JJCO Japanese Journal of Clinical Oncology

Jpn J Clin Oncol 2012;42(8)730–741
doi:10.1093/jjco/hys092
Advance Access Publication 21 June 2012

Validation Study on Pfetin and ATP-dependent RNA Helicase DDX39 as Prognostic Biomarkers in Gastrointestinal Stromal Tumour

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Received March 26, 2012; accepted May 20, 2012

Objective: This study aimed to validate two prognostic biomarkers, pfetin and adenosine triphosphate-dependent RNA helicase DDX39 (DDX39), in gastrointestinal stromal tumour. Prognostic biomarkers have long been required for the optimal use of kinase inhibitors in gastrointestinal stromal tumour.
Methods: The expression level of pfetin was immunohistochemically examined in 72 gastrointestinal stromal tumour cases, being correlated with the clinicopathological parameters. Meta-analysis of the prognostic value of pfetin was performed in a total of 371 cases. The prognostic utility of the combination of pfetin and DDX39 was examined in the 72 gastrointestinal stromal tumour cases.
Results: Immunohistochemical study demonstrated the disease-free survival rate to be 94.7% for pfetin-positive patients and 20.0% for pfetin-negative patients among the 72 gastrointestinal stromal tumour cases ($P < 0.0001$). In the 371 cases, the disease-free survival rate was 93.8% for pfetin-positive patients and 40.6% for pfetin-negative patients ($P < 0.0001$). Both univariate and multivariate analyses revealed that pfetin expression was an independent prognostic factor ($P < 0.0001$). When evaluated in combination with pfetin and DDX39, the disease-free survival rates were 0.0% for the pfetin-negative and DDX39-strong patients.
Conclusions: These results established the clinical utility of pfetin as a novel prognostic biomarker for gastrointestinal stromal tumour. The combined use of pfetin and DDX39 appeared to have powerful prognostic value. These biomarkers will be useful in deciding whether to administer adjuvant therapy after surgery.

Key words: orthopaedics/sarcoma-basic – patho-molecular – gastrointestinal (GI) medicine

INTRODUCTION

Gastrointestinal stromal tumour (GIST) is the most common mesenchymal tumour in the digestive tract, with an incidence of ~15 per 1 000 000 people (1,2). GIST originates from the intestinal cells of Cajal, and is characterized by the presence of gain-of-function mutations in the c-kit or the platelet-derived growth factor receptor (1). These mutations result in the constitutive activation of signalling pathways. A small molecule tyrosine kinase inhibitor, namely imatinib mesylate (STI571, Novartis), is the standard treatment for metastatic GISTs, resulting in 75% response rates (1–4). The prognosis for GIST spans a wide spectrum, from cases with curable disease to those with fatal tumours due to metastasis and recurrence. Approximately, 10–30% of GISTs are malignant, showing recurrence and distant metastasis (2). Therefore, only a limited number of patients may benefit from imatinib mesylate treatment. In addition, a recent report demonstrated that non-continuous use of imatinib mesylate resulted in tumour progression in most patients (1). Thus, a prognostic biomarker after surgery would be beneficial for selecting patients most likely to develop recurrence, and who would benefit from treatment with imatinib mesylate. Tumour location, risk classification and certain molecular aberrations have been shown to correlate with the prognosis and responsiveness to imatinib mesylate treatment (5–10). However, these biomarkers have not been found to be clinically applicable.

Previously, we reported a novel application of pfetin (potassium channel tetramerization domain containing 12, KCTD12) as a prognostic biomarker for GIST using a proteomic approach (11). Pfetin was originally cloned as a gene highly expressed in the foetal cochlea and brain (12). Pfetin belongs to the family of proteins containing the potassium channel tetramerization domain (12). In a recent report, pfetin was identified as an auxiliary gamma-aminobutyric acid type B ($GABA_B$) receptor subunit that determines the pharmacology and kinetics of the receptor response (13,14). Besides such functional studies, its contributions to carcinogenesis and cancer progression have yet to be elucidated. As described in our previous report, an immunohistochemical study of pfetin expressions in 210 cases from the National Cancer Center Hospital revealed that a 5 year disease-free survival rate was 93.9% for pfetin-positive patients and 36.2% for pfetin-negative patients using polyclonal antibody against pfetin (11). We succeeded in developing a novel monoclonal antibody against pfetin, and thereby confirmed the prognostic value of pfetin in 159 GIST cases from the National Cancer Center Hospital, 100 GIST cases from the Niigata University Medical and Dental Hospital and 40 cases from the Juntendo University Shizuoka Hospital (15,16). Meta-analysis revealed that the disease-free survival rate was 92.4% for pfetin-positive patients and 60.8%for pfetin-negative patients in these 299 patients (15,16).

Recently, we also reported adenosine triphosphate (ATP)-dependent RNA helicase DDX39 (DDX39) as a novel prognostic biomarker in GIST using two-dimensional difference gel electrophoresis with a large format electrophoresis device (17). Pfetin expression was reduced in the advanced GIST cases. In contrast, DDX39 was increased in the patients with metastasis after surgery. DDX39 was originally reported as a novel growth-associated RNA helicase (18), and overexpressed in lung squamous cell carcinoma (19). We reported that DDX39 was an independent prognostic factor of GIST by immunohistochemistry and that the disease-free survival rate was 90.2% for DDX39-weak, and 50.4% for DDX39-strong, cases (17).

