The cpk model of recessive PKD shows glutamine dependence associated with the production of the oncometabolite 2-hydroxyglutarate

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Hwang VJ, Kim J, Rand A, Yang C, Sturdivant S, Hammock B, Bell PD, Guay-Woodford LM, Weiss RH. The cpk model of recessive PKD shows glutamine dependence associated with the production of the oncometabolite 2-hydroxyglutarate. Am J Physiol Renal Physiol 309: F492–F498, 2015. First published July 8, 2015; doi:10.1152/ajprenal.00238.2015.—Since polycystic kidney disease (PKD) was first noted over 30 years ago to have neoplastic parallels, there has been a resurgent interest in elucidating neoplasia-relevant pathways in PKD. Taking a nontargeted metabolomics approach in the B6(Cg)-Cys1263Stop (cpk) mouse model of recessive PKD, we have now characterized metabolic reprogramming in these tissues, leading to a glutamine-dependent TCA cycle shunt toward total 2-hydroxyglutarate (2-HG) production in cpk compared with B6 wild-type kidney tissue. After confirmation of increased 2-HG expression in immortalized collecting duct cpk cells as well as in human autosomal recessive PKD tissue using targeted analysis, we show that the increase in 2-HG is likely due to glutamine-sourced α-ketoglutarate. In addition, cpk cells require exogenous glutamine for growth such that inhibition of glutaminase-1 decreases cell viability as well as proliferation. This study is a demonstration of the striking parallels between recessive PKD and cancer metabolism. Our data, once confirmed in other PKD models, suggest that future therapeutic approaches targeting this pathway, such as using glutaminase inhibitors, have the potential to open novel treatment options for renal cystic disease.

ARPKD; glutamine; metabolomics; oncometabolite; reprogramming

THE POLYCYSTIC KIDNEY DISEASES (PKD) are disorders characterized by, among other signaling events, dysregulated renal tubular epithelial (RTE) cell proliferation. While the concept that PKD is a “neoplasia in disguise” was initially suggested by Grantham in 1990 (9), it is becoming increasingly clear that the cystic renal diseases have marked biochemical similarities with many aspects of the malignant process (22). Consequently, PKD is now being investigated in the context of tumor metabolism with an eye toward discovery of new therapies and/or repurposing of currently approved or pipeline oncology drugs. Indeed, recent studies in our and other laboratories have shown connections between the cyclin-dependent kinases (1, 17, 18) and nuclear transport (26) inhibitors in PKD, as well as a striking parallel of metabolic reprogramming related to glycolysis and the Warburg effect, which was shown to occur in an autosomal dominant PKD model (21).

Given the current emphasis on the metabolic changes associated with oncogenesis, and since we have successfully utilized metabolomics to discover biomarkers and altered metabolic pathways in renal cell carcinoma (RCC) (8, 30), we took a nontargeted metabolomics approach to compare cancer with autosomal recessive PKD (ARPKD) metabolic pathways. We evaluated the three “matrices” (kidney tissue, serum, and urine) in the B6(Cg)-Cys1263Stop (cpk) mouse model of recessive PKD (10) and then validated the changes in human ARPKD tissue and unaffected controls. We then utilized mTERT-immortalized collecting duct cells derived from B6-cpk and B6 wild-type (WT) kidneys (24) and show that these cpk cells require glutamine and that an exogenous supply of this amino acid is required for production of the oncometabolite 2-hydroxyglutarate (2-HG) by these cells.

This is the first description of a striking parallel between PKD and cancer in both glutamine addiction and oncometabolite production, which, once confirmed in other PKD models and renal cystic diseases, holds the potential to uncover novel therapeutic approaches. This includes the consideration of glutaminase inhibitors and the repurposing of existing cancer drugs for treatment of renal cystic disease.

