Original Article

Associations between Expression Levels of O-GlcNAc Transferase (OGT) and Chemo-Response in Osteosarcoma

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Objective: The present study aimed to investigate global levels of O-GlcNAcylation (O-GlcNAc), expressions of O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) in osteosarcoma and to explore a prognostic marker.

Materials and Methods: The authors studied in three independent experiments. Western blot analysis was performed to detect the expressions of O-GlcNAcylated proteins, OGT, and OGA in cell lines derived from seven patients with osteosarcoma stage IIB. The band intensities were analyzed using Scion Image software. The correlations between clinicopathologic characters of osteosarcoma cases and expression levels of O-GlcNAcylated proteins, OGT, and OGA were analyzed by using Student's t-test.

Results: The results demonstrated a tendency of higher OGT expression in primary osteosarcoma cells. Furthermore, the level of OGT was elevated in patients who poorly responded to chemotherapy and had shorter survival time ($p<0.05$). The present study revealed a promising use of OGT as a prognostic marker in osteosarcoma.

Conclusion: The present study showed significant higher levels of OGT in osteosarcoma patients who poorly responded to chemotherapy. Furthermore, levels of OGT relate to survival time of osteosarcoma. These findings suggest a role for OGT in chemo-response of osteosarcoma cells. The present study was performed in a limited number of cases, thereby the finding requires further validation in a larger cohort.

Keywords: Osteosarcoma, O-GlcNAcylation, O-GlcNAc transferase (OGT), O-GlcNAcase (OGA)

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Osteosarcoma is the primary malignant bone tumor that commonly affects children, adolescents, and young adults. Osteosarcoma usually occurs in the metaphysis of long bones, and most commonly occurs in the distal femur, proximal tibia, or humerus[(1)]. The incidence has been reported at 1.6 to 2.8 per million of children under 15 years and are more common in male than female at a ratio of 1.6:1[(2)]. Due to the complexity of the genomic background and heterogeneity, the causes of osteosarcoma remain unclear. The current treatment strategy for osteosarcoma includes neoadjuvant chemotherapy, followed by surgical removal of the primary tumor along with all clinically evident metastatic disease, plus the addition of adjuvant chemotherapy.

O-GlcNAcylation (O-GlcNAc) is one of post-translation modifications (PTM) of protein. This PTM play important roles in regulating various functions of proteins through an addition of N-acetylglucosamine (GlcNAc) to hydroxyl group of serine or threonine residues of target proteins. O-GlcNAc is regulated by a pair of enzymes, O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA), which add and remove a

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Table 1. Clinicopathologic characteristics of osteosarcoma cases

Case	Age at diagnosis (years)	Sex	Enneking stage	Chemotherapy	Tumor necrosis (%)	Status	Metastasis (lung)
OS1	9	Female	IIb	Carbo/Doxo	90.0	Alive	No
OS2	15	Female	IIb	Adria/Cis	90.0	Alive	No
OS3	17	Female	IIb	Cis/Adria	80.0	Alive	No
OS4	14	Male	IIb	Carbo/Doxo	90.0	Alive	No
OS5	21	Male	IIb	Cis/Adria	15.0	Death	Yes
OS6	61	Female	IIb	Cis/Adria	35.5	Death	Yes
OS7	5	Female	IIb	Carbo/Adria Cis/Doxo HD-MTX	10.0	Death	Yes

OS=osteosarcoma primary cells; Carbo=carboplatin; Doxo=doxorubicin; Adria=adriamycin; Cis=cisplatin; HD-MTX=high-dose methotrexate

