

# Methylation analysis of cell cycle control genes *RB1*, *p14<sup>ARF</sup>* and *p16<sup>INK4a</sup>* in human gliomas

## Research Article

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**Abbreviations:** central nervous system, (CNS); methylation-specific polymerase chain reaction, (MSP); neurofibromatosis 1, (NF1); polymerase chain reaction, (PCR); World Health Organisation, (WHO)

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## Summary

Aberrant methylation of CpG islands located in promoter regions is one of the major mechanisms for silencing cancer-related genes in tumor cells. We determined the frequency of aberrant CpG island methylation for three cell cycle control-associated genes *RB1*, *p14<sup>ARF</sup>* and *p16<sup>INK4a</sup>* in 198 glioma biopsies consisting of: 16 pilocytic astrocytomas (World Health Organisation grade I), 26 low-grade diffuse astrocytomas (WHO grade II), 23 anaplastic astrocytomas (WHO grade III), 53 glioblastomas (WHO grade IV: 43 primary and 10 secondary), one giant cell astrocytoma, 24 oligodendrogliomas (WHO grade II), 16 anaplastic oligodendrogliomas (WHO grade III), six oligoastrocytomas (WHO grade II-III), two WHO grade I ependymomas, 24 ependymomas (WHO grade II), five anaplastic (WHO grade III) ependymomas, and two ependymoblastomas (WHO grade IV) as well as in two non-neoplastic brain samples, using methylation-specific polymerase chain reaction (MSP) and sequencing. The three tumor-related genes were unmethylated in the two normal brain control samples. In contrast, 106 of 198 (54%) of the tumors had an abnormal methylation pattern in at least one of the target genes. The overall methylation frequencies for all three genes were: 13% (26/198) for *RB1*; 21% (42/198) for *p14<sup>ARF</sup>*, and 37% (74/198) for *p16<sup>INK4a</sup>*. Some differences may be established regarding the methylation profiles of specific genes and tumor types: pilocytic astrocytomas showed hypermethylation in 44% for *p16<sup>INK4a</sup>* gene and in only 6% of the *p14<sup>ARF</sup>*. Low-grade astrocytomas had two genes (*RB1* and *p16<sup>INK4a</sup>*) with methylation rates >30% and *p14<sup>ARF</sup>* had a lower hypermethylation rate (15%). There were also differences between primary and secondary glioblastomas: *p16<sup>INK4a</sup>* and *RB1* have higher methylation rates in the latter group (60% and 40%, respectively) than in the primary glioblastomas (37% and 12%, respectively). No methylation at all was detected for *RB1* in pure oligodendrogliomas, whereas *p14<sup>ARF</sup>* was hypermethylated at significant rates (46-50%) in both low-grade and anaplastic oligodendrogliomas. In contrast, *p16<sup>INK4a</sup>* was hypermethylated more frequently in low-grade than in anaplastic oligodendrogliomas. Ependymal tumors primarily displayed *p14<sup>ARF</sup>* methylation and lower values for the other two genes. We conclude that methylation is a common mechanism that contributes to inactivating cell cycle control-related genes in glial neoplasms because these genes present a high frequency of aberrant methylation of the 5' CpG island in this study. This aberration seems to occur early in the carcinogenesis process since it is already present in the low-grade forms.

## I. Introduction

Primary brain tumors are neoplasms that originate from various intracranial tissues. About 17,000 new cases occur annually and primary cancer of the central nervous system (CNS) is the cause of death of approximately 13,000 individuals per year (Surawicz et al, 1998). More than 60% of all brain tumors have a glial origin, including pilocytic astrocytoma, low-grade astrocytoma, anaplastic astrocytoma, glioblastoma, oligodendroglioma, anaplastic oligodendroglioma, mixed oligoastrocytoma and low-grade and anaplastic ependymomas (Kleihues and Cavenee, 2000).

