Initiation of Human Astrocytoma by Clonal Evolution of Cells with Progressive Loss of p53 Functions in a Patient with a 283H TP53 Germ-line Mutation: Evidence for a Precursor Lesion

Giulia Fulci, Nobuaki Ishii, Daniela Maurici, Kim M. Gernert, Pierre Hainaut, Balveen Kaur, and Erwin G. Van Meir

Laboratory of Molecular Neuro-Oncology, Department of Neurosurgery, Hematology/Oncology and Winship Cancer Institute [G. F., B. K., E. G. V. M.] and BIMCORE [K. M. G.], Emory University School of Medicine, Atlanta, Georgia 30322; Laboratory of Tumor Biology and Genetics, 1011 Lusanne, Switzerland [G. F.; N. I., E. G. V. M.]; and Mechanisms of the Carcinogenesis Unit, IARC, 69372 Lyon, cedex 08, France [D. M., P. H.]

ABSTRACT

Little is known about the genetic and molecular events leading to the early stages of human astrocytoma formation. To examine this issue, we analyzed the significance of sequential accumulation of two somatic point mutations (R267W and E258D) in the TP53 gene during the initiation of astrocytoma in a patient born with a single germ-line p53 point mutation (R283H). We adapted a p53 transcriptional assay in yeast to establish the temporal occurrence and allelic distribution of the p53 mutations present in the patient and characterized these mutations through functional assays and structural modeling.

Our results show that the first somatic mutation occurred at codon 267 on the p53 allele harboring the germ-line mutation R283H, whereas the second somatic mutation occurred in the remaining wild-type (wt) allele at codon 258. These two mutations induced the formation of tumor cells with the genotype p53R267W/283HWT, which comprised 70% of the cells in the primary WHO grade II astrocytoma. Another 8% of cells within the tumor had the partially mutated genotype p53R267W/283HWT and represented the remnants of a clinically undetectable intermediate stage of astrocytic neoplastic transformation. The remaining 22% of cells had the constitutive p53R283H/WT genotype and likely consisted of nontumor cells. Functional analysis of the p53 alleles present in the patient’s tumor indicated that the germ-line p53R283H could transactivate the CDKN1A(p21)/BAX gene and retained the ability to induce growth arrest of human glioblastoma cells. The p53R267W/283H and p53R267W/283HWT were incapable of transactivating either promoter or inducing growth arrest. Modeling of p53 interaction with DNA suggests that R283H mutation may weaken the sequence-specific interaction of p53 with the BAX gene but not the CDKN1A p53-responsive elements.

Taken together, these results have characterized, for the first time, the genetic events defining a clinically undetectable precursor lesion leading to a grade II astrocytoma. They also suggest that astrocytoma initiation in this patient resulted from monoclonal evolution driven by a sequential loss of proapoptotic and growth arrest functions of p53.

INTRODUCTION

The early events of human tumorigenesis are poorly understood. The relevance of the accumulation of point mutations in single tumor suppressor genes in this process has not been examined. Tumor formation is driven by clonal expansion of cells containing genetic alterations (1). Analysis of these mutations provides important clues in cancer etiology. Whereas most missense point mutations found in tumor suppressor genes result in inactive proteins, some lead to only a partial loss of function. We hypothesized that the latter might allow us to identify the importance, at different stages of tumorigenesis, of distinct functions mediated or controlled by tumor suppressors. Understanding when functions such as loss of apoptosis induction or cell cycle control may occur is important to comprehend the mechanisms that start tumor formation, to help define new stage-specific targets for therapy, and to define the appropriate available treatment at a given progression stage.

Diffusely infiltrating astrocytomas progress from low-grade (WHO grade II) lesions to grade III anaplastic astrocytoma and grade IV glioblastoma (2). No lesion preceding grade II astrocytoma has been identified to date, and the sequence of molecular alterations leading to this tumor are unknown. Although the brain does not allow for easy examination of preneoplastic lesions (such as adenomas in the colon), the presence of clinical symptoms before brain tumor detection suggests that such lesions might exist. Recent studies on animal models for gliomas have identified hyperplastic regions in the brain before tumor establishment (3, 4). We hypothesized that remnants of such precursor lesions might still exist in some low-grade tumors. The finding of a minor cell population in a grade II tumor that contained only part of the genetic changes present in the majority population of the tumor would support the existence of such lesions in humans.

The involvement of p53 mutation in clonal expansion of tumor cells was shown previously (5–9), and this process occurs early in astrocytomas (10, 11). The importance of p53 in tumor suppression is explained by its multiple functions, including induction of apoptosis, growth arrest, and regulation of angiogenesis (Refs. 10 and 12–14 and the references therein). p53 accomplishes most of its functions acting as a transcription factor. It transactivates the cell cycle inhibitor gene CDKN1A and proapoptotic genes, including BAX, p53AIP1, and PUMA (reviewed in Refs. 12 and 13). In most tumors, these functions are lost through deletion of one allele (LOH) and loss of function of the gene product of the other allele via a missense point mutation. However, in some tumors, more than one mutation is present in one TP53 allele. Some missense mutations only partially disrupt the functions of p53, preventing transactivation of some p53 targets (15–21).

We assumed that tumor initiation and early progression might require loss of different functions of p53. As a result, a partial loss of p53 function might predispose a cell to preneoplastic and early neoplastic development, and additional events eliminating the remaining p53 functions would be required for further progression. To examine this issue, we first analyzed the distribution of TP53 mutations in...
tumors with single and multiple TP53 alterations using the IARC database of somatic TP53 mutations (22). Secondly, we screened the IARC database of germ-line TP53 mutations to identify patients with multiple TP53 alterations in a single allele that would allow us to study progressive p53 inactivation in tumorigenesis.

