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Pathological and Molecular Advances in Pediatric Low Grade Astrocytoma

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Abstract

Pediatric low grade astrocytomas are the commonest brain tumors in children. They sometimes have similar microscopic and clinical features, making accurate diagnosis difficult. For patients whose tumors are in locations that do not permit full resection, or those with an intrinsically aggressive biology, more effective therapies are required. Until recently, little was known about the molecular changes that drive the initiation and growth of pilocytic and other low grade astrocytomas beyond the association of a minority of cases, primarily in the optic nerve, with neurofibromatosis type 1. Over the last several years, a wide range of studies have implicated the *BRAF* oncogene and other members of this signaling cascade in the pathobiology of pediatric low grade astrocytoma. In this review, we attempt to summarize this rapidly developing field, and discuss the potential for translating our growing molecular knowledge into improved diagnostic and prognostic biomarkers and new targeted therapies.

Keywords

Pilocytic astrocytoma; BRAF; NF1; RAF1

CLINICAL AND DEMOGRAPHIC FEATURES

Pediatric low grade astrocytomas (PLGA) arise throughout the central nervous system (CNS), but are found most often in the cerebellum, followed by the cerebrum and deep midline structures, optic pathways, brainstem and spinal cord (1). According to 2012 CBTRUS data, pilocytic astrocytomas (PA) are the second most common brain tumor (after embryonal neoplasms) in the 0–4 year age group, the commonest tumors in children 5–14 years of age, and the second most common tumors (after pituitary neoplasms) in 15–19 year-olds (<http://www.cbtrus.org>). A graphical representation of primary pediatric brain tumor diagnoses based on current CBTRUS data is shown in Figure 1. Most non-infiltrative PLGA (i.e. pilocytic/pilomyxoid astrocytoma, pleomorphic xanthoastrocytoma, subependymal giant cell astrocytoma) show variable contrast enhancement on imaging studies, while infiltrating diffuse low grade astrocytomas are generally non-enhancing.

Outcomes for this group of tumors are good overall, but vary depending on the extent of surgical resection and histopathological classification. In one large institutional series of

children with low grade astrocytomas, 5 year overall survival (OS) was 96% for PA patients and 48% for diffuse astrocytoma patients (2). Gross total resection has consistently been strongly prognostic of long-term survival, but can often only be achieved when tumors are localized to the cerebellum or superficial cerebrum (3–5). It has also been suggested that PA behave in a more aggressive fashion when arising in adults(6).

HISTOLOGICAL CLASSIFICATION

Pilocytic Astrocytoma (Grade I)

PA are generally characterized by a biphasic growth pattern including both compacted bipolar cells (Figure 2A) and regions with microcysts and more loosely textured cells (Figure 2B). Characteristic elements include Rosenthal fibers and eosinophilic granular bodies (Figure 2A, B). Oligodendroglial-appearing cells are often encountered in PA, and in some cases can comprise a significant portion of the tumor (7), making diagnosis difficult in small samples. Most lesions are cytologically bland, although some atypia can be present. Mitotic figures are only present in the minority of cases, and the lack of infiltration, as well as the presence of more specific features such as eosinophilic granular bodies, allows most cases to be differentiated from high grade astrocytomas (8).

A number of potential histological and immunohistochemical prognostic markers have been investigated in PA. It has been suggested that tumors with an elevated Ki67 proliferation index are associated with worse progression-free survival (9, 10), although in other studies proliferation was not prognostic (11–13). Anaplastic features, including cytological atypia, hypercellularity, high mitotic activity, and necrosis, can also portend worse outcomes (14). Morphological or immunohistochemical evidence of oligodendroglial differentiation may also predict aggressive behavior in PA patients (7, 11).

Pilomyxoid Astrocytoma (Grade II)

Pilomyxoid astrocytomas are comprised of monomorphous bipolar cells, often arrayed around vessels, with a mucoid background matrix, and lack Rosenthal fibers and eosinophilic granular bodies (Figure 2C). Referred to in early reports as “infantile” pilocytic astrocytomas, their distinct microscopic features and more aggressive clinical behavior were highlighted in 1999 by Tihan and colleagues (15), leading to their recognition as a potentially distinct variant in the 2007 World Health Organization (WHO) classification (8). They are often found in hypothalamus, optic chiasm, and around the third ventricle, although other sites can also be involved. Recently, transitional tumors with mixed pilocytic and pilomyxoid features have been described, as well as cases in which a pilomyxoid astrocytoma “matured” into a classic pilocytic astrocytoma over time (16), suggesting that these lesions represent part of a spectrum rather than completely distinct entities.

Pleomorphic Xanthoastrocytoma (PXA, Grade II)

PXA generally show greater cellularity and atypia than other entities discussed in this review, and can sometimes be misdiagnosed as high grade glioma. They occur most frequently in teenagers and young adults, often involve superficial cortex and meninges, and can contain lipidized “xanthomatous” astrocytes (8). Eosinophilic granular bodies as well as spindled and pleomorphic glial elements are all common, and the tumors frequently attract a lymphocytic infiltrate and contain abundant extracellular reticulin (Figure 2D). CD34 expression has been proposed as a useful immunohistochemical feature (17).

Subependymal Giant Cell Astrocytoma (SEGA, Grade I)

These benign tumors are tightly associated with the autosomal dominant inherited condition tuberous sclerosis, and arise almost exclusively in the walls of the lateral ventricles (8).

Spindled, gemistocytic and ganglion-cell like elements can be present, with the latter cells representing the most characteristic finding (Figure 2E). Immunohistochemical and ultrastructural studies have shown mixed glial and neuronal differentiation in tumor cells (18–20), and despite their designation as an “astrocytoma” these are probably best regarded as mixed glial-neuronal neoplasms.

Diffuse Astrocytoma (Grade II)

Infiltrating fibrillary astrocytomas in children are morphologically similar to their WHO grade II counterparts in adults. They are characterized by modest cellularity, diffuse infiltration of normal brain elements, and a lack of significant mitotic activity, vascular proliferation or necrosis (Figure 2F)(8). Because of their ability to spread diffusely, these tumors are difficult to completely resect and have worse outcomes than PA in children (2). As in adults, they can progress to high grade glioma, although this often does not occur. Emerging data suggest that diffuse astrocytomas in the pediatric population are molecularly distinct compared to their morphologically similar adult counterparts.

Diagnostic Difficulties in PLGA

Tumors which are difficult to classify are unfortunately all too often encountered among the spectrum of PLGA. In one institutional study of 278 consecutive PLGA resected between 1965 and 1996, 75 cases (27%) did not clearly fit into a WHO diagnostic category. A more recent review of 1,670 pediatric brain tumors of all types diagnosed at our institution between 2003 and 2008 identified 302 cases which could not be classified, and 7 of the 10 most common problems with diagnosis involved low grade gliomas (personal communication, Dr. Peter Burger). In some cases diagnostic dilemmas arise due to small biopsies, but in others they reflect inability of the current scheme to fit a heterogeneous spectrum of lesions. Molecular studies have the potential to help with these problematic issues, and as described below are beginning to shed light on some diagnostic difficulties.

