Clinicopathological significance of glucose transporter protein-1 overexpression in human osteosarcoma

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Abstract. Although previous studies have demonstrated that Glut-1 is the predominant glucose transporter, is significantly overexpressed in various types of tumor and is correlated with poor prognosis, the potential function and clinical value of Glut-1 expression in osteosarcoma remains largely unclear. In particular, the prospective associations between Glut-1 expression levels and clinicopathological factors remains to be elucidated. In the present study, immunohistochemistry was performed to detect Glut-1 protein expression in 51 paired osteosarcoma specimens and adjacent non-cancerous tissues, and reverse transcription-quantitative polymerase chain reaction analysis was performed to examine Glut-1 mRNA expression levels in 6 pairs of these tissues. Statistical analyses were conducted to determine the associations between Glut-1 expression and various clinicopathological parameters. Glut-1 protein was revealed to be overexpressed in 38 (74.5%) osteosarcoma tissues, but only in 6 (11.8%) adjacent non-cancerous tissues. Glut-1 mRNA levels were also upregulated in osteosarcoma tissues compared with adjacent non-cancerous tissues. While there were no clear statistical relationships between Glut-1 expression and patient sex, resection, tumor location, size, T stage and adjuvant treatment, Glut-1 expression levels were significantly associated with age, tumor-node-metastasis stage, lymph node metastasis and survival. The median survival time in patients with low Glut-1 expression levels was longer than in patients with a high expression level. Glut-1 was significantly overexpressed in osteosarcoma tissues, and Glut-1 expression was associated with clinicopathological factors which upregulate the invasion and metastasis of osteosarcoma, and may be a potential predictor of survival in patients with osteosarcoma.

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Introduction

Osteosarcoma is one of the most common pediatric malignancies and accounts for up to 15% of childhood cancers (1). Osteosarcoma is the major form of bone and soft tissue primary malignant tumor, and is characterized by specific tumor cell proliferation, early and rapid metastasis that also occurs at the local primary site, and a high mortality rate (2). Despite the development of novel treatments for osteosarcoma, including neo-adjuvant chemotherapy combined with wide excision of tumors or the amputation of the affected limbs, which has resulted in improved survival rates in patients who present with non-metastatic osteosarcoma in their extremities, the survival rate in patients with osteosarcoma in general has only demonstrated slight improvements (3). In particular, early metastasis is the key risk factor responsible for the low survival rate. Previous studies have also demonstrated that ~30% of patients with no evidence of metastasis at diagnosis who were treated with wide tumor resection and intensive adjuvant chemotherapy may develop lung metastases later (4,5), leading to poor survival. Therefore, more effective and earlier diagnosis of osteosarcoma is critical for the early initiation of treatment and resultant improved survival of patients. In conjunction with traditional factors that influence patient survival, including age, sex, tumor location, size, differentiation and lymph node metastasis, molecular genetics technology has been employed to predict prognosis in osteosarcoma diagnosed at an earlier stage (6-9).

Enhanced glucose metabolism is one of the principal alterations observed in malignant tissue, and malignant cells often exhibit augmented expression levels of glucose transport genes. Glucose transporters (Gluts) are a group of proteins expressed on the cytoplasmic side of the plasma membrane, which are involved in energy-independent glucose transport. As a member of the Glut family, Glut-1 is the most common form of human glucose transporter and is crucial for glucose metabolism (10,11). Glut-1 expression has been demonstrated to be associated with enhanced glucose uptake, resulting improved glucose metabolism which provides additional

energy to meet the requirements tumor cells as they proliferate and adapt to severe microenvironments (12-14). In addition, previous studies have demonstrated that Glut-1 is the predominant glucose transporter that is significantly overexpressed in various types of tumor cell, and its expression is correlated with poor prognosis (15-17).

