

**Fig. 2.** PML-RAR $\alpha$  regulates endogenous RAR $\beta$  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  $\mu$ M) to the culture medium. After 2 days of incubation, total RNA was extracted and RT-PCR using primers for RAR $\beta$ , CYP26, and  $\beta$ -actin (control) was performed. In the presence of PML-RAR $\alpha$ , the expression levels of endogenous RAR $\beta$  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-RAR $\alpha$  (Fig. 3). F-PML-RAR was transiently expressed in 293T cells and incubated for 2 days with or without 1  $\mu$ M of ATRA. After crosslinking by formaldehyde, chromatin solution was obtained. ChIP assay against endogenous RAR $\beta$  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-RAR $\alpha$  contains the DNA binding domain of RAR $\alpha$  and is also able to bind to RARE region as a heterodimer with RXR or a homodimer [26]. Endogenous RXR $\alpha$  expression in 293T cells was confirmed by RT-PCR (data not shown).

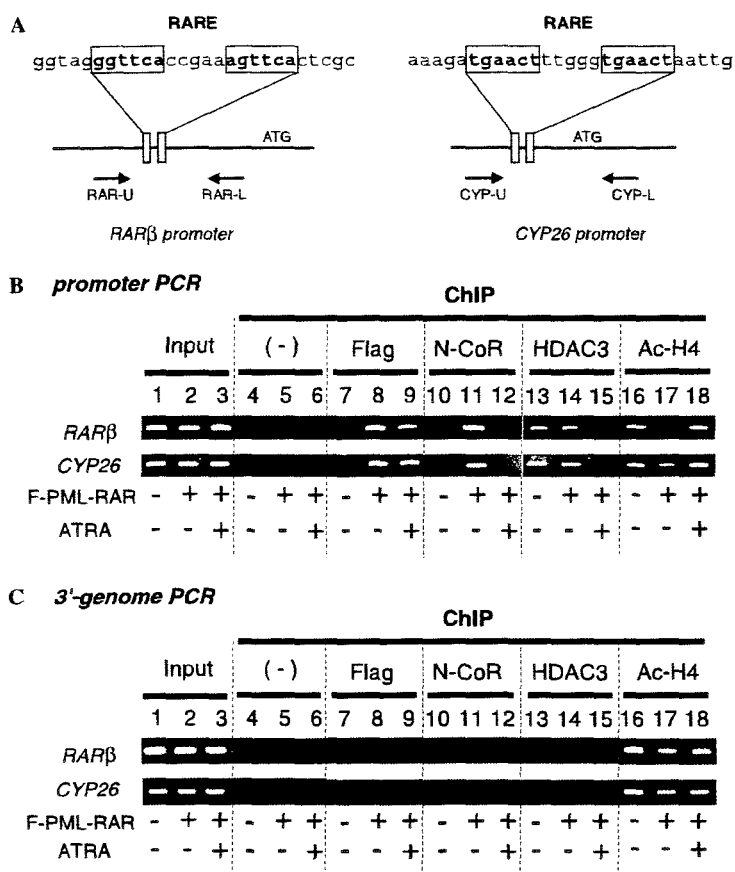
PML-RAR $\alpha$  bound to endogenous RAR $\beta$  and CYP26 promoter constitutively with and without ATRA (Fig. 3B, lanes 8 and 9). Endogenous N-CoR and HDAC3 were recruited to the promoters in the absence of ATRA (lanes 11 and 14). In contrast, N-CoR-HDAC3 recruitment to the target gene promoters was dissociated by adding ATRA (lanes 12 and 15). Interestingly, HDAC3 was found to be associated with both promoters even in the absence of PML-RAR $\alpha$  (lane 13). In the presence of PML-RAR $\alpha$ , 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 RAR $\beta$  and CYP26 genes, ChIP assay using anti-acetylated histone H4 antibody (Upstate) was performed. If PML-RAR $\alpha$  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-RAR $\alpha$  binds to RAR $\beta$  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 RAR $\beta$  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-RAR $\alpha$ , 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-RAR $\alpha$ *in vivo*

To determine whether HDAC3 is critical for the transcription repression by PML-RAR $\alpha$  *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 target 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

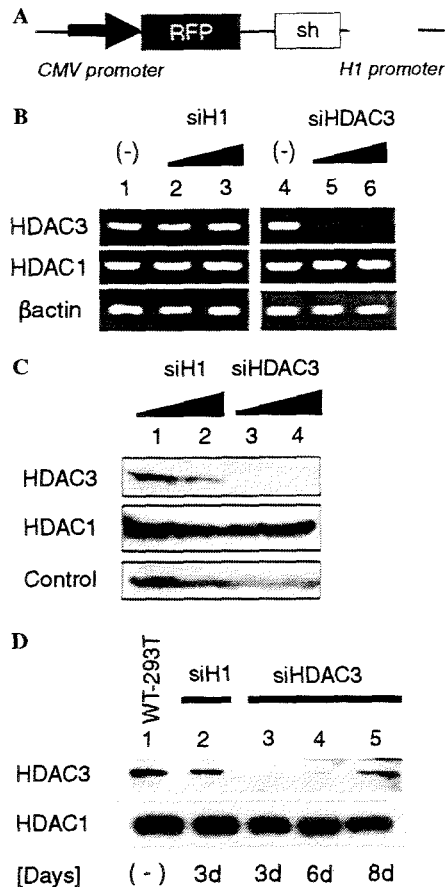


**Fig. 3.** Endogenous N-CoR and HDAC3 proteins are recruited to PML-RAR $\alpha$  target gene promoters *in vivo*. (A) Schematic representation of RAR $\beta$  and CYP26 promoters. Retinoic acid receptor response element (RARE) is located at the promoter site as indicated. Detailed nucleic acid sequences 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  $\mu$ M) 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 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

