

Expression of glucose transporter-1 is correlated with hypoxia-inducible factor 1 α and malignant potential in pancreatic neuroendocrine tumors

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Abstract. The present study aimed to investigate the prognostic usefulness of the expression of glucose transporter type 1 (GLUT-1) and GLUT-2, hypoxia-inducible factor 1 α (HIF-1 α) and insulin-like growth factor II messenger RNA-binding protein 3 (IMP3) in pancreatic neuroendocrine tumors (pNETs). Immunohistochemical staining for GLUT-1, GLUT-2, HIF-1 α and IMP3 was performed in 70 pNET specimens. The expression of GLUT-1 and HIF-1 α was significantly higher in the World Health Organization grade 2 (G2), neuroendocrine carcinoma cases and mixed-type pNETs compared with the G1 cases. Vessel invasion, a high Ki-67 labeling index and a high mitotic count were significantly more frequent in the GLUT-1- and HIF-1 α -positive cases compared with the negative cases. Lymph node metastasis was significantly higher in the GLUT-1-positive cases than in the negative cases. Insulin expression was significantly higher in the IMP3-positive cases than the negative cases. The GLUT-1 expression group experienced a significantly poor disease-free survival rate compared with the negative GLUT-1 expression group. HIF-1 α expression was significantly correlated with poor disease-free survival and overall survival rates. A multivariate analysis revealed that lymph node metastasis was an independent risk factor for disease-free survival in all cases. In the G1/G2 group, tumor size and lymph node metastasis were independent risk factors for disease-free survival.

Overall, the results suggested that GLUT-1 is a useful prognostic biomarker for pNETs.

Introduction

Pancreatic neuroendocrine tumors (pNETs) are a rare clinical entity with an annual incidence of 1.09-5.25 cases per million individuals (1), representing a small percentage of all pancreatic neoplasms; however, their incidence is rising (2,3). These tumors are generally slow-growing and exhibit indolent behavior. However, distant metastasis is possible, worsening the prognosis (4).

It is well recognized that compared with non-neoplastic cells, malignant cells exhibit an accelerated metabolism, a high glucose requirement and an increased uptake of glucose. Glucose transporters (GLUTs) facilitate the entry of glucose into cells. GLUTs are passive carriers that function as an energy-independent system to transport glucose down a concentration gradient (5). GLUT type 1 (GLUT-1) is a high-affinity GLUT that is expressed in normal human tissues, including red blood cells, the endothelium of the blood-brain barrier and the placenta (6,7).

GLUT overexpression is frequently observed in cancer, and it is associated with a high metabolism and the rapid growth of cells in often-hypoxic tumor areas (8). Increased levels of GLUT-1 expression have been demonstrated to be associated with a range of carcinomas, including those of the breasts (9), head and neck (10), bladder (11), colorectum (12) and lungs (13), and pulmonary neuroendocrine carcinomas (NECs) (14). GLUT-2 was previously suggested to be overexpressed in hepatic tumors (15), and breast (16) and gastric cancers (17).

It is known that hypoxia-inducible factor 1 (HIF-1) is a master regulator of the transcriptional responses of mammalian cells to hypoxia. HIF-1 plays a critical role in the expression of a number of genes that control angiogenesis, glucose metabolism, cell proliferation, cell survival and metastasis in response to hypoxia (18,19). Elevated expression of HIF-1 α is associated with a poor prognosis in numerous types of solid tumors, including lung, breast, colorectal, brain, pancreatic, ovarian,

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renal and bladder cancer (20). Downstream HIF-1 targets, such as GLUT-1, play critical roles in cellular metabolism and glucose transport, where enhanced glucose metabolism is observed following the upregulation of their respective genes by hypoxia (18).

Insulin-like growth factor II messenger RNA-binding protein 3 (IMP3) plays an important role in RNA trafficking and stabilization, cell growth and cell migration during the early stages of embryogenesis (21,22). The expression of IMP3 is found in malignant tumors as an oncofetal protein that promotes cell proliferation, and the adhesion and invasion of malignant neoplasms (23). IMP3 expression has been studied in neuroendocrine tumors of the lung (24), but to the best of our knowledge, no studies have examined IMP3 expression in pNETs.

It is difficult to evaluate the malignant potential of pNETs, as recurrence or distant metastasis is occasionally observed in the group of low-grade pNETs. The aim of the present study was to clarify the usefulness of the expression of GLUT-1, GLUT-2, HIF-1 α and IMP3 in pNETs, and their clinicopathological correlation for evaluating the malignant potential of pNETs.

