Missense mutations in PML-RARA are critical for the lack of responsiveness to arsenic trioxide treatment

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Arsenic trioxide (As2O3) is a highly effective treatment for patients with refractory/relapsed acute promyelocytic leukemia (APL), but resistance to As2O3 has recently been seen. In the present study, we report the findings that 2 of 15 patients with refractory/relapsed APL treated with As2O3 were clinically As2O3 resistant. Leukemia cells from these 2 patients harbored missense mutations in promyelocytic leukemia gene–retinoic acid receptor-α gene (PML-RARA) transcripts, resulting in amino acid substitutions of A216V and L218P in the PML B2 domain. When wild-type or mutated PML-RARA (PR-WT and PR-B/L-mut, respectively) were overexpressed in HeLa cells, immunoblotting showed SUMOylated and/or oligomerized protein bands in PR-WT but not in PR-B/L-mut after As2O3 treatment. Protein-localization analysis indicated that PR-WT in the soluble fraction was transferred to the insoluble fraction after treatment with As2O3, but PR-B/L-mut was stably detected in fractions both with and without As2O3. Immunofluorescent microscopy analysis showed PR-WT localization as a microgranular pattern in the cytoplasm without As2O3 and as a macrogranular pattern with As2O3. PR-B/L-mut was diffusely observed in the cytoplasm with and without As2O3. Nearly identical localization patterns were observed in patients’ primary cells. Therefore, B2 domain mutations may play an important role in aberrant molecular responses to As2O3 and may be critical for As2O3 resistance in APL. (Blood. 2011;118(6):1600-1609)

Introduction

Acute promyelocytic leukemia (APL) is characterized by the reciprocal chromosomal translocation t(15;17)(q22;q21), leading to fusion of the promyelocytic leukemia gene (PML) on chromosome 15 and the retinoic acid receptor-α gene (RARA) on chromosome 17.1 PML-RARA fusions are detectable in >95% of patients with APL. In 1985, all-trans retinoic acid (ATRA) was introduced for the treatment of APL as a differentiation therapy, and a dramatic improvement in the overall survival of patients with APL has been obtained.2-4 However, approximately 10%-30% of patients eventually relapse after treatment with combination chemotherapies with ATRA.5-7

Arsenic trioxide (As2O3) is a critical drug for the treatment of APL and is clinically effective even in ATRA-resistant patients.8 As2O3 is a natural substance that has been used medically for over 2400 years. In the 1970s, a group in China identified As2O3 as a component of an anticancer reagent.9 Over the last 18 years, clinical trials conducted worldwide have demonstrated the efficacy of As2O3 for the treatment of relapsed patients with APL.10,11 Recently, it was also reported that As2O3 improves event-free survival and overall survival of adult APL when As2O3 is used as a consolidation treatment after obtaining the first remission.12 Currently, the role of As2O3 in frontline therapy is under investigation.13,14

Rapid degradation of PML-RARA via targeting of PML has been reported as a molecular mechanism for the effectiveness of As2O3.14 Furthermore, As2O3 induces posttranslational modifications of PML-RARA with small ubiquitin-related modifier (SUMO) and ubiquitin, resulting in the transfer of PML-RARA from the soluble fraction to the insoluble nuclear matrix14 and the degradation of both PML and PML-RARA.14-17 In addition to the significant clinical effectiveness of As2O3 for patients with APL, acquired resistance to As2O3 therapy has been recognized in clinical practice.18 Several studies have indicated that arsenic-resistant NB4 cells in vitro show higher glutathione levels than in parental cells.19-21 However, the detailed molecular mechanisms of resistance to As2O3 remain unclear.

Very recently, 2 studies reported that As2O3 binds directly to cysteine residues in zinc fingers located within the RBCC motif that contains 3 cysteine-rich zinc-binding domains, a RING-finger (R), 2 B-box motifs (B1 and B2), and a coiled-coil (CC) domain,22,23 in PML-RARA and PML.24,25 An intriguing hypothesis is that impairment of As2O3 binding to PML-RARA due to conformational changes may result from genetic mutations and/or abnormal posttranslational modifications. These events may be related to resistance to As2O3 therapy.

