รายงานวิจัยฉบับสมบูรณ์

การเปลี่ยนแปลงของ *O*-GlcNAcylation ในเซลล์มะเร็ง: การใช้เทคนิคทางโปรติโอมิกส์ เพื่อค้นหาโปรตีนบ่งชี้ชนิดใหม่ของโรคมะเร็ง

วรรัตน์ แช่มพัฒนชัย

1 กรกฎาคม 2557

สัญญาเลขที่ TRG5580006

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สถาบันวิจัยจุฬาภรณ์

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย และสถาบันวิจัยจุฬาภรณ์

กิตติกรรมประกาศ

โครงการวิจัยนี้ได้รับการสนับสนุนจาก

สำนักงานกองทุนสนับสนุนการวิจัย
และสถาบันวิจัยจุฬาภรณ์

ปีงบประมาณที่ได้รับทุน ปีงบประมาณ 2555

Abstract

Project Code:	TRG5580006
Project Title:	Alteration of O-GlcNAcylation in cancer cell lines: A proteomic approaches to identify novel protein biomarkers in cancer
Investigator:	**Mr.Voraratt Champattanachai, Chulabhorn Research Institute**
E-mail Address:	voraratt@cri.or.th
Project Period:	3 years (extension for 1 year)

Several lines of scientific research report that increasing *O*-GlcNAcylation, a single *N*-acetylglucosamine (GlcNAc) attachment of cytoplasmic and nuclear proteins, is associated with malignant development in many types of cancer. The main goal of this study was to seek *O*-GlcNAc-modified proteins which may play roles in cancer cell growth and development using cancer cell lines as study models. Human cancer cell lines explored in the study were breast adenocarcinoma (MDA231 and MCF-7), colorectal adenocarcinoma (SW480 and HT29), and hepatocarcinoma (HepG2 and SK-Hep-1). The levels of *O*-GlcNAcylation and its controlling enzymes, *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA) were determined using immunoblotted analysis. We found that all cancer cell lines displayed the similar pattern of *O*-GlcNAc-modified proteins but they were differed in the level of *O*-GlcNAcylation. In addition, cancer cell lines tested had higher *O*-GlcNAcylation level than those in their normal cells. Transient knockdown of *O*-GlcNAylation by RNA interference (RNAi) against *OGT* gene was performed to study the effect of *O*-GlcNAylation reduction on cancer cells. siOGT treated cells of all six cancer cell lines showed an obvious reduction of both OGT and *O*-GlcNAcylation levels in comparison with those with si-scramble control cells. Time course of 5 days of cancer cells treated with either OGT knockdown or siScramble on adherent monolayer conditions reveled unclear whether it causes any effect on cell viability. However, when cultured in soft agar cultures (anchorage independent growth), treatment of siOGT reduces the colony formation and colony sizes of all cancer cells tested. In addition, cultured on poly-hema coated plates (anoikis resistant growth), siOGT treated cells of MCF-7, SW480, and SW620 revealed a decrease in viability. Proteomic and mass spectrometric analysis revealed that si-OGT treated MCF-7 under non-adherent conditions caused up-expression levels of proteins involved in cell signaling, antioxidant and stress responses, transportation of macromolecule within intercellular, and lipid, energy, and nucleic acid metabolism. Among of them, heat shock protein 27 (Hsp27) showed the highest expression level when OGT silencing under non-adherent condition. Taken together, our results indicate that *O*-GlcNAcylation is strongly required for malignant transformation and development. Moreover, these identified proteins may involve in controlling of cancer cell formation.

Keywords: Anchorage independent growth, anoikis resistant growth, cancer, heat shock protein 27, *O*-GlcNAcylation

บทคัดย่อ

รหัสโครงการ : TRG5580006

ชื่อโครงการ : การเปลี่ยนแปลงของ *O*-GlcNAcylation ในเซลล์มะเร็ง: การใช้เทคนิคทางโปรติโอมิกส์เพื่อค้นหาโปรตีนบ่งชี้ชนิดใหม่ของโรคมะเร็ง

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ระยะเวลาโครงการ : 3 ปี (ขยายเวลาสัญญา 1 ปี)

