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ORIGINAL PAPER

Isocitrate dehydrogenase gene mutations and 2-hydroxyglutarate accumulation in esophageal squamous cell carcinoma

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Abstract

Isocitrate dehydrogenase 1 and 2 (IDH1 and IDH2) are key metabolic enzymes that convert isocitrate to α-ketoglutarate. Somatic point mutations in *IDH1/2* confer a gain-of-function in cancer cells, resulting in overproduction of an oncometabolite, 2-hydroxyglutarate (2HG). 2HG interferes with cellular metabolism and epigenetic regulation, contributing to oncogenesis. Given that IDH1 and IDH2 are attracting attention as promising therapeutic targets, better evaluation of the incidence of *IDH1* and *IDH2* mutations and 2HG level in human cancers is clinically important. This is the first study to assess their incidence in esophageal squamous cell carcinomas (ESCCs). First, we established pyrosequencing assays for *IDH1* and *IDH2* mutations and revealed that these mutations were absent in 10 ESCC cell lines and 96 ESCC tissues. Second, utilizing IDH1 and IDH2 overexpression vectors, we demonstrated that LC-MS/MS assays can accurately evaluate 2HG level and found that some ESCC cases presented a high level of 2HG. In conclusion, *IDH1* or *IDH2* mutations play a limited role in the development of ESCC. 2HG is potentially synthesized to high levels in the absence of *IDH1* and *IDH2* mutations, and this may correlate with progression of ESCCs.

Keywords Esophageal squamous cell carcinoma · Isocitrate dehydrogenase · 2-Hydroxyglutarate · α-Ketoglutaric acid · Oncometabolite

Abbreviations

IDH	Isocitrate dehydrogenase (IDH)
2HG	2-Hydroxyglutarate
α-KG	α-ketoglutarate

Introduction

Esophageal squamous cell carcinoma (ESCC) is the major histological type of esophageal cancer in Eastern Asian countries. Although multimodal techniques, such as combined surgery, chemotherapy and radiotherapy, have developed during the last several decades, the prognosis of advanced ESCC patients remains poor [1, 2].

Recently, 18F-fluorodeoxyglucose positron emission tomography (FDG-PET) has become available for assessing the range of ESCC progression and predicting prognoses [3–5]. As FDG-PET can detect the glucose uptake of cancer cells, it may reveal a crucial link between cancer metabolism and malignancy in ESCC. Therefore, identifying critical factors implicated in cancer progression and metabolism

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is important for the development of innovative therapeutic strategies for treating ESCC, especially those that are molecularly targeted.

Renewed interest in cellular metabolism in cancer has more recently followed the discovery of germline or somatic mutations in specific genes coding for metabolic enzymes. Isocitrate dehydrogenase 1 and 2 (IDH1/2) are key metabolic enzymes that convert isocitrate to α-ketoglutarate (α-KG). Somatic point mutations in *IDH1/2* confer a gain-of-function on cancer cells, resulting in the accumulation and secretion in vast excess of an oncometabolite, D-2-hydroxyglutarate (D-2HG) [6–8]. *IDH1/2* mutations have been observed in several types of human tumors including low-grade glioma [9], glioblastoma [10], chondrosarcoma [11], intrahepatic cholangiocarcinomas [12], hematologic malignancies [8, 13, 14] and other solid tumors [15–17]. D-2HG modulates the activity of α-KG-dependent dioxygenases, which are a family of enzymes requiring oxygen and iron that are involved in proline hydroxylation [18], histone demethylation [19] and epigenetic DNA modifications [20] and that contribute to oncogenesis. D-2HG is a potential biomarker for the detection of *IDH1/2* mutations. Importantly, inhibitors of IDH1 and IDH2 are in early phase clinical trials, and early results from these trials involving hematologic malignancies or solid cancers have yielded promising results. However, there is no published evaluation of the prevalence of *IDH1/2* gene mutations and 2HG accumulation in ESCCs.

In this study, we assessed the precision of a polymerase chain reaction (PCR) pyrosequencing assay for identifying *IDH1/2* mutations and of a liquid chromatography-tandem mass spectrometry (LC–MS/MS) assay for measuring 2HG level. Second, we determined *IDH1/2* mutations and 2HG levels in ESCC cell lines and ESCC tissues. We show that *IDH1* and *IDH2* mutations were absent in the dataset of 96 ESCCs, but some cases demonstrated a high level of 2HG independent of *IDH1* and *IDH2* mutations.

Materials and methods

Study subjects

A total of 96 patients with ESCCs who underwent curative resection at Kumamoto University Hospital between April 2005 and December 2015 were enrolled onto this study. We used formalin-fixed paraffin-embedded (FFPE) tissues of ESCCs ($n=96$) for *IDH1* and *IDH2* mutation analysis, and frozen tissues of ESCCs and matched normal mucosa ($n=30$) for 2HG analysis. Informed consent for this retrospective analysis was obtained by the opt-out method, and this study was approved by the institutional review board at Kumamoto University (approved number #812). Informed consent was obtained from all individual participants included in the study. All experiments were performed in accordance with the Declaration of Helsinki.

DNA extraction

Genomic DNA extraction from normal and tumor FFPE tissues was performed as previously described [21, 22]. DNA was also extracted from cell lines using a QIAamp DNA FFPE Mini Kit (QIAGEN).

Cell lines and plasmid transfections

Human cell lines were obtained from RIKEN BioResource Center (Japan), Japanese Collection of Research Bioresources Cell Bank (Japan), American Type Culture Collection (USA). GSC03U#5, GSC07U#7 and GSC03A#40 were a gift from the Department of Tumor Genetics and Biology, Graduate School of Medical Sciences, Kumamoto University, Kumamoto [23].

Cell lines were cultured in medium supplemented with 10% FBS in a 5% CO_2 atmosphere at 37 °C. For expression of wild-type and mutant IDH1/2 in TE-1 and TE8 cells, transfection was performed with Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. The cells were collected 48 h after plasmid transfection and analyzed as described below.

Vectors

Primers for gene-specific reverse transcription, amplification of wild-type *IDH1/2* and site-directed mutagenesis are listed in Table S1. Human *IDH1* cDNA (CCDS2381.1, wild type) and *IDH2* cDNA (CCDS10359.1, wild type) were obtained using gene-specific RT primers and PCR primers from the RNA of MCF7 cells. *IDH1* and *IDH2* cDNAs were subcloned into the modified pIRESpuro3 using *Nhe*I and *Xho*I. Arginine residues IDH1 R132, IDH2 R140 and IDH2 R172 were mutated to histidine (c.395G>A), glutamine (c.G419G>A) and lysine (c.515G>A) by site-directed mutagenesis using the appropriate primers and confirmed by sequencing.

Immunoblot analysis

Cells were rinsed with ice-cold PBS and lysed in RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with Halt Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Proteins were separated by SDS-PAGE and transferred to a PVDF membrane, blocked in 5% nonfat milk in TBS containing 0.1% Tween-20, probed with primary antibodies and detected with horseradish peroxidase-conjugated secondary antibody (Santa Cruz, USA, anti-rabbit: sc-2004; anti-mouse: sc-2005), and chemiluminescence

was detected using Amersham ECL Western Blotting Detection Reagent (GE Healthcare, GBR). Primary antibodies used were IDH1 antibody (Proteintech, USA, 23309-1-AP), IDH2 antibody (Abcam, GBR, ab55271), GFP tag antibody (Thermo Fisher Scientific, A-11122) and β-actin antibody (Cell Signaling Technologies, USA, #4967).

PCR and pyrosequencing for IDH1 and IDH2 mutation analysis

The PCR amplification primers for pyrosequencing are listed in Table S2. Each PCR mix contained 1 X AmpliTaq Gold 360 Buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP with dUTP, forward and reverse primers (0.2 μM each), 0.625 U of AmpliTaq Gold 360 DNA polymerase, 0.25 U of AmpErase UNG and 10 ng of template DNA in a total volume of 25 μL. The PCR conditions consisted of activation AmpErase UNG at 50 °C (10 min), activation AmpliTaq Gold 360 at 95 °C (10 min); 35 cycles of 95 °C (15 s), annealing (30 s; 60 °C for IDH1 R132 and IDH2 R172, 55 °C for IDH2 R140), and 72 °C (30 s); and a final extension at 72 °C (7 min). The PCR products were electrophoresed through an agarose gel to confirm successful amplification of the 74 bp (IDH1 R132), 104 bp (IDH2 R140) and 77 bp (IDH2 R172) products.

These PCR products were integrated into a TOPO TA Cloning Kit with PCR2.1 TOPO (Thermo Fisher Scientific) for positive control as *IDH1/2* wild type. The mutations of *IDH1* c.395G>A, *IDH2* c.419G>A and *IDH2* c.515G>A were introduced by site-directed mutagenesis using the appropriate primers and confirmed by sequencing for positive control as mutant IDH1/2.

The primers and the dispensation orders for IDH1 R132, IDH2 R140 and IDH2 R172 pyrosequencing are shown in Table S2, and sequencing was performed using the PyroMark Q24 System (Qiagen, Germany) according to the manufacturer's instructions. A part of pyrosequencing results was confirmed by dideoxy sequencing.

LC-MS/MS analysis of 2HG

We used D-α-hydroxyglutaric acid disodium salt (Sigma, USA, H8378) and L-α-hydroxyglutaric acid disodium salt (Sigma, 90790) for making a standard curve and recovery test.

Cells were collected by centrifugation of 2×10^6 cells and suspended in 40 μL of ice-cold PBS. The cells were lysed by adding 160 μL of chilled methanol and placed at − 80 °C for 30 min, and 120 μL of ice-cold water was then added.

Frozen tissue (approximately 2 mm^3) was placed in a microfuge tube, and 100 μL/5 mg chilled 80% methanol/water was added. The frozen tissues were homogenized using a tissue grinder with pestle for a test tube (AS ONE, JPN) at 3000 rpm for 30 s, on ice. Lysates were incubated for 30 min at − 80 °C and then centrifuged at 20,600×*g* at 4 °C for 5 min. The 100 μL supernatant was mixed with 60 μL of ice-cold water.

Subsequently, these cell (320 μL) or tissue (160 μL) lysates were applied to an OASIS MAX μElution Column (waters) that had been preconditioned with 200 μL of methanol and 200 μL of water. The column was then washed with 400 μL of 50% methanol/water, and the analytes were eluted with 100 μL of 2% formic acid in methanol. The eluent was mixed with an equal amount of water, and 10 μL of the mixture was injected into an LC–MS/MS system consisting of 1290 Infinity (Agilent Technologies) and 6460 Triple Quad LC/MS (Agilent Technologies). The analytes were separated from foreign substances using a Hypercarb column (outer diameter 2.1 mm × length 150 mm, particle size 5 μm, Thermo Fisher Scientific) 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 200 μL/min was used as the mobile phase. The analytes were ionized by ESI negative mode, and multiple reaction monitoring (MRM) data were acquired using the following MRM transitions: 147.0 > 57.3, 147.0 > 85.2, 147.0 > 101.1, 147.0 > 129.1 [24].

Results

Validation of the pyrosequencing analysis for IDH1/2 mutation detection

Pyrosequencing is a useful methodology in molecular diagnostics and large-scale epidemiological studies. We first examined the validity of the pyrosequencing method using five positive control vectors, into which PCR products of *IDH1* wild type, *IDH2* wild type, *IDH1* c.395G>A (IDH1 R132H), *IDH2* c.419G>A (IDH2 R140Q) and *IDH2* c.515G>A (IDH2 R172K) were inserted. These positive control vectors were also validated by the dideoxy sequencing (Fig. 1), and the results were consistent. These findings demonstrated that the pyrosequencing method was reliable for the detection of *IDH1* and *IDH2* mutations.

IDH1/2 mutational status in different types of cancer cell lines

Next, utilizing this pyrosequencing method, we analyzed the mutational status of *IDH1* c.395G, *IDH2* c.419G and *IDH2* c.515G in several types of cancer cell lines: esophageal cancer [TE-1, TE-4, TE-6, TE-8, TE-9, TE-10, TE-11, TE-14 TE-15 and KYSE30], gastric cancer [AGS, Kato III, MKN1, MKN7, MKN45, NCI-N87, NUGC-3 and NUGC-4], cervical cancer [HeLa], a breast cancer cell line [MCF7], cholangiocarcinoma [K100, M055, M213, M213 L5, M214 and M214 L5], glioma [A172, U87MG, U251 and

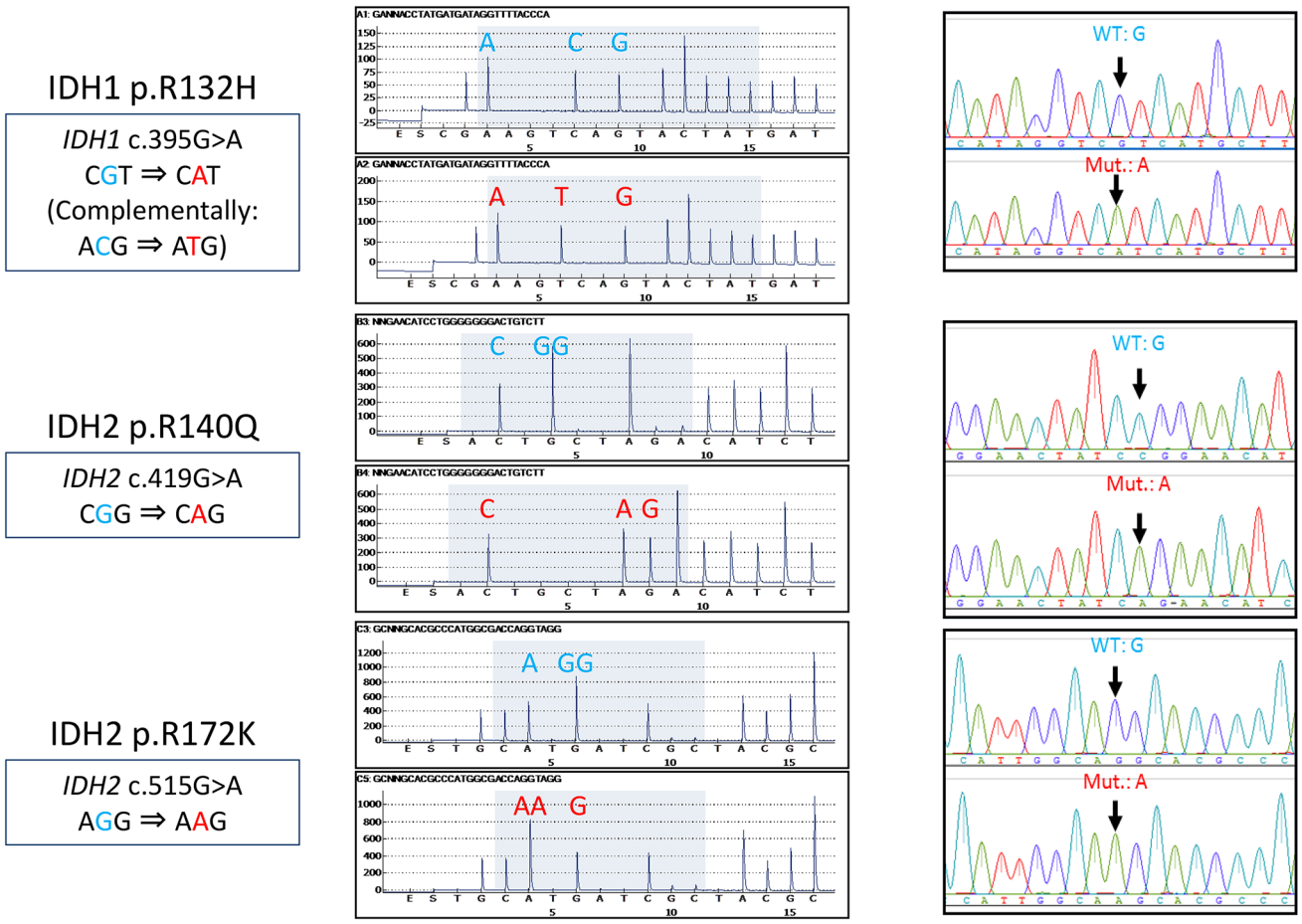

Fig. 1 Detection of *IDH* mutations by dideoxy sequencing and pyrosequencing. IDH1 wild type, IDH1 R132H, IDH2 wild type, IDH2 R140H and IDH2 R172K were analyzed by pyrosequencing (left panels) and dideoxy sequencing (right panels). The pyrosequencing nucleotide dispensation order is shown below each pyrogram, and the numerical position for each nucleotide is indicated at the bottom. Arrows indicate the presence of mutant alleles

U373] and glioma stem cells [GSC03U#5, GSC07U#7 and GSC03A#40]. Furthermore, glioma and glioma stem cell lines were analyzed by dideoxy sequencing. Data for these mutations in 21 cell lines [TE-1, TE-4, TE-6, TE-9, TE-10, TE-11, TE-14 TE-15, KYSE30, AGS, Kato III, MKN1, MKN7, MKN45, NCI-N87, NUGC-3, NUGC-4, HeLa, MCF7, A172, U87MG] were also acquired on the COSMIC Cell Lines Project (http://cancer.sanger.ac.uk/cosmic). These results were consistent, revealing that all cancer cell lines were wild type in *IDH1* c.395G, *IDH2* c.419G and *IDH2* c.515G (Table 1).

IDH1/2 mutational status in ESCC tissues

Pyrosequencing analysis of IDH1 R132H, IDH2 R140Q and IDH2 R172K was successful for all 96 ESCC paraffin-embedded tissues. We found that these specimens were all wild type in *IDH1* c.395G, *IDH2* c.419G and *IDH2* c.515G (Table 2).

Standard curve and recovery test

Two distinct forms of 2HG have been reported: L-2-hydroxyglutarate (L-2HG) and D-2-hydroxyglutarate (D-2HG). These metabolites are normal endogenous metabolites found throughout the human body. Although these metabolites are identical in their physical properties, they are different entities in biochemical properties [25, 26]. Our LC–MS/MS detected D-2HG and L-2HG near a retention time of 3 min. D-2HG and L-2HG were not detected separately; thus, we detected D-2HG and L-2HG as total 2HG (Fig. 2a).

We performed a product ion scan and obtained four MRM transitions: m/z 147.0 > 57.3, 147.0 > 85.2, 147.0 > 101.1 and 147.0 > 129.1. We measured each MRM transition using diluted 2HG standard and obtained four standard curves. The results are displayed in Fig. 2b. At a collision energy of 5 V, MRM transitions of m/z 147.0 > 129.1 were more efficient than the others. The standard curve of m/z 147.0 > 129.1 was $y = 530.65x - 259.86$, and the correlation coefficient was

Table 1 Comparative analysis of database search on COSMIC Cell Lines Project (http://cancer.sanger.ac.uk/cosmic), pyrosequencing and dideoxy sequencing

Cell line name	Type	*IDH1*	*IDH2*	
		R132	R140	R172
TE-1	Esophageal cancer	WT/WT/–	WT/WT/–	WT/WT/–
TE-4	Esophageal cancer	WT/WT/–	WT/WT/–	WT/WT/–
TE-6	Esophageal cancer	WT/WT/–	WT/WT/–	WT/WT/–
TE-8	Esophageal cancer	WT/WT/–	WT/WT/–	WT/WT/–
TE-9	Esophageal cancer	WT/WT/–	WT/WT/–	WT/WT/–
TE-10	Esophageal cancer	WT/WT/–	WT/WT/–	WT/WT/–
TE-11	Esophageal cancer	WT/WT/–	WT/WT/–	WT/WT/–
TE-14	Esophageal cancer	–/WT/–	–/WT/–	–/WT/–
TE-15	Esophageal cancer	WT/WT/–	WT/WT/–	WT/WT/–
KYSE30	Esophageal cancer	WT/WT/–	WT/WT/–	WT/WT/–
AGS	Gastric cancer	WT/WT/–	WT/WT/–	WT/WT/–
Kato III	Gastric cancer	WT/WT/–	WT/WT/–	WT/WT/–
MKN1	Gastric cancer	WT/WT/–	WT/WT/–	WT/WT/–
MKN7	Gastric cancer	WT/WT/–	WT/WT/–	WT/WT/–
MKN45	Gastric cancer	WT/WT/–	WT/WT/–	WT/WT/–
NCI-N87	Gastric cancer	WT/WT/–	WT/WT/–	WT/WT/–
NUGC-3	Gastric cancer	WT/WT/–	WT/WT/–	WT/WT/–
NUGC-4	Gastric cancer	WT/WT/–	WT/WT/–	WT/WT/–
HeLa	Cervical cancer	WT/WT/–	WT/WT/–	WT/WT/–
MCF7	Breast cancer	WT/WT/–	WT/WT/–	WT/WT/–
K100	Cholangiocarcinoma	–/WT/–	–/WT/–	–/WT/–
M055	Cholangiocarcinoma	–/WT/–	–/WT/–	–/WT/–
M213	Cholangiocarcinoma	–/WT/–	–/WT/–	–/WT/–
M213 L5	Cholangiocarcinoma	–/WT/–	–/WT/–	–/WT/–
M214	Cholangiocarcinoma	–/WT/–	–/WT/–	–/WT/–
M214 L5	Cholangiocarcinoma	–/WT/–	–/WT/–	–/WT/–
A172	Glioma	WT/WT/WT	WT/WT/WT	WT/WT/WT
U87MG	Glioma	WT/WT/WT	WT/WT/WT	WT/WT/WT
U251	Glioma	–/WT/WT	–/WT/WT	–/WT/WT
U373	Glioma	–/WT/WT	–/WT/WT	–/WT/WT
GSC03U#5	Glioma stem cell	–/WT/WT	–/WT/WT	–/WT/WT
GSC07U#7	Glioma stem cell	–/WT/WT	–/WT/WT	–/WT/WT
GSC03A#40	Glioma stem cell	–/WT/WT	–/WT/WT	–/WT/WT
		(COSMIC/pyrosequencing/dideoxy sequencing)		

For detection of *IDH1* and *IDH 2* mutations in 30 cell lines

Table 2 *IDH1* and *IDH 2* mutation status of clinical specimens of ESCCs by pyrosequencing

Gene	Codon	Wild type	Mutation
IDH1	R132	96 (100%)	0 (0%)
IDH2	R140	96 (100%)	0 (0%)
	R172	96 (100%)	0 (0%)

$R^2 = 1$ in the range of 0–250 pmol. We used MRM transitions of m/z 147.0 > 129.1 to measure the level of 2HG.

We added 2HG standard (0, 0.5, 5, 50, 500 pmol in 10^5 cells) to TE-1 and extracted 2HG from the cell line. The extracted 2HG level was then measured by LC–MS/MS. The area value for cells with no added 2HG was subtracted from the area values for cells with added 2HG. A standard curve of 2HG recovery using the subtractions was calculated $y = -0.2117x^2 + 925.77x$, and the correlation coefficient was $R^2 = 0.9999$. The amount of recovered 2HG was the same as the amount of added 2HG standard (Fig. 2c).

Level of 2HG in cell lines of ESCCs and expression of IDH1 and IDH2 in cell lines

To confirm the reliability of LC–MS/MS for 2HG detection, we performed an in vitro assessment. First, we evaluated

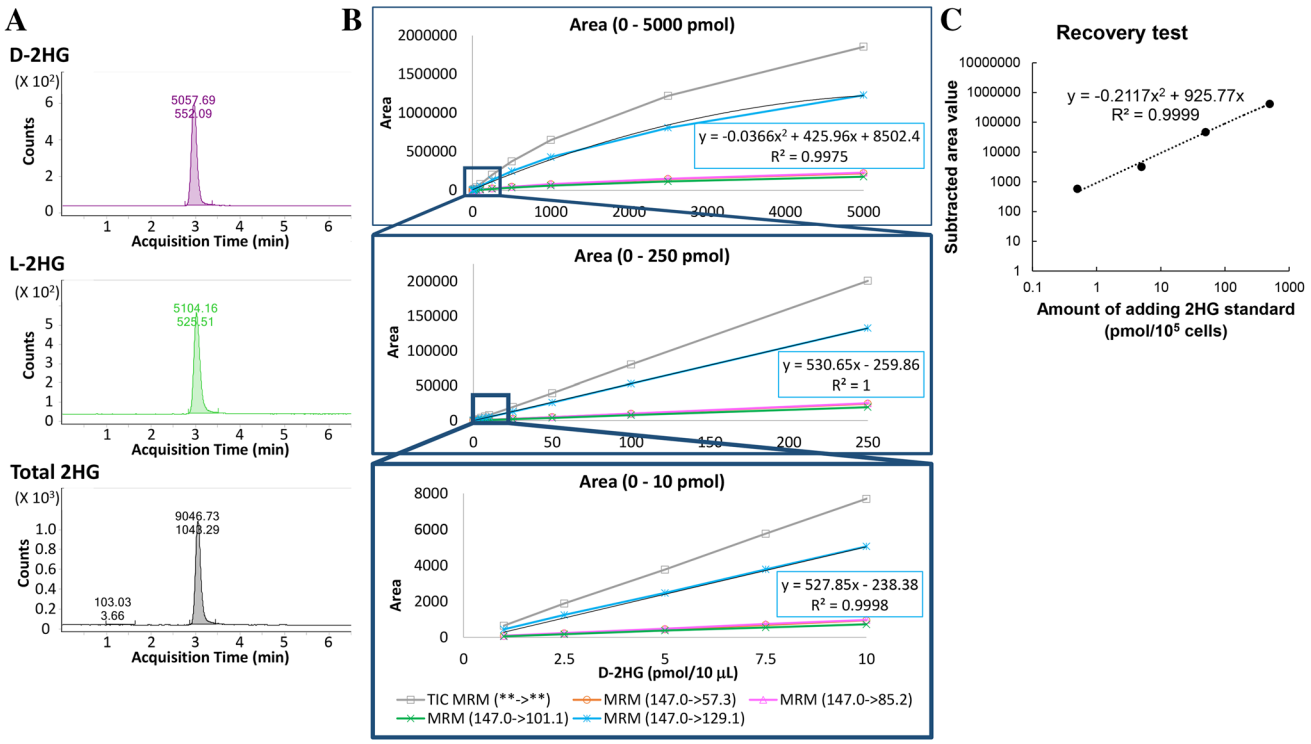

Fig. 2 Measuring the level of 2HG by LC-MS/MS. **a** Mass fragmentograms of D- and L-2-HG. Shown are D-2HG standard, L-2HG standard and a mixture of D- and L-2HG standards (total 2HG). D-2HG, L-2HG and total 2HG displayed a peak at 3.0 min. **b** Standard curves of D-2HG. Total ion chromatogram (TIC) and multiple reaction monitoring (MRM) of 147.0 > 57.3, 147.0 > 85.2, 147.0 > 101.1 and 147.0 > 129.1. **c** Recovery of exogenous D-2HG in the esophageal cell line TE-1

the level of 2HG in ten cancer cell lines using LC-MS/MS (Fig. 3a). We then transfected vectors encoding AcGFP1 (Aequorea coerulescens GFP)-tagged IDH1 wild type, IDH2 wild type, IDH1 R132H, IDH2 R140Q or IDH2 R172K into TE-1 and TE-8 cells which the 2HG expression level is very low. The transfected cell lines were evaluated by western blotting using specific antibodies (Fig. 3b). We could confirm that the levels of 2HG in IDH1 R132H-, IDH2 R140Q- and IDH2 R172K-expressing cells were higher than those in wild-type IDH1/2-expressing cells (Fig. 3c), consistent with previous human genetics studies [10–12].

Level of 2HG in ESCCs and matched normal mucosa

Finally, we examined the level of 2HG in 30 ESCC specimens and matched normal esophageal mucosa utilizing our reliable LC–MS/MS assay. The levels of 2HG in ESCCs (median: 1435.7; mean: 1953.5; SD: 1978.0) were higher than those in matched normal mucosa (median: 1328.0; mean: 1518.9; SD: 693.8) (Fig. 4), although this difference was not statistically significant. None of these cancer tissues had *IDH1* or *IDH2* mutations. Notably, some ESCC tissues (e.g., cases 029, 011, 009 and 007) presented are higher than in the matched normal tissue sample, thereby supporting the importance of 2HG in the development and progression of ESCC.

Discussion

Mutations in *IDH1* or *IDH2*, whose gene products are essential for cellular respiration in the tricarboxylic acid cycle, are prevalent in several types of human cancers. These mutations confer a gain-of-function on cancer cells and result in overproduction of the oncometabolite 2HG, leading to oncogenesis [6, 7, 27]. Importantly, IDH1 and IDH2 are attracting attention as a promising therapeutic target: inhibitors of IDH1 and IDH2 are in early phase clinical trials involving hematologic malignancies or solid cancers. The early results from these trials have yielded promising results [28]. Therefore, a better understanding of *IDH1/2* mutation status and 2HG level in cancer tissues is increasingly important. To our knowledge, this is the first study evaluating *IDH1* and *IDH2* mutations and 2HG level in ESCCs. We developed our own PCR pyrosequencing assay and LC-MS/MS assay and found that *IDH1* and *IDH2* mutations were absent in ESCC cell lines and tissues, but that some cases displayed a high level of 2HG.

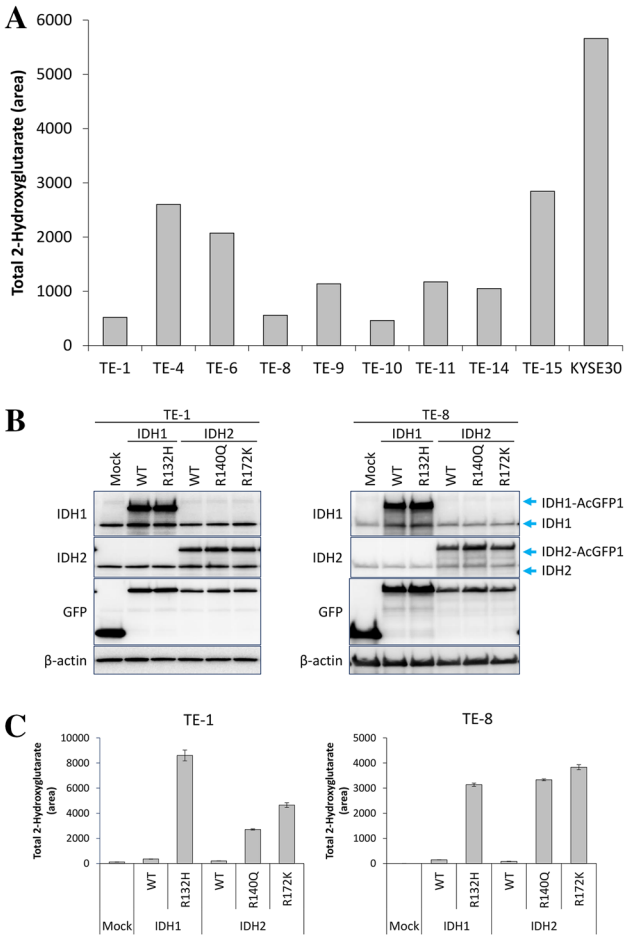

Fig. 3 **a** Levels of total 2HG in ten esophageal cancer cell lines. Mutant IDH1 or IDH2 expression increases the level of 2HG. TE-1 and TE-8 cells were mock-transfected or transfected with wild-type or mutant IDH vectors at day 1. **b** Cells were lysed at day 2, and the cell lysates were analyzed by immunoblotting with anti-IDH1, anti-IDH2, anti-GFP and anti-β-actin antibodies. **c** Cells were collected at day 2, and 2HG levels were measured. The data represent means ± S.D. Average amounts of 2HG are shown above each bar

Accumulating evidence supports the possibility of exploiting *IDH1/2* mutations as a prognostic biomarker in human neoplasms. In addition, 2HG is a biomarker that is suitable for the detection of *IDH1/2* mutations at diagnosis and is predictive of the clinical response. We analyzed the *IDH1* and *IDH2* mutations in various types of cancer cell lines (ESCC, gastric cancer, cervical cancer, breast cancer, cholangiocarcinoma, glioma and glioma stem cells) and in ESCC tissue samples ($n = 96$) by pyrosequencing. However, there were no mutations in *IDH1* or *IDH2*. Our data matched with those in the COSMIC Cell Lines Project database. In agreement with a previous report [6–8], *IDH1* and *IDH2* mutations correlated with an increased level of 2HG in esophageal cell lines.

2HG is a competitive inhibitor of multiple α-KG-dependent dioxygenases, including histone demethylases [19] and the TET family of 5mC hydroxylases [20], and is associated with poor prognosis in various cancers. 2HG analysis using LC-MS/MS showed variable levels of total 2HG in esophageal cell lines, without *IDH1* or *IDH2* mutation. In clinical ESCC samples ($n = 30$), although some specimens had no *IDH* mutation, the level of total 2HG was higher than in matched normal tissue. 2HG comprises two types [25], D-2HG and L-2HG, which are synthesized by different metabolism. D-2HG is formed by mutant IDH [6, 7], 3-phosphoglycerate dehydrogenase [29] and hydroxyacid-oxoacid transhydrogenase [30], while L-2HG is formed by malate dehydrogenase [31] or hypoxia [32]. D-2-hydroxyglutarate dehydrogenase [30] and L-2-hydroxyglutarate dehydrogenase [33, 34], respectively, reduce D-2HG and L-2HG level [26]. Our results raise the possibility that mutant forms of these enzymes cause abnormal 2HG metabolism in esophageal cancer.

In summary, we established pyrosequencing assays for *IDH1* and *IDH2* mutations and revealed that these mutations were absent in 10 ESCC cell lines and 96 ESCC tissues. Second, utilizing IDH1 and IDH2 overexpression vectors, we demonstrated that 2HG assays with LC-MS/MS can accurately assess 2HG level and found that some proportion of ESCC tissues displayed high levels of 2HG. In conclusion, *IDH1* or *IDH2* mutations play a limited role in the development of ESCC. 2HG may be synthesized independent of *IDH1* and *IDH2* mutations and may be related to progression of ESCCs. We currently plan a further study focusing on the relationship between *IDH1/2* mutations, 2HG status, environmental risk factors (e.g., smoking, alcohol) and patient survival in ESCC.

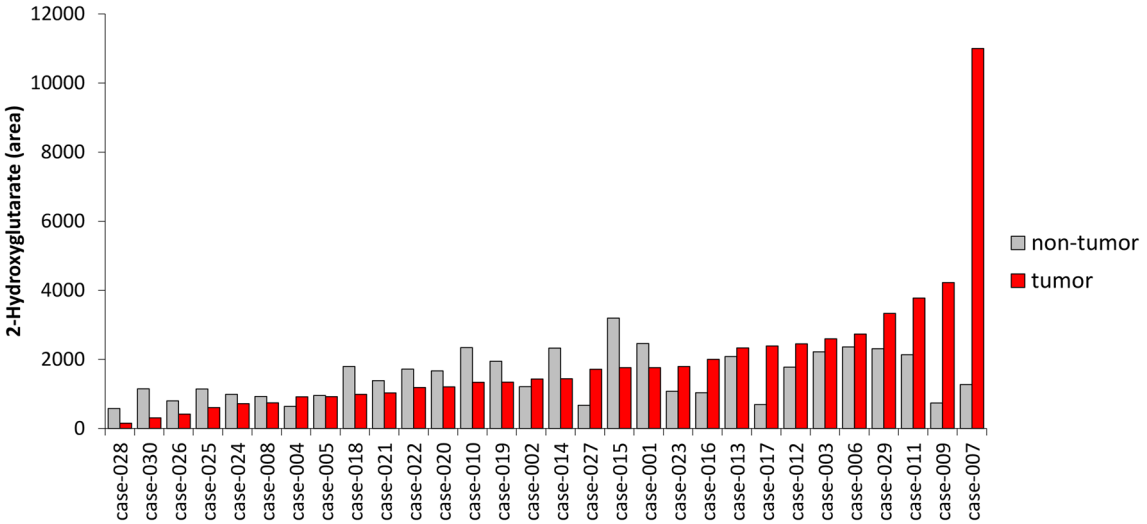

Fig. 4 Level of total 2HG in esophageal cancers (right bar) and matched normal mucosa (left bar)

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest.

References

1. Lehnert T. Multimodal therapy for squamous carcinoma of the oesophagus. Br J Surg. 1999;86(6):727–39.
2. Katlic MR, Wilkins EW Jr, Grillo HC. Three decades of treatment of esophageal squamous carcinoma at the Massachusetts General Hospital. J Thorac Cardiovasc Surg. 1990;99(5):929–38.
3. Flamen P, Van Cutsem E, Lerut A, et al. Positron emission tomography for assessment of the response to induction radiochemotherapy in locally advanced oesophageal cancer. Ann Oncol. 2002;13(3):361–8.
4. Brucher BL, Weber W, Bauer M, et al. Neoadjuvant therapy of esophageal squamous cell carcinoma: response evaluation by positron emission tomography. Ann Surg. 2001;233(3):300–9.
5. Downey RJ, Akhurst T, Ilson D, et al. Whole body 18FDG-PET and the response of esophageal cancer to induction therapy: results of a prospective trial. J Clin Oncol. 2003;21(3):428–32.
6. Dang L, White DW, Gross S, et al. Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. Nature. 2009;462(7274):739–44.
7. Ward PS, Patel J, Wise DR, et al. The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting alpha-ketoglutarate to 2-hydroxyglutarate. Cancer Cell. 2010;17(3):225–34.
8. Gross S, Cairns RA, Minden MD, et al. Cancer-associated metabolite 2-hydroxyglutarate accumulates in acute myelogenous leukemia with isocitrate dehydrogenase 1 and 2 mutations. J Exp Med. 2010;207(2):339–44.
9. Yan H, Parsons DW, Jin G, et al. IDH1 and IDH2 mutations in gliomas. N Engl J Med. 2009;360(8):765–73.
10. Parsons DW, Jones S, Zhang X, et al. An integrated genomic analysis of human glioblastoma multiforme. Science. 2008;321(5897):1807–12.
11. Amary MF, Bacsi K, Maggiani F, et al. IDH1 and IDH2 mutations are frequent events in central chondrosarcoma and central and periosteal chondromas but not in other mesenchymal tumours. J Pathol. 2011;224(3):334–43.
12. Borger DR, Tanabe KK, Fan KC, et al. Frequent mutation of isocitrate dehydrogenase (IDH)1 and IDH2 in cholangiocarcinoma identified through broad-based tumor genotyping. Oncologist. 2012;17(1):72–9.
13. Mardis ER, Ding L, Dooling DJ, et al. Recurring mutations found by sequencing an acute myeloid leukemia genome. N Engl J Med. 2009;361(11):1058–66.
14. Marcucci G, Maharry K, Wu YZ, et al. IDH1 and IDH2 gene mutations identify novel molecular subsets within de novo cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study. J Clin Oncol. 2010;28(14):2348–55.
15. Cerami E, Gao J, Dogrusoz U, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov. 2012;2(5):401–4.
16. Lu J, Xu L, Zou Y, et al. IDH1 p.R132 mutations may not be actively involved in the carcinogenesis of hepatocellular carcinoma. Med Sci Monit. 2014;20:247–54.
17. Li WL, Xiao MS, Zhang DF, et al. Mutation and expression analysis of the IDH1, IDH2, DNMT3A, and MYD88 genes in colorectal cancer. Gene. 2014;546(2):263–70.
18. Koivunen P, Lee S, Duncan CG, et al. Transformation by the (R)-enantiomer of 2-hydroxyglutarate linked to EGLN activation. Nature. 2012;483(7390):484–8.
19. Chowdhury R, Yeoh KK, Tian YM, et al. The oncometabolite 2-hydroxyglutarate inhibits histone lysine demethylases. EMBO Rep. 2011;12(5):463–9.
20. Xu W, Yang H, Liu Y, et al. Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of alpha-ketoglutarate-dependent dioxygenases. Cancer Cell. 2011;19(1):17–30.

21. Iwagami S, Baba Y, Watanabe M, et al. Pyrosequencing assay to measure LINE-1 methylation level in esophageal squamous cell carcinoma. Ann Surg Oncol. 2012;19(8):2726–32.
22. Iwagami S, Baba Y, Watanabe M, et al. LINE-1 hypomethylation is associated with a poor prognosis among patients with curatively resected esophageal squamous cell carcinoma. Ann Surg. 2013;257(3):449–55.
23. Niibori-Nambu A, Midorikawa U, Mizuguchi S, et al. Glioma initiating cells form a differentiation niche via the induction of extracellular matrices and integrin alphaV. PLoS ONE. 2013;8(5):e59558.
24. Matsunaga H, Futakuchi-Tsuchida A, Takahashi M, Ishikawa T, Tsuji M, Ando O. IDH1 and IDH2 have critical roles in 2-hydroxyglutarate production in D-2-hydroxyglutarate dehydrogenase depleted cells. Biochem Biophys Res Commun. 2012;423(3):553–6.
25. Struys EA. 2-Hydroxyglutarate is not a metabolite; D-2-hydroxyglutarate and L-2-hydroxyglutarate are! Proc. Natl. Acad. Sci. USA. 2013;110(51):E4939.
26. Kranendijk M, Struys EA, Salomons GS, Van der Knaap MS, Jakobs C. Progress in understanding 2-hydroxyglutaric acidurias. J Inherit Metab Dis. 2012;35(4):571–87.
27. Cairns RA, Mak TW. Oncogenic isocitrate dehydrogenase mutations: mechanisms, models, and clinical opportunities. Cancer Discov. 2013;3(7):730–41.
28. Fujii T, Khawaja MR, DiNardo CD, Atkins JT, Janku F. Targeting isocitrate dehydrogenase (IDH) in cancer. Discov Med. 2016;21(117):373–80.
29. Zhao G, Winkler ME. A novel alpha-ketoglutarate reductase activity of the serA-encoded 3-phosphoglycerate dehydrogenase of Escherichia coli K-12 and its possible implications for human 2-hydroxyglutaric aciduria. J Bacteriol. 1996;178(1):232–9.
30. Achouri Y, Noel G, Vertommen D, Rider MH, Veiga-Da-Cunha M, Van Schaftingen E. Identification of a dehydrogenase acting on D-2-hydroxyglutarate. Biochem J. 2004;381(Pt 1):35–42.
31. Linster CL, Van Schaftingen E, Hanson AD. Metabolite damage and its repair or pre-emption. Nat Chem Biol. 2013;9(2):72–80.
32. Intlekofer AM, Dematteo RG, Venneti S, et al. Hypoxia Induces Production of L-2-Hydroxyglutarate. Cell Metab. 2015;22(2):304–11.
33. Rzem R, Van Schaftingen E, Veiga-da-Cunha M. The gene mutated in l-2-hydroxyglutaric aciduria encodes l-2-hydroxyglutarate dehydrogenase. Biochimie. 2006;88(1):113–6.
34. Shim EH, Livi CB, Rakheja D, et al. L-2-Hydroxyglutarate: an epigenetic modifier and putative oncometabolite in renal cancer. Cancer Discov. 2014;4(11):1290–8.