A Homozygous Deletion in a Small Cell Lung Cancer Cell Line Involving a 3p21 Region with a Marked Instability in Yeast Artificial Chromosomes

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ABSTRACT

All types of lung carcinoma are characterized by a high frequency of loss of sequences from the short arm of chromosome 3, the smallest region of overlap containing D3F1SS2 in band p21. Here we characterize a 440-kilobase segment from this region, which we found homozygously deleted in one of our small cell lung cancer-derived cell lines. The homozygous deletion maps between UBE1L and ZnF16, just centromeric to D3F1SS2. Yeast artificial chromosomes with inserts originating from the deleted region are very unstable and readily lose parts of their insert.

INTRODUCTION

Both by cytogenetic analysis (1, 2) and by loss of heterozygosity studies (3–5), the region 3p21–3p22 of the short arm of chromosome 3 has been identified as the region most frequently involved in loss of constitutional heterozygosity of the various types of lung cancer. This strongly suggests that this chromosomal region is the location of a lung-specific tumor suppressor gene. The finding that a human-mouse hybrid cell line containing a 2-megabase DNA fragment from 3p21 had reduced tumorigenicity as compared with the parent mouse fibrosarcoma cell line (6) further supports this suggestion.

Homozygous deletions in lung cancer as well as in other types of tumors are usually of a substantial size, and their common region of overlap is still large. Homozygous deletions are detected at a much lower frequency, and the homozygously deleted region is usually small. Therefore, mapping homozygous deletions may be of considerable help for the fine localization and identification of tumor suppressor genes. The identification of the RB1 and WT1 genes is illustrative in this respect (7–9). Homozygous deletions in 3p21–22, the common region of overlap of the heterozygous deletions, have been described in two recent reports (10, 11).

When screening a number of SCLC3-derived cell lines with a new probe from the distal 3p21 region, we found that its homologous sequences were completely missing from one of the cell lines. Moreover, YACs containing inserts from the region, which was homozygously deleted, showed a marked instability not found in YACs with inserts from the adjacent nondeleted regions.

MATERIALS AND METHODS

Cell Lines. Lung cancer cell lines were established from biopsies of primary tumors by one of us (L. d. L.). Culture conditions were as described (12).

Probes and Primers. Primers were made in this laboratory by the phosphorite triester method using a commercial oligonucleotide synthesizer (Gene Assembler Plus; Pharmacia). Primer sequences for D3S643, D3S647, D3S663, D3S771, D3S917, D3S1227, GNA12, and D3S1225 were as published (13, 14). DD1 primers were 5′-GGCTCTGTCCTGCCTTG-TG-3′ and 5′-TTGGAACAGCCCTGCCCTGAGG-3′ (229-base pair product); H1R primers were 5′-AGGTAGGTTAGGTTGC-3′ and 5′-TCAGAACATTTGTGACCC-3′ (113-base pair product); HIL primers were 5′-AGTCTACAGAACCCTGG-5′ and 5′-ATGGAGCATGGAGGAACG-3′ (150-base pair product).

Southern Analysis. DNA was digested with restriction enzymes under conditions recommended by the suppliers. Agarose gel electrophoresis was carried out at low voltage using 0.7% gels. The DNA was transferred from the gel onto Hybond-N+ filters (Amersham) in 0.4 M NaOH for 8 h. Filters were hybridized with probes labeled by random hexanucleotide priming in a solution consisting of 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA, and 7% sodium dodecyl sulfate (w/v) at 65°C for 16 h.

PCR Analysis. PCR was carried out in a total volume of 30 μl using 10 ng genomic DNA or 25 ng yeast DNA and 150 ng of each primer in a reaction buffer consisting of 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl2, 50 mM KCl, 0.01% (w/v) gelatin, 0.1% Triton X-100, 200 μM of each nucleotide, and 0.13 units Taq-polymerase (Sphaerob; HT Biotechnology, Ltd.). Amplification was carried out for 30 cycles, each cycle consisting of denaturation at 92°C for 30 s (except for the first cycle 2 min) and elongation at 72°C for 60 s (except for the last cycle 5 min) for all primer sets. Annealing was carried out at a temperature specific for the primers used for 60 s.

