ฤทธิ์ต้านมะเร็งของตัวยับยั้ง glucose transporter 1, phloretin, ในเซลล์มะเร็งท่อน้ำดี

Anti-cancer activity of glucose transporter 1 inhibitor, phloretin, in cholangiocarcinoma cells

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บทคัดย่อ

การแสดงออกที่เพิ่มขึ้นของ glucose transporter 1 (GLUT1) ถูกรายงานในมะเร็งหลายชนิด โดย GLUT1 มีบทบาทสำคัญในการกระบวนการเพิ่มจำนวน, การแพร่ลุกลามและการตายของเซลล์มะเร็ง ตัวอย่างเช่น มะเร็งตับ มะเร็งลำไส้และมะเร็งเต้านม เป็นต้นอย่างไรก็ตามในปัจจุบันยังไม่มีข้อมูลที่บ่งชี้ถึงการแสดงออกและบทบาทของ GLUT1 ในมะเร็งท่อน้ำดีที่สัมพันธ์กับการติดเชื้อพยาธิใบไม้ตับ การศึกษานี้เป็นการศึกษาการแสดงออกของ GLUT1 และฤทธิ์ต้านมะเร็งของตัวยับยั้ง GLUT1 ที่มีชื่อว่า phloretinในเซลล์มะเร็งท่อน้ำดีที่สัมพันธ์กับการติดเชื้อพยาธิใบไม้ตับ การวิเคราะห์การแสดงออกของ GLUT โดย serial analysis of gene expression พบว่ามีการแสดงออกของ GLUT1 เพิ่มขึ้นในผู้ป่วยมะเร็งท่อน้ำดีเมื่อเทียบกับเนื้อเยื่อตับปกติและยังพบว่า GLUT1 มีการแสดงออกสูงในเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีชนิดKKU-213L5 และ KKU-214L5 ทั้งในระดับ mRNA และระดับโปรตีน นอกจากนี้ยังพบว่าphloretin สามารถยับยั้งการเจริญเติบโตของเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีได้อย่างมีนัยสำคัญทางสถิติ การศึกษานี้แสดงให้เห็นถึงความสำคัญของ GLUT1ต่อการเจริญของเซลล์มะเร็งท่อน้ำดี และความเป็นไปได้ในการใช้ GLUT1 เป็นเป้าหมายสำหรับการรักษาผู้ป่วยมะเร็งท่อน้ำดี

คำสำคัญ: glucose transporter1 มะเร็งท่อน้ำดี phloretin

Abstract

Overexpression of glucose transporter 1 (GLUT1) has been reportedin various cancers. GLUT1plays significant roles in many cellular processes including proliferation, metastasis, and apoptosis of several cancers such as cancer of liver, colon and breast. However, the expression and roles of GLUT1 in *Opisthorchisviverrini* (*Ov*)-associated cholangiocarcinoma (CCA) is still unknown.The purpose of this study was to investigate GLUT1expression and anti-cancer activity of glucose transporter 1 inhibitor, namelyphloretin,in *Ov*-associated CCA. The expression of GLUTs from serial analysis of gene expression demonstrated thatGLUT1 was overexpressed in CCA tissues. Similar results were also observed in CCA cell lines using real time-PCR and western blotting techniques. In addition, the inhibition of GLUT1 activity by phloretin significantly inhibited growth of CCA cell lines in adose-dependent fashion. These results suggest the possibility of GLUT1 as a therapeutic target for CCA patients.

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Introduction

Cholangiocarcinoma (CCA) is a bile duct cancer that found both intra- and extra- hepatic biliary epithelia.[1] The incidence of CCA has been increased and the highest incidence of 96 cases per 100,000 men was reported in Thailand.[2] The prognosis of CCA patients is generally poor, because the lacking of early detection and effective therapeutic treatments.Therefore, it is an urgent need for searching a novel treatment that may target on the aberrantly expressed genes in the carcinogenesis and/or progression of CCA.

