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Increased Expression of *ras* Genes in Non-Hodgkin's Lymphomas Is not Associated with Oncogenic Activation of Those Genes by Point Mutation

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Twenty-three cases of non-Hodgkin's lymphoma (NHL) were analyzed for expression of *ras* genes by *in situ* hybridization utilizing biotinylated DNA probes. Increased expression of Ki-*ras*, Ha-*ras* and N-*ras* genes was observed in 12 cases, 6 cases and 1 case of NHL, respectively. Genomic DNA extracted from these 23 cases of NHL was region-specifically amplified by means of polymerase chain reaction to examine the presence of point mutations at the 12th, 13th and 61st codons of Ki-, Ha- and N-*ras* genes. Dot hybridization assays with appropriate oligonucleotide probes showed no evidence of point mutation in any case of NHL examined. These results indicate that increased expression of *ras* genes in NHL is not associated with *ras* gene activation by point mutation.

Key words: Lymphoma — *ras* — *In situ* hybridization — Point mutation — Polymerase chain reaction

Increased expression of *ras* proto-oncogene products or expression of aberrant gene products due to the point mutation of *ras* has been observed in various human cancers.[1,2] A series of experiments have shown that both changes might lead to cellular transformation, indicating essential roles of *ras* genes in human oncogenesis.

Our previous analyses of non-Hodgkin's lymphomas (NHL) have shown that certain cases of NHL are associated with increased expression of *ras* genes. Analyses have been performed by immunohistochemical stainings with anti-*ras* p21 monoclonal antibodies, and also by *in situ* hybridization assays with biotinylated DNA probes of oncogenes.[3–5]

In this study we examined whether those cases of NHL with increased expression of *ras* genes are associated with activation of *ras* genes by point mutation. Twenty-three cases of NHL were typed for the expression of each *ras* gene by *in situ* hybridization and also were analyzed for the presence of the point mutation of *ras* genes by utilizing oligonucleotide probes and cellular DNA amplified by polymerase chain reaction (PCR).

Biopsied lymphoma tissues were maintained at −80°C until use. Parts of specimens were utilized for histopathological diagnosis and also for typing of immunological surface markers. *In situ* hybridization to detect expression of *ras* genes was performed as previously described.[5] DNA was extracted from frozen tissue samples and was amplified so as to analyze the presence of mutations at codons 12, 13 and 61 of c-Ki-*ras*, c-Ha-*ras* and c-N-*ras* by the PCR method as described by Saiki *et al.*[6] The round of amplification was repeated 35 to 40 times. The presence of the point mutation of *ras* genes was analyzed by dot hybridization assays using oligonucleotide probes.[7] Tables I and II show the primers used for the gene amplification and oligonucleotide probes used for dot hybridization assays.

Expression of Ki-*ras* was increased in 12 of the 23 cases examined (2 cases each of follicular type and small lymphocytic type, 4 cases each of diffuse small cleaved type, diffuse mixed type, diffuse large cell type and large cell immunoblastic type, and 3 cases of lymphoblastic type lymphomas according to the International Working Formulation), and those of Ha-*ras* and N-*ras* were increased in 6 cases and 1 case, respectively. Figure 1 shows examples of *in situ* hybridization and Table III summarizes the results. No correlation of increased expression of *ras* genes with histological type of NHL was observed.

Specificity of the *in situ* hybridization assays was, as previously reported, supported by the following considerations[5]: expression of *TCRβ* and *IgH* genes in NHL detected by *in situ* hybridization was consistent with the results of immunological typings using monoclonal antibodies; positive reactions were observed in cytoplasma of NHL; the results of *in situ* hybridization were compatible with those of dot hybridization per-

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Table I. PCR Primer Sequences

