Genetic aberrations leading to MAPK pathway activation mediate oncogene-induced senescence in sporadic pilocytic astrocytomas

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Translational relevance:

Pilocytic astrocytomas are the most common brain tumour in children. Despite an overall good prognosis, children may experience significant morbidity due to tumor location and/or the side-effects of adjuvant therapies in case of residual disease. Our study provides a comprehensive molecular profiling of the effect of the recently uncovered, genetically-driven, MAPK pathway activation in PA. It shows prominent hallmarks of oncogene-induced senescence (OIS) that is potentially at the origin of clinical behavior and overall good prognosis of PA. Moreover, artificial acceleration of this OIS program could present an interesting therapeutic possibility in patients with progressive disease. In this area of research the strength of associations defines the translational implications of the findings. In this regard, the combined results from three independent cohorts as well as our in vitro data provide inherent validation for the mechanisms we propose are limiting the potential for transformation in this tumor.
Abstract

Purpose: Oncogenic BRAF/Ras or NF1 loss can potentially trigger oncogene-induced-senescence (OIS) through activation of the Mitogen-Activated Protein Kinase (MAPK) pathway. Somatic genetic abnormalities affecting this pathway occur in the majority of pilocytic astrocytomas (PA), the most prevalent brain neoplasm in children. We investigated whether OIS is induced in PA.

Experimental design: We tested expression of established senescence markers in 3 independent cohorts of sporadic PA. We also assessed for OIS in vitro using forced expression of wild-type and V600E-mutant-BRAF in 2 astrocytic cell lines: hTERT-immortalized astrocytes and fetal astrocytes.

Results: Our results indicate that PA are senescent as evidenced by marked SA-beta-Galactosidase activity, low KI-67 index and induction of p16INK4a but not p53 in the majority of 52 PA samples (46/52, 88.5%). Overexpression of a number of senescence associated genes (CDKN2A (p16), CDKN1A (p21), CEBPB, GADD45A and IGFBP7) was demonstrated at the mRNA level in two independent PA tumor series. In vitro, sustained activation of wild-type or mutant-BRAF induced OIS in both astrocytic cell lines. Loss of p16INK4a in immortalized astrocytes abrogated OIS, indicative of the role of this pathway in mediating this phenomenon in astrocytes. OIS is a mechanism of tumor suppression that restricts the progression of benign tumors. We show that it is triggered in PA through p16INK4a pathway induction following aberrant MAPK activation.

Conclusions: OIS may account in PA for the slow growth pattern, the lack of progression to higher grade astrocytomas, and for the high overall survival of affected patients.
Introduction

Primary brain tumors are the second most common type of cancer in children (after leukemia), and the leading cause of cancer-related mortality and morbidity in young patients (1, 2). Astrocytomas account for 50% of all central nervous system (CNS) tumors and are commonly regrouped into low-grade (WHO grade I and II, LGA) or high-grade (WHO grade III and IV, HGA) tumors (3). Pilocytic astrocytomas (PA) are WHO grade I tumors. They are the predominant histological subtype in LGA and the most prevalent CNS neoplasm in childhood, accounting for 23% of all pediatric brain tumors (2). They occur sporadically throughout childhood and in 15-40% of children affected with neurofibromatosis type 1 (NF1) (4). These tumors arise throughout the CNS, but have a predilection for the cerebellum and the optic pathways (5). PA are slow growing tumors, which often harbor a cystic component. They exhibit distinct features, readily distinguishable from other LGAs, including an improved clinical course and prognosis in children, limited proliferation index, and no TP53 mutations or PDGFA/PDGFRα amplification (6-10). Maximal surgical resection is the mainstay of therapy, and failure to achieve it remains the main therapeutic concern (3). While cerebellar PA are usually readily amenable to complete surgical resection, residual tumor in other less anatomically accessible locations can require adjuvant therapies for tumor control (9, 11). Thus, despite an excellent 5-year overall survival of over 90% and progression-free survival of over 65% for PA (1, 11, 12), affected children may experience significant morbidity due to the tumor location and/or the side-effects of adjuvant therapies in case of residual disease.

