Advances in Brief

Homozygous Deletions at Chromosome 9p21 and Mutation Analysis of *p16* and *p15* in Microdissected Primary Non-Small Cell Lung Cancers

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Abstract

Loss of heterozygosity on chromosome 9p has been detected in many primary human tumors and cell lines, suggesting that this chromosomal arm harbors one or more tumor suppressor genes. The recently cloned *p16* and *p15* genes, mapped to 9p21, are likely candidates for such tumor suppressors. To map the deletion at chromosome 9p21 in non-small cell lung tumors, we analyzed DNA from 25 tumors and matching normal DNAs at six microsatellite markers that flank the region occupied by the *p16* and *p15* genes. Loss of heterozygosity of at least one microsatellite marker on chromosome 9p21 was detected in 13 (52%) of 25 tumors, including one tumor that exhibited homozygous deletion of both human IFNα and D9S171. Six tumors analyzed by a comparative multiplex PCR technique showed homozygous deletions of the sequence tag site marker c5.1 (within *p16*). Screening for mutations in *p16* and *p15* revealed one tumor with a non-sense mutation in exon 2 of *p16*, but no mutations were detected in *p15* in any of the tumors. Thus, in these analyses approximately one-half of the non-small cell lung tumors had loss of heterozygosity at chromosome 9p21, and of these tumors, one-half had homozygous deletions of the region that includes *p16*. This appears to confirm the importance of a locus in this region critical to growth control in lung. The apparent lack of other mutations in *p16* and *p15* in the tumors with loss of heterozygosity leaves open the possibility of an unidentified gene in this region that may function as a tumor suppressor.

Introduction

Lung cancer is the leading cause of cancer mortality in the United States today, with over 149,000 deaths per year (1). The steps leading to the development of lung cancer are not well defined, but LOH[2] on several Chrs including 3p, 3q, 5q, 9p, 9q, and 17p are common in non-small cell lung tumors (2–4). These findings suggest the presence of tumor suppressor genes in these regions, the loss of which may define critical stages in tumor development.

Chr 9p21 is often deleted in several tumor types including lung (5, 6). This region contains two known genes, *MTS1/p16-INK4* (*p16*; Refs. 7 and 8) and *MTS2/p15-INK4B* (*p15*; Refs. 9 and 10), which are likely candidates for tumor suppressor genes since they encode proteins that inhibit regulation of cyclin-dependent kinases. Loss of this function could cause the cell cycle to become unregulated, thereby leading to uncontrolled cell growth. Tumor cell lines show frequent homozygous deletions of *p16*, although primary tumors appear to have a lower frequency of homozygous deletions or mutations of *p16* (11–13). The role of *p16* in primary tumor formation remains unclear, and deletions in this region could be due to unidentified tumor suppressor genes in this region.

To better define the locus in Chr 9p21 lost in lung cancer, we screened 25 microdissected primary non-small cell lung tumors for alterations in this region. Six microsatellite markers surrounding the *p16/p15* region were used to investigate LOH, and sequence tag site markers within the *p16/p15* region were used to do finer mapping of loss within the critical region that lacks useful polymorphic markers. In addition, exons one and two of both *p16* and *p15* genes were screened for mutations.

Materials and Methods

Primary frozen NSCLC tumors and corresponding normal lung tissues were obtained from Saint Mary's Hospital (Grand Junction, Colorado). The lung tumors included 9 adenocarcinomas, 12 squamous cell carcinomas, 1 large cell carcinoma, 2 bronchioalveolar carcinomas, and 1 adenosquamous carcinoma.

For extraction of DNA, about 0.1 g tissue was minced, added to 0.5 ml digesting solution (1X TNE, 10 mg/ml Pronase, and 10% SDS), and incubated overnight at 37°C. Following digestion, DNA was isolated by phenol chloroform extraction and ethanol precipitation (14).

For each patient, genomic DNA from primary tumors was compared to matching normal DNA for LOH at multiple loci on Chr 9p. Amplification primers for microsatellite markers D9S171, D9S126, D9S169, D9S162, D9S156, and HIFNα (15) were obtained from Research Genetics (Huntsville, AL). Prior to PCR (16), one primer from each set was end labeled with [γ-^{33}P]ATP using T4 polynucleotide kinase. After 25–27 cycles of PCR, the amplified products were separated on a 6% polyacrylamide gel that contained 8 M urea and formamide (17). Gels were dried and exposed to X-ray film overnight.

