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Abnormalities of *p51*, *p53*, *FLT3* and *N-ras* Genes and Their Prognostic Value in Relapsed Acute Myeloid Leukemia

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Abstract

To clarify the role of the genetic mutations in the clinical course of acute myeloid leukemias (AML), we analyzed for *p51*, *p53*, *FLT3* and *N-ras* gene mutations and the expression of the *p51* gene in relapsed AML. Paired samples obtained from patients with AML at both stages of diagnosis and first relapse were analyzed. Twenty-four patients with relapsed AML survived for 6 to 81 months (median 24 months) from diagnosis. In one patient, no point mutation of the *p51* gene was detected, but loss of the *p51* gene expression was observed at both stages. Point mutations of the *p53* gene were positive at both stages (+/+) in two patients and negative at diagnosis and positive at relapse (-/+) in two patients. Tandem duplication of the *FLT3* gene was detected in five patients at both stages (+/+). *N-ras* gene mutations at both stages (+/+) were detected in three patients. Mutant *p53* at relapse was associated with short survival in patients with relapsed AML ($P < 0.014$). Our findings show that *p53* mutations were, at least in part, associated with the mechanism of relapse in AML, while new *p51*, *FLT3* and *N-ras* gene alterations did not occur at relapse. Loss of the *p51* gene expression in *de novo* AML has not been reported yet.

(J Nippon Med Sch 2004; 71: 270–278)

Key words: gene mutation, acute myeloid leukemia, relapse, prognosis

Introduction

Although there has been progress in the therapeutic strategies for leukemia, for many patients acute myeloid leukemia (AML) is fatal. Recently, chemotherapy achieves a high complete remission (CR) rate, but the survival rate among patients who are less than 65 years of age is only 40%¹. Approximately half of patients who achieve CR eventually relapse. Generally, relapsed leukemia is resistant to chemotherapy and is associated with short survival². A number of molecular diagnostic

assays, including cytogenetics, fluorescence in situ hybridization and polymerase chain reaction-based assays, have been applied to the analysis of leukemic relapse. Although they have shown that relapse of AML is accompanied by karyotype changes³ and genetic alterations such as of *p53*⁴ and *FLT3*⁵, the biologic mechanisms behind the evolution of leukemic blasts from the time of initial diagnosis to relapse remain unclear.

Mutations or deletions of tumor suppressor genes relating to cell-cycle regulators such as *p53* and its family genes are found in leukemic cells. It is generally accepted that loss of the function of tumor

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suppressor genes is associated with tumorigenesis. The *p51* gene, one of the *p53* family genes, expresses at least six major subtypes. One of them, *p51A/TAp63γ*, is considered to be a tumor suppressor gene⁶. Mutations of the *p51* gene occur especially as hematopoietic neoplasms become more malignant, such as at the time of blast crisis of chronic myeloid leukemia (CML)⁷. In AML, mutations of the *p53* gene were detected in 5~10% of patients⁸, which is less frequent than the rate of more than 50%⁹ *p53* mutations seen in non-hematological malignancies. Mutations of the *p53* gene are significantly associated with a poor prognosis and resistance to chemotherapy^{9,10}. *FLT3* is expressed by early hematopoietic progenitors and induces a growth control signal in normal hematopoiesis¹¹. Mutations of the *FLT3* gene were reported in 25% of adult AML, and the mutated gene usually presented internal tandem duplication (ITD) of the juxtamembrane domain-coding sequence^{12,13}. The mutant *FLT3* gene with ITD (*FLT3*-ITD) was associated with significant shortening of relapse-free and overall survival in AML¹⁴. Mutations of *N-ras* at codons 12, 13, and 61 result in activation of the *N-ras* oncogene. Mutant *N-ras* is one of the most commonly observed genetic aberrations in AML, with the reported incidence ranging from 20 to 30%¹⁵. However, its prognostic significance is a matter of controversy^{15,16}.

The associations of these four genes with relapse of AML remain unclear. Recent studies show that a molecular multi-step process, including activation of oncogenes and inactivation of tumor suppressor genes, contributes to oncogenesis^{17,18}. In this study we examined alterations of the four genes in paired AML samples obtained at initial diagnosis and at relapse, and investigated their prognostic value.

