Alterations in the RB1 pathway in low-grade diffuse gliomas lacking common genetic alterations

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Running title: Alterations in the RB1 pathway in diffuse gliomas

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Abstract
We recently reported that the vast majority (>90%) of low-grade diffuse gliomas (diffuse astrocytoma, oligoastrocytoma and oligodendroglioma) carry at least one of the following genetic alterations: IDH1/2 mutation, TP53 mutation or 1p/19q loss. Only 7% of cases were triple-negative (i.e. lacking any of these alterations). In the present study, array CGH in 15 triple-negative WHO grade II gliomas (8 diffuse astrocytomas and 7 oligodendrogliomas) showed loss at 9p21 (p14ARF, p15INK4b, p16INK4a loci) and 13q14–13q32 (containing the RB1 locus) in 3 cases and 2 cases, respectively. Further analyses in 31 triple-negative cases as well as a total of 160 non-triple-negative cases revealed that alterations in the RB1 pathway (homozygous deletion and promoter methylation of the p15INK4b, p16INK4a, and RB1 genes) were significantly more frequent in triple-negative (26%) than in non-triple-negative cases (11%; P=0.0371). Multivariate analysis after adjustment for age, histology and treatment showed that RB1 pathway alterations were significantly associated with unfavorable outcome for patients with low-grade diffuse glioma [hazard ratio, 3.024 (1.279–6.631); P=0.0057]. These results suggest that a fraction of low-grade diffuse gliomas lacking common genetic alterations may develop through a distinct genetic pathway, which may include loss of cell-cycle control regulated by the RB1 pathway.

INTRODUCTION
The present World Health Organization (WHO) classification recognizes three main histologic types of WHO grade II low-grade diffuse glioma: diffuse astrocytoma, oligoastrocytoma, and oligodendroglioma (14). We have recently reported that at least one of the following alterations – IDH1/2 mutation, TP53 mutation or 1p/19q loss – is present in the vast majority (93%) of low-grade diffuse gliomas; the most frequent combinations were IDH1/2 mutations plus TP53 mutations, IDH1/2 mutations plus 1p/19q loss, or IDH1/2 mutations only (11). As a means to predict patient survival, molecular classification based on these alterations showed similar power to histological classification (11). In 7% of all cases, none of these genetic alterations were detected (triple-negative cases) (11), suggesting the possibility of alternative genetic pathway(s) in the development of low-grade diffuse glioma.

In the present study, we used array comparative genomic hybridization (aCGH) to screen 15 triple-negative cases in order to identify chromosomal loci associated with their development. Since the results showed loss at 9p21 (p14ARF, p15INK4b, p16INK4a loci) and 13q14–13q32 (containing the RB1 locus), we then screened for alterations in the RB1 pathway (homozygous deletion and promoter methylation of the p15INK4b, p16INK4a and RB1 genes) and alterations in the p14ARF gene (homozygous deletion and promoter methylation), a member of the TP53 pathway in a larger number of triple-negative (31 cases) and non-triple-negative (160 cases) low-grade diffuse gliomas.
MATERIALS AND METHODS

Tumor samples

Tumor samples were obtained from the Department of Neuropathology, University Hospital Zurich, Switzerland (88 cases); the Department of Neuropathology, University Hospital Frankfurt, Germany (45 cases); the Institute of Neuropathology and Department of Neurosurgery, University Hospital Muenster, Germany (24 cases); the Department of Neuropathology and Neurosurgery, University Hospital Essen (16 cases), Germany; the Department of Pathology, Gunma University, Japan (6 cases); the Institute of Neuroscience, Bordeaux, France (6 cases); and the Department of Neurosurgery, University Hospital Bern, Switzerland (6 cases). We collected clinical data, including age and sex of the patient, location of tumors, histological diagnosis, date of surgical resection, extent of surgery, other treatment (radiotherapy, chemotherapy), date of last follow-up or last contact, and date of death. Patients who died within four months after surgery were excluded from the survival analysis in order to eliminate cases in which death was attributable to surgery and surgical complications. Before genetic analyses, histology review was carried out as previously described (11). All tumors were subjected to gross total or subtotal resection, without therapy prior to surgery. Needle-biopsy cases were not included in this study.

