

Original Article

Prolactin-induced protein as a potential therapy response marker of adjuvant chemotherapy in breast cancer patients

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Abstract: Many studies are dedicated to exploring the molecular mechanisms of chemotherapy-resistance in breast cancer (BC). Some of them are focused on searching for candidate genes responsible for this process. The aim of this study was typing the candidate genes associated with the response to standard chemotherapy in the case of invasive ductal carcinoma. Frozen material from 28 biopsies obtained from IDC patients with different responses to chemotherapy were examined using gene expression microarray, Real-Time PCR (RT-PCR) and Western blot (WB). Based on the microarray results, further analysis of candidate gene expression was evaluated in 120 IDC cases by RT-PCR and in 224 IDC cases by immunohistochemistry (IHC). The results were correlated with clinical outcome and molecular subtype of the BC. Gene expression microarray revealed *Prolactin-Induced Peptide (PIP)* as a single gene differentially expressed in BC therapy responder or non-responder patients ($p < 0.05$). The level of PIP expression was significantly higher in the BC therapy responder group than in the non-responder group at mRNA ($p = 0.0092$) and protein level ($p = 0.0256$). Expression of PIP mRNA was the highest in estrogen receptor positive (ER+) BC cases ($p = 0.0254$) and it was the lowest in triple negative breast cancer (TNBC) ($p = 0.0336$). Higher PIP mRNA expression was characterized by significantly longer disease free survival (DFS, $p = 0.0093$), as well as metastasis free survival (MFS, $p = 0.0144$). Additionally, PIP mRNA and PIP protein expression levels were significantly higher in luminal A than in other molecular subtypes and TNBC. Moreover significantly higher PIP expression was observed in G1, G2 vs. G3 cases ($p = 0.0027$ and $p = 0.0013$, respectively). Microarray analysis characterized PIP gene as a candidate for BC standard chemotherapy response marker. Analysis of clinical data suggests that PIP may be a good prognostic and predictive marker in IDC patients. Higher levels of PIP were related to longer DFS and MFS but not with OS.

Keywords: PIP, adjuvant chemotherapy, breast cancer

Introduction

Breast cancer (BC) is one of the most common malignancies among women. The high morbidity of BC together with its significant mortality rate, make it exceptionally important medically. Furthermore, there are great personal and social expenses involved [1].

In everyday clinical practice the heterogeneous group of BC is classified according to the immunohistochemical (IHC) expression of estrogen

receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and Ki-67 antigen. The majority of the diagnosed BC cases (ca. 70%) demonstrate ER and PR immunoreactivity, whereas ca. 20% present HER2 overexpression. These antigens are regarded as predictive factors before the decision of employing anti-estrogen and/or trastuzumab therapy [2]. However, the remaining 10-20% cases of BC, so called "triple negative" (TNBC: ER-, PR-, HER2-), are considered to have poor prognosis regarding their response

to conventional therapies [3]. Although significant progress has been made in the last years in BC diagnosis and therapy, reliable prognostic and predictive tools are lacking in the most aggressive BC cases.

Prolactin-Induced Protein (PIP), known also as Gross Cystic Disease Fluid Protein (GCD-FP15), extra-parotid glycoprotein, gp17 or Seminal Actin-Binding Protein (SABP) is a small 15-17 kDa glycoprotein, first described as one of the major components of cystic disease fluid. PIP is regarded as a specific secretory marker of apocrine cells and localizes typically in the cytosol of apocrine epithelia in all major organs as well as in regions of apocrine metaplasia and cancers of various origins-especially in those with apocrine features [4, 5]. PIP is also an important secretory component of various body fluids including saliva, tears, sweat, milk and seminal plasma [6]. The physiological function of PIP is not fully known but most probably differs between particular organs and body fluids. Considering the role of PIP in tumor biology, different posttranslational variants of the protein were demonstrated to interact with CD4-a molecule crucial for antigen recognition in the context of MHC class II by T cells [7, 8]. It has been suggested that PIP interaction with CD4 may inhibit the apoptotic potential of T cells, in mechanisms associated with Bcl2 expression [9]. Additionally, PIP aspartyl proteinase activity, allowing to cleave fibronectin, represents another feature possibly influencing BC progression and spreading. Fibronectin fragments through activation of integrin- β 1 receptor promotes cell invasion and induces key signaling pathways such as MAPK/ERK and PI3K/Akt involved in cell proliferation [10, 11]. It has also been shown that incubation of immortalized breast epithelial cell lines with exogenous PIP exerted mitogenic effects *in vitro* [12].

The expression of PIP in human BC cell lines was shown to be up-regulated by lactogens (prolactin, PRL and the human growth hormone, HGH), glucocorticosteroids and androgens, whereas, estrogens inhibited PIP expression [13-16]. The production of PIP by BC cell lines in response to androgen and glucocorticosteroids could be further differentially regulated by immune factors such as IL1 and IL6 [17, 18]. On the subcellular level the signal transducer and activator of transcription 5 (Stat5),

modified upon PRL binding, cooperates with androgen receptors in the process of PIP gene regulation [19].

Although, detection of PIP mRNA has been described in normal tissues, including breast ductal cells, salivary glands and skin, it has been suggested that the level of expression differs between normal breast tissue, primary breast cancer and metastatic carcinoma [20, 21]. Since PIP expression seems to be preserved in many cases of metastatic tumors, PIP was also exploited (together with mammoglobin) as a marker of BC nodal micrometastases, as a differentiation factor of distant metastases of unknown origin or even as a marker of BC cells circulating in peripheral blood [20, 22, 23]. Moreover, various levels of PIP expression were demonstrated in particular human BC cell lines, BC histopathological types and, most recently, molecular subtypes. Especially high PIP expression was shown in luminal A and molecular apocrine subtypes of BC, whereas TNBC and basal like BC were characterized by low PIP expression levels [24-26].

Resistance to chemotherapy is a major cause of treatment failure in patients with advanced cancer. Even though cases of BC can be the same, in terms of clinical, histological and receptor status, they react differently to chemotherapy. Despite significant advances in the diagnosis and treatment of breast cancer it is still necessary to search for new prognostic and predictive factors of BC. The aim of this study was typing the candidate genes associated with the response to standard chemotherapy in the case of IDC. Following the selection of the corresponding genes our goal was to confirm the results on a larger group of IDC cases. Furthermore, we decided to determine whether the expression level of PIP has prognostic and predictive significance.