We report the clinical significance and frequency of As2O3 resistance in patients with APL. Fifteen patients with APL were treated with As2O3 after combination chemotherapy with ATRA, and 2 patients showed clinical As2O3 resistance. Interestingly, in both of these As2O3-resistant patients, missense genetic mutations in the PML-RARA fusion transcript were observed in the leukemia cells. We demonstrated that the mutations, which were located in the PML RBCC region, were critical for PML localization and As2O3 responsiveness in vitro. Our observations suggest that acquired genetic mutations in the PML-RARA transcript may be a critical molecular mechanism of resistance to As2O3 therapy.

Methods

Patients

From January 2000 to December 2008 at Nagoya University Hospital, Japan, 15 patients with APL who showed relapse or disease progression...
Table 1. APL patients treated with \( \text{As}_2\text{O}_3 \) at Nagoya University Hospital

<table>
<thead>
<tr>
<th>No.</th>
<th>Age, y</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Treatments prior to ( \text{As}_2\text{O}_3 )</th>
<th>Disease status at ( \text{As}_2\text{O}_3 )</th>
<th>Treatments after ( \text{As}_2\text{O}_3 )</th>
<th>Outcome</th>
<th>Survival after ( \text{As}_2\text{O}_3 )</th>
<th>( \text{As}_2\text{O}_3 ) resistance</th>
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<tr>
<td>1</td>
<td>61</td>
<td>M</td>
<td>M3v</td>
<td>A + CT</td>
<td>Rel1</td>
<td>(−)</td>
<td>D</td>
<td>6 y, 1 mo</td>
<td>+</td>
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<td>35</td>
<td>M</td>
<td>M3</td>
<td>A + CT</td>
<td>Rel1</td>
<td>sib-PBSCT</td>
<td>A</td>
<td>3 y, 8 mo</td>
<td>−</td>
</tr>
<tr>
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<td>30</td>
<td>M</td>
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<td>A + CT</td>
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<td>A</td>
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<td>A</td>
<td>5 y, 8 mo</td>
<td>−</td>
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<td>A + CT, HD-Ara-C</td>
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<td>A + CT</td>
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Fifteen patients were treated with \( \text{As}_2\text{O}_3 \) at Nagoya University Hospital during the period of January 2000–December 2008. Outcomes were confirmed on December 1, 2009. Patients 5, 6, and 13 received cord blood transplantation after \( \text{As}_2\text{O}_3 \). Patients 5 and 13 died of complications of transplantation without any relapse sign. Patient 6 died of relapse just after transplantation. Patient 11 died of brain bleeding due to APL relapse with disseminated intravascular coagulation.

Ralt through 3 indicates the first to third relapse; A + CT, ATRA with combination chemotherapy; PBSCT, peripheral blood stem cell transplantation; CBT, cord blood transplantation; BMT, bone marrow transplantation; D, dead; and A, alive.

after treatment with chemotherapy with ATRA were treated with \( \text{As}_2\text{O}_3 \) (Table 1). The diagnosis of APL and its relapse were confirmed by bone marrow morphology according to the FAB classification, chromosomal abnormality (t(15;17) in peripheral blood and/or bone marrow cells, positive RT-PCR assay for \( \text{PML-RARA} \) transcripts, and/or FISH analysis of \( \text{PML} \) and \( \text{RARA} \). \( \text{As}_2\text{O}_3 \) was diluted in 500 mL of 5% dextrose and administered intravenously over 2 hours at a dose of 0.15 mg/kg daily for a cumulative maximum of 60 days.

Patient 1 (Table 1 and Figure 1A) was diagnosed with APL (the microgranular variant M3v) in October 1998 (Figure 1A), and complete remission (CR) was obtained after combination chemotherapy with ATRA (45 mg/m²/d). However, relapse with insufficient response to ATRA was observed (Figure 1A) after the end of consolidation therapy in August 1999. \( \text{As}_2\text{O}_3 \) (0.15 mg/kg/d) was started as a salvage chemotherapy, and a molecular response was obtained. The effectiveness of \( \text{As}_2\text{O}_3 \) gradually decreased during the patient’s 7-year clinical course. Am80 (6 mg/m²/d) was started in July 2005 in addition to \( \text{As}_2\text{O}_3 \) therapy. However, the effectiveness was poor, and Am80 was discontinued in October 2005. At the terminal stage of his clinical course, only \( \text{As}_2\text{O}_3 \) was used. At the terminal stage of his clinical course, only \( \text{As}_2\text{O}_3 \) was used.