PFGE. Agarose plugs containing 5 μg DNA were made as described starting from either blood lymphocytes or cultured cell lines (15, 16). The plugs were digested with 40 units of the individual restriction enzyme as starting from either blood lymphocytes or cultured cell lines (15, 16). The plugs were digested with 40 units of the individual restriction enzyme as recommended by the manufacturers for 4 h. The DNA plugs were applied onto a 1% agarose gel and electrophoresed in a Pulsaphor CHEF apparatus (Pharmacia). Electrophoresis was at 150 V for 36 h using ramped pulse times. DNA was transferred onto Hybond N+ filters (Amersham) in 0.4 M NaOH. Filters were hybridized as above.

Screening and Analysis of YACs. A copy of the CEPH YAC library was screened as described (17) using a PCR-based screening strategy (18). Culturing of yeast cells, isolation of DNA from them (18), and generation of plugs were performed as described (19). The restriction digestion of the plugs was carried out as described previously (16) using conditions recommended by the manufacturer (Pharmacia). PFGE and DNA transfer onto nylon membranes were carried out as described above. YAC-DNA was hybridized with both the 2.67-kilobase and the 1.69-kilobase PvuII × BamHI fragment from pBR322 to define left and right end-fragments, respectively. YAC endclones were amplified by vectorette PCR according to Riley et al. (20) and sequenced.

Fluorescence in situ Hybridization. In situ hybridization was carried out essentially as described previously for cosmids (21) and chromosome libraries (22). Chromosomes were identified in R-banded metaphases (23) using a filter for propidium iodide fluorescence. In all cases, at least 50 chromosomes or 100 interphase nuclei were analyzed.
RESULTS

From a 3p14-specific microdissection library (kindly provided by G. Senger, H-J. Lüdecke, U. Claussen, and B. Horsthemke, Institute Für Humangenetik, Essen, Germany), we isolated a few clones that turned out to map far outside 3p14. One of these hybridized to DNA from the human-Chinese hamster hybrid cell line D125.6. Since this cell line contains part of 3p21 as its only chromosome 3 material (16), the microdissection clone must map to this chromosomal subregion. By screening a cosmid library with the clone, we obtained a cosmid from which we isolated a 2.9-kilobase single copy BamHI fragment, DD1. When DD1 was used in a Southern analysis of 15 SCLC-derived cell lines, one of these, GLC20, completely failed to give a signal (Fig. 1). Rehybridization with probes from other loci in 3p21, namely UBE1L (16), ZnF16 (24), and D3S32 (25) resulted in positive hybridization signals with all cell lines. Also, upon PCR amplification with primer sets for MYL3, D3S1235, D3S643, D3S647, D3S663, D3S917, D3S771, and D3S1227 that obtained after hybridization of the same filter with a genomic probe from the UBE1L gene (Fig. 2a). Both probes detect the same 360-kilobase NotI fragment in control DNA, but in GLC20 DNA again a 680-kilobase NotI fragment. The same clone recognizes a 110-kilobase control NotI fragment both in control DNA and in GLC20 DNA. Thus, one end of the homozygous deletion appears to map within the 360-kilobase NotI fragment detected by ZnF16, not in the 110-kilobase NotI fragment. Detailed pulsed field analysis, including double digests with the enzymes used to construct the map (see Fig. 1c), indicated that the 360-kilobase NotI fragment is adjacent to the 110-kilobase NotI fragment detected by DD1 and GNAI2. Thus, in control DNA, the two NotI sites flanking the homozygous deletion span three NotI fragments of 650, 110, and 360 kilobases, respectively, i.e., 1120 kilobases in total. In GLC20, they define a single NotI fragment of 680 kilobase. This implies that the size of the deletion in GLC20 must be 440 kilobases. Its approximate position (the exact breakpoints have not yet been determined) is indicated with a hatched bar in Fig. 2c.