Analyses showed that thirty-one tumors were triple-negative, i.e. lacked IDH1/2 mutations, TP53 mutations, and 1p/19q loss (11). Histologically, these tumors were classified as diffuse astrocytoma WHO grade II (16 cases), oligoastrocytoma WHO grade II (2 cases) and oligodendroglioma WHO grade II (13 cases). The clinical and histological features of these triple-negative low-grade diffuse gliomas are shown in Table 1. These triple-negative cases, as well as a total of 160 non-triple-negative cases (50 cases with IDH1/2 mutation only, 54 cases with IDH1/2 plus TP53 mutations, and 56 cases with IDH1/2 mutation plus 1p/19q loss; or, as classified histologically, 77 diffuse astrocytomas, 43 oligoastrocytomas, 40 oligodendrogliomas) published previously (11), were screened for alterations in the RB1 and TP53 pathways.

DNA extraction

DNA was extracted from formalin-fixed paraffin-embedded histological sections as previously described (9). Briefly, tumor areas were scraped from the histological slide, and were deparaffinized in xylene for 15 min and then in 100% ethanol for 10 min. The pellets were dried in acetone and washed with 0.4% Tween 20 solution and then with PBS (pH 7.4), before being dissolved in 400 μl 1M NaSCN solution. After overnight incubation at 37 °C, samples were suspended in 400 μl of DNA extraction buffer, composed of 360 μl of ATL buffer and 40 μl of proteinase K (DNeasy Mini kit, Qiagen, Valencia, CA), and were incubated overnight at 55 °C. Additional proteinase K (40 μl) was added 12 hours and 24 hours later, with a total incubation time of 60 hours. After incubation with 8 μl RNase (100 mg/ml) for 10 min at room temperature (RT), 420 μl ATL buffer was added and the samples
were separated into two parts (each 450 μl). Each part was mixed with 450 μl AL buffer and 450 μl 100% ethanol, and incubated at RT for 5 min. The samples were loaded into DNeasy Mini spin columns (Qiagen). After washing the columns with AW1 buffer and drying the column membrane with 80% ethanol, the purified genomic DNA was eluted with 25 μl nuclease-free H2O. The DNA concentration was determined by spectrophotometer (NanoDrop Technologies, Wilmington, USA). Absorption was measured at 230, 260 and 280 nm and the DNA quality was evaluated by A260/A230 and A260/A280 ratios.

**Array CGH**

Array comparative genomic hybridization (aCGH) analysis was carried out as described previously (9). Briefly, the genomic profile changes of paired DNA samples were compared using a 2× 105K CGH oligonucleotide microarray (Agilent Technologies, Santa Clara, CA; 15.0 Kb average probe resolution) according to the manufacturer’s instructions. Briefly, the sample (1 μg) and the sex-matched reference DNA were chemically labeled with ULS-Cy5 and ULS-Cy3, respectively, at 85°C for 30 min using an Oligo aCGH labeling kit for FFPE samples (Agilent). The labeled samples were purified with the genomic DNA purification module (Agilent), combined, mixed with human Cot-1 DNA, denatured at 95°C using a Oligo aCGH hybridization kit (Agilent), and were applied to microarrays. After hybridization at 65°C for 40 hours, microarrays were washed in Oligo aCGH wash buffer 1 at RT for 5 min and in wash buffer 2 at 37°C for 1 min. After drying, the microarrays were scanned with a DNA microarray scanner G2565BA (Agilent) and data (log2) were extracted from the raw microarray image files using Feature Extraction software version 9. Data were analyzed by DNA Analytics software (version 3.5) with default filter settings. The aberration detection method 2 (ADM2) algorithm with fuzzy zero correction was used to define aberrant intervals. The log2 ratio of < -1.0 at the region of interest was considered to represent homozygous loss, and values of -1.0 to -0.2 were considered to represent heterozygous loss (24).

