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# Overexpression of Pyruvate Carboxylase Is Correlated With Colorectal Cancer Progression and Supports Growth of Invasive Colon Cancer HT-29 Cell Line

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**Abstract.** *Background/Aim: Pyruvate carboxylase (PC) is a major anaplerotic enzyme for generating oxaloacetate for the TCA cycle and also a key enzyme in gluconeogenesis, de novo fatty acid and amino acid synthesis in normal cells. Recent studies have identified PC overexpression in different cancers, such as breast and lung. However, the involvement of PC in colorectal cancer (CRC) is unclear. Our purpose was to investigate the PC expression levels and its correlations with potentially relevant clinical-pathological parameters in CRC. Materials and Methods: PC expression levels in tissues from 60 Thai CRC patients were investigated by immunohistochemistry while a clonogenic assay was performed for determining cell growth of HT-29 cells with PC knockdown. Results: Our results showed for the first time that high PC expression levels were significantly correlated with late stage of the cancer, perineural invasion and lymph node metastasis. The overexpression of PC was also significantly associated with poor overall and disease-free survival times of CRC patients. In addition, suppression of cancer cell growth was found in PC-deficient cell lines using CRISPR-Cas9. Conclusion: The overexpression levels of PC were correlated with CRC progression and survival times. Therefore, PC might serve as a potential clinical prognostic marker for colorectal cancer.*

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Colorectal cancer (CRC) is the third most common cancer which affects people worldwide (1). CRC is caused by the transformation of normal epithelial cells lining in the colon or rectum to cancerous cells (1). This cancer often presents no sign or symptoms in the early stage, but becomes more aggressive as the disease progresses. Like other cancers, CRC can metastasize to distal organs including liver, lung and others, causing death (2). Genome instability, mutations of oncogenes and alteration of growth factor signaling are important underlying mechanisms of both hereditary and sporadic CRC (3). In response to mitogenic signaling, cancer cells reprogram their metabolism to meet high bioenergetic and anabolic demand during rapid proliferation. Altered cellular metabolism including increased glucose uptake, aerobic glycolysis and biosynthetic pathways of nucleotides, lipids and amino acids are important metabolic hallmarks of many cancers, including CRC (4, 5).

During high anabolic demand, the tricarboxylic acid (TCA) cycle plays an important role in biosynthetic pathways because its intermediates such as citrate, α-ketoglutarate and malate are used as biosynthetic precursors for lipids, nucleotides and amino acids. To maintain TCA cycle activity, the levels of its intermediates require the replenishment *via* glutaminolysis or pyruvate carboxylation (6). Glutaminolysis converts glutamine to α-ketoglutarate *via* glutamate and is catalyzed by glutaminase. Pyruvate carboxylation converts pyruvate to oxaloacetate by pyruvate carboxylase (PC), a member of the biotin-dependent carboxylase family. Depending on the genetic background and stress conditions, most cancers rely on either of these two anaplerotic reactions to maintain the levels of TCA cycle intermediates. PC has been reported to be overexpressed in several cancers including low grade glioma (7), non-small cell lung cancer (8), thyroid (9), paraganglioma and renal clear cell carcinoma (10, 11) as well as pancreatic

ductal adenocarcinoma (12) where it supports the growth of primary tumor and/or metastasis. In breast cancer patients, overexpression of PC is associated with tumor size, advanced stage (13) and poor survival (14). Inhibition of PC expression or its activity in different models of cancer reduces growth and/or metastasis, indicating that PC is an attractive anti-tumor target (8, 12-16).

Here, we show for the first time that PC is overexpressed in cancerous colon tissue of CRC patients, and its expression level is associated with several clinicopathological parameters, such as advanced stage, lymph node and perineural invasion and poor prognosis. Knocking out the PC gene in the invasive colon cancer cell line HT-29 also inhibits clonogenic growth, suggesting an important role for PC in supporting tumor growth in CRC patients.

## Materials and Methods

*Tissue specimens*. Formalin-fixed paraffin-embedded blocks of CRC tissues of 60 Thai patients comprising of 25 females and 35 males, with an average age of 63.45±12.07 years were collected and prepared as approved by the Siriraj Institution Review Board (Si.677/2018). Of these patients, 37 were diagnosed with colon cancer and 23 were diagnosed with rectal cancers. Histological examination of CRC identified patients into four stages (I-IV) based on the tumor-node-metastasis staging system (17). All clinical investigations were conducted according to the declaration of Helsinki and good clinical practice. Written informed consent was obtained from each patient. The clinical data of patients and clinical follow-up data were also included.

