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Myc and loss of p53 cooperate to drive formation of choroid plexus carcinoma

Jun Wang¹, Diana M. Merino^{2,3}, Nicholas Light^{3,4}, Brian L. Murphy⁵, Yong-Dong Wang⁶, Xiaohui Guo⁷, Andrew P. Hodges⁷, Lianne Q. Chau¹, Kun-Wei Liu¹, Girish Dhall⁸, Shahab Asgharzadeh⁸, Erin N. Kiehna⁸, Ryan J. Shirey⁹, Kim D. Janda⁹, Michael D. Taylor¹⁰, David Malkin^{3,11,12}, David W. Ellison¹³, Scott R. VandenBerg¹⁴, Charles G. Eberhart¹⁵, Rosalie C. Sears¹⁶, Martine F. Roussel⁵, Richard J. Gilbertson¹⁷, and Robert J. Wechsler-Reya^{1,*}

¹Tumor Initiation and Maintenance Program, NCI-Designated Cancer Center, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA, USA

²Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada

³Program in Genetics and Genome Biology, The Hospital for Sick Children, Toronto, Ontario, Canada

⁴Institute of Medical Science, University of Toronto, Toronto, Ontario, Canada

⁵Department of Tumor Cell Biology, St. Jude Children's Research Hospital, Memphis, TN

⁶Department of Computational Biology, St. Jude Children's Research Hospital, Memphis, TN

⁷Bioinformatics Core Facility, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA

⁸Division of Hematology, Oncology and Blood & Marrow Transplantation, Children's Center for Cancer and Blood Diseases, Children's Hospital Los Angeles

⁹Departments of Chemistry, Immunology and The Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA

¹⁰Division of Neurosurgery and Program in Developmental and Stem Cell Biology, The Hospital for Sick Children, Toronto, Ontario, Canada

¹¹Departments of Pediatrics and Medical Biophysics, University of Toronto

¹²Division of Hematology/Oncology, The Hospital for Sick Children, Toronto, Ontario, Canada

¹³Department of Pathology, St. Jude Children's Research Hospital, Memphis, TN

¹⁴UCSF Helen Diller Family Comprehensive Cancer Center, University of California San Francisco, San Francisco, CA

¹⁵Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD

¹⁶Molecular and Medical Genetics Department, Oregon Health and Sciences University, Portland, OR

*Address correspondence to rwreya@sbpdiscovery.org.

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¹⁷Cancer Research UK Cambridge Centre, CRUK Cambridge Institute, Li Ka Shing Centre, Cambridge, UK.

Abstract

Choroid plexus carcinoma (CPC) is a rare brain tumor that occurs most commonly in very young children and has a dismal prognosis despite intensive therapy. Improved outcomes for CPC patients depend on a deeper understanding of the mechanisms underlying the disease. Here we developed transgenic models of CPCs by activating the *Myc* oncogene and deleting the *Trp53* tumor suppressor gene in murine neural stem cells or progenitors. Murine CPC resembled their human counterparts at a histological level, and like the hypodiploid subset of human CPC, exhibited multiple whole-chromosome losses, particularly of chromosomes 8, 12 and 19. Analysis of murine and human CPC gene expression profiles and copy number changes revealed altered expression of genes involved in cell cycle, DNA damage response, and cilium function. High-throughput drug screening identified small molecule inhibitors that decreased the viability of CPC. These models will be valuable tools for understanding the biology of choroid plexus tumors and for testing novel approaches to therapy.

Introduction

Choroid plexus tumors are rare pediatric neoplasms that arise around the ventricles of the brain, and account for up to 20% of brain tumors in children under 1 year of age. These tumors can be divided into 3 subgroups based on histology: choroid plexus papillomas (CPPs, WHO grade I), atypical choroid plexus papillomas (aCPPs, WHO grade II) and choroid plexus carcinomas (CPCs, WHO grade III). CPPs have a favorable prognosis after surgical resection and rarely require additional treatment. CPCs, in contrast, usually require surgical removal followed by radiation and chemotherapy. Despite aggressive treatments, the 5-year overall survival rate is less than 60% (1,2) and the median progression-free survival (PFS) is only 13 months (3). Patients who do survive often suffer devastating side effects from the therapy, including neurocognitive deficits, endocrine disorders and secondary cancers. Effective treatments for CPC are lacking due to poor understanding of CPC biology and the paucity of patient specimens and animal models for studying the disease.

The pathogenesis of choroid plexus tumors is not well understood. Mutations in the *TP53* tumor suppressor gene are present in 50% of CPCs and have been associated with poor prognosis (4). However, whole genome sequencing of CPC patient specimens has not identified other recurrent single nucleotide variants, insertions/deletions, or focal copy number alterations (5). Rather, CPCs exhibit frequent chromosomal imbalances, with some tumors exhibiting multiple large chromosomal gains (hyperdiploid) and others showing predominantly large chromosomal losses (hypodiploid) (6-8). These studies suggest that copy number alterations might be oncogenic drivers of CPC.

To understand the pathogenesis of CPC, we have created mouse models of hypodiploid CPC by activating the *Myc* oncogene and inactivating the *Trp53* tumor suppressor in neural stem cells or progenitors. The resulting models are useful for understanding the biology of CPC and for testing novel therapies.