Several consecutive papers, including from our groups, recently showed aberrant activation of the Mitogen-Activated Protein Kinase (MAPK) pathway to be the main molecular abnormality in
PA(13-18). Indeed, gene fusions and activating mutations involving key regulators of the MAPK pathway have been found in the majority of PA tested. Duplication of 7q34 leading to in-frame KIAA1549–BRAF fusions and constitutive BRAF activation have been identified in up to 65% of sporadic PA tested (13-18). KRAS activating mutations affecting codons 12, 13, and 61 have been identified in approximately 4–7% of PA(13, 19-21) and similarly low incidence rates have been found for the BRAF V600E activating mutation in PA(13, 14, 16, 17). RAF1 fusions leading to constitutive activation of RAF1 have been detected in four PA to date(13, 15) and a novel BRAF activating mutation was also recently identified at low frequency in PA(15, 22, 23).

Moreover, in NF1 patients, NF1 gene inactivation leads to increased Ras activity and subsequent MAPK pathway activation. All of these observations converge to indicate a major role of MAPK pathway activation in the clinical course and biology of PA.

Senescence is a physiological phenomenon characterized by a permanent cell cycle arrest. Oncogene-induced senescence (OIS) was first reported when the expression of oncogenic Ras in primary human or rodent cells resulted in a permanent G1 arrest, and was accompanied by accumulation of p53 and p16(24). Collectively, investigators have subsequently observed that mutations in KRAS, BRAF, PTEN and NF1 can trigger cellular senescence in vivo, and have implied that OIS is a mechanism of tumor suppression that restricts the progression of benign tumors in the absence of additional cooperating mutations(25-28). Human melanocytic nevi, which are common benign lesions of cutaneous melanocytes that rarely progress to melanoma, constitute an intriguing example. Somatic mutations leading to constitutive activation of BRAF (V600E) are present in up to 80% of melanocytes from this benign tumor, and lead to growth arrest of cells through OIS(27, 29). Human neurofibromas developing in the context of Neurofibromatosis Type-I also show senescence in vivo due to loss of NF1 activity(25).
The natural history of PA is unique as these tumors are self-containing, almost never progress to higher grade astrocytomas, and their initial growth is usually followed by a decrease in tumor activity, after which they enter a dormant phase and either remain quiescent or restart cycle(s) of growth followed by dormancy, similar to what is observed in OIS in vitro. Based on these observations, we hypothesized that activation of the MAPK pathway leads to OIS in PA, potentially accounting for the benign nature of these tumors. Thus, we investigated a number of established senescence markers in 52 primary sporadic pediatric PA, including expression levels of p16\(^{INK4a}\), p53, and KI-67, a marker of proliferation index, in archival tissues as well as SA-β-galactosidase activity in fresh tissue samples where available (30). Gene expression profiling in two independent PA cohorts also strongly supported the presence of an activated senescence programme in PA.

In addition, we overexpressed wild-type or V600E mutant BRAF in human immortalized astrocytes and in fetal astrocytes and assessed for OIS induction in vitro in these surrogate astrocytic models. Our results indicate that MAPK activation triggers OIS in PA. As MAPK activation is likely the major abnormality driving proliferation in PA, OIS may be responsible for the tendency of PA for spontaneous growth arrest. Furthermore, the absence of additional genetic alterations and the presence of functional cell cycle control mechanisms could explain the lack of transformation of PA into higher grade tumors, accounting for the relatively benign course of this grade I tumor when considering overall survival.
Material and Methods

Sample Characteristics and Pathological Review.

All samples were obtained with informed consent after approval of the Institutional Review Board of the respective hospitals they were treated in and were independently reviewed by senior paediatric neuropathologists according to the WHO guidelines(3, 31). Patients’ characteristics in the Montreal, Cambridge, and Heidelberg series are detailed in Table 1 and in supplementary Tables 1 and 2.