Target	Sequence		Fragment amplified (bp)
N-*ras* 12, 13	GACTGAGTACAAACTGGTGG	(sense)	109
	CTCTATGGTGGGATCATATT	(antisense)	
N-*ras* 61	GGTGAAACCTGTTTGTTGGA	(sense)	103
	ATACACAGAGGAAGCCTTCG	(antisense)	
H-*ras* 12, 13	CTCTATAGTGGGGTCGTATT	(sense)	111
	ATGACGGAATATAAGCTGGT	(antisense)	
H-*ras* 61	AGACGTGCCTGTTGGACATC	(sense)	73
	CGCATGTACTGGTCCCGCAT	(antisense)	
K-*ras* 12, 13	GACTGAATATAAACTTGTGG	(sense)	108
	CTATTGTTGGATCATATTCG	(antisense)	
K-*ras* 61	TTCCTACAGGAAGCAAGTAG	(sense)	128
	CACAAAGAAAGCCCTCCCCA	(antisense)	

Table II. The Oligomers Used to Analyze *ras* Gene Mutations

Probe (oligomer)			Sequence (sense)							
N-*ras*	12	wt	GGA	GCA	GGT	GGT	GTT	GGG	AA	
		P1			nGT					n=A,C,T
		P2			GnT					n=A,C,T
	13	P1				nGT				n=A,C,T
		P2				GnT				n=A,C,T
	61	wt	ACA	GCT	GGA	CAA	GAA	GAG	TA	
		P1				nAA				n=A,G
		P2				CnA				n=C,T,G
		P3				CAn				n=C,T
H-*ras*	12	wt	GTG	GGC	GCC	GGC	GGT	GTG	GG	
		P1				nGC				n=A,C,T
		P2				GnC				n=A,C,T
	13	P1					nGT			n=A,C
		P2					GnT			n=A,C,T
	61	wt	ACC	GCC	GGC	CAG	GAG	GAG	TA	
		P1				nAG				n=A,G
		P2				CnG				n=G,C,T
		P3				CAn				n=C,T
K-*ras*	12	wt	GTT	GGA	GCT	GGT	GGC	GTA	GG	
		P1				nGT				n=A,C,T
		P2				GnT				n=A,C,T
	13	P1					nGC			n=A,C,T
		P2					GnC			n=A,C,T
	61	wt	ACA	GCA	GGT	CAA	GAG	GAG	TA	
		P1				nAA				n=A,G
		P2				CnA				n=G,C,T
		P3				CAn				n=C,T

wt, wild type.