Pilocytic astrocytoma (a slow-growing tumor with a World Health Organization (WHO) grade I) is considered to be the most common glioma in children, accounting for 10% of cerebral and 85% of cerebellar astrocytomas. It constitutes the principal CNS neoplasm in neurofibromatosis 1 (NF1) (Burger et al, 2000). Cytogenetic analysis of pilocytic astrocytoma has revealed normal karyotypes or a variety of aberrations, primarily involving gains of chromosomes 7 and 8 (Rey et al, 1987; Karnes et al, 1992; White et al, 1995). Allelic losses at 1p36 or at 17q have been identified in a few cases (von Deimling et al, 1993; Bello et al, 1995), and comparative genomic hybridization analysis identified gains of chromosomes 19, 22 and 9q34.1-qter, and losses of chromosome 19 (Sanoudpu et al, 2000). Regarding *TP53* gene mutations discordant data are available; early studies identified sequence changes in a few tumors (von Deimling et al, 1993), whereas 35% of samples (7 of 20) analyzed by Hayes et al. (1999) displayed mutations of this gene. The only consistent gene alteration described in this astrocytoma subtype is a loss of *NF1* alleles that occurs in up to 90% of informative NF1-associated cases, in contrast to only 4% of sporadic tumors (Burger et al, 2000). LOH analysis at 1p, 10, 17 and 19q, and mutation detection at *TP53*, *p16<sup>INK4a</sup>* and *EGFR* has been performed on 12 samples, including three NF1-associated tumors (Tada et al, 2003). None of the genetic abnormalities commonly detected in higher-grade astrocytomas were found in the sporadic cases. In contrast, LOH 10 and 17q (including the *PTEN* and *NF1* regions, respectively) and homozygous deletion of *p16<sup>INK4a</sup>* were identified in the NF1-associated samples. These data support the hypothesis that some NF1-associated pilocytic astrocytomas would differ genetically from sporadic cases.

Diffuse astrocytic gliomas are the most common primary neoplasm occurring in the CNS and are histologically classified as WHO grade II astrocytomas, WHO grade III anaplastic forms, and WHO grade IV glioblastoma (Kleihues and Cavenee, 2000). Low grade (WHO grade II) tumors and anaplastic grade III astrocytomas usually occur in adults and show a strong tendency toward progression. Glioblastoma, the most malignant subtype of glioma, may develop either from diffuse or anaplastic tumors (secondary glioblastoma) or *de novo* (primary glioblastoma) without a defined prior tumor lesion. Multiple genetic alterations have been identified in these astrocytic neoplasms; these alterations primarily involve inactivation or amplification/over-expression of *TP53*, *p16<sup>INK4a</sup>*, *RBI*, *PTEN*, *MDM2*, and

*EGFR* genes (Kleihues and Cavenee, 2000). Several other non-random anomalies are also characteristic features of these gliomas, including loss of heterozygosity at 1p, 10p, 10q, 11p, 19q and 22q, although the putative tumor suppressing genes remain unidentified. A distinct pattern of involvement of these genes and chromosomal regions characterizes both forms of glioblastoma. The main differences consist of *EGFR* gene amplification and *TP53* mutations, which respectively characterize primary and secondary glioblastomas (Kleihues and Cavenee, 2000).

Tumors with a major oligodendroglial component account for 4% of all primary brain tumors and represent between 5% and 18% of all intracranial gliomas, including oligodendroglioma (WHO grade II), anaplastic oligodendroglioma (WHO grade III) and mixed oligoastrocytoma (Kleihues and Cavenee, 2000). They arise preferentially in the cerebral hemispheres of adult patients with a mean age at diagnosis of ~40 years. Low-grade oligodendrogliomas are characterized by a high incidence of loss of chromosome arms 1p and 19q, and anaplastic forms accumulate allelic losses on the short arm of chromosome 9 and on chromosome 10 (for review see Kleihues and Cavenee, 2000).

Ependymomas represent 3-9% of all intracranial brain tumors and about 60% of spinal tumors, and commonly arise in children (Kleihues and Cavenee, 2000). Cytogenetic and molecular biology studies have demonstrated a preferential involvement of chromosome 22 (by losses), parallel to the inactivation of the *NF2* gene (located at 22q12), primarily in sporadic cord tumors. Additional genomic abnormalities include chromosome 7 gains and overrepresentation of chromosomes 2, 5, 9, 12, 15, 18, 20q and X, and proportional losses of 13q. Losses of 6q and 9p, with gains of 1q, have primarily been found in intracranial ependymomas (Weremowicz et al, 1992; Rubio et al, 1994; Ebert et al, 1999; Hulsebos et al, 1999; Rousseau-Merk et al, 2000; Kraus et al, 2001; Alonso et al, 2002). These findings, thus, suggest that intracranial and spinal cord ependymomas progress along different genetic pathways that may influence differences in the clinical behavior of these gliomas.