Materials and Methods

Animals

Atoh1-Cre (B6.Cg-Tg(Atoh1-cre)1Bfri/J, stock number 01104) and p53LoxP (B6.129P2-Trp53tm1Brn/J, stock number 008462) mice, Nestin-Cre (B6.Cg-Tg(Nes-cre)1Kln/J, stock number 003771) and GFAP-Cre (FVB-Tg(GFAP-cre)25Mes/J, stock number 004600) were purchased from JAX. Blbp-Cre mice were purchased from NCI mouse repository (B6;CB-Tg(Fabp7-creLacZ)3Gtm/Nci, strain number: 01XM9). LSL-MycT58A mice were kindly provided by Rosalie Sears at Oregon Health and Science University. All animals were maintained in the animal facilities at SBP. All experiments were performed in accordance with national guidelines and regulations, and with the approval of the animal care and use committees at SBP, at the University of California San Diego (UCSD) and at St Jude Children's research hospital.

Histology and immunohistochemistry

For histological analysis, animals were perfused with PBS followed by 4% paraformaldehyde (PFA). Brains were removed and fixed in 4% PFA overnight and then transferred to 70% ethanol and embedded in paraffin. Sections were then stained with hematoxylin and eosin or with Ki67 antibody (Abcam: ab15580) or MYC antibody (Abcam: ab32072). For immunofluorescent staining, brains from PFA-perfused animals were fixed overnight in 4% PFA, cryoprotected in 30% sucrose, frozen in Tissue Tek-OCT (Sakura Finetek), and cut into 12 µm sagittal sections. Sections were blocked and permeabilized for 1 hr with PBS containing 0.1% Triton X-100 and 10% normal donkey serum, stained with primary antibodies (anti-Otx2: Millipore AB9566; anti-Aqp1: Santa Cruz SC-20810; anti-Ki67: Abcam ab15580; pH2A.X: Cell Signaling 9718P) overnight at 4°C, and incubated with secondary antibodies for 1 hr at room temperature. Sections were counterstained with DAPI and mounted with Fluoromount-G (Southern Biotech) before being visualized using a Zeiss LSM 700 confocal microscope.

Quantitative RT-PCR

RNA was isolated using Qiagen RNeasy mini kit. Reverse transcription was done using iScript cDNA synthesis kit (Bio-Rad). Primers for qPCR are listed below. *Myc* (forward: 5'-ATGCCCCCTCAACGTGAACCTTC-3', reverse: 5'-CGCAACATAGGATGGAGAGCA-3'); *Trp53* (forward: 5'-CACAGCGTGGTGGTACCTTA-3', reverse: 5'-TCTTCTGTACGGCGGTCTCT-3'); *Hspa1b* (forward: 5'-GAGATCGACTCTCTGTTCGAGG-3', reverse: 5'-GCCCCGTTGAAGAAGTCCTG-3')

Viability assay

Cells were plated in 384 well plates prior to drug treatment. 48 hours after treatment, cell viability was analyzed by CellTiter Glo assay (Promega) and results were collected on a Perkin Elmer Envision plate reader.

Western blot

Protein lysates were prepared in RIPA buffer (Milipore) and protein concentration was determined by BCA assay (Bio-Rad). Proteins were transferred to nitrocellulose membrane (Invitrogen) after SDS-PAGE electrophoresis and blocked in non-fat dry milk for 30min. Membrane was incubated with primary antibodies (anti-MYC: Cell Signaling 5605S; anti-GAPDH: Cell Signaling 5174S; anti-phospho-RB: Cell Signaling 8516T; anti-RNAPII: Cell Signaling 2629) overnight and washed three times in TBST. Membrane was then incubated with secondary antibody for 1 hour at room temperature and washed three times in TBST. Membrane was incubated with Bio-Rad Clarity Western substrate before being imaged on a Bio-Rad ChemiDoc MP imaging system.

Array CGH

Array CGH analysis was performed at St. Jude Children's Research Hospital on Agilent SurePrint G3 Mouse Genome CGH microarrays using genomic DNA samples prepared from mouse normal choroid plexus, choroid plexus papillomas and choroid plexus carcinomas. DNA copy number changes were visualized in Integrated Genome Viewer (IGV). Raw data of array CGH have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) and are accessible through the GEO Series accession number GSE126408.

Gene expression profiling and pathway enrichment analysis

Gene expression profiling was done using Affymetrix Gene Chip Mouse Gene ST 2.0 microarrays. RMA normalization was applied and differentially expressed genes were generated using R/Limma package (cut off: fold change > 2 and p value < 0.05). Pathway enrichment analyses were determined using Metascape ([Metascape.org](https://metascape.org)). Chromosome enrichment analysis was done in DAVID Bioinformatics 6.8 (<https://david.ncifcrf.gov/>). Raw data of microarray have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) and are accessible through the GEO Series accession number GSE126327.

Sanger sequencing of TP53

Exons 2 to 11 of TP53 were PCR amplified from the genomic DNA of CHLA-CPC02 and CHLA-CPC03 patient specimen. PCR products were column purified and Sanger sequencing were performed to determine the mutational status of TP53. Mutation calls were made with Mutation Surveyor software.

