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ORIGINAL ARTICLE

# Pilocytic astrocytomas: *BRAFV600E* and *BRAF* fusion expression patterns in pediatric and adult age groups

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## Abstract

**Purpose** Pilocytic astrocytomas (PCAs) are characterized by two dominant molecular alterations of the *BRAF* gene, i.e., *BRAFV600E* mutation and *KIAA1549-BRAF* fusions which show a differential pattern of frequency across different age-groups.

**Methods** Formalin-fixed paraffin-embedded tissues of 358 (pediatric 276 and adult 82) consecutive PCAs were evaluated for *BRAFV600E* mutation by Sanger sequencing and *KIAA1549:BRAF* fusion transcripts (*KIAA1549:BRAF* 16-9, *KIAA1549:BRAF* 15-9, and *KIAA1549:BRAF* 16-11) by reverse transcriptase polymerase chain reaction, which were correlated with different clinicopathological features.

**Results** *BRAFV600E* mutation was detected in 8.9% pediatric and 9.75% adult PCAs, whereas 41.1% and 25.7% of pediatric and adult cases showed *KIAA1549-BRAF* fusions respectively. *BRAFV600E* did not show any statistically significant correlation with any of the clinical parameters (age, location, and gender). *KIAA1549:BRAF* fusions showed a significant statistical association with the pediatric age group and cerebellar location. *KIAA1549-BRAF* 16-9 was the commonest variant and was predominantly associated with cerebellar location than non-cerebellar whereas fusion variant 15-9 negatively correlated with cerebellar locations.

**Conclusions** The present study showed overall frequency of 53.5% and 37.3% *BRAF* alterations in pediatric and adult PCA cases respectively. *BRAF* fusion in PCA cases showed a different distribution pattern across age groups and locations; while no such differential pattern was observed for *BRAFV600E*.

**Keywords** *BRAFV600E* · *KIAA1549-BRAF* fusion · Sanger sequencing · RT-PCR

## Introduction

Pilocytic astrocytoma (PCA) is the most frequent pediatric low-grade glioma and is also not uncommon in young adults, accounting for 15.3% among children and adolescents [1, 2]. It is WHO grade I circumscribed glioma classified under other astrocytic tumors in the current 2016 WHO classification [3]. It commonly occurs in the cerebellum and the optic pathway but can occur throughout the neuroaxis [4]. It is predominantly sporadic but is the most common familial syndrome associated glial tumor, especially in neurofibromatosis 1 (NF1). PCAs are of favorable prognosis with > 95% of 10-year overall survival [1].

PCAs are mediated by constitutive activation of mitogen-activated protein kinase (MAPK) pathway through the in-frame fusion between oncogene *BRAF* and *KIAA1549* genes (resulting from breakpoint at multiple sites of *KIAA1549* and *BRAF* generating different fusion variants), through

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oncogenic *BRAFV600E* mutation and uncommonly through alterations in fibroblast growth factor receptor 1 (*FGFR1*). *KIAA1549* Exon 16-*BRAF* Exon 9 fusion is the commonest followed by *KIAA1549* Exon 15-*BRAF* Exon 9 and *KIAA1549* Exon 16-*BRAF* Exon 11 [5–7]. Other uncommon reported fusion variants are Exon 18-10, 19-9, 16-10, 15-11, 17-10 of *KIAA1549* and *BRAF* [5, 6]. Rare non-*KIAA* gene fusion variants such as *SRGAP3-BRAF* [8], *FAM131B:BRAF* [9, 10], *GIT2I-BRAF* [11], *TMEM106B-BRAF* fusions [12], and *BRAF* gain of function mutation are also reported [13]. While *BRAFV600E* mutation results in constitutive activation of *BRAF* even in its monomeric form and also induces the same effect on MAPK pathway activation as in *BRAF* fusions [14]. *FGFR1* alterations also induce MAPK/ERK pathway upregulation through intragenic duplications of the FGFR1 tyrosine kinase domain resulting in *FGFR1* autophosphorylation. However, this is relatively frequent in grade II diffuse gliomas (24%) than PCAs and LGGs (7.4%) [15].

Although both the *BRAF* (fusions and *V600E*) alterations exert their effect of tumorigenesis through the MAPK pathway, but their frequency pattern varies according to age of diagnosis and location. *KIAA1549-BRAF* fusions are more common in younger age and in cerebellar location, whereas *BRAFV600E* is more common in non-cerebellar location [13, 16, 17]. *KIAA1549-BRAF* fusion was reported in around 60–70% of all PCAs, whereas *BRAFV600E* mutation in < 10% of both pediatric and adult cases [17–19].

This is a single institutional study aimed at evaluating the frequency of *BRAFV600E* mutation and *KIAA1549-BRAF* (16-9, 15-9, and 16-11) fusions in PCAs and to correlate it with clinicopathological features.

## Materials and methods

### Tumor samples

The study is approved by our institutional review board, comprised of 358 consecutive (2011–2018) histologically diagnosed cases of PCA (including pilomyxoid astrocytomas (PMA)). Cases with syndromic association (especially NF1) and inadequate tissue were excluded. Formalin-fixed paraffin-embedded (FFPE) tumor tissues were used for the study, which were essentially primary samples at diagnosis; 205 (pediatric 157/276, adult 48/82) were in-hospital and 153 (pediatric 119/276, adult 34/82) were referrals. Clinical and demographic details were collected from electronic medical records.

### Immunohistochemistry

p53 (Dako, D07, 1:50 dilution)and ATRX protein (1:750; Polyclonal; Sigma; USA) expression was performed on 4 μm representative FFPE sections using the Ventana Benchmark XT autoimmunostainer by polymer detection kit.

p53 protein expression was graded as positive (> 50% tumor cells with strong intensity nuclear staining), focal positive (10–50% with strong nuclear staining/> 10% tumor cells with moderate intensity staining) and negative (absence of any staining or staining in < 10% tumor cells). ATRX expression was interpreted as retained (nuclear staining in tumor cells), loss (no staining in tumor cells with reactivity of native cells like endothelial and/or neurons as an internal control), and non-contributory (no staining in internal control).

