Global Metabolic Profiling Identifies a Pivotal Role of Proline and Hydroxyproline Metabolism in Supporting Hypoxic Response in Hepatocellular Carcinoma

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Abstract

Purpose: Metabolic reprogramming is frequently identified in hepatocellular carcinoma (HCC), which is the most common type of liver malignancy. The reprogrammed cellular metabolisms promote tumor cell survival, proliferation, angiogenesis, and metastasis. However, the mechanisms of this process remain unclear in HCC.

Experimental Design: The global nontargeted metabolic study in 69 paired hepatic carcinomas and adjacent tissue specimens was performed using capillary electrophoresis-time of flight mass spectrometry–based approach. Key findings were validated by targeted metabolomic approach. Biological studies were also performed to investigate the role of proline biosynthesis in HCC pathogenesis.

Results: Proline metabolism was markedly changed in HCC tumor tissue, characterized with accelerated consumption of proline and accumulation of hydroxyproline, which significantly correlated with α-fetoprotein levels and poor prognosis in HCC.

In addition, we found that hydroxyproline promoted hypoxia- and HIF-dependent phenotype in HCC. Moreover, we demonstrated that hypoxia activated proline biosynthesis via upregulation of ALDH1A1, subsequently leading to accumulation of hydroxyproline via attenuated PRODH2 activity. More importantly, we showed that glutamine, proline, and hydroxyproline metabolic axis supported HCC cell survival through modulating HIF1α stability in response to hypoxia. Finally, inhibition of proline biosynthesis significantly enhanced cytotoxicity of sorafenib in vitro and in vivo.

Conclusions: Our results demonstrate that hypoxic microenvironment activates proline metabolism, resulting in accumulation of hydroxyproline that promotes HCC tumor progression and sorafenib resistance through modulating HIF1α. These findings provide the proof of concept for targeting proline metabolism as a potential therapeutic strategy for HCC. Clin Cancer Res; 24(2); 474-85. ©2017 AACR.

Introduction

Reprogrammed metabolism is a hallmark of cancer cells and a perpetual topic of interest for researchers (1, 2). Cancer cells reprogram their cellular metabolisms to consume more energy and materials for proliferation, invasion, and metastasis (3, 4). Despite these common features among different tumor types, many intrinsic genetic lesions and extrinsic nongenetic influences may also contribute to abnormal metabolism in a tumor-dependent manner (1). To increase survival of patients with cancer, a better understanding of the deregulated metabolism associated with a specific malignancy is necessary to provide promising opportunities for early clinical diagnosis and potential therapeutic interventions.

We have previously demonstrated metabolic alterations in hepatocellular carcinoma (HCC) tumors, including elevated glycolysis, gluconeogenesis, and β-oxidation with a reduced tricarboxylic acid (TCA) cycle (5). However, the role of amino acid metabolism in HCC pathogenesis has not been identified. Oncogenic MYC can promote glutamine/proline metabolism through regulating enzymes in this pathway, suggesting an involvement of proline metabolism in tumorigenesis (6). In fact, recent studies have shown that proline biosynthesis from glutamine might be critical for cell growth (7).
inhibition of delta-1-pyrroline-5-carboxylate synthetase (ALDH18A1, P5CS), a key enzyme in the proline metabolic pathway, significantly decreased cell viability in melanoma cell lines, suggesting that proline biosynthesis might be a potential therapeutic target for cancer (8). One biological function of synthesized proline is to produce collagen, which accounts for the majority of the extracellular matrix (ECM; ref. 9). The incorporated proline is utilized to generate hydroxyproline, which is catalyzed by prolyl 4-hydroxylase (PH; ref. 10). Intracellular hydroxyproline can originate from procollagen digested by prolidase, the only known cytosolic dipeptidase, which breaks down dipeptides to yield free proline and hydroxyproline (11). Furthermore, hydroxyproline may function as a regulatory hub in low-oxygen conditions and stabilize hypoxia-induced factor-1α (HIF1α) in response to hypoxia (12, 13). However, little is known about the role of proline metabolism as a regulatory hub in low-oxygen conditions and stabilize hypoxia-induced factor-1α (HIF1α) in response to hypoxia (12, 13). We further demonstrated that hydroxyproline was discovered and validated in two HCC tumor tissues of patients with primary liver cancer compared with normal liver tissues. Accumulation of hydroxyproline was discovered and validated in two HCC cohorts. Cellular hydroxyproline levels positively correlated with α-fetoprotein (AFP) levels and poor prognosis. Importantly, we showed that hydroxyproline promoted angiogenesis and cell growth in xenograft tumors, supporting a HIF1α-dependent mechanism. Hypoxia promotes proline biosynthesis and hydroxyproline production, which are crucial for HCC cell survival. We further demonstrated that hydroxyproline modulates HIF1α stability, resulting in enhanced cellular response to hypoxia. Inhibition of proline biosynthesis significantly induced apoptosis and sensitized HCC cells to sorafenib, which is a tyrosine kinase inhibitor that has been used in the clinics for advanced liver cancer. Our findings demonstrated that glutamine, proline, and hydroxyproline metabolic axis promoted HCC pathogenesis and sorafenib resistance through modulating HIF1α in HCC.