Cell culture

CPC tumor tissue was isolated from the mouse ventricle and dissociated with papain for 30 minutes at 37 degree. Dissociated CPC cells were plated for subsequent experiments in Neurocult Basal Medium (STEMCELL Technologies) with proliferation supplement (STEMCELL Technologies) and Penicillin Streptomycin.

High throughput drug screening

A total of 7902 compounds from drug libraries including StemSelect (EMD), Spectrum (Microsources), LOPAC (Sigma), FDA/IN drugs (Microsources), Prestwick chem library (Prestwick), LeadGen collection (Enzo Life Science), InhibitorSelect Pathway (EMD), NIH clinical collection (NIH), NCI oncology drugs (NCI), Epigenetics library (Enzo Life Science), Kinase inhibitors (EMD), Kinase inhibitors (Cayman), Kinase inhibitors (SeleckChem, Drug Reposition Library (various vendors). Compounds were loaded into 384 well plates at a concentration of 1 μ M and 5000 CPC cells were seeded into each well. Celltiter Glo assay was used to determine the effect of drugs on cell viability 48 hours later. More than 50% reduction in viability compared to vehicle control (DMSO) treated cells was used as a cut off to select candidate compounds.

Small molecule compounds

Dinaciclib, Flavopiridol, Abemaciclib were purchased from SelleckChem. Triptolide was purchased from Cayman Chemical. 10058-F4 was purchased from Sigma. CD-532, Meriolin 3, Cdk/Crk inhibitor were obtained from the Conrad Prebys Center for Chemical Genomics at SBP.

Statistical analysis

Statistical analysis was performed in GraphPad Prism software and Limma R package. Monte Carlo simulation was performed by randomly generating 1712 human genes and 113 mouse genes respectively from the 19,676 possible human-mouse orthologous genes and the overlap was calculated across 1,000,000 simulations.

Drug Synergy analysis

Drug synergy was tested using CompuSyn software (www.combosyn.com). $CI < 0.5$ is highly synergistic, $0.5 < CI < 0.9$ is synergistic, $0.9 < CI < 1.1$ is additive, $CI > 1.1$ is antagonistic.

Results

Ectopic expression of *Myc* and loss of *Trp53* in neural progenitor cells leads to choroid plexus carcinoma

We previously generated mouse models of MYC-driven medulloblastoma by using retroviruses to overexpress *Myc* and inactivate *Trp53* in purified cerebellar stem cells or granule neuron precursors (GNPs) and transplanting those cells into the cerebellum of naïve mice (9,10). In an effort to create a transgenic version of these models, we crossed mice carrying a Cre-inducible allele of *Myc* (Rosa-LoxP-Stop-LoxP-Myc^{T58A}, hereafter called LSL-Myc) (11) and a Cre-deletable allele of *Trp53* (LoxP-Trp53-LoxP, or Trp53-flox) with animals that express Cre recombinase in neural stem or progenitor cells, including Atoh1-Cre (12), Blbp-Cre (13) and hGFAP-Cre (14) (Figure 1A and Figure S1A). Within 13 to 28 weeks, mice developed signs of increased intracranial pressure and neurological symptoms and had to be sacrificed (Figure 1B and Figure S1B, S1C). Unexpectedly, fewer than 5% of mice had cerebellar tumors that resembled medulloblastoma; the remaining mice had tumors in the choroid plexus, and these exhibited histological characteristics of CPP or CPC.

Animals in which *Myc* was activated but *Trp53* expression was retained (**A**toh1-Cre; LSL-**M**yc (hereafter referred to as AM); **G**fap-Cre; LSL-**M**yc (GM) and **B**lbp-Cre; LSL-**M**yc (BM)) developed tumors that showed increased numbers of cells but maintained architecture of normal choroid plexus, with a single layer of epithelial cells surrounding a fibrovascular core; histologically, these tumors resembled CPPs (Figure 1C). In contrast, tumors induced by activation of *Myc* and deletion of both copies of *Trp53* (**A**toh1-Cre; LSL-**M**yc; **T**rp53-flox (AMP), **G**fap-Cre; LSL-**M**yc; **T**rp53-flox (GMP) and **B**lbp-Cre; LSL-**M**yc; **T**rp53-flox (BMP)) exhibited frequent nuclear pleomorphism and loss of papillary structure, and resembled CPCs (Figure 1D). CPCs had increased numbers of mitotic figures and much higher proliferative indices than CPPs as shown by Ki67 staining (Figures 1E and F). Mice that developed CPPs survived much longer (median survival = 400 days for AM) than those that developed CPCs (median survival = 131 days for AMP) (Figure 1B). Interestingly, tumors induced by Atoh1-Cre and Gfap-Cre developed in the fourth ventricle whereas Blbp-Cre induced tumors were found in all four ventricles.

Normal choroid plexus epithelial cells express Aquaporin 1 (Aqp1) and Orthodenticle Homeobox 2 (Otx2) (15,16). While murine CPPs retain expression of these markers (Figures 1G and H), murine CPCs have reduced expression of Aqp1 and Otx2 (Figures 1I and J), suggesting that they are relatively undifferentiated. Furthermore, leptomeningeal metastasis was observed in a subset of mouse CPCs (Figure S2A-D). Because inactivation of p53 often results in genomic instability (17), we examined DNA double-stranded breaks by staining with antibodies against Phospho-Histone H2A.X (pH2A.X). In animals as young as 4 weeks old, we observed pH2A.X staining in CPCs (Figure 1K) but not in CPPs (Figure 1L) or in normal choroid plexus (Figure 1M). We also observed higher mitotic indices in CPCs (Figure 1N) than that in CPPs (Figure 1O) and normal choroid plexus (Figure 1P). These studies demonstrate that activation of *Myc* and loss of *Trp53* can cooperate to generate aggressive choroid plexus tumors in mice.

