Microsatellite Instability in Lung Cancer Patients 40 Years of Age or Younger

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Lung cancer in the young, which has the characteristics of a higher incidence of adenocarcinoma, lower male-to-female ratio of the patients, and less frequent smoking history in the patients, may possibly be associated with genetic predisposition to cancers. We studied six microsatellite loci (D2S123, D3S659, D3S966, D5S346, WT1, and TP53) in 18 surgically treated lung cancer patients aged 25–40 years and nine control patients aged 62–74 to determine the presence of microsatellite instability (MSI) and to correlate its occurrence with clinicopathological characteristics. Of the 18 patients, 11 were female and seven were non-smokers. There were 15 adenocarcinomas and three squamous cell carcinomas, 15 (83%) of which had vascular invasion. MSI was positive in seven (39%) of 18 young patients and one (11%) of nine control patients. Moreover, MSIs in a half or more of six loci examined were demonstrated in five (28%) young patients, whereas no control patients showed such a high frequency of MSI. We observed no significant differences in clinical or pathologic parameters between cases with and without MSI. This result suggests that genetic factors play an important role in the development of lung cancer in young adults.

Key words: Lung carcinoma — Genetic instability — Microsatellite — Replication error — Young patient

Genetic instability, which is thought to be critical in multistep carcinogenesis,[1, 2] has recently been demonstrated in families with hereditary non-polyposis colorectal cancer (HNPCC), and in patients with a subset of sporadic cancers in many different organs.[3, 4] In lung cancer, however, there has been little evidence of a host factor linked to tumor progression,[5] although epidemiologic studies show increased familial risk.[6–8]

Since a number of genetic changes are required for a normal progenitor cell to acquire neoplastic characteristics, most cancers usually develop at the age of 50 or older.[9] Conversely, early onset of cancer may be associated with a genetic factor leading to increased mutation rate, the accelerated accumulation of such alterations, and an enhanced potential for tumor development. Lung cancer in the young has characteristics that distinguish it from cancer in older patients, including a higher incidence of adenocarcinoma, lower male-to-female ratio of the patients, and decreased smoking history in the patients.[10, 11]

We investigated microsatellite instability (MSI), a useful marker of genetic instability, and the histopathological characteristics of lung carcinomas in young people suspected of being predisposed to the disease.

MATERIALS AND METHODS

Paraffin-embedded tissues of lung carcinoma and corresponding mediastinal lymph nodes without metastasis were obtained from 18 patients aged 40 years or younger, and 9 patients aged 60 years or older as negative controls, who were treated surgically at our hospital from 1974 to 1995. None of them received chemotherapy or radiotherapy before surgery. Each block was sectioned into 5-μm slices; one of every 5 slices was stained with hematoxylin and eosin to determine the areas to be selected. The circumscribed tumor or lymph node tissue, about 100 mm^2 in surface area, was scraped off the slide with a 18G needle and placed into a 1.5 ml microfuge tube. After deparaffinization with xylene, the tissues were incubated for 12 to 24 h at 48°C in a digestion buffer consisting of 10 mM Tris (pH 8.0), 100 mM NaCl, 25mM EDTA, 0.5% sodium dodecyl sulfate, 200 μg/ml proteinase K. DNA was extracted with phenol/chloroform and precipitated with 80% ethanol.[12]

Genetic alterations were studied at six microsatellite loci, and the sequences of primers used were: D2S123, 5'-ACA TTG CTG GAA GTT CTG GC-3' and 5'-CCT TTC TGA CTT GGA TAC CA-3'; D3S659, 5'-CTG CAA GGT CTG TTT AAC AG-3' and 5'-ATT CAG GGA CAA GTT CC-3'; D3S966, 5'-TAC TCT CAC TGT TTC ATA TTA G-3' and 5'-CAC ATA GTA TGT CTC GGC TAA CAG-3'; D5S346, 5'-ACT CAC

