Proceeding

Effect of High Glucose on Expression of Glycolytic Enzymes in Cholangiocarcinoma Cells

Salak Thaenkaew[1*], *Wunchana Seubwai*[2,4], *Chawalit Pairojkul*[3,4], *Sopit Wongkham*[1,4]

[1]*Departments of Biochemistry,* [2]*Forensic Medicine,* [3]*Pathology, Faculty of Medicine, Khon Kaen University, Khon Kaen, 40002 Thailand.* [4]*Cholangiocarcinoma Research Institute, Faculty of Medicine, Khon Kaen University*

Background and Objective: Most of cholangiocarcinoma (CCA) patients are presented with metastasis which is the major cause of death of CCA patients. Understanding of factors mediated metastasis may lead to a novel treatment for CCA. Cancer cells produced most of their ATP via aerobic glycolysis. Increasing of enzymes in glycolytic pathways were reported in several cancers. The association of high glucose and CCA progression have been demonstrated previously. Thus, the aim of this study was to investigate whether high glucose condition could affect expression of glycolytic enzymes in CCA.

Methods: Three CCA cell lines, KKU-213, -214, and -055, in normal glucose condition (NG; 5.6 mM) were established by sequentially reduced glucose concentration in the media from high glucose (HG; 25 mM) to 5.6 mM. The increase of O-GlcNAcylated proteins was used as the marker indicating adaptation of cells in low or high glucose condition. Expression of glycolytic enzymes were determined using western blot.

Results: Three CCA cell lines, KKU-213, -214, and -055, in NG and HG were successfully established. All NG cells had lower levels of O-GlcNAcylated proteins than HG cells. The western blot analysis of the key regulatory enzymes: HKII, PFK-1, PKM2, LDHA and MCT1 and MCT4 were compared between NG and HG cells. The results showed that HKII and PFK1 were increased in HG cells of both KKU-055 and KKU-213 cells.

Conclusion: High glucose could promote expression of HKII and PFK1 in CCA cells. This may be one of the mechanisms that high glucose activated progression of CCA.

Keywords: bile duct cancer, high glucose, glycolytic enzymes, metabolism

Introduction

The incidence of cholangiocarcinoma (CCA), a cancer of bile duct epithelium, is increasing worldwide. Thailand has been reported to have the highest world incidence of CCA[1]. The infection of a carcinogenic liver fluke, *Opisthorchis viverrini*, is a major risk factor of CCA in this region. At present, surgical resection is still the main treatment of choice for cure of CCA. As CCA has no particular symptom and sign at the beginning and the limitation of early diagnostic methods, most of CCA patients are frequently presented with metastatic stage where surgery cannot be offered. Currently, there is no standard protocol for an effectively treatment of CCA.

Therefore, finding of a novel treatment targeted on genes associated with carcinogenesis or/and progression of CCA is urgently needed.

Deregulating of cellular energetics is one of the cancer hallmarks, of which cancer cells produced most of their ATP via glycolysis under aerobic condition, known as "Warburg effect" or "aerobic glycolysis"[2]. The activation of oncogenes or loss of tumor suppressors have been linked to the continuing activation of aerobic glycolysis and consequently advancing of cancer progression. In CCA, Hexokinase II (HKII), Lactate dehydrogenase A (LDHA) and Monocarboxylate transporter (MCT) 1 and MCT4 were shown to be overexpressed and related to increasing of cell proliferation, cell migration and invasion[3, 4].

Diabetes mellitus (DM) is a group of metabolic diseases indicating by high glucose condition or hyperglycemia. Recently, DM and hyperglycemia have been described not only as a risk factor[5] but also as a poor prognostic factor of many cancers, including CCA[6]. The association of DM and CCA has been reviewed recently[7]. The data collected in 2014 from the Bureau of policy and strategy, Ministry of Public Health of Thailand, showed that northeast region with a high mortality rate of DM also had a high mortality rate of liver and biliary tract cancers (Figure 1). The aim of this study was to verify whether high glucose could mediate expression levels of glycolytic enzymes in CCA cell lines.

