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O-GlcNAcylation is a novel regulator of lung and colon cancer malignancy Wenyi Mi[#], Yuchao Gu[#], Cuifang Han, Haiyan Liu, Qiong Fan, Xinling Zhang, Qi Cong, and Wengong Yu^{*}

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Key Words: O-GlcNAc, lung cancer, colon cancer, immunohistochemistry

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Abstract

O-GlcNAc is a monosaccharide attached to serine or threonine hydroxyl moieties on numerous nuclear and cytoplasmic proteins; O-GlcNAcylation is dynamically regulated by O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA). Although recent studies have shown that O-GlcNAcylation plays essential roles in breast cancer progression, it is also necessary to know whether O-GlcNAcylation is involved in other types of human cancer. In this study, O-GlcNAcylation levels and the expressions of OGT and OGA in human lung and colon cancer tissues were examined by immunohistochemistry analysis. We found that O-GlcNAcylation as well as OGT expression was significantly elevated in the cancer tissues compared with that in the corresponding adjacent tissues. Additionally, the roles of O-GlcNAcylation in the

malignancy of lung and colon cancer were investigated *in vitro*. The results showed that O-GlcNAcylation markedly enhanced the anchorage-independent growth of lung and colon cancer cells; O-GlcNAcylation could also enhance lung and colon cancer invasion in a context-dependent manner. All together, this study suggests that O-GlcNAcylation might play important roles in lung and colon cancer formation and progression, and may be a valuable target for diagnosis and therapy of cancer.

Research highlights

The levels of O-GlcNAc and OGT were elevated in the lung and colon cancer tissues. OGT increase may be a main cause of O-GlcNAc elevation in tumor tissues. O-GlcNAc plays important roles in lung and colon cancer malignancy.

1. Introduction

O-linked β -N-acetylglucosamine (O-GlcNAc) is a monosaccharide attached to serine or threonine hydroxyl moieties on nuclear and cytoplasmic proteins. Similar to protein phosphorylation, O-GlcNAcylation can be attached or removed dynamically in response to changes in the cellular environment triggered by stress, hormones, or nutrients [1]. O-GlcNAc transferase (OGT) catalyzes the transfer of GlcNAc from UDP-GlcNAc to serine/threonine residues of target proteins while O-GlcNAcase (OGA) catalyzes removal of O-GlcNAc. This dynamic and reversible modification is emerging as a key regulator of various cellular and disease processes [1-3].

It has been proposed that O-GlcNAcylation might be a regulator of cancer, based on the O-GlcNAcylation of many oncogenes and tumor suppressors [4-6]. In recent years, the roles of O-GlcNAcylation in breast cancer have been investigated.

Reginato's group have reported that the metastatic breast cancer cell lines showed an increase in OGT protein expression and O-GlcNAcylation, suggesting that higher O-GlcNAcylation level might be beneficial to breast cancer cells [7]. In our group, immunohistochemistry analysis indicated that the global O-GlcNAcylation level in breast tumor tissues is elevated significantly, compared with that in the corresponding adjacent tissues; moreover, O-GlcNAcylation was significantly enhanced in the metastatic lymph nodes compared to their corresponding primary tumor tissues [8]. It has also been demonstrated that O-GlcNAcylation could enhance breast cancer tumorigenesis, invasion and metastasis in some breast cancer cells [7, 8].

Colon cancer and lung cancer are the ones of most common cancers in the world. To investigate whether O-GlcNAcylation also plays roles in colon and lung cancers, O-GlcNAcylation level was examined in human lung cancer tissues and colon cancer tissues by immunohistochemistry analysis. Additionally, the global O-GlcNAcylation level was altered through OGA inhibition and OGT silencing in lung and colon cancer cells, and the effects of O-GlcNAcylation on cancer malignancy were investigated.

2. Materials and methods

2.1. Cell cultures

Human lung epithelial carcinoma cell line A549 was cultured in F12K medium supplemented with 10% fetal bovine serum (FBS). The human non-small cell lung carcinoma cell line H1299 was cultured in RPMI-1640 medium supplemented with 10% FBS. The human colon tumor cell line HT29 was maintained in DMEM medium supplemented with 10% FBS. To elevate O-GlcNAcylation level, cells were treated

with 5 μM Thiamet-G (a selective OGA inhibitor, synthesized as described previously[9]) for 24 h or the indicated time period.

