

# Circulating Oncometabolite 2-hydroxyglutarate as a Potential Biomarker for Isocitrate Dehydrogenase (*IDH1/2*) Mutant Cholangiocarcinoma

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## ABSTRACT

Isocitrate dehydrogenase (IDH) enzymes catalyze the decarboxylation of isocitrate to alpha-ketoglutarate ( $\alpha$ KG). *IDH1/2* mutations preferentially convert  $\alpha$ KG to R-2-hydroxyglutarate (R2HG), resulting in R2HG accumulation in tumor tissues. We investigated circulating 2-hydroxyglutarate (2HG) as potential biomarkers for patients with *IDH-mutant (IDHmt)* cholangiocarcinoma (CCA).

R2HG and S-2-hydroxyglutarate (S2HG) levels in blood and tumor tissues were analyzed in a discovery cohort of patients with *IDHmt* glioma and CCA. Results were validated in cohorts of patients with CCA and clear-cell renal cell carcinoma.

The R2HG/S2HG ratio (rRS) was significantly elevated in tumor tissues, but not in blood for patients with *IDHmt* glioma,

while circulating rRS was elevated in patients with *IDHmt* CCA. There were overlap distributions of circulating R2HG and total 2HG in patients with both *IDHmt* and *wild-type (IDHwt)* CCA, while there was minimal overlap in rRS values between patients with *IDHmt* and *IDHwt* CCA. Using the rRS cut-off value of 1.5, the sensitivity of rRS was 90% and specificity was 96.8%.

Circulating rRS is significantly increased in patients with *IDHmt* CCA compare with patients with *IDHwt* CCA. Circulating rRS is a sensitive and specific surrogate biomarker for *IDH1/2* mutations in CCA. It can potentially be used as a tool for monitoring *IDH*-targeted therapy.

## Introduction

Cholangiocarcinoma (CCA) is a heterogeneous group of hepatobiliary tumors that have a poor prognosis. Advanced CCA is traditionally subclassified by anatomic sites into intrahepatic cholangiocarcinoma (iCCA) and extrahepatic cholangiocarcinoma (eCCA). Recently, genomic advances have partially unveiled the complex molecular landscape of CCA, shedding new light on novel therapeutic opportunities and ushering in the era of precision oncology for 40%–55% of patients with CCA (1). Among these putatively actionable alterations, mutations in the isocitrate dehydrogenase (*IDH1/2*) genes are detected in 15% iCCA and <5% of eCCA (2–4). *IDH1/2* mutations are also seen in other cancers including low-grade glioma (80%), acute myeloid leukemia (20%), and central chondrosarcoma (80%) (5, 6). The majority of *IDH1* and *IDH2* point mutations occur at residues arginine 132 (R132) or 172 (R172), respectively. *IDH* is an essential enzyme in the tricarboxylic acid cycle that catalyzes the decarboxylation of isocitrate

to alpha-ketoglutarate ( $\alpha$ KG) (6–8). However, *IDH1/2* mutations preferentially convert  $\alpha$ KG to R-2-hydroxyglutarate (R2HG) (9, 10). The aberrant R2HG production from *IDH1/2* mutations results in R2HG accumulation, and its concentration in *IDH-mutant (IDHmt)* tumor tissues can reach 5–30 mmol/L, far higher than its physiologic concentrations ( $\approx$ 100  $\mu$ mol/L) (11). Interestingly, the S-enantiomer (S2HG) concentrations remain low in both *IDHmt* and *wild-type* cancers.

Because 2HG is a membrane-diffusible small molecule, multiple attempts have been undertaken to explore the feasibility of circulating total 2-hydroxyglutarate (t2HG) or R2HG concentrations as alternative diagnostic tools for identifying *IDH1/2* mutations in various *IDHmt* malignancies. Studies have demonstrated elevated levels of circulating t2HG and/or R2HG in *IDHmt* cancers, including CCA (12–20). We previously reported that tissue ratios of R2HG over S2HG (rRS) are a more sensitive biomarker for *IDH1/2* mutations in patients with glioma than R2HG (21). In this study, we investigated circulating R2HG, t2HG, and rRS as potential biomarkers for *IDH1/2* mutations in patients with CCA.

