WW Domain Containing Oxidoreductase Gene Expression Is Altered in Non-Small Cell Lung Cancer


ABSTRACT

WWOX (WW domain containing oxidoreductase), a putative tumor suppressor gene that maps to the common fragile site FRA16D on chromosome 16q23.3-24.1, is altered in breast, esophageal, and ovarian cancer. Because the FRA3B/FHIT locus at 3p14.2 is a preferential target for genetic changes caused by tobacco smoke, we intended to evaluate the status of the FRA16D/WWOX gene in non-small cell lung cancer; we have analyzed 27 paired normal and tumor lung tissues and 8 lung cancer cell lines for WWOX alterations by reverse transcriptase-PCR, loss of heterozygosity, and mutation analysis. Transcripts missing WWOX exons were detected in 7 primary tumors (7 of 27; 25.9%) and 5 of 8 cell lines. In addition, loss of heterozygosity at the WWOX locus was observed in 10 primary tumors (10 of 27; 37.0%). We conclude that WWOX alterations occur in a significant fraction of lung cancers and may contribute to the pathogenesis of non-small cell lung cancer.

INTRODUCTION

Lung cancer is the leading cause of cancer mortality in the United States, accounting for 169,500 deaths in 2001 alone (1). NSCLC comprises 80% of all lung cancer. Surgery remains the mainstay of therapy for NSCLC patients; however, a significant fraction of patients are not candidates for a curative resection (2). Additional elucidation of the genetic alterations and molecular mechanisms involved in lung cancer may allow the development of novel therapeutic strategies.

Lung cancer is strongly associated with exposure to environmental carcinogens, particularly tobacco smoke. It has been suggested that carcinogen-induced alterations are more frequent at fragile sites and that genes located at these fragile sites are frequently altered because of such exposure. If these genes are tumor suppressor genes, such alterations may provide an etiologic mechanism for carcinogenesis (3, 4). A common, early, and consistent alteration found in lung cancer is that genes located at these fragile sites are frequently altered because carcinogen-induced alterations are more frequent at fragile sites and because of the strong association of tobacco smoking with lung cancer, we hypothesized that the WWOX gene at FRA16D may be altered in a significant fraction of NSCLCs.

MATERIALS AND METHODS

Cell Lines and Human Tissue. Tumors and the corresponding normal lung tissue were obtained from patients undergoing surgery at the Hospital of the University of Pennsylvania. Tissue was frozen as soon as possible at −80°C. Human lung cancer cell lines A549, NCI-H460, NCI-H23, NCI-H1573, NCI-H1299, NCI-H522, NCI-H1650, and Calu-3 were obtained from the American Type Culture Collection. All cell lines were maintained in RPMI 1640 with 10% fetal bovine serum at 37°C and 5% CO2. DNA was obtained by digestion of tissue with proteinase K overnight at 50°C and extraction with phenol/chloroform/isooamy alcohol and chloroform/isooamy alcohol, followed by precipitation with alcohol. RNA was extracted with the Trizol reagent (Invitrogen Life Technologies, Inc., Carlsbad, CA) as per the manufacturer’s recommendations. cDNA was made from 2 μg of total RNA using the SuperScript first strand synthesis system (Invitrogen Life Technologies, Inc.).

Reverse Transcriptase-PCR. Nested reverse transcriptase-PCR was performed using 1 μl of cDNA. The first and second amplifications were performed with nested primers (forward primer 5′-AGTTCCCTGACGGTG-GACC-3′; reverse primer 5′-TTACTTTTCAACAGGCCACCAC-3′) and (forward primer 5′-AGGTGCCCCTACAGCT-3′; reverse primer 5′-GTGT-GTGGCCCTACCGCTCT-3′), respectively. Both reactions were carried out in a volume of 50 μl containing 20 pmol of each primer, 2.5 mM MgCl2, 0.2 mM deoxynucleotide triphosphate mix, 1X PCR buffer, and 2.5 units of AmpliTaq Gold (Perkin-Elmer, Life Sciences Inc., Boston, MA). The PCR program was set for one cycle of 95°C for 8 min followed by 35 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min with an extension step of 72°C for 5 min in a Perkin-Elmer Gene Amp PCR System 9600. One μl of the amplification product from the first reaction was used for the second reaction. After running the products on a 1.5% agarose gel, the normal and abnormal bands were excised and purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA) and sequenced using Applied Biosystems Prism 377 DNA sequencing system using appropriate primers. Glyceraldehyde-3-phosphate dehydrogenase amplification served as a control for cDNA quality.

Exon PCR and Mutation Analysis. PCR amplification of individual exons from genomic DNA of lung cancer cell lines and paired normal lung and tumor tissue was performed using primers described previously (GenBank Accession Nos. AF325423-AF325432; Ref. 10). The reaction conditions were: 50 ng of genomic DNA template; 10 pmol of each primer; 2.5 mM MgCl2; 1.5 mM deoxynucleotide triphosphate mix; 1X PCR buffer; and 0.5 unit of AmpliTaq Gold (Perkin-Elmer, Boston, MA) in a 20-μl final volume in a Perkin-Elmer Gene Amp PCR system 9600. PCR cycles used were one cycle at 95°C for 12 min, succeeded by 10 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min and 35 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min with an extension step of 72°C for 5 min in a Perkin-Elmer Gene Amp PCR System 9600. PCR cycles used were one cycle at 95°C for 12 min, succeeded by 10 cycles at 94°C for 15 s, 55°C for 15 s, and 72°C for 30 s. This was followed by 25 cycles at 89°C for 15 s, 55°C for 15 s, and 72°C for 30 s. An elongation cycle of 72°C for 10 min completed the sequence. After purification of the PCR products using the QIAquick purification kit (Qiagen, Inc.), sequencing was performed using an Applied Biosystems Prism 377 DNA sequencing system (Applied Biosyst., Inc.).