*Cell culture*. The human CRC cell line HT-29 (ATCC HTB-38™) was kindly provided by Dr. Arthit Chairongdua from the Department of Physiology, Faculty of Science, Mahidol University, Thailand. HT-29 cells were cultured in Dulbecco's modified Eagle medium with F12 (1:1) (DMEM/F12) (Gibco, Carlsbad, CA, USA), supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco) and 100 units/ml penicillin, 100 μg/ml streptomycin (Gibco). Cells were grown at 37˚C in a humidified incubator with 5% $CO_2$.

*Generation of PC knockout colorectal cancer cell lines by CRISPR Cas9*. Two guide RNAs (gRNA) targeted to the human PC gene (GeneBank accession No; U30891.1) were designed using the SYNTHEGO CRISPR Design program – (http://www.synthego.com). The gRNA sequences were generated by cloning double oligonucleotide cassettes corresponding to nucleotides 243-262 of the human PC coding region. The double stranded oligonucleotides corresponding to these gRNAs were ligated into pSpCas9(BB)-2AGFP (Addgene, Watertown, MA, USA) and transformed into DH5α *Escherichia coli* cells. These two gRNA constructs were sequenced and transfected to HT-29 cells. PC knockout HT29 clones were selected as described previously (18).

*Western blotting*. $1\times10^6$ cells of PC KO or scrambled control HT-29 cell lines were plated into 35-mm dishes containing DMEM/F12. The cells were cultured at 37˚C with 5% $CO_2$ until they reached 80-90% confluency. Approximately $2\times10^6$ cells were suspended in 50 μl of radioimmuno-precipitation assay buffer (RIPA), containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, 1% (v/v) NP-40, 1 mM DTT and 1× protease inhibitor cocktail (Roche, Basel, Switzerland). Protein concentrations were determined by Bradford assay. 40 μg of total protein samples were separated by SDS-polyacrylamide gel electrophoresis and Western blotting. The blot was incubated with 1:5,000 dilution of rabbit anti-yeast PC polyclonal antibody (19) or 1:40,000 dilution of mouse anti-actin monoclonal antibody (Sigma-Aldrich, St Louis, MO, USA). The blots were washed and incubated with 1:5,000 dilution of goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (DAKO, Santa Clara, CA, USA) or 1:10,000 dilution of sheep anti-mouse antibody conjugated with HRP (GE Healthcare, Chicago, IL, USA). The chemiluminescence bands were detected using chemiluminescence HRP detection reagent (Millipore, Burlington, MA, USA), and images were captured using an enhanced chemiluminescence imaging system (Syngene, Frederick, MA, USA).

*Clonogenic assay*. Clonogenic assays were performed by plating 500 cells of scrambled control or PC KO HT-29 clones in 35 $mm^2$ culture dishes containing minimal essential medium supplemented with 10% fetal bovine serum (Gibco) and 100 units/ml penicillin, 100 μg/ml streptomycin (Gibco), respectively and cultured at 37˚C for 10 days. Colonies were fixed with 4% (v/v) paraformaldehyde (Sigma-Aldrich) before being stained with 0.5% (w/v) crystal violet (Sigma-Aldrich) and counted.

*Immunohistochemistry*. The paraffin in tissue sections was removed with xylene, before rehydrating in absolute ethanol and 95% (v/v) ethanol. Antigens were retrieved by incubating slides with 10 mM citrate buffer, pH 6.0, at 95˚C for 1 h and cooled down for 20 min before rinsing with distilled water. The endogenous peroxide was inactivated with 3% (v/v) hydrogen peroxide in methanol for 30 min before blocking with 3% (w/v) bovine serum albumin (Sigma-Aldrich) for 1 h. The tissue sections were incubated with 1:500 dilution of anti-PC polyclonal antibody (19) for 16 h, at 4˚C. Following rinsing with PBS, the tissue sections were incubated with anti-rabbit EnVision+system with HRP labelled polymer (K4003 DAKO, USA) at room temperature for 30 min. The secondary antibody was removed by soaking in 1xPBS for 10 min. The immune staining was detected by adding 3,3-diaminobenzidine (DAB, Sigma-Aldrich) solution onto each slide for 10 min at room temperature. The nucleus was counter-stained with Mayer's hematoxylin and 1% (w/v) lithium carbonate, respectively. All slides were rinsed with tap water for 5 min, followed by rehydrating with ethanol, acetone and cleared in xylene, respectively. The tissue sections were mounted with Permount™ (Thermo Fisher Scientific, Hampton, NH, USA) and observed under a microscope. Immunohistochemistry grading scores were evaluated as semi-quantitative scores based on the staining intensity and percentage of PC positive cells. An intensity score of 0 was assigned for negative or unstained cells; 1 for slightly; 2, for intermediate and 3 for strongest staining. The percentage of PC positive cells was graded as follows: 0, negative; 1, 1-25%; 2, 26-50%; 3, 51-75% and 4, 76-100%. Intensity was then multiplied with the percentage of PC positive cells to obtain the total score ranging from 0 to 12. An IHC score equal to 6 was used as the cut off value to classify the expression level as low (≤6) or high (>6).

