Monitoring of Minimal Residual Disease in Children with Acute Promyelocytic Leukemia by RT-PCR Detecting PML/RAR α Chimeric Gene: A Retrospective Study of Clinical Feasibility

HOSHIRO SUZUKI,¹ MASUE IMAIZUMI,^{1,2} ATSUSHI SATO,¹ MIYAKO YOSHINARI,¹ TAKESHI RIKIISHI,¹ MIKIYA ENDO,³ TOSHIKUNI TAKANO,³ TOSHIYUKI SHIMIZU,⁴ YOSHIRO HATAE,⁵ TAKEO FUJIMOTO,⁶ YUTAKA HAYASHI² and KAZUIE INUMA¹

¹*Department of Pediatrics and*

²*Department of Pediatric Hematology and Oncology, Tohoku University School of Medicine, Sendai 980-8574,*

³*Department of Pediatrics, Iwate Medical School, Morioka 020-8505,*

⁴*Department of Pediatrics, Yamagata University School of Medicine, Yamagata 990-8959,*

⁵*Department of Pediatrics, National Sapporo Hospital, Sapporo 003-0804, and*

⁶*Department of Pediatrics, Aichi Medical School, Aichi 480-1195*

SUZUKI, H., IMAIZUMI, M., SATO, A., YOSHINARI, M., RIKIISHI, T., ENDO, M., TAKANO, T., SHIMIZU, T., HATAE, Y., FUJIMOTO, T., HAYASHI, Y. and INUMA, K. *Monitoring of Minimal Residual Disease in Children with Acute Promyelocytic Leukemia by RT-PCR Detecting PML/RAR α Chimeric Gene: A Retrospective Study of Clinical Feasibility.* Tohoku J. Exp. Med., 2001, **193**(2), 127–139 —

We studied retrospectively the clinical feasibility of minimal residual disease (MRD) monitoring by reverse transcription-polymerase chain reaction (RT-PCR) detecting the PML/retinoic acid receptor α (RAR α) chimeric gene in children with acute promyelocytic leukemia (APL). MRD monitoring of APL was performed with standard and nested RT-PCR for PML/RAR α gene, the sensitivity of which was 1 leukemic cell in 10^3 – 10^4 and 1 in 10^4 – 10^5 cells, respectively. Patients were nine children with APL (average age: 8.3 year; average period of follow-up: 69.2 months) who, after achieving remission with all-*trans* retinoic acid (ATRA), received treatment either with multidrug chemotherapy or with a combination of chemotherapy and ATRA. Out of six patients treated with multidrug-combined chemotherapy, two patients exhibited PCR positivity after six months of post-

Received October 10, 2000; revision accepted for publication February 13, 2001.

Address for reprints: Masue Imaizumi, M.D., Department of Pediatric Hematology and Oncology, Tohoku University School of Medicine, 1-1 Seiryomachi, Aoba-ku, Sendai 980-8574, Japan.

e-mail: mimaizumi@ped.med.tohoku.ac.jp

remission therapy, which shifted from the detectable range of the nested PCR to that of the standard PCR. These two patients subsequently relapsed and, together with two of the other patients receiving multidrug-combined chemotherapy, underwent allogeneic bone marrow transplantation. No MRD was detected in these patients after transplantation. In the remaining three patients who underwent cyclic treatment with alternative chemotherapy and ATRA, two showed positive RT-PCR at the nested or standard level, respectively, after six months of combined therapy, and one of them relapsed. Overall, three of four patients with MRD detected in post-remission period ultimately relapsed, while all of five patients without detectable MRD had a good prognosis. These findings suggest that impending relapse may be predicted by the detection of preceding PCR positivity with an increasing quantity of the PML/RAR α mRNA that appears beyond six months of post-remission chemotherapy, with or without combined ATRA therapy. ——— APL; MRD monitoring; PML/RAR α ; RT-PCR

© 2001 Tohoku University Medical Press

Monitoring of minimal residual disease (MRD) by detecting a disease-specific gene using reverse transcription-polymerase chain reaction (RT-PCR) is a potential approach to predicting patients at risk of impending relapse of malignant disease (Campana and Pui 1995). However, the clinical significance of MRD monitoring of disease-specific genes seems to vary depending on the hematological malignancy and the nature of the chimeric gene (Campana and Pui 1995). In acute promyelocytic leukemia (APL), the PML/retinoic acid receptor- α (RAR α) chimeric gene is characterized by distinct isoforms with variable splicing, low levels of expression and instability as compared with other well defined chimeric genes, such as AML-1/ETO or BCR/ABL (Miyamura et al. 1992; Satake et al. 1995; Jurlander et al. 1996; Radich et al. 1997; Diverio et al. 1998; Lo Coco et al. 1998). These characteristics lead to a lower level of sensitivity in the RT-PCR detecting the PML/RAR α chimeric transcripts and, therefore, MRD of APL (Grimwade et al. 1996). Interestingly, despite this relatively low sensitivity of PML/RAR α detection, MRD monitoring seems to be positively correlated with the clinical course of the disease (Mandelli et al.

