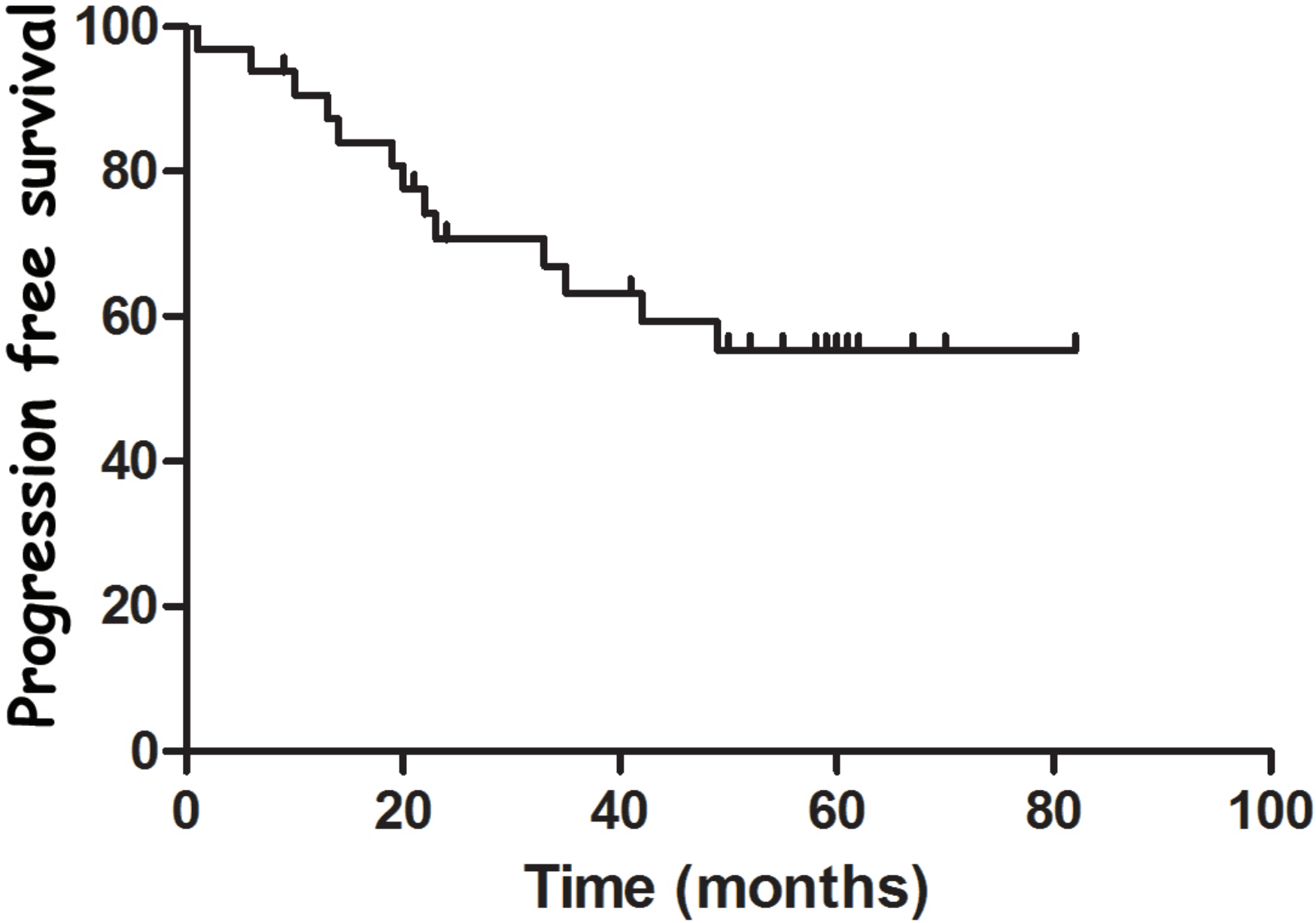
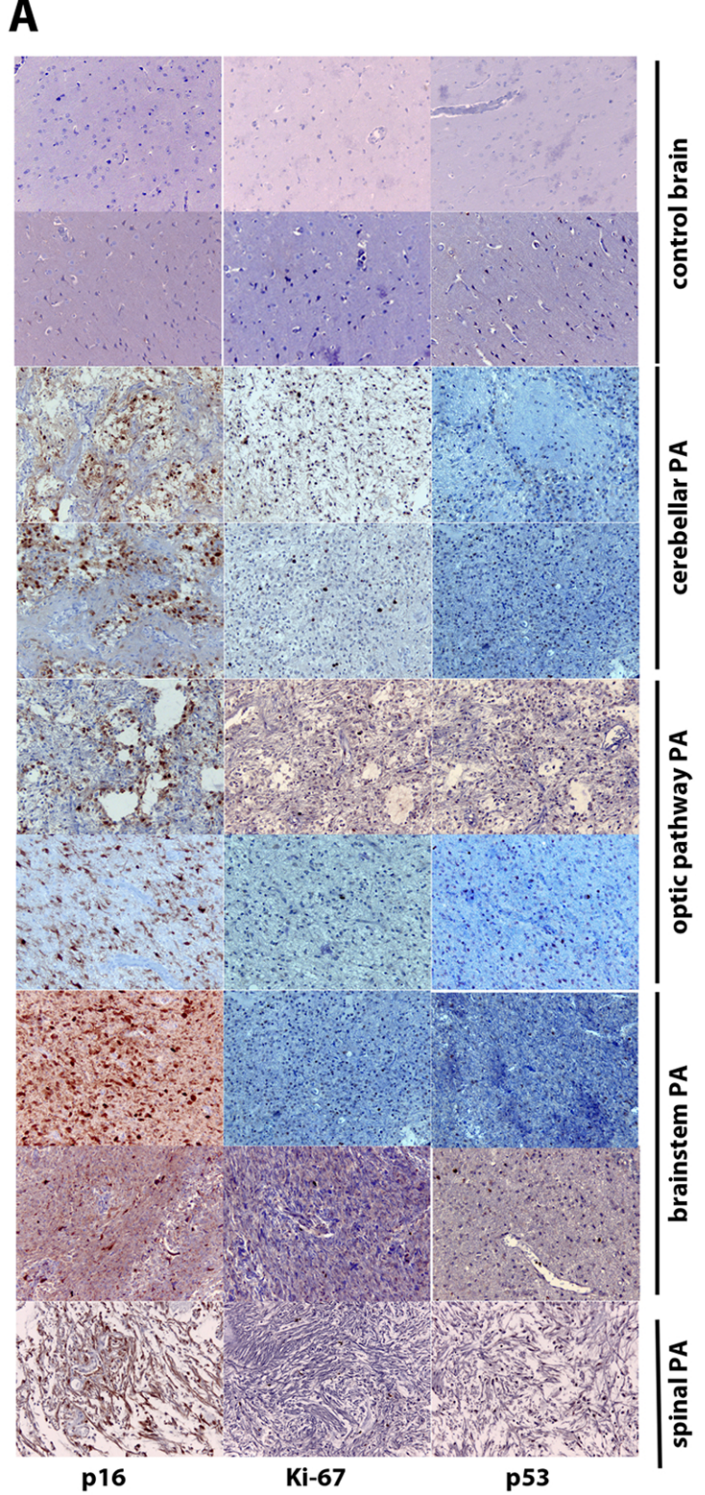