### Molecular analysis: *BRAF* alterations

Selected FFPE blocks were subjected for detection of *BRAF* mutation by Sanger sequencing and *KIAA1549-BRAF* fusion transcript detection by Reverse transcriptase PCR (RT-PCR) for three different fusion transcripts; *KIAA1549-BRAF* 16-9 (124 bp), *KIAA1549-BRAF* 15-9 (159 bp) and *KIAA1549-BRAF* 16-11 (Table 1: primer sequences). See Appendix for the detailed methodology of *BRAFV600* sequencing and *BRAF* fusion RT-PCR.

### Validation of *KIAA1549*: *BRAF* fusion transcripts

Sanger sequencing was performed on 10% of the *KIAA1549-BRAF* fusion-positive samples to validate and confirm the presence of *KIAA1549-BRAF* fusion transcripts (Appendix). The sequences were aligned and compared with the reference sequences using the BLAST alignment tool.

### Statistical analysis

The data was analyzed using SPSS 20.0 software. The *BRAF* mutation and *KIAA1549-BRAF* fusion results were correlated with the clinical and histological features using Fisher's test and $p$ value < 0.05 was considered statistically significant.

## Results (Table 2)

### Sample size ($n = 358$)

214 were males and 144 were females (M:F ratio of 1.5:1). Age range was 1–50 years (interquartile range [IQR] 6–18 years; median10 years) with 276 pediatric cases (≤ 18 years) and 82 adults (19–39 years,77; >39 years, 5).

**Table 1** Primer sequences

| Gene | Primer sequence | | Product size |
|---|---|---|---|
| *ACTB* | Forward<br>Reverse | 5′-CGGGACCTGACTGACTACCT-3′<br>5′-TGCCAATGGTGATGACCTG-3’ | 208 bp |
| *BRAF* Ex 15 | Forward<br>Reverse | 5′-GGTGATTTTGGTCTAGCTACAG-3′<br>5′-AGTAACTCAGCAGCATCTCA GG-3′ | 173 bp |
| *KIAA1549* Ex 16<br>*BRAF* Ex 9 | 5′-AAACAGCACCCCTTCCCAGG-3′<br>5′-CTCCATCACCACGAAATCCTTG-3′ | | 124 bp |
| *KIAA1549* Ex 15<br>*BRAF* Ex 9 | 5′-CGGAAACACCAGGTCAACGG-3′<br>5′-CTCCATCACCACGAAATCCTTG-3′ | | 159 bp |
| *KIAA1549* Ex 16<br>*BRAF* Ex 11 | 5′AAACAGCACCCCTTCCCAGG-3′<br>5′-GTTCCAAATGATCCAGATCCAATTC-3′ | | 182 bp |

## Pediatric group ($n = 276$)

### Clinical

M:F was 1.4:1 and IQR was 5–13 years (0–3 years, 29; 4–14 years, 203; 15–18 years, 44) with median age of 8 years.The most common location was cerebellar (*n* 123; 44.6%) followed by 3rd ventricular (*n* 68; 24.6% (suprasellar, 53; 3rd ventricle,11; hypothalamic, 3; pineal, 1)), cerebrum (*n* 25; 9.1%), spinal (*n* 17; 6.2%), optic nerve and chiasma (*n* 15; 5.4%), thalamic (*n* 14; 5.1%), and brainstem (*n* 14; 5.1%). Histologically, 270 were PCA while 6 were PMAs.

### *BRAF* alterations

Interpretable *BRAFV600E* was in 246 cases, while the remaining 30 were uninterpretable (17 could not be amplified for beta-actin gene and 13 showed non-readable sequencing data). *BRAFV600E* was observed in 22 (8.9%) cases. All cases showed typical heterozygous *BRAFV600E* substitution replacing adenine in place of thymine nucleotide at codon 600 (Fig. 1).

*KIAA1549-BRAF* fusion PCR could be performed in 239 pediatric cases (37 failed due to degraded RNA), 99 (41.1%) cases showed fusion transcripts. Sixty-six cases were positive for *KIAA1549-BRAF* 16-9 transcripts (Fig. 1) whereas *KIAA1549-BRAF* 15-9 and *KIAA1549-BRAF* 16-11 fusion transcripts were detected in 21 and 12 cases respectively. None of the cases showed the presence of more than one fusion transcript and were mutually exclusive with *BRAFV600E*.

Of the 276 cases, both *BRAF* mutation and *KIAA1549-BRAF* fusion transcript expression could be analyzed in 226 cases with 53.5% (121/226) of cases showing *BRAF* alterations. 43.8% (*n* 99) were *KIAA1549-BRAF* and only 9.7% cases demonstrated the presence of *BRAFV600E*. All PMA cases within the group were negative for *BRAF* alterations.

### p53 ($n = 190$) and ATRX protein ($n = 272$) expression

p53 protein expression was not available in 86 cases, 17 (8.9%) were positive, 47 (23.9%) were focal positive and 126 (66.3%) were negative. All 272 cases showed retained ATRX expression (4 were non-contributory).

## Adult group ($n = 82$)

### Clinical

M:F was 1.9:1 and age range was 19–50 years with IQR of 21–28 years (19–39 years, 77; > 39 years, 5), and median age 24 years. Cerebellum was the commonest location (*n* 27; 32.9%), followed by cerebrum (*n* 16; 19.5%), 3rdventricular (*n* 13; 15.9% (suprasellar, 8; 3rd ventricle, 2; hypothalamic, 2; pineal, 1)), spinal (*n* = 12; 14.6%), thalamic (*n* 6; 7.3%), brainstem (*n* 4; 4.9%), and optic nerve/chaisma (*n* 4; 4.9%).