These analyses first revealed a significant difference between the TP53 somatic mutation spectra derived from tumors with single versus multiple TP53 mutations, including a 48% reduction in frequency of “hot spot” TP53 mutations. We further adapted a p53 transcriptional assay in yeast to determine the order by which multiple p53 mutations can occur in patients. We validated this method by establishing the mutation sequence of three TP53 mutants in a unique patient with a germ-line p53 mutation (R283H) and astrocytoma progression. This genetic analysis also established the existence and distribution of genetically distinct cell populations in the primary tumor; one represents the remnant of a precursor lesion that we called “preastrocytoma.” We further established the transcriptional activity of these mutants toward the proapoptotic gene BAX and the cell cycle-arrest gene CDKN1a. We propose a molecular model explaining why the germ-line p53 mutant 283H cannot transactivate the BAX gene but can activate CDKN1a and induce growth arrest in human glioblastoma cells. Overall, these findings improve our understanding of the molecular mechanisms at the origin of human tumor initiation and progression.

MATERIALS AND METHODS

Statistical Analysis of the IARC Database of TP53 Somatic Mutations.

The tumors reported in the database were split in three groups according to the number of TP53 mutations (single, double, and multiple). The codon distributions in each group were analyzed by pairwise comparison with the asymptotic Kolmogorov-Smirnov two-sample test (SASv8.0 software; SAS Institute, Inc.). Because the codon distributions of mutations in tumors with double and multiple mutations were not significantly different, we combined them for the next analysis. The proportions of nine TP53 mutants were calculated in single versus double/multiple mutant groups with a 95% CI (23).

Patient (41 yrs old, male) amniasis did not reveal a family history of cancer (patient 3; Ref. 9). His brain was irradiated with 5400 cGy after removal of the first tumor, and tumor recurrence occurred 28 months later. The irradiation did not contribute to TP53 mutations because they were already present in the first tumor.

p53 transcriptional assays in yeast were performed as described previously (19, 24). The yeast strains used were YIG397 (MATa ade2-1 leu2-3,112 trpl-1 his3-11,15 can1-100 ura3-1 URA3 3X RGC; pCYCI:ADE2), YPH-p21 (MATa ura3-52 lys2-801 ade2-101 trpl-1 Delta3 his3-D200 leu-200 URA3 p21::pCYCI:ADE2), and YPH-bax (MATa ura3-52 lys2-801 ade2-101 trpl-1 Delta3 his3-D200 leu-200 URA3 p21::pCYCI:ADE2). The pLS76 yeast-expressing vector (CEN6/ARS4, LEU2) containing TP53WT was used as a control. The cDNA coding for each of the mutants TP53267W, TP53283H, TP53273H, TP53283H, and TP53267W+283H was cloned by homologous recombination in pSS16. TP53 cDNA rescue from yeast and sequencing were as described previously (9). Six white and pink and 50 red colonies were sequenced/tumor. This assay underestimates the frequency of white (but not red or pink) colonies by ~3–5% (25). This experimental error is likely because of the introduction of random point mutations in the WT p53 coding sequence during the RT-PCR step, which disrupts p53 transactivation function (24).

Statistical Error for the Sampling of TP53 Alleles in the Tumor Using the p53 TP53 Transcriptional Assay in Yeast. The SE of the frequency of each type of yeast colony (corresponding to one TP53 allele) was calculated with a normal approximation confidence for a binomial proportion (see the Fig. 2C legend).

LOH Analysis at the TP53 Locus. The heterozygosity status of locus 17p13.1 was analyzed as described previously (26) using genomic DNA extracted from patient lymphocytes and tumors.

Construction of the Mammalian Expression Vectors Coding for the Mutants TP53267W, TP53283H, and TP53267W+283H. A plasmid pc53-SNC expressing p53W under a CMV promoter (27) was used for site-directed mutagenesis (QuickChange site-directed mutagenesis kit; Stratagene) using the following primers: (a) 283H-f, 5’-ccttggaacaagacagcacaggaagaa-tct-3’; (b) 283H-r, 5’-gatcctcctctgctgctcctctcagggc-3’; (c) 267W-f, 5’-gtaatcagctggtagcagtgggtgagttc-3’; (d) 267W-r, 5’-cactcaacgctctcactacagatagtac-3’; (e) 283D-f, 5’-catcacagcacgccactcggtaatta-3’; and (f) 283D-r, 5’-gattaccaacgcctcaatgtctgagttg-3’. Mutations were verified by sequencing.

Reporter Gene Luciferase Assays. Transcriptional activity of mutant p53s was tested in glioblastoma cell line LN-Z308, which is p53-null, PTEN mutated, and wt for p14ARF and p16 (25, 28). The cells were transiently cotransfected with each p53 expression vector, a luciferase reporter plasmid driven by p53REs from either the human CDKN1a or BAX genes, and a β-gal expression vector. The transfections were performed in 6-well dishes using 105 cells/well. 5 μg of GenePorter (Gene Therapy Systems), and 1.01 μg of total DNA (10 ng of CMV-p53, 900 ng of p53RE-luciferase, and 100 ng of CMV-LacZ) for each reaction. Subsequently, cells were grown for 48 h at 37°C and lysed in 200 μl of lysis buffer (Tropix) containing 1 mm EDTA, 1 mm EGTA, and 1 mm phenylmethylsulfonil fluoride. Cell extracts (10 μl) were tested for luciferase and β-gal activities using a dual light chemiluminescence assay (Dual Light Kit; Tropix). β-gal activity was used to verify transfection efficiency. The results were normalized to lysate protein concentrations. The levels of transfected p53 were determined by Western blot using anti-WT/mutant p53 antibody D07 diluted 1:1000 (DAKO) and antiamouse IgG horseradish peroxidase (Promega). Equal protein loading was verified by membrane staining with red Ponceau and detection of α-actin (goat polyclonal antibody I-19 (Santa Cruz Biotechnology)) with antihorseradish peroxidase (Roche 605275). The assays were repeated three times in triplicate.

