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# Investigation of human holocarboxylase synthetase function in MCF-7 breast cancer cell line by gene knockdown การศึกษาบทบาทของเอนไซม์โฮโลคาร์บอกซิเลสซินธีเทสในเซลล์มะเร็งเต้านมชนิด MCF-7 ด้วยวิชี gene knockdown

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# ABSTRACT

Holocarboxylase synthetase (HCS) is an enzyme that catalyzes the covalent attachment of biotin to the biotin-dependent carboxylases (BDCs). All of which are crucial for intermediary metabolism in human. Several studies clearly showed that certain BDCs are overexpressed to support growth and survival of many cancers. We purpose that disruption of BDCs simultaneously through suppressing HCS expression would impair breast cancer growth using MCF-7 cell line as a model. The short hairpin RNA constructs (shRNA) targeted to various regions of human HCS have been generated and stably transfected to MCF-7 cell line. HCS knockdown MCF-7 cells showed marked reduction of HCS expression accompanied by the reduction of biotinylation of BCDs. Furthermore, HCS knockdown MCF-7 also showed marked reduction of cell growth, indicating that HCS is crucial for breast cancer cells to support their growth through the maintenance of BDCs activity.

# บทคัดย่อ

เอนไซม์โฮโลการ์บอกซิเลสซินซีเทส (HCS) เป็นเอนไซม์ที่เร่งปฏิกิริยาการเติมหมู่ใบโอตินให้เอนไซม์ใบ โอตินการ์บอกซิเลส (BCDs) ซึ่งเป็นเอนไซม์ที่สำคัญต่องบวนการเมตาบอลิซึมพื้นฐานในเซลล์ มีการรายงานว่าระดับ การแสดงออกงองเอนไซม์กลุ่ม BCDs บางตัวมีระดับสูงขึ้นเพื่อเกื้อหนุนต่อการมีชีวิตรอดในเซลล์มะเร็งหลายชนิด จึง นำไปสู่สมมติฐานกือ การยับยั้งการทำงานของเอนไซม์กลุ่ม BDCs ผ่านการยับยั้งการแสดงออกงองเอนไซม์ HCS สามารถยับยั้งการเพิ่มจำนวนของเซลล์มะเร็งเต้านมได้ คณะผู้วิจัยจึงทำการสร้างเซลล์มะเร็งเต้านมชนิด MCF-7 ที่ถูก ยับยั้งเอนไซม์ HCS อย่างถาวรโดยใช้ shRNA จากการวิจัยพบว่าการยับยั้งการแสดงออกงองเอนไซม์ HCS ส่งผลให้ ระดับไบโอตินในเอนไซม์กลุ่ม BDCs ลดลงและสอดกล้องกับการเพิ่มจำนวนของเซลล์ที่ลดลง จากผลข้างต้นนี้ สามารถสรุปได้ว่าเอนไซม์ HCS อาจสำคัญต่อการเพิ่มจำนวนและการมีชีวิตรอดของเซลล์มะเร็งเต้านมผ่านการทำงาน ของเอนไซม์กลุ่ม BDCs

# Keywords: HCS, BDCs, Breast Cancer คำสำคัญ: โฮโลการ์บอกซิเลสซินธีเทส ใบโอตินการ์บอกซิเลส มะเร็งเต้านม

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## Introduction

Breast cancer is the most prevalent cancer in women with estimation of one million cases diagnosed every year and remains one of the cancer types that cause a high mortality rate worldwide including Thailand (Welch et al., 2015). These malignancies are resulted from uncontrolled cellular proliferation. Similar to other cancers, breast cancer requires the synthesis of macromolecules such as nucleic acids, amino acids and lipids to maintain structural integrity of the new cells. To meet these high anabolic demands, breast cancer cells have to reprogram their cellular metabolism in order to provide the sources of those biosynthetic precursors (Schulze, Harris, 2012). These biochemical adaptations make breast cancer cells become metabolically distinct from normal cells. Hence inhibition of metabolic pathways specifically active in cancer cells as novel anti-cancer therapeutic strategy.

