Isolation of DNA sequences deleted in lung cancer by genomic difference cloning

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ABSTRACT To identify DNA sequences that are deleted in human lung cancer, genomic subtraction hybridization was used to construct plasmid libraries that are enriched for DNA sequences deleted in the small cell lung carcinoma cell line SK-LC-17. The clones of the libraries contained predominantly single copy sequences, allowing direct screening of normal and tumor DNA by genomic Southern blotting. Of 150 clones tested, three independent clones (del-27, del-118, and del-109) were identified that specifically hybridized with normal human DNA but not with tumor DNA from the cell line SK-LC-17. The corresponding DNA sequences are localized on human chromosomes 5, 8, and X/Y. The DNA regions identified by del-109 and del-118 were also found to be deleted in several other lung carcinoma cell lines. Moreover, del-118 was deleted in a freshly isolated lymph node metastasis of a human lung adenocarcinoma. It is therefore reasonable to speculate that the identified clones are derived from independent genetic loci encoding potential tumor suppressor genes.

According to current hypotheses, tumor development is due to genomic alterations including deletions, rearrangements, and point mutations of relevant genes. In some tumors, loss of genes at defined chromosomal regions is responsible for primary cancer susceptibility, while secondary genetic changes associate with tumor progression (1, 2). For instance, three to six genetic changes accumulate during progression from premalignant to malignant colorectal tumors, and a greater number of allelic deletions correlate with poor prognosis (3). In lung cancer, the sequence of genetic events leading from premalignant lesions to a malignant tumor is less clear. Deletions on chromosomes 3 and 11 and mutations of the tumor suppressor genes encoding RB and p53 occur early in lung cancer development, but the frequency of these genetic changes is different between the various histological types (4-11). Amplification of MYC genes as well as further genetic changes are implicated in later pathogenesis (4, 12). Since lung cancer is a major cause of cancer death in the human population, it is of interest to gain further insight into the multistep process of genetic alterations that occur in the course of its development.

Several proven or putative tumor suppressor genes—for example, genes encoding RB and NF-1—are particularly prone to being deleted in tumors (13, 14). Accordingly, probes from chromosomal regions lost in tumor cells should help to identify and clone potential tumor suppressor genes. Such probes can, in principle, be obtained by genomic difference cloning. In recent strategies, consecutive subtraction hybridizations were combined with a PCR amplification step resulting in considerable enrichment of critical target sequences of genomic human DNA (15), yeast DNA (16), and cDNA (17, 18). In the present study, this approach was used to isolate DNA probes that are deleted in the lung carcinoma cell line SK-LC-17. These DNA probes were then used to screen other tumor cell lines and freshly isolated human lung carcinomas for loss of the corresponding genomic sequences.

MATERIALS AND METHODS

Preparation of Genomic Difference Libraries. High molecular weight DNA was isolated from human placenta (normal DNA) and from human lung carcinoma cell line SK-LC-17. This cell line was chosen because it shows a highly malignant phenotype (12) and, thus, tumor-specific genomic deletions could be expected. Genomic difference libraries were constructed according to the procedure of Wieland et al. (15). Briefly, tumor DNA was randomly sheared by sonication to an average size of 500 base pairs (bp) and size fractionated. Normal DNA mixed with two copies of bacteriophage λ DNA per haploid genome was cleaved with Sau3A, size fractionated, and biotinylated by a fill-in reaction. A double-stranded oligonucleotide tag that served as a primer template in the PCR was ligated to both ends of the restriction fragments. Sheared tumor DNA and modified normal DNA were mixed at a mass ratio of 200:1, heat-denatured, and reannealed. Single-stranded DNA was isolated by hydroxyapatite fractionation and introduced into the next subtraction hybridization step with fresh tumor DNA. After three rounds, the subtracted single-stranded normal DNA was purified by biotin/avidin affinity chromatography and amplified by PCR using primers complementary to the oligonucleotide tags. The PCR products were cloned into the plasmid vector pUC118. The resulting libraries contained >109 independent colonies and consisted of >80% recombinant clones.

Isolation and Characterization of Plasmid Clones. Plasmid DNA was isolated and single-stranded DNA was prepared as described (15). Inserts were sequenced by the dideoxynucleotide chain-termination method (19) using a 35S sequencing kit (Pharmacia LKB). For the chromosomal assignment, the National Institute of General Medical Sciences human/rodent somatic cell hybrid mapping panel 1 (Human Genetic Mutant Cell Repository, Camden, NJ) was used in Southern blotting experiments. Southern blotting was carried out as described (15).

Isolation and PCR of Human Tumor Specimens. Tumor islets of human lung carcinomas were isolated as described (31). Briefly, 10-μm cryostat sections were placed on a UV-sterilized plastic supporter foil and stained with hematoxylin and eosin. Tumor islets consisting of 50–200 cells were microscopically identified and excised with a small scalpel blade. Particular care was taken to obtain tumor islets that were free of necrosis, leukocytes, stroma, or surrounding normal tissue. The tumor islets were then digested in 50 μl of K buffer (0.1 μg of proteinase K per μl/0.25% Nonidet P-40/1 mM EDTA/10 mM Tris Cl, pH 9) at 55°C for 1 h.