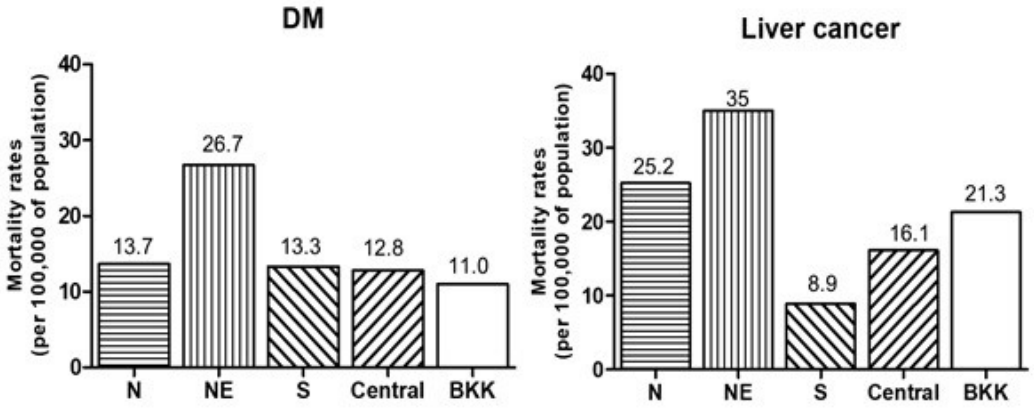

Figure 1 Mortality rates of DM and CCA patients in each region of Thailand, 2014. N = north; NE = northeast; S = south; BKK = Bangkok; DM = diabetes mellitus (Ref. the Bureau of policy and strategy, Ministry of Public Health of Thailand)

Materials and Methods

CCA cell lines

Human CCA cell lines, KKU-055, KKU-213 and KKU-214 were established from the primary tumors of CCA patients of Srinagarind Hospital, Khon Kaen University by Dr. Banchob Sripa (Department of Pathology, Faculty of Medicine, Khon Kaen University, Thailand) and kindly provided from Cholangiocarcinoma Research Institute, Khon Kaen University. CCA cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) with normal (NG; 5.56 mM) or high (HG;

25 mM) concentrations of glucose and supplemented with 10% fetal bovine serum and 1% antibiotic-antimyotic and incubated in a 37°C, 5% CO_2, humidified incubator. Cells were cultured in the NG or HG medium for at least 5 passages to allow cell adaptation prior to use in the experiment. As level of cellular O-GlcNAcylation is varied according to the level of glucose in the culture media[8], levels of O-GlcNAcylated proteins (OGPs) was used as an indicator of cell adaptation.

SDS-PAGE and Western blot

Cells were lyzed with urea lysis buffer containing 7M urea, 2M thiourea, 4% CHAPs and protease inhibitors. Total protein concentration is determined using Bradford method (9). The protein samples were treated with SDS-PAGE sample buffers and bolied for 5 minutes. Equivalent amounts of protein (10-20 µg per lane) were subjected to a 10-12% SDS-PAGE. After electrophoresis, proteins in the gel were transferred onto a PVDF membrane by wet electro-transferred and then non-specific proteins were blocked in a solution of 5% BSA and 0.3% Tween-20 in PBS pH 7.4 for 1 hour at room temperature. The blot was incubated in the primary antibody and the secondary antibody for 1 hour each, at room temperature. Antigen-antibody complex signal was developed with ECL Prime Western blotting Detection System. The images of ECL signals were taken with ImageQuant 400 image analyzer and analyzed using ImageQuant™ TL analysis software (GE health care).

Results and Discussion

To establish CCA cells in NG condition, cells were sequentially cultured in a decreasing concentration of glucose media until getting cells in NG. Cells in NG were cultured further in NG media for at least 5 passages prior to the experiment (Figure 2). The levels of OGPs of the 3 cell lines in high glucose condition were increased compared to those cultured in normal glucose condition (Figure 3). The results were similar to those observed by Phoomak[8]. High glucose can promote expression of HKII in the glycolytic pathway and gluocosamine-fructose-6-phosphate amidotransferase in the hexosamine biosynthesis pathway that consequently enhance OGPs level in CCA cells.

