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Loss of heterozygosity on chromosomes 3, 13, and 17 in small-cell carcinoma and on chromosome 3 in adenocarcinoma of the lung

(lung cancer/allelic deletion/mapping/*RB* locus/DNA polymorphisms)

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ABSTRACT By a molecular genetic approach using polymorphic DNA markers that detect allelic deletion of specific chromosomal regions, we analyzed for possible loss of chromosomal heterozygosity in five different histological types of lung cancers obtained from 47 patients. In small-cell carcinomas, the incidence of allelic deletions at three different chromosomal loci was extremely high; loss of heterozygosity was detected on chromosomes 3p in 7 of 7 patients (100%), 13q in 10 of 11 patients (91%), and 17p in 5 of 5 patients (100%). The deletions at these loci in small-cell carcinomas were observed even in the tumors without any clinical evidence of metastasis. Furthermore, loss of heterozygosity on chromosomes 3p and 13q occurred prior to *NMYC* amplification and chromosome 11p deletion. Loss of heterozygosity on chromosome 3p was also detected with high frequency in adenocarcinomas [5 of 6 patients (83%)]. Heterozygosity of chromosomes 13q and 17p was lost in 10 of 31 patients (32%) and in 3 of 12 patients (25%), respectively, of lung cancers other than small-cell carcinomas. These results indicate that recessive genetic changes involving sequences on chromosomes 3p, 13q, and 17p may play important roles in the genesis of small-cell carcinoma, and those on chromosome 3p may play an important role in the genesis of adenocarcinoma.

Although lung cancer is a common malignancy in adults, little is known about the genetic changes that may contribute to tumor development. In small-cell carcinoma (SCC), two types of genetic alterations have been reported to date. One is the amplification of viral *myc*-related human genes *MYC*, *NMYC*, and *LMYC* (1, 2), and the other is the deletion of chromosome 3p (3–5). However, because most of these analyses have been performed with cell lines and metastatic cells, it is still unclear whether these changes play important roles in the development of SCC. Furthermore, almost nothing is known about the genetic changes in other histological types of lung cancers.

Loss of genes at specific chromosomal loci is implicated in the development of certain childhood tumors (6–13). Since such a loss occurs not only in hereditary but also in nonhereditary tumors, it is possible that gene loss on specific chromosomes is associated with the genesis of common adult tumors. Availability of probes to detect allelic deletion of specific chromosomal regions by restriction fragment length polymorphisms (RFLPs) has allowed us to study possible loss of chromosomal heterozygosity in lung cancer. Using 24 polymorphic DNA markers that identify RFLPs determined by loci on 16 different chromosomes, we examined 64 fresh lung tumors of five different histological types from 47 patients.

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MATERIALS AND METHODS

Human Tissue Samples. Forty-five primary tumors, 19 metastatic tumors, and 47 normal lung tissues were obtained from 47 lung cancer patients at surgery or autopsy. Histological diagnoses of tumors were made according to the Histological Typing of Lung Cancers defined by the World Health Organization (14); the 47 cases represented 11 SCCs, 18 adenocarcinomas (AdCs), 12 squamous-cell carcinomas (SqCs), 4 large-cell carcinomas (LCCs) and 2 adenosquamous carcinomas (ASCs). The tumors were staged according to the TNM Classification of Malignant Tumors defined by the International Union Against Cancer (15); of the 47 cases, 13 were classified as being stage I, 2 as stage II, 13 as stage III, and 19 as stage IV.

DNA Isolation and Southern Blot Analysis. High molecular weight DNA was prepared by proteinase K digestion and phenol/chloroform extraction as described (16). DNA (10 μg) was digested with appropriate restriction enzymes, fractionated by 0.8% agarose gel electrophoresis, transferred to nitrocellulose filters, and hybridized to ^{32}P-labeled probes prepared by nick-translation. The filters were hybridized repeatedly with probes homologous to several different chromosomal loci to exclude the possibility of mispairing of the samples or unequal loss of DNA from the filters. The DNA segments homologous to various polymorphic human chromosomal loci used in this study are listed in Table 1 (2, 17, 18). In all cases, the allele lengths observed were identical to those published.