2.2. Plasmid construction

The shRNA-expressing lentiviral vector pLKO.1-puro, $\Delta 8.2$ packaging plasmid construct (encoding gag, pol, rev) and VSV-G plasmid were provided by Dr. William C. Hahn (Harvard Medical School and Dana-Farber Cancer Institute, Boston, MA). The human shOGT-targeting sequence is GGATGCTTATATCAATTTAGG. The synthesized oligos were annealed and inserted into the AgeI/EcoRI double digested pLKO.1-puro vector. A control shRNA oligo, which does not match with any known human coding cDNA, was used as control (named shCtrl).

2.3. Viral production and infection of target cells

Lentiviruses of pLKO.1-based vectors were produced by cotransfection with $\Delta 8.2$ and VSV-G plasmids into HEK293T cells. The stable infected cells were selected with 8 µg/ml puromycin for 2 weeks.

2.4. Soft agar assay

The soft agar assay was performed as described [10]. Briefly, cells (1×10^4) were suspended in 1 ml top agar medium (the corresponding medium supplied with 0.4% agar). The cell suspension was then overlaid onto 1.5 ml bottom agar medium (the corresponding medium supplied with 0.8% agar) in six-well tissue culture plates in triplicate. Fresh medium was added to plates every 3 days as a feeder layer. On the day 15, the colonies were captured at \times 40 magnifications.

2.5. Immunoblotting

Cells were lysed in lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP40,

1 mM EDTA, 1 mM Na₃VO₄, 10 mM NaF] containing a protease inhibitor cocktail (Roche, NJ, USA) and 5 μM PUGNAc (Toronto Research Chemicals Inc., Canada). Protein samples (50 μg) were separated by 12% SDS-PAGE and transferred to Immobilon-P membranes (Millipore, MA, USA). Antibodies to O-GlcNAcylation (CTD110.6; Abcam, Cambridge, UK), OGT (F-12; Santa Cruz, CA, USA), and GAPDH (Santa Cruz, CA, USA) were used for detection by ECL-detecting reagent (Amersham Biosciences, Buckinghamshire, England).

2.6. Immunohistochemical studies

A lung cancer tissue microarray (TMA, OD-CT-RsLug03-002; Shanghai Outdo Biotech Co., China) was constructed with formalin-fixed paraffin-embedded 31 lung cancer tissues and their corresponding adjacent lung tissues. A colon cancer TMA (OD-CT-DgCol03-002; Shanghai Outdo Biotech Co.) was constructed with formalin-fixed paraffin-embedded 31 colon cancer tissues and their corresponding adjacent lung tissues.

Immunohistochemistry was performed on the TMAs by using DAKO Liquid DAB Substrate Chromogen System (Dako Cytomation, CA, USA) and a monoclonal mouse antibody against O-GlcNAc (RL2; Affinity Bioreagents, CO, USA; 1:200), OGA (L-14; Santa Cruz, CA, USA; 1:50), and OGT (F-12; Santa Cruz, CA, USA; 1:100). Fromowitz standard was used to semi-quantitatively assess the staining of O-GlcNAcylation, OGA, and OGT [11, 12].

2.7. Cell invasion assay

These procedures were performed as described [10]. Cell invasion was assayed

using Transwell chambers (6.5 mm; Corning, NY, USA) with 8 μ m pore membranes, the upper face of the membrane was covered with 70 μ l Matrigel (1mg/ml) (BD Biosciences, NJ, USA). The lower chamber was filled with 600 μ l lower medium (medium with 20% FBS). Cells (2 × 10⁴) were suspended with 100 μ l upper medium (medium with 1% FCS) and plated into the upper chamber. After 20 hours, the number of cells appearing by crystal violet staining on the undersurface of the polycarbonate membranes was scored visually in five random fields at × 100 magnification.

