BRAF-KIAA1549 Fusion Predicts Better Clinical Outcome in Pediatric Low-Grade Astrocytoma

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Abstract

Purpose: Recent studies have revealed that the majority of pediatric low-grade astrocytomas (PLGA) harbor the BRAF-KIAA1549 (B-K) fusion gene resulting in constitutive activation of the RAS/MAPK pathway. However, the clinical significance of this genetic alteration is yet to be determined. We aimed to test the prognostic role of the B-K fusion in progression of incompletely resected PLGA.

Experimental Design: We retrospectively identified 70 consecutive patients with incompletely resected “clinically relevant” PLGA. We added 76 tumors diagnosed at our institution between 1985 and 2010 as controls. We examined BRAF alterations by reverse transcriptase PCR, FISH, and single-nucleotide polymorphism array analysis and correlated that with progression-free survival (PFS).

Results: Overall, 60% of tumors were B-K fusion positive. All patients with B-K fused PLGA are still alive. Five-year PFS was 61% ± 8% and 18% ± 8% for fusion positive and negative patients, respectively (P = 0.0004). B-K fusion resulted in similarly significant favorable PFS for patients who received chemotherapy. Multivariate analysis revealed that B-K fusion was the most significant favorable prognostic factor in incompletely resected PLGA and was independent of location, pathology, and age. In vitro, BRAF overexpression resulted in growth arrest associated with DNA damage (γH2AX expression). Five-year PFS was 68% ± 15% and 0% for patients with B-K fused and γH2AX-expressing PLGA versus negative tumors (P = 0.001).

Conclusion: These data suggest that B-K fusion confers a less aggressive clinical phenotype on PLGA and may explain their tendency to growth arrest. Combined analysis of B-K fusion and γH2AX expression can determine prognosis and may be a powerful tool to tailor therapy for these patients. Clin Cancer Res; 17(14); 4790–8. ©2011 AACR.

Introduction

Pediatric low-grade astrocytomas (PLGA) are the most prevalent pediatric brain neoplasm. These encompass both pilocytic astrocytomas (WHO grade I) and diffuse astrocytomas (WHO grade II). In some cases, particularly with small biopsies necessitated by the location of the tumor, accurate grading is not possible and the more generic term low-grade astrocytoma is used. Unlike in adults where low-grade diffuse astrocytomas almost inevitably progress to higher grade lesions, this is only rarely the case in pediatrics (1). Most posterior fossa PLGAs can be completely resected, allowing for excellent progression-free survival (PFS; ref. 2). However, for PLGAs located in strategic locations such as the optic pathways, brainstem, and spinal cord, gross total resection is usually not possible and, if attempted, can have devastating morbidity (3, 4). For many years, conventional radiation was used as the primary treatment for local tumor control but concerns about long-term sequelae have resulted in a more conservative approach with low-dose chemotherapy and debulking surgeries as the primary approach to the disease (5, 6). Unlike malignant astrocytomas (WHO grades III and IV) of childhood, which progress relentlessly, PLGAs have a heterogeneous clinical course, ranging from prolonged periods of growth arrest to continuous progression (7). Moreover, because more than half of PLGAs will progress after initial chemotherapy (8), requiring multiple chemotherapy courses and other modalities, there is an urgent need for clinical and biological risk stratification for these children.

Until recently, the only clue to the genetic pathways involved in the development of PLGA was the observation...
of a high rate of optic pathway PLGAs among individuals with neurofibromatosis type 1, suggesting RAS-MAPK pathway activation. Then, in a seminal paper, by using genomic and molecular genetic tools to study PLGA, Pfister and colleagues (9) uncovered a novel duplication in chromosome 7q34 which included the BRAF gene, a downstream gene in the RAS-MAPK pathway (10). Later the same year, a comprehensive study by the Collins laboratory showed that this gain is a result of a tandem duplication between BRAF and KIAA1549 (B-K), producing a novel fusion oncogene (11). Several groups have subsequently confirmed the findings and extended the spectrum of RAS-MAPK activation in pediatric gliomas (12–17).

It is now clear that the B-K fusion is common in pilocytic astrocytomas but not in adult low-grade gliomas (17). However, the fusion does not seem to be specific to WHO grade I astrocytomas, as the B-K fusion is seen in other PLGAs, such as pilomyxoid and diffuse astrocytomas (12). In addition to oncogenic fusions involving the BRAF gene, BRAF mutations (V600E) can be found in pediatric gangliogliomas, pleomorphic xanthoastrocytomas, and rarely in other PLGAs (18, 19). Alterations in other genes in the pathway have also been reported, providing further evidence for the importance of the RAS-MAPK pathway in PLGA.

