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L-myc Restriction Fragment Length Polymorphism in Japanese Patients with Esophageal Cancer

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L-myc polymorphism is a representative genetic trait related to an individual's susceptibility to several cancers. However, there have been no reports concerning the association between esophageal cancer and L-myc polymorphism. To analyze the distribution of polymorphism in Japanese patients with esophageal cancer, a molecular genotyping method using a polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) was used. Based on an analysis of 65 Japanese patients with esophageal cancer and 107 healthy control subjects, a significant difference was observed in either the distribution of genotypes (P=0.012) or of allele frequencies between the two groups (P=0.004). The relative risk of esophageal cancer for genotypes including the shorter allele was 2.9 compared to the longer allele homozygote. Furthermore, the patients with S-allele had a tendency for poor prognosis among those with three genotypes. A significant difference between the distribution of genotypes and the incidence of lymph node metastasis was found based on the clinicopathological features of the cancers. These results suggest that L-myc polymorphism may be implicated as a genetic trait affecting an individual's susceptibility to esophageal cancer, at least among Japanese patients.

Key words: Esophageal cancer — L-myc — RFLP — Cancer susceptibility

L-myc polymorphism has been documented to be a genetic trait which influences an individual's susceptibility to cancers. In an effort to find a suitable genetic marker that may allow us to detect a predisposition to the initiation or progression of cancer, restriction fragment-length polymorphism (RFLP) of the L-*myc* gene has been investigated in various types of cancers.[1–4)]

L-myc RFLP is known to be caused by an *Eco*RI restriction site at the nucleotide position (nt) 2886 in intron 2, which produces two types of alleles, the longer allele (L) and the shorter allele (S), with three genotypes (LL, LS, and SS types). Previous studies have reported that patients having the S-allele showed a much higher incidence of metastasis and a poor prognosis for lung and renal cancer.[1, 2, 4)] We have very recently shown the relationship between genetic susceptibility to gastric cancer and L-myc polymorphism.[5)] Consequently, these results suggested that L-myc polymorphism had some influence on the susceptibility to cancer. No study on the relevance of L-myc polymorphism to esophageal carcinogenesis, however, has yet been reported.

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We recently developed a simple and rapid genotyping method by using polymerase chain reaction (PCR)-based RFLP (PCR-RFLP) that discriminates three genotypes according to the combinations of these alleles. In the present study, we applied this method to analyze the distribution of L-myc polymorphism in patients with esophageal cancer and a large group of healthy Japanese people in an attempt to resolve the debate as to whether any relationship exists between polymorphism and the risk of developing this neoplasm.

MATERIALS AND METHODS

Subjects Sixty-five randomly selected esophageal cancer patients who underwent surgery at the Medical Institute of Bioregulation Hospital, Kyushu University, Oita Prefectural Hospital and Saitama Cancer Center Hospital between 1994 and 1995, and 107 healthy volunteers as a control group were included in this study. Of the 65 cancer cases, 62 had squamous cell carcinoma and 3 had small cell carcinoma, histologically. The control group showed no difference from the patient group in age, sex, or ethnic origin, and no history of inbreeding or specific exposure to carcinogens. Informed consent was obtained from all participants.

Determination of the L-myc genotype The genomic DNA obtained from normal esophageal mucosa of the esophageal cancer patients and from the peripheral blood cells of the healthy volunteers, approximately 100 ng per sample, was amplified by PCR. The amplification of genomic DNA was carried out by PCR in a total volume which included a 10× PCR buffer (100 m*M* Tris-HCl, pH 8.3, 500 m*M* KCl, 15 m*M* $MgCl_2$, 1% Triton X-100), 25 m*M* dNTP (mixed dATP, dCTP, dGTP, and dTTP, each 100 m*M*), 25 m*M* of each L-myc primer, and 1 unit of *Taq* DNA polymerase (Promega, Madison, WI). The oligonucleotide primers used for amplification were: upstream primer 5′-ACGGCTGGTGGAGTGGTAGA-3′; downstream primer 5′-AAGCTTGAGCCCCTTTGTCA-3′, which were synthesized in our laboratory specifically to amplify part of intron 2. The conditions for the PCR were: 30 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 2 min, and extension at 72°C for 2 min. The amplified DNAs were all digested with 5 units of *Eco*RI at 37°C for 1 h. After the digested DNA fragments were separated by 2.0% agarose gel electrophoresis, the responsible L-myc RFLP alleles were identified in each sample. The genotypes were classified into three types according to the combination of L-myc alleles as follows: a homozygote of the L-allele which had no *Eco*RI restriction site, a homozygote of the S-allele which had an *Eco*RI restriction site, and a heterozygote of L- and S-alleles.