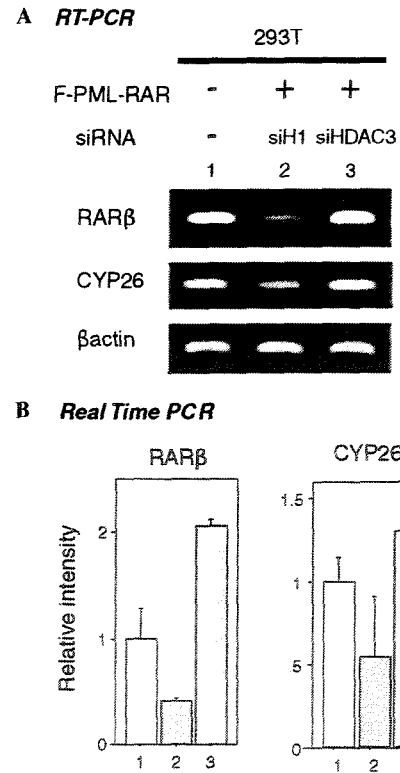
after transfection. pS65R-shH1 or -shHDAC3 were transfected into 293T cells, and whole cell lysates were obtained after incubation for different days as indicated in Fig. 4D. HDAC3 protein expression was significantly repressed until 6 days after transfection (lanes 3 and 4), at which time faint bands 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  $\beta$ -actin were also amplified as controls. Different amounts of plasmid DNA were transfected (0.6  $\mu$ g (lanes 2 and 5) and 1.2  $\mu$ g (lanes 3 and 6) for  $5 \times 10^5$  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  $\beta$ -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.

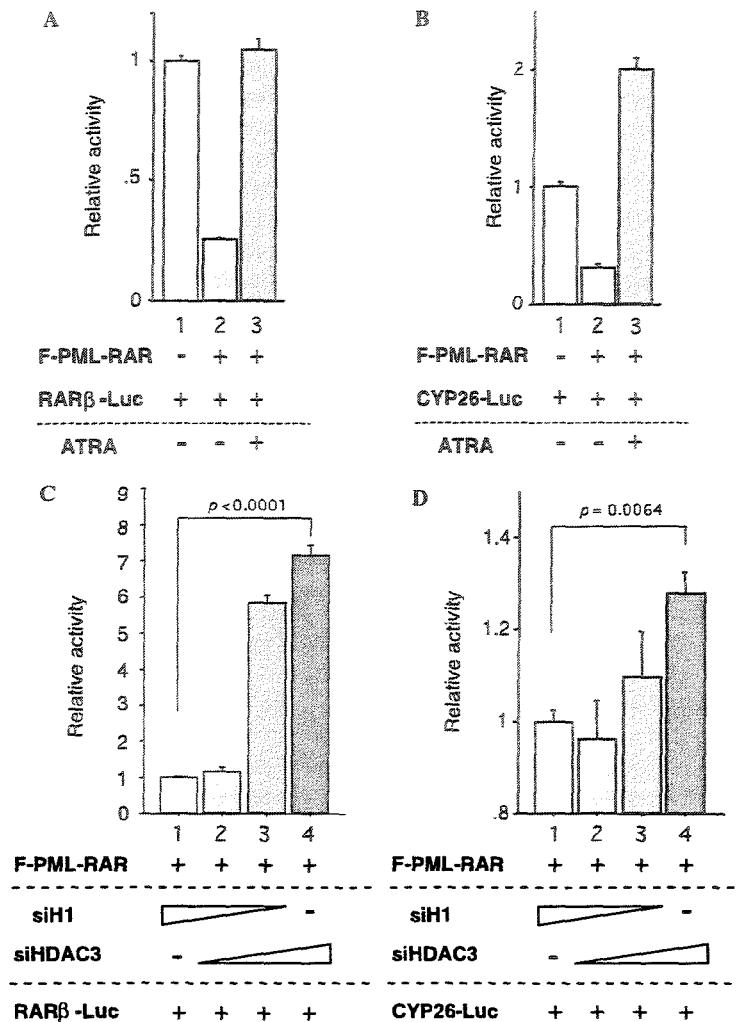
another 2 days. Total RNA was extracted and conventional RT-PCR for RAR $\beta$ , CYP26, and  $\beta$ -actin was performed (Fig. 5A). As shown in Fig. 2 (lane 2 vs. lane 1), F-PML-RAR repressed the expression of endogenous RAR $\beta$  and CYP26 genes. When siH1 (negative control) was expressed in PML-RAR $\alpha$  expressing 293T cells, target



**Fig. 5.** Endogenous HDAC3 protein knockdown induces de-repression of PML-RAR $\alpha$  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 RAR $\beta$ , CYP26, and  $\beta$ -actin primers. (B) Real-time PCR was performed to measure the expression levels of RAR $\beta$  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.

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-RAR $\alpha$ -induced transcription repression of the target genes RAR $\beta$  and CYP26.

Furthermore, we carried out a luciferase reporter assay on 293T cells in the presence or absence of siHDAC3. Human RAR $\beta$  and CYP26 promoters were cloned into luciferase reporter vector (RAR $\beta$ -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 RAR $\beta$ -Luc was significantly repressed without ATRA (Fig. 6A, lane 2 vs. lane 1). By adding 1  $\mu$ M ATRA, the repressed



**Fig. 6.** Transcription repression by PML-RAR $\alpha$  is released by HDAC3 knockdown in luciferase transcription assay. RAR $\beta$  and CYP26 promoters were cloned into luciferase reporter vectors as indicated in Materials and methods. RAR $\beta$ -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-RAR $\alpha$  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-RAR $\alpha$ , 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  $\mu$ g. Concentration of siHDAC3 expression vector is 0  $\mu$ g (lane 1), 0.1  $\mu$ g (lane 2), 0.5  $\mu$ g (lanes 3), and 1  $\mu$ g (lane 4), respectively. Twenty-four hours after transfection of shRNA expression vectors, PML-RAR $\alpha$  expression vector and RAR $\beta$ -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-RAR $\alpha$  induced transcription repression was significantly de-repressed in the presence of siHDAC3 (lane 4 vs. lane 1,  $p = 0.0064$ ). Error bar indicates  $\pm 1$  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-RAR $\alpha$  in 293T cells.

Next, siHDAC3 was introduced in the transcription assay to confirm whether endogenous HDAC3 is involved in PML-RAR $\alpha$ -induced transcription repression (Fig. 6C and D). Twenty-four hours after transfection of increasing amounts of pS65-shHDAC3 into 293T cells, the F-PML-

RAR $\alpha$  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 RAR $\beta$  by PML-RAR $\alpha$  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-RAR $\alpha$ -dependent transcription repression of target genes, supporting the data of the transcription assay using endogenous gene promoters in Fig. 5.

