ARTICLE

DOI: 10.1038/s41467-018-02826-8

A recurrent kinase domain mutation in PRKCA defines chordoid glioma of the third ventricle

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Chordoid glioma is a rare brain tumor thought to arise from specialized glial cells of the lamina terminalis along the anterior wall of the third ventricle. Despite being histologically low-grade, chordoid gliomas are often associated with poor outcome, as their stereotypic location in the third ventricle makes resection challenging and efficacious adjuvant therapies have not been developed. Here we performed genomic profiling on 13 chordoid gliomas and identified a recurrent D463H missense mutation in PRKCA in all tumors, which localizes in the kinase domain of the encoded protein kinase C alpha (PKCα). Expression of mutant PRKCA in immortalized human astrocytes led to increased phospho-ERK and anchorage-independent growth that could be blocked by MEK inhibition. These studies define PRKCA as a recurrently mutated oncogene in human cancer and identify a potential therapeutic vulnerability in this uncommon brain tumor.

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Chordoid glioma of the third ventricle is characterized by its stereotypic location in the anterior third ventricle and its chordoid cellular architecture composed of glial fibrillary acidic protein (GFAP)-positive tumor cells embedded within a myxoid matrix, often accompanied by a dense lymphoplasmacytic inflammatory infiltrate. Due to their intraventricular location, they often obstruct flow of cerebrospinal fluid, leading to symptoms associated with obstructive hydrocephalus, such as intracranial hypertension, headache, nausea, vomiting, ataxia, and cognitive changes. Despite being histologically low-grade (classified as grade II in the 2016 WHO Classification of Tumors of the Central Nervous System) and well circumscribed without substantial invasion of surrounding brain tissue, chordoid gliomas are associated with substantial morbidity and mortality. Their proximity and adherence to critical regional structures makes them difficult to safely resect. While gross total resection remains a goal and can be curative, it can result in hypothalamic dysfunction or peri-operative complications including death.

Subtotal resection is often accompanied by tumor recurrence. Optimal adjuvant therapy for subtotally resected or recurrent tumors has not been established.

Chordoid gliomas were recently demonstrated to share expression of the homeobox transcription factor TTF-1 (also known as NKX2.1) with the organum vasculosum of the lamina terminalis, suggesting a cellular origin of this neoplasm from these specialized ependymal cells located along the anterior wall of the third ventricle. However, the molecular alterations that drive these tumors are unknown. Here we report genomic analysis on a cohort of chordoid gliomas that was performed with the goal of identifying new diagnostic and prognostic biomarkers, as well as potential targets for molecularly tailored therapy for these rare brain tumors.

Results

Chordoid glioma patient cohort. To investigate the molecular pathogenesis of this rare brain tumor entity, we assembled a cohort of archival tissue specimens from 13 patients diagnosed with chordoid glioma of the third ventricle at medical centers around the world. Clinical features of these 13 patients are listed in Supplementary Table 1. The 6 male and 7 female patients ranged in age at time of biopsy or resection from 34 to 67 years (median 48 years). All tumors were located in the anterior third ventricle and ranged in size from 2 to 6 cm. Representative imaging features are shown in Fig. 1a and Supplementary Figure 1. Pathologic review confirmed the diagnosis of chordoid glioma for each case. Representative histologic and immunohistochemical features are shown in Fig. 1b and Supplementary Figures 2 and 3. Patient outcomes ranged from early post-operative death from surgical complications to mortality from tumor recurrence within one year following subtotal resection to long term recurrence-free survival.