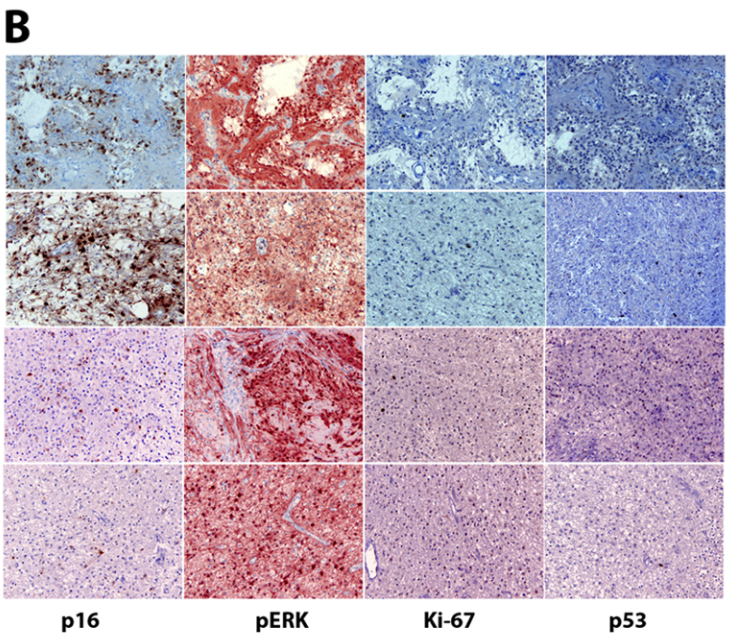
Supplementary Figure 1: Progression free survival in the Montreal cohort.



