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Close correlation between restriction fragment length polymorphism of the L-*MYC* gene and metastasis of human lung cancer to the lymph nodes and other organs

(cancer diagnosis/cellular oncogene)

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ABSTRACT **Restriction length fragment polymorphism of the L-*MYC* gene was examined in DNAs from lung cancer tissues and normal tissues of 51 Japanese patients with lung cancer. In individual patients, no difference was seen between the restriction length fragments of the two alleles of L-*MYC* [6-kilobase (kb) and 10-kb fragments in *Eco*RI digests] in lung cancer tissues and normal tissues. But a striking correlation was found between the restriction length fragment polymorphism pattern of L-*MYC* and the extent of metastasis, particularly to the lymph nodes at the time of surgery: Patients with only the L band (10 kb) had few lymph node metastatic lesions, whereas patients with either the S band (6 kb) or the S and L bands almost always had lymph node metastatic lesion. A similar correlation was found between the presence of the S band and metastases to other organs. This correlation was particularly marked in cases of adenocarcinoma. These results indicate a clear genetic influence on metastases and a consequent poor prognosis for certain patients of lung cancer; L-*MYC* restriction length fragment polymorphism is thus shown to be a useful marker for predicting the metastatic potential of human lung cancer.**

Some cancers, such as familial polyposis, retinoblastoma, and Wilms tumor are considered hereditary cancers (1, 2); but even in other cancers, genetic background may relate to cancer susceptibility and to progression of the disease. Restriction fragment length polymorphism (RFLP) (3) of some cellular oncogenes—that is, genetic predisposition—may relate to the incidence or progression of a particular human cancer. When so, RFLP analysis of these oncogenes should prove useful in diagnosis and treatment. However, in extensive studies in several laboratories, close correlation between RFLP and severity of cancer has not been found (4–8).

We report here that RFLP of L-*MYC* is a good marker for determining predisposition of patients with lung cancer for metastasis to lymph nodes and other organs.

MATERIALS AND METHODS

Isolation of DNA from Surgical Specimens and Peripheral Blood Leukocytes. Primary lung tumors and normal parts of resected lung, as controls, were obtained immediately after cancerous tissue removal during surgery. An adrenal gland with a metastasis was resected from one patient at the time of lung lobectomy. Operations were done at the National Cancer Center Hospital, Tokyo, between August 1986 and August 1987. Leukocytes were obtained by fractionation of peripheral blood from normal adults and patients with lung cancer in Ficoll-Paque solution. Samples were frozen and stored at −80°C until isolation of DNA. High-molecular weight DNA was prepared from tissues and leukocytes as described by Perucho *et al.* (9).

Southern Blot Analysis of the L-*MYC* Gene. DNA was digested with the restriction endonuclease *Eco*RI under standard conditions. A sample of about 10 μg of the digest DNA was subjected to electrophoresis on 0.8% agarose gel, and the fractionated DNA was transferred from the gel to a nitrocellulose membrane as described by Southern (10). A ^{32}P-labeled human L-*MYC* fragment was prepared using Amersham's multiprime DNA labeling system (11). The nitrocellulose filters were heated at 80°C for 4 hr and were then preincubated for 4 hr at 42°C in 50% formamide/5× SSPE (1× SSPE is 0.15 M NaCl/10 mM NaH_2PO_4/1 mM EDTA, pH 7.4)/5× Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin)/sonicated salmon sperm DNA at 0.1 mg/ml. Then filters were incubated for 18 hr at 42°C in 50% formamide/5× SSPE/2× Denhardt's solution/10% dextran sulphate containing sonicated salmon sperm DNA at 0.1 mg/ml and 50–100 ng of ^{32}P-labeled L-*MYC* fragment (specific activity, 5×10^5 cpm/ng). After the hybridization reaction, filters were washed at 65°C first with 2× SSC (1× SSC is 0.15 M sodium chloride/0.015 M sodium citrate)/0.1% $NaDodSO_4$ and then washed with 0.1× SSC/0.1% $NaDodSO_4$. For autoradiography, the filters were exposed to Kodak XAR-5 film with an intensifying screen at −80°C.

Human L-*MYC* Probe. The 1.8-kilobase (kb) human L-*MYC* DNA fragment used as probe was prepared from a recombinant plasmid (pJB327) that contains this fragment (*Sma* I–*Eco*RI); the plasmid, a gift from J. D. Minna (12), was obtained through the Japanese Cancer Research Resources Bank, Tokyo.

