

การแสดงให้เห็นเพิ่มขึ้นของ Pyruvate Kinase M2 ในมะเร็งท่อน้ำดี

Upregulation of Pyruvate Kinase M2 in Cholangiocarcinoma

อัญชลี ต้นศรี^{1,4}, วันชนะ สืบไว^{2,4}, ศักดา วราอัสวปติ³, อุบล ชาอ่อน^{1,4}

Unchalee Thonsri^{1,4}, Wunchana Seubwai^{2,4}, Sakda Waraasawapati³, Ubon Cha'on^{1,4*}

บทคัดย่อ

โรคมะเร็งท่อน้ำดีเป็นมะเร็งที่เกิดจากเยื่อบุของท่อน้ำดี และถือเป็นปัญหาด้านสาธารณสุขที่สำคัญของประเทศไทย โดยเฉพาะที่ภาคตะวันออกเฉียงเหนือของประเทศไทย จากคุณสมบัติของ เซลล์มะเร็ง ที่มีเมแทบอลิซึมแตกต่างจากเซลล์ปกติ เมแทบอลิซึมของกลูโคสจัดเป็นวิถีที่มีการศึกษาอย่างกว้างขวางและเป็นที่ยอมรับกันดี โดยพบว่า เซลล์มะเร็งมีกระบวนการสร้างพลังงานที่แตกต่างจากเซลล์ปกติ เพื่อการมีชีวิตรอด รวมถึงกระบวนการพัฒนาและการแพร่ลุกลามของเซลล์มะเร็ง (progression and metastasis) การทำความเข้าใจกระบวนการเมแทบอลิซึมของเซลล์มะเร็งจึงน่าจะเป็นแนวทางหนึ่งเพื่อพัฒนาการรักษาโรคมะเร็งที่มีความจำเพาะและมีประสิทธิภาพ แต่เนื่องจากการศึกษาบทบาทของเอนไซม์สำคัญในวิถีไกลิโคไลซิสในมะเร็งท่อน้ำดีที่สัมพันธ์กับการติดเชื้อมาโดยบังเอิญไม่ได้เป็นที่ยอมรับกันดี ดังนั้นผู้วิจัยจึงมีวัตถุประสงค์ที่จะศึกษารูปแบบการแสดงออกของ PKM2 ในชิ้นเนื้อผู้ป่วยมะเร็งท่อน้ำดี (n = 30) ด้วยวิธีอิมมูโนฟลูออโรเซนซ์ และวัดระดับ PKM2 ในซีรัม ด้วย ELISA kit ผลการศึกษาพบว่า การแสดงออกของ PKM2 มีความสัมพันธ์กับการเกิดมะเร็งท่อน้ำดี และพบวาระดับของ PKM2 ในซีรัมผู้ป่วยมะเร็งท่อน้ำดี (n = 26) สูงกว่าในกลุ่มควบคุม (n = 18) แต่ยังไม่ถึงนัยสำคัญทางสถิติ ดังนั้นเพื่อให้ได้ข้อมูลที่ชัดเจนขึ้นเกี่ยวกับบทบาทของ PKM2 ในการวินิจฉัยและการรักษามะเร็งท่อน้ำดี จึงจำเป็นต้องศึกษาในจำนวนตัวอย่างที่มากขึ้น

คำสำคัญ: cholangiocarcinoma pyruvate kinase M2 cancer metabolism glycolysis

^{1,4} นิสิตปริญญาโท, ภาควิชาชีวเคมี ^{2,4}ผู้ช่วยศาสตราจารย์, ภาควิชานิติเวชวิทยา ³ นายแพทย์, ภาควิชาพยาธิวิทยา, ^{1,4} ผู้ช่วยศาสตราจารย์, ภาควิชาชีวเคมี คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น 40002

¹ Departments of Biochemistry, ² Departments of Forensic Medicine, ³ Departments of Pathology, Faculty of Medicine, Khon Kaen University, Thailand, 40002, ⁴ Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Thailand, 40002.

*Corresponding author. Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Thailand, 40002

Tel: +66-43-348-386, E-mail: ubocha@kku.ac.th.



Abstract

Cholangiocarcinoma (CCA), a cancer of the bile duct that arising from biliary epithelium, is a major public health problem of Thailand, especially in the Northeastern Thailand. Based on the hallmarks of cancer, glucose metabolism is the well establishment of metabolic differences between normal and cancer cells. Alteration of energy metabolism in cancer cells is necessary to cell survive, progression and metastasis. Understanding of this hallmark may help to identify a potential target for cancer therapies. Since the roles of key glycolytic enzymes in CCA are limited, we aimed to investigate pyruvate kinase M2 (PKM2) expression in Ov-associated CCA tissues using immunohistochemistry and determine serum PKM2 levels in CCA patients using ELISA kit. Results indicated that PKM2 expression was associated with CCA carcinogenesis in human CCA tissues (n = 30). Although the changes did not reach statistical significant, serum PKM2 levels in CCA patients (n = 26) was apparently higher compared to healthy controls (n = 18). Greater number needed to be further evaluated to support the significant role of PKM2 in CCA diagnosis and treatment.