DNA extraction and RT-PCR

Total RNA was isolated from each sample using TRIzol (Invitrogen). cDNA was synthesized from 5 µg of total RNA using Superscript II reverse transcriptase (Invitrogen) as described previously.26,27 PCR was performed using LA-Taq polymerase (Takara) under the following conditions: one cycle of 95°C for 4 minutes, followed by 40 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 60 seconds. PCR primers for amplification of the coding sequences of \( \text{PML-RARA} \) are as follows: forward PR-U619: 5’-TGT TCC AAC CCG CTG T-3’, reverse PR-L2189: 5’-CAT CTT CAG CGT GAT CA-3’. Amplified PCR fragments were purified with a Wizard PCR prep DNA purification kit (Promega) and cloned into the pCR2.1-TOPO cloning vector (Invitrogen). At least 20 clones were sequenced with an ABI 310 automated DNA sequencer (Applied Biosystems). Genetic mutations were confirmed using MacVector Version 10.5.1 software.

Expression vectors

The coding sequence of \( \text{PML-RARA} \) was amplified with PCR, and the Flag sequence was added with the forward primer as described previously.26,28 The PCR fragment was cloned into the pcDNA4-His-Max-TOPO mammalian expression vector (Invitrogen) to generate the following expression vectors: pcDNA4-XF-PR-B/L-mut for Xpress-tagged wild-type \( \text{PML-RARA} \), pcDNA4-XF-PR-B/L-mut for Xpress-tagged \( \text{PML-RARA} \) with mutations resulting in A216V and G391E substitutions, pcDNA4-XF-PR-B2-mut for \( \text{PML-RARA} \) with the A216V substitution, and pcDNA4-XF-PR-L2-mut2 for the long-form \( \text{PML-RARA} \) with the L218P substitution. To express the long form of the wild-type \( \text{PML-RARA} \) protein, pcDNA4-XF-PR-L was used as described previously.27 Expression vectors pcDNA-F-Ubc9, pcDNA-F-SUMO, and pcDNA-HA-PML for Flag-tagged Ubc9 and SUMO1, and HA-tagged PML, respectively, have also been described previously.30

Cell culture

Cells of the human cervical cancer cell line HeLa were cultured in DMEM containing 10% FCS. U937 cells, a human monocytic leukemia cell line, were cultured in RPMI containing 10% FCS.
Protein extraction and antibodies for immunoblotting

HeLa cells (1.0 × 10^5/well) were cultured in a 12-well plate for 12 hours before transfection. Transfection of the expression vectors was carried out using Effectene (Invitrogen) according to the manufacturer’s instructions. The cells were washed with DMEM 24 hours after transfection, and then incubated for 8 hours with or without 10μM As2O3 (Sigma-Aldrich) until protein extraction. Whole-cell protein samples for immunoblotting were obtained using 250 μL of Laemmli sample buffer (200mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.004% bromophenol blue). After boiling for 5 minutes, samples were subjected to SDS-PAGE.

U937 cells were cultured in a 12-well plate for 12 hours before transfection. Transfection of the expression vectors pcDNA4-XF-PR-WT and pcDNA4-XF-PR-B/L-mut was carried out using Nucleofector Kit C (Lonza) according to the manufacturer’s instructions. After 12 hours, immunofluorescent analysis was performed.

To separate PML-RARA protein into soluble and/or insoluble fractions, cells were lysed in 200 μL of RIPA lysis buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.2mM PMSF; and a complete mini protease inhibitor tablet [Roche]). After centrifugation at 10 000 g for 10 minutes, the supernatants were placed into new tubes, and 200 μL of 2× SDS sample buffer was added (soluble fraction), PBS (20 μL) and 200 μL of 2× SDS sample buffer were added to the pellets (insoluble fraction). After boiling for 5 minutes, samples were analyzed by SDS-PAGE followed by immunoblotting. Antibodies used in this assay are as follows: rabbit anti-hemagglutinin (anti-HA; Sigma-Aldrich), mouse anti-Xpress tag (Invitrogen), and mouse anti–FLAG-M2 (Sigma-Aldrich).