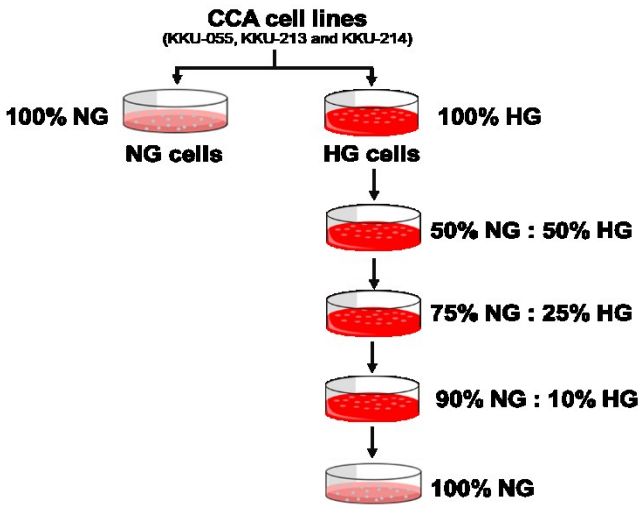

Figure 2 Establishment of CCA cell lines in normal and high glucose conditions. CCA cells were cultured by stepwise decreasing of glucose concentration in the media until getting cells in 100% of normal glucose media. NG = normal glucose; HG = high glucose

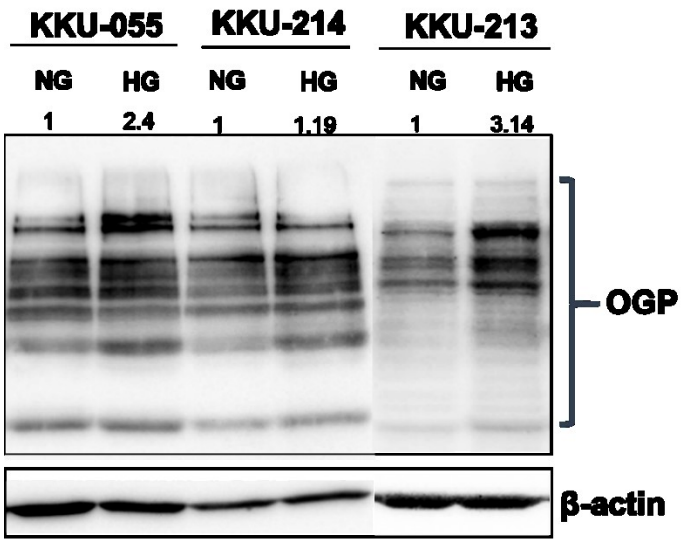

Figure 3 Expression of OGPs in three CCA cells. Comparison of OGP expression between NG and HG cells.

NG cells and HG cells of KKU-055 and KKU-213 were cultured in the specified media for 48 hours and levels of glycolytic enzymes i.e., HKII, *Phosphofructokinase-1 (PFK-1), Pyruvate kinase M2 (PKM2), LDHA* and MCT1 and MCT4 were determined using Western blot. As shown in Figure 4, expression of HKII and PFK1 in both cell lines were higher in HG cells than NG cells. The expressions of PKM2 and MCT1, however, were higher in HG than NG cells only in KKU-213. The MCT1, a transporter that preferentially pumps LDH into intracellular was not changed in NG and HG cells of KKU-055. The MCT4 of KKU-055 cells could not be detected in both NG and HG cells.

