0023-6837/01/8101-077$03.00/0
LABORATORY INVESTIGATION
Vol. 81, No. 1, p. 77, 2001
Printed in U.S.A.

Promoter Hypermethylation of the *RB1* Gene in Glioblastomas

Mitsutoshi Nakamura, Yasuhiro Yonekawa, Paul Kleihues, and Hiroko Ohgaki

International Agency for Research on Cancer (MN, PK, HO), Lyon, France; and Department of Neurosurgery (YY), University Hospital, Zürich, Switzerland

SUMMARY: Loss of expression of the retinoblastoma gene (*RB1*) has been shown to occur in up to 25% of glioblastomas (WHO Grade IV). To elucidate the underlying mechanism, we assessed *RB1* promoter hypermethylation using methylation-specific polymerase chain reaction and *RB1* expression by immunohistochemistry in 35 primary (de novo) glioblastomas and in 21 secondary glioblastomas that had progressed from low-grade diffuse astrocytoma (WHO Grade II) or anaplastic astrocytoma (WHO Grade III). Promoter hypermethylation was significantly more frequent in secondary (9 of 21, 43%) than in primary glioblastomas (5 of 35, 14%; $p = 0.0258$). There was a clear correlation between loss of *RB1* expression and promoter hypermethylation. In the majority of glioblastomas with loss of *RB1* expression, there was promoter hypermethylation (11 of 13, 85%), whereas 93% of tumors with *RB1* expression had a normal *RB1* gene status ($p < 0.0001$). In three glioblastomas, areas with and without *RB1* expression were microdissected; promoter hypermethylation was detected only in areas lacking *RB1* expression. In patients with multiple biopsies, methylation of the *RB1* promoter was not detectable in the less malignant precursor lesions, ie, low-grade diffuse and anaplastic astrocytoma. These results indicate that promoter hypermethylation is a late event during astrocytoma progression and is the major mechanism underlying loss of *RB1* expression in glioblastomas. (*Lab Invest 2001, 81:77–82*).

Glioblastoma multiforme (WHO Grade IV) is the most frequent and malignant neoplasm of the human nervous system. The majority of glioblastomas develop rapidly in older patients (mean age, approximately 55 years) after a short clinical history and without clinical or histological evidence of a less malignant precursor lesion (primary or de novo glioblastoma). Secondary glioblastomas manifest in younger patients (mean age, approximately 40 years) through progression from low-grade diffuse astrocytoma (WHO Grade II) or anaplastic astrocytoma (WHO Grade III) (Kleihues and Ohgaki, 1999). Recent studies have shown that these glioblastoma subtypes develop through different genetic pathways (Biernat et al, 1997; Kleihues and Ohgaki, 1999; Lang et al, 1994; Tohma et al, 1998; von Deimling et al, 1992; Watanabe et al, 1996). Primary glioblastomas are characterized by *EGFR* amplification/overexpression, *PTEN* mutations, $p16^{INK4a}$ homozygous deletion, and loss of heterozygosity (LOH) on chromosomes 10p and 10q (Biernat et al, 1997; Fujisawa et al, 2000; Kleihues and Ohgaki, 1999; Tohma et al, 1998; Watanabe et al, 1996), whereas secondary glioblastomas contain frequent *p53* mutations and show LOH preferentially on chromosomes 19q and 10q (Fujisawa et al, 2000; Kleihues and Ohgaki, 1999; Nakamura et al, 2000b; Watanabe et al, 1996, 1997).

