A novel germline gain-of-function HIF2A mutation in hepatocellular carcinoma with polycythemia

Jiong Yu[1,*], Xiaowei Shi[1,*], Chunzhang Yang[2], Petra Bullova[3], Christopher S. Hong[2], Cody L. Nesvick[2], Pauline Dmitriev[2], Karel Pacak[3], Zhengping Zhuang[2], Hongcui Cao[1], Lanjuan Li[1]

[1]State Key Laboratory for the Diagnosis and Treatment of Infectious Diseases, National Clinical Research Center for Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou City, China
[2]Surgical Neurology Branch, National Institute of Neurological Disorders and Stroke (NINDS), National Institute of Health (NIH), Bethesda, MD 20892, USA
[3]Department of Molecular Medicine, Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovakia
*Equal contribution

Correspondence to: Hongcui Cao; **email:** hccao@zju.edu.cn
Keywords: hypoxia-inducible factors, mutation, germline, Von Hippel-Lindau, hepatocellular carcinoma
Received: November 16, 2019   **Accepted:** January 27, 2020   **Published:** April 1, 2020

ABSTRACT

Hypoxia-inducible factors (HIFs) regulate oxygen sensing and expression of genes involved in angiogenesis and erythropoiesis. Polycythemia has been observed in patients with hepatocellular carcinoma (HCC), but the underlying molecular basis remains unknown. Liver tissues from 302 HCC patients, including 104 with polycythemia, were sequenced for *HIF2A* mutations. A germline *HIF2A* mutation was detected in one HCC patient with concurrent polycythemia. Three additional family members carried this mutation, but none exhibited polycythemia or were diagnosed with HCC. The gain-of-function mutation resulted in a HIF-2α protein that was transcribed normally but resistant to degradation. HIF-2α target genes *EDN1*, *EPO*, *GNA14*, and *VEGF* were significantly upregulated in the tumor bed but not in the surrounding liver tissue. Polycythemia resolved upon total resection of the tumor tissue. This newly described *HIF2A* mutation may promote HCC oncogenesis.

INTRODUCTION

Appropriate cellular responses to oxygen levels are essential for tissue homeostasis. Hypoxia-inducible factors (HIFs) are evolutionarily-conserved proteins that govern cellular response to low oxygen tension by initiating the transcription of genes associated with angiogenesis, erythropoiesis, and metabolism. While HIFs are essential for metabolic regulation in both adults and embryos, they also play key roles in pathologic processes such as tumorigenesis, metastasis, tumor-associated angiogenesis, and congenital erythrocytosis [1]. Germline and somatic mutations in genes involved in the hypoxia-sensing pathway, including Von Hippel-Lindau (VHL) and Egl-9

homolog 1 (EGLN1; also known as prolyl hydroxylase domain-containing protein 2 [PHD2]), have been described in neuroendocrine tumor syndromes associated with polycythemia [2–6]. Recently, somatic gain-of-function mutations in the oxygen-dependent domain (ODD) of HIF-2α were discovered in the context of multiple paraganglionomas, somatostatinomas, and polycythemia [7–9]. Dysregulation of the oxygen-sensing pathway occurs in many neuroendocrine tumors, but the exact mechanisms by which this dysregulation promotes oncogenesis remain largely unknown.

The liver is a primary site of hematopoiesis in embryos and extramedullary hematopoiesis in adults [10], and

circulating hematopoietic stem cells may be capable of hepatocyte differentiation [11]. Polycythemia has been observed in patients with hepatocellular carcinoma (HCC) and is thought to result from the tumor's secretion of erythropoietin [12, 13]. HCC is a relatively rare disease with an incidence between 3 and 18 per 100,000 in the United States [14, 15], but it may be as frequent as 24 per 100,000 in endemic countries [16]. Major risk factors for HCC include infection with oncogenic hepatitis B virus as well as chronic hepatitis due to hepatitis C virus infection, alcoholism, smoking, non-alcoholic fatty liver disease, and aflatoxin B1 intake [17–24]. In light of recent findings that *HIF2A* mutations can cause polycythemia in certain tumor syndromes, polycythemia in HCC might also involve genetic alternations in hypoxia-induced signaling pathways. In this study, we therefore sought to identify mutations in *HIF2A* in HCC patients presenting with polycythemia. We also examined in depth a previously undescribed germline c.G1645A (p.E549K) mutation in a critical oxygen-sensing region identified in one of the 302 HCC patients screened; this patient also exhibited polycythemia that resolved upon tumor resection.

RESULTS

HIF2A mutational analysis in HCC patients

Three hundred and two HCC patients underwent genomic DNA sequencing to identify mutations in *HIF2A*. Of the 302 patients, 104 exhibited polycythemia, while the remaining 198 patients did not. A unique heterozygous c.G1645A (p.E549K) mutation in the *HIF2A* gene was identified in the tumor DNA of only one patient (index patient) who exhibited concurrent polycythemia.

Clinical history of index patient

The index patient was a 43-year-old male who was referred to the Division of Hepatobiliary and Pancreatic Surgery clinic at the First Affiliated Hospital of Zhejiang University in August 2012 for a suspected hepatic hemangioma identified by abdominal computed tomography (CT). The patient presented with non-radiating abdominal pain on the right side below the xyphoid process that did not worsen with eating or changing position. He also complained of mild general weakness but denied any nausea or vomiting. Physical exam revealed no jaundice. Laboratory results are presented in Table 1. A viral hepatitis panel was positive for hepatitis B surface (HBs) antigen and antibodies to hepatitis B core antigen (HBc) and envelope antigen (HBe); there were no detectable antibodies to hepatitis C virus (HCV). Serum α-fetoprotein (AFP) was elevated at 89.5 ng/mL

(reference range: < 20 ng/mL). A complete blood count (CBC) revealed a hemoglobin level of 188 g/L (reference range: 131-172 g/L), a red blood cell count of 5.8×10^{12}/L (reference range: 4.09-5.74×10^{12}/L), and a hematocrit of 54.2% (reference range: 38.0-50.8%). Abdominal ultrasonography revealed a hypo-echoic mass in the right liver and possible cholecystitis. A subsequent non-contrast abdominal CT revealed a 5.2 cm × 3.4 cm hypodense mass at the same location (Figure 1A). Hepatectomy was performed to remove the tumor.

