

Platelet isoform of phosphofructokinase accelerates malignant features in breast cancer

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Abstract. The platelet isoform of phosphofructokinase (PFKP) is one of the key enzymes in the glycolytic pathway. *PFKP* is highly expressed in several cancers, and it has been reported to be involved in the progression of cancer cells. However, its oncological role in breast cancer (BC) remains unclear. The present study aimed to evaluate the function of PFKP in BC cells and its expression level in patients with BC. Firstly, the mRNA and protein expression of PFKP was evaluated in BC and non-cancerous mammary cell lines. Polymerase chain reaction (PCR) array analysis was conducted to evaluate the correlation between *PFKP* and 84 cancer-related genes. Then, *PFKP* knockdown was conducted using small interfering RNA, and cell proliferation, invasiveness and migration were analyzed. Furthermore, the association between *PFKP* mRNA expression and clinicopathological factors was investigated in 167 patients with BC. *PFKP* was highly expressed in estrogen receptor-negative and human epidermal growth factor receptor 2-negative BC cell lines. PCR array analysis demonstrated that the expression level of *PFKP* was significantly correlated with that of transforming growth factor- β 1 and MYC proto-oncogene. *PFKP* knockdown significantly decreased the proliferation and invasiveness of MCF7, SK-BR-3, and MDA-MB-231 cells. Furthermore, cell migration was inhibited in SK-BR-3 and MDA-MB-231 cells. In the clinical specimens, patients with T2/T3/T4, lymph node metastasis, or stage II/III/IV exhibited higher expression of *PFKP* mRNA than patients with less severe disease. In conclusion, the present findings indicated that PFKP is involved in promoting tumor-progressive oncological roles in BC cells

across different subtypes and is considered a possible novel therapeutic target for BC.

Introduction

Breast cancer (BC) is the most common malignant tumor among women throughout the world (1). The development of adjuvant therapy has improved the prognosis of patients with BC. Indeed, the 5-year overall survival (OS) rate of BC patients without metastasis currently exceeds 80% (2). However, 20-30% of patients with BC develop metastases after primary tumor treatment (3). Patients with recurrent BC are classified according to the immunohistochemical detection of conventional target molecules such as the estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2). Although various drugs have been developed and are available for the treatment of patients with recurrent BC, they are still insufficient to cure and only 5% of those patients achieve long-term disease control (4). From this point of view, development of new biomarkers or therapeutic target molecules for the purpose of improving the prognosis of BC patients is required.

Phosphofructokinase (PFK), which catalyzes the formation of fructose 1,6-bisphosphate and adenosine diphosphate from fructose 6-phosphate and adenosine triphosphate, is one of the key regulating enzymes in the glycolytic pathway (5). PFK is a complex tetrameric enzyme that has three isoforms: Liver (PFKL), muscle (PFKM), and platelet (PFKP) (6). The activity of PFK is regulated by quantitative and isozymic changes secondary to altered gene expression during neoplastic transformation (7). Among the three isoforms, the expression and regulatory mechanisms of *PFKP* have been studied in several malignancies, including brain tumor, renal and bladder cancer, in which the increased expression of *PFKP* has been associated with the progression of cancer cells (8-10).

In BC cells, hypoxia inducible factor 1 subunit α , a major transcriptional regulator of the cellular response to hypoxia, and kruppel-like factor 4, a transcription factor that regulates the expression of several genes involved in cell cycle regulation and differentiation, activated the transcription of *PFKP* and enhanced glycolytic metabolism (7,11). Furthermore, in a triple-negative BC cell line, PFKP regulated extracellular

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lactate production via lactate dehydrogenase A enzyme (12). However, it has not been evaluated whether PFKP promotes malignant features of BC across subtypes. The present study aimed to investigate the functional roles of PFKP in BC cells and the significance of *PFKP* expression in patients with BC.

Materials and methods

Sample collection. A total of 13 BC cell lines (BT-20, BT-474, BT-549, HCC1419, HCC1954, Hs578T, MCF7, MDA-MB-231, MDA-MB-361, MDA-MB-415, MDA-MB-468, SK-BR-3, and ZR-75-1) and two non-cancerous breast epithelial cell lines (MCF-10A and MCF-12A) were obtained. BT-549, HCC1419, HCC1954 and Hs578T cell lines were purchased from the Japanese Collection of Research Bioresources Cell Bank. BT-474, MCF7, and MCF-12A were kindly provided by Professor David Sidransky from Johns Hopkins University (Baltimore, USA). The other cell lines were all purchased from the American Type Culture Collection. All cell lines were stored at -80°C using a cell preservation solution (CELLBANKER®; Mitsubishi Chemical Medicine Corporation) and cultured in RPMI-1640 medium (Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal bovine serum (FBS; Corning, Inc.) and incubated in an atmosphere of 5% carbon dioxide at 37°C (13).

Primary BC and non-cancerous specimens were also collected from 167 patients histologically diagnosed with BC after undergoing surgery at Nagoya University Hospital (Nagoya, Japan) from March 2002 to May 2007. Surveillance data for more than five years after surgery for all 167 patients were available. All specimens were immediately resected to a diameter of approximately 1.5 mm and stored at -80°C. Non-cancerous specimens were resected ≥ 3 cm from the edge of the tumor (14). All specimens were histologically diagnosed as BC and classified using the Union for International Cancer Control (UICC) staging system (8th edition). Postoperative adjuvant therapy was determined on the basis of the condition of the patient, pathological features, cancer subtype, and discretion of physicians (14).

A total of 167 female patients were enrolled in the present study; there were no male participants. The median age was 52 years (range, 26-78 years). The median follow-up duration was 100 months (range, 8-155 months), including fatalities. The tumor (T) categories were Tis (ductal carcinoma *in situ*), 7; T1, 70; T2, 75; T3, 9; and T4, 6. A total of 82 patients (49%) had lymph node metastases. The UICC stages were as follows: Stage 0, 7; stage I, 47; stage II, 78; stage III, 34; and stage IV, 1. Among the 167 patients, 127 (76%) were ER-positive and 40 (24%) were ER-negative. There were 115 (69%) PgR-positive and 52 (31%) PgR-negative patients. A total of 39 patients (23%) were HER2-positive and 119 patients were (71%) HER2-negative. A total of 9 patients had unknown HER2 status. Of the 167 patients, 12 patients received neoadjuvant chemotherapy, such as anthracycline and taxane. Tumor response to neoadjuvant chemotherapy was assessed with the use of Response Evaluation Criteria in Solid Tumors, version 1.1 (15); partial response occurred in 8 patients and stable disease occurred in 4 patients. None of the patients had a pathological complete response.

