A Codon 248 p53 Mutation Retains Tumor Suppressor Function as Shown by Enhancement of Tumor Growth by Antisense p53

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ABSTRACT

Codon 248 in domain IV of the highly conserved region of the p53 gene is a frequent site of mutations associated with sporadic cancers and the familial cancer syndrome (Li-Fraumeni syndrome). Therefore, a characterization of the functional significance of a codon 248 mutation is of interest. We used antisense RNA methodology to study the role of the wild-type and mutated p53 gene in cell growth and tumorigenesis. We introduced wild-type p53 complementary DNA in sense or antisense orientation under control of a ß-actin promoter into human non-small cell lung cancer cell line H322a which has a codon 248 mutation (G to T) and WTH226b which has wild type p53. The biological properties and p53 expression of stable G418-resistant clones were analyzed. We observed that in both cell lines antisense RNA expression significantly reduced p53 mRNA and protein production; it also caused increases in growth rate in cell cultures and in tumorigenicity in nu/nu mice for both cell types, suggesting that the mechanism by which p53 suppresses cell proliferation and tumorigenesis is not always abrogated by a codon 248 mutation.

INTRODUCTION

The p53 gene encodes a complex protein that mediates several properties that appear to be primarily involved in the regulation of cell growth (for review see Ref. 1). These properties include DNA binding, oligomerization, protein binding, and transcriptional activation (2–5). Mutations and deletions of p53 frequently occur in many human cancers (6, 7), and these mutations appear to completely or partially inhibit some of the functions ascribed to the p53 protein (2, 8–12). However, normal development and function can occur in mice that overexpress a mutant (codon 135) p53 allele; in one study only 20% of mice with this mutation developed tumors, and those tumors had a long latent period and low penetrance (13). The recent identification of germline mutations of the p53 gene in members of cancer-prone families with Li-Fraumeni syndrome suggests that p53 mutations can contribute to cancer susceptibility. Other studies have suggested that p53 mutations confer a dominant negative transforming ability to the gene product (12). We studied the function of an endogenous mutated p53 in families with Li-Fraumeni syndrome suggests that p53 mutations may contribute to cancer susceptibility. Other studies have suggested that p53 mutations confer a dominant negative transforming ability to the gene product (12). We studied the function of an endogenous mutated p53 using an antisense construct. The mutation studied may contribute to cancer susceptibility. Other studies have suggested that p53 mutations confer a dominant negative transforming ability to the gene product (12). We studied the function of an endogenous mutated p53 using an antisense construct. The mutation studied involved codon 248, which is one of the most common codons mutated in sporadic tumors and in the germline mutations in Li-Fraumeni families (7, 14). We found that reduction in the expression of this mutant enhanced tumor cell proliferation and tumorigenicity indicating that this p53 mutant retained significant tumor suppressor function.

MATERIALS AND METHODS

Cell Culture and DNA Transfection. Human non-small cell lung cancer cell lines H322a, H358, and WTH226b were derived and characterized as described previously (15). H322a is a human adenocarcinoma lung cancer cell line, and WTH226b is a human squamous cell lung cancer cell line. Cell cultures were maintained in RPMI medium supplemented with 5% heat-inactivated fetal calf serum. Cells were electroporated using 10 μg purified plasmid DNA; 48 h after electroporation, 300 μg/ml (Gentisin; Sigma Chemical Co., St. Louis, MO) were added to the cultures. Individual G418-resistant clones were grown separately for further analysis.

SSCP and DNA Sequencing. The p53 mutations in the cell lines were examined by SSCP analysis as described elsewhere (16). Exons 5 to 8 of the each cell line were examined for the presence of mutation by SSCP analysis from two independent PCR products. DNA was labeled directly by adding [α-32P]dCTP to the PCR reaction during the last 10 cycles. One μl of the reaction mix was denatured and run on a 10% nondenaturing polyacrylamide gel in TBE buffer. The gel was exposed to X-ray film overnight. The PCR-amplified DNA sample which was positive on the SSCP gel was further subcloned into a BlueScript vector for sequencing. To confirm the mutational status of these cell lines, the complete 1.2-kilobase cDNA from these cell lines was analyzed by reverse transcriptase/PCR followed by DNA sequencing. Complete cDNA from these cell lines was subcloned into a BlueScript plasmid and sequencing was performed by the modified dideoxy chain termination method using Sequenase (United States Biochemicals) enzyme according to the manufacturer’s instructions.