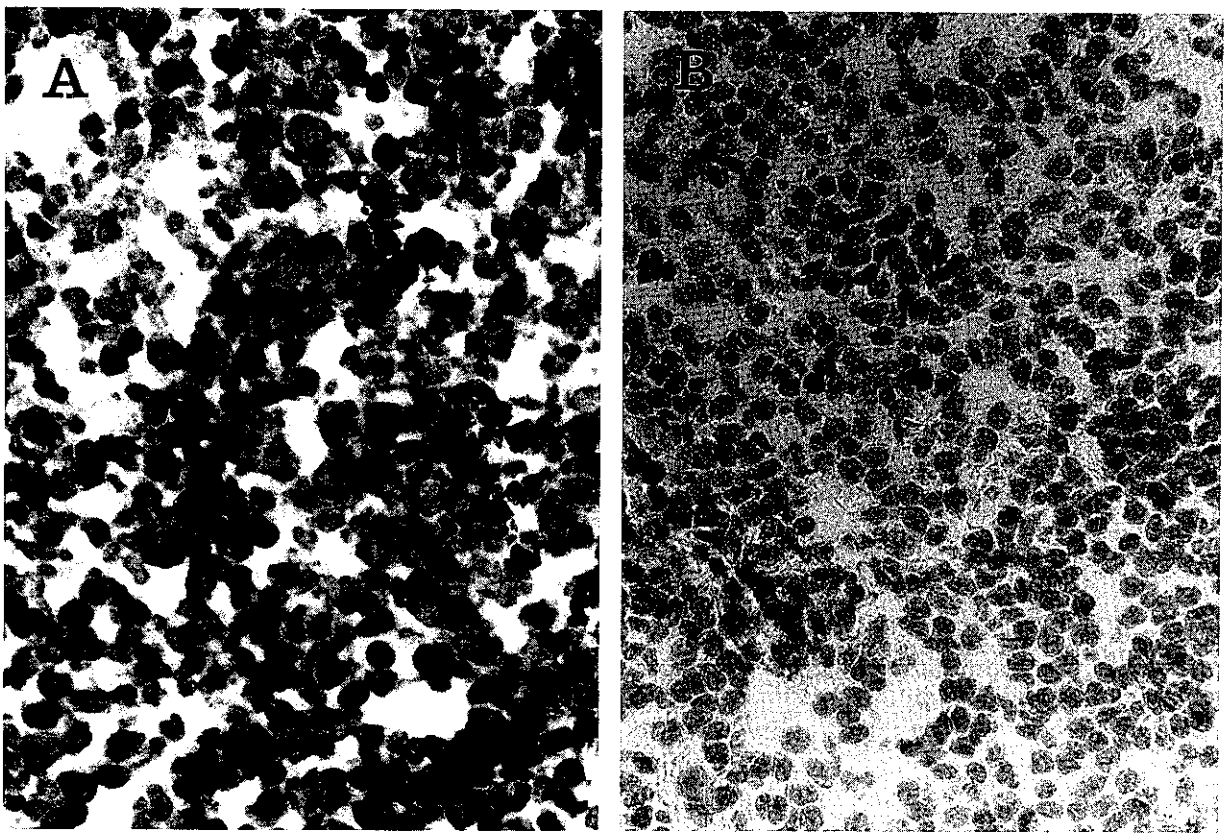

Fig. 1. Examples of *in situ* hybridization of NHL with Ki-*ras* gene probes. A, NHL scored as positive (+～++); B, NHL scored as negative (−). The comparative analyses of *in situ* hybridization assays and dot hybridization assays indicate that samples scored as (+) in the *in situ* hybridization assays have 8 to 16 times greater copy numbers of mRNA when compared with samples scored as (−). Samples scored as (±) generally have 2 to 4 times greater copy numbers of mRNA.

formed with RNA extracted from the same samples. In addition, in this study 12 samples were also analyzed by immunohistochemical stainings utilizing anti-ras p21 monoclonal antibodies we previously prepared.[4] The results of the two assays are essentially compatible as shown in Table IV.

The presence of the point mutation at the 12th, 13th and 61st codons of Ki-, Ha- and N-*ras* genes was also examined by dot hybridization assays with appropriate oligonucleotide probes. The regions around the 12th, 13th and 61st codons of each *ras* gene were preamplified by means of PCR. Point mutation was not found in any of the 23 cases of NHL. Examples of dot hybridization assays are shown in Fig. 2.

These results, taken together, indicate that increased expression of *ras* genes observed in NHL is independent of point mutation and also that the point mutation of *ras* genes at the 12th, 13th and 61st codons is rare, if it occurs at all, in NHL.

Table III. Expression of *ras* Genes in 23 Cases of NHL Tested by *in situ* Hybridization

Grade of expression	Ki-*ras*	Ha-*ras*	N-*ras*
+～++[a)]	6[b)]	3	1
±	6	3	0
−	11	17	22

a) Scored according to criteria shown in Fig. 1.
a) No. of cases.

Growing evidence indicates an essential role of the *ras* genes in human oncogenesis. Activated *ras* genes with transformation activity have been detected in a wide variety of cancers. More recent analyses with oligonucleotide probes to directly detect mutated *ras* genes have shown that significant proportions of cancer cases are