The present study was conducted in accordance with the principles of the Declaration of Helsinki and was approved

(approval no. 2019-0028) by the Institutional Review Board and Ethics Committee of Nagoya University Hospital. All patients provided written informed consent for the use of their clinical samples and data.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). *PFKP* mRNA expression levels were evaluated by RT-qPCR. RNA was extracted from cell lines (8×10^6 cells per cell line) using RNeasy Mini Kit (Qiagen GmbH), as well as from BC and non-cancerous specimens from 167 patients. cDNA was synthesized as previously described (13). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were quantified to normalize expression levels. The primers specific for each gene were as follows: *PFKP* forward, 5'-GGGCCAAGGTGTACTTCATC-3' and reverse, 5'-TGGAGACACTCTCCCAGTCG-3' (which generated a 90-bp product); GAPDH forward, 5'-GAAGGTGAAGGT CGGAGTC-3' and reverse, 5'-GAAGATGGTGATGGGATT TC-3' (which generated a 226-bp product) (14). RT-qPCR was performed using an ABI StepOnePlus real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) as previously described (13). Each cell line sample was examined three times. The mRNA relative expression level of *PFKP* was obtained by dividing the value of each sample by the corresponding GAPDH value (13).

PCR array analysis. To determine the correlation between the expression levels of *PFKP* and 84 cancer-related genes in BC cell lines, PCR array analysis was conducted using RT² Profiler PCR Array Human Oncogenes and Tumor Suppressor Genes (cat. no. 330231; GeneGlobe ID PAHS-502Z; Qiagen GmbH) according to the manufacturer's protocol. The relative expression levels of these genes in each sample were obtained by dividing them by the corresponding GAPDH value.

PFKP knockdown using PFKP-specific small interfering RNAs (siRNAs). For *PFKP* knockdown, MCF7, SK-BR-3, and MDA-MB-231 cell lines were transfected with three types of siRNAs specific for *PFKP*, named 'si*PFKP*'. Their sequences were as follows: si*PFKP*-1, 5'-UAU UAAUGUCAUAUACGUG-3'; si*PFKP*-2, 5'-GGAGCAAUUGAUACCCAAATT-3'; and si*PFKP*-3, 5'-GGAUCACUGCAAAACUCAATT-3' (Hokkaido System Science Co., Ltd.). AccuTarget™ Fluorescein-labeled Negative Control siRNA (cat. no. SN-1023; Cosmo Bio Co., Ltd.) served as the control nontargeting siRNA, named 'siControl'. BC cells were seeded in antibiotic-free RPMI-1640 medium supplemented with 10% FBS; a total of 24 h after seeding, cells were transfected with the corresponding siRNAs (80 pmol for 6-well plates and 400 pmol for 10-cm dishes) in the presence LipoTrust EX Oligo (Hokkaido System Science Co., Ltd.). After transfection, cells were cultured in antibiotic-free RPMI-1640 medium with 10% FBS in an atmosphere of 5% carbon dioxide at 37°C for 72 h. Knockdown efficiency was determined using RT-qPCR.

Western blot analysis. Western blotting was performed by the Simple Western technique using the WES instrument (ProteinSimple), according to the manufacturer's protocol. Cells were incubated in RIPA lysis buffer, and the lysates

were stored at -30°C . Protein concentration was assessed using a BCA protein assay kit (Thermo Fisher Scientific, Inc.). Using a 12-230 kDa Separation module (cat. no. SM-W004; ProteinSimple), protein samples ($6\text{ }\mu\text{g}/\text{lane}$), biotin ladder, primary antibody, secondary antibody, blocking reagent, chemiluminescent substrate, and wash buffer were prepared and dispensed into the assay plate. Antibody Diluent II (cat. no. 042-203; ProteinSimple) attached to the Detection module was used as a blocking reagent. Then, the assay plate was loaded into the instrument, and the protein was separated into individual capillaries. Using the instrument, the protein was anchored to the inner wall of the capillary. Protein separation and detection was performed automatically on individual capillaries. The duration of incubation of the primary and secondary antibodies was 30 min at room temperature. Detection was performed by chemiluminescence with luminol (cat. no. 043-311) and peroxide (cat. no. 043-379; both from ProteinSimple) attached to the Detection module. Anti-PFKP antibody (1:50; product no. 12746; Cell Signaling Technology, Inc.) and anti- β -actin antibody (1:50; cat. no. ab6276; Abcam) were used as primary antibodies. Streptavidin HRP (cat. no. 042-414) and anti-mouse or anti-rabbit secondary antibodies (anti-mouse, cat. no. 042-205; and anti-rabbit, cat. no. 042-206) (all from ProteinSimple) were selected according to the corresponding primary antibody (16,17).

Proliferation assay. Cell proliferation was evaluated using the Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc.). MCF7 (3×10^3 cells/well), SK-BR-3 (3×10^3 cells/well), and MDA-MB-231 (3×10^3 cells/well) cells, which were transfected with siPFKP or siControl, were seeded into 96-well plates with RPMI-1640 medium containing 10% FBS and 1% antibiotic [Antibiotic-Antimycotic (100X); Thermo Fisher Scientific, Inc.]. Each sample was applied to six wells, and the optical density (450 nm) of each well was measured 2 h after adding 10 μl of CCK-8 solution up to 5 days after seeding (14).

