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Molecular Heterogeneity of the *PML* Gene Rearrangement in Acute Promyelocytic Leukemia: Prevalence and Clinical Significance

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We determined the breakpoints of the *RAR-α* and *PML* genes in acute promyelocytic leukemia (APL) cells from 40 patients using Southern blot analysis. We also analyzed the relationship between the location of breakpoints, the clinical features of APL and the response to all-*trans* retinoic acid (ATRA). While the breakpoints of the *RAR-α* gene were consistently within intron 2, we found two major clusters in the breakpoints of the *PML* gene. The two breakpoint clusters in the *PML* gene were separated by 10 kb; 5′ breakpoints were in intron 3, and 3′ breakpoints were around introns 5 and 6. Twenty percent of the patients had 5′ breakpoints in the *PML* gene and 70% had 3′ breakpoints. No rearrangement was observed in the remaining 10% of patients in spite of the presence of t(15;17) translocation. There was no relationship between the location of the *PML* breakpoints, the clinical features at diagnosis and the response to ATRA.

Key words: Acute promyelocytic leukemia — Gene rearrangement — *PML* — All-*trans* retinoic acid

The t(15;17) translocation, which is a cytogenetic hallmark of acute promyelocytic leukemia (APL), is reported to be a reciprocal rearrangement between the *PML* gene in the 15q22 region and the gene in the 17q12-21 region.[1–4] The direction of transcription of both genes is from centromere to telomere, and both are broken within introns.[2–5] The resultant *PML/RAR-α* fusion mRNA appears to encode a chimeric protein, and in supposedly associated with leukemogenesis in a dominant negative manner.[6–8] While the functions of the normal *PML* and *RAR-α* proteins in myelopoiesis are not obscure, the disruption of the *PML* and *RAR-α* genes and the production of the chimeric *PML/RAR-α* protein may impair granulocytic differentiation in APL cells. Administration of high-dose all-*trans* retinoic acid (ATRA) seems to force the leukemia cells to differentiate, and successfully induces complete remission (CR).[9–11]

The disruption of the *RAR-α* gene in APL cells has been well studied. The breakpoints of these *RAR-α* genes were localized within intron 2 (formerly called intron 1) in the majority of cases.[3, 12, 13] However, there has been little study of breakpoints in the *PML* gene. Recently, it has been reported that *PML* breakpoints are clustered in restricted regions, and that the resultant chimeric transcripts are unexpectedly heterogeneous.[14, 15]

In this paper, we determined the breakpoints of the *RAR-α* and *PML* genes in APL cells from 40 patients using Southern blot analysis. We also analyzed the relationship between the location of breakpoints, the clinical features of APL, and the response to ATRA.

MATERIALS AND METHODS

Patients and leukemia cells Bone marrow was aspirated from 40 patients with APL after obtaining informed consent. Leukemia cells were separated by hemolysis with ammonium chloride solution: 0.13 *M* NH_4Cl, 1 m*M* NH_4HCO_3 in phosphate-buffered saline (PBS). Samples were washed in PBS, and cryopreserved in a deep freezer until analysis. Thirty-two of the 40 patients were cases registered to the Kouseisho APL Study Group, and were treated with ATRA 45 mg/m^2 *per os* daily. The other 8 cases were treated with chemotherapy. All cases were diagnosed as M3 except one (case #36) which was an M3 variant, according to the French-American-British classification system.[16] There were 18 males and 22 females, and the age ranged from 8 to 72 with a median of 40. Twenty-three were newly diagnosed cases and 17 were relapsed cases. Chromosomal analysis was carried out in 33 cases, and 27 (82%) had the t(15;17) translocation. Eight of 27 had additional chromosomal changes (Table I).

Southern blot analysis of the *RAR-α* and *PML* genes High-molecular-weight DNA was extracted from frozen leukemia cells by the standard method,[17] digested with the restriction enzymes *Eco*RI, *Bam*HI and *Hin*dIII, electrophoresed on a 0.9% agarose gel, blotted onto nylon membrane and hybridized as described.[17] After hybridization with probes, the filters were washed under

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stringent conditions (0.1×standard saline citrate at 50°C) and exposed to X-OMAT AR film (Kodak, Rochester, NY). The filters were recycled for use with other probes by complete stripping of the probe.