2.8. Statistic analysis

Data were analyzed by the Student's t-test using the SPSS 11.0 software program. P < 0.05 was considered statistically significant. Data are present as the mean \pm standard error of the mean (SEM).

3. Results

3.1. O-GlcNAcylation level is elevated in the lung and colon tumor tissues

To investigate the role of O-GlcNAcylation in lung and colon cancer malignancy, a lung cancer TMA and a colon cancer TMA were firstly used to analyze the O-GlcNAcylation by immunohistochemistry. The lung cancer TMA was constructed with formalin-fixed paraffin-embedded 31 lung squamous cell carcinoma tissues and their corresponding adjacent lung tissues (Supplementary Table S1). The colon cancer TMA was constructed with formalin-fixed paraffin-embedded 31 colon adenocarcinoma tissues and their corresponding adjacent colon tissues (with mucosa chronic inflammation) (Supplementary Table S2). The representative tissue sections stained with an antibody to O-GlcNAc (RL2) were shown in the left and middle

panels of Figure 1A and B. The intensity of O-GlcNAcylation immunostaining was analyzed by Fromowitz standard, and the results indicated that O-GlcNAcylation was markedly enhanced in lung and colon cancer tissues compared with that in the adjacent tissues (the right panels of Fig. 1A and B).

3.2. The expression of OGT is elevated in the lung and colon tumor tissues

To reveal the mechanism underlying the elevation of O-GlcNAcylation in lung and colon tumor tissues, the expressions of OGT and OGA were analyzed by immunohistochemistry in the same lung and colon tumor tissues and their corresponding adjacent tissues. The results indicated that OGT expression was conspicuously elevated in both lung and colon tumor tissues compared with their corresponding adjacent tissues (Fig. 2A and B); then, we searched the Oncomine database (www.oncomine.org) and found that OGT mRNA levels were also significantly elevated in both lung and colon tumor tissues (data not shown) [13-16]. However, the expression of OGA showed no significant difference between the tumor tissues and their corresponding adjacent tissues (Fig. 2C and D).

3.3. O-GlcNAcylation is elevated or decreased in the lung and colon cancer cell lines

To determine whether O-GlcNAcylation played an important role in lung and colon cancer malignancy, the level of O-GlcNAcylation was decreased by the silencing of OGT and elevated by the inhibition of OGA in the lung and colon cancer cell lines. The expression of OGT shRNA (shOGT) drastically reduced OGT expression as well as O-GlcANcylation level in A549, H1299 and HT29 cells (Fig. 3A). On the other hand, a highly potent and selective OGA inhibitor (Thiamet G) could markedly

elevate the O-GlcNAcylation of A549, H1299 and HT29 cells (Fig. 3B). These two approaches enabled us to identify the effects of O-GlcNAcylation on the malignant properties of lung and colon cancer cells.

3.4. O-GlcNAcylation enhances the anchorage-independent growth of lung and colon cancer cells

To investigate whether O-GlcNAcylation affected proliferation and anchorage-independent growth of lung and colon cancer cells, MTT and soft agar colony assays were performed. OGT silencing and Thiamet-G treatment didn't significantly affect the proliferation of A549, H1299 and HT29 (data not shown). Soft agar colony assays showed that OGT silencing markedly reduced the colony formation ability, and Thiamet-G treatment significantly increased colony formation ability of A549, H1299 and HT29 cells (Fig. 4). These results indicated O-GlcNAcylation was important for maintaining the anchorage-independent growth of lung and colon cancer cells.

3.5. The effects of O-GlcNAcylation on the invasion of lung and colon cancer cells

Recently, our and others' labs have found that O-GlcNAcylation could enhance the invasion and metastasis of breast cancer cells. To investigate whether O-GlcNAcylation affects lung and colon cancer cells invasion, invasion assays were carried out. As shown in Figure 5, the invasion abilities of A549 (Fig. 5A), but not H1299 and HT29 cells (Fig. 5B and C), was significantly enhanced by Thiamet-G treatment and inhibited by OGT silencing, suggesting that O-GlcNAcylation might play important roles in lung and colon cancer metastasis in a context-dependent

manner.