*Analysis of PC transcription levels in CRC samples from TCGA*. Publicly available transcript levels of the HLCS and PC genes in

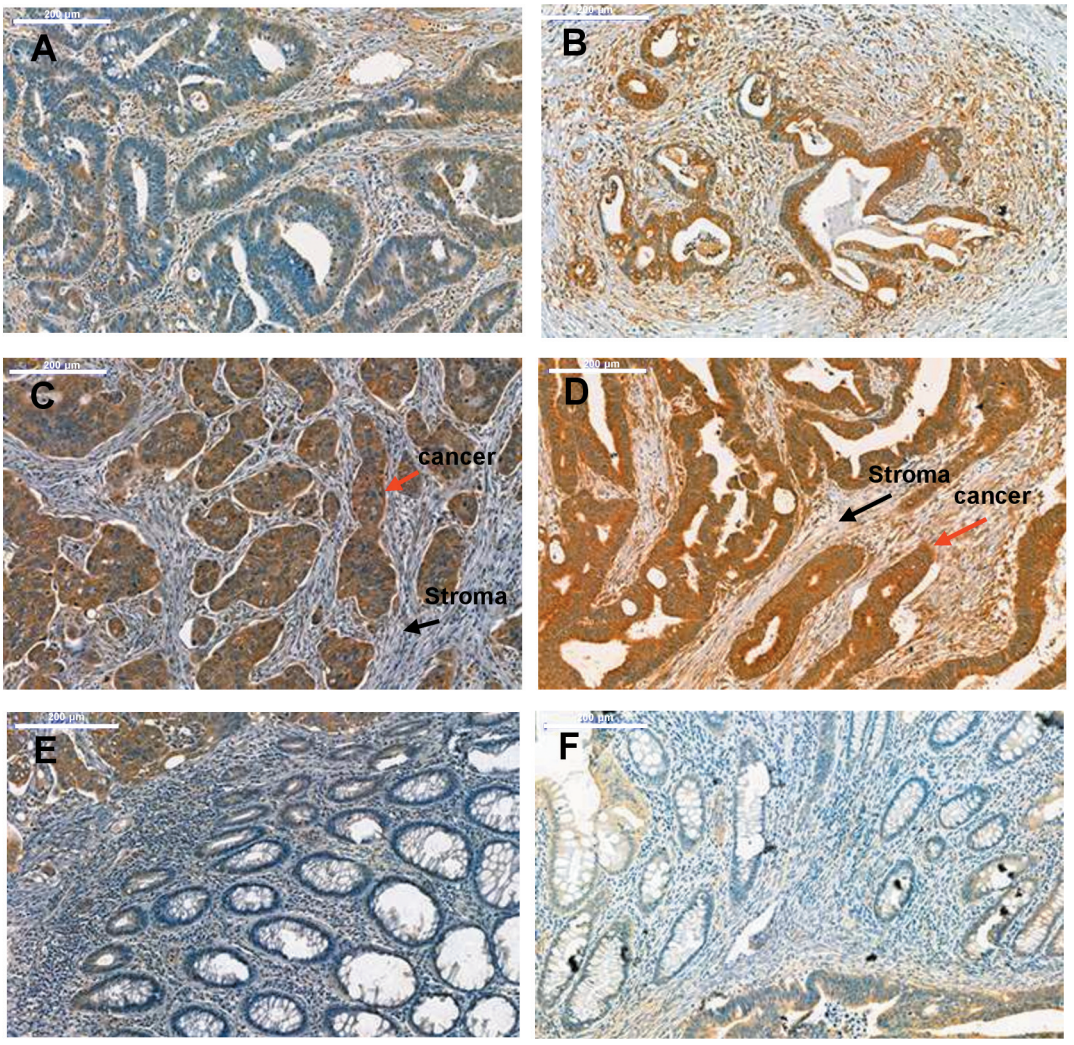


Figure 1. *Expression of PC in paraffin-embedded tissues of CRC patients with stage I (A), II (B), III (C) and IV (D) by IHC staining. (E) and (F) indicate the non-cancerous area adjacent to the cancerous area in CRC stage III and IV, respectively. Original magnification 20×. Scale bar; 200 μm. Red arrows indicate cancer and black arrows indicate stromal area within tissues with CRC stages III and IV.*