Considering the clinical and genetic heterogeneities of GIST, a further validation study was needed before the application of pfetin in the clinical setting, and the combined use of pfetin and DDX39 should be of interest. In this study, we examined the correlations of pfetin expression with clinicopathological parameters in 72 newly enrolled GIST cases from the Juntendo University Hospital. We performed the meta-analysis on the prognostic value of pfetin in 371 GIST cases including 72 cases from the Juntendo University Hospital, 159 cases from the National Cancer Center Hospital, 100 cases from the Niigata University Medical and Dental Hospital and 40 cases from the Juntendo University Shizuoka Hospital. Finally, we also examined the combination of pfetin and DDX39 in 72 newly enrolled cases from the Juntendo University.

PATIENTS AND METHODS

Patients

We examined the primary tumour tissues of 72 GIST patients who underwent surgery at the Juntendo University Hospital during the period from 2000 to 2009. All patients underwent resection with curative intent and were not given adjuvant treatments, including imatinib mesylate. Diagnosis was based on the WHO classification system for soft-tissue tumours: tumour size, presence of necrosis, differentiation, mitotic rate, mindbomb homolog-1 positive rate (MIB-1 index) and presence of epithelioid cells (20). Risk classification was based on tumour size and the MIB-1 index (21). C-kit expression in all GIST samples was confirmed using immunohistochemical staining (CD117 antibody, DAKO Japan Corp., Tokyo, Japan). Clinicopathological features of the 72 GIST patients are listed in Table 1 and summarized in Table 2.

We performed meta-analysis by integrating the data of pfetin expressions in these 72 cases and the other 299 patients reported in our previous studies: 159 patients underwent surgery at the National Cancer Center Hospital between 1974 and 2005, 100 cases at the Niigata University Medical and Dental Hospital between 1982 and 2005 and 40 patients at the Juntendo University Shizuoka Hospital between 1995 and 2009 (15,16). In our previous study, we immunohistochemically examined pfetin expressions in the primary tumours of these 299 cases using the same monoclonal antibody as in the present study. The clinical features

Table 1. Clinico-pathological features of the 72 gastrointestinal stromal tumour (GIST) cases from the Juntendo University Hospital

Sample no	Age	Gender	Site	Size (cm)	Necrosis	Risk classification[a]	Metastatic site/recurrence (first development)	Disease-free survival (months)	Pfetin positivity	DDX39 strong/weak
1	46	F	Stomach	4.8	–	Low	–	96	+	+
2	55	F	Small intestine	2	–	Low	–	45	+	–
3	67	M	Stomach	1.7	–	Low	–	105	+	+
4	68	F	Stomach	2	–	Low	–	61	+	–
5	49	F	Small intestine	12	–	Intermediate	Liver	0	–	–
6	59	M	Stomach	4	–	Low	–	1	+	+
7	67	M	Stomach	1.3	–	Low	Liver	12	+	–
8	61	F	Stomach	4	–	Low	–	0	+	–
9	65	F	Stomach	4	–	Low	–	82	+	+
10	53	M	Stomach	2.5	–	Low	Liver	3	–	+
11	65	M	Stomach	0.8	–	Low	–	110	+	–
12	44	F	Stomach	7	–	Intermediate	–	12	+	–
13	78	M	Stomach	1.3	–	Low	Liver	36	–	+
14	43	M	Small intestine	17.5	–	High	Liver	36	+	–
15	58	F	Stomach	4.8	+	Low	Liver	3	–	+
16	58	F	Stomach	1.6	–	Low	Liver	15	–	+
17	66	M	Stomach	4	–	Low	–	105	+	–
18	62	F	Stomach	5.4	–	Low	–	105	+	–
19	42	M	Stomach	4.2	–	Low	–	48	+	–
20	44	M	Stomach	4	–	Low	–	103	+	–
21	44	M	Rectum	15	–	High	Liver	16	–	+
22	80	M	Colon	2.5	–	Low	–	87	+	–
23	18	M	Stomach	8	–	Intermediate	–	92	+	+
24	54	M	Stomach	2.5	–	Low	–	25	+	–
25	49	M	Stomach	9	–	Intermediate	Liver	48	–	–
26	63	M	Stomach		–	Low	–	1	–	–
27	68	M	Stomach	4	–	Low	–	90	–	–
28	65	M	Stomach	16	–	High	Liver	48	–	+
29	63	M	Stomach	3	+	High	–	1	+	+
30	59	M	peritoneal dissemination	1.8	–	Low	–	1	+	–
31	65	F	Stomach	4.6	–	Low	–	79	+	+
32	57	M	Stomach	3.5	–	Low	–	77	+	–
33	71	F	Small intestine	5	+	Intermediate	–	13	+	–
34	13	M	Stomach	5.5	+	Intermediate	Liver	79	+	+
35	40	M	Small intestine	2.5	–	Low	–	78	+	–
36	39	M	Small intestine	8	–	Intermediate	–	4	+	+
37	41	M	Stomach	2	–	Low	–	2	+	–
38	68	M	Stomach	2.9	+	Low	–	75	–	–
39	36	F	Small intestine	3.5	+	Low	–	70	+	–
40	74	M	Stomach	8	–	High	Liver	71	+	+
41	68	F	Stomach	3.5	–	Low	–	66	+	–
42	67	M	Stomach	6.5	+	Low	Liver	41	–	+

