Identification of a Novel Region of Homozygous Deletion on Chromosome 9p in Squamous Cell Carcinoma of the Lung: The Location of a Putative Tumor Suppressor Gene


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Abstract

Cytogenetic and molecular studies have implied the presence of tumor suppressor genes (TSGs) on chromosome 9p that are critical in the development of lung and other cancers. The p16/CDKN2 gene, a cyclin dependent kinase inhibitor, is a well-defined TSG on chromosome 9p. Although the frequency of mutations in the p16/CDKN2 gene has been detected in approximately 30% of non-small cell lung cancer, loss of heterozygosity on 9p has been observed in greater than 70% of non-small cell lung cancers. These and other deletion mapping studies have suggested the existence of additional TSGs on 9p. This study examined chromosome 9p for TSG loci by analyzing 23 squamous cell carcinomas of the lung with 21 microsatellite markers. Loss of heterozygosity was detected in all of the tumors, and homozygous deletions of the p16/CDKN2 gene were observed in approximately 30% of tumors in this series. In addition to p16/CDKN2 and located between D9S265 and D9S259.

Introduction

Lung cancer is one of the leading causes of cancer death in the world. In 1993, more than 140,000 individuals died of lung cancer in the United States, and the incidence of this disease is rising. The high mortality rate for lung cancer probably results, at least in part, from the absence of standard clinical procedures for diagnosis of the disease at early and more treatable stages compared to breast, prostate, and colon cancers (1, 2). The delineation of genetic alterations that occur in lung tumorigenesis may aid in both the development of molecular markers for early detection and in the prediction of response to chemotherapy.

Cytogenetic and molecular genetic studies have shown that mutations in proto-oncogenes and TSGs are critical in the multistep development and progression of lung tumors. Although mutations in the K-ras proto-oncogene occur in approximately 30% of pulmonary adenocarcinomas (3) and frequent overexpression and/or amplification of cyclin D1 (4) and the myc family of proto-oncogenes (5) have been observed in some lung tumors, inactivation of TSGs are by far the most common mutational events documented during the development of lung cancer. For example, loss of function of the Rb and/or p53 genes have been detected in over 50% of SCLCs and NSCLCs. In addition, allelic loss analyses have implicated the existence of other TSG loci on 9p, as well as 3p, 5q, 8q, 9q, 11p, 11q, and 17q.

A high frequency of genetic alterations has been detected on 9p in numerous tumor types. Specifically, Merlo et al. (6, 7) used polymorphic microsatellite markers to detect LOH on 9p21 in 76% of NSCLCs (6) and 52% of SCLCs (7). Deletions of the same region of 9p have also been observed in other tumor types, including head and neck squamous cell carcinomas (8), bladder tumors (9), melanomas (10), mesotheliomas (11), and gliomas (12). The p16/CDKN2 TSG is located on 9p21 (13), and a high percentage of alterations in this gene have been observed in some tumor types with LOH on 9p, including pancreatic adenocarcinomas (14) and glioblastomas (15). In contrast, the frequency of p16/CDKN2 mutations in human lung tumors is lower than the frequency of LOH on 9p (16–18). Moreover, deletion-mapping studies of various tumor types suggest that more than one TSG may exist on 9p (11, 19–26). Thus, in addition to p16/CDKN2, another TSG(s) may reside on 9p that contributes to the development of lung and other tumor types (17, 27).

We report here the results of LOH, multiplex PCR, and FISH analysis on 9p for 23 surgically resected squamous cell carcinomas of the lung. Our results suggest the presence of a novel TSG locus proximal to p16/CDKN2, localized to a chromosomal region between D9S265 and D9S259.

Materials and Methods

Microdissection of Lung Tumor Specimens. Surgically resected squamous cell carcinomas of the lung and distal normal lung tissue were collected at St. Mary's Hospital, Grand Junction, CO. Two 5-μm sections were stained with H&E and used as guides in the microdissection. Ten to twelve 10-μm

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sections of paraffin-embedded tumor tissues were microdissected to enrich for neoplastic cells using a previously described method (28).

