

ORIGINAL ARTICLE

BRAF Alterations in Primary Glial and Glioneuronal Neoplasms of the Central Nervous System With Identification of 2 Novel *KIAA1549:BRAF* Fusion Variants

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Abstract

Recent studies highlight the importance of *BRAF* alterations resulting in mitogen activated protein kinase (MAPK/ERK) pathway activation in low-grade CNS tumors. We studied 106 low-grade CNS neoplasms in a cohort of primarily pediatric patients to identify the prevalence and clinicopathologic significance of these alterations. Polymerase chain reaction testing identified *KIAA1549:BRAF* fusions in 51 (48%) tumors overall, including 42 (60%) pilocytic astrocytomas, 4 (17%) unclassifiable low-grade gliomas, 4 (36%) low-grade glioneuronal/neuroepithelial tumors, 0 (of 5) pleomorphic xanthoastrocytomas, 0 (of 4) diffuse astrocytomas (World Health Organization grade II), and 1 (of 3, 33%) pilomyxoid astrocytoma. *KIAA1549:BRAF* gene fusions confirmed by sequencing included the previously reported ones involving exons 1–16/9–18 (49%), 1–15/9–18 (35%), and 1–16/11–18 (8%) and 2 fusions with novel breakpoints: 1–15/11–18 (6%) and 1–17/10–18 (1%). DNA sequencing identified *BRAF*^{V600E} mutations in 8% of tumors. *BRAF*^{G468A} mutations were absent. *KIAA1549:BRAF* fusions were significantly more frequent in infratentorial (57%) and optic pathway (59%) tumors versus supratentorial (19%) tumors ($p = 0.001$). We did not identify significantly improved progression-free survival in tumors with fusions. In summary, *KIAA1549:BRAF* fusions predominate in pilocytic astrocytomas but are also present in some low-grade unclassifiable gliomas and glioneuronal tumors. The prognostic and therapeutic significance of this alteration is unclear and merits further study.

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INTRODUCTION

Pilocytic astrocytomas (PAs) are primary CNS tumors classified as grade I astrocytomas by the World Health Organization (WHO). They occur predominantly in children and have a better prognosis than higher-grade astrocytomas, with a 10-year survival rate as high as 96% (1). However, PA is the most frequent primary brain neoplasm in children and young adults (2), and recurrent or progressive disease occurs in approximately one fifth of patients (3), necessitating further therapy, which may be associated with significant morbidity. The goal of therapeutic interventions using various chemotherapy modalities is maintaining disease stability rather than cure. Although the use of standard chemotherapy has been validated in this setting, treatment-associated adverse effects are common, and the availability of novel, less toxic treatments would be desirable. Therefore, understanding the molecular basis of these tumors, with the aim of developing targeted therapies, remains a priority.

Several large-scale multi-institutional efforts, including the ongoing Cancer Genome Atlas Project, have dissected the molecular genetic abnormalities associated with more aggressive gliomas such as glioblastoma in detail (4), but less is known about the biology of pediatric low-grade gliomas and glioneuronal tumors. Recently, independent high-resolution genomic studies have identified a tandem duplication of *BRAF* at 7q34 that leads to various *KIAA1549:BRAF* gene exon fusions in the majority (53%–72%) of PA (5–10). In low-grade gliomas arising in patients with neurofibromatosis type 1 (NF-1), genetic inactivation of *NF1* results in K-RAS hyperactivation (11). In a smaller proportion of sporadic low-grade gliomas, activating point mutations in *BRAF* and alternative rearrangements of *BRAF* family members (e.g. *RAF1*) have also been reported (12). The frequency of these alterations may vary depending on histologic subtype, that is, gangliogliomas and pleomorphic xanthoastrocytomas demonstrate an increased frequency of *BRAF*^{V600E} point mutations in some studies (13, 14).

These genetic alterations, which are commonly seen in low-grade gliomas, invariably result in MAPK/ERK pathway activation. The RAS/RAF/MEK/ERK intracellular signaling

cascade mediates cellular response to growth stimuli, and the pathway is frequently activated in human cancers, including gliomas. Here, we evaluated the clinical and pathologic relevance of *BRAF* alterations in a multi-institutional cohort of low-grade glioma and glioneuronal tumors.