RESULTS

Loss of Heterozygosity Occurs Frequently on Several Chromosomal Loci in Lung Cancer. To test whether loss of heterozygosity occurs in the specific region of a certain chromosome in lung cancer, we examined a total of 24 loci on 16 chromosomes. Loss of heterozygosity was found at loci on 13 different chromosomes: chromosomes 1, 3, 5, 6, 9, 11, 12, 13, 14, 16, 17, 18, and 20. The frequency of loss at individual loci ranged between 1 of 11 (9%) at *D5S2* on chromosome 5 and 12 of 15 (80%) at *D3S2* on chromosome 3p (Table 1). No loss of heterozygosity was detected on chromosomes 8, 15, and 19. Loss of heterozygosity at one or more loci was detected in 57% (27 of 47) of the lung cancers: 11 of 11 SCCs, 8 of 18 AdCs, 5 of 12 SqCs, 2 of 4 LCCs and 1 of 2 ASCs. Since the incidence of loss of heterozygosity was high at *D3S2* on 3p, at *D13S1*, *D13S2*, *D13S3*, and *D13S4* on 13q and at *D17S1* on 17p, we examined in more detail for types and stages of lung cancers with loss of heterozygosity at these loci.

Abbreviations: RFLP, restriction fragment length polymorphism; SCC, small-cell carcinoma; AdC, adenocarcinoma; SqC, squamous-cell carcinoma; LCC, large-cell carcinoma; ASC, adenosquamous carcinoma.

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Table 1. Loss of chromosomal heterozygosity in lung cancer

Marker locus	Chromosome location	Enzyme	Cases, no.	Heterozygosity: Constitutional	Heterozygosity: Loss in tumor
LMYC	1p	*Eco*RI	46	20	2
D3S2	3p	*Msp* I	40	15	12
D3S3	3p	*Msp* I	47	0	0
SST	3q	*Eco*RI	10	4	0
		*Bam*HI	28	8	0
D5S2	5	*Msp* I	46	11	1
FMS	5q	*Eco*RI	4	2	0
MYB	6q	*Eco*RI	47	18	3
TG	8q	*Taq* I	34	0	0
ASSP3	9q	*Hin*dIII	41	14	2
HRAS1	11p	*Bam*HI	46	13	3
INS	11p	*Taq* I	34	12	3
D11S24	11q	*Bam*HI	43	2	0
KRAS2	12p	*Taq* I	21	3	1
D13S1	13q	*Msp* I	44	19	8
		Taq I	47	1	0
D13S2	13q	*Msp* I	44	21	12
D13S3	13q	*Msp* I	47	24	9
		*Hin*dIII	47	23	7
D13S4	13q	*Msp* I	41	19	10
D14S1	14q	*Eco*RI	45	23	2
D15S1	15q	*Msp* I	33	8	0
HP	16q	*Eco*RI	46	20	5
D17S1	17p	*Msp* I	46	17	8
D18S5	18	*Taq* I	47	28	3
D19S7	19p	*Msp* I	45	17	0
D20S4	20	*Msp* I	47	10	1

Loss of Heterozygosity on Chromosome 3. Analysis of the abnormality on chromosome 3 was performed by using three different polymorphic DNA markers: *D3S2* (3p14–p21) (19), *D3S3* (3p) (19), and *SST* (3q28) (gene for somatostatin) (20). Fifteen of 40 patients showed heterozygous genotypes in their normal tissues for the polymorphic DNA marker at *D3S2* on 3p and were informative for determining whether heterozygosity at this locus was lost in their respective tumor tissue. Twelve of 15 cases (80%) showed marked reduction in the intensity of one of two allelic fragments in their tumor tissues on Southern blot analysis, suggesting loss of heterozygosity in the tumors. Loss of heterozygosity on 3p was observed in 7 of 7 SCC tissues. Heterozygosity on 3p was also lost in 5 of 6 AdC tissues (Table 2). None of the 47 patients was heterozygous in their normal tissues at the *D3S3* locus. Loss of heterozygosity at the *SST* locus on 3q was not detected in 12 cases with constitutional heterozygosity at this locus.

Table 2. Histological types of tumors with loss of heterozygosity at loci on chromosomes 3p, 13q, and 17p

Type of tumor	Loss of heterozygosity on chromosomes: 3p	13q	17p
SCC	7/7	10/11	5/5
AdC	5/6	4/15	2/7
SqC	0/1	4/11	1/4
LCC	0/1	1/3	0/0
ASC	0/0	1/2	0/1
Total	12/15	20/42	8/17

Tumors were histologically classified according to the criteria described in the text. The incidence of loss of heterozygosity in each histological type of tumor is given as the no. of patients with loss of heterozygosity in tumors/no. of patients with constitutional heterozygosity.

