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RESEARCH ARTICLE

Frequent BRAF Gain in Low-Grade Diffuse Gliomas with 1p/19q Loss

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Keywords
BRAF gain, *BRAF-KIAA1549* fusion gene, *BRAF*^{V600E} mutation, diffuse astrocytoma, oligodendroglioma.

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Abstract

Chromosomal 7q34 duplication and *BRAF-KIAA1549* fusion is a characteristic genetic alteration in pilocytic astrocytomas. 7q34 gain appears to be common in diffuse astrocytomas, but its significance is unclear. We assessed *BRAF* gain and *BRAF* mutations in 123 low-grade diffuse gliomas, including 55 diffuse astrocytomas, 18 oligoastrocytomas and 50 oligodendrogliomas. Quantitative polymerase chain reaction (PCR) revealed *BRAF* gain in 17/50 (34%) oligodendrogliomas, a significantly higher frequency than in diffuse astrocytomas (7/55; 13%; $P = 0.0112$). *BRAF* gain was common in low-grade diffuse gliomas with 1p/19q loss (39%) and those lacking any of the genetic alterations analyzed (31%), but was rare in those with *TP53* mutations (2%). Logistic regression analysis showed a significant positive association between 1p/19q loss and *BRAF* gain ($P = 0.0032$) and a significant negative association between *TP53* mutations and *BRAF* gain ($P = 0.0042$). Fluorescence *in situ* hybridization (FISH) analysis of 26 low-grade diffuse gliomas with *BRAF* gain additionally revealed *BRAF-KIAA1549* fusion in one oligodendroglioma. Sequencing of cDNA in 17 low-grade diffuse gliomas showed *BRAF-KIAA1549* fusion in another oligodendroglioma. A *BRAF*^{V600E} mutation was also detected in one oligodendroglioma, and a *BRAF*^{A598V} in one diffuse astrocytoma. These results suggest that low-grade diffuse gliomas with 1p/19q loss have frequent *BRAF* gains, and a small fraction of oligodendrogliomas may show *BRAF-KIAA1549* fusion.

INTRODUCTION

Pilocytic astrocytoma [World Health Organization (WHO) grade I], a relatively circumscribed, slowly growing, often cystic astrocytoma occurring in children and young adults (22), is genetically characterized by frequent (>60%) fusion of the *BRAF* and *KIAA1549* genes, which are closely associated with duplication of the *BRAF* gene at 7q34 (14, 18). *BRAF*^{V600E} mutations were also reported in a small fraction of pilocytic astrocytomas (up to 7%) (14, 29, 30).

BRAF-KIAA1549 fusion has not been detected in any of 50 diffuse astrocytomas WHO grade II by fluorescence *in situ* hybridization (FISH) (18), 11 diffuse astrocytomas by reverse-transcriptase polymerase chain reaction (RT-PCR) (29) or 3 diffuse astrocytomas by single-nucleotide polymorphism (SNP) array (20). However, gain of 7q34 without evidence for *BRAF-KIAA1549* fusion appears to be common in diffuse astrocytomas, although frequencies vary significantly among different studies (12, 13, 18, 25, 31). Korshunov *et al* (18) showed gain of 7q34 in 31 of 50 (62%) diffuse astrocytomas by FISH. In array comparative genome hybridization (CGH) analyses, Pfister *et al* (25) reported 7q34 duplication in 2 of 13 (15%) diffuse astrocytomas, and Jeuken *et al*. (13) showed gain at the *BRAF* locus at 7q34 in 4/9 (44%) diffuse astrocytomas. Sievert *et al* (31) found 7q34 duplication in

three of six pediatric fibrillary astrocytomas by FISH, whereas Jacob *et al* (12) reported the lack of 7q34 duplication in 27 diffuse astrocytomas by SNP array and quantitative PCR.

It has been reported that gliomas with *BRAF* gain showed significantly increased levels of BRAF mRNA compared with tumors without gain (25). Furthermore, silencing of BRAF or pharmacological inhibition of its downstream phosphorylation targets suppressed proliferation of low-grade glioma cells (25). These findings suggest that activation of the mitogen-activated protein kinase (MAPK) pathway due to *BRAF* gain may play a role in the pathogenesis of a fraction of low-grade diffuse gliomas.