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TCT AGT GAT AAA TCG GG-3′ and 5′-AGC AGA TAA GAC AGT ATT ACT AGT T-3′; *WT1*, 5′-AAT GAG ACT TAC TGG GTG AGG-3′ and 5′-TTA CAC AGT AAT TTC AAG CAA CGG-3′; and *TP53*, 5′-ACT GCC ACT CCT TGC CCC ATT C-3′ and 5′-AGG GAT ACT ATT CAG CCC GAG GTG-3′. The loci were selected on the basis of two criteria: (i) a relatively high frequency of MSI was reported in the lung cancer specimens, and (ii) instability of the region may be involved in the carcinogenesis of lung cancer.[13-15] The polymerase chain reaction (PCR) primer sequences for these markers were obtained from the Genome Data Base. The PCR was performed in 20-μl volumes of a mixture containing 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM MgCl$_2$, 50 μM dNTP, 0.1 μM concentrations of each Cy 5-end labeled primer (Pharmacia Biotech, Uppsala), 0.3 U of Taq polymerase (Takara Biomedicals, Ohtsu), and 1 μl of DNA sample. The reaction mixtures were heated to 95°C for 2 min and then cycled 40 times in a DNA Thermal Cycler (Perkin Elmer, California); each cycle consisted of 30 s at 94°C for denaturation, 1–2 min at 50 to 60°C for annealing, 1 min at 72°C for

strand elongation, and 7 min at 72°C for final elongation. The PCR products were diluted with a loading buffer consisting of 95% formamide, 20 mM EDTA (pH 8.0) and Dextran Blue, and denatured for 5 min at 98°C. The solution was electrophoresed on 6% polyacrylamide gels containing 8.3 M urea for 4 h at 34 W using an ALFred DNA sequencer. The data were processed by Fragment Manager (Pharmacia Biotech). To confirm the reproducibility of the experiment, all cases showing MSI were examined at least twice by an independently performed PCR and electrophoresis. MSI was defined as an additional two or more bands in the tumor sample than observed in the normal tissue samples (Fig. 1), and MSI was defined positive only when two or more MSI were detected in a patient.

Histologic slides for routine pathologic diagnosis were reviewed in each case. The MSI was compared with various pathologic factors including histologic type, differentiation, and vascular invasion. The nuclear atypia and mitotic index were also evaluated in adenocarcinoma. The nuclear atypia was categorized into three grades: mild, nuclei that were uniform in size and equal
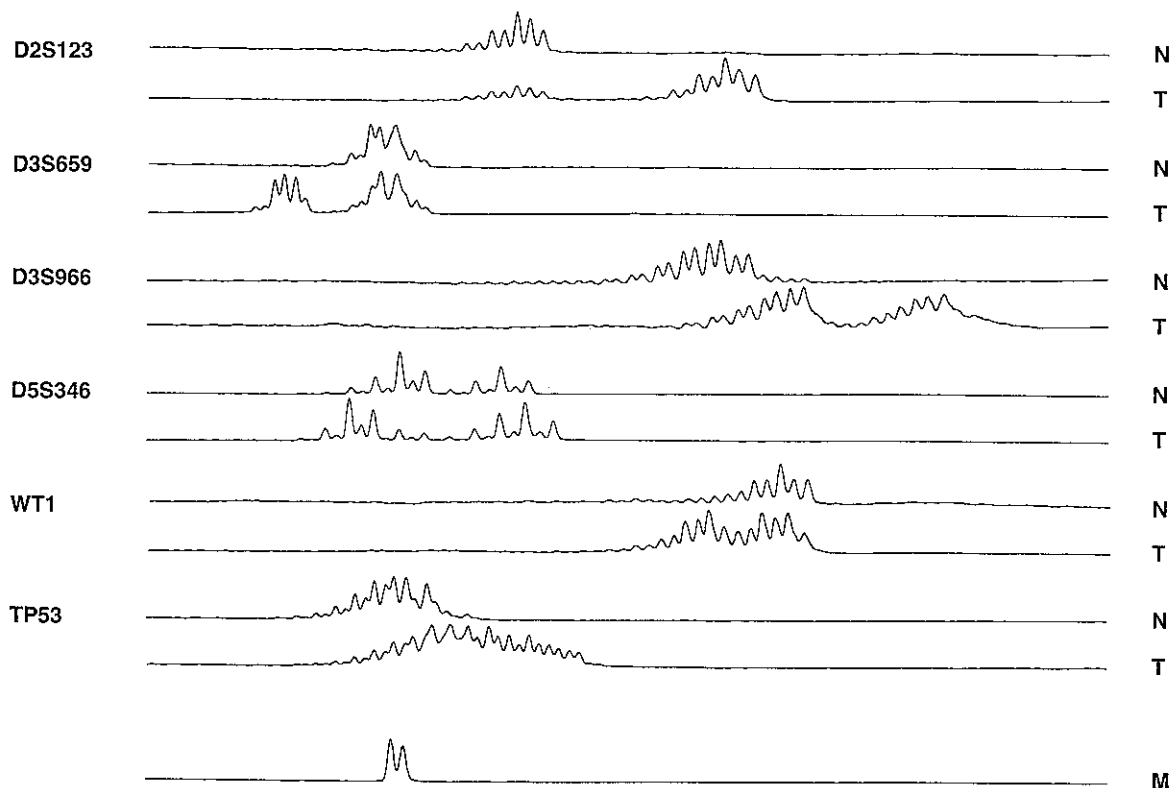