Materials and methods

Study population

Tumor samples and clinicopathological data of patients with IDC were obtained from the Department of Tumor Pathology, Center of Oncology Maria Skłodowska-Curie Memorial Institute, Cracow Branch. Twenty-eight specimens of frozen biopsies obtained from IDC patients, divided according to their response to

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Table 1. Clinical and pathological characteristics of 28 patients: 14 therapy non-responders-NR and 14 therapy responders-R. DOX (doxorubicin), AT (adriamycin, docetaxel), CMF (cyclophosphamide, methotrexate, 5-fluorouracyl), MTX (methotrexate)

Parameters	%
Age	(37-81)
Menopausal status	
Yes	19
No	9
pT	
T1	4
T2	22
T3	1
T4	1
pN	
pN-	10
pN+	18
pTNM	
I	1
II	21
III	6
IV	0
ER	
Neg.	18
Pos.	10
PR	
Neg.	18
Pos.	10
HER2	
Neg.	21
Pos.	7
ER-/PR-/HER2-	
Yes	12
No	16
Radiotherapy	
Yes	25
No	3
Hormonotherapy	
Yes	11
No	17
Chemotherapy	
DOX - 4×	11
DOX - 6×	14
DOX - 4×, AT - 4×	1
CMF - 6×, MTX - 2×	2

chemotherapy (n=14 therapy responders, R and n=14 therapy non-responders, NR), were

examined with gene expression microarray, Real-Time PCR and Western blot. Clinical data of the 28 patients are presented in **Table 1**. Molecular investigations (RT-PCR) were performed on frozen IDC fragments, sampled from 120 patients diagnosed from 2002 to 2007 (**Table 2**). The material for IHC investigations involved 224 paraffin blocks of IDC patients diagnosed from 2000 to 2007, aged between 27 and 84 years (mean age: 57 years). Clinical and pathological traits of the patients are presented in **Table 2**. Histopathological evaluation of the hematoxylin and eosin (H&E) stained slides was used to determine the type and the malignancy grade of the tumors (G) according to WHO criteria [27]. In 199 (88.8%) cases of IDC, adjuvant chemotherapy was applied. Adjuvant hormonotherapy was administered to 141 (62.5%) patients and 118 (52.7%) patients were treated with adjuvant radiotherapy. Patients were followed up for 1-145 months. During this period 85 (37.9%) patients showed progression and 52 (23.21%) died of the disease. The study protocol was approved by the Bioethical Committee of the Wroclaw Medical University.

Gene expression microarray

A screening study using gene expression microarray (Illumina Gene Expression Direct Hybridization Assay-Human HT-12 v4 Beadchip; San Diego, CA, USA), targeting more than 47,000 transcripts was performed (Acumen Research Laboratories, Singapore) on biopsies obtained from 28 IDC patients, of which 14 suffered a disease relapse within two years following chemotherapy based on doxorubicin and cyclophosphamide (non-responders, NR) and the other 14 remained relapse free during this period (responders, R). Total RNA from 28 IDC samples was extracted with RNeasy Mini Kit (Qiagen, Valencia, CA, USA) as described in the manufacturer's protocol. Analysis of the yield and purity of the samples were performed through measurement of RNA concentrations and the absorbance at 230, 260 and 280 nm using the NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The mean concentration of total RNA was 963 ng/ μ l, while A260/A280 ratios indicated that all samples were of sufficient quality for microarray analysis (2-2.1). All RNA samples were analyzed by 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) using the RNA 6000 Nano LabChip,

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Table 2. Clinical and pathological characteristics of studied patients

Parameters	All patients			
	IHC N=224	%	PCR N=120	%
Age				
≤50	162	72.3	30	25
>50	62	27.7	90	75
Menopausal status				
Pre	81	36.2	93	78
Post	143	63.8	27	22
Tumor grade				
G1	10	4.5	6	5
G2	131	58.5	46	38
G3	83	37	68	57
Tumor size				
pT1	104	46.4	29	24
pT2	106	47.3	86	72
pT3	11	4.9	4	3
pT4	3	1.4	1	1
Lymph nodes				
pN0	82	36.6	41	34
pN1-pN3	142	63.4	79	66
Stage				
I	43	19.2	16	13
II	124	55.4	64	54
III	56	25	40	33
IV	1	0.4	0	0
ER				
Neg.	66	29.5	48	40
Pos.	158	70.5	72	60
PR				
Neg.	84	37.5	58	48
Pos.	140	62.5	62	52
HER2				
Neg.	190	84.8	92	77
Pos.	34	15.2	28	23
Molecular tumor types				
Luminal A	76	33.9	12	10
Luminal B	86	38.4	62	51.6
HER2 type	17	7.6	20	16.7
Triple negative (TNBC)	45	20.1	26	21.7
Ki-67				
Low proliferation ≤25	145	64.7	88	73.3
High proliferation >25	79	35.3	32	26.7

following the manufacturer's protocol to estimate the integrity of total RNA samples. The RNA Integrity Number (RIN) of the samples

ranged between 7.0 and 9.2. The samples were measured in duplicates. RNA underwent microarray gene chip hybridization and subsequent RT-PCR validation experiments (described below). All arrays were scanned with the Illumina Bead Array Reader and read with Illumina GenomeStudio® software (version 1.1.1) (San Diego, CA, USA).

Real Time PCR (RT-PCR)

Additionally, total RNA from 120 samples of IDC were extracted with RNeasy Mini Kit (Qiagen) as described in the protocol. From total RNA 400 ng were used to synthesize cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Auburn, NY, USA). The reaction mixtures were incubated for 10 min. at 25°C and then for 120 min. at 37°C and terminated for 5 min. at 85°C. PCR amplifications of total cDNA were carried out for 30 cycles. Specific primers for PIP (Hs01-114172_m1) and reference gene SDHA (Hs00188166_m1) were obtained from Applied Biosystems. cDNA was amplified in TaqMan Gene Expression Master Mix (Applied Biosystems) with gene-specific primers and probe on the 7500 Real-Time PCR System (Applied Biosystems). Thermal cycling conditions were: 60°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Data were analyzed using the 7500 Real-Time PCR System. Expression of each gene was normalized against mRNA expression of the house-keeping gene SDHA, as previously described. Real-Time PCR experiments for each gene were done in triplicates.

Western blot (WB)

Frozen BC samples of 4 responders and 6 non-responders were thawed in CellLytic MT Cell Lysis Solution (Sigma Aldrich, Munich, Germany) with the addition of protease inhibitors and 0.2 mM PMSF. Whole protein cell lysates containing equal amounts of total protein (30 µg) were determined by BCA protein assay (Pierce, Rockford, IL, USA). Protein samples were mixed with sample buffer and dithiothreitol (DTT) and resolved by SDS-PAGE electrophoresis. After

