GLUT1 and PKM2 may be useful prognostic predictors in patients with non-small cell lung cancer following curative R0 resection

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Abstract. Lung cancer has a poor prognosis despite recent progresses being made regarding its treatment. In addition, there is a paucity of reliable and independent prognostic predictors for non-small cell lung cancer (NSCLC) following curative resection. Glycolysis is associated with the malignancy and proliferation of cancer cells. Glucose transporter 1 (GLUT1) promotes glucose uptake, whereas pyruvate kinase M2 (PKM2) promotes anaerobic glycolysis. The present study aimed to evaluate the relationship between the expression of GLUT1 and PKM2 and the clinicopathological features of patients with NSCLC, and to identify a reliable prognostic factor for NSCLC following curative resection. Patients with NSCLC who underwent curative surgery were retrospectively enrolled to the present study. GLUT1 and PKM2 expression was assessed using immunohistochemistry. Subsequently, the association between the clinicopathological features of patients with NSCLC and the expression of GLUT1 and PKM2 was assessed. Of the 445 patients with NSCLC included in the present study, 65 (15%) were positive for both GLUT1 and PKM2 expression (G+/P+ group). GLUT1 and PKM2 positivity was significantly associated with sex, absence of adenocarcinoma, lymphatic invasion and pleural invasion. Furthermore, patients with NSCLC in the G+/P+ group presented significantly poorer survival rates than those expressing other markers. G+/P+ expression was significantly associated with poor disease-free survival. In conclusion, the findings of the present study indicated that the combination of GLUT1 and PKM2 may be considered a reliable prognostic factor for patients with NSCLC following curative resection, especially in patients with stage I NSCLC.

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Introduction

Lung cancer is the leading cause of cancer death worldwide, and has a poor prognosis in spite of recent advances in therapy (1). Approximately 85% of lung cancers are non-small cell lung cancer (NSCLC). The outcome of curative surgery in patients with NSCLC associated with several clinicopathological prognostic features, such as smoking history, gene mutations, and pathological stage (2,3). However, no reliable prognostic factor for NSCLC after curative resection has been identified yet.

Glucose metabolism plays an important role in the proliferation in cancer cells (4,5). Cancer cells rely more on anaerobic glycolysis than mitochondrial oxidation, even in the presence of ample oxygen; this is known as the Warburg effect (6). This suggests that cancer malignancy might be influenced by enzymes involved in glucose metabolism, especially those involved in anaerobic glycolysis within the cancer cells (7,8).

Glucose transporters (GLUTs) are responsible for glucose uptake through the cell membrane to compensate for the increased glucose metabolism in cancer cells. GLUT1 is overexpressed in both solid and hematological cancers (9-11). The relationship between GLUT1 and various cancers, including gastric cancer, hepatic cancer, prostate cancer, thyroid cancer, head and neck cancer, and NSCLC, has been reported (12). A meta-analysis reported that GLUT1 overexpression in NSCLC is associated with a poor prognosis (13). In contrast, other studies suggest that GLUT1 affects the prognosis in patients with NSCLC and has a different effect in complete resection (14-17). Thus, the relationship between GLUT1 and NSCLC following curative R0 operation remains unclear.

Pyruvate kinase M2 (PKM2) is a glucose metabolic enzyme that promotes anaerobic glycolysis, and its selective expression plays an important role in the Warburg effect (18). Guo *et al* reported a correlation between PKM2 expression and good prognosis in patients with lung adenocarcinoma as well as a correlation between higher expression of PKM2 with shorter overall and disease-free survival (19). In contrast, Rzechonek *et al* reported that PKM2 has a low specificity and its utility in NSCLC diagnosis or evaluation of cancer progression is limited (20). Thus, the role of PKM2 as a prognostic marker in NSCLC remains controversial.

GLUT1 is overexpressed in hypoxic environments and its overexpression is associated with increased glucose metabolism through anaerobic glycolysis in cancer cells (21). PKM2 also plays an important role in anaerobic glycolysis in cancer cells. Thus, these two enzymes involved in glucose uptake and metabolism may have a significant effect on tumor malignancy. However, to best of our knowledge, there is no studies that examined the correlation between the glucose uptake affected by GLUT1 and glucose metabolism pathway by PKM2 on the clinicopathological features and prognosis associated with NSCLC. The purpose of this study is to identify a reliable glucose metabolic enzyme-based prognostic predictor for NSCLC following curative R0 surgery.

Materials and methods

Patient selection. This single-center retrospective cohort study was conducted with clinical course of 665 patients with NSCLC who underwent surgical procedure at the Osaka City University Hospital, Osaka, Japan, between January 2010 and December 2016. We excluded patients from the investigation who underwent R1 or R2 surgery, who received preoperative chemotherapy and/or radiation therapy, who did not undergo curative resection procedures such as segmentectomy, wedge resection or lobectomy without mediastinal lymph node dissection. A total of 445 patients were enrolled in this study, and all of whom were diagnosed with histologically confirmed stage 0 to IIIA primary NSCLC and who underwent radical resection (other than lobectomy and mediastinal lymph node dissection). We determined pathological findings according to the 8th edition of the Union for International Cancer Control TNM classification. The regimen of adjuvant chemotherapy was determined in consultation with of surgeons, radiologists, and oncologists. All patients underwent follow-up examinations every 2-6 months, which involved chest radiography, computed tomography, and assessment of tumor markers.

This study was conducted in accordance with the Declaration of Helsinki and approved by the Osaka City University Ethics Committee (approval number 2019-006). Written informed consent was obtained from all the patients prior to the operative procedure. All procedures involving humans were performed in accordance with the relevant guidelines and regulations.