Overexpression of Glut-1 may be associated with clinical outcome in bone and soft tissue sarcomas (18), and expression levels of Glut-1 may be negatively associated with survival time and tumor microvessel density in patients with osteosarcoma (19). Furthermore, Glut-1 protein is positively overexpressed in osteosarcoma, and downregulation of Glut-1 has the capacity to inhibit the formation, growth and invasion of osteosarcoma cells *in vitro* and *in vivo* (20,21), further indicating the potential of using Glut-1 to assess the malignancy of bone tumors and as a predictor of survival in patients with osteosarcoma. However, the potential function and clinical value of Glut-1 expression in osteosarcoma still remains unclear, particularly in terms of the prospective association between Glut-1 expression and clinicopathological factors. To the best of our knowledge, no previous studies have investigated the association between Glut-1 expression and other pathological variables including age, sex, tumor location, size, differentiation, T stage, lymph node metastasis, tumor-node-metastasis (TNM) stage, inner metastasis, recurrence and reaction to chemotherapy. It is possible to use this information to demonstrate the relationships between Glut-1 expression levels and the prognosis of patients with osteosarcoma.

In the present study, to evaluate the potential value of Glut-1 in predicting the prognosis of osteosarcoma patients, 51 paired human osteosarcoma specimens and adjacent non-cancerous tissues from the last ten years were retrospectively collected and analyzed to investigate the associations between Glut-1 expression levels and clinicopathological variables.

Materials and methods

Patients. A total of 51 patients with osteosarcoma with complete clinical data who underwent surgical resection in the Orthopedic Department of Tongji Hospital, Tongji University (Shanghai, China) between April 1993 and March 2012 were retrospectively reviewed. The surgical specimens included paraffin-embedded primary osteosarcoma tissues and paired control tissues adjacent to the carcinoma specimens. The 51 specimens of osteosarcoma were from 28 male and 23 female patients between 13.3-71.2 years old (average age, 34.6 years). All patients received adjuvant chemotherapy, including conventional doxorubicin in combination with methotrexate treatment without radiotherapy prior to surgery. The adjuvant chemotherapy consisted of 30 mg doxorubicin combined with 40 mg methotrexate once a week and continued for three weeks as one period of treatment. Each period was had interval of 3 weeks and three periods were usually used for each patient. The histological responses to adjuvant chemotherapy were determined by the Huvos grading scale (22). Surgical procedures consisted of wide or marginal resection as described by Enneking *et al* (23). Age, sex, tumor location, size, differentiation, T stage, lymph node metastasis, TNM stage, inner metastasis, recurrence and reaction to chemotherapy were recorded prior to surgery (Table I). The TNM stage was determined according to the American Joint Committee on Cancer (24). A total of 15 patients were diagnosed with inner metastasis and metastases, including lung (n=8), liver (n=5) and bone (n=2). All patients were followed with chest X-ray or computed tomography scans every 3 months during the first year following the completion of treatment, then every 6 months for at least 5 years to investigate the recurrence and survival of these cases. Survival time was defined as the period from diagnosis to mortality from any cause except emergency traffic accidents or physical diseases of numerous patients following identification of the tumor and developed during the study period. The follow-up duration was dated from the day of diagnosis, and the median follow up time was 6 years 5 months (range, 62-242 months). The postoperative pathology specimens were all confirmed for osteosarcoma. The present retrospective study was approved by the Institutional Human Research Ethics Review Board of Tongji Hospital.