At present, the metabolism of glucose in cancer cells is now gaining attention as a potentialtarget for cancer therapy.[3] In 1924, Otto Warburg described glucose metabolism in cancer cells called Warburg effect or aerobic glycolysis[4]which different from normal cells.Cancer cells aberrantly expressed some specific proteins involve in glucose metabolism such as glucose transporter 1 (GLUT1), and hexokinase 2 (HKII).[5]Glucose transporter 1 is a transmembrane protein used for uptakinghexose. Overexpression of GLUT1 is reported in several cancers such as ovarian,[6] liver,[7]and colon.[8]High levels of GLUT1 were associated with poor outcomes of non-*Opisthorchisviverrini*(*Ov*) associated CCApatients, such as tumor size, metastasis, and short survival rates.[9]Moreover, the inhibition of GLUT1using its inhibitor, namely phloretin, effectively suppressed the proliferation and metastasis of liver cancer cells in *in-vitro* and *in vivo*.[10]However, theinformation on GLUT1 in *Ov*-associated CCA is limited.

The aim of the present study is to investigate the expression of GLUT1 in *Ov*associated CCA cell lines. In addition, the efficacies of phloretin in growth of CCA cell lines were investigated.

Materials and Methods

Serial analysis of gene expression (SAGE).The SAGE Differential Gene Expression Display (DGED) tool was used to compare SAGE data between the pools of CCA samples and normal human liver. The Data were provided on the CGAP website (http://cgap.nci.nih.gov) and results were reported only when odds ratio (fold change) was significantly greater than 2 or significantly lesser than 0.5. The odds ratio was calculated by the belowing formula.

(Tag count sum/ total tag count of CCA libraries)/ (Tag count/ total tag count of normal liver library)[11]

Cell culture.Human CCA cell lines, KKU-213L5 and KKU-214L5 were used. These 2 cell lines were established from theparental KKU-213 and KKU-214 by Professor Sopit Wongkham, Khon Kaen University and Professor Seiji Okada, Kumamoto University, Japan.Both CCA cell lines were cultured in HAM-F12 (Gibco/BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin (Life Technologies, Inc.) and incubated at 37°C with 5% CO_2.

Cell viability assay. Cell number was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Invitrogen, Carlsbad, CA) as previously described.[12]CCA cellsof 1.5 x 10^3 cells/well were cultured in a 96-well plate (Corning, Lowell, MA) for 18 h, then treated with 50-250 μM of phloretin(Sigma-Aldrich, St. Louis, MO) for 72 h. Cells treated with vehicle (0.125% DMSO) were used as control.

Realtime-PCR. The expression ofGLUTs and the internal control gene, beta actin,wereperformed using the LightCycle 480® real-time PCR system (Roche Diagnostic). Each PCR reaction was 1X LightCycle 480® SYBR green I master mix, and 50 ng of cDNA. The amplification was initiated by pre-incubation step with one cycle at 95 °C for 4 min followed by the amplification step with 40 cycles of 95°C 10 sec, 60°C 10 sec, and 72°C 10 sec. The accumulated PCR products were detected by monitoring the increasing of fluorescent intensity of the reporter dye from dsDNA binding with SYBR green. Each sample wasanalyzedin duplicates.

SDS-PAGE and Western blot. Whole cell lysate was obtained using NP-40 lysis buffer and protein concentration was determined according to Lowry method.[13]Protein (30 µg/well) was subjected to a 12% SDS-polyacrylamide gel electrophoresis and transferred to a Hybond™-P PVDF membrane (GE Healthcare, Buckinghamshire, UK) by wet electro-transferred at 10 Voltage, for 2 h using Bolt&Marhoney buffer.[14] After blocking with 3% BSA in 0.3% Tween-20 in TBS, the blot was incubated with 1:500 of anti-GLUT1 (SPM498) (Abcam, Bridge, MA) at 4ºC for overnight following by HRP-conjugated secondary antibody (GE Healthcare) for 1 h at room temperature. Signal was developed with ECL Prime Western Blotting Detection System (GE healthcare). The images of ECL signals were taken with ImageQuant 400 image analyzer and analyzed using ImageQuant™ TL analysis software (GE healthcare).