Quantitative Real-time RT-PCR. LN-Z308 and U251MG glioma cells were transfected transiently with expression constructs for wt p53, p53267W, or p53267W+283H using GenePorter as described above. Cells were harvested 48 h after transfection, and total RNA was prepared using Trizol (Life Technologies, Inc.). The primers for CDKN1a (annealing temperature, 55°C) were 5’-GTTCTTTTGTGGACCCGAGC-3’ (sense) and 5’-GGTACACAGCTG-GACAGGTCT-3’ (antisense). Quantitative real-time RT-PCR for CDKN1a mRNA was performed using the Titan One Tube RT-PCR system (Roche Molecular Biochemicals) and SYBR Green 1 (Molecular Probes) on an iCycler (Bio-Rad). The Ct (threshold cycle) was defined as a fractional cycle when the fluorescence generated by the PCR product and SYBR Green 1 crosses a fixed threshold value in each reaction (horizontal orange line).

Theoretical Modeling. The individual mutations in protein and DNA were modeled in the p53WT-DNA crystal structure (Protein Databank file ID, ITSCR; Ref. 29) using Sybyl mutate side chain function (Sybyl 6.5 Tripos, Inc.). This was performed on each mutant to highlight regions of structural instability. Parameters for the minimization included the Tripos force field, Kollman charges, and no water. DNA nucleotide substitutions resulted in the structural instability of C8, A9, and K120 in the p53-BAX structure and A11 and T12 in the p53-CDKN1a structure. The side chain search for possible K120 positions was performed on the mutated yet unminimized structures. However, bp were manually corrected for optimal bp hydrogen bonding and stacking.

Using Sybyl systematic search command, the following searches were calculated: (a) K120/R283-CDKN1a; (b) K120/R283-CDKN1a; (c) K120/R283-BAX; and (d) K120/R283-BAX; with atomic radii at 95% or 85% and an angle rotation of 5 degrees for each χ angle of K120. The number of unique conformations for each search was recorded. No difference was seen between side chain searches including electrostatics calculations and those that did not include electrostatics calculations.

Growth Inhibition Assay. LN-Z308 cells were transfected and selected with 800 μg/ml Geneticin for 10 days in six replicates. Resistant clones were counted 7 days later. The test was repeated twice.

RESULTS

Analysis of p53 Mutation Spectra in Tumors with Single and Multiple p53 Mutations. Screening of the R3 (April 1999) version of the IARC p53 mutations database indicated that 7.9% of 7160 tumors harbored more than one mutation. Some tumors (6.1%) had...
two mutations, either distributed in both alleles or accumulated in a single allele (database does not discriminate). Other tumors (1.8%) had more than two mutations, suggesting that one TP53 allele underwent several mutational events. The spectra of mutations in tumors with double and multiple TP53 mutations could not be distinguished (P = 0.17), but both were significantly different from the distribution of single TP53 mutations (P = 0.013 and P = 0.002, respectively). This included a decreased prevalence of 48% (95% CI, 42.5–54.6%) for the nine most frequent TP53 mutations found in tumors and known to completely abrogate p53 function (Ref. 20; Fig. 1). Consequently, infrequent TP53 mutations are more common in tumors with multiple TP53 mutations than in those with single mutation. Several studies have suggested that infrequent mutations are rare because they induce only partial inactivation of p53 functions (17, 18, 20). These findings are compatible with the hypothesis that, in alleles with double mutations, the initial mutation might have induced partial loss of function and that during tumor progression a second mutation was necessary to fully inactivate p53.

Determination of the Sequence and Timing of Two Somatic TP53 Mutations during Astrocytoma Initiation and Progression in a Patient with Germ-line TP53 Mutation. To dissect the function of sequential accumulation of TP53 mutations in tumor initiation and progression, we screened the IARC database for patients with germ-line mutations (193 patients) to identify those with multiple TP53 mutations and a clinical history of tumor progression. Only one patient suitable for our analysis was found (patient 3; Ref. 9). He was initially operated on for a low-grade astrocytoma (WHO grade II) and reoperated on 28 months later for a glioblastoma (WHO grade IV) in the same location, suggesting tumor progression. The patient carried a germ-line TP53 mutation (R283H), and the primary and recurrent tumors acquired two somatic mutations (R267W and E258D).

We first established by LOH analysis that TP53 alleles had been inactivated exclusively by point mutations and that no allelic deletions had occurred. TP53 alleles present in DNA from patient blood and tumors were analyzed by PCR using primers flanking a polymorphic site in intron 1 of the gene (26). A difference in the number of tandem repeats allowed us to distinguish the size of maternal and paternal alleles by electrophoresis. We found two fragments of equal intensity in all three samples (Fig. 2A), suggesting that tumor formation did not involve genetic events leading to TP53 allele loss but rather resulted from mononuclear evolution of cells that sequentially accumulated two somatic point mutations.