Loss of Heterozygosity on Chromosome 13. As shown in Table 2, 42 of the 47 patients were heterozygous in their normal tissue for at least one of the four polymorphic DNA markers on chromosome 13q: *D13S1* (13q12), *D13S2* (13q22), *D13S4* (13q22), and *D13S3* (13q33) (6, 7). Twenty of 42 cases (48%) showed loss of heterozygosity at one or more loci on chromosome 13 in the tumors: 10 of 11 SCCs, 4 of 15 AdCs, 4 of 11 SqCs, 1 of 3 LCCs, and 1 of 2 ASCs. Thus, loss of heterozygosity on chromosome 13 was observed in five different histological types of lung cancers, and the frequency was extremely high in SCCs.

Since chromosome 13q14 is a commonly deleted region in retinoblastoma (6, 7), we determined the deleted region in each tumor. Among 20 cases including 10 SCCs that showed loss of heterozygosity on chromosome 13, the constitutional heterozygosity on all informative loci from 13q12 to 13q33 was lost in 16 cases. The results are consistent with the loss of one chromosome 13 complement or a large deletion involving almost the entire chromosome 13q. A typical case (SCC 6) is shown in Fig. 1. However, in 4 cases (SCC 4, SCC 7, SqC 3, and LCC 1 in Fig. 1 and Table 3), only a part of chromosome 13 was deleted. In SCC 4, the *D13S1* (13q12) locus showed loss of heterozygosity, while the *D13S2* (13q22) locus remained heterozygous. Thus, the region between 13pter and 13q22 was deleted in this case. In SCC 7, the deleted region was between 13pter and 13q33, since the

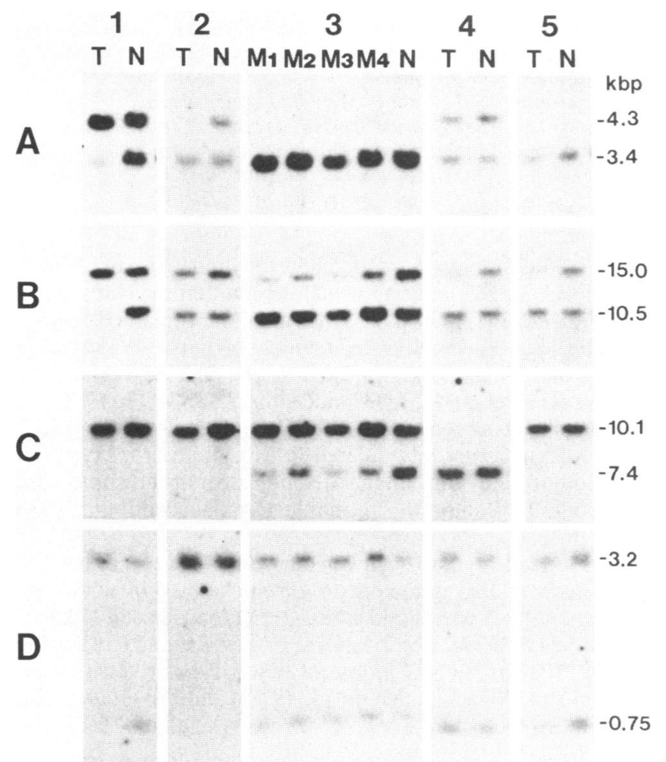

FIG. 1. Loss of heterozygosity at loci on chromosome 13q in lung cancers. Five typical cases are shown: SCC 6 (lanes 1), SCC 4 (lanes 2) SCC 7 (lanes 3), SqC 3 (lanes 4), and LCC 1 (lanes 5). DNA was isolated from primary tumors (lanes T), metastases (lanes M1–M4), and corresponding normal tissues (lanes N). Approximately 10 μg of DNA was digested with *Msp* I (*A*, *B*, and *C*) or *Hin*dIII (*D*), fractionated by 0.8% agarose gel electrophoresis, and transferred to nitrocellulose filters. Filters were hybridized to the following ^{32}P-labeled DNA probes to detect the loci (in parentheses) on chromosome 13: p7F12 (*D13S1*) (*A*), p9D11 (*D13S2*) (*B*), p1E8 (*D13S4*) (*C*), and p9A7 (*D13S3*) (*D*). Numbers to the right of autoradiographs indicate the molecular size of the two polymorphic alleles in kilobases; the larger and smaller restriction fragments correspond to alleles 1 and 2 in Table 3, respectively.