The patient was stable post-operatively and his recovery was unremarkable. Histological examination of the tumor specimen revealed tumor cells with prominent clear cytoplasm arranged in a trabecular growth pattern (Figure 1B). Tumor cells were positive for AFP and Hep par 1 (HEP) stains but negative for cytokeratin 19 (CK19). The tumor also contained numerous capillary channels positive for CD34 staining. These observations supported a diagnosis of hepatocellular carcinoma, clear cell variant. On post-operative day four, CBC revealed a hemoglobin level of 135 g/L, a red blood cell count of 4.1×10^{12}/L, and a hematocrit of 37.5%.

Up-regulation of hypoxia-related gene expression

Expression of canonical hypoxia-induced genes in the tumor tissue of the index patient was examined using real-time PCR. Peritumoral liver tissue was used as a control. HIF-2α transcriptional targets *EDN1*, *EPO*, *GNA14*, and *VEGFA* were significantly upregulated in the tumor bed but not the surrounding liver tissue (Figure 1C), indicating hyperactivation of the hypoxia signaling pathway within the tumor.

Germline mutational analysis in DNA of index patient and family

To determine whether this was a somatic or germline mutation, additional *HIF2A* analyses were performed on DNA obtained from normal peritumoral tissue and blood lymphocytes of the index patient. Sanger sequencing confirmed the presence of the p.E549K mutation in both normal liver tissue and blood (Figure 2A), confirming a germline etiology.

Subsequently, additional studies were performed to identify this mutation in family members of the index patient. Blood lymphocytes from the proband's family members revealed the p.E549K mutation was present in two sisters and one niece (Figure 2A). However, CBCs failed to demonstrate polycythemia in any family member, and none were diagnosed with HCC. These findings are illustrated in a genetic pedigree (Figure 2B).

Table 1. Laboratory values of the index patient and family members.

| | Index case | | | | | | |
	Pre-operative	4 days post-operative	7 weeks post-operative	Sister (II-C)	Sister (II-E)	Niece (III-C)	Reference range
Serum α-fetoprotein (ng/ml)	89.5	Undetectable	Undetectable	2.9	ND	ND	< 20 ng/mL
Viral hepatitis panel				ND	ND	ND	
HBs Ag	Positive						
HBs Ab	Negative						
HBc Ab	Positive						
HBe Ag	Negative						
HBe Ab	Positive						
HCV Ab	Negative						
Complete blood count							
Erythrocytes $\times 10^{12}$/L	5.8	4.1	5.18	4.38	4.44	4.16	4.09 – 5.74
Hematocrit (%)	54.2	37.5	48.3	39.7	35.3	37.8	38.0 – 50.8
Hemoglobin (g/L)	188	135	165	143	115	133	131 – 172
Leukocytes $\times 10^{9}$/L	7.7	20.7	7.3	5.1	4.4	5.4	4 – 10
Platelets $\times 10^{9}$/L	227	186	178	217	379	314	83 – 303

The reference ranges presented are those used at the Massachusetts General Hospital.

P53 mutational analysis in tumor of index patient

Because none of the index patient's family members had been diagnosed with HCC, further studies were performed to identify any additional mutations that had accumulated in the tumor. Mutations in the p53 tumor suppressor gene are common in HCC [25–27]. As such, Sanger sequencing for *p53* was performed on DNA obtained from the tumor and blood lymphocytes of the index patient. A c.G270T (p.R90S) mutation was identified in the tumor DNA but not in the blood, confirming a somatic etiology (Figure 2C).

Localization of HIF2A mutation

Having established a germline origin for the p.E549K mutation, additional studies were undertaken to elucidate the function of this genetic alteration. Multiple peptide sequence alignment demonstrated that E549 of HIF-2α is located proximal to the protein's primary
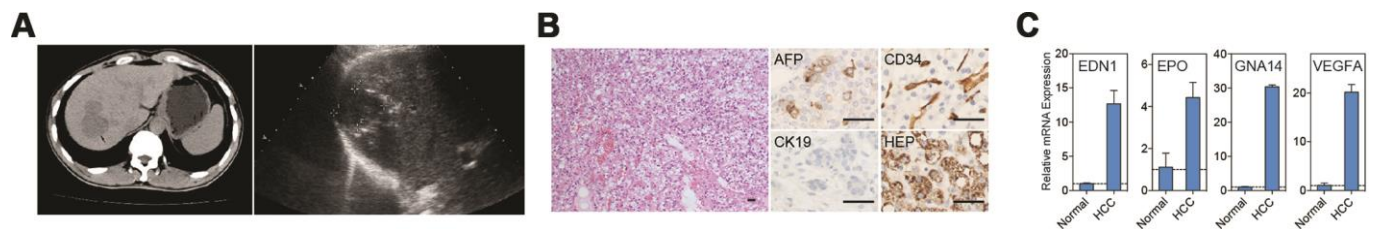

Figure 1. Imaging, histopathology, and mutant HIF2A signaling in the index patient. (**A**) Abdominal CT imaging and ultrasound revealed a hypodense and hypoechoic mass in the patient's right liver. (**B**) Hematoxylin and eosin staining of the resected tumor revealed hepatocellular carcinoma, clear cell variant, with prominent angiogenesis. Tumor cells were positive for AFP and HEP and negative for CK19 by immunohistochemistry; endothelial cells stained positively for CD34. (**C**) Real-time PCR demonstrated overexpression of the HIF2A transcriptional targets *EDN1*, *EPO*, *GNA14*, and *VEGFA* within the tumor bed. Scale bars: 50 µm.

hydroxylation domain and is conserved across species (arrow, Figure 2D). An amino acid substitution at this site could therefore potentially affect the conformation of the HIF-2α hydroxylation center and alter its recognition by VHL.

Stability of the mutant HIF-2α protein

To determine the effect of the c.G1645A mutation on HIF-2α protein stability, we performed an immunoprecipitation assay to analyze HIF-2α ubiquitination and von Hippel-Lindau protein (pVHL) recognition (Figure 3A). Under normoxic conditions, HIF-2α is hydroxylated and subsequently ubiquitinated by pVHL. The mutant E549K exhibited lower affinity for VHL, preventing its ubiquitination and subsequent degradation by the proteasome. A CHX assay confirmed that the stabilization of mutant HIF-2α extended the half-life of the mutant peptide (Figure 3B).

