Relationship between O-GlcNAcase Expression and Prognosis of Patients with Osteosarcoma

Thamonwan Sombutthaweesri

Chiang Mai University Faculty of Dentistry

Shuangjiang Wu

Chiang Mai University Faculty of Dentistry

Nutchapon Chamusri

Chiang Mai University Faculty of Dentistry

Jongkolnee Settakorn

Chiang Mai University Faculty of Medicine

Dumnoensun Pruksakorn

Chiang Mai University Faculty of Medicine

Parunya Chaiyawat

Chiang Mai University Faculty of Medicine

Thanapat Sastraruji

Chiang Mai University Faculty of Dentistry

Suttichai Krisanaprakornkit

Chiang Mai University Faculty of Dentistry

Chayarop Supanchart (✉ chayarop.supanchart@cmu.ac.th)

Chiang Mai University Faculty of Dentistry <https://orcid.org/0000-0003-1894-8863>

Research

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Abstract

Background: Several studies have demonstrated a role of *O*-GlcNAcylation (*O*-GlcNAc) in tumorigenesis of various carcinomas by modification of tumor-associated proteins. However, its implication in the pathogenesis of osteosarcoma remains unclear. This study aimed to investigate the levels of *O*-GlcNAc and the expressions of *O*-linked *N*-acetylglucosamine transferase (OGT) and *O*-GlcNAcase (OGA) in human osteosarcoma tissues and to find correlations between the levels or expressions and several clinicopathologic parameters.

Methods: Immunohistochemistry was conducted in 109 formalin-fixed, paraffin-embedded bone tissue sections that were derived from 109 first diagnosed osteosarcoma patients, including Enneking stage IIB (n=70) and III (n=39). Correlations between the immunoreactive score (IRS) and clinicopathologic parameters, overall survival, and metastasis-free survival were evaluated.

Results: A positive correlation was found between the IRS of OGA and the percentage of post-chemotherapeutic tumor necrosis ($r=0.308$; $P=0.017$). Univariate analysis revealed significantly lower OGA IRS in metastatic patients ($P=0.020$) and poor chemotherapeutic-responder patients ($P=0.001$). By multivariate analysis, presence of tumor metastasis ($P=0.002$) and lower OGA IRS ($P=0.004$) was significantly associated with shorter overall survival. Subgroup analysis in stage IIB osteosarcoma (n=70) demonstrated that male gender ($P=0.019$), presence of tumor recurrence ($P=0.026$), poor chemotherapeutic responder ($P=0.022$), and lower OGA IRS ($P=0.019$) were significantly correlated with short metastasis-free survival. But, lower OGA IRS was the only independent predictor for short metastasis-free survival ($P=0.006$).

Conclusions: *O*-GlcNAc pathway, especially OGA, involved in pathogenesis and aggressiveness of osteosarcoma. Low level of OGA expression may be used as a poor prognostic indicator.

Background

O-GlcNAcylation (*O*-GlcNAc) is a post-translational modification of proteins associated with regulation of various protein functions by an addition of *N*-acetylglucosamine (GlcNAc) to the hydroxyl group of serine or threonine residues of the target proteins. This modification is a dynamic and reversible process, strictly regulated by two important enzymes, *O*-linked *N*-acetylglucosamine transferase (OGT) and *O*-GlcNAcase (OGA), which add and remove the GlcNAc moieties, respectively.^{1–5} *O*-GlcNAc is involved in many cellular processes, such as signal transduction, transcription, cell cycle control, and epigenetic control of gene expressions in response to stress and nutrient imbalance.^{6,7} In this regard, *O*-GlcNAc homeostasis needs to be maintained in order to achieve proper cellular functions.

