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Overexpression of PBK/TOPK relates to tumour malignant potential and poor outcome of gastric carcinoma

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Background: PDZ-binding kinase/T-LAK cell-originated protein kinase (PBK/TOPK) is a serine–threonine kinase and overexpressed in various types of cancer by inhibiting the transactivation activities of p53 and PTEN. We tested whether PBK/TOPK acts as a cancer-promoting gene through its activation/overexpression in gastric cancer (GC).

Methods: We analysed five GC cell lines and 144 primary tumours, which were curatively resected in our hospital between 2001 and 2003.

Results: Overexpression of the PBK/TOPK protein was frequently detected in GC cell lines (4 out of 5 lines, 80.0%) was detected in primary tumour samples of GC (24 out of 144 cases, 16.6%) and was significantly correlated with venous invasion, tumour depth and recurrence rate. PDZ-binding kinase/T-LAK cell-originated protein kinase-overexpressing tumours had a worse survival rate than those with non-expressing tumours ($P=0.0009$, log-rank test). PDZ-binding kinase/T-LAK cell-originated protein kinase positivity was independently associated with a worse outcome in multivariate analysis ($P<0.0001$, hazard ratio 6.40 (2.71–14.49)). In PBK/TOPK-overexpressing GC cells, knockdown of PBK/TOPK inhibited the cell proliferation through the p53 activation in a TP53 mutation-dependent manner and inhibited the migration/invasion through the PTEN upregulation in a TP53 mutation-independent manner.

Conclusions: These findings suggest PBK/TOPK plays a crucial role in tumour malignant potential through its overexpression and highlight its usefulness as a prognostic factor and potential therapeutic target in GC.

Gastric cancer (GC) is one of the most common causes of death from cancer worldwide (Siegel *et al*, 2013). Although recent progress in diagnostic and treatment techniques have contributed to early detection, less invasive treatments and decreased mortality rate, patients with advanced disease still frequently develop recurrent disease, despite extended radical resections and consequently present extremely poor survival rates (Martin *et al*, 2002).

Because understanding the molecular mechanisms of carcinogenesis and identifying the molecular targets for treatment may

contribute to the improvement of survival of patients with GC, only a few molecular targets with frequent targets have been identified (Ushijima and Sasako, 2004), such as gene amplifications of *MET* and *ERBB2*; hypermethylation of *p16* (Oue *et al*, 2002; Ding *et al*, 2003); mutations of *TP53*, *APC* and *E-cadherin* (Becker *et al*, 1994; Maesawa *et al*, 1995; Lee *et al*, 2002); oncogenic activation of β -catenin and *K-ras* (Park *et al*, 1999); and inactivation of the mismatch repair gene *hMLH1*, which is associated with microsatellite instability (Fang *et al*, 2003).

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However, in clinical settings, only a few genes have been used as diagnostic biomarkers and/or molecular therapeutic targets (Bang *et al*, 2010; Wilke *et al*, 2014). These findings prompted us to identify novel genes associated with the progression of GC.

PDZ-binding kinase/T-LAK cell-originated protein kinase (PBK/TOPK) encodes a serine/threonine protein kinase and is highly expressed in various types of human cancer, such as breast and lung cancers (Park *et al*, 2006; Shih *et al*, 2012; O Leary *et al*, 2013). Physiologically, PBK/TOPK plays a positive regulatory role in proper chromosomal separation and cytokinesis through phosphorylation of various targets (Matsumoto *et al*, 2004; Park *et al*, 2010). PDZ-binding kinase/T-LAK cell-originated protein kinase gene expression is regulated by the cell cycle-specific transcription factors *E2F* and *CREB/ATF* (Nandi and Rapoport, 2006). PBK/TOPK prevents cancer cell death by impairing processes in the DNA damage-induced apoptosis pathway (Ayllon and O'Connor, 2007; Nandi *et al*, 2007; Hu *et al*, 2010), such as tumour suppressor p53 and p38-MAPK activities. Also, PBK/TOPK promotes cell migration by modulating PI3K/PTEN/AKT-dependent signalling (Shih *et al*, 2012). Although more recently, the prognostic significance and some functional analyses of PBK/TOPK in GC were reported (Kwon *et al*, 2016), to date, there has been no report on the prognostic significance of PBK/TOPK as an independent prognostic factor and its molecular mechanisms through the p53 and PI3K/AKT pathways that contributes to the tumour development of GC. Therefore, we closely wished to investigate the effects of PBK/TOPK overexpression and activation in GC.