1997; Diverio et al. 1998). Furthermore, distinct isoforms of chimeric oncoproteins may affect the prognosis of APL patients receiving all-trans retinoic acid (ATRA) therapy due to differences in retinoic acid binding (Benedetti et al. 1997; Vahdat et al. 1994).

Therapeutic modality is another factor affecting both MRD positivity as well as its persistency. For patients with APL, ATRA therapy is highly effective in producing remission by inducing APL cells to differentiate (Breitman et al. 1980; Imaizumi and Breitman 1987; Huang et al. 1988; Castaigne et al. 1990; Warrell et al. 1991). However, when used alone it is insufficient in ensuring PCR negativity or in maintaining the patient in a state of remission (Chen et al. 1991; Warrell 1993). Therefore, post-remission multidrug-combined chemotherapy is required in order to prevent relapse. Even so, a proportion of patients with APL who receive intensive chemotherapy following ATRA therapy ultimately relapse (Diverio et al. 1998; Guglielmi et al. 1998). MRD monitoring is necessary to identify these patients at risk of impending relapse before it becomes overt (Grimwade et al. 1996). Furthermore, several groups have recently been

investigating the clinical efficacy of a combination of ATRA and chemotherapy in the induction and maintenance of remission (Fenaux et al. 1994; Diverio et al. 1998; Hirota et al. 1997). It may be useful to utilize MRD monitoring to evaluate the effect of this combined treatment on the maintenance of remission as compared to other therapeutic modalities.

In this study, we have applied MRD monitoring using RT-PCR for the PML/RAR α chimeric gene to the clinical courses of nine children with APL who were in remission following ATRA therapy. As post remission therapy, they received either a multidrug-combined chemotherapy regimen (alone or with bone marrow transplantation [BMT]), or alternatively a combination of chemotherapy and ATRA administration. This serial monitoring study may be useful for investigating the clinical feasibility of MRD monitoring by RT-PCR detecting PML/RAR α chimeric gene in children with APL.

MATERIALS AND METHODS

Patients and treatment

Nine children (four boys and five girls) with average age of 8.3 years (range: four to fifteen years old) were diagnosed with de novo APL according to the French-American-British classification and the disease-specific cytogenetic abnormality, t(15;17) translocation (Table 1). For remission induction, patients were successfully treated with ATRA therapy (40 to 50 mg/m²/day, administered orally) for four to six weeks. In treatment after achieving complete remission with ATRA, patients were treated either with multidrug combined chemotherapy (Uzuka et al. 1976; Kigasawa et al. 1998) (cases 1, 3, 4, 5, 7 and 8, four of whom received allogeneic bone marrow transplantation (BMT) thereafter), or with a combination of alternative chemotherapy and ATRA administration every eight weeks (Hirota et al. 1997) (cases 2, 6 and 9). The observation period for these children ranged

from 18 to 94 months after the initiation of treatment (average of 63.2 months).

Cell preparation and RNA extraction

Bone marrow aspirates were obtained from the patients at onset before treatment and, thereafter, serially during therapy or follow-up periods. Mononuclear cells were separated by a density gradient centrifugation with Ficoll-Paque (density 1.077; Pharmacia, Uppsala, Sweden). Cells were lysed in a solution containing 4 M guanidinium thiocyanate, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol and 25 mM sodium citrate (pH 7.0) (Wako Pure Chemical, Osaka), and then total RNA was extracted from the cell lysate using an acid guanidinium thiocyanate-phenol-chloroform extraction method as described (Chomczynski and Sacchi 1987).

RT-PCR

Amplification of PML/RAR α chimeric gene was performed by PCR as described (Saiki et al. 1988). Briefly, total RNA was reverse transcribed to cDNA by incubating 20 μ l of reaction mixture containing 50 mM NaCl, 20 mM Tris (pH 7.4), 8 mM MgCl₂, 500 mg/ml oligo dT, 4 mM dithiothreitol, 4 U/ml RNasin (Takara Shuzo, Osaka), 20 U of AMV-reverse transcriptase (Life Science, St. Petersburg, FL, USA). Reaction mixture (20 μ l) including 0.2 μ l of cDNA, 1 mM oligonucleotide primers, 200 mM dNTPs, 2.5 U of Taq polymerase (Takara Shuzo), 20 mM Tris (pH 8.4), 0.5 mM MgCl₂, 25 mM KCl was then applied to a Gene Amp system 2400 (Perkin Elmer Cetus, Norwalk, CT, USA). The conditions of the standard PCR were 35 cycles, 30 seconds at 94°C (denaturation), 30 seconds at 62°C (annealing) and 1 minutes at 72°C (elongation). For the second-round of PCR (nested PCR), the system is the same as for the standard procedure except that 1.0 μ l of the standard PCR products were used instead of cDNA products. PCR products (10 μ l) were separated using a 2% agarose gel