Statistical analysis for comparison between patients with cancer and the control subjects The significance of variations in the L-myc genotypes and allele frequencies between the patients and control subjects was determined by using the χ^2 test and logistic regression analysis for all subjects. Additional analysis was performed after subdividing the patients with esophageal cancer according to their clinicopathological factors using the χ^2 test. *P* values of less than 0.05 were considered to be significant in all analyses.

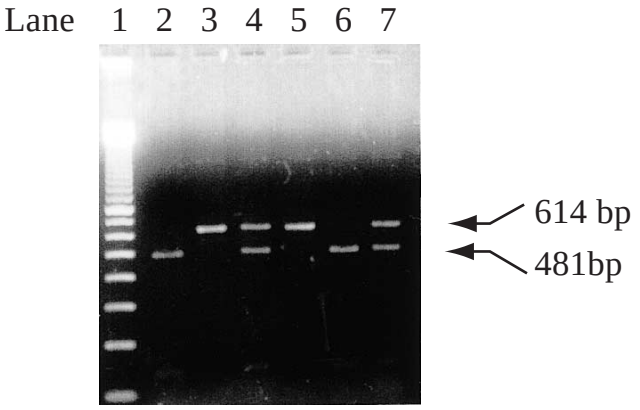

Fig. 1. L-myc RFLP analysis of genomic DNA. The amplified DNA fragments were digested with *Eco*RI, then applied to 2.0% agarose gel containing ethidium bromide. Lane 1, a 100-base ladder as a molecular marker; lanes 2 and 6, the homozygote for the S-allele (481 bp); lanes 3 and 5, the homozygote for the L-allele (614 bp); lanes 4 and 7, the heterozygote for the S- and L-allele.

Table I. Distribution of L-myc RFLP Genotypes in Patients with Esophageal Cancer and Healthy Subjects

	n	Number of genotypes (%)			Odds ratio[a] (95%CI)	*P*[b]
		SS	LS	LL		
Patients	65	20 (30.8)	36 (55.4)	9 (13.8)		
					2.90 (0.54−6.54)	0.012
Control	107	18 (16.8)	55 (51.4)	34 (31.8)		

a) Odds ratio was ascertained based on the distribution of two groups (SS plus LS versus LL).
b) *P* value was calculated according to the χ^2 test.

RESULTS

L-myc polymorphism in genomic DNAs isolated from the patients with esophageal cancer and normal populations was examined by the PCR-RFLP method. The *Eco*RI digestion of PCR products of the DNA revealed 614 bp (L) and 481 bp (S) fragments that were homologous to the L-myc sequence and gave rise to three genotypes (Fig. 1).

The incidences of L-myc genotypes are summarized in Table I. The distribution of each genotype in the two populations closely fits the Hardy-Weinberg equilibrium. There was a statistically significant difference in the distribution of the genotypes (χ^2=9.29, *P*=0.012) between all the patients and the control subjects. The relative risk (odds ratio) of esophageal cancer for the LS and SS genotypes compared to the LL genotype was 2.90 (95% confidence interval (CI): 0.54−6.54) (Table I). When the genotypes were classified into two groups as "SS genotype" and "LS plus LL genotypes," the "SS genotype" also showed higher risk than "LS plus LL genotype" (odds ratio : 2.20, 95% CI: 0.59−4.57).

