HE retinoblastoma protein (pRB) is well documented as being of central importance to the regulation of cell proliferation (reviewed in 1994 by Hinds and Weinberg11). Events that favor pRB phosphorylation result in pRB inactivation and promote cell division, whereas the activity of dephosphorylated pRB is associated with cell quiescence. In addition to its phosphorylation, pRB inactivation can also be achieved through corresponding gene alterations, and inactivating mutations of the RB gene have been reported in many types of tumors (reviewed in 1993 by Hamel and coworkers8). Alterations of genes whose products modulate pRB activity have also been demonstrated in several cancers. In malignant astrocytomas, homozygous deletions of P16ink4, an apparent tumor suppressor gene at chromosomal location 9p21, appear to be the most frequent among these.7,9,14,16,19,22 The corresponding protein of this gene binds to and prevents the formation of active cyclin-dependent kinase 4 (cdk4)–cyclin D complexes that phosphorylate pRB.17,23 Significantly, CDK4 gene amplification has been shown to be an alternative mechanism to P16ink4 and CDK4 gene alterations associated with each type of tumor. Southern hybridization analysis revealed homozygous P16ink4 deletions in one (5%) of 20 PNETs and in seven (35%) of 20 malignant astrocytomas. The CDK4 gene amplification was evident in two additional astrocytomas, but not in any of the PNETs. In total, nine astrocytomas (45%) exhibited homozygous P16ink4 deletion or CDK4 gene amplification, but only one PNET (5%) demonstrated either gene alteration. These results indicate that the incidence of P16ink4 and CDK4 gene alterations in these two groups of tumors is different and suggest distinct pathogenetic etiologies may be associated with each neoplasm.

Key Words • tumor suppressor • oncogene • retinoblastoma protein • cyclin • cyclin-dependent kinase

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By demonstrating an absence of P16ink4 homozygous deletions in primitive neuroectodermal tumor (PNET) xenografts, a previous report suggests that this gene alteration does not occur in PNETs and thereby implies that the p16-cdk4–cyclin D axis may not be involved in PNET development.14 In the current study, we have examined the frequency of P16ink4, CDK4, and CYCLIN D1 gene alterations in 20 malignant astrocytomas and 20 PNETs to understand more fully the pathogenetic mechanisms associated with each type of tumor. Our results suggest that these mechanisms are, in fact, distinct.

Materials and Methods

Tumor Specimens

Except in cases of recurrent malignant astrocytoma (Cases 1, 8, 11, 17, 18, and 20; Table 1), tumor samples were removed surgically from the brain before the patient underwent radiation or chemotherapy. All specimens were immediately frozen in liquid nitrogen and stored at −80°C until used for DNA extraction. His-
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A:

inactive cdk4

\[ \text{cdk4}^+ + \text{cyclin D} \rightarrow \text{activated complex} \]

\[ \text{cdk4} + \text{cyclin D} \rightarrow \text{pRb} \]

B:

inactive cdk4

\[ \text{p16}^+ + \text{inactive complex} \]

\[ \text{p16}^+ + \text{pRb} \rightarrow \text{cell quiescence} \]

\[ \text{Phosphorylated, inactive pRb} \rightarrow \text{cell proliferation} \]

FIG. 1. Models displaying interactions of the cell cycle proteins that modulate retinoblastoma protein (pRb) activity. When bound to p16, cyclin D1 is prevented from forming a catalytically active heterodimer with cyclin-dependent kinase 4 (cdk4); phosphorylation of pRb is thereby prevented. Hypophosphorylated or active pRb binds and inactivates DNA transcription factors, blocking cell proliferation in the G1 phase of the cell cycle. Hyperphosphorylated or inactive pRb is unable to bind transcription factors, allowing DNA synthesis and progression through the S phase of the cell cycle to occur.

topathological diagnosis of each tumor was determined according to the three-tier scheme of Burger, et al.,2 and all samples used for DNA analysis were judged to contain more than 75% neoplastic cells.

Nucleic Acid Extraction and Analysis

Tumor DNA was isolated and purified in a manner previously described.3 The DNAs were treated with a restriction enzyme (EcoRI or HindIII), electrophoresed through an 0.8% agarose gel, transferred to a nylon-based nitrocellulose membrane, and fixed to that membrane by baking 2 hours in a vacuum oven set at 80˚C. The DNA filters were hybridized with 32P-labeled probes from the following loci: P16ink4 (exon 1 and exon 2 probes polymerase chain reaction (PCR)–amplified from normal genomic DNA14); D9S19 (4.4-kb EcoRI fragment from clone CRI-L1623 (American Type Culture Collection, Rockville, MD)); CDK4 (genomic probe PCR-synthesized18); and CYCLIN D1 (256-bp complementary DNA probe synthesized by reverse transcriptase–PCR (RT-PCR)9). After hybridization, the filters were washed and exposed to x-ray film. Each filter was successively analyzed with each of the probes listed above; the treatment of DNA filters for multiple, sequential hybridizations has been described previously.12,13

Gene Expression Analysis

The optical density of restriction fragments identified by each probe was determined using scanning x-ray films with an imaging densitometer (model 670; Bio-Rad, Hercules, CA) and analyzing the resulting images with the appropriate computer software (Molecular Analyst; Bio-Rad). Autoradiographic signal responses for P16ink4 hybridizations were normalized against those associated with syntenic (D9S19) and nonsyntenic (CYCLIN D1) loci. Tumor DNAs for which the normalized P16ink4 gene expression was reduced by more than 70% of that determined for control DNA obtained from nonneoplastic tissue (peripheral blood leukocytes) were scored as having homozygous deletions (gene dosage 0) and those showing a normalized P16ink4 gene expression reduced by 35% to 70% were scored as having hemizygous deletions (gene dosage 1).