Tumorigenesis of gliomas seems to be a multi-step process composed of genetic and epigenetic alterations involving tumor suppressor genes, cell cycle regulatory genes, oncogenes, and as yet unidentified genes located at specific chromosomal regions (Kleihues and Cavenee, 2000). Transcriptional silencing by hypermethylation of CpG islands located in the promoter regions is considered a common epigenetic mechanism for inactivation of tumor-related genes (Esteller, 2003). CpG islands are 0.5-2 Kb regions rich in cytosine-guanine dinucleotides, present in the 5' region of about half of all human genes (Baylin et al, 1998). Little information is available on the CpG island methylation status of neurogenic neoplasms. Isolated previous studies focus on high-grade astrocytomas, primarily the anaplastic forms and glioblastoma multiforme (Costello et al, 1996; Park et al, 2000; Nakamura et al, 2001a; 2001b; Yin et al, 2002; Gonzalez-Gomez et al, 2003a, 2003b; Uhlmann et al, 2003) and less frequently on low-grade astrocytomas (Costello et al, 2000; Gonzalez-Gomez et al, 2003a;

2003b; 2003c; Uhlmann et al, 2003), oligodendrogliomas (Watanabe et al, 2001a; Wolter et al, 2001; Yin et al, 2002; Alonso et al, 2003; Hong et al, 2003; Uhlmann et al, 2003), pilocytic astrocytoma (Gonzalez-Gomez et al, 2003c; Uhlmann et al, 2003) and ependymomas (Rousseau et al, 2003; Alonso et al, 2004).

In the present study we determined the frequency of methylation of three genes: *RBI*, *p14<sup>ARF</sup>* and *p16<sup>INK4a</sup>* in a series of 198 gliomas, including astrocytic, oligodendroglial and ependymal tumors, and in two normal brain tissue samples, using polymerase chain reaction (PCR)-based techniques involving sodium bisulfite modification of DNA (MSP) and sequencing of the PCR products.

## II. Materials and methods

### A. Sample collection and DNA preparation

Fresh tumor tissues were obtained from 198 patients with malignant gliomas, including 16 WHO grade I pilocytic astrocytomas; 26 WHO grade II diffuse astrocytomas; 23 WHO grade III anaplastic astrocytomas; 53 WHO grade IV glioblastomas multiformes (43 primary and 10 secondary); one giant cell astrocytoma; 24 WHO grade II oligodendrogliomas; 16 WHO grade III anaplastic oligodendrogliomas, six WHO grade II-III mixed oligo-astrocytomas; two WHO grade I ependymomas, 24 WHO grade II ependymomas, five anaplastic (WHO grade III) ependymomas, and two ependymoblastomas (WHO grade IV) as well as two nonneoplastic brain samples. Tumors were diagnosed according to the WHO guidelines (Kleihues and Cavenee, 2000), and the tumor cell content was estimated by histologic examination to be approximately 75-90%. DNA was prepared from frozen tissues using standard methods, as described (Rey et al, 1992).

### B. Bisulfite treatment of DNA, methylation-specific polymerase chain reaction (MSP) and sequencing

Bisulfite modification of genomic DNA was performed as reported (Herman et al, 1996). Briefly, 2µg of genomic DNA was denatured with 2mol/L NaOH (37°C for 10 min), followed by incubation with 3mol/L sodium bisulfite (pH 5.0) at 55-56°C

