Mutation profiling of anaplastic ependymoma grade III by Ion Proton next generation DNA sequencing [version 1; peer review: 1 approved, 1 approved with reservations]

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Abstract

Background: Ependymomas are glial tumors derived from differentiated ependymal cells. In contrast to other types of brain tumors, histological grading is not a good prognostic marker for these tumors. In order to determine genomic changes in an anaplastic ependymoma, we analyzed its mutation patterns by next generation sequencing (NGS).

Methods: Tumor DNA was sequenced using an Ion PI v3 chip on Ion Proton instrument and the data were analyzed by Ion Reporter 5.6.

Results: NGS analysis identified 19 variants, of which four were previously reported missense variants; c.395G>A in IDH1, c.1173A>G in PIK3CA, c.1416A>T in KDR and c.215C>G in TP53. The frequencies of the three missense mutations (PIK3CA c.1173A>G, KDR c.1416A>T, TP53, c.215C>G) were high, suggesting that these are germline variants, whereas the IDH1 variant frequency was low (4.81%). However, based on its FATHMM score of 0.94, only the IDH1 variant is pathogenic; other variants TP53, PIK3CA and KDR had FATHMM scores of 0.22, 0.56 and 0.07, respectively. Eight synonymous mutations were found in FGFR3, PDGFRA, EGFR, RET, HRAS, FLT3, APC and SMAD4 genes. The mutation in FLT3 p.(Val592Val) was the only novel variant found. Additionally, two known intronic variants in KDR were found and intronic variants were also found in ERBB4 and PIK3CA. A known splice site mutation at an acceptor site in FLT3, a 3’-UTR variant in the CSF1R gene and a 5’_UTR variant in the SMARCB1 gene were also identified. The p-values were below 0.00001 for all variants and the average coverage for all variants was around 2000x.

Conclusions: In this grade III ependymoma, one novel synonymous mutation and one deleterious missense mutation is reported. Many of the variants reported here have not been detected in ependymal tumors by
NGS analysis previously and we therefore report these variants in brain tissue for the first time.

Keywords
Ependymoma, Palisading necrosis, Perivascular psuedorossettes, Ion Proton, Next Generation DNA sequencing, Glioma, pediatric brain tumors, Anaplastic Ependymoma

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**Introduction**

Ependymal cells are macroglial cells which line the ventricles, the central canal of the spinal cord and form the blood-cerebrospinal fluid barrier, being involved in producing the cerebrospinal fluid^{14}. These tumors account for only 4–8% of gliomas and, after astrocytomas and oligodendrogliomas, ependymomas are the least common^1^. Nearly one-third of brain tumors in patients younger than three years old are ependymomas and constitute around 5%–9% of all neuroepithelial malignancies^1^4. These tumors are also found in the choroid plexus and may occur at any age, from one month to 81 years and without any gender preference^1^5. In pediatric cases, the location of the tumor is intracranial, while adult ependymal tumors can have either an intracranial or a spinal localization^6^7. The prognosis is better in older children as compared to young infants but nonetheless, in children with intracranial ependymomas, event-free survival after five years is less than 50%^6^8. In adults, about 50% to 60% intracranial ependymomas are supratentorial; however, pediat- ric supratentorial ependymomas account for 25% to 35% of all ependymomas^9^8. Adults present better prognosis with a 5-year survival of around 90%, while in the pediatric population it is around 60%. The five-year survival rate for supratentorial, infratentorial, and spinal cord ependymomas is 62%, 85%, and 97%, respectively, and for grade I, II, and III spinal cord ependymomas the five-year overall survival rate is 92%, 97% and 58%, respectively^10^11^–^12.

Ependymoma tumors are well circumscribed, soft, tan-red masses and may be associated with hemorrhage. Their microscopic appearance shows hypercellularity and distinct infiltrative margins with surrounding parenchyma, consisting of monomorphi-c cells with nuclear atypia and brisk mitotic activity. They may also have intramural or glomeruloid vascular proliferation, pseudopalisading necrosis, perivascular pseudo rosettes (5–10% cases), calcifications and hyalinized vessels^1^2^3^6. Other diagnostic hallmarks include areas of fibrillary and regressive changes such as myxoid degeneration, palisading necrotic areas and the formation of true rosettes, composed of columnar cells arranged around a central lumen^16^9. Immunologically, they are positive for epithelial membrane antigen (EMA), glial fibrillary acidic protein (GFAP) and S-100. According to the 2016 updated World Health Organization (WHO) classification of brain tumors, ependymomas are divided into 4 types based on the basis of histologic appearance: (1) grade I subependymomas, (2) grade I myxopapillary ependymomas, (3) grade II ependymomas, (4) grade II or III RELA fusion-positive ependymomas and grade III anaplastic ependymomas^1^3^–^1^4^.

Previous studies have shown the use of comparative genomic hybridization (CGH) arrays to distinguish intracranial ependymomas from spinal ependymomas^1^5. In contrast to other types of brain tumors, histological grading is not a good prognostic marker for outcome for ependymomas^1^6^–^1^7^3^7. Several gene expression studies have been helpful in differentiating between intracranial and extra cranial ependymomas, but have not had clinical significance in directing therapy and their role in tumor origin and prognosis is not clear^1^8^–^1^9^4. Studies using cDNA micro-arrays have shown that gene expression patterns in ependymomas correlate with tumor location, grade and patient age^1^6^–^1^9^. Cytogenetic studies have shown that chromosomal abnormalities are relatively common in ependymomas^2^1. Loss of 22q has been the commonest abnormality found in ependymoma and, in some other tumors, gain of 1q or loss of 6q was observed^2^1^–^2^2^.

To date, there is a lack of information regarding the mutational signatures which distinguish the various subgroups of ependymomas. Another ependymoma cohort study found very few mutations and gene amplifications but a high expression of multi-drug resistance, DNA repair and synthesis enzymes^2^3. Intracranial ependymomas differ from spinal ependymomas in the expression of these proteins, and protein expression is also dependent on the ependymoma grade^2^3. For both intracranial and spinal ependymomas, very few mutations were reported by using whole exome sequencing^2^4. In another study, profiling of NGS mutations was carried out for one case of grade II ependymoma^2^5 using a GlioSeq panel, which contains a total of 30 genes. In order to determine the mutational patterns of grade III anaplastic ependymoma, we have sequenced DNA from this ependymoma tumor using the Ion Proton system for next generation DNA sequencing with the Ion Torrent’s AmpliSeq cancer HotSpot panel. This panel contains 50 genes, only 15 of which also appear in the GlioSeq panel used in previous research. These data provided an evaluation of mutational signatures of this anaplastic ependymoma which differs from the previous two studies, but confirms their conclusions about finding very few mutations in cancer driver genes, helping to direct diagnosis and therapy for ependymomal tumors.

**Methods**

**Ethical statement**

This study was performed in accordance with the principles of the Declaration of Helsinki. This study was approved by the Institutional Review Board (IRB) bioethics committee of King Abdullah Medical City (KAMC), Makkah, Kingdom of Saudi Arabia (IRB number 14-140). A written informed consent was obtained from the parent of this patient before starting the study.

**Clinical specimen**

Specimens from all patients willing to give written informed consent and diagnosed with gliomas were eligible to be included in this study. Specimens that cannot be unambiguously identified (no label or specimen number, and/or sample and requisition do not match), specimens with hematomas and blood clots, or specimens from patients who refused inclusion in the study were not eligible. The single patient’s tumor tissue (FFPE sections in PCR tubes) used in this NGS analysis was obtained from the histopathology laboratory of Al-Noor Specialty Hospital Makkah, after tumor excision and left frontal craniotomy in the neurosurgery department. The tumor was classified based upon similarity to the constituent cells of the central nervous system, such as astrocytes, oligodendrocytes and ependymal, glial cells, mitosis and cell cycle-specific antigens, used as markers to
evaluate proliferation activity and biological behavior (the WHO grading system)\(^5\). The final diagnosis was made following radiological, histopathological and immunological examinations.

