PIBF1 in breast cancer:

Candidate for predictive factor and effect on taxane-based chemotherapy response

유방암에서 PIBF1 의 예측인자에 대한 가능성과 탁센 기반

항암치료 반응성과의 연관성에 관한 연구

울산대학교대학원

의 학 과

신 은 주

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이 논문을 의학박사 학위 논문으로 제출함

2023년 10월

울산대학교대학원

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Contents

Abstract

Background

   With the increasing incidence of breast cancer and the array of available treatment choice, it is imperative to investigate tumor cell-associated markers that can predict the disease's nature. Progesterone-induced blocking factor 1 (PIBF1), originally associated with pregnancy-induced immunity, is produced in tumors to evade maternal immunity. PIBF1 is revealed to overexpressed in several cancers, including breast, cervical and lymphoma. However, little research exists on PIBF1' role in breast cancer and its clinical outcomes. We thus studied the link between PIBF1 expression and prognosis, as well as its influence on chemotherapy response.

Materials and Methods

   Samples, obtained from 231 high risk TNBC breast cancer patients underwent surgery between 2008 and 2013, with lymph node metastasis, received taxane-based adjuvant chemotherapy and selected 238 non TNBC patients matched with TNBC patients were selected. Anti-PIBF1 antibody was used for immunohistochemical detection of the PIBF1 protein in tissues, whose sections were analyzed with cut-off value of 3 (intensity plus proportion). Kaplan-Meier survival analysis was used to assess the probability of overall survival (OS). Utilizing the colonogenic unit assay and employing knockdown methodologies in breast cancer cell lines, we examined the correlation between PIF1 expression and chemosensitivity.

Results

   In a study of 469 breast cancer patients, both non-TNBC (n = 238) and TNBC (n = 231), those with PIBF1 expression manifested a lower histologic grade ($p < 0.001$), reduced p53 ($p < 0.001$) and decreased Ki-67 ($p < 0.001$) compared to their non-expressing counterparts. This differential was particularly pronounced in non-TNBC subset. Survival analysis revealed a significant difference in OS for patients with PIBF1, with non-TNBC patients showing superior outcomes. In multivariable

analysis with OS, PIBF1 expression shows relation with better prognosis and the statistical significance was borderline (hazard ratio = 0.44, 95% confidence interval = 0.18-1.11, p = 0.082). *In vitro* experiments revealed a correlation between PIBF1 expression in breast cancer cell lines (BT549, HCC70, BT20, HS578T) and their sensitivity to paclitaxel, with certain cell lines showing significant viability reductions and also resisting the treatment after PIBF1 knockdown.

Conclusions

We observed a correlation between PIBF1 expression and better prognosis in breast patients with nodal metastasis undergoing taxane-based chemotherapy, especially in non-TNBC cohort. Additionally, we discerned a relationship between PIBF1 and chemosensitivity in our *in vitro* studies. These insights suggest the potential utility of PIBF1 as a predictive marker in determining therapeutic approaches.

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Introduction

Breast cancer remains a global health concern, impacting a vast majority of individuals each year. With its complex and diverse pathology means that patients present with a broad range of clinical manifestations and prognosis, there is a clear challenge in stratifying patents to the most suitable treatment option, with each therapeutic approach bearing its unique set of toxicities[1]. Also, this emphasizes the necessity for development and validation of new tumor markers which could provide insights into refining treatment strategies and prognosis determination[1-3].

Progesterone-induced blocking factor 1 (PIBF1), initially identifies as a secretory product of lymphocytes during human pregnancy, has been characterized as an immunoregulatory molecule pivotal for the sustenance of pregnancy. Current research revealed an elevated expression of PIBF1 in tumor cells relative to their normal counterparts across diverse malignancies. Despite its potential significance, comprehensive studies about the molecular mechanisms of PIBF1 remain sparse. Hypothesized mechanisms through which PIBF1 might influence tumorigenesis include modulating anti-neoplastic immune responses, instigating apoptosis via p53 upregulation, and orchestrating cell cycle dynamics[4]. It is noteworthy to mention that the 35 kDa secretory variant of PIBF1 is predominant during pregnancy. In contrast, the 90 kDa full-length version, which is associated with centrosomes, is prevalent in cancerous states. Given the association of several tumorigenic proteins with centrosomes and the resultant chromosomal missegregation due to compromised centrosome functionality, this observation gains importance[5]. Moreover, the genomic mapping of the PIBF1 gene on chromosome 13 corresponds to a region implicated in breast cancer susceptibility. Interestingly, breast carcinoma cells exhibit autonomous PIBF1 production, independent of progesterone, manifesting a pronounced expression in comparison to unaltered breast tissues[3, 5, 6].

PIBF1, primarily recognized for its immune modulation activities, appears to have potential influence on cancer cell growth. Investigations into its exact mechanism reveal its involvement in regulating

cytokine production, which in turn may affect cellular processes such as proliferation and apoptosis[2, 3]. Its involvements in several other malignancies, such as cervical, lymphoma, and leukemia has been the subject for ongoing investigations, with preliminary findings underscoring its potential as a valuable tumor marker[7-9]. Also, in a previous study undertaken by Ro et al., the role of PIBF1 in modulating the ATR/CHK1 signaling pathway and its inhibitory effects on the proliferation and migration of TNBC cell lines was investigated[3]. These findings revealed the oncogenic role of PIBF1, offering novel perspectives on its functional attributes and associated molecular mechanisms in breast cancer.

Nowadays, with the advancement of chemotherapeutic agents and their efficacy, the role of chemotherapy is becoming increasingly significant in breast cancer management. The number of patients undergoing chemotherapy is on the rise, especially among those with axillary metastasis. Even in cases without metastasis, genetic testing has enabled some patients to receive chemotherapy as adjuvant therapy, when demonstrated beneficial for preventing recurrence. Consequently, investigating factors associated with chemotherapy, such as chemosensitivity or predictive response markers, is crucial for tailoring appropriate therapeutic strategies.

The primary objective of this study revolves around exploring the clinicopathologic attributes of PIBF1 in breast cancer and to assess its therapeutic implication by examining the association between PIBF1 and chemosensitivity through a comprehensive approach, employing both immunohistochemistry (IHC) and cell-line experiments. We aim to unearth the utility of PIBF1 as a prognostic tool within the breast cancer spectrum, potentially aiding in anticipating the treatment response and consequently optimizing patients' management[5, 6].

Materials and Methods

Patients

In this retrospective investigation, tissue specimens were procured from a cohort comprising 469 high-risk patients. These patients underwent surgical interventions at the Asan Medical Center, Seoul, Korea, within the timeframe of January 2008 to December 2013. All included patients exhibited pathologically lymph node positive and subsequently received taxane-based adjuvant systemic chemotherapy. For analytical purposes, 231 TNBC breast cancer patients were selected with the condition and 238 non-TNBC patients were matched. Pertaining to the systemic chemotherapy administered, a taxane-based regimen was universally adopted for all patients. Standard therapeutic interventions were adhered to: individuals underwent either breast-conserving surgery or mastectomy coupled with axillary procedures. Patients diagnosed with HR+ breast cancer received treatments with tamoxifen or aromatase inhibitors, with optional ovarian function suppression. Furthermore, those identified with HER2+ neoplasms were administered adjuvant targeted therapies, and those who opted for breast-conserving surgeries underwent subsequent radiotherapy. The ethical facets of this research were scrupulously addressed, with approval procured from the Institutional Review Board of the Asan Medical Center (Approval No. 2021-0004). Given the retrospective nature of the data underpinning this study, the necessity for informed consent was dispensed with.

Clinicopathologic analysis

Clinicopathological data encompassing factors such as age, surgical approach to the breast and axilla, and the TNM staging were meticulously collated. Histopathological parameters, inclusive of histologic grading, lympho-vascular invasion status, breast cancer subtyping, and Ki-67, were obtained. Additionally, the utilization of radiotherapy as an adjuvant therapeutic modality was documented.