MATERIALS AND METHODS

Patients and Tissue Specimens

This study was conducted under protocols approved by the Institutional Review Boards of Johns Hopkins University Medical Center and NYU Langone Medical Center. We obtained a total of 106 primary low-grade (WHO grade I–II) gliomas or glioneuronal tumors with available frozen tissue from tissue banks at both institutions. Demographic and clinical information was obtained through a retrospective chart review. The study cohort was composed of 61 males and 43 females (sex unknown in 2). Median age at first tissue diagnosis was 10 years (range, 1–29 years). A gross total resection was obtained in 57% of patients. Postoperative therapy after initial surgery included chemotherapy in 22% and irradiation in 8% of patients. Tumor histology was classified according to the most recent WHO criteria (15), with more than 80% of cases of the combined cohort classified by consensus of at least 2 neuropathologists (P.C.B., C.G.E., and F.J.R.), and more than 80% of cases from the NYU cohort reviewed by an additional neuropathologist (D.Z.). A subset of tumors was difficult to classify according to the WHO criteria (often because of limited diagnostic tissue), and these tumors are referred to as “low-grade gliomas.” In addition, 5 high-grade astrocytomas, 4 medulloblastomas, 2 ependymomas, and 1 dysembryoplastic neuroectodermal tumor were used as controls. Clinical follow-up was available in 94 (89%) of 106 patients. Progression-free survival (PFS) was the main outcome measure of interest and was measured from the time of first tissue diagnosis of the tumor until tumor progression occurred or the time of last follow-up brain scan. Progression was defined as objective evidence of tumor growth in follow-up imaging or progressive neurologic symptoms requiring additional tumor-specific treatment.

KIAA1549:BRAF Fusions

RNA was isolated from frozen tumor tissue using TRIzol (Invitrogen, Carlsbad, CA), followed by purification through RNeasy columns (Qiagen, Valencia, CA), according to the manufacturers’ instructions and converted to complementary DNA (cDNA) using a M-MLV Kit (Applied Biosystems, Foster City, CA). Screening for various *KIAA1549:BRAF* fusions was performed by polymerase chain reaction (PCR) on 10 ng of cDNA in a 20- μ L reaction. Polymerase chain reaction was performed in a iCycler (Bio-Rad, Hercules, CA), with initial denaturation at 94°C for 10 minutes, amplification for 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 68°C for 45 seconds, and final extension at 68°C for 10 minutes. The primer sequences were as follows: 5′-CGGAAACACCAGGTCAACGG-3′ (*KIAA1549* exon 15, forward) and 5′-GTTCCA AATGATCCAGATCCAATT-3′ (*BRAF* exon 11, reverse) as previously reported by Jones et al (7). The size of the PCR

product was determined on a 2% Sodium Borate (SB) agarose gel. Purified PCR products (QIAquick PCR Purification Kit; Qiagen) were sent for Sanger sequencing at the Synthesis & Sequencing Facility at Johns Hopkins.

BRAF Point Mutations

Genomic DNA from frozen tumor samples was extracted with the Blood & Tissue DNA Mini Kit (Qiagen). Screening for mutations in *BRAF* exons 11 and 15 was performed by PCR in a 50- μ L reaction containing 10 ng of DNA solution, 5 μ L of 10 \times PCR buffer containing 15 mmol/L MgCl₂ (Qiagen), 0.2 mmol/L dNTPs (New England Biolabs, Ipswich, MA), 1 U of Taq DNA polymerase (Qiagen), and 0.1 μ mol/L of both forward and reverse primers. Polymerase chain reaction was performed in a Bio-Rad iCycler with initial denaturation at 95°C for 8 minutes, amplification for 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55.8°C for 1 minute, extension at 72°C for 45 seconds, and final extension at 72°C for 8 minutes. Primer sequences for exon 11 were as follows: 5′-AAGGTAATGTA-CTTAGGGTCAAACA-3′ (forward) and 5′-CGAACAGTGAATATTTCTTTGA-3′ (reverse) and for exon 15 were 5′-TCATAATGCTT-GCTCTGATAGGA-3′ (forward) and 5′-GGCCAA-AAATTTAATCAGTGGGA-3′ (reverse). Polymerase chain reaction products were purified with a PCR purifications kit (Qiagen), followed by Sanger sequencing using the amplification primers.

Quantitative Reverse Transcription–PCR

Quantitative reverse transcription (RT)–PCR was performed as previously described (16). In brief, quantitative RT-PCR for *BRAF*, *KIAA1549*, and *KIAA1549:BRAF* was performed in separate tumors representative of each *KIAA1549:BRAF* fusion type identified using cDNA obtained as described above. SYBR green chemistry was used for detection. Samples were run in triplicate. Expression differences were evaluated using ΔC_t values with β -actin as an internal control. Specific primers identifying each specific fusion transcript type are provided in a supplementary figure (Supplementary Digital Content 1, <http://links.lww.com/NEN/A293>).

Statistical Analysis

Clinicopathologic and molecular characteristics were described using frequencies, medians, ranges, and interquartile ranges as appropriate. Progression-free survival was illustrated using Kaplan–Meier survival curves and analyzed with log rank or Wilcoxon tests as appropriate. The χ^2 or Fisher exact tests were used to compare proportions. All tests were 2-sided with $p < 0.05$ considered statistically significant. Statistical analyses were performed using JMP software (SAS Institute, Inc., Cary, NC).