## Materials and Methods

Tissue and blood samples were collected from patients who were participating in studies approved by the Research Ethics Board at University Health Network, Toronto, Canada. Written informed consent was obtained from each patient before biospecimen collection. These studies were conducted in accordance with the Declaration of Helsinki.

The study consisted of two cohorts of patients (Fig. 1). The discovery cohort comprised of patients with low-grade glioma or metastatic CCA. These patients were known to be *IDHmt*. The presence of *IDH1/2* mutations was determined via either IHC for patients with glioma or next-generation sequencing for patients with CCA as a part of standard clinical care. Matched

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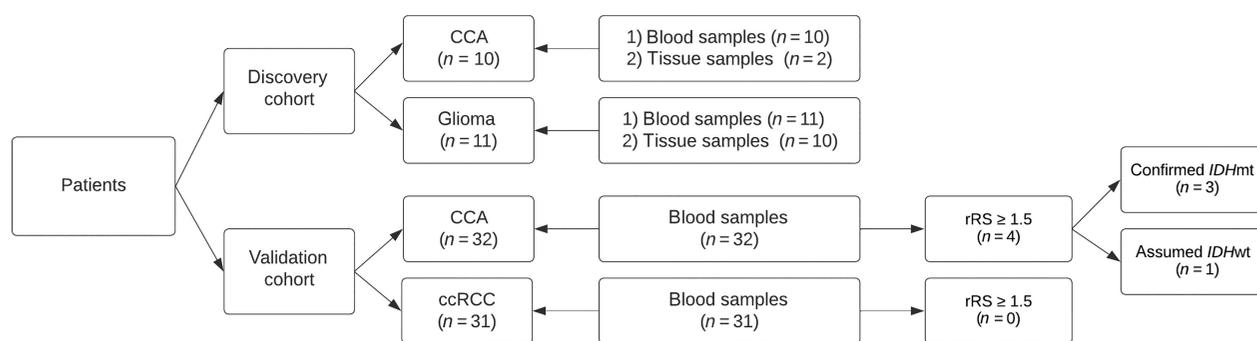
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**Figure 1.**

Study flow diagram. CCA: Cholangiocarcinoma; ccRCC: clear-cell renal cell carcinoma; 2HG: 2-Hydroxylutarate; rRS: ratio of R2HG/S2HG; *IDHmt*: *IDH* mutated; *IDHwt*: *IDH* wild type.

tissue and blood samples were collected where available from these patients.

The validation cohort consisted of a second group of patients with metastatic CCA, whose blood samples were collected as a part of institutional biobanking initiatives. The mutational status of *IDH1/2* in this group of patients with CCA was unknown except for one patient who was known to be *IDHmt* based on testing as part of routine clinical care. For patients who were suspected of harboring *IDH1/2* mutations based on the elevated circulating rRS results, mutational analysis using circulating tumor DNA (ctDNA) was performed in a Clinical Laboratory Improvement Amendments–certified laboratory. In addition, a group of patients with clear-cell renal cell carcinoma (ccRCC) was included as a control group because *IDH1/2* mutations have not been reported in the ccRCC population.

Tissue and blood samples collected were analyzed for R2HG and S2HG using a validated high-performance liquid chromatography (HPLC) tandem mass spectrometry method as reported previously (21).

Summary statistics were presented for R2HG, S2HG, t2HG, and rRS. The t2HG (t2HG = R2HG + S2HG) and rRS (rRS = R2HG/S2HG) values were calculated on the basis of measured levels of S2HG and R2HG. R2HG, S2HG, t2HG, and rRS were compared using *t* tests or one-way ANOVA between patients with glioma and CCA, between tissue and circulating 2HGs, or among cohorts using Prism (Version 9.5.1, GraphPad Software). Results were considered statistically significant if  $P \leq 0.05$ .

#### Data availability

The data generated in this study are available upon request from the corresponding author.

## Results

In the discovery cohort, a total of 21 patients with *IDHmt* tumors were included (glioma = 11, CCA = 10). Blood samples were available for all 21 patients, while sufficient tissue samples were available for 10 patients with glioma but only 2 patients with CCA. The validation cohort included 32 patients with CCA. There were no significant differences in demographic characteristics between patients with CCA enrolled in the discovery and validation cohorts (Table 1).