Immunofluorescence microscopy

HeLa cells expressing Flag- and Xpress-tagged PML-RARA and its mutated proteins were cultured on Chamber Slides (Lab-Tek) with or without 10μM As2O3. U937 cells expressing Flag- and Xpress-tagged PML-RARA and its mutated proteins and the primary leukemia cells were placed on slide glasses using Cytospin (Shandon Southern Products), air dried, and fixed in acetone/methanol for 10 minutes at −20°C. Cells were then blocked with 1% BSA (Sigma-Aldrich) in PBS for 1 hour, incubated with primary antibodies for 3 hours, and incubated with Alexa Fluor 488 (green)- or 568 (red)-conjugated secondary antibodies for 1 hour at room temperature. Antibodies used in this assay are as follows: rabbit anti–human PML (Santa Cruz Biotechnology), mouse anti–FLAG-M2 (Sigma-Aldrich), rabbit Alexa Fluor 488 (Invitrogen), and mouse Alexa Fluor 568 (Invitrogen). The slides were examined with an Axioskop 2 fluorescence microscope (Carl Zeiss), photos were taken and analyzed with AxioVision FRET Release 4.5, and images were processed with Adobe Photoshop CS3 software.

Results

Two of 15 patients showed clinical As2O3 resistance

From January 2000 to December 2008 in Nagoya University Hospital, 15 relapsed patients with APL, including 3 with M3v,21 were treated with As2O3 (Table 1). As a first-line treatment, combination chemotherapies with ATRA were administered to all patients. Thirteen patients received autologous or allogeneic stem cell transplantation after treatment with As2O3, and long-term remission (range, 44-81 months) was confirmed in 10 patients. One patient (patient 1, see also “Patients”) showed resistance to As2O3 after repeated therapy. Another patient (patient 6; see also “Patients”) showed resistance to the first course of As2O3 therapy. Disease progression (elevation of the blast count in the peripheral blood) was observed in patient 1 after long-term treatment with As2O3, and primary refractory disease that was resistant to As2O3 was confirmed in patient 6. Both patients died after disease progression.

Acquired missense mutations in PML-RARA transcripts observed in the 2 patients with As2O3 resistance

To determine the molecular mechanisms of the resistance to As2O3, we first focused on patient 1 who had a long clinical course and whose clinical samples had been preserved several times at each disease stage.

Total RNA was extracted from each sample, and RT-PCR for PML-RARA transcripts was performed. DNA sequencing analysis using the sample obtained from the terminal stage was performed first (Figure 1A sample 5). The PML-RARA transcript was a short-form type (bcr32) that lacked the nuclear localizing signal (NLS) in PML. Missense mutations resulting in the A216V substitution in the PML-B2 domain and the G391E substitution in the RARA ligand-binding domain (LBD; Figure 1B) were detected. The predicted mutated PML-RARA protein resulting from these mutations is shown in Figure 2A.

We then determined the sequence of PML-RARA in patient #6, who showed primary refractory disease to As2O3. Only a limited clinical sample obtained at 27 days after starting the As2O3 treatment was available for genomic analysis. A missense mutation resulting in an L218P substitution in the PML-B2 domain was confirmed in 2 of 20 clones with PCR cloning using genomic DNA PCR (Figure 1C). The mutations in the PML-B2 domain are indicated as B2-mut and B2-mut2 in Figure 1D. The predicted mutated PML-RARA protein designated as PR-L-B2-mut2 is also shown in Figure 2A. The location of these mutations is very close to the As2O3-binding cysteine-cysteine (CC) motif reported by Jeanne et al.25

Clonal expansion of PR-B/L-mut at the terminal stage of APL disease progression

To confirm the clonal expansion of the genetic mutations in PML-RARA, we performed sequencing analysis using the RT-PCR fragments of PML-RARA transcripts from the serial clinical samples obtained from patient 1 (samples 1-5 in Figure 1A). PCR fragments were cloned into the vector, and at least 20 clones were picked for sequencing analysis. Genetic mutations resulting in PR-B2-mut, PR-LBD-mut, and PR-B/L-mut (Figure 2A) were confirmed in samples 4 and 5 obtained at this patient’s terminal stage when As2O3 resistance and the expansion of the blast count were clinically observed (Figure 2B). These clones were not confirmed in samples 1-3, and the partial response to As2O3 treatment was confirmed at the periods 2-3. The samples 2 and 3 had blasts showing FISH-positive PML-RARA clones (33.9% and 74.0%, respectively). This result strongly suggests that these mutations were closely related to disease progression during As2O3 treatment.

