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Clonal Analysis of Multiple Point Mutations in the N-*ras* Gene in Patients with Acute Myeloid Leukemia

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We have screened mutations of the N-*ras* gene at codons 12, 13, and 61 in leukemia cells obtained from 100 patients with acute myeloid leukemia (AML), and found mutated N-*ras* alleles in 9 patients. We further analyzed the polyclonality of multiple N-*ras* gene mutations in 4 AML patients. One patient, who had the monoclonal karyotype, t(11;17), had two types of double missense mutations at codons 13 and 61 in the same allele. Each of the remaining three patients, one of whom had t(15;17) with a monoclonal rearrangement of the retinoic acid receptor alpha and PML genes, carried two missense mutations in a relatively small population of leukemia cells. We have demonstrated that multiple clonality of the N-*ras* gene is occasionally observed in leukemia with a monoclonal karyotype. These findings indicate that the N-*ras* mutations may not always be characterized simply by an accumulative process and that the activated N-*ras* gene alone is not sufficient to cause leukemia.

Key words: AML — N-*ras* oncogene — Point mutation — Clonal analysis

Molecular analyses have revealed that nucleotide substitutions and chromosomal translocations are the two major genetic alterations associated with leukemia.[1,2] An example of the former is the mutated N-*ras* gene. Twenty to 40% of patients with acute myeloid leukemia (AML) reportedly have mutated N-*ras* genes.[1] Sequential analyses of the same patients with AML or myelodysplastic syndrome (MDS), however, have provided puzzling data regarding the association of mutated *ras* genes with leukemogenesis, progression and relapse.[3–5] In some patients with MDS, clinical progression to secondary AML appeared to be correlated with N-*ras* gene mutations,[6] though conflicting data have also been presented.[7] A considerable percentage of AML patients who had N-*ras* mutations at initial presentation had lost the mutations at relapse.[4,8] Additionally, not all leukemia cells carried mutant alleles,[9] and some leukemia cells contained multiple point mutations.[10] Thus, it should be clarified in which stage of leukemogenesis the *ras* mutation is involved, and whether the mutation alone is sufficient to induce leukemia. One explanation is that the *ras* gene mutation is associated with the progression of leukemia, but is not a prerequisite for leukemogenesis, although in animals the mutation appears to be an initial step of carcinogenesis.[11] An alternative explanation is that the *ras* mutation occurs early in the leukemogenesis, and that an evolutional clone with additional genetic alterations occasionally deletes the mutated *ras* gene during the progression.[12]

To study the significance of *ras* mutation in leukemogenesis, we focused on the polyclonality of the N-*ras* mutations found in leukemia cells from 4 AML patients. The clonality of the mutation and its association with chromosomal translocation were analyzed.

MATERIALS AND METHODS

Patients and leukemic cell samples Mononuclear cells were separated from heparinized peripheral blood or bone marrow by Ficoll-Conray density gradient centrifugation, which enriched the specimens to over 80% blast cells. All samples were cryopreserved in liquid nitrogen until analysis. From October 1980 to April 1991, leukemia cells from 120 patients with AML, who had given their informed consent, were obtained and preserved in our laboratory. The DNA in 100 of these samples, for which full clinical data and sufficient cells were available, was analyzed. Samples were from 52 males and 48 females, with ages ranging from 3 to 81 years (median, 51). AML subtypes were determined according to the French-American-British (FAB) classification[13] as follows: 27 were M1, 39 were M2, 8 were M3, 14 were M4, 11 were M5, and one was M6. Secondary AML cases with clinically antecedent MDS were not

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included. DNA and RNA were prepared by standard procedures.[14]

Polymerase chain reaction (PCR) of N-*ras* gene To amplify the sequences spanning codons 12 and 13 and codon 61, oligonucleotide primers were prepared on a DNA synthesizer (Model 380B; Applied Biosystems, Foster City, CA) according to the published sequences[15] as follows: the 5′ primer for codons 12, 13 (named NA12), 5′-GACTGAGTACAAACTGGTGG-3′; the 3′ primer for codons 12, 13 (NB12), 5′-CTCTATGGTGGGATCATATT-3′; the 5′ primer for codon 61 (NA61), 5′-GGTGAAACCTGTTTGTTGGA-3′ and the 3′ primer for codon 61 (NB61), 5′-ATACACAGAGGAAGCCTTCG-3′. PCR was performed according to the published method.[15,16] Genomic DNA was amplified by 40 PCR cycles (denaturation for 60 s at 92°C, annealing for 60 s at 55°C, and elongation for 60 s at 72°C). The product of amplification was evaluated by agarose gel electrophoresis, and only the reactions resulting in a band of the expected size were further analyzed.