## Discussion

PML-RAR $\alpha$  has long been implicated to play a critical role in APL. Many *in vitro* studies on PML-RAR $\alpha$  and co-repressor complexes suggest that PML-RAR $\alpha$  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-RAR $\alpha$  *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-RAR $\alpha$  *in vivo*.

First, using IP assay followed by immunoblotting, we showed that unliganded PML-RAR $\alpha$  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-RAR $\alpha$  (Flag), N-CoR, HDAC3, and acetylated-histone H4 antibodies indicated that N-CoR-HDAC3 is recruited to the target gene promoters (RAR $\beta$  and CYP26) through PML-RAR $\alpha$  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-RAR $\alpha$  in the absence of ligand and the interaction is dissociated by adding ATRA [17]. In the last 5 years, N-CoR/SMRT-TBLR1/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-RAR $\alpha$ -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-RAR $\alpha$  [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-RAR $\alpha$  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-RAR $\alpha$ . Our study and previous data [17] show that inhibition of the function of endogenous HDAC3 and TBLR1 induces transcription repression of PML-RAR $\alpha$  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 TBLR1/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-RAR $\alpha$ . 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-RAR $\alpha$ -dependent transcription repression in APL cells. While preparing this manuscript, MBD1 (methyl-CpG binding domain protein 1)-HDAC3 complex is reported as a PML-RAR $\alpha$  interacting co-repressor complex [30]. The report may support our findings that Complex A is critical for PML-RAR $\alpha$  target gene repression.

One surprising finding from our ChIP assay is that endogenous HDAC3, but not N-CoR, is associated with RAR $\beta$  and CYP26 promoters in the absence of PML-RAR $\alpha$  (Fig. 3B, lane 13 vs. lane 10). In the presence of PML-RAR $\alpha$  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-RAR $\alpha$ -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-RAR $\alpha$ . It is unclear how HDAC3 alone associates with the promoters in the absence of PML-RAR $\alpha$ . One explanation for HDAC3 interaction with target gene promoters without PML-RAR $\alpha$  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-RAR $\alpha$ , 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-RAR $\alpha$  interacting protein complexes, including TRAP/DRIP [32], SWI/SNF, and histone acetyltransferase (HAT) complexes [20], such that even the PML-RAR $\alpha$ -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-RAR $\alpha$ . This is further supported directly *in vivo* by our HDAC3 knockdown experiments.

Almost 90% of APL patients have the aberrant chimeric transcription factor PML-RAR $\alpha$ . Besides this chimeric protein, several RAR $\alpha$  chimeric proteins from APL patients including PLZF-RAR $\alpha$ , NPM-RAR $\alpha$ , and NuMA-RAR $\alpha$  have been reported. Recently, using the same IP strategy, we also confirmed that PLZF-RAR $\alpha$  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-RAR $\alpha$  may thus provide new avenues for improving the cure rate of patients, especially those with ATRA resistant, mutated-PML-RAR $\alpha$ .

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

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### 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 ( $\geq 50\%$  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. © 2006 Elsevier Inc. All rights reserved.

### 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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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 <sup>a</sup>	Int-2	76	No	6	No
4 <sup>c</sup>	M/70	RA	47,XY,del(20)(q11),+del(20)(q11)[20]		Int-1	100	No	34 <sup>b</sup>	Yes
5	M/74	RA	47,XY,+8,del(20)(q11)[20]		Int-1	100	No	46 <sup>b</sup>	No
6	F/66	RA	47,XX,+9,del(20)(q11)[18]		Int-1	100	No	14 <sup>b</sup>	No
7 <sup>c</sup>	M/69	RA	45,XY,+1,der(17)(q10;p10),-20[2]/46,XY,idem,+20,del(20)(q11)[12]/46,XY[7]	Mult <sup>a</sup>	Int-2	55	No	43 <sup>b</sup>	No
8	M/67	RA	46,XY,+1,der(17)(q10;p10)[7]/46,XY,idem,del(20)(q11)[13]		Int-2	65	Yes	60 <sup>b</sup>	No
9 <sup>c</sup>	M/72	RA	46,XY,+1,der(17)(q10;p10)[9]/46,XY,idem,del(20)(q11)[6]/46,XY[5]		Int-2	30	Yes	13 <sup>b</sup>	No
10 <sup>c</sup>	M/65	RA	46,XY,+1,der(17)(q10;p10)[4]/46,XY,idem,del(20)(q11)[17]		Int-2	81	No	13 <sup>b</sup>	No
11	M/57	RA	46,XY,+1,der(17)(q10;p10),del(20)(q11)[20]/46,XY,idem,add(18)(p11)[2]	Mult <sup>a</sup>	Int-1	100	No	9	No
12	M/46	RA	46,XY[19]		Int-1		No	30 <sup>b</sup>	Yes
13	M/68	RA	46,XY,del(7)(q31)[3]/46,XY,idem,del(20)(q11)[9]/46,XY[7]	Late <sup>d</sup>	Int-1	47	Yes	30 <sup>b</sup>	Yes
14	M/59	RA	44,XY,-4,-11,del(20)(q11)[2]/46,XY[19]	Late <sup>d</sup>	Int-1	10	No	22 <sup>b</sup>	No
15 <sup>c</sup>	M/68	RA	46,XY,del(7)(q22)[17]/46,XY,idem,del(20)(q11)[3]	Late <sup>d</sup>	Int-2	15	No		No
16 <sup>c</sup>	M/76	RAEB	46,XY,+1,der(17)(q10;p10),del(20)(q11)[2]/46,XX[8]	Late <sup>d</sup>	Int-1	20	No	22 <sup>b</sup>	Yes
17	M/86	RAEB	47,XY,-20,+del(20)(q11)XZ[10]		Int-2	100	No	44 <sup>b</sup>	Yes
18	M/83	RAEB	46,XY,del(7)(q22),del(20)(q11)[20]		Int-2	100	No	22 <sup>b</sup>	Yes
19	M/74	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 <sup>a</sup>	High	90	No	9 <sup>b</sup>	Yes
20	M/56	RAEB	46,XY,del(20)(q11)[17]/45,idem,-14,del(20)(q11)[3]/46,XY[1]		High	95	No	24 <sup>b</sup>	Yes
21	M/83	CMML	40-44,XY,-5,-7,-17,-21,del(20)(q11),2-4near[21]/46,XY[3]	Mult <sup>a</sup>	High	88	No	10 <sup>b</sup>	Yes
22	F/75	CMML	46,XY,del(20)(q11)[4]/46,XY,idem,t(3;2)(q21;q21),q21;q22[14]		Int-1	100	No	16 <sup>b</sup>	No
			46,XX,del(16q)[3]/46,XX[4]		Low		No	35 <sup>b</sup>	No
			46,XX,del(20)(q11)[3]/46,XX[17]	Late <sup>d</sup>		15	No		No

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

<sup>a</sup> Multiple: > 1 abnormality in addition to del(20q).