Materials and methods

Case selection. The study used 70 formalin-fixed paraffin-embedded tissue samples of pNETs that had been obtained from surgical resection samples and diagnosed at the Department of Anatomical Pathology (Pathological Science, Graduate School of Medical Science, Kyushu University, Fukuoka, Japan) between June 1991 and May 2011. All samples were classified into four groups according to the World Health Organization classification (2010) (25): G1 (mitotic count of <2 and/or \leq 2% Ki-67 index; n=47), G2 (mitotic count of 2-20 and/or 3-20% Ki-67 index; n=18), NEC (large- or small-cell type; n=4) and mixed adenoneuroendocrine carcinoma (mixed type, n=1). Available clinical follow-up data were obtained from 50 of the pNET patients. This study was approved by the Institutional Review Board of Kyushu University and conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Immunohistochemical staining and evaluation. All specimens were fixed in 10% formalin and processed routinely. Hematoxylin and eosin staining was also performed on 4- μ m thick sections of formalin-fixed paraffin-embedded tissue. The sections were deparaffinized in xylene and rehydrated in ethanol. Endogenous peroxidase activity was blocked by incubation in methanol containing 0.3% H₂O₂ for 30 min. Antigen retrieval was achieved by microwave heating in 10 mM citrate buffer (pH 6.0) for 20 min (for GLUT-1 and GLUT-2) or in Target Retrieval Solution (pH 9.0; Dako, Carpinteria, CA) for 20 min (for IMP3), or through use of BORG Decloaker solution (Biocare Medical, Walnut Creek, CA, USA) and a Decloaking Chamber (Biocare Medical) for ~30 min (for HIF-1 α).

The sections were incubated overnight at 4°C with the following primary antibodies: Rabbit polyclonal anti-GLUT-1 (1:300 dilution; cat. no. ab15309; Abcam, Cambridge, UK), mouse monoclonal anti-GLUT-2 (1:1,000 dilution; cat. no. ab85715; Abcam), mouse

monoclonal anti-HIF-1 α (1:500 dilution; cat. no. NB100-105; Novus Biologicals, Littleton, CO, USA), mouse monoclonal anti-IMP3 (1:100 dilution; cat. no. M3626; Dako) and mouse monoclonal anti-insulin (1:1 dilution; cat. no. ab6995; Abcam). The labeled antigens were detected with an EnVision+ system -Horseradish Peroxidase-Labeled Polymer system (Dako) and visualized using 3,3'-diaminobenzidine tetrahydrochloride as a chromogen. Counterstaining was then performed with hematoxylin.

Samples of clear cell renal cell carcinoma, normal liver tissue, colon cancer and normal tonsil tissue were used as the positive controls of GLUT-1, GLUT-2, HIF-1 α and IMP3, respectively. Immunoreactivities were assessed in the membranous staining for GLUT-1, the cytoplasmic staining for GLUT-2, IMP3 and insulin, and the nuclear staining for HIF-1 α , and were defined as positive for any extent of expression. Islets of Langerhans or red blood cells were used as internal controls of GLUT-1 and GLUT-2, respectively. All stained slides were reviewed independently by two pathologists.

Statistical analysis. All statistical analyses were performed using JMP 9.0.2 software (SAS Institute, Cary, NC, USA). Clinicopathological comparisons were conducted using the Pearson, χ^2 and Fisher's exact tests. Survival curves were calculated by the Kaplan-Meier method, and the survival data were examined by the log-rank test. P<0.05 was considered to indicate a significant difference.

Results

GLUT-1, GLUT-2, HIF-1 α and IMP3 expression, and clinicopathological findings. The correlations between the immunohistochemical results and clinicopathological findings are summarized in Table I. In the GLUT-1-positive cases, positive cells were distributed along the periphery to the center of the tumor, accompanied by moderate to severe fibrosis or necrosis. Membranous staining of GLUT-1 was found in 8 out of the 47 (17%) G1 tumors, in 8 out of the 18 (44%) G2 tumors, and in all of the NEC and mixed-type tumors (100%) (Fig. 1). The expression of GLUT-1 was significantly higher in the G2, NEC and mixed-type cases compared with the G1 cases (P=0.0007). Vessel invasion (P=0.0007), lymph node metastasis (P=0.026), a high Ki-67 labeling index (P=0.0019) and high mitotic counts (P=0.0002) were significantly more frequent in the GLUT-1-positive cases (Table I).