RESULTS

KIAA1549:BRAF Fusion Frequency Varies by Pathology and Anatomic Location

The distribution of molecular *BRAF* alterations in the total of 106 tumors is shown in Figure 1. *BRAF* alterations included *KIAA1549:BRAF* fusions in 51 (48%) and *BRAF*

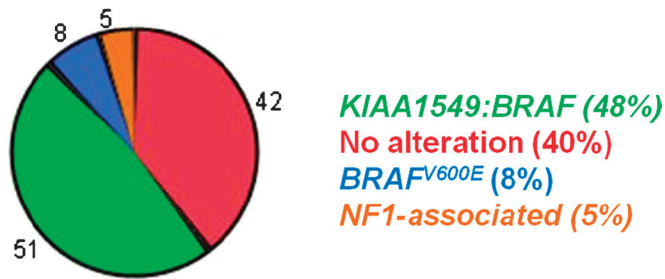


FIGURE 1. *KIAA1549:BRAF* is the most frequent genetic alteration in low-grade gliomas/glioneuronal tumors. *KIAA1549:BRAF* fusions were the most common genetic alteration in the 106 tumors examined. Pie chart represents the numbers of cases with the indicated specific alteration.

exon 15 (*BRAF^{V600E}*) point mutations in 8 (8%). No exon 11 point mutations (*BRAF^{G468A}*) were identified in any tumors. Five patients (5%) had a clinical diagnosis of neurofibromatosis and all of these lacked *BRAF* alterations. A single low-grade astrocytoma had a concomitant *KIAA1549:BRAF* fusion (1–15/9–18 exons) and a *BRAF^{V600E}* mutation. By comparison, no *KIAA1549:BRAF* fusions were identified in the 5 high-grade astrocytomas, 4 medulloblastomas, 2 ependymomas, or 1 dysembryoplastic neuroectodermal tumor tested. A single anaplastic astrocytoma had a *BRAF^{V600E}* mutation.

Review of array comparative genomic hybridization data available in 24 PA of this current series and reported in a previous study was performed (5). Among 6 tumors negative for *BRAF* duplication, 2 demonstrated *BRAF^{V600E}* mutations and 1 patient had a clinical diagnosis of NF-1. Neither *RAF1* duplications nor the recently described deletion leading to a *BRAF* and *FAM131B* fusion (17) was identified on review of the array

comparative genomic hybridization data. Two (of 24) cases demonstrated whole chromosome 7 gain, 1 with *BRAF* fusion, and 1 with a *BRAF^{V600E}* mutation. This frequency of whole chromosome 7 gain was similar to that of conventional PA in a previous study (~10%) (18).

We next searched for any molecular differences by anatomic location or pathology. The frequency of *KIAA1549:BRAF* fusion was similar in optic pathway (59%) and infratentorial tumors (57%) but was significantly lower in supratentorial (hemispheric) tumors (19%) ($p = 0.001$) (Fig. 2A). In addition, *KIAA1549:BRAF* fusion distribution varied by pathologic subtype, with a higher frequency in PA (60%) compared with non-PA tumors (22%, $p < 0.001$; Fig. 2B). Conversely, *BRAF^{V600E}* mutations were identified in a total of 8 tumors, 6 in non-PA tumors, and 2 in PA; this difference was statistically significant ($p = 0.005$). Both PA with *BRAF^{V600E}* mutations presented in hemispheric locations. Also, there were no significant differences by age in *KIAA1549:BRAF* fused and nonfused tumors ($p > 0.05$).

***KIAA1549:BRAF* Fusions With Novel Breakpoints**

Using the RT-PCR primers reported by Jones et al (7), we identified the previously reported 392-, 536-, and 710-bp products; by direct sequencing, these were confirmed to correspond to the fusions involving *KIAA1549* and *BRAF* exons 1–15/9–18, 1–16/11–18, and 1–16/9–18, respectively. In addition, 2 PCR products of approximately 210 and 800 bp in size were identified in 3 patients and in 1 patient, respectively; these resulted in *KIAA1549:BRAF* fusions with novel breakpoints involving 1–15/11–18 and 1–17/10–18 exons (Fig. 3). Interestingly, the *KIAA1549:BRAF* 1–16/11–18 fusion was only present in infratentorial tumors with PA histology,