Lack of multimerization of PR-B/L-mut with and without As2O3

Posttranslational modification of PML, including SUMOylation, is reported to be critical for the responsiveness to As2O3.24,25,33 To confirm the functional difference between PR-WT and its mutant, we performed an in vitro SUMOylation assay in HeLa cells. HA-tagged PML, Xpress-tagged PR-WT, PR-B/L-mut, and SUMO1/Ubc9 were expressed in HeLa cells with or without 10μM As2O3 (Figure 3A-B). PML, PR-WT, and PR-B/L-mut were detected with immunoblotting. SUMO1/Ubc9 is coexpressed with PML, and therefore, SUMOylated PML bands were observed (indicated with black triangles in Figure 3A lane 2). The intensity of the mobility-shifted bands was increased with As2O3 treatment (Figure 3A lane 3). When using PR-WT, multimerized/SUMOylated...
PR-WT bands were confirmed with and without As2O3 (Figure 3B lanes 4-6 black triangles). However, when using PR-B/L-mut, oligomerized/SUMOylated PML–RARA protein was not observed with SUMO1/Ubc9 with or without As2O3 (Figure 3B lanes 8 and 9). When PR-B2-mut was used in the same assay system, nearly the same result was obtained (Figure 3B lanes 11 and 12). These results indicate that the genetic mutation leading to amino acid alteration of the PML-B2 domain is critical for the appropriate posttranslational modification of the PML–RARA protein, including SUMOylation and oligomerization.

**Distinct cellular localization of PR-WT and PR-B/L-mut in soluble/insoluble fractions**

Recently, it has been reported that As2O3 promotes PML–RARA multimerization via disulfide-mediated covalent binding, leading to formation of PML nuclear bodies; these multimers are subsequently SUMOylated.25 To confirm the cellular localization (soluble/insoluble fractions, see also “Methods”) of PML, PR-WT, and PR-B/L-mut with or without As2O3, we performed immunoblotting using HeLa cells that were transiently overexpressing each protein. Cells were lysed using RIPA buffer, as described previously,14,24 and the whole-cell lysate was separated into 2 fractions, soluble (supernatant) and insoluble (pellet). In the absence of As2O3, PML was localized in both the soluble and insoluble fractions (Figure 4A lanes 1 and 3). With As2O3, PML was detected mostly in the insoluble fraction (Figure 4A lanes 2 and 4). Nearly the same results were obtained with PR-WT (short form of PML–RARA, Figure 4B lanes 5, 6, 11, and 12) and PR-long (long form of PML–RARA, Figure 4B lanes 9, 10, 15, and 16). Conversely, PR-B/L-mut was localized in both the soluble and insoluble fractions with and without As2O3, and protein modification including multimerization was not observed with this assay system (Figure 4B lanes 7, 8, 13, and 14). Protein expression levels were quantitated with BioMax software, and the relative intensity is depicted in Figure 4C. These findings indicate that As2O3 did not affect PR-B/L-mut compared with PML and PR-WT in this assay system, and this phenomenon was closely related to insufficient SUMOylation (Figure 3).

**Distinct cellular localization of PR-WT and PR-B/L-mut with or without As2O3 in HeLa and U937 cells**

We then performed immunofluorescent (IF) staining to examine the cellular localization pattern of wild-type PML, PR-WT, and PR-B/L-mut in HeLa cells (Figure 5). Wild-type PML was overexpressed in HeLa cells, which were incubated with or without As2O3. PML localization was confirmed by IF staining using an anti-PML antibody. PML was localized in PML nuclear bodies showing a speckled pattern without As2O3 (Figure 5Ai,iii). In the presence of As2O3, the localization pattern was clearly altered to a macrogranular pattern (Figure 5Ai,iv,vi).

Similar analyses using PR-WT- and PR-B/L-mut–expressing HeLa cells were also performed (Figure 5B-C). Anti-PML antibody was used to detect endogenous PML, overexpressed PR-WT, and PR-B/L-mut, and anti-Flag antibody was used to detect PR-WT and PR-B/L-mut. In the absence of As2O3, PR-WT, a PML–RARA short form without an NLS, was detected around the nucleus as a microgranular pattern (Figure 5Bi,ii,iv), and as a macrogranular pattern in the presence of As2O3 (Figure 5Bv,vi, and vii). Surprisingly, PR-B/L-mut was localized differently in the cytoplasm as a diffuse pattern without As2O3 (Figure 5Ci,ii,iv), and the localization of PR-B/L-mut was not altered in the presence of As2O3 (Figure 5Cv,vi, and vii). With As2O3 treatment, endogenous PML was confirmed in the nucleus with the macrogranular pattern (Figure 5Cv,vi, and vii). Transfected PR-B/L-mut (red fluorescence) could not be confirmed in the nucleus (Figure 5Cvi). The localization of PR-B2-mut showed almost the same pattern as PR-B/L-mut (data not shown). These data strongly suggest that the point mutation in the PML-B2 domain disrupts or inhibits PML body formation and the responsiveness to As2O3 treatment.

