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Mutations of N-*ras* Oncogene in Myelodysplastic Syndromes and Leukemias Detected by Polymerase Chain Reaction

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By using polymerase chain reaction and differential hybridization, point mutations of N-*ras* oncogene were investigated among 43 patients with hematopoietic malignancies. Six cases were revealed to carry mutational N-*ras* oncogenes. One case with acute myelocytic leukemia was found to carry a mutation at codon 13, although this case was in complete remission.

Key words: N-*ras* oncogene — Myelodysplastic syndrome — Leukemia — Polymerase chain reaction

The *ras* gene family, composed of N-*ras*, K-*ras* and H-*ras*, is believed to be involved in neoplastic changes of several tissues. The genes are activated by single nucleotide alterations in codon 12, 13 or 61 with corresponding amino acid substitutions of the protein product, p21.[1-3] Mutational activations of N-*ras* oncogene have been frequently detected in hematopoietic malignancies, such as acute myelocytic leukemia (AML)[2] and myelodysplastic syndrome (MDS).[4,5] A part of MDS is presumed to be a preleukemic state. Up to 40% of cases with MDS develop leukemias. The mechanism of malignant progression from MDS to overt leukemia is not yet known. Activation of N-*ras* oncogene, however, may play a role in this process.[4] To gain further information about the role of N-*ras* oncogene in leukemogenesis, we have investigated the activation of N-*ras* oncogene in MDSs and leukemias, using the polymerase chain reaction (PCR),[6] which amplifies short stretches of target DNA *in vitro*, and differential dot-blot hybridization with synthetic oligonucleotides as probes.

Bone marrow samples were obtained from 19 patients with MDS, 10 patients with AML, 2 patients with ALL, 10 patients with CML and 2 patients with CLL. They were subgrouped according to the French-American-British (FAB) classification. Details of these patients are shown in Table I and Table II. High-molecular-weight DNAs were isolated from those samples as described elsewhere,[7] and then sheared, and extracted successively with phenol/chloroform (1:1), chloroform and finally water-saturated ether. As positive controls, we used DNAs of NIH-3T3 cells transformed by the transfection of DNAs from an AML patient carrying N-*ras* mutation at codon 12[8] and an MDS patient carrying N-*ras* mutation at codon 13.[4] For the positive control of codon 61, DNA of 4N-1-4 cell line, which carries lysine at codon 61 of N-*ras* oncogene instead of glutamine, was used (provided by Dr. M. Nagao). Oligonucleotides for primers and probes were synthesized by an Applied Biosystems model A380 DNA synthesizer; they are listed in Table III. Target sequences of sample DNAs were amplified *in vitro* by a modification of the method described by Saiki *et al.*[6] The sizes of target sequences are 97 bp and 92 bp for codons 12 and 13, and for codon 61, respectively. Five hundred nanograms of each DNA was mixed with 100 pmol of upstream ("5′-") and downstream ("3′-") primers complementary to the sequences surrounding the codon to be screened, in 100 μl of 50 m*M* Tris-HCl, pH 8.5, 15 m*M* $(NH_4)_2SO_4$, 10 m*M* $MgCl_2$, 10 m*M* β-mercaptoethanol and 0.2 mg/ml of bovine serum albumin. After incubation at 95°C for 7 min, 2 U of *Taq* polymerase (Biotec International Ltd.) and the four deoxynucleotide triphosphates (to a final concentration of 1 m*M* each) were added to the mixture. PCR was started by incubation at 65°C for 1 min, followed by denaturation at 90°C for 30 s, then annealing at 50°C for 30 s. After 40 cycles of amplification, 1 U of *Taq* polymerase was added to the mixture and another 20 cycles of amplification were carried out. Aliquots (10 μl) of the mixtures were dot-blotted onto nylon filters (Gene Screen Plus, New England Nuclear). The filters were prehybridized at 50°C for 2 h in 1 *M* NaCl, 50 m*M* Tris-HCl, pH 7.4, 1% SDS, and 100 μg/ml heat-denatured salmon sperm DNA, and hybridized overnight

Abbreviations: PCR, polymerase chain reaction; AML, acute myelocytic leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myelocytic leukemia; CLL, chronic lymphocytic leukemia; MDS, myelodysplastic syndrome; RA, refractory anemia; RAEB, refractory anemia with excess of blasts; RAEB in T, refractory anemia with excess of blasts in transformation; BMT, bone marrow transplantation; NCC, nucleated cell count; FAB, French-American-British.