Fig. 1.   Representative results of microsatellite instability at six loci in a tumor from one patient with squamous cell carcinoma of the lung (case #1). N, normal tissue DNA; T, tumor DNA; M, DNA size marker (114 base pair).

to or slightly larger than those of reactive type II alveolar epithelial cells; moderate, nuclei that were of uniform size and up to twice the size of reactive type II alveolar epithelial cells; and marked, nuclei of various sizes, and the presence of giant tumor cells. The mitotic index was divided into three grades: low, number of mitotic cells up to 5/10 high-power fields (HPF); moderate, 6 to 15/10 HPF; and high, 16 or more/10 HPF.[16]

Statistical significance was tested by the χ^2 test with Yates' correction.

RESULTS

The 18 patients' characteristics are summarized in Table I. We noted no patients with HNPCC. The frequency of MSI was 22% for D2S123, 28% for D3S659, 39% for D3S966, 28% for D5S346, 11% for WT1, and 28% for TP53. MSI was positive in seven (39%) of 18 patients. Of the seven patients, five showed MSI in three or more of six loci tested (Table II). Loss of heterozygosity was detected only at the D2S123 locus in a patient with MSI and at D3S966 in a patient without MSI. We also examined MSI in nine old (60 years or more) patients as negative controls. Their median (range) age was 64 (62–74) years, six were female and three male, and all patients had adenocarcinoma histology. MSI was detected in one locus of one patient and two loci of another patient. Thus, MSI was positive in 11% of the control patients. When calculating the frequency in the total microsatellite loci examined, the frequency of MSI in young patients was higher than that in old patients: 26% (28/108) in young patients and 6% (3/54) in old patients (P-value=0.0038).

The predominant histological type was adenocarcinoma, which was observed in 15 (83%) of 18 patients (Table I). Marked nuclear atypia was observed in four of the 15 adenocarcinomas, and high mitotic index was observed in two. There were four poorly differentiated,

nine moderately differentiated, and five well differentiated tumors. Vascular invasion was observed in 15 (83%) of 18 tumors. Female-to-male ratios were 0.8 in MSI-negative and 6.0 in positive patients, but no statistical significance was obtained. There was no difference in other clinical or pathologic characteristics between MSI-negative and positive patients (Table III).

Table I. Characteristics of Young Lung Cancer Patients

Age	
median (range)	37 (25–40)
Sex	
female/male	11/7
Pack-years smoked	
0	7
$0 < PY^{a)} < 20$	9
$20 \leq PY$	2
Family history of cancer	
positive	5
negative	12
unknown	1
Second primary cancer	
none	17
lung and esophagus	1
History of exposure to industrial dust	
negative	16
unknown	2
Histology	
adenocarcinoma	15
squamous cell carcinoma	3
Pathological stage	
I	5
II	1
IIIa	3
IIIb	3
IV	6

a) Pack-years.

Table II. Lung Carcinoma with Microsatellite Instability in the Young[a]

Case	Histology	Stage	D2S123	D3S659	D3S966	D5S346	WT1	TP53
1	squamous cell	I	MSI	MSI	MSI	MSI	MSI	MSI
2	adeno	I	LOH	N	MSI	MSI	N	MSI
3	adeno	IIIA	MSI	MSI	MSI	MSI	MSI	N
4	adeno	IIIB	MSI	MSI	N	MSI	N	N
5	adeno	IIIB	N	N	MSI	N	N	N
6	adeno	IIIB	N	N	MSI	N	N	N
7	adeno	IV	MSI	MSI	MSI	MSI	N	MSI
8	adeno	IV	N	MSI	N	N	N	MSI
9	adeno	IV	N	N	MSI	N	N	MSI

a) Only cases with microsatellite instability are shown. N, normal; MSI, microsatellite instability.