RESULTS

Nau *et al.* (12) have previously reported that *Eco*RI digests of human genomic DNA contain two L-*MYC*-related fragments (10.0 kb and 6.6 kb). They showed that the formations of these two *Eco*RI fragments is due to *Eco*RI restriction site polymorphism from the presence of two alleles for L-*MYC*. In addition, they frequently observed amplification and enhanced transcription of the L-*MYC* gene in human small-cell lung cancer cell lines (12).

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Abbreviations: RFLP, restriction fragment length polymorphism; L-L, patient homozygous for 10-kb L-*MYC* fragment; S-S, patient homozygous for 6-kb L-*MYC* fragment; L-S, patient heterozygous for L-*MYC*.

Table 1. Data on patients with lung cancer and L-*MYC* RFLP patterns

	DNA pattern of L-*MYC*, no. of cases		
	L-L	L-S	S-S
Total number	10	18	23
Age, yr			
<49	4	3	3
50–69	4	9	16
>70	2	6	4
Sex			
Male	7	16	21
Female	3	2	2
Size of tumor, cm			
<4	1	6	10
4.1–8	5	10	10
>8.1	4	2	3
Diagnosis			
Adenocarcinoma	4	5	15
Squamous cell carcinoma	3	8	3
Large-cell lung cancer	2	1	1
Small-cell lung cancer	0	2	3
Squamous cell carcinoma and adenocarcinoma	0	1	0
Squamous cell carcinoma and large-cell lung cancer	0	1	0
Other	1	0	1
Grade of differentiation			
Well	2	3	2
Moderate	4	7	12
Poor	2	5	5
Unknown	2	3	4

L-L, patient homozygous for 10-kb L-*MYC* fragment; S-S, patient homozygous for 6-kb L-*MYC* fragment; L-S, patient heterozygous for L-*MYC*.

Therefore, in this work we analyzed L-*MYC*-associated RFLP in primary lung cancer tissues (adenocarcinoma, squamous cell carcinoma, and small-cell and large-cell lung cancers) as well as in adjacent normal lung tissues from 51 Japanese patients with lung cancer to determine whether L-*MYC* RFLP is related to the type and/or stage of lung cancer. The patients were 44 men and 7 women of ages 31–81 yr. Data on these patients, such as age, sex, and pathological diagnosis of their resected lung tumors, are summarized in Table 1. The tumor characteristics seem evenly distributed among the patients classified for type of L-*MYC* RFLP. Fig. 1 shows typical patterns of RFLP of L-*MYC* DNA from patients with lung cancer. Southern blot analysis showed two alleles of L-*MYC* (10 kb and 6 kb after *Eco*RI digestion, designated as fragment L and S, respectively in the DNAs from lung cancer tissues and their normal counterparts, thus confirming the report by Nau *et al.* (12). Scarcely any differences were found between the RFLPs or intensities of the two fragments in the lung cancer tissues and normal lung tissues of any individual patient thus far examined. However, it is noteworthy that in 3 of 17 patients who were heterozygous with respect to L-*MYC*, the L-*MYC* fragment S was amplified in lung tumor cells, as shown for patient 13 in Fig. 1.‡ Furthermore, in all cases examined, the pattern of L-*MYC* RFLP of DNAs from other organs, such as adrenal tissue and leukocytes from peripheral blood, were also found identical with that of lung cancer tissue (data not shown). These results indicate that L-*MYC* RFLP is genetically fixed, and no deletion or change of either alleles occurred in lung cancer cells so far examined.

Table 2. Correlation of L-*MYC* RFLP with metastasis to lymph nodes and other organs in 51 patients with lung cancer

	Fragments		
	L-L	L-S	S-S
Total cases	10	18	23
N−	8	2	3
N+	2	16	20
M−	9	15	15
M+	1	3	8

N−, no metastasis to lymph nodes; N+, metastasis to lymph nodes; M−, no metastasis to other organs; M+, metastasis to other organs.

Table 2 summarizes data on the relationship between metastasis to the lymph node and/or other organs at the time of surgery and the L-*MYC* RFLP pattern. Surprisingly, 8 of the 10 patients with only fragment L (homozygous with respect to the 10-kb L-*MYC* fragment, L-L), had no lymph node metastasis, whereas many patients with only fragment S (homozygous with respect to the 6-kb L-*MYC* fragment, S-S) or with both fragments (heterozygous with respect to L-*MYC*, L-S) had lymph node metastases. A similar correlation was found in cases of metastasis to other organs: Only one of 10 patients with fragment L-L had a metastasis to the brain, but 11 of 41 patients with fragment S-S or L-S had metastases to the brain, liver, bone, and kidney. Thus a strong correlation was found between the pattern of L-*MYC* RFLP and metastasis of lung cancer by Fisher's exact probability test. Only L-L versus S-S and L-S showed significantly lower incidences of lymph node metastasis ($P = 0.000$), and only S-S versus L-S and L-S showed significantly higher incidences of metastases to other organs ($P = 0.051$).