**Differential PCR**

The analysis for homozygous deletion of the p14ARF, p15 INK4b, p16 INK4a (at 9p21) and the RB1 (at 13q14) genes were carried out by using differential PCR, as previously reported (20). The CF gene sequence was used as a reference (25). For the detection of homozygous deletion of the p14ARF, p15 INK4b, p16 INK4a and RB1 genes, the following primers were used: 5’-ACC CCG CTT TCG TAG TTT-3’ (sense) and 5’-AAA TGG ACA TTT ACG GTA GTG G-3’ (antisense) for p14ARF and p16 INK4a (PCR product, 101 bp), 5’-AAT TTT TGG AAC AAA GAT AAT GGA A-3’ (sense) and 5’-CCT CTA ATG ATT GAG TGC TTA AGT GA-3’ (antisense) for p15 INK4b (PCR product, 100 bp), 5’-AAA ACT GTA CAT TTA AAA TTT TGT CTA TG-3’ (sense) and 5’-CAC AAC ATC AGA CCA TTA AGA CTC-3’ (antisense) for RB1 (PCR product, 104 bp) and 5’-GGC ACC ATT AAA GAA AAT ATC ATC TT-3’ (sense) and 5’-TGT GGC ATG CTT TGA TGA CGC TTC-3’ (antisense) for CF.
(PCR product, 79 bp). PCR was carried out with 28 cycles, with an annealing temperature at 55°C. The PCR products were loaded on 8% acrylamide gels and stained with ethidium bromide. The mean ratio of signal of $p14^{ARF}$, $p15^{INK4b}$, $p16^{INK4a}$ and $RB1$ to $CF$ of normal DNA (14 samples of normal tissue) was approximately 1.0. Samples with a ratio of ≤ 0.2 were considered to present deletions (28).

**Methylation-specific PCR**

Screening with methylation-specific PCR for promoter methylation of $p14^{ARF}$, $p15^{INK4b}$, $p16^{INK4a}$ and $RB1$ was performed as described previously (16, 17). Briefly, approximately 300 ng of DNA extracted from paraffin sections was modified with sodium bisulfite using an EZ DNA Methylation Kit™ (Zymo Research, Orange, CA, USA). Briefly, DNA was denatured with dilution buffer at 37 °C for 15 min, incubated with CT conversion reagent at 50 °C for 16 hours, and cleaned up and desulfonated by using columns. The primer sequences of $p14^{ARF}$, $p15^{INK4b}$, $p16^{INK4a}$ and $RB1$ for the methylated and unmethylated PCR have been reported previously (16, 17). DNA extracted from normal blood samples were used as negative controls, and CpGenome™ Universal methylated DNA (Chemicon International Inc., Temecula, Calif, USA) was used as a positive control for methylated DNA, as previously described (16, 17).

**Statistical analyses**

The $\chi^2$ test or the Fisher’s exact test was conducted to analyze the significance of the association between triple-negative status and alterations in the RB1 and TP53 pathways. Statistical analysis was performed with Stat-View for Windows 5.01 software® (SAS Institute Inc., Cary, NC). For survival analyses, the Kaplan-Meier method was used. Multivariate analyses were performed with adjustment for age, sex, and treatment (surgery and radiotherapy, surgery only).

**RESULTS**

**Array CGH**

Array CGH was carried out for 15 triple-negative low-grade diffuse gliomas. Chromosomal imbalances observed in at least two cases and confirmed by differential PCR were loss at 9p21, containing the $p14^{ARF}$, $p15^{INK4b}$, and $p16^{INK4a}$ loci (three cases), and loss at 13q14–13q32, containing the $RB1$ locus (two cases) (Fig. 1). In one case, array CGH showed loss at 10p13 (C10orf30), which was confirmed by differential PCR (data not shown).

**Alterations in the $p14^{ARF}$, $p15^{INK4b}$, $p16^{INK4a}$ and $RB1$ genes**

Homozygous deletion and promoter methylation of the $p14^{ARF}$, $p15^{INK4b}$, $p16^{INK4a}$ and $RB1$ genes were assessed in 31 triple-negative and 160 non-triple-negative low-grade diffuse gliomas. Alterations in the RB1 pathway were more frequent in triple-negative than in non-
triple-negative cases (26% vs 11%; \( P = 0.0371 \); Table 2). In particular, homozygous deletion of \( p15^{\text{INK4b}} \) was significantly more frequent in triple-negative than in non-triple-negative cases (13% vs 1%; \( P = 0.0069 \); Table 2).