The retinoblastoma gene (*RB1*) at chromosome 13q14 was originally identified as the gene responsible for the development of retinoblastomas (Lee et al, 1987). It functions as a negative regulator of cell proliferation at the G1/S checkpoint of the cell cycle by complexing with cellular proteins, such as the transcriptional factor E2F (Harbour and Dean, 2000; Weinberg, 1995). LOH at the *RB1* locus has been found in 25–45% of glioblastomas (Burns et al, 1998; Henson et al, 1994; Ichimura et al, 1996; Nakamura et al, 2000b; Ueki et al, 1996); however, sequencing of all 27 exons of the *RB1* gene revealed inactivating mutations in only 5–12% of cases (Henson et al, 1994; Ichimura et al, 1996, 2000; Ueki et al, 1996), suggesting that other tumor suppressor gene(s) are present on chromosome 13q14. Like *RB1* mutations in retinoblastomas (Hogg et al, 1993), most *RB1* mutations in glioblastomas result in a truncated pRB protein (Henson et al, 1994; Ueki et al, 1996) that does not enter the nucleus. Homozygous deletions in the *RB1* locus have been reported in a small fraction of glioblastomas (3 of 120, 3%) (Ichimura et al, 1996). Loss of *RB1* expression was detected by immunohistochemistry in 5–27% of glioblastomas (Biernat et al, 1997; Burns et al, 1998; Henson et al, 1994; Nakamura et al, 1996; Ueki et al, 1996); however, there was no clear correlation between loss of *RB1* expression and LOH at the *RB1* locus (Burns et al, 1998; Henson et al, 1994; Ueki et al, 1996).

The objective of the present study was to assess *RB1* promoter hypermethylation in primary and sec-

Received September 12, 2000.
This study was supported by a grant from the Foundation for Promotion of Cancer Research, Japan.
Address reprint requests to: Dr. Hiroko Ohgaki, Unit of Molecular Pathology, International Agency for Research on Cancer, 69372 Lyon, France. E-mail: ohgaki@iarc.fr

ondary glioblastomas as a potential underlying mechanism of loss of *RB1* expression. To assess the timing of these alterations during astrocytoma progression, we also analyzed less malignant precursor lesions, ie, low-grade diffuse astrocytomas and anaplastic astrocytomas, from the same patients.

Results

RB1 Promoter Hypermethylation

RB1 promoter hypermethylation was detected in 14 of 56 (25%) glioblastomas (Tables 1 and 2). Promoter methylation was significantly more frequent in secondary than in primary glioblastomas (14% versus 43%, $p = 0.0258$, Tables 1 and 2).

RB1 methylation was not detected in any of 15 low-grade diffuse astrocytomas or 10 anaplastic astrocytomas (Table 2). Methylated and unmethylated control DNAs showed the expected fragment size of 163 bp (Fig. 1).

pRB Immunohistochemistry

In 28 of 35 (80%) primary and 15 of 21 (71%) secondary glioblastomas, pRB immunoreactivity was detected, but the fraction of positive tumor cells varied considerably (Table 1). Six (17%) primary and four (19%) secondary glioblastomas showed loss of pRB expression throughout the section (Table 1).

Correlation between RB1 Methylation and pRB Immunoreactivity

Of 13 glioblastomas with loss of *RB1* expression, 11 tumors (85%) showed promoter hypermethylation, whereas 40 of 43 (93%) cases with pRB immunoreactivity did not show *RB1* methylation (Table 1, $p < 0.0001$).

In three glioblastomas (Table 1, Cases 292, 59, and 70), most tumor areas showed *RB1* expression, but there were also focal areas with neoplastic cells that lacked *RB1* expression (Fig. 2). In all three cases, promoter hypermethylation was detected only in areas lacking pRB immunoreactivity but not in the areas with *RB1* expression (Fig. 1, Table 1).

Discussion

The essential promoter region of *RB1* lies 185–206 bp upstream of the initiation codon and contains putative binding sites for the transcription factors RBF-1, Sp1, ATF, and E2F (Ohtani-Fujita et al, 1993). It contains CpG islands that are frequent sites of methylation (Ohtani-Fujita et al, 1993; Stirzaker et al, 1997). Hypermethylation in the *RB1* promoter region has been reported in 13% of unilateral retinoblastomas (Greger et al, 1994; Sakai et al, 1991) and 27% of pituitary adenomas (Simpson et al, 2000), suggesting that promoter hypermethylation is an alternative mechanism for mutational loss of *RB1* expression.

In this study, one quarter of the glioblastomas showed promoter hypermethylation in the *RB1* gene. *RB1* methylation was detected more frequently in secondary than in primary glioblastomas. There was a clear correlation between loss of pRB expression detected by immunohistochemistry and promoter hypermethylation: the majority of glioblastomas with loss of *RB1* expression had *RB1* promoter hypermethylation (11 of 13, 85%), whereas the majority (93%) of tumors with *RB1* expression had normal *RB1* gene status. These results indicate that promoter hypermethylation is the major mechanism underlying the loss of *RB1* function in glioblastomas. This was supported by the finding that in three glioblastomas, promoter hypermethylation was detected only in areas lacking pRB immunoreactivity but not in the areas where *RB1* was expressed.

