Clinicopathological effects of protein phosphatase 2, regulatory subunit A, alpha mutations in gastrointestinal stromal tumors

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Recently, several studies have reported that dysfunctions in protein phosphatase 2A (PP2A) caused by alterations in protein phosphatase 2 regulatory subunit A, alpha (*PPP2R1A*) are responsible for tumorigenesis and tumor progression in several types of cancers. The impact of *PPP2R1A* mutations remains unknown in gastrointestinal stromal tumors (GISTs), although mutations in *KIT* and *PDGFRA*, which result in constitutive activation of the receptor tyrosine kinase pathway, are important in GIST tumorigenesis. In this study, we performed mutation analysis of *PPP2R1A* to examine the frequency of *PPP2R1A* mutations and their clinicopathological correlation in 94 GIST cases. In addition, we performed an *in vitro* analysis to investigate the effects of *PPP2R1A* mutations on cell proliferation and kinase phosphorylation in GIST cells. Seventeen GIST cases (18%) harbored mutations in *PPP2R1A*. All but one of these 17 cases harbored a *KIT, PDGFRA, HRAS, NRAS,* or *KRAS* mutation as the oncogenic driver mutation, and the remaining case was immunohistochemically negative for succinate dehydrogenase B (SDHB). Multivariate analysis showed that larger tumor size, higher mitotic rate, and *PPP2R1A* mutation are independent prognostic factors for overall survival; however, *PPP2R1A* mutation was not an independent prognostic factor for disease-free survival. The transduction of GIST cells with mutant *PPP2R1A* induced an accelerated growth rate via increased phosphorylation of Akt1/2, ERK1/2, and WNK1, a kinase associated with angiogenesis. In addition, the transduction of GIST cells with mutant *PPP2R1A* caused increased c-kit phosphorylation, suggesting that c-kit is also a target of PP2A, reinforcing the tumorigenic capabilities of c-kit. Furthermore, the transducing GIST cells with wild-type PP2A dephosphorylated mutant c-kit. This study provides a new insight into the biology of GISTs and their phosphatase activity, and activated PP2A could be a therapeutic target in GISTs.
Modern Pathology (2016) **29,** 1424–1432; doi:10.1038/modpathol.2016.138; published online 29 July 2016

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors in the gastrointestinal tract. Approximately 85–90% of GISTs harbor oncogenic mutations in either *KIT* or *PDGFRA*, leading to the downstream activation of the RAS-RAF-MAPK and PI3K-AKT-mTOR pathways.[1–7] A subset of the remaining *KIT* and *PDGFRA* mutation-negative GISTs have inactivating mutations in genes encoding subunits of succinate dehydrogenase (SDH) or activating mutations in *BRAF*, *HRAS*, *NRAS*, *KRAS,* or *PIK3CA*.[1,8,9] These mutations are also expected to cause constitutive downstream activation of the KIT/PDGFRA signaling pathway.

Many cellular processes depend on the phosphorylation or dephosphorylation of signal transduction pathways. However, relatively little attention has been paid to phosphatase. Protein phosphatase 2A (PP2A), a serine/threonine phosphatase, is a tumor suppressor that plays important roles in regulating the cell cycle, survival, and differentiation.[10–20] The A subunit, encoded by protein phosphatase 2, regulatory subunit A, alpha (*PPP2R1A*) is a scaffolding unit containing 15 HEAT repeats.[21] Mutations in *PPP2R1A*

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Received 17 April 2016; revised 13 June 2016; accepted 13 June 2016; published online 29 July 2016

are responsible for tumorigenesis in several types of cancer.[10,13,15,22,23] In addition, irreversible phosphorylation plays an important role in the progression of ovarian and uterine carcinomas.[22] PP2A dysfunction could constitutively activate various signal transduction pathways, including downstream receptor tyrosine kinase pathways. Therefore, *PPP2R1A* mutations might substitute for driver mutations in *KIT*/*PDGFRA* mutation-negative GISTs. Although PP2A is a serine/threonine phosphatase, PP2A activation by FTY720 causes dephosphorylation of the mutant c-kit receptor and its downstream signaling targets pAkt, pSTAT5, and pERK1/2.[24] Therefore, reactivation of PP2A might be a therapeutic strategy for patients with drug-resistant c-kit mutation-positive cancers.[24] However, the effects of *PPP2R1A* mutations in GISTs have not yet been elucidated.

