**ORIGINAL ARTICLE**

**Sensitivity of MLL-rearranged AML cells to all-trans retinoic acid is associated with the level of H3K4me2 in the RARα promoter region**

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All-trans retinoic acid (ATRA) is well established as differentiation therapy for acute promyelocytic leukemia (APL) in which the PML–RARα (promyelocytic leukemia-retinoic acid receptor α) fusion protein causes blockade of the retinoic acid (RA) pathway; however, in types of acute myeloid leukemia (AML) other than APL, the mechanism of RA pathway inactivation is not fully understood. This study revealed the potential mechanism of high ATRA sensitivity of mixed-lineage leukemia (MLL)-AF9-positive AML compared with MLL-AF4/Sq31-positive AML. Treatment with ATRA induced significant myeloid differentiation accompanied by upregulation of RARα, C/EBPα, C/EBPε and PU.1 in MLL-AF9-positive but not in MLL-AF4/Sq31-positive cells. Combining ATRA with cytarabine had a synergistic antileukemic effect in MLL-AF9-positive cells in vitro. The level of dimethyl histone H3 lysine 4 (H3K4me2) in the RARα gene-promoter region, PU.1 upstream regulatory region (URE) and RUNX1 + 24/ + 25 intronic enhancer was higher in MLL-AF9-positive cells than in MLL-AF4-positive cells, and inhibiting lysine-specific demethylase 1, which acts as a histone demethylase inhibitor, reactivated ATRA sensitivity in MLL-AF4-positive cells. These findings suggest that the level of H3K4me2 in the RARα gene-promoter region, PU.1 URE and RUNX1 intronic enhancer is determined by the MLL-fusion partner. Our findings provide insight into the mechanisms of ATRA sensitivity in AML and novel treatment strategies for ATRA-resistant AML.

**Blood Cancer Journal** (2014) 4, e205; doi:10.1038/bcj.2014.25; published online 25 April 2014

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**INTRODUCTION**

Differentiation therapy using all-trans retinoic acid (ATRA) is well established for the treatment of acute promyelocytic leukemia (APL)\(^1\,^2\) in which the PML–RARα (promyelocytic leukemia-retinoic acid receptor α) fusion protein causes blockade of the retinoic acid (RA) pathway.\(^3\,^4\) There has been significant success using ATRA to treat APL, but there has been little research into the use of ATRA in a non-APL acute myeloid leukemia (AML) setting. In addition, ATRA does not induce myeloid differentiation in non-APL AML cells. Myeloid differentiation is regulated via the activation of RARα gene.\(^5\,^6\) Studies of the molecular mechanism blocking differentiation in non-AML AML cells indicate that expression of RARα is diminished in non-AML AML cells and that its restoration can induce differentiation of human AML cells.\(^6,^7\) These observations suggest that a lack of RARα expression impairs the RA pathway in non-AML AML cells, blocking myeloid differentiation. In addition, these studies also revealed that epigenetic mechanisms, including DNA methylation, histone methylation and acetylation, are involved in the repression of RARα expression in non-AML AML cells.\(^6,^7\) Based on these findings, recent studies have focused on the development of differentiation therapies for non-AML AML using ATRA combined with epigenetic modifiers.\(^8\,^9\) Recently, it has been reported that inhibition of lysine-specific demethylase 1 (LSD1), which demethylates dimethyl histone 3 lysine 4 (H3K4me2) to silence expression of its target gene, reactivates the RA pathway in non-AML AML.\(^10\,^11\) Rearrangements of the mixed-lineage leukemia (MLL) gene, which lies on chromosome 11, can involve any of the 79 potential translocation partner genes.\(^12\) MLL-rearranged leukemia is a heterogeneous group, and clinical outcome depends on the translocation partner gene involved.\(^13\,^14\) AML patients with t(9;11) and t(11;19) translocations are classified as intermediate risk, while those with all other MLL-fusion partners are classified as high risk.\(^15\) In addition, patients with MLL-rearranged AML grouped according to the translocation partner gene involved can be further stratified by the gene expression signature and promoter methylation pattern of their leukemic cells.\(^16\,^17\) In terms of induction of myeloid differentiation in MLL-rearranged AML, we have previously described that the demethylating agent 5-aza-2′-deoxycytidine enhanced the sensitivity of MLL-AF9-expressing AML cells to ATRA.\(^8\) and Iijima et al.\(^8\) have also reported that the combination of the histone deacetylase inhibitor trichostatin A and ATRA was effective as a growth inhibitor and differentiation enhancer in MLL-AF9-expressing leukemia cells. In addition, Niitsu et al.\(^21\) demonstrated that ATRA sensitivity of MLL-rearranged AML cells were varied and that might be related to p16 expression level. These findings suggest that chromatin remodeling, such as histone modification by methylation or acetylation, is an important factor for ATRA sensitivity in MLL-rearranged AML, and the epigenetic priming of RA pathway is necessary for the differentiation therapy in MLL-rearranged AML.