In patients with *IDHmt* glioma, S2HG levels were significantly lower in tumor tissues than in the blood, while R2HG levels were not statistically different (Table 2; Fig. 2). As a result, rRS was significantly higher in tumor tissue than in blood ( $46.4 \pm 42.3$  vs.  $1.1 \pm 0.5$ ,  $P = 0.002$ ). Because of limited tissue availability for patients with CCA, comparisons between tissue and blood 2HG levels were not performed. For 2 patients with CCA with sufficient tumor tissues for analysis, their tissue rRS levels were 144.8 and 244.9, respectively. Comparing patients with glioma and patients with CCA, circulating R2HG was significantly higher in patients with CCA while there was no difference seen in S2HG (Fig. 2). Consequently, circulating t2HG and rRS were significantly higher in patients with CCA than those in patients with glioma.

Among 32 patients with CCA in the validation cohort, 1 patient (Patient 460) was known to harbor *IDH1R132C* mutation (Table 3). Circulating 2HG analysis revealed additional 2 patients with abnormally high R2HG and rRS levels (Patients 376 and 468).

The distribution of S2HG, R2HG, and t2HG was wide in both patients with glioma and patients with CCA (Figs. 2 and 3). Although circulating R2HG and t2HG were significantly higher in patients with *IDHmt* CCA than those in *IDHwt* patients, there was considerable overlap in R2HG and t2HG concentrations in these patients. However,

**Table 1.** Characteristics of patients across cohorts.

Characteristic	Discovery cohort		Validation cohort	
	Glioma	CCA	CCA	ccRCC
<b>Number of patients</b>	11	10	32	31
<b>Sex, male (%)</b>	8 (73%)	5 (50%)	17 (53%)	26 (84%)
<b>Age, years</b>				
Median (range)	42.0 (23.2–66.6)	63.7 (48.0–78.9)	64.5 (42.3–78.1)	60 (30.0–81.0)

Abbreviations: CCA: Cholangiocarcinoma; ccRCC: clear cell renal cell carcinoma.

**Table 2.** Tissue and circulating S2HG, R2HG, t2HG, and rRS mean levels for patients with glioma and CCA in the discovery cohort.

