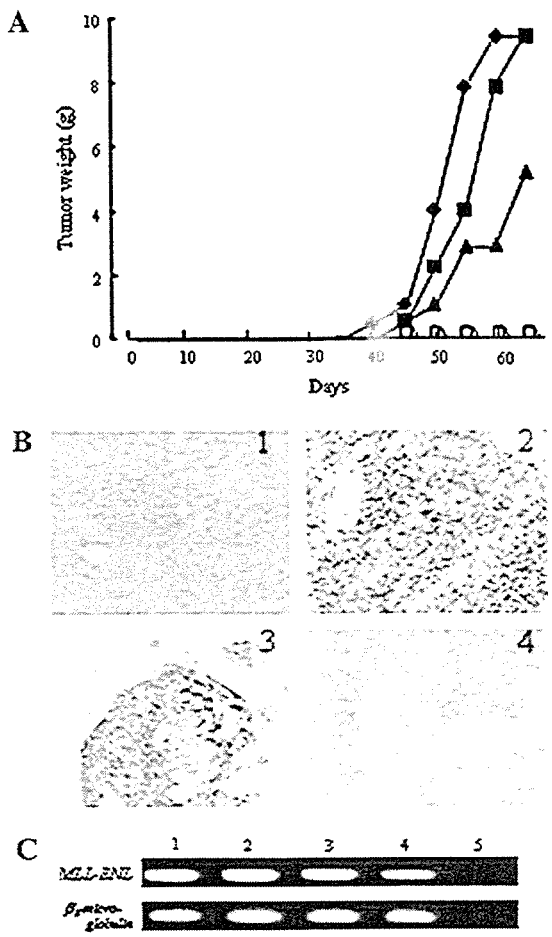

Fig. 7. TRL-01 required the coexistence of stroma cells for tumor formation in vivo. (A) Engraftment of BM MNC (2×10^6 cells) from the AML M0 leukemia patient and HESS-5 cells (6×10^4 cells) into dorsal regions of NOD/scid/γ_c^{null} (NOG) mice (close symbols, $n = 3$). As controls, either TRL-01 alone (squares; $n = 3$) or HESS-5 alone (triangles; $n = 3$) was similarly injected. Engrafted NOG mice were observed for 60 days. (B) Pathological findings of the subcutaneous tumor from NOG mice ($n = 3$) 60 days after the transplantation. The specimens from the subcutaneous tumor bearing NOG mice were sectioned and stained with H&E (panel 1, subcutaneous tumor) or developed human CD45 with DAB (panel 2, subcutaneous tumor; panel 3, bone marrow; panel 4, spleen). Original magnification: ×400. (C) RT-PCR of the *MLL-ENL* cDNA synthesized from the RNA in the subcutaneous tumor cells of NOG mice. Primers were *MLL* primer 4 and *ENL* primer 1, which gave PCR products at 438 bp. Lane 1, BM MNC at diagnosis; lane 2–4, cells collected from subcutaneous tumor of NOG mice ($n = 3$); lane 5, H_2O. As a positive control, primers for β_2-microglobulin were used.

mutation of *FLT3* (data not shown). Instead, the expression level of KIT/CD117 was high, although the *KIT* gene was not mutated (data not shown).

Another important characteristic of TRL-01 is the dependence on BM stroma cells in vivo and in vitro. Within 1 month after intravenous injection of TRL-01 into the tail, TRL-01 was exclusively found in the endosteal region of the BM and not in the other organs of mice. Subcutaneous injection of TRL-01 resulted in tumors only when HESS-5 was coinjected.

The HESS-5 stroma cell line has been used for ex vivo expansion of human hematopoietic progenitor cells, suggesting that HESS-5 cells have similar properties to human hematopoiesis-supporting cells in the BM [25,26]. HESS-5-conditioned medium was able to maintain the growth and viability of cells transiently. Soluble molecules produced by HESS-5 cells have been studied, and murine SCF, G-CSF, TPO, and nerve growth factor (NGF) are reported [26]. In the culture of TRL-01 without HESS-5, recombinant human SCF or GM-CSF promoted the proliferation of TRL-01 but did not maintain it. Given that murine SCF and GM-CSF do not cross-react with human receptors, these molecules are unlikely to have a key role in the proliferation of TRL-01.

Direct contact with HESS-5 was essential for the continuous survival of the cell line. Furthermore, proliferation of TRL-01 is also upregulated by direct contact with HESS-5 (data not shown). It is known that the fate of leukemia cells as well as hematopoietic stem cells (HSCs) is significantly affected by the adhesion mechanism [27]. Many adhesion molecules (such as integrin families, CD44, CD34, selectins, and immunoglobulin-like adhesion molecules) are present on the leukemia cell surface [28,29], although their roles are not fully understood. TRL-01 expresses VLA-4 and -5, and the apoptosis is partially blocked by the fibronectin-coated plate. Some studies also suggested that adhesion to fibronectin inhibited apoptosis of leukemia samples separated from the BM [30]. Accordingly, extracellular signaling via VLA-4 and -5 might be important for leukemia survival. Recent reports suggested that distinct intercellular interactions between the BM niche and HSCs are important for self-renewal, quiescence, and differentiation [31]. Leukemia cells may divide and survive in a similar way to HSCs.

Intravenously injected TRL-01 was located in the endosteal region of the NOG mouse after xenotransplantation. The mechanisms underlying the homing and lodging of TRL-01 in specific microenvironments is unknown. Currently, the chemokine SDF-1 and its receptor CXCR4 are known to play a critical role in the homing of normal hematopoietic progenitor cells to the BM [32,33]. TRL-01, however, does not express CXCR4 (data not shown). Furthermore, it remains unclear whether injected TRL-01 was localized to the murine BM endosteal region using the same mechanism by which it adheres to HESS-5. In our study, the coinjection of TRL-01 and HESS-5 resulted in a subcutaneous tumor in mice. HESS-5 might secrete a migration-inhibitory factor for TRL-01, which is different from SDF-1. Therefore, we believe that the self-organization of the tumor outside the BM might provide an interesting model to study homing of leukemia, leukemia-stroma interaction, angiogenesis, and drug development.

In conclusion, TRL-01 would be a useful cell line for studying not only the biology of *MLL-ENL* but also

the intercellular association between leukemia and the microenvironment.

Acknowledgments

This study was supported by grants-in-aid from the Ministry of Health and Welfare and from the Ministry of Education, Culture, Sports, Science and Technology, and was performed in cooperation with Research on the Human Genome, Tissue Engineering, Food Biotechnology from the Ministry of Health and Welfare. We thank Ms. Satomi Yamaji for technical assistance, and Ms. Manami Kira for secretarial assistance.

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Histone deacetylase 3 (HDAC3) is recruited to target promoters by PML-RARα as a component of the N-CoR co-repressor complex to repress transcription *in vivo*

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Received 18 April 2006
Available online 15 May 2006

Abstract

PML-RARα is a chimeric transcription factor tightly associated with acute promyelocytic leukemia. PML-RARα plays an important role in the aberrant transcription repression on the target genes of wild-type retinoic acid receptors. Here, we demonstrated that HDAC3, one component of the N-CoR transcription repressor complex, is a key regulator of the transcription repression by PML-RARα *in vivo*. Using immunoprecipitation, we demonstrated that PML-RARα interacts with N-CoR/HDAC3 *in vivo* without ligand. Next, using chromatin immunoprecipitation (ChIP) assay, this N-CoR/HDAC3 co-repressor complex was recruited to the endogenous target promoters (RARβ and CYP26) through PML-RARα. The neighboring histones were de-acetylated and gene expression was repressed. When HDAC3 protein was knocked down by RNA interference in PML-RARα-expressing cells, the endogenous target genes were significantly activated, which was also confirmed by promoter-luciferase reporter assay. These results provide evidence to show that the N-CoR/HDAC3 co-repressor complex is involved in the aberrant transcription regulation in PML-RARα-expressing cells.

Keywords: PML-RARα; N-CoR; HDAC3; Acute promyelocytic leukemia (APL); Transcription repression; Chromatin; SMRT

Transcription repression by the PML-RARα chimeric transcription factor plays an important role for the leukemogenesis in APL [1–3]. Recently, it has become widely recognized that all-*trans* retinoic acid (ATRA) is an excellent molecular targeting reagent for APL [4,5]. ATRA works directly on PML-RARα protein *in vivo*, and the aberrant transcription repression on many target genes can be released by administration of ATRA at the pharmacological concentration. Transcription de-repression on PML-RARα target genes is thought to be a key mechanism of the molecular targeting therapy and "transcription therapy" in APL.

Transcription repression by PML-RARα involves nuclear receptor co-repressor/silencing mediator for retinoid and thyroid hormone receptors (N-CoR/SMRT) co-repressor protein complexes that interact with PML-RARα in the absence of ligand [6]. N-CoR and SMRT are known to be key molecules for the transcription repression of nuclear receptors including retinoic acid receptor (RAR) and thyroid hormone receptor (TR) [7]. In the late 1990s, it had been reported that N-CoR/SMRT forms large protein complexes that contain other functional proteins including transcription co-repressors Sin3 and HDAC1 (histone deacetylase 1) [4,5,8,9]. In the last 5 years, numerous N-CoR/SMRT complexes other than Sin3/HDAC1 complex have been reported [10]. N-CoR/SMRT-TBL1/R1 (transducin beta like protein 1/related protein)-HDAC3 complex was purified from human nuclear extract by using a biochemical strategy [11–14]. N-CoR/SMRT-TBL1/R1-HDAC3 proteins exist in a large complex in human cells with an estimated size of 1.5–2 MDa. TBL1/R1, HDAC3, Kaiso [15], GPS2 [12] (G-protein pathway suppressor 2),

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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.05.047

and very recently JMJD2A [16] were identified as components of the N-CoR/SMRT complexes. TBL1/R1, which has WD40 repeats known to be a histone-binding motif, is critical for transcription repression [13,14,17,18]. Transcription repression introduced by these proteins is closely related to binding with de-acetylated histone on chromatin [19]. HDACs catalyze de-acetylation of specific lysine residues antagonizing histone acetyl transferases such as p300, PCAF, and GCN5 [20]. De-acetylation of histones in chromatin is closely related to transcription repression of target genes.