MOLECULAR ADVANCES

Aside from PA arising in neurofibromatosis type 1 (NF1) patients, and SEGA in children and young adults with tuberous sclerosis, for many years little was known about the molecular underpinnings of PLGA. Sporadic PA do not inactivate *NF1*, and generally lack changes to the oncogenes and tumor suppressors altered in adult diffuse astrocytomas (8, 21). Early cytogenetic studies of PA were notable for a lack of detectable chromosomal alterations, with largely normal karyotypes in the more than 100 cases initially studied (8). Mutations in *IDH1* have been identified in the majority of low grade gliomas in adults, but interestingly are almost never detected in pediatric low or high grade glioma, and when present have mostly been reported in children at least 14 years old (22–24). This suggests that adolescents with *IDH1*-mutant tumors may represent the youngest patients with “adult” gliomas. Over the last few years, however, it has become clear that most non-syndromic PLGA harbor genomic alterations which affect the function of BRAF, and that mitogen activated protein kinase (MAPK) represents the dominant genetically altered pathway in these tumors (Figure 3).

BRAF Fusions and MAPK Activation in PLGA

In 2008, five different groups identified gains at 7q34 approximately 2 megabases in size in most PLGA using array-based comparative genomic hybridization (25–29) (Figure 4A). Fluorescent in situ hybridization (FISH) and other molecular analyses showed that these represented segmental duplications in the region (Figure 4B)(25, 28). In these initial studies, between 53% and 77% of the PLGA examined (mostly PA) contained the duplication at

7q34, with relatively few other chromosomal alterations detected, suggesting this represented the dominant change in PA.

In two of the papers, the authors demonstrated that 7q34 duplication resulted in expression of a novel fusion transcript between the *KIAA1549* locus and *BRAF* that included the *BRAF* kinase domain but lacked the inhibitory N-terminal regulatory region of this oncogene (Figure 4C)(28, 29). The fusion showed constitutive kinase activity and was able to transform NIH 3T3 cells (29). *BRAF* is known to induce signaling in the MAPK pathway, and activated targets including phosphorylated MEK (pMEK) and/or ERK (pERK. Figure 4D) were identified in the majority of PLGA specimens examined in these initial studies (25, 27, 28).

A large number of additional reports have confirmed these exciting findings, and firmly establish *KIAA1549:BRAF* duplication/fusion as the commonest genetic change in PA (30–39). Specific breakpoints between *KIAA1549* and *BRAF* can vary, but all lead to loss of the *BRAF* autoregulatory domain (Figure 5). In one recent study of 106 pediatric low grade brain tumors, five types of *KIAA1549:BRAF* gene fusions were identified, involving exons 1–16/9–18 (49%), 1–15/9–18 (35%), 1–16/11–18 (8%), 1–15/11–18 (6%) and 1–17/10–18 (1%)(39). In this study the 1–16/11–18 fusion was limited to infratentorial sites, and the 1–15/11–18 fusion to supratentorial locations, but it will be necessary to examine larger numbers of cases before firm conclusions can be drawn regarding associations between fusion genotype and tumor phenotype. Genetic mapping of the breakpoints involved have highlighted enrichment for microhomologous DNA sequences, suggesting microhomology-mediated break-induced replication as a possible mechanism for the rearrangements (40).

The *NFI* gene product acts to inhibit RAS and *BRAF* activity, and the discovery of fusions activating *BRAF* therefore links syndromic and sporadic PA to the same oncogenic signaling cascade (Figure 3). Some patients with Noonan syndrome, in which MAPK signaling is activated by mutations in *PTPN11*, *SOS1* and *KRAS*, have also been reported to have PA (41–43), providing further support for the central role of this pathway. A rare rosette forming glioneuronal tumor of the posterior fossa demonstrating strong pERK immunoreactivity has also been reported in a case of Noonan syndrome (44).

Novel genetic mechanisms driving activation of the MAPK pathway in PLGA continue to be discovered. Two groups identified oncogenic fusions between *SRGAP3* and *RAF1* predicted to give rise to unregulated kinase activity similar to that seen with *KIAA1549:BRAF* (Figure 5)(30, 45). Rare 3 base pair insertions at position 599 have also been described in PA (32, 45–47). This alteration (*BRAF^{insT}*) results in duplication of a threonine residue, and causes over 6-fold increases in kinase activity *in vitro* (47). An interstitial deletion causing fusions between *FAM131B* and *BRAF* has also been identified (37). However, both the *SRGAP3* and *FAM131B* fusions and the *BRAF^{insT}* are much less common than *KIAA1549:BRAF* fusions in PLGA.

Point Mutations in *BRAF*, *RAF* and *RAS*

MAPK signaling can also sometimes be activated in PLGA by point mutations in various pathway members. Indeed, prior to the discovery of *BRAF* gene duplications, rare oncogenic mutations in *KRAS* had been described (48, 49). Subsequent studies have confirmed the presence of occasional *KRAS* mutations in PA. Forshev and colleagues sequenced *HRAS*, *KRAS* and *NRAS* in 50 PLGA and found an activating *KRAS*G12A mutation in one cerebellar PA which lacked *BRAF* and *RAF1* fusions (30). Cin and colleagues examined *HRAS*, *KRAS*, *NRAS*, *PTP11*, and *RAF1* in 125 primary PA samples and identified oncogenic *KRAS* mutations in 2 tumors (37). Thus approximately 2% of PA have mutations activating *KRAS*. Point mutations in *RAF1* may not be selected for due to

its lower basal activity as compared to *BRAF* (45, 50), but it is not clear why mutations in *HRAS* and *NRAS* are not present in PLGA.

The commonest point mutation in PLGA occurs in *BRAF* itself at codon 600, and results in substitution of valine by glutamic acid. The *BRAF*^{V600E} mutation was first described in extra-CNS tumor cell lines; it is most frequent in melanoma, but can also be found in a range of other neoplasms (51). In PLGA, *BRAF*^{V600E} mutations were identified in 4/66 (6%) of the tumors examined by Pfister et al. (25), including 3 PA and 1 diffuse astrocytoma. Subsequent studies have also readily identified *BRAF*^{V600E} mutations in PLGA (28, 30, 32, 37–39, 46, 47), although as discussed below these are most common in tumors other than PA.