for 16 hours in the dark. After treatment, DNA was purified using the DNA clean-up Kit (Promega, Madison, WI) as recommended by the manufacturer, incubated with 3mol/L NaOH (room temperature for 5 min), precipitated with 10mol/L ammonium acetate and 100% ethanol, washed with 70% ethanol and re-suspended in 30 µl distilled water. The primer sequences of these genes for the methylated and unmethylated reactions were as reported (Xing et al, 1999; Simpson et al, 2000). PCR was performed for the methylated and unmethylated alleles using a thermal cycler in standard conditions with variable (55-66°C) annealing temperatures. Each PCR reaction (20µl) was loaded directly onto non-denaturing 6% polyacrylamide gels or 2-3% agarose gels, stained with ethidium bromide, and visualized under UV illumination. Samples giving signals approximately equivalent to the positive control were designated as methylated. As positive control for methylated alleles, we used DNA (from lymphocytes of healthy volunteers) treated with SssI methyltransferase (New England Biolabs), then subjected to bisulfite treatment. To verify the identity of PCR products, they were purified and sequenced (after PCR re-amplification with the same primer set) using the ABI PRISM Big-Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems) on the Applied Biosystem model 3100 or 377 DNA sequencers. Each amplicon was sequenced bidirectionally.

## III. Results

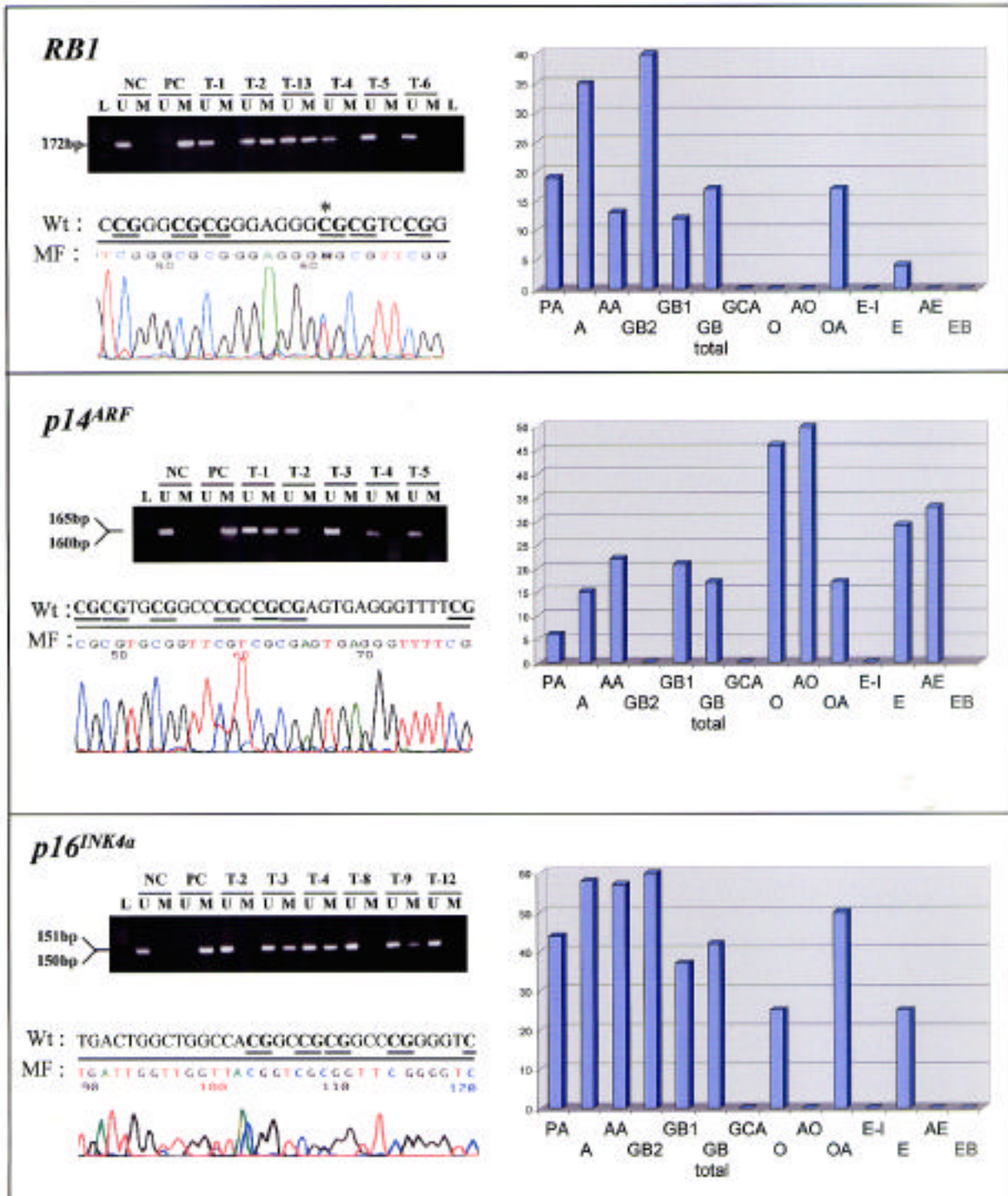
Examples of methylation of all three genes, sequences from methylated alleles aligned against the corresponding wild type sequence, and bar diagrams that illustrate the methylation frequency of each gene in the different histological glioma subtypes are shown in **Figure 1**.