PRKCA D463H mutation defines chordoid glioma. We performed targeted next-generation sequencing as previously described on genomic DNA isolated from the 13 tumors, as well as matched normal tissue when available (two cases). In each tumor, a G>C transversion mutation in the PRKCA gene was identified causing a c.1387G>C, p.D463H substitution (reference transcript NM_002737; Supplementary Table 2). This missense mutation was verified to be somatic in both tumors with matched normal tissue available for sequencing (CG-UCSF-1 and CG-UCLA-1; Supplementary Figure 4). The mutant allele frequency for this PRKCA D463H variant ranged from 12 to 42% in the 13 tumors, consistent with a somatic heterozygous mutation in all cases. The cases with the highest PRKCA mutant allele frequencies had genomic DNA that was isolated from areas histologically visualized to contain a high tumor cell content. The cases with lower PRKCA mutant allele frequencies had genomic DNA that was isolated from areas that were visualized to contain a more abundant lymphoplasmacytic inflammatory cell infiltrate. The PRKCA locus is located at chromosome 17q24.2. No chromosomal gains, losses, or copy-neutral loss of heterozygosity involving this chromosomal locus were identified in any of the tumors (Supplementary Figure 5, Supplementary Table 3). Therefore, these data suggest that this PRKCA D463H mutation is likely to be a clonal alteration in chordoid gliomas (i.e., present in all tumor cells), indicating that it is probably an early or initiating event in tumorigenesis.

Besides PRKCA mutation, no somatic nonsynonymous mutations, amplifications, deletions, or rearrangements were identified in any of the other 478 genes sequenced in the 13 chordoid gliomas (Supplementary Data 1). Ten of the tumors demonstrated a balanced diploid genome, while the remaining three tumors contained isolated whole chromosome or arm-level gains and losses, without focal regions of amplification or deletion (Supplementary Figure 5, Supplementary Table 3). Specifically, there were no alterations identified involving NF2 or RELA, indicating that chordoid gliomas are genetically distinct from most spinal and supratentorial ependymomas. Additionally, there were no alterations identified involving IDH1, IDH2, TP53, ATRX, TERT, CIC, FUBP1, and NOTCH1, indicating that chordoid gliomas are genetically distinct from the vast majority of diffuse lower-grade gliomas in adults. Also, there were no alterations identified involving TSC1 or TSC2, indicating that chordoid gliomas are genetically distinct from subependymal giant cell astrocytomas, another subtype of intraventricular glioma that may show morphologic overlap with chordoid glioma of the third ventricle (Supplementary Figure 2e).

PRKCA encodes protein kinase C alpha (PKCa), a cytoplasmic serine/threonine kinase whose activity is modulated through calcium and diacylglycerol binding domains (Fig. 1c). Only 95 tumors with confirmed somatic nonsynonymous mutations of PRKCA are present amongst the 30,367 tumors with available sequencing data for PRKCA in the version 81 release of the Catalogue of Somatic Mutations in Cancer (COSMIC) database (Supplementary Data 2). The mutations in these 95 tumors are scattered throughout the gene without clustering in the kinase or other functional domain, and none are affecting codon D463 as seen in chordoid gliomas (Supplementary Figure 6). These 95 tumors are predominantly from cancer types with conspicuously high somatic mutational burden such as melanoma and microsatellite unstable gastrointestinal carcinoma, suggesting that a majority of these PRKCA variants in tumors other than chordoid gliomas are likely to be passenger or bystander mutations.