BRAF and *RAF1* fusions are generally mutually exclusive with other genetic alterations activating MAPK signaling, but some exceptions to this have been reported. Cin and colleagues identified 2 PA patients with concomitant *BRAF*^{V600E} mutation and *BRAF* fusion, one of whom also had NF1 syndrome (37). In another study, 6 tumors (out of 198) had both *BRAF* fusion and *BRAF*^{V600E} mutation (13).

Genetic Alterations and Tumor Site

Tandem duplications involving *BRAF* do not occur uniformly in PLGA at all sites in the CNS (Figure 6). The percentage of cerebellar/posterior fossa PA with molecular alterations at 7q34 is particularly high, ranging from 63% to 94% in various reports (27, 28, 30, 31, 37, 52). In one study of 32 posterior fossa PAs, 30 had *KIAA1549:BRAF* fusions, 1 had a *SRGAP3:RAF1* fusion, and 1 had a mutation in *KRAS* (30). In contrast, the frequency of *BRAF* duplication/fusion in PA arising in the cerebral cortex is quite low, ranging from 0% to 50% (27, 29, 31–33, 37, 52–54). A total of 72 cerebral cases were reported in these studies, with 18 (25%) showing *BRAF* duplication or fusion (Figure 6). Our review of published cases yielded percentages of *BRAF* duplications at other sites higher than cerebral cortex but lower than posterior fossa. This included the optic pathways (42/83, 51%), deep grey matter (17/41, 41%), brainstem (56/95, 59%) and spinal cord (6/11, 55%).

The limited number of other molecular changes in PA makes it harder to definitively assess their spatial distribution. However, it seems that *BRAF*^{V600E} and *KRAS* mutations are more common in PA arising outside the posterior fossa. In one series, 10/49 (20%) of non-cerebellar PA had point mutations in one of these two oncogenes, as compared to 3/76 (4%) of cerebellar lesions, a statistically significant difference (37). Schindler and colleagues also found a statistically significant association between *BRAF*^{V600E} and extracerebellar location of PA ($p = 0.009$) (46). In contrast, the handful of *SRGAP3:RAF1* and *FAM131B:BRAF* fusions reported to date have largely been in PA arising in the cerebellum (30, 37).

The cause of the increased incidence of *BRAF* duplication in the posterior fossa is not clear, but it may reflect increased susceptibility of the tumor cell(s) of origin at this site. It has also been suggested that *BRAF* rearrangements in PA are less common in adult patients (55), which could reflect changes in tumor histogenesis as patients' age. Regional differences in expression of the fusions could also play a role. While the *KIAA1549*, *SRGAP3* and *FAM131B* genes are all expressed in the CNS, little is known regarding their precise levels in various regions or cell types. It has recently been demonstrated that *KIAA1549:BRAF* fusions are expressed at roughly equivalent levels in PA as the endogenous *KIAA1549* gene (39).

Senescence in PLGA

One interesting feature of PLGA is the propensity of some tumors, particularly PA, to occasionally spontaneously stop growing or even regress (56, 57). A similar pattern of initial

neoplastic proliferation followed by growth arrest is often seen in benign melanocytic nevi of the skin, which also commonly contain genetic alterations in *BRAF* (58). This process has been termed oncogene induced senescence (OIS), with senescence defined as an irreversible growth arrest. OIS has been shown to result from induction of the p16/Rb, p14^{ARF}/p53 and/or DNA damage response pathways by BRAF and other oncogenes (59, 60). Markers of OIS include enlargement and flattening of cells, expression of p16 and p53, and activation of acidic senescence-associated β -galactosidase (SA- β -Gal). These are frequently found in premalignant lesions, but are essentially absent in malignant tumor cells, suggesting the latter have found ways to bypass or escape senescence. One such mechanism is deletion of *p16*, which is frequently identified in malignant melanoma (59, 60).

Given the importance of BRAF activation in PA, and their often indolent growth pattern, it is not surprising that several groups have examined the potential role of OIS. In Raabe et al. and Jacob et al., the investigators show that OIS markers including p16, p53 and SA- β -Gal are expressed in both primary PA samples and low passage tumor cultures (61, 62). The groups also found that introducing *BRAF*^{V600E} into either human neural stem cells, hTERT-immortalized astrocytes or fetal astrocytes results in growth arrest and the induction of p16 and SA- β -Gal. OIS has also been documented in benign cutaneous neurofibromas driven by *NFI* loss (63).

The role of p16 in escape from OIS and aggressive tumor growth has attracted particular attention. Jacob and colleagues found that loss of p16 was required for isolation of astrocyte clones stably expressing *BRAF*^{V600E}. Raabe and colleagues examined p16 expression in 66 PA cases using immunohistochemistry, and found that the 9 patients whose tumors were p16 negative had significantly shorter overall survival. Additional support for a prognostic clinical role comes from FISH studies by two groups. Homozygous *p16* deletions were selectively identified in more aggressive anaplastic PA in Rodriguez et al. (52). Horbinski and colleagues examined a large cohort of 198 PLGA, and found that *p16* deletion was the second strongest predictor of adverse outcome (after midline location) in the group overall, and also correlated with significantly shorter progression-free survival in tumors with *BRAF* rearrangement (13). Taken together, these data strongly support the concept that OIS contributes to the sometimes indolent behavior of PA, and that *p16* loss can contribute to escape from senescence and clinically aggressive tumor growth (Figure 7). It remains to be seen if OIS also plays a role in the pathobiology of other PLGA.

Another potential contributor to the slowing or arrest of growth in PLGA is replicative senescence mediated by shortening of telomeres. Using the PCR-based telomeric repeat amplification assay, Chong and colleagues documented telomerase activity in many high grade gliomas but not in the 16 PA and 2 PXA examined (64). Tabori and colleagues subsequently confirmed a lack of telomerase activity in 11 pediatric low grade gliomas (65), as well as a significant decrease in telomere length over time, leading them to propose that lack of telomere maintenance may contribute to growth arrest or regression of PLGA (65, 66). They also found that longer telomere length is inversely correlated with survival, further supporting this concept. A telomerase-independent process known as alternative lengthening of telomeres (ALT) has recently been shown to be active in almost half of pediatric glioblastoma (67). However ALT is very rare or absent in PA (65, 67), although ALT-associated promyelocytic leukemia bodies have been identified in some cases (68).