Invasiveness assay. Invasiveness in Matrigel was determined using BioCoat Matrigel Invasion Chambers (pore size $8\text{-}\mu\text{m}$; Corning Inc.) according to the manufacturer's protocol. Each chamber was precoated with 500 μl of serum-free RPMI-1640 medium in an atmosphere of 5% carbon dioxide at 37°C for 2 h. After transfection, MCF7 (2.5×10^4 cells/well), SK-BR-3 (2.5×10^4 cells/well), and MDA-MB-231 (2.5×10^4 cells/well) cells were suspended in 750 μl of serum-free RPMI-1640 medium and seeded in the upper chambers. The lower chamber was filled with 750 μl of RPMI-1640 medium containing 10% FBS and 1% antibiotics. After 72 h of incubation in an atmosphere of 5% carbon dioxide at 37°C , cells were fixed with 99% methanol for 5 sec at room temperature, and stained with Solution I and II in Diff Quik (cat. no. 16920; Sysmex) for 5 sec at room temperature. Cells on the membrane were counted in ten randomly selected microscopic fields with a magnification of $\times 100$ using an upright microscope (Olympus Corporation) (14).

Migration assay. Migration of MCF7, SK-BR-3, and MDA-MB-231 cells was determined using a gap closure assay. After transfection, MCF7 (4.9×10^4 cells/well), SK-BR-3 (4.9×10^4 cells/well), and MDA-MB-231 (4.9×10^4 cells/well) cells were seeded into each well of a 35-mm dish with a culture

insert with a cell-free gap width of $500\text{-}\mu\text{m}$ (Ibidi GmbH) using RPMI-1640 medium containing 10% FBS and 1% antibiotics. Because siPFKP-transfected cells did not proliferate sufficiently under serum starvation, cells were cultured in 10% FBS. After 24 h, in the state of 100% confluence, the insert was removed and images of the wound were captured after 0, 4, 8, 12, 18, 24, 36 and 48 h. Wound widths at each time-point were assessed 20 times/well at $100\text{-}\mu\text{m}$ intervals using an inverted microscope at a magnification of $\times 40$ magnification (Olympus Corporation) (14).

Kaplan-Meier survival analysis using Kaplan-Meier Plotter. The website of the Kaplan-Meier Plotter (<http://kmplot.com/analysis/index.php?p=background>) was used to analyze relapse-free survival (RFS) and OS for patients with BC with respect to the expression of PFKP by classifying its expression levels into the upper quartile or to other quartiles (18).

Statistical analysis. Numeric variables between two groups were compared using the Mann-Whitney test. Comparisons between multiple groups were performed using ANOVA followed by Tukey's post hoc test and the Kruskal-Wallis test for parametrical and non-parametrical continuous variables, respectively. All values obtained from each cell line were used to compare the PFKP expression levels between the two groups. Spearman's rank correlation test was performed to evaluate the correlation between PFKP and cancer-related gene expression levels in the PCR array analysis. Data are presented in each graph as the mean \pm standard error of the mean (SEM). The ratio of PFKP mRNA expression levels between cancerous and non-cancerous specimens was presented as mean \pm standard deviation (SD). The association between PFKP mRNA expression and clinicopathological factors were analyzed using the χ^2 test. Disease-free survival (DFS) and OS were calculated using the Kaplan-Meier method, and survival curves were compared using the log-rank test. Although RT-qPCR was conducted three times, proliferation, invasion, and migration assays were performed once. All statistical analyses were performed using JMP 15 software (SAS Institute, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

PFKP mRNA expression levels in BC and non-cancerous cell lines and its association with cancer-related genes in BC cell lines. PFKP mRNA expression levels in 13 BC cell lines and two non-cancerous cell lines are demonstrated in Fig. 1A. ER, PgR, and HER2 statuses of the cell lines have been evaluated in previous studies (19,20). PFKP mRNA expression levels in ER-negative cell lines were significantly higher than those in ER-positive BC cells ($P=0.003$). In addition, PFKP in triple-negative cell lines revealed higher mRNA expression levels than that in the other cell lines ($P=0.038$). Subsequent PCR array analysis revealed that PFKP mRNA expression levels were positively correlated with those of several well-known oncogenes, such as transforming growth factor- $\beta 1$ (*TGFBI*) (correlation coefficient 0.758, $P=0.003$) and MYC proto-oncogene (*MYC*) (correlation coefficient 0.648, $P=0.017$) (Fig. 1B). The correlation between PFKP mRNA expression levels and those of 84 cancer-related genes is revealed in Table SI.