### *BRAF* alterations

Ten were uninterpretable for *BRAFV600E* (7 had suboptimal DNA preservation and 3 showed noisy sequencing data); 9.7% (7/72 (19–39, 7/67; > 39, 0/5)) were *BRAFV600E* mutant (Fig. 2).

*KIAA1549-BRAF* fusion was interpretable in 70 cases (12 had degraded RNA). Of these, 25.7% (18/70 (19–39, 16/65; > 39, 2/5)) were positive for *KIAA1549-BRAF* fusion (*KIAA1549-BRAF* 16-9, 11; *KIAA1549-BRAF* 15-9, 5; and *KIAA1549-BRAF* 16-11, 2) (Fig. 2).

Sixty-seven cases were interpretable for both *BRAF* alterations, of which 25 (37.3%) *BRAF* alterations (25/67; *BRAFV600E*, 7; *BRAF* fusion, 18).

### p53 ($n = 59$) and ATRX ($n = 78$) protein expression

p53 was positive in 13.6% (8/59) cases, focal positive in 20.3% (12/59) and 66.1% (39/59) negative cases. One case showed a mosaic pattern of ATRX expression (which

**Table 2** *BRAFV600E* and *KIAA1549:BRAF* fusion with clinical and histological features

| Parameter | | Subtype | *KIAA1549:BRAF* ($n = 309$) | | | | | | | *BRAF* mutation ($n = 318$) | | | | | *BRAF* Alterations ($n = 293$) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | N | 16-9 | 15-9 | 16-11 | Negative | % positivity | *p* value | N | BRAFV600E | Wild type | % positivity | *p* value | N | Positive | Negative | % positivity |
| Gender | Pediatric (≤ 18) | Male | 141 | 45 | 13 | 6 | 77 | 45.34 | 0.15* | 146 | 13 | 133 | 8.6 | 0.84* | 136 | 77 | 59 | 56.6 |
| | | Female | 98 | 21 | 8 | 6 | 63 | 35.7 | | 100 | 9 | 91 | 9.0 | | 90 | 44 | 46 | 48.9 |
| | Adults (> 18) | Male | 48 | 7 | 5 | 2 | 34 | 29.2 | | 50 | 4 | 46 | 8.0 | | 47 | 18 | 29 | 38.3 |
| | | Female | 22 | 4 | 0 | 0 | 18 | 18.2 | | 22 | 3 | 19 | 13.6 | | 20 | 7 | 13 | 35.0 |
| Age group (years) | Pediatric (≤ 18) | 0–3 | 25 | 9 | 2 | 2 | 12 | 52.0 | 0.02* | 125 | 0 | 25 | 0 | 0.82* | 23 | 13 | 10 | 56.5 |
| | | 4–14 | 176 | 51 | 18 | 9 | 98 | 44.3 | | 180 | 17 | 163 | 9.4 | | 166 | 95 | 71 | 57.2 |
| | | 15–18 | 38 | 6 | 1 | 1 | 30 | 21.1 | | 41 | 5 | 36 | 12.2 | | 37 | 13 | 24 | 35.1 |
| | Adults (> 18) | 19–39 | 65 | 9 | 5 | 2 | 49 | 24.6 | | 67 | 7 | 60 | 10.4 | | 62 | 23 | 39 | 37.1 |
| | | > 39 | 5 | 2 | 0 | 0 | 3 | 40.0 | | 5 | 0 | 5 | 0 | | 5 | 2 | 3 | 40.0 |
| Tumor location | Pediatric (≤ 18) | Cerebellar | 109 | 43 | 5 | 7 | 54 | 50.5 | 0.01** | 110 | 7 | 103 | 6.4 | 0.26** | 102 | 62 | 40 | 60.8 |
| | | 3rd Ventricular | 56 | 7 | 7 | 1 | 41 | 26.8 | | 58 | 9 | 49 | 15.5 | | 53 | 24 | 29 | 45.3 |
| | | Cerebrum | 20 | 2 | 4 | 1 | 13 | 35.0 | | 22 | 2 | 20 | 9.1 | | 19 | 9 | 10 | 47.4 |
| | | Thalamic | 12 | 3 | 1 | 1 | 7 | 41.7 | | 13 | 2 | 11 | 15.4 | | 12 | 7 | 5 | 58.3 |
| | | Brainstem | 13 | 6 | 1 | 0 | 6 | 53.8 | | 13 | 2 | 11 | 15.4 | | 13 | 9 | 4 | 69.2 |
| | | Spinal | 16 | 2 | 3 | 1 | 10 | 37.5 | | 17 | 0 | 17 | 0 | | 16 | 6 | 10 | 37.5 |
| | | Optic Nerve/chiasma | 13 | 3 | 0 | 1 | 9 | 30.8 | | 13 | 0 | 13 | 0 | | 11 | 4 | 7 | 36.4 |
| | Adults (> 18) | Cerebellar | 24 | 7 | 1 | 1 | 15 | 37.5 | 0.15** | 24 | 4 | 20 | 16.7 | 0.21** | 23 | 13 | 10 | 56.5 |
| | | 3rd Ventricular | 12 | 2 | 1 | 1 | 8 | 33.3 | | 12 | 0 | 12 | 0 | | 12 | 4 | 8 | 33.3 |
| | | Cerebrum | 12 | 0 | 2 | 0 | 10 | 16.7 | | 14 | 2 | 12 | 14.3 | | 11 | 4 | 7 | 36.4 |
| | | Thalamic | 4 | 0 | 0 | 0 | 4 | 0 | | 5 | 0 | 5 | 0 | | 4 | 0 | 4 | 0 |
| | | Brainstem | 4 | 1 | 0 | 0 | 3 | 25.0 | | 4 | 0 | 4 | 0 | | 4 | 1 | 3 | 25.0 |
| | | Spinal | 10 | 1 | 1 | 0 | 8 | 20.0 | | 10 | 1 | 9 | 10.0 | | 10 | 3 | 7 | 30.0 |
| | | Optic nerve/chiasma | 4 | 0 | 0 | 0 | 3 | 0 | | 3 | 0 | 3 | 0 | | 3 | 0 | 3 | 0 |