Cell lines, Antibodies, and Transfections. hTERT-immortalised human astrocytes (kind gift of Dr A Guha, Labbatt Brain Tumour Research Centre, Ontario, Canada) were grown as previously described(32). Human fetal astrocytes were obtained from 18-weeks-old to 22-weeks-old fetal brain specimens provided by the Human Fetal Tissue Repository (Albert Einstein College of Medicine, Bronx, NY) following approved guidelines from the Canadian Institutes of Health Research (CIHR) and processed as described in Supplementary Table 1. Primary antibodies were from Cell Signaling (GFAP/c-Myc-tag/β-actin) and from Santa Cruz Biotechnology (pERK/BRAF/p53/p16INK4a). Cells were transfected with wild-type Myc-tagged BRAF or V600E-BRAF (kind gift of Dr Richard Marais, Cancer Research, London, UK) as previously described (33). Transfection efficiency was assessed using immunofluorescence and western blot analysis as previously described (33).
**BRAF Tandem Duplication Screening.** RT-PCR to detect the BRAF fusion transcripts was performed as previously described(16).

**Gene expression profiling.**

*Cambridge series:* Samples were analysed on the Illumina HT12 v3 expression platform. Selected genes were assessed for differential expression between tumor and control samples using a 2-tailed Mann-Whitney U test. *Heidelberg series:* Samples were analysed on the Illumina WG6 v3 expression platform. The mean of the normal brain expression was subtracted from the expression value for each sample and for each gene of interest, to give a log₂ fold-change value. Detailed overview is provided in Supplementary Table 1.

**Analysis of SA β–Galactosidase Activity.** Cells were plated in a 6 well plate at 50% confluence overnight prior to the assay. A detailed overview of the experimental procedure is provided in Supplementary Table 1. The appearance of a blue color was determined under the microscope as previously described (27, 30).

**Immunofluorescence, Immunohistochemical analysis, Western Blot and Cell Cycle Analysis.** Immunofluorescence and immunohistochemical staining as well as western blot analysis were performed and scored as previously described (24, 28, 34). Detailed overview of the experimental procedures and data analysis using the FlowJow software are provided in Supplementary Table 1. All experiments were performed at least 3 independent times in triplicate and at least 40,000 cells were acquired from each sample for FACS analysis.
Results

Genetic alterations in the MAPK pathway in PA included in the Montreal Cohort

We first confirmed the nature of the genetic abnormality affecting the MAPK pathway in the cohort of 52 sporadic PA from the Montreal series investigated in this study. To this end, we used specific exonic primer pairs for PCR amplification of KIAA1549-BRAF fusion products. Fusion transcripts were detected in 37/52 (71.1%) PA samples (24/28 posterior fossa, 4/7 brainstem, 5/8 optic pathway, 1/4 cerebrum, 3/5 spinal PA). We also sequenced BRAF exons 11 and 15 in 50/52 PA, and identified the hotspot V600E mutation in 2 of the 15 tumors negative for KIAA1549–BRAF fusion and 1 in a PA with KIAA1549–BRAF fusion. We then screened for mutations of exons 2-7 of RAF1, exons 2-3 of KRAS and NRAS, and exon 13 of PTPN11, as well as for SRGAP3-RAF1 fusion, and identified 2 samples with mutated KRAS (2 posterior fossa) in the 13 samples that were negative for KIAA1549–BRAF fusion or V600E BRAF mutation (Table 1A, Supplementary Table 2). This data, as well as overall survival and event-free survival in our patient cohort (supplementary Figure 1) are in concordance with current literature, demonstrating the preponderance of KIAA1549–BRAF fusions as MAPK activating events in PA(1, 11, 12, 35).