Statistical analysis. The results are representative data from three separated experiments. Data are presented as the mean ± standard deviation (SD) of all three experiments. Differences between groups were compared using one-way ANOVA and *t-test*; *P* values < 0.05 were considered significant. All analysis were performed using SPSS 17.0 software (SPSS, Chicago, IL).

Results

GLUT1 is overexpressed in CCA tissues.

The SAGE data indicated that GLUT1 was overexpressed in many cancers such as stomach, pancreas, and CCA tissues (Figure 1A). GLUT1 was observed 16 tags per 200,000 tags in metastatic nodules and 8 tags per 200,000 tagsin primary tumor while normal bile duct was detect 3 tags per 200,000tags. However, GLUT2 and GLUT4 were not detected in CCA tissues. GLUT3 was also detected in CCA tissues with 3-4 tags per 200,000tags in theprimary tumor and metastatic nodules. Howerver, GLUT3 was not detected in thenormal bile duct (Figure 1B).

GLUT1 is overexpressed in CCA cell lines.

In the current study, human CCA cell lines, KKU-213L5 and KKU-214L5, were selected to assess the roles of GLUT1 in CCA cells. The expression levels of GLUTs in CCA cell lines were determined using real time-PCR and western blot. Both CCA cell lines expressed GLUT1 with different basal levels. GLUT1 was expressed higher in KKU-213L5than in KKU-214L5 at mRNA level (Figure 2A). However, there was no significant difference in protein levels of GLUT1 between these two cell lines(Figure 2B).