Reverse transcriptase-polymerase chain reaction (RT-PCR) Because codons 12 and 13 of N-*ras* are encoded by exon 1, and codon 61 by exon 2,[17] RT-PCR was performed to clone the allele with the mutations spreading over the two exons. RT-PCR was performed as follows. Complementary DNA (cDNA) was synthesized from 500 ng of RNA using 100 ng of random hexamer (Boehringer Mannheim Yamanouchi, Tokyo), 80 U of RNase-inhibitor (Promega, Madison, WI), and 200 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Lab., Gaithersberg, MD) in the buffer recommended by the manufacturer. The reaction proceeded for 60 min at 37°C, and the synthesized cDNA was used as a template for PCR amplification for 40 cycles as described above, using the 5′ primer (NA12-*Hin*dIII) 5′-ACAAGCTTGTGGTGGTTGG-3′ and the 3′ primer (NB61-*Bam*HI) 5′-CGGATCCTCATGTATTGG-3′. As shown by underlines, each primer was modified to produce convenient restriction sites (*Hin*dIII, *Bam*HI) for cloning into the M13mp18 vector.[14]

DNA sequencing To clone the mutated N-*ras* gene at codons 12 and 13 into the M13mp18 vector, NA12 and NB12 primers were modified to produce convenient restriction sites (*Eco*RI, *Bam*HI) as follows: NA12-*Eco*RI, 5′-GACTGAATTCAAACTGGTGG-3′ and NB12-*Bam*HI, 5′-CTCTATGGTGGGATCCTATT-3′. Similarly, to clone the mutated N-*ras* gene at codon 61, modified primers were prepared as follows: NA61-*Eco*RI, 5′-GGTGAATTCTGTTTGTTGGA-3′ and NB61-*Bam*HI as described above. The PCR or RT-PCR amplified products were isolated from polyacrylamide gels, then digested with *Eco*RI/*Bam*HI or *Hin*dIII/

Table I. Synthetic Oligonucleotide Probes Used to Analyze N-*ras* Gene Mutations

Probe[a]	Sequence (5′···3′)[b]	Amino acid[c]
N-*ras* 12 (GGT)	GTTGGAGCAGGTGGTGTTGG	Gly (wt)
N-*ras* 12 (AGT)	____AGT____	Ser
N-*ras* 12 (TGT)	____TGT____	Cys
N-*ras* 12 (CGT)	____CGT____	Arg
N-*ras* 12 (GAT)	____GAT____	Asp
N-*ras* 12 (GTT)	____GTT____	Val
N-*ras* 12 (GCT)	____GCT____	Ala
N-*ras* 13 (GGT)	GGAGCAGGTGGTGTTGGGAA	Gly (wt)
N-*ras* 13 (AGT)	____AGT____	Ser
N-*ras* 13 (TGT)	____TGT____	Cys
N-*ras* 13 (CGT)	____CGT____	Arg
N-*ras* 13 (GAT)	____GAT____	Asp
N-*ras* 13 (GTT)	____GTT____	Val
N-*ras* 13 (GCT)	____GCT____	Ala
N-*ras* 61 (CAA)	ACAGCTGGACAAGAAGAGTA	Gln (wt)
N-*ras* 61 (GAA)	____GAA____	Glu
N-*ras* 61 (AAA)	____AAA____	Lys
N-*ras* 61 (CCA)	____CCA____	Pro
N-*ras* 61 (CTA)	____CTA____	Leu
N-*ras* 61 (CGA)	____CGA____	Arg
N-*ras* 61 (CAC)	____CAC____	His
N-*ras* 61 (CAT)	____CAT____	His

a) Probes are 20 bases long.
b) Underlines represent wild type sequences.
c) Amino acids encoded by the mutated codons. wt: wild type.

