Advances in Brief

Lack of p16INK4 or Retinoblastoma Protein (pRb), or Amplification-associated Overexpression of cdk4 Is Observed in Distinct Subsets of Malignant Glial Tumors and Cell Lines

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Abstract

In this study the expression of p16INK4, retinoblastoma protein (pRb), and cdk4 proteins have been examined in 18 malignant glioma cell lines and in 45 malignant glial tumors. Loss of p16INK4 expression associated with p16INK4 gene homozygous deletion was evident in 12 cell lines and in 10 primary tumors. Lack of p16INK4 expression was also evident in five tumors for which there was no evidence of p16INK4 gene homozygous deletion. Two of the cell lines and six of the primary tumors in which p16INK4 was present were determined to overexpress cdk4 in association with CDK4 gene amplification. Absence of pRb was determined in two of the cell lines and in ten of the tumors. In total, 16 of 18 cell lines and 25 of 45 tumors showed either a lack of p16INK4 or pRb or amplification-associated overexpression of cdk4. Two additional tumors showed an absence of pRb and p16INK4, and one tumor showed a lack of pRb combined with amplification-associated overexpression of cdk4. These results suggest a common growth-regulatory mechanism that is disrupted in gliomas by either suppressing the expression of p16INK4 or pRb or by increasing the expression of cdk4.

Introduction

Control of cell proliferation is managed by a series of checkpoints that regulate cell cycle progression (1). pRb function is central to at least one of these checkpoints, and loss of pRb function is known to occur through inactivating RB gene mutations in a number of cancers (reviewed in Ref. 2). Recently, alternative genetic mechanisms that would presumably act to negatively regulate pRb function have been described. One of these, amplification of the CDK4 gene, the protein product of which inactivates pRb by phosphorylation, has been shown in soft tissue sarcomas and in glial tumors (3–6). Alternatively, inactivating mutations of the p16INK4 gene, the protein product of which prevents pRb phosphorylation by negatively regulating cdk4, has been demonstrated in many types of human tumors and tumor cell lines (4–9).

Because the RB, p16INK4, and CDK4 gene alterations that have been described in human cancers would each result in the loss or suppression of pRb function, it is reasonable to infer that multiple disruptions of the growth-regulatory mechanism defined by these proteins would be redundant, and that the lack of pRb or p16INK4 or the increased expression of cdk4 would occur in distinct subsets of tumors. Several recent reports indicate this to be the case. For instance, the absence of p16INK4 protein or the overexpression of cdk4 has been shown among different groups of malignant gliomas (6); this relationship had been suggested previously by studies demonstrating the occurrence of CDK4 amplification as an alternative mechanism to p16INK4 gene homozygous deletion in glial tumors and cell lines (4, 5). In addition, a reciprocal relationship has been demonstrated between pRb inactivation and a lack of p16INK4 expression in primary lung cancers and in lung cancer cell lines (10, 11); this relationship has been shown in other types of tumor cell lines as well (12–14).

Limited information is available, however, regarding relationships between the expression of cdk4, pRb, and p16INK4 within the same set of tumors or cell lines. Here we have examined the expression of these proteins in 45 malignant gliomas and in 18 cell lines established from gliomas. Our results indicate that the absence of p16INK4 or pRb or the amplification-associated overexpression of cdk4 most often occurs singularly and in distinct subsets of these tumors, thereby, suggesting that multiple disruptions of this growth-regulatory mechanism are of an infrequent nature.

Materials and Methods

Cell Lines and Tumors. The identities and sources of the cell lines used in this study are: U87, U118, U138, U373, A172, H4, T98G, HS683, SW1783, and CCFSTTGl (ATCC); TP265, TP483, and TP365 (V. P. Collins, Tumor Pathology, Karolinska Hospital, Stockholm, Sweden); D32 and D37 (D. D. Bigner, Department of Pathology, Duke University, Durham, NC); A1235 (S. A. Aaronson, Rutenberg Cancer Center, Mount Sinai Medical Center, New York, NY); and MO67 (R. S. Day, Cross Cancer Institute, Edmonton, Alberta) and U251 (J. Ponten, Uppsala University, Uppsala, Sweden). The cell lines were established from malignant gliomas, all but three of which (HS683, H4, and D32) were classified as glioblastomas, the most common and malignant of glial tumors. Tumor specimens were obtained directly from samples taken during surgery at Emory University Hospital, frozen in liquid nitrogen, and stored at −80°C for periods of time between 1 week and 6 months before use for DNA or protein extraction. All tumors analyzed in this study were classified as glioblastomas.

Nucleic Acid Extraction and Analysis. Cell line RNAs were isolated as described previously (4), electrophoresed through formaldehyde-agarose gels (1.0%), and blot-transferred and fixed to a nylon-based nitrocellulose membrane (nitroplus; Micron Separations, Inc.) by baking in a vacuum oven at 80°C for 2 h. Tumor DNAs were isolated, digested with HindIII, electrophoresed through 0.8% agarose, and transferred and fixed to nitrocellulose membranes (15).