GLUT1 and PKM2 immunostaining. Immunohistochemical staining was performed on paraffin-embedded sections of primary lesions obtained from 445 patients with NSCLC. We deparaffinize the slides with a thickness of 4 μm in xylene and hydrated in decreasing concentrations of ethyl alcohol and incubate the sections with 3% hydrogen peroxide to block endogenous peroxidase activity. Then, we heated the sections in Target Retrieval Solution (DAKO, Carpinteria, CA, USA) for 10 min at 105˚C using an autoclave. Nonspecific binding was blocked by incubating the sections with 10% normal rabbit serum for 10 min. The specimens were incubated with anti-GLUT1 antibodies (sc-377228; 1:150; Santa Cruz Biotechnology, Dallas, TX, USA; RRID: AB_2716767) for 30 min at 24˚C and with anti-PKM2 antibodies (sc-365684; 1:200; Santa Cruz Biotechnology; RRID: AB_2716767) at 4˚C overnight. Subsequently, these sections were incubated with a mouse linker for 10 min, and a peroxidase-labeled polymer solution (Histofine SAB-PO(M), #424022, Nichirei Biosciences Inc., Tokyo, Japan) for 5 min, then counterstained with Mayer's hematoxylin (#131-09665; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) for 30 sec at 24˚C.

Immunohistochemical analysis. In this study, we evaluate immunostaining intensity score by visual scoring with a BZ-X710 microscope (Keyence, Osaka, Japan). Positive immunostaining of GLUT1 and PKM2 were evaluated based on the intensity of membranous staining in the innermost part of the tumor and the proportion of immunoreactive cells. The immunostaining intensity score was defined as follows: 0, negative; 1+, weakly positive; 2+, positive; 3+, strongly positive (Fig. 1). The immunostaining proportion score was determined by estimating the proportion of positive cells and defined as follows: 0, no immunoreactive cells; 1+, <30% immunoreactive cells; 2+, 40-70% immunoreactive cells; 3+, >80% immunoreactive cells. We calculated the final numerical score by summing up the two scores and ranged from 0 to 6; both GLUT1 and PKM2 expressions were considered positive when the total score was ≥4. We evaluated the association between clinicopathological features and the expression levels of GLUT1 and PKM2.

Bioinformatics analysis. The ProggeneV2 (http://genomics.jefferson.edu/proggene/) database sourced the relevant data (22). The GSE 42127 dataset was used to evaluate the prognostic value of GLUT1 and PKM2 in NSCLC survival (23). Kaplan-Meier plots were used for overall survival rates, then compared with the Log rank test with GSE 42127 dataset.

Statistical analysis. The χ^2 test was used to determine significant differences between covariates. Survival duration was constructed using the Kaplan-Meier method and analyzed using the log-rank test. The Cox proportional hazards model was used for multivariate analysis. Multivariate analysis was performed between variables with significant difference in univariate analysis. $P<0.05$ was considered statistically significant. All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interface of R (version 2.13.0) and a modified version of the R commander (version 1.6-3) (The R Foundation for Statistical Computing, Vienna, Austria) (24).

Results

Relationship between GLUT1 and PKM2 expression and clinicopathological features. The clinicopathological features of all 445 patients based on GLUT1 and PKM2 expression are summarized in Table I. The median age was 69 years (range, 34-91 years). In total, 106 (24%) and 190 (43%) specimens were GLUT1-positive and PKM2-positive, respectively. Thus, there were 65 (15%) GLUT1- and PKM2-positive (G+/P+ group) patients. GLUT1-positivity significantly associated with sex ($P<0.001$) and the presence of squamous cell carcinoma ($P<0.001$), lymphatic invasion ($P<0.001$), venous invasion ($P=0.005$), pleural invasion ($P<0.001$), and depth of invasion ($P<0.001$), as opposed to GLUT1 negativity. PKM2 positivity was not significantly associated with any of the clinicopathological features of patients with NSCLC. There