In the present study, to provide further information on the frequencies of *BRAF* alterations in low-grade diffuse gliomas and to correlate these with other common genetic alterations, we assessed *BRAF* gain, *BRAF-KIAA1549* fusion, and *BRAF* mutations in low-grade diffuse gliomas with different histology (diffuse astrocytomas, oligoastrocytomas and oligodendrogliomas) and genetic features (*IDH1/2* mutations, *TP53* mutations and 1p/19q loss).

MATERIALS AND METHODS

Tumor samples

A total of 123 low-grade diffuse gliomas of WHO grade II (109 tumors in patients older than 20 years, and 14 cases in those younger than 20 years) were obtained from the Department of Neuropathology, University Hospital Zurich, Switzerland; the Department of Neuropathology, University Hospital Frankfurt, Germany; the Departments of Neuropathology and Neurosurgery, University Hospital Essen, Germany; the Department of Pathology, Gunma University, Japan; the Institute of Neuropathology and Department of Neurosurgery, University Hospital Munster, Germany; the Institute of Neuroscience, Bordeaux, France; and the Department of Neurosurgery, University Hospital Bern, Switzerland.

Histologically, these tumors were classified as diffuse astrocytoma (55 cases), oligoastrocytoma (18 cases) and oligodendroglioma (50 cases). Genetic alterations in these tumors have been published previously (16). Thirty-four cases had *IDH1/2* plus *TP53* mutations, 27 cases had *IDH1/2* mutation plus 1p/19q loss, 12 cases showed *IDH1/2* mutation only, 7 cases had *TP53* mutations only and 14 cases had 1p/19q loss only. Twenty-nine cases lacked any of these changes (*IDH1/2* mutations, *TP53* mutations and 1p/19q loss).

DNA extraction

DNA was extracted from typical tumor areas that were manually scraped off from formalin-fixed, paraffin-embedded (FFPE) tissue sections as previously described (16). DNA concentration was determined by spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Absorption was measured at 230, 260 and 280 nm and DNA quality was evaluated by A_{260}/A_{230} and A_{260}/A_{280} ratios.

BRAF gain

BRAF gain was assessed by quantitative PCR, using three reference sequences at different chromosomal locations (*CF* at 7q31.2, β-globin at 11q15.5 and *GAPDH* at 12p13.31) (24, 26, 33, 34). Primer sequences were as follows: 5′-TTC ATG AAG ACC TCA CAG TAA AAA-3′ (sense) and 5′-CCA CAA AAT GGA TCC AGA CA-3′ (antisense) for *BRAF* (PCR product, 107 bp), 5′-GGC ACC ATT AAA GAA AAT ATC ATC TT-3′ (sense) and 5′-GTT GGC ATG CTT TGA TGA CGC TTC-3′ (antisense) for the CF (PCR product, 79 bp), 5′-GTG CAT CTG ACT CCT GAG GAG A-3′ (sense) and 5′-CCT TGA TAC CAA CCT GCC CAG-3′ (antisense) for the β-globin (PCR product, 102 bp), and 5′-TCA AGA AGG TGG TGA AGC AG-3′ (sense) and 5′-TGT CGC TGT TGA AGT CAG AG-3′ (antisense) for the GAPDH (PCR product, 96 bp). Quantitative PCR was carried out in a total volume of 20 µL with 10 µL of iQ™ SYBR green (Bio-Rad, Hercules, CA, USA), 6.4 µL of primers (1.25 µmol/L of each primer) and approximately 20 ng of DNA with initial denaturation at 95°C for 12 minutes followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 20 s and extension at 72°C for 45 s. PCR was performed in triplicate on a 96-well optical plate with an iCycler iQ5 Detection System (Bio-Rad). The copy-number calculation was carried out using the comparative Ct (threshold cycle) method, as described previously (3, 24). Results using quantitative PCR with three different references were concordant in >92% of cases. Tumors were considered to have *BRAF* gain when PCR reactions using two or three references showed significant copy-number gain of *BRAF*.

BRAF mutation

The mutational hotspot codons of *BRAF* were amplified by PCR. Primer sequences were as follows: 5′-TGC TTG CTC TGA TAG GAA AAT G-3′ (sense) and 5′-CCA CAA AAT GGA TCC AGA CA-3′ (antisense) (PCR product, 173 bp) (30). PCR amplification products were subjected to the direct sequencing on ABI 3100 PRISM DNA sequencer (Applied Biosystems, Foster City, CA, USA) with the Big Dye Terminator cycle sequencing kit (ABI PRISM, Applied Biosystems).