DNA Isolation. DNA from the resected normal lung tissue was phenol extracted and ethanol precipitated, rehydrated in Tris EDTA, quantitated spectrophotometrically, and diluted to approximately 0.2 µg/ml. The microdissected tumor tissue DNA was isolated by incubation at 55°C for 2 h in a digestion buffer that consisted of 1 X PCR buffer, 0.45% NP40, 0.45% Tween 20, and 0.5 µg/ml proteinase K. The protease was then inactivated by incubating at 95°C for 10 min. The microdissected DNA was quantitated by comparing the band intensities of the PCR products from approximately 200 pg of normal DNA and various dilutions of the tumor DNA. The tumor DNA was subsequently diluted to approximately 0.2 µg/ml.

LOH Assays. The 21 microsatellite markers used in this study are indicated in Fig. 1. Primer pairs were obtained commercially (Research Genetics, Huntsville, AL) or synthesized (Applied Biosystems, Emeryville, CA). All sequences are available through the Genome Data Base. PCR amplifications were performed in 10-µl reaction volumes, including approximately 200–300 pg of genomic DNA, 1.0 µM of an unlabeled primer, 1.0 µM of a biotinylated primer, and 0.25 units of Taq polymerase (Perkin-Elmer Cetus, Inc.). A "touchdown" PCR program was used (29), and the cycles were as follows: 94°C for 15 s, 30 s at the annealing temperature, and 72°C for 30 s for a total of 30 cycles and a final extension at 72°C for 10 min. Following amplification, the PCR products were resolved on 8% denaturing polyacrylamide gels containing 8 M urea, transferred to Immobilon filters (Millipore Corp., Bedford, MA), and visualized by chemiluminescence (New England Biolabs). For informative cases, allelic loss was scored if the intensity of the signal from one allele was significantly reduced in the tumor DNA when compared to the normal DNA.

Comparative Multiplex PCR. Homozygous deletions were confirmed by comparative multiplex PCR involving the amplification of two different sets of primer pairs in the same reaction mixture (30). A control primer set was selected for a locus outside the area of suspected homozygous deletion and a test primer set for the locus of suspected homozygous deletion. For example, the D9S199 primer pair was used as a control to test for homozygous deletion at the D9S126 locus. Two primer sets were used in the same reaction to ensure that it is possible to amplify the DNA and to normalize the amount of product from the tumor DNA relative to that of the normal DNA for both the test and control primers. The PCR reaction was as described above except for the following modifications: (a) Taq polymerase concentration was increased from 0.25 to 0.50 units; (b) total number of cycles were decreased from 30 to 28; and (c) extension times were increased from 30 to 90 s. The concentrations of template DNAs were sometimes adjusted to obtain similar (and comparable) band intensities between the control products visualized in the tumor and normal lanes. Homozygous deletions were scored when the test primer signal in the tumor lane was less than 10% of the signal in the normal lane.

FISH Analysis. FISH was also used to confirm homozygous deletions. FISH was performed on touch preparations or on nuclei isolated from frozen tumor blocks. To prepare touch preparations, frozen tissue blocks at the melting point were touched to a silane-coated glass slide, fixed in Carnoy's solution overnight, air dried, and stored at ~70°C until hybridization. To isolate nuclei, a modification of the Hedley technique was used (31). The morphology of these preparations was initially assessed by Wright staining to ensure that a sufficient number of well-preserved tumor cells or tumor cell nuclei were present to permit reliable quantification of FISH signals. Touch preparations and isolated nuclei from normal frozen lung tissue were used as controls.

Cosmid clones c25 and c72 (32) and two P1 plasmids, 8559 and 8660 (Genome Systems Inc., St. Louis), all containing the D9S126 sequence, were used as probes in FISH analyses. The DNAs were biotin labeled using a sequence-independent amplification method described previously (33). The labeled probes were coprecipitated with salmon sperm and human Cot-1 DNA and dissolved in hybridization buffer (50% formamide, 10% dextran sulfate, 1% v/v Tween 20, 2X SSC, pH 7.0) at a final concentration of 20 ng of DNA/µl. The DNA mixture was then denatured at 75°C for 5 min and incubated at 37°C for 30 min to allow for reannealing of repetitive sequences. A pericentromeric digoxigenin-labeled classical-satellite DNA (D9Z1, Oncor, Gaithersburg, MD) was also used to control for chromosome 9 copy number. Five µl of the control probe were dissolved in 26 µl of Hybrisol V1 (Oncor), denatured at 75°C for 5 min, and quenched on ice. Pericentromeric and cosmid or P1 probes were mixed together in proportions of 3 (5 ng of control DNA):10 µl (200 ng of P1 or cosmid DNA), hybridized to touch preparation and nuclear cytopsin slides, and washed to a high stringency as described previously (34). Dual color detection (biotin-FITC, digoxigenin/rhodamine) was performed at 37°C using a mixture of FITC-avidin and rhodamine-sheep antidigoxigenin antibodies. Slides were then counterstained with a 0.1% 4',6-diamidino-2-phenyldihyde solution.