Keywords: cholangiocarcinoma, pyruvate kinase M2, cancer metabolism, glycolysis

Introduction

Cholangiocarcinoma (CCA) is an epithelial bile duct cancer. It is apparently low incidence rate worldwide, however, high incidence rate is found in the Southeastern countries, especially in Thailand ¹. The pathogenesis of this cancer is mostly linked to the infection of the liver fluke (*Opisthorchis viverrini*). CCA is difficult to diagnose which most of CCA patients are the clinically silent until reaching an advanced stage of disease lead to late diagnosis and short survival ². So far, surgery is the only curative treatment for CCA patients but only a minor fraction can be cured ³. Therefore, discovery of a specific tumor marker for diagnosis and therapeutic modalities to improve the effective treatment and outcome of CCA are urgently required.

Targeting cancer metabolism for cancer therapy is increasing favorable during the past few years. One of the hallmarks of cancer is defined as reprogramming energy metabolism. Most cancer cells display an altered energy

metabolism with the higher rate of glucose breakdown followed by increased lactate production to generate the energy compared to the normal cells. This unique of cancer metabolism was firstly observed by Otto Warburg in 1924 ⁴ that the cancer cells prefer to generate ATP via glycolysis compared to that of oxidative phosphorylation even when the oxygen is sufficient. Among of metabolic alterations, glucose metabolism is an important to maintain cancer survives with the benefit of providing intermediate precursors for biosynthesis of macromolecules ⁵. Therefore, targeting the glycolytic enzymes may be a promising strategy to conquer the cancer. We targeted on pyruvate kinase M2 (PKM2), one of the rate-limiting steps of glycolytic pathway which converts the phosphoenol pyruvate to pyruvate. More attention has been drawn to PKM2 as a tumor marker detected in serum ⁶, plasma, bile ⁷ and feces ⁸.

This study was aimed to investigate the PKM2 expression profiles in human CCA tissues as well as serum PKM2 levels. Results of this



study indicated that PKM2 can be the potential targeted molecules for an effective treatment of CCA.

Materials and Methods

Human CCA tissues

Paraffin embedded tissues from CCA patients (n = 30) who were admitted and underwent liver surgical resection at Srinagarind hospital, Faculty of Medicine, Khon Kaen University were obtained from the specimen bank of the Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Thailand. The informed consent was obtained from each subject and the protocol has been reviewed and approved by the Ethics Committee for Human Research of Khon Kaen University (HE571283) based on the Declaration of Helsinki and ICH-Good Clinical Practice Guidelines. All tissues were histologically proven to be CCA.

Serum samples

Serum samples (n = 55) were obtained from LFCRC. Serum samples were divided into three groups; healthy control (n = 18), benign biliary disease (BBD) (n = 11), and patients with CCA (n = 26), to determined serum PKM2 levels using Human-PKM2 ELISA kit.

PKM2 immunohistochemistry (IHC)

Paraffin embedded tissues were sliced into 4 μ m in thick and IHC was performed using standard immunohistochemistry protocol. The sections were incubated with primary antibody against PKM2 (dilution 1:600; Cell signaling Technology, Boston, USA) at room temperature for 1 hour. Following washing with 1X PBS (2 times, 5 minutes), tissue sections were incubated

with EnVision+System-HRP labeled anti rabbit (Dako, Carpinteria, USA) for 1 hour at room temperature. Then the sections were washed in PBS. The peroxidase activity was observed using diaminobenzidine tetrahydroxy chloride (DAB) solution (Dako, Glostrup, Denmark) as the substrate. The sections were counterstained with hematoxylin for 3- to 5 minutes and mounted with per mounted on glass slide.

The evaluation of immunohistochemical staining was based on intensity and frequency staining. The staining intensity for PKM2 expression were scored as 1 (weak); 2 (moderate); and 3 (strong). The percentages of positive cells were graded as follows; grade0, negative; grade1, 1–25%; grade2, 26–50%; and grade3, more than 50%. The immunohistochemistry score of PKM2 expression of each section will be calculated as intensity multiplied by frequency. PKM2 expression levels were categorized as low expression (score < median, 6) and high expression (score \geq 6 median, 6).