To understand the role each mutation had played during tumor initiation and early progression, it was important to first establish the order in which they had occurred. This could have proceeded in two different ways (see Fig. 2B). If the first mutation occurred at codon 267, then we have scenario 1; if it occurred at 258D, then we have scenario 2. Each scenario defines three genetically distinct cell types that we refer to as X (left), Y (middle), and Z (right) and defines the number of alleles contained in each cell type as 2x, 2y, and 2z. Because all three types of mutated TP53 alleles (283H, 267W+283H, and 258D) were found in the grade II astrocytoma (9), we can assume that all three cell types could be present in the primary tumor, albeit in different proportions. The total relative number of alleles in the primary tumor will be 2x + 2y + 2z = 100%. How can we determine which mutation occurred first? It is important to notice that the number of alleles of each type (wt, 283H, 267W+283H, and 258D) will vary depending on the scenario. Indeed, in scenario 1, two cell types (X and Y) contain wt alleles (in white), whereas only X contains 283H alleles (in pink). In scenario 1, there will be x + y% wt alleles and x + y% 283H alleles (e.g., more white than pink). In contrast, in scenario 2 it will result in x% of wt alleles and x + y% 283H alleles (e.g., more pink than white). Similar predictions can be made for the 258D and 267W+283H alleles (in red). On the basis of these calculations, we wished to establish a method that would allow us to quantify the different percentages of alleles present in the tumors. Such a method would aid in establishing the correct scenario and mutation order.

For this purpose, we adapted a p53 transcriptional assay in yeast (24). In this assay, tumor-derived TP53 mRNA is reverse transcribed, and the resulting cDNA is transformed into an ADE2-deficient yeast strain. The expression of an ectopic ADE2 gene in this yeast is controlled by a p53RE derived from the human RGC (30). Each transformed yeast cell expresses a single cDNA representing the TP53 status of one TP53 allele from the tumor. If the TP53 cDNA encodes p53 wt, the yeast cell will form a white colony on agar plates containing limiting amounts of adenine. If it encodes a mutant p53, adenine insufficiency results in a red yeast colony. The frequency of white and red colonies reflects the proportion of TP53 alleles in the analyzed tissue because the presence of a missense mutation does not alter the expression or stability of TP53 mRNA. Using this assay, we determined wt and mutant TP53 allele proportions in the microdissected primary and recurrent tumors and were able to infer the temporal occurrence of the two somatic mutations.

The assay showed three types of yeast colonies in both tumors: (a) white; (b) red; and (c) pink. Sequencing of the TP53 cDNA found in white colonies showed wt sequence, whereas pink colonies contained the 283H germ-line mutation. The pink color was interpreted as an intermediate phenotype resulting from a partial loss of the capacity to transactivate the RGC p53RE controlling the ADE2 gene (24). The red colonies harbored two different alleles, either TP53267W+283H or TP53258D (indicating that mutation 267W occurred in the germ-line TP53283H allele and mutation 258D occurred in the wt allele). The red color indicates that these two mutants have totally lost the capacity to transactivate the RGC p53RE. The relative frequencies of the colonies in the grade II astrocytoma were as follows: (a) 15 ± 1.96%, white; (b) 11 ± 1.72%, pink; and (c) 74 ± 2.41%, red (Fig. 2C). The higher percentages of white as compared with pink colonies suggest that the first scenario is the most probable (Fig. 2D). Astrocytomas are diffusely infiltrating tumors; therefore, this tumor probably contained some normal cells (astrocytes, vascular cells, and immune cells). This does not affect the difference in the percentages of pink and white colonies because normal cells of this patient will generate equal amounts of each. To further confirm scenario 1, we examined the relative numbers of TP53267W+283H and TP53258D alleles in the tumors. The first scenario predicts an excess of TP53267W+283H [(y + z)%] over TP53258D alleles (x%) in the primary tumor (Fig. 2B). Because both mutants give red colonies in this assay, we sequenced TP53 cDNA extracted from 50 red colonies/tumor to derive an estimate of the relative frequency of each. In the primary tumor, the

![Fig. 1. Frequency of nine hot spot p53 mutations in tumors with single versus two or more p53 mutations. We observed a decrease of 48% (95% CI, 42.5–54.6%) in tumors with two or more p53 mutations for nine p53 mutations known to completely abrogate p53 function.](image-url)
frequency of TP53<sup>267W+283H</sup> was 40%, whereas that of TP53<sup>258D</sup> was 34%, figures compatible with the first scenario (Fig. 2D).

With progression to glioblastoma (WHO grade IV), an increased frequency of red colonies was observed: (a) 96 ± 0.66% were red (TP53<sup>267W+283H</sup> or TP53<sup>258D</sup>); (b) 1.7 ± 0.44%, were pink (TP53<sup>283H</sup>); and (c) 2.3 ± 0.51% were white (TP53<sup>wt</sup>; Fig. 2C). Sequencing of the two red alleles in 50 colonies revealed equal frequencies (48%) for each (Fig. 2C). These data are compatible with both scenarios (Fig. 2D).

In conclusion, these data suggest that the first mutation occurred at codon 267 of the TP53<sup>267H</sup> germ-line allele and that the TP53<sup>wt</sup> allele was subsequently hit at codon 258.

**Determination of the Percentages of Genetically Different Cell Populations in the Primary and Recurrent Tumor.** Next, we were interested in determining the proportions of the three cell populations with differing TP53 genotypes in primary and recurrent tumors. The predominant population would have likely determined the tumor diagnosis. Consequently, its TP53 genotypic status would be indicative of the number of mutations necessary to reach that stage. Minor populations in the tumor might represent remnants of earlier stages that had a competitive disadvantage during clonal evolution, or they could be newly evolved, representing the first stages of progression. It was also of interest to know how many “normal” cells (defined as cells with the constitutive TP53 genotype) might be intermingled with tumor cells in both tumors despite their microdissection.

