A Candidate Tumor Suppressor Gene, H37, from the Human Lung Cancer Tumor Suppressor Locus 3p21.3

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ABSTRACT

Frequent allelic loss and homozygous deletions within chromosome 3p in human lung cancers have suggested that the 3p21.3 (370-kb) region contains a critical tumor suppressor gene(s) (TSG). With the exact identity/characteristics of such a gene(s) still unconfirmed, a lack of inactivating structural mutations in the expressed genes contained within this region may indicate that the 3p TSG(s) do not fit into the classical “two-mutation” model. This report characterizes a candidate 3p TSG, H37, located within the 370-kb region. Reduced expression of the H37 transcript was found in 9 of 11 (82%) of primary non-small cell lung cancers (NSCLCs) when compared with adjacent normal tissues. Generation of an H37 antibody followed by immunohistochemical analysis of primary NSCLC specimens demonstrated that 46 of 62 (73%) of these cancers contain reduced levels of H37 protein when compared with adjacent normal bronchial cells. Moreover, introduction of the H37 cDNA into human breast cancer cells deleted of 3p21–22 reduced both anchorage-dependent and -independent cell growth in vitro. Subsequent transfection of H37 cDNA into one of the human lung cancer cell lines homogeneously deleted in this region resulted in a very low yield of H37-expressing clones. H37 also suppressed anchorage-dependent and -independent growth of A9 mouse fibrosarcoma cells and inhibited tumor formation in nude mice. These data indicate a potential role for H37 as one of the 3p TSGs in human lung cancer.

INTRODUCTION

Lung cancer accounts for >160,000 new cancer diagnoses and >150,000 deaths each year, making it the leading cause of cancer mortality in the United States (1). In addition, worldwide epidemiological data show it to be a significant medical problem outside the United States. As a result, a major research effort has been undertaken by a number of laboratories directed at understanding the molecular pathogenesis of this disease. Allelic loss of a portion of chromosome 3p is observed in >90% of SCLCs and in 50–80% of NSCLCs (2, 3), suggesting that this region may contain one or more critical TSGs. The 3p deletion has also been reported in a variety of other human malignancies including breast (4), cervix (5), kidney (6), and head and neck (7) cancers, making the putative TSG(s) in this region of general significance. Additional supporting evidence for TSG function in this region comes from data that transfer of a normal chromosome 3 to human cancer cells results in tumor suppression (8–10).

A significant research effort undertaken by several investigators (The International Lung Cancer Chromosome 3p21.3 TSG Consortium; Ref. 11) over the past ~18 years has narrowed the region on chromosome 3 down to an area of ~370 kb at “3p21.3” (12). This region was identified by the study of three human lung cancer cell lines with homozygous deletions at this location, i.e., NCI-H740, NCI-H1450, and GLC 20 (12). To date, however, none of the ~35 genes contained in the region of minimal overlap demonstrate structural mutations in actual human lung cancer specimens, leading the Consortium to speculate that the TSG(s) within the region may not conform to the classical “two-hit” criterion, i.e., mutations in both alleles (11). Similar phenomena have now been identified for other TSG(s) found in the genome such as transforming growth factor-β1 and p27/Kip1 (13, 14). Thus far, the only gene located at 3p21.3 reported to be significantly altered in human lung cancer is a human RAS effector homologue (RASSF1), the promoter of which is highly methylated in 40% of primary lung tumors (15).

The H37 gene (GenBank accession no. AF103802) was initially identified in our laboratory during differential gene expression profiling to determine genes whose expression levels change in association with HER-2/neu proto-oncogene overexpression (16). HER-2/neu overexpression is observed in 25–30% of human breast cancers and is associated with a poor clinical outcome (17, 18). On further analysis, H37 was found to match LUCAL5/RBM5 (12, 19), which is 1 of the ~35 genes located in the region of interest on 3p21.3. Here we describe the characterization of H37 as a candidate 3p tumor suppressor gene. We report reduction of H37 mRNA and protein expression in primary NSCLCs compared with adjacent normal control tissues as well as in vitro and in vivo growth suppression exerted by H37.