DNA and RNA filters were hybridized with 32P-labeled probes from the following loci: RB (clone pLRbRNL; ATCC); β-actin (clone HBFCC83; ATCC); p16INK4 (exon 1 probe PCR synthesized from normal genomic DNA by using conditions and primers described in Ref. 7); CDK4 (genomic probe PCR synthesized by using conditions and primers described in Ref. 3); and D9S19 (4.4-kb EcoRI fragment form clone CRI-L1623; ATCC). After hybridization, filters were washed and exposed to X-ray film (15).

p16INK4 Gene Dosage Analysis. The absorbance of the HindIII restriction fragment identified by the p16INK4 probe was determined by scanning X-ray films with a Bio-Rad model 670 imaging densitometer and analyzing resulting images with the Molecular Analyst (Bio-Rad) software program. Autoradiographic signal responses for p16INK4 hybridizations were normalized against...
those associated with the syntenic D9S19 locus. Tumor DNAs for which the normalized pl6INK4 expression for abrogating pRb function. Two cell lines were examined by Northern analysis for the lack of expression patterns of the proteins in question (Fig. 1B). Twelve of these proteins (Table 1). examined showed aberrant expression of the types noted for one of ones failing to show RB transcript. The specimens constituting each of amplification; and cell lines not expressing pRb protein were the only showed high-level overexpression of CDK4 mRNA and CDK4 gene tions; cell lines showing high-level overexpression of cdk4 protein were the only ones having incurred pl6INK4 gene homozygous dele protein were the only ones not expressing pl6INK4 mRNA, which pl16INK4, and CDK4 gene products in glioma cell lines. A, 10-µg samples of total RNA were electrophoresed through a denaturing 1.0% agarose gel and blot transferred to a nitrocellulose membrane that was hybridized with a probe from the retinoblastoma locus. The autoradiogram shows a lack of RB transcript in two cell lines (SW1783 and D32). The filter was rehybridized with a probe for β-actin to assess the uniformity of sample loading. B, 100-µg quantities of total protein were electrophoresed through a denaturing 8–16% polyacrylamide gel and electrobotted onto a nitrocellulose membrane. The filter was incubated with anti-p16INK4 antibody and then incubated with appropriate secondary antibody, the binding of which was detected by enhanced chemiluminescence. The filter was stripped of primary-secondary antibody complexes and the procedure was repeated with antibodies against each of the remaining proteins indicated. The reciprocal nature existing between the lack of p16INK4 expression, lack of pRb expression, or high-level overexpression of cdk4 is evident.
A, 100-μg quantities of total tumor protein were analyzed as described in Fig. 1B. The with a CDK4 probe shows high level CDK4 gene amplification in samples 11-16. The analysis) >/6INK4 signal response relative to that associated with D9S19, a syntenic specimens 1-10 as revealed by their decreased (>80% as determined by densitometric instances (two of which, 2 and 13, are shown here). B, 5-μg quantities of HindIII-digested tumor DNA was electrophoresed through a 0.8% agarose gel and blot transferred to a nitrocellulose membrane. The filter was sequentially analyzed with probes from each of the three loci indicated. The autoradiograms show homozygous p16INK4 deletions in specimens 1–10 as revealed by their decreased (>80% as determined by densitometric analysis) p16INK4 signal response relative to that associated with D9S19, a syntenic marker located in band 9p21 that we had determined previously to reside outside of the region of 9p21 homozygous deletion in glioma cell lines (21). Rehybridization of the filter with a CDK4 probe shows high level CDK4 gene amplification in samples 11–16. The results in Lanes 1-7 and 13-16 correspond with those shown in Lanes 1-7 and 10-13, respectively, in A.

Table 1. Expression of p16INK4, pRB, and cdk4 in glioblastomas and cell lines

<table>
<thead>
<tr>
<th>Cell lines (n = 18)</th>
<th>p16INK4-</th>
<th>Rb-</th>
<th>cdk4+++</th>
<th>p16INK4+/cdk4+++</th>
<th>p16INK4- / pRB-</th>
<th>pRB-/cdk4+++</th>
<th>p16INK4+/pRB+/cdk4++</th>
</tr>
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<tbody>
<tr>
<td>Total (n = 63)</td>
<td></td>
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"- lack of expression; +, low or moderate expression; ++, high-level, amplification-associated expression.

Gliomas were examined by Western blot analysis. Lack of p16INK4 expression was evident in 15 cases, high-level overexpression of cdk4 was apparent in 6 specimens, and 10 of these tumors did not express pRB (examples shown in Fig. 2A; Table 1 for data summary).

With respect to genetic mechanisms associated with the patterns of p16INK4 expression, diminished p16INK4 gene content suggesting p16INK4 gene homozygous deletion was apparent in 10 of the 15 tumors for which a lack of p16 protein was evident (Fig. 2B). In three additional cases for which no p16INK4 protein was detected, densitometric analysis of Southern blot autoradiograms indicated p16INK4 gene hemizygous deletions (data not shown). For cdk4, each of the six cases showing high-level overexpression of this protein were determined to have CDK4 gene amplification (Fig. 2B); there was complete concordance between the detection of this gene alteration and the detection of high-level overexpression of cdk4.

