Correlation between O-GlcNAcylation and Expression of OGT and OGA in Postoperative Osteosarcoma Chemotherapy

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Abstract

O-GlcNAcylation is a post-translational modification of a protein that plays an important role in cancer pathogenesis and chemoresistance mechanisms. Osteosarcoma is the most common malignant bone tumor that occurs in children and young adults. If the cancer is diagnosed at the localized stage, the 5-year survival rate is > 65%; however, the rate decreases in metastatic cancer and poor responder chemotherapy. The genomic background is complex, and the implications of O-GlcNAc remain unclear in osteosarcoma. Aberrant O-GlcNAc was demonstrated in osteosarcoma using immunohistochemistry of O-GlcNAcylated modified proteins, O-GlcNAc transferase, and O-GlcNAcase to assess preoperative and postoperative chemotherapeutic specimens derived from the first 20 diagnosed osteosarcoma patients. The specimens were fixed in formalin and paraffin-embedded bone tissue sections. Differentiation expression levels of the O-GlcNAcylated proteins, O-GlcNAc transferase, and O-GlcNAcase between preoperative and postoperative chemotherapeutic specimens were analyzed using the Wilcoxon matched-pairs signed-rank test. Results presented significantly lower immunoreactivity scores of O-GlcNAcylated proteins (p=0.0083), while a lower O-GlcNAc transferase expression (p=0.2634) was observed in post-chemotherapeutic osteosarcoma specimens. In contrast, O-GlcNAcase expression showed higher levels in post-chemotherapeutic osteosarcoma specimens (p=0.7680). The findings indicated that O-GlcNAc was possibly related to tumorigenesis and may be useful for describing chemoresistance mechanisms of osteosarcoma.

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

1. Introduction

Osteosarcoma is the most common malignant osseous neoplasm that affects children, adolescents, and young adults. Osteosarcoma presents in the metaphysis of long bones as the distal femur (43%), proximal tibia (23%), or humerus (10%) (Isakoff, Bielack, Meltzer, & Gorlick, 2015). An incidence of osteosarcoma has been reported at 1.6-2.8 per one million children under 15 years and is more common in males than females at a ratio of 1.6:1 (Craft, 1992). The 5-year survival rate of people with osteosarcoma is > 65% if the cancer is diagnosed at the localized stage (Misaghi, Goldin, Awad, & Kulidjian, 2018). The genomic background and heterogeneity of osteosarcoma are complex, and the causes of the disease remain unclear. The current treatment for osteosarcoma includes neoadjuvant chemotherapy, followed by surgical removal of the primary tumor along with additional adjuvant chemotherapy.

O-GlcNAcylation is an important post-translational modification that plays an important role in regulating various functions of proteins by the addition of N-acetylglucosamine (GlcNAc) to the hydroxyl group of serine or threonine residues of target proteins. O-GlcNAc is operated by a pair of enzymes, O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA), which add and remove an O-GlcNAc molecule from

the proteins, respectively. A modification of O-GlcNAc has been implicated in many human diseases such as Type II diabetes mellitus, neurodegenerative diseases, and cancers (Butkinaree, Park, & Hart, 2010; Hart, Slawson, Ramirez-Correa, & Lagerlof, 2011; Zachara & Hart, 2006). Differentiation expressions of OGT, OGA, and levels of UDP-GlcNAc are associated with the modification of O-GlcNAc and reported to be related to the development and progression of many cancers. Caldwell et al. reported that O-GlcNAcylation levels and an expression of OGT were upregulated in breast cancer cells (Caldwell et al., 2010). Champattanachai et al. found that O-GlcNAcylation level and OGT expression increased in breast cancer tissues compared with benign breast tumors, and upregulated OGT was related to the degree of cell differentiation. OGT knockdown of breast cancer cell lines resulted in decreased colony formation but not affected cell viability or cell proliferation (Champattanachai et al., 2013). Kamigaito et al. found that overexpression of O-GlcNAc in prostate cancer reduced the overall survival, and knockdown OGT in the prostate cancer cell led to a decrease in cell growth and invasion but did not alter E-cadherin expression (Kamigaito et al., 2014). Phoomak et al. found that cholangiocarcinoma tissues showed elevated O-GlcNAcylation levels and OGT expression but decreased the OGA expression under histologic examination (Phoomak et al., 2012). However, insufficient information exists concerning O-GlcNAc and its role in osteosarcoma. Our previous study suggested that OGT played an important role in the chemotherapeutic response of osteosarcoma cells with significantly higher levels of OGT in osteosarcoma patients who responded poorly to chemotherapy, while levels of OGT related to the survival time of osteosarcoma (Rattanakuntee et al., 2020). This study involved a small sample number, and larger sample size is needed to gain further insights. Here, the expressions of OGT and OGA and levels of O-GlcNAc were examined in 20 bone specimens, histopathologically diagnosed as osteosarcoma using immunohistochemistry, and the correlations were determined between pretreatment and post-chemotherapy treatment osteosarcoma.

