IDH1 p.R132 mutations may not be actively involved in the carcinogenesis of hepatocellular carcinoma

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Background: Recent studies have identified prevalent isocitrate dehydrogenase 1 (IDH1) codon 132 mutations (p.R132) in gliomas and acute myeloid leukemia (AML). The IDH1 mutations lead to a loss of its normal enzymatic activity and acquisition of neomorphic activity in production of \(\alpha\)-ketoglutarate (\(\alpha\)-KG) and 2-hydroxyglutarate (2-HG), which finally cause alterations of multiple gene expression of tumorigenesis-associated \(\alpha\)-KG-dependent enzymes. The aim of this study was to determine whether IDH1 p.R132 mutations are involved in the carcinogenesis of hepatocellular carcinoma.

Material/Methods: A total of 87 Han Chinese patients with primary hepatocellular carcinoma (HCC) were analyzed by direct DNA sequencing for IDH1 p.R132 mutations. The expression levels of multiple \(\alpha\)-KG-dependent enzymes and associated genes were quantified in HepG2 cells overexpressing IDH1 p.R132 mutants by Western blotting and real-time PCR.

Results: None of 87 Han Chinese patients with HCC harbored any IDH1 p.R132 mutations. The protein levels of HIF-1\(\alpha\) and histone methylation marker (H3K4me3 and H3K79me2) were determined in HepG2 cells overexpressing IDH1 p.R132 mutants by Western blotting and real-time PCR. Measurement of mRNA expression levels of VEGF, GLUT1, and HOXA genes also showed no significant difference between cells overexpressing IDH1 wild-type and p.R132 mutants.

Conclusions: Our negative results, together with some previous reports of the absence of IDH1 p.R132 mutations in HCC tissues, suggests that IDH1 p.R132 mutations are not actively involved in the development of HCC.

MeSH Keywords: Carcinoma, Hepatocellular • Mutation – genetics • Carcinogenesis • Histones

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Background

Hepatocellular carcinoma (HCC) is the fifth most common cause of cancer, causing about 700,000 deaths worldwide per year [1–3]. Despite great progress in understanding the origin, development, and treatment of HCC in recent decades, the detailed molecular mechanisms have not been well characterized [2,4–9]. Identification of novel molecular biomarkers of HCC is crucial to the treatment and cure of this disease [6].

Recently, mutations in the isocitrate dehydrogenase 1 (IDH1) gene, which encodes the enzyme to catalyze the oxidative decarboxylation of isocitrate to α-ketoglutarate (α-KG), were identified in a variety of tumor types (Table 1). Almost all of the mutations in the IDH1 gene were heterozygous and affected the 132nd residue (IDH1 p.R132) [10,11]. Additionally, mutations in the homologous residues p.R172 and p.R140 in the IDH2 were also frequently observed in many types of human cancers [11–13]. IDH1 mutants acquired neomorphic enzymatic activity to catalyze α-KG into 2-hydroxylglutarate (2-HG), which resulted in the accumulation of 2-HG. To date, all the detected samples with IDH1 (p.R132) and IDH2 (p.R140 and p.R172) mutations invariably have shown significant accumulation of 2-HG [14–18]. Further studies showed that 2-HG competitively inhibited multiple α-KG-dependent enzymes, including histone demethylases, prolyl hydroxylases (PHD) and members of the ten-eleven translocation (TET) family of proteins [19–22].

Based on observations that IDH1 mutations were presented in a variety of tumors (Table 1) and IDH1 activity was coordinately regulated with the cholesterol and fatty acid biosynthetic pathways in hepatic cells [23], we hypothesized that IDH1 mutations might play an active role in HCC. Previous studies failed to detect any IDH1 p.R132 mutations in patients with HCC [24–26], but this may be due to the limited number of patients analyzed.

In this study, we aimed to characterize the possible role of IDH1 p.R132 mutations in the carcinogenesis of HCC. We first collected cancerous tissues from 87 Chinese patients with primary HCC and screened for the presence of IDH1 p.R132 mutations. We then overexpressed IDH1 p.R132 mutants in HepG2 cells and quantified the expression levels of multiple α-KG-dependent enzymes and associated genes.