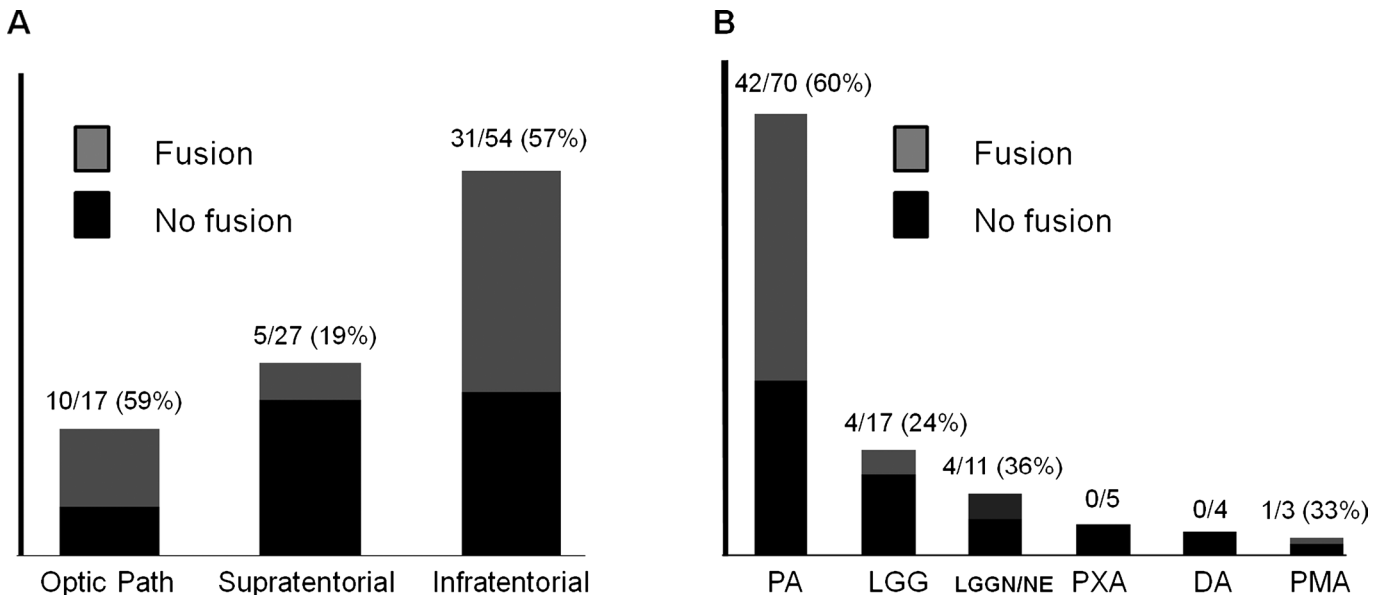


FIGURE 2. *KIAA1549:BRAF* distribution varies by location and histologic subtype. **(A)** *KIAA1549:BRAF* fusions were relatively more frequent in tumors located in the optic pathways (path) and infratentorial locations compared with the supratentorial compartment. **(B)** *KIAA1549:BRAF* fusions were most frequent in PA versus tumors with other histologic features. DA, diffuse astrocytoma, WHO grade II; LGG, low-grade glioma, indeterminate type; LGGN/NE, low-grade glioneuronal or neuroepithelial tumor; PA, pilocytic astrocytoma; PMA, pilomyxoid astrocytoma; PXA, pleomorphic xanthoastrocytoma.