O-GlcNAc molecule from the proteins, respectively. Uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) is a substrate of O-GlcNAc process and an end-product of hexosamine biosynthesis pathway (HBP). HBP pathway is one of the glucose metabolic pathways in which about 2% to 5% of glucose enters the HBP[3,4]. Glucose is imported into the cell via a glucose transporter, then phosphorylated by hexokinase to be glucose-6-phosphate (Glc-6-P) and then converted into fructose-6-phosphate (Fruc-6-P) by Glc-6-P isomerase. Glutamine donates the amino group to form glucosamine-6-phosphate (GlcN-6-P), using glucosamine:fructose-6-phosphate aminotransferase (GFAT). Acetyl-CoA is added to GlcN-6-P by glucosamine-6-phosphate N-acetylglucosamine transferase to generate N-acetylglucosamine-6-phosphate (GlcNAc-6-P) and converted to N-acetylglucosamine-1-phosphate (GlcNAc-1-P) by phosphoacetylglucosamine mutase. Finally, uridine-5'-triphosphate (UTP) is phosphorylased by UDP-GlcNAc pyrophosphorylyse (UAP) to create UDP-GlcNAc. The rate limiting enzyme of HBP is GFAT1. This is due to feedback inhibition by the products including both GlcN-6-P and the final product: UDP-GlcNAc[5].

O-GlcNAc governs diverse intracellular processes such as translation, transcription, cell cycle regulation, and epigenetic control of gene expression in response to nutrient and cellular stress[6,7]. Balance of the enzymes regulating O-GlcNAc is required to maintain normal cellular function. High level of O-GlcNAc has been found in many diseases such as diabetes, Alzheimer disease, and cancers.

In various types of cancers, increased level of O-GlcNAc seem to be involved with tumor invasion and distant metastasis. Several lines of research suggested that decreasing levels of O-GlcNAc by OGT inhibition or OGT knockdown could reduce migration and invasion of cancer cells in vitro and decrease distant metastasis in vivo in breast, prostate, and colorectal cancer. Champattanachai et al[8] showed an increase of OGT protein and O-GlcNAc levels relating to the histological grade of breast tumors. Furthermore, Slawson et al[9] showed an association of increased OGA activity and breast cancer tumor grade.

Until now, there is a lack of information about O-GlcNAc-modified proteins and their roles in osteosarcoma. In the present study, the authors studied the level of O-GlcNAc, expression of OGT and OGA in patient-derived osteosarcoma cells using immunoblotting analysis. The authors also explored an association of O-GlcNAc profile and its regulating enzymes with clinical outcomes of the patients.

Materials and Methods

Patients characteristics

Primary osteosarcoma cells were derived from seven selected osteosarcoma patients diagnosed as stage IIB osteosarcoma and treated at Maharaj Nakorn Chiang Mai Hospital, Thailand. Patients had been treated with a standard neoadjuvant regimen or underwent surgery at Maharaj Nakorn Chiang Mai Hospital. Exclusion criteria were patient who died from other systemic disease or accidental trauma and patient who had been diagnosed with serious systemic diseases that restricted tumor surgery.

The Research Ethics Committee of the Faculty of Medicine, Chiang Mai University, approved the present study. Clinicopathologic characteristics, including age, gender, Enneking staging, chemotherapy, percentage of tumor necrosis, and metastasis status is shown in Table 1. The response of treatment, defined by the tumor necrosis in the previous study, showed

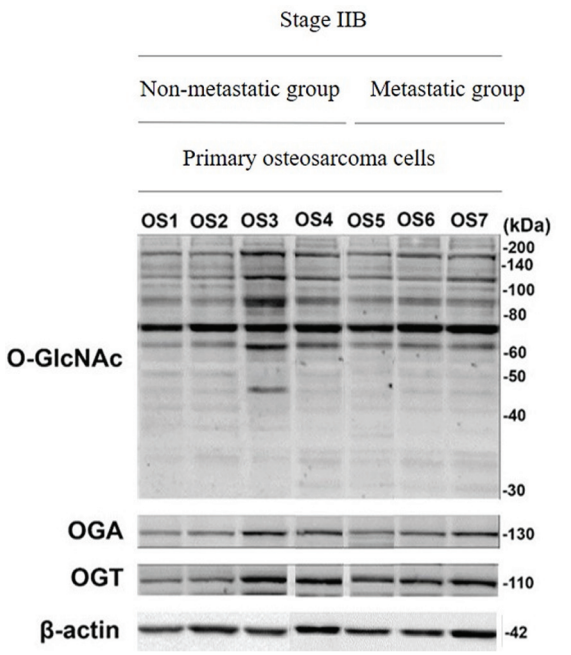

Figure 1. Immunoblots of O-GlcNAcylation, OGT, and OGA.