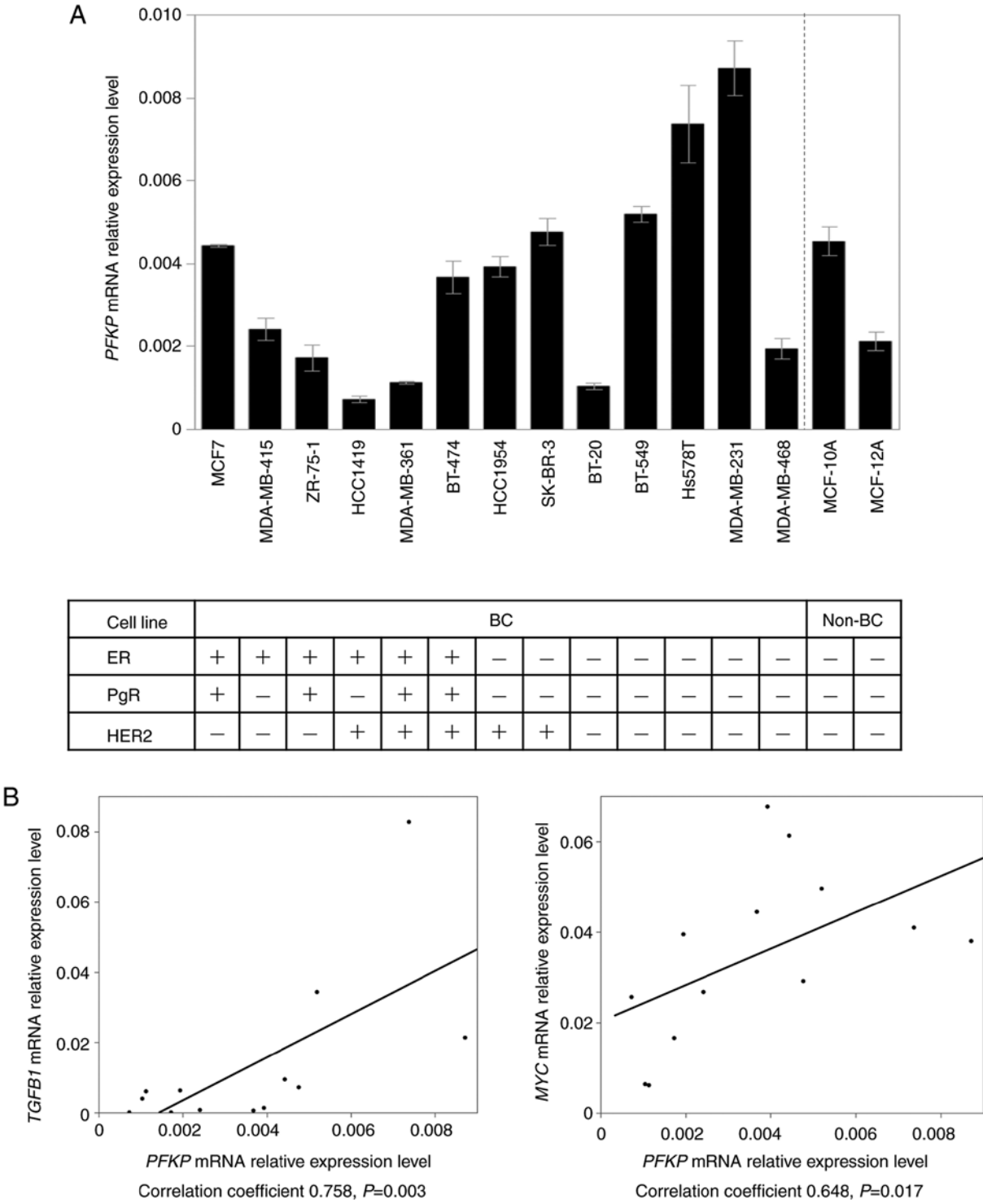


Figure 1. *PFKP* mRNA expression in 13 breast cancer and two non-cancerous cell lines, and the correlation between *PFKP* and cancer-related gene expression in PCR array analysis. (A) *PFKP* mRNA expression levels in ER-negative cell lines were significantly higher than those of ER-positive cells. Error bars, mean \pm SEM. (B) The mRNA expression level of *PFKP* exhibited a significant positive correlation with the levels of *TGFBI* and *MYC* in various cell lines. *PFKP*, platelet isoform of phosphofructokinase; BC, breast cancer; non-BC, non-cancerous breast; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; *TGFBI*, transforming growth factor- β 1; *MYC*, *MYC* proto-oncogene; PgR, progesterone receptor.

Effects of PFKP knockdown in BC cell lines. Considering the results of *PFKP* mRNA expression levels, *PFKP* protein expression was evaluated in representative BC cell lines to differentiate cell lines between high and low *PFKP* levels. Among these cell lines, MCF7 represents ER-positive/HER2-negative, SK-BR-3 represents ER-negative/HER2-positive and MDA-MB-231 represents triple-negative cells. HCC1419, which expressed the

lowest mRNA expression level, was used as a negative control (Fig. 2A). Knockdown cells tended to exhibit decreased levels in *PFKP* mRNA expression (Fig. 2B), which was confirmed with the protein expression levels (Fig. 2C).

To determine the tumor-progressive roles of *PFKP* in BC cells, cell proliferation, invasiveness and migration were evaluated in the knockdown cells. Compared with the untransfected