*Bam*HI (Boehringer Mannheim Yamanouchi), respectively. The digested fragments were inserted into the M13mp18 vector and transfected into *E. coli* strain JM 109. Individual clones were isolated, and single-stranded DNA phage templates were prepared. DNA was sequenced by the dideoxy chain termination method using *Taq* polymerase (Promega) and fluorescently tagged M13 universal primers (Applied Biosystems). Sequencing gel electrophoresis and data analysis were performed on a Applied Biosystems model 373A DNA sequencer (Applied Biosystems).

Dot blot and oligonucleotide hybridization Dot blots and oligonucleotide probe hybridization were performed as previously described.[15, 16] Briefly, either 100 μg of DNA amplified by PCR or cloned double-stranded DNA that was ligated into the M13mp18 vector and propagated in JM109 as described above, was transferred to nylon filter membranes (Hybond-N; Amersham Japan, Tokyo) using a 96-well filtration manifold (Bethesda Research Lab.). Blotted DNA was crosslinked by UV illumination and the membranes were hybridized with [γ-^{32}P]oligonucleotide probes. The oligonucleotide panel included probes specific for the wild type allele and all possible amino acid substitutions at codons 12, 13 and 61 of the N-*ras* gene (Table I). Prehybridization, hybridization and washing of membranes were performed according to the published conditions.[15] The membranes were finally exposed to Kodak XAR 5 films at -70°C using intensifying screens.

Chromosome analysis After 24 h *in vitro* culture, AML cells were incubated with 0.016 μg/ml colcemid for 2 h and treated with 0.05 *M* KCl for 20 min at room temperature, followed by fixation with methanol-acetic acid (3:1). Slides were made by an ordinary air drying method. The chromosome preparations were stained by a modification of the trypsin-Giemsa banding method.[18]

Southern blot analysis DNA was digested with the restriction endonucleases: *Eco*RI, *Bam*HI and *Hin*dIII, electrophoresed in 0.7% agarose gel, blotted and hybridized as described.[14] After hybridization, the filters were washed under stringent conditions and exposed to X-ray film.

To detect the retinoic acid receptor alpha (RAR-α) gene rearrangement, the *Rsa*I-*Rsa*I fragment (309 bp), which corresponded to exons 2, 3 and 4, was subcloned from a full-sized RAR-α cDNA clone[19] and used as the probe. To obtain the PML probe, the truncated cDNA of the PML gene was amplified from the cDNA of HL60 cells by PCR using the 5′ primer 5′-CTCAGCTCTTGCATCACCCAGGGGAAA-3′ and the 3′ primer 5′-AACACCACTAGTCGTCGAGCC-3′. These primers were synthesized according to the published sequences.[20] The amplified product (530 bp) was confirmed to be derived from the PML gene[20] by DNA sequencing. This probe gave germline bands of 7, 5.5 and 9 kb in *Eco*RI, *Bam*HI and *Hin*dIII digests, respectively (unpublished results).

RESULTS

Leukemia cells from 100 patients with AML were screened for the presence of the mutated N-*ras* gene at codons 12, 13 and 61. Differential hybridization and sequencing revealed mutated N-*ras* alleles in 9 patients (Table II). Although no significant association was observed between the presence of the mutated N-*ras* gene and age or FAB classification, females were significantly dominant compared with males (P=0.011 by Fisher's exact test; 2-tailed). It was difficult to determine the correlation between the *ras* mutation and clinical outcome, because the number of patients with the mutated N-*ras* gene was small, and the therapeutic regimens were not the same among these 100 patients.

Six patients had mutations at codon 12, one at codon 13, one at codon 61, and one (patient YY) at codons 12, 13 (2 mutant types) and 61. Deletion of a normal allele was not observed in any of the leukemic cells with mutated N-*ras* genes. The most common nucleotide substitution was a G-to-A transition [6/15: ratio of G→A to total number of nucleotide substitutions], followed by a G-to-C transversion [4/15]. No particular amino acid substitution was observed, but the frequency of aspartic acid at codon 12 was moderately high [4/9: ratio of aspartic acid to total number of mutated codons].

Four of the 9 patients with mutated N-*ras* genes presented multiple point mutations; one patient (YY) had four different mutations and three patients (ST, YN and KK) had two mutations. In order to estimate the cell population containing each N-*ras* gene mutation, and to investigate the allelic relationship of the mutations, the amplified N-*ras* gene was cloned and analyzed by dot hybridization.