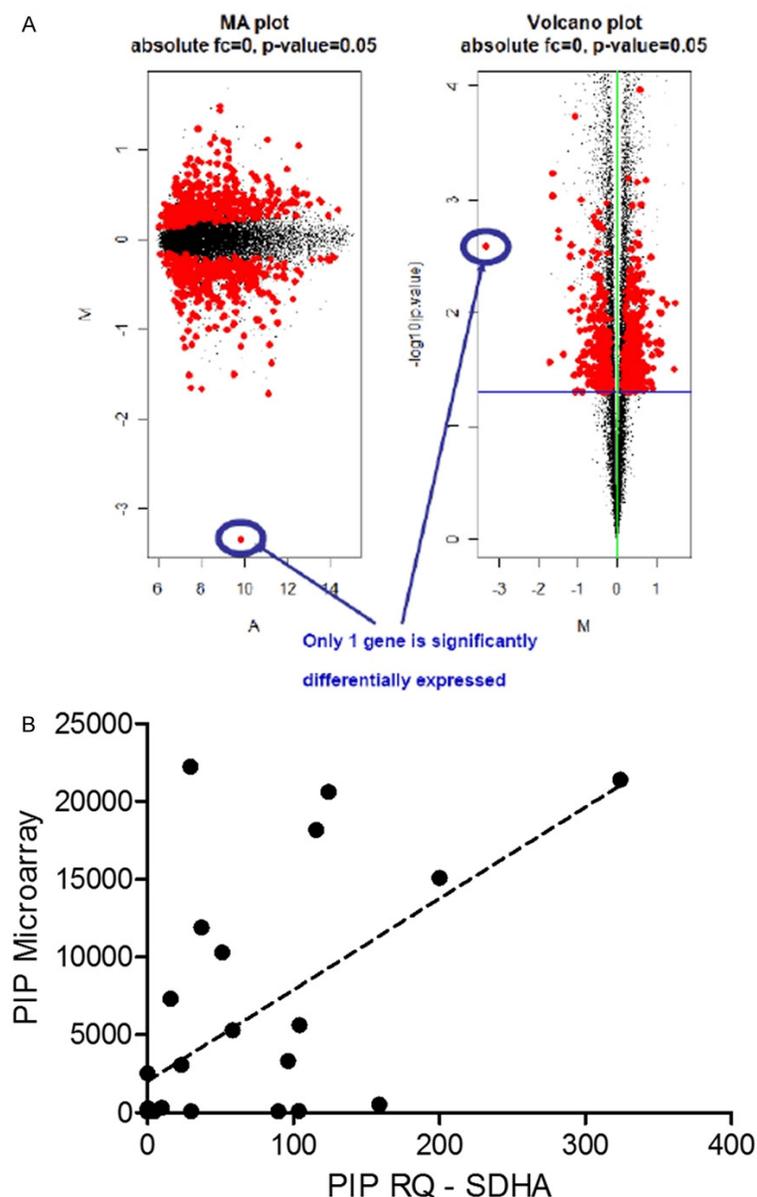


Figure 1. A. The analysis of genes from the expression arrays. The MA and Volcano plots showed the spread of the differentially expressed genes and *PIP* gene as the only gene differentially expressed in patients with non-responder or responder status. B. Real-time PCR analysis of *PIP* gene expression significantly correlated positively with microarray method ($r=0.66$; $p=0.001$).

the completion of electrophoresis, samples were transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon; Millipore, Bedford, MA, USA) and incubated in 4% BSA solution in TBS with 0.1% Tween-20. The membranes were incubated with anti-human monoclonal antibody PIP, clone EP-15824 (Novus Biologicals, Abingdon, UK), diluted 1:200 overnight at 4°C. Finally, the membranes were incubated with peroxidase-conjugated donkey anti-rabbit secondary antibody, clone 711-035-152,

diluted 1:3000 (Jacksons Immunoresearch, Suffolk, UK) for 1 h, rinsed, and incubated with the Immun-Star-HRP Chemiluminescent Substrate (Biorad, Hercules, CA, USA). Protein quantifications were based on β -actin expression with the use of primary antibody anti-human β -actin (Cell Signaling, Danvers, MA, USA).

Immunohistochemistry (IHC)

Expression of PIP antigen was studied by IHC. All samples ($n=224$) were initially fixed in 4% buffered formalin solution and embedded in paraffin. IHC reactions were performed on 4- μ m thick paraffin sections using DAKO Autostainer Link48 (Dako, Glostrup, Denmark). In order to deparaffinize, rehydrate and unmask the antigens the sections were boiled in Target Retrieval Solution, High pH (97°C, 20 min.) using Pre-Treatment Link Platform and, subsequently, cooled in EnVision FLEX Wash Buffer. The activity of endogenous peroxidase was blocked by incubation in EnVision FLEX Peroxidase-Blocking Reagent 5 min. at room temperature (RT). Then, the sections were washed in EnVision FLEX Wash Buffer. Detection of PIP expression was conducted using anti-GCDFP-15 antibody (clone 23A3, ready to use, RTU) for 20 min. at RT. After washing the sections in EnVision FLEX

Wash Buffer, EnVision FLEX/HRP (horseradish peroxidase) a secondary antibody was applied (20 min RT). Subsequently, sections were washed in EnVision FLEX Wash Buffer and, the substrate for peroxidase, diaminobenzidine was applied and the sections were incubated for 10 min. at RT. Finally, all the sections were counterstained with FLEX Hematoxylin for 7 min. at RT, dehydrated in alcohol (70%, 96%, 99.8%) and xylene. Subsequently, the preparations were mounted in SUB-X Mounting Medium.

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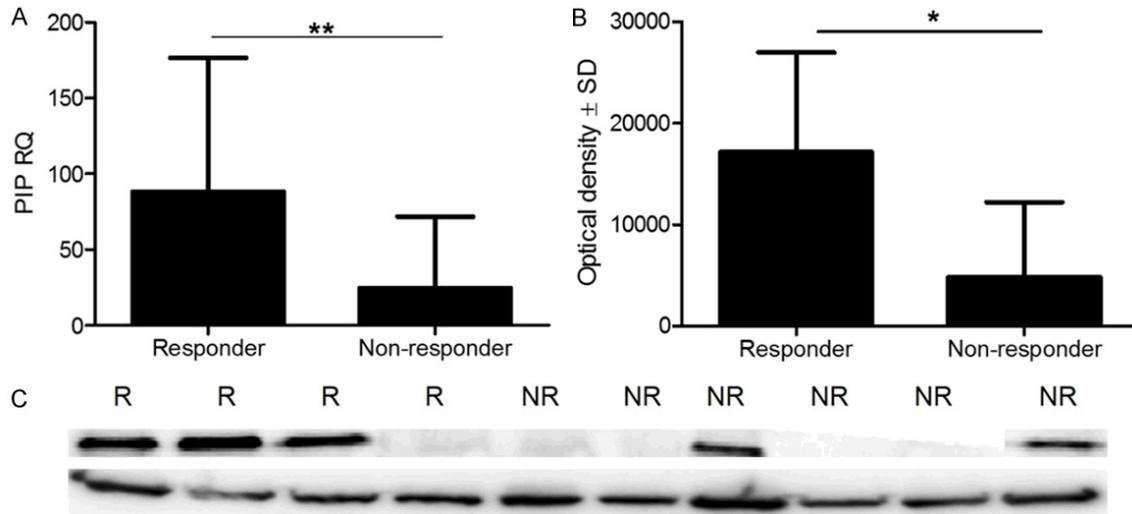


Figure 2. A. *PIP* mRNA relative expression (RQ) is significantly higher in the breast cancer (BC) therapy responder group than in the non-responder group ($**p=0.0092$). B. Densitometry analysis of IDC cases of therapy responder group (R) as compared to non-responder (NR) demonstrates significantly higher *PIP* protein level in the group of R vs. NR ($*p=0.0256$). C. Western blot of 4 responders and 6 non-responders BC samples shows the differences in the amount of *PIP* protein.

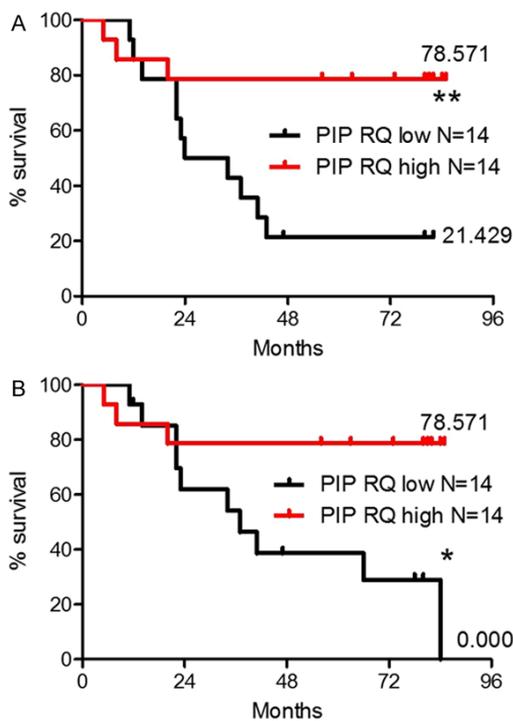


Figure 3. A. BC patients with higher *PIP* expression are characterized by a significantly longer disease free survival (DFS; $**p=0.0093$). B. BC patients with higher *PIP* expression are characterized by longer metastasis free survival (MFS; $*p=0.0144$).