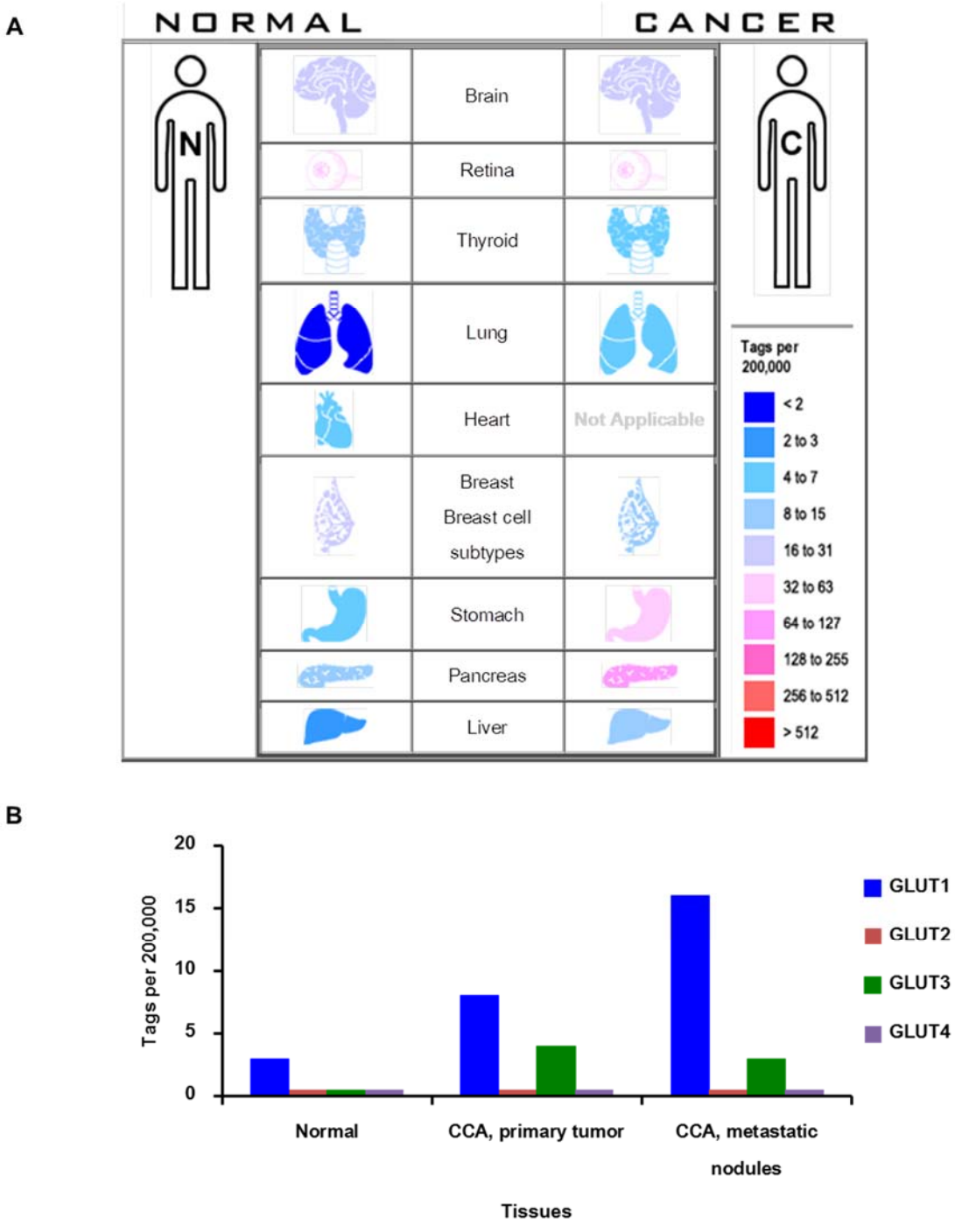


Figure 1 Expression levels ofGLUTs in CCA and normal liver tissues.(A)The comparison of GLUT1 expression from SAGE in normal and cancertissues. (B) GLUTs expression in normal liver, CCA primary and secondary tumors.