Table 3. Genotypes in lung tumor DNA at loci on chromosome 13

	Marker locus				
Patient	*D13S1**	*D13S2*†	*D13S4*‡	*D13S3*§	*D13S3*¶
SCC 1	–	–	–	2	1
SCC 2	–	1	2	1	2
SCC 3	–	–	–	2	1
SCC 4	2	1,2	–	–	–
SCC 5	2	1	2	–	–
SCC 6	1	1	–	2	1
SCC 7	–	2	1	1,2	1,2
SCC 8	1	–	1	–	1
SCC 9	–	1	–	–	–
SCC 10	–	–	–	–	2
AdC 1	–	1	–	–	–
AdC 2	2	–	–	–	–
AdC 3	1	2	2	–	–
AdC 4	–	1	2	1	2
SqC 1	2	2	2	–	–
SqC 2	–	–	1	1	2
SqC 3	1,2	2	–	–	1,2
SqC 4	–	2	2	–	–
LCC 1	–	2	–	1,2	1,2
ASC 1	2	–	–	1	2

The restriction fragment length alleles present in tumor tissue at loci that were constitutionally heterozygous are indicated by 1 and 2; 1 indicates loss of the smaller sized constitutional allele, 2 indicates loss of the larger sized constitutional allele, 1,2 indicates that heterozygosity remained in the tumor, and a minus sign indicates constitutional homozygosity.

*Chromosome, 13q12; probe, p7F12; enzyme, *Msp* I.
†Chromosome, 13q22; probe, p9D11; enzyme, *Msp* I.
‡Chromosome, 13q22; probe, p1E8; enzyme, *Msp* I.
§Chromosome, 13q33; probe p9A7; enzyme, *Msp* I.
¶Chromosome, 13q33; probe p9A7; enzyme, *Hin*dIII.

D13S3 (13q33) locus remained heterozygous. In SqC 3, only the *D13S2* (13q22) locus showed loss of heterozygosity, while the *D13S1* (13q12) and *D13S3* (13q33) loci remained heterozygous. Thus, the deleted region must be between 13q12 and 13q33, including 13q22. In LCC 1, the deleted region was between 13pter and 13q33, since the *D13S3* (13q33) locus remained heterozygous. Therefore, the region between 13q12 and 13q22 is commonly deleted in SCCs and other types of lung tumors carrying allelic deletion on chromosome 13q. This region contains the retinoblastoma-susceptibility (*RB*) locus (6, 7).

Loss of Heterozygosity on Chromosome 17. Loss of heterozygosity was also detected on chromosome 17p with high frequency. Loss was found at the *D17S1* locus in 8 of 17 cases (47%): 5 of 5 SCCs, 2 of 7 AdCs, 1 of 4 SqCs, and 0 of 1 ASC (Table 2). Thus, in SCCs, loss of heterozygosity occurs not only on 3p (100%) but also on 13q (91%) and 17p (100%). We were able to detect loss of heterozygosity at all of these three chromosomal loci in two SCCs, SCC 5 and SCC 7, because these two patients were heterozygous in their normal tissues at loci on chromosomes 3p, 13q, and 17p.

Loss of Heterozygosity on Chromosomes 3, 13, and 17 Occurs Early in the Development of SCCs. According to the stage grouping defined by the TNM Classification of Malignant Tumors (15), stage I is defined as "no evidence of regional lymph node and distant metastasis." Among 13 of the stage I samples, loss of heterozygosity was observed in 4 cases: 3 of 3 SCCs (SCC 3, SCC 6, and SCC 11) and one of 2 LCCs (LCC 1). Loss of heterozygosity on chromosome 3p was detected in SCC 6 (Fig. 2*A*) and SCC 11 (Fig. 2*C*). Heterozygosity on chromosome 13q was lost in SCC 3 (Fig. 2*B*) and SCC 6 (Fig. 1). Loss of heterozygosity on chromosome 17p was detected in SCC 11 (Fig. 2*D*). Therefore, three