BRAF-KIAA1549 fusion by FISH

Tumors showing *BRAF* gain according to quantitative PCR were further screened for the *BRAF-KIAA1549* fusion gene by FISH using previously published probes and methods with minor modifications (18). Two-color interphase FISH analysis was performed on 5-micron thick paraffin tissue sections pairing two home brew locus-specific probes: FITC-labeled locus-specific probe RP11-355D18 (CHORI BACPAC Resources Center, Oakland, CA, USA) corresponding to fluorescein isothiocyanate (FITC) labeled *KIAA1549* (green) and rhodamine-labeled locus-specific probe 726N20 corresponding to *BRAF* (red). Pretreatment of slides, hybridization, posthybridization processing and signal detection were performed as reported elsewhere (25). Samples showing sufficient FISH efficiency (>90% nuclei with signals) were evaluated, and signals were scored in at least 100 nonoverlapping, intact nuclei. Non-neoplastic brain biopsy specimens were used as controls. Chromosomal gains at 7q34 region were defined as >5% of nuclei containing three or more signals for both locus-specific probes. The *BRAF-KIAA1549* fusion gene was scored in cases showing 7q34 gain in combination with overlap of at least one red signal and one green signal, resulting in a yellow signal. Because these two probes are normally in close proximity, signals were designated as fused only when the red and green signals were completely or nearly completely overlapping. Based on the median

number of fusion signals encountered in control specimens plus three standard deviations, we scored cases as positive for *BRAF-KIAA1549* fusion when >25% of cells had both yellow fusion signals and associated copy-number gains (at least three green and/or red signals). Additionally, in order to distinguish polysomy 7 from a more specific gain of the *BRAF* region, a second FISH analysis was performed pairing the *BRAF* probe with a commercial SpectrumGreen labeled centromere enumerating probe (CEP7; Abbott Laboratories, Abbott Park, IL, USA). Copy-number gains associated with an overall *BRAF* to CEP7 ratio >1.15 were considered *BRAF* specific gains, while the remaining cases were classified as polysomy 7.

BRAF-KIAA1549 fusion by sequencing

RNA was extracted from paraffin sections of 17 cases for which sufficient materials were available (eight diffuse astrocytomas, nine oligodendrogliomas). For preparation of RNA extraction from FFPE samples, the RNA RNeasy FFPE kit (QIAGEN GmbH, Hilden, Germany) was used according to the manufacturers' recommendations. cDNA was constructed with Superscript® II RT (Invitrogen, Carlsbad, CA, USA). Primer sequences were as follows: 5′-GCG ATG GCA CCT ACA GGA-3′ (sense) for *KIAA1549* exon 15, 5′-CAG TGG GGG TCC TTC TAC AG-3′ (sense) for *KIAA1549* exon 16, 5′-TGC CAG AGG GAT CTA CTC G-3′ (sense) for *KIAA1549* exon 18 and 5′-CCT TCG TAC GGG GAG GAC-3′ (sense) for *KIAA1549* exon 19, and 5′-CCA CGA AAT CCT TGG TCT CT-3′ (antisense) for *BRAF* exon 9, 5′-GGG GGT AGC AGA CAA ACC T-3′ (antisense) for *BRAF* exon 10 and 5′-TCA CTC GAG TCC CGT CTA CC-3′ (antisense) for *BRAF* exon 11. The sizes of the PCR products were 88 bp for *KIAA1549* exon 15–*BRAF* exon 9, 80 bp for *KIAA1549* exon 16–*BRAF* exon 9, 80 bp for *KIAA1549* exon 16–*BRAF* exon 11, 97 bp for *KIAA1549* exon 18–*BRAF* exon 10 and 70 bp for *KIAA1549* exon 19–*BRAF* exon 9.

RT-PCR was performed with 40 cycles of denaturation for 50 s at 94°C, annealing for 45 s at 58°C and extension for 50 s at 72°C. PCR products were visualized by 8% acrylamide-gel electrophoresis, staining with gel red. RT-PCR products were subjected to direct sequencing on an ABI PRISM®3100 DNA sequencer (Applied Biosystems) with the BigDye® Terminator Cycle Sequencing kit (ABI PRISM, Applied Biosystems).