In each case the negative control was included with Primary Negative Control.

Determination of the Ki-67, ER and PR antigen expression was performed according to the standard IHC procedure, as recommended by the manufacturer of the EnVision kit, FLEX, High pH. Ki-67 was detected using mouse monoclonal primary antibody (clone MIB-1; RTU) incubated for 20 min. at RT. Expression of ER and PR were determined using mouse monoclonal antibodies (clone ID5 and PR626, respectively, RTU). HER2 receptor was detected using Hercept Test and in cases of equivocal results (+2) additional verification was carried out using HER2 FISH pharmDxe Kit in line with recommendations of the producer. All reagents were obtained from Dako.

Evaluation of IHC reactions

Evaluation of IHC was conducted by two independent investigators (PD, BP) using the BX-41 light microscope (Olympus, Tokyo, Japan). For the evaluation and comparison of *PIP* expression the semi-quantitative IRS scale of Remmele and Stegner [28] was used. The scale takes into account the percentage of cells with a noticeable reaction (A) and intensity of the reaction color (B). The final score represents the sum of the two values, ranging from 0 to 12 pts. The cut-off scores of *PIP* expression used for Kaplan-Meier overall survival curves were: 0-3 pts vs. 4-12 pts. Intensity of Ki-67 antigen expression in tumor cells was evaluated accord-

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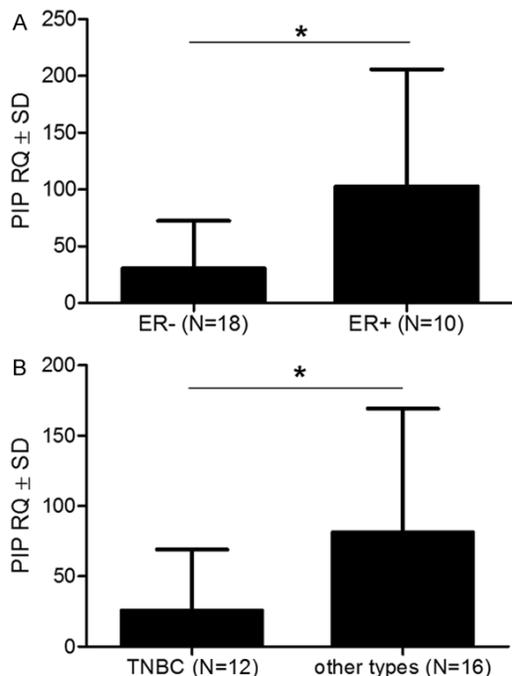


Figure 4. A. *PIP* mRNA relative expression (RQ) is significantly higher in the BC cases with positive estrogen receptor status ER+ than in ER- cases (* $p=0.0254$). B. *PIP* mRNA relative expression (RQ) is significantly lower in the TNBC (* $p=0.0336$).

ing to the percentage of positive tumor cells as compared to all tumor cells: 0 pts-no reaction, 1 pt-1-10%, 2 pts-11-25%, 3 pts-26-50%, 4 pts >50% [29]. The Ki-67 index indicated low expression when the percentage of positive cells was $\leq 25\%$, while high expression was recognized when it was $>25\%$. The status of ER and PR receptors was scored from 0 to 3 points, depending on the percentage of positive cells: 0 (no positive cells), 1 (1-10% positive cells), 2 (11-50% positive cells), and 3 (51-100% positive cells). The reaction was considered to be positive when the percentage of positive cells was $\geq 1\%$ and negative when it was $<1\%$ [3]. Expression of HER2 receptors was evaluated using a scale taking into account both, intensity of the membrane reaction and percentage of positive tumor cells [30].

Microarray data analysis

The Limma (linear models for microarray data) package was used for the analysis of microarray gene expression data. Moderated Student's t-test and Benjamini-Hochberg's method was used to determine differentially expressed genes from the arrays based on their grouping

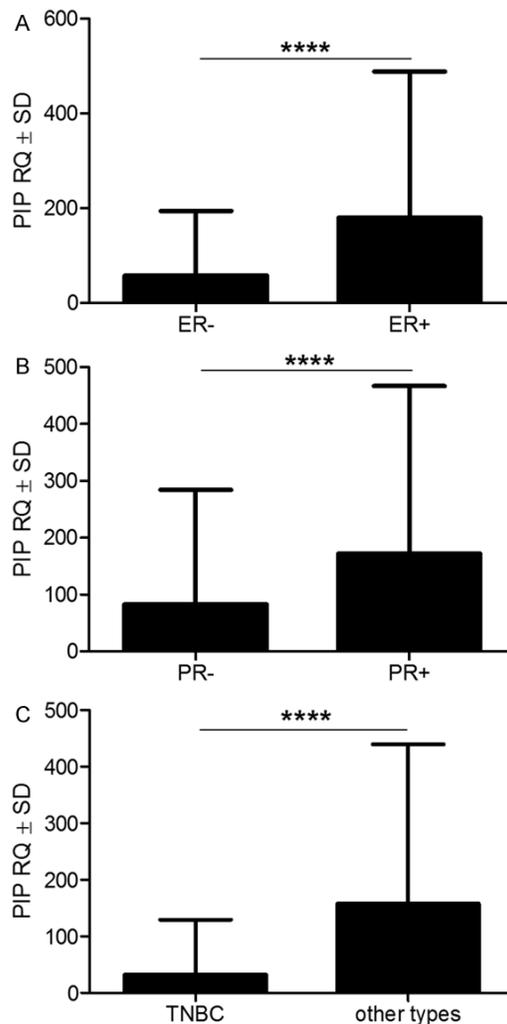


Figure 5. Association of *PIP* mRNA level with clinicopathological features. A. *PIP* mRNA relative expression (RQ) is significantly higher in the group of ER+ than in ER- IDC cases (**** $p < 0.0001$). B. PR+ cases showed significantly higher levels of *PIP* mRNA expression than PR- IDC (**** $p < 0.0001$). C. TNBC cases were characterized by significantly lower *PIP* mRNA RQ than in other cases (**** $p < 0.0001$).

into R and NR status of patients. Those genes with adjusted p -value < 0.05 were selected as differentially expressed genes. Raw intensity values were exported from GenomeStudio® software (version 1.1.1) for data processing and analysis in R (<http://www.R-project.org>) and Bioconductor (<http://www.bioconductor.org>).