Knowing the mutation order and the allele distribution in both tumors, establishing the cell proportions was a straightforward task. Indeed, when expressed in percentages, the relative number of each cell type is twice that of its corresponding allele. In the primary tumor, we have 22% (2 × 11%) TP53<sup>267Hwt</sup> cells, 8% (2 × 4%) TP53<sup>267W+283Hwt</sup> cells, and ~70% (2 × ~35%) TP53<sup>267W+283H</sup> cells (see Fig. 3A, grade II encoding transcriptionally active p53 can synthesize adenine and grow as white yeast colonies. Those with inactive p53 accumulate an intermediate product of the defective adenine synthesis pathway and form red yeast colonies. For the primary tumor, 332 yeast colonies were analyzed [50 were white (15 ± 1.96%), 37 were pink (11 ± 1.72%; arrows), and 245 were red (74 ± 2.41%)], and the sampling error of the frequency was calculated as the normal approximation confidence for a binomial distribution using the operation square root of [percentage of colonies of one color × percentage of colonies of the other color ÷ total number of colonies analyzed]. For the recurrent tumor, 870 colonies were analyzed: (a) 20 colonies were white (2.3 ± 0.51%); (b) 15 colonies were pink (1.7 ± 0.44%); and (c) 835 colonies were red (96 ± 0.66%). In addition, one has to consider that the frequency of white colonies is underestimated by ~5% because of the experimental error of the p53 transcriptional assay in yeast (see “Materials and Methods”). Taking this into account would increase the frequency of white colonies to 15.75 ± 1.96% and further augment the difference between white and pink colonies. Sequencing of TP53 cDNA revealed WT alleles in white yeast colonies, TP53<sup>267H</sup> in pink yeast colonies, and TP53<sup>258D</sup> or TP53<sup>267W+283H</sup> in red yeast colonies.

The proportion of TP53<sup>258D</sup> and TP53<sup>267W+283H</sup> alleles among each tumor was estimated by sequencing TP53 cDNA from 50 randomly picked red colonies/tumor. The estimated frequencies of TP53<sup>258D</sup> and TP53<sup>267W+283H</sup> alleles were 46% ± 7% and 54% ± 7%, respectively, in the primary tumor, and 70% ± 7% each in the recurrent one. D. Evolution of cell populations in scenarios 1 and 2. Under each circle, the calculated frequencies of each TP53 allele are indicated. In both panels the first row represents the situation in the normal brain (50% white yeast colonies for the TP53<sup>wt</sup> and 50% pink yeast colonies for TP53<sup>258D</sup> and TP53<sup>267W+283H</sup>). The second row represents the first somatic mutation that led to the neoplastic change (2nd hit). Because no biopsy was available for this stage of tumor progression, we could not count the frequencies of TP53 alleles (indicated by ?). The third row represents the situation in the primary tumor (Astro II). The observed frequencies of each type of yeast colony are compatible only with the first scenario. Indeed, in this scenario, the counted frequencies of white colonies are 35% (x% = 15%), pink (y% = 11%), and red (z% = 54%); TP53<sup>258D</sup> and TP53<sup>267W+283H</sup> alleles (%), which is not the case. Incompatibilities in the second scenario are highlighted in green. These discrepancies cannot be resolved by proportionally increasing or decreasing the number of the three cell types that are present in the primary tumor. The small differences (0.5–1%) in scenario 1 between the estimated frequencies of white colonies for the primary tumor are within the SE (-1.72%–1.96%, see text). Allele frequencies (x%, y%, and z%) in the recurrent tumor (fourth row, Astro IV) are compatible with both scenarios. The small difference between white (2.3%) and pink (1.7%) alleles for the recurrent tumor is within the SE (-1.04–1.51%, see text).
**LOSS OF p53 FUNCTIONS DURING TUMOR PROGRESSION**

**A. Clonal evolution of cell populations in tumorigenesis:**

![Diagram showing clonal evolution of cell populations](image)

- **Normal cancer predisposition**
  - Astrocytoma
  - Pre-astrocytoma
  - WT
  - Mutant

- WHO grade II astrocytoma
  - WT
  - Mutant

- WHO grade IV glioblastoma
  - WT
  - Mutant

**B. Sequential accumulation of p53 mutations:**

- 283H in germline
- 267W
- 283D

**C. Composition of p53 tetramers:**

- 100% wild type
- 6.2% mutant
- 6.2% heterogeneous

**D. Progressive loss of p53 functions:**

- Partial loss of apoptosis
- Partial loss of apoptosis and growth arrest
- 100% loss of apoptosis and growth arrest

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**Fig. 3. Clonal evolution of tumor cell populations and model of sequential loss of p53 functions in a patient.**