Neurofibromatosis type 1

NF1 is caused by germline mutations in the gene at 17q11.2 encoding for neurofibromin, a tumor suppressor that works as a GTPase activating protein to deactivate RAS (8). PA are the most frequent NF1-associated CNS tumors, and it is estimated that approximately 15% of pediatric NF1 patients develop optic pathway gliomas (69, 70). Most molecular studies

have demonstrated *BRAF* alterations to be mutually exclusive with NF1 clinical status (29, 32, 39, 53). Conversely, the neurofibromin gene is almost never altered in sporadic PA (71, 72), and NF1-associated and sporadic PA have distinct global gene expression patterns (70, 73). However, in rare instances NF1-associated tumors have been reported with additional activating mutations in *BRAF*. Such cases reported include *KIAA1549:BRAF* fusion in a NF1-associated pilomyxoid astrocytoma (30), a *BRAF*^{V600E} point mutation in a NF1-associated PA (47), and an interesting “triple hit” with concomitant NF1 syndrome, *BRAF*^{V600E}, and *KIAA159-BRAF* fusion (37).

While a major molecular consequence of neurofibromin loss is MAPK pathway activation, additional signaling nodes contribute to tumorigenesis in NF1, including mTOR pathway activation (74). Indeed, activation of the PI3K/AKT/mTOR signaling axis is a prominent feature of the rare PA that develop anaplastic change (52), 24% of which are NF1-associated. Recent studies have also highlighted a role for the non-neoplastic stromal microenvironment in optic glioma development in NF1 model systems. *Nf1* heterozygous microglia are required for glioma formation in these models, in addition to *Nf1* homozygous loss in neoplastic astrocytes. Recent evidence suggests that stromal derived factors (e.g. CXCL12) lead to altered cAMP levels, and facilitate tumor formation in this setting (75).

AKT/mTOR

Another important signaling pathway operating downstream from neurofibromin and RAS is AKT/mTOR, which leads to increased protein translation, cell growth and survival through two multiprotein complexes (mTORC1 and mTORC2) that vary in their sensitivity to rapamycin (76–78). The prototypical low grade glioma in which mTOR activation is an intrinsic molecular property is the tuberous sclerosis-associated SEGA (79). Tuberous sclerosis is characterized by germline mutations in the tumor suppressor genes *TSC1* or *TSC2*, which regulate AKT activation through RHEB, and recent clinical success has been described with mTOR pathway inhibitors (80). Some studies have also highlighted a role for mTOR signaling in NF1-associated tumors, in particular PA (81). Examination of *Nf1* deficient mouse models has suggested anatomical variation in neuroglial progenitor proliferation through AKT activation (82). Recent studies have also suggested a possible role for differential mTOR activation in subsets of NF1 associated low grade gliomas difficult to classify by traditional criteria (83).

Outside of the syndrome-associated low grade gliomas, little is known at the present time regarding mTOR pathway activation in tumorigenesis or progression in PLGA. However, immunohistochemical studies suggest the pathway is active in at least some tumors. Of interest, mutations in *PIK3CA* have been reported in 3 (of 4) rosette-forming glioneuronal tumors, rare low grade neoplasms with a frequent pilocytic astrocytoma-like component (84).

Gene Expression Analysis

Early studies suggested that PA have expression profiles distinct from those of high grade gliomas (85–87), and that PA could also be differentiated from diffuse astrocytoma (88) oligodendroglioma and normal white matter (89). Additional studies identified MBP and Matrilin as potential markers of poor outcome (90, 91). Gene expression analysis has also been used to suggest potential cells of origin for PA, which may be region-specific (73, 92). However, unlike other childhood brain tumors such as medulloblastoma and ependymoma, large-scale studies integrating pathology, clinical factors, and mutations/copy number change with mRNA and miRNA expression have not yet been published. For pediatric medulloblastoma and adult glioblastoma, such correlations have been critical for parsing clinically and genetically meaningful tumor subgroups, potential cells of origin, and

generating molecularly relevant classification schemes (93). Hopefully similar integrated data will soon be available for PLGA.

CLINICAL AND THERAPEUTIC IMPLICATIONS

Diagnostic Utility of *BRAF* Alterations

Distinguishing between the various types of PLGA can sometimes be difficult, thus testing for *BRAF* alterations could potentially be of great use diagnostically. In the first report in which *KIAA1549:BRAF* fusions were identified, Jones and colleagues noted that almost all cases were PA (29). A few PLGA not originally diagnosed as PA contained the alteration, but because they were all cerebellar and associated with survival of greater than 12 years the authors suggested that they might represent PA which had been misclassified. A number of subsequent investigations have confirmed a strong association between PA histology and *BRAF* duplication/fusion (31, 34, 54), but some exceptions exist. In a study including 27 pediatric diffuse astrocytoma, Jacob and colleagues did not detect duplication of 7q34 (31), but the same group later found some diffuse astrocytomas with *KIAA1549:BRAF* fusions (53). Forshev and colleagues reported a *KIAA1549:BRAF* fusion in 1 of 11 pediatric diffuse astrocytoma in their study (30), while Sievert and colleagues identified the duplication in 3 of 6 of these tumors (28).

BRAF duplication/fusion events occur fairly frequently in pilomyxoid astrocytoma (30, 39, 53) supporting a recent study suggesting that pilocytic and pilomyxoid astrocytoma may be part of a single disease spectrum (16). However, the few PXA examined to date have not shown these alterations (30, 39). *BRAF* fusions have also not been identified in high grade pediatric gliomas. Finally, *BRAF* fusions were recently reported in a few pediatric low grade glioneuronal tumors (39). Taken together, these reports of rare fusions in diffuse astrocytoma and other non-pilocytic lesions suggests that such molecular changes involving *BRAF* are highly enriched in PA, but not absolutely specific for this diagnosis.

In contrast to *BRAF* duplication/fusion, point mutations in *BRAF* are most common in low grade pediatric brain tumors other than PA, and are also found in higher grade gliomas (54). Dougherty and colleagues identified *BRAF*^{V600E} mutations in 9/18 (50%) of gangliogliomas as well as several PXA (94). A study of 1,320 nervous system tumors found that *BRAF*^{V600E} was most common in PXA (57/87, 66%) and WHO grade I ganglioglioma (14/77, 18%)(46). Another group also identified common *BRAF*^{V600E} mutations in PXA (12/20, 60%)(95).

Prognostic Utility of *BRAF* Alterations in PLGA

Another potential clinical application of molecular testing for alterations in *BRAF* and other MAPK pathway members is to determine which PLGA are more aggressive, allowing more precise delivery of therapy. Neither our group nor Cin and colleagues found better outcomes in PLGA patients whose tumors contained *BRAF* fusions (37, 39). Hawkins and colleagues focused on a “clinically relevant” subgroup of PLGA cases defined as non-NF1 patients with non-cerebellar tumor location and subtotal resection. They reported that *KIAA1549:BRAF* fusions were significantly associated with better outcome in a cohort of 70 PLGA patients meeting these criteria (53). However, when we examined an analogous subgroup within our cohort we did not identify any trend towards better outcome with *BRAF* fusion (39).

