Jpn. J. Cancer Res. **91**, 589–596, June 2000

Three New Regions on Chromosome 17p13.3 Distal to p53 with Possible Tumor Suppressor Gene Involvement in Lung Cancer

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We investigated loss of heterozygosity (LOH) at the distal portion of the *p53* gene on the short arm of chromosome 17 in lung cancers in order to search for new tumor suppressor genes. The roles of the putative genes were also studied in terms of pathological features. One hundred and forty-five resected non-small cell lung cancers were examined for LOH using 11 markers mapped on, and distal to the p53 locus, and deletion maps were constructed. Four commonly deleted regions were found: one from TP53 to ENO3, where the *p53* gene resides, and three others from ENO3 to D17S1566, D17S379 to D17S1574 and distal to ABR, with LOH frequencies almost the same as, or higher than, at the TP53 locus. Examination of the relationship between LOH of the latter three regions and histopathological parameters of adenocarcinomas (genetically negative for p53 mutation) revealed allelic losses on D17S379 to be associated with advanced lesions, while D17S513 was more frequently deleted in poorly differentiated tumors. These results indicate that new tumor suppressor gene(s) may reside on these three distinctly deleted regions on chromosome 17p13.3 distal to the *p53* gene in lung cancer, with possible roles in progression and differentiation of adenocarcinomas.

Key words: Tumor suppressor gene — 17p13.3 — Non-small cell lung cancer — LOH — Prognosis

The specific chromosomal deletions which have been reported in various tumors are highly suggestive of the presence of tumor suppressor genes.[1)] Examples such as MTS1, RB and p53, which play important roles in the genesis of lung cancers, have been identified at distinct regions on chromosomes 9p, 13q and 17p, respectively, all of which show a high frequency of allelic loss.[2–6)] Previously we looked for allelic loss on virtually all chromosome arms in surgically resected non-small cell lung cancers, and found this to be frequent on chromosome 17p with the variable number of tandem repeats (VNTR) marker, YNZ22, mapped to 17p13.3, distal to the p53 locus.[7, 8)] At that time, we considered that the loss of the chromosome region implied involvement of the *p53* gene itself. However, frequent loss of heterozygosity (LOH) on 17p13.3, distal to p53, has subsequently been reported in a number of different tumor types other than lung cancer,[9)] suggesting the presence of another tumor suppressor gene(s). Recently, new candidate tumor suppressor genes for breast, colon and ovarian cancers have been identified on 17p13.3.[10, 11)] These findings suggest that a new tumor suppressor gene(s) for lung cancer may also be present distal to the *p53* gene. To investigate this hypothesis, we examined allelic loss on chromosome 17p using many markers mapped distal to the *p53* gene and succeeded in identifying two distinctly deleted regions in lung cancers. The relationship between allelic loss and pathological parameters was also examined to provide a basis for speculation as to the function of the putative tumor suppressor gene(s) *in vivo*.

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MATERIALS AND METHODS

Tumor samples and histological classification One hundred and forty-five non-small cell lung cancer cases (35 squamous cell carcinoma and 110 adenocarcinoma), along with corresponding normal lung tissues, resected consecutively at the Cancer Institute Hospital, Tokyo, were examined. None of the patients had received any preoperative treatment. All the materials had been used in the allelotype studies reported previously.[7, 8)] Histological classification of the tumors based on the WHO classification was performed by one of the authors (ET).[12)] Only adenocarcinomas were examined for the relationship between allelic loss and pathological characteristics. Stages (p-stages) were determined according to the TNM classification of Malignant Tumors defined by the International Union Against Cancer.[13)]

Isolation of DNA and LOH analysis Extraction of DNA from the tissues was carried out according to the methods described previously.[7)]