Material and Methods

Patients and mutational analysis

Tissue samples from a total of 87 Han Chinese patients with primary HCC were collected at the YouAn Hospital of Capital Medical University. Among them, 27 patients had both primary cancerous and adjacent normal liver tissues and 60 patients only had cancerous tissues. The criteria for pathological diagnosis of HCC were based on the results after surgical resection by 2 independent pathologists. The study conformed to the tenets of the Declaration of Helsinki and written informed consent was obtained from all patients prior to participation in the study. The institutional review boards of the Kunming Institute of Zoology and Capital Medical University approved this study.

Genomic DNA was isolated from paraffin-embedded tumor tissues (N=87) and normal tissues (N=27) that were dissected from the hematoxylin-eosin stained slides using FFPE DNA kits (Omega Bio-tek, Inc. USA). We amplified a short fragment with a size of 269 bp using primer pair hiDH1f (5’-TGCTGCGAAGCTATAAAGAG-3’) [27]/hiDH1r (5’-GCAATACATTATGCCAAC-3’). PCR products were sequenced using the amplification primers and the Big Dye Terminator v. 3.1 Cycle Sequencing Kit ( Applied Biosystems, CA, USA) on an ABI Prism 3730 DNA sequencer (Applied Biosystems, CA, USA) according to the manufacturer’s manual.

Plasmid construction, cell culture, and transfection

The wild-type human IDH1 and p.R132H mutant (c.395G>A) constructs were obtained as generous gifts from Drs Kun-Liang Guan and Yue Xiong (Fudan University, China). We generated two IDH1 p.R132 mutants (p.R132C, c.394C>T and p.R132G, c.394C>G) based on the wild-type IDH1 by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). All constructs were verified by sequencing.