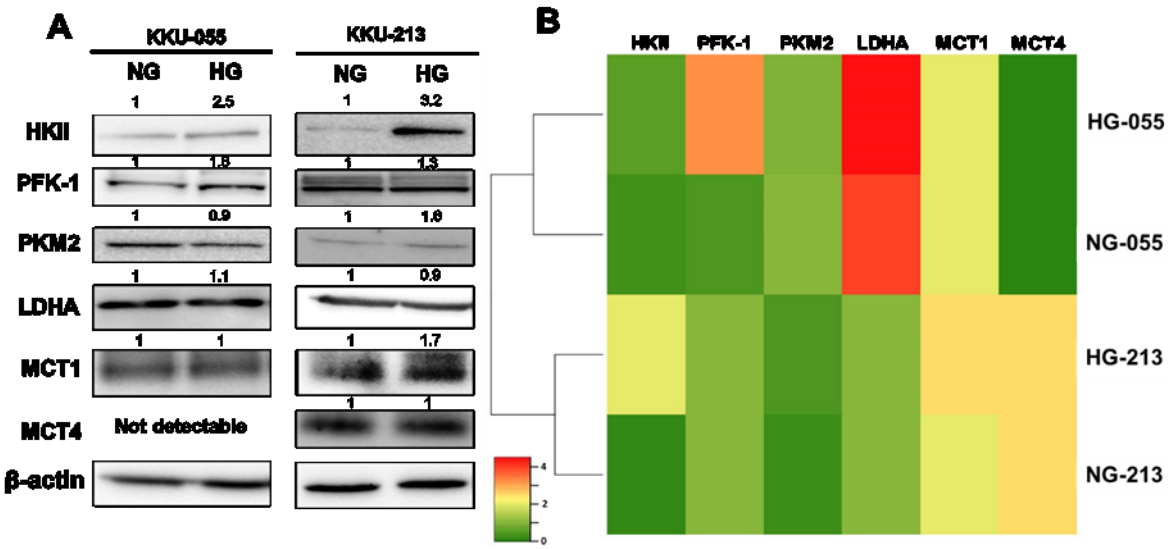

Figure 4 The expression of glycolytic enzymes in NG and HG cells. The key enzymes in glycolytic pathway of NG and HG cells of KKU-055 and KKU-213 were determined using Western blot analysis (A). The numbers indicate the protein intensities normalized by β-actin by giving those of NG cells = 1. The expression levels were compared as shown in (B). Heatmap was generated by heatmapper online program (http://www2.heatmapper.ca/).

High glucose could enhance glycolytic rate in both KKU-055 and KKU-213 cells by increasing expression of key regulatory enzymes—namely HKII and PFK1. Increasing of glycolytic rate, especially HKII can promote biosynthesis of macromolecules and hexosamine biosynthesis pathway. HKII catalyzed glucose to glucose-6-phosphate, the input for hexosamine biosynthesis and pentose phosphate pathways which generates biomolecules necessary for proliferation and progression of cancer cells (Figure 5). It has been shown recently that high glucose could promote growth, migration and invasion ability of CCA cells via activation of STAT3 and epithelial mesenchymal transition process[10]. In addition, high glucose condition also increased O-GlcNAcylation in CCA cells. The mechanism was shown to be via activation of hexosamine synthesis which generated UDP-GlcNAc, the substrate of O-GlcNAcylation[8]. The lack of MCT4 in KKU-055 is of interest as MCT4 is essential for transportation of LDH out of cells. KKU055 may use MCT1 and other transporters in transporting LDH extracellularly. Further experiments are needed to elucidate the mechanism by which glucose regulates expression of these enzymes.

Conclusion

CCA cell lines in NG and HG conditions were established and shown to be appropriate as the model for studying the effect of glucose on CCA cell metabolism and functions. High glucose condition enhances glycolytic rate of CCA cells by increasing the expression of key regulatory enzymes; HKII and PFK1.

