A Gene from Human Chromosome Region 3p21 with Reduced Expression in Small Cell Lung Cancer

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ABSTRACT

A combination of cytogenetic and molecular studies has implicated the p21 region of human chromosome 3 as the probable site of a gene that is the loss of which contributes to the development of small cell lung cancer. We report here the isolation of a gene from this region which is expressed in normal lung tissue and in cell lines derived from a number of different types of tumor, but the expression of which in small cell lung cancer cell lines is undetectable by RT-PCR analysis. Although the more sensitive polymerase chain reaction did detect transcripts, a novel quantitative polymerase chain reaction assay showed that their concentration in small cell lung cancer cell lines is less than 3% of that in normal lung.

INTRODUCTION

Characteristic chromosome deletions are found in a number of different tumor types and are thought to signal the presence of a gene or genes whose loss contributes to tumorigenesis (1–3). Such genes have been termed “tumor suppressor” genes. Deletions may be homo- or heterozygous and have been found both in cancers with a strong genetic component and in those without. In either case, where the deletion affects only one homologue of a chromosome pair in the tumor, submicroscopic events appear to have inactivated the tumor suppressor gene on the other homologue (4, 5). In cancers with an inherited predisposition, a chromosome carrying a mutant allele or, in rare cases, a microscopically visible deletion, is transmitted through the germ line. The mutation is therefore constitutional and heterogeneous in the tumor through the (functional) loss of the remaining active allele. It has been postulated (1) that the same genes are inactivated or lost in both inherited and sporadic forms of the same tumor type.

Microscopically visible deletions of the short arm of chromosome 3 have consistently been found in SCLC (6–8) with the shortest region of overlap in 3p21. The pattern of allele loss in tumors at loci for which the patients are heterozygous confirms these findings, which suggest that a likely position for a lung tumor suppressor gene is in 3p21 (8–11). The locus most consistently involved in allele loss in SCLC is D3F15S2 (formerly DNF15S2), which has been assigned by somatic cell hybrid and in situ hybridization studies to 3p21 (8, 12, 13). Rare cases of SCLC have been found (14–16) in which heterozygosity is retained at other loci similarly assigned to 3p21, suggesting that a putative SCLC tumor suppressor gene might lie close to D3F15S2. The pattern of expression of genes localized to this region is therefore of interest, particularly in normal lung as compared to SCLC tumors and cell lines. Here we report a gene in 3p21 which is expressed in normal adult lung and in cell lines derived from a wide variety of tumor types but which is consistently and specifically not expressed or underexpressed in SCLC cell lines.

MATERIALS AND METHODS

Library Screening. Cosmid libraries in the vector Lorist B were screened with radiolabeled probes as described (17), and the resulting clones were assembled into contigs by restriction mapping. Regions enriched in the dinucleotide CpG were identified in the contig by digesting individual cosmids with restriction enzymes with a recognition sequence that includes one or more copies of this dinucleotide. CDNA libraries in λgt10 or λgt11 were screened with single-copy probes isolated from the vicinity of CpG-enriched regions.

Fluorescent in Situ Hybridization. In situ hybridization was carried out essentially as described (18). For use as probe somatic cell hybrid DNA was amplified using the Alu-IV primer (19). Human chromosomes were identified in R-banded metaphases (20) using a filter for propidium iodide fluorescence.

Northern Blot Analysis. Polyadenylated RNA was isolated using guanidium HCl (21), electrophoresed on a 1.4% agarose gel, blotted to Hybond N (Amersham), and hybridized to 3P-labeled CDNA inserts.

Pulsed Field Gel Electrophoresis. Agarose plugs containing 5 µg lymphocyte DNA were made as described (22). The plugs were digested for 4 h with 40 units of the indicated restriction enzyme in a buffer recommended by the manufacturers. The DNA plugs were applied to a 1% agarose gel and electrophoresed in a Pulsaphor CHEF apparatus (LKB-Pharmacia). Electrophoresis was for 18 h at 200 V, using ramped pulse times. DNA was transferred to Hybond N-plus (Amersham) in alkaline 1.5 M NaCl. Filters were hybridized in 0.5 M sodium phosphate, pH 7.5/1 mM EDTA/7% sodium dodecyl sulfate with probes labeled by random hexanucleotide priming.