HepG2 cells were bought from the Kunming Cell Bank, Kunming Institute of Zoology, which was initially introduced from ATCC. Cells were cultured in DMEM medium with 10% fetal bovine serum (Gibco-BRL, Gaithersburg, MD) at 37°C in 5% CO₂. In brief, cells (5×10⁴ per well) were seeded in 12-well plates and grown to 80% confluence. HepG2 cells were transfected with pcDNA3.1 empty vector, IDH1 wild-type and p.R132 mutants (p.R132H, c.395G>A; p.R132C, c.394C>T and p.R132G, c.394C>G), respectively. For each well, a mixture of 1 μg plasmid DNA and 2.5 μL FuGENE® HD Transfection Reagent (Roche, Indianapolis, IN, USA) in a volume of 50 μL was incubated at room temperature for 20 min. Meanwhile, culture medium from cells was removed and washed once with Opti-MEM medium (Gibco-BRL, Gaithersburg, MD). DNA/FuGENE HD complex was added to each well, together with an additional 450 μL Opti-MEM. After incubation for 6 h, 1 mL of growth medium was added to each well. Cells were then incubated at 37°C for another 42 h until performance of the protein and gene expression assay. All cells were harvested 48 h later after transient transfection. To mimic hypoxia, untransfected cells were washed once with phosphate-buffered saline and incubated in 200 μM CoCl₂ (Sigma-Aldrich, USA) for 6 h before harvesting.
| Diseases                     | Total number of samples* | Samples with mutation | Mutation frequency | Frequency range | Number of IDH1-related reports ** |
|------------------------------|--------------------------|-----------------------|-------------------|----------------|----------------------------------|
| **Brain tumors**             |                          |                       |                   |                |                                  |
| Gliomas                      | 18297                    | 6432                  | 35.2%             | 0.0–100.0%     | 168                              |
| Non-glioma brain tumors      | 2232                     | 675                   | 30.2%             | 0.0–100.0%     | 30                               |
| **Hematological malignancies** |                          |                       |                   |                |                                  |
| AML                          | 15509                    | 1053                  | 6.8%              | 0.0–25.0%      | 68                               |
| MDS/MPD/MPN                  | 4376                     | 82                    | 1.9%              | 0.0–18.8%      | 28                               |
| NHL                          | 640                      | 1                     | 0.2%              | 0.0–0.8%       | 4                                |
| ALL                          | 567                      | 7                     | 0.5%              | 0.0–3.2%       | 6                                |
| CML                          | 479                      | 0                     | 0.0%              | 0.0%           | 7                                |
| HL                           | 122                      | 0                     | 0.0%              | 0.0%           | 3                                |
| MM                           | 92                       | 0                     | 0.0%              | 0.0%           | 2                                |
| CLL                          | 18                       | 0                     | 0.0%              | 0.0%           | 2                                |
| AMML                         | 3                        | 0                     | 0.0%              | 0.0%           | 1                                |
| **Others**                   |                          |                       |                   |                |                                  |
| Mesenchymal tumour           | 1200                     | 74                    | 6.2%              | 6.2%           | 1                                |
| Colorectal cancer            | 926                      | 3                     | 0.3%              | 0.0–2.9%       | 8                                |
| Breast cancer                | 603                      | 1                     | 0.2%              | 0.0–100.0%     | 6                                |
| Lung cancer                  | 517                      | 0                     | 0.0%              | 0.0%           | 6                                |
| Sarcoma                      | 529                      | 1                     | 0.2%              | 0.0–100.0%     | 3                                |
| Pheochromocytoma             | 214                      | 9                     | 0.0%              | 0.0%           | 2                                |
| Prostate cancer              | 387                      | 7                     | 1.8%              | 0.0–2.7%       | 6                                |
| Pancreatic cancer            | 293                      | 0                     | 0.0%              | 0.0%           | 4                                |
| Thyroid Cancer               | 504                      | 19                    | 3.8%              | 0.0–15.7%      | 6                                |
| Cholangiocarcinoma           | 482                      | 45                    | 9.3%              | 7.1–14.9%      | 3                                |
| GIST                         | 150                      | 0                     | 0.0%              | 0.0%           | 7                                |
| Enchondroma and related      | 278                      | 103                   | 37.1%             | 1.0–90.0%      | 4                                |
| Gastric cancer               | 190                      | 0                     | 0.0%              | 0.0%           | 3                                |
| Ovarian cancer               | 176                      | 0                     | 0.0%              | 0.0%           | 4                                |
| Hepatocellular carcinoma     | 159                      | 0                     | 0.0%              | 0.0%           | 3                                |
| Paraganglioma                | 155                      | 1                     | 0.6%              | 0.0–0.8%       | 2                                |
| Renal cancer                 | 161                      | 0                     | 0.0%              | 0.0%           | 5                                |
| Melanoma                     | 173                      | 3                     | 1.7%              | 0.0–5.1%       | 4                                |
| Squamous cell carcinoma (oral)| 90                       | 0                     | 0.0%              | 0.0%           | 1                                |
| Biliary tract cancer         | 87                       | 9                     | 10.3%             | 10.3%          | 1                                |

Table 1. Summary of previously reported IDH1 p.R132 mutations in human diseases.
Table 1 continued. Summary of previously reported IDH1 p.R132 mutations in human diseases.

| Diseases                                      | Total number of samples* | Samples with mutation | Mutation frequency | Frequency range | Number of IDH1-related reports ** |
|-----------------------------------------------|--------------------------|-----------------------|--------------------|----------------|----------------------------------|
| Esophageus cancer                             | 73                       | 0                     | 0.0%               | 0.0%           | 2                                |
| Bladder cancer                                | 38                       | 0                     | 0.0%               | 0.0%           | 1                                |
| Fibrous histiocytoma                          | 36                       | 0                     | 0.0%               | 0.0%           | 1                                |
| Urothelial carcinoma                          | 28                       | 0                     | 0.0%               | 0.0%           | 1                                |
| Gallbladder cancer                            | 25                       | 0                     | 0.0%               | 0.0%           | 3                                |
| Squamous cell carcinoma (skin)                | 19                       | 0                     | 0.0%               | 0.0%           | 1                                |
| Mesothelioma                                  | 18                       | 0                     | 0.0%               | 0.0%           | 3                                |
| Endometrial                                   | 18                       | 0                     | 0.0%               | 0.0%           | 1                                |
| Cervical cancer                               | 11                       | 0                     | 0.0%               | 0.0%           | 2                                |
| NPC                                           | 7                        | 0                     | 0.0%               | 0.0%           | 1                                |
| HNSCC                                         | 1                        | 0                     | 0.0%               | 0.0%           | 1                                |