*Pediatric vs adults; **cerebellar vs non-cerebellar

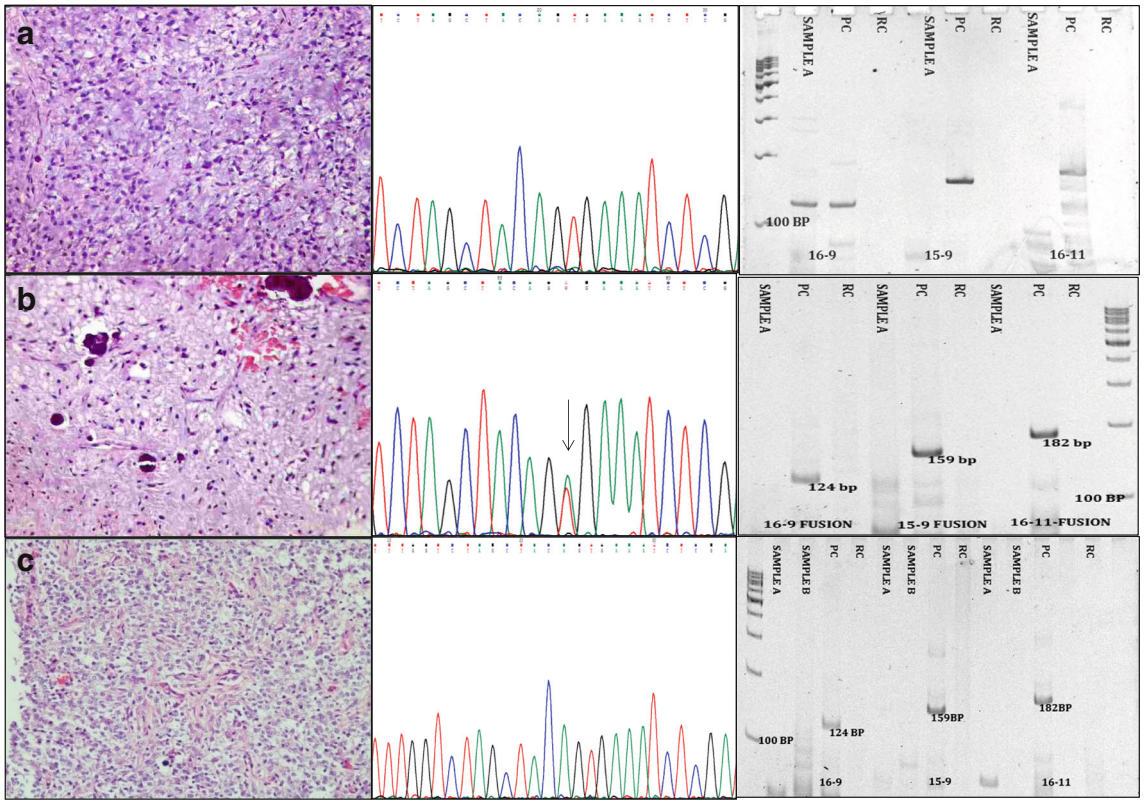


**Fig. 1** Representative photomicrographs of pediatric PCA. **a** Cerebellar PCA (H&E; × 200) with electropherograms showing *BRAFV600* and gel picture depicting *KIAA1549-BRAF 16-9* fusion positive. **b** 3rdventricularPCA (H&E; × 200) with electropherograms showing *BRAFV600E* mutant (black arrow) and gel picture depicting *KIAA1549-BRAF* fusion negative. **c** Suprasellar PCA (H&E; × 200) with electropherograms showing *BRAFV600* wild type and gel picture depicting *KIAA1549-BRAF* fusion negative

was negative for *BRAF* alterations) and 77(98.7%) showed retained ATRX protein.

## Validation of *KIAA1549: BRAF* fusion transcripts

12/117 (10%) of *KIAA1549-BRAF* fusion-positive cases were confirmed by Sanger sequencing for all three fusion variants and their breakpoints (Fig. 3).

## Correlation of *BRAF* alterations with clinicopathological parameters

### Clinical

*BRAFV00E* was detected in the age range of 5–33 years (median age13). No significant difference ($p$ value 0.82) was observed between *BRAFV600E* and age group (pediatric 8.9% and adult group 9.7%) and also for gender ($p$ value 0.84). Of 117 *KIAA1549-BRAF* fusion positive cases, 78 were males and 39 were females with a median age of 8 years. There was no statistically significant difference observed for *KIAA1549-BRAF* fusion and gender ($p$ value 0.15). *KIAA1549-BRAF* fusions were more common in the pediatric age group as compared with adults and this preponderance in ≤ 18 years (99/239; 41.4%) is statistically significant ($p$ value 0.02) as compared with that in adults (> 18 years 18/70; 25.7%). Within the adult group, two cases were > 39 years (42 years of cerebellar location and 46 years of cervicomedullary location) showed presence of *KIAA1549-BRAF* 16-9 fusions.