In this study, we performed mutation analysis of *PPP2R1A* in GISTs to elucidate the frequency and clinicopathological effects of mutations in this gene. We found that *PPP2R1A* mutations are associated with adverse clinical outcomes for GISTs. The transduction of GIST cells with mutant *PPP2R1A* cells induces accelerated growth via increased phosphorylation of Akt, ERK, and WNK1. These phenotypic changes might be associated with the activation of angiogenesis. Therefore, *PPP2R1A* mutations could be a novel biomarker for deciding whether to use second-line tyrosine kinase inhibitors in patients with GISTs.

Materials and methods

Patients

Ninety-four cases of GISTs with prognostic information were collected from the files of the Department of Human Pathology, Juntendo University Hospital, Tokyo, Japan. All patients were treated at the Juntendo University Hospital between 2000 and 2013. These cases were diagnosed by the WHO classification system[25] for soft-tissue tumors and by classification using the modified risk classification.[26] In addition, diagnoses were confirmed by immunohistochemical analysis of c-kit, DOG1, and succinate dehydrogenase B (SDHB) expression. Clinicopathologic data of the 94 patients are shown in Supplementary Table 1. The follow-up period ranged from 0.3 to 164 months (mean: 65 months). The patients were treated with surgical resection without a pre-adjuvant treatment such as imatinib. In 92 of the cases, the tumors were completely resected. In the remaining two cases (cases 25 and 29), incomplete resections were performed owing to large tumor size.

Mutation Analysis of *PPP2R1A*, *KIT*, *PDGFRA, HRAS, NRAS,* and *KRAS*

Genomic DNA was extracted from each of the 94 formalin-fixed and paraffin-embedded GIST samples. Genomic DNA was also extracted from corresponding non-tumor tissue to confirm that mutations found in the tumor-derived DNA were not found in normal tissue samples. Mutation analysis of *PPP2R1A* was performed from exon 5 to 6 by PCR and direct sequencing. PCR cycle conditions were as follows: 94 °C for 2 min followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final hold at 72 °C for 2 min. Cases with *PPP2R1A* mutations were also tested for mutations in *KIT* (exons 9, 11, 13, and 17), *PDGFRA* (exons 10, 12, 14, and 18), *BRAF* (V600E), *NRAS* (exon 2 and 3), and *KRAS* (exons 2, 3, and 4). The primer sequences used are listed in Supplementary Table 2.

Preparation of Retrovirus and Transduction of Cell Lines

For retrovirus production, the pCX4[27] and pGEM (Promega) vector systems were used. A plasmid encoding human *PPP2R1A* (Origene) was used to generate constructs, which were subcloned into pGEM. cDNA encoding the *PPP2R1A* Val201Ala (*PPP2R1A*-T602C) and Glu238Lys (*PPP2R1A*-G712A) mutants was generated using the QuickChange II Site-Directed Mutagenesis kit (Agilent Technologies), and these constructs were subcloned into pCX4bleo. These two mutants were selected from the mutations detected in our GIST samples, together with the control vectors (GFP and wild-type PPP2R1A (WT)). Retroviruses were generated using αφ cells as packaging cells and were used to infect the T1 cell line (kindly provided by Dr Taguchi). GIST T1 cells had wild-type sequences in exons 5 and 6 of *PPP2R1A*. Infected cells were selected in 500 μg/ml Zeocin (Invitrogen). We established four GIST cell lines and named them as T1-G712A, T1-T602C, T1-GFP, and T1-WT.

Human Phospho-Kinase Array Analysis

We performed a human phospho-kinase array analysis using our four cell lines. The relative phosphorylation levels of 39 selected proteins on the array were acquired by using the Proteome Profiler Human Phospho-Kinase Array Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The expression levels of phosphorylation proteins were quantified by the Fuji Film Multi Gauge software (Tokyo, Japan).

Cell Proliferation Assay

Cells (1×10^6) from each cell line were plated in 100-mm-diameter culture dishes with 8 ml of RPMI supplemented with 10% calf serum and antibiotics (SM and PC). Cells were counted in triplicate using a TC20 Automated Cell Counter (BIO-RAD) after 24, 72, and 120 h.