Immunohistochemical analysis of Glut-1 protein expression. Paired specimens of osteosarcoma and tissues adjacent to the carcinoma were routinely embedded in paraffin and sectioned (5 μm). The fresh sections were subsequently dewaxed with xylene and dehydrated with a graded ethanol series (100 and 70%) two times for 10 min each. Endogenous peroxidase was blocked by incubating the sections with 3% hydrogen peroxide in 50% methanol for 30 min at room temperature. Pre-warmed Dako target retrieval solution (pH 6; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) was used for the antigen retrieval and non-specific protein binding was blocked by incubation with 10% normal rabbit serum (Dako; Agilent Technologies, Inc.) in 1% bovine serum albumin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany)/PBS for 1.5 h in a humidified chamber at room temperature. Subsequent to washing with PBS, the slides were incubated at 4˚C overnight with polyclonal Glut-1 antibodies (dilution, 1:200; catalog no. MA5-11315; Thermo Scientific Lab Vision; Thermo Fisher Scientific, Inc., Waltham, MA, USA), after which they were incubated with horseradish peroxidase-conjugated secondary antibody (dilution, 1:500; catalog no., PA1-28587, Thermo Scientific Lab Vision) for 1 h at room temperature. These slides were then processed for 3,3'-Diaminobenzidine (DAB) substrate solution (Sigma-Aldrick; Merck KGaA) reaction following the manufacturer's protocol. Ten random fields of view from each section were examined and analyzed using an imaging system (catalog no. HMIAS-2000; Champion Medical Imaging Co., Wuhan, China). Cells with characteristic membranous and/or cytoplasmic staining were identified as Glut-1 positive (Glut-1$^+$) cells. The Glut-1$^+$ staining intensity was also expressed as the number of Glut-1$^+$ cells/the total number of cells x100, and was divided into three categories: <10%, negative; 10-50%, weak positive; >50%, strong positive, as previously described (18).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR was performed to further verify the expression levels of Glut-1 in 6 paired specimens of osteosarcoma and tissue adjacent to the carcinoma, in which the Glut-1$^+$ staining intensities were identified as positive (>10%) by immunohistochemistry. Extraction and purification of

Table I. Polymerase chain reaction primer sequences used in the present study.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Glut-1	CCATCCACCACACTCACCAC	GCCCAGGATCAGCATCTCAA
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG

Glut-1, glucose transporter-1.

Table II. Qualitative analysis of glucose transporter protein-1 immunostaining in osteosarcoma and tissue adjacent to carcinoma.

Cases	Osteosarcoma tissue	Tissue adjacent to carcinoma
Total number of cases, n	51	51
Cases with negative staining, n (%)	13 (25.5)	45 (88.2)
Cases with weak positive staining, n (%)	19 (37.3)	6 (11.8)
Cases with strong positive staining, n (%)	19 (37.3)	0 (0)

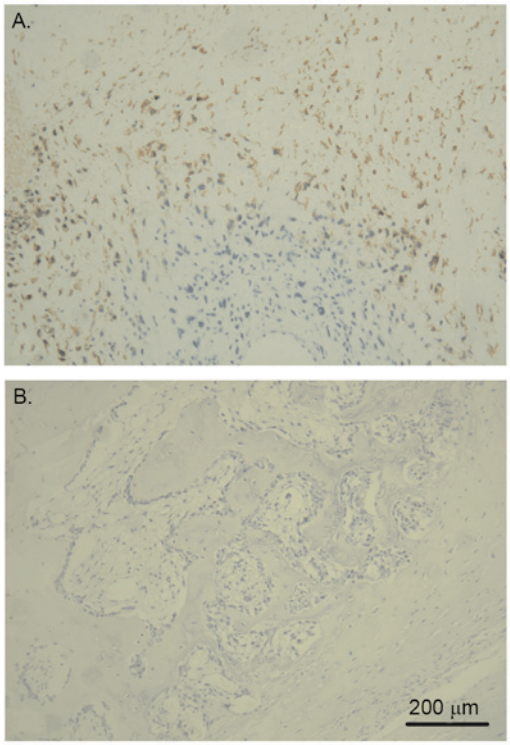

Figure 1. Immunohistochemical analysis of Glut-1 expression in osteosarcoma and tissue adjacent to carcinoma. (A) Positive Glut-1 staining was primarily observed in osteosarcoma samples, with positive staining intensity being stronger with increased distance from the stromal blood vessel. (B) In contrast, the majority of the tissues adjacent to carcinoma did not stain positive for Glut-1. Glut-1, glucose transporter-1. Scale bars in A and B=200 μm.