4. Discussion

Here we have demonstrated that O-GlcNAcylation of lung and colon cancer tissues is significantly elevated compared with that in the corresponding adjacent tissues, consistent with our previous results and others' that O-GlcNAcylation was elevated in the primary breast tumor tissues and breast cancer cells [7, 8]. One unanswered question is what is responsible for the augmentation of O-GlcNAcylation in the cancer tissues. Theoretically, an increase of O-GlcNAcylation level could be caused by elevated UDP-GlcNAc concentration, reduced OGA expression as well as its catalytic activity, and/or increased OGT expression and activity. A physiological hallmark of tumors is the use of aerobic glycolysis (also known as the Warburg effect) instead of oxidative phosphorylation to produce ATP [1]. This metabolic pathway has elevated rates of glucose uptake [17]. Additionally, cancer cells are addicted to glutamine; the rate of glutamine consumption in tumors is 10-fold higher than in normal cells [18, 19]. Approximately 2–3% of total cellular glucose is funneled into the hexosamine biosynthetic pathway (HBP) to produce UDP-N-acetylglucosamine (UDP-GlcNAc) [20]. The HBP shares its first two steps with glycolysis; first, hexokinase phosphorylates glucose to produce glucose 6-phosphate, which is then converted into fructose 6-phosphate by phosphoglucoseisomerase. At this point the pathways diverge, GFAT1 catalyzes the irreversible transfer of the amino group from glutamine and the isomerization of fructose 6-phosphate into glucosamine 6-phosphate and glutamate [1]. Intuitively, increased cellular glucose as well as

UDP-GlcNAc glutamine shunted the HBP would increase into levels. O-GlcNAcylation is catalyzed by OGT, whose activity is tightly dependent on the substrate concentration of UDP-GlcNAc in the cell. Previously, Reginato's group reported tumor cell lines that mimic metastatic tumors showed an increase in OGT protein expression; moreover, they found that OGT mRNA levels were significantly elevated in breast tumor tissues [7]. Our data indicate that OGT expression is markedly enhanced in cancer tissues compared with that in corresponding adjacent tissues; coordinately we found that OGT mRNA levels were also significantly elevated in both lung and colon tumor tissues. The OGT expression elevation as well as probably raised activity could results in O-GlcNAcylation elevation in tumor tissues. At the same time, the opposing enzyme (OGA) was evaluated at the protein level. The results show that the expression of OGA has no significant difference between the tumor tissues and their corresponding adjacent tissues. Unfortunately, the difficulty of the amount of available fresh resection samples limited our study to the UDP-GlcNAc concentration, OGT and OGA activity in cancer tissues. This is an important issue that we are attempting to address in future studies. Nonetheless, our data indicate that the increase of OGT expression may be one of the main causes of O-GlcNAcylation elevation in tumor tissues.

Several studies have shown that O-GlcNAcylation acts as a key cellular regulator in cell cycle and survival. Deletion of OGT in ES cells is lethal [21], and OGT tissue-specific mutation results in the loss of O-GlcNAcylation in specific tissues and causes T-cell apoptosis and fibroblast growth arrest [22]. It was also reported that

OGT inhibition prevented G2/M transition in Xenopus laevis oocytes [23]. Recently, Caldwell et al. showed that reduction of O-GlcNAcylation through RNA interference of OGT in breast cancer cells leads to inhibition of tumor growth both *in vitro* and *in vivo* [7]. Anchorage-independent growth is thought to be among the fundamental properties of malignant cells [10]. In the present study we demonstrated that O-GlcNAcylation significantly enhanced anchorage-independent growth of lung and colon cancer cells, suggesting that O-GlcNAcylation increase might initiate and promote lung and colon cancer formation. Additionally, our data indicate O-GlcNAcylation enhances the invasion of some types of cancer cells. So, the roles and mechanisms of O-GlcNAcylation in cancer invasion might be context-dependent and influenced by cell type and oncogenic events acquired during the course of tumor evolution.