**Radiology and histopathological analysis**

A CT scan of the brain was performed by a multi-slice CT (MSCT), using a 64-detector-row scanner. The use of computed tomography (CT) allowed visualization of detailed images of the soft tissues in the body in 3D as well as in multilplanar reconstructions. Images were acquired with 5mm slice thickness throughout on a GE Medical Systems, light speed VCT, 64-slice multidetector CT (MDCT). High quality images were processed at low dose performance on Volara™ digital DAS (Data Acquisition System).

The excised tumor was fixed in 4% buffered formaldehyde, routinely processed and paraffin embedded. Four-micrometer-thick sections were prepared on clear ground glass microscope slides with ground edges and routinely stained using Dako Reagent Management System (DakoRMS) with hematoxylin and eosin (H and E) on a Dako Coverstainer (Agilent). For immunohistochemistry, sections were collected on Citoglas adhesion microscope slides (Citotest). Mouse monoclonal beta-catenin (14) (Sigma-Aldrich, cat. no. 224M-1), mouse monoclonal EMA (E29) (Sigma-Aldrich, cat. no. 247M-9), rabbit monoclonal EGFR (SP84) (Cell Marque, cat. no. 41R-16-ASR), mouse monoclonal Vimentin (vim 3B4) (Ventana-Roche, cat. no. 760-2512), GFAP EP672Y rabbit monoclonal (Ventana-Roche, cat. no. 760-4345) and E-cadherin (36) mouse monoclonal (Ventana-Roche, cat. no. 790-4497) and mouse monoclonal anti-Ki-67 (Leica Biosystems, cat. no. Ki67-MM1-L-CE) antibodies were used for immunohistochemistry. Briefly, the tissue sections were deparaffinized with EZ Prep (Ventana, cat. no. 950-102) at 60°C for 1 hr. Immunohistochemistry was performed with the Ventana BenchMark XT automated stainer (Ventana, Tucson, AZ). After inactivation of the endogenous peroxidase using a UV-inhibitor for 4 min at 37°C, the primary antibody was added for 16 min at 37°C, followed by the application of HRP Universal Multimer for 8 min, and detected using the ultraView Universal DAB Detection Kit (cat. no. 760-500) for 38 min. Slides were counterstained with hematoxylin for 8 min and bluing reagent for 4 min before mounting with cover slips. Following staining, images were acquired using NIKON Digital Microscope Camera - DS-Ri1, with image software NIS Elements v.4.0. Appropriate positive controls for all of the studied antibodies were used.

**DNA isolation and NGS analysis**

DNA isolation was carried out using the QIAamp DNA FFPE Kit (50), Cat. No. 56404, 5-10 Formalin-Fixed Paraffin-Embedded sections of 5 microns were deparaffinized using xylene, treated with ethanol to remove the xylene, and the pellet was dried at 65°C for 5 mins. The pellets were resuspended in ATL buffer then treated with protease K. The remaining steps were carried out according to the user manuals. DNA concentration was measured using Nanodrop2000C and 10 ng of DNA was used for NGS analysis. DNA was sequenced using the Ion PI v3 Chip Kit (Cat no. A25771, Thermo Fisher Scientific, USA) with the Ion Proton System (Cat no. 4476610, Thermo Fisher Scientific, USA)\(^6\). Libraries were prepared using Ion AmpliSeq cancer HotSpot Panel v1 (Cat no. 4471262, Thermo Fisher Scientific, USA) primer pools. The Ion AmpliSeq Library Kit 2.0 (Cat no. 4475345, Thermo Fisher Scientific, USA) and Ion PI Hi-Q OT2 200 Kit (Cat no. A26434, Thermo Fisher Scientific, USA) was used for library and template preparation respectively. Sequencing was carried out using Ion PI Hi-Q Sequencing 200 Kit (Cat no. A26433, Thermo Fisher Scientific, USA) reagents and libraries were tagged with Ion Express Barcode Adapters 1-16, Cat. No. 4471250 (Thermo Fisher Scientific, USA). After sequencing, amplicon sequences were aligned to the human reference genome GRCh37 (hg19) (Accession no. GCA_000001405.1) in the target region of the cancer HotSpot panel using the Torrent Suite Software v.5.0.2 (Thermo Fisher Scientific, USA). Variant call format files (vcf files) were generated by running the Torrent Variant Caller Plugin v5.2. Variant calling and creation of vcf files can also be carried out using non-proprietary software such as SAMtools\(^3\) or VarScan\(^2\), which also provide coverage analysis. The vcf file data were analyzed using Ion Reporter v5.6 (ThermoFisher Scientific, USA), which calculated allele coverage, allele frequency, allele ratio, variant impact, clinical significance, PolyPhen 2 scores, Phred scored, SIFT scores, Grantham scores and FATHMM scores. This vcf file analysis was also carried out by Advaita Bioinformatics' iVariantGuide. PolyPhen2, SIFT, variant impact and clinical significance can be calculated using non-proprietary software SnpEff\(^7\) and SnpSift\(^8\). FATHMM scores can also be predicted using fathmm\(^1\) and Grantham scores according to the formula as described in Grantham, 1974\(^9\). The heat map was generated by the clustering of predicted variant impact scores by Ion Reporter v5.6. The most deleterious score was picked for every gene to generate the heat map; thereafter, hierarchical clustering was conducted. The color codes indicate the following variant impacts using score values 0-8: (0) unknown; (1) synonymous; (2) missense; (3) non-frameshift block substitution; (4) non-frameshift indel; (5) nonsense; (6) stop-loss; (7) frameshift block substitution or indel; (8) splice variant.

**Results**

**Clinical presentation and radiology**

A six-year-old female patient presented with a history of right facial palsy for few months with ataxia and right-sided weakness. The patient had a chronic headache, vomiting and had repeatedly been treated for sinusitis. Unenhanced computed tomography (CT) of the brain was performed (Figure 1, panels A, B and C). A large lesion (5.4 x 7.5cm) was noticed in the left cerebral frontoparietal region. There was an indication of a predominant cystic component and large, eccentric clump of coarse calcification. Additionally, mass effect resulting in midline shift, along with mild scalloping of the internal cortex of the parietal bone, was noted. No hydrocephalic changes or intrinsic hemorrhagic focus were seen (Figure 1).

Histopathological examination revealed sheets of neoplastic cells with round to oval nuclei and abundant granular chromatin. A variable dense fibrillary background and endothelial proliferation was also noted. Hematoxylin and eosin (H&E) staining results are shown in Figure 2 and Figure 3. Panels A and
Figure 1. Grade III ependymoma unenhanced computed tomography (CT) of the brain. A large lesion (5.4 x 7.5cm) in the left cerebral frontoparietal location with predominantly cystic components (panel A, green arrow), and a large, eccentric clump of coarse calcification (panel B; yellow arrow). Mass effect and midline shift (panel C, red arrow) can also be seen. No hydrocephalic changes or intrinsic active hemorrhagic focus were observed.