Table 1 *PPP2R1A* and driver mutation status in GISTs

Case no.	*Risk clasification*	*Gene alteration status*						
		PPP2R1A		KIT	PDGFRA		KRAS	
5	High	p.W257***	c.770 G>A	c.1678_1734del57				ND
6	High	p.V195M	c.583 G>A				p.A130T	c.388 G>A
8	High	p.E197G	c.590 A>G		p.L661P	c.1982 T>C		
		p.K266R	c.797 A>G					
9	High	p.E204V	c.611 A>T	c.1678_1734del57				ND
10	Low	p.V201A	c.602 T>C	c.1678_1734del57				ND
		p.A232T	c.694 G>A					
		p.E238K	c.712 G>A					
13	Very low	p.F209L	c.625 T>C		p.G829R	c.2485 G>A		
15	High	p.E238K	c.712 G>A	c.1678_1734del57				ND
25	High	p.N211D	c.631 A>G	c.1678_1734del57				ND
		p.S214P	c.640 T>C					
26	Low	p.L173P	c.518 T>C	c.1678_1734del57				ND
29	High	p.L234P	c.701 T>C					(–)
		p.L248Q	c.743 T>A					
33	High	p.K194R	c.581 A>G	c.1651_1662del12				ND
		p.E204G	c.611 A>G					
		p.I206T	c.617 T>C					
35	High	p.A184D	c.551 C>A	c.1735_1737del3				ND
36	Low	p.M208T	c.623 T>C	c.1674_1675ins3				ND
38	High	p.V225M	c.673 G>A	c.1651_1662del12				ND
		p.V244M	c.730 G>A					
		p.A252T	c.754 G>A					
40	High	p.Y261H	c.781 T>C	c.1651_1662del12				ND
60	High	p.A193T	c.577 G>A	c.1669_1671del3				ND
		p.V201I	c.601 G>A					
84	Very low	p.Q233H	c.699 G>C	c.1678_1734del57				ND

Abbreviation: ND, not determined.

Western Blotting

Proteins were extracted from our four GIST cell lines (T1-G712A, T1-T602C, T1-GFP, and T1-WT) and were separated via SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with one of the following antibodies: rabbit polyclonal antibodies against c-kit (dilution 1:200, Santa Cruz, sc-168), phospho-c-kit (Tyr568-/570, dilution 1:200, Santa Cruz, sc-18076), phospho-c-kit (Tyr721, dilution 1:200, Santa Cruz, sc-101659), or mouse monoclonal antibody against GAPDH (dilution 1:500, Santa Cruz, sc-32233). After incubation, membranes were washed three times with Tris-EDTA buffer and then reacted with the appropriate horseradish peroxidase-conjugated secondary antibody (1:1000 dilution, GE Healthcare Biosciences).

Statistical Analysis

The χ^2-test and Mann–Whitney *U*-test were used to examine associations between *PPP2R1A* mutations and clinicopathological features. The impact of *PPP2R1A* mutation on disease-free survival rate and overall survival rate was calculated using Kaplan–Meier analysis with the log-rank test.

Results

PPP2R1A Mutations

Of the 94 GIST cases, 17 (18%) harbored *PPP2R1A* mutations. (Table 1, Figure 1). In total, 27 *PPP2R1A* mutations were detected in the 17 cases, with some cases having multiple mutations in exons 5 and 6 of *PPP2R1A*. Besides a single point mutation, E238K, that was common between cases 10 and 15, all other *PPP2R1A* mutations were different between cases. In total, 26 types of mutations were detected in this study. We detected the W257STOP mutation in case 5, DNA derived from both tumor tissue and normal tissue; thus, we considered it a germline mutation or somatic mosaicism. This mutation was excluded from the following statistical analysis.

Mutation Analysis of *KIT*, *PDGFRA,* and *KRAS*

To evaluate the relationship with the driver mutations in GISTs, we examined the mutation status of *KIT*, *PDGFRA, HRAS, NRAS*, and *KRAS* in *PPP2R1A* mutation-positive tumors. Among the 17 cases with *PPP2R1A* mutations, 13 cases (77%) harbored *KIT* mutations and 2 cases (12%) harbored *PDGFRA* mutations in a mutually exclusive fashion. One case among the remaining two cases harbored a