### Translational Relevance
Cancer cells underlie metabolic reprogramming to adapt the cellular stress conditions, including nutrient deprivation, drug treatment, and hypoxia for their survival. However, the mechanism of metabolic adaptation in hepatocellular carcinoma (HCC) is still poorly understood. In this study, by analyzing the metabolic signatures of 69 paired primary HCC specimens, we showed that proline biosynthesis was the most significantly altered amino acid metabolism. Hypoxia microenvironment activates proline biosynthesis via ALDH18A1, subsequently leading to accumulation of hydroxyproline in HCC. Activation of proline and hydroxyproline production promoted HIF1α stability and metabolic adaptation of HCC cells that were critical for cell survival under hypoxia. In contrast, disruption of proline production significantly inhibited tumor growth and sensitized HCC cells to sorafenib by inducing apoptosis. Our findings suggest that proline biosynthesis may be an attractive therapeutic target in HCC.

### Materials and Methods

#### Ethics statement
Tumor samples were obtained from patients with liver cancer with written informed consent under the approval of the Harbin Medical University (Harbin, China) and Zhongshan Hospital of Xiamen University (Xiamen, China), according to the Declaration of Helsinki. All animal procedures were performed in accordance with the NIH Guide on the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the Dalian Medical University (Dalian, China).

#### Clinical samples
A total of 69 human hepatic tissues for untargeted metabolomics discovery and 66 hepatic tissues for targeted external validation were recruited from the Affiliated Hospital of Harbin Medical University (Harbin, China) and Zhongshan Hospital of Xiamen University (Xiamen, China), respectively. Detailed clinical information on the subjects providing the samples is summarized in Supplementary Tables S1 and S2. The sample preparation procedure for the metabolomics analysis is provided in the Supplementary Experimental Procedures.

#### Metabolomics analysis
Untargeted metabolic profiling was performed using a CE-TOF/MS system (Agilent) equipped with the coaxial sheath liquid interface. The targeted metabolomics analysis was performed using Gas chromatography–mass spectrometry Selective Ion Monitoring (GC-MS SIM) mode and LC/MS with multiple reaction monitoring methods. Details of the analytical method are described in the Supplementary Experimental Procedures.

#### Cell culture, siRNA transfection, and viral infection
HCC cell lines (SMMC-7721, HepG2) were purchased from the ATCC and cultured in DMEM containing 10% FBS and 2 mmol/L glutamine under a humidified atmosphere with 5% CO2 at 37°C, as described previously (14). Cell lines were confirmed by short tandem repeat profiling with the PowerPlex1.2 System (Promega) and tested for mycoplasma every 2 weeks. After getting cells from liquid nitrogen, the cell lines were passaged once prior to use in experiments. si-MYC (SR303025), si-HIF1α (SR302102), and si-ALDH18A1 (SR303932) were purchased from Origene. For siRNA transfection, Lipofectamine 2000 (Life Technologies) was used according to the standard protocol. The lentiviral shALDH18A1 was purchased from Origene (TL314847, Origene) and cotransfected with the packaging plasmid (TR30022, Origene) to generate the viral supernatant as previously described (15).

#### Reagents
Antibodies against HIF1α (cat# 14179s), OH-HIF1α (cat# 3434s), PARP1 (cat# 9542p), Myc (cat# 5605s), and MMP9 (cat# 13667) were purchased from Cell Signaling Technology. Antibodies against PRODH (cat# ab93210) were purchased from Abcam Inc. PRODH2 antibody (cat# PA-42039) was purchased from Invitrogen. ALDH18A1 antibody (cat# NRP1-83324) was purchased from NOVUS Biologicals. Actin antibody (Santa Cruz Biotechnology) was used as protein loading control. Sorafenib (cat# s7397) was purchased from Selleck. N-Carbobenzoxy-l-proline (CBZ-pro, cat# 114811-14) was purchased from Bachem.