Abbreviation: SCLC, small cell lung carcinoma.

To whom reprint requests should be addressed.

The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M99171 and M99172).
Proteinase K was inactivated at 95°C for 10 min. Debris was pelleted at 10,000 x g for 2 min, and aliquots of the supernatant were subjected to PCR amplification with Tag polymerase (Boehringer Mannheim). A 272-bp sequence of exons 8 and 9 of the p53 gene and a 356-bp sequence of del-27 were amplified for 40 cycles. For amplification of the 181-bp sequence of del-118, a “nested primer” approach (20) with 30 and 27 cycles was chosen. PCR primers for amplifying the p53 sequence were 5'-GACGGAACAGCTTTGAGGTG-3' from exon 8 and 5'-TCTCTACCCAGTGGTTTTC-3' from exon 9; primers for amplifying del-27 and del-118 are indicated in Fig. 2. PCR products were electrophoresed on 2% agarose gels.

Cell Lines. Human tumor cell lines SK-LC-12 and SK-LC-17 were kindly provided by M. Wigler (Cold Spring Harbor Laboratory, NY); Calu-3 (HTB 55) was obtained from the American Type Culture Collection (ATCC); and A-427, A-549, LXF289, and SK-MES-1 (21) were obtained from the Deutsches Krebsforschungszentrum (Heidelberg, F.R.G.). Human lung fibroblast cell line MRC-5 was purchased from Flow Laboratories. Cells were grown in Dulbecco's modified Eagle's medium and 10% fetal calf serum under standard cell culture conditions.

RESULTS

Analysis of Genomic Difference Libraries. The differential genomic plasmid libraries were first analyzed for enrichment of a known target sequence (two copies of bacteriophage λ per haploid genome) included into the system. In one library, 3 of 200 recombinant clones examined contained known target sequences (λ DNA), which is a 450-fold enrichment for single copy sequences present in the normal DNA but absent in the tumor DNA [calculated as described (15)]. The insert sizes of recombinant clones in this plasmid library ranged from 200 to 1500 bp with 85% of the inserts being 300–600 bp long. Of 150 clones that hybridized to Southern blots of normal human DNA, 114 clones identified single copy sequences, 8 hybridized to multiple copy sequences (3–20 copies), 10 hybridized to highly repeated sequences, and 18 gave no signal (Table 1).

Isolation and Characterization of DNA Probes Deleted in the Small Cell Lung Carcinoma (SCLC) Cell Line SK-LC-17. Of the 114 clones identifying single copy sequences, three specifically failed to hybridize with DNA from the tumor cell line SK-LC-17 initially used in the subtraction hybridizations (Fig. 1A). The inserts of these clones were sequenced. Del-27 has an insert of 420 bp and del-118 has an insert of 522 bp (Fig. 2). The insert size of del-109 is 450 bp and was only partially sequenced. Comparison with known DNA sequences of two data bases (European Molecular Biology Laboratory and GenBank) revealed no significant homology to any recorded DNA sequence. When the three probes were hybridized to a zool blot with DNA from dog, cow, hamster, rat, mouse, African green monkey, and human, probes del-27 and del-109 crosshybridized only with African green monkey DNA, whereas del-118 did not hybridize with nonhuman DNA (data not shown). This suggests that all three deletion probes are not highly conserved during evolution. Hybridization of the three probes to a human/rodent somatic cell hybrid panel revealed location of del-27 on human chromosome 5 (94% concordance), del-118 on chromosome 8 (94% concordance), and del-109 on homologous sites on chromosomes X and Y (100% concordance; Fig. 1B).

Loss of Deletion Sequences in Other Human Lung Carcinoma Cell Lines. Several lung carcinoma cell lines were examined for loss of the three deletion sequences by Southern blot analysis. Del-109 was deleted in four of six lung carcinoma cell lines tested (in addition to SK-LC-17)—i.e., Calu-3, SK-LC-12, LXF289, and SK-MES-1 (Fig. 3; Table 2). Del-118 was deleted in SK-LC-17 as well as in the lung carcinoma cell line A-549 and LXF289 (Fig. 3). These cell lines are derived from poorly differentiated lung carcinomas and exhibit a poorly differentiated phenotype in tissue culture (Table 2; ref. 21). Of all the cell lines tested, del-27 was only deleted in SK-LC-17 (Fig. 3). Taken together, these results demonstrate loss of del-118 and del-109 in up to two-thirds of the lung carcinoma cell lines tested, while loss of del-27 occurred at low frequency (Table 2). No homozygous deletion of the sequences was detectable in normal DNA from 15 blood donors (data not shown).