MATERIALS AND METHODS

Antibody Generation. Recombinant protein was produced in 801-K bacterial cell lines (New England Biolabs, Inc., Beverly, MA) by transformation with pMAL-c2 vector (New England Biolabs) coding for fusion between bacterial maltose binding protein and the COOH-terminal fragment of H37 (amino acids 408–815). After isopropyl-1-thio-β-galactopyranoside induction, the bacterial cells were collected by centrifugation, resuspended in column buffer (20 mM Tris-HCl, 200 mM NaCl, and 1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride, and lysed by multiple freeze and thaw cycles and sonication. Cellular proteins were recovered in the supernatants after centrifugation at 9000 g at 4°C for 20 min. The MBP-H37 fusion proteins were purified via amyllose resin column and eluted in column buffer containing 10 mM maltose. The fusion proteins were injected s.c. into rabbits using Freund’s complete adjuvant (Life Technologies, Inc., Rockville, MD) for an initial round, followed by multiple boosts using Freund’s incomplete adjuvant.

Tumor Specimens. Primary NSCLC tumors and adjacent normal tissues used in the Northern blot analysis were specimens obtained from various hospitals during 1988–1989. After surgical removal, all of the samples were immediately snap-frozen in liquid nitrogen and stored at ~80°C until total RNA was extracted by guanidinium/cesium chloride ultracentrifugation. Primary NSCLC tumors used in immunohistochemical staining were obtained from patients undergoing surgical resection at the UCLA Medical Center from 1995 to 2000.

Immunohistochemical Staining. Specimens containing both tumor and adjacent normal tissues were fixed in formalin and embedded in paraffin block. Four-μm-thick sections cut from blocks were baked, deparaffinized in xylene, and rehydrated through a series of ethanol. Antigen retrieval was performed by incubating the sections at 100°C in 10 mM sodium citrate buffer (pH 6.0) for 20 min. Endogenous peroxidase activity was quenched by treating with 3% hydrogen peroxide.
hydrogen peroxide solution for 15 min. Slides were incubated with 2% BSA to block nonspecific antibody binding and then reacted with the primary rabbit polyclonal anti-H37 serum (1:2500 dilution) at 4°C overnight. After washed in PBS, slides were incubated with the streptavidin-biotin-peroxidase complex (Dako Co., Carpinteria, CA). Sections were visualized by 3,3'-diaminobenzidine chromagen (Dako Co.). Normal rabbit IgG at the same concentration as the primary antibodies served as negative controls.

Construction of Recombinant Plasmids and Transfections. The full-length H37 cDNA was subcloned in the pBK-CMV eukaryotic expression vector (16). The gene is transcribed via a CMV immediate-early promoter. The plasmid was transfected into cells by Lipofectamine (Life Technologies, Inc.) according to the manufacturer’s protocol. Cells were grown in RPMI 1640; supplemented with 10% fetal bovine serum, 2 mM glutamine, and 1% penicillin G/streptomycin/fungizone solution; and selected in G418, 600 µg/ml for MCF-7, 350 µg/ml for HBL-100, and 300 µg/ml for A9 cells, respectively. Even after the 2-week initial selection, the transfected cells were strictly maintained in the same concentrations of G418 to avoid reversion of the cells. As a control, cells were also transfected with the empty vector pBK-CMV. In MCF-7 cells, neo(1H37.1) versus neo2(1H37.2) cell populations are different according to different lipid:DNA ratios used during transfection following the manufacturer’s guidelines. HBL-100/1H37.1 versus 1H37.2 are different in a similar manner. For the floating cells (NCI-H740), the pBK-CMV/H37 plasmid was transfected in DMRIE-C (Life Technologies, Inc.) according to the manufacturer’s protocol. Cell lumps were dissociated to single cells in 0.1% pluronics solution (Life Technologies, Inc.) before transfection. The transfected cells were selected in 100 µg/ml G418 for up to 1 month, after which the antibiotic was removed. For A9 cells, after selection in G418 for 2 weeks, cells were plated into 10-cm dishes at a very low density, and individual colonies were selected by use of cloning rings and expanded. During the selection and expansion, all of the transfected cells were strictly maintained in G418.

