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AMPLOTYPING OF MICRODISSECTED, METHANOL-FIXED LUNG CARCINOMA BY ARBITRARILY PRIMED POLYMERASE CHAIN REACTION

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The arbitrarily primed polymerase chain reaction (AP-PCR) was used to detect somatic genetic alterations in lung carcinomas. DNA fingerprints generated by a single arbitrary primer were compared between normal and tumor tissues of the same individuals. We adapted the technique to the use of tissue fixed with methanol, which allowed the analysis of small areas of tissue by microdissection. This improvement of the fingerprinting technique permitted the study of tumors at early stages of progression. Loss of sequences from chromosome 7 was detected in 41.7% of adenocarcinomas and from chromosome 22 in 84.6% of small-cell carcinomas. Gains of sequences from chromosomes 1, 8 and 13 were detected in more than 40% of adenocarcinomas and in chromosome 2 in 63.3% of squamous-cell carcinomas. Our results indicate that allelic imbalances at these chromosomal regions are common genetic abnormalities in lung carcinomas. Loss of sequences from chromosome 22q13.3, found in 11 of 13 small-cell carcinomas, were confirmed by microsatellite PCR analysis. We show that the use of our improved AP-PCR fingerprinting permits the detection of both losses and gains of novel chromosomal regions early during lung cancer development. Our results indicate that early-stage tumors tend to have more allelic imbalances than relatively advanced tumors, suggesting a high tumor genetic heterogeneity in the early stages of lung tumor progression. *Int. J. Cancer (Pred. Oncol.)* 89:19–25, 2000.
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Lung carcinoma is one of the most common malignancies in human adults. There have been many previous molecular biological studies of lung carcinogenesis (Jonson and Kelley, 1993). Loss of heterozygosity (LOH), which is associated with the recessive inactivation of tumor-suppressor genes, has been extensively examined (Shiseki *et al.,* 1996; Tamura *et al.,* 1997) using Southern blot hybridization analysis of restriction fragment-length polymorphism (RFLP) and polymerase chain reaction (PCR) amplification of highly informative microsatellite loci. In this manner, LOH on several chromosomes, such as 2q, 3p, 5q, 9p, 11p, 13q, 17p, 18q and 22q, has been reported (Lugwig *et al.,* 1991; Merlo *et al.,* 1994; Shiseki *et al.,* 1994; Tamura *et al.,* 1997; Wieland and Bohn, 1994).

With recent advances in molecular genetics, powerful techniques, such as representation differential analysis (RDA), comparative genomic hybridization (CGH) and arbitrarily primed PCR (AP-PCR) (Welsh and McClelland, 1990), have been developed that also permit the detection of gains of genetic material. AP-PCR is a PCR-based DNA fingerprinting technique that uses single primers of arbitrarily chosen sequences and several initial cycles of low stringency. AP-PCR was instrumental in the identification of microsatellite mutator phenotype in colon cancer (Ionov *et al.,* 1993). For genetic analysis, AP-PCR DNA fingerprinting has several advantages over other techniques. The previous determination of the chromosomal origin of many of the fingerprint bands (Welsh and McClelland, 1990) permits the simultaneous detection of multiple genetic abnormalities in a cancer at the subchromosomal level. The quantitative nature of AP-PCR fingerprinting also allows the detection of allelic losses and gains in tumor cells by the reduction or increase in intensity of tumor fingerprint bands, respectively. In this manner, DNA fingerprinting by AP-PCR generates a molecular karyotype that has been denominated amplotype (Malkhosyan *et al.,* 1998). In addition, AP-PCR DNA fingerprinting does not require specialized instrumentation and can be applied as a screening method for genetic alterations with only a small amount of DNA. However, AP-PCR does not generate reproducible fingerprints with formalin-fixed archival tissue.

Lung carcinoma usually contains many stromal cells and lymphocytes in the tumor lesions, and many investigators tend to underestimate the importance of these contaminated non-tumorous cells in the molecular biological studies of LOH. Microdissection of formalin-fixed tissue in combination with PCR has allowed a refined molecular analysis of the oncogenic alterations in tumors (Moskaluk and Kern, 1997; Shibata *et al.,* 1993). We have previously shown that methanol fixation is quite useful for both molecular biological and morphological analyses (Noguchi *et al.,* 1997). We show here that methanol-fixed tissue is adequate for AP-PCR fingerprinting and that combination of these 2 methods facilitates the detection of unknown chromosomal abnormalities in lung carcinoma.