Tissue specimens were fixed using 10% Buffered Formalin (Sigma-Aldrich, St. Louis, Missouri) and

subsequently embedded in paraffin (Sigma-Aldrich). From each case, a singular tissue block embedded in paraffin was procured. These blocks were then sectioned into 4-micron thick slices. Ensuing paraffin removal, these sections underwent rehydration and were subsequently treated with a target retrieval solution. Treatment with 3% H_2O_2 (Sigma-Aldrich) was employed to quench endogenous peroxidase activity, followed by a blockade of nonspecific immunoglobulin binding using 10% goat serum (Sigma-Aldrich). Primary rabbit polyclonal anti-PIBF antibody[7] (Sigma-Aldrich, AE030801) was utilized for section incubation at a dilution of 1:300. Post-incubation, sections were subjected to washes using phosphate-buffered saline (Sigma-Aldrich), followed by incubation with secondary antibodies (Sigma-Aldrich) and 3,3'-diaminobenzidine (Sigma-Aldrich). Counterstaining was accomplished employing hematoxylin and eosin (Sigma-Aldrich). Expert pathologists, leveraging polarized light microscopy (Nikon Eclipse Ni-E; Nikon, Japan), meticulously evaluated each section. For analytical considerations, sections displaying the zenith of tumor cell staining were chosen. The expression levels of PIBF were quantified using the quick score, integrating both general staining intensities (0: negative; 1+: mild intensity; 2+: moderate intensity; 3+: intense staining) and percentages of positive tumor cell staining (1+: 1%-20%; 2+: 21%-50%; 3+: >50%). Digital documentation of the preparations was executed using a camera (Nikon DS-Fi2; Nikon).

Cell lines and cultures

Protein extracts were prepared using human breast cancer cell lines, BT549, MM231, HCC70, MCF7, BT20, ZR-75-1, SK-BR-3, HCC1395, and HS578T cell were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco® Life Technologies, USA) and 1% penicillin/streptomycin solution (Gibco® Life Technologies, USA) in a humidified incubation of 37°C and 5% CO_2.

Plasmid and Generation of cell lines with stable overexpression using a lentiviral infection system

Human PIBF1 cDNA (GeneBank accession No. NM_001349655) was PCR amplified and the complete nucleotide sequences were cloned into the pCMV delta R8.2. The vector was transformed primary embryonic kidney cells (293FT; Invitrogen) then used for packaging lentiviruses (cotransfection of pRSV-Rev, pMDLg/pRRE and pMD2.G; 3rd generation transfer plasmids, Addgene) for 36 hours. Viral particles were then concentrated from 293FT host cells using a Lenti-X™ concentrator (Clontech). HS578T cells were infected with the particles to produce cell lines with stable overexpression of PIBF1.

Measurement of mRNA expression

Total RNA was extracted from breast cancer cell lines using TRIzol reagent (Invitrogen), and 1 μg total RNA from each sample was transcribed. RT-PCR, consisting of 27-29 cycles at 94°C for 30 sec, 57°C for 50 sec, and 72°C for 1 min, was performed using the PIBF1 specific primer (NM-001349655), sense 5'-ccgagaagcaagggataatg-3', antisense 5'-aaaagaacccttcagcctc-3'. As an internal standard, glyceraldehyde 3-phosphate dehydrogenase was amplified and analyzed under identical conditions, using the following specific primers: sense 5'-gaccccttcattgacctc-3' and antisense 5'-gctaagcagttggtggtg-3'.

Immunoblotting (IB) assay

Protein extracts were prepared using pro-prep lysis buffer (Intron Biotech., Sungnam, Korea) with additional protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA). The proteins were separated on an 10% SDS-PAGE gel, transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Freiburg, Germany). The blotting was performed using pibf1 (1:1000, Cat ab72118, abcam, Cambridge, UK) and β-actin (1:5000, Santa Cruz, California, USA). Proteins of

expression were visualized using an enhanced chemiluminescence system (Amersham Biosciences, Little Chalfont Buckinghamshire, UK).

Cell viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (TACS MTT kit, #4890-25-02, TREBIGEN® Instruction) was used to assess cell viability in proliferation assay context according to the manufacturer's instructions.

Colony formation assay

Un treated control and 2nM paclitaxel-treated cells were plated at a low density (200~600 cells/well) in RPMI1640 media. Fresh medium of finial 2nM paclitaxel was added once every three days. After two weeks of culture, cells were fixed with 4% formaldehyde for 10min and stained with 0.05% crystal violet for 2 hours.

Statistical analysis

We established an IHC cut-off score at 3 (comprising both intensity and proportion) was delineated as the median value for categorization. Scores at or above this threshold were classified as high PIBF1 expression, while scores below this were considered representative of low PIBF1 expression,

To discern the ramifications of PIBF1 in the context of breast cancer, we scrutinized two pivotal outcomes: Overall Survival (OS) and Disease-Free Survival (DFS) across both TNBC and non-TNBC patient cohorts, bifurcated based on PIBF1 expression. DFS was delineated as the interval commencing from the surgery date to the earliest manifestation of local or regional recurrence, distant metastasis, or any mortality. Conversely, OS signified the duration from the breast cancer diagnosis date leading up

to any mortality, irrespective of its association with breast cancer.

Baseline variables, segregated by the presence or absence of PIBF1, underwent rigorous statistical analyses. We employed the chi-squared test, Fisher's exact test, and Mann–Whitney U test to ascertain the significance of our findings. Survival outcomes, namely OS and DFS, were graphically represented through the Kaplan–Meier product-limit method, accompanied by the computation of the log-rank p-value. To evaluate the prognostic implications of the clinicopathological factors, hazard ratios, 95% confidence intervals, and p-values were gleaned via the Cox proportional hazards model. All statistical evaluations were two-tailed, and an alpha level below 0.05 was designated as the threshold for statistical significance. All computational analyses were orchestrated utilizing the Statistical Package for the Social Sciences (SPSS, ver. 20, Armonk, NY, USA).

Clonogenic unit assay and knockdown of breast cancer cell lines

Cell lines were cultivated in the 24-well plates supplemented with complete RPMI-1640 medium (10% FBS and 1% antibiotic). After 24-hour incubation, cells were subjected with 2 or 5 nM paclitaxel, and a combination of both for 5 days at 37℃, 5% CO_2 in a water-jacketed incubator. After air drying the cells for 60 minutes at ambient temperature, they were stained with crystal violet solution for 5 minutes. Excess dye was gently rinsed with water, and the plates were air-dried inverted. Imaging was performed, and colonies with a diameter exceeding 0.5mm were enumerated. The colonogenic assay was replicated thrice across all groups.

To further elucidate this association, PIBF1 knock-down was achieved using 200pmole pooling served- three siRNAs of PIBF1 (NM_001349655.1: cat no. 10464-1, 10464-2, 10464-3; Bioneer, Korea). H578T cells were transfected using the Lipofectamin® RNAiMAX (Invitrogen, Carlsbad, CA, USA) reagent according to the manufacturer's instructions.

Cell viability was ascertained using the EZ-Cytox Cell Viability Assay Kit, measured at 450 nm. The implications of PIBF1 manipulation were then deduced by comparing the relative cell viability of the knock-down cohort to its control counterpart.

Results

Patients' characteristics

Table 1 provides a comparison of clinical and histopathological parameters in a cohort of 469 breast cancer patients, categorized them into non-TNBC (n = 238) and TNBC (n = 231) categories. Age distribution was remarkably consistent across both groups, with a mean age of 47.4 ± 9.1 years. In terms of surgical interventions, breast-conserving surgery was opted for by 55.0% of the non-TNBC population, in contrast to a larger 70.0% in the TNBC category. On the flip side, 45.0% of non-TNBC patients elected for a total mastectomy, compared to a lesser 30.0% in the TNBC. This divergence in surgical choices was statistically significant ($p = 0.001$). With tumor stages, there were significant discrepancies ($p = 0.015$) observed. Most notably, T2 tumors were predominantly diagnosed in the TNBC subset (60.2%) as against 48.3% in their non-TNBC counterparts. Exclusively in the non-TNBC category, receptor statuses revealed that 77.3% were ER-positive, 60.9% were PGR-positive, and a significant 66.7% were HER2-negative. A rigorous analysis of tumor grades unveiled that TNBC patients predominantly had G3 histologic (82.0%) and nuclear (82.5%) grades, in stark contrast to the 35.3% and 36.6%, respectively, in the non-TNBC category. These grading variations bore statistical significance ($p < 0.001$). Expression analyses showed a marked upregulation of P53 in TNBC, with a pronounced strong expression in 43.0% of cases, dwarfing the 14.9% observed in non-TNBC. These expression differentials were statistically meaningful ($p < 0.001$). Furthermore, elevated Ki-67 expression (>20%) was predominantly seen in the TNBC group at 83.3%, overshadowing the 43.2% in the non-TNBC group ($p < 0.001$). In therapeutic interventions, radiotherapy was administered to 84.0% of TNBC patients, which was significantly higher than the 74.4% in the non-TNBC group ($p = 0.010$).