colon cancers obtained by the Cancer Genome Atlas (TCGA) program were retrieved through National Cancer Institute's Genomic Data Commons Data Portal (20). The samples were filtered for "colon" as a primary site, and then "adenomas and adenocarcinomas" as a disease type. As a result, "HT-seq FPKM" values were retrieved from 539 primary tumor samples and 47 solid tissue normal samples. Statistical comparisons between the primary tumor and normal samples were performed by Wilcoxon test using R (3.6.0) (21), and visualized by the ggplot2 (3.3.0) package (22).

*Statistical analysis*. The correlation between PC expression levels and clinicopathological factors of CRC patients was investigated using logistic regression analysis. The overall and metastasis-free survival rates were calculated using Kaplan-Meier Methods and compared with the Log-rank test. The prognostic analysis was performed using univariate and multivariate COX regression analysis to determine the clinicopathological variable with the patients' survival times and was also used to estimate hazard ratios (HR). The 95% confidence intervals (CIs) were used to quantify correlation between PC expression level and clinicopathological factors ($p<0.05$ indicating statistical significance). All statistical analyses were performed using SPSS 23.0 (SPSS Inc, Chicago, IL, USA).

## Results

*Expression of PC in colorectal cancer tissues*. The levels of PC protein expression in paraffin-embedded colorectal tissue sections from 60 CRC patients with different stages (I-IV) were determined by immunohistochemistry. PC expression was detected in all patients, and the staining was mainly localized in the cytoplasm as shown in Figure 1. The

Table I. *Correlation between PC expression levels and clinico-pathological parameters in CRC patients (n=60).*

| Variable | Low PC expression n (%) | High PC expression n (%) | Univariate | Multivariate |
|---|---|---|---|---|
| | | | *p*-Value | *p*-Value |
| Gender | | | | |
| Female | 13 (50.0) | 12 (35.3) | | |
| Male | 13 (50.0) | 22 (64.7) | 0.254 | 0.31 |
| Age group (years) | | | | |
| ≤60 | 6 (23.1) | 17 (50.0) | | |
| >60 | 20 (76.9) | 17 (50.0) | 0.037* | 0.044* |
| Cancer site | | | | |
| Rectum; NOS | 9 (34.6) | 14 (41.2) | | |
| Colon; ascending | 2 (7.7) | 1(2.9) | 0.382 | 0.957 |
| Colon; hepatic flexure | 2 (7.7) | 1(2.9) | 0.382 | 0.619 |
| Colon; sigmoid | 11 (42.3) | 17(50.0) | 0.991 | 0.297 |
| Colon; transverse | 2 (7.7) | 1(2.9) | 0.382 | 0.56 |
| Staging | | | | |
| Stage I-II | 20 (76.9) | 10 (29.4) | | |
| Stage III-IV | 6 (23.1) | 24 (70.6) | 0.001* | 0.002* |
| Histological type | | | | |
| Well-differentiate | 4 (15.4) | 2 (5.9) | | |
| Moderated differentiate | 22 (84.6) | 32 (94.1) | 0.24 | 0.28 |
| Tumor size (cm) | | | | |
| ≤2 | 1 (3.8) | 2 (5.9) | | |
| >2 | 25 (96.2) | 32 (94.1) | 0.722 | 0.2 |
| Perineural invasion | | | | |
| Absence | 25 (96.2) | 17 (51.5) | | |
| Presence | 1 (3.8) | 16 (48.5) | 0.003* | 0.004* |
| Lymphovascular invasion | | | | |
| Absence | 19 (76.0) | 23 (69.7) | | |
| Presence | 6 (24.0) | 10 (30.3) | 0.595 | 0.482 |
| Lymph node invasion | | | | |
| Absence | 23 (88.5) | 11 (32.4) | | |
| Presence | 3 (11.5) | 23 (67.6) | <0.001* | 0.001* |
| Metastasis | | | | |
| Absence | 26 (100.0) | 19 (55.9) | | |
| Presence | 0 (0.0) | 15 (44.1) | 0.998 | - |