OS=osteosarcoma primary cells

a significant greater result of 90% tumor necrosis[10]. Metastasis is indicated if the cancer was identified in distant organ by computed tomography.

Primary cells and culture condition

All primary osteosarcoma cells were derived from musculoskeletal, sciences, and Translational Research Biobank. Primary osteosarcoma cells were isolated from chemo-naïve tissues of the seven patients[11] at Maharaj Nakorn Chiang Mai Hospital. All cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Invitrogen™) and 1% penicillin and streptomycin (Invitrogen™). All primary cells were incubated at 37°C in a humidified chamber with 5% CO_2. Culture medium was refreshed every two days until 80% confluence.

Protein extraction and western blotting analysis

Whole cell lysates were extracted using radio-immunoprecipitation assay (RIPA) buffer supplemented with protease inhibitor and Thimet-G. Total protein concentration in the whole cell lysates was first determined by Bradford assay according to the manufacturer's protocol. The absorbance is then measured by using microplate reader (Sunrise™, Tecan). Each whole cell lysate sample was denatured by heating at 100°C for five minutes in a sampling buffer. Ten µg of protein of whole cell lysates reduced for titrate error were separated on sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. After transferring, the membranes were blocked with 1% casein in phosphate buffered saline (PBS), buffer at room temperature for one hour with gentle agitation. Then, the membranes were incubated with primary antibody against β-Actin (Santa Cruz Biotechnology, Inc.), O-GlcNAc (RL2, Santa Cruz Biotechnology, Inc.), OGT (F-12, RL2, Santa Cruz Biotechnology, Inc.) or OGA (ab124807, Abcam) at 4°C for 16 to 18 hours. The membranes were washed three times with PBS buffer for 15 minutes each time. Secondary antibodies in 1% casein PBS, including horseradish peroxidase (HRP)-conjugated antibodies against mouse immunoglobulins or rabbit immunoglobulins, where then added into the membranes at dilution 1:5,000 for one hour at room temperature. After washing with PBS buffer, reserve chemiluminescent reagent (KPL, Gaithersburg, MD, USA) was used as a substrate. The signal was later captured by a CCD camera attached to the ChemiDoc XRS gel documentation system (Bio-Rad Laboratories). This protocol was repeated three times.

Image analysis

Scion Image software was used to quantify the intensities of all O-GlcNAcylated protein, OGT, and OGA bands in the same level by using line and measured the area under the graph. Level of O-GlcNAcylated proteins, OGT, and OGA of each sample was normalized by β-Actin expression.

Statistical analysis

Statistical analysis was performed using GraphPad Prism, version 8.0.1 (GraphPad Software, Inc., San Diego, CA, USA). The different levels of O-GlcNAc, OGT, and OGA among osteosarcoma cells and association to clinicopathological characteristic of patients were tested using Student's t-test.