Loss of *RB1* expression has been found to correlate well with LOH at the *RB1* locus in hepatocellular carcinomas (Zhang et al, 1994), bladder carcinomas (Xu et al, 1993), and malignant neuroendocrine lung carcinomas (Gouyer et al, 1994). However, it did not correlate with LOH at the *RB1* locus in other tumors, including glioblastomas (Burns et al, 1998; Ueki et al, 1996), pituitary tumors (Pei et al, 1995; Simpson et al, 1999), and carcinomas of the breast (Borg et al, 1992), prostate (Cooney et al, 1996), ovary (Dodson et al, 1994; Kim et al, 1994), and head and neck (Yoo et al, 1994). Simpson et al (2000) recently showed that the majority of pituitary adenomas with loss of *RB1* expression without LOH on the *RB1* locus had promoter hypermethylation. The present study confirms this mechanism and suggests that the lack of correlation between *RB1* expression and LOH on the *RB1* locus in glioblastomas frequently reflects promoter methylation as the underlying cause of loss of *RB1* expression.

Hypermethylation has been considered one of the mechanisms of inactivation of the *RB1* gene in the two-hit theory, because the majority of retinoblastomas with promoter hypermethylation showed LOH on the *RB1* locus (Greger et al, 1994; Sakai et al, 1991). However, there is recent evidence that hypermethylation without LOH at this locus may be sufficient to cause loss of *RB1* expression. A majority of pituitary adenomas with hypermethylation showed loss of *RB1* expression without LOH at the *RB1* locus (Simpson et al, 2000). We correlated *RB1* expression with LOH on *RB1* locus in 24 previously published glioblastomas (Nakamura et al, 2000b). Six of nine (67%) glioblastomas with hypermethylation showed loss of *RB1* expression, and all of these retained heterozygosity on the *RB1* locus (data not shown).

Loss of *RB1* expression appears to be a prognostic factor in several human neoplasms, including glioblastomas. The mean survival of patients with glioblastomas showing *RB1* expression was 11 months, whereas for those lacking *RB1* expression it was 6 months (Nakamura et al, 1996). Similarly, the mean survival of patients with nonsmall cell lung carcinomas with loss or altered *RB1* expression was 18 months and therefore significantly shorter than that of patients with *RB1* expression (32 months) (Xu et al, 1994). Kornblau et al (1998) reported that the level of expres-

Table 1. pRB Expression and *RB1* Promoter Hypermethylation in Glioblastomas

Patient ID	Age/Sex	Location	pRB IHC[a]	*RB1* promoter hypermethylation
Primary (de novo) glioblastoma				
93	65/F	T	+	—
94	50/M	T	+++	—
95	58/M	O	+++	—
96	61/F	TO	+++	—
97	35/F	FT	−	—
98	68/F	F	−	Methylation
99	34/M	T	−	—
101	46/F	TO	++	—
103	48/M	P	+++	—
104	39/F	T	+++	—
106	73/M	FT	+	—
107	51/M	FT	++	—
111	70/F	FT	+++	—
112	69/F	TO	+++	—
132	58/M	FP	++	—
133	55/F	T	+++	—
134	58/F	F	+	—
135	63/M	T	+	—
136	63/M	F	+++	—
138	63/F	T	−	Methylation
139	73/M	PO	++	—
140	47/M	P	++	—
141	58/F	TPO	+++	—
233	59/M	T, BG	+++	—
256	56/F	T	++	—
257	50/M	F, BG	+++	—
258	65/F	F	+++	—
288	69/M	P	+++	—
292	62/M	P	+++[b]	Methylation[c]
294	36/M	P	+++	—
296	63/F	VE	−	Methylation
300	71/F	TO	+++	—
301	68/F	TP	+++	—
314	40/M	F	−	Methylation
344	47/M	P	++	—
Secondary glioblastoma				
11	33/F	VE	+	—
25	44/F	T	+	Methylation
26	32/F	F	++	—
33	47/M	F	−	Methylation
35	53/M	T	++	—
51	40/F	F	++	—
57	26/M	F	++	—
58	33/M	FT	−	Methylation
59	40/M	FT	+++[b]	Methylation[c]
60	31/F	T	+++	—
65	46/M	T	+	—
68	52/F	TPO	+++	—
70	52/M	PO	++[b]	Methylation[c]
72	28/F	F	+	Methylation
85	41/F	TP	+	—
295	47/M	FT	−	Methylation
3	23/F	T	+++	—
10	45/F	T	−	Methylation
13	30/F	FP	+	Methylation
37	74/M	T	+	—
79	25/F	TP	+	—