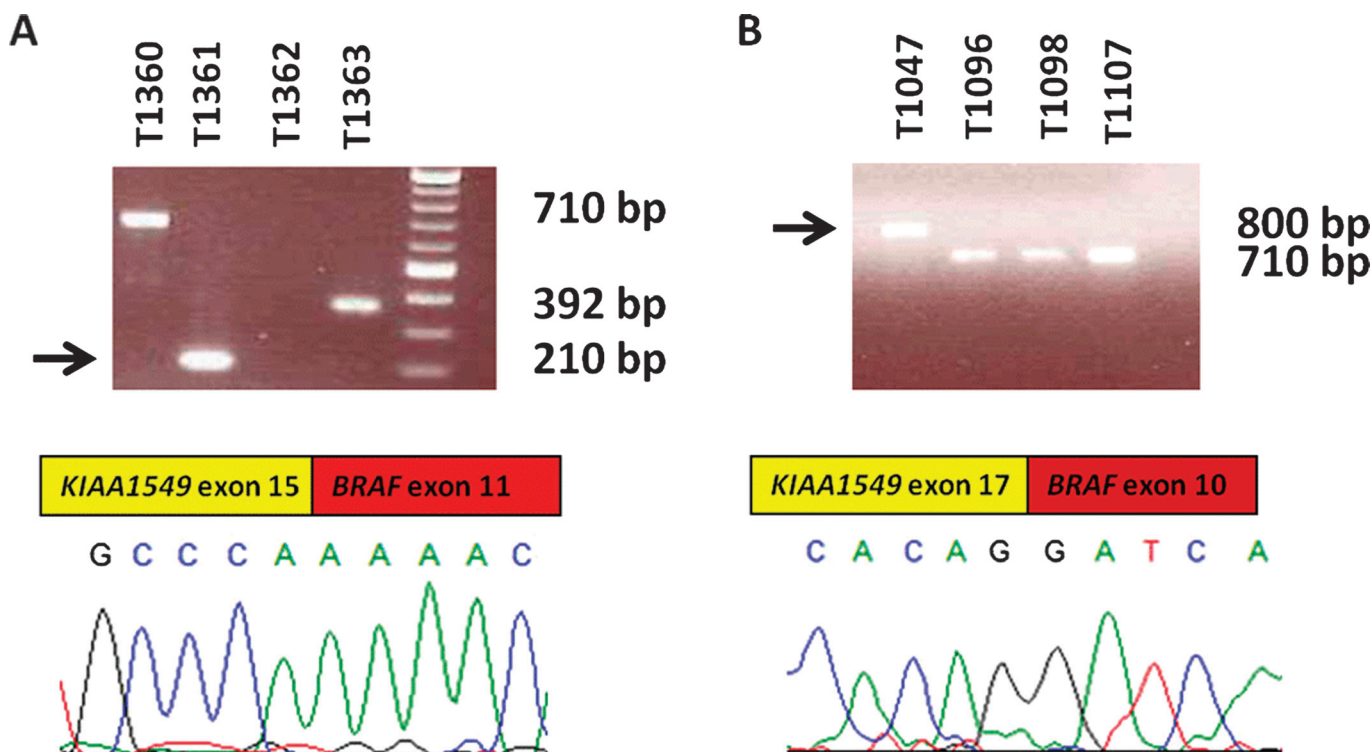


FIGURE 3. Novel *KIAA1549:BRAF* fusion breakpoints. Polymerase chain reaction (PCR) identified 2 novel *KIAA1549:BRAF* fusion products with sizes corresponding to 210 nucleotide base pairs (bp) (**A**) and 800 nucleotide base pairs (bp) (**B**). Sanger sequencing confirmed novel breakpoints involving *KIAA1549* exons 1–15/*BRAF* exons 11–18 and *KIAA1549* exons 1–17/*BRAF* exons 10–18, respectively (**A**, **B**, bottom panel).

whereas the novel 1–15/11–18 fusion was present in 3 non-infratentorial tumors. The relative frequencies of all 5 fusion variants are illustrated in Figure 4.

KIAA1549:BRAF* Fusions Are Expressed at Similar Levels as *KIAA1549

Next, we asked if *KIAA1549:BRAF* fusion expression levels are different from *KIAA1549* and *BRAF* or if they vary by fusion type. Quantitative PCR analysis was performed in individual tumors representing each fusion type. Comparisons of ΔC_t values revealed that *KIAA1549:BRAF* fusion transcripts were expressed at similar or slightly lower levels than *KIAA1549*, whereas *BRAF* was expressed at higher levels (Fig. 5).

Clinical Outcome Analysis

Clinical progression was documented in a total of 37 patients of the 94 patients with clinical follow-up data available (39%) after a median follow-up time of 21 months. The median time to progression was 16.5 months. Progression-free survival was less in patients who had a subtotal resection versus patients with a gross total resection ($p = 0.0001$; Fig. 6A), in patients less than 5 years ($p = 0.04$; Fig. 6B), and in patients with noninfratentorial tumor location ($p = 0.02$). There was a nonsignificant trend toward longer PFS in patients with *KIAA1549:BRAF* fusions ($p = 0.15$; Fig. 6C). Restricting our analysis to the “clinically relevant group” described by Hawkins et al (i.e. patients with subtotal resection, noncerebellar

locations, absence of NF-1, and monitored for at least 1 year after surgery [19]) resulted in a cohort of 35 patients. We did not find any prognostic significance for fusion status in this group ($p = 0.46$; Fig. 6D). We also found no differences in PFS in a comparison of PA tumors with non-PA tumors or *BRAF* alteration subtypes (fusion vs point mutation vs lack of fusion) ($p > 0.05$). However, 4 of 7 patients with *BRAF*^{V600E} mutation progressed after a median follow-up of 20 months.

Only 3 patients died of disease in this study: 2 patients with grade II gliomas and 1 with a pleomorphic xanthoastrocytoma. None of the tumors in these patients had *KIAA1549:BRAF* fusions, but the pleomorphic xanthoastrocytoma had a *BRAF*^{V600E} mutation.

DISCUSSION

Until recently, specific genetic alterations underlying low-grade glioma biology remained elusive. Modern high-resolution genomic studies have provided detailed insight into the molecular genetics of these neoplasms, and there is now a general consensus that MAPK pathway activation is a fundamental feature of many, if not all, low-grade pediatric gliomas, mediated in most instances by activation of RAF family members through rearrangements and/or point mutations (20).