2. Objectives

This study explored the expression of OGT and OGA and levels of O-GlcNAc in 20 osseous specimens diagnosed as osteosarcoma using immunohistochemistry protocol before and after adjuvant chemotherapy.

3. Materials and Methods

3.1 Formalin-fixed paraffin-embedded tissues

Twenty formalin-fixed paraffin osseous specimens were obtained from the osteosarcoma patients who were treated and diagnosed between 1999 and 2020 at the Department of Orthopedic Surgery, Maharaj Nakorn Chiang Mai Hospital, Thailand.

All patients received standard treatments including surgical tumor resection, followed by neoadjuvant chemotherapy. Tumor staging was classified following the Enneking classification (Enneking, Spanier, & Goodman, 1980).

3.2 Determination of immunoreactive score (IRS)

The expression levels of OGT, OGA, and O-GlcNAc were examined using the standard protocol of immunohistochemistry (IHC) staining. Each 3-μm osseous specimen section was detected using a Ventana automated stainer and Ultraview Universal DAB Detection Kit (Ventana Medical Systems, Tucson, AZ, USA). The osseous specimen sections were incubated with anti-OGT (11576-2-AP, Protein Technologies, Inc., Tucson, AZ, USA), anti-OGA (ab124807, Abcam Cambridge, MA, USA), and anti-O-GlcNAc (MA1-072, Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 32 minutes at a room temperature. Each sample was first oriented using a bright field microscope (Carl Zeiss, Oberkochen, Germany) at 10X magnification, and three images were taken of each section using an attached digital camera (Axiocam ICc5, Carl Zeiss) at 40X magnification. The positive control was derived from colon, breast, and lung cancers (Chaiyawat et al., 2018). The immunoreactive score (IRS) gives a range of 0-12 from the multiplication between staining intensity score (0-3) as 0 = no staining, 1 = weak (light yellow), 2 = moderate (brown), and 3 = strong (dark brown), whereas positive cells proportion score (0-4) as 0 = 0%, 1 = <10%, 2 = 10-50%, 3 = 51-80%, and 4

= >80%. All specimens were examined by two independent observers before IRS analysis (Fedchenko & Reifenrath, 2014).

3.3 Statistic analysis

Statistical analysis was performed using GraphPad Prism, version 9.0.0 (GraphPad Software, Inc., San Diego, CA, USA). Mean immunoreactive scores (IRS) were compared between pretreatment specimens and post-chemotherapeutic specimens using the Wilcoxon matched-pairs signed-rank test.

4 Results and Discussion

Levels of O-GlcNAcylation decreased in post-chemotherapeutic tumors

To investigate the role of O-GlcNAc in the treatment protocol of osteosarcoma, the levels of O-GlcNAcylated proteins in 20 osseous specimens were analyzed using immunohistochemistry, as shown in Figure 1. A weak immunoreactivity of O-GlcNAc was observed in almost every post-chemotherapeutic specimen with an immunoreactivity score of O-GlcNAcylated proteins significantly lower than the pretreated osseous specimens.