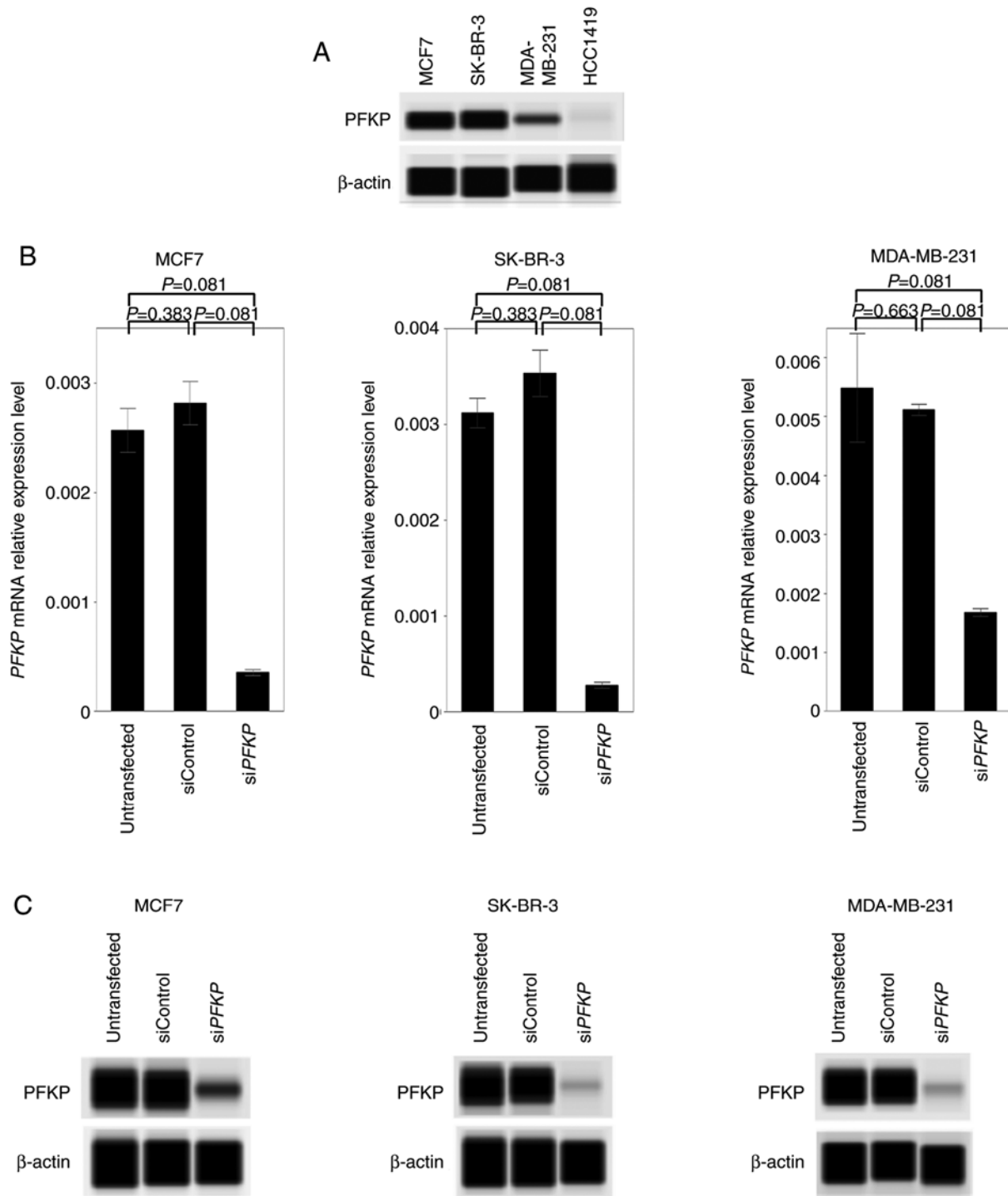


Figure 2. PFKP expression, and knockdown of *PFKP* mRNA and PFKP protein with *siPFKP* in BC cell lines. (A) PFKP expression in representative BC cell lines. PFKP expression was observed in MCF7 (ER-positive/HER2-negative), SK-BR-3 (ER-negative/HER2-positive), and MDA-MB-231 (triple-negative), whereas PFKP was not detected in HCC1419 cells, which exhibited the lowest expression level of *PFKP*. (B) Validation of *PFKP* knockdown efficacies in mRNA expression levels. Knockdown cells tended to exhibit lower *PFKP* expression levels in MCF7, SK-BR-3, and MDA-MB-231 cell lines. Error bars, mean \pm SEM. Kruskal-Wallis test. (C) Western blotting using the Simple Western technique confirmed the inhibition of PFKP in MCF7, SK-BR-3 and MDA-MB-231 cell lines. PFKP, platelet isoform of phosphofructokinase; si, small interfering; BC, breast cancer; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2.

and siControl-transfected cells, proliferation was significantly inhibited in *siPFKP*-transfected MCF7 and SK-BR-3 cells during the entire study period ($P < 0.001$). Proliferation of MDA-MB-231 cells transfected with *siPFKP* resulted in significant inhibition on day 5 ($P < 0.01$; Fig. 3A). In the invasiveness assay, fewer *siPFKP*-than siControl-transfected or

untransfected MCF7, SK-BR-3, and MDA-MB-231 cells passed the Matrigel ($P < 0.001$; Fig. 3B). Moreover, the migration ability of SK-BR-3 and MDA-MB-231 cells was inhibited after *siPFKP* transfection ($P < 0.01$; Fig. 3C). *siPFKP*-transfected MCF7 cells did not exhibit sufficient proliferation to perform the migration assay, as revealed in Fig. 3A.