The most frequent RAF family alteration present in PA is the *KIAA1549:BRAF* fusion (7). Pilocytic astrocytoma is also the most frequent primary CNS tumor occurring in the setting of NF-1 (21), a genetic syndrome characterized by

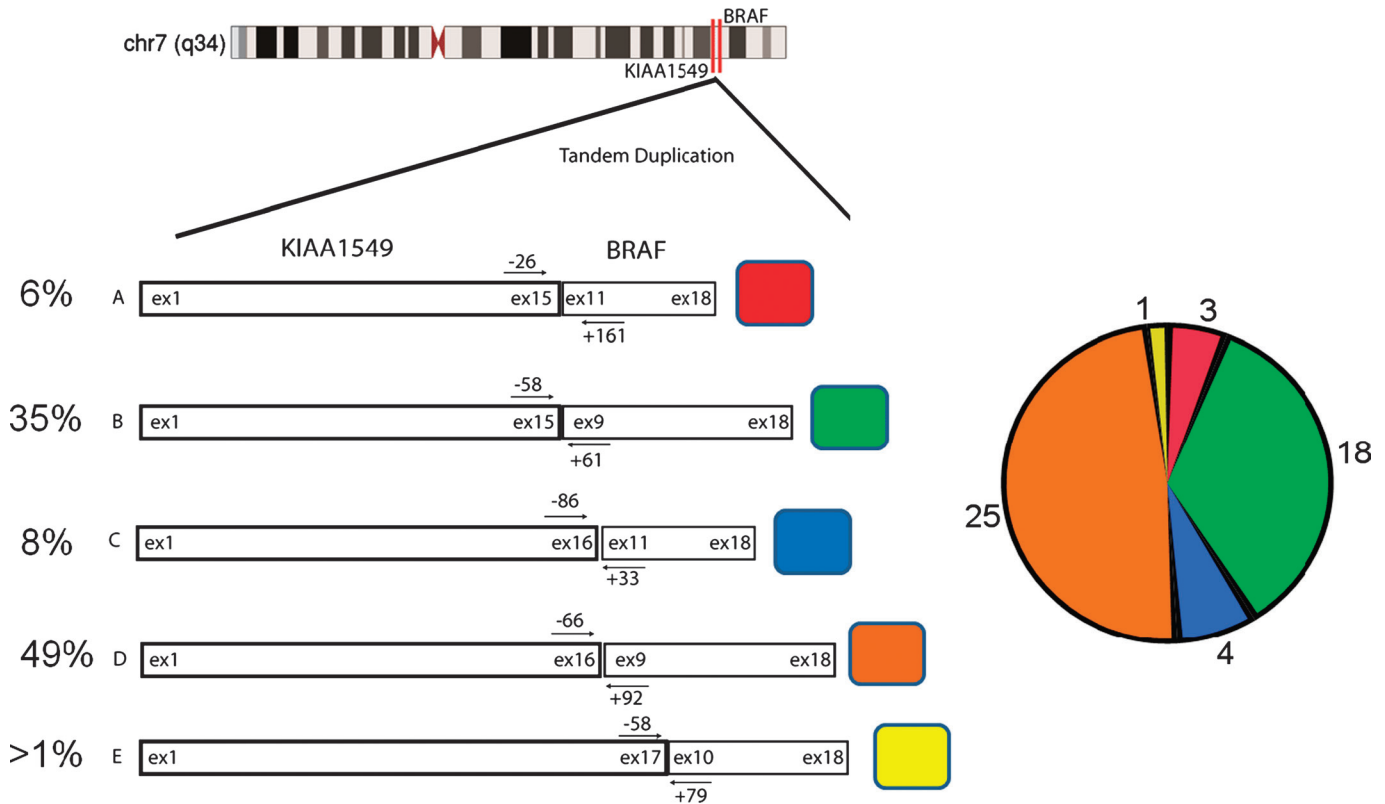


FIGURE 4. Relative distribution of *KIAA1549:BRAF* fusion subtypes. The distribution of all *KIAA1549:BRAF* fusions types identified by percentage (left). The specific *KIAA1549* and *BRAF* exons involved in the different fusion types are labeled within the schematic boxes. Actual tumor numbers with each specific fusion type are indicated in the pie chart (right).

constitutive MAPK/ERK activation. Frequency of *KIAA1549:BRAF* fusion in low-grade glioma/PA varies in the literature, ranging from 60% to 73% (7, 10, 17, 19, 22), although the prevalence may exceed 90% in cerebellar PA (22).

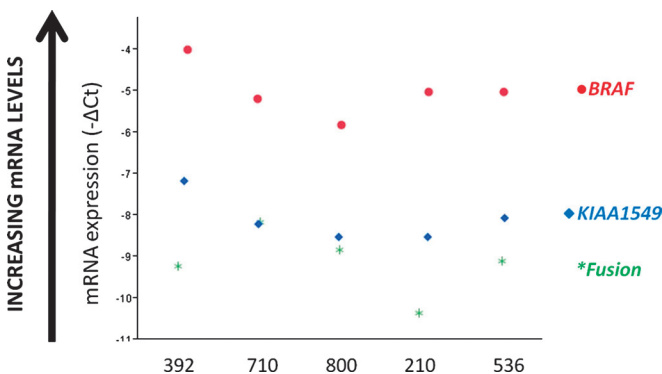


FIGURE 5. Expression levels of *KIAA1549:BRAF* fusion are similar or slightly lower than endogenous *KIAA1549*. Quantitative RT-PCR analysis demonstrated *KIAA1549:BRAF* fusion and *KIAA1549* levels to be similar, but lower than endogenous *BRAF*. mRNA expression levels are expressed as $-\Delta C_t$ values (higher levels in the direction of the arrow), with matched β -actin levels used as internal control. The PCR product sizes (bp) representing the 5 different *KIAA1549:BRAF* fusion variants are indicated in the x axis.