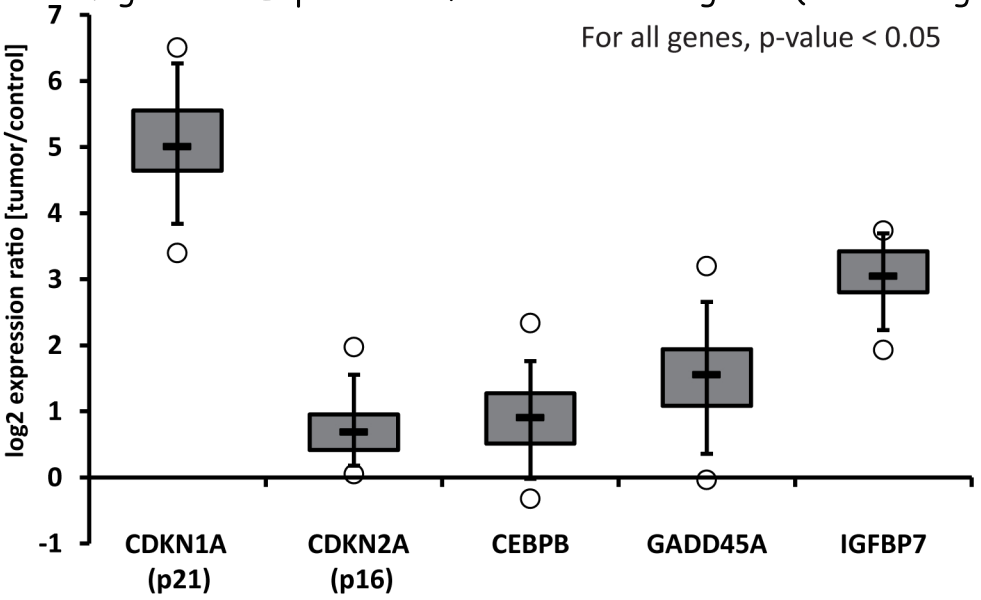




Supplementary Figure 2:

Immunohistochemical analyses of p16, Ki-67 and p53 was performed on control brains, and 52 pediatric pilocytic astrocytomas (PA) samples (Montreal cohort). (A) A representative staining of 2 control brain and 7 PA from different regions within the brain is shown demonstrating low KI67 and p53 nuclear positivity in PA samples. Most of the PA samples had increased p16 expression.
(B). In the 52 samples tested by immunohistochemistry for P16 expression, 6 had low p16 levels and still showed an active MAPK pathway (positive pERK staining) low KI-67 levels, and negative p53 nuclear staining. A representative staining of 2 of the 6 samples is shown.

Figure S3: Expression of OIS candidate genes (Heidelberg series)



Supplementary Table 1:

Characteristics of patients included in the three independent cohorts:

Montreal series: Fifty-two sporadic PAs from pediatric patients between the age of 1 and 18 years were included in the study. Clinical characteristics of PA patients are summarized in Table 1. No pilomyxoid tumors were included. Samples were taken at the time of the first surgery, prior to further treatment (where needed). Six previously described control brain samples from pediatric patients, obtained from surgical procedures on pediatric patients with epilepsy or congenital malformations and reviewed by the neuropathologist to assess for astrocytic content, were also included in the study (37, 38). Tissues were obtained from the London/Ontario Tumor Bank, the Montreal Children's Hospital and from collaborators in Hungary.

Cambridge series: Tumor tissues (n=29) were collected at the Karolinska Hospital, Stockholm and the Sahlgrenska University Hospital, Gothenberg, Sweden. The included samples were: PA3, PA5, PA7, PA8, PA9, PA12, PA14, PA15, PA16, PA18, PA20, PA23, PA24, PA25, PA27, PA30, PA32, PA33, PA40, PA43, PA44, PA47, PA48, PA51, PA54, PA57, PA65, PA67 and PA68, with clinical parameters as previously described (17). No pilomyxoid tumors were included. In addition, 10 control samples were obtained commercially, including: 4 adult whole brain (AM6050, Ambion/Applied Biosystems, Warrington, UK; samples R1234035-50 and R1234035-P from BioChain/AMS Biotechnology, Abingdon, UK and #636530 from Clontech, Basingstoke, UK), 2 fetal whole brain (R1244035-50, BioChain and #636526, Clontech), 3 adult cerebellum (AM6820, Ambion; #636535, Clontech and #540007, Strategene/Agilent) and 1 fetal cerebellum (R1244039, BioChain).