Nuclear receptor co-repressor null mice show abnormality in erythrocyte and thymocyte hematopoiesis [10], and disrupting N-CoR function using a dominant negative form expressed in immature leukemia cells induces cell differentiation [21]. Furthermore, interference of enzymatic activity of HDACs by administration of HDAC inhibitor, such as butyrate, trichostatin A (TSA), and valproic acid, induces differentiation in leukemia cells [22]. Taking these results into consideration, N-CoR-HDAC complexes are essential for hematopoiesis in development and are likely involved in cell differentiation in adult hematopoieses and pathogenesis in specific types of leukemia including APL. The determination of which N-CoR complex is utilized for the transcription regulation by PML-RARα is important for not only understanding of mechanisms of leukemogenesis, but also developing new molecular targeting therapies in APL. Here, we demonstrate directly *in vivo* that N-CoR-HDAC3 is recruited to endogenous target promoters through PML-RARα to repress PML-RARα target gene transcription. Our data indicate that N-CoR-HDAC3 complexes play an important role for the transcription repression in PML-RARα-expressing leukemic cells, and may be good candidates for molecular targets in the treatment of patients with APL.

Materials and methods

Cell culture. A human embryonic kidney cell line 293T cells was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum.

Plasmids. The coding sequence of PML-RARα was amplified by PCR and the Flag sequence was added in the forward primer as shown previously [17]. The PCR fragment was directly cloned into pcDNA4-His-Max-TOPO mammalian expression vector (Invitrogen, Carlsbad, CA). For the expression of short-hairpin RNA (shRNA) against HDAC3, pS65R-shHDAC3 that has CMV promoter for the expression of red fluorescent protein (RFP) was generated as follows. RFP sequence of pDsRed2-C1 (Stratagene, La Jolla, CA) was amplified using primers; RFP-forward 5′-GCCGCCACCGGTATGGCCTCCTCCGAGAAC-3′, reverse 5′-GC CGCCAGATCTTCACAGGAACAGGTGGTGGCG-3′. After digestion by *Age*I and *Bgl*II, the RFP fragment was inserted into *Age*I–*Bgl*II sites of pS65T-C1 vector (Stratagene). Short-hairpin RNA sequence with H1 promoter from shRNA expression vector p*Silencer* 3.0-H1 (Ambion, Austin, TX) was amplified by PCR using the following primers; shH1-forward 5′-GCCGCCCTTAAGGATATCTTCCCAGTCACGACGTT GTA-3′, reverse 5′-GCCGCCCTTAAGGATATCCACAGGAAACAGC TATGACC-3′. This fragment was inserted into *Afl*II site after blunting (pS65R-siH1). For shRNA sequence for HDAC3, two oligos (forward 5′-GATCCGATGCTGAACCATGCACCTTTCAAGAGAAGGTGCA TGGTTCAGCATCTTTTTTGGAAA-3′, reverse 5′-AGCTTTTCCAA AAAAGATGCTGAACCATGCACCTTCTCTTGAAAGGTGCATG GTTCAGCATCG-3′) generated by Nippon Gene Laboratory (Sendai, Japan) were annealed and inserted into *Bam*HI–*Hin*dIII sites of pS65R-shH1 (pS65R-shHDAC3). The sequence for the siHDAC3 and its specificity were published previously [31]. RARβ and CYP26 promoter sequences were amplified by PCR using genomic DNA obtained from the author's white blood cells. PCR primers were designed based on the genomic DNA sequences from GenBank (RARβ; X56849, CYP26; AL358613), and the primer sequences are; RARβ-pro-forward 5′-GCCG CCGGTACCCCAGAACACACAGCTGGTAA-3′, reverse 5′-GCCGC CAAGCTTGATCTCCCTTGCACTGAATG-3′, CYP26-pro-forward 5′-GCCGCCAAGCTTGTACAGATGGAGCCGGGCTC-3′, reverse 5′-GCCGCCAAGCTTGGCGCGCCGCGACCTCCCG-3′. These primers amplify the promoter sequences just before the translation start codon (ATG), and the lengths of those fragments are 1313 and 1000 bp, respectively. Those fragments were cut by *Kpn*I and *Hin*dIII, and inserted into the *Kpn*I–*Hin*dIII sites of pGL2-basic luciferase reporter vector (Promega).

RNA extraction and RT-PCR. Total RNA was isolated from 293T cells and complementary DNA was obtained by using SuperScriptII reverse transcriptase (Invitrogen). PCR was carried out as follows; 95 °C 3 min 1 cycle, 94 °C 30 s–55 °C 30 s–72 °C 40 s 25 to 30 cycles for semi-quantitative PCR. PCR primers for the cording sequences of RARβ, CYP26, and HDAC3 are as follows; RARβ-forward 5′-ATGTTTGACTGTATGG ATGTTC-3′, reverse 5′-CCCACTTCAAAGCACTTCTG-3′, CYP26-forward 5′-GATGAAGCGCAGGAAATACG-3′ and reverse 5′-ATGGC GATTCGGAACATGAG-3′, HDAC3-forward 5′-CTGGCTTCTGCTA TGTCAAC-3′, HDAC3-reverse 5′-ACATATTCAACGCATTCCCCA-3′, HDAC1-forward 5′-GAGATGACCAATACCACAG-3′, HDAC1-reverse 5′-TATCCCGTAGGTCCCCAGT-3′. As an internal control, β-actin primers were used, β-actin-forward 5′-TCACTCATGAAGATCC TCA-3′, reverse 5′-TTCGTGGATGCCACAGGAC-3′.

Quantitative RT-PCR. Quantitative PCR (TaqMan real-time PCR) was performed using ABI Prism 7000 (Applied Biosystems). Probes and primers for RARβ (Hu00233507) and CYP26 (Hs00175627) were purchased from Applied Biosystems. As an internal control, endogenous GAPDH mRNA was measured (Hu GAPDH).

Antibodies. Affinity purification of N-CoR antiserum was described previously [17]. Anti-human HDAC3, HDAC1, and Sin3 were purchased from Abcam (Cambridge, UK). Acetylated histone H4 antibody was purchased from Upstate (Waltham, MA). Anti-Flag antibody was purchased from Sigma (St. Louis, MO).

Immunoprecipitation (IP) and immunoblotting. Flag-tagged PML-RARα expression vector (2 μg) was transfected into 293T cells (5×10^5) using FuGene6 (Roche). After 2 days of incubation, cells were lysed in 400 μl lysis buffer (50 mM Tris–HCl, pH 8.0, 1.5 mM $MgCl_2$, 1 mM EGTA, 5 mM KCl, 10% glycerol, 0.5% NP-40, 300 mM NaCl, 0.2 mM PMSF, 1 mM DTT, and Complete-mini protease inhibitor tablet (Roche)). After centrifugation, the supernatant was put into a new tube, to which was added 400 μl of dilution buffer (50 mM Tris–HCl, pH 8.0, 1.5 mM $MgCl_2$, 1 mM EGTA, 5 mM KCl, 10% glycerol, 0.2 mM PMSF, 1 mM DTT, and protease inhibitor tablet). IP was performed using Flag-M2 affinity beads (Sigma) as indicated previously [18,23]. After IP, agarose beads were washed three times in wash buffer (50 mM Tris–HCl, pH 8.0, 1.5 mM $MgCl_2$, 1 mM EGTA, 5 mM KCl, 10% glycerol, 0.05% NP-40, 150 mM NaCl, 0.2 mM PMSF, 1 mM DTT, and protease inhibitor tablet), and the purified protein was eluted with 100 mM glycine. Eluted proteins were analyzed by immunoblotting using specific antibodies.

Chromatin immunoprecipitation (ChIP) assay. Chromatin immunoprecipitation assay was performed using 293T cells (1×10^6 cells for one assay) as indicated on the manufacturer's instruction manual (Upstate) and previous report [17]. Wash buffers I to IV were prepared as follows; ChIP I (0.1% sodium deoxycholate, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.0, and 150 mM NaCl), ChIP II (0.1% sodium deoxycholate, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.0, and 500 mM NaCl), ChIP III (0.25 M LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, and 10 mM Tris–HCl, pH 8.0), ChIP IV

(10 mM Tris–HCl, pH 8.0, and 1 mM EDTA). All washing buffers contain 0.2 mM PMSF, 1 mM DTT, and protease inhibitor tablet (Roche). After washing and reverse-crosslinking, immunoprecipitated DNA was purified and PCR was performed. The following PCR primers were used to detect the immunoprecipitated promoter DNA; RARβ promoter forward 5′-TCCTGGCTGTCTGCTTTTGT-3′, reverse 5′-CAAAAAGCCTTCC GAATGCG-3′, CYP26 promoter forward 5′-TAAAGATTTTGGGCA GCGCC-3′, reverse 5′-CATCTGCAAGGTTTCCCCAA-3′, RARβ gene 3′ region forward 5′-CCCTGGTTATCTGTCATAGC-3′, reverse 5′-ACT GAAGGTACTGGGGAATG-3′, and CYP26 gene 3′ region forward 5′-GGGGTTTCAGTGCTTTTTGC-3′, reverse 5′-CAATGCTGGAAA CCTGGC-3′.

Luciferase reporter assay. 293T cells (5×10^4 cells/one assay) were plated in 12-well plates and incubated for one day. Then, pS65R-shH1 or pS65R-shHDAC3 (concentration is indicated in legends) were transfected. Twenty-four hours after the first transfection, pcDNA4-F-PML-RAR (0.5 μg) and luciferase reporter plasmids RARβ-Luc or CYP26-Luc (1 μg) were transfected. Cells were incubated for 2 days with or without 1 μM ATRA and lysed for luciferase assay. Dual-luciferase assay kit (Promega) was used for this assay, and chemoluminescence signal was measured by Luminometer AB-2250 (ATTO, Tokyo, Japan).

Results

PML-RARα interacts with N-CoR and HDAC3 in the absence of ligand

To determine whether N-CoR-HDAC3 complexes are involved in the transcription regulation of PML-RARα *in vivo*, we first tried to co-IP complex components from 293T cells (Fig. 1). The expression vector for Flag-tagged PML-RARα (F-PML-RAR) was transiently overexpressed in 293T cells and incubated at 37 °C with (lanes 2–4 and 7–9) or without ATRA (lanes 1, 5, 6, and 10). Two days after transfection, IP using Flag-M2 affinity beads was performed on whole cell lysate. Each eluted protein sample was immunoblotted using affinity purified rabbit anti-N-CoR antiserum [17] and rabbit anti-HDAC3 antibody (Abcam). F-PML-RAR interacts with endogenous N-CoR and HDAC3 in the absence of ATRA (lane 6). In the presence of ATRA, N-CoR and HDAC3 were dissociated from F-PML-RAR in a dose-dependent manner (lanes 7–9). We also tried to detect F-PML-RAR interacting with endogenous HDAC1 and Sin3, which were reported to be components of N-CoR co-repressor complexes [24]. In this assay system, we could not detect endogenous Sin3-HDAC1 interaction with F-PML-RAR (lanes 6–10). Longer exposure failed to detect Sin3-HDAC3 interaction with F-PML-RAR (data not shown). Expression levels of endogenous N-CoR, HDAC3, HDAC1, Sin3, and exogenous F-PML-RAR are indicated in lanes 1–5 (Pre-IP). Almost the same amount of those proteins was expressed with or without ATRA. These data indicate that endogenous N-CoR-HDAC3 interacts with PML-RARα without ligand, and the affinity of those interactions is strictly regulated by ATRA.