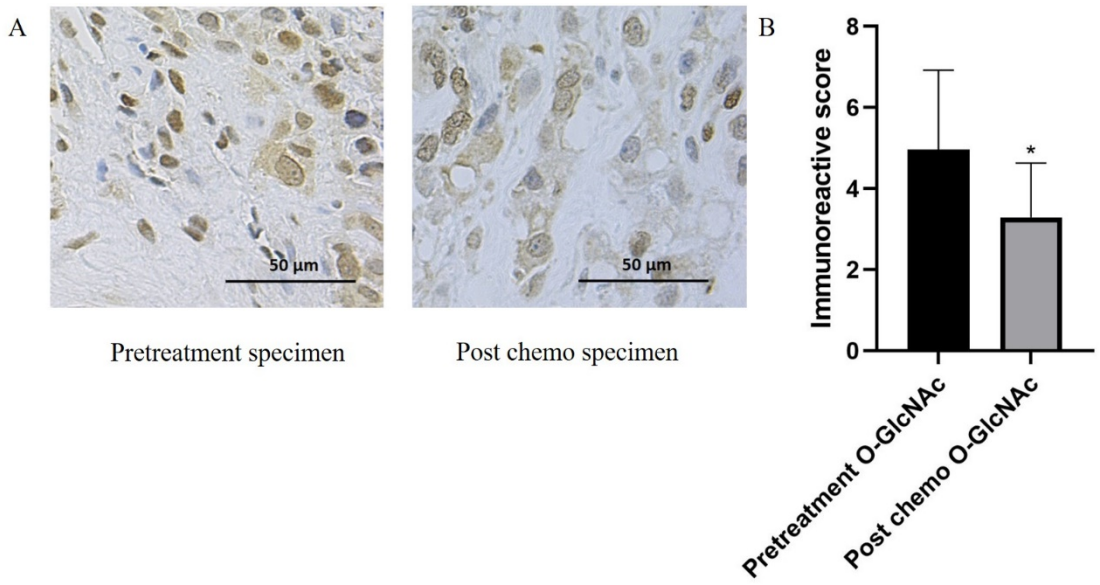

Figure 1 A reduction of O-GlcNAcylation in the post chemotherapeutic osteosarcoma specimens. A scale bar represents 50 µm. *P < 0.05.

The levels of O-GlcNAc were determined in 20 specimens before and after the adjuvant chemotherapy for osteosarcoma using immunohistochemistry staining of O-GlcNAcylated proteins. (A) The post-chemotherapeutic osteosarcoma specimens showed a weak immunoreactivity of O-GlcNAcylated protein whereas those of the pretreated osseous specimens had a high O-GlcNAcylated protein. (B) Immunoreactivity scores of O-GlcNAcylated proteins observed in the post-chemotherapeutic osteosarcoma specimens were significantly lower than observed in the pretreated osseous specimens. The data are mean ± SD (*p =0.0083, Wilcoxon matched-pairs signed-rank test).

A reduction of O-GlcNAcylation in the post-chemotherapeutic specimens associated with a tendency of low OGT expression but high OGA expression.

The expressions of O-GlcNAcylated proteins, OGT, and OGA were examined in 20 cases of osteosarcoma cell pretreatment and post-chemotherapeutic treatment using immunohistochemistry as shown in Table 1. The results showed a tendency of lower OGT but higher OGA expressions of the immunoreactivity in the post-chemotherapy osseous biopsy as shown in Figures 2 and 3.