The cDNA from patient YY, who had N-*ras* mutations in exons 1 and 2, was amplified by RT-PCR. Amplified samples were cloned into the M13mp18 vector, and the presence of mutations in 90 clones was analyzed by oligonucleotide hybridization (Fig. 1). The results are summarized in Table III. Forty clones had a single mutation either at 12, 13 or 61, and two clones contained double mutations at codons 13 and 61. The remaining 48 clones had the wild type N-*ras* gene. The dot hybridization data were confirmed by sequencing each of these 90 clones. The sequences of the clones with double mutations are presented in Fig. 2.

Of the 3 patients with two point mutations (ST, YN and KK), significant signals were given with the probe for the wild type N-*ras* gene, compared with that for two mutant genes (data not shown). To semi-quantify the

Table II. N-*ras* Point Mutations Identified in Nine Patients with AML

Patients	Age(yr)/Sex	Subtype[a]	Karyotype	Point mutation[b]
OR	64/F	M1	46,XX	12:GAT (Asp)
KE	70/M	M2	46,XY	12:GCT (Ala)
YT	50/F	M2	46,XX	12:GCT (Ala)
ST	52/F	M2	not done	12:GAT (Asp) 12:GTT (Val)
YN	24/F	M3	46,XX, t(15;17)	12:CGT (Arg) 12:GAT (Asp)
UK	35/F	M4	46,XX	12:TGT (Cys)
HF	54/F	M3	49,XX,+8, t(8;12),+12, +13,+15,−16, i(17q)	13:GAT (Asp)
KK	26/F	M2	46,XX, inv(9)[c]	61:AAA (Lys) 61:CAT (His)
YY	9/F	M1	46,XX, t(11;17)	12:GAT (Asp) 13:CGT (Arg) 13:GAT (Asp) 61:CAC (His)

a) FAB subtype.
b) Mutated codons (amino acids encoded by the mutated codons); underlines indicate the substituted nucleotides.
c) The inv(9) is not associated with leukemia but is a normal variant.