Continued

Table 1. *Continued*

Sample no	Age	Gender	Site	Size (cm)	Necrosis	Risk classification[a]	Metastatic site/recurrence (first development)	Disease-free survival (months)	Pfetin positivity	DDX39 strong/weak
43	63	F	Stomach	5	−	Low	−	64	+	−
44	62	M	Stomach	8	+	Intermediate	−	57	+	−
45	56	M	Stomach	4	+	Low	−	61	+	−
46	27	F	Small intestine	2.5	−	Low	−	61	+	−
47	65	M	Stomach	6	−	Intermediate	−	57	+	−
48	54	M	Stomach	8.3	−	Intermediate	−	30	+	−
49	70	F	Small intestine	7	+	High	−	29	+	−
50	60	M	Stomach	7	−	Intermediate	−	30	+	−
51	68	M	Stomach	11	−	High	−	1	+	−
52	36	M	Stomach	6.5	−	Intermediate	−	31	+	+
53	70	M	Stomach	1.5	−	Low	−	30	+	−
54	60	F	Small intestine	3.7	−	Low	−	11	+	−
55	53	F	Small intestine	7	+	High	−	26	+	−
56	72	M	Small intestine	1.2	−	Low	−	18	+	−
57	70	M	Small intestine	6.5	+	High	Liver	12	−	+
58	72	M	Stomach		−	Low	Lung	8	+	−
59	59	F	Stomach	6.5	−	Intermediate	−	22	+	−
60	68	F	Stomach	4.5	+	Low	−	21	+	−
61	54	M	Stomach	6.5	−	Intermediate	−	20	+	+
62	47	F	Stomach	4.8	−	Low	−	19	+	−
63	64	F	Stomach	1.5	−	Low	−	13	+	−
64	53	M	Stomach	2.7	−	Low	−	25	+	−
65	65	M	Stomach	4	−	Low	−	13	+	−
66	59	M	Small intestine	3	−	Low	−	14	+	−
67	59	F	Stomach	0.7	−	Low	Liver	4	+	−
68	69	M	Stomach	2.7	−	Low	−	13	+	+
69	65	M	Small intestine		+	Low	−	1	+	−
70	67	F	Stomach	3	+	High	−	12	+	−
71	79	F	Stomach	7	+	Intermediate	−	6	+	−
72	59	M	Small intestine	3.5	−	Low	−	10	+	−

+, present; −, absent.
[a]Risk classification based on tumour size and MIB-1 grade (21).

of GIST patients in these 299 cases are summarized in Table 3. The institutional review boards of the National Cancer Center Hospital, the Niigata University Medical and Dental Hospital, the Juntendo University Shizuoka Hospital and the Juntendo University Hospital approved this study. Written informed consent was obtained from all patients examined.

Immunohistochemistry

Pfetin and DDX39 expression was examined immunohistochemically using paraffin-embedded tissues, as described in our previous report (11,15–17). In brief, 4 μm-thick tissue sections were deparaffinized through xylene and rehydrated with ethanol. Endogenous peroxidase was blocked with 1% H_2O_2 diluted in methanol for 30 min at room temperature. The slides were autoclaved in 10 mmol/l citrate buffer (pH 6.0) at 121° for 30 min and incubated with the antibody against pfetin (1:1000 dilutions) and the antibody against DDX39 (dilution, 1:15, BMR00389, Bio Matrix Research, Chiba, Japan) at room temperature. Immunostaining was carried out by the streptavidin–biotin peroxidase method using the Strept ABC Complex/horseradish peroxidase kit (DAKO). One pathologist (A.Y.) and one clinician (D.K.)

Table 2. Univariate and multivariate analysis and the relationship between clinicopathological variables and pfetin and DDX39 expression of the 72 GIST cases from the Juntendo University Hospital

Variable	Number of cases	Pfetin positive	Pfetin negative	Correlation (pfetin) χ^2 *P* value	DDX39 strong	DDX39 weak	Correlation (DDX39) χ^2 *P* value	Disease-free survival		Multivariate analysis of disease-free survival by Cox regression		
								Rate (%)	Log-rank (*P* value)	*P* value	Relative risk	95% confidence interval
Age												
<60	34	27	7	0.597	11	23	0.38	76.47	0.444			
≥60	38	30	8		10	28		81.58				
Sex												
F	26	23	3	0.122	5	21	0.13	73.91	0.241			
M	46	34	12		16	30		88.46				
Site												
Stomach	53	41	12	0.588	18	35	0.251	79.25	0.4869			
Small intestine	16	14	2		2	14		81.25				
Other	3	2	1		1	2		66.67				
Histology												
Spindle	63	51	12	0.495	17	46	0.082	80.95	0.8071			
Epithelioid	7	5	2		2	5		71.43				
Mixed	2	1	1		2	0		50				
Size (cm)												
<5	46	38	8	0.05	11	35	0.222	86.96	0.0064	0.353	1.88	0.496–73118
5–15	23	18	7		8	15		73.91				
≥15	3	1	2		2	1		0				
Necrosis												
Present	16	11	5	0.299	5	11	0.531	80.36	0.4567			
Absent	56	46	10		16	40		75				
Risk classification[a]												
Low	47	39	8	0.266	11	36	0.225	85.11	0.006	0.635	0.769	0.260–2.275
Intermediate	15	12	3		5	10		80				
High	10	6	4		5	5		50				
DDX39												
Strong	21	11	10	0.001	21	0	0.004	90.2	0.004	0.609	1.397	0.387–5.045
Weak	51	46	5		0	51		52.38				
Pfetin												
+	57	57	0		11	46	0.001	94.7	<0.0001	0.001	0.092	0.023–0.374
–	15	0	15		10	5		20				