MATERIAL AND METHODS

Patients and genomic DNA extraction

Forty-nine lung carcinomas (12 adenocarcinomas, 11 squamous-cell carcinomas, 13 small-cell carcinomas and 13 large-cell carcinomas, including 18 stage I, 14 stage II, 12 stage IIIA and 5 stage IIIB carcinomas) from patients undergoing surgical resection between 1995 and 1997 at the National Cancer Center Hospital, Tokyo, Japan, were analyzed. Resected specimens, tumor lesions and corresponding non-tumorous tissues were fixed with methanol and embedded in paraffin. Additionally, for several cases, fresh tumor tissues and corresponding non-tumorous tissue were also frozen and DNAs were extracted as usual. Two to three 5-μm-thick methanol-fixed and paraffin-embedded sections were deparaffinized, and all sections were subsequently stained with hematoxylin and eosin. The stained sections were dried, and tumor lesions and the corresponding normal lung tissue were selectively scratched up using 3-axis mechanical manipulator with microdissection needle, 10 -μm to 30-μm in diameter (Moskaluk and Kern, 1997). Precautions were taken to avoid contaminating the tumor tissue with non-neoplastic inflammatory and stromal cells (Fig. 1). After microdissection, genomic DNA was extracted from the scratched fragments by digesting with proteinase K overnight at 37°C. After heat denaturation for 7 min, eluted DNA was purified with a Glass MAX DNA Isolation Spin Cartridge System (GIBCO BRL, Gaithersburg, MD) following the manufacturer's instructions.

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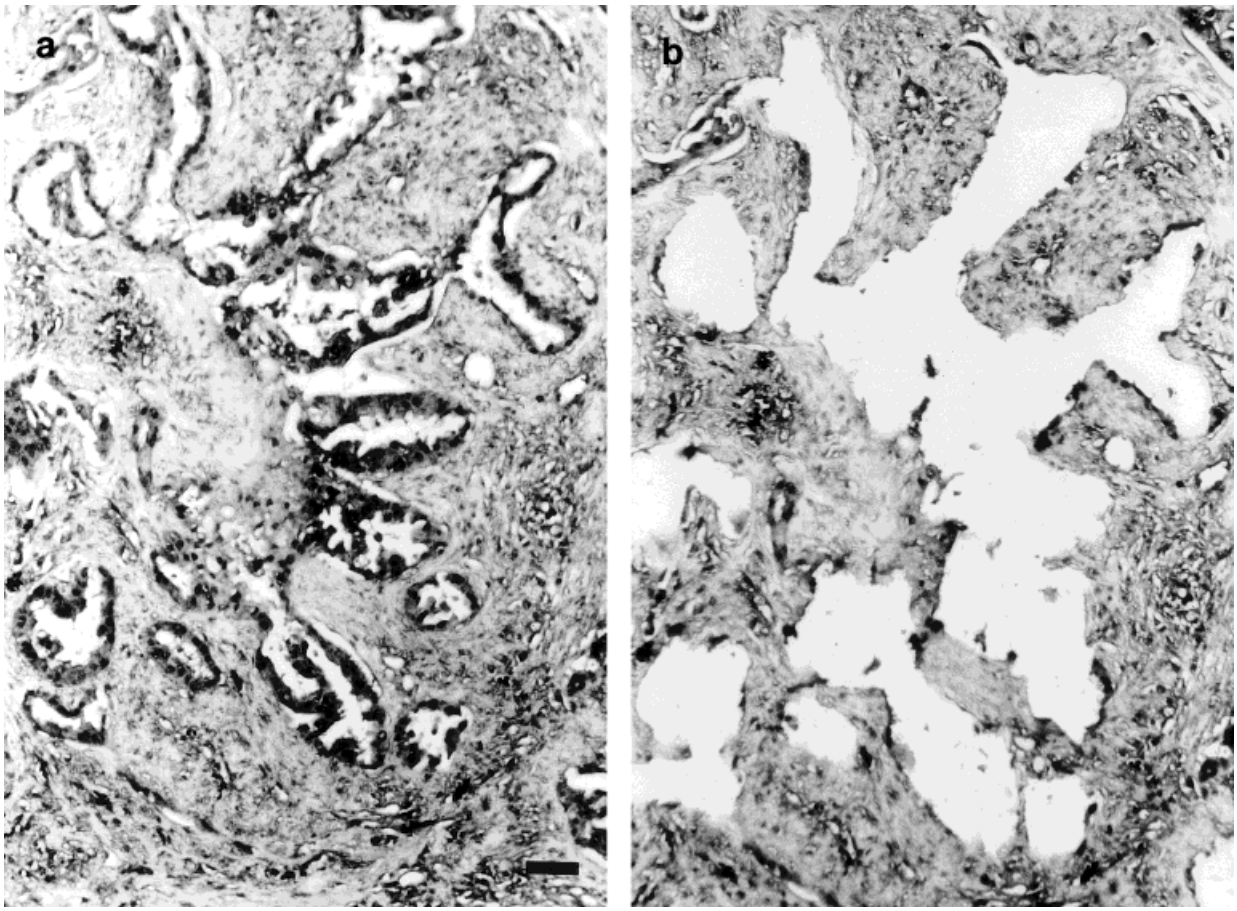

FIGURE 1 – Histology of the adenocarcinoma (AD11) (H and E) before *(a)* and after *(b)* tissue microdissection. Material was fixed with methanol and embedded in paraffin. Scale bar = 20 μm.