Atoh1 expressing neural progenitor cells contribute to the choroid plexus epithelial lineage

Most previous studies have suggested that Atoh1 is expressed in GNPs of the cerebellum and in progenitor cells destined to give rise to the deep cerebellar nuclei (18). The induction of choroid plexus tumors by Atoh1-Cre suggested that Atoh1-expressing progenitors also contribute to the choroid plexus. To test whether this was the case, we performed lineage tracing by crossing Atoh1-Cre mice with Cre-inducible fluorescent reporter mice (Rosa-CAG-LoxP-stop-LoxP-tdTomato), in which tdTomato expression is permanently induced upon excision of the stop sequence (Figure 2A). Brains were collected from progeny of these crosses at embryonic day 15.5 and postnatal day 7. In Atoh1-Cre; LSL-tdTomato mice, tdTomato expression was detected in a subset of choroid plexus epithelial cells in the fourth ventricle as early as E15.5. These tdTomato-expressing cells also expressed the choroid plexus epithelial cell markers Aqp1 and Otx2 (Figure 2B). To determine whether tumor cells in AMP mice are derived from Atoh1+ CP progenitors, we crossed these animals with tdTomato reporter mice to generate tumor-bearing mice in which Atoh1+ cells and their progeny are labeled with tdTomato (AMP-tdTomato). These mice developed CPCs and most of the tumor cells expressed tdTomato, indicating that CPCs are derived from Atoh1+

progenitors (Figure 2C). Similar studies performed using *Blbp-Cre* mice showed that *Blbp*-expressing progenitor cells give rise to choroid plexus epithelial cells in both the forebrain and the hindbrain (Figure S3). These studies revealed that CPCs can arise from *Atoh1*-expressing or *Blbp*-expressing progenitors in the choroid plexus.

Choroid plexus tumors are dependent on *Myc*

Although previous studies have shown that *TP53* mutations are common in CPC, the role of *MYC* in CPC pathogenesis has not been studied in detail. To confirm the overexpression of *Myc* and loss of *p53* in murine tumors, we performed Western blotting. As expected, *Myc* was highly expressed in murine CPPs and CPCs (Figure 3A), whereas loss of *Trp53* expression was only observed in CPCs (Figure 3B). We also observed high *MYC* expression in primary human CPPs and CPCs compared to normal fetal and adult brain tissue (Fig 3C and Fig S4A-D). These results suggest that *MYC* is highly expressed in murine and human choroid plexus tumors.

To determine whether *Myc* is required for tumor cell survival, we treated mouse CPCs with compounds that have been shown to inhibit *Myc* function. KJ-Pyr-9 (19) and 10058-F4, both of which disrupt *Myc*/Max dimerization, decreased CPC cell viability in a dose dependent manner (Figure 3D, E). CD-532, a novel compound that destabilizes *Myc* by preventing its interactions with Aurora kinase (20), also potently reduced mouse choroid plexus tumor cell viability (Figure 3F). To determine whether human CPC cells are sensitive to *MYC* inhibition, we obtained primary tumor cells from CPC patients. These samples (CHLA-CPC02 and CHLA-CPC03) both contained polymorphisms in the *TP53* locus (P72R (rs1042522) and intron 3 duplication (rs17878362) respectively) that are associated with increased cancer risk. Consistent with the results from murine tumors, the viability of human CPC cells was decreased when treated with compounds that disrupt *MYC* function (Figure 3G). These studies suggest that *MYC* is required for survival of murine and human CPCs.

Mouse choroid plexus carcinomas are characterized by frequent losses of chromosomes 8, 12 and 19

Human choroid plexus tumors frequently exhibit large chromosomal gains (hyperdiploid tumors) or losses (hypodiploid tumors). To investigate if mouse choroid plexus tumors share these features, we performed array-based comparative genomic hybridization (aCGH) on normal choroid plexus (ChP), CPPs from AM mice, and CPCs from AMP and BMP mice. While no significant chromosomal aberrations were found in CPPs, CPCs exhibited multiple losses similar to those observed in human CPCs (8). Specifically, we observed recurrent losses of mouse chromosome 8 (syntenic with human chromosomes 8, 13, 16 and 19), mouse chromosome 12 (syntenic with human chromosomes 2, 7 and 14) and mouse chromosome 19 (syntenic with human chromosomes 9 and 10) respectively (Figure 4A-D).

To identify pathways that are altered during the course of choroid plexus tumor formation, we performed gene expression profiling using Affymetrix Gene Chip Mouse Gene ST 2.0 microarrays to compare CPCs with CPPs and normal choroid plexus cells. Principal component analysis (PCA) indicated that CPCs and CPPs are distinct from one another, and very different from normal choroid plexus (Figure 5A). Hierarchical clustering confirmed

the results observed using PCA (Figure 5B), indicating that CPCs and CPPs are related but distinct tumors. Using a cutoff of fold change > 2 and P-value < 0.05 , we identified 2579 genes differentially expressed between CPCs and normal choroid plexus, 2194 genes differentially expressed between CPPs and normal choroid plexus, and 637 genes differentially expressed between CPCs and CPPs.