IHC, immunohistochemistry; F, frontal; T, temporal; P, parietal; O, occipital; VE, paraventricle; BG, basal ganglia.

[a] The results of pRB immunohistochemistry were recorded as −, positive staining in < 5% of the tumor cells; +, positive staining in 5%–25% of the tumor cells; ++, positive staining in 25%–50% of the tumor cells; and +++, positive staining in > 50% of the tumor cells.

[b] Indicates the presence of areas with clusters of tumor cells lacking pRB expression.

[c] Methylation was detected in only the tumor areas lacking pRB expression.

Table 2. Promoter Hypermethylation of the *RB1* Gene in Astrocytic Brain Tumors

	WHO grade	No. of cases	No. of cases with promoter hypermethylation
Primary glioblastoma	IV	35	5 (14%)
Secondary glioblastoma	IV	21	9 (43%)*
Glioblastoma total	IV	56	14 (25%)
Anaplastic astrocytoma	III	10	0
Low-grade diffuse astrocytoma	II	15	0

* Significantly more frequent than in primary glioblastomas ($p = 0.0258$).

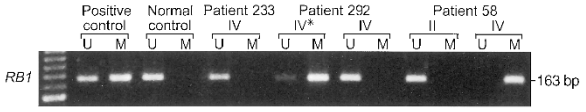

Figure 1.

Methylation-specific PCR of CpG islands of the *RB1* promoter in low-grade diffuse astrocytomas (II) and glioblastomas (IV). In glioblastoma 233, only unmethylated DNA (U) was present. In glioblastoma 292, *RB1* methylation (M) was restricted (*asterisk*) to areas lacking pRB immunoreactivity (Figure 2B). In Patient 58, the low-grade diffuse astrocytoma biopsy showed unmethylated status, whereas the glioblastoma derived from it contained a hypermethylated promoter region. Positive control for unmethylated and methylated DNA; normal control, DNA from a normal blood sample.

sion of pRB determined by immunohistochemistry was a strong prognostic factor in acute myelogenous leukemia, inferior survival rates being associated with no or low *RB1* expression. In invasive transitional carcinomas of the bladder, loss of *RB1* expression, together with p53 protein accumulation, was associated with significantly shorter survival (Cote et al, 1998). Non-small cell lung cancer with pRB−/p53+ also showed significantly shorter survival (5 year survival 20%) in comparison with pRB+/p53− tumors (5 year survival 73%) (Dosaka-Akita et al, 1997).

It has been reported that loss of *RB1* expression is associated with a higher grade of malignancy in several human neoplasms. The majority of parathyroid carcinomas (88%) showed abnormal *RB1* expression, whereas none of parathyroid adenomas analyzed had loss of *RB1* expression (Cryns et al, 1994). A fraction of endometrial carcinomas lacked *RB1* expression, whereas all hyperplastic lesions showed normal *RB1* levels (Tsuda et al, 2000). In the present study, we observed *RB1* promoter methylation only in glioblastomas and not in the less malignant precursor lesions, ie, low-grade diffuse and anaplastic astrocytoma, suggesting that loss of *RB1* expression is a late event during astrocytoma progression.

