Protein tyrosine phosphatase 1B expression contributes to the development of breast cancer[*]

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Abstract: The protein tyrosine phosphatase 1B (PTP1B) is an important regulator of metabolism. The relationship between PTP1B and tumors is quite complex. The purpose of this study is to explore the expression pattern and role of PTP1B in breast cancer. The expression of PTP1B was detected in 67 samples of breast cancer tissue by Western blot. Cell growth assay, Transwell migration assay, and Scratch motility assay were used to examine the proliferation and migration of MCF-7 with and without PTP1B. The total levels and phosphorylated levels of signal transduction and activator of transcription 3 (STAT3) and the expression of C-C motif chemokine ligand 5 (CCL5) were also examined by Western blot. PTP1B was overexpressed in over 70% of breast cancer tissues, correlating with patients with estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and human epidermal growth factor receptor 2 (HER2)-positive tumors. The data also showed that both tumor size and lymph node metastasis were significantly higher in patients with a higher level of PTP1B. The proliferation and migration of MCF-7 cells were found to be inhibited after knocking down the gene of *PTP1B*. Our data also showed that PTP1B could up-regulate the dephosphorylated level of STAT3, which could increase the expression of CCL5. These phenomena indicated that PTP1B may play a crucial role in the development of breast cancer.

Key words: Protein tyrosine phosphatase 1B (PTP1B); Signal transduction and activator of transcription 3 (STAT3); Breast cancer; Tumorigenesis

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1 Introduction

Tyrosyl phosphorylation is a reversible process. It plays an important role in a fundamental mechanism of eukaryotic cells. It is closely related to human health and disease (Mustelin *et al.*, 2002). Many cellular functions, such as proliferation and metastasis, are under its control. Protein tyrosine phosphorylation and dephosphorylation can be regulated by both the protein tyrosine kinases and the protein tyrosine phosphatases (PTPs). PTPs comprise a large superfamily. Among them, protein tyrosine phosphatase 1B (PTP1B) is the first PTP purified from human placenta by Fischer *et al.* (1991). PTP1B, localized on the surface of endoplasmic reticulum, is an abundant cytoplasmic enzyme (Dubé and Tremblay, 2004). Down-regulating the signaling pathways of insulin and leptin is mainly contributed by PTP1B. The gene of *PTPN1*, encoding PTP1B, is located in the region from q13.1 to q13.2 in the human chromosome of No. 20. This region is found frequently amplified in cervical, gastric, and breast cancers, and is also closely related to poor prognosis (Bjorge *et al.*, 2000; Scotto *et al.*, 2008; Buffart *et al.*, 2009). Recently, it was found that PTP1B may be involved with many kinds of tumor, but the specific function is controversial. Some research proposed that PTP1B may have the potential to

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inhibit the proliferation and development of tumor cells by counteracting the activity of protein tyrosine kinases. Research showed that tyrosine phosphorylation of some oncogenes, such as ErbB2 and Bcr-Abl, was suppressed by the overexpression of PTP1B in fibroblasts (Woodford-Thomas *et al.*, 1992; LaMontagne *et al.*, 1998; Liu *et al.*, 1998), and Warabi *et al.* (2000) reported that PTP1B expression in esophageal cancer cells significantly decreased in comparison with normal esophageal tissue. All these suggested that PTP1B may act as a brake to metabolic and proliferative signals. However, recent data implied a positive role of PTP1B in growth signaling in some cancer cells, notably in a mice model of breast cancer which was induced by ErbB2 (Bentires-Alj and Neel, 2007). Some research also reported that the expression of PTP1B in gastric and colon tumor cells is higher than that in surrounding normal tissues (Zhu *et al.*, 2007; Wang *et al.*, 2012). Therefore, the relationship between PTP1B and tumors remains to be determined.