TABLE 1. *Clinical, hematological and cytogenetic profiles of the patients with APL in this study*

UPN	Age (y)/sex	FAB	Karyotype	WBC (μl^{-1}) (% blast)	Remission induction		Post-remission treatment	Relapse	Outcome
					Therapy	Response			
1	14	F	M3	t(15; 17)	2100 (64)	ATRA ^a	Yes	Chemotherapy ^b → BMT	(-) + 100M, 1CR
2	15	M	M3	t(15; 17)	2600 (62)	ATRA	Yes	Chemotherapy + ATRA ^c	(-) + 54M, 1CR
3	5	F	M3	t(15; 17) 9q-	4000 (31)	ATRA	Yes	Chemotherapy ^c	(-) + 83M, 1CR
4	4	M	M3	t(15; 17)	8400 (52)	ATRA	Yes	Chemotherapy ^d → BMT	+ 51M, 2CR
5	4	F	M3	t(15; 17)	17 600 (76)	ATRA	Yes	Chemotherapy ^c → BMT	+ 49M, 1CR
6	10	M	M3v	t(15; 17)	43 600 (90)	ATRA	Yes	Chemotherapy + ATRA ^e	BM 18M, Death
7	6	F	M3	t(15; 17)	4100 (57)	ATRA	Yes	Chemotherapy ^b → BMT	(-) + 68M, 1CR
8	10	F	M3	t(15; 17)	4200 (54)	ATRA	Yes	Chemotherapy ^c	(-) + 53M, 1CR
9	7	M	M3	t(15; 17)	15 600 (29)	ATRA	Yes	Chemotherapy + ATRA ^e	(-) + 51M, 1CR

^a, ATRA therapy (40 to 50 mg/m²/day, orally) for four to six weeks; ^b, Chemotherapy (Uzuka et al. 1976) with adriamycin (ADR), cytarabine (Ara-C), 6-mercaptopurine (6-MP), prednisolone (PSL) and L-asparaginase (L-ASP); ^c, Chemotherapy (Kigasawa et al. 1988) with Ara-C, etoposide (VP-16), tetrahydropyranil adriamycin (THP-ADR), aclarubicin (ACR) and vincristin (VCR); ^d, Chemotherapy with AraC, daunomycin, VCR, PSL and ADR; ^e, Combined therapy (Hirota et al. 1997) of ATRA and chemotherapy.

Fig. 1. Schematic diagram of PML/RAR α chimeric gene and primer designs for MRD monitoring by RT-PCR. (A) Long (L) type PML/RAR α chimeric mRNA. (B) Short (S) type PML/RAR α chimeric mRNA. Oligonucleotide primers are C (sense) and D (antisense) for the standard RT-PCR, E (sense) and G (antisense) for nested PCR of L type, and F (sense) and G (antisense) for nested PCR of S type PML/RAR α chimeric gene.

containing 1 mg/ml ethidium bromide.

Oligonucleotide primers for PCR

For the long (L) type PML/RAR α , as shown in Fig. 1, a nucleotide fragment (680 bp) was amplified by standard RT-PCR using sense oligonucleotide primer C (5'-CCGATGGCTTC GACGAGTTC-3' in exon 3 of PML) and antisense primer D (5'-CTCACAGGCGCTGAC CCCAT-3' in exon 3 of RAR α), and a fragment (151 bp) by nested RT-PCR using sense primer E (5'-AACAGCAACCACGTGGCCAG-3' in exon 6 of PML) and antisense primer G (5'-AGCCTGAGGACTTGTCCTGA-3' in exon 3 of RAR α). For the short (S) type PML/RAR α , a fragment (206 bp) was amplified by standard RT-PCR using the same primers C and D, and a fragment (163 bp) by nested RT-PCR sense primer F (5'-TTCAAGGTGCGCCTGCAGGA-3' in exon 3 of RAR α) and antisense primer G. As the intrinsic control transcript, a nucleotide fragment (226 bp) of the human glyceraldehyde-3-phosphate dehydrogenase

(GAPDH) mRNA was amplified by RT-PCR using oligonucleotide primers; 5'-GAAGGTGA AGGTCCGAGTC-3' (sense in exon 1 of GAPDH) and 5'-GAAGATGGTGATGGGAT TTC-3' (antisense in exon 3 of GAPDH).

Allele specific oligonucleotide hybridization

Allele specific oligonucleotide (ASO) hybridization was performed as described (Imazumi et al. 1998). Briefly, separated PCR products were transferred onto nylon membranes, hybridized with a 3' end-labeled oligonucleotide probe using the ECL 3'-oligolabelling and detection system (Amersham International, Buckinghamshire, UK) and exposed to autoradiography film for 30 minutes according to the manufacturer's instructions. The oligonucleotide probes for ASO hybridization were P6/R3 (5'-GGGGAGGCAGCCATTG AGAC-3') for detecting the L type fusion point between PML exon 6 and RAR α exon 3, and P3/R3 (5'-CAGGGGAAAGCCATTGAGAC-3') for the S type fusion point between PML exon

3 and RAR α exon 3. The sensitivity of this method for detecting APL cells was evaluated using APL patients' marrow with more than 90% of leukemia cells in morphology, which were serially diluted in a ten-fold manner with HL-60 cells not having the PML/RAR α chimeric gene.