Herein, to determine whether the different partner gene translocations of MLL-rearranged AML affect the sensitivity of ATRA, we investigated the relationship between the level of H3K4me2 in the RARα gene-promoter region, PU.1 gene upstream regulatory region (URE) and RUNX1 gene intronic enhancer and sensitivity to ATRA, and whether, by inhibiting H3K4me2 demethylation in ATRA-resistant cells, sensitivity could be induced in MLL-rearranged human AML cells and murine immortalized cells expressing MLL-AF9 or MLL-AF5q31.
MATERIALS AND METHODS

Cell lines and culture

Human AML cell lines, THP-1 (American Type Culture Collection, Manassas, VA, USA) and MOLM-13 (kindly provided by Dr Bert A. van der Reijden, Radboud University Nijmegen Medical Centre, Nijmegen, Netherlands) bearing the MLL-AF9 gene fusion and KOCL-48 (kindly provided by Dr Kenji Sugita, Yamanashi University, Kofu, Japan) bearing MLL-AF4 were cultured in suspension in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (10 mg/ml) at 37 °C in a 5% CO2 humidified atmosphere.

Reagents

ATRA (Sigma-Aldrich, St. Louis, MO, USA), dissolved in dimethyl sulfoxide, and cytarabine (Sigma-Aldrich), dissolved in water, were stored as 1 mM stock solutions in small aliquots at −20 °C. Tranexymycin (TCP), a nonreversible LSD1 inhibitor purchased from Sigma-Aldrich, was prepared as a 10 mM stock solution in dissolved dimethyl sulfoxide and stored at −20 °C.

Retroviral constructs and transduction of Lin− murine hematopoietic progenitors

Retroviral constructs encoding either human MLL-AF9 or MLL-AF5q31 fusion genes were used to establish murine cell lines expressing MLL-AF9 or MLL-AF5q31 fusion proteins as described previously.26

Clinical AML cell samples

Primary leukemic cells expressing the MLL-AF9 fusion were obtained from the diagnostic bone marrow samples of two pediatric AML patients following informed parental consent, in accordance with the revised Helsinki protocol. The AML cells were purified from bone marrow as mononuclear cells using Ficoll density-gradient centrifugation and stored in liquid nitrogen. On thawing, cells were plated in methylcellulose (MethoCult H4434, Stem Cell Technologies, Vancouver, BC, Canada) at 1.0 × 10^6/ml and cultured with ATRA (1 μM).

Morphological studies

Cells at 1.0 × 10^6/ml were cultured with ATRA (1 μM) and/or TCP (10 μM) for 3 days. Cytospin preparations were stained with May-Grünwald Giemsa.

Nitroblue tetrazolium (NBT) reduction test for detection of myeloid differentiation

After culture with ATRA (1 μM) and/or TCP (10 μM) for 3 days, cells were subjected to a NBT reduction test using the NBT Reduction Kit (Sigma-Aldrich) according to the manufacturer’s instructions. The percentage of cells containing the precipitated formazan particles was determined by light microscopy. At least 200 cells were counted per sample.

Cytotoxicity assay

ATRA, TCP and cytarabine cytotoxicity were measured by cell viability using a WST assay (Cell Count Reagent SF, Nacalai Tesque Inc., Kyoto, Japan). The concentration of each drug causing 50% growth inhibition (IC50) was determined. The effect of ATRA and cytarabine and of ATRA and TCP cytotoxic interactions were determined from the combination index (CI); CI < 1, CI = 1 and CI > 1 indicate a synergistic, an additive and an antagonistic effect, respectively. The CI was calculated using the following equation: CI = (D1/D1) + (D2/D2). (D1)1 and (D2)2 are concentrations of each drug when each drug inhibits cell proliferation at the 50% growth level individually. (D1)1 and (D2)2 are concentrations of each drug when combination treatment of two drugs inhibit cell proliferation at the 50% growth level.