### Location

None of the tumor location showed significant statistical correlation for *BRAFV600E* mutation in pediatric and/or adult age group. None of the optic nerve/chiasma cases showed *BRAF* mutation. In the pediatric group, *BRAF* fusions were significantly associated with cerebellar as compared with non-cerebellar location ($p = 0.01$). It was the commonest location within fusion positive cases. 55/99 (55.6%) fusion positive pediatric cases were from cerebellar location. Among the three variants of fusion transcripts, *KIAA1549-BRAF* 16-9 correlated significantly with cerebellar location than other two fusion transcripts ($p = 0.03$) whereas fusion 15-9 transcript showed significant association with non-cerebellar location and negatively correlated with cerebellar location ($p = 0.00$).Adult cases also showed cerebellar (37.5%) as the predominant

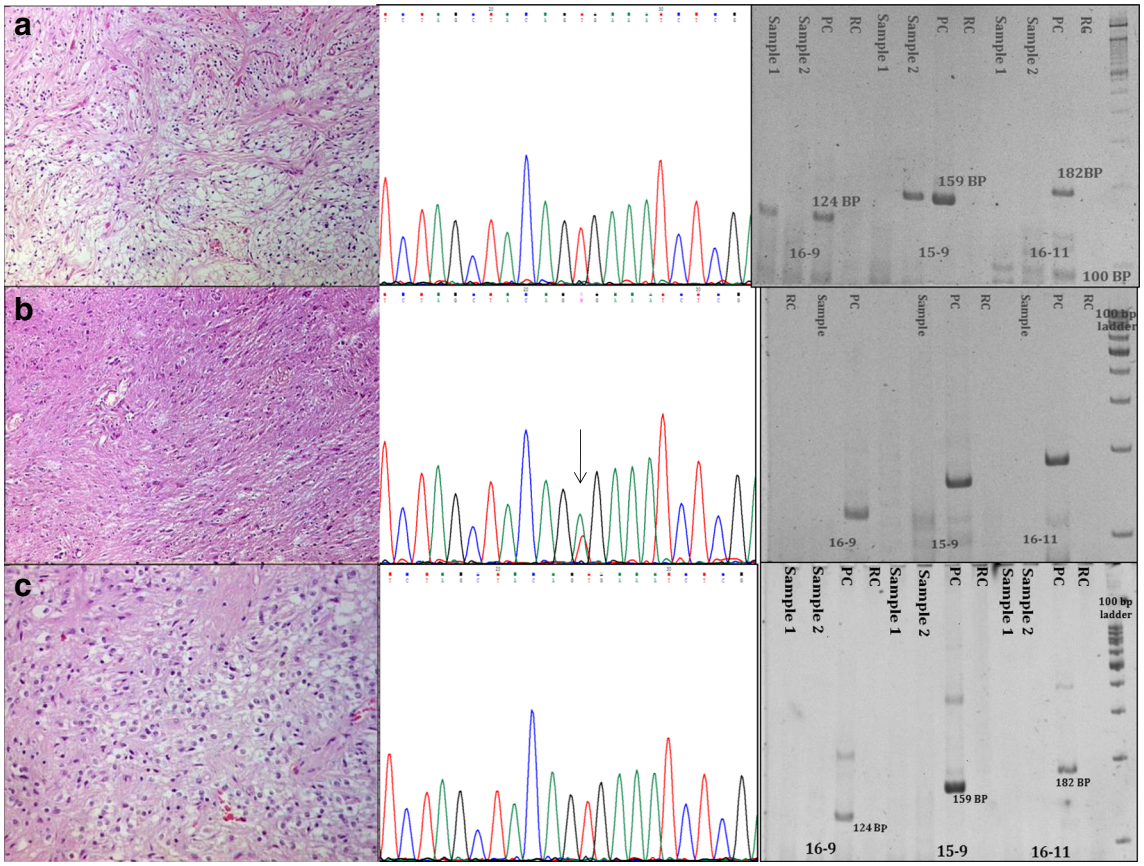


**Fig. 2** Representative photomicrographs of adult PCA. **a** Cerebellar PCA (H&E; × 100) with electropherograms showing *BRAFV600* and gel picture depicting *KIAA1549:BRAF 15-9* fusion positive. **b** Suprasellar PCA (H&E; × 100) with electropherograms showing *BRAFV600E* mutant (black arrow) and gel picture depicting *KIAA1549:BRAF* fusion negative. **c** spinal PCA (H&E; × 200) with electropherograms showing *BRAFV600* wild type and gel picture depicting *KIAA1549:BRAF* fusion negative

location for *BRAF* fusion but did not show a statistical significant association ($p = 0.15$).

### p53 and ATRX protein expression

8.3% of *BRAFV600E* were in p53 positive and 8.9% were in focal positive while 11.5% were in negative cases. *BRAF* fusion transcripts were present in 33.3% p53 positive, 37.7% focal positive and 43.6% in p53 negative cases. No statistically significant correlation was observed. All except one case showed ATRX retained expression and thus no further comparative evaluation was done.

## Discussion

PCAs are characterized by constitutive activation of the MAPK pathway through two major *BRAF* gene alterations—*BRAF* fusion and *BRAFV600E* mutation. The *BRAF* gene located at chromosome 7 (7q34) encodes for serine/threonine RAF kinase which acts as an intracellular transducer of the RAS/RAF/MEK/ERK pathway where activated *RAS* induces dimerization and activation of *BRAF* leading to MAPK activation, which causes tumorigenesis [20, 21]. The most common mechanism of alteration of MAPK pathway in PCAs is tandem duplication of approximately 2 MB 7q34 region with subsequent fusion resulting in *KIAA1549-BRAF* fusion variant [5]. The event of *KIAA1549-BRAF* fusion results in the replacement of the N-terminal regulatory region of *BRAF* with the N-terminal end of *KIAA1549* resulting in constitutive activation of *BRAF* kinase domain and upregulation of the MAPK pathway. Another mechanism of constitutive MAPK activation is *BRAFV600E* mutation, the most common hotspot mutation observed across various cancers including glial tumors and is a missense substitution in exon 15 of *BRAF* gene at nucleotide 1799 replacing valine to glutamic acid at codon 600 within the activation segment coding region resulting in constitutive activation of *BRAF* in its monomeric form [20, 22]. *BRAF* fusion is the most common genetic event and characteristic of PCAs whereas *BRAFV600E* is uncommon. The latter is more frequent in non-PCA other astrocytic tumors like pleomorphic xanthoastrocytomas and gangliogliomas [17].