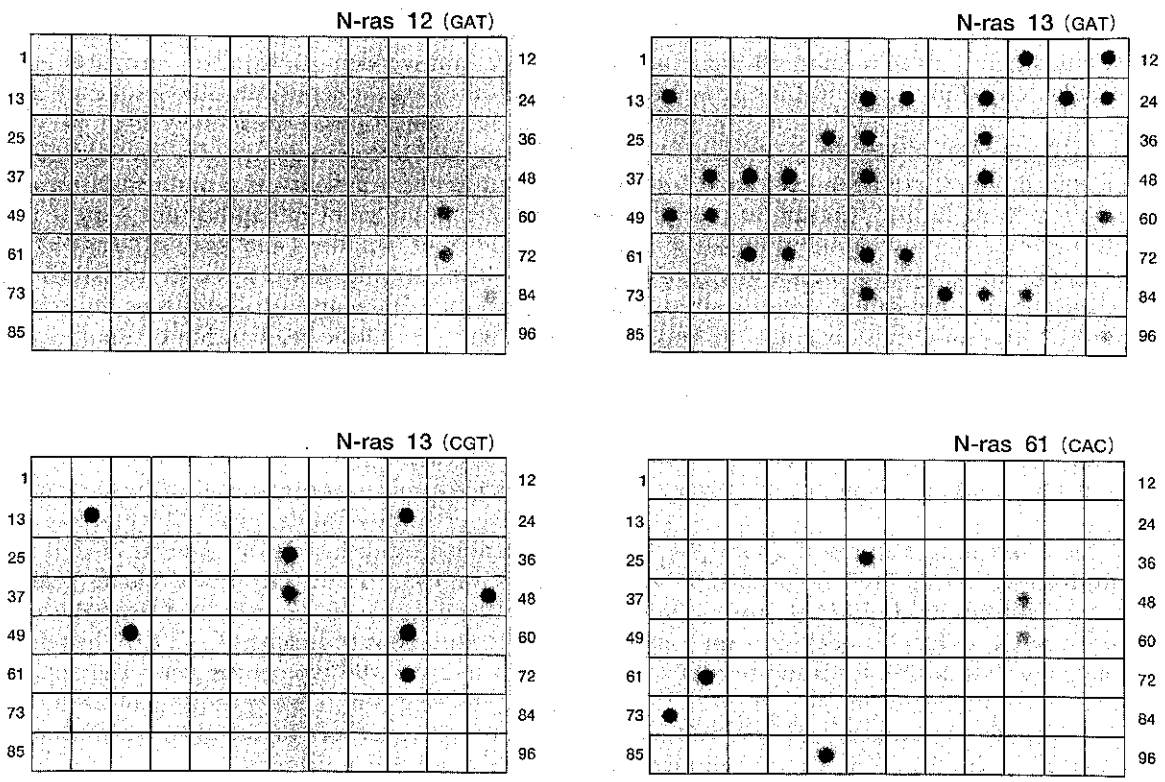

Fig. 1. Clonal analysis of N-*ras* point mutations by oligonucleotide hybridization in patient YY. Dots 1–90, clones derived from AML cells of YY; 91–94, clones derived from a B-cell line of YY; 95, a clone derived from a rhabdomyosarcoma cell line, RMS-YM[29] as a wild type control; 96, cDNA of YY amplified by RT-PCR as a mutation-positive control. Data using probes for the wild type N-*ras* gene at codons 12, 13 and 61 are not shown.

mutant and normal alleles, each genomic DNA was amplified and cloned. Forty-eight clones were analyzed by oligonucleotide hybridization. The population of the mutant clones from each patient was smaller than that of the wild type. The results are summarized in Table IV.

As regards *ras* mutation, these AML cells seemed to be biclonal or polyclonal. However, leukemia cells from patients YN and YY presented chromosomal translocations, t(15;17) and t(11;17), respectively, in all 20 mitoses analyzed. To confirm the clonality of t(15;17),

Table III. Summary of Clonal Analysis for the Presence of N-*ras* Point Mutations by Oligonucleotide Hybridization and Sequencing in Patient YY

Point mutation[a)]	Number of clones[b)]
Wild type	48
Codon 12:GAT	3
Codon 13:CGT	7
Codon 13:GAT	26
Codon 61:CAC	4
Codons 13:CGT & 61:CAC	1
Codons 13:GAT & 61:CAC	1

a) Underlines indicate the substituted nucleotides.
b) A total of 90 clones was analyzed.

Table IV. Clonal Analysis of N-*ras* Point Mutations in Patients ST, YN, and KK

Patient	Point mutation[a)]	Number of clone[b)]
ST	Wild type	39
	Codon 12:GAT	5
	Codon 12:GTT	4
YN	Wild type	41
	Codon 12:CGT	2
	Codon 12:GAT	5
KK	Wild type	36
	Codon 61:AAA	11
	Codon 61:CAT	1

a) Underlines indicate the substituted nucleotides.
b) A total of 48 clones was analyzed per patient.