AP-PCR DNA fingerprinting

Genomic DNA (0.5–2.0 ng) was subjected to AP-PCR amplification in 15 μl of reaction mixture: 0.6 unit of Taq DNA polymerase (Perkin-Elmer-Cetus, Norwalk, CT), 125 μM of each dNPT, 1 μCi of (α-^{32}P)dCTP (Amersham, Aylesbury, UK), 10 mM of Tris-HCI (pH8.3), 50 mM of KCI, 4.5 mM of $MgCl_2$, 0.1% gelatin and 1 mM of MCG1 (5'-AACCCTCACCCTAACCCCAA-3') as arbitrary primer (Yasuda *et al.,* 1996). Each experiment was performed in duplicate. The AP-PCR conditions were as previously described with 25 high-stringency cycles after 5 low-stringency cycles (Yasuda *et al.,* 1996). The AP-PCR products were electrophoresed in a 5.5% polyacrylamide gel at 55W for 6 hr. The dried gels were exposed to X-ray film (X-OMAT AR, Kodak, Rochester, NY) at room temperature for 12 to 16 hr. Each AP-PCR product amplified with the arbitrary primer MCG1 were previously named as corresponding to the name of the bands in the fingerprint: A, $B_{0,1,2}$, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P, Q_0, Q_1, R_0 and R_1. The chromosomal origins of these AP-PCR products were also determined previously as chromosome 1 (fragments Q_1 and Q_0), 2 (fragments B, F and N), 7 (fragments I and R_1), 8 (fragments D and O), 9 (fragments A and L), 10 (fragments C and F), 11 (fragments B, F, L and N), 12 (fragment K), 13 (fragments E and J), 19 (fragment N), 20 (fragment C), 22 (fragments F, H and M) and X (fragment G) by simultaneous hybridization of arbitrarily primed PCR DNA fingerprinting products (SHARP) method (Yasuda *et al.,* 1996). Evaluation of the fingerprint bands were performed by 3 individuals (YA, TT and MN). The results were expressed as gain or loss when their estimations coincided.

Determination of fragment M sequences of AP-PCR fingerprint

To determine the sequences of fragment M, the band was cut out from the dried gels and extracted with 500 μl of distilled water. The extracted DNA fragments were reamplified with the same MCG1 primer and cloned with the TA cloning system (Invitrogen, Carlsbad, CA) following the manufacturer's instruction. To confirm the authenticity of the cloned fragment, they were used as probes in Southern blot analysis of AP-PCR gel. The correct clones were selected after comparison of the fingerprint and blotting patterns. The BLAST program on the World Wide Web (http://www.ncbi.nlm.gov/) was used for the homology search (Altschul *et al.,* 1997).

To confirm the loss of intensity in fragment M in AP-PCR fingerprints and the loss of sequences from the chromosome, including sequences of fragment M, we examined 6 microsatellite markers selected from the Whitehead Institute database (http://www-genome.wi.mit.edu/). The markers are D22S1140, D22S1160, D22S1170, D22S1161, D22S922 and D22S295, all located around 22q13.3. Each primer was labeled with γ-^{32}P ATP. PCR was carried out with 30 cycles of 30 sec at 95°C, 30 sec at 55°C and 1 min at 72°C. PCR products were electrophoresed on 4% acrylamide gel and subjected to autoradiography.

RESULTS

AP-PCR fingerprinting (amplotyping) with methanol-fixed tissue.

First, we estimated the effect of methanol fixation on the results of AP-PCR DNA fingerprinting using both DNAs extracted from fresh tissue and from tissue fixed with methanol and embedded in

paraffin. Bands A_0, A and $B_{0,1,2}$, which are longer than 800 bp (Malkhosyan *et al.,* 1998), could not be amplified reproducibly from the DNAs extracted from paraffin sections fixed with methanol. These results suggest that high-mw genomic DNA was slightly degraded during the methanol fixation and paraffin embedding. The rest of the bands of smaller size were reproducibly amplified. In this study, we estimated the chromosomal alterations of AP-PCR products named B, C, D, E, F, G, H, I, J, K, L, M, N, O, P, Q_0, Q_1, R_0 and R_1. We found that some gains and losses that could not be detected on AP-PCR fingerprints using fresh frozen sections could be detected in our samples from paraffin sections. This is probably because the admixtures of non-tumorous cells were avoided in the microdissected tumor specimens.

Amplotyping of lung cancer from methanol-fixed tissues

Figure 2 shows representative AP-PCR fingerprints of each histological subtype of lung tumors. Most tumors showed more than one alteration (gains and losses of some bands in the AP-PCR fingerprints). Results are summarized in Figure 3. Loss of sequences from chromosomes 7 (fragment I) were detected in 41.7% of adenocarcinomas. Loss of sequence from chromosome 22 (fragment M) were also detected in 84.6% of small-cell carcinomas. A frequent loss of band F was also observed in all tumors. Loss of sequences from chromosome 13 (band J) was also relatively common. Gains of chromosomes 1 (fragment Q_o), 8 (fragment D) and 13 (fragment E) were detected in more than 40% of adenocarcinomas, and gains of chromosome 2 (fragment H) were detected in 63.3% of squamous-cell carcinomas.