Breast cancer is the most common tumor and the second leading cause of cancer death among women (Baselga and Norton, 2002). In America, the incidence of breast cancer is just a little lower than that of non-melanomatous skin cancers and its mortality rate is second to that of lung cancer (ACS, 2010). The incidence of breast cancer has increased rapidly over the last 20 years, especially in Asia. However, the molecular mechanisms of development and metastasis in breast cancer are not yet clear. In order to clarify the role of PTP1B in breast cancer, we examined the expression of PTP1B in human breast cancer tissue, and used genetic approaches to investigate the function of PTP1B. Our data indicate that PTP1B represents a major mechanism contributing to tumorigenesis in breast cancer.

2 Materials and methods

2.1 Antibodies and reagents

Anti-PTP1B, anti-C-C motif chemokine ligand 5 (anti-CCL5), unphosphorylated signal transduction and activator of transcription 3 (U-STAT3) antibodies, anti-tubulin and phospho-STAT3 antibodies were purchased from Cell Signaling Technology (CST Co., Ltd., Danvers, MA, USA). Rabbit and mouse special secondary antibodies were purchased from Santa Cruz Biotechnology (SCB Co., Ltd., Santa Cruz, CA, USA).

2.2 Tissue sample collection

From January 2009 to August 2010, 67 samples of breast cancer tissues and surrounding normal tissues were collected from the Department of Breast and Thyroid Surgery in the Renmin Hospital and Zhongnan Hospital of Wuhan University, China. All patients with breast cancer were diagnosed by pathological examination, and the pathologic tumor staging was developed by the American Joint Committee on Cancer's Tumor-Node-Metastasis Classification in 2003. All patients involved in this study have signed informed consent, and this study has been approved by the Ethics Committee at Wuhan University, China.

2.3 Cell culture

The human breast cancer cell line MCF-7 was maintained in RPMI 1640 medium (HyClone, Thermo Scientific, Logan, UT, USA) supplemented with 10% (v/v) of fetal calf serum and 1% (v/v) of penicillin and streptomycin at 37 °C with an atmosphere of 95% air/5% CO_2 in a humidified incubator.

2.4 Lentivirus infections

The lentiviral particle productions, PTP1B-RNAi-LV and NC-RNAi-LV respectively containing PTP1B short hairpin RNA (shRNA) and non-target control shRNA, were purchased from GeneChem Co., Ltd. (Shanghai, China). Lentiviral knockdown of PTP1B proteins was achieved by infecting 30%–50% confluent MCF-7 cells with 1.2×10^4 TU/μl (TU: transducing unit) lentivirus in the presence of 10 μg/ml polybrene. The infection medium was replaced after 18 h. Then culture with fresh medium was supplemented with 5 μg/ml puromycin for 4 weeks to allow for efficient PTP1B protein knockdown. Stably transfected clones were collected for use in this study.

2.5 Western blot

Proteins were extracted as described by Boute *et al.* (2003). Protein samples were resolved by 10% (0.1 g/ml) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes. The PVDF membranes were blocked by 1% (0.01 g/ml) bovine serum albumin (BSA) and hybridized with corresponding primary antibody at 4 °C overnight, and incubated with specific secondary antibody for

2 h at room temperature. They were detected by a chemiluminescence system. The density of autoradiograms was quantified by Quantity One software (Bio-Rad, USA). Relative protein levels were calculated by reference to the amount of tubulin protein.

2.6 Cell growth assay

MCF-7 cells were respectively planted at about 2×10^3 per well in 96-well plates in quintuplicate. The cell number was counted every 12 h using Cell-Counting Kit-8 (R & D Systems, Minneapolis, MN, USA). The optical density (OD) values of absorbance were measured by a microplate reader (Multiskan FC, Thermo Scientific, USA), and reported as mean± standard deviation (SD).

2.7 Transwell migration assay

Serum-free media with 5×10^4 suspension cells were added into the upper chamber of 24-well Transwell plates, and 500 µl RPMI 1640 medium with 10% fetal calf serum was added to the bottom chamber as chemoattractant. After incubation at 37 °C with 5% CO_2 for 24 h, cells on the upper chamber were removed by sterile swabs, and cells in the lower chamber were fixed by methanol for 20 min and stained with crystal violet staining solution for 30 min, and three preset fields per insert were photographed. Then they were eluted by 33% (v/v) acetic acid, and microplate reader was used to measure the OD values of absorbance in staining-positive cells.