PA express senescence markers

Activation of the INK4a/ARF locus by oncogenes is well documented as a tumor suppressive mechanism, notably through induction of OIS. Senescent cells have been shown to display increased expression of p16INK4a, p21CIP or p53, while having a low to null mitotic index(36-38). The p16INK4a protein is a major tumor suppressor, often highly expressed in senescent cells in vitro and inactivated in a variety of human cancers, including 30–70% of HGA(39, 40). OIS is
characterized by cycle arrest, which is accompanied by the induction of both \(p16^{\text{INK4a}}\) and senescence-associated acidic beta-galactosidase (SA-beta-Gal) activity, a commonly used senescence marker(27, 28, 37). We investigated whether senescence was triggered in PA \textit{in vivo} using SA-beta-Gal activity and levels of \(p16^{\text{INK4a}}\), p53, and KI-67 (a marker of cellular proliferation). Fresh PA tumor samples from 6 consecutive patients including lesions from the cerebellum (N=4), brainstem (N=1) and spinal cord (N=1) were markedly positive for SA-beta-Gal (Figure 1, Montreal Cohort). Five of these samples (PA 2-3-7-12) had KIAA1549-BRAF fusions and one (PA 37) had no identified genetic abnormality of the MAPK pathway (Table 1, supplementary table 2). We then investigated fixed material from the 52 PA samples included in our cohort, including these 6 samples for which we obtained fresh material at initial surgery, for the expression of \(p16^{\text{INK4a}}\), p53 and KI-67 using specific antibodies targeting these proteins. Staining for \(p16^{\text{INK4a}}\) showed markedly increased expression in 46/52 (89.5%) PA compared to normal brain (Figure 2, Table 1B). PA were positive for \(p16^{\text{INK4a}}\) regardless of their anatomical location and regardless of the presence or type of the genetic alteration affecting the MAPK pathway. Immunopositivity for \(p16^{\text{INK4a}}\) was both cytoplasmic and nuclear. Its extent varied widely, both in terms of the fraction of positive cells and its intensity (Table 1B) and was found predominantly in the non-pilocytic component, particularly in those PA that had the classical biphasic “loose-dense” pattern (Figure 2). Six PA had \(p16^{\text{INK4a}}\) staining in 5% of cells or less counted across any field (Figure 2, Table 1B). These PA had an active MAPK pathway as shown by the positive pERK staining on the respective slides (Supplementary Figure 2B). No PA showed increased nuclear p53 expression consistent with previous publications indicating absence of \(TP53\) mutations in PA(reviewed in 9). As also expected in PA, Ki-67 index was low in all cases (1-2%; rarely up to 5%). The MAPK pathway was triggered in all PA cases (14) as shown by the positivity of the antibody against the phosphorylated (activated) forms of ERK1/2.
(pERK) (Supplementary Figure 2B, and (14)). Slides from normal control brain were negative for p16<sup>INK4a</sup>, p53 and Ki-67 expression (Supplementary Figure 2A, Table 1B).

Taken together, these data demonstrate that at the protein level, PAs display features of OIS. Several key publications show central roles for both CDKN2A (p16) and CDKN1A (p21) in OIS, and these are now widely accepted markers of this phenomenon (e.g.(24, 41)). More recently, IGFBP7 has been proposed as being a central mediator of BRAF-induced OIS in melanocytes, and also as having growth regulatory properties in thyroid carcinogenesis (a tumour which also closely associated with BRAF mutation)(42, 43). CEBPB, encoding CCAAT/enhancer binding protein beta, has also recently been shown to have a role in MAPK activation-mediated OIS in fibroblasts (44, 45), and GADD45A has been linked with apoptosis and growth arrest in Ras-driven breast cancer and UV-induced skin cancer (46, 47). To investigate the transcriptional changes underlying this senescent behaviour, we examined transcriptome data from two independent tumor series (Cambridge and Heidelberg) to look at these specific candidate genes with well-defined or putative roles in senescence. Strikingly, both datasets provided strong evidence for overexpression of these genes in the tumors compared with normal brain (Figure 3, Supplementary Figure 3). In the Cambridge series, all genes showed significantly increased expression in primary tumours compared with normal brain controls (p<0.0001 for all comparisons, Mann-Whitney U-test, Figure 3). In the Heidelberg series, the median log<sub>2</sub> fold change of tumor vs control ranged from 0.69 – 5.01, with all differences also significant at p<0.05 (2-tailed Mann-Whitney U test, Supplementary Figure 3). Thus, the alterations in behaviour and in tumor protein expression seen in the Montreal cohort are closely mirrored by a
program of OIS gene expression changes in two additional PA cohorts assayed on two different platforms.