Loss of chromosome 22q13 sequences in small-cell lung carcinoma

We studied in more detail the loss of sequences from chromosome 22 (fragment M) (Yasuda *et al.,* 1996) in small-cell carcinomas (Fig. 4). These losses were found in 11 of 13 tumors (84.6%), as shown in Figure 3. The fragment M was cloned and sequenced. A database search revealed that the nucleotide sequence of fragment M was essentially identical to a portion of the sequence of a human P1 artificial chromosome (PAC) clone, 111J24 (Genbank accession number: Z83836), mapped to chromosome 22q13.3 (the Sanger Center chromosome 22 mapping group, http://www.sanger.ac.uk/). The deduced primer annealing sites were similar to the MCG1 sequence. The PAC clone has a characterized STS in its sequence (D22S660, WI-220), which was mapped to chromosome 22q13 at the Whitehead Institute (Hudson *et al.,* 1995). Our previous study showed that the fragment M was assigned to chromosome 22 by SHARP analysis (Yasuda *et al.,* 1996). Therefore, we conclude that fragment M is derived from the genomic sequence corresponding to the insert of the PAC clone 111J24 and localized to chromosome 22q13.

To examine whether the signal decreases of fragment M observed in the AP-PCR fingerprints of the small-cell lung carcinomas were associated with the loss of the corresponding chromosomal regions, we analyzed allelic imbalances of the region using microsatellite polymorphic markers characterized previously (Hudson *et al.,* 1995; Dib *et al.,* 1996). The D22S660 has been physically mapped by several groups (Collins *et al.,* 1995; Dib *et al.,* 1996). A computer-assisted integrated map between genetic and physical mappings is available through the Internet (Collins *et al.,* 1996). By integrating these data, we determined the relative position of the fragment M in the chromosome 22q region. We found very good correlation between the loss of fragment M and the allelic imbalances at the markers (Figs. 5 and 6).

Comparative amplotype of microdissected lung cancer

The total number of chromosomal imbalances (gains and losses) in the lung cancer amplotypes were examined. Patients were divided into 2 groups. One group revealed 0 to 5 gains or losses in each tumor and the other revealed more than 5 gains or losses in each tumor. There were no differences between histological sub-