Transcriptional activity of mutant HIF-2α

Transcription of the mutant HIF-2α peptide was determined using real-time PCR (Figure 4A) and a luciferase assay (Figure 4B). Transcription of mutant HIF-2α increased under normoxic conditions and was maintained during hypoxia, demonstrating that the p.E549K mutation results in a stable HIF-2α peptide without affecting its transcription.

DISCUSSION

This report documents a novel germline gain-of-function *HIF2A* mutation in a family in which one individual developed HCC and polycythemia. Interestingly, the germline mutation itself was not associated with erythrocytosis; none of the affected family members who did not develop HCC had a history of polycythemia or associated symptoms. Furthermore, although all cells carried the mutation, only tumor cells from the index patient exhibited upregulation of the transcriptional targets of HIF-2α. Removal of the tumor resolved his polycythemia.

Several *HIF2A* mutations are known to cause polycythemia. All of them result in amino acid substitutions or in-frame deletions in the primary hydroxylation domain, thereby subverting normal oxygen sensing and driving tumor formation [7–9]. The first such mutation discovered was p.G537W, which was originally reported as a germline mutation found across three generations in a single family that causes Familial Erythrocytosis Type 4 per Online Mendelian Inheritance in Man (OMIM) [28]. Since then, numerous somatic gain-of-function mutations have been identified, including p.P534L, p.M535V, p.M535I, p.G537W, p.G537R, and p.D539E, all of which are located in the ODD of *HIF2A* [29]. Like these mutations, the p.E549K mutation lies in the
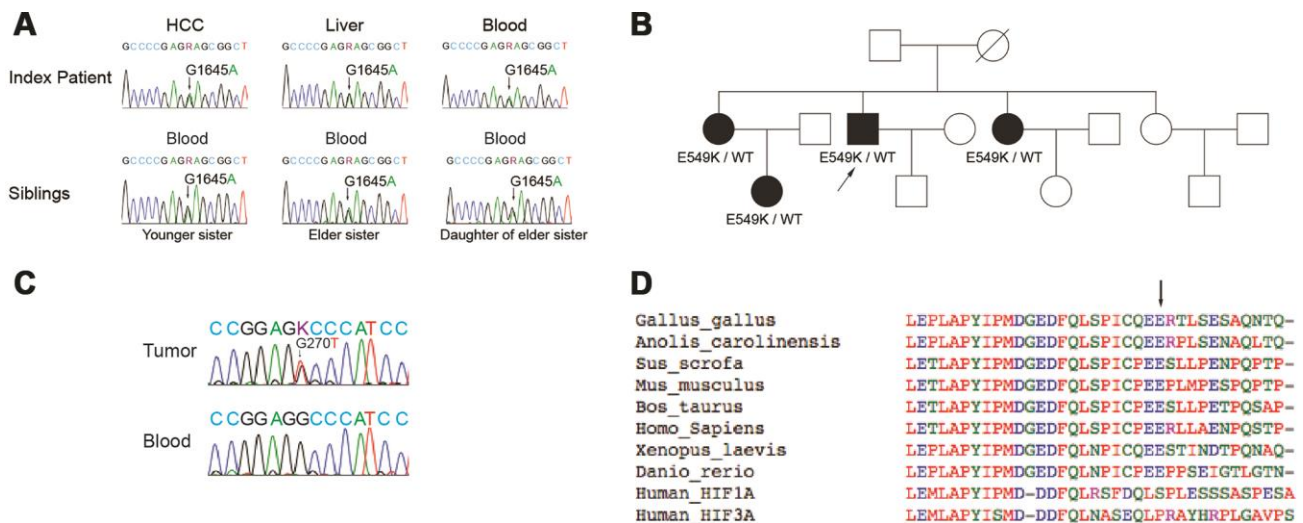

Figure 2. Sanger sequencing, genetic pedigree, and localization of germline mutation in the index patient and family members. (A) Direct Sanger sequencing results are shown for the *HIF2A* gene in the index patient and affected family members. The patient's blood lymphocytes as well as the tumor and surrounding liver tissue carried a G1645A mutation in the gene coding for HIF-2α. This mutation was also present in the blood lymphocytes of two of the patient's sisters and one niece. (B) A family pedigree is shown. (C) Sanger sequencing of the *p53* gene in the tumor tissue and blood lymphocytes of the index patient is shown. The *p53* mutation was present only in the index patient's tumor tissue. (D) Multiple peptide sequence alignment was performed; the E549 site was critically conserved across species (arrow). As such, a mutation at this site could potentially alter HIF-2α signaling.

evolutionarily-conserved primary hydroxylation site of HIF-2α. This precludes oxygen-dependent HIF-2α hydroxylation by PHD enzymes, ubiquitination by the pVHL-E3 ligase complex, and subsequent proteasomal degradation, resulting in constitutive hypoxia signaling in the presence of normal oxygen tension.