DNA sequencing

PCR products were subcloned into PBS and sequenced by the Sanger dideoxynucleotide-mediated chain-termination method using a Sequenase kit (U.S. Biochemical, Cleveland, OH, USA) as described (Sanger et al. 1977).

RESULTS

Sensitivity for detection of PML/RAR α chimeric mRNA

With the fresh APL cells of case 2, the sensitivity for detecting the S type PML/RAR α mRNA was 1 leukemic cell in 10^4 cells (10^{-4}) by the standard RT-PCR and 1 in 10^5 cells (10^{-5}) by the nested RT-PCR combined with ASO hybridization, respectively (Fig. 2). In contrast, the sensitivity for detecting APL cells (case 7) having the L type PML/RAR α chimeric gene was 10^{-3} by the standard PCR and 10^{-4} by the nested RT-PCR, respectively

Fig. 2. Sensitivity of the standard or nested PCR. Sensitivity of RT-PCR was determined using patient's bone marrow cells containing more than 90% leukemia cells (case 3). Marrow cells were mixed in a ten-fold dilution manner with HL-60 cells serially from lane 1 to lane 7, and no APL cells in lane 8. Sensitivity for the S type PML/RAR α mRNA was of 1 leukemic cell in 10^4 cells (10^{-4}) by the standard PCR (A) and 1 in 10^5 cells (10^{-5}) by the nested PCR combined with ASO hybridization (B), respectively. RT-PCR detection of GAPDH mRNA was performed as an internal control (C).

(data not shown).

Detection of PML/RAR α chimeric mRNA at the onset of APL

Either a single or several major DNA fragments were amplified by the standard RT-PCR in all of the patients (Figs. 3 and 4). Two of the patients (cases 3 and 6) showed a positive hybridization for P3/R3 oligonucleotide probe,

Fig. 3. Representative result of RT-PCR detecting the L or S type of PML/RAR α . Three DNA fragments were amplified for the L type PML/RAR α (lane 1), and a single DNA fragment was amplified for the S type PML/RAR α (lane 2). No DNA fragment was amplified for HL-60 (lane 3).

and the others (cases 1, 2, 4, 5, 7, 8 and 9) were positive for P6/R3 oligonucleotide probe. By DNA sequence analysis, we confirmed that the single pattern of PCR was derived from the S type PML/RAR α chimeric mRNA, and the other pattern with several DNA fragments was from the L type chimeric mRNA with variable splicing (data not shown).

MRD monitoring during the clinical course

As shown in Fig. 5, successful hematological remission was obtained in all patients within six weeks of the start of ATRA therapy. During this time PML/RAR α chimeric transcripts remained detectable in all patients, and thereafter MRD became undetectable by the standard RT-PCR approximately six months after the start of therapy. As for post-remission therapy, six patients (cases 1, 3, 4, 5, 7 and 8) received multidrug-combined chemotherapy (Uzuka et al. 1976; Kigasawa et al. 1998). Four of these patients also received allogeneic BMT, either during the first (cases 1 and 7) or second remission following overt relapse (cases 4 and 5). For these latter two cases, MRD was detected by the standard PCR after six months of post-remission chemotherapy and clinical

Fig. 4. RT-PCR detection of PML/RAR α chimeric mRNA in nine patients with APL. (A) Electrophoresis and ethidium bromide staining of standard PCR products. (B) ASO hybridization with P6/R3 oligonucleotide probe (L type). (C) ASO hybridization with P3/R3 oligonucleotide probe (S type). Two patients (cases 3 and 6) were of S type PML/RAR α , and others (cases 1, 2, 4, 5, 7, 8 and 9) were of L type PML/RAR α . N, negative control; M, Marker.

Fig. 5. RT-PCR monitoring of nine patients with APL. Symbols on the solid lines are the results of standard PCR and ASO hybridization, and symbols on the broken lines are of nested PCR and ASO hybridization. Closed or open circles represent positive or negative detection of PML/RAR α chimeric mRNA. Closed or hatched squares represent the period of ATRA therapy or chemotherapy, respectively.

relapse occurred thereafter. After receiving BMT, no MRD was detected in any of the four patients. The three remaining patients (case 2, 6 and 9) received post-remission therapy comprised of a combination of ATRA and chemotherapy (Children's Cancer and Leukemia Study Group [CCLSG] APL protocol). Of these, two patients had MRD detected after six months of combined therapy, and one of them relapsed with acquired ATRA-resistance (case 6).