‡No amplification of L-*MYC* fragment L was seen in patients with heterozygous L-*MYC* alleles. The extent of amplification of the L-*MYC* gene was difficult to determine exactly in patients homozygous for fragment L or fragment S.

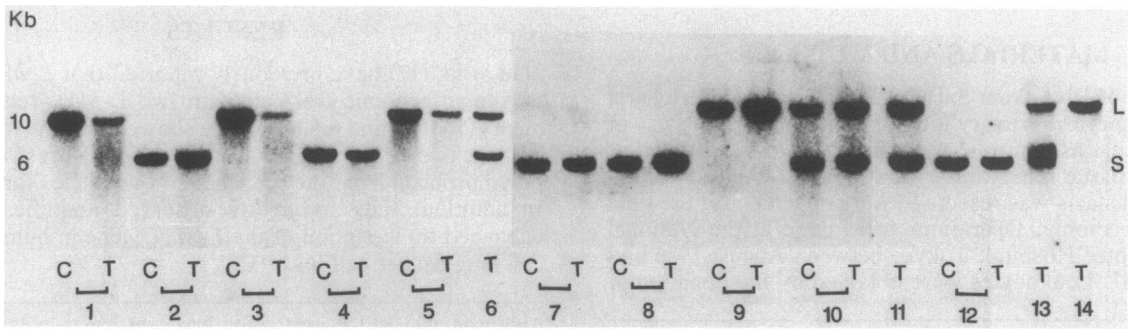

FIG. 1. Southern blot analysis of DNA from patients (numbered) with lung cancer. T, DNA from lung tumor tissue; C, DNA from adjacent normal lung tissue.

Table 3. Relationship between L-*MYC* RFLP and metastasis to lymph nodes or other organs in patients with lung cancer classified according to histological type of cancer

	L-L				L-S				S-S			
	N−	N+	M−	M+	N−	N+	M−	M+	N−	N+	M−	M+
Adenocarcinoma	3	1	3	1	1	4	4	1	2	13	9	6
Squamous cell carcinoma	2	1	3	0	1	7	8	0	1	2	3	0
Large-cell lung cancer	2	0	2	0	0	1	1	0	0	1	1	0
Small-cell lung cancer	0	0	0	0	0	2	0	2	0	3	1	2
Squamous cell carcinoma and adenocarcinoma	0	0	0	0	0	1	1	0	0	0	0	0
Squamous cell carcinoma and large-cell lung cancer	0	0	0	0	0	1	1	0	0	0	0	0
Other	1	0	1	0	0	0	0	0	0	1	1	0

Table 3 shows the profiles of L-*MYC* RFLP and metastases of the 51 patients with lung cancer classified according to histological subgroups. The correlation was especially marked in cases of adenocarcinoma and possibly also of squamous cell carcinoma. In other types of lung cancer, such as large-cell lung cancer and small-cell lung cancer, too few patients were examined to obtain any definite conclusion. Most patients with lung cancer examined were men—only seven were women. Of these women, three had the L-L type [one adenocarcinoma (N−, M+), one squamous cell carcinoma (N−, M−), and one large-cell lung cancer (N−, M−)], two had the L-S type [one adenocarcinoma (N+, M−) and one squamous cell carcinoma (N+, M−)], and two had the S-S type [one adenocarcinoma (N−, M−) and one large-cell lung cancer (N+, M−)]. Thus the correlation between the L-*MYC* RFLP pattern and metastasis to lymph nodes was also seen in women patients. However, no definite conclusion concerning a correlation in both male and female patients with lung cancer is valid until more women with lung cancer have been examined.

The relative ratios of fragments L-L, L-S, and S-S are similar in healthy individuals and patients with lung cancer (compare Fig. 2 with Table 1). Therefore, L-*MYC* RFLP is probably unrelated to incidence of lung cancer, although more analyses are needed for a firm conclusion.