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**Translational Relevance**

Cancer cells underlie metabolic reprogramming to adapt the cellular stress conditions, including nutrient deprivation, drug treatment, and hypoxia for their survival. However, the mechanism of metabolic adaptation in hepatocellular carcinoma (HCC) is still poorly understood. In this study, by analyzing the metabolic signatures of 69 paired primary HCC specimens, we showed that proline biosynthesis was the most significantly altered amino acid metabolism. Hypoxia microenvironment activates proline biosynthesis via ALDH18A1, subsequently leading to accumulation of hydroxyproline in HCC. Activation of proline and hydroxyproline production promoted HIF1α stability and metabolic adaptation of HCC cells that were critical for cell survival under hypoxia. In contrast, disruption of proline production significantly inhibited tumor growth and sensitized HCC cells to sorafenib by inducing apoptosis. Our findings suggest that proline biosynthesis may be an attractive therapeutic target in HCC.
Cell viability and invasion assay
For cell viability assays, 3,000 cells were plated in 96-well plates and cultured overnight. Compounds were then added in serial dilutions. Cellular ATP levels were determined after 48, 96, and 120 hours by the Cell Titer-Glo Luminescence Cell Viability Assay (cat# G7570; Promega). Plates were measured on a THERMO max microplate reader. Cell invasion assays were performed using Transwell membranes coated with Matrigel (BD Bio). The lower chamber was filled with 10% FBS. After 48 hours, cells remaining in the upper chamber were removed with cotton swabs, whereas invading cells were fixed with 3% paraformaldehyde (Santa Cruz Biotechnology) and stained with crystal violet (Solarbio). Cells that penetrated through the polyethylene terephthalate membrane were counted in 10 representative microscopic fields (∗200 magnification).

Western blotting
Immunoblotting was carried out using standard techniques. Briefly, cells were lysed in ice-cold 1x RIPA lysis buffer, and protein concentrations were determined. Aliquots (50 μg) of protein were denatured in Laemmli loading buffer and separated on precast 4% to 10% NuPAGE Novex 4% to 12% Bis-Tris Protein Gels (Novex-Invitro). The proteins were then transferred to polyvinylidene difluoride membranes, which were blocked and probed with primary antibodies, then detected using appropriate horseradish peroxidase–labeled secondary antibodies. Proteins were visualized using enhanced chemiluminescence (Thermo-Fisher) on Hyperfilm (GE Healthcare).

In vivo xenograft mice
To generate tumor xenografts, 3 million SMMC-7721 cells were injected randomly into the flanks of BALB/c immunodeficient nude mice (2 months old, male; eight tumors for each group), which were unable to produce T cells due to the lack of a thymus (Charles River). Tumors were measured with Vernier calipers in two dimensions, and the volumes were calculated using the following equation: \( \pi/6 \times \text{length} \times \text{width}^2 \). Note that 1% hydroxyproline was administered orally with water 1 week after injection of SMMC-7721 cells. Sorafenib was administered at 40 mg/kg i.p. These doses were selected based on the previous publication with these agents (16). All of the mice were sacrificed after 3 weeks of treatment.

In vitro \(^{13}\)C tracer analysis
For isotopic labeling experiments, cells were cultured in glutamine-free DMEM (Sigma) containing 10% FBS, with 4 μmol/L [U-\(^{13}\)C]-glutamine (Cambridge Isotope Labs) for 3 and 6 hours under normoxic and hypoxic conditions, respectively. Cells were rinsed with 10 mL PBS twice and quenched with liquid nitrogen. Note that 1 mL of ice-cold 80% methanol-water (v/v) was added to each dish, and cells were scraped and collected in a tube by pipette. The extracts were sonicated and vortexed, followed by a centrifugation at 13,500 rpm for 15 minutes. Prior to derivatization, 750 μL of supernatant was lyophilized in a Labconco Free Zone 4.5 Freeze-Dry System. The dry residue was dissolved in 40 μL of methoxyamine solution (20 mg/mL in pyridine) and placed in a 37°C water bath for 1.5 hours. Subsequently, the sample was held at 55°C for 1 hour after adding 40 μL N-methyl-N-trifluoracetamide (MTBSTFA) with 1% tert-butyl(dimethyl)silane (TBDMS). Ultimately, the derivatized samples were centrifuged at 14,000 g for 10 minutes, and the supernatant was transferred to a conical insert in 2-mL glass vial for subsequent GC/MS analysis. GC/MS analysis was performed using a Shimadzu QP 2010GC tandem quadrupole MS equipped with Agilent DB-5 MS fused silica capillary column. The GC oven temperature was held at 100°C for the first 3 minutes and then increased at 3.5°C/min to 310°C for 10 minutes. One microliter of the derivatized sample was injected to the GC-MS system. The helium carrier gas flow rate was set to 1.16 mL/min, and the temperatures of the interface and the ion source were adjusted to 280 and 230°C, respectively. Mass isotopomer distributions were corrected for natural isotope abundance by using the in-house–developed software.