To detect the *RAR-α* gene, a partial cDNA probe corresponding to exons 2, 3 and 4 was subcloned from a full-sized *RAR-α* cDNA clone provided by Prof. Pierre Chambon (INSERM, Strasbourg, France).[18] To clarify whether novel bands hybridized to exon 2 or to exons 3 and 4, a 477 bp fragment comprising the 3′ side of intron 2 and exon 3 was cloned from placental DNA by using the polymerase chain reaction (PCR). The primers were synthesized on a DNA synthesizer (model 381A; Applied Biosystems, Inc., Foster City, CA) according to the published sequence[4] as follows: 5′ primer 5′-AGGAGCTTTAGAATCAGGGTGACC-3′, and 3′ primer 5′-CTTGCAGCCCTCACAGGCGCTGA-3′. The amplified fragment was inserted into a pUC19 plasmid vector.

To obtain the *PML* probe, two *PML* cDNAs corresponding to exon 3, and exons 5 and 6 were amplified from the cDNA of HL60 cells by PCR using the 5′ primer 5′-GCGGTACCAGCGCGACTACGAGGAGAT-3′ and the 3′ primer 5′-TTTCCCCTGGGTGGGATTGCAAGAGCTGAG-3′ for the amplification of exon 3. The 5′ primer 5′-GAGAGAGTGAAGGCCCAGGT-3′ and the 3′ primer 5′-CCGAGCTGCTGATCACCACAA-3′ were used for the amplification of exons 5 and 6. These primers were synthesized according to the published sequences.[6, 7, 15] The *PML* gene origins of the amplified products were confirmed by DNA sequencing.

Isolation of the genomic *PML* gene A genomic library was constructed by Dr. M. Seto (Aichi Cancer Center, Nagoya) from normal placental DNA. This library was screened with the *PML* probe described above. Genomic library screening and plaque purification were performed by standard procedures.[17] After screening 10^6 clones, six positive clones were obtained and restriction enzyme maps were established for each.

RESULTS

Rearrangement of the *RAR-α* gene The genomic structure of the *RAR-α* gene is shown in Fig. 1.[4, 12, 15] Since the translocation breakage occurs within the second intron of the *RAR-α* gene, we divided the second intron into 7 subregions bordered by *Eco*RI, *Bam*HI and *Hin*dIII sites. The *RAR-α* probe, which corresponded to exons 2, 3 and 4, detected 5.5 and 6.6 kb bands in *Eco*RI-digested germline DNA, 7.5 and 11.5 kb bands in *Bam*HI-digested DNA, and a 17 kb band in *Hin*dIII-digested DNA (Fig. 2A). The probe for exon 3 of the *RAR-α* gene detected 5.5 kb, 7.5 kb and 17 kb bands in *Eco*RI-, *Bam*HI- and *Hin*dIII-digested DNA, respectively (Fig. 2B). The use of a combination of these two probes made it easy to map breakpoints within the *RAR-α* gene. For example, in case #34, the exon 3 probe showed a novel 19 kb band in *Hin*dIII digests only. On the other hand, the exon 2, 3 and 4 probe hybridized to a novel band at 9 kb in the *Bam*HI digest in addition to the 19 kb band in the *Hin*dIII digest, and the intensity of the germ line bands