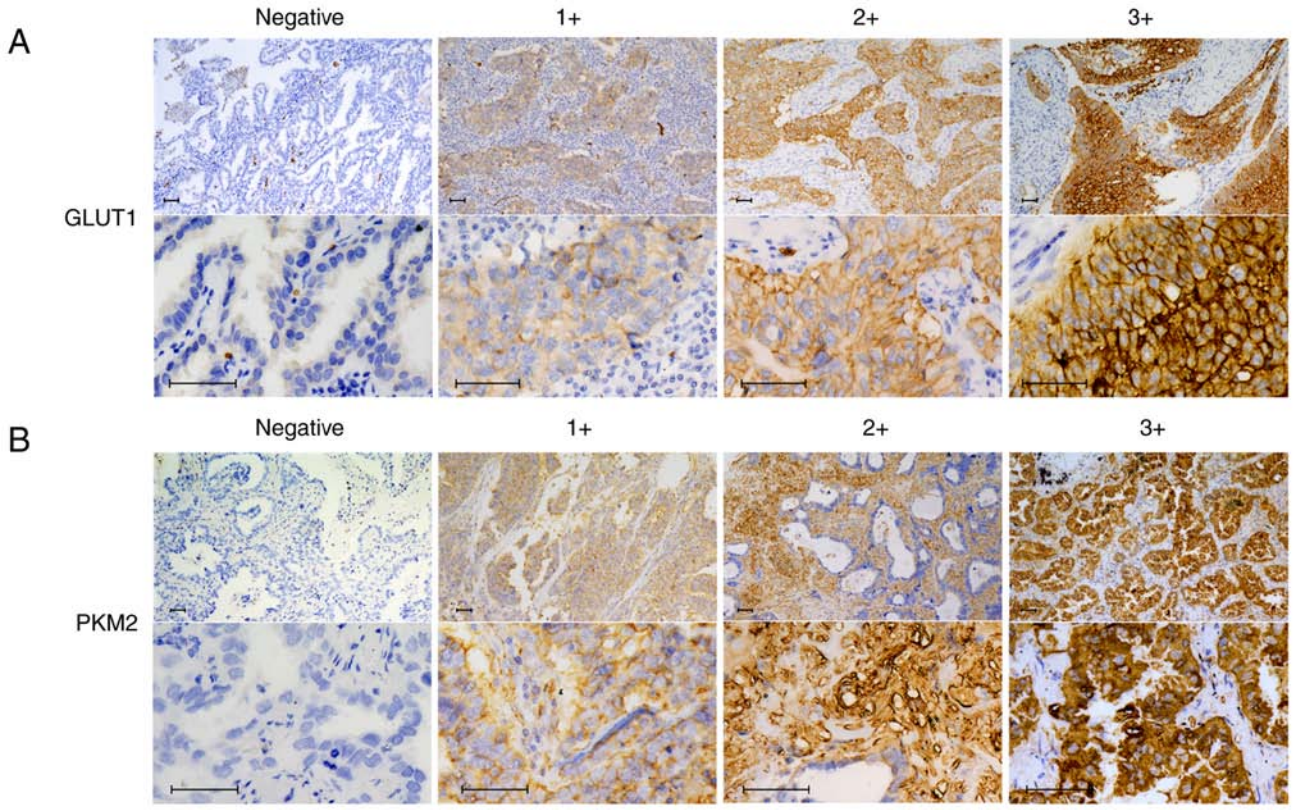

Figure 1. Representative images of immunostaining intensities of GLUT1 and PKM2 expression in patients with non-small cell lung carcinoma. The immunostaining intensity of (A) GLUT1 and (B) PKM2. Intensity score: 0, negative; 1+, weakly positive; 2+, positive; 3+, strongly positive. Scale bar: 50 μm. GLUT1, glucose transporter 1; PKM2, pyruvate kinase M2.

was a significant positive association between GLUT1 and PKM2 expression (P<0.001). GLUT1- and PKM2-positivity significantly associated with sex (P=0.016) and the absence of adenocarcinoma (P<0.001), lymphatic invasion (P=0.004), and pleural invasion (P=0.014). In patients with stage 0-I NSCLC, 59 patients had squamous cell carcinoma. Table II shows the relationship between expression of GLUT1 and clinicopathologic features in patients with squamous cell carcinoma and non-squamous cell carcinoma. There was no significant difference in any of clinicopathological features in patients with squamous cell carcinoma. Compared to all of 445 patients with NSCLC, patients with non-squamous cell carcinoma showed similar result except lymph node metastasis. GLUT1-positivity significantly associated with lymph node metastasis (P=0.017) as opposed to GLUT1-negativity. We compared the maximum standardized uptake value (SUVmax) of [^{18}F]fluorodeoxyglucose positron emission tomography/computed tomography (^{18}F-FDG-PET) of primary tumor between patients with G+/P+ expression and other expressions, but there was no significant difference (data not shown).

Association between GLUT1 expression and survival of patients with NSCLC. Comparison of the 5-year overall survival rate between GLUT1-positive and GLUT1-negative patients with NSCLC is presented in Fig. 2. GLUT1-positive patients with NSCLC had significantly poorer overall survival rates (P<0.001) than GLUT1-negative patients. The similar result was found from the dataset GSE42127 in PROGgeneV2 database (Fig. S1A).

Comparison of the 5-year disease free survival rate between GLUT1-positive and -negative patients with NSCLC is presented in Fig. 3A. GLUT1-positive patients with NSCLC presented significantly poorer disease-free survival rates (P<0.001) than those who were GLUT1-negative. Regarding tumor pathological stage, the 5-year disease-free survival rate of GLUT1-positive patients with stage I NSCLC was significantly poorer than that of GLUT-1 negative patients (P<0.001). In contrast, no significant difference in the 5-year disease-free survival rate was found between patients with stage II and III NSCLC with positive and negative GLUT1 expressions.

There was no significant difference in overall survival and disease-free survival rates according to the adjuvant chemotherapy in patients with GLUT1 positive stage II-III NSCLC (Fig. S2A and D).

Association between PKM2 expression and survival of patients with NSCLC. Comparison of the 5-year overall survival rate between PKM2-positive and PKM2-negative patients with NSCLC is presented in Fig. 2. There was no significant difference in the survival rate (P=0.274) between PKM2-positive and PKM2-negative patients with NSCLC. The similar result was found from the dataset GSE42127 in PROGgeneV2 database (Fig. S1B).

Comparison of the 5-year disease free survival rate between PKM2-positive and -negative patients with NSCLC is presented in Fig. 3B. There was no significant difference in the disease-free survival rate (P=0.227) between PKM2-positive and -negative patients with NSCLC. In terms of tumor

Table I. Relationship between expression of GLUT1 and/or PKM2 and clinicopathologic features in 445 patients with NSCLC.