Materials and Methods

Tumor Samples and DNA Extraction

The surgical specimens of brain tumors were obtained from patients treated between 1977 and 1994 in the Department of Neurosurgery, University Hospital of Zürich, Switzerland. Tumors were fixed in buffered formalin and embedded in paraffin. Pathological diagnosis was made according to the WHO classification (Kleihues and Cavenee, 2000). Thirty-five patients with primary glioblastoma had a preoperative clinical history of less than 3 months (mean, 1.5 months) and histologic diagnosis of a glioblastoma at the first biopsy, without any evidence of a less malignant precursor lesion. Twenty-one patients with secondary glioblastoma had at least 2 biopsies, with clinical and histologic evidence of progression from low-grade diffuse astrocytoma (16 cases, WHO Grade II) or anaplastic astrocytomas (5 cases, WHO Grade III). Low-grade diffuse astrocytomas (15 cases) and anaplastic astrocytomas (5 cases) from the same patients with secondary glioblastomas as well as five additional anaplastic astrocytomas were also examined. DNA was extracted from paraffin sections as described previously (Brüstle et al, 1992). In three glioblastomas (Cases 292, 59, and 70), pRB immunopositive or negative tumor areas were clearly recognized. These areas were carefully microdissected and analyzed separately.

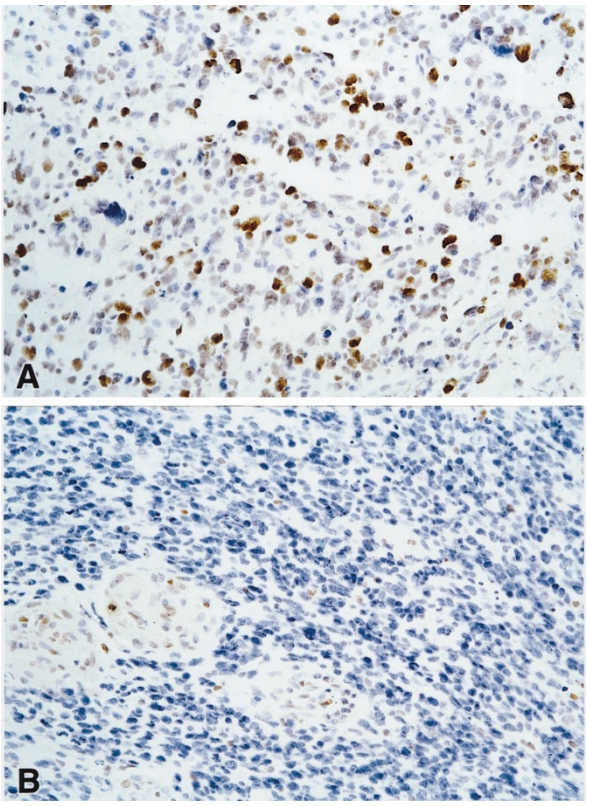

Figure 2.

A, pRB immunohistochemistry showing nuclear immunoreactivity in the majority of glioblastoma cells (Case 70). B, Loss of *RB1* expression in clusters of undifferentiated neoplastic cells in a glioblastoma (Case 70), whereas vascular endothelial cells show *RB1* expression. Magnification, ×155.

Methylation-Specific Polymerase Chain Reaction for RB1 Promoter Hypermethylation

DNA methylation patterns in the CpG islands of the *RB1* gene were determined by methylation-specific

polymerase chain reaction (MSP) (Herman et al, 1996). Sodium bisulfite modification was performed using the CpGenome DNA Modification Kit (Intergen, Oxford, United Kingdom) as described previously (Nakamura et al, 2001). Primer sequences of *RB1* for the methylated and unmethylated reaction were as previously reported (Simpson et al, 2000). The PCR was carried out in a 10 μl volume containing PCR buffer (20 mM Tris pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, dNTPs (250 μM each), primers (4 pmol each), 0.5 unit of PLATINUM *Taq* DNA polymerase (GIBCO BRL, Cergy Pontoise, France), and approximately 40 ng bisulfite-modified DNA. Amplification was carried out in a DNA Thermal Cycler 480 (Perkin-Elmer Cetus, Norwalk, Connecticut) with initial denaturing at 95° C for 5 minutes followed by 35 cycles of denaturing at 95° C for 1 minute, annealing for 45 seconds at 65° C (for methylated *RB1*) or 61° C (for unmethylated *RB1*), extension for 1 minute at 72° C, and then a final extension for 5 minutes at 72° C. Amplified products were electrophoresed on a 3% agarose gel and were visualized with ethidium bromide.