Materials and Methods

Patients and Leukemia Cells

After obtaining written informed consent for each examination, we analyzed 48 bone marrow (BM) samples obtained from 24 patients with *de novo* AML at initial diagnosis and at relapse. AML was diagnosed according to the French-American-British

(FAB) classification. The 24 patients, comprised of 12 men and 12 women, were diagnosed from 1993 to 1999. The mean age was 54 years (range, 34~78). The patients were treated with a protocol consisting of N⁴-behenoyl-1-beta-D-arabinofuranosyl-cytosine (BHAC), daunorubicin, 6-mercaptopurine and prednisolone (BHAC-DMP regimen)¹⁹. Patients with M3 subtype were treated with all-trans retinoic acid. After achieving CR, all patients received two courses of consolidation chemotherapy followed by six courses of intensification chemotherapy. CR was defined as <5% blasts in normo-cellular bone marrow with normal peripheral neutrophil and platelet counts. The CR duration was defined as the time interval from the date of CR to relapse. The duration of follow-up ranged from 6 to 81 months (median, 19 months).

Cytogenetic Analysis

Cytogenetic analysis was performed after culturing the cells for 24~48 hours. Twenty metaphase cells of BM were analyzed after Trypsin-Giemsa banding²⁰.

Extraction of RNA and DNA

Mononuclear cells (MNCs) were obtained by Ficoll-Hypaque centrifugation (Lymphoprep, Neegard, Norway). The total RNA of BM-MNCs was extracted with an RNazol kit (Biotex Laboratories, Inc., Houston, TX, USA), using a modification of the technique described previously¹⁹. Total cellular DNA was extracted from BM-MNCs by protease K digestion, phenol-chloroform extraction and ethanol precipitation²⁰.

Reverse-transcription Polymerase Chain Reaction Single-strand Conformation Polymorphism (RT-PCR-SSCP) of *p51* gene

cDNA was prepared from 0.5 μg of total RNA using a random hexamer primer according to our original protocol¹⁹. Then the synthesized cDNA was subjected to PCR-SSCP analysis to search for mutations of the *p51* gene. To prepare primers for analysis we modified eight sets of primers designed by Sunahara *et al.*²¹, covering the entire coding regions of both *p51A/TAp63γ* and *p51B/TA p63α*.

Each fragment was amplified using the following primer pairs: *p51A* 1F 5'-AAAGAAAGTTATTACCGATC-3' and *p51A* 1R 5'-CGCGTGGTCTGTGTTA-TAGG-3'; *p51A* 2F 5'-CATGGACCAGCAGATTCA-GA-3' and *p51A* 2R 5'-CATCACCTTGATCTGGAT-G-3'; *p51A* 3F 5'-CCACCTGGACGTATTCCACT-3' and *p51A* 3R 5'-TGGCTCATAAGGTACCAGCA-3'; *p51A* 4F 5'-GATCCCATCACAGGAAGAC-3' and *p51A* 4R 5'-TCTGATGCTATCTTCATCCG-3'; *p51A* 5F 5'-ATGAACCGCCGTCCAATT-3' and *p51A* 5R 5'-GTGCTGAGGAAGGTACTG-3'; *p51A* 6F 5'-GGC-CGTGAGACTTATGAAAT-3' and *p51A* 6R 5'-CAC-TTAAAATATAGAGATAGGGC-3'; *p51B* 7F 5'-AC-CAGCACTTACTTCAGAAA-3' and *p51B* 7R 5'-CA-TGGAGTAATGCTCAATCT-3'; and *p51B* 8F 5'-A-TCATGTCTGGACTATTTCA-3' and *p51B* 8R 5'-A-GGAAGAGCTCACATGGTGA-3'. PCR for six sets of *p51A* was performed as follows: preheating at 96°C for 3 min, followed by 35 cycles of 96°C for 30 sec, 53°C for 30 sec and 72°C for 1 min, with a final extension at 72°C for 5 min. The PCR products were denatured at 98°C for 5 min. PCR for two sets of *p51B* was performed as follows: preheating at 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 5 min. The PCR products were denatured at 94°C for 5 min.

SSCP analysis was performed according to the method of Orita *et al*²². The 5'-ends of primers (100 pmol) were labeled with [γ -³²P] (3,000 Ci/mmol, NEN) and polynucleotide kinase (5 U, Boehringer-Mannheim) in 10 μ l of 50 mM Tris-HCl, pH 8.3, 10 mM MgCl₂ and 5 mM DTT at 37°C for 30 min. The PCR mixture contained 10 pmol of each of the labeled primers, 2 nmol each of four deoxynucleotides, 0.1 μ g of sample cDNA or DNA, and 0.25 U of *Taq* polymerase in 10 μ l of the buffer specified in the GeneAmp kit. The PCR products were mixed with 10 volumes of a loading buffer containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% Xylene cyanol, denatured at 94°C or 96°C for 5 min, quenched on ice and applied (1 μ l/lane) to a 10% polyacrylamide gel containing 90 mM Tris-borate, pH 8.3, 4 mM EDTA and 10% glycerol. Electrophoresis was performed at 40 W for 3 h at 18°C with cooling using

a water jacket. The gel was dried on a filter paper and exposed to X-ray film at -80°C for 1~24 h with an intensifying screen.