Biotin-dependent carboxylases (BDCs) are important metabolic enzymes that require biotin as a cofactor to perform catalysis. Mammals possess five BDCs namely, acetyl-CoA carboxylase 1 (ACC1), acetyl-CoA carboxylase 2 (ACC2), methylcrotonyl-CoA carboxylase (MCC), propionyl-CoA carboxylase (PCC) and pyruvate carboxylase (PC). All these BCDs catalyze the various intermediary metabolism ranging from fatty acid synthesis, glucose synthesis, energy generating pathway, oxidation of fatty acids and amino acids (Tong, 2013). The attachment of biotin onto a specific lysine residue within the active site of all five BDCs is performed by a single enzyme, holocarboxylase synthethase (HCS) and mutation of biotin binding site in these BCDs absolutely inactivate their catalytic activity (Polyak, Chapman-Smith, 2013).

Pharmacological inhibiting BDCs activities would significantly affect the metabolisms in cancer cells while minimizing of those in normal cells. Most cancer researchers have currently paid attention to the BDCs, especially ACC1 and PC in several cancers while there is no report on the implication of HCS in cancers. As BDCs require the attachment of biotin cofactor by HCS, inhibiting HCS expression by gene silencing or gene knockdown would be the most effective way to reduce or diminish the activities of all BDCs, simultaneously which should theoretically reduce cancer growth.

# Objectives of the study

To investigate the necessity of HCS in supporting cancer cell growth in MCF-7 cells by generating stable HCS knockdown cells.

#### Methodology

#### **Cell culture**

MCF-7 cells were cultured in Dulbecco's modified Eagle high glucose medium (DMEM) (Gibco) supplement with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin and incubated at  $37^{\circ}$ C in a CO<sub>2</sub> incubator.

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#### Designation of HCS shRNA constructs and generation of HCS knockdown MCF-7 cells

Three different shRNA constructs targeted to human HCS gene coding sequence (NM\_000411.6) were designed using "siRNA Wizard v 3.1". The two complementary oligonucleotides corresponding to the shRNA sequences with *Bam*HI or *Hind*III restriction sites overhang at at 5'- and 3'-ends were synthesized by Biobasic, and their sequences are shown in Table 1. Firstly, the annealed insert was generated by phosphorylation at 5'-end, followed by annealing of each pair of oligonucleotides. The constructs carrying shRNA sequences were then ligated at the *Bam*HI and *Hind*III sites of the modified pSilencer 2.1-U6 puro TOL2 vector (Phannasil et al., 2017). These constructs were sequenced to confirm the correct ligation before performing transfection. Similarly, the scrambled shRNA sequences (5'-ACTACCGTTGTTATAGGTG-3') were ligated into the same vector to serve as a control.

Name	Sequence (5'-3')	Length (bp)
HCS_868	5'-GATCCTCGACCTTCCCTACGATTATAGCTGTGC	66
(forward)	TTGCTATAATCGTAGGGAAGGTCTTTTTGGAAA -3'	
HCS_868	5'-AGCTTTTCCAAAAAGCTTGGACTTACGTTTATT	66
(reverse)	GTAAGCACAACAATAAACGTAAGTCCAAGCGAG -3'	
HCS_1950	5'-GATCCTCGCAGGAAATGGGCTTAATAGTTGTG	66
(forward)	CTTACTATTAAGCCCATTTCCTGCTTTTTGGAAA -3'	
HCS_1950	5'- AGCTTTTCCAAAAAGCAGGAAATGGGCTTAAT	66
(reverse)	AGTAAGCACAACTATTAAGCCCATTTCCTGCGAG -3'	
HCS_2922	5'-GATCCTCGCTTGGACTTACGTTTATTGTTGTGC	66
(forward)	TTACAATAAACGTAAGTCCAAGCTTTTTGGAAA -3'	
HCS_2922	5'- AGCTTTTCCAAAAAGCTTGGACTTACGTTTATT	66
(reverse)	GTAAGCACAACAATAAACGTAAGTCCAAGCGAG -3'	