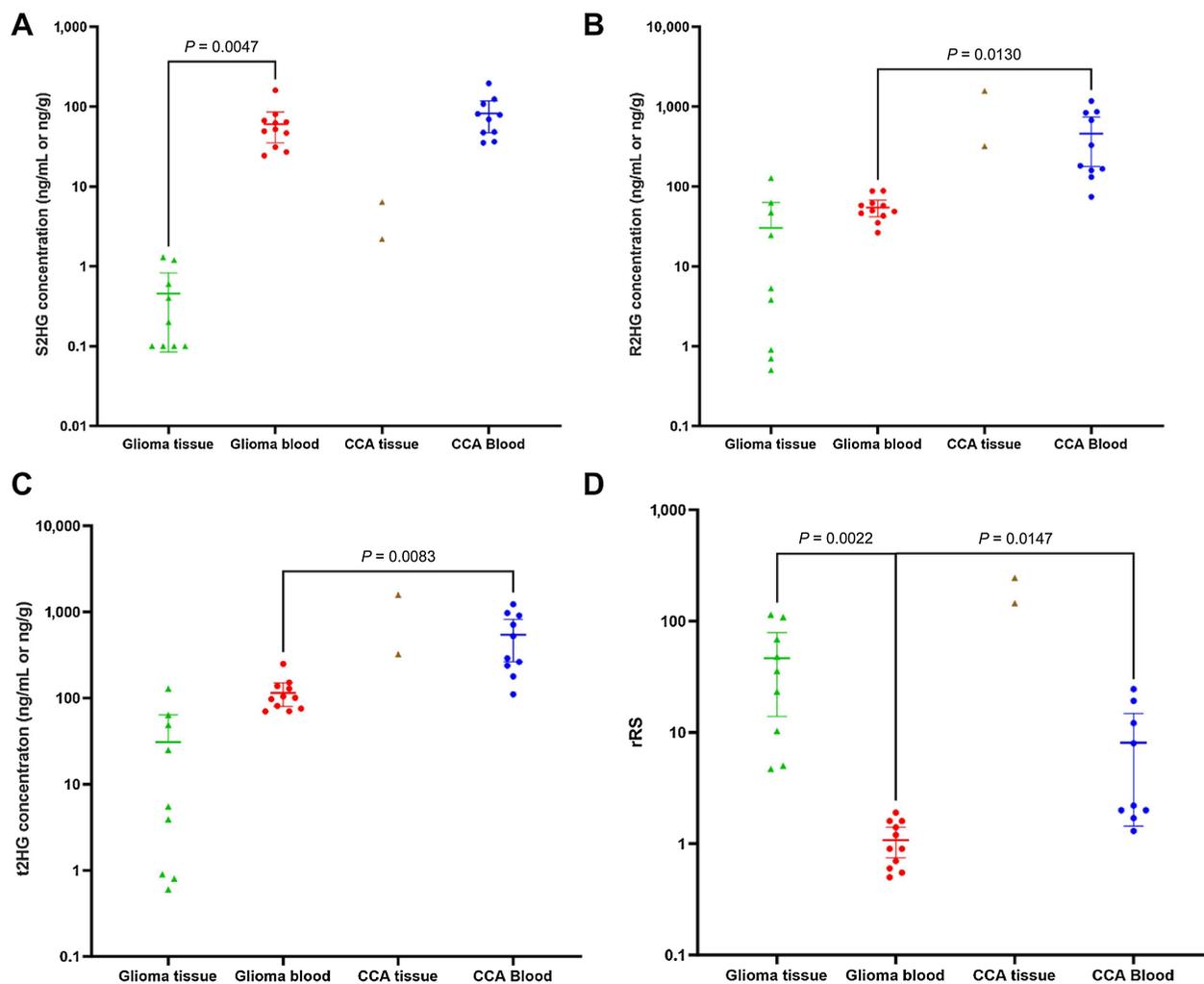
Biomarkers	Glioma			Cholangiocarcinoma	
	Serum (ng/mL) (n = 11)	Tissue (ng/g) (n = 10)	P value	Serum (ng/mL) (n = 10)	Tissue (ng/g) (n = 2)
<b>S2HG</b>	60.3 ± 37.4	0.4 ± 0.5	<b>0.005</b>	82.3 ± 49.4	4.3 ± 3.0
<b>R2HG</b>	54.7 ± 19.3	30.3 ± 42.9	NS	458.8 ± 392.9	945.0 ± 883.9
<b>t2HG</b>	114.9 ± 52.1	30.7 ± 43.2	<b>NS</b>	541.0 ± 338.8	949.3 ± 886.9
<b>rRS</b>	1.1 ± 0.5	46.4 ± 42.3	<b>0.002</b>	7.6 ± 8.3	194.9 ± 70.8

Abbreviations: R2HG, R-2-hydroxyglutarate; rRS, ratio of R2HG/S2HG; S2HG, S-2-hydroxyglutarate; t2HG, total of R2HG+S2HG.

the circulating rRS distributed tightly around  $0.8 \pm 0.2$  (range: 0.45–1.55) in *IDHwt* patients (Table 3). For the cohort of patients with ccRCC, the mean rRS was  $0.8 \pm 0.3$ , ranging from 0.2 to 1.5.

rRS was significantly higher among patients with *IDHmt* CCA in both discovery and validation cohorts than that for patients with *IDHwt* CCA. In the discovery cohort, only 1 patient with *IDHmt* CCA had a rRS value lower than 1.5 (1.3), while only 1

*IDHwt* patient in the validation cohort had a rRS value higher than 1.5 (1.55). In addition to Patient 460, 2 patients with CCA in the validation cohort had rRS values above 1.5 (Table 3). Subsequent ctDNA analysis confirmed that both patients harbored *IDH1R132C* mutations. Using a cut-off rRS value of 1.5, the sensitivity of rRS for predicting *IDH1/2* mutation status was 90%, and the specificity was 96.8%.

