATGTAAGAGGAAAGTGTAGTT	CTCCATAAAATACCTAAAATAC	54	48	528
NR4A3-Region 2	−37 to +485	47	AGGTATTTTATGGAGAG	AAAAATCACAACTACTATCC	54.4	40	522
NR4A3-Region 3	+6151 to +6535	15	TTTGTTTTTGAGAGATTTTTTTT	AATAACTACTAATACTACTAC	53.2	45	385
NR4A3-Region 4	+7116 to +7432	9	GAGGGTTGTAAGGGTTTTTT	CAAATCCTTTTAAAACTCTAC	57.7	40	317