All but 92 study samples displayed CpG island hypermethylation in at least one gene (54%). The methylation frequency of the *RBI*, *p14<sup>ARF</sup>* and *p16<sup>INK4a</sup>* genes was 13%, 21%, and 37%, respectively. In contrast, methylation of these genes did not occur in either of the two normal brain samples. The methylation frequencies for the three genes in every tumor type studied are shown in **Table 1**.

**Table 1:** Methylation frequencies of cell-cycle control genes in histological subtypes of gliomas

Tumor type	<i>RBI</i>	(%)	<i>p14<sup>ARF</sup></i>	(%)	<i>p16<sup>INK4a</sup></i>	(%)
PA	3/16	(19)	1/16	(6)	7/16	(44)
A	9/26	(35)	4/26	(15)	15/26	(58)
AA	3/23	(13)	5/23	(22)	13/23	(57)
Secondary GB	4/10	(40)	0/10	(0)	6/10	(60)
Primary GB	5/43	(12)	9/43	(21)	16/43	(37)
Total GB	9/53	(17)	9/53	(17)	22/53	(42)
GCA	0/1	(0)	0/1	(0)	0/1	(0)
O	0/24	(0)	11/24	(46)	6/24	(25)
AO	0/16	(0)	8/16	(50)	2/16	(12.5)
OA	1/6	(17)	1/6	(17)	3/6	(50)
E-I	0/2	(0)	0/2	(0)	0/2	(0)
E	1/24	(4)	2/6	(33)	6/24	(25)
AE	0/5	(0)	1/3	(33)	0/5	(0)
EB	0/2	(0)	N.D.	--	0/2	(0)

PA: Pilocytic Astrocytoma; A: grade II Astrocytoma; AA: Anaplastic Astrocytoma; GB: Glioblastoma; GCA: Giant Cell Astrocytoma; O: grade II Oligodendroglioma; AO: Anaplastic Oligodendroglioma; OA: Mixed Oligo-astrocytoma; E-I: grade I Ependymoma; E: grade II Ependymoma; AE: Anaplastic Ependymoma; EB: Ependymoblastoma. N.D. Not done



**Figure 1.** Examples of MSP analysis for *RB1*, *p14<sup>ARF</sup>*, and *p16<sup>INK4a</sup>* in gliomas. Lane U, amplified product with primers recognizing unmethylated sequence; lane M, amplified product with primers recognizing methylated sequence. NC: negative control of methylation; PC: positive control of methylation; L: ladder. The forward sequences of representative methylated tumors are shown. Sequences are aligned against the wild-type (Wt) sequences showing a change of C to T by bisulfite treatment. C in CpG dinucleotides that were methylated remains unaffected. The frequency of aberrant methylation for all three genes in every glioma subgroup is shown on the right. (The tumor nomenclature is the same described in Table 1).

