PFKP Accelerates Malignant Features in Breast Cancer

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Abstract

Purpose 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. This study aimed to evaluate the function of PFKP in BC cells and its expression level in patients with BC.

Methods 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. *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.

Results *PFKP* was highly expressed in estrogen receptor-negative and human epidermal growth factor receptor 2-negative BC cell lines. PCR array analysis demonstrated that *PFKP* expression level significantly correlated with that of transforming growth factor beta 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 showed higher expression of *PFKP* mRNA than patients with less severe disease.

Conclusions PFKP is involved in promoting tumor-progressive oncological roles in BC cells across different subtypes. PFKP 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 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 demanded.

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 cancer, 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 alpha, 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, activate the transcription of *PFKP* and enhance glycolytic metabolism [7,11]. However, whether PFKP promotes malignant features in BC has not been evaluated. This 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

We obtained 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). BT-549, HCC1419, HCC1954, and Hs578T cell lines were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). BT-474, MCF7, and MCF-12A were kindly provided by Prof. David Sidransky from Johns Hopkins University (Baltimore, MD, USA). Other cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). All cell lines were stored at −80°C using a cell preservation solution (Cell Banker; Mitsubishi Chemical Medicine Corporation, Tokyo, Japan) and cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) and incubated in an atmosphere of 5% carbon dioxide at 37°C [12].

We also collected primary BC and non-cancerous specimens from 167 patients histologically diagnosed with BC after undergoing surgery at Nagoya University Hospital 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 [13]. 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 patient's condition, pathological features, cancer subtype, and physicians' discretion [13].

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

PFKP mRNA expression levels were evaluated by qRT-PCR. RNA was extracted from cell line (8.0×10^6 cells per cell line), as well as from BC and non-cancerous specimens from 167 patients. cDNA was synthesized as described previously [12]. 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'-GAAGGTGAAGGTCGGAGTC-3' and reverse 5'-GAAGATGGTGATGGGATTTC-3', which generated a 226-bp product [13]. qRT-PCR was performed using an ABI StepOnePlus real-time PCR System (Applied Biosystems, Foster City, CA, USA) as described previously [12]. The mRNA expression level of *PFKP* was obtained dividing each sample's value by the corresponding *GAPDH* value [12].

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 RT^2 Profiler PCR Array Human Oncogenes & Tumor Suppressor Genes (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

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 kinds of siRNAs specific for *PFKP*, named "si*PFKP*". Their sequences were si*PFKP*-1: 5'-UAUUAAUGUCAAUAAUACGUG-3'; si*PFKP*-2: 5'-GGAGCAAUUGAUACCCAAATT-3'; and si*PFKP*-3: 5'-GGAUCACUGCAAAACUCAATT-3' (Hokkaido System Science, Sapporo, Japan). AccuTarget^{TM} Negative Control siRNA Fluorescein-labeled (Cosmo Bio Co. Ltd., Tokyo, Japan) served as control nontargeting siRNA, named "siControl". BC cells were seeded in antibiotic-free RPMI-1640 supplemented with 10% FBS; 24 h after seeding, cells were transfected the corresponding siRNAs in the presence LipoTrust EX Oligo (Hokkaido System Science). After transfection, cells were cultured in antibiotic-free RPMI-1640 with 10% FBS for 72 h. Knockdown efficiency was determined using qRT-PCR.

Simple Western analyses

Simple Western analyses was performed using the WES machine (ProteinSimple, San Jose, CA, USA) according to the manufacturer's recommendations. Cells were incubated in RIPA lysis buffer, and the lysates were stored at –30°C. Protein concentration was measured using a BCA protein assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Protein samples, biotin ladder, primary antibody, secondary antibody, blocking reagent, chemiluminescent substrate, and wash buffer were prepared and dispensed into the assay plate. Then, the assay plate was loaded into the instrument, and the protein was separated into individual capillaries. Protein separation and detection was performed automatically on individual capillaries. Anti-*PFKP* antibody (1:50 dilution) (Cell Signaling Technology, Beverly, MA, USA) and anti-beta actin antibody (1:50 dilution) (Abcam, Cambridge, UK) were used as primary antibodies. Streptavidin HRP and anti-mouse or anti-rabbit secondary antibodies (ProteinSimple) were selected according to the corresponding primary antibody [14,15].

Proliferation assay

Cell proliferation was evaluated using the Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). MCF7 (3.0×10^3 cells per well), SK-BR-3 (3.0×10^3 cells per well), and MDA-MB-231 (3.0×10^3 cells per well) cells, which transfected with si*PFKP* or siControl, were seeded into 96-well plates with RPMI-1640 containing 10% FBS and 1% antibiotic. Each sample was applied to six wells, and the optical density of each well was measured 2 h after adding 10 μL of CCK-8 solution up to 5 days after seeding [13].