Consequently, we demonstrated that PBK/TOPK was frequently overexpressed in GC cell lines and primary GCs. Overexpression of PBK/TOPK was a poor prognosticator independent of other prognostic factors. We also demonstrated that knockdown of PBK/TOPK expression in PBK/TOPK-overexpressing GC cells suppressed the cell proliferation through the activation of the p53 pathway in a *TP53* mutation-dependent manner, and suppressed migration and invasion through the upregulation of PTEN in a *TP53* mutation-independent manner. Our results provided evidence that PBK/TOPK could be an important molecular marker for determining the malignant properties and a target for molecular therapy in patients with GC.

MATERIALS AND METHODS

Cell lines and primary tissue samples. A total of five GC cell lines (Kato III, NUGC4, HGC27, MKN45 and MKN74 cells), a *TP53*-null osteosarcoma cell line (SaOS2), a wild-type *TP53* osteosarcoma cell line (U2OS) and the fibroblast cell line (WI-38) were used in this study. All cell lines were purchased from the RIKEN Cell Bank (Tsukuba, Japan) and the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan), and all cell lines were authenticated using STR genotyping. HGC27 and U2OS cells were cultured in Dulbecco's Minimum Essential Medium. SaOS2 cells were cultured in HyClone McCoy's 5A Minimum (GE Healthcare Life Sciences, Amersham, UK). The other cells were cultured in Roswell Park Memorial Institute-1640 medium (Sigma, St Louis, MO, USA). All mediums were purchased from Sigma and supplemented with 100 ml⁻¹ FBS (Trace Scientific, Melbourne, Australia). All cell lines were cultured in 50 ml⁻¹ carbon dioxide at 37 °C in a humidified chamber. Primary tumour samples of GC were obtained from 144 consecutive GC patients, who has undergone curative gastrectomy at the Division of Digestive Surgery, Department of Surgery, Kyoto Prefectural University of Medicine (Kyoto, Japan) between 2001 and 2003. Each sample was embedded in paraffin after 24 h of formalin fixation. Relevant clinical and survival data were available for all

patients. Written consent was always obtained in the formal style and after approval by the local ethics committee. None of these patients had undergone endoscopic mucosal resection, palliative resection, preoperative chemotherapy or radiotherapy, and none of them had synchronous or metachronous multiple cancers in other organs. Disease clinical and pathological stage was defined in accordance with the International Union Against Cancer tumour-lymph node-metastases (TNM) classification (Sobin and Compton, 2010). The median follow-up period for surviving patients was 80.0 months (ranging from 0.5 to 165.9 months).

Quantitative real-time RT-PCR. Single-stranded complementary DNA generated from total RNA was amplified with primers specific for each gene, as described below. The abundance of messenger RNA was measured with a quantitative real-time fluorescence detection method (ABI StepOnePlus Sequence Detection System; Applied Biosystems, Foster City, CA, USA) using TaqMan Gene Expression Assays (Hs00902992_m1 for PBK/TOPK, Hs00355782_m1 for p21 and Hs02621230_s1 for PTEN; Applied Biosystems) according to the manufacturer's instructions. The total RNA of normal organs was purchased from Takara Bio Inc., Shiga, Japan (Human Total RNA Master Panel II (Cat. No. 636643) and Human Liver Total RNA (Cat. No. 636531)) and BioChain Institute Inc., CA, USA (Human Oesophagus Total RNA (Cat. No. R1234106-50)). The results of gene expression were calculated as a ratio between PBK/TOPK and expression of an internal reference gene (Hs01060665_g1 for β -actin; Applied Biosystems) that provides a normalisation factor for the amount of RNA isolated from a specimen. This assay was performed in duplicate for each sample.

Western blotting. Anti-PBK/TPOK mouse polyclonal antibody (sc-293028) and anti-ACTB antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PDZ-binding kinase/T-LAK cell-originated protein kinase is an affinity purified mouse polyclonal antibody raised against a recombinant protein of PBK/TOPK. Anti-p53 (DO7) antibodies were from Novocastra Laboratories (Newcastle upon Tyne, UK), and anti-p53 (DO1) and anti-p21 were from Santa Cruz Biotechnology. Anti-PTEN, anti-AKT and anti-phospho-AKT (pAKT) were from Cell Signaling Technology (Cell Signaling, Massachusetts, USA). Cells were lysed and their proteins were extracted by M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, Waltham, MA, USA).