Measuring of serum PKM2 level by ELISA

Enzyme-linked immunosorbent assay kit (catalog No. E-EL-H1089, Elabscience, USA) was used for determination of PKM2 levels according to the manufacturer's protocol. In brief, diluted samples (1:50) were added to 96-well plates captured with anti-PKM2 antibody. The samples were incubated for 1 hr and several rinses before they were incubated with a biotin-conjugated anti-PKM2 monoclonal antibody for another 30 min. PKM2 molecule was detected with a streptavidin-coupled horseradish peroxidase reaction and signal was detected at 450 nm using automatic ELISA plate reader (Tecan Austria GmbH). All samples were assayed in duplicate.

Statistical analysis

Serum PKM2 expression levels of three groups, healthy control, benign biliary disease, and CCA were using t-test analysis. *P* value of less than 0.05 was considered as a statistical significant. The results were presented as median and standard deviation (SD) value.

Results

Upregulation of PKM2 expression in human CCA tissues

PKM2 expression was determined using immunohistochemistry in human CCA tissues from 30 cases of CCA patients. PKM2 was less

expressed in normal bile duct. Only 18% (5/28) of normal bile duct had positive PKM2 staining (Figure 1-1A). In contrast, the up-regulation of PKM2 was observed in bile duct hyperplasia with 58% (7/12) positive staining (Figure 1-1B) and 93% (28/30) in CCA area. PKM2 expression was classified as low (< median) and high (\geq median) level of expression which 47% (14/30) of low and 53% (16/30) of high PKM2 expression, respectively (Figure 1-1C and D). PKM2 was expressed in both cytoplasm and nucleus of CCA cells. In most cases of the study, PKM2 was more frequently observed in the cytoplasm of CCA cells.

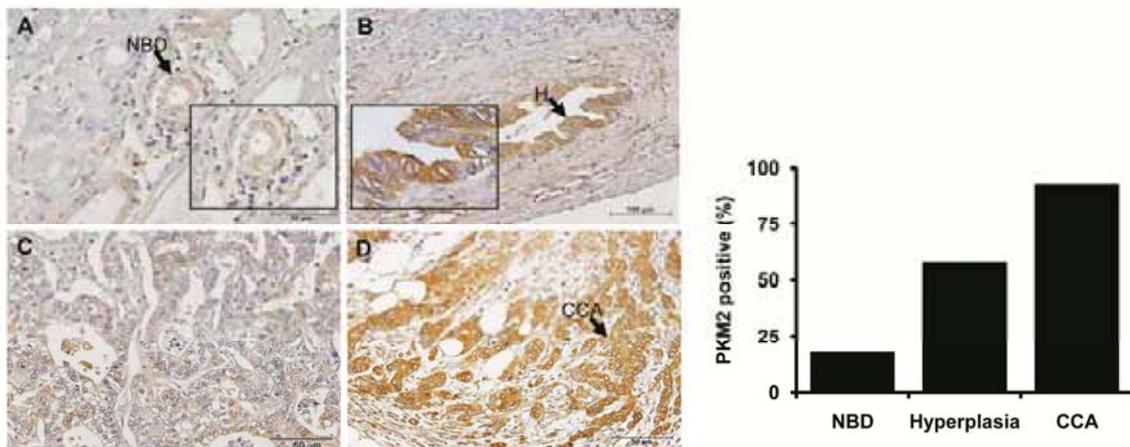


Figure 1-1 Representative immunohistochemistry staining of PKM2 expression in human CCA tissues. (A) PKM2 was minimal expressed in normal bile duct (17%). (B) Higher PKM2 expression was observed in bile duct hyperplasia (23%). PKM2 was highly expressed in CCA area (93%) which categorized as follows; (C) low PKM2 expression in CCA tissues and (D) high PKM2 expression in CCA tissues. PKM2 was increased during carcinogenesis of CCA from normal to hyperplasia and develop to cancer as show in bar graph. The data are expressed as n/N (%), n = number of positive cases of PKM2 expression, NBD = normal bile duct, (Magnification x200)



Serum PKM2 levels in healthy control, benign biliary disease (BBD) and CCA

Serum PKM2 levels were determined to explore its role in diagnostic and prognostic marker of CCA patients using ELISA kit assay. There are three groups of the experiment including the group of healthy control (n = 18), BBD (n = 11), and CCA (n = 26). Result indicated

that serum PKM2 levels increased in BBD group compared to the healthy controls (Figure 1-2A). However, there was no significant difference in serum PKM2 between healthy controls and CCA patients. No significant difference was observed in serum PKM2 of CCA patients comparing with pre-operative and post-operative status (n = 15 of each), as show in the Figure 1-2B.