Plasmid Constructs. A complete 2.3-kilobase human p53 cDNA with 5’ and 3’ untranslated sequences was placed under control of the ß-actin promoter in either sense or antisense orientation in a Apr-1-neo vector carrying a neo-selectable marker (a kind gift from Dr. Larry Kedes of the University of California, Los Angeles). The orientation of the p53 cDNA in the plasmid was confirmed by DNA sequencing using the vector primer (5’-GCA GGA TCA GTC GAC CTG CA) preceding the cloning site. The entire cDNA was sequenced to verify the presence of wild-type p53. A 118-base pair p53 DNA fragment containing exon 7 in a BlueScript vector and a 248-base pair human ß-actin cDNA in a PGM vector (Promega Corp, Madison, WI) were used to synthesize RNA probes for the RNase protection assay. Antisense ß-actin was used as an internal control for the assay.

RNase Protection Assay. The plasmid containing a 118-base pair exon 7 p53 cDNA fragment was linearized with HindIII, and T3 RNA polymerase was used with 100 μCi of [α-32P]UTP (800 Ci/mmol) for in vitro transcription. A 32P-labeled antisense RNA probe was hybridized with 10 μg of total RNA in an 80% formamide/piperazine-N,N-bis(2-ethanesulfonic acid) buffer (pH 6.4) at 51°C for 16 h, and single-strand RNA was digested with RNase A and T1 (Boehringer-Mannheim Biochemicals, Indianapolis, IN). The PGM vector containing ß-actin cDNA was linearized with EcoRI, and SP6 RNA polymerase was used to make an antisense actin riboprobe used for control experiments. Protected RNA was resolved in 8% acrylamide/7 M urea gels. Gels were dried and exposed to Kodak X-AR film.

Northern Blot and RNA-PCR Analysis. Total RNA was isolated from the cell lines using guanidinium isothiocyanate (17). Twenty μg of total RNA was size-fractionated in a 1.4% denaturing formaldehyde gel in morpholinoopropanesulfonic acid buffer. The RNA was transferred onto a GeneScreen membrane (NEN; DuPont), and the membrane was hybridized with the 32P-labeled 118-base pair sense riboprobe corresponding to exon 7 of the p53 cDNA fragment according to the manufacturer’s instructions. Genomic or cDNA sequences were amplified by PCR as described elsewhere (18).

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The abbreviations used are: SSCP, single-strand conformation polymorphism; PCR, polymerase chain reaction; cDNA, complementary DNA.

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Western Blot Analysis of p53 Protein. Cells were directly solubilized in Laemmli sample buffer without β-mercaptoethanol. Solubilized cells were boiled at 100°C for 5 min and spun in a microfuge for 15 min at 12,000 rpm. The supernatant was collected, and its protein content was measured by BCA kit (Pierce Chemical Co., Rockford, IL). Equal amounts of each protein were taken, and β-mercaptoethanol was added to a final concentration of 5%. Samples were again boiled for 2 min and separated on 12.5% polyacrylamide/ sodium dodecyl sulfate gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane using a semidry transfer apparatus (Hopper, taken, and β-mercaptoethanol was added to a final concentration of 5%. PLEXES were detected by an ECL Kit (Amersham Corp., Arlington Heights, IL). Blocking solution containing 0.3% Tween 20 and 5% nonfat dry milk in phosphate-buffered saline overnight at 4°C and probed with p53 monoclonal antibody PAB 1801 (Clonetech Laboratory, Inc., Palo Alto, CA). Immunocomplexes were detected by an ECL Kit (Amerham Corp., Arlington Heights, IL). After appropriate exposure to X-ray film, the blots were used to remove the first antibody and were reprobed with anti monoclonal antibody to ensure equal loading of the protein in all lanes.

Growth Rate Analysis and Tumorigenicity in nu/nu Mice. Cells (10⁶) were plated on 60-mm culture dishes. The cultures were replenished with fresh medium every 48 h, and cells were counted daily for 7 days. For each day cell count, cells were trypsinized and counted on hemocytometer. The data are presented as the mean of triplicate samples. The H322a and WTH226b cells and their p53-transfected clones were each injected s.c. into five replicate nude mice. Each mouse received 10⁶ cells, which were injected into the shoulder. Untransfected cells and those transfected with the unaltered vector alone, which served as controls, were also injected into five mice each. The mice were then monitored for tumor appearance and growth; the tumors that appeared were measured externally in cross-sectional diameter every 5 days for 35 days. Tumor volumes were calculated as described previously (18).