Statistical analyses

The χ^2 test or the Fisher's exact test was conducted to analyze the significance of the association of age, histology or genetic features with *BRAF* gain. Logistic regression analysis was carried out to assess associations between different genetic alterations. Statistical analysis was performed with StatView® for Windows 5.01 software (SAS Institute Inc., Cary, NC, USA).

RESULTS

BRAF gain

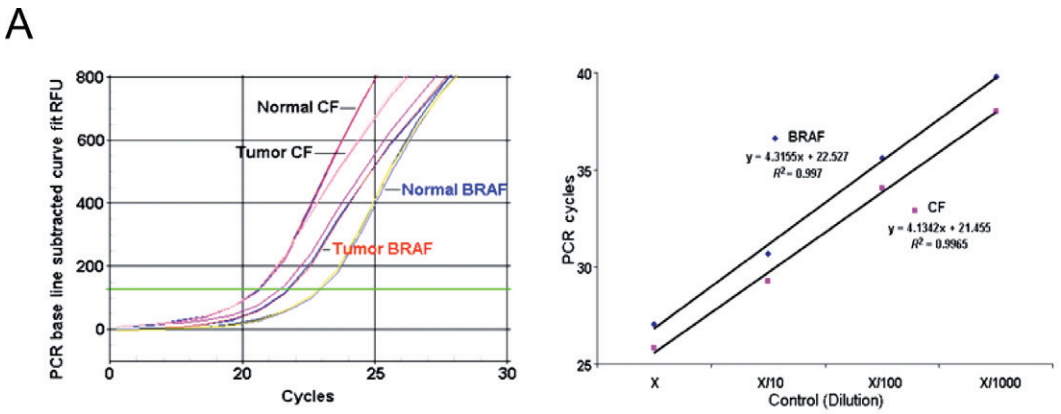
Quantitative PCR revealed *BRAF* gain in a total of 28 of 123 (23%) low-grade diffuse gliomas (Figure 1A). *BRAF* gain was significantly more frequent in oligodendrogliomas than in diffuse astrocytomas (34% vs. 13%; $P = 0.0112$; Table 1). *BRAF* gain was common in low-grade diffuse gliomas with 1p/19q loss (16/41; 39%) and in those lacking any of the common genetic alterations (9/29; 31%). In contrast, only 1 of 41 (2%) low-grade diffuse gliomas with *TP53* mutation showed *BRAF* gain (Table 1). Logistic regression analysis showed a significant positive association between 1p/19q loss and *BRAF* gain [OD = 3.733 (1.553–8.973); $P = 0.0032$], and a significant negative association between *TP53* mutations and *BRAF* gain [OD = 0.051 (0.007–0.391); $P = 0.0042$].

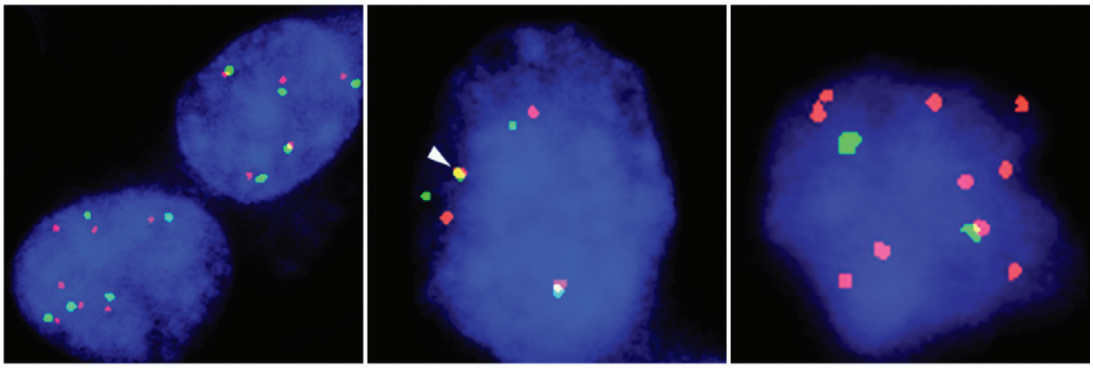
Patients with low-grade diffuse glioma with *BRAF* gain tended to be younger, but the age difference was significant only among pediatric patients (<20 years; Table 2). The median survival of patients was not significantly different between cases with and without *BRAF* gain (data not shown).