Loss of function by small interfering RNA and cell growth analysis. For loss-of-function analysis by knocking down endogenous gene expression, each of the small interfering RNAs (siRNAs) targeting PBK/TOPK (Stealth RNAi siRNA #HSS183331; Invitrogen Corporation, Carlsbad, CA, USA), p53 (Stealth RNAi siRNA #HSS186390; Invitrogen Corporation) a negative control (Stealth RNAi siRNA Cat. No.12935-112; Invitrogen Corporation) or Luciferase (Luc) 5'-CGUACGCGAAUACUUCGA-3' (Sigma, Tokyo, Japan) was transfected into cells (10 nmol l⁻¹) using Lipofectamine RNAiMAX (Invitrogen Corporation) according to the manufacturer's instructions. The knockdown of a target gene was confirmed by western blotting. For measurements of cell growth, the number of viable cells at various time points after transfection was assessed by the colorimetric water-soluble tetrazolium salt assay (Cell counting kit-8; Dojindo Laboratories, Kumamoto, Japan). Cell cycle phase was investigated 72 h after transfection by fluorescence-activated cell sorting (FACS), as described elsewhere (Komatsu *et al*, 2009).

Transwell migration and invasion assays. Transwell migration and invasion assays were carried out in 24-well-modified Boyden chambers (transwell-chamber, Becton Dickinson and Company, Franklin Lakes, NJ, USA). The upper surface of 6.4 mm diameter filters with 8- μ m pores was precoated with (invasion assay) or

without (migration assay) Matrigel (Becton Dickinson and Company). The siRNA transfectants (8×10^5 cells per well) were transferred into the upper chamber. Following 22 h of incubation, the migrated or invasive cells on the lower surface of the filters were fixed and stained with Diff-Quik stain (Sysmex, Kobe, Japan), and stained cell nuclei were counted directly in triplicate. We assessed the invasive potential according to the protocol of Corning BioCoat Matrigel Invasion Chamber (Catalogue No. 354480) by calculating the number of cells, which is the ratio of the percentage invasion through the Matrigel-coated filters relative to the migration through the uncoated filters of test cells over that in the control counterparts as described elsewhere (Kashimoto *et al*, 2012; Nishimura *et al*, 2013; Komatsu *et al*, 2015).

Immunohistochemistry. Primary tumour samples were fixed with 10% formaldehyde in phosphate buffered saline and routinely embedded in paraffin. The horseradish peroxidase (HRP) staining method was performed. In brief, after deparaffinization, antigen retrieval was performed by heating the samples in 10 mmol l^{-1} citrate buffer (pH 6.0) at 95°C for 1 h. Endogenous peroxidases were quenched by incubating the sections for 20 min in 3% H_2O_2 . After treatment with Block Ace (Dainippon Sumitomo Pharmaceutical, Osaka, Japan) for 30 min at room temperature, sections were incubated at room temperature for 1 h with an anti-PBK/TOPK (1:500) or at 4°C overnight with an anti-p53 (DO7, Novocastra Laboratories; 1:200). Phosphate buffered saline was used for all dilutions and washings. The bound primary antibody was detected with EnVision+ Horse Radish Peroxidase Systems (EnVision+ dual link System-HRP; Dako North America, Inc., Carpinteria, CA, USA). Horseradish peroxidase labelling was visualised using colour development with diaminobenzidine tetrahydrochloride. Slides were counterstained with Mayer's haematoxylin. A formalin-fixed GC cell line-overexpressing PBK/TOPK (HGC27 cells), in which $>50\%$ of cells showed staining of PBK/TOPK protein, was used as a positive control, whereas HGC27 cells incubated without the PBK/TOPK antibody were used as a negative control.

For scoring PBK/TOPK expression, the intensity (intensity score: 0 = negative, 1 = weak, 2 = moderate, 3 = strong) and percentage of the total cell population (proportion score: $0 < 10\%$, $10\% \leq 1 < 30\%$, $30\% \leq 2 < 60\%$, $60\% \leq 3 \leq 100\%$) that expressed PBK/TOPK was evaluated for each case. Expression of PBK/TOPK was graded as high expression (intensity plus proportion scores ≥ 5 of tumour cells showing immunopositivity) or low expression (intensity plus proportion scores ≤ 4 of tumour cells showing immunopositivity) using high-power ($\times 200$) microscopy (Tsuda, 2008).

For scoring p53 expression, the intensity (intensity score: 0 = negative, 1 = weak, 2 = moderate, 3 = strong) and percentage of the total cell population (proportion score: $0\% \leq 0 < 30\%$, $30\% \leq 1 \leq 100\%$) that expressed p53 was evaluated for each case. A distinct nuclear immunoreaction in both $3+$ and $\geq 30\%$ of the cancer cells was judged positive. The remaining cases were judged negative.

Statistical analysis. Clinicopathological categorical variables pertaining to the corresponding patients were analysed for significance by the χ^2 -test or Fisher's exact test. Differences of non-categorical variables between subgroups were tested with the non-parametric Mann-Whitney U test. For the analysis of survival, Kaplan-Meier survival curves were constructed for groups based on univariate predictors, and differences between the groups were tested with the log-rank test. Univariate and multivariate survival analyses were performed using the likelihood ratio test of the stratified Cox proportional hazards model. Differences were assessed with a two-sided test and considered significant at the $P < 0.05$ level.