MATERIALS AND METHODS

Materials. Human ARPKD tissues provided by Dr. Lisa Guay-Woodford and Dr. Darren Wallace were obtained following approved Institutional Review Board protocols at the University of Alabama, Birmingham, the University of Kansas, and the University of California, Davis. The two human controls were male (21 min old) and female (10 mo old). ARPKD samples were male (or unknown) and between 26 and 35 days old. Dr. Lisa Guay-Woodford provided 14-day-old B6-cpk and B6-wt kidneys (10) obtained from animals under appropriate Institutional Animal Care and Use Committee approval at the Children’s National Health System, and the cpk cell lines were mTERT immortalized in the laboratory of Dr. P. Darwin Bell at the Medical University of South Carolina (24). Primary normal human RTE cells (NHK) were from Lonza, and the human ADPKD WT9-7 cell line was from the American Type Culture Collection.

Cell culture. NHK and WT9-7 cell lines were maintained as described (26); cpk cell lines were maintained in DMEM/F-12 with similar supplements and included 0.2 μg/ml dexamethasone, 10 nM triiodothyronine, and 1× insulin-transferrin-sodium selenite. Bis-2-(5-
Fig. 1. Metabolomic analysis shows that the TCA cycle is directed toward increased glutamine metabolism to 2-hydroxyglutarate (HG). A: nontargeted metabolomic analysis was performed on 8 wild-type (wt) and 8 cpk kidneys. B: targeted analysis was performed in 4 human autosomal recessive polycystic kidney disease (ARPKD) tissues and 2 unaffected controls. The metabolites increased (red) and decreased (green) are indicated. *P < 0.05, **P < 0.01, ***P < 0.001.
performed in DMSO, and the final concentration for all treatments was 0.01%. Glutamine-deficient experiments were performed in glutamine-free DMEM with supplements described.

**Metabolomic analysis.** For nontargeted metabolomic analysis, kidneys were snap frozen in liquid nitrogen immediately after nephrectomy and subjected to metabolomic analysis as previously described (4, 8).

Analysis of 2-HG in cpk kidney tissues followed the liquid chromatography-tandem mass spectrometry (LC-MS/MS) procedure adapted from Dang et al. (2). Quantification of 2-HG was confirmed by standard addition of a pure metabolite standard at known concentrations. All metabolites were scaled to each metabolite’s median value. Analysis of 2-HG, citrate, isocitrate, malate, fumarate, and glutamate and glutamate on human tissues as well as the cpk and WT cell lines were analyzed by gas chromatography-mass spectrometry (GC-MS) using the Agilent 7890A GC system. Metabolites were detected with an Agilent 5977A MSD equipped with an Agilent EI source. MS data were acquired in selective ion monitoring scan mode with electron multiplier gain set to 2.0.

**Immunoblotting.** Immunoblotting was performed as previously described (12). Isocitrate dehydrogenase (IDH) 2 (Abcam) was probed at 1:1,000, glutaminase (GLS)-1 (Abcam) was probed at 1:5,000, glutaminase (GLS)-2 (Abcam) was probed at 1:1,000, GLS-2 (Abcam) was probed at 1:1,000, and β-actin (Cell signaling) was probed at 1:2,000.

**Mutational analysis.** Mutational analysis was performed following methods described (14). Primers were designed from genomic sequences at least 100 bases upstream and downstream of the codons for IDH1 amino acid 132 and IDH2 amino acids 140 and 172. For IDH1 analysis, PCR was performed with the forward primer 5′-ACCAATGGCACCACAAA-3′ and reverse primer 5′-TTCATACCCTGGTTAATGGGTGT-3′ for amplification, and primer 5′-CGGTCTTCAGAGAAGCCATT-3′ for sequencing. For IDH2 analysis, PCR was performed with the forward primer 5′-GTCTGGCTGTGG-3′ and reverse primer 5′-CAGAGACAAGAGGATG-GCTAGG-3′ for amplification, and the same forward primer for sequencing. Amplicons were sequenced using standard methods.