After analysis for LOH, which showed allele imbalances but few clear losses, it was apparent that many of the lung tumors contained some normal cell contamination. Histological examination demonstrated areas of normal fibrous tissue and

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[2] The abbreviations used are: LOH, loss of heterozygosity; NSCLC, non-small cell lung cancer; HIFN, human IFN; Chr, chromosome.

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infiltrates of mononuclear cells in many of the tumor samples. To reduce contamination with normal cells, tumors containing greater than 20% normal tissue by histological examination were microdissected. Frozen tissues were embedded in OCT media, and one 6–10-μm tissue section was cut in a cyrostat and stained immediately with hematoxylin and eosin. This section was examined microscopically to assess the proportion of normal tissue contamination and find areas which were mostly tumor (>80%). Then 20-μm sections were cut and stained with hematoxylin and eosin without fixation. These sections were examined under a dissecting microscope, and tumor areas were scraped away from normal cells with a razor blade. Tumor tissue from three serial sections was scraped into a 1.5-μl microfuge tube containing digestion solution (see above). During the microdissection procedure, boxes of slides and undissected tissue blocks were stored on dry ice.

Comparative multiplex PCR was used to assess homozygous deletions in the region of the *p16* and *p15* genes (18). Primer pairs from the *HIFNγ* gene, which resides on Chr 12, were included in the same PCR reaction mix along with the sequence tag site marker primer c5.1 (7). Multiplex PCR with HIFNγ was also performed with microsatellite markers HIFNα and D9S171 to define the region of homozygous deletion. One primer from each pair was end labeled with [^{33}P]ATP as described above. PCR products were loaded onto denaturing gels containing formamide as described previously (17). Amplified tumor and normal DNA from each patient were loaded into adjacent lanes, and signals were quantified by densitometry on a phosphorimaging screen (Molecular Dynamics, Sunnyvale, CA). Additionally, ratios of c5.1:HIFNγ were determined for 5, 10, 20, 50, and 100% normal tissue added to DNA from a cell line deleted at this marker (19). From the standard curve generated for the c5.1 marker, it was determined that deletions could be detected if there was less than about 30% normal tissue contamination. A homozygous deletion was scored if the c5.1 band representing the normal DNA was 3-fold greater than that from the tumor DNA. Correction for amplification was made using HIFNγ primers as an internal PCR standard. This comparative PCR analysis was repeated for those samples that appeared to have losses of c5.1. Other sequence tag site markers in this region, R2.7 and RN1.1 (7), were also used in multiplex PCR with HIFNγ to define the region of homozygous deletion. However, the standard curves for these markers indicated that homozygous deletions could not be determined if more than 10–20% normal tissue was present. Most of the tumors analyzed had approximately this amount of normal tissue and therefore could not be scored using these markers.

Amplified DNA products of exon 2 of *p16* were directly sequenced as described previously (20). DNAs from the microdissected tumors were screened for mutations in exon 1 of the *p16* gene and exons 1 and 2 of the *p15* gene by single-strand conformation analysis as previously described (21). Primers for amplification of exons 1 and 2 of the *p16* gene and exon 2 of the *p15* gene were from Kamb *et al.* (7), and sequences for primers for exon 1 of the *p15* gene were as follows: 5′-TCC GCA CCC TGC GGC CAG A-3′ and 5′-GAT CGC GCG CCT CCC GAA-3′. The PCR products were labeled by incorporation of [α-^{33}P]dATP and then incubated with a single cutter restriction enzyme before denaturation and loading onto two 0.5 X MDE gels (±10% glycerol) in 0.6X Tris-borate EDTA buffer. The restriction enzymes (New England Biolabs, Beverly, MA) used were *Ava*I (exon 1, *p16*), *Hga*I (exon 1, *p15*), and *Hph*I (exon 2, *p15*). The gels were run at 6–8 W for 16 to 18 h at room temperature before drying and exposure to X-ray film.