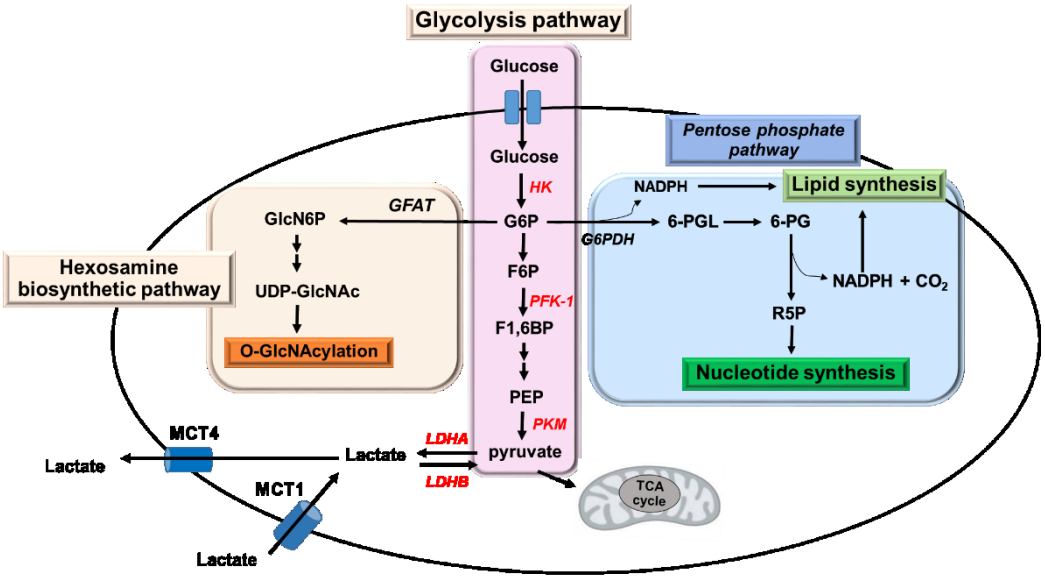

Figure 5 Glycolysis and linked pathways. Glucose is converted to pyruvate via glycolysis pathway. Glucose-6-phosphate (G6P) can be catalyzed into hexosamine biosynthesis or pentose phosphate pathway to generate NADPH for lipid synthesis, and 6-phosphogluconate (6-PG) for nucleotide synthesis. (Originated by Salak Thaenkaew)

Acknowledgements

This project was co-supported by the Thailand Research Fund and Medical Research Council-UK (Newton Fund) (DBG5980004) and Invitation Research Grant, Faculty of Medicine, Khon Kaen University (IN60330).

References

1. Vatanasapt V, Sriamporn S, Vatanasapt P. Cancer control in Thailand. Jpn J Clin Oncol 2002;32 Suppl:S82-91.
2. Warburg O. On the origin of cancer cells. Science 1956; 123(3191): 309-14.
3. Thamrongwaranggoon U, Seubwai W, Phoomak C, Sangkhamanon S, Cha'on U, Boonmars T, et al. Targeting hexokinase II as a possible therapy for cholangiocarcinoma. Biochem Biophys Res Commun 2017; 484(2): 409-15.
4. Thonsri U, Seubwai W, Waraasawapati S, Sawanyawisuth K, Vaeteewoottacharn K, Boonmars T, et al. Overexpression of lactate dehydrogenase A in cholangiocarcinoma is correlated with poor prognosis. Histol Histopathol, 2016: 11819.
5. Palmer WC, Patel T. Are common factors involved in the pathogenesis of primary liver cancers? A meta-analysis of risk factors for intrahepatic cholangiocarcinoma. J Hepatol 2012; 57(1): 69-76.
6. Liu RQ, Shen SJ, Hu XF, Liu J, Chen LJ, Li XY. Prognosis of the intrahepatic cholangiocarcinoma after resection: hepatitis B virus infection and adjuvant chemotherapy are favorable prognosis factors. Cancer Cell Int 2013; 13(1): 99.
7. Saengboonmee C, Seubwai W, Wongkham C, Wongkham S. Diabetes mellitus: Possible risk and promoting factors of cholangiocarcinoma: Association of diabetes mellitus and cholangiocarcinoma. Cancer Epidemiol 2015; 39(3): 274-8.
8. Phoomak C, Vaeteewoottacharn K, Silsirivanit A, Saengboonmee C, Seubwai W, Sawanyawisuth K, et al. High glucose levels boost the aggressiveness of highly metastatic cholangiocarcinoma cells via O-GlcNAcylation. Sci Rep 2017; 7: 43842.
9. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248-54.
10. Saengboonmee C, Seubwai W, Pairojkul C, Wongkham S. High glucose enhances progression of cholangiocarcinoma cells via STAT3 activation. Sci Rep 2016; 6: 18995.