2.8 Scratch motility assay

Cells of 5×10^5 per well were seeded into six-well plates and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum at 37 °C with 5% CO_2 overnight. Next day, the monolayer was scratched with a pipette tip, washed with phosphate-buffered saline (PBS) three times to remove floating cells, and further cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. The scratched area was photographed at 0 and 24 h with 10× objective lens. The average scratch width was calculated at five random points and reported as mean±SD.

2.9 Statistical analysis

SPSS 18.0 software was used for statistical analysis. Results are expressed as mean±SD. The chi-squared test was used to test the correlation be-

tween PTP1B expression in tumors and clinicopathologic variables, and the *t*-test was used to test the difference for the independent samples.

3 Results

3.1 Expression of PTP1B in breast cancer tissue

To explore the role of PTP1B in breast cancer development, we detected the expression of PTP1B in 67 samples of breast cancer tissue and surrounding normal tissue by Western blot. Forty-nine of 67 patients had a high expression of PTP1B in breast cancer tissue, and the level of PTP1B in breast cancer tissue was lower than that in surrounding normal tissue in the other 18 patients. When the densities of autoradiograms were quantified by Quantity One software (Fig. 1), we found that the level of PTP1B was much higher in breast cancer tissue than in the surrounding normal tissue and the difference has significance ($P<0.05$, $n=67$).
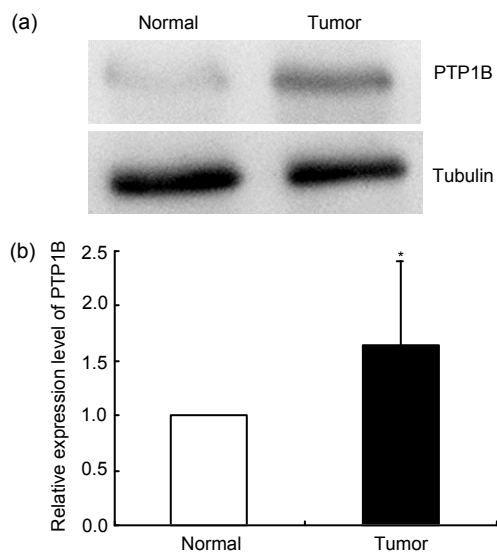

Fig. 1 Expression of PTP1B in breast cancer tissue
(a) The expression of PTP1B in breast cancer tissue and the surrounding normal tissue was examined by Western blot; (b) Quantifications of PTP1B autoradiograms are represented as mean±SD ($n=67$). * $P<0.05$, vs. normal tissue

In order to assess the relationship between PTP1B expression level and clinicopathologic factors in breast cancer, 67 samples of tumor tissue and surrounding normal tissue were analyzed. Patients' clinicopathologic conditions are presented in Tables 1 and 2. Our research showed that the positive rate of

Table 1 Expression of PTP1B in breast cancer tissues and its correlation with clinicopathologic characteristics

Variable	PTP1B[*]		Overall P
	Negative	Positive	
Tumor stage (T)			0.032
T1	7 (50%)	7 (50%)	
T2	9 (23%)	30 (77%)	
T3	2 (18%)	9 (82%)	
T4	0 (0%)	3 (100%)	
Lymph node (N)			0.014
N0	12 (48%)	13 (52%)	
N1	2 (10%)	18 (90%)	
N2	4 (27%)	11 (73%)	
N3	0 (0%)	7 (100%)	
Metastasis (M)			0.388
M0	18 (28%)	47 (72%)	
M1	0 (0%)	2 (100%)	
TNM stage			0.018
I	7 (54%)	6 (46%)	
II	7 (24%)	22 (76%)	
III	4 (17%)	19 (83%)	
IV	0 (0%)	2 (100%)	
ER status			0.001
Positive	15 (47%)	17 (53%)	
Negative	3 (9%)	32 (91%)	
PR status			0.002
Positive	11 (52%)	10 (48%)	
Negative	7 (16%)	37 (84%)	
Unknown	0 (0%)	2 (100%)	
HER2 status			<0.001
Positive	7 (14%)	42 (86%)	
Negative	11 (61%)	7 (39%)	