Nearly identical analyses using U937 cells were performed to confirm the significance in hematopoietic cells. As indicated in Figure 5D, PR-WT was observed in the cytoplasm with the microgranular pattern (Figure 5Di,ii,iv), and PR-B/L-mut showed a diffuse pattern, as observed in HeLa cells (Figure 5Dv,vi, and viii).

**Distinct cellular localization of PR-L and PR-L-B2-mut2 with or without As2O3 in HeLa cells**

We also performed a similar analysis using PR-L and PR-L-B2-mut2 to show the molecular significance of the L218P mutation.
in the B2 domain confirmed in patient 6 (Figure 6). PR-L, a PML-RARA long form with an NLS, was detected in the nucleus as a microgranular pattern without As2O3 (Figure 6Ai,ii,iv) and as a macrogranular pattern with As2O3 (Figure 6Av,vii,viii). In contrast, PR-L-B2-mut2 was localized in the nucleus as a diffuse pattern without As2O3 (Figure 6Bi,ii,iv). The localization was not altered in
the presence of As$_2$O$_3$ (Figure 6Bv,vi,viii). These data strongly suggest that the L218P mutation in the PML-B2 domain contributes to the aberrant PML body formation and disrupts responsiveness to As$_2$O$_3$ treatment.

Cellular localization of endogenous PR-B/L-mut without As$_2$O$_3$ in primary cells from a patient

To show the localization of PR-WT and PR-B/L-mut protein in primary leukemia cells, IF staining using an anti-PML antibody was performed (Figure 7). Primary leukemia cells from patient 1 obtained at diagnosis and at the terminal stage were used to detect PR-WT and PR-B/L-mut, respectively. Nearly the same localization pattern as seen in Figure 5 was confirmed in this assay. The PML bodies were observed as a granular pattern at diagnosis (PR-WT), but the pattern was significantly altered to become diffuse at the terminal stage (PR-B/L-mut). These data strongly suggest that PR-B/L-mut protein was expressed and functional in primary leukemia cells, and may contribute to different responsiveness to As$_2$O$_3$ treatment.

Discussion

In the present study, we have shown acquired missense mutations in $PML$-$RARA$ that are closely related to resistance to As$_2$O$_3$ treatment. In 2 patients, we detected A216V and L218P substitutions in the B2 domain of PML. The B2 domain is part of the RBCC motif, which is thought to be critical for PML homo/
heterodimerization, oligomerization, and As$_2$O$_3$ binding. Recent studies have indicated that As$_2$O$_3$ binds directly to cysteine residues in zinc fingers in the RBCC domain, especially C77/80 and C88/91 in the RING domain and C212 and C213 in the B2 domain. Binding of As$_2$O$_3$ in the RBCC domain appears to be critical for the effect of As$_2$O$_3$ on PML-RARA. Interestingly, the mutations described herein were located just adjacent to the CC motif (C212/213) in the B2 domain, which is thought to be critical for As$_2$O$_3$ binding (Figure 1D). These findings suggest that substitutions at A216 and L218 may affect proper As$_2$O$_3$ binding, resulting in aberrant responsiveness to As$_2$O$_3$ through aberrant subcellular localization, insufficient SUMOylation, and/or multimerization.