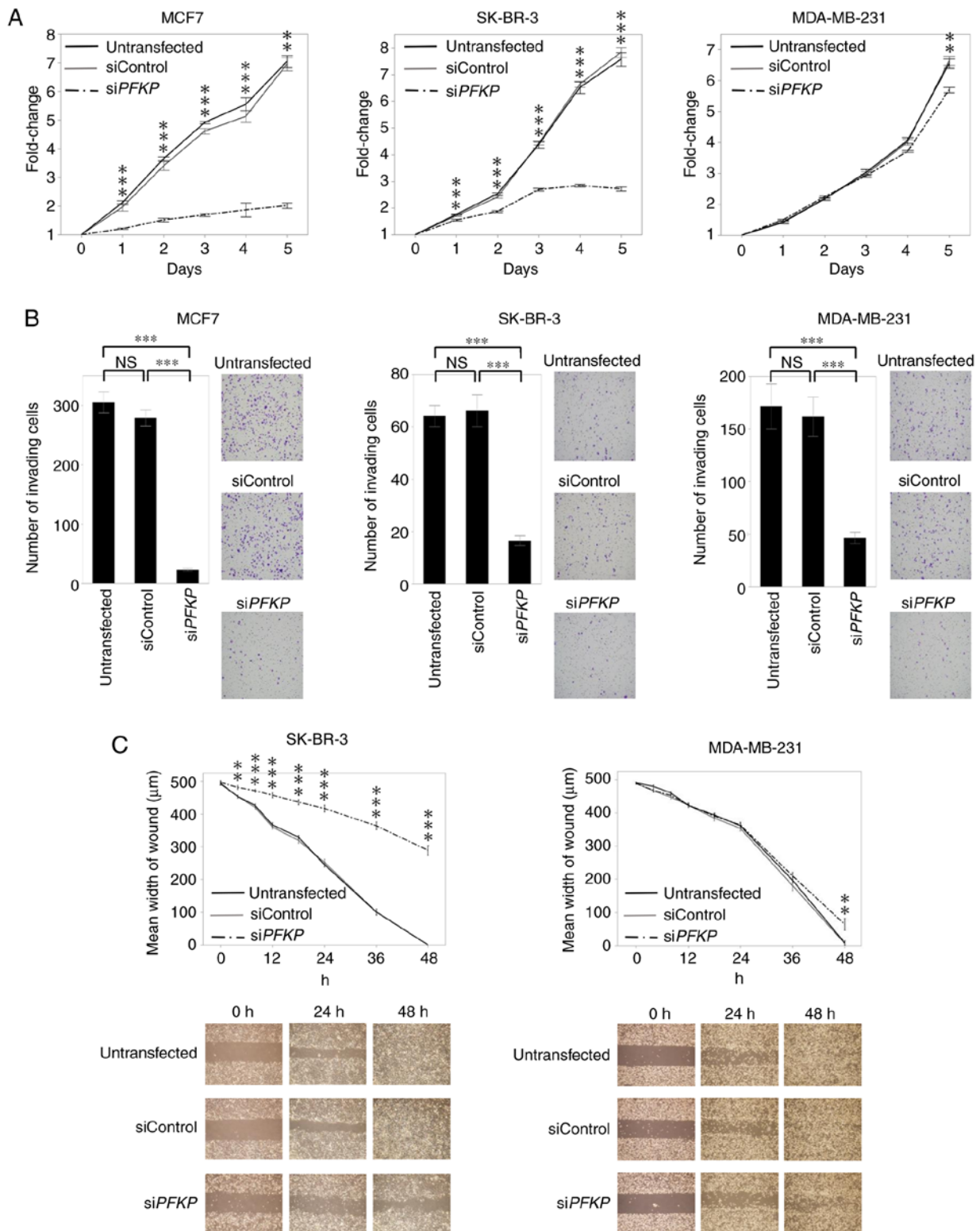


Figure 3. Functional analysis in breast cancer cell lines using knockdown cells. (A) Proliferation assay: siPFKP cells revealed significantly decreased proliferation in MCF7, SK-BR-3, and MDA-MB-231 cells, compared with untransfected and siControl cells. (B) Invasiveness assay: Inhibiting PFKP in MCF7, SK-BR-3, and MDA-MB-231 cells significantly decreased the number of invading cells. (C) Migration assay: The migration ability was inhibited in siPFKP cells in SK-BR-3 and MDA-MB-231 cell lines. Error bars mean \pm SEM. ANOVA followed by Tukey's post hoc test (for A, B, and C). **P<0.01 and ***P<0.001. PFKP, platelet isoform of phosphofructokinase; N.S., not significant; si, small interfering.

Association between PFKP mRNA expression levels and clinicopathological factors. PFKP mRNA expression levels in both BC and non-cancerous specimens were evaluated. The ratio of PFKP mRNA expression levels between cancerous and non-cancerous

specimens was defined as the 'C/N ratio'. Accordingly, the mean C/N ratio (\pm SD) was 1.82 ± 3.26 , and the C/N ratio was >1 in 69 patients (41%). PFKP mRNA expression levels in patients with T2/T3/T4 (n=90) were significantly higher than those

Table I. Associations between *PFPK* mRNA expression and the clinicopathological characteristics of 167 patients with breast cancer.

Characteristics	Expression of <i>PFPK</i>		P-value
	High- <i>PFPK</i> group (n=42)	Medium-low <i>PFPK</i> group (n=125)	
Age (years)	52 (27-76)	52 (26-78)	0.813
Histology			0.794
DCIS	1 (2.4%)	6 (4.8%)	
IDC	38 (90.5%)	110 (88.0%)	
ILC	3 (7.1%)	3 (2.4%)	
Other	0 (0%)	6 (4.8%)	
UICC T factor			0.023 ^a
Tis/T1	1/12 (31.0%)	6/58 (51.2%)	
T2/T3/T4	23/4/2 (69.0%)	52/5/4 (48.8%)	
Lymph node status			0.055
Positive	26 (61.9%)	56 (44.8%)	
Negative	16 (38.1%)	69 (55.2%)	
UICC pathological stage			0.001 ^a
0/I	1/4 (11.9%)	6/43 (39.2%)	
II/III/IV	27/10/0 (88.1%)	51/24/1 (60.8%)	
ER status			0.004 ^a
Positive	25 (59.5%)	102 (81.6%)	
Negative	17 (40.5%)	23 (18.4%)	
PgR status			<0.001 ^a
Positive	19 (45.2%)	96 (76.8%)	
Negative	23 (54.8%)	29 (23.2%)	
HER2 status			0.118
Positive	13 (31.0%)	26 (20.8%)	
Negative	25 (59.5%)	94 (75.2%)	
Unknown	4 (9.5%)	5 (4.0%)	
Adjuvant therapy			0.214
Endocrine therapy alone	13 (30.9%)	44 (35.2%)	
Chemotherapy alone	12 (28.6%)	18 (14.4%)	
Endocrine and chemotherapy	13 (31.0%)	51 (40.8%)	
None	4 (9.5%)	12 (9.6%)	

Data are expressed as the median (range) or number (%). ^aP<0.05. *PFPK*, platelet isoform of phosphofructokinase; DCIS, ductal carcinoma *in situ*; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; PgR, progesterone receptor; Tis, tumor *in situ*; UICC, Union for International Cancer Control.

revealed in patients with Tis/T1 (n=77; P=0.049). Similarly, patients with lymph node metastases (n=82) exhibited higher *PFPK* mRNA expression levels than those without lymph node metastases (n=85; P=0.048; Fig. 4A). Furthermore, patients with stage II/III/IV (n=113) exhibited higher *PFPK* expression levels than those with stage 0/I (n=54; P=0.011; Fig. 4A). Regarding conventional biomarkers, ER-negative specimens (n=40) revealed higher *PFPK* mRNA expression levels than ER-positive specimens (n=127; P=0.002). PgR-negative specimens (n=52) exhibited significantly higher *PFPK* mRNA expression than PgR-positive specimens (n=115; P<0.001; Fig. 4B). There was no significant difference between the HER2-positive (n=39)

and HER2-negative specimens in terms of their *PFPK* mRNA expression (n=119; P=0.088; Fig. 4B).

The patients were grouped in the highest quartile of *PFPK* mRNA expression into a 'high-*PFPK* group' (n=42) and the remaining patients in other quartiles to a 'medium-low *PFPK* group' (n=125). The association between clinicopathological factors and *PFPK* expression is revealed in Table I. As anticipated, the high-*PFPK* group included more patients with T2/T3/T4 (P=0.023) and with more advanced UICC pathological stages (P=0.001). In addition, the high-*PFPK* group had more ER-negative and PgR-negative patients than the medium-low *PFPK* group (P=0.004 and P<0.001, respectively).