More recently, *HIF2A* mutations have been implicated in unique clinical syndromes in patients with neuroendocrine tumors associated with polycythemia. These include the somatic mutations p.A530V [30] and p.A530E [31], somatic mosaic mutations p.L529P and p.L542P [32], and the germline mutation p.F374Y [33]. While HIF2A overexpression promotes oncogenesis in renal cell carcinoma [34], the p.E549K mutation described in the index patient here is the first *HIF2A* mutation to be identified in HCC. Previous studies have shown that prolonged HIF activation may accelerate the development of fibrosis, promote viral replication in chronic liver disease, and promote tumor cell growth and metastasis [35–38]. Hepatocellular carcinoma is typical of metabolically heterogeneous cancers in that tumor
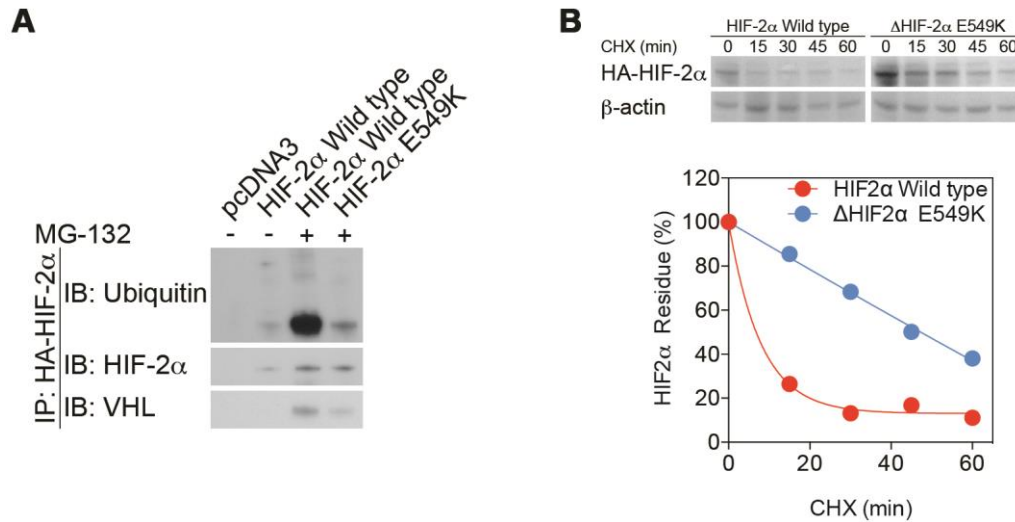

Figure 3. Stability is increased, while enzymatic function is maintained, in mutant HIF-2α protein. (**A**) An immunoprecipitation assay demonstrated lower affinity of mutant HIF-2α protein for binding to VHL and subsequent decreases in ubiquitination and proteasome degradation. (**B**) A cycloheximide assay confirmed that stabilization of mutant HIF-2α extended the half-life of the mutant protein.
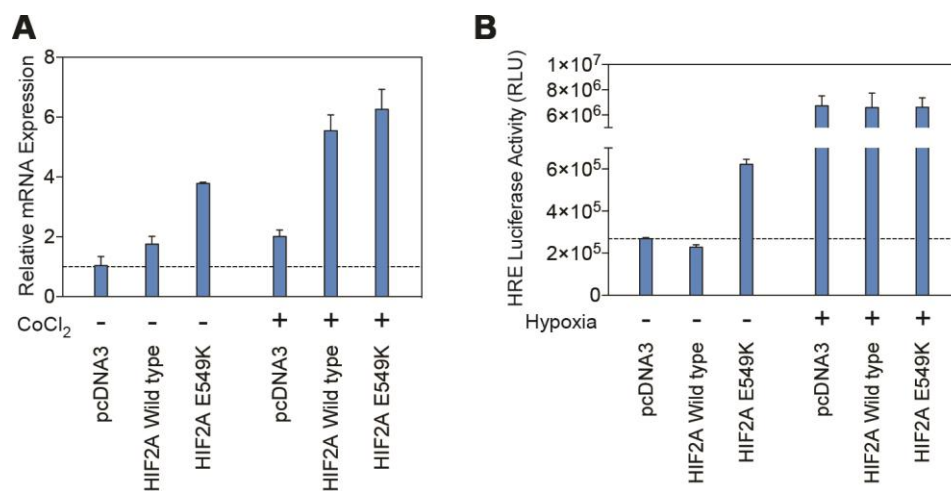

Figure 4. Transcription of the mutant HIF-2α protein is maintained under normoxic and hypoxic conditions. (**A**) Results from real-time PCR demonstrated similar transcription levels for the wildtype and mutant *HIF2A* genes under conditions of normoxia and simulated hypoxia. (**B**) A luciferase assay using Hep3B cells transfected with wildtype and p.E549K *HIF2A* confirmed these findings.

cells often generate energy through aerobic glycolysis rather than relying on oxidative phosphorylation in the mitochondria, even when oxygen supplies are adequate [23, 24]. This phenomenon, known as the Warburg effect, promotes tumor cell growth not only by providing an energy source for rapid cell proliferation, but also by minimizing the production of reactive oxygen species [39, 40]. The p.E549K mutation observed here stabilized the mutant peptide, which may transcriptionally up-regulate many genes encoding glycolytic enzymes [39]. Additionally, the HIF2α transcriptional targets EDN1, EPO, GNA14, and VEGFA were significantly upregulated in the tumor and might also promote tumor progression. Interestingly, despite its germline inheritance, the p.E549K mutation upregulated HIF-2α targets in the tumor but not the surrounding tissue, indicating that it selectively activated hypoxia signaling within the tumor. Moreover, the lack of obvious necrosis in the tumor suggests aberrant hypoxia signaling. Why HIF2A expression was not upregulated in the patient's normal cells and why the other affected family members did not exhibit polycythemia remain unclear. These findings suggest that this mutation may show incomplete penetrance in causing the polycythemia phenotype. Well-known examples of gain-of-function mutations with incomplete penetrance and tumor predisposition include RET mutations in multiple endocrine neoplasia (MEN) syndromes and GNAS1 mutations in McCune-Albright syndrome. Like these syndromes, tumor development arising from a germline p.E549K mutation likely requires additional mutagenic changes, such as the p53 mutation reported in the index patient.

The mechanisms underlying potential carcinogenic interactions between the p53 tumor suppressor pathway and HIF expression are not fully understood. HIF expression is associated with increased genomic instability [41] and can promote malignant transformation through antagonism of p53-mediated tumor suppression [42, 43]. In clear-cell renal cell carcinoma, for instance, HIFs act upon the p53 pathway to mediate the DNA damage response [44], and overexpression of HIF2A suppresses Hdm2-mediated p53 activity, conferring chemotherapeutic resistance [43]. Likewise, inhibition of HIF-2α activity abrogates p53 activity, significantly sensitizing clear-cell renal carcinoma cells to radiation-induced apoptosis [45]. Therefore, further studies are needed to elucidate any potential synergy or causality between p53 mutations and gain-of-function mutations in hypoxia induced signaling pathways. In the case of the index patient, it is unclear whether selective activation of HIF2A signaling was attributable to or further propagated by the p53 mutation.