Statistical analysis

The obtained results were subjected to statistical analysis using Prism 5.0 software

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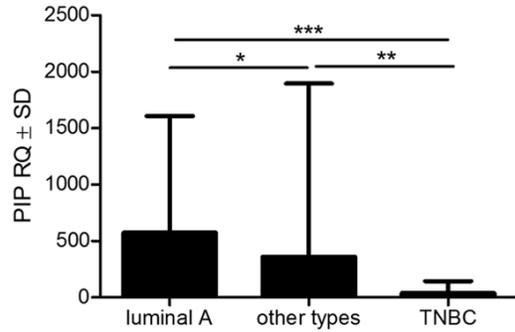


Figure 6. Association of *PIP* mRNA expression with BC molecular subtypes. *PIP* mRNA RQ was significantly higher in luminal A than in other molecular subtypes and to the TNBC as well as in other molecular subtypes to the TNBC (* $p=0.0268$, *** $p=0.0005$ and ** $p=0.0041$ respectively).

(GraphPad, La Jolla, CA, USA). Clinicopathological data utilizing Kruskal-Wallis, Mann-Whitney tests, and Unpaired t Test were used to compare differences of *PIP* expression in analyzed groups. Correlation analysis was performed using Spearman's rank correlation test. The Kaplan-Meier method and the log-rank test were used to determine the significance of patients' overall survival, disease free survival (DFS) and metastasis free survival (MFS). Univariate analysis was performed using the Cox regression model. For each variable, the hazard ratio and 95% confidence interval (CI) were determined. The results were considered statistically significant with values of $p < 0.05$ in all the analyses.

Results

Screening for candidate genes according to chemotherapy response

Gene expression microarray: The analysis of genes from expression arrays, based on their groupings into clinically non-responder or responder status of patients, revealed the *PIP* gene as the only gene differentially expressed for BH-adjusted p -value of 0.05. The MA and Volcano plots (**Figure 1A**) show the spread of the differentially expressed genes, taking a 0 fold-change and unadjusted p -value of 0.05 as the cut off. It can be seen that there is a single gene (*PIP* gene) with a fold change >3 on a log-scale, and the rest are hovering around the 1-2 level, the best p -values are around 10^{-4} , which unadjusted is definitely within the noise range.

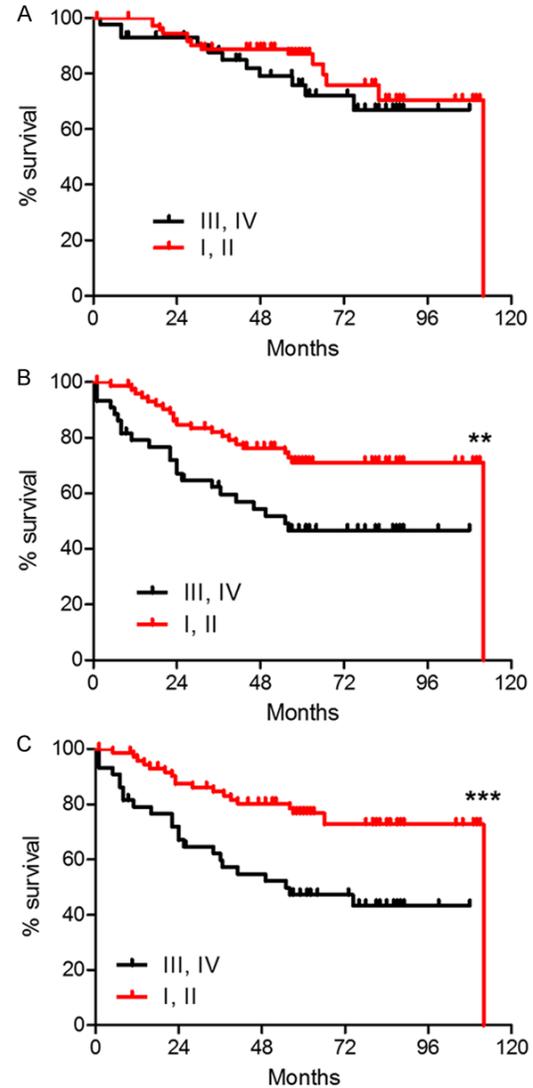


Figure 7. Univariate survival analysis of *PIP* mRNA of 120 IDC patients. (A) Mantel-Cox test demonstrates no significant association of overall survival with pTNM staging. (B) Lower pTNM staging correlates significantly with longer disease free survival (DFS; III-IV vs. I-II; ** $p=0.0046$) as well as with (C) longer metastasis free survival (MFS; III-IV vs. I-II; *** $p=0.0007$).

Real-Time PCR: The results of gene expression microarray of 28 IDC (responder and non-responder) cases were confirmed in further analysis using RT-PCR. The results of RT-PCR analysis of *PIP* gene expression showed significant positive correlation with the microarray method ($r=0.66$; $p=0.001$, Spearman correlation test, **Figure 1B**). Moreover, the level of *PIP* mRNA relative expression (RQ) was significantly higher in the BC biopsies obtained from the therapy responder group than from the non-

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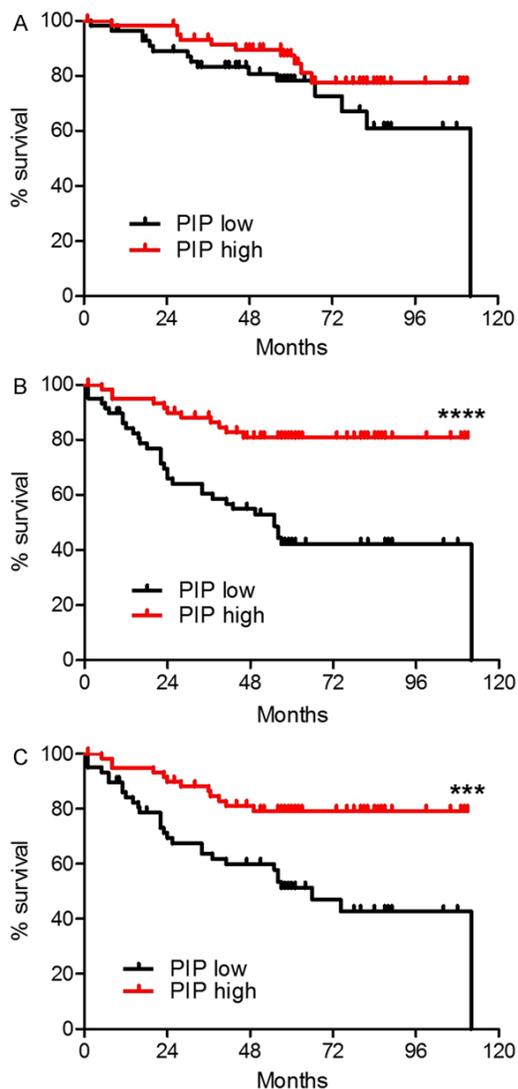


Figure 8. Survival analysis of *PIP* mRNA. (A) *PIP* mRNA expression showed no significant association with overall survival. (B) Higher level of *PIP* mRNA expression strongly correlates with longer disease free survival (DFS; **** $p < 0.0001$) as well as with (C) longer metastasis free survival (MFS; *** $p = 0.0006$).

responder group ($p = 0.0092$, Mann-Whitney test, **Figure 2A**).

Western blot: To compare the amounts of PIP at the protein level in the BC biopsy material obtained from therapy responders and non-responders, we performed Western blot analysis. Densitometry analysis of examined cases showed a significantly higher level of PIP protein in responder than in non-responder groups ($p = 0.0256$, Unpaired t Test, **Figure 2B, 2C**).