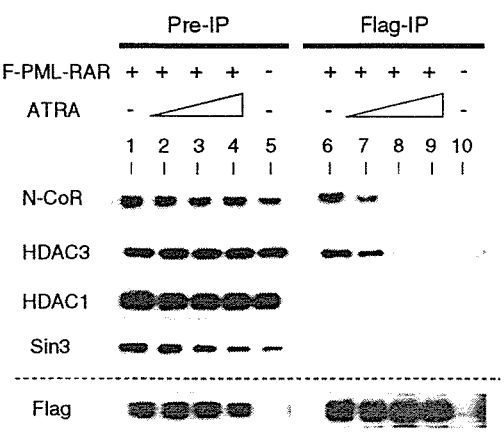

Fig. 1. Endogenous HDAC3 and N-CoR interact with PML-RARα in the absence of ligand. Flag-tagged-PML-RARα (F-PML-RAR) was transiently overexpressed in 293T cells and incubated for 2 days. Different concentrations of ATRA (lane 2, 10 nM; lane 3, 100 nM; lane 4, 1 μM) were added during incubation. Cells were lysed and subjected to immunoprecipitation using Flag-M2 affinity beads. Cell lysate (pre-IP) and IP (Flag-IP) samples were used for Western blotting. Detection was performed using anti-Flag, N-CoR, HDAC3, HDAC1, and Sin3 antibodies. Note that N-CoR/HDAC3 dissociated from F-PML-RAR in the presence of ATRA (lane 9). F-PML-RAR was successfully immunoprecipitated by Flag affinity beads (Flag; lanes 6–9).

Exogenous PML-RARα regulates target gene expression in vivo

To determine whether the overexpressed F-PML-RAR chimeric protein is functional *in vivo*, we analyzed RARα target gene expression by RT-PCR using 293T cells. Two RARα target genes [25], RARβ and CYP26, were selected for this assay. F-PML-RAR was overexpressed in 293T cells and incubated for 1 day with or without increasing amounts of ATRA as indicated in Fig. 2A. Total RNA was purified and RT-PCR using RARβ, CYP26, and β-actin specific primers were performed. These primers were designed for the coding sequences that are located on different exons to eliminate amplifying genomic DNA contamination in the PCRs. As shown in Fig. 2A, endogenous RARβ and CYP26 mRNA expression is significantly repressed in the presence of F-PML-RAR (lane 2 vs. lane 1). In contrast, RARβ and CYP26 expression is induced by ATRA in a dose-dependent manner (lanes 3–5). To compare the expression level in each sample, quantitative PCR (real-time PCR) was performed (Fig. 2B). The same cDNA samples in Fig. 2A were used for this assay. Repressed expression of RARβ and CYP26 by PML-RARα (lanes 2, adjusted as 1) was significantly relieved as ATRA concentration increased. These data indicate that exogenous F-PML-RAR in 293T cells regulates endogenous RARα target gene expression. F-PML-RAR works as repressor in the absence of ligand and as an activator with ligand.

Endogenous N-CoR-HDAC3 is recruited to PML-RARα target gene promoters in the absence of ligand

Next, we performed ChIP assay in 293T cells to show recruitment of N-CoR-HDAC3 complexes to target gene

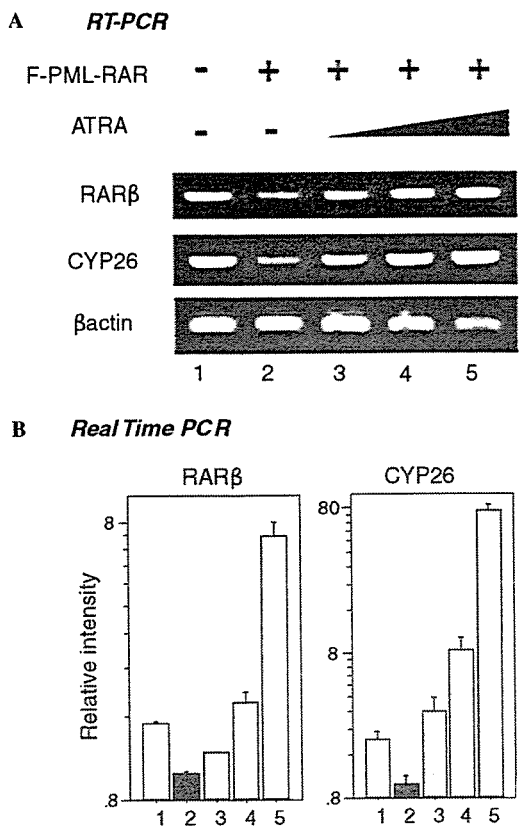

Fig. 2. PML-RARα regulates endogenous RARβ and CYP26 gene expression in the presence or absence of ligand. (A) F-PML-RAR was overexpressed in 293T cells and different concentrations of ATRA were added (lane 3, 10 nM; lane 4, 100 nM; lane 5, 1 μM) to the culture medium. After 2 days of incubation, total RNA was extracted and RT-PCR using primers for RARβ, CYP26, and β-actin (control) was performed. In the presence of PML-RARα, the expression levels of endogenous RARβ and CYP26 gene were significantly repressed (lane 2). In the presence of ATRA, their expression levels were stimulated in a dose-dependent manner (lanes 3–5). This experiment was repeated three times. (B) To confirm the dose-dependent effect of ATRA on the promoter activities, quantitative PCR (real-time PCR) was performed. Relative expression levels were indicated, and the intensity of lane 2 was set to 1.

promoters through PML-RARα (Fig. 3). F-PML-RAR was transiently expressed in 293T cells and incubated for 2 days with or without 1 μM of ATRA. After crosslinking by formaldehyde, chromatin solution was obtained. ChIP assay against endogenous RARβ and CYP26 promoters that contain RARE (retinoic acids responsive element; DR5) sequences (Fig. 3A) [25] was performed. PCR primers for ChIP were designed to cover the RARE sequences on the target gene promoters. As previously reported, RAR/RXR (retinoid X receptor) heterodimer or RAR/RAR homodimer binds to RARE sequence through the DNA binding domains of those nuclear hormone receptors [25]. The chimeric transcription factor PML-RARα contains the DNA binding domain of RARα and is also able to bind to RARE region as a heterodimer with RXR or a homodimer [26]. Endogenous RXRα expression in 293T cells was confirmed by RT-PCR (data not shown). PML-RARα bound to endogenous RARβ and CYP26 promoter constitutively with and without ATRA (Fig. 3B, lanes 8 and 9). Endogenous N-CoR and HDAC3 were recruited to the promoters in the absence of ATRA (lanes 11 and 14). In contrast, N-CoR-HDAC3 recruitment to the target gene promoters was dissociated by adding ATRA (lanes 12 and 15). Interestingly, HDAC3 was found to be associated with both promoters even in the absence of PML-RARα (lane 13). In the presence of PML-RARα, HDAC3 remained associated with the promoters in the absence but not the presence of ATRA (lanes 14 and 15). To determine the acetylation level of histones around the RARE region of RARβ and CYP26 genes, ChIP assay using anti-acetylated histone H4 antibody (Upstate) was performed. If PML-RARα were expressed, histone acetylation levels of the target gene promoters were significantly decreased (lane 17). In the presence of ATRA, histone acetylation levels of those promoters were significantly increased (lane 18). These data indicate that PML-RARα binds to RARβ and CYP26 promoters containing RARE sequences and recruits N-CoR-HDAC3 in the absence of ligand to reduce the histone acetylation level around those promoters. It was previously reported that histone de-acetylation is closely related to the transcription repression [27].

Furthermore, PCRs using primer pairs that amplify the 3′ regions of RARβ and CYP26 genes were performed as negative controls (Fig. 3C). The same ChIP DNA with Fig. 3B was also used for this amplification. The cycle number of each PCR was the same with the experiment in Fig. 2B. Almost the same amplification efficiencies by these primer pairs were observed using the input samples (lanes 1–3). The band intensities of Flag, N-CoR, and HDAC3 (lanes 8, 9, 11, 13, and 14) were obviously lower than that in Fig. 2B. These data suggest that PML-RARα, N-CoR, and HDAC3 interact specifically with the target promoters.

Thus, consistent with these data in Figs. 1–3, it is strongly suggested that N-CoR-HDAC3 is recruited to the target gene promoters to repress the transcription through histone de-acetylation by HDAC3.

HDAC3 works as a transcription repressor for PML-RARα in vivo

To determine whether HDAC3 is critical for the transcription repression by PML-RARα *in vivo*, we used RNA interference in 293T cells. An expression vector was designed for this assay that expresses a short-hairpin RNA (short-interfering RNA; siRNA) for HDAC3 (pS65R-shHDAC3) under control of the H1 promoter and red fluorescent protein (RFP) under control of the constitutively active CMV promoter (Fig. 4A). A vector that expresses siRNA without specific target RNA was also prepared (pS65R-shH1) as a negative control. These vectors were transfected into 293T cells, and the efficiency of blocking mRNA and protein expression was determined by semi-quantitative RT-PCR and immunoblotting using whole cell lysates (Fig. 4B and C). Transfection efficiency