Although a substantial amount of data has been collected in a very short period of time, the clinicobiological implication of the B-K fusion in PLGA is still unclear. Furthermore, activation of the RAS-MAPK pathway in PLGA fails to explain the unique tendency of PLGAs to manifest a less aggressive clinical course. To test this hypothesis, we utilized a large group of biopsies and matching clinical data from patients with PLGAs who underwent only partial resection of their tumors because of their location in strategic areas of the nervous system. By using reverse transcriptase PCR (RT-PCR), FISH, and DNA-based single-nucleotide polymorphism (SNP) arrays, we examined the role of B-K fusion and DNA damage as prognostic markers in these tumors.

Patients and Methods

By using the Hospital for Sick Children (SickKids) low-grade glioma database, we retrospectively identified 70 patients who had non-cerebellar PLGA tumors diagnosed between 1985 and 2010 for whom both biopsy material and adequate clinical follow-up was available (Table 1, Supplementary Table S1) and which were not completely resected and had been either treated or monitored for more than 1 year. These represented our main “clinically relevant” group for survival analysis. For clinical analysis of these patients, only primary tumor resections were used. FISH and γH2AX staining were conducted on 38 samples where slides were available. An additional 76 cases from other locations in the central nervous system (CNS) and for whom both frozen and formalin-fixed paraffin-embedded tissue was available were used as control samples. This group allowed for comparison between the various methods of fusion detection, namely, FISH versus PCR versus microarray. Patients with neurofibromatosis type 1 were not included in the clinical analysis. All cases were pathologically reviewed and categorized as pilocytic astrocytoma (WHO grade I), pilomyxoid astrocytoma (WHO grade II), or diffuse astrocytoma (WHO grade II), where adequate material was available to accurately assign the tumor to a particular category by using WHO criteria. For some midline tumors, where the amount of tumor tissue was too small to be accurately assigned to one category or another (n = 4), the tumor was given the more generic designation, low-grade astrocytoma. All clinically relevant patients (n = 70) received less than 75% resection of their tumors and 68/70 received less than 50% resection. For analysis of patients who received chemotherapy, we included only patients who were treated on modern protocols since 1995 (n = 45). Treatment regimens included carboplatin-based regimens (n = 37), vinblastine (n = 7), and TPCV (Thioguanine, Procarbazine, CCNU (1-β-Chloroethyl)-3-Cyclohexyl-1-Nitrosourea) and Vincristine) as per CCG9952B (n = 1). PFS was calculated from initial diagnosis. Progression was defined as more than 25% growth in tumor volume on consecutive MRI studies as per recent Children’s Oncology Group clinical trials.

Molecular analysis of BRAF-KIAA1549 fusion

For tumors where sufficient tissue was available, we conducted RT-PCR for the B-K fusion genes as published by Jones and colleagues (ref. 11; n = 118). For tumors where only slides were available, FISH was used as previously
described (ref. 17; n = 38). In addition, we conducted SNP array analysis to detect BRAF gene amplification as previously described by our group (ref. 22; n = 26). Immuno-histochemistry for γH2AX (clone JBW301, Millipore/Upstate) was conducted on available slides as previously described (23). Details of experimental design are available in Supplementary Methods.

Analysis of BRAF overexpressing astrocyte cell line

BRAF overexpressing human telomerase reverse transcriptase (hTERT)-immortalized human astrocytes were previously well characterized and published by our group (14). Additional data are available in Supplementary Methods. For the long-term treatment study, the cells were seeded at 1 × 10^5 per 10-cm dish and fed with Imetelstat (5 μmol/L, Geron Corp) containing medium twice a week. The cells were counted by cell counter ViCELL XR (Beckman Coulter) every week to determine population doublings and replated in the presence of fresh drug during the course of 8 weeks of treatment. Population doublings were calculated as log (the number of cells collected/the number of cells plated)/log 2. Telomerase inhibition was achieved by treatment with Imetelstat (5 μmol/L, Geron Corp). Mismatch (MIS) scrambled RNA served as treatment control as previously reported (24). Further information of experimental procedures, β-galactosidase activity, and immunofluorescence are available in Supplementary Methods.