RESULTS

Overexpression of PBK/TOPK in GC cell lines. Quantitative RT-PCR analysis was performed to test whether *PBK/TPOPK* is overexpressed in GC cell lines compared with the normal organs and the fibroblast cell line WI-38 (Figure 1A). *PBK/TPOPK* mRNA overexpression was observed in almost all GC cell lines (4 out of 5 lines, 80.0%) and testis compared with the other organs and the fibroblast cell line WI-38, suggesting this gene to be a cancer-testis antigen and a target for activation in GC. Expression of PBK/TOPK protein detected by the PBK/TOPK-specific antibody (4 out of 5 lines, 80.0%) correlated with that of PBK/TOPK mRNA in GC cell lines (Figure 1B). Because suppression of p53 activity through the DNA-binding domain (DBD) by PBK/TOPK was reported previously (Hu *et al*, 2010), we used representative osteosarcoma cell lines (SaOS2; *TP53*-null, U2OS; wild-type *TP53*) and confirmed the expression of p53-DO7 associated with the status of *TP53* mutation in the GC cell lines, including these osteosarcoma cell lines by western blotting. These statuses of *TP53* mutation in various cell lines are positively associated with their reported status of *TP53* mutation in the database (<http://p53.free.fr/index.html>; W: wild-type *TP53*, M: mutant *TP53*). Although wild-type *TP53* may be expected to be a more suitable substrate than mutant *TP53* for PBK/TOPK, expression of PBK/TOPK mRNA and protein was not correlated with the mutation status and expression of p53 (Figure 1B).

Immunohistochemical analysis of PBK/TOPK expression in primary tumours of GC. Because the PBK/TOPK protein was overexpressed in almost all GC cell lines, it was hypothesised that PBK/TOPK was also highly expressed in GC tissues and would be associated with carcinogenesis and malignant outcomes. We examined the prognostic and clinicopathological significance of PBK/TOPK expression in primary tumour samples of GC based on the immunohistochemical staining pattern of this protein. PDZ-binding kinase/T-LAK cell-originated protein kinase protein was observed in both the cytoplasm and nucleus of cancer cells (Figure 1C). We classified 144 GC samples into positive and negative groups according to the intensity and proportion of PBK/TOPK staining among tumour cells, as described in Materials and Methods. In primary cases, PBK/TOPK protein expression was negative in the non-tumorous gastric mucosal cell population (Figure 1C), and it was weakly detected in the lymphoid follicles. Supplementary Table 1 shows the distribution of patients based on the intensity and proportion scores of PBK/TOPK immunoreactivity of tumour samples. Kaplan-Meier survival estimates showed that PBK/TOPK immunoreactivity in tumour cells was associated with a worse cause-specific survival according to the extent in each intensity and proportion scoring (Supplementary Figure S1A and B).

In the total scores of intensity plus proportion (Supplementary Table 1), the high-expression group of PBK/TOPK presenting scores ≥ 5 tumour cells showing immunopositivity presented significantly poorer prognosis than the low-expression group ($P = 0.0009$, log-rank test; Figure 1D). Five-year survival rates of patients with PBK/TOPK high- and low-expression cancer in each stage were 98.4% vs 100% ($P = 0.7077$) in stage I, 9.2% vs 34.7% ($P = 0.0013$) in stage II + III, respectively.

Association between PBK/TOPK protein abundance and clinicopathological characteristics in primary cases of GC. The relationship between the expression of the PBK/TOPK protein and clinicopathological characteristics is summarised in Table 1. Protein expression of PBK/TOPK was significantly associated with venous invasion, depth of invasion and higher recurrence rate, and tended to be associated with pStage in the TNM classification, whereas other characteristics, including histological grade were not.

Figure 1. Expression of PBK/TOPK was correlated with poor prognosis in gastric cancer patients. **(A)** Expression of PBK/TOPK mRNA in five GC cell lines compared with that of the normal organs and the fibroblast cell line WI-38. **(B)** Expression of PBK/TOPK mRNA and protein in five GC cell lines and osteosarcoma cell lines, such as TP53-null SaOS2 and TP53 wild-type U2OS, compared with that in the fibroblast cell line WI-38. The status of TP53 mutation in each cancer cell line was evaluated by western blotting. The status of TP53 mutation was positively associated with the reported status of TP53 mutation in the database (<http://p53.free.fr/index.html>, W: wild-type TP53, M: mutant TP53). **(C)** Specific immunostaining of PBK/TOPK in a representative primary tumour sample. On the basis of this result, the intensity scores for PBK/TOPK staining were determined as follows: 0 = negative, 1 = weak, 2 = moderate, 3 = strong. Magnification: $\times 200$; Scale bar, 20 μm . **(D)** Cause-specific survival rates of GC patients (as determined by Kaplan–Meier plots) depending on the combination scores of intensity and proportion in PBK/TOPK expression.