Among triple-negative cases, 6 diffuse astrocytomas and 2 oligodendrogliomas showed alterations in the RB1 pathway, whereas 10 diffuse astrocytomas, 2 oligoastrocytomas, and 11 oligodendrogliomas lacked alterations in the RB1 pathway (Table 1). Gemistocytic components were observed in 4 out of 6 triple-negative diffuse astrocytomas (67%) with an alteration in the RB1 pathway, although the fraction of gemistocytes did not reach > 20% to fulfill the diagnostic criteria of gemistocytic astrocytomas (14). In contrast, gemistocytic components were not recognized in any of the 10 triple-negative diffuse astrocytomas without alterations in the RB1 pathway (Table 1) and in only 5 out of 77 (6%) non-triple negative diffuse astrocytomas.

Promoter methylation of the \( p14^{\text{ARF}} \) was significantly less frequent in triple-negative than in non-triple-negative tumors (3% vs 21%; \( P = 0.0199 \); Table 2). Overall, alterations in the TP53 pathway were also significantly less frequent in triple-negative than in non-triple-negative cases (6% vs 49%; \( P = 0.0001 \); Table 2). This tendency was consistent when we analyzed each histologic type separately, but a significant difference was observed only in diffuse astrocytoma (data not shown).

Clinical outcome
The mean survival of patients with triple-negative and non-triple-negative tumors was not markedly different (63.0 ± 57.9 months vs 67.6 ± 48.8 months; \( P = 0.6603 \)). Among all low-grade diffuse gliomas, multivariate analysis after adjusting for age, sex, histology, and treatment showed that alterations in the RB1 pathway were significantly associated with shorter survival [hazard ratio, 3.024 (1.279–6.631); \( P = 0.0057 \)].

Among triple-negative tumors, median survival of patients with diffuse astrocytoma with an alteration in the RB1 pathway was 14.0 months, significantly shorter than that of patients with diffuse astrocytoma lacking RB1-pathway alterations (49 months, \( P = 0.0187 \); Fig. 2). Among triple-negative cases without alterations in the RB1 pathway, patients with oligodendroglioma or oligoastrocytoma had a significant longer survival than those with diffuse astrocytoma (median, 74 vs 49 months; \( P = 0.0475 \); Fig. 2).

DISCUSSION
The cyclin D-CDK4,CDK6/INK4/RB1/E2F pathway plays a key role in controlling cell growth by integrating multiple mitogenic and anti-mitogenic stimuli (19). The CDK4-cyclin D1 complex phosphorylates the RB1 protein, thereby inducing release of the transcription factor E2F, which activates genes involved in the G1→S transition (3, 23). The members of the INK4 family (\( p16^{\text{INK4a}} \), \( p15^{\text{INK4b}} \), \( p18^{\text{INK4c}} \), and \( p19^{\text{INK4d}} \)) block the progression of the cell cycle by binding to either CDK4 or CDK6 and inhibiting the action of cyclin D (5). In addition
to their capacity to arrest cells in the G1 phase of the cell cycle, the INK4 proteins participate in a number of cellular processes, including senescence, apoptosis, and DNA repair (5). Since the RB1 signaling pathway is deregulated by genetic and epigenetic alterations in a variety of human tumors (7, 18), certain molecules in the pathway are being considered as targets for cancer therapy (6).

In contrast to glioblastoma, in which frequent alterations in the RB1 pathway (40–70% of cases) have been reported (4, 18), there are few such reports for low-grade diffuse glioma. Alterations in the RB1 pathway have been reported in 6 out of 46 (13%) diffuse astrocytomas (RB1 methylation in one case, p16INK4a methylation in 3 cases, p15INK4b methylation or homozygous deletion in 3 cases) (27). We have previously shown that alterations in the RB1 pathway are rare in oligodendrogliomas (WHO grade II) (4%), but frequent (65%) in anaplastic oligodendrogliomas (WHO grade III) (29).

In order to identify novel genetic pathways to triple-negative low-grade diffuse gliomas, which lack IDH1/2 mutation, TP53 mutation or 1p/19q loss, we first carried out array CGH analyses. Chromosomal imbalances that were observed in at least two cases were loss at 9p21 (p14ARF, p15INK4b, p16INK4a loci) and loss at 13q14–13q32 (containing the RB1 locus). Since these two chromosomal loci contain genes involved in the RB1 signaling pathway (p15INK4b, p16INK4a, RB1) (5), we further assessed alterations in these genes in triple-negative as well as non-triple negative low-grade diffuse gliomas. We found that approximately one quarter (26%) of triple-negative low-grade diffuse gliomas carry alterations in the RB1 pathway, as represented by p15INK4b homozygous deletion, homozygous deletion or promoter methylation of the p16INK4a, or RB1 homozygous deletion. Such alterations were significantly more frequent than in non-triple negative cases (17/160; 11%).