Figure 2. Hematoxylin and eosin (H&E) staining showing anaplastic ependymoma features. (A) Focal calcification areas (blue arrow), palisading necrosis (yellow arrow) and perivascular pseudo-rosettes (white arrow). (B) Pseudo palisading necrosis, characterized by a garland-like structure of hypercellular tumor nuclei (black arrow) lining up around irregular foci of tumor necrosis (blue arrow). (C) The cellular tumor exhibiting glomeruloid vascular proliferation (black arrow). (D) Extensive palisading necrosis (green arrow) and true rosettes (yellow arrow).
B of Figure 2 show the tumor exhibiting delicate cytoplasmic processes, perivascular rosettes characteristic of ependymoma, focal calcification areas and pseudo palisading necrosis, characterized by a garland-like structure of hypercellular tumor nuclei lining up around irregular foci of tumor necrosis. Panel C shows glomeruloid vascular proliferation and panel D shows extensive palisading necrosis and true rosette formation. The exhibition of a true rosette with a central lumen and the formation of pseudo-palisading necrotic areas is also clear from Figure 3 (panel A). Panel B shows focal areas with numerous tumor giant cells and the presence of a brisk mitotic activity, vascular formation and pseudo-palisading necrotic areas. Formation of true rosettes surrounding the microvascular proliferation within ependymal tumors usually signifies anaplastic transformation which is characteristic of ependymomas.

Figure 3. Hematoxylin and eosin (H&E) staining showing anaplastic ependymoma features. (A) Pseudo palisading necrotic areas, exhibiting true rosettes with central lumen (yellow arrow). (B) Focal areas with numerous tumor giant cells and the presence of a brisk mitotic activity (green arrows). (C) Tumor with vascular formation (yellow arrows) and pseudo palisading necrotic areas. (D) Formation of true rosettes (green arrows) surrounding the microvascular proliferation within ependymal tumors, usually signifies anaplastic transformation which is characteristic of ependymomas.

NGS data analysis variant identification and variant statistics
Alignment to the target regions (CHP2. 20131001.designed) of the reference genome (hg 19) was performed by the Ion Torrent Suite software v.5.0.2. For this tumor, NGS generated 6,252,341 mapped reads using the Ion PI v3 Chip, with more than 90% reads on target. Amplicon and target base read coverages for the sequencing are shown in Table 1. All 207 amplicons were sequenced with Ion AmpliSeq Cancer HotSpot Panel primer pool. As shown in Table 1, for this sample sequencing the uniformity of amplicon coverage was 95.17%, and the uniformity of base coverage on target was 94.81%. The average reads per amplicon was 34, 179, and the average target base coverage depth was 31,771. 100% of amplicons had at least 500 reads and the percentage of amplicons read end-to-end was 89.37% (Table 1).
Figure 4. Photomicrographs of Ki-67, vimentin, GFAP, and EMA immunostaining of the ependymal tumor. (A) Ki-67 immunostaining indicates a high proliferation index in the tumor (70%). (B) Vimentin stain is positive. (C) GFAP stain is positive. (D) EMA stain is positive and shows punctate cytoplasmic (perinuclear dot-like) staining, fairly diagnostic of the ependymal nature of the tumor cells.

Figure 5. Photomicrographs of beta-Catenin and E-Cadherin immunostaining of the ependymal tumor. Immunostaining is strongly positive for beta-Catenin (panel A 20x, panel B 40x) and true rosettes (red arrows) and palisading cells (blue arrow) are clearly visible. E-Cadherin stain is also positive in this tumor. Red and blue arrows indicate tumor cells arranged in true rosettes and formation of palisading structures, respectively (panel C 20x, panel D 40x).
Table 1. Coverage analysis of the tumor DNA sequencing on Ion Proton.

<table>
<thead>
<tr>
<th>Amplicon Read Coverage</th>
<th>Target Base Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of amplicons</td>
<td>207</td>
</tr>
<tr>
<td>Bases in target regions</td>
<td>22,027</td>
</tr>
<tr>
<td>Percent assigned amplicon reads</td>
<td>97.53%</td>
</tr>
<tr>
<td>Percent base reads on target</td>
<td>89.69%</td>
</tr>
<tr>
<td>Average reads per amplicon</td>
<td>34, 179</td>
</tr>
<tr>
<td>Average base coverage depth</td>
<td>31,771</td>
</tr>
<tr>
<td>Uniformity of amplicon coverage</td>
<td>95.17%</td>
</tr>
<tr>
<td>Uniformity of base coverage</td>
<td>94.81%</td>
</tr>
<tr>
<td>Amplicons with at least 1 read</td>
<td>100%</td>
</tr>
<tr>
<td>Target base coverage at 1x</td>
<td>100%</td>
</tr>
<tr>
<td>Amplicons with at least 20 reads</td>
<td>100%</td>
</tr>
<tr>
<td>Target base coverage at 20x</td>
<td>100%</td>
</tr>
<tr>
<td>Amplicons with at least 100 reads</td>
<td>100%</td>
</tr>
<tr>
<td>Target base coverage at 100x</td>
<td>100%</td>
</tr>
<tr>
<td>Amplicons with at least 500 reads</td>
<td>100%</td>
</tr>
<tr>
<td>Target base coverage at 500x</td>
<td>100%</td>
</tr>
<tr>
<td>Amplicons with no strand bias</td>
<td>97.58%</td>
</tr>
<tr>
<td>Target bases with no strand bias</td>
<td>96.35%</td>
</tr>
<tr>
<td>Amplicons reading end-to-end</td>
<td>89.37%</td>
</tr>
<tr>
<td>Percent end-to-end reads</td>
<td>86.80%</td>
</tr>
</tbody>
</table>

Initial analysis by the Ion Reporter 5.6 program found that a total of 1652 variants passed all filters. Initial analysis by Advaita’s iVariantGuide software showed 100% (1633) of variants passed all filters (see Extended data). The filter flags signify variants which do not meet certain criteria during variant calling. The flags refer to the quality or confidence of the variant call. The parameters of flags were read in from the input vcf file. If a variant passes all filters, it is marked as having passed. Six hundred and fifteen variants were identified using a filter for clinical significance that identifies drug response, likely to be pathogenic and pathogenic variants. The distribution of these variants, based on chromosomal position, region within the gene, variant class, functional class, variant impact and clinical significance, are shown in doughnut charts A – F (Figure 6). As shown in doughnut chart A, chromosome 17 has the highest number of variants (26%) and chromosome 8 has lowest number of variants (0.8%). 98.7% of variants are exonic and, according to variant class distribution, 73.8% are SNPs, 70.2% are missense variants, 25.4% are high impact variants and 46.8% are pathogenic. We have considered true mutations to be those with a Phred score above 20 and significant mutations called by Ion Reporter software were those with a p-value below 0.05.