<sup>b</sup> Patient died.

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

<sup>d</sup> 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  $\geq 50\%$  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 ( $\geq 50\%$ )	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  $\geq 3$  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 ( $\geq 3$  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

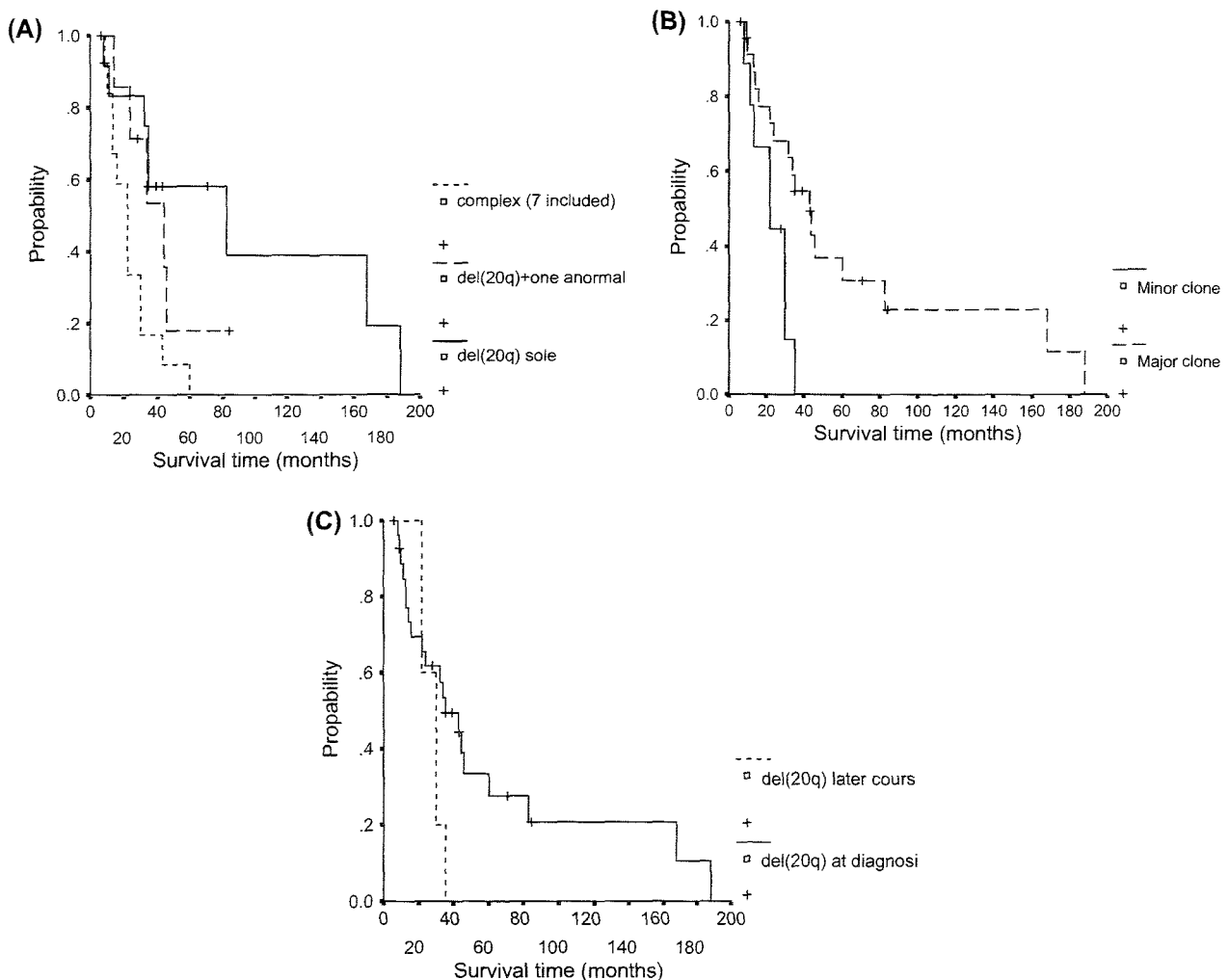


Fig. 1. The overall survival in all MDS patients with del(20q). (A) Categorized by sole del(20q), del(20q) with one anomaly, or complex abnormality (log-rank  $P = 0.005$  in overall survival,  $P = 0.017$  in leukemia-free survival [not present]). Only patients with sole del(20q) have better survival. (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 (log-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.

### 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

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

	<i>n</i>	Odds ratio	<i>P</i>
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.