[a]Risk classification based on tumour size and MIB-1 grade (21).

reviewed the sections stained with anti-pfetin and anti-DDX39 antibody. Both were blinded to the clinical data (age, sex, anatomic site and outcome). Cases in which >20% of tumour cells were stained with the anti-pfetin antibody were considered to be pfetin-positive, whereas those in which <20% tumour cells were stained with the anti-pfetin antibody were considered to be pfetin-negative, in accordance with our previous report (11,15,16). Strongly stained cells of DDX39 were defined as those with staining intensity equivalent to or higher than that of lymphocytes (17). In most cases, the difference was quite obvious and the two reviewers concurred as to the results.

Table 3. Clinical and histopathological variables concerning the GIST cases examined according to the institution of origin

Variable	Number of cases				Correlation
	NCCH	Niigata University	Juntendo Shizuoka	Juntendo University	χ^2 (P value)
Age					
<60	65 (40.9%)	32 (32%)	6 (15%)	34 (47%)	0.003
≥60	94 (59.1%)	68 (68%)	34 (85%)	38 (53%)	
Sex					
Female	76 (47.8%)	56 (56%)	19 (47.5%)	56 (64%)	0.084
Male	83 (52.2%)	44 (44%)	21 (52.5%)	26 (36%)	
Site					
Stomach	140 (88.1%)	84 (84%)	32 (80%)	53 (74%)	0.029
Non-gastric	19 (11.9%)	16 (16%)	8 (20%)	16 (22%)	
				3 (4%)	
Histology					
Spindle	124 (80.0%)	81 (81%)	36 (90%)	63(88%)	0.059
Epithelioid	12 (7.5%)	6 (6%)	3(7.5%)	7 (10%)	
Mixed	23 (14.5%)	11 (11%)	1(2.5%)	2 (2%)	
Unknown		2 (2%)			
Size (cm)					
<5	94 (59.1%)	51 (51%)	20 (50%)	44 (61%)	0.686
5–15	57 (35.8%)	44 (44%)	19 (47.5%)	25 (35%)	
≥15	8 (5.1%)	5 (5%)	1 (2.5%)	3 (4%)	
Necrosis					
Present	15 (9.4%)	44 (44%)	8 (20%)	16 (22%)	0.0001
Absent	144 (90.6%)	54 (54%)	32 (80%)	56 (78%)	
Unknown		2 (2%)			
Risk classification[a]					
Low	88 (55.4%)	50 (50%)	21 (52.5%)	46 (64%)	0.284
Intermediate	32 (20.1%)	28 (28%)	7 (17.5%)	16 (22%)	
High	39 (24.5%)	22 (22%)	12 (30%)	10 (14%)	
Recurrence					
+	134 (84.3%)	14 (14%)	8 (20%)	57 (80%)	0.568
−	25 (15.7%)	86 (86%)	32 (80%)	15 (20%)	

NCCH, National Cancer Center Hospital.
[a]Risk classification based on tumour size and MIB-1 grade (21).

Statistical Analysis

All statistical analyses were carried out using the χ^2 test or Fisher's exact test in cross tables to assess the relationships between pfetin expression and clinicopathological factors. The tumour-specific and disease-free survivals were calculated from the initial resection of the primary tumour until death from tumour-specific causes or until first evidence of metastasis and recurrence, respectively. All time-to-event endpoints were computed by the Kaplan–Meier method (22). Patients who died of causes unrelated to GIST were censored at the time of death. Potential prognostic factors were identified by univariate analysis using the log-rank test. Independent prognostic factors were evaluated using Cox's proportional hazards regression (23) model with significant variables at the univariate level ($P < 0.05$). Calculations were carried out using the SPSS software statistical package (SPSS Japan Inc., Tokyo, Japan).

RESULTS

Localization and Expression of Pfetin in the Primary GIST

We examined pfetin expressions in the primary tumours of the newly enrolled 72 GIST patients managed at the Juntendo University Hospital, applying our original monoclonal antibody (15). Immunohistochemistry showed diffuse staining of the membranes and cytoplasm of tumour cells with the anti-pfetin monoclonal antibody (Fig. 1). The typical pfetin expression patterns are exhibited in Fig. 1. A high expression of pfetin was observed in sample no. 1, obtained from a patient with a good outcome (Fig. 1A), while a low expression of pfetin was observed in sample no. 21 from a patient with a poor outcome (Fig. 1B). These observations were consistent with those in our previous study (11,15,16).