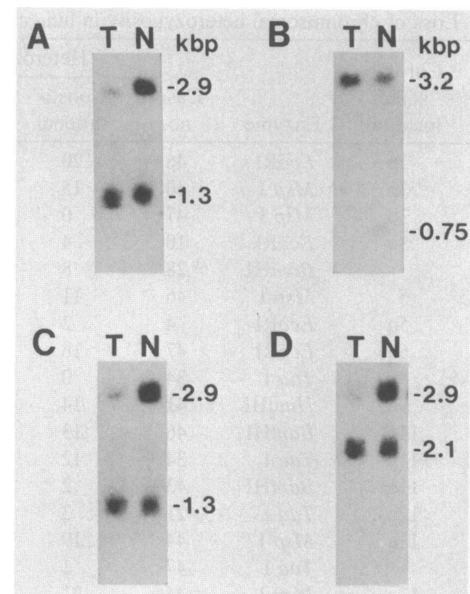

FIG. 2. Loss of heterozygosity on chromosomes 3p, 13q, and 17p in stage I SCCs of the lung. DNA was isolated from primary tumors (lanes T) and normal lung tissues (lanes N) from patients SCC 6 (*A*), SCC 3 (*B*), and SCC 11 (*C* and *D*). DNA was digested with *Msp* I (*A*, *C*, and *D*) or *Hin*dIII (*B*). The filters were hybridized with probes pHF12-32 (*D3S2*) (*A* and *C*), p9A7 (*D13S3*) (*B*), or pHF12-2 (*D17S1*) (*D*).

different genetic events, deletions of 3p, 13q, and 17p, occur prior to the development of clinically evident metastasis in SCCs. In LCC 1, loss of heterozygosity on chromosome 13q was also detected (Fig. 1).

In one case of SCC (SCC 9), both amplification of *NMYC* and loss of heterozygosity on chromosomes 3 and 13 were observed. In this case, we obtained a primary tumor and four different organ metastases at the same time. As shown in Fig. 3 *A*, *B*, and *C*, amplification of *NMYC* was found only in the primary tumor, hilar lymph node metastasis, and pleural metastasis, but not in liver metastasis or paraaortic lymph node metastasis, while loss of heterozygosity on chromosomes 3 and 13 was observed in all tumors taken from five different regions. This result clearly shows that amplification of *NMYC* occurred in a small portion of the cells during the tumor progression, and tumors both with and without amplified *NMYC* metastasized to different organs. In another case of SCC (SCC 10), loss of heterozygosity on chromosomes 3 and 13 was observed in both primary tumor and hilar lymph node metastasis, while deletion of the *HRAS1* locus on chromosome 11p was detected only in metastasis (Fig. 3 *D*, *E*, and *F*). Thus, loss of heterozygosity on chromosomes 3 and 13 must have occurred before that on chromosome 11 in this tumor.

DISCUSSION

Information on the cytogenetics of fresh human lung cancer is limited mainly because of difficulties in preparing the chromosome for karyotyping. The existence of specific chromosomal changes is still controversial, though some papers pointed out that structural abnormality of chromosome 3p and loss of chromosome 13 are frequent in SCC cell lines (3–5). We applied the RFLP analysis to obtain definitive information on chromosome abnormalities in fresh lung tumors rather than in cultured cell lines. Three pieces of unique evidence were obtained from this study. First, heterozygosity on chromosomes 3p, 13q, and 17p is lost in nearly 100% of SCCs. Second, loss of heterozygosity on chromosomes 3p, 13q, and 17p occurs relatively early in the