PCR-SSCP of *p53* gene

Mutations of the *p53* gene were detected by our standard protocol, as described previously⁷. DNA was subjected to PCR-SSCP analysis to search for mutations in exons 5~8 of the *p53* gene. Primer sets for amplification of four exons of the *p53* gene were designed: exon 5 5F 5'-TTCTCTTCCTACAGTACTCC-3' and 5R 5'-GCCCCAGCTGCTCACCATCGC-3'; exon 6 6F 5'-CACTGATTGCTCTTAGGTCTG-3' and 6R 5'-AGTTGCAAACCAGACCTCAGG-3'; exon 7 7F 5'-CCAAGGCGCACTGGCCTCATC-3' and 7R 5'-TCAGCGGCAAGCAGAGGCTGG-3'; and exon 8 8F 5'-CCTATCCTGAGTAGTGTAAT-3' and 8R 5'-GTCCTGCTTGCTTACCTCGCT-3'. PCR was performed as follows: exon 5, 6 and 8 for 35 cycles at 94°C for 30 sec, 60°C for 1 min, and 72°C for 1 min; and exon 7 for 35 cycles at 94°C for 30 sec, 70°C for 1 min, and 72°C for 1 min. The PCR products were denatured at 80°C for 5 min. SSCP analysis was performed as described above.

PCR of *FLT3* gene

The PCR was performed according to the procedure of Kiyoi *et al*²³. After genomic PCR for internal tandem duplication (TD) and aspartic acid at codon 835 (Asp 835) as described previously¹⁹, the PCR products were visualized directly in ethidium bromide-stained agarose gels and photographed.

PCR-SSCP of *N-ras* gene

Mutations of the *N-ras* gene were detected by our standard protocol as described previously²⁴.

Sequence Analysis of *p51*, *p53* and *N-ras* genes

After the RT-PCR products were separated on 2% agarose gels and stained with ethidium bromide, the amplified fragment was excised from the gel, electroeluted, purified with phenol and precipitated with ethanol. The fragments were subcloned into the EcoRV site of the pGEM-5 Zf (+/-) cloning vector. The transfected cells were plated onto Luria-Beriani (LB)-ampicillin agar plates containing 5-

bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal), isotransferred to fresh LB-ampicillin agar plates containing X-Gal and isopropylthio- β -D-galactoside, and cultured overnight for secondary selection. White colonies were transferred into 150 μ l of LB medium containing ampicillin at 100 μ g/ml and cultured at 37°C for 4 h. The cultures were sedimented by centrifugation, resuspended in 20 μ l of water and heated at 98°C for 10 min. After centrifugation, the supernatants were amplified by PCR using T7 or SP6 primers. Three to five independent PCR clones were sequenced using a Model 377 ABI sequencer with dye terminators (Perkin Elmer, Warrington, U.K.). All sequences were confirmed in both orientations. The *p51*, *p53* and *N-ras* genes were defined as having a mutation when two or more clones from the independent PCR products showed the same abnormality of the base sequence.

Statistical Methods

Statistical analyses were performed with StatView (Brain Power Inc., Calabasas, CA) software package. Comparisons of groups were analyzed using the Mann-Whitney test or Fisher's exact test when appropriate. Survival probabilities were estimated by the Kaplan-Meier method, and differences in the survival distributions between the mutation-positive and -negative groups were evaluated by the log-rank test. For these analyses, the *P* values were two-tailed, and a *P* value of <0.05 was considered to indicate statistical significance.