In response to multiple forms of injury, cells remodel their metabolic and signaling pathways to promote survival – the so-called cellular stress response [24, 25]. As a stress sensor, O-GlcNAcylation levels increase rapidly and dynamically in multiple mammalian cell lines in response to numerous forms of cellular stress (heat stress, oxidative stress, hypoxia, ischemia reperfusion injury, and trauma hemorrhage injury) [26]. Notably, raising O-GlcNAcylation levels by either inhibition of O-GlcNAcase, overexpression of OGT, rendered cells more thermotolerant [27]. or O-GlcNAcylation is protective for cells under oxidative stress through FOXO4 O-GlcNAcylation and enhancing its transcriptional activity [28]. Conversely, lowering levels of O-GlcNAcylation by altering OGT levels or blocking the HBP

sensitized cells to apoptotic stimuli [26]. The inappropriate proliferation of primary tumor cells is challenged by multiple layers of mechanisms that suppress tumor formation. Several of these barriers include reactive oxygen species, extracellular matrix components, basement membranes, the limited availability of nutrients and oxygen, and attack by the immune system [29]. Furthermore, tumor progression toward metastasis requires a further defense against microenvironmental death stimuli, such as nutrient deprivation and hypoxia, alterations in extracellular adhesions, changes in cell shape during invasion and exposure to novel stromal microenvironments [29]. Therefore, O-GlcNAcylation elevation might be "beneficial" for cancer cells to protect against a wide range of apoptotic stimuli, promoting tumor formation and progression. At the same time, the protective effects of O-GlcNAcylation may also reduce tumor sensitivity to ionizing radiation and a number of DNA damaging chemotherapeutic agents. Thus, O-GlcNAcylation suppression, in combination with radiation and the conventional antitumor agents, may be of clinical interest for treatment of certain types of tumors.

5. Conclusion

In this study, immunohistochemical studies indicated that O-GlcNAcylation and OGT of lung and colon cancer tissues were significantly elevated compared with that in the corresponding adjacent tissues. Furthermore, soft agar assay demonstrated that O-GlcNAcylation could enhance the anchorage-independent growth of lung and colon cancer cells. However, O-GlcNAcylation might play different roles in invasion depending on cancer cellular context. Altogether, these results suggest that

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O-GlcNAcylation might play important roles in lung and colon cancer formation and progression, and may be a valuable target for diagnosis and therapy of cancer.

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Fig. 1. O-GlcNAcylation level is elevated in the lung and colon tumor tissues. Representative lung (A) and colon tumor tissue sections (B) stained with an antibody to O-GlcNAc (RL2). The staining intensity was graded by an experienced pathologist and analyzed by Fromowitz standard. ***P < 0.001 (paired sample t-test), mean \pm SEM.





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Fig. 2. The expressions of OGT and OGA were examined in the lung and colon tumor tissues. (A and B), Representative lung (A) and colon tumor tissue sections (B) stained with an antibody to OGT. (C and D), Representative lung (C) and colon tumor tissue sections (D) stained with an antibody to OGA. The staining intensity was graded by an experienced pathologist and analyzed by Fromowitz standard. ***P < 0.001 (paired sample t-test), mean \pm SEM.



Fig. 3. O-GlcNAcylation was decreased by OGT silencing or elevated by Thiamet-G treatment. (A), The expression of OGT and O-GlcNAcylation level in OGT silenced A549, H1299, and HT29 cells were detected by immunoblotting with antibody to OGT and to O-GlcNAc (RL2). (B), A549, H1299, and HT29 cells were pre-treated or not with 5 μ M Thiamet-G for 24 h and applied for immunoblotting with an antibody to O-GlcNAc (RL2).



Fig. 4. O-GlcNAcylation enhances anchorage-independent growth of lung and colon cancer cells. The anchorage-independent growth of A549 (A), H1299 (B) and HT29 (C) cells were analyzed by soft agar assay. $(5 \times 10^3 \text{ cells/well})$



Fig. 5. The effect of O-GlcNAcylation on lung and colon cancer cells invasion. (A) Thiamet-G treatment significantly enhanced the cell invasion of A549 cells, and OGT silencing inhibited the cell invasion. (B and C), Thiamet-G treatment and OGT silencing have no effect on the cell invasion of H1299 (B) and HT29 cells (C). **P <