We then performed pathway analysis using Metascape (metascape.org) (21) and Gene Set Enrichment Analysis (GSEA) (22,23) to look for pathways enriched in mouse CPCs compared to normal choroid plexus. MYC targets, as well as regulators of ribosome biogenesis and metabolic processes, which are often associated with MYC overexpression, were highly enriched in CPCs. Cell cycle related genes and regulators of the E2F, AURORA B and PLK1 pathways were also highly expressed (Figure 5C). Genes related to cilium function, which were recently reported to play key roles in choroid plexus tumor formation (24), were significantly downregulated (Figure 5D).

To assess whether the changes in gene expression in CPCs were due to copy number alterations, we integrated our microarray and aCGH data. Interestingly, when we looked at the downregulated genes (LogFC < 0.2 , p-value < 0.05 , FDR < 0.05) in murine CPCs compared to CPPs, we found that these genes were enriched on three mouse chromosomes: 8 (17.4% of all chr8 genes exhibited decreased expression), 12 (9.1% of all chr12 genes exhibited decreased expression) and 19 (8.2% of all chr19 genes exhibited decreased expression) (Figure S5A); notably, all three of these chromosomes were deleted in CPCs based on aCGH analysis. These data suggest that chromosomal losses contribute significantly to the differences in gene expression between CPP and CPC.

Analysis of the genes exhibiting copy-number driven decreases in gene expression revealed enrichment of metabolic pathways, such as lipid modification and phospholipid metabolism, carbohydrate metabolism and alpha-amino acid metabolism (Figure S5B). Genes involved in phosphatidylinositol signaling, mitochondrion organization and apoptosis were also altered in CPCs compared to CPPs. To determine if these genes are also lost in human CPC, we compared our data to copy number analysis and gene expression data from patients with CPP and CPC (8). Among the genes showing copy number driven downregulation of gene expression in murine tumors, 30 also showed copy number driven loss in human CPCs (Table 1). To test if the overlap between human and murine genes in our study is significant, we performed Monte Carlo simulation. The average number of genes predicted to overlap by chance was 9 (maximum = 26), suggesting that the overlap of genes between human and murine CPCs is significant ($p < 1E-6$) (Fig S6).

The overlapping genes encode adhesion molecules (cadherin related family member 3, integrin subunit beta 8), transporters (solute carrier family 22 member 8, solute carrier family 5 member 5), kinases (NIMA related kinase 5, adenylate kinase 7), calcium binding proteins (calmodulin 1 (phosphorylase kinase, delta), and regulators of cilia and intracellular transport (Bardet-Biedl Syndrome 1, dynein light chain roadblock-type 2, tubulin polymerization promoting protein family member 3). Although few of these genes have well-studied roles in cancer, the fact that they are lost in both murine and human CPCs

suggests that they might represent tumor suppressor genes that are involved in initiation or progression of choroid plexus tumors.

High throughput drug screening identifies potential therapies for CPCs.

To identify novel therapies for CPC, we performed high throughput drug screening on murine CPC cells using libraries containing 7902 biologically active compounds. Preliminary screening identified 51 compounds that inhibited viability by 50% compared to vehicle treated control cells. Among these, 18 were also able to inhibit the growth of primary patient CPC cells. To eliminate compounds with broad toxicity, we tested these compounds on normal granule neurons. Of the 18 compounds tested, 9 exhibited minimal toxicity to granule neurons (Figure 6A); notably, five of these were inhibitors of cyclin dependent kinases (CDKs).

To determine if CDK inhibitors might be effective for treatment of CPC, we performed dose-response studies. As shown in Figure 6B, pan-CDK inhibitors all potently inhibited viability of CPCs, with IC₅₀'s ranging from 5 – 96 nM. As expected, CDK inhibitors reduced phosphorylation of their common target, the retinoblastoma protein (Rb), in a dose-dependent manner (Figure 6C). Notably Dinaciclib also reduced levels of Myc protein, as has been demonstrated previously (25,26). Interestingly, Abemaciclib, an inhibitor of CDK4 and CDK6, was much less potent at inhibiting growth of CPCs (Figure S7A). These studies suggest that broadly acting CDK inhibitors may be useful for therapy of CPC.

In addition to CDK inhibitors, another compound that inhibited viability of CPCs was Triptolide. Triptolide is a natural product that was originally isolated from the thunder god vine, and has been shown to inhibit the growth of pancreatic cancer through downregulation of heat shock protein 70 (Hsp70) and RNA Polymerase II (Pol II). Triptolide decreased the viability of mouse CPC cells with an IC₅₀ of 25.82 nM (Figure 6D). To confirm that Triptolide targets CPCs through Hsp70 and Pol II, we performed qRT-PCR to check the expression of Hspa1b, the gene that encodes Hsp70. Hspa1b expression was inhibited in a dose dependent manner 3 hours after mouse CPC cells were treated with Triptolide (Fig 6E). Likewise, the expression of Pol II decreased after Triptolide treatment (Figure 6F). Importantly, we observed strong synergy between Dinaciclib and Triptolide (Figure S7B). These studies suggest that CDK inhibitors and Triptolide might be candidates for therapy of choroid plexus tumors.