RT-PCR Analysis and Quantitative PCR. First strand cDNA synthesis was carried out on 30 µg total cellular RNA using 32 units of Moloney murine leukemia virus reverse transcriptase after priming with 450 ng random 14-mer primers, under conditions recommended by the manufacturer (Pharmacia). Without further purification, a volume corresponding to 1 µg total cellular RNA was amplified with a set of specific primers. Amplification was for 33 cycles in a total volume of 30 µl; of this 15 µl were analyzed on a gel consisting of 2% w/v Nussieve agarose (Research Organics) and 1% agarose (Pharmacia). A recombinant CDNA was constructed by ligating a 102-base pair restriction fragment of pUC19 into the insert of a cDNA clone. After linearization of this construct, recombinant RNA was synthesized in vitro using T7 polymerase. Mixtures of varying amounts of in vitro-synthesized recombinant RNA and 5 µg of cellular RNA were subjected to a RT-PCR analysis, using the same primer set in each case; because of the insertion, the PCR product from the in vitro-synthesized RNA is larger than that from the native mRNA. The amount of specific mRNA in the cellular RNA sample is determined by comparing the intensities of the two bands. Due to competition for primers, there is some reduction in the amount of product from the cellular RNA sample when high concentrations of the in vitro-synthesized RNA are included in the PCR. The detailed method will be published elsewhere.
RESULTS

The locus most consistently reduced to hemizygosity in SCLC is D3F15S2. However, in a systematic search for expressed sequences in 100 kilobases of normal genomic sequence cloned from around this locus only a single gene was found. This was the previously identified acylpeptide hydrolase, APEH (15, 23, 24), formerly known as D3S48E.

No other genes were found within the approximately 100 kilobases around the D3F15S2 locus. Therefore, in order to isolate further sequences from 3p21, a genomic library was constructed from DNA of the Chinese hamster-human hybrid DIS2.6. This hybrid contains the human D3F15S2 locus (25), but not the human RAF1, ERBA2, D3S2, D3S3, or D3S6 loci (not shown), all of which have been regionally assigned on the short arm of chromosome 3 (26). The amount of human chromosome 3 material present in this hybrid was assessed, on the basis of blot hybridization to these probes, as not more than 3p21–p24. The absence of the human D3S2 locus from this hybrid is of particular interest inasmuch as it has also been assigned to 3p21, indicating that the rearrangement of human chromosome 3 in the hybrid occurred within this band. In situ hybridization of PCR-amplified single-copy DNA from the DIS2.6 hybrid to banded human lymphocyte metaphases revealed that a small part of band p21 is the only chromosome 3 material present in the hybrid (Fig. 1a). Some other human sequences, including about 5% of the short arm of chromosome 1 (12), known to be present in this hybrid from gene expression and DNA blot hybridization assays, were also detected by this method.

Human genomic clones were isolated from the DIS2.6 library and assigned to human chromosomes using somatic cell hybrids and in situ hybridization. A number were found which mapped to the short arm of chromosome 3, among them clone D2-λ8. In situ hybridization using both tritium-labeled and fluorescent D2-λ8 probes (Fig. 1b) shows that this clone originates from the 3p21 fragment in the hybrid. Because both the D3F15S2 locus and genomic sequences homologous to the D2-λ8 clone lie within the same small part of 3p21, an attempt was made to determine the physical distance between them by pulsed field gel electrophoresis. As shown in Fig. 2 the two loci are separated by a distance of about 140 kilobases. As expected from their proximity, quantitative DNA blot analysis (not shown) showed a concomitant reduction in copy number of D3F15S2 and D2-λ8 sequences in all 15 SCLC cell lines examined.

A single-copy fragment obtained from the D2-λ8 clone was found which hybridized, under stringent conditions, to DNA from a wide variety of species (Fig. 3a). The restricted evolutionary divergence of this sequence is consistent with the presence of coding sequences within the fragment, and it was therefore used to screen a lung cDNA library. A single clone was isolated which was used to isolate further clones from human pre-B-cell, T-cell, and liver cDNA libraries. Of the several clones isolated, the longest was 3.3 kilobases. All clones detected a 3.5-kilobase transcript by gel blot (Northern) analysis of normal adult lung RNA (Fig. 3b). We refer to this gene as D8.

Polyadenylated RNAs from various normal and tumor cell lines were hybridized, after electrophoresis and blotting, to D8 cDNA probes. A 3.5-kilobase transcript was detected in a wide variety of cell types, both normal and tumor (Fig. 4a; Table 1). However, under the same conditions, the transcript was undetectable in all 23 SCLC lines examined. Hybridization of the same RNA blots to the APEH/D3S48E cDNA was carried out either subsequently or concurrently, inasmuch as this gene is also included in the SCLC deletion and can be used for standardization not only of RNA loading and integrity but also of possible quantitative differences in transcript abundance due to reduced gene dosage.