total RNA was conducted using the TRIzol RNA isolation kit (Invitrogen; Thermo Fisher Scientific, Inc.). All RNA samples were diluted to 1 μg/l and were reverse-transcribed using the PrimeScript RT-PCR kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's instructions. The PCR primers for Glut-1 were obtained from Fermentas; Thermo Fisher Scientific, Inc. (Table I). PCR assays were run in a Real-Time PCR System (ABI 7500; Applied Biosystems; Thermo Fisher Scientific, Inc.) using iTaq Universal SYBR-Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA). PCR was conducted as follows: 95˚C for 10 sec; 40 cycles of 95˚C for 5 sec; and 60˚C for 34 sec. Analysis of RT-PCR data was performed using the comparative Cq ($2^{-\Delta}$Cq) method to calculate levels of gene expression relative to the internal control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as previously described (25).

Statistical analysis. Statistical analysis was performed using the statistical software R (version 3.01, Nokia Bell Labs, Murray Hill, NJ, USA). A paired Student's t-test was used to identify significant differences in Glut-1 mRNA expression levels between osteosarcoma and tissues adjacent to carcinoma. Fisher's test was used to test the association between Glut-1 expression levels and clinicopathological variables. Cumulative survival rate was estimated using the Kaplan-Meier method, log-rank tests were performed to test the survival time difference, and univariate and multivariate proportional hazards (Cox) regressions were used to test the associations between survival time, and clinicopathological variables and Glut-1 expression. In the multivariate Cox regression, those associated variables were step-wisely selected according to Akaike's information criterion (26). $P<0.05$ was considered to indicate a statistically significant difference. In the figures, the symbols * and ** represent $P<0.05$ and $P<0.01$, respectively.

Results

Glut-1 protein expression in osteosarcoma and tissues adjacent to carcinoma. In general, Glut-1 protein was revealed to primarily be expressed in osteosarcoma cell membranes and cytoplasm, with the immunostaining having a focal or diffuse distribution pattern. The intensity of Glut-1^{+} cellular staining in osteosarcoma was significantly higher than that in paired tissue adjacent to carcinoma. In 38 (74.5%) of 51 patients with osteosarcoma, the expression of Glut-1 was positive. Indeed, half (19) of these patients demonstrated strong expression (Table I). On the other hand, only 6 (11.8%) of 51 patients had positive expression of Glut-1 in tissue adjacent to carcinoma and none of them had a strong expression intensity (Table II).

Table III. Relationship between glucose transporter protein-1 expression and clinicopathological characteristics.

Clinicopathological characteristic	n	Postive (n, %)	Negative (n, %)	P-value
Sex				
Male	28	22 (43.1)	6 (11.8)	0.529
Female	23	16 (31.4)	7 (13.7)	
Age				
<30 year	28	23 (45.1)	5 (9.8)	0.207
≥30 year	23	15 (29.4)	8 (15.7)	
Tumor site				
Distal femur	26	20 (39.2)	6 (11.8)	0.755
Proximal tibia	25	18 (35.3)	7 (13.7)	
Tumor volume				
<3 cm	5	4 (7.8)	1 (2.0)	0.012
≥3 cm	46	37 (72.5)	9 (17.6)	
Differentiation				
Well-differentiated	13	5 (9.8)	8 (15.7)	0.001
Moderately differentiated	38	33 (64.7)	5 (9.8)	
T stage				
T1+T2	20	13 (25.5)	7 (13.7)	0.513
T3	23	18 (35.3)	5 (9.8)	
T4	8	7 (13.7)	1 (2.0)	
Lymph node metastasis				
N0	16	8 (15.7)	8 (15.7)	0.013
N1	35	30 (58.8)	5 (9.8)	
TNM stage				
I	13	5 (9.8)	8 (15.7)	0.001
II	29	24 (47.1)	5 (9.8)	
III	9	9 (17.6)	0 (0.0)	
Inner metastasis				
No	36	25 (49.0)	11 (21.6)	0.297
Yes	15	13 (25.5)	2 (3.9)	
Recurrence				
No	14	13 (26.5)	1 (2.0)	<0.001
Yes	35	34 (69.4)	1 (2.0)	
Reaction to chemotherapy				
No	23	17 (34.0)	6 (12.0)	0.510
Yes	27	20 (40.0)	7 (14.1)	

TNM, tumor, node, metastasis.