Upon categorizing patients based on PIBF1 status (Table 2), no statistically significant variations were observed in the TNM stage. However, salient differences in receptor status, tumor grade, and molecular markers, specifically P53 and Ki-67, were apparent in relation to PIBF1 status. The estrogen

receptor (ER) and progesterone receptor (PGR) were notably less prevalent in low PIBF1 patients, documented at 10.3% and 6.9%, respectively. This contrasted with high PIBF1 patients where the figures were substantially higher: 61.3% for ER and 49.2% for PGR. Both distinctions were statistically significant ($p < 0.001$). Furthermore, the HER2-negative phenotype was more commonly exhibited among low PIBF1 patients at 45.2% compared to 71.7% in the high PIBF1 cohort. This discrepancy was statistically significant ($p = 0.001$). In terms of histologic gradation, low PIBF1 patients demonstrated a pronounced proclivity for G3 histologic (78.5%) and nuclear (79.0%) grades, which was significantly higher than the corresponding figures in high PIBF1 patients: 42.9% and 44.0% ($p < 0.001$). Finally, molecular marker evaluation revealed an intensified strong P53 expression in low PIBF1 patients (39.3%) compared to their high PIBF1 counterparts (20.8%), a difference underlined by a p-value <0.001. Additionally, a high Ki-67 expression (>20%) was predominantly identified in low PIBF1 patients (82.4%) relative to high PIBF1 patients (46.5%), a distinction that was also statistically significant ($p < 0.001$).

On stratification of patients into either TNBC or PIBF expression, some characteristics showed even more clear statistical significance in the non-TNBC subgroup (Table 3). In the non-TNBC cohort, PIBF1 expression was related to a lower nodal stage ($p = 0.025$), lower pathologic stage ($p = 0.024$), and lower histologic grade ($p = 0.006$). But these differences were not distinct in TNBC subgroup.

Survival outcomes according to PIBF

Figure 1 displays the Kaplan–Meier curves for OS and DFS, stratified by the high or low of PIBF1 expression within the total study population. Significant differences in OS between the two groups were observed ($p = 0.010$), additionally, while there was a result of better DFS in high PIBF1 group, its statistical significance approached the threshold of validation ($p = 0.055$). Among the 105 patients who exhibited recurrence, 15 events of local recurrence, 11 events of regional recurrence, and 79 events of distant metastasis were reported within a median follow-up period of 98.2 months (5.5–140.7 months).

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Notably, 76 patients died during this period. We also found that patients with PIBF1 had a five-year OS rate of 92.5% compared to those without PIBF1 who had an OS rate of 84.7% ($p = 0.010$) (Fig. 1a). Also, the five-year DFS rate was 92.7% for patients with PIBF1 and 86.7% for patients without PIBF1 ($p = 0.055$). (Fig. 1b).

Based on the analysis segmented into TNBC and non-TNBC cohorts, the non-TNBC cohort mirrored the overall population's outcomes. Specifically, the group with PIBF1 demonstrated superior outcomes in both OS and DFS, and these differences were statistically significant ($p = 0.013$ for OS and $p = 0.025$ for DFS) as depicted in Figures 2a and 2b. Conversely, in the TNBC cohort, no statistical significance was observed, as shown in Figures 3a and 3b.

According to the univariate analysis of the non-TNBC cohort, pT, ER, HER2 status, and histologic grade were not statistically significant, but pN stage (hazard ratio = 5.70, 95% confidence interval = 2.44-13.33, $p < 0.001$), PGR (hazard ratio = 0.40, 95% confidence interval = 0.18-0.90, $p = 0.026$), lymphovascular invasion (hazard ratio = 4.68, 95% confidence interval = 1.86-11.79, $p = 0.001$), p53 (hazard ratio = 2.53, 95% confidence interval = 1.07-5.98, $p = 0.035$) and presence of PIBF1 (hazard ratio = 0.35, 95% confidence interval = 0.15-0.83, $p = 0.017$) were statistically significant predictors of the OS (Table 4). In the multivariate analysis of the non-TNBC cohort, PIBF1 emerged as a favorable prognostic factor for OS, though this association did not reach statistical significance (hazard ratio = 0.44, 95% confidence interval = 0.18-1.11, $p = 0.082$).

Association of PIBF1 with Paclitaxel sensitivity: insights from breast cancer cell line experiments

All patients in the study underwent taxane-based chemotherapy, prompting our focus on the potential relationship between PIBF1 expression and chemotherapy sensitivity. To elucidate this, we conducted *in vitro* experiments using various breast cancer cell lines. Specifically, BT-549 and BT-20 (both from ATCC, Manassas, VA, USA) exhibited elevated PIBF1 expression, while HCC70 and HS578T (also

from ATCC, Manassas, VA, USA) displayed reduced PIBF1 expression (Fig. 5).

In the cell viability assays, upon administration of 5nM paclitaxel, BT549 and BT20 demonstrated significant reductions in cell viability (BT549: 13.6%; BT20: 15.0%), in contrast to HCC70 and HS578T, which retained over 80% viability (Fig. 6).

In the colonogenic assays, the BT549 cell line exhibited a 69.8% reduction in colonogenic count, with BT20 showing complete colony eradication. Conversely, the HS578T cell line demonstrated a mere 26.7% decline, and interestingly, the HCC70 cell line exhibited an increase in colony count (Fig. 7)

To further substantiate the relationship between PIBF1 expression and paclitaxel sensitivity, we conducted an siRNA-mediated knockdown of PIBF1 in the BT20 cell line. Following this genetic intervention, the knock-down cell lines demonstrated a diminished response to paclitaxel treatment compared to controls (Control: $100 \pm 51.5\%$ decreasing to $32.43 \pm 12.5\%$; Knock-down: $99.9 \pm 29.6\%$ decreasing to $68.35 \pm 18.7\%$) (Fig. 8).
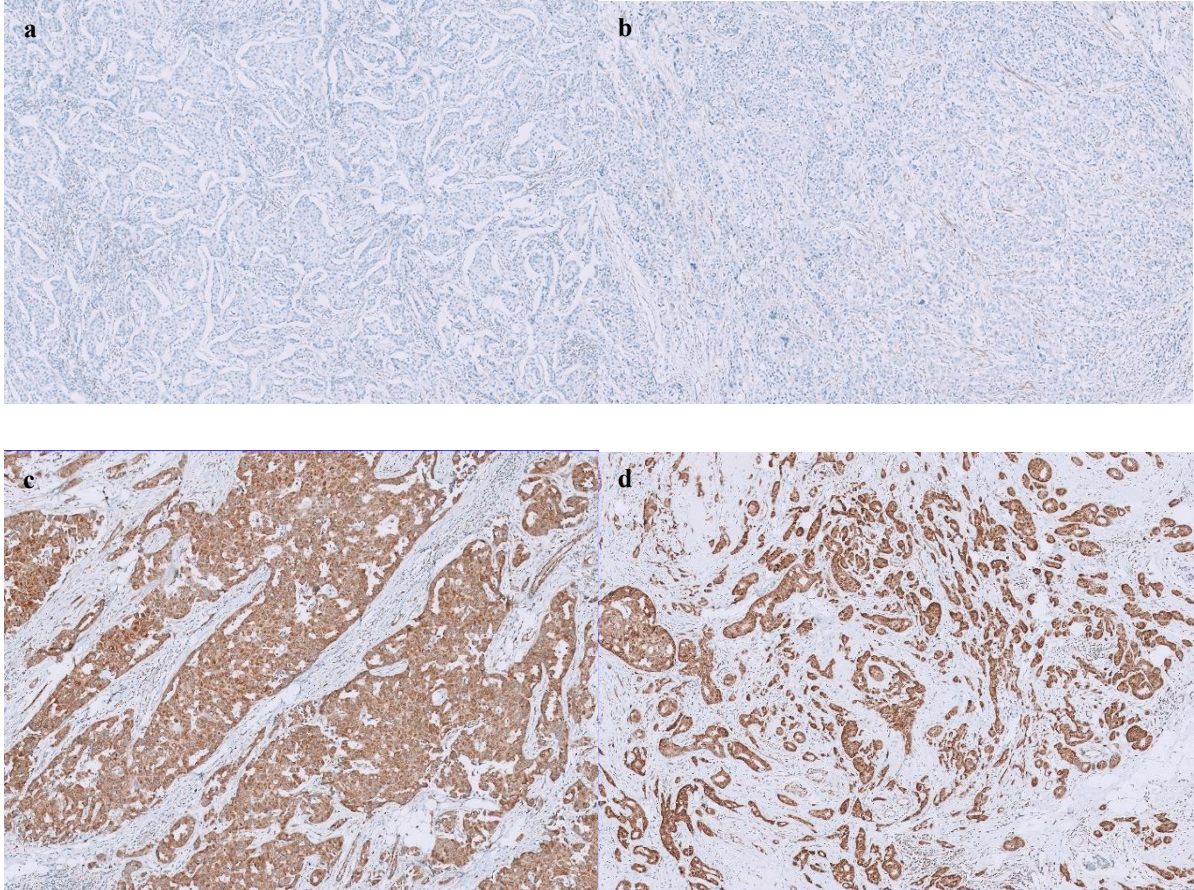
Fig. 1. Representative pathologic images of PIBF1 expression in breast cancer IHC