GLUT-2 staining was detected in the cytoplasm of the tumor cells in 4 out of the 47 (8.5%) G1 cases, in 2 out of the 18 (11%) G2 cases, in 1 out of the 4 (25%) NEC cases and in the single (100%) mixed-type case (Fig. 2A). GLUT-2 expression exhibited no correlation with any clinicopathological factors (Table I).

HIF-1 α expression was detected in the nucleus of the tumor cells in 4 out of the 47 (8.5%) G1 cases, in 4 out of the 18 (22%) G2 cases, in 1 out of the 4 (25%) NEC cases and in the single (100%) mixed-type case (Fig. 2B). HIF-1 α expression was also significantly higher in the G2, NEC and mixed-type groups compared with the G1 group (P=0.048). The vessel invasion (P=0.048), high Ki-67 labeling index (P=0.012) and high mitotic counts (P=0.038) were each significantly correlated with HIF-1 α expression (Table I). There was a significant

Table I. Association of GLUT-1, GLUT-2, HIF-1 α and IMP3 expression with clinicopathological variables.

Variables	n	GLUT-1		GLUT-2		HIF-1 α		IMP3	
		Positive/ negative	P-value	Positive/ negative	P-value	Positive/ negative	P-value	Positive/ negative	P-value
Total cases	70	21/49		8/62		10/60		10/60	
Mean age, years			0.3208		0.8634		0.6250		0.6250
<55	33	8/25		4/29		4/29		4/29	
\geq 55	37	13/24		4/33		6/31		6/31	
Gender			0.6296		0.9473		0.5475		0.4226
Female	43	12/31		5/38		7/36		5/38	
Male	27	9/18		3/24		3/24		5/22	
Tumor size, cm			0.1244		0.6228		0.4561		1.0000
\geq 3.0	21	9/12		3/18		4/17		3/18	
<3.0	49	12/37		5/44		6/43		7/42	
Vessel invasion			0.0007 ^a		0.7664		0.0484 ^a		0.0965
+	23	13/10		3/20		6/17		1/22	
-	47	8/39		5/42		4/43		9/38	
Lymph node metastasis			0.0261 ^a		0.5131		0.4755		0.0745
+	15	8/7		1/14		3/12		0/15	
-	55	13/42		7/48		7/48		10/45	
Necrosis			0.6903		0.6228		0.4561		0.4561
+	21	7/14		3/18		2/19		2/19	
-	49	14/35		5/44		8/41		8/41	
Functioning			0.1307		0.4239		0.8399		0.8399
+	26	5/21		4/22		4/22		4/22	
-	44	16/28		4/40		6/38		6/38	
Insulin			0.8644		0.1896		0.4561		0.0253 ^a
+	21	6/15		4/17		2/19		6/15	
-	49	15/34		4/45		8/41		4/45	
Ki-67 index, %			0.0019 ^a		0.1224		0.0116 ^a		0.8263
>2	19	11/8		4/15		6/13		3/16	
\leq 2	51	10/41		4/47		4/47		7/44	
Mitotic count			0.0002 ^a		0.1045		0.0383 ^a		0.5174
\geq 2	12	9/3		3/9		4/8		1/11	
<2	58	12/46		5/53		6/52		9/49	
WHO classification			0.0007 ^{a,b}		0.2727 ^b		0.0484 ^{a,b}		0.8354 ^b
G1	47	8/39		4/43		4/43		7/40	
G2	18	8/10		2/16		4/14		2/16	
NEC	4	4/0		1/3		1/3		1/3	
Mixed-type	1	1/0		1/0		1/0		0/0	

^aP<0.05. ^bG1 compared with G2, NEC and mixed-type. WHO, World Health Organization; G, grade; NEC, neuroendocrine carcinoma; GLUT, glucose transporter; HIF-1 α , hypoxia-inducible factor 1 α ; IMP3, insulin-like growth factor II messenger RNA-binding protein 3; functioning, insulinoma, glucagonoma, somatostatinoma, gastrinoma and VIPoma.

correlation between GLUT-1 expression and HIF-1 α expression (P=0.025) (Table II).

IMP3 expression was recognized in the cytoplasm of the tumor cells. IMP3 was expressed in 7 out of the 47 (15%) G1 cases, in 2 of the 18 (11%) G2 cases, and in 1 out of the 4 (25%) NEC cases, but not in the single (0%) mixed type case.

Insulin expression was significantly more frequently observed in the IMP3-positive cases compared with the IMP3-negative cases (P=0.025) (Table I).