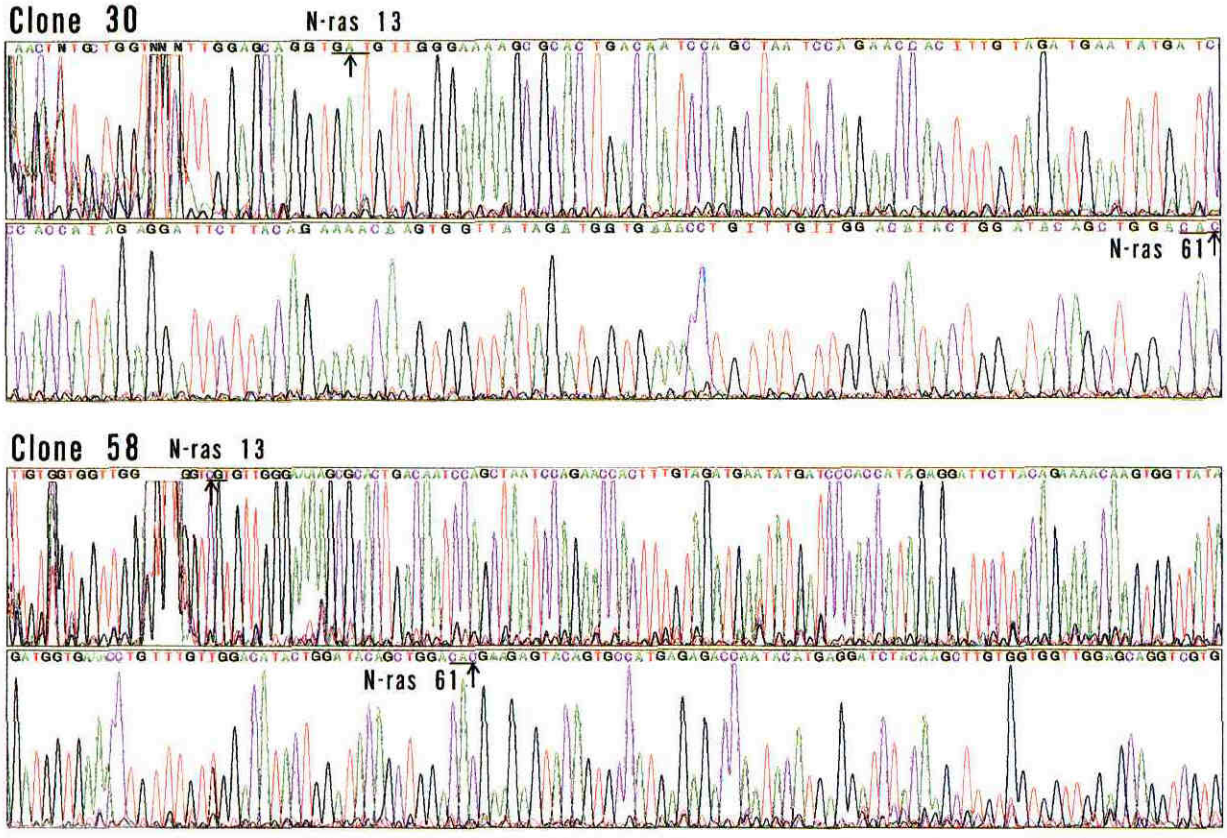

Fig. 2. cDNA sequences of the N-*ras* gene in AML cells from patient YY. Two clones containing double mutation loci at codons 13 and 61 are indicated by dot blot hybridization. These sequences were analyzed as described in "Materials and Methods." Arrows indicate the substituted nucleotides.

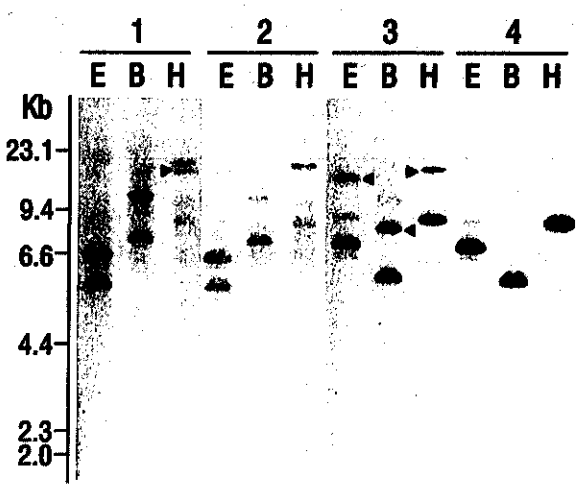

Fig. 3. Southern blot analysis of the PML gene in patient YN with t(15;17). The genomic DNA from patient YN (lanes 1 and 3) and normal placental DNA (lanes 2 and 4) were digested with *Eco*RI (E), *Bam*HI (B), *Hin*dIII (H) and hybridized with the RAR-α probe (lanes 1 and 2) and the PML probe (lanes 3 and 4). The rearranged bands are indicated by arrowheads.

Southern blot analysis of the RAR-α and PML genes was performed. The RAR-α gene gave a rearranged band of 15 kb in the *Hin*dIII digest. The PML gene was rearranged at 14, 7 and 15 kb in the *Eco*RI, *Bam*HI and *Hin*dIII digests, respectively (Fig. 3). Since the intensity of the rearranged band was the same as the germ line band in each digest, it was concluded that the sample was monoclonal for the RAR-α and PML gene rearrangements.