In addition, in osteosarcoma samples, more intensely positive staining was observed in the center of the tumor tissue, with the positive intensity becoming stronger with increased distance from the stromal blood vessel (Fig. 1).

Glut-1 mRNA expression in fresh specimens of osteosarcoma and tissues adjacent to carcinoma. To determine the differences

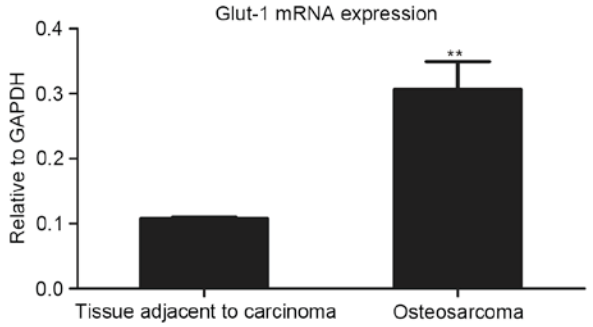

Figure 2. Glut-1 mRNA expression levels in osteosarcoma and the corresponding tissue adjacent to the carcinoma. Reverse transcription-quantitative polymerase chain reaction analysis of relative Glut-1 mRNA expression was performed in osteosarcoma and tissues adjacent to carcinoma. **P<0.01 vs. tissue adjacent to carcinoma. Glut-1, glucose transporter-1.

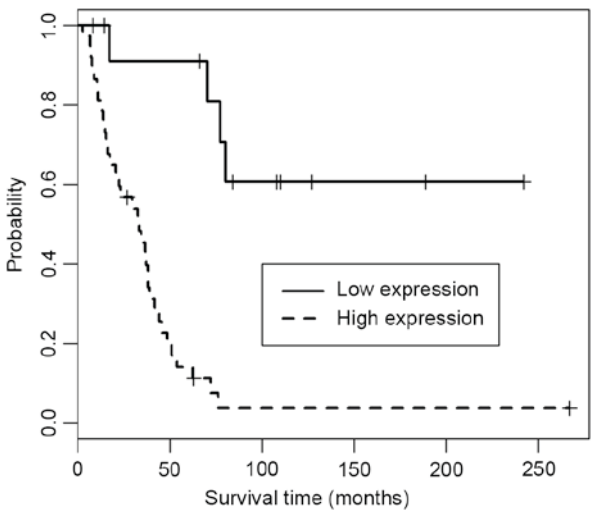

Figure 3. Kaplan-Meier curves of the survival times for patients with high and low glucose transporter-1 high expression levels.

in Glut-1 mRNA expression levels within or adjacent to carcinoma tissues, RT-qPCR analysis was conducted with freshly frozen specimens. The mRNA expression levels of Glut-1 in osteosarcoma tissues were significantly higher than those in tissues adjacent to carcinoma (P<0.01; Fig. 2).

Associations between Glut-1 expression and osteosarcoma clinicopathological parameters. Fisher's test was conducted to identify associations between Glut-1 expression and clinicopathological parameters. Sex, age, tumor site, T stage, inner metastasis and reaction to chemotherapy were not associated with Glut-1 expression (Table III). On the other hand, tumor volume, differentiation, lymph node metastasis, TNM stage and recurrence were observed to have a significant association with Glut-1 expression (Table II). Due to missing data in a few patients, the sample sizes for recurrence and reaction to chemotherapy were 49 and 50, respectively.

Association between Glut-1 expression and postoperative survival of osteosarcoma patients. While the survival time was the period between diagnosis and death for patients with tumor recurrence, in a few cases, patients died of other

Table IV. Single Cox regression analysis.