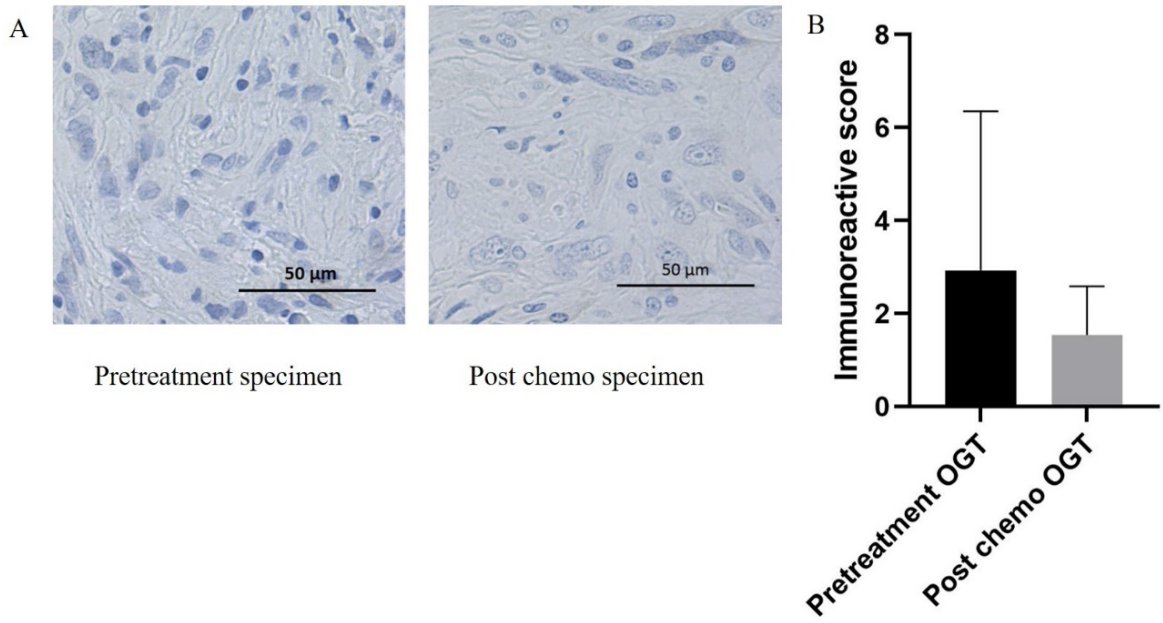

Figure 2 A reduction of OGT in the post chemotherapeutic osteosarcoma specimens. A scale bar represents 50 µm.

The levels of OGT were determined in pretreatment and posttreatment with the adjuvant chemotherapy for 20 osteosarcoma specimens using immunohistochemistry staining. (A) The post-chemotherapeutic osteosarcoma specimens showed a weak immunoreactivity of OGT whereas pretreated osseous specimens had a high OGT expression. (B) The immunoreactivity of OGT observed in the post-chemotherapeutic osteosarcoma specimens was lower than in the pretreated osseous specimens. The data are mean ± SD ($p = 0.2634$, Wilcoxon matched-pairs signed-rank test).

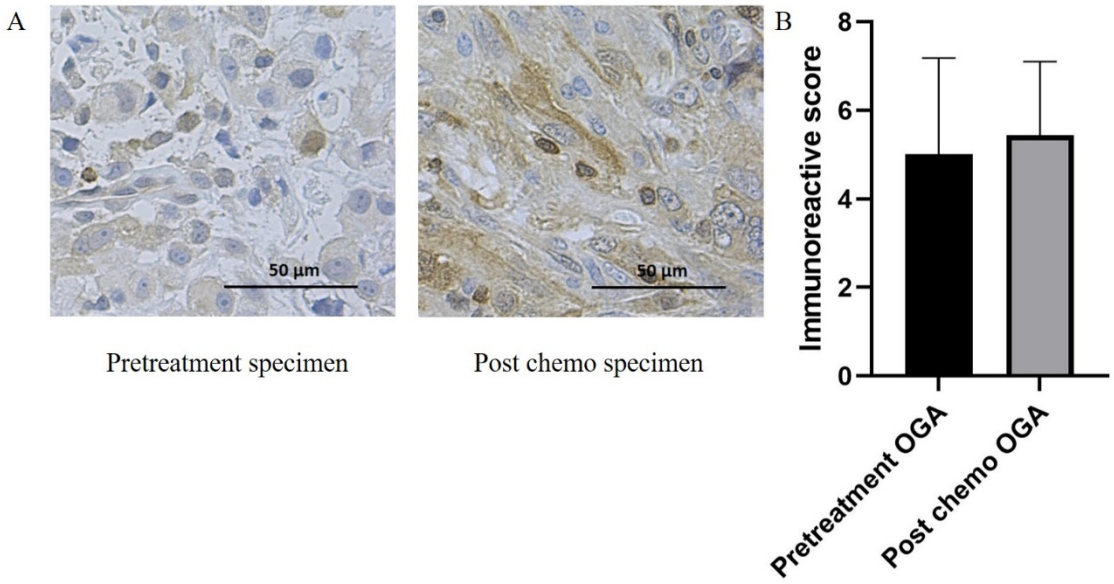

Figure 3 An elevation of OGA in the post chemotherapeutic osteosarcoma specimens. A scale bar represents 50 µm.