Results

Detection of Mutations of the *p51*, *p53*, *FLT3* and *N-ras* genes

The results of the mutation analyses are summarized in **Table 1**. While point mutations of the *p51* gene were not observed in 21 patients, loss of *p51* gene expression was observed at both stages in one patient (Patient 11). Mutations of the *p53* gene were found in the region from codon 248 to 282, where the C terminal of the DNA binding domain is located. Among 23 examined patients, point mutations of the *p53* gene were positive at

both stages (+/+) in two patients (Patients 13 and 15), and negative at initial diagnosis and positive at relapse (-/+) in two patients (Patients 8 and 14). Deletions of the *p53* gene were observed at both stages in two patients (Patient 3 and 8). Neither mutation nor deletion of the *p53* was observed in these patients during remission (data not shown). *FLT3-ITD* was detected in five patients (Patients 3, 5, 6, 9 and 10) at both stages (+/+). Mutations of the *N-ras* gene were found in one patient (Patient 21) at codon 12 and three patients (Patients 2, 13 and 22) at codon 13. No mutation was detected at codon 61. The *N-ras* gene was mutated in both stages (+/+) in three patients (Patients 2, 21 and 22), positive at least at relapse (ND/+) in one patient (Patient 13), and negative in both stages (-/-) in the remaining 19 patients. Differences in gene mutation between at diagnosis and at relapse were observed in only two cases (Patients 8 and 14) showing mutations of the *p53* gene (-/+).

Karyotype Analysis

The karyotype was analyzed at diagnosis and relapse. Among 20 evaluable patients, seven (35%) patients showed karyotype changes between diagnosis and relapse (**Table 2**). In Patients 9, 11 and 13, a normal karyotype at diagnosis changed to abnormal at relapse. Patients 18, 20 and 21 showed an abnormal karyotype at diagnosis and had become more complex at relapse. Clonally unrelated karyotypes, which were completely different abnormalities at the two stages, were found only in Patient 23. Loss of the chromosomal translocation was observed at relapse stage in Patient 23.

Clinical Significance of the Gene Mutations

We analyzed the clinical significance of the *p53*, *FLT3* and *N-ras* gene mutations by the Kaplan-Meier method (**Fig. 1**). Mutation (+) was defined as the detection of gene mutation at anytime during the clinical course. Twenty-four patients with relapsed AML were studied, and their duration of survival from diagnosis ranged from 6 to 81 months (median 24 months). The *N-ras* gene mutation was unrelated to the survival duration from initial diagnosis (*P* = 0.99) (**Fig. 1-C**). The *FLT3* mutation had borderline

Table 1 Summary of 24 patients with relapsed AML

No	Age/Sex	FAB	<i>p53</i> D/R	<i>p51</i> D/R	<i>FLT3</i> D/R	<i>N-ras</i> D/R	Survival (months)	<i>p53</i> mutation	<i>N-ras</i> mutation
1	68/M	M0	- / -	- / -	- / -	- / -	12		
2	56/M	M1	- / -	ND/ND	ND/ND	+ / +	17 +		codon 13, GGT → AGT
3	55/M	M1	del/del	- / -	+ / +	- / -	7		
4	58/M	M1	- / -	- / -	- / -	- / -	12		
5	63/M	M1	- / -	- / -	+ / +	- / -	15		
6	45/F	M1	- / -	- / -	+ / +	- / -	21		
7	56/M	M2	- / -	- / -	- / -	- / -	24 +		
8	60/F	M2	del/mut + del	- / -	- / -	- / -	24	codon 282, CGG → GGG	
9	43/F	M2	- / -	- / -	+ / +	- / -	24		
10	61/M	M2	- / -	- / -	+ / +	- / -	7		
11	61/F	M2	- / -	LE/LE	- / -	- / -	37		
12	40/F	M2	- / -	- / -	- / -	- / -	27		
13	46/M	M2	+ / +	- / -	- / -	ND/ +	9	codon 248, CGG → TGG	codon 13, GGT → CGT
14	35/F	M2	- / +	ND/ND	ND/ND	- / -	10	codon 272, GTG → ATG	
15	64/F	M2	+ / +	ND/ND	ND/ND	- / -	11	codon 277, TGT → TAT	
16	70/M	M3	- / -	- / -	- / -	- / -	13		
17	60/M	M3	- / -	- / -	- / -	- / -	38		
18	78/F	M3	- / -	- / -	- / -	- / -	24		
19	57/M	M3	- / -	- / -	- / -	- / -	58		
20	34/F	M3	- / -	- / -	- / -	ND/ND	81 +		
21	37/M	M4	- / -	- / -	- / -	+ / +	34		codon 12, GGT → GAT
22	45/F	M4	ND/ND	- / -	- / -	+ / +	15		codon 13, GGT → GAT
23	61/F	M5	- / -	- / -	- / -	- / -	6		
24	43/F	M5	- / -	- / -	- / -	- / -	23		