	GLUT1 expression			PKM2 expression			GLUT1 and PKM2 expression		
Variables (N)	Positive (n=106)	Negative (n=339)	P-value	Positive (n=190)	Negative (n=255)	P-value	G+/P+ (n=65)	Others (n=380)	P-value
Age, years									
<65 (136)	32 (30.2%)	104 (30.7%)	1	59 (31.1%)	77 (30.2%)	0.917	18 (27.7%)	118 (31.1%)	0.663
≥65 (309)	74 (69.8%)	235 (69.3%)		131 (68.9%)	178 (69.8%)		47 (72.3%)	262 (68.9%)	
Sex									
Female (155)	19 (17.9%)	136 (40.1%)	<0.001	60 (31.6%)	95 (37.3%)	0.228	14 (21.5%)	141 (37.1%)	0.016
Male (290)	87 (82.1%)	203 (59.9%)		130 (68.4%)	160 (62.7%)		51 (78.5%)	239 (62.9%)	
Smoking									
Yes (313)	68 (64.2%)	245 (72.3%)	0.115	135 (71.1%)	178 (69.8%)	0.834	45 (69.2%)	268 (70.5%)	0.883
No (132)	38 (35.8%)	94 (27.7%)		55 (28.9%)	77 (30.2%)		20 (30.8%)	112 (29.5%)	
Histology									
Adenocarcinoma (299)	39 (36.8%)	260 (76.7%)	<0.001	126 (66.3%)	173 (67.8%)	0.894	29 (44.6%)	270 (71.1%)	<0.001
Squamous cell carcinoma (120)	58 (54.7%)	62 (18.3%)		52 (27.4%)	68 (26.7%)		30 (46.2%)	90 (23.7%)	
Others[a] (26)	9 (8.5%)	17 (5.0%)		12 (6.3%)	14 (5.5%)		6 (9.2%)	20 (5.3%)	
Lymphatic invasion									
Negative (305)	56 (52.8%)	249 (73.5%)	<0.001	123 (64.7%)	182 (71.4%)	0.149	34 (52.3%)	271 (71.3%)	0.004
Positive (140)	50 (47.2%)	90 (26.5%)		67 (35.3%)	73 (28.6%)		31 (47.7%)	109 (28.7%)	
Venous invasion									
Negative (366)	77 (72.6%)	289 (85.3%)	0.005	157 (82.6%)	209 (82.0%)	0.901	48 (73.8%)	318 (83.7%)	0.077
Positive (79)	29 (27.4%)	50 (14.7%)		33 (17.4%)	46 (18.0%)		17 (26.2%)	62 (16.3%)	
Pleural invasion									
Negative (331)	63 (59.4%)	268 (79.1%)	<0.001	145 (76.3%)	186 (72.9%)	0.444	40 (61.5%)	291 (76.6%)	0.014
Positive (114)	43 (40.6%)	71 (20.9%)		45 (23.7%)	69 (27.1%)		25 (38.5%)	89 (23.4%)	
Depth of invasion									
T1 or T2 (384)	80 (75.5%)	304 (89.7%)	<0.001	162 (85.3%)	222 (87.1%)	0.581	51 (78.5%)	333 (87.6%)	0.053
T3 or T4 (61)	26 (24.5%)	35 (10.3%)		28 (14.7%)	33 (12.9%)		14 (21.5%)	47 (12.4%)	
Lymph node metastasis									
N0 (346)	76 (71.7%)	270 (79.6%)	0.108	144 (75.8%)	202 (79.2%)	0.421	45 (69.2%)	301 (79.2%)	0.078
N1 or N2 (99)	30 (28.3%)	69 (20.4%)		46 (24.2%)	53 (20.8%)		20 (30.8%)	79 (20.8%)	
pStage									
0-II[b] (365)	83 (78.3%)	282 (83.2%)	0.25	154 (81.1%)	211 (82.7%)	0.708	50 (76.9%)	315 (82.9%)	0.293
III (80)	23 (21.7%)	57 (16.8%)		36 (18.9%)	44 (17.3%)		15 (23.1%)	65 (17.1%)	

Table I. Continued.

Variables (N)	GLUT1 expression			PKM2 expression			GLUT1 and PKM2 expression		
	Positive (n=106)	Negative (n=339)	P-value	Positive (n=190)	Negative (n=255)	P-value	G+/P+ (n=65)	Others (n=380)	P-value
PKM2 expression									
Negative (255)	41 (38.7%)	214 (63.1%)	<0.001						
Positive (190)	65 (61.3%)	125 (36.9%)							

[a]Others, adenosquamous cell carcinoma, large cell carcinoma, pleomorphic carcinoma, and mucoepidermoid carcinoma [b]Stage 0, in TNM classification of lung cancer in UICC 8, stage 0 means TisN0M0 patient. When the cancer is carcinoma *in situ*, the T factor is 'Tis'. GLUT1, glucose transporter 1; PKM2, pyruvate kinase M2; G+/P+, GLUT1 positive and PKM2 positive; NSCLC, non-small cell lung carcinoma.

pathological stage, the 5-year disease-free survival rate of PKM2-positive patients with stage I NSCLC was significantly poorer than that of PKM2-negative patients (P=0.017). In contrast, no significant difference in the survival rate was found between patients with stage II and III NSCLC with positive and negative PKM2 expression.

There was no significant difference in overall survival and disease-free survival rates according to the adjuvant chemotherapy in patients with PKM2 positive stage II-III NSCLC (Fig. S2B and E).

Association between GLUT1 and/or PKM2 expression and survival of patients with NSCLC. The 5-year overall survival rate based on GLUT1 and/or PKM2 expression in all the 445 patients is presented in Fig. 2. Patients in the GLUT1-positive and PKM2-positive (G+/P+) group presented significantly poorer overall survival rates (P<0.001) than those in the GLUT1-negative and PKM2-negative ('other expressions') groups.

The 5-year disease-free survival rate based on GLUT1 and/or PKM2 expression in all the 445 patients is presented in Fig. 3C. Patients in the G+/P+ group presented significantly poorer disease-free survival rates (P<0.001) than those in the 'other expressions' group. Regarding tumor pathological stage, the 5-year disease-free survival rate of patients with stage I NSCLC in the G+/P+ group was significantly poorer than that of patients in the 'other expressions' group (P<0.001). In contrast, no significant difference in survival was found between patients with stage II and III NSCLC in the G+/P+ group and those in the 'other expressions' group (Fig. S3).

There was no significant difference in overall survival and disease-free survival rates according to the adjuvant chemotherapy in patients with G+/P+ stage II-III NSCLC (Fig. S2C and F).

Univariate and multivariate analyses. Table III shows the results of the univariate and multivariate analyses for overall survival. Univariate analysis revealed that poor overall survival was significantly associated with GLUT1 positivity (P<0.001), GLUT1 and PKM2 positivity (P=0.001), male (P<0.001), smoking history (P=0.049), histological type of adenocarcinoma (P=0.001), lymphatic invasion (P=0.006), pleural invasion (P=0.002), pathological T3/4 (P=0.02), and lymph node metastasis (P=0.014) are significantly associated with poor overall survival. Multivariate analysis including the significant factors mentioned above showed that the male (P<0.001) was significantly associated with poor overall survival.