Survival Analyses of Pfetin and DDX39 Expression in 72 GIST Cases

In these 72 cases, Kaplan–Meier survival analysis revealed pfetin expression to correlate significantly and inversely with metastasis and recurrence (Fig. 2). Disease-free survival rates were higher in pfetin-positive than in pfetin-negative cases; the disease-free survival rate was 94.7% (57/72) for pfetin-positive and 20.0% (15/72) for pfetin-negative cases ($P < 0.0001$; log-rank test). Previously, we reported the DDX39 expression in these 72 cases from the Juntendo University Hospital (17). The disease-free survival rate was 90.2% (21/72) for ATP-dependent RNA helicase DDX39-weak and 50.38% (51/72) for ATP-dependent RNA helicase DDX39-strong cases (Table 2) (17).

Univariate and Multivariate Analyses of Pfetin Expression

We performed univariate and multivariate analyses for pfetin in the 72 newly enrolled cases from the Juntendo University Hospital. According to the univariate analysis, tumour size, risk classification (21), DDX39 and pfetin were significantly correlated with disease-free survival. Then, in the multivariate analysis, pfetin expression was only an independent significant predictor of disease-free survival. [$P < 0.0001$; relative risk = 0.092; 95% confidence interval (CI), 0.023–0.374; Table 2].

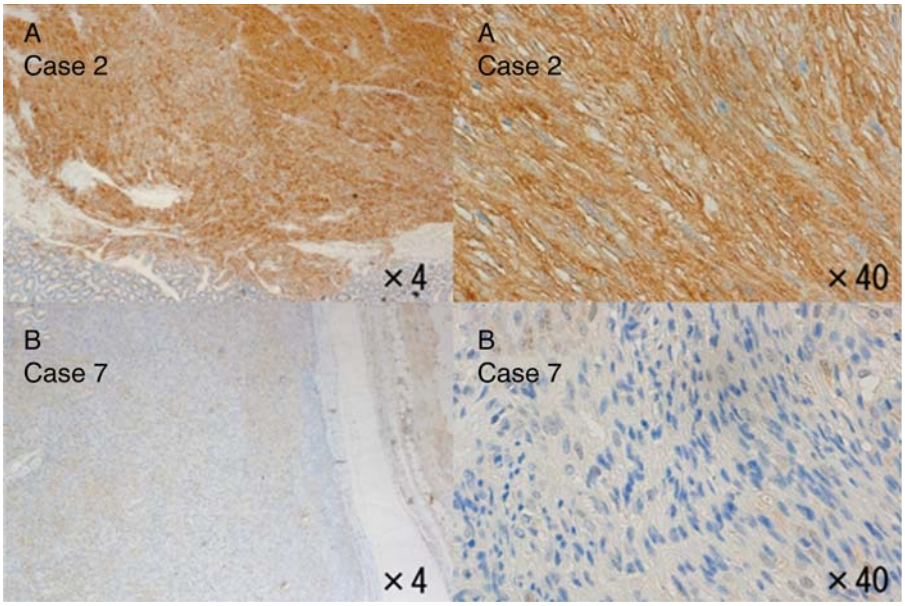

Figure 1. Immunohistochemical staining for pfetin. Typical images of pfetin-positive (A) and -negative (B) cases were demonstrated. The localization of pfetin was consistent with our previous report (11,15,16).

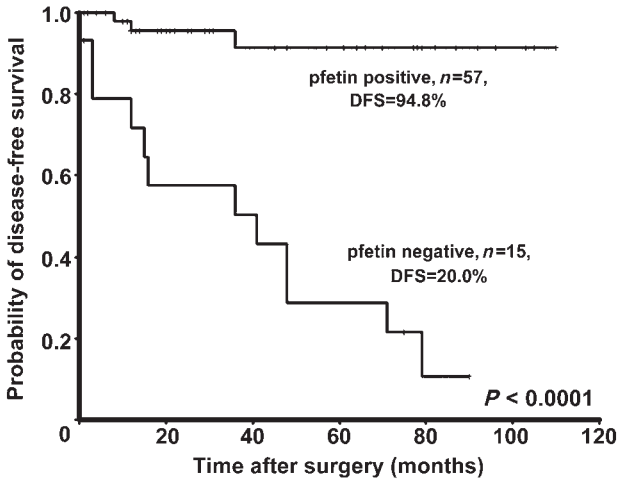

Figure 2. The Kaplan–Meier estimated disease-free survival curves are illustrated for the 72 patients in the Juntendo University Hospital based on pfetin expression. Statistically significant differences in disease-free survival periods were observed between pfetin-positive and -negative cases.