การวิจัยทางวิทยาศาสตร์จากแหล่งต่างๆได้รายงานว่าการเพิ่มระดับปริมาณ โอ-กลูเน็คไซเลชั่น ซึ่งเป็นการดัดแปลงเติมน้ำตาลกลูเน็คจำนวนหนึ่งโมเลกุลที่โปรตีนต่างๆในไซโตพลาซึมและในนิวเคียส มีความเกี่ยวกับสัมพันธ์กับการพัฒนาการของเนื้อร้ายในโรคมะเร็งหลายชนิด งานวิจัยนี้ มีวัตถุประสงค์เพื่อค้นหาโปรตีนที่ถูกดัดแปลงด้วยน้ำตาลกลูเน็ค (*O*-GlcNAc-modified proteins)ซึ่งน่าจะมีบทบาทสำคัญในการพัฒนาการและการเติบโตของเซลล์มะเร็ง โดยใช้เซลล์มะเร็งเพาะเลี้ยงเป็นโมเดลในการศึกษานี้ เซลล์มะเร็งเพาะเลี้ยงที่ใช้ในการทดลองนี้ ได้แก่ มะเร็งเต้านม (MDA231 และ MCF-7) มะเร็งลำไส้ (SW480 และ HT29) และมะเร็งตับ (HepG2 และ SK-Hep-1) ระดับของ โอ-กลูเน็คไซเลชั่น และเอนไซม์ที่ควบคุมการดัดแปลงโปรตีน คือ โอ-กลูเน็คทรานสเฟอเรส (OGT) และ โอ-กลูเน็คเคส (OGA) ถูกตรวจวัดโดยใช้การวิเคราะห์ทางอิมมูโนวิทยา ผลการทดลองพบว่าเซลล์มะเร็งเพาะเลี้ยงทั้งหกชนิดมีการแสดงออกที่คล้ายคลึงกันแต่แตกต่างกันที่ระดับปริมาณของ *O*-GlcNAc-modified proteins นอกจากนี้ ยังพบว่าเซลล์มะเร็งเพาะเลี้ยงทุกชนิดมีระดับของโอ-กลูเน็คไซเลชั่นที่สูงกว่าเซลล์ร่างกายปกติ การยับยั้งกระบวนการโอ-กลูเน็คไซเลชั่นแบบชั่วคราวโดยใช้อาร์เอ็นเออินเตอร์เฟอเรนซ์ (RNAi) กับยีน OGT ถูกนำมาใช้เพื่อศึกษาผลของการลดลงของโอ-กลูเน็คไซเลชั่นต่อเซลล์มะเร็ง เซลล์มะเร็งทั้งหกชนิดที่ได้รับRNAi (siOGT) มีการลดลงของระดับ OGT และ โอ-กลูเน็คไซเลชั่น อย่างชัดเจนเมื่อเปรียบเทียบกับเซลล์มะเร็งทั้งหกชนิดที่ได้รับRNAi ชุดควบคุม (siScramble) เมื่อเลี้ยงเซลล์มะเร็งบนเพลทอาหารปกติ (adherent monolayer) เป็นเวลา 5 วัน โดยเปรียบเทียบระหว่างชุดที่ได้รับ siOGT หรือ siScramble พบว่าไม่สามารถสรุปผลได้ชัดเจนว่ามีผลการการเติบโตของเซลล์มะเร็งหรือไม่ อย่างไรก็ตาม เมื่อเลี้ยงเซลล์ในอาหารวุ้นอ่อน (soft agar) ซึ่งเป็นลักษณะการเติบโตแบบไม่มีที่เกาะ (anchorage independent growth) การลดลงของ OGT มีผลทำให้จำนวนโคโลนีและขนาดของเซลล์มะเร็งทุกชนิดลดลง นอกจากนี้ เมื่อเลี้ยงเซลล์บนเพลทที่เซลล์ไม่สามารถเกาะได้ (poly-hema coated pates) ซึ่งเป็นลักษณะการเติบโตที่สามารถต่อต้านการตายแบบอนอยคิส (anoikis resistant growth) การลดลงของ OGT มีผลทำให้การเติบโตของเซลล์มะเร็งชนิด MCF-7 SW480 และ SW620 ลดลง การวิเคราะห์ผลทางโปรติโอมิกและแมสสเป็กโตรมิเตอร์ แสดงให้เห็นว่า การลดลงของ OGT ของเซลล์มะเร็งชนิด MCF-7 ภายใต้สภาวะการเติบโตแบบไม่มีที่เกาะนี้ ทำให้มีการเพิ่มระดับการแสดงออกของโปตีนหลายชนิดที่เกี่ยวข้องกับสัญญาณในเซลล์ แอนตี้ออกซิดั้นท์และสภาพวะเครียด การขนส่งโมเลกุลขนาดใหญ่ภายในเซลล์ และโปรตีนที่เกี่ยวกับของกระบวนการเมตาบอลิซึมของไขมัน พลังงาน และกรดนิวคลีอิก จากโปรตีนกลุ่มนี้ ฮีทช็อคโปรตีน 27 (Hsp27) มีระดับการแสดงออกที่สุดที่สุด เมื่อมีการลดลงของ OGT ภายใต้สภาวะการเติบโตแบบไม่มีที่เกาะนี้ ผลการทดลองทั้งหมดนี้ ชี้ให้เห็นว่า โอ-กลูเน็คไซเลชั่น เป็นกระบวนการที่สำคัญสำหรับการเปลี่ยนแปลงและการพัฒนาของเซลล์มะเร็ง นอกจากนี้โปรตีนที่วิเคราะห์ได้นี้น่าจะเกี่ยวข้องกับการควบคุมการเติบโตของเซลล์มะเร็ง

คำหลัก : Anchorage independent growth, anoikis resistant growth, cancer, heat shock protein 27, *O*-GlcNAcylation

Introduction

Cancer is a top leading cause of death worldwide. According to World Health Organization (WHO), an estimated 14.1 million new cancer cases and 8.2 million cancer deaths were reported in 2012 [1]. The most commonly diagnosed cancers were lung (1.82 million), breast (1.67 million), and colorectal (1.36 million) whereas the most common causes of cancer death were lung cancer (1.6 million deaths), liver cancer (745,000 deaths), and stomach cancer (723,000 deaths), respectively. In Thailand, based on cancer registry at National Cancer Institute, new cancer patients admitted in 2011 is 3,341 (5.54% or 60,261 cases who was examined) and most cancer patients are in advanced stages [2]. Although public campaigns are rising for cancer prevention and control in many areas, detection of cancer at the early stage is of importance to get better chance of healthy life.

Cancer is an abnormal system of cell growth resulting in uncontrolled proliferated cells which can invade and spread to other organs of the body. Altered carbohydrate metabolism is one of the major characteristic of cancer cells. This metabolic shift, termed the 'Warburg effect' [3], involves an elevated rate of glucose uptake through glycolysis for predominantly energy and biosynthetic producing in most cancer cells. Although these metabolic changes are not the fundamental defects that cause cancer, several lines of evident have revealed that the multiple genetic alterations that cause tumor development directly affect glycolysis, the cellular response to hypoxia and the ability of tumor cells to recruit new blood vessels. The increased glucose flux by malignant tumors is also the foundation for the detection and monitoring of human cancers by imaging uptake of 2-F-2-deoxyglucose (FDG) with positron emission tomography (PET) to monitor the location of malignant cell mass in the body. Changes in tumor glucose uptake and metabolic shift also alter distinct nutrient signaling pathways including hexosamine biosynthesis pathway (HBP). Normally, the majority of glucose goes to glycolysis pathway whereas approximately 2-5% of glucose enters the HBP resulting in the end product, uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc). This active sugar is a substrate for classical *N*-linked and *O*-linked glycosylation and *O*-GlcNAcylation.

O-GlcNAcylation is a monosaccharide attachment to the hydroxyl group of serine or threonine residues of nuclear and cytoplasmic proteins [4]. Unlike classic glycosylation that occurs in endoplasmic recticulum and golgi apparatus, this glycosylation takes place in cytoplasm and nucleus. *O*-GlcNAcylation is a dynamic reversible process regulated by two key enzymes; *O*-GlcNAc transferase (OGT) [5] and *O*-GlcNAcase (OGA) [6] which add in and remove an UDP-GlcNAc from proteins, respectively. Like phosphorylation, this glycosylation has the wide ranging effects on cellular function and signaling. A number of studies reported that

many oncogenes and tumor suppressors were modified by *O*-GlcNAc [for review, see [7]]. This further suggests that *O*-GlcNAcylation is beneficial to cancer cells and might be a novel signature of cancer phenotype.

One of characteristics of cancer is an ability to invade and metastasize from its primary sites to other distant sites in the body through the blood and lymphatic system. Metastatic cancer cells must detach its primary sites and escape from normal defensive mechanisms including cell cycle arrest and apoptosis. This adaptive process is also known as anoikis resistance. In addition, they have to proliferate and grow in the absence of anchorage to the extracellular matrix (ECM) and their neighboring cells, a process termed as anchorage independent growth. Extensive studies on anoikis resistance mechanisms have been performed in cancers to find the molecular targets for preventing metastasis [8-10]. In addition, anchorage-independent cell growth (colony forming capacity in soft agar media) of cancer cells *in vitro* has been used to predict the tumor phenotype, particularly with respect to potential for metastasis in primary breast and lung tumors [11].