In most cases, a lack of p16INK4 or pRB, or amplification-associated overexpression of cdk4 was observed to occur singularly and in distinct subsets of tumors (25 of 45 cases; see specimens 1 and 3–12 in Fig. 2A). In one case, a lack of p RB was observed with amplification-associated overexpression of cdk4 (specimen 13 in Fig. 2A), and in two cases a lack of p16INK4 and pRB was observed (see specimen 2 in Fig. 2A). In total, 28 of the 45 tumors showed aberrant p16INK4, pRB, and/or cdk4 expression of the types noted (Table 1).

Discussion

The p16INK4 protein acts as a negative regulator of cell growth and proliferation through its binding to cdk4 and by preventing it from forming an active complex with cyclin D proteins (16). In its active state, cdk4 phosphorylates the retinoblastoma protein, which results in pRB inactivation and, thereby, promotes transit through the G1 phase of the cell cycle (1, 17). As a consequence of the proposed interactions between the p16INK4, cdk4, and pRB, several recent studies have sought to address the nature of the patterns of expression of these proteins in various types of tumors and tumor cell lines. Currently, reciprocal relationships have been demonstrated between the lack of p16INK4 expression and overexpression of cdk4 in glioblastomas (6) and the lack of p16INK4 expression and the functional inactivation of pRB (including lack of pRB expression) in various types of tumor cell lines, as well as in lung carcinomas (10–14).

Here we have examined the expression of each of these three proteins in a series of gliomas and cell lines. Among the cell lines, lack of p16INK4 or pRB expression or amplification-associated overexpression of cdk4 was observed in 16 of 18 cases (89%), and there was no overlap between the members constituting each group. Among the 45 tumors examined, lack of p16INK4 expression, lack of pRB expression, and/or high-level overexpression of cdk4 was observed in 28 of 45 cases (62%). Of the 44 tumors and cell lines showing aberrant expression of at least one of these proteins (70% of all specimens tested), 41 involved pRB, p16INK4, or cdk4 only, whereas 3 involved pRB with either p16INK4 or cdk4. The propensity for the occurrence of singular (95%) rather than combined aberrant expression of pRB, p16INK4, or cdk4 in these specimens supports the concept that these proteins do interact to form a critical cell cycle checkpoint and that the suppression of pRB or p16INK4 or the increased expression of cdk4 is sufficient to disrupt this regulatory mechanism in a
manner that favors cell proliferation. The fact that combined aberrant expression was observed in three cases is not entirely unexpected because certain mutations in this regulatory pathway may lead to diminution rather than loss of function. Although such mutations would be favored during tumor growth, a second alteration in the pathway resulting in complete loss of function would not be truly redundant. Alternatively, the determination of aberrant pRb expression combined with the aberrant expression of p16INK4 or cdk4 may indicate that p16INK4 and cdk4 have other effects on cell proliferation distinct from and/or in addition to those mediated by pRb.

Examination of the Western blot data for the tumors and cell lines with detectable p16INK4 revealed that its expression was generally higher in specimens lacking pRb or showing amplification-associated overexpression of cdk4. Increased expression of p16 in the absence of functional pRb has been noted previously and interpreted as indicating that pRb activity inhibits p16INK4 synthesis (11, 12, 18). This explanation could be extended to account for the increased levels of p16INK4 in cases showing amplification-associated overexpression of cdk4 for which pRb activity would presumably be suppressed.

For 13 of the 15 tumors showing a lack of p16INK4 expression, Southern analysis of corresponding DNAs revealed either homozygous (ten cases) or hemizygous (three cases) p16INK4 gene deletion. In two cases there was no evidence of p16INK4 gene deletion, thereby indicating the existence of other mechanisms for suppressing the expression of p16INK4 in gliomas (6). For cdk4, high-level overexpression of this protein kinase was always associated with gene amplification (six cases). Southern analysis of the DNAs from the ten tumors and cell lines showing a lack of pRb expression failed to reveal RB gene homozygous deletions or rearrangements in any case (data not shown). We have shown previously, however, that the RB region of chromosome 13 is a frequent site of loss of heterozygosity in malignant gliomas (15). Gross deletion of the RB gene as indicated by loss of heterozygosity, combined with more subtle RB gene alterations (e.g., point substitutions, microrearrangements, etc.), could lead to loss of pRb expression. In fact, others have shown that inactivating RB genes mutations resulting in suppressed expression of pRb occurs in a significant proportion of malignant gliomas (19, 20).

In total, the data presented here and in other studies (4, 15, 19, 20) indicate that a corresponding genetic alteration is usually associated with the aberrant expression of pRb, p16INK4, or cdk4 in gliomas. The frequency at which such aberrations occur, in combination with the proposed model relating the interactions of these proteins, suggests that inactivation of pRb function is important to, and perhaps necessary for, the development of malignant glioma.

Acknowledgments

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References

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