A summary of the all missense mutations found in the grade III tumor is shown in Table 2. In this tumor, NGS data analysis identified 19 variants, four of which were missense mutations, eight were synonymous mutations and seven were intronic variants. Known missense mutation c.395G>A; p.(Arg132His) in exon 4 of the IDH1 gene, c.1173A>G; p.(Ile391Met) in exon 7 of the PIK3CA gene, c.215T>C; p.(Val72Val) in exon 11 of the KDR gene and c.257C>T; p.(Pro84Pro) in exon 4 of the TP53 gene were found in this tumor. The frequency, allele coverage, allele ratio, p-value and Phred score for these mutations is shown in Table 3. The p-values and Phred scores were significant for all of these mutations. The frequencies of the three missense mutations, namely PIK3CA c.1173A>G, KDR c.1416A>T and TP53 c.215C>G, were high, suggesting that these are germ line variants, whereas the IDH1 variant frequency was low (4.81%). As shown in Table 2, eight synonymous mutations were found in this tumor, in exon 14 of FGFR3 p.(Thr651Thr), exon 12 of PDGFRA p.(Pro356Pro), exon 20 of EGFR p.(Gln278Gln), exon 13 of RET p.(Leu769Leu), exon 2 of HRAS p.(His27His), exon 14 of FLT3 p.(Val592Val), exon 16 of APC p.(Thr1493Thr) and exon 9 of SMAD4 p.(Phe362Phe). The synonymous mutation in FLT3 (c.1776T>C; p.(Val592Val)) detected in this tumor was a novel variant, while the other variants were previously reported. Additionally, two known intronic variants were identified in KDR (c.798+54G>A and c.2615-36A>CA) (Table 2). A known splice site mutation (c.1310-3T>C) was highlighted by a Phred score of 0.94. As described in the COSMIC database, variants which do not meet certain criteria during variant calling the flags refer to the quality or confidence of the variant call. The parameters of flags were read in from the input vcf file. If a variant passes all filters, it is marked as having passed. Six hundred and fifteen variants were identified using a filter for clinical significance that identifies drug response, likely to be pathogenic and pathogenic variants. The distribution of these variants, based on chromosomal position, region within the gene, variant class, functional class, variant impact and clinical significance, are shown in doughnut charts A – F (Figure 6). As shown in doughnut chart A, chromosome 17 has the highest number of variants (26%) and chromosome 8 has lowest number of variants (0.8%). 98.7% of variants are exonic and, according to variant class distribution, 73.8% are SNPs, 70.2% are missense variants, 25.4% are high impact variants and 46.8% are pathogenic. We have considered true mutations to be those with a Phred score above 20 and significant mutations called by Ion Reporter software were those with a p-value below 0.05.

Table 2. Summary of missense mutations in the grade III tumor.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Variant Type</th>
<th>Missense Mutation</th>
<th>p-Value</th>
<th>Phred Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDH1</td>
<td>Missense</td>
<td>c.395G&gt;A; p.(Arg132His)</td>
<td>0.001</td>
<td>78.94</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>Missense</td>
<td>c.1173A&gt;G; p.(Ile391Met)</td>
<td>0.002</td>
<td>77.85</td>
</tr>
<tr>
<td>KDR</td>
<td>Missense</td>
<td>c.1416A&gt;T; p.(Pro472Pro)</td>
<td>0.003</td>
<td>76.74</td>
</tr>
<tr>
<td>TP53</td>
<td>Missense</td>
<td>c.215C&gt;G; p.(Val72Val)</td>
<td>0.004</td>
<td>75.63</td>
</tr>
<tr>
<td>FGFR3</td>
<td>Synonymous</td>
<td>c.1776T&gt;C; p.(Val592Val)</td>
<td>0.005</td>
<td>74.52</td>
</tr>
<tr>
<td>PDGFRA</td>
<td>Synonymous</td>
<td>c.125C&gt;G; p.(Pro42Pro)</td>
<td>0.006</td>
<td>73.41</td>
</tr>
<tr>
<td>EGFR</td>
<td>Synonymous</td>
<td>c.278G&gt;A; p.(Gln93Gln)</td>
<td>0.007</td>
<td>72.31</td>
</tr>
<tr>
<td>RET</td>
<td>Synonymous</td>
<td>c.769G&gt;A; p.(Leu259Leu)</td>
<td>0.008</td>
<td>71.20</td>
</tr>
<tr>
<td>HRAS</td>
<td>Synonymous</td>
<td>c.27C&gt;G; p.(His9His)</td>
<td>0.009</td>
<td>70.10</td>
</tr>
</tbody>
</table>

Discussion

Ependymomas are brain tumors that arise throughout the central nervous system, within the supratentorial areas, the posterior fossa and the spinal cord. Histologic low-grade (WHO grade I) tumors, such as subependymomas and myxopapillary ependymomas, are usually slow progressing variants of ependymomas. In contrast, grade III ependymomas display anaplastic features like hypercellularity, high mitosis, proliferation of endothelial cells and palisading necrosis. Histopathological evaluation of ependymoma tissue reveals pseudo-rosette formation, high
Figure 6. iVariant analysis of variant characteristics. Distribution of variants according to filters, showing characteristics including the relative number of variants located on each chromosome, variant class, substitution type and the functional consequences of each variant, in order to interpret and score the severity and impact of variants and therefore predict the severity of the disease. Doughnut charts in panels shows variants passed for each individual filter for (A) Chromosomal distribution, (B) Region in the gene, (C) Variant class, (D) Variant effect on the protein structure, (E) Variant impact on the protein function and (F) Clinical significance of the variants as annotated on the ClinVar database.

Table 2. Variants found in the grade III ependymoma tumor.