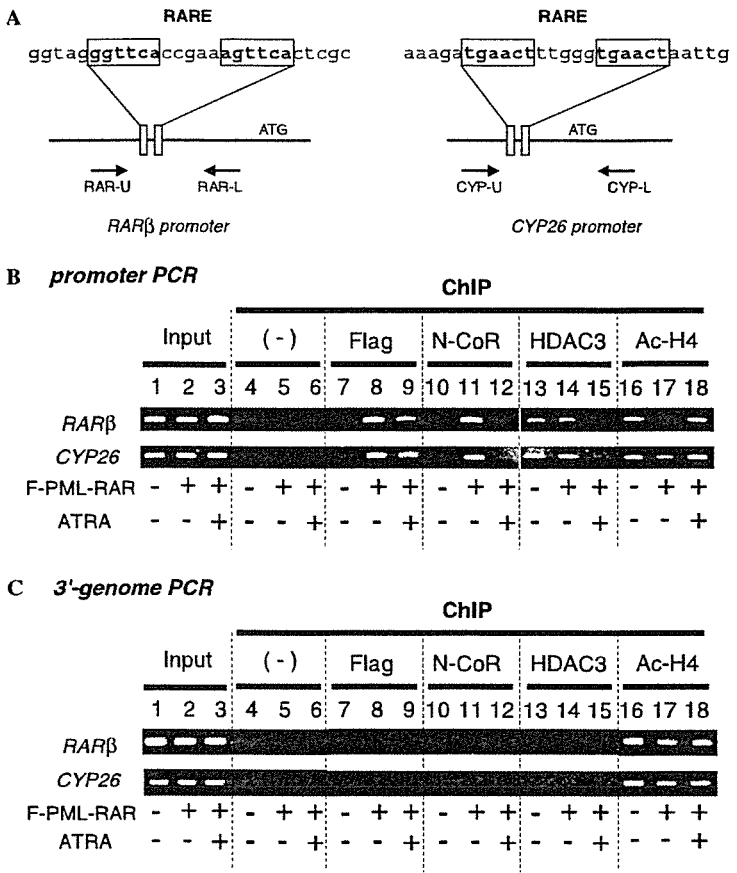

Fig. 3. Endogenous N-CoR and HDAC3 proteins are recruited to PML-RARα target gene promoters *in vivo*. (A) Schematic representation of RARβ and CYP26 promoters. Retinoic acid receptor response element (RARE) is located at the promoter site as indicated. Detailed nucleic acid sequences of RARE are also shown. Two consensus sequences were separated by 5 nucleic acids (DR5). Translation start sites are indicated as ATG, and the primers for ChIP assay are indicated by black arrows. (B) ChIP assay was performed using F-PML-RAR expressed in 293T cells. Extracted chromatin samples were used directly in the PCR for the input control (lanes 1–3). Rabbit preimmune serum was used as a negative control (lanes 4–6). For the immunoprecipitation of F-PML-RAR, Flag-M2 affinity beads were used (lanes 7–9). Antibodies for N-CoR (lanes 10–12), HDAC3 (lanes 13–15), and acetylated-histone H4 (Ac-H4, lanes 16–18) were used for immunoprecipitation of the endogenous proteins. ATRA (1 μM) was added as indicated (lanes 3, 6, 9, 12, 15, and 18). Semi-quantitative PCR was performed to confirm the protein interaction with the target promoters. Note that F-PML-RAR is recruited to the target promoters with or without ligand (lanes 8 and 9). In contrast, endogenous N-CoR and HDAC3 are recruited to the promoters without ligand (lanes 11 and 14) and are dissociated from the promoter in the presence of ligand (lanes 12 and 15). In the presence of PML-RAR, histone acetylation was significantly decreased without ligand (lane 17). This experiment was performed three times and the same tendency was observed. (C) For the negative control, PCR using primer pairs which amplify the 3′ regions of each gene (at least 5 kb downstream from the promoter regions) were performed. Note that acetylated histone H4 antibody only recognized the 3′ regions (lanes 16–18). Flag, N-CoR, and HDAC3 antibody could not precipitate the 3′ regions (lanes 7–15). For semi-quantitative PCR of ChIP assay in (B) and (C), the following PCR cycles were used; 28 cycles for Input, Flag, and Ac-H4, 32 cycles for (−), N-CoR and HDAC3.

was determined by counting the number of RFP positive cells using a fluorescence microscope. At least 80% of the cells expressed RFP signal at 3 days after transfection, and the number of RFP positive cells gradually decreased as cells proliferated (data not shown). Increasing amounts of pS65R-shH1 and -shHDAC3 were transfected, and the expression of HDAC3 mRNA and protein was successfully knocked down (Fig. 4B, lanes 5 and 6, and 4C lanes 3 and 4). To check the specificity of siHDAC3, RT-PCR and Western blotting for HDAC1 were also performed. HDAC1 expression levels were not changed in the presence of siHDAC3 (Fig. 2B, lanes 5 and 6, and 4C lanes 3 and 4). Next we tried to check how long the siRNA effect lasts after transfection. pS65R-shH1 or -shHDAC3 were transfected into 293T cells, and whole cell lysates were obtained after incubation for different days as indicated in Fig. 4D. HDAC3 protein expression was significantly repressed until 6 days after transfection (lanes 3 and 4), at which time faint bands were seen (lane 4). As an internal control, HDAC1 protein was also detected, and no significant differences were observed. Based on these data, we decided to perform transcription assays using siRNA expression vectors within 3 days of transfection.

One day after transfection of pS65R-shH1 or -shHDAC3 into 293T cells, the same cells were transfected with the F-PML-RAR expressing vector and incubated for

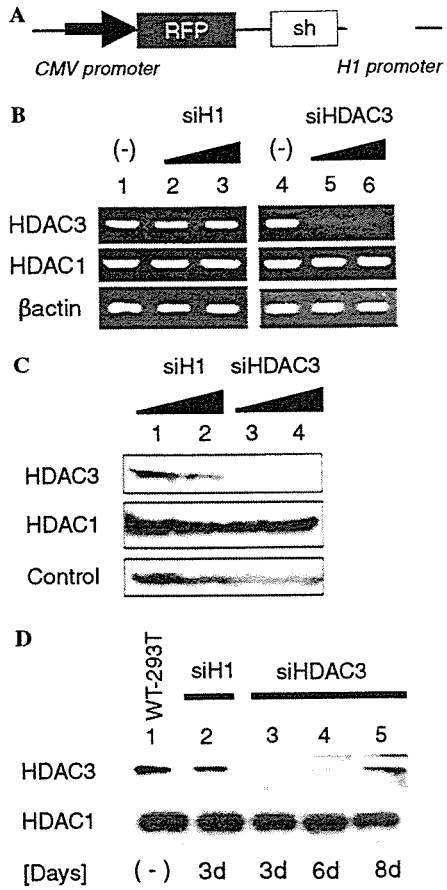

Fig. 4. Endogenous HDAC3 protein knockdown using HDAC3 short-hairpin RNA (shRNA) expressing plasmid vector. (A) Design of shRNA expressing plasmid vector. Red fluorescent protein (RFP) expressing DNA sequence was located downstream of the constitutively active CMV promoter. The shRNA expressing sequence was under the H1 promoter. These two fragments were cloned into pS65T1 mammalian expression vector. (B) RNA interference was observed using pS65R-shHDAC3 vector. shRNA was digested by endogenous enzymes, and short-interfering RNA (siRNA) was generated *in vivo*. pS65R-shHDAC3 or a negative control vector (pS65R-shH1) was transfected transiently into 293T cells and the total RNA was purified after 72 h incubation. Semi-quantitative RT-PCR was performed using HDAC3 forward and reverse primers. To exclude the possibilities of nonspecific knockdown against cellular RNAs, HDAC1 and β-actin were also amplified as controls. Different amounts of plasmid DNA were transfected (0.6 μg (lanes 2 and 5) and 1.2 μg (lanes 3 and 6) for 5×10^5 cells). (C) The whole cell lysate of siRNA-expressing cells was also obtained after 72 h incubation. Those samples were subjected to Western blotting using anti-HDAC3, HDAC1, and β-actin antibody. Note that HDAC3 protein expression was specifically knocked down by siHDAC3. (D) pS65R-shHDAC3 was transfected transiently, and the cells were lysed after different times (from 3 to 8 days). Whole cell lysate was subjected to Western blotting, and endogenous HDAC3 and HDAC1 were detected.

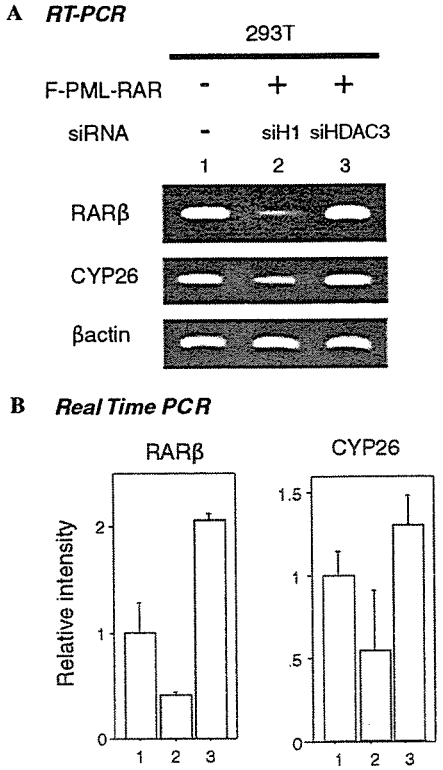

Fig. 5. Endogenous HDAC3 protein knockdown induces de-repression of PML-RARα target genes. (A) siH1 (lane 2) and siHDAC3 (lane 3) were introduced into 293T cells, and 24 h later, F-PML-RAR was transiently transfected (lanes 2 and 3). Total RNAs were extracted at 72 h after transfection of shRNA expressing vectors and subjected to RT-PCR using RARβ, CYP26, and β-actin primers. (B) Real-time PCR was performed to measure the expression levels of RARβ and CYP26 mRNA. The cDNAs used in A were also used in this assay. Note that repressed target gene expression was recovered by HDAC3 knockdown (lane 3) but not in siH1 (lane 2). This experiment was repeated three times and almost the same results were obtained.

another 2 days. Total RNA was extracted and conventional RT-PCR for RARβ, CYP26, and β-actin was performed (Fig. 5A). As shown in Fig. 2 (lane 2 vs. lane 1), F-PML-RAR repressed the expression of endogenous RARβ and CYP26 genes. When siH1 (negative control) was expressed in PML-RARα expressing 293T cells, target gene expression was still repressed (Fig. 5A, lane 2). In contrast, when siHDAC3 was expressed, repression of the target genes was relieved (lane 3). The cDNA used in Fig. 5A was also subjected to real-time PCR to quantitate this de-repression, and we observed the same tendency as with semi-quantitative RT-PCR in Fig. 5A. These data indicate that HDAC3 is critical for PML-RARα-induced transcription repression of the target genes RARβ and CYP26.