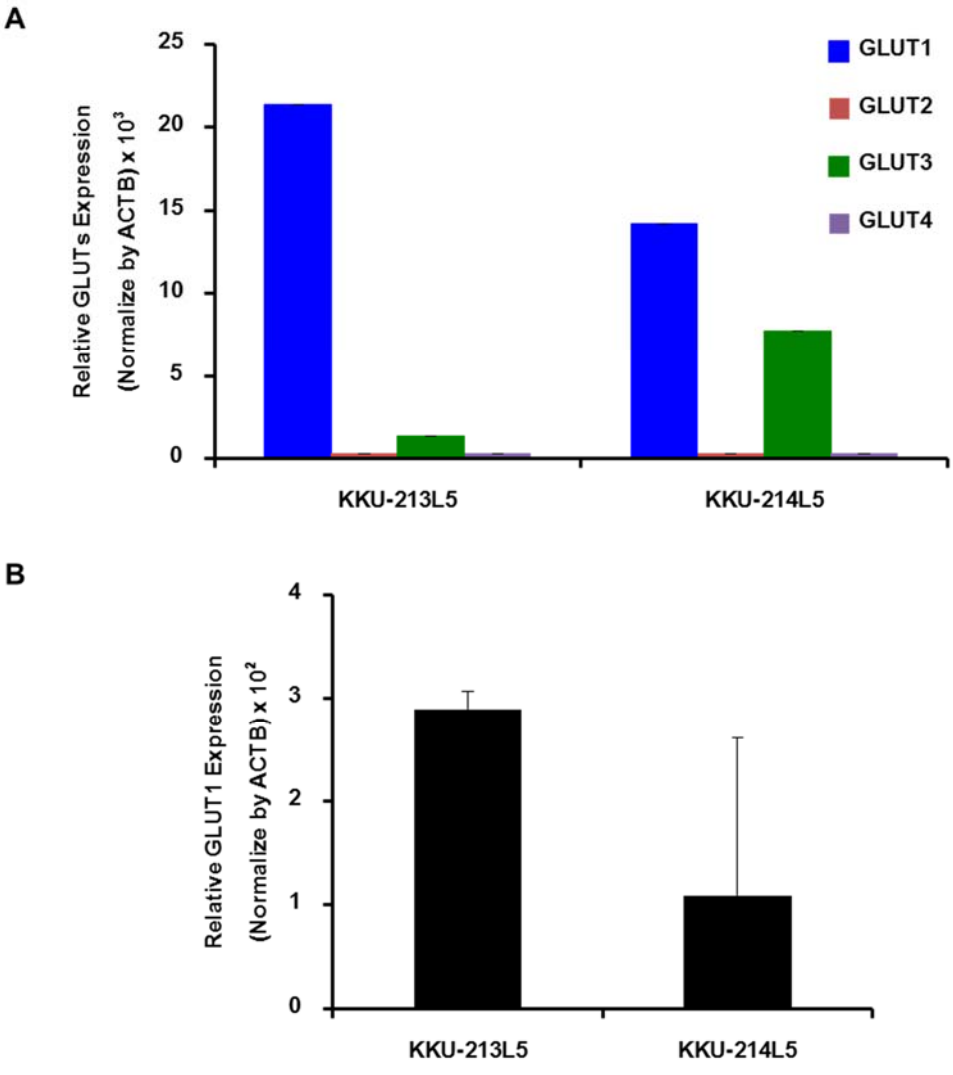
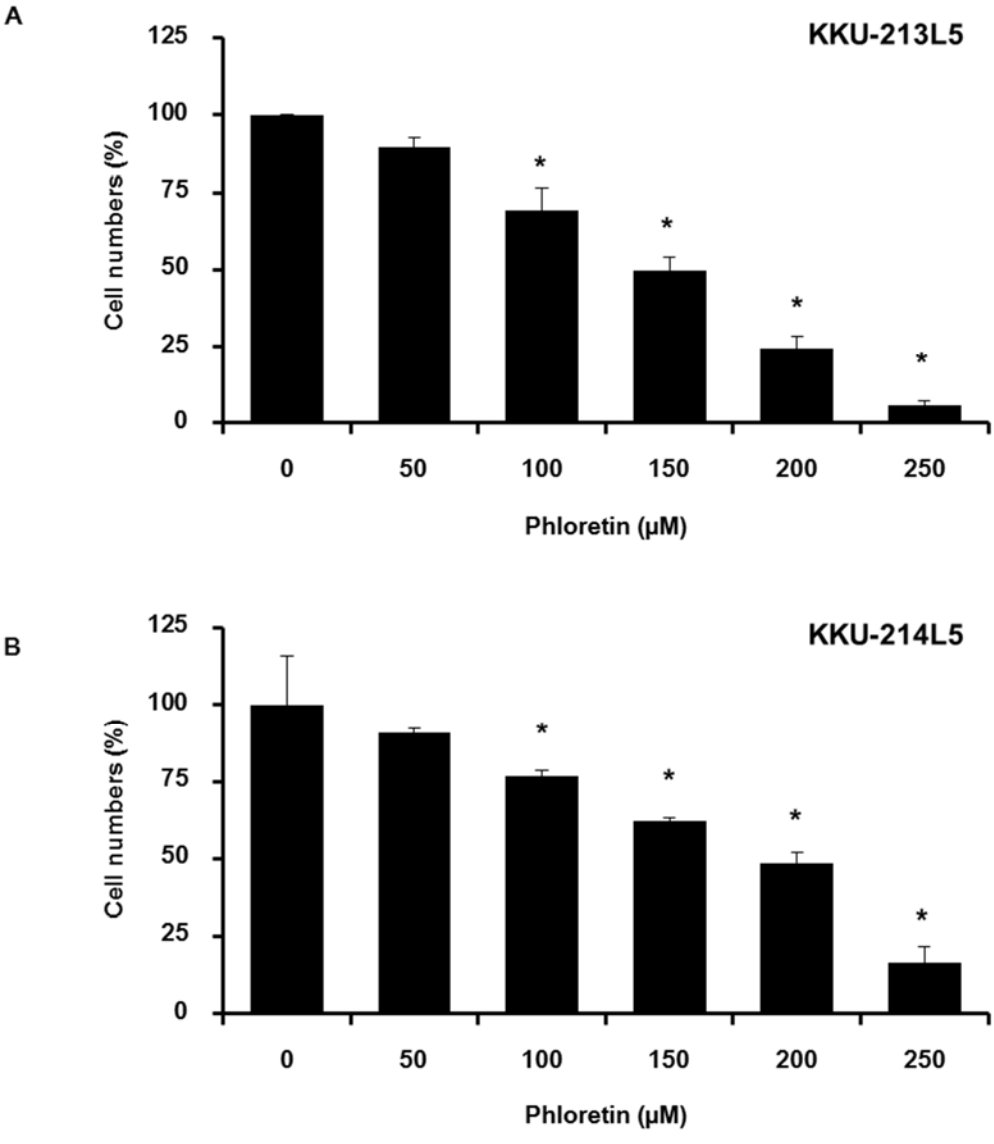