(Original magnification x200) a. PIBF1 low expression in non-TNBC tumor b. PIBF1 low expression TNBC tumor c. PIBF1 high expression in non-TNBC tumor d. PIBF1 high expression in TNBC tumor
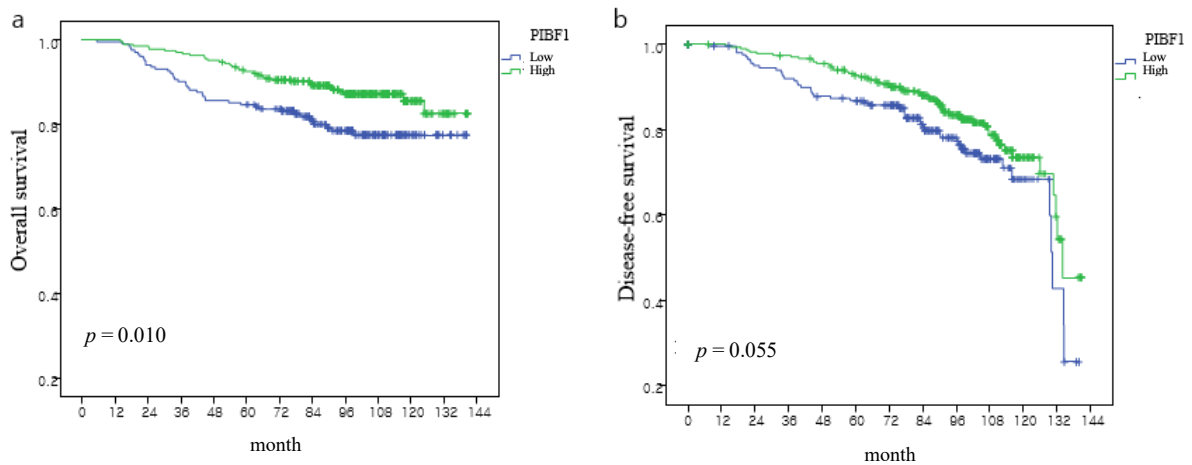
Fig. 2. Kaplan-Meier curves showing survival outcomes and disease-free survival according to PIBF1 expression in overall population

This demonstrates enhanced OS(a) and DFS(b) in the cohort with high PIBF1 expression but a statistically significant difference was only in OS ($p = 0.010$).
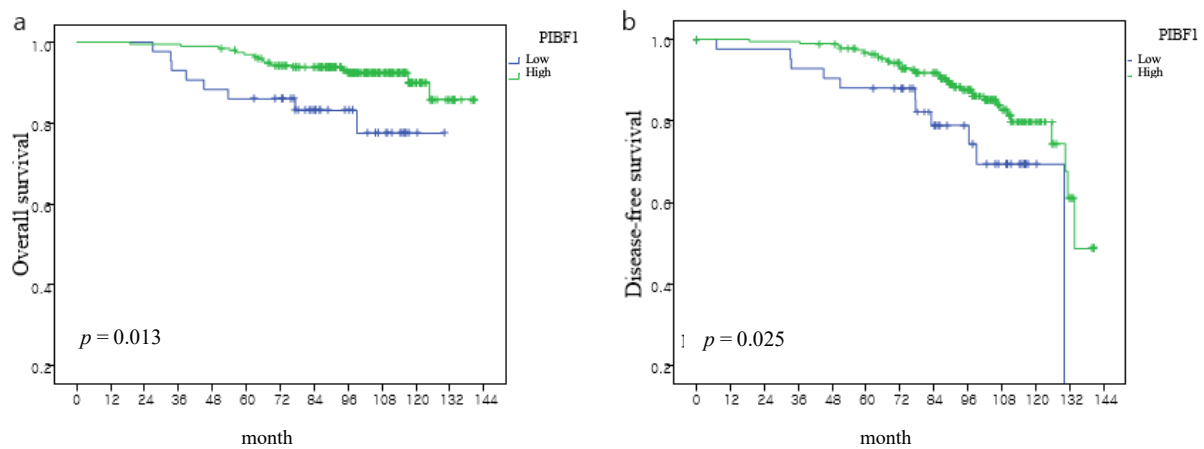
Fig. 3. Kaplan-Meier curves showing survival outcomes and disease-free survival according to PIBF1 expression in non-TNBC cohort

This demonstrates enhanced OS(a) and DFS(b) in the cohort of non-TNBC patients. There were also improved OS and DFS with high PIBF1 expression and in this cohort both show statistically significant differences (OS: $p = 0.013$, DFS: $p = 0.025$).

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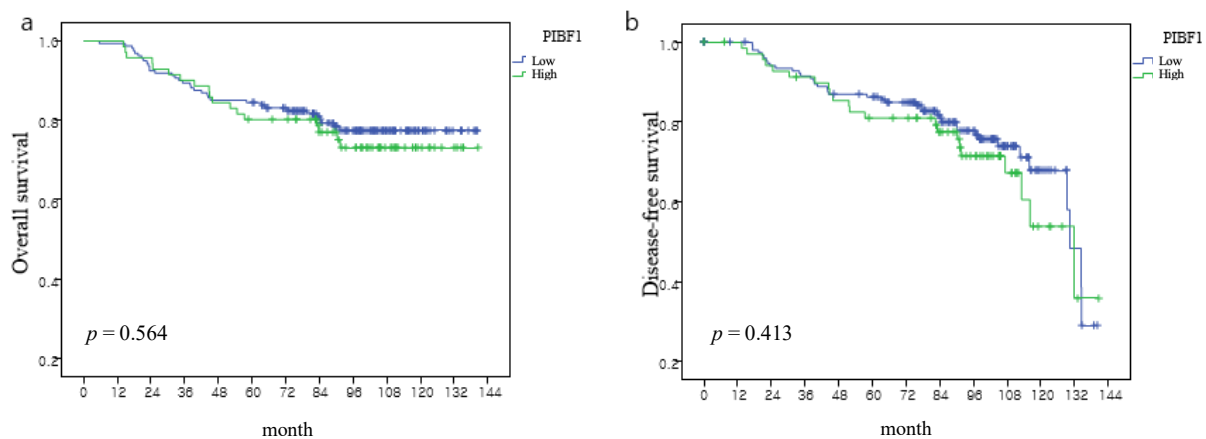
Fig. 4. Kaplan-Meier curves showing survival outcomes and disease-free survival according to PIBF1 expression in TNBC cohort