By definition, triple-negative cases do not have TP53 mutations. In the present study, we also noted that promoter methylation of the p14ARF gene, another component of the TP53 pathway was significantly less frequent in triple-negative than in non-triple-negative low-grade diffuse gliomas (3% vs. 21%; P=0.0199). Such infrequent alterations in the TP53 pathway thus appear to be typical of triple-negative low-grade diffuse gliomas.

Alterations in RB1 pathway-related genes have been reported to be associated with shorter survival (median survival, 1.4 vs 5.8 years; P=0.0009) in patients with anaplastic astrocytoma (2). Abnormalities in the p15INK4b, p16INK4a, RB1, or CDK4 genes are also associated with shorter survival in patients with glioblastoma (P = 0.0002) (1). However, the prognostic value of alterations in the RB1 signaling pathway has not been studied in low-grade diffuse gliomas.

In the present study, among all low-grade diffuse gliomas, multivariate analysis after adjusting for age, histology, and treatment showed that alterations in the RB1 pathway were significantly associated with shorter survival [hazard ratio, 3.024 (1.279–6.631); P=0.0057]. Among triple-negative diffuse astrocytomas, survival of patients with tumours
carrying RB1 pathway alterations was significantly shorter than that of patients with tumours without RB1 pathway alterations (Fig. 2). It was of interest to note that 4 out of 6 triple-negative diffuse astrocytomas with RB1 pathway alterations but none of 10 cases lacking RB1 pathway alterations showed gemistocytic components (Table 1). This may at least in part explain the poorer outcome of patients with RB1 pathway alterations, as gemistocytic astrocytomas or astrocytomas containing a significant fraction of gemistocytes are characterized by a tendency for rapid recurrence and malignant progression (12, 13, 15, 22, 26) and shorter patient survival (13, 26).

It is well established that oligodendroglialomas with 1p/19q loss have a favorable prognosis (8, 10, 14, 21). In the present study, among triple-negative tumors without alterations in the RB1 pathway, survival of patients with oligodendroglialomas or oligoastrocytomas was significantly longer than for those with diffuse astrocytoma, further presenting evidence that, even without 1p/19q loss, outcome for patients with oligodendroglial tumors was significantly better than for patients with diffuse astrocytomas (Fig. 2).

In summary, we present evidence that alterations in the RB1 pathway are common in triple-negative low-grade diffuse gliomas, and that genetic alterations in the RB1 pathway are associated with unfavorable outcome for the patient. These results suggest that a fraction of low-grade diffuse gliomas lacking common genetic alterations (IDH1/2 mutation, TP53 mutation and 1p/19q loss) may develop through a distinct genetic pathway, which may include loss of cell-cycle control regulated by the RB1 pathway.
Figure legends

Fig. 1
Array CGH showing chromosomal loss at 9p21 (p14^{ARF}, p15^{INK4b}, p16^{INK4a} loci) in a “triple-negative” diffuse astrocytoma and 13q14–13q32 (containing the RB1 locus) in a “triple-negative” oligodendroglioma.

Fig. 2
Median survival of diffuse astrocytoma patients with RB1 pathway alteration was 14 months, significantly shorter than that of diffuse astrocytoma patients lacking RB1 pathway alteration (49 months, \( P=0.0187 \)). Note that among triple-negative cases without RB1 pathway alteration, oligodendroglioma and oligoastrocytoma patients have significant longer survival than those with diffuse astrocytoma (median, 74 vs 49 months; \( P=0.0475 \)).
References


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**Table 1** Clinical and histological features of triple negative low-grade diffuse gliomas with and without alterations in the RB1 pathway