Heidelberg series: Tumor tissues (n=51) were collected at the Department of Neuropathology, Burdenko Neurosurgical Institute, Moscow, Russia. Informed consent was obtained for collection of specimens and scientific use. No pilomyxoid tumors were included. Gender distribution was 32M:19F. The median age of the patients was 9yrs, range 2-22yrs. Distribution of tumour location was as follows: cerebellum 36/51, diencephalon 7/51, cerebral hemisphere 6/51 and brain stem 2/51. In addition, two commercial control samples, one whole brain (#636530, Clontech) and one cerebellum (#636535, Clontech, pool of 24 cases) were also included in the analysis.

Processing of Human Fetal Astrocytes: 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) (collaboration with Dr J. Antel, Montreal Neurological Institute). Fetal brain tissues were washed with cold PBS containing 0.6% glucose. Tissues were digested with 0.1% papain (Worthington, NJ) for 1-hour at 37_C followed by DNase (10 lg/ml) treatment for 15 minutes. Cells were cultured as free floating neurospheres in 75-cm2 flasks with growth medium containing Dulbecco's modified Eagle's medium (DMEM; high glucose, L-glutamine)/Ham's F12 and supplemented with 15 mM HEPES, 0.6% D-glucose, 9.6 mg/ml putrescine, 6.3 ng/ml progesterone, 5.2 ng/ml sodium selenite, 0.025 mg/ml insulin, 0.1 mg/ml, heparin, 2 mM L-glutamine, penicillin and streptomycin, 20 ng/ml recombinant human EGF (Peprotech, Rocky Hill, NJ, http://www.peprotech.com), 10 ng/ml recombinant human bFGF (Peprotech), 10 ng/ml recombinant human LIF

(Chemicon, Temecula,CA, http://www.chemicon.com). GFAP staining was performed to ascertain for astrocytic content prior to transfection assays.

Human Tumor Dissociation and Analysis of SA -β–Galactosidase Activity.

Fresh specimen tissue was washed in PBS twice, minced into small pieces and incubated in a solution containing collagenase dispase (Roche, Mississauga, ON) for 15 min at 37^0C. The sample was then centrifuged at 500 rpm for 5 min. The supernatant was discarded and the tissue sample was resuspended in DMEM containing 10% FBS (Wisent, St-Bruno, QC). SA-β-galactosidase assay was performed following an overnight incubation for optimal adhesion. Cells were fixed in 2% formaldehyde and 0.2% glutaraldehyde and incubated overnight at 37°C with staining solution containing 1 mg/ml X-gal substrate in 40mM citric acid/sodium phosphate buffet at pH 6, 150 mM NaCl and 2mM MgCl2, 5nM potassium ferrocyanide and 5nM potassium ferricyanide for 16-18h, according to the manufacturer's protocol (Cell Signalling, Danvers, MA, USA). The percentage of positive cells was assessed by counting the number of blue cells in a X20 microscopic field and dividing the number by the total number of cells in the field ([3]27, 30).

Immunohistochemical staining: Immunohistochemistry for p16, p53 and Ki-67 was performed using the Ventana Benchmark systems (Ventana Medical Systems Inc, Tucson, Arizona) on FFPE sections cut at 4 mm thick. As a pre-treatment step, tissues underwent heat-induced epitope retrieval with the Cell Conditioning 1 (CC1) solution (Ventana) for 60 min for p16 and p53 and 30 min for Ki-67. Standardized staining protocols were provided by Ventana using pre-diluted antibodies for the CINtec p16INK4a Histology kit (clone E6H4, mouse monoclonal, MTM Laboratories Inc, Westborough, Massachusetts) and p53 antibody (clone Bp-53-11, mouse monoclonal, Ventana Medical Systems Inc., Tucson Arizona) and Ki-67 (clone 30-9, rabbit monoclonal, Ventana Medical Systems Inc., Tucson Arizona). Controls were included in each assay, comprising of positive tissue controls and negative controls. All IHC slides

were additionally reviewed and positivity of staining assessed by a senior pathologist (S.A) blinded to the clinical outcome.

Gene expression profiling.

Cambridge series: Samples were analysed on the Illumina HT12 v3 expression platform. All tumor pieces selected for RNA extraction had high tumor cell content (minimum of 70%, generally >90%) as estimated by histological examination. RNA was extracted and cleaned with an RNeasy® kit (Qiagen, Crawley, UK) according to manufacturer's protocols. All samples had a RIN score >5.5 as assessed using a 2100 Bioanalyzer (Agilent, Wokingham, UK). Array probes scored as absent in all samples were excluded, and the remaining probes (21,632) were transformed and normalised according to the defaults of the *lumi* package in the R statistical environment(33). Selected genes were assessed for differential expression between tumor and control samples using a 2-tailed Mann-Whitney U test. P-values <0.05 were considered significant. Where multiple probes existed for a given gene, the highest quality probe (greatest number of 'present' calls) was selected as representative.