This demonstrates enhanced OS(a) and DFS(b) in the cohort of TNBC patients. There were no differences in both groups.
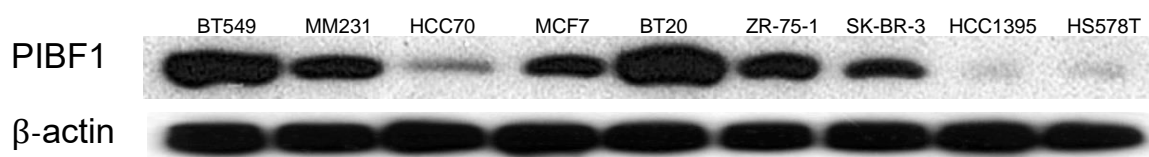
Fig. 5. PIBF1 expression in several cell lines

In the cell line, BT 549 and BT20 showed elevated PIBF1 expression, where as HCC70 and HS578T showed reduced expression.
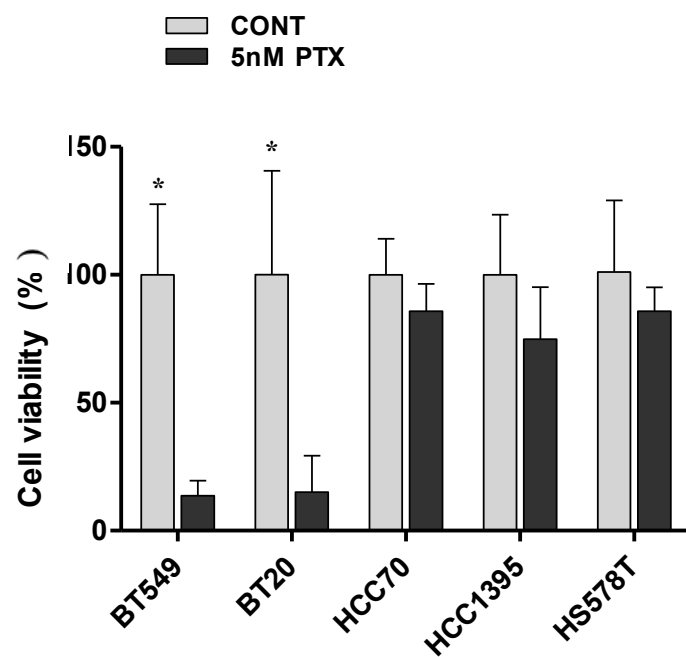
Fig. 6. Cell viability assays using paclitaxel

BT549 and BT20 displayed reductions in cell viability, whereas HCC70 and HS578T retained viability.
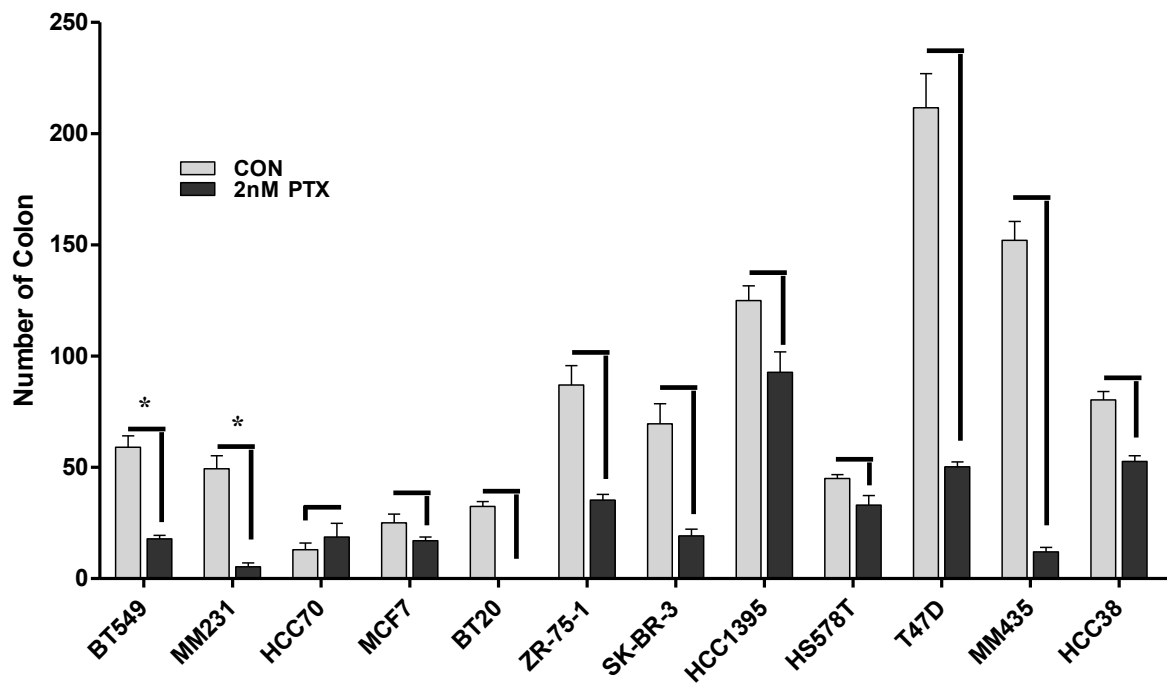
Fig 7. Colonogenic assays using paclitaxel

BT549 and BT20 displayed a substantial reduction in colonogenic count, but HS578T displayed a small decline, and in HCC70, there was even an increase in colony count.
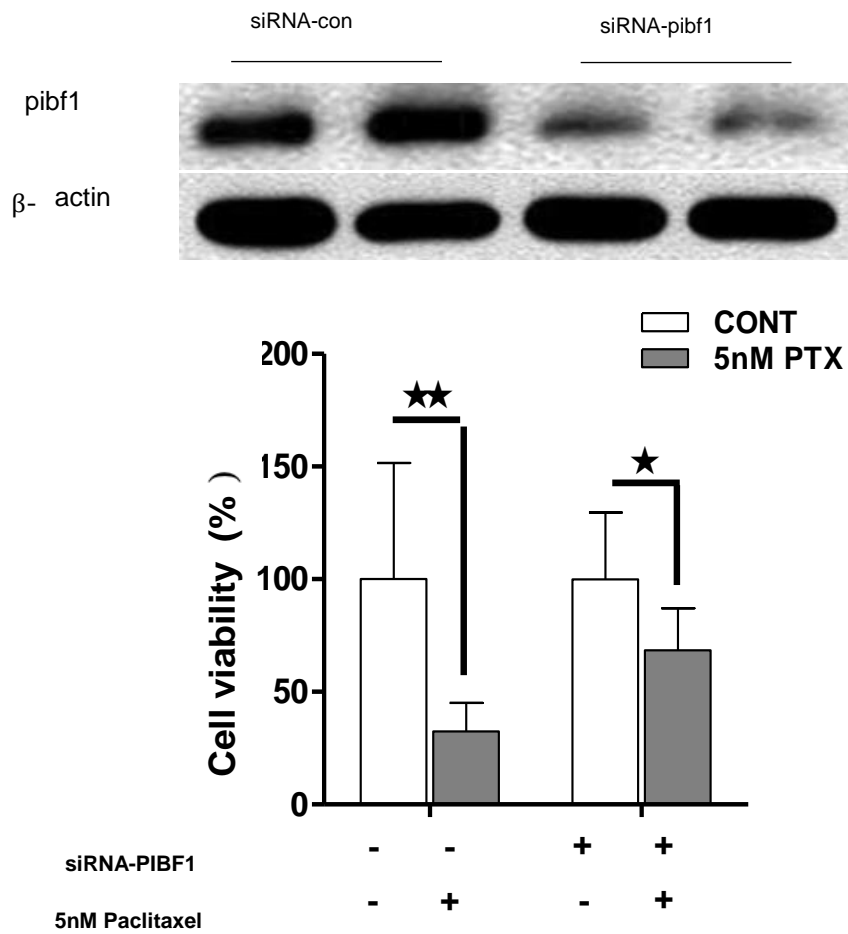
Fig 8. PIBF1 knockdown and paclitaxel sensitivity

siRNA mediated knock-down of PIBF1 was conducted and the knock-down cell lines demonstrated a diminished response to paclitaxel.