Immunohistochemistry
Immunohistochemistry (IHC) was performed according to the general protocol. Detailed experimental procedures are described in the Supplementary Experimental Procedures.

Data processing and statistical analysis
Raw metabolomics data processing was carried out as previously described. Before statistical analysis, normalization to the weight and the area of internal standards was conducted for all tissues. Statistical analyses were carried out using SIMCA-P software (Umetrics), Multi Experiment Viewer (MeV; open-source genomic analysis software created by the MeV development team), an in-house–developed program using Matlab software (The MathWorks). Heatmaps were generated with MeV. Metabolic pathway analysis was carried out using MetaAnalyst (http://www.metaboanalyst.ca), the Kyoto Encyclopedia of Genes and Genomes, and the Human Metabolome Database. To determine the statistical significance between groups in metabolic study, Wilcoxon–Mann–Whitney test, Benjamini–Hochberg FDR correction, and matched pairs sign test were carried out. To determine the statistical significance in biological experiments, two-tailed Student t test for comparison between two groups or one-way ANOVA for multiple group comparison was used by GraphPad Prism (GraphPad Software). A value of \( P < 0.05 \) was considered statistically significant. Details of the data processing and statistical analysis procedures are described in the Supplemental Information.

Results
Global metabolic profiling identifies hydroxyproline accumulation in primary liver cancer
In this study, 69 hepatic tissue samples of HCC were surgically resected from patients, including 23 “normal tissues” (borderline tissues acquired from patients with benign hepatic tumor) representing baseline metabolome levels and 18 borderline tissues and 28 carcinoma tissues (Supplementary Table S1). Untargeted metabolic analysis based on CE-MS was performed for the discovery portion of the study (Supplementary Fig. S1A). To obtain insight into the comprehensive metabolic changes in these tissues, multivariate pattern recognition was performed. In a partial least squares discriminant analysis (PLS-DA) model (Fig. 1A), the carcinoma tissue samples were located together, apart from the borderline and normal groups, along the direction of the first principal component. Despite the similar metabolic characteristics of the borderline and normal groups obtained from the PLS-DA model, a slight discriminatory trend between these two groups was also present in the direction of the second principal
component. Univariate analysis was conducted to quantify these differences. A Venn diagram (Fig. 1B) analysis revealed 49 "carcinoma versus normal," 49 "carcinoma versus Border- line," and 49 "borderline versus normal" metabolites that met the significance criterion ($P < 0.05$) in each comparison pair based on the distribution of fold change ($\log_{10}$). On the basis of the 68 significant differential metabolites associated with carcinoma tissue (the union of sets of a and b), a total of 68 significantly different metabolites associated with carcinoma tissue (the union of sets of a and b) were clustered based on the analysis of Pearson correlation coefficients. A heatmap across three groups. A total of 68 significantly different metabolites associated with carcinoma tissue (the union of sets of a and b) were clustered based on the analysis of Pearson correlation coefficients. E, Metabolic characterization of hepatic carcinoma based on the pathway enrichment analysis. All data are presented as mean ± SEM. *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$. The black * means the statistical significance between the normal tissue group and other groups. The red * means the statistical significance between the carcinoma tissue group and borderline tissue group.