The expression of the D8 gene was further examined using the more sensitive PCR. Sufficient sequence information (to be published in full elsewhere) was obtained from cDNA clones to enable us to design a set of oligonucleotide primers for a combined RT-PCR analysis in which uncloned cDNA, reverse transcribed from normal and SCLC cell line RNA, was amplified. The primers originated from the 3' region of the D8 gene and spanned at least one intron. In order to quantitate D8 mRNA levels, various amounts of recombinant D8 mRNA, synthesized in vitro from a cDNA clone enlarged by insertion of a 102-base pair plasmid fragment into the D8 insert, were

Fig. 1. a, human DNA in the hybrid DIS 2.6. Single-copy human sequences from the hybrid DIS 2.6 were obtained by Alu-PCR (19), biotin labeled, and hybridized to normal lymphocyte chromosome preparations as described (18). Arrow, band 3p21, which is strongly labeled by the DIS 2.6 probe. b, chromosome assignment of a cosmid isolated with the D2-λ8 probe by fluorescent in situ hybridization. Fluorescent signals accumulate only over band 3p21.
GENE FROM 3p21 WITH REDUCED EXPRESSION IN SCLC

Fig. 2. Long-range physical map showing the physical relationship of the D3F15S2/D3S48E (APEH) and D8 loci. In a, gel transfers of pulsed-field gel electrophoresis experiments were hybridized to single-copy probes isolated from either side of the MluI and NruI sites in genomic clones containing the D8 and D3F15S2/D3S48E (APEH) regions. Autoradiographs produced by the probes B4.1 (from a D2-X8 cosmid) and H3E4 (which detects the D3S48E/APEH locus) are shown for illustration. Left, both probes recognize a 620-kilobase (Kb) NotI fragment; right, NruI site within this fragment separates the two probes. The position of the Saccharomyces cerevisiae chromosome markers is indicated between the autoradiographs; their sizes are (top to bottom) 760, 580, 430, 340, 270, and 235 kilobases. Asterisk, compression zone. b, long-range physical map. The map, derived from experiments such as those in a, is shown as a continuous horizontal line; below it is a representation of the regions cloned, with the positions of D2-X8 and D3F15S2 marked. A high-resolution map around the D3F15S2 locus appeared in the paper of Welch et al. (17).

Fig. 3. Conserved and expressed sequences within the D2-X8 clone. a, hybridization of a single-copy sequence from the D2-X8 clone to DNA from different organisms. Genomic DNA (10 μg), isolated from tissues of the indicated species, was digested with TaqI, electrophoresed, blotted, and hybridized to the B4.1 probe (see Fig. 2). b, hybridization of the D8 lung cDNA clone to human adult lung RNA. The position of the 28S and 18S rRNA bands is shown. In the experiment shown here, the probe was the insert of a 1.6-kilobase (Kb) clone isolated from a T-cell cDNA library.

added to a fixed amount of total RNA isolated from the cell line under test. Subsequent RT-PCR analysis of the RNA mixtures gave rise to two products of different sizes. An analysis of the ratio of these two products in different gel lanes (Fig. 4b) made it possible to estimate the amount of endogenous D8 mRNA. The results, summarized in Table 2, show that the D8 mRNA concentration in SCLC cell lines is 0.5–3% of that in normal lung tissue.

DISCUSSION

We have localized a new gene to the chromosomal region 3p21 and studied its expression in SCLC cell lines. This gene lies within a region of the genome which both cytogenetic and molecular studies have identified as a possible site for a putative SCLC-specific tumor suppressor gene. This is the fourth gene assigned to this region which has been studied in this way. The
aminoacylase gene (ACY1) shows reduced expression in about 20% of SCLC cell lines (15, 27), but the significance of this finding for a gene whose product participates in general cellular metabolism is unclear. The APEH (D3S48E) gene, also described here, has been found in a single study (15) to be underexpressed in one of four SCLC cell lines studied; the exceptional cell line was also deficient in ACY1 expression. Again, the significance of this is not clear. In the present study we found consistently high levels of expression of this gene in SCLC cell lines. In one study (23), some reduction in the expression of the APEH gene has been found in renal cell carcinomas. A protein-tyrosine phosphatase (PTPG) has also recently been assigned to this region and is deleted in 5 of the 10 primary non-SCLC tumors and one of the two SCLC cell lines examined (28). This gene was apparently well expressed in all the lung tumor cell lines examined (histopathological type not specified).

With regard to the D8 gene described here, the question naturally arises as to whether the observed pattern of expression, namely strong expression in normal lung and dramatically reduced expression in SCLC, indicates its primary involvement in the origin and/or progression of this tumor. It may be, for example, that the reduced expression of this gene in SCLC cell lines reflects a differentiated state that they share with a sub-