<table>
<thead>
<tr>
<th>Chromosomal Position</th>
<th>Ref</th>
<th>Observed Allele</th>
<th>% Frequency</th>
<th>Gene</th>
<th>Coding</th>
<th>COSMIC/dbSNP</th>
<th>AA Change</th>
<th>Exon</th>
</tr>
</thead>
<tbody>
<tr>
<td>chr2:209113112</td>
<td>CG</td>
<td>TG</td>
<td>4.81</td>
<td>IDH1</td>
<td>c.395G&gt;A</td>
<td>COSM28746</td>
<td>p. (Arg132His)</td>
<td>4</td>
</tr>
<tr>
<td>chr2:212812097</td>
<td>T</td>
<td>C</td>
<td>100</td>
<td>ERBB4</td>
<td>c.421+58A&gt;G</td>
<td>rs839541</td>
<td>p.?</td>
<td></td>
</tr>
<tr>
<td>chr3:178917005</td>
<td>A</td>
<td>G</td>
<td>47.97</td>
<td>PIK3CA</td>
<td>c.352+40A&gt;G</td>
<td>rs372674</td>
<td>p.?</td>
<td></td>
</tr>
<tr>
<td>chr3:178927410</td>
<td>A</td>
<td>G</td>
<td>54.25</td>
<td>PIK3CA</td>
<td>c.1173A&gt;G</td>
<td>COSM328028</td>
<td>p. (Lle391Met)</td>
<td>7</td>
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<tr>
<td>chr4:1807894</td>
<td>G</td>
<td>A</td>
<td>100</td>
<td>FGFR3</td>
<td>c.1953G&gt;A</td>
<td>rs7688609</td>
<td>p. (Thr651Thr)</td>
<td>14</td>
</tr>
<tr>
<td>chr4:55141050</td>
<td>AGGCCAGA</td>
<td>AGCCCGGA</td>
<td>100.00</td>
<td>PDGFA</td>
<td>c.1701A&gt;G</td>
<td>rs1873778</td>
<td>p. (Pro566Pro)</td>
<td>12</td>
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<tr>
<td>chr4:55962545</td>
<td>T</td>
<td>TG</td>
<td>43.46</td>
<td>KDR</td>
<td>c.2615-36A&gt;CA</td>
<td>rs34085292</td>
<td>p.?</td>
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<tr>
<td>chr4:55972974</td>
<td>A</td>
<td>G</td>
<td>51.35</td>
<td>KDR</td>
<td>c.1416A&gt;T</td>
<td>rs1870377</td>
<td>p. (Gln472His)</td>
<td>11</td>
</tr>
<tr>
<td>chr4:55980239</td>
<td>C</td>
<td>T</td>
<td>98.35</td>
<td>KDR</td>
<td>c.798+54G&gt;A</td>
<td>rs7692791</td>
<td>p.?</td>
<td></td>
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<tr>
<td>chr5:112175769</td>
<td>CGG</td>
<td>CAG</td>
<td>100</td>
<td>APC</td>
<td>c.4479G&gt;A</td>
<td>COSM3760869</td>
<td>p. (Thr1493Thr)</td>
<td>16</td>
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<tr>
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<td>TG</td>
<td>GA</td>
<td>100</td>
<td>CSF1R, HMGXB3</td>
<td>c.*1841TG&gt;GA, c.2954_2955delCAinsTC</td>
<td>rs2066934</td>
<td>p.?</td>
<td></td>
</tr>
<tr>
<td>chr7:55249063</td>
<td>G</td>
<td>A</td>
<td>71.04</td>
<td>EGFR, EGFR-AS1</td>
<td>c.2361G&gt;A</td>
<td>rs1050171</td>
<td>p. (Gln778Gln)</td>
<td>20</td>
</tr>
<tr>
<td>rs1800861</td>
<td>G</td>
<td>T</td>
<td>100</td>
<td>RET</td>
<td>c.2307G&gt;T</td>
<td>COSM4418405</td>
<td>p. (Leu769Leu)</td>
<td>13</td>
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<tr>
<td>chr11:534242</td>
<td>A</td>
<td>G</td>
<td>49.07</td>
<td>HRAS</td>
<td>c.81T&gt;C</td>
<td>rs12628</td>
<td>p. (His27His)</td>
<td>2</td>
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<tr>
<td>chr13:28608280</td>
<td>A</td>
<td>G</td>
<td>53.5</td>
<td>FLT3</td>
<td>c.1776T&gt;C</td>
<td>Novel</td>
<td>p. (Val592Val)</td>
<td>14</td>
</tr>
<tr>
<td>chr13:28610183</td>
<td>A</td>
<td>G</td>
<td>100</td>
<td>FLT3</td>
<td>c.1310-3T&gt;C</td>
<td>rs2491231</td>
<td>p.?</td>
<td></td>
</tr>
<tr>
<td>chr17:7579472</td>
<td>G</td>
<td>C</td>
<td>47.94</td>
<td>TP53</td>
<td>c.215C&gt;G</td>
<td>rs1042522</td>
<td>p. (Pro72Arg)</td>
<td>4</td>
</tr>
<tr>
<td>chr18:48591923</td>
<td>T</td>
<td>C</td>
<td>63.63</td>
<td>SMAD4</td>
<td>c.1086T&gt;C</td>
<td>rs1801250</td>
<td>p. (Phe362Phe)</td>
<td>9</td>
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<tr>
<td>chr22:24176287</td>
<td>G</td>
<td>A</td>
<td>52.5</td>
<td>DERL3, SMARCB1</td>
<td>c.1119-41G&gt;A, c.*727C&gt;T</td>
<td>rs5030613</td>
<td>p.?</td>
<td></td>
</tr>
</tbody>
</table>
mitotic activity, vascular proliferation and necrosis, EMA staining with perinuclear dot-like structures and with diffuse GFAP immunoreactivity. Immunological staining with GFAP and vimentin is very helpful for the differential diagnosis of ependymomas from other non-ependymal tumors, such as astrocytic and choroid plexus tumors, and also in differentiating between the various grades of ependymomas. It has been reported that the GFAP expression correlates with a loss of E-cadherin expression in anaplastic ependymomas, although in this case there was E-cadherin expression. Changes in E-cadherin expression promote tumor invasion and metastasis. Overexpression of EGFR is known to correlate with tumor grades in ependymomas (100%, 50%, and 0% in grade I, II and III, respectively). The tumor in our case is grade III anaplastic ependymoma and it stained negatively for EGFR, confirming this observation.

Based upon the expression profiles of numerous angiogenesis genes (HIF-1a signaling, VEGF signaling, cell migration) and signaling pathway genes (PDGF signaling, MAPK signaling, EGFR signaling), posterior fossa ependymomas are subdivided into two groups. In this case, a diagnosis of anaplastic ependymoma (WHO grade III) was made upon the observation of the above characteristics for the tumor. The pathology of the resected tissue demonstrated a hypercellular tumor with areas of perivascular pseudo rosettes, consistent with a diagnosis of ependymoma.

Despite several investigations, the correlation between histological grading of ependymoma tumors and their prognosis is unclear. Apart from histopathological grading, previous studies have focused on gross deletions and chromosomal abnormalities through cytogenetic studies and array-CGH profiling of ependymomas. These studies helped to distinguish between intracranial and spinal cord ependymomas. Around 70% of supratentorial ependymas are known to carry a fusion gene which produces the C11orf95/RELA fusion transcript and the prognosis is poor for this tumor. Ion Torrent PGM sequencing of a grade II ependymoma demonstrated MET and ATRX copy number gain. Overexpression of L1 cell adhesion molecule (L1CAM), 1q25 copy number gain and a homozygous deletion in CDKN2A was also reported in some aggressive supratentorial
ependymomas. However, in the present case we did not detect any MET or CDKN2A mutations using the Ion AmpliSeq Cancer HotSpot panel.

Patients with neurofibromatosis type 2 are predisposed to the development of ependymomas, and the gene for neurofibromatosis type 2 (NF2) maps to chromosome 22 (q1216,17). Mutations in the NF2 gene are uncommon in sporadic ependymomas and appear to be restricted to spinal tumors. For spinal cord ependymomas, four out of eight tumors were found to have an NF2 mutation and all eight tumors had loss of heterozygosity (LOH) of chromosome 22, where the NF2 locus is found. However, five out of eight intracranial tumors exhibited LOH of chromosome 22 but no NF2 mutations. A high rate of truncating mutations such as nonsense and frameshift mutations in the NF2 gene were also reported previously in spinal ependymomas. Unfortunately, in the Ion AmpliSeq cancer HotSpot panel primer pool used in the present study NF2 gene was not included.

We have verified all mutations in various databases (COSMIC, ExAc and dbSNP) to confirm whether variants are novel. Only one detected in our case, a synonymous variant found in FLT3 (c.1776T>C; p.(Val592Val), is a novel variant. The IDH1 mutation c.395G>A; p.(Arg132His) we detected in this tumor is a substitution missense mutation which has been reported previously (COSM28746) in glioma tumors. In this codon, another missense G>T mutation (COSM28750), and a compound substitution c.394_395CG>GT (COSM28751) are also known. Somatic IDH1 mutations in this codon have been found with greater frequency in diffuse astrocytomas, oligodendrogliomas, oligoastrocytomas and secondary glioblastomas. However, several grade II and grade III ependymal tumors tested did not show this mutation in the IDH1 gene. For astrocytic tumors, the presence of this mutation is known to be associated with younger patients. This observation supports our findings for this ependymoma tumor as the patient is six years-old. This mutation is pathogenic, having a FATHMM score of 0.94. Other variants detected in this tumor, such as those in FGFR3, PDGFRA, KDR (c.1416A>T), CSF1R, EGFR, RET, HRAS, PIK3CA, FLT3 (c.1310-3T>C), and SMAD4, are benign. Variants detected in this tumor have also been reported in other cancers: PDGFRA mutations in cervical adeno-squamous cell carcinoma.
carcinomas; ERBB4 mutations in lung adenocarcinomas; FGFR3 mutations in breast, endometrial and ovarian cancers; CSF1R mutations in prostate cancer; EGFR mutations in lung adenocarcinomas; RET mutations in thyroid carcinomas; HRAS mutations in melanomas; and SMAD4 mutations in breast cancer. However, with the exception of the KDR variant c.1416A>T, this is the first time the above variants are reported in a brain tumor.

We found an intronic variant in PIK3CA and one missense mutation in this gene. This missense mutation was also reported previously in hemangioblastoma and in colon adenocarcinomas. Missense mutations in PIK3CA are known to promote glioblastoma tumor progression. Mutations of the PTEN gene are rare in ependymomas and we have also not detected any PTEN mutations in this tumor.