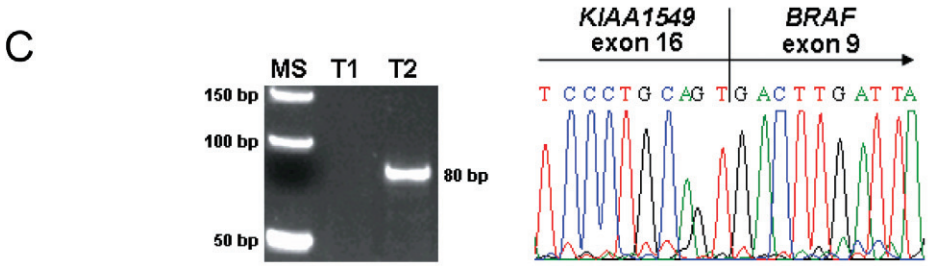
BRAF-KIAA1549 fusion

FISH analyses were carried out in 26 of 28 cases in which *BRAF* gain was detected by quantitative PCR. In all cases, *BRAF* gain was confirmed by FISH (Figure 1B left). Furthermore, FISH detected a *BRAF–KIAA1549* fusion in one oligodendroglioma (male, aged 40 years; frontal right location; with 1p/19q loss and *BRAF* gain but no *IDH1/2* mutation) with 65% of cells showing *BRAF-KIAA1549* fusion signals in addition to copy-number gains (Figure 1B middle). Additionally, CEP7/*BRAF* FISH studies demonstrated that at least six of the positive cases were associated with specific gains of the *BRAF* region, rather than polysomy 7 (Figure 1B right). Sequencing of cDNA in 17 low-grade diffuse gliomas (11 cases with *BRAF* gain and 6 cases without gain) showed *BRAF-KIAA1549* gene fusion in one oligodendroglioma (female aged 22 years; thalamus; with *BRAF* gain but no *TP53* mutations, no 1p/19q loss and no *IDH1/2* mutations) (Figure 1C).

Figure 1. A. Quantitative PCR showing *BRAF* gain in low-grade gliomas. Note that normal *CF* (normal brain) and tumor *CF* show similar CTs, while *BRAF* in a low-grade diffuse glioma (tumor) shows a significantly smaller CT compared with *BRAF* in normal brain; this indicates a *BRAF* gain in tumor DNA (left). Relative CT values for *BRAF* and *CF* at different concentrations of normal control DNA are shown. The slopes of the curves are similar, suggesting equal efficiencies of the two PCR reactions at CT (right). **B.** FISH analysis of *BRAF* gain and *BRAF-KIAA1549* fusion. Most cases demonstrated gain of *BRAF* and *KIAA-1549* without any evidence of fusion (left). In one oligodendroglioma with 1p/19q co-deletion, FISH demonstrated fusion of *BRAF* (red) and *KIAA1549* (green) signals, resulting in a yellow signal for the fusion gene (arrow) in addition to copy-number gains (middle). FISH in another case demonstrates selective gain of the red *BRAF* in comparison with green CEP7 signals (right). **C.** RT-PCR showing *KIAA1549* exon 16–*BRAF* exon 9 fusion in an oligodendroglioma (left). MS, molecular size marker; T1, fusion-negative tumor; T2, fusion-positive oligodendroglioma. Sequencing confirming a *KIAA1549* exon 16–*BRAF* exon 9 fusion (right). **D.** $BRAF^{V600E}$ mutation in an oligodendroglioma (left), and $BRAF^{A598V}$ mutation in a diffuse astrocytoma (right). CT = cycle threshold; PCR = polymerase chain reaction; RT-PCR = reverse-transcriptase PCR.