Previously, we reported that the levels of *O*-GlcNAcylation and OGT were increased in primary breast and colorectal cancer tissues and many proteins were selectively modified by *O*-GlcNAc in both cancers [12, 13]. In this study, we examined the levels of *O*-GlcNAcylation and *O*-GlcNAc cycling enzymes of breast (MCF-7 and MDA-MB-231), colorectal (SW480 and SW620), and liver (SK-Hep1 and HepG2) cancer cell lines in comparison to their normal cells. In addition, a reduction of *O*-GlcNAcylation using OGT knockdown was performed and the effects of altered *O*-GlcNAcylation on cell malignancy were investigated in cancer cell lines cultured in monolayer, soft agar, and anoikis resistance conditions. Moreover, proteins associated to non-adherent cultures were identified in both siOGT and siScramble control cells using 2D gel based proteomic and mass spectrometric analysis.

Methodology

Human adenocarcinoma cell lines and normal cells including breast cancer (MDA231 and MCF-7), colorectal cancer (SW480 and HT29), liver cancer (HepG2 and SK-Hep-1), normal colon epithelial cell (CCD 841 CoN) and normal liver epithelial cell (THLE-3) were purchased from the American type Culture Collection (ATCC). Human normal mammary epithelial cell (HMEC) and its medium were purchased from LONZA. MDA-MB- 231 and MCF-7 were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). SW480 and SW620 were maintained in RPMI-1640 supplemented with 10% FBS. HMEC was cultured in medium from LONZA as recommendation by the manufacturer. CCD 841 CoN was

cultured in DMEM supplemented with 10% FBS and 1% L-glutamine while THLE-3 was cultured in DMEM supplemented with 10% FBS and 25 mmol/L HEPES. All cells were supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin and cultured in media as mentioned above in humidified atmosphere, 95% air, 5% CO_2 at 37^{O}C.

Assessment of *O*-GlcNAc transferase (OGT), *O*-GlcNAcase (OGA), and *O*-GlcNAcylation levels

The levels of *O*-GlcNAc, OGT, and OGA were determined by immunoblotting using the anti-*O*-GlcNAc antibody RL2 (Abcam), OGT (sigma), and OGA (Abcam) as previously described [13]**.** Briefly, cells were lysed in RIPA buffer containing 1% protease inhibitor cocktail (Sigma) and 20 μM of *O*-GlcNAcase inhibitor, Thiamet-G (Sigma). Protein samples (20 μg) were separated by 10% SDS-PAGE, transferred to PVDF membranes (Millipore), and probed with RL2 (1:1,000), OGT (1:1000), and OGA (1:5,000), respectively. β-actin (cell signaling) was used to compare protein loading of cell lysates. Immunoblots were developed with ECL substrate and the signal was recorded using ImageQuant™ LAS 4000 digital imaging system (GE Healthcare).

RNA interference

The Stealth™ siOGT targeting *OGT* gene was as follows: sense 5'-UAAUCAUUUCAAUAACUGCUUCUGC-3', and antisense 5'-GCAGAAGCAGUUAUUGAAAUGAUUA-3' (Invitrogen). Stealth™ scrambled siRNA medium GC duplex (Invitrogen) was used as a negative control. For monolayer cultures, transfection of the siRNA oligos into cancer cells was carried out using Lipofectamine 2000 in forward transfection mode as described previously [12]. For soft agar and non-coated plate cultures, cells were transfected using reverse transfection mode as described in soft agar and poly-hema coated plate cultures, respectively.

Cell viability assay

For monolayer cultures, cell viability was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. Briefly, cell suspensions were seeded in 96-well plates, cultured for 24 hours, and transfected with RNA interference. Transfected cells were further cultured for 24-96h. Then, the wells were replaced and incubated with fresh culture media containing 0.5 mg/mL MTT (Sigma) for 2 hours at 37^{O}C. Finally, the media are removed and DMSO are added to the wells (100 μL/well), and absorbance are measured at 550 nm in a microplate reader, subtracted with absorbance at 650 nm. The number of viable cells is determined from the absorbance.

Soft agar assay (Anchorage independent growth)

Briefly, 5 x 10^3 cells were transfected with siScramble and siOGT and suspended in 500 µl of 2X antibiotic-free medium and mixed with 500 µl 0.4% top agar. The cell mixture was then overlaid onto 1.5 ml of the bottom agar medium with 0.8% agar. Fresh growth medium was added on top of the upper agar and add up every 3 days as a feeder layer. To stop culturing and count the total colony cells, the colonies were stained with 0.005% crystal violet and 25% methanol for 1 h. Excess dye was washed by adding 1 ml of distilled water and gently agitated until the background was faded. The colonies were then counted at x4 magnifications. Colony number and size were determined using the analyze particle software, Image J 1.42l (NIH).

Poly-hema coated plate cultures (Anoikis resistance)

Poly-hema coated plate or anoikis resistance culture was performed as descried previously with some modifications [9]. Briefly, 3x10^5 cells (OGT knockdown and scramble cells using reverse infection conditions) were cultured in the complete medium on poly-hema coated plates. The polyHEMA-coated plates were prepared by soaking of 30 mg/mL poly-HEMA in 95% ethanol onto plates and drying at 37^{o}C in an incubator, followed by extensive washing with water and UV sterilization. After culturing for 3 days, cells were centrifuged at 1,000 X g for 10 min, resuspended with PBS buffer, and incubated with 0.05% trypsin for 10 min. Dissociated cells were corrected for measurement of OGT and *O*-GlcNAc modified proteins levels as descried above.

To test cell viability on poly-hema coated plate cultures, trypan blue exclusion assay was used. At the selected times, cell suspensions were harvested using centrifugation at 1,000 X g for 10 mins. After centrifugation, cell pellets were resuspended with PBS buffer and incubated with 0.05% trypsin (Sigma) for 10 min and the reaction was stopped by adding the complete medium. 10 µL of trypsized cells was mixed with 10 µL of 0.04% Trypan blue. Viable and non-viable cells from four microscopic viewing areas were counted using a hemocytometer.

Two-dimensional gel electrophoresis (2DE): 2DE were performed as described previously [13]**.** Briefly, cancer cells (10^7) were lysed with 2D lysis buffer supplemented with protease inhibitor cocktail and Thiamet-G. Proteins (100 µg) were separated by isoelectric focusing (IEF) using 70 mm nonlinear pH 3-10, immobilized pH gradient (IPG) strips (GE Healthcare). The IPG strips were then applied to the second dimension and run on 10% SDS-PAGE, stained by 0.1% coomassie brilliant blue R-250 (CBB) for total protein staining. Densitometric analysis was performed using ImageMaster 2D Platinum version 7.0 (GE healthcare).