Statistical and survival analysis

Overall survival and PFS rates were estimated by using the Kaplan–Meier method and significance testing (P < 0.05) conducted on the basis of the log-rank test. Multivariate analysis was done by using multivariate Cox proportional hazards models and significance testing (α = 0.05) based on the Wald test. Correlation between parameters was assessed by using the Pearson χ^2 and Fisher’s exact tests, when applicable. Data were analyzed by using SPSS version 15.0 (SPSS). Because γH2AX as a marker of DNA damage may change over time for PLGA, we analyzed time to progression from the specific biopsy and not from the time of initial diagnosis.

Results

B-K fusion studies were conducted on 146 pediatric low-grade astrocytomas from 125 patients. This represents 70 patients with clinically relevant tumors (see below) which were our study group and additional 76 tumors which were used to establish reproducibility of the B-K fusion in repeated samples and different tumor locations in the brain. For 118 tumors, RNA was available and RT-PCR to detect the fusion was conducted. For the other 28 tumors, material was insufficient for RNA extraction and FISH was conducted. For 16 tumors, both RT-PCR and FISH were conducted in parallel to test concordance of the 2 methods. In 26 samples, SNP arrays were also done to

<table>
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<tr>
<th>Table 1. Patient and tumor characteristics</th>
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<td><strong>B-K fusion</strong></td>
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<tr>
<td>Tumors examined</td>
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<tr>
<td>Patients</td>
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<tr>
<td>Tumor location</td>
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<tr>
<td>Optic pathway non NF1</td>
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<tr>
<td>Optic pathway NF1</td>
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<td>Brainstem</td>
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<td>Posterior fossa</td>
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<td>Lobar</td>
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<td>Disseminated</td>
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<td>Pathology subtype</td>
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<tr>
<td>Pilocytic astrocytoma</td>
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<td>Low-grade astrocytoma</td>
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<td>Pilomyxoid astrocytoma</td>
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<td>Diffuse astrocytoma</td>
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<td>Females</td>
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<td>Males</td>
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<td>Age</td>
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<td>More than 5</td>
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<td>Less than 1.5</td>
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Abbreviation: NF1, neurofibromatosis type 1.
*Three of 5 patients are dead, all progressed.
look for duplication of the 7q34 locus. Overall, there was excellent correlation between RT-PCR and FISH results and with array results (see Supplementary Table S1). In addition, we conducted PCR for the known BRAF V600E mutation on 109 tumors (15). Three (2.7%) pilocytic astrocytomas were positive for the mutation, 2 of which had concomitant fusion of the gene.

Extent of B-K fusion in PLGA subsets
Overall, a B-K fusion was found in 60% of tumors (Table 1). Midline PLGAs (optic pathway, brainstem, posterior fossa, and spinal PLGAs) harbored the B-K fusion in 65% of cases as opposed to only 11% of lobar tumors ($P = 0.0002$). Sixty-two percent of pilocytic astrocytomas had the B-K fusion, with similar frequency observed in pilomyxoid astrocytomas (67%). No fusions were found in pilocytic astrocytomas from patients with neurofibromatosis type 1. No significant difference was found in the frequency of B-K fusions as stratified by age or gender (Table 1). Survival curve for the whole group is available in Supplementary Figure S1.

Clinical significance of B-K fusion in PLGA
We then conducted survival analyses on 70 patients who had clinically relevant PLGA (i.e., incompletely resected optic pathway, brainstem, or spinal cord tumors). Thirty-seven patients had B-K fused tumors, all of whom are alive at a mean follow up of 5.4 years. Of the 33 patients with nonfused tumors, 4 patients (12%) have died. Five-year overall survivals were 100% and 88% ± 6% for patients with B-K fused and nonfused tumors, respectively ($P = 0.002$, Fig. 1A). Five-year PFS were 61% ± 14% and 10% ± 9% for fusion positive and negative tumors, respectively ($P = 0.001$, Fig. 1D). For patients with brainstem tumors, 5-year PFS was 69% ± 13% and 25% ± 12% for fusion positive and negative tumors, respectively ($P = 0.06$). Finally, for patients with spinal PLGA, 2 patients had fusion negative tumors. Both progressed, compared with only 1 of 5 patients with fusion positive tumors. Taken together, patients with incompletely resected tumors had better PFS if their tumor harbored the B-K fusion, irrespective of their tumor location or grade, or whether they received chemotherapy at initial diagnosis. Of particular interest is the observation that for patients less than 1.5 years of age, tumors which lacked the B-K fusion constituted a specifically high-risk group. Of these 5 patients, all have experienced tumor progression and 3 have died of their disease (Table 1).