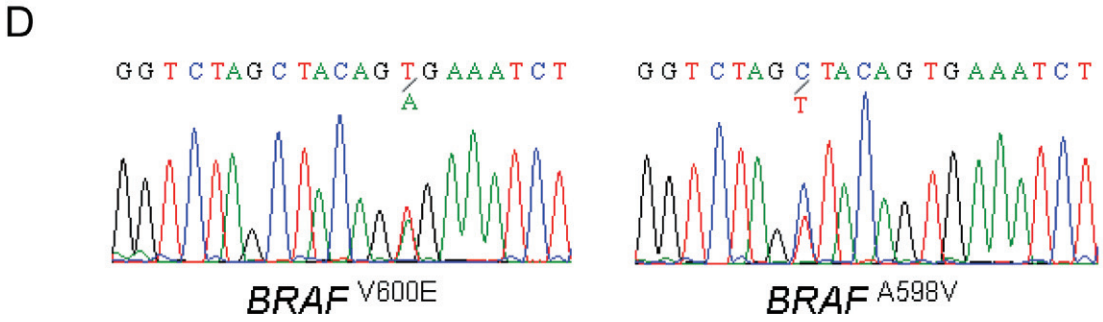


Table 1. *BRAF* gain in low-grade gliomas.

	No. of cases with *BRAF* gain
Histology	
Diffuse astrocytoma ($n = 55$)	7 (13%)*
Oligoastrocytoma ($n = 18$)	4 (22%)
Oligodendroglioma ($n = 50$)	17 (34%)*
Genetic alterations	
TP53 mutation ± *IDH1/2* mutation ($n = 41$)	1 (2%)†‡
1p/19q loss ± *IDH1/2* mutation ($n = 41$)	16 (39%)†
IDH1/2 mutation only ($n = 12$)	2 (17%)
No alteration§ ($n = 29$)	9 (31%)‡

*$P = 0.0112$; †$P = 0.0001$; ‡$P = 0.0011$.
§Absence of *IDH1/2* mutations, *TP53* mutations, 1p/19q loss.

BRAF ^{V600E} mutation

Sequencing analyses revealed a *BRAF*^{V600E} mutation in one oligodendroglioma that lacked *BRAF* gain. This tumor was located in the right occipital lobe and had 1p/19q loss, but lacked *IDH1/2* mutation (Figure 1D). A rare *BRAF*^{A598V} mutation was also detected in a diffuse astrocytoma that lacked *BRAF* gain (temporal location; with *TP53* mutation plus *IDH1* mutation) (Figure 1D).

DISCUSSION

The MAPK/extracellular signal-regulated kinase (ERK) pathway regulates a wide range of biological activities, including cell differentiation, proliferation, senescence and survival (6, 7, 15, 27). This pathway consists of a small GTP protein of the RAS family that is activated in response to extracellular signaling to recruit a member of the RAF kinase family to the cell membrane (6). Mutations in the *BRAF* or *RAS* genes have been found as activating mutations in approximately 30% of all human cancers (6).

BRAF^{V600E}, the most common mutation in this gene, is frequent in hairy cell leukemias (100%) (32), melanomas (60%–80%) (4, 9, 23, 28) and papillary thyroid cancers (35%–70%) (8, 17). *BRAF* mutations are additionally associated with *BRAF* gene amplification in melanomas (10, 21). Lin *et al* (21) showed copy-number gain at 7q34 in 65% of melanomas and activating *BRAF*^{V600E} mutations in 56% of cases, and a co-occurrence of these two events was observed in 46% of cases, suggesting that the mutated *BRAF* gene may be amplified in melanomas. CGH analysis by Bastian *et al* (1) also showed frequent gain of the *BRAF* gene in 16/32 (50%) melanomas.

In pilocytic astrocytomas, the *BRAF*^{V600E} mutation is present in only a small fraction (up to 7%) (2, 14, 29, 30), but *BRAF* fusion genes (>60%) are the most common genetic alterations leading to abnormal activation of the MAPK/ERK pathway (14, 18). *BRAF* fusion in pilocytic astrocytomas is considered to occur as a result of tandem *BRAF* duplication at chromosome 7q34 (14, 18). Several reports have suggested that gain at 7q34 (60%–80%) (13, 18, 25, 31) and gain/amplification of the *BRAF* gene (50%–80%) (11, 14, 18, 25) are frequent genetic alterations in pilocytic astrocytomas.