Horbinski and colleagues examined *BRAF* status in 118 unselected PA with outcome data, but did not identify significantly different outcomes in cases with and without the duplication in their initial study (35). In a subsequent larger study of 198 cases by this group, of which 143 were PA, they found a trend towards improved progression free

survival in patients with low grade gliomas whose tumors had *BRAF* rearrangements ($p = 0.06$)(13). They also noted that the only patients with *BRAF* rearrangements who died had midline tumors. In contrast, patients in their cohort with *BRAF*^{V600E} had a trend towards increased risk of progression ($p = 0.07$)(13). Given the somewhat conflicting data in these various studies, it seems that while *BRAF* fusions may portend better outcomes, examination of a larger cohort, preferably from a controlled clinical trial, will be necessary to definitively determine the prognostic role of this alteration.

Clinical Testing for BRAF Alterations

To date, no standard approach to testing for the various alterations affecting MAPK signaling in PLGA has been developed. Because fresh tissue is not always available, assays which can work with formalin fixed, paraffin-embedded (FFPE) specimens will be of greatest practical utility. Given the frequent presence of *BRAF*^{V600E} point mutations in cutaneous melanoma and other common neoplasms arising outside the CNS, many clinical labs have standard tests for this alteration based on sequencing or hybridization (96). A monoclonal antibody specific for the *BRAF*^{V600E} protein has also recently been developed, and will be useful in small specimens (97).

A range of approaches to identify *BRAF* fusions have also been reported. Many laboratories have used duplication of the 7q34 region as a marker for this change. This can be assessed by FISH, which has the advantages of working well in FFPE tissues, spatially localizing the change, and detecting it in small groups of cells. As costs have dropped, array CGH is increasingly being used in a diagnostic capacity in both fresh and FFPE samples, and this technology can detect changes across the genome rather than only those at 7q34, including for example loss of the *p16* locus. Direct identification of fusion transcripts using reverse transcription polymerase chain reaction (RT PCR) is highly specific and yields information on fusion breakpoints, but only pre-determined regions can be assessed (39). While this method has traditionally been performed using RNA extracted from frozen tumors, it has recently been shown that 97% sensitivity and 91% specificity can be achieved using fusion transcripts isolated from FFPE tissue (38). Finally, pyrosequencing has been used to detect fusions and changes in *BRAF* gene dosage in FFPE specimens, and may identify some alterations not found using PCR primers specific for various fusions (98).

Preclinical Testing Models

The development of effective new therapies for PLGA would be greatly assisted by cell or animal based models which accurately reflect the molecular biology and pathology of these tumors. Unfortunately, PLGA models are not as advanced overall as is true for high grade gliomas, although transgenic mice with NF1-associated optic gliomas have been generated and used for preclinical testing (99, 100). Another genetically engineered mouse model which may prove useful in preclinical PLGA testing was recently reported by Gronych and colleagues (101). They found that *BRAF* activation alone in nestin-positive murine neural progenitors was sufficient to induce the formation of cerebral low grade gliomas, but this was achieved by a combination of V600E mutation and deletion of the negative regulatory region. It therefore still remains to be seen if fusions of the *BRAF* kinase domain with *KIAA1549* or other partners analogous to those in humans will be sufficient to drive PLGA tumorigenesis.

Cultures of human PLGA represent an additional potential platform for preclinical testing. It has been difficult to develop useful lines from PA and other indolent PLGA, but some have been reported (25, 102). The recent description of OIS in low passage cultures may account at least in part for these problems (61, 62). It may be possible to develop more robust cultures by maintaining them as neurospheres in serum-free media, or by manipulating

expression of p16 and other OIS factors. Now that signature genetic defects in *BRAF* have been discovered, it will be critical to demonstrate that any cell lines developed carry the molecular markers of the tumors from which they are derived.

Some preclinical testing has been done with the models currently available. Using their *Nf1* mutant optic glioma model, Banerjee and colleagues have explored various rapamycin doses affecting mTOR signaling, and shown that not all pathway biomarkers accurately reflect effective pathway response or changes in tumor growth (74). Proliferation of one human PA culture was inhibited by pharmacological MAPK blockade (25). Finally, murine neurospheres transduced with active BRAF are responsive to Sorafenib (101), and engineered human neural stem and progenitor cell systems (61, 62) could also be used in this fashion.

Therapeutic Possibilities

Given the high frequency of BRAF activation via duplication/fusion and mutation in pilocytic and pilomyxoid astrocytomas, there is considerable interest in targeted inhibition of the MAPK pathway as therapy for these tumors (Figure 7). Sorafenib (Nexavar, Bayer and Onyx Pharmaceuticals) is an inhibitor of BRAF which has less potency against *BRAF*^{V600E}. Currently, Sorafenib is in phase II studies against recurrent and chemotherapy-refractory PLGA (NCT01338857 ClinicalTrials.gov). AZD6244 (Selumetinib, AstraZeneca and Array BioPharma) is a potent inhibitor of MEK also currently in phase I trials against PLGA (NCT01386450, NCT01089101 ClinicalTrials.gov).

BRAF^{V600E} mutations are rare among PLGA overall, but are relatively common in PXA, gangliogliomas and a subset of extra-cerebellar pilocytic astrocytomas (46, 54, 94, 95). Vemurafenib is a competitive small molecule that was designed to bind to and inhibit the ATP binding domain of the *BRAF*^{V600E} mutant, but not other forms of BRAF (103, 104). Following impressive albeit transient responses of recurrent melanoma to Vemurafenib (104), the United States Food and Drug Administration (FDA) has approved it for the treatment of *BRAF*^{V600E} mutation positive, inoperable, or metastatic melanoma. It is anticipated that there will be a clinical trial of Vemurafenib against *BRAF*^{V600E} mutant low-grade gliomas in the near future.

The AKT/mTOR pathway has been implicated in several types of PLGA, including SEGA and PA. Clinical trials have demonstrated that mTOR inhibitors including Sirolimus and Everolimus (RAD-001 or Afinitor; Novartis Pharmaceuticals) have activity against SEGA (105), and Everolimus has received approval by the FDA for the treatment of SEGA that cannot be surgically resected. A recent reported phase I/II study of Sirolimus (Rapamune, Pfizer) and Erlotinib (Tarceva, Genentech) examined 16 patients with recurrent PLGA (106). Of the 7 children with NF1 in this clinical trial, all patients had either stable disease or tumor responses. A phase II study of Everolimus against recurrent and chemotherapy-refractory PLGA has recently been completed (NCT00782626); results of this study are pending. As agents targeting the MAPK and AKT/mTOR pathways are tested, it will be critical to also search for molecular biomarkers which are predictive of response.