When the patients with cancer were subdivided according to the clinicopathological factors, such as histology, location, depth of invasion, lymph node metastasis, lymphatic involvement, vascular involvement and clinical stage, a significant difference between the distribution of genotypes and the incidence of lymph node metastasis was found (Table II).

The allele frequency in each group is shown in Table III. The S-allele was more frequent than the L-allele in the patient group, compared with the distribution of each allele in the normal control group (significantly different, χ^2=7.60, *P*=0.004).

Table II. Relationship between L-myc Genotypes and Clinicopathological Features of Esophageal Cancer

Variable	No. of subjects (%)	L-myc genotypes (%)			$P^{a)}$
		LL	LS	SS	
Histology					
ifferentiated	35 (100)	2 (6)	22 (63)	11 (31)	0.21
Undifferentiated	13 (100)	3 (23)	7 (54)	3 (23)	
Location					
Lower	16 (100)	2 (13)	8 (50)	6 (38)	0.67
Middle	28 (100)	2 (7)	19 (68)	7 (25)	
Upper	4 (100)	1 (25)	2 (50)	1 (25)	
Depth of invasion					
No adventitial invasion	15 (100)	1 (7)	11 (73)	3 (20)	0.47
With adventitial invasion	33 (100)	4 (5)	18 (63)	11 (32)	
Lymph node metastasis					
Positive	40 (100)	5 (8)	21 (56)	14 (36)	0.04[b)]
Negative	8 (100)	0 (0)	8 (100)	0 (0)	
Lymphatic involvement					
Positive	42 (100)	4 (10)	25 (60)	13 (31)	0.72
Negative	6 (100)	1 (17)	4 (67)	1 (17)	
Vascular involvement					
Positive	38 (100)	3 (8)	23 (61)	12 (32)	0.48
Negative	10 (100)	2 (20)	6 (60)	2 (20)	
Clinical stage					
TNM I	3 (100)	0 (0)	3 (100)	0 (0)	0.54
II	14 (100)	2 (14)	9 (64)	3 (21)	
III	31 (100)	3 (10)	17 (55)	11 (35)	

a) *P* value was calculated according to the χ^2 test.
b) The distribution of genotype between patients subdivided according to lymphnode metastasis is statistically significant (χ^2=6.29).

We also analyzed the association between the genotype of L-myc polymorphism and overall survival. The survival curves plotted by the method of Kaplan-Meier are shown in Fig. 2. Statistical analysis of the results by means of the log rank (Mantel-Cox) test revealed that patients with genotype SS tended to have the poorest prognosis and the survival rate of LL type patients was highest among three genotypes, although these associations were not significant (*P*=0.09).

Table III. Distribution of L-myc Alleles in Patients with Esophageal Cancer and Healthy Subjects

	n	Number of alleles (frequency)		RR (95%CI)	$P^{a)}$
		S	L		
Patients	130	76 (0.585)	54 (0.415)	1.90 (1.06–2.95)	0.004
Control	214	91 (0.425)	123 (0.575)		

a) The *P* value was calculated according to the χ^2 test.
RR, relative risk; 95%CI, 95% confidence interval.

DISCUSSION

The L-*myc* gene is a nuclear oncogene thought to be activated late in tumorigenesis,[6)] and was initially isolated on the basis of its high level amplification in a subset of human small-cell lung cancers and its homology to a conserved region of exon 2 of other *myc* genes, such c- and N-*myc*.[7)] Since a study of the L-myc RFLP of Japanese lung cancer patients was first reported in 1988,[1)] many investigators have analyzed the L-myc RFLP types in cases of lung cancer and other malignant tumors. These studies have shown that L-myc polymorphism is linked to both cancer susceptibility and prognosis. The role in cancer susceptibility has been analyzed by means of studies examining differences in genotype distribution between cancer patients and controls.[5, 8)] As regards the role of L-