Survival analysis. Patients in the positive GLUT-1 expression group showed significantly poor disease-free survival

Table II. Association between HIF-1 α and GLUT-1/GLUT-2.

Expression	HIF-1 α		P-value
	Positive (n=10)	Negative (n=60)	
GLUT-1			
Positive/negative	6/4	15/45	0.025
GLUT-2			
Positive/negative	2/8	6/54	0.357

GLUT, glucose transporter; HIF-1 α ; hypoxia-inducible factor 1 α .

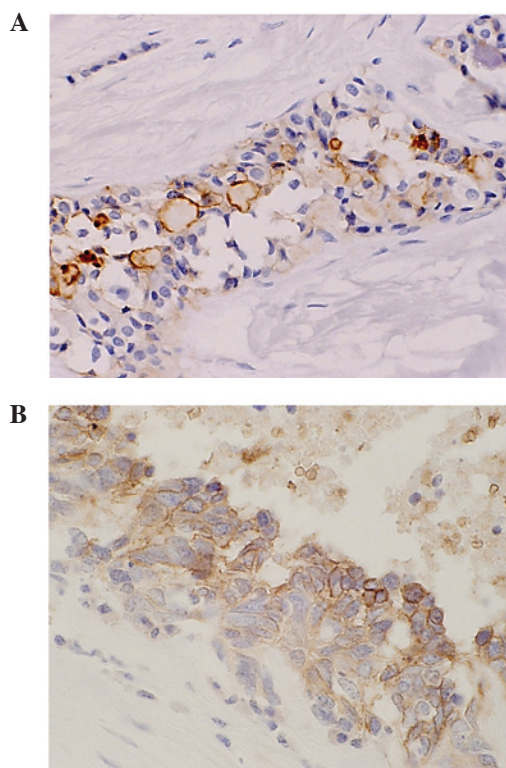


Figure 1. GLUT-1 expression in pancreatic neuroendocrine tumors. GLUT-1 showing a membranous staining pattern in (A) grade 1 and (B) neuroendocrine carcinoma small cell type tumors. Magnification, x400. GLUT-1, glucose transporter type 1.

rates compared with those in the negative group ($P=0.0039$; Fig. 3A). Among the G1/G2 tumors, the patients with positive GLUT-1 expression ($n=12$) showed significantly poor disease-free survival rates compared with those in the negative GLUT-1 expression group ($n=36$) ($P=0.035$; Fig. 3B). The patients with HIF-1 α expression ($n=5$) showed significantly poor disease-free survival and overall survival rates ($P=0.047$ and $P=0.0071$, respectively) (Fig. 3C and D). GLUT-2 and IMP3 expression did not affect disease-free survival or overall survival rates.

Multivariate analysis. The significant factors revealed by univariate analysis were assessed using a Cox proportional hazard model. A multivariate analysis revealed that lymph

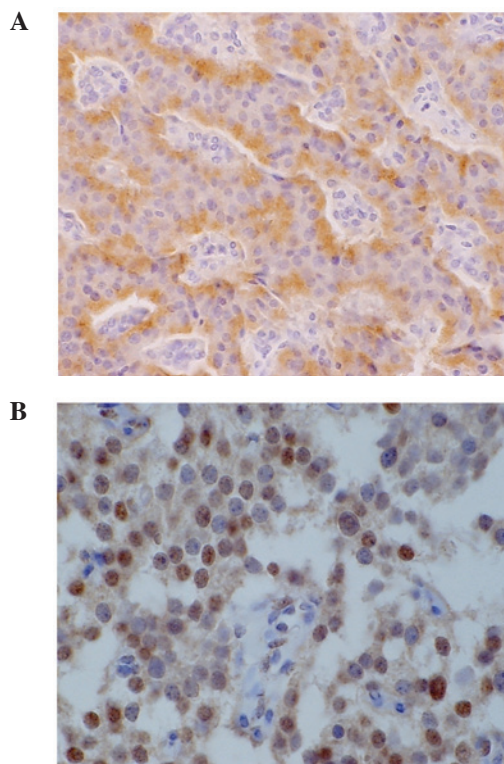


Figure 2. GLUT-2 and HIF-1 α expression in pancreatic neuroendocrine tumors. (A) GLUT-2 showing a cytoplasmic staining pattern in a G1 tumor, and (B) HIF-1 α showing a nuclear staining pattern in a G1 tumor. Magnification, x400. GLUT-2, glucose transporter type 2; HIF1 α , hypoxia-inducible factor 1 α .

node metastasis was an independent risk factor for disease-free survival in all cases ($P=0.0107$) (Table III). In the G1/G2 group, tumor size ($P=0.0479$) and lymph node metastasis ($P=0.0214$) were shown to be independent risk factors for disease-free survival (Table IV).