Table 1. Patients characteristics between non-TNBC and TNBC group

Characteristics	Total(n=469)	nonTNBC(n=238)	TNBC(n=231)	*p*-value
Age (yr) (Mean ± SD)	47.35 ± 9.12	47.24 ± 8.98	47.46 ± 9.28	0.999
Breast operation				0.001
Breast conserving surgery	292(62.4)	131(55.0)	161(70.0)	
Total mastectomy	176(37.6)	107(45.0)	69(30.0)	
Unknown	1	0	1	
Axillary operation				0.322
No	2(0.4)	2(0.8)	0(0.0)	
Sentinel node biopsy	67(14.3)	36(15.1)	31(13.4)	
Axillary lymph node dissection	400(85.3)	200(84.0)	200(86.6)	
pT stage				0.015
T0/is	4(0.9)	0(0.0)	4(1.7)	
T1	178(38.0)	105(44.1)	73(31.6)	
T2	254(54.2)	115(48.3)	139(60.2)	
T3	30(6.4)	16(6.7)	14(6.1)	
T4	3(0.6)	2(0.8)	1(0.4)	
pN stage				0.412
N0	7(1.5)	4(1.7)	3(1.3)	
N1	326(69.5)	167(70.2)	159(68.8)	
N2	388(17.5)	45(18.9)	37(16.0)	
N3	360(11.5)	22(9.2)	32(13.9)	
Pathologic stage				0.992
I	17(3.6)	8(3.4)	9(3.9)	
II	298(63.5)	152(63.9)	146(63.2)	
III	152(32.4)	77(32.4)	75(32.5)	
IV	2(0.4)	1(0.4)	1(0.4)	
ER			-	-
Negative	54(22.7)	54(22.7)	-	-
Positive	184(77.3)	184(77.3)	-	-
PGR			-	-
Negative	93(39.1)	93(39.1)	-	-
Positive	145(60.9)	145(60.9)	-	-
HER2 status			-	-
Negative	148(66.7)	148(66.7)	-	-
Positive	74(33.3)	74(33.3)	-	-
Unknown	16	16	-	-
Histologic grade				<0.001
G1/2	195(41.8)	154(64.7)	41(18.0)	
G3	271(58.2)	84(35.3)	187(82.0)	
Unknown	3	0	3	
Nuclear grade				<0.001
G1/2	191(41.0)	151(63.4)	40(17.5)	
G3	275(59.0)	87(36.6)	188(82.5)	
Unknown	3	0	3	
Lymphovascular invasion				0.539
Absent	276(60.3)	139(58.9)	137(61.7)	
Present	182(39.7)	97(41.1)	85(38.3)	
Unknown	11	2	9	

P53				<0.001
Negative	275(59.1)	168(71.5)	107(46.5)	
Weak	31(6.7)	21(8.9)	10(4.3)	
Intermediate	25(5.4)	11(4.7)	14(6.1)	
Strong	134(28.8)	35(14.9)	99(43.0)	
Unknown	4	3	1	
Ki-67				<0.001
Low ($\leq$20%)	72(35.5)	54(56.8)	18(16.7)	
High(>20%)	131(64.5)	41(43.2)	90(83.3)	
Unknown	266	143	123	
Radiotherapy				0.010
No	98(20.9)	61(25.6)	37(16.0)	
Yes	371(79.1)	177(74.4)	194(84.0)	

Data shown are number (%) not otherwise specified.

SD = standard deviation; HER2 = human epidermal growth factor receptor 2; ER = estrogen receptor; PGR = progesterone receptor; G = grade

Table 2. Patients characteristics according PIBF1 status

Characteristics	Total(n=469)	PIBF1		p-value
		Low(n=203)	High(n=266)	
Age (yr) (Mean ± SD)	47.35 ± 9.12	47.76 ± 8.87	47.03 ± 9.32	0.953
Breast operation				0.705
Breast conserving surgery	292(62.4)	128(63.4)	164(61.7)	
Total mastectomy	176(37.6)	74(36.6)	102(38.3)	
Unknown	1	1		
Axillary operation				0.849
No	2(0.4)	1(0.5)	1(0.4)	
Sentinel node biopsy	67(14.3)	31(15.3)	36(13.5)	
Axillary lymph node dissection	400(85.3)	171(84.2)	229(86.1)	
pT stage				0.387
T0/is	4(0.9)	3(1.5)	1(0.4)	
T1	178(38.0)	70(34.5)	108(40.6)	
T2	254(54.2)	113(55.7)	141(53.0)	
T3	30(6.4)	15(7.4)	15(5.6)	
T4	3(0.6)	2(1.0)	1(0.4)	
pN stage				0.725
N0	7(1.5)	3(1.5)	4(1.5)	
N1	326(69.5)	140(69.0)	186(69.9)	
N2	388(17.5)	33(16.3)	49(18.4)	
N3	360(11.5)	27(13.3)	27(10.2)	
Pathologic stage				0.428
I	17(3.6)	8(3.9)	9(3.4)	
II	298(63.5)	127(62.6)	171(64.3)	
III	152(32.4)	66(32.5)	86(32.3)	
IV	2(0.4)	2(1.0)	0(0.0)	
ER				<0.001
Negative	285(60.8)	182(89.7)	103(38.7)	
Positive	184(39.2)	21(10.3)	163(61.3)	
PGR				<0.001
Negative	324(69.2)	189(93.1)	135(50.8)	
Positive	144(30.8)	14(6.9)	131(49.2)	
HER2 status				0.001
Negative	148(66.7)	19(45.2)	129(71.7)	
Positive	74(33.3)	23(54.8)	51(28.3)	
Unknown	16	1	15	
Histologic grade				<0.001
G1/2	195(41.8)	43(21.5)	152(57.1)	
G3	271(58.2)	157(78.5)	114(42.9)	
Unknown	3	3	0	
Nuclear grade				<0.001
G1/2	191(41.0)	42(21.0)	149(56.0)	
G3	275(59.0)	158(79.0)	117(44.0)	
Unknown	3	3	0	
Lymphovascular invasion				0.128
Absent	276(60.3)	126(64.6)	150(57.3)	
Present	182(39.7)	70(35.7)	112(42.7)	

Unknown	11	7	4	
P53				<0.001
Negative	275(59.1)	97(48.3)	178(67.4)	
Weak	31(6.7)	13(6.5)	18(6.8)	
Intermediate	25(5.4)	12(6.0)	13(4.9)	
Strong	134(28.8)	79(39.3)	55(20.8)	
Unknown	4	2	2	
Ki-67				<0.001
Low (≤20%)	72(35.5)	18(8.9)	54(53.5)	
High(>20%)	131(64.5)	84(82.4)	47(46.5)	
Unknown	266	101	165	
Radiotherapy				0.214
No	98(20.9)	37(18.2)	61(22.9)	
Yes	371(79.1)	166(81.8)	205(77.1)	

Data shown are number (%) not otherwise specified.

SD = standard deviation; HER2 = human epidermal growth factor receptor 2; ER = estrogen receptor; PGR = progesterone receptor; G = grade

Table 3. Comparison of characteristics of PIBF1 expression according to TNBC

Characteristics	Non-TNBC			TNBC		
	No PIBF1	PIBF1	*p*-value	No PIBF1	PIBF1	*p*-value
Age (yr) (Mean ± SD)	49.91± 7.95	46.65± 9.11	0.370	47.19± 9.03	48.07± 9.86	0.967
Breast operation			0.009			0.827
Breast conserving surgery	16(37.2)	115(59.0)		112(70.4)	49(69.0)	
Total mastectomy	27(62.8)	80(41.0)		47(29.6)	22(31.0)	
Unknown	0	0		1	0	
Axillary operation			0.480			0.290
No	1(2.3)	1(0.5)		0(0.0)	0(0.0)	
Sentinel node biopsy	7(16.3)	29(14.9)		24(15.0)	7(9.9)	
Axillary lymph node dissection	35(81.4)	165(84.6)		136(85.0)	64(90.1)	
pT stage			0.315			0.865
T0/is	0(0.0)	0(0.0)		3(1.9)	1(1.4)	
T1	17(39.5)	88(45.1)		53(33.1)	20(28.2)	
T2	20(46.5)	95(48.7)		93(58.1)	46(64.8)	
T3	5(11.6)	11(5.6)		10(6.3)	4(5.6)	
T4	1(2.3)	1(0.5)		1(0.6)	0(0.0)	
pN stage			0.025			0.224
N0	0(0.0)	4(2.1)		3(1.9)	0(0.0)	
N1	26(60.5)	141(72.3)		114(71.3)	45(63.4)	
N2	8(18.6)	37(19.0)		25(15.6)	12(16.9)	
N3	9(20.9)	13(6.7)		18(11.3)	14(19.7)	
Pathologic stage			0.024			0.276
I	0(0.0)	8(4.1)		8(5.0)	1(1.4)	
II	23(53.5)	129(66.2)		104(65.0)	42(59.2)	
III	19(44.2)	58(29.7)		47(29.4)	28(39.4)	
IV	1(2.3)	0(0.0)		1(0.6)	0(0.0)	
ER			< 0.001			
Negative	22(51.2)	32(16.4)		-	-	
Positive	21(48.8)	163(83.6)		-	-	
PGR			< 0.001			
Negative	29(67.4)	64(32.8)		-	-	
Positive	14(32.6)	131(67.2)		-	-	
HER2 status			0.001			
Negative	19(45.2)	129(71.7)		-	-	
Positive	23(54.8)	51(28.3)		-	-	
Unknown	1	15		-	-	
Histologic grade			0.006			0.051
G1/2	20(46.5)	134(68.7)		23(14.6)	18(25.4)	
G3	23(53.5)	61(31.3)		134(85.4)	53(74.6)	
Unknown	0	0		3	0	
Nuclear grade			0.004			0.088
G1/2	19(44.2)	132(67.7)		23(14.6)	17(23.9)	
G3	24(55.8)	63(32.3)		134(85.4)	54(76.1)	
Unknown	0	0		3	0	
Lymphovascular			0.359			0.285