Correlation of prolactin-induced protein mRNA expression with clinicopathological data: The

correlation of *PIP* gene expression with clinical data of BC patients demonstrated that *PIP* expression values, higher than the median RQ of the whole group, were characterized by significantly longer disease free survival (DFS, $p = 0.0093$, Mantel-Cox test, **Figure 3A**), as well as metastasis free survival (MFS, $p = 0.0144$, Mantel-Cox test, **Figure 3B**). Furthermore, the level of *PIP* mRNA relative expression (RQ) was significantly higher in the BC cases with positive estrogen receptor status (ER+) ($p = 0.0254$, Mann-Whitney test, **Figure 4A**) and the lowest in the TNBC cases ($p = 0.0336$, Mann-Whitney test, **Figure 4B**).

Association of prolactin-induced protein gene expression in IDC cases with clinicopathological data

In order to further investigate and confirm the level of *PIP* mRNA expression, the IDC samples obtained from 120 patients were analyzed with RT-PCR.

***PIP* mRNA expression pattern in BC molecular subtypes:** The analysis revealed significant differences among particular molecular subtypes of IDC. The level of *PIP* mRNA relative expression (RQ) was significantly higher in the whole group of ER+ IDC cases ($p < 0.0001$; Mann-Whitney test; **Figure 5A**). Similarly, cases expressing PR+ showed significantly higher levels of *PIP* mRNA expression than PR- IDC ($p < 0.0001$; Mann-Whitney test; **Figure 5B**). The TNBC cases were characterized by significantly lower *PIP* mRNA RQ ($p < 0.0001$; Mann-Whitney test; **Figure 5C**). *PIP* mRNA RQ was significantly higher in luminal A (ER+ and/or PR+, HER2-, low Ki-67) than in other molecular subtypes and the TNBC ($p = 0.0268$ and $p = 0.0005$, respectively; Mann-Whitney test, **Figure 6**). Moreover, *PIP* mRNA RQ was also significantly higher in other molecular subtypes than in the TNBC ($p = 0.0041$; Mann-Whitney test, **Figure 6**).

Patients' survival analysis according to *PIP* mRNA expression: The analysis with Mantel-Cox test performed in the group of 120 patients, revealed no significant association of overall survival with pTNM staging (**Figure 7A**). However, higher pTNM staging correlated significantly with shorter disease free survival (DFS; III-IV vs. I-II; $p = 0.0046$, **Figure 7B**), as well as with shorter metastasis free survival (MFS; III-IV vs. I-II; $p = 0.0007$, **Figure 7C**). *PIP* mRNA expression showed no significant association

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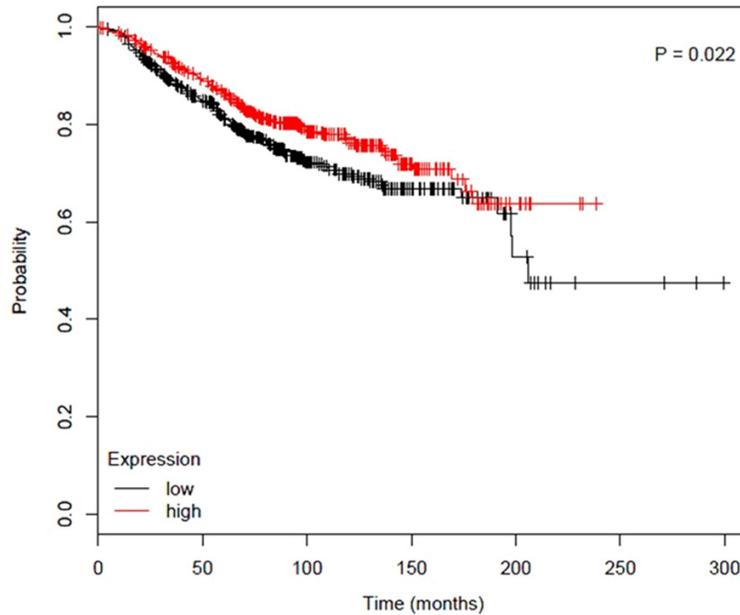


Figure 9. Survival analysis with using online analysis tool on 1,115 cases of BC. The good prognostic effect of high *PIP* expression is related to longer OS [31].

with overall survival (**Figure 8A**). Most importantly, *PIP* mRNA expression level in this patient group correlated strongly with DFS ($p < 0.0001$, **Figure 8B**) and MFS ($p=0.0006$, **Figure 8C**). An online survival analysis tool based on the microarray data of 1,115 cases showed that higher *PIP* expression was associated with longer overall survival ($p=0.022$, **Figure 9**) [31].

Immunohistochemical analysis of prolactin-induced protein expression in BC and its pattern in BC molecular subtypes

Based on the results obtained in the screening study, immunohistochemistry (IHC) was employed to assess the *PIP* expression in 224 paraffin-

embedded cases of IDC. Expression of *PIP* was observed in the cytoplasm of 142 (63.4%) cases with low intensity (1-6 pts) in 118 cases (**Figure 10A**) and high intensity (7-12 pts) in 24 cases (**Figure 10B**), while 82 (36.6%) cases presented no positive immunostaining. The mean value of *PIP* expression in IRS scale was 2.6 ± 3.1 pts.

Analysis of *PIP* expression with IDC tumor malignancy grade (G) showed that significantly higher *PIP* expression level was observed in G1 and G2 than in G3 cases ($p=0.0027$ and $p=0.0013$, respectively; Mann-Whitney test, **Figure 11**). Importantly, PR+ tumors showed significantly higher levels of *PIP* expression than PR- cases ($p < 0.05$; Mann-Whitney test, **Figure 12A**), whereas TNBC cases exhibited lower levels of *PIP* expression than with other molecular subtypes of IDC ($p < 0.001$; Mann-Whitney test, **Figure 12B**). A negative correlation was noted between expression of *PIP* and Ki-67 proliferation marker in BC samples ($r=-0.254$; $p=0.0001$; Spearman correlation test, **Figure 12C**). *PIP* expression was significantly higher in luminal A than in other molecular subtypes and TNBC ($p=0.0003$ and $p < 0.0001$, respectively, **Figure 13**). Moreover, *PIP* expression was also significantly higher in other molecular subtypes to TNBC ($p=0.0343$, **Figure 13**).

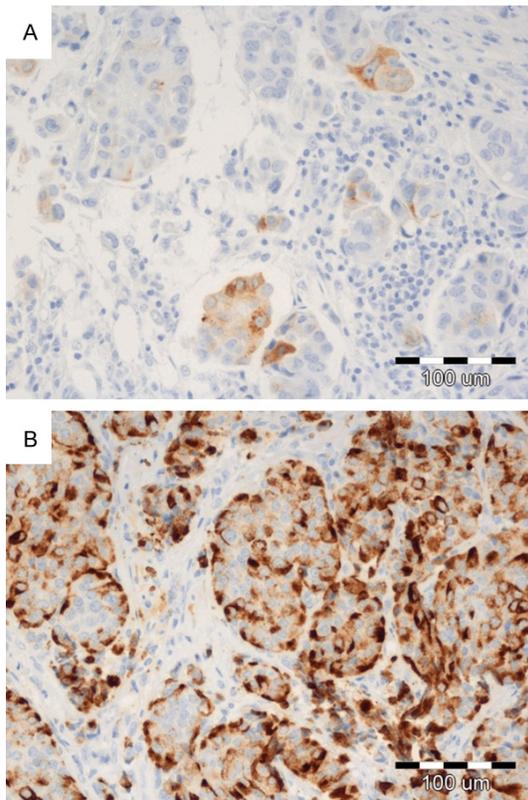


Figure 10. Immunohistochemical expression pattern of *PIP* in IDC cells. Expression of *PIP* was localized only in cytoplasm of tumor cells. A. Low *PIP* expression. B. High intensity of *PIP* expression. Magnification $\times 200$.