Differential MRD positivity for the standard or nested RT-PCR in patients with relapse

MRD of patients without relapse became

undetectable by the standard RT-PCR within six months from the onset of disease. By contrast, three patients who relapsed (cases 4, 5 and 6) had MRD detected by the standard RT-PCR after six months of post-remission therapy. We hypothesized that MRD detection using the standard and nested RT-PCR with different sensitivity may be useful for the prediction of impending relapse. As summarized in Fig. 5, case 4 patient showed positive MRD at the nested, but not the standard, RT-PCR level 20 months after the onset of disease and he had MRD detected again by the standard PCR one month before the overt relapse. In case 5 patient, MRD was detected nine months after

onset of APL by the nested RT-PCR when the patient was still in the state of hematological and clinical remission. The disease then progressed and exhibited positive MRD by the standard RT-PCR, followed by hematological relapse. In case 6, who relapsed with ATRA-resistance 16 months after the onset of the disease, the recurrence was so rapid that we were unable to detect MRD prior to the overt relapse, despite MRD monitoring performed in every four months.

DISCUSSION

Previous studies have reported the clinical application of RT-PCR for the monitoring of PML/RAR α minimal residual disease in APL (Miller et al. 1993; Diverio et al. 1994; Laczika et al. 1994; Mandelli et al. 1997; Diverio et al. 1998). It is generally thought that, in most patients with APL, persistent PCR positivity can predict relapse, while achievement of PCR negativity is associated with a good prognosis. Because a proportion of patients with APL ultimately relapse even with intensive chemotherapy following ATRA therapy (Diverio et al. 1998; Guglielmi et al. 1998), MRD monitoring needs to predict these patients with potential risk of relapse so as to alter the therapeutic modality before recurrence becomes overt. However, it remains inconclusive whether or not the MRD monitoring for this purpose would be clinically feasible for practical use (Diverio et al. 1998). In addition to this question, the limitations of MRD detection also need to be understood in clinical trials.

To be sensitive and unambiguous are necessary requirements for the effective application of MRD monitoring (Tobal and Liu Yin 1998). In this study the RT-PCR sensitivity for the detection of the L type chimeric gene was approximately 1 log lower than that for the S type chimeric gene. Therefore, the clinical significance of PCR positivity may not be interpreted uniformly among patients with different isoforms. ASO hybridization combined with

RT-PCR for identifying PML/RAR α chimeric fusion points was useful for avoiding ambiguity particularly for detecting the L type PML/RAR α transcripts that had multiple transcripts due to variable splicing patterns.

Apart from these properties associated with the chimeric gene, therapeutic modality can also affect the persistency of MRD as well as the prognosis of APL patients. Since ATRA therapy alone rarely leads to PCR negativity, additional chemotherapy is needed to eliminate the remaining APL cells with the PML/RAR α chimeric gene and to prolong remission (Fenaux et al. 1994; Mandelli et al. 1997; Lo Coco et al. 1998). However, a proportion of patients ultimately relapse even after multidrug-combined therapy following ATRA (Fukutani et al. 1995; Grimwade et al. 1996). Therefore, alteration of therapy may need to be considered for patients with a conversion of PCR positivity as was experienced in case 5 (Lo Coco et al. 1999), even though BMT is not currently recommended in the first clinical remission for APL.

Overall, three out of the four patients who exhibited conversion to PCR positivity after six months of maintenance therapy ultimately relapsed. In comparison MRD was undetected in those patients with a good prognosis. Therefore, this MRD monitoring study indicates that a conversion of PCR positivity after six months of maintenance therapy may be predicting a potential relapse, particularly when an increase in quantity of the PML/RAR α gene occurs. This conclusion is supported by the recent work of Diverio et al. (1998) who showed that in a prospective study of 163 APL patients, 20 of 21 patients who showed conversion to PCR positivity during remission underwent subsequent relapse. These findings indicate that frequent evaluation of any alteration in PML/RAR α gene status using PCR would be of great help in the precise prediction of impending relapse in children with APL.

Recently, the question has been raised as to whether or not chemotherapy combined with

ATRA during maintenance therapy is as effective as, or superior to, chemotherapy alone for APL. We have approached this issue in regard to MRD monitoring and in this study could not find any difference between these two therapeutic modalities in the PCR positivity for PML/RAR α . In comparison, patients receiving BMT showed continuous PCR negativity after treatment. Recently, Deverio et al. (1998) have also shown that there is no difference in PCR positivity among 165 patients who were randomized into maintenance chemotherapy with or without ATRA administration. More recently, Hirota et al. (1999) reported that 25 patients treated with CCLSG APL-ATRA protocol showed 67% event-free survival at 42 months from onset, while relapse tended to occur after one year of therapy. Further investigations utilizing MRD monitoring are needed in order to obtain a definitive conclusion.