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invasion					
Absent	28(65.1)	111(57.5)		98(64.1)	39(56.5)
Present	15(34.9)	82(42.5)		55(35.9)	30(43.5)
Unknown	0	2		7	2
P53			0.048			0.529
Negative	24(57.1)	144(74.6)		73(45.9)	34(47.9)
Weak	4(9.5)	17(8.8)		9(5.7)	1(1.4)
Intermediate	2(4.8)	9(4.7)		10(6.3)	4(5.6)
Strong	12(28.6)	23(11.9)		67(42.1)	32(45.1)
Unknown	1	2		1	0
Ki-67			0.049			0.015
Low (≤20%)	9(39.1)	45(62.5)		9(11.4)	9(31.0)
High (>20%)	14(60.9)	27(37.5)		70(88.6)	20(69.0)
Unknown	20	123		81	42
Radiotherapy			0.706			0.807
No	12(27.9)	49(25.1)		25(15.6)	12(16.9)
Yes	31(72.1)	146(74.9)		135(84.4)	59(83.1)

Data shown are number (%) not otherwise specified.

SD = standard deviation; HER2 = human epidermal growth factor receptor 2; ER = estrogen receptor; PGR = progesterone receptor; G = grade

Table 4. Univariable and multivariable regression analysis in OS of non-TNBC subset

Variables	Univariable HR (95% CI)	P-value	Multivariable HR (95% CI)	P-value
Age	1.011(0.966-1.059)	0.630		
Breast operation		0.359		
Breast conserving surgery	1(ref)			
Total mastectomy	1.457(0.652-3.253)			
Axillary operation		0.730		
No/SNB only	1(ref)			
Axillary lymph node dissection	1.237(0.369-4.149)			
pT stage		0.283		
T0-1	1(ref)			
T2-4	1.594(0.681-3.732)			
pN stage		< 0.001		0.005
N0-1	1(ref)		1(ref)	
N2-3	5.701(2.438-13.327)		3.800(1.510-9.562)	
ER		0.375		
Negative	1(ref)			
Positive	0.671(0.278-1.620)			
PGR		0.026		0.091
Negative	1(ref)		1(ref)	
Positive	0.397(0.176-0.898)		0.432(0.163-1.144)	
HER2 status		0.725		
Negative	1(ref)			
Positive	1.167(0.494-2.760)			
Histologic grade		0.514		
G1/2	1(ref)			
G3	1.311(0.582-2.952)			
Nuclear grade		0.598		
G1/2	1(ref)			
G3	1.244(0.552-2.801)			
Lymphovascular invasion		0.001		0.001
Absent	1(ref)		1(ref)	
Present	4.678(1.857-11.789)		5.217(1.883-14.455)	
P53		0.035		0.434
Negative-Weak	1(ref)		1(ref)	
Intermediate-Strong	2.525(1.067-5.975)		1.458(0.566-3.756)	
Ki-67		0.896		
Low (≤20%)	1(ref)			
High (>20%)	1.092(0.293-4.069)			
Radiotherapy		0.058		
No	1(ref)			
Yes	4.062(0.954-17.285)			
PIBF1 expression		0.017		0.082
Low	1(ref)		1(ref)	
High	0.353(0.150-0.832)		0.444(0.178-1.108)	

HER2 = human epidermal growth factor receptor 2; ER = estrogen receptor; PGR = progesterone receptor; G = grade

Discussion

Breast cancer stands as a formidable adversary in the global fight against malignancies. The heterogeneity of its pathology presents both challenges and opportunities. Its diverse clinicopathologic manifestations emphasize the paramount need for refining diagnostic and prognostic tools that can aid clinicians in tailoring treatment modalities to individual patient profiles, thereby ensuring optimized therapeutic outcomes[1]. Our study concentrated on the potential of Progesterone-induced blocking factor 1 (PIBF1) as on such marker.

Initially distinguished in the realm of pregnancy immunology, PIBF1's transition to oncology research was appeared to be surprising at first glance. However, its increased expression in various cancers compared to their normal tissue adds credence to its potential role in tumor progression and possibly in treatment modulation. Our study's focal point was to unravel PIBF1's relevance and characteristics in the breast cancer panorama. Given the inherent chromosomal significance of its gene location and its elevated expression in breast cancer cells, this protein emerged as another probable keystone in breast cancer pathophysiology[2, 4, 9].

The immune modulatory role of PIBF1, coupled with its interplay in cellular events such as proliferation and apoptosis, underpins its importance in tumorigenesis[2, 3]. Although its exact mechanisms remain under exploration, preliminary findings from other malignancies set a promising stage for its implications in breast cancer[7, 8, 10].

In our study, garnered from a sizable patient cohort, there were some highlighted insights with PIBF1 and breast cancer. In the analysis of clinicopathological features, patients manifesting PIBF1 expression exhibited a notable association with a lower histologic and nuclear grade compared to those lacking PIBF1 expression. Furthermore, in relation to the Ki-67 index, the presence of PIBF1 expression was concomitantly linked to a decreased in Ki-67 relative to the PIBF1 negative cohort. This pattern was

accentuated within the non-TNBC cohort when categorizing patients into TNBC and non-TNBC subsets. Such findings underscore that PIBF1 expression is potentially indicative of more favorable clinicopathological attributes within breast cancer pathophysiology.

Additionally, PIBF1 expression was correlated with hormone receptor positivity, suggesting that its expression not only associated with favorable prognostic indicators but may also be intrinsically linked to hormone-related characteristics of the cancer (Table 2, 3). Within this study cohort, a quantitative evaluation was conducted on the relationship between the ER Allred score and PIBF1 expression, revealing a positive correlation. These findings potentially underscore the hormone-related nature of PIBF1, warranting more extensive research to fully elucidate this aspect.

Regarding survival analysis, the broader patient population evidenced enhanced overall survival with PIBF1 expression, an improved OS that achieved statistical significance ($p = 0.010$). On stratification into TNBC and non-TNBC subsets, the statistically significant improvement in survival was exclusively observed within the non-TNBC cohort ($p = 0.013$). Subsequent multivariable analysis concerning overall survival within the non-TNBC cohort indicated that PIBF1 expression might correlate with a reduced risk of adverse outcomes. While this association approached statistical significance, it did not conclusively attain it (hazard ratio = 0.44, 95% confidence interval = 0.18-1.11, $p = 0.082$). However, these associations may vary with the expansion of the study cohort to include a larger patient population. The survival analysis unveiled compelling distinctions predicated on PIBF1 expression, particularly prominent in non-TNBC cohort. Collectively, our findings intimate that PIBF1 expression is favorably related to survival outcomes among high-risk breast cancer patients who have been administered chemotherapy.