BRAF overexpressing astrocytes show early senescence
Because constitutive RAS activation can cause oncogene-induced senescence (21, 25), we hypothesized that this mechanism could explain the earlier tumor growth arrest and better PFS of patients with BRAF-activated (i.e., B-K fused) PLGA. To test this hypothesis, we overexpressed BRAF in hTERT-immortalized human astrocytes, as previously published by our group (14), and reversed the hTERT immortalization by telomerase inhibition (ref. 24, Fig. 2). BRAF-overexpressing cells showed time-dependent growth arrest, accompanied by evidence of senescence (beta-galactosidase positivity) and DNA damage ($\gamma$H2AX expression; Fig. 2) compared with the vector-only controls. This suggests an association of DNA damage and early senescence in cells with BRAF activation.

$\gamma$H2AX expression predicts tumor progression irrespective of B-K fusion
Given that DNA damage was associated with BRAF activation and early senescence in culture, we hypothesized that increased DNA damage ($\gamma$H2AX positivity) would be associated with improved PFS in PLGA. Thus, we examined the prognostic value of $\gamma$H2AX as a marker of DNA damage in a cohort of 38 clinically relevant PLGA. Five-year PFS was 56% ± 13% and 19% ± 10% for patients with $\gamma$H2AX positive and negative tumors, respectively ($P = 0.007$, Fig. 1F). Further analysis revealed that PFS for patients with B-K fused tumors was 68% and 22% for $\gamma$H2AX positive and negative PLGAs, respectively ($P = 0.02$). Furthermore, 5-year PFS for patients with B-K nonfused tumors was 22% and 0% for $\gamma$H2AX positive and negative

Table 2. Cox regression model for multivariate analysis ($n = 70$)

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<tr>
<th>Covariable</th>
<th>HR (CI)</th>
<th>$P$</th>
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<tr>
<td>BRAF fusion (+ vs. –)</td>
<td>0.28 (0.14–0.58)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pathology subtype</td>
<td>1.17 (0.89–1.55)</td>
<td>0.28</td>
</tr>
<tr>
<td>Tumor location</td>
<td>0.82 (0.52–1.27)</td>
<td>0.37</td>
</tr>
<tr>
<td>Age (&lt; 5 years vs. &gt; 5 years)</td>
<td>0.81 (0.41–1.60)</td>
<td>0.54</td>
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Pathology subtypes: Pilocytic astrocytoma versus pilomyxoid variant and low-grade astrocytoma NOS.
Tumor location: Optic pathway versus brainstem and spinal tumors.
patients, respectively ($P = 0.05$). Taken together, patients with B-K fused tumors and $\gamma$H2AX expression had excellent tumor control, whereas all patients with nonfused tumors and lack of $\gamma$H2AX expression had tumor progression.

**Discussion**

This study is a continuation of the exciting discoveries of the last 2 years and has both biological and clinical implications. Our observations provide insight into a broader understanding of the role of RAS-MAPK activation and the unique benign behavior of some PLGAs. Specifically, we have shown that B-K fusion is common in midline PLGAs, a group of tumors in which complete resection is often impossible, and which constitutes the largest group of clinically relevant PLGA. Our observations also suggest that B-K fusion is associated with less aggressive tumor behavior possibly because of DNA damage oncogene-induced senescence.

Most previous molecular PLGA studies were comprised largely of completely resected posterior fossa tumors with very few (<10) clinically relevant tumors per study. This combined with the high overall survival of such tumors did not allow for sufficient power to look at outcome in these patients. The particular aim of this study was to test the potential prognostic significance of $BRAF$ fusion status in a group of clinically relevant, incompletely resected patients. Both overall survival and
PFS were examined with the latter being the primary endpoint of the study, as it is the more relevant clinical outcome measure for these children. We found that children with incompletely resected but B-K fusion positive tumors had a much better PFS than their BRAF fusion negative counterparts. Interestingly, Horbinski and colleagues (26) reported a trend to a less aggressive phenotype among B-K fused PLGA even in posterior fossa completely resected tumors. For the noncerebellar tumors, they reported no difference in adverse events between B-K fused and nonfused patients. How this compares with our data is unclear as no formal survival analysis was done. Previous work from our group suggested a survival difference and was the basis of this study (14).