Mutations in cancer driver genes such as TP53, CDKN2A, and EGFR, which are frequently affected in gliomas, have been shown to be rare in ependymomas[20,21]. We have detected a TP53 mutation (c.215C>G, p.Pro72Arg, rs1042522) in this tumor with a frequency of 47.94%. This mutation p.(Pro72Arg) has also been reported previously in a medulloblastoma tumor in a young patient[22]. Previous studies have shown that out of 15 ependymoma tumors tested, only one case, a patient with a malignant ependymoma of the posterior fossa, had a mutation in exon 6 of the TP53 gene, which was silent, and in another study only one out of 31 ependymoma tumors tested contained a mutation in the TP53 gene[23,24]. However, in another study, out of 15 ependymoma tumors, none had a mutation in the TP53 gene, suggesting that this gene does not play an important role in the pathogenesis and development of ependymomas, unlike other brain tumor types[25,26]. Miller et al., (2018) through whole-exome sequencing of an anaplastic ependymoma tumor, have shown mutations in several cancer-related genes, as well as genes related to metabolism, neuro-developmental disorder, epigenetic modifiers and intracellular signaling[27]. These authors have shown resistance-promoting variant expression in a single ependymoma case at different stages of recurrence. However, these genes were not present in the cancer panel we used in this study. Using the human exome capture on Illumina, Bettegowda et al., (2013) have reported that in one out of eight grade III intracranial ependymomas, tumors have mutations in PTEN and TP53, and one tumor with HIST1H3C mutations[28]. The HIST1H3C p.(Lys27Met) mutation has also been reported previously in posterior fossa ependymomas[29]. Ependymomas may in fact represent a very heterogeneous class of tumors, each with distinct molecular profiles and, even within posterior fossa ependymomas, there are at lest two distinct gene expression patterns, as demonstrated by Witt et al., (2011)[20,21]. Overall, in previous studies, a very low frequency of mutations was observed in both intracranial and spinal ependymomas and our findings also supports this observation.

The Ion AmpliSeq Cancer HotSpot Panel consists of 207 primers in 1 tube, targeting 50 oncogenes and tumor suppressor genes that are frequently mutated in several types of cancers. The detected mutations were found to have high accuracy; 100% amplicons had at least 500 reads and 500x target base coverage was also 100%. This high level of accuracy and the high depth of coverage achieved with the Ion Proton system allowed us to reliably detect low frequency mutations with high confidence. Allele coverage in most of the variants is around 2000x, the p-value was 0.00001 and the Phred score was very high for all the variants, indicating high confidence in the variants found in this tumor. Apart from its use in whole-exome sequencing, cancer panel analysis has also become common practice for Ion Proton[30]. The Ion Proton instrument has the advantage of pooling samples using barcodes and the Ion PI chip. For pooled samples, sequencing enables a high throughput up to 15 Gb of data, with more than 60–80 million reads passing read filtering. The purpose of read filtering is to discard the reads that contain low quality sequences, to remove polyclonal reads, remove reads with an off-scale signal, remove reads lacking a sequencing key, remove adapter dimers, and remove short reads etc. If the computed mean read length from all the reads and the minimum total mapped reads in the sample is less than the specified threshold, then sample does not pass the quality control.

Recent molecular diagnostics research had helped in subdividing glioblastomas, oligodendrogliomas and oligoastrocytomas into genetically diverse groups of tumors, and these mutational markers may help in predicting the prognosis and response to therapy[31]. However, such a strategy for the molecular subdivision of ependymomas has been not successful so far using mutational profiling. Epigenetic markers and fusion protein analysis have also helped in identifying new groups of supratentorial ependymomas tumors and in spite of the histopathological signs of malignancy, a small set of ependymomas had a very good prognosis, suggesting that this subgroup of tumors should not be diagnosed as classic ependymomas. However, another study showed that methylation profiling did not identify a consistent molecular class within the supratentorial tumors, but successfully sub-classified posterior fossa ependymoma into two subgroups[32].

In conclusion, we have identified four known missense mutations, eight synonymous and seven intronic, in this grade III ependymoma. Out of these, only one mutation in FLT3 (c.1776T>C, synonymous) is novel, and only one mutation in IDH1 (c.395G>A, missense) is deleterious, with all other mutations benign. Many of the variants we reported here were not detected in the ependymal tumors analyzed by NGS previously. HRAS c.81T>C, PIK3CA c.1173A>G, RET c.2307G>T, KDR c.1416A>T, APC c.4479G>A, EGFR c.2361G>A and FLT3 c.1310-3T>C variants have not been previously reported in brain tissue, as verified in COSMIC data base, although they have been reported in other tissues like lung and breast. Further studies are warranted, using NGS methods in all three grades of intracranial ependymomas to identify the genetic signatures that may distinguish between these tumors at the molecular genetic level.

Data availability
Underlying data
Raw sequence reads for this tumor on Sequence Read Archive, Accession number SRP192752: https://identifiers.org/insdc.sra/SRP192752
Open Science Framework: Mutation profiling of anaplastic ependymoma grade III by Ion Proton next generation DNA sequencing. https://doi.org/10.17605/osf.io/y9sfg

This project contains the following underlying data:

- All variants before filtration.xlsx (spreadsheet of all annotated variants)
- Final Variant Calls using HotSpot filter.xlsx (spreadsheet of annotated variants called using HotSpot filter)
- TSV_C_variants_IonXpress_013.vcf (file containing all variants (un-annotated) in vcf format)
- TSV_C_variants_IonXpress_013.vcf.gz (file containing all variants (un-annotated) in vcf.gz format)
- heatmap-gr3 ion rep.csv (spreadsheet containing impact scores used to generate heat map)
- EGFR Figure neg.jpg (images for EGFR staining)
- Fig2A.jpg – Figur4D.jpg (raw image files used in Figure 2—Figure 5)
- Radiology Fig.1.jpg – Radiology Figure 1 (2).jpg (raw image files used in Figure 1)

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Extended data

Open Science Framework: Mutation profiling of anaplastic ependymoma grade III by Ion Proton next generation DNA sequencing. https://doi.org/10.17605/osf.io/y9sfg

This project contains the following extended data:

- Gr-3 Advaia Final report.pdf (Advaia iVariant analysis report)

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Grant information

This study is supported by a grant from The Deanship of Scientific Research, Umm-Al-Qura University, Makkah to Dr. MM. Taher (Code. No. 43509008).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

We appreciate the technical help of Mrs. Rowa Abbas Bakhsh, Histopathology Division, Al-Noor Hospital, Makkah. The authors would like to thank the staff of Science and Technology Unit at Umm Al-Qura University for the continuous support.

References

Ependymoma. Is 11q13 a recurring breakpoint in ependymomas?

Intracranial ependymomas in children.

Systematic comparison of amino acid substitutions using hidden Markov Model for Genotoxic Chemical Mutational Studies with a New Program.

Targeted next generation sequencing of solid tumors in a clinical laboratory.

Targeted next-generation sequencing of selected cancer-associated genes in resected adenocarcinomas: the role of EGFR, PDGFRA and VEGFR2 in cervical adenosquamous carcinoma.

Immunohistochemistry and associations in a series of brain tumors.

Analysis of chromosome arm 17p and chromosome arm 22q DNA sequences embedded in formalin-fixed paraffin-embedded lung adenocarcinoma samples.

Identification of germline alterations in the IDH1 (K130, R132) and IDH2 (R172) genes in sporadic gliomas.