**Short term overexpression of BRAF\(^{WT}\) or BRAF\(^{V600E}\) induces features typical of OIS in two independent astrocytic cell line models**

A wealth of data support a model in which somatic cells in culture possess at least two independent mechanisms limiting their lifespan and leading to senescence: First, a telomere-dependent pathway registers the cumulative number of cell divisions and triggers senescence following telomere attrition. Second, an INK4a/ARF dependent mechanism reacts to the exposure of cells to mitogenic stimulation and may in some cases trigger a permanent cell cycle arrest\(^{(36)}\). Activation of this INK4a/ARF pathway can be accelerated by introducing high levels of promitogenic oncogenes\(^{(36)}\). We elected to use normal human hTERT-immortalized astrocytes (NHA), where telomere shortening-induced senescence is inhibited by constitutive overexpression of human telomerase reverse transcriptase (hTERT), thereby resulting in immortalization. We used these cells and fetal astrocytes as models to determine *in vitro* if increased wild-type BRAF (BRAF\(^{WT}\)) or V600E mutant BRAF (BRAF\(^{V600E}\)) expression levels induce OIS and increased levels of p16\(^{\text{INK4a}}\) in cells from the astrocytic lineage, similar to what has been observed in fibroblasts and melanocytes and as we observed in primary PA.

NHA and fetal astrocytes were transiently transfected with BRAF\(^{WT}\) and its mutant form BRAF\(^{V600E}\). Transfection efficiency was estimated by immunofluorescence analysis against the Myc-tag to be approximately ~60% at 48, 72, and 96 hours (Figure 4A). No foci of transformation were observed in cells transfected with either construct, while morphological
changes including an enlarged cytoplasm and rounded flattened shape consistent with a senescent phenotype were noted in cells transfected with the BRAF constructs (Figure 4B). As we had previously described(14), proliferation assays revealed limited changes in the growth of cells overexpressing BRAFWT or BRAFV600E compared to empty-vector transfectants. Increased SA-beta-Gal activity in BRAFWT and BRAFV600E transfectant cells compared to empty-vector controls was also observed (Figure 4B). Western blotting analysis showed induction of p16INK4a expression in BRAFWT and BRAFV600E transfected NHA and fetal astrocytes, while p53 levels remained unchanged (Figure 4C) compared to the empty vector. Moreover, forced BRAFWT and BRAFV600E expression in NHA and fetal astrocytes induced a growth arrest at the G1/M phase as shown by increased G1 and decreased S phase in these cells compared to empty vector transfectant cells following cell cycle analysis (Figure 4D).

These results demonstrate that forced overexpression of WT and mutant BRAF induces p16INK4a expression, SA-beta-Gal activity and cell cycle arrest in two astrocytic cell lines. The maintenance of telomere length through h-TERT immortalization of NHA argues for an active oncogene-driven senescence process, rather than a loss of replicative potential in these cells, which can normally be serially passaged successfully.

**Loss of p16 abrogates the senescent features of NHA cells stably overexpressing BRAFWT or BRAFV600E**

To better understand the cellular responses to expression of BRAF, we sought to generate stable NHA clones overexpressing BRAFWT or BRAFV600E. In several independent experiments, we observed unusually low transfection efficiency and were only able to select 4 clones
overexpressing BRAF\textsuperscript{WT} from 75 that were screened, and 2 clones overexpressing BRAF\textsuperscript{V600E} from 60 screened. Interestingly, when we investigated p16\textsuperscript{INK4a} and p53 levels in clones, we observed that p16\textsuperscript{INK4a} expression was lost in all BRAF-overexpressing clones, while p53 expression remained unchanged (Figure 5A). This suggests that inactivation of p16\textsuperscript{INK4a} in NHA may allow the cells to bypass BRAF-induced senescence and to divide enough to constitutively incorporate the DNA plasmid and become transformed. In keeping with this hypothesis, sustained expression of BRAF\textsuperscript{WT} and BRAF\textsuperscript{V600E} did not induce senescent-associated features in NHA as was seen following transient overexpression. Indeed, cells had no morphological changes indicative of senescence no significant induction of SA-beta-Gal activity (Figure 5B), showed foci of transformation (Figure 5C), and were able to grow and form colonies in anchorage independent conditions (soft agar) compared to EV transfectants (Figure 5D). This data indicates that p16\textsuperscript{INK4a} may protect against BRAF-driven proliferation, and that its loss may diminish the physiological protection provided by this cell cycle regulator through induction of senescence.
Discussion