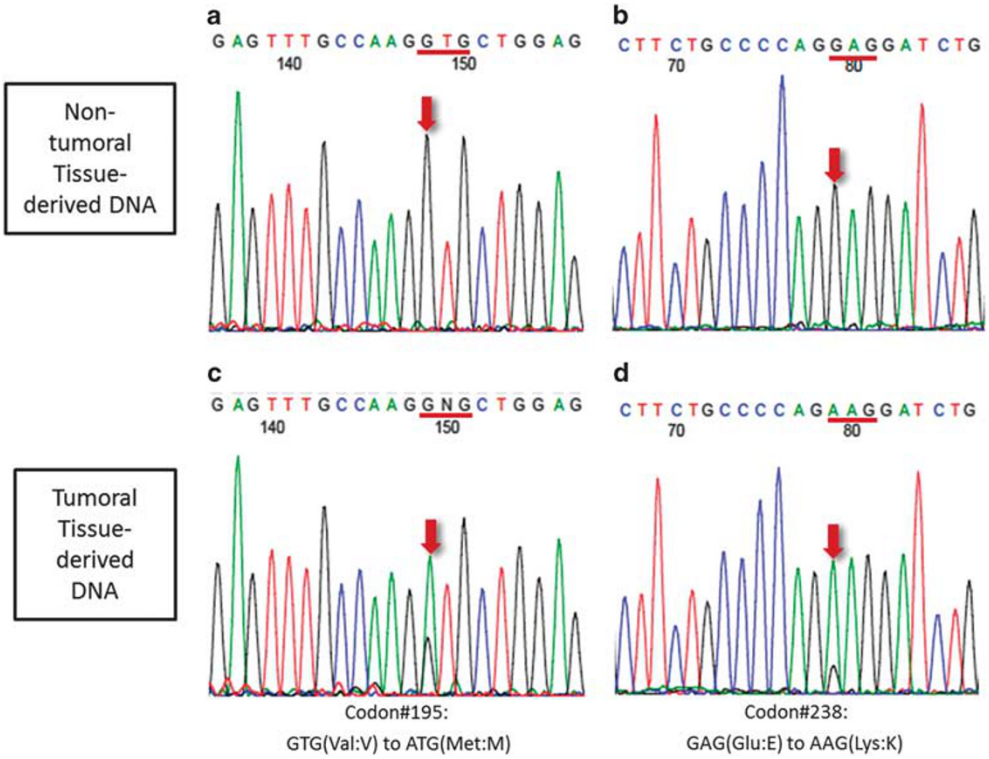

Figure 1 Examples of *PPP2R1A* mutations. The left two panels (**a** and **c**) show mutations detected in exon 5 of case #6, and the right two panels (**b** and **d**) show mutations detected in exon 6 of cases #10 and #15. These mutations were tumor-specific. (**a** and **b**) DNA derived from non-tumor tissue. (**c**) DNA derived from tumor tissue samples was examined and a *PPP2R1A* variant at codon 195 GTG > ATG (V195M) was detected. (**d**) DNA from tumor tissue was examined and a *PPP2R1A* variant at codon 238 GAG > AAG (E238K) was detected.

KRAS mutation. All *KIT* mutations were located in exon 11. The most common *KIT* mutation was a 57 base pair deletion from codons 560 to 578. Two *PDGFRA* mutations were located in exons 14 and 18. One of the 17 cases with mutated *PPP2R1A* was negative for *KIT, PDGFRA, HRAS, NRAS,* and *KRAS* mutations, although this case was negative for SDHB as determined by immunohistochemistry.

Impacts of *PPP2R1A* Mutations on GIST Clinicopathological Factors

We analyzed associations between *PPP2R1A* mutations and clinicopathological factors like age, gender, tumor site, tumor size, presence of necrosis, mitotic rates, and risk classification. We also examined the impact of recurrence and metastases on overall survival during follow-up periods. High-risk groups as determined by risk classification and higher mitotic rates had a statistically significant association with presence of a *PPP2R1A* mutation (mitotic rates $P < 0.05$, risk classification $P < 0.05$) (Table 2). Regarding the risk classification, 5 of the 45 cases (11%) in the very low- or low-risk group had a *PPP2R1A* mutation, none of the 14 cases (0%) in the intermediate-risk group had a *PPP2R1A* mutation, and 12 of the 35 cases (34%) in the high-risk group had a *PPP2R1A* mutation. In the very low/low-risk group, of the 5 cases harboring *PPP2R1A* mutations, only one was fatal. None of the remaining patients in the very low/low-risk group died. Regarding prognosis, univariate analysis revealed that patients with *PPP2R1A* mutations had significantly lower rates of both overall survival and disease-free survival compared to patients with wild-type *PPP2R1A* (Table 3: overall survival $P < 0.05$, disease-free survival $P < 0.05$) (Figure 2a and b). Furthermore, larger tumor size, presence of necrosis, higher mitotic rate, and higher risk classification (very low/low *vs* intermediate/high) were associated with shorter disease-free survival. Gastric location, larger tumor size, and higher mitotic rate were also associated with shorter overall survival. In addition, recurrence and metastasis during follow-up periods was significantly associated with shorter overall survival. Multivariate analysis showed that, larger tumor size, higher mitotic rate, and *PPP2R1A* mutation were independent prognostic factors for overall survival (Table 3). However, only larger tumor size and higher mitotic rate were independent prognostic factors for disease-free survival. Furthermore, regarding the 17 patients with *PPP2R1A* mutations, 6 of these patients received postoperative imatinib. Among these six patients, three died of disease, two have remained disease-free, and one is alive with recurrent disease. There was no

difference in prognosis according to the post-operative imatinib status in patients with *PPP2R1A* mutation. In addition, there was no difference in prognosis in this series of cases according to post-operative imatinib status (data not shown).