Aberrations of *O*-GlcNAc are associated with the pathogenesis of many human diseases, including diabetes, neurodegenerative diseases and cancers.^{6,8–11} With respect to cancer pathogenesis and progression, several key players, such as p53, c-Myc, Forkhead box M1 (FoxM1) etc., have been shown to

be modified via the *O*-GlcNAc pathway.^{12–15} Recently, several cancer studies have reported that elevated *O*-GlcNAc is associated with tumorigenesis and cancer cell metastasis.^{12, 16–25} Moreover, several previous studies have shown that hyper-*O*-GlcNAc and overexpression of OGT were associated with worse clinicopathological outcomes in various cancers.^{17, 21, 26, 27}

Osteosarcoma is most commonly found in children, adolescents and young adults, accounting for approximately 20% of all primary bone tumors.³² At present, the treatment strategy for osteosarcoma includes surgical resection of primary bone tumor combined with polychemotherapy.^{28–32} In patients with non-metastatic osteosarcoma, the 5-year survival rate has reported as 71%.³³ However, the 5-year survival rate is as low as approximately 30% in metastatic cases.³⁴ The clinical prognostic indicators for osteosarcoma include extent of disease (tumor staging) at the time of diagnosis, size and location of the tumor, surgical remission and/or responses to chemotherapy, and the presence of metastasis after treatment.^{30, 35, 36}

Although there are many studies that have investigated the relationship between *O*-GlcNAc and various cancers, the role of *O*-GlcNAc in the pathogenesis of osteosarcoma remains largely unknown. Based on our previous study,³⁷ it was found that *O*-GlcNAc levels and OGT expression were increased in primary cancer cells isolated from patients with osteosarcoma in association with metastasis and poor response to chemotherapy. Furthermore, the degrees of OGT expression were significantly higher in these cancer cells derived from poor responders than in those from good responders. However, due to a small sample size in that study³⁷ as well as very limited knowledge regarding the role of *O*-GlcNAc together with that of the two relevant enzymes in the pathogenesis and prognosis of osteosarcoma, a study with a larger sample size is required to gain further insights. In this study, the expressions of OGT and OGA, and the levels of *O*-GlcNAc were, therefore, examined in 109 bone specimens, histopathologically diagnosed as osteosarcoma using immunohistochemistry, and the correlations were determined between the *O*-GlcNAc profiles and several clinicopathological characteristics at the time of diagnosis as well as patients' treatment outcomes.

Methods

Tissue samples

The study cohort included bone biopsies taken from 109 patients with osteosarcoma, who were diagnosed and treated at the Maharaj Nakorn Chiang Mai Hospital, Thailand, between 1999 and 2020. All patients were followed-up for survival analysis until 11 January 2020. According to the Enneking tumor staging,³⁸ all of these cases were classified as either stage IIB or III (Table 2). Of 109 patients, 73 cases (66.9%) had received standard treatment, comprising surgical tumor resection in combination with neoadjuvant chemotherapy. The remaining 36 patients (33.1%) refused either chemotherapy (n=9), surgical therapy (n=21) or both (n=6). All specimens were fixed in formalin and embedded in paraffin before being obtained from tissue archive of the Department of Pathology, Faculty of Medicine, Chiang

Mai University. Demographic and clinicopathological features were retrieved from hospital records and are summarized in Table 2. Using the previously published protocol,^{39, 40} the percentage of post-chemotherapeutic tumor necrosis was calculated by certified pathologists. Of 73 cases who received both chemical and surgical therapies, tumor necrosis data of 60 cases were only available for determination of their correlation with the levels of the three molecules.

Immunohistochemistry and scoring

Immunohistochemistry protocol was described as in previous study.⁴¹ It was performed on the 3- μ m tissue sections using the Ventana automated stainer (Ventana Medical Systems, Tucson, AZ, USA) and Ultraview Universal DAB Detection Kit (Ventana Medical Systems, Tucson, AZ, USA). Optimized conditions of each antibody are shown in Table 1. The section was first oriented using a bright field microscope (Carl Zeiss, Oberkochen, Germany) at 10X magnification, and three images that represented the immunostaining of each section were taken using an attached digital camera (Axiocam ICc5, Carl Zeiss) at 40X magnification. Tissue sections from breast, lung and colon cancers were used as a positive control (Additional Fig. S1).^{12, 17-19, 21, 23, 25}