Table I. Details of Patients with MDS

Patient		Age (yr)	Sex	FAB type	Mutated codon
1	T.N.	23	F	RA	61
1′	same patient			→post-BMT	n
2	J.W.	74	M	RA	n
3	K.O.	63	M	RA	n
4	Y.S.	17	M	RA	n
5	S.K.	13	M	RA	n
6	E.H.	18	M	RA	n
7	S.K.	35	M	RA	n
8	Y.K.	50	M	RA	n
9	S.M.	41	F	RAEB	n
10	K.K	72	M	RAEB	n
11	J.K.	40	M	RAEB in T	n
11′	same patient			→leukemic transformation	n
12	A.S.	74	F	RAEB in T	12
13	Y.N.	76	M	RAEB in T	n
14	T.S.	52	M	RAEB in T	n
15	S.H.	39	M	leukemic transformation	n
16	Y.O.	52	M	leukemic transformation	n
17	Y.M.	21	F	leukemic transformation	n
18	K.H.	73	M	leukemic transformation	61
19	K.O.	84	M	leukemic transformation	n

M, male; F, female; n, negative.

at 50°C in the same solution containing oligonucleotide probes end-labeled with [γ-^{32}P]ATP (Amersham: 3000 Ci/mmol). The specific activity of probes was more than 5×10^5 cpm/pmol. The filters were washed twice at room temperature in 1×SSC (1×SSC=0.15 *M* NaCl, 0.015 *M* Na citrate), 0.1% SDS, and once at 50°C in TMAC washing solution (3*M* tetramethylammonium chloride, 50 m*M* Tris-HCl, pH 8.0, 2 m*M* EDTA, 0.1% SDS) for 40 min, and finally once at 64°C in TMAC washing solution for 10 min. The exposure to Kodak XAR films was performed for 2 h at −70°C with intensifying screens (Du Pont, Lightning Plus). By this protocol, we could detect mutations when at least 1% of total cells carried mutational N-*ras* oncogene (Fig. 1). To check whether the amplification by PCR was successful or not, we tested the hybridization signal with the corresponding upstream primer for each PCR. In the hybridization with the primers, the filters were stringently washed as in the case of other probes. Therefore, the primers were presumed to hybridize to the target PCR-product precisely. We also checked the amplification of PCR by gel-electrophoresis for many samples. The results of gel-analysis showed a good correlation with those of the primer-hybridization. To use wild-type probes for the control of PCR, however, was thought to be unsuitable, because the copy number of wild-type N-*ras* oncogene was different from one sample to another.

As shown in Tables I and II and Fig. 2, point mutations of N-*ras* oncogene were detected in six out of 43 patients. The ratios of patients with AML and MDS carrying N-*ras* mutations are 30% and 16%, respectively. Among them, two cases should be noted. One was a 23-year-old female with RA (No. 1 patient in Table I). She had an allogenic bone marrow transplantation (BMT) from her identical twin. Before and after BMT, her bone marrow aspirations showed: NCC=15×10^4 cells/μl, %blast =1.5%, and NCC = 8×10^4 cells/μl, % blast = 1.0%, respectively. Morphologically it was hard to distinguish whether her BMT was successful or not, because her pre-BMT bone marrow had almost the same percentage of blasts as that of her post-BMT. The result of our study, however, indicated that she had received a successful BMT, because PCR of her pre-BMT bone marrow revealed a point mutation at codon 61 of N-*ras* oncogene, but that of her post-BMT bone marrow did not. The other case which should be noted was an 86-year-old male with AML, M5 (No. 9 AML patient in Table II). His bone marrow sample was obtained after a successful chemotherapy, when he was clinically in "complete remission." The PCR-differential hybridization method, however, revealed a mutation of N-*ras* oncogene at codon 13. Because of the high sensitivity of our protocol, morphologically indistiguishable malignant cells could be recognized as mutated.