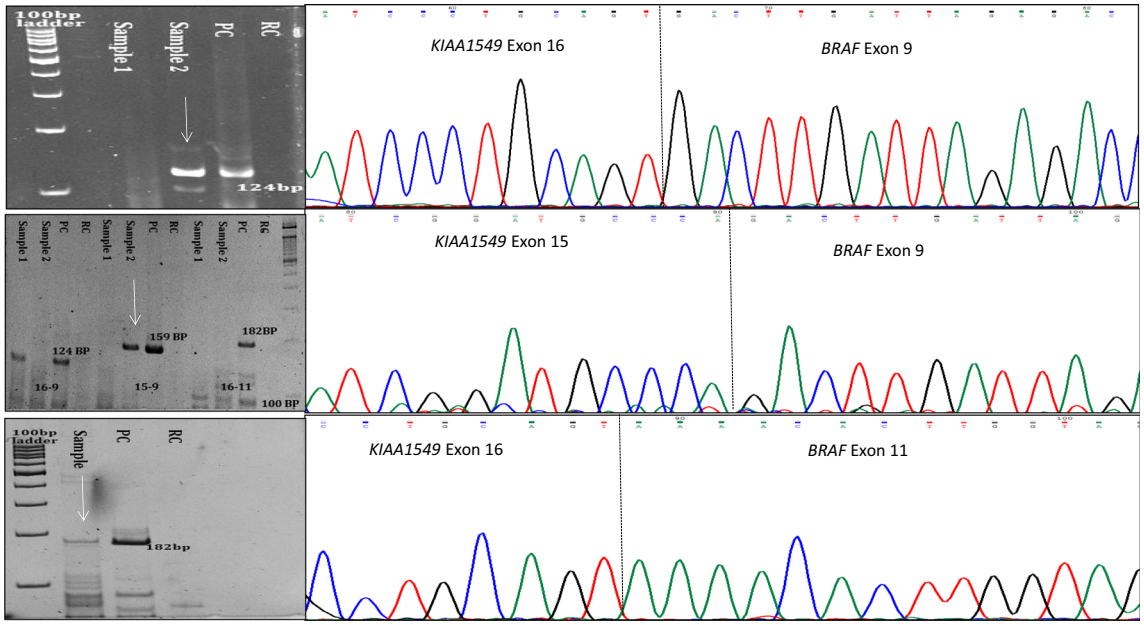


**Fig. 3** Representative gel images and electropherograms of validated cases of *KIAA1549:BRAF* 16-9, 15-9 and 16-11 fusion variants

Detection of these *BRAF* alterations has a diagnostic value and has become an integral part of routine diagnostic practice in neuro-oncology. *KIAA1549-BRAF* fusion transcripts were detected using the most popular RT-PCR due to its ease to perform, cost-effectiveness, easy interpretation. However, RT-PCR requires good-quality RNA and a different set of primers for different variants with well optimization of each set [23]. The other method of detection is fluorescent in situ hybridization (FISH), this method is challenging especially in FFPE section. In view of the low cellularity of these tumors compounding with nuclear truncation usually result in variability in the signal pattern leading to difficulty in interpreting of fusion signals and is also endowed with inability to identify definite fusion variant [24]. However, a well-optimized FISH assay can also be used as an alternative method for *BRAF* fusion detection in diagnostic setting with skilled personnel for interpretation.Recent more accurate and sensitive techniques have also been developed based on droplet digital PCR, NanoString technology and Pyromark system but use of these techniques are restricted due to unavailability of these platforms in routine setups [25–27]. *BRAF* mutation can also be detected with high resolution melting curve analysis, allele-specific SNaP shot and by immunohistochemistry (IHC) with *BRAF* mutation-specific VE-1 antibody. The detection of *BRAFV600E* with VE-1 antibody is a robust, cheaper, and less time-consuming method and also showed high concordance with Sanger sequencing, but in some instances, it may yield non-specific staining necessitating the confirmation with sequencing [19, 28]. This study employed RT-PCR and Sanger sequencing for detection of limited *BRAF-KIAA* fusions (16-9, 15-9, and 16-11) and *BRAFV600E* in pediatric and adult groups.

The frequency of fusion transcripts within the PCA group varies from 50 to 80% across various studies (Table 3). The frequency of *BRAF* fusions in the present study was 41.1% in pediatric cases which is comparatively lower than reported, even in comparison with the three fusion variants evaluated. This possibly reflects the inherency of this population or probably due to the lesser sensitivity of the RT-PCR though only the cases with adequate representation of the tumor were selected [23, 24]. *BRAF* fusions were observed to be less frequent in adult PCAs and the frequency was observed to be 25.7% which is similar to previous studies [16, 29].

*KIAA1549-BRAF* fusion transcripts are predominantly seen in PCAs and PMAs, although the presence of these fusion transcripts are also reported in few non-PCA/PMA tumors like in 70–80% of low-grade glioneuronal tumors, diffuse leptomeningeal glioneuronal tumors [30–32], pediatric oligodendroglial tumors [33], and few reports of pediatric diffuse gliomas, NOS [17, 34–37]. However, none (although the number is small) of the PMA cases in the present study showed the presence of *BRAF* fusion.