Constitutive activation of the RAS-MAPK oncogenic pathway is involved in many cancers, including most adult gliomas (27, 28). Mutations in RAS, neurofibromin, or other downstream targets are thought to be an early event in these cancers (29). It is therefore intriguing that in vitro evidence is mounting that activation of this oncogenic pathway can push cells into senescence and apoptosis (30). Furthermore, outside the CNS, mutations in BRAF are associated with low-grade and benign lesions, such as melanocytic nevi (31). Over the last several years, the concept of oncogene-induced senescence has gained increasing recognition as an explanation of these controversial findings. Bartkova and colleagues (21, 25) showed that activation of these oncogenic pathways in precancerous and early cancers in colon, breast, and bladder neoplasms, without concomitant disregulation of tumor suppressors such as p53 or Rb, leads to bifurcation fork collapse and overwhelming DNA damage response and hence to senescence and apoptosis.
apoptosis. Because PLGA, as opposed to adult low-grade gliomas, do not have abrogation of the TP53 or RB genes, it is tempting to speculate that this may be the explanation for why these tumors rarely progress to high-grade malignancies and can undergo growth arrest or even spontaneous regression.

Our findings support this concept both in vivo and in vitro. PLGAs with B-K fusion had better PFS regardless of their location, treatment, or pathologic subtype (Fig. 1). It is known that patients with neurofibromatosis type 1 have less aggressive optic pathway pilocytic astrocytomas than non-neurofibromatosis type 1 patients. We recently summarized the experience of our institution (7) and found that PFS for our neurofibromatosis patients was very similar to the B-K fused PLGA in Figure 1D and E, further supporting this concept as both neurofibromin and Braf are regulators of the RAS-MAPK pathway and may thus be driving oncogene-induced senescence.

By using an astrocytic model of Braf overexpression, we were able to show earlier growth arrest in Braf overexpressing immortalized cells following telomerase inhibition. This phenomenon was associated with higher DNA damage and senescence, supporting the role of Braf in oncogene-induced senescence in PLGA. We have previously shown that in contrast to high-grade gliomas, PLGAs lack telomerase which may be associated with their tendency for spontaneous growth arrest (32). Therefore, although other pathways need to be abrogated, one may speculate that telomerase inhibition by using a novel drug Imetelstat that was recently shown to be effective in high-grade gliomas (33) may reduce these high-grade tumors to low-grade ones and expose them to oncogene-induced senescence.

Clinically, PLGA represents a chronic disease in which the timing and modality of intervention, especially at progression, are still controversial. Furthermore, as in some cases diagnoses are made on small biopsies, it can sometimes be difficult to accurately subclassify these tumors. Our findings suggest that objective genetic and molecular tools can help clinicians predict the risk of tumor progression and the need for a more aggressive approach or careful observation. B-K nonfused tumors constitute a high-risk PLGA group, even for grade I tumors, with less than 20% 5-year PFS after treatment with chemotherapy.

This article has the classical limitations of a retrospective study and should therefore be interpreted as such, setting the stage for future prospective ones. Furthermore, as many midline PLGAs are not biopsied but instead are treated according to their imaging and clinical characteristics, the patients included in this study (i.e., biopsied patients) may represent a biased higher-risk group (34). It is important to note that even given this potential bias, the presence of a B-K fusion identifies a group of patients with a better outcome. Finally, as both γH2AX immunostaining and FISH can be conducted on slides from paraffin-embedded tissue and can separate PLGA into clinically important groups (Fig. 3), we may be witnessing a change in the current paradigm in the near future. Biopsies of PLGA may be encouraged upfront but also at further progression to determine treatment decisions. A similar debate exists in diffuse intrinsic pontine gliomas in which the generation of clinically relevant biological data may necessitate tumor biopsy upfront (35, 36).

If indeed these results are confirmed by other studies, there will be a need for consensus statements about which methods should be used for clinical testing (FISH, RT-PCR, or others). Furthermore, rigorous assessment of the right primers and probe sets to use will be required to move forward to prospective clinical trials.

In summary, this study supports the hypothesis that B-K fusion has an important prognostic role in PLGA and furthers our understanding of the causes of growth arrest in PLGA. Further prospective studies are needed to define the right modality to diagnose B-K fusion and to better define the role of RAS/MAPK gene alterations in PLGA.
Disclosure of Potential Conflicts of Interest

This study has not been presented elsewhere; the authors have nothing to disclose.

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References


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