Results

Level of O-GlcNAc, OGT, and OGA expression levels in osteosarcoma cells

Levels of O-GlcNAc, OGT, and OGA were examined in seven cases of primary osteosarcoma cells using immunoblotting technique as shown in Figure 1. The results revealed variable levels of O-GlcNAc in the osteosarcoma cells. The results showed a tendency of higher OGT expression in the

Table 2. Level of O-GlcNAc and expression of OGT and OGA in primary osteosarcoma cells

Factor	Total patients	Expression levels; mean±SD					
		O-GlcNAcylated proteins	p-value	OGT	p-value	OGA	p-value
Distant invasion			0.20		0.17		0.52
Non-metastasis	4	5.32±1.57		0.51±0.26		0.58±0.26	
Metastasis	3	6.96±1.25		0.76±0.10		0.69±0.06	
Chemoresistance			0.06		0.02*		0.04*
Good responders (tumor necrosis ≥90%)	3	4.77±1.37		0.41±0.21		0.47±0.15	
Poor responders (tumor necrosis <90%)	4	6.97±1.25		0.77±0.08		0.75±0.13	
Status			0.20		0.17		0.52
Alive	4	5.32±1.57		0.51±0.26		0.58±0.26	
Died	3	6.96±1.25		0.76±0.10		0.69±0.06	

OGT=O-GlcNAc transferase; OGA=O-GlcNAcase

* p<0.05 is considered significant

metastasis primary osteosarcoma cells compared with the non-metastasis primary osteosarcoma.

Correlation of levels of O-GlcNAc, OGT, OGA, and clinicopathological factors

O-GlcNAc and OGT levels were elevated in metastasis, poor responders, and dead group (Table 2). Furthermore, the osteosarcoma patients who poorly responded to chemotherapy (tumor necrosis of less than 90%) expressed higher levels of OGT compared with the good responders (tumor necrosis of 90% or more) (p=0.04) as shown in Table 2.

Discussion

From the current knowledge, the present study is the first study that investigated levels of O-GlcNAcylated proteins, OGT, and OGA in osteosarcoma. Levels of O-GlcNAc as well as expression of OGT and OGA were observed in primary osteosarcoma cells derived from chemo-naïve tissues of osteosarcoma patients.

To minimize the inconsistence of treatment decision and clinical outcome, the study focused to observe protein expression in osteosarcoma with clinical stage IIB, which is the most commonly detected.

Accumulating evidences revealed increasing levels of O-GlcNAc and OGT in various diseases including cancers. The study of pancreatic tumors indicated higher O-GlcNAc and OGT levels, together with down-regulated expression of OGA in cancerous tissues in comparison to normal tissues[12]. Lynch et al showed that O-GlcNAc and OGT levels were higher in prostate cancer cells compared with normal cells lines[13]. Furthermore, Itkonen et al suggested that OGT inhibitor could be used as a target therapy for prostate cancer, in that OGT was upregulated in prostate cancer and inhibition of OGT reduced c-MYC protein stability[14]. Besides from prostate cancer, it is reported that O-GlcNAc levels and an expression of OGT was upregulated in breast cancer cells[8,15]. Moreover, Gu et al also found that level of O-GlcNAc was upregulated in breast cancer tissues and metastatic lymph node[16]. Interestingly, Champattanachai et al found that upregulated OGT was related with degree of cell differentiation and OGT knockdown of breast cancer cell lines resulting in decrease colony formation, but not affecting cell viability or cell proliferation[8].

The present study revealed that OGT level was significantly associated with chemo-response in stage IIB osteosarcoma patients, in which expression of OGT was higher in patients who poorly responded to chemotherapy compared to good responders. Roles of OGT on an aggressiveness of cancers have been widely studied. Krzeslak et al measured O-GlcNAc cycling enzymes messenger RNA (mRNA) levels in 76 endometrial cancer patients using real-time reverse transcription polymerase chain reaction (RT-PCR) analysis. Both OGT and OGA mRNA expression were significantly higher in high grade tumors compared with low grade groups. They also showed an association between OGT and OGA expression and myometrial invasion[17], indicating that OGT was related to aggressiveness and prognosis of cancer cells. Furthermore, a significant decrease of in vitro cell migration and invasion upon OGT knockdown was observed in three different breast cell lines[16].