Given that the index patient's polycythemia resolved upon total tumor resection, it is probable that the erythrocytosis in this case was due to upregulation of HIF2A targets by the novel mutant protein. However, this mutation was found in only one of 104 total HCC patients with concurrent polycythemia, suggesting that genetic alterations in HIF2A are involved in only a subset of HCC patients with erythrocytosis. There are numerous other possible etiologies for polycythemia, including mutations in PHD enzymes, VHL proteins, alterations in angiotensin II signaling, upregulation of insulin-like growth factor-1 signaling, and constitutive activation of the JAK/STAT pathway [46–50]. Additional investigations are needed to determine the molecular mechanisms and therapeutic significance of polycythemia in HCC patients.

MATERIALS AND METHODS

Patients

302 patients with HCC including 104 with polycythemia were referred to the Division of Hepatobiliary and Pancreatic Surgery, Department of Surgery at the First Affiliated Hospital of Zhejiang University and fulfilled the diagnostic criteria for hepatocellular carcinoma described by the American Association for the Study of Liver Diseases (AASLD) [51]. Patients with hematological system disorders were excluded from the study. Polycythemia was defined as an elevated hematocrit or red blood cell count that did not meet the 2007 World Health Organization (WHO) criteria for diagnosis of polycythemia vera [52].

Study oversight

This study was approved by the institutional review board of the First Affiliated Hospital, College of Medicine, Zhejiang University (Reference number: 2014-272). Pedigree investigation participants provided written informed consent for participation in this study. Written informed consent was not obtained from other screened patients because this study used only preexisting data and pathological tissues; in these cases, we informed the participants of their right to refuse enrolment via telephone interview. These informed consent and enrolment procedures are in accordance with the detailed regulations described in the guidelines.

Laboratory studies

Immunohistochemistry
Formalin-fixed, paraffin-embedded tissue sections were probed with primary antibodies against AFP (mouse monoclonal; Zhongshan Golden Bridge Biotechnology Company Limited, Beijing, China), CD34 (mouse

monoclonal; Long Island Biotechnology Company Limited, Shanghai, China), CK19 (mouse monoclonal; Zhongshan), or HEP (mouse monoclonal; Zhongshan). Slides were then stained with 3,3'-diaminobenzidine (DAB) and visualized using a Leica DM LB light (Solms, Germany) microscope at magnifications of 100× or 400× as indicated.

Mutation analysis

Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Venlo, Netherlands). *HIF2A* exons were amplified by polymerase chain reaction (PCR) using previously described primer sets [7]. Likewise, *P53* exons were amplified by PCR using previously described primer sets [53]. Amplicon sequences were then determined by Sanger sequencing.

Plasmids and mutagenesis

A pcDNA3 plasmid (Addgene, Cambridge, MA; plasmid 18950 [7]) containing the human *HIF2A* coding sequence was used for wild-type HIF2A expression analysis. E549K-mutant *HIF2A* was cloned and inserted into an expression plasmid using a Quikchange Lightning Site-directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). Sanger sequencing was used to confirm successful insertion.

Real-time PCR

Total RNA was extracted from microdissected tumor samples using an RNeasy Plus Mini Kit (Qiagen). Messenger RNA (mRNA) expression of HIF target genes was examined by real-time polymerase chain reaction (PCR) using an Eco Real-Time PCR System (Illumina, San Diego, CA). Gene-specific primer sets included endothelin-1 (EDN1; Qiagen QT00088235), erythropoietin (EPO; Origene, Rockville, MD; HP200740), guanine nucleotide binding protein alpha 14 (GNA14; Qiagen QT00099379), vascular endothelial growth factor A (VEGFA; Qiagen QT01682072), and beta-actin (ACTB; Qiagen QT00095431).

Immunoprecipitation

HIF2A-transfected HEK293 cells were treated with MG-132 (10 μM) for 6 hr. Total protein was extracted using immunoprecipitation (IP) lysis buffer supplemented with a protease inhibitor cocktail (Thermo, Waltham, MA). According to the manufacturer's instructions, 500 μg total protein extract was incubated with 3 μg monoclonal anti-hemagglutinin (HA) antibody (Covance, Princeton, NJ) and a Dynabeads Protein G Immunoprecipitation Kit (Life Technologies, Carlsbad, CA) overnight at 4°C.

The eluent was then subjected to Western blot and probed with antibodies for ubiquitin (Abcam, Cambridge, UK), HIF-2α (Novus Biologicals, Littleton, CO), or VHL protein (Cell Signaling Technology, Danvers, MA).

Cycloheximide assay

HIF2A-transfected HEK293 cells were treated with cycloheximide (CHX) (50 μM, Sigma-Aldrich, St. Louis, MO) for 0, 15, 30, 45, or 60 min as indicated. Total protein was extracted using RIPA lysis buffer supplemented with a protease inhibitor cocktail (Thermo). HIF-2α protein levels were then determined by Western blot using an anti-HA antibody (Covance).

Luciferase assay

Hep3B cells stably expressing HRE-luciferase (Promega, Madison, WI) were transfected with *HIF2A* recombinants. Cells were incubated in 21% or 1% O_2 for 12 hr and luciferase activity was determined using the ONE-Glo Luciferase assay system (Promega).

Abbreviations

HIF: hypoxia-inducible factor; VHL: Von Hippel-Lindau; EGLN1: Egl-9 homolog 1; PHD2: prolyl hydroxylase domain-containing protein 2; ODD: oxygen-dependent domain; HCC: hepatocellular carcinoma; AASLD: American Association for the Study of Liver Diseases; WHO: World Health Organization; DAB: 3:3'-diaminobenzidine; mRNA: messenger RNA; PCR: polymerase chain reaction; EDN1: endothelin-1; EPO: erythropoietin; GNA14: guanine nucleotide binding protein alpha 14; VEGFA: vascular endothelial growth factor A; ACTB: beta-actin; IP: immunoprecipitation; hemagglutinin: HA; CHX: cycloheximide; CT: computed tomography; HBs: hepatitis B surface antigen; HBc: hepatitis B core antigen; HBe: hepatitis B envelope antigen; HCV: hepatitis C virus; AFP: α-fetoprotein; CBC: complete blood count; HEP: Hep par 1; CK19: cytokeratin 19; pVHL: Von Hippel-Lindau protein; OMIM: Online Mendelian Inheritance in Man; MEN: Multiple Endocrine Neoplasia.