Protein identification by mass spectrometry (MS): Proteins on CBB gels between siOGT and siScramble cells displayed significantly different were identified using MS analysis. The protein bands were excised from the gel, destained and enzymatically digested by trypsin

(Promega). Digested peptides from 2DE gels were identified using Nanoflow liquid chromatography coupled with the amaZon speed ion trap mass spectrometry (Bruker) as previously described [14]. Protein identification was performed using MASCOT search engine with NCBInr databases (http://www.matrixscience.com). Proteins with molecular weight and p*I* consistent to spot on 2DE gel with p-value $\leq$0.05 were considered positively identified.

Data analysis

The data from densitometric analysis was presented as means ± SE throughout that normalized by averaged intensity of all normal samples. The statistical analysis was analyzed using unpaired Student's t-test to test for the difference between two groups, and statistically significant differences between groups were defined as $P < 0.05$.

Results

Augmentation of *O*-GlcNAcylation and *O*-GlcNAc cycling enzymes in cancer cell lines

To determine whether the levels of *O*-GlcNAcylation and *O*-GlcNAc cycling enzymes were aberrant in cancer cells; immunobolts of *O*-GlcNAc, OGT and OGA were performed. Breast cancer cells (MCF-7 and MDA-MB 231) had increased in *O*-GlcNAcylation, OGT and OGA levels in comparison to breast normal cells (HMEC) (Figure 1A). Similar manner was also found in colorectal cancer cells (SW480 and SW620) when compared to colorectal normal cells (CCD-841 CoN) (Figure 1B). Interestingly, it showed that *O*-GlcNAcylation level was increased in liver cancer cells (SK-Hep1 and HepG2) but OGT and OGA expression levels were not different between cancer cells and normal liver cells (THLE-3), respectively (Figure 1C).

Transient knockdown of *O*-GlcNAc transferase

As we found that O-GlcNAc level was up-regulated in all cancer cells, therefore, transient knockdown of *O*-GlcNAylation by RNA interference against OGT was performed in six cancer cell lines. As shown in Figure 2, si-OGT treated cells of all six cancer cell lines showed a reduction of both OGT and *O*-GlcNAcylation expression levels in comparison with si-scramble cells.

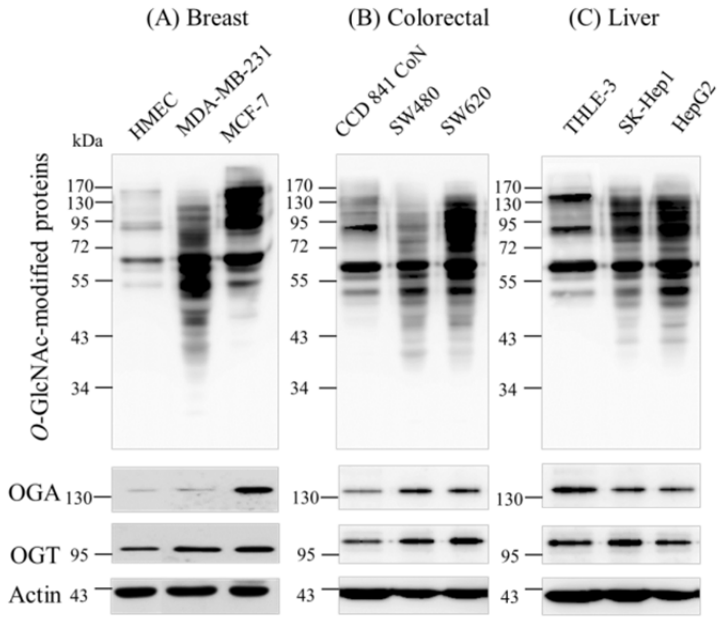

Figure 1 Immunoblots of *O*-GlcNAcylation, OGT, and OGA expression levels in normal and cancer cells of breast (HMEC, MDA-MB-231 and MCF-7), colorectal (CCD841 CoN, SW480 and HT29), and liver (THLE-3, HepG2 and SK-Hep-1) in comparison to their normal breast cells (HMEC). Proteins (10 µg) were run in 10% SDS-PAGE, transferred to PVDF membranes and immunoblotted with antibodies against *O*-GlcNAc (RL2), OGT, OGA, and actin as loading control.

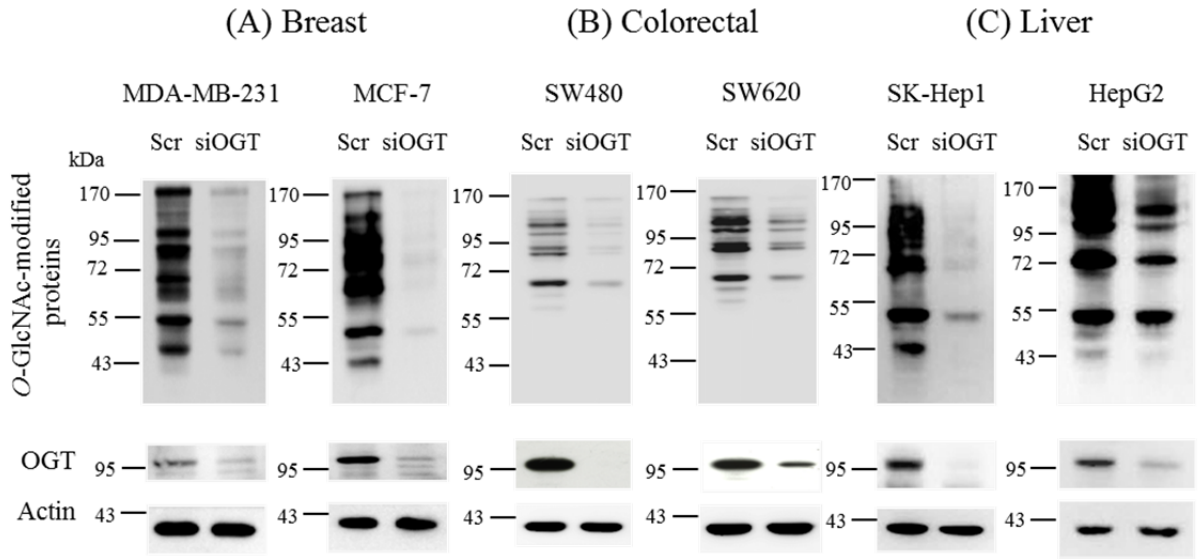

Figure 2 OGT Knockdown led to the reduction of O-GlcNAc-modified proteins and OGT levels in breast, colorectal and liver cancer cell lines. si-OGT or si-sramble was transfected into (A) breast adenocarcinoma (MDA231 and MCF-7), (B) colorectal adenocarcinoma (SW480 and HT29), and (C) hepatocarcinoma (HepG2 and SK-Hep-1) using lipofectamine 2000. Approximately 2 days following transfection, OGT and *O*-GlcNAc protein levels were determined by immunoblotting with OGT and *O*-GlcNAc (RL2) antibodies. Actin immunoblots were used as a protein loading control.