Discussion

Various studies have shown a close association between GLUT-1 expression and tumor aggressiveness and poor prognosis in a number of carcinomas (9-14). However, little is known about GLUT-1 expression in pNETs.

Ozbudak *et al* demonstrated that GLUT-1 expression was associated with an increased risk of mortality among patients with pulmonary NECs (14), and that GLUT-1 expression was strongly correlated with neuroendocrine differentiation/grade, but not with other clinicopathological variables. In the present study, GLUT-1 expression was similarly significantly increased in the high-grade pNET group. Unlike in the pulmonary NECs, the present pNET cases with GLUT-1 expression were correlated with markers of tumor aggressiveness, including vessel invasion, lymph node metastasis, a high Ki-67 labeling index and a high mitotic count.

These findings indicate that GLUT-1 expression in pNETs is a more useful marker of malignant potential than that in pulmonary NECs. Moderate to severe fibrosis and/or necrosis is observed in pNETs, suggesting the presence of a hypoxic area in pNETs (17). In response to hypoxia, HIF-1

Table III. Univariate and multivariate analysis of disease-free survival in all cases.

Variable	Univariate analysis		Multivariate analysis	
	P-value	Hazard ratio	95% confidence interval	P-value
Age (≥ 55 years)	0.0175	0.291	0.014-1.822	0.2099
Tumor size (≥ 3.0 cm)	0.0048	3.959	0.682-33.63	0.1267
Vassel invasion (+)	0.0012	0.381	0.020-5.720	0.4891
Lymph node metastasis (+)	<0.0001	18.591	1.797-414.0	0.0107
HIF-1 α (+)	0.047	1.998	0.257-11.68	0.4679
GLUT-1 (+)	0.0078	3.081	0.458-24.39	0.2490

GLUT, glucose transporter; HIF-1 α ; hypoxia-inducible factor 1 α .

Table IV. Univariate and multivariate analysis of disease-free survival in the G1/G2 group.

Variable	Univariate analysis		Multivariate analysis	
	P-value	Hazard ratio	95% confidence interval	P-value
Gender (male/female)	0.0377	1.515	0.169-16.02	0.7035
Tumor size (≥ 3.0 cm)	0.0302	8.328	1.018-182.3	0.0479
Vassel invasion (+)	0.0085	0.261	0.004-6.712	0.4448
Lymph nodes metastasis (+)	0.0003	32.486	1.522-2667	0.0214
HIF-1 α (+)	0.3360	12.327	0.386-459.9	0.1363
GLUT-1 (+)	0.0509	2.436	0.354-19.75	0.3654

GLUT, glucose transporter; HIF-1 α ; hypoxia-inducible factor 1 α ; G, grade.

