**BRAF-KIAA1549** fusion predicts better clinical outcome in pediatric low grade astrocytoma

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Pediatric low grade astrocytomas (PLGA) are the most common pediatric CNS neoplasm. PLGA represents a chronic disease in which the timing and modality of intervention, especially at progression, are still controversial. Recent studies have revealed that the majority of PLGA harbor the \textit{BRAF-KIAA1549} (B-K) fusion gene resulting in constitutive activation of the \textit{RAS/MAPK} pathway. However, the clinical significance of this genetic alteration is yet to be determined. We demonstrate 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. Combining B-K fusion and measurement of DNA damage can segregate these tumors into 4 different clinically relevant groups. This study represents a change in the current paradigm since biopsies of PLGA may be encouraged upfront but also at further progression to determine treatment decisions for these devastated children.
Abstract:

Purpose: Recent studies have revealed that the majority of pediatric low grade astrocytomas (PLGA) harbor the \textit{BRAF-KIAA1549} (B-K) fusion gene resulting in constitutive activation of the \textit{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 1985-2010 as controls. We examined \textit{BRAF} alterations by RT-PCR, FISH and SNP array analysis and correlated that with progression-free survival.

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. Multiivariate 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. \textit{In vitro}, BRAF overexpression resulted in growth arrest associated with DNA damage (\textit{γH2AX} expression). Five-year PFS was 68\%/15\% and 0\% for patients with B-K fused and \textit{γH2AX} expressing PLGA vs. 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 \textit{γH2AX} expression can determine prognosis and may be a powerful tool to tailor therapy for these patients.
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. Most posterior fossa PLGAs can be completely resected, allowing for excellent progression free survival. 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. 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. 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. Moreover, since more than half of PLGAs will progress after initial chemotherapy, 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 I, suggesting RAS-MAPK pathway activation. Then, in a seminal paper using genomic and molecular genetic tools to study PLGA, Pfister et al uncovered a novel duplication in chromosome 7q34 which included the BRAF gene, a downstream gene in the
RAS-MAPK pathway\textsuperscript{10}. Later the same year, a comprehensive study by the Collins laboratory demonstrated that this gain is a result of a tandem duplication between \textit{BRAF} and \textit{KIAA1549}, producing a novel fusion oncogene\textsuperscript{11}. Several groups have subsequently confirmed the findings and extended the spectrum of RAS-MAPK activation in pediatric gliomas\textsuperscript{12-17}.

It is now clear that the \textit{BRAF} and \textit{KIAA1549} (B-K) fusion is common in pilocytic astrocytomas but not in adult low grade gliomas\textsuperscript{17}. However, The fusion does not seem to be specific to WHO grade I astrocytomas, since the B-K fusion is seen in other PLGAs such as pilomyxoid and diffuse astrocytomas\textsuperscript{12}. In addition to oncogenic fusions involving the \textit{BRAF} gene, \textit{BRAF} mutations (V600E) can be found in pediatric gangliogliomas, pleomorphic xanthoastrocytomas and rarely in other PLGAs\textsuperscript{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 clinico-biological 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 PLGA to growth arrest. Since the concepts of oncogene-induced senescence and/or replicative senescence could explain the mechanism of RAS activation leading to tumor growth arrest\textsuperscript{20,21}, we hypothesized that in PLGA the B-K fusion results in increased DNA damage, driving PLGAs with this fusion to undergo early growth arrest and thus 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 due to their location in strategic areas of the nervous system. Using RT-PCR, FISH and DNA-based SNP arrays we examined the role of B-K fusion and DNA damage as prognostic markers in these tumors.
Patients and methods:

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 1), 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 performed on 38 samples where slides were available. An additional 76 cases from other locations in the central nervous system 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 vs PCR vs microarray. Patients with NF-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 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 as per CCG9952B (n=1). Progression free survival was calculated from initial diagnosis.
Progression was defined as more than 25% growth in tumor volume on consecutive MRI imaging studies as per recent Children’s Oncology Group clinical trials.