LOH studies were performed using polymerase chain reaction (PCR) amplification of 10 microsatellite repeat markers (TP53, ENO3, D17S513, D17S1566, D17S379, D17S525, D17S1574, ABR, D17S926, D17S643) available through the Genome Database, and a VNTR marker, YNZ22. The orders of the markers and the spacing between adjacent markers were decided by use of the Stanford G3 Radiation hybrid (RH) panel (Research Genetics, Huntsville, AL) (Fig. 1). Statistical analysis of RH data was conducted with RHMAP version 2.0 (Michael Boehnke, Ann Arbor, MI, http://www.sph.umich.edu/group/statgen/software/). Each PCR was performed in a 10 μl mixture containing 50 ng of genomic DNA; 10 pmol each of primer (one was end-labeled with [γ-^{32}P]-ATP); 250 μM each of dATP, dGTP, dCTP, and dTTP; and 0.25 units of *Taq* DNA polymerase (Boehringer Mannheim, Mannheim, Germany). The mixture was subjected to 35 PCR cycles with a Perkin Elmer (Norwalk, CT) GeneAmp PCR System 9600, employing annealing temperatures that ranged from 54 to 64°C. Loading buffer was added to each product before it was heat-denatured and electrophoresed in a 6% denaturing polyacrylamide gel. Gels were dried and exposed to Kodak (Rochester, NY) film overnight, and allelic loss was determined visually. In almost all cases that were ambiguous for LOH with the microsatellite markers, PCR was repeated one or more times until the results were considered to be reliable.

For LOH analysis data with YNZ22, the results of previous studies were used.[7, 8)]

p53 mutation analysis and DNA sequencing Genomic DNAs of adenocarcinomas used for LOH analysis were examined. Exons 4–8 and 10 of the *p53* gene were analyzed by the PCR-SSCP (single-strand conformation polymorphism) method and sequenced in order to characterize *p53* gene mutations as described previously.[14)]

Statistical analysis In considering the relationship between LOH status and the pathological parameters of adenocarcinomas, Fisher's exact test and the Mann-Whitney *U* test were used for statistical analysis of the results.

RESULTS

Representative autoradiograms illustrating interstitial or partial deletions are shown in Fig. 2. The results of autora-

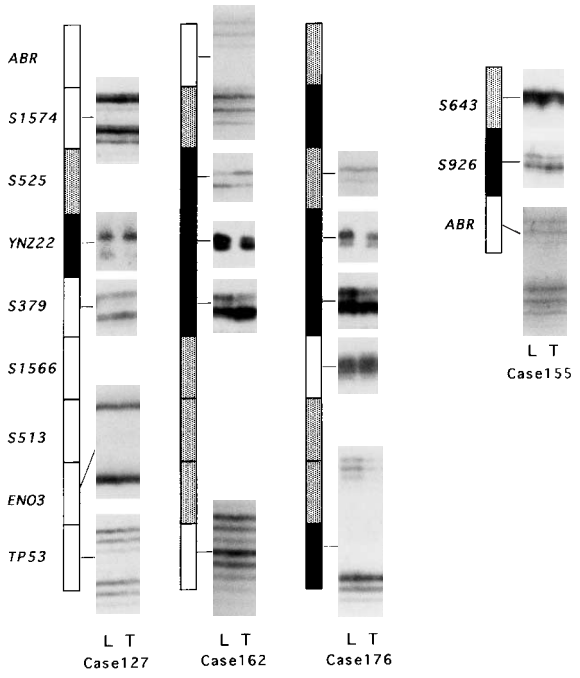

Fig. 2. Representative findings for the heterozygosity status of DNA from normal lung tissue (L) and tumors (T) in four cases. The markers used are given on the left side of the boxes. White boxes indicate retention of heterozygosity, black boxes LOH, and gray boxes not-informative cases.

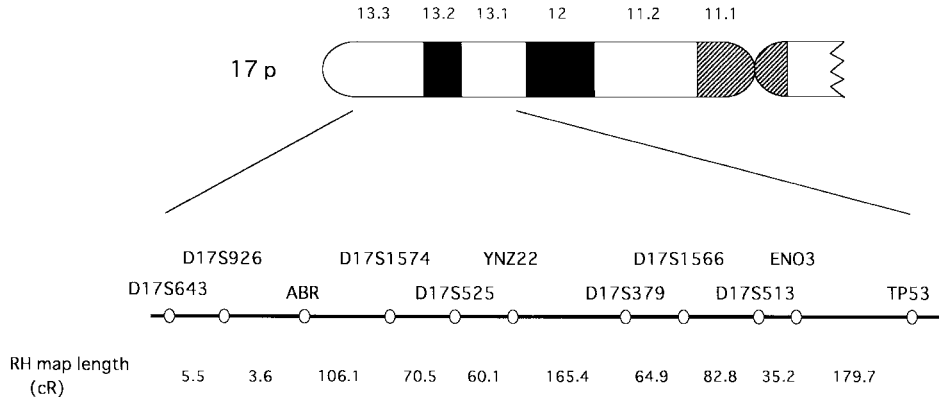

Fig. 1. Order and spacing of markers on chromosome band 17p13 within the region from TP53 to the telomere. cR, centi ray 8000.