Invasiveness assay

Invasiveness in Matrigel was determined using BioCoat Matrigel Invasion Chambers (Corning Inc., Corning, NY, USA) according to the manufacturer's protocol. After transfection, MCF7 (2.5×10^4 cells per well), SK-BR-3 (2.5×10^4 cells per well), and MDA-MB-231 (2.5×10^4 cells per well) cells were suspended in serum-free RPMI-1640 and seeded in the upper chambers. After 72 h of incubation, cells on the membrane surfaces were fixed, stained, and counted in ten randomly selected microscope fields [13].

Migration assay

Migration of MCF7, SK-BR-3, and MDA-MB-231 cells was determined using a wound-healing assay. After transfection, MCF7 (4.9×10^4 cells per well), SK-BR-3 (4.9×10^4 cells per well), and MDA-MB-231 (4.9×10^4 cells per well) cells were seeded into each well of a 35-mm dish with culture insert (Ibidi, Martinsried, Germany) using RPMI-1640 containing 10% FBS and 1% antibiotic. After 24 h, the insert was removed, and wound widths were measured 20 times per well at 100-μm intervals [13].

Kaplan–Meier survival analysis using Kaplan–Meier Plotter

We used the website of the Kaplan–Meier Plotter (http://kmplot.com/analysis/index.php?p=background) to analyze relapse-free survival (RFS) and OS for patients with BC with respect to expression of *PFPK* by classifying its expression levels into the upper quartile and others [16].

Statistical analysis

Numeric variables between two groups were compared using the Mann–Whitney test. Spearman's rank correlation test was performed to evaluate the correlation between *PFKP* and cancer-related gene expression levels in the PCR array analysis. We analyzed the association between *PFKP* mRNA expression and clinicopathological factors 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. All statistical analyses were performed using JMP 15 software (SAS Institute Inc., Cary, NC, USA), and statistical significance was defined as $P < 0.05$.

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 shown in Fig. 1a. ER, PgR, and HER2 statuses of the cell lines have been evaluated in previous studies [17,18]. *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 showed higher mRNA expression levels than that in the other cell lines. (P = 0.038). Subsequent PCR array analysis showed that *PFKP* mRNA expression levels were positively correlated with those of several well-known oncogenes, such as transforming growth factor beta 1 (*TGFB1*) (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 shown in Supplementary Table S1.

Effects of PFKP knockdown in various BC subtypes

Considering the results of *PFKP* mRNA expression levels, PFKP protein expression was evaluated in representative BC cell lines. Among these cell lines, MCF7 represents the ER-positive/HER2-negative subtype, SK-BR-3 represents the ER-negative/HER2-positive subtype, and MDA-MB-231 represents the triple-negative subtype. HCC1419, which expressed the lowest mRNA expression level, was used as negative control (Fig. 2a). PFKP knockdown was confirmed at both mRNA and protein expression levels (Fig. 2b and 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 and siControl-transfected cells, proliferation was significantly inhibited in si*PFKP*-transfected MCF7 and SK-BR-3 cells during the entire study period (P < 0.05). Proliferation of MDA-MB-231 cells transfected with si*PFKP* resulted significantly inhibited on days 4 and 5 (P < 0.05; Fig. 3a). In the invasiveness assay, fewer si*PFKP*- 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 si*PFKP* transfection (P < 0.01; Fig. 3c). si*PFKP*-transfected MCF7 cells did not show enough proliferation to perform the migration assay, as shown in Fig. 3a.

Patient characteristics

A total of 167 female patients were enrolled in this 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. Eighty-two 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 patients (69%) PgR-positive and 52 (31%) PgR-negative. Thirty-nine patients (23%) were HER2-positive and 119 (71%) HER2-negative. Nine patients had unknown HER2 status.

Association between PFKP mRNA expression levels and clinicopathological factors

PFKP mRNA expression levels in patients with T2/T3/T4 (n = 90) were significantly higher than those found in patients with Tis/T1 (n = 77; *P* = 0.049). Likewise, patients with lymph node metastases (n = 82) showed higher *PFKP* 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) showed higher *PFKP* expression levels than those with stage 0/I (n = 54; *P* = 0.011; Fig. 4a). Regarding conventional biomarkers, ER-negative specimens (n = 40) showed higher *PFKP* mRNA expression levels than ER-positive specimens (n = 127; *P* = 0.002), and PgR-negative specimens (n = 52) showed significantly higher *PFKP* 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 *PFKP* mRNA expression (n = 119; *P* = 0.088; Fig. 4b).