 Table 1
 Oligonucleotide sequences used for generation of shRNA constructs

## Generation of stable HCS-knockdown MCF-7 cells

 $5 \times 10^{5}$  MCF-7 cells were plated in 6-well plate and maintained in complete media for 24 h. Cells were then transfected with 2 µg of shRNA construct targeted to human HCS, and 1 µg of TOL2 plasmid, using Lipofectamine 3000. After 24 h, the transfected cells were selected in the complete medium containing 0.5 µg/ml of puromycin. The puromycin resistant colonies were formed after about 20 days of selection. The selected cells were expanded and maintained in selection media at all times. The success of the knockdown was examined by quantitative real-time PCR (qRT-PCR) and Western blotting, respectively.

#### Reverse transcription polymerase chain Reaction (RT-PCR)

Total RNA was extracted from cells using TRIzol Reagent (Gibco) following the manufacturer's instructions. 2 µg of total RNA was mixed with 200 ng of random hexamers in a 10 ml-reaction mixture and pre-



heated at 70°C for 5 min to generate the random hexamers-primed RNA. Reverse transcription was initiated by adding 10  $\mu$ l of mixture containing 1xImProm-II reaction buffer, 3 mMMgCl<sub>2</sub>, 0.5 mM dNTP mix and 1U/ $\mu$ l ImProm-II reverse transcriptase (Promega), to the primed-RNA mixture and the reaction was incubated at 25°C for 5 min, 42°C for 60min and 70°C for 15min, respectively. The cDNA was stored at -20°Cuntil used.

#### Quantitative real-time PCR

Quantitative real time PCR was performed using SYBR green. 2  $\mu$ l of cDNA was mixed with 10  $\mu$ l of master mix containing 1x KAPA SYBR FAST qPCR Master mix universal, 200 nM of forward and reverse primers for HCS in a 12  $\mu$ l-reaction mixture. The thermal profiles consisted of initial reaction at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 30 sec and annealing at 60°C for 30 sec and extension at 72°C for 30 secs in MX3000P Q-PCR system (Agilent Technologies). Expression of HCS mRNA was normalized with that of 18s ribosomal RNA gene and shown as the relative gene expression. Fold change was calculated using the comparative CT method).

#### Western blot analysis

MCF-7 cells grown in T-75 cm<sup>2</sup> flask were trypsinized with 0.05%(v/v) trypsin-EDTA. The detached cells were centrifuged at 3,000 xg for 5min, cell pellet was re-suspended in RIPA buffer [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, 1% NP-40, 1 mM DTT and 1x protease inhibitor cocktail (Roche)]. Protein concentrations were measured by Bradford assay (Bradford, 1976). 30-40 g of protein lysate was subjected to SDS-PAGE under reduction conditions (Laemmli, 1970). The proteins were transferred from the gel to a polyvinylidenedifluoride (PVDF) membrane by Semi-Dry Transfer Cell (BIO-RAD) at constant 12 V for 90 min. The membrane was blocked with 5% skim milk or 3% BSA, depending on antibody in 1x TBS-T (50 mM Tris-Cl pH 7.6, 150 mM NaCl, and 0.1% Tween 20) at  $4^{\circ}$ C overnight. For detecting HCS protein, the blot was incubated with 1.5  $\mu$ g of anti- rabbit human HCS (Bailey et al., 2010) for 1 h. The blot was then washed in 1x TBS-T before incubating with 1:10,000 anti-rabbit IgG conjugated with horseradish peroxidase (HRP) for 1 h. For detection of biotinylated BDCs, 1:5,000 dilution of streptavidin-HRP conjugate (Sigma) was used to detect the holo form of BCDs. For detecting each BCD, the following dilutions of antibody against each BCDs were used as follows: 1:10,000 dilution of anti-rabbit ACC monoclonal antibody (Cell signalling) or 1:10,000 of anti-PC polyclonal antibody (Rohde et al., 1991) were used to detect ACC and PC, respectively. For detection of beta-actin, 1:40,000 dilution of anti-mouse actin monoclonal antibody and 1:10,000 dilution of anti-mouse IgG conjugated with HRP (GE healthcare) were used for primary and secondary antibodies, respectively. The immunoreactive bands were detected using the chemiluminescent HPR substrate (Immobilon Western from Merck Millipore). The images were captured using an enhanced chemiluminescence imaging system (Syngene).