[*] Data are expressed as number (percent)

PTP1B was increased with the degree of malignancy. From Table 1, we can see that with the increase of tumor stage from T1 to T4, the positive rate of PTP1B significantly increased from 50% to 100% (P=0.032). With the increase of lymph node stage from N0 to N3, the positive rate of PTP1B also significantly increased from 52% to 100% (P=0.014). Moreover, PTP1B was highly expressed in all breast cancer patients with distant metastasis. However, no significant correlation was found between the level of PTP1B and the patients with metastasis (P=0.388). From Table 2, the PTP1B level in T2+T3+T4 was higher than that in T1 (P=0.029, relative risk (RR)=3.818, 95% confidence interval (CI) 1.105–13.198). The patients with lymph node metastasis (N1+N2+N3) have a higher PTP1B level than those without lymph node metastasis (P=0.003, RR=5.538, 95% CI 1.724–17.797). We also found that advanced breast cancer (II+III+IV) tissue had a higher PTP1B level than the early stage breast cancer tissue. The data from Table 1 also showed that the higher PTP1B level was correlated with estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and human epidermal growth factor receptor 2 (HER2)-positive tumors.

3.2 Effect of PTP1B on the proliferation of breast cancer cells

To delineate the proliferation role of PTP1B in human breast cancer cells, we used lentiviral delivery of shRNA to knock down *PTP1B*. As shown in Figs. 2a and 2b, effective and selective knockdown of *PTP1B* was achieved. The expression of PTP1B in MCF-7/PTP1B shRNA was lower than that in MCF-7 cells. The proliferation of MCF-7 cells was much faster than that of MCF-7/PTP1B shRNA cells. The proliferation ability decreased with time, especially after 24 h (Fig. 2c).

Table 2 Relationship between PTP1B expression and TNM stage in breast cancer

Variable	PTP1B[*]		Overall P	RR	95% CI
	Negative	Positive			
Tumor stage (T)			0.029	3.818	1.105–13.198
T1	7 (50%)	7 (50%)			
T2+T3+T4	11 (21%)	42 (79%)			
Lymph node (N)			0.003	5.538	1.724–17.797
N0	12 (48%)	13 (52%)			
N1+N2+N3	6 (14%)	36 (86%)			
TNM stage			0.015	4.561	1.273–16.334
I	7 (54%)	6 (46%)			
II+III+IV	11 (20%)	43 (80%)			

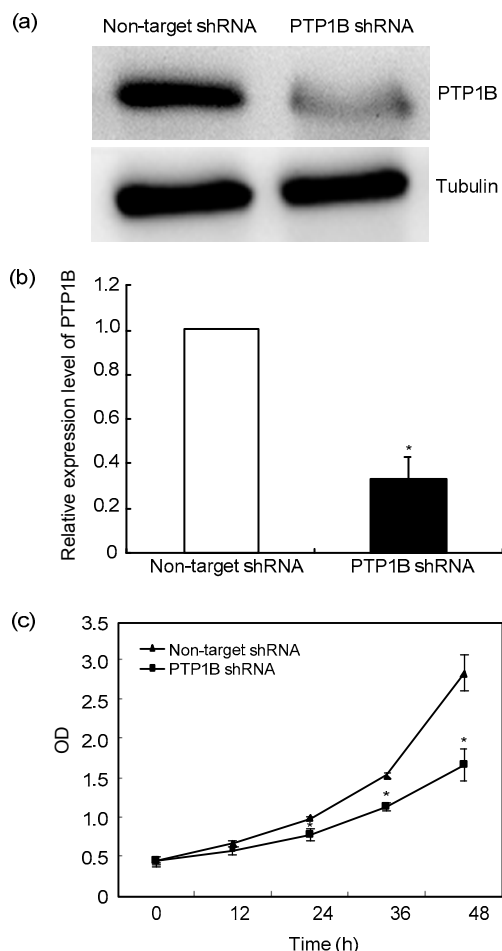[*] Data are expressed as number (percent)