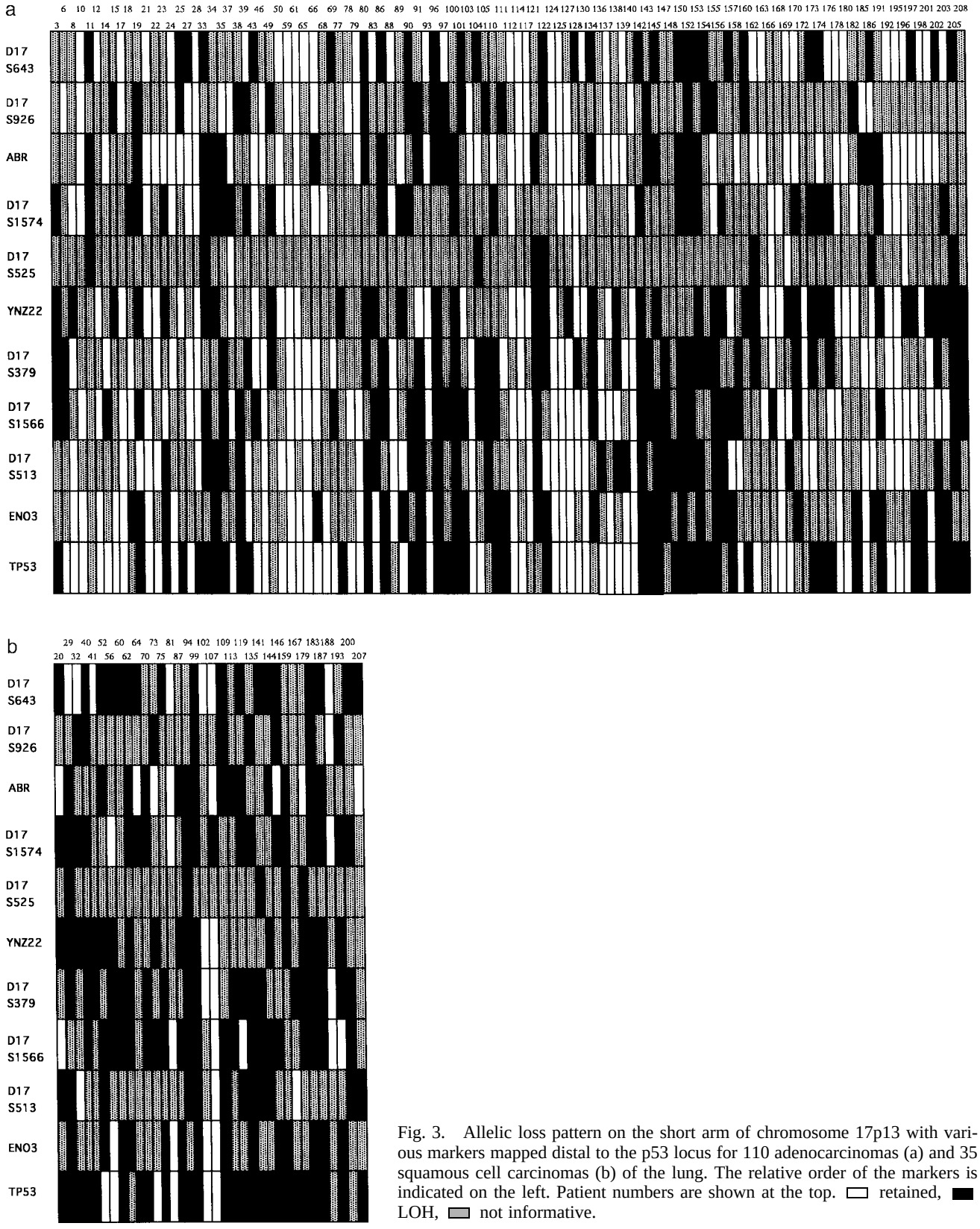

Fig. 3. Allelic loss pattern on the short arm of chromosome 17p13 with various markers mapped distal to the p53 locus for 110 adenocarcinomas (a) and 35 squamous cell carcinomas (b) of the lung. The relative order of the markers is indicated on the left. Patient numbers are shown at the top. ☐ retained, ■ LOH, ▭ not informative.