*Statistical significance, $p \leq 0.05$.

expression levels of PC were varied in different CRC samples in which 43% (26/60) possessed low PC expression levels and 57% (34/60) possessed a high level of PC expression. Interestingly, 29.4% of those having low PC expression were in stages I and II while 70.6% of those having a high PC level were in stages III and IV. Figure 1A, B shows representative IHC images of CRC tissue of stages I and II patients that possessed low PC expression, respectively, while Figure 1C, D represents the CRC tissue of stages III and IV patients, respectively. While the IHC staining for PC was mainly localized in the cancerous area, weak staining was observed in the stroma cells of stage IV (Figure 1C). The glandular cells in the normal areas adjacent to the cancerous areas of stages III and IV showed no PC expression (Figure 1E, F).

*Correlation between PC expression levels, clinicopathological parameters and survival time*. Logistic regression analysis was used to assess the association between PC level and clinicopathological parameters. As shown in Table I, PC expression levels were significantly correlated with age ($p$=0.037), late stages (3 and 4) ($p$=0.001), perineural invasion ($p$=0.003) and lymph node metastasis ($p$<0.001). Other parameters including histological type, gender, diagnosed cancer site, tumor size, lymphovascular invasion and local metastasis (liver, lung or ovary) did not appear to correlate to the PC expression levels ($p$>0.05). In addition, the patients with higher PC expression level had poor overall survival, as shown in Figure 2A ($p$<0.003). Moreover, the metastasis-free survival rate of patients with high PC expression was also poorer than those with low PC expression ($p$<0.001; Figure