CONCLUSIONS

It is now clear that alterations affecting the *BRAF* oncogene and other members of the MAPK cascade represent the main genetic defects in PLGA. Segmental duplications resulting in fusions between *KIAA1549* and *BRAF* are most common, and result in a novel protein with constitutive kinase activity. Tumors lacking *KIAA1549:BRAF* fusions often have other changes with similar functional effects, including *SRGAP3:RAF1* and *FAM131B:BRAF* fusions and mutations and insertions activating *BRAF*. These findings

link syndromic and sporadic PA, and suggest that MAPK is the dominant signaling pathway to target therapeutically. The detection of BRAF activation as a cardinal feature of PLGA has also led to the discovery that oncogenic induction of senescence may account for the spontaneous growth arrest or regression of some tumors.

The challenge now is to translate these new discoveries in improved diagnostic, prognostic and predictive markers, and to develop targeted therapies for patients with clinically aggressive tumors. PLGA are a heterogeneous group of lesions, and while *BRAF* fusions are not entirely specific for one entity they are almost always encountered in PA, thus molecular BRAF testing may help us to distinguish these tumors from other pediatric gliomas. The prognostic role of *BRAF* alteration is still not clear, and analysis of large, uniformly treated cohorts will likely be required to definitively assess associations with outcome. Finally, it will be critical to determine if specific genetic changes predict response to therapies targeting BRAF or other pathway members. The recent initiation of several clinical trials of MAPK inhibitors in PLGA patients should provide some initial insights into this key question.

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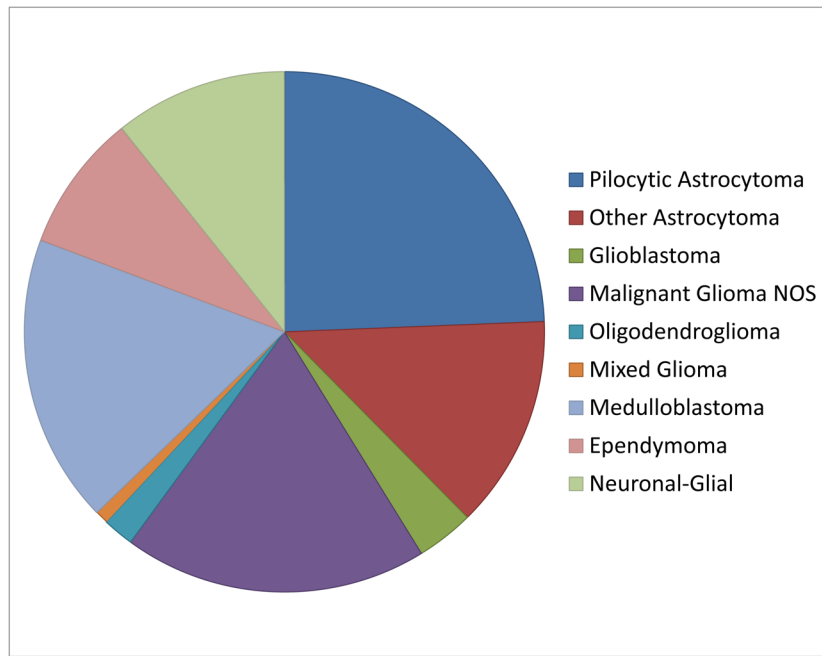


Figure 1. Primary CNS tumor distribution in children ages 0–14 based on CBTRUS data.

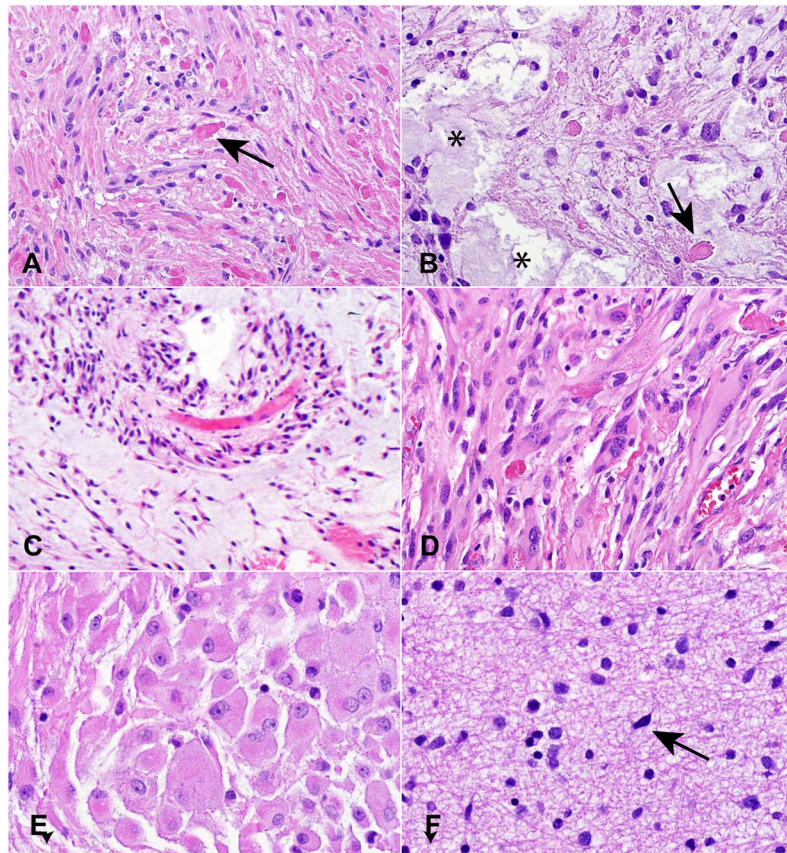


Figure 2. Histopathological features of PLGA. (A) PA with compacted glial cells and Rosenthal fibers (arrow). (B) Microcysts (asterisks) and eosinophilic granular bodies (EGB, arrow) in a looser region of a PA. (C) Pilomyxoid astrocytoma comprised of monomorphous cells with a pronounced perivascular growth pattern. (D) Spindled, pleomorphic cells in a PXA. (E) Enlarged ganglion-cell like elements in a SEGA. (F) Diffuse astrocytoma are only modestly hypercellular with scattered atypical cells.