Effect on Cell viability of transient OGT knockdown in monolayer cultures

Cell viability by MTT was determined in order to study the consequent effect of *O*-GlcNAc reduction. Surprisingly, in six cancer cells tested for 5 days, siOGT knockdown were likely to have no or little effects in siOGT treated cells in comparison to those in siScramble cells (Figure 3). Although some time points between siOGT and siScramble treatments showed a statistically significant different, they were inconsistent whether siOGT caused increasing or decreasing in cellular viability.

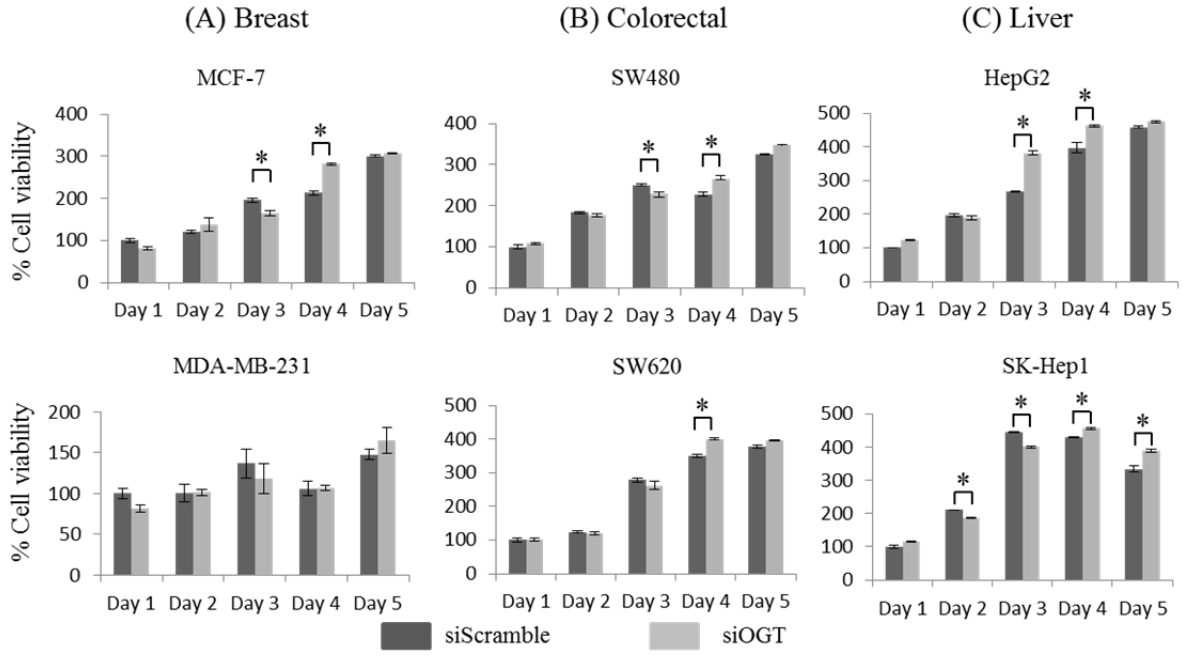

Figure 3 Effect on cell viability of OGT knockdown in six cancer cell lines. (A) Breast adenocarcinoma (MDA231 and MCF-7), (B) colorectal adenocarcinoma (SW480 and SW620), and (C) hepatocarcinoma (HepG2 and SK-Hep-1) were transfected with siScramble or siOGT and cultured in 96 well-plates for 1-5 days. Cell viability was measured by MTT assay. **P* <0.05 vs. siScramble control cells at indicated time points.

Effect on colony formation of transient OGT knockdown in soft agar cultures

Since OGT knockdown had no or little effects on cellular viability under the monolayer cultures, we asked whether it may affect in colony formation in soft agar cultures. As shown in Figure 3, siOGT treatment led to a decreased in colony formation of MDF-7, MDA-MB-231, SW460, and HepG2 with significantly different in comparison to siScramble cells. In SW480 and SK-Hep1cells, although the average number of colony formation was lower in siOGT treatment in comparison to those in siScramble controls, they were not statistically different. However and interestingly, colony size determination revealed that si-OGT treated cells of all cancer cell lines had the average colony sizes smaller than those in siScramble cells.

