Loss of p53 but not ARF Accelerates Medulloblastoma in Mice Heterozygous for patched

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Abstract

Brain malignancies represent the most common solid tumors in children, and they are responsible for significant mortality and morbidity. The molecular basis of the most common malignant pediatric brain tumor, medulloblastoma, is poorly understood. Mutations in several genes including the human homologue of the Drosophila segment polarity gene, patched (PTCH), the adenomatous polyposis coli gene (APC), β-catenin, and p53 have been reported in subsets of hereditary and sporadic medulloblastoma. Inactivation of one Ptc allele in mice results in a 14% incidence of medulloblastoma. Here, we report a dramatic increase in the incidence (>95%) and accelerated development (prior to 12 weeks of age) of medulloblastoma in mice heterozygous for Ptc that lack p53. The acceleration of tumorigenesis in Ptc+/- mice is specific for loss of p53, because no change in tumor incidence was observed in Ptc+/- mice carrying a mutation in APC (Min+/-) or in Ptc+/- mice deficient in INK4a-ARF. Thus, there is a specific interaction between p53 and heterozygosity of Ptc that results in medulloblastoma. This may be a consequence of increased genomic instability associated with loss of p53 function that may enhance the rate of acquisition of secondary mutations. Ptc+/-;p53+/- mice provide a useful model for investigation of the molecular bases of medulloblastoma and for evaluation of the efficacy of therapeutic intervention strategies in a spontaneously arising endogenous brain tumor.

Introduction

Medulloblastoma arises from the primitive neuroectoderm in the posterior fossa of children generally between the ages of 3 and 9. The tumors are derived from cerebellar granule precursor cells that undergo a dramatic proliferative expansion during the early phases of postnatal brain development. Several gene mutations have been described in medulloblastoma, although they occur in small subsets of tumors (1). A low frequency of mutations in patched (PTCH; Ref. 2), p53 (3), the adenomatous polyposis coli (APC) gene (4), and β-catenin (5, 6) have been reported in subsets of sporadic medulloblastoma. In addition, brain tumors, including medulloblastomas, have been reported to occur more frequently in patients carrying germline mutations in PTCH, APC, or p53 (7–9). In the case of heterozygous loss of PTCH, which is associated with Gorlin syndrome, also known as basal cell nevus syndrome (OMIM 109400), the incidence of medulloblastoma increases from 2 per million to 4 per hundred in children <18 years of age (10, 11). Thus, it is likely that there are several unknown prevalent mutations in these tumors that contribute to disease progression.

Progress in understanding the etiology of medulloblastoma has been hampered by the lack of an appropriate animal model. Recently, a mouse strain was generated in which Ptc was mutated by targeted disruption (12, 13). Homozygous deletion of Ptc results in embryonic lethality, whereas mice heterozygous for Ptc exhibit several features of Gorlin syndrome, including an increased propensity to develop tumors in the brain and soft tissues. Histological analysis of the brain tumors showed that they closely resemble human medulloblastoma (12, 14). However, only 14% of mice heterozygous for Ptc develop medulloblastoma over a period of 10 months, indicating that it is likely that additional genetic lesions are required for oncogenic transformation.

Ptc functions as a component of the receptor complex that transduces a signal from Hedgehog (Hh) through a complex pathway that was first described in Drosophila (7). The interaction of Shh, the mammalian orthologue of Hh, with Ptc relieves suppression of smoothened (Smo), resulting in increased transcription of Gli1 and other target genes (7). During cerebellar development, Shh, produced by Purkinje cells, functions as a mitogen to stimulate proliferation of granule cell precursors (15). Ptc does not function as a classic tumor suppressor gene in medulloblastomas in Ptc+/- mice because the normal allele is not lost, and it continues to be expressed in tumors (14, 15).

p53 functions as a transcription factor that transduces signals elicited by physiological stress and DNA damage to regulate cell proliferation and apoptosis. Abrogation of p53 function attenuates both of these responses (17). The mouse tumor suppressor gene p19ARF (p14ARF in humans) is the product of an alternative reading frame encoded by the INK4a-ARF locus. ARF functions as a sensor of normal proliferative signals upstream of p53 by interfering with Mdm2, a negative regulator of p53 function (18). Thus, loss of p19ARF diminishes p53 activity and promotes tumor formation (19). Mice deficient in p53 do not develop brain tumors, although they are predisposed to develop tumors in several other tissues by 5 months of age (20, 21). Approximately 10% of ARF-null mice develop glial tumors by 6 months of age (19).