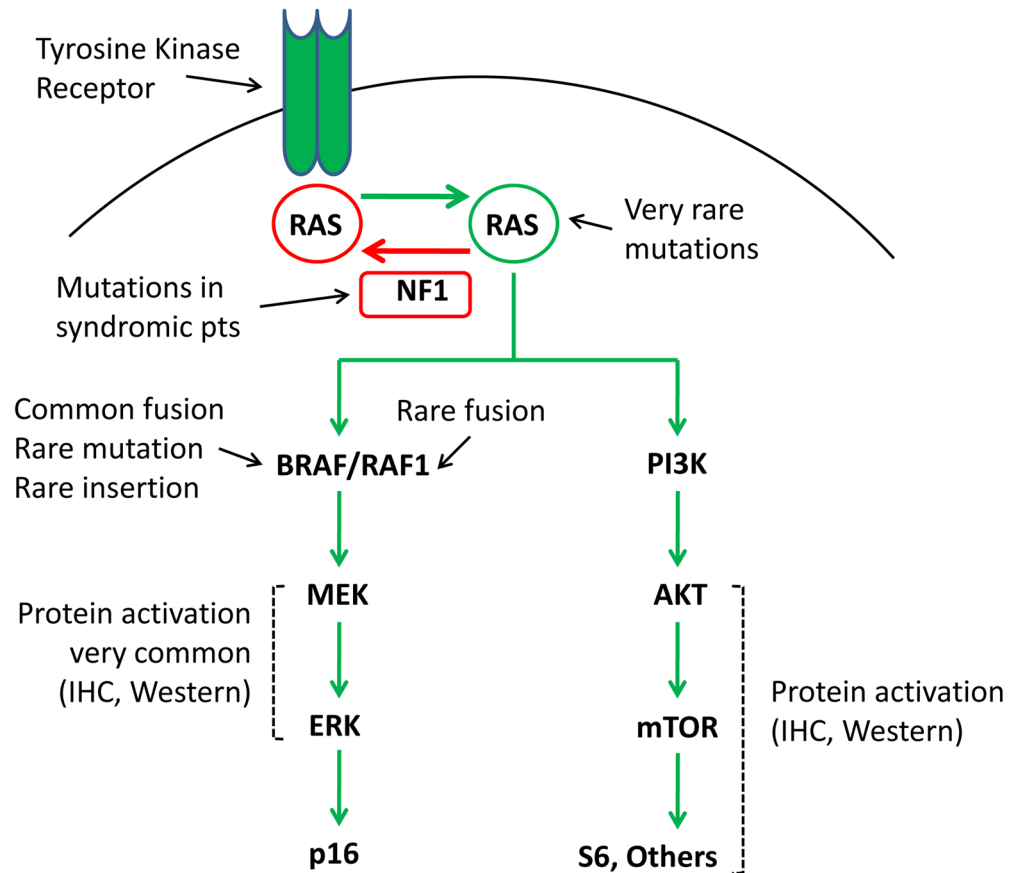


Figure 3.
Signaling pathways commonly activated in PLGA.

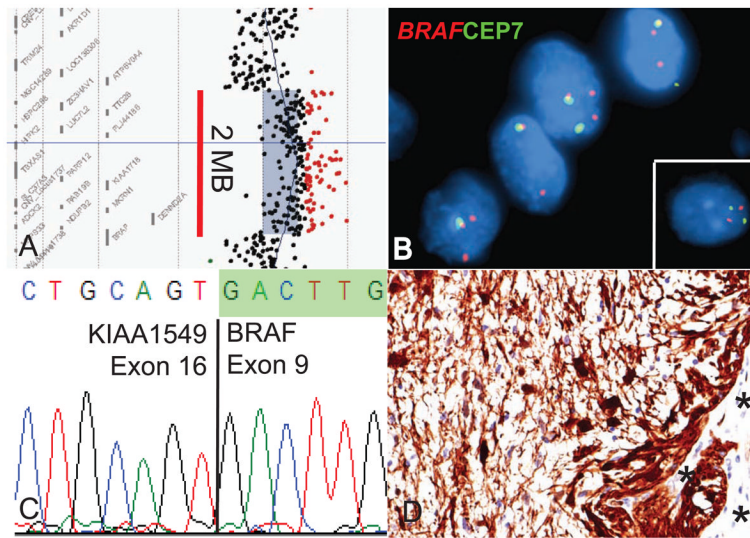


Figure 4.

Molecular alterations involving *BRAF*. (A) Segmental gains at 7q34 approximately 2 megabases in size are commonly identified in PA using array CGH. (B) FISH reveals duplication in this region, with an extra copy of the region encoding *BRAF*. A normal control is shown in the inset. (C) Sequencing of a *KIAA1549:BRAF* fusion product between exons 16 and 9 of the two genes. (D) PA contain abundant active phospho-ERK. Normal vessels serve as an internal negative control (asterisks).