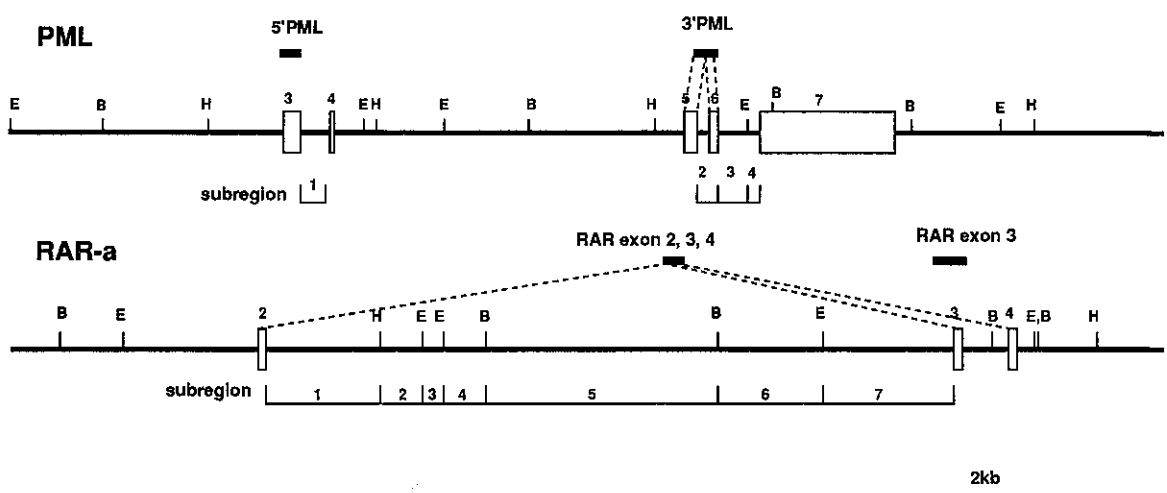

Fig. 1. Restriction map of the *PML* and *RAR-α* genes according to the literature[4, 12, 15] and our results. White boxes indicate exons, and exon numbers are given above the box. The probes used in this study are indicated as solid lines above the map. The breakpoint clustering regions were divided into subregions bordered with restriction enzyme sites and exon/intron boundaries. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII.

corresponding to exon 2 was slightly reduced (Fig. 2A and 2B), but the *Eco*RI digests gave no rearrangements. Therefore, the breakpoint in this case was localized to subregion 3 or 4. No rearrangement was observed in 11 of 40 cases, although our Southern blot analysis could detect breaks within a long region that included the full sequence of intron 2. This result probably arose because the novel bands often overlapped with the germline bands. Some cases seemed to share a common rearrangement pattern of the *RAR-α* gene. In cases #25 to #31, breakpoints were localized to subregion 7, and novel bands in *Hin*dIII-digested DNA were invariably 0.5 to 1 kb smaller than the novel bands in *Bam*HI-digested DNA, and 3 to 4 kb smaller than in *Eco*RI-digested DNA. Similarly, in cases #22, #23 and #24, whose *RAR-α* breakpoints were localized to subregion 6, the patterns of novel bands in *Hin*dIII- and *Bam*HI-digested DNA were identical to those in cases #25 to #31. In other cases, no regularity was observed in the *RAR-α* rearrangement pattern.

In cases #25 to #31, in which *RAR-α* rearrangements were observed with exon 3 probe in each of the three digests, the disruption occurred within intron 2. This was demonstrated because the *PML/RAR-α* chimeric DNA transcripts could be amplified using reverse transcriptase-PCR (RT-PCR) with primers corresponding to *PML* exon 5 and *RAR-α* exon 3 (data not shown).

Isolation of the *PML* genomic clone To elucidate the genomic configuration of the *PML* gene, genomic clones were obtained using *PML* amplified fragments as probes.