Table IV shows the results of the univariate and multivariate analyses for disease-free survival. Univariate analysis revealed that GLUT1 positivity (P<0.001), GLUT1 and PKM2 positivity (P<0.001), male (P<0.001), histological type of adenocarcinoma (P=0.03), lymphatic invasion (P<0.001), venous invasion (P<0.001), pleural invasion (P<0.001), pathological T3/4 (P<0.001), and lymph node metastasis (P<0.001) are significantly associated with poor disease-free survival. Multivariate analysis including the significant factors mentioned above showed that GLUT1 and PKM2 positivity (P=0.039), male (P=0.003), pleural invasion (P=0.004), and lymph node metastasis (P<0.001) were significantly associated with poor disease-free survival.

Table II. Relationship between expression of GLUT1 and clinicopathologic features in 445 patients with squamous cell carcinoma and non-squamous cell carcinoma.

	GLUT1 expression in squamous cell carcinoma			GLUT1 expression in non-squamous cell carcinoma		
Variables (N)	Positive (n=58)	Negative (n=62)	P-value	Positive (n=48)	Negative (n=277)	P-value
Age, years						
<65 (136)	15 (25.9%)	9 (14.5%)	0.170	17 (35.4%)	95 (34.3%)	0.871
≥65 (309)	43 (74.1%)	53 (85.5%)		31 (64.6%)	182 (65.7%)	
Sex						
Female (155)	10 (17.2%)	7 (11.3%)	0.435	9 (18.8%)	129 (46.6%)	<0.001
Male (290)	48 (82.8%)	55 (88.7%)		39 (81.2%)	148 (53.4%)	
Smoking						
Yes (313)	36 (62.1%)	42 (67.7%)	0.568	32 (66.7%)	203 (73.3%)	0.383
No (132)	22 (37.9%)	20 (32.3%)		16 (33.3%)	74 (26.7%)	
Lymphatic invasion						
Negative (305)	35 (60.3%)	43 (69.4%)	0.341	21 (43.8%)	206 (74.4%)	<0.001
Positive (140)	23 (39.7%)	19 (30.6%)		27 (56.2%)	71 (25.6%)	
Venous invasion						
Negative (366)	44 (75.9%)	52 (83.9%)	0.362	33 (68.8%)	237 (85.6%)	0.007
Positive (79)	14 (24.1%)	10 (16.1%)		15 (31.2%)	40 (14.4%)	
Pleural invasion						
Negative (331)	38 (65.5%)	47 (75.8%)	0.234	25 (52.1%)	221 (79.8%)	<0.001
Positive (114)	20 (34.5%)	15 (24.2%)		23 (47.9%)	56 (20.2%)	
Depth of invasion						
T1 or T2 (384)	41 (70.7%)	50 (80.6%)	0.286	39 (81.2%)	254 (91.7%)	0.035
T3 or T4 (61)	17 (29.3%)	12 (19.4%)		9 (18.8%)	23 (8.3%)	
Lymph node metastasis						
N0 (346)	44 (75.9%)	41 (66.1%)	0.315	32 (66.7%)	229 (82.7%)	0.017
N1 or N2 (99)	14 (24.1%)	21 (33.9%)		16 (33.3%)	48 (17.3%)	
pStage						
0-II[a] (365)	48 (82.8%)	50 (80.6%)	0.817	35 (72.9%)	232 (83.8%)	0.100
III (80)	10 (17.2%)	12 (19.4%)		13 (27.1%)	45 (16.2%)	
PKM2 expression						
Negative (255)	28 (48.3%)	40 (64.5%)	0.097	13 (27.1%)	174 (62.8%)	<0.001
Positive (190)	30 (51.7%)	22 (35.5%)		35 (72.9%)	103 (37.2%)	

[a]Stage 0, In TNM classification of lung cancer in UICC 8, stage 0 means TisN0M0 patient. When the cancer is carcinoma *in situ*, the T factor is 'Tis'. GLUT1, glucose transporter1; PKM2, pyruvate kinase M2; G+/P+, GLUT1 positive and PKM2 positive; NSCLC, non-small cell lung carcinoma.

Discussion

In this study, we revealed that GLUT1 positivity was significantly associated with sex, the histological type of squamous cell carcinoma, lymphatic invasion, venous invasion, pleural invasion, depth of invasion, and poor prognosis, but was not an independent prognostic factor. However, the combination of GLUT1 and PKM2 expression was found to be a reliable independent prognostic predictor for patients with NSCLC.

The activities of GLUT1 and PKM2 are associated with various cancers; however, no study has evaluated the relationship between GLUT1 and PKM2 expression and the clinicopathological features associated with NSCLC to date. In this study, positive expression of both GLUT1 and PKM2 in NSCLC significantly associated with the male sex, squamous cell carcinoma, lymphatic invasion, and pleural invasion. Compared with adenocarcinoma, squamous cell carcinoma is associated with low oxygen-containing environments (25). This may be due to lower microvessel density compared to that of adenocarcinoma (26). The lower vessel density of squamous cell carcinoma leads to hypoxic microenvironment relative to tumor oxygen demand, and may affect subsequent upregulation of anaerobic glucose metabolic markers. Under aerobic