Discussion

In this study, we generated novel mouse models of choroid plexus tumors by conditionally expressing Myc and/or deleting *Trp53* in progenitor cells that give rise to choroid plexus epithelial cells. Our CPCs exhibit deletions on chromosomes 8, 12, 19 and resemble the hypodiploid subtype of human CPC, which is characterized by large chromosomal deletions. High throughput drug screening identified CDK inhibitors and triptolide as potential therapies for CPC.

Our previous studies (9,10) demonstrated that overexpression of Myc and inactivation of Trp53 in purified hindbrain progenitors allowed these cells to give rise to tumors that

resemble Group 3 medulloblastoma. The fact that inducing the same events in transgenic mice resulted in CPC instead of medulloblastoma was unexpected. One possible explanation is the timing of Myc induction. In our retrovirus-based models, Myc was overexpressed in neonatal hindbrain progenitors, which may have undergone more extensive lineage commitment and differentiation than the embryonic progenitors targeted by the Atoh1-Cre and Blbp-Cre transgenes used in the current study. However, the fact that in utero electroporation of Myc at the embryonic stage results in medulloblastoma with a small percentage of CPCs (27) suggests that embryonic neural progenitors are also capable of giving rise to medulloblastoma. An alternative explanation is that higher levels of Myc are required for development of medulloblastoma than are required for development of CPC. Both the retroviral and electroporation-based models express very high levels of *Myc*, whereas in our CPC model, *Myc* expression is driven by the Rosa26 promoter, which results in relatively weak expression in the brain (28). Further studies will be necessary to determine if transgenic mice expressing higher levels of Myc develop medulloblastoma.

Our observation that overexpression of Myc leads to CPP and that concomitant deletion of *Trp53* results in CPC has implications for the relationship between these diseases. Although CPP and CPC are often described as distinct diseases (29), our results suggest that loss of TP53 could result in malignant progression of CPP to CPC. In fact, there have been several reports of malignant progression of CPP to CPC (30,31). The mechanisms by which such progression can occur remain to be determined, but it is possible that loss of TP53 could result in deregulation of proliferation, survival or genomic stability. The fact that hypodiploid CPCs lose multiple chromosomes supports the notion that genomic instability is one key factor in progression.

The finding that CPCs can be generated from Atoh1+, Blbp+ and GFAP+ cells also raises interesting questions about the origin of CPC. One interpretation of these results is that a subpopulation of progenitors in the choroid plexus is particularly susceptible to transformation, and these cells co-express Atoh1, Blbp and GFAP. Alternatively, it is possible that each of these genes marks a distinct cell type or stage of differentiation, and that a number of different progenitors can function as cells of origin for CPC. Finally, we cannot rule out the possibility that overexpression of Myc and loss of p53 can reprogram a variety of cell types and thereby render them susceptible to transformation. Further analysis of the progenitors marked by Atoh1, Blbp and GFAP may shed light on this issue.

Our models are distinct from previously described mouse models of choroid plexus tumors. More than two decades ago, it was found that expression of simian virus 40 (SV40) large tumor antigen in transgenic mice was sufficient to induce proliferation in choroid plexus epithelial cells (32). SV40 large tumor antigen inactivates the Rb and p53 tumor suppressors, and it was subsequently shown that inactivation of Rb could also induce choroid plexus tumor formation in transgenic mice. Notably, in this model, deletion of p53 was required for tumor progression, but it did not act by promoting chromosomal instability (33). In contrast, our CPCs exhibit chromosomal instability, including losses of chromosomes 8, 12 and 19, and resemble the hypodiploid type human CPCs. In a recent cross-species genomics study using mouse choroid plexus carcinomas generated by simultaneously knocking out p53, Rb and Pten in embryonic choroid plexus epithelial cells, a cluster of CPC oncogenes (TAF12,

NYFC and RAD54L) located on human chromosome 1p32-35.3 were identified (5). Our CPCs do not exhibit recurrent gains of these oncogenes. Interestingly, however, GSEA analysis indicates that syntenic human chromosome 1 genes are overexpressed in our CPCs, indicating that activation of some pathways may be shared between hypodiploid and hyperdiploid CPCs.

Recent studies from Shannon et. al (34) demonstrated that overexpressing Myc alone in Nestin-positive neural progenitor cells led to formation of choroid plexus carcinomas and ciliary body tumors. In our model, Myc alone is not sufficient to induce CPC. The difference may be due to different levels of *Myc* expression. As mentioned previously, the Rosa26 promoter used in our studies often drives weak expression in the brain, whereas the CAG promoter used in Shannon et al. results in higher expression of *Myc*. Another study from El Nagar et. al, (35) found that overexpression of MycT58A and deletion of *Trp53* in Otx2-expressing cells resulted in CPC formation in all ventricles. Pathway analysis in this model pointed to cell cycle deregulation in these tumors, similar to that seen in our model. Although DNA copy number changes were not reported in these studies, it will be interesting to find out if our models share similarities at DNA level.