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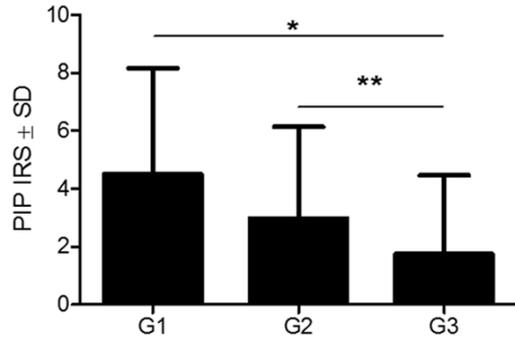


Figure 11. PIP expression in BC cells. Significantly higher PIP expression level is observed in G1 and G2 as compared to G3 cases (* $p=0.027$ and ** $p=0.0013$, respectively).

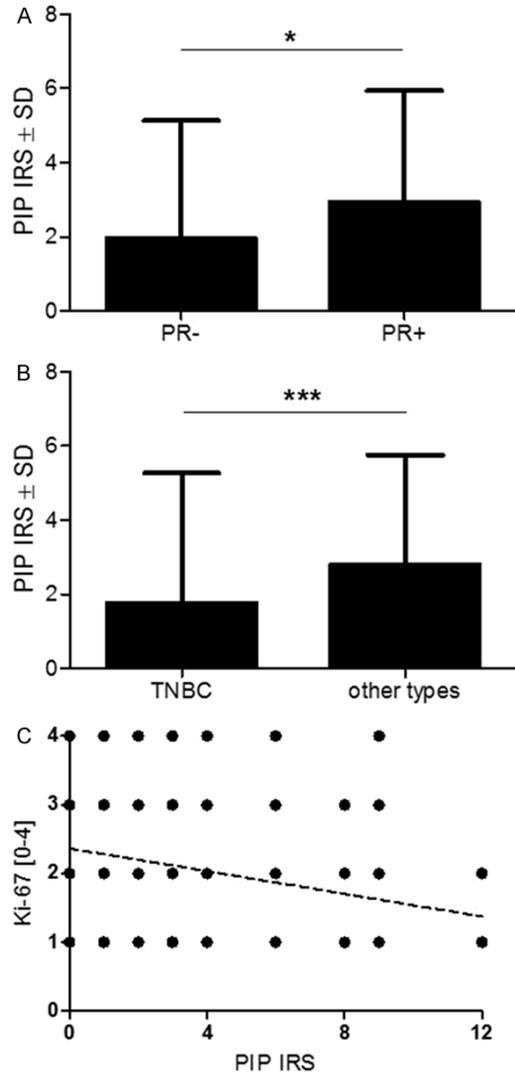


Figure 12. Association of PIP expression (IHC) with clinicopathological traits. A. PR positive (PR+) tumors show significantly higher level of PIP expression than

PR negative (PR-) cases (* $p < 0.05$). B. Triple negative (TNBC) cases exhibited lower level of PIP expression as compared with other molecular subtypes (** $p < 0.001$). C. PIP expression negatively correlates with Ki-67 proliferation marker in BC samples ($r = -0.254$; $p = 0.0001$).

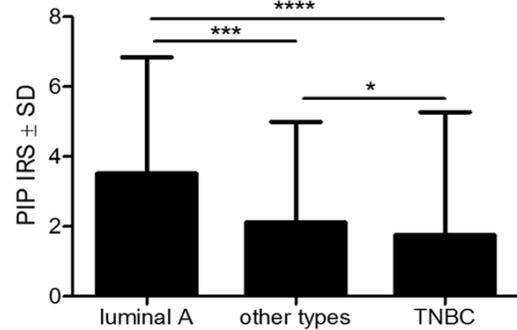


Figure 13. Association of PIP (IHC) expression with BC molecular subtypes. Significantly higher PIP expression in luminal A as compared with the other molecular subtypes and with the TNBC as well as other molecular subtypes to TNBC (** $p = 0.0003$; **** $p < 0.0001$; * $p = 0.0343$, respectively).

Association of immunohistochemical prolactin-induced protein expression of IDC with clinical outcome

In 224 examined patient samples univariate analyses using the Mantel-Cox test demonstrated significant association of shorter overall survival with higher pTNM staging (III-IV vs. I-II $p = 0.0075$, **Figure 14A**), with higher malignancy grade (G3 vs. G1 and G2; $p = 0.0037$, **Figure 14B**) and with high Ki-67 expression in the cancer cells ($p = 0.0469$, **Figure 14C**). However, PIP expression level assessed by IHC in IDC showed no significant association with OS ($p = 0.1581$, **Figure 14D**).

Discussion

Breast cancer is a heterogeneous group of tumors which can be classified according to its morphological and biological features, clinical picture as well as response to therapy [3, 32]. Traditional prognostic factors used to predict tumor clinical course are insufficient to characterize the whole clinical and genetic heterogeneity of BC and to adjust the therapy for each individual patient. In recent years, the development of molecular research, such as gene microarrays technology has revolutionised the

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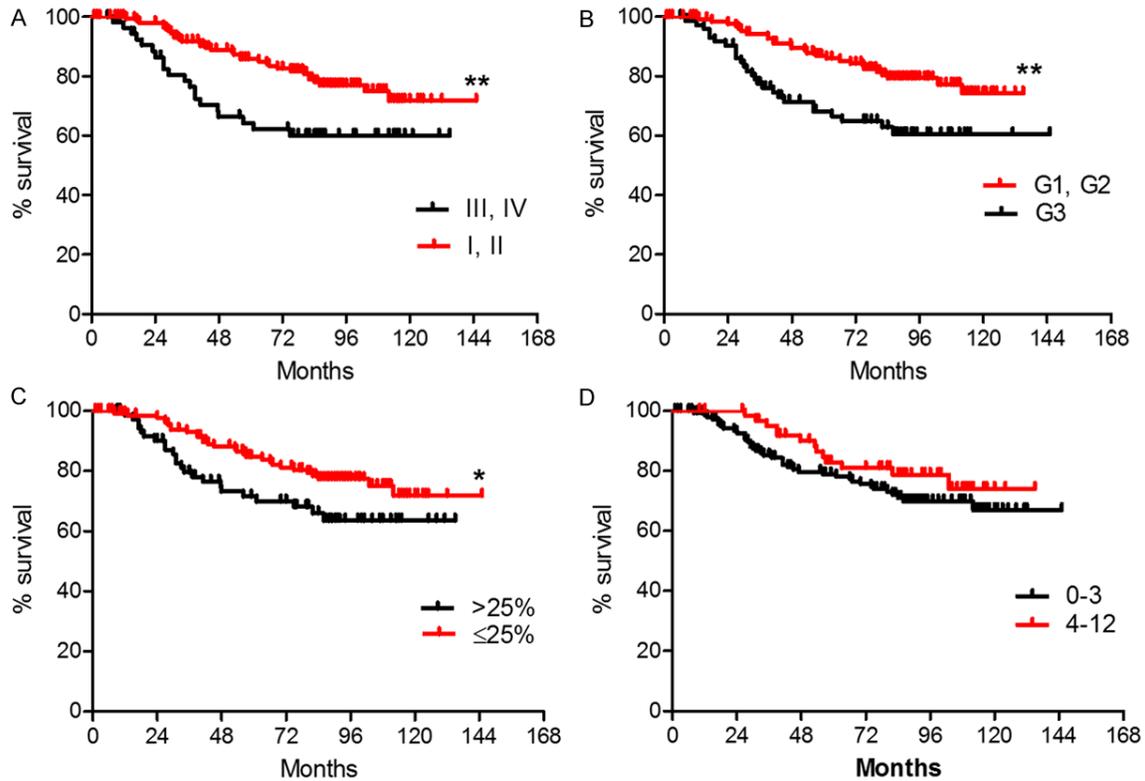