We grouped the patients in the highest quartile of *PFKP* mRNA expression into a “High *PFKP* group” (n = 42) and the remaining patients into a “Others” (n = 125). The association between clinicopathological factors and *PFKP* expression is shown in Table 1. As expected, the high *PFKP* 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 *PFKP* group had more ER-negative and PgR-negative patients than the others (*P* = 0.004 and *P* < 0.001, respectively).

When prognosis was evaluated in our cohort, there were no significant differences in terms of DFS or OS between these two groups (Fig. 5a). Because the small sample size was concerned in our cohort, we subsequently investigated the impact of *PFKP* expression on prognosis using the Kaplan–Meier Plotter website. Similarly, when patients were assigned either to the upper quartile (High *PFKP* group) or to other quartiles (Others), the high *PFKP* group showed significantly worse RFS (n = 3951; *P* < 1E–16) and OS (n = 1402; *P* = 2.6E–06; Fig. 5b).

Discussion

This 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 showed 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 [19,20]. *PFKP* upregulation increases glycolytic flux and promotes tumor cell proliferation and tumor growth [9]. In hepatocellular carcinoma, *PFKP* is regulated by Tat-activating regulatory DNA-binding protein via microRNA 520 [21]. In glioblastoma, phosphorylation of PFKP S386 via AKT activation promotes aerobic glycolysis and tumor growth [8]. In addition to the transcription factors that directly upregulate *PFKP*, there is crosstalk between glycolysis and oncogenic signaling [22]. From these notions, we investigated the expression and functional roles of PFKP in BC.

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 testing negative for PgR were also significantly higher than those found in patients with PgR-positive results. These results are consistent with a previous report showing that triple-negative BC is more dependent on glycolysis by upregulating several key glycolytic enzymes and transporters, including PFK and the glucose transporter [22]. To analyze the interactions between *PFKP* and several oncogenic signaling pathways, we investigated the correlation between the expression levels of cancer-related genes and those of *PFKP* in BC cell lines using a PCR array. Accordingly, *TGFB1* and *MYC* were coordinately expressed with *PFKP* in BC cell lines. In fact, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) promotes the synthesis of fructose 2,6-bisphosphate, which is the most potent allosteric stimulator of PFKP, and induced by TGFB1, it is involved in the activation of glycolysis observed in glioblastoma [23]. *Myc* suppresses the level of thioredoxin-interacting protein, which is a negative regulator of glucose uptake and glycolysis gene expression, and activates aerobic glycolysis in BC [24]. Although further mechanistic investigation is warranted, these results would provide important insights into understanding the involvement of *PFKP* in signaling pathways associated with BC progression.

In this 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 lower than that in MCF7 and SK-BR-3 cells, as shown in Fig. 2a, the impact of PFKP inhibition on cell proliferation in MDA-MB-231 cells was also lower than that in MCF7 and SK-BR-3 cells. In clinical samples, *PFKP* expression levels were higher in patients with larger tumor sizes, positive lymph node metastases, or more advanced stages. A previous study on PFK isoenzyme patterns in BC tissue revealed a positive correlation between increased pathological stages and the expression of PFKP [25], suggesting 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 others in our cohort, the analysis using the public database demonstrated that patients with high *PFKP* expression showed poorer RFS and OS. This discrepancy could be due to the small sample size in our cohort and the impact of adjuvant therapy. In summary, our results suggest 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 target glycolytic enzymes. These results suggest that PFKP could be a new therapeutic target molecule in BC.

This study had some limitations. First, the mechanism of *PFKP* expression involved in tumor progression has not been fully elucidated. Second, as noted above, 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 using public databases. Finally, further *in vivo* studies are required to prove the potential therapeutic targets of PFKP.

In conclusion, this study showed 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.

Declarations

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Conflicts of interest: The authors declare that they have no conflict of interest.

Data availability: The datasets analysed 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 the acquisition and interpretation of patient data. TIc, MK, MH, IS, DT, YT, NT, YK, and TK reviewed and revised the manuscript. The final manuscript was read and approved by all authors.

Ethics approval: The present study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Institutional Review Board and Ethics Committee of Nagoya University Hospital (approval number: 2019–0028).

Informed consent: Informed consent was obtained from all patients included in the study.