FISH signals were scored in a blind fashion for at least 400 single, intact nuclei from each specimen. Copy numbers of probes (green) and control (red) signals were determined for each cell and nuclei were scored only when at least one bright control signal was present. For documentation, separate gray scale

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Fig. 1. Patterns of allelic loss on the short arm of chromosome 9 for twenty-three squamous cell carcinomas of the lung. The relative order of the microsatellite markers is listed on the left. LOH, retention of heterozygosity; noninformative; homozygous deletion at informative locus; homozygous deletion at noninformative locus; ND, not done; tel, telomeric; cen, centromeric. The tumor stage for each case is shown at the top.
Results

Normal and tumor DNAs from 23 NSCLC patients were screened for LOH on 9p with 21 microsatellite markers. The summary of the LOH data is presented in Fig. 1, and examples of the LOH analysis are illustrated in Fig. 2 for tumors 30 and 58. Twenty-two of the 23 tumors had allelic loss at more than one informative locus, and the remaining tumor (tumor 24) exhibited LOH only at D9S126. Ten of the tumors had allelic loss at all informative markers and 4 other tumors (tumors 37, 39, 58, and 59) had allelic loss at all but one informative marker. On the remaining nine tumors, extensive allelic loss was observed except for tumors 24, 60, and 61. Tumors 24 and 60 revealed a relatively small region of loss between markers D9S285 and D9S162 (approximately 4 cM) and tumor 61 harbored a hemizygous loss that included the region containing D9S126. Tumor 60 also harbored a hemizygous deletion of the p16 locus. Fig. 1 also presents the tumor staging of the 23 squamous cell tumors. Because the total number of samples was small, it was not possible to correlate staging with hemi- or homozygous deletions.

The 23 squamous cell carcinomas were examined for homozygous deletions at the p16/CDKN2 locus using the criteria and markers described by Cairns et al. (16). The detection of homozygous deletion is based on the apparent retention of one or more closely spaced microsatellite markers flanked by regions of clear LOH. This apparent retention of heterozygosity for markers within a region of LOH could result from amplification of a small amount of normal DNA contamination because the homozygously deleted region within the tumor DNA does not provide a template for amplification. This criterion has been confirmed by Reed et al. (35) using FISH and immunohistochemical staining. The p16 gene is localized between markers 1069 and D9S1748, and homozygous deletion was detected in six tumors (tumors 23, 26, 43, 44, 45, and 64).

There were four tumors (tumors 30, 52, 58, and 64) of particular interest that showed apparent homozygous deletions at D9S126 (tumors 52 and 58) and/or D9S259 (tumors 30 and 64) with LOH of the surrounding informative markers (see Fig. 2 for tumors 30 and 58). The possibility of homozygous deletions for these four tumors was suggested because the amplified tumor DNA produced very weak or absent signals for both alleles at D9S126 and/or D9S259 (Fig. 2). Also, as mentioned above, regions of homozygous deletion have been identified where apparent retention of heterozygosity for one or more closely spaced markers is flanked by regions of LOH (16, 30, 35).

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The minimum region of homozygous deletion was determined using markers that are immediately distal (D9S265) and proximal (D9S259) to D9S126 by comparative multiplex PCR. None of the six tumors with a homozygous loss at D9S126 exhibited homozygous loss at D9S265 (Figs. 1 and 3B). Specifically, the intensity of the bands in the corresponding bands in the normal lanes was similar to the relative intensities for the control marker, D9S199, was similar in intensity in both the normal and tumor lanes. In addition, this analysis was used to examine the remaining tumors and revealed homozygous deletions of D9S126 in three additional tumors (tumors 22, 24, and 64; Fig. 3A). All of the comparative multiplex PCRs for the tumors with homozygous deletions were reproduced at least three times. Thus, comparative multiplex PCR confirmed that 6 of the 23 tumors were homozygously deleted at the D9S126 locus. It should also be noted that only tumor 64 contained a homozygous deletion at both p16 and D9S126.