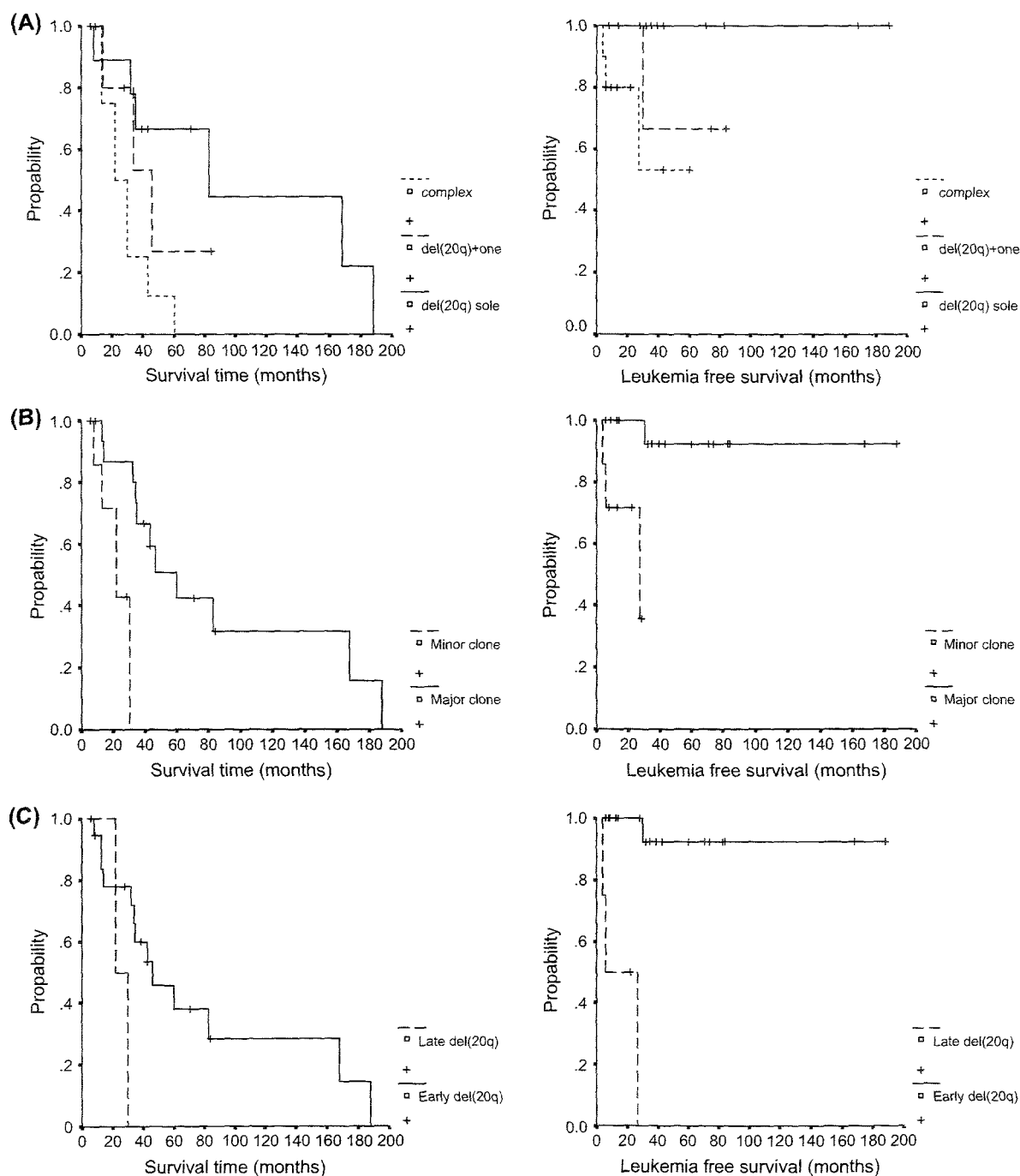


Fig. 2. The overall survival and leukemia-free survival in MDS-RA patients with del(20q). (A) Categorized by sole del(20q), del(20q) with one anomaly, or complex del(20q) abnormality (log-rank  $P = 0.021$  in overall survival and  $P = 0.075$  in leukemia-free survival). (B) Categorized according to 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.

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## Derivative (1;7)(q10;p10) in multiple myeloma. A sign of therapy-related hidden myelodysplastic syndrome

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### Abstract

Therapy-related myelodysplastic syndrome (MDS) is a major problem in long-term cancer survivors, therefore early detection and prevention of therapy-related secondary neoplasia is an important issue. We searched for therapy-related MDS and analyzed cytogenetic changes in 155 patients with multiple myeloma (MM) from a single institution. Of the total 155 MM patients with cytogenetic results, 7 patients showed de novo appearance of myeloid-related cytogenetic changes, and 5/7 had  $-7/7q-$ , including 3 with  $der(1;7)(q10;p10)$ : 3 patients developed MDS (i.e. 2 patients with  $der(1;7)(q10;p10)$  and 1 with a complex abnormality including  $-5$  and  $7q-$ ). Among five patients receiving more than 2 g of melphalan, three developed MDS, and two of them showed  $der(1;7)(q10;p10)$  before or at the time of MDS diagnosis. Although morphologic identification of MDS was difficult in some cases, we concluded that the presence of  $7q-$ , specifically  $der(1;7)(q10;p10)$ , during chemotherapy involving melphalan for MM patients might indicate hidden MDS status and appropriate therapeutic options should be considered for such patients. © 2006 Elsevier Inc. All rights reserved.

### 1. Introduction

Multiple myeloma (MM) patients have heterogeneous responses to therapy and varying survival. A new scoring system to identify survival probability, however, has been currently proposed [1]. For MM patients, therapeutic strategies, including stem cell transplantation or immuno-modulator agents, are available based on the risk of each patient [2–4]. However, alkylating agents (e.g., melphalan) are still a mainstay in the treatment of MM, especially for elderly patients [4]. Moreover, many MM patients have been treated with alkylating agents, and they are at risk for developing secondary hematologic malignancies [i.e., therapy-related myelodysplastic syndromes (MDS) and/or acute myeloid leukemia (AML)] [5]. The risk of secondary MDS/AML in melphalan-treated MM patients is well known, and cytogenetic changes related to secondary MDS/AML in MM patients have been reported [6–10]. Amiel et al. [11] reported that  $5q-$ , rather than  $7q-$ , was frequently detectable before

the overt development of MDS/AML, by using fluorescence in situ hybridization (FISH) analysis. In contrast, Nilsson et al. reported a high incidence of myeloid-related abnormalities [i.e.,  $+8$  or  $del(20)(q11)$  in patients with treated MM or monoclonal gammopathy of undetermined significance] [8].

Since patients with therapy-related MDS/AML have unfavorable prognoses and no effective therapeutic approaches for these patients are available at present, prevention for secondary MDS/AML in patients receiving genotoxic agents might be an important issue. The susceptibility of genotoxic agents might partly depend on racial differences, possibly because of different patterns of single-polymorphism nucleotides (SNP) [12]. Additional cytogenetic changes related to genotoxic exposure in Asian patients could be different from those of Caucasians.