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Metzger AK, Sheffield VC, Duyk G, et al.: Identification of a germ-line mutation in...
Open Peer Review

Current Peer Review Status: ?

Version 1

Reviewer Report 26 February 2020

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Firoz Ahmad
Research and Development Division, SRL Ltd, Mumbai, Maharashtra, India

The authors presented report on their NGS study on anaplastic ependymoma grade III by Ion Proton next generation DNA sequencing.

The article looks interesting, and is well written. Since this report is from a single case, a necessary correction has to be made in the material section. Also, the authors need to mention the tumor content of the FFPE block which was taken for the NGS analysis.

I feel, the author need to cut down on the text significantly while explaining NGS data analysis and variant identification statistics. There is too much of information which is not required. The authors have already shared the coverage analysis report which is sufficient.

Except for IDH1 mutation, other variants are non significant and hence do not add much value clinically. Identification of FLT3 p.(Val592Val) does not carry any meaning unless and until the authors show the functional impact of this variant.

Overall, good article but the text needs to be significantly cut down considering this to be a single case report.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes
Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Molecular Diagnostics, Cancer Genetics, Next Generation Sequencing.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

---

**Reviewer Report 17 October 2019**

https://doi.org/10.5256/f1000research.20502.r53778

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**Luni Emdad**
Department of Human and Molecular Genetics, School of Medicine, Virginia Commonwealth University, Richmond, VA, USA

In this study authors analyzed mutation patterns by next generation sequencing (NGS) in order to determine genomic changes in an anaplastic ependymoma. Authors identified one novel synonymous mutation and one deleterious missense mutation in this grade III ependymoma.

**Comments:**

1. In the clinical specimen section authors state “Specimens from all patients willing to give written informed consent and diagnosed with gliomas were eligible to be included in this study.” However, the specimen used in this study for NGS analysis was obtained from a single patient’s tumor tissue. What are the other samples and in what study they were used?

2. Figure 2A: authors indicate yellow arrow showing palisading necrosis; is this the correct location?

3. Authors conclude they identified four known missense mutations, eight synonymous and seven intronic, in this grade III ependymoma. How global these signature molecules in the context of grade III ependymoma? Can the authors add additional correlation analysis by analyzing TCGA or other bioinformatics based data?

4. Authors indicate FLT3 (c.1776T>C, synonymous) is novel, which is interesting; is this mutation reported in other cancer than ependymoma? What is the clinical and functional significance of this mutation in ependymoma progression?

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
No source data required

Are the conclusions drawn adequately supported by the results?
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Cancer Biology and Molecular Oncology, Genetics, Targeted experimental therapeutics development

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

---

**Author Response 22 Oct 2019**

Mohiuddin Taher, Umm-Al-Qura University, Makkah, Saudi Arabia

**1st Reviewer’s Comments:**
We are thankful to this reviewer (Dr. Luni Emdad, Virginia Commonwealth University, Richmond, USA) for her helpful suggestions and comments. Our answers to the comments are listed below.

**Comment:**
1. In the clinical specimen, section authors state “Specimens from all patients willing to give written informed consent and diagnosed with gliomas were eligible to be included in this study.” However, the specimen used in this study for NGS analysis was obtained from a single patient’s tumor tissue. What are the other samples and in what study they were used?

**Answer:**
The present study is a part of the project on mutation profiling of brain tumors in the Saudi population which is supported by a grant from Umm-Al-Qura University (Code. No. 43509008). For this project, various brain tumors were collected such as GBMs all grades, pilocytic astrocytomas, medulloblastomas, craniopharyngiomas, and meningiomas, etc., also. The NGS analysis is ongoing for many other tumors, the authors like to publish first this grade III ependymoma NGS analysis.

We have made general statements in this manuscript to describe the inclusion and exclusion criteria for the patient’s selection. As the present communication deals with a single case, this statement will be deleted or will be changed whichever is appropriate in the revised manuscript.
Comment:
2. Figure 2A: Authors indicate yellow arrow showing palisading necrosis; is this the correct location?

Answer:
As suggested by the reviewer the corrections are made in this figure, and new figure-2 and figure legend will be added at the time of the revision.

Comment:
3. Authors conclude they identified four known missense mutations, eight synonymous and seven intronic, in this grade III ependymoma. How global these signature molecules in the context of grade III ependymoma? Can the authors add additional correlation analysis by analyzing TCGA or other bioinformatics-based data?

Answer:
As suggested by the reviewer we have searched various databases including TCGA, and summarize our answer to the reviewer's comments as below. A relevant section of this summary will be included in the final revision of the manuscript.

In the TCGA projects (https://www.intogen.org/search?cancer) genes such as, TP53, IDH1, KDR, and EGFR in GBM, TP53, IDH1, PIK3CA, and EGFR in Low-Grade Glioma, TP53 and PIK3CA in medulloblastoma are detected as a mutational cancer driver genes.

In the NCI's Genomic Data Commons (GDC) portal (https://portal.gdc.cancer.gov/) 19,144 cases are reported in 4 projects of gliomas. Only in Brain projects, for IDH1 6 mutations were found in 423 cases, 369 cases were affected with 239 mutations in TP53; 118 cases were affected with 59 mutations in PIK3CA; 90 mutations in 283 cases were found in EGFR. 0 mutations in 57 cases in HRAS, 38 cases with 20 mutations in RET, and in FGFR3 40 cases with 8 mutations, 32 cases with 15 mutations for ERBB4, in APC 33 mutations in 38 cases, in PDGFRA 110 cases with 45 mutations, in SMAD4 23 cases with 3 mutations, in SMARCB1 24 cases with 6 mutations, in CSF1R 35 cases with 16 mutations, in KDR 102 cases with 28 mutations, in FLT3 63 cases with 20 mutations.

In the TCGA database the missense IDH1 mutation p.(R132H) affected cases are 90.07% (381/423) VEP impact (Ensembl database) is moderate for this, and Shift impact is deleterious low confidence (score 0.01), and the PolyPhen impact is also possibly damaging (score 0.813).

In the NCI's Genomic Data Commons (GDC) 423 cases (36.94%) were affected in the IDH1 gene out of 1137 cases. IDH1 c.395G>A p.(R132H) mutation (rs121913500), Clin variant database, #VCV000156444 variant is reported in 2 cases of oligodendroglioma grade II, astrocytoma grade IV respectively (PUBMED ID 28125199). Also, this variant is reported in 2 cases of anaplastic ependymoma in this database. This variant also reported in AML as an adverse prognostic factor (PUBMED ID 20368538).

IDH1/IDH2 but not TP53 mutations predict prognosis in glioblastoma patients (PUBMED ID 24868540). The variant we found in the present ependymoma case such as in TP53, HRAS, SMAD4, PIK3CA not in TCGA. However, in the ClinVar database SMAD4 synonymous variant is reported (Accession: VCV000132693.2, Variation ID: 132693).

TP53 mutations in GBM mostly point mutations that lead to a gain of function (GOF) of the oncogenic variants of the p53 protein (Zhang Y, et al., 2018). The TP53 variant rs1042522 we reported in the present case [(c.215C>G, p. (Pro72Arg))] was also reported in anaplastic astrocytoma grade-III (Pessoa IA, et al., 2019), but not in ependymoma cases. In the COSMIC database, this variant is reported in several types of cancers. A mutation in Exon 5 of the TP53
gene was reported in one anaplastic ependymoma out of three cases (Tominaga T, et al., 1995). Whereas in our case the mutation was found in exon 4.