Figure 2 Expression of GLUT1 in CCA cell lines. The expression levels of GLUT1 at(A)mRNAand(B) proteinlevels in KKU-213L5 and KKU-214L5 cell lines.

GLUT1 inhibitor, phloretin, inhibits growth of CCA cell lines.

As GLUT1 was overexpressed in both CCA tissues and cell lines, we hypothesized that GLUT1 may serve as a target for therapy of CCA. We,therefore, analyzed the efficacy of GLUT1 inhibitor, phloretin,on growth of CCA cells. KKU-213L5 and KKU-214L5 were treated with various concentrations (50-250 μM) of phloretin for 3 days, and numbers of viable cells were determined by MTT assay. Phloretin inhibited growth of both CCA cell lines as a dose-dependent manner compared to the vehicle controls (Figure. 3A, B). Based on the IC_{50} of phloretin, KKU-213L5 was more sensitive to phloretinthan KKU-214L5.



C IC_{50} of phloretin

Cell lines	Phloretin (μM ± SD)
KKU-213L5	117.4 ± 0.5
KKU-214L5	210.5 ± 3.6

Figure 3 Effect of phloretin on cell growth of CCA cell lines.Cell numbers (%) was determined using the MTT assay in (A) KKU-213L5 and(B) KKU-214L5 with various concentrationd ofphloretin for 72 h. Cells without phloretin was used as control. (C)The IC_{50} was calculated.

Discussion

Tumor growth and survival are supported by the reprogramming of glucose metabolism known as Warburg effect. The association of high glycolytic rate in cancers and aggressive phenotypes has also been reported. Thereby, targeting tumor glycolysis is a novel strategy for diagnosis and selective anti-cancer therapies. In this study, GLUT1 was investigated for their possible involvement in CCA progression andthe target treatment of CCA.

Associations between GLUT1 expression and tumor aggressiveness have been shownin tumor tissues of pancreatic, pulmonary, colorectal, hepatocellular, ovarian and squamous cell carcinoma.[15-19] The overexpressions of GLUT1 in CCA tissues were previously reported with varied positive percentages, i.e.,81% in Korean CCA patients [20],46.3% in intrahepatic CCA[9], 58.3% in ampular of Vator[21] and 76.9% in extrahepatic CCA[22].

In this study, GLUT1 was overexpressed in primary tumor and metastatic nodules comparing with other GLUTs.This suggested that GLUT1 is the major glucose transporter in *Ov*-associated CCA. Therefore,GLUT1 may be a potential target for CCAtherapy.

Phloretin is a natural compound occuring in apple and apricot and was shown to inhibit glucose transporter. The antitumor activity of phloretin was reported in liver cancer both *in vitro* and *in vivo*. Phloretininduced apoptosis in HepG2 cell line and retarded tumor growth in HepG2 xenograft mice.[10] Here we tested the antitumor activity of phloretin in the highly metastatic CCA cells, KKU-213L5 and KKU-214L5.Phloretin significantly suppressed growth of CCA cell lines in adose-dependent manner. The average $IC_{50}$of phloretin for KKU-213L5 was 117.4 μM and KKU-214L5 was 210.5 μM. The anti-tumor efficacy of phloretin seems to vary based on cell types and the demand of GLUT1 expression.

GLUT1 may bea good and specific target for CCA treatment because it is overexpressed in CCA cells and the inhibition of GLUT1 using its inhibitor,phloretin, can suppress growth of high metastasis CCA cell lines. In conclusion, this study enhances the knowledge on the therapeutic targets for CCA in which targeting of GLUT1 maylimit the metastasis of CCA cells and hence extend the survival of CCA patients.

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