To address the possible involvement of tumor suppressor genes in medulloblastoma and to accelerate the incidence of these tumors, we crossed Ptc+/- mice with mice carrying mutations in other tumor suppressor genes. We selected APC because it regulates the levels of β-catenin, which functions in the Wnt signaling pathway (22). Humans with brain tumor-polyposis, or Turcot’s syndrome, carry germ-line mutations in APC, and they have an increased incidence of tumors arising in colon and brain (8). In addition, mutations in β-catenin have been reported in spontaneous medulloblastoma, albeit at a low frequency (5, 6). We also crossed the Ptc+/- mice with mice carrying inactivating mutations in two major tumor suppressor genes that are defective in more than half of all human cancer, p53 and ARF (18). These genes serve critical functions in the regulation of cell proliferation, apoptosis, and response to DNA damage (18, 23).
Materials and Methods

Animals. The Ptc<sup>+/-</sup> mice used in this study were generated and maintained on a mixed C57Bl/6 × 129Sv background, as described previously (14), and crossed with mice carrying targeted disruptions in p53, APC (C57BL/6J-Min<sup>1</sup>); Jackson Laboratories, Bar Harbor, ME), and ARF<sup>−/−</sup> (19) to generate the following cohorts of mice: Ptc<sup>+/-</sup> p53<sup>+/−</sup> (n = 440), Ptc<sup>+/-</sup> p53<sup>−/−</sup> (n = 68), Ptc<sup>+/-</sup> p53<sup>−/−</sup> ARF<sup>−/−</sup> (n = 40), and Ptc<sup>+/-</sup> Min<sup>−/−</sup> (n = 16). Cohorts of mice were observed for tumor formation for a minimum of 6 months after birth. All mice were observed daily for signs of increased intracranial pressure and for evidence of enlarged occipital prominence three times weekly for at least 24 weeks. Animals were euthanized when they were moribund according to NIH-approved institutional guidelines or when they showed signs of increased intracranial pressure or when extracranial tumors were evident. Brains were removed from the surrounding calvarium, and tumor tissue was carefully separated from surrounding brain parenchyma under a dissecting microscope. In every mouse, the presence of tumor was confirmed by gross examination of the brain. If the mouse was not available for examination or if no tumor was detected, the cause of death was attributed to “unknown causes.” Fresh tissue was snap frozen and stored at −80°C for later extraction of RNA, DNA, and protein. For histochemical analyses, animals were deeply anesthetized and perfused transcardially with 4% paraformaldehyde in 0.1 M PBS and processed for immunohistochemical analyses as described previously (14).

RNA Isolation and Northern Analysis. Total cellular RNA was isolated from 11 mouse medulloblastomas using Trizol (Ambion, Inc., Austin, TX) according to the manufacturer’s directions. Five to 10 μg of total RNA were electroeluted on a 0.8% agarose-formaldehyde gel, transferred to a nitrocellulose filter (Hybond N+; Amersham Pharmacòa; Buckinghamshire, United Kingdom), and hybridized under stringent conditions (18 h at 68°C in 5× SSPE, 50% formamide, 5× Denhardt’s solution, 1% SDS, and 0.1 mg/ml denatured salmon sperm DNA) with a 32P-labeled RNA probe. Filters were washed (twice × 20 min in 0.1 SSC, 0.1% SDS at 68°C) and exposed to film (Eastman Kodak) for 12–72 h at −80°C. Control and tumor tissues were analyzed by hybridization with 32P-labeled RNA probes specific for mouse Ptc (12), Gli1 (mouse EST clone 38654), and mdm2.