AUTHOR CONTRIBUTIONS

H.C. and Z.Z., the coprincipal investigators, designed and supervised the study. Z.Z. and H.C. obtained the funding. J.Y. and X.S. assisted with recruitment, clinical management, and data collection and completed immunohistochemistry and mutation analysis. C.Y., P.D., C.L.N., and C.S.H. detected gene function and performed statistical analyses. K.P. and P.B. provided technical support. H.C., Z.Z., L.L., and C.Y. drafted the article, and all authors reviewed and approved the final version.

ACKNOWLEDGMENTS

REFERENCES

1. Jochmanová I, Yang C, Zhuang Z, Pacak K. Hypoxia-inducible factor signaling in pheochromocytoma: turning the rudder in the right direction. J Natl Cancer Inst. 2013; 105:1270–83.
https://doi.org/10.1093/jnci/djt201
PMID:23940289

2. Da Silva JL, Lacombe C, Bruneval P, Casadevall N, Leporrier M, Camilleri JP, Bariety J, Tambourin P, Varet B. Tumor cells are the site of erythropoietin synthesis in human renal cancers associated with polycythemia. Blood. 1990; 75:577–82.
https://doi.org/10.1182/blood.V75.3.577.577
PMID:2297568

3. Krieg M, Marti HH, Plate KH. Coexpression of erythropoietin and vascular endothelial growth factor in nervous system tumors associated with von Hippel-Lindau tumor suppressor gene loss of function. Blood. 1998; 92:3388–93.
https://doi.org/10.1182/blood.V92.9.3388
PMID:9787178

4. Percy MJ, Zhao Q, Flores A, Harrison C, Lappin TR, Maxwell PH, McMullin MF, Lee FS. A family with erythrocytosis establishes a role for prolyl hydroxylase domain protein 2 in oxygen homeostasis. Proc Natl Acad Sci USA. 2006; 103:654–59.
https://doi.org/10.1073/pnas.0508423103
PMID:16407130

5. Taïeb D, Barlier A, Yang C, Pertuit M, Tchoghandjian A, Rochette C, Zattara-Canoni H, Figarella-Branger D, Zhuang Z, Pacak K, Metellus P. Somatic gain-of-function HIF2A mutations in sporadic central nervous system hemangioblastomas. J Neurooncol. 2016; 126:473–81.
https://doi.org/10.1007/s11060-015-1983-y
PMID:26514359

6. Yang Y, Zhang YJ, Zhu Y, Cao JZ, Yuan ZY, Xu LM, Wu JX, Wang W, Wu T, Lu B, Zhu SY, Qian LT, Zhang FQ, et al. Prognostic nomogram for overall survival in previously untreated patients with extranodal NK/T-cell lymphoma, nasal-type: a multicenter study. Leukemia. 2015; 29:1571–77.
https://doi.org/10.1038/leu.2015.44 PMID:25697894

7. Zhuang Z, Yang C, Lorenzo F, Merino M, Fojo T, Kebebew E, Popovic V, Stratakis CA, Prchal JT, Pacak K. Somatic HIF2A gain-of-function mutations in paraganglioma with polycythemia. N Engl J Med. 2012; 367:922–30.
https://doi.org/10.1056/NEJMoa1205119
PMID:22931260

8. Yang C, Sun MG, Matro J, Huynh TT, Rahimpour S, Prchal JT, Lechan R, Lonser R, Pacak K, Zhuang Z. Novel HIF2A mutations disrupt oxygen sensing, leading to polycythemia, paragangliomas, and somatostatinomas. Blood. 2013; 121:2563–66.
https://doi.org/10.1182/blood-2012-10-460972
PMID:23361906

9. Pacak K, Jochmanova I, Prodanov T, Yang C, Merino MJ, Fojo T, Prchal JT, Tischler AS, Lechan RM, Zhuang Z. New syndrome of paraganglioma and somatostatinoma associated with polycythemia. J Clin Oncol. 2013; 31:1690–98.
https://doi.org/10.1200/JCO.2012.47.1912
PMID:23509317

10. Swain A, Inoue T, Tan KS, Nakanishi Y, Sugiyama D. Intrinsic and extrinsic regulation of mammalian hematopoiesis in the fetal liver. Histol Histopathol. 2014; 29:1077–82.
https://doi.org/10.14670/HH-29.1077
PMID:24526457

11. Theise ND, Nimmakayalu M, Gardner R, Illei PB, Morgan G, Teperman L, Henegariu O, Krause DS. Liver from bone marrow in humans. Hepatology. 2000; 32:11–16.

https://doi.org/10.1053/jhep.2000.9124
PMID:10869283

12. Kew MC, Fisher JW. Serum erythropoietin concentrations in patients with hepatocellular carcinoma. Cancer. 1986; 58:2485–88.
https://doi.org/10.1002/1097-0142(19861201)58:11<2485::AID-CNCR2820581122>3.0.CO;2-N
PMID:2429757

13. Sakisaka S, Watanabe M, Tateishi H, Harada M, Shakado S, Mimura Y, Gondo K, Yoshitake M, Noguchi K, Hino T, Nohno R, Majima Y, Hirai K, et al. Erythropoietin production in hepatocellular carcinoma cells associated with polycythemia: immunohistochemical evidence. Hepatology. 1993; 18:1357–62.
https://doi.org/10.1002/hep.1840180612
PMID:7694895

14. El-Serag HB, Davila JA, Petersen NJ, McGlynn KA. The continuing increase in the incidence of hepatocellular carcinoma in the United States: an update. Ann Intern Med. 2003; 139:817–23.
https://doi.org/10.7326/0003-4819-139-10-200311180-00009
PMID:14623619

15. Davila JA, Morgan RO, Shaib Y, McGlynn KA, El-Serag HB. Hepatitis C infection and the increasing incidence of hepatocellular carcinoma: a population-based study. Gastroenterology. 2004; 127:1372–80.
https://doi.org/10.1053/j.gastro.2004.07.020
PMID:15521006