Soft Agar Assay. Cells were plated in 0.4% (bottom) and 0.2% (top) soft agar layers containing 600 µg/ml G418 for MCF-7 and 300 µg/ml for A9 cells. Cells were plated in triplicate in 60-mm dishes (2 × 10⁴ cells/dish) for MCF-7 cells (mass transfection) and in 35-mm dishes (4000 cells/dish) for A9 single cell expanded clones. Cells were incubated at 37°C for up to 3 weeks before staining in tetrazolium dye for counting and photography.

RESULTS

Sequence Analysis and Tissue Expression of H37. The H37 cDNA was isolated in our laboratory from a library prepared from MCF-7 human breast adenocarcinoma cells (16). This cDNA is 3091 bp in size including 126 nucleotides of 5’ and 520 nucleotides of 3’ untranslated sequence and an open reading frame encoding 816 amino acids (Fig. 1A). The putative H37 protein contains functional motifs that include two RNA binding domains, two zinc finger motifs, and a bipartite nuclear localization signal (Fig. 1A). Homology studies demonstrated that H37 is related to Drosophila Sex-lethal (20), mouse S1-1 (21), and the human DEF-3 proteins (22) sharing RNA recognition motif domains (Fig. 1B). Northern blot analysis comparing H37 gene expression in various human tissues indicated that it is expressed mainly in heart, skeletal muscle, kidney, and placental tissues (Fig. 2). Two different transcripts representing alternate splice variants of 3.1 and 6.5 kb were detected in cells expressing the gene (Fig. 2).

Reduction of H37 mRNA and Protein Expression in Primary NSCLCs. Consistent with the Consortium’s report supporting alternative tumor-suppressive mechanisms for the potential 3p TSGs (11), sequence analysis of RNA from the actual lung tumor specimens in our study failed to show any mutation in the coding region of H37. Nevertheless, our study evaluating H37 transcript levels in 11 malignant versus adjacent normal tissue from NSCLC specimens demonstrated that 9 of 11 cases (82%) contained easily detectable decreases in H37 RNA expression in tumor as compared with adjacent normal tissue (Fig. 3). These tumors included 4 adenocarcinomas (patient samples 2, 5, 6, and 10), 6 squamous cell carcinomas (samples 1, 3, 4, 7, 8, and 11), and a large cell carcinoma (sample 9). In sample 9, the tumor sample expressed more H37 RNA than levels found in the corresponding normal sample. This occasional reversal of expression pattern for a putative TSG has been observed with other well-recognized TSGs such as BAX and p21 and may be related to alterations upstream of the target sequence. For example, occasional up-regulation of the BAX and p21 TSGs in thyroid cancer is associated with dysregulation of wild-type p53 (23).

To expand these studies by immunohistochemical analysis, a polyclonal anti-H37 antibody was generated, and the specificity of the antibody was demonstrated using NCI-H740 cells, which are homozygous H37 deletants and which failed to show the presence of the H37 protein by immunohistochemical analysis when compared with other various positive control cell lines. We further confirmed the specificity of the immunostaining studies by demonstrating that the recombinant H37 protein blocks immunostaining (Fig. 4C). We then performed immunohistochemical analysis to evaluate the H37 protein expression in a larger series of actual NSCLC specimens. A total of 46 of 62 (73%) of the specimens studied showed reduced H37 protein expression in malignant compared with normal bronchial epithelial cells, whereas 16 of 62 (27%) showed similar H37 immunoreactivity between tumor and normal elements (Table 1; Fig. 4). H37 expression was reduced in 32 of 39 (82%) of the adenocarcinomas (which represent the most common type of NSCLCs) and 11 of 17 (65%) of
the squamous cell carcinomas (Table 1). Tumors with decreased immunoreactivity were further classed into “strongly reduced (−)” versus “moderately reduced (−)” based on subjective evaluation of the degree of staining (Table 1). In adenocarcinomas, “strongly reduced” expression was observed most commonly in the poorly differentiated subtypes (11 of 21; 52%). In squamous cell carcinoma, the relative frequency and degree of decrease was less than that seen in adenocarcinomas. Only six samples of large cell undifferentiated carcinoma were evaluated in this study, and of these, 3 (50%) showed markedly reduced immunoreactivity, whereas 3 showed no difference between normal and tumor cells.