diograms and the frequencies of LOH on 11 loci are summarized in Fig. 3 and Table I. In squamous cell carcinomas, the lowest frequency of LOH was 64%, observed on ABR, and the highest was 100% on D17S525, although the number of informative cases was very small. TP53 showed 81% allelic loss, with values for the other markers being between 88% and 72%. In adenocarcinomas, frequencies of LOH were about 30–40% lower on each locus than those of squamous cell carcinomas, but the pattern of allelic loss with the markers was almost the same as that of squamous cell carcinomas. The overall frequency of TP53 was 55%, and those of the markers from ENO3 to D17S1574 were approximately the same or slightly higher (53 to 72%). ABR showed the lowest frequency (40%), and telomeric to ABR, the frequency again increased to 55% on D17S926 and 51% on D17S643. We considered the presence of new tumor suppressor genes likely on loci where the frequency of LOH was approximately the same as or higher than that of TP53, and two such regions, from TP53 to D17S1574 and distal to ABR, were identified.

Table I. Frequency of LOH[a] on Chromosome 17p13 as Assessed with Eleven Markers

Markers	Allelic loss/informative cases (%)		
	Sq[b]	Ad[c]	Total
D17S643	18/25 (72)	29/68 (43)	47/93 (51)
D17S926	9/10 (90)	21/45 (47)	30/55 (55)
ABR	14/22 (64)	22/71 (31)	36/93 (39)
D17S1574	18/21 (86)	27/54 (50)	45/75 (60)
D17S525	5/5 (100)	8/13 (62)	13/18 (72)
YNZ22	21/24 (88)	42/80 (53)	63/104 (61)
D17S379	20/23 (87)	34/67 (51)	54/90 (60)
D17S1566	21/27 (78)	38/83 (46)	59/110 (54)
D17S513	12/15 (80)	29/63 (46)	41/78 (53)
ENO3	14/18 (78)	36/67 (54)	50/85 (59)
TP53	26/32 (81)	44/96 (46)	70/128 (55)

a) Loss of heterozygosity.
b) Squamous cell carcinoma.
c) Adenocarcinoma.

Deletion mapping from TP53 to D17S1574 and distal to ABR The deletion map was created on the assumption that if uninformative loci are present between a locus with LOH and a locus with retention of heterozygosity, such loci should be considered as deleted. Fifty of 145 cases showing partial or interstitial deletions at loci from TP53 to D17S1574 were analyzed to identify the commonly deleted regions (Fig. 4). Three independently deleted regions were identified, the first from TP53 to ENO3, the second from ENO3 to D17S1566 (because there are several cases in which both p53 and D17S379 were retained and the loci between these two loci were specifically deleted), and the third from D17S379 to D17S1574. In a deletion map distal to ABR (figure is not shown), the region distal to ABR was commonly deleted.