Structural modeling of mutant PKCa. Amino acid D463 within the kinase domain of PKCa functions as the proton acceptor during the ATP hydrolysis reaction and is highly conserved from yeast to humans (Fig. 1d). Structural modeling of PKCa illustrates that substitution of aspartate with histidine at this codon (D463H) results in a roughly isosteric side chain that contains a proton acceptor in approximately the same location of one of the oxygens in aspartate, if the imidazole ring is not doubly protonated (Fig. 1e and Supplementary Figure 7). However, the proton affinities of aspartate and histidine are markedly different (pKa of 4.0 and 6.5, respectively), and the histidine side chain is commonly, but not always, doubly protonated at physiological pH. This substitution is therefore likely to alter the activity of the kinase function by perturbing the ATP hydrolysis.
d 421 NGGDLYHIQQVGRKPRQAFCVAEISSGFLFHLKRGIIYRDRLNMLDEGHKIA 480 (Homo sapiens)
521 NGGDLYHIQQVGRKPRQAFCVAEISSGFLFHLKRGIIYRDRLNMLDEGHKIA 480 (Pan troglodytes)
520 NGGDLYHIQQVGRKPRQAFCVAEISSGFLFHLKRGIIYRDRLNMLDEGHKIA 479 (Loxodonta africana)
579 NGGDLYHIQQVGRKPRQAFCVAEISSGFLFHLKRGIIYRDRLNMLDEGHKIA 438 (Ovis aries)
279 NGGDLYHIQQVGRKPRQAFCVAEISSGFLFHLKRGIIYRDRLNMLDEGHKIA 480 (Mus musculus)
421 NGGDLYHIQQVGRKPRQAFCVAEISSGFLFHLKRGIIYRDRLNMLDEGHKIA 480 (Rattus norvegicus)
423 NGGDLYHIQQVGRKPRQAFCVAEISSGFLFHLKRGIIYRDRLNMLDEGHKIA 482 (Gallus gallus)
419 NGGDLYHIQQVGRKPRQAFCVAEISSGFLFHLKRGIIYRDRLNMLDEGHKIA 478 (Danio rerio)
278 NGGDLYHIQQVGRKPRQAFCVAEISSGFLFHLKRGIIYRDRLNMLDEGHKIA 337 (Drosophila melanogaster)
475 NGGDLYHIQQVGRKPRQAFCVAEISSGFLFHLKRGIIYRDRLNMLDEGHKIA 534 (Caenorhabditis elegans)
reaction. Prior in vitro biochemical analysis of PKCα has shown that synthetic isoforms harboring alanine or asparagine substitution at codon 463 (D463A or D463N) are kinase dead and lack the ability to phosphorylate a peptide substrate relative to the wildtype protein. However, the functional consequence of the aspartate to histidine substitution at this codon found in chordoid gliomas (D463H) has not been evaluated to date, and we are unaware of any other protein kinases or pseudo-kinases with histidine at this position in the enzymatic pocket.

PRKCA D463H is an oncogenic mutation that drives anchorage independent growth. To determine the functional effects of this PRKCA mutation during tumorigenesis, we generated a lentiviral expression vector for the human PRKCA cDNA. We then used site-directed mutagenesis to introduce the D463H mutation observed in chordoid gliomas, and additionally generated a separate expression vector harboring a D463A mutation. As the amino acid side chain of alanine cannot function as a proton acceptor, we hypothesized that this PRKCA D463A construct may function as a kinase-dead mutant. Immortalized human astrocytes were transduced with lentivirus and then grown in soft agar to assay anchorage-independent growth, a classic measure of cellular transformation. Expression of wild-type PRKCA led to only a small number of colonies, whereas D463H mutant PRKCA yielded numerous colonies, indicating a potent oncogenic effect of this mutation (Fig. 2a). No colonies were observed in immortalized human astrocytes after infection with empty vector or D463A mutant PRKCA. Lentiviral expression of D463H mutant PRKCA was also sufficient to drive anchorage-independent growth of NIH-3T3 cells (Supplementary Figure 8). These results indicate that D463H is an oncogenic, gain-of-function mutation.

PRKCA D463H mutation induces activation of the MAP kinase signaling pathway. To study the mechanisms by which PRKCA D463H mutation drives tumorigenesis of chordoid gliomas, we transiently overexpressed wildtype and mutant PRKCA in 293T cells and immortalized human astrocytes. Reverse transcription-PCR analysis after ectopic expression confirmed expression of wild-type and mutant PRKCA transcripts (Supplementary Figure 9). No differences in phosphorylated ERK were observed in either cell type during in vitro growth in media supplemented with 10% fetal bovine serum (Fig. 2b). In addition, no increase in phosphorylated MARCKS, a known substrate of PKCα kinase activity, was observed (Fig. 2b). However, when stably transduced immortalized human astrocytes were serum starved for 24 h, a significant increase in phospho-ERK was observed for the D463H mutant relative to the wildtype and D463A mutant isoforms (Fig. 2c). This was accompanied by a less robust increase in phospho-Akt after serum starvation (Fig. 2c), presumably related to the increased proliferative signaling through the MAP kinase pathway. As opposed to the over-expression of PKCα seen after transient transfection (Fig. 2b), a significant increase in PKCα levels was not observed after stable lentiviral transduction in immortalized human astrocytes (Fig. 2c), possibly due to only low levels of overexpression or auto-regulatory feedback mechanisms controlling the expression of PKCα.