RESULTS

The sequence of the 1.2-kilobase p53 wild-type cDNA and the p53 coding regions in all cell lines was confirmed by the dideoxy chain termination method. The complete p53 cDNA coding the p53 protein was amplified by reverse transcriptase/PCR followed by subcloning of the PCR product into a BlueScript vector according to the strategy shown in Fig. 1A. In addition, to eliminate the possibility of errors by Taq polymerase, we have analyzed exons 5 to 8 of each cell line and doubly confirmed the presence of the mutation (codon 248 mutation was amplified by reverse transcriptase/PCR followed by subcloning of the PCR product into a BlueScript vector according to the strategy shown in Fig. 1A. In addition, to eliminate the possibility of errors by Taq polymerase, we have analyzed exons 5 to 8 of each cell line and doubly confirmed the presence of the mutation (codon 248 mutation in H322a cell line; CGG > CTG, Arg to Leu) by SSCP analysis and sequencing of the individual exon DNA from the two independent PCR products (Fig. 1B and D). WTH226b cells have a homozygous wild-type p53. Two β-actin expression vectors containing the human 2.3-kilobase full-length p53 cDNA with 5' and 3' untranslated sequences in either sense or antisense orientation were constructed (Fig. 1C). The plasmid DNA constructs were electroporated into H322a and WTH226b cells to generate neo-resistant colonies. All G418-resistant clones transfected with sense or antisense p53 were analyzed for the presence of exogenous p53. Total genomic DNA from the transfected cells was used to amplify a 234-base pair cDNA fragment carrying exons 6 and 7 to show the presence of exogenous p53 cDNA (Fig. 2). WTH226b clones showing integration of both sense and antisense p53 were recovered. H322a antisense clones were recovered, but we could not recover any H322a sense clones. This is consistent with earlier reports of similar transfection experiments indicating that after transfection of exogenous wild-type p53 into cells with a mutant p53 there is a selection preference against recovery of cells expressing wild-type p53 (19). We obtained equal numbers of G418 resistant clones of H358 cells (deleted p53) transfected with either vector alone or antisense p53 constructs indicating that the antisense p53 did not influence the growth of cells with a homoygous p53 deletion as measured by colony formation (data not shown).

The unusual growth pattern of the H322a antisense-transfected cells prompted us to examine the expression of exogenous sense and antisense p53 RNA in selected transfectants. Expression of antisense RNA was assessed by Northern blot analysis (Fig. 3A). A 118-base pair sense RNA probe synthesized in vitro from a BlueScript vector was used as a probe for detection of the antisense p53 message. A 6-kilobase RNA species expressing representation of a segment of the 4.8-kilobase β-actin promoter sequence linked to the 2.3-kilobase p53 cDNA was observed in the WTH226b antisense clone but was undetectable in the sense clones or the parental cells. The probe also hybridized nonspecifically with 28S rRNA, which served as an internal control for equal RNA loading. Accumulation of antisense RNA
resulted in a significant decrease in the total cellular p53 RNA. Total RNA from the cell line clones expressing either sense or antisense RNA was analyzed by an RNase protection assay using an antisense 118-base pair exon 7 RNA probe (Fig. 3B). As expected, we observed a reduction in cellular p53 RNA in the clones expressing antisense RNA and an increase in the total p53 message in the sense clones. The same amount of RNA was used with an actin probe as an internal control (Fig. 3C). To determine whether the increase in p53 mRNA in the sense clones was due to mRNA synthesized from the vector construct, we used a reverse transcriptase/PCR assay since our p53 cDNA had a molecular weight similar to that of the endogenous p53 that we could not differentiate by Northern blot analysis. We synthesized a 20-base pair amplimer (GCA GGA TCA GTC GAC CTG CA) corresponding to the β-actin promoter sequence (Applied Biosystems, Foster City, CA). Total RNA from four WTH226b sense clones was reverse transcribed, and the resulting cDNA was used to amplify a part of the exogenous p53 RNA using 5' β-actin vector primer and 3' p53 exon 3 cDNA primers (3R). A 670-base pair amplified cDNA fragment was detected that corresponded to the predicted size of the amplified product containing the 5' untranslated region of the exogenous p53 cDNA (Fig. 4A). An equal amount of cDNA was used to amplify total p53 message, using a 5' primer which includes the p53 ATG start site and a 3' 3R exon 3 primer. A 167-base pair cDNA fragment that corresponds to both the exogenous and endogenous p53 RNA populations was also amplified (Fig. 4B). The WTH226b sense clones analyzed for exogenous p53 showed a level of expression comparable to that in wild type and was consistent in several stable clones. In all cases, the level of the endogenous p53 RNA was higher than that of the RNA synthesized from our construct. We observed a moderate increase in the wild-type p53 RNA in the sense clones. The exogenous complete cDNA was retrieved from the WTH226b transfectants by reverse transcriptase PCR and subcloned into the BlueScript vector and sequenced as shown in Fig. 1A. Results indicated that clones were carrying wild-type exogenous p53 cDNA.

We did Western blot analysis to examine the quantitative differences in the p53 protein content in the different clones (Fig. 5A). Antisense clones of both cell lines showed very low levels of p53 protein. The expression of wild-type p53 level is in general low in normal cells but the amount of the protein varies from one cell type to another. We have overexposed the film to detect the very low level of the endogenous p53 protein present in the antisense clones. The blot was reprobed with an anti-actin antibody (Fig. 5B) to show equal protein loaded in each lane. Since the amount of the protein present in the H322a C4 clone was comparable to that associated with wild-type p53, we sequenced the genomic p53 exon 7 DNA to determine

Fig. 3. Expression of antisense (AS) and sense (S) RNA in the control and transfected cell lines were examined. A, expression of p53 antisense RNA was detected by Northern blot analysis. Total RNA was size fractionated in 1.4% agarose/formaldehyde/morpholinopropanesulfonic acid gel and blotted onto GeneScreen membrane (NE). RNA was hybridized with antisense p53 exon 7 riboprobe. Lane 1, H226b; Lane 2, H226b C5-AS; Lane 3, H226 C12-S. Arrow, position of antisense p53 RNA. Lane 4, H322a C4-AS; Lane 5, H226h C6-AS; Lane 6, H358. In C, the same RNA samples were used for RNase protection assay. 

Fig. 4. Reverse transcriptase/PCR analysis showing exogenous p53 expression in WTH226b transfectants. (A) cDNA was made from total RNA using p53 exon 3 reverse primer (3R as shown in Fig. 1A). An equal amount of cDNA was amplified by PCR using 5' vector primer and 3' 3R p53 cDNA primer to show the level of exogenous p53 expression. Four sense (S) WTH226b clones (a, C2-S; b, C5-S; c, C9-S; and d, C12-S) show 670-base pair (bp) reverse transcriptase/PCR-amplified p53 DNA; in B, the same cDNA product was used to amplify a 167-base pair p53 fragment using 5' 1F and 3' 3R primers to demonstrate total level of both exogenous and endogenous p53 expression. 

Fig. 5. Western blot analysis of p53 protein in H322a and H226b cell lines and their transfectants. Ten μg of the total protein were run on 12.5% polyacrylamide/SDS gel and transferred onto nitrocellulose membrane. A, p53 monoclonal antibody PAB 1801 was used to probe the membrane (in which S is sense and AS is antisense); Lane 1, H322a; Lane 2, H322a C4-AS; Lane 3, H226b; Lane 4, H226b C5-AS; Lane 5, H226b C6-AS; Lane 6, H226b C2-S; Lane 7, H226b C9-S; Lane 8, H226b C12-S; in B, the same blot was reprobed with actin monoclonal antibody to ensure equal protein loading.
whether the mutation was still present. The genomic DNA of the
H322a C4 clone was isolated, and the endogenous p53 exon 7 carry-
ing the 248-codon mutation was selectively amplified using intron-
specific primers, subcloned into BlueScript vector and sequenced.
Results indicated that the H322a C4 antisense clone was still carrying
the codon 248 mutation.