16. Skolnick AA. Armed with epidemiologic research, China launches programs to prevent liver cancer. JAMA. 1996; 276:1458–59.
https://doi.org/10.1001/jama.1996.03540180012005
PMID:8903245

17. Tsukuma H, Hiyama T, Tanaka S, Nakao M, Yabuuchi T, Kitamura T, Nakanishi K, Fujimoto I, Inoue A, Yamazaki H, Kawashima T. Risk factors for hepatocellular carcinoma among patients with chronic liver disease. N Engl J Med. 1993; 328:1797–801.
https://doi.org/10.1056/NEJM199306243282501
PMID:7684822

18. Trichopoulos D, Bamia C, Lagiou P, Fedirko V, Trepo E, Jenab M, Pischon T, Nöthlings U, Overved K, Tjønneland A, Outzen M, Clavel-Chapelon F, Kaaks R, et al. Hepatocellular carcinoma risk factors and disease burden in a European cohort: a nested case-control study. J Natl Cancer Inst. 2011; 103:1686–95.
https://doi.org/10.1093/jnci/djr395
PMID:22021666

19. El-Serag HB, Tran T, Everhart JE. Diabetes increases the risk of chronic liver disease and hepatocellular carcinoma. Gastroenterology. 2004; 126:460–68.
https://doi.org/10.1053/j.gastro.2003.10.065
PMID:14762783

20. Ascha MS, Hanouneh IA, Lopez R, Tamimi TA, Feldstein AF, Zein NN. The incidence and risk factors of hepatocellular carcinoma in patients with nonalcoholic steatohepatitis. Hepatology. 2010; 51:1972–78.
https://doi.org/10.1002/hep.23527
PMID:20209604

21. Yu MC, Tong MJ, Govindarajan S, Henderson BE. Nonviral risk factors for hepatocellular carcinoma in a low-risk population, the non-Asians of Los Angeles County, California. J Natl Cancer Inst. 1991; 83:1820–26.
https://doi.org/10.1093/jnci/83.24.1820
PMID:1660542

22. Chen CJ, Wang LY, Lu SN, Wu MH, You SL, Zhang YJ, Wang LW, Santella RM. Elevated aflatoxin exposure and increased risk of hepatocellular carcinoma. Hepatology. 1996; 24:38–42.
https://doi.org/10.1002/hep.510240108
PMID:8707279

23. Cassim S, Raymond VA, Dehbidi-Assadzadeh L, Lapierre P, Bilodeau M. Metabolic reprogramming enables hepatocarcinoma cells to efficiently adapt and survive to a nutrient-restricted microenvironment. Cell Cycle. 2018; 17:903–16.
https://doi.org/10.1080/15384101.2018.1460023
PMID:29633904

24. Cassim S, Raymond VA, Lacoste B, Lapierre P, Bilodeau M. Metabolite profiling identifies a signature of tumorigenicity in hepatocellular carcinoma. Oncotarget. 2018; 9:26868–83.
https://doi.org/10.18632/oncotarget.25525
PMID:29928490

25. Lunn RM, Zhang YJ, Wang LY, Chen CJ, Lee PH, Lee CS, Tsai WY, Santella RM. p53 mutations, chronic hepatitis B virus infection, and aflatoxin exposure in hepatocellular carcinoma in Taiwan. Cancer Res. 1997; 57:3471–77.
PMID:9270015

26. Cleary SP, Jeck WR, Zhao X, Chen K, Selitsky SR, Savich GL, Tan TX, Wu MC, Getz G, Lawrence MS, Parker JS, Li J, Powers S, et al. Identification of driver genes in hepatocellular carcinoma by exome sequencing. Hepatology. 2013; 58:1693–702.
https://doi.org/10.1002/hep.26540
PMID:23728943

27. Guichard C, Amaddeo G, Imbeaud S, Ladeiro Y, Pelletier L, Maad IB, Calderaro J, Bioulac-Sage P, Letexier M, Degos F, Clément B, Balabaud C, Chevet E, et al. Integrated analysis of somatic mutations and focal copy-number changes identifies key genes and

pathways in hepatocellular carcinoma. Nat Genet. 2012; 44:694–98.
https://doi.org/10.1038/ng.2256
PMID:22561517

28. Percy MJ, Furlow PW, Lucas GS, Li X, Lappin TR, McMullin MF, Lee FS. A gain-of-function mutation in the HIF2A gene in familial erythrocytosis. N Engl J Med. 2008; 358:162–68.
https://doi.org/10.1056/NEJMoa073123
PMID:18184961

29. Lee FS, Percy MJ. The HIF pathway and erythrocytosis. Annu Rev Pathol. 2011; 6:165–92.
https://doi.org/10.1146/annurev-pathol-011110-130321
PMID:20939709

30. Taïeb D, Yang C, Delenne B, Zhuang Z, Barlier A, Sebag F, Pacak K. First report of bilateral pheochromocytoma in the clinical spectrum of HIF2A-related polycythemia-paraganglioma syndrome. J Clin Endocrinol Metab. 2013; 98:E908–13.
https://doi.org/10.1210/jc.2013-1217
PMID:23539726

31. Toyoda H, Hirayama J, Sugimoto Y, Uchida K, Ohishi K, Hirayama M, Komada Y. Polycythemia and paraganglioma with a novel somatic HIF2A mutation in a male. Pediatrics. 2014; 133:e1787–91.
https://doi.org/10.1542/peds.2013-2419
PMID:24819565

32. Buffet A, Smati S, Mansuy L, Ménara M, Lebras M, Heymann MF, Simian C, Favier J, Murat A, Cariou B, Gimenez-Roqueplo AP. Mosaicism in HIF2A-related polycythemia-paraganglioma syndrome. J Clin Endocrinol Metab. 2014; 99:E369–73.
https://doi.org/10.1210/jc.2013-2600
PMID:24276449

33. Lorenzo FR, Yang C, Ng Tang Fui M, Vankayalapati H, Zhuang Z, Huynh T, Grossmann M, Pacak K, Prchal JT. A novel EPAS1/HIF2A germline mutation in a congenital polycythemia with paraganglioma. J Mol Med (Berl). 2013; 91:507–12.
https://doi.org/10.1007/s00109-012-0967-z
PMID:23090011