Fig. 2 Effect of PTP1B on the proliferation of MCF-7 cells

(a) Western blotting analysis showed that PTP1B protein expression in MCF-7 cells was much higher than that in MCF-7/PTP1B shRNA cells; (b) Quantifications of PTP1B autoradiograms are represented as mean±SD (* $P<0.05$, vs. non-target shRNA); (c) Cell growth (mean±SD) was assessed with a cell-counting kit 8 (CCK8). Knocking down the PTP1B expression decreased the proliferation of cells (* $P<0.05$, vs. non-target shRNA). All results were representative of three independent experiments

3.3 Effect of PTP1B on migration of breast cancer cells

To investigate the effect of PTP1B on migration of breast cancer cells, we used two different methods, scratch motility assay and Transwell migration assay. For the scratch motility assay, the wound closure was observed after the monolayer cell was scratched for 24 h. A significantly enhanced wound closure was observed in MCF-7 cells (Fig. 3a). The motility of MCF-7 was almost twice of that in MCF-7/PTP1B

shRNA cells (MCF-7 vs. MCF-7/PTP1B shRNA cells: 13.13 μm/h vs. 7.24 μm/h). In addition, Transwell assay also indicated that the invasiveness and migration had association with PTP1B (Fig. 3c). The migration and invasiveness of MCF-7/PTP1B shRNA cells reduced by 51.78% compared to MCF-7 cells.

3.4 Effect of PTP1B on the production of CCL5

In the previous study, we found that the proliferation and migration of breast cancer are closely correlated with CCL5 (Zhang *et al.*, 2009). Research has also shown that U-STAT3 can effectively compete with non-phosphorylated inhibitory κB (IκB) in binding with nuclear factor (NF)-κB to form a new transcription factor. The new transcription factor could induce the expression of CCL5 (Yang *et al.*, 2005; 2007; Yang and Stark, 2008; Zhang *et al.*, 2014). Therefore, we detect the expressions of phosphorylated STAT3, U-STAT3, and CCL5 in both MCF-7 cells and MCF-7/PTP1B shRNA cells by Western blot. The results are shown in Fig. 4. When the gene of *PTP1B* was knocked down, the phosphorylated level of STAT3 was significantly increased, but the levels of U-STAT3 and CCL5 were significantly decreased. Therefore, we postulated that the expression of CCL5 might be mediated through PTP1B.