Results

Southern hybridization analysis was used to determine the frequency of P16ink4, CDK4, and/or CYCLIN D1 gene alterations in 20 malignant astrocytomas and 20 pediatric PNETs. Among the PNETs, only one of 20 tumors showed a homozygous deletion of P16ink4, as compared with seven such deletions in the 20 malignant astrocytomas (Table 1; examples shown in Fig. 2). Den-sitometric analysis also revealed four instances of hemi-zygous P16ink4 deletions among the malignant astrocytomas (Cases 1, 10, 15, and 19; Table 1), whereas no hemizygous deletions were evident among the PNETs. Analysis of a second 9p21 locus centromere proximal to P16ink4, D9S19,3 revealed no instances of deletion in any of the tumor DNAs. These results are consistent with those previously derived from an analysis of glioblastoma cell lines9,13 and support the probability that 9p21 alterations in malignant astrocytomas are specifically targeted toward the deletion of P16ink4.

Normalized gene expression for CDK4 and CYCLIN D1 probes was also analyzed. Among the 40 specimens, only two cases were identified in which the tumor DNA gene expression showed an increase that was two or more times that of control samples. These two, malignant astrocytoma Cases 9 and 11, showed increases that were nine and five times the levels of CDK4 gene amplification, respectively. No instances of CDK4 amplification were detected among the PNETs (Fig. 2). Amplification of CYCLIN D1 was not detected in either group of tumors (Fig. 2; Table 1).

Discussion

Data from studies involving the analysis of RB, P16ink4, CYCLIN D1, and CDK4 loci indicate that alteration of any one of these genes is associated with the development and progression of many types of cancer. A recent report indicates that the number of inactivating mutations of the RB gene may be appreciable in malignant astrocytomas.10 In addition, evidence is accumulating that supports the theory that frequent functional inactivation of pRB in astrocytomas is a result of the occurrence of gene alterations affecting the expression of accessory proteins. For example, overexpression of cdk4 as a result of gene amplification may occur in as many as 15% of high-grade
Our finding of CDK4 amplification in two of the 20 astrocytomas studied and their occurrence in tumors that do not show inactivating mutation/deletion of P16ink4 is consistent with previously published observations, which may be evidence for the oncogenic potential of this kinase in the absence of other perturbations affecting pRB function. The amplification of CYCLIN D1, which has been described in other types of cancer, was not apparent in any of the tumors examined here.

In addition to CDK4 gene amplification, the inactivation of genes encoding cdk-inhibitory proteins could also elevate the amount of enzymatically active cyclin D–cdk4 complex in tumor cells. The P16ink4 gene is the most notable of these and is located on the short arm of chromosome 9, a region long known to be involved in glial tumor development. Homozygous deletion or inactivating mutation of P16ink4 has been demonstrated in a high proportion of tumor-derived cell lines and in a significant proportion of primary malignant astrocytomas, pancreatic adenocarcinomas, acute lymphoblastic leukemias, malignant mesotheliomas, and nonsmall cell lung cancers.

### TABLE 1

<table>
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<tr>
<th>Case No.</th>
<th>Patient Age (yrs)</th>
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* Abbreviations: PNET = primitive neuroectodermal tumor; + = alteration present; – = alteration absent.

† Malignant astrocytoma Cases 1 through 6 include patients with anaplastic astrocytomas and Cases 6 through 20 patients with glioblastomas.

‡ P16ink4 gene expression was determined as previously described by Hinds and Weinberg for IFN gene deletions. Tumor DNAs for which the normalized P16ink4 gene expression (gene expression 2) was reduced by more than 70% were scored as homozygous deletions (gene expression 0) and those showing a normalized P16ink4 gene expression reduced by more than 35% were scored as having hemizygous deletions (gene expression 1).

§ Gene alterations refer to gene expression greater than two times that determined in control tissues.

FIG. 2. Southern hybridization analysis of primitive neuroectodermal tumor (PNET; left) and malignant astrocytoma (MA; right) DNAs for gene alterations affecting the p16–cdk4 growth-regulatory axis. Tumor DNAs were digested with HindIII, electrophoresed through an 0.8% agarose gel, transferred to a nitrocellulose membrane, and successively hybridized with probes from the loci indicated. The autoradiographic response patterns shown represent the only restriction fragments identified by each probe. The P16ink4 gene homozygous deletions are evident in one PNET (Case 18) and three malignant astrocytomas (Cases 18, 20, and 6). Gene expression was normalized to the hybridization signal for D9S19. Hybridization with a CDK4 probe revealed amplification in two malignant astrocytomas (Cases 9 and 11) but none of the PNETs. No instances of CYCLIN D1 amplification were evident in either group.
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carcinomas. This type of gene alteration was observed in seven of 20 malignant astrocytomas examined in the present study. Interestingly, the nine cases that exhibited P16ink4a homozygous deletion or CDK4 amplification were restricted to the adult cases in this series; these gene alterations were not evident in any of the four pediatric malignant astrocytomas.

Although investigators have found no instances of P16ink4a homozygous deletions among 14 medulloblastomas studied previously, we detected a single case of P16ink4a homozygous deletion in this series of PNETs. Consequently, these results indicate that P16ink4a gene inactivation may be of importance to the development of a small subgroup of these tumors. In contrast to the malignant astrocytomas, however, disruptions of the p16–cdk4 growth-regulatory pathway appear to be uncommon in PNETs, and our results suggest that distinct genetic mechanisms are associated with each group of tumors.

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References

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