Human Phospho-Kinase Array Analysis

To identify activated cellular signaling pathways associated with *PPP2R1A* mutations in GISTs, we performed a human phospho-kinase array analysis of 46 specific Ser/Thr or Tyr phosphorylation sites on 39 selected proteins, using T1-G712A, T1-T602C, T1-GFP, and T1-WT cell lines. The expression levels of the phosphorylated proteins in each GIST cell line transduced with a *PPP2R1A* mutation were compared with the protein expression levels in cells transduced with GFP to identify proteins with altered phosphorylation (Figure 3a).

In T1-WT cells, the levels of phosphorylated Akt1/2/3, ERK1/2, and WNK1 were significantly reduced compared with those in the control as follows: Akt1/2/3 (0.66 ± 0.02), ERK1/2 (0.56 ± 0.02), and WNK1 (0.80 ± 0.00). Both T1-T602C and T1-G712A cells had significantly higher Akt1/2/3 phosphorylation levels (*PPP2R1A*-T602C: 1.41 ± 0.01, *PPP2R1A*-G712A: 1.22 ± 0.03) and WNK1 phosphorylation levels (*PPP2R1A*-T602C: 1.32 ± 0.00, *PPP2R1A*-G712A: 1.38 ± 0.00). The level of phosphorylated ERK1/2 increased in T1-G712A cells (1.18 ± 0.03) and decreased in T1-T602C cells (0.81 ± 0.01).

Table 2 Correlation between *PPP2R1A* mutations and clinicopathological factors

Parameters		PPP2R1A *mutation* +	PPP2R1A *mutation* –	*Total*	P-*value*
Age		59.0 (46.0–65.0)	63.0 (53.0–68.0)		0.13
Sex	M	10	44	54	0.899
	F	7	33	40	
Site	Stomach	9	58	67	0.064
	others	8	19	27	
Size (cm)		4.0 (3.5–8.0)	4.5 (2.7–7.0)		0.545
Risk classification	Very low	2	12	14	0.012*
	Low	3	28	31	
	Intermediate	0	14	14	
	High	12	23	35	
Necrosis	+	7	23	30	0.365
	–	10	54	64	
Mitosis	≦5	5	58	63	< 0.001
	5 <	12	19	31	
Metastatic or reccurence	+	4	12	16	0.244
	–	13	63	76	
Total		17	77	94	

Abbreviations: F, female; M, male. *$p < 0.05$.

Cell Proliferation Assay

To elucidate the impact of *PPP2R1A* mutations on the behavior of GISTs, we conducted a cell proliferation assay using T1-G712A, T1-T602C, T1-GFP, and T1-WT cell lines. Both T1 lines containing *PPP2R1A* mutations (T1-G712A and T1-T602C) had significantly higher growth rates compared with growth rates of T1-GFP and T1-WT cells (Figure 3b). There was no difference in proliferation rate between T1-GFP and T1-WT cells.

Western Blotting

The c-kit phosphorylation status in T1 cells expressing *PPP2R1A* mutations (T1-G712A and T1-T602C) was also evaluated. In T1 cells transduced with these *PPP2R1A* mutations, phosphorylation at Tyr721 of c-kit was increased compared with those transduced

Table 3 Univariate and multivariate analysis for prognostic factors

OS (n = *94*)	*OS*			*DFS*		
Factor	*Univariate analysis*		*Multivariate analysis*	*Univariate analysis*		*Multivariate analysis*
	Hazard ratio (95% CI)	P-*value*	*Hazard ratio (95% CI)*	*Hazard ratio (95% CI)*	P-*value*	*Hazard ratio (95% CI)*
Age > 65	0.209 (0.026–1.678)	0.141		0.339 (0.096–1.191)	0.091	
Sex (female)	0.619 (0.150–2.553)	0.507		0.616 (0.214–1.772)	0.369	
Site (stomach)	0.237 (0.059–0.955)	0.043	—	0.403 (0.151–1.076)	0.070	
Size > 5 cm	9.655 (1.207–77.22)	0.033	5.232 (0.613–44.671)	22.81 (3.002–173.2)	0.003	13.04 (1.655–102.8)
Necrosis (+)	3.388 (0.897–12.80)	0.072		3.320 (1.233–8.939)	0.018	—
Mitosis > 6	14.75 (1.835–118.6)	0.011	5.866 (0.653–52.676)	11.42 (3.244–40.21)	0.000	6.217 (1.723–22.434)
PPP2R1A mutation	5.421 (1.438–20.44)	0.013	3.102 (0.786–12.237)	2.944 (1.059–8.187)	0.038	—
Risk factor (intermediate or high)	6.796 (0.848–54.45)	0.071		15.64 (2.064–118.5)	0.008	—

Abbreviations: OS, overall survival; DFS, disease-free survival; CI, confidence interval.