The clinical significance of MRD cannot be interpreted precisely without a highly sensitive, quantitative assessment. Although this study used semi-quantitative differential detection of the PML/RAR α gene by the standard or nested RT-PCR, more precise evaluation of the quantity of PCR products may be needed for this purpose (Mensink et al. 1998). For two of the three patients who relapsed, MRD monitoring with differential assessment was predictive of impending relapse. However, for the other patient who relapsed rapidly, MRD monitoring performed every four months failed to detect PCR positivity before relapse. From a prospective study of 163 APL patients with MRD monitoring, it has been reported that patients underwent hematological relapse after an average of three months (range: 1 to 14 months) from the first conversion to PCR positivity. This diversity in periods of MRD progression is one of the characteristics of APL, which may limit the clinical feasibility of MRD monitoring.

In conclusion, this study suggests that further precautionary treatment may be

required in those patients at risk of impending relapse as shown by PCR positivity after six months of maintenance therapy (with or without combined ATRA therapy). It is difficult to draw a definite conclusion from this study because of a retrospective study analyzing a small number of patients with heterogeneous treatments. However, this study implicates some practical aspects that may be useful to design MRD monitoring for APL in children, and it is suggested that MRD monitoring using RT-PCR detection of PML/RAR α may be feasible for predicting patients with APL who are at risk of impending relapse.

Acknowledgments

The authors are grateful to Dr. Ian Ponting for his careful reading of paper. This work was supported by grants from the Ministry of Education, Science, Sports and Culture, and the Ministry of Health, Sports and Public Welfare, Japan.

References

- Benedetti, L., Levin, A.A., Scicchitano, B.M., Grignani, F., Allenby, G., Diverio, D., Lo Coco, F., Avvisati, G., Ruthardt, M., Adamo, S., Pelicci, P.G. & Nervi, C. (1997) Characterization of the retinoid binding properties of the major fusion products present in acute promyelocytic leukemia cells. *Blood*, **90**, 1175-1185.
- Breitman, T.R., Selonick, S.E. & Collins, S.J. (1980) Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. *Proc. Natl. Acad. Sci. USA*, **77**, 2936-2940.
- Campana, D. & Pui, C.H. (1995) Detection of minimal residual disease in acute leukemia: methodologic advances and clinical significance. *Blood*, **85**, 1416-1434.
- Castaigne, S., Chomienne, C., Daniel, M.T., Ballerini, P., Berger, R., Fenaux, P. & Degos, L. (1990) All-*trans* retinoic acid as a differentiation therapy for acute promyelocytic leukemia. I. Clinical results. *Blood*, **76**, 1704-1709.
- Chen, Z.X., Xue, Y.Q., Zhang, R., Tao, R.F., Xia, X.

