Identification of Three Distinct Tumor Suppressor Loci on the Short Arm of Chromosome 9 in Small Cell Lung Cancer

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Abstract

Deletion at 9p21 is frequent in many tumor types. A candidate tumor suppressor gene, p16\textsuperscript{INK4a}, was mapped to this region and is inactivated by several different mechanisms in many tumor types, including non-small cell lung cancer, but not in small cell lung cancer (SCLC). p16 functions as a cyclin/CDK inhibitor to prevent phosphorylation of pRB. It has been demonstrated that most SCLCs have lost pRB but retained p16, and the inactivation of pRB excludes the inactivation of p16 and vice versa. To determine the potential existence of other tumor suppressor genes on the short arm of chromosome 9 in SCLC, we tested 46 primary SCLCs by microsatellite analysis. We found that more than 89% of the tumors exhibited loss of heterozygosity (LOH) at 9p with three distinct minimal deleted areas. Among those areas, LOH at 9p21 was most frequent (86%), with a peak at a marker 150 kb telomeric to p16\textsuperscript{INK4a}. LOH was also observed in more than 50% of the tumors at two other regions, 9p22 and 9p13. Our data strongly suggest the presence of at least three novel tumor suppressor loci at 9p in SCLC, and further investigations to clone candidate tumor suppressor genes are warranted.

Introduction

Lung cancer is the leading cause of cancer death in industrialized countries, and SCLC\textsuperscript{3} accounts for more than 20% of this tumor type. SCLC carries an extremely poor prognosis. The development of SCLC is believed to occur through a multistep process in which the genetic alterations are accumulated, promoting tumorogenesis. Inactivation of tumor suppressor genes plays an important role in this process and involves both alleles of the genes (1, 2). Recently, p16\textsuperscript{INK4a}, a candidate tumor suppressor gene, was mapped to 9p21 (3). Intensive study of the gene and its product has revealed frequent alterations that can inactivate the function of this gene through at least three mechanisms: mutations, homozygous deletion, and methylation of the promotor region (4—6). p16 is an inhibitor of the cyclin/CDK complex and plays an essential role in the progression of cells into S phase by preventing phosphorylation of pRB (7).

Although frequent inactivation of p16 has been observed in NSCLC, only a small fraction of SCLC lost wild-type p16 (5, 6, 9). It has been demonstrated that loss of pRB is frequent in SCLC and that inactivation of pRB or of p16 excluded the other in SCLC and other tumor types (9—12). It is believed that p16 and pRB function in the same pathway; therefore, inactivation of both proteins may be unnecessary. Merlo et al. (13) found that 58% of 33 SCLCs exhibited LOH at 9p21—22, raising the question of whether a tumor suppressor gene other than p16\textsuperscript{INK4a} might exist and play an important role in SCLC in this region. However, one-third of those SCLCs displayed the replication error-positive phenotype, preventing accurate interpretation of LOH in that study (14). Furthermore, the majority of the tumors that exhibited LOH at 9p lost all of the 9p markers that were tested (14), making it difficult to accurately determine the minimal deleted regions. To determine the potential presence of other tumor suppressor genes on the short arm of chromosome 9 in SCLC, we tested 46 primary SCLCs by intensive microsatellite analysis.

Materials and Methods

Primary Tumors and Control Tissues. A total of 91 patients with SCLC were identified based on tissue blocks available from autopsy, biopsy, or surgery at The University of Texas M. D. Anderson Cancer Center from 1978 to 1995. The 46 primary SCLCs (41 from autopsy and 5 from surgical resection of primary tumors) were chosen for this study because of the availability of both primary tumor tissue and corresponding control tissue. The quality of tumors (with fewer contaminating normal cells within the tumor area) was also a factor. The control tissues were kidneys or spleens without evidence of tumor cell infiltration. Paraffin-embedded tissue blocks were sectioned using a microtome. A 4-μm section from each block was stained with H&E and reviewed by pathologists to confirm the diagnosis. The paraffin sections were then stored in microtubes at −80°C. Normal control tissues were disected with a scalpel under a stereomicroscope to ensure the accuracy of the dissection. We used only the tumor blocks in which tumor cell could be dissected with less than 30% normal cell contamination. Normal control tissues were dissected using the same approach.

SCLC Cell Lines. Genomic DNA from 40 SCLC cell lines was selected randomly from our cell line DNA bank. pRB and p16 status of 35 cell lines was available in the literature (9, 11, 12). Thirty-one (89%) cell lines contained an abnormal p16, whereas the other four (11%) lines had a normal p16. Thirty-one cell lines with an abnormal p16 had normal p16, whereas one (H1417) contained homozygous deletion of p16. All four cell lines with a normal p16 did not have p16 protein. p16 exon 1, p16 exon 2, p16β exon 1, and p15 exon 2 were amplified using exon-specific primers as described previously (15). Amplified products were separated on 1% agarose gel and visualized by ethidium bromide staining.