As a first step in the analysis of the region homozygously deleted from GLC20, the CEPH YAC library was screened with DD1-specific primers. Thirty individual yeast colonies, all originating from the same DD1-positive library address 181H1, contained YACs varying in length from 60—340 kilobases (Fig. 3). FISH analysis with the longest YAC proved that it was nonchimeric. By applying a vectorette-PCR protocol to four of these YACs, we could demonstrate that they had identical endclones. For 19 individual 181H1 YACs, we constructed an MluI, NotI, and NotI restriction map. A comparison of these maps with the pulsed-field map showed that the left endclone (H1L) of all 181H1 YACs mapped at 80 kilobases from DD1 in the 110-kilobase genomic NotI fragment, i.e., within the GLC20 deletion. Indeed, amplification of GLC20 DNA with primers derived from H1L failed to produce a PCR product. The right endclone (H1R) mapped at 45 kilobases from the NotI/MluI site between UBE1L and APEH. Thus, in control DNA, the endclones of 181H1 span a genomic region of about 475 kilobases, implying that even the longest (340-kilobase) YAC we obtained still carries a deletion of some 135 kilobases. In 8 of 15 YACs, the deletion included the DD1 homologous sequence. We subsequently screened the YAC library with primers specific for H1L, H1R, and UBE1L, respectively. This resulted in four more YACs. 407A11 (nonchimeric; 300 kilobases) is positive for H1R and UBE1L but not for DD1. This YAC also overlaps with the loci APEH and D3F15S2, and thus extends away from DD1. 191D3 (chimeric) is positive for H1R but not for UBE1L. As this YAC had already been reported to be positive for D3F15S2 (14), it also maps away from DD1. YAC 529B2 is positive for UBE1L but not for H1R. Although this YAC is only 60 kilobases long, it contains a sequence homologous to H1L, the most distant endclone of the 181H1 YAC. Therefore, this YAC, too, most likely has been derived from a larger YAC which lost part of its human insert. 181B3 was isolated with H1L. This 160-kilobase YAC was positive for GNAI2 as well. Although by FISH analysis it appeared to be nonchimeric, its left endclone, isolated by vectorette PCR, failed to hybridize to single human chromosome 3 hybrid DNA. Since primers derived from the right endclone of 181B3...
were able to amplify both 181H1 and GLC2O DNA, this end of the YAC should map between UBE1L and DD1. Sequences homologous to DD1 were absent from this YAC, indicating that it must have undergone some rearrangement(s).

In order to determine the orientation of the pulsed-field map with respect to the chromosome 3 centromere, we applied bicolor fluorescent in situ hybridization using combinations of two of three YACS, namely 407A11 and 181B3, isolated with the endclones of 181H1, plus one at a larger distance, for which we used the nonchimeric YAC 264C5, isolated with MYL3. By first hybridizing YACS 407A11 and 264C5 to prometaphase chromosomes from blood lymphocytes, YAC 407A11 containing APEH and UBE1L turned out to map centromeric to 264C5, containing MYL3 (Fig. 4). From a subsequent interphase FISH analysis of 407A11 and 181B3 in combination with 264C5 (results not shown), the order of the YACs from telomere to centromere could be established as 264C5–407A11–181B3. This implies that the UBE1L locus flanks the homozygous deletion at the telomeric end, whereas the ZnF16 locus flanks the deletion at its centromeric end. Using cosmid clones from these loci in a bicolor FISH analysis on interphase nuclei from normal lymphocytes in combination with a 4.0-kilobase BamH1 fragment of ZnF16 (Z), respectively. The restriction enzymes used for DNA digestion are indicated above the lanes. Each digestion was carried out on lymphocyte DNA from two unrelated individuals.

The finding of a homozygous deletion in a region so frequently affected by heterozygous losses corroborates the idea of the involvement of a tumor suppressor gene. Moreover, it clearly restricts the area in which to search for such a gene. Also, for known tumor suppressor genes like RB1 and TP53, homozygous deletion has been reported as an inactivating mechanism in a number of cases (32, 33).

For lung tumors, there have been some previous reports on homozygous deletions. In the SCLC-derived cell line SK-C-17, homozygous 3p21 deletion in an SCLC cell line.