We checked the status of the patients with lung cancer with regard to metastases and prognosis in August 1987. Of the 10 patients who had only fragment L (L-L), one died from infection with Gram-positive bacteria, but the other 9 are in relatively good condition. The brain tumor of one female patient was later moved surgically, and her condition is thus far good. No further metastases have been detected in this group. On the other hand, 8 of the 41 patients with S-S or L-S fragments have died from metastatic lesions, and 6 other patients (2 L-S and 4 S-S), who had no metastasis to other organs than lymph nodes at the time of surgery, have since been found to have metastasis to the brain or bone. Moreover, one patient (N−, M+), who had no metastasis to lymph nodes at the time of surgery, was later found to have such metastatic lesions. Therefore, it is evident that a correlation of L-*MYC* RFLP with the occurrence of metastasis in patients with lung cancer persists even after surgery. In the patients with lung cancer, average time from surgery to August 1987, was 218 days for the L-L type, 230 days for the L-S type, and 206 days for the S-S type. Therefore, the poorer prognoses of patients with the L-S and S-S types were not due to longer follow-up periods after surgery.

DISCUSSION

In this work we found a close correlation between L-*MYC* RFLP and metastasis of lung cancer. All Japanese patients with lung cancer examined were Mongolian; because size and frequency of L-*MYC* RFLPs of Caucasians are the same as those of Mongolians (12), the same correlation probably will be found in lung cancer patients of all races.

Between August 1986, and August 1987, an approximate total of 150 patients with lung cancer underwent surgical operation at the National Cancer Center Hospital. Of these patients, 51 were chosen at random for the present study—the only bias in selection being exclusion of a substantial number of patients with small-cell lung cancer who were treated by chemotherapy without surgical operation. Preliminary analyses of L-*MYC* RFLP and metastases were done on 50 more patients with lung cancer. Data on these cancer patients were found to be comparable with the results reported here. However, no close correlation was seen between L-*MYC* RFLP and metastases in patients with lung cancer who came to the National Cancer Center Hospital one year or more after their initial diagnosis in other hospitals and were not considered to be surgically operative (unpublished results). The most likely explanation for this discrepancy is that L-*MYC* RFLP is not the only factor determining incidence of metastasis but influences speed and extent of the metastasis. Namely, metastasis may develop much faster in patients with lung cancer with the L-S or S-S type than in those with the L-L type. Therefore, even in lung cancer patients with the L-L type, metastatic lesions may eventually develop when the primary tumor is not removed soon enough.

Table 1 shows that the average size of lung tumors was larger in patients with the L-L type than in those with the L-S or S-S type. It can be argued that the correlation of

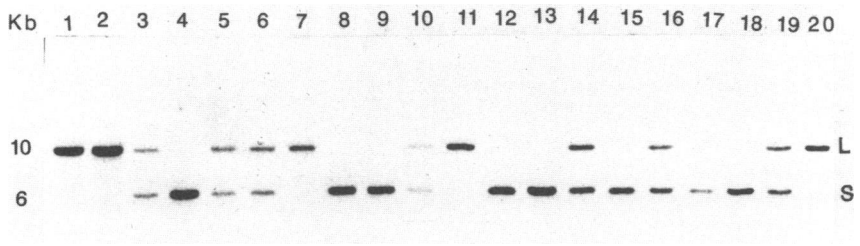

FIG. 2. L-*MYC* RFLP of healthy individuals (numbered).

metastasis was actually not with L-*MYC* RFLP but with the size of tumors at the time of surgery. This possibility is, however, unlikely for the following reasons. First, no tendency for larger tumors to be of the L-L type was found in the 24 patients with adenocarcinomas of the lung: the average tumor size was 5.2 cm for the L-L type, 5.5 cm for the L-S type, and 3.7 cm for the S-S type. Second, it is difficult to explain biologically why larger tumors should have less tendency to metastasize.

An interesting question is how the presence of one of the alleles of L-*MYC* could be related to metastasis of lung cancer. Possible explanations include the following. (*i*) Another gene(s) that is closely associated with the L-*MYC* RFLP in the chromosome has a direct role in metastasis of lung cancer. (*ii*) The two L-*MYC* genes differ in structure (13), and a portion of the protein coded by the gene of L-*MYC* corresponding to fragment S differs functionally from that coded by the L-*MYC* gene of fragment L and plays a crucial role in metastasis to lymph nodes and other organs. (*iii*) The regulatory regions for transcription of the two L-*MYC* genes differ, and the mode of expression of the L-*MYC* fragment S-S is important for metastasis. Total nucleotide sequences of both L-*MYC* genes must be determined to clarify the mechanism by which one of the two L-*MYC* alleles is involved in metastasis. But irrespective of the reason why fragment S is associated with metastasis, our results indicate that RFLP analysis of L-*MYC* could be useful for predicting the presence of, or tendency for, metastases, which affect the prognosis of patients with lung cancer. L-*MYC* RFLP analysis of the DNA of leukocytes from patients before surgery or chemotherapy should aid in the design of better treatments for individual patients.

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