Flow cytomteric analysis

Cells (1.0 × 10^6) were harvested, washed twice with PBS and incubated for 30 min with phycoerythrin-conjugated anti-human and anti-mouse Mac-1 (murine CD11b) antibody (BD Biosciences, Sparks, MD, USA) and analyzed on a FACS Calibur (BD Biosciences) with the FlowJo software (Treestar, San Carlos, CA, USA).

Cell-cycle analysis

Cells were harvested, washed twice with PBS and incubated for 30 min with propidium iodide (PI) to stain DNA. Propidium iodide fluorescence was analyzed using a FACS Calibur, and the cell-cycle phase was determined on the basis of DNA content using the ModFit LT software (Verity Software House, http://www.vsh.com/).

Real-time reverse transcription–PCR (RT-PCR)

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Venio, The Netherlands) according to the manufacturer’s instructions. The SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) was used to synthesize cDNA according to the manufacturer’s instructions, and real-time RT-PCR was performed using the 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR Green 1 Master Mix (Takara Bio, Tokyo, Japan). Relative expression of target mRNA was determined using the comparative threshold (ΔCt) method. Glyceraldehyde-hyde-3-phosphate dehydrogenase was used as an internal control. The primer pairs used in this study are listed in Supplementary Table S1. A standard curve analysis with stepwise sample dilution demonstrated that all primer pairs had similar efficiency (data not shown).

Immunoblot analysis

Immunoblot analysis was performed as described previously.27 The following primary antibodies were used: anti-RARα (diluted 1:200), anti-C/EBPβ (diluted 1:750), anti-C/EBPδ (diluted 1:200) and anti-PU.1 (diluted 1:500). All antibodies were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA.

Chromatin immunoprecipitation (ChIP)

ChIP was carried out using the Simple ChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology Inc., Danvers, MA, USA) according to the manufacturer’s instructions. IP was performed with the following antibodies: anti-H3K4me2 (Abcam, Tokyo, Japan) and anti-histone H3 (Cell Signaling Technology Inc.). IP DNA in each IP sample of murine cell lines was analyzed by quantitative RT-PCR using RARα gene-specific primers covering the promoter and 5’ untranslated region (5’UTR); from −1000 to +1000 bp from the transcriptional start site.28,29 PU.1 URE1 and RUNX1 + 241 + 25 intronic enhancer.29,30 Also, IP DNA in each IP sample of murine cell lines was analyzed by quantitative RT-PCR using Rarα gene-specific primers covering the promoter and 5’UTR from −1000 to +1000 bp from the transcriptional start site, Sfpi.1 URE and Runx1 + 241 + 25 intronic enhancer.29,30 The list of primer sequences are shown in Supplementary Tables 2 and 3. All expression values were normalized against histone H3.

Statistical analysis

Statistical analysis was performed using the Student’s t-test. A P-value <0.05 was considered statistically significant.

RESULTS

ATRA induces myeloid differentiation and growth inhibition with cell-cycle arrest in human MLL-AF9-positive AML cells

First, we evaluated whether ATRA could induce myeloid differentiation, as observed in APL, in three human AML cell lines with MLL rearrangements. ATRA induced morphological changes, such as irregularly shaped nuclei, extended cytoplasm and the appearance of fine granules, more markedly in THP-1 and MOLM-13 cell lines, which bear the MLL-AF9 fusion gene, than in the MLL-AF4-expressing THP-1 and MOLM-13 cell lines. (Figure 1a). These morphological changes corresponded with a reduction in NBT, which indicated an increase in mature myeloid cell function (for example, phagocytosis activity), and the induction of CD11b on flow cytometric analysis (Figures 1b and c). Furthermore, ATRA induced G0/G1 arrest more clearly in the MLL-AF9-positive cell lines than in the MLL-AF4-positive cells and had a much lower IC50 with the MLL-AF9-positive cell lines (THP-1 and MOLM-13) than with the MLL-AF4 (KOCL-48) positive cells (3.91 ± 0.87 and 1.24 ± 0.70 vs 77.2 ± 7.37 μM; Supplementary Figure S1a and Figure 1d). Taken together, these data demonstrate that the effects of ATRA on the induction of myeloid differentiation were more apparent, and that the effects of ATRA on growth inhibition were significantly greater, on the MLL-AF9-positive AML cell line than
The RA pathway is more profoundly impaired in human MLL-AF4-positive cells than in MLL-AF9-positive cells.