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Tables

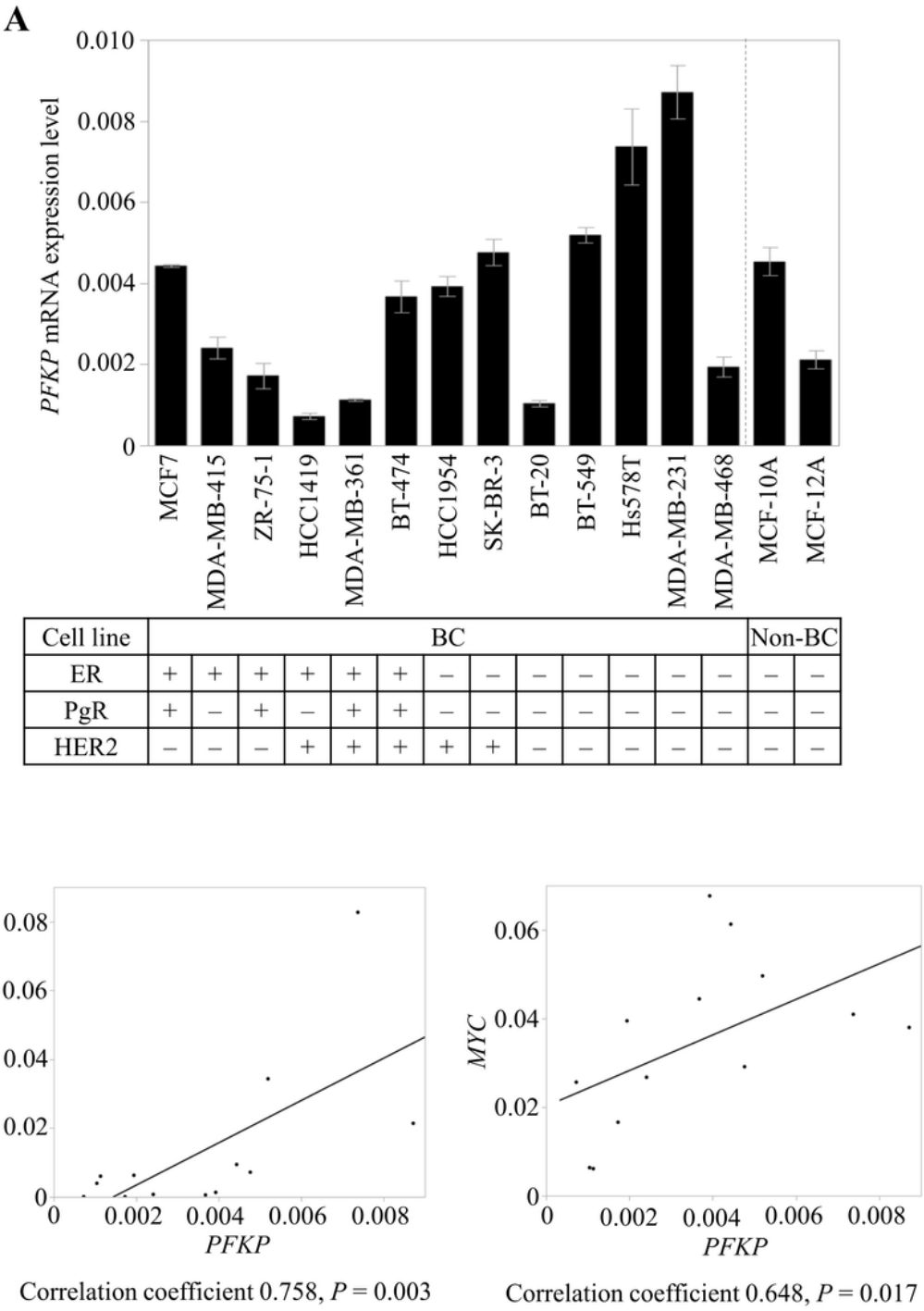
Table 1 Associations between *PFKP* mRNA expression and the clinicopathological characteristics of 167 patients with breast cancer

	High *PFKP* group (n = 42)	Others (n = 125)	*P* value
Age (years)	52 (27–76)[a]	52 (26–78)[a]	0.813
Histology			0.794
DCIS	1	6	
IDC	38	110	
ILC	3	3	
Other	0	6	
UICC T factor			**0.023**[b]
Tis/T1	1/12	6/58	
T2/T3/T4	23/4/2	52/5/4	
Lymph node status			0.055
Positive	26	56	
Negative	16	69	
UICC pathological status			**0.001**[b]
0/I	1/4	6/43	
II/III/IV	27/10/0	51/24/1	
ER status			**0.004**[b]
Positive	25	102	
Negative	17	23	
PgR status			**< 0.001**[b]
Positive	19	96	
Negative	23	29	
HER2 status			0.118
Positive	13	26	
Negative	25	94	
Unknown	4	5	
Adjuvant therapy			0.214