- M., Li, C., Wang, W., Zu, W.Y., Yao, X.Z. & Ling, B.J. (1991) A clinical and experimental study on all-*trans* retinoic acid-treated acute promyelocytic leukemia patients. *Blood*, **78**, 1413-1419.
- Chomczynski, P. & Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162**, 156-159.
- Diverio, D., Pandolfi, P.P., Rossi, V., Biondi, A., Pelicci, P.G. & Lo Coco, F. (1994) Monitoring of treatment outcome in acute promyelocytic leukemia by RT-PCR. *Leukemia*, **8**, 1105-1107.
- Diverio, D., Rossi, V., Avvisati, G., De Santis, S., Pistilli, A., Pane, F., Saglio, G., Martinelli, G., Petti, M.C., Santoro, A., Pelicci, P.G., Mandelli, F., Biondi, A. & Lo Coco, F.L. (1998) Early detection of relapse by prospective reverse transcriptase-polymerase chain reaction analysis of the PML/RAR α fusion gene in patients with acute promyelocytic leukemia enrolled in the GIMEMA-AIEOP multicenter "AIDA" trial. *Blood*, **92**, 784-789.
- Fenaux, P., Chastang, C. & Degos, L. (1994) Treatment of newly diagnosed acute promyelocytic leukemia (APL) by a combination of all-*trans* retinoic acid (ATRA) and chemotherapy. French APL Group. *Leukemia*, **8**, S42-S47.
- Fukutani, H., Naoe, T., Ohno, R., Yoshida, H., Miyawaki, S., Shimazaki, C., Miyake, T., Nakayama, Y., Kobayashi, H., Goto, S., Takeshita, A., Kobayashi, S., Kato, Y., Shiraiishi, K., Sasada, M., Ohtake, S., Murakami, H., Kobayashi, M., Endo, N., Shindo, H., Matsushita, K., Hasegawa, S., Tsuji, K., Ueda, Y., Tominaga, N., Furuya, H., Inoue, Y., Takeuchi, J., Morishita, H., Iida, H. & the Leukemia Study Group of the Ministry of Health and Welfare (Kohseisho) (1995) Isoforms of PML-retinoic acid receptor alpha fused transcripts affect neither clinical features of acute promyelocytic leukemia nor prognosis after treatment with all-*trans* retinoic acid. *Leukemia*, **9**, 1478-1482.
- Grimwade, D., Howe, K., Langabeer, S., Burnett, A., Goldstone, A. & Solomon, E. (1996) Minimal residual disease detection in acute promyelocytic leukemia by reverse-transcriptase PCR: evaluation of PML-RAR alpha and RAR alpha-PML assessment in patients who ultimately relapse. *Leukemia*, **10**, 61-66.
- Guglielmi, C., Martelli, M.P., Diverio, D., Fenu, S., Vegna, M.L., Cantu-Rajoldi, A., Biondi, A., Cocito, M.G., Del Vecchio, L., Tabilio, A., Avvisati, G., Basso, G. & Lo Coco, F. (1998) Immunophenotype of adult and childhood acute promyelocytic leukaemia: correlation with morphology, type of PML gene breakpoint and clinical outcome. A cooperative Italian study on 196 cases. *Br. J. Haematol.*, **102**, 1035-1041.
- Hirota, T., Fujimoto, T., Katano, N., Tsurusawa, M., Eguchi, H., Nakadate, N., Kamitamari, A., Kawakami, K., Nishikawa, K., Asami, K., Shirahata, A., Koizumi, S., Mugishima, H., Gushiken, T., Hyakuna, N., Imaizumi, M., Komatu, K., Takitani, K., Miyake, M. & Yanase, T. (1997) II Treatment results of intermittent and cyclic regimen with ATRA and chemotherapy in childhood acute promyelocytic leukemia. *Jpn. J. Clin. Hematol.*, **38**, 1177-1182. (in Japanese with English abstract)
- Hirota, T., Fujimoto, T., Katano, N., Tsurusawa, M., Hatae, Y., Kamitamari, A., Nishikawa, K., Asami, K., Yanase, T., Koizumi, S., Mugishima, H., Hyakuna, N., Imaizumi, M., Watanabe, A., Takitani, K. & Miyake, M. (1999) Treatment results of CCLSG APL-ATRA protocol in childhood acute promyelocytic leukemia (abstract). *Jpn. J. Clin. Hematol.*, **40**, 1006. (in Japanese)
- Huang, M.E., Ye, Y.C., Chen, S.R., Chai, J.R., Lu, J.X., Zhao, L., Gu, L.J. & Wang, Z.Y. (1988) Use of all-*trans* retinoic acid in the treatment of acute promyelocytic leukemia. *Blood*, **72**, 567-572.
- Imaizumi, M. & Breitman, T.R. (1987) Retinoic acid-induced differentiation of the human promyelocytic leukemia cell line, HL-60, and fresh human leukemia cells in primary culture: a model for differentiation inducing therapy of leukemia. *Eur. J. Haematol.*, **38**, 289-302.
- Imaizumi, M., Suzuki, H., Yoshinari, M., Sato, A., Saito, T., Sugawara, A., Tsuchiya, S., Hatae, Y., Fujimoto, T., Kakizuka, A., Konno, T. & Inuma, K. (1998) Mutations in the E-domain of RAR α portion of the PML/RAR α chimeric gene may confer clinical resistance to all-*trans* retinoic acid in acute promyelocytic leukemia. *Blood*, **92**, 374-382.