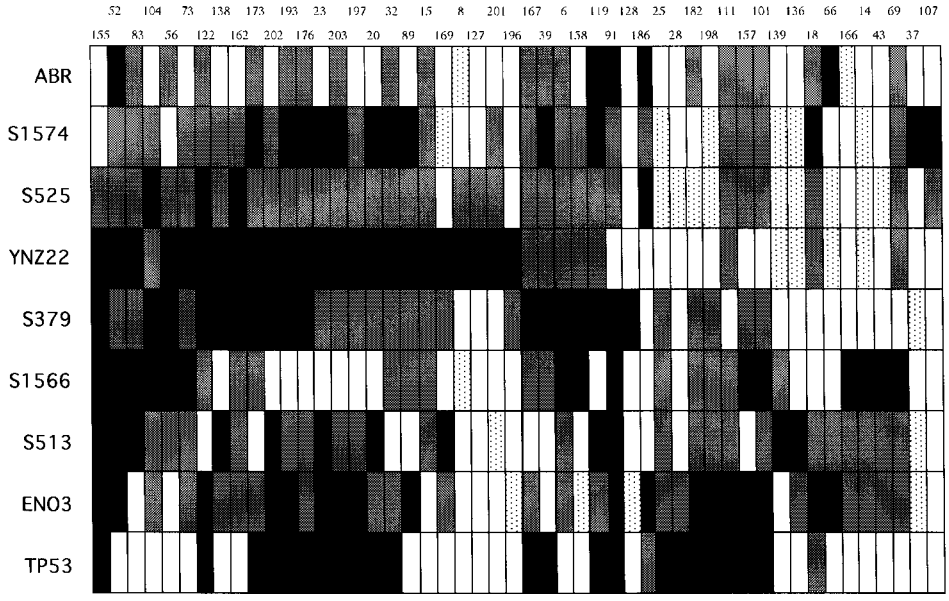

Fig. 4. Deletion maps at the loci from TP53 to D17S1574 on chromosome 17p for 42 adenocarcinomas and eight squamous cell carcinomas showing partial or interstitial deletions. When uninformative loci were present between a locus with LOH and a locus with retention of heterozygosity, such loci were considered as being deleted. ☐ retained, ■ LOH, ▩ hypothetic LOH, ⁘ uncertain region.

Table II. Clinicopathological Parameters of Adenocarcinomas without p53 Mutation and Informative for Seven Markers

Markers	No. of cases	Sex[a]		Age (average±SD)	Subtypes[b]				Differentiation[c]			p-stages	
		M	F		A	P	BA	S	W	M	P	I	II≤
S643	40	19	22	61±9.0	6	29	5	0	18	20	2	23	17
S926	23	14	9	63±9.8	5	15	2	1	12	7	4	15	8
YNZ22	39	17	22	62±11.0	5	26	7	1	15	21	3	22	17
S379	44	24	20	61±9.9	7	28	8	1	21	19	4	28	16
S1566	52	27	25	62±10.2	10	32	9	1	23	23	6	34	18
S513	34	16	18	61±9.5	8	19	6	1	18	13	3	19	15
ENO3	37	16	21	60±8.8	8	25	3	1	16	18	3	20	17

a) M, male; F, female.
b) A, acinar; P, papillary; BA, bronchiolo-alveolar; S, solid carcinoma with mucus formation.
c) W, well; M, moderately; P, poorly.

Table III. Relationship between LOH[a] Status with Seven Markers and Pathological Parameters of No p53 Mutation Adenocarcinomas

Markers	LOH	No. of cases	Subtypes[b]				Differentiation[c]			p-stages	
			A	P	BA	S	W	M	P	I	II≤
S643	+	12	4	8	0	0	3	7	2	5	7
	–	28	2	21	5	0	15	13	0	18	10
S926	+	6	2	3	0	1	2	2	2	3	3
	–	17	3	12	2	0	10	5	2	12	5
S379	+	14	3	10	1	0	3	11	0	7	7[e]
	–	25	2	16	6	1	12	10	3	15	10
S1566	+	19	4	12	2	1	8	9	2	8	11
	–	25	3	16	6	0	13	10	2	20	5
S513	+	18	5	9	3	1	6	8	4[d]	11	7
	–	34	5	23	6	0	17	15	2	23	11
ENO3	+	12	4	7	0	1	3	7	2	6	6
	–	22	4	12	6	0	15	6	1	13	9
YNZ22	+	14	5	7	1	1	4	8	2	6	8
	–	23	3	18	2	0	12	10	1	14	9

a) Loss of heterozygosity.
b) A, acinar; P, papillary; BA, bronchiolo-alveolar; S, solid carcinoma with mucus formation.
c) W, well; M, moderately; P, poorly.
d) P=0.0320, Mann-Whitney U test.
e) P=0.0113, Fisher's exact test.

Possible role of the new candidate gene(s) in adenocarcinomas From the above results, three regions, ENO3 to D17S1566, D17S379 to D17S1574, and distal to ABR, were considered as likely sites of new tumor suppressor genes for lung cancer. To cast light on the impact of alterations in these genes on biological behavior, we examined the relationship between LOH status for each locus involved in the new commonly deleted regions, and histological subtypes, differentiation and p-stages of adenocarcinomas.

It is well known that *p53* gene abnormalities have a marked influence on the biological behavior of carcinomas *in vivo*. Therefore, for analysis of target gene functions in adenocarcinomas, p53 mutated cases (46 out of 110) were excluded[14] and the remaining 64 (58%) were analyzed. Of these, the number of informative cases for each

marker (except D17S525 because of its insufficient size), is shown with respect to the clinicopathological parameters in Table II.

Allelic loss at D17S513 was observed more frequently in less differentiated adenocarcinomas (P=0.0320, Mann-Whitney U test) (Table III). Division was into two stages, with group I consisting of p-stage I and group II including p-stage II and more advanced cases. LOHs were observed more frequently in group II for D17S379 (P=0.0113, Fisher's exact test).