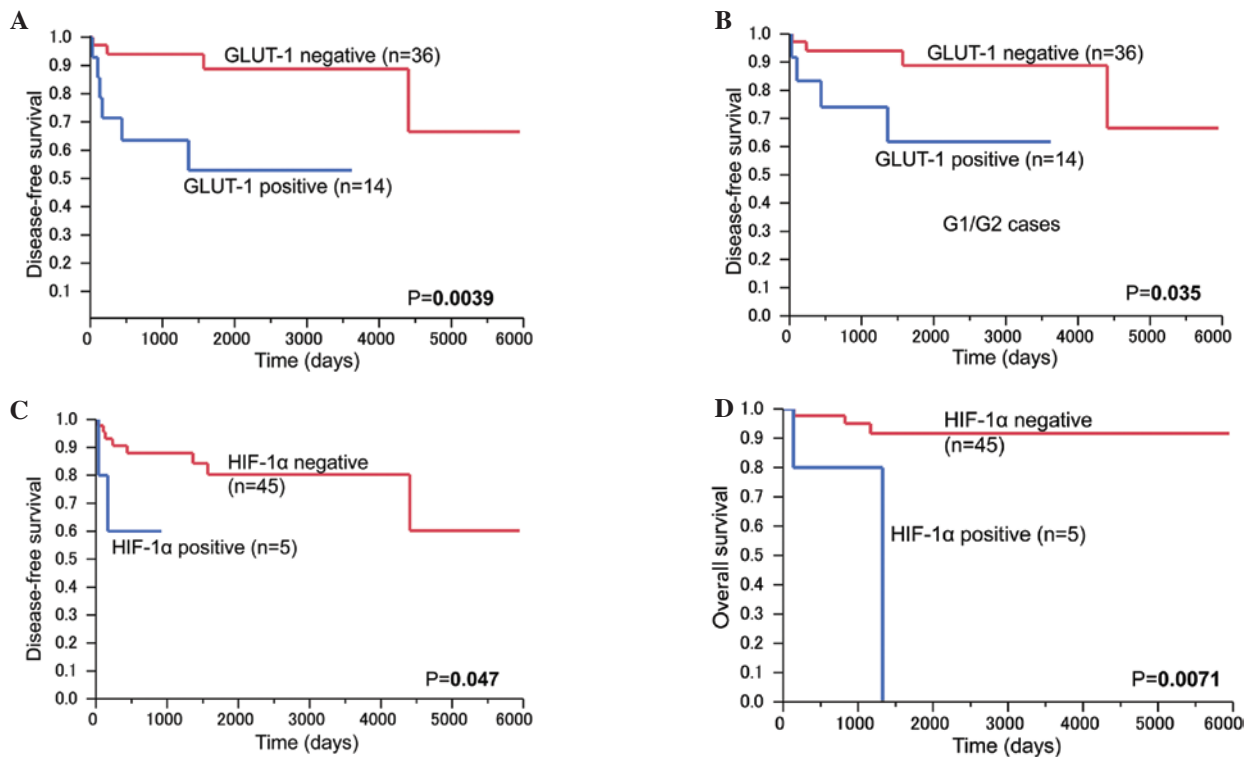


Figure 3. Kaplan-Meier analysis for disease-free survival and overall survival by GLUT-1 and HIF-1 α expression. (A) Among the 50 cases, GLUT-1 expression group showed significantly poor disease-free survival ($P=0.0039$). (B) Among the 48 of G1/G2 cases, GLUT-1 expression group showed significantly poor disease-free survival ($P=0.035$). (C) Among the 50 cases, HIF-1 α expression group showed significantly poor disease-free survival ($P=0.047$). (D) Among the 50 cases, HIF-1 α expression group showed significantly poor overall survival ($P=0.0071$).

plays a critical role in the expression of a number of genes that control angiogenesis, cell proliferation, cell survival, metastasis and glucose metabolism. GLUT-1 plays critical roles in cellular metabolism and glucose transport, where enhanced glucose metabolism is observed following the upregulation of their respective genes by hypoxia (17). The present study found that the expression of GLUT-1 was significantly correlated with HIF1- α expression ($P=0.025$) in pNETs, indicating the possibility of the induction of GLUT-1 by HIF1- α in the hypoxic condition.

The patients in the present positive GLUT-1 expression group showed significantly poor disease-free survival rates compared with those in the negative GLUT-1 expression group. In addition, the GLUT-1-positive cases in the G1/G2 group showed significantly poor disease-free survival rates ($P=0.035$) compared with the negative GLUT-1 expression group. These findings suggest that the expression of GLUT-1 is one of the factors that can be used for the prognostic assessment of pNETs.

Frendrich *et al* reported that GLUT-2 expression was detectable in normal islet cells and pancreatic intraepithelial neoplasia 1B lesions or higher grade lesions (26). The present study observed the expression of GLUT-2 in normal islet cells, but only in 8 out of the 70 (11%) cases of pNET. These data suggest that elevated glucose metabolism occurs in pNETs via GLUT-1, but not GLUT-2.

IMP3 is expressed in malignant neoplasms, including intraductal papillary mucinous neoplasms (27), pancreatic adenocarcinoma (28,29) and hepatocellular carcinoma (30). In these tumors, IMP3 expression is a useful diagnostic marker for distinguishing the malignant phenotype from benign lesions and is a prognostic biomarker associated with poor survival. The present study showed no association between IMP3 expression and malignant characteristics or prognosis in pNET. However, it was found that GLUT-1 expression is associated with a poor disease-free survival rate, indicating that GLUT-1 is a useful biomarker rather than IMP3 in pNET. Among the 18 cases of insulinoma (18/70; 26%), 15 cases were positive for insulin; IMP3 expression exhibited a close association with insulin expression, but the mechanism underlying this association is not yet known.

In conclusion, the findings of the present study indicated that GLUT-1 expression is correlated with malignant potential and that its overexpression is a prognostic biomarker for pNET.

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