The average immunoreactive score (IRS) from three representative fields of each stained section was used to semi-quantitatively assess immunohistochemical staining. The IRS (0-12) in each digitized image was determined by multiplication of the percentage score of positive cells as follows, 0 = 0%, 1 = <10%, 2 = 10-50%, 3 = 51-80%, 4 = >80%, with the intensity score as follows, 0 = no staining, 1 = weak (light yellow), 2 = moderate (brown), 3 = strong (dark brown). All immunostained specimens were examined by two independent observers (T.S. and N.C.), who were trained and calibrated with J.S., a Thai Board-certified pathologist, before IRS analysis. The intraclass correlation coefficients for intra- and inter-observer calibration were equal to 0.93 and 0.81, respectively.

Statistical analysis

The statistical analysis was performed using IBM[®] SPSS[®] Statistics version 23 (Chicago, IL, USA). The demographic and clinicopathologic features were compared between the two tumor stages (IIB versus III) using Independent samples *t*-test or chi-square analysis. The mean IRSs were compared between the two tumor stages using Independent samples *t*-test. Correlations between the IRS and the clinicopathologic characteristics of patients were analyzed using Pearson correlation or Spearman Rank correlation analysis. Univariate analyses of overall and metastasis-free survival in association with the IRS were performed using the Kaplan–Meier method together with the log-rank test. Multivariate survival analysis was carried out by Cox regression of proportional hazards to indicate significance at the 95% confidence interval (CI) using Stata version 16 (StataCorp, College Station, TX, USA). The results with *P*-values less than 0.05 were considered statistically significant (**P*-value < 0.05; ** *P*-value < 0.01)

Results

Positive correlation between the immunoreactive scores of OGA expression and the percentages of tumor necrosis

Varying intensities from weak to strong immunostaining of *O*-GlcNAc levels, OGT and OGA expressions were observed in 109 osteosarcoma specimens as shown by their representative images in Figure 1. Between the two stages, No significant differences were found in any of the clinicopathologic parameters (Table 2) and in the mean IRS of the three biomolecules (Additional Fig. S2). By univariate analysis (Table 3), four significant associations between the IRS of *O*-GlcNAc levels, that of OGT expression, or that of OGA expression and the clinicopathologic parameters were noted, including the *O*-GlcNAc IRS and tumor locations ($P = 0.029$), the OGT IRS and the presence of recurrence during the follow-up period ($P = 0.001$), the OGA IRS and the presence of metastasis during the follow-up period ($P = 0.020$), and the OGA IRS and the percentage of post-chemotherapeutic tumor necrosis ($P = 0.001$). In particular, a moderate and positive correlation was found between the IRS of OGA expression and the percentage of post-chemotherapeutic tumor necrosis in 60 cases ($r = 0.308$, $P = 0.017$; Figure 2), whereas the *O*-GlcNAc IRS or the OGT IRS was not found to be correlated with the percentage of post-chemotherapeutic tumor necrosis.

Survival analysis reveals significant association between the immunoreactive scores of OGA expression and patients' overall survival

Using the Kaplan–Meier method and the log-rank test, the IRS cut-off points for *O*-GlcNAc, OGT and OGA, derived from receiver operating characteristic (ROC) curves, were 6, 2.5 and 5, respectively. By univariate analysis of patients' overall survival, high degrees of OGA expression (IRS > 5; Fig. 3C), but not high *O*-GlcNAc levels (Fig. 3A) or OGT expressions (Fig. 3B), were found to be a significant positive predictor in patients with osteosarcoma ($P = 0.002$, Table 4). In addition to the IRS of OGA, good chemotherapeutic response with the percentages of tumor necrosis ≥ 90 , the absence of metastasis, and the low Enneking stage were found to be a significant positive predictor in patients with osteosarcoma ($P = 0.008$, $P < 0.001$, and $P < 0.001$, respectively; Table 4). By multivariate analysis, the low IRS of OGA and the presence of metastases were found to be an independent poor prognostic marker for patients' overall survival with a hazard ratio of 2.07 (95% CI: 1.26-3.42, $P = 0.004$) and 3.33 (95% CI: 1.56-7.10, $P = 0.002$), respectively (Table 5).