**Figure 2.**

S2HG (A), R2HG (B), t2HG (C), and rRS (D) in tissue and blood samples from the discovery cohort. S2HG: S-2-hydroxyglutarate; R2HG: R-2-hydroxyglutarate; t2HG: total of R2HG+S2HG; rRS: ratio of R2HG/S2HG. Y-axis is in logarithmic scale.

**Table 3.** Circulating R2HG, S2HG, t2HG, and rRS for patients with CCA and ccRCC in the validation cohort.

Characteristics	S2HG (ng/mL)	R2HG (ng/mL)	t2HG (ng/mL)	rRS
<b>All patients with CCA (n = 32)</b>	166.1 ± 74.4	316.8 ± 625.1	482.9 ± 654.6	1.7 ± 2.9
Mean ± SD (range)	(79.7–356)	(67.4–2,570)	(148.8–2,747)	(0.5–14.5)
<b>CCA with IDH1/2 mutations (n = 3)</b>				
Patient 376	242	2,290	2,532	9.5
Patient 468	177	2,570	2,747	14.5
Patient 460	324	1,730	2,054	5.3
Mean ± SD (range)	247.7 ± 73.7 (177–324)	2196.7 ± 427.7 (1,730–2,570)	2444.3 ± 354.7 (2,054–2,747)	9.8 ± 4.6 (5.3–14.5)
<b>IDH wild-type CCA (n = 29)</b>				
Mean ± SD (range)	157.7 ± 70.4 (79.7–356)	122.3 ± 41.4 (67.4–226)	280 ± 103 (148.8–538)	0.8 ± 0.2 (0.5–1.6)
<b>All patients with ccRCC (n = 31)</b>	89.0 ± 70.9	60.2 ± 19.8	149.2 ± 85.4	0.8 ± 0.3
Mean ± SD (range)	(35.1–365)	(34.7–124)	(72.1–451.2)	(0.2–1.5)

Abbreviations: R2HG, R-2-hydroxyglutarate; rRS, ratio of R2HG/S2H; S2HG, S-2-hydroxyglutarate; t2HG, total of R2HG+S2HG.

## Discussion

CCA is characterized by overlapping, low-penetrance genomic alterations that span diverse signaling pathways (22). Identification of *IDH1/2* mutations has enabled targeted therapy in a subset of patients with CCA. Numerous agents targeting *IDHmt* cancers, such as ivosidenib, vorasidenib, ceralasertib, olutasidenib, enasidenib, and BAY1436032, have either received regulatory approvals or are in various stages of clinical development (23–25). The ClarIDHy trial evaluated ivosidenib in patients with refractory *IDHmt* CCA (24). Ivosidenib provides a modest, but statistically significant improvement in progression-free survival of 1.3 months, compared with placebo (24).

Obtaining tumor tissue samples for molecular profiling remains a clinical challenge in patients with CCA. Some patients are diagnosed on the basis of bile duct brushing during endoscopic retrograde cholangiopancreatography. Even with core biopsies, there is a considerable failure rate in yielding sufficient tumor DNA content for accurate pathologic diagnosis or comprehensive molecular profiling (26, 27). This limitation can be attributed to the challenging characteristics of CCA, including its desmoplastic and necrotic nature, as well as the presence of intratumoral heterogeneity (28–30).

The intriguing aspect of *IDHmt* tumors is the aberrant production and accumulation of the circulating oncometabolite 2HG, specifically R2HG rather than S2HG (7, 9–11, 31). Under physiologic conditions, R2HG and S2HG exist at low but equal levels. Both are small molecules that can readily diffuse across cellular membranes (11). There has been a significant interest in the evaluation of circulating 2HG as a biomarker for *IDH1/2* mutations in various hematologic and solid malignancies (12–20, 32). Janin and colleagues found that elevated serum 2HG concentrations reliably diagnosed *IDHmt* acute myeloid leukemia, achieving 100% sensitivity and 79% specificity when compared with wild-type cases (20). Lombardi and colleagues showed urinary 2HG concentrations were not elevated in patients with *IDHmt* glioma (13). Similarly, Capper and colleagues concluded that circulating 2HG were not elevated in patients with *IDHmt* glioma (14). For other solid tumors, circulating 2HG concentrations were reported to be a surrogate biomarker of *IDHmt* (12, 15, 16). In addition, circulating 2HG levels were shown to correlate with tumor burden in patients with *IDHmt* CCA (16).