Factors	Coding	Hazard ratio	2.5% limit	97.5% limit	P-value
Sex	Male vs. female	0.924	0.488	1.749	0.808
Age		0.360	0.182	0.715	0.004
Site	Distal femur vs. proximal tibia	0.887	0.468	1.684	0.715
Size	≥3 cm vs. <3 cm	3.329	0.793	13.983	0.101
Differentiation	Poor vs. well	4.458	1.824	10.897	0.001
T stage	T4>T3>T1&T2	4.982	2.713	9.150	<0.001
Lymph	N1 vs. N0	9.590	3.608	25.490	<0.001
TNM stage	III>II>I	6.780	3.196	14.383	<0.001
Inner metastasis	Yes vs. no	2.598	1.283	5.261	0.008
Recurrence	Yes vs. no	57.158	7.434	439.195	<0.001
Reaction to chemotherapy	Poor vs. good	1.137	0.597	2.168	0.696
Glut-1 expression	High vs. low	8.75007	2.902	26.386	<0.001

Glut-1, glucose transporter protein-1; TNM, tumor, node, metastasis.

Table V. Multivariate Cox regression analysis.

Factors	Hazard ratio	2.5% limit	97.5% limit	z statistics	P-value
Age	0.301	0.136	0.611	0.0294	0.003
T stage	4.916	1.968	12.282	3.409	<0.001
Lymph	14.473	2.875	72.858	3.241	0.001
TNM stage	8.519	3.194	22.722	4.280	<0.001
Glut-1 expression	22.351	4.479	111.521	3.788	<0.001

Glut-1, glucose transporter protein-1; TNM, tumor, node, metastasis.

diseases during the follow-up phase, and this was considered as a truncated event. In the present study, the median survival time was defined as the time of 50% cumulative survival rates, following which half of the patients were still living. Kaplan-Meier survival curves were presented for the osteosarcoma patients with high or low Glut-1 expression (Fig. 3). From the survival curves, the median survival time for patients with low Glut-1 expression was observed to be 540 days, while for patients with high Glut-1 expression it was 317 days. In addition, Glut-1 overexpression was observed to be associated with a poor survival time. The survival curve of the patients with high expression significantly differed from that of the patients with low expression ($P=1.39x10^{-05}$, as determined by the log-rank test).

Single and multivariate Cox regression analyses of prognosis and survival. Single proportional hazards (Cox) regression analysis revealed that sex, tumor site, tumor size and reaction to chemotherapy were not significantly associated with survival time (P>0.05; Table III). On the other hand, age, differentiation, inner metastasis, recurrence, T stage, lymph, TNM stage and Glut-1 expression were revealed to have a significant association with survival time (P<0.05; Table III). However, in the following multivariate Cox regression analysis, the effects of differentiation, inner metastasis and recurrence were masked by the other risk factors due to collinearity. T stage, lymph, TNM stage and Glut-1 expression were still observed to be associated with survival time (P<0.05; Tables IV and V).

Discussion

Glut-1 is an essential carrier responsible for glucose transportation across the plasma membrane of cells. Cellular regulation of glucose intake is dependent on Glut-1 expression and function, either through active transport or facilitated diffusion and even under the circumstance of a low glucose concentration. Previous studies have demonstrated that Glut-1 is usually expressed at low levels in mammalian embryos and mature tissues, providing basic energy for normal cell growth and function. On the other hand, Glut-1 is typically expressed at a high level in multiple types of malignant carcinoma tissue and in atypical hyperplasia tissues with a high cancer risk, and this is believed to meet the requirements for increased absorption and utilization of glucose of the tumor cells (27). Although Glut-1 expression levels have been investigated in various types of tumor (15-17), no further literature has reported the association between Glut-1 expression and osteosarcoma beyond those of Endo *et al* (18), Kubo *et al* (19) and the present study. In the present study, in 51 paired human osteosarcoma specimens and adjacent non-cancerous specimens collected

between April 1993 and March 2012, Glut-1 expression levels were examined using immunohistochemistry and RT-qPCR. In total, 74.5% of osteosarcoma tissues stained positive for Glut-1, but only 11.8% adjacent tissues stained positively for Glut-1. The mRNA expression level of Glut-1 was also higher in osteosarcoma compared with non-cancerous tissues. These results were consistent with the results obtained by Endo *et al* (18). Furthermore, the associations between the Glut-1 expression and clinicopathological parameters of osteosarcoma were investigated.