*RB1* was primarily methylated in astrocytic and mixed tumors, with frequencies ranging from 13% (in anaplastic astrocytoma) to 40% in secondary glioblastoma. On the other hand, pure oligodendrogliomas and ependymomas presented 0-4% aberrant *RB1* methylation. In contrast, *p14<sup>ARF</sup>* hypermethylation rates were higher in

oligodendroglial tumors (46% and 50% in low-grade and anaplastic oligodendrogliomas, respectively), and values of about 20-30% characterized *p14<sup>ARF</sup>* in anaplastic astrocytomas and ependymomas. On the other hand pilocytic astrocytomas and secondary glioblastoma displayed a 0-6% methylation rate for this gene. The

*p16<sup>INK4a</sup>* gene was hypermethylated by >35% of each subtype of astrocytic tumor, including pilocytic astrocytomas. However, the methylation rate for this gene was lower than 25% in pure oligodendrogliomas and ependymal tumors.

As shown in **Table 2**, simultaneous promoter methylation of two or three genes was demonstrated in some tumors as follows: *RB1* and *p16<sup>INK4a</sup>* in 7% (14/198) of cases, *p16<sup>INK4a</sup>* together with *p14<sup>ARF</sup>* in 8% (15/198) of cases, and *RB1* plus *p14<sup>ARF</sup>* in 0.5% (1/198) of cases. Three gliomas showed hypermethylation of all three genes (1.5%). Promoter methylation of either *RB1* or *p16<sup>INK4a</sup>* was present in 41% of samples (82/198).

In most tumors, gene hypermethylation was always accompanied by amplification of the unmethylated reaction (**Figure 1**). This finding was expected, since the tumor specimens were macroscopically isolated samples that contained both tumor and a small fraction of non-malignant tissues. Alternatively, cytosine hemimethylation, that is the cytosine on one chromosome is methylated but its homologue on the other chromosome is not, might also explain the finding. Sequencing of the corresponding methylated PCR products demonstrated the presence of invariable CpG as was expected (**Figure 1**).

#### IV. Discussion

The results presented here clearly demonstrate that CpG island hypermethylation of cell-cycle control genes is a frequent event in gliomas, as methylation of one or more genes was observed in 54% of the analyzed samples (106 of 198). Our study shows that hypermethylation rates vary by gene, and that it occurs in most instances at early stages of gliomagenesis, because it is already present in the low-grade forms. This was primarily evident for *p16<sup>INK4a</sup>*, which presented methylation rates >40% in low-grade astrocytic tumors such as pilocytic astrocytoma and WHO grade II tumors. For its part *p14<sup>ARF</sup>* was hypermethylated in <15% of these low-grade astrocytomas.

*RB1* is located on the long arm of chromosome 13 (13q14) and is the classical example of a tumor suppressor gene. It encodes a nucleoprotein (pRB) that plays a key role in the cell cycle regulation complexes that govern the G1-S transition of cells, thus allowing mitosis and cell division (Friend et al, 1986; Lee et al, 1987).

**Table 2:** Isolated and concurrent methylation frequencies of cell-cycle control genes in gliomas

Gene	Frequency	
<i>RB1</i>	13%	(26/198)
<i>p14<sup>ARF</sup></i>	21%	(42/198)
<i>p16<sup>INK4a</sup></i>	37%	(74/198)
<i>RB1 + p14<sup>ARF</sup></i>	0.5%	(1/198)
<i>RB1 + p16<sup>INK4a</sup></i>	7%	(14/198)
<i>p14<sup>ARF</sup> + p16<sup>INK4a</sup></i>	8%	(15/198)
<i>RB1 + p14<sup>ARF</sup> + p16<sup>INK4a</sup></i>	1.5%	(3/198)
<i>RB1 or p16<sup>INK4a</sup></i>	41%	(82/198)