Next we performed immunohistochemistry for phospho-ERK on the cohort of chordoid gliomas with PRKCA D463H mutation. In each of the six chordoid gliomas that were assessed by immunohistochemistry, robust staining for phospho-ERK was observed, which was specific to the tumor cells and not present in non-neoplastic endothelial cells and admixed inflammatory cells (Fig. 3). The high level of phospho-ERK expression in chordoid gliomas was equivalent to that seen in other glioma types harboring known genetic alterations within the MAP kinase signaling pathway, including pleomorphic xanthoastrocytoma with BRAF V600E mutation and pilocytic astrocytoma with KIAA1549-BRAF gene fusion (Fig. 3). Minimal phospho-ERK staining was observed in sections of normal brain as well as other glioma types harboring genetic alterations not associated with MAP kinase pathway activation (Fig. 3), including clear cell ependymoma with RELA gene fusion, which activates the nuclear factor-kappa B signaling pathway. Thus, PRKCA D463H mutation is likely to drive chordoid glioma, at least in part, by activation of the MAP kinase signaling pathway, although this may be a downstream consequence rather than direct phosphorylation by mutant PKCα.

MEK inhibition blocks the oncogenic effects of mutant PRKCA. We next tested the hypothesis that MEK inhibition might be an effective therapy for chordoid glioma of the third ventricle. Immortalized human astrocytes transduced with D463H mutant PRKCA alongside a series of malignant glioma cell lines harboring wildtype PRKCA alleles were grown in soft agar containing either DMSO vehicle or 5 μM trametinib, a small molecule inhibitor of MEK that is FDA-approved for the treatment of melanoma. Trametinib effectively blocked the colony formation of immortalized human astrocytes expressing D463H mutant PRKCA, while causing only a mild reduction in colony formation for the malignant glioma cell lines with wild-type PRKCA alleles (Fig. 4).

Discussion

Together, these results identify PRKCA as a novel, recurrently mutated oncogene in human cancer. PRKCA D463H mutation appears to define chordoid glioma of the third ventricle and genetically distinguishes it from all other brain tumor types that have been studied to date. Thus, evaluation for this mutation may help to distinguish chordoid glioma from other tumor entities during pathologic assessment of suprasellar and intraventricular neoplasms that are difficult to classify based on histologic features alone.

The protein kinase C (PKC) family has been intensely investigated in the context of cancer ever since the discovery that it is a receptor for phorbol esters, the potent tumor promoting compounds derived from the seed oil of the Croton tiglium plant.
This led to the hypothesis that the PKC genes are proto-oncogenes whose activation by phorbol esters, endogenous ligands, or genetic alteration promotes tumorigenesis. However, recurrent somatic mutations in the genes encoding the various PKC isoforms have not been found in any of the common human cancer types studied to date. Only a small number of tumors with confirmed somatic nonsynonymous mutations of PRKCA are present amongst the greater than 30,000 tumors with available sequencing data for PRKCA in the version 81 release of the COSMIC database. These PRKCA variants are scattered throughout the gene without clustering in the kinase or other functional domain and are predominantly from cancer types with conspicuously high somatic mutational burden such as melanoma and microsatellite unstable gastrointestinal carcinoma (Supplementary Figure 6), suggesting that a majority of these PRKCA variants in tumors other than chordoid gliomas are likely to be passenger or bystander mutations. Our study is the first to demonstrate frequent somatic mutations in a PKC gene in a human cancer subtype.