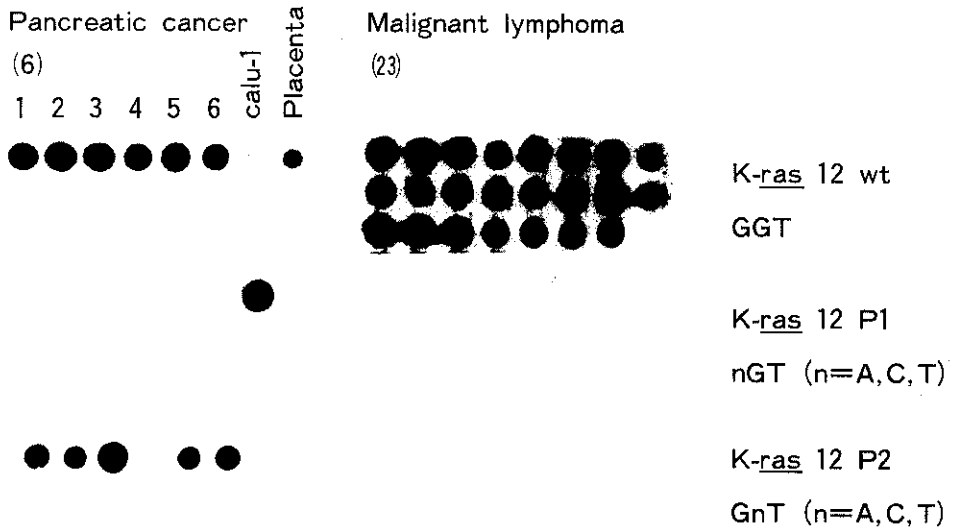

Fig. 2. Examples of dot hybridization assays to characterize mutations at codon 12 of the Ki-*ras* gene. Twenty-three cases of NHL, 6 cases of pancreatic cancer and a lung cancer cell line, Calu-1 were examined by using an oligonucleotide probe with the normal *ras* gene (wild type; wt) and also oligonucleotide probes to detect point mutations of the first and second nucleotides. Five cases of pancreatic cancer have point mutations in the second nucleotide and Calu 1 has one in the first nucleotide. Calu 1 is known to lack a normal allele. Though examples show dot hybridization assays with mixed oligonucleotide probes for detection of mutations, analyses have been done with individual oligonucleotide probes as well.

Table IV. Comparative Analysis of 12 Cases of NHL by *in situ* Hybridization Assays and Immunohistochemical Stainings

Cases of NHL	Expression of *ras* genes detected by			
	in situ hybridization			immunohisto-chemistry
	Ki-*ras*	Ha-*ras*	N-*ras*	by RASK-3[a]
B cell type				
L-3	−	−	−	−[b]
L-12	+	+	−	+
L-29	+	−	−	±
L-48	−	−	−	−
L-51	−	−	−	±
L-52	−	±	−	±
L-73	−	−	−	−
T cell type				
L-1	+	±	−	+
L-17	−	−	−	±
L-19	±	−	−	+
L-64	+	±	−	+
L-65	−	+	−	±

a) RASK-3 is a monoclonal antibody reactive with all of Ki-, Ha- and N-*ras* p21.[4]
b) Scored according to ref. 4.

associated with the point mutation of *ras* genes.[8-13] These include cancers of pancreas, colon, lung, breast and thyroid. In hematological malignancy, point mutation particularly of N-*ras* has been found in some 30% of acute myelogenous leukemia.[14,15] The mutated *ras* genes, however, have only rarely been found in acute lymphoblastic leukemias.[15] Recently, a paper by Neri *et al.* was published reporting an analysis of the *ras* gene mutation in 178 cases of lymphoid malignancy.[16] Out of 88 cases of NHL none showed the point mutation and 6 out of 33 cases of acute lymphoblastic leukemia had mutations involving codons 12 or 13 of N-*ras* gene. Our analysis of 23 cases of NHL including those with apparently increased expression of *ras* genes, revealed no case with point mutation, which indicates their scarcity. Therefore, the contribution of activated *ras* genes to oncogenesis in lymphoid tissues might be rather limited.

Increased expression of *ras* proto-oncogenes in NHL, on the other hand, must be influential to determine the cellular characteristics of these NHL, though details are still unknown. It is therefore important to have precise profiles of expression of *ras* genes in NHL and to compare them with other biological characteristics of NHL such as histopathology, immunological markers and clinical course.