4 Discussion

Previous studies of PTP1B expression in different kinds of cancer and tumor cells have found variable expression levels and conflicting effects of this phosphatase in oncogenesis (Warabi *et al.*, 2000; Dubé *et al.*, 2005; Julien *et al.*, 2007; Zhu *et al.*, 2007). Up to now, the function of PTP1B is still unclear. Some studies have found that PTP1B is required for HER2/*Neu*-induced breast cancer, but the correlation between the expression of PTP1B and clinicopathologic factor was not clarified in breast cancer. To answer this question, we designed this experiment. We found PTP1B was overexpressed in over 70% breast cancer tissues. The result also provides evidence that the expression of PTP1B may adversely alter the presentation of breast cancer. The data from our study showed that both tumor size and lymph node metastasis have a significantly higher stage in
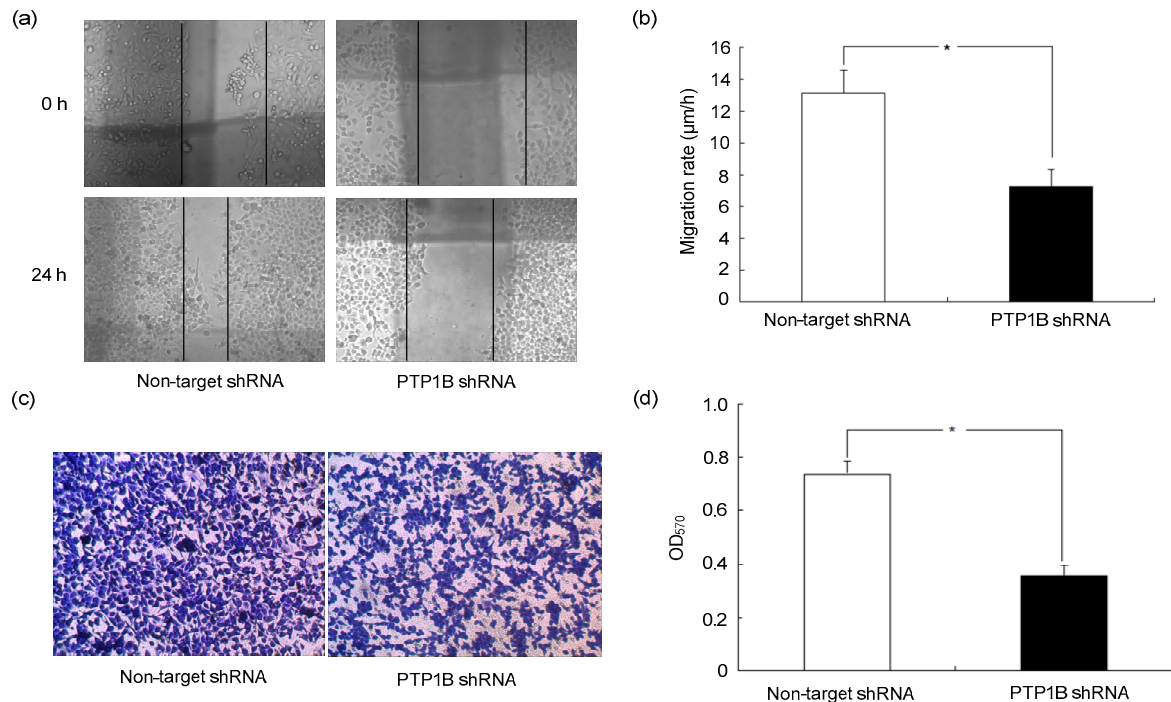
Fig. 3 Effect of PTP1B on the migration of breast cancer cells

Low level of PTP1B decreased the migration of breast cancer cells. (a) Scratch motility assay; (b) The velocity of cell migration into the scratched area was counted at five randomly selected field and represented as mean±SD; (c) Transwell migration assay; (d) Optical density values at 570 nm (OD_{570}) in staining positive cell were represented as mean±SD. * $P<0.05$. All results were representative of three independent experiments
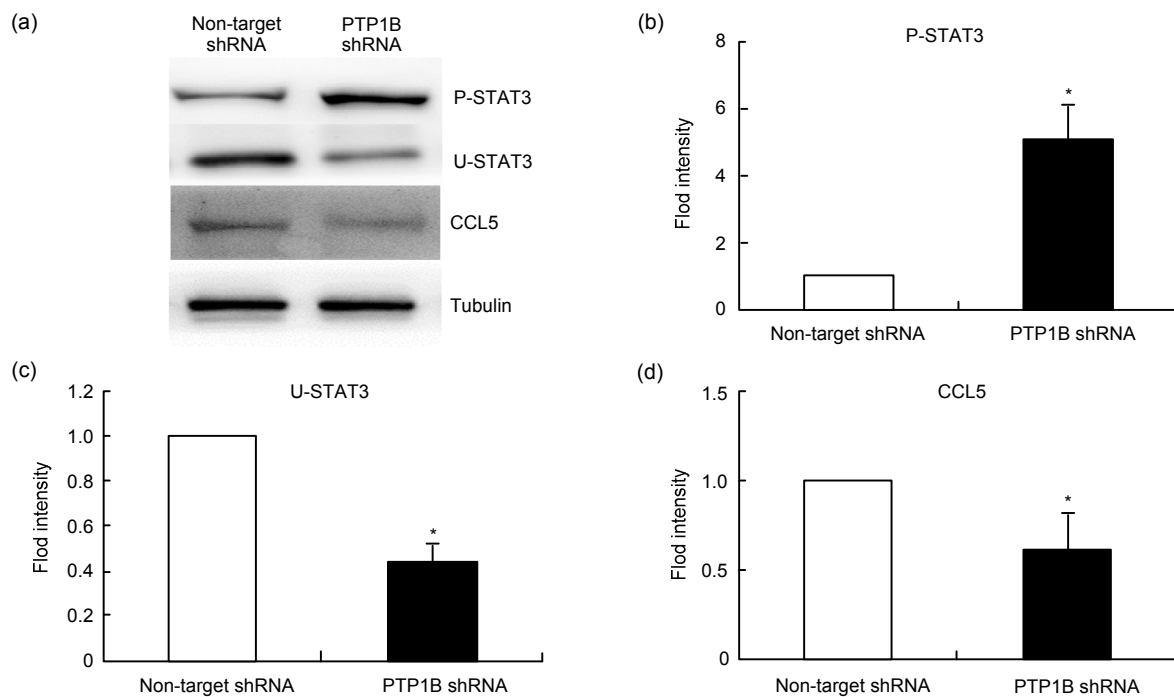