Furthermore, we carried out a luciferase reporter assay on 293T cells in the presence or absence of siHDAC3. Human RARβ and CYP26 promoters were cloned into luciferase reporter vector (RARβ-Luc and CYP26-Luc, respectively) as indicated in Materials and methods. To determine whether these two reporter vectors worked appropriately, we performed the transcription assay using the F-PML-RAR expression vector and the reporter vector with or without ATRA (Fig. 6A and B). In the presence of F-PML-RAR, basal luciferase activity of RARβ-Luc was significantly repressed without ATRA (Fig. 6A, lane 2 vs. lane 1). By adding 1 μM ATRA, the repressed

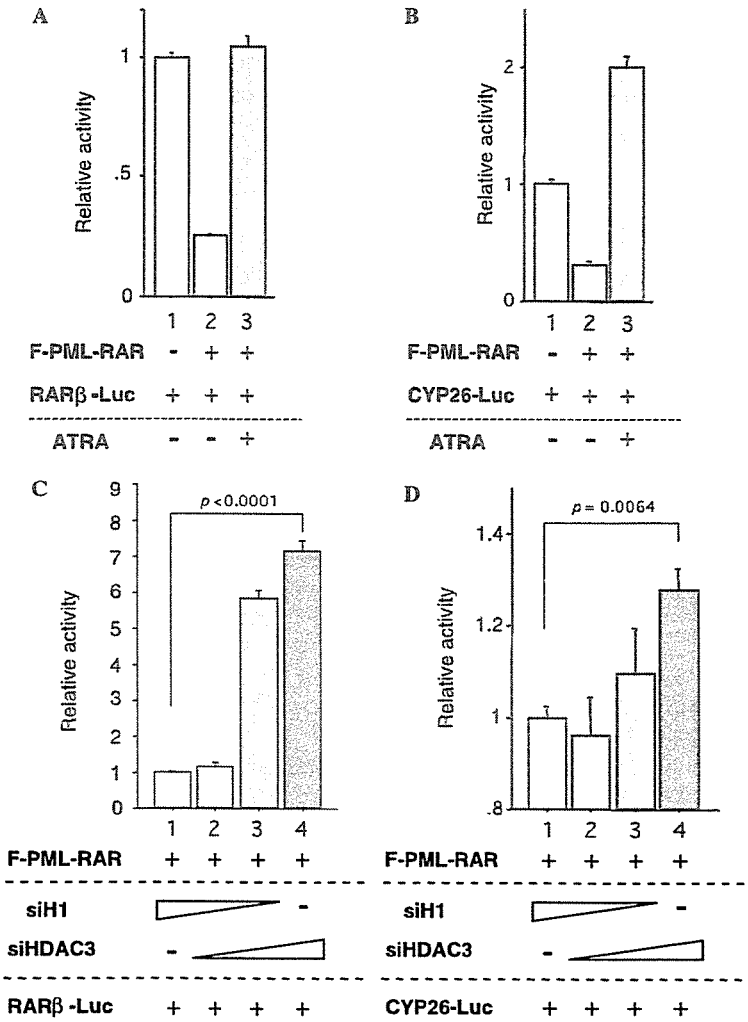

Fig. 6. Transcription repression by PML-RARα is released by HDAC3 knockdown in luciferase transcription assay. RARβ and CYP26 promoters were cloned into luciferase reporter vectors as indicated in Materials and methods. RARβ-Luc (A and C) and CYP26-Luc (B and D) reporter vectors were used in these transcription assays. (A,B) Luciferase reporter vectors and PML-RARα expressing vector were transfected into 293T cells as indicated and incubated for 2 days with (lane 3) or without (lanes 1 and 2) ATRA. Lane 1 shows the basal activity of these reporter vectors. In the presence of PML-RARα, the transcription activity was significantly repressed in the absence of ATRA (lane 2). On the other hand, the repressed transcription activity was activated in the presence of ATRA (lane 3). (C) shRNA expression vector for siHDAC3 and siH1 (negative control) were transfected into 293T cells at different concentration. Total DNA amounts for pS65R-shRNA vectors were adjusted to 1 μg. Concentration of siHDAC3 expression vector is 0 μg (lane 1), 0.1 μg (lane 2), 0.5 μg (lanes 3), and 1 μg (lane 4), respectively. Twenty-four hours after transfection of shRNA expression vectors, PML-RARα expression vector and RARβ-Luc reporter vector were transfected. Forty-eight hours after the last transfection, cells were lysed and used for the luciferase transcription assay. Transcription de-repression was observed in the presence of siHDAC3 in a dose-dependent manner (lanes 2–4) (lane 4 vs. lane 1, $p < 0.0001$). (D) CYP26-Luc reporter vector was used for the same assay as in C. PML-RARα induced transcription repression was significantly de-repressed in the presence of siHDAC3 (lane 4 vs. lane 1, $p = 0.0064$). Error bar indicates ±1 standard error. Each analysis was done in triplicate and repeated at least three times.

transcription activity was significantly activated (lane 3). Almost the same result was obtained when CYP26-Luc reporter vector was used (Fig. 6B). These data indicate that these two reporter vectors work appropriately as target genes of PML-RARα in 293T cells.

Next, siHDAC3 was introduced in the transcription assay to confirm whether endogenous HDAC3 is involved in PML-RARα-induced transcription repression (Fig. 6C and D). Twenty-four hours after transfection of increasing amounts of pS65-shHDAC3 into 293T cells, the F-PML-RARα expression vector was transfected. As a negative control, pS65R-shH1 was used. Three days after the first transfection, cells were lysed and used in the luciferase transcription assay. As shown in Fig. 6C, the repression of RARβ by PML-RARα was significantly released if endogenous HDAC3 was knocked down (lanes 2–4). When using CYP26-Luc reporter vector, transcription de-repression was also observed by HDAC3 knockdown (lanes 2–4). Unpaired *t*-test revealed that the transcription activity in lane 4 (Fig. 6D) is significantly higher than that in lane

1 ($p = 0.0064$). This experiment was repeated three times and almost the same results were obtained. These data also indicate that HDAC3 is required for unliganded PML-RARα-dependent transcription repression of target genes, supporting the data of the transcription assay using endogenous gene promoters in Fig. 5.

Discussion

PML-RARα has long been implicated to play a critical role in APL. Many *in vitro* studies on PML-RARα and co-repressor complexes suggest that PML-RARα may repress RAR target genes aberrantly by recruiting N-CoR/SMRT corepressor complexes. Both N-CoR and SMRT form multiple HDAC-containing complexes and little is known about which complex(es) is utilized by PML-RARα *in vivo*. Here, we have provided several lines of direct *in vivo* evidence to show that N-CoR-HDAC3 complexes play a critical role in regulating target gene expression by PML-RARα *in vivo*.

First, using IP assay followed by immunoblotting, we showed that unliganded PML-RARα physically interacts with N-CoR-HDAC3 *in vivo*. This interaction is dissociated by ATRA in a dose-dependent manner (Fig. 1). Furthermore, ChIP assay using anti-PML-RARα (Flag), N-CoR, HDAC3, and acetylated-histone H4 antibodies indicated that N-CoR-HDAC3 is recruited to the target gene promoters (RARβ and CYP26) through PML-RARα in the absence of ligand, and likely HDAC3 induces the de-acetylation of neighboring histone tails resulting in transcription repression of target genes (Fig. 3). Previously, we have reported using IP and ChIP assay that N-CoR-TBLR1 complexes interact with target gene promoters through PML-RARα in the absence of ligand and the interaction is dissociated by adding ATRA [17]. In the last 5 years, N-CoR/SMRT-TBL1/R1-HDAC3 (designated as Complex A) has been purified from human cell lines in independent laboratories by using biochemical strategies [11–14]. Consistent with these reports and our data, N-CoR-TBLR1-HDAC3 complexes are involved in unliganded PML-RARα-induced transcription repression *in vivo*. As previously reported, N-CoR/SMRT-Sin3-HDAC1 (designated as Complex B) plays an important role for the transcription repression by PML-RARα [5,6], although Complex B has not yet been purified from human cells as a large protein complex by using biochemical strategies. We also could not show PML-RARα interaction with endogenous Sin3-HDAC1 using IP in human cell lines (Fig. 1) and a frog oocyte assay system [17]. In previous studies, numerous efforts to confirm the existence of Complex B had been carried out. Interaction assays using overexpressed proteins of N-CoR/SMRT, Sin3, and HDAC1 in cell lines (or *in vitro* assay using GST-tagged bacterial proteins) showed the interaction of these three proteins and nuclear hormone receptors including RAR and TR [8,24], suggesting that Complex B may interact with nuclear hormone receptors in cells. Possible explanations for the discrepancies of the existence of Complex B *in vivo* and *in vitro* are, (1) Complex A is more stable than Complex B *in vivo*, and the purification of Complex B is technically more difficult, (2) the condition of purification is not optimized for Complex B, and (3) the expression ratio of Complex A and B is different from each tissue or cell line, and the detection of Complex B is quite difficult when using the specific tissues or cells. One important question about the two N-CoR complexes is which complex is more critical for the transcription repression by PML-RARα. Our study and pervious data [17] show that inhibition of the function of endogenous HDAC3 and TBLR1 induces transcription derepression of PML-RARα target gene expression *in vivo* (Figs. 5 and 6). Several reports also support our data that the transcription repression by unliganded RAR (also TR and ER) is restored by interfering with the protein function of TBL1/R1 and HDAC3, as well as N-CoR [13,18,28,29]. These reports indicate that Complex A seems to be more critical for the transcription repression by PML-RARα. Although, one possibility still remains that each N-CoR complex (or SMRT complex) may be utilized differently on different target gene promoters in distinct tissues or different time points of cell growth and development. Further investigation is needed to confirm the utilization of Complex A and B on PML-RARα-dependent transcription repression in APL cells. While preparing this manuscript, MBD1 (methyl-CpG binding domain protein 1)-HDAC3 complex is reported as a PML-RARα interacting co-repressor complex [30]. The report may support our findings that Complex A is critical for PML-RARα target gene repression.

One surprising finding from our ChIP assay is that endogenous HDAC3, but not N-CoR, is associated with RARβ and CYP26 promoters in the absence of PML-RARα (Fig. 3B, lane 13 vs. lane 10). In the presence of PML-RARα without ligand, HDAC3 continues to associate with target genes, and addition of ATRA dissociates HDAC3 from the promoters (lane 15). Since we have established *in vivo* the physical interaction of HDAC3 with PML-RARα-N-CoR in the absence of ligand (Fig. 1, lane 6), it is fair to say that N-CoR-HDAC3 likely interacts with target genes through PML-RARα. It is unclear how HDAC3 alone associates with the promoters in the absence of PML-RARα. One explanation for HDAC3 interaction with target gene promoters without PML-RARα is the existence of an unknown, N-CoR-independent HDAC3 complex that interacts with those target promoters. Interestingly, despite HDAC3 interaction with target genes, the de-acetylation of histone H4 tail is not induced (Fig. 3, lane 16). To explain this phenomenon, previous reports suggest that DAD (deacetylase activating domain), which is located at the N-terminus between repression domain 1 and 2 of N-CoR, is required for the HDAC activity [31]. Thus, in the presence of PML-RARα, N-CoR-HDAC3 instead of HDAC3 alone is recruited to the promoters, leading to histone de-acetylation. In the presence of ligand (Fig. 3B, lane 15), interaction of HDAC3 with target promoters is completely dissociated, suggesting that the ligand not only leads to the dissociation of N-CoR-HDAC3 complexes but also the alteration of

local chromatin structure, likely through PML-RARα interacting protein complexes, including TRAP/DRIP [32], SWI/SNF, and histone acetyltransferase (HAT) complexes [20], such that even the PML-RARα-independent association of HDAC3 is also removed. This leads to histone acetylation and gene activation, suggesting a role of HDAC3 in gene regulation by PML-RARα. This is further supported directly *in vivo* by our HDAC3 knockdown experiments.