We show in vivo and in vitro that PA display classical hallmarks of senescence, suggesting that MAPK pathway activation, whatever the genetic defect at its origin, drives OIS partly through the induction of p16\textsuperscript{INK4a} pathway activation in astrocytes. Fresh samples from pediatric PA were invariably positive for SA-beta-Gal, and we observed a clear induction of p16\textsuperscript{INK4a} protein in 46/52 (89.5%) primary pediatric PA samples. Tumor samples also had a low mitotic index indicative of a limited growth potential and no increased p53 nuclear expression, suggesting that it is primarily the INK4a/RB cell-cycle control pathway which is being triggered in these tumors through induction of p16\textsuperscript{INK4a} (48). Similar to what we observed in vivo, we show that transient BRAF\textsuperscript{WT} or mutant BRAF\textsuperscript{V600E} overexpression in human astrocytes (NHA and fetal astrocytes) induced morphological changes evocative of senescent cells, which was accompanied by the induction of p16\textsuperscript{INK4a}, SA-beta-Gal activity and cell cycle arrest. Conversely, we also show that loss of p16 \textsuperscript{INK4a} expression in NHA abrogated OIS, promoted cellular transformation and increased proliferation following sustained BRAF\textsuperscript{WT} and BRAF\textsuperscript{V600E} expression in vitro. This is of particular interest given a recent study reporting deletion of p16 in a subset of clinically aggressive PA(49). Moreover, in mice, loss of p16\textsuperscript{INK4a} allowed cultured astrocytes to grow without senescing(37). This data is concordant with our results on the role for p16\textsuperscript{INK4a} pathway activation in the induction of OIS in PA.

Our data may provide a rationale for the lack of progression to higher grade tumors of PA, in contrast to WHO grade II and III astrocytomas. Senescence is not triggered by a single, linear series of events, but instead is regulated by a complex signalling network including the p53 and retinoblastoma (Rb) tumor suppressor pathways, which serve as critical cell-cycle checkpoints.
that mediate both replicative and oncogene-induced senescence. In contrast to PA, nearly all grade II to IV astrocytomas exhibit alterations in genetic loci related to the p53 and Rb pathways governing G1 arrest, including loss of the INK4a-ARF or Rb loci, or gain of CDK4(37, 38).

In this study, 6 PA samples from the Montreal cohort of 52 samples showed limited p16<sup>INK4a</sup> expression. These samples had marked pERK positivity, limited proliferation index, no p53 nuclear staining and did not belong to a given age group. Three harbored KIAA1549-BRAF fusions and were typical cerebellar PA. In naevi, additional factors to p16<sup>INK4a</sup> contribute to the protection of melanocytes against BRAF<sup>V600E</sup>-driven proliferation (27). Senescence in these 6 PA as well as in all of the other PA samples investigated in this study could be additionally triggered through other mechanisms. Indeed, at the transcriptional level, a number of genes associated with OIS showed significant upregulation in PA compared with normal brain. These included CDKN2A (p16) and CDKN1A (p21) which are widely accepted markers of OIS (24, 41), as well as IGFBP7, CEBPB and GADD45A, which have also been associated with MAPK-induced OIS (42-46, 50). Furthermore, telomerase activity has been shown to be low in LGA, and telomere attrition has also been shown to increase with recurrence in LGA including PA (51). Together, these observations indicate that there may be a more widespread senescence program which is activated in PA more than solely p16<sup>INK4a</sup> induction, and that there may be a degree of functional redundancy in these tumours.