The associations between Glut-1 expression and clinicopathological parameters have previously been investigated in certain other types of malignant tumor. Glut-1 expression in lung cancer was demonstrated to be associated with its malignant stage, with more advanced stages typically being accompanied with higher expression levels of Glut-1 (28). Expression of Glut-1 in endometrial lesions has also been demonstrated to be associated with cancer differentiation, and it is possible to use Glut-1 expression to effectively distinguish the malignant tendency from a benign tissue to atypical hyperplasia of the endometrium (29). Similarly, in pancreatic ductal adenocarcinoma and laryngeal cancer, Glut-1 expression levels have been demonstrated to be positively correlated with the clinical malignant stage (30). However, the associations between Glut-1 expression and clinicopathological parameters of osteosarcoma have not previously been reported. In the present study, based on the recorded clinical pathological characteristics for the collected specimens, the associations between Glut-1 expression levels, pathological variables and survival of the patients were examined with statistical methods, to evaluate the value of Glut-1 expression levels as a predictor of prognosis in osteosarcoma. The results revealed that the expression levels of Glut-1 were positively associated with osteosarcoma tumor volume, differentiation, lymph node metastasis, TNM stage and recurrence, indicating that Glut-1 is involved in the incidence of osteosarcoma. Since Glut-1 provides an energy supply for the rapid progression of malignant osteosarcoma, higher expression levels of Glut-1 are consistent with the malignant status. The data from the present study also demonstrated that Glut-1 expression levels are associated with cancer recurrence and metastasis.

In the present study, the median survival time of patients with positive expression of Glut-1 was decreased compared with those with negative expression of Glut-1. From the univariate analysis, patients with high Glut-1 expression and patients with low Glut-1 expression were revealed to have significant differences in survival rate. Multivariate analysis also revealed that the hazard ratio of the patients with high expression of Glut-1 was 22.4 times (95% confidence interval=4.5-111.5; $P=1.51x10^{-4}$) higher compared with patients with low expression. These results suggested that decreased survival time caused by the proliferative and invasive behaviors of malignant cells was significantly associated with Glut-1 overexpression. Therefore, Glut-1 has the potential to be a prognostic marker for osteosarcoma.

Aside from Glut-1 expression, there were other potential prognostic factors observed by the present study to be associated with survival time, including age, T stage, lymph and TNM stage, which is similar to the results of Endo *et al* (18). However, in clinical practice, while the adoption by surgeons of these factors as prognostic indicators may be more subjective, determining Glut-1 expression levels by immunohistochemistry is comparatively more objective and reliable. Therefore, Glut-1 expression levels may provide us with an independent and valuable reference that distinguishes high risk patients and develops an adapted therapeutic strategy (based on the levels of risk) for osteosarcoma treatment.

Although the present study is based on clinical data, other studies concerning the effects of inhibiting glucose transport in osteosarcoma have been conducted. The results obtained from these *in vivo* and in osteosarcoma cell *in vitro* studies are consistent with those from the present study (4,16). They demonstrated that Glut-1 expression is a key and independent prognostic factor for survival in osteosarcoma patients, supporting the idea that assessment of Glut-1 expression should be performed prior to treatment to predict the potential clinical effects.

The present study contains a few notable limitations. Due to the individual differences of the patient's physique and treatment, it is challenging to obtain clinicopathological data under the same circumstances. Meanwhile, the judgment of certain clinical pathological parameters is subjective and may lead to deviations. In addition, the follow-up phase for evaluating patient survival rates and the total number of patients remains limited, which may also affect the results. Therefore, to provide more definitive conclusions, further multi-institution studies are required with longer follow-up durations and larger patient populations.

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