Meta-analyses of Prognostic Value of Pfetin Expression in Multiple Clinical Facilities

In our previous report, we demonstrated the possible prognostic value of pfetin in 159 GIST cases from the National Cancer Center Hospital, 100 GIST cases from the Niigata University Medical and Dental Hospital and 40 GIST cases from the Juntendo University Shizuoka Hospital, using the same monoclonal antibody as in this study. The all disease-free survival rates were 90.8, 94.6 and 92.0% for pfetin-positive patients and 67.5, 61.5 and 25.0% for pfetin-negative patients in the 159 cases from the National Cancer Center Hospital, in the 100 cases from the Niigata University Medical and Dental Hospital and in the Juntendo University Shizuoka Hospital, respectively. By combining all these data, we obtained pfetin expressions in a total of 371 cases in four hospitals. In these such 371 cases, Kaplan–Meier survival analysis revealed pfetin expression to correlate significantly with metastasis and recurrence; the disease-free survival rate was 93.8% (208/225) for pfetin-positive cases and 40.6% (45/74) for pfetin-negative cases ($P < 0.0001$, log-rank; Fig. 3A). Univariate and multivariate analyses of the total 371 cases revealed risk classification and pfetin expression to be significant predictors of disease-free survival ($P < 0.001$, relative risk = 2.376, 95% CI = 1.490–3.790; $P < 0.001$, relative risk = 0.262, 95% CI = 0.148–0.464; Table 4).

Survival Analysis for Pfetin in Each Risk Classification Category

We investigated the relationship between the risk classification (21) and disease-free survival. Metastasis and recurrence after surgery were observed in 11 of 206 patients (5.3%) in the low-risk group, 9 of 82 (11.0%) in the intermediate-risk group, and 41 of 83 (49.4%) in the high-risk group (Fig. 3B). There was a significant correlation between the risk classification and postoperative metastasis and recurrence of GIST (Table 4). Then we analysed the pfetin expression in each risk classification group. The disease-free survival rate was significantly higher in the pfetin-positive than in the pfetin-negative group within each risk group. In the low-risk group, the disease-free survival rate was 97.7% for pfetin-positive and 77.4% for pfetin-negative patients ($P < 0.0001$, log-rank; Fig. 3C). In the intermediate-risk group, the disease-free

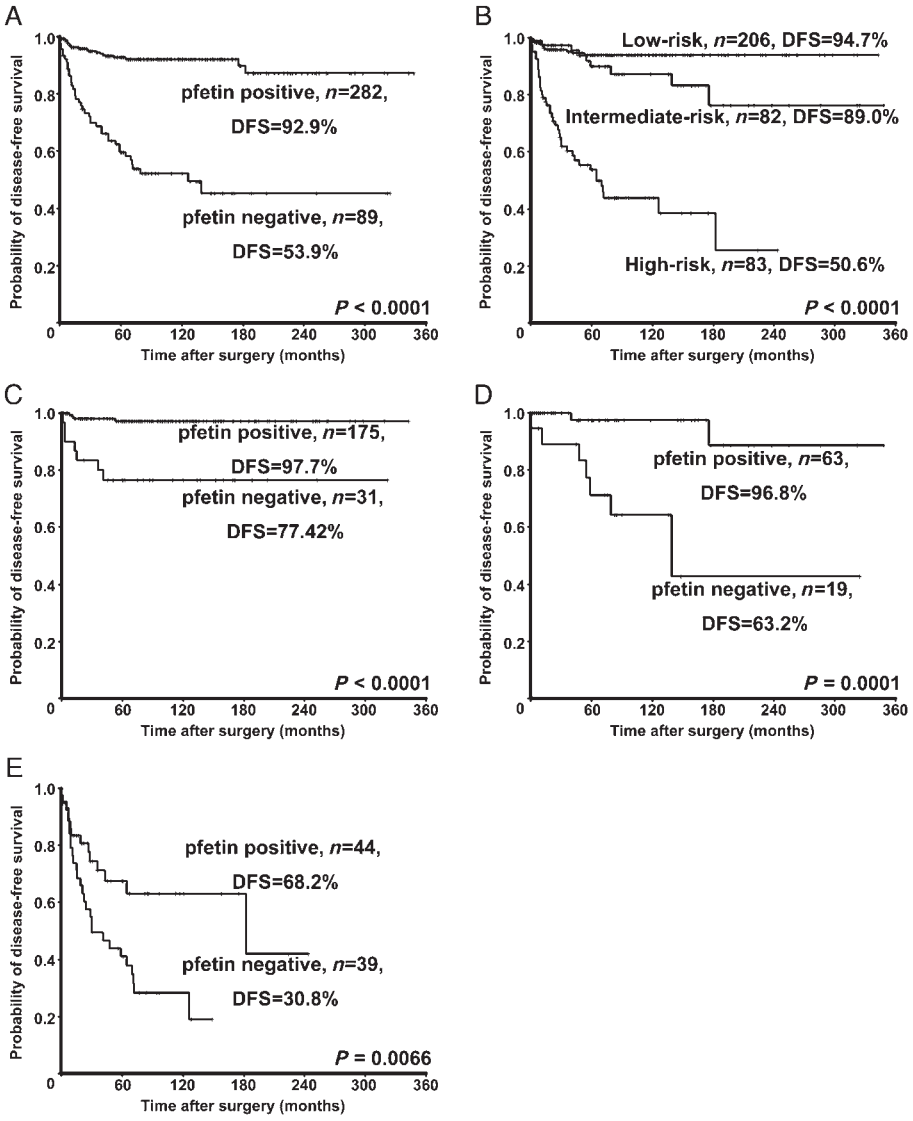

Figure 3. The Kaplan–Meier estimated disease-free survival curves are illustrated for 371 patients based on pfetin expression. Statistically significant differences in disease-free survival periods were observed between the pfetin-positive and -negative cases in the 371 cases (A). The curve is also illustrated for 371 patients based on risk classification group (21). Statistically significant differences were observed among the each risk group (B). The Kaplan–Meier estimated disease-free survival curves illustrated based on pfetin expression in low-, intermediate- and high-risk groups (C, D and E, respectively).

survival rate was 96.8% for the pfetin-positive and 63.2% for the pfetin-negative patients ($P = 0.0001$, log-rank; Fig. 3D). In the high-risk group, the disease-free survival rate was 68.18% for the pfetin-positive and 30.8% for pfetin-negative patients ($P = 0.0066$, log-rank; Fig. 3E).