Fig. 4 Effect of PTP1B on the expressions of proteins P-STAT3, U-STAT3, and CCL5

(a) Western blotting analysis showed that knocking down the PTP1B increased the phosphorylation levels of STAT3 and decreased the levels of U-STAT3 and CCL5. (b, c, d) Quantifications of P-STAT3, U-STAT3, and CCL5 autoradiograms are represented as mean±SD. * $P<0.05$, vs. non-target shRNA. All results were representative of three independent experiments. P-STAT3: phosphorylated STAT3; U-STAT3: unphosphorylated STAT3; CCL5: C-C motif chemokine ligand 5

patients with a higher level of PTP1B; the RR is 3.818 and 5.538, respectively. We also found tumors at an advanced stage (II+III+IV) expressed a high level of PTP1B. When we compared the expression of PTP1B and the clinicopathologic characteristics of 67 breast cancer patients, the results also showed that a higher level of PTP1B was correlated with patients with ER-negative, PR-negative and HER2-positive tumors. These phenomena indicated that PTP1B may play a crucial role in the development of breast cancer. To investigate the role of PTP1B in proliferation and migration of breast cancer, we knocked down the gene of *PTP1B* in MCF-7 cells. Our results showed that knocking down the gene of *PTP1B* will inhibit the proliferation and migration of MCF-7 cells. From the current study, we consider that PTP1B could up-regulate the dephosphorylated level of STAT3, which could increase the expression of CCL5.

Some studies also found that PTP1B could promote the development of breast cancer. Bentires-Alj and Neel (2007) have reported that transgenic overexpression of an activated form of HER2 (NeuNT) in the PTP1B$^{+/+}$ mice mammary gland, under the control of mouse mammary tumor virus (MMTV) promoter (MMTV-NeuNT mice), causes glandular hyperplasia followed by mammary adenocarcinoma in fewer than 13 months on average, but PTP1B$^{-/-}$ mice developed mammary tumors over a 3-year period. Julien *et al.* (2007) found that inhibiting the expression of PTP1B might prevent the occurrence of breast cancer. His study also indicated that PTP1B has association with the lung metastasis of breast cancer. This phenomenon was also found in other tumors, such as gastric and colon cancers (Zhu *et al.*, 2007; Wang *et al.*, 2012).

Currently, there are several mechanisms by which PTP1B could promote tumor development. Some studies have found that high expression of PTP1B will abnormally activate some oncogenes (Nanney *et al.*, 1997). Another mechanism is that PTP1B could catalyze Src dephosphorylation and Src Tyr530 phosphorylation could reduce Src activation. Therefore, PTP1B might promote the activation of Src. The activity of Src was overexpressed in several types of human cancers and played critical roles in a variety of cellular signal transduction pathways (Zhu *et al.*, 2007; Arias-Romero *et al.*, 2009). In the study of Arias-Romero *et al.* (2009), their results suggested