Almost 90% of APL patients have the aberrant chimeric transcription factor PML-RARα. Besides this chimeric protein, several RARα chimeric proteins from APL patients including PLZF-RARα, NPM-RARα, and NuMA-RARα have been reported. Recently, using the same IP strategy, we also confirmed that PLZF-RARα physically interacts with endogenous N-CoR-HDAC3 *in vivo* (Tomita et al., unpublished data). To determine which protein is critical for the transcription repression by leukemia-related chimeric transcription factors is required for the exploration of new molecular targeting and transcription therapies for leukemia patients expressing aberrant transcription factors. Our study of the involvement of N-CoR-HDAC3 complexes in gene repression by PML-RARα may thus provide new avenues for improving the cure rate of patients, especially those with ATRA resistant, mutated-PML-RARα.

Acknowledgments

We thank Dr. Shi Y.B. and Dr. Buchholz D.R. in NICHD/NIH for critical reading. We also thank Ms. Kira, Ms. Yamaji, Mr. Ninomiya, Mr. Koyama and Mr. Nakano for helping our lab work. This work is supported by Grants-in-Aid from the Scientific Research of Education, Culture, Sports, Science and Technology, Japan, and the Public Trust Haraguchi Memorial Cancer Research Fund, Japan.

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Cancer Genetics and Cytogenetics 171 (2006) 9–16

CANCER GENETICS AND CYTOGENETICS

Risk factor analysis in myelodysplastic syndrome patients with del(20q): prognosis revisited

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Received 5 May 2006; received in revised form 6 June 2006; accepted 8 June 2006

Abstract The deletion of the long arm of chromosome 20, or del(20q), is a common cytogenetic abnormality in various myeloid disorders and is known to be a favorable prognostic factor in myelodysplastic syndromes (MDS) when it is the sole change. However, del(20q) occurs with one or more cytogenetic changes when it is associated with disease progression. Here, we analyzed 33 patients with MDS and del(20q) to ascertain the risk factors in MDS. We categorized del(20q) into two groups: one with the del(20q) clone (≥50% marrow metaphases), corresponding to genomic integrity, and the other with a late appearance of a minor del(20q) clone (<50% metaphases) with additional cytogenetic changes, representing genomic instability. Of the MDS patients with del(20q) at initial presentation, the negative factors in predicting prognosis on survival are (i) INT-2/High risk according to the International Prognostic Scoring System, (ii) any additional cytogenetic changes, or (iii) minor del(20q) clone. The late appearance of del(20q) at any phase is linked to a significantly unfavorable prognosis, thus indicating the clinical and biological heterogeneity of del(20q) in MDS.

1. Introduction

The deletion of the long arm of chromosome 20, del(20q), represents a relatively common cytogenetic abnormality in various myeloid disorders, including myeloproliferative disorders (MPD) [1,2], myelodysplastic syndromes (MDS) [3–7], and acute myeloid leukemia (AML) [8,9]. Clinically, the presence of del(20q) has a prognostic value in different myeloid disorders: the International Prognostic Scoring System (IPSS) for evaluating prognosis of MDS revealed a relative good prognosis [10].

The del(20q) with or without other abnormalities can occur as either an early or a late event in the evolution of myeloid disorders [11,12], but the exact biological significance is still unresolved. It has been suggested that the deletion may result in the loss of one or more target genes that perturb the regulation of multipotent hematopoietic progenitors [1,6,13,14]. At present, the identified myeloid tumor suppressor gene on 20q is unknown; nevertheless, several researchers have reported candidate genes in the specific common deleted region [13,14]. In MDS and MPD, an interstitial deletion of 20q has been found as an isolated karyotypic change at the time of diagnosis [1–7], and is often shown to be a predominant clone by cytogenetic analysis [15]. In de novo AML, the appearance of del(20q) as a major cytogenetic abnormality at diagnosis is less common than in MDS and MPD [8,9], and it may develop later in the disease course, with or without disease progression [12]. The de novo appearance of del(20q) as either a predominant clone in MDS or a more minor clone in disease progression implies different roles of del(20q) in pathogenesis. We analyzed 33 patients with del(20q) in MDS to identify and clarify the risk factors.

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0165-4608/06/$ – see front matter
doi:10.1016/j.cancergencyto.2006.06.003

Table 1
Clinical features and cytogenetic data for MDS patients with del(20q) not as the sole anomaly

No.	Sex/Age, yr	Dx	Karyotype	Note	IPSS	del(20q)%	Genotoxic exposure?	Overall survival, mo	Leukemia transformation
1	M/73	RA	46,XY,del(20)(q11)[7]/45,X,−Y[3]/46,XY[13]		Int-1	30	No	28	No
2	M/71	RA	46,XY,del(16)(q23),del(20)(q11)[20]		Int-1	100	No	84	No
3	F/69	RA	48,XX,+del(1)(p10),t(3;4)(p21;q34),del(20)(q11),+del(20)(q11)[16]/46,XX[5]	Mult[a]	Int-2	76	No	6	No
4[c]	M/70	RA	47,XY,del(20)(q11),+del(20)(q11)[20]		Int-1	100	No	34[b]	Yes
5	M/74	RA	47,XY,+8,del(20)(q11)[20]		Int-1	100	No	46[b]	No
6	F/66	RA	47,XX,+9,del(20)(q11)[18]		int-1	100	No	14[b]	No
7[c]	M/69	RA	45,XY,+1,der(1;7)(q10;p10),−20[2]/46,XY,idem,+20,del(20)(q11)[12]/46,XY[7]	Mult[a]	Int-2	55	No	43[b]	No
8	M/67	RA	46,XY,+1,der(1;7)(q10;p10)[7]/46,XY,idem,del(20)(q11)[13]		Int-2	65	Yes	60[b]	No
9[c]	M/72	RA	46,XY,+1,der(1;7)(q10;p10)[9]/46,XY,idem,del(20)(q11)[6]/46,XY[5]		Int-2	30	Yes	13[b]	No
10[c]	M/65	RA	46,XY,+1,der(1;7)(q10;p10)[4]/46,XY,idem,del(20)(q11)[17]		Int-2	81	No	13[b]	No
11	M/57	RA	46,XY,+1,der(1;7)(q10;p10),del(20)(q11)[20]/46,XY,idem,add(18)(p11)[2]	Mult[a]	Int-1	100	No	9	No
12	M/46	RA	46,XY[19]		Int-1		No	30[b]	Yes
			46,XY,del(7)(q31)[3]/46,XY,idem,del(20)(q11)[9]/46,XY[7]	Late[d]		47			
13	M/68	RA	46,XY[14]		Int-1		Yes	30[b]	Yes
			44,XY,−4,−11,del(20)(q11)[2]/46,XY[19]	Late[d]		10			
14	M/59	RA	46,XY,del(7)(q22)[19]/46,XY[1]		Int-2		No	22[b]	No
			46,XY,del(7)(q22)[17]/46,XY,idem,del(20)(q11)[3]	Late[d]		15			
15[c]	M/68	RA	46,XX[15]		Int-1		No	22[b]	Yes
			46,XY,+1,der(1;7)(q10;p10),del(20)(q11)[2]/46,XX[8]	Late[d]		20			
16[c]	M/76	RAEB	47,XY,−20,+del(20)(q11)XZ[10]		Int-2	100	No	44[b]	Yes
17	M/86	RAEB	46,XY,del(7)(q22),del(20)(q11)[20]		Int-2	100	No	22[b]	Yes
18	M/83	RAEB	43,XY,del(5)(q12),del(7)(q22),add(9)(p21),add(12)(p12),−16,−17,del(20)(q11),−22[19]/46,XY[2]	Mult[a]	High	90	No	9[b]	Yes
19	M/74	RAEB	46,XY,del(20)(q11)[17]/45,idem,−14,del(20)(q11)[3]/46,XY[1]		High	95	No	24[b]	Yes
20	M/56	RAEB	40~44,XY,−5,−7,−17,−21,del(20)(q11),2~4mar[21]/46,XY[3]	Mult[a]	High	88	No	10[b]	Yes
21	M/83	CMML	46,XY,del(20)(q11)[4]/46,XY,idem,t(3;2;21)(q21;p21;q22)[14]		Int-1	100	No	16[b]	No
22	F/75	CMML	46,XX,del(16q)[3]/46,XX[4]		Low		No	35[b]	No
			46,XX,del(20)(q11)[3]/46,XX[17]	Late[d]		15			

Abbreviations: CMML, chronic myelomonocytic leukemia; Int, intermediate; IPSS, International Prognostic Scoring System; MDS, myelodysplastic syndrome; RA, refractory anemia; RAEB, refractory anemia with excess blasts.

[a] Multiple: > 1 abnormality in addition to del(20q).

[b] Patient died.

[c] Reported previously: Patients no. 4 and 16 (Ohyashiki et al., 1992 [29]) and patients no. 7, 9, 10, and 15 (Hsiao et al., 2006 [18]).

[d] Late appearance of del(20q), detected in follow-up cytogenetic studies prompted by change in disease condition.

2. Materials and methods

2.1. Patients

Out of 515 MDS patients seen in our hospital between 1993 and 2005, 33 were found to possess the del(20q) anomaly and served as our study sample. Most of the study patients were male (24/33, or 72.7%), and the median age was 70 years (range: 46–92). The MDS diagnoses were as follows: 24 patients with refractory anemia (RA), 7 with RAEB (RA with excess blasts), and 2 with chronic myelomonocytic leukemia (CMML). Among them, 28 had del(20q) at the time of diagnosis: 28/33 of the study sample (84.8%) and 28/515 (5.4%) of all MDS patients.

Before 2001, the FAB classification for myeloid disorders was used; after 2001, the WHO classification [16]. We recorded the gender, age, disease status at the time of diagnosis, IPSS score, initial complete blood cell count, previous genotoxic exposure, occurrence of other malignancies, leukemia-free survival (LFS), and overall survival (OS) of all patients. The genotoxic exposure included prior history of radiation or cytotoxic agents. We analyzed clinical features of MDS patients using this database after obtaining informed consent.

2.2. Cytogenetic study

The cytogenetic examinations were performed with a quinacrine–banding technique using short-term cultured bone marrow cells without addition of any mitogens. For clonality, we followed the ISCN 1995 [17] definition, an aberration appearing in at least two metaphases (in three cells, for a missing chromosome). Although interphase fluorescence in situ hybridization (FISH) might detect a correct percentage of del(20q) clone, we used percentage of metaphases with del(20q), because cytogenetic study using marrow metaphases is commonly performed in hematologic malignancies. The major del(20q) clone was tentatively defined when ≥50% marrow metaphases had del(20q), and we designated it as a minor del(20q) clone if it was seen in <50% metaphases. If the disease condition changed, we performed subsequent cytogenetic studies as possible.