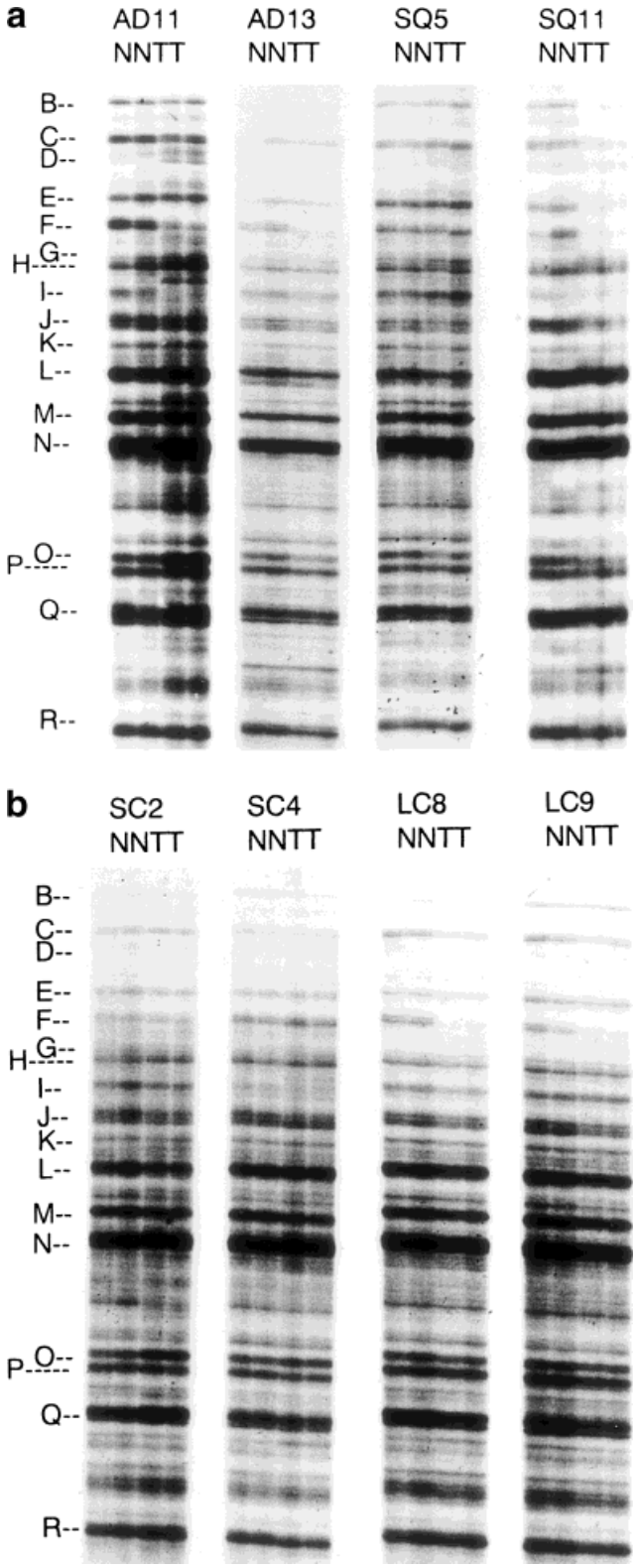

FIGURE 2 – *(a)* AP-PCR DNA fingerprints of lung adenocarcinoma (AD) and squamous-cell carcinoma (SQ) using arbitrary primer MCG-1. *(b)* AP-PCR DNA fingerprints of lung small-cell carcinoma (SC) and large-cell carcinoma (LC) using arbitrary primer MCG-1. N: non-tumor; T: tumor; B-R: AP-PCR bands. See Material and Methods for the chromosomal assignment of each fragment. In case of AD11, the tumor shows increase of intensity (gains) in bands D and O and decrease of intensity (losses) in bands F and I.

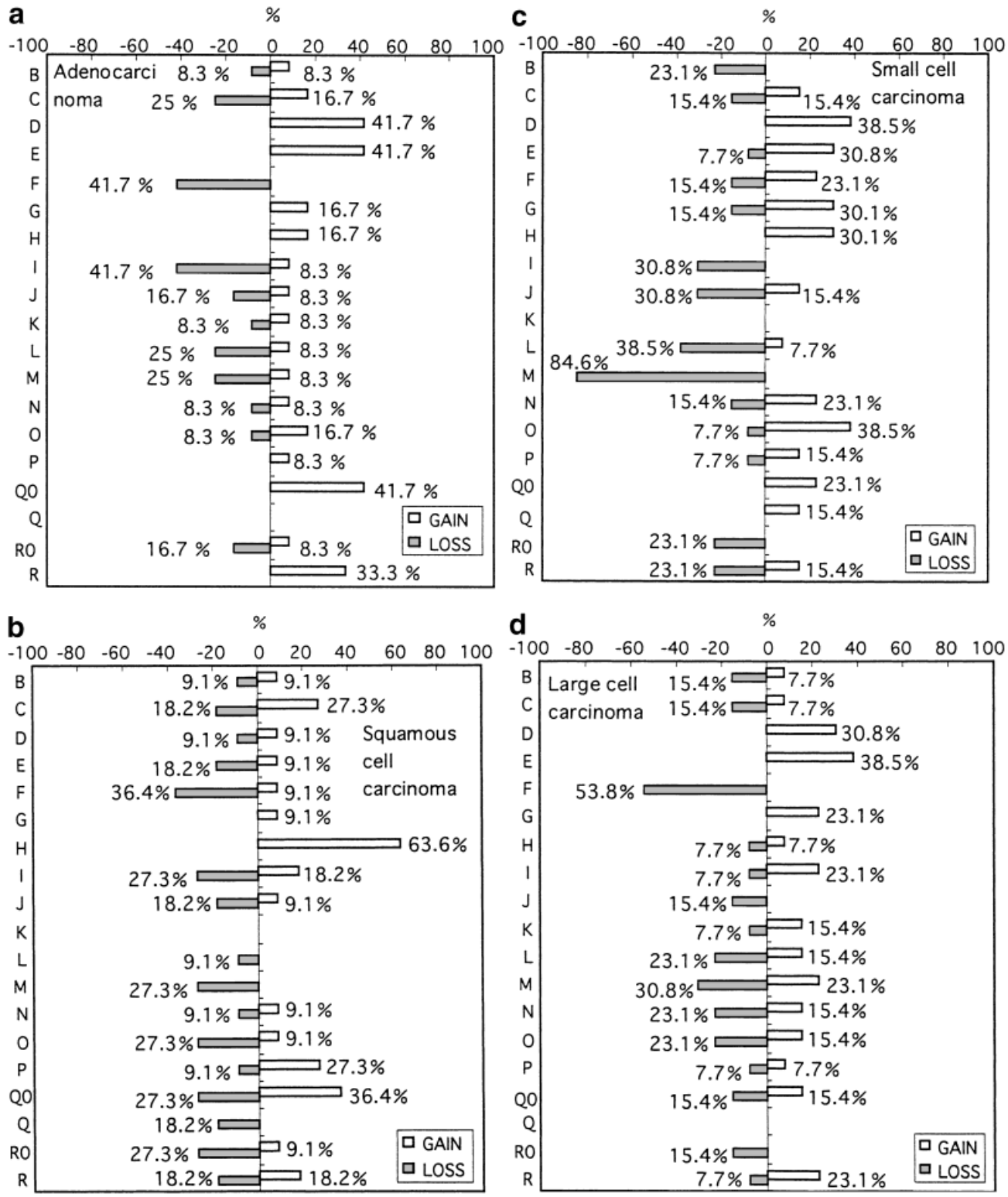

FIGURE 3 – Frequency of gains of sequences (right) and losses of sequences (left) in lung cancer. The graph represents the combined, average values of the analysis of 12 adenocarcinomas *(a)*, 11 squamous-cell carcinomas *(b)*, 13 small-cell carcinomas *(c)*, and 13 large-cell carcinomas *(d)*.