The immunostaining confirmed subcellular localization of H37 protein to the nucleus as predicted from the presence of nuclear protein structural motifs. This implies that H37 may function in some aspect of transcriptional regulation similar to a number of other tumor suppressor genes, i.e., p53, RB, and BRCA1.

Effects of H37 Overexpression in Human Breast Cancer Cells. Concurrent efforts to functionally characterize biological effects of this novel gene in relation to HER-2/neu gene overexpression phenotype were undertaken by analyzing the growth properties of MCF-7 human breast cancer cells overexpressing H37. Analysis of the MCF-7 parental cells using comparative genomic hybridization has demonstrated that like the human lung cancer cells, these cells have lost chromosome 3p21–22 (24). The full-length H37 cDNA subcloned in the pBK-CMV eukaryotic expression vector was transfected into MCF-7 cells. Simultaneous studies were performed with the empty vector controls.

Fig. 3. Northern blot analysis of the H37 transcript in lung cancer specimens and adjacent normal tissues. Total RNA was extracted from frozen samples of 11 primary NSCLC tumors (T) and patient-matched normal lung tumor (N). Expression of H37 was analyzed by Northern hybridization using the entire H37 coding region as a probe. 18S RNase is shown as a loading control.

Fig. 4. Histology and immunohistochemical staining for H37 in normal and cancer cells of the three different subtypes of NSCLC. A–C, normal bronchial epithelium. The same area was stained with H&E (A), H37 antibody showing intense nuclear staining of H37 protein (B), and antiserum blocked with the H37 recombinant protein as a negative control (C). D and E, adenocarcinoma. H&E (D) and immunostaining (E) of the corresponding area shows reduced H37 protein in the tumor cells compared with adjacent normal epithelium. F, H&E staining of the tumor area shown in H. G, F, normal bronchial epithelium. H, E, adenocarcinoma. I–K, large cell undifferentiated carcinoma comparing immunostaining of H37 in normal epithelia (J) versus reduced staining of the protein in tumor cells (K), both fields from the same slide. L, H&E staining of the tumor area shown in K. M, normal bronchial epithelium. N, adenocarcinoma. O, large cell undifferentiated carcinoma.
vector as a negative control. At the end of the 2-week selection in G418, pooled populations of two negative control cell lines (neo1 and neo2) and two \( H37 \) transfected lines (\( H37.1 \) and \( H37.2 \)) were assayed for growth characteristics. By day 14, growth of \( H37.1 \) cells was suppressed by 61\% compared with the neo1 level, and \( H37.2 \) was suppressed by 32\% compared with neo2 (Fig. 5A).

