Activating *ACVR1* mutations in posterior fossa ependymoma

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Molecular profiling of ependymal tumors has resolved a previously histologically defined entity into clinically meaningful tumor types driven by gene fusions, copy number variants, and epigenetic alterations. Moreover, the distinct relationship between neuroanatomic compartment (i.e., supratentorial, posterior fossa, spine), molecular alteration, and clinical outcome[10] now forms the basis for their classification in the 2021 WHO “Blue Book”[6]. In contrast to other CNS tumors, recurrent mutations are uncommon in ependymal tumors, but instead harbor specific gene fusions (e.g., *ZFTA*, *YAP1*) or copy number alterations (*MYCN* amplification, chromosome 6 loss). In posterior fossa (PF) ependymomas, recurrent somatic alterations are particularly uncommon[7, 8, 10]. However, a recent study demonstrated that a small subset of posterior fossa group A (PFA) ependymomas harbor recurrent missense mutations in the *EZHIP*/*CXorf67* gene[9]. Histone H3 K27M mutations, seen in ~~the majority of~~most diffuse midline gliomas (DMG), have also been detected in a small proportion of PFA ependymomas[4, 9]. *TERT* promoter mutations were recently shown to identify a group of clinically aggressive posterior fossa ependymal tumors with hybrid histologic and epigenetic features of ependymoma and subependymoma (EPN/SE)[13]. Exceptionally, *ZFTA* (*C11orf95*) fusions have also been detected in PF ependymomas[5]. The lack of recurrent alterations in PF ependymomas has likely hindered the development of effective targeted therapies. Here, we report the occurrence of heterozygous coding mutations in the activin receptor type I (*ACVR1*) gene in a rare subset of PF ependymomas. Furthermore, we show that, in the context of retained H3K27me3 expression, *ACVR1*-mutant PF ependymomas exhibit a methylation signature distinct from other PF ependymomas.

We initially identified an uncharacterized group of ependymal tumors (n=7) through unsupervised clustering of DNA methylation array data from approximately 16,000 CNS tumors. Sequencing of tumors from this group revealed pathogenic *ACVR1* missense mutations in all cases for which sufficient material was available (n=6) (Fig. 1a, herein referred to as PF-ACVR1). Evaluation with the Heidelberg classifier (v11b4/12.5) failed to match these tumors to a known DNA methylation class (calibrated score < 0.9). We also identified two methylation-defined PFA ependymomas harboring oncogenic *ACVR1* mutations (Fig. 1a, PFA-ACVR1). Clustering analysis of PF-ACVR1 among established PF ependymal tumor types (PFA, PFB, EPN/SE) revealed a distinct DNA methylation signature associated with these tumors (Fig. 1b), confirming findings on non-linear dimensionality reduction. In contrast to *ACVR1*-mutant DMG, *ACVR1*-mutant PF ependymomas lacked co-occurring alterations in *PIK3CA*, *PIK3R1*, and *PPM1D* (Fig. 1c). One case harbored deleterious mutations in *TP53* and *RB1* and a single case contained a *TERT* promoter mutation. In the majority of PF-ACVR1 (6/7), cCopy number analysis revealed broad gains and losses ~~in the majority of PF-ACVR1 (6/7)~~, resembling profiles that are often observed in PFB (Supplementary Fig. 1, online resource). The median age at diagnosis (32 years) was similar to PFB (29 years), but significantly older than PFA (median age) ($p < 0.01$) and younger than EPN/SE (median age) ($p < 0.05$) (Fig. 1b; Supplementary Fig. 2, online resource).

Review of histology from available cases showed characteristic morphologic features of ependymoma (e.g., perivascular pseudorosettes) and a compatible immunohistochemical profile (i.e., GFAP+, OLIG2-, perinuclear dot-like EMA). Interestingly, we observed focal papillary or pseudopapillary features in most cases

(Supplemental Fig. 3, online resource). Immunohistochemical staining of PF-ACVR1 tumors showed retained H3K27me3 and an absence of EZHIP/CXorf67 expression (Supplemental Fig. 3, online resource). The two cases clustering with PFA (PFA-ACVR1) occurred in young children and demonstrated loss of H3K27me3 with concurrent EZHIP expression in tumor cells (Supplementary Fig. 3, online resource).

