Biomedicine & Pharmacotherapy 69 (2015) 277–284

Available online at
ScienceDirect
www.sciencedirect.com

Elsevier Masson France
EM|consulte
www.em-consulte.com/en

Original article

Pyruvate kinase M2 affects liver cancer cell behavior through up-regulation of HIF-1α and Bcl-xL in culture

Tianfu Dong [a,1], Youde Yan [b,1], Hao Chai [c], Shenglin Chen [c], Xinkui Xiong [c], Daoyi Sun [c], Yue Yu [d], Lei Deng [d], Feng Cheng [c,*]

[a] General Surgery Department, Lianyungang First People's Hospital, Lianyungang, Jiangsu Province, China
[b] Department of Infectious Diseases, First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu Province, China
[c] Liver Transplantation Center, First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu Province, China
[d] Key Laboratory of Living Donor Liver Transplantation, Ministry of Public Health, Nanjing, Jiangsu Province, China

ARTICLE INFO

Article history:
Received 11 August 2014
Accepted 9 December 2014

Keywords:
Hypoxia inducible factor-1α
Pyruvate kinase M2
Warburg effect

ABSTRACT

Cancer cells consume large amounts of glucose to produce lactate, even in the presence of ample oxygen. This phenomenon is known as the Warburg effect. The pyruvate kinase promotes aerobic glycolysis, and the pyruvate kinase M2 isoform (PKM2) is highly expressed in many cancer cells. Although the Warburg effect is a hallmark of cancer, the mechanism by which PKM2 contributes to the Warburg effect, and its role in tumor growth remain to be defined. We proposed that PKM2 activates transcription of hypoxia inducible factor-1α (HIF-1α) by phosphorylating STAT3 (signal transducer and activator of transcription 3) at Y705 (tyrosine 705) as a plausible mechanism for liver cancer cell proliferation. In the current study, we observed that PKM2 was over-expressed in hepatocellular carcinoma (HCC) tissues compared to adjacent normal tissues. The experiments further indicate that nuclear PKM2 is an active protein kinase in cultured cells. Knockdown of PKM2 affected the levels of HIF-1α and Bcl-xL (B-cell lymphoma-extra large), suggesting that PKM2 plays an important role in promoting cell proliferation. In conclusion, the current findings demonstrate that PKM2 is an active protein kinase, and promotes liver cancer cell proliferation by up-regulating HIF-1α and Bcl-xL expression.

1. Introduction

Most cancer cells consume higher amounts of glucose, and produce much more lactate than normal cells, even in the presence of ample O_2. This phenomenon, known as aerobic glycolysis or the Warburg effect, supports tumor cell growth [1]. Aerobic glycolysis is a key metabolic feature that distinguishes cancer cells from normal cells. Pyruvate kinase (PK) catalyzes the last, and a rate-limiting step in aerobic glycolysis. The human genome encodes two distinct PK genes, PKLR and PKM (formerly PKM2). The latter has four PK isoforms: L, R, M1, and M2 [2]. Tissue-specific promoters drive expression of the PKL or PKR isoforms from the PKLR gene. PKR expression is exclusive to red blood cells, whereas PKL is expressed primarily in the liver, with low expression in the kidney [2,3]. PKM1 and PKM2 are encoded by the same but differentially spliced gene [4]. Over the past several decades, although some studies have investigated the role of PKM2 in HCC, its function has not been fully elucidated.

STAT3 mediates diverse cellular processes initiated by extracellular signals, and plays a central role in the progression of HCC [5]. STAT3 is constitutively activated in many human tumors such as prostate, liver, brain, breast and lung cancer [5–9]. The downstream genes including cyclin D1, c-myc, HIF-1α and Bcl-xL are potentially up-regulated by phosphorylated STAT3. Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcription factor that is a key regulator of transcriptional responses to reduced O_2 availability (hypoxia). HIF-1 consists of an O_2-regulated HIF-1α subunit and a constitutively expressed HIF-1β subunit [10,11]. In addition to the key role of HIF-1α in the Warburg effect (the shift from oxidative to glycolytic metabolism), HIF-1α has been shown to interact with and stimulate the transcription of downstream genes. Over-expression of HIF-1α plays an important role in critical aspects of cancer biology, including cell immortalization, vascularization [12], glucose metabolism [13], immune evasion, invasion, metastasis [14] and radiation resistance [15]. B cell CLL/lymphoma-2 (BCL-2) and its

* Corresponding author at: Liver Transplantation Center, First Affiliated Hospital of Nanjing Medical University, 300 Guangzhou Road, Nanjing 210029, China. Tel.: +86 025 83718836 6476; fax: +86 025 83762106.
E-mail address: docchengfeng@njmu.edu.cn (F. Cheng).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.biopha.2014.12.010
0753-3322/

relatives comprise the BCL-2 family of proteins which are originally characterized with respect to their roles in controlling outer mitochondrial membrane integrity and apoptosis. Previous studies have revealed that Bcl-xL is over-expressed in tumor tissues compared to normal tissues [16]. In addition, STAT3 acts as a transcription factor, and promotes cancer cell proliferation by up-regulation of Bcl-2, Bcl-xL [17]. Thus, the essential functions of PKM2 in cancer cells have attracted a great deal of attention.

The aim of the current study was to assess PKM2 expression in HCC, and determine the effects of alterations of PKM2 expression on proliferation, and the mechanisms involved in cultured liver cancer cells.