DISCUSSION

On the short arm of chromosome 17, there are two regions where a high frequency of allelic loss is reported in different tumors. One of these is TP53 at 17p13.1, encompassing the tumor suppressor gene, *p53*, which is most frequently mutated in cancers.[15] The other is a region at 17p13.3, telomeric from the TP53 locus in several types of tumor where the presence of new tumor suppressor gene(s) is indicated: hepatocellular carcinoma, malignant astrocytoma, pediatric primitive neuroectodermal tumors, breast carcinoma, high grades of astrocytic tumors and ovarian cancer.[16–22] In lung cancers, the existence of 17p13 deletions has long been known. However, a detailed analysis to define the extent of the deletion was only conducted very recently.[23] One of the reasons for this is that the rate of cases showing partial or interstitial deletion in the region is rather low,[24, 25] as also found in our study (34%). Thus, large numbers of difficult-to-collect carcinomas are needed for deletion map analysis. In the present study of a large series, we identified four commonly deleted regions: from TP53 to ENO3, ENO3 to D17S1566, D17S379 to D17S1574, and distal to ABR. The first deleted region includes the *p53* gene, while new tumor suppressor gene(s) for lung cancer might reside in the other commonly deleted regions.

A very recently published report suggested the presence of putative tumor suppressor genes for lung cancer in two regions.[23] One is between D17S379 and D17S695, with the smallest overlapping deleted region from D17S379 to D17S5 (YNZ22), and the other is telomeric to D17S695. Our second region, ENO3 to D17S1566, did not overlap with any of these regions, having a more centromeric location, and might contain a tumor suppressor gene for lung cancer. Our third region, D17S379 to D17S1574, overlapped to some extent with the smallest region of the first, although it was a little larger (130.6 cR) in the telomeric direction. Our fourth region, distal to ABR spanned from the telomeric region of the first one to the second one in the published report.

Our third region includes a commonly deleted region in early ovarian cancers,[20] and D17S379 was found to be deleted in higher-grade astrocytic tumors.[21] Furthermore, in breast carcinomas two deleted regions which are the most likely sites of putative suppressor genes have been reported: one ranging from our second to fourth regions,[19] and the other overlapping with our fourth region.[25] Judging from these results, putative tumor suppressor genes in these two regions may play important roles in the genesis of various kinds of tumors.

In the commonly deleted region from D17S379 to D17S1574 (the third region), four candidate tumor suppressor genes have been identified so far. The first one, called Rox/Mnt, is a novel *myc* antagonist gene located in D17S379.[26] However, no mutations were found in lung cancers.[27] The second, *HIC-1* gene (hypermethylated in cancer gene), is located very near YNZ22 (at a distance of less than 11.0 kb) and is thought to be related to tumorigenesis in the brain, breast and colon.[10] The last two genes, named OVCA1 and OCCA2, located distal to D17S5 (YNZ 22) and proximal to D17S28, may contribute to ovarian cancers.[11] These three genes should be examined for possible associations with lung cancer.

It is important to find genes or chromosome loci whose abnormalities are related to malignant progression of tumors, so that patients can be selected who will benefit from adjuvant therapy. So far, some molecular markers, for example p53, MDM2, and p27, have been shown to be prognostic factors for non-small cell lung cancer.[14, 28–31] However, the prognostic significance of these gene abnormalities is still controversial, or at best uncertain.[32] In our study, higher frequencies of allelic loss on D17S379 were observed in more advanced adenocarcinomas, and recent reports have found that LOH of the D17S379 locus, independently of p53, is associated with higher grades of astrocytic tumors.[21] Therefore, the putative tumor suppressor genes at these loci may be related to tumor progression. Additional studies, especially analyses of large numbers of cases with the same histology and at the same pathological stage, are needed for further evaluation of these results.

At D17S513, more frequent LOHs were observed in less differentiated adenocarcinomas, which suggests that the gene(s) on the locus may be related to differentiation.

ACKNOWLEDGMENTS

We thank Drs. H. Sugano and Y. Miki (Cancer Institute, Tokyo) for their helpful advice and discussions. The technical assistance of T. Yoshikawa and Y. Yamaoka is gratefully acknowledged. This study was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan, as well as by grants from the Vehicle Racing Commemorative Foundation, and the Smoking Research Foundation.

(Received December 14, 1999/Revised March 23, 2000/Accepted March 28, 2000)

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