Our integrated gene expression profiling and aCGH revealed decreased expression of genes on chromosomes 8, 12 and 19 associated with DNA copy number losses. These results indicate that CPCs with p53 deficiency may undergo large chromosomal losses, leading to decreased expression of tumor suppressor genes and driving tumor formation. Identification and validation of these tumor suppressor genes may help us understand more about the mechanisms of tumorigenesis and point to novel therapeutic targets.

Our high throughput drug screening identified CDK inhibitors as promising therapeutic agents against CPCs, consistent with our finding that cell cycle genes are significantly upregulated in CPCs. Although Dinaciclib and Flavopiridol target multiple CDKs, CDK1 and 2 are the only two enzymes that are common targets of these two compounds (36,37). Furthermore, the CDK4/6 specific inhibitor Abemaciclib did not affect the viability of CPC cells (Fig S6A). Therefore, CDK 1 and 2 are likely to be responsible for the efficacy of these compounds. Dinaciclib and Flavopiridol have been used in clinical trials for treating other cancers such as acute myeloid leukemia (NCT01349972), gastric cancer (NCT00991952), breast cancer (NCT01676753, NCT01624441), chronic lymphocytic leukemia (NCT02684617) and pancreatic cancer (NCT01783171). Additional studies will be necessary to determine whether Dinaciclib or Flavopiridol, or other CDK inhibitors, can benefit CPC patients as well.

We also identified Triptolide as an effective inhibitor of CPC survival. Triptolide acts by inhibiting heat shock protein HSP70 and RNA Polymerase II. Its poor solubility in aqueous solution limits its use in preclinical and clinical studies. A water-soluble derivative of Triptolide, called Minnelide, was successfully used to treat pancreatic cancer in preclinical models (38) and is currently in clinical trials for that disease. Our studies suggest that CPC patients may benefit from Triptolide or Minnelide, alone or in combination with CDK inhibitors. Further studies using animal models such as the ones we have described here will

provide insight into the biology of choroid plexus tumors and help identify effective therapies for this disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Expression of Myc and loss of p53 induce choroid plexus carcinoma. **A.** Mice used in this study. Crossing Atoh1-Cre mice to lox-STOP-lox (LSL) Myc^{T58A} mice results in progeny that develop choroid plexus papilloma (CPP); further crossing of these animals to p53-flox mice results in progeny that develop choroid plexus carcinoma (CPC). **B.** Kaplan-Meier survival curve shows median survival times for murine CPC (Atoh1-Cre;Myc/Myc;p53fl/fl) and CPP are 131.5 and 387 (Atoh1-Cre;Myc/Myc;p53fl/+) or 400 (Atoh1-Cre;Myc/Myc) days respectively, whereas mice that have p53 loss without Myc^{T58A} expression or mice that do not express Atoh1-Cre do not develop tumors. **C** and **D.** Hematoxylin and eosin staining shows histology of murine CPP and CPC. **E** and **F.** Ki67 staining shows proliferation in murine CPP and CPC. **G-J.** Aqp1 and Otx2 immunostaining on brain sections of murine CPP and CPC. * indicates normal choroid plexus, dotted line separates normal choroid plexus from the tumor. **K-P.** p-H2A.X and Ki67 immunostaining shows DNA damage and proliferation status in murine CPC, CPP or normal choroid plexus.

Figure 2.

Murine CPCs originate from the *Atoh1*+ lineage. A. Schematic shows breeding strategy used to generate mice for lineage tracing of *Atoh1*+ progenitors. In *Atoh1*-Cre mice x Rosa26-LoxP-Stop-LoxP-tdTomato mice, *Atoh1*+ progenitors and their progeny express tdTomato. B. Immunohistochemistry shows that at embryonic stage E15.5 and postnatal day 7, cells originating from *Atoh1*+ progenitors express choroid plexus epithelial cell markers Aquaporin1 (Aqp1) and Orthodenticle homolog 2 (Otx2). Insets show magnified regions of choroid plexus tissue. C. Murine CPCs (*Atoh1*-Cre; Myc/Myc; p53flox/flox; Rosa26-LoxP-Stop-LoxP-tdTomato) express tdTomato reporter ubiquitously. Dotted line highlights the boundary between tumor (left) and normal choroid plexus tissue (right).

Figure 3.

Choroid plexus tumors are dependent on Myc. A, B. Quantitative RT-PCR showing Myc and p53 expression in choroid plexus carcinoma (CPC) and choroid plexus papilloma (CPP) compared to normal mouse cerebellum (CB) and normal choroid plexus (ChP). Error bars represent standard error of the mean (SEM) C. Western blot showing MYC expression in a collection of human CPC and CPP specimens. D. Effects of Myc inhibitor 10058-F4 on viability of murine CPC cells. E. Effects of Myc inhibitor KJ-Pyr-9 on viability of murine CPC cells. F. Effects of CD532 on viability of murine CPC cells. G and H. Effects of various

compounds that inhibit MYC on viability of human CPC cells (CHLA-CPC02 in G and CHLA-CPC03 in H). Error bars represent standard error of the mean (SEM).

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Figure 4.

Mouse choroid plexus carcinomas exhibit large chromosomal losses. A. Array CGH analysis of normal mouse choroid plexus (ChP) (n=3), choroid plexus papilloma (CPP) (n=5) and choroid plexus carcinoma (CPC) (n=10). B-D. High resolution image showing DNA copy number status on mouse chromosomes 8, 12 and 19. DNA copy number status is shown in integrative genomics viewer.