In addition to benign melanocytic nevi, the BRAF V600E mutation has been identified in the majority of melanomas. It is unclear to date as to what leads to cellular transformation in this tumor type, as senescence can still be partly triggered in tumor cells (29). Limiting signals affecting induction and levels of OIS may be dictated by the initiating genetic alteration but also the threshold (intensity and duration) of the signalling and/or the tissue type (cell of origin,
micro-environment including the stroma and immune response). The lack of progression to higher grade tumors in senescent PA may be due to the nature of the oncogenic aberration, as KIAA1549-BRAF fusions predominate and the V600E mutation accounts for only 4-7% of all genetic abnormalities in PA, or to any of these other factors. Additional studies using larger cohorts of patients or engineered mouse-models generated to that effect(26) are needed to tease out the impact of these variables in PA.

In summary, our study suggests that aberrant MAPK signalling leads to OIS in PA. This can explain the dichotomy between activation of an oncogenic pathway, which was recently found to be constitutively activated in 75-100% of PA, and their tendency towards growth arrest. This unique phenomenon is at least partly due to the induction of p16INK4a in cells and may account for the lack of progression to higher grade astrocytomas, which is a unique feature of PA compared to other LGA, and for the better survival of patients with this tumor. Further studies are warranted to determine whether the nature of the genetic alteration, the cell of origin or the micro-environment play an additional role in the maintenance of OIS and in the striking lack of potential for transformation seen in PA. Finally, artificial acceleration of this OIS program could also present an interesting therapeutic possibility in this tumor type.
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Figure legends

Figure 1. SA-beta-galactosidase activity is increased in primary pilocytic astrocytomas (PA). Six fresh tissue samples from patients with PA (Montreal Cohort, Table 1, Supplementary Table 2) were processed as described(27) and showed marked SA-beta-galactosidase activity. Control immortalized astrocytes (NHA), cells from a primary cell line established from a pediatric glioblastoma patient (SF188) or a fresh tissue sample obtained from a pediatric patient with glioblastoma (GBM) were processed similarly and did not show SA-beta-galactosidase activity (negative controls).

Figure 2. p16\textsuperscript{INK4A} is induced in the majority of pilocytic astrocytomas (PA). Immunohistochemical analyses of p16\textsuperscript{INK4A} (p16) was performed on 52 pediatric PA samples (Montreal cohort) and control brains (CB) from age-matched children. A representative staining of 3 CB and 18 PA from different regions within the brain is shown. PA1-PA15 had marked p16 induction whereas PA16-PA18 and the CB had limited p16 staining.

Figure 3. Expression of OIS-related candidate genes. Normalised log\textsubscript{2} expression values in PA tumors (dark grey, n=29) and control brain samples (light grey, n=10) showing clear upregulation of these genes in the tumor samples. ***, p < 0.0001, two-tailed Mann-Whitney U test.

Figure 4. Transient overexpression of wild-type (WT)-BRAF and V600E-BRAF induces OIS in immortalized astrocytes (NHA) and fetal astrocytes (FA). (A) Transfection efficiency at 48h of NHA and FA following transient overexpression of cmyc-tagged (red staining) WT BRAF (upper panel, green staining for BRAF) and V600E-BRAF (lower panel). DAPI staining (blue) is provided to show the total number of cells per field. (B) Morphological changes and
induction of SA-beta-Gal activity were seen in FA (upper panel) and in NHA (lower panel) cells transiently overexpressing WT- or mutant V600E-BRAF compared to empty vector controls (EV). The percentage of cells showing positive SA-beta-gal activity following transient BRAF (WT and V600E) induction was statistically significantly increased compared to EV controls (p<0.001, Fisher’s exact test). (C) A mean of 4-fold induction in p16^{INK4a} expression was seen in NHA (left panel) and FA (right panel) following overexpression of either WT or mutant BRAF. (D). Cell cycle analysis of NHA and FA cell lines transiently overexpressing WT or mutant V600E-BRAF shows an increase in G1 and a decrease in the S phase in these cells compared to cells transfected with EV. This is suggestive of a G1/M cell cycle arrest induced by BRAF overexpression in both astrocytic cell lines. Results are representative of three independent experiments performed in triplicates for each cell line.