#### **Proliferation assay**

Cell proliferation was determined by counting the viable cells.  $1.5 \times 10^5$  cells of the HCS knockdown and the scrambled control MCF-7 cell lines were plated into 35mm dishes and grown in complete MEM for 2, 4, 6 and 8 days, at 37°C in a CO<sub>2</sub> incubator. At each time point, the cells were trypsinized, subsequently stained with 0.4% trypan blue and counted under a microscope. The number of viable cells was calculated by the following formula

Number of viable cells (cells/ml) = Number of viable cells/square× dilution factor  $\times 10^4$ 

#### MTS proliferation assay

Cell proliferation was also determined by MTS proliferation assay (Promega) at days 2, 4, 6 and 8. 4,000 cells of the HCS knockdown and the scramble control cell lines were plated into 96-well plate and grown in complete MEM. At each time point, the medium was replaced with 100  $\mu$ l of fresh medium. 20  $\mu$ l of CellTiter one solution reagent were added to the cells and incubated at 37°C in CO<sub>2</sub> incubator for 1 h. The absorbance of soluble formazan was measured using 96 well plate reader at 540 nm.

#### Statistical analysis

All values were showed as mean  $\pm$  S.D. The statistical analysis was performed using one-way analysis of variance (one-way ANOVA) followed by Student's t-test using SigmaStat version 3.5 (Systat Software, Inc., California).

#### Results

#### Expression of HCS in stable HCS knockdown MCF-7 cells

After the selection of stable clones in medium containing puromycin, three stable lines, namely, HCS-868, HCS-1950 and HCS-2922 were obtained.

Suppression levels of HCS expression in various clones were found different even among the clones that were transfected with the same construct. Clones HCS-868#1, 2, 3 and HCS-1950#2 retained HCS mRNA levels approximately 15-25% relative to that of the scrambled control (p < 0.01, p < 0.001). In contrast, clone HCS-2922 retained HCS mRNA levels about 70-80% of that of the scrambled control (p < 0.01) (Figure 1A). To confirm whether the level of HCS protein was also down-regulated in agreement with HCS mRNA, Western blot analysis was also performed. Consistent with HCS mRNA levels, these four strong HCS knockdown clones contained the lowest amount of HCS protein while the others retained HCS protein approximately half of the scramble control (Figure 1B).

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Figure 1 Expression of HCS mRNA and protein in various stable HCS knockdown MCF-7 clones. (A) Results of qRT-PCR is showed as relative mRNA expression and the scramble control was arbitrarily set as 1. The results were obtained from two independent experiments and statistic was analyzed by student's t-test where \*p< 0.05, \*\*p<0.01, \*\*\*p<0.001. (B) 40 µg of protein were subjected to Western blot analysis. Results of Western blot analysis showed different intensity of HCS band.</p>

#### Effect of HCS knockdown on biotinylated BCDs

To examine whether down-regulation of HCS also affected its target (BCDs), Western blot analysis using streptavidin-conjugate HRP to detect biotinylated enzymes (holo-form) was performed. In theory, suppression of BCD expression should also lower biotinylation of BCDs which could be detected using streptavidin. As shown in Figure 2, suppression of HCS in four strongest HCS knockdown clones resulted in decrease of biotinylated ACC, PC, PCC and MCC compared to the scramble control.