Endocrine therapy alone	13	44
Chemotherapy alone	12	18
Endocrine and chemotherapy	13	51
None	4	12

[a]median (range). [b]*P* < 0.05. 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 ductal carcinoma in situ, UICC Union for International Cancer Control

Figures



Cell line	BC													Non-BC	
ER	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–
PgR	+	–	+	–	+	+	–	–	–	–	–	–	–	–	–
HER2	–	–	–	+	+	+	+	+	–	–	–	–	–	–	–

Figure 1

PFKP mRNA expression in 13 BC and two non-cancerous cell lines, and the correlation between PFKP and cancer-related genes expression in PCR array analysis. a PFKP mRNA expression levels in ER-negative cell lines were significantly higher than those of ER-positive cells. b PFKP mRNA expression level showed significant positive correlation with those of TGFB1 and MYC in various cell lines. BC breast cancer cell lines, non-BC non-cancerous breast cell lines, ER estrogen receptor, HER2 human epidermal growth factor

receptor 2, PFKP platelet isoform of phosphofructokinase, TGFB1 transforming growth factor beta 1, MYC MYC proto-oncogene

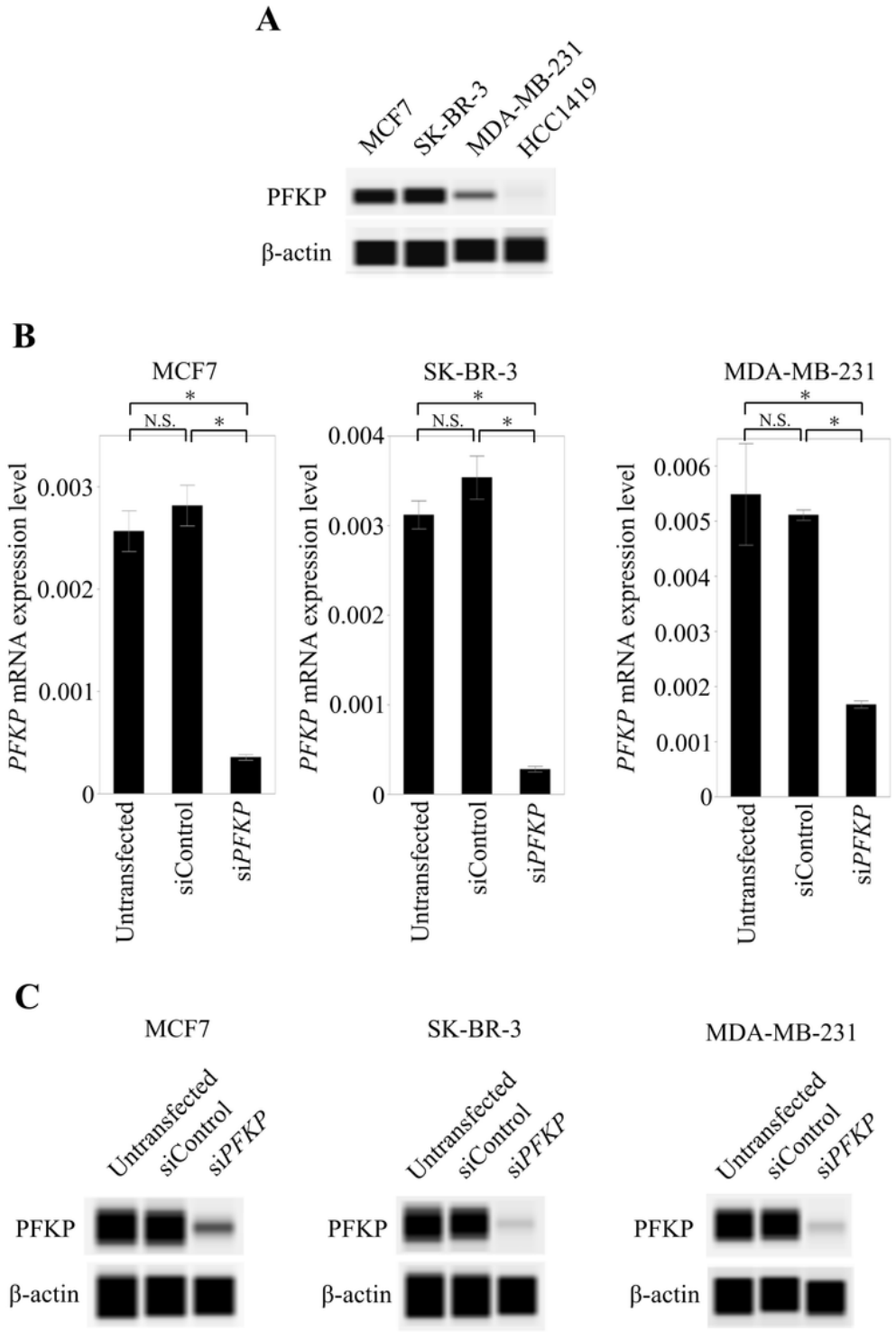


Figure 2

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, which expressed the lowest expression level of PFKP. b Validation of PFKP knockdown efficacies in mRNA expression levels. PFKP expression levels were significantly inhibited with siPFKP in MCF7, SK-BR-3, and MDA-MB-231 cell lines. c Simple Western analyses confirmed the inhibition of PFKP in MCF7, SK-BR-3, and MDA-MB-231 cell lines. PFKP platelet isoform of phosphofructokinase. P < 0.05