2. Materials and methods

2.1. Reagents

Antibodies used were: anti-PKM2 (sc-65176) and anti-HIF-1α (sc-53546) (Santa Cruz Biotechnology, Texas, CA, USA); anti-STAT3, anti-phospho-STAT3^{Tyr705}, anti-phospho-STAT3^{Ser727} (#9136), anti-p65 (#8242), anti-phospho-p65 (#3033) and anti-Bcl-xL (#2764) (Cell Signaling Technology, Boston, MA, USA); anti-GAPDH (KangChen Bio-tech, Shanghai, China).

2.2. Tissue samples

One hundred and fifteen patients with HCC who had undergone routine surgery at the First Affiliated Hospital of Nanjing Medical University (Nanjing, China) from January 2011 to July 2013 were recruited into this study. Informed consent for patient sample analysis was obtained from each subject before surgery, and the study was approved by the Institutional Ethics Committee of the First Affiliated Hospital of Nanjing Medical University. Portions of HCC tissues and the adjacent liver tissues were collected from all patients, then immediately snap frozen in liquid nitrogen, and stored at −80 °C until RNA extraction. Other tissue samples were fixed in 4% paraformaldehyde, and cut from paraffin blocks into 5 μm thick sections for immunohistochemistry. Adjacent tissue was located within 1 cm of the tumor margin and was confirmed to be non-tumor tissue by pathological examination. Detailed patient information is listed in Table S1.

2.3. Cell culture

293T and HepG2 cell lines were purchased from Shanghai Institute for Biological Science (Shanghai, China). All cells were maintained by passaging twice a week in DMEM supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (10 mg/mL) at 37 °C in an atmosphere containing 5% CO_2. Each cell line was passaged when they reached 70–80% confluence. IKK inhibitor PS1145 dihydrochloride (sc-301621A; Santa Cruz Biotechnology, Texas, CA, USA) was added to HepG2 cells at 20 or 40 μM for 72 h.

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from frozen liver tissues using Trizol solution (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. mRNA was reverse transcribed to cDNA, and qRT-PCR analysis was carried out using ABI 7900 Fast Real-Time PCR system and ABI Prism7500 Sequence Detector System (Applied Biosystems, CA, USA). Thermal cycler conditions were as follows: 30 min at 48 °C, then 15 min at 95 °C, followed by 40 cycles of 15 sec at 95 °C, 1 min at 59 °C. Relative expression levels of PKM2, HIF-1α and Bcl-xL mRNA were measured by SYBR Green real-time PCR (RT-PCR), and normalized to GAPDH levels. Oligonucleotide primers (Table 1) for qRT-PCR were purchased from Invitrogen (Invitrogen Life Technologies, Shanghai, China).

2.5. Immunohistochemistry

Tissue samples that were fixed in 4% paraformaldehyde and sectioned were deparaffinized. Antigen retrieval was performed by heating the slides in an autoclave for 3 min using citrate buffer (pH 6.0). The slides were incubated with primary antibody (1:400 dilution) overnight at 4 °C. Negative controls were prepared by replacing the primary antibody either with serum or with antibody dilution buffer. The primary antibodies were PKM2 and HIF-1α. All slides were then counterstained with hematoxylin. Brown-yellow staining was considered to indicate positive staining in the cells. Photographs were taken using a light microscope (ECLIPSE 50i; Nikon, Tokyo, Japan) and software NIS-Elements v4.0.

2.6. Plasmid construction and cell transduction

The human PKM2 mRNA sequence was obtained from GenBank (Accession number NM_002654). PKM2 gene was synthesized (Genscript, Nanjing, Jiangsu, China) by restriction digestion using H*pa* I and X*ho* I (New England Biolabs, Ipswich, MA, USA), and subcloned into pLV-GFP plasmid (Vector 1, a gift from D. Beicheng Sun, University of Nanjing Medical University, China) [18], and named pLV-GFP-PKM2 (PKM2). To generate a plasmid expressing PKM2-shRNA, double-stranded oligonucleotides were cloned into pLL3.7 plasmid (Vector 2, a gift from Dr. Yun Chen, University of Nanjing Medical University, China) and named pLL3.7-PKM2-shRNA (PKM2-shRNA). Recombinant lentivirus was generated from 293T cells using calcium phosphate precipitation, and transduced into 293T and HepG2 cells using polybrene (8 μg/ml) [19]. Representative pictures of wild-type and transfected cells are shown in Fig. S1.

2.7. Cell proliferation and anchorage-dependent colony formation assays

Cells were seeded into 96-well plates at 3000 cells/well. A CCK-8 proliferation assay kit (C0038; Beyotime Institute of Biotechnology, Nanjing, Jiangsu, China) was used. 10 μL 2-(2-methoxy-4-(phenyl)-3-(4-(phenyl)-5-(2,4-sulpho benzene)-2)h-tetrazolium) monosodium salt (WST-8) (0.5 mg/mL) was added into each well [20]. At each time-point, the medium in each well was changed to 200 μl fresh medium mixed with 10 μL CCK-8 solution. The absorbance of each well was read on a spectrophotometer (Thermo, Pittsburgh, PA, USA) at 450 nm (A_{450}). The data are presented as mean ± SEM, derived from triplicate samples of at least 3 independent experiments. Cell anchorage-dependent colony formation assays were performed as described previously [21].

2.8. Apoptosis assay

Forty-eight hours after transfection, apoptosis in cultured cells was evaluated using annexin V labeling.[22] Alexa Fluor-647

Table 1
Primer sequences.

Target gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
PKM2	ATGTCGAAGCCCCATAGTGAA	TGGGTGGTGAATCAATGTCCA
HIF-1α	GAACGTCGAAAAGAAAAGTCTCG	CCTTATCAAGATGCGAACTCACA
Bcl-Xl	GAGCTGGTGGTTGACTTTCTC	TCCATCTCCGATTCAGTCCCT