DISCUSSION

This study showed the presence of N-*ras* mutation in leukemia cells from 9 out of 100 patients with AML. Our data differ from those of previous reports[1, 4, 5, 21] in some respects. First, in this study, the prevalence of the N-*ras* mutation (9%) was lower than those of previous reports (15 to 27%). This may result from the fact that we have analyzed only *de novo* AML and excluded AML that arose from proven MDS, which is frequently associated with N-*ras* mutations. Second, the incidence of the N-*ras* mutations was higher in females than in males. No association of mutation was observed with age, FAB classification, clinical course or chromosomal abnormality, although a predominance of the M4 subtype has been noted in AML patients with the N-*ras* mutation.[5, 21] All studies, including ours, were retrospective and performed on heterogeneous populations in AML. Furthermore, there may be sampling biases in collection or cryopreservation of leukemia cells. Therefore, as Bartram *et al.* previously pointed out, the clinical significance of *ras* gene mutations must be precisely studied in prospective ways.[5]

Multiple *ras* gene mutations in a single tumor have been noted in many types of neoplasms. Forrester *et al.* have reported activated N- and K-*ras* oncogenes in a preinvasive villous adenoma of the colon.[22] Farr *et al.* and Neri *et al.* have found multiple mutations of N-*ras* gene in AML and acute lymphoblastic leukemia (ALL), respectively.[4, 23] In AML, the NIH/3T3 focus-forming assay has revealed that mutations were present in different alleles.[4] In ALL, when slot-blot hybridization analysis yielded a weaker signal for the N-*ras* mutation at codon 13 than that for the one at codon 12, it was argued that the two mutations were present not only in two distinct alleles but also in two different cell populations.[23] Mariyama *et al.* reported multiple mutations in pancreatic carcinoma,[24] and Levi *et al.* described multiple K-*ras* codon 12 mutations in cholangiocarcinomas.[25] The latter two studies yielded more details about multiple mutations by application of cloning and sequencing. However, they did not clarify whether multiple mutations existed within a single tumor cell or in different cells, or whether tumor cells containing multiple mutations were derived from a monoclonal clone or not. To study the former issue directly, a single tumor cell needs to be cloned, and its sequence analyzed. In regard to the latter, we studied the cells from patients YN and YY who carried t(15;17) and t(11;17), respectively.

Leukemia cells from patient YN had monoclonal RAR-α and PML gene rearrangement, while not all leukemia cells carried the *ras* mutations. This case presented a unique opportunity to determine the temporal relationship between *ras* mutation and chromosomal translocation. Here, we present four possible models for the development of acute promyelocytic leukemia (APL) in patient YN (Fig. 4). First, if the *ras* mutation occurred after the chromosomal translocation, t(15;17), model A could be proposed. Second, if the *ras* mutations occurred prior to the translocation and the mutant alleles were deleted stepwise during the progression, models B and C could apply. Model B represents a serial mutation of *ras* gene, and model C a biclonal mutation of the gene. However, in model B, the leukemia cells must carry only the mutant alleles, and this is inconsistent with our findings. In model C, the same translocation must occur in the three clones, which seems highly improbable. Third, if the mutations occurred before and after the translocation, model D would apply. This model is consistent with our findings, if the mutant allele, which appeared before the translocation, is deleted and the normal allele remains in the cell. We favor model A or D. That is, the N-*ras* mutation is involved mostly after the