### 2. Materials and methods

We obtained cytogenetic data on 155 MM patients treated between 1993 and 2004 at our institute: 97 patients showed normal karyotypes at the time of MM diagnosis, and the remaining 58 patients had abnormal karyotypes. Of the

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97 MM patients with normal karyotypes at the time of MM diagnosis, 17 patients showed de novo appearance of clonal cytogenetic changes during their courses, and 7/17 patients showed myeloid-related cytogenetic changes (Table 1). Of the seven patients with myeloid-related cytogenetic changes, 5 had  $-7/7q-$  anomalies, including three with  $der(1;7)(q10;p10)$  [hereafter referred to as  $der(1;7)$ ] (Table 1). Of the 155 MM patients, 3 were given a diagnosis of MDS during the course of their disease: 2 had  $der(1;7)$  and the remaining patient showed complex abnormalities, including  $-5$  and  $7q-$  [45,XY,del(3)(p21),-5,del(7)(q11),t(9;14)(q11;q11),t(11;11)(p15;q13),del(17)(p11) by spectral karyotyping analysis]. We then focused on MM patients with  $der(1;7)$  anomaly.

### 3. Results

#### 3.1. Cytogenetic results in MM patients with $der(1;7)(q10;p10)$

**Case 1:** At the time of MM diagnosis, cytogenetic analysis of bone marrow cells revealed a normal female karyotype [13/13 cells], and until March 1998, repeated cytogenetic studies yielded normal results. In June 1999,  $der(1;7)$  was detectable [12/19 cells] when pathologically identifiable myeloma progression was evident [total dose of melphalan before the appearance of  $der(1;7)$  was 2,100 mg]. After that time, a minor population with  $der(1;7)$  was continuously detected, but the patient was not given a diagnosis of concomitant MDS after reassessment of the marrow films (Table 2).

**Case 2:** Cytogenetic study demonstrated a normal female karyotype (20/20 cells). In October 2001, the clonal nature of  $der(1;7)$  was evident without obvious myelodysplastic features or myeloma cell proliferation (1.2%) in the marrow. In December 2004, 18/22 cells had  $der(1;7)$  and 2 cells had  $t(3;4)(p14;q32)$  with myelodysplastic features (Table 2). The total dose of melphalan before the appearance of  $der(1;7)$  was 2,018 mg.

**Case 3:** Cytogenetic study at the time of MM diagnosis in December 1996 revealed a normal male karyotype [20/20 cells], but repeated analysis in June 2004 when he had MM/MDS diagnosis showed the presence of clonal  $der(1;7)$  in 3/20 marrow cells. The most current bone marrow sample showed a normal male karyotype [20/20 cells] after administration of thalidomide (Table 2).

#### 3.2. Detection of $7q-$ by FISH study in MM with $der(1;7)(q10p10)$

We used the D7S486 SpectrumOrange/ CEP 7 SpectrumGreen probe (Vysis, Downers Grove, IL) to detect the missing long arm of chromosome 7 [13]. Carnoy's fixed cells were hybridized with the probe according to the manufacturer's instructions, and at least 100 interphases were counted to identify  $7q-$  cells corresponding to  $der(1;7)$ -positive cells. The incidence of interphase cells with  $7q-$  in our MM patients were shown in Table 2. We anticipated that the  $7q-$  FISH technique could detect cells with  $der(1;7)$  before the time of detection of metaphases with  $der(1;7)$ , but could not do so in the marrow. Moreover, the incidence of interphase cells with  $7q-$  detected by FISH was lower than that of metaphases with  $der(1;7)$ . The dissociation of

Table 1  
De novo appearance of cytogenetic changes in multiple myeloma

Patient no.	Myeloid-related cytogenetic changes	Myeloma-associated cytogenetic changes	unknown	MDS
1	46,XX,+1,der(1;7)(q10;p10)[12/19]			No
2	46,XX,+1,der(1;7)(q10;p10)[5/21]			Yes
3	46,XY,+1,der(1;7)(q10;p10)[3/20]			Yes
4	45,X,-Y[8/14]/46,XY,del(20)(q11)[1/14]			No
5	45,X,-Y[6/18]			No
6	49,X,-Y,add(2)(p?),del(8)(q?), +3,+5,-7,+9,+11,+13[10/21]			No
7	45,XY,del(3)(p21),-5,del(7)(q11), t(9;14)(q11;q11),t(11;11)(p15;q13), del(17)(p11)[10/21]			Yes
8		44,XX,-7,-11,der(16)t(11;16)(q13;q22)[3]		No
9		45,XX,t(11;14)(q13;q32),-13,-16,+mar[12]		No
10		43,X,-X,del(1)(p21?),-4,-8,t(1;14)(q11;q32),t(1;21)(q11;q22)[10]		No
11		46,XX,t(11;14)(q13;q32)[2]		No
12		46,XX,t(11;14)(q13;q32)[3]		No
13		46,XY,add(12)(p13),-13,add(14)(q32)[8]		No
14		55,XY,+5,add(7)(q31?),+9,+9,+der(11)(p15?), +15,+17,+19,+21[3]		No
15		49~54,XX,+3,+11,-12,add(15)(p11?),+2~7mar[3]		No
16			45,XY,-20[3/21]	No
17			49,XY,+1,+i(1)(q10)+3[2/18]	No

The numbers in brackets indicate metaphase with abnormal karyotypes/examined metaphases.

Table 2  
Cytogenetic results of multiple myeloma with der(1;7)(q10;p10)