**Molecular analysis of **BRAF-KIAA1549** fusion:** For tumors where sufficient tissue was available, we performed RT-PCR for the B-K fusion genes as published by Jones et al\(^{11}\) (n=118). For tumors where only slides were available, fluorescent in situ hybridization (FISH) was used as previously described\(^{17}\) (n=38). In addition, we performed SNP array analysis to detect BRAF gene amplification as previously described by our group\(^{22}\) (n=26). Immunohistochemistry for γH2AX (clone JBW301, Millipore/Upstate, Mississauga, Canada) was performed on available slides as previously described\(^{23}\). Details of experimental design are available as supplementary methods.

**Analysis of BRAF over-expressing astrocyte cell line:** BRAF overexpressing hTERT-immortalised human astrocytes were previously well characterized and published by our group\(^{14}\). Additional data is available in the supplemental methods. For the long-term treatment study, the cells were seeded at 1 x 10\(^5\) per 10-cm dish and fed with Imetelstat (5 uM, GERON corp, Ca, USA) containing medium twice a week. The cells were counted by cell counter Vi-CELL XR (Beckman Coulter, Mississauga, Ontario) 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 uM, GERON corp, Ca, USA). 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 the supplementary methods.
**Statistical and survival analysis:** Overall and progression-free survival rates were estimated using the Kaplan-Meier method and significance testing (p<0.05) performed on the basis of the log-rank test. Multivariate analysis was done using multivariate Cox proportional hazards models and significance testing (α = 0.05) based on the Wald test. Correlation between parameters was assessed using the Pearson Chi-square and Fisher’s exact tests, when applicable. Data were analyzed using SPSS version 15.0 (SPSS, Chicago, IL). Since 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:**

*BRAF-KIAA1549* (B-K) fusion studies were performed 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 performed. For the other 28 tumors, material was insufficient for RNA extraction and FISH was performed. For 16 tumors, both RT-PCR and FISH were performed in parallel to test concordance of the two methods. In 26 samples, SNP arrays were also done to look for duplication of the 7q34 locus. Overall, there was excellent correlation between RT-PCR and FISH results and with array results (see supplemental Table 1). In addition, we performed PCR for the known *BRAF* V600E mutation on 109 tumors. Three (2.7%) pilocytic astrocytomas were positive for the mutation, two 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=0.002). 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 supplemental Figure 1.

Clinical significance of B-K fusion in PLGA

We then performed survival analyses on 70 patients who had clinically relevant PLGA (ie 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 non-fused tumors, 4 patients (12%) have died. Five year overall survivals were 100% and 88%/6% for patients with B-K fused and non-fused tumors, respectively (p=0.07, Figure 1A). Five year progression free survivals (PFS) were 61%/8% and 18%/8% for fusion positive and negative patients, respectively (p=0.0004) (Figure 1B). Cox Regression multivariate analysis (including tumor location, pathology subtype, patient age and B-K fusion status) revealed that the presence of the B-K fusion was the single most significant risk factor with hazard ratio of 0.28 (p<0.001) for fusion-positive patients.

In order to better define clinically relevant risk groups we performed a separate survival analysis on patients who received chemotherapy as their first line of treatment at initial diagnosis (n=45). Of these patients, 25(55%) harbored the B-K fusion. Five year PFS was 48%/10% and 6%/6%
for fusion positive and negative tumors, respectively (p=0.0018, Figure 1C). Analysis of the 58 patients with pilocytic astrocytomas revealed 5 year PFS of 65+/-9% and 17+/-8% for fusion positive and negative tumors, respectively (p=0.002, Figure 1E). We then stratified the patients by tumor location. Patients with optic pathway tumors had 5 year PFS of 61+/-14% and 10+/-9% for fusion positive and negative tumors, respectively (p=0.001, Figure 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, two patients had fusion negative tumors. Both progressed, compared to only one of five 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 over-expressing astrocytes demonstrate early senescence.**

Since 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 \textit{BRAF} activated (i.e. B-K fused) PLGA. To test this hypothesis, we over-expressed \textit{BRAF} in hTERT-immortalized human astrocytes as previously published by our group$^{14}$, and reversed the hTERT immortalization by telomerase inhibition$^{24}$ (Figure 2). BRAF over-expressing cells demonstrated time dependent, growth arrest accompanied by evidence of senescence (beta-galactosidase positivity) and DNA damage (\textit{\gamma}H2AX expression) (Figure 2) compared to the
vector-only controls. This suggests an association of DNA damage and early senescence in cells with BRAF activation.