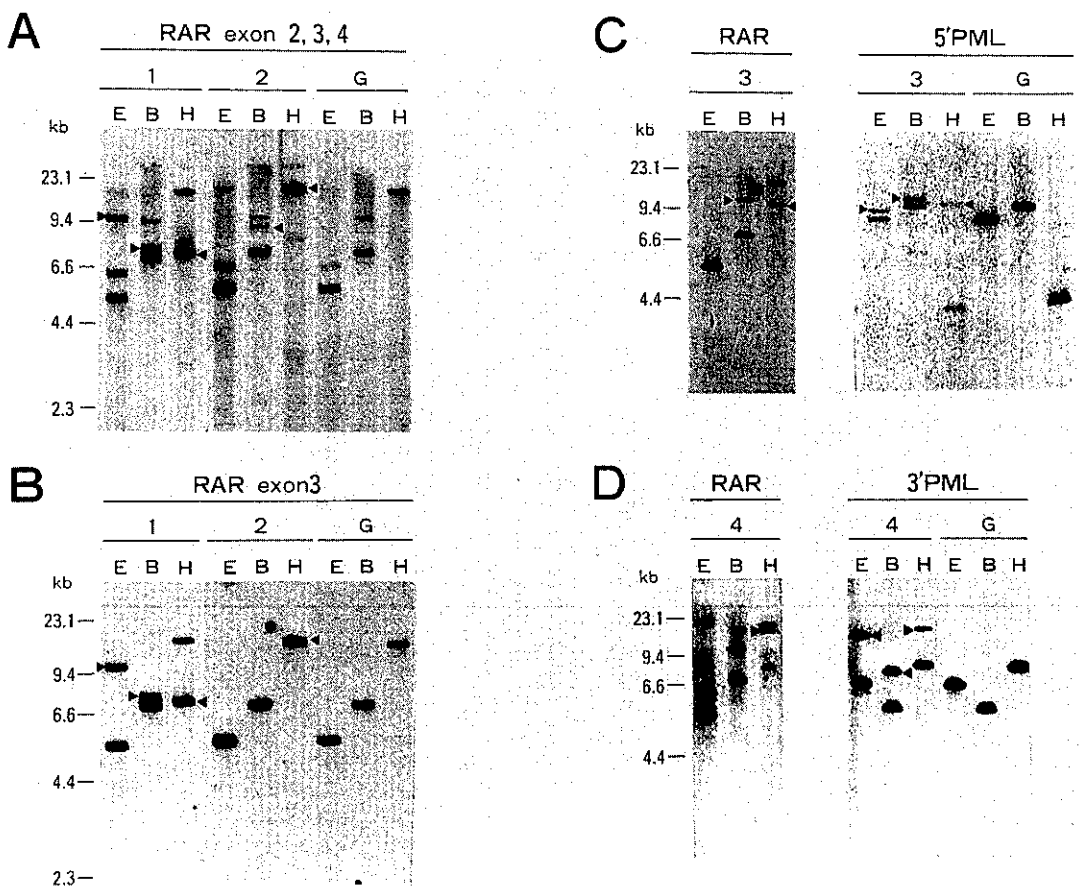

Fig. 2. Southern blot analysis of the *PML* and *RAR-α* genes in representative cases. The same filter was hybridized with the *RAR-α* exons 2, 3 and 4 probe (A), and *RAR-α* exon 3 probe (B). In lane 1 (case #30), rearranged bands were detected with both probes. However, in lane 2 (case #34), the rearranged band in *Bam*HI digests was detected only with the former probe. In C, the rearranged bands of 5′ *PML* were detected at the same position as those of *RAR-α* in *Bam*HI and *Hin*dIII digests. Similarly, in D, the rearranged band of 3′ *PML* overlapped with that of *RAR-α* in *Hin*dIII digest. Arrowheads indicate novel bands. Lane 1, case #30; lane 2, case #34; lane 3, case #6; lane 4, case #21; lane G, placental DNA; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII.

The clones were characterized by restriction enzyme analysis and Southern blotting. A 20 kb region of the *PML* gene is shown in Fig. 1.

To date, at least two *PML/RAR-α* chimeric mRNAs have been reported in which the *PML* sequences differ in size. A recent study suggested that the shorter transcript