types or extent of differentiation and the distribution of these classes of tumors. However, as indicated in Table I, cases of pathological stage I tended to be more frequent in the latter group, in which each tumor showed more than 5 gains or losses of amplotype bands.

DISCUSSION

Recently developed DNA fingerprinting techniques such as AP-PCR are being applied to screen for chromosomal abnormalities in carcinomas (Ionov *et al.*, 1993). In this study, we have shown that

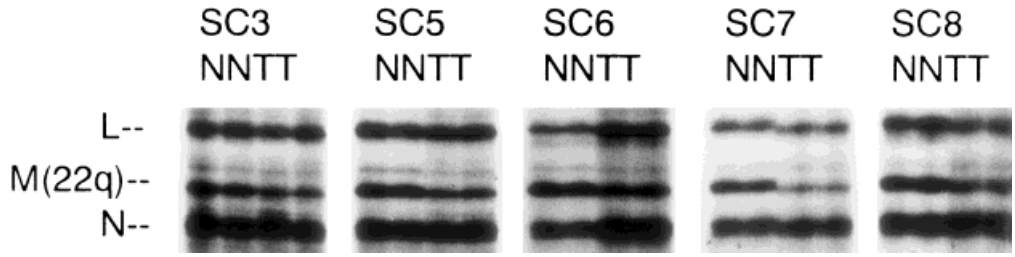

FIGURE 4 – Representative results of AP-PCR amplification of fragment M (chromosome 22q) in small-cell carcinoma. SC: small-cell carcinoma; N: non-tumor; T: tumor. All the tumors showed a decrease of intensity in the AP-PCR fragment M.

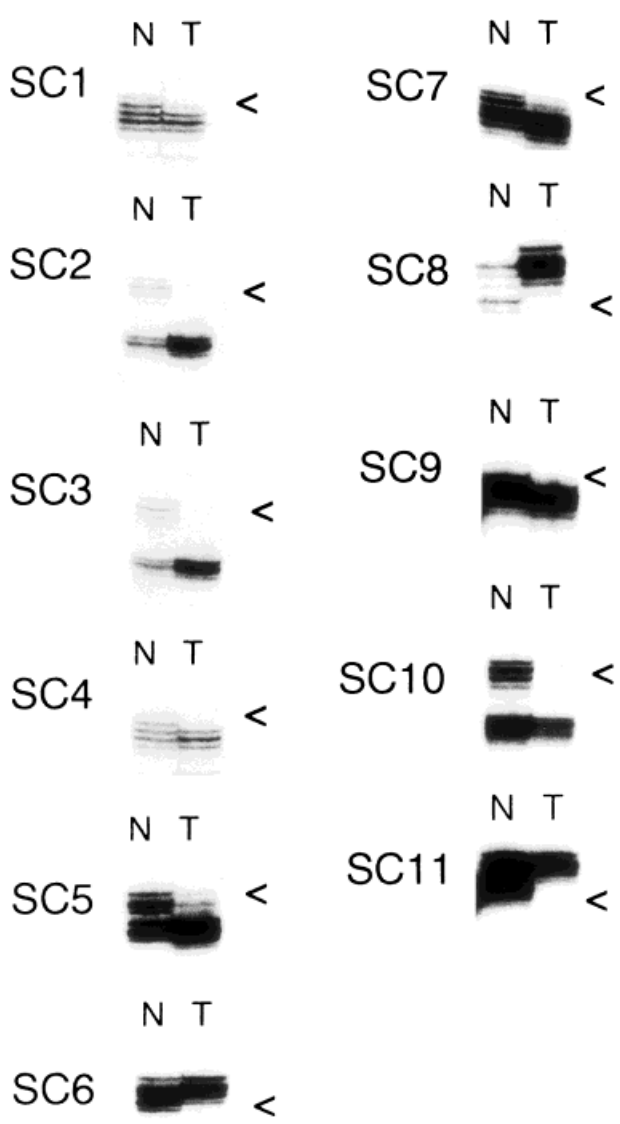

FIGURE 5 – Representative results of CA-microsatellite analysis showing allelic losses on chromosome 22q13.3 (D22S1140;SC 1, 2, 3, 4, 7, 9 and 10, D22S1170;SC8, D22S1161;SC5, 6 and 11). Lost alleles are indicated by arrowheads (<); SC1–11: small-cell carcinoma case number 1–11; N: non-tumor; T: tumor.