**Cell assays.** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and methylene blue assays were performed as described (12). For small interfering (si) RNA knockdown, transfections were performed with Lipofectamine RNAiMAX using siRNAs targeting IDH2 (Dharmacon) or a nontargeting control (Dharmacon) following the manufacturer’s instructions.

**Statistical analysis.** Student’s t-tests were used for statistical analyses.

**Results and Discussion**

Nontargeted metabolomics analysis was performed in three matrices from eight 14-day-old cpk and 8 age-matched WT mice, demonstrating an increase in total 2-HG in cpk kidneys compared with WT kidneys that was concomitant with significant increases in acetyl-CoA and citrate. There was marked downregulation of the downstream TCA-cycle metabolites succinate, fumarate, and malate, as well as glutamine and glutamate (Fig. 1A), suggesting high utilization of glutamine and glutamate as well as a shunt in the TCA cycle toward the production of 2-HG. This is the first description of an oncometabolite present in a nonmalignant disease.

A parallel increase in 2-HG was seen in cpk compared with WT serum (fold-change 1.54; P < 0.05). Additionally, in cpk urine, glutamine (fold-change 0.36; P < 0.001), glutamate (fold-change 0.42; P < 0.05), and fumarate (fold-change 0.47; P < 0.001) were significantly decreased in cpk compared with WT, but no other TCA metabolites were found to be significantly different. These data are consistent with our previous
Fig. 3. Isocitrate dehydrogenase (IDH2) and glutamine (Gln) play a role in 2-HG production. Cells were grown in media deprived of Gln or treated with 10 μM BPTES for 9 h, and 2-HG levels were assessed in cpk conditioned media (A) and cpk cells (B). C: knockdown of wt Idh2 in cpk cells was confirmed and resulted in decreased levels of 2-HG in conditioned media (D). E: overexpression of wt IDH2 and mutant (mut)-IDH2 R172K was confirmed and resulted in increased production of 2-HG in conditioned media (F). Small interfering (si) Control, scrambled siRNA control; siIDH2, siRNA targeted against IDH2; CMV-GFP, transfection control plasmid; wt IDH2, wt IDH2 plasmid. Shown are representative values of at least 3 experiments. ***P < 0.001. **P < 0.05. *P < 0.01.

Fig. 4. Cpk cells and a human ADPKD cell line are dependent on exogenous Gln for survival and proliferation. A: Cpk and wt cells were subjected to immunoblotting with GLS1 and GLS2 antibodies. Cpk and WT9-7 and their respective controls were incubated for 72 h in Gln-depleted media and subsequently assayed by MTT (cell viability, B and D) or methylene blue (relative growth to day 0, C and E). Shown are representative values of at least 3 experiments. ***P < 0.001. **P < 0.05. *P < 0.01.
work which showed that tissue metabolomics is the most sensitive of the three matrices for uncovering biological changes (8).

To determine whether this metabolomic profile is also evident in human ARPKD, we performed targeted metabolomics analysis of four ARPKD kidney tissues (between 26 and 35 days old) and two available unaffected neonatal controls (21 min and 10 mo old). We found increases in levels of 2-HG (fold-change 6.5; \( P < 0.02 \)), citrate (fold-change 23.46; \( P < 0.001 \)), isoositrate (fold-change 8.2; \( P < 0.001 \)), and \( \alpha \)-KG (fold-change 1.2; \( P < 0.24 \)), and decreases in levels of glutamate (fold-change 0.74; \( P < 0.03 \)), glutamine (fold-change 0.56; \( P < 0.04 \)), fumarate (fold-change 0.55; \( P < 0.02 \)), and malate (fold-change 0.5; \( P < 0.2 \)) in ARPKD tissue compared with control tissue (Fig. 1B). While the levels of \( \alpha \)-KG and malate in human ARPKD tissue were not found to be significantly different from normal tissues, the significant changes of these metabolites in the larger cohort of cpk tissue suggest that these observations represent a valid biological phenomenon in cystic disease. Interestingly, in RCC, citrate was also increased and malate and fumarate were decreased in all grades compared with normal control (30), further demonstrating the similarities between cancer and PKD (22). Overall, our metabolomic analyses are consistent in cpk and human ARPKD kidneys and indicate metabolic reprogramming leading to a glutamine-dependent TCA cycle shunt toward total 2-HG production; this data also indicate that the cpk cells and tissues can serve as a viable model for further study of ARPKD metabolism.