Loss of *RB1* function has been described in a variety of tumor types, and hypermethylation of the promoter is recognized as an important *RB1* silencing mechanism (Ohtani-Fujita et al, 1993). *RB1* methylation was most frequent in astrocytic neoplasms in our tumor series and, in agreement with previous reports (Nakamura et al, 2001a; Gonzalez-Gomez et al, 2003a), we found *RB1* promoter methylation more frequently in secondary (40%) than in primary (12%) glioblastomas. The non-random frequencies (13-35%) of aberrant *RB1* methylation that we detected in the low-grade and anaplastic astrocytomas might be indicative of a subgroup of astrocytic tumors that further develop towards secondary glioblastoma, thus displaying a more aggressive biological behavior. In fact, loss of *RB1* expression has been associated with a higher grade of malignancy in several human neoplasms (Cryns et al, 1994; Tsuda et al, 2000). In oligodendroglial and ependymal tumors we found very low rates or no methylation of this gene. Since *RB1* inactivating mutations are also infrequent in these tumors (Gonzalez-Gomez et al, 2003a), the inactivation of the *RB1* cell cycle control pathways should occur through the silencing of another alternative genes. However, oligo-astrocytomas presented a 17% rate of aberrant *RB1* methylation, probably corresponding to the astrocytic component in these mixed tumors.

Low rates of *p14<sup>ARF</sup>* hypermethylation were found in the pilocytic astrocytoma group (6%) and low-grade astrocytomas (15%) and this frequency increased slightly in anaplastic astrocytomas (22%). Loss of *p14<sup>ARF</sup>* expression has been shown to be an important event in the genetic pathways for the development of both primary and secondary glioblastoma (Nakamura et al, 2001b; Ghimenti et al, 2003). Although promoter hypermethylation of this gene has been reported as more frequent in secondary tumors (Nakamura et al, 2001b), we found 21% *p14<sup>ARF</sup>* methylation in primary glioblastoma and no secondary glioblastoma in our series displayed this anomaly. This finding may be due to the small number of secondary tumors included in our study; *p14<sup>ARF</sup>* promoter methylation has been proposed as an early event in a subset of low-grade astrocytomas (as occurs in our tumor series) that may undergo malignant progression to secondary glioblastoma. Some of these highly malignant (secondary) tumors display homozygous deletion of *p14<sup>ARF</sup>* (Nakamura et al, 2001b). With regard to oligodendroglial tumors, previous studies on *p14<sup>ARF</sup>* methylation have shown variable results: Dong et al (2001) found 2%, whereas Wolter et al (2001) found 41%, and Watanabe et al (2001b) detected *p14<sup>ARF</sup>* methylation in 21% of grade II oligodendrogliomas and 15% of the anaplastic forms. Our data show 46% and 50% in grade II and grade III forms, respectively, whereas 17% of our mixed tumors showed epigenetic alteration in this gene; these figures are closer to those we detected in astrocytomas. The epigenetic inactivation of *p14<sup>ARF</sup>* is a frequent alteration in ependymal tumors, since about 30% of the samples (low-grade and anaplastic forms) displayed this alteration in our series. These findings agree with the data recently provided by Rousseau et al (2003), who detected 21% epigenetic change in this gene in this glioma subtype and, in

agreement with their data, aberrant  $p14^{ARF}$  hypermethylation was more frequently identified in the intracranial ependymal tumors. An inverse correlation has been reported for  $p14^{ARF}$  and  $TP53$  mutations in glioblastomas (Ichimura et al, 2000). Since oligodendrogliomas and ependymomas rarely present  $TP53$  inactivating mutations (for review see Kleihues and Cavenee, 2000), silencing of the  $p14^{ARF}$  gene through aberrant promoter methylation may be a mechanism to inactivate the  $p14^{ARF}/MDM2/TP53$  cell-cycle signaling pathway in these glioma subtypes. Recent data have demonstrated a high percentage of  $p14^{ARF}$  inactivation in glioblastomas with classical, astrocytic or oligodendroglial differentiation areas (Ghimentì et al, 2003), suggesting again that silencing this gene is an important step in tumorigenesis and/or progression of distinct glioma subtypes. Aberrant methylation was the molecular gene silencing mechanism in some of those cases.