Hydroxyproline correlates with HCC clinical pathogenesis

Twenty metabolites met both the univariate requirement ($P < 0.05$ in both of two comparisons between the carcinoma and noncarcinoma groups) and the multivariate criterion [VIP (1) >1 in both of two PLS-DA models between the carcinoma and noncarcinoma groups]. These metabolites were subjected to hierarchical clustering to visualize their variation tendencies (Fig. 1D). A suite of metabolic processes was observed in tumor cells to facilitate proliferation and metastasis. Pathway enrichment analysis showed that alanine, aspartate, and glutamate metabolism, purine metabolism, the TCA cycle, taurine and hypotaurine metabolism, and glycerophospholipid metabolism were significantly altered in tumor tissues (Fig. 1E). These metabolic perturbations were involved in (i) carbohydrate metabolism, (ii) glycerophospholipid metabolism, and (iii) amino acid metabolism (Supplementary Fig. S2).
Among these metabolites, 13 were selected with an FDR < 0.05 (Fig. 2A). Subsequently, the statistical significance of 18 pairs of carcinoma and borderline tissues (a subset of HMU samples) was internally validated. Hydroxyproline exhibited a progressive trend of a significant increase from normal to borderline tissues (P < 0.05) and then to carcinoma tissues (P < 0.05), suggesting its great potential in tumorigenesis (Fig. 2A). Moreover, among the amino acids showing accumulation in borderline tissues, hydroxyproline is unique from the perspective of pathway analysis. To determine if the increase in hydroxyproline was due to liver injury caused by hepatitis or cirrhosis, we analyzed the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum. However, we did not observe any correlation between hydroxyproline and ALT or AST (Supplementary Fig. S4), suggesting that the increase in hydroxyproline was derived from intact liver cells, rather than damaged liver tissues. To validate our profiling results, we analyzed another cohort of 33 pairs of HCC and borderline tissues from another clinical site (Zhongshan Hospital Xiamen University, XMU). Targeted metabolomics analysis confirmed the elevation of hydroxyproline in both HCC cohorts based on the comparison of borderline and tumor tissues (P < 0.05, Fig. 2B and C). In agreement, hydroxyproline levels were significantly upregulated in the groups with high AFP level (Fig. 2D). Notably, a positive correlation was also observed between the levels of hydroxyproline and poor prognosis in this HCC cohort.
Hydroxyproline supports hypoxia-dependent phenotype in vitro and in vivo

To determine if hydroxyproline supported hypoxia-dependent phenotype, we carried out the cell invasion assay, showing the enhanced cell motility in the presence of exogenous hydroxyproline (Fig. 3A). In addition, we found that treatment of hydroxyproline significantly enhanced level of HIF1α under hypoxia (Fig. 3B). Furthermore, knockdown of HIF1α by siRNA inhibited hydroxyproline-induced HCC cell survival under hypoxia (Fig. 3C). The knockdown efficiency for HIF1α was determined by immunoblotting (Fig. 3C). The findings above led us to hypothesize that hydroxyproline contributed to tumor development through modulating the HIF1α-dependent phenotype in mice model. Therefore, we subcutaneously injected SMMC-7721 cells into immunodeficient nude mice (2 groups, n = 5 in each group). Hydroxyproline was given 1 week after injection for up to 3 weeks. Xenograft tumors were observed every 3 days after cell inoculation. As expected, the size of xenograft tumors in the hydroxyproline-treated group increases twofold compared with the control group at 3 weeks after implantation (Fig. 3D). In addition, hydroxyproline-treated mice exhibited higher levels of Ki-67 immunoreactivity relative to untreated mice, indicating the proliferative function of hydroxyproline in HCC (Supplementary Fig. S5).

We next examined the effect of hydroxyproline on angiogenesis in HCC xenografts. As expected, CD31 IHC showed more blood vessels formed in the hydroxyproline-treated xenografts compared with the controls (Fig. 3E). In consistence with this observation, level of HIF1α was significantly augmented in the hydroxyproline-treated group (Fig. 3F). Taken together, these findings indicate that hydroxyproline promotes hypoxia and HIF-dependent tumor phenotypes.

Hypoxia activates proline biosynthesis

Recent metabolic profiling study showed that level of intracellular hydroxyproline is associated with hypoxic response in tumor cell lines, which led us to examine if proline metabolism is activated in low-oxygen condition. Indeed, we found that expression of ALDH18A1, the key enzyme responsible for proline production, significantly increased in a time course manner under hypoxia (Fig. 4A). In agreement with previous findings, expression of proline dehydrogenase 1 (PRODH) increased with hypoxia. While expression of proline dehydrogenase 2 (PRODH2), an enzyme to catabolize hydroxyproline, remarkably decreased under hypoxia. Altered ALDH18A1 and PRODH2 expressions by hydroxyproline were also confirmed by qR-PCR assay (Supplementary Fig. S6). Targeted metabolic analysis was also performed to show the accumulation of hydroxyproline, which is consistent with attenuated PRODH activity under hypoxia (Fig. 4B). The cellular hypoxic response was also confirmed by induction of HIF1α and matrix metalloproteinase 9 (MMP9), which is a typical downstream gene of HIF1α to digest the component of ECM (Fig. 4B). To determine if hydroxyproline enhances proline production in HCC cells, we carried out 13C-glutamine tracer analysis and found increased conversion of glutamine to glutamate and proline under hypoxia in 3 to 6 hours. (Supplementary Fig. S7). In addition, because it has been shown that oncogenic Myc is frequently activated in HCC and regulate glutaminolysis via glutaminase (GLS; refs. 17, 18), we hypothesized that MYC may contribute to the enhanced proline production under hypoxia via glutamine catabolism. To determine this, we knocked down Myc by siRNA in SMMC-7721 cells and showed that loss of Myc significantly decreased glutamate, proline, and hydroxyproline production in response to hypoxia in 3 hours (Fig. 4C). In consistent with previous finding, we found that knockdown of Myc impaired the expression of ALDH18A1 (Fig. 4D), but had no effect on prolidase expression (Supplementary Fig. S8). Notably, we showed that hydroxyproline level positively correlated with protein levels of Myc, HIF1α, and ALDH18A1 in primary HCC tissues (Fig. 4E).