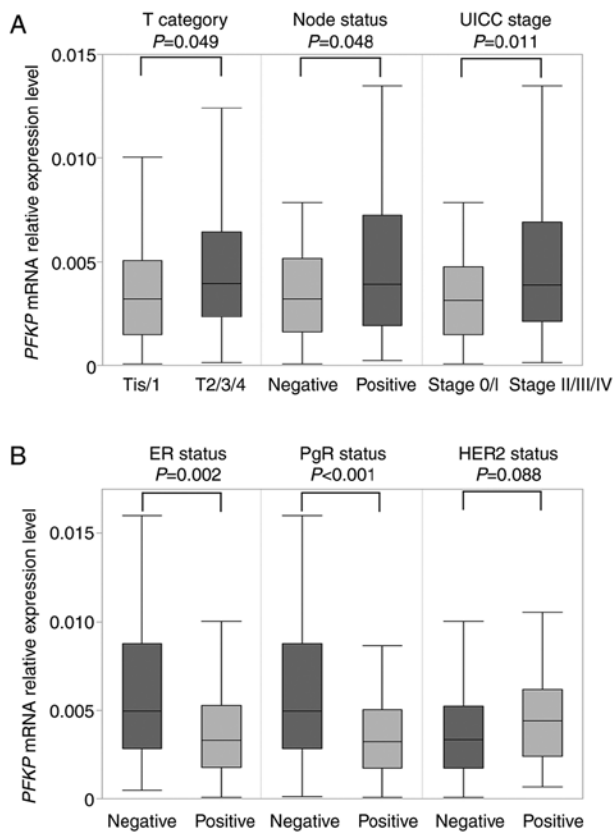


Figure 4. Association between *PFKP* mRNA expression and clinicopathological factors. (A) The mRNA expression level of *PFKP* was significantly higher in the patients with T2/T3/T4, lymph node metastases, or stage II/III/IV than those with Tis/T1, without lymph node metastases, or stage 0/I, respectively. (B) ER-negative and PgR-negative specimens exhibited higher *PFKP* mRNA expression than ER-positive and PgR-positive specimens, respectively, but no significant difference was observed in HER2 status. *PFKP*, platelet isoform of phosphofructokinase; Tis, tumor *in situ*; UICC, Union for International Cancer Control; ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

When prognosis was evaluated in our cohort, there were no significant differences in terms of DFS or OS between these two groups (Fig. 5A). Due to the small sample size of our cohort, the effect of *PFKP* expression on prognosis was subsequently investigated using the Kaplan-Meier Plotter website. Similarly, when patients were assigned either to the upper quartile (high-*PFKP* group) or to other quartiles (medium-low *PFKP* group), the high-*PFKP* group exhibited significantly worse RFS (n=3951; $P<1E-16$) and OS (n=1402; $P=2.6E-06$; Fig. 5B).

Discussion

The present study demonstrated that *PFKP* expression contributes to tumor progression by promoting cellular proliferation, invasiveness and migration in various subtypes of BC cell lines. Furthermore, analysis of clinical samples revealed that *PFKP* mRNA expression levels were higher in patients with advanced pathological stage, which supported our *in vitro* results.

The activity of glycolytic enzymes, such as hexokinase, PFK, and pyruvate kinase, is several folds higher in cancer cells than that in normal cells (21,22). *PFKP* upregulation has been revealed to increase glycolytic flux and promote

tumor cell proliferation and tumor growth (10). In hepatocellular carcinoma, *PFKP* was revealed to be regulated by Tat-activating regulatory DNA-binding protein via microRNA 520 (23). In glioblastoma, phosphorylation of *PFKP* S386 via AKT activation promoted aerobic glycolysis and tumor growth (9). In addition to the transcription factors that directly upregulate *PFKP*, there is crosstalk between glycolysis and oncogenic signaling (24). From these insights, the expression and functional roles of *PFKP* in BC were investigated.

Regarding *PFKP* mRNA expression levels in BC cell lines and non-cancerous cell lines, ER-negative BC cell lines had significantly higher *PFKP* mRNA expression levels than ER-positive BC cell lines. In addition, triple-negative BC cell lines expressed higher levels of *PFKP* mRNA than the other cell lines. Similarly, analysis of our clinical samples demonstrated that *PFKP* mRNA expression levels in ER-negative patients were significantly higher than those in ER-positive patients, and its expression levels in patients negative for PgR were also significantly higher than those found in patients with PgR-positive results. These results are consistent with a recent report revealing that triple-negative BC is more dependent on glycolysis by upregulating several key glycolytic enzymes and transporters, including PFK and the glucose transporter (24). To analyze the interactions between *PFKP* and several oncogenic signaling pathways, the correlation between the expression levels of cancer-related genes and those of *PFKP* in BC cell lines were investigated using a PCR array. Accordingly, several already known oncogenes were coordinately expressed with *PFKP* in BC cell lines, such as *BAX*, *JUN*, *MYC*, *PRKCA*, and *TGFB1*. Among them, previous studies demonstrated that *TGFB1* and *MYC* may be correlated with *PFKP* (25,26). *TGFB1* was revealed to induce 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 expression through activation of p38 MAPK and PI3K/AKT signaling pathways that complement and converge with Smad signaling activation (25), which promotes the synthesis of fructose 2,6-bisphosphate, an activator of PFKP (27). *Myc* has been revealed to suppress the level of thioredoxin-interacting protein, which is a negative regulator of glucose uptake and glycolysis gene expression, and activates aerobic glycolysis in BC (26). Although further mechanistic investigation is required, these results would provide important insights into understanding the involvement of *PFKP* in signaling pathways associated with BC progression.

In the present study, *PFKP* inhibition suppressed cellular proliferation, invasiveness and migration in various subtypes of BC cell lines, such as MCF7, SK-BR-3 and MDA-MB-231. Because *PFKP* protein expression in MDA-MB-231 cells was relatively low compared with that in MCF7 and SK-BR-3 cells, the effects of *PFKP* inhibition on cell proliferation and migration in MDA-MB-231 cells was lower than those in MCF7 and SK-BR-3 cells, indicating that cellular proliferative and migrating capacities are proportional to *PFKP* expression levels. In clinical samples, *PFKP* expression levels were higher in patients with larger tumor sizes, positive lymph node metastases, or more advanced stages. Aerobic glycolysis provides a material basis for growth and proliferation of tumor cells (28), and large amounts of lactic acid cause invasion of tumor tissues to the normal adjacent tissues (29). In non-small cell lung cancer (NSCLC) cell lines, *PFKP* was revealed to promote