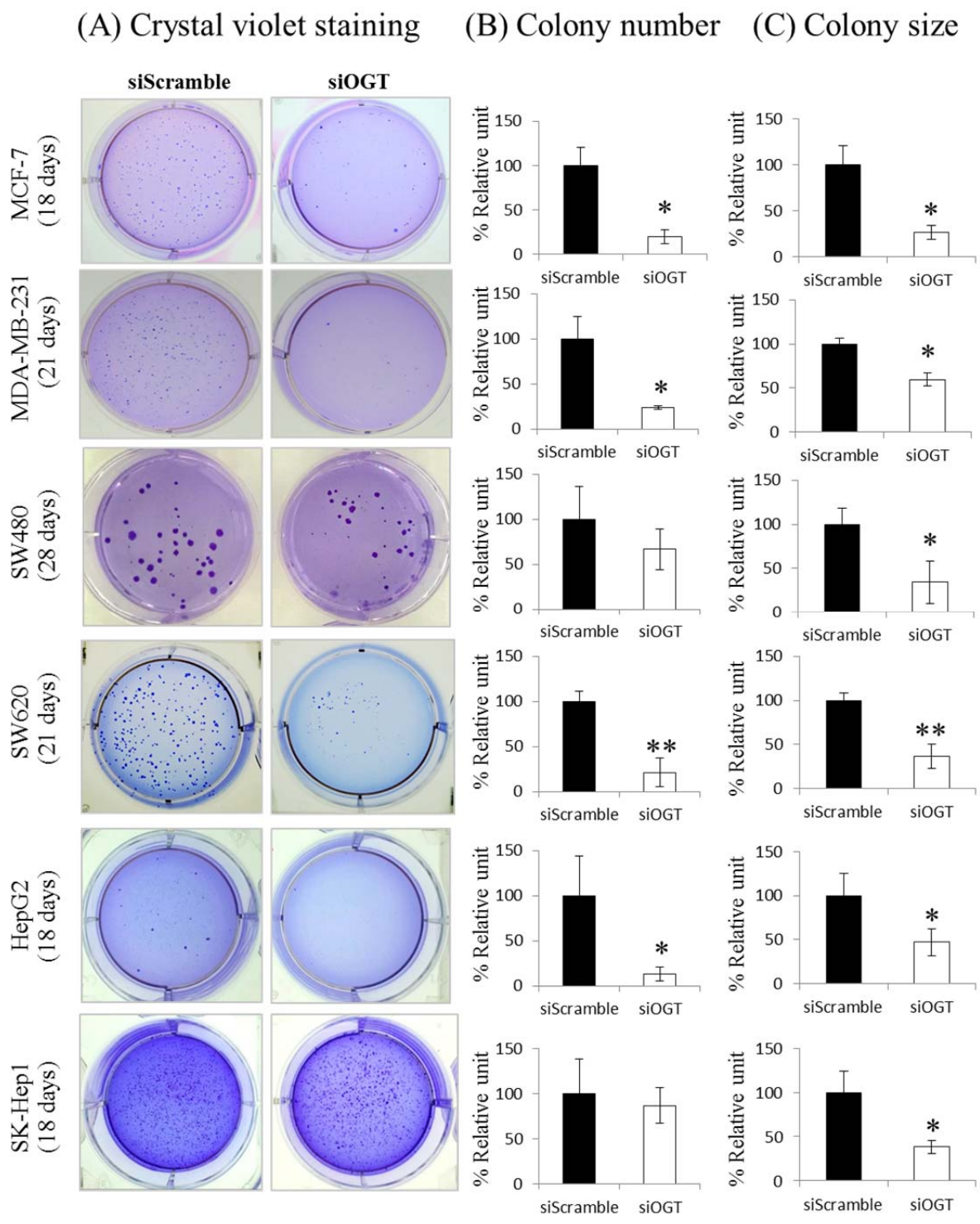

Figure 4 Effect of OGT knockdown on colony formation of MCF-7, MDA-MB-231, SW460, SW480, HepG2, and SK-Hep1 in soft agar cultures. All cancer cells tested were transfected with siScramble or siOGT and cultured into soft agar of 6 well-plates as indicated times (days shown below cancer cells). Cell viability was measured by crystal violet staining. Colony number and size were determined using the analyze particle software, Image J 1.42I (NIH). **P* <0.05, ** *P* <0.01 vs. siScramble control cells.

Effect on spheroid formation of transient OGT knockdown in poly-hema coated plates

Since siOGT knockdown reveals a decrease in colony formation, we therefore ask whether OGT treatment has similar effects on another non-adherent culture, anoikis resistance condition in which cells cultured on poly-hema coated plates. As shown in Figure 4, siOGT treatment had no effects on spheroid formation of all six cancer cells. However, siOGT treated cells of MCF-7, SW480, and SW620 revealed a decrease in viability with significantly different in comparison to those in their siScramble treated cells.

Because cells cultured on poly-hema coated plates can be harvested, we therefore collected cells and examined if OGT knockdown may cause the global protein alteration and what kinds of proteins that may play important roles in cancer cell formation. 2D gel based proteomic and mass spectrometric analysis was used to explore the differential protein expression between siScramble and siOGT treated MCF-7 cell cultured on poly-hema coated plates. To increase the biological and statistical significance of the results, 3 independent experiments were performed. Overall, image analysis revealed that 10 protein spots were differentially expressed in siOGT treated cells compared to that in siScramble cells (Figure 6). As shown in Table 1, 10 protein spots were identified by LC-MS/MS analysis. All spots were highly expressed or up-regulated in siOGT treated MCF-7 cells including nucleoside diphosphate kinase A (NDK A), peroxiredoxin-2 (PRDX2), heat shock protein beta-1 or heat shock protein 27 (HSP27), catechol-O-methyltransferase (COMT), endoplasmic reticulum resident protein 29 (ERp29), inorganic pyrophosphatase, phospatidylcholine transfer protein (PCTP)-like protein, protein disulfide-isomerase A3 (PDIA3) and heat shock protein 60 (Hsp60).