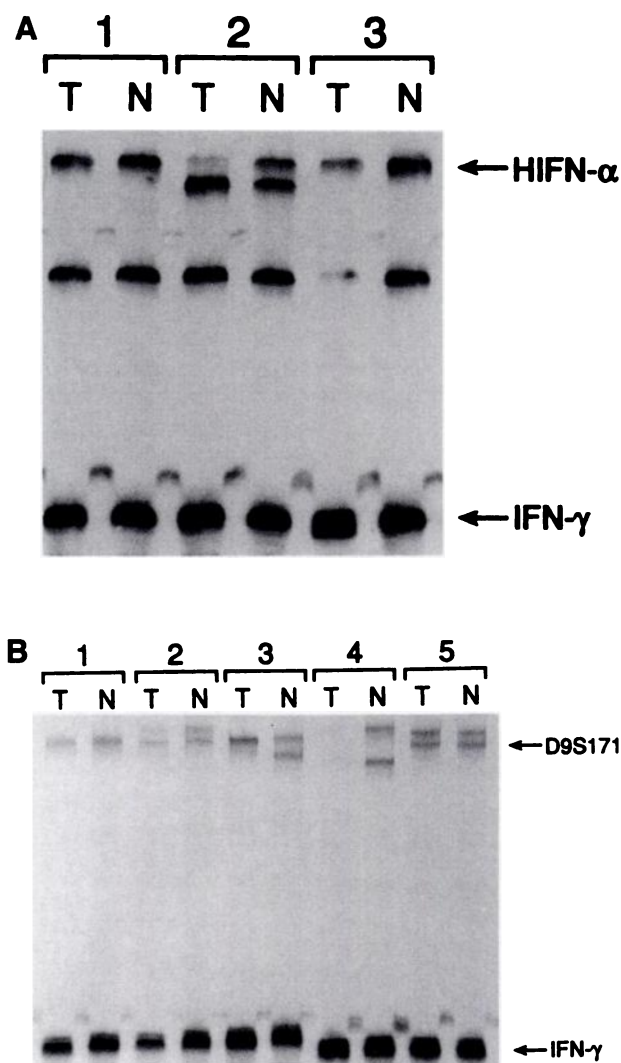

Fig. 1 LOH of microsatellite markers on Chr 9p in NSCLC. DNA derived from normal tissue (*N*) and from tumor tissue (*T*). *A*, LOH of HIFNα with HIFNγ (Chr 12) as control for amplification. Tumor 1, noninformative; tumor 2, LOH; tumor 3, homozygous deletion. Tumor *lanes 1–3* correspond to *lanes 5, 7,* and *1*, respectively, in Fig. 2. *B*, LOH for D9S171 and HIFNγ markers. Tumor 1, noninformative; tumor 2, LOH; tumor 3, LOH; tumor 4, homozygous deletion; tumor 5, both alleles retained. Tumor *lanes 1–5* correspond to *lanes 3, 5, 7, 1,* and *6* in Fig. 2.

Results and Discussion

Recently, the putative tumor suppressor genes *p16* and *p15* were identified and mapped to Chr 9p21 (7–10). To define the smallest deleted region at 9p21 and assess the status of these genes in primary lung tumors, we first examined 25 NSCLC