- **A.** Clonal expansion of cell populations during tumor progression in the patient. Cells are represented with WT or mutant TP53 alleles as described in the Fig. 2 legend. The percentage of each cell population in the primary (grade II) and recurrent (grade IV) tumors was calculated based on the distribution of alleles in Fig. 2. The first clonally detectable tumor (astrocytoma grade II) already has 70% of cells with three p53 mutations. This tumor likely evolved from a clonally undetected hyperplastic lesion that we called preastrocytoma. **B.** Accumulation of p53 mutations in TP53 alleles during tumorigenesis. Each allele is represented by a vertical bar in the color it would generate after expression in the yeast assay described in Fig. 2. Point mutations are indicated by circles. **C.** Different types of p53 tetramers formed (four circles) within a cell after each mutation is shown. Each p53 molecule is represented by a circle. The circle colors define the different homomers and heteromers between WT and mutant forms of p53. The activity of tetramers with a mix of WT and mutant p53 molecules is unknown. **D.** The above data suggest that the various mutants identified differ in their ability to bind specific p53REs. To examine the structural basis for these differences, we used computer visualization softwares to perform three-dimensional modeling of the p53-DNA interactions based on the published crystal structure of the p53RE-DNA complex (Protein DataBank ID, IJTS: Ref. 29). Localization within the p53 tertiary structure of the three mutated amino acids revealed that only R283 is located at the protein-DNA interface (Fig. 5A). Therefore, substitution R-H at position 283 could directly modify p53 binding to DNA. This residue has two important roles in efficient and specific binding to DNA. First, it stabilizes the p53-DNA complex through non-sequence-specific interactions with the phosphatic backbone of DNA (green arrow, Fig. 5B). Second, it influences sequence-specific binding of p53 to the DNA through its interaction with K120 (red arrow, Fig. 5B). K120 is one of the three p53 residues involved in sequence-specific interactions with the nitrogen bases of DNA (yellow arrow; Ref. 29). The 4.25 Å distance between K120 and R283 creates a Van der Waals interaction that stabilizes K120 in the correct orientation for sequence-specific interaction with DNA. Substitution R283H allows incorrect conformations of K120, which in turn loses the direct protein-to-base interactions with DNA (Fig. 5C). The model predicts during tumor initiation and progression, we expressed them in yeast and in the p53-null glioma cell line LN-Z308 (28). We examined their ability to transactivate reporter genes (ADE2 or luciferase, respectively) under the control of p53RE from the CDKN1A and BAX promoters.

The transcriptional assay in yeast indicated that p53 283H had WT transactivating activity toward the CDKN1a promoter (white colonies) but strongly reduced activity toward the BAX promoter (mostly red colonies; Fig. 4A). p53 267W + 283H and p53 283D failed to induce either promoter (red colonies; data not shown). However, p53 267W alone could transactivate the CDKN1A but not the BAX promoter (data not shown).

Transcriptional activity in glioblastoma cells (Fig. 4B) showed that p53 283H transactivated the CDKN1A promoter but had a reduced activity on the BAX promoter (100% and 42% of wt activity; P = 0.0009), p53 267W + 283H and p53 283D completely lost the capacity to induce CDKN1A [5% (P < 0.0001) and 1% (P < 0.0001) of wt activity, respectively] and BAX [1.2% (P < 0.0001) and 0.7% (P < 0.0001) of wt activity, respectively]. p53 267W had substantially reduced the capacity to induce transcription from the BAX promoter (23% of wt; P = 0.0016) and had a markedly reduced capacity to activate the CDKN1A promoter (22% of wt activity; P < 0.0001). The control p53 273H was inactive on both promoters. Western blot analysis indicated that differences in transcriptional activity were not because of a deficiency of exogenous p53 protein to be expressed within the cells (Fig. 4B). Separate immunocytochemistry experiments also showed that these mutants are expressed predominantly in the nucleus (data not shown). To confirm that transcriptional activation also occurred on the endogenous CDKN1A promoter, we transfected glioma cells with the different p53 DNA expression vectors and measured the activation of the endogenous CDKN1A gene by quantitative real-time RT-PCR (Fig. 4C). The Cts for amplification were identical for p53 WT and p53 283H, indicating equal ability to activate transcription. The Cts for p53 267W + 283H and mock-transfected cells were identical, confirming that the second mutation abrogated p53 transactivation ability.

**Taken together,** these results suggest that p53 283H had a strongly reduced ability to induce transcription from the BAX promoter but maintained transcription of the CDKN1A promoter. Accumulation of a second mutation at R267W abrogated this activity. p53 283D was functionally similar to hot spot mutant p53 273H because it eliminated p53 transactivation on both promoters.

**Modeling of Mutant p53-DNA Interactions.** The above data suggest that the various mutants identified differ in their ability to bind specific p53REs. To examine the structural basis for these differences, we used computer visualization softwares to perform three-dimensional modeling of the p53-DNA interactions based on the published crystal structure of the p53RE-DNA complex (Protein DataBank ID, IJTS: Ref. 29). The 4.25 Å distance between K120 and R283 could directly modify p53 binding to DNA. This residue has two important roles in efficient and specific binding to DNA. First, it stabilizes the p53-DNA complex through non-sequence-specific interactions with the phosphatic backbone of DNA (green arrow, Fig. 5B). Second, it influences sequence-specific binding of p53 to the DNA through its interaction with K120 (red arrow, Fig. 5B). K120 is one of the three p53 residues involved in sequence-specific interactions with the nitrogen bases of DNA (yellow arrow; Ref. 29). The 4.25 Å distance between K120 and R283 creates a Van der Waals interaction that stabilizes K120 in the correct orientation for sequence-specific interaction with DNA. Substitution R283H allows incorrect conformations of K120, which in turn loses the direct protein-to-base interactions with DNA (Fig. 5C). The model predicts
were used. Yeast were grown at 35 °C but not by p53 267W cytochemistry in a separate transfection experiment. The transcriptional activities of the three 48 h, cell extracts were prepared and analyzed for luciferase activity, and p53 protein levels p53 WT, p53 283H, and p53 273H were expressed predominantly in the nucleus (data not shown). CDKN1a under the regulation of the p53REs of TP53 283H transcriptional assay in yeast using allele and CDKN1a cells (LN-Z308). Luciferase reporter genes under the control of p53REs derived from human reporter assays of TP53 CDKN1a promoter (5) Lane 3 from cells that are mock transfected (5, E and F). These changes are likely to globally impair p53-DNA binding irrespective of sequence specificity. This interpretation is compatible with our observation of loss of transactivation for both mutants on the CDKN1A, BAX, and RGC promoters. Furthermore, the predicted physical distance between amino acids 267 and 283 does not support the existence of any trivial cooperation between mutations 283H and 267W that would lead to synergistic inactivation of p53 in the double mutant. The p53 molecule with R267W and R283H mutations is likely to adopt a grossly misfolded conformation, leading to reduced or abrogated DNA binding.