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>Histology</th>
<th>Tumor location</th>
<th>Ki-67 Index</th>
<th>Survival (months)</th>
<th>Alterations in the RB1 pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>WP31</td>
<td>28</td>
<td>M</td>
<td>Diffuse astrocytoma*</td>
<td>-</td>
<td>7%</td>
<td>14</td>
<td>+</td>
</tr>
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<td>38</td>
<td>F</td>
<td>Diffuse astrocytoma*</td>
<td>Left temporal</td>
<td>&lt;1%</td>
<td>12.7</td>
<td>+</td>
</tr>
<tr>
<td>PB718</td>
<td>54</td>
<td>F</td>
<td>Diffuse astrocytoma*</td>
<td>Left temporal and parietal</td>
<td>&lt;1%</td>
<td>20.1</td>
<td>+</td>
</tr>
<tr>
<td>PB678</td>
<td>61</td>
<td>M</td>
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<td>Left temporal</td>
<td>&lt;1%</td>
<td>31.3</td>
<td>+</td>
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<tr>
<td>M01</td>
<td>61</td>
<td>F</td>
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<td>Right frontal</td>
<td>2%</td>
<td>-</td>
<td>+</td>
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<td>PB659</td>
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<td>M</td>
<td>Diffuse astrocytoma*</td>
<td>Left frontal</td>
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<td>4.4</td>
<td>+</td>
</tr>
<tr>
<td>M54</td>
<td>7</td>
<td>F</td>
<td>Oligodendrogloma</td>
<td>Left frontal</td>
<td>1%</td>
<td>47</td>
<td>+</td>
</tr>
<tr>
<td>AV13</td>
<td>56</td>
<td>F</td>
<td>Oligodendrogloma</td>
<td>Temporal</td>
<td>12%</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>M44</td>
<td>5</td>
<td>M</td>
<td>Diffuse astrocytoma</td>
<td>Right thalamus</td>
<td>&lt;5%</td>
<td>56</td>
<td>-</td>
</tr>
<tr>
<td>M08</td>
<td>24</td>
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<td>Diffuse astrocytoma</td>
<td>Left frontal</td>
<td>&lt;1%</td>
<td>85</td>
<td>-</td>
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<tr>
<td>PB773</td>
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<td>F</td>
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<td>&lt;1%</td>
<td>120</td>
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<td>41</td>
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<td>Diffuse astrocytoma</td>
<td>Crus cerebri / Pons area</td>
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<td>13.8</td>
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<td>BA39</td>
<td>43</td>
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<td>Diffuse astrocytoma</td>
<td>-</td>
<td>nd</td>
<td>63</td>
<td>-</td>
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<tr>
<td>M38</td>
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<td>M</td>
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<td>Left temporal</td>
<td>&lt;1%</td>
<td>42</td>
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</tr>
<tr>
<td>M43</td>
<td>49</td>
<td>M</td>
<td>Diffuse astrocytoma</td>
<td>Left insular</td>
<td>&lt;1%</td>
<td>40</td>
<td>-</td>
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<td>M33</td>
<td>52</td>
<td>M</td>
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<td>Right temporal and parietal</td>
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<td>-</td>
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<td>F</td>
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<td>-</td>
<td>nd</td>
<td>90</td>
<td>-</td>
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<td>M</td>
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<td>Right temporal</td>
<td>5%</td>
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<td>-</td>
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<td>DNB247</td>
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<td>M</td>
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<td>Basal ganglia</td>
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<td>43.3</td>
<td>-</td>
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<td>PB760</td>
<td>3</td>
<td>M</td>
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<td>Right temporal, thalamus, insular</td>
<td>&lt;1%</td>
<td>52.4</td>
<td>-</td>
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<tr>
<td>M49</td>
<td>4</td>
<td>F</td>
<td>Oligodendroglia</td>
<td>Right frontal</td>
<td>1%</td>
<td>-</td>
<td>-</td>
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<tr>
<td>DNB4951</td>
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<td>F</td>
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<td>Left temporal</td>
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<td>214.5</td>
<td>-</td>
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<td>Oligodendroglia</td>
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<td>&lt;1%</td>
<td>149.5</td>
<td>-</td>
</tr>
<tr>
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<td>21</td>
<td>M</td>
<td>Oligodendroglia</td>
<td>Right parietal</td>
<td>1%</td>
<td>21</td>
<td>-</td>
</tr>
<tr>
<td>PB745</td>
<td>22</td>
<td>F</td>
<td>Oligodendroglia</td>
<td>Thalamus 4/5-7/8</td>
<td>&lt;1%</td>
<td>25.5</td>
<td>-</td>
</tr>
<tr>
<td>PB1002</td>
<td>29</td>
<td>M</td>
<td>Oligodendroglia</td>
<td>Left temporal</td>
<td>nd</td>
<td>190.3</td>
<td>-</td>
</tr>
<tr>
<td>M57</td>
<td>30</td>
<td>F</td>
<td>Oligodendroglia</td>
<td>Left temporal</td>
<td>&lt;1%</td>
<td>32</td>
<td>-</td>
</tr>
<tr>
<td>M51</td>
<td>31</td>
<td>M</td>
<td>Oligodendroglia</td>
<td>Cerebellum</td>
<td>2%</td>
<td>96</td>
<td>-</td>
</tr>
<tr>
<td>PB778</td>
<td>41</td>
<td>M</td>
<td>Oligodendroglia</td>
<td>Left frontal</td>
<td>&lt;1%</td>
<td>144.6</td>
<td>-</td>
</tr>
<tr>
<td>DNB524</td>
<td>48</td>
<td>F</td>
<td>Oligodendroglia</td>
<td>Temporal and parietal</td>
<td>&lt;1%</td>
<td>124.5</td>
<td>-</td>
</tr>
</tbody>
</table>

