RGC Ref.: M-CUHK406/13
(please insert ref. above)

The Research Grants Council of Hong Kong
SRFDP & RGC ERG Joint Research Scheme
Completion Report

(Please attach a copy of the completion report submitted to the Ministry of Education by the Mainland researcher)

Part A: The Project and Investigator(s)

1. Project Title

Impact of hypoxia-inducible factor 1 alpha on EpCAM+ hepatic cancer stem cells in hepatocellular carcinoma

2. Investigator(s) and Academic Department/Units Involved

	Hong Kong Team	Mainland Team
Name of Principal Investigator *(with title)*	Professor George G Chen	Professor Fan Jia
Post	Professor, Laboratory Director	President of Zhongshan Hospital, Fudan University; Professor, Chairman of Liver Cancer Institute, Fudan University; Head of Hepatic Surgery Department, Zhongshan Hospital, Fudan University
Unit / Department / Institution	Department of Surgery, The Chinese University of Hong Kong	Department of Liver Surgery, Zhongshan Hospital & Liver Cancer Institute, Fudan University
Co-investigator(s) *(with title and institutions)*	Professor Lai Paul Bo-San Department of Surgery, The Chinese University of Hong Kong	Dr Yang Xinrong Department of Liver Surgery, Zhongshan Hospital & Liver Cancer Institute, Fudan University
PhD student(s) (with period of involvement)	Name: Mr Yang Shengli Institution: The Chinese University of Hong Kong Period from 1 January 2014 to 31 July 2015	Mr Sun Yunfan Department of Liver Surgery, Zhongshan Hospital & Liver Cancer Institute, Fudan University Period from 1 January 2014 to 31 July 2015

Note: The Hong Kong project team must involve at least one research postgraduate student pursuing a Doctor of Philosophy degree at the UGC-funded university (PhD student) at any time throughout the project period.

3. Project Duration

	Original	Revised	Date of RGC/ Institution Approval *(must be quoted)*
Project Start date	1 January 2014		
Project Completion date	31 Dec 2016		
Duration *(in month)*	36		
Deadline for submission of Completion Report	31 Dec 2017		

Part B: The Completion Report

5. Project Objectives

5.1 Objectives as per original application

1. To confirm the relationship between hypoxia and HIF1a in HCC. This is a necessary step for the subsequent tests.
2. To determine the role of HIF1a in the development of hCSC. This will explore the role of HIF1a in the formation of EpCAM+ hCSC.
3. To assess the impact of changes in HIF1a levels on hCSC development and their features. This will explore the regulatory effects of HIF1a on key markers of hCSC, migration, invasion, tumorigenic capacity and other relevant features of hCSC *in vitro* and *in vivo*.

5.2 Revised Objectives

Date of approval from the RGC: _____

Reasons for the change: _____

_____

1.
2.
3. ....

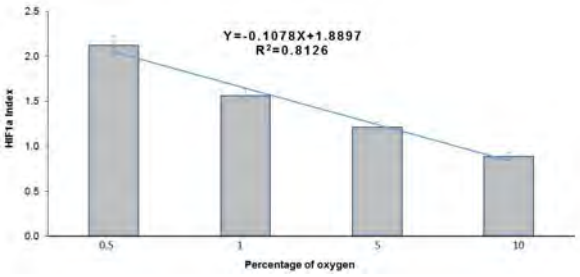
6. Research Outcome

Major findings and research outcome

(maximum 1 page; please make reference to Part C where necessary)

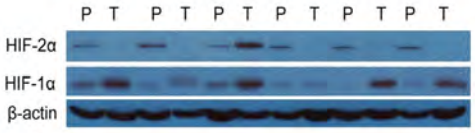
1. The level of HIF1a is decreased when the concentration of oxygen is increased (Fig 1).

Fig 1. The impact of oxygen levels on the expression of HIF1a. HepG2 cells were cultured in different levels of oxygen as indicated in the figure. After the incubation of 24 hours, cells were harvested and total proteins were isolated for Western blot of HIF1a and actin that was used as an equal loading and control. The density of the positive bands was determined and the ratio of HIF1a to actin was calculated as the expression index of HIF1a.



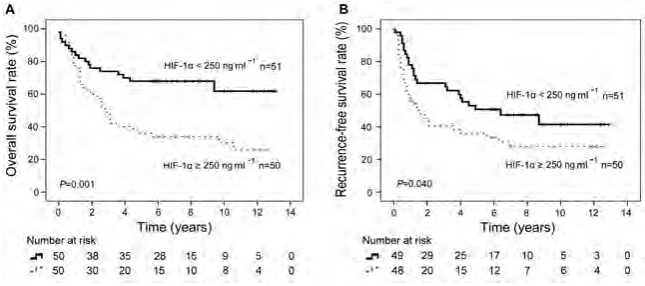
2. The levels of HIF1a and HIF2a were opposite in HCC and paired peritumoral tissues and the result is confirmed in immunohistochemical staining of HCC tissues (138 cases) as well as by Western blot of protein expression. A representative result of Western blot was shown in Fig 2.