Immunoblot Analysis. Protein extracts were prepared by Dounce homogenization of 80–100 mg of snap-frozen tumor or normal tissue as described (14). Extracts were clarified by microcentrifugation at 18,000 × g, and equal volumes with 10% SDS-PAGE. Samples were electrophoresed, detected, and analyzed on PE/ABI model 373, 370, Stretch, or 377 DNA sequencers (Perkin-Elmer Applied Biosystems, Inc.). Sequence analysis was performed using Sequencher (Gene Codes Corp., Ann Arbor, MI) software.

Results and Discussion

The Loss of p53 Dramatically Accelerated the Age of Onset and the Incidence of Medulloblastoma in Ptc<sup>+/-</sup> Mice. Tumors were apparent as early as 4 weeks, and all of the Ptc<sup>+/-</sup> p53<sup>−/−</sup> mice developed either brain tumors or died from unknown causes prior to 12 weeks of age (Fig. 1). Within this Ptc<sup>+/-</sup> p53<sup>−/−</sup> cohort, 95% of mice were confirmed by gross and histological analysis to have tumors in the posterior fossa. In 5% of the mice, no brain tumor was apparent by gross examination at the time of death. A very low incidence of extracranial soft tissue sarcomas were noted to arise at similar frequencies in all cohorts of Ptc<sup>+/-</sup> mice. There was no acceleration in the incidence or time of onset of the extracranial tumors (soft tissue sarcomas, basal cell carcinomas, and lymphomas) described in Ptc<sup>-/+</sup>/p53<sup>-/-</sup> mice (Fig. 1). Thus, complete loss of p53 synergizes with haploinsufficiency of Ptc to produce a very high frequency of medulloblastoma in young mice.

The brain tumors in Ptc<sup>+/-</sup> p53<sup>−/−</sup> mice arose in the same anatomical location in the posterior fossa, and they exhibited histology similar to the medulloblastomas found in Ptc<sup>−/+</sup> mice that did not carry p53 mutations (14). Furthermore, the normal Ptc allele was expressed in all tumors examined, as described previously for tumors in Ptc<sup>-/-</sup> mice (14). Gli1 mRNA was present at much higher levels in the tumors than in the control tissues (Fig. 2A). Transcription of ready reaction kits with AmpliTaq DNA polymerase FS (Perkin-Elmer Applied Biosystems, Inc., Foster City, CA) and synthetic oligonucleotides complementary to regions covering the entire open reading frame of p53. Samples were electrophoresed, detected, and analyzed on PE/ABI model 373, model 37 Stretch, or model 377 DNA sequencers (Perkin-Elmer Applied Biosystems, Inc.). Sequence analysis was performed using Sequencher (Gene Codes Corp., Ann Arbor, MI) software.

![Fig. 1. Medulloblastoma incidence is increased and age of tumor onset is decreased in Ptc<sup>+/-</sup> mice lacking a normal p53 gene. Greater than 95% of Ptc<sup>+/-</sup>/p53<sup>-/-</sup> mice spontaneously developed tumors in the posterior fossa between 10 and 12 weeks of age, compared with a 14% incidence of tumors in Ptc<sup>-/-</sup> mice by 10 months of age. A small percentage (3%) of the Ptc<sup>-/-</sup>/p53<sup>-/-</sup> mice died within the first 3–4 weeks of life, with no tumor detected by gross examination of the brain. All Ptc<sup>+/-</sup>/p53<sup>-/-</sup> mice died by 12 weeks of age. Brains were removed and examined for the presence of tumor. No significant acceleration in tumorigenesis was noted in Ptc<sup>-/-</sup>/p53<sup>-/-</sup>, Ptc<sup>−/-</sup>/ARF<sup>−/−</sup>, or in Ptc<sup>-/-</sup>/Min<sup>−/−</sup> mice.](image-url)
Gli1 is normally repressed by Ptc, and in adult, nonproliferating tissues, Gli1 expression is not readily detected. However, during development, when cells are rapidly proliferating, repression of Gli1 transcription is abrogated by the interaction of Shh with the Ptc/Smo receptor complex (7). When Ptc is mutated or absent, intrinsic signaling by Smo is not suppressed, resulting in increased transcription of Gli1 and other downstream genes. A single copy of Ptc was sufficient to maintain repression of Gli1 in the non-tumor containing Ptc<sup>+/−</sup> cerebellum, because no increases in Gli1 levels were detected in control brain tissues compared with the dramatic increases in Gli1 mRNA seen in the tumors (Fig. 2A). However, in medulloblastomas that arise in Ptc<sup>−/−</sup> mice, there is persistent expression of the wild-type Ptc allele, suggesting that derepression of Gli1 expression does not require complete loss of Ptc (14). These data indicate that genes other than Ptc may influence Gli1 expression in the cerebellum. Expression of p53 mRNA was found to be elevated in all tumors (Fig. 2B). This is consistent with prior reports of elevated p53 expression in populations of rapidly dividing cells during development and tumorigenesis (23, 24).