Table III. Clinical and Pathological Characteristics of Microsatellite Instability-negative and -positive Patients 40 Years of Age or Younger

	MSI-negative[a] (n=11)	MSI-positive (n=7)
Age		
median (range)	36 (30–40)	38 (25–40)
Sex		
female	5	6
male	6	1
Family history		
yes	4	1
no	6 (unknown 1)	6
Second primary		
yes	1	0
no	10	7
Smoking history		
yes	7	4
no	4	3
Pack-years smoked		
median (range)	5 (0–57)	4 (0–20)
Pathological stage		
I	3	2
II	1	0
IIIA	2	1
IIIB	2	1
IV	3	3
Histology		
adeno ca	9	6
squamous cell ca	2	1
Differentiation		
poorly	2	2
moderately	6	3
well	3	2
Nuclear atypia		
mild	1	0
moderate	5	5
marked	3	1
Mitotic index		
low	1	1
moderate	7	4
high	1	1
Vascular invasion		
yes	10	5
no	1	2

a) Microsatellite instability.

DISCUSSION

Exogenous carcinogens found in cigarette and industrial dusts have been focused on in an attempt to understand carcinogenesis in the lung; there is little evidence that inherent factors are associated with the etiology, except for familial risk of the disease.[6-8] In this study, however, only two patients had a history of heavy smoking, and none was heavily exposed to industrial dusts. In addition, the predominant histology was adenocarcinoma, which is not thought to be associated with ciga-

rette smoking. Thus, environmental agents probably had little influence upon the occurrence of lung cancer in these patients.

In this study the frequencies of MSI positivity were 39% (7/18) in the young patients group, and 11% (1/9) in the control group, which is comparable to the frequency in other reports.[13, 14, 17, 18] In addition, the frequency of MSI in the total microsatellite loci examined was higher in young patients than in old patients with statistical significance. These results are consistent with the observation by Ryberg et al. of a higher incidence (38%) of MSI in patients aged 50 years or younger than that (19%) in older patients.[15] Moreover, MSI was observed in a half or more of the examined loci in five (28%) young patients, whereas no patient showed such a high frequency of MSI in the control group. This kind of high MSI positivity was also found in HNPCC tumors, 50% of which had mutations in the hMSH2 or hMLH1 gene, whereas only low MSI positivity was found in non-HNPCC tumors, which rarely had mutations in these genes.[19] Thus, our results suggested that the DNA mismatch repair function might be impaired in young lung cancer patients with a high frequency of MSI.

The reasons why patients in this study developed cancer at an early age might include either exogenous factors from the environment severely affecting the lung epithelium or genetic factors hastening the malignant transformation of the epithelium, or both. The present study, however, suggests that genetic rather than environmental factors were primarily involved in the development of cancer in these patients. MSI was negative in eleven patients. Even in these patients, however, MSI might be found if other loci were studied; other genes which do not induce MSI but accelerate carcinogenesis might be involved.

Although the incidence of family history of cancer in MSI-negative patients (40%) appeared to be higher than that in MSI-positive patients (14%), this difference was not statistically significant. In addition, the frequency of cancer among relatives of young patients was possibly low because other family members were also young. Thus, it is difficult to assess the relevance of family history of cancer in this study population.

Colon cancer typically occurs at an early age in HNPCC patients.[3, 4] Furthermore, young colorectal cancer patients who do not have a family history of cancer were recently shown to have MSI phenotype and germline mutations of mismatch repair gene.[20] Thus, the early onset of the disease is possibly an example of predisposition to cancers.

The decreased degree of tumor differentiation is one of the characteristics of lung carcinoma in the young.[21] This tendency was also found in this series; well-differentiated tumor was observed only in five of 18 cases.

Moreover, vascular invasion was prominent; it was observed in 15 (83%) of 18 tumors.

In conclusion, the key to understanding the early development of lung cancer in young adults seems to lie in genetic rather than environmental factors. We believe that analyzing this kind of cohort, which seems to have susceptibility to cancers, may provide a good insight into carcinogenesis in the lung.