In the Cox proportional hazard regression model (Table 2), univariate analyses demonstrated that PBK/TOPK protein expression, age, location, macroscopic appearance, tumour size, venous invasion, lymphatic invasion, pT category and pN category were significantly correlated with cause-specific survival. When data were stratified for multivariate analysis using both the forward and backward stepwise Cox regression procedures, PBK/TOPK immunoreactivity in tumour cells remained significant at $P < 0.0001$ (hazard ratio, 6.40 (2.71–14.49)) for cause-specific survival in all patients, suggesting that immunoreactivity may be an independent predictor of cause-specific survival.

Suppression of cell proliferation by knockdown of PBK/TOPK and the knockdown effect according to TP53 mutation status.

To gain an insight into the potential role of PBK/TOPK as an oncogene whose overexpression could be associated with gastric tumorigenesis, we first performed a cell proliferation assay using several siRNAs specific to PBK/TOPK to investigate whether knockdown of PBK/TOPK would suppress proliferation of GC cells that overexpress PBK/TOPK. In all TP53 wild-type cell lines, such as NUGC4 (Figure 2A), MKN45 (Supplementary Figure S2A) and U2OS (Supplementary Figure S2B), expression of the PBK/TOPK protein was more efficiently knocked down 24–72 h after the transient introduction of a PBK/TOPK-specific siRNA (siRNA-PBK/TOPK) than with the control siRNA (siRNA-Control;

Figure 2A). The proliferation of all these cell lines was significantly lower than with controls after the knockdown of endogenous PBK/TOPK expression. Whereas in all TP53 mutant cell lines, such as HGC27 (Figure 2B) and SaOS2 (Supplementary Figure S2C), the suppression of the cell proliferation was not detected despite the complete knockdown of the PBK/TOPK protein.

Cell cycle analysis by downregulation of PBK/TOPK expression using FACS.

Fluorescence-activated cell sorting analysis demonstrated that transfection of TP53 wild-type NUGC4 cells with siRNA-PBK/TOPK resulted in an accumulation of cells in the G0–G1 phase compared with transfection with control siRNA (Figure 2A). In TP53 mutant HGC27 cells, however, there was no difference of FACS results between siRNA-PBK/TOPK and control siRNA cells (Figure 2B). Specifically, in NUGC4 cells, p21 and PTEN level was increased, and pAKT was decreased at 72 h protein level in siRNA-PBK/TOPK transfected cells (Figure 2A). However, in TP53 mutant HGC27 cells, p21 level was not changed, but PTEN level was increased and pAKT was decreased at 72 h in siRNA-PBK/TOPK transfected cells (Figure 2B). These findings suggested that PBK/TOPK might be related to the cell proliferation in a TP53 mutation-dependent manner.

Suppression of cell migration and invasion by downregulation of PBK/TOPK expression and the knockdown effect according to TP53 mutation status. Next, transwell migration and invasion

Table 1. Association between clinicopathological characteristics and PBK/TOPK expression				
	PBK/TOPK immunoreactivity			P-value ^a
	n	Positive (%)	Negative (%)	
Total	144	24	120	
Gender				
Male	97	15 (62%)	82 (68%)	0.5995
Female	47	9 (38%)	38 (32%)	
Age (years)				
< 60	55	9 (38%)	46 (38%)	0.9388
≥ 60	89	15 (63%)	74 (62%)	
Location				
Upper	19	4 (17%)	15 (13%)	0.5740
Middle	74	10 (42%)	64 (44%)	
Lower	51	10 (42%)	41 (34%)	
Histological grade				
Differentiated	69	15 (62%)	54 (45%)	0.1162
Undifferentiated	75	9 (38%)	66 (55%)	
Macroscopic appearance				
Type 0	80	13 (54%)	69 (57%)	0.2952
Type 1/2/3/4	64	11 (46%)	51 (43%)	
Tumour size (mm)				
< 60	103	16 (67%)	87 (73%)	0.5680
≥ 60	41	8 (33%)	33 (27%)	
Venous invasion				
Negative	102	10 (42%)	92 (77%)	0.0010
Positive	42	14 (58%)	28 (23%)	
Lymphatic invasion				
Negative	77	11 (46%)	66 (55%)	0.4117
Positive	67	13 (54%)	54 (45%)	
TNM classification				
pT categories				0.0062
T1	80	11 (45%)	69 (58%)	
T2	12	0 (0%)	12 (10%)	
T3	22	9 (38%)	13 (11%)	
T4	30	4 (17%)	26 (21%)	
pN categories				0.4481
N0	93	13 (54%)	80 (67%)	
N1	18	5 (21%)	13 (11%)	
N2	10	1 (4%)	9 (8%)	
N3	23	5 (21%)	18 (15%)	
pStage				0.0792
I	87	11 (46%)	76 (63%)	
II	16	6 (25%)	10 (8%)	
III	41	7 (29%)	34 (28%)	
Rucurrence				
Absent	112	14 (58%)	98 (82%)	0.0178
Present	32	10 (42%)	22 (18%)	
p53 (DO7)				
Positive	38	10 (42%)	27 (23%)	0.1047
Negative	106	14 (58%)	82 (77%)	
Abbreviations: PBK/TOPK = PDZ-binding kinase/T-LAK cell-originated protein kinase; TNM = tumour-lymph node-metastases. NOTE: statistically significant values are in bold type.				
^a P values are from χ^2 or Fisher's exact test and were statistically significant at <0.05.				