Survival Analysis for the Combination of Pfetin and DDX39

We previously reported that DDX39 expression was significantly associated with metastasis and poor clinical outcome in a group of 72 GIST patients from the Juntendo University. The disease-free survival rate was 90.2% for DDX39-weak and 50.38% for DDX39-strong cases (17).

Finally, we performed a survival analysis with the combined use of pfetin and DDX39 for 72 cases from the Juntendo University Hospital. The disease-free survival rates were 93.48% for pfetin-positive and DDX39- weak patients, 100% for pfetin-positive and DDX39-strong patients, 60% for pfetin-negative and DDX39- weak patients and 0% for pfetin-negative and DDX39-strong patients, respectively ($P < 0.0001$, log-rank; Fig. 4). The disease-survival rate was significantly lower in the pfetin positive and DDX39-weak patients. Multivariate analyses revealed the combination of pfetin and DDX39 to be significant predictors of disease-free survival ($P = 0.004$, relative risk = 2.113, 95% CI = 1.277–3.496; Table 5).

DISCUSSION

A biomarker predicting postoperative metastasis and recurrence would be a useful indicator allowing the optimal therapeutic strategy for GIST to be selected. Previously, we found

Table 4. Univariate and multivariate analysis and the relationship between clinicopathological variables and pfetin expression of 371 GIST cases

Variable	Number of cases	Pfetin positive	Pfetin negative	Correlation (pfetin) χ^2 *P* value	Disease-free survival		Multivariate analysis of disease-free survival by Cox regression		
					Rate (%)	Log-rank (*P* value)	*P* value	Relative risk	95% confidence interval
Age									
<60	137	98	39	0.132	80.29	0.318			
≥60	234	184	50		85.47				
Sex									
F	194	145	49	0.627	86.44	0.0932			
M	177	137	40		80.93				
Site									
Stomach	309	235	74	0.157	87.11	<0.001	0.232	1.27	0.858–1.881
Small intestine	45	37	8		58.62	0.9587			
Other	17	10	7		92.86	0.0646			
Histology									
Spindle	304	235	69	0.361	85.2	0.4383	0.454	0.138	0.812–1.594
Epithelioid	28	18	10		78.57	0.0421			
Mixed	37	27	10		72.97	0.64637			
Unknown	2	2	0						
Size (cm)									
<5	209	175	34	<0.001	94.26	<0.001	0.123	1.493	0.897–2.485
5–15	145	103	42		75.17	<0.001			
≥15	17	4	13		23.53	<0.001			
Necrosis									
Present	83	51	32	0.001	67.47	<0.001	0.229	1.393	0.812–2.392
Absent	288	231	57		88.19				
Unknown									
Risk classification[a]									
Low	205	174	31	<0.001	95.12	0.0271	<0.001	2.376	1.490–3.790
Intermediate	83	64	19		87.95	<0.001			
High	83	44	39		50.6	<0.001			
Pfetin									
+	282	282	0		92.91	<0.001	<0.001	0.262	0.148–0.464
–	89	0	89		53.93				

[a]Risk classification based on tumour size and MIB-1 grade (21).

the prognostic value of pfetin expression in GISTs using a proteomic approach (11), and later confirmed it in the independent sample sets (15,16). Pfetin contains a voltage-gated potassium (K+) channel tetramerization domain (12), and a component of auxiliary $GABA_B$ receptor subunits, with possible biophysical and pharmacological properties of receptor response (13,14). However, its function in the process of cancer development and progression is still unknown. In this study, we further confirmed the utilities of pfetin as a prognostic biomarker in the newly enrolled 72 GIST cases. Even when the previous data from another 299 cases were combined, pfetin expression correlated significantly with the disease-free survival of GIST patients.

Although the risk classification showed a high correlation with prognosis, clinical outcomes differed significantly according to pfetin expressions in each risk classification

category. Determining pfetin expression may be beneficial, especially for low-risk and intermediate-risk patients, who are more likely to have metastasis and recurrence, and thus should receive imatinib mesylate treatment.