**FIG. 2.** a–c, PFGE analysis and resulting long-range physical map of the region containing the APEH, UBE1L, DD1, GNAJ2, and ZnF16 loci. a, successive hybridizations of a single PFGE filter with a 4.0-kilobase BamH1 fragment from UBE1L (U), the 1.2-kilobase BamH1 × Nol fragment from DD1 (L), and the 1.7-kilobase Nol × BamH1 fragment from DD1 (R), respectively. The restriction enzymes used for DNA digestion are indicated above the lanes. Each digestion was carried out on lymphocyte DNA from two unrelated individuals. UBE1L and DD1-L detect the same fragments; DD1-R detects different ones. b, successive hybridizations of a single PFGE filter with a 4.0-kilobase BamH1 fragment of UBE1L (U) and with a 2.3-kilobase EcoRI fragment of ZnF16 (Z), respectively. The restriction enzymes used for DNA digestion are indicated above the lanes. The lanes contain either GLC2O DNA (S) or lymphocyte DNA (L). c, long range map derived from PFGE analysis as described in the text and shown above. The position of the loci is indicated by small blocks (not drawn to scale). Restriction sites for Mnl, Nol, and Nru are indicated by M, N, and R, respectively. Numbers indicate the length of the Nol fragments in kilobases. The Nru site indicated by an asterisk is consistently partially digested, as can be seen below a and b. The approximate position of the region homozygously deleted in GLC20 is indicated by a hatched bar below the map.
gous deletions occurred on chromosomes 5, 8 and X/Y, respectively (34). The latter two were found repeatedly in a small number of cell lines. The X/Y deletion was also detected in a lymph node metastasis. Since it did not occur in the primary tumor, it cannot be a primary event. Instead, this kind of homozygous deletion may reflect an increased genomic instability during progression of lung cancer in vivo or in vitro. Homozygous deletions on chromosome 9 were detected in 4 of 26 non-SCLC-derived cell lines but not in 6 SCLC-derived cell lines (35). This is in line with the relatively high frequency of loss of heterozygosity of 9p in non-SCLC (36). The smallest region of overlap of these homozygous deletions on chromosome 9 is between the MTAP gene and the IFN gene cluster in band p22. From this region, a gene has been isolated which shows mutations in a variety of tumors, including non-SCLC (37, 38). A large homozygous deletion at 3p12–13 has been described to occur in the SCLC cell line U2020 (14, 39). As this deletion is not in 3p21, the most commonly deleted region in lung cancer, its significance is unclear.

More recently, homozygous 3p21 deletions have been reported in lung cancer-derived cell lines (10, 11). A cosmid that was mapped to 3p21.3 by in situ hybridization detected homozygous deletions in 5 of 36 lung cancer cell lines (10). Thirty-nine more cosmids from 3p21.3–p22 did not detect any homozygous deletions. Since no reference markers had been included, the position of the homozygous deletions relative to ours cannot be determined. A homozygous deletion in the SCLC cell line NCI-H740 encompasses the loci GNAI2 and mfd93, defining D3S1235 (11). FISH analysis with a YAC positive for the latter locus showed that it lies centromeric to ZnF16. Since D3S1235 is not deleted in GLC2O, this implies that, at the centromeric side, the homozygous deletion of cell line NCI-H740 extends much further than our homozygous deletion. The region deleted from NCI-H740

4 Unpublished results.
overlaps with a human chromosome 3 fragment of approximate 2 megabases translocated into a mouse fibrosarcoma cell line by microcell fusion (11). The resulting hybrid cell line, HA(3)BB9F, had a much lower tumorigenicity than its parental mouse fibrosarcoma cell line (6). Our homozygously deleted region shares GNA12 with both the region homozygously deleted in NCI-H740 and the chromosome 3 fragment present in the hybrid cell line HA(3)BB9F. These various independent indications for the presence of a tumor suppressor gene in the same small region of band p21 of chromosome 3 warrant a detailed genomic analysis of this region. The construction of a YAC contig overlapping the homzygous deletion revealed a marked instability of this region in the YACs. Whether this may be considered as a reflection of an inherent instability of this region has to await further analysis.

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REFERENCES


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