Fig. 4. Expression of the D8 gene in SCLC cell lines. a, Northern blot analysis of D8 mRNA levels in SCLC and other tumor cell lines. JW2 is a colorectal carcinoma cell line, EJ is a bladder carcinoma, and GCT27 is an embryonal carcinoma cell line (see Table 1). All other cell lines are derived from SCLC. Polyadenylated RNA gel transfers were hybridized concurrently with the D3S48E/APEH cDNA (lower signal in all lanes) and with the insert of a 1.6-kilobase D8 cDNA (upper signal in non-lung tumor lanes and absent in SCLC samples). A, quantitative RT-PCR analysis of D8 mRNA levels in SCLC cell lines. In vitro-synthesized recombinant D8 RNA (1 ng, 0.2 ng, 40 pg, 8 pg, and 1.6 pg, respectively) and total cellular RNA were mixed and subjected to the quantitative RT-PCR protocol outlined in “Materials and Methods.” One-half of the PCR product was analyzed on a 3% agarose gel. For each cell line, the amount of D8 mRNA present is estimated by determining the amount of recombinant RNA that will give an intensity similar to the cellular RNA sample. From the relative lengths of the recombinant and cellular D8 RNAs, we calculate that 1 pg of recombinant RNA is equivalent to 1.7 pg of cellular message (about 10^6 copies). bp, base pairs.
population of normal lung cells the phenotypic profile of which is not apparent when unfractionated lung tissue is examined. This issue is difficult to resolve, particularly because the normal cell type from which SCLC arises is not known with certainty. However, the SCLC cell lines used here are phenotypically heterogeneous (7, 29-33) and display to very different extents the "putative SCLC suppressor. Although the location of the D8 gene in the 3p21 chromosomal region, close to the D3P15S2 locus, and its dramatically reduced expression in SCLC are consistent with its role as an SCLC tumor suppressor gene, in the absence of a functional assay these should for the moment be regarded as circumstantial evidence for such a role.

**ACKNOWLEDGMENTS**

We wish to acknowledge the contribution made in the early stages of this work by the late Alan D. Jonas. We thank P. Twentyman, L. de Leij, B. Wainwright, T. H. Rabbitts, and A. Bernards for cell lines and cDNA libraries and T. G. Draaijers for carrying out the Alu-PCR of DIS 2.6.

**REFERENCES**


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**Table 1** Expression of the D8 gene in SCLC and other cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Name</th>
<th>3.5-kilobase transcript</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diploid skin fibroblast</td>
<td>HFL121</td>
<td>+</td>
</tr>
<tr>
<td>B-lymphoblastoid</td>
<td>MGL8B2</td>
<td>+</td>
</tr>
<tr>
<td>Tumor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervical carcinoma</td>
<td>HeLa</td>
<td>±</td>
</tr>
<tr>
<td>Bladder carcinoma</td>
<td>EJ</td>
<td>+</td>
</tr>
<tr>
<td>Colorectal carcinoma</td>
<td>JW2</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CaCO2</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Colo320</td>
<td>+</td>
</tr>
<tr>
<td>Breast carcinoma</td>
<td>MCF7</td>
<td>+</td>
</tr>
<tr>
<td>Burkitt's lymphoma</td>
<td>Raji</td>
<td>+</td>
</tr>
<tr>
<td>Embryonal carcinoma</td>
<td>GCT27</td>
<td>+</td>
</tr>
<tr>
<td>SCLC</td>
<td>23 independent lines</td>
<td></td>
</tr>
</tbody>
</table>

* +, positive hybridization signal at 3.5 kilobases using D8 cDNA probes; -, no detectable 3.5-kilobase signal on Northern blots.

* Two different strains of HeLa (in London and Groningen) gave quantitatively different results, one having consistently higher mRNA levels than the other.

* The colorectal carcinoma cell line Colo320 contained very low levels of D8 mRNA as assessed by Northern blot hybridization. Methods were as in Fig. 3.

**Table 2** Quantitative analysis of D8 mRNA levels

<table>
<thead>
<tr>
<th>Cell line</th>
<th>pg D8 mRNA/μg total RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control tissues</td>
<td></td>
</tr>
<tr>
<td>Fibroblasts (2)</td>
<td>25-35</td>
</tr>
<tr>
<td>Lymphocytes (4)</td>
<td>1.5-3</td>
</tr>
<tr>
<td>Long</td>
<td>50</td>
</tr>
<tr>
<td>Lymphoblastoid line</td>
<td>250</td>
</tr>
<tr>
<td>SCLC cell lines</td>
<td></td>
</tr>
<tr>
<td>GLC1</td>
<td>0.7</td>
</tr>
<tr>
<td>GLC2</td>
<td>1.4</td>
</tr>
<tr>
<td>GLC4</td>
<td>0.7</td>
</tr>
<tr>
<td>GLC7</td>
<td>0.17</td>
</tr>
<tr>
<td>GLC8</td>
<td>1.4</td>
</tr>
<tr>
<td>GLC20</td>
<td>0.7</td>
</tr>
<tr>
<td>GLC28</td>
<td>0.7</td>
</tr>
<tr>
<td>GLC42</td>
<td>0.35</td>
</tr>
<tr>
<td>GLC44</td>
<td>0.7</td>
</tr>
</tbody>
</table>

* Number of different samples.

* Range of values.


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