D, at diagnosis; R, at relapse; +, mutation positive; -, mutation negative; del, deletion; ND, not done; LE, loss of expression

Table 2 Karyotype changes at diagnosis and relapse

Pt. No.	At diagnosis	At relapse
9	46, XX	46, XX, t(1; 11) (p34~36; p11), t(2; 8) (p11; p23), inv(3) (p25; q12), t(7; 12) (q11; p13)
11	46, XX	46, XX, del(3) (q12; q21), del(9) (q22)
13	46, XY	45, XY, -17
18	46, XX, t(15; 17) (q22; q11~12)	46, XX, der(15)t(15; 17) (q22; q11~12)ider(17)t(15; 17)
20	46, XX, t(15; 17) (q22; q11~12)	46, XX, -6, t(15; 17) (q22; q11~12), +8
21	48, XY, +8, inv(16) (q13q22), +22	48, Y, t(X; 13) (q13; q12), +8, inv(16) (q13q22), del(17) (q12), +22
23	del(17) (q12), +22	46, XX

significance ($P=0.06$) (Fig. 1-B). On the other hand, *p53* mutations or deletions were related to short survival from initial diagnosis ($P<0.014$) (Fig. 1-A). Because factors other than *p53* mutations or deletions might influence survival, we compared the characteristics of patients with the wild type *p53* gene to those with a mutant or deleted *p53* gene (Table 3). The age, WBC count at initial diagnosis, number of remission induction regimens and the karyotype at initial diagnosis were similar in both groups.

Discussion

In the context of the leukemogenesis of AML, a number of oncogenes and tumor suppressor genes have been studied. Mutations of *p53*, *FLT3* and *N-ras* have been well-studied in leukemias. While *p53* and *FLT3* gene mutations are associated with a poor prognosis^{9,10,14}, the prognostic significance of *N-ras* gene mutations remains unclear^{12,16}. We analyzed these three genes and the *p51* gene in patients with relapsed AML. The present study found that: (1) loss of *p51* gene expression occurred during the disease course of one patient, (2) mutations or deletions of the *p53* gene were associated with short survival in relapsed AML, (3) mutations of the *p51*, *FLT3* and *N-ras* genes at onset were unchanged at relapse of AML and (4) in two patients, acquisition of a *p53* gene mutation was detected at relapse.

p53 mutations predominate in relapsing or advanced hematological malignancies. This is particularly obvious in CML, low-grade lymphoma, CLL and T-ALL. In AML, the rate of *p53* mutations was reported to be the same at diagnosis and

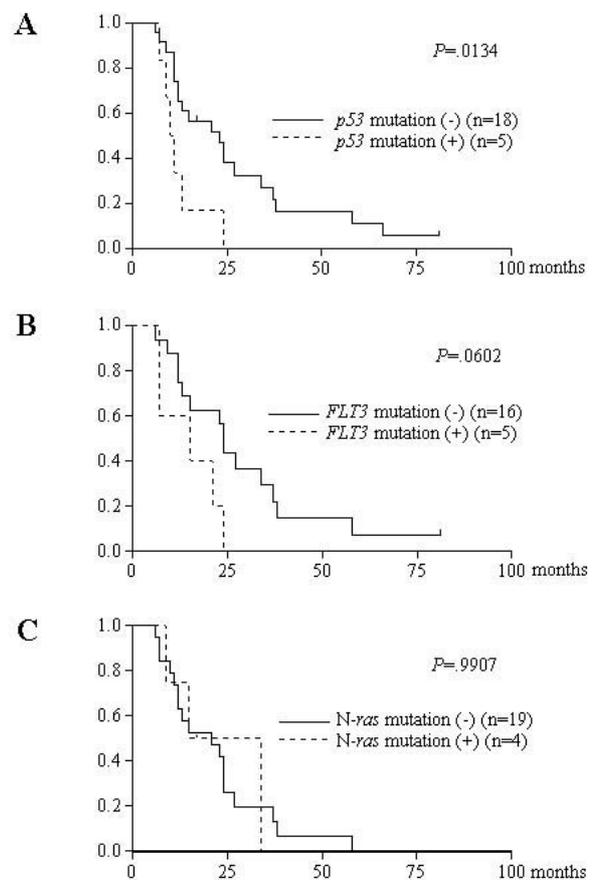


Fig. 1 Overall survival in patients with relapsed AML according to the (A) *p53* gene status, (B) *FLT3* gene status and (C) *N-ras* gene status. Mutation (+) was defined as the detection of the gene mutation at diagnosis and/or relapse. Statistical significance was evaluated by the log-rank test.