Perhaps one of the most enlightening aspects of this study was the exploration of PIBF in the context of taxane-based chemotherapy. With all patients in our cohort being subjected to this regimen, discerning a potential correlation with PIBF1 was paramount. Our *in vitro* assays, encompassing both viability and colonogenic assessments, elucidated the differential responses based on PIBF1 expression.

The tangible reductions in cell viability and colony formation in high PIBF1 expressing cell lines, such as BT-549 and BT20, upon paclitaxel administration, mirrored the inherent biology observed in the patient cohort. Moreover, our genetic knockdown experiments further reinforced the influence of PIBF1 on chemotherapy sensitivity.

Kim et al.[11] identified that the larger isoform of PIBF1, primarily associated with the centrosome, functions as a pericentriolar satellite protein to integrity of the mitotic spindle pole, and have named this protein as CEP90. Taxanes are known to inhibit the dynamic behavior of microtubules, leading to the induction of multipolar mitotic spindles and the redistribution of the microtubule network from the centrosomes to the cell cortex[12]. Given the impact of PIBF1 on spindle pole conformation, it may exert a synergistic effect with taxane-based chemotherapy. This interaction could hypothetically contribute to the observed enhancement in chemotherapy response and outcomes evidenced in the study.

In an era of personalized medicine, given the multifaceted treatment paradigms within the realm of breast cancer, the identification, and validation of markers like PIBF1 could pave the way for more refined and individualized therapeutic strategies, aligning with the overarching goal of optimizing patient outcomes in the challenging landscape of breast cancer.

Our study establishes a preliminary but robust foundation for PIBF1's significance in breast cancer prognosis and treatment strategies. However, there were some limitations with this study, primarily hinged on its retrospective nature and single-center design. Furthermore, given that the cohort predominantly consisted of high-risk patients who had received chemotherapy, there are inherent limitations in extrapolating the natural attributes of PIBF1 to the broader breast cancer population. Further investigation is required to elucidate the oncogenic mechanisms of PIBF1 and its effect on patients, including those who have not undergone chemotherapy and those treated with other therapeutic modalities.

In conclusion, the identification and validation of biomarkers such as PIBF1 hold the promise of enhancing personalized medicine withing breast cancer treatment, offering a potential pathway to more

nuanced and patient-specific therapeutic strategies. Our study adds to the body of evidence supporting the value of PIBF1 as a marker for breast cancer prognosis and prediction of chemotherapy response, paving the way for improved patient management in this complex disease landscape.

References

1.   Walker, R.A., *Immunohistochemical markers as predictive tools for breast cancer.* J Clin Pathol, 2008. **61**(6): p. 689-96.

2.   CHECK, J.H. and D. CHECK, *Therapy Aimed to Suppress the Production of the Immunosuppressive Protein Progesterone Induced Blocking Factor (PIBF) May Provide Palliation and/or Increased Longevity for Patients With a Variety of Different Advanced Cancers – A Review.* Anticancer Research, 2019. **39**(7): p. 3365-3372.

3.   Ro, E.J., et al., *PIBF1 suppresses the ATR/CHK1 signaling pathway and promotes proliferation and motility of triple-negative breast cancer cells.* Breast Cancer Res Treat, 2020. **182**(3): p. 591-600.

4.   Balassa, T., et al., *The effect of the Progesterone-Induced Blocking Factor (PIBF) on E-cadherin expression, cell motility and invasion of primary tumour cell lines.* J Reprod Immunol, 2018. **125**: p. 8-15.

5.   Szekeres-Bartho, J. and B. Polgar, *PIBF: the double edged sword. Pregnancy and tumor.* Am J Reprod Immunol, 2010. **64**(2): p. 77-86.

6.   Lachmann, M., et al., *PIBF (progesterone induced blocking factor) is overexpressed in highly proliferating cells and associated with the centrosome.* Int J Cancer, 2004. **112**(1): p. 51-60.

7.   González-Arenas, A., et al., *Progesterone-induced blocking factor is hormonally regulated in human astrocytoma cells, and increases their growth through the IL-4R/JAK1/STAT6 pathway.* J Steroid Biochem Mol Biol, 2014. **144 Pt B**: p. 463-70.

8.   DiAntonio, G., et al., *Serum levels of the immunomodulatory protein, the progesterone induced blocking factor (PIBF) which is found in high levels during pregnancy is not higher in women with progesterone (P) receptor (R) positive vs. negative breast cancer.* Clinical and Experimental Obstetrics & Gynecology, 2017. **44**(2): p. 187-189.

9.   Kabel, A.M., *Tumor markers of breast cancer: New prospectives.* Journal of Oncological Sciences, 2017. **3**(1): p. 5-11.

10. Halasz, M., et al., *Progesterone-induced blocking factor differentially regulates trophoblast and tumor invasion by altering matrix metalloproteinase activity.* Cell Mol Life Sci, 2013. **70**(23): p. 4617-30.

11. Kim, K. and K. Rhee, *The pericentriolar satellite protein CEP90 is crucial for integrity of the mitotic spindle pole.* J Cell Sci, 2011. **124**(Pt 3): p. 338-47.

12. Hornick, J.E., et al., *Live-cell analysis of mitotic spindle formation in taxol-treated cells.* Cell Motil Cytoskeleton, 2008. **65**(8): p. 595-613.

국문요약

유방암에서 PIBF1 의 예측인자에 대한 가능성과
탁센 기반 항암치료 반응성과의 연관성에 관한 연구

유방암의 발생률이 증가하고 다양한 치료 선택지의 발전과 더불어 환자들의 치료 전략을 세움에 있어 환자가 가지고 있는 암세포 각각의 예후와 치료 반응을 예측하는 종양 인자(Tumor marker)에 대한 중요성이 대두되었다. 프로게스테론 유도 차단 인자 1 (PIBF1)은 원래 임신 유지 면역과 관련이 있으나, 또한 암세포에서 증가되어 암세포에 작용하는 면역반응을 억제하여 종양 형성에 관여하는 것으로 알려져 있다. 유방암 외에 자궁경부암, 림프종, 백혈병에서도 PIBF1의 과발현이 확인되었고 그 기전과 역할에 대한 연구가 진행중이나, 유방암에서 PIBF1의 역할 및 임상 결과에 대한 연구는 거의 없다. 그래서 우리는 PIBF1 발현과 임상적 특성, 그리고 화학요법 반응성과의 연관성을 연구하였다.

469명의 겨드랑이 임파선 전이가 있고 taxane 기반의 항암치료를 받은 469명의 고위험 환자들을 삼중음성유방암 (TNBC) 군과 그렇지 않은 군(non-TNBC)으로 나누었다. 면역조직화학적으로 PIBF1의 발현을 분석하였고 PIBF1 발현에 따른 임상병리학적 특성을 분석하고 생존분석을 실시하였다. 그리고 유방암 세포주에서 colonogenic unit assay와 knockdown을 통해 PIBF1 발현과 항암치료 민감도 사이의 상관 관계를 조사하였다.

전체 환자군에서 PIBF1 발현은 대조군에 비해 낮은 조직학적 등급, p53, Ki-67을 보였고($p < 0.001$), 이 차이는 특히 non-TNBC 군에서 두드러졌다. 생존분석에서는 PIBF1이 있는 환자들의 전체 생존기간 (OS)이 유의한 차이로 좋았으며, 이 또한 non-TNBC에서 두드러졌다. 이와 더불어 다변량 분석에서 PIBF1 발현은 더 좋은 예후와 관련이 있었으나 통계적 유의성에는 못 미쳤다(HR = 0.44, p = 0.082). 체외 실험에서는 유방암 세포주의 PIBF1발현과 Paclitaxel 민감도 사이의 상관관계가 있었고, PIBF1 knockdown 후에는 민감도 저하를 보였다.

본 연구에서는 겨드랑이 림프절 전이가 있고 항암화학요법을 받은 유방암 환자에서 PIBF1 발현이 좋은 예후와 상관관계가 있는 것을 밝혀내었다. 또한 세포주 실험을 통해 PIBF1발현과 taxane 기반의 항암화학요법에 대한 민감도가 이러한 상관관계에 기여할 수 있음을 추론하였다. 이러한 결과를 바탕으로 PIBF1발현의 유무를 통해 유방암 환자에서 치료적 접근 방법을 결정하는 예측 마커로서의 잠재적인 유용성이 있음을 시사하고 있다.