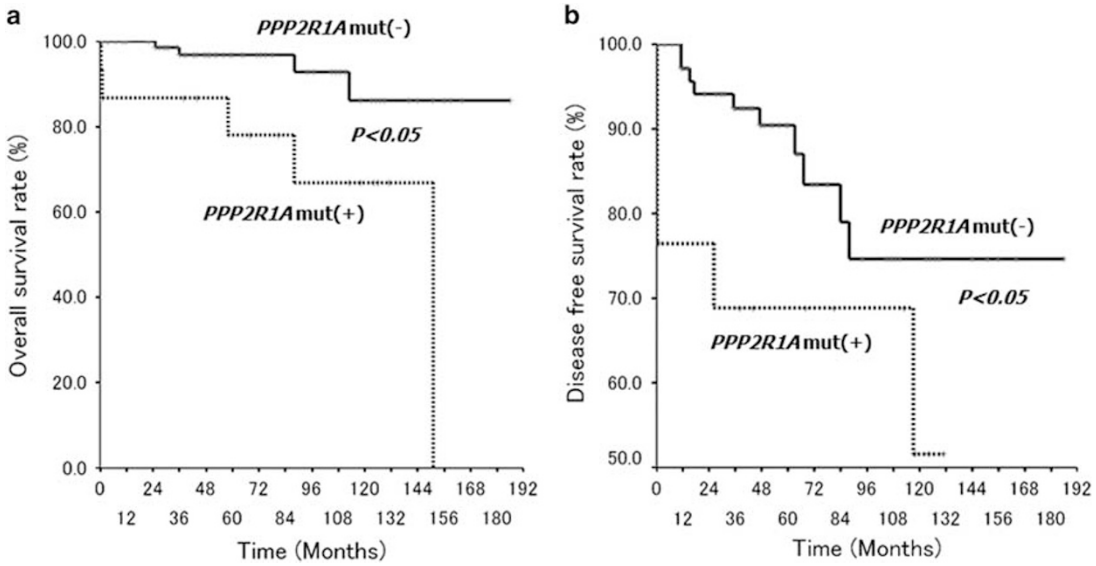

Figure 2 Prognostic impact of *PPP2R1A* mutation in GISTs. Both overall survival (**a**) and disease-free survival (**b**) were significantly different between the mutation-positive and mutation-negative cases (overall survival $P < 0.05$, disease-free survival $P < 0.05$).

with wild-type *PPP2R1A* or GFP (Figure 4). The same phenomenon was observed regarding phosphorylation of Tyr568/570; however, this trend was not as strong as the trend for Tyr721 (Figure 4). This is probably due to the position of the *KIT* mutation in T1 cells having KIT del570–578. On the other hand, in wild-type transduced cells, phosphorylation was decreased compared with T1-GFP (Figure 4). These findings suggested that wild-type PP2A could dephosphorylate mutant c-kit in GIST.

Discussion

Mutations in *PPP2R1A* have been reported in many cancers such as lung cancer, melanoma, and breast cancer,[10] and are important in the pathogenesis of some uterine[15,22,23] and ovarian cancers.[13,22,23] In addition, reduced expression of subunit A of PP2A, which is encoded by *PPP2R1A,* has been reported in glioma.[14] In this study, we detected *PPP2R1A* mutations in 18% of GISTs, which is relatively higher rate than is seen in other cancers.[10,13,15,22,23] All *PPP2R1A* mutations except one were missense mutations and were heterozygous, consistent with previous findings.[10,22] Previously reported somatic *PPP2R1A* mutations are located in both HEAT5 and HEAT7 motifs near the interface of subunits A and B.[22,23] Most reported *PPP2R1A* missense mutations occur in recurrent hotspots.[22,23] However, most *PPP2R1A* mutations that we detected were not recurrent, although all were located in HEAT5 to HEAT7. The *in vitro* assay performed using a GIST cell line transduced with either of two *PPP2R1A* mutants selected from the mutations detected in this study showed drastic phenotypic changes. Thus, *PPP2R1A* mutations detected in this study are expected to reduce the enzymatic activity of PP2A.