Growth suppression was also tested using anchorage-independent growth assays. When colonies of >20 cells were counted at the end of a 3-week observation period, significantly reduced numbers (as well as size) of the colonies resulted from \( H37 \) transfection. The \( H37.1 \) clones were reduced by 76\% and \( H37.2 \) by 78\% compared with their respective control transfectants (Fig. 5B). The same assays were also performed for MCF-7 cells transfected with a number of other genes identified during our initial differential screening studies using the same vector and selection methods as those used for \( H37 \) (16). Only \( H37 \) suppressed MCF-7 cell growth on soft agar, whereas the other genes either had no effect or alternatively increased cell growth (data not shown). This suggests that the growth suppression observed with \( H37 \) gene transfection is specific to this gene and is not the result of transfection artifact or selection induced by the experimental system. In addition, to address the possibility that the suppression seen with \( H37 \) may be attributable to general nonspecific toxicity of the protein in proliferating cells as opposed to specific suppression of tumor cell growth, we performed additional studies. In these studies, \( H37 \) was transfected into HBL-100 immortalized, proliferating human cells that share characteristics similar to normal cells and distinct from malignant cells (25). Comparative gene hybridization has shown previously that there are no deletions in chromosome 3p in HBL-100 (24). In our growth assay, HBL-100 cells stably transfected with pBK-CMV/\( H37 \) and/or vector control (neo) were counted at the end of the 2-week selection in G418. Growth of two separate pooled populations of \( H37 \) transfectants (\( H37.1 \) and \( H37.2 \)) were found to be at 114 and 122\% of the control transfectant growth level (Fig. 5C), demonstrating that \( H37 \) overexpression in HBL-100 does not suppress growth of this cell line. This supports the concept that the \( H37 \)-induced growth suppression in malignant cells is not simply a result of toxicity of this gene in proliferating cells.

Lastly, expression levels of the \( H37 \) mRNA and protein were evaluated in control and \( H37 \)-transfected MCF-7 cells and compared with various cell lines to confirm that transfected expression levels do not exceed the “normal” range. \( H37 \) transcript and protein levels obtained in the MCF-7 transfectants were no higher than in some of the lung cancer cell lines examined (none of which are homozygously deleted at 3p21.3). These expression profiles were demonstrated by both Northern and Western blot analyses (Fig. 5D, upper two and lower two panels, respectively). On Western blot analysis, the only band identified by the \( H37 \) antibody in the lung cancer cell lines as well as in the breast cancer cells was the expected \( M_r \sim 90,000 \) band.

**Inhibition of Tumor Formation in Nude Mice by \( H37 \) Transfection.** Subsequently, the \( H37 \) gene was introduced into NCI-H740 cells, which is one of the three lung cancer cell lines known to be homozygously deleted at 3p21.3. Introduction of \( H37 \) resulted in a very low yield of \( H37 \)-expressing clones in NCI-H740 (Fig. 6A), making it technically challenging to obtain a sufficient quantity of cells to examine the \( H37 \) expression level.

Similar to other cells that grow in suspension, the NCI-H740 cells do not grow well in soft agar nor form s.c. xenografts in nude mice. To further evaluate the growth-inhibitory effects of the \( H37 \) gene in vivo, we used the A9 mouse fibrosarcoma cells that do form xenografts and that are the same cells originally used to obtain the initial functional evidence, demonstrating that chromosome 3p contains tumor suppressive activity (10). In published reports, in vivo growth of A9 cells in nude mice was inhibited by transfecting the cells with a 2-megabase piece of human chromosome 3 encompassing 3p21–22 (which includes the \( H37 \) gene). In the current study, single-cell-expanded A9 clones of the \( H37 \) transfected (\( H37.1 \) and \( H37.2 \)) and vector controls (neo1 and neo2) were developed, and \( H37 \) expression levels in the individual clones were tested by both Northern and Western blot analyses (Fig. 6B). In the initial in vitro growth assays, both \( H37.1 \) and \( H37.2 \) cell growth was suppressed by 51 and 39\%, respectively, compared with vector control cells (Fig. 6C). Also, \( H37 \) overexpressors consistently formed smaller size colonies of significantly reduced numbers (Fig. 6C). These in vitro growth assays were also performed for A9 cells transfected with the various plasmid constructs (described for MCF-7 cells above), and again we confirmed specificity of growth suppression induced by \( H37 \) (data not shown).

Subsequently, the \( H37 \)-transfected A9 cells were further evaluated by s.c. injection into nude mice. As reported previously, A9 control cells formed relatively large tumors within a short period of time. In contrast, animals injected with \( H37.1 \) and \( H37.2 \) cells exhibited a significant suppression of tumor development, reaching only 22 and 11\%, respectively of the growth of control xenografts at the end of a 4-week period (Fig. 6D).