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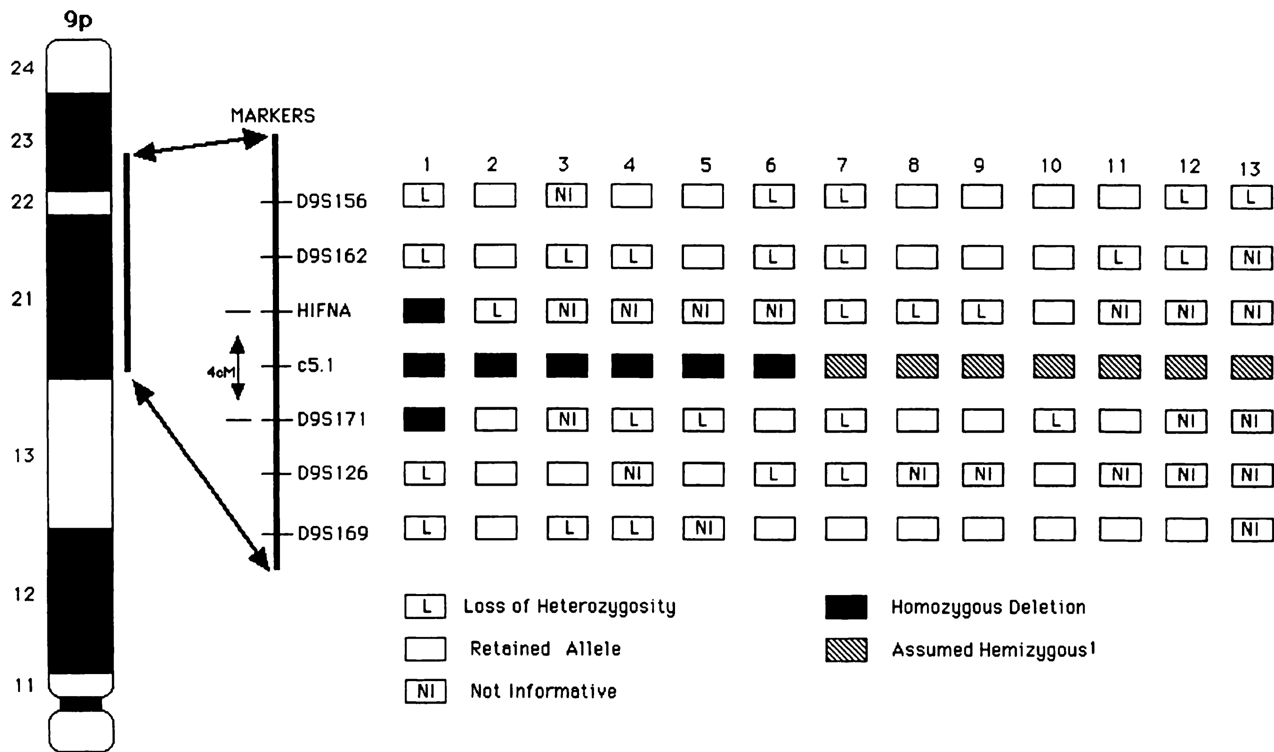

Fig. 2 Summary of LOH analysis showing patterns of deletions on Chr 9p in the 13 cases of primary NSCLC with losses. LOH analysis at D9S156, D9S162, D9S126, and D9S169 microsatellite markers was performed with DNA from tumors that were not microdissected. Apparent inconsistencies in retention of both alleles of D9S126 and D9S171 in tumors 3 and 6, respectively, may be due to undetectable homozygous deletions.

normal/tumor pairs for LOH at six microsatellite markers within and surrounding this region. About one-half of these tumors appeared to have allelic imbalances, showing deviations from the expected 1:1 ratio at some of the markers. However, clear LOH could not always be ascribed because of contamination of neoplastic tissue with normal cells. Therefore, the tumors with more than about 20% normal tissue were microdissected. The LOH analysis was repeated on the 25 microdissected tumors and their normal DNA pairs at the two markers HIFNα and D9S171, this time multiplexed with HIFNγ (Chr 12) to detect homozygous deletions as well as allelic loss. LOH or homozygous deletion was observed in 5 (33%) of 15 informative cases at the *HIFNα* locus and 5 (25%) of 20 informative cases at the *D9S171* locus (Fig. 1). Moreover, 13 (52%) of the 25 tumors showed LOH for at least one microsatellite marker at chromsome 9p21 (Fig. 2), suggesting that the functional loss of a lung tumor suppressor gene in this region occurred in more than one-half of the tumors examined. These data are similar to those reported by Merlo *et al.* (5), who found LOH of Chr 9 in 55% of primary NSCLCs, and Olopade *et al.* (6), who detected LOH on Chr 9p in a similar proportion of NSCLC cell lines.