pRB Immunohistochemistry

pRB immunohistochemistry was performed using the pRB monoclonal antibody (clone G3–245, which recognizes *RB1* exons 9–12; PharMingen, San Diego, California) as previously described (Biernat et al, 1997) for Cases 233–344 and 295. The results of pRB immunohistochemistry in other cases were previously reported (Biernat et al, 1997). Fractions of positive cells were recorded as follows: positive in 5–25% tumor cells (+), positive in 25–50% tumor cells (++), or positive in >50% tumor cells (+++). pRB immunoreactivity in less than 5% of tumor cells or presence of clusters of tumor cells showing complete loss of *RB1* expression was regarded as negative (Biernat et al, 1997).

References

Biernat W, Tohma Y, Yonekawa Y, Kleihues P, and Ohgaki H (1997). Alterations of cell cycle regulatory genes in primary (de novo) and secondary glioblastomas. Acta Neuropathol 94:303–309.

Borg A, Zhang QX, Alm P, Olsson H, and Sellberg G (1992). The retinoblastoma gene in breast cancer: Allele loss is not correlated with loss of gene protein expression. Cancer Res 52:2991–2994.

Brüstle O, Ohgaki H, Schmitt HP, Walter GF, Ostertag H, and Kleihues P (1992). Primitive neuroectodermal tumors after prophylactic central nervous system irradiation in children. Association with an activated K-*ras* gene. Cancer 69:2385–2392.

Burns KL, Ueki K, Jhung SL, Koh J, and Louis DN (1998). Molecular genetic correlates of p16, cdk4, and pRb immunohistochemistry in glioblastomas. J Neuropathol Exp Neurol 57:122–130.

Cooney KA, Wetzel JC, Merajver SD, Macoska JA, Singleton TP, and Wojno KJ (1996). Distinct regions of allelic loss on 13q in prostate cancer. Cancer Res 56:1142–1145.

Cote RJ, Dunn MD, Chatterjee SJ, Stein JP, Shi SR, Tran QC, Hu SX, Xu HJ, Groshen S, Taylor CR, Skinner DG, and Benedict WF (1998). Elevated and absent pRb expression is associated with bladder cancer progression and has cooperative effects with p53. Cancer Res 58:1090–1094.

Cryns VL, Thor A, Xu HJ, Hu SX, Wierman ME, Vickery AL, Jr, Benedict WF, and Arnold A (1994). Loss of the retinoblastoma tumor-suppressor gene in parathyroid carcinoma. N Engl J Med 330:757–761.

Dodson MK, Cliby WA, Xu HJ, DeLacey KA, Hu SX, Keeney GL, Li J, Podratz KC, Jenkins RB, and Benedict WF (1994). Evidence of functional RB protein in epithelial ovarian carcinomas despite loss of heterozygosity at the *RB* locus. Cancer Res 54:610–613.

Dosaka-Akita H, Hu SX, Fujino M, Harada M, Kinoshita I, Xu HJ, Kuzumaki N, Kawakami Y, and Benedict WF (1997). Altered retinoblastoma protein expression in nonsmall cell lung cancer: Its synergistic effects with altered ras and p53 protein status on prognosis. Cancer 79:1329–1337.

Fujisawa H, Reis RM, Nakamura M, Colella S, Yonekawa Y, Kleihues P, and Ohgaki H (2000). Loss of heterozygosity on chromosome 10 is more extensive in primary (de novo) than in secondary glioblastomas. Lab Invest 80:65–72.

Gouyer V, Gazzeri S, Brambilla E, Bolon I, Moro D, Perron P, Benabid AL, and Brambilla C (1994). Loss of heterozygosity at the *RB* locus correlates with loss of RB protein in primary malignant neuro-endocrine lung carcinomas. Int J Cancer 58:818–824.

Greger V, Debus N, Lohmann D, Hopping W, Passarge E, and Horsthemke B (1994). Frequency and parental origin of hypermethylated RB1 alleles in retinoblastoma. Hum Genet 94:491–496.