Next, we asked whether mutations in *PPP2R1A* could be a driver oncogene in *KIT/PDGFRA* mutation-negative GISTs. Of the ~20% of GIST cases for which we detected *PPP2R1A* mutations, we also examined the mutation status of *KIT*, *PDGFRA*, *BRAF, HRAS, NRAS,* and *KRAS*. The frequencies of these mutations within GISTs with *PPP2R1A* mutations are consistent with previously reported values.[1] These findings suggest that mutations in *PPP2R1A* and reported driver genes including *KIT* and *PDGFRA* are not mutually exclusive. Therefore, mutations in *PPP2R1A* are not another driver mutation of GISTs.

The prognostic impact of *PPP2R1A* mutations in malignant tumors has not been investigated, despite many reports describing the frequency of these mutations in various tumor types. *PPP2R1A* mutations might be one of many molecular genetic alterations that contribute to tumor progression in undifferentiated uterine and ovarian low-grade endometrioid carcinomas.[28] Mutations in *PPP2R1A* can affect the binding of other subunits or substrate recognition and constitutively disrupt PP2A function.[12,29] Thus, *PPP2R1A* mutations could cause constitutive activation of various signal transduction pathways, including the receptor tyrosine kinase pathway.

In this study, the presence of *PPP2R1A* mutations was associated with higher risk classification and higher mitotic rate (>5/50 HPFs). Among the 17 GIST cases with *PPP2R1A* mutations, 5 cases were classified as very low or low risk. One of these five patients died despite the low-risk classification. Furthermore, *PPP2R1A* mutations were determined to be adverse prognostic factors for disease-free

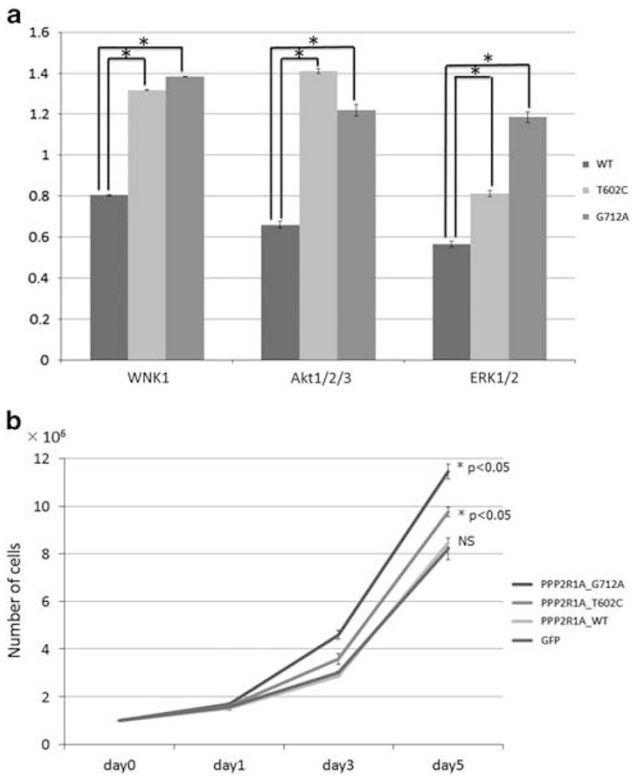

Figure 3 (**a**) Human phospho-kinase array analysis. Levels of phospho-kinases were assessed using a horseradish peroxidase-conjugated phospho-kinase antibody, which was followed by chemiluminescence detection. In T1-WT cells, the levels of phosphorylated Akt1/2/3, ERK1/2, and WNK1 were significantly reduced. Both T1-T602C and T1-G712A cells had significantly higher levels of phosphorylated Akt1/2/3 and WNK1. The level of phosphorylated ERK1/2 increased in T1-G712A cells and decreased in T1-T602C cells. $*P < 0.05$ compared with cells expressing *PPP2R1A*-WT. (**b**) Evaluation of *in vitro* cell growth. For each experiment, 1.0×10^6 cells were cultured in RPMI and counted at 24, 72, and 120 h. $*P < 0.05$ compared with cells expressing GFP; NS, not significant compared with cells expressing GFP.

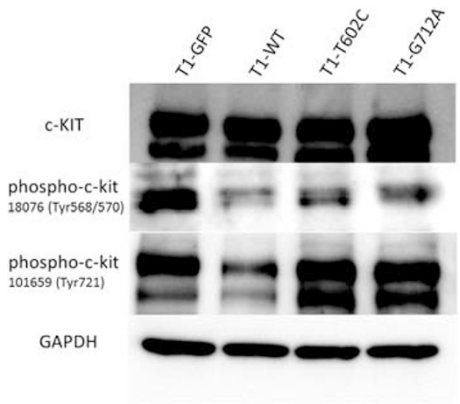

Figure 4 c-kit phosphorylation status according to the transduction of mutant *PPP2R1A* in T1 cells. In T1 cells transduced with mutant *PPP2R1A*, phosphorylation at Tyr721 was increased compared with those transduced with wild-type *PPP2R1A* or GFP. The same phenomenon was observed regarding phosphorylation of Tyr568/570, however, this trend was weaker than Tyr721. On the other hand, in wild-type-transduced cells, phosphorylation was decreased compared with T1-GFP.