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Figure 2 Western blot analysis of various HCS knockdown MCF-7 clones using streptavidin-HRP and antibodies against ACC1, PC, and PCC. ACC, acetyl-CoA carboxylase; PC, pyruvate carboxylase; PCC, propionyl-CoA carboxylase and MCC, methylcrotonyl-CoA carboxylase.

#### Effect of chronic HCS suppression on cell growth

To examine whether HCS suppression in MCF-7 cells affect its growth phenotype, Cell proliferation was determined by proliferation or MTS assay. For proliferation assay, clone HCS-868#3 showed the highest degree of reduction in growth approximately 60% at day 4 (p < 0.05), day 6 and day 8 (p < 0.01). In contrast, clones HCS-868#2 and HCS-1950#2 significantly reduced in cell proliferation at day 8 (p < 0.05) (figure 3A). Consistent with proliferation assay, clone HCS-868#3 showed the lowest number of viable cells at day 4, day 6 (p < 0.001) and day 8 (p < 0.01) while the other two clones showed a significant reduction in growth at day 8 (p < 0.001) (figure 3B).



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Figure 3 Proliferation rate of various HCS knockdown MCF-7 cell lines. The result was obtained from proliferation assay (A) and MTS assay (B) at day 2, 4, 6 and 8, respectively. The means values and S.D. were obtained from two independent experiments and statistic was analyzed by student's t-test where \*p< 0.05, \*\*p<0.01, \*\*\*p<0.001.</p>

#### **Discussion and Conclusions**

As a result of rapid proliferation, cancer cells require macromolecules such as nucleic acids, amino acids and lipids as the structural components of the new cells. To meet these high anabolic demands, they have to reprogram their cellular metabolism so that the sources of those biosynthetic precursors are satisfied. BDCs are important enzymes that catalyze various biosynthetic pathways of the above macromolecules. Several evidences show that certain BDCs are overexpressed in certain cancers to support their growth and survival. For example, ACC1 is required to maintain the *de novo* fatty acid synthesis for supporting growth and viability of breast cancer (Chajes et al., 2006), prostate cancer (Beckers et al., 2007) and non-small-cell lung cancer (Svensson et al., 2016). PC is highly expressed and essential in supporting anaplerosis that required for cell proliferation in lung cancer (Fan et al., 2009), glioblastomas (Cheng et al., 2011) and breast cancer (Phannasil et al., 2015). Therefore, HCS is the one most excellent candidate as a target because fully functional BDCs require the attachment of biotin to them by HCS. Inhibition of HCS activity would be the most effective way to knockdown the activities of ACC1, ACC2, PC, PCC and MCC, simultaneously. Understanding the molecular and biochemical mechanisms of HCS in supporting cell growth can lead to the development of novel anti-cancer drugs.

To investigate the role of HCS enzyme in supporting growth, three stable HCS knockdown MCF-7 cell lines with various degrees of HCS suppression were successfully generated for studying the defective growth phenotype in a long term. Clones HCS-868 and HCS-1950 show levels of HCS suppression rather than clones HCS-2922. These different degrees of HCS suppression may be influenced by the siRNA-binding sites. There are three siRNA-binding sites on HCS mRNA. HCS-868 and HCS-1950 bind to target sequences at positions coding for N-terminal and catalytic domains, respectively. In contrast, HCS-2922 binds to its target at position which is non-coding sequences (Suzuki et al., 2005; Sternicki et al., 2017). However, the transcriptional, post-transcriptional, and translational mechanisms that regulate expression of HCS in mammals are currently unknown.

The various levels of decreases in HCS mRNA and protein were strongly correlated with biotinylated BDCs and slow proliferation rate. In conclusion, inhibition of HCS can retard growth of MCF-7 breast cancer cells, suggesting its potential as an anti-cancer drug target. Other defective phonotype of HCS knockdown cells such as cell motility, apoptosis and cell cycle arrest are currently under further investigation.

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