Table I. Clinical Data and Southern Blot Analysis of 40 APL Patients

Case	Age	Sex	Karyotype	*RAR-α*			5′ *PML*			3′ *PML*			Breakpoint subregion		Response to ATRA
				E	B	H	E	B	H	E	B	H	*PML*	*RAR*	
1	8	M	2	G	G	G	10	12	4	G	G	G	1	1	CR
2	9	F	0	G	G	14	13	7	14*	ND	ND	ND	1	5	CR
3	30	F	0	G	G	14	13	7	14*	G	G	G	1	5	CR
4	72	F	2	G	G	15	14	6	15*	G	G	G	1	5	CR
5	68	M	1	ND	G	18	ND	12	18*	G	G	G	1	5	CR
6	59	F	1	G	11	10	9	11*	10*	G	G	G	1	6	CR
7	20	F	ND	G	12	11	10	12*	11*	ND	ND	ND	1	6	PR
8	45	F	0	11	9	7	11*	9*	7*	G	G	G	1	7	ND
9	67	M	1	G	11	19	G	G	G	8	7, 11*	19*	2	3, 4	CR
10	38	F	ND	G	G	G	G	G	G	15	9, 2	17,*10	2	5	CR[a]
11	59	M	ND	6	G	20	ND	ND	ND	9	9	2.8	3	1	CR[a]
12	52	M	1	ND	ND	22	G	G	G	9	9	3	3	1	CR
13	57	M	ND	G	G	15	G	G	G	10	5	15*	3	4	CR
14	31	F	0	G	G	G	G	G	G	17	10	18*	3	4	ND
15	30	F	ND	G	G	G	G	G	G	17	10	18*	3	4	ND
16	60	M	ND	G	G	15	ND	ND	ND	14	9	15*	3	5	CR
17	45	M	2	G	G	16	G	G	G	14	6	16*	3	5	CR[a]
18	13	F	2	G	G	16	ND	ND	ND	15	6	16*	3	5	CR
19	49	F	1	G	G	16	ND	ND	ND	15	9	16*	3	5	CR
20	38	F	1	G	G	G	G	G	G	14	9	17*	3	5	CR
21	24	F	1	G	G	G	ND	ND	16	14	9	16*	3	5	ND
22	40	F	1	G	8.5	8	ND	ND	ND	11	8.5*	8*	3	6	ND
23	25	F	1	G	12	11	G	G	G	9	12*	11*	3	6	CR
24	34	M	ND	G	12	11	G	G	G	9	12*	11*	3	6	CR
25	50	F	1	9	5	4.5	G	G	G	9*	5*	4.5*	3	7	CR
26	19	F	0	9	G	5.5	ND	ND	ND	9*	G	5.5*	3	7	ND
27	51	M	2	9	G	5.5	G	G	G	9*	G	5.5*	3	7	CR
28	54	M	1	12	7.5	7	G	G	G	12*	G	7*	3	7	CR
29	46	F	1	12	G	7	G	G	G	12*	G	7*	3	7	F
30	38	M	2	11	8	7.5	G	G	G	11*	8*	7.5*	3	7	CR
31	13	M	1	12	9	8	G	G	G	12*	9*	8*	3	7	ND
32	49	M	1	ND	ND	18	ND	ND	G	ND	ND	18*	3	–	F
33	45	M	1	7.5	11	G	ND	ND	ND	G	G	7.5	4	1	CR[a]
34	23	F	1	G	9	19	G	G	G	G	7	19*	4	3, 4	CR
35	36	M	2	G	G	G	G	G	G	G	7	20	4	5	ND
36	25	M	1	G	10	9.5	ND	ND	ND	G	10*	9.5*	4	6	CR
37	55	F	2	G	G	G	G	G	G	G	G	G	0	0	CR
38	40	F	1	G	ND	G	G	G	G	G	ND	G	0	0	CR
39	49	M	0	G	G	G	G	G	G	G	G	G	0	0	PR
40	23	F	1	G	G	G	G	G	G	G	G	G	0	0	CR

Abbreviations; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; ND, not done; G, germline band; CR, complete remission; PR, partial remission; F, failure. In the karyotype column, numbers 0, 1 and 2 indicate normal, t(15;17) and t(15;17) plus additional abnormalities, respectively. In the *RAR-α*, 5′ *PML* and 3′ *PML* columns, numbers indicate the length (kilobases) of the novel bands. An underline in case #34 indicates that the band was not detected with the exon 3 probe. In the breakpoint subregion, 0 indicates an unknown position and 1–7 correspond to Fig. 1. *Overlapping of the band with normal *RAR-α* band.
a) In combination with chemotherapy.

was generated by splicing exon 3 of the *PML* gene to exon 3 of the *RAR-α* gene, and that the longer transcript was produced by splicing exon 6 of the *PML* gene to exon 3 of the *RAR-α* gene.[15] To identify the location of the breakpoint within the *PML* gene, we used two probes corresponding to exon 3 and to exons 5 and 6 of the *PML* gene. In most cases, the *PML* gene was disrupted around these regions.[15]

Rearrangement of the *PML* gene The exon 3 probe (named the 5′ *PML* probe) hybridized to germ line DNA bands of 8.5, 10 and 4 kb in *Eco*RI, *Bam*HI and *Hin*dIII digests, respectively (Fig. 2C). No polymorphic bands were observed in any of the DNA samples from 40 patients with APL, or from 5 normal subjects. Novel bands were detected in cases #1 to #8. In cases #2, #3, #4, #5, #7 and #8, the novel bands overlapped with the *RAR-α* bands. These overlaps are marked by asterisks in Table I. The majority of these cases seemed to have a breakpoint within intron 3, because a chimeric mRNA could be detected using RT-PCR with primers corresponding to *PML* exon 3 and *RAR-α* exon 3 (manuscript in preparation).