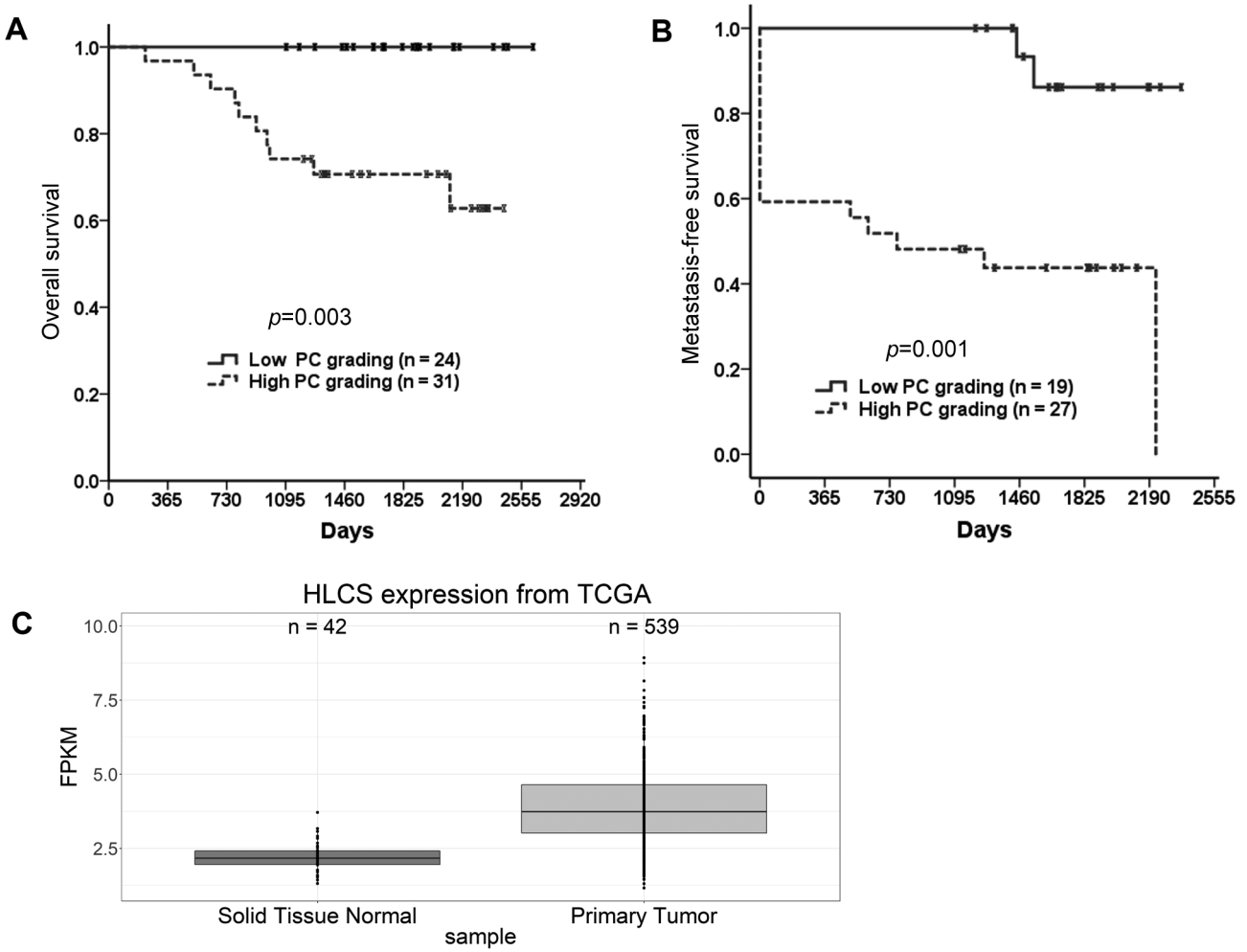


Figure 2. *Overall survival (A) and metastasis-free survival (B) times of colorectal cancer patients using Kaplan-Meier analysis, categorized as low and high PC expression (n=60). The dots indicate three-year survival time for each patient. (C) Box plots of HLCS mRNA expression levels (as fragments per kilobase per million of transcript values from RNA-seq experiments) in normal and primary CRC tissues available from the Cancer Genome Atlas (TCGA) dataset, *p<0.0001 by Wilcoxon test.*

2B). Transcriptional analysis of PC expression in an independent dataset, as available from the cancer genome atlas (TCGA), consisting of 539 CRC samples and 42 normal colon tissues, did not show significant association between the PC transcript level and any clinicopathological parameters, as observed with PC protein levels (data not shown), suggesting that the post-transcriptional regulation of the protein might contribute to regulation of PC in the patients with different clinicopathological characteristics. Interestingly, although there was no association between PC transcript levels and the aforementioned clinical parameters, a significant increase of the transcript encoding the holocarboxylase synthetase (HLCS), an enzyme that post-translationally regulates the PC activity (Figure 2C; $p<0.0001$) was observed in the CRC samples as compared to normal controls, based on the same TCGA dataset. This could suggest that the increased expression of HLCS mRNA may support the PC activity in cancerous tissues.

*Prognostic value of PC expression for clinical outcome in CRC patients*. Univariate and multivariate COX regression analysis of PC expression level and clinicopathological parameters with metastasis-free survival time for patients showed that higher PC expression level had an adverse prognostic impact on several parameters such as gender ($p=0.006$), cancer stage ($p=0.005$), lymph node invasion ($p=0.003$), lymphovascular invasion ($p=0.019$) and perineural invasion ($p<0.001$), as shown in Table II. These data indicate that PC can be used as a prognostic marker for disease aggressiveness.

Table II. *Univariate and multivariate COX regression analysis of PC expression level and clinicopathological parameters with metastasis-free survival time for colorectal cancer patients (n=60).*

| Variable | Univariate survival analysis | | | Multivariate survival analysis | | |
|---|---|---|---|---|---|---|
| | HR | 95% CI | *p*-Value | HR | 95%CI | *p*-Value |
| IHC grading (low, high) | 7.979 | 1.812-35.130 | 0.006* | 10.274 | 2.178-48.473 | 0.003* |
| Gender (Female, male) | 5.045 | 1.433-17.763 | 0.012* | 8.466 | 2.100-34.122 | 0.003* |
| Age (≤60, >60 years) | 1.19 | 0.460-3.074 | 0.72 | 2.242 | 0.770-6.529 | 0.139 |
| Cancer stage (I-II, III-IV) | 8.532 | 1.942-37.495 | 0.005* | 12.483 | 2.691-57.908 | 0.001* |
| Histological type (well, moderately) | 21.957 | 0.002-275148.95 | 0.521 | - | - | - |
| Lymph node invasion (Absence, Presence) | 6.871 | 1.966-24.016 | 0.003* | 7.236 | 1.951-26.840 | 0.003* |
| Lymphovascular invasion (Absence, Presence) | 3.283 | 1.214-8.880 | 0.019* | 4.145 | 1.447-11.873 | 0.008* |
| Perineural invasion (Absence, Presence) | 7.486 | 2.521-22.234 | <0.001* | 8.219 | 2.572-26.266 | <0.001* |

CI, confidence interval; *p*-Value, Pearson's $\chi^2$ test. *indicates statistical significance.