AML – acute myeloid leukemia; MDS/MPD/MPN – myelodysplastic/myeloproliferative diseases/neoplasms; NHL – non-Hodgkin lymphoma; ALL – acute lymphocytic leukemia; CML – chronic myeloid leukemia; HL – Hodgkin lymphoma; MM – multiple myeloma; CLL – chronic lymphocytic leukemia; AML – acute mixed lineage leukemia; GIST – gastrointestinal stromal tumor; NPC – nasopharyngeal carcinoma; HNSCC – head and neck squamous cell cancer. * The last PubMed search was performed on March 8, 2013. ** Papers with redundant data were not included in this table. Due to the limit of reference list for the paper, we have to present the entire list of all 293 references at http://www.mitotool.org/lab/pdf/IDH1_Table_1.pdf.

Table 2. Primers used for quantitative real-time PCR.

| Primer name | Sequence (5’-3’) | Product length (bp) | GenBank accession number | Annealing temperature (°C) |
|-------------|------------------|---------------------|-------------------------|---------------------------|
| GAPDH_F*    | CAACTACATGGTCTTACATGTTC | 181                | –                       | 55                        |
| GAPDH_R*    | GCCAGTGACTCCACGAC  | –                   |                         |                           |
| GLUT1_F**   | GATTGGCTCTTCTCTGTGG | 129                | –                       | 55                        |
| GLUT1_R**   | TCAAAGGACTGGCCAGGTT | –                   |                         |                           |
| VEGF_F**    | AGGAGAGGGCGAGAATCATCA | 74                 | –                       | 55                        |
| VEGF_R**    | CTGGGATTGGATGGCAAGCT | –                   |                         |                           |
| HOXA2_F     | AACCTAACACACACACCC  | 116                | NM_006735.3             | 55                        |
| HOXA2_R     | ATGCCATCCTAATGTTATA | –                   |                         |                           |
| HOXA4_F     | CTGTAGGTGAGTTGAC    | 80                  | NM_002141.4             | 55                        |
| HOXA4_R     | AGGGAGCACAGAATCCT   | –                   |                         |                           |
| HOXA5_F     | TTGATGCAATGTTGTT    | 88                  | NM_019102.2             | 55                        |
| HOXA5_R     | TAAACGCTTGGAGCTT    | –                   |                         |                           |
| HOXA6_F     | AGGGCCGGCGAGTAGATGC | 100                 | NM_024014.2             | 65                        |
| HOXA6_R     | CGGGGGAGAAAATGTGGG  | –                   |                         |                           |
| HOXA7_F     | TGGGGGACCTTGTAGCA   | 89                  | NM_006896.3             | 60                        |
| HOXA7_R     | AGGAGTGAAAGGGCATG   | –                   |                         |                           |

* These primers are taken from Zhou et al. [33]; ** These primers are taken from Zhao et al. [28].

Quantitative real-time PCR

1 μg total RNA was isolated from HepG2 cells 48 h post-transfection using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was isolated from HepG2 cells 48 h post-transfection using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). 1 μg total RNA was used to synthesize cDNA using M-MLV Reverse Transcriptase (Promega Corp., Madison, WI, USA) with an oligo(dT) 18 primers. Quantitative real-time PCR was performed on MyiQ2 Two-Color Real-Time PCR Detection system.
(BioRad Laboratories, Hercules, CA, USA) with SYBR® Premix Ex Taq™ II kit (TaKaRa Biotechnology Co., Ltd. Dalian, China) according to the manufacturer's instructions. The primers for GLUT1, VEGF, HOXA2, HOXA4, HOXA5, HOXA6, and HOXA7 genes are shown in Table 2. The mRNA expression of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control for normalization.