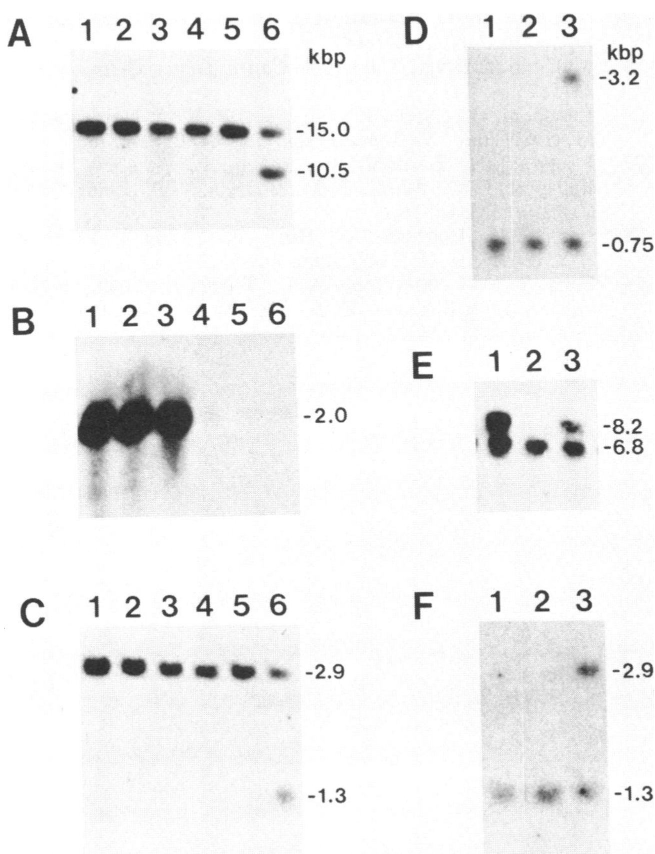

FIG. 3. Loss of heterozygosity on chromosomes 13q and 3p in both primary and metastatic lung cancers. (*A–C*) DNA was isolated from primary tumor (lanes 1), hilar lymph node metastasis (lanes 2), pleural metastasis (lanes 3), liver metastasis (lanes 4), paraaortic lymph node metastasis (lanes 5), and normal lung tissue (lanes 6) from patient SCC 9. (*D–F*) DNA was isolated from primary tumor (lanes 1), hilar lymph node metastasis (lanes 2), and normal lung tissue (lanes 3) from patient SCC 10. DNA was digested with *Msp* I (*A*, *C*, and *F*), *Eco*RI (*B*), *Hin*dIII (*D*), or *Bam*HI (*E*). The filters were hybridized with probes p9D11 (*D13S2*) (*A*), N-*myc* (*NMYC*) (*B*), pHF12-32 (*D3S2*) (*C* and *F*), p9A7 (*D13S3*) (*D*), or c-Ha-*ras* (*HRAS1*) (*E*).

development of SCCs and may be critical in the genesis of the tumor rather than being a secondary event that takes place during the progression of the tumor, such as *NMYC* amplification and chromosome 11p deletion. Third, loss of heterozygosity on chromosome 3p occurs not only in SCCs but also in AdCs with high frequency.

A recent molecular genetic approach has revealed that loss of heterozygosity on a specific chromosome occurs not only in childhood tumors (6–13) but also in common adult tumors (21–26). These results strongly suggest that unmasking of recessive mutation on a specific chromosome may have pathogenetic significance in a variety of tumors. Loss of heterozygosity at the same loci on chromosomes 3, 11, and 13 has been observed to date in various types of tumors: chromosome 3 in renal cell carcinoma (22), SCC, and AdC of the lung (ref. 21 and this study); chromosome 11 in Wilms' tumor (4–7), hepatoblastoma (13), rhabdomyosarcoma (13), and bladder cancer (23); and chromosome 13 in retinoblastoma (6, 7), osteosarcoma (8), breast cancer (26), and SCC of the lung (this study). Therefore, it is possible that these chromosomal alterations represent a common pathogenetic mechanism for different histological types of tumors. Recently the *RB* gene has been cloned independently by three groups (27–29). Structural analysis of the *RB* gene in SCC will clarify whether or not the same gene is involved in the development of different types of tumors. Furthermore, loss of heterozygosity has been detected on some chromosomes other than chromosomes 3, 11, and 13: chromosome 2 in uveal melanoma (25), chromosome 22 in acoustic neuroma (24), and chromosome 17 in SCC of the lung (this study). Future studies should focus on the cloning of genes in these regions, and the structural analysis of these genes in tumors will clarify the biological significance of loss of chromosomal heterozygosity in the tumor development. Our finding that heterozygosity is frequently lost in three different chromosomal loci in SCC assures that these studies will also help us to identify which chromosomal loss is really involved in the genesis of SCC. Furthermore, these studies will clarify whether chromosome 3p deletion has common pathogenetic mechanisms in the development of two histologically different types of lung cancers, SCC and AdC.

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