Clinical outcome of the GIST patients differed between the clinical facilities. The disease-free survival rates for pfetin-negative patients in the National Cancer Center Hospital, the Niigata University Medical and Dental Hospital, the Juntendo University Shizuoka Hospital and the Juntendo University Hospital were 67.5, 61.5, 25.0 and 20.0%, respectively. These differences may be a part of the differences in the various clinical outcomes. These differences in disease-free survival rates may be associated with (i) number of patients, (ii) average period of disease-free survival and (iii) the presence of the cases that developed metastasis/recurrence after a long interval. First, the number of patients used in this analysis may affect the difference in the disease-free survival rate. The patient population of the Juntendo University Shizuoka Hospital (40 patients) was smaller than the other facilities (National Cancer Center Hospital: 159 patients, Niigata University Medical and Dental Hospital: 100 patients, Juntendo University Hospital: 72 patients). Second, the average period of disease-free survival in the Juntendo University Shizuoka Hospital (35 months) was shorter than in other facilities (Juntendo University Hospital: 38 months, National Cancer Center Hospital: 113 months, Niigata University Medical and Dental Hospital: 87 months). Third, there were few cases that developed recurrence/metastasis after a long interval. Although there were no cases that developed metastasis/recurrence after 5 years in the Juntendo University Shizuoka Hospital, 3 of 24 patients from the National Cancer Center Hospital, 4 of 14 patients in the Niigata University Medical and Dental Hospital and 2 of 16 patients in the Juntendo University Hospital developed metastasis/recurrence after 5 years. Although there are such variations in patients' backgrounds among the four facilities, pfetin expression was statistically and significantly correlated with the prognosis of GIST patients in all four facilities examined. Because of the clinical and genetic heterogeneity of GIST, our data sets were important to evaluate the novel biomarker before using clinical application.

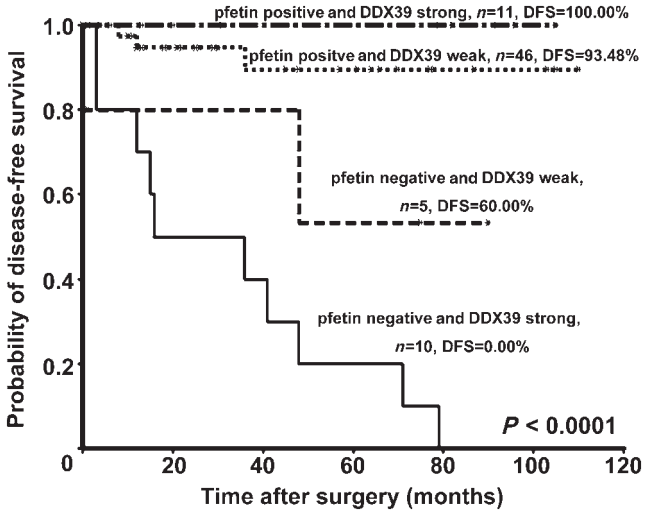

Figure 4. The Kaplan–Meier estimated disease-free survival curves are illustrated for 72 patients from the Juntendo University Hospital based on pfetin and DDX39 expression. Statistically significant differences in disease-free survival periods were observed between the pfetin-positive and DDX39-weak and pfetin -negative and DDX39-strong cases.

DDX39 was originally reported as a novel growth-associated RNA helicase (18). Overexpression of DDX39 was observed in lung squamous cell carcinoma, and up-regulation of this enzyme stimulated colony formation (19). Yoo and Chung (24) reported that DDX39 was required for global genome integrity as well as telomere protection. They found that overexpression of DDX39 in telomerase-positive human cancer cells led to progressive telomere elongation and depletion of endogenous ATP-dependent RNA helicase DDX39 by small hairpin RNA, resulting in telomere shortening (24). In our previous report, DDX39 was significantly correlated to worse prognosis of GIST, and multivariate analysis revealed that DDX39 was an

Table 5. Univariate and multivariate analysis and the relationship between the combination of pfetin and DDX39 expression of the 72 GIST cases from the Juntendo University Hospital

Variable	Disease-free survival				Multivariate analysis of disease-free survival by Cox regression		
	Number of cases	Number of metastasis and recurrence	Rate (%)	Log-rank (*P* value)	*P* value	Relative risk	95% confidence interval
Pfetin and DDX39							
Pfetin+ and DDX39 weak	46	3	93.48	<0.0001	0.004	2.113	1.277–3.496
Pfetin+ and DDX39 strong	11	0	100.00				
Pfetin– and DDX39 weak	5	2	60.00				
Pfetin– and DDX39 strong	10	10	0.00				

independent prognostic factor. In this study, we examined the combination of pfetin and DDX39 in 72 cases from the Juntendo University Hospital. Univariate and multivariate analysis revealed that the combination of pfetin and DDX39 expression was an independent prognostic factor in GIST. Interestingly, all cases with pfetin negative and DDX39 strong caused metastasis and recurrence. Although the relationship between pfetin and DDX39 expression remains to be defined, it is considered better to treat the cases with pfetin-negative and DDX39-strong primary tumour with imatinib mesylate.

We anticipate that our study results will lead to a novel clinically applicable prognostic marker in routine diagnosis and evaluation of the malignancy risk of GIST. Larger-scale validation studies and a functional investigation of pfetin and DDX39 will be our next challenges.

Acknowledgements

We would greatly appreciate Prof. Kajiyama, Prof. Watanabe, Prof. Sakamoto and Prof. Kawasaki from the Juntendo University Hospital to collect the clinical samples.

Funding

This work was supported by a grant from the Ministry of Health, Labor and Welfare and by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation of Japan and the National Cancer Center Research and Development Fund (23-A-10).

Conflict of interest statement

None declared.

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Appendix

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