**DISCUSSION**

Lung cancer is the number one cause of death from cancer for both men and women worldwide (1, 26). Despite improvements in cytotoxic drug development, radiotherapy, and patient care, the overall survival rate for lung cancer patients has remained unchanged in the
last 20 years. An important step toward developing more effective diagnosis and treatment for lung cancer patients is a clear understanding of its genetic pathobiology. The most frequent genetic alterations in lung cancer are deletions of the short arm of chromosome 3 (3p), suggesting that one or more TSG(s) present in this region may act as a gatekeeper for lung carcinogenesis. Discovery of such genes may serve as particularly useful diagnostic/prognostic markers because the 3p alterations occur in the early stages of lung cancer, such as in bronchial dysplasia and metaplasia (27).

In the present studies, we report characterization of a candidate 3p TSG, H37, located in the minimal overlap of homozygous deletions in 3p in lung cancers (3p21.3). Reduced expression of H37 mRNA and protein was demonstrated in ~75% of primary lung cancer specimens compared with their adjacent normal control tissues. Introduction of H37 cDNA results in suppression of both anchorage-dependent and -independent cell growths in vitro as well as inhibition of tumor formation in nude mice. H37, also called LUCA15/RBM5 by others (12, 19), has amino acid sequence homology with its immediate neighboring gene, LUCA16/RBM-6/DEF-3 (22), suggesting that these two genes arose from gene duplication event, and they belong to the same gene family (11). Interestingly, both LUCA15 and LUCA16 have been found to be autologous serum antigens in kidney and lung cancer patients, respectively (30, 31). Also, it has been reported that overexpression of an alternative RNA splice variant of LUCA15 retards proliferation of Jurkat T cells and expedites CD95-mediated apoptosis (32), implicating the LUCA15 gene locus in the regulation of apoptosis (33). H37 may be functionally related to Sex-lethal, S1-1, and DEF-3 proteins because they all contain RNA recognition motifs and show in vitro RNA binding activity (20–22). Therefore, it is possible that H37 may suppress tumor growth by playing a role in posttranscriptional regulation, i.e., RNA splicing, polyadenylation, message stability, translation, and others. In support of this, the molecular function of at least one of the above proteins (Sex-lethal) is known. It controls sex development of Drosophila by posttranscriptional regulation of sex-related genes (20).

A potential mechanism by which a “non-structurally mutated” gene may be inactivated or altered includes disturbances in regulatory elements (34). A good example of this is promoter hypermethylation as shown for the recently characterized 3p21.3 gene RASSF1 (15), which is believed to be of lesser importance in NSCLC (35). Alternatively, a tumor suppressor may function by the relatively novel concept, “haploinsufficiency,” characterized by having an intact re-
remaining allele (36). The current data show that \( H37 \) expression is decreased in tumors by more than half of their adjacent normal tissue in \( \sim 75\% \) of samples tested. The mechanism involved in this decrease in expression in a remaining wild-type allele requires further elucidation, and these studies are currently underway. Studies are also underway to determine the significance of the interaction of \( H37 \) with the HER-2/neu oncogene.

Taken together, our results suggest that \( H37 \) may be one of the 3p TSGs found in the 3p21.3 region. During preparation of the manuscript, in support of our data, others reported that \( H37/LUCA15 \) is one of the down-regulated genes in ras-transformed cells, and transfection of this gene in human fibrosarcoma cells suppressed the cell growth (37). The significant reduction or in some instances absence of \( H37 \) protein expression in actual primary lung cancers observed in our study underscores its potential value as a candidate TSG. This gene may be useful in early detection of malignancies in screening programs as well as an indicator of response in cancer chemoprevention trials. Results from further screening studies of \( H37 \) expression in larger cohorts of human tumor specimens as well as premalignant lesions will be required to evaluate the prognostic and/or predictive value of this gene. In addition, further characterizations of \( H37 \) protein function as well as evaluation of genes whose expression may be affected by \( H37 \) may lead to the development of new therapeutic strategies for malignancies carrying this alteration.

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REFERENCES


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