The PRKCA mutations in chordoid gliomas are all heterozygous missense mutations that cluster at a mutational hotspot within a critical functional domain is strongly suggestive that these are oncogenic, gain-of-function mutations, as opposed to inactivating, loss-of-function events that are typically truncating mutations scattered throughout a gene and accompanied by loss of heterozygosity. Thus, the genetic evidence indicates that PRKCA is likely to function as an oncogene, rather than a tumor suppressor gene, in chordoid gliomas. Additionally, our functional data show that this D463H mutation is an oncogenic mutation sufficient to drive anchorage independent growth of immortalized human astrocytes. This is in contrast to the wildtype and kinase-dead D463A mutant isoforms that did not promote anchorage independent growth. Together, we believe this combination of genetic and functional data prove that the D463H variant is an oncogenic, gain-of-function mutation in PRKCA that drives chordoid gliomas.

While the precise mechanism by which this PRKCA mutation drives gliomagenesis remains to be elucidated, it does result in increased MAP kinase pathway activation that may render chordoid gliomas sensitive to MEK inhibitors. Overexpression of the D463H mutant isoform was not found to increase phosphorylation of MARCKS, one of the known kinase substrates of PKCα. We thus speculate that the PRKCA D463H mutation does
Fig. 3  Chordoid gliomas demonstrate high levels of phospho-ERK, equivalent to levels seen in other glioma types harboring oncogenic alleles of Ras-Raf-MAP kinase pathway components. Shown are representative images of hematoxylin and eosin (H&E) staining and phospho-ERK immunohistochemistry on a chordoid glioma with PRKCA D463H mutation (CG-UVA-1), normal brain, pleomorphic xanthoastrocytoma with BRAF V600E mutation, and clear cell ependymoma with CTORF95-RELA gene fusion. Scale bar, 40 μm

Fig. 4  MEK inhibition is sufficient to block the oncogenic effects of mutant PRKCA. Colony formation in soft agar was assessed for immortalized human astrocytes transduced with D463H mutant PRKCA alongside multiple malignant glioma cell lines, all of which harbor wildtype PRKCA alleles. DMSO vehicle or 5 μM trametinib was added to the top agar layer during casting. Images of representative wells (left) and quantitation of the percent reduction in colony number of trametinib treated wells relative to DMSO-treated wells (right) are shown. Error bars represent standard deviation from the mean of six replicates derived from two independent experiments performed in triplicate.
not function to simply increase the kinase activity of PKCo for its canonical substrates, but rather causes a novel gain of function. Possibilities for this neomorphic function that we speculate include changing the specificity of the kinase activity for new phosphorylation substrates, altering the pH at which the kinase domain is active, or abolishing the kinase activity entirely but promoting tumorigenesis through substrate trapping, acting as a protein scaffold, or other novel kinase-independent mechanisms. Additional studies are ongoing in our laboratory to assess the cellular mechanisms by which this novel genetic alteration causes this tumor, and identified a potential new therapeutic strategy based on the presence of this defining genetic mutation.

**Methods**

**Study population and tumor specimens.** This study was approved by the Committee on Human Research of the University of California, San Francisco, with a waiver of patient consent. Thirteen chordoid gliomas were retrieved from the pathology archives of our respective institutions, spanning years 2001 to 2015. All tumor specimens had been fixed in 10% neutral buffered formalin and embedded in paraffin. Clinical information regarding patient outcomes was obtained from the electronic medical records of our respective institutions. Pathologic re-review of all tumor samples was performed to confirm the diagnosis by two expert neuropathologists (AP and DAS).

**Immunohistochemistry.** Immunohistochemistry was performed on whole formalin-fixed, paraffin-embedded tissue sections using the following antibodies: GFAP (Dako, cat# Z0334, polyclonal, 1:3000 dilution), TTF-1 (Dako, cat# IS056, clone G8G3/1, 1:500 dilution), and phospho-p44/42 ERK Thr202/Tyr204 (Cell Signaling, cat #4370, clone D13.14.4E, 1:10,000 dilution). All immunostaining was performed in a Leica Bond-Max automated stainer. Following antigen retrieval, primary antibody was applied for 15 min followed by Bond-Max polymer for 15 min. Diaminobenzidine was used as the chromogen, followed by hematoxylin counterstain.