No Acceleration of Medulloblastoma Formation Was Observed in Ptc<sup>+/−</sup>Min<sup>+/−</sup> Mice. Only 1 of 16 of these mice (6%) developed a posterior fossa tumor by 22 weeks of age. This tumor was pheno-

typically similar to the medulloblastomas found in Ptc<sup>+/−</sup> mice. These data suggest that genetic lesions in the PTCH and APC pathways found in subsets of sporadic human medulloblastoma do not act synergistically in mice.

**p53 Loss Is Not Required for Medulloblastoma Formation in Ptc<sup>−/−</sup> Mice.** The dramatic acceleration of medulloblastoma formation in Ptc<sup>−/−</sup> mice prompted us to investigate the status of p53 in tumors arising in Ptc<sup>−/−</sup> mice in which there is no germ-line mutation of p53. Interestingly, these tumors contained high levels of p53 mRNA compared with control tissues. In contrast, there was no consistent difference in Mdm2 mRNA levels between normal and tumor tissues (Fig. 2B). The Mdm2 gene product acts to repress p53 activity, and amplification of Mdm2 inactivates p53 in a subset of astrocytomas (25, 26). Wild-type p53 protein has a short half-life, and it is not readily detected in populations of nonproliferating cells unless it has been stabilized by mutation (23). Therefore, we performed immunoblotting analysis to look for evidence of p53 inactivation in tumors from Ptc<sup>−/−</sup> mice. As shown in Fig. 3, despite the increase in p53 mRNA, p53 protein was present at significantly lower levels in medulloblastomas from Ptc<sup>−/−</sup> mice, compared with those observed in a mouse lymphoma with an E254G substitution mutation in p53 (CR246). However, expression of p53 protein and mRNA were higher in the tumors than in control brain tissue, which contains relatively few proliferating cells (Figs. 2A and 3). Nucleotide sequence analysis of p53 revealed no mutations in any of the seven tumor mRNAs examined. Thus, although germ-line loss of p53 accelerates tumorogenesis in Ptc<sup>−/−</sup> mice, mutation of p53 is not required for medulloblastoma formation. This contrasts with a report of increased medulloblastoma formation in mice carrying homozygous mutations in both the retinoblastoma (Rb) and p53 genes. In this mouse model, brain tumors were not detected in mice in which only one of these genes was disrupted (27).

**No Accelerated Tumor Formation Was Noted in Ptc<sup>+/−</sup>p53<sup>+/−</sup> or in Ptc<sup>+/−</sup>ARF<sup>−/−</sup> Mice.** The lack of acceleration of medulloblastoma in Ptc<sup>−/−</sup>p53<sup>+/−</sup> mice may be attributed to the very limited time window in which the presumed tumor precursor cells are proliferating. Granule cell precursors undergo rapid expansion in the external germinal layer of the cerebellum during the first 2 weeks of postnatal life.