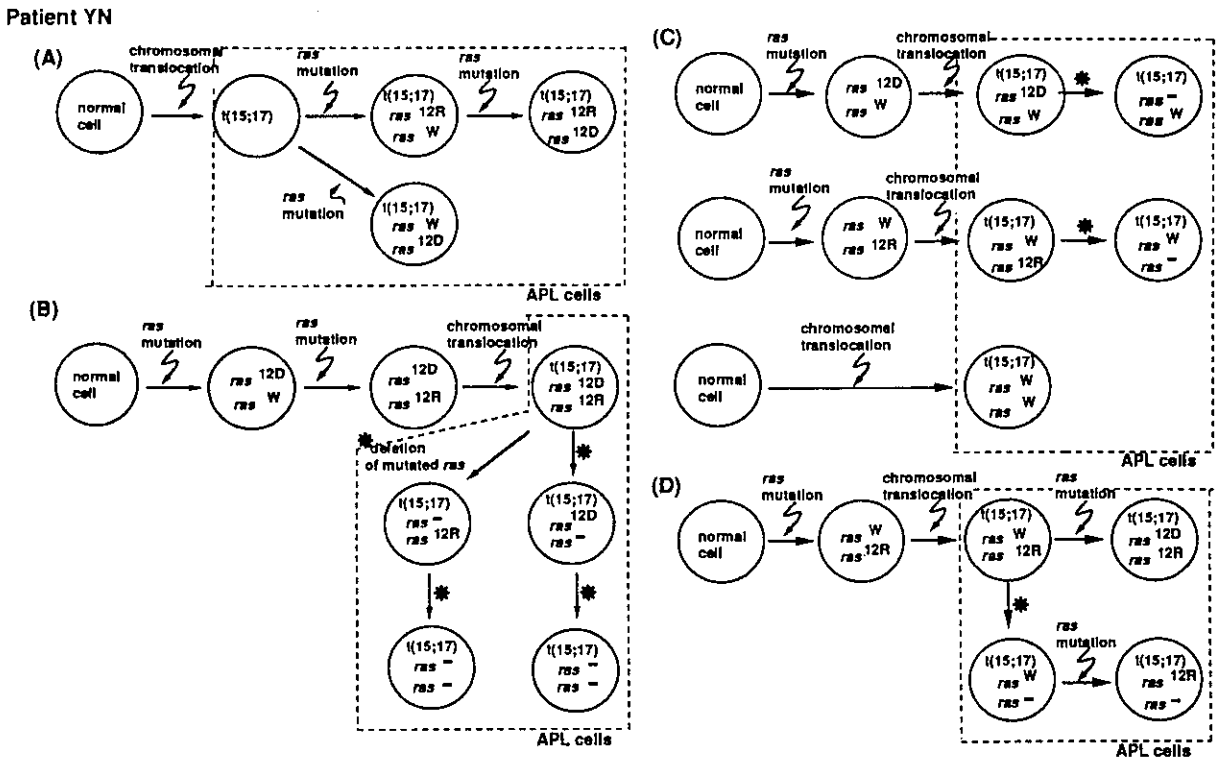

Fig. 4. Models of the development of AML in patient YN from the perspective of molecular alterations. Model A is based on the assumption that the *ras* mutation is involved posterior to the chromosomal translocation. Models B and C are based upon the hypothesis that the mutations occur prior to the translocation and that the mutant alleles are deleted in the late stage. Model D is based on the assumption that the mutations occur at any stage during development and can be deleted after translocation. W, wild type; 12D, mutation from Gly to Asp at the 12th amino acid; 12R, mutation from Gly to Arg at the 12th amino acid; *ras*$^-$, deletion of the N-*ras* gene.

chromosomal translocation, or the mutation occurs during any stage of leukemogenesis and the mutated alleles can be deleted after chromosomal translocation. Recently Pandolfi *et al.* suggested that APLs lacked *ras* and p53 mutations and were caused by a single cytogenetic abnormality.[26] Further data regarding the association between *ras* mutation and t(15;17) are required.

In patient YY, who carried four different mutations at the three codons, we think that model A in Fig. 4 is most suitable to explain the complexity of the ras mutations. Interestingly, we detected two clones with two mutant loci in the same allele. The possibility of cloning or sequencing error due to the lack of *Taq* polymerase fidelity can be ruled out for the following three reasons. First, the nucleotide substitution in the two clones was identical with that in single locus mutants. Second, we obtained two independent clones containing two mutant loci. Finally, the error rate of *Taq* polymerase during DNA amplification is estimated to be below 6.6×10^{-5} misincorporations per base at the low dNTP and Mg^{2+} ion concentrations (200 μM dNTP and 1.5 mM $MgCl_2$)[27] which we used in our experiments. This was the first observation of double-locus mutant in the same allele, which suggested stepwise accumulation of *ras* mutations after chromosomal translocation in a single clone. The biological significance of the clone with two mutant loci is unknown. If sequential samples were obtained from this patient, the dominant clone would be expected to be selected at the time of relapse. DNA transfection using NIH/3T3 cells also revealed differences in focus-forming activity among the mutant alleles.[28] However, even if the double-locus mutant shows higher transforming activity than a single-locus mutant, the heterogeneity of *ras* mutations present in a single tumor indicates that the *ras* mutations do not always confer a significant growth advantage.

We have demonstrated that multiple clonality of the N-*ras* gene is occasionally observed in leukemia with a

monoclonal karyotype. These findings indicate that the N-*ras* mutations may not always be characterized simply in terms of a hierarchically ordered process, and that the activated N-*ras* gene alone is not sufficient to cause leukemia.

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