*KIAA1549-BRAF* fusion variant of 16-9 (pediatric 66.7%, adults 61.1%) is the commonest followed by fusion 15-9 (pediatric 21.2%, adults 27.8%) and fusion 16-11 (pediatric 12.1%, adults11.1%), which is concordant with the predominant literature [5, 38]. However, one Egyptian study reported a higher incidence of 15-9 fusion variant [34]. Faulkner et al. and Kondo et al. have shown the presence of more than one type of fusion variant in PCA cases but none of the PCA cases of the present study showed the presence of more than one variant [23, 25]. *KIAA1549-BRAF* fusions were detected in both pediatric and adult cases ranging from 1 to 46 years of age but were significantly associated with the pediatric age

**Table 3** Frequency of *BRAF* alterations in PCAs across previous studies

| Study group | Year | Sample size | Age range | *KIAA1549:BRAF* fusion | | *BRAFV600E* mutation | |
|---|---|---|---|---|---|---|---|
| | | | | Technique used | Frequency | Technique used | Frequency |
| Horbinski et al. | 2012 | 143 | 30 days–18.8 years (median age 7.6 years) | FISH | 79.6% (90/133) | Fluorescence melting curve analysis | 9.1% (10/110) |
| Theeler et al. | 2014 | 127 | Adults 18–72 years (median age 29 years) | FISH | 20% (9/ 45) | Mass spectrometry array | 0% (0/40) |
| Roth et al. | 2016 | 116 | 5 months–23 years | SNP array, FISH, and RT-PCR | 74% (86/116) | SNP array analysis | 6% (6/103) |
| Hasselblatt et al. | 2011 | 105 | 1–74 years (median age 17 years) | RT-PCR (16-9, 15-9, 16-11) | 51% (53/105) | – | – |
| Schindler et al. | 2011 | 97 | – | – | – | | 9/97 (95) |
| Zhang et al. | 2013 | 92 | 0.5–18 years (mean age of 7.7) | Whole genome sequencing (WES) | 59% | WES | 6.0% |
| Cruz et al. | 2014 | 65 | 0–22 Years | RT-PCR | 52.3% (34/65) | Sanger sequencing | 7.7% (5/65) |
| Becker et al. | 2015 | Fusion 64; mutation 42 | 0.3 to 53.4 years (median age 9.1 years) | FISH | Pediatric 33/55; adult 4/9 | Capillary sequencing | Pediatric 2/42; adult 0/6 |
| Johnson et al. | 2017 | 46 | – | NGS | 61% (28/46) | NGS | 13.0% (6/46) |
| Tian et al. | 2011 | 41 | – | qRT-PCR (16-9, 15-9, 16-11) | 87.5% (35/40) | Pyrosequencing | 0% (0/21) |
| Antonelli et al. | 2015 | 35 | Pediatric (median age 11 years) | RT-PCR (16-9, 15-9, 16-11) | 63.6% (21/35) | – | – |
| Faulkner et al. | 2015 | 32 | 6 months to 17 years 4 months | RT-PCR (16-9, 15-9, 16-11) | 75% (24/32) | IHC | 0% (0/32) |
| Taha et al. | 2015 | 31 | 1 to 18 years (median of 7 years) | RT-PCR (16-9, 15-9, 16-11) | 74.2% (23/31) | – | – |
| Badiali et al. | 2012 | 13 | Median age 26 years | RT-PCR (16-9, 15-9, 16-11) | 46.1%/ (6/13) | – | – |
| Kondo et al. | 2018 | 10 | 3–13 years | Pyrosequencing (16-9, 15-9, 16-11) | 90% (9/10) | – | – |
| Present study | 2019 | 358 | Pediatric 0–18 (median age 8 years); adults 19–50 (median age 24 years) | RT-PCR (16-9, 15-9, 16-11) | Pediatric 41.1% (99/239); adults 25.7% (18/70) | Sanger sequencing | Pediatric 8.9% (22/246); adults 9.7% (7/72) |

group than adults, which is consistent with previous studies [16, 17, 35].

*BRAFV600E* was seen in 8.9% and 9.7% in pediatric and adult groups respectively which is consistent with the current reported literature [17, 19, 39]. A recent study has also showed the presence of novel somatic duplication mutation in *BRAF* gene (p.V504_R506dup) in five PCA cases [40]; however, the current study did not detect non-*BRAFV600E* mutations. Behling et al. and Schindler et al. have reported a high frequency of *BRAF* mutation in ≥ 30 years; however, in the present study, *BRAFV600E* was more common in < 30 years with a median age of 13 years [17, 19].

Schindler et al. [17] in their large cohort ($n = 1320$) showed an association of *BRAF* mutation with non-cerebellar origin of tumors whereas the present study did not show any significant difference of *BRAF* mutation between cerebellar (11/134, 8.2%) and non-cerebellar locations (18/186, 9.7%), while Horbinski et al. reported a higher frequency of *BRAF* mutation for cerebral hemispheric location [39]; but that was not the case in the present study; however, the number of cerebral hemispheric cases in the present study was very small ($n = 25$).

In the study under discussion, 55.6% of *BRAF* fusions in pediatric cases were cerebellar; among the three variants of fusion transcripts, fusion 16-9 was strongly associated with cerebellar location whereas fusion 15-9 showed a significant correlation with non-cerebellar location (16/21; 76.2%). The association of fusion 15-9 with non-cerebellar tumors was also demonstrated by Faulkner et al. in their study of 32 cases of PCAs [23]. Fusion 16-11 did not show any specific pattern for location.