Oligodendrogliomas and ependymomas showed a lower rate of  $p16^{INK4a}$  promoter hypermethylation than astrocytic tumors. We found a 25% rate of  $p16^{INK4a}$  aberrant hypermethylation in oligodendrogliomas and a 12.5% rate in the anaplastic forms, as well as a 25% rate in the low-grade ependymomas. Dong et al, (2001), Walter et al (2001) and Watanabe et al, (2001) also showed variable results in methylation studies of this gene. They respectively report rates of 12%, 32% and 0%. Rates of about 21% aberrant methylation in the  $p16^{INK4a}$  gene were identified in ependymomas (Rousseau et al, 2003; Alonso et al, 2004). Variable frequencies of  $p16^{INK4a}$  hypermethylation have also been reported in secondary glioblastoma, and range from 70% to <5% (Costello et al, 1996; Fueyo et al, 1996; Hegi et al, 1997; Burns et al, 1998; Nakamura et al, 1998; Schmidt et al, 1998; Park et al, 2000; Yin et al, 2002; Gonzalez-Gomez et al, 2003). Our results corroborate the proposal that a high rate of  $p16^{INK4a}$  CpG island hypermethylation is characteristic of both primary and secondary glioblastoma subtypes and also of the lower grade astrocytic forms. A 44% rate for  $p16^{INK4a}$  CpG island hypermethylation was found in the pilocytic astrocytomas included in our series. The mixed oligo-astrocytoma group was different from the pure oligodendrogliomas, perhaps due to their astrocytic component. Interestingly, an association between a worse prognosis and  $p16^{INK4a}$  inactivation either by deletion/mutation or aberrant promoter methylation has been recently reported in oligodendrogliomas (Bortolotto et al, 2000).

Concurrent aberrant hypermethylation was identified in several cases; for instance methylation occurred in both  $p16^{INK4a}$  and either  $RBI$  or  $p14^{ARF}$  in 7-8% of cases, whereas only one sample (0.5%) displayed concurrent  $RBI$  plus  $p14^{ARF}$  methylation. Accordingly, promoter hypermethylation of the  $p14^{ARF}$  gene seems to be independent of the methylation status of  $p16^{INK4a}$  even though their promoters are very close to each other on 9p21 and they share two exons, albeit in different reading frames (Sherr et al, 1996; Kamijo et al, 1998), and they are frequently co-deleted in glioblastoma (Newcomb et al, 2000). The transcriptional activity of  $p14^{ARF}$  is regulated independently of  $p16^{INK4a}$  and participates in a regulatory

feedback loop with  $p53$  and  $MDM2$  (Kamijo et al, 1998). The  $p16^{INK4a}$  protein function is closely related to  $RBI$  in cell cycle regulation; it regulates G1-S phase transition by inhibiting the activity of cyclin-dependent kinases CDK4 and CDK6. This cell-cycle control pathway was inactivated in 41% of the gliomas we studied, since methylation of both or either the  $RBI$  or  $p16^{INK4a}$  gene occurred. Some cases in our series displayed a concurrent aberrant hypermethylation of both  $RBI$  and  $p16^{INK4a}$  that might represent a redundant epigenetic alteration of this cell-cycle control pathway.

In conclusion, this study demonstrates that CpG island methylation of cell-cycle control genes is a common event in gliomas. It generally occurs at early stages of carcinogenesis, since it is already present in the low-grade forms. Some differences in the pattern of gene methylation were observed: hypermethylation of  $p14^{ARF}$  occurred more frequently in oligodendrogliomas and ependymomas than in astrocytic tumors. On the other hand  $p16^{INK4a}$  and  $RBI$  were frequently methylated in astrocytic gliomas. In agreement with previous data (Gonzalez-Gomez et al, 2003c; Uhlmann et al, 2003) our findings suggest an important role for epigenetic changes in the development of pilocytic astrocytoma, a glial tumor in which no consistent genetic alteration has been identified previously. Finally, epigenetic inactivation of the cell-cycle control genes in some glioma subtypes might be indicative or predictive of an aggressive behavior:  $RBI$  or  $p14^{ARF}$  in astrocytic tumors (Nakamura et al, 2001a; 2001b), or  $p16^{INK4a}$  in oligodendrogliomas (Bortolotto et al, 2000). Accordingly, therapies addressed to promoting re-expression of these genes (Swanton, 2004) might be useful in the management of glioma patients. An accurate glioma-subtype histological diagnosis together with an unequivocal identification of samples displaying promoter hypermethylation, in combination with gene mutational/expression analyses, will contribute to optimizing the clinical application of molecular characterization of gliomas; this should lead to a firm establishment of predictive prognostic factors and specific therapies.

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