DNA Extraction and Microsatellite Analysis. Dissected tissues were digested in 200 μl of 50 mM Tris-HCl (pH 8.0) containing 1% SDS-proteinase K and incubated at 42°C for 12—24 h. Digested samples were purified, and DNA was precipitated as described previously (16). For microsatellite analysis, 14 microsatellite markers on the short arm of chromosome 9 (including D9S269, D9S274, RPS6, D9S156, D9S157, D9S152, IFN-α, D9S174, D9S171, D9S169, D9S126, D9S161, D9S104, and D9S1853) were obtained from Research Genetics (Huntsville, AL). One of the primers for each marker was end-labeled with [γ-32P]ATP (4500 Ci/mmol; ICN Biomedicals, Costa Rica) and used as the radiolabeled primer. Digested DNA samples (1 μg) were amplified in a 1× PCR buffer (10 mM Tris-HCl, 16 mM (NH4)2SO₄, 0.01% (w/v) gelatin, 1.5 mM MgCl₂, and 0.025 units of Taq polymerase) using primers at 400 μM concentration. Each reaction was incubated for 3 min at 94°C and then subjected to 30 cycles of amplification. Each cycle consisted of 20 s at 94°C, 30 s at 55°C, and 1 min at 72°C. The amplified products were separated on 1% agarose gels, and the gels were stained with ethidium bromide.
Mesa, CA) and T4 DNA polynucleotide kinase (New England Biolabs, Beverly, MA). PCR reactions were carried out in a 12.5-μl volume containing 20 ng of genomic DNA, 1% DMSO, 200 μM dNTP, 1.5 mM MgCl2, 0.4 μM PCR primers, including 0.1 μM γ-32P-labeled primer, and 0.5 units of Taq DNA polymerase (Life Technologies, Inc., Gaithersburg, MD). DNA was amplified for 35 cycles at 95°C for 30 s, 52–60°C for 60 s, and 70°C for 60 s in a temperature cycler (Hybaid; Omnigene, Woodbridge, NJ) in 500-μl plastic tubes, followed by a 5-mm extension at 70°C. The PCR products were separated on a 6% polyacrylamide-urea-formamide gel, which was then autoradiographed. LOH was defined as a >50% reduction of the intensity by visual inspection in either of the two alleles as compared with those in normal control panels. Shifted bands were determined by the appearance of clear novel alleles in SCLC and to pinpoint minimal deleted regions, we selected 46 primary SCLCs from autopsy tissue samples, making this one of the largest panels of primary small cell lung tumors yet studied. Using microdissection, a tumor cell population of more than 80% purity was reached in most tumor samples to ensure accurate analysis. We used 14 highly polymorphic microsatellite markers between 9p13 and 9p23. The order of these markers is shown in Fig. 1. We found that 41 (89%) of 46 tumors exhibited LOH in at least one of these microsatellite markers, representing at least three distinct regions (Table 1). Eight (20%) of the 41 lost a larger area, exhibiting LOH in almost all informative distal markers tested. The other 33 tumors exhibited LOH in a small region or several separated regions.

Thirty-six (86%) of 42 informative tumors showed LOH at markers between IFN-α and D9S171. Six of these tumors lost only this small region and retained heterozygosity at all other informative markers. These observations indicate that a tumor suppressor gene or genes in this region must play an important role in SCLC. There are at least four candidate tumor suppressor genes, p16INK4a, p15INK4b, p16β, and MTAP, in this region (13, 15, 17, 18). A few SCLC cell lines showed inactivation of p16INK4a by methylation of the gene (5), but there is little evidence to support the theory that inactivation of p16INK4a plays a major role in SCLC. Furthermore, no alterations were found in p15INK4b and p16β in the previous studies (15, 19). Because of the sample availability and quality, we were unable to determine pRB status in these primary tumors. However, by examination of 40 SCLC cell lines, we found that 2 (5%) of the cell lines contained homozygous deletions, including p16INK4a, p15INK4b, and pRB, and that pRB and p16 status were available in 35 of 40 cell lines. Thirty-one of 35 cell

**Results and Discussion**

To determine the presence of novel tumor suppressor genes on 9p in SCLC and to pinpoint minimal deleted regions, we selected 46 microsatellite markers exhibiting retention patterns, tumors with retentions at markers D9S162 or D9S171, flanked by markers with LOH at both sides, were selected. A microsatellite marker (D18S46) in 18q21 was used as a reference marker. For the determination of potential homozygous deletions, one test marker and the reference marker were added in the same PCR reaction tube, and DNA was amplified for 30 cycles. PCR products were separated on a 6% polyacrylamide-urea-formamide gel and visualized by exposure on film.