Table II. Details of Patients with Leukemias

Diagnosis	Patient	Age (yr)	Sex	FAB type	% Blast in BM	Mutated codon
AML						
1	S.G.	79	F	M1	94	n
2	M.W.	21	F	M1	94	n
3	S.T.	41	F	M1	91	n
4	K.W.	40	F	M1	86	n
5	M.G.	26	F	M2	80	13
6	J.M.	56	F	M2	78	n
7	T.M.	55	F	M2	11	n
8	A.Y.	46	M	M4	74	n
9	I.W.	86	M	M5	1	13
10	S.O	45	M	M5	80	13
10′	same patient				→1.2	n
ALL						
1	Y.A.	12	M		96	n
2	M.S.	53	F		95	n
CML						
1	M.M.	59	F	accelerated	6	n
2	Y.K.	29	M	accelerated		n
3	K.M.	77	M	accelerated		n
4	Y.W.	41	M	accelerated	5.2	n
4′	same patient			→blastic crisis	46	n
5	M.K.	27	M	accelerated	4.4	n
6	T.H.	68	F	blastic crisis	39	n
7	K.O.	55	M	blastic crisis	55	n
8	M.Y.	31	M	blastic crisis	75	n
9	K.N.	42	F	blastic crisis	60	n
10	S.W.	32	M	blastic crisis		n
CLL						
1	S.K.	71	M		2.1	n
2	A.I.	42	F		92	n

M, male; F, female; BM, bone marrow; n, negative.

Table III. Oligonucleotides for Primers and Probes

	Name	Sequence		Strand
Probes	wild for 12, 13	5′-TTCCCAACACCACCTGCTCC-3′		a
	N-12 mix	———————N———	N=A, G, T	a
		———————N———	N=A, G, T	a
	N-13 mix	———————N———	N=A, G, T	a
		———————N———	N=A, G, T	a
	wild for 61	5′-ACAGCTGGACAAGAAGAGTA-3′		s
	N-61 mix	———————N———	N=A, G	s
		———————N———	N=G, C, T	s
		———————N———	N=C, T	s
Primers	5′-primer for 12, 13	5′-CTTGCTGGTGTGAAATGACT-3′		s
	3′-primer for 12, 13	5′-ACAAAGTGGTTCTGGATTAG-3′		a
	5′-primer for 61	5′-GGTGAAACCTGTTTGTTGGA-3′		s
	3′-primer for 61	5′-AAGCCTTCGCCTGTCCTCAT-3′		a

s, sense strand; a, anti-sense strand. Thin lines represent wild-type sequences.

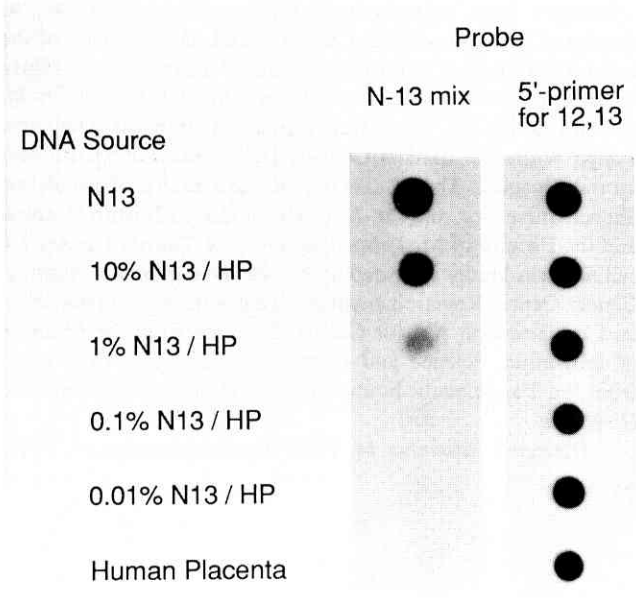

Fig. 1. Sensitivity of PCR and differential dot-blot hybridization. Genomic DNA which has a point mutation at codon 13 of the N-*ras* oncogene (N13)[4] was diluted with human placental DNA (HP) to the concentration indicated at the left side of the panel. Each DNA mixture was amplified *in vitro* with 5′- and 3′-primers for codons 12 and 13, and dot-blotted onto nylon filters. The filters were then hybridized with oligonucleotide probes designated at the top of the panel, washed and visualized as described in the text.

