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IDH1 and IDH2 have critical roles in 2-hydroxyglutarate production in D-2-hydroxyglutarate dehydrogenase depleted cells

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ABSTRACT

D-2-hydroxyglutaric aciduria (D-2HGA) is a hereditary metabolic disorder characterized by the elevated levels of D-2-hydroxyglutaric acid (D-2HG) in urine, plasma and cerebrospinal fluid. About half of the patients have autosomal recessive mutations in D-2-hydroxyglutarate dehydrogenase (D2HGDH) gene. To analyze the origin of D-2HG in D2HGDH-depleted cells, we used small interfering RNA (siRNA) techniques. We found that knockdown of D2HGDH in MCF7 cells increased the levels of 2HG, mimicking D2HGDH mutant cells. Additional knockdown of isocitrate dehydrogenase 1 (IDH1) or isocitrate dehydrogenase 2 (IDH2) decreased the level of 2HG in D2HGDH knockdown MCF7 cells. Conversely, ectopic expression of IDH1 or IDH2 increased 2HG in MCF7 cells. These results suggest that IDH1 and IDH2 have roles in production of D-2HG in cells.

1. Introduction

D-2-hydroxyglutaric aciduria (D-2HGA) is a rare hereditary neurometabolic disorder (OMIM ID: 600721). Clinical symptoms of patients with D-2HGA are developmental delay, epilepsy, hypotonia, and dysmorphic features. All patients have consistently increased D-2-hydroxyglutarate (D-2HG) levels in urine, plasma and cerebrospinal fluid. For type I D-2HGA patients, accounting for about 50% of the disorder, autosomal recessive mutations of D2HGDH gene are the cause of the disease [1–3]. D2HGDH encodes D-2-hydroxyglutarate dehydrogenase, which converts D-2HG to 2-oxoglutarate and decreases the level of D-2HG.

However, the source of D-2HG is not fully understood. Struys and colleagues [4] analyzed the origin of D-2HG with lymphoblasts from type I D-2HGA patients, suggesting that D-2HG was derived from 2-oxoglutarate. They also revealed that hydroxyacid oxoacid transhydrogenase enzyme (HOT) produce D-2HG from 2-oxoglutarate as a coupling reaction with oxidation of gamma-hydroxybutyrate to succinic semialdehyde [5]. However, there is no data on amino acid sequence of HOT, and the molecular mechanism remains to be understood.

Recently, isocitrate dehydrogenase 2 (IDH2) mutations were found in the D-2HGA patients without D2HGDH mutation whose conditions are categorized as type II D-2HGA [6]. Wild type IDH2 is a metabolic enzyme that converts isocitrate to 2-oxoglutarate. In these patients, IDH2 mutant protein, either IDH2R140Q or IDH2R140G, catalyzes 2-oxoglutarate to D-2HG. Thus, in the type II patients, mutant IDH2 proteins produce D-2HG.

Interestingly, IDH2 and its paralog isocitrate dehydrogenase 1 (IDH1) were reported to be mutated in several neoplasms such as glioma [7,8], acute myelogenous leukemia [9,10], myelodysplastic syndrome [11,12], and chondrosarcoma [13–15]. Almost all mutations were restricted to IDH1R132, IDH2R140 or IDH2R172 residues and the mutant proteins catalyze 2-oxoglutarate to D-2HG, resulting in high D-2HG production in mutant tumor cells [10,16]. Therefore, these mutant IDH1/IDH2s gain much attention in cancer area [17].

In the analysis of enzymatic activity of IDH1, Pietrak et al., showed that wild type IDH1 is able to convert 2-oxoglutarate to D-2HG, although mutant IDH1 produce D-2HG much more efficiently [18].

From these reports, we hypothesized that wild type IDH1 and IDH2 might have roles in D-2HG production in type I D-2HGA patients. Here we showed that IDH1 and IDH2 are responsible of

Abbreviations: D-2HGA, D-2-hydroxyglutaric aciduria; D-2HG, D-2-hydroxyglutarate; 2HG, 2-hydroxyglutarate; D2HGDH, D-2-hydroxyglutarate dehydrogenase; IDH1, isocitrate dehydrogenase 1; IDH2, isocitrate dehydrogenase 2; HOT, hydroxyacid oxoacid transhydrogenase.

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D-2HG production in D2HGDH depleted cells. Our data indicate that inhibiting endogenous IDH1/2 activity could be a novel medication for type I D-2HGA patients.