**Multiplex PCR.** For the determination of potential homozygous deletions at microsatellite markers exhibiting retention patterns, tumors with retentions at markers D9S162 or D9S171, flanked by markers with LOH at both sides, were selected. A microsatellite marker (D18S46) in 18q21 was used as a reference marker. For the determination of potential homozygous deletion of p16INK4a, tumors with LOH at only the marker D9S174 were selected. The marker D9S1748 (located between the p16 and p15 genes) was used to represent the region, and, the marker D14S51 (located at 14q32), was used as a reference. Primers (one from each marker) were labeled as described above. One test marker and the reference marker were added in the same PCR reaction tube, and DNA was amplified for 30 cycles. PCR products were separated on a 6% polyacrylamide-urea-formamide gel and visualized by exposure on film.
Table 1. LOH on 9p in small cell lung cancer

<table>
<thead>
<tr>
<th>Regions</th>
<th>Tumors, LOH/informative</th>
<th>% of LOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-α ↔ D9S171</td>
<td>36/42</td>
<td>86</td>
</tr>
<tr>
<td>D9S156 ↔ D9S157</td>
<td>24/41</td>
<td>59</td>
</tr>
<tr>
<td>D9S104 ↔ D9S1853</td>
<td>19/58</td>
<td>50</td>
</tr>
<tr>
<td>Total</td>
<td>41/46</td>
<td>89</td>
</tr>
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Fig. 2. Examples of deletions observed in primary SCLCs. A, order of microsatellite markers used in this study. Dashed line, approximate location of RPS6. B, LOH at markers D9S156 (9p22), D9S171 (9p21), D9S169, and D9S1853 (9p13) with retention at markers between them in tumor SCLC20. C, LOH at markers D9S156, D9S171, and D9S161 and retentions at other markers in tumor SCLC29.

Furthermore, the presence of another tumor suppressor gene (rather than p16$^{INK4a}$ and p15$^{INK4b}$ at 9p21 region) had been suspected by others (22). Because this region has been studied in detail, available data and materials should allow more specific identification of a smaller deletion region and may allow candidate genes to be cloned in the near future.

Two other distinct regions also exhibited frequent LOH (Table 1). Twenty-four (52%) of the 46 tumors showed LOH in two or three distinct regions on 9p (Fig. 1), indicating the presence of more than one tumor suppressor locus in this chromosome arm. Eleven (46%) of the 24 tumors lost three separate regions (Fig. 1). The examples of LOH at different regions are shown in Fig. 2. A possible reason for the observed retentions between markers with LOH may be small homozygous deletion and normal cell contamination (6). However, it is unlikely that this is the case in the present study for the following reasons. Most of the tumors we used contained a minimal amount of contaminating normal cells. We used the multiplex PCR strategy to test 10 tumors selected from those with retention at either marker D9S162 or marker D9S171, flanked by markers with LOH at both sides, and found no evidence of significant changes in the ratio of density between normal tissues and tumors (Fig. 3). Six tumors with LOH at D9S1747 only were also selected for the examination of potential homozygous deletion of p16$^{INK4a}$ region, and no homozygous deletion was observed (Fig. 3).

Our data also show that one of the minimal deleted regions was located at 9p22, between D9S156 and D9S157 (Fig. 1). Twenty-four (59%) of 41 informative tumors exhibited LOH at one or both of the markers. The estimated distance between the two markers is about 1 cm (21). Another minimal deleted region was narrowed to 9p13 between D9S104 and D9S1853 (Fig. 1). Nineteen (50%) of 38 informative tumors exhibited LOH at one or both of the markers. The estimated distance between the two markers is about 3 cm (Research Genetics). The talin gene was recently mapped to 9p, between D9S156 and D9S104 (23). Talin is critical in the formation of focal adhesions, and inactivation of talin may induce fibroblasts to round up and disassemble many of the adhesions (24). Talin, therefore, is a possible candidate gene that may be responsible for the morphological phenotype and metastatic potential of SCLC. Although frequent microsatellite instability was observed in SCLC in a previous report (14), we observed only six shifted bands in six (15%) individual tumors in this study. Instability occurred in only 1% (7 of 644) of the loci tested and did not resemble the replication error-positive phenotype. Taken together, our data strongly indicate that at least three tumor suppressor
Further intensive studies to clone candidate tumor suppressor genes roles in the tumor development and progression processes in SCLC. Further intensive studies to clone candidate tumor suppressor genes are warranted.

Acknowledgments

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References

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