From the study of Caldwell, inhibition of OGT for treatment in MCF-10A-ErbB2 and MDA-MB-231 breast tumor cells by using Transwell assays shows a decrease of cell migration[15]. OGT knockdown in the PC3-ML prostate tumor cell line also reduced their ability to migrate compared with control cells[13]. Another study in prostate tumor cells demonstrated that OGT knockdown decreased invasiveness of LNCaP cells[18].

Correlation of levels of O-GlcNAc, OGT as well as OGA and status of patient after diagnosis were investigated in the present study. The results showed a relationship between OGT and O-GlcNAc level with status after diagnosis of stage IIB-osteosarcoma patients but not OGA. Higher level of OGT and O-GlcNAc were associated with dead patients. Elevated O-GlcNAc and altered expression of its cycling enzymes were previously observed in nearly all cancer types. OGT overexpression was associated with prostate cancer progression and recurrence, and high O-GlcNAc immunohistochemistry (IHC) staining was an independent prognostic factor for poor survival[18]. Lin et al showed that the high-OGT group of patients with lung adenocarcinoma had shorter recurrence free survival and overall survival in comparison with the low-OGT[19]. Pham et al also showed that patients with high OGT expression in diffuse large B-cell lymphoma had the worst progression free survival compared with patients with low or intermediate OGT mRNA expression[20]. Recent study from Xu et al that the 3-year overall survival rate in patient with high OGT expression was lower than that with low expression[21].

The present study was performed in limited number of cases, thereby the finding required further validation in a larger cohort.

Conclusion

From a previous study, increased OGT levels were used as prognostic factors in various type of cancer but there are still lack of information about OGT levels and their roles in osteosarcoma. The present study showed significant higher levels of OGT in osteosarcoma cells line isolated from patients who poorly responded to chemotherapy. Furthermore, level of OGT relates to aggressiveness of osteosarcoma. These findings suggest that the intensive study of O-GlcNAc in specific protein might reveal the pathogenesis of osteosarcoma.

What is already known on this topic?

Several studies reported the O-GlcNAc protein and the increased OGT levels were used as prognostic factors in various type of cancer.

What this study adds?

This study showed significant higher levels of OGT in osteosarcoma patients that poorly responded to chemotherapy.

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Ethics approval and consent to participate

Ethical approval and clearance was obtained from the Research Ethics Committee of Faculty of Medicine, Chiang Mai University (Research ID: 2717, Study code: ORT-2557-02717).

Conflicts of interest

The authors declare no conflict of interest.

References

1. Isakoff MS, Bielack SS, Meltzer P, Gorlick R. Osteosarcoma: Current treatment and a collaborative pathway to success. J Clin Oncol 2015;33:3029-35.
2. Craft AW. Osteosarcoma and chondrosarcoma. In: Voûte PA, Barrett A, Lemerle J, editors. Cancer in children. Berlin, Heidelberg: Springer; 1992. p. 282-94.
3. Marshall S, Bacote V, Traxinger RR. Discovery of a metabolic pathway mediating glucose-induced desensitization of the glucose transport system. Role of hexosamine biosynthesis in the induction of insulin resistance. J Biol Chem 1991;266:4706-12.
4. Bouché C, Serdy S, Kahn CR, Goldfine AB. The cellular fate of glucose and its relevance in type 2 diabetes. Endocr Rev 2004;25:807-30.
5. Broschat KO, Gorka C, Page JD, Martin-Berger CL, Davies MS, Huang Hc HC, et al. Kinetic characterization of human glutamine-fructose-6-