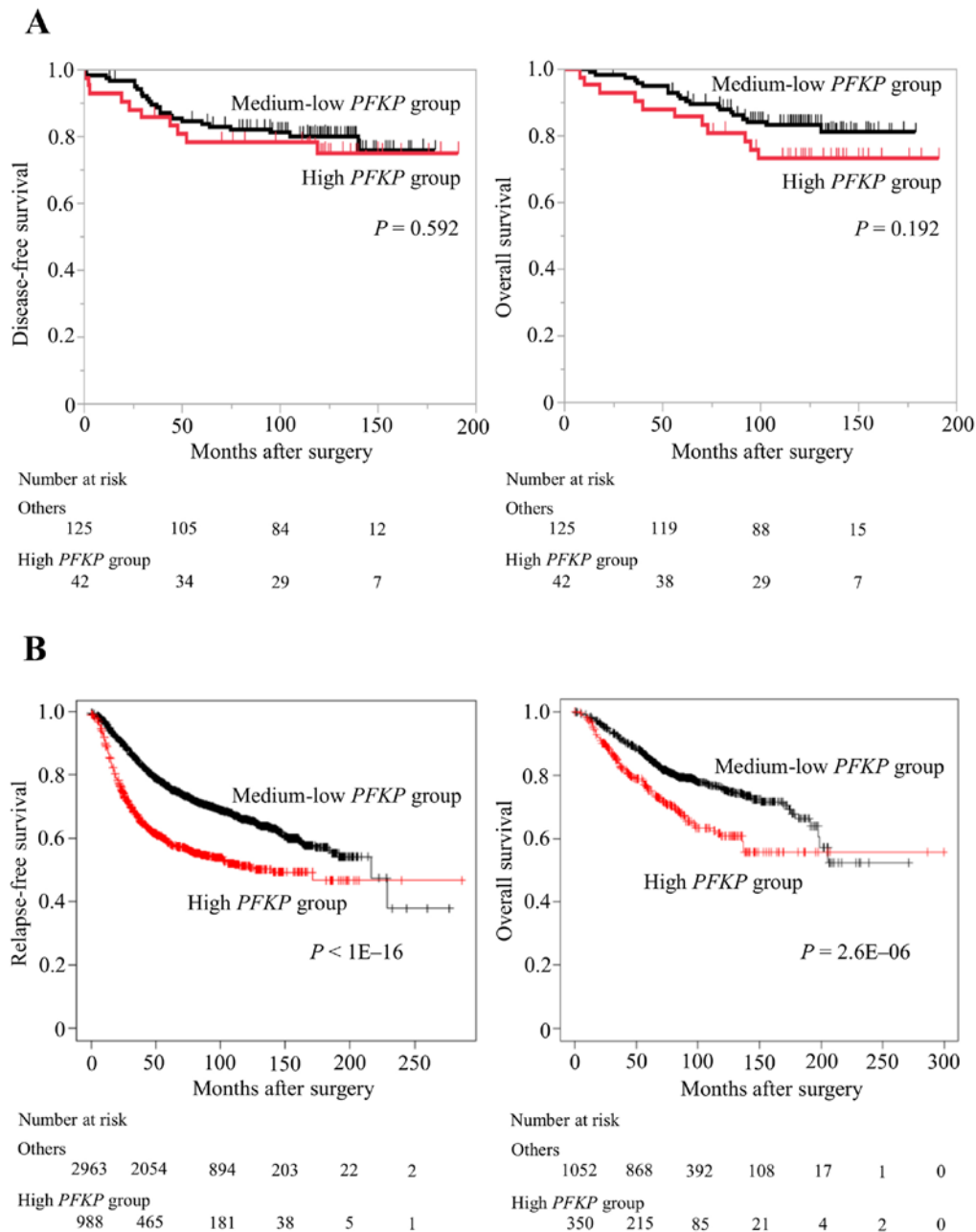


Figure 5. Prognosis according to *PFKP* expression level. (A) There were no significant differences between the two groups in either disease-free survival or OS in our cohort. (B) According to the Kaplan-Meier Plotter, patients in the high-*PFKP* group exhibited decreased relapse-free survival and OS than the medium-low *PFKP* group. *PFKP*, platelet isoform of phosphofructokinase; OS, overall survival.

proliferation, migration, invasion, epithelial-mesenchymal transition, and glycolysis (30). Furthermore, *PFKP* mRNA expression was associated with lymph node metastasis in NSCLC tissues (30). A similar phenomenon could be caused by *PFKP* in BC as it promotes lymph node metastasis, leading to a more malignant phenotype. A previous study on PFK isoenzyme patterns in BC tissue revealed a positive correlation between increased pathological stages and the expression of *PFKP* (31), indicating that *PFKP* is involved in promoting the malignant phenotype of BC regardless of the BC subtype. Regarding prognosis, although there was no significant difference in DFS or OS between the high-*PFKP* group and medium-low *PFKP* group in our cohort, the analysis using the public database demonstrated that patients with high *PFKP*

expression exhibited poorer RFS and OS. This discrepancy could be due to the small sample size of our cohort and the effect of adjuvant therapy. Interestingly, the high-*PFKP* group in our cohort included patients with more advanced T factors and pathological stages, which indicated an association between *PFKP* and tumor progression. In summary, our results indicated that *PFKP* promotes malignant cellular features and contributes to a more advanced pathological stage, which leads to poor prognosis. Noticeably, there is no drug approved for BC that targets glycolytic enzymes. These results indicated that *PFKP* could be a new therapeutic target molecule in BC.

However, the present study had some limitations. Firstly, the mechanism of *PFKP* expression involved in tumor progression has not been fully elucidated. Secondly, as aforementioned, due

to the small number of patients in our study and use of adjuvant medication therapy such as endocrine therapy, chemotherapy and molecular targeted therapy, the results of the prognostic analysis in our cohort data did not coincide with those in public databases. Finally, further *in vivo* studies are required to demonstrate the potential therapeutic targets of PFKP.

In conclusion, the present study revealed the tumor-progressive roles of PFKP in various subtypes of BC cells expressing PFKP. These data support the possibility of PFKP as a therapeutic target in BC.

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Availability of data and materials

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

Authors' contributions

TIn and MS conceived and designed the study. TIn, MS, TIc, and IS conducted the experiments. TIn and MS analyzed the data and wrote the manuscript. MS, DT, YT, NT, and TK contributed to the acquisition of patient data. TIc, MK, MH, IS, DT, YT, NT, YK, and TK contributed to the interpretation of the comprehensive data and revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board and Ethics Committee of Nagoya University Hospital (approval no. 2019-0028). Informed consent was obtained from all patients included in the study.

Patient consent for publication

Participants in the present study provided written informed consent for publication required by the Institutional Review Board and Ethics Committee of Nagoya University Hospital.

Competing interests

The authors declare that they have no competing interests.

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