The results presented in Fig. 1 are consistent with the data presented in Fig. 3. The remaining 17 tumors also tested negative for homozygous deletion at D9S265 by comparative multiplex PCR (data not shown). Similarly, the region of homozygous deletion was bounded proximally by D9S259. As shown in Fig. 3C, tumors 24 and 58 did not contain a homozygous deletion at D9S259, whereas tumor 30 harbored a homozygous deletion at this marker. Tumor 64 also showed a homozygous deletion at D9S259; however, homozygous deletions were not detected at D9S259 in any
Fig. 3. Comparative multiplex PCRs of selected squamous cell carcinomas of the lung. A, analysis for homozygous deletions at locus D9S126; D9S199 was used as the control primer set. B, analysis for homozygous deletions at locus D9S265 for cases 24, 30, and 58; D9S285 was used as the control primer set. C, analysis for homozygous deletions at locus D9S259 for cases 24, 30, and 58; D9S285 was used as the control primer set.

of the other tumors (data not shown). Thus, the minimum region of homozygous deletion is between D9S265 and D9S259, a distance of approximately 1 Mb.4

FISH analysis was also used to confirm the homozygous deletions detected at D9S126. FISH has the distinct advantage of being able to identify chromosomal deletions in individual tumor cells and can, therefore, distinguish between LOH and homozygous deletions with greater confidence than PCR based methods. Touch preparation or nuclear cytopsin slides constructed from frozen tumor tissue were used in these analyses. Slides that contained a sufficient number of tumor cells or tumor cell nuclei were generated for analysis of 16 cases, including five cases (tumors 22, 24, 30, 58, and 64) thought to contain a homozygous deletion of D9S126 by molecular analysis and three tumors (tumors 27, 45, and 50) without homozygous deletion. Cases could be divided into those with hemizygous loss, homozygous loss, or a mixture of hemizygous and homozygous loss. For each case examined, 40–50% of the nuclei counted contained two red signals (centromeric probe) and two green signals (D9S126 probe). These cells are presumed to be contaminating normal cells because our morphological analysis of Wright stained slides suggested that 40–60% of the intact cells on the slides were normal. In all five cases with molecular evidence of homozygous loss, homozygous deletion was detected at D9S126 (1, 2, or 3 red signals and no green signal) in the tumor cells at a high frequency: 82% for tumor 30, 75% for tumor 24, 60% for tumor 64, and 50% for tumors 22 and 58. Hemizygous deletion was also frequently observed in some nuclei. In our control slides generated from normal lung tissue, single cosmid or P1 probe signals were observed in 8–12% of the nuclei counted. This 8–12% of cells from normal lung tissue with hemizygous deletions could have arisen from two sources: (a) a small number of cells with overlapping P1 or cosmid probe signals (i.e., only one signal was visible); or (b) poor hybridization efficiencies of the cosmid or P1 probes in this small percentage of cells. This implies that some of the hemizygous deletions observed by FISH in the tumor specimens could have originated from normal cells, thereby correspondingly increasing the percentage of tumor cells with homozygous deletion. Examples of the FISH analysis for homozygous deletion are presented in Fig. 4, A and B, for tumors 64 and 30, respectively. An example of hemizygous deletion is presented in Fig. 4C for case 45, in which two tumor cells are shown with two classical satellite signals and one P1 signal. In each panel, a single normal nuclei is included in the field to document hybridization of the cosmid or P1 probe. The presence of two centromeric signals in one of the cells in Fig. 4A could indicate duplication of the allele that had a homozygous deletion at D9S126. A single centromeric signal in each of the tumor cells in Fig. 4B also indicated loss of one copy of chromosome 9, which was previously suggested in the microsatellite analyses (Fig. 1). The data in Fig. 4C imply 9p loss without monosomy, which is also consistent with the results of microsatellite analysis (Fig. 1).