Harbour JW and Dean DC (2000). Rb function in cell-cycle regulation and apoptosis. Nat Cell Biol 2:E65–E67.

Henson JW, Schnitker BL, Correa KM, von Deimling A, Fassbender F, Xu HJ, Benedict WF, Yandell DW, and Louis DN (1994). The retinoblastoma gene is involved in malignant progression of astrocytomas. Ann Neurol 36:714–721.

Herman JG, Graff JR, Myöhänen S, Nelkin BD, and Baylin SB (1996). Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 93:9821–9826.

Hogg A, Bia B, Onadim Z, and Cowell JK (1993). Molecular mechanisms of oncogenic mutations in tumors from patients with bilateral and unilateral retinoblastoma. Proc Natl Acad Sci USA 90:7351–7355.

Ichimura K, Bolin MB, Goike HM, Schmidt EE, Moshref A, and Collins VP (2000). Deregulation of the $p14^{ARF}$/MDM2/p53 pathway is a prerequisite for human astrocytic gliomas with G_1-S transition control gene abnormalities. Cancer Res 60: 417–424.

Ichimura K, Schmidt EE, Goike HM, and Collins VP (1996). Human glioblastomas with no alterations of the *CDK2A* (*$p16^{INK4A}$*, *MTS1*) and *CDK4* genes have frequent mutations of the retinoblastoma gene. Oncogene 13:1065–1072.

Kim TM, Benedict WF, Xu HJ, Hu SX, Gosewehr J, Velicescu M, Yin E, Zheng J, D'Ablaing G, and Dubeau L (1994). Loss of heterozygosity on chromosome 13 is common only in the biologically more aggressive subtypes of ovarian epithelial tumors and is associated with normal retinoblastoma gene expression. Cancer Res 54:605–609.

Kleihues P and Cavenee WK, editors (2000). Pathology and genetics of tumours of the nervous system, 2nd ed. Lyon: International Agency for Research on Cancer Press.

Kleihues P and Ohgaki H (1999). Primary and secondary glioblastomas: From concept to clinical diagnosis. J Neuro-Oncol 1:44–51.

Kornblau SM, Andreeff M, Hu SX, Xu HJ, Patel S, Theriault A, Koller C, Kantarjian H, Estey E, Deisseroth AB, and Benedict WF (1998). Low and maximally phosphorylated levels of the retinoblastoma protein confer poor prognosis in newly diagnosed acute myelogenous leukemia: A prospective study. Clin Cancer Res 4:1955–1963.

Lang FF, Miller DC, Koslow M, and Newcomb EW (1994). Pathways leading to glioblastoma multiforme: A molecular analysis of genetic alterations in 65 astrocytic tumors. J Neurosurg 81:427–436.

Lee WH, Bookstein R, Hong F, Young LJ, Shew JY, and Lee EY (1987). Human retinoblastoma susceptibility gene: Cloning, identification, and sequence. Science 235:1394–1399.

Nakamura M, Konishi N, Hiasa Y, Tsunoda S, Fukushima Y, Tsuzuki T, Takemura K, Aoki H, Kobitsu K, and Sakaki T (1996). Immunohistochemical detection of CDKN2, retinoblastoma and p53 gene products in primary astrocytic tumors. Int J Oncol 8:889–893.

Nakamura M, Watanabe T, Klangby U, Asker C, Wiman K, Yasuhiro Y, Kleihues P, and Ohgaki H (2001). *P14*^{Arf} deletion and methylation in genetic pathways to glioblastomas. Brain Pathol 11:159–168.

Nakamura M, Yang F, Fujisawa H, Yonekawa Y, Kleihues P, and Ohgaki H (2000b). Loss of heterozygosity on chromosome 19 in secondary glioblastomas. J Neuropathol Exp Neurol 59:539–543.

Ohtani-Fujita N, Fujita T, Aoike A, Osifchin NE, Robbins PD, and Sakai T (1993). CpG methylation inactivates the promoter activity of the human retinoblastoma tumor-suppressor gene. Oncogene 8:1063–1067.

Pei L, Melmed S, Scheithauer B, Kovacs K, Benedict WF, and Prager D (1995). Frequent loss of heterozygosity at the retinoblastoma susceptibility gene (*RB*) locus in aggressive pituitary tumors: Evidence for a chromosome 13 tumor suppressor gene other than *RB*. Cancer Res 55:1613–1616.