To date, *ACVR1* mutations in gliomas have been restricted to specific amino acid residues within the TGF-β glycine-serine-rich (GS) (codon 206), protein kinase catalytic (PKc; codons 258, 328) and protein kinase catalytic-like (PKc-like; codon 356) domains[1, 3, 12, 14]. The p.Gly328Gln (c.982G>T, n=2) and p.Arg375Cys (c.982G>T, n=1) substitutions described here have not been previously reported in CNS tumors (Fig. 1d; Supplemental table 2, online resource). Germline mutations in *ACVR1* are associated with fibrodysplasia ossificans progressiva (FOP) and result in ligand-independent upregulation of bone morphogenic protein (BMP) pathway signaling[3, 11]. Interestingly, the p.G328Q variant reported here has not been reported in a somatic malignancy or in FOP (https://cancer.sanger.ac.uk/cosmic, queried Feb. 22, 2022). In DMG, *ACVR1* mutations are associated ~~correlate~~ with distinct clinical and molecular features: they frequently co-occur with the less common histone H3.1 (*H3C4*) K27M mutations, are more common in females, and have been associated with longer overall survival (OS)[14]. While survival differences were not observed in the current series, the small sample size and short follow-up time may limit interpretation of outcome. Notably, the 7 patients with ACVR-1 mutations were all alive, with a median follow-up of 153 months ~~all patients were living at last follow-up with a median overall survival of 153 months~~ (Supplemental Table 1; Supplemental Fig.4, online resource).

Despite a relative lack of recurrent somatic mutations, recent evidence suggests that leveraging 'super-enhancer' dependency may be an effective therapeutic approach in ependymal tumors[7]. This approach has identified subtype-specific transcriptional dependencies in ependymomas that may be responsive to small molecule inhibitors. Thus, the identification of lineage- or molecular-based groups will be increasingly important in directing future therapeutic approaches in ependymoma. A recent study also revealed clinical benefit of repurposing a receptor tyrosine kinase inhibitor (vandetanib) in combination with an mTOR inhibitor in patients with *ACVR1*-mutant DMG[2]. Vandetanib, a multi-RTK inhibitor, has demonstrated a synergistic effect with everolimus in inhibiting *ACVR1* downstream effector signaling (i.e., SMAD), as well as improved CNS penetration in this context[2], thus raising the possibility of this approach in *ACVR1*-mutant PF ependymoma. Our findings indicate further study is needed to delineate the biologic and clinical implications of *ACVR1* mutations in PF ependymomas.

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Data availability

The raw methylation array data (IDAT format) has been made available for download at the Gene Expression Omnibus (GEO) repository under the accession number GSE196013 (https://www.ncbi.nlm.nih.gov/geo/).

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Author contributions

All authors provided meaningful contributions to the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1. Genetic and epigenetic characterization of *ACRV1*-mutant PF ependymomas. Unsupervised embedding of DNA methylation array data from representative samples from ependymal tumors across all neuroanatomic compartments (**a**); also included are K27-altered diffuse midline gliomas (DMG) with and without *ACVR1* mutations. Notably, PF ependymomas with *ACVR1* mutations and retained H3K27me3 expression (labeled me3+) form a distinct epigenetic group. Heatmap of DNA methylation array data from PF ependymal tumors confirming four distinct tumor types with hierarchical clustering (**b**). Oncoplot of co-occurring oncogenic or likely oncogenic somatic alterations from *ACVR1*-mutant PF ependymomas (left) and diffuse midline gliomas (DMG, right) (**c**). Lollipop plot of the distribution of AVCR1 mutations in PF ependymomas (top) and DMG (bottom) (**d**); novel missense mutations are labeled arrows. Abbreviations: DMGK27, diffuse midline glioma, K27-altered; ZFTA, supratentorial ependymoma, *ZFTA* fusion-positive; EPN, SPINE, spinal ependymoma; YAP1, supratentorial ependymoma, *YAP1* fusion-positive; MPE, myxopapillary ependymoma; EPN/SE, PF, posterior fossa ependymoma/subependymoma[13]; SUBEPN, SPINE, spinal ependymoma; SUBEPN, ST, supratentorial subependymoma; PFA, posterior fossa ependymoma, group A; PFB, posterior fossa ependymoma, group B; MYCN, spinal ependymoma, *MYCN*-amplified.

Supplemental Figure 1. Copy number profiles derived from DNA methylation data. Sample-specific profiles for PF-ACVR1 showed frequently broad gains and losses. Proportion-based plots for other PF ependymoma types are shown on the right.

Supplemental Figure 2. Age distribution of PF ependymoma types and statistical differences when compared to PF-ACVR1. ns = $p > 0.05$, * = $p \leq 0.05$, ** = $p \leq 0.01$

Supplemental Figure 3. Representative histologic and immunophenotypic (H3K27me3, EZHIP) features of PF-ACVR1 (cases 1-5, 7) and PFA-ACVR1 (cases 8 and 9) included in this series.

Supplemental Figure 4. Kaplan-Meier curves of OS and PFS stratified by PF ependymoma tumor type (the exact log rank test is a permutation test based on 100,000 random permutations). Pairwise comparison of the other groups vs. PF-ACVR1 ~~groups~~ did not reveal significant differences in survival distributions; this is likely a result from extremely unbalanced sample sizes (i.e., 7 vs ≥ 47) in pairwise comparisons with PF-ACVR1.