Date of examination	Status	Cytogenetics [no. of cells examined]	Frequency of 7q- by FISH	Cumulative melphalan dose (mg)
Case 1				
Sept. 30, 1991	MM at diagnosis	46,XX[13]	0%	0
Dec. 10, 1991	MM	46,XX[6]	0%	72
Mar. 12, 1993	MM	46,XX[17]	0%	216
May 29, 1995	MM	46,XX[20]	0%	616
Aug. 4, 1995	MM	46,XX[16]	0%	712
Sept. 8, 1995	MM	46,XX[8]	0%	724
Jun. 6, 1996	MM	46,XX[17]	0%	1,164
Mar. 12, 1998	MM	46,XX[6]	0%	1,748
Jun. 2, 1999	MM	46,XX,+1,der(1;7)(q10;p10)[12]/46,XX[7]	33%	2,100
Dec. 12, 2000	MM	46,XX,+1,der(1;7)(q10;p10)[1]/46,XX[19]	6%	2,356
Apr. 26, 2001	MM	46,XX,+1,der(1;7)(q10;p10)[3]/46,XX[19]	5.9%	2,488
Case 2				
Apr. 25, 1996	MM at diagnosis	46,XX[20]	1%	0
Aug. 14, 1996	MM	46,XX[16]	0%	192
Sept. 22, 1997	MM	46,XX[19]	0%	416
Oct. 22, 2001	MM	46,XX,+1,der(1;7)(q10;p10)[5]/46,XX[16]	16%	2,018
Dec. 27, 2004	MM with MDS	46,XX,+1,der(1;7)(q10;p10)[18]/46,XX[2] 46,XX,t(3;12)(p14;q32)[2]	69%	2,786
Case 3				
Dec. 01, 1996	MM at diagnosis	46,XY[20]	NA	0
Feb. 17, 1999	MM	46,XY[20]	NA	640
Apr. 4, 2000	MM	46,XY[20]	NA	696
Jun. 23, 2004	MM with MDS	46,XY,+1,der(1;7)(q10;p10)[3]/46,XY[17]	4%	3,010
Jan. 12, 2005	After thalidomide therapy	46,XY[20]	3%	3,010

the incidence of interphase 7q- and metaphase der(1;7) might result from the mixed cell population in the marrow. Our data, however, suggest the possibility that detection of 7q- change in the peripheral neutrophils by FISH could be a useful tool to detect the progression of MDS.

### 3.3. Occurrence of MDS and -7/7q- in treated MM

During the current decade, we had 155 MM patients with cytogenetic results, and we surveyed the occurrence of MDS each year after MM diagnosis and calculated the cumulative doses of melphalan in MDS patients or those with -7/7q-. All three MDS cases following MM had 7q- abnormality, and two of them with der(1;7) were given diagnoses of MDS. In contrast, two patients showed de novo appearance of complex abnormalities, including -7, within 1 year after the MM diagnosis, and they received less than 0.5 g of melphalan. One patient also had a myeloma-associated cytogenetic change [14] [i.e., t(11;16)(q13;q22)].

According to survey results, 1/20 (5%) patients with a 7-year follow-up and 2/17 (11.7%) patients with an 8-year follow-up after the diagnosis of MM developed MDS (Table 3). Clonal der(1;7) was detectable in 1/34 (3%) patients at 6 years and in 2/20 (10%) patients at 8 years after the diagnosis of MM (Table 3). The cumulative doses of melphalan among these three MDS cases or those with the appearance of der(1;7) were more than 2 g (Table 3). Most cases of MM treated with 2 g or more of melphalan

developed MDS with 7q-: 1/1 patient with 2.0–2.5 g melphalan, 2/3 patients with 2.5–3.0 g melphalan, and 1/1 patient with 3 g or more melphalan had either the development or appearance of clonal der(1;7) anomaly, thus indicating that the critical cumulative melphalan dose might be 2 g.

## 4. Discussion

We encountered three cases of der(1;7) during the course of MM, and two of them developed MDS. One patient did not show any apparent myelodysplastic features when der(1;7) was detected, though the patient had persistent pancytopenia. Since all patients in this study received multiple chemotherapies, we cannot completely rule out the possibility that the appearance of -7/7q- could occur in MM cells as additional cytogenetic changes. It is likely, however, that der(1;7) in MM patients might be derived from non-MM cells, possibly resulting from genotoxic agents for MM, because the timing of the de novo appearance of der(1;7) was not always associated with the progression of MM. Furthermore, three patients with MM had de novo appearance of -7/7q- other than der(1;7), and one of them developed MDS.

Additional cytogenetic changes in treated MM patients were identified (e.g., 5q-, +8, or 20q-) [8,11]. These additional myeloid-related changes were found not only at the time of secondary MDS/AML, but also during the stable

Table 3  
Cumulative occurrence of myelodysplastic syndromes in multiple myeloma

Followed up periods after MM diagnosis	No. of patients	No. of patients developing MDS	No. of patients with der(1;7)	No. of patients with-7/7q-other than der(1;7)
~1 yr	155	0	0	2
1 yr < < 2 yr	91	0	0	0
2 yr < < 3 yr	62	0	0	0
3 yr < < 4 yr	48	0	0	0
4 yr < < 5 yr	40	0	0	0
5 yr < < 6 yr	34	0	1	0
6 yr < < 7 yr	25	0	0	0
7 yr < < 8 yr	20	1	2	0
8 yr < < 9 yr	17	2 <sup>a</sup>	0	1 <sup>a</sup>
9 yr	15	0	0	0
Total melphalan doses in multiple myeloma and MDS				
Cumulative melphalan doses	No. of patients	No. of patients developing MDS	No. of patients with der(1;7)	No. of patients with-7/7q-other than der(1;7)
< 0.5 g	123	0	0	2
0.5–1.0 g	19	0	0	0
1.0–1.5 g	6	0	0	0
1.5–2.0 g	2	0	0	0
2.0–2.5 g	1	1	1	0
2.5–3.0 g	3	1 <sup>a</sup>	1	1 <sup>a</sup>
> 3.0 g	1	1	1	0

<sup>a</sup> The MDS patient with complex abnormality, including 7q-, after MP therapy. One MDS patient with complex abnormality, including 7q-, after MP therapy.

phase preceding MM [11], thus indicating that the appearance of such anomalies may be linked to pre-existing myelodysplastic conditions in patients receiving alkylating agent treatments. Fonseca et al. [15] also reported on a patient with primary systemic amyloidosis showing der(1;7) without any evidence of myelodysplasia, and they suggested a possibility that der(1;7) in the myeloid cells may link to myeloid stem cell damage, presumably from exposure to melphalan or other alkylating agents [15]. Nevertheless, all three MDS patients in our study showed 7q-, including two with der(1;7). Rodjer et al. [16] reported that 6/11 therapy-related MDS/AML in MM patients had -7/7q-, and all patients received oral melphalan ranging from 0.5 to 4.1 g (median dose, 0.9 grams).