Fig 2. The expression of HIF-1α and HIF-2α proteins in HCC tumor tissues and the paired peritumorial tissues. Total proteins were isolated from tissues. Western blot was perform to detect HIF1a, HIF2a and the control actin. P: peritumorial tissues and T: tumor tissues.



3. The expression of HIF-1α protein is negatively associated with overall survival (OS) and recurrence-free survival (RFS) (Fig 3).

Fig 3. Correlation of HIF-1α with HCC prognosis. The levels of HIF1a in HCC tumor tissues were determined by ELISA and the Kaplan–Meier analysis was performed to analyze OS (**A**) and DFS (**B**). HIF-1α expression of HIF-1α <250 ng/ml (black line) was regarded as the high expression group while HIF-1α ≥ 250 ng/ml as the low expression group (gray line).



4. The expression of HIF1a enhances self-renewal, proliferation, migration and invasion of hCSC whereas HIF2a inhibits these features. Consistent with these findings, HIF1a is positively associated with EpCAM+ hCSC but the level of HIF2a is negatively related to EpCAM+ hCSC. Therefore, the over-expression of HIF2a suppresses HCC tumor growth and induces apoptosis rate in HepG2-cell xenografts in nude mice (Fig 4).

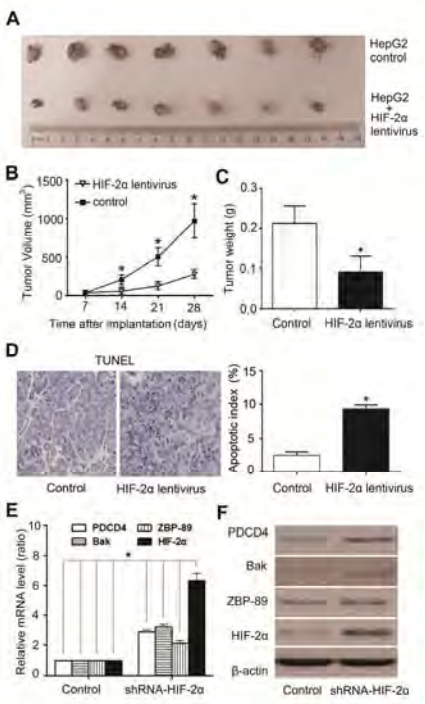


Fig 4 Over-expression of HIF-2α induced HCC growth arrest and high apoptosis rate in HepG2-cell xenografts in nude mice. (A) Tumors formed by implanted cells with different expression levels of HIF-2α. (B) Tumor volume. Tumor volumes were calculated and data were plotted using the geometric mean for each group vs. time. Each point represents the mean tumor volume (± sd) of measurements from the 7 mice in each treatment group. ***P*<0.01, two-tailed test. (C) The weight of tumors was significantly lower in HIF-2α lentivirus-infected group than the control group. ***P*<0.01, two-tailed test. (D) Cells positive for TUNEL staining were statistically increased in HIF-2α-over-expressing tumors, compared with the control (***P* < 0.01, Student's t test). Real-time PCR (E) and western blotting analysis (F) of HIF-2α, ZBP-89, Bak, PDCD4 expression in xenograft tumors after mouse sacrifice.

Potential for further development of the research and the proposed course of action *(maximum half a page)*

The novel finding of this study is that the opposite roles of HIF1a and HIF2a on hCSC and the growth of HCC. This important result may have therapeutic impact on HCC as the inhibition of HIF1a or/and the upregulation of HIF2a can result in the inhibition of HCC. However, the in vivo model of this study is a mouse subcutaneous model, the environment of which may not be the same as in liver. Thus, further test of this conclusion/concept in an orthotopic HCC model is needed.

The finding that the expression of HIF1a protein is negatively associated with overall survival (OS) and recurrence-free survival (RFS) may also have some clinical impacts as it suggests a prognostic value of HIF1a in HCC. However, this may need to be further confirmed in a large case of HCC (the current result is based on 138 cases of HCC).