The exons 5 and 6 probe (named the 3′ *PML* probe) hybridized to germline bands of 7.5, 6, and 9 kb in *Eco*RI, *Bam*HI and *Hin*dIII digests, respectively (Fig. 2D). As in the 5′ *PML* probe analysis, no polymorphic bands were detected by the 3′ *PML* probe. A heterogeneous pattern of the rearrangements was observed by Southern blot analysis. The 3′ *PML* probe hybridized to a short novel band in *Hin*dIII digests, when the breakpoint was located in subregion 1 of the *RAR-α* gene. On the other hand, when the breakpoint was located in any of subregions 2 to 7, the novel bands overlapped with the *RAR-α* bands in *Hin*dIII digests. When the breakpoints were within subregion 6, the novel bands in *Bam*HI digests overlapped with the normal bands. When the breakpoints were within subregion 7, the novel bands in *Bam*HI and *Eco*RI digests were the same in size.

In cases #8 and #9, the 3′ *PML* probe revealed two novel bands. This was thought to be because the *PML* gene was disrupted within intron 5 or in exon 6 (subregion 2) and the probe recognized both DNA fragments, *PML/RAR-α* and *RAR-α/PML*.

In cases #34, #35 and #36, no novel bands were detected by *Eco*RI digestion, while rearrangements were revealed by *Bam*HI and *Hin*dIII digests. Their breakpoints were thought to be localized to the 5′ side of the *Eco*RI site within intron 6 (subregion 4).

In 4 of 40 cases, there was no observable rearrangement of the *PML* and *RAR-α* genes, although the samples from these patients contained sufficient APL cells, and the t(15;17) translocation was observed in 3 of the 4 cases.

Analysis of clinical variables Our data from Southern blot analysis indicated that the translocation breakpoints in APL patients were clustered in two regions of the

Table II. Summary of Clinical Variables and Responses of ATRA

	Locus of the *PML* breakpoint		
	5′ side (N=8)	3′ side (N=28)	*P* value
Age	37.5 (8–72)	39 (13–67)	0.87
Sex (M/F)	2/6	15/13	0.15
RBC (10^{12}/liter)	2.645 (1.46–3.55)	2.88 (1.64–4.72)	0.54
WBC (10^9/liter)	1.25 (0.5–32)	2.25 (0.4–47.4)	0.64
% Blast+Pro	30.3 (0–80)	30.8 (0–95)	0.48
Platelets (10^9/liter)	29 (14–222)	31 (4–153)	0.59
Bone marrow hyperplasia	5/8	17/28	0.63
% Blast+Pro	85.8 (65–97.5)	86.2 (41.2–99)	0.87
Chromosomal abnormality			
t(15;17)	4/7	20/22	0.07
additional change	2/4	5/20	0.33
FDP (μg/dl)	60 (10–210)	63 (2.5–280)	0.63
Fibrinogen (mg/dl)	141 (55–328)	52 (21–642)	0.73
Clinial DIC	5/8	26/28	0.06
CR responder to ATRA	6/7	15/17	0.66

Values in the table are medians, and parentheses indicate ranges. *P* values were based on the two-tailed Wilcoxon rank-sum test, except for the comparison of proportions with sex, bone marrow hyperplasia, chromosomal abnormality, clinical DIC and CR responder to ATRA, for which Fisher's exact test was used.

PML gene; 5′ breakpoints were in intron 3, and 3′ breakpoints were mainly in intron 6 and rarely in intron 5. To elucidate the clinical differences between the two molecular subtypes of APL patients, pretreatment variables and the respones to ATRA were compared between the cases with 5′ breaks of the *PML* gene and those with 3′ breaks (Table II). Patients with 5′ and 3′ breaks had similar absolute leukocyte, erythrocyte and platelet counts, and similar differentials in peripheral leukocytes and bone marrow cells. There was no difference between the two subtypes in fibrin degradation product (FDP) level, fibrinogen concentration or degree of disseminated intravascular coagulopathy (DIC). Seven of 8 patients with 5′ breaks were treated with ATRA and 6 (75%) achieved CR. Seventeen of 28 patients with 3′ breaks were treated with ATRA and 16 (88%) had CR. Three of 4 cases with no observable rearrangement achieved CR by ATRA therapy, and one had partial remission.