LOH Analysis. Allelic losses were analyzed by PCR amplification of polymorphic microsatellites internal to WWOX at loci D16S3029, D16S3096, D16S504, and D16S518 as described elsewhere (12). Briefly, primers were obtained from the Genome database and labeled using 5′-fluorescein phos-
phoramidite or 5'-tetrachlorofluorescein phosphoramidite, as described by Ishii et al. (16). PCR was performed on 50 ng of DNA for each sample using conditions described for the mutation search. PCR products were denatured in formamide for 5 min at 95°C and then loaded on a 6% denaturing gel on the Applied Biosystems 373 DNA sequencer. LOH was analyzed by using the Applied Biosystems Prism Genescan and the Applied Biosystems Prism Genetyper Analysis software (Perkin-Elmer/Applied Biosystems). Cases were defined as exhibiting LOH when an allele peak signal from DNA was reduced by 30% compared with the normal counterpart.

RESULTS

Tumor Characteristics. Of 36 tumor samples obtained, confirmed diagnosis of non-small cell carcinoma was established in 27. These 27 paired normal and malignant samples were analyzed in this study. Of these 27 samples, 10 (37.0%) were adenocarcinomas, 11 (40.7%) were squamous cell carcinomas, 4 (14.8%) were poorly differentiated carcinomas, and 2 (7.4%) samples had other histologies. The distribution by stage was stage I, 17 (62.9%); stage II, 7 (25.9%); and stage III, 3 (11.1%).

Aberrant WWOX Transcripts in Tumor Cell Lines and Tumors. As reported previously, WWOX mRNA expression is too low to be detectable routinely by Northern blot of RNA from small tumor samples (12). Hence, reverse transcriptase-PCR amplification was performed to analyze WWOX expression. Of 27 cases of NSCLC studied, 7 showed transcripts with missing exons. Of these 7, 2 tumor samples (cases B-83 and B-86) did not exhibit a normal sized transcript. In all 27 pairs, corresponding normal tissues demonstrated normal size transcripts without aberrant transcripts. Sequence analysis of the reverse transcriptase-PCR amplification products showed the alterations summarized in Table 1. Of 8 cell lines studied by reverse transcriptase-PCR, aberrant transcripts were found in 5 (Fig. 1). Interestingly, sequencing of several of these aberrant amplification products (in cell lines NCI-H23, NCI-H1650, and NCI-H1573) showed frameshifts in the open reading frame (Fig. 2).

Deletion and Mutation Analysis of Cell Lines and Tumors. To evaluate the frequency of detection of homozygous deletions or point mutations at the WWOX locus in NSCLC, mutation analysis for all exons was performed in all 27 normal and tumor samples and 8 human lung cancer cell lines. This was done by direct sequencing of the PCR products of amplification of each exon. All of the exons were amplified in all 8 cell lines and 27 tumor and normal samples. Homozygous deletion of exons was not detected, suggesting that aberrant reverse transcriptase-PCR products might be explained by hemizygous deletions or biallelic nonoverlapping deletions within the WWOX locus. No point mutations were identified in the clinical samples. Among 8 cell lines, we identified a variant in exon 6 in the cell line NCI-H23. The alteration involves a G to A transition at nucleotide 547 and results in an aspartic acid to asparagine substitution at amino acid 183 (GAC to AAC). This variant amino acid is located in an oxidoreductase domain; whether it represents a rare polymorphism or a missense mutation is not known.

LOH Analysis. To evaluate the frequency of allelic loss of WWOX in NSCLC, we performed a search for LOH using four polymorphic microsatellite markers within the gene. The loci D16S3029, D16S3096, and D16S504 are located in intron 7 and the locus D16S518 is located in exon 1. All of the samples were informative for at least one marker. Among 27 paired normal and tumor samples, LOH was detected in 10 samples (37.0%); 4 of 11 (36.4%) squamous cell cancers; and 3 of 10 (30.0%) adenocarcinomas (Table 2).

It is interesting to note that in three samples (B-55, B-64, and B-43)
01-36), LOH is not observed at locus D16S518 located in intron 1, whereas it is observed in the other three loci studied. This is consistent with the presence of aberrant transcripts that retain exon 1 while demonstrating loss of other telomeric exons.

**DISCUSSION**

In this study, we have demonstrated that WWOX is altered by deletion and/or aberrant expression in a significant fraction of NSCLC tumors (51.8%; 14 of 27). Among the primary NSCLC samples studied, reverse transcriptase-PCR showed aberrant transcripts (25.9%; 7 of 27), and the WWOX locus exhibited LOH (10 of 27; 37.0%). This is similar to the frequency of alterations found in breast cancer samples previously (11). The frequency of alterations of WWOX observed in the primary tumors is a low estimate because tumors with no expression of WWOX would only express the normal transcript from contaminant nontumor cells.

Our data are consistent with the pattern of WWOX alterations described by others. Bednarek et al. (10) have demonstrated that 10% of primary NSCLC samples studied, reverse transcriptase-PCR showed aberrant transcripts (25.9%; 7 of 27), and the WWOX locus exhibited LOH (10 of 27; 37.0%). This is similar to the frequency of alterations found in breast cancer samples previously (11). The frequency of alterations of WWOX observed in the primary tumors is a low estimate because tumors with no expression of WWOX would only express the normal transcript from contaminant nontumor cells.

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