assays were performed to examine the ability of *TP53* wild-type NUGC4 and *TP53* mutant HGC27 cells transfected with siRNA-PBK/TOPK to move through pores under different conditions. Uncoated membrane was used for the migration assays, whereas Matrigel-coated membrane was used for invasion assays. In Figure 3, the number of both NUGC4 (Figure 3A) and HGC27 (Figure 3B) cells that migrated into the lower chamber was significantly lower for siRNA-PBK/TOPK-transfected cells than for

Table 2. Cox proportional hazard regression analysis for overall survival				
Factor	Univariate ^a		Multivariate ^b	
	P-value ^c	HR	95%CI	P-value ^c
Gender				
Male vs female	0.6793		—	
Age (years)				
≥ 60 vs < 60	0.0294		—	
Location				
Upper vs middle/lower	0.0296	2.776	1.125–6.27	0.0282
Histological grade				
Undifferentiated vs differentiated	0.8722		—	
Macroscopic appearance				
Type 3/4/5 vs type 0/1/2	<0.0001			
Tumour size (mm)				
≥ 60 vs < 60	<0.0001	2.842	1.336–6.44	0.0063
Venous invasion				
Positive vs negative	<0.0001		—	
Lymphatic invasion				
Positive vs negative	<0.0001		—	
pT-stage				
pT 2/3/4 vs pT 1	<0.0001	14.48	1.611–310.70	0.0164
pN-stage				
pN 1/2/3 vs pN 0	<0.0001	4.14	1.242–23.31	0.0174
PBK/TOPK expression ^d				
High vs low	0.0009	6.403	2.712–14.49	<0.0001
p53 (DO7)				
Positive vs negative	0.5273			
Abbreviations: CI = confidence interval; HR = hazard ratio. Statistically significant values are in boldface type.				
^a Kaplan and Meier method, and the statistical significance was determined by log-rank test.				
^b Multivariate survival analysis was performed using Cox's proportional hazard model.				
^c P-values were from two-sided tests and were statistically significant at <0.05.				
^d PBK/TOPK expression was evaluated by immunohistochemical analysis as described in Materials and Methods.				

siRNA-control-transfected cells under both conditions, suggesting that PBK/TOPK may increase the ability of GC cells to migrate and invade in a *TP53* mutation-independent manner.

Relationship between protein expression of PBK/TOPK and p53 in primary cases of GC. A previous report indicated that PBK/TOPK physically interacts with the tumour suppressor p53 through its DBD (Hu *et al*, 2010). Therefore, we examined the relationship between the protein expression of PBK/TOPK and p53 in primary tumour cells (Figure 4A). Because a good correlation is known to exist between p53-DO7 protein accumulation and the presence of *TP53* gene mutations, especially missense mutations (Bennett *et al*, 1991; Wagata *et al*, 1993), we used immunohistochemistry to evaluate *TP53* mutation status. As a result, of 144 cases of GC, tumours showing positive p53-DO7 immunoreactivity were detected in 38 cases (26.4%, Table 1). Expression of p53 was not observed in non-tumorous gastric mucosal cells. No significant correlations were found between PBK/TOPK and p53-DO7 immunohistochemical staining patterns ($P=0.1407$; Table 1). Kaplan–Meier survival estimates showed that the expression of p53-DO7 was not associated with cause-specific survival ($P=0.5273$, log-rank test; Figure 4B). However, in both p53-DO7-negative and p53-DO7-positive groups, the cause-specific survival rate was significantly poorer in patients with PBK/TOPK expression than in those without ($P=0.0228$, log-rank test in four groups; Figure 4C). Five-year survival rates of PBK/TOPK high expression vs low expression were: 46.2% vs 81.3% in p53-DO7-

Figure 2. Effects of PBK/TOPK knockdown by siRNA-PBK/TOPK compared with those of control siRNA in NUGC4 and HGC27 cell lines.