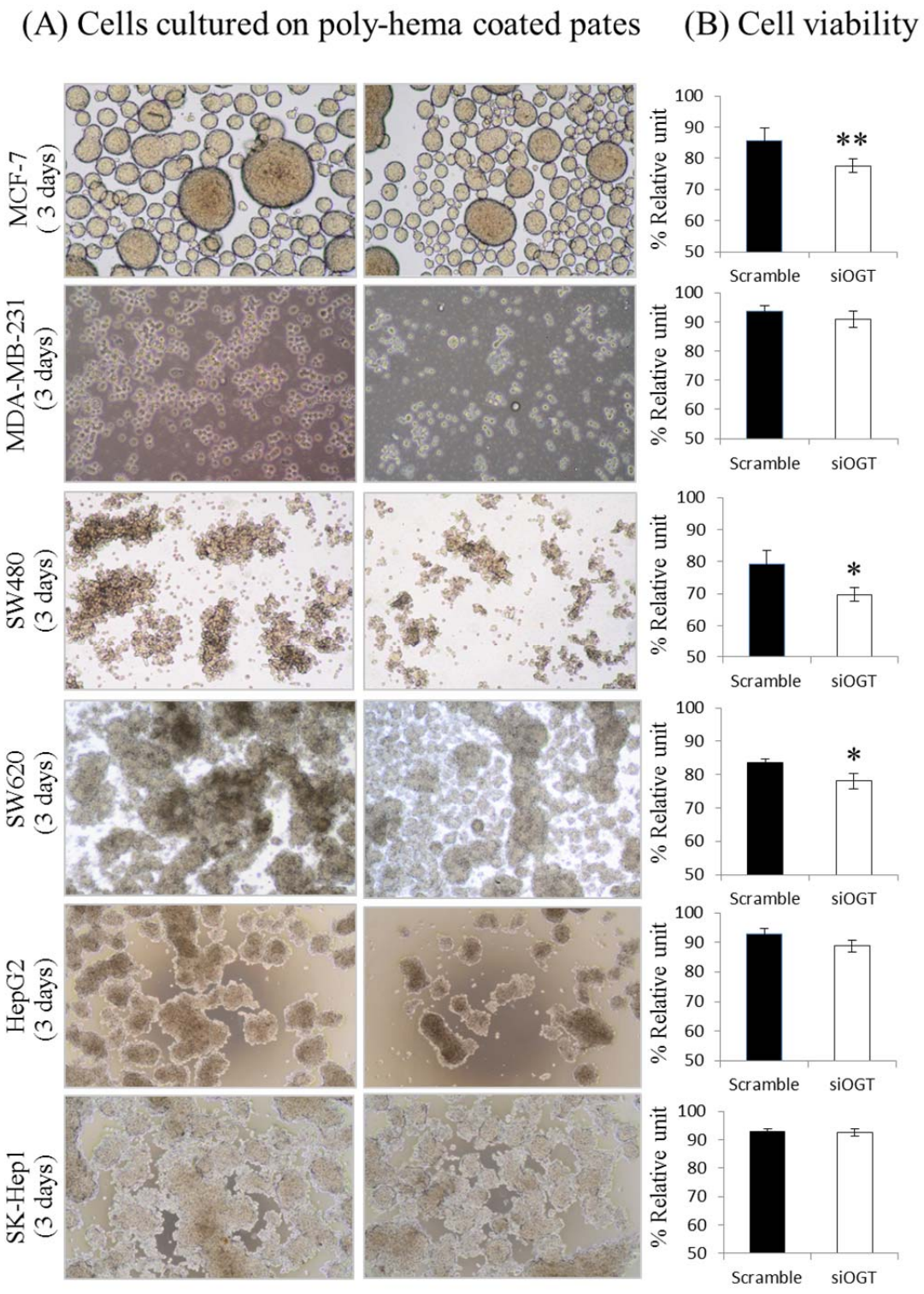

Figure 5 Effects of OGT treatment on spheroid formation and their viability of MCF-7, MDA-MB-231, SW460, SW480, HepG2, and SK-Hep1 cultured on poly-hema coated plates. All cancer cells tested were transfected with siScramble or siOGT and cultured under non-adherent condition (poly-hema coated plates) for 3 days. (A) Spheroid formation and (B) Cell viability measured by crystal violet staining*P <0.05, ** P <0.01 vs. siScramble control cells.

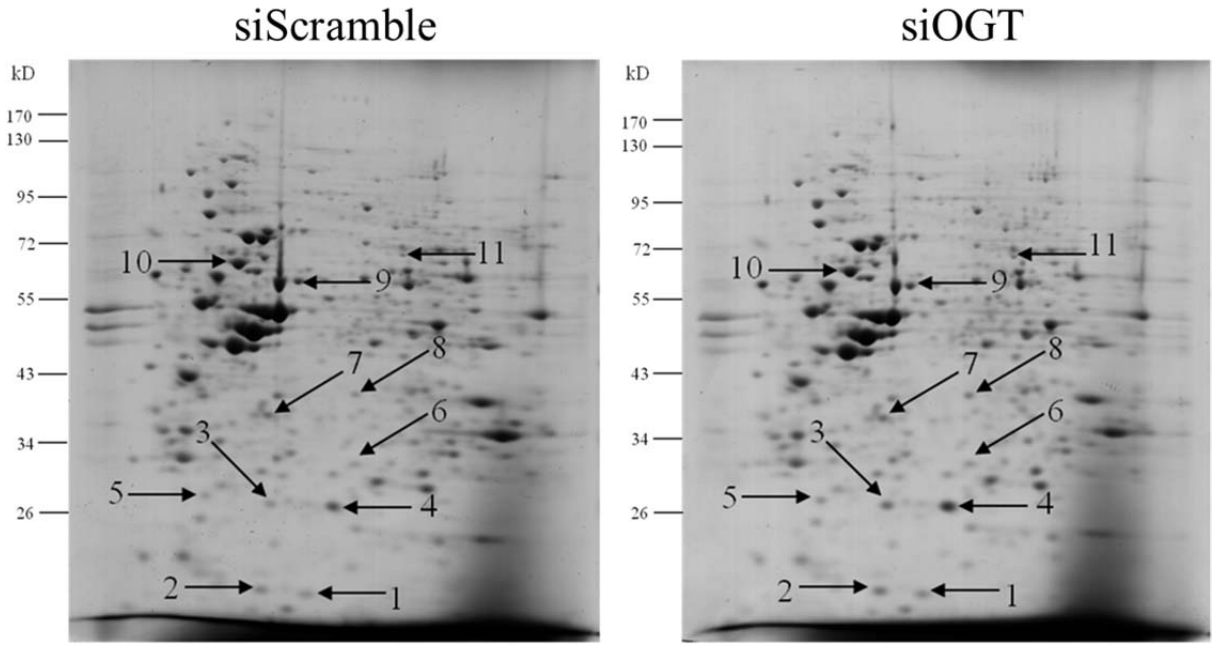

Figure 6 2D-IEF/PAGE of siScramble and siOGT treated MCF-7 cells cultured under non-adherent condition. Proteins (100 µg) were separated in IEF strips (pH3-10) and 10% SDS-PAGE, and stained using coomassie brilliant blue R-250. Arrows with numbers indicate up-regulated protein spots between siScramble and siOGT treated cells using ImageMaster 2D Platinum software.

Spots	Protein Names	Accession number [(a)]	MW/pI (kDa/pI)[(b)]	Peptide Matches[(c)]	Score[(d)]	Fold change[(e)]	*p*-value
1	Nucleoside diphosphate kinase A	gi|35068	17.138/5.83	4	148	1.48	
2	Peroxiredoxin-2	gi:32189392	22.049/5.66	3	89	1.35	< 0.05
3	Heat shock protein 27	gi|4504517	22.826/5.98	8	398	2.22	< 0.05
4	Heat shock protein 27	gi|4504517	22.826/5.98	5	207	2.01	< 0.05
5	Catechol-O-methyltransferase	gi|179955	30.433/5.26	5	92	1.34	< 0.05
6	Endoplasmic reticulum resident protein 29	gi|5803013	29.032/6.77	3	153	1.34	< 0.05
7	Inorganic pyrophosphatase	gi|11056044	33.095/5.54	2	92	1.51	< 0.05
8	Phospatidylcholine transfer protein (PCTP)-like protein	gi|116812600	33.427/6.67	3	103	1.50	< 0.05
9	Protein disulfide-isomerase A3	gi|21361657	57.146/5.98	7	160	1.24	< 0.05
10	Heat shock protein 60	gi|306890	61.157/5.71	16	647	1.38	< 0.05

a) Protein sequence GI number using NCBInr database
b) Theoretical molecular weight and pI from MASCOT database
c) The number of unique peptides identified by LC-MS/MS
d) MASCOT scores
e) Volume ratio of protein spot intensity calculated by ImageMaster 2D Platinum software

Discussion

O-GlcNAcylation is unique type of post-transitional protein modification that is thought to implicate in the wide range effects in metabolic diseases and cancer. Growing evidence from our groups and others revealed that certain cellular proteins were abnormally modified by *O*-GlcNAc in cancers, indicating that *O*-GlcNAc alteration was associated with cancer malignancy. Therefore, in this study, we investigated the consequent effects of decreasing *O*-GlcNAc levels using various cancer cell lines as models. These cancer cells are MCF-7 (low aggressive breast cancer) MDA-MB-231 (high aggressive breast cancer), SW480 (primary colorectal cancer) and SW620 (metastatic colorectal cancer), HepG2 (low invasive liver cancer), and SK-Hep1 (high invasive liver cancer).