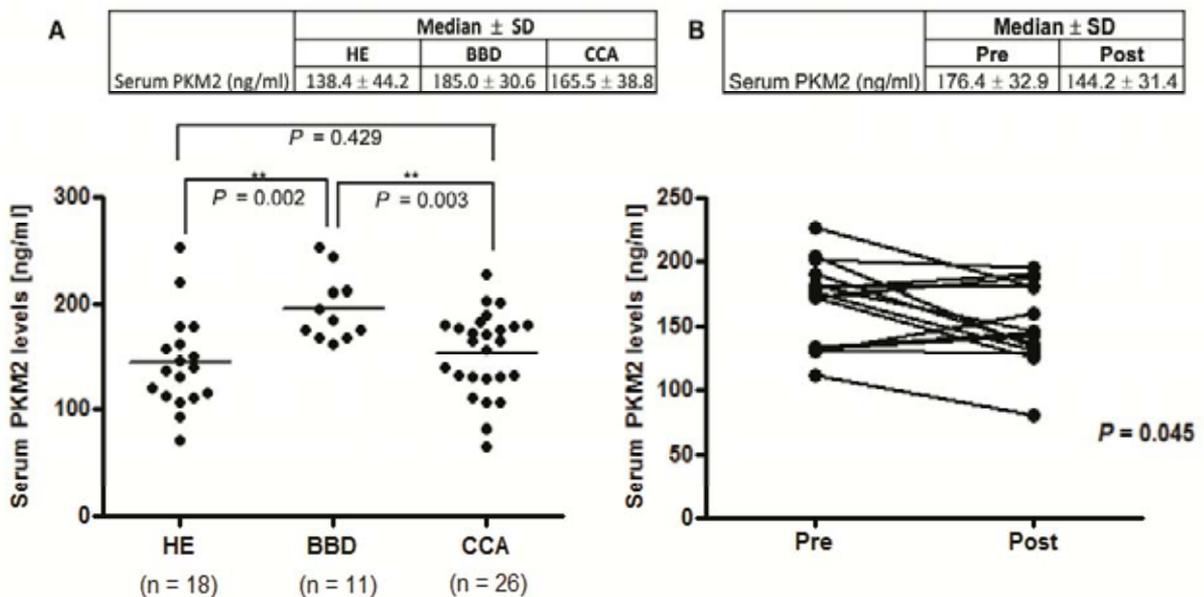


Figure 1-2 A. Serum PKM2 of healthy control, patients with benign biliary disease and patient with cancer are shown. B. comparison of PKM2 levels between pre- and post-operative conditions. HE = healthy control, BBD = benign biliary disease, Pre = pre-operative, Post = post-operative.

Discussion

Upregulation of PKM2 expression was confirmed in the human CCA tissues in this present study. In the majority of cases, the CCA areas had high expression of PKM2 compared to that of normal bile duct adjacent tissues. In agreement of previous studies that PKM2 is upregulated in several cancer tissues such as cancer of colon⁹, gastric¹⁰, breast¹¹, thyroid¹², and lung¹³. This indicated that overexpression of PKM2 is a common features in cancerous tissues. In the present study, we determined serum PKM2 levels

to mediate for diagnostic and prognostic marker of CCA patients using ELISA assay kit. The preliminary data suggested that there was a trend of increasing PKM2 level in CCA patients compared to healthy controls. This also observed in serum of patients with post-operative comparing with pre-operative state, that PKM2 level was decreased in post-operative group. A greater sample size needed to be investigated since the different did not reach the statistical significant.

Excreted PKM2 can be detected in different body fluids such as pleural fluid, plasma,

serum, and stool. Several diagnostic studies have evaluated the clinical efficacy of PKM2 as a biomarker for diagnosis such as gastric ¹⁴, cervical ¹⁵, and skin cancer ¹⁶ as well as biliary tract cancer ¹⁷. Moreover, results from previous study indicated that plasma and bile PKM2 had correlated with tumor aggressiveness and poor prognosis¹⁷. Importantly, serum PKM2 concentration has been identified as a diagnostic and prognostic biomarker with equivalent of serum CA19-9 in cholangiocarcinoma ¹⁸. Therefore, PKM2 levels in plasma or serum is still challenged.

Conclusion

This study indicated that PKM2 expression was increased with the carcinogenesis of Ov-associated CCA. PKM2 was highly expressed in CCA area but not in normal bile duct. Therefore, PKM2 could be a potential therapeutic target for CCA treatment. Serum PKM2 levels may use for diagnostic and prognostic markers of CCA.

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