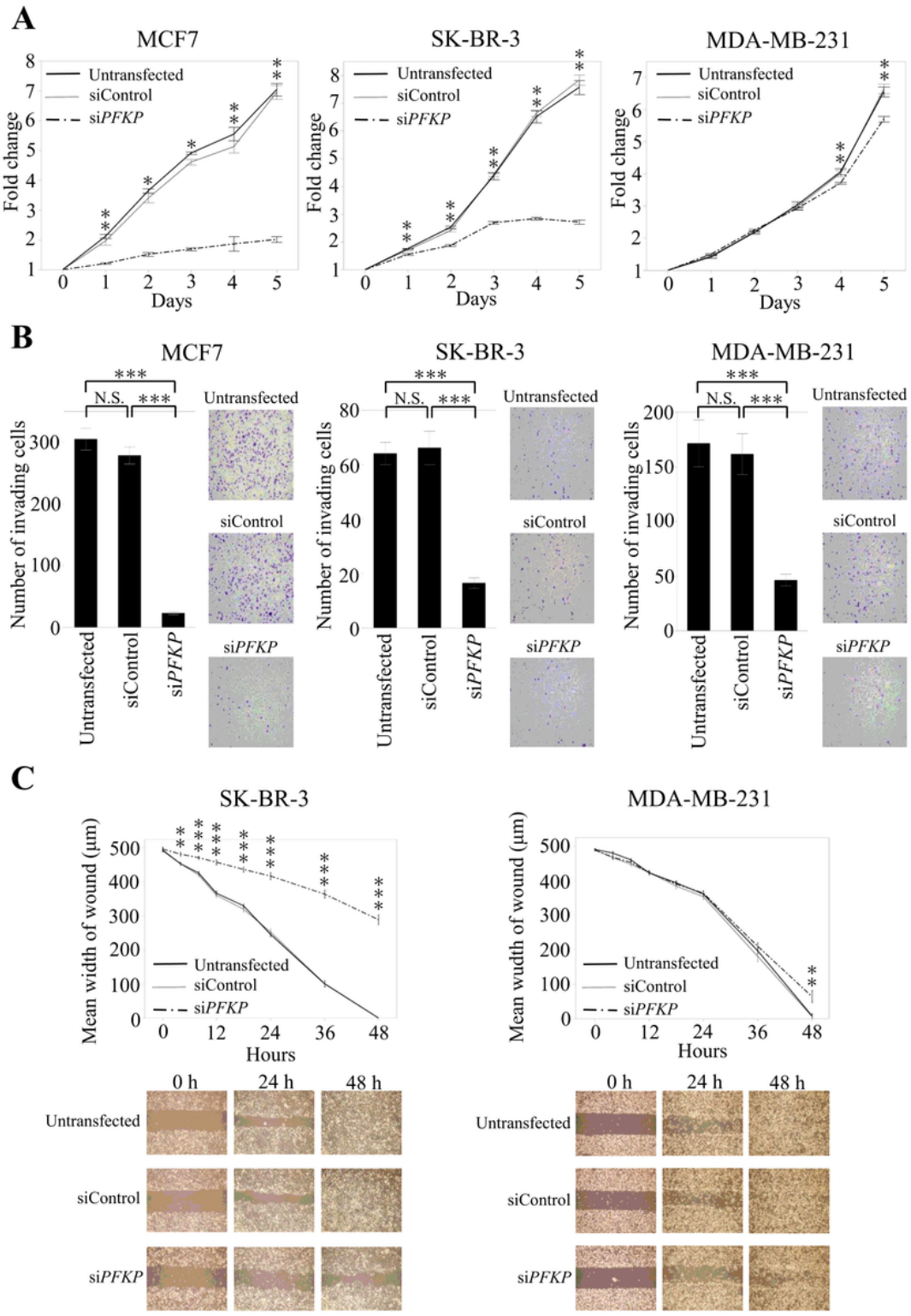


Figure 3

Functional analysis in BC cell lines using knockdown cells. a Proliferation assay: siPFKP cells showed 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 numbers of invading cells. c Migration assay: The migration ability was inhibited in siPFKP cells in SK-BR-3 and MDA-MB-231 cell lines. PFKP platelet isoform of phosphofructokinase, N.S. not significant. P < 0.05, P < 0.01, P < 0.001

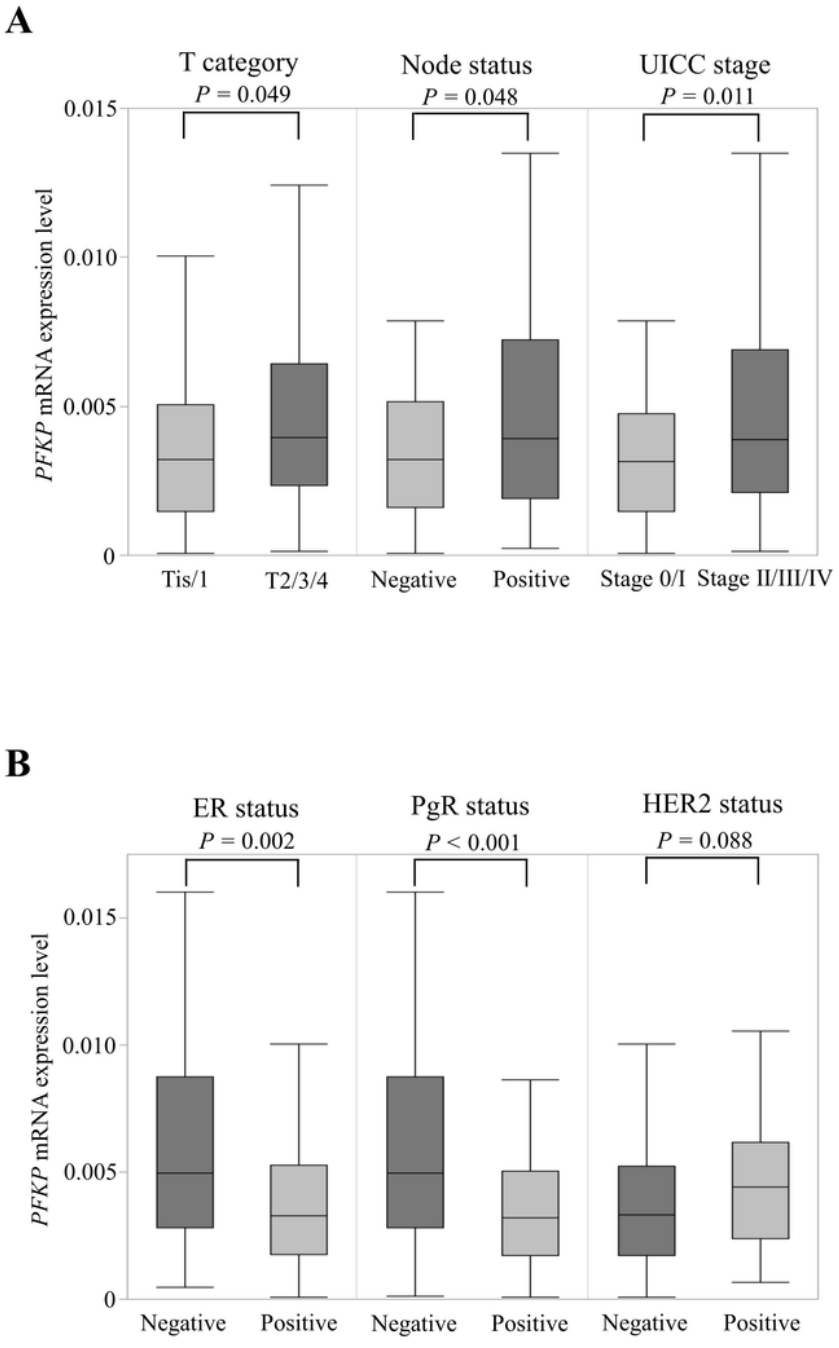


Figure 4

Association between PFKP mRNA expression and clinicopathological factors. a PFKP mRNA expression level 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 showed higher PFKP mRNA expression than ER-positive and PgR-positive specimens, respectively, but no significant difference in HER2 status. PFKP platelet isoform of phosphofructokinase, UICC Union for International Cancer Control, ER estrogen receptor, PgR progesterone receptor, HER2 human epidermal growth factor receptor 2