*PC knockout HT-29 colon cancer cells show impaired clonogenic growth*. To examine the functional role of PC in supporting growth of colon cancer, two PC KO colorectal cancer cell lines, HT-29 (PC KO HT-29) were generated from two independent guide RNAs, targeted to different exonic regions of the human PC gene. As shown in Figure 3A, both PC KO clones had PC mRNA levels equal to 5% and 31% of the WT cell line (Figure 3A) and barely detectable PC protein levels (Figure 3B). Both PC KO HT-29 clones showed 40-45% reduction of growth, as assessed by clonogenic assay. This result indicates that PC supports growth of HT-29 cells, in agreement with the association between PC and several clinical parameters in CRC patients.

## Discussion

PC is an anaplerotic enzyme that plays a role in replenishing oxaloacetate, one of the crucial TCA cycle intermediates (23). Recent studies have shown that PC protein is highly expressed in various types of cancer (7, 13, 24). However, the involvement of PC in CRC is unknown. Herein, we showed that PC expression was correlated with late stages of CRC, which is similar to that reported in breast cancer patients (13). The association between PC expression and perineural and lymph node invasion suggests that PC may support invasion during metastasis. Shinde *et al*. (2018) showed that primary breast cancer bearing a PC knockout gene failed to metastasize to murine lung tissue, demonstrating the essential role of PC for metastasis. Likewise, an association between high PC expression and low survival rate of CRC patients has been reported in human breast cancer (14). The impaired clonogenic growth of PC KO HT-29 colon cancer cell line lends further support for a role of PC as a pro-proliferative enzyme as in other types of cancers (7, 8, 13). Weak staining of PC observed in stromal cells surrounding cancerous tissues of stage III and IV patients suggests that PC could also be important in this cell type. Linares *et al*. (2016) showed that PC is essential to support asparagine biosynthesis in p62-deficient prostate cancer associated fibroblasts under glutamine-depleted growth conditions. CAFs-derived asparagine is in turn assimilated by prostate cancer cells to support their growth (25).

It is currently unknown what the molecular mechanism underlying overexpression of PC in CRC is. Accumulating evidence indicate that aberrant expression of some oncogenes such as c-Myc and K-ras can drive expression of key metabolic genes to support the invasive phenotype of many cancers (26, 27). Lao-on *et al*. (2020) have recently shown that overexpression of c-Myc is a key driver for overexpression of PC in highly invasive breast cancer cell lines (28). The same mechanism may also apply to CRC, where more than 66% of aggressive cancers have aberrant overexpression of c-Myc (29, 30). The positive association between several clinicopathological parameters with the level of PC protein but not PC mRNA may suggest a tight regulation of PC at the post-transcriptional level (31). Pinweha *et al*. (2019) have recently shown that PC mRNA expression is subject to posttranscriptional regulation by miRNA-143 in invasive breast cancer (32). It is possible that the same mode of regulation is present in CRC. Alternatively, different genetic backgrounds of the subjects in TCGA could also contribute to this result.

HLCS catalyzes posttranslational attachment of biotin to various biotin-dependent carboxylases, converting them into the catalytic form. The parallel increase of HLCS transcript levels in cancerous tissues over the normal tissue of CRC patients in the TCGA data set has also been reported in breast cancer patients (33), indicating a co-regulation between HLCS and PC expression. Similarly, the association between overexpression of PC and lymph node invasion has also been reported in other biotin enzymes including acetyl-CoA carboxylase 1 in melanoma patients (34) and methylcrotonyl-CoA carboxylase in breast cancer patients (35).