### Western blotting

Cells were collected at 48 h post-transfection and proteins were extracted by cell lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China). Protein samples were separated on 8–12% SDS-PAGE gel and transferred onto polyvinylidene fluoride (PVDF) membranes (Roche Diagnostics, USA) using standard procedures. The membranes were blocked at room temperature for 2 h with 5% nonfat dry milk. Membranes were incubated with primary antibody against FLAG (1:2000, EneGene Biotech Co. Ltd, China), HIF-1α (1:1000, Novus Biologicals, Kittketon, Co, USA), H3K4me3 (1:5000, Abcam, UK), H3K79me2 (1:3000, Active Motif, Inc. Japan), GAPDH (1:5000, EneGene Biotech Co. Ltd, China), β-actin (1:100000, Enogene Biotech Co. Ltd, China), and tubulin (1:10000, Enogene Biotech Co. Ltd, China) overnight at 4°C. Membranes were washed 3 times for 10 min each and incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (1:10000, KPL, Gaithersburg, MD, USA). The proteins were detected using enhanced chemiluminescence (ECL) reagents (Millipore, Billerica, MA, USA).

### Statistical analysis

For measurement of the expression levels of multiple α-KG-dependent enzymes and associated genes in HepG2 cells overexpressing IDH1 p.R132 mutants, each assay was independently performed at least 3 times to validate the consistency of the results. Data was presented as mean ±SD of 3 independent tests. Statistical analysis was performed with GraphPad software (GraphPad Software, La Jolla, CA, USA) with unpaired Student’s t-test.

### Results

#### Absence of IDH1 p.R132 mutations in 87 HCC patients

We sequenced the region covering IDH1 p.R132 mutations in a total of 87 Chinese patients with HCC. Among them, 27 patients were analyzed for both cancerous tissues and paired normal tissues. None of the analyzed samples were found to harbor any IDH1 p.R132 mutations (Figure 1). This observation is consistent with previous reports for patients from Korea and America [24–26].

#### Overexpression of IDH1 p.R132 mutants in HepG2 cells did not influence the expression of α-KG-dependent enzymes and downstream target genes

Previous studies have characterized the potential mechanism of the IDH1 mutations in carcinogenesis [14,20,28] (Figure 2). To further explore whether the IDH1 p.R132 mutations (c.395G>A, c.394C>T and c.394C>G) would have an effect on the development of HCC, we first determined the level of HIF-1α protein and the mRNA expression levels of GLUT1 and VEGF genes, which were indirectly regulated by PHD and associated with the activation of the HIF-1α signaling pathway [28]. Compared with cells expressing IDH1 wild-type, all 3 mutants did not significantly increase protein expression levels of HIF-1α in HepG2 cells.

![Figure 1. Representative sequencing electrophoregrams of the wide-type IDH1 codon 132 in the paired cancerous and normal tissues from a Chinese patient with primary hepatocellular carcinoma.](image)

| Codon | Amino Acid | Cancerous tissue | Matched normal tissue |
|-------|------------|------------------|----------------------|
| 130   | I          | A                | A                    |
| 131   | G          | T                | T                    |
| 132   | R          | G                | G                    |
| 133   | H          | T                | T                    |
| 134   | A          | A                | A                    |

![Figure 2. Summarization of the role of IDH1 mutants and 2-HG signaling in cellular pathway. IDH1 mutants inhibit its normal catalytic activity and acquire the ability to convert α-ketoglutarate (α-KG) to 2-hydroxyglutarate (2-HG). 2-HG competitively inhibited multiple α-KG-dependent enzymes, including prolylhydroxylases (PHD) and histone demethylases.](image)
The real-time PCR results showed that overexpression of all 3 mutants did not significantly increase mRNA expression levels of GLUT1 and VEGF genes in HepG2 cells (Figure 3B). These negative results led us to speculate whether IDH1 mutations affect the production of 2-HG in HepG2 cells. Recent reports showed that elevated 2-HG increased histone methylations and altered the expression level of HOXA genes family in cells that were overexpressed IDH1 p.R132H mutant [20,21]. We measured the H3K4me3 and H3K79me2 proteins and the HOXA mRNA level in HepG2 cells expressing IDH1 p.R132 mutants, but discerned no effect of any of the 3 mutations on change of the histone methylation protein level in HepG2 cells (Figure 3).
Discussion

Since the initial observation that IDH1 gene mutations presented in glioblastoma multiforme [10], a series of studies have been performed to detect the presence of such mutations in a variety of tumors. These prior studies showed that almost all mutations in the IDH1 gene were IDH1 p.R132, and none of the analyzed samples with HCC harbored IDH1 p.R132 mutations (Table 1). Additionally, subsequent functional assays found that the mutations in the IDH1 gene might contribute to carcinogenesis via accumulation of 2-HG, which could be substantially attributed to the inhibition of α-KG-dependent enzymes.