survival and overall survival by univariate analysis. *PPP2R1A* mutations, larger tumor size, and higher mitotic rate were independent prognostic factors for overall survival, as determined by multivariate analysis. *In vitro* analysis also supported these findings. Human phospho-kinase array analysis revealed that phosphorylation of Akt and ERK significantly increased in mutant *PPP2R1A*-transduced cell lines compared to the WT *PPP2R1A*-transduced cell line. PP2A is involved in signaling pathways, including Akt and ERK, as a dephosphorylation enzyme.[10–20] These findings confirm that mutations in *PPP2R1A* disrupt PP2A function, leading to elevated phosphorylation of its substrates. In addition, the proliferation of mutation-expressing T1 cell lines was significantly accelerated compared with control cell lines. PP2A is a tumor suppressor and has an important role in regulating cell cycle progression, survival, and differentiation.[10–20] Together with these findings, GISTs with loss of PP2A function would likely acquire a more aggressive phenotype, and *PPP2R1A* mutations could act in a dominant negative manner in GISTs. Alternatively, biallelic inactivation might be necessary for the functional reduction or loss of PP2A, because 7 out 17 cases with *PPP2R1A* mutations harbored multiple mutations. In addition, the acquisition of a more aggressive GIST phenotype upon *PPP2R1A* mutation could be attributed to the enhanced phosphorylation of mutant c-kit.

Angiogenesis is also essential for GIST growth, invasion, and metastasis.[30–32] Among the phosphorylated proteins analyzed in our phosphorylation array, phosphorylation of WNK1, which is involved in regulating ion transport systems in the distal nephron,[33] was significantly elevated. Recently WNK has been considered a causative gene of pseudohypoaldosteronism type II.[33] In addition, WNK1 is involved in angiogenesis in gliomas[34] and in *in vivo* examination of zebrafish.[35–37]

Emergence of a tyrosine kinase inhibitor that targets phosphorylated receptor tyrosine kinases would drastically improve the prognosis of GIST patients. However, advanced GISTs may acquire secondary resistance or even have primary resistance to tyrosine kinase inhibitors.[38] Interestingly, our phospho-kinase array revealed that phosphorylation of Akt1/3, downstream kinases of vascular endothelial growth factor (VEGF) signaling, and WNK1, involved in angiogenesis, was increased in mutant PPP2R1A expressing GIST cells. These findings suggest that VEGF signaling could be activated in a ligand-independent manner and be involved in tumor progression in GIST cells with *PPP2R1A* mutation. VEGF signaling is a therapeutic target of sunitinib, which is currently used as a second-line therapy for patients with imatinib-resistant GISTs. Furthermore, increased microvessel density and

angiogenesis by VEGF signaling affects GIST patients prognosis.[32] Our findings suggest that as tyrosine kinase inhibitors targeting the VEGF signaling pathway, such as sunitinib, might be less effective for GISTs with *PPP2R1A* mutations, *PPP2R1A* mutations could be potential biomarkers and indicators for the application of second-line tyrosine kinase inhibitors.

Finally, drugs that directly activate PP2A do not exist. However, drugs that remove endogenous PP2A inhibitors, such as SET and CIP2A, already exist.[39–42] These drugs could be used for advanced GISTs with primary or secondary resistance to tyrosine kinase inhibitors as an adjuvant therapy.

In conclusion, *PPP2R1A* mutations occur in a subset of GISTs and are associated with a high malignant potential that leads to decreased disease-free survival and overall survival. The functional disorders of PP2A caused by *PPP2R1A* mutations promote phosphorylation of specific kinases, including those involved in angiogenesis, leading to the activation of signaling pathways that involve PP2A.

Acknowledgments

This work was supported in part by a Grant-in-Aid for General Scientific Research from the Ministry of Education, Science, Sports, and Culture (#26670286 to Tsuyoshi Saito and #15H04964 to Yoshiyuki Suehara), Tokyo, Japan.

Author contributions

TS, KM, and TY diagnosed and histologically evaluated GIST samples. MT-I, KA, and TO performed DNA extraction and sequencing analysis. MT-I, KM, DK, and SK performed *in vitro* experiments. MT-I, KA, KM, and TS contributed to data analysis. MT-I, YS, TT, KK, TY, and TS wrote the manuscript.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol)