Altered Structure and Expression of the p53 Gene in Human Neuroepithelial Tumors

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Abstract

The p53 gene, located on chromosome 17p13.1, may be important in the pathogenesis of human neuroepithelial tumors, because it is a tumor suppressor gene and genetic alteration is essential for certain human cells to acquire the neoplastic phenotype. The structure and expression of the p53 gene were investigated in cultured human glioma cells and biopsied specimens of neuroepithelial tumors. Immunocytochemical examination of p53 gene expression revealed positive nuclear staining in six of seven glioma cell lines tested. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis demonstrated unequivocal heterogeneity of migration rate in p53 bands. Pulse-chase analysis clearly showed an increased half-life of p53 in cultured human glioma cells. These abnormalities are presumably due to genetic alterations in the p53 gene. Nucleotide substitutions in exon 5, 7, or 8 of the p53 gene could be detected by polymerase chain reaction-single strand conformational polymorphic analysis in four of seven (57%) human glioma cell lines, and nine of 29 (31%) biopsied specimens of neuroepithelial tumors examined. The present results indicate that genetic alterations in the p53 gene are responsible for the tumorigenesis of at least some human neuroepithelial tumors.

Key words: glioma, medulloblastoma, p53, tumor suppressor gene, genetic alteration

Introduction

Several genetic alterations are associated with the development of certain human gliomas.^{13,18,19,22,23,39,47,50} The common alterations are deletions on the short arm of chromosome 17 (17p13.1) and the long arm of chromosome 10. Allelic deletions of chromosome 17p occur in a wide variety of human neoplasms^{1,8,15,36,46} such as colon, breast, and lung cancers, strongly suggesting that anti-oncogenes (tumor suppressor genes) on 17p are involved in the neoplastic process of tumors, and probably in some human glial tumors.^{13,19,23,39}

The p53 gene, a postulated tumor suppressor gene, is located on chromosome 17p and codes for a cellular 53-kd phosphoprotein (p53) now believed to be essential in the regulation of cell division, as it is required for the transition from phase G₀ to G₁ of the cell cycle.^{9,21,25,26,32} The p53 was first identified through its interaction with the large T antigen of Simian virus 40, and was thought to be a dominant oncogene enabling full transformation of ver-

tebrate somatic cell in combination with an activated *ras* gene.³⁸ However, it has recently been shown that the p53 in the neoplastic cells is all mutant and the wild-type p53 suppresses transformation of normal cells.^{2,14} The p53 may therefore act as a tumor suppressor, like the product of the retinoblastoma susceptibility gene. The p53 gene is possibly responsible for the development of human neuroepithelial tumors.

Here, we report our investigation of the structure and expression of the p53 gene in cultured human glioma cells and biopsied specimens of neuroepithelial tumors.

Materials and Methods

I. Cell lines and tumor specimens

Seven established human glioma cell lines (U-251MG, U-373MG, T98G, KMG4, A172, U-118MG, and U-87MG) were used in this study. U-251MG, U-373MG, T98G, U-118MG, and U-87MG were obtained from the American Type Culture Collection (Rockville, Md., U.S.A.), and A172 from the Japan Cancer Research Bank (Tokyo). KMG4 was kindly

donated by Dr. Y. Ushio (Kumamoto University Medical School, Kumamoto). All glioma cells were grown in Modified Eagle's Medium (MEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C in a humidified 5% CO₂ atmosphere.

The 29 neuroepithelial brain tumor tissues obtained at craniotomy were fixed with neutral formalin and embedded in paraffin. The histological types of the specimens were eight astrocytomas, 16 glioblastomas, and five medulloblastomas.

II. Immunocytochemistry

Subconfluent monolayers of cultured cells were fixed in absolute methanol and air dried. Immunological investigation used mouse anti-p53 monoclonal antibody (MoAb) (ab-2; Oncogene Sci. Inc., Manhasset, N.Y., U.S.A.). Mouse anti-rabbit immunoglobulin G MoAb (Sigma Chemical Co., St. Louis, Mo., U.S.A.) was also used as a negative staining control. Antibody binding was visualized by the streptavidin-biotin immunoperoxidase method using a commercial kit (DAKO-Japan, Kyoto).

III. Immunoprecipitation and gel electrophoresis

For metabolic labeling, subconfluent cells were suspended in 2 ml of methionine-free MEM supplemented with 10% dialyzed FBS. ³⁵S-methionine (250 μCi) was added and the suspension incubated at 37°C for 3 hours. Radiolabeled cells were lysed in buffer containing 150 mM NaCl, 50 mM Tris (pH 7.5), 1% NP-40, 1% sodium deoxycholate, and 1 mM phenyl methyl sulfonyl fluoride. The cell lysates were incubated with mouse anti-p53 MoAb (ab-1; Oncogene Sci. Inc.) at 4°C overnight with constant rotation. Packed protein A sepharose (50 μl; Pharmacia, Uppsala, Sweden) was added and the lysate kept at 4°C for 30 minutes with rotation. The immune complex was resuspended in protein sample buffer, heated at 70°C for 10 minutes, and then applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Two-dimensional PAGE (2D-PAGE) subjected the immunoprecipitates to isoelectric focusing electrophoresis followed by SDS-PAGE.

IV. Pulse-chase analysis

Plastic culture plates (10 cm in diameter) containing equivalent numbers of cells from different cell lines were labeled with ³⁵S-methionine (250 μCi) for 90 minutes. The radioactive medium was then replaced with medium containing non-radioactive methionine, and the label was chased for the indicated period. At the end of the specific chase

period, the cells were processed as described above.

V. Deoxyribonucleic acid (DNA) preparation and polymerase chain reaction (PCR) amplification

High-molecular weight DNA was extracted from the 1% NP-40 treated nuclear fraction of each cell culture. About 10⁸ cultured cells were collected and treated with 7.5 ml of buffer containing 5 mM NaCl, 5 mM Tris-HCl (pH 7.5), and 0.5 mM MgCl₂ for 3 minutes on ice. Ten percent NP-40 was added to 1/10 volume and gently mixed. Immediately the sample was centrifuged at 3500 rpm for 1 minute, and the pellet dissolved in 10 ml of proteinase K solution [100 μg/ml proteinase K, 0.2% SDS, 10 mM Tris-HCl (pH 7.5), and 10 mM ethylenediaminetetraacetic acid (EDTA)], and incubated at 25°C for 24 hours. The solution was extracted with an equal

Fig. 1 Scheme of the p53 gene structure and conserved domains. Solid boxes indicate conserved domains, where nucleotide sequences are homologous throughout the species. These domains are supposedly mutational hot regions in a variety of tumors. Three pairs of alleles represent the PCR primers used to amplify the DNA fragments containing the conserved domains. The nucleotide sequences are as follows: p5A, 5'TTCGAATTCCTGCAGTACTCCCCTG; p5B, 5'TAGGATCCGCCCCAGCTGCTCACC; p7A, 5'GTTGAATTCTAGGTGGCTCTGAC; p7B, 5'CAAGTGGATCCTGACCTGGAGTCTTC; p8A, 5'CGGAATTACCTATCCTGAGTAGTGG; and p8B, 5'ATGGATCCTGCTTGCTTACCTCGC. a.a: amino acid, mRNA: messenger ribonucleic acid, ORF: open reading frame.

volume of phenol, followed by extraction with an equal volume of chloroform/isoamylalcohol (24:1). Two volumes of ethanol and final 150 mM concentration of NaCl were added. The high-molecular weight DNA was isolated and dissolved in 1 ml of 10 mM Tris-HCl (pH 7.5) and 10 mM EDTA.

For biopsied specimens, three slices of 10 μ m thick section were cut and deparaffinized with 3 $\times$ 300 μ l xylene washes. The samples were rinsed three times with 300 μ l of 70% ethanol, and the centrifuged pellet was dissolved in 300 μ l proteinase K solution (100 μ g/ml) and incubated for 24–48 hours. DNA was extracted and precipitated as described above. Nearly 5 μ g of DNA was obtained by this procedure. Extracted DNA was utilized for the PCR consisting of 40 cycles of 94°C (1 min), 62°C (1 min), and 72°C (1 min).⁴⁰⁾ The six oligonucleotides were synthesized as PCR primer according to the nucleotide sequences of the introns just anterior and posterior to exon 5, 7, and 8 of the human p53 gene, which are known to be highly conserved domains throughout the species: p5A, p5B, p7A, p7B, p8A, and p8B (Fig. 1). The PCR reagents were 1.5 mM MgCl₂, 10 mM Tris-HCl, 50 mM KCl, 200 μ M each of deoxy-adenosine triphosphate (ATP), guanosine 5'-triphosphate, cytidine triphosphate, and thymidine 5'-triphosphate, 100 pM of γ -³²P-deoxy-ATP-labeled primers, and 0.25 U Taq polymerase (Promega Corp., Madison, Wis., U.S.A.).

VI. PCR-single strand conformational polymorphic (SSCP) analysis

The SSCP analysis of PCR products was carried

out as follows. Each PCR product was diluted 100 fold with 95% formamide dye and heated at 95°C for 3 minutes to denature the DNA, and then 2 μ l of sample was loaded on 6% neutral polyacrylamide gel (40 $\times$ 20 $\times$ 0.03 cm). Electrophoresis was carried out at 4°C under 40 W constant power. The gel was transferred to a 0.33 mm thick cellulose paper (Whatman Ltd., Maidstone, England) and dried, and then autoradiography performed overnight.³⁷⁾

Results

I. Immunocytochemistry

Table 1 shows that human glioma cell lines revealed a variety of immunostaining reactions to p53. U-87MG cells showed equivocal nuclear staining for p53, while U-118MG, KMG4, and A172 cells were weakly positive. U-251MG, U-373MG, and T98G

Table 1 Altered expression of p53 in human glioma cell lines

Cell line	p53 expression*	p53 half-life
U-87MG	+	ND
T98G	+++	prolonged
U-118MG	++	ND
A172	++	prolonged
U-251MG	+++	prolonged
U-373MG	+++	prolonged
KMG4	++	ND

*Immunocytochemical positivity: +, 5–49%; ++, 50–89%; +++, 90–100%. ND: not determined.

Fig. 2 Immunocytochemical localization of p53 in cultured U-251MG (left), U-373MG (center), and T98G (right) cells. Immunoperoxidase method without counterstain, $\times$ 400.

Fig. 3 SDS-PAGE analysis of p53 immunoprecipitates from human glioma cell lines. p53 is detected in all glioma cell lines except for U-87MG. The positivity for p53 in U-87MG is equivocal, but a faint band is discernible with longer exposure. There is evident heterogeneity in the migration rates of p53 in a slab gel (arrows). Note A172 cells showing doublet bands.

cells displayed intensely positive nuclear staining for p53 (Fig. 2). The cytoplasm of cultured human glioma cell appeared negative.

II. Immunoprecipitation and gel electrophoresis

The SDS-PAGE analysis detected p53 in all human glioma cell lines except for U-87MG. The migration rates of p53 were slightly different between cell lines (Fig. 3). U-251MG and U-373MG showed a single band for p53, which migrated faster than those of T98G and U-118MG. A172 in particular revealed doublet bands for p53. The findings indicate that p53 expressed in these human glioma cells has heterogeneous molecular conformation and antigenic activity. Further evidence was obtained from 2D-PAGE analysis (Fig. 4). Several spots, all of the same molecular weight, were identified as p53. With A172 cells, there were six different spots for p53, consisting of three in the fast and three in the slow components, suggesting different isoelectric points.

Fig. 4 2D-PAGE analysis of p53 immunoprecipitates from U-251MG (upper), T98G (middle), and A172 (lower) cell lines. Multiple spots for p53 occur with different isoelectric points (arrows), but the same molecular weight. A172 reveals six different spots for p53, consisting of three in the fast and three in the slow migration components. The isoelectric points of these spots are different.

Fig. 5 Pulse-chase analysis of p53 in A172 (left) and T98G (right) cells. Both p53 doublet bands of A172 (arrows) and the single band of T98G continue to maintain radioactivity even after 60 minutes of chase, indicating the prolonged half-life of p53 in these cells.

III. Pulse-chase analysis

The pulse-chase analysis was performed to determine whether the intense expression of p53 in glioma

cells is due to its increased half-life. Figure 5 shows both p53 doublet bands of A172 cells and the single band of T98G cells maintained radioactivity even after 60 minutes, suggesting marked prolongation of the half-life of p53 in these cells.

IV. PCR-SSCP analysis

Regions of the human p53 gene (Fig. 1) were selectively amplified by PCR using each pair of primers. As shown in Fig. 6, mobility shifts of the DNA fragments, which reflected the conformational changes of the p53 gene secondary to nucleotide substitutions or deletions, were observed in exon 5

Fig. 6 PCR-SSCP analysis of conserved domains of the p53 gene in human glioma cell lines. Each lane consists of two bands, which are complementary single strands of DNA fragments. Mobility shift is considered due to nucleotide substitution or deletion. The p53 gene displays altered mobility bands for KMG4 in exon 5 (*upper*), T98G in exon 7 (*middle*), and U-251MG and U-373MG in exon 8 (*lower*).

Fig. 7 PCR-SSCP analysis of the biopsied specimens. *upper*: For exon 7 of the p53 gene, NS067, a case of glioblastoma, shows aberrant bands due to nucleotide substitution or deletion. *lower*: For exon 8 of the p53 gene, NS083, a case of medulloblastoma, also reveals abnormal bands indicating mutation of the p53 gene.

Table 2 Alteration in the p53 gene detected by PCR-SSCP analysis

Histology	No. of cases tested	No. of cases showing mutation
Astrocytoma	8	3 (38%)
Glioblastoma	16	5 (31%)
Medulloblastoma	5	1 (20%)
Total	29	9 (31%)

from KMG4 cells, in exon 7 from T98G cells, and in exon 8 from both U-251MG and U-373MG cells. The DNA fragments prepared by PCR from the nine formalin-fixed tumor tissues (3 astrocytomas, 5 glioblastomas, and 1 medulloblastoma) also displayed mobility shifts in either exon 7 or 8 of the p53 gene (Fig. 7). However, no altered mobility was observed in other biopsied specimens examined. Table 2 summarizes the results obtained from 29 surgical specimens.

Discussion

This study clearly demonstrated heterogeneities in the conformation and expression of p53 in cultured human glioma cells. Immunocytochemical examination of p53 expression revealed positive nuclear staining in six of seven glioma cell lines. SDS-PAGE analysis showed unequivocal heterogeneity in the migration rate of p53 bands. Previous studies^{31,48)} indicate that the mutant forms of p53 may be distinguished from the wild-type p53 by abnormal migration on polyacrylamide gels, and by an increased half-life causing accumulated mutant proteins in the nucleus. Even a single amino acid change in p53 may cause an abnormal migration on SDS-PAGE or prolongation of half-life.⁶⁾ Indeed, elevated levels and prolonged half-life of p53 frequently occur in transformed cell lines.^{10,30)} The half-life of the wild-type p53 is 6–30 minutes.¹⁷⁾ The pulse-chase analysis clearly revealed the increased half-life of p53 expressed in both A172 and T98G cells, presumably due to genetic alterations in the p53 gene. Nucleotide substitutions or deletions in exon 5, 7, or 8 of the p53 gene were successfully detected by PCR-SSCP analysis in four of seven (57%) glioma cell lines. Substitutions of amino acids in the conserved domains of p53 may result in loss of function and prolongation of half-life, or impairment of the autoregulation of p53. The present study, of course, cannot exclude the presence of subtle alterations in other coding or promoter regions of the p53 gene in the other three cell lines.

Multiple subspecies of p53 with different isoelectric points were observed by 2D-PAGE, though little is known about their structural properties or behavioral activities. However, differential phosphorylation of p53 is most likely since a recent study has shown that three serine residues of human p53 are phosphorylated at the 309, 315, and 386 codons. Interestingly, serine-315 is phosphorylated by p60-cdc2 and cyclin B-cdc2 complex,⁴⁾ and this is considered essential for controlling the cell cycle. In the present study, the electrophoretic patterns of p53 were similar for wild and mutant types, indicating that the observed mutations in the conserved domains do not affect the phosphorylation of p53.

Biological potential differs fundamentally between *in vivo* and *in vitro* populations of glioma cells. Therefore, we cannot conclude whether the altered structure and expression of the p53 gene are primary characters of gliomas or a secondary sequence in established glioma cell lines. However, we did detect mutations of the p53 gene in the biopsied specimens of various neuroepithelial tumors: three of eight

(38%) astrocytomas, five of 16 (31%) glioblastomas, and one of five (20%) medulloblastomas. These results strongly suggest that mutations of the p53 gene may be closely related to the tumorigenesis in one third of human neuroepithelial tumors. In contrast, medulloblastoma infrequently displays p53 gene mutation.⁴¹⁾

The role of tumor suppressor gene in the oncogenesis of human glioma has not yet been studied in detail.^{11,21,29)} Recently, allelic deletions of chromosome 17p have been found in certain human astrocytic tumors^{13,19,22,23,50)} and in other tumors^{5,12,20,42,43)} such as lung and colorectal cancers. Genetic analyses of these tumors have demonstrated deletions or mutations of the p53 gene located on chromosome 17p. Although the function of p53 in non-transformed cells is not yet well understood, it is generally believed that the wild-type p53, like the retinoblastoma gene product, acts as a tumor suppressor probably by negative regulation of cell division.^{2,7,14,16,34)} Gene transfection studies showed that the wild-type p53 gene inhibited the transforming ability of mutant p53 in rat embryo fibroblasts.^{14,16)} In contrast, microinjected MoAbs against p53 inhibited the transition of Swiss 3T3 cells from G₀ to S phase, but had no effect on the progression of these cells from S phase to mitosis.³³⁾ Therefore, wild-type p53 is probably an important negative regulator of cell cycle, and mutations of the p53 gene result in loss of suppression or co-operate with other oncogenes such as *ras* or *myc* to stimulate neoplastic growth.

Certain oncogenes are frequently activated or amplified in human gliomas.^{3,24,27,45)} We cannot now explain the pathogenic relationship between the altered structure of the p53 gene and the activation of certain oncogenes such as *erbB-1* in glial tumors. Probably most of the activation or amplification of oncogenes in glioma are due to a secondary event associated with the neoplastic transformation of glial cells. Recently, germ-line mutations of the p53 gene have been shown to underlie the Li-Fraumeni syndrome which predisposes the development of brain tumor and leukemia, in addition to breast cancer, sarcoma, and other malignancies.^{28,44,49)} In fact, mutations of the p53 gene appear to be the most common genetic alterations in human cancers. For example, an interesting case of ependymoma was associated with a germ-line mutation of the p53 gene,³⁵⁾ suggesting that the p53 mutations predispose intracranial malignancy. However, further investigation including locating the mutations or deletions within the p53 gene of glioma tissues are necessary to define the causal relationship between the alterations in the p53 gene and transformation of glial cells to

the neoplastic phenotype.

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