DNA extractions from tissues fixed with methanol are also applicable to the AP-PCR DNA fingerprinting method. The methanol-fixed materials are very useful in PCR-based genetic analyses such as allelotyping with microsatellite markers (Noguchi *et al.*, 1997). By using methanol-fixed and paraffin-embedded sections, neoplastic cells could be specifically collected under a lightmicroscope connected to a tissue microdissection system. In this study, we focused on lung carcinomas contaminated with many non-tumorous cells, which may mask the detection of tumor-specific genetic alterations by the currently available techniques.

In AP-PCR fingerprints using methanol-fixed tissues, sequences longer than 800 bp, such as bands A and $B_{0,1,2,}$ could not be sufficiently amplified. The results may indicate that DNA cleavage during methanol fixation is the major cause of the insufficient amplification. However, we could analyze more than 20 independent DNA fragments in each AP-PCR experiment. Therefore, the AP-PCR fingerprinting is useful to analyze multiple loci at one time from very small amounts of microdissected tissue.

Most of the tumors showed several chromosomal imbalances (gains and losses) regardless of their histological type or differentiation (Figs. 2 and 3), showing that allelic imbalances are common in lung carcinomas. With the single arbitrary primer used, we found frequent losses of sequences from chromosomes 7 and 22 in adenocarcinoma and small-cell carcinoma, respectively. Frequent losses of chromosome 13 were relatively common in all types of lung cancer. The common losses of band F are difficult to interpret because this band has been shown to be a composite of sequences from several chromosomes (Malkhosyan *et al.*,1998; Welsh and McClelland, 1990). One possibility is that the methanol-fixed tissue favors the amplification of only one of the sequences, which is the chromosomal region undergoing the losses in the tumors. Analysis is underway to determine which of the sequences from band F is the one suffering the frequent losses in all types of lung cancer. Frequent gains were observed of sequences from chromosomes 1, 8 and 13 in adenocarcinoma and from chromosome 2 in squamous-cell carcinoma.

Our results show that some of the changes are tumor tissue specific, while other changes are shared among different tumor types. For instance, gains of bands D and E, from chromosomes 8 and 13, respectively, have been shown to be common in colorectal cancer (Malkhosyan *et al.,* 1998). Thus, adenocarcinoma, small-cell carcinoma and large-cell carcinoma of the lung but not squamous-cell carcinoma of the lung behave in a similar manner to colorectal cancer relative to these sequences. On the other hand, band J, also from chromosome 13, undergoes gains in colon cancer (Malkhosyan *et al.,* 1998) but losses in lung cancer. These results show the ability of the amplotype approach to identify chromosomal regions frequently altered in lung cancer that have not been described by other allelotyping or cytogenetic approaches (Lugwig *et al.,* 1991; Merlo *et al.,* 1994; Shiseki *et al.,* 1994, 1996; Suzuki *et al.,* 1990; Tamura *et al.,* 1997; Wieland and Bohm, 1994).

Thus, we found frequent losses of sequences from chromosome 22 in small-cell carcinoma (11 of 13 cases). The fragment M sequence was found to originate from 22q13.3. Decreased intensity of fragment M in AP-PCR fingerprinting was confirmed by allelotyping using 6 highly polymorphic microsatellite markers mapped at flanking regions of the fragment M. Several reports have detected frequent LOH in chromosome 22q, which is known