**γH2AX 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 (γH2AX positivity) would be associated with improved PFS in PLGA. Thus, we examined the prognostic value of γH2AX 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 γH2AX positive and negative tumors, respectively (p=0.007, Figure 1F). Further analysis revealed that PFS for patients with B-K fused tumors was 68% and 22% for γH2AX positive and negative PLGAs, respectively (p=0.02). Furthermore, 5 year PFS for patients with B-K non-fused tumors was 22% and 0% for γH2AX positive and negative patients, respectively (p=0.05). Taken together, patients with B-K fused tumors and γH2AX expression had excellent tumor control while all patients with non-fused tumors and lack of γH2AX 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 due to 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- and progression-free survival were examined with the latter being the primary end-point 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 progression-free survival than their BRAF-fusion negative counterparts. Interestingly, Horbinski et al\textsuperscript{26} reported a trend to a less aggressive phenotype among B-K fused PLGA even in posterior fossa completely resected tumors. For the non-cerebellar tumors they reported no difference in adverse events between B-K fused and non-fused 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\textsuperscript{14}.

Constitutive activation of the \textit{RAS-MAPK} oncogenic pathway is involved in many cancers including most adult gliomas\textsuperscript{27,28}. Mutations in RAS, neurofibromin or other downstream targets are thought to be an early event in these cancers\textsuperscript{29}. It is therefore intriguing that \textit{in vitro} evidence is mounting that activation of this oncogenic pathway can push cells into senescence and apoptosis\textsuperscript{30}. Furthermore, outside the CNS, mutations in \textit{BRAF} are associated with low grade and benign lesions such as melanocytic nevi\textsuperscript{31}. Over the last several years the concept of oncogene-induced senescence has gained increasing recognition as an explanation of these controversial
findings. Bartkova et al\textsuperscript{21,25} demonstrated 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. Since 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 \textit{in vivo} and \textit{in vitro}. PLGAs with B-K fusion had better PFS regardless of their location, treatment or pathological subtype (Figure 1). It is known that patients with neurofibromatosis type 1 have less aggressive optic pathway pilocytic astrocytomas than non-NF1 patients. We recently summarized our institution’s experience\textsuperscript{7} and found that PFS for our neurofibromatosis patients was very similar to the B-K fused PLGA in Figure 1-D,E, further supporting this concept since both neurofibromin and $BRAF$ are regulators of the $RAS$-$MAPK$ pathway and may thus be driving oncogene-induced senescence.

Using an astrocytic model of $BRAF$ over-expression, we were able to demonstrate earlier growth arrest in $BRAF$ over-expressing 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\textsuperscript{32}. Therefore, although other pathways need to be abrogated, one may speculate that telomerase inhibition using a novel drug Imetelstat, that was recently shown to be effective in high grade gliomas\textsuperscript{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, since 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 non-fused tumors constitute a high risk PLGA group, even for grade I tumors, with less than 20% 5 year PFS after treatment with chemotherapy.

This report has the classical limitations of a retrospective study and should therefore be interpreted as such, setting the stage for future prospective ones. Further, 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. 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, since both γH2AX immunostaining and FISH can be performed on slides from paraffin embedded tissue and can separate PLGA into clinically important groups (Figure 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 up-front.

If indeed these results are confirmed by other studies, there will be a need for consensus statements regarding which methods should be used for clinical testing (FISH, RT-PCR or other). 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.
References:

Table 1: Patient and tumor characteristics:

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<th></th>
<th>Negative (%)</th>
<th>Positive (%)</th>
<th>Total</th>
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<tr>
<td><strong>B-K fusion</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumors examined</td>
<td>58(40)</td>
<td>88(60)</td>
<td>146</td>
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<tr>
<td>Patients</td>
<td>51(41)</td>
<td>74(59)</td>
<td>125</td>
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<tr>
<td><strong>Tumor location</strong></td>
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<td></td>
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<tr>
<td>Optic pathway non NF1</td>
<td>11(39)</td>
<td>17(61)</td>
<td>28</td>
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<tr>
<td>Optic pathway NF1</td>
<td>4(100)</td>
<td>0(0)</td>
<td>4</td>
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<tr>
<td>Brainstem</td>
<td>19(53)</td>
<td>17(47)</td>
<td>36</td>
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<tr>
<td>Posterior fossa</td>
<td>7(18)</td>
<td>31(82)</td>
<td>38</td>
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<tr>
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<td>2(25)</td>
<td>6(75)</td>
<td>8</td>
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<tr>
<td>Lobar</td>
<td>8(89)</td>
<td>1(11)</td>
<td>9</td>
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<tr>
<td>Disseminated</td>
<td>1(50)</td>
<td>1(50)</td>
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<tr>
<td><strong>Pathology subtype:</strong></td>
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<td></td>
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<tr>
<td>Pilocytic astrocytoma</td>
<td>40(38)</td>
<td>65(62)</td>
<td>105</td>
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<tr>
<td>Low grade astrocytoma</td>
<td>2(50)</td>
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<td>4</td>
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<td>Pilomyxoid astrocytoma</td>
<td>2(33)</td>
<td>4(67)</td>
<td>6</td>
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<td>Diffuse astrocytoma</td>
<td>8(63)</td>
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<td>10</td>
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<td>Females</td>
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<td>38(54)</td>
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<tr>
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<td>18(33)</td>
<td>36(67)</td>
<td>54</td>
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<tr>
<td>Age</td>
<td></td>
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<tr>
<td>More than 5</td>
<td>36(44)</td>
<td>27(56)</td>
<td>81</td>
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<tr>
<td>Less than 5</td>
<td>16(36)</td>
<td>28(64)</td>
<td>44</td>
</tr>
<tr>
<td>Less than 1.5</td>
<td>5*(41)</td>
<td>7(59)</td>
<td>12</td>
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NF1-Neurofibromatosis type 1.
*Three of five patients are dead, all progressed.
Table 2: Cox regression model for multivariate analysis (n=70)

<table>
<thead>
<tr>
<th>Covariable</th>
<th>Hazard Ratio (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>
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<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>
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<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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</table>

Pathology subtypes: Pilocytic astrocytoma vs pilomyxoid variant and low grade astrocytoma NOS. Tumor location: Optic pathway vs brainstem and spinal tumors.
Figure 1: **Kaplan Meier estimates for PLGA subtypes.** A. Overall survival for all clinically relevant patients (n=70). B. Progression free (PFS) survival for all patients (n=70). C. PFS for patients who received chemotherapy upfront (n=45). D. PFS for patients with optic pathway tumors (n=28). E. PFS for patients with Pilocytic astrocytoma (n=58). F. PFS for tumors by γH2AX expression (n=38).

**Figure 2: BRAF over-expressing astrocytes undergo early growth arrest.**

hTERT immortalized astrocytes were stably transfected by BRAF or empty vector (EV11). In order to mimic PLGA, cells were treated with the telomerase inhibitor Imetelstat. BRAF over-expressing cells manifested complete growth arrest after 7 weeks (A). This was associated with senescence as demonstrated by β-galactosidase activity (B) and higher degree of DNA damage measured by γH2AX expression (C).

**Figure 3: Immunohistochemistry and FISH analysis of progression in PLGA.**

Using 2 robust methods performed on slides from paraffin embedded PLGA, one can predict progression free survival for these patients. Tumor which harbored the B-K fusion and stained positive for gH2AX had excellent PFS while all tumors which lacked the B-K fusion and did not did not express gH2AX progressed (p=0.001). PFS- progression free survival.
A

![Graph showing cell population doubling over weeks of treatment for EV-11, BRAF-untreat, BRAF-mis, and BRAF-Imetelstat.](image1)

B

![Images showing untreated and Imetelstat-treated EV-11 and BRAF cells.](image2)

C

![Images showing the number of H2AX foci per cell for EV-11 and BRAF2-5 under untreated, Mis, and Imetelstat treatments.](image3)
FISH

γH2AX

5yr PFS

0%  22+/−8%  22+/−5%  68+/−15%

**p=0.001
BRAF-KIAA1549 fusion predicts better clinical outcome in pediatric low grade astrocytoma


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