(A) shows that the knockdown of PBK/TOPK overexpression in *TP53* wild-type NUGC4 cells downregulated expression of pAKT and upregulated that of p21, which resulted mainly in G0–G1 arrest. The proliferation of NUGC4 cell was significantly lower than with controls after the knockdown of endogenous PBK/TOPK expression. However, (B) shows that there was no difference of cell proliferation and FACS results between siRNA-PBK/TOPK and control siRNA cells in *TP53* mutant HGC27 cells. The p21 level was not changed, but pAKT level was decreased and PTEN level was increased at 72 h in siRNA-PBK/TOPK-transfected cells. Including the results of Supplementary Figure S2, these results suggested that PBK/TOPK might be related to the cell proliferation in a *TP53* mutation-dependent manner.

negative patients and 58.3% vs 72.1% in p53-DO7-positive patients. Notably, a strong significant difference of the cause-specific survival was found between patients positive and negative for PBK/TOPK in the p53-DO7-negative group ($P = 0.0027$, log-rank test). However, no significant difference was found between patients positive and negative for PBK/TOPK in the p53-DO7-positive group ($P = 0.4519$, log-rank test), although the PBK/TOPK positive group presented poorer prognosis than the negative group.

DISCUSSION

The PBK/TOPK gene is upregulated in various types of cancer and tumours such as bladder cancer, brain tumour, breast cancer, liver cancer, lung cancer and sarcoma (Park *et al*, 2006; Herrero-Martin *et al*, 2009; He *et al*, 2010; Shih *et al*, 2012; O Leary *et al*, 2013; Singh *et al*, 2014; Joel *et al*, 2015). PDZ-binding kinase/T-LAK cell-originated protein kinase was initially identified as a mitotic protein kinase (Gaudet *et al*, 2000), assisting the recruitment of cdk1/cyclin B to mitotic spindles, where PBK/TOPK plays a role in the formation of the spindle midzone and cytokinesis (Matsumoto *et al*, 2004). Previous studies have identified various oncogenic functions of PBK/TOPK related to multiple signalling pathways,

such as the mitogen-activated protein kinase (MAPK) pathway (Ayllon and O'Connor, 2007), PI3K/AKT pathway (Abe *et al*, 2000; Zhu *et al*, 2007; Shih *et al*, 2012) and p53 pathway (Nandi *et al*, 2007; Hu *et al*, 2010). Through the MAPK pathway, PBK/TOPK functions as mediator of growth factor activation of p38 with a role in motility and as part of the DNA damage-sensing machinery and is necessary for the phosphorylation of histone H2AX (Ayllon and O'Connor, 2007). Regarding the PI3K/AKT pathway, PBK/TOPK decreases the PTEN level and regulates PI3K/AKT-stimulated migration (Shih *et al*, 2012). Most importantly, by impairing the DNA damage-induced apoptotic pathway, p53 pathway, PBK/TOPK prevents cancer cell death (Nandi *et al*, 2007). PDZ-binding kinase/T-LAK cell-originated protein kinase physically interacts with p53, specifically through its DBD and promotes tumour cell survival and resistance to chemotherapy-induced apoptosis through suppression of p21 (Hu *et al*, 2010). Thus, PBK/TOPK has promising oncogenic functions, and these findings prompted us to clarify the clinicopathological and independent prognostic significance of PBK/TOPK overexpression/activation through molecular mechanisms associated with the p53 and PI3K/AKT pathways in primary GC.

Here we hypothesised that overexpression/activation of PBK/TOPK may promote tumour cell proliferation and/or poor survival

Figure 3. Regarding the cell migration and invasion, the results of the increase of PTEN and/or the decrease of pAKT in siRNA-PBK/TOPK-transfected cells suggested that PBK/TOPK may increase the ability of GC cells to migrate and invade via the PI3K/Akt pathway activation in a *TP53* mutation-independent manner. (A) NUGC wild-type *TP53* and (B) mutant-type *TP53*.

of GC patients. To verify this hypothesis, we firstly examined the expression status of PBK/TOPK in cell lines and primary tumours of GC, and its clinicopathological and prognostic significance were evaluated. As a result, we demonstrated that PBK/TOPK was overexpressed in 16.7% (24 out of 144) of primary GCs, as well as in 80.0% (4 out of 5) of GC cell lines, and this overexpression was an indicator of poor prognosis independent of other prognostic factors in both an intensity and proportion-dependent manner (Supplementary Figure S1A and B). In combination with p53, there was no relationship between the expression levels of PBK/TOPK and p53 in primary tumours and cell lines, although one previous report suggested these positive correlations in lung cancer (Lei *et al*, 2015). These results suggest that immunoreactivity to PBK/TOPK may be useful as an independent prognosticator in patients with GC.