Immunoblots of *O*-GlcNAc-modified proteins and *O*-GlcNAc cycling enzyme (OGT and OGA) revealed that breast (MCF-7 and MDA-231) and colorectal (SW-480 and SW620) cancer cell lines had higher *O*-GlcNAc and OGT but lower OGA levels than those in their normal cells (Figure 1). However, liver cancer cells (SK-Hep1 and HepG2) showed higher *O*-GlcNAc and OGA but lower OGT levels in comparison to normal liver cells (Figure 1). Augmentation of the two *O*-GlcNAc cycling enzyme levels may be important and, at least in part, control the overall *O*-GlcNAc level in these cancer cells. Consistent with these results, *O*-GlcNAcylation level was previously reported to increase in breast cancer cells and breast cancer tissues [15-17], colorectal cancer tissues [13, 18], liver cancer tissues [19].

Transient OGT knockdown was performed in these six cancer cell lines. We found that OGT treatment led to a decrease of OGT and *O*-GlcNAc levels (Figure 2). It was reported that embryonic deletion of OGT was lethality and abolishment of *O*-GlcNAc level led to cell death and apoptosis [20]. However, other studies reported that, in cancer cells, OGT knockdown did not alter cell growth and proliferation [15, 16], but it markedly reduced the anchorage-independent growth and inhibited cancer cells invasion [16]. Similar with these findings, OGT silencing in these cancer cell lines had no or little effects on cell viability in monolayer culture conditions (Figure 3). Interestingly, we found that OGT knockdown decreased the colony number and size of breast, colorectal, and liver cancer cells cultured in soft agar (the anchorage-independent growth) (Figure 4). To mimic non-adherent conditions in soft agar, cells cultured on poly-hema coated plates, termed anoikis resistant model were used. Unfortunately, siOGT had no effects on spheroid formation cultured on poly-hema coated plate conditions for 3 days of all cancer cells tested (Figure 5). OGT knockdown caused a decrease in cell viability and only siOGT treated cells of MCF-7, SW480, and SW620 had lower cell viability when compared to those in siScramble treated cells. Cell viability in siOGT of MDA-MB-231, Sk-

Hep1, and HepG2 tended to decrease in comparison to those in siScramble cells but they were not statistically different.

Since OGT knockdown of MCF-7 showed decreasing both in colony formation cultured in soft agar and in cell viability cultured in non-adherent conditions, it was chosen to study proteins associated to cancer cell formations. The results form 2D-IEF/PAGE from non-adherent condition indicated that total 10 protein spots were highly expressed or up-regulated after OGT reduction (Figure 6). These proteins can be largely classified based on their functions into (1) proteins involved in chaperone and stress response including heat shock protein 27 (Hsp27), Hsp60 ERp29, PDIA3, and PRDX2 and (2) proteins involved in biosynthesis including NDK A, COMT, PPase and PCTP-like protein. Among these, Hsp27 showed highest up-regulated (fold-change $\geq$ 2) after OGT silencing in non-adherent condition (Table 1).

Currently, physiological roles of Hsp27 in cancer progression have been extensively studied. It functions as a molecular chaperone which is constitutively expressed in mammalian cells, especially in the pathological conditions. This chaperone protein is believed to play vital roles in cellular protection against a variety of toxic stimuli, stress inducing condition or oxidative inflammation condition [21]. In addition, it also exhibits anti-apoptotic properties to prevent apoptotic cell death triggered by various stimuli and existed its tumorigenic properties when expressed in cancer cells [22]. However, there is little information about *O*-GlcNAcylation in Hsp27. Guo et al. reported that Hsp27 was modified by *O*-GlcNAc in HCC cells. Furthermore, this special type glycosylation implied in the entry of Hsp27 into the nucleus [23]. Moreover, Hsp27 has been reported to be modified by *O*-GlcNAc in breast cancer cells, however, underlying effect of this modification on chaperones and stress response function is still unclear [24]. Therefore, elucidating dynamic post-translational modification of Hsp27 should improve the understanding on Hsp27 related cell survival under stressful conditions.

In conclusion, we demonstrated that *O*-GlcNAc level was increased in breast, colorectal, and liver cancer cells when compared to their normal cells. Transient OGT knockdown had no or little effects on viability of cancer cells cultured in monolayer conditions. However, in soft agar cultures, it reduces the colony formation and colony sizes of all cancer cells tested. In addition, on poly-hema coated plate cultures, siOGT treated cells of MCF-7, SW480, and SW620 revealed a decrease in viability. 2D proteomic analysis showed that si-OGT treated MCF-7 under non-adherent condition led to increasing expression levels of proteins associated to chaperone and stress responses as well as biosynthesis. Among of these, Hsp27 showed the higher up-regulation in si-OGT treated cells. Taken together, our results indicate that *O*-

GlcNAcylation is strongly required for malignant transformation and these identified proteins may involve in controlling of cancer cell formation. However, it still needs to validate data from mass spectrometric analysis using other techniques such as immunodetection in order to draw a strong discussion and conclusion.

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

1.1 Chaiyawat P, Netsirisawan P, Svasti J, Champattanachai V. Aberrant O-GlcNAcylated Proteins: New Perspectives in Breast and Colorectal Cancer. Front Endocrinol (Lausanne). 2014 Nov 11;5:193. doi: 10.3389/fendo.2014.00193. eCollection 2014.

1.2 Netsirisawan P, Chaiyawat P, Lirdprapamongkol K, Chokchaichamnankit D,Srisomsap C, Svasti J, Champattanachai V. Reduction of *O*-GlcNAcylation demonstrated increasing expression levels of proteins associated to stress response and metabolic enzymes in cancer cells under non-adherent condition (In preparation)

2. การนำผลงานวิจัยไปใช้ประโยชน์

- วิทยากร เรื่อง "*O*-GlcNAcylated proteins กับการเกิดมะเร็งเต้านมระยะแรก" งานวิจัยวิชาการและนวัตกรรม คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล
- Oral presentation: Aberrant *O*-GlcNacylated proteins: a novel biomarker candidate of breast and colorectal cancer. The 7th Asia Oceania Human Proteome Organization (AOHUPO) Congress and the 9th International Symposium of the Protein Society of Thailand (PST), Miracle Grand Hotel, Bangkok, Thailand. 6-8 Aug, 2014.