*with gemistocytic components; nd, not determined
<table>
<thead>
<tr>
<th>Alteration in the RB1 pathway</th>
<th>Triple negative* (n=31)</th>
<th>Non-triple negative ** (n=160)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>At least one alteration in the RB1 pathway</td>
<td></td>
<td></td>
<td>0.0371</td>
</tr>
<tr>
<td>$p15^{INK4b}$ homozygous deletion</td>
<td>4 (13%)</td>
<td>2 (1%)</td>
<td>0.0069</td>
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<tr>
<td>$p15^{INK4b}$ promoter methylation</td>
<td>0</td>
<td>7 (4%)</td>
<td>ns</td>
</tr>
<tr>
<td>$p16^{INK4a}$ homozygous deletion</td>
<td>1 (3%)</td>
<td>2 (1%)</td>
<td>ns</td>
</tr>
<tr>
<td>$p16^{INK4a}$ promoter methylation</td>
<td>2 (6%)</td>
<td>4 (3%)</td>
<td>ns</td>
</tr>
<tr>
<td>RB1 homozygous deletion</td>
<td>2 (6%)</td>
<td>5 (3%)</td>
<td>ns</td>
</tr>
<tr>
<td>RB1 promoter methylation</td>
<td>0</td>
<td>0</td>
<td>ns</td>
</tr>
<tr>
<td>At least one alteration in the TP53 pathway</td>
<td>2 (6%)</td>
<td>79 (49%)</td>
<td>0.0001</td>
</tr>
<tr>
<td>$p14^{ARF}$ homozygous deletion</td>
<td>1 (3%)</td>
<td>2 (1%)</td>
<td>ns</td>
</tr>
<tr>
<td>$p14^{ARF}$ promoter methylation</td>
<td>1 (3%)</td>
<td>34 (21%)</td>
<td>0.0199</td>
</tr>
<tr>
<td>TP53 mutations</td>
<td>0</td>
<td>54 (34%)</td>
<td>0.0001</td>
</tr>
<tr>
<td>No alterations</td>
<td>23 (74%)</td>
<td>72 (45%)</td>
<td>0.0032</td>
</tr>
</tbody>
</table>

* Cases without any of IDH1/IDH2 mutation, TP53 mutation, or 1p/19q loss
** Non-triple negative cases included 54 cases (IDH1/2 plus TP53 mutations), 56 cases (IDH1/2 mutation plus 1p/19q loss) and 50 cases (IDH1/2 mutation only).

ns, not significant
Chromosome 9p

Fig. 1

Chromosome 9p

Chromosome 13q

Green: loss
Red: gain

$p14^{ARF}/p16^{INK4a}$ (log2 ratio: -0.3 -- -1.1)
$p15^{INK4b}$ (log2 ratio: -1.1 -- -1.9)

$RB1$ (log2 ratio: -0.5 -- -2.2)
Fig. 2

- Oligodendroglioma/oligoastrocytoma without RB1 pathway alteration (Median, 74 mo, n=12)
- Diffuse astrocytoma with RB1 pathway alteration (Median, 14 mo, n=5)
- Diffuse astrocytoma without RB1 pathway alteration (Median, 49 mo, n=10)