Glutamine, proline, and hydroxyproline metabolic axis stabilizes HIF1α under hypoxia

The results above led us to examine if proline and hydroxyproline metabolism contributed to hypoxic response and HIF induction in low-oxygen condition. We noted that cellular proline can be directly produced from glutamine and utilized to synthesize proteins (19, 20). Intracellular hydroxyproline can be released from peptides after proline hydroxylation by prolidase (21). Therefore, to determine if this process is critical for hypoxic response, we measured HIF1α level in SMMC-7721 cell line in response to hypoxia after treatment with CBZ-pro, a specific prolidase inhibitor. As expected, HIF1α level was remarkably abolished due to the inhibition of prolidase activity (Fig. 5A). Importantly, we found that hydroxyproline inhibited hydroxylation of HIF1α in a dose-dependent manner and attenuated the binding of HIF1α to Von Hippel–Lindau (VHL) tumor-suppressor protein in hypoxic condition (Fig. 5B and C). Because Myc regulated glutamine–hydroxyproline metabolism, we attempted to examine if exogenous hydroxyproline had any effect on HIF1α stability in the absence of Myc. Indeed, the attenuated level of HIF1α by Myc knockdown could be restored by additional hydroxyproline in low-oxygen condition (Fig. 5D). Moreover, either ALDH18A1 knockdown or glutamine starvation attenuated HIF1α induction, which could be restored by additional hydroxyproline (Fig. 5E and F). More importantly, glutamine starvation decreased HCC cell viability in low-oxygen condition, which could be completely recovered by exogenous hydroxyproline (Fig. 5G). These findings strongly suggest that glutamine–proline conversion is important for hypoxic response through regulating HIF1α.

Proline metabolism is critical for HCC cell survival and sorafenib resistance under hypoxia

Because it has been shown that hypoxia contributed to sorafenib resistance in HCC (22, 23), we tried to examine the potential role of proline biosynthesis in hypoxia-mediated sorafenib resistance. We showed that knockdown of ALDH18A1 by lentiviral shRNA inhibited HCC cell survival and enhanced the cytotoxicity of sorafenib in both normoxic and hypoxic conditions (Fig. 6A and B). In normoxic condition, loss of ALDH18A1 synergized with sorafenib to induced apoptosis (Fig. 6C and D). In hypoxic condition, ALDH18A1 deficiency could induce HCC cell death regardless of sorafenib treatment (Fig. 6D; Supplementary Fig. S9). In addition, we found that inhibition of proline production by shRNA against ALDH18A1 could significantly enhance the cytotoxic effect of sorafenib on inhibiting HCC tumor growth in vivo (Fig. 6E).
Figure 3. Hydroxyproline promotes hypoxia-related tumor phenotypes. A, Cell invasion was evaluated by in vitro Transwell assays. Cells were starved for 12 hours and treated with or without 2 mmol/L hydroxyproline in normal or low-oxygen conditions. Cell viability was assessed by crystal violet staining. The mean ± SEM is shown for triplicate experiments. B, SMMC-7721 and HepG2 cells were cultured under hypoxia and normoxia for 1.5 hours with or without 2 mmol/L hydroxyproline. Total cell lysates were extracted and subjected to the indicated antibodies. The intensities of HIF1α levels were assessed by Image J software. The ratio of HIF1α/Actin was normalized to hydroxyproline-untreated groups as mean ± SEM. C, SMMC-7721 cells were transfected with scrambled or anti-HIF1α siRNA, and then subsequently cultured under 1% O2 in the presence or absence of 2 mmol/L hydroxyproline. Total cell lysates were extracted and subjected to the indicated antibodies. Cell viability was determined via crystal violet staining for triplicates and plotted as mean ± SEM. D, 3 × 10^6 SMMC-7721 cells were injected into the flanks of BALB/c nude mice. Hydroxyproline was orally administered 1 week after injection. All the mice were sacrificed in 3 weeks. Tumor volume data are shown as the mean ± SEM (n = 7). E, IHC against CD31 was performed to assess the effect on blood vessels formation in xenograft tumors treated with either vehicle or hydroxyproline. Scale bar, 50 μm. Representative images of anti-CD31 IHC from seven tumor xenografts were shown. F, Tumor xenografts were lysed and subjected to anti-HIF1α and anti-Actin antibodies. Numbers of 1–7 indicated seven tumor xenografts from four mice with tumor implantations on both sides each. All data are presented as mean ± SEM; **, P < 0.01 between indicated groups.
HCC is characterized by aberrant metabolic reprogramming. We have previously shown that amino acid (AA) metabolism is activated in HCC compared with adjacent tissue (5). For instance, significant increases in glutamine, glutathione, and branched-chain amino acids, including valine, leucine, and isoleucine, were identified in primary HCC tissues. However, a few studies have addressed whether AA metabolism is crucial for HCC tumor progression. Arginine and proline metabolisms are the central pathway for the biosynthesis of AAs from glutamate (24). Recently, proline metabolism has been shown to be critical for tumor progression.