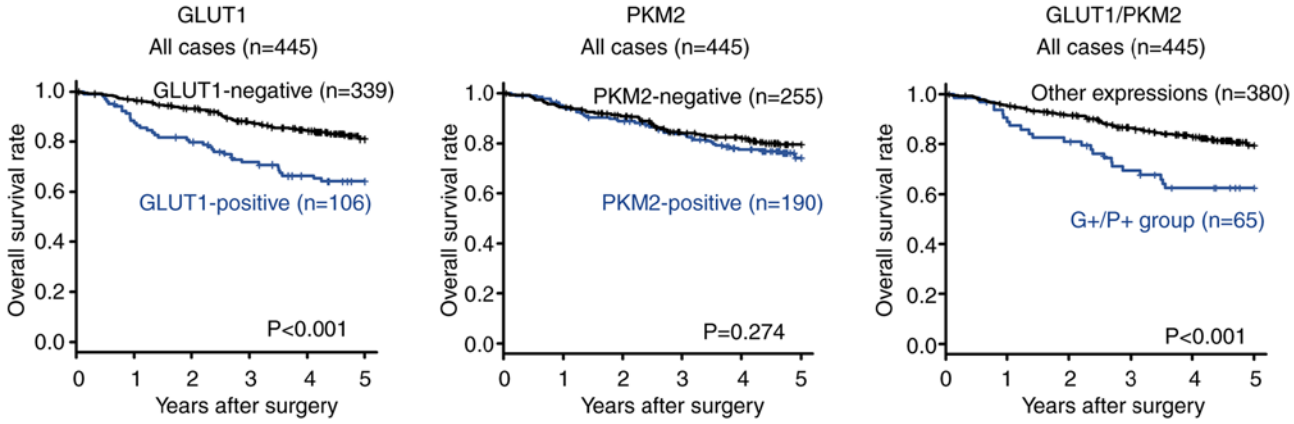

Figure 2. Five-year overall survival rate of patients with non-small cell lung carcinoma based on GLUT1 and/or PKM2 expression. GLUT1, glucose transporter 1; PKM2, pyruvate kinase M2; G+/P+, positive GLUT1 and PKM2 expression.

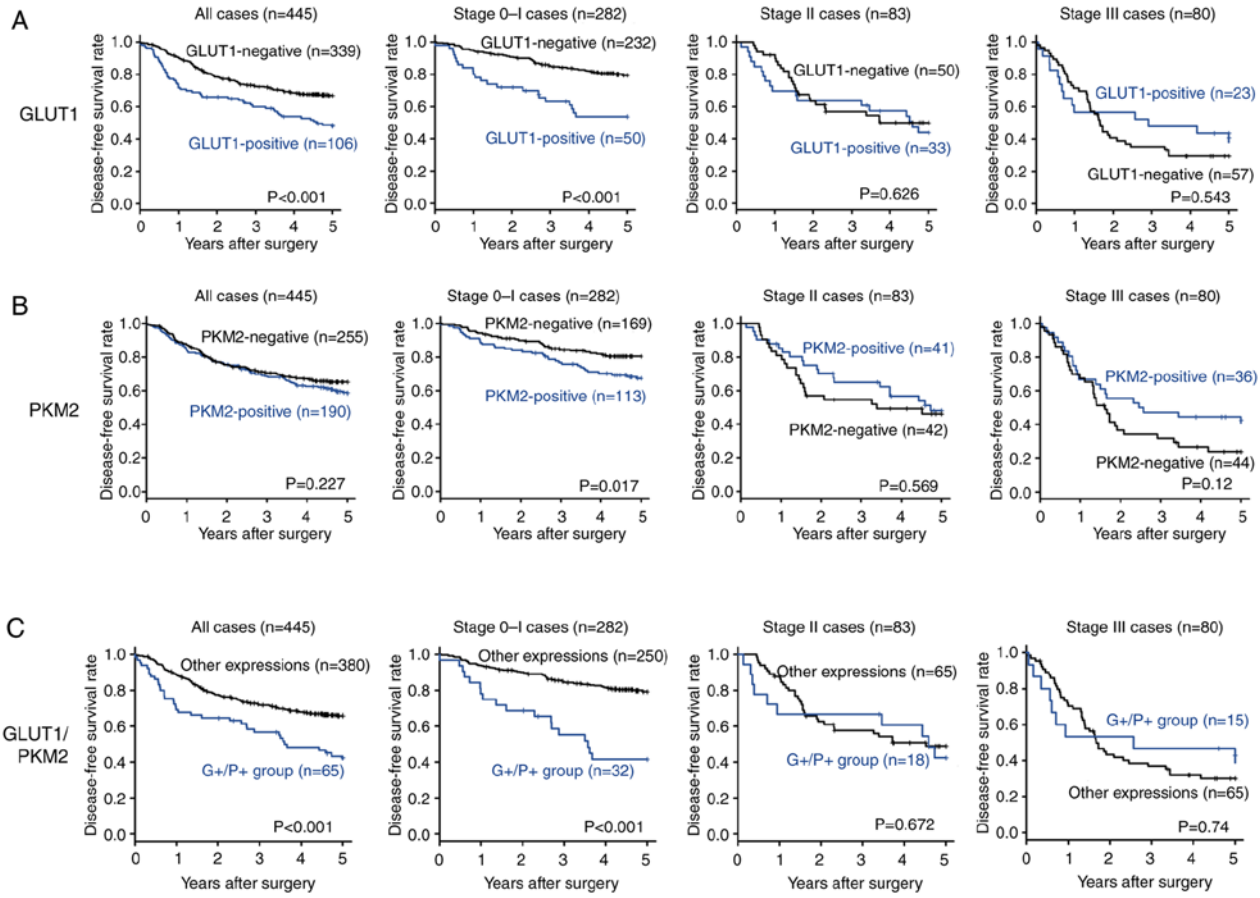

Figure 3. Disease-free survival rate in patients with non-small cell lung carcinoma based on GLUT1 and/or PKM2 expression. (A) Relationship between the disease-free survival rate and GLUT1 expression. (B) Relationship between the disease-free survival rate and PKM2 expression. (C) Relationship between the disease-free survival rate and GLUT1 and/or PKM2 expression. GLUT1, glucose transporter 1; PKM2, pyruvate kinase M2; G+/P+, positive GLUT1 and PKM2 expression.

conditions, normal cells obtain energy from glucose via mitochondrial oxidative phosphorylation (27). Upregulation of anaerobic glycolysis in cancer cells and squamous cell carcinoma in hypoxic conditions might increase the expression of PKM2 (18,25). In addition, GLUT1 expression may have been upregulated owing to increased glucose consumption. GLUT1 expression can be enhanced by the hypoxia-inducible factor-1α (HIF-1α), which is upregulated in hypoxic environments (28). These findings imply that owing to the relatively lower oxygen levels in the squamous cell carcinoma environment, squamous cell carcinoma might be more common than adenocarcinoma in patients with positive expressions of both GLUT1 and PKM2. Since both GLUT1 and PKM2 are glucose metabolism enzymes, we examined the association between diabetes and the expressions of these enzymes and observed no significant association (data not shown).