The expression of RARα, C/EBPα, C/EBPε, and PU.1 genes is modulated in myeloid differentiation by RA. Using western blotting analysis, we found that RARα and C/EBPα expression levels, which are directly regulated by ATRA, were increased in response to ATRA in the MLL-AF9-positive cell line THP-1 and MOLM-13 but not in the MLL-AF4-positive cell line KOCL-48. In addition, expression of C/EBPα and PU.1, which are important transcriptional factors in myeloid differentiation, was also increased only in MLL-AF9-positive cells (Figure 1e).

The effect of ATRA on primary MLL-AF9-positive AML cells

To confirm these effects of ATRA on myeloid differentiation, we evaluated the expression levels of RARα, C/EBPα, C/EBPε, and PU.1 in two primary AML samples bearing MLL-AF9 fusion using real-time RT-PCR analysis. In accordance with the results of the western blotting analysis of human MLL-AF9-positive AML cell lines, the expression levels of RARα, C/EBPα, C/EBPε, and PU.1 were upregulated in both primary MLL-AF9-positive AML samples (Supplementary Figure S2). Collectively, these results showed clearly that ATRA was able to induce myeloid differentiation and activate the RA pathway in MLL-AF9-positive AML cells. In contrast, ATRA did not induce myeloid differentiation or activate the RA pathway in MLL-AF4-positive cells.

The effect of ATRA on murine MLL-rearranged immortalized cells

To confirm that the particular MLL-fusion partner (AF9 or AF4) directly determined the activity of the RA pathway, our aim was to generate immortalized murine cells expressing MLL-AF9 or MLL-AF4; however, we were unable to transform murine hematopoietic progenitor cells using MLL-AF4. Therefore, AF5q31, a member of the AF4 family of genes, fused with MLL was used as an alternative. Similar to the results of the experiments with human AML cells, ATRA induced morphological changes only in the murine MLL-AF9-expressing cells (Figure 2a), and these changes were accompanied by a reduction in NBT and upregulation of Mac 1 expression (Figures 2b and c). In addition to these effects, the percentage of cells in G0/G1 was greater among the MLL-AF9-expressing cells (Supplementary Figure S1b), and the ATRA IC50 was much lower for MLL-AF9-expressing cells than for MLL-AF5q31-expressing cells (2.01 ± 0.39 vs 32.6 ± 14.5 μM; Figure 2d). The expression levels of Rara, Cebpα, Cebpε, and Sfpi1 determined by real-time RT-PCR were significantly upregulated in response to ATRA in the MLL-AF9-expressing cells but were unchanged in the MLL-AF5q31-expressing cells (Figure 2e), suggesting that murine Lin− hematopoietic progenitor cells expressing MLL-AF9, but not those expressing MLL-AF5q31, remain sensitive to ATRA. Thus, it could be concluded that particular MLL-fusion partners affect sensitivity to ATRA.

A synergistic antileukemic effect between cytarabine and ATRA in MLL-AF9-positive AML cells

To explore the possibility of a synergistic antileukemic effect between ATRA and cytarabine, one of the major drugs used to
treat AML, we treated human and murine MLL-AF9-positive cells (THP-1 and MOLM-13 human AML cell lines and the immortalized murine MLL-AF9-expressing cells) with a titrating dose of cytarabine in combination with 1 μM ATRA or without ATRA. We found that the presence of ATRA decreased the cytotoxic IC50 in THP-1 and MOLM-13 cell lines and in the murine MLL-AF9-expressing cells (cytarabine IC50 of 3.69 ± 1.00, 0.042 ± 0.030 and 0.060 ± 0.012 μM, respectively, without ATRA, vs 0.17 ± 0.075, 0.0038 ± 0.0031 and 0.015 ± 0.0026 μM, respectively, with ATRA). The combined effects of cytarabine and ATRA on cell-growth inhibition were clearly synergistic in the three cell lines (Figures 3a and b). ATRA in combination with cytarabine was therefore, more effective than cytarabine alone for ablating MLL-AF9-positive cells in vitro.