- Jurlander, J., Caligiuri, M.A., Ruutu, T., Baer, M.R., Strout, M.P., Oberkircher, A.R., Hoffmann, L., Ball, E.D., Frei-Lahr, D.A., Christiansen, N.P., Block, A.M., Knuutila, S., Herzig, G.P. & Bloomfield, C.D. (1996) Persistence of the AML1/ETO fusion transcript in patients treated with allogeneic bone marrow transplantation for t(8;21) leukemia. *Blood*, **88**, 2183-2191.
- Kigasawa, H., Akiyama, Y., Ohira, M., Tukimoto, I. & Nagao, T. (1998) Prospective comparison between postinduction chemotherapy and various types of stem-cell transplantations for childhood acute myeloid leukemia using Japan Cooperative Protocol ANLL91 (abstract). *Med. Pediatr. Oncol.*, **31**, 225.
- Laczika, K., Mitterbauer, G., Korninger, L., Knobl, P., Schwarzhinger, I., Kapiotis, S., Haas, O.A., Kyrle, P.A., Pont, J., Oehler, L., Purscher, B., Thalhammer, F., Lechner, K. & Jaeger, U. (1994) Rapid achievement of PML-RAR alpha polymerase chain reaction (PCR)-negativity by combined treatment with all-*trans*-retinoic acid and chemotherapy in acute promyelocytic leukemia: a pilot study. *Leukemia*, **8**, 1-5.
- Lo Coco, F., Nervi, C., Avvisati, G. & Mandelli, F. (1998) Acute promyelocytic leukemia: a curable disease. *Leukemia*, **12**, 1866-1880.
- Lo Coco, F., Diverio, D., Avvisati, G., Petti, M.C., Meloni, G., Pogliani, E.M., Biondi, A., Rossi, G., Carlo-Stella, C., Selleri, C., Martino, B., Specchia, G. & Mandelli, F. (1999) Therapy of molecular relapse in acute promyelocytic leukemia. *Blood*, **94**, 2225-2229.
- Mandelli, F., Diverio, D., Avvisati, G., Luciano, A., Barbui, T., Bernasconi, C., Brocchia, G., Cerri, R., Falda, M., Fioritoni, G., Leoni, F., Liso, V., Petti, M.C., Rodeghiero, F., Saglio, G., Vegna, M.L., Visani, G., Jehn, U., Willemze, R., Muus, P., Pelicci, P.G., Biondi, A. & Lo Coco, F. (1997) Molecular remission in PML/RAR alpha-positive acute promyelocytic leukemia by combined all-*trans* retinoic acid and idarubicin (AIDA) therapy. Gruppo Italiano-Malattie Ematologiche Maligne dell'Adulto and Associazione Italiana di Ematologia ed Oncologia Pediatrica Cooperative Groups. *Blood*, **90**, 1014-1021.
- Mensink, E., van de Locht, A., Schattenberg, A., Linders, E., Schaap, N., Geurts van Kessel, A. & De Witte, T. (1998) Quantitation of minimal residual disease in Philadelphia chromosome positive chronic myeloid leukaemia patients using real-time quantitative RT-PCR. *Br. J. Haematol.*, **102**, 768-774.
- Miller, W.H., Jr., Levine, K., DeBlasio, A., Frankel, S.R., Dmitrovsky, E. & Warrell, R.P., Jr. (1993) Detection of minimal residual disease in acute promyelocytic leukemia by a reverse transcription polymerase chain reaction assay for the PML/RAR-alpha fusion mRNA. *Blood*, **82**, 1689-1694.
- Miyamura, K., Tanimoto, M., Morishima, Y., Horibe, K., Yamamoto, K., Akatsuka, M., Kodera, Y., Kojima, S., Matsuyama, K., Hirabayashi, N., Yazaki, M., Imai, K., Onozawa, Y., Kanamaru, A., Mizutani, S. & Saito, H. (1992) Detection of Philadelphia chromosome-positive acute lymphoblastic leukemia by polymerase chain reaction: possible eradication of minimal residual disease by marrow transplantation. *Blood*, **79**, 1366-1370.
- Radich, J., Gehly, G., Lee, A., Avery, R., Bryant, E., Edmands, S., Gooley, T., Kessler, P., Kirk, J., Ladne, P., Thomas, E.D. & Appelbaum, F.R. (1997) Detection of bcr-abl transcripts in Philadelphia chromosome-positive acute lymphoblastic leukemia after marrow transplantation. *Blood*, **89**, 2602-2609.
- Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. & Erlich, H.A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, **239**, 487-491.
- Sanger, F., Nicklen, S. & Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA*, **74**, 5463-5467.
- Satake, N., Maseki, N., Kozu, T., Sakashita, A., Kobayashi, H., Sakurai, M., Ohki, M. & Kaneko, Y. (1995) Disappearance of AML1-MTG8 (ETO) fusion transcript in acute myeloid leukaemia patients with t(8;21) in long-term remission. *Br. J. Haematol.*, **91**, 892-898.
- Tobal, K. & Liu Yin, J.A. (1998) RT-PCR method with increased sensitivity shows persistence of PML-RARA fusion transcripts in patients in long-term remission of APL. *Leukemia*, **12**, 1349-1354.
- Uzuka, Y., Liong, S. K. & Yamagata, S. (1976) Treatment of adult acute non-lymphoblastic leukemia using intermittent combination

- chemotherapy with daunomycin, cytosine arabinoside, 6-mercaptopurine and prednisolone-DCMP two step therapy. *Tohoku J. Exp. Med.*, **118**, 217-225.
- Vahdat, L., Maslak, P., Miller, W.H., Jr., Eardley, A., Heller, G., Scheinberg, D.A. & Warrell, R.P., Jr. (1994) Early mortality and the retinoic acid syndrome in acute promyelocytic leukemia: impact of leukocytosis, low-dose chemotherapy, PML/RAR-alpha isoform, and CD13 expression in patients treated with all-*trans* retinoic acid. *Blood*, **84**, 3843-3849.
- Warrell, R.P., Jr., Frankel, S.R., Miller, W.H., Jr., Scheinberg, D.A., Itri, L.M., Hittelman, W.N., Vyas, R., Andreeff, M., Tafuri, A., Jakubowski, A., Gabrilove, J., Gordon, M.S. & Dmitrovsky, E. (1991) Differentiation therapy of acute promyelocytic leukemia with tretinoin (all-*trans*-retinoic acid). *N. Engl. J. Med.*, **324**, 1385-1393.
- Warrell, R.P., Jr. (1993) Retinoid resistance in acute promyelocytic leukemia: new mechanisms, strategies, and implications. *Blood*, **82**, 1949-1953.
-