Table III. Univariate and multivariate analysis of 5-year overall survival of 445 patients with NSCLC.

	Univariate analysis			Multivariate analysis (GLUT1 and PKM2 separately)			Multivariate analysis (combination of GLUT1 and PKM2)		
Variables	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value
GLUT1									
Positive vs. Negative	2.27	1.49-3.45	<0.001	1.45	0.92-2.33	0.114			
PKM2									
Positive vs. Negative	1.25	0.83-1.89	0.275	1.10	0.73-1.67	0.643			
GLUT1/PKM2									
G+/P+ vs. Other expressions	2.17	1.36-3.47	0.001				1.58	0.97-2.58	0.067
Sex									
Male vs. Female	4.99	2.66-9.37	<0.001	3.95	2.06-7.56	<0.001	4.03	2.11-7.71	<0.001
Age, years									
≥65 vs. <65	1.37	0.87-2.17	0.175						
Smoking									
Yes vs. No	0.66	0.44-2.29	0.049	0.73	0.48-2.22	0.146	0.71	0.34-1.09	0.111
Histology									
Adenocarcinoma vs. Others	0.52	0.69-0.77	0.001	0.87	0.56-1.35	0.531	0.83	0.54-1.28	0.397
Lymphatic invasion									
Positive vs. Negative	1.78	1.18-2.67	0.006	1.10	0.68-1.76	0.702	1.13	0.71-1.80	0.608
Venous invasion									
Positive vs. Negative	1.38	0.84-2.29	0.206						
Pleural invasion									
Positive vs. Negative	1.94	1.27-2.94	0.002	1.45	0.90-2.32	0.123	1.43	0.90-2.29	0.131
Pathological T									
3 or 4 vs. 1 or 2	1.82	1.10-3.01	0.02	1.08	0.63-1.85	0.771	1.10	0.65-1.88	0.721
Pathological N									
1 or 2 vs. 0	1.74	1.12-2.70	0.014	1.30	0.81-2.09	0.273	1.27	0.79-2.03	0.325

GLUT1, glucose transporter1; PKM2, pyruvate kinase M2; G+/P+, GLUT1 positive and PKM2 positive; NSCLC, non-small cell lung carcinoma; CI, confidence interval; HR, hazard ratio.

A correlation between GLUT1 expression and sex was reported in patients with NSCLC (13,29,30); however, no correlation has been reported between PKM2 expression and sex. Moreover, we found a significant association between sex and GLUT1 expression, but not between sex and PKM2 expression. Squamous cell carcinoma is closely associated with a history of smoking, and a large proportion of Japanese men have a history of smoking (31), which could be the reason for the high frequency of squamous cell carcinoma in the G+/P+ group.

The importance of glycolysis in lymphangiogenesis has been well established (32). The glucose metabolic enzyme, PKM2, plays an important role in anaerobic glycolysis and has been reported to promote lymphangiogenesis and the proliferation and migration of lymphatic endothelial cells (33). In addition, high GLUT1 expression was associated with lymph node metastasis in patients with lung cancer (34), indicating that high GLUT1 expression in tumors correlates with lymphatic invasion. These results indicate that patients in the G+/P+ group have a significantly higher lymphatic invasion rate than those in the 'other expressions' group.

GLUT1 expression is associated with large tumor sizes in patients with lung cancer (13,34). Tumor growth activity is partially regulated by PKM2-initiated tumor angiogenesis (35,36). As tumor size increases, the tumor edge can reach the visceral pleura. Both GLUT1 and PKM2 have been reported to promote the invasive ability of tumors (37,38). These findings suggest that the patients in the G+/P+ group have a high rate of pleural invasion than those in the 'other expressions' group.

There was no significant difference in lymph node metastasis according to GLUT1 expression in patients with squamous cell carcinoma, whereas patients with non-squamous cell carcinoma had significant association between GLUT1 expression and lymph node metastasis. Positive expression of GLUT1 has been reported to be associated with lymph node metastasis in patients with lung cancer (34), and relationship between lymph node metastasis and GLUT1

Table IV. Univariate and multivariate analysis of 5-year disease-free survival of 445 patients with NSCLC.

	Univariate analysis			Multivariate analysis (GLUT1 and PKM2 separately)			Multivariate analysis (Combination of GLUT1 and PKM2)		
Variables	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value
GLUT1									
Positive vs. Negative	1.85	1.35-2.56	<0.001	1.33	0.92-1.92	0.129			
PKM2									
Positive vs. Negative	1.20	0.88-1.64	0.228	1.03	0.75-1.41	0.854			
GLUT1/PKM2									
G+/P+ vs. Other expressions	2.07	1.43-2.98	<0.001				1.49	1.02-2.19	0.039
Sex									
Male vs. female	2.42	1.66-3.54	<0.001	1.80	1.21-2.69	0.004	1.81	1.22-2.70	0.003
Age, years									
≥65 vs. <65	1.29	0.92-1.83	0.144						
Smoking									
Yes vs. No	0.78	0.56-1.08	0.122						
Histology									
Adenocarcinoma vs. Others	0.70	0.51-0.96	0.03	1.20	0.85-1.72	0.299	1.20	0.83-1.67	0.345
Lymphatic invasion									
Positive vs. Negative	2.36	1.74-3.22	<0.001	1.18	0.82-1.70	0.367	1.19	0.83-1.71	0.34
Venous invasion									
Positive vs. Negative	2.28	1.61-3.22	<0.001	1.24	0.85-1.81	0.268	1.26	0.86-1.83	0.239
Pleural invasion									
Positive vs. Negative	2.53	1.84-3.46	<0.001	1.72	1.19-2.47	0.004	1.71	1.19-2.45	0.004
Pathological T									
3 or 4 vs. 1 or 2	2.31	1.60-3.34	<0.001	1.38	0.91-2.09	0.133	1.39	0.92-2.11	0.118
Pathological N									
1 or 2 vs. 0	3.10	2.26-4.27	<0.001	2.33	1.65-3.29	<0.001	2.30	1.63-3.24	<0.001