*BRAFV600E* has been shown to be a negative prognostic marker in pediatric LGGs. Lassaletta A et al. have shown that *BRAFV600E* positive cases showed significantly poor progression-free survival (27%) as compared with *BRAF* wild type (60.2%) and showed a higher tumor recurrence rate after conventional chemotherapy [41]. However, *BRAF* fusion has been shown to be a positive prognostic marker and is dependent on the clinicopathological features such as age of patient and tumor location [42]. In addition to its diagnostic and prognostic significance, it also represents a good candidate for targeted therapy as both *BRAF* fusion and *BRAFV600E* constitutively activated the MAPK pathway. In a phase I trial of the MEK inhibitor selumetinib, Banerjee et al. demonstrated a promising effect showing 20% (5/23) of recurrent LGG children with sustained partial response. Among these 5 cases with partial response, authors could perform *BRAF* alterations in 4 and all were positive for *BRAF* alterations [43]. *BRAFV600E* has also been shown as an effective target in progressive optic pathway glioma (OPG) where biopsy and its molecular evaluation is not a routine clinical practice. Upadhyaya et al. (2018) in their case series demonstrated an excellent positive effect of vemurafenib on progressive *NF1*-negative OPG cases who did not respond to frontline chemotherapy and also highlighted a potential impact of *BRAF* alterations in OPG cases [44]. In the present study, 5.4% of cases were OPGs and 30.8% showed the presence of BRAF alterations; however, due to the small number of OPGs, this may lead to underrepresentation of the frequency. This biological implication does suggest the need for timely biopsy and the molecular evaluation in these refractory LGG cases to identify the subjects for targeted therapy. Drobysheva et al. and Miler et al. also demonstrated a positive response and improvement in cases with PCA when treated with MAPK inhibitors, thus providing supporting evidence for *BRAF* alterations as a candidate for MAPK targeted therapy [45–47]. The present study is majorly limited by the lack of clinical follow-up; thus, it does not address the issue of prognostic significance.

In summary, this is the largest study from our subcontinent, essentially aimed at determining the frequency and distribution pattern of *BRAF* alterations in PCAs across all age groups. *BRAF* alterations were seen in 53.5% pediatric and 37.3% adult PCA cases. In keeping with the previous studies, *BRAF* fusion was found to be a key event in PCA than *BRAF* mutation and was strongly associated with PCA and cerebellar location. Interestingly, no significant difference was observed for *BRAV600E* for location and age group. This study will also serve as a baseline reference for other subsequent subcontinental studies.

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## Compliance with ethical standards

**Conflict of interest** The authors declare no conflicts of interests in the study.

## Appendix. Detailed methodology of nucleic acid extraction, PCR with subsequent sequencing for *BRAFV600E* and RT-PCR for *BRAF* fusions

### DNA and RNA extraction

Selected FFPE blocks were subjected to genomic DNA and total RNA extraction from four sections each of 10 μm thickness. Sections were deparaffinized with limonene (Sigma Aldrich, USA) followed by overnight digestion. DNA was extracted using QIAamp DNA mini kit (Qiagen) as per manufacturer's instructions. Extracted DNA was checked for quality (260:280 ratio) and quantity by Nanodrop (Thermo Scientific, USA). The integrity of the DNA was assessed by

PCR for beta-actin (*ACTB*-208 bp) housekeeping gene (Table 1) and the positive samples were then subjected to PCR for *BRAF* Exon 15. Total RNA extraction was performed using RecoverAll total nucleic acid isolation kit (Ambion, Thermo Scientific, USA). RNA was quantitated using Nanodrop (Thermo Scientific, USA) and complementary DNA (cDNA) was synthesized from 100 ng RNA using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, USA). The quality of cDNA was determined using *ACTB* housekeeping gene PCR and cDNA amplifiable for *ACTB* gene were subjected to RT-PCR for *KIAA1549-BRAF* fusion.

### *BRAF* polymerase chain reaction and sequencing

Amplification was carried out in a total volume of 20 μl reaction containing 10 μl of 2× master mix (Thermo Scientific, USA), 1 μl each of 10 pmol forward and reverse primer (Table 1), and 100 ng of template DNA. PCR conditions were initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, annealing at 54 °C for 45 s, 72 °C for 45 s, and a final extension at 72 °C for 10 min. PCR products were electrophoresed on 1.5% agarose gel and purified with EXOSAP-IT (USB, Affymetrix). Direct DNA sequencing was performed on the purified PCR products using BigDye v3.1 cycle sequencing kit (Thermo Scientific, USA) followed by purification with BigDye × terminator kit. The purified products were sequenced on ABI3500 Genetic Analyzer (Thermo Scientific, USA) and sequences analyzed using Chromas Lite software and compared with the reference sequence for *BRAFV600E* mutation and other adjoining mutations.

### *KIAA1549-BRAF* fusions: reverse transcriptase–polymerase chain reaction

RT-PCR was performed for 3 different fusion transcripts; *KIAA1549-BRAF* 16-9 (124 bp), *KIAA1549-BRAF* 15-9 (159 bp) and *KIAA1549-BRAF* 16-11 (182 bp). PCR reaction contained 2 μl of template cDNA, 5 μl of 2X PCR master mix (Thermo Scientific, USA), and 0.5 μl of forward and reverse primers specific for each fusion transcript (Table 1) in a total volume of 10 μl. PCR program was as follows; 94 °C for 3 min followed by 40 cycles of 94 °C for 45 s, 57–61 °C for 45 s, and 72 °C for 45 s, and completed with an extension step at 72 °C for 10 min. Annealing temperature for *KIAA1549-BRAF* 16-9 (124 bp), *KIAA1549-BRAF* 15-9 (159 bp) and*KIAA1549-BRAF* 16-11 (182 bp) fusion transcripts were 57 °C, 59 °C, and 61 °C respectively. PCR products were separated on 10% polyacrylamide gel, stained with ethidium bromide, and visualized under UV illumination (Alpha Imager, Bioscreen, USA).

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