Heidelberg series: Samples were analysed on the Illumina WG6 v3 expression platform. Total RNA extraction was performed from snap-frozen tumor tissues using TRIZOL (Invitrogen, Carlsbad, USA). RNA was analyzed on both NanoDrop (ND-1000, Thermo Scientific, Wilmington, USA) and Bioanalyzer (Agilent Technologies, Böblingen, Germany) for quality assessment. Only samples with a RNA integrity number (RIN) >5.0 and no evidence of ribosomal degradation were included in the study. Low intensity probes were excluded, and the data were then transformed via variance stabilization and subjected to robust spline normalization with the *lumi* R package. The mean of the

normal brain expression was then subtracted from the expression value for each sample and for each gene of interest, to give a $\log_2$ fold-change value.

Cell Cycle Analysis. Data analysis was performed using FlowJow software. All experiments were performed in triplicates and at least 40,000 cells were acquired from each sample for FACS analysis. Cells were plated in triplicated in a 6cm² dish and collected at 80% confluence. Cells were washed in PBS, fixed using 75% ethanol and stored at -20°C. For the analysis of cell cycle, 100 ul of FBS was added to the cells. Cells were then centrifuged, washed with PBS, and resuspended in a 300 ul solution containing 4ug/ul of propidium iodine (Sigma), 200ug/ul RNase (Sigma) and PBS. Samples were incubated for 1 hour at 37°C. Cells were analysed using a FACS Calibur (BD biosciences) and the FlowJo program (Tree Star software).

Supplementary table 2: Genetic alterations of the MAPK pathway identified in the Montreal's serie

ID	MAPK pathway alteration	fusion breakpoint
PA01	KIAA1549-BRAF	Ex 15-9
PA02	KIAA1549-BRAF/BRAF^{V600E}	Ex 15-9
PA03	KIAA1549-BRAF	Ex 15-9
PA04	KIAA1549-BRAF	Ex 15-9
PA05	KIAA1549-BRAF	Ex 15-9
PA06	KIAA1549-BRAF	Ex 16-11
PA07	KIAA1549-BRAF	Ex 16-11
PA08	KIAA1549-BRAF	Ex 16-11
PA09	KIAA1549-BRAF	Ex 16-11
PA10	KIAA1549-BRAF	Ex 16-11
PA11	KIAA1549-BRAF	Ex 16-11
PA12	KIAA1549-BRAF	Ex 16-9
PA13	KIAA1549-BRAF	Ex 16-9
PA14	KIAA1549-BRAF	Ex 16-9
PA15	KIAA1549-BRAF	Ex 16-9
PA16	KIAA1549-BRAF	Ex 16-9
PA17	KIAA1549-BRAF	Ex 16-9
PA18*	KIAA1549-BRAF	Ex 16-9
PA19*	KIAA1549-BRAF	Ex 16-9
PA20*	KIAA1549-BRAF	Ex 16-9
PA21*	KIAA1549-BRAF	Ex 16-9
PA22*	KIAA1549-BRAF	Ex 16-9
PA23*	KIAA1549-BRAF	Ex 16-9
PA24*	KIAA1549-BRAF	Ex 15-9
PA25*	KIAA1549-BRAF	Ex 15-9
PA26*	KIAA1549-BRAF	Ex 16-9
PA27*	KIAA1549-BRAF	Ex 16-9
PA28*	KIAA1549-BRAF	Ex 16-9
PA29*	KIAA1549-BRAF	Ex 16-9
PA30*	KIAA1549-BRAF	Ex 16-9
PA31*	KIAA1549-BRAF	Ex 16-9
PA32*	KIAA1549-BRAF	Ex 16-9
PA33*	KIAA1549-BRAF	Ex 16-9
PA34*	KIAA1549-BRAF	Ex 15-9
PA35*	KIAA1549-BRAF	Ex 15-9
PA36*	KIAA1549-BRAF	Ex 16-11
PA37*	NAD	-
PA38*	NAD	-
PA39*	NAD	-
PA40*	NAD	-
PA41*	NAD	-
PA42*	NAD	-
PA43*	NAD	-
PA44*	NAD	-

PA45*	NAD	-
PA46	NAD	-
PA47	$BRAF^{V600E}$	-
PA48	NAD	-
PA49*	NAD	-
PA50*	KRAS	-
PA51	KRAS	-
PA52	$BRAF^{V600E}$	-

NAD: no known genetic alteration detected in the MAPK pathway.

*: samples previously reported in Jacob et al. 2009 (33)