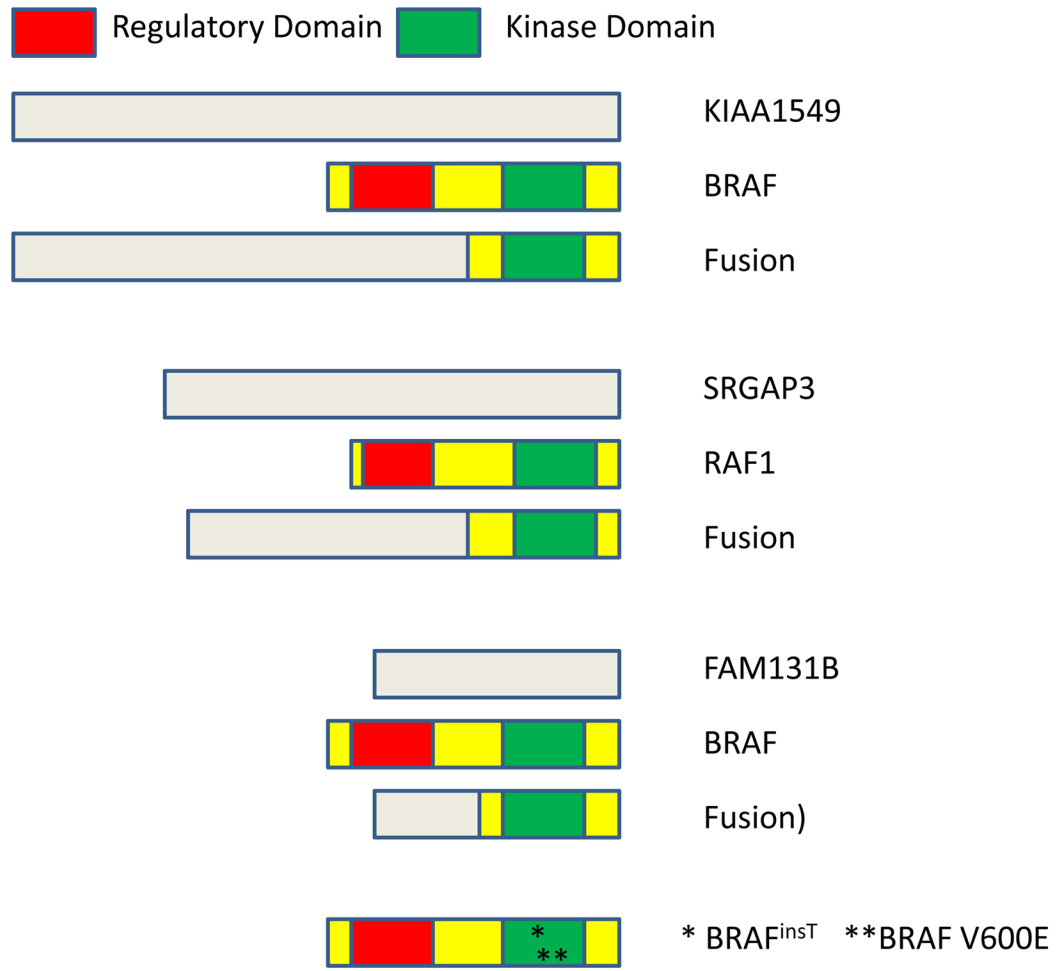


Figure 5.
Mechanisms of BRAF and RAF1 activation in PLGA

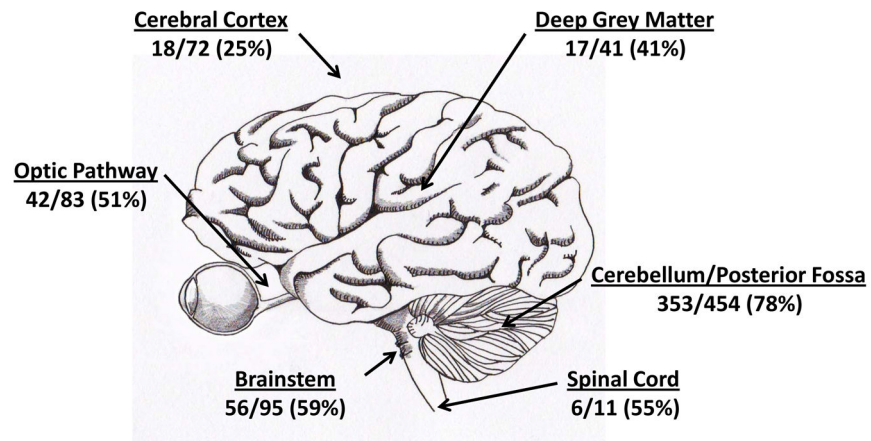


Figure 6. Localization of *BRAF* duplication/fusion in PA. Summary of tumor localization from published cases, including patients with NF1.

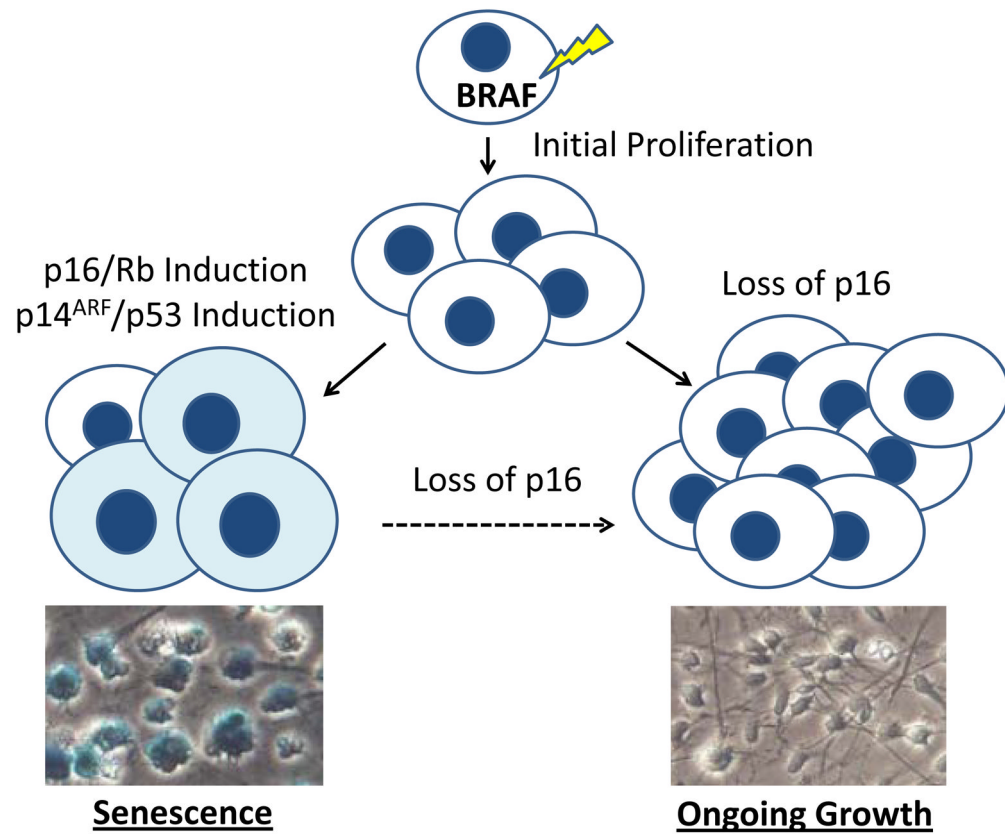


Figure 7. Oncogene induced senescence in PA. Senescence-associated β -galactosidase stains of *BRAF*^{V600E} and vector transduced human neural stem cells are shown in the bottom panels.

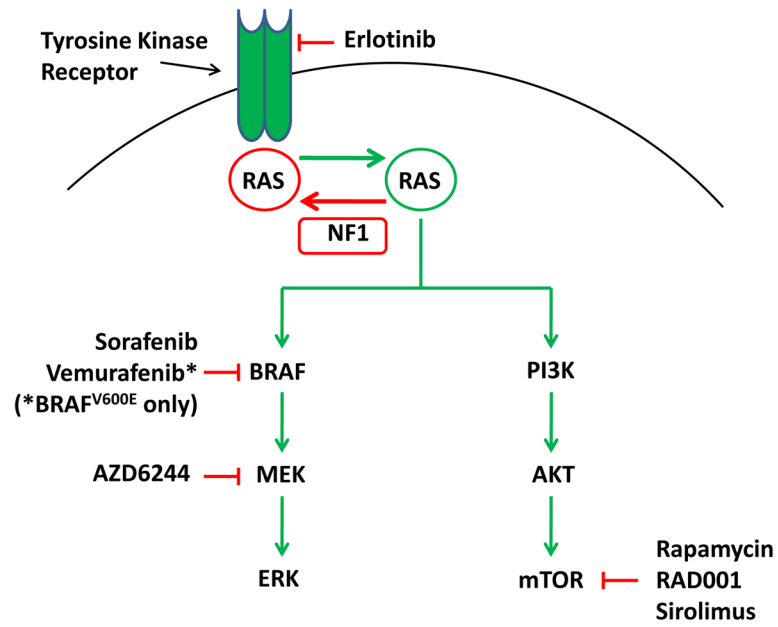


Figure 8.
Therapeutic agents targeting BRAF/RAF1 and AKT/mTOR pathways