![Fig. 2. Analysis of mRNA expression of medulloblastomas arising in Ptc<sup>+/−</sup> and Ptc<sup>−/−</sup>p53<sup>−/−</sup> mice. A, persistent expression of Ptc and Gli1 mRNA in tumors arising in Ptc<sup>−/−</sup>p53<sup>−/−</sup> mice. Total RNA was prepared from the following tumors and control tissues: postnatal day 7 C57BL/6 mouse brain (P7 brain), C57BL/6 adult cerebellum (Ptc<sup>−/−</sup>p53<sup>−/−</sup>), non-tumor-bearing adult Ptc<sup>−/−</sup> cerebellum (Ptc<sup>−/−</sup>p53<sup>+/−</sup>), non-tumor-bearing adult p53<sup>−/−</sup> cerebellum (Ptc<sup>−/−</sup>p53<sup>−/−</sup>), tumor from one Ptc<sup>−/−</sup> mouse (199), and tumors from 10 Ptc<sup>−/−</sup>p53<sup>−/−</sup> mice. Three transcripts (5.8, and 12.5 kb) were detected with the Ptc probe. The major 8-kb Ptc transcript was decreased in all tumors examined compared with control tissues, whereas the 5.8 and 12.5-kb transcripts were expressed more robustly in tumors than in control tissues. A single Gli1 mRNA transcript of 4 kb was detected in all tumors from Ptc<sup>−/−</sup> as well as from Ptc<sup>+/−</sup>p53<sup>−/−</sup> mice at higher levels than those in control tissues. B, increased expression of wild-type p53 mRNA in tumors from Ptc<sup>−/−</sup>p53<sup>−/−</sup> mice. Northern analysis of total RNA prepared from medulloblastoma and control brain tissues revealed increased expression of p53 mRNA and no increase in mdm2 mRNA in tumor tissues.

![Fig. 3. p53 expression in medulloblastomas arising in Ptc<sup>−/−</sup>p53<sup>−/−</sup> mice. Immunoblot analysis of protein extracts from medulloblastomas arising in Ptc<sup>−/−</sup> mice revealed very low levels of p53 expression in the medulloblastoma tissues compared with a murine B-cell leukemia (CR246) that expresses mutated p53 (E251G). In contrast, p53 expression was much lower in B-cell leukemia cells lacking p53 mutations (CR205). p53 protein was not detected in control tissues (normal brain and medulloblastoma arising in Ptc<sup>−/−</sup>p53<sup>−/−</sup> mice). Sequence analysis of RT-PCR products amplified from these tumors revealed no mutations in p53.](cancerres.aacrjournals.org)
In mice, these cells differentiate and migrate to their mature positions in the internal granular layer by the third week of postnatal development (28). In humans, this process is completed by the ninth postnatal month (29). Thus, Ptch<sup>+/−</sup> precursor cells have a limited number of cell divisions in which to acquire the additional mutation(s) that contribute to medulloblastoma formation.

Loss of p53 leads to accumulation of cytogenetic abnormalities (23, 30). Indeed, we observed a much higher incidence of random chromosome loss in tumors from Ptch<sup>−</sup>/− p53<sup>−</sup>/− mice compared with those from Ptch<sup>+/−</sup> p53<sup>+/−</sup> mice. In p53<sup>−</sup>/− mice, there may be an insufficient number of cell generations to lose the remaining p53 allele and to acquire other genetic changes. Additionally, no acceleration in tumorigenesis was noted in Ptch<sup>−</sup>/− ARF<sup>−</sup>/− mice. This may be because ARF does not increase genomic instability, and therefore, the tumor precursor cells may be less prone to sustain DNA damage than cells deficient in p53. It is likely that the genomic instability associated with complete loss of p53 function accelerates the mutation rate in granule cell precursors. This may synergize with the effects of reduced Ptch expression in these mice to increase the incidence of medulloblastoma.

Survivors of pediatric brain tumors have significant morbidity as a direct consequence of the therapy required to eradicate tumor cells from the developing brain of a child. Genetic mutations have been detected only in small subsets of medulloblastoma, and the molecular basis of the majority of these tumors remains to be elucidated. The high frequency and rapid onset of tumors in Ptch<sup>+/−</sup> p53<sup>−</sup>/− mice provide a useful model to investigate other molecules that influence the balance between proliferation and cell death in the nervous system.

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References

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