2-HG has been of particular interest as it is the first oncometabolite described in many cancers, including RCC (23), and has been proposed to contribute to tumor growth and cell proliferation through epigenetic modifications (6, 14, 33). Using GC/LC-MS with a known 2-HG standard (Fig. 2A), we validated the production of 2-HG and confirmed that there are higher 2-HG levels in cpk mutants compared with WT in tissues, cells, and cell-conditioned media (Fig. 2, B–D).

The majority of 2-HG overproduction in cancer has been linked to mutant IDH1 or -2 in cancer, producing 2-HG from glutamine-sourced citrate (32). To rule out the possibility that known mutations are occurring in the ARPKD tissues and cells tested causing 2-HG production in this manner, we evaluated the relevant genes and proteins. No change in D2HGDH protein expression was observed in either human ARPKD tissue and cpk kidney tissue (data not shown); sequencing of \( IDH1 \) and \( IDH2 \) in cpk cells revealed the absence of any variants compared with the murine reference genome available at GenBank (19, 34). PHGDH does not produce 2-HG from \( \alpha \)-KG in the murine cells (5).

Glutamine can contribute to 2-HG synthesis through conversion to \( \alpha \)-KG, via glutaminase (GLS-1) and glutamate dehydrogenase in cancer cells (20, 28, 32). Therefore, we next evaluated this pathway in our PKD model. cpk cells grown in glutamine-free media or treated with the specific GLS-1 inhibitor BPTES resulted in lower levels of 2-HG in conditioned media (Fig. 3A) and cells (Fig. 3B). Additionally, as knockdown of WT \( IDH2 \) in breast cancer resulted in decreased levels of 2-HG, we also investigated the impact of siRNA knockdown on 2-HG production in recessive PKD (28). siRNA knockdown of WT \( Idh2 \) resulted in a decrease in 2-HG (Fig. 3, C and D) while overexpression of the WT \( IDH2 \) plasmid resulted in an increase in 2-HG in cpk conditioned media, although considerably less than with the mutant \( IDH2 \) R172K plasmid, which served as a positive control (Fig. 3, E and F). Thus it is likely that 2-HG in recessive PKD arises from glutamine-sourced \( \alpha \)-KG, a finding that is supported by low levels of glutamine and glutamate in cpk and human ARPKD renal tissue (see Fig. 1). These data suggest manipulation of glutamine transport or GLS-1 inhibition as a possible therapeutic approach to cystic disease.

Concluding Remarks

In this study, we demonstrate a striking parallel between recessive PKD and cancer metabolism using the cpk mouse model with validation in human ARPKD tissue. These data suggest that recessive PKD cells are reprogrammed to utilize exogenous supply of this amino acid presumably required for high levels of synthesis of protein and nucleotides (11, 30, 31). Given the similarity between many of the hallmarks of cancer and PKD (22), we asked whether glutamine is required for growth and viability of these cells. We first evaluated the levels of GLS1 and -2, which catalyze the most proximal step of glutamine metabolism, and found that WT and cpk cells express both isoforms (13) (Fig. 4A). Cells incubated for 3 days in glutamine-free media showed significant decreases in viability as well as proliferation in cpk, but not in WT cells (Fig. 4, B and C). To begin to evaluate these findings as possibly a general phenomenon of human cystic disease, we evaluated the glutamine requirement of the ADPKD human cell line WT9-7 compared with primary normal human proximal tubule epithelial (NHK) controls and found a similar glutamine dependence (Fig. 4, D and E).

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AUTHOR CONTRIBUTIONS


DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