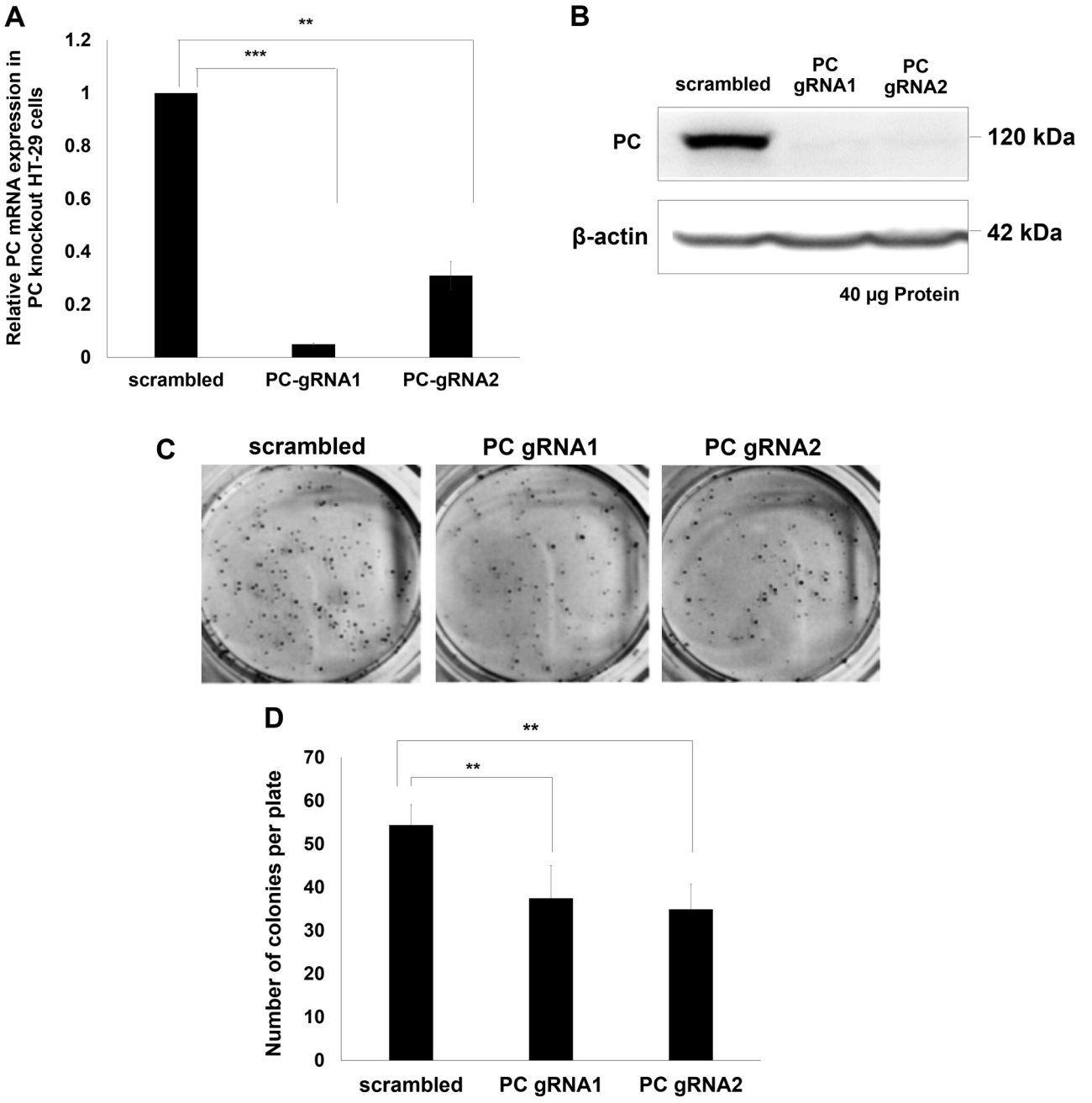


Figure 3. *(A) Relative expression of PC mRNA determined by quantitative real-time PCR and (B) Western blot analysis of PC protein expression in two PC KO (gRNA1 and gRNA2) and control cell lines. (C) Clonogenic growth of two PC KO HT-29 and control cell lines. (D) Average number of colony of PC KO HT-29 and control cell lines obtained from C. Results are shown as mean±standard deviation of three independent experiments. Statistical analysis was performed by one-way ANOVA. **p<0.01 and ***p<0.001.*

In conclusion, our results presented herein demonstrate that a high PC expression level is significantly associated with certain clinical parameters including stage, lymph node metastasis and perineural invasion in CRC. In this regard, PC could be used as an effective biomarker in CRC from Thai patients. However, the exact functional role of PC in cancer development in CRC is still unknown, but the potential biomarker could be used for monitoring the patients' further management in order to prolong survival time.

## Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

## Authors' Contributions

J.N. designed and performed experiments. J.N., C.T., P.T., P.K. and V.C. performed data analysis. C.T. provided tissue samples and clinicopathological data. J.N., C.T., V.C. and S.J. wrote the article. K.L. provided some reagents. C.T. and S.J. designed and supervised all experiments.

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