Gain or amplification of the *BRAF* gene has also been reported in other gliomas, including diffuse astrocytomas (15%–62%) (13, 18, 25), oligoastrocytomas (14%) (13), anaplastic oligoastrocytomas (56%) (13), anaplastic oligodendrogliomas (18%) (13) and glioblastomas (76%) (13). In the present study, we present evidence suggesting that *BRAF* gain is common in oligodendrogliomas (34%) and in low-grade diffuse gliomas with 1p/19q loss (39%). In contrast, *BRAF* gain is infrequent in diffuse astrocytoma (13%) and very rare in low-grade diffuse gliomas with *TP53* mutations (2%). We found a significant positive association between 1p/19q loss and *BRAF* gain ($P = 0.0032$), and a significant negative association between *TP53* mutations and *BRAF* gain ($P = 0.0042$). The finding of infrequent *BRAF* gain in diffuse astrocytomas or in low-grade diffuse gliomas with *TP53* mutations in the present study was consistent with the results of a study by Jacob *et al* (12), in which quantitative PCR revealed absence of 7q34 duplication in the 27 diffuse astrocytomas analyzed (12). In contrast, several previous studies using FISH with centromere probes showed frequent 7q polysomy in diffuse astrocytomas (62%–76%) (5, 18, 19), while other studies using array CGH showed gain at the *BRAF* locus in 15%–44% of diffuse astrocytomas (13, 25). Discrepancies of frequencies of *BRAF* gain in different studies may be at least in part due to variation in the specificity and sensitivity of the different methods used.

BRAF gain may be due to polysomy 7 or specific gain at the *BRAF* region. As we used *BRAF*-specific primers for quantitative PCR, the results in the present study indicate the specific gain of the *BRAF* gene. Our FISH analysis using the CEP7/*BRAF* probe demonstrated that at least 6 of 26 cases with *BRAF* gain were associated with specific gain of the *BRAF* region, rather than polysomy 7. Irrespective of the mechanisms involved, *BRAF* gain itself appears to have significant biological implications. In

Table 2. Age and *BRAF* gain in low-grade gliomas.

	No. of cases (%)	Mean age ± SD (years)	*P*-value
All patients (n = 116)			
With *BRAF* gain	28 (24)	34.3 ± 18.2	0.0802
Without *BRAF* gain	88 (76)	40.4 ± 15.2	
Pediatric patients (<20 years; n = 14)			
With *BRAF* gain	5 (35)	4.8 ± 1.5	0.0038*
Without *BRAF* gain	9 (65)	13.3 ± 5.1	
Adult patients (≥20 years; n = 102)			
With *BRAF* gain	23 (22)	40.7 ± 12.8	0.3570
Without *BRAF* gain	79 (77)	43.5 ± 12.7	

*Statistically significant.

thyroid tumors, *BRAF* gain detected by FISH analysis was associated with higher levels of BRAF protein as detected by Western blot; *BRAF* gain and *RAS* mutations were mutually exclusive (6).

The present study also shows that, although rare, *BRAF* fusion genes and *BRAF* mutations may be present in low-grade diffuse gliomas. FISH analysis showed the *BRAF-KIAA1549* gene fusion to be present in one oligodendroglioma. In addition, RT-PCR analysis revealed *KIAA1549* exon 16–*BRAF* exon 9 fusion in another oligodendroglioma with *BRAF* gain. We also found $BRAF^{V600E}$ mutation in an oligodendroglioma, and a rare $BRAF^{A598V}$ mutation in a diffuse astrocytoma. This is consistent with the results of a previous study on 162 low-grade diffuse gliomas, which showed that only one oligodendroglioma had a $BRAF^{V600E}$ mutation (30).

It has been shown that in pilocytic astrocytomas, younger patients more frequently show 7q34 duplication or *BRAF* rearrangement (11, 25, 31). In the present study, we assessed the relationship between age and *BRAF* gain in low-grade diffuse gliomas. In adults, there was no significant correlation between age and *BRAF* gain, whereas among pediatric cases (aged <20 years), *BRAF* gain was associated with the youngest patients (mean 4.8 vs. 13.3 years; $P = 0.0038$) (Table 2).

In summary, this study suggests that *BRAF* gain is common in oligodendrogliomas and in low-grade diffuse gliomas with 1p/19q loss, suggesting that activation of the MAPK signaling pathway may be involved in their pathogenesis. As with pilocytic astrocytomas, this finding raises potential therapeutic implications.

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