A novel aspect of our study is the discovery of 2 new *KIAA1549:BRAF* fusion variants. In the initial study by Jones et al (7), 3 fusion variants were identified, and Forsheew et al (22) reported 2 additional ones involving *KIAA1549* exons 18 and 19 and *BRAF* exons 10 and 9, respectively. The new sequence-verified variants we identify have breakpoints involving exons 1–15/11–18 and 1–17/10–18 and add to the growing list of possible *KIAA1549:BRAF* fusions. In addition, Cin et al (17) recently reported a novel fusion involving *BRAF* and *FAM131B* mediated by an interstitial deletion. Recent insights into these rearrangements support a role for sequence microhomology and the mechanism of “microhomology-mediated break-induced replication” (23).

We also performed quantitative PCR studies for the various fusion transcripts and demonstrated that the *KIAA1549:BRAF* fusion transcripts are expressed at similar levels as endogenous *KIAA1549*. In line with the study by Jones et al (7), these results suggest that the fusion product is transcribed from a *KIAA1549* promoter but may, in addition, raise the possibility that low levels of the fusion transcript are sufficient for driving neoplastic transformation. One possible explanation is that having appropriate levels of the fusion is important for tumorigenesis, but excess levels of mutant *BRAF* protein products may interfere with tumorigenesis and/or progression. This is supported by recent studies demonstrating that activating genetic alterations in *BRAF* lead to the process of oncogene-induced senescence (24, 25), a process that may