34. Gordan JD, Simon MC. Hypoxia-inducible factors: central regulators of the tumor phenotype. Curr Opin Genet Dev. 2007; 17:71–77.
https://doi.org/10.1016/j.gde.2006.12.006
PMID:17208433

35. Ju C, Colgan SP, Eltzschig HK. Hypoxia-inducible factors as molecular targets for liver diseases. J Mol Med (Berl). 2016; 94:613–27.
https://doi.org/10.1007/s00109-016-1408-1
PMID:27094811

36. Rankin EB, Giaccia AJ. Hypoxic control of metastasis. Science. 2016; 352:175–80.
https://doi.org/10.1126/science.aaf4405
PMID:27124451

37. Chen C, Lou T. Hypoxia inducible factors in hepatocellular carcinoma. Oncotarget. 2017; 8:46691–703.
https://doi.org/10.18632/oncotarget.17358
PMID:28493839

38. Chen J, Chen J, Huang J, Li Z, Gong Y, Zou B, Liu X, Ding L, Li P, Zhu Z, Zhang B, Guo H, Cai C, Li J. HIF-2α upregulation mediated by hypoxia promotes NAFLD-HCC progression by activating lipid synthesis via the PI3K-AKT-mTOR pathway. Aging (Albany NY). 2019; 11:10839–60.
https://doi.org/10.18632/aging.102488
PMID:31796646

39. Chen Z, Zuo X, Zhang Y, Han G, Zhang L, Wu J, Wang X. MiR-3662 suppresses hepatocellular carcinoma growth through inhibition of HIF-1α-mediated Warburg effect. Cell Death Dis. 2018; 9:549.
https://doi.org/10.1038/s41419-018-0616-8
PMID:29748591

40. De Matteis S, Scarpi E, Granato AM, Vespasiani-Gentilucci U, La Barba G, Foschi FG, Bandini E, Ghetti M, Marisi G, Cravero P, Gramantieri L, Cucchetti A, Ercolani G, et al. Role of SIRT-3, p-mTOR and HIF-1α in Hepatocellular Carcinoma Patients Affected by Metabolic Dysfunctions and in Chronic Treatment with Metformin. Int J Mol Sci. 2019; 20:E1503.
https://doi.org/10.3390/ijms20061503
PMID:30917505

41. Koshiji M, To KK, Hammer S, Kumamoto K, Harris AL, Modrich P, Huang LE. HIF-1alpha induces genetic instability by transcriptionally downregulating MutSalpha expression. Mol Cell. 2005; 17:793–803.
https://doi.org/10.1016/j.molcel.2005.02.015
PMID:15780936

42. Obacz J, Pastorekova S, Vojtesek B, Hrstka R. Cross-talk between HIF and p53 as mediators of molecular responses to physiological and genotoxic stresses. Mol Cancer. 2013; 12:93.
https://doi.org/10.1186/1476-4598-12-93
PMID:23945296

43. Sermeus A, Michiels C. Reciprocal influence of the p53 and the hypoxic pathways. Cell Death Dis. 2011; 2:e164.
https://doi.org/10.1038/cddis.2011.48
PMID:21614094

44. Selvarajah J, Nathawat K, Moumen A, Ashcroft M, Carroll VA. Chemotherapy-mediated p53-dependent DNA damage response in clear cell renal cell

carcinoma: role of the mTORC1/2 and hypoxia-inducible factor pathways. Cell Death Dis. 2013; 4:e865.
https://doi.org/10.1038/cddis.2013.395
PMID:24136229

45. Bertout JA, Majmundar AJ, Gordan JD, Lam JC, Ditsworth D, Keith B, Brown EJ, Nathanson KL, Simon MC. HIF2alpha inhibition promotes p53 pathway activity, tumor cell death, and radiation responses. Proc Natl Acad Sci USA. 2009; 106:14391–96.
https://doi.org/10.1073/pnas.0907357106
PMID:19706526

46. Mrug M, Stopka T, Julian BA, Prchal JF, Prchal JT. Angiotensin II stimulates proliferation of normal early erythroid progenitors. J Clin Invest. 1997; 100:2310–14.
https://doi.org/10.1172/JCI119769
PMID:9410909

47. Shih LY, Huang JY, Lee CT. Insulin-like growth factor I plays a role in regulating erythropoiesis in patients with end-stage renal disease and erythrocytosis. J Am Soc Nephrol. 1999; 10:315–22.
PMID:10215331

48. Witthuhn BA, Quelle FW, Silvennoinen O, Yi T, Tang B, Miura O, Ihle JN. JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. Cell. 1993; 74:227–36.
https://doi.org/10.1016/0092-8674(93)90414-L
PMID:8343951

49. Ang SO, Chen H, Hirota K, Gordeuk VR, Jelinek J, Guan Y, Liu E, Sergueeva AI, Miasnikova GY, Mole D, Maxwell PH, Stockton DW, Semenza GL, Prchal JT. Disruption of oxygen homeostasis underlies congenital Chuvash polycythemia. Nat Genet. 2002; 32:614–21.
https://doi.org/10.1038/ng1019
PMID:12415268

50. Semenza GL. Oxygen sensing, homeostasis, and disease. N Engl J Med. 2011; 365:537–47.
https://doi.org/10.1056/NEJMra1011165
PMID:21830968

51. Bruix J, Sherman M, and American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update. Hepatology. 2011; 53:1020–22.
https://doi.org/10.1002/hep.24199 PMID:21374666

52. Tefferi A, Thiele J, Vardiman JW. The 2008 World Health Organization classification system for myeloproliferative neoplasms: order out of chaos. Cancer. 2009; 115:3842–47.
https://doi.org/10.1002/cncr.24440
PMID:19472396

53. Hsueh C, Wang H, Gonzalez-Crussi F, Lin JN, Hung IJ, Yang CP, Jiang TH. Infrequent p53 gene mutations and lack of p53 protein expression in clear cell sarcoma of the kidney: immunohistochemical study and mutation analysis of p53 in renal tumors of unfavorable prognosis. Mod Pathol. 2002; 15:606–10.
https://doi.org/10.1038/modpathol.3880573
PMID:12065773