We examined the growth properties of the transfected cell lines. The
antisense clones had a significant growth advantage over their parental
control cells (Fig. 6). The increased proliferation rate of WTH226b
antisense cells could be explained by inhibition of the growth suppressor function of the wild-type p53. Surprisingly, we found that
H322a cells transfected with the antisense construct also had a faster
rate of proliferation than the untransfected or mock-transfected H322a
cells. We hypothesized that the similarity of effects of the antisense
construct on growth of the H322a and WTH226b cells indicates that
the mutation at codon 248 did not abolish the growth suppressor
function of the p53 gene in H322a cells. H226b cells transfected with
wild-type p53 cDNA (sense clones) showed a marginal decrease in
their growth rate. At this moment, we do not know the threshold
amount of exogenous wild-type p53 required for significant growth
reduction. Moreover, we could not detect a higher level of p53 protein
in our sense clones either because of the short half-life of the protein
or alternatively, or because the wild-type p53 protein could regulate its
expression as reported earlier (20), thereby keeping a steady state
level of the protein in the cell without cell growth. In our retroviral
mediated p53 gene transfer studies we have noticed that expression of
the wild-type p53 gene in H358 (p53 negative) cell line caused sig-
nificant growth inhibition (20). Thus, the p53 protein can have dif-
erential effect on cell growth depending on the genetic composition
of the cells.

We injected these cell lines s.c. into nu/nu mice and looked for
tumor development. The experiment was repeated three times using
five animals in each group with similar results. The parental cell lines
were not tumorigenic within the time period measured, but the anti-
sense clones readily formed tumors in 2 weeks (Fig. 7).

DISCUSSION

Previous studies have shown that p53 mutants vary in the extent of
their loss of wild-type function. For example, mutants may differ in
their ability to cooperate in a ras transformation assay, in protein
binding, and loss of transcriptional activity (4, 10, 11, 21). Although
previous reports have shown retention of some wild-type character-
istics by various p53 mutants, we believe this is the first report of
retention of tumor suppressor function by a mutant.

Interestingly, a mouse codon 245 mutation (Arg to Trp), which
corresponds to the human codon 248 mutation, retains some wild-type
functions (10, 22). This mutant retained trans-activating function in a
GAL4 fusion assay but could not trans-activate the muscle creatinine
kinase promoter. However, this mutant retains many wild-type func-
tions such as formation of oligomers, localization to the nucleus,
retention of wild-type conformation as measured by antibody binding,
and retention of the ability to bind SV40 but not hsc70 proteins. Direct
extrapolation of these results to the human mutant is problematic because the primary sequence of p53 differs between mouse and human.

Another study showed that a codon 248 mutation (Arg to Trp) which occurs in germ-line p53 mutations did not suppress colony formation in cells with a homozygous p53 deletion (23). Our study differs in several significant aspects compared to the this study. The mutation in our study was a G to T transversion occurring in the second base compared to a C to T transition in the third base for the Li-Fraumeni mutation. The type and location of amino acid substitutions in the p53 protein can result in distinct local "informational changes" in the protein (24). For example, in mouse cell lines, the temperature-sensitive mutation at codon 135 of the p53 gene encodes a protein which adopts a wild-type function at 32°C and blocks cells at a specific stage of the cell cycle, while at 37°C the protein displays a mutant form which does not stop cell division (25). Furthermore, the introduction of p53 into a cell that has already lost p53 function may differ from a situation in which expression of endogenous mutant p53 is reduced. Different cell types could also vary in their response to a reduction in endogenous p53 protein expression.

Does this mutation contribute to the malignant phenotype of the cell even though it retains some tumor suppressor function? High levels of the mutant protein are expressed in H322a cells suggesting that the codon 248 mutation stabilizes the p53 protein and increases its half-life in the cell. It is possible that high levels of the mutant protein may mediate transcription of some additional proteins at levels higher than normal. These as yet unidentified proteins may contribute to transformation. Preliminary studies show that this mutation retains transactivating function, subsequent genetic events, such as deletions or additional mutations, that further reduce the expression of mutant proteins that retain wild-type function could enhance characteristics of the malignant phenotype of the cell by eliminating residual wild-type functions.

This study has important implications for therapies that are based on modulation of p53 expression. Introduction of the wild-type p53 into lung cancer cells consistently reduces proliferation and tumorigenicity (19, 26). In some systems p53 can function as a dominant transforming gene by cooperating with an activated ras gene (27). Thus, one might expect reduction of mutant p53 expression to reduce proliferation and tumorigenicity. Our result indicates that this does not always occur. The functional status of mutant p53 proteins will need to be considered in applying antisense strategies to the therapy of cancer.

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