Deletion of del-118 in a Human Lung Carcinoma. Loss of del-27 and del-118 was analyzed by PCR in freshly isolated primary tumors of 20 patients with lung cancer. In 3 of them, lymph node metastases were also examined. Tumor islets completely free of nontumor cells were isolated and the DNA was amplified by PCR. Del-27, del-118, and exons 8 and 9 of the p53-encoding gene were present in normal tissue and primary tumors. However, in one patient with lung adenocarcinoma, cells from a lymph node metastasis did not contain del-118, while it was present in normal tissue and the primary tumor (Fig. 4). The same PCR results were reproduced several times from different sections of the same metastasis. This demonstrates homozygous deletion of del-118 in the metastasis.

DISCUSSION

With the procedure of genomic subtraction hybridization (15), we isolated three single copy DNA sequences (del-27, del-118, and del-109) that are homozygously deleted in the SCLC cell line SK-LC-17. With this procedure, target sequences could be enriched 450-fold by the subtraction hybridization steps and another 50-fold by screening for differentially hybridizing clones using the Southern blotting technique. In total, the deletion sequences were thus enriched

Table 1. Analysis of genomic difference library

<table>
<thead>
<tr>
<th>Total clones tested</th>
<th>150 (100%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single copy</td>
<td>114 (76%)</td>
</tr>
<tr>
<td>Multiple copies</td>
<td>8 (5%)</td>
</tr>
<tr>
<td>Repeats</td>
<td>10 (7%)</td>
</tr>
<tr>
<td>No signal</td>
<td>18 (12%)</td>
</tr>
<tr>
<td>Deleted</td>
<td>3 (2%)</td>
</tr>
</tbody>
</table>

Insert sizes ranged from 200 to 1500 bp (85% with 300–600 bp).
Sequence del-27 was homozgyously deleted in three lung carcinoma cell lines—i.e., SK-LC-17, A-549, and LXF289. All three lines exhibit characteristics of advanced tumor progression (Table 2; refs. 12 and 21). Moreover, loss of del-118 was found in a freshly isolated lymph node metastasis of a patient with lung adenocarcinoma but not in the primary tumor or in normal tissue (Fig. 4). Thus, loss of del-118 may play a role in tumor progression.

Sequence del-118 maps to homologous sites on the human X and Y chromosomes. Human sex chromosomes share small regions with >95% similarity, and alleles in these regions are pseudoautosomal (29). Loss of del-109 was found in two lung carcinoma cell lines (SK-LC-17 and LXF289), and deletion of only the Y chromosome homologous region occurred in three cell lines (SK-LC-12, Calu-3, and SK-MES-1). A cytogenetic study has shown that numerical losses of the sex chromosomes are very common in SCLC (24). Frequent loss of the Y chromosome has also been observed in prostate cancer (27). Furthermore, loss of either the X or Y chromosome occurs in 25% of M2-type acute myeloid leukemias, and it has been suggested that this may involve loss of a tumor suppressor gene (30).

DNA probes from chromosomal regions 3p, 13q, and 17p that have a high incidence of allelic loss in lung cancer were...
not obtained with this genomic difference cloning strategy. A prerequisite for our procedure is that deletions are homozygous and amount to at least 50 kb. Homozygous deletions on chromosome 3p have rarely been reported (for review, see ref. 4), and it is not clear how frequent homozygous deletions of the putative tumor suppressor genes actually occur in lung cancer. In the case of the gene for RB on chromosome 13q and the gene for p53 on chromosome 17p altered mRNA expression and point mutations rather than large deletions are predominately observed in lung cancer (9, 11). In the cell line SK-LC-17 used for constructing the genomic difference library, no homozygous deletion of these two genes has been detected (unpublished data). This explains why no DNA probes were obtained from these loci.

This study demonstrates that genomic difference cloning is an efficient method to isolate genomic DNA sequences specifically deleted in tumor cells. The three deletion sequences isolated are homozygously lost from chromosomes implicated to play a role in tumor development (del-27 and del-109) or are nonrandomly associated with tumor progression (del-118). This suggests that the deletion sequences are derived from independent genetic loci encoding potential tumor suppressor genes.

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Table 2. Loss of deletion sequences in lung carcinoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Morphology</th>
<th>Del-27</th>
<th>Del-109</th>
<th>Del-118</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-LC-17*</td>
<td>F</td>
<td>Del</td>
<td>Del</td>
<td>Del</td>
</tr>
<tr>
<td>Calu-3</td>
<td>E</td>
<td>+</td>
<td>Y-Del1</td>
<td>+</td>
</tr>
<tr>
<td>SK-LC-12†</td>
<td>E</td>
<td>+</td>
<td>Y-Del1</td>
<td>+</td>
</tr>
<tr>
<td>A-427†</td>
<td>E/F</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A-549†</td>
<td>F/E</td>
<td>+</td>
<td>+</td>
<td>Del</td>
</tr>
<tr>
<td>LXZ289†</td>
<td>F</td>
<td>+</td>
<td>Del</td>
<td>Del</td>
</tr>
<tr>
<td>SK-MES-11</td>
<td>F</td>
<td>+</td>
<td>Y-Del1</td>
<td>+</td>
</tr>
</tbody>
</table>

*Epithelioid; †fibroblastoid; +, sequence not deleted; Del, sequence homozygously deleted.