2. Materials and methods

2.1. Cell culture, transfection of siRNAs and plasmids

MCF7 cells were obtained from ATCC. MCF7 cells were maintained in RPMI1640 (Life Technologies) supplemented with 10% FBS (Hyclone). For the knockdown experiments, we used siRNAs from Dharmacon. Catalog numbers are D-001810-01-20 (siControl), J-184309-09-0005 (siD2HGDH#1), J-184309-12-0005 (siD2HGDH#2), J-008294-10-0005 (siIDH1), and J-004013-09-0005 (siIDH2). We used Lipofectamine RNAiMax (Life Technologies) to transfect siRNAs according to the manufacture's instruction. Each siRNA was used at 5 nM throughout this study. For the D2HGDH/IDH1/IDH2 knockdown experiments, MCF7 cells were harvested four days after siRNA transfection, and then 2HG were extracted by the method described below.

For the ectopic expression experiments, MCF7 cells were first treated with siControl or siD2HGDH#1. On the next day, plasmids were transfected to the same number of cells using the Neon transfection system (Life Technologies). Cells were collected three days after transfection of plasmids and their 2HG level were analyzed as described below.

2.2. Extraction of 2HG

Cells were collected and the volume was adjusted to 80 μL with the medium. The cells were lysed by adding 320 μL of chilled methanol and placed at −80 °C for 20 min, then 240 μL of ice-cold water was added.

Subsequently, these cell lysates were pretreated with an OASIS MAX μElution plate (Waters Corp.). To 80 μL of the lysate samples, 100 μL of d5–3HG were added as an internal standard in addition to 20 μL of 50% methanol/water. The mixtures were applied to the extraction plate which had been pre-conditioned with 200 μL of methanol and 200 μL of water. Then, the extraction plate was washed with 400 μL of 50% methanol/water and the analyte was eluted with 100 μL of 2% formic acid in methanol. The eluent was mixed with 100 μL of water, and 10 μL of the mixture was injected to an LC–MS/MS system consisted of Alliance 2795 (Waters Corp.) and Quattro Premier XE (Waters Corp.). The analyte and internal standard were separated from foreign substances using a Hypercarb column (2.1 mm O.D. × 150 mm L, particle size: 5 μm, Thermo Fisher Scientific Inc.) in a column oven set at 40 °C. Isocratic methanol/water/formic acid (497.5/497.5/5, v/v/v) at a flow rate of 300 μL/min was used as mobile phase. The analyte and the internal standard were ionized by ESI negative mode and detected with the MRM transitions of *m/z* 147 to 129 and *m/z* 152 to 89, respectively.

2.3. Plasmid construction

We used the cDNAs of IDH1 and IDH2 obtained from Thermo Fisher Scientific as the templates. IDH1 and IDH2 were subcloned into *p*3*FLAG-CMV14 (Sigma) to make C-terminally 3*FLAG-tagged constructs using NotI and BamHI sites introduced by PCR primers.

2.4. Quantitative RT-PCR

RNA was extracted with RNeasy mini kit (QIAGEN), and cDNA was synthesized from the same amount of total RNA with a high capacity cDNA reverse transcription Kit (Life technologies). Quantitative PCR was performed with Fast SYBR Green Master Mix (Life technologies) with ABI 9700 system (Life Technologies). Signals were normalized to beta-ACTIN. The sequences of the primers were as follows: D2HGDH-F, 5′-acggtgtccatcttgtgtcc-3′; D2HGDH-R, 5′-tctgcagaacctcagcaaag-3′; L2HGDH-F, 5′-tcaaaaattcatccctgaaattact-3′; L2HGDH-R, 5′-ctaccagatttccatctctatccag-3′; IDH1-F, 5′-ggtgacatacctggtacataactttg-3′; IDH1-R, 5′-gtgtgcaaaatcttcaattgactt-3′; IDH2-F, 5′-gcatgtacaacaccgacgag-3′; IDH2-R, 5′-ccatttcttctggatggcata-3′; bACTIN-F, 5′-ccaaccgcgagaagatga-3′; bACTIN-R, 5′-ccagaggcgtacagggatag-3′.

2.5. Western Blot analysis

Cells were rinsed with ice-cold PBS and lysed in buffer A (50 mM Tris, pH7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 1% TritonX-100) supplemented with phosphatase inhibitor 2 (Sigma), phosphatase inhibitor 3 (Sigma), protease inhibitor (Sigma) and Complete protease inhibitor cocktail (Roche). Proteins were separated by SDS–PAGE and transferred to a PVDF membrane. After blocking, the membrane was probed with horseradish-peroxidase-conjugated FLAG-tag antibody (Sigma), and the chemiluminescence was detected using ECLpro (GE Healthcare). The same membrane was re-probed with beta-actin antibody (Cell Signaling Technologies) followed by horseradish-peroxidase-conjugated secondary antibody (GE Healthcare), and analyzed the chemiluminescence.

2.6. Statistical analysis

The results are expressed as the mean ± SD. Statistical significance was analyzed using the Student's *t*-test, and a level of probability of 0.01 was used as the criterion for significance.

3. Results

3.1. Knockdown of IDH1/IDH2 decreased the level of 2HG in D2HGDH deficient MCF7 cells

To mimic D2HGDH deficient cells, we conducted knockdown experiments using MCF7 cells (Fig. 1A). As expected, knockdown of D2HGDH significantly increased the level of 2HG in MCF7 cells (Fig. 1B). Though we did not separate D-2HG from L-2HG and we only detected whole D/L-2HG, depletion of D2HGDH is thought to affect only the level of D-2HG, not L-2HG [4]. Thus, we suppose that the increase of 2HG in this experiment was derived from D-2HG.

When IDH1 or IDH2 were knocked down together with D2HGDH (Fig. 2A), the levels of 2HG were lower than that of siD2HGDH alone (Fig. 2B). Moreover, the level of 2HG of cells treated with all three genes (D2HGDH, IDH1, and IDH2) was much lower, close to the background level (Fig. 2B).

3.2. Expression of IDH1 and IDH2 increased the level of D-2HG

Next, to see whether IDH1/2 can enhance 2HG production or not, we ectopically expressed IDH1 and IDH2 in MCF7 cells (Fig. 3A). The 2HG levels of IDH1 or IDH2 expressed cells were 2-3 times higher than that of control (Fig. 3B). Even under the condition of D2HGDH knockdown, overexpression of IDH1 and IDH2 had an additive effect on 2HG production. We also confirmed that 2HG level of IDH2(R140Q)-expressing cells was by far higher than the level of wild type IDH2-expressing cells (Fig. 3B), consisting with previous reports of human genetics [10].

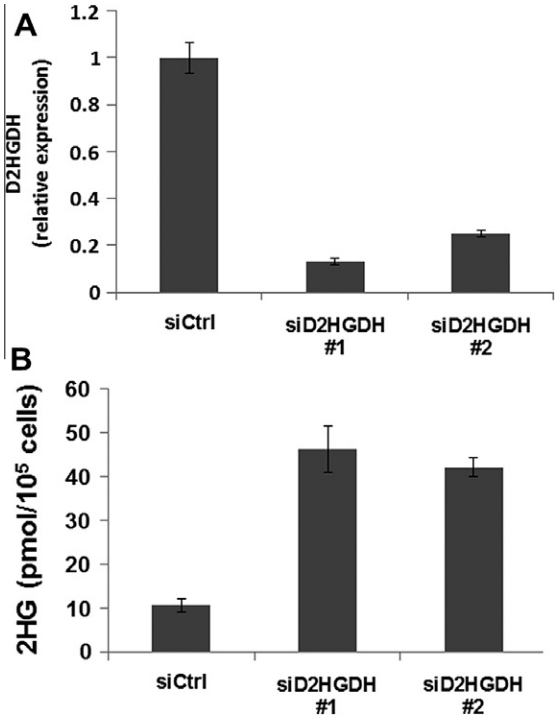

Fig. 1. Knockdown of D2HGDH increases the level of 2-hydroxyglutarate in MCF7 cells. MCF7 cells were treated with indicated siRNAs. (A) Quantity of D2HGDH and beta-actin was measured by quantitative RT-PCR analysis, and relative abundances of D2HGDH were calculated. (B) Cells were collected four days after siRNA transfection for 2HG extraction and measurement. The data represent means ± S.D.

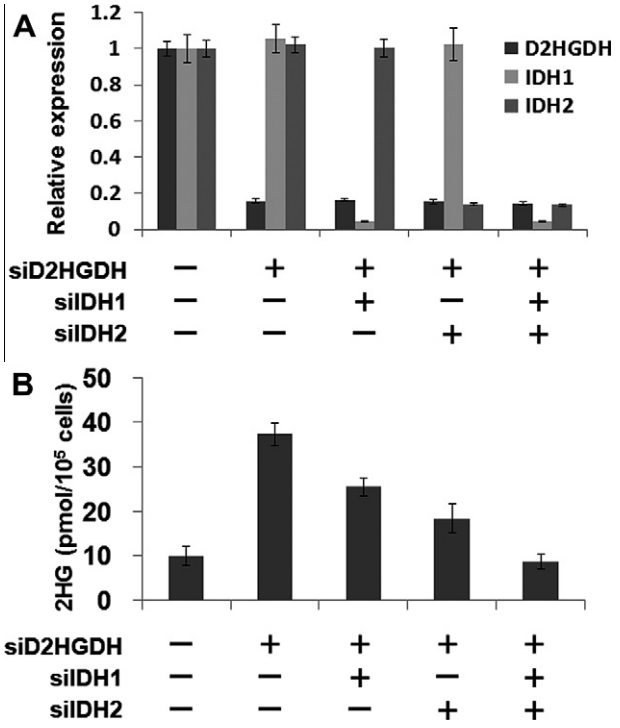

Fig. 2. Knockdown of IDH1 and/or IDH2 reduces the level of 2HG in D2HGDH depleted MCF7 cells. MCF7 cells were treated with indicated siRNAs. (A) Quantitative RT-PCR analysis was conducted with cells at day 1. (B) 2HG levels were measured with cells at day 4. The data represent means ± S.D.

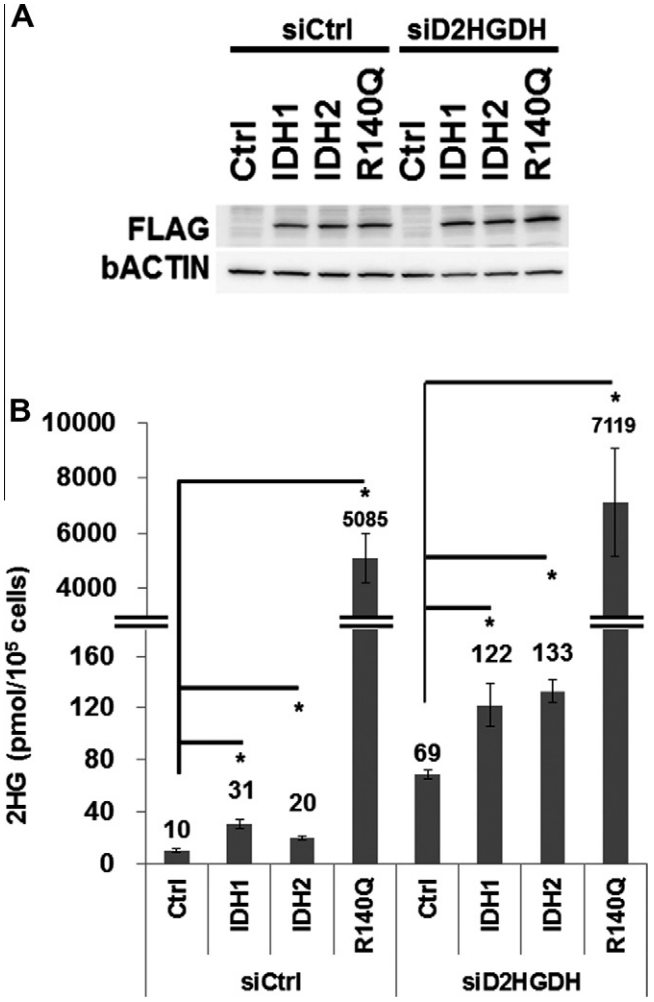

Fig. 3. Ectopic expression of IDH1 or IDH2 increases the level of 2HG. MCF7 cells were treated with control siRNA or D2HGDH siRNA at day 0. Flag-tagged expression vectors were transfected at day 1. (A) Cells were lysed at day 4 and the cell lysates were analyzed by immunoblotting with anti-FLAG and anti-beta actin antibodies. (B) Cells were collected at day 4 and 2HG levels were measured. The data represent means ± S.D. Average amounts of 2HG are shown above each bar. $^{*}p < 0.01$.

4. Discussion

The previous work showed recombinant wild type IDH1 can produce D-2HG in addition to catalyzing isocitrate to 2-oxoglutarate [18]. In this study, we showed (1) siIDH1 or siIDH2 decreased the level of 2HG in D2HGDH depleted cells, (2) expression of IDH1 or IDH2 increased the level of 2HG, and 2HG level was much higher in the D2HGDH depleted cells. These results strongly suggest that IDH1 and IDH2 produce D-2HG in D2HGDH depleted conditions. Especially, knockdown of both IDH1 and IDH2 with D2HGDH decreased the level of 2HG nearly to the background level, indicating that these two proteins are the main source of D-2HG in D2HGDH down-regulated cells.

We also observed that IDH2R140Q produced much higher level of 2HG than wild type IDH2 or IDH1 in MCF7 cell. Thus our result is basically consistent with the previous work [10], in which the authors proposed that IDH2R140Q mutation acquired neomorphic activity to produce 2HG. Although the activities of wild type IDH1 and IDH2 to produce 2HG seem to be weak, these relatively weak enzymatic activities are critical in D2HGDH depleted conditions.

We could not analyze the role of HOT enzyme in our model because its exact entity is not known thus far. And we are aware

of the limitation of our system to fully mimic the physiological condition of D-2HGA patients. However, our work shed new light on the mechanism of D-2HGA. Importantly, our results indicate that targeting wildtype IDH1 and IDH2 would be a medication for type I D-2HGA patients. Certainly, further work needs to be done to study the role of IDH1, IDH2 and HOT enzyme in D-2HGA patients. Especially analyses with cells from type I D-2HGA patients would be informative.

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