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REFERENCES

1) Barbacid, M. *Ras* genes. *Ann. Rev. Biochem.*, **56**, 779–827 (1987).
2) Bos, J. L. The *ras* gene family and human carcinogenesis. *Mutat. Res.*, **195**, 255–271 (1988).
3) Mitani, S., Sugawara, I., Shiku, H. and Mori, S. Expression of c-*myc* oncogene product and *ras* family oncogene products in various human malignant lymphomas defined by immunohistochemical techniques. *Cancer*, **62**, 2085–2093 (1988).
4) Yoshida, K., Hamatani, K., Koide, H., Ikeda, H., Nakamura, N., Akiyama, M., Tsuchiyama, H., Nakayama, E. and Shiku, H. Preparation of anti-*ras* Mr 21,000 protein monoclonal antibodies and immunohistochemical analyses on expression of *ras* genes in human stomach and thyroid cancers. *Cancer Res.*, **48**, 5503–5509 (1988).
5) Hamatani, K., Yoshida, K., Kondo, H., Toki, H., Okabe, K., Motoi, M., Ikeda, S., Mori, S., Shimaoka, K., Akiyama, M., Nakayama, E. and Shiku, H. Histological typing of non-Hodgkin's lymphomas by *in situ* hybridization with DNA probes of oncogenes. *Blood*, in press.
6) Saiki, R., Sharf, S., Faloona, F., Mullis, K., Horn, G., Ehrlich, H. A. and Arnheim, N. Enzymatic amplification of β-globin genetic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science*, **23**, 1350–1353 (1985).
7) Verlaan-de Vries, M., Bogaard, M. E., van den Elst, H., van Boom, J. H., van der Eb, A. J. and Bos, J. L. A dot-blot screening procedure for mutated *ras* oncogenes using synthetic oligodeoxynucleotides. *Gene*, **50**, 313–320 (1986).
8) Almoguera, C., Shibata, D., Forrester, K., Martin, J., Arnheim, N. and Perucho, M. Most human carcinomas of the exocrine pancreas contain mutant c-K-*ras* genes. *Cell*, **53**, 549–554 (1988).
9) Bos, J. L., Fearon, E. R., Hamilton, S. R., Verlaan-de Vries, M., van Boom, J. H., van der Eb, A. J. and Vogelstein, B. Prevalence of *ras* gene mutations in human colorectal cancers. *Nature*, **327**, 293–297 (1987).
10) Forrester, K., Almoguera, C., Han, K., Grizzle, W. E. and Perucho, M. Detection of high incidence of K-*ras* oncogenes during human colon tumorigenesis. *Nature*, **327**, 298–303 (1987).
11) Rodenhuis, S., Slebos, R. J. C., Boot, A. J. M., Evers, S. G., Mooi, W. J., Wagenaar, S. S., van Bodegom, P. C. and Bos, J. L. Incidence and possible clinical significance of K-*ras* oncogene activation in adenocarcinoma of the human lung. *Cancer Res.*, **48**, 5738–5741 (1988).
12) Groften, L. Y. J. and Valenzuela, D. M. c-Ki-*ras* mutations in human carcinomas occur preferentially in codon 12. *Oncogene*, **1**, 315–318 (1987).
13) Suarez, H. G., Duvillard, J. A., Callow, B., Schlumberger, M., Tubiana, M., Parmentier, C. and Monier, R. Detection of activated *ras* oncogenes in human thyroid carcinomas. *Oncogene*, **2**, 403–406 (1988).
14) Farr, C. J., Saiki, R. K., Erlich, H. A., Mocormick, F. and Marshall, C. J. Analysis of RAS gene mutations in acute myeloid leukemia by polymerase chain reaction and oligonucleotide probes. *Proc. Natl. Acad. Sci. USA*, **85**, 1629–1633 (1988).
15) Senn, H-P., Tran-Thang, C., Wodnar-Filipowicz, A., Jiricny, J., Fopp, M., Gratwohl, A., Signer, E., Weber, W. and Moroni, Ch. Mutation analysis of the N-*ras* proto-oncogene in active and remission phase of human acute leukemias. *Int. J. Cancer*, **41**, 59–64 (1988).
16) Neri, A., Knowles, D. M., Greco, A., McCormick, F. and Dalla-Favera, R. Analysis of *ras* oncogene mutations in human lymphoid malignancies. *Proc. Natl. Acad. Sci. USA*, **85**, 9268–9272 (1988).