relapse⁹. To study the mechanism of leukemic relapse, comparative analyses of samples between at diagnosis and at relapse have been performed³⁻⁵. These studies showed frequent clonal changes, indicating that relapse is not merely a regrowth of

Table 3 Clinical characteristics of the patients with or without p53 mutation

	p53		P value
	normal (n = 18)	abnormal (n = 5)	
Age at initial diagnosis			.6280
mean	55.1	52	
(range)	(34 ~ 78)	(35 ~ 64)	
WBC at initial diagnosis			.9406
mean	14,288	18,782	
(range)	(600 ~ 179,300)	(2,600 ~ 50,100)	
Number of remission induction regimens			> .9999
1	14 (77.8%)	4 (80.0%)	
2 or more	4 (22.2%)	1 (20.0%)	
Chromosome			.9703
favorable	4 (22.2%)	0	
intermediate	7 (38.9%)	4 (80.0%)	
unfavorable	7 (38.9%)	1 (20.0%)	

the initial leukemic blast. Genetic alterations detected at relapse indicate additional changes in the initial clone or appearance of a completely new clone. The first case is considered a clonal shift, while the second is considered to be a secondary leukemia. In our study, the acquisitions of a *p53* mutation at relapse in two cases appear to have been clonal shifts, in view of the clinical courses. We found that *p53* mutations were, at least in part, associated with the mechanism of relapse of AML, while *p51*, *N-ras* and *FLT3* gene alterations contributed to leukemogenesis only in the early stage. These findings suggest that *p53* gene mutations might affect the leukemic development from the initiation of AML to relapse. However, further studies will be needed to confirm these findings.

In CML, mutations of the *p51* gene were observed in 11.8% of patients in blast crisis and in 1.5% of chronic-phase patients, which suggest that *p51* gene mutations may act as a genetic alteration responsible for the progression of CML⁷. In our present study, while point mutation of the *p51* gene was not observed in AML, loss of *p51* gene expression was observed at both stages in one patient. Yamaguchi *et al.* showed that the *p51* gene was not detected in 6 of 80 cases of CML⁷. To our knowledge, ours is the first report of loss of *p51* gene expression in *de novo* AML. In our patient, leukemic blasts had a normal karyotype, and no

mutations of *p53*, *FLT3* or *N-ras* were detected. Therefore, loss of *p51* gene expression may have played important roles in leukemogenesis in this patient.

Nakano *et al.* reported molecular analysis of the *p53*, *FLT3* and *N-ras* genes in 28 patients with relapsed AML⁵. They found that the incidences of molecular alterations (loss or acquisition of mutation) of *FLT3* and *N-ras* were more frequent than *p53*. They reported prognostic significance for *FLT3* mutations as well as *p53* mutations in relapsed AML⁵. Recent studies of *FLT3* mutations in relapsed AML reported frequent observations of loss or acquisition of mutations or changes in mutation patterns of *FLT3*^{25,26}.

In our study, the aberrant *p53* population had a significantly worse prognosis. Relapsed AML is associated with short survival. Mutation of the *p53* gene is one of the most unfavorable prognostic factors in patients with relapsed AML. While *FLT3* mutations did not correlate with overall survival in our study, several studies showed that *FLT3* mutations in AML^{12,14} and in relapsed AML⁵ were associated with a worse outcome. A larger-scale study might clarify the prognostic impact of mutant *FLT3* in relapsed AML.

In conclusion, the present results showed that abnormalities of the *p51*, *FLT3* and *N-ras* genes may contribute to leukemogenesis at an early stage but may not play significant roles in the progression or

relapse of AML. In view of the karyotype complexity, other molecular abnormalities may contribute to the disease progression. To confirm the present findings, it will be necessary to analyze a much larger number of samples from relapsed AML patients. However, abnormalities of the *p53* gene may contribute to leukemogenesis not only at an early stage but also at late progression of AML. *p53* mutation is an independent risk factor for survival of AML^{9,10,19}. This study found a similar prognostic impact of *p53* mutation in relapsed AML. Thus, AML patients with the *p53* mutation should be treated using a different strategy than that for patients with normal *p53*. It is hoped that these findings will provide a means of predicting and treating leukemic relapse. Finally, this is the first report of loss of *p51* gene expression in *de novo* AML.

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(Received, January 21, 2004)

(Accepted, April 5, 2004)