One possible explanation for the high levels of circulating 2HG levels in *IDHmt* CCA, but not in patients with glioma, is due to the

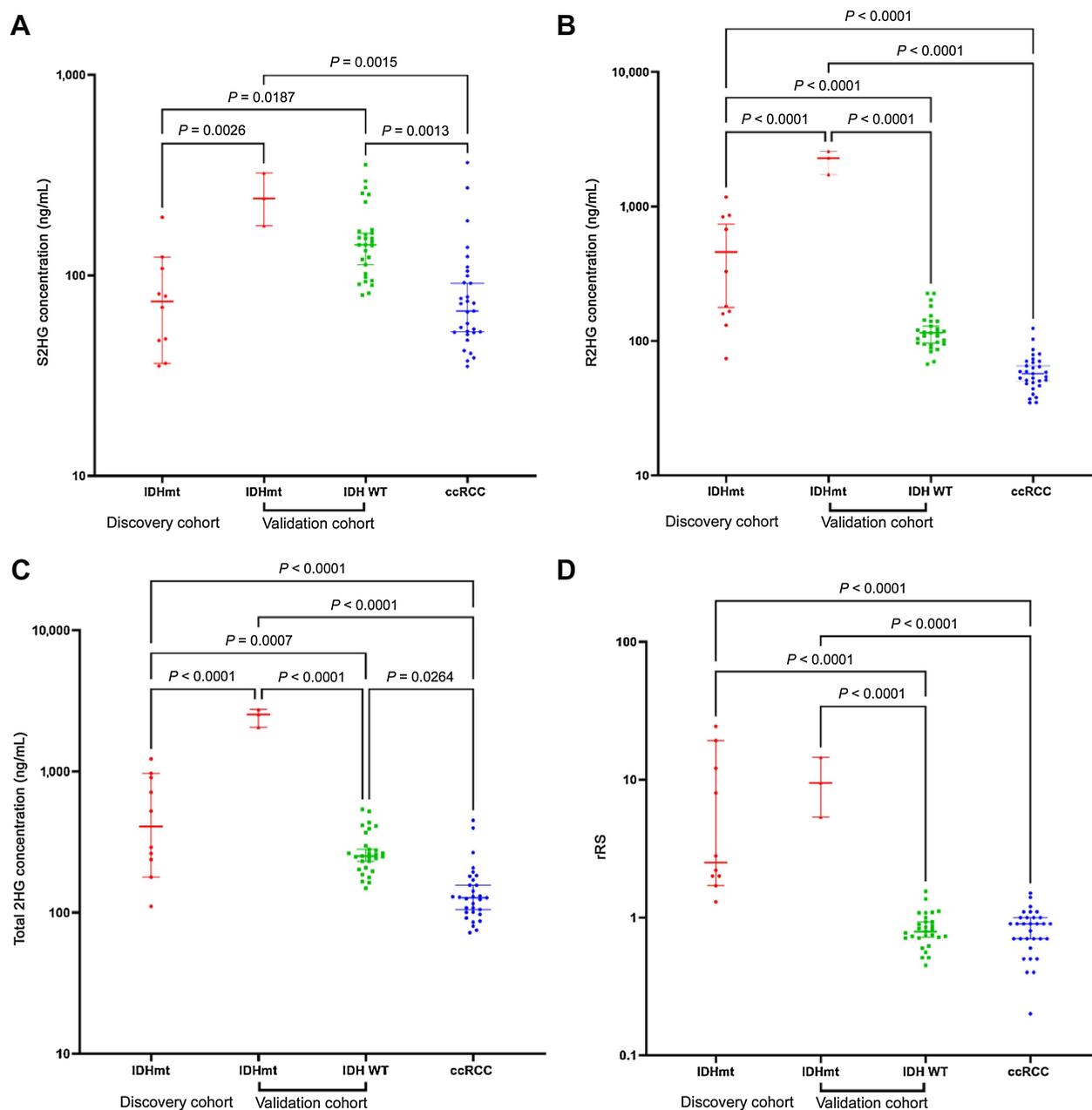
relative volumes of the circulatory system and brain tumor lesions. Any 2HG released from glioma lesions is diluted, making it harder to detect elevated 2HG in the peripheral blood of these patients.