**Targeted next-generation DNA sequencing and mutational analysis.** Genomic DNA was extracted from formalin-fixed, paraffin-embedded blocks of tumor tissue from 13 patients with chordoid glioma of the third ventricle using the QIAamp DNA FFPE Tissue Kit (Qiagen). Genomic DNA was also extracted from leukocytes in a peripheral blood sample from one of the patients (CG-UCSF-1) and a non-neoplastic gastric biopsy from specimen of the patients (CG-UCCLA-1). Capture-based next-generation DNA sequencing was performed at the University of California, San Francisco Clinical Cancer Genomics Laboratory, using an assay that targets all coding exons of 479 cancer-related genes, select introns of 47 genes, harboring targets all coding exons of 479 cancer-related genes, select introns of 47 genes, and the mutagenesis reactions were as follows: PRKCA D463H Fwd: 5′-GGAACATTTATCGACTCAACTTAAAATGATTCC-3′, PRKCA D463H Rev: 5′-TGTACTAATCAACCTGAGTGTGCT-3′, PRKCA D463A Fwd: 5′-GGAACATTTCTGACGGTAACTGA TAA-3′, PRKCA D463A Rev: 5′-GGTATTCAATCTCAAGGCTAATGATTCC-3′.

**Imortalized human astrocytes and cell culture.** Human astrocytes immortalized by retroviral transduction of hTERT, E6, and E7 proteins were generously provided by Dr. Russ Peeper (University of California, San Francisco). 293 T and NIH-3T3 cells were obtained directly from ATCC and were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C in 5% CO₂. Glioblastoma and other malignant glioma cell lines (GATK; Appistry v2015.1.1 – 22. Single nucleotide variants and insertions/deletions were identified using Integrated Genome Viewer. Genome-wide copy number analysis based on on-target and off-target reads was performed by CNVkit. Copy number EGAS00001002733. All sequence data from the 13 chordoid gliomas have been deposited in the University of California-San Francisco (supported by NIGMS P41-GMI103311) for its use and distribution, or available from the authors upon request.

**PKCo structural modeling.** The crystal structure of the kinase domain for human PKCo has been previously resolved (pdb: 4RA4)[19, except for a loop corresponding to amino acids 617–633. A structural model incorporating this missing loop was built using Protein Local Optimization Program (PLOP)[20] and homology with the human protein kinase A (PKA) crystal structure bound to a non-hydrolysable ATP analog (pdb: 3OTL)[21], which was subjected to a short minimization (deepest descent). A model ATP was then built into the minimized PKCo by modifying the AMP-PNP molecule. D463 from the resulting model was mutated in the pdb file to D463H protonated at the epsilon position. Figures were produced using UCSF Chimera, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, and published online: 23 February 2018.
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Acknowledgements

This study was supported by NIH Director’s Early Independence Award (DP5 OD021403) and Career Development Award from the UCSF Brain Tumor SPORE (P50 CA097257) to D.A.S. We thank Dr. Russ Pieper for generously contributing the immortalized human astrocytes used in this study.

Author contributions

B.G. and M.H. performed the DNA extractions, PKRCA expression vector construction, site-directed mutagenesis, and in vitro functional studies. G.M. performed the soft agar colony forming assays and RT–PCR analysis. D.G.R. and M.F.P. performed structural modeling analysis. Y.-H.L. and A.P.W. performed proteomics analysis. N.M.I., J.V.Z., C.O., E.T., J.P.G., I.Y., B.C.B., and D.A.S. performed the targeted next-generation sequencing and genomic analysis. I.H.H., M.S., D.J.B., B.K.-D.M., F.J.R., D.N.L., W.H. Y., M.B.L., M.K.R., N.B., T.T., A.W.R., A.P., and D.A.S. procured tumor specimens and performed pathologic assessment. J.P.P. performed immunohistochemistry on tumor tissue. D.A.S. conceptualized the study, reviewed all data, and prepared the figures. All authors assisted with drafting and critically revising the manuscript.

Additional information

Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-018-02826-8.

Competing interests: The authors declare no competing financial interests.

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