ChIP assays of H3K4me2 on MLL-rearranged human AML cell lines and murine MLL-rearranged immortalized cells

Next, we carried out ChIP assays on MLL-rearranged human AML cell lines to investigate whether inactivation of the RA pathway was correlated with decreased H3K4me2 in the RARz-promoter region, which contained the RA response elements.23 IP DNA was analyzed using RARz gene-specific primers mapping to the promoter and 5′UTR from −1000 to +1000bp from the transcriptional start site. The levels of H3K4me2 normalized against histone H3 were lower in the MLL-AF9-positive cell line, KOCL-48, than in the MLL-AF9-positive cell lines, THP-1 and MOLM-13. These findings revealed that ATRA sensitivity correlates with the H3K4me2 level in the RARz gene in MLL-rearranged AML cell lines (Figure 4a). In addition, we evaluated the histone modification status at the PU.1 proximal and distal URE and RUNX1 +24/+25 intronic enhancer, which were also associated with myeloid differentiation.25,26 The levels of H3K4me2 at these regions were lower in the MLL-AF4-positive cell line, KOC-48, than in the MLL-AF9-positive cell lines, THP-1 and MOLM-13 (Figure 4b). Collectively, MLL-AF9-positive cell lines bear higher H3K4me2 level in the RARz gene, PU.1 URE and RUNX1 intronic enhancer than MLL-AF4 positive cell line, resulting in efficient induction of myeloid differentiation in MLL-AF9-positive cell lines by ATRA.

To confirm the particular MLL-fusion partner (AF9 or AF4) directly modified the histone modification, such as the H3K4me2 level, we investigated the levels of H3K4me2 at RARz gene-promoter, Sphi.1 URE, and Runx1 intronic enhancer in immortalized murine cells expressing MLL-AF9 or MLL-AF5q31. Consistent with the result of human AML cell lines, the levels of H3K4me2 normalized against histone H3 were lower in the murine cells expressing MLL-AF5q31 than in the murine cells expressing MLL-AF9 at RARz gene-promoter, Sphi.1 URE, and Runx1 intronic enhancer (Figures 4c and d).

TCP restores ATRA sensitivity in the MLL-AF4-positive and ATRA-resistant cell line, KOCL-48

LSD1 demethylates H3K4me2, silencing the target gene; inhibition of LSD1 leads to an increase in H3K4me2. To determine whether the H3K4me2 status of the RARz promoter region and 5′UTR affected the RA pathway, KOCL-48 cells were treated with TCP,
Inhibitor of LSD1. A combination of 1 μM ATRA and 10 μM TCP (ATRA/TCP) induced more marked morphological changes and caused a bigger reduction in NBT than either ATRA or TCP alone (Figures 5a and b). Furthermore, ATRA/TCP induced higher-intensity expression of CD11b and increased the proportion in G0/G1 cell-cycle arrest (Figure 5c and Supplementary Figure S1c). Western blotting analysis revealed that the expression of RARα and PU.1 increased in response to ATRA/TCP (Figure 5d), suggesting that the combination of ATRA and TCP reactivates the RA pathway. We therefore treated KOCL-48 cells with a titrating dose of ATRA/TCP (Figure 5e), and the CI showed that ATRA and TCP had a synergistic effect (Figure 5e, m).37-39 The imposition of H3K4me2 in the translocation breakpoint region, such as RUNX1 and PU.1 were closely interacted, and these interactions resulted in the inactivation of the RA pathway and that inhibition of sensitivity. Our results showed that loss of H3K4me2 levels in positive AML cells is corroborated in vivo 

**DISCUSSION**

In this study, we have examined the mechanisms of ATRA sensitivity in non-APL AML cells. We found that MLL-AF9-positive cells were sensitive to ATRA, in vitro. Previous studies have shown that ATRA induces cell-cycle arrest and apoptosis in nucleophosmin 1 (NPM1)-mutated AML cells and that the NPM1 mutation sensitizes the AML cells to ATRA and cytarabine in vitro.35,36 However, several subsequent studies have failed to reveal any clinical benefit of combining ATRA with conventional chemotherapy.37-39 Thus it will be important to determine whether the synergistic antileukemic effect of cytarabine and ATRA seen in MLL-AF9-positive AML cells in vitro is corroborated in vivo.