Our present study has demonstrated that PCR is of great value in detecting the mutational activation of the N-*ras* oncogene. The method is much simpler and faster than previous methods such as DNA transfection assay and sequencing. It has a further advantage in that many samples can be analyzed simultaneously. Not only activated N-*ras* oncogene but also other mutated genes can be investigated using this method. Previously we reported mutations at codon 13 of N-*ras* oncogene in three MDS patients using *in vivo* transfection assay.[4] In the present investigation, however, we found N-*ras* mutations at codon 13 in three AML patients and at codon 12 or 61 in three MDS patients. Using PCR, every point mutation at codon 12 or 13 or 61 has been reported in both AML and MDS patients.[4, 5, 8–11] Therefore the positions of mutation may have little, if any, relationship with the diagnosis of acute leukemia, or leukemia transformed from MDS. We previously postulated that N-*ras* activation is critical in the progression from MDS to overt leukemia.[4] In our present study, the ratios of the number of patients with overt leukemia from MDS to that of patients with MDS are 33% and 19% in those carrying mutated N-*ras* oncogenes and those carrying wild-type N-*ras* oncogenes, respectively. The result seems to be compatible with the hypothesis, though the difference of the ratio is statistically not significant. A further extensive and prospective study in progress will clarify the significance of N-*ras* activations in the process of

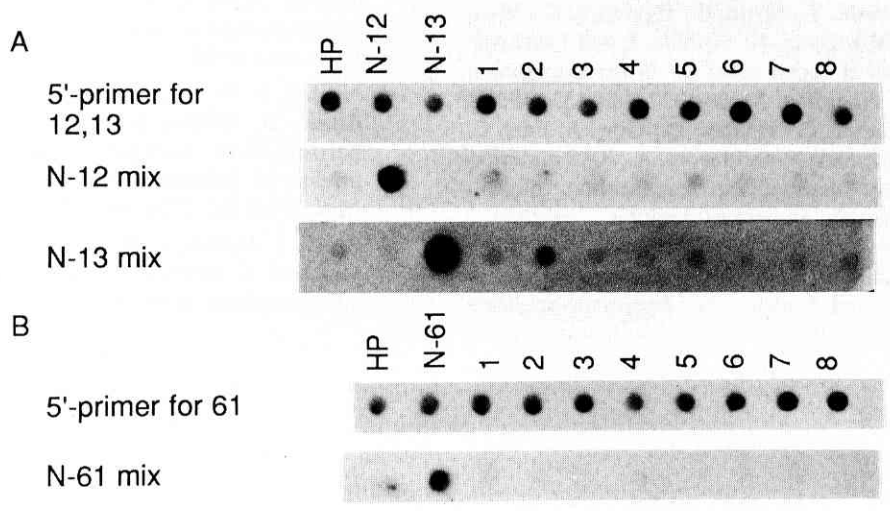

Fig. 2. Detection of N-*ras* mutations. Samples of examined cases are shown. Sources of DNAs were: HP, human placenta; N-12,[8] N-13[4] and N-61, positive controls which have N-*ras* oncogenes mutated at codons 12, 13 and 61, respectively; No. 1 and 2, AML patients No. 8 and 9 in Table II; No. 3–8, MDS patients No. 3–8 in Table I. Each DNA was amplified *in vitro* with 5′- and 3′-primers for codons 12, 13 (A), and 61 (B), and hybridized with the oligonucleotide probe shown at the left side of the panel as described in the text. A point mutation at codon 13 was detected in No. 9 AML patient.

leukemogenesis. The use of PCR and differential dot-blot hybridization is also useful to evaluate the clinical condition of leukemia patients after treatment. Clinical diagnosis of complete remission is simply based on the number of morphologically determined blast cells in the bone marrow. Therefore, it is sometimes difficult to estimate the nature of remaining blast-like cells after treatment, as in the case of No. 9 AML patient. By utilizing PCR, however, a small malignant population in this case could be recognized. Because of the high sensitivity and the short period required for the whole procedure (only three days are needed), this method is expected to be highly useful in the clinical context such as in the estimation of patients' status, the prediction of prognosis and the choice of treatment.

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