REFERENCES

1) Loeb, L. A., Springgate, C. F. and Battula, N. Errors in DNA replication as a basis of malignant change. *Cancer Res.,* **34**, 2311–2321 (1974).
2) Nowell, P. The clonal evolution of tumor cell populations. *Science,* **194**, 23–28 (1976).
3) Rüschoff, J., Bocker, T., Schlegel, J., Stumm, G. and Hofstaedter, F. Microsatellite instability: new aspects in the carcinogenesis of colorectal carcinoma. *Virchows Arch.,* **426**, 215–222 (1995).
4) Marra, G. and Boland, C. R. Hereditary nonpolyposis colorectal cancer: the syndrome, the genes, and historical perspectives. *J. Natl. Cancer Inst.,* **87**, 1114–1125 (1995).
5) Reif, A. E. Heredity as a determining factor in which smokers die of lung cancer. *J. Natl. Cancer Inst.,* **83**, 64–66 (1991).
6) Tokuhata, G. K. and Lilienfeld, A. M. Familial aggregation of lung cancer in humans. *J. Natl. Cancer Inst.,* **30**, 289–312 (1963).
7) Ooi, W. L., Elston, R. C., Chen, V. W., Bailey-Wilson, J. E. and Rothschild, H. Increased familial risk for lung cancer. *J. Natl. Cancer Inst.,* **76**, 217–222 (1986).
8) Shaw, G. L., Falk, R. T., Pickle, L. W., Mason, T. J. and Buffler, P. A. Lung cancer risk associated with cancer in relatives. *J. Clin. Epidemiol.,* **44**, 429–437 (1991).
9) Dix, D. The role of aging in cancer incidence: an epidemiological study. *J. Gerontol.,* **44**, 10–18 (1989).
10) McCambridge, M. M. and Eliasson, A. H. Lung cancer in the young. *Ann. Thorac. Surg.,* **55**, 809–810 (1993).
11) Green, L. S., Fortoul, T. I., Ponciano, G., Robles, C. and Rivero, O. Bronchogenic cancer in patients under 40 years old. The experience of a Latin American country. *Chest,* **104**, 1477–1481 (1993).
12) Shimizu, H. and Burns, J. C. Extraction of nuclear acids: sample preparation from paraffin-embedded tissues. *In* "PCR Strategies," ed. M. A. Innis, D. H. Gelfand and J. J. Sninsky, pp. 32–38 (1995). Academic Press, San Diego.
13) Shridhar, V., Siegfried, J., Hunt, J., del Mar Alonso, M.
and Smith, D. I. Genetic instability of microsatellite sequences in many non-small cell lung carcinomas. *Cancer Res.,* **54**, 2084–2087 (1994).
14) Adachi, J., Shiseki, M., Okazaki, T., Ishimura, G., Noguchi, M., Hirohashi, S. and Yokota, J. Microsatellite instability in primary and metastatic lung carcinomas. *Genes Chromosomes Cancer,* **14**, 301–306 (1995).
15) Ryberg, D., Lindstedt, B. A., Zienolddiny, S. and Haugen, A. A hereditary genetic marker closely associated with microsatellite instability in lung cancer. *Cancer Res.,* **55**, 3996–3999 (1995).
16) Kurokawa, T., Matsuno, Y., Noguchi, M., Mizuno, S. and Shimosato, Y. Surgically curable "early" adenocarcinoma in the periphery of the lung. *Am. J. Surg. Pathol.,* **18**, 431–438 (1994).
17) Mao, L., Lee, D. J., Tockman, M. S., Erozan, Y. S., Askin, F. and Sidransky, D. Microsatellite alterations as clonal markers for the detection of human cancer. *Proc. Natl. Acad. Sci. USA,* **91**, 9871–9875 (1994).
18) Fong, K. M., Zimmerman, P. V. and Smith, P. J. Microsatellite instability and other molecular abnormalities in non-small cell lung cancer. *Cancer Res.,* **55**, 28–30 (1995).
19) Konishi, M., Kikuchi-Yanoshita, R., Tanaka, K., Muraoka, M., Onda, A., Okumura, Y., Kishi, N., Iwama, T., Mori, T., Koike, M., Ushio, K., Chiba, M., Nomizu, S., Konishi, F., Utsunomiya, J. and Miyaki, M. Molecular nature of colon tumors in hereditary nonpolyposis colon cancer, familial polyposis, and sporadic colon cancer. *Gastroenterology,* **111**, 307–317 (1996).
20) Liu, B., Farrington, S. M., Petersen, G. M., Hamilton, S. R., Parsons, R., Papadopoulos, N., Fujiwara, T., Jen, J., Kinzler, K. W., Wyllie, A. H., Vogelstein, B. and Dunlop, M. G. Genetic instability occurs in the majority of young patients with colorectal cancer. *Nat. Med.,* **1**, 348–352 (1995).
21) Kyriakos, M. and Webber, B. Cancer of the lung in young men. *J. Thorac. Cardiovasc. Surg.,* **67**, 634–648 (1974).