We also demonstrated that the level of H3K4me2 in the Ran promote region was closely associated with ATRA sensitivity. Our results showed that loss of H3K4me2 levels in RA resulted in the inactivation of the RA pathway and that inhibition of LSD1 could reactivate the RA pathway in ATRA-resistant MLL-AF4-positive cells. In addition, we revealed that the levels of H3K4me2 in the PU.1 URE and RUNX1 intronic enhancer were also upregulated in the presence of TCP (Figure 5g). These findings suggest that inactivation of the RA pathway and myeloid differentiation block are induced by a decrease in the level of H3K4me2 in the RA promoter region, the PU.1 URE and RUNX1 intronic enhancer.

**Figure 3.** Synergistic cytotoxic effects between ATRA and cytarabine in MLL-AF9-positive cells. (a) THP-1, MOLM-13 and murine MLL-AF9-expressing immortalized cells were cultured with a range of concentrations of cytarabine (0-10 μM) with 1 μM ATRA or without. After incubation for 72 h in MLL-AF9-expressing immortalized cells and 96 h in human cell lines (THP-1 and MOLM-13), the number of viable cells was assessed by a WST assay. (b) Cytarabine IC50 with or without 1 μM ATRA values were determined for each cell line.
leukomogenesis. In addition, the demethylating agent or histone deacetylase inhibitor enhance the ATRA-mediated myeloid differentiation in MLL-AF9-expressing AML cells. These observations suggest that epigenetic modification is an important factor for leukomogenesis in some AML subtypes, including MLL-rearranged AML.

LSD1, which demethylates histone H3 at lysine 4 (H3K4) and lysine 9 (H3K9), was the first histone demethylase to be identified. LSD1 is overexpressed in various cancers, and recent researches showed that this epigenetic modifier is associated with the development of drug resistance. Schenk et al. reported that the LSD1 inhibitor TCP can reactivate ATRA sensitivity in the ATRA-resistant TEX cell line in vitro and diminish the engraftment of primary AML samples in vivo. In addition, they demonstrated that the combination of ATRA and TCP led to upregulation of gene expression associated with the myeloid differentiation program and apoptosis, accompanied by increased amounts of H3K4me2 near the transcriptional start sites.

In conclusion, we found that MLL-AF9-positive AML cells were sensitive to ATRA and that a high level of H3K4me2 in the promoter region of the RARα gene, PU.1 URE or RUNX1 intronic enhancer is associated with ATRA sensitivity in this subtype.

Figure 4. H3K4me2 modification of the RARα, Rara gene, PU.1/Sfpi.1 URE and RUNX1/Runx1 intronic enhancer in human AML cell lines and murine immortalized cell lines. After cells were incubated with ATRA (1 μM) for 72 h, ChIP assays were performed using antibodies to H3K4me2 and histone H3 (H3). Precipitated DNA was analyzed by real-time RT-PCR using primers mapping to within the promoter region and 5′UTR) of the RARα gene (a) and PU.1 URE or RUNX1 +24/+25 intronic enhancer (b), the promoter region and 5′UTR of the Rara gene (c) and Sfpi.1 URE or Runx1 +24/+25 intronic enhancer (d). Results represent the mean ± s.d. of two independent ChIP experiments and a total of four independent PCR analysis.
Addition of ATRA to cytarabine had a synergistic antileukemic effect on MLL-AF9-positive AML cells in vitro, suggesting that in cases of AML with high levels of H3K4me2 in the RARx promoter region, PU.1 URE and RUNX1 intronic enhancer, ATRA would be able to sensitize leukemic cells to cytarabine; an in vivo study is now required to confirm this. Furthermore, our findings indicate that epigenetic modifiers, such as LSD1 inhibitors, are potentially useful for treating ATRA-resistant AML, including MLL-rearranged AML.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**ACKNOWLEDGEMENTS**

This work was supported by grants for Clinical Cancer Research and Research on Measures for Intractable Diseases from the Japanese Ministry of Health, Labor and Welfare and by grants-in-aid for scientific research from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

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