The in vitro SUMOylation assay indicated that PR-B/L-mut and PR-B2-mut mostly lack SUMO1-Ubc9–induced SUMOylation and dimerization/multimerization (Figure 3B), and this was not changed in the presence of As$_2$O$_3$. It has been suggested that degradation of the PML-RARA protein with As$_2$O$_3$, followed by SUMOylation and oligomerization, may also be inhibited by mutations in the B2 domain. Furthermore, PR-B/L-mut was localized in both the soluble and insoluble fractions, and the localization was not changed in the presence of As$_2$O$_3$. Nearly the same result was obtained with IF staining, indicating that PR-B/L-mut and PR-B2-mut were localized in the cytoplasm with a diffuse pattern that was not altered in the presence of As$_2$O$_3$ (Figure 5C). Furthermore, PR-L-B2-mut2 was localized in the nucleus as a diffuse pattern with or without As$_2$O$_3$ (Figure 6B). These results strongly suggest that amino acid substitution in the B2 domain is...
whether the mutation contributes to clinical resistance to As2O3 RARA according to the FISH analysis. Careful evaluation of clonal expansion of PML-RARA with the L218P substitution may explain the cytoplasmic localization during the disease progression are required. Unfortunately, DNA obtained at the disease onset and the terminal stage (Figure 2B lanes 4 and 5). Therefore, an APL variant phenotype with bcr3 of the PML–RARA protein may be related to a poor prognosis, showing that insufficient ATRA effectiveness and the LBD mutation are related to a consistent phenotype of ATRA resistance. PR-B/L-mut is localized mainly in the cytoplasm, and the effectiveness of ATRA may not be anticipated. Further analyses are needed.

PR-B/L-mut also contains a mutation in the LBD of RARA (G391E). Previous studies have indicated that the LBD mutation is closely related to ATRA resistance, and patient 1 in this study also showed a clinically refractory response to ATRA in the late stage of his disease progression and resistance to Am80 in the terminal stage. The LBD mutation G391E was detected only in the terminal stage (Figure 2B). Therefore, an APL variant phenotype with bcr3 of the PML–RARA protein may be related to a poor prognosis, showing that insufficient ATRA effectiveness and the LBD mutation are related to a consistent phenotype of ATRA resistance. PR-B/L-mut is localized mainly in the cytoplasm, and the effectiveness of ATRA may not be anticipated. The function of PR-WT and PR-B/L-mut as transcription factors to control activation and/or repression by recruiting coregulators, such as p300/CBP, SMRT/N-CoR-TBL1/R1, and histone deacetylases, should be analyzed to confirm the responsiveness to ATRA. Conversely, the probability of the effects of the LBD mutation on As2O3 resistance may be relatively low based on our experiment, because the diffuse localization pattern in the cytoplasm of PR-B2-mut without an LBD mutation in the presence of As2O3 showed nearly the same pattern as PR-B/L-mut (data not shown). Furthermore, a previous study indicated that ATRA-resistant NB4 clones with mutations in the PML–RARA LBD domain were fully sensitive to As2O3 treatment.

Interestingly, leukemia cells from patient 1 contained minor clones of PR-B2-mut and PR-LBD-mut in addition to the major clone of PR-B/L-mut at the terminal stage (Figure 2B time points 4 and 5), which is difficult to explain. One possibility is that one allele of the wild-type PML and RARA genes originally had genetic mutations in the B2 and LBD domains, and PR-WT, PR-B2-mut, PR-LBD-mut, and PR-B/L-mut were generated at the early stage of disease progression. Another explanation is that the PML–RARA fusion gene with mutations in the B2 and LBD domains translocated again with wild-type PML or RARA genes during the disease progression. Further analyses are required using several strategies including allele-specific PCR, FISH, and/or single nucleotide polymorphism analysis.

Mutations in the B2 domain that result in insufficient responsiveness to As2O3 therapy were confirmed in 2 of 15 (13%) patients with APL treated with As2O3. Recently, As2O3 was introduced as a consolidation treatment, and the event-free survival at 3 years was significantly improved compared with conventional consolidation treatment with ATRA and daunorubicin (80% vs 63%). However, almost 5% of patients with APL treated with As2O3 show As2O3 refractory disease. Because it is possible that a PML B2 mutation may be partly related to the As2O3 refractory phenotype, repeated genetic analyses at several time points of the clinical course may be useful for predicting patients at high risk for a poor
response to As₂O₃ therapy. Further investigation is required to confirm the clinical significance.

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Authorship

Contribution: E.G., A.T., and F.H. designed the experiments, performed the research, and the analyzed data; E.G. and A.T. wrote the manuscript; A.A. prepared the clinical samples and performed the research; and A.T., H.K., and T.N. interpreted the data and supervised the experiments.

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References


Missense mutations in PML-RARA are critical for the lack of responsiveness to arsenic trioxide treatment

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