DISCUSSION

Rearrangements of the *PML* and *RAR-α* genes in APL cells were analyzed. There have been several reports that breakpoints within the *RAR-α* gene are consistently clustered within intron 2.[3, 12, 13] We have confirmed these findings by Southern blot analysis and PCR. On the other hand, heterogeneity of the *PML* gene rearrangements has emerged from recent studies.[14, 15] However, the prevalence and clinical significance of the *PML* gene rearrangements have not been elucidated yet. We demonstrated that 20% of 40 Japanese APL patients had a 5′ break in the *PML* gene and 70% had a 3′ break. The majority (93%) of the patients with a 3′ break had a breakpoint within intron 6. In Chinese patients, Tong *et al.* reported that two (7%) of 28 cases with APL had a 5′ break, and that 20 (71%) had a 3′ break.[14] On the other hand, Pandolfi *et al.* localized breakpoints in the *PML* gene to three breakpoint cluster regions (bcr): bcr 1 in intron 6; bcr 2 in exon 5, intron 5 and exon 6; and bcr 3 around exon 3 and exon 4.[15] They found that bcrs 1 and 2 (3′ breaks), and bcr 3 (5′ breaks) were involved in 60% and in 40% of cases, respectively, in Italian patients. Miller *et al.* described two types of chimeric transcript detected by RT-PCR in APL cells. They observed a short transcript in 11 (31%) of 36 U.S. patients and a long one in 21 (58%).[19] The length of the transcript may be affected by the position of the breakpoint. Thus, the distribution of the breakpoints within the *PML* gene might be different in the West and in the Far East, although this finding is not statistically significant due to the small number of patients analyzed.

Due to the variable locations of breakpoints within the *PML* gene and the result of alternative splicing, heterogeneous *PML/RAR-α* proteins are presumably produced in APL cells.[15] If a breakpoint is located in intron 3 (5′ break), 83 and 87 kDa chimeric proteins would be the predominant products. If a break occurs within intron 6, 105 and 100 kDa proteins would be the principal products. A similar phenomenon is observed in the Philadelphia chromosome, t(9;22), in which there are two breakpoint cluster regions.[19–23] The breakpoint clusters in the major bcr and the minor bcr are associated with chimeric proteins of 210 and 190 kDa, respectively. Furthermore, the former is observed in chronic myeloid leukemia but rarely in acute lymphoblastic leukemia (ALL),[24] while the later is detected in ALL only. Therefore, we studied the relationship between the location of the *PML* breakpoints, the clinical features at diagnosis, and the response to ATRA therapy. However, no difference between the two subtypes was detected. The explanation for this may be that when the *PML* gene is disrupted in intron 3, a shorter chimeric mRNA is formed. This fused protein would have a shorter α-helix domain than a chimeric protein generated by the disruption in intron 6 of the *PML* gene.[6, 7, 15] However, in both types of chimeric protein, the proline-rich domain, the cysteine-rich domain and the leucine zipper region derived from the *PML* gene on the N-terminal side as well as the B to F domain derived from the *RAR-α* gene on the C-terminal side are expected to be conserved. Since both chimeric proteins have a putative sequence-specific DNA binding domain from the *PML* protein and a retinoic acid-binding domain from the *RAR-α* protein, there may be no biological or oncogenic difference between the two proteins. Furthermore, both may act in the same manner as a transcriptional factor, and this action may be either dependent on or independent of ATRA.

In 4 cases, no rearrangement was observed in either of the *RAR-α* and *PML* genes. It is not clear why we could not detect the rearrangement. The percentage of APL cells in the samples might have been too low to be detected by Southern blot analysis, although the smear samples contained significant numbers of APL cells. Alternatively, our Southern blot analysis may have failed to detect the rearrangements. The use of other enzymes or probes may have allowed detection of rearrangements in these patients. These cases may carry a new type of rearrangement. Three (cases #37, #38, and #39) of 4 were relapsed cases. If additional genetic alteration had occurred at relapse, the leukemic clones might have lost the t(15;17) translocation. However, the response to ATRA in all these cases tends to rule out this possibility.

The restricted localization of breakpoints in the *PML* gene as well as in the *RAR* gene will enable us to identify *PML/RAR-α* fused transcripts for the definite diagnosis of APL, to evaluate therapeutic outcome of chemotherapy or ATRA, and to detect minimal residual disease by means of RT-PCR in APL patients in CR.

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