7. The Layman's Summary

(describe <u>in layman's language</u> the nature, significance and value of the research project, in no more than 200 words)

Hepatocellular carcinoma (HCC) is often resistant to chemotherapy. Hepatic cancer stem cells (hCSC) appear to play a key role in the aggression, invasion and resistance of HCC and thus effective anti-HCC treatments need to target and remove hCSC. Accumulating evidence suggests that hypoxia-inducible factors (HIFs) have a key role in the development of cancer stem cells (CSC) in solid tumors. This study demonstrates that HIF1a plays a positive role in the development of hCSC whereas HIF2a has an inhibitory role in the formation/maintenance of hCSC. We have also found that the expression of HIF1a protein is negatively associated with overall survival (OS) and recurrence-free survival. This finding may also have some clinical impacts as it suggests a prognostic value of HIF1a in HCC. Collectively, this study has

demonstrated that HIF1a promotes HCC while HIF2a inhibits it, and the level of HIF1a may have a prognostic value in HCC.

Part C: Research Output

8. Peer-reviewed journal publication(s) arising directly from this research project

(Please attach a copy of each publication and/or the letter of acceptance if not yet submitted in the previous progress report(s). All listed publications must acknowledge RGC's funding support by quoting the specific grant reference.)

The Latest Status of Publications				Author(s) *(**bold** the authors belonging to the project teams and denote the corresponding author with an asterisk*)*	Title and Journal/ Book *(with the volume, pages and other necessary publishing details specified)*	Submitted to RGC *(indicate the year ending of the relevant progress report)*	Attached to this report *(Yes or No)*	Acknowledged the support of this Joint Research Scheme *(Yes or No)*	Accessible from the institutional repository *(Yes or No)*
Year of publication	Year of Acceptance *(For paper accepted but not yet published)*	Under Review	Under Preparation *(optional)*						
2016				**Yang SL**, Liu LP, **Sun YF, Yang XR, Fan J**, Ren JW, **Chen GG*, Lai PB**.	Distinguished prognosis after hepatectomy of HBV-related hepatocellular carcinoma with or without cirrhosis: a long-term follow-up analysis. J Gastroenterol. 2016 Jul;51(7):722-32. doi: 10.1007/s00535-015-1146-0.	not	yes	yes	yes
2016				**Yang SL,** Liu LP, Niu L, **Sun YF, Yang XR, Fan J,** Ren JW, **Chen GG*, Lai PB.**	Downregulation and pro-apoptotic effect of hypoxia-inducible factor 2 alpha in hepatocellular carcinoma. Oncotarget. 2016 Jun 7;7(23):34571-81. doi: 10.18632/oncotarget.8952.	not	yes	yes	yes

2017				**Sun Y,** Guo W, Xu Y, Shi YH, Gong Z, Ji Y, Du M, Zhang X, Hu B, Huang A**, Chen GG, Lai PBS,** Cao Y, Qiu SJ, Zhou J**, Yang XR*, Fan J*.**	Circulating Tumors Cells from Different Vascular Sites Exhibit Spatial Heterogeneity in Epithelial and Mesenchymal Composition and Distinct Clinical Significance in Hepatocellular Carcinoma. Clin Cancer Res. 2017 Oct 25. pii: clincanres.1063.2017. doi: 10.1158/1078-0432.CCR-17-1063.	not	yes	yes	Not yet

9. Recognized international conference(s) in which paper(s) related to this research project was/were delivered *(Please attach a copy of each delivered paper. All listed papers must acknowledge RGC's funding support by quoting the specific grant reference.)*

Month/Year/ Place	Title	Conference Name	Submitted to RGC *(indicate the year ending of the relevant progress report)*	Attached to this report *(Yes or No)*	Acknowledged the support of this Joint Research Scheme *(Yes or No)*	Accessible from the institutional repository *(Yes or No)*
Sept/2016/ Chicago, USA	Differential levels of hypoxia-inducible factor-1a and 2a in hepatocellular carcinoma	International Society of Oncology and Biomarkers	not	yes	yes	Not yet
Oct/2016/ Baltimore, USA	Interaction between ZBP-89 and oncogenic molecules in hepatocellular carcinoma	International Conference on Cancer Research and Targeted Therapy	not	yes	yes	Not yet
Nov/2017/ Atlanta, USA	ZBP-89 and hypoxia-inducible factor 1a in hepatocellular carcinoma	Oncology and Biomarkers Summit 2017	not	yes	yes	Not yet

10. Student(s) trained *(Please attach a copy of the title page of the thesis.)*

Name	Degree registered for	Date of registration	Date of thesis submission/ graduation
Shengli Yang	PhD	Aug 2012	July 2015

11. Other impact *(e.g. award of patents or prizes, collaboration with other research institutions, technology transfer, etc.)*

None