Evaluation of Cell Growth Inhibition of Mutants p53283H, p53267W+283H, and p53258D Using a Colony Formation Assay. p53 acts as a suppressor of neoplastic growth by inducing cell growth arrest and/or apoptosis. To functionally characterize the in vitro growth-inhibitory properties of the mutant proteins found in the patient, we have tested their capacity to inhibit clonal cell growth in monolayer cultures using a colony formation assay. LN-Z308 human glioblastoma cells (p53-null) were chosen because expression of wt p53 in these cells is known to induce growth arrest but not apoptosis (31, 32). Cells were transfected with expression vectors for each of the p53 mutants and neomycin resistance (p53283H, p53273H, and the empty vector served as controls). Fig. 6 shows that p53283H inhibits clonal cell growth of LN-Z308 cells similarly to p53 wt. However, the presence of both mutations (267W+283H) in the same p53 molecule abrogated this function and yielded the same amount of colonies as p53258D, p53273H, or the control vector.

A significant number of colonies (10%) was formed after transfection of the TP53 wt and TP53283H cDNA constructs. It has been previously found for TP53 wt CDNA transfectants that these colonies lose or rearrange exogenous TP53 sequences (27). To evaluate this issue in the case of TP53283H, we isolated these independent colonies, expanded them into viable clones, and examined p53 expression by Western blot and immunocytochemistry. As controls, we also expanded colonies from p53258D, p53267W+283H, p53267W, p53273H, and p53 wt. Most of the colonies (95%) obtained with p53 wt or p53283H were not viable because they could not be expanded, and those few (5%) that did expand failed to express p53 (data not shown).

DISCUSSION

The initiation steps of human gliomagenesis are poorly understood. The timing and sequence of the progressive loss of single tumor

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suppressor functions by accumulation of point mutations have not yet been investigated in this process.

We found a general decrease in the frequency of hot spot TP53 mutations in human tumors containing multiple TP53 mutations and, consequently, an increase of rare TP53 mutants in this group. The presence of multiple TP53 mutations in tumors might be due in part to mismatch repair defects leading to alleles with multiple mutations, to artifacts of PCR amplification, and/or to sample contaminations. The latter two would favor the presence of hot spot mutations because these are a more frequent source for sample contamination, and polymerase mutation frequency in vitro is higher on these codons (33). Although we recognize the inherent limits of the p53 database, the finding that tumors with multiple mutations have more infrequent mutations is compatible with the idea that progressive loss of p53 functions may have occurred in these tumors. Infrequent mutants can lead to only partial loss of p53 function (17–20).

Tumor initiation and progression might require abrogation of several p53 functions that could be lost at once by a “hot spot” mutation or progressively within the same tumor through multiple partial loss of function mutations. To test this hypothesis, we established the chronological order by which three TP53 mutations occurred during human astrocytoma development and progression in a germ-line TP53283H patient. We showed that the primary tumor contained three cell populations differing in their TP53 genotype: (a) normal cells (TP53283H/WT; 22% of cells); (b) grade II tumor cells (TP53267W/283H/258D; 70% of cells); and (c) cells with an intermediate genotype (TP53267W/283H/WT; 8% of cells) from which the tumor cells derived (Fig. 3A). This provides the first evidence for monoclonal evolution in a WHO grade II astrocytoma and suggests that this tumor derived from a precursor lesion (called preastrocytoma in Fig. 3A) that was not detected clinically. Complete inactivation of a single TP53 allele by the germ-line and somatic 267 mutation (and potential effects of TP53 mutations on tumor initiation and progression are represented in Figs. 5, 6A, and 6B.)
induce transcription of the wt transcriptional activity toward the CDKN1a p53 258D and p53 267W mutation in a low-grade tumor can lead to tumor progression (5). Previous studies have shown that acquisition of a tumor suppressor gene mutations in these tumors, but this is rare in brain tumors and is mismatch repair deficiency contributed to the accumulation of multiple mutations in two alleles. We did not examine whether a -signaling proteins may have contributed to the accumulation of alterations in DNA repair enzymes and/or DNA damage-sensing and function as the result of point mutation in one allele and loss of the other allele. Previous works have shown that the ratio of p53WT tetramers in the cells. The activity of the 87.5% of p53 mutants, as is frequently observed, might reduce even further to the ones obtained with empty vector or p53273H.

To verify that the ability of p53283H to transactivate CDKNA1 has functional relevance, we examined its capacity to inhibit colony formation from transfected human glioma cells. p53283H inhibited colony formation, consistent with its ability to activate the p21 growth arrest pathway. In contrast, p53283H and p53267W–283H had totally lost this capacity, leading to the formation of a number of clones similar to the ones obtained with empty vector or p53273H.