Discussion

Deletion of 9p is a very common event in lung cancer as well as numerous other types of cancer (6–12). We have demonstrated a novel region of homozygous deletion on 9p in 6 of 23 (26%) squamous cell carcinomas of the lung by using both comparative multiplex PCR and FISH analyses. The minimum region of homozygous deletion has been narrowed to 1 Mb and is localized between D9S265 and D9S259. In all six cases, the D9S126 marker, which resides between D9S265 and D9S259, is homozygously deleted. This region is at least 2.5 cM proximal to and exclusive of the region containing the p16/CDKN2 gene. Overall, 22 of 23 tumors analyzed in this study har-

4 J. Fountain, unpublished results.
bored either a hemi- or a homozygous deletion of D9S126. Thus, a TSG in the region between D9S265 and D9S259 could contribute to the tumorigenesis of a high percentage of squamous cell carcinomas of the lung.

Findings reported in a recent 9p deletion analysis in melanoma (19) have also suggested that the D9S126 region contains a TSG and that the smallest region of loss was based on interstitial losses in 5 of 54 tumors examined. Whereas these investigators placed D9S265 proximal to D9S126, we have determined that D9S265 is located between D9S171 and D9S126 by physically mapping it on a yeast artificial chromosome contig that spans this entire region. If the order of D9S265 and D9S126 given in Puig et al. (19) is reversed, then this homozygous deletion is reversed either a hemi- or a homozygous deletion of D9S126. Thus, a TSG in the region between D9S265 and D9S259 could contribute to the tumorigenesis of a high percentage of squamous cell carcinomas of the lung.

Several conflicting studies have been reported regarding the frequency of p16/CDKN2 alterations in primary NSCLCs. Hayashi et al. (36) reported p16/CDKN2 alterations in 19 of 64 NSCLCs; 14 tumors contained missense mutations, and the other 5 contained homozygous deletions. In contrast, Okamoto et al. (18) reported no alterations in 25 primary NSCLCs and 6 alterations (2 insertions and 4 homozygous deletions) in 22 metastatic NSCLCs. Vos et al. (17) detected alterations in p16/CDKN2 in 4 of 34 NSCLCs, including 1 mutation and 3 homozygous deletions. When Cairns et al. (16) used five microsatellite markers in close vicinity to p16/CDKN2, homozygous deletions were detected in only 5 of 39 NSCLCs and 2 of 39 SCLCs. We also screened the 23 squamous cell carcinomas for homozygous deletions in the p16/CDKN2 locus using the microsatellite markers developed by Cairns et al. (16) and detected homozygous deletions in 6 of 23 (26%) squamous cell carcinomas. Also of interest is that only one of the six tumors harbored homozygous deletions at both D9S126 and p16/CDKN2 (see Fig. 1 for tumor 64). Also, it has been suggested that methylation of the 5' CpG island may suppress p16/CDKN2 expressions in human tumors, including NSCLCs (37).

In addition to the data in this study with NSCLCs and to the above mentioned analysis by Puig et al. (19) in melanoma, several other reports have implicated the presence of a TSG(s) on 9p other than p16/CDKN2. Tarmin et al. (25) observed localized LOH at D9S165 in esophageal tumors, and Ichimura et al. (22) and Schmidt et al. (24) observed localized LOH in markers proximal to D9S171, including D9S126, in several malignant gliomas. The region between the markers D9S156 and D9S162 has also been implicated to contain a TSG (23, 25, 26). Our data shows localized LOH in this region for tumors 24 and 60 (Fig. 1). Overall, allele loss was observed between markers D9S156 and D9S259 in 20 of 23 tumors examined. Thus, loss of this chromosomal region by deletion of the entire 9p arm, a large telomeric deletion, or a large interstitial deletion, could simultaneously inactivate one allele of two or more chromosome 9 TSGs during tumorigenesis.

In all cases in which homozygous deletion at D9S126 was identified by molecular analysis, large populations of tumor cells also exhibited homozygous deletions when assayed by FISH. This evidence suggests that the D9S126 region contains a TSG(s) that is targeted during the development of squamous cell carcinoma of the lung. Further fine mapping and cloning efforts should help identify candidate TSGs in this region.

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References


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