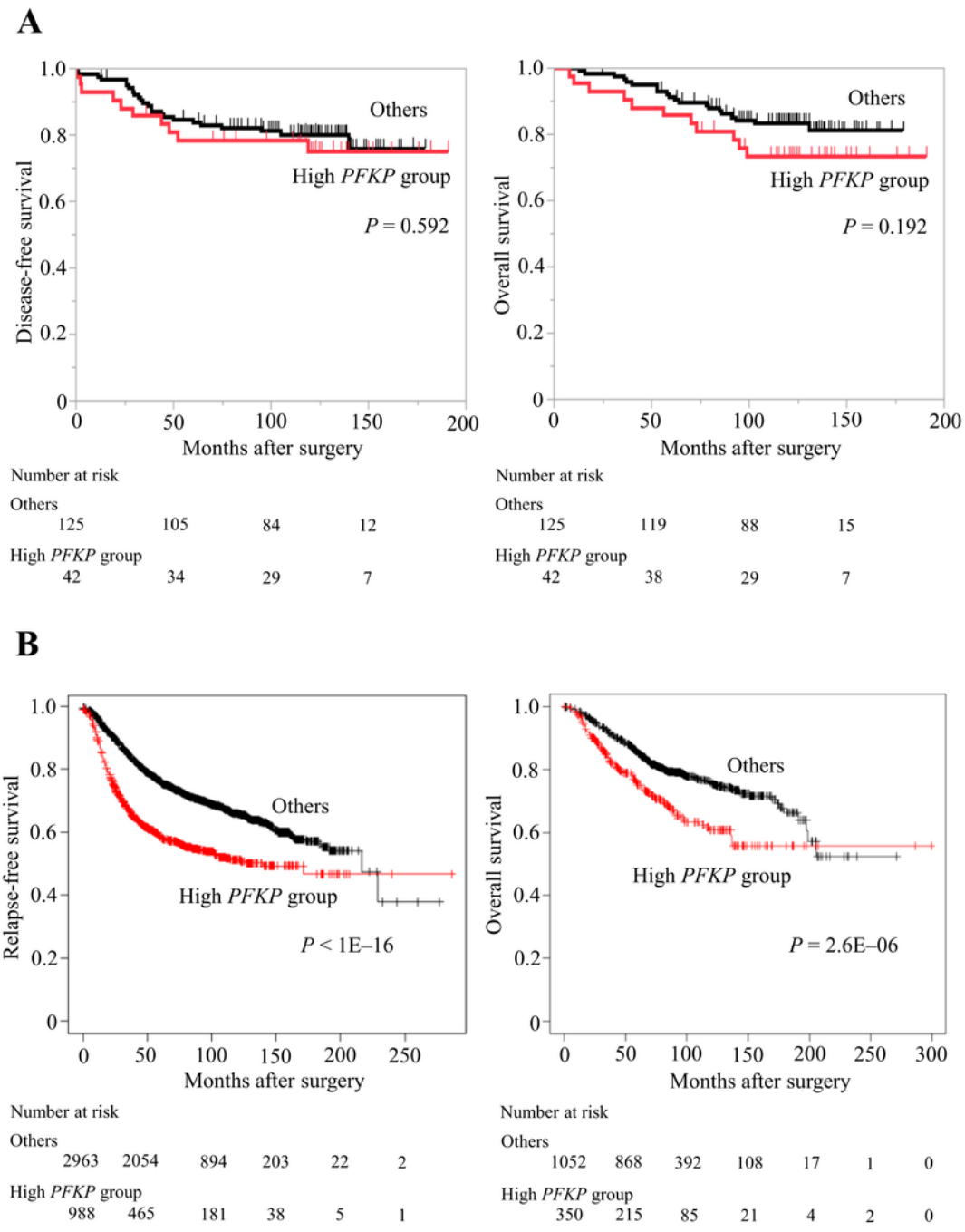


Figure 5

Prognosis according to PFKP expression level. a There were no significant differences between the two groups in either DFS or OS in our cohort. b According to the Kaplan–Meier Plotter, patients in the high PFKP group showed inferior RFS and OS than others. PFKP platelet isoform of phosphofructokinase