GLUT1, glucose transporter1; PKM2, pyruvate kinase M2; G+/P+, GLUT1 positive and PKM2 positive; NSCLC, non-small cell lung carcinoma; CI, confidence interval; HR, hazard ratio.

expression differs in patients with adenocarcinoma and non-adenocarcinoma (17). Thus, the effect of GLUT1 on lymph node metastasis may differ between squamous and non-squamous cell carcinoma.

While there was no significant difference in prognosis between PKM2 expression, GLUT1-positive patients with NSCLC exhibited a significantly poorer overall survival rate and disease-free survival rate than GLUT1-negative patients. We found the similar result from the dataset GSE42127 in PROGgeneV2 database (22,23) (Fig. S2). GLUT1-positive patients with stage I NSCLC exhibited poorer disease-free survival rate than GLUT1-negative patients. This significant difference in disease-free survival among patients with stage I NSCLC might be attributed to enhanced cancer malignancy induced by GLUT1 expression (12,13). On the other hand, the absence of a significant difference in disease-free survival between patients with stages II and III NSCLC might be attributed to the high rate of tumor recurrence, regardless of GLUT1 expression in patients with stage III NSCLC.

Patients with NSCLC who were positive for both GLUT1 and PKM2 had significantly poorer survival rates than those in the 'other expressions' group. The combination of GLUT1 and PKM2 shows potential as an independent prognostic factor for disease-free survival in patients with NSCLC who underwent R0 resection. These results illustrate the importance of evaluating GLUT1 and PKM2 expression in patients with NSCLC. There are several reports about the prognostic significance of GLUT1 (13-15) and of PKM2 (19,20). Osugi *et al* reported the prognostic significance of the combination of GLUT1 and adenosine triphosphate-citrate lyase (ACLY) (16) Meijer *et al* reported that the combination of GLUT1 and monocarboxylate transporter 4 (MCT4) as a useful prognostic marker (17). These two enzymes are related to glucose metabolism, but not the enzyme of glucose metabolism pathway itself. The novelty of this study is to examine the association between the glucose uptake affected by GLUT1 and glucose metabolism pathway by PKM2 on the clinicopathological features and prognosis associated with NSCLC. Cancer cells that positively express

both GLUT1 and PKM2 are upregulated during anaerobic glycolysis and are associated with high malignancy. Malignant tumors cause hypoxic environments because they grow more rapidly than they undergo angiogenesis. Cancer cells have robust anaerobic glycolysis; thus, the Warburg effects increase the glucose requirement of cancer cells (6). GLUT1 overexpression promotes glucose uptake, whereas PKM2 overexpression promotes anaerobic glycolysis. These findings suggest that cancer cells positively express both GLUT1 and PKM2 may uptake larger amounts of glucose and switch to anaerobic glycolysis owing to increased glucose metabolism rates. We found significantly poor prognosis in G+/P+ group especially in stage 0-I. This may be due to the activation of malignancy of cancer cells in early hypoxic area, which was caused by upregulated glycolysis by GLUT1 and PKM2. We did not find the significant difference of SUVmax in primary tumor between patients with G+/P+ expression and other expressions in this study. In lung cancer, however, a high SUVmax score of ^{18}F-FDG-PET, which reflects glucose metabolism in tumors, is considered an indicator of malignancy (39). The combination of GLUT1 and PKM2 expression might be a useful prognostic marker for lung cancer following curative R0 operation and possibly serve as a potential treatment target and an adjuvant chemotherapeutic regimen for patients with NSCLC.

However, this study had some limitations. First, owing to the retrospective nature of the study, not all parameters were analyzed in the patients; moreover, some patients dropped out and were unavailable for follow-up. Second, GLUT1 and PKM2 expression were evaluated via immunohistochemistry alone; thus, future studies should perform alternate methods to assess GLUT1 and PKM2 expression to validate our findings.

In conclusion, both of GLUT1 and PKM2 positive expression have a higher lymphatic invasion rate. The combination of GLUT1 and PKM2 is a reliable prognostic predictor in patients with NSCLC following curative resection and may be used as a clinical target for NSCLC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RI conceived and designed the study, provided administrative support and the materials or patients in the study, collected and assembled the data, analyzed and interpreted the data, and drafted the manuscript. MY conceived and designed the study, provided administrative support, analyzed and interpreted the data, and made critical revisions to the manuscript. TT prepared the paraffin-embedded sections, performed immunostaining, analyzed and interpreted the data. NI conceived and designed the study, and prepared the materials and patients in the study. HK prepared the paraffin-embedded sections, performed immunostaining, and analyzed and interpreted the data. HI prepared the paraffin-embedded sections, performed immunostaining, collected and assembled the data, and analyzed and interpreted the data. YY collected and assembled the data. NN prepared the paraffin-embedded sections, performed immunostaining, and made critical revisions to the manuscript. RI and MY confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki and approved by the Osaka City University Ethics Committee (approval number 2019-006). Written informed consent was obtained from all the patients prior to the operative procedure. All procedures involving humans were performed in accordance with the relevant guidelines and regulations.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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