We previously demonstrated that tissue rRS represents a highly sensitive and specific biomarker for *IDH1/2* mutations in glioma, compared with R2HG or t2HG levels (21). The absolute levels of R2HG and/or t2HG are influenced by tumor volumes, cellularity and different point mutations in the *IDH1/2* genes. These limitations can be mitigated by using S2HG as a readily available internal standard because R2HG and S2HG share the same physiochemical properties except chirality. As demonstrated in this study, there is considerable overlap of circulating R2HG or t2HG between patients with *IDHwt* and *IDHmt* CCA, while there is minimal overlap in rRS between patients with *IDHwt* and *IDHmt* CCA. Therefore, the circulating rRS could offer a more sensitive and specific biomarker for identifying *IDH1/2* mutations in patients with CCA, particularly in cases where traditional tissue-based methods are not feasible or practical.

The majority of previous studies on circulating 2HG only measured t2HG. We employed an analytic method that can quantitate R2HG and S2HG enantiomers separately, enabling the interpretation of rRS as a biomarker for *IDH1/2* mutations in CCA. Our discovery cohort in this study is limited by the small number; however, the difference between tissue and serum 2HG levels in patients with glioma and the elevated circulating rRS in patients with *IDHmt* CCA are striking. Only 2 patients with CCA in the validation cohort were selected for subsequent ctDNA analysis. This decision was made based on the cost of performing ctDNA analysis.

Recent advances in ctDNA technology have revolutionized genomic analysis by eliminating the need for tumor tissue samples. However, the analysis of circulating 2HG has advantages as it requires a small volume of blood (<0.5 mL) and it can be completed within 30 minutes (33, 34). The cost is also a fraction of the cost of ctDNA analysis. The ClarIDHy trial demonstrated that ivosidenib inhibited 2HG production and reduced circulating 2HG levels in responding patients (35, 36). Hence, circulating 2HG analysis represents a cost-effective method for serially monitoring responses to *IDHmt* targeted therapies.

Our results show that circulating R2HG, t2HG, and rRS are elevated in patients with *IDHmt* CCA, and rRS is a sensitive and specific biomarker for *IDHmt*, consistent with the biology of *IDH1/2* mutations. The potential of circulating rRS as a biomarker

**Figure 3.**

Circulating S2HG (A), R2HG (B), t2HG (C), and rRS (D) for patients with CCA in the discovery and validation cohorts. S2HG: S-2-hydroxyglutarate; R2HG: R-2-hydroxyglutarate; t2HG: total of R2HG+S2HG; rRS: ratio of R2HG/S2HG. Y-axis is in logarithmic scale.

for response to *IDH1/2* targeted therapies should be incorporated in future studies of *IDHmt* targeting agents.

### Authors' Disclosures

G.M. O'Kane reports personal fees from AstraZeneca, Servier, MSD, Incyte; and grants and personal fees from Roche outside the submitted work. W.P. Mason reports personal fees from Merck; grants and personal fees from Servier and Novocure; grants from Hoffman La Roche; and personal fees from Ont Therapeutics outside the submitted work. A.R. Hansen reports grants from Roche,

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## Authors' Contributions

**C.L. Lee:** Conceptualization, data curation, formal analysis, investigation, visualization, writing—original draft, writing—review and editing. **G.M. O'Kane:** Data curation, writing—review and editing. **W.P. Mason:** Data curation, writing—review and editing. **W.-j. Zhang:** Formal analysis, writing—review and editing. **P. Spiliopoulou:** Data curation, writing—review and editing. **A.R. Hansen:** Data curation, writing—review and editing. **R.C. Grant:** Data curation, writing—review and editing. **J.J. Knox:** Data curation, writing—review and editing. **T.L. Stockley:** Formal analysis, writing—review and editing. **G. Zadeh:** Data curation, writing—review and editing. **E.X. Chen:** Conceptualization, resources, data curation,

supervision, funding acquisition, visualization, project administration, writing—review and editing.

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