Sakai T, Toguchida J, Ohtani N, Yandell DW, Rapaport JM, and Dryja TP (1991). Allele-specific hypermethylation of the retinoblastoma tumor-suppressor gene. Am J Hum Genet 48:880–888.

Simpson DJ, Hibberts NA, McNicol AM, Clayton RN, and Farrell WE (2000). Loss of pRb expression in pituitary adenomas is associated with methylation of the *RB1* CpG island. Cancer Res 60:1211–1216.

Simpson DJ, Magnay J, Bicknell JE, Barkan AL, McNicol AM, Clayton RN, and Farrell WE (1999). Chromosome 13q deletion mapping in pituitary tumors: Infrequent loss of the retinoblastoma susceptibility gene (*RB1*) locus despite loss of *RB1* protein product in somatotrophinomas. Cancer Res 59:1562–1566.

Stirzaker C, Millar DS, Paul CL, Warnecke PM, Harrison J, Vincent PC, Frommer M, and Clark SJ (1997). Extensive DNA methylation spanning the *Rb* promoter in retinoblastoma tumors. Cancer Res 57:2229–2237.

Tohma Y, Gratas C, Biernat W, Peraud A, Fukuda M, Yonekawa Y, Kleihues P, and Ohgaki H (1998). *PTEN (MMAC1)* mutations are frequent in primary glioblastomas (de novo) but not in secondary glioblastomas. J Neuropathol Exp Neurol 57:684–689.

Tsuda H, Yamamoto K, Inoue T, Uchiyama I, and Umesaki N (2000). The role of p16-cyclin D/CDK-pRb pathway in the tumorigenesis of endometrioid-type endometrial carcinoma. Br J Cancer 82:675–682.

Ueki K, Ono Y, Henson JW, Efird JT, von Deimling A, and Louis DN (1996). CDKN2/p16 or RB alterations occur in the majority of glioblastomas and are inversely correlated. Cancer Res 56:150–153.

von Deimling A, von Ammon K, Schoenfeld DA, Wiestler OD, Seizinger BR, and Louis DN (1993). Subsets of glioblastoma multiforme defined by molecular genetic analysis. Brain Pathol 3:19–26.

Watanabe K, Sato K, Biernat W, Tachibana O, von Ammon K, Ogata N, Yonekawa Y, Kleihues P, and Ohgaki H (1997). Incidence and timing of *p53* mutations during astrocytoma progression in patients with multiple biopsies. Clin Cancer Res 3:523–530.

Watanabe K, Tachibana O, Sato K, Yonekawa Y, Kleihues P, and Ohgaki H (1996). Overexpression of the EGF receptor and *p53* mutations are mutually exclusive in the evolution of primary and secondary glioblastomas. Brain Pathol 6:217–224.

Weinberg RA (1995). The retinoblastoma protein and cell cycle control. Cell 81:323–330.

Xu HJ, Cairns P, Hu SX, Knowles MA, and Benedict WF (1993). Loss of RB protein expression in primary bladder cancer correlates with loss of heterozygosity at the RB locus and tumor progression. Int J Cancer 53:781–784.

Xu HJ, Quinlan DC, Davidson AG, Hu SX, Summers CL, Li J, and Benedict WF (1994). Altered retinoblastoma protein expression and prognosis in early-stage non-small-cell lung carcinoma. J Natl Cancer Inst 86:695–699.

Yoo GH, Xu HJ, Brennan JA, Westra W, Hruban RH, Koch W, Benedict WF, and Sidransky D (1994). Infrequent inactivation of the retinoblastoma gene despite frequent loss of chromosome 13q in head and neck squamous cell carcinoma. Cancer Res 54:4603–4606.

Zhang X, Xu HJ, Murakami Y, Sachse R, Yashima K, Hirohashi S, Hu SX, Benedict WF, and Sekiya T (1994). Deletions of chromosome 13q, mutations in *Retinoblastoma 1*, and retinoblastoma protein state in human hepatocellular carcinoma. Cancer Res 54:4177–4182.