2.3. Statistical analysis

Cytogenetic data were compared by the chi-square test and Fisher's exact test. Continuous data were compared with two-sample *t*-test and one-way ANOVA for approximately normally distributed data, and the nonparametric Wilcoxon rank-sum test for other distributions. Kaplan–Meier life tables were constructed for survival data, and data were compared by the log-rank test. To identify poor prognostic factors for patients with del(20q), we performed multivariate Cox proportional hazards regression analysis. All statistics was performed using SPSS 11.5 software (SPSS, Chicago, IL). A *P*-value of <0.05 was considered statistically significant.

3. Results

3.1. del(20q) in myelodysplastic syndromes

Cytogenetic results other than sole del(20q) are summarized in Table 1.

We first divided MDS patients into two groups, major or minor del(20q) clone. The del(20q) in MDS tended to appear as a major clone (24/33, or 73%), and also in MPD (7/13, or 54%), which was significantly different from AML (2/9, or 22%) in our experience ($P = 0.016$) (data not shown). The major del(20q) clone in MDS was noted

Table 2
Patients with del(20q) in MDS

	del(20q) at diagnosis, no.		del(20q) at late stage, no.	
	All MDS	MDS with RA	All MDS	MDS with RA
Total patients	28	20	5	4
Cytogenetics				
del(20q) as sole anomaly	11	9	1	0
del(20q) with other anomalies				
Complex or with −7/7q−	10	6	4	4
Others	7	5	0	0
IPSS				
Low	4	4	1	0
Int-1	13	11	3	3
Int-2	8	5	1	1
High	3	0	0	0
Percentage of del(20q) clone				
Major (≥50%)	24	17	0	0
Minor (<50%)	4	3	5	4
Prior genotoxic exposure				
Yes	5	5	1	1
No	23	15	4	3

Abbreviations: Complex, involvement of ≥3 chromosomes; Int, intermediate; IPSS, International Prognostic Scoring System; MDS, myelodysplastic syndrome; RA, refractory anemia.

only at the diagnosis of MDS, and sometimes appeared as a sole anomaly (9/24 patients). By contrast, the minor del(20q) clone often appeared during the later courses (5/9 patients), and most of them exhibited as a complex anomaly (≥3 chromosomes involved) or with −7/7q− changes (4/5 patients) (Table 2) (P = 0.001). In all MDS, patients with the major del(20q) clone or with sole del(20q) anomaly had significant favorable OS (log-rank P = 0.009 and P = 0.005, respectively) and tended to have better LFS (log-rank P = 0.176 and P = 0.017, respectively) (Figs. 1A and 1B). Of the patients with other cytogenetic changes in addition to del(20q), a single additional chromosomal anomaly did not have a benefit in terms of OS or LFS (Fig. 1A).

3.2. Myelodysplastic syndromes with del(20q) at diagnosis

To exclude the influence of the late occurrence of del(20q), we analyzed MDS patients with del(20q) at MDS diagnosis, including 20 RA patients. In all MDS patients, there was no significant benefit in OS and LFS (log-rank P = 0.154 and P = 0.128, respectively) regardless of whether the del(20q) appeared at the time of diagnosis or late (Fig. 1C). There were 11 patients with sole del(20q) and 17 with additional abnormalities, including 10 with complex changes or −7/7q−. MDS patients with sole del(20q) had better OS (log-rank P = 0.018), but again a single additional chromosome change did not have any benefit for outcome. Of note is that 2 of 5 MDS patients with del(20q) along with der(1;7)(q10;p10) had a prior history of genotoxic exposure [18]. Moreover, patients with the major del(20q) clone at the diagnosis of MDS had significantly longer OS (log-rank P = 0.021), but not LFS (log-rank P = 0.639). These results indicate that negative factors in predicting prognosis in patients showing del(20q) are (i) more progressive disease status, (ii) any additional cytogenetic changes, and (iii) <50% of del(20q) in the

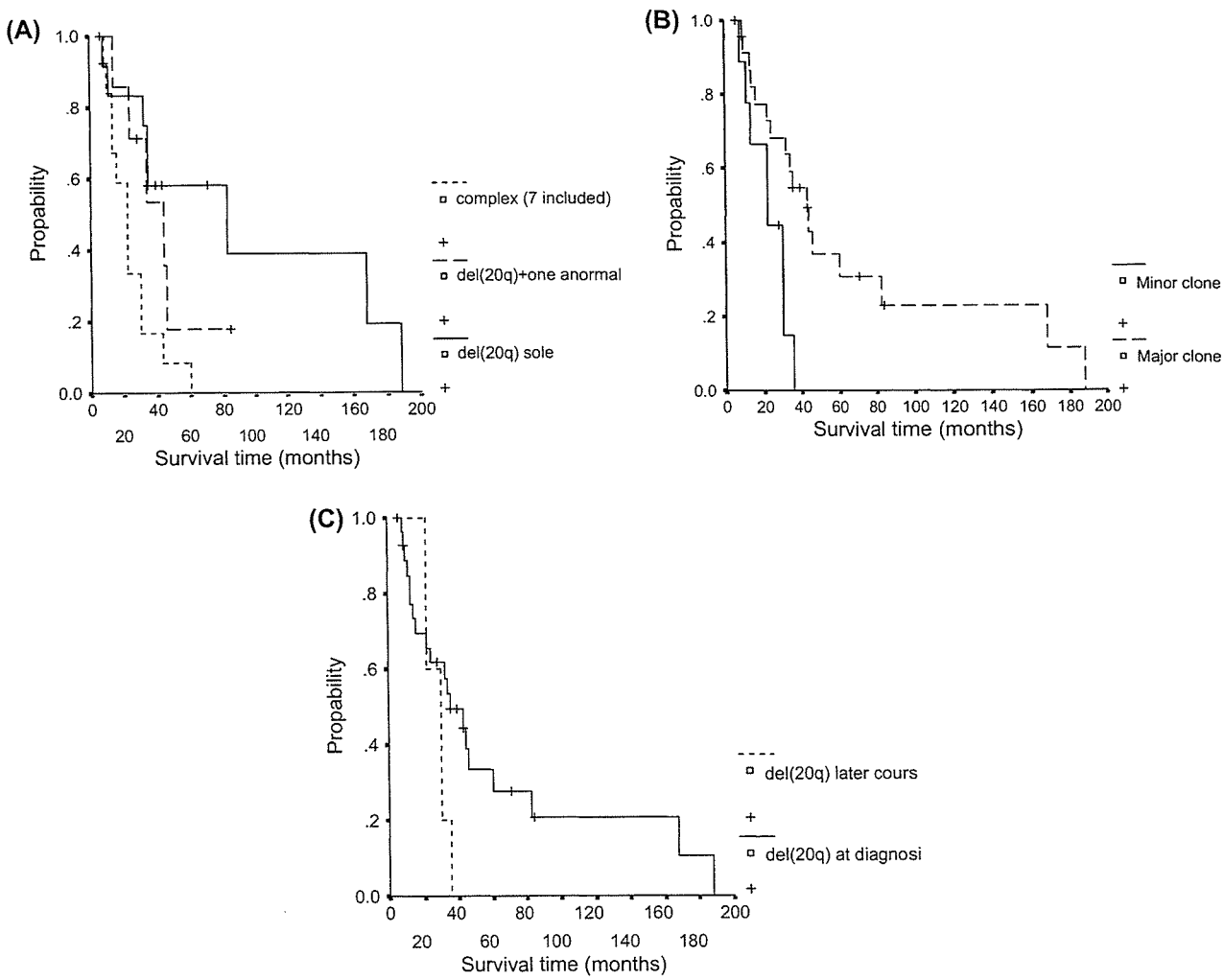

Fig. 1. The overall survival in all MDS patients with del(20q). (A) Categorized by sole del(20q), del(20q) with one anomaly, or complex abnormality (log-rank P = 0.005 in overall survival, P = 0.017 in leukemia-free survival [not present]). Only patients with sole del(20q) have better survival. (B) Categorized by the major and minor del(20q) clone (log-rank P = 0.009 in overall survival, P = 0.176 in leukemia-free survival [not present]). MDS patients with major del(20q) have good prognosis. (C) Categorized by early (at the time of diagnosis) and late appearance of del(20q) (log-rank P = 0.154 in overall survival and P = 0.128 in leukemia-free survival [not present]).

marrow metaphases. The presence of a prior history of genotoxic agents might be a lesser factor, however, such patients fitted the above negative factors.

The results of multivariate analysis with Cox proportional hazards regression model in MDS with del(20q) are shown in Table 3. Although the number of patients in this study is too small to yield a statistically significant difference, the presence of complex cytogenetic change or −7/7q− anomaly (odds ratio: OR = 4.998), minor del(20q) clone (OR = 2.914), higher IPSS score (OR = 2.748), and female gender (OR = 2.624) might be risk factors for unfavorable prognosis.

3.3. del(20q) in refractory anemia

Sole del(20q) in RA patients again showed long OS (log-rank $P = 0.021$) and trend on LFS (log-rank $P = 0.075$) (Fig. 2A). RA patients with the major del(20q) had statistically significantly better OS (log-rank $P = 0.001$) and LFS (long- rank $P = 0.001$) than those with minor del(20q) clone (Fig. 2B). There was also significant better OS (log-rank $P = 0.018$) and LFS (log-rank $P = 0.001$) in RA patients with del(20q) at diagnosis (Fig. 2C). Based on these analyses, the negative factors in predicting survival in RA patients are (i) any additional cytogenetic changes, (ii) minor del(20q) clone, or (iii) late appearance of del(20q). This implies that in RA patients, the del(20q) with a sole major clone and early appearance had a likelihood of good survival and had no correlation with leukemic transformation.

Table 3
Multivariate analysis with Cox proportional hazards regression model in MDS with del(20q)

	n	Odds ratio	*P*
Age, years	33	1.021	0.520
Sex			
Male	24		
Female	9	2.624	0.304
Diagnosis			
RA	24		
RAEB or CMML	7/2	2.106	0.293
IPSS			
Low	5		
Int-1	16	1.263	0.813
Int-2/High	12	2.748	0.436
Percentage of del(20q) metaphases			
del(20q) clone, major	24		
del(20q) clone, minor	9	2.914	0.065
Cytogenetics			
del(20q) as sole anomaly	12		
del(20q) and one other anomaly	7	1.744	0.574
del(20q) with complex anomalies or with −7/7q−	14	4.998	0.093
Prior history of genotoxic exposure			
Yes	6	0.502	0.446
No	27		
Nonhematologic malignancy			
Yes	8	1.130	0.875
No	25		

Abbreviations: CMML, chronic myelomonocytic leukemia; Int, intermediate; IPSS, International Prognostic Scoring System; MDS, myelodysplastic syndrome; RA, refractory anemia; RAEB, refractory anemia with excess blasts.