Figure 5.

CPC and CPP exhibit distinct gene expression profiles. A. Principal component analysis shows normal choroid plexus (ChP), choroid plexus papilloma (CPP) and choroid plexus carcinoma (CPC) cluster separately. B. Heat map of gene expression data shows CPP and CPC share common gene expression signatures but are distinct from each other. C, D. Pathway analysis reveals enrichment (C) or downregulation (D) of pathways in CPC compared to ChP.

Figure 6.

High throughput drug screening identifies compounds active against choroid plexus tumors.

A. Compounds identified in high throughput drug screening using mouse choroid plexus carcinoma cells. Percent viability (normalized to vehicle treat control) of mouse CPC, mouse CPP and patient-derived human CPC cells treated with each compound is shown. B. Effects of CDK inhibitors on viability of mouse CPC cells; IC50's are shown in the legend. C. Effects of CDK inhibitors Flavopiridol and Dinaciclib on phosphorylation of Rb at Ser 807/811. D. Effects of Triptolide on viability of mouse CPC cells. Error bars represent standard error of the mean (SEM). E. qRT-PCR showing effects of triptolide on Hspa1b, 3

hours after treatment of mouse CPC cells. Error bars represent standard error of the mean (SEM) F. Western Blot showing effects of triptolide on RNA polymerase II expression in mouse CPC cells.

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Table 1.
Genes that show decreased copy number and decreased expression in CPC

Genes listed showed significantly lower expression in murine CPC compared to murine CPP or ChP (expression fold change < -2.0) and exhibited copy number loss in both murine and human CPC (copy number fold change < -1.4)

Mouse Gene Symbol	Human Gene Symbol	Mouse CPC/CP Expression FC	Mouse CPC/ChP Expression FC	Human CPC Copy Number FC	Description
Lrrc9	LRRC9	-1.07257	-12.4918	-9.07091	leucine rich repeat containing 9
Nek5	NEK5	1.67621	-8.75255	-5.46295	NIMA related kinase 5
Dynlrb2	DYNLRB2	1.0788	-11.6175	-5.32223	dynein light chain roadblock-type 2
Ak7	AK7	1.04765	-18.8911	-5.20001	adenylate kinase 7
Cdhr3	CDHR3	-1.18149	-2.76221	-5.18214	cadherin related family member 3
Ccdc113	CCDC113	1.17339	-6.91464	-4.65974	coiled-coil domain containing 113
Slc22a8	SLC22A8	1.39518	-6.48663	-4.0717	solute carrier family 22 member 8
Tppp3	TPPP3	1.35701	-3.82637	-3.49017	tubulin polymerization promoting protein family member 3
Slc5a5	SLC5A5	-1.50415	-3.24411	-2.25699	solute carrier family 5 member 5
Aldh3b1	ALDH3B1	-1.89726	-4.22038	-1.98439	aldehyde dehydrogenase 3 family member B1
Tsnaxip1	TSNAXIP1	1.05724	-5.47203	-1.93413	translin associated factor X interacting protein 1
Aktip	AKTIP	-1.38847	-2.79752	-1.85756	AKT interacting protein
Fam92b	FAM92B	-1.86128	-2.01077	-1.81121	family with sequence similarity 92 member B
Slc16a12	SLC16A12	1.29146	-3.77031	-1.8044	solute carrier family 16 member 12
Itgb8	ITGB8	-2.00707	-2.85899	-1.75865	integrin subunit beta 8
1700030J22Rik	C16orf46	-1.1477	-3.54304	-1.71946	chromosome 16 open reading frame 46
Bbs1	BBS1	-1.37233	-2.9617	-1.6732	Bardet-Biedl syndrome 1
Aga	AGA	-2.02572	-1.8463	-1.66193	aspartylglucosaminidase
Prdx5	PRDX5	-1.53174	-2.13576	-1.63915	peroxiredoxin 5
Wdr35	WDR35	-1.31549	-2.51214	-1.59151	WD repeat domain 35
Rtn1	RTN1	-2.85685	-8.09233	-1.52236	reticulon 1
Calm1	CALM1	-1.86345	-2.17705	-1.49998	calmodulin 1 (phosphorylase kinase, delta)
Pla2g16	PLA2G16	-1.51443	-2.08918	-1.49705	phospholipase A2 group XVI
Lpcat2	LPCAT2	1.19479	-2.48945	-1.44088	lysophosphatidylcholine acyltransferase 2
Gabarap12	GABARAP12	-1.3509	-2.03335	-1.43023	GABA type A receptor associated protein like 2
Tusc3	TUSC3	-1.34314	-2.51689	-1.42215	tumor suppressor candidate 3
9030617003Rik	C14orf159	-4.91235	-1.67544	-1.41798	chromosome 14 open reading frame 159
Sorbs1	SORBS1	-1.47707	-2.09523	-1.41353	sorbin and SH3 domain containing 1
Erlin2	ERLIN2	-1.11342	-3.26837	-1.41209	ER lipid raft associated 2
Tmco3	TMCO3	-1.39928	-2.26471	-1.41063	transmembrane and coiled-coil domains 3