that Src was a key target in mediating the positive role of PTP1B in ErbB2 oncogenesis. Furthermore, PTP1B is also proposed to dephosphorylate the scaffolding adapter p62DOK, which binds p120 Ras GTPase-activating protein (RasGAP) and promotes Ras/extracellular signal-regulated kinase (ERK) pathway inactivation (Dubé *et al.*, 2004; Bentires-Alj and Neel, 2007). Bentires-Alj and Neel (2007) found that PTP1B mainly exists in HER2-positive breast cancer tissue, which is similar to our conclusion, and they considered that ErbB2 is another target of PTP1B in contributing to tumorigenesis in breast cancer. Moreover, PTP1B can also regulate insulin-like growth factor 2 (IGF-2)-induced MCF-7 cell migration. Herein, we described a novel mechanism: PTP1B could increase the expression of U-STAT3, which can effectively compete with non-phosphorylated IκB in binding with NF-κB to form a new transcription factor, and a new transcription factor could induce the expression of CCL5 Yang *et al.*, 2005; 2007; Yang and Stark, 2008). It has been reported that the proliferation and migration of breast cancer have a close relationship with CCL5, and CCL5 is considered as an independent prognostic factor in breast cancer (Karnoub *et al.*, 2007; Pinilla *et al.*, 2009; Zhang *et al.*, 2009).

In conclusion, PTP1B was overexpressed in breast cancer tissues, and the level of PTP1B in breast cancer was significantly correlated with the tumor and node stage. Our results also showed that the deficiency of PTP1B will inhibit the proliferation and migration of MCF-7 cells. Our study indicated that the expression of PTP1B may adversely alter the presentation of breast cancer. In recent years, several pharmaceutical companies have programs to develop PTP1B inhibitors to treat obesity, diabetes, and other important medical disorders (Klaman *et al.*, 2000; Bence *et al.*, 2006). Our study suggests that a PTP1B inhibitor can be used for the treatment of breast cancer.

Compliance with ethics guidelines

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中文概要

题　目：蛋白酪氨酸磷酸酶 1B 在乳腺癌进展中的作用

目　的：讨论蛋白酪氨酸磷酸酶 1B（PTP1B）在乳腺癌中的表达模式，及其在肿瘤进展中所起的作用。

创新点：首次证明了 PTP1B 能够增加非磷酸化信号转导与转录激活因子 3（STAT3）的水平，而非磷酸化 STAT3 与核转录因子（NF-κB）形成新的转录因子介导 C-C 型趋化因子配体 5（CCL5）的表达，从而促进肿瘤细胞增殖和迁移，导致肿瘤恶性程度增加。

方　法：收集 67 名乳腺癌患者的癌组织和癌旁组织标本，并记录患者临床病理学特征，使用蛋白质免疫印迹法（Western blot）检测乳腺癌组织及癌旁组织 PTP1B 含量；研究 PTP1B 水平与患者临床病理特征关系；制作 PTP1B 基因敲除 MCF-7 细胞系，分别使用细胞生长实验、Transwell 细胞迁移实验和划痕活力实验检测基因敲出组与对照组细胞增殖及迁移能力，使用 Western blot 检测两组细胞中磷酸化与非磷酸化 STAT3 表达水平和 CCL5 表达水平。

结　论：大约 70%乳腺癌患者高表达 PTP1B，并且随着患者 TNM 分期增加，患者 PTP1B 表达水平不断增加，同时 PTP1B 高表达与 ER−、PR−和 HER2+ 相关（表 1 和 2）；与对照组相比，PTP1B 基因敲除组中肿瘤细胞增殖及迁移能力明显下降（图 2 和 3），同时 PTP1B 可以通过上调 STAT3 非磷酸化水平来提高 CCL5 的表达，从而加快乳腺癌发生及发展（图 4）。总之，PTP1B 对乳腺癌的发生起到至关重要的作用。

关键词：蛋白酪氨酸磷酸酶 1B（PTP1B）；信号转导与转录激活因子 3（STAT3）；乳腺癌；肿瘤发生