Survival analysis reveals significant association between the immunoreactive scores of OGA expression and metastasis-free survival in stage IIB

Analysis of metastasis-free survival was only performed in stage IIB cases ($n = 70$). By univariate analysis, high degrees of OGA expression ($\text{IRS} > 5$) were found to be a significant positive predictor of metastasis-free survival ($P = 0.019$; Fig. 4C, Table 6), whereas neither high *O*-GlcNAc levels (Fig. 4A) nor OGT expressions (Fig. 4B) were found to be associated with metastasis-free survival. Moreover, good chemotherapeutic response with the percentages of tumor necrosis ≥ 90 was found to be a significant positive predictor ($P = 0.022$; Table 6), whereas male patients and the presence of recurrence during the follow-up period were found to be negatively associated with metastasis-free survival ($P = 0.019$; $P = 0.026$, respectively; Table 6). By multivariate analysis, low degrees of OGA expression ($\text{IRS} \leq 5$) were the only independent poor prognostic factor for metastasis-free survival in the stage IIB with a hazard ratio of 2.85 (95% CI: 1.36–6.00, $P = 0.006$; Table 7).

Discussion

In this study, the mean IRS of these three biomolecules were not found to be different between the two stages. However, four significant associations between the IRS of the three biomolecules and some clinicopathological parameters were found by univariate analysis. Particularly, high expressions of OGA, but not those of *O*-GlcNAc or OGT, were found to be positively associated with the percentage of post-chemotherapeutic tumor necrosis. Consistently, the high OGA expressions were only found to be significantly associated with increased overall survival and with a longer metastasis-free period in stage IIB. In addition, by multivariate analysis, the low OGA expression was found to be an independent prognostic factor for a decrease in both overall and metastasis-free survivals of patients with osteosarcoma.

In carcinoma, elevated levels of *O*-GlcNAc and high degrees of OGT expression, whilst decreased expression of OGA, are demonstrated in cancerous tissues of pancreas,⁴² whereas only the *O*-GlcNAc levels and the OGT expressions are reported to be increased in lung, prostate, breast, colon and gall bladder cancers.^{17, 20, 21, 24, 26, 43} However, from our recent study of *O*-GlcNAcylation in oral cancer, the *O*-GlcNAc levels have not been found to be significantly enhanced upon increased histopathologic severities of oral squamous cell carcinoma, nor have the OGT or the OGA expressions.⁴⁴ Consistent with these findings, the *O*-GlcNAc levels, the OGT or the OGA expressions were not found to be different between the two distinct clinical severities of osteosarcoma. Therefore, involvement of *O*-GlcNAcylation along with OGT and OGA expressions in the pathogenesis of osteosarcoma still requires further investigations.

By univariate analysis, the present study showed that high degrees of OGA expression, but not those of *O*-GlcNAc or OGT, were found to be correlated with increased overall survival with osteosarcoma. Moreover, high OGA expressions were found to be associated with increased metastasis-free survival in patients with stage IIB. These findings are consistent with the results of a previous retrospective study⁴⁵ in liver cancer that showed high OGA expressions in patients with hepatocellular carcinoma, who exhibited 3-year cumulative recurrence-free survival longer than those with low OGA expressions, implying that OGA

expression in the specimens might be useful as a prognostic indicator of tumor-free survival in both hepatocellular carcinoma and osteosarcoma.

By multivariate analysis, OGT overexpression was found to be associated with prostate cancer progression and recurrence, and high *O*-GlcNAc levels were found to be an independent prognostic factor for poor survival.^{24,27} Increased *O*-GlcNAcylation levels were also associated with poor survival in patients with cholangiocarcinoma.²⁶ The *O*-GlcNAc levels and the OGT expressions were up-regulated in breast cancer cells, in which these two biomolecules appeared to contribute to tumor cell growth and invasion.^{12,21} In lung cancer, high OGT expressions were associated with poor survival of patients and with short recurrence-free survival.¹⁷ In contrast to these findings from various types of carcinoma, our study using multivariate analysis instead demonstrated that OGA expression in osteosarcoma specimens is an independent prognostic indicator. This discrepancy may be due to different origins of tumor cells.

Several studies have shown that the histological response to pre-surgical chemotherapy, as a standard intervention for patients with osteosarcoma, is one of the most important predictors for clinical outcomes and prognosis of osteosarcoma.^{46,47} By comparison with poor responders, good responders have significantly higher overall and disease-free survival rates. In agreement with those studies, our data showed that a good chemotherapeutic response with the percentages of tumor necrosis ≥ 90 was found to be a significant positive predictor in both overall survival of all patients with osteosarcoma and metastasis-free survival in patients with stage IIB. In addition, high OGA expressions in osteosarcoma specimens were positively correlated with the good chemotherapeutic response with the percentages of tumor necrosis ≥ 90 . Consequently, it is suggested by this study that both OGA expression, as a molecular marker, and the good chemotherapeutic response, as a clinical indicator, are useful for prognosis of patients with osteosarcoma. Consistently, inhibition of OGA expressions by treatment with Thiamet-G remarkably enhanced cancer cell migration and invasion in lung, colon, thyroid and ovarian cancers,^{19,48,49} which may likely decrease a patients' survival rate.

Conclusions

Our findings demonstrate that the high degrees of OGA expression are associated with good clinical outcomes and prognosis of patients with osteosarcoma. Low level of OGA expression may be useful as a poor prognostic marker for overall and metastasis-free survival in patients with osteosarcoma. To better understand the molecular mechanism(s) of OGA in association with specific tumor-associated protein(s) in osteosarcoma for patients' survival enhancement, additional *in vitro* investigations are required to address a possibility for OGA, functioning to reduce growth, migration and invasion of tumor cells, isolated from osteosarcoma lesions.

Abbreviations

IRS: Immunoreactive score

OGA: *O*-GlcNAcase

O-GlcNAc: *O*-GlcNAcylation

OGT: *O*-linked *N*-acetylglucosamine transferase

ROC: Receiver Operating Characteristic

Declarations

Ethics approval and consent to participate

The study protocol was approved by The Research Ethics Committee of the Faculty of Medicine, Chiang Mai University (No. 062/2558).

Consent for publication

Not applicable.

Availability of data and materials

Data supporting the findings of this work are available within this article and its Additional Information files. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

T.S. and C.S. designed the study. T.S., S.W., N.C. and J.S. evaluated IHC staining results. D.P., J.S. and P.C. collected tissue samples and clinical data. P.C. and T.S. performed statistical analyses. S.K. and C.S.

contributed essential reagents or tools. T.S., S.W., S.K. and C.S. composed the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1
List of antibodies

Antibody	Immunoreactivity	Cat./ company	Dilution	Incubation (min)
<i>O</i> -GlcNAc	Nucleus/ cytoplasm	MA1-072/ Thermo Fisher Scientific, Inc., Waltham, MA, USA	1:500	32
OGT	Nucleus/ cytoplasm	11576-2-AP/ Protein Technologies, Inc., Tucson, AZ, USA	1:200	32
OGA	Nucleus/ cytoplasm	ab124807/ Abcam Cambridge, MA, USA	1:1000	32
<i>O</i> -GlcNAc = <i>O</i> -GlcNAcylation; OGT = <i>O</i> -linked <i>N</i> -acetylglucosamine transferase; OGA = <i>O</i> -GlcNAcase				

Table 2
Clinicopathologic characteristics of 109 osteosarcoma cases

Characteristics	Enneking stage		<i>P</i> -value
	IIB (n = 70) ^a	III (n = 39) ^a	
Age			
- Mean ± SD (years)	22.21±15.91	19.30±16.25	0.360 ^b
Gender			
- Male	32 (45.7%)	23 (58.9%)	0.171 ^c
- Female	38 (54.3%)	16 (41.1%)	
Tumor location			
- Extremities	63 (90%)	34 (87.2%)	0.419 ^c
- Axial	7 (10%)	5 (12.8%)	
Neoadjuvant chemotherapy			
- Cis/Doxo based ^d	51 (72.9%)	29 (74.4%)	0.997 ^c
- Others ^e	9 (12.9%)	5 (12.8%)	
- None	10 (14.2%)	5 (12.8%)	
Surgical resection			
- Yes	56 (80%)	26 (66.7%)	0.075 ^c
- No	14 (20%)	13 (33.3%)	
Lung metastasis (later detected during a follow-up)			
- Yes			N/A
- No	34 (48.6%)	N/A	
	36 (51.4%)	N/A	
Bone metastasis (later detected during a follow-up)			
- Yes			N/A
- No	3 (4.3%)	N/A	
	67 (95.7%)	N/A	
Recurrence			
- Yes	8 (11.4%)	4 (10.3%)	0.836 ^c
- No	62 (88.6%)	35 (89.7%)	

Tumor necrosis after chemotherapy (n = 60)				
- ≥ 90%				
- < 90%	9 (20.9%)	3 (17.6%)	0.678 ^c	
	34 (79.1%)	14 (82.4%)		

^a Stage IIB is a high-grade and extra-compartmental osteosarcoma lesion without distant metastases at the diagnostic period, while stage III is an osteosarcoma lesion diagnosed as any grade with distant metastases. ^b Independent samples *t*-test; ^c two-tailed Pearson's chi-squared test. ^d Common drugs used in chemotherapy for 94 cases, including Carbo = Carboplatin; Cis = Cisplatin; Doxo = Doxorubicin. ^e Others = metrotrexate, ifosfamide, etoposide, docetaxel and gemcitabine.

N/A = not applicable; SD = standard deviation.

Table 3
Characteristics of 109 patients with osteosarcoma

Factor	Profiles of O-GlcNAc, OGT, OGA [immunoreactive scores (IRS), mean ± SD]						
	N	O-GlcNAc	P-value	OGT	P-value	OGA	P-value
Age at diagnosis, years [mean = 21.16 ± 16.024, median = 15 (range 5-75)]							
≤ 15	59	5.97 ± 2.16	0.547	3.22 ± 1.71	0.135	5.63 ± 2.01	0.429
> 15	50	5.74 ± 1.94		2.78 ± 1.35		5.34 ± 1.88	
Gender							
- Male	55	5.83 ± 2.01	0.861	3.06 ± 1.55	0.777	5.21 ± 1.99	0.111
- Female	54	5.90 ± 2.11		2.97 ± 1.59		5.80 ± 1.87	
Enneking stage							
- IIB	70	5.88 ± 2.13	0.892	2.85 ± 1.48	0.143	5.56 ± 1.82	0.630
- III	39	5.83 ± 1.94		3.31 ± 1.68		5.38 ± 2.18	
Site							
- Extremities	97	6.02 ± 2.00	0.029*	2.99 ± 1.52	0.744	5.60 ± 1.88	0.137
- Axial	12	4.70 ± 2.17		3.15 ± 1.91		4.74 ± 2.37	
Metastasis (during follow-up period, n = 70)							
- Yes	34	5.72 ± 1.92	0.540	2.79 ± 1.46	0.734	5.06 ± 1.74	0.020*
- No	36	6.04 ± 2.34		2.91 ± 1.51		6.06 ± 1.78	
Recurrence							
- Yes	12	5.20 ± 1.97	0.238	2.25 ± 0.59	0.001**	4.92 ± 1.55	0.276
- No	97	5.94 ± 2.06		3.11 ± 1.62		5.57 ± 1.99	

Chemoresistance (n = 60)							
- Good responders (Tumor necrosis $\geq$ 90%)	12	5.78 $\pm$ 1.96	0.431	2.98 $\pm$ 1.21	0.711	6.99 $\pm$ 1.03	0.001**
- Poor responders (Tumor necrosis < 90%)	48	5.30 $\pm$ 1.81		2.80 $\pm$ 1.55		5.04 $\pm$ 1.88	
<i>O</i> -GlcNAc = <i>O</i> -GlcNAcylation; OGT = <i>O</i> -linked <i>N</i> -acetylglucosamine transferase; OGA = <i>O</i> -GlcNAcase <i>P</i> -values were calculated with Independent samples <i>t</i> -test. * <i>P</i> -value < 0.05; ** <i>P</i> -value < 0.01							

Table 4
Median survival analyses of patients with osteosarcoma (n = 109)

Factor	Patients	Events (Death)	Median survival (months)	P-value
Age at diagnosis (years)				
≤ 15	59	38	20.5	0.347
> 15	50	37	15.6	
Gender				
- Male	55	42	17.1	0.277
- Female	54	33	20.5	
Enneking stage				
- IIB	70	42	24.8	< 0.001**
- III	39	33	12.5	
Site				
- Extremities	97	66	18.5	0.204
- Axial	12	9	10.5	
Metastasis				
- Yes	73	63	15.2	< 0.001**
- No	36	12	Undefined	
Recurrence				
- Yes	12	12	15.2	0.262
- No	97	62	17.4	
Chemoresistance (n = 60)				
- Good responders	12	3	Undefined	0.008**
- Poor responders	48	33	20.6	
O-GlcNAc				
- High (immunoreactive score > 6)	50	33	17.7	0.561
- Low (immunoreactive score ≤ 6)	59	42	17.1	
OGT				

- High (immunoreactive score > 2.5)	58	41	15.6	0.651
- Low (immunoreactive score ≤ 2.5)	51	34	20.6	
OGA				
- High (immunoreactive score > 5)	59	33	27.8	
- Low (immunoreactive score ≤ 5)	50	42	13.1	0.002**
<i>O</i> -GlcNAc = <i>O</i> -GlcNAcylation; OGT = <i>O</i> -linked <i>N</i> -acetylglucosamine transferase; OGA = <i>O</i> -GlcNAcase				
<i>P</i> -values were calculated with the log-rank test, ** <i>P</i> -value < 0.01				

Table 5
Multivariate analysis of overall survival in patients with osteosarcoma (n = 109)

Factor	Overall survival	
	HR (95% CI)	<i>P</i> -value
Age at diagnosis (years)		
≤ 15	1.00	0.874
> 15	1.04 (0.62-1.75)	
Gender		
- Male	1.00	0.249
- Female	1.35 (0.81-2.25)	
Enneking stage		
- IIB	1.00	0.211
- III	1.41 (0.82-2.43)	
Site		
- Extremities	1.00	0.108
- Axial	1.86 (0.87-3.98)	
Recurrence		
- No	1.00	0.780
- Yes	0.91 (0.47-1.75)	
Metastasis		
- No	1.00	0.002**
- Yes	3.33 (1.56-7.10)	
OGA		
- High (immunoreactive score > 5)	1.00	0.004**
- Low (immunoreactive score ≤ 5)	2.07 (1.26-3.42)	
CI = confidence interval, HR = hazard ratio, OGA = <i>O</i> -GlcNAcase, ** <i>P</i> -value < 0.01		

Table 6
Metastasis-free survival analyses of patients with stage IIB osteosarcoma (n = 70)

Factor	Patients	Events of metastasis (n)	Median of metastasis-free period (months) ^a	P-value
Age at diagnosis (years)				
≤ 15	33	21	101.8	0.058
> 15	37	13	24.8	
Gender				
- Male	32	13	24.8	0.019*
- Female	38	21	157.4	
Site				
- Extremities	63	32	38.3	0.933
- Axial	7	2	Undefined	
Recurrence				
- No	62	26	62.3	0.026*
- Yes	8	8	20.9	
Chemoresistance (n = 43) ^b				
- Good responders	9	2	101.8	0.022*
- Poor responders	34	24	27.1	
O-GlcNAc				
- High (immunoreactive score > 6)	32	12	157.4	0.091
- Low (immunoreactive score ≤ 6)	38	22	27.8	
OGT				
- High (immunoreactive score > 2.5)	34	16	56.2	0.806
- Low (immunoreactive score ≤ 2.5)	36	18	27.8	
OGA				
- High (immunoreactive score > 5)				

- Low (immunoreactive score ≤ 5)	37	13	157.4	0.019*
	33	21	20.9	

O-GlcNAc = *O*-GlcNAcylation; OGT = *O*-linked *N*-acetylglucosamine transferase; OGA = *O*-GlcNAcase

^a median of metastasis-free period (months) from available data of either 70 or 43 patients;

^b available data of the percentage of post-chemotherapeutic tumor necrosis in patients with stage IIB

P-values were calculated with the log-rank test, * *P*-value < 0.05

Table 7
Multivariate analysis of metastasis-free survival in patients with stage IIB osteosarcoma (n = 70)

Factor	Metastasis-free survival	
	HR (95% CI)	<i>P</i> -value
Age at diagnosis (years)		
≤ 15	1.00	0.222
> 15	1.60 (0.75-3.38)	
Gender		
- Male	1.00	0.067
- Female	0.50 (0.24-1.05)	
Site		
- Extremities	1.00	0.893
- Axial	1.11 (0.25-4.89)	
Recurrence		
- No	1.00	0.137
- Yes	1.85 (0.82-4.17)	
OGA		
- High (immunoreactive score > 5)	1.00	0.006**
- Low (immunoreactive score ≤ 5)	2.85 (1.36-6.00)	
CI = confidence interval, HR = hazard ratio, OGA = <i>O</i> -GlcNAcase, ** <i>P</i> -value < 0.01		

Figures

Figure 1

Representative images of O-GlcNAc (O-GlcNAcylation) levels, OGT (O-linked N-acetylglucosamine transferase) and OGA (O-GlcNAcase) expressions from different osteosarcoma specimens, categorized as stage IIB or III according to the Enneking's classification, showing distinct immunostaining intensities,

scored as strong, moderate or weak intensity. Negative = specimens reacted without the primary antibodies; scale bar = 50 μ m.

Figure 2

A moderately positive correlation between the immunoreactive scores (IRS) of OGA (O- GlcNAcase) and the percentage of post-chemotherapeutic tumor necrosis.

Figure 3

The immunoreactive scores (IRS) of O-GlcNAc (O-GlcNAcylation) or those of OGT (O-linked N-acetylglucosamine transferase) were not significantly associated with increased overall survival (A and B, respectively), whereas the higher OGA (O-GlcNAcase) IRS than its cut-off value was significantly associated with increased overall survival (C). The cut-off value of each biomolecule was estimated from the time-dependent ROC curve and its P-value was derived from the log-rank test. * $P < 0.05$

Figure 4

The immunoreactive scores (IRS) of O-GlcNAc (O-GlcNAcylation) or those of OGT (O-linked N-acetylglucosamine transferase) were not significantly associated with a period of metastasis-free survival in stage IIB (A and B, respectively), whereas the higher OGA (O-GlcNAcase) IRS than its cut-off value was significantly associated with a longer period of metastasis-free survival than the lower OGA IRS (C). The cut-off value of each biomolecule was estimated from the time-dependent ROC curve and its P-value was derived from the log-rank test. * $P < 0.05$

Supplementary Files

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