We searched der(1;7) in Mitelman's database (<http://www.gap.nci.nih.gov/Chromosomes/Mitelman>). Eighteen patients with MM or monoclonal gammopathy, including our three patients, were found to have der(1;7) (Table 4) [17–28]. All patients showed der(1;7) after chemotherapy, including melphalan, and 14/18 of them developed MDS/AML. Moreover, 7/18 MM patients with der(1;7) were Japanese. Susceptibility to genotoxic agents may vary, depending on ethnic factors, possibly owing to differences of SNP. We do not know the exact difference on melphalan sensitivity for normal hematopoiesis, but one plausible explanation might be that the difference of myeloid-related cytogenetic abnormalities in treated MM patients might result from differences in genetic background. The common agent for the association is melphalan, and the

duration between MM diagnosis and the appearance of der(1;7) ranged from 1.8 months to 9 years (mean, 57.2 months, Table 4). All three of our MM patients received more than 2 g of melphalan. Among the 18 reported MM patients with der(1;7), 4 were not given a diagnosis of secondary MDS at the time of der(1;7) appearance, thus we can speculate that the appearance of der(1;7) might be a predictive indicator for a hidden myelodysplastic condition, especially in Asian populations. Therefore, careful follow-up, including repeated cytogenetic study of the marrow cells, is required when treating MM patients with der(1;7).

In this study, we surveyed MM patients with cytogenetic results in our institute, and the appearance of 7q-, including der(1;7), was found in MM patients who had survived for more than 5 years and had a therapeutic history of more than 2 grams of melphalan. Some MM patients had eventually been treated with  $\alpha$ -interferon, and the occurrence of der(1;7) at 5 years was 1/34, the incidence of der(1;7) increased to 10% in those surviving for more than 7 years, and the incidence of MDS development increased dramatically around this period (Table 3). This notion is also supported from another view that most patients receiving more than 2 grams of melphalan developed MDS with the 7q- anomaly, including der(1;7). Thus, sequential cytogenetic study on marrow cells or neutrophil FISH study using the 7q probe(s) might detect the hematologic condition earlier, before the development of MDS.

Immuno-modulator agents, including thalidomide, have been widely used in recent years, not only for MM, but also

Table 4  
Clinical features of multiple myeloma with der(1;7)(q10;p10)

Age/sex	Type of myeloma	Treatment before der(1;7) appearance	Duration before der(1;7)	Status at appearance of der(1;7)	Cytogenetics of der(1;7) appearance	No MDS diagnosis	Duration of der(1;7) and MDS diagnosis	Follow-up period after der(1;7)	References
1 53/F	IgG-κ	MP/MCNU-VMP/ VAD/α-IFN	93 m	MM in relapse	46,XX,+1,der(1;7)(q10;p10)[12]/46,XX[7]	0	24 m	Case 1	
2 51/F	BJP-λ-type	MCNU-VMP/ α-IFN/MP	66 m	MM stable	46,XX,+1,der(1;7)(q10;p10)[5]/46,XX[16]	38	44 m	Case 2	
3 61/M	IgG-κ	MCNU-VMP/MP	90 m	MDS-RA	46,XY,+1,der(1;7)(q10;p10)[3]/46,XX[17]	0	10 m	Case 3	
4 71/F	MM	MP	5 yr	MDS-RAEB	47,XX,+1,der(1;7)(q10;p10),+2[5]	ND	3 m	Case 8 in [17]	
5 61/M	MGUS	MP	5 yr	MM stable	46,XY,+1,der(1;7)(q10;p10)[5]/46,XY[27]	ND	32 m	Case 11 in [17]	
6 67/F	MM	MP	4 yr	MM stable	46,XX,+1,der(1;7)(q10;p10)[11]/46,XX[22]	ND	7 m*	Case 12 in [18]	
7 53/F	MM	Melphalan/ radiotherapy	3 yr	MM with dyserythropoiesis	46,XX,+1,der(1;7)(q10;p10)[6]/46,XX[15]	ND	13 m*	Case 3 in [17]	
8 82/F	IgG-κ + IgA-κ	Melphalan/CPA/ VDS/PSL	81 m	MDS	46,XX,+1,der(1;7)(q10;p10),-7,+8[19]/46,XX[11]	ND	11 m	[19]	
9 55/F	MM	ABCm	Survival 658 d	MDS-RA	46,XX,t(1;7)(p18)/46,XX[7]	ND	ND	Case 25 in [11]	
10 74/F	IgA	MP	45 m later	MDS-RAEB	48,XX,+t(1;7),-7,+11,+13/45,XX,-7	ND	ND*	Case 19 in [21]	
11 76/M	IgG	CPA/XCR/PSL/ MEVP	32 m	AML-M1		0	10 d	Case 1 in [20]	
12 57/M	MM	ND	ND	MDS	46,XY,+1,der(1;7)(q10;p10)/46,XY	ND	ND	Case 24 in [23]	
13 72/M	IgA-κ	MP	32 m	AML		0	9 m	[24]	
14 50/F	MM	CPA	ND	Dyshematopoiesis	46,XX,-7,+t(1;7)(p11;p11)/46,XX	ND	ND	Case 2 in [25]	
15 75/F	MM	Melphalan	ND	MDS-RAEB	46,XX,-7,+t(1;7)(p11;p11)/46,XX	ND	ND	Case 22 in [25]	
16 74/M	MM	Melphalan/CPA/ VCR	9 yr	MDS/AML	46,XY,t(3;10)(q21;q24),+t(1;7)(p11;q11)/48,XX,+Y,+8,t(3;10),+der(1)	ND	ND	Case 2079 in [27]	
17 55/F	MM	ND	ND	ND	46,XX,+1,der(1;7)(q10;p10)[3]/46,XX[27]	ND	ND	[27]	
18 66/F	MM	MP	4 yr	MDS-RAEBt	47,XX,+1,der(1;7)(q10;p10),+8[10]/46,XX[10]	ND	ND	Case 7 in [28]	

Cytogenetics are based on the original descriptions.

Diagnosis: MM, multiple myeloma; BJP, Bence-Jones protein; MGUS, monoclonal gammopathy undetermined significance.

Cytogenetics: modified recent description. Parentheses indicate number of cells examined.

Therapy: MP, melphalan + prednisolone; MCNU-VMP, MUNU + vincristine + melphalan + prednisolone; α-IFN, α interferon; CPA, cyclophosphamide; VDS, vindesine; PSL, prednisolone; ABCM, adriamycin + BCNU + cyclophosphamide + melphalan.

ND, no description.

Asterisks indicate patients who expired.