In our *in vitro* analyses, knockdown of PBK/TOPK overexpression in wild-type *TP53* cells induced cell cycle G0–G1 arrest, but knockdown of PBK/TOPK overexpression in mutant *TP53* cells did not (Figure 2; Supplementary Figure S2). This suggests that, in tumours with wild-type *TP53*, p53 may be an important substrate for PBK/TOPK. Indeed, particular in the p53-DO7-negative group, patients with tumours positive for PBK/TOPK expression showed significantly worse survival than those with tumours lacking PBK/TOPK expression (Figure 4C), suggesting that a combination of p53 and PBK/TOPK immunoreactivity might be a more useful predictor for survival of patients with GC, because p53 and its target molecules, which regulate the cell cycle and trigger apoptosis after DNA damage, play a key role in a wide range of human cancers, including GC (Caelles *et al*, 1994; Fenoglio-Preiser *et al*, 2003; Bellini *et al*, 2012).

Next, we investigated further the molecular mechanism affecting the malignant potential in tumour cells with both overexpression

of PBK/TOPK and mutant *TP53*. As a result, knockdown of PBK/TOPK suppressed invasion and migration through PTEN upregulation in *TP53* mutant GC cells, as well as wild-type *TP53* cells (Figure 3A and B). Indeed, as shown in our immunohistochemical analysis, overexpression of PBK/TOPK is related to poor survival even in patients with putative mutant *TP53* GC. Thus, *in vitro* findings strongly supported our *in vivo* results of immunohistochemical analysis and strongly suggested that PBK/TOPK plays a pivotal role in the malignant potential of GC. We presented our hypothetical model of the overexpression/activation of PBK/TOPK in GC (Figure 4D).

Recently, PBK/TOPK-specific inhibitors such as OTS514, OTS964 (OncoTherapy Science Inc., Japan; Matsuo *et al*, 2014; Ikeda *et al*, 2016) and HI-TOPK-032 (Kim *et al*, 2012; Joel *et al*, 2015) have been developed. Particularly, the PBK/TOPK-specific inhibitor OTS964 was proved to be extremely effective in treating lung cancer in a xenograft mouse model without showing any adverse reactions (Matsuo *et al*, 2014). Regarding p53 status, Kim *et al* (2012) reported that the administration of PBK/TOPK-specific inhibitor, HI-TOPK-032 suppressed tumour growth in *TP53* wild-type HCT116 colon cancer xenograft model. Whereas, Ikeda *et al* (2016) more recently reported that oral administration of PBK/TOPK-specific inhibitor, OTS514 significantly elongated overall survival in *TP53* mutant-type ES-2 abdominal dissemination xenograft model, compared with vehicle control in ovarian cancer. These data indicated that the molecular targeting to PBK/TOPK may work for tumours of both *TP53* wild type and mutant type in various cancers. Therefore, the PBK/TOPK-specific inhibitors may be a key molecule for the treatment of patients with GC-developed PBK/TOPK overexpression. These inhibitors are currently being evaluated *in vitro* and *in vivo* at our institute.

Figure 4. Relationship between protein expression of PBK/TOPK and p53 in primary cases of gastric cancer. **(A)** Specific immunostaining of PBK/TOPK in a representative primary tumour sample. On the basis of this result, the intensity scores for p53 (DO7) staining were determined as follows: 0 = negative, 1 = weak, 2 = moderate, 3 = strong. Magnification: $\times 40$; Scale bar, 500 μm . **(B)** Kaplan–Meier curves for cause-specific survival rates of patients at all stages according to the expression of p53. There was no difference of survival between the patients positive and negative for p53-DO7. **(C)** Postoperative overall survival curves according to a combination of the expression of PBK/TOPK and p53. A significant difference in survival was observed among the patients positive (+) or negative (–) for p53-DO7 and PBK/TOPK ($P = 0.0228$, log-rank test). Notably, in the p53-DO7-negative group, the patients with PBK/TOPK expression had a markedly worse outcome than those with no PBK/TOPK expression. **(D)** Hypothetical model of the overexpression/activation of PBK/TOPK in GC cells.

In conclusion, this is the first report to show that PBK/TOPK has a pivotal oncogenic role through molecular mechanisms associated with the p53 and PI3K/AKT pathways and is a potential therapeutic target in GC. We clearly demonstrated the frequent overexpression of PBK/TOPK protein and its prognostic value in patients with GC. Although studies of larger cohorts are needed to validate these findings before moving to clinical settings, our results provide evidence that PBK/TOPK could be a crucial molecular marker to determine the malignant properties of GC cells and that it could be a target for molecular therapy in patients with GC.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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