In this study, we first detected IDH1 p.R132 mutations in Han Chinese patients with HCC, to test whether IDH1 mutations were presented in HCC. Our screening for cancerous tissues or paired cancerous and normal tissues from 87 patients showed no IDH1 p.R132 mutations (Figure 1). The absence of the IDH1 p.R132 mutations in HCC analyzed here and elsewhere indicate that changes at this position may not be actively involved in the pathogenesis of HCC [24–26]. Recent studies have found recurrent IDH1 mutations in cholangiocarcinoma [26,29,30]. However, due to the relatively limited sample size analyzed in this and prior studies [24–26], it is still hard to draw a definitive conclusion that IDH1 p.R132 mutations are not presented in HCC. Moreover, recent studies showed that all detected samples with IDH1 p.R132 mutations presented the accumulation of 2-HG [14–18]. Simultaneously, mutant IDH1 might also activate histone methylations and PHD downstream target genes, including HOXA genes, GLUT1, and VEGF genes [20,28]. Based on these observations (Figure 2), we tested whether overexpression of IDH1 p.R132 mutants would lead to upregulation of those genes associated with the activation of the 2-HG signaling pathway, to further determine if IDH1 mutations are involved in the development of HCC. However, our measurement revealed no essential change in the expression levels of HIF-1α target genes or histone methylation marker genes in HepG2 cells with overexpression of IDH1 p.R132 mutants and wild-type (Figure 3). A limitation of this cellular observation is that the changes in histone methylation protein and mRNA expression levels of VEGF, GLUT1 and HOXA were, at best, secondary effects of mutant IDH1 activity. An evaluation of mutant IDH1 activity should have included the measurement of 2-hydroxyglutarate levels in cells and media to discern the direct downstream effect. Although we did not quantify the level of 2-HG in transfected cells and culture medium due to lack of required equipment, we think that it might not be altered, because no 2-HG induced genes were changed. Taken together, our results indicate that IDH1 p.R132 mutations may not play an active role in HCC. Another limitation of the current cellular assay is that we only analyzed HepG2 cells. It may be more proper to work on human primary hepatocytes [31,32].

One equally important issue regarding the role of the IDH1 gene in the development of solid tumors is whether the haplotype and/or rare variant(s) of this gene are a risk factor for cancer susceptibility and development. According to the latest released HapMap phase 3 data (http://hapmap.ncbi.nlm.nih.gov/), there are 5 haplotypes of the IDH1 gene that were characterized by 5 SNPs (rs6730955, rs16840798, rs6435435, rs3769521, and rs1437410) in all 255 East Asian samples or samples of East Asian origin. It would be rewarding to genotype all these SNPs in patient samples and to identify the potential association between the onset of cancer and the allele/haplotype of the IDH1 gene. Detecting rare variant(s) requires extremely larger sample size and deep sequencing of the genomic region covering the IDH1 gene. Unfortunately, we used up the tiny amount of DNA from the hematoxylin/eosin stained slides and could not test the above speculation in this study. In addition, accumulating evidence has shown that IDH2 mutations (p.R140 and p.R172) exhibit similar tumor-promoting roles as IDH1 p.R132 mutations [15,18,29]. We did not test whether these IDH2 hotspot mutations were involved in our samples due to the lack of sufficient DNA. Further research should be carried out to clarify this issue.

Conclusions

Taken together, we detected no IDH1 p.R132 mutation in 87 Chinese HCC samples. IDH1 p.R132 mutants might not lead to alteration of expression of these genes involved in the previously well characterized cellular pathways in HepG2 cells. Our results suggest that IDH1 p.R132 mutations are not be actively involved in HCC. Further studies should be carried out to characterize the exact role of the IDH1 and IDH2 genes in HCC and to validate our negative observations.

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