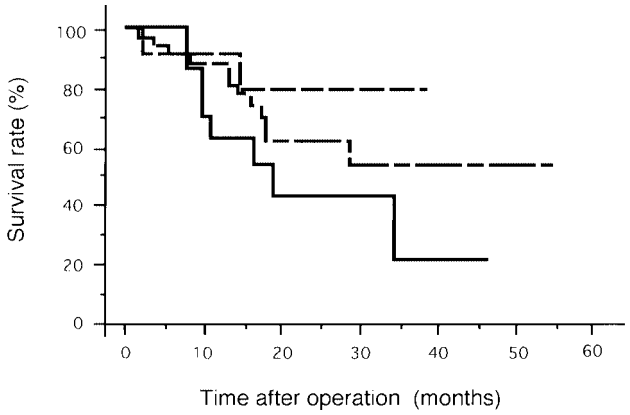

Fig. 2. Survival curves in 65 patients with esophageal cancer according to L-myc genotype. The patient group with the S-allele tended to have a poorer prognosis than that with the L-allele. *P* values were obtained using the log rank test ($P=0.09$). – – – LL, – – – LS, —— SS.

myc in prognosis, studies on lung,[4] kidney,[2] stomach,[5] bone or soft tissue sarcomas,[8] and breast[9] showed that the presence of the S-allele (either LS or SS genotype) was associated with earlier lymph node involvement or metastasis, or a poorer prognosis than that of the LL genotype. However, a clear correlation between the L-myc RFLP and susceptibility to cancer or prognosis was found only in certain types of cancers or restricted populations. For example, no correlation was found in colorectal cancer,[3] breast cancer, melanoma or hepatoma,[10] bladder cancer,[11] or gastric cancer in Russia,[12] while even in cases of lung cancer, an association may not exist in some types of small-cell lung cancer.[4] In contrast, a protective effect of the SS genotype was reported in hepatocellular carcinoma.[13, 14]

The present study indicated that there was a significant difference in the distribution of L-myc RFLP between patients with esophageal cancer and healthy subjects. This positive finding was also observed for patients classified into two genotypes "LS type plus SS type" and "LL type," and the group of "LS plus SS type," which showed a high value of relative risk, was thought to be a higher risk group compared with the group of "LL type." L-myc polymorphism in esophageal cancer patients also showed a unique relationship between the clinical prognosis and the genotype. Our results, therefore, support the idea that, at least for Japanese patients, the L-myc locus is involved in a genetic predisposition to esophageal cancer and imply that L-myc RFLP analysis may be a useful marker for predicting the progression of esophageal cancer. These findings are compatible with the findings for Japanese lung cancer patients. On the other hand, several studies on lung cancer in different ethnic populations have obtained conflicting results.[15, 16] A possible explanation for these contradictory results may be differences in environmental factors or in the ethnic origins of the patients analyzed. The cancer incidence of many, if not all, tumor types will also vary due to both environmental and genetic factors. Further studies of ethnic differences in allele distributions need to be made. For example, black Americans show a higher frequency of the SS genotype of L-myc.[15]

In this study, we adopted a molecular genotyping method using PCR-RFLP. From a clinical viewpoint, this method appears to be an accurate and reliable approach for the mass screening of a large number of patients, because this analysis can be rapidly and simply performed by examining only a small amount of genomic DNA. It has been reported that cigarette smoking, alcohol intake and family history are important factors influencing the occurrence of esophageal cancer.[17] Therefore, this method should be applied to high risk groups with such individual risk habits as smoking, drinking and a positive family history.

The polymorphic site of the L-*myc* gene exists in the intron, and the reason why RFLP of the L-*myc* gene is related to the progression of human cancer is also an interesting problem. No functional difference between the proteins encoded by S and L alleles has yet been described and the role of L-myc in esophageal cancer remains unclear. It is possible that the polymorphism of L-myc might have no biological effect except as a marker in linkage disequilibrium with an as-yet-unknown susceptibility gene. It is, of course, possible that the L-*myc* oncogene itself is directly involved. The L-*myc* gene has been shown to undergo alternative splicing into several different mRNAs in some small cell lung cancer cell lines. A substantial portion of intron 2 is included in some transcripts.[18] The role of L-myc RFLP in esophageal cancer will require further study.

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