Figure 14. The univariate analyses of 224 cases of IDC patients. (A) Lower pTNM staging (III-IV vs. I-II $**p=0.0075$) demonstrated significant association with the longer overall survival, (B) Lower malignancy grade (G1 and G2 vs. G3; $**p=0.0037$) correlates significantly with the longer OS and (C) low Ki-67 expression in the BC cells correlates significantly with the longer OS ($\leq 25\%$; $*p=0.0469$). (D) PIP expression level (IHC) in BC cells showed no significant association with OS. The IHC cut-off scores for OS: 0-3 pts vs. 4-12 pts.

predictive and prognostic tools in the clinical settings of BC. Currently, several diagnostic protocols employ molecular assays based on microarray or RT-PCR analysis (e.g.: OncotypeDX, MammaPrint, Tailorx) to identify the high-risk patients who take advantage of the chemotherapy [33, 34]. So far, several studies have shown the overexpression of PIP in primary and metastatic BCs indicated the potential PIP prognostic value but none of these studies have considered PIP as a marker of response to chemotherapy [5, 6, 35].

In the present paper we have characterized for the first time *PIP* gene as a potential marker of response to the therapy in IDC. Analysis of more than 47,000 transcripts indicated that *PIP* gene is the only gene differentially expressed in IDC patients responding to cyclophosphamide and doxorubicin therapy as compared to the group of non-responding patients. The microarray data were supported by RT-PCR

and Western blot analyses in which was detected higher PIP expression in IDC patients responding to the therapy at the level of mRNA and protein, respectively. Importantly we have showed that patients responding to therapy and demonstrated higher levels of *PIP* mRNA were characterized by significantly longer DFS and MSF. These results were confirmed in a large group of IDC patients at the mRNA level. This is consistent with the results of Pagani et al. who found longer DFS in patients with increased *PIP* gene expression [5]. Similarly Fritzsche et al. indicated that low PIP expression was significantly associated with shortened DFS in univariate and multivariate analyses [35]. Higher PIP expression of mRNA and protein (IHC) showed a trend to be associated with longer OS but this correlation was not statistically significant. Nevertheless, it corresponds with the results obtained with survival analysis online tool on 1,115 cases of BC and points to the prognostic utility of *PIP* gene

expression in BC prognosis [31]. Additionally, Darb-Esfahani et al. demonstrated that PIP positive tumors had more favourable prognosis with respect to DFS and OS. However, multivariate analysis showed that PIP expression was not an independent prognostic factor for either OS or DFS [36].

Baniwal et al. revealed the requirement of PIP for the proliferation of tamoxifen-resistant BC cells suggesting that PIP may be targeted in the treatment of BC patients non-responding to hormonal therapy. They observed that PIP silencing inhibited the proliferation of the tamoxifen-resistant T47D breast cancer cells [26]. The enhancing effects of PIP on proliferation and invasion of BC cells may be ascribed to the asparthyl protease activity [10, 11, 37]. In this process secreted PIP cleaves fibronectin to release fragments that bound integrin β -1 receptors. Activation of integrin β -1 receptor mediates the induction of signalling pathways related to BC cell proliferation and invasion [10, 11]. Lines of evidence suggest that this pathway and fibronectin expression may be responsible for the observed tamoxifen and chemotherapy resistance of BC cells [38, 39]. Moreover, treatment of human BC cell lines with purified PIP enhanced their proliferation, whereas PIP silencing in ER+ and ER- BC cell lines inhibited cell proliferation and invasion through extracellular matrix [11, 26, 40]. Recently, the study of Naderi et al. also demonstrated that PIP expression was associated with cell cycle genes and concluded that PIP is required for BC cells progression [41]. Furthermore, available data suggest that PIP expression regulates the process of cell adhesion in BC [42].

Based on the results obtained in the microarray screening study, we investigated PIP expression using RT-PCR and IHC in a large and well-characterized group of IDC. So far PIP expression has been shown in BC cells in numerous studies. However, depending on the type of the lesion (primary vs. metastatic) or on molecular type, varying number of positive tumor cells could be noted (14% to 74%) [25, 35, 43-45]. We have shown that expression of PIP mRNA and protein was higher in ER+ and PR+ BC than in TNBC cases. These data are consistent with the results presented in an earlier study of Darb-Esfahani et al. who showed patients sub-

jected to anthracycline/taxane-based neoadjuvant chemotherapy [36]. Analyzing the IDC literature, the ER+ cases exert the highest, whereas TNBC the lowest PIP expression [25]. Taking into account that in our study we observed an inverse correlation between PIP and Ki-67 antigen it can be assumed that higher PIP expression was associated with luminal A molecular subtype of BC which is characterized by ER+ and/or PR+, HER2-, low Ki67 [11, 34]. This could be confirmed by results obtained by Lewis et al. who reported that PIP staining was observed in 65% (11/17) of luminal A tumors and in only 3% of TNBC cases [46]. Similar results were shown by Huo et al. who reported low percentages of PIP positive cases in primary and metastatic TNBC [25]. Baniwal et al. also emphasizes higher PIP expression in BC cell lines T47D and MDA-MB-453, representing luminal A molecular subtype of BC [26]. Genomic studies have revealed that PIP was overexpressed in luminal A and molecular apocrine subtypes of BC [11, 26]. The importance of the *PIP* gene expression in molecular apocrine signature was confirmed in meta-analyses of microarray studies performed on a larger group of ER- BC specimens and breast cancer cell lines [11, 47, 48].

Our results also indicate decreased PIP expression in tumors with increasing malignancy grade (G) which supports previous findings suggesting an association of PIP expression with features of good prognosis in BC tumors [26, 35, 46]. Darb-Esfahani et al. showed a relationship of high PIP expression with favourable tumor characteristics, such as a low G and the negative nodal status [36]. The results of our study can suggest the role of PIP as a potential prognostic and predictive factor in BC therapy.

In this study microarray analysis characterized PIP gene as a candidate for therapy response marker in BC. Our results suggest PIP as a factor differentiating patients responding to cyclophosphamide and doxorubicin chemotherapy. The fact that higher levels of PIP mRNA expression are associated with longer DFS and MFS seems to be of great importance. Additionally, PIP might be considered as a marker for the luminal A molecular BC subtype. Low expression of PIP in high malignancy tumors and TNBC cases indicate association of PIP expression with features of good prognosis in IDC. The

results of this study may point to a potential prognostic and predictive significance of PIP in BC therapy.

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Disclosure of conflict of interest

None.

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