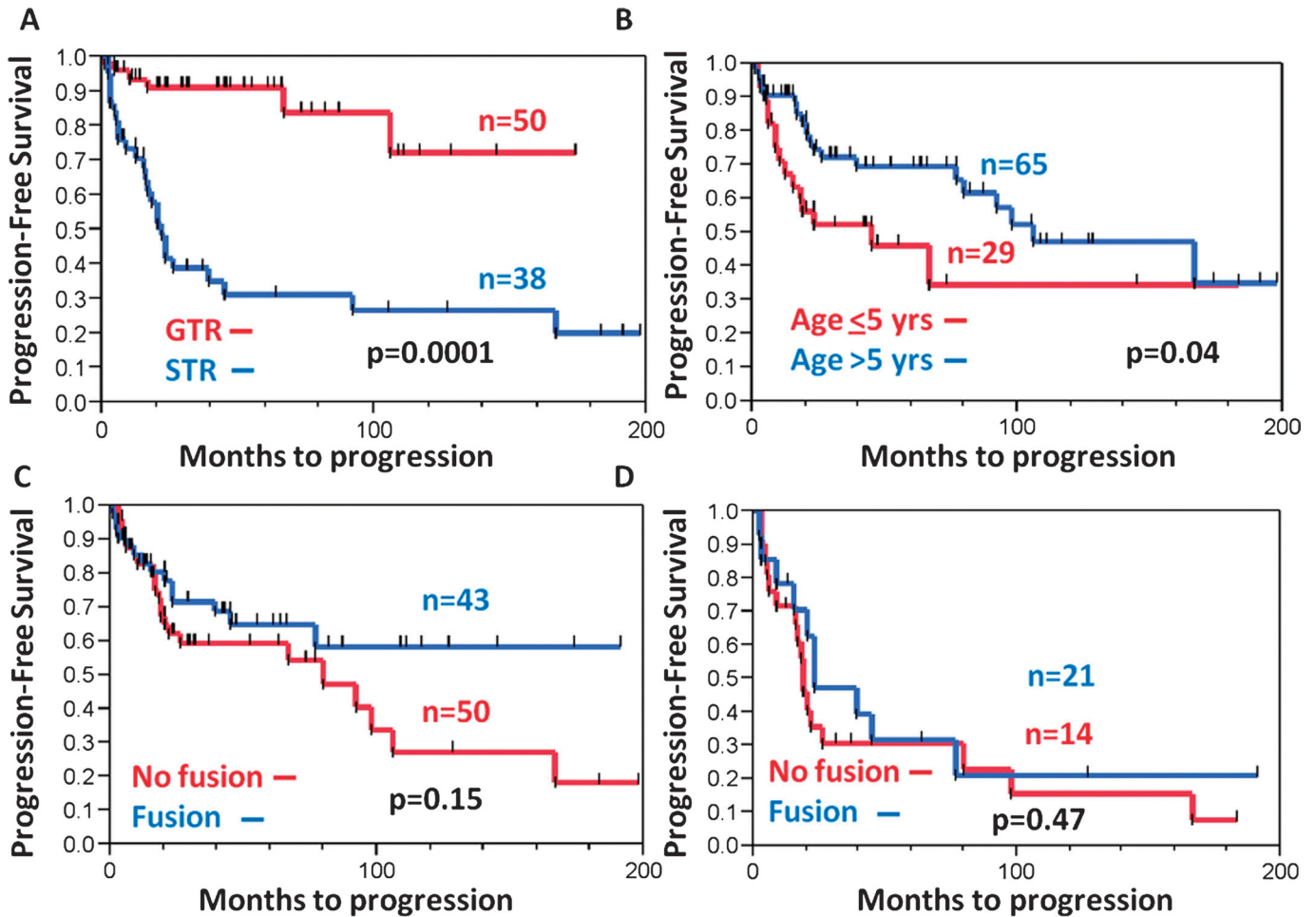


FIGURE 6. Extent of resection and age are significantly associated with progression-free survival (PFS) in low-grade glioma/glioneuronal tumors. **(A–D)** Kaplan Meier curves illustrate PFS comparisons by extent of surgical resection (gross total resection [GTR], red; subtotal resection [STR], blue) **(A)**, age at surgical resection (>5 years, blue; ≤5 years, red) **(B)**, *KIAA1549:BRAF* fused (blue) versus nonfused tumors in the whole group **(C)**, and the clinically relevant group described by Hawkins et al (19) **(D)**. The p values were obtained by log-rank analysis.

antagonize the proliferative advantage provided by oncogene activation.

The strongest clinicopathologic associations we found in the present study were of the *KIAA1549:BRAF* fusion with PA histology and location in the posterior fossa or optic pathways. Some investigators have reported *KIAA1549:BRAF* fusion to be specific for PA (26); however, we found that a subset of difficult-to-classify “low-grade gliomas,” as well as low-grade glioneuronal tumors, also have it. Some of these could represent PA that could not be diagnosed because of limited tissue; however, several of these tumors had significant tissue available for review, suggesting that a subset of non-PA also may have the fusion and that it is not 100% specific for a single diagnostic group. The lower frequency of *KIAA1549:BRAF* fusion in supratentorial PA has been previously noted by another group (10), and our findings help to verify this association. In addition, a recent study found that the frequency of this rearrangement in PA was lower with increasing patient age when taking into account adult patients (27). In our pediatric cohort, we did not identify any correla-

tions with *KIAA1549:BRAF* fusion status and age. Whether specific *KIAA1549:BRAF* rearrangements have biologic significance is unclear. We found that the 1–16/11–18 fusion was limited to infratentorial locations, and the 1–15/11–18 fusion to supratentorial locations, but this should be interpreted with caution in view of the small numbers studied.

Why *KIAA1549:BRAF* fusions occur at a higher frequency at specific anatomic locations (optic pathway/cerebellum) and in association with PA histology is unclear. In a previous publication focusing on anaplastic subsets of PA, *BRAF* duplication was identified by FISH in approximately 60% of cerebellar tumors but in none of the extracerebellar examples (18). Specific biologic differences have been reported in pediatric tumors by anatomic location. Pilocytic astrocytomas and ependymomas, in particular, demonstrate similar gene expression profiles to putative anatomic specific precursors, despite of a lack of histologic differences by site (28, 29). These findings suggest that *BRAF* alterations are tumorigenic when they occur in specific precursors, but these alterations in alternative precursors may not result in tumors

because of the lack of a proper environment or the mechanism of oncogene-induced senescence (24, 25).

The clinical significance of *BRAF* alterations remains unclear. Most studies have not found a significant association with outcome (7, 17, 30). Here, we found a trend toward increased PFS in patients with *KIAA1549:BRAF* fusion, but this did not reach statistical significance. Hawkins et al (19) recently focused on the role of *KIAA1549:BRAF* fusions in a “clinically relevant” subgroup of pediatric low-grade astrocytoma patients. They defined this group as non-NF-1 patients with noncerebellar tumor location and subtotal resection and found that fusions were significantly associated with better outcome in their cohort of 70 patients. In contrast, when we examined outcome in the 35 patients in our series who met the same criteria, we did not identify a trend toward improved survival, although the smaller number of patients in our study was certainly a limiting factor.

In summary, the results of our study are largely in keeping with previously published reports identifying *BRAF* alterations as a frequent event in pediatric low-grade glioma/neuroepithelial tumors, in particular tumors with PA histology, and those involving the cerebellum and optic pathways. We report 2 novel *KIAA1549:BRAF* fusion breakpoints, which add to a growing literature suggesting that these rearrangements are highly heterogeneous. In contrast to the study of Hawkins et al (19), we found no association between *KIAA1549:BRAF* fusions and clinical outcome in either our cohort as a whole or the “clinically relevant” subset, with our analysis limited by a smaller number of patients. Future studies should expand on these observations and continue to define the heterogeneous genetic and biologic features of pediatric low-grade gliomas and the role of *BRAF* alterations in the pathogenesis and clinical behavior of these tumors.

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