Taken together, these data are compatible with the model illustrated in Fig. 3, B–D. The germ-line mutation R283H in one TP53 allele may result in a reduction of p53-dependent apoptosis because we show that this mutant does not efficiently transactivate BAX. This partial loss of function phenotype, initiated by a 50% reduction in TP53283H gene dosage, is theoretically expected to reduce p5335 tetramers by ~94% in the cells, if one assumes equal transcription, translation, stability, and ability to form tetramers for the mutant and wt p53 proteins. Increased stability of p53 mutants, as is frequently observed, might reduce even further the ratio of p53WT tetramers in the cells. The activity of the 87.5% of p53WT/mutant heteromers is unknown and will likely vary according to the ratio of wt and mutant p53 molecules/tetramer (Fig. 3C). Because clonal expansion of cells with two additional p53 mutations occurred during the tumorigenic process, it is likely that there was a selective advantage of eliminating residual wt activity in these heteromers. p53 is known to induce apoptosis in cells sustaining DNA damage, thus a decreased overall proapoptotic activity of p53 tetramers in cells containing damaged DNA might increase their viability and survival. Acquisition of mutation 267W on the TP53283H allele and subsequent acquisition of mutation 258D on the remaining TP53283H allele progressively disrupted the capacity of p53 tetramers to induce growth arrest through the p21 pathway. This would have conferred an increasing proliferative advantage and led the cells to clonal expansion. It is likely that the increased genetic instability associated with p53 loss leads to the accumulation of additional genetic defects and ultimately malignant progression (Fig. 3B).

Although this model hinges on data from a single patient, it allows us to make several interesting predictions. First, the early stages of tumorigenesis and progression can occur through progressive decreases in wt p53 activity. p53 activity is subject to multiple pathways of regulation, and the importance of progressive loss of p53 functions in tumor formation is emerging (16). Second, a decrease in expression...
of p53-regulated apoptosis mediators (such as BAX, PUMA, and/or p53AIP1) can precede loss of p53 cell cycle-regulatory function (p21) and promote early tumor formation. BAX has been demonstrated previously to be involved in tumor initiation (39, 40) but not in tumor progression because for the latter the cells need to acquire a proliferative advantage (41). Understanding that loss of apoptosis induction might precede loss of cell cycle control during tumorigenesis in a subset of patients has significance beyond the mechanisms that start tumor formation. It could also help define new stage-specific targets for the development of novel treatments and define the most appropriate currently available therapy. Clinical therapies that induce DNA damage and cell death by p53-dependent apoptotic response might not be appropriate for such patients. Third, clonal analysis of tumor evolution will likely uncover subtle steps that have not been described in the current histological classification of tumors such as the preastrocytoma lesion we found within the grade II astrocytoma of this patient. Finally, studying tumor suppressor genes with multiple mutations in single tumors could be particularly informative to understand the progressive loss of function of their products during tumorigenesis. These predictions need to be further validated experimentally when other such cases become available, and it remains to be established whether they will be generally applicable to tumors losing p53 function by a combination of mutation in one allele and loss of the other.

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References

Announcements

MEETING OF THE RADIATION RESEARCH SOCIETY

The annual meeting of the Radiation Research Society will be held at the State University of Iowa, Iowa City, on June 22–24, 1955. The Society will be the guest of the University, and all meetings will be held on the campus. The program will consist of: (1) Two symposia, one on "The Effects of Radiation on Aqueous Solutions," which includes the following speakers: E. S. G. Barron, Edwin J. Hart, Warren Garrison, J. L. Magee, and A. O. Allen. The second is "Physical Measurements for Radiobiology" and companion talks by Ugo Fano, Burton J. Moyer, G. Failla, L. D. Marinelli, and Payne S. Harris. (2) On Monday night, June 22, a lecture by Dr. L. W. Alvarez on meson physics has been tentatively scheduled. On Tuesday night, June 23, Dr. L. H. Gray of the Hammersmith Hospital, London, will speak on a topic to be announced. Dr. Gray's lecture is sponsored by the Iowa Branch of the American Cancer Society. Those desiring to report original research in radiation effects, or interested in attending or desiring additional information, please contact the Secretary of the Society, Dr. A. Edelmann, Biology Department, Brookhaven National Laboratory, Upton, L.I., New York.

ERRATUM

The following correction should be made in the article by Beck and Valentine, "The Aerobic Carbohydrate Metabolism of Leukocytes in Health and Leukemia. I. Glycolysis and Respiration," November, 1955, page 891; substitute for the last paragraph:

The data in Table 5 permit several interesting calculations. If one compares the amount of glucose actually disappearing with the sum of the amount equivalent to lactic acid produced plus that equivalent to O₂ consumption, it is seen that the amount of glucose "cleavage products" exceeds the amount of glucose utilized by 12 per cent in N and 27 per cent in CML and is exceeded by the glucose utilized by 16 per cent in CLL. If the assumption is made that, in this respect, the myeloid and lymphoid cells of leukemia are similar to those of normal blood, it may be that the computed normal figure represents a summation of the myeloid (M) and lymphoid (L) cells that make up the normal leukocyte population. Thus, if \( M = +0.27 \) and \( L = -0.16 \) and the normal differential is 65 per cent M and 35 per cent L, then

\[
0.65(+0.27) + 0.35(-0.16) = +0.12
\]

a figure identical to the observed +0.12 for normal leukocytes.
Initiation of Human Astrocytoma by Clonal Evolution of Cells with Progressive Loss of p53 Functions in a Patient with a 283H TP53 Germ-line Mutation: Evidence for a Precursor Lesion

Giulia Fulci, Nobuaki Ishii, Daniela Maurici, et al.


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