**Figure 5. Loss of p16^{INK4A} decreases BRAF mediated OIS in NHA and promotes cellular transformation.** (A) Stable clones overexpressing wild-type (WT) or mutant V600E-BRAF were obtained only from cells that lost p16 expression. (B) Loss of p16 decreased SA-beta-Gal activity in NHA clones despite sustained WT-BRAF or V600E expression. It promoted the formation of foci of transformation (C) and allowed for anchorage independent growth (D) as shown by increased colony formation in soft agar of clones stably overexpressing WT-BRAF or V600E-BRAF compared to empty vector transfectant NHA cells (* p<0.05, Fisher’s exact test). Numbers are representative of three independent experiments.
References

Table 1A: Patient characteristics and genetic alterations affecting the MAPK pathway (Montreal cohort).

<table>
<thead>
<tr>
<th>Gender</th>
<th>24M; 28F</th>
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<tr>
<td>Age (average and (range), years)</td>
<td>6.22 (0.33 - 18)</td>
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<tr>
<td>Tumor location</td>
<td></td>
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<tr>
<td>Posterior fossa</td>
<td>28 (53.8%)</td>
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<tr>
<td>Optic pathway</td>
<td>8 (15.4%)</td>
</tr>
<tr>
<td>Brainstem</td>
<td>7 (13.5%)</td>
</tr>
<tr>
<td>Cerebrum</td>
<td>4 (7.7%)</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>5 (9.6%)</td>
</tr>
<tr>
<td>MAPK pathway aberration</td>
<td></td>
</tr>
<tr>
<td>KIAA1549-BRAF fusion</td>
<td>37 (71.2%)</td>
</tr>
<tr>
<td>BRAF V600E</td>
<td>2 (3.8%)</td>
</tr>
<tr>
<td>SRGAP3–RAF1</td>
<td>0</td>
</tr>
<tr>
<td>KRAS mutation</td>
<td>2 (3.8%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>11 (21.2%)</td>
</tr>
</tbody>
</table>

Table 1B: Levels of Ki-67, nuclear p53 and p16INK4A staining in all 52 primary PAs and in control brain samples.

<table>
<thead>
<tr>
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<th>All PA samples</th>
<th>Pilocytic Astrocytoma (PA)</th>
<th>Control Brain</th>
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<tr>
<td></td>
<td>n=52</td>
<td>KIAA1549-BRAF fusion = n=37</td>
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<tr>
<td>Ki67</td>
<td></td>
<td>BRAFV600E = n=2</td>
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<tr>
<td>&lt;1%</td>
<td>9 (18.8%)</td>
<td>7 (13.4%)</td>
<td>6 (100%)</td>
</tr>
<tr>
<td>1-5%</td>
<td>39 (78.4%)</td>
<td>32 (66.3%)</td>
<td></td>
</tr>
<tr>
<td>&gt;5%</td>
<td>0 (%)</td>
<td>2 (100%)</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td>p53</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>0%</td>
<td>42 (80.7%)</td>
<td>32 (66.3%)</td>
<td></td>
</tr>
<tr>
<td>1-20%</td>
<td>7 (13.5%)</td>
<td>4 (11%)</td>
<td>6 (100%)</td>
</tr>
<tr>
<td>&gt;20%</td>
<td>0</td>
<td>2 (100%)</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td>p16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10%</td>
<td>6 (11.5%)</td>
<td>3 (8%)</td>
<td>6 (100%)</td>
</tr>
<tr>
<td>10-50%</td>
<td>30 (57.7%)</td>
<td>20 (54%)</td>
<td></td>
</tr>
<tr>
<td>≥50%</td>
<td>16 (30.8%)</td>
<td>14 (37.8%)</td>
<td></td>
</tr>
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</table>
Figure 1
Figure 4
# Clinical Cancer Research

## Genetic aberrations leading to MAPK pathway activation mediate oncogene-induced senescence in sporadic pilocytic astrocytomas

Karine Jacob, Dong-Anh Khuong Quang, David T.W. Jones, et al.

*Clin Cancer Res* Published OnlineFirst May 24, 2011.

<table>
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</tr>
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