3.4. Late appearance of del(20q)

Given that del(20q) as a sole anomaly is a good prognostic factor in MDS patients, this raises the question about progression in MDS patients who had normal karyotypes at MDS diagnosis but in whom del(20q) appeared during the follow-up period. The present study included 5 MDS patients (4 RA and 1 CMML) with late appearing del(20q) (Table 2): all of them expired within 3 years after the MDS diagnosis, and 3 RA patients developed leukemia. All except one of 5 MDS patients with late appearance of del(20q) had a complex abnormality or −7/7q−, and all 5 had a minor del(20q) clone. In contrast, sole del(20q) was only detectable at MDS-RA diagnosis and none of those patients progressed to the acute leukemia phase, but they expired due to bone marrow failure (Fig. 2A).

4. Discussion

The del(20q) anomaly often appears as a major clone in MDS patients at diagnosis, and the sole del(20q) as a major clone is associated with good prognosis in MDS, in keeping with previous observations [5–7,10,15]. However, the del(20q) anomaly sometimes develops as a minor clone in the late stages of MDS, MPD, and AML, and is associated with poor prognosis [11,12]. Considering the pathogenesis of various myeloid diseases, a clonal hematopoietic stem cell disorder characterized by a step-wise genetic progression model has been proposed [5,6,19,20]. In MDS with the major del(20q) clone, it may involve the early stage of pathogenesis following unknown initial events on progenitor cells. The del(20q) anomaly can also appear as an additional and related cytogenetic change accompanied with the original abnormalities and is associated with karyotype evolution. It also implies that, if the del(20q) develops as an additional change, even only as a minor clone in all myeloid diseases, this may indicate disease progression and poor prognosis. The allelic loss of 20q was found in 23% of MDS patients who progressed to AML [21]. This suggests that cytogenetically normal MDS cells may have accumulated genetic damage and additional changes [19,20,22,23], including minor del(20q) clone, during disease progression, resulting in short survival with leukemia progression. Another plausible explanation might be that late appearance of del(20q) may result from the cytogenetic expression of genetic instability related to disease progression [24,25]. In the present study, the late appearance of del(20q) with complex abnormality or with −7/7q−, even in RA patients, may be closely linked to leukemic

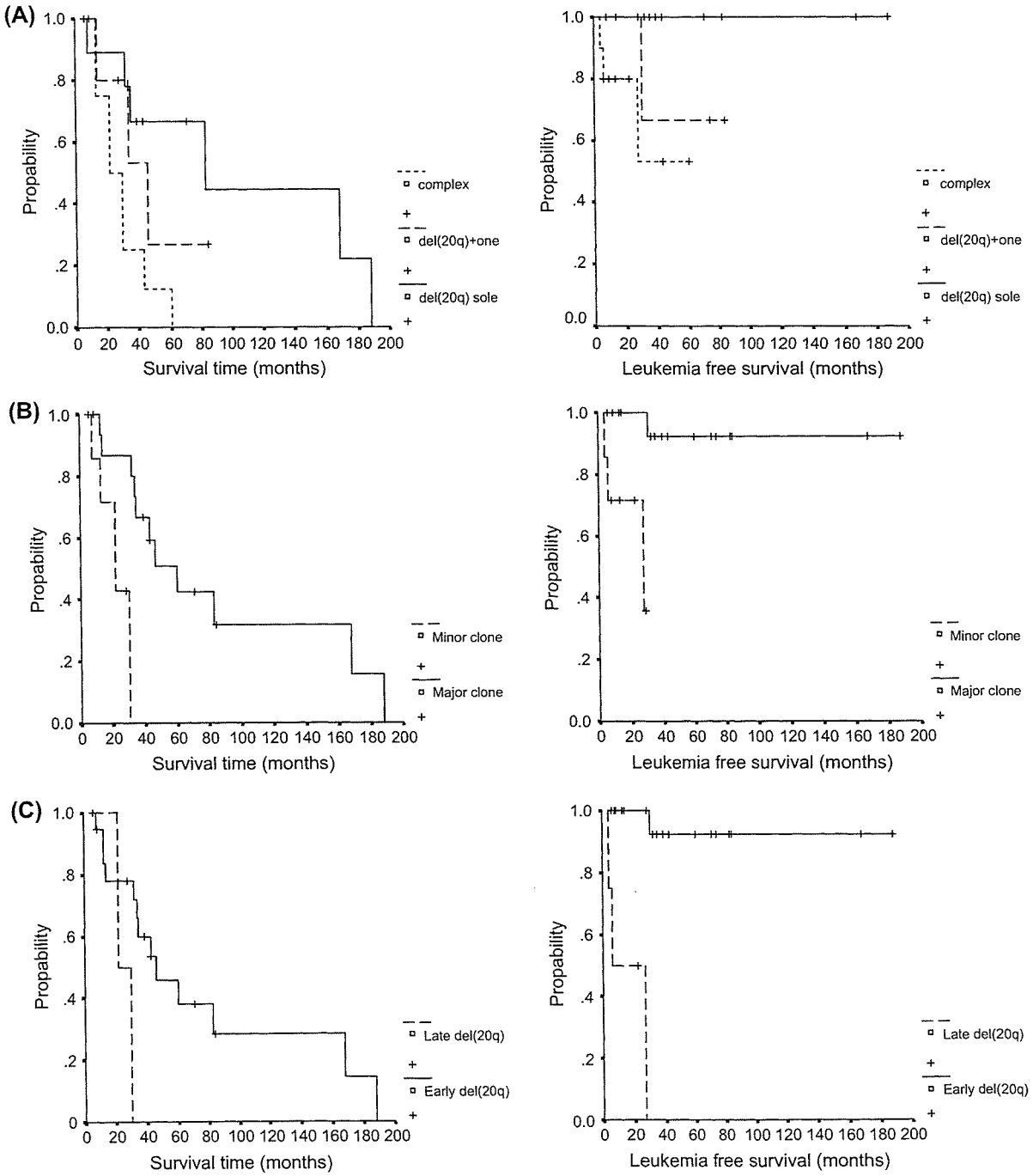

Fig. 2. The overall survival and leukemia-free survival in MDS-RA patients with del(20q). (A) Categorized by sole del(20q), del(20q) with one anomaly, or complex del(20q) abnormality (log-rank $P = 0.021$ in overall survival and $P = 0.075$ in leukemia-free survival). (B) Categorized according to the major and minor del(20q) clone (log-rank $P < 0.001$ in overall survival and $P = 0.001$ in leukemia-free survival). (C) Categorized according to the early and late appearance of del(20q) (log-rank $P = 0.018$ in overall survival and $P < 0.001$ in leukemia-free survival). MDS patients with sole del(20q), major clone (>50% metaphase occupancy), and early appearance of del(20q) have better survival.

transformation; sole del(20q) in RA at any time may represent marrow failure, rather than leukemic development.

The identification of the genes involved in the 20q deletion remains unclear. Interstitial deletions of variable size in the 20q deletion have been described. Several research groups have identified an interstitial commonly deleted region, including a 2.9-Mb span in MPD and a 3-Mb span in AML and MDS, with an overlapping myeloid commonly deleted region spanning ~2 Mb. [13,14,26]. These comprise a gene-rich region, and the candidate genes within this region may have a variety of functions, including transcription factors, components of

signal transduction pathways, RNA transcription modulator, and regulator of apoptosis [14].

The influence of dysregulation and dysfunction in these genes may result in different presentations of del(20q) in primary and secondary MDS and AML. Recently, the human *L3MBTL* gene, which lies in this region, was implicated as a candidate tumor suppressor gene. No mutations were detected, however, and the methylated allele was not associated with a reduction of *L3MBTL* mRNA level [26,27]. It is still unresolved whether the del(20q) at different stages of disease may involve the same target gene or genes or leukemogenesis. Given that the clinical significance of del(20q) at diagnosis (major clone with sole del(20q) anomaly) is quite different from that appearing at later stages (minor clone with other cytogenetic changes), some of the biological and clinical implications of the del(20q) in these situations may be different.

In del(20q) MDS, patients with sole del(20q) and the major clone have significantly better overall survival. Regarding the major del(20q) clone with better survival in all MDS, as well as in MDS-RA patients, the interpretation should be made cautiously, because of the limited case number of minor del(20q) clone in our MDS patients. Further study will be necessary to prove this survival difference. Nevertheless, when the del(20q) appears de novo as an additional cytogenetic clone, even as a minor clone, in all myeloid malignancies, hematologic conditions should be followed carefully, because a high percentage of these patients develop leukemia with an unfavorable prognosis, even in RA.

A relatively high percentage of nonhematologic malignancies (6/33, or 18%) was found in this study, and five of these were found before MDS diagnosis. The incidence of sole del(20q) anomaly in therapy-related MDS and AML is less common [28], however, therapy-related MDS with del(20q) as one among several cytogenetic changes may exist: two patients with der(1;7)(q10;p10) and two with other cytogenetic abnormalities were found to have prior exposure of genotoxic agents in this study. The additional cytogenetic changes and prior genotoxic exposure was found to be linked to unfavorable prognostic predictors in MDS and AML patients with der(1;7)(q10;p10) [18]. Possibilities such as the cumulative probability of mutational events or genetic instability or age-related predisposition to secondary malignancy need to be explored regarding the genesis of del(20q).

In conclusion, the clinical significance of del(20q) differs, depending on conditions. The presence of additional cytogenetic changes, minor del(20q) clone, and higher IPSS score might be risk factors for unfavorable prognosis. A possible cause may be the involvement at different steps in the pathogenesis of myeloid disease and progression. In MDS patients with del(20q) at diagnosis, the sole del(20q) and the major del(20q) clone are related to significantly better survival. When the del(20q) appears as an additional change (especially as a minor clone) at anytime, it indicates clonal evolution toward leukemia, and poor prognosis. The results in this study should be confirmed using a large number of MDS and AML patients.

Acknowledgments

The authors are indebted to Prof. J Patrick Barron of the International Medical Communications Center of Tokyo Medical University for his review of this manuscript. This work was supported by "High-Tech Research Center" project for private universities matching fund subsidy from the MEXT (Ministry of Education, Culture, Sports, Science, and Technology,) (to KO) and by the "University-Industry Joint Research Project" for private universities matching fund subsidy from the MEXT (to KO). This work was also supported by Intractable hematopoietic diseases from the Ministry of Health and Welfare (to KO).

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