phosphate amidotransferase I: potent feedback inhibition by glucosamine 6-phosphate. J Biol Chem 2002;277:14764-70.
6. Hart GW, Slawson C, Ramirez-Correa G, Lagerlof O. Cross talk between O-GlcNAcylation and phosphorylation: roles in signaling, transcription, and chronic disease. Annu Rev Biochem 2011;80:825-58.
7. Ma Z, Vosseller K. O-GlcNAc in cancer biology. Amino Acids 2013;45:719-33.
8. Champattanachai V, Netsirisawan P, Chaiyawat P, Phueaouan T, Charoenwattanasatien R, Chokchaichamnankit D, et al. Proteomic analysis and abrogated expression of O-GlcNAcylated proteins associated with primary breast cancer. Proteomics 2013;13:2088-99.
9. Slawson C, Pidala J, Potter R. Increased N-acetyl-beta-glucosaminidase activity in primary breast carcinomas corresponds to a decrease in N-acetylglucosamine containing proteins. Biochim Biophys Acta 2001; 1537:147-57.
10. Raymond AK, Chawla SP, Carrasco CH, Ayala AG, Fanning CV, Grice B, et al. Osteosarcoma chemotherapy effect: a prognostic factor. Semin Diagn Pathol 1987;4:212-36.
11. Pruksakorn D, Teeyakasem P, Klangjorhor J, Chaiyawat P, Settakorn J, Diskul-Na-Ayudthaya P, et al. Overexpression of KH-type splicing regulatory protein regulates proliferation, migration, and implantation ability of osteosarcoma. Int J Oncol 2016;49:903-12.
12. Ma Z, Vocadlo DJ, Vosseller K. Hyper-O-GlcNAcylation is anti-apoptotic and maintains constitutive NF-kappaB activity in pancreatic cancer cells. J Biol Chem 2013;288:15121-30.
13. Lynch TP, Ferrer CM, Jackson SR, Shahriari KS, Vosseller K, Reginato MJ. Critical role of O-Linked beta-N-acetylglucosamine transferase in prostate cancer invasion, angiogenesis, and metastasis. J Biol Chem 2012;287:11070-81.
14. Itkonen HM, Minner S, Guldvik IJ, Sandmann MJ, Tsourlakis MC, Berge V, et al. O-GlcNAc transferase integrates metabolic pathways to regulate the stability of c-MYC in human prostate cancer cells. Cancer Res 2013;73:5277-87.
15. Caldwell SA, Jackson SR, Shahriari KS, Lynch TP, Sethi G, Walker S, et al. Nutrient sensor O-GlcNAc transferase regulates breast cancer tumorigenesis through targeting of the oncogenic transcription factor FoxM1. Oncogene 2010;29:2831-42.
16. Gu Y, Mi W, Ge Y, Liu H, Fan Q, Han C, et al. GlcNAcylation plays an essential role in breast cancer metastasis. Cancer Res 2010;70:6344-51.
17. Krześlak A, Wójcik-Krowiranda K, Forma E, Bieńkiewicz A, Bryś M. Expression of genes encoding for enzymes associated with O-GlcNAcylation in endometrial carcinomas: clinicopathologic correlations. Ginekol Pol 2012;83:22-6.
18. Kamigaito T, Okaneya T, Kawakubo M, Shimojo H, Nishizawa O, Nakayama J. Overexpression of O-GlcNAc by prostate cancer cells is significantly associated with poor prognosis of patients. Prostate Cancer Prostatic Dis 2014;17:18-22.
19. Lin YC, Lin CH, Yeh YC, Ho HL, Wu YC, Chen MY, et al. High O-linked N-acetylglucosamine transferase expression predicts poor survival in patients with early stage lung adenocarcinoma. Oncotarget 2018;9:31032-44.
20. Pham LV, Bryant JL, Mendez R, Chen J, Tamayo AT, Xu-Monette ZY, et al. Targeting the hexosamine biosynthetic pathway and O-linked N-acetylglucosamine cycling for therapeutic and imaging capabilities in diffuse large B-cell lymphoma. Oncotarget 2016;7:80599-611.
21. Xu D, Wang W, Bian T, Yang W, Shao M, Yang H. Increased expression of O-GlcNAc transferase (OGT) is a biomarker for poor prognosis and allows tumorigenesis and invasion in colon cancer. Int J Clin Exp Pathol 2019;12:1305-14.