Targeted therapy is being studied for the treatment of childhood ependymoma and other brain tumors utilizing the genomic data. NCI supported Clinical Trials for Ependymomal brain tumors 4 clinical trials are listed (https://www.cancer.gov/types/brain/patient/child-ependymoma-treatment-pdq). One study is enrolling the patients for drug targets (Carboplatin and Bevacizumab for Recurrent Ependymoma) that inhibit VEGF-promoted angiogenesis. Based on the interesting results observed in the reported small series of patients with recurrent ependymomas treated with bevacizumab, as well as on the evidence of VEGF-promoted angiogenesis in these tumors, we designed a phase II study to test the efficacy of bevacizumab in patients with recurrent ependymoma. Cabozantinib, a multi-kinase inhibitor of FLT3, MET, VEGFR2, and KIT, respectively, and clinical trials are undergoing for Non-Small Cell Lung Cancer, thyroid cancer, AML, and GBM treatment.

Amplification of PDGFRA, VEGFR2 (KDR), and EGFR in gliomas are reported (Puputti et al., 2006), and VEGFR2 plays a key role in neovascularization and tumor initiation by glioma stem-like cells (Yao et al., 2013).

We have searched The NCI's Genomic Data Commons (GDC) and found 28 mutations in 102 affected cases with KDR mutations in all TCGA GBM projects. The expression of the KDR gene is increased in endothelial cells during tumor angiogenesis, and missense mutations cause constitutive activation of VEGFR2 in hemangioma. Patients with infantile capillary hemangioma are known to have constitutive activation of VEGFR2 signaling and carry a germline mutation (C482R) in the KDR gene (Jinnin, et al., 2008).

In non-small cell lung cancer patients, a SNP (Q472H), is associated with increased VEGFR2 activity, and it was correlated with increased microvessel density (Glubb DM et al., 2011). This missense mutation is observed in the present ependymoma case also by us, this missense mutation was not known in ependymomas previously. This mutation is reported in colorectal cancer, melanoma, non-small cell lung cancer, and it's an important target for drugs like Avastin (Bevacizumab), Afibircept, and drugs reported in the database at (http://atlasgeneticsoncology.org/Genes/GC_KDR.html).

FLT3 is also expressed in the human brain, though its activating mutations were found mostly in AML (Metzeler KH, et al., 2016). Several insertions, missense, and duplication mutations are known in AML at Val592 codon, for example, FLT3 missense mutation, c.1775T>A p.(V592D) is a pathogenic one in AML. However, in the present ependymoma case, we have observed a mutation in c.1776T>C p. (Val592Val) is a synonymous one. One study reported that this gene is down-regulated and it's associated with favorable clinical outcomes in glioma patients, thus this driver gene might be potential prognostic biomarkers for glioma patients. (Liang A., et al., 2017). In another study, it was reported that two target genes (FLT1, FLT3) of the experimental drug sorafenib were recurrently deleted, whereas another target (KDR) of sorafenib was recurrently amplified in glioblastoma multiforme. (Tran HV, et al., 2018).

The FLT3 intronic variant c.1310-3T>C (rs2491231) was identified in 84% of triple-negative breast cancer cases (Usangca-Perales et al., 2019). This variant was not reported in ependymomas previously, this is the first time we report it here.

SMARCB1 mutation in c.1119-41G>A (rs5030613) is reported in Schwannomatosis. This disease is the third major form of neurofibromatosis, clinically and genetically distinct from
neurofibromatosis type 1 (NF1) and neurofibromatosis type 2 (NF2). SMARCB1 germline mutations contribute to 10% of sporadic schwannomatosis (Rousseau et al., 2011). Patients with neurofibromatosis type 2 are predisposed to the development of ependymomas, and the NF2 and SMARCB1 genes map to chromosome 22. However, this SNP rs5030613 was not reported previously in ependymomas.

Lo Iacono M, et al., (2016) have reported the same mutations we found in ependymoma case viz., in TP53 p.(P72R), KDR p.(Q472H), PIK3CA p.(I391M), and a SNP in CSFR1 (rs2066934), in prostate cancer tumor also, and these variants were shown to play a role in progression and aggressiveness of the tumor.

SMAD4 mutation in c.1086T>C (rs1801250) reported in breast cancer (Tram E, et al., 2011); HRAS mutation in c.81T>C rs12628, reported in Chronic Myeloid Leukemia (Mir R, et al., 2015), PIK3CA mutation in c.352+40A>G, rs3729674 in breast cancer (Arsenic et al., 2015); ERBB4 mutation in c.421+58A>G, rs839541 in brain tissue (Mothersill O et al., 2012).

Ion Torrent software analysis uses a variety of information from external sources to interpret variants and gives data about each variant. The following annotation sources were used in this analysis by Ion Reporter; Prediction of the functional effect of a variant on a protein SIFT; Prediction of the functional effect of a variant on a protein PolyPhen2; Prediction of the functional consequences of a variant, FATHMM; Protein domain families in the coded protein PFAM; Single Nucleotide Polymorphism database dbSNP; Database of Genomic Variants: A curated database of human genomic structural variation DGV; UCSC Common SNPs; Exome Aggregation Consortium—Database catalog of variant frequencies ExAC; Catalog of somatic mutations in tumor tissue COSMIC; Standardized ontology for gene and gene products, for example, functional role or localization, Gene Ontology; Disease Research Area DRA; List of drugs known to target the gene(s) affected by the variant DrugBank; Assessment of the impact of the variant observed from NCBI ClinVar database ClinVar; Population frequency information from the 5000 exomes project, (NHLBI ESP); and Population frequency information from the 1000 genomes project MAF. All these analysis data are shown in the Excel file uploaded in the supplementary data section ([Final Variant Calls using HotSpot filter.xlsx (spreadsheet of annotated variants called using HotSpot filter)]).

References:


https://www.cbioportal.org/results/legacy_submission


Comment:
4. Authors indicate FLT3 (c.1776T>C, synonymous) is novel, which is interesting; is this mutation reported in other cancer than ependymoma? What is the clinical and functional significance of this
mutation in ependymoma progression?

**Answer:**

FLT3 is also expressed in the human brain, though its activating mutations were predominant in acute myeloid leukemia (AML). It is known that high expression of FLT3 is a risk factor in leukemia, it has been reported the most common form of FLT3 mutation is in internal tandem duplication (ITD) region of the juxta-membrane domain, which occurs in 15–35% of patients with acute myeloid leukemia (AML). In the database of Genomics of Drug Sensitivity in Cancer, https://www.cancerrxgene.org/compound, Mutations in FLT3 are associated with altered sensitivity to the following 5 drugs: CX-5461, Elesclomol, Linifanib, Quizartinib, and Cabozantinib, and these drugs are tested in several cell types, viz., neuroblastoma, glioma, lung NSCLC adenocarcinoma, and melanoma. In AML patients with FLT3 mutations ‘Gilteritinib,’ targeted therapy showed promising results (https://www.medscape.com).

This driver gene (FLT3) down-regulation is related to favorable clinical outcome in glioma patients. This might be a potential prognostic biomarker for glioma patients also. Interestingly, several mutations were reported at Val592 coding positions c.1774 and c.1775 in the COSMIC database but all in AML. However, the FLT3 synonymous mutation found in our case [c.1776T>C; p. (Val592Val)] within the juxta-membrane domain in exon 14, which codes the same amino acid valine, hence we don’t anticipate to have any functional and clinical impact of this mutation. In 922 glioma cases tested FLT3 mutations found in only 26 samples including ependymoma cases also, and a different mutation in Val592 codon [c.1774G>A; p.(V592I)] was found in 2 cases of Astrocytoma Grade IV. But the novel FLT3 mutation described in this manuscript was not reported.

**References:**


**Competing Interests:** The authors declare no competing interests.
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