Since the putative tumor suppressor genes *p15* and *p16* are located within the minimal region of loss between HIFNα and D9S171, we analyzed the 25 lung tumors for mutations in these two genes. No band shifts indicative of mutations were detected by single-strand conformation analysis in exons 1 or 2 of *p15* or in exon 1 of *p16* (data not shown). By sequence analysis, one of the 25 tumors was found to have a mutation in exon 2 of *p16*, a G to T transversion at the first base of codon 112, converting glutamine to a stop codon. This sample also exhibited LOH at surrounding microsatellite markers (Fig. 2, *Lane 7*), indicating that the normal allele was lost as well. Other recent reports also have demonstrated a paucity of mutations in *p16* in various types of human primary tumors including lung (11, 22).

We also examined the NSCLC cases for homozygous deletions of *p16* by using the sequence tag site marker c5.1, located within *p16* (7) in comparative multiplex PCR reactions with HIFNγ as a control for amplification. Six (24%) of the 25 lung tumors appeared to have homozygous deletions at this marker (Figs. 2 and 3). Seven other tumors, including the one with the *p16* mutation, exhibited LOH at one or more adjacent microsatellite markers, suggesting that they also have lost at least one allele of the putative tumor suppressor gene. No correlations with a single tumor histology type (adenocarcinoma *versus* squamous cell carcinoma) were found in the number of Chr 9p21 deletions detected.

Our results with these NSCLC cases are consistent with those of others showing common homozygous deletions of *p16* in lung tumor cell lines but rare intragenic *p16* mutations in either cell lines or primary lung tumors (11, 22). Although mutation of one allele and deletion of the normal allele appear to be a common mechanism for inactivation of some tumor suppressor genes such as *p53*, this mechanism may not account for loss of all tumor suppressor genes. In the present study, *p16*

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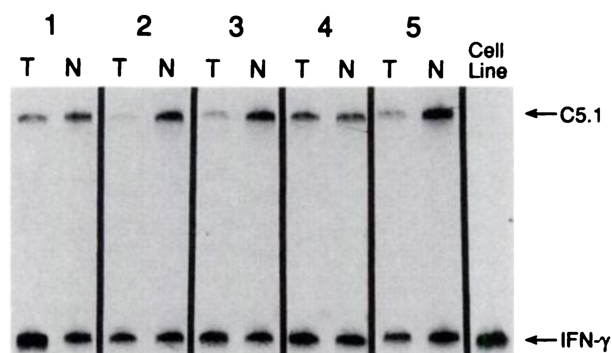

Fig. 3 Homozygous deletions of sequence tag site marker c5.1 on Chr 9p21 in primary NSCLC using a comparative multiplex PCR with HIFNγ as a control for amplification. DNA derived from normal lung tissue (*N*) or tumor tissue (*T*). By phosphorimager analysis (see "Materials and Methods"), tumor DNAs 1–3 and 5 have homozygous deletions. Tumor *lanes 1–5* correspond to *lanes 4, 3, 5, 7,* and *6*, respectively, in Fig. 2. Hemizygous deletions could not be detected at this marker since there was little difference in amplification between 50% normal and 100% normal tissue (see "Materials and Methods").

appears to be a likely candidate tumor suppressor gene for lung cancer because of its deletion in over one-half of the cases which exhibited LOH at adjacent markers on Chr 9p21. *p16* appears to be important in regulating cyclin-dependent kinase 4, and its loss may trigger uncontrolled growth (8). As more laboratories have reported failure to detect intragenic mutations in *p16* in tumors (11, 12), there has been speculation that the newly identified and related *p15* gene might be one of the putative tumor suppressor genes (9, 10). However, we did not detect mutations in this gene in the 25 NSCLC tumors examined in this study.

On the other hand, we cannot rule out the existence of another locus in this region that is involved in lung cancer. The Chr 9p21 region defined by HIFNα and D9S171 is a 4-cM segment that includes *p15* and *p16* and may include other genes. The lack of mutations in *p16* and *p15* in the tumors with LOH argues against the involvement of these genes in lung cancer. Although one-quarter of the tumors in this study showed homozygous loss of the c5.1 marker within *p16*, this loss could also be reflecting deletion of an adjacent locus. Since very few mutations have been identified in *p15* or *p16* in various human primary tumor types (11, 12), it is possible that there is an unidentified gene in this region with a major role in growth control.

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