The levels of OGA were determined in pretreatment and posttreatment with the adjuvant chemotherapy for 20 osseous specimens using immunohistochemistry staining. (A) The post-chemotherapeutic osteosarcoma specimens showed a stronger immunoreactivity than the initial biopsy specimens. (B) The immunoreactivity of OGA was higher than in the initial biopsy specimens. The data are mean ± SD (p = 0.7680, Wilcoxon matched-pairs signed-rank test)

Table 1 Immunoreactive scores of O-GlcNAc, OGT, and OGA in 20 primary osteosarcoma specimens.

Factor	Profiles of O-GlcNAc, OGT, OGA [immunoreactive scores (IRS), mean ± SD]						
	All patients	O-GlcNAcylated proteins	*p* -value	OGT	*p* -value	OGA	*p* -value
Specimens							
- Biopsy tissue							
- Post-chemotherapeutic	20 20	4.96±1.96 3.29±1.34	0.0083*	2.93±3.42 1.53±1.05	0.2634	5.01±2.17 5.43±1.66	0.7680

OGT = O-GlcNAc transferase; OGA = O-GlcNAcase * p<0.05 is considered significant

Discussion

Previous evidence revealed increasing levels of O-GlcNAc and OGT in various diseases, including cancers. A study of pancreatic tumors indicated higher O-GlcNAc and OGT levels, together with a down-regulated expression of OGA in cancerous tissues as compared with normal tissues (Ma, Vocadlo, & Vosseller, 2013). Lynch et al. showed that O-GlcNAc and OGT levels were higher in the prostate cancer cells compared with normal cell lines (Lynch *et al*., 2012). Phoomak et al. revealed that inhibition of O-GlcNAc might be a therapeutic target for cholangiocarcinoma, while the OGT expression level might also be a helpful prognostic indicator for this cancer. The study showed an *in situ* lower expression in O-GlcNAc modified

proteins and OGT but a higher expression of OGA in normal bile ducts using immunohistochemistry protocol. In contrast, O-GlcNAc modified proteins were strongly expressed in cholangiocarcinoma tissues, together with the up-regulation of OGT and down-regulation of OGA (Phoomak et al., 2012). Several studies have shown a correlation between O-GlcNAc and successful postoperative chemotherapy for cancer. Zhou et al. suggested that decreased ability of OGT action enhanced cisplatin-induced autophagy via SNAP-29 that is related to cisplatin resistance in ovarian cancer. They used an immunohistochemistry protocol to determine the expression of OGT, OGA, and O-GlcNAc in chemoresistant and chemosensitive ovarian cancer tissues. The results showed that the O-GlcNAc and OGT levels were significantly lower in the chemoresistant ovarian cancer than in the chemosensitive tissues but the OGA levels showed no difference between the specimens (Zhou et al., 2018). Liu et al. revealed that increased activation of O-GlcNAc through the hexosamine biosynthesis pathway resulted in cell resistance and survival during chemotherapy. They concluded that the hexosamine biosynthesis pathway played an important role in cancer cell chemoresistance by controlling O-GlcNAc. The increased resistance of the cancer cells to chemotherapy was related to an increase in the levels of O-GlcNAc (Liu et al., 2018).

A correlation was identified between O-GlcNAc and postoperative chemotherapy for osteosarcoma. A significant reduction of O-GlcNAc was recorded in the postoperative chemotherapy tumors associated with the tendency of low OGT expressions but high OGA expressions. These results concurred with our previous study that investigated the levels of O-GlcNAcylated proteins, OGT, and OGA in osteosarcoma cells derived from chemo-naïve tissues of osteosarcoma patients. Our previous study also revealed that the OGT level was significantly associated with chemo-response in stage IIB osteosarcoma patients, and the expression of OGT was higher in the patients who poorly responded to chemotherapy, compared with good responders (Rattanakuntee et al., 2020).

5. Conclusions

A modification of O-GlcNAc plays an important role in postoperative chemotherapy for many cancers but the information is still lacking, concerning the role of O-GlcNAc in the postoperative chemotherapy for osteosarcoma. Our previous study revealed that O-GlcNAc was significantly associated with chemo-response in stage IIB osteosarcoma patients. Here, the results showed significantly lower levels of O-GlcNAc with lower OGT and higher OGA in the postoperative chemotherapeutic osteosarcoma specimens. These findings suggested that the modification of O-GlcNAc in poor responder osteosarcoma might further reveal its role in osteosarcoma and cancer cell chemoresistance.

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