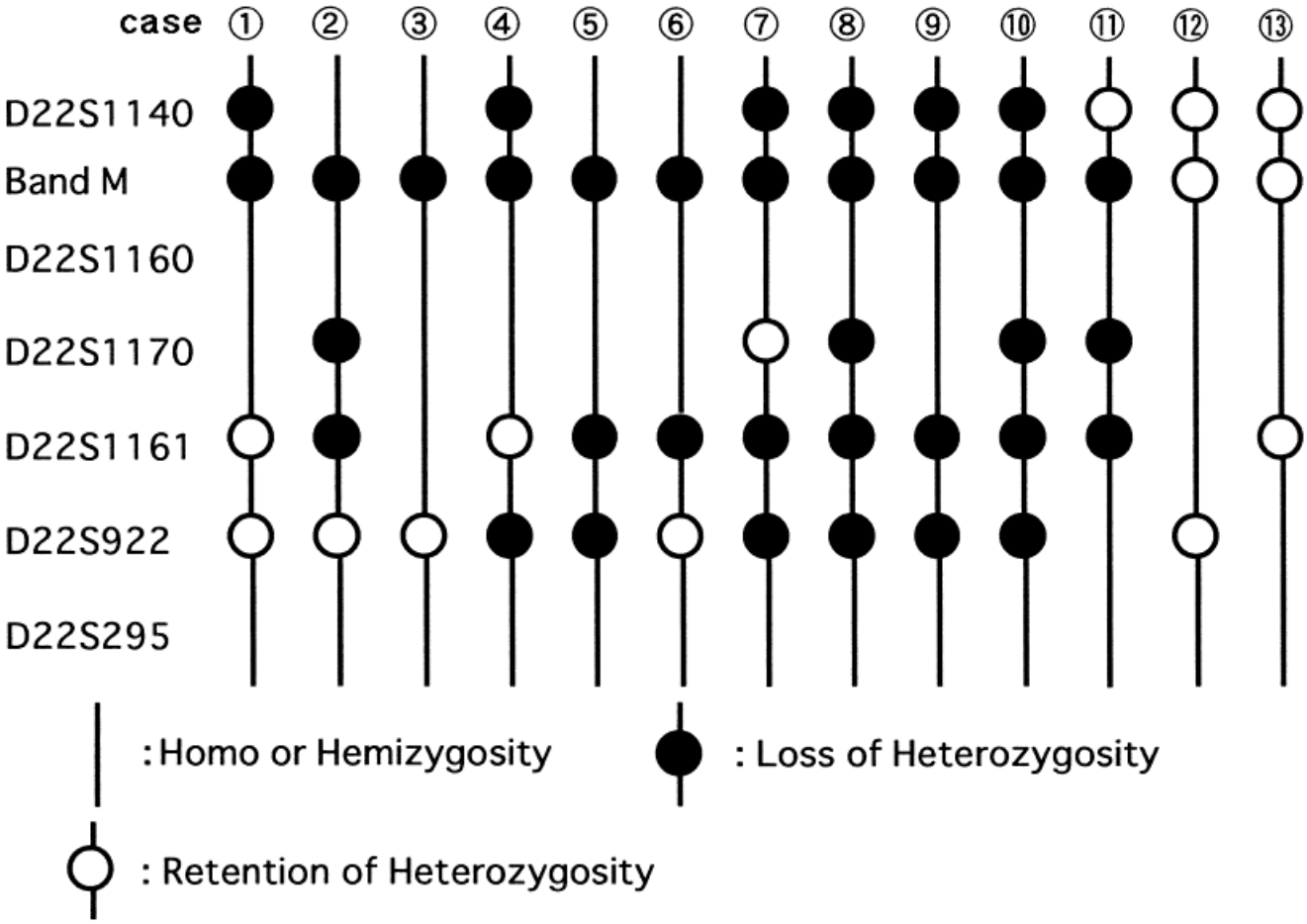

FIGURE 6 – Deletion map of 22q in small-cell carcinomas. Tumor numbers are shown above, and markers are shown on the left. Locations of homozygosity are not informative since only one allele was present in the normal tissue.

TABLE I – PATHOLOGICAL STAGE AND ALLELIC IMBALANCES

	Total number of allelic imbalances		
Pathological stage	0–5	6 or more	Total
Stage I	5[1]	13	18
Stage II	9	5	14
Stage IIIA	6	6	12
Stage IIIB	4	1	5
Total	24	25	49

[1]Case number.

to carry the NF2 tumor-suppressor gene, in non-small-cell lung carcinoma and human gliomas (Rey *et al.,* 1992; Shiseki *et al.,* 1994). However, the region that we report here is located more telomeric relative to the loci reported previously. These results suggest the presence of a novel tumor-suppressor gene(s) on chromosome 22q, which may contribute to the carcinogenesis or progression of the vast majority of small-cell carcinoma of the lung.

In stepwise carcinogenesis, it is thought that genetic alterations accumulate in the course of tumor progression. However, our results show that early-stage tumors tend to have more chromosomal imbalances than relatively advanced tumors. This apparent paradox might be explained by the following possibility. Allelic imbalances (LOH and gains) that occur in early-stage tumors and are detected by AP-PCR in some critical chromosomes may be masked by duplication of the same chromosome or by gains of other chromosomes in the course of tumor progression. As a result, the total number of allelic imbalances detectable by AP-PCR might decrease in the more advanced tumors because this technique, in contrast with the detection of LOH by microallelotyping, cannot detect the loss of an allele followed by the duplication of the remaining allele (Malkosyan *et al.,* 1998). This scenario is reminiscent of the situation observed in colon cancer, with some adenomas consisting of a heterogenous population of neoplastic cells with different genetic alterations, which are homogenized in the carcinoma after clonal expansion (Shibata *et al.,* 1993). Alternatively, the early neoplastic lesions may be different entities from the clones that eventually from the advanced cancers.

In summary, using AP-PCR DNA fingerprinting on small regions of tumor tissue specimens isolated by a tissue microdissection method of methanol-fixed tissue, we have examined genomic quantitative alterations in 49 lung carcinomas. The following conclusions have been drawn: 1. imbalances of several chromosomal regions, both losses and gains, were detected in lung carcinoma of all histological types and extent of differentiation; 2. we localized the chromosomal region of fragment M to chromosome 22q13.3 and found that most small-cell carcinomas (11 of 13 cases, 84.6%) exhibited LOH in microsatellites near this locus; 3. finally, frequent chromosomal imbalances are detectable in the relatively early stages of lung carcinogenesis and tend to decrease during the course of tumor progression.

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