**Discussion**

Hypoxia activates proline biosynthesis. A, SMMC-7721 cells were cultured under normoxia and hypoxia for 24 and 48 hours, respectively. The cells were subsequently lysed and subjected to the indicated antibodies. B, SMMC-7721 and HepG2 cells were cultured in low-oxygen conditions. Hydroxyproline levels were determined at 0.5, 1.5, and 3 hours by GC/MS. Total cell lysates were extracted and subjected to the indicated antibodies. C, SMMC-7721 cells were transfected with scrambled or anti-MYC siRNA for 48 hours. The cells were cultured under 20% O2 and 1% O2 conditions for 3 hours, and then harvested and subjected to targeted metabolic analysis. Data were shown as mean ± SEM (n = 3); *, P < 0.05 and **, P < 0.01 between indicated groups. D, SMMC-7721 cells were transfected with scrambled or anti-Myc siRNA for 48 hours. Total cell lysates were extracted and subjected to the indicated antibodies. E, Primary tumor tissues of HCC patients were lysed and subjected to the indicated antibodies. The two groups (n = 5 of each) were established based on levels of hydroxyproline that were identified in this study.
Glutamine, proline, and hydroxyproline metabolism is critical for stability of HIF-1α and cell survival in HCC. A, SMMC-7721 cells were treated with the prolidase inhibitor CBZ-Pro for overnight and cultured in 1% O2 condition. Hydroxyproline was then added, followed by culturing for additional 3 hours. Total cell lysates were extracted and subjected to the indicated antibodies. B, SMMC-7721 cells were cultured in 20% O2 or 1% O2, respectively. Hydroxyproline was then added at the same time point for additional 24 hours. Total cell lysates were extracted and subjected to the indicated antibodies. C, SMMC-7721 cells were transiently transfected with myc-tagged-VHL plasmid in the presence or absence of 0.5 mmol/L OH-pro for 24 hours in normoxic or hypoxic conditions, respectively, followed by immunoprecipitation using anti-myc antibody. D, SMMC-7721 cells were transfected with scrambled or anti-Myc siRNA for 48 hours. The cells were subsequently cultured in 20% or 1% O2 condition. Hydroxyproline was then added, followed by culturing for additional 3 hours. The cells were subsequently lysed and subjected to the indicated antibodies. E, Cells stably expressing scrambled or anti-ALDH18A1 shRNA were cultured in 20% or 1% O2 condition, respectively. Hydroxyproline was then added at the same time, followed by culturing for additional 3 hours. Cell viability was determined by Cell Titer-Glo assay. Data were shown as mean ± SEM (n = 3); **, P < 0.01 and *** P < 0.001 between indicated groups.
Figure 6.
Proline metabolism supports HCC cell survival under hypoxia. A, Cells that stably expressed scramble or anti-ALDH18A1 shRNA were cultured under 20% O2 and 1% O2 in the presence or absence of 10 μmol/L sorafenib for 48 hours. Cell viability was determined by Cell Titer-Glo assay. Data were shown as mean ± SEM (n = 3); ** P < 0.01 between indicated groups. B, The cell viability in A was assessed by crystal violet staining. C, Cells were treated as in A for 24 hours. Total cell lysates were extracted and subjected to the indicated antibodies. D, Cells in C were also stained by PI/Annexin V to evaluate apoptosis effect using flow cytometry. The mean ± SEM was shown for triplicate experiments. E, 3 × 10^6 SMMC-7721 cells that stably expressed scramble or anti-ALDH18A1 shRNA were injected into BALB/c nude mice. After the subcutaneous tumors reached a volume of 300 mm^3, mice were randomly subjected to vehicle or sorafenib (10 mg/kg) given every 2 to 3 days. Data of tumor volume were shown as mean ± SEM (n = 5). F, Proposed model for role of glutamine, proline, and hydroxyproline metabolic axis in supporting of HIF-dependent hypoxic response. Hypoxic tumor microenvironment activates glutamine and proline metabolism via ALDH18A1. Attenuated PRODH2 activity by hypoxia results in accumulation of hydroxyproline, which stabilizes HIF1α and promotes HIF-dependent cell survival and sorafenib resistance in HCC.
growth in human cancer (7). Inhibition of proline metabolism by knockdown of ALDH1A1 significantly inhibits melanoma cell growth (8). Moreover, proline starvation inhibits mTOR-dependent tumor clonogenicity (25). To our knowledge, the abnormal regulation and importance of proline metabolism have not yet been studied in HCC.

Intracellular hydroxyproline can only originate from proline, which is hydroxylated by PHs. In this context, proline and hydroxyproline could be released from procollagen by prolidase (26). However, the role of hydroxyproline metabolism in human cancer remains unclear (13). Our metabolic profiling results strongly indicate a hypoxic microenvironment in HCC. Indeed, the hypoxia-induced expression of Rab11-FIP4 promotes the HIF1α-dependent metastasis of HCC (27). In addition, interactions of HMGB1 and mtDNA activate TLR9 signaling to induce tumor growth during hypoxia (28). These findings led us to speculate that hydroxyproline accumulation may promote the HCC pathogenesis by supporting cellular hypoxic responses. Our results regarding the correlation of hydroxyproline with AFP levels and poor prognosis are quite important, providing the clinical evidence of the association of hydroxyproline with HCC progression, although the significance of the analysis may be needed to be further investigated in more HCC cohorts.

In this study, we demonstrated that hydroxyproline supports hypoxia and HIF-dependent phenotypes in HCC, suggesting a mechanistic link between proline metabolism and hypoxia. Indeed, it has recently been shown that mechanism between proline metabolism and hypoxia. Hypoxia and HIF-dependent phenotypes in HCC, suggesting a further investigated in more HCC cohorts.

Indeed, we showed that knockdown of HIF1α by blockade of proline biosynthesis could be restored by treatment with hydroxyproline in HCC cell lines, suggesting that the glutamine, proline, and hydroxyproline metabolic axis significantly contributes to cellular hypoxic response and stabilization of HIF1α under hypoxia. Because it has been reported that MYC posttranscriptionally modulates HIF1α (35), our findings provide an alternative interpretation that MYC might stabilize HIF1α through regulating glutamine, proline, and hydroxyproline metabolism.

Based on previous findings and our results, disruption of either proline anabolism or catabolism could reduce HCC cell viability under hypoxia. Here, we demonstrated that hydroxyproline production from proline is quite critical for tumor survival by stabilizing HIF1α under hypoxia. These findings indicate that targeting proline anabolism, rather than catabolism, may offer a better therapeutic outcome in HCC. This could be supported by the fact that inhibition of PRODH did not induce apoptosis (29), whereas we showed that knockdown of ALDH1A1 significantly induce apoptosis of HCC cells in hypoxic condition. Furthermore, as hypoxia and HIF significantly contribute to sorafenib resistance in HCC (36), we demonstrated that inhibition of ALDH1A1 activity sensitized HCC cells to sorafenib by inducing apoptosis and significantly impaired HCC cell survival under hypoxia.

In summary, our results showed that hypoxia activates glutamine, proline, and hydroxyproline metabolism in HCC. Aberrant accumulation of hydroxyproline by attenuated PRODH2 activity promotes HCC pathogenesis through stabilizing HIF1α (Fig. 6F). More importantly, our findings strongly suggest a hypoxic tumor microenvironment presented in HCC. HCC cells may respond to hypoxia by sensing the intracellular level of hydroxyproline, suggesting that targeting proline biosynthesis might be an attractive therapeutic strategy for HCC tumors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Hypoxia Activates Proline Biosynthesis

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Acknowledgments
Special thanks are given to Dr. Guangzu Ye for the metabolomics validation analysis. The study is supported by the National Key R&D Program of China (2017YFC0906/00) and the Key Foundation of the National Natural Science Foundation of China (21435006; to G. Xu). This study is also supported by Hundred Talents Program of